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Identification et caractérisation des phosphoprotéines phosphatases, PP4 et PP2A, comme nouveaux régulateurs négatifs de la voie IMD chez la Drosophile

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Abstract

NF-κB pathways are highly conserved key regulators of the innate immune response in metazoans. However, their excessive activation is highly detrimental and is associated with the development of chronic inflammatory diseases. A keen interest is thus attributed to the characterization of the processes which ensure the proper duration and intensity of NF-κB signaling. Here, using *Drosophila melanogaster* as a model, we aimed at investigating the role of phosphoprotein phosphatases in the fine-tuning of the IMD-NF-κB pathway. This pathway is akin to mammalian tumor necrosis factor receptor signaling pathway and controls *Drosophila* immune defenses to Gram-negative bacterial infections through the activation of the NF-κB transcription factor Relish. We identify the highly conserved PP2A and PP4 as bona fide new negative regulators of IMD. By combining genetic and biochemical approaches, we show that PP4 and PP2A act at the level of the IKK signalosome and Relish respectively to modulate IMD signaling. Altogether, these data provide the first evidence of the regulation of the IMD pathway by phosphatases and emphasize the high conservation of the role of PP2A and PP4 in the regulation of NF-κB pathways. Our results set the bases for new perspectives for the characterization of the molecular processes controlling the IMD intracellular cascade.

Keywords: Innate immunity, NF-κB pathways, phosphatases, negative regulation, *Drosophila melanogaster*

Résumé

Les facteurs de transcription NF-κB sont des régulateurs essentiels de la réponse immunitaire innée hautement conservée au cours de l'évolution. Leur activation excessive est hautement délétère et est associée au développement de maladies inflammatoires chroniques. Il est donc particulièrement intéressant de caractériser les mécanismes moléculaires régulant leur activation. Au cours de ce travail, nous avons exploré le rôle de phosphoprotéines phosphatases dans la régulation de la voie IMD-NF-κB chez la drosophile. Homologue de la voie de signalisation activée en aval du récepteur TNFα et des récepteurs Toll-like chez les mammifères, cette voie contrôle les infections bactériennes chez la drosophile à travers l'activation du facteur de transcription de type NF-κB, Relish. Nous avons identifié les phosphatases PP2A et PP4 comme de nouveaux régulateurs négatifs de la voie IMD. En combinant des approches génétiques et moléculaires nous avons montré que ces phosphatases agissent au niveau du complexe IKK et du facteur de transcription Relish respectivement, pour la régulation fine de la voie IMD. Ainsi ce travail fournit la première preuve de la régulation négative de la voie IMD par des phosphoprotéines phosphatases et met l'accent sur la haute conservation des fonctions de PP2A et de PP4 dans la régulation des voies NF-κB. Nos résultats offrent ainsi de nouvelles perspectives pour la caractérisation des mécanismes moléculaires régulant la voie IMD.

Mots clefs : Immunité innée, voies NF-κB, phosphatases, régulation négative, Drosophile

Abbreviation

A

AcCoA: Acetyl co-enzyme A
AGO2: Argonaut-2
ALR: AIM2-like receptors
AMP: Antimicrobial Peptides
Ankrd: Ankyrin Repeat Domain
ASK1: Apoptotic Signal-regulating Kinase 1
Asp or D: Aspartate
ATF2: Activating Transcription Factor
ATP: Adenosine Triphosphate

B

BDSC: Bloomington Drosophila Stock Center

C

Can B: Calcineurin B
Cas9: CRISPR Associted protein 9
CD: cluster Differentiation
CDK: Cyclin Dependent Kinase
CDRE: Caudal-protein DNA recognition elements
Cka: Connector to kinase to AP-1
CLR: C-type lectin receptors
CRISP: Clustered Regularly Interspaced Short Palindromic Repeats
CYLD: Cylindromatosis

D

DAMP: Damage Associated Molecular Patterns
DAP: Diaminopimelic acid
dAP-1: *Drosophila* activator protein 1
Dcr-2: Dicer-2
DD: Death Domain
DIAP2: *Drosophila* inhibitor of apoptosis 2
DIF: Dorsal-related Immunity Factor
DGRC: Drosophila Genomics Resource Center
DNA: Deoxyribonucleic Acid
Dnr: Defense repressor 1
Dome: Domeless
DREDD: Death related ced-3/Nedd2-like protein
Dscam: Down Syndrome Cell Adhesion Molecule
DSK: Dual-Specificity Kinase
DSP: Dual Specificity Protein Phosphatases
dSR-CI: Scavenger Receptor Family
dsRNA: double stranded RNA
DUOX: dual oxidase

E

EC: Enterocyte
EEC: Enteroendocrine Cell
EGF: Epithelial Growth Factor
ERK: Extracellular Regulated Kinase

F

FADD: FAS associated Death Domain
FCP/SCP: TFIIF-associating component of RNA polymerase II CTD phosphatase/small CTD phosphatase
Flfl: falafel
FOXO: Forkhead Box O transcription factor

G

Gcm: Glial cell missing
GNBP3: Glucan Binding Protein 3
GR: Glucocorticoid Receptor
Grass: Gram positive Specific Serine Protease

H

HDAC1: Histone deacetylase 1
HEAT (huntingtin- elongation-A subunit-TOR
Hep: Hemipterous

Hh: Hedgehog
His or H: Histidine
HKE: Heat Killed E. coli
HMG: High Mobility Group
HP: Hematopoietic Pockets
Hpo: Kinase protein Hippo
HSP: Heat-Shock Proteins

I

IKK: I-kappa Kinase
IKK: Inhibitor of NF-κB Kinase
IL: Interleukin
IL-1: Interleukin 1
IMD: Immune Deficiency
IRAK: IL-1R–Associated Kinase
IRC: immune responsive catalase
IRD5: Immune Response deficient 5
ISC: intestinal Stem Cell

J

JAK/STAT: Kinase/Signal Transducer and Activator of Transcription
JAK: Janus Kinase
JNKK: JNK Kinase
JNKKK: JNK Kinase Kinase

K

KD: Knock Down
KDa: Kilodalton

L

LMPTP: Low Molecular weight PTP
LPS: Lipopolysaccharide
LRR: Leucine-Rich Repeat
Lys: Lysine

M

MAMPs: Microbial Associated Molecular Patterns
MAP: c-mitogen activated protein
MAPKKK: MAP Kinase Kinase Kinase
MAPKP: MAP kinase phosphatase
MEKK1: MEK Kinase 1
MLK2: Mixed Lineage Kinase 2
ModSP: Modular Serine Protease
MP1: Melanization protease
mRNA: messenger RNA
mts: microtubule star
MyD88: Myeloid differentiation primary response gene 88

N

NADPH: Nicotinamide Adenine Dinucleotide Phosphate
Nec: Necrotic
NFAT: Nuclear Factor of Activated T Cells
NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells
NIG: National Institute of Genetics
NimC1: Nimrod C1
NLR: NOD-Like Receptors
Nox1: NADPH oxidase 1
NSC: Neuronal stem cells

P

PCR: Polymerase Chain Reaction
PDGF: Platelet-Derived Growth Factor
PGN: Peptidoglycan
PGRP: Peptidoglycan Recognition Protein
PIMS: PGRP-LC Interacting Inhibitor of IMD Signaling
PIRK: Poor immune response upon knock-in
PM: Peritrophic Matrix
POSH: Plenty of SH3s
PP: protein phosphatase
PP1: phosphatase 1
PPAE: pro-phenoloxidase activating enzyme
PPMs: Metal-dependent Phosphatases
PPO: Pro-phenoloxydase
PPP: Phosphoprotein Phosphatase
PRR: Pattern recognition receptors
PSer: Phosphoserine
Psh: Persephone
PSP: Serine/Threonine phosphatases
pThr: Phosphothreonine
PTKs Tyr kinases

PTP: Protein Tyrosine Phosphatases
pTyr: Phosphotyrosines
PVR: Receptor –related pathway
pyMT: polyoma middle T
pyST: polyoma small T

R
R subunit: Regulatory Subunit
Ras: Ribosomal protein S6 kinase
Rdgc: Retinal degeneration C
RHD: Rel homology domain
RHIM: RIP Homotypic Interaction Motif
RIG-Like Receptors (RLRs)
RIP1: receptor-interacting protein 1
RISC: RNA-induced silencing complex
RNA: Ribonucleic Acid
RNAi: RNA interference
ROS: Reactive Oxygen Species
RT-qPCR: Real Time quantitative PCR

S
S2: Schneider 2
SAP: Sit4-associated protein
Ser or S: Serine
Ser/Thr: Serine / Threonine
SILAC: Stable Isotope Labeling of Amino Acids in Cell Culture
siRNA: short-interfering RNA
SLE: Systemic lupus erythematosus
Smo: Smoothened
SPE: Spätzle Processing Enzyme
Spn: Serpin
ssRNA: single-stranded RNA
ST: SV40 small T
SUMO: Small Ubiquitin-like Modifier

T
TAB2: TAK1-associated binding protein 2
TAK1: TGF-β Activated Kinase 1
TCR: T Cells Receptor
TCT: Tracheal Cytotoxin
TEP: Thioester-containing motif protein
TGF-β: Transforming growth factor beta
Thr or T: Threonine
TIR: Toll/IL-1R
TLR: Toll-like receptors
TNFR: Tumour-Necrosis Factor-Receptor
TNFα: Tumor necrosis factor alpha
TOR: Target of rapamycin
Tot: Turandot
TPR: Tetratricopeptide Repeat
TRAF2: TNF receptor-associated factor 2
Tregs: Regulatory T cells
Tws: Twins
Tyr or Y: Tyrosine

U
UAS: Upstream Activating Sequence
Uev1a: Ubiquitin-conjugating enzyme variant 1A
Upd: unpaired
UTR: Untranslated Transcribed Region
UV: Ultraviolet

V
VDRC: Vienna *Drosophila* RNAi Center
VEGF: Vascular Endothelial Growth
Vtd: Verthand

W
Wdb: Widerborst
WntD: Wnt inhibitor of Dorsal
Wrd: Well-rounded

Z
Zfh: Zinc finger homeodomain 1

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Preamble

NF-κB pathways play crucial roles in innate immunity in all metazoans. Activated downstream of pattern recognition receptors, NF-κB pathways control the expression of numerous genes encoding for cytokines, chemokines, effector and co-stimulatory molecules responsible for the activation and orientation of adaptive immunity (1). Therefore, NF-κB pathways are essential modulators of inflammatory responses in vertebrates. However, an exacerbated NF-κB signaling is detrimental to the host. Indeed, over-activation of NF-κB is linked to chronic inflammatory and auto-immune diseases, as well as to the development and progression of tumors (2). Hence, the challenge of current research is to progress towards a better understanding of the mechanisms governing the fine-tuning of NF-κB activation for the onset of more efficient therapeutic strategies. Phosphorylation has been shown to regulate the various steps in NF-κB signaling (3, 4) a process that is controlled by kinases and phosphatases with opposing roles. Numerous kinases have been demonstrated to be involved in the phosphorylation of distinct components in the NF-κB pathways; in contrast to the roles of phosphatases in NF-κB signaling which remain less explored.

The aim of my PhD research is to improve our understanding of the regulation of NF-κB signaling by protein phosphatases, using *Drosophila melanogaster* as a model system. By the power of its genetic and molecular tools, the latter is particularly well suited for the characterization of NF-kB pathways due to their high conservation from insects to mammals. To fulfill this goal, I characterized the molecular function of two protein phosphatases, Protein phosphatase 4 (PP4) and protein phosphatase 2 A (PP2A), implicated in the modulation of the NF-κB-IMD pathway of *Drosophila*.

In this manuscript, a review of literature composed of two chapters will be presented before exposing the set of the obtained results. The first chapter presents a broad overview of the known mechanisms of *Drosophila* innate immune responses and the second revolves around protein phosphatases, the essential effectors mediating reversible protein phosphorylation. This literature part is followed by two chapters containing our main results and findings regarding the functions of PP4 and PP2A, respectively, in fine-tuning the IMD

mediated immune response in *Drosophila*. Finally, new perspectives that will allow us to further dissect the roles of protein phosphatases in orchestrating NF-κB signaling are revealed.

Review of literature

Chapter 1: *Drosophila melanogaster* as a model to study the innate immunity

I- Innate immunity: an ancestral defense system

The evolution of various animal phyla required the development of powerful defense mechanisms to oppose infectious microorganisms that represent a threat to all metazoans. These mechanisms involve a wide variety of sensors that discriminate between self and non-self for the recognition of invaders thus activating efficient immune reactions. The general aspects of these defense processes, highly conserved from insects to humans, constitute the innate immune system (5-7). The engaged molecular sensors, called "PRRs" for "Pattern Recognition Receptors" recognize repeated patterns of molecular structures that are specific to microorganisms and absent from eukaryotic cells (8). These patterns are termed "MAMPs" for "Microbial Associated Molecular Patterns" (1, 8). Once activated, the sensors are engaged in molecular pathways, including NF-kB cascades, rapidly driving the expression of genes encoding effector molecules such as cationic antimicrobial peptides (AMPs) which target microbial cell membranes (9-11). *Drosophila melanogaster* can be largely credited for our current knowledge on innate immunity, particularly for the discovery of Toll receptor that played a crucial role in the antifungal response, uncovered by the team of Pr. Jules Hoffmann in 1996 (12). Following this finding, the hypothetical PRRs postulated by Charles Janeway were found to be homologues of Toll and thus were called "Toll like receptors" (TLR). The first TLR discovered in mammals was TLR4, identified as a receptor to bacterial lipopolysaccharide (LPS) (13). All of these discoveries have been critical for our knowledge on sensing and signaling in response to an infection. These studies were crowned with the Nobel Prize for Medicine dedicated to Jules Hoffmann, Ralph Steinman and Bruce Beutler in 2011.

Until now, ten members of the TLR family have been identified in humans. These receptors, following their dimerization or their association with other membrane molecules, allow the detection of a variety of microbial particles. In addition, by their localization at the level of the cytoplasmic or endosomal membranes, these receptors enable a constant

surveillance on extracellular infections. For example, TLR1, TLR2, TLR4, TLR5 and TLR6 are localized at the level of the cytoplasmic membrane and recognize MAMPs such as lipids, lipoproteins, LPS or proteins, while TLR3, TLR7, TLR8 and TLR9 are found at the level of the endosomal membrane and are involved in the recognition of nucleic acids (14). In addition to TLRs, the large family of mammalian PRRs also includes intracellular receptors such as NOD-Like Receptors (NLRs) that recognize bacterial peptidoglycan (PGN) and RIG-Like Receptors (RLRs) sensing nucleic acid fragments (15).

In addition to the discrimination between self and the infectious non-self, mediated by PRRs and MAMPs, another model for the activation of innate defense mechanisms was proposed by Polly Matzinger in 1994 (16). This model states that an immune response is triggered for self-protection against any danger threatening the integrity of its tissues, whether it is infectious or not. The model proposes that following cell or tissue damage, self-molecules that are not normally exposed to the immune system, can be released thus transmitting a "danger signal" activating immune reactions (16). These molecules have been designated by "Damage Associated Molecular Patterns", or DAMPs (16). Several self-molecules were subsequently certified as "DAMP"; these include: (i) intracellular proteins such as heat-shock proteins (HSPs) and the High Mobility group box-1 (HMGB1); (ii) proteins derived from the extracellular matrix such as hyaluronan fragments, fibronectin A and fibrinogen; (iii) non-protein DAMPs including uric acid, sulfate heparin and DNA (17, 18). Interestingly, these molecules induce several members of the PRR superfamily such as TLRs, NRLs, RLRs, C-type lectin receptors (CLR) and AIM2-like receptors (ALR) (17, 18).

In *Drosophila*, recognition of microbes is achieved through the sensing of bacterial derived PGN and fungal-derived β-(1,3)-glucan by specific PPRs, the peptidoglycan recognition proteins (PGRPs) and Glucan-binding proteins (GNBPs), respectively. The induction of these receptors results in the production of AMPs. Seven families of AMPs have been characterized in *Drosophila*; they comprise Attacin, Diptericin, Cecropin and Drosocin that are effective against Gram-negative bacterial infections; Defensin that target Gram-positive bacteria; Drosomycin and Metchnikowin that have antifungal activities (10). AMPs are the main effectors of the humoral response in *Drosophila*. They are produced upon immune challenge by the fat body, a

tissue required for lipid storage with functional equivalence to the mammalian liver, and secreted in the hemolymph (the insect blood) (5, 19). The expression of the AMPs encoding genes is regulated at the transcriptional level by two distinct signaling pathways, Toll and immune deficiency pathway (IMD) that activate NF-κB trancscription factors. These pathways exhibit high similarities with the signaling cascades activated downstream of the TLRs, IL-1 (Interleukin -1) and TNFα (Tumor Necrosis Factor alpha) receptors, hallmarks of the innate immune response in vertebrates (6).

Innate immunity provides immediate reactions that are essential for containing and counterstriking the infectious microorganisms. The vast majority of living organisms solely rely on these defense mechanisms (5). In vertebrates, the innate immune system is also essential for the initiation and the orientation of adaptive immunity (20). The latter is characterized by its long-lasting specific response and the set-up of an immunological memory (21). Adaptive immune responses appeared more recently, in the ancestors of cartilaginous fish, thus exist only in gnathostome vertebrates (5). The high specificity of its response relies on a large repertoire of receptors clonally expressed on highly differentiated B and T lymphocytes. These receptors are encoded by somatically rearranged gene fragments which allow an enormous diversification of their antigen binding domains. Antigen binding triggers the clonal selection and expansion of the lymphocytes thus driving the specificity of the ensued immune response (21). Beside the development of an efficient reaction that fights the invading pathogen, this specific recognition leads to the establishment of a memory response. This last characteristic sets the basis of the generation of vaccines, which represents, together with the antibiotic treatments, one of the major achievements of contemporary bio-medical research.

II- Methods for studying *Drosophila* immune defenses

Similarly to all invertebrates, *Drosophila* exclusively relies on innate immunity to fight infections, to which it is highly resistant despite its constant contact with microorganisms, making this model organism well suited for the investigation of innate immune responses (22, 23). In addition, *Drosophila* disposes advanced genetic and molecular tools, a fully sequenced genome (24) and a short generation time (8-10 days) (**Figure 1**) giving this model organism a

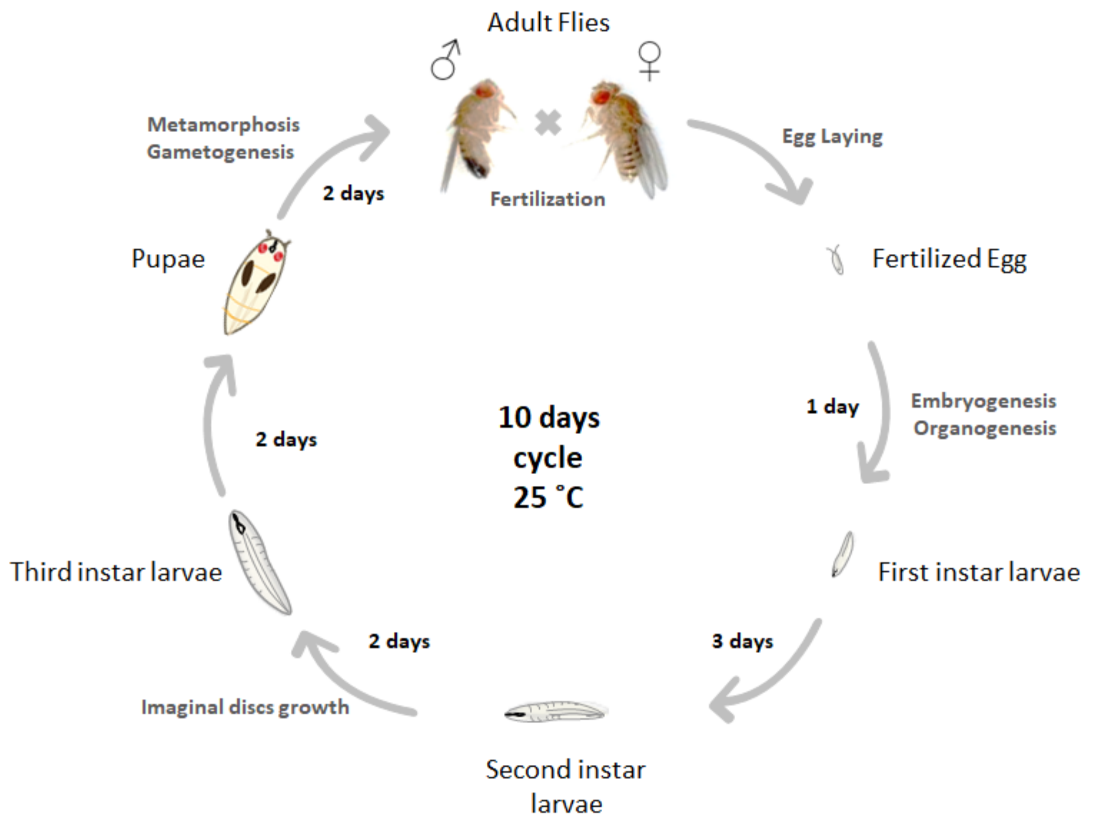

Figure 1: The life cycle of *Drosophila melanogaster*

particular advancement for deciphering the fundamental mechanisms underlying the innate immune response.

A collection of methods and protocols have been developed for the infection of flies both systemically and orally. These methods allowed extensive research for the characterization of *Drosophila* humoral, cellular, and epithelial responses to infections by a variety of microorganisms (25-27). A systemic infection can be induced by: i) pricking anesthetized flies, in the thorax or abdomen using a needle dipped into bacterial, viral, or fungal solutions; ii) by microinjection in which exact doses of microbes or immune elicitors are introduced in the fly general cavity; iii) by coating flies with fungal spores upon their placement on the sporulating lawn of a fungal culture plate. Oral infection is performed by feeding adult flies over contaminated media (27).

In addition to the collection of methods and protocols developed to challenge the flies, *Drosophila* displays an array of genetic and molecular tools that facilitate the studies of basic molecular mechanisms and physiological responses. The development of genetic research in *Drosophila* particularly benefited from the discovery of the transposable element P. Indeed, this element was extremely exploited for insertional transgenesis, for the induction of rapid and efficient mutagenesis in *Drosophila* (28). More recently, the development of RNA interference technology (RNAi) has provided an additional powerful tool to exploit the *Drosophila* model. RNAi is a cellular mechanism triggered by the detection of double-stranded RNA (dsRNA) sequences, which leads to the degradation of messenger RNA (mRNA) by sequence homology (29). This mechanism became a tool of choice for simultaneously characterizing gene functions and intracellular signaling mechanisms (30). The adaptation of this technology in *Drosophila* has made it possible to perform genome-wide screens both in cultured cells as well as in specific tissues *in vivo*, in flies (30). To perform screens in cultured cells, dsRNA is introduced into the cells by transfection or by simple incubation, allowing the subsequent inactivation of the gene of interest (31-33). Schneider 2 cells (S2), derived from embryonic hemocytes, are the most used in high throughput RNAi screens (34-36). These cells have been adapted for the identification of genes involved in the cellular response against several infectious agents (34). Furthermore, the *in vivo* application of RNAi is carried out by

the generation of transgenic fly lines carrying cloned repeated and inverted DNA sequences allowing the expression of a hairpin dsRNA. The temporal and spatial conditioned expression of the dsRNA is achieved by the use of the yeast transcriptional system, the UAS-GAL4 system (UAS: Upstream Activating Sequence). Gal4 protein is a yeast transcription factor that binds to the UAS regulatory region (37). The activation of this system requires crossing two transgenic fly lines, the driver-Gal4 line expressing Gal4 and the line carrying a specific dsRNA sequence for the gene of interest under the control of the UAS sequence (38). The off-spring of this cross have both transgenes but the dsRNA will only be produced in cells or tissues expressing Gal4 (38). Today, a vast collection of RNAi lines are available from the "Transgenic RNAi project" in the stock centers: "Vienna *Drosophila* RNAi Center" (VDRC), Bloomington *Drosophila* Stock Center (BDSC) and National Institute of Genetics (NIG). The details on all fly lines and phenotypes are listed online in "RNAi Stock Validation and Phenotype" (www.flyrnai.org/rsvp) and in the database FlyBase (39). Finally, thanks to the development of CRISPR-cas9 technology (Clustered Regularly Interspaced Short Palindromic Repeats) in *Drosophila*, it has recently become possible to produce new alleles for candidate genes to confirm the phenotypes observed in RNAi screens (30). CRISPR, was initially discovered in prokaryotes as a defense mechanism against phages. This technology has the advantage of generating null mutants allowing the validation of the hypomorphic phenotypes observed with the RNAi system (30).

In the following section, I will present an overview of our current knowledge on *Drosophila* immunity with a particular focus on the detection of microorganisms and the regulation of the subsequent response through the NF-κB pathways.

III- *Drosophila* innate immune system

Like all multicellular organisms, insects have evolved an array of strategies to defend themselves against pathogens since they live in a world where they are constantly exposed to an unquantifiable amount of micro-organisms. Particularly, insect larvae develop on decomposing organic matter and adults carry micro-organisms for which they serve as vectors, causing plant and animal diseases. Hence, the fruit fly *Drosophila melanogaster* relies on different innate defense reactions, many of which are shared with higher organisms (22, 23,

40). Systemic defense responses in *Drosophila* include cellular responses mediated by hemocytes, namely phagocytosis or encapsulation of foreign material and a potent humoral systemic response. The latter encloses the activation of proteolytic cascades in the hemolymph that lead to melanization of the invading microbe at the site of injury, and to hemolypmh clotting inhibiting pathogen dissemination from a wound. The hallmark of the systemic humoral reactions is the challenge-induced synthesis and secretion of AMPs that accumulate in the hemolymph, opposing invading pathogens. The early discovery of this inducible response in insects gained momentum in the early 1980s with the isolation from bacteria-challenged giant silk-moth *Hyalophora cecropia* of two groups of inducible AMPs that were shown to be effectors of this response (41, 42). As previously mentioned, distinct AMPs were subsequently identified in many insect species, including *Drosophila melanogaster*. The identification of these inducible molecules and the cloning of their corresponding genes in the early 1990s were rapidly followed by analysis of the challenge-induced control of their expression. NF-κB binding sites were found in the AMP gene promoters and were shown to be crucial for their induction and tissue specific expression (43, 44). Of great interest was the discovery that the activation of the *Drosophila* NF-κB transactivators, DIF and Relish, in response to fungal and bacterial infections occurred through two distinct signaling cascades, the Toll and the IMD pathways, respectively (12, 45). Before encountering *Drosophila's* systemic immune responses, micro-organisms need to cross physical barriers including the body cuticule, the peritrophic chitinous membrane lining the gut lumen and the underlying epithelium. In addition, a set of defense mechanisms specific to some tissues, so-called the local immune responses, are sufficient to contain most microorganisms. These responses are mediated by epithelial layers, which, beside from constituting physical barriers, fight against invading microorganisms by producing AMPs and reactive oxygen species (ROS) (46, 47). A full description of the systemic and local responses will be presented in the following sections.

1- Systemic immune responses

1.1- Cellular immune responses

The body cavity of *Drosophila* is filled with hemolymph that contains both free-floating and sessile cells, named hemocytes (22). These are divided into three cell types on the basis of their structural and functional features (34, 48, 49):

- Plasmatocytes are monocyte-like cells involved in phagocytosis of apoptotic bodies and microorganisms, cellular-mediated encapsulation and coagulation (50). These 10 µM professional phagocytes are the most abundant cellular form in the larvae representing 90% to 95% of the cell population. In addition, these cells contribute to the humoral response, by secreting extracellular matrix proteins and AMPs following an infection (51, 52). Furthermore, several studies have shown that plasmatocytes emanate signals that modulate infection-induced immune responses to inform distant tissues of a microbial invasion (53-57).

- The crystal cells are required for melanization and coagulation. These non-phagocytic cells contain crystalline inclusions, composed of Pro-phenoloxydase (PO), which are oxidoreductases related to hemocyanins that mediate melanization. Upon activation, they disrupt and release their contents into the hemolymph (58, 59). Under normal conditions, the crystal cells represent the minority population of hemocytes in larvae (5%).

- The lamellocytes are only observed in larvae infected with parasites. They are involved in the encapsulation of targets that are too large to be phagocytosed (60). These cells differentiate massively from the lymph gland in larvae after parasitization by wasps (50).

Hematopoiesis in *Drosophila* is a spatiotemporal triphasic process allowing the embryo, larvae and adults to be populated with mature blood cells. It eventually gives rise to hemocytes derived from two distinct lineages, embryonic or tissue hemocytes and lymph gland hemocytes; and both can be found in adult flies (34). The first hematopoietic wave occurs during early embryogenesis when hemocyte progenitors emerge from the procephalic mesoderm generating a limited number of hemocytes, which subsequently differentiate into plasmatocytes and a few crystal cells (61). While these differentiated crystal cells remain around their points of origins and populate specific localizations, the differentiated embryonic plasmatocytes migrate to spread along the embryonic tissues (62). They are required for embryonic development, particularly for the phagocytosis of apoptotic cells and the morphogenesis of the central nervous system (34, 62, 63). In the larval stages, these cells are

found along the dorsal vessel, in the proventriculus and in specialized microenvironments of the subepidermal layers of the body cavity, the hematopoietic pockets (HPs) (61, 64). These sessile hemocytes expand during the second larval hematopoietic wave within the HPs in response to signals delivered by the peripheral nervous system (65). They differentiate into plasmatocytes, lamellocytes upon parasitization of *Drosophila* larvae with a wasp egg, and also into crystal cells through a Notch-dependent transdifferentiation process (64, 66, 67). The third hematopoietic wave takes place in the third stage larvae, in a specialized organ situated in the dorsal aorta, namely the lymph gland (68), which holds pro-hemocytes, progenitors. Plasmatocytes and crystal cells differentiate and are dispersed during the dissociation of the lymph gland, at the onset of the larva to pupa transition. Lamellocytes, however, do not differentiate in normal developmental conditions but only in response to immune challenges such as wasp parasitism or stress conditions such as an increase of ROS (50, 66, 69).

The differentiation of all these hemocytes arising from a common pro-hemocyte precursor is regulated by distinct signaling pathways. The identity and the maintenance of the precursors are defined by the GATA transcription factor Serpent (70); their proliferation state is modulated by Platelet-Derived Growth Factor (PDGF)/Vascular Endothelial Growth (VEGF) Receptor –related pathway (PVR) (71), the ribosomal protein S6 kinase (Ras)/Extracellular signal-regulated kinase (ERK) pathway (72), the JAK/STAT pathway (73) and potentially the Toll pathway (74). The differentiation of pro-hemocytes to plamatocytes requires the transcription factors Glial cell missing (Gcm) 1 and Gcm2 (75) while crystal cells differentiation requires the activation of Notch pathway and the subsequent transcriptional activity of Lozenge transcription factor (75). The differentiation of lamellocytes requires the activation of JAK/STAT and the Toll-dependent Dorsal transcription factor (76).

1.1.1- Phagocytosis

Phagocytosis is a multi-step process that requires first the attachment of the phagocytic cell to the targeted particle which subsequently triggers cytoskeletal modification, internalization and finally destruction of the engulfed target within a highly complex organelle, the phagosome (34). *Drosophila* plasmatocytes are able to eliminate both micro-organisms and

apoptotic cells. They have the capacity to internalize bacteria, yeast, latex beads and double stranded RNAs (dsRNAs) within minutes.

To date, phagocytosis in *Drosophila* has been shown to implicate several types of receptor proteins. These include members of the scavenger receptor family (dSR-CI) (77); the Epithelial Growth Factor (EGF)-domain proteins, Eater (78, 79); Nimrod C1 (NimC1) (80); and Draper (81, 82). These EGF-domain containing receptors possess EGF-like sequences in the extracellular region called NIM repeats, located immediatly after a CCXG(Y/W) amino acid motif (83). The molecular function of such motifs is potentially linked with recognition of MAMPs. The immune function of phagocytosis receptors is well documented in the case of Eater, which is expressed exclusively on plasmatocytes (and prohemocytes). Indeed, eater-deficient flies show a severe reduction of phagocytosis of Gram-negative (*Escherichia coli*, *Serratia marcescens* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus*) (78). Besides Eater, NimC1 and dSR-CI have been shown also to be required for plasmatocyte-mediated phagocytosis of *E. coli* (77-80). In addition to the phagocytosis of apoptotic cells, Draper mediates the phagocytosis of *S. aureus* via a specific binding to lipoteichoic acid (84).

Moreover, members of the (PGRP) family, PGRP-SC1 and PGRP-LC were shown also to be involved specifically in the phagocytic response against Gram-positive and Gram-negative bacteria, respectively (85, 86). Other potential receptors, such as the Down Syndrome Cell Adhesion Molecule (Dscam), have been proposed as mediators of phagocytosis in *Drosophila*. Dscam encodes a member of the Ig superfamily (IgSF) with a gene that comprises a cluster of variable exons flanked by constant exons, which can theoretically generate 19,000 isoforms by alternative slpicing. Secreted isoforms of Dscam were detected in the hemolymph, and hemocyte specific *Dscam* silencing reduces the phagocytic uptake of bacteria (87).

The *Drosophila* genome harbors six genes coding for thioster-containing motif (TEP) proteins (TEP 1 to 6). The TEP family members possess a signal peptide indicating that they are secreted, and three of them (TEP1, TEP2 and TEP4) are up-regulated following an immune challenge with a mixture of Gram-negative and Gram-positive bacteria (88). The TEP proteins are close homologs of vertebrate complement factors C3/C4/C5 and the α2-macroglobulin

family of protease inhibitors. It has been proposed that TEPs function as opsonins to promote phagocytosis and/or protease inhibitors (22).

The clearance of apoptotic cells is also driven by plasmatocytes via their scavenger's receptors. Draper and Croquemort, the CD36-related receptor, have been shown to be implicated in this process (82, 89). Among the receptors that mediate the engulfment of apoptotic cells is the integrin αPS3/βν heterodimer (90).

1.1.2- Encapsulation

Encapsulation is a defense reaction against invading parasites that is only observed in invertebrates (48). It only occurs in insects' larvae and is mainly mediated by lamellocytes with the concerted activity of plasmatocytes and crystal cells. The encapsulation response has been analyzed using wasps that lay their eggs into the hemocoel of larvae (48, 60). Plasmatocytes that exert a permanent immune surveillance in circulation are able to detect wasp eggs (91). They attach to the egg and induce signaling molecules that lead to an increase in proliferation and a massive differentiation of pro-hemocytes to lamellocytes in the lymph gland (69). Lamellocytes are released from the lymph gland and then form a multilayered capsule around the invader that is then melanized through the release of PPO (Prophenoloxydase) by crystal cells (the melanization response is described below in section 1.2.2). Within the capsule, the parasite is eventually killed, either by the local production of cytotoxic products such as superoxide anions (92), or by asphyxiation.

1.2- Humoral immune responses

Microorganisms getting access to the insect body cavity rapidly trigger a humoral systemic immune response characterized by the synthesis and secretion in the hemolymph of powerful effector molecules that counteract the infection. Prominent among these are the AMPs (10, 93). Additionally, large-scale analyses, at the transcriptome and proteome levels, have revealed that in addition to AMPs, production of many peptides and proteins is upregulated after septic injury (94-96). Among these are putative immune effectors belonging to the DIM family (*Drosophila* immune molecules) which are small peptides of unknown functions secreted by the fat body. The Clotting or coagulation of the hemolymph at the site of the wound, and the deposition of melanin, known as melanization constitute secondary

barriers that immobilize bacteria and promote their killing. These reactions are triggered by the release of immune molecules by hemocytes upon the infection.

1.2.1- Coagulation

Clotting is critical to restrict pathogen dissemination from a wound and to limit hemolymph loss in insects as in vertebrates. It also serves as a secondary barrier that immobilizes bacteria thus facilitating their killing. The clot contains various proteins that form characteristic filaments which cross-link bacteria (23). These have been identified by proteomic analysis of the larval hemolymph clot (97). One particular protein, Hemolectin, encoded by a hemocyte-specific gene, has been demonstrated to be essential for efficient clot formation in *Drosophila* (98, 99). This protein is a major component of the fibers; it contains several domains found in other clotting factors. Additional proteins have also been identified including Fondue, an abundant hemolymph protein regulated by the Toll pathway (100). Knockdown of *fondue* by RNAi reduced aggregation activity of larval hemolymph and affected wound closure. In contrast to hemolectin, Fondue is not involved in the formation of primary clot fibers, but rather in the subsequent cross-linking of these fibers (100). This clotting reaction is independent of melanization because it still occurs in pro-Phenoloxydase (PPO)-deficient mutants (see melanization below) (99). However, crosslinking enzymes, such PPO and transglutaminase, may be involved in hardening of clots. Subsequent steps in wound closure include melanization and epithelial movements (101, 102).

1.2.2- Melanization

This reaction is immediate and considered as the earliest response against invading pathogens. It is observed by a blackening at the site of injury and on the surface of pathogens and parasites. It results from the synthesis and deposition of melanin, which plays a role in wound healing, encapsulation of parasites and sequestration of pathogens. In addition, the intermediates of the reaction are speculated to directly kill invading microorganisms (103). Melanization is triggered by the activation of an enzyme that catalyzes the oxidation of mono and di-phenols to orthoquinones that polymerize to melanin, termed Phenoloxidase (PO). PO exists as inactive zymogen Pro-PO (PPO) that is activated upon cleavage by a serine protease known as pro-phenoloxidase activating enzyme (PPAE). The latter itself is cleaved by other

serine proteases (Melanization protease 1, MP1 and MP2), a process induced upon infection or injury (104). The recognition of an injury derived or a microbial component is speculated to be mediated by members of the PGRP and GNBP families namely PGRP-LC, PGRP-LE and GNBP3 (GNBP3) (103-105). PGRP-LC and –LE were shown to mediate melanization in response to Gram-negative bacteria while GNBP3-dependent melanization was observed during fungal infections (106-108). In addition, local wounds rapidly induce activation of a novel circulating haemolymph serine protease, Hayan, which in turn converts PPO to phenoloxidase PO. The Haemolymph Hayan-PO cascade is required for activation of the c-Jun N-terminal kinase (JNK)-dependent cytoprotective program in neuronal tissues (109). Interestignly, a recent study study has revealed a disconnection between the melanization process occurring during resistance to infection and the blackening of the wound site. Although a mutation in Hayan leads to the almost complete loss of the blackening reaction in adults, Hayan mutants do not share the susceptibility of PPO1 and 2 mutant flies against S. *aureus* (110).

The *Drosophila* genome harbors three *PPO*s expressing genes, two expressed in crystal cells (*DoxA1* and *CG8193*) and one in lamellocytes (*DoxA3*) and as a consequence may participate in melanization during encapsulation (111). Two Serine protease inhibitors (Serpins), Spn27A (112, 113) and Spn28Dc (114) have been shown to negatively regulate the PPO activation pathway after an infection or wounding, respectively, preventing from excessive melanization.

1.2.3- Production of AMPs

As previously mentioned, AMPs constitute potent effectors of the humoral response. These are small cationic peptides that exhibit a broad range of activities against bacteria (Gram-negative and Gram-positive) and/or fungi (10). They are expressed in evolutionarily diverse organisms, from prokaryotes to invertebrates and vertebrates, and to plants (115). Due to its large size and its location inside the open circulatory system of the insect hemocoel, the Drosophila fat body represents a powerful organ for the production of AMPs into the hemolymph, where they reach their effective concentrations. Septic injury also triggers the expression of AMPs via the IMD pathway in circulating plasmatocytes (116). Their contribution to the hemolymph AMPs is probably minimal (22). Twenty immune-inducible

AMPs, which can be grouped into seven families, have been identified in Drosophila. These can be further can be further classified in three groups depending on their main microbial targets; we distinguish thus Attacin (4 genes), Cecropin (4 genes), Drosocin (1 gene), and Diptericin (2 genes) which are effective against Gram-negative bacteria (117, 118); Defensin (2 genes) targets Gram-positive germs (119); whereas Drosomycin (7 genes) and Metchnikowin (1 gene) have antifungal properties (120, 121). Upon infection, the concentration of these molecules in the hemolymph ranges from 0.5 μM for diptericin, to 100 for μM Drosomycin (10, 122). The majority of AMPs have a positive net charge at physiological pH because of the presence of a high content of arginine and lysine which is suited to interacting with negatively charged membranes of microbes, resulting in their permeabilization and ensuing cell death (122). The sensing and signaling events leading to AMP expression via the Toll and IMD pathways are detailed in section IV. The IMD pathway is rapidly induced following an infection, within few minutes, whereas the Toll pathway peaks 24 to 48 hours after immune stimulation, as monitored by the expression of *Drosomycin* (12, 22, 123). AMPs are also produced locally by epithelial cells, but their expression is exclusively dependent on the IMD pathway (124).

2- Local immune responses

Barrier epithelia are in continuous exposure to a large number of microorganisms; thus these surfaces must contain efficient systems for microbial recognition and control. Indeed, surface epithelia constitute physical and chemical barriers that separate internal tissues and organs from the surrounding environment. The most studied epithelial tissue is the gastrointestinal tract. The gut lumen is a hostile environment for microbial colonization due to its physical and physiological properties. In addition to the gastrointestinal tract, epithelial responses have also been reported in the trachea and the male genital plates.

The structure and the function of the cellular components of *Drosophila* gastrointestinal tract are also conserved during evolution. Although this tract is simpler than that of mammals, its cell components share several properties with their equivalents in mammals (125). The gastrointestinal tract is characterized by a highly compartmentalized structure containing different cell types with distinct functions. This structural complexity reflects the existence of a multitude of defense and regulatory mechanisms that synchronize together to ensure local

homeostasis (125). Indeed, the intestinal epithelium is equipped with physical and biochemical barriers to provide a natural barrier against infectious agents. Beyond the latter, the intestinal epithelium is provided with an inducible immune response encompassing three main reactions: (i) the secretion of PAM following the activation of the IMD pathway; (ii) the production of ROS; and (iii) tissue repair due to damage caused by the pathogen toxins secreted during the infectious process or by the side effects of the immune response (126-128). This last activated mechanism following infections is known by the term of "resilience" (46). Indeed, damaged enterocytes are replaced by the multiplication and differentiation of ISCs involving several signaling pathways and molecular events such as: Wingless, JAK-STAT, JNK, Hippo and Epidermal Growth Factor Receptor (EGFR) pathways (126, 129-132). In this section, I describe the intestinal anatomy as well as the functional compartmentalization of *Drosophila* intestine, and then I detail all the immune responses that allow the system to remain responsive to acute infectious challenges and inhibit pathogen proliferation while tolerating commensal and harmless microbes (47, 133-135).

2.1- Anatomical and functional regions of *Drosophila*'s gut

The gut is subdivided into three regions, foregut, midgut, and hindgut **(Figure 2)**, each is a tubular epithelium composed of a monolayer of cells surrounded by visceral muscles, nerves and trachea (47, 134, 136).

The foregut and hindgut are of ectodermal origin; they are protected on the apical side by an impermeable cuticle, and epithelial cells are sealed by septate junctions. The foregut encompasses the proboscis, a feeding and drinking appendage; the pharynx; the esophagus; the crop, a food storage organ; and the proventriculus that regulates ingested food passage to the midgut **(Figure 2)**. The crop contains a variety of immune proteins, including thioester containing proteins (TEPs) (88).

The midgut epithelium is of endodermal origin and exhibits smooth septate junctions. The midgut is protected by a peritrophic matrix (PM) which is a semi-permeable membrane allowing the passage of nutrients and enzymes, but not microbes (47). Ingested food passes the pharynx and is either stored in the crop, or sent to the midgut where the digestion starts (46). It is here that food absorption occurs. The midgut is further divided into the anterior

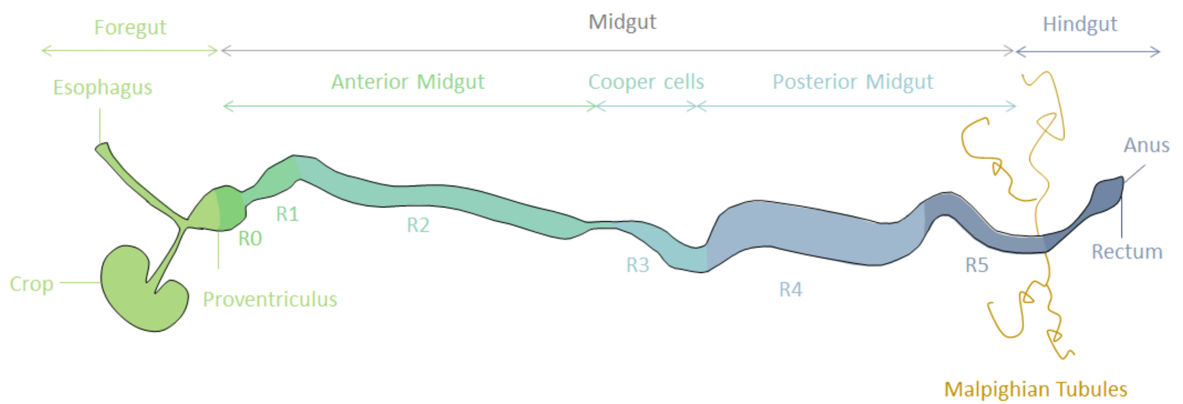

Figure 2: Structural compartmentalization of *Drosophila*'s gut

The gut of *Drosophila* is subdivided into three principle regions: the foregut (green), the midgut (light blue) and the hindgut (dark blue). The midgut is probably the most complex portion of the intestine and is further sub-divided into six distinct regions: R0, R1 and R2 being part of the anterior midgut; R3 comprises the copper cells region; R4 and R5 give rise to the posterior midgut.

midgut, the acidic copper cells region and the posterior midgut. A study distinguished six anatomically distinct compartments in the midgut (anteriorly to posteriorly named R0 to R5), that remain stable from young to old adult flies, associated with distinct metabolic and digestive functions (137) **(Figure 2)**. At the median of the midgut, the R3 region contains highly differentiated cells, the copper cells. These cells secrete H+ in exchange of Adenosine Triphosphate (ATP). As a result, this acidic region is considered the equivalent of the mammalian stomach, where the acidic PH (<4) provides the adequate conditions for protein denaturation and for the enzymatic activity of some proteases. In addition, this acidity constitutes a natural barrier that protects from pathogens (138).

The posterior part of the gut, the hindgut, is composed of pylorus, ileum and rectum **(Figure 2)**. In the hindgut, water and salt re absorption from food bolus takes place, as well as excretion of metabolized nutrients. In addition, structures associated to the gut and are equivalent to the salivary glands and kidneys have been characterized in *Drosophila*. Particularly, malphighian tubules which function as hemolymph filtering organs are attached to the gastro-intestinal tract at the hindgut region (139).

The epithelium of adult *Drosophila*'s midgut is constantly renewed throughout its lifespan. It is maintained by pluripotent intestinal stem cells (ISCs) that divide and self-renew, giving rise to two cells: a new ISC and a post-mitotic progenitor cell, called the enteroblast (140, 141) **(Figure 3)**. Enteroblasts are maintained transiently in the epithelium or differentiate into one of two cell types: large enterocytes (ECs) or small secretory enteroendocrine cells (EECs). ECs are large polyploid cells that secrete digestive enzymes and absorb nutrients. Their differentiation depends on a strong activation of the Notch pathway in enteroblasts (142) **(Figure 3)**. In contrast, EECs are small diploid cells that represent only 5% of the differentiated cellular population. These cells exhibit neural-like functions by regulating intestinal physiology (143). A lower induction of Notch pathway in enteroblasts mediates the EEC commitment (140) **(Figure 3)**. The differentiation of enteroblasts via Notch pathway-dependent processes is initiated through the local Notch Receptor ligand, Delta, by ISCs. Interestingly, signals independent of Notch pathway activity may account for EEC

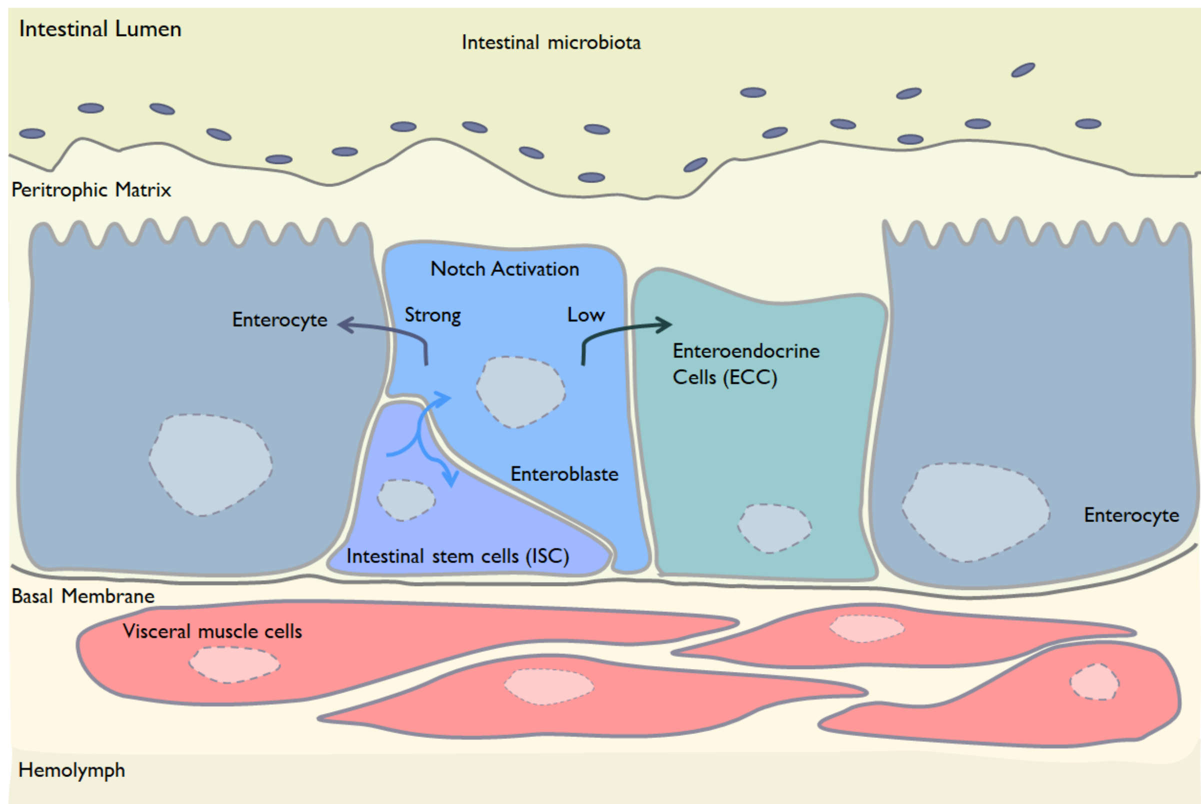

Figure 3: Cellular organization of *Drosophila*'s midgut

The gut of *Drosophila* is a tubular epithelium composed of a monolayer of cells surrounded by visceral muscles, nerves and trachea. A basal membrane separates the epithelium from the underlying muscle cells. At the apical side, intestinal cells are protected from the lumen by a semi-permeable membrane, the peritrophic matrix. The epithelium of adult *Drosophila*'s midgut is renewed constantly by pluripotent intestinal stem cells (ISCs) that divide and self-renew, giving rise to two cells: a new ISC and a post-mitotic progenitor cell, the enteroblast. The differentiation of enteroblasts depends on Notch pathway signaling: high induction of Notch signaling stimulates their differentiation to enterocytes whereas a lower induction of Notch pathway in enteroblasts mediates the entero-endocrine commitment.

differentiation, as the depletion of Notch or Delta in ISCs does not inhibit EECs commitment (140).

