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# Deciphering regulatory mechanisms of the IMD pathway activation during the innate immune response in

## *Drosophila*

François Bonnay

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UPR9022 CNRS Réponse Immunitaire et Développement chez les Insectes

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**François Bonnay**

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Spécialité : Aspects moléculaires et cellulaires de la Biologie

**Caractérisation des mécanismes de régulation de la voie IMD au cours  
de la réponse immunitaire chez *Drosophila melanogaster***

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## IV. Abbreviations

20E: 20-hydroxyecdysone

AA: amino acid

AD: Adherens junction

AgAkirin: *Anopheles gambiae* Akirin

AMP: Anti-microbial peptide

ANT-C: Antennapedia complex

AP1: Activator protein 1

APC: Adenomatous polyposis coli

ARID: AT-rich interaction domain

ATF2: Activating transcription factor 2

ATP: Adenosine triphosphate

Att: Attacin

BAFs: Brahma-associated factors

BAP complex: OSA-associated  
Brahma complex

Bap170: Brahma-associated protein  
170 kDa

Bap55: Brahma-associated protein  
55kDa

Bap60: Brahma-associated protein  
60kDa

Bbg: Big-bang

Bcl3: B-cell lymphoma 3

BCR: B-cell receptor

Bfl-1: Bcl2-related gene expressed in  
fetal liver 1

Brg-1: Brahma-related gene 1

Brm: Brahma

BX-C: Bithorax complex

C106: Spätzle 106 amino acids C-  
terminal fragment

C3PO: Component 3 promoter of RISC

Caspase: Cysteine-dependent  
aspartate-directed protease

CD: Crohn's disease

CDRE: Caudal protein DNA  
recognition element

CeAkirin: *Caenorhabditis elegans*  
Akirin

Cec: Cecropin

Chd: Chromodomain-helicase DNA  
binding

ChIP: Chromatin Immunoprecipitation

CK1 $\alpha$ : Casein kinase 1 $\alpha$

CrPV: Cricket paralysis virus

CYLD: Cylindromatosis

DAG: Diacylglycerol

DAMP: damage-associated molecular  
pattern

DAP-type: meso-diaminopymelic-type

DAV: Drosophila A virus

DCE: Dopachrome conversion enzyme

DCHS: Dachshaus

Dcr-2: Dicer-2

DCV: Drosophila C virus

Dcy: Drosocrystalin

DD: Death domain

DDC: DOPA decarboxylase enzyme

DED: Death effector domain

DFV: Drosophila F virus

DIAP2: Drosophila inhibitor of  
apoptosis 2

## Abbreviations

DIF: Dorsal-related immunity factor	Faf: Fat facets
Dilp: Drosophila insulin-like peptide	FHV: Flock-house virus
<i>DmAkirin</i> : <i>Drosophila melanogaster</i> Akirin	FOXO: Forkhead box O
DMAP1: DNA methyl transferase 1	FT: Fat
Dnr-1: Defense repressor 1	Fz: Frizzled
DOPA: 3,4-dihydroxyphenylalanine	G707: Gluconobacter sp. strain EW707
Dpt: Diptericin	Gcm: Glial cell missing
DPV: Drosophila P virus	GNBP: Gram-negative bacteria binding protein
Dredd: Death related ced-3/Nedd2-like protein	GPCR: G protein-coupled receptor
Drs: Drosomycin	Gprk2: G protein-coupled receptor kinase 2
Drsl: Drosomycin-like	Grass: Gram-positive specific serine protease
Dsh: Dishevelled	GSK3: Glycogen synthase kinase 3
Dsp1: Dorsal-switch protein 1	GTP: Guanosine triphosphate
dSR-CI: Drosophila scavenger receptor CI	Gaq: G protein $\alpha$ q sub-unit
dsRNA: double-stranded RNA	H3K4: Histone 3 Lysine residue 4
DSS: Dextran sodium sulfate	H3K4ac: H3K4 acetylation
Duox: Dual oxidase	H3K4me: H3K4 methylation
DXV: Drosophila X virus	H3K4me3: H3K4 tri-methylation
EB: Enteroblast	HAT: Histone acetyl transferase
EC: Enterocyte	HDAC: Histone deacetylase
EcR: Ecdysone receptor	HMG: High mobility group
EEC: entero-endocrine cell	HPO: Hippo
EGF: Epidermal growth factor	hTRIF: human TIR domain containing adapter inducing interferon- $\beta$
EGF: Epidermal growth factor	IAP: Inhibitor of apoptosis
EGFR: Epidermal growth factor receptor	IBD: Inflammatory bowel disease
ER: Endoplasmic reticulum	IBM: IAP2 binding motif
ERK: Extracellular regulated kinase	IFN: Interferon
ESC: Embryonic stem cell	Ig: Immunoglobulin
ET: Eye transformer	IIV6: Invertebrate iridescent virus 6
FADD: FAS associated death domain	IKK: Inhibitor of NF- $\kappa$ B Kinase



## Abbreviations

IL: Interleukin	Mya: Million years ago
IMD: Immune deficiency	Myd88: Myeloid differentiation primary response gene 88
Ino80: Inositol auxotroph 80	NADPH: Nicotinamide adenine dinucleotide phosphate
InR: Insulin receptor	NF- $\kappa$ B: Nuclear factor kappa-light-chain-enhancer of activated B cells
IP <sub>3</sub> : Inositol 1,4,5-triphosphate	NimC1: Nimrod C1
IRC: Immune-regulated catalase	NLS: Nuclear localization signal
Ird5: Immune-response deficient 5	Nt: nucleotide
<i>IsAkirin: Ixodes scapularis</i> Akirin	PBAP complex: Polybromo-associated Brahma complex
ISC: Intestinal stem cell	Pdk1: Phosphatidylinositol-dependent kinase 1
Iswi: Immitation switch	PDZ: PSD-95, Discs-large, ZO-1
I $\kappa$ B: Inhibitor of NF- $\kappa$ B	PEST: Proline, Glutamate, Serine, Threonine-rich
JAK/STAT: Janus Kinase / Signal Transducers and Activators of Transcription	PGN: Peptidoglycan
JNK: Jun N-terminal kinase	PGRP: peptidoglycan-recognition protein
KD: Knock-down	Pias : Protein inhibitor of activated STAT
Key: Kenny	PIP <sub>2</sub> : Phosphatidylinositol 4,5-biphosphate
KO: Knock-out	Pirk: Poor immune response upon knock-in
LPS: Lipopolysaccharide	PLC- $\beta$ : Phospholipase C- $\beta$
LRR: Leucin-Rich-Repeat	PM: Peritrophic matrix
Lys-PGN: Lys-type peptidoglycan	PMA: Phorbol 12-myristate 13 acetate
Mad: Mothers against Dpp	PO: Phenoloxydase
MAMP: Microbial-associated molecular pattern	Posh: Plenty of SH3s
MAP: Mitogen associated protein	PP2A: Protein phosphatase 2A
MAPK: MAP kinase	pPA: PPO activating enzyme
MAPKK: MAPK kinase	PPO: Prophenoloxydase
MAPKKK: MAPKK kinase	
MATS: Mob as tumor suppressor	
MEKK1: MEK kinase 1	
MHC: Major histocompatibility complex	
ModSP: Modular serine protease	
MP1/2: Melanization protease 1/2	
MPDZ: Multiple PDZ domain protein	