2.2- Natural and biochemical barriers of the gut

The gut is the major route for microorganisms, whether pathogens, members of the microbiota or dietary microbes. Consequently, gut epithelia are heavily shielded to resist microbial aggression by different layer of physical barriers. These include tight junctions that seal the single cell layer, an impermeable cuticle that covers the foregut and the hindgut, and the PM, a semi permeable grid-like structure of chitin polymers and proteins, that lines the midgut and limits the passage of luminal contents and microbes while allowing the passage of digestive enzymes (144). Layers of chitin fibrils and glycoproteins (notably peritrophins) are secreted by the proventriculus and are further compressed by muscular contraction of muscle cells to form two layers as they enter the midgut (145, 146). The PM constitutes the first protection layer of the gut from abrasive food particles, digestive enzymes, and infectious pathogens as the PM pores do not allow the penetration of components larger than 10 nm or proteins larger than 200 KDa (144, 146). The protective role of the PM is demonstrated by the study of *drosocrystallin* mutants. Drosocrystallin is a chitin-binding protein that is over-expressed during an oral infection. Indeed, Kuraishi and colleagues showed that *drosocrystallin* mutants exhibit a reduction in PM thickness, an increased susceptibility to infections by *Pseudomonas entomophila* and *S. marcescens* pathogens and a decreased lifespan (147). These data suggest that the PM is dynamically involved in intestinal host defense. Another physical barrier is provided by a mucus layer, composed of polysaccharides and proteins (mucins), which is located between the peritrophic matrix and the epithelium, but its functional relevance has not been investigated (134). In addition, epithelial integrity of the gut relies on the tight junctions that seal epithelial cells. This is in agreement with the phenotype of mutants for the *big-bang* gene that is required for the establishment of septate junctions in the apical side of epithelial cells. These mutants display an increased susceptibility to oral infections concomitant with a constitutive activation of the immune response and a shortened lifespan (148). This phenotype can be rescued by the clearance of gut microbiota by antibiotics treatment, indicating that intact intestinal cell junctions are required for immune tolerance of the gut microbiota.

In addition to physical barriers, the *Drosophila* is endowed with several antibacterial enzymes that together with the midgut acidity provide a biochemical protective shield of its epithelium. Indeed, catalytic members of the PGRPs as well as bactericidal enzymes, lysozymes, are produced in *Drosophila*'s midgut. The expression of catalytic PGRPs is part of the induced response and their function is described later in Section IV 2.1.2. Lysozymes are constitutively expressed catalytic enzymes that hydrolyze the β-1,4 glycosidic bonds linking glucidic monomers of PGN, leading to bacterial death by membrane instability (149, 150) **(Figure 7)**. The bactericidal activity of lysozymes can be also attributed to their positive charge rendering them highly attractive to negatively-charged bacterial membranes, causing membrane instability by the activation of autolysins (149, 150). Seven lysozymes encoding genes have been identified in *Drosophila* (151).

Like most animals, larvae and adults *Drosophila* guts harbor a community of gut bacteria and yeasts that is much simpler than that of vertebrates and which are mostly are cultivable (152). This community of yeasts and primarily lactic acid and acetic acid bacteria acquired from the environment is reflective of the fermentative substrates in which flies live and feed (153, 154). 16S rRNA sequencing-based studies on wild flies and laboratory stocks identified up to 30 bacterial species in the fly gut (155-160). The most common members of the *D. melanogaster* microbiome belong to the *Lactobacillus* and *Acetobacter/ Gluconobacter* genera, with *Lactobacillus plantarum*, *Lactobacillus brevis* and *Acetobacter pomorum* and *Acetobacter pasteurianus* as the most consistently associated species. Bacterial flora seems to be necessary for optimal larval growth as axenic cultures of *Drosophila* larvae showed elongated developmental times (23, 161). In addition, the lifespan of adult flies under axenic conditions was reduced, a phenotype that was restored upon reintroducing bacteria during the first week of adult life (162). Later, it was demonstrated that *Lactobacillus plantarum* is sufficient on its own to recapitulate the natural microbiota growth-promoting effect, by modulating the target of rapamycin (TOR)-dependent host nutrient sensing system controlling hormonal growth signaling (163). A second mono-association study showed that *A. pomorum* exhibits a positive influence on larval growth by inducing insulin signaling (164). Whether the natural microbiota of the adult gut provides any sort of protection against pathogenic infection remains an open question. However, the role of the microbiota in inducing a basal immune

response is largely attested. Remarkably, this induced response is marked by the expression of IMD negative regulators (165, 166). Importantly, the impact of the microbiota on gut immunity and intestinal tissue homeostasis translates into host fitness and lifespan modulation (167). Guo *et al.* have confirmed that ageing fly guts bear increased bacterial load that causes excessive proliferation and abnormal differentiation of the intestinal stem cells (ISCs); consequently, gut homeostasis is disrupted and life-span is reduced (168).

2.3- Active immune response of the gut

Beyond the production of amidases and lysozymes, a potent immune response is induced in the gut. This includes the production of Reactive Oxygen Species (ROS) and AMPs.

2.3.1- Local production of antimicrobial peptides (AMPs)

In contrast to the systemic immune response in *Drosophila* where AMP production is regulated by both NF-κB pathways Toll and IMD, the local expression of AMP encoding genes in the gut epithelium relies mostly on the IMD pathway (47, 169-171). Similarly to the compartmentalization of the gastro-intestinal tract, the localized immune response correlates with a clear regionalization of the expression of PGRPs receptors that recognize bacterial-derived PGN, and their negative regulators along the length of the gut (126, 165). Hence, PGRP-LE is the predominant intracellular receptor for monomeric PGN in the midgut. However, the membrane receptor PGRP-LC acts in the proventriculus and the hindgut, and concomitantly with PGRP-LE in the ventriculus for the detection of both monomeric and polymeric PGN (165, 172). The important role of this AMP mediated response is highlighted by the increased susceptibility of flies with an impaired AMP production in the gut to pathogenic infections by *Erwinia carotovora* Ecc15 (126, 173), *P. aeruginosa* PA14 (174) and *S. marcescens* (175). This phenotype was rescued by the over-expression of a single AMP encoding gene, *Diptericin* (170, 175, 176).

A tight balance in the gut maintains a homeostatic relationship between microbiota and the host immune response. Dominant bacteria in the *Drosophila* gut carry Diaminopimelic acid (DAP)-type PGN, the specific ligand of PGRP-LC and PGRP-LE. Hence the IMD pathway is constitutively activated at basal levels (165). A central role in bacterial tolerance of the gut has been attributed to negative regulators, some of which alter the initial steps of sensing and

signaling processes by targeting either the bacterial inducer or the host receptor **(Figure 9)**. The detailed mechanisms ensuring this regulation are described in section IV-2.3.3. Any dysregulation of the IMD pathway alters this balance. Indeed, Ryu and colleagues demonstrated that upregulation of AMP gene expression in the gut induces intestinal dysbiosis, marked by the overgrowth of *Gluconobacter morbifer*, which is normally only a minor component of the gut microbiota (159). An excessive activation of the IMD pathway in the gut also results in a shortening of *Drosophila*'s lifespan (177, 178). Notably, the up-regulation of NF-κB pathways upon intestinal infection is similarly observed in mammals (126). Like in *Drosophila*, an exacerbated mammalian NF-κB response in the gut is pathologic and correlates with Inflammatory Bowel Diseases (IBD) (179).

2.3.2- Production of reactive oxygen species (ROS)

In addition to the production of AMPs, a balanced redox system is essential for the host defense and maintenance of gut homeostasis in *Drosophila*. In eukaryotic immune system, the production of ROS is a key response as it targets all types of microorganisms (180). In *Drosophila,* natural infections with bacteria also induce rapid ROS synthesis in the gut, and the dynamic cycle of ROS generation and elimination appears to be vital. Over the last ten years, extensive research focused on ROS production by enterocytes in *Drosophila*'s gut as a crucial defense mechanism. Oral infection of adult flies is associated with the rapid production of ROS generated by the enzyme dual oxidase (DUOX), a member of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family, localized at the apical side of the intestinal epithelium and preferentially expressed in the foregut and hindgut (181). The bactericidal activity of ROS is not targeted to specific microbial structures; they damage proteins, lipids and nucleic acids (180, 182). Intestinal bacterial infections stimulate the expression of Duox and consequently ROS production (183). The vital role of ROS in fighting an infection is confirmed in *Duox* RNAi flies that rapidly succumb to an oral infection by the Gram-negative bacteria *E. carotovora*, and this lethality is associated with an inability to control bacterial growth (181). However, an oxidative burst can be deleterious to host tissues, inducing enterocytes cell death in the presence of *P. entomophila* infection (127). Recently, a study demonstrated that *Lactobacillus* bacteria, the major component of intestinal microbiota, induce the endogenous production of ROS that is essential for the proliferation of ISC. This ROS production, absent in

axenic flies, does not requires DUOX, but is dependent on another enzyme belonging to family of NADPH oxidase, Nox1 (NADPH oxidase 1) (184). Indeed, mono-association of axenic flies' guts by *L. plantarum* induces ROS production in the absence of DUOX, but not Nox1. These observations suggest that the mechanisms leading to the production of ROS discriminate between commensal bacteria and infectious pathogens, mediating thus microbiota tolerance (184). It was shown that pathogen derived uracil modulates Drosophila DUOX-dependent immunity whereas *L. plantarum*-derived lactic acid is the main trigger for the activation of Nox in the gut (185, 186).

Both the expression and activity of DUOX are enhanced upon infection. The DUOX enzyme is calcium dependent and is regulated by the Gαq phospholipase Cβ (PLC-β) (187). Its activity is induced by the sensing of uracil, abundantly produced by pathogenic bacteria, by an unknown receptor (188). PLC-β hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2) to inositol 1,4,5-triphosphate (IP3), which mobilizes Ca2+ from the endoplasmic reticulum (RE), required for DUOX activity. DUOX expression is regulated through the uracil-dependent activation of the mitogen activated protein (MAP) kinase pathway, including MEKK1, MKK3, p38 kinases and the Activating Transcription Factor (ATF2). Two microbial recognition pathways, the Gαq-PLC-β pathway and an atypical NF-κB-independent IMD pathway would lead to its activation, although precise mechanisms of such activation are unclear (183, 187) **(Figure 4)**.

Excessive ROS production, which is deleterious to the host, is prevented in *Drosophila* by immune responsive catalase (IRC) which is constitutively expressed. Silencing of *IRC* by RNAi results in higher ROS production and fly lethality, indicating that IRC provides an antioxidant defense system in *Drosophila* (182) **(Figure 4)**. Under normal conditions, expression of DUOX is inhibited by calcineurin B (Can B) which activates MKP3, an inhibitor of the MAP kinase pathway (182).

3- Intrinsic antiviral response

Like all living organisms, insects are continually exposed to viruses and have developed efficient defense mechanisms. Over the last 12 years, a number of groups have started to investigate the genetic basis of antiviral resistance in *Drosophila*. It is now well established that

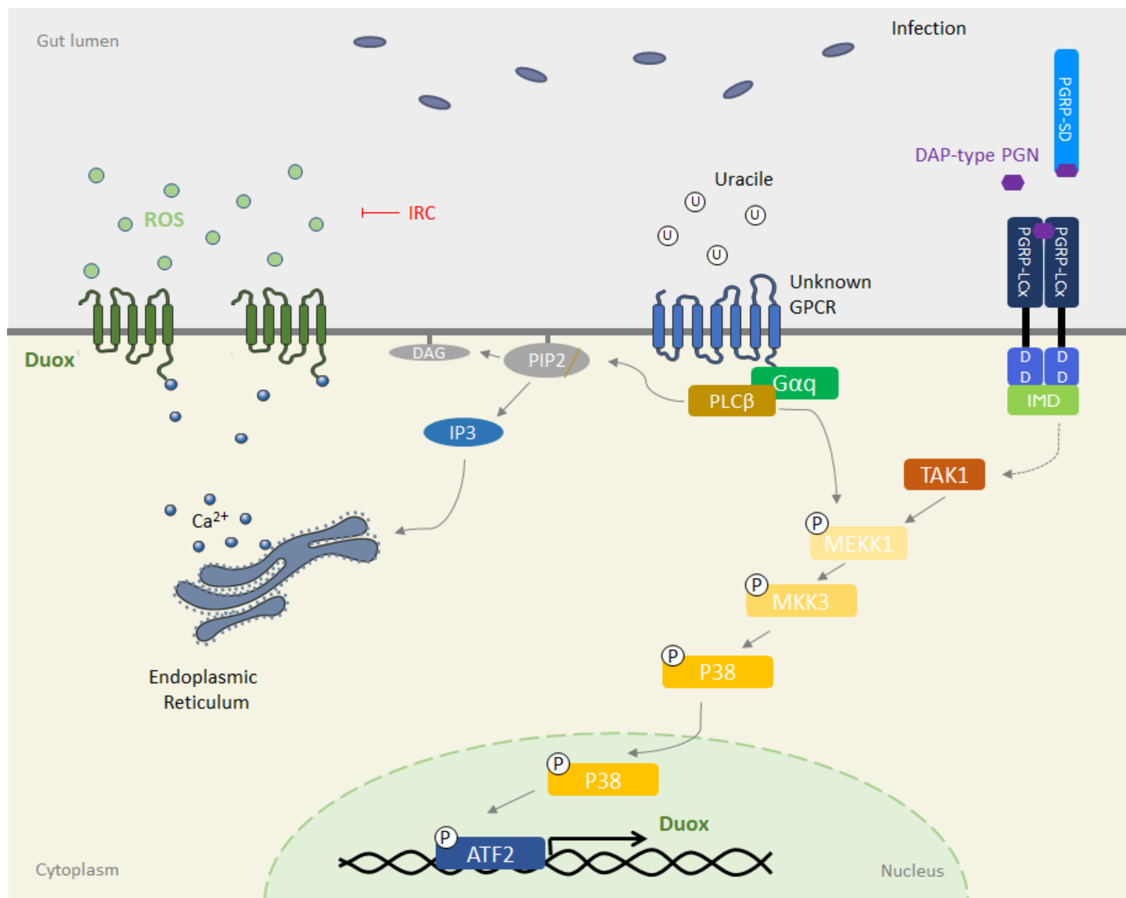

Figure 4: ROS production through Duox activation and expression in the gut of *Drosophila melanogaster*

Upon an oral ingestion of microbes, the production of ROS in the gut is submitted to two levels of regulation: the induced expression of DUOX encoding gene and the regulation of its catalytic activity. Both of the mechanisms are induced upon the detection of uracile, produced by pathogenic bacteria. This detection is mediated by an unknown G-protein coupled receptor. The subsequent activation of the phosphplopase C-β (PLC-β) leads to the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) to inositol 1,4,5-triphosphate (IP3). IP3 causes the release of calcium (Ca^{2+}) from the endoplasmic reticulum, essential for the catalytic activity of DUOX. The expression of DUOX is dependent on the p38 MAP kinase pathway, activated by the detection of uracile. The pathway implicates the kinases MEKK1, MKK3, p38 and the transcription factor ATF2 (activating transcription factor 2). The expression of DUOX also integrates signals emanating from the receptor PGRP-LC upon the detection of PGN. The activity of DUOX is negatively regulated by antioxidant system implicating the activity of the catalase IRC (Immune regulatory catalase).

the cell intrinsic mechanism of RNA interference (RNAi) plays a central role in the control of viral infections in flies, as it does in plants and other invertebrates. Unlike in mammals, in which the anti-viral immunity is deeply dependent of the systemic release of Interferon cytokines, the intrinsic RNAi mechanism helps *Drosophila* fight the molecular steps of virus replication within host cells. This intrinsic defense, required against all types of viral infections in flies, relies mostly on the short-interfering RNA (siRNA) pathway (189).

In insect cells, the siRNA pathway is triggered by the recognition of cytosolic dsRNAs which is an uncommon cellular component under normal physiological conditions. Upon a viral infection and incorporation of the virus inside host cells, dsRNA fragments may arise from viral genome of dsRNA viruses (e.g., *Drosophila* X virus), viral replication intermediates of single-stranded RNA (ssRNA) viruses or from endogenous transposons. The detection of viral dsRNAs and its processing are mediated by the Ribonuclease (RNAse) III enzyme Dicer-2 (Dcr-2) into 21 nucleotides long siRNA duplexes bearing 5' monophosphates and 2nt 3' hydroxyl overhangs (190, 191). These are loaded onto the Argonaut-2 (AGO2) protein to form a pre-RISC complex, with the help of R2D2, a dsRNA binding co-factor of Dicer-2 (191, 192). One strand of the duplex, the passenger strand, is then ejected, leading to the formation of a mature RISC complex. The remaining strand, known as the guide strand, is stabilized by 2'-O-methylation of the 3' nucleotide by the protein Hen1, and then targets the RNAs containing complementary sequence, which will be cleaved by the AGO2 slicer activity, inhibiting the formation of new viruses. Several reports have underlined the importance of this mechanism in the *Drosophila* antiviral response. Mutations in *Ago-2* or *Dicer 2*, which affect the RNAi pathway, increased the susceptibility of flies to a large number of RNA viruses including *Drosophila* C Virus, Flock House virus, Sinbis, and *Drosophila* X virus (193, 194).

IV- Immune signaling pathways in *Drosophila*

In this section I will describe the molecular immune pathways that modulate both local and systemic immune responses after the detection of an infection. As previously mentioned, these include the NF-κB dependent pathways, IMD and Toll, and two NF-κB independent pathways, the JNK and the JAK/STAT pathways. Since my PhD work is mainly focused on NF-κB pathways, I will start with a brief summary of the JNK and JAK/STAT pathways and I will

then detail our current knowledge of the sensing and signaling mechanisms underlying Toll and IMD immune responses in *Drosophila.*

1- The NF-κB independent immune pathways in *Drosophila*

1.1- The JNK pathway

Jun N-terminal kinase (JNK) pathway is an evolutionary conserved eukaryotic signaling pathway, from yeast to mammals, that has been the focus of studies over the last 15 years. This pathway constitutes one of the three *Drosophila* MAP Kinase signaling pathways, also including the extracellular regulated kinase (ERK) and the p38 pathways (195). The JNK pathway plays a fundamental role in developmental processes such as embryonic dorsal closure (196) and cell elongation (197). Indeed, null mutations in JNK signaling components are typically embryonic lethal in flies (196, 197). Besides, it has been linked to cell migration, apoptosis, and immune responses in both insects and mammals (198-201). In addition, this pathway is one of the most crucial responses induced by stress in adult animals and can be activated by numerous stimuli such as UV irradiation, reactive oxygen species, DNA damage, heat, infections and inflammation (202).

These stress stimuli selectively activate a member of the JNK Kinase Kinase (JNKKK) family, which then phosphorylates and activates a dual-specificity Kinase of the JNK Kinase (JNKK) family that phosphorylates JNK on Serine/Threonine and Tyrosine residues. In *Drosophila*, Several known JNKKK are known including the Mixed Lineage Kinase 2 (MLK2, also named Slipper) (203), the Apoptotic Signal-regulating Kinase 1 (ASK1) and MEK Kinase 1 (MEKK1) activated in response to Sodium arsenite and Cadmium toxic metals exposure (204) **(Figure 5)**. Moreover, two JNKK Kinases have been identified that act downstream of JNKKK, Hemipterous (Hep) (205), and dMKK4 (206), involved especially during immune responses (207). JNKK finally phosphorylates the JNK factor, Basket (208). Basket itself has a number of nuclear and cytoplasmic targets, most prominently transcription factors, including the AP-1 family members Jun and Fos and the Forkhead Box O transcription factor (FOXO) (209, 210) **(Figure 5)**. Additionally, Silverman and colleagues have shown that the JNK pathway is triggered in response to bacteria (211) **(Figure 5)**. The proposed model states that IMD

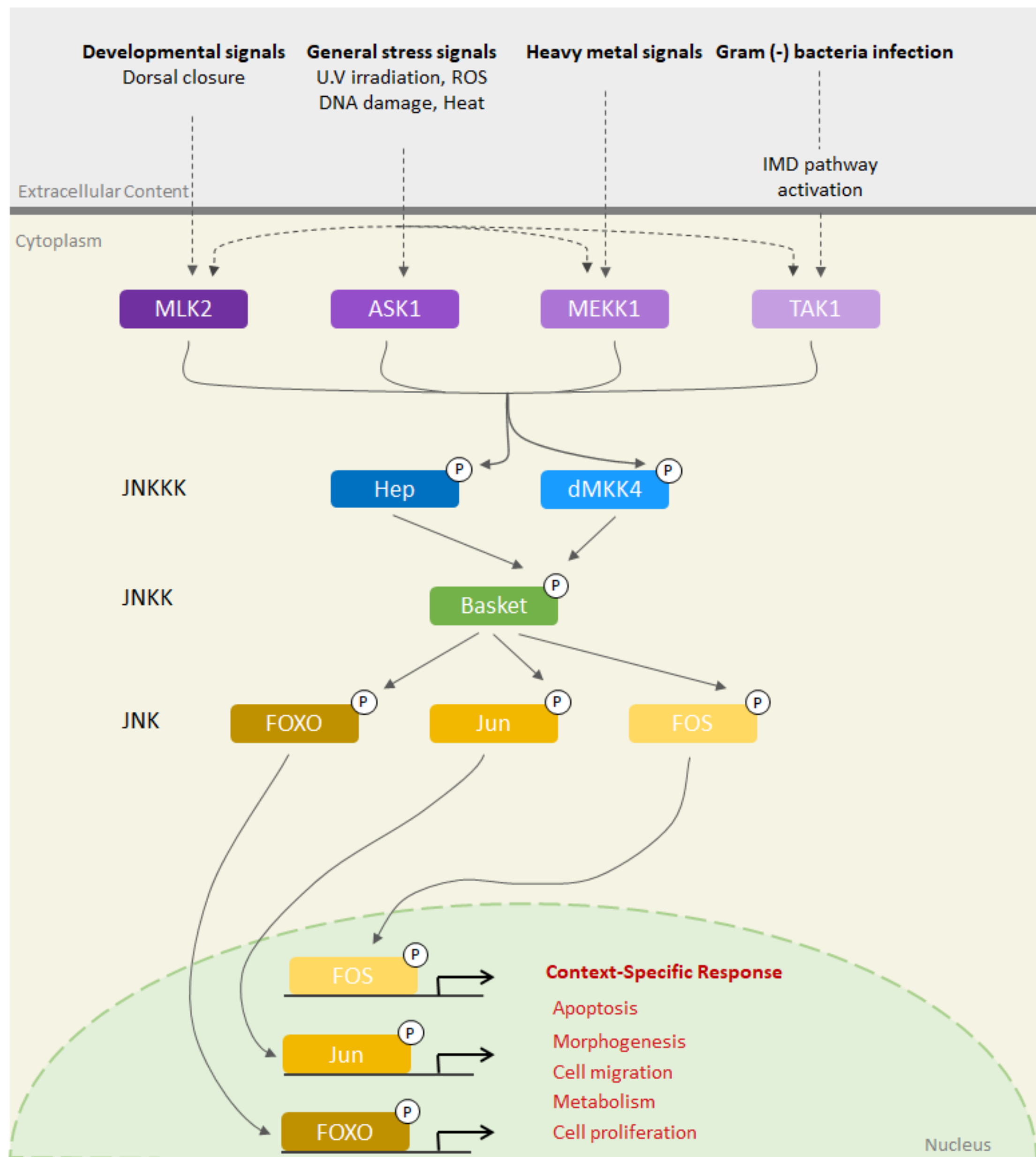

Figure 5: The JNK pathway in *Drosophila*

The JNK pathway is activated by variable signals including developmental processes, stress signals, heavy metal exposure and bacterial infections. These signals lead to the activation of a kinase cascade that finally activates the JNK basket, mediating the phosphorylation of transcription factors Activating Protein 1 (AP-1), Fos and Jun, and Foxo. Once in the nucleus, these three transcription factors induce the transcription of genes mediating distinct events such as apoptosis, morphogenesis, cell migration, metabolism and proliferation (see chapter I, section IV-1.1).

signaling bifurcates downstream of the Transforming growth factor beta (TGF-β)-activated kinase 1 (TAK1) (211).

Once in the nucleus, AP-1 and FOXO transcription factors induce the expression of numerous target genes mediating different responses ranging from morphogenesis, cell migration, metabolism, cell proliferation and apoptosis. Notably, some JNK-dependent immune genes encode many proteins involved in cytoskeleton remodeling, in keeping with a role in hemocyte activation (200). Additionally, a role for the JNK pathway in AMP genes expression by the fat body has been proposed (212).

1.2- The JAK/STAT pathway

The importance of Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling in the regulation of mammalian immunity has been recognized for decades. JAK/STAT signaling has been linked to several aspects of the innate immune system, including the control of inflammatory responses and wound repair, as well as the activation of neutrophils and macrophages (213). This pathway is highly conserved in evolution and was shown to control several biological processes in both *Drosophila* embryos and adults (214). These include embryonic patterning (215), formation of the wing and eye (216, 217) and maintenance of stem cells in their niches (218, 219). This pathway also directly mediates the immune and stress responses by activating infection-induced genes (200, 220), particularly the response to viral infection (221).

In *Drosophila*, the known JAK/STAT pathway ligands consist of only three cytokine-like proteins called unpaired (upd) (222) upd2 (223) and upd3 (224). Unpaired ligands seem to be specific to *Drosophila* but share some homology with leptins, a family of hormones regulating fat storage in mammals (222). All three upd molecules are induced locally in response to tissue damage such as wounding; upd3 expression is induced in adult hemocytes upon bacterial challenge, and both upd2 and upd3 are induced in response to viral infections (189, 224) **(Figure 6)**. The upd molecules bind and signal via a single receptor, Domeless (Dome) a transmembrane receptor sharing functional and sequence similarities with the Interleukin-6 Receptor (IL-6R) (225) **(Figure 6)**. This binding activates the *Drosophila* Janus Kinase (JAK) Hopscotch (226), and the *Drosophila* STAT transcription factor, Stat92E (216). Activated JAKs

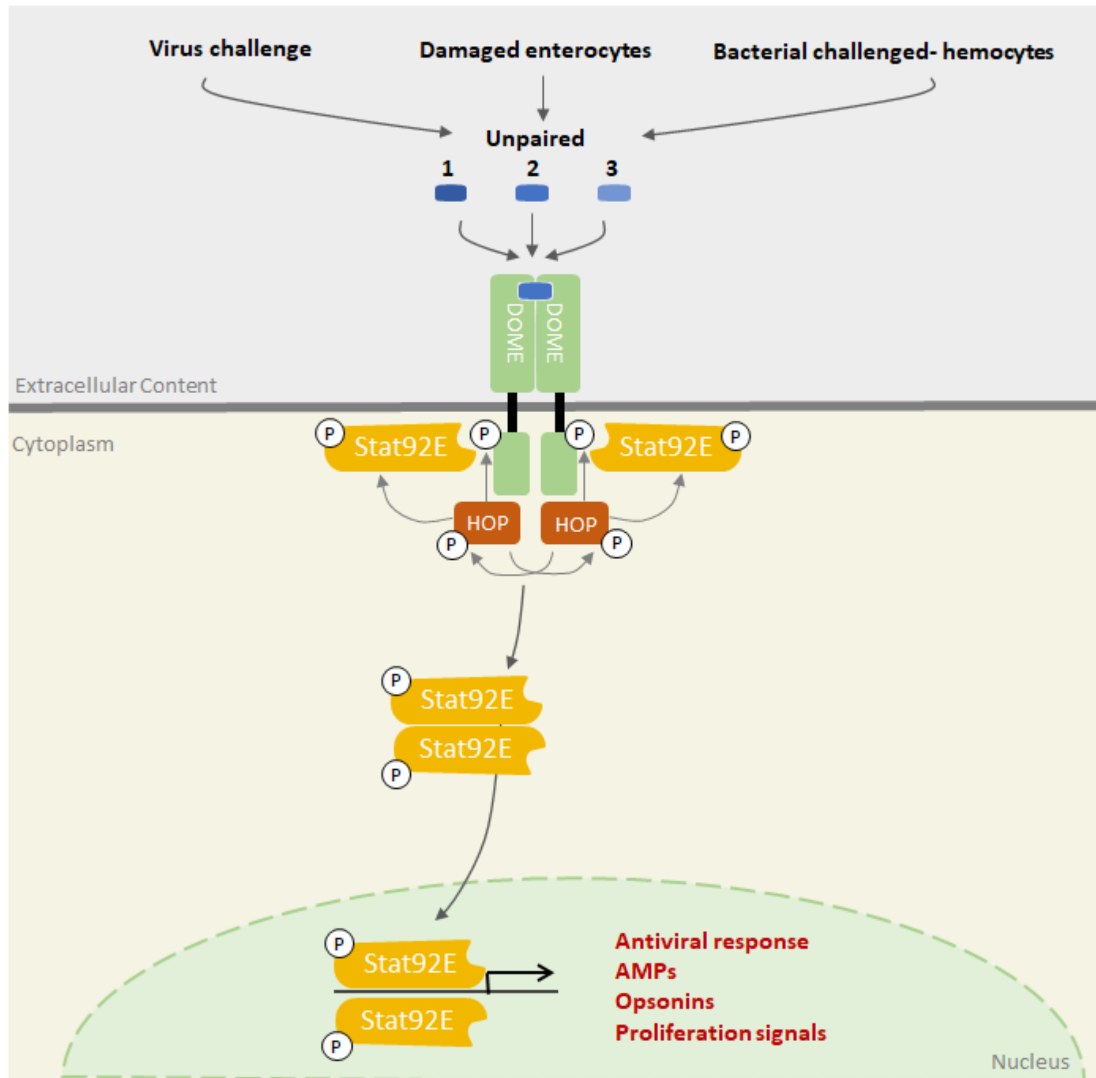

Figure 6: The JAK/STAT pathway in *Drosophila*

The JAK/STAT pathway is activated upon the binding of unpaired cytokines (Upd1, 2 and 3) to the receptor domeless. Unpaired molecules are produced in response to stress signals, derived from viral infections, damaged enterocytes or bacterial challenged hemocytes. Upon Upd binding, Domeless receptors dimerize which activates the *Drosophila* Janus Kinase (JAK) Hopscotch and the STAT transcription factor, Stat92E. Phosphorylated Stat92E translocates to the nucleus and activates the expression of target genes including antiviral effectors, anti-microbial peptides (AMPs), opsonins and cell-proliferation signals (see chapter I, section IV-1.2).

phosphorylate each other, specific tyrosine residues on the cytoplasmic part of the receptor and the STATs transcription factors, which subsequently dimerize and translocate into the nucleus, where they bind the promoters of their target genes **(Figure 6)**.

The JAK/STAT pathway is responsible for the expression of several immune-related proteins, including cytokines and stress response proteins in the fat body. This is induced by the JAK/STAT pathway ligand upd3, which is produced by the hemocytes in response to septic injury or other stress stimuli (227). One example of such stress-induced genes is the Turandot (Tot) family, whose expression is dependent on both the JAK/STAT and IMD pathways and is found in large quantities in the hemolymph (228). Another example of *Drosophila* immune responsive genes that are regulated by this pathway is the complement-like TEP2 proteins, opsonization molecules involved in phagocytosis (220). In addition, *Drosophila* JAK/STAT signaling controls hemocyte proliferation and the maintenance and differentiation of intestinal stem cells in the gut which is essential for its regeneration and homeostasis (131).

Besides, this pathway has been shown to play a crucial role in *Drosophila* viral response. Indeed, its target genes, such as TotM, as well as upd2 and upd3, are induced by multiple viruses, including Flock House virus (FHV), Vesicular stomatitis virus (VSV) and *Drosophila* X virus (189). Deficiencies in the pathway activation result in increased DCV viral loads and higher mortality of flies (221). Even though the mechanism of viral detection remains unknown, an indirect activation mechanism has been suggested for the JAK/STAT pathway, where stress signals sent by infected and damaged cells would be recognized by surrounding uninfected cells.

2- The NF-κB pathways: sensing and signaling

In the early nineties, it became apparent that NF-κB factors play a role in the antimicrobial host defense of *Drosophila* by modulating the expression of AMP encoding genes (44, 229, 230). The *Drosophila* genome codes for three NF-κB family members. Dorsal and DIF (Dorsal-related Immunity Factor) are 70 kDa proteins, with a typical Rel homology domain, which is 45% identical to that of the mammalian counterparts c-Rel, Rel A, and Rel B (231). Both Dorsal and DIF are retained in the cytoplasm by binding to the same 54-kDa inhibitor protein Cactus, which is homologous to mammalian I-κBs (232). Relish, is a 100-kDa protein with an amino-

terminal Rel domain and a carboxy-terminal extension with typical ankyrin repeats, as found in Cactus and mammalian I-κBs (43). Relish is similar to mammalian p100 and p105 (231). In adult flies, DIF is mainly activated in response to fungal and Gram-positive bacterial infection via the Toll pathway, whereas Relish is preferentially activated upon IMD pathway activation by Gram-negative bacteria infection (6). Both pathways play a fundamental role in the defense against invasive microbes by triggering the massive release of AMPs. So far, the functions of these pathways have been mostly characterized in three main immune tissues: i) the fat-body, the main inducer of *Drosophila* systemic immune responses ii) the hemocytes (described above), and iii) the digestive tract (described above). By the end of the 1990s the *D. melanogaster* proteins that sense invading microbes were functionally characterized. Remarkably, these recognition proteins seem to be derived from phylogenetically ancient amidases and glucanases. Consequently, the main microbial inducers that have been found to date are various forms of PGN and glucans (233, 234). A general picture of this immune inducible response covering the microbial triggers, the host receptors as well as the activated signaling events are presented in the following section.

2.1. Microbial detection

2.1.1- Structure of microbial molecular patterns

In *Drosophila*, recognition of bacteria is achieved through the sensing of specific forms of PGN, by specific PPRs, PGRPs. PGN is a polymer of sugar and amino acids that is restricted to the cell wall of both Gram-negative and Gram-positive bacteria. PGN plays essential functions in protecting bacteria from osmotic pressure and maintaining the rigidity and integrity of bacterial cells. It consists of long glycan chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues that are cross-linked to each other by short peptide bridges **(Figure 7).** PGN is a highly complex molecule found in all bacteria but can present differences from one bacterium to another. PGN from Gram-negative bacteria differs from most Gram-positive PGN by the replacement of lysine (lys) with *meso*-diaminopimelic acid (DAP) at the third position in the peptide chain **(Figure 7).** However, a subclass of Gram-positive bacteria including *Bacillus* species produce DAP-type PGN (6, 233). Another major difference between Gram-negative and Gram-positive PGN is its localization within the cell wall. Gram-negative PGN consists of a single layer and is hidden in the periplasmic space under the outer

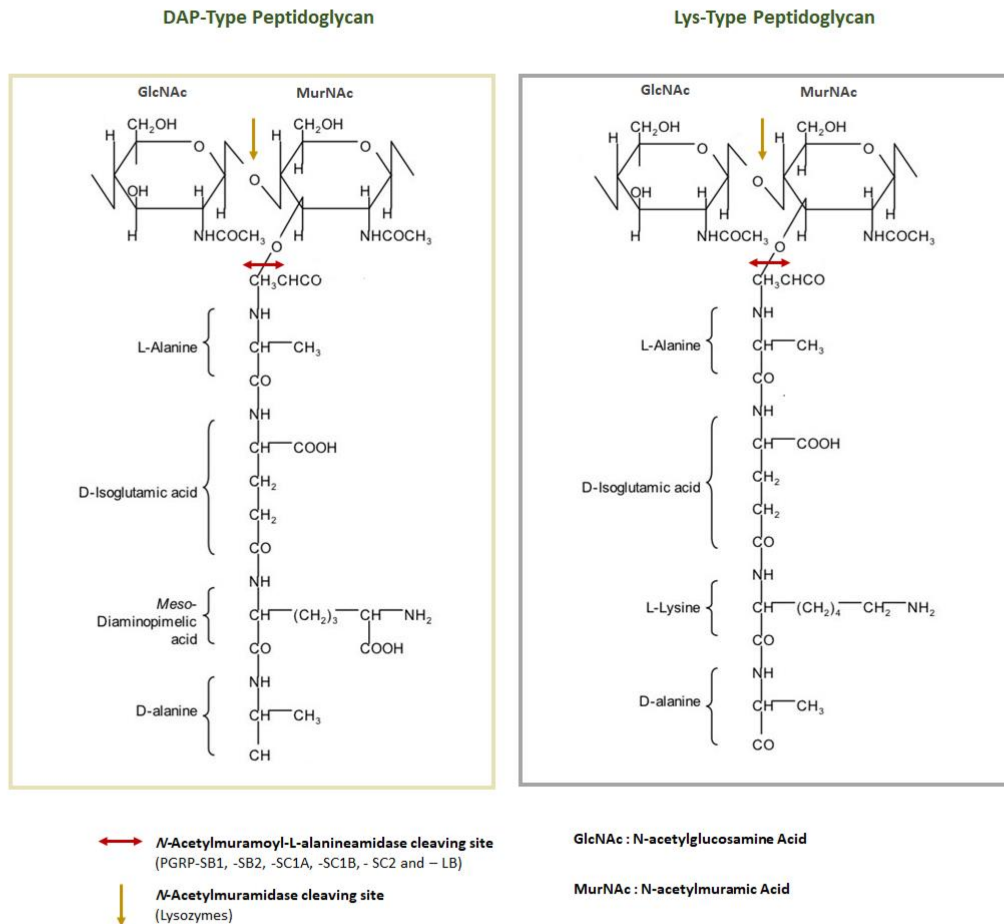

Figure 7: Structure of bacterial DAP- and Lys–type Peptidoglycan

PGN is a complex heteropolymer consisting of long glycan chains of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues, connected by short tetrapeptide bridges. PGN from Gram-negative bacteria differs from most Gram-positive PGN by the replacement of lysine (lys) with *meso*-diaminopimelic acid (DAP) at the third position in the peptide chain. Lysosymes with a N-acetylmuramidase activity catalyze the cleavage of the glycosidic bond between the MurNAc and GlcNAc (yellow arrow), while amidase PGRPs (PGRP-SB1. –SB2, -SC1A, -SC1B, -SC2 and –LB) (red arrow) remove the peptidic bridge from the sugar backbone with a N-acetylmuramoyl-L-alanineamidase activity (Adapted from Humann and Lenz 2009).

Humann, J., and Lenz, L.L. (2009). Bacterial peptidoglycan degrading enzymes and their impact on host muropeptide detection. J Innate Immun 1, 88-97.

membrane and lipopolysaccharide (LPS) layer, whereas PGN from Gram-positive bacteria is multilayered and exposed at the bacterial surface (22). The PGN is a dynamic structure constantly renewed during bacterial growth and proliferation. Polymeric PGN as well as Gram-negative monomeric PGN fragments, called tracheal cytotoxin (TCT), are able to induce an immune response in the host (22, 235). Furthermore, fungal-derived β-(1,3)-glucan, a major component of the fungal cell wall, is detected by GNBP.

2.1.2- General features of recognition receptors

PGRPs are highly conserved from insects to mammals, sharing a characteristic 160 amino acid PGRP domain. The latter exhibits similarities with the bacteriophage T7 lysozyme, a zinc-dependent N-acetylmuramoyl-l-alanine amidase, some members have retained this enzymatic activity and are referred to as catalytic PGRPs. By contrast, other PGRPs have lost crucial residues that are essential for catalysis, thus serving recognition proteins (236-238). Recognition PGRPs can distinguish between the lys-type and the DAP-type PGN of Gram-positive and negative bacteria, respectively. This feature allows the discrimination between two large groups of pathogens and to subsequently trigger distinct signaling cascades and gene expression programs. The genome of *Drosophila* harbors 13 genes encoding at least 17 independent PGRPs isoforms through alternative splicing. They are classified into small-sized (182 to 203 amino-acids), PGRP-S and long-sized (215 to 520 amino-acids) PGRP-L receptors (238). Among the PGRP-S group are seven proteins secreted in the hymolymph (PGRP-SA, PGRP-SB1 and SB2, PGRP-SC1A, SC1B and SC2 and PGRP-SD). The PGRP-L class comprises seven proteins with a transmembrane domain (PGRP-LAa, PGRP-Lab, PGRP-LCa, LCx and LCy, PGRP-LD and PGRP-LF) and three other proteins lacking this domain and are thus intracellular or extracellular (PGRP-LAc, PGRP-LB and PGRP-LE) (238).

The PGRPs members that have retained the amidase activity are PGRP-SB1, -SB2, -SC1A, -SC1B, - SC2 and –LB. These catalytic enzymes hydrolyze the bond between the glucidic chain and the stem peptide **(Figure 7)**. This feature confers to catalytic PGRPs the capacity to act as negative regulators of the immune response, as they degrade the PGN into non-stimulatory fragments (168, 177, 239, 240). In contrast non-catalytic PGRPs serve as PRRs for the

detection and activation of immune signaling cascades (PGRP-SA, -SD, LA, LC, LE), or down-regulate the immune response via distinct mechanisms (PGRP-LE and –LF) (241-246).

On a different note, GNBPs that detect fungal infections display a significant overall homology to bacterial glucanases (247). They contain an N-terminal domain that binds to β(1,3)-glucan and a C-terminal domain that is homologous to the catalytic domain of β-glucanase. However, the absence of conserved key residues in the catalytic site renders the glucanase domain non-functional (248). The *Drosophila* genome contains three consensus members of the GNBP family (GNBP 1,2 and 3). Two among these are known to be required in the immune response, GNBP 1 and 3 (249). GNBPs are present in most invertebrates but have not been found in vertebrates (6).

2.2. The Toll pathway

The Toll pathway, the first characterized NF-κB pathway in *Drosophila*, is an evolutionarily conserved signaling cascade that plays a key role in the establishment of the dorso-ventral axis of the *Drosophila* embryo, as well as in several other developmental processes (250). In embryos, the Toll pathway regulates the activation of Dorsal for the set-up of the dorso-ventral axis whereas in adult immune-depndent Toll response is dependent on the activation of DIF (251, 252). The crucial role of Toll signaling in D*rosophila*'s immune response against Gram-positive and fungal infections was discovered years after its initial characterization as a developmental regulator. Lemaitre and colleagues discovered that flies deficient in Toll signaling succumb more rapidly to Gram-positive and fungal infections (12). Importantly, this discovery has strongly accelerated the characterization of Toll-like Receptors (TLR), one of the most potent families of pattern-recognition receptors in mammals. Toll signaling has also some parallels to the mammalian signaling cascades downstream of the interleukin 1 receptor (IL-1R) (6, 22).

2.2.1- Toll pathway activation

Unlike mammalian TLRs, the Toll receptor in *D. melanogaster* is not activated by interacting directly with microbial ligands thus does not function as a PRR. The initiating event for Toll signaling is the cleavage of the cytokine-like, Spätzle (spz), and the binding of its C-terminal fragment to the leucine-rich repeats (LRR) of the Toll receptor (253). As previously

mentioned, The Toll pathway is triggered upon Gram-positive bacterial and fungal infections, thus two MAMPs have been identified as ligands for the PRRs acting upstream of spz. This mechanism of sensing defines the MAMPs pathway upstream of Toll. Indeed, the Toll pathway was also shown to be activated upon the sensing of microbial virulence factors, in secreted fungal and bacterial proteases. These define the so-called Danger pathway for Toll activation as described in the following **(Figure 8)** (234, 254, 255).

2.2.1.1- The PRR recognition pathway

The PRRs acting upstream of the Toll receptor belong to the PGRP and GNBP families. Namely, PGRP-SA and GNBP1 are involved in the sensing of Lys-type PGN from Gram-positive bacteria (246, 256), while circulating GNBP3 binds specifically to fungal β-1,3-glucans (234) **(Figure 8)**. It has also been proposed that PGRP-SD recognizes Lys-PGN from Gram-positive bacteria (241). More recently, this model has been confronted by a study indicating the role of PGRP-SD in the detection of DAP-PGN. Interestingly, a structural study suggests that PGRP-SD can also recognize DAP-PGN, implying that the Toll pathway can also be activated by Gram-negative bacteria through its PRR recognition pathway (257). In contradiction with these two studies, Iatsenko et al. Showed that PGRP-SD acts upstream of PGRP-LC as a co-receptor and recognizes DAP-type PGN (258).

GNBP1 binds to a more restricted range of Lys-type PGN than does PGRP-SA (248), and functions together with PGRP-SA in sensing some Gram positive bacterial strains (256). One proposed model is that GNBP1 cleaves PGN using its glucanase activity into shorter dimeric or tetrameric muropeptides that bind to PGRP-SA (248). Indeed, GNBP1-digested Lys-type PGN can induce the Toll pathway in a GNBP1- independent, but PGRP-SA-dependent manner (248). A contradictory study showed that GNBP1 did not have such enzymatic activity but instead acted as a linker between PGRP-SA and the downstream signaling component ModSP (Modular Serine Protease) (259). Alternatively, PGRP-SD was also shown to recognize Lys-type PGN from Gram-positive bacteria (241). However, a structural study suggested that PGRP-SD can bind to DAP-type PGN, implying that Toll pathway may also be able to recognize Gram-negative bacteria through its PRR recognition pathway (257). In contrast, a recent study demonstrated that PGRP-SD acts upstream of PGRP-LC as an extracellular co-receptor. This

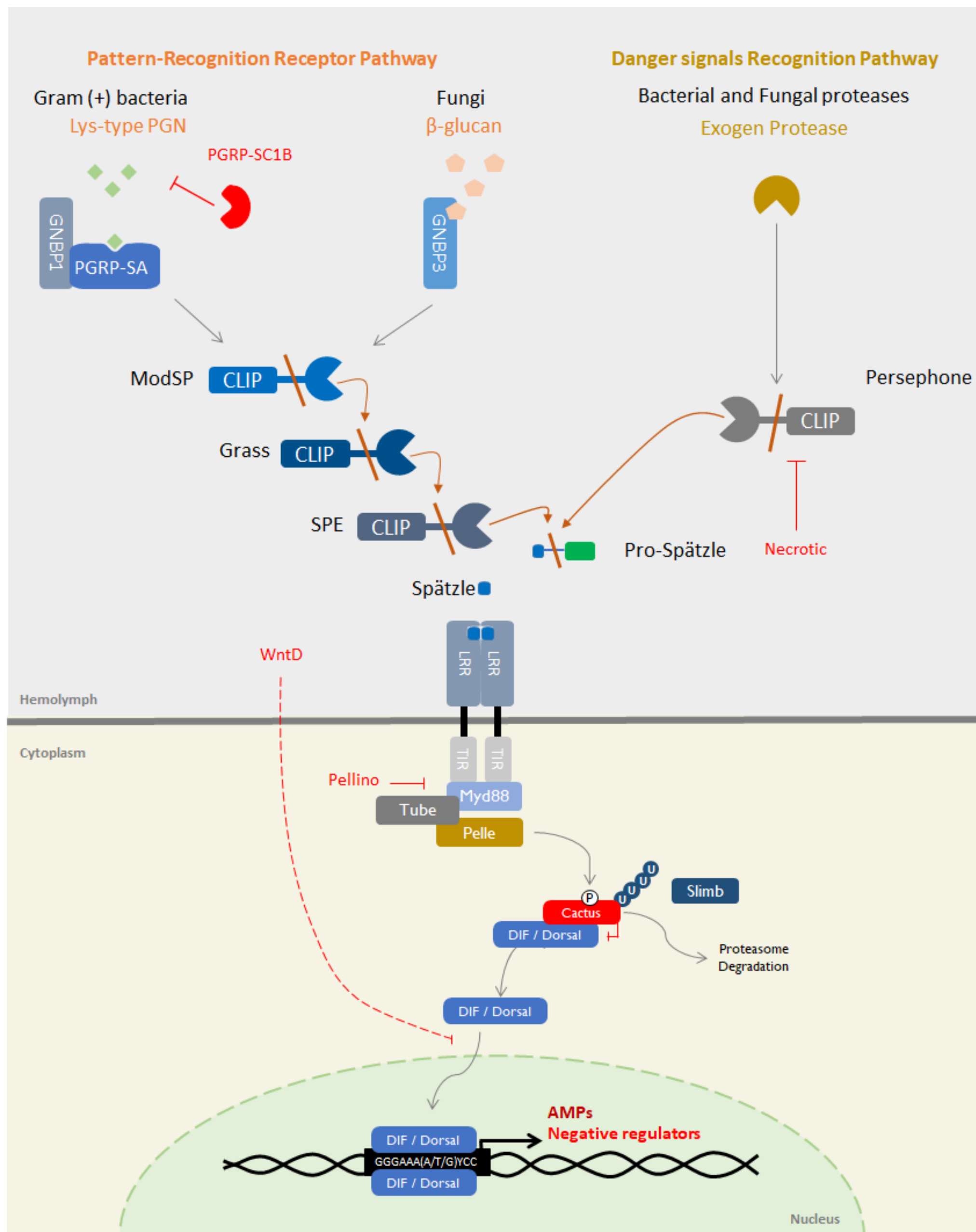

Figure 8: Summary of Toll signaling pathway

The Toll receptor is activated upon binding with a cleaved form of Spätzle that is processed via two activation pathways: The Pattern recognition receptor pathway (PRR) and the Danger signals recognition pathway. The first one is activated by secreted recognition molecules (PGRP-SA, PGRP-SD, GNBP1, GNBP3) via two recognition modules that involve the sensing of Lys-type PGN from Gram-positive bacteria by PGRP-SA, PGRP-SD and GNBP1, and fungi-derived β-Glucan by GNBP3. PGRP-SD can also sense DAP-type PGN of Gram-negative bacteria. These recognitions lead to the activation of a CLIP-domain serine protease cascade leading to the activation of Spätzle processing enzyme (SPE) that cleaves Spätzle. Alternatively, in the Danger signal recognition pathway, SPE can be activated by Persephone, another CLIP-domain serine protease that is activated in the presence of protease from Gram-positive bacteria and fungi. Mature Spätzle binds as a dimer to Toll, thereby inducing its dimerization at the plasma membrane. The intracellular signaling leads to the nuclear translocation of Dif/Dorsal transcription factors that activate the expression of AMPs and Toll pathway negative regulators (see chapter I-section IV-2.2)

study suggested that PGRP-SD recognizes DAP-type PGN and facilitates the induction of the IMD pathway by presenting it to the PGRP-LC receptor (258).

Binding of recognition proteins to either class of PAMP triggers activation of the Modular Serine Protease (ModSP) (259). The latter in turn activates another serine protease, Grass (Gram positive Specific Serine Protease) (254, 260). The serine protease cascade continues with activation of Spätzle processing enzyme (SPE), which cleaves Spz, generating a functional Toll ligand (261) **(Figure 8)**. RNAi-based experiments indicate that the pathway linking Grass to SPE likely involves additional serine protease family members, including Spirit (serine protease immune response integrator), Spheroide, and Sphinx (the Sphinx1 and Sphinx2 proteins) (260). All the mentioned Serine proteases are characterized by a CLIP domain found only in insects, and exist in the hymolymph as zymogen precursors, activated in cascade to generate mature enzymes, similarly to the complement system in vertebrate's immunity.

2.2.1.2- The danger recognition pathway

MAMP-based recognition is complemented by the sensing of bacteria and fungi through the activation of the Serine-Protease, Persephone (Psh) (113, 234, 254, 255). The latter senses danger signals such as secreted proteases of fungi and bacteria. Psh is produced as an inactive zymogen that requires activation by exogenous protease cleavage to give a catalytically active Serine protease **(Figure 8)**. Proteases known to proteolytically trigger the maturation of Psh are the secreted fungal virulence factor PR1 derived from *Beauveria bassiana* and *Metarhizium anisopliae* (234) and Gram-positive bacterial virulence factors (254). Microbes use such proteases to degrade adherence junctions, enabling penetration of the epithelial barrier and the external cuticule. Some Gram-negative bacteria, including those belonging to the genera *Pseudomonas* and *Serratia*, secrete proteolytic enzymes, activating thus the Toll pathway (262). Issa and colleagues showed that Psh is an immune sensor that harbors a specific domain serving as a cleavage site to all exogenous proteases. This cleavage constitutes the first step to the full maturation of Psh, which requires the consecutive recruitment of a circulating cathepsin (255). Once activated, Psh directly cleaves and activates SPE.

Persephone-mediated detection of danger signals and the PRR recognition and response to MAMPs both have a significant *in vivo* role in regulating Toll responses. Whereas Toll signaling remains readily observable in mutants lacking either Psh or grass function, knocking out both systems eliminates Toll pathway activity (254).

2.2.2- The core Toll pathway signaling events

Toll receptors are transmembrane proteins composed of a composite Leucine-rich repeat (LRR)-containing extracellular ectodomain. Binding of the cleaved Spz fragment to the Toll extracellular N-terminal LRRs induces a conformational change, generating an active form of the Toll dimer (263) **(Figure 8)**. To date, nine genes encoding Toll-related receptors have been identified in the *Drosophila* genome. Toll, or Toll-1, was the first Toll identified and is responsible for AMP induction via the Toll pathway. However, Toll-5 and Toll-9 may also play in the immune response since their overexpression is sufficient to induce the *Drosomycin* and *Metchnikowin* target genes (264-267). Toll-9 has an extracellular structure very similar to mammalian TLRs (268). *Drosophila* Tolls and the IL-1Rs in mammals share a cytosolic homology domain called Toll/IL-1R (TIR) domain, which interacts with adaptor molecules, thereby activating downstream events (268).

Once activated, Toll signals via a cytoplasmic TIR domain, which forms a homotypic interaction with the TIR domain of the adaptor protein Myeloid differentiation primary response gene 88 (MyD88) (269, 270). Upon this interaction, MyD88, Tube, and the kinase Pelle are recruited to form a MyD88-Tube-Pelle heterotrimeric complex through death domain (DD)-mediated interactions (271, 272). MyD88 and Pelle do not bind with each other; instead, two distinct DD surfaces in the adaptor protein Tube separately bind MyD88 and Pelle (271). Notably, a highly conserved Pelle/IL-1R–associated kinase (IRAK) interacting protein Pellino was shown to act as a positive regulator of Toll signaling, possibly by promoting a signaling poly-ubiquitinylation of Pelle. *Drosophila* Pellino mutants have impaired *Drosomycin* expression and reduced survival against Gram-positive bacteria (273). From the trimeric MyD88-Tube-Pelle complex, the signal proceeds to the phosphorylation and degradation of the *Drosophila* I-κB factor Cactus. In un-stimulated conditions, Cactus binds and sequestrates the NF-kB transcription factor(s) Dorsal and/or Dif in the cytoplasm. The degradation of Cactus

requires its phosphorylation in two distinct N-terminal motifs (274). This is achieved by the kinase Pelle because its kinase activity is required for Cactus phosphorylation (275, 276). After phosphorylation, the subsequent degradation of polyubiquitinated Cactus is mediated by a member of the β-TrCP ubiquitin ligase family, Slimb (276). Finally, nuclear translocation of Dorsal/DIF leads to activation of the transcription of several sets of target genes (277) **(Figure 8)**.

Dorsal and DIF harbor a Rel homology domain (RHD) responsible for their NF-κB transcription factor activity. They can function in homo-dimers (Dorsal/Dorsal, DIF/DIF) or in heterodimers (Dorsal/DIF) (278). While Dorsal is effective for both Toll-dependent embryonic patterning and immune response in larvae and embryonic S2 cells, only DIF mediates Toll-dependent induction of the antifungal peptide gene *Drosomycin* in *Drosophila* adults (251, 279). In the nucleus, DIF and Dorsal bind to their specific κB response element, GGGAAA(A/T/G)YCC, to trans-activate the transcription of hundreds of target genes (22). The most well characterized effectors of Toll pathway activation are AMPs, notably the antifungal Drosomycin and Metchnikowin and the anti-Gram-positive bacterial Defensin peptides (6) **(Figure 8)**.

2.2.3- Negative regulation of the Toll pathway

The Toll pathway must be tightly modulated particularly during embryonic development, to allow the dorso-ventral axis to be established, and during the adult's immune responses. Hence, Toll signaling is subject to negative regulation at several points.

The MAMPs recognition pathway is modulated by the activity of the amidase PGRP-SC1B that is capable of cleaving both DAP-type and Lys-type PGN (236) **(Figure 8)**. In addition, the serpin Spn1 (Spn42Dd) has a significant antagonistic role in the response to fungal cell wall components, most likely targeting ModSP(280). Alternatively, the danger recognition pathway is regulated by the activity of serpin Necrotic (nec or Spn43Ac) that helps maintain Persephone in an inactive state in the absence of infection (281) **(Figure 8)**. Loss of Necrotic constitutively activates the Toll pathway and is detrimental to flies, leading to general melanization and a reduced lifespan in a Psh dependent manner (282). At the intracellular level, Ji and colleagues have described an antagonistic role for Pellino in the negative

regulation of Toll by destabilizing the adaptor Myd88. This study shows that upon Toll activation, Pellino accumulates at the cytoplasmic membrane in comlex with Myd88, leading to the poly-ubiquitination and subsequent proteasomal degradation of Myd88 (283). Cactus, the inhibitor of Dif and Dorsal, also acts in a negative feed-back regulation, as its expression is induced by the Toll pathway. Newly synthesized cactus sequestrates Dorsal and Dif in the cytoplasm, overpowering the activation signals (284). Finally, WntD (Wnt inhibitor of Dorsal), is a member of the Wnt family in *Drosophila* that also acts in a negative feedback loop for down-regulating Toll activation. WntD seems to block the nuclear translocation of Dif and Dorsal upon infection, in a yet unknown mechanism (285) **(Figure 8)**.

2.3. The IMD pathway

The immune deficiency (IMD) pathway, which regulates the activity of a third *Drosophila* NF-κB protein called Relish, controls the expression of most of the *Drosophila* AMPs and thus, is indispensable for normal immunity (45). This pathway was initially defined by the identification of a mutation named *IMD* (286). Adult flies carrying this mutation alone had impaired production of most AMPs following a mixed infection with *E. coli* and *Micrococcus luteus*, however the antifungal *Drosomycin* remained inducible (12, 45). The IMD pathway controls the immune response to Gram-negative bacteria but can be highly detrimental and a source of pathologies in flies when over-activated (177, 178, 287).

The IMD pathway is similar to the tumour-necrosis factor-receptor (TNFR) and TRIF-dependent TLRs pathways in mammals, as several molecules within the signaling cascade were either homologous or very similar to members of pathways initiated by TNFR (6, 214, 288). The latter represents one of the most potent pathways involved in inflammation (289). The high degree of conservation demonstrates the relevance of the IMD pathway as a model for analyzing the molecular processes regulating NF-kB signaling in innate immunity and inflammation.

2.3.1- The IMD pathway recognition events

The IMD pathway is induced by Gram-negative bacteria and Gram-positive *Bacilli*; and controls the host defense against these infections. Its activation is mediated by the recognition

of DAP-type PGN derived from the cell wall of these bacteria. Bacterial determinants are sensed through two PRRs: PGRP-LC and PGRP-LE (172) **(Figure 9)**.

2.3.1.1- PGPRs involved in the activation of the IMD pathway

PGRP-LC is a transmembrane receptor that preferably binds DAP–type PGN found on Gram-negative bacteria and certain Gram-positive bacteria, such as *Bacillus* spp (233, 235). It functions as the principal receptor for mediating the activation of the IMD pathway in a systemic infection and locally in the anterior part of the midgut and in the hindgut (165, 290). The PGRP-LC mRNA has threesplice isoforms, a, x and y, that share the same intracellular signaling domain but each code for a distinct extracellular PGRP domain (238). PGRP-LCy lacks a functional PGN-recognition domain and may therefore act as a negative regulator of other PGRP-LC isoforms (172, 291). PGRP-LE encodes a PGRP with affinity to DAP-type PGN and is expressed both extra- and intracellularly, lacking a transmembrane domain (292). PGRP-LE is crucial for the activation of the IMD pathway locally in the midgut (165, 172).

2.3.1.2- Efficient bacterial sensing

Different combinations of extracellular sensing by distinct isoforms of PGRP-LC receptor and intracellular sensing through PGRP-LE provide adapted mechanisms to detect and differentiate between infections by different DAP-type bacteria (172). PGRP-LCx homodimers recognize polymeric PGN; PGRP-LCa does not directly bind PGN, but it acts as a co-receptor with PGRP-LCx to bind monomeric PGN, the TCT (235, 293, 294). The predicted model is that signaling is achieved by association of at least two PGRP-LC molecules in close proximity through binding of polymeric PGN. Such an interaction cannot occur with monomeric PGN, and in this case PGRP-LCa is expected to act as an adaptor (295, 296).

PGRP-LC mutant flies are still responsive to TCT fragments. This is due to PGRP-LE, which is found in two forms (172, 244). The short form corresponding to the PGRP domain alone is secreted in the hymolymph and binds PGN to enhance PGRP-LC-mediated PGN recognition on the cell surface. It is thought to assist IMD signaling by presenting PGN to PGRP-LC (292). The full-length PGRP-LE remains in the cytoplasm, where it is believed to recognize TCT fragments that gain access to the cell. Binding of TCT leads to the oligomerization of cytoplasmic PGRP-LE, which activates the IMD pathway (294).

In addition to PGRP-LC and –LE, a role for the extracellular secreted PGRP-SD in IMD activation has been recently proposed. PGRP-SD was shown to enhance PGRP-LC-dependent immune activation by promoting PGN localization to the cell surface. Interestingly, PGRP-SD counterbalances the action of PGRP-LB, an extracellular negative regulator, to modulate the intensity of the immune response (258).

2.3.2- IMD intracellular cascade

2.3.2.1- Linking PGN sensing to IKK activation

Activation of the IMD pathway in response to bacterial challenge is rapid. Once bound to PGN, the recognition receptors likely dimerize or multimerize (296) and the intracellular signal is rapidly transmitted. Deletion analysis of both PGRP-LC and PGRP-LE signaling domains identified a conserved region critical for signal transduction in the N-terminal signaling domains of both receptors. This short-conserved motif resembles the RIP Homotypic Interaction Motif (RHIM motif) found in mammalian receptor-interacting protein 1 (RIP1) (235). PGRP-LC and –LE's interaction with the adaptor protein IMD requires this core motif but is not direct, suggesting the involvement of a third unknown molecule (297). IMD is a 25-kDa protein with a death domain that has strongest similarities to that of mammalian RIP1 (TNF-receptor-interacting protein) (286, 288). However, in contrast to RIP, the IMD protein has no kinase domain. A recent study has shown that the receptors PGRP-LC and PGRP-LE form amyloid fibrils via their RHIM motifs with the adaptor protein IMD, both *in vitro* and in cells. The amyloid fibrils formation is required for the activation of IMD signaling (298, 299). IMD interacts via its death domain with the *Drosophila* homolog of FADD (FAS associated Death domain) called dFADD (300). dFADD in turn recruits the mammalian caspase-8 homolog DREDD (Death related ced-3/Nedd2-like protein) to the signaling complex via a homotypic Death-effector domain (301)**(Figure 9).**

Whereas K48-linked polyubiquitin chains targets the proteins to proteasomal degradation, K63-linked chains often result in the activation of target proteins thus mediating intracellular signaling (302). The IMD pathway is likely regulated by both K48 and K63 polyubiquitination, but to opposing purposes. A tetrameric ubiquitin-ligase complex formed by the E3 ubiquitin ligase *Drosophila* inhibitor of apoptosis 2 (DIAP2) and the E2 ubiquitin

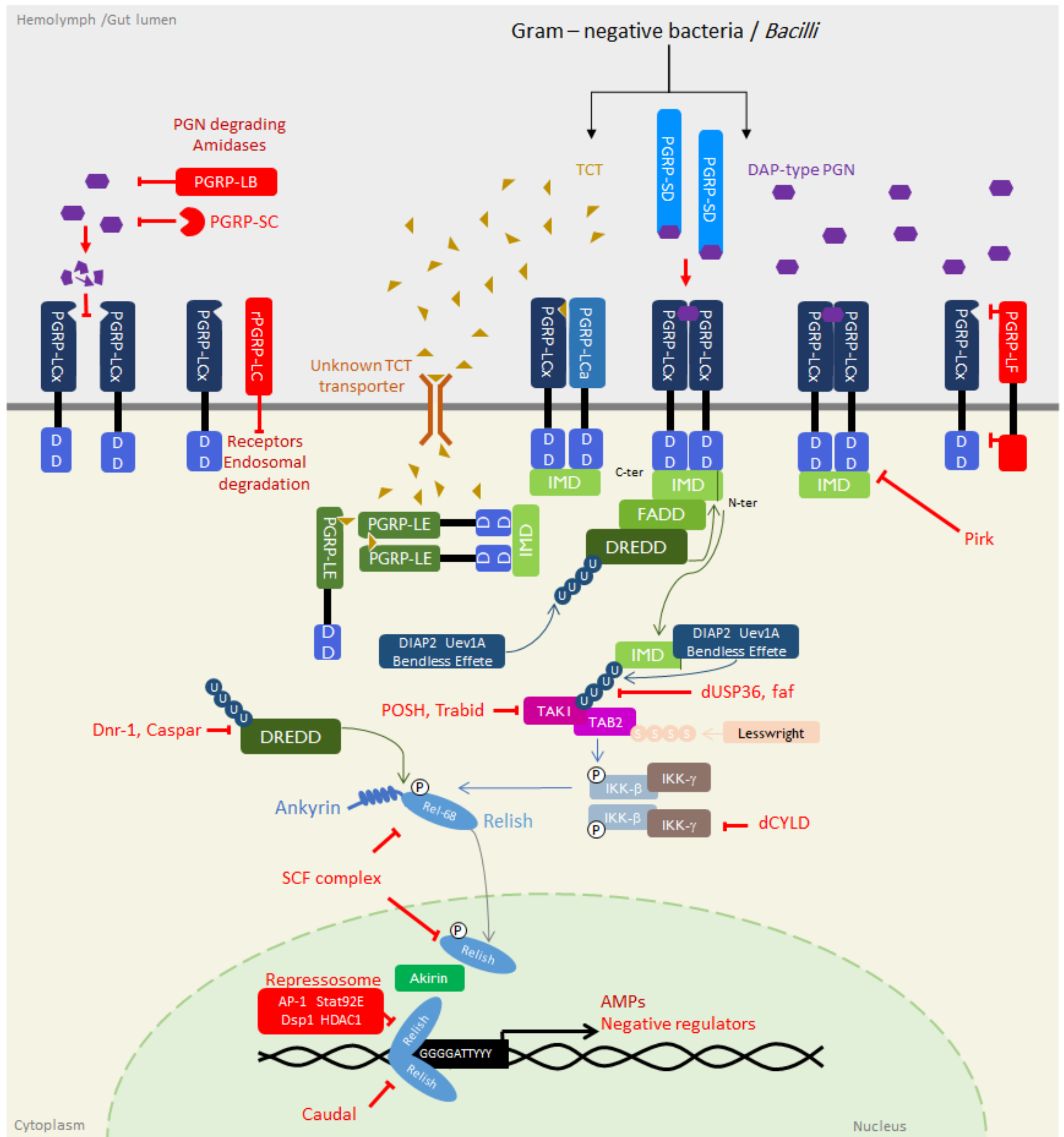

Figure 9: A schematic representation of the *Drosophila* IMD pathway

Diaminopimelic acid (DAP)-type peptidoglycans (PGNs) from the cell wall of Gram-negative bacteria and *Bacillus* species are sensed by the plasma membrane-bound pattern-recognition receptor PGRP-LC or cytosolic receptor PGRP-LE (sensor of monomeric PGN, called TCT). Recognition of bacterial determinants initiates immune signal transduction, involving IMD, FADD, Dredd, TAK1 and the IKK complex, and leads to the activation and cleavage of the NF-κB transcription factor Relish. The N-terminal part of Relish then translocates into the nucleus to activate transcription of IMD target genes, including AMPs and IMD negative regulators (see chapter I, section IV-2.3).

conjugating Ubiquitin-conjugating enzyme variant 1A (Uev1a) together with Bendless (Ubc13), and Effete (Ubc5) activates Dredd by K63-linked poly-ubiquitinylation (303). In this respect the IMD pathway signaling events resemble the mammalian tumor necrosis factor receptor (TNFR) signaling pathway, where Uev1a and Ubc13 have been reported to participate in the activation of TNF receptor-associated factor 2 (TRAF2) via K63-linked ubiquitination of RIP (304) **(Figure 9).**

DREDD, the cysteine-dependent aspartate-directed protease, is critical for the cleavage of two proteins in the IMD pathway. First, it cleaves IMD, which exposes a binding site for the ubiquitin E3 ligase IAP2 (305). Second, DREDD is also required for the cleavage of the NF-κB precursor Relish (301, 306-308). Once K63-polyubiquitinated, IMD likely recruits the MAP (Mitogen Activated protein) kinase kinase kinase (MAPKKK) Transforming growth factor beta (TGF-β)-activated kinase 1 (TAK1) and TAK1-associated binding protein 2 (TAB2) (309). TAK1 further activates the Inhibitor of I-κB Kinase (IKK) complex (211, 310). TAB2/TAK1 likely phosphorylates and activates the IKK complex as it is described in mammals (304). As Previously mentioned, TAK1 is also involved in the activation of JNK signaling (211) and also the MAPK p38 pathway for ROS production in the local immune response in the gut, potentially by phosphorylating MEKK1 (183) **(Figure 9).**

The *Drosophila* IKK complex consists of two subunits, the catalytic kinase subunit (IKKβ) and the regulatory subunit IKKγ also designated Immune response deficient 5 (IRD5) and Kenny referring to their first identified mutants in *Drosophila* respectively (251, 311). The IKK complex is required for the activation of Relish. Interestingly, it was demonstrated that Ird5 requires Small Ubiquitin-like Modifier (SUMO) ligation on its K152 residue to be mediate IMD pathway activation (312) **(Figure 9).**

2.3.2.2- Relish post-translational activation and transcriptional activity

Relish, the third NF-κB protein in *Drosophila*, is the critical transcription factor in the IMD pathway responsible of AMP gene induction. Unlike the other two NF-κB proteins DIF and Dorsal, Relish consists of either an N-terminal Rel homology (or NF-κB) domain and a C-terminal ankyrin-repeat/IκB-like domain responsible for its sequestration in the cytoplasm, thus resembling the mammalian NF-κB precursors p100 and p105 (313). The N-terminal

transcription factor domain (Rel68) is released by endoproteolytic cleavage, after which it translocates in the nucleus and initiates the transcription of its target genes. The C-terminal IκB (Rel49) domain remains in the cytoplasm, where its functions are so far unclear (307, 308, 314) **(Figure 9).**

Relish cleavage is mediated by the Caspase DREDD; it occurs at residue D545 following the recognition of the caspase cleavage motif (308, 315). The IKK complex controls Relish activation through at least two distinct mechanisms. On the one hand, both IKK subunits are crucial for AMP gene induction and both are required for Relish cleavage (308, 311). However, Relish cleavage occurs normally in flies carrying a catalytically inactive ird5, but in this case, AMP induction is drastically reduced. In addition, the *Drosophila* IKK was shown to directly phosphorylate Serine residues 528 and 529 of Relish, which are part of the mature Rel68 cleavage fragment (306). Thus, these phosphorylations seem to be essential for the full activation of Relish and AMP gene induction **(Figure 9)**.

Nuclear Rel-68 dimerizes and binds to its cognate cis-elements, named κB response elements, contained in the promoter of hundreds of genes, including *Diptericin*, *Attacin* and *Cecropin* (231). One additional factor implicated in Relish dependent AMP gene induction is Akirin, a nuclear protein with no other recognizable motifs (316). Knock out of Akirin2 in mammalian fibroblasts results also in defects in their ability to induce NF-κB dependent cytokines (316). In *Drosophila*, Akirin seems to modulate Relish transcriptional output by selectively regulating the expression of AMP immune effectors but not the negative regulators. Bonnay and colleagues showed that Akirin is a NF-κB cofactor required for the activation of a subset of Relish-dependent genes correlating with the presence of histone H3K4ac epigenetic marks, but dispensable for the transcription of genes that are negative regulators of the innate immune response. This study proposed a first line of evidence of a transcriptional selectivity modulating the activity of NF-κB factors in *Drosophila*'s immune response (317).

2.3.3- IMD pathway negative regulation

Constitutive immune activation is harmful to *Drosophila* and can have detrimental outcomes recalling chronic inflammatory diseases in mammals. Therefore, tightly adjusting the intensity and duration of the immune response in a way to match the level of host immune

activation to the level of immune stimulus enables flies to simultaneously fight pathogenic microorganisms, tolerate the endogenous flora and prevent the deleterious effects of an over-activated response. Numerous studies have highlighted the complexity of the mechanisms that modulate IMD activity. A striking feature is the existence of multiple negative regulators interfering with IMD signaling by acting at different levels and through different mechanisms (318). Figure 9 summarizes the modulatory factors that keep this response in check.

2.3.3.1- Control of PGN recognition and catalytic PGRPs

The first level of negative regulation of the IMD pathway acts at the level of ligand-receptor binding. This is mediated by enzymatic degradation of PGN into smaller fragments with greatly diminished immune-stimulatory activity (236, 239, 319). Five of the *Drosophila* PGRPs, PGRP-LB, PGRP-SB1, PGRP-SB2, PGRP-SC1, and -SC2 have amidase activity. PGRP-SC1 and -SC2 are capable of degrading both DAP-and Lys-type PGN, while PGRP-LB seems to be specifically degrading DAP-type PGN (237, 319). Genetic analyses showed that all these amidases downregulate the IMD pathway activation *in vivo* (177, 236, 239, 240, 319). In addition, both PGRP-LB and PGRP-SC2 are induced by the IMD signaling, thus acting in a regulatory feedback loop (94, 320).

PGRP-LB is produced by the fat body cells and secreted in the hemolymph and was shown to be required for the negative regulation systemic AMP (319). Moreover, both PGRP-LB and PGRP-SC1/2 are expressed in the gut epithelium and released into the lumen. These scavenging amidases have a particular importance in the gut as they dampen the PGN released by the microbiota, to inhibit a constitutive activation of the immune response. Indeed, flies exhibit an over-production of AMPs in the absence of PGRP-LB and SC1/2 during infection and also under physiological conditions (239, 319) **(Figure 9).**

Non-catalytic PGRP proteins can also execute a negative regulation on IMD activation as is the case of PGRP-LF (245, 321, 322). PGRP-LF is a transmembrane protein, which has a very short cytoplasmic tail thus lacking a signaling function. PGRP-LF seems to act as a competitive inhibitor of PGRPLC dimerization. It can strongly interact with the ectodomain of PGRP-LC blocking the formation of the active PGRP-LC homodimer, thereby blocking signaling (245, 321). Moreover, rPGRP-LCx (regulatory PGRP-LCx), an alternative splice variant encoded by

the PGRP-LC locus lacking the capacity to trigger IMD signaling, selectively dampens immune activation in response to polymeric PGN through efficient endocytosis of PGRP-LC receptor and termination of signaling via the ESCRT pathway, an endosomal protein sorting complex (323). The signaling receptor PGRP-LE was also shown to have a negative regulator role in the proventriculus of the *Drosophila* foregut most likely by promoting the expression of genes encoding negative regulators such as PGRP-LB, PIRK (detailed below) and PGRP-SC1 (172) **(Figure 9).**

PIRK (*Poor immune response upon knock-in)*, also referred to as PIMS (*PGRP-LC Interacting Inhibitor of IMD Signalin*g) and Rudra is an additional negative regulator that acts at the receptor level (324-326). It was originally identified as a target of the IMD pathway with an expression that peaks much earlier than AMPs, around an hour after the immune stimulation, in a Relish dependent manner. Biochemically, PIRK was shown to interact with both PGRP-LC and PGRP-LE, as well as with IMD (324-326). Hence, it was proposed that PIRK might act by interfering with the formation of the PGRP-LC-IMD signaling complex (324, 325). Indeed, PIRK was shown to inhibit the formation of amyloid fibrils by interfering with the complex PGRP-LC/IMD (298) **(Figure 9).**

2.3.3.2- IMD-IKK signaling control

The ubiquitination state of various pathway components is directly related to the pathway activity (305, 327). Consequently, many steps of IMD signal transduction are targeted by de-ubiquitination enzymes to shut down its transmission. While IMD is activated by conjugation with K63-polyubiquitin chains by the E3 ligase dIAP2, it also seems to be de-ubiquitinated in order to suppress the IMD pathway signaling. An ubiquitin-specific protease, scrawny or dUSP36 reduced K63-polyubiquitination of IMD, which in turn increased its proteasomal degradation (328). Fat facets (faf) is a de-ubiquitinase that was also shown to negatively regulate IMD pathway, probably by modulating IMD ubiquitination and stability state (329). Moreover, an E3 ubiquitin ligase, Plenty of SH3s (POSH), poly-ubiquitinates TAK1 mediating its targeting for proteasomal degradation (330). Finally, the de-ubiquitinase Cylindromatosis (CYLD) is reported to down-regulate IMD activation (331). In flies, CYLD was shown to interact with Kenny, or IKKγ. Flies deficient for CYLD exhibit increased AMP gene

expression (331). Interestingly, mammalian CYLD suppresses NF-κB signaling by removing K63-linked ubiquitin chains from TRAF2, TRAF6, and NEMO/IKKγ (332, 333), demonstrating the functional conservation of this immune modulator **(Figure 9).**

2.3.3.3- Control of Relish: cleavage, stability and transcriptional activity

The caspase-8 like DREDD is responsible for the cleavage and activation of Relish. Therefore, DREDD is tightly controlled and the target of negative regulation. Defense repressor 1 (Dnr1) has been proposed to inhibit the activity of DREDD (334, 335). Dnr1 encodes an evolutionarily conserved protein harboring a RING (Really interesting new gene) finger domain, which suggests that DNR1 might function as an E3 ligase. The absence of Dnr1, both in cells and in flies, results in enhanced activation of IMD signaling, both in the absence or following infection (334, 335). Dnr1 interacts physically with DREDD but the mechanism of the inhibition still remains unknown. According to the Guntermann study, Dnr1 is probably involved in DREDD proteasomal degradation since the RING finger domain of DNR1 is required for the suppression of the IMD pathway signaling (335). Another modulator of IMD signaling acting on DREDD inhibition is Caspar, a multiple ubiquitin related domain protein. Depletion of Caspar results in elevated transcription of antimicrobial peptide genes both with and without infection (336). Caspar has been shown to inhibit DREDD-dependent cleavage of Relish *in vivo* but its target remains unknown (336). Caspar shares homology with the human Fas associated factor 1 (FAF1) which has been reported to negatively regulate mammalian NF-κB signaling (337) **(Figure 9).**

Relish stability was shown to be modulated by an ubiquitin-proteasome pathway, another layer of IMD signaling regulation. A genetic screen identified skpA, a component of Skp/Cullin/F-box protein (SCF)-E3 ubiquitin ligase complex as a negative regulator of the IMD pathway (338). RNAi silencing of skpA was shown to increase the levels of Relish suggesting that the SCF complex might regulate the stability of Relish and thereby modulate the IMD pathway activity (338).

Once cleaved and translocated to the nucleus, the transcriptional activity of Rel68 is submitted to different layers of regulation. Transcription factors of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) and the JNK pathways have been

shown to have a negative influence on the transcriptional activity of Relish. In particular, *Drosophila* activator protein 1 (dAP-1) and Stat92E, the specific transcription factors of the JNK and JAK/STAT pathways, respectively, have been suggested to form a repressosome complex. The latter also includes a *Drosophila* High mobility group (HMG) protein called Dorsal switch protein 1 (DSP1), and associates in response to continuous immune signaling (339). Depletion of either dAP-1, Stat92E or DSP1 by loss of function mutation or RNAi increased transcription of AMP genes *in vivo*, in a Relish-dependent manner, but decreases flies' survival upon an infection pointing out the harmfulness of unresolved immune responses in *Drosophila*. This complex was shown to function by replacing Relish at overlapping cis-regulatory elements and recruiting a histone deacetylase 1 (HDAC1) to close chromatin and inhibit transcription of Relish target genes (339).

Another transcriptional regulator, Zinc finger homeodomain 1 (Zfh1) was also identified as a negative regulator of the IMD pathway signaling (340). RNAi knock-down of ZFH1 hyperactivates the IMD response cell lines, but curiously *in vivo* RNAi targeting of this gene caused elevated AMP gene expression only for *Cecropin B* and *Attacin A*. ZFH1 includes zinc finger domains, a homeodomain as well as a nuclear localization signal but its function in mediating IMD pathway suppression remains obscure (340).

Finally, the homeobox transcription factor Caudal, was shown to negatively regulate the expression of AMPs in the *Drosophila* gut. Importantly, flies deficient for the expression of caudal were shown to over-express AMPs, displayed an altered composition of the commensal microbiota and subsequently a shortened life-span (159). Molecular mechanisms underlying Caudal's activity in controlling Relish transcription have not been described but Caudal is predicted to bind to Caudal-protein DNA recognition elements (CDRE) that are found in AMP promoters (341) **(Figure 9).**

In defiance of all these described IMD negative regulators acting via distinct molecular mechanisms, no activity of specific protein phosphatases towards NF-κB-Relish-dependent transcription had been described. In this context, my PhD work on *Drosophila* phosphoprotein phosphatases aimed at better understanding their mode of action during NF-κB-activated

immune responses for the counteraction of the different phosphorylation events required for the proper activation.

Chapter 2: Protein phosphorylation and dephosphorylation in the regulation of cell signaling

I- Protein Kinases and the role of protein phosphorylation in cellular signaling

The capacity of a cell to efficiently adapt to external changes is a vital requirement for its survival in a dynamic environment. To acquire this ability, cellular proteins undergo numerous post-translational modifications which imply a drastic influence on their structure, charge and enzymatic activity allowing them to rapidly adapt to external stimuli. The inventory of protein modifications includes changes in ubiquitination, phosphorylation, glycosylation, methylation, acetylation, etc. Reversible protein phosphorylation was the first identified modification that is able to alter the enzymatic activity of a protein. The concept of protein phosphorylation was first discovered in the early 1950s by Edmond Fischer and Edwin Krebs, by the studies of the interconversion of glycogen phosphorylase from an inactive form "b" to an active form "a" (342-344). In the *in vitro* conversion of phosphorylase b to phosphorylase a, Fisher and Krebs have demonstrated a dual requirement for Adenosine triphosphate (ATP) and a "converting enzyme", which was later called phosphorylase kinase. This enzyme transfers a phosphate group (PO_4) from ATP to phosphorylase b, and the resulting phosphorylase *a* was found to be a phosphorylated protein (342, 343). Now, six decades since that first discovery, concerted research in this domain highlighted that nearly all proteins undergo phosphorylation by multiple kinases at several sites, allowing the cells to process distinct physiological signals (345). Indeed, protein phosphorylation has been established as a major control mechanism for most aspects of eukaryotic physiology, such as the regulation of metabolism, proliferation, apoptosis, subcellular trafficking, inflammation, growth and differentiation (346). This reversible mechanism can produce changes in the conformation of substrate proteins that switch them either on or off. The addition of a phosphate group to the polar chain of various amino acids modifies the polarity of the protein from hydrophobic apolar to hydrophilic polar, resulting in changes in its conformation. Hence, the phosphorylated amino acid acquires

different biochemical properties allowing it to bind with other proteins and consequently assemble and detach protein complexes (346).

Most of the protein phosphorylation events occurs on serine (Ser or S), threonine (Thr or T), and tyrosine residues (Tyr or Y) (347). Early studies of phosphoamino acid analysis estimated that Phosphotyrosines residues (pTyr) recorded < 1% with the remainder of phosphorylation on the Thr and Ser residues (345). Afterwards, the development of more sophisticated phospho-analysis approaches of approximately 2000 phosphoproteins, based on stable isotope labeling of amino acids in cell culture (SILAC) and mass spectrometry, allowed a more precise distribution of phospho-groups (348). These approaches allowed the identification of 1.8 % pTyr-containing proteins, with pThr and pSer accounting for 98.2 % of the total phosphosites in mammalian Hela cells. More precisely, the phosphorylated residues of Ser are 86.4 %, followed by residues of Thr accounting for 11.8 % (349, 350). In addition, the cell signaling technology PhosphoSitePlus (www.phosphosite.org) database, based mainly on shotgun mass spectrometry, certifies nearly 170 000 pSer, 70 000 pThr and 44 000 pTyr containing sites (345). Altogether, these data highlight that the majority of phosphorylation events on eukaryotic proteins occur on Ser and Thr amino acids. Tyr phosphorylation is a signature of the EGFR family that harbors a domain called tyrosine kinase (346). Indeed, a tenfold increase in pTyr content occurred in cells transformed by Rous sarcoma virus, which drew attention to the importance of this modification in cancer biology (351, 352). Besides, some phosphorylation events on histidine (His or H) and aspartate (Asp or D) residues have also been reported, but these are shown to be less stable than those of the previously mentioned amino acids (346).

In eukaryotic cells, there is a constant balance between phosphorylation and de-phosphorylation events, mediated by kinases and phosphatases respectively **(Figure 10)** (346, 353). This balance is crucial for the control of biological processes and for the maintenance of physiological conditions. For example, the P53 protein, the DNA-binding and stress-activated transcription factor, is activated by phosphorylation in order to enhance the expression of genes that inhibit the cell cycle, activate DNA repair and block apoptosis (354). In 2009, Macalaine and colleagues have shown that an imbalance in the mechanism of phos-

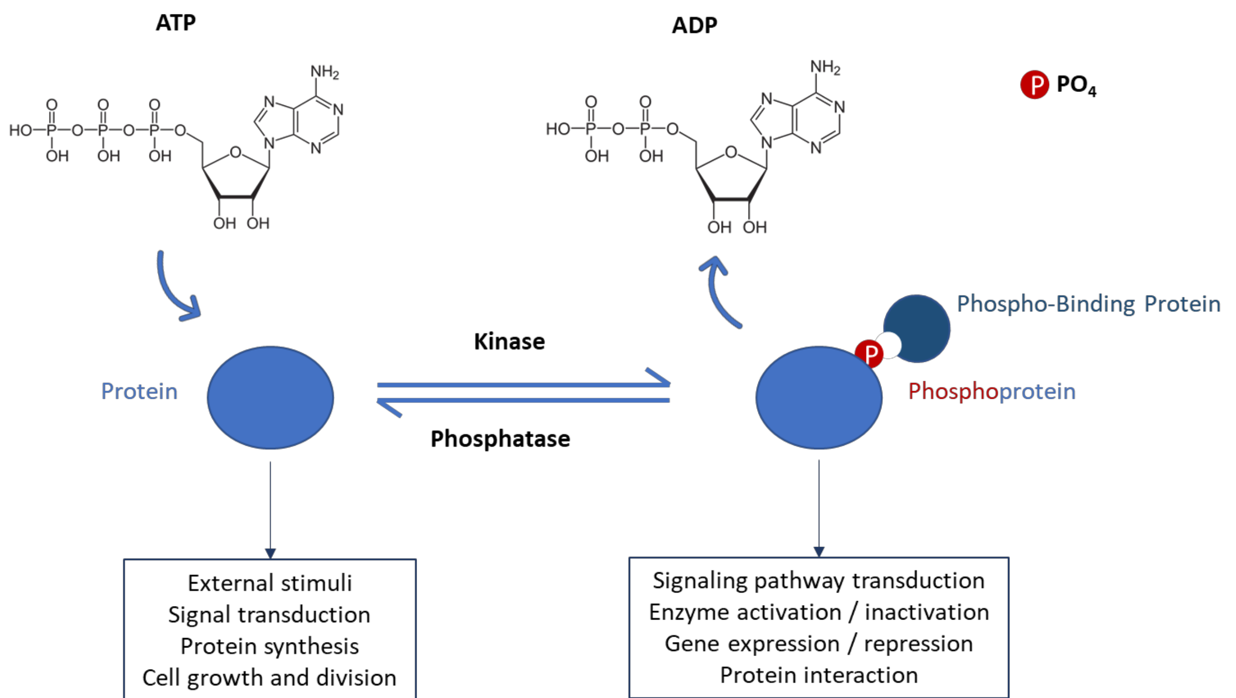

Figure 10: Balance in the reversible phosphorylation of proteins

The mechanism of phosphorylation regulation is mediated by kinases and phosphatases. For example, phosphorylation is activated by external stimuli, intracellular signaling events, protein synthesis and mechanisms involved in cell growth and division. Consequently, the protein receives a phosphate group by adenosine triphosphate (ATP) hydrolysis catalyzed by the enzymatic activity of kinases. This phosphorylation event is a reversible process due to the activity of phosphatases. Phosphorylation and de-phosphorylation are a molecular switch and, in particular, a phosphoprotein can stimulate a signaling pathway transduction, lead to the activation or inactivation of an enzymatic activity, activate or repress gene expression and interact with a phospho-binding protein that binds to the phosphate group.

phorylation/dephosphorylation of the p53 factor can lead to a chronic inactivation of the protein, which in turn can induce an oncogenic transformation of the cell, and also trigger organism ageing(354).

The fully sequenced human genome contains 518 putative protein kinases (355-357), that can be classified into two families: 90 Tyr kinases (PTKs) and 428 Ser/Thr kinases (PSKs) (344). Moreover, some kinases have been found to act on all three residues, these are called the dual-specificity kinases (DSKs) (346, 358, 359). In addition, studies in eukaryotes models have described kinases that specifically phosphorylate small molecules, including lipids and sugars (360-362). The alignment of the primary amino acid sequence and crystal structures revealed that kinases share conserved features in their kinase catalytic domain (363, 364). Indeed, the overall structural organization of the 300 residues of the protein kinase domain is conserved with 10 key residues mediating the core functions of the enzyme (365-367). The catalytic domain of protein kinases has 2 subdomains, N- and C-terminal, that consist of a mostly β-stranded N-lobe, linked by a short hinge region to a larger α-helical C-lobe. These subdomains bind the ATP in the cleft between the N- and C-terminal lobes of the kinase domain where the adenine group of ATP is sandwiched between hydrophobic residues and makes contact via hydrogen bonds to the hinge region (368-370). The catalytic domain is unavailable when it is active because propellers of the N- and C-terminal subdomains rotate inward (346). Nevertheless, protein kinases exhibit variability in other parts of the catalytic domain; they may contain additional domains, additional subunits or both (363). Besides, protein kinases harbor different phosphorylation sites located within the N-terminal or C-terminal polypeptide chain, outside the catalytic domain. They may also be phosphorylated on residues located in the center of the kinase domain. This regulation mechanism is termed the "activation segment" (363). These differences serve as regulatory or targeting modules. Activation or deactivation of kinases occur in different ways: (i) through the kinase itself with a cis-phosphorylation/auto-phosphorylation or phosphorylation by other kinases; (ii) control by additional subunits or domains that respond to second messengers, (iii) control by regulatory subunits whose expression can vary depending on the stimuli, (iv) control by additional domains that target the kinase to different subcellular localization and substrates (363). Besides transferring the gamma phosphate group of ATP onto hydroxyl groups of substrates,

protein kinases may also exhibit non-catalytic functions for scaffolding, relocation, allosteric effects, subcellular targeting, DNA binding as well as protein–protein interactions (371).

The discovery of reversible protein phosphorylation that occurred on the enzyme phosphorylase showed that this latter became activated following its phosphorylation. The activation of this enzyme was dependent on allosteric metabolites such as glucose and AMP, which triggered a conformational change in the enzyme that was restored following de-phosphorylation (372). This observation emphasized the fact that the conformational change triggered by the phosphorylation of a protein modifies the enzyme activity and affinity towards its substrates. Indeed, many reported examples of protein phosphorylation show a subsequent activation of cellular enzymes; among these are many protein kinases including Raf, MEK, MAPK, AKT, AMPK, TGFβ-R, and IKK (345). However, some phosphorylation events act on inactivating many enzymes, such as glycogen synthase (373), Acetyl co-enzyme A (AcCoA) carboxylase (374), src family of kinases (375), and cyclin-dependent kinase (CDKs) (376). These enzymes are reactivated by their de-phosphorylation that is catalyzed by protein phosphatases. Besides enzyme activation, phosphorylation of Ser/Thr residues can also generate docking sites for a subsequent phospho-dependent protein-protein interaction. Specific binding modules such as 14-3-3, FHA, Polo-box..., bind specifically sequences containing pSer or pThr residues (377, 378). This phosphorylation-dependent binding provides a basis for the recruitment and assembly of various proteins with other enzymes, transcription factors, adaptors, and regulatory molecules into large protein complexes, a fundamental requirement for cell signaling. Binding of these pSer/pThr recognition proteins protects phosphorylated sites from being dephosphorylated by phosphatases, regulating thus the persistence of pSer/pThr sites. This concept reflects the competition that occurs between pSer/pThr-binding partners and the protein phosphatases that dephosphorylate these specific sites (345). Protein phosphorylation is not a stable event; it is highly dynamic such as the half-lives of some phosphosites is in order of seconds (379, 380). This high cycling of phosphosites is essential for the tuning of cellular signals. Indeed, mathematical analyses propose that an increased cellular sensitivity and responsiveness to physiological stimuli occur with rapid kinase/phosphatase cycles. In addition, they suggest that high phosphatase activity does not only function as a shutoff mechanism. In fact, it allows an accurate proofreading of

phosphorylation events and acts as a negative regulation that is required to achieve effective termination of a signaling response (381). Another mathematical model of kinase signaling suggests that kinases may be regulators of the amplitude of a physiological response, whereas phosphatases may have a more dominant function in determining the speed and duration of a signal response (382). Afterwards, the essential role of protein phosphatases was highlighted by Gelens and colleagues, who insist on the equal contribution of both kinases and phosphatases, cooperatively, in dictating the intensity, timing, directionality and localization of signaling during physiological cellular processes such as mitosis. They emphasize on the important role of protein phosphatases in the origination and transmission of cellular signaling, instead of being viewed as simply negative regulators that shut down the cellular signal (383).

In this chapter, I focus on the major phosphatases that catalyze de-phosphorylation of proteins, with the emphasis on pSer/pThr phosphoprotein phosphatases family in controlling eukaryotic cell physiology.

II- Protein Phosphatases: classification and features

As described above, reversible protein phosphorylation is an essential element of nearly all aspects of cell life. It allows the communication and integration of numerous stimuli across a cell's surface and subsequently causes changes in the activities, functions and associations of intracellular proteins. Changes in the state of protein phosphorylation are regulated by two types of enzyme activities: the kinase and the reverse activity of the protein phosphatases. The latter removes the phosphate group from phosphoproteins by hydrolyzing phosphoric acid monoesters into a phosphate group and a molecule with a free hydroxyl group (384, 385) **(Figure 10)**. As mentioned earlier, it has been established that nearly all protein Kinases, with rare exceptions, exhibit a common 3D structure involving a β-sheet N-terminal lobe that binds the ATP and a α-helical C-terminal lobe associating the peptide substrate. The reaction catalyzed by kinases is relatively fast; it involves a conformational change of the enzyme upon binding to the substrate and a subsequent fast phosphotransfer and product release. In this regard, protein kinases have a fairly simple biochemistry, operating with the same mechanism and exhibiting the same structure (386). In contrast to kinases, protein phosphatases are

highly diverse; they evolved distinct biochemistry with different 3D structures, active sites and various mechanisms of regulation and hydrolysis. Based on these biochemical properties, protein phosphatases are subdivided into separate superfamilies and subfamilies as detailed below.