## Abbreviations

PRR: Pattern-recognition receptor	siRNA: short-interfering RNA
Psh: Persephone	SJ: Septate junction
pSJ: pleated septate junction	Snr1: SNF5-related 1
PTEN: Phosphatase and tensin homolog	Soc36E: Suppressor of cytokine signaling 36E
Ptp61F: Protein tyrosine phosphatase 61F	SP: Sex peptide
Pvf2: PDGF- and VEGF-related factor 2	SPE: Spätzle processing enzyme
Pvr: Platelet-derived Growth Factor (PDGF)-Vascular Endothelial Growth Factor (VEGF) Receptor	Spn: Serpin, Serine-protease inhibitor
RAG: Recombination-activating gene	Spz: Spätzle
RanBP : Ras-like guanine nucleotide-binding protein	SRR: Serin-rich region
RHD: Rel-homology domain	sSJ: Smooth septate junction
RING: Really interesting new gene	ssRNA: single-stranded RNA
RIP: Receptor interacting protein	SUMO: Small Ubiquitin-like Modifier
RISC: RNA-induced silencing complex	SWI/SNF: Mating-type switching / Sucrose non-fermentable
RNAi: RNA-interference	TAB2: TAK1-associated binding protein 2
RNAse: Ribonuclease	TAK1: TGF- $\beta$ -activated kinase 1
ROS: Reactive Oxygen Species	Tcf: T-cell factor
SANT: Swi3, Ada2, N-CoR, TFIIB	TCR: T-cell receptor
SAPK: Stress-activated protein kinase pathway	TCT: Tracheal cytotoxin
SAV: Salvador	TEP: Thioester-containing protein
Sayp: Supporter of activation of Yellow protein	TGF- $\beta$ ; Transforming growth factor beta
SC: Synaptonemal complex	Tip60: TAT-interactive protein 60kDa
Scr: Sex-comb reduced	TIR: Toll/interleukin-1 receptor
Sd: Scalloped	TJ: Tight junction
Serpin: Serine protease inhibitor	TLR: Toll-like receptor
SIGMAV: Sigma virus	TNF: Tumor necrosis factor
SINV: Sindbis virus	TNFR: Tumor-necrosis factor receptor
	Tor: Target of rapamycin
	Tot: Turandot
	trxG: Trithorax group
	TSC1/2: Tuberous sclerosis 1/2

## *Abbreviations*

TSS: Transcription start site

UC: Ulcerative colitis

Uev1a: Ubiquitin-conjugating enzyme variant 1a

Upd: Unpaired

Usp: Ultraspiracle

V,D,J: Variable, Joining, Diversity

VLR: Variable lymphocyte receptor

VMC: Visceral muscle cell

vsiRNA: viral siRNA

VSV: Vesicular stomatitis virus

Wbp2: WW-binding protein 2

Wg: Wingless

WTS: Warts

Yki: Yorkie

Zfh1: Zinc-finger homeodomain 1

ZO1: Zonula occludens 1

κB-RE: κB response element

## **V. General Introduction**

During evolution, metazoans have established a powerful immune system to survive pathogenic invading microorganisms. There are two main types of defense systems: innate and adaptive.

The innate immune system predates the adaptive response and consists of a package of defense mechanisms that has been conserved for more than a billion years within the animal kingdom. The innate immune system involves a wide variety of cells, effectors and molecular pathways that give a robust and immediate response to immune challenge. An active innate immune mechanism requires three categories of molecules: i) Sensors, able to discriminate and detect microbial pattern or danger signal and to engage a downstream signaling pathway. ii) Adaptors, constituting the molecular pathways driving the sensing signal to the production of the effectors. iii) The induced effector molecules, which can directly (e.g. Anti-microbial Peptides (AMPs), Reactive Oxygen Species (ROS)) or indirectly (e.g. Cytokines, Fever) counteract microbial challenges.

The adaptive immune system appeared more recently on an evolutionary scale, around 650 Million years ago (Mya), among the ancestors of jawless fishes (Kasahara and Sutoh, 2014). This adaptive system is based on antigen-specific recognition and maintains a memory of the response. This last property enabled the development of vaccines, which represents, together with the discovery of antibiotics, one of the major achievements of contemporary bio-medical research. As far as is known, two main branches of the adaptive immune system have diverged from these ancestral vertebrates, based on T-Cell and B-Cell receptors (TCRs and BCRs) in gnathostomes or based on variable lymphocyte receptors (VLRs) in jawless vertebrates (e.g. lampreys, hagfishes). In jawed vertebrates, TCRs and BCRs are expressed clonally on lymphocytes and recognize a wide variety of antigens (Tonegawa, 1983). To possess such plasticity in the recognition motif, vertebrate genes encoding these receptors somatically recombine the Variable (V), Joining (J), or V, Diversity (D) and J genes fragments through double-stranded DNA breaks induced by the recombination-activating gene (RAG) nuclease (Schatz and Swanson, 2011). A third component of this adaptive immune system, the Major

## General Introduction

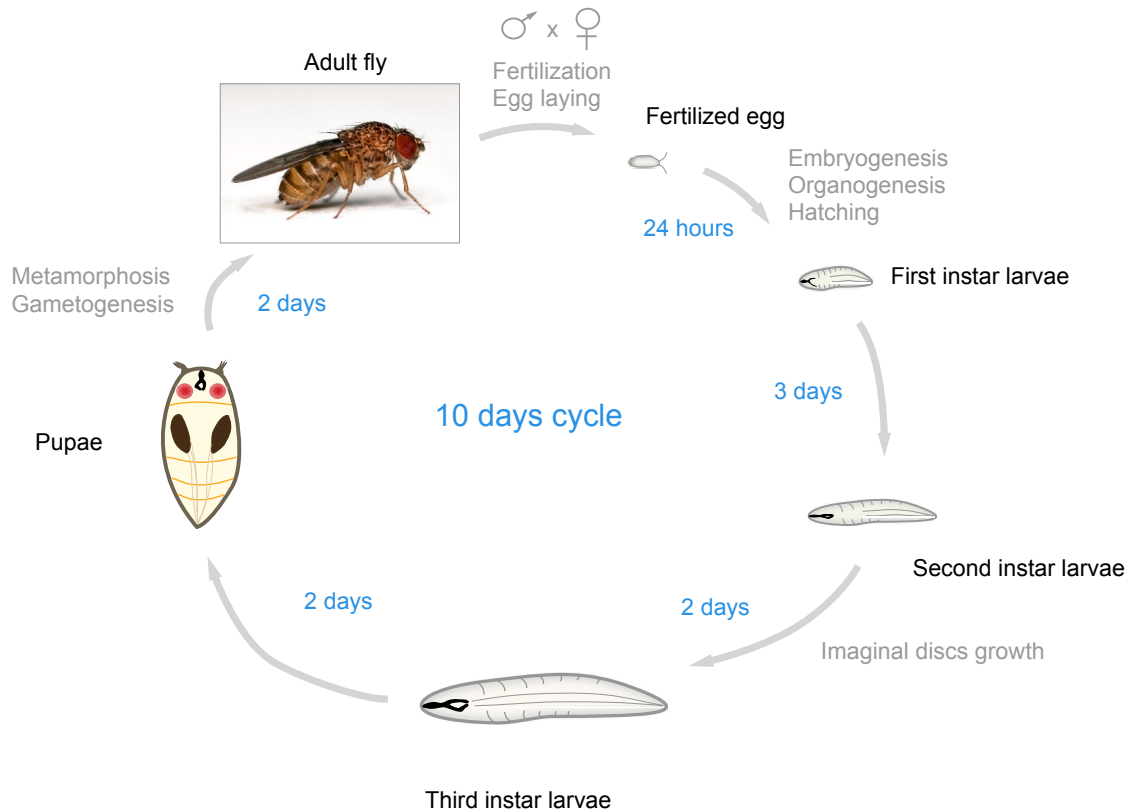
Histocompatibility Complex (MHC) molecules, is required for antigen recognition by the  $\alpha\beta$  subset of TCRs (Klein and Sato, 2000). In jawless vertebrates, the diversity of antigen recognition is produced by the assembling of variable Leucin-Rich-Repeat (LRR) modules encoding the VLRs in lymphocyte lineages (Nagawa et al., 2007). Importantly, the activation of the adaptive immune system strongly relies on concomitant innate immune responses (Fearon and Locksley, 1996).

The scientific context of my PhD was the exploration of innate immune mechanisms and I will therefore focus the rest of the manuscript on this aspect. In humans the innate immune system is required to defend against microbial challenges. When abnormally regulated however, innate immune responses contribute to a range of pathologies including autoimmune diseases, chronic inflammation and cancer (Maeda and Omata, 2008). Chronic inflammation-related pathologies such as atherosclerosis, type II diabetes or inflammatory-bowel diseases (IBDs) are difficult to cure with currently available anti-inflammatory therapeutic molecules and have become a major health problem (Tabas and Glass, 2013). The understanding in fine-tuning mechanism as well as deciphering the innate immune pathways cannot be dissociated from the unraveling of the next generation of therapeutic molecules.

*Drosophila melanogaster* is a small fly that has been widely used during the past hundred years to investigate complex biological questions, notably in genetics and developmental biology. The advanced genetic tools and the short generation time of *Drosophila* (8-10 days) (**Figure 1**) contributed to its success as a model organism. Importantly, *Drosophila* and humans share many genes and molecular pathways with similar functions (Rubin, 2000). *Drosophila* is well suited for deciphering the fundamental mechanisms underlying the innate immune response as unlike in vertebrates, the defense mechanisms of invertebrates rely entirely on innate immune responses. Although *Drosophila* and humans diverged more than 800 Mya in evolution, they share many molecular pathways underlying the activation of their innate immune systems (Hoffmann and Reichhart, 2002). Massive efforts during the past twenty years to describe the *Drosophila* innate immune system has largely contributed to the characterization of mammalian Toll-like Receptors (TLRs) and NF-

## General Introduction

$\kappa$ B pathways (Leulier and Lemaitre, 2008) and demonstrated the relevance of this model to study innate immunity.



**Figure 1** The life cycle of *Drosophila melanogaster*.

Additionally, work on *Drosophila* innate immunity may valuably contribute to understanding the biology of other arthropods human diseases vectors. Such vectors include other Diptera such as the sand fly (Phlebotominae, vector of Leishmaniasis), the buffalo gnat (Simuliidae, vector of Onchocerciasis), the *Anopheles* and *Aedes* mosquitoes (vector of malaria and filariasis, dengue fever, yellow fever and chikungunya). These pest species represent a major and growing threat for human health.

The aim of my PhD research was to improve our understanding of the innate immune response activation using *Drosophila melanogaster* as a model system. To fulfill this goal, I characterized the molecular function of two genes, *big-bang* (*bbg*)

and *akirin*, respectively implicated in the intestinal and systemic immune system of *Drosophila*. The following introduction sections present a broad overview of the known mechanisms of *Drosophila* innate immune responses, with an emphasis on NF- $\kappa$ B pathways biology.

## **VI. Chapter 1: Innate immune responses in *Drosophila melanogaster***

In the wild, *Drosophila* lives in a microorganisms-rich environment including decaying fruits. As a consequence, this organism is constantly exposed to microbial threats during nutrition and had to develop a powerful innate immune system. The first layer of *Drosophila* innate immune response is located at the putative entry sites of natural microbial infections: the gut, the trachea and the genital plates. To prevent a potential invasion in its internal cavity (the hemocoel), *Drosophila* has developed a set of defense mechanisms specific to these tissues, so-called the local immune responses, described thereafter (3.). This local immune system is sufficient to contain most microorganisms, but some pathogenic species such as *Pseudomonas aeruginosa* are able to cross the epithelial barriers and spread in the hemocoel (Limmer et al., 2011a). When such entomophagous pathogens invade the internal cavity of flies, or following septic injury, a second layer of defense mechanisms is activated in the hemocoel: the systemic immune response (4.).

For clarity, I will first describe the main molecular immune pathways of *Drosophila* that influence both local and systemic immune responses. These include the NF- $\kappa$ B-dependent pathways Immune Deficiency (IMD) and Toll (1.) and two NF- $\kappa$ B-independent additional molecular pathways: the Jun N-terminal Kinase (JNK) and the Janus Kinase / Signal Transducers and Activators of Transcription (JAK/STAT) pathways (2.). Finally, I will describe the intrinsic defense mechanisms deployed by *Drosophila* to fight viral infections (5.).

### **1. NF- $\kappa$ B pathways in *Drosophila***

In *Drosophila*, the Toll and the Immune deficiency (IMD) pathways play a fundamental role in the defense against invasive microbes by triggering the massive release of anti-microbial peptides. These pathways are able to recognize, discriminate and fight three main pathogen families of flies: Gram-negative bacteria, Gram-positive bacteria and fungi. So far, the functions of these pathways have been mostly characterized in three main immune tissues: i) the fat-body, a pseudo-epithelial tissue required for lipid storage with functional equivalence to the mammalian liver, but also the most potent organ of *Drosophila* systemic immune responses ii) the hemocytes, specialized phagocytic cells and iii) the digestive tract. This section of the introduction describes the current knowledge of the Toll and IMD NF- $\kappa$ B pathways without tissue restriction. Additional details about specific local NF- $\kappa$ B pathways activations are provided in the section **3**.

## 1.1 The IMD pathway

The IMD pathway controls the expression of a set of anti-microbial peptides, one of the stronger arms of *Drosophila* immune effectors. The absence of a functional IMD pathway activation leads to a high susceptibility of flies to Gram-negative bacterial infections, but not to Gram-positive bacterial or fungal infections (Lemaitre et al., 1995a). Conversely, when over-activated, the IMD pathway is a source of pathologies in flies (Paredes et al., 2011a). This section describes the known mechanisms of IMD pathway in flies as well as the numerous regulatory mechanisms blocking this activation (**Figure 2**). Note that a large portion of the proteins involved in IMD pathway signaling have a close ortholog in the mammalian Tumor-Necrosis Factor Receptor (TNFR) pathway (Hoffmann, 2003), one of the primary pathway involved in inflammation (Locksley et al., 2001). This high degree of conservation validates the relevance of studying of *Drosophila* IMD pathway for bio-medical research.

### 1.1.1 IMD pathway recognition events

The IMD pathway is initiated through the recognition of meso-diaminopymelic-type (DAP-type) peptidoglycan. This microbial-associated molecular pattern (MAMP)



is contained in Gram-negative bacteria and some Gram-positive bacilli. Two pattern-recognition receptors (PRR), members of the peptidoglycan-recognition proteins (PGRPs) family are involved in such recognition: PGRP-LC, PGRP-LE (Neyen et al., 2012).

**(a) General features of PGRPs**

PGRP family of receptors is conserved from invertebrates to mammals and is composed in *Drosophila* of 13 genes encoding at least 17 independent PGRPs isoforms through alternative splicing (Werner et al., 2000). PGRP receptors are classified into small-sized (182 to 203 amino-acids (AA)) PGRP-S and long-sized (215 to 520 AA) PGRP-L receptors. All PGRPs proteins have a PGRP domain, closely related to the T7 bacteriophage type II amidases secreted enzymes involved in PGN degradation (Kang et al., 1998).

In *Drosophila*, six members of PGRP family possess a PGRP domain bearing a functional PGN-degrading amidase activity: PGRP-SB1, -SB2, -SC1A, -SC1B, -SC2 and -LB. The amidase activity of these receptors gives them roles in the negative regulation of immune responses via the scavenging of immune-potent PGN (Bischoff et al., 2006; Guo et al., 2014; Paredes et al., 2011a; Zaidman-Rémy et al., 2011). By contrast, the seven other *Drosophila* PGRPs (PGRP-SA, -SD, -LA, -LC, -LD, -LE, and -LF) do not have an amidase activity and are involved either in sensing and signal transduction to immune pathways (PGRP-SA, -SD, -LA, -LC, -LE), or in the negative regulation of immune responses (PGRP-LE, -LF) (Bischoff et al., 2004; Choe et al., 2005a; Gendrin et al., 2013; Kaneko et al., 2006; Maillet et al., 2008; Michel et al., 2001). All PGRP-Ss have a secretion signal peptide in their N-terminal part and are therefore exclusively located outside of the cell. Finally, PGRP-Ls are either trans-membrane (PGRP-LA, -LC, -LD, -LF), intracellular (PGRP-LE) or secreted (PGRP-LE, -LB) proteins (Werner et al., 2000).

**(b) PGRPs involved in the activation of the IMD pathway**

Among PGRP family members, PGRP-LC is the main contributor for IMD signaling in the systemic immune system. PGRP-LC encodes three isoforms through alternative splicing: PGRP-LCa, PGRP-LCx and PGRP-LCy. These isoforms differ in their PGN-recognition domain (Neyen et al., 2012). PGRP-LCy lacks a functional PGN-recognition domain and may therefore act as a negative regulator of other PGRP-LC isoforms. By contrast, the PGRP-LCx isoform is necessary and sufficient to respond to Gram-negative and Gram-positive bacterial challenge as the PGRP-LCx homodimer can recognize DAP-type PGN multimers. By contrast, PGRP-LCx/PGRP-LCa heterodimers recognize the monomeric PGN known as tracheal cytotoxin (TCT).