Eukaryotic protein phosphatases are structurally and functionally diverse enzymes. Based on the catalysis mechanism and the nature of the de-phosphorylated residue, they can be divided into two main superfamilies: the protein tyrosine phosphatases (PTPs) and the Serine/Threonine phosphatases (PSPs). The PTPs catalyze de-phosphorylation by the use of a cysteinyl-phosphate enzyme intermediate. In contrast, the PSPs are metalloenzymes that de-phosphorylate their substrates in a single reaction step using a metal-activated nucleophilic water molecule. A subfamily of the PTPs, the dual-specificity phosphatases, dephosphorylates all three phosphoamino acids (386)**(Figure 11)**. The specificity of signaling and the reversibility of phosphorylation would suggest that there is a similar number of cellular protein kinases and phosphatases. However, there are only 107 putative protein Tyr phosphatases in the human genome (387) and far less protein Ser/Thr phosphatases (~ 40) (345). Whereas the numbers of PTKs and PTPs roughly match each other, the number of catalytic subunits of PSPs is at least 10 times lower than the number of genes that encode mammalian protein Ser/Thr kinases. Functional analyses defined further three broad families of PSPs: phosphoprotein phosphatases (PPPs), metal-dependent phosphatases (PPMs) and the aspartate-based phosphatases constituted by FCP/SCP (TFIIF-associating component of RNA polymerase II CTD phosphatase/small CTD phosphatase) (344, 386) **(Figure 11)**. Within each family, the catalytic domains are highly conserved, with functional diversity endowed by regulatory domains and subunits. The PPP family includes protein phosphatase 1 (PP1), PP2A, PP2B (commonly known as calcineurin), PP4, PP5, PP6, and PP7. The PPM family is represented by protein phosphatases that require metal ions manganese/magnesium (Mn^{2+}/Mg^{2+}), such as PP2C and pyruvate dehydrogenase phosphatase (386). For several members of the PPP family, the catalytic subunit associates with a great variety of regulatory subunits, in contrast to PPMs that do not have regulatory subunits but contain instead additional domains and conserved sequence motifs that may help determine substrate specificity (344). Both PPP and PPM require metal ions in their catalytic mechanisms for the

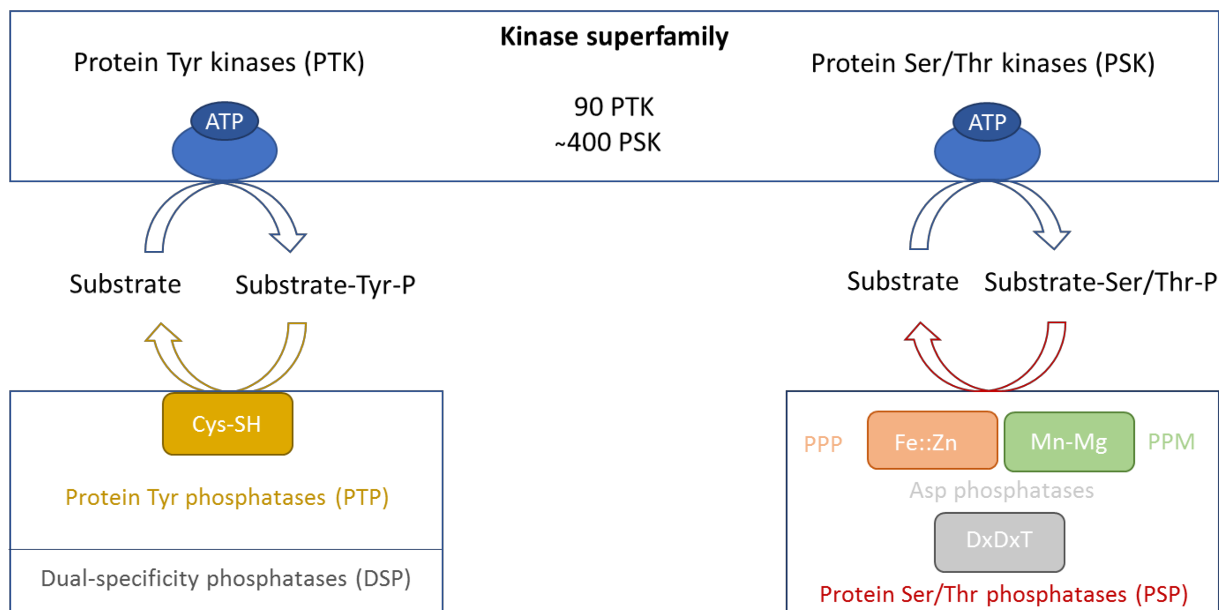

Figure 11: Protein kinases and protein phosphatases

Protein kinases (top box) are separated into Tyr (PTK) and Ser / Thr (PSK) specific types, represented by two-lobed structures with ATP in the N-terminal lobe. Protein phosphatases are divided into four families, with the protein Tyr phosphatases (PTP) family containing a Cys-based active site (lower left) subdivided into different groups (PTP, DSP). The Ser / Thr phosphatases are shown in three families, with active sites depicted as bimetallic iron-zinc for the PPP family, Mn-Mg for the PPM family dependent on added metal ions, and DxDxT for the family that utilizes an Asp-phospho-intermediate in hydrolysis.

activation of a water molecule underlying the de-phosphorylation reaction. In contrast, FCP/SCP uses an aspartate-based catalysis mechanism (386).

The striking imbalance in the numbers of kinases and phosphatases led to the speculation that phosphatases are non-specific enzymes that de-phosphorylate multiple substrates. They were thought to be, unlike kinases, much less specific in their recognition and interaction with phosphoproteins. However, it became clear later that PPPs rarely exist as free catalytic subunits in the cells. Instead, they are incorporated into multi-subunit complexes (holoenzymes) that combine the catalytic subunit with one or more regulatory subunits, dictating substrate specificity, subcellular localization and regulation of the phosphatase' activity (344, 386, 388-391). So far, more than 200 regulators for PP1 and more than a dozen genes and isoforms for B regulatory subunits of PP2A have been discovered (detailed below). Hence, the number of PPP holoenzymes approximately equals the number of Ser/Thr kinases. However, given the tremendous number of phosphorylation sites in the human genome, that was estimated to 38000 (392), and the fact that several phosphoproteins are modified at multiple Ser/Thr sites, every kinase and every phosphatase should be expected to have numerous substrates.

1- Protein tyrosine phosphatases

The cellular balance of tyrosine phosphorylation is achieved through the complementary actions of PTKs and PTPs. A disruption in this balance causes a plethora of human diseases attesting of the importance of a tightly controlled activity of these groups of enzymes. PTPs are recognized by a signature active-site motif HC(X)5R (393). Studies on PTPs demonstrated that they exhibit exquisite substrate selectivity. It has been shown that both the catalytic and non-catalytic domains of the PTPs contribute to substrate specificity *in vivo*. Whereas non-catalytic N- and C-terminal segments target the phosphatase to defined intracellular compartments leading it to the substrate (394, 395), the catalytic domains confer site-selective recognition of both the phosphotyrosine residue to be dephosphorylated and its flanking amino acids (393).

The active-site sequence HC(X5)R defining the PTP family is referred to as the 'PTP signature motif'. Residues in this motif are essential for the catalysis and form the phosphate-binding loop of the active-site (393). All the PTPs employ the same catalytic mechanism that is

dependent on a nucleophilic cysteine within the active site, with a low pKA forming a thiophosphate intermediate during the catalysis. This latter also requires stabilization by an invariant arginine residue and a catalytic acid/base aspartate. The active site of these enzymes is formed by highly conserved loops allowing an optimal arrangement of the mentioned essential residues (396).

Based on the sequences of their active domains, human PTPs can be subdivided into three distinct families: class I PTPs containing ~100 proteins (that will be described in the following), class II PTPs which includes a single protein, the pTyr specific low molecular weight PTP (LMPTP) (397), and class III PTPs with 3 members of which the pThr/pTyr specific CDC25s (394, 396). The class I of PTPs can be divided into two groups, the classical phosphotyrosine specific PTPs (37 human genes) that have a strict activity towards tyrosine residues, and the VH1-like or dual specificity protein phosphatases (DSP) (63 genes). These have a diverse activity; they de-phosphorylate substrates on tyrosine, serine and threonine as well as lipid substrates (393, 396). DSPs constitute the most diverse group of PTKs; some known representatives of this family include the pThr/pTyr c-mitogen activated protein (MAP) kinase phosphatase (MAPKP), the p-Ser/pThr specific CDC14, the PTENs which de-phosphorylate phosphatidylinositol (398-400). The p-Tyr specific PTPs can be further sub-divided into the trans-membrane receptor-like PTPs and the intracellular non-receptor-like PTPs (394, 398).

2- Protein Serine/Threonine phosphatases

2.1- Metal-dependent protein phosphatases (PPM)

This highly conserved family of protein phosphatases includes members dependent on manganese/magnesium ions (Mn^{2+}/Mg^{2+}), such as PP2C and pyruvate dehydrogenase phosphatase (344). In contrast to PPPs, the members of this family are not sensitive to inhibition by okadaic acid, which is a hallmark of the PSPs belonging to the family of PPPs. PPM is a large family with at least 16 representative genes in mammals giving rise to at least 22 distinct protein isoforms (344, 401). PPMs are widely expressed across prokaryotic and eukaryotic species, with 80 and 78 genes in the model plant *Arabidopsis thaliana* and rice, respectively (402). Thus, PPMs are a major family of Ser/Thr phosphatases in plants where

they transduce hormonal signals as a subunit of the abscisic acid receptor and modulate a variety of stress responses (403-405).

The architecture of PPM catalytic domains is highly conserved, characterized by a central β-sandwich, with each β sheet flanked by a pair of α-helices (406). This arrangement generates a cleft for the placement of two metal ions that bind to a water molecule, similarly to the catalysis mechanism of PPPs. More recent kinetic and structural evidences show these PPM phosphatases actually use three metal ions at their active sites. The third ion binds with relatively low affinity, consistent with the dependence on millimolar levels of added Mg2+ for optimal activity (407, 408). These monomeric enzymes do not bind to regulatory subunits; they contain additional domains and sequences that determine specificity (344). The primary function of PPMs appears to be the regulation of stress signaling, although it also plays a role in cell differentiation, growth, survival, apoptosis, and metabolism (80, 409).

2.2- Aspartate based protein phosphatases

This family of phosphatases is represented by FCP/SCP. Two main features characterize these enzymes rendering them different from all other Ser/Thr phosphatases. The first is their reliance on the aspartic acids of the sequence motif DxDxT/V for phosphatase activity. The second feature is that FCP/SCP has only one primary substrate which is the (C-terminal domain) CTD of RNA polymerase II, which contains tandem repeats of the sequence YSPTSPS (410, 411). There are eight putative CTD phosphatases in the human genome (410). The level and the pattern of the CTD phosphorylation oscillate during transcription, with hypo-phosphorylation during the pre-initiation complex and hyper-phosphorylation during transcription elongation. Distinct regulatory proteins are essential for the orchestration of these phosphorylation patterns (412, 413), constituting the “CTD code” (414). FCP1 is one of these factors, main phosphatase for the pSer in the second and fifth position of the tandem repeat.

2.3- Phosphoprotein Phosphatases

2.3.1- Distinct families of PPPs and classification

The major protein phosphatases that catalyze de-phosphorylation of most pSer and pThr residues and are required in controlling the majority of cellular functions belong to this

family of enzymes. Early studies on protein phosphatases separated these into two major groups, namely type-1 and type2, depending on their *in vitro* activity towards selected substrates and their sensitivity to certain inhibitors (415). This classification scheme assigned one type of PPP as PP2C, but years later this turned out to be the founding member of the separate PPM family, with sequences distinct from those of PPPs (415). Later molecular cloning and further functional analyses separated the PPPs family of phosphatases from the two other Ser/Thr phosphatases clades, the PPMs and the aspartate-based phosphatases. This separation was based on their primary sequence, structure and their mechanisms of catalysis (344). In mammals, the cloning of PPPs catalytic subunits revealed seven distinct types: PP1, PP2A, PP3 (also known as PP2B or calcineurin), PP4, PP5, PP6, and PP7. PP7 is also known as RdgC-like phosphatase, following identification of the *Drosophila RdgC* gene, whose loss of function leads to retinal degeneration (416, 417). The PPP family contains three characteristic sequence motifs within the conserved 30 kDa catalytic domain: GDxHG, GDxVDRG, and GNHE (344). Several members of this family are characterized by their sensitivity to the natural toxin okadaic acid inhibitor, and their association with distinct regulatory subunits to form "holoenzymes" (386). In *Drosophila*, the genome contains 19 genes coding for PPPs catalytic subunits (416, 418-439) listed in **Table 1**. The seven families of PPPs are described in the section below.

2.3.1.1- <u>Protein Phosphatase 1 (PP1)</u>

PP1 represents the original identified PPP and the historical phosphorylase phosphatase (386). It is a major protein Ser/Thr phosphatase, ubiquitously expressed in all eukaryotic cells. PP1 is required in a wide range of cellular processes, including meiosis and cell division, apoptosis, protein synthesis, metabolism, cytoskeletal reorganization, and the regulation of membrane receptors (440). A milestone accomplishment in the PPP description was the discovery of the crystal structure of PP1 catalytic subunit (441, 442). This latter adopts a compact α/β fold, with two metal ions, identified as Mn^{2+} and Fe^{2+} (iron) located in the active site (441, 442). Coordination of these two metal ions is provided by three highly conserved residues in all members of the PPP family, which suggests a common mechanism of metal-catalyzed reaction in the protein family (344). The two metal ions are thought to be required for the catalysis reaction by binding and activating a water molecule, which initiates a

Table 1: Phosphoprotein phosphatases catalytic subunits of *Drosophila melanogaster*

Phosphatase	Coding Gene (CG) number	Family	References
PP1-13C	CG9156	PP1	(421, 423)
PP1-87B	CG5650	PP1	(418, 419)
PP1-96A	CG6593	PP1	(420, 421)
PP1-9c	CG2096	PP1	(420, 422)
PpY-55A	CG10930	PP1	(424)
PpN-58A	CG3245	PP1	(425)
PpD5	CG10138	PP1	(426)
PpD6	CG8822	PP1	(426)
Pp1-Y1	CG41534	PP1	(426)
Pp1-Y2	CG40448	PP1	(426)
CanA1	CG1455	PP2B	(427-429)
PP2B-14D	CG9842	PP2B	(429, 430)
CanA-14F	CG9819	PP2B	(429, 431)
PP2Ac (mts)	CG7109	PP2A	(432, 433)
PP4-19c	CG32505	PP4	(434, 435)
CG11597	CG11597	-	(436)
PpD3	CG8402	PP5	(438)
Ppv	CG12217	PP6	(437)
Rdgc	CG44746	PP7	(415)

nucleophilic attack on the phosphorous atom (441, 442). Each functional PP1 enzyme consists of a catalytic subunit and a regulatory subunit (R subunit). Early studies suggested PP1 binds to a very specific RVxF/W (R, arginine; V, valine; x, any amino acid; F, phenylalanine; W, tryptophan) sequence motif that most R subunits contain. This notion was supported by the crystal structure of PP1 bound to a peptide containing the sequence RRVSFA (S, serine; A, alanine) (443). To date, at least 100 putative PP1-binding R subunits have been identified, based on PP1 binding assays that yielded 78 novel RVxF-containing proteins, with many more expected to be found (444). These R subunits target the PP1 catalytic subunit to specific subcellular compartments and substrates or serve as substrates themselves (386).

The representative members of this family in *Drosophila* are: Pp1-13C, Pp1-87B, Pp1-96A and PP1-9c (424). The functions of PP1 phosphatases in *Drosophila* are diverse; they include the regulation of cellular division, Hedgehog signaling, wing development and muscle functions and attachments (420, 422, 423, 445). In addition, there are 6 novel members: PpY-55A, PpN-56A, PpD5, PpD6, Pp1-Y1, and Pp1-Y2, which are specific to *Drosophila* and exclusively expressed in the testis of male flies (427).

2.3.1.2- Protein phosphatase 2B (PP2B or calcineurin)

Calcineurin, also known as PP2B or PP3, is an essential calcium and calmodulin dependent family of phosphatases that play an important role in numerous biological processes, including memory and neurodevelopment, muscle development, cardiac hypertrophy and immune response (446). Purified calcineurin is a heterodimer consisting of a catalytic subunit, calcineurin A (CNA), and a regulatory subunit, calcineurin B (CNB) (344, 447)). CNA contains an N-terminal phosphatase domain, a helical CNB-binding domain, a calcium-calmodulin binding motif and an autoinhibitory element that blocks access to the catalytic center (386). The phosphatase is inactive alone and only gains activity once associated to calcium-calmodulin. The phosphatase domain of CNA is structurally similar to the catalytic subunit of PP1, with the same pattern of metal ion coordination (Zn^{2+} and Fe^{3+}) (448). The role of calcineurin was extensively studied in T cells, as it de-phosphorylates the nuclear factor of activated T cells (NFAT), allowing its nuclear import and activation of transcription. Calcineurin is a *bona-fide* target of the drugs FK-506 and cyclosporine which

have been used clinically for many years to inhibit host versus graft response following transplantation (449-451). The calcineurin/Pp2B/PPP3 Ca^{2+}-regulated protein phosphatases are represented by 3 closely related genes in *Drosophila*: *CanA1*, *Pp2B-14D* and *CanA-14F* (428, 429, 431). Interestingly, a role for these phosphatases in the regulation of *Drosophila* NF-κB pathways has been proposed. More precisely, CanA1 regulates the non-canonical IMD pathway activation by nitric oxide (NO) in *Drosophila* larvae. A depletion in *CanA1* expression suppressed immune induction in larvae upon infection or upon treatment with NO donors, whereas a gain-of-function CanA1 transgene activated immune responses in untreated larvae (429). Additionally, Li and Dijkers have demonstrated an involvement of Pp2B-14D and CanA-14F in Toll mediated immune signaling. In cell culture, inhibition of *Pp2B-14D/CanA-14F* expression, but not *CanA1*, decreased Toll-dependent Dorsal/Dif activity (430).

2.3.1.3- Protein phosphatase 2A (PP2A)

PP2A is a ubiquitously and highly expressed protein Ser/Thr phosphatase. Along with PP1, it accounts for over 90 % of all Ser/Thr phosphatase activity in the cell (344, 390). It engages in numerous functions: development, cell proliferation and death, cell mobility, cytoskeleton dynamics, the control of the cell cycle, and the regulation of several signaling pathways (452); it is also likely to be an important tumor suppressor (453, 454). Although once perceived as a single broad specificity phosphatase, this notion now seems oversimplified. PP2A represents a family of holoenzyme complexes with different activities and diverse substrate specificities. PP2A exists in eukaryotic cells in two general forms: a heterodimeric core enzyme and a heterotrimeric holoenzyme (344). The PP2A core enzyme consists of a catalytic subunit (C subunit) and a scaffold subunit (also known as the A or PR65 subunit). The holoenzyme complex is formed by the association of the core enzyme with a third regulatory B subunit that could be very variable (390, 455) **(Figure 12).** The diverse functions of PP2A derive from the fact that cells can assemble over 200 biochemically distinct complexes containing different combinations of A, B and C subunits each targeting the phosphatase to a different substrate, or even different phosphorylated residues on the same protein (456).

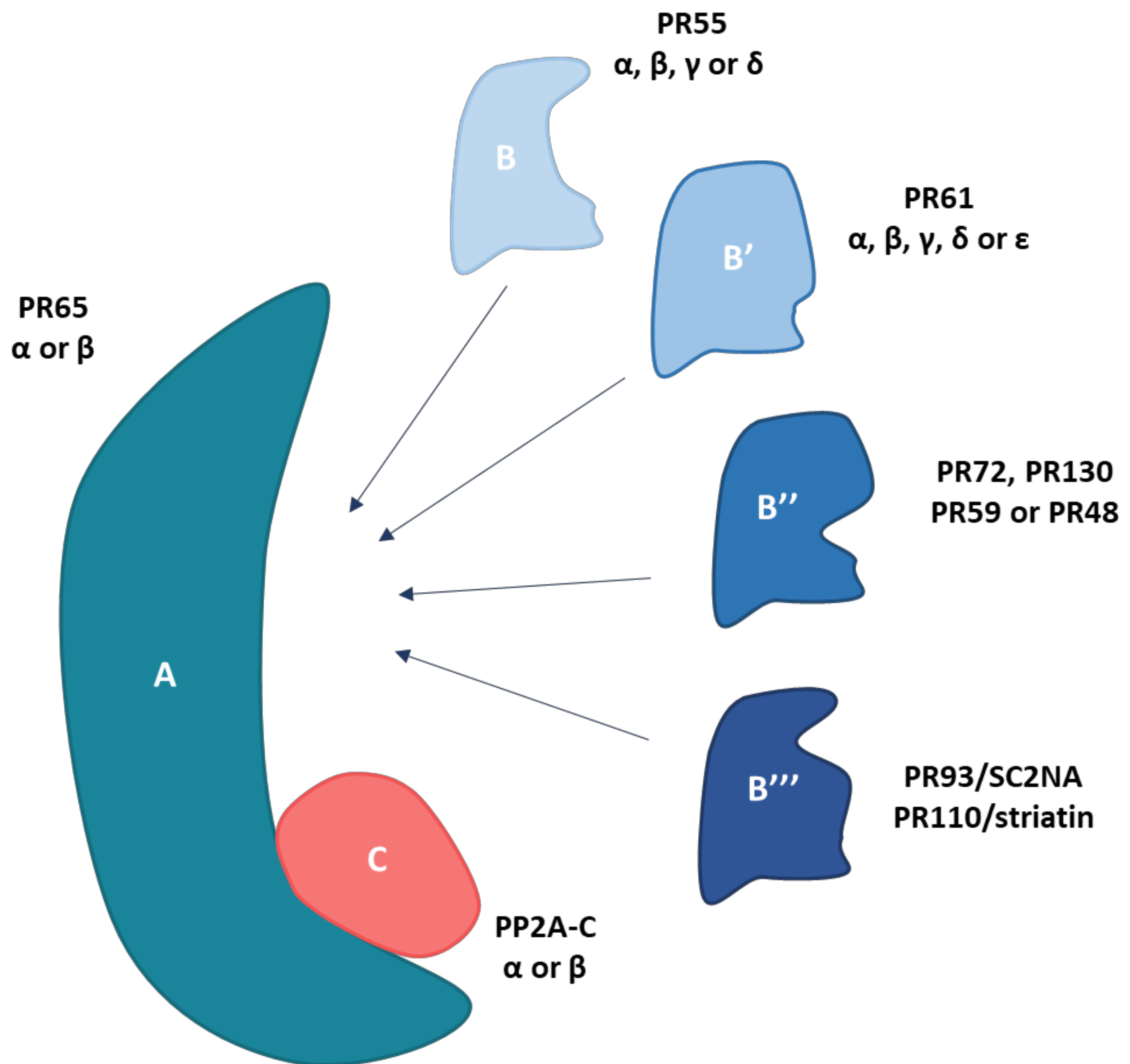

Figure 12: Structure of protein phosphatase 2A (PP2A)

PP2A holoenzyme contains the catalytic subunit C (pink), a structural or scaffold subunit A (left, blue), and a third variable regulatory subunit, that can be a member of four different families B/B'/B''/B''' which are structurally unrelated. In mammals, A and C are encoded by two genes (α and β); the B/PR55 subunits are encoded by four related genes (α, β, γ and δ); the B'/PR61 family are encoded by five related genes (α, β, γ, δ andε), the B'' family contains three related genes, encoding PR48, PR59 and the splice variants PR72 and PR130; PR93 (SG2NA) and PR110 (Striatin) form the B''' subunit family.

- The catalytic subunit of PP2A (PP2A-C):

Mammalian PP2A catalytic subunit is encoded by two distinct ubiquitously expressed genes ($C\alpha$ and $C\beta$) with the α being about 10-fold less expressed than the β due to a stronger promoter (344, 457, 458). The C subunit encoding genes share 97% sequence identity and are both 35 kDa in size (390, 457). The levels of expression of *PP2A-C* are tightly controlled at the translational and post-translational levels (459). The catalytic subunit has a conserved active domain that contains a bimetallic active site for phosphor-ester hydrolysis. It also exhibits a characteristic conserved sequence ^{304}TPDYFL^{309} at the C-terminus that undergoes post-translational modifications (386). This domain resides at a critical interface between the scaffold A subunit and the regulatory B subunit (460-462). Essentially, the recruitment of the B subunit is dependent on the methylation and phosphorylation patterns of the C-terminus (462-466), thus adding another dimension to holoenzyme regulation. Structural analysis revealed that the catalytic subunit recognizes specific motifs in the scaffold subunit, and specific residues are required for the interaction, and although other PPP family members share extensive sequence similarity with the catalytic subunit of PP2A, they do not associate with the PP2A scaffold subunit (344, 467). In *Drosophila*, there is one gene encoding for the PP2A catalytic subunit (418, 434). Numerous studies have linked PP2A to the regulation of cellular division, cell survival and apoptosis, cell fate determination and embryogenesis in *Drosophila.* The roles attributed to PP2A in these functions are detailed in chapter II of the results, section II.

- The scaffold subunit of PP2A (PP2A-A):

The scaffold A subunit is a structural subunit tightly associated with PP2Ac, forming a platform to which the appropriate B subunit can bind. The same overlapping sites within the A subunit are involved in the interaction with the B subunits, which explains why binding of the B subunits is mutually exclusive (468, 469). The PP2A scaffold subunit contains 15 tandem HEAT (huntingtin- elongation-A subunit-TOR) repeats, which form an elongated, horseshoe-shaped structure (470). These repeats are required for the interaction with PP2A-C giving rise to the core enzyme, where the scaffold protein folds in on itself forming a more horseshoe shape-like structure (460, 461). This mechanism forbids the catalytic subunit to get access to the PP2A substrate, which is recruited to the holoenzyme by the B regulatory subunit.

Similarly, as the catalytic subunit, two distinct mammalian PR65 isoforms are present in the cells, α and β, sharing 86% sequence identity (471). Most PP2A holoenzymes contain the PR65α isoform; while only a small fraction (10%) contain the PR65β that is expressed at much lower levels than PR65α in adult tissues. Despite sequence similarity, PR65β has unique biochemical properties and is not redundant with PR65α in mice, differing in its ability to bind with regulatory subunits (472). Interestingly, PR65β has been identified as a putative human tumour suppressor (473). Somatic alterations in the gene encoding PR65β were discovered in 15% of primary lung and colon tumour-derived cell lines. Afterwards, mutations in the gene encoding the PR65α isoform were detected in human melanomas and breast and lung carcinomas (390, 474). Same as the catalytic subunit, *Drosophila*'s genome contains one gene encoding for the scaffold subunit of PP2A (434).

- The regulatory B subunits of PP2A:

The PP2A core enzyme interacts with a variable regulatory subunit to assemble into a holoenzyme. The regulatory subunits comprise four families: B (also known as B55 or PR55), B' (B56 or PR61), B'' (PR48/PR72/PR130), and B''' (PR93/PR110/STRIATIN) (344). Each family of B subunits consists of different genes encoding two to five isoforms, some have different splice variants. To date, the human genome was found to contain 15 genes encoding at least 26 different alternative transcripts and splice variants of the PP2A B subunits (390). The striking features of the B subunits are their diversity, their differential expression in tissues and the lack of sequence similarities between the different families even though they recognize similar domains of the A subunit. While the scaffold Aα can interact with all mentioned regulatory B subunits, the Aβ scaffold is unable to interact with the B/PR55 family of B subunits and shows a preference for binding to PR72 (472). The B subunits are proposed to mediate substrate specificity of the PP2A holoenzyme complex (452). For example, the B' subunit, but not B or B'', was found to bind specifically to shugoshin, a centromeric protein required for chromosome segregation (475, 476). In contrast, the B, but not B' or B'' subunit was responsible for the de-phosphorylation of the microtubule-binding protein Tau, a process that was found to be altered in Alzheimer's disease (477, 478). Hence, the identification of the

subunits forming the PP2A holoenzyme in each physiological context seems to have a particular interest, unveiling major insight into PP2A function and specificity.

In Mammals, the B 55 kDa subunit is encoded by four genes (*PR*55α, *PR*55β, *PR*55γ and *PR*55δ), giving rise to at least six members; each gene is expressed in a tissue specific manner (479, 480). Both PR55α and PR55δ are expressed almost ubiquitously while PR55β and PR55γ are highly enriched in the brain. Substrate regulation by PR55 family members exhibits several features. First, substrate binding by the PR55 subunits appears to be dependent on a stretch of five degenerate WD40 repeats in its sequence, which are conserved 40 amino acid sequences that end with a characteristic tryptophan-aspartate (WD), mediating directly protein–protein interactions (481). Secondly, the PP2AC subunit of the core enzyme needs to be methylated on Leu^{309} and dephosphorylated at Thr^{304} for its interaction with the PR55 regulatory B subunits (462, 482).

The B' family contains at least five isoforms, α, β, γ, δ, and ε (483, 484). The human β gene encodes two isoforms, β1 and β2. The γ isoform has at least three different splice variants called γ1, γ2, and γ3. All B' family members exhibit a highly conserved central region with 80% sequence identity; however, the C- and N- termini are significantly more divergent. Hence, the conserved central region seems to be required for the interaction with the core enzyme while the ends may mediate subcellular targeting and substrate specificity (452).

The B'' members PR72 and PR130 were the first identified representatives of this family of regulatory subunits. It was suggested that they might arise from the same genes by alternative splicing, with differences in the N-terminus (485). PR130 is ubiquitously expressed while PR72 is mainly expressed in heart and skeletal muscle tissues. Afterwards, other family members were identified by a yeast two hybrid screen, the PR59, and PR48 which was identified as an interacting partner of Cdc6, a protein required for the initiation of DNA replication (486).

The fourth family of regulatory B''' subunits contains PR110, also known as Striatin, and PR93, or SG2NA, which were identified based on a conserved epitope with the B' subunits (487). Like the PR55 family, Striatin and SG2NA hold WD-40 repeats and interact with the

PP2A core dimer. Both proteins are also able to bind to Calmodulin in a calcium-dependent manner.

PP2A is an essential and multifarious player regulating distinct functions and signaling pathways in the cells. Initial understanding of PP2A was primarily based on general inhibitors such as okadaic acid that inhibits the activity of the catalytic subunit. As such, the precise roles of the PP2A holoenzymes and their specific roles in each signaling pathway were undisclosed. Afterwards, the advances in gene knock down technology have been able to dissect the individual roles of the PP2A holoenzymes. Hence, the current prospective is to elucidate the functions of the catalytic and other individual subunits that give rise to the holoenzyme, allowing to decipher the precise role and substrate of the PP2A phosphatase in each physiological context. In *Drosophila,* representatives of each of the mentioned families of PP2A regulatory subunits are found (422, 434, 488-503). They are discussed in the second chapter of results, section II. A summary of their functions and respective targets is given in **Table 2**.

2.3.1.4- Protein phosphatase 4 (PP4)

Originally called PPX when it was first cloned (435); PP4 is most closely related to PP2A among the PPPs. However, PP4 does not bind to the scaffold subunit of PP2A and few years later, it was found to have its own dedicated subunits (504). This phosphatase was localized to centrosomes and was shown to be implicated in centrosome duplication (436, 505). Afterwards, several studies demonstrated the fundamental roles of PP4 in cell signaling, separately from other PPPs, giving this phosphatase recognition for its numerous functions. In 2007, Shui et al reported that the knockout of PP4 in mice is embryonic lethal and PP4 null thymocytes exhibit decreased proliferation and enhanced apoptosis (506). PP4 was also found to regulate C-Jun N-terminal kinase via Tumor Necrosis Factor-α (472); and to de-phosphorylate histone H2AX during DNA replication and in response to DNA damage (507, 508). Like its closely affiliated phosphatase PP2A, PP4 functions as a heterotrimeric complex, consisting of one catalytic subunit (PP4c) that associates with a structural protein and a regulatory subunit (507, 509).

Table 2: Summary of the regulatory B subunits and their functions in *Drosophila*

Regulatory B subunit	Cellular mechanism and or signaling pathway	Substrate	Regulatory effect	Reference
Twins (Tws, PR55, aar, B55, cg6235)	Cell cycle progression, centrosome attachment to nuclei	-	Activate	(487)
	Mitosis, Greatwall-Endos axis	-	Inhibit	(488)
	Mitosis and wing Growth	Verthandi	Activate	(489)
	DNA damage repair	Ku70	Activate	(490)
	Wnt signaling	β-catenin	Activate	(491)
Widerborst (wdb, B', cg5643)	Planar cell polarity, hair outgrowth	-	Activate	(492)
	Survival and inhibition of apoptosis	-	Inhibit	(433)
	Insulin receptor signaling cascade and neuronal stem cells reactivation	Akt	Inhibit	(493)
	Insulin like Growth factor and lipid metabolism	Ci	Activate	(494)
	Hedgehog signaling	Smo	Inhibit	(421)
	Hedgehog signaling	Akt	Inhibit	(495)
	TOR pathway and autophagy	-	Inhibit	(496)
Well rounded (wrd, B', B56, cg7913)	Survival and inhibition of apoptosi	-	Inhibit	(433)
	Insulin/TOR signaling	S6K	Inhibit	(498)
	TOR pathway and autophagy	Atg		(496)
	Synaptic growth at neuromuscular junction	-	Activate	(499)
PR72 (B'', cg4733)	Wnt signaling	-	Activate	(500)
Cka (connector of Kinase to AP-1, B''', Striatin)	Hippo signaling	Hp	Inhibit	(501)
	Hippo signaling and neuronal stem cells reactivation	Hpo	Inhibit	(493)
	Microtubule organization and neuronal morphogenesis	-	Activate	(502)
	JNK pathway and spermatogenesis	-	Inhibit	(497)

- The catalytic subunit of PP4 (PP4c)

The sequence of the mammalian PP4c was deduced from several cDNAs cloning and by its amino acid identity with PP2Acα and PP2Acβ isoforms (65% sequence homology). Due to the high degree of conservation of this phosphatase, it was likely to have distinct functions from PP2A (436, 510, 511). The functional redundancy between PP2A and PP4 was observed with the PP4 orthologue in *Saccharomyces cerevisiae*, PpH3p (512, 513), but was opposed in later studies on PP4 in *Drosophila melanogaster.* Particularly, a role for PP4 in microtubule nucleation was proposed, independently of PP2A, where embryos deficient in PP2A were still able to nucleate and elongate microtubules from centrosomes (436). Regarding their structure, Ppp4c and Pph3 were shown to belong to the PPP family of Ser/Thr phosphatase, as they contain all the indicative characteristics of this family and were found to be closely related to PP6 (509). PP2Ac, PP4c and PP6c are the only catalytic subunits among the PPP family that undergo methyl-esterification at their carboxy-termini, which regulates their functions and interaction with regulatory subunits (466, 514). *PP4-19c* is the PP4 catalytic subunit encoding gene in *Drosophila melanogaster*. This phosphatase exhibits crucial roles in cellular division and is associated to the regulation of developmental signaling pathways. The functions played by PP4-19c in *Drosophila* are detailed in chapter I of the results, section I-2.

- The structural subunits of PP4

In 1999, the structural subunit of PP4 was identified using gel filtration chromatography followed by mass spectrometry analysis. Kloeker *et al* found that in mammalian cells, PP4 exists in high molecular mass complexes containing two major proteins: the PP4 catalytic subunit (35 KDa) plus a protein, termed PP4 regulatory subunit 1 (PP4R1). The amino-acid sequence of the 105 KDa protein was used to isolate a human cDNA clone that was tagged and expressed in the cells, to show that PP4c co-immunoprecipitates with PP4-R1. Analysis of the predicted amino acid sequence revealed a "PP2A-A-like" structure containing 14 ''heat'' repeats (504). An isoform of R1, termed R_{MEG} was also reported. It presents a small insertion near the N-terminus (515). Following the same approach, Hastie *et al,* in 2000, have identified a second scaffold protein termed PP4-R2, a highly asymmetrical protein with a 55 KDa molecular mass (516). PP4-R1 and PP4-R2 do not bind PP2Ac (507, 509). One orthologue of *PP4-R2,* also named R2 or *CG2890,* exists in *Drosophila* (516, 517).

- The regulatory subunits of PP4

Several proteins were identified that associate to PP4, but whether these proteins represent *bona fide* regulatory subunits remains unclear. Indeed, PP4c-R2 dimer was found to bind Gemin3 and Gemin4 proteins, components of the Survival of Motor Neurons complex, enhancing the temporal localization of small nuclear ribonucleoproteins (518). In addition, HDAC3, histone deacetylase, copurifies with PP4c-PP4R1 complex which controls HDAC3 activity (519). Some of the interacting proteins were also able to associate with other PPPs catalytic subunits. For example, PPP4c, PP2Ac and PPP6c have been identified in a yeast two-hybrid screen as proteins that interact with α4, a mammalian protein related to Tap42p in *S. cerevisiae* which appears to be controlled by TOR protein kinase, the target of an immunosuppressant drug, rapamycin (520, 521). Beside these PP4-interacting proteins, the major form of the trimeric holoenzyme, conserved from yeast to humans, comprises PP4c with the structural subunit R2 and a regulatory subunit R3 (517). PP4R3 is the orthologue of Psy2 in *Saccharomyces cerevisiae* (507). Two isoforms of R3 (also known as SMEK) exist in humans, α and β, sharing sharing 67% sequence identity and 77% homology at the amino acid level (507). PP4R3-like proteins are conserved throughout eukaryotes: The *Drosophila* falafel (*flfl*) protein shares 58% identity with R3-α at the amino acid level, and the *S. cerevisiae* Psy2 protein exhibits 27% identity with R3α (507). R3 proteins are thought to be substrate targeting subunits that provide specificity of the PP4 phosphatase complex (517).

2.3.1.5- Protein phosphatase 5 (PP5)

PP5 is encoded by a single gene throughout eukaryotes (344). Uniquely among the PPP family, the canonical PP5 carries the catalytic and the regulatory domains within the same polypeptide (522). The regulatory domain is localized at its N-terminus and contains the tetratricopeptide repeat (TPR) domain, a known protein-protein interaction motif (522). This latter, together with a C-terminal helix, maintain free PP5 in an auto-inhibited conformation by blocking the access to the active site (523). Interactions with the TPR domain lead to ligand-induced conformational change activating PP5 and allowing it to respond to a number of cellular factors (524); thus, attesting of the importance of this domain in regulating PP5 function. Indeed, interactions with the TPR domain by Hsp90 and fatty acids such as arachidonic acid lead to release of autoinhibition (525). PP5 is ubiquitously expressed in

mammalian tissues and regulates cellular proliferation, differentiation, migration, survival and death, and DNA damage repair (526). In particular, PP5 plays an important role in hormone and stress-induced signaling, as it modulates glucocorticoid receptor (GR) signaling through direct interaction with hsp90-GR complex (527). *PpD3* in *Drosophila* is the orthologue of mammalian PP5 (439). The functions of *PpD3* in *Drosophila* are not well documented, but it is highly expressed in the embryo than at later developmental stages, which suggests a role in cellular division and development (439).

2.3.1.6- Protein phosphatase 6 (PP6)

PP6 was first discovered in saccharomyces *cerevisiae*, named Sit4, as one of the suppressors of initiation of transcription defects, with a sequence resembling that of PP2A (528). Human PP6 was cloned in 1996 and was shown to be the functional homologue of Sit4 (529). In yeast, Sit4 is required for the progression through the cell cycle, by regulating the expression of G1 cyclins (530). Regulatory subunits for this phosphatase, termed Sit4-associated protein (SAP) were also isolated and shown to be required for its function (530). Sequence alignment with the core domain of the yeast SAPs allowed for the separation of specific subunits for PP6 in humans (531). The human SAPS bind Sit4 in yeast and restore SAP function, showing that the catalytic and regulatory subunits are conserved (532). In vertebrates, PP6 has three subunits of the Ankrd (ankyrin repeat domain) protein family (531). In analogy with PP2A, the catalytic subunit of PP6 is thought to form a heterotrimeric holoenzyme, with SAP domain-containing scaffold subunit and an ankyrin repeat subunit that likely serves as the regulatory subunit (531). In 1993, Mann et al showed that PpV is the *Drosophila* ortholog of budding yeast SIT4 and that the N-terminal 50 residues determined specificity (438). Ppv is associated to the negative regulation of the JNK pathway, where its disruption promotes tumor growth via a JNK-dependent manner (533). In addition, this phosphatase is linked to the AMPK pathway for the regulation of lipid metabolism (534).

2.3.1.7- Protein phosphatase 7 (PP7)

PP7 is a phosphatase unique to plants (344). It was first cloned and identified in *Arabidopsis thaliana* as a novel protein Ser/Thr phosphatase, with a sequence unrelated to any of the identified PPPs (535). Afterwards, PP7 was characterized in a bacterial system where

PP7 was shown to contain three insertions in its phosphatase domain that kept it inactive. The recombinant protein gained phosphatase activity only after cleavage of the longest insertion, suggesting an auto-inhibitory role (536). PP7 was resistant to okadaic acid and was stimulated by Mn^{2+} or Fe^{2+} (536). Afterwards, PP7 was shown to interact with Calcium-calmodulin in *Arabidopsis thaliana*, but in contrast to calcineurin, this interaction appears to inhibit the phosphatase activity (537). In *Drosophila*, Retinal degeneration C (*rdgC*) was discovered as a Calcium-calmodulin regulated protein phosphatase protecting retina from light-induced degeneration. It is similar to PP7 (416).

2.3.2- Evolutionary conservation of PPPs

PPPs encoding genes exhibit sequences that appear in all eukaryotes as well as in bacteria and archaebacteria, highlighting their remarkable conservation through evolution (538, 539). Members of the PPP family show a remarkable level of sequence identity across species (approximately 80%), which is among the highest degree of conservation for any enzyme. Indeed, mammalian Pp4c and *Drosophila* PP4-19c share 94% amino acid identity (509). PPPs are essential factors as their loss is detrimental on viability particularly in simple eukaryotes that hold only a single gene for any given PPP (540). This could explain the expansion of genes encoding distinct isoforms of these enzymes that can fulfill overlapping functions in animal cells, avoiding lethality due to loss of PPPs. Each individual subtype of PPP shows remarkable specificity in functional complementation across species (345, 386). For example, mutations in *S. cerevisiae* Sit4 are complemented by *Drosophila* Ppv and human PP6, establishing the functional equivalency of PPPs from divergent species (438, 529). However, loss of Sit4 is not complemented by another subtype of the PPP, such as PP4 or PP5, thereby reinforcing the functional specificity of each family of phosphatases. The outstanding conservation does not only cover catalytic subunits, but also regulatory subunits for each distinct family. The ability of human PP4 regulatory subunits SAPs to bind to yeast Sit4 and to functionally complement the yeast SAPs shows that these regulatory subunits have an equally critical role for the function of the phosphatase in different species (532). Therefore, complementary structures in catalytic and regulatory subunits mediate the assembly of distinctive multisubunit holoenzymes, and these features are conserved across evolution.

2.4- Protein phosphatases as drug targets

Supporting their essential roles in eukaryotic physiology, PPPs are targeted by several xenobiotics, such aspolyketides (okadaic acid, calyulin A) and cyclic peptides (microcystin, nodularin) that bind to PPP active sites. By inhibiting PPPs, these molecules are cytotoxic (541). Since their discovery, these toxins were widely used to identify the broad repertoire of PPP substrates and, in turn, elucidate signaling pathways for biological functions for years, before the set-up of molecular cloning and gene silencing techniques (542, 543). They served as useful tools to inhibit PPPs *in vivo* because some can penetrate into cells and do not inhibit PPMs or Aspartate based phosphatases. PP1 and PP2A subfamilies were the first identified to be the targets of natural toxins. PP4, PP5 and PP6 were later shown to be effectively inhibited by these compounds with different affinities. However, the catalytic subunits of PPP have broad substrate specificity. It is only upon association with regulatory and/or targeting subunits that PPP holoenzymes exhibit substrate preferences. Therefore, selective inactivation of these enzymes needs to be enforced at the holoenzyme level rather than at the catalytic site itself. Indeed, compounds that prevent the assembly of particular holoenzymes will only affect a subset of the substrates, achieving the desired selective pharmacological inhibition towards PPPs, without causing tremendous side effects. Hence, it is crucial to attain a better understanding on the holoenzyme complexes and the specific substrate implicated in each molecular mechanism. The best and most successful examples for inhibition of PPP proteins are cyclosporin and FK506 that inhibit calcineurin. Although insensitive to PPP toxins, PP2B is inhibited by these compounds that have been clinically used as immunosuppressive drugs to counteract graft-versus-host disease following organ transplantation (449-451). The success of these drugs raises the possibility that inhibitors targeting individual PPPs may be developed to treat other human diseases.

Objectives

Aims and objectives of the PhD study

The general aim of my PhD project was to explore the fine-tuning of NF-κB-IMD signaling by protein Serine/Threonine phosphatases in *Drosophila*. It has been known that several core components of IMD pathway undergo inducible phosphorylations mediated by protein kinases (318). Given the increasing complexity of IMD pathway negative regulation in *Drosophila*, we question the role of reverting phosphatases, mediating an effective regulatory mechanism to modulate IMD signal activities. In contrast to kinases that have been extensively studied in these phosphorylation events, the counter-acting phosphatases obtained much less emphasis in the past. However, in recent years, increasing evidence has implicated that phosphatases play crucial and specific roles in the context of immune signaling in mammals.

In an attempt to identify new regulators of the IMD pathway, a high- throughput RNAi screen was previously conducted a in *Drosophila* S2 hemocyte- like cells in the laboratory (316). This screen identified Akirin that was characterized as a nuclear protein driving selectivity of Relish transcriptional activity (317). This screen also identified other potential candidates for both positive and negative regulators of the IMD pathway of which our first candidate encoding the catalytic subunit of a Serine Threonine Phosphatase, PP4c (544). The characterization of this phosphatase's function in the immune response led us to the identification of another phosphoprotein phosphatase that is also required for fine-tuning IMD signaling, protein Phosphatase 2A (PP2A). In the course of my PhD work, different approaches were used in order to have a better understanding of its role in the regulation of IMD signaling. These approaches are the following:

I- Tests on S2 cells

These tests included genetic analyses using RNAi, overexpression, epistasis analyses, observation of the cellular localization by confocal microscopy, biochemical tests for co-immunoprecipitation experiments and lastly biochemical analysis of protein phosphorylation state.

II- Tests *In-vivo*:

In order to further evaluate the function of these phosphatases in the regulation of the immune response, the results obtained from S2 cells were confirmed in adult flies using the UAS-Gal4 system to drive an RNAi-mediated depletion **(Figure 13)** or overexpression of these phosphatses.

The following chapters expose a review of the literature relative to the functions of each of these phosphatases together with our results concerning their function in the IMD pathway.

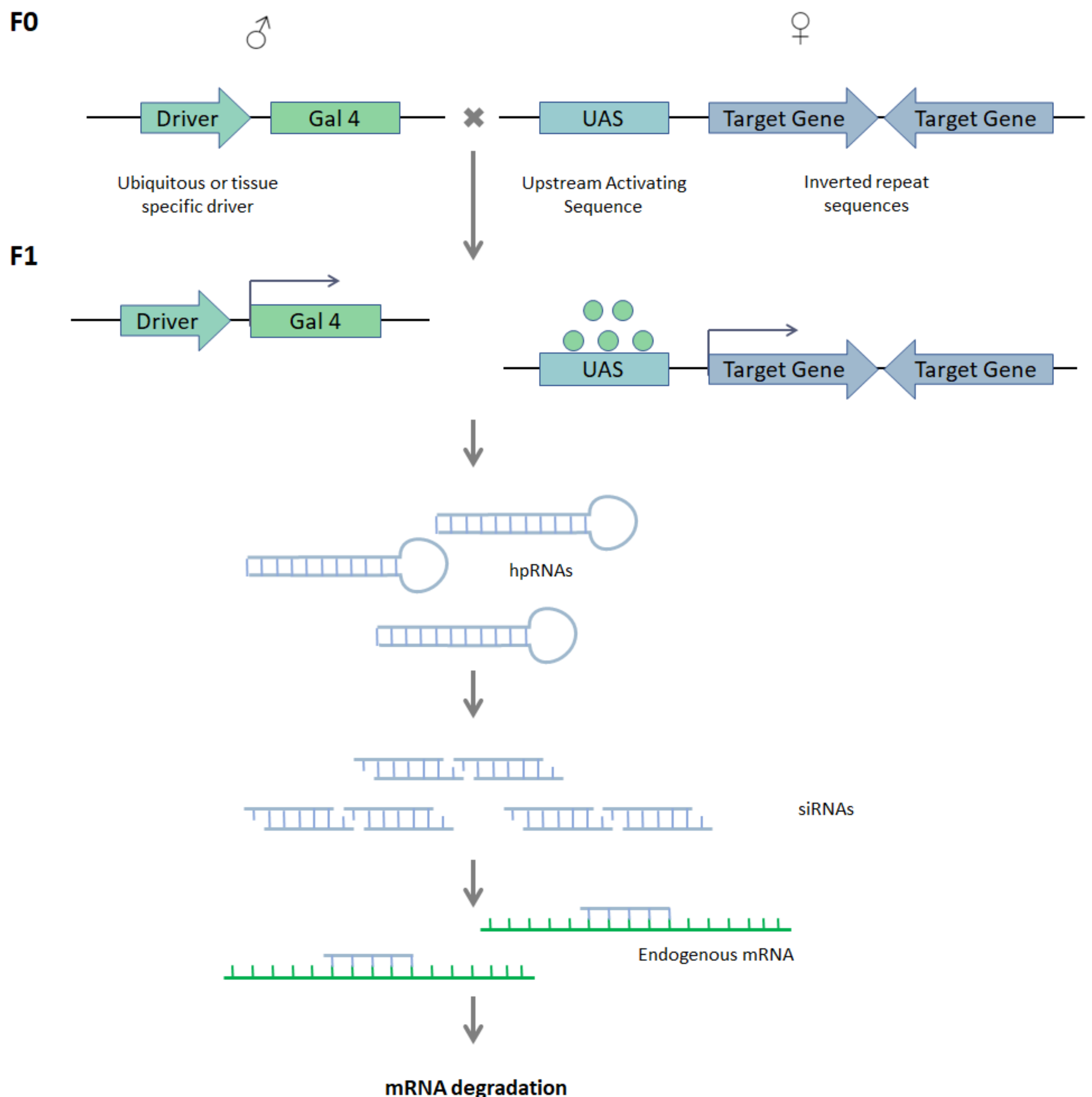

Figure 13: Inhibition of the Expression of a Target Gene in *Drosophila* using the UAS-GAL4 / RNA Interference System

Activation of the UAS-Gal4 system requires genetic cross between two transgenic lines. The first expresses the Gal4 protein, a yeast transcription factor, under the control of a ubiquitous or specific tissue driver. The expressed Gal4 protein becomes efficient when it binds to the DNA regulatory region, UAS (Upstream Activation Sequence). The second transgenic line carries inverted repeats DNA sequences of the target gene allowing the expression of double stranded RNA (dsRNA) under the control of the UAS sequence. The offspring of this cross possess both transgenes, but the dsRNA will only be produced in Gal4 expressing cells or tissues. The dsRNA produced is in the form of a hairpin (hairpin RNA: hpRNA) activating the RNAi system. Activation of this system leads to the cleavage of hpRNA into siRNA (single interfering RNA) by a ribonuclease III called Dicer. Afterwards, a strand of siRNA (the guide strand) will guide a degradation machinery called RISC (RNA-induced silencing complex) that will target the mRNA of the target gene to cleave it. Once cleaved, the target mRNA will be degraded.