Unlike PGRP-LC, PGRP-LE recognizes only TCT. Moreover, PGRP-LE is crucial for the local activation of IMD in the midgut while it is dispensable for immune activation in the fat-body (Bosco-Drayon et al., 2012a). Thus, pattern-recognition receptors are expressed in region-specific patterns along the length of the fly intestine. On one hand, the trans-membrane PGRP-LC receptor plays a predominant role in the foregut and the hindgut. On the other hand the intracellular PGRP-LE's sensing function is required in the midgut (Bosco-Drayon et al., 2012b; Neyen et al., 2012). Interestingly, as intracellular PGRP-LE is the only sensor in the midgut, it suggests that a yet-unidentified trans-membrane TCT transporter is involved in such recognition. The indirectness of PGRP-LE-dependent midgut recognition of bacteria is thought to prevent undesired and potentially harmful over-activation of the IMD pathway at this very location, where the permeability towards external components is greater than the other sections of the gut (further detailed in **3.1.1.**).

Intriguingly, PGRP-LC is required for the IMD pathway activation in the foregut proventriculus, while PGRP-LE selectively promotes the expression of negative regulators of the IMD pathway (Bosco-Drayon et al., 2012b). Finally, a recent study, indicates that the trans-membrane PGRP-LA protein activates the IMD pathway activation in the gut. The mode of action of PGRP-LA is unclear as this protein has neither a predicted PGN-binding domain nor amidase enzymatic activity (Gendrin et al., 2013).

### 1.1.2 IMD pathway signaling events

#### (a) Establishment of the IMD-IKK signalosome

DAP-type PGN is recognized by PGRP-LC and -LE proteins at their C-terminal domains (Chang et al., 2006; Lim et al., 2006). Following this recognition, PGRP-LC or -LE oligomerize and transduce the activation signal through their N-terminal domain (Choe et al., 2005b). The signal transduction is mediated through a specific AA sequence named the “core motif” in the N-terminal domain. The core motif is conserved between PGRP-LC and -LE (Kaneko et al., 2006) and shares a strong homology with the human Toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon- $\beta$  (hTRIF) protein. Interestingly, hTRIF is also involved in Toll-like Receptor pathway at the level of signal transduction by the pattern-recognition receptors (Meylan et al., 2004). The activation of PGRP-LC and -LE allows the recruitment of the adaptor molecules Immune Deficiency (IMD) and FAS associated Death domain (FADD) plus Death related ced-3/Nedd2-like protein (Dredd) (Leulier et al., 2000, 2002; Naitza et al., 2002).

To establish the formation of a signaling complex, the first interaction occurs between PGRP-LC or -LE and IMD. IMD possesses a death-domain (DD), a protein-protein interaction domain homologous to the mammalian receptor interacting proteins (RIP) (Georgel et al., 2001). PGRP-LC and -LE's interaction with IMD requires the core motif but is not direct, suggesting the involvement of a third unknown molecule involved in this process (Kaneko and Silverman, 2005). Subsequently, FADD is recruited onto IMD. FADD contains a DD and another protein-protein interaction domain, Death Effector Domain (DED). Dredd, is further recruited into this complex and also carries a DED domain through which it interacts with FADD.

Dredd, is a cysteine-dependent aspartate-directed protease (caspase) ortholog to the mammalian Caspase-8. Besides its role in the IMD pathway activation, Dredd acts as an effector of Reaper, Grid and Hid-mediated apoptosis in *Drosophila* (Chen et al., 1998). A tetrameric ubiquitin-ligase complex formed by the

E3 ubiquitin ligase *Drosophila* inhibitor of apoptosis 2 (DIAP2) and the E2 ubiquitin conjugating Ubiquitin-conjugating enzyme variant 1A (Uev1a) together with Bendless and Effete activates Dredd by K63-linked poly-ubiquitylation (Meinander et al., 2012). Once poly-ubiquitylated, Dredd is able to cleave IMD at aspartic residue 30, hence truncating the 30 N-terminal amino acids. The cleaved N-terminal of IMD exposes an IAP2 binding motif (IBM) that allows the recruitment of the tetrameric DIAP2, Uev1a, Bendless and Effete complex (Zhou et al., 2005). This complex will add K63-linked ubiquitin chains on cleaved IMD, which will serve as a scaffold to recruit the MAP kinase kinase kinase (MAPKKK) Transforming growth factor beta (TGF- $\beta$ )-activated kinase 1 (TAK1) and TAK1-associated binding protein 2 (TAB2) (Kleino et al., 2005).

The resultant heptameric protein complex can activate i) the MAPK p38 pathway to sustain ROS production possibly by phosphorylating MEKK1 (further detailed in **3.1.2(a)**) (Ha et al., 2009a), ii) the Jun N-terminal kinase (JNK) pathway to promote stress response and wound healing by phosphorylating the JNK kinase (JNKK) Hemipterous (further detailed in **2.1.**) (Silverman et al., 2003) and iii) the Inhibitor of NF- $\kappa$ B Kinase (IKK) complex (Silverman et al., 2003; Vidal et al., 2001). IKK complex activation by TAK1 and TAB2 likely involves a phosphorylation event on the IKK complex, as it is described in mammals (Wang et al., 2001), but this event was not described so far in *Drosophila*.

*Drosophila* IKK complex contains the catalytic subunit IKK $\beta$  (also named immune-response deficient 5 (Ird5)) and the regulatory subunit IKK $\gamma$  (also named Kenny (Key)). Together, Ird5 and Kenny mediate the phosphorylation of the NF- $\kappa$ B factor Relish, the final player of the IMD pathway and an ortholog of the mammalian p100 and p105 NF- $\kappa$ B factors. This step is mandatory for Relish activation (Ertürk-Hasdemir et al., 2009). Interestingly, a recent study demonstrated that Ird5 kinases require Small Ubiquitin-like Modifier (SUMO) ligation on their K<sub>152</sub> residue to be functional in the IMD pathway activation (Fukuyama et al., 2013). This study also showed that Lesswright (also named Ubc9), a putative SUMO-conjugating enzyme is required in the SUMOylation of Ird5.

**(b) Post-translational activation of Relish**

Relish is a 110kDa protein with functionally distinct N-terminal and C-terminal portions (Dushay et al., 1996). Following proteolytic cleavage of its C-terminal I $\kappa$ B-like domain, the N-terminal domain of Relish can translocate from the cytoplasm to the nucleus, where it acts as a NF- $\kappa$ B transcription factor. On one hand, the N-terminal portion of Relish (Rel-68) contains a Rel-homology domain (RHD), responsible for the transcription factor activity of the protein, two serine-rich regions (SRR) and a nuclear localization signal (NLS). On the other hand, the C-terminal portion of Relish (Rel-49) contains an I $\kappa$ B-like domain containing multiple Ankyrin repeats, responsible for Relish cytoplasmic sequestration by hindering NLS accessibility and a PEST (Proline, Glutamate, Serine, Threonine-rich) domain. Of note, functional study of Relish domains indicated that the SRR and PEST domains were negative regulators of Relish activation that prevent the full-length protein from entering the nucleus. In particular, the removal of the SRR between S<sub>29</sub> and S<sub>45</sub> converts Relish to a constitutively active form, Relish $\Delta$ S<sub>29</sub>-S<sub>45</sub> (Stoven et al., 2003).

When the IMD pathway is activated, Relish N-terminal (Rel-68) and C-terminal (Rel-49) portions are separated through a Dredd-mediated cleavage. This cleavage occurs at residue D<sub>545</sub> following the recognition of the L<sub>542</sub>Q<sub>543</sub>H<sub>544</sub>D<sub>545</sub>G<sub>546</sub> caspase cleavage motif (Kim et al., 2014; Stoven et al., 2003). While Rel-68 is immediately imported to the nuclear compartment to act as a transcription factor, Rel-49 is stably maintained in the cytoplasm, with no known function (Stoven et al., 2003). IKK complex-mediated phosphorylations occur on serine residues S<sub>528</sub> and S<sub>529</sub> precisely at the very end of the N-terminal portion. These phosphorylations are required for Relish-mediated RNA polymerase-II recruitment and subsequent gene activation. Nonetheless, these phosphorylations are dispensable for Dredd-mediated Relish cleavage and Relish subsequent nuclear translocation, although activated IKK complex participates in Relish cleavage in a non-catalytic way (Ertürk-Hasdemir et al., 2009; Stoven et al., 2003). Nuclear Rel-68 binds to its cognate cis-elements, named  $\kappa$ B response elements ( $\kappa$ B-REs). Relish  $\kappa$ B-REs are contained in the promoter of hundreds of genes, including anti-microbial peptide-coding genes such as *diptericin*, *attacin* and *cecropin* described further (1.1.3.) (Hetru and Hoffmann, 2009).