Results

Chapter 1: PP4-19c, a new negative modulator of the IMD pathway targeting the IKK complex

I- Context of the study

Prolonged immune response triggered by spontaneous activation of NF-κB pathways is detrimental to the host (159, 239, 245, 339). Hence, it is vital that IMD signaling is subject to negative regulation, at various levels. In an attempt to isolate new modulators of the IMD pathway, a functional genome-wide RNAi screen was performed in *Drosophila* hemocyte-like S2 cells, prior to my arrival to the lab (316). Briefly, cultured S2 cells were treated with 21,306 RNAi probes and the ones that induced a moderate to marked effect on the expression of the IMD pathway dependent *AttacinA* gene, monitored by a luciferase reporter activity, induced by a Heat killed *Escherichia coli* (HKE) infection, were selected (316). The selected candidate genes were then divided into two groups, the positive regulators for which RNAi suppressed IMD-dependent Attacin induction and negative regulators that enhanced IMD activation when silenced by RNAi. One of the essential components of the IMD pathway named Akirin encoding a nuclear protein that was later characterized and shown to orchestrate Relish transcriptional selectivity, was initially identified in that screen (316, 317). Among the isolated negative regulators, we selected the gene encoding for the catalytic subunit of protein phosphatase 4 complex, *cg32505* or *pp4-19c* (544). Since it is well established that different components of the IMD pathway undergo reversible phosphorylation during the progression of the IMD intracellular signaling and that inappropriate regulation of this mechanism can have a profound effect on the signaling's outcome, this particular candidate gene presented an exceptional interest that intrigued us to further characterize its function in the regulation of IMD activation.

II- Protein Phosphatase 4 activity in *Drosophila*

Protein phosphatase 4 (PP4 also known as PPX)(435) is an essential and ubiquitous Serine/Threonine phosphatase that has gained recognition since its discovery over a decade and a half. It regulates many cellular functions independently of other related protein

phosphatases in the PPP family, including chromatin biology, DNA repair and cell cycle progression (509, 519, 545, 546). Like other members of PPP family, the catalytic subunit of PP4 (PP4c) interacts with regulatory proteins, which specify substrate targeting and intracellular localization. The identification of these regulatory proteins is, therefore, key to fully understanding the function of this enzyme class (507, 509). The major form of PP4, conserved from yeast to human cells, consists of one evolutionarily conserved catalytic subunit (PP4c) that associates with two types of regulatory subunits: a structural or scaffold protein, PP4R2 (R2, and R1 in mammals), and a regulatory 3 (R3) subunit, PP4R3 (also known as Falafel (Flfl) in *Drosophila*, homolog to of SMEK1 and SMEK2, the two known isoforms in mammals) (507, 509, 517) **(Figure 14).** However, other complexes have also been found in metazoans in which PP4c can associate with other regulatory proteins that presumably have distinct substrates and biological roles (for example, R4, HDAC3, α4 or Gemin4) (507, 509, 547). The catalytic subunit's amino acid sequence of PP4 is extremely conserved throughout evolution, with 94% identity between *Drosophila* and mammalian sequences (509).

The functions of PP4 in *Drosophila* started to gain recognition when it was found to be essential for centrosome maturation. Initially, a role of an okadaic acid sensitive Serine/Threonine phosphatase in microtubule nucleation was proposed, although the phosphatase involved was believed to be PP2A (548, 549). However, later studies have shown that *Drosophila* embryos deficient in PP2A are able to nucleate and elongate microtubules from centrosomes, while being unable to connect them to Kinetochore (433). Similarly to PP2A, PP4 was found to be sensitive to okadaic acid and was shown to localize at centrosomes (435). Therefore, PP4 seemed to be more likely a candidate for regulation of microtubules. In 1998, Helps and colleagues showed that a mutation in the PP4c encoding gene by a P-element insertion, gives rise to a homozygous strain termed *centrosome minus microtubule (cmm)* (436). Interestingly, this strain which was found to exhibit a decreased amount of PP4c mRNA and ~25% of wild type protein has a semi-lethal phenotype, with only 10% viability in certain conditions. Early *cmm* embryos displayed areas containing centrosomes with no radiating microtubules. In these regions, the mitotic spindles were either absent or aberrant and unconnected to the centrosome, and nuclei blocked in mitosis with condensed DNA. As nucleation of γ-tubulin was found to be essential for the nucleation of microtubules in

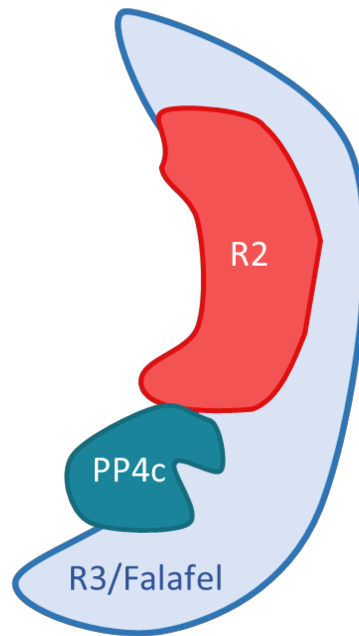

Figure 14: Schematic representation of the structure of protein phosphatase 4 (PP4) in *Drosophila*

A hypothetic model demonstrating that PP4 activity is mediated by the regulatory subunit 3 (R3), Falafel that directly interacts with the cellular target and the core enzyme, consisting of the catalytic subunit (PP4c) and the scaffold protein R2.

Drosophila (550), a role of PP4c in ensuring a conformational change in γ-tubulin or a relocation of γ-tubulin to the centrosomes was proposed. Since a decrease in immunostaining of γ-tubulin in *cmm* embryos was observed, it was suggested that PP4 is required for the initiation of microtubules nucleation, growth and/or stabilization (436). Afterwards, it was found that knockdown of either the catalytic or scaffold subunit of PP4 in S2 cells (PP4c and PP4-R2) leads to an accumulation of large 4C non-mitotic cells, blocking the G2 progression in the cell cycle (546). In 2008, Sousa-nunes and colleagues demonstrated a role of the complex PP4c-R2-R3 in the regulation of neuroblasts asymmetric division. In this study, Falafel (Flfl) was shown to be a key mediator for the specific localization of Miranda (Mira) and associated cell fate determinants during both interphase and mitosis. A cortical localization of Mira and associated proteins is detrimental for the divison of neuroblasts. Attenuating the function of either the catalytic subunit PP4c or of PP4R2 leads to similar defects in the localization of Mira and associated proteins, as observed in falafel mutants' neuroblasts (551). However PP4's substrate in the process of cell division regulation was still unknown. In 2015, Lipinszki et al showed that Flfl directly binds to the centromeric protein C (CENP-C) bringing thus PP4 activity to centromeres. CENP-C is the key centromeric protein required for kinetochore assembly and association to the mitotic centromere, a crucial step for proper chromosome segregation. They also dissected the binding surfaces between Flfl and CENP-C and showed the first crystal structure of the EVH1 domain of falafel subunit in complex with CENP-C. They further showed that PP4 activity is required for the dephosphorylation of both Flfl and CENP-C (517). Afterwards, a third PP4-inetracting regulatory subunit was revealed, named PTPA for phosphotyrosyl phosphatase activator. This factor was reported to act as a regulator for the effective basal localization of the Mira complex during mitosis of larval neuroblasts asymmetric divisions. It was shown that PTPA functions with the PP4 complex to promote the cortical association of Mira, through dephosphorylation of the amino acid residue T591 (552).

Besides its crucial role in cellular division, PP4 was associated to the regulation of developmental signaling pathways. Using an *in-vivo* RNAi screen, PP4 was identified as a phosphatase that influences Hedgehog (Hh) signaling by regulating the transducing transmembrane protein Smoothened (Smo). RNAi knockdown of PP4 elevates Smo phosphorylation and accumulation, leading to an increased Hh signaling activity (495).

Furthermore, in a screen for modifiers of Wingless (Wg) signaling in the *Drosophila* wing imaginal disc, the three components of PP4 complex, PP4c, PP4R2 and falafel, were found to reduce Wg target genes following their knockdown through RNAi (501). This novel PP4 function was further dissected in 2017 and was shown to be caused by effects on wg transcriptional regulation by Notch. PP4 complex was reported to function in the nucleus to promote Notch signaling (553). In addition, a role of Falafel in the negative regulation of TNF-JNK signaling-induced cell death *in vivo* was proposed (554).

These crucial and intriguing roles played by PP4 in cellular signaling and regulation are conserved through evolution. In addition to these functions reported in *Drosophila*, few studies have shown the implication of PP4 in the modulation of NF-κB mediated responses in mammals (509), though the molecular mechanisms underlying these observations and the precise targets are not very clear. For example, PP4 was identified in a two hybrid screen with c-Rel as bait and was shown the bind to c-Rel, NF-κB p65 and p50 by immunoprecipitation (555). In addition, TNFα is associated with an increase in transcriptional activity of NF-κB and was observed to activate endogenous PP4c about 4-fold (556). Furthermore, in 2012 Brechmann *et al* demonstrated that the PP4R1-PP4c complex is an important negative regulator of canonical NF-κB signaling in T cells. They discovered that in activated T cells PP4R1 directly interacted with IKKα (557). In *Drosophila,* no previous studies have implicated PP4 in NF-κB signaling modulation.

Based on the initial phenotype obtained after the KD of *PP4c* in the screen, we investigated the function of this phosphatase as a potential regulator of the immune response. In this chapter, I discuss the characterization of PP4 holoenzyme, as a new negative regulator of the IMD pathway. Our results show that PP4c with its two regulatory subunits, PP4R2 and Falafel, are implicated in fine-tuning the IMD pathway activation profile, both in S2 cells and in adult flies upon an immune stimulation. Furthermore, using genetic and biochemical interaction studies, we show that PP4 inhibits the activation of IMD signaling cascade at the level of the IKK complex, and specifically interacts with the latter. Taken together, we have identified a novel role for PP4 in regulating IMD-NF-κB immune response in *Drosophila*.

III- Results

1. **Manuscript: The PP4 phosphatase targets the IKK complex for the downregulation of the IMD-NF-κB pathway in *Drosophila* immune response (submitted in the journal of immunology).**

The PP4 phosphatase targets the IKK complex to downregulate the IMD-NF-κB pathway during the *Drosophila* immune response

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Abstract

The evolutionarily conserved IMD signaling pathway shields *Drosophila* against bacterial infections. It regulates the expression of antimicrobial peptides (AMP) encoding genes through the activation of the NF-κB transcription factor Relish. Tight regulation of the signaling cascade ensures a balanced immune response which is otherwise highly harmful. Several phosphorylation events mediate intracellular progression of the IMD pathway. In particular, phosphorylation of the Inhibitor of κB kinase complex (IKK) is essential for stimulating Relish transcriptional activity. However, signal termination by dephosphorylation remains largely elusive. Here, we identify the highly conserved Protein Phosphatase 4 (PP4) complex as a *bona fide* negative regulator of the IMD pathway. RNAi-mediated gene silencing of *PP4-19c*, *PP4R2* and *Falafel,* which encode the catalytic and regulatory subunits of the phosphatase complex respectively, caused a marked up-regulation of bacterial-induced AMP gene expression both in cell culture and in adult flies. *PP4* deficient flies also exhibit an inflammatory-like state that is marked by a reduced lifespan in the absence of any infection. In contrast, flies overexpressing this phosphatase are highly sensitive to bacterial infections. Our data show that PP4 specifically interacts with the IKK complex for the subsequent inhibition of IMD activation. Altogether, our results highlight an evolutionary conserved function of PP4c in the regulation of NF-κB signaling from *Drosophila* to mammals.

30 # Introduction

31 Since the discovery of NF-κB transcription factors in 1986 (1), concerted research activities
32 have provided considerable progress in elucidating the triggers and the components of their
33 signaling cascades as well as in characterizing their functions. A particular interest for the
34 characterization of NF-κB signaling stems from their central role in the regulation of
35 inflammation and innate immune reactions. Indeed, NF-κB factors control the expression of
36 genes encoding effector and co-stimulatory molecules as well as inflammatory cytokines that
37 are essential for the onset of an efficient immune response against invading microorganisms.
38 However, besides their beneficial effects in controlling the infections, exacerbated NF-κB
39 signaling is highly detrimental. Accordingly, the intensity and duration of their signaling are
40 tightly controlled and their deregulation is frequently associated with chronic inflammatory
41 diseases, tissue damage and autoimmune diseases, as well as the development and
42 progression of tumors (2-6). A keen interest is thus attributed for the identification and the
43 characterization of the regulatory processes, which ensure the proper modulation of NF-κB
44 signaling profiles.

45 NF-κB pathways are highly conserved and the *Drosophila* model has provided a prominent
46 insight into their role in the regulation of the innate immune response in metazoans (7, 8).
47 Two NF-κB pathways: the Toll and the Immune deficiency (IMD) pathways, play a crucial
48 role in controlling the *Drosophila* immune response. More precisely, these pathways regulate
49 the expression of antimicrobial peptides (AMPs) encoding genes, which constitute the
50 principal effectors of the humoral response (9-12). Both pathways share considerable
51 similarities with NF-κB cascades controlling innate immunity and inflammation in mammals.
52 Notably, the Toll receptor is the founding member of the Toll-Like Receptors family in
53 mammals and its downstream signaling cascade is analogous to the Myeloid differentiation

54 factor 88 (MyD88)-dependent TLR signaling cascade (7, 13). The IMD pathway is akin to
55 the Tumor Necrosis Factor Receptor (TNFR) signaling pathway and also resembles the TIR-
56 domain-containing adapter-inducing interferon-β (TRIF) -dependent TLR signaling in
57 mammals (7, 14-16).

58 The IMD pathway is activated upon the sensing of Diaminopimelic acid (DAP)-type
59 peptidoglycan by the PeptidoGlycan Recognition Proteins (PGRP)-LC and PGRP-LE on the
60 cell membrane or in the cytosol respectively (17-27). Ligand binding triggers receptor
61 multimerization and proto-amyloid formation through the conversion of cryptic Receptor
62 Homotypic Interaction Motif (cRHIM). This receptor agglomeration in turn seeds fibrils
63 formation of the adaptor protein IMD which sequence also carries cRHIM motifs (28, 29).
64 Via its death domain, that is homologous to that of the mammalian Receptor Interacting
65 Protein (RIP1), IMD further recruits a signaling complex including, the *Drosophila* Fas
66 Associated Death Domain (dFADD) adaptor and the caspase 8 homologue, Death related
67 ced-3/Nedd2-like protein (DREDD) (30-33). Upon its ubiquitinylation by the E3 ligase
68 *Drosophila* Inhibitor of Apoptosis 2 (dIAP2), the latter cleaves IMD at its N-terminus thus
69 exposing an evolutionarily conserved IAP binding motif (34-39). Consequently, with the
70 concerted activity of the E2 conjugating enzymes Bendless (Ubc13), Uev1a as well as Effete
71 (Ubc5), dIAP2 further targets IMD for K63-linked ubiquitin chains (39). These connect IMD
72 to the Transforming growth factor β Activated protein Kinase 1(TAK1), via its associated
73 protein TAK1-Binding protein 2 (TAB2) and to the IKK signalosome which includes a
74 regulatory subunit (Kenny) and a catalytic subunit (Immune response deficient (Ird5)), both
75 homologous to mammalian Ikkγ and Ikkβ, respectively (37, 40-44). The establishment of this
76 ubiquitin-dependent signaling platform is presumed to activate TAK1 which in turn
77 phosphorylates Ikkβ; itself required for the phosphorylation of the *Drosophila* NF-κB
78 transcription factor Relish on serine residues in its Rel homology domain (28, 45). Like its

79 mammalian counterpart's p100 and p105, Relish is also characterized by a C-terminal
80 ankyrin-repeat IκB domain. This domain is cleaved by DREDD in an IKK dependent fashion
81 (45-48). Whereas the phosphorylation of Relish is not required for its cleavage and nuclear
82 translocation, this modification is crucial for the optimal expression of Relish-dependent
83 AMPs encoding genes such as *Attacin* (45, 48-50).

84 Several signal terminators have been shown to negatively regulate the IMD pathway by
85 acting at different levels and through different mechanisms (16, 51-53). These include
86 catalytic PGRPs, which degrade peptidoglycan into small entities of low immunostimulatory
87 potential. In addition, the non-amidase membrane-associated PGRP-LF receptor and the
88 alternatively spliced regulatory isoforms of PGRP-LC (rPGRP-LC), which lack the
89 intracellular cRHIM domain, likely act as decoy receptors preventing the intracellular
90 progression of the signaling cascade (54-66). At the intracellular level, Pirk, also known as
91 Rudra or PIMs, most likely interrupts the IMD amyloid fibrils signaling platform (29, 67-69).
92 Finally, several ubiquitinating and deubiquitinating enzymes were described to promote K48-
93 linked ubiquitination and subsequent proteasomal degradation of IMD pathway signaling
94 intermediates or to interrupt the formation of K63-linked ubiquitin chains which are
95 perquisite for signal transmission (70-79). In particular, IMD is targeted to proteasomal
96 degradation upon its phosphorylation by TAK1 a process that likely triggers both the removal
97 and the addition of K-63 of and K-48 polyubiquitin chains respectively (42). Despite the
98 identification of several protein kinases in the IMD-NF-κB cascades, far less is known about
99 negative regulators operating signal termination by de-phosphorylation.

100 In this article, we report the characterization of the Protein Phosphatase 4 (PP4) holoenzyme,
101 as a new negative regulator of the IMD pathway. We show that PP4c and its two regulatory
102 subunits, PP4R2 and PP4R3, also known as Falafel, are required for the proper down-

103 regulation of the IMD pathway following an immune stimulus. Moreover, our results indicate
104 that flies deficient for the expression of *PP4c* exhibit an inflammatory-like state that is
105 marked by a reduced lifespan in the absence of any infection. Finally, we show that PP4
106 specifically interacts with the IKK complex for the inhibition of Relish activation. Overall,
107 our results highlight an evolutionary conserved function of PP4c in the regulation of NF-κB
108 signaling from *Drosophila* to mammals.

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123 # Materials and Methods

124 **Plasmid constructs**

125 A complementary DNA clone for PP4-19c (FMO03839) was obtained from the *Drosophila*
126 Genomics Resource Center. This clone contains a metallothionein promoter and Flag-HA tag
127 C-terminal fusion. Metallothionein promoter expression plasmids encoding FLAG-tagged
128 PP4-19c (wild-type and phosphatase inactive mutant) and FLAG-tagged FLFL were a kind
129 gift from Zoltan Lipinszki (80). pAC-PGRP-LC, pAC-IMD, pAC-Rel (ΔS29-S45) constructs
130 were described previously (81, 82).

131 **Fly strains**

132 Stocks were raised on standard cornmeal–yeast–agar medium at 25°C with 60% humidity.
133 relishE20 (46) and Dif^{1} (83) flies were used as mutant deficient for the IMD and Toll
134 pathway, respectively. Flies carrying an UAS-RNAi against Pp4-19c (25317), R2 (1053999),
135 R3 (103793) were obtained from the Vienna *Drosophila* RNAi Center
136 (http://stockcenter.vdrc.at/control/main). Flies carrying a UAS-RNAi transgene against GFP
137 (397-05) were obtained from the *Drosophila* Genetic Resource Center (Kyoto, Japan; http://
138 www.dgrc.kit.ac.jp/index.html). Flies carrying an UAS-PP4-19c construct (F001063) (84)
139 was obtained from FlyORF (http://flyorf.ch/index.php). Flies carrying an UAS-GFP construct
140 (BL#5431) were obtained from Bloomington *Drosophila* stock center. Flies carrying Gal4
141 driver C564 (6982) used to express UAS constructs in the fat body (85) were obtained from
142 Bloomington *Drosophila* Stock Center (Bloomington,USA; http://flystocks.bio.indiana.edu/).
143 Flies carrying Gal4 driver Yolk (33) used to express UAS constructs in the fat body. Gal4-
144 driven RNAi and ORF expression was enhanced by incubating 2-3 days-old flies for six
145 further days at 29°C.

146

147 **Microbial strains and infections**

148 We used *Escherichia coli* strain DH5αGFP, *Enterobacter cloacae*, and *Micrococcus luteus*
149 (CIPA270) bacteria for septic injuries (86). The *E. coli* strain DH5αGFP was generated in our
150 laboratory. Bacteria were grown in Luria broth (LB) (*E. coli*, *E. cloacae*) or brain–heart
151 infusion broth (BHB) (*M. luteus*) at 30°C (*E. cloacae, M. luteus*) or 37°C (*E. coli*). Survival
152 experiments were performed on 15–25 females infected by *E. cloacae* septic injury at 29°C
153 three independent times. Control survival experiments were made by sterile injury (86). qRT–
154 PCR experiments were performed on 10–20 nine-day-old females not infected and infected
155 with *E. coli* for 4 h and 16h or *M. luteus* for 24 h, by septic injury at 29°C, three times
156 independently.

157 **Cell culture, transfection and luciferase reporter assay**

158 S2 cells were cultured at 25°C in Schneider's medium (Biowest) supplemented with 10%
159 fetal calf serum (FCS) (Thermo scientific, lot RUF35205), 8mM penicillin/streptomycin
160 (Gibco) and 100U/mL L-Glutamin (Gibco). For transient transfection, S2 cells were seeded
161 in a 24-well plate at 0.5x106/ml. Transfection was performed by the calcium phosphate co-
162 precipitation method. Each plate was transfected with 10 µg of indicated plasmids. After 12-
163 16 h, the cells were washed with PBS and incubated in fresh medium. For expression of
164 recombinant proteins by expression vectors containing a metallothionein promoter (pMT),
165 CuSO4 was added. 48 h later, cells were infected with heat-killed *E. coli* for IMD activation.
166 Luciferase reporter assays were performed for IMD pathway activation measurements.
167 Briefly, S2 cells were transfected with Attacin-luciferase reporter (87) and Actin-Renilla and
168 the pathway was induced with HKE 60h after transfection. 24h later, S2 cells were harvested
169 by centrifugation and lysed in Passive Lysis Buffer (Promega). Luciferase and Renilla
170 activities were measured using standard procedures.

171 **Immunofluorescence**

172 Cells were seeded on eight-wells Lab-Tek® Chamber Slide, rinced with PBS 1x and fixed
173 with 2% paraformaldehyde. Cells were then permeabilized with 0.1% Triton X-100, saturated
174 with 3% BSA, incubated 1h with HA mouse antibody ab18181 (abcam), then with Cy3 goat
175 anti mouse secondary antibody A10521 (life technologies). Slides were mounted in a solution
176 of Vectashield/DAPI and samples were observed using a Zeiss LSM780 confocal
177 microscope. Images were processed using ImageJ and Adobe Photoshop.

178 **Immunoprecipitation and Western Blot**

179 The cells were harvested 72 hours after transfection, washed in PBS, and lysed in 500 ul of
180 buffer containing 30 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM MgAc, 2 mM DTT, 1% NP-
181 40, and complete protease inhibitor cocktail (Roche). Immunoprecipitations were performed
182 overnight with rotation at 4°C, using mouse monoclonal anti-flag or anti-HA antibodies
183 coupled with agarose beads (Sigma). Proteins from total cell lysates and immunoprecipitates
184 were resolved by SDS-PAGE and detected by Western blotting using rabbit anti-flag
185 (abcam), rabbit anti-HA (abcam) or Rat anti-HA-HRP linked (Roche), mouse anti-actin
186 (Milipore), and rabbit anti-PP4R2 (gift from Zoltan Lipinszki). The secondary antibodies
187 used are Mouse-HRP linked (NA931 GE HEALTHCARE), Rabbit-HRP linked (NA934 GE
188 HEALTHCARE) or Rabbit-HRP (W401B Promega).

189 **Gene knock down in S2 cells**

190 **dsRNA preparation**

191 DNA Templates for dsRNA preparation were PCR-derived fragments flanked by two T7
192 promoter sequences (TTAATACGACTCACTATAGG). Fragment for *GFP* is *GFP*
193 (nucleotides 35–736, GenBank accession L29345). The other fragments were generated from
194 genomic DNA templates using oligonucleotides designed for use with DKFZ Genome-RNAi
195 libraries. The corresponding references are: HFA21251 and BKN23059 for *PP4-19c,*
196 DRSC27825 for *PP4-R2*, 64410 for *PP4-R3* and DRSC37194 for *Relish*. Single-stranded

197 RNAs were synthesized with the MEGAscript T7 transcription kit (Ambion). Annealed
198 dsRNAs were ethanol precipitated and dissolved in sterile deionized water.

199 **dsRNA bathing**

200 Cultured S2 cells were pelleted and washed once in PBS to remove fetal calf serum (FCS)
201 supplemented Schneider's medium and resuspended in serum-free Schneider's medium
202 (Biowest) supplemented with 8mM penicillin/streptomycin (Gibco) and 100U/mL L-
203 Glutamin (Gibco) at 1,5 x 10^6 cells/ml. 30 µl of this cell suspension (45 x10^3 cells) was added
204 to 10 µl of dsRNA (500 ng/µl) and incubated at 24 °C for one hour in a U-shape 96-wells
205 plate. 160 µl of FCS-supplemented Schneider's medium was then added and cells were
206 incubated for six days at 24 °C. Cells were stimulated with heat-killed *E. coli* for 4 or 16
207 hours and frozen prior to RNA extraction.

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209 **Quantitative RT-PCR**

210 For quantitative analysis of *Attacin A, PP4-19c*, *R2*, *R3* and *rp49*, RNA from cells was
211 extracted and treated with DNAse, using Total RNA isolation NucleoSpin® 96 RNA
212 (Macherey-Nagel). RNA extraction from flies and dissected fat-bodies were extracted with
213 TRI Reagent® RT (Molecular Research Center) and BAN (4-bromoanisole) (Molecular
214 Research Center) after mechanical lysis by 1,4 mm ceramic beads using a Precellys®24
215 tissue homogenizer. cDNAs were synthesized using the Biorad iScriptTM cDNA Synthesis
216 kit and quantitative PCR was performed using Biorad iQ™ SYBR® Green. Real-time PCR
217 was performed in 384-well plates using CFX384 Touch™ Real-Time PCR Detection System
218 (Biorad). The amount of mRNA was normalized to control *rp49* mRNA values. Primers used
219 for Q-RT-PCR are for *Attacin A* (GGCCCATGCCAATTTATTCA-Forward and
220 AGCAAAGACCTTGGCATCCA-Reverse), *Drosomycin*
221 (CGTGAGAACCTTTTCCAATATGATG-Forward and TCCCAGGACCACCAGCAT-

222 Reverse), *Rp49* (GACGTTCAAGGGACAGTATCTG-Forward and
223 AAACGCGGTTCTGCATGAG-Reverse), *PP4-19c* (CCTTCACCTCGTTCTCCTTG-
224 Forward and ATGTCCGACTACAGCGACCT-Reverse), *PP4-R2*
225 (CGGTAACGCCGATGAGGGCT-Forward and CATTGTCGTCCGAACGCGGG-Reverse
226 for RNA extracted from S2 cells and CGATCCTCGGAAGCAGTA-Forward and
227 GATCGATTGTGCTAACCACTA-Reverse for RNA extracted from flies), *R3*
228 (ACAACAATGTCATGAAATCCGT-Forward and TGTGTGGCGGAGAGGAT-Reverse)
229 and *Relish* (CCACCAATATGCCATTGTGTGCCA-Forward and
230 TTCCTCGACACAATTACGCTCCGT-Reverse).
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243 # Results

244 **PP4c negatively regulates the IMD pathway**

245 In order to identify new regulators of the IMD pathway, we have previously conducted a
246 high- throughput RNAi screen in *Drosophila* S2 hemocyte- like cells (88). This screen
247 identified Akirin that we characterized as a nuclear protein driving selectivity of Relish
248 transcriptional activity (82, 88). In this study, we re-explored the results of this screen and
249 focused on genes inducing an over-activation of the IMD pathway when silenced by RNAi.
250 We thus selected the *CG32505*, which encodes the catalytic subunit of the phospho- serine-
251 threonine phosphatase 4 (PP4-19c) (89). To confirm this result, we used two non-overlapping
252 double stranded RNA (dsRNA) constructs, *dsPP4-1* and *dsPP4-2*, for silencing the *PP4-19c*
253 gene and monitored the *Attacin A* expression profile in S2 cells. We first confirmed that both
254 constructs efficiently silence the *PP4-19c* transcript as compared to the *dsGFP* control
255 (supplementary figure 1A). *PP4-19c* knock-down leads to a constitutive activation of the
256 IMD pathway in S2 cells (Figure 1A). Moreover, *dsPP4-19c* treated cells exhibit an
257 enhanced and prolonged activation of the IMD pathway 4 and 16 hours upon their stimulation
258 with heat-killed *Escherichia coli* (HKE) as compared to *dsGFP* control cells (Figure 1A).
259 Conversely, the overexpression of the wild-type *PP4-19c* construct significantly inhibits IMD
260 pathway activation following HKE induction (Figure 1B and supplementary Figure 1B). This
261 phenotype is strictly dependent on its catalytic activity since the overexpression of a
262 phosphatase dead-mutant (*PP4-PD*) construct does not alter *Attacin A* expression in immune
263 induced cells. Altogether, these results indicated that PP4-19c is a negative regulator of the
264 IMD pathway *ex-vivo*, in *Drosophila* S2 cells and prompted us to investigate its role in the
265 control of the immune response of adult flies.

266 PP4c is essential for centrosome maturation in the *Drosophila* embryo and loss of function
267 mutants exhibit a high lethality rate (90). Therefore, we took advantage of yeast UAS-Gal4
268 system to selectively drive a restricted expression of a dsRNA targeting the *PP4-19c*
269 transcript in the fat body of adult flies, the main immune organ of *Drosophila*, using either
270 the *yolk-Gal4* (91) or the *c564-Gal4* transgenes (85). In both cases, the expression of the
271 *PP4-19c* transcript was significantly reduced in the fat-body of the flies (supplementary
272 Figure 2A). Compared to *dsGFP* control flies, ds*PP4-19c* flies show an enhanced and
273 prolonged expression of *Attacin A* at 4 and 16 hours following their infection with *E. coli*
274 (Figure 2A and 2B). We show that, conversely to the IMD pathway, the Toll pathway is not
275 altered in flies deficient for the expression of *PP4-19c* as shown by the quantification of the
276 *Drosomycin* transcript, a conventional readout of the Toll pathway, 24 hours after the
277 infection of flies with *Micrococcus luteus* (Figure 2C). These results indicate that PP4-19c is
278 specifically involved in the regulation of the IMD pathway in *Drosophila*.

279 Impairing the expression of IMD pathway negative regulators is known to result in a
280 shortening of *Drosophila* lifespan. This is reminiscent to NF-κB dependent chronic
281 inflammatory diseases in mammals (57, 70, 92). We show that *dsPP4-19c* flies recapitulate
282 this shortened-lifespan phenotype (figure 2D) that correlates with an exacerbated activation
283 of the IMD pathway in the ageing flies (Figure 2E). In a complementary approach, we
284 overexpressed *PP4-19C* in the fat body of adult flies using the *yolk-Gal4* driver
285 (supplementary figure 2B) and checked for the IMD pathway activation 4 hours after their
286 infection with *E. coli*. Compared to control flies overexpressing *GFP*, the *Attacin A*
287 expression is significantly reduced in flies overexpressing *PP4-19c* (figure 2F). This impaired
288 IMD pathway activation is most probably accounting for the susceptibility of yolk-
289 Gal4>UAS:PP4-19c flies to an infection with the Gram-negative bacterium *Enterobacter*

290 *cloacae,* as is the case for the IMD pathway mutant Rel^{E20} (figure 2G). In sum, these results

291 further confirm the role of PP4-19C as a negative regulator of the IMD pathway *in-vivo*.

292 **The PP4R2 and PP4R3 regulatory subunits are required for the modulation of the IMD**

293 **pathway**

294 The major form of PP4, conserved from yeast to mammals comprises PP4c and two

295 regulatory subunits: a core protein, PP4R2 and a regulatory protein, PP4R3. Nevertheless,

296 several other proteins were shown to bind PP4c and additional mutually exclusive complexes

297 have been described in metazoans (93-95). In *Drosophila*, PP4R2 and PP4R3, also known as

298 Falafel (*Flfl*), are requisite for PP4c function in the regulation of developmental signaling

299 pathways such as hedgehog, c-Jun N terminal Kinase (JNK) and wingless, during centrosome

300 maturation and neuroblast asymmetric division as well as for the coordination of glial cells

301 recruitment and phagocytosis of degenerating axons from the central nervous system (80, 96-

302 101). To check whether these PP4c regulatory subunits are also required for modulating the

303 IMD pathway activation profile, we used specific dsRNA constructs to silence their

304 expression in S2 cells and further checked for the *Attacin A* expression 4 and 16 hours

305 following their stimulation with HKE. We first confirmed that the dsRNA constructs

306 targeting *PP4-R2* (*dsPP4-R2*) and *Flfl* (*dsFlfl*) efficiently reduced the expression of their

307 targeted transcripts (supplementary Figure 3). Similarly to the attenuation of *PP4-19c* by

308 ds*PP4-1*, impairing the expression of *PP4R2* and of *Flfl* leads to a significant increase in

309 *Attacin A* expression in HKE induced S2 cells (Figure 3A). Collectively these results indicate

310 that both PP4R2 and Flfl are required with PP4-19c for the negative regulation of the IMD

311 pathway in *Drosophila S2* cells. We further validated these results in transgenic adult flies in

312 which the RNAi-mediated knock-down of *PP4R2* and *Flfl* using either the *c564-Gal4* or the

313 *yolk-Gal4* drivers, leads to a systemic over-activation of the IMD pathway following an

314 infection with *E. coli* (Figure 3B and 3C).

315 **PP4c acts at the level of the IKK complex in the IMD pathway**

316 In an attempt to identify the cellular target of PP4-19c in the IMD pathway, we first undertook
317 epistasis analysis. To this aim, we overexpressed either PGRP-LC, IMD or a constitutively
318 active form of Relish (with a short internal truncation RelishΔS29-S45) (49) in S2 cells with
319 or without a concomitant overexpression of the full-length catalytically active form of PP4-
320 19c. We then monitored the IMD pathway activation using an *Attacin A- Luciferase* reporter.
321 As previously described, the overexpression of PGPR-LC and the IMD adaptor protein
322 triggers a constitutive activation of the IMD pathway (67, 81) (Figure 4A, 4B and 4C). The
323 activity of the *Attacin A* reporter is significantly reduced in cells co-transfected with the
324 *dsPP4-19c* construct (Figure 4A and 4B). Conversely, the induction of the *Attacin A* -
325 *Luciferase* reporter by the cells overexpressing *RelishΔS29-S45* is not affected whether they
326 are co-transfected with PP4-19c or not (Figure 4C). These observations indicate that PP4c acts
327 downstream of IMD and upstream of Relish. Additionally, confocal microscopy analysis
328 indicated a strictly cytoplasmic localization of the tagged PP4c-FLAG-HA in S2 cells that is
329 not changed whether the cells are stimulated or not by IMD pathway agonist (figure 4D).
330 Altogether, these data suggests that PP4 targets a cytoplasmic component of the IMD
331 pathway, acting downstream of IMD and upstream of Relish. In this context, we reasoned that
332 TAK1 and the IKK complex both constitute *bona-fide* candidates for the presumed target.
333 Indeed, these proteins are phosphorylated during the progression of the IMD intracellular
334 cascade and thus their de-phosphorylation is an appropriate mechanism for fine-tuning the
335 duration and the intensity of the signaling (40, 53).

336 To identify the target(s) of PP4-19c in the IMD pathway, we performed immunoprecipitation
337 experiments on protein extracts from S2 cells co-transfected with the tagged *HA- Flag-PP4-*
338 *19c* and *Flag-PP4-19c* and *Flag-TAK1*, *HA-IKKβ* or *HA-IKKγ*. Transfections were performed
339 with or without a PGRP-LC expression vector in order to analyze protein interactions in the

340 presence or the absence of an IMD activating stimulus respectively. As shown in Figure 5A
341 and B, no interaction between the tagged versions of TAK1 and PP4-19c recombinant
342 proteins is observed. In contrast, our results revealed both components of the IKK complex as
343 co-immunoprecipitant with the PP4-19c recombinant protein (figure 5B).Using an anti-R2
344 antibody, we also detected the endogenous PP4R2 protein in the PP4-IKK co-
345 immunoprecipitated complexes (figure 5B). These results indicate an interaction between PP4
346 and the IKK complex and support the hypothesis that PP4 inhibitory mechanism would
347 operate through the targeting of the IKK complex.

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363 ## Discussion

364 NF-κB transcription factors are key regulators of innate immunity and inflammation from
365 insects to mammals. However, their activation comes with a significant cost on fitness, tissue
366 homeostasis and lifespan (57, 70, 92, 102, 103). Therefore, the intensity and duration of NF-
367 κB signaling are tightly regulated in physiological conditions. Several negative regulators of
368 the *Drosophila* IMD-NF-κB pathway have been previously identified acting at multiple
369 levels and by different means. These include the interruption of the initial signaling trigger
370 and receptor activation, the disruption of supramolecular signaling complexes and the
371 proteasomal degradation of signaling intermediates upon K48 linked ubiquitination (28). In
372 this study, we introduce the PP4 phosphatase as a new negative regulator of the IMD pathway
373 and provide a first evidence for its negative regulation through the interruption of protein
374 phosphorylation that is essential for the regulation of NF-κB-Relish transcriptional activity.
375 Our results show that, RNAi mediated knock-down of *PP4-19c*, *PP4R2* and *Flfl*, which
376 encode the catalytic, scaffold and regulatory subunits of the PP4 complex respectively, lead
377 to an enhanced and prolonged activation of the IMD pathway both in S2 cells and in adult
378 flies. In a complementary approach, overexpression of PP4-19c significantly limits the IMD
379 pathway activation in S2 cells following their induction by HKE. This phenotype is strictly
380 dependent on its catalytic activity. Similarly to the IMD pathway mutants, flies
381 overexpressing *PP4-19c* display a compromised Relish-dependent AMP gene expression and
382 are susceptible to the infections by Gram-negative bacteria. Moreover, as is the case for IMD
383 pathway negative regulators, RNAi mediated silencing of *PP4-19c* leads to a shortened
384 lifespan of adult flies which correlates with a progressively intensified activation of the IMD
385 pathway in the ageing flies compared to wild-type flies. Altogether, these results provide
386 evidence of an important role of PP4 in the modulation of the IMD pathway signaling.
387 Genetic analysis placed PP4 downstream of the IMD adaptor protein and upstream of Relish.

388 Using immunoprecipitation approach, we show that PP4-19c and its PP4R2 regulatory
389 subunit specifically interact with the IKK complex. Therefore, our results provide genetic and
390 biochemical evidences for PP4 acting as a *bona-fide* negative regulator of the IMD pathway
391 by acting at the level of the IKK signalosome. The identification of PP4 as a new negative
392 regulator of the IMD pathway demonstrates that the IKK signalosome is targeted by different
393 mechanisms for the fine-tuning of its signaling. These include deubiquitinilation, autophagic
394 degradation and now dephosphorylation (75, 102). It remains to be clarified how these
395 processes are regulated and coordinated upon immune challenge. In all cases, the costly
396 effects of exacerbated IMD signaling are attested by a loss of tissue homeostasis and /or
397 reduced lifespan of the flies.

398 Many of the previously identified IMD negative regulators act in a negative feedback loop
399 (57-59, 67-69). Our preliminary data show that the expression of the genes encoding *PP4-19c*
400 and its regulatory subunits are not induced upon *E. coli* infection (supplementary Figure 4).
401 Further experiments will be needed to decipher the complete process that leads to the
402 activation and recruitment of the PP4 complex upon IMD pathway activation. Another
403 question pertains to the molecular mechanisms underlying the interaction of PP4 with its
404 target in the IMD cascade. As is the case for all Phosphoprotein Phosphatases (PPP), it is
405 generally accepted that the PP4 functional profile can be diversified by the combinatorial
406 association of its catalytic subunit with distinct scaffold and regulatory subunits driving its
407 activity towards different cellular targets (80, 93, 104-106). Although our results provide the
408 first evidence of PP4's function in the *Drosophila* IMD pathway, its role in the regulation of
409 cell division as well as many developmental processes in *Drosophila* is well documented.
410 The Flfl regulatory subunit was previously shown to be required for PP4's function in the
411 regulation of cell cycle progression, asymmetric neuroblast division, proper glial responses to
412 nerve injury in the adult brain and the regulation of Wingless and Notch pathways in the wing

413 imaginal disc (80, 90, 96, 97, 100, 101, 107-110). Flfl belongs to the highly conserved family
414 of PP4R3 orthologues that is characterized by a well-defined domains organization (80, 93,
415 94). This comprises a succession of an amino-terminal Pleckstrin Homology (PH)
416 superfamily-like domain and a Smk-1/DUF625 domain followed by a variable number of
417 ARM (armadillo/HEAT repeats) and finally a carboxy-terminal unstructured low complexity
418 region (80, 94, 100). In a recent study, Lipinski *et al* showed that Flfl directly binds the key
419 Centromeric Protein C (CENP-C) via its EVH1 domain (which belongs to PH-like domains)
420 thus recruiting PP4-19c to centromeres and that this interaction is critical for regulating the
421 integrity of the mitotic centromeres (80). The EVH1 domain of Flfl was also shown to bind
422 Mira for the regulation of neuroblast asymmetric division (100). Our attempts to detect an
423 interaction between R3 and the IKK complex have been unsuccessful so far. Presently, we
424 cannot exclude that other ancillary proteins might be required for the targeting of IKK by the
425 PP4 complex.

426 Conversely to *Drosophila*, in mammals, several research activities have lately indicated a role
427 of PP4, in the regulation of NF-κB signaling (111-114). A decline in PP4 expression is
428 associated with aberrant NF-κB, sustained malignancy as well as enhanced metastasis of T
429 cell lymphomas and lung cancer cells (111, 115). In addition, the PP4R1 subunit in
430 mammals, that is homologous to PP4-R2 in *Drosophila*, is targeted by the merckel
431 polyomavirus to subvert the NF-κB- dependent antiviral response (93, 115) . These studies
432 attest of the central role of PP4 in the regulation of NF-κB mediated immune responses in
433 mammals. Notably, PP4 was shown to interact with several components of the NF-κB
434 cascades including members of the NF-κB transcription factors, the E3 ubiquitinases TRAF2
435 and 6 (PP4R1) as well as the IKK signalosome (111, 112, 114, 116). The pleiotropic
436 functions of PP4 might be explained by the coaction of different regulatory subunits.
437 Remarkably, the PP4/PP4R1 complex was shown to target the IKK complex for the

438 suppression NF-κB signaling in Jurkat T cells and primary T lymphocytes (111). Thus, our
439 current study reveals the evolutionarily conserved function of PP4 for the modulation of NF-
440 κB signaling via the targeting of the IKK signalosome from insects to mammals. SMEK, the
441 human homolog of Flfl, was shown to be required for targeting Par3 dephosphorylation by
442 PP4 during neuronal differentiation (117). However, no immune function of SMEK has been
443 reported to date. Given the high conservation of NF-κB signaling from insects to mammals, it
444 is tempting to speculate a similar role of SMEK in their regulation. The innate immune
445 response conserved among metazoan is the first and unique line of defense for invertebrates
446 against pathogens. Whereas highly potent to counterstrike or prevent microbial infections,
447 deregulation of NF-κB signaling could be considered as a shared evolutionary threat. The
448 paradox between the necessity of these pathways and the danger implied by their
449 deregulation underlies their tight regulation by conserved factors, such as PP4.

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Figures

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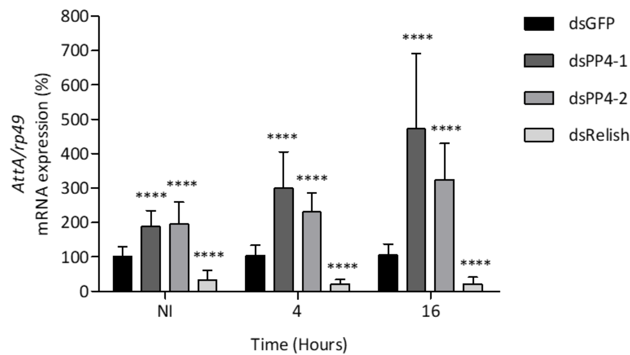

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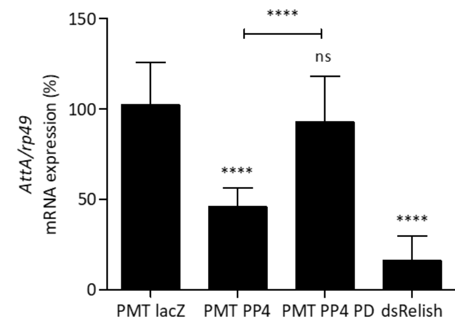

Figure 1: **PP4-19c negatively regulates the IMD pathway in *Drosophila* S2 cells.**

(A) RNAi mediated Knock down of *PP4-19c* increases IMD pathway activation and expression of *Attacin A (AttA)*, compared with *GFP* controls. S2 cells were soaked with two different dsRNA constructs (ds*PP4-1* and ds*PP4-2*) targeting *PP4-19c* mRNA. *GFP* dsRNA and NF-κB *Relish* dsRNA were used as negative and positive controls, respectively. The IMD pathway was induced 6 days later by adding Heat Killed *E. coli* (HKE), and the expression of *AttA* antimicrobial peptide was measured by Real-time quantitative PCR (RT-qPCR) at 4 and 16 hours post-induction, and in the absence of HKE induction, and normalized to ribosomal protein 49 (*rp49*) transcript levels.
(B) Overexpression of *PP4-19c* reduces IMD signaling in Drosophila S2 cells. S2 cells were transiently transfected with a metallothionein promoter–driven transgene expressing wild type *PP4-19c* (PMT PP4) and a phosphatase dead mutant allele (PMT PP4 PD). CuSO4 was added for 48 hours and then IMD pathway activation was stimulated with HKE for 4 hours. *AttA* expression levels were compared to cells transfected with *lacZ* expression vector (PMT lacZ), or *Relish* dsRNAs and normalized to *rp49* transcript levels
Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01< p < 0.05$; **:$0.001< p < 0.01$; ***: $0.0001< p < 0.001$; ****: $p < 0.0001$).

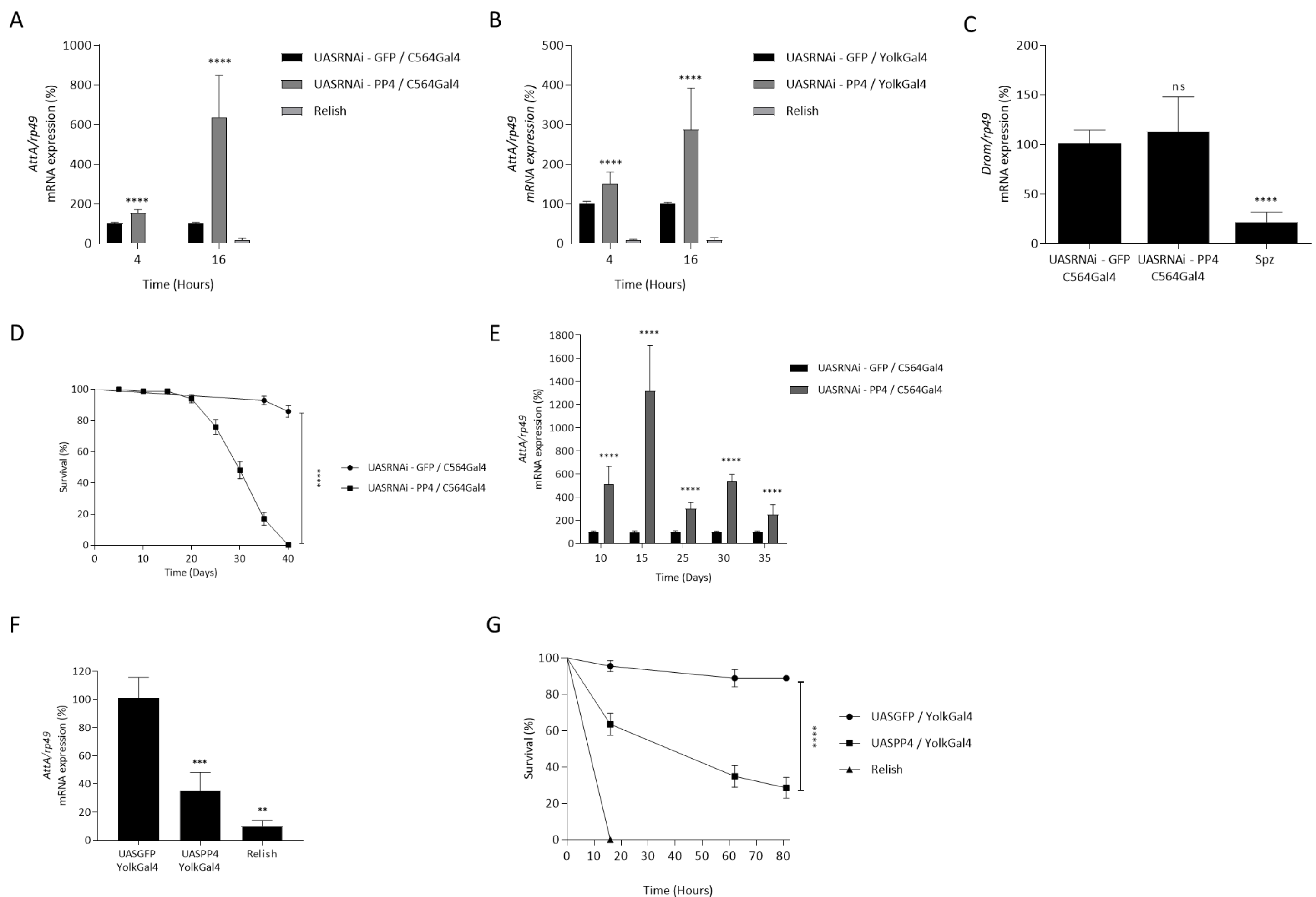

Figure 2: PP4-19c is required for the regulation of adult flies' immune response

(A) and (B) *PP4-19c* in vivo RNAi increases IMD pathway activity. Real-time RT-PCR analysis of *AttA* from the offspring of *PP4-19c* RNAi line crossed with *c564-Gal4* (A) or *yolk-Gal4* (B) drivers that was infected with *E. coli* for 4 and 16 hours. A fly line expressing RNAi *GFP* was used as a wild-type control.
(C) PP4-19c does not affect Toll pathway activity. Flies expressing RNAi *PP4-19c* under the control of *c564-Gal4* driver were infected with *Micrococcus luteus* to activate the Toll pathway. After 24 hours, total RNA was extracted and the expression levels of *drosomycin (Drom)* antimicrobial peptide gene were measured by RT-qPCR. *Spaetzle* mutants (Spz) and RNAi *GFP* were used as positive and negative controls, respectively.
(D) *PP4-19c* RNAi reduces the lifespan of uninfected flies. The survival rate of flies expressing RNAi *PP4-19c* under the control of *c564-Gal4*, and incubated at 29°C, was followed. The number of surviving flies was counted every 5 days, for a period of 40 days.
(E) Expression levels of *AttA* antimicrobial peptide gene were measured by RT-qPCR, following total RNA extraction from the survival flies.
(F) Overexpression of *PP4-19c* reduces IMD pathway activation in vivo. UAS-*PP4* transgenic fly line was crossed with *yolk-GAL4* driver flies. The IMD pathway was activated with *E. coli*. Expression levels of *AttA* were analyzed by qRT-PCR, 4 hours after the infection. Flies expressing UAS *GFP* were used as control.
(G) Survival assays were performed following infection of UAS *GFP* and UAS *PP4* expressing flies with *E. cloacae*. Infected flies were incubated at 29°C and the number of surviving flies was counted every 24 hours.
Data obtained from three independent experiments are combined in single value (mean ± sd). Log Rank test for the survival assays and Mann-Whitney test for the RT-qPCR data within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

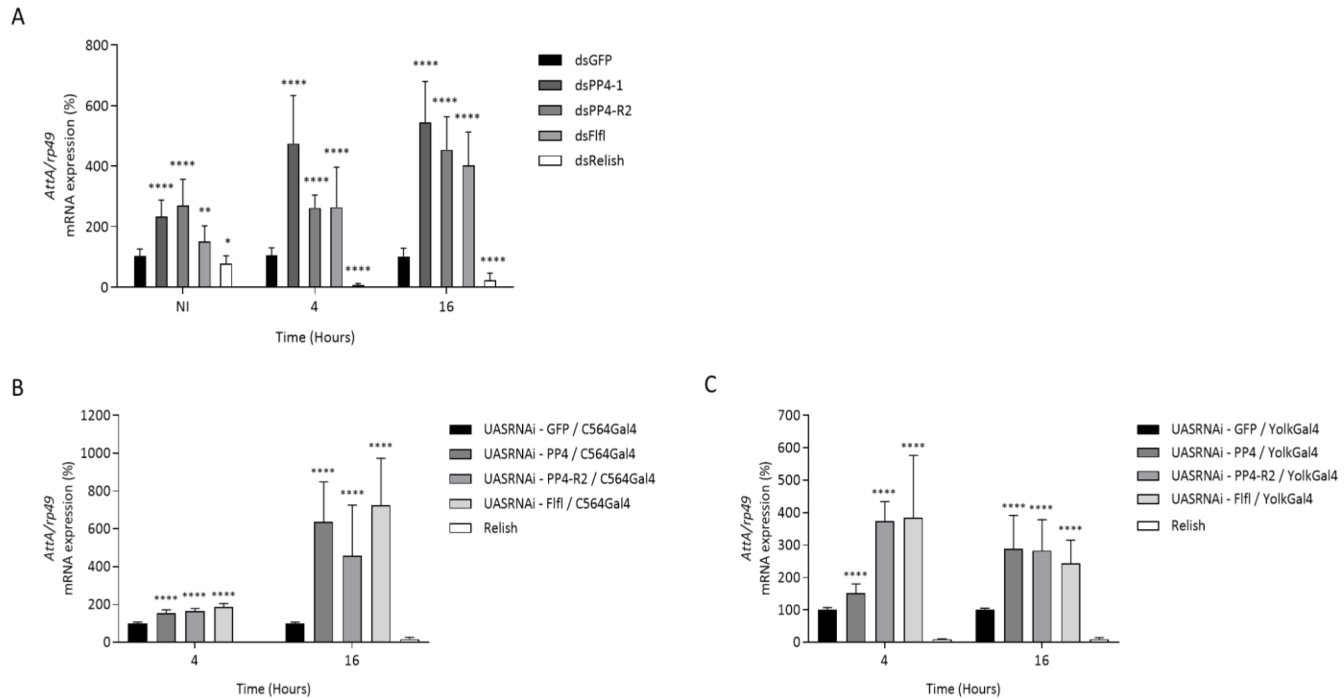

Figure 3: The R2 and Flfl regulatory subunits of PP4 are required for IMD pathway negative regulation in S2 cells and in adult flies

(A) RNAi mediated Knock down of *pp4R2* and *Falafel* (*Flfl*) regulatory subunits increases IMD pathway activation and expression of *AttA*, compared with ds*GFP* controls. S2 cells were soaked with dsRNAs targeting specifically *pp4R2* and *Flfl* transcripts. *GFP* dsRNA and *Relish* dsRNA were used as negative and positive controls, respectively. The IMD pathway was induced 6 days later by adding HKE, and the expression of *AttA* antimicrobial peptide was measured by Real-time quantitative PCR (RT-qPCR) at 4 and 16 hours post-induction, and in the absence of HKE induction, and normalized to *rp49* transcript levels.
(B)-(C) RNAi silencing of *PP4-R2* and *Flfl* genes in the fat body using *c564-Gal4* (B) or *yolk-Gal4* (C) drivers. *AttA* mRNA level was measured by RT-PCR normalized to the expression of *rp49* and presented relative to the expression in RNAi *GFP* flies, sets arbitrary as 1 (control), 4 and 16 hours following an infection with *E. coli*.

Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

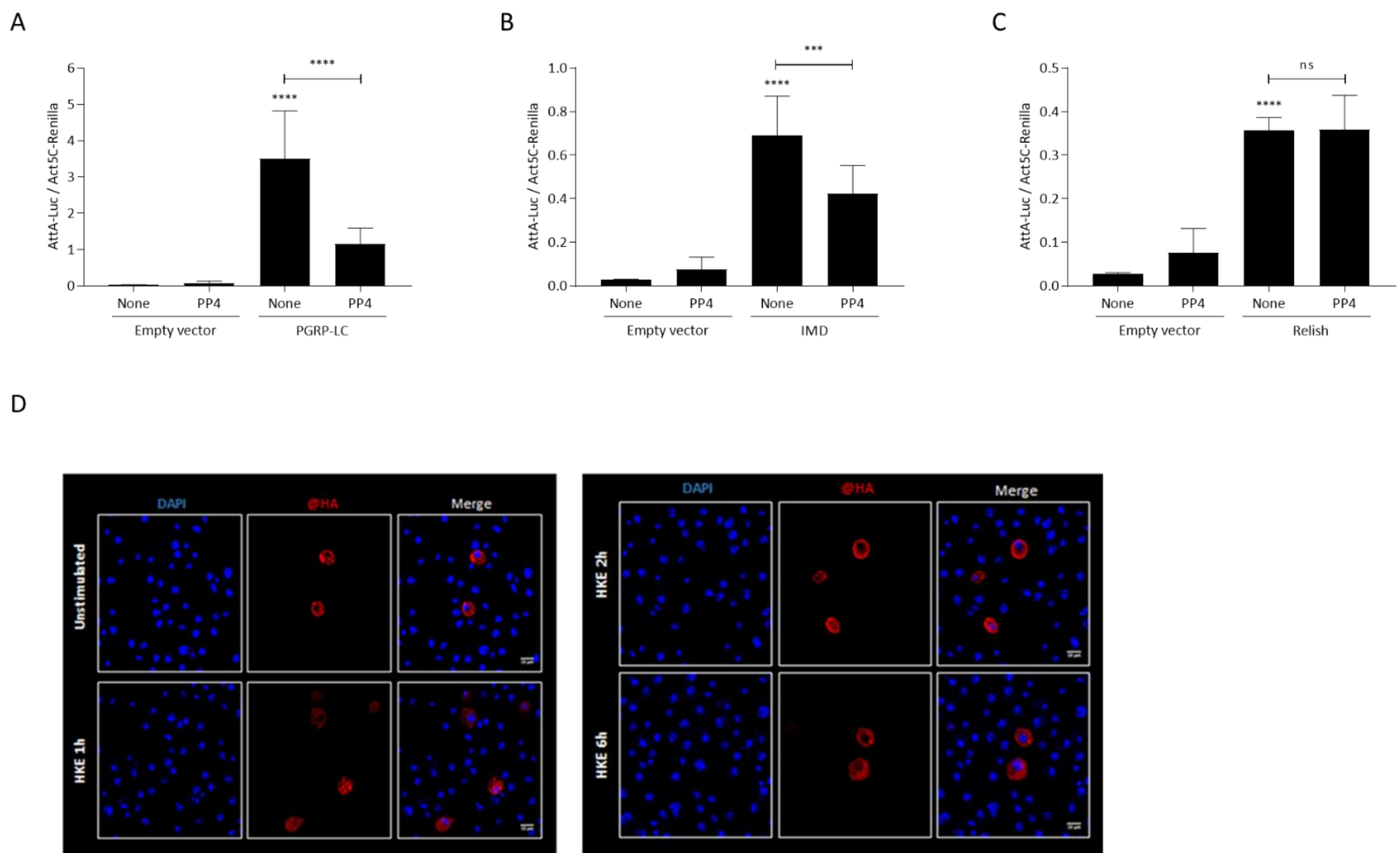

Figure 4: PP4 acts upstream of Relish in the IMD cytoplasmic cascade

(A) S2 cells were transfected with vectors overexpressing PGRP-LC (TM+Intra), (B) IMD, (C) Rel (ΔS29-S45) a Serine rich region deleted Relish. Cells transfected with an empty vector were used as control. Together with these plasmids, the cells were transfected with a vector overexpressing *PP4-19c*. IMD pathway activation was monitored with the *AttA*-Luciferase reporter gene. *Actin*-5C-Renilla activity was measured to normalize transfection efficiency.

Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

(B) PP4-19C is localized in the cytoplasm. Confocal microscopy of S2 cells showing the cellular localization of PP4-19C at 1, 2 and 6 hours after adding HKE. The cells were transfected with a PP4-FLAG- HA expressing vector. For cell staining, nuclei were visualised using DAPI (Blue). Scale bar, 10 µmData obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

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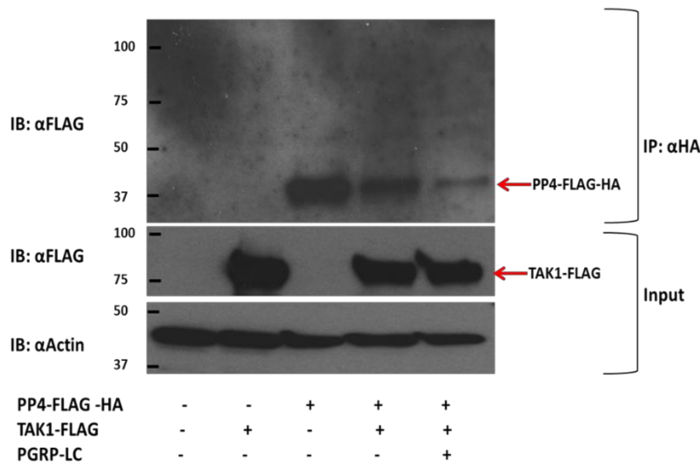

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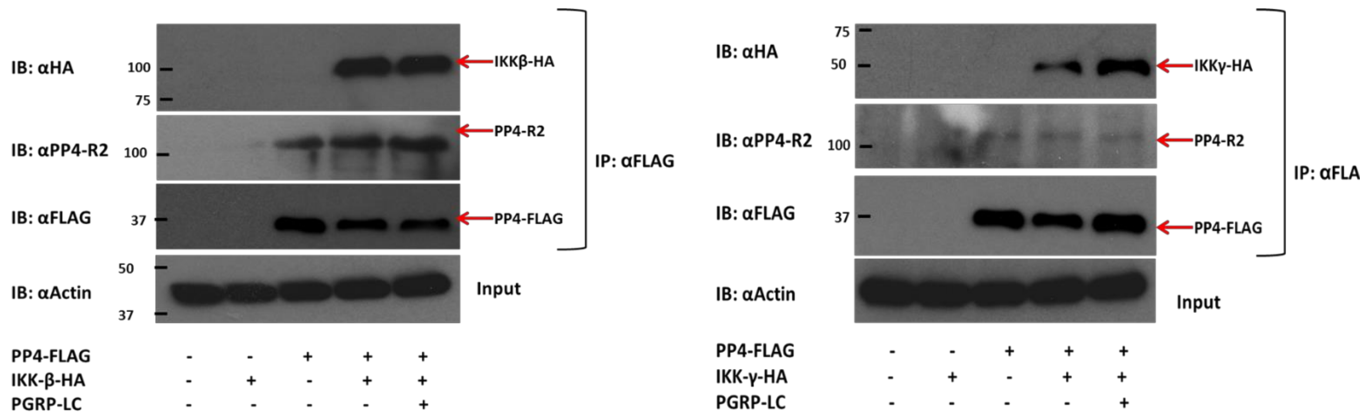


Figure 5: PP4-19c acts at the level of the IKK signalosome in the *Drosophila* IMD pathway.

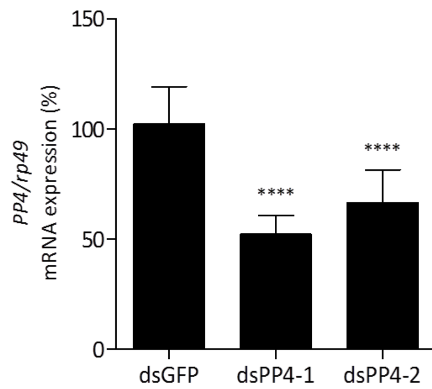
(A) Immunoprecipitation (IP) was performed using anti-HA Antibody coupled to agarose beads, and immunoblotting (IB) with anti-FLAG, and anti-Actin antibodies. Lysates were obtained from S2 cells transiently transfected with metallothionein promoter expression plasmids encoding PP4-FLAG-HA and TAK1 FLAG.

(B) Immunoprecipitation (IP) was performed using anti-FLAG Antibody coupled to agarose beads, and immunoblotting (IB) with anti-HA, anti-FLAG, anti-PP4R2 and anti-Actin antibodies. Lysates were obtained from S2 cells transiently transfected with PP4-FLAG, IKK-β-HA and IKK-γ-HA expression plasmids.

Data obtained from three independent experiments.

Supplementary materials

A



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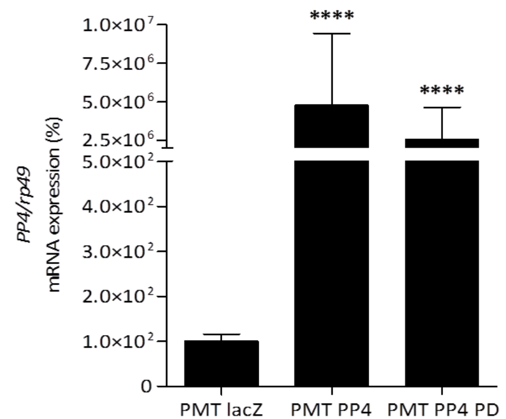

Supplementary figure 1: Knock down and overexpression of *PP4-19c* in S2 cells

(A) Two different non-overlappning dsRNA (*dsPP4-1* and *dsPP4-2*) targeting *PP4-19c* mRNA were constructed and used to knock down the expression of *PP4-19c* in S2 cells. ds*GFP* constructs were used as negative controls. The levels of *PP4-19c* transcripts were measured by RT-qPCR and normalized to *rp49* transcript levels.

(B) S2 cells were transiently transfected with a metallothionein promoter–driven transgene expressing wild type *PP4-19c* (*PMT PP4*) and a phosphatase dead mutant allele (*PMT PP4 PD*). CuSO4 was added for 48 hours. *PP4-19c* expression levels were compared to cells transfected with lacZ expression vector (*PMT lacZ*), and normalized to *rp49* transcript levels.

Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

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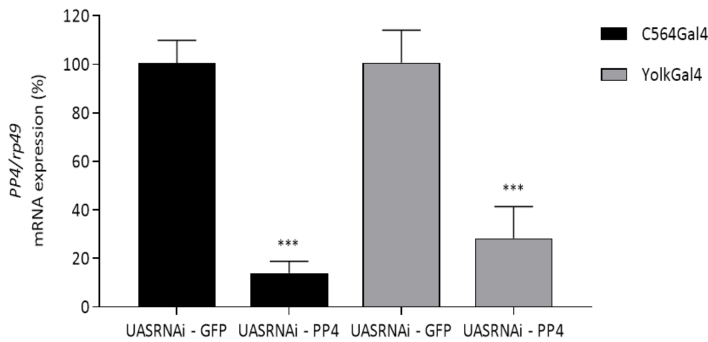

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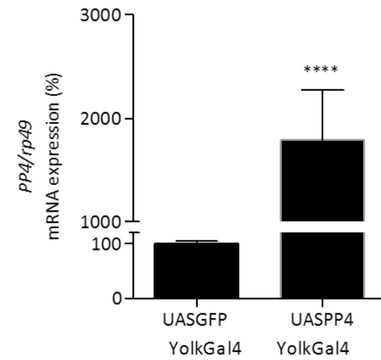
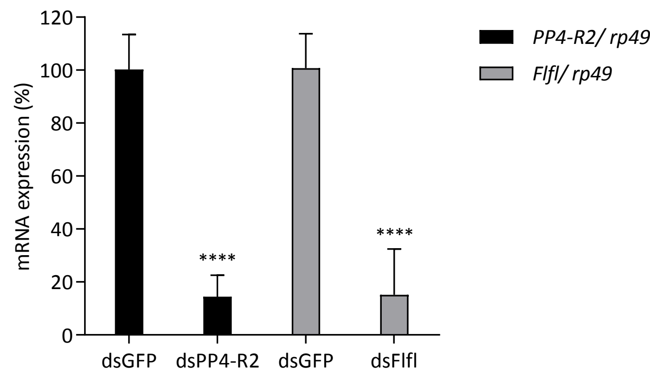


Supplementary figure 2: Knock down and overexpression of *PP4-19c* in the fat body of adult flies.

(A) Total RNA was extracted from the fat body of adult flies expressing RNAi targeting *PP4-19c* transcripts using *C564-Gal4* or *Yolk-Gal4* drivers. *PP4-19c* mRNA level was measured by RT-qPCR normalized to the expression of *rp49* and presented relative to the expression in UAS RNAi *GFP* flies, sets arbitrary as 1 (control).

(B) Total RNA was extracted from the fat body of adult flies expressing UAS- *PP4-19c* transcripts using *Yolk-Gal4* driver. *PP4-19c* mRNA level was measured by RT-qPCR normalized to the expression of *rp49* and presented relative to the expression in UAS *GFP* flies, sets arbitrary as 1 (control).

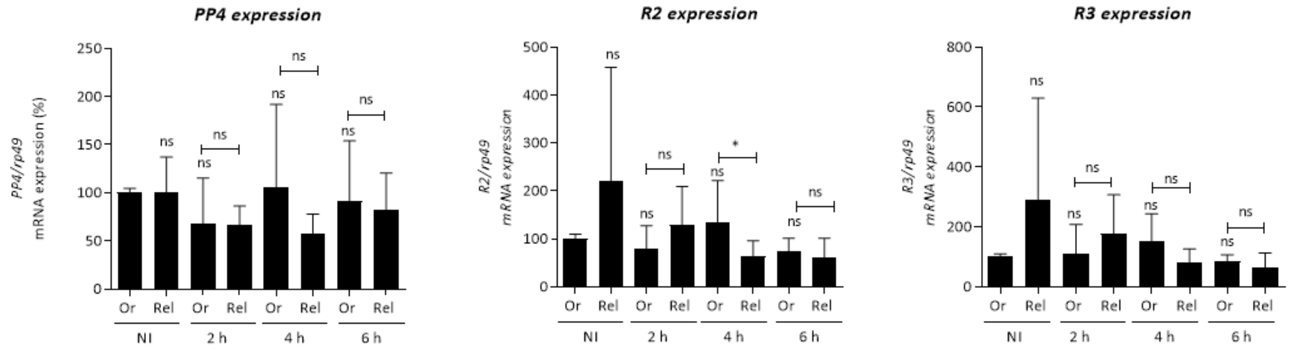
Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).



Supplementary figure 3: Knock down of *PP4-R2* and *Flfl* in S2 cells

S2 cells were soaked with dsRNAs targeting specifically *PP4R2* and *Flfl* transcripts. *GFP* dsRNA were used as negative control. The expression level of each gene was measured by RT-qPCR and normalized to *rp49* transcript levels.

Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).



Supplementary figure 4: *PP4-19c, PP4-R2 and Flfl* are not induced after Gram-negative bacterial infection.

To induce the IMD pathway, wild type (Oregon, Or) and *Relish* mutant flies were infected by *E. coli*. After the infection, RNA from dissected fat bodies were extracted and the expression level of *PP4-19c (PP4), PP4-R2 (R2) and Flfl* (R3) transcripts was measured using RT-qPCR, at 0, 2,4 and 6 h after infection.
Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

Table S1. List of oligonucleotides used to generate dsRNA for RNAi in S2 cells

Gene	dsRNA reference	Forward	Reverse	Size
pp4-19c	HFA21251	GGTTGGCGGCGATGTGCCCG	GGTGTGCACGGCAAATCATG	515
pp4-19c	BKN23059	AATTCTCAGGGTCTTGGGCT	CTAAGACGCTTCCGTTCCTG	473
r2	DRSC27825	GAGGGAGGAGGACAAAAAGG	CTTCTCGACTGACTCCTGGG	505
r3	64410	GTGTGAGGACCTGGACAATACC	GTTCATCACGAGAGTGAGTTCC	586
relish	DRSC37194	TGCCATGTGGAGTGCATTAT	GCCATCCAGACGATACAGGT	411
ptpa	BKN60634	TTCTCTCCAAGTTTCCCGTG	ACCATTGGCCAACGCTATTA	448

Table S2. List of oligonucleotides used for quantitative real-time PCR.

Gene	Forward	Reverse
Attacin A	GGCCCATGCCAATTTATTCA	AGCAAAGACCTTGGCATCCA
Drosomycin	CGTGAGAACCTTTTCCAATATGATG	TCCCAGGACCACCAGCAT

rp49	GACGTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG
pp4-19c	CCTTCACCTCGTTCTCCTTG	ATGTCCGACTACAGCGACCT
r2	CGGTAACGCCGATGAGGGCT	CATTGTCGTCCGAACGCGGG
r2	CGATCCTCGGAAGCAGTA	GATCGATTGTGCTAACCACTA
ptpa	TAAGATGTACCAAAAGGAGATT	GGCTCGAATGTCATCAGT
r3	ACAACAATGTCATGAAATCCGT	TGTGTGGCGGAGAGGAT
relish	CCACCAATATGCCATTGTGTGCCA	TTCCTCGACACAATTACGCTCCGT

2- Complementary results: PP4-19C interacts with DREDD in the *Drosophila* IMD pathway.

In a view to test whether PP4 is able to interact with other components of the IMD pathway that match with its cellular localization and the epistasis analysis results, we looked for an association with the adaptor protein IMD and the caspase DREDD by co-immunoprecipitation. Therefore, we co-transfected S2 cells with the tagged *FLAG-PP4-19c* and *myc-DREDD* and *V5-IMD.* These transfections were concomitant, or not, with the transfection of *PGRP-LC* for the induction of the IMD pathway. The preliminary results, that require further confirmation, presented in the complementary Figure 15A show that DREDD is able to co-immunoprecipitate with PP4-19C, with and without the induction of the pathway, in contrast to IMD **(Figures 15A and 15B).** Hence, beside its interaction with the IKK complex, PP4 seems to physically associate with DREDD.

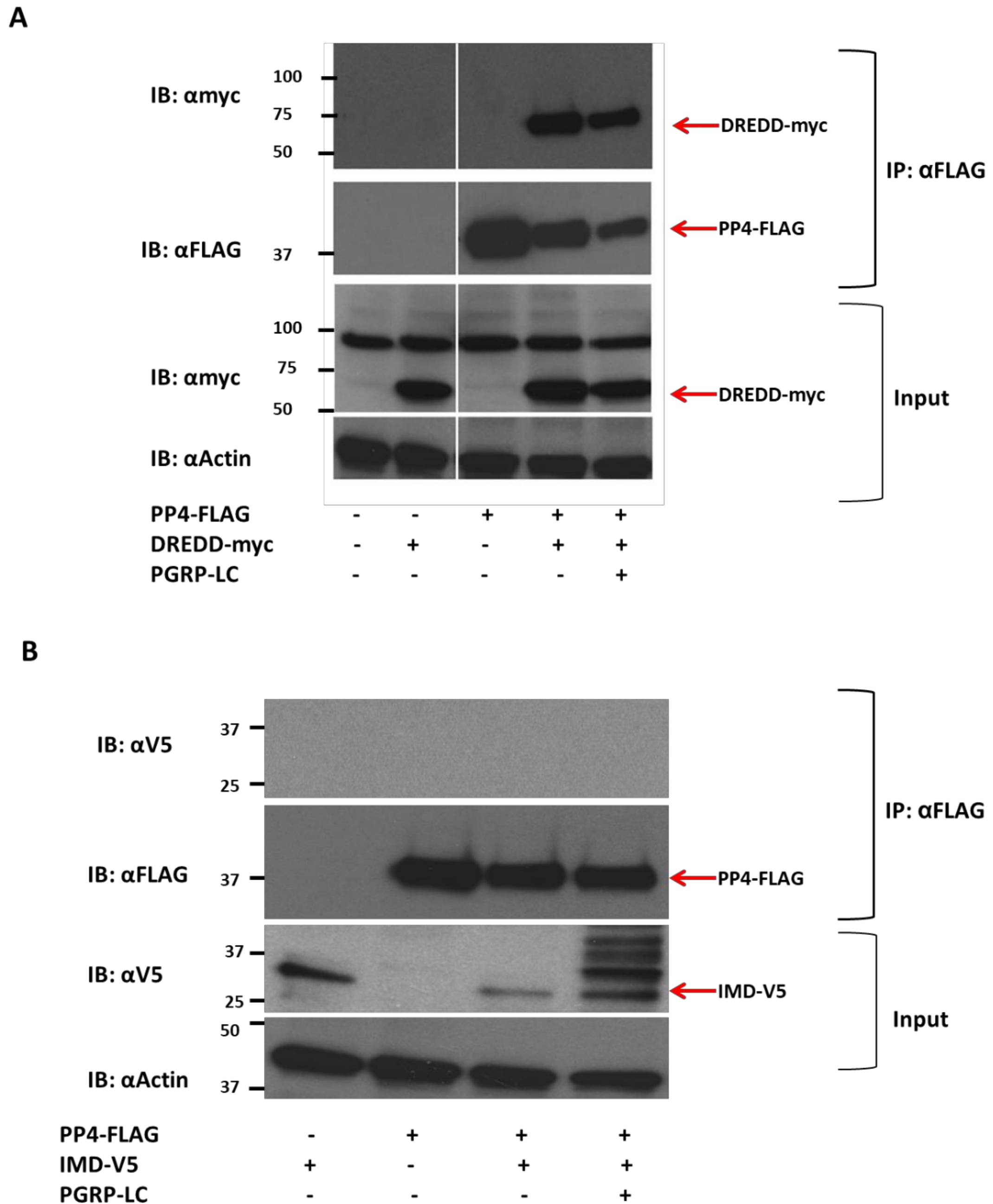


Figure 15: PP4-19C interacts with DREDD in the *Drosophila* IMD pathway

(A) Immunoprecipitation (IP) was performed using anti-FLAG Antibody coupled to agarose beads, and immunoblotting (IB) with anti-FLAG, anti-myc and anti-Actin antibodies. Lysates were obtained from S2 cells transiently transfected with metallothionein promoter expression plasmids encoding PP4-FLAG, DREDD-myc and PGRP-LC.

(B) Immunoprecipitation (IP) was performed using anti-FLAG Antibody coupled to agarose beads, and immunoblotting (IB) with anti-V5, anti-FLAG and anti-Actin antibodies. Lysates were obtained from S2 cells transiently transfected with PP4-FLAG, IMD-V5 and PGRP-LC expression plasmids.

Chapter 2: PP2A fine-tunes the activation of the IMD pathway by acting at the level of the NF-κB factor Relish

I- Context of the study

In the previous chapter, I discussed the characterization of PP4-19c as a new negative regulator of the IMD pathway acting at the level of the kinase IKK complex. This discovery highlighted a new mechanism for IMD signaling modulation driven by the activity of a phosphatase opposing an intracellular phosphorylation event. Besides, it raised the question of whether other PPPs could be implicated in fine-tuning this pathway's response, operating signal termination by de-phosphorylation. Hence, we sought to check whether impairing the expression of other PPP's catalytic subunits could also have an effect on the activation of the IMD pathway. As previously mentioned, *Drosophila'*s genome contains 19 genes coding for PPP catalytic subunits (418). Among them, we selected first PP2A and PP1-87B encoded by *CG7109* and *CG5650*, respectively, that account together for over 90 % of all Ser/Thr phosphatase activity in the cell (390). The implication of these enzymes in IMD modulation was analysed using RNAi mediated knock-down in S2 cells. While the KD of *PP1-87B* in the cells did not affect the activation of the IMD pathway, cells impairing the expression of *PP2A* catalytic subunit showed an enhanced and prolonged activation of this pathway in S2 cells, uncovering a new potential candidate of IMD signaling negative regulator **(Figures 17 A and C).**

II- PP2A in the control of a diverse array of cellular processes in *Drosophila*

The protein phosphatase 2A (PP2A) represents a family of holoenzyme complexes, highly conserved from yeast to humans, and constitutes one of the most abundant Ser/Thr phosphatase in the cell with different activities and diverse substrate specificities (390). Indeed, this ubiquitously expressed phosphatase regulates multiple fundamental cellular mechanisms including embryogenesis, tumorigenesis, DNA damage response and mitosis (558-565). This functional diversity of PP2A is mainly depending on the association of different components forming the holoenzyme complexes. Three distinct functional components constitute the holoenzyme complex: the catalytic subunit (PP2A-C) that interacts

with the structural core subunit or scaffold subunit (PP2A-A) giving rise to the core of the enzyme **(Figure 16).** The *Drosophila* genome harbors two genes, *CG7109* and *CG17291* encoding the PP2A-C and the PP2A-A, respectively. The association with a wide variety of B regulatory subunits to the core enzyme results in the formation of functionally distinct heterotrimers, thus mediating substrates diversity of PP2A holoenzyme complexes (566). Hence, the functional characterization of PP2A requires the deciphering of the holoenzyme complexes composition by the identification of its components. By the limited number of PP2A regulatory B subunits, *Drosophila* presented itself for the functional studies of PP2A in diverse cellular processes. Five regulatory B subunits are encoded in *Drosophila* genome: Twins (Tws) representing B55 family, Widerborst (Wdb) and Well-rounded (Wrd) belong to B56 family, and CG4733 is the member of PR72 family (491, 567). The fly genome also contains the connector to kinase to AP-1 gene (*cka*) that encodes a protein homologous to mammalian B‴/STRN (502). Thereby, the implicated regulatory B subunits as well as their targeted substrate for each below described PP2A functions are summarized in the Table 2.

The high degree of conservation of PP2A allowed the cloning of the genes encoding for its catalytic and the scaffold subunits in *Drosophila* by sequence similarity to their mammalian counterparts (568, 569). Ever since, numerous studies have linked PP2A to the regulation of cellular division, cell survival and apoptosis, cell fate determination and embryogenesis. Indeed, mutations of PP2A catalytic subunit were shown to alter photoreceptor development by affecting Ras-/Raf/MAP kinase mediated signaling (570). Moreover, PP2A was shown to be involved in planar cell polarity for hair outgrowth in the *Drosophila* wing (493). The essential role of PP2A during mitosis in *Drosophila* was outlined by several studies, emphasizing on the tight link between PP2A and centrosomes. The first line of evidence was the discovery of "*mts*" mutant embryos that carry a P element in the promoter of the PP2A catalytic subunit gene. In this study, Snaith and colleagues have shown that PP2A is required for the attachment of microtubules to chromosomal DNA at the kinetochore. Indeed, *mts* die in embryogenesis around the time of cellularisation, exhibiting over-condensed chromatin and a block in mid-mitosis. Accordingly, all cellularised embryos displayed disorganised microtubules arrays emanating from centrosomes in all directions, resulting in a star shape; for which *PP2A* mutants owe their name "*microtubule star*" or "*mts*" (433). Later, other studies have

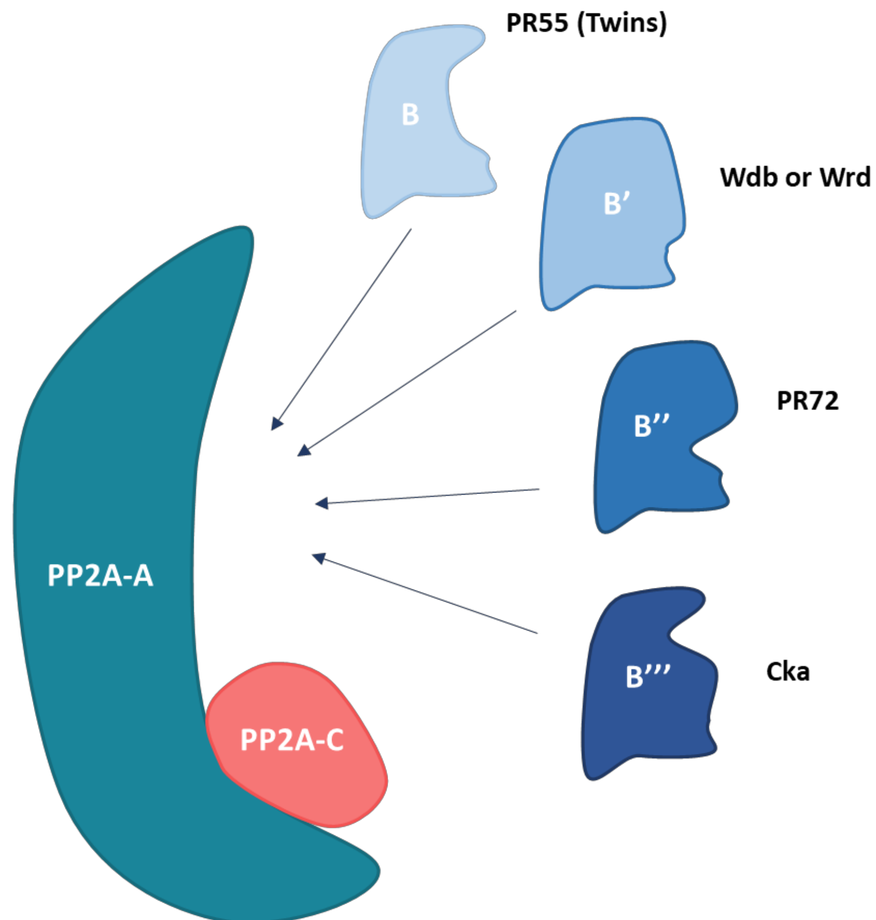


Figure 16: Schematic representation of the structure of protein phosphatase 2A (PP2A) in *Drosophila*

PP2A holoenzyme contains the catalytic subunit C (pink), a structural or scaffold subunit A (left, blue), and a third variable regulatory subunit, that can be a member of four different families B/B'/B''/B''' which are structurally unrelated. In *Drosophila*, A and C are encoded by single genes; the B family is represented by PR55 (Twins); the B' family is represented by weiderborst (wdb) and well-rounded (Wdb) , the B'' family contains PR72; and Cka represents the B''' subunit family.

highlighted the requirement of PP2A for the mitosis progression. In 2011, a study has shown that PP2A is involved in the centrosome attachment to nuclei and the cell cycle progression in syncytial stage during early embryogenesis (488). Recently, Kim and colleagues have reported that PP2A is a regulator of Rad21/Verthandi (Vtd), essential component of the Cohesin complex that controls separation and sister chromatid cohesion during mitosis. Mutations or knock-down of both *PP2A* and *vtd* cause spindle defects and nuclear loss during nuclear division in early embryos. This regulatory mechanism was explained by the requirement of PP2A for the maintenance of Vtd protein level by a proteasome-dependent pathway, which is essential for Vtd stability and wing growth (490). Besides exerting a general control on mitotic progression and centrosome-related functions, PP2A is also implicated in the maintenance of genome integrity and DNA double-strand breaks repair (491). Furthermore, PP2A promotes cell survival and protects from apoptosis by de-phosphorylating a specific substrate upstream of dp53, homologue to mammalian p53 anti-apoptotic factor (434). In addition, PP2A was also demonstrated to be associated to the regulation of distinct signaling pathways controlling cell differentiation and tissue growth such as Hippo, Wnt/Wingless and Hh signaling pathways. A mass spectrometry-based analysis combined with a genome-wide RNAi screening identified PP2A as a negative regulator of Hippo signaling in *Drosophila*. It was proposed that PP2A controls Hippo pathway activity by reverting the activating phosphorylation of the Kinase protein Hippo (Hpo) (502). Recently, a study showed that PP2A contributes to neuronal stem cells (NSCs) quiescence by orchestrating Hippo signaling and insulin receptor signaling in NSCs (494). In addition, PP2A was shown to be required for the regulation of Wnt signaling, via the modulation of β-catenin phosphorylation and degradation *in vivo* (492). Besides, a role of PP2A in both positive and negative regulation of Hh signaling pathway has been suggested by different studies. The positive effect on Hh signaling was first suggested in a genome-wide RNAi screen in which PP2A was selected as Hh regulator (571). Later, PP2A was found in a genetic screen for genomic regions that modulate the activation of Hh signaling. This screen was performed by analysing the impact of genomic deficiencies on the wing vein development in flies impairing the expression of the seven-transmembrane domain protein Smoothened (Smo), the key transducer of Hh signaling pathway. Deficiency in PP2A chromosomal region enhances the Smo knock-down phenotype, suggesting that PP2A acts as positive regulator of

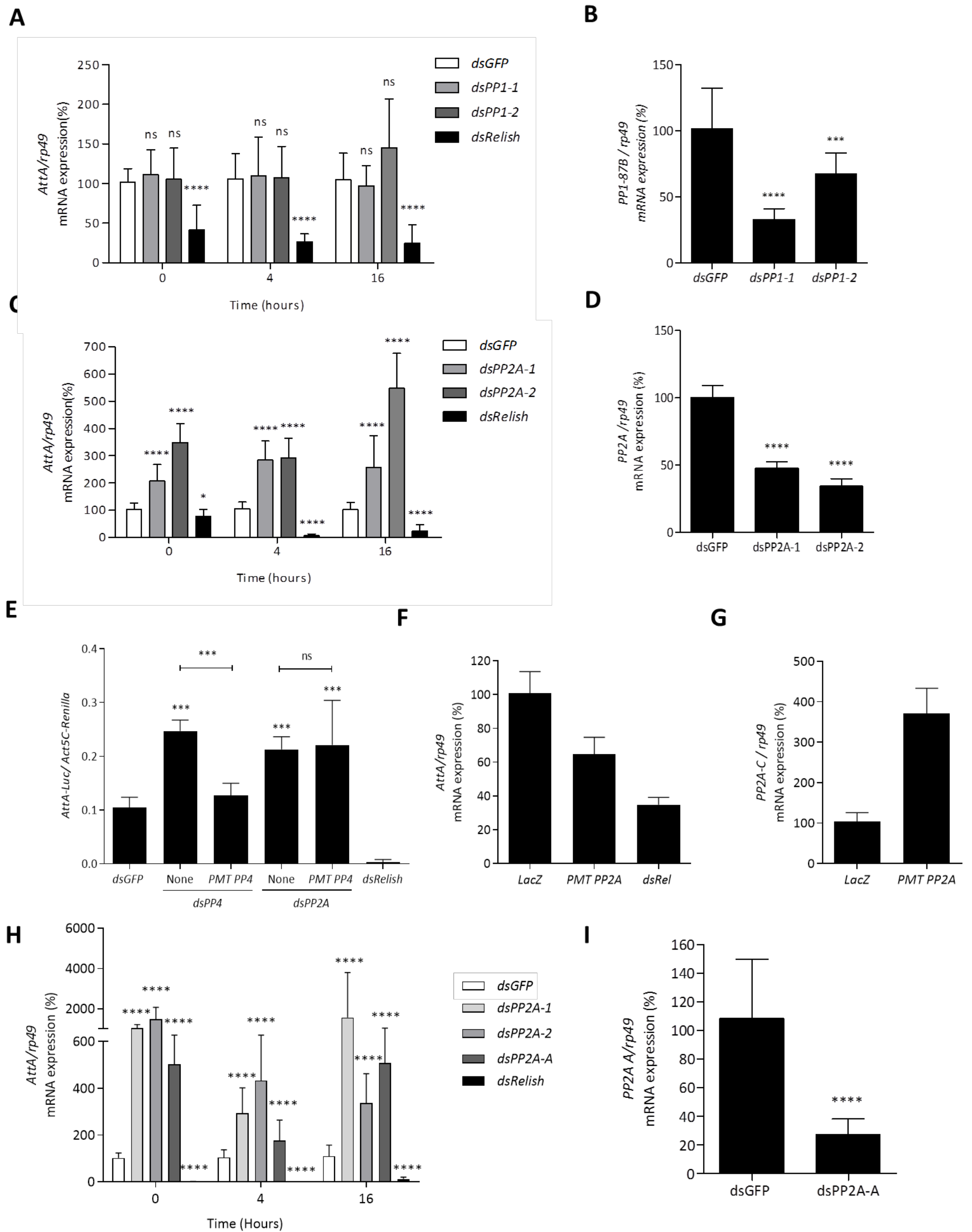


Figure 17: PP2A is a potential candidate for the negative regulation of IMD pathway

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Hh signaling (572). Afterwards, a genetic interaction was revealed between PP2A and Cubitus interruptus kinase (Ci), showing a direct role of PP2A in Ci de-phosphorylation, thus activating Hh signaling (495). In 2012, Su and colleagues revealed a negative influence of PP2A on Hh signaling by showing a specific activity of PP2A on Smo phosphorylation, to restrict signaling by high concentrations of Hh (422).

On a different note, PP2A has been reported to have distinct regulatory functions in metabolic mechanisms. In a study on lipid metabolism regulated by insulin-like growth factor signaling, PP2A was shown to negatively regulate the subcellular pool of activated and phosphorylated protein kinase B (Akt), altering lipid droplet size and the expression of lipid storage protein LSD2 (496). In addition, a role of PP2A in insulin/TOR signaling pathway was also described. It was shown to dephosphorylate S6K, an important effector of the insulin/TOR pathway inducing ribosome biogenesis and inhibiting apoptosis (499). Another study also suggested an effect of PP2A on TOR signaling, by acting at different levels for the regulation of starvation-induced autophagy (497).

Consistent with all these fundamental cellular functions, accumulating evidence indicates that PP2A loss of function is associated to the development of many types of cancer advancing its function as as a tumor suppressor in mammalian systems (390, 573, 574). Indeed, several tumor-promoting viruses are capable of displacing the B regulatory subunits from the core enzyme resulting in altered enzymatic activity towards PP2A substrates (559, 575, 576). Alternatively, virus-derived proteins, such as polyoma small T (pyST) antigen, polyoma middle T (pyMT) antigens and the simian virus SV40 small T antigen (ST), function as B subunits leading to deregulation of PP2A modulated pathways that regulate cell proliferation and apoptosis (559, 577). In *Drosophila* model, this mechanism was also obtained for SV40 ST expression, where ST induces supernumerary centrosomes, increased microtubule stability, chromosome segregation errors, defective assembly of actin into cleavage furrows, cleavage failure, a rise in cyclin E levels and embryonic lethality. All these phenotypes are dependent on ST's interaction with PP2A (578). In addition, mutations have been identified in different components of the PP2A holoenzyme complex, which have been linked to a variety of primary human tumors, neurodegenerative and autoimmune diseases (579-582). Indeed, somatic

Continuation of the Figure 17

Figure 17: PP2A is a potential candidate for the negative regulation of IMD pathway

(A) RNAi mediated Knock down of *PP1-87B* has no effect on IMD pathway activation and expression of *Attacin A (AttA)*, compared with *dsGFP* controls. S2 cells were soaked with two different dsRNA constructs (ds*PP1-1* and ds*PP1-2*) targeting *PP1-87B* mRNA. *GFP ds*RNA and NF-κB *Relish* dsRNA were used as positive and negative controls, respectively. The IMD pathway was induced 6 days later by adding Heat Killed *E. coli* (HKE), and the expression of *AttA* antimicrobial peptide (AMP) was measured by Real-time quantitative PCR (RT-qPCR) at 4 and 16 hours post-induction, and in the absence of HKE induction, and normalized to ribosomal protein 49 (*rp49*) transcript levels.

(B) Knock down of *PP1-87b* in S2 cells. Two different non-overlappning dsRNA (*dsPP1-1* and *dsPP1-*2) targeting *pp1-87b* mRNA were constructed and used to knock down the expression of *pp1-87b* in S2 cells. *dsGFP* constructs were used as positive controls. The levels of *pp1-87b* transcripts were measured by RT-qPCR and normalized to *rp49* transcript levels.

(C) Knock down of *PP2A* enhances IMD pathway activation and expression of *AttA*. S2 cells were soaked with two different dsRNA constructs (*dsPP2A-1* and *dsPP2A-2*) targeting *PP2A* mRNA. *GFP* dsRNA and NF-κB *Relish* dsRNA were used as positive and negative controls, respectively. The IMD pathway was induced 6 days later by HKE, and the expression of *AttA* was measured by RT-qPCR at 4- and 16-hours post-induction, and in the absence of HKE induction, and normalized to *rp49* transcript levels.

(D) Knock down of *PP2A* in S2 cells. Two different non-overlappning dsRNA (*dsPP2A-1* and *dsPP2A-2*) targeting *PP2A* mRNA were constructed and used to knock down the expression of *PP2A* in S2 cells. *dsGFP* constructs were used as positive controls. The levels of *PP2A* transcripts were measured by RT-qPCR and normalized to *rp49* transcript levels.

(E) Overexpression of PP4-19c does not rescue the PP2A RNAi phenotype. S2 cells were transfected with a metallothionein promoter–driven transgene expressing wild type PP4-19c (PMT PP4), after the treatment with *dsPP4-1* and *dsPP2A-2*. Cells treated with dsGFP and dsRelish were used as control. IMD pathway activation was monitored with the Attacin A-Luciferase reporter gene. Actin-5C-Renilla activity was measured to normalize transfection efficiency.

(F) – (G) Overexpression of *PP2A* reduces IMD signaling in *Drosophila* S2 cells. S2 cells were transiently transfected with a metallothionein promoter–driven transgene expressing wild type *PP2A* (PMT PP2A). CuSO4 was added for 48 hours and then IMD pathway activation was stimulated with HKE for 4 hours. *AttA* (F) and *PP2A* (G) expression levels were compared to cells transfected with lacZ expression vector (PMT lacZ), or *Relish* dsRNAs and normalized to *rp49* transcript levels. Data was obtained from one biological experiment.

(H) – (I) Knock down of *PP2A* and *PP2A-A* in S2 cells. (H) Two different dsRNA (*dsPP2A-1, dsPP2A-2*) targeting *PP2A* mRNA were constructed and used to knock down the expression of *PP2A* in S2 cells. (I) One dsRNA targeting *PP2A-A* mRNA was constructed and used to knock down the expression of *PP2-A* in S2 cells. *dsGFP* constructs were used as positive controls. The levels of expression of each gene were measured by RT-qPCR and normalized to rp49 transcript levels.

Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

alterations of the PP2A structural subunit Aβ (PPP2R1B) have been found in colon, lung and breast cancers (474, 583). Moreover, it was also shown that the high expression levels of PP2Ac in T cells from systemic lupus erythematosus (SLE) patients, an autoimmune disease, play a significant role in decreased IL-2 expression, as IL-2 levels could be restored upon silencing of the mRNA expression of PP2Ac (580). In 2016, Apostolidis and colleagues have reported that regulatory T cells (Tregs) require PP2A for keeping their suppressive capabilities in mice (584). In murine model, the deficiency of PP2A in cells leads to development of a severe lymphoproliferative and autoimmune disorder with spontaneous activation of the immune system and production of autoantibodies that were also against lupus-associated nuclear autoantigens (584). In addition, Breuer and colleagues have outlined a role of PP2A regulatory subunit B56γ in regulating TCR-mediated NF-κB singling pathway (585). Altogether, these observations firmly establish PP2A as an important regulator of signaling pathways involved in oncogenesis and the control of the immune reactions in mammalian models.

In this chapter, I report the characterization of PP2A, as an additional new phosphatase fine- tuning the signaling of *Drosophila* IMD pathway. Our results show that impairing the expression of PP2A results in a hyper-activated AMP expression in *Drosophila* S2 cells as well as in adult flies conferring resistance to microbial infections. Two of the PP2A regulatory B subunits were shown to be implicated in modulating IMD pathway activation. Epistasis analysis indicated that PP2A acts at level of the NF-κB factor Relish. Co-immunoprecipitation experiments revealed an interaction between PP2A and Relish putting forward the hypothesis that Relish might be the target of this phosphatase.