**(c) Relish transcriptional activity in the nucleus**

NF- $\kappa$ B factors work as dimers to recognize a  $\kappa$ B-REs composed of the consensus sequence 5'-GGGRNWYYCC-3' (R: purine (G or A); N: any nucleotide; W: A or T; Y: pyrimidine derivative (C or T)) (Gilmore, 2006). During NF- $\kappa$ B trans-activation events, the RHD domain of each NF- $\kappa$ B monomer mediates base-specific contacts through the DNA major groove to one half site wherein the flanking (G)GG/(C)CC sequences are contacted by conserved residues among NF- $\kappa$ B family members. By contrast, the inner more variable sequence (RNWY) is recognized by more specific regions of each NF- $\kappa$ B member. Work on mammalian NF- $\kappa$ B factors demonstrated that the variable central nucleotide (N) plays a crucial role in determining the binding specificity of different NF- $\kappa$ B-dimers as well as the outcome of such binding (Wang et al., 2012). For example, dimers of the NF- $\kappa$ B factor that initiates the mammalian inflammatory response, RelA preferentially bind central (A/T)-containing  $\kappa$ B-REs to activate transcription. By contrast, when bound to a central (A/T)-containing  $\kappa$ B-REs, the p52:B-cell lymphoma 3 (Bcl3) atypical NF- $\kappa$ B dimers recruit the histone deacetylase HDAC3 to repress transcription. When bound to a central (C/G)-containing  $\kappa$ B-REs however, these same p52:Bcl3 dimers recruit instead the histone acetyl transferase (HAT) TAT-interactive protein 60kDa (Tip60) to activate transcription. This binding specificity makes sense, as p52:Bcl3 dimers have repressive functions on inflammation and as repressed (A/T)-containing  $\kappa$ B-RE are found in pro-inflammatory genes (Interleukin-23 (IL-23), IL-6, IL-8) whereas promoted (C/G)-containing  $\kappa$ B RE are found in anti-inflammatory cytokines (IL-10) (Wang et al., 2012).

The *Drosophila* genome encodes three NF- $\kappa$ B factors: Relish, Dorsal and Dorsal-related immunity factor (DIF), Dorsal and DIF being primarily involved in the second NF- $\kappa$ B pathway, the Toll pathway mostly directed against Gram-positive bacteria and fungi (further detailed in 1.2.). Upon IMD pathway activation, Relish forms homo-dimers that induce the expression of IMD pathway target genes. Relish homo-dimers recognize preferentially a sequence of four Gs followed by a three nucleotide A/T-rich stretch and three pyrimidine bases (GGGGATTYYY). Upon Toll

pathway activation, DIF homo-dimers preferentially bind a sequence of three Gs followed by four to five A/T-rich nucleotides (GGGAAA(A/T/G)YCC). Additionally, perfect palindromic GGGAATTCCC and GGGGAAAACCCC sequences are efficiently bound by both Relish and DIF homo-dimers (Busse et al., 2007). Moreover, a study demonstrated that, upon the activation of both Toll and IMD pathways, Relish can form hetero-dimers with DIF or Dorsal and activate both Toll and IMD pathway target genes (Tanji et al., 2010a). Another study identified the response element of such heterodimers as GGGA(A/T)TC(C/A)C (Senger et al., 2004).

#### **(d) Positive regulators of Relish transcriptional activity**

During the immune response, several transcription factors or other nuclear proteins may act together with or in parallel of Relish to sustain the transcriptional activation of Relish targets. These transcriptional “helpers” are described below.

First, the GATA transcription factor family, which binds GATA sequences, was shown to positively influence Relish-targeted transcription in tissue-specific contexts during larval stages (Petersen et al., 1999; Senger et al., 2006). This family of factors contains five members in *Drosophila*: Pannier, Serpent, Grain, dGATA<sub>d</sub> and dGATA<sub>e</sub>. GATA motifs, (A/T)GATA(A/G) are present in proximity to κB RE in a large number of insect immune-related genes (Kadalayil, 1997). However, only Serpent has been shown to be required for the expression of the *cec-A1* gene, one of the target of Relish, in the larval but not the adult fat-body (Petersen et al., 1999) while dGATA<sub>e</sub> was shown to participate in Relish-dependent transcription in the larval midgut (Senger et al., 2006).

Second, the steroid hormone 20-hydroxyecdysone (also named 20E) produced during *Drosophila* metamorphosis at larval and pupal stages is a potent positive regulator of both IMD and Toll-pathway immune responses (Dimarcq et al., 1997; Flatt et al., 2008; Meister and Richards, 1996; Zhang and Palli, 2009). 20E first binds to heterodimers of the Ecdysone Receptor (EcR) and Ultraspiracle (Usp) nuclear receptors (Yao et al., 1993). This signaling complex induces the expression

of multiple target genes, many of which are themselves transcription factors themselves, creating a complex cascade of signaling events (Thummel, 1996). A recent study analyzed 20E-provoked immune up-regulation in S2 cells and identified four transcription factors required in this process: EcR, Broad, Serpent and Pannier (Rus et al., 2013). This work also demonstrated that 20E was able to induce the production of PGRP-LC in an immune-stimulation-independent manner to further sustain all IMD signaling outputs.

Finally, a novel player of Relish-mediated transcription, the nuclear protein Akirin, has been shown to be required in the transcription of *attacin-A* and *dipthericin-A* in *Drosophila* (Goto et al., 2008). Interestingly, this gene is well conserved among animal species, from mosquitoes to vertebrates, in which the gene was duplicated (akirin-1 and akirin-2). In mice, Akirin-2, the closest homolog of *Drosophila* Akirin was shown to be required for a key NF- $\kappa$ B targets transcription upon immune challenges (Goto et al., 2008). However, molecular mechanisms of Akirin's mode of action towards NF- $\kappa$ B-dependent transcription had not been described. In this context, my PhD work on *Drosophila* Akirin (Bonney et al., 2014) and a parallel study performed in mice by our collaborators (Tartey et al., 2014) aimed at better understanding Akirins' mode of action during NF- $\kappa$ B-activated immune responses.

### **1.1.3 IMD pathway effectors**

The best-characterized induced effectors of the IMD pathway are anti-microbial peptides (AMPs). These small secreted peptides (mostly less than 10kDa, with the exception of Attacins) play a central role in the defense of procaryotes, vertebrates, plants and other invertebrates against micro-organisms (Toke, 2005). *Drosophila melanogaster* has seven AMP families: Dipterocins (Dpt-A and -B), Attacins (Att-A, -B, -C and -D), Cecropins (Cec-A1, -A2, -B, -C and Andropin), Drosomycins (Drosomycin (Drs), Drs-like (Drsl) -1, -2, -3, -4, -5 and -6), Metchnikowin, Defensin and Drosocin. IMD pathway activation induces transcription of all these AMP families.



The AMP families can be subdivided into three groups based on their specific microbicidal activity: i) Drosomycins and Metchnikowins have fungicidal activities, ii) Defensin is effective against Gram-positive bacteria and iii) Attacins, Cecropins, Dipterocins and Defensin fight Gram-negative bacteria (Bulet, 1999). All of these peptides have a positive net charge at physiological pH and mostly bear amphiphilic  $\alpha$ -helices or hairpin-like  $\beta$ -sheets in their structure. The predicted activity of AMPs is to perforate microbial cell walls, although their precise mode of action remains to be investigated (Bulet, 1999). In addition to AMPs, the IMD pathway induces a few hundred of other molecules via Relish transcriptional activity (Levy et al., 2004). These genes encode proteins with diverse immune functions such as microbial-recognition, phagocytosis, melanization, production of reactive oxygen species or iron sequestration (Ferrandon et al., 2007).

#### **1.1.4 IMD pathway negative regulation**

When inappropriately regulated, the IMD pathway is associated with pathologies in flies. For example, in the brain, uncontrolled activation of IMD leads to brain damage and neurodegeneration that are directly linked with the production of AMPs (Cao et al., 2013). Upregulated IMD pathway activations in the gut, which is in constant contact with microorganisms can lead to a premature death (Guo et al., 2014; Lhocine et al., 2008; Maillet et al., 2008; Paredes et al., 2011b). To prevent inappropriate microbial activation, flies have developed a battery of negative regulators that fine-tune the IMD pathway. Inhibitory proteins have been identified at almost all the key steps of IMD pathway activation: **(a)** DAP-type PGN recognition, **(b)** IMD-IKK signaling platform, **(c)** Relish cleavage and **(d)** Relish activity in the nucleus.

##### **(a) Control of DAP-type PGN recognition**

Four Peptidoglycan Recognition Proteins inhibit the initiation of the pathway directly at the level of DAP-type PGN recognition: PGRP-LB, -LE, -LF and -SC. The PGRPs with functional amidase activity (PGRP-LB and PGRP-SCs) probably scavenge available bacterial PGN. The resultant lowering in PGN activity will reduce

PGN binding by the non-catalytic PGRP-LC and -LE PRRs, which would downregulate the IMD pathway (Guo et al., 2014; Paredes et al., 2011a; Zaidman-Rémy et al., 2006, 2011). Secondly, PGRP-LF acts a competitive inhibitor of PGRP-LC dimerization. PGRP-LF is unable to bind PGN itself and also lacks an intracellular signaling domain but PGRP-LF association with PGRP-LC blocks formation of the active PGRP-LC homodimer, which is required for IMD signaling (Basbous et al., 2011; Maillet et al., 2008). In the proventriculus of the *Drosophila* foregut, PGRP-LE acts as a negative regulator by promoting the expression of PGRP-LB, PIRK (further detailed below) and PGRP-SC1 (Neyen et al., 2012).

An additional negative regulator acting at the level of PGRP proteins, Poor immune response upon knock-in (Pirk) protein has been identified. Pirk interacts with PGRP-LC to change its sub-cellular localization from the cytoplasmic membrane to perinuclear structures, therefore preventing PGN recognition (Lhocine et al., 2008). With the exception of PGRP-LF, all these negative regulators of IMD pathway are induced upon IMD pathway activation and therefore work as negative feedback loops of the IMD pathway activation. Finally, the Toll 8 member of Toll receptors (also called Tollo) constitutively down-regulates IMD pathway activation in the larval tracheal epithelium (Akhouayri et al., 2011a). Tollo binds the ligand Spätzle2 (also known as Neutrophin 1 or DNT1) and Ectoderm-expressed 4 (Ect4), a putative Toll/interleukin-1 receptor (TIR)-domain adaptor to mediate a negative regulation of IMD signaling at the level of PGRP-LC and IMD.

## **(b) Control of IMD-IKK signaling**

The ubiquitination of IMD is a crucial step in the activation of the pathway, and this step is the target of multiple ubiquitinating and de-ubiquitinating enzymes. First, the ubiquitin-specific protease dUSP36 (also called Scrawny) degrades the K63-linked ubiquitin chain of IMD required for signaling, while promoting the formation of K49-linked ubiquitin chains, which target IMD for proteasome degradation. As a consequence, Scrawny blocks IMD signaling and provokes the degradation of IMD by the proteasome (Thevenon et al., 2009). Another ubiquitin-specific protease, fat

facets (faf) was also demonstrated to have a negative impact on IMD pathway, probably by modulating IMD ubiquitination and/or stability state (Yagi et al., 2013).

Third, an E3 ubiquitin ligase, Plenty of SH3s (POSH) poly-ubiquitinates TAK1, targeting TAK1 for proteasomal degradation, and therefore diminishing the activation of the IKK complex (Tsuda et al., 2005). Additionally, a recent study demonstrated that TAK1 K63-poly-ubiquitylation was required for IMD pathway signaling and that this step was targeted by a regulatory mechanism involving the ubiquitin protease Trabid (Fernando et al., 2014). The absence of Trabid constitutively activates the IMD pathway, leading in particular to intestinal damages. However, the (Fernando et al., 2014) study does not document how TAK1 activation poly-ubiquitylation initially occurs. Finally, the *Drosophila* homolog of Cyldromatosis (CYLD), a known de-ubiquitinating enzyme down-regulating the Tumor Necrosis Factor (TNF) Receptor pathway in mammals (Trompouki et al., 2003), dCYLD has also been shown to down-regulate the IMD pathway by interacting with the IKKγ Kenny protein. Although the molecular event establishing this negative regulation has not been identified, these results suggests that Kenny would require ubiquitination for signaling (Tsichritzis et al., 2007).

### (c) Control of Relish cleavage and stability

Two proteins, Defense repressor 1 (Dnr-1) and Caspar have been shown to interfere with Dredd-mediated Relish cleavage. Dnr-1 was first shown to act as a negative regulator of the IMD-dependent Dipterucin-LacZ transgene in *Drosophila* S2 cells (Foley and O'Farrell, 2004). This study also demonstrated that Dnr-1 was stabilized upon IMD pathway activation, further establishing this protein as a *bona fide* retro-controlling protein. A more recent study demonstrated that Dnr-1 blocks IMD pathway activation by interacting with through the C-terminal RING domain of Dredd. The RING (Really Interesting New Gene) domain, usually found on inhibitor of apoptosis (IAP) caspase inhibitors (Guntermann et al., 2009; Vaux and Silke, 2005). According to the Guntermann study, Dnr-1 is probably involved in Dredd proteasomal degradation since IAP family members inhibit their targeted caspase by poly-ubiquitination and proteasome addressing (Guntermann et al., 2009).

Relish cleavage by Dredd can also be inhibited by Caspar, a multiple ubiquitin-related domain protein (Kim et al., 2006a). Although the molecular mechanism of such inhibition has not been investigated in *Drosophila*, its closest human homolog, hFAF1 has been shown to activate the ubiquitin-proteasome pathway (Song et al., 2005). Since Caspar is genetically required at the level of Relish cleavage, it is tempting to speculate that Caspar would target Dredd for proteasomal degradation (Kim et al., 2006a). The IMD pathway activation can also be fine-tuned by the regulation of Relish protein pool. In particular, Relish stability is directly affected by the E3 ubiquitin ligase Skpa, dCullin, F-box (SCF) complex, promoting Relish proteasomal degradation (Khush et al., 2002).

#### **(d) Control of Relish activity in the nucleus**

Once cleaved and imported in the nucleus, the activated Rel-68 may encounter an additional layer of inhibition from specific nuclear factors before being able to trans-activate its cognate target genes. Five transcription factors in particular, Activator protein 1 (AP1), Signal Transducer and Activator of Transcription (STAT) 92E, Dorsal-switch protein 1 (Dsp1), Zinc-finger homeodomain 1 (Zfh1) and Caudal are able to block the activation of the IMD pathway at the level of Relish (Kim et al., 2007, 2005; Myllymäki and Rämetsä, 2013; Ryu et al., 2008). AP1 (also called Jun-related Antigen, Jra or Jun) is a transcription factor activated by the JNK signaling pathway (further detailed in 2.) while STAT92E is at the top of the activation of the Janus Kinase (JAK)/STAT pathway (further detailed in 2.). Alternatively to their role in trans-activating their own target genes transcription, these two transcription factors can form a repressosome complex with the High mobility group (HMG) protein Dsp1 and the histone deacetylase 1 (HDAC1) to down-regulate Relish-dependent transcription (Kim et al., 2007). Dsp1 works as the nucleating factor linking all the members of this complex and specifies its binding to Relish-target promoters, likely in the close proximity of Relish  $\kappa$ B Response elements, as suggested by the binding specificity of the mammalian homolog of Dsp1, HMGB1 (Goodbourn et al., 1986). Consequently, Relish is displaced from its response-element and is no longer able to induce transcription. This displacement seems to occur in wild-type flies rapidly after