III- Results

1- PP2A, a new negative regulator of the IMD pathway

The initial finding that PP4 knock down triggers a deregulated IMD signalling in *Drosophila* S2 cells prompted us to verify the specificity of the observed phenotype. Indeed, the specificity of phosphatase activity has long been challenged and therefore we aimed to confirm our results by verifying whether the knock-down of other highly expressed cellular phosphatases would give a similar phenotype **(Figure 17).** Hence, we measured the IMD induction level after

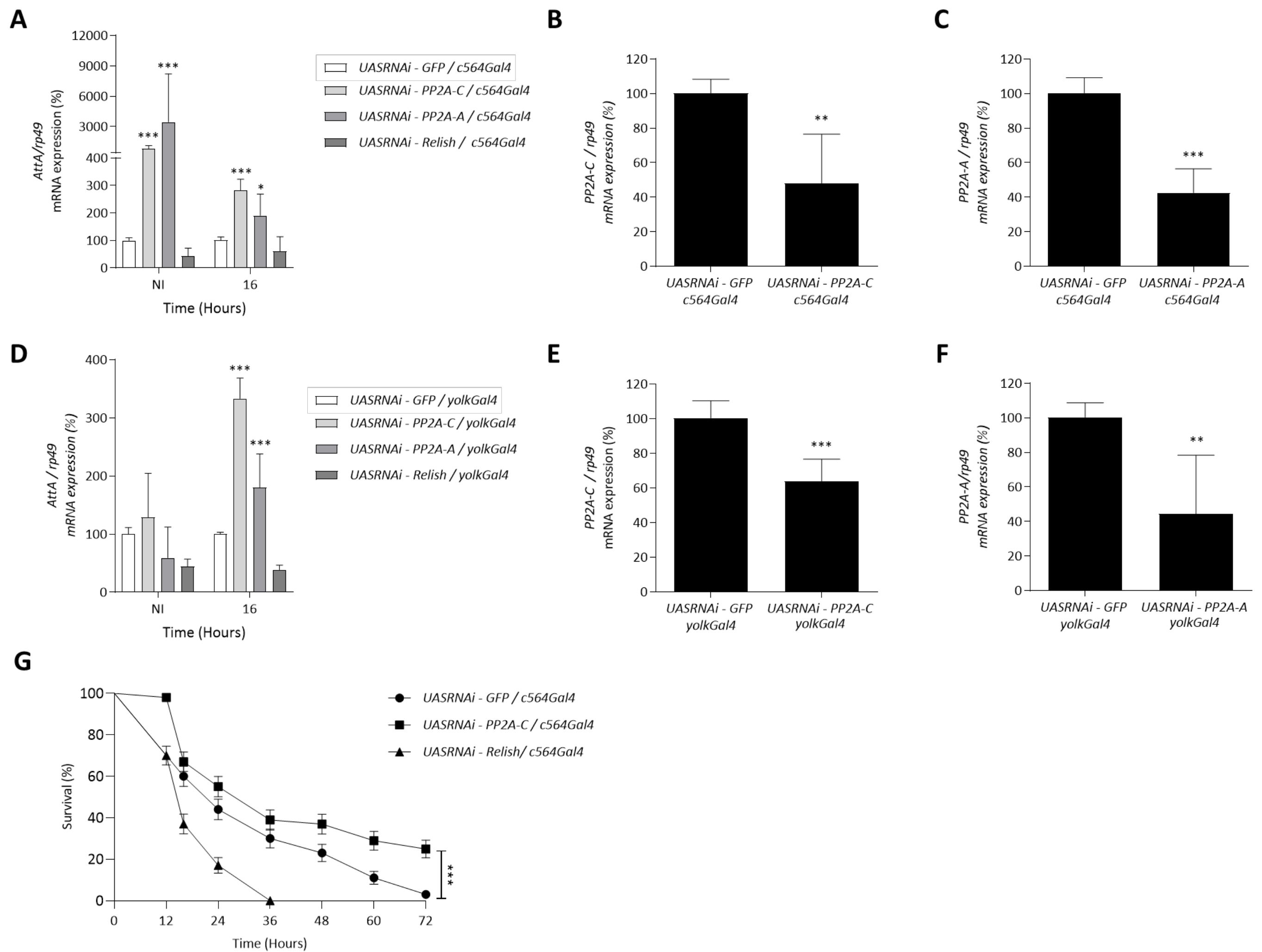

Figure 18: PP2A negatively regulates IMD pathway activity *in vivo*

(A) – (D) *PP2A-C and PP2A-A in vivo* RNAi increases IMD pathway activity. Real-time RT-qPCR analysis of *AttA* from the offspring of *PP2A-C* and *PP2A-A* RNAi lines crossed with c564-GAL4 (A) or Yolk-Gal4 (D) drivers. Total RNA was extracted from unchallenged flies (NI) or flies infected with *E. coli* for 16 hours. Flies expressing RNAi *GFP* and RNAi *Relish* were used as control. *AttA* expression levels were normalized to *rp49* transcript levels.

(B) – (C) – (E) – (F) Knock down of *PP2A-C* and *PP2A-A* in the fat body of adult flies. RNA was extracted from the fat body of adult flies expressing RNAi targeting *PP2A-C* and *PP2A-A* transcripts using c564-gal4 (B and C) or yolk-gal4 (E and F) drivers. *PP2A-C* and *PP2A-A* mRNA levels were measured by RT-qPCR and normalized to the expression of *rp49* and presented relative to the expression in UAS RNAi *GFP* flies, sets arbitrary as 1 (control).

(G) KD of *PP2A-C* in flies enhances their resistance against Gram-negative bacterial infections. Survival assays were performed following infection of flies expressing RNAi *PP2A-C* under the control of c564-GAL4 driver with *Pseudomonas aeruginosa* (PA14). Infected flies were incubated at 29°C and the number of surviving flies was counted every 12 hours. Flies expressing RNAi *GFP* and RNAi *Relish* were used as control.

Data obtained from three independent experiments are combined in single value (mean ± sd). Log Rank test for the survival assays and Mann-Whitney test for the RT-qPCR data within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

impairing the expression of two among 19 PPPs encoding genes, *PP2A* and *PP1-87B,* which are closely related to *PP4-19c* and highly expressed in S2 cells. For that, we constructed two *dsRNA*s constructs to knock-down each of these genes, namely: *dsPP1-1*, *dsPP1-2*, *dsPP2A-1* and *dsPP2A-2* for targeting the transcripts of *PP1-87B* and *PP2A-C*, respectively. The effect of the KD of these two phosphatases on the IMD pathway activation was analysed following the verification of the efficiency of the RNAi mediated knock down of their relative transcripts by RT-qPCR **(Figures 17B and 17D).** IMD signalling was monitored by the quantification of the *Attacin A* in dsRNA treated S2 cells with or without prior stimulation by Heat-killed *E. coli* (HKE). *DsRNA* targeting *GFP* and the NF-κB factor *relish* transcripts were used as positive and negative controls respectively. As shown in Figure 17A, the levels of *Attacin A* expression in *dsPP1-1* and *dsPP1-2* treated cells were comparable to control *dsGFP* treated cells. This result clearly indicates that this PPP is not involved in the regulation of the IMD pathway. In contrast, a significant increase in *Attacin A* expression was observed following HKE stimulation in the cells treated with *dsRNA* constructs targeting the *PP2A* catalytic subunit (*PP2A-C*) transcripts **(Figure 17C).** Moreover, these cells exhibit a constitutive activation of the IMD pathway in the absence of any bacterial induction **(Figure 17C)** alike the *PP4-19c* KD phenotype (Chapter 1 – Manuscript).

Since PP4 and PP2A share considerable sequence similarities, we owed to an additional verification of the specificity of the observed phenotype. Therefore, we performed a rescue experiment by overexpressing PP4c coding sequence in *dsPP2A-2* treated cells. As shown in Figure 17E, the transient overexpression of PP4-19c driven by a copper inducible promoter (*pMT PP4*), does not attenuate the enhanced activity of an Attacin A-luciferase reporter in *dsPP2A-2* treated cells following their stimulation with HKE. This contrast with the situation of *dsPP4* treated cells in which the transient overexpression of PP4 completely restored Attacin A-luciferase reporter activity to the level observed in *dsGFP* treated cells. These results advance PP2A-C as a new negative regulator of the IMD pathway. To further test the functional significance of this negative regulation, we owed to two additional experiments. First, we measured the activation of IMD signaling in S2 cells overexpressing *PP2A-C* following HKE stimulation (*PMT PP2A*). As shown in Figure 17F, this results in a significant reduction in *Attacin A* expression as compared to control cells (transfected with a *lacZ* over-expressing

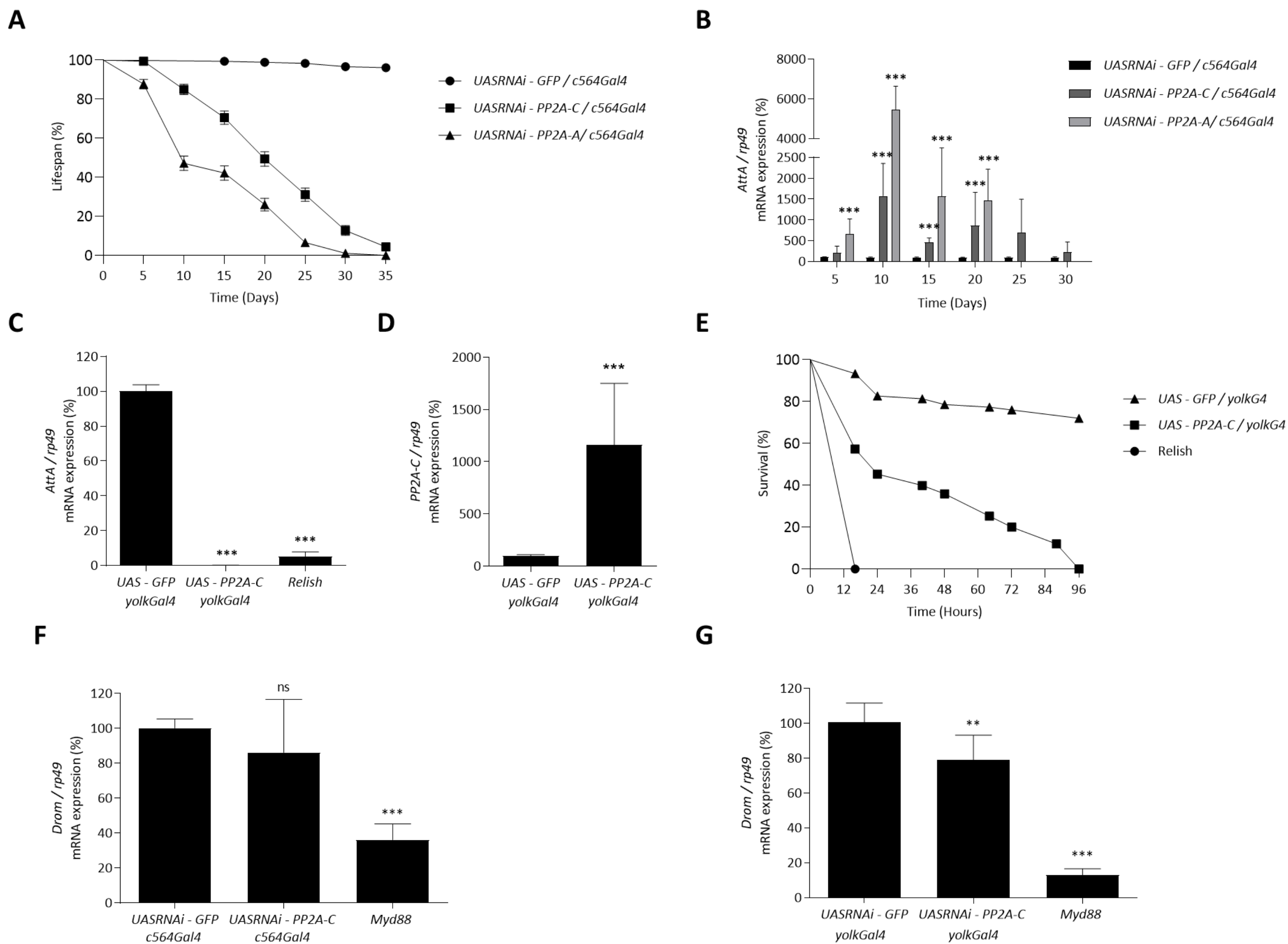

Figure 19: PP2A fine-tunes IMD pathway activity *in vivo*

(A) PP2A RNAi reduces the lifespan of uninfected flies. The survival rate of flies expressing RNAi *PP2A-C* and *PP2A-A* under the control of c564-GAL4 driver, and incubated at 29°C, was followed. Flies expressing RNAi GFP were used as control. The number of surviving flies was counted every 5 days.

(B) PP2A RNAi induces a constitutive activation of the IMD pathway in ageing flies. Expression levels of *AttA* were measured by RT-qPCR, following total RNA extraction from ageing flies expressing RNAi PP2A-C and PP2A-A under the control of c564-GAL4 driver. Flies expressing RNAi GFP were used as control. AttA expression levels were normalized to *rp49* transcript levels.

(C) – (D) Overexpression of *PP2A* inhibits IMD pathway activation *in vivo*. *UAS-PP2Ac* transgenic fly line was crossed with Yolk-GAL4 driver flies. The expression levels of (D) *PP2A-C* and (C) *AttA* upon infection of the flies with *E. coli* for 4 hours, were analyzed by RT-qPCR and normalized to *rp49* expression levels. Flies expressing UAS GFP were used as control.

(E) Overexpression of *PP2A-C* increases the sensitivity of the flies upon infection with *Enterobacter cloacae.* Survival assays were performed following infection of UAS *GFP* and UAS *PP2A-C* expressing flies with E. cloacae. Relish mutant flies were used as control. Infected flies were incubated at 29°C and the number of surviving flies was counted every 8 hours.

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vector) **(Figures 17F and 17G).** Second, since PP2A-C is known to function in a multimueric complex including the constant PP2A-A scaffold subunit, we checked whether the knock down of the gene encoding this protein would also affect IMD pathway signaling in S2 cells. As shown in Figure 17H, similarly to *PP2A*-C (*dsPP2A-1* and *dsPP2A-2*), the KD of *PP2A-A* (*dsPP2A-A*) leads to a significant increase in *Attacin A* expression compared to ds*GFP* control cells **(Figures 17H and 17I)***.* Altogether, these results indicate that PP2A is a new negative regulator of the IMD pathway *ex-vivo*, in *Drosophila* S2 cells and prompted us to investigate its role in the control of the immune response of adult flies.

2- PP2A fine-tunes the systemic IMD pathway activity in adult flies

Looking at the results obtained in S2 cells, we questioned whether PP2A has the same impact on the modulation of the IMD-dependant immune response *in vivo*. PP2A is known to be associated with mitosis and organ development in *Drosophila*, with evidence supporting that *PP2A* loss of function results in severe morphological changes and mitotic defects in early embryos (434, 490). Therefore, we exploited the yeast UAS/GAL4 system to exclusively express short hairpin RNA (shRNAs) targeting *PP2A-C* and *PP2A-A* transcripts in the adult fly's fat body, using either *c564-Gal4* or *yolk-Gal4* drivers. In both cases, the expression of the *PP2A-C* **(Figures 18B and 18E)** and *PP2A-A* **(Figures 18C and 18F)** transcripts were significantly reduced in the fat-body of the flies. These RNAi flies (UASRNAi PP2A-C and UASRNAi PP2A-A) were then challenged by *E. coli* in order to trigger IMD signaling. Interestingly, *PP2A-C* and *PP2A-A* RNAi flies show an enhanced expression of *Attacin A* at 16 hours following their infection with *E. coli* compared to RNAi-*GFP* control flies **(Figures 18A and 18D).** An increase in *Attacin A* expression was also only observed in the *c564-Gal4* driven RNAi uninfected flies **(Figures 18A and 18D).** This observation could be explained by the added activity of this driver to the hemocytes that also express AMPs following an infection (22). Next, we sought to check the consequence of this increased *Attacin A* expression profiles on *Drosophila* immune response. For this purpose, we infected RNAi flies by septic injury with the pathogenic Gram-negative bacterium *Pseudomonas aeruginosa*, strain PA14, that is known to be fatal to wild type flies (586, 587). The survival curves of infected adult flies presented in Figure 18G clearly shows that RNAi-*GFP* flies succumb to PA14 infection within 72 hours, whereas RNAi-*PPA2-C* exhibit significantly higher survival rates **(Figure 18G).** RNAi-*relish* flies, used as a negative

Continuation of the Figure 19

Figure 19: PP2A fine-tunes IMD pathway activity *in vivo*

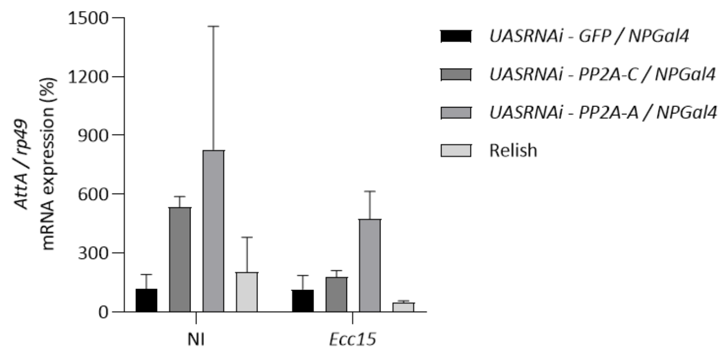
(F) – (G) PP2A's activity is specific to the IMD pathway. Flies expressing RNAi *PP2A-C* under the control of (F) c564-GAL4 or (G) Yolk-GAL4 drivers were infected with *Enterococcus faecalis* to activate the Toll pathway. After 24 hours, total RNA was extracted and the expression levels of *drosomycin* (Drom) AMP gene were measured by RT-qPCR and normalized to *rp49*. Myd88 mutants and RNAi GFP were used as negative and positive controls, respectively.

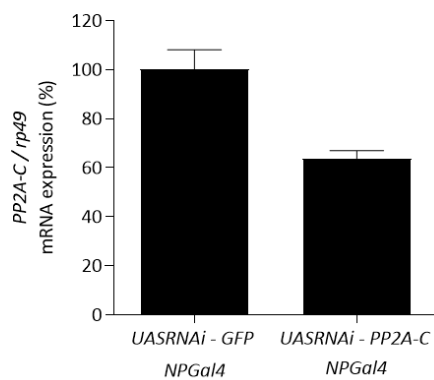
Data obtained from three independent experiments are combined in single value (mean ± sd). Log Rank test for the survival assays and Mann-Whitney test for the RT-qPCR data within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **: $0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

control, display a complete lethality 36 hours following the infection **(Figure 18G).** Altogether, these observations indicate that the KD of *PP2A* in flies enhances the activation of IMD pathway, conferring resistance to Gram-negative bacterial infections.

On a different note, an over-expression of antimicrobial peptides in *Drosophila* is known to result in cytotoxic effects, thus contributing to a shortened lifespan of the flies (588). This over-expression of AMPs could be induced by a constitutive activation of the immune response, triggered by a negative regulation defect (177, 178, 326). Interestingly, the flies impairing the expression of either *PP2A-C* or *PP2A-A* exhibited a striking shortened longevity compared to RNAi-*GFP* control flies **(Figure 19A).** This phenotype was indeed linked to a significant over-expression of *Attacin A* that was measured in the ageing flies, demonstrating a constitutively active IMD pathway when the expression of one of the core components of the PP2A complex is impaired **(Figure 19B).** Next, we asked whether the activity of PP2A is sufficient to inhibit IMD pathway activation upon infection. Therefore, we checked for the immune induction of the IMD response in the fat body of flies overexpressing *PP2A-C* under the control *of yolk-Gal4* driver line **(Figures 19C and 19D)** following *E. coli* infection. As shown in Figure 19C, IMD activation was strikingly inhibited by the over-expression of *PP2A-C*, as monitored by *Attacin A* expression that was almost abolished in these flies 4 hours post-infection **(Figure 19C).** To further study the effect of this over-expression on the immune response, we assessed whether it could reduce the survival rate of the infected flies. As expected, the fat-body specific over-expression of *PP2A-C* resulted in a decrease in the viability of the flies compared to flies overexpressing *GFP* following their infection with *Enterobacter cloacae* **(Figure 19E)***.* Collectively, these data underline the modulatory function of PP2A on the regulation of the systemic activation of IMD pathway.

Next, we sought to check the specificity of PP2A modulatory function to the IMD pathway. Hence, we measured the activation of the Toll pathway in the flies lacking PP2A function. For that, the *Drosomycin* expression level, a conventional readout of the Toll pathway, was evaluated in RNAi-*PP2A-C* flies following their infection with Gram-positive *Enterococcus faecalis.* As shown in Figure 19F, the *c564Gal4*-driven KD of *PP2A-C* in the flies' fat body does not affect the activation of Toll pathway upon *E. faecalis* infection **(Figure 19F).** A slight





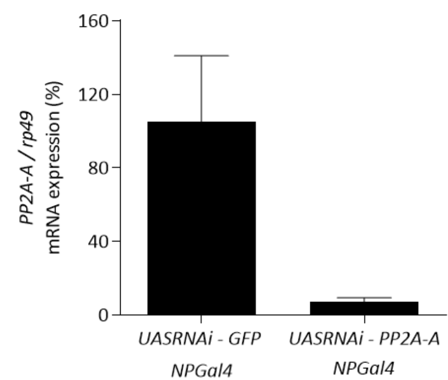


Figure 20: PP2A negatively regulates IMD activation in the gut

(A) *PP2A-C* and *PP2A-A* RNAi induces IMD activation in the gut. Real-time RT-qPCR analysis of *AttA* from the offspring of *PP2A-C* and *PP2A-A* RNAi lines crossed with NP-GAL4 drivers. RNA was extracted from the guts of unchallenged flies (NI) or flies infected orally with *Erwinia carotovara carotovora* for 6 hours. Flies expressing RNAi *GFP* and *Relish* mutants were used as control. *AttA* expression levels were normalized to *rp49* transcript levels.

(B) – (C) Knock down of *PP2A-C* and *PP2A-A* in the gut of adult flies. RNA was extracted from the gut of adult flies expressing RNAi targeting (B) *PP2A-C* and (C) *PP2A-A* transcripts using NP-GAL4 driver. *PP2A-C* and *PP2A-A* mRNA levels were measured by RT-qPCR and normalized to the expression of *rp49* and presented relative to the expression in UAS RNAi *GFP* flies, sets arbitrary as 1 (control).

Data was obtained from one biological experiment.

decrease in *Drosomycin* expression is observed when the RNAi expression was driven by *yolk-Gal4* driver **(Figure 19G).** Altogether, these data indicate that PP2A is required for the regulation of the immune response in *Drosophila* mainly targeting the IMD pathway.

3- PP2A negatively regulates IMD activation in the adult flies' gut

The immune response in the gut is complex, as its strength and timing must be tightly controlled in order to maintain a homeostatic host-microbe interaction (326, 589). An important role is thus attributed to the negative regulators of immune reactions that permit an adjusted activation to ingested pathogenic bacteria while keeping a controlled activation threshold, granting an immune tolerance to commensal microbiota (159, 326). To investigate the role of PP2A in regulating the IMD pathway activation in the gut, we analyzed the basal expression levels of *Attacin A,* after impairing the expression of the genes encoding both components forming the core enzyme using NP-Gal4 gut-specific driver **(Figures 20B and 20C).** Interestingly, RNAi-mediated KD of both *PP2A-C* and *PP2A-A* resulted in ectopic expression of *Attacin A* that was observed in the absence of any infection, and to an increased *Attacin A* expression upon *Erwinia carotovora carotovora 15* (ECC15) oral infection, compared to the RNAi-*GFP* control flies **(Figure 20A).** This preliminary result suggests a role of PP2A in maintaining an adjusted IMD-activation in the gut.

4- Identification of the regulatory subunits required for the regulation of PP2A activity during the immune response

As mentioned previously, the substrate specificity and the subcellular localization of PP2A heterotrimeric complex are determined by diverse regulatory B subunits. In order to explore which of the five *Drosophila*-encoded regulatory B subunits (described in section II) is involved in the negative regulation of IMD pathway, we evaluated the activation of IMD signaling in S2 cells impairing the expression of the genes encoding these subunits separately. In addition to the dsRNAs targeting the five known regulatory B subunits transcripts in *Drosophila*, we constructed an additional one targeting the transcript of the *strip* gene. The latter encodes striatin-interacting protein (STRIP) that is known to be a component of the STRIPAK-PP2A complex containing the PP2A core dimer and CKa (498, 503, 590). After treating S2 cells with these constructs and measuring the efficiency of the KD of each of the corresponding gene

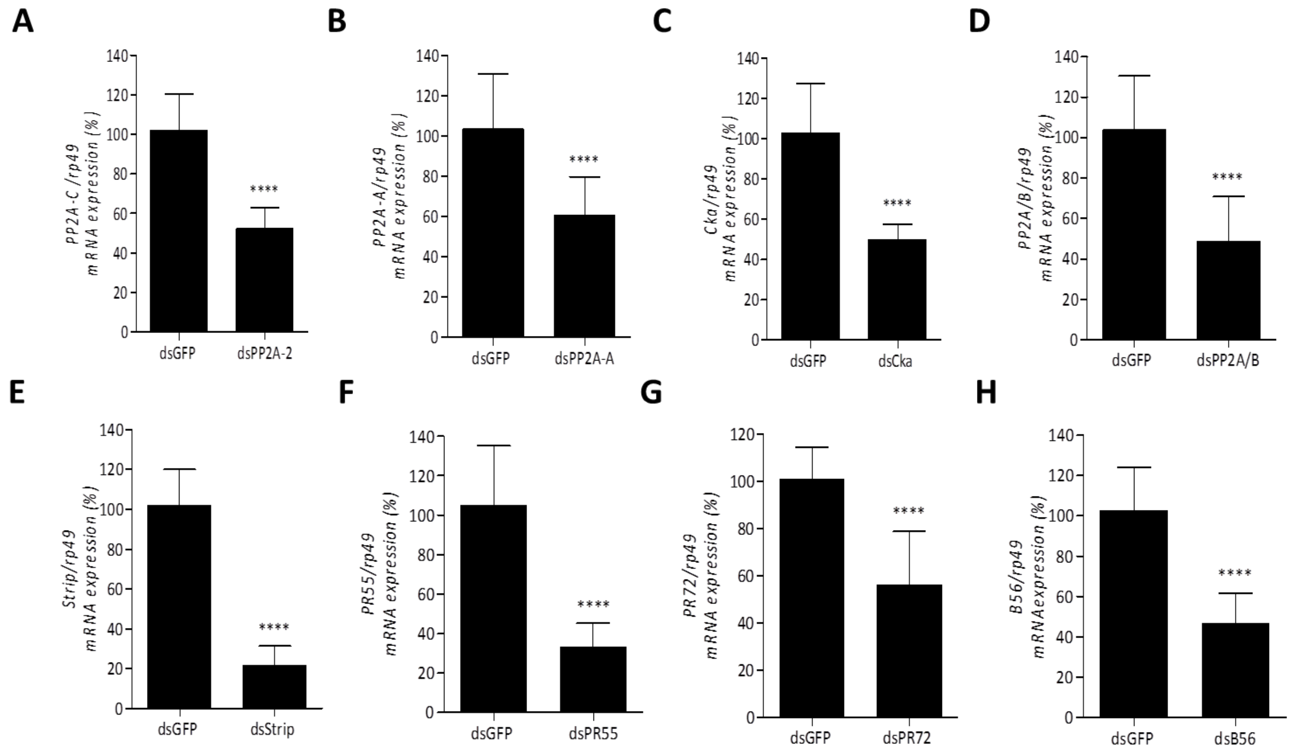


Figure 21: Identification of the regulatory subunits required for the regulation of PP2A activity during the immune response

KD efficiency of the dsRNA constructs used to silence the expression of PP2A regulatory subunits. *S*2 cells were soaked with (A) *dsPP2A* (B) *dsPP2A-A* (C) *dsCKA* (D) *dsPP2A-B* (E) *dsStrip* (F) *dsPR55* (G) *dsPR72* (H) B56 targeting *PP2a, PP2A-A, CKA, wdb, Strip, PR55, PR72 and wrd*, respectively. ds*GFP* were used as control. The expression level of each was measured by RT-qPCR and normalized to *rp49.*

Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

(Figure 21), *Attacin A* expression levels were monitored upon HKE stimulation. Out of the screened regulatory B subunits, PR55, CKa and Strip influenced the activation of the IMD pathway **(Figures 22A and 22B).** The KD of these genes resulted in an over-expression of *Attacin A* following HKE induction, compared to cells treated with *dsGFP* **(Figure 22A).** A constitutive expression of *Attacin A* was also noticed in the uninduced cells impairing the expression of these regulatory B subunits, similarly to the phenotype obtained after knocking-down the expression of the genes encoding the catalytic and the scaffold subunits in the cells **(Figure 22A).** Additionally, we used transgenic flies expressing UAS-dsRNA constructs driven by the *c564-gal4* to impair the expression of their corresponding transcripts in the fat body. IMD pathway activation was monitored on whole fly RNA extracts 16 hours following their infection by *E. coli*. Alike the results obtained in S2 cells, the KD of both CKa and Strip in the flies leads to a significant increase of *Attacin A* expression following an immune challenge with *E. coli* **(Figures 22C and 22D).** This observation contrasts with the phenotype observed in the flies impairing the expression of PR55 and all the other regulatory subunits, where *Attacin A* expression was comparable to UASRNAi-*GFP* control flies. Collectively, these data give evidence on the implication of both components of the STRIPAK complex, together with the PP2A core dimer, for the negative regulation of the IMD pathway both in S2 cells and in adult flies.

5- The NF-κB factor Relish is a direct interacting partner of PP2A

We previously described the role of the phosphatase PP4 in the negative regulation of the IMD pathway by acting at the level of the IKK complex. Genetic evidence reported in the previous chapter suggested that PP4 and PP2A act at different levels for the modulation of the IMD pathway. Therefore, we next sought to map the activity level of PP2A for the control of signal transduction, by conducting epistasis analysis in S2 cells. As shown by previous studies, an ectopic expression of some of the known IMD pathway components can trigger a constitutive expression of AMP encoding genes (316, 324, 336). This is particularily the case for *PGRP-LC*, *IMD* and a constitutively active form of *Relish*, Rel ΔS29–45. Therefore, a *PP2A-C* overexpressing vector was co-transfected with plasmids expressing the coding sequence of these genes in S2 cells. IMD signaling activation was monitored by the activity of an *Attacin A* – Luciferase reporter. As expected, the expression of the mentioned IMD pathway components

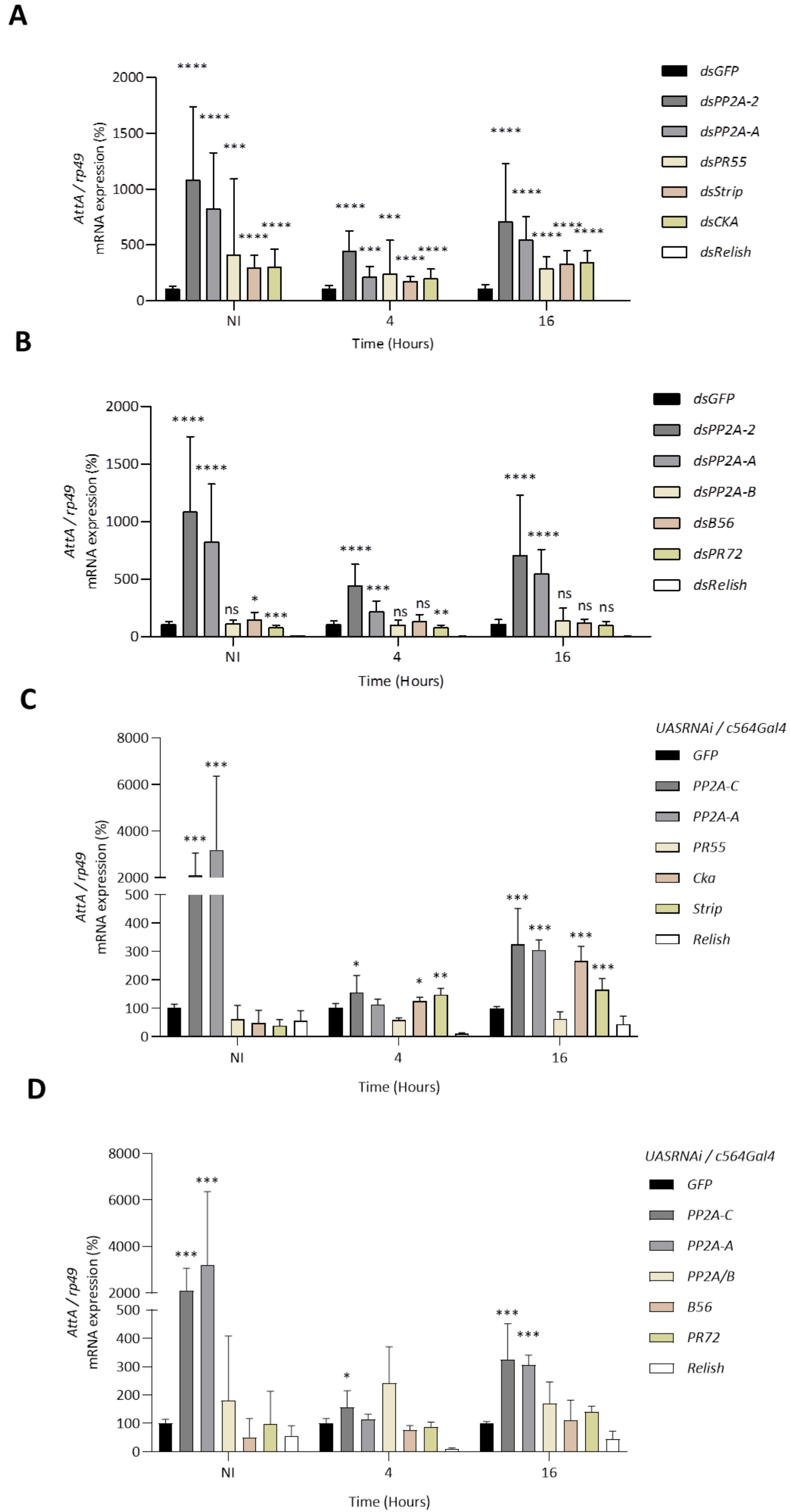


Figure 22: Identification of the regulatory subunits required for the regulation of PP2A activity during the immune response in S2 cells and *in vivo*

Continued on the next page

resulted in a robust *Attacin A* induction in the cells, compared to cells transfected with a LacZ expressing vector **(Figures 23A, 23B and 23C).** Interestingly, this phenotype was reversed by the concomitant over-expression of *PP2A-C* **(Figure 23A, 23B and 23C).** These results suggest that PP2A functions at the same level or downstream of the NF-κB factor Relish. Knowing that the phosphorylation of Relish is an essential event to trigger IMD-dependent gene expression (306, 311), these data lead us to hypothesize that Relish could potentially be the target of the phosphatase PP2A.

To further investigate this possibility, co-immunoprecipitation experiments were performed with lysates from S2 cells co-transfected with *PP2A-FLAG-HA*, *Relish-myc* and *PGRP-LC*. We immunoprecipitated PP2A using anti-FLAG antibodies- coated beads. As shown in Figure 23D, Relish is co-immunoprecipitated with PP2A in both IMD-induced or non-induced conditions **(Figure 23D).** This result is in agreement with the hypothesis that PP2A might be targeting Relish for the down regulation of the IMD signaling.

Continuation of the Figure 22

Figure 22: Identification of the regulatory subunits required for the regulation of PP2A activity during the immune response in S2 cells and *in vivo*

(A) – (B) RNAi mediated Knock down of *PR55*, *CKA* and *Strip* enhaces IMD pathway activation in S2 cells. S2 cells were soaked with dsRNA constructs targeting the five known regulatory subunits of PP2A in *Drosophila*: (A) *dsPR55, dsCKA* and *dsStrip* targeting *PR55*, *CKA* and *Strip* transcripts, respectively; (B) *dsPP2A-B*, *dsB56* and *dsPR72* targeting *wdb, wrd and PR72* transcripts, respectively. *GFP* dsRNA and *Relish* dsRNA were used as positive and negative controls, respectively. *dsPP2A* and *dsPP2A-A* were used as control for the experiment. The IMD pathway was induced 6 days later by adding HKE, and the expression of *AttA* was measured by Real-time quantitative RT-qPCR at 4- and 16-hours post-induction, and in the absence of HKE induction, and normalized to ribosomal protein 49 (*rp49*) transcript levels.

(C)- (D) *CKA* and *Strip in vivo* RNAi increases IMD pathway activity. Real-time RT-qPCR analysis of *AttA* from the offspring of (C) *PR55, CKA and Strip* RNAi lines and (D) PP2A-B, B56 and PR75 RNAi lines; crossed with c564-GAL4 driver. Total RNA was extracted from unchallenged flies (NI) or flies infected with *E. coli* for 4 or 16 hours. Flies expressing RNAi *GFP*, RNAi *Relish,* RNAi *PP2A-C* and RNAi *PP2A-A* were used as control. *AttA* expression levels were normalized to *rp49* transcript levels.

Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **: $0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

Discussion and Perspectives

Discussion and perspectives

Drosophila innate immune humoral response relies on NF-κB signaling pathways Toll and IMD that induce transcriptional programs leading to the expression of antimicrobial peptides encoding genes, main effectors of this response (6). Tight regulation of NF-κB pathways is vital, since a properly balanced activation is important for health and longevity (588, 591) and over-exuberant and prolonged responses are detrimental to the host (239, 339). NF-κB intracellular signaling progresses by post-translational modifications of the NF-κB factor and other pathway proteins (318). Hence, reverting these modifications seems to be a relevant modulatory mechanism to keep this response in check. Protein phosphorylation has been revealed to play a critical role in the activation of several core components of the *Drosophila* IMD pathway that harbors many kinases within its signaling players (211, 306, 311, 318). Upon the binding of IMD pathway receptors, PGRPL-LC and PGRP-LE (86, 172, 233, 235, 242, 244, 290, 293, 295) to bacterial-derived PGN and the recruitment of receptor-proximal components, the intracellular signal is transmitted to downstream effectors, in particular to the Kinase TAK1 (211, 251, 310, 592), responsible of phosphorylating and activating the I-κB Kinase complex IKK (211, 318). In its turn, the IKK complex is required for a full activation of the NF-κB factor Relish, by catalyzing a direct phosphorylation on Serine residues 528 and 529, which seems to be required for the proper activation of Relish and AMP gene induction (306). All of these phosphorylation events exhibit high conservation between *Drosophila* and vertebrate NF-κB pathways (593-595). Although these inducible phosphorylations significantly contribute to IMD signaling outcome and the executing kinases have been studied, relatively little is known about the opposing protein phosphatases. During my PhD thesis, I have studied the role of two protein phosphatases, namely PP4 and PP2A in fine-tuning the activation of the IMD pathway in *Drosophila*.

I- PP4 and PP2A negatively regulate the IMD-dependent immune response

RNAi mediated silencing of *PP4* and *PP2A* expression both in *Drosophila* S2 cells and in the fat body of adult flies enhanced IMD-dependent *Attacin A* expression. This elevated level of IMD signaling lead to an increased survival of the RNAi *pp2a* flies after their infection with the pathogenic Gram-negative bacteria *P. aeruginosa* PA14. Similar phenotypes were reported for

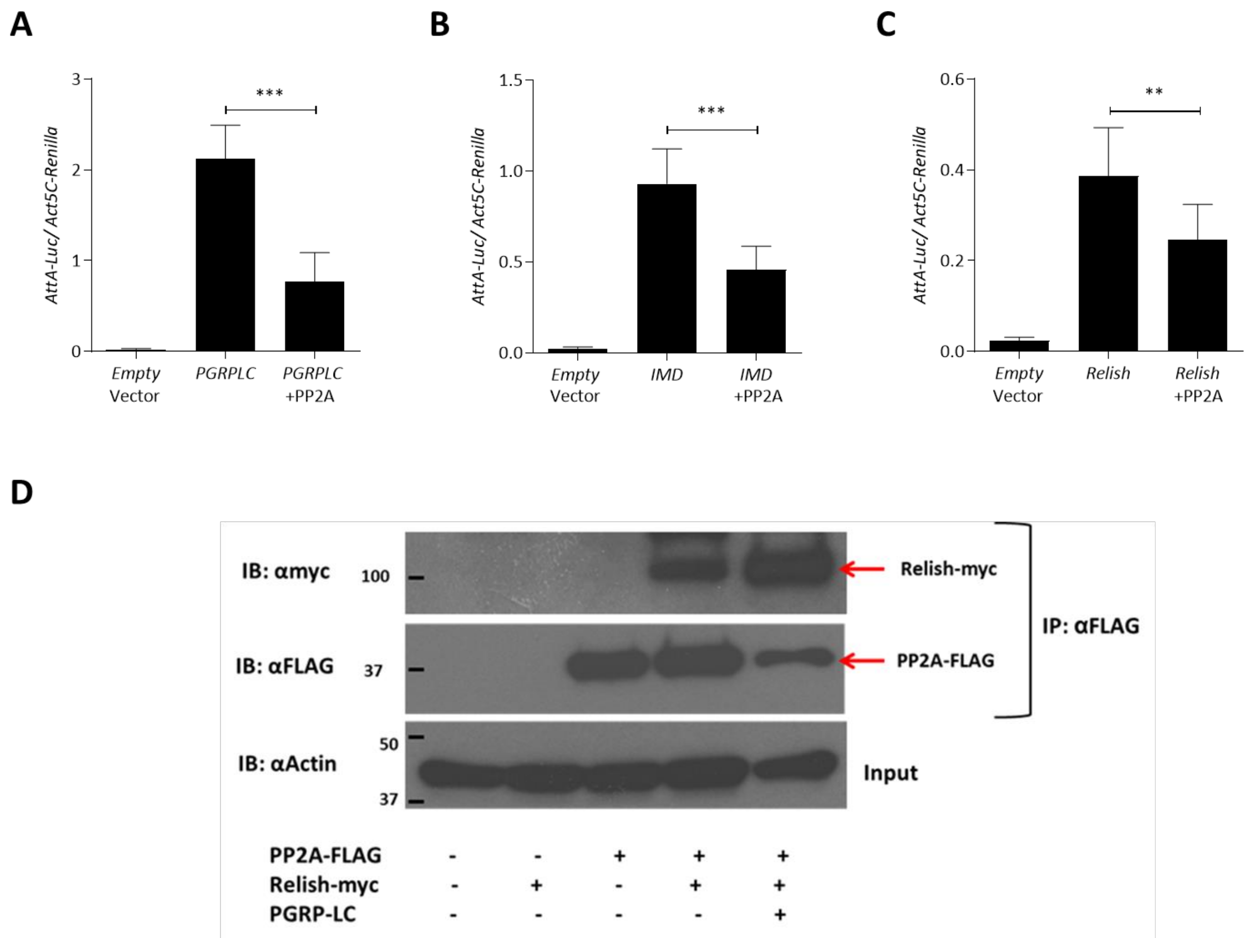


Figure 23: The NF-κB factor Relish is a direct interacting partner of PP2A

(A) - (B) – (C) PP2A acts at the same level or downstream or Relish. S2 cells were transfected with vectors overexpressing (A) PGRP-LC, (B) IMD, (C) Relish (ΔS29-S45). Cells transfected with an empty vector were used as control. Together with these plasmids, the cells were transfected with a vector overexpressing PP2A. IMD pathway activation was monitored with the AttA-Luciferase reporter gene. Actin-5C-Renilla activity was measured to normalize transfection efficiency.

Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

(D) PP2A interacts with Relish in the IMD pathway. Immunoprecipitation (IP) was performed using anti-FLAG Antibody coupled to agarose beads and immunoblotting (IB) with anti-myc, anti-FLAG and anti-Actin antibodies. Lysates were obtained from S2 cells transiently transfected with PP2A-FLAG-HA, Relish-myc and PGRP-LC expression plasmids.

mutants lacking the expression of IMD negative regulators. This was the case of the *Caspar* mutants, a negative regulator of IMD that exerts its function by inhibiting DREDD-mediated cleavage of Relish (336). Likewise, loss of function of Rudra induced over-expression of AMPs and resistance to infections (324). Selective RNAi-mediated knock down of both phosphatases using the *c564gal4* driver also resulted in a reduced lifespan of the flies in the absence of infection. In this context, the importance of negative regulation of the IMD pathway in fly fitness was underlined in several studies, showing that constitutive IMD activity predisposes flies to a reduced lifespan by different means. To give but some examples, a targeted over-expression of AMPs in *Drosophila* neuronal tissue or a decrease in the expression of some IMD negative regulators, which also results in a significant increase in AMP levels, lead to neurodegenerative effects and shortening of lifespan (287, 596). Loss of Trabid, negative regulator acting at the level of TAK1, also leads to a dramatically reduced life span of flies (178). In this case, a stress response accompanied by disrupted gut homeostasis was observed. However, rearing the flies on germ free conditions does not ameliorate the flies' lifespan meaning that the deleterious effects are more largely from the host rather than a disturbance in microbiota. This contrasts with other situations, where the reduced lifespan of IMD negative regulators is associated to an imbalanced microbiota (159) or to chronic activation of the IMD pathway by the indigenous bacteria (177). In our study, the expression of *PP2A* and *PP4* was specifically inhibited in the fat body and hemocytes of the flies. The reduced lifespan of these flies is accompanied by a progressive systemic over-expression of AMPs. Although it is known that immunity factors and molecules can strongly induce cell death, the exact mechanisms underlying these observations are not established (597). Notably, our observations are in agreement with a study showing that a global or fat body induced over-expression of AMPs result in shortening of lifespan of the flies by exerting cytotoxic effects (588). We are currently confirming the immune related effect of the reduced lifespan phenotype by examining whether a mutation in *Relish* would have a beneficial effect on RNAi flies. Given the multiple cellular functions played by PP2A and PP4, as mentioned above, the KD of their expression could lead to cellular defects affecting either the fat-body physiology or the hemocytes function. It would be interesting to investigate whether one or the other might be accounting for the lifespan-reduced phenotype. In collaboration with Dr Stanislava Chtarbanova, we are also checking

whether this enhanced immune response affects age-related neurodegeneration and whether PP2A and/or PP4 are also involved in the regulation of IMD signaling in glial cells (598). Finally, it is also important to study the role of these phosphatases in the regulation of the gut immune response where a fine-tuned immune reaction is essential for the tolerance of indigenous bacteria as discussed below.

In addition to these loss-of-function results, the over-expression of *pp2a* strongly suppressed signaling through IMD pathway both in S2 cells and in adult flies. These flies were then highly susceptible to an infection with Gram-negative bacteria *E. cloacae*. In order to investigate whether the bacterial infection accounts for the lethality phenotype, the survival of clean injured UAS *pp2a* flies was also followed. These flies survived normally after the injury. In addition, a CFU count should be performed on fly extracts, to check for an increase in bacterial colony formation in *pp2a* over-expressing flies. In order to confirm that this function is dependent on the catalytic activity of PP2A regulating IMD pathway signaling through dephosphorylation, we decided to develop a catalytically inactive form of *pp2a*. Analysis of the active sites of all phosphoprotein phosphatases family members shows that they are highly similar with conserved sequence motifs (599). Two metal ions identified as Mn^{2+} and Fe^{2+} are located in the active site. Coordination of these two metal ions is provided by three histidines, two aspartic acids, and one asparagine. These residues are highly conserved in all members of the PPP family (344). In order to design a catalytically inactive PP2A, we aligned the primary sequences of the catalytic subunits of PP2A and PP4, which identified highly conserved residues in the active site **(Figure 24).** We then mutated the wild-type transgene carried in the clone used for the overexpression of *PP2A* in the cells with a site directed mutagenesis, by the substitution of the residue D85 with N and H118 with N, which should render the phosphatase catalytically inactive. The same approach was applied by Lipinszki *et al*, to construct a phosphatase-dead mutant of PP4 catalytic subunit in order to study its effect on mitotic centromeres (517). The PP2A phosphatase-dead expressing clone will be further used to evaluate the effect of the catalytically inactive PP2A on IMD pathway activation. In addition, this clone will be exploited in rescue experiments, to further examine the specificity of *PP2A* RNAi in S2 cells by testing if the over-expression of wild-type *PP2A* and catalytically inactive *PP2A* could rescue its RNAi phenotype.

```
PP4c   ---MSDYSDLDRQIEQLKRCEIIKENEVKALCAKAREILVEEGNVQRVDSPVTVCGDIHG   57
PP2A    MEDKATTKDLDQWIEQLNECNQLTETQVRTLCDKAKEILSKESNVQEVKCPVTVCGDVHG   60

PP4c   QFYDLKELFKVGGDVPEKNYLFMGDFVDRGYYSVETFLLLLALKVRYPDRITLIRGNHES  117
PP2A   QFHDLMELFRIGGKSPDTNYLFMGDYVDRGYYSVETVTLLVALKVRYRERITILRGNHES  120

                               D85N                            H118N
```

Figure 24: Protein sequence alignment of the catalytic subunits of *Drosophila* PP4-19c and PP2Ac

The protein sequence alignment of the first 117 amino acids (a.a) of PP4-19c and 120 a.a of PP2Ac shows the conserved amino acids of the active site. Residues D85 and H118 of PP2Ac were mutated to N85 and N118 to produce a catalytically phosphatase dead mutant of this enzyme.