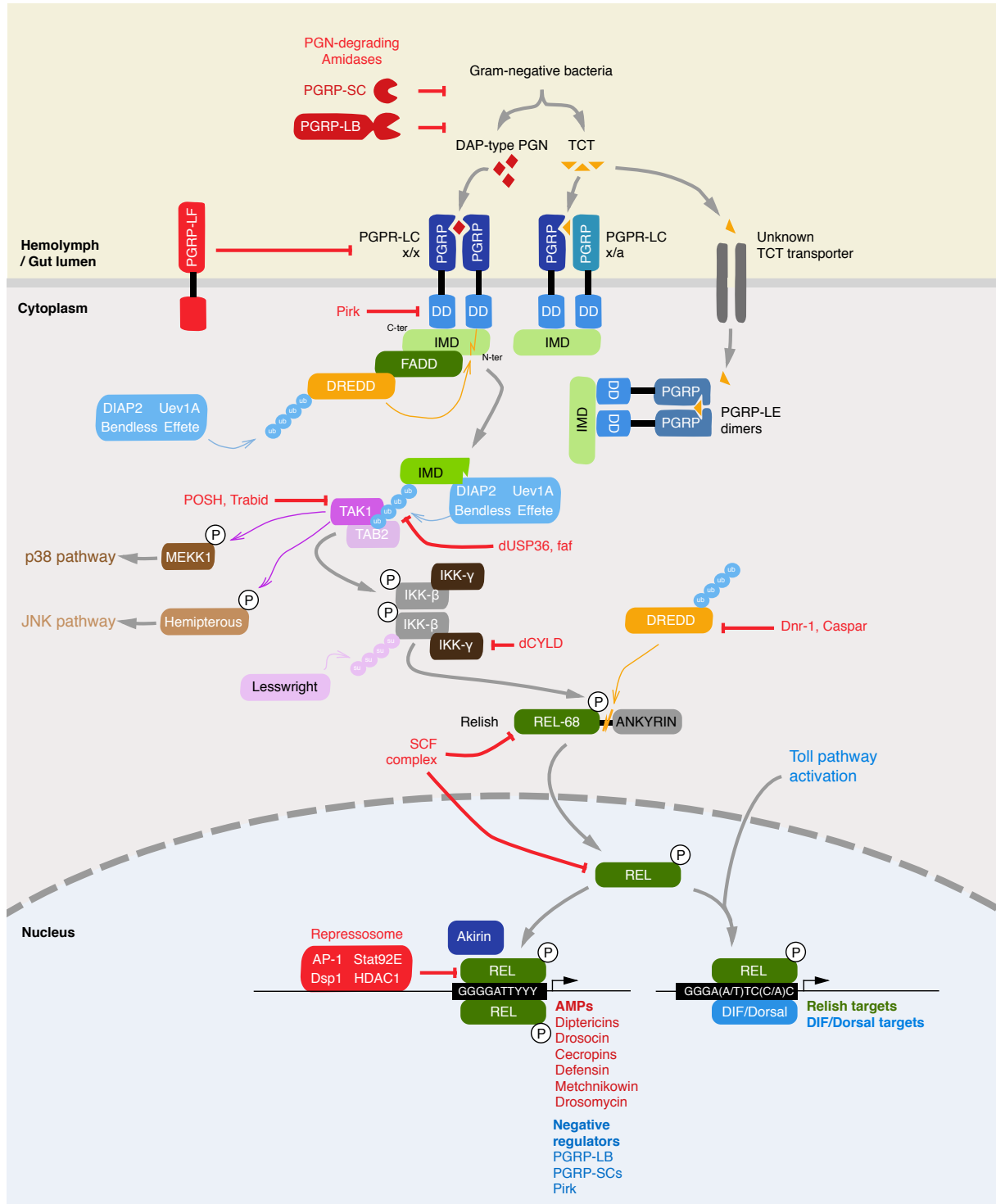
immune challenge (15min) and is more pronounced at later time points (8h), suggesting that this mechanism is required for a proper termination of the IMD pathway immune response. Intriguingly, the removal of a member of this repressosome complex increases Relish-target AMPs production in response to a Gram-negative bacterial septic injury but decreases flies' survival to such an infection in a Relish-dependent manner, pointing out the harmfulness of unresolved immune responses in *Drosophila* (Kim et al., 2007).

Another transcription factor, Zinc-finger homeodomain 1 (Zfh1) also functions as a negative regulator of Relish target genes expression in S2 cells and in the adult fat body. However, Zfh1 only represses a subset of Relish-target genes *in vivo*, as its absence leads to an up-regulation of *attacin-A* and *cecropin-B*, but did not change the expression of *diptericin-B*, *attacin-B* and *attacin-D* transcription upon immune stimulation. Zfh1 contains multiple Zinc finger domains and one homeobox domain allowing this protein to interact both with DNA and other transcription factors or inhibitors. Nonetheless, the mechanism by which Zfh1 mediates its repression remains unknown and could be indirect as no interaction with Relish was detected and no putative binding site of this factor was found on targeted promoters (Myllymäki and Rämetsä, 2013).

Finally, in the *Drosophila* gut, another homeobox transcription factor, Caudal, was found to play a crucial role in dampening a subset of Relish-target genes, specifically AMPs, in the proventriculus and the posterior part of the midgut. Importantly, caudal deficient flies' gut were shown to over-express AMPs, displayed an elevated number of apoptotic epithelial cells and carried an altered microbiota favoring the proliferation of a pathogenic commensal, *Gluconobacter* sp. strain EW707 (G707). As a consequence, conditional KD of caudal in the gut was sufficient to decrease the lifespan of flies in a microbiota-dependent manner, as antibiotics treatment partially rescued this phenotype (Ryu et al., 2008). Caudal is predicted to bind to Caudal-protein DNA recognition elements (CDRE) that are found in AMP promoters (Ryu et al., 2004). Molecular mechanisms by which Caudal would repress Relish transcription however, have not been described. Note that Caudal can also act as an activator to express the basal level of expression of AMP genes such as

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*cecropin* and *drosomycin* in specific *Drosophila* tissues such as S2 cells, the trachea, the salivary glands and the ejaculatory duct epithelia (Ryu et al., 2004).



**Figure 2** The IMD pathway of *Drosophila melanogaster*.

IMD is specifically activated through the recognition of Gram-negative bacteria-derived meso-diaminopymelic-type (DAP-type) peptidoglycan (PGN) and tracheal cytotoxin (TCT) by the Peptidoglycan recognition (PGRP) domain of Peptidoglycan recognition protein -LC and -LE (PGRP-LC, -LE). PGRP-LC isoforms x homodimerize to recognize DAP-type PGN, while PGRP-LC isoform x

and a heterodimerize to recognize TCT. PGRP-LE dimers recognize only TCT. PGRP-LC and -LE death-domains recruit Immune deficiency (IMD), FAS associated Death domain (FADD) and Death related ced-3/Nedd2-like protein (Dredd). An ubiquitin-ligase complex formed by the E3 ubiquitin ligase *Drosophila* inhibitor of apoptosis 2 (DIAP2) and the E2 ubiquitin conjugating Ubiquitin-conjugating enzyme variant 1A (Uev1a), Bendless and Effete activates Dredd by K63-linked poly-ubiquitylation. Activated Dredd cleaves IMD N-terminal domain. Cleaved IMD is further K63-polyubiquitylated by DIAP2-Uev1a-Bendless-Effete complex and recruit Transforming growth factor beta (TGF- $\beta$ )-activated kinase 1 (TAK1) and TAK1-associated binding protein 2 (TAB2). Consequently, TAK1 is able to activate the p38 pathway by phosphorylating MEKK1, the Jun N-terminal Kinase (JNK) pathway by phosphorylating Hemipterous, or the Inhibitor of NF- $\kappa$ B (I $\kappa$ B) Kinase (IKK) complex formed of IKK $\beta$  and IKK $\gamma$  subunits. Phosphorylated IKK $\beta$  is sumoylated by Lesswright and consequently phosphorylates the N-terminal portion of the NF- $\kappa$ B factor Relish to enable its transcriptional activity. Relish is separated from its I $\kappa$ B-like C-terminal ankyrin repeats region by Dredd through proteolytic cleavage.

The NLS-containing N-terminal portion of Relish (Rel-68) is then imported to the nucleus while the I $\kappa$ B-like C-terminal portion (Rel-49) remains in the cytoplasm. Phosphorylated Rel-68 homodimerize or heterodimerize with Dorsal-related immunity factor (DIF) or Dorsal if both Toll and IMD pathway are activated. Rel68 homodimers bind their cognate  $\kappa$ B Response element, the consensus sequence 5'-GGGGATTYYY-3' (Y: C or T) and activate IMD-pathway target genes with the help of the nuclear protein Akirin. Relish-target genes include antimicrobial-peptides (AMPs) and negative regulators retro-controlling the activation of the pathway. Rel68/DIF or Rel68/Dorsal bind to the  $\kappa$ B Response element 5'-GGGA(A/T)TC(C/A)C-3' and are able to activate both IMD and Toll pathways target genes. Negative regulators, highlighted in red, act at almost every step of the pathway activation and are described more in detail in the main text. Tissue-specific negative regulators of the IMD pathway were not included in the scheme but detailed in the main text.