II- A role for PP2A in IMD regulation in the *Drosophila* gut

Previous studies have uncovered the vital role of IMD modulators in down-regulating the IMD pathway in the adult gut under unchallenged conditions to prevent its constitutive activation by commensal bacteria thus providing tolerance to this microbial community (148, 159, 177, 326, 341). In addition to the local immune over-activation, individuals with depleted levels of IMD negative regulators in the gut significantly activate the IMD pathway systemically, in unchallenged conditions and upon a bacterial oral infection (177, 326). This phenotype is due to the presence of commensal microbiota, since it was rescued when these flies were raised under sterile conditions (326). It has been proposed that a systemic reaction to a local infection is mediated by translocation of peptidoglycan fragments across the gut epithelium (177, 319, 600). However, our preliminary results show that PP2A functions as an immune modulator in the gut. Even in the absence of infection, flies impairing the expression of *PP2A* display expression of *Attacin A* in the gut. Moreover, upon ingestion of nonpathogenic bacteria *Ecc15, PP2A* KD flies exhibit an enhanced IMD activation compared to wild-type flies. Similar results were obtained for PP4 **(Figure 25)**. A key question regarding PP2A and PP4's implication in immune tolerance is their significance on the viability of non-infected and infected flies. To address this question, we will assay the lifespan of flies impairing the expression of these phosphatases in the gut under sterile conditions (germ-free) and following an oral bacterial infection. Moreover, we will investigate the role of these phosphatases in the context of immune regulation in the gut, by analyzing the effect of an oral infection on AMPs expression not only locally, but also systemically in flies depleted for their expression.

III- PP4 targets the IKK complex in the IMD pathway

Our results indicate that PP4 targets IKK for the regulation of IMD signaling. Indeed, PP4c and its R2 scaffold subunit co-immunoprecipitate with both components of the IKK complex. Biochemical studies also reveal a variation in the phosphorylation state of the IKK catalytic subunit upon the overexpression of PP4 in immune-challenged S2 cells. In additional experiments, we investigated whether PP4 also interacted with other components of the IMD pathway that match with its cellular localization and the epistasis analysis results. Thus, we checked for an association with the adaptor protein IMD and the caspase DREDD by co-immunoprecipitation. Therefore, we co-transfected S2 cells with the tagged *FLAG-PP4-19c*,

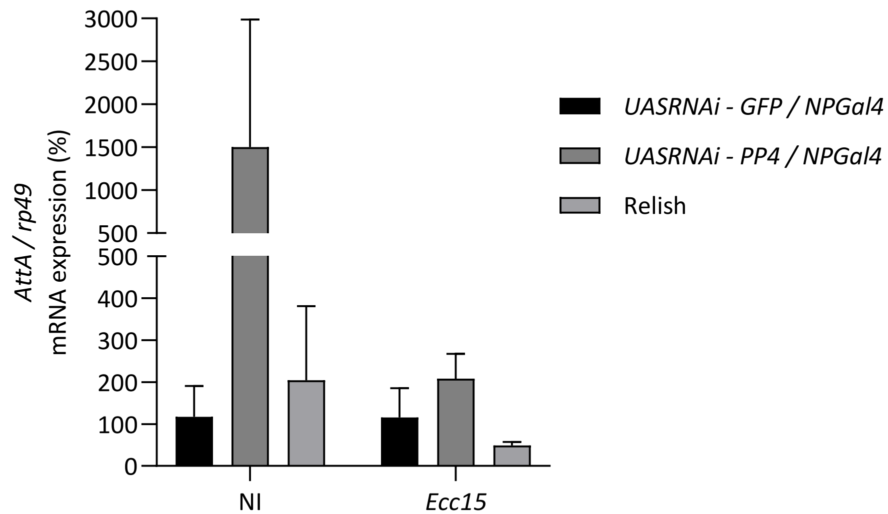


Figure 25: PP4-19c negatively regulates IMD activation in the gut

PP4-19c RNAi induces IMD activation in the gut. Real-time RT-qPCR analysis of *AttA* from the offspring of *PP4-19c* RNAi lines crossed with NP-GAL4 drivers. RNA was extracted from the guts of unchallenged flies (NI) or flies infected orally with *E. carotovara carotovora* for 6 hours. Flies expressing RNAi *GFP* and *Relish* mutants were used as control. *AttA* expression levels were normalized to *rp49* transcript levels.

Data was obtained from one biological experiment.

myc-DREDD and *V5-IMD.* These transfections were concomitant, or not, with the transfection of *PGRP-LC* for the induction of the IMD pathway. The preliminary results, that require further confirmation, presented in the complementary Figure 15A show that DREDD is able to co-immunoprecipitate with PP4-19C, with and without the induction of the pathway, in contrast to IMD **(Figure 15B)**. As previously described, both IKK and DREDD are required for Relish cleavage and robust AMP gene induction (308, 311). However, Relish processing by DREDD does not require the catalytic activity of IKK (306). Hence it was proposed that IKK complex may function as a scaffold or adaptor in controlling the cleavage of Relish by DREDD (306). This latter may therefore bind indirectly to PP4, likely via its interaction with IKK complex. In this context, one of our additional experiments is to verify both the phosphorylation and the cleavage of Relish following the overexpression of PP4. Knowing that one of the main outcomes of IKK complex activation is the subsequent phosphorylation of Relish to enhance its transcriptional activity, an indirect manner to observe the inhibitory effect of PP4 on IKKβ is to verify the phosphorylation state of Relish. In addition, even though Relish phosphorylation and cleavage seem to be two independent mechanisms (306) it would be interesting to test if Relish processing occurs normally in PP4-overexpressing conditions. Therefore, we aim to perform western blot analysis to observe the Relish cleavage fragments (Rel-68) as well as its phosphorylated form.

In a recent study, Tusco and colleagues have shown that IKKγ is a selective autophagic receptor that mediates the degradation of the IKK complex. They further show that a phosphorylated form of IKKγ accumulates in autophagy mutants (601). We have not so far considered the IKKγ phosphorylation state in PP4 depleted cells or fat bodies. However, this would be an interesting perspective to check. We can also verify whether IKK accumulates in autophagosomes in these conditions. These analyses aim at the consideration of a potential link between the negative regulation of IKK by phosphorylation/dephosphorylation and autophagy mediated degradation.

We clearly demonstrate the conservation of PP4's function as a modulator of NF-κB mediated immune response from insects to mammals. In addition to the implication of the catalytic and the scaffold subunits of the PP4 phosphatase complex in the regulation of NF-κB signaling observed in mammals (555, 557, 602, 603), our results represent the first evidence of

the involvement of the targeting subunit PP4-R3 or Falafel in *Drosophila* in this modulatory process. The molecular mechanisms underlying the assembly, recruitment and activation of the trimeric holoenzyme complex upon immune induction remain to be clarified. So far, we did not detect a robust physical interaction between Falafel and the IKK complex. Hence, a time scale co-immunoprecipitation experiments of Falafel and the IKK complex following an immune challenge could allow us to perceive an association in case this interaction is transitory. Moreover, PPPs are known to be modulated by post-translational modifications that either stimulate or inhibit their activity depending on the context (345). These modifications that include phosphorylation, methyl-esterification, acetylation and ubiquitination could target either the catalytic or the regulatory subunits of the phosphatase complex. Hence, focusing on the modifications occurring on PP4c, PP4-R2 and Falafel upon an immune stimulation, by mass spectrometry, could give us mechanistic insights on the activation of the complex and its implication in fine-tuning IMD signaling.

IV- Cellular functions of PP2A in the regulation of the IMD pathway

Epistasis analysis placed PP2A at the level of Relish. This result was further supported by co-immunoprecipitation experiments which revealed interaction between both proteins. These data support the hypothesis that PP2A could interfere with Relish phosphorylation. Indeed, phosphorylation of Relish is critical for an efficient signal-dependent transcriptional activation of target genes. Serines 528 and 529 have been identified as targets of IKKβ phosphorylation (306). Because this phosphorylation event takes place in the cytoplasm, this is consistent with our observations indicating a cytoplasmic localization of PP2A in S2 cells, independently of the immune induction **(Figure 26)**. In this study, I have shown that the knock-down of PP2A is sufficient to prompt a constitutive activation of IMD signaling in the absence of any infection. It is possible that PP2A acts on the de-phosphorylation of basal levels of phosphorylated Relish preventing deleterious side effects of unnecessarily induced or hyperactivated immune responses. This hypothesis is in agreement with our data showing an absence of *PP2A* induction upon an immune stimulation **(Figure 27).** This result suggests that *PP2A* is continuously expressed and permanently present in the cells serving as prerequisite for IMD inhibition. This contrasts with some other characterized IMD negative regulators that are rapidly induced following infection, in an IMD-dependent manner (177, 240, 319, 324-

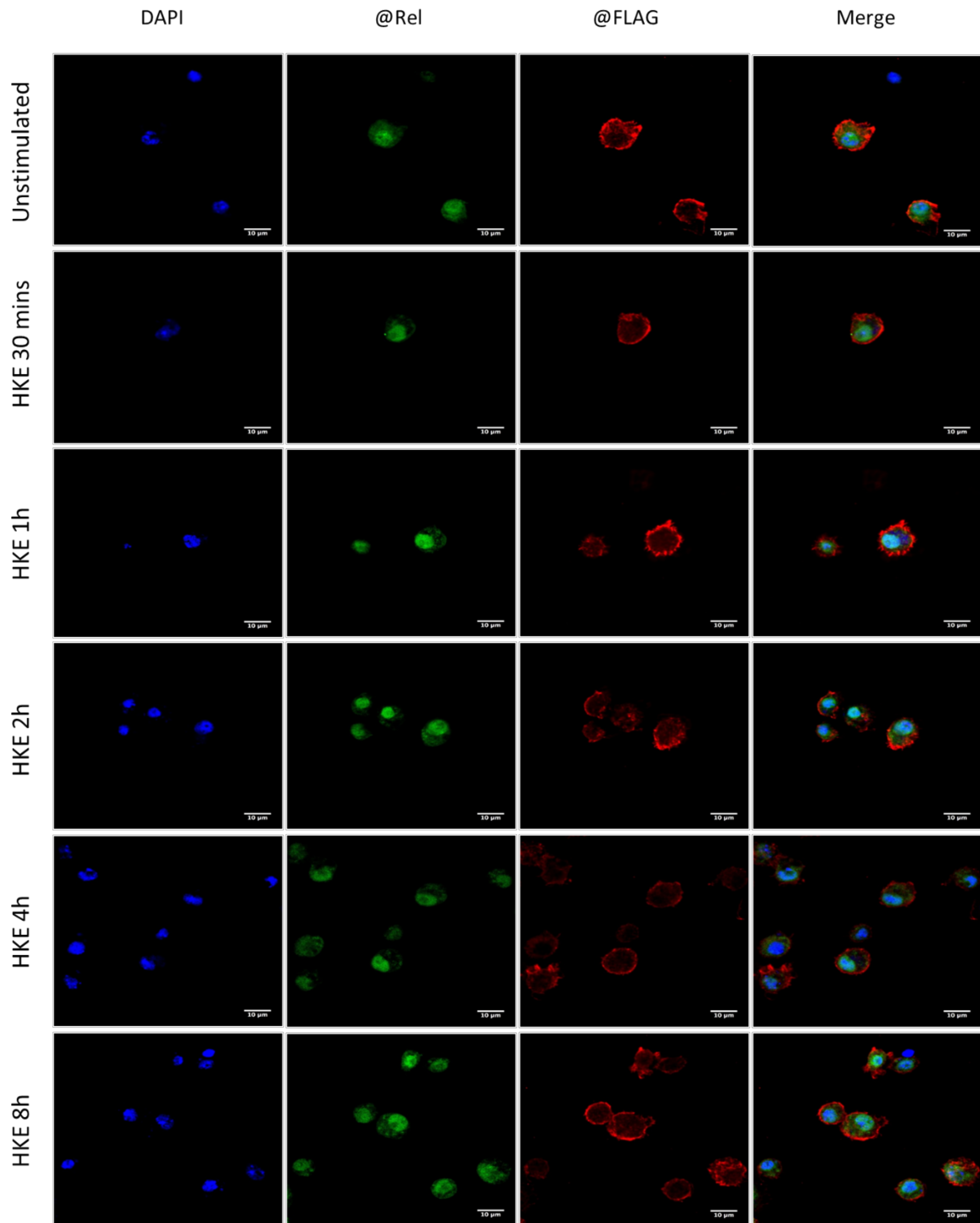


Figure 26: A time scale induction of S2 cells showing that PP2Ac is localized in the cytoplasm

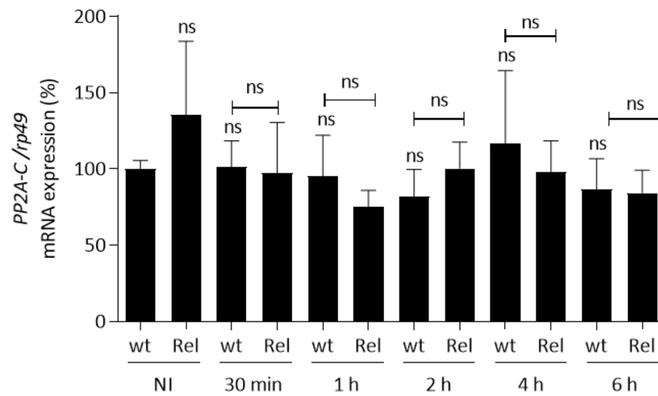
Confocal microscopy of S2 cells showing the cellular localization of PP2Ac at 30 minutes, 1, 2, 4 and 8 hours after adding HKE to S2 cells. The cells were transfected with a PP2Ac-FLAG-HA expressing vector and PP2Ac localization was followed using and anti-FLAG (@FLAG antibody). Relish localization was followed by using an N-terminal anti-Relish (@Rel) antibody. For cell staining, nuclei were visualised using DAPI (Blue). Scale bar, 10 µm.

326). A further characterization of Relish phosphorylation status is required in order to assess whether it is directly de-phosphorylated by PP2A. Our current results provide evidence for an interaction between PP2A and Relish. Relish phosphorylation state will be further investigated in protein extracts from cells over-expressing wild-type or phosphatase dead PP2A using a phospho S528-529 specific antibody by immunoblot assay (306).

Efficient employment of protein Serine/Threonine phosphatases in counteracting the activity of numerous kinases is achieved by forming distinct multimeric holoenzymes with other interacting partners, each with its own mode of regulation. This concept of holoenzyme has been well illustrated for PP2A (390). PP2A holoenzyme is a heterotrimeric complex, composed of a scaffolding subunit, a regulatory subunit and a catalytic subunit, of which the number of regulatory subunits is higher than that of scaffolding or catalytic subunit (604). In *Drosophila*, five regulatory subunits are encoded: Twins representing B55 family, Widerborst (Wdb) and Well-rounded (Wrd) belonging to B56 family, CG4733 representing PR72 family, and CKa orthologous to mammalian B''' or Striatin (502). In this study, we have shown that both the catalytic and the scaffold subunits of PP2A core enzyme have the same impact on IMD activation, proving further the specificity of the phenotype associated to this phosphatase complex. In the absence of PP2A-A, the IMD cascade is active at a significant level in the absence of exogenous bacterial infection and is enhanced following an immune stimulation, both in S2 cells and in flies. Moreover, we showed that the IMD pathway activation due to the loss of PP2A-A function is sufficient to mediate a reduced lifespan of the RNAi flies. The lack of induction of *PP2A-A* upon infection of the flies **(Figure 27)** shows that this structural subunit is constitutively expressed and acts together with the catalytic subunit for IMD inhibition.

Studies of the individual regulatory B subunits are critical for teasing apart the various functions and substrate specificity of PP2A (499). Therefore, besides uncovering the contribution of the PP2A core enzyme in fine-tuning the activation of IMD signaling, we discovered here the regulatory subunits mediating the specific activity of the holoenzyme. In an unbiased RNAi screen in S2 cells for PP2A regulatory subunits affecting IMD signaling profile, we found a phenotype associated to the KD of the regulatory subunits B (PR55) and Cka (B''') with its interacting protein Strip. An involvement of both Cka and Strip, but not PR55, in the modulation of the IMD pathway *in vivo* is also revealed, when their expression is

A



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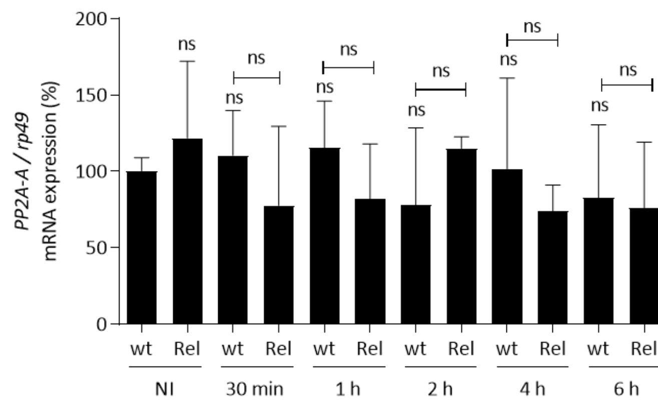

Figure 27: PP2Ac and PP2A-A are not induced after Gram-negative bacterial infection

To induce the IMD pathway, wild type (wt) and Relish mutant flies were infected by *E. coli*. After the infection, RNAs from dissected fat bodies were extracted and the expression level of *PP2A-C* and *PP2A-A* transcripts was measured using RT-qPCR, at 0, 0.5, 1, 2,4 and 6 h after infection. Data obtained from three independent experiments are combined in single value (mean ± sd). Statistical tests were performed using the Mann-Whitney test within Prism software (ns: $p > 0.05$; * $0.01 < p < 0.05$; **:$0.001 < p < 0.01$; ***: $0.0001 < p < 0.001$; ****: $p < 0.0001$).

impaired in the fat body of the flies. Taken together, our data support the implication of the STRIPAK complex, Cka and Strip, PP2A –A and PP2Ac, in the immune response. As the regulatory subunit PR55 has also been revealed to have an effect on the activation of IMD pathway in S2 cells, we cannot exclude the fact that PP2A could target more than one component within the IMD pathway, thereby mediating distinct de-phosphorylation events via different regulatory subunits. Indeed, this situation has been reported in the context of Hedgehog signaling regulation by PP2A. It was shown that PP2A acts as both positive and negative regulator of Hh signaling, via its subunits PR55 and Wdb that play distinct roles to modulate PP2A substrate and localization (422, 567, 571). This was also reported for PP2A–mediated NF-κB signaling in human embryonic kidney derived cells (605). The regulatory event mediated by PR55 may not be limiting for IMD modulation in adult flies. Genetic epistasis experiments have to be performed in order to place the dSTRIPAK complex in the IMD pathway. In addition, we should test whether the components of the dSTRIPAK complex, Cka and Strip, were able to physically associate with Relish, by conducting co-immunoprecipitation experiments. Noteworthy, Cka has been found in genetic experiments as well as large-scale RNAi screens to play critical roles in JNK signaling, and Hippo pathway regulation (502, 606, 607). Additionally, Strip was associated to microtubules stability regulating neuronal morphogenesis (503). These observations indicate distinct roles for dSTRIPAK complex in *Drosophila.* The present study is the first report that demonstrates the contribution of dSTRIPAK to the NF-κB mediated host defense. Interestingly, Strip seems to act as a molecular linker required for endosome formation and clustering (503), an essential mechanism for spatio-temporal regulation of the amount of cellular receptors. Consistent with this report, our cellular localization data show a cytoplasmic confinement of PP2Ac in S2 cells, with a cortical concentration towards the cytoplasmic membrane **(Figure 26)**. Additionally, recent studies have shown a novel function of an isoform of the receptor PGRP-LC, termed rPGRPLC. This latter presents a regulatory capacity that allows an adequate adjustment to the immune response to the level of threat, by down-regulating IMD pathway activation. It was shown that the dimerization of rPGRPLC with activating PGRPLC leads to efficient receptor endocytosis and termination of signalling via the ESCRT pathway (323). Therefore, it will be interesting to consider a role of the dSTRIPAK-PP2A complex in the regulation of this

endocytic modulatory mechanism. We could imagine an interplay in which STRIPAK provides a molecular scaffold allowing the de-phosphorylation of a certain substrate by PP2A leading to receptor recycling. Whether Relish could be targeted by PP2A at this level remains an open question. The current model of IMD mediated intracellular signaling implies the formation of an amyloid supramolecular signaling complex at the level of the PGRP-LC receptor recruiting both DREDD and the IKK signalosome (299). However, it remains unclear how Relish is recruited to this signaling platform. On the basis of our observations, it would be tempting to speculate that the STRIPAK complex could provide a scaffold bringing Relish into proximity with DREDD and IKK in this signaling platform. Studying the subcellular localization of these components would be useful to tackle this hypothesis. A potential role of PP2A/STRIPAK in the dissociation of this signaling platform is also an interesting question. Indeed, the elaborate regulation of PP2A activity by different regulatory subunits highlights a potential paradigm in which differential PP2A complexes could act at various levels on IMD pathway modulation.

V- Final thoughts

So far, our studies confirmed that both PP4 and PP2A are required for fine-tuning IMD pathway activation. Nevertheless, the high specificity of phosphatases towards cellular targets and their responsiveness to different stimuli suggest that other phosphoprotein phosphatases may modulate the IMD mediated response. For example, PP1 and PP2A dephosphorylate the same substrate, Par-3, to regulate cell polarity in the specification of neuroblast cell fate (608, 609). Similarly, PP2A and PP4 respond to different DNA damage signals to dephosphorylate γ-H2AX, for the repair of DNA double-strand breaks (508, 610). Therefore, we thought to test the implication of other PPPs in the regulation of IMD pathway. Subsequently, among the 19 phosphoprotein phosphatases encoding genes in *Drosophila*, we selected the ones that are expressed in S2 cells and in the fat body of adult flies. We then constructed dsRNA constructs targeting each one of the selected genes. Theses constructs will be used for performing a mini screen in S2 cells in order to find candidate phosphatases. In addition, our findings raised the question of whether the NF-κB response mediated by the Toll pathway is similarly counter-regulated by the activity of PPPs. Indeed, the activation of Dif, the NF-κB factor specific to the Toll pathway, requires phosphorylation events (275, 277). In our future perspectives, we aim to identify the candidate regulatory phosphatases involved in the modulation of this pathway.

Conclusion

Conclusion

During the last two decades, the *Drosophila* model has deeply served in expanding our knowledge and understanding of innate immune processes. Similarly to mammals, a malfunctioning immune system in *Drosophila* can cause severe damage to the target tissue, emanating the importance of intricate regulatory mechanisms in maintaining the immune response in check. The highly conserved NF-κB signaling which is of paramount importance in mediating the immune response must thus be delicately controlled. In *Drosophila* as well as in mammals, NF-κB activation is regulated by post-translational modifications of NF-κB and upstream pathway proteins, with reversible protein phosphorylation being one of the essential regulatory modifications. Cells use this post-translational modification to alter the activity or localization of key regulatory proteins required for NF-κB efficient activation. Protein phosphatases, together with kinases, set the phosphorylation state of signaling and effector proteins and thereby play a large role in controlling cellular responses. Inappropriate or defective phosphatase or kinase activity leads to aberrant patterns of phosphorylation contributing to disorders such as cancer and autoimmunity. Activation of NF-κB upon stimulation by phosphorylation is well studied; however, termination and fine-tuning of the signaling by de-phosphorylation are only partially understood.

In this study, we report the first characterization of IMD pathway negative regulation by dephosphorylation. We found that highly conserved PP4 and PP2A phosphoprotein phosphatases complexes are essential for IMD pathway adjusted signaling. Both complexes regulate the IMD mediated immune signaling in response to Gram-negative bacteria, and by inhibiting a constitutive activation of the response in the absence on an immune stimulation. By applying genetic and biochemical approaches we showed that PP4 acts at the level of the IKK complex. Our current data place PP2A at the level of the NF-κB factor, Relish. These Serine/threonine phosphatases act in trimeric complexes consisting of a promiscuous catalytic subunit, a scaffolding subunit, and a specificity-mediating regulatory subunit. Hence, we identified the specific phosphatase heterotrimeric complex components acting for the modulation of the IMD pathway for each of PP4 and PP2A. Our data highlight an evolutionarily

conserved function of these phosphatases in the regulation of NF-kB signaling from *Drosophila* to mammals.

With a high degree of conservation of NF-κB signaling pathways between *Drosophila* and mammals, a complete understanding of the roles of PP4 and PP2A in the control of the immune response should allow us to better design modulators targeting their activity. Indeed, NF-κB signaling abnormalities are relevant in many malignancies, autoimmune and inflammatory diseases, where regulators of NF-κB are mutated or differentially expressed. The results presented here demonstrate that each of the regulatory phosphatases act at a different level for the modulation of IMD signaling, in a complex combining distinct subunits. Therefore, in addition to directly modulating the activity of a phosphatase, targeting a regulatory subunit of holoenzyme complexes can be an alternative approach. Such strategy can be highly effective in medical applications as it may limit the devastating toxic side effects of inhibiting the activity of a promiscuous and multi-functional phosphatase.

Materials and Methods

Materials and Methods

Most experimental procedures have been described in detail in the manuscript: The PP4 phosphatase targets the IKK complex for the downregulation of the IMD-NF-κB pathway in *Drosophila* immune response (see results, chapter I, manuscript). Additional materials and protocols are described in this section.

Plasmid constructs

A complementary DNA clone for PP2Ac (FMO02385) was obtained from the *Drosophila* Genomics Resource Center (DGRC). This clone contains a metallothionein promoter and Flag-HA tag C-terminal fusion. PP2AcD85NH118N (Phosphatase Dead) entry clone was created by standard mutagenesis using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). pAC-PGRP-LC, pAC-IMD, pAC-Rel (ΔS29-S45) constructs were described previously (316, 317), a metallothionein promoter containing vector expressing full length Relish with a myc tag was used for Co-immunoprecipitation experiments. An anti-Relish (gift from Tony Ip) was used for Relish staining.

Mutagenesis

In vitro site-directed mutagenesis of PP2Ac expressing vector (FMO02385) was performed using QuikChange II XL Site-Directed Mutagenesis Kit from Agilent Technologies according to the manufacturer's protocol. Briefly, 2 pairs of complimentary oligonucleotides containing the desired mutations (D85NH118N) were synthesized, flanked by unmodified nucleotide sequence. The mutagenesis reaction contains: 20ng of plasmid template, 125 ng of forward and reverse primers, dNTPs mix, 10×reaction buffer, QuikSolution, H_2O and PfuUltra HF DNA polymerase (2.5 U/μl). The reaction was cycled using the cycling parameters described in the table 3 (7 minutes of elongation time were applied); then the Dpn I restriction enzyme (10 U/μl) was added directly to the amplification reaction and incubated at 37°C for 1 hour to digest the parental (i.e., the non-mutated) supercoiled dsDNA. Four μl of the Dpn I-treated DNA from the sample reaction were added to XL10-Gold ultracompetent cells for transformation. Two rounds of mutagenesis were performed for the insertion of two substitution mutations. The resulting mutated clone was verified by DNA sequencing.

Table 3: Cycling Parameters for the QuikChange II XL Method

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	1 minute/kb of plasmid length
3	1	68°C	7 minutes

Table 4: List of oligonucleotides used to generate dsRNA for RNAi in S2 cells

Gene	dsRNA reference	Forward	Reverse	Size
PP2Ac-1	BKN28006	TCGTGTCGTCGTCTCTTTTG	TTGCATTCGTTCAACTGCTC	320
PP2Ac-2	DRSC30716	ACACACACAGCGCGTTCTTA	CGATTCAATTTTGACGGCTT	289
PP2Ac-3	DRSC03574	AATGCCCGGTGACAGTGT	GAGGCGAGATTCCCCAGC	535
PP2A-A	DRSC31394	TCGAGTACATGCCTGCTCTG	ATATCTGTGCCGCAGACCTC	378
PP2A-B	DRSC30985	CACGTGAACGTGATTTCCTG	GCCATTATGATGCTCCGTTT	240
PP2A-B56	HFA14163	GTGGACTCGACACGCATTTT	CAACGGATTCGCCTTGCCCC	360
PR55	BKN60465	TCCAACAGCACTCGAACTTG	ACTCGAGCTCTAAGGGCACA	475
PR72	BKN60652	CAGCCAAAACAAGGGAATGT	ATTGGCAGAAGACCATCACC	402
Strip	BKN22135	CACAAAGCTATCGCCACTCA	CAAGACGGAGAGCATCAACA	389
Cka	AMB31793	ATGGAACGGTTAAGCTGTGG	GGGTGTGACACCACCTTGTT	293

Table 5: List of oligonucleotides used for quantitative real-time PCR

Gene	Forward	Reverse
PP2Ac	CAACACAAACGGCCTGACAC	CACATTGCGATCGTGACACC
PP2A-A	ACAAGGCTGTGGAATCTCTACG	AAACCAATCGCTGCAGTGTC
PP2A-B	GCTTTGCTCTGCCGCTTAAG	AATAGGTGAGCTGCGGATGG
PP2A-B56	CAAGACGCCAAACTCAACCG	CTGGTCGTTTGTTGCGTGTC
PR55	ACAACTTCTTCCGCGTCTTC	TGGCTTAAGCACCGTTTTCG
PR72	TCAGCGAGCTTATTCAGCAG	GATGTGGTTGCTGTTGATGC
Strip	AAACGACGATTGGGCCTTTG	TGCCGTTGTTTCCACACTTC
Cka	ACAATAAGACGTGCGTGCAG	ATGTACGGCTTTGTGGCATG

Transgenic flies

Flies carrying an UAS-RNAi against PP2Ac (41924), PP2A-A (49672), PP2A-B (107057), PP2A-B56 (101406), PR55 (104167), PR72 (107621), Strip (106184) Cka (106971) and Relish (108469) were obtained from VDRC (http://stockcenter.vdrc.at/control/main). Fly stocks were cultured at 25°C on standard *Drosophila* food. Flies carrying an UAS-PP2Ac construct (F001123) were obtained from FlyORF (http://flyorf.ch/index.php). Flies carrying Gal4 driver NP (3084) used to express UAS constructs in the gut were obtained from BDSC (Bloomington, USA; http://flystocks.bio.indiana.edu/).

Microbial strains and infections

We used *P. aeruginosa* strain PA14 with a final optical density (OD_{600}) of 0.5 and *E. faecalis* (OG1RF) with a final OD_{600} of 0.1 for septic injuries; and *E. carotovora* (Ecc15) with a final concentrated OD_{600} of ~100 for oral infections. Bacteria were grown in Luria broth (LB) (*E. carotovora*) or brain–heart infusion broth (BHB) (*E. faecalis and P. aeruginosa*) at 37°C. Survival experiments were performed on 15–25 females infected by *P.aeruginosa* septic injury at 29°C three independent times; with a thin tungsten needle previously dipped in the microorganism suspension diluted in PBS at the indicated concentrations. Control survival experiments were made by sterile injury. qRT–PCR experiments were performed on 10–20 nine-day-old females not infected and infected with *E. faecalis* for 16h by septic injury at 29°C, three times independently. For the oral infection of flies by *E. carotovora,* an overnight bacterial culture was centrifuged and prepared to a final OD_{600} ~ 100 in a 50mM sucrose solution. The challenged flies were incubated on 29 °C for 6 h and the *Attacin A* expression was quantified in RNA extracts of 5 to 10 dissected guts by RT-qPCR.

Gene knock down in S2 cells

DNA Templates for dsRNA preparation were PCR-derived fragments flanked by two T7 promoter sequences (TTAATACGACTCACTATAGG) and synthetized as described above. Oligonucleotides were generated based on DKFZ Genome-RNAi libraries and are listed in the following Table 4.

Real Time quantitative PCR

This method was described in detail in the manuscript: "The PP4 phosphatase targets the IKK complex for the downregulation of the IMD-NF-κB pathway in *Drosophila* immune response". Additional primers used are listed in table 5.

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Résumé étendu en Français

I- Introduction

Le système immunitaire inné constitue l'unique moyen de défense de la vaste majorité des espèces vivantes. Seuls les vertébrés ont développé des mécanismes de défense supplémentaires constituant le système immunitaire adaptatif (Hoffmann and Reichhart 2002). Grâce à la puissance de ses outils génétiques et la haute conservation des mécanismes de défense entre les insectes et les mammifères, la mouche du vinaigre, *Drosophila melanogaster*, a émergé comme un organisme modèle adapté pour étudier l'immunité innée. En effet, les études effectuées chez la drosophile ont largement contribué à nos connaissances actuelles de l'immunité innée surtout dans le domaine de la détection et de la signalisation suite aux infections (Lemaitre and Hoffmann, 2007).

L'une des réponses immunitaires les mieux caractérisées chez la drosophile est la production de peptides antimicrobiens (PAM) par les cellules du corps gras, l'équivalent fonctionnel du foie des mammifères (Lemaitre & Hoffmann, 2007). L'expression inductible des gènes codant les PAM est régulée au niveau transcriptionnel par deux voies de signalisation de type NF-κB, la voie Toll et la voie IMD (*Immune Deficiency).* Cette dernière présente de fortes homologies avec la cascade de signalisation activée en aval du récepteur TNF-α, l'une des principales voies de signalisation régulant la réponse inflammatoire chez les mammifères (Ferrandon, Imler et al. 2007). La voie IMD est activée suite à la détection du peptidoglycane renfermant l'acide méso diaminopimélique (DAP-PGN), caractéristique de la paroi des bactéries à Gram négatif et à Gram positif de type bacille, par des récepteurs appartenant à la famille de PGRP (*Peptidoglycan Recognition Proteins).* Il s'agit en particulier de PGRP-LC et PGRP-LE qui sont localisés au niveau de la membrane cytoplasmique et le cytosol, respectivement (Kaneko, Yano et al., 2006). Le signal intracellulaire est transmis à la protéine IMD recrutant par la suite dFADD (*Drosophila Fas-Associated protein with Death Domain),* la caspase DREDD (*Death related ced-3/Nedd2-like protein)* et la Kinase TAK1 (*TGF-β activated kinase1)* par l'intermédiaire de chaînes d'ubiquitine K63 (Leulier, Rodriguez et al, 2000). TAK1 active le complexe IKK (*inhibitor of κB kinase*) par phosphorylation. Ce dernier comprend une sous-unité régulatrice, IKKγ ou Kenny ; et une sous unité catalytique, IKKβ ou Ird5 (*Immune response deficient*) (Kleino & Silverman, 2014, Silverman, Zhou et al, 2000). Cette cascade de signalisation aboutit à la translocation nucléaire du facteur NF-κB, Relish*,* suite au clivage de son domaine inhibiteur C-terminal par la caspase DREDD. La phosphorylation de Relish sur des résidus sérine par le complexe IKK constitue également une étape critique pour promouvoir son activité transcriptionnelle (Kleino & Silverman, 2014).

II- Contexte de l'étude

Les voies de signalisation NF-κB sont les régulateurs clés de la réponse immunitaire innée. Cependant, une activation excessive ou incontrôlée de NF-κB est fréquemment associée au développement de maladies inflammatoires chroniques et de cancers (Gamble, McIntosh et al, 2012). En conséquence, de nombreux mécanismes de régulation capables d'interférer avec l'activation des voies NF-κB ont évolué, assurant ainsi une réponse proprement ajustée. Dans ce contexte, plusieurs régulateurs négatifs de la voie IMD ont été caractérisés. Ceux-ci agissent à différents niveaux et impliquent des mécanismes moléculaires variés limitant le stimulus

activant, entre autre la dégradation du PGN issu des bactéries et la dé-ubiquitination (Bischoff, Vignal et al., 2006, Mellroth, Karlsson et al., 2003, Tsichritzis, Gaentzsch et al., 2007). Toutefois, bien que plusieurs protéines kinases soient impliquées dans l'activation de la voie IMD, aucun phénomène d'inhibition par déphosphorylation n'a été décrit. Le présent travail vise à explorer des mécanismes de régulation agissant à ce niveau.

Au cours d'un crible génétique par ARN interférence (ARNi) effectué en culture de cellules S2 dérivées d'hémocytes de drosophile à l'UPR 9022, nous avons identifié *CG32505,* codant pour la sous unité catalytique du complexe de la phosphoprotéine phosphatase 4 (PP4c) comme un nouveau régulateur négatif de la voie IMD. Mon travail sur la caractérisation de la fonction de PP4 a mené consécutivement à l'identification d'une autre phosphatase impliquée dans la régulation négative de la voie IMD, la protéine phosphatase 2Ac (PP2Ac) codée par le *CG7109*. Toutefois, la spécificité d'action des phosphoprotéines phosphatases, dont le nombre est relativement restreint, est assurée par une variété de sous unités régulatrices ciblant l'activité du complexe à un substrat spécifique. Par conséquent, cette étude vise à l'identification des sous unités régulatrices associées à PP4c et PP2Ac soutenant la spécificité de régulation. Ainsi, le travail de ma thèse a visé la caractérisation de ces complexes de phosphatases en vue d'une meilleure compréhension de la régulation fine de la voie IMD.

III- Résultats

1- Le complexe PP4 est un nouveau modulateur de la voie IMD agissant au niveau du complexe IKK

La spécificité du phénotype attribué à l'atténuation de l'expression de *pp4c* au cours du crible en cellules S2, a été confirmée par la reproduction des résultats obtenus en utilisant deux nouvelles constructions d'ARN double brin (*dsRNA*), non chevauchant, ciblant spécifiquement *pp4c*. L'activation de la voie IMD a été évaluée par le dosage de l'expression de l'*Attacine A,* un gène codant pour un PAM, par la technique de PCR quantitative en temps réel (RT-qPCR). En comparaison à des cellules contrôles traitées par des *dsRNA* ciblant le gène *gfp,* les cellules traitées par ds*pp4c* montrent une augmentation dans l'expression d'*attacine A*, suite à leur stimulation par des bactéries *Escherichia coli* tuée par la chaleur (HKE). Dans le but de confirmer ces résultats *in vivo,* j'ai utilisé le système UAS-Gal4 afin d'exprimer des constructions d'ARN double brins pour atténuer l'expression de *pp4c* sélectivement dans le corps gras des drosophiles adultes. Similairement aux résultats obtenus en cellules, une augmentation de l'expression d'*Attacine A* est observée chez les drosophiles transgéniques, ARNi *pp4c,* suite à leur infection par *E. coli.* D'une façon intéressante, la longévité de ces drosophiles est fortement réduite. Ce phénotype, associé à une activation constitutive et progressive de la voie IMD chez les drosophiles âgées, rappelle le développement des maladies inflammatoires chroniques chez les mammifères. Par une approche génétique complémentaire, la surexpression de *pp4c* dans le corps gras des drosophiles engendre une réduction significative de l'expression d'*Attacine A* suite à l'infection. Cette altération dans l'activation de la voie IMD se traduit par une susceptibilité accrue de ces drosophiles à une infection par *Enterobacter cloacae*. Aucune altération du profil d'activation de la voie Toll n'est observé chez les drosophiles déficientes pour l'expression de *pp4c*. Ce résultat atteste de la spécificité d'action de PP4 au sein de la voie IMD.

La forme majeure du complexe enzymatique PP4, la plus décrite dans la littérature, inclut la sous unité catalytique (PP4c) ainsi que deux sous-unités régulatrices : une protéine structurale ou PP4R2 et une protéine régulatrice, PP4R3 (Lipinszki, Lefevre et al, 2015). Ce complexe agit dans la régulation de la signalisation Hedgehog, la maturation des centrosomes et la division des neuroblastes chez la drosophile (Hall, Pradhan-Sundd et al., 2017, Jia, Liu et al, 2009, Lipinszki et al, 2015). D'autres complexes agissant dans des contextes différents ont également été décrits. Entre autres le complexe incluant la sous unités PTPA assurant la localisation corticale du complexe MIRA (Zhang, Huang et al., 2016). Dans le but de vérifier si ces sous-unités régulatrices seraient impliquées dans la modulation de la voie IMD, j'ai suivi la même approche par ARNi pour atténuer leur expression en cellules S2 et en drosophiles adultes. Les résultats obtenus confirment l'implication des deux sous-unités PP4R2 et PP4R3 dans la régulation négative de la voie IMD chez la drosophile.

Dans l'objectif de caractériser le rôle de PP4 dans la régulation négative de le la voie IMD, j'ai effectué une série d'expériences afin d'identifier sa potentielle cible cellulaire. Pour cela, j'ai d'abord montré, par microscopie confocale, que PP4 a une localisation cytoplasmique invariante que les cellules soient stimulées ou pas par HKE. Ensuite, j'ai réalisé des expériences d'épistasie. Pour cela, j'ai transfecté les cellules S2 par des constructions permettant la surexpression de différentes composantes de la voie IMD. Il s'agit en particulier du récepteur PGRP-LC, de l'adaptateur IMD et de la forme constitutivement active du facteur NF-κB, Relish dont la surexpression provoque une activation constitutive de la voie IMD en absence de tout stimulus. L'activation de la voie IMD, suivie par l'activité du rapporteur p-Attacine A – Luciférase, a été comparée à celles relevée sur des cellules co-transfectées par un vecteur permettant l'expression d'une forme catalytiquement active de PP4c que nous avons montré efficace pour l'inhibition de l'activation de la voie IMD. Les résultats de ces expériences ont montré que PP4c agit en aval de PGRP-LC et IMD et amont de Relish. A ce niveau de la cascade de signalisation IMD, agissent la kinase TAK1 et le signalosome IKK. Leur phosphorylation suivant un stimulus activant la voie IMD supporte l'hypothèse que l'une des deux composantes serait éventuellement la cible présumée de la phosphatase PP4. Pour tester cette possibilité, j'ai réalisé des expériences de co-immunoprécipitation. Les résultats obtenus supportent une interaction entre PP4 et les deux composantes du complexe IKK, qui serait la *bona-fide* cible de cette phosphatase. J'ai également vérifié l'état de phosphorylation de IKKβ dans les cellules S2 surexprimant PP4 par des expériences de « band-shift ». Les résultats préliminaires, qui restent à valider, supportent la déphosphorylation de IKKβ dans ces cellules. Pour aller plus loin, j'ai effectué des analyses d'interactomes pour PP4c et PP4R3 par spectrométrie de Masse, à partir de cellules S2. Les résultats préliminaires confirment l'interaction des deux unités du complexe avec NEMO ou IKKγ, et révèlent une interaction avec une E3-ubiquitin ligase impliquée dans l'activation de la voie IMD.

2- Le complexe PP2A régule négativement la voie IMD en agissant au niveau du facteur NF-κB Relish

En caractérisant le rôle de PP4 au sein de la voie IMD, et dans le but de confirmer la spécificité du phénotype attribué à PP4, j'ai arbitrairement testé le rôle de deux autres phosphoprotéines phosphatases connues, *pp2ac* et *pp1-87b*. Suite à une stimulation des cellules S2 par des HKE, l'atténuation de l'expression de *pp1-87b* n'affecte pas l'activation de la voie IMD, alors que celle de *pp2ac* résulte en un phénotype similaire à *pp4c*. J'ai vérifié qu'il ne

s'agit pas d'une redondance entre les deux protéines appartenant à la même famille de Sérine/Thréonine Phosphatases. En effet, l'atténuation de l'expression de *pp4c* et de *pp2ac,* simultanément, aboutit à une surexpression d'*Attacine A* plus intense, par comparaison à celle obtenues à partir des cellules traitées par des constructions de *dsRNA* ciblant l'une des deux phosphatases. En plus, la co-transfection des cellules par une construction sur-exprimant *pp4c* n'a pas pu sauver le phénotype lié au KD de *pp2ac*. Pour mieux comprendre le rôle de PP2A dans la régulation de la voie IMD, j'ai réalisé des tests d'épistasie en suivant la même approche décrite préalablement. Les résultats obtenus indiquent que PP2Ac agit au même niveau que le facteur NF-κB Relish. L'ensemble de ces données indique que PP4c et PP2Ac agissent à différents niveaux pour la régulation négative de la voie IMD. Le rôle de PP2Ac dans la régulation de la voie IMD a également été confirmé *in vivo* par les mêmes moyens que ceux décrits pour PP4.

Similairement à PP4c, les diverses fonctions de PP2Ac sont assurées par une multitude de sous unités régulatrices. Ainsi, le complexe enzymatique PP2A consiste en une sous unité structurale nommée PP2A-A et une sous unité régulatrice B variable selon le substrat et le contexte moléculaire (Cegielska, Shaffer et al, 1994, Eichhorn, Creyghton et al, 2009). En appliquant l'approche ARNi en cellules et chez les drosophiles adules, j'ai confirmé l'implication de la sous-unité structurale PP2A-A dans la régulation négative de la voie IMD. En plus, l'inhibition de l'expression des gènes codant les sous-unités régulatrices B employées lors de la réponse immunitaire en cellule a suggéré un rôle des sous-unités régulatrices PR55 ou Twins et le complexe Striatin.
En accord avec les résultats des analyses d'épistasie suggérant un rôle de PP2A au niveau de Relish, une interaction entre ces deux protéines a été révélée par des analyses d'interactomes que j'ai effectuées par spectrométrie de masse.

IV- Conclusions

Les travaux de recherches effectués dans le cadre de cette thèse s'inscrivent dans la compréhension des mécanismes de régulations de voies de signalisations NF-κB en utilisant la drosophile comme un organisme modèle. Une meilleure caractérisation de la régulation négative de la voie IMD, dont les défaillances rappellent les situations d'inflammations chroniques impliquant une dérégulation de la voie TNFα chez les mammifères, pourrait fournir des indices sur la façon de concevoir de nouvelles molécules ou stratégies thérapeutiques et ainsi contribuer à minimiser ou retarder l'inflammation. En effet, bien que la signalisation intracellulaire au sein de la voie IMD implique plusieurs processus de phosphorylation, les connaissances actuelles sont très mineures quant à sa régulation négative par déphosphorylation. En combinant des approches de génétique, de biologie moléculaire et de biochimie, le travail réalisé au cours de cette thèse a permis de mettre en avant deux complexes enzymatiques de protéines phosphatases, PP4 et PP2A, essentiels à la régulation négative de la voie IMD. Les résultats obtenus montrent que le complexe moléculaire formé par la protéine phosphatase 4 (PP4c), et deux sous-unités régulatrices, assure la régulation négative de la voie IMD en ciblant le signalosome IKK. Par ailleurs, la protéine phosphatase 2A (PP2A) cible le facteur de transcription NF-κB, Relish. L'ensemble de ces résultats soulignent complexité de régulation de la voie IMD.

En outre, ces résultats soulèvent plusieurs questions quant à l'implication d'autres protéines phosphatases dans la régulation des voies IMD et Toll de la Drosophile.

Les facteurs NF-κB étant les régulateurs principaux de la réponse inflammatoire chez l'homme, j'espère qu'une meilleure compréhension du mode d'action des protéines phosphatases au sein de ces voies trouve ses applications dans le développement de nouvelles stratégies pour le traitement des maladies inflammatoires. PP2A étant un suppresseur de tumeur connu chez les mammifères, je souhaite que mes données fournissent de nouvelles pistes de recherche sur le lien entre la signalisation de NF-κB, l'inflammation et le développement et la progression des tumeurs.

Présentation d'un poster:

"A phosphoprotein phosphatase negatively regulates the Drosophila IMD-NF-κB pathway"

25th European Drosophila Research Conference 2017, London 2017/2018

"A phosphoprotein phosphatase negatively regulates the Drosophila IMDNF-κB pathway"

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Prix du meilleur poster.

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Identification et caractérisation des phosphoprotéines phosphatases, PP4 et PP2A, comme nouveaux régulateurs négatifs de la voie IMD chez la Drosophile

Résumé

Les facteurs de transcription NF-κB sont des régulateurs essentiels de la réponse immunitaire innée hautement conservée au cours de l'évolution. Leur activation excessive est hautement délétère et est associée au développement de maladies inflammatoires chroniques. Il est donc particulièrement intéressant de caractériser les mécanismes moléculaires régulant leur activation. Au cours de ce travail, nous avons exploré le rôle de phosphoprotéines phosphatases dans la régulation de la voie IMD-NF-κB chez la drosophile. Homologue de la voie de signalisation activée en aval du récepteur TNFα et des récepteurs Toll-like chez les mammifères, cette voie contrôle les infections bactériennes chez la drosophile à travers l'activation du facteur de transcription de type NF-κB, Relish. Nous avons identifié les phosphatases PP2A et PP4 comme de nouveaux régulateurs négatifs de la voie IMD. En combinant des approches génétiques et moléculaires nous avons montré que ces phosphatases agissent au niveau du complexe IKK et du facteur de transcription Relish respectivement, pour la régulation fine de la voie IMD. Ainsi ce travail fournit la première preuve de la régulation négative de la voie IMD par des phosphoprotéines phosphatases et met l'accent sur la haute conservation des fonctions de PP2A et de PP4 dans la régulation des voies NF-κB. Nos résultats offrent ainsi de nouvelles perspectives pour la caractérisation des mécanismes moléculaires régulant la voie IMD.

Mots clefs : Immunité innée, voies NF-κB, phosphatases, régulation négative, Drosophile

Résumé en anglais

NF-κB pathways are highly conserved key regulators of the innate immune response in metazoans. However, their excessive activation is highly detrimental and is associated with the development of chronic inflammatory diseases. A keen interest is thus attributed to the characterization of the processes which ensure the proper duration and intensity of NF-κB signaling. Here, using Drosophila melanogaster as a model, we aimed at investigating the role of phosphoprotein phosphatases in the fine-tuning of the IMD-NF-κB pathway. This pathway is akin to mammalian tumor necrosis factor receptor signaling pathway and controls Drosophila immune defenses to Gram-negative bacterial infections through the activation of the NF-κB transcription factor Relish. We identify the highly conserved PP2A and PP4 as bona fide new negative regulators of IMD. By combining genetic and biochemical approaches, we show that PP4 and PP2A act at the level of the IKK signalosome and Relish respectively to modulate IMD signaling. Altogether, these data provide the first evidence of the regulation of the IMD pathway by phosphatases and emphasize the high conservation of the role of PP2A and PP4 in the regulation of NF-κB pathways. Our results set the bases for new perspectives for the characterization of the molecular processes controlling the IMD intracellular cascade.

Keywords: Innate immunity, NF-κB pathways, phosphatases, negative regulation, Drosophila melanogaster