### 1.2 The Toll pathway

The Toll pathway was the first characterized NF- $\kappa$ B pathway in *Drosophila*. Its discovery was initiated by genetic screens to identify genes involved in early embryonic development. These screens, conducted by Christiane Nüsslein-Volhard and Eric Wieschaus, identified 15 genes controlling embryonic segmentation (Nüsslein-Volhard and Wieschaus, 1980). This work constituted the basis for the discovery of dorso-ventral patterning genes, including most of the known members of the Toll pathway (Belvin and Anderson, 1996).



Besides its role in the establishment of dorso-ventral axis formation during embryogenesis, the Toll pathway plays a crucial role in *Drosophila* immunity against Gram-positive bacteria and fungi. Flies deficient in this pathway succumb more rapidly to Gram-positive bacterial and fungal infections (Lemaitre et al., 1996). Of note, unlike the IMD pathway, the known Toll pathway spectrum of action goes wider than just AMP production. Indeed, the Toll pathway was shown to play an important role in the cellular immune response (hemocyte differentiation and proliferation; melanization) in larvae, which provides a defense line against both unicellular microorganisms and pluricellular parasites (Bettencourt et al., 2004a; Lemaitre et al., 1995b; Qiu et al., 1998; Sorrentino et al., 2004; Zettervall et al., 2004). The terminal signaling molecules of this pathway are two closely related NF- $\kappa$ B members (more than 45% of identity) to mammalian c-Rel, Rel-A and Rel-B NF- $\kappa$ B factors (Hetru and Hoffmann, 2009): DIF and Dorsal (Ip et al., 1993; Lemaitre et al., 1995b; Rutschmann et al., 2000).

Importantly, the discovery of an immune function for the Toll receptor, (Lemaitre et al., 1996) has strongly influenced and accelerated the characterization of Toll-like Receptors (TLR), one of the most potent family of pattern-recognition receptors in mammals. Hereafter are described the molecular events leading to *Drosophila* Toll pathway activation and its negative regulation (**Figure 3**).

### **1.2.1 Toll pathway recognition events**

The Toll pathway is able to sense fungi, Gram-positive bacteria and some Gram-negative bacteria through two categories of recognition mechanisms: the recognition of microbe-associated molecular patterns (MAMPs) by PRRs (so-called PRR pathway) and the recognition of so-called “danger-signal”. This last term was introduced by Polly Matzinger to define deleterious molecules from self or non-self produced in the case of infection or sterile damage (Matzinger, 1994). During PRR pathway activation, a set of pattern recognition receptors recognizes Lys-type peptidoglycan (Lys-PGN) from Gram-positive bacteria and  $\beta$ -glucans from fungi (**a**). Alternatively, danger signals, in this case, proteases produced by fungi, Gram-positive bacteria and possibly some Gram-negative bacteria, are sensed by a proteolytically activable protease engaging the “danger-signal” pathway (**b**).

**(a) The PRR recognition pathway**

In contrast to the IMD pathway, the Toll pathway PRRs are secreted proteins circulating in the hemolymph. These PRRs belong to the Peptidoglycan recognition protein (PGRP, previously described in **1.1.1a**) and Gram-negative bacteria binding proteins (GNBP) families. GNBP proteins are characterized by their N-terminal  $\beta$ -glucan-binding protein domain and a C-terminal enzymatic  $\beta$ -glucanase domain (Ochiai and Ashida, 2000). Of note, this family of PRR is conserved in most invertebrates but has not been identified in vertebrates so far.

Gram-positive bacterial Lys-type PGN is recognized by GNBP1, PGRP-SA and PGRP-SD (Gobert et al., 2003; Michel et al., 2001; Pili-Floury et al., 2004) while fungal  $\beta$ -glucans are recognized specifically by GNBP3 (Gottar et al., 2006). During Lys-type PGN recognition events, PGRP-SA and GNBP1 are physically bound in a complex. Upon the formation of this complex, GNBP1  $\beta$ -glucanase domain hydrolyzes the Lys-type PGN, resulting in the formation of glycan reducing ends further recognized by PGRP-SA (Wang et al., 2006). 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 (Buchon et al., 2009a). Alternatively, PGRP-SD was also shown to recognize Lys-type PGN from Gram-positive bacteria (Bischoff et al., 2004). Interestingly, a structural study suggested that PGRP-SD can recognize DAP-type PGN, further implying that Toll pathway may also be able to recognize Gram-negative bacteria through its PRR recognition pathway (Leone et al., 2008).

**(b) The danger signal recognition pathway**

In addition to the PRR pathway, bacteria and fungi can be sensed through the activation of the Serine-Protease Persephone (Psh) (El Chamy et al., 2008; Gottar et al., 2006). Psh is first produced as an inactive zymogen that requires activation by exogenous protease cleavage to give a catalytically active Serine protease. Identified proteases provoking Psh cleavage are the cuticle-degrading PR1 subtilisin-like

proteases released by the entomopathogenic fungi *Beauveria bassiana* (*B. bassiana*) and *Metarhizium anisopliae* (*M. anisopliae*) (Gottar et al., 2006) and proteases from *Bacillus subtilis* Gram-positive bacterium and *Aspergillus oryzae* fungi (El Chamy et al., 2008). Of note, secreted proteases from pathogenic Gram-negative bacteria such as *Pseudomonas aeruginosa* may potentially also be recognized by this mechanism, as the Toll pathway is induced and required for survival following *P. aeruginosa* infection (Lau et al., 2003; Limmer et al., 2011a). Finally, a recent study reports that Psh-dependent Toll pathway activation would play a role in the recognition of endogenous damage-associated molecular patterns (DAMPs) from non-apoptotic cell death, in a model of apoptosis-deficient flies (Ming et al., 2014).

### 1.2.2 Toll pathway signaling events

Following microbial recognition, Toll pathway signaling is initiated through an extracellular proteolytic signaling cascade leading to the activation of the transmembrane Toll receptor, which is the starting point of the intracellular pathway. Upon recognition Lys-type PGN or  $\beta$ -glucans by the PGRP-SA-GNBP1, PGRP-SD or GNBP3 receptors, a proteolytic cascade is initiated. The cascade includes Modular serine Protease (ModSP) and Gram-positive Specific Serine Protease (Grass) and ends with Spätzle-processing enzyme (SPE) (Buchon et al., 2009a; El Chamy et al., 2008; Kellenberger et al., 2011). Alternatively, following microbial protease cleavage, Psh may directly process and activate SPE (El Chamy et al., 2008; Gottar et al., 2006). Once activated, SPE processes the Pro-spätzle ligand to its active Toll-binding form Spätzle (Spz). Spz is a member of the cysteine knot family of growth factor cytokines. Of note, in dorso-ventral patterning, Spz is processed through a different Serine-protease cascade composed of Nudel, Gastrulation Defective, Snake and Easter (Chasan et al., 1992; Hong and Hashimoto, 1995).

Pro-spätzle circulates as an inactive dimeric precursor that is unable to bind its cognate receptor, the Toll receptor (Hu et al., 2004). Toll receptors are transmembrane proteins composed of a composite Leucine-rich repeat (LRR)-containing extracellular ectodomain, a single-span transmembrane region and an intra-cellular signaling domain referred as to Toll / Interleukine-1 Receptor (TIR)-domain (Imler

and Hoffmann, 2001). SPE-mediated cleavage of Pro-spätzle frees the active 106 C-terminal AA fragment (also named C106). Spz-C106 dimers bind to the N-terminal extracytoplasmic domain of Toll and provokes the crosslinking of two Toll receptors ectodomains. This ligand binding event remodels the conformational structure of the receptor and the dimerized Toll receptor then activates intra-cellular signaling (Weber et al., 2003).

Nine Toll-related receptors (Toll-1 to -9) have been identified so far in the *Drosophila*. Toll-1, the first identified member of this protein family is the main receptor for NF- $\kappa$ B-dependent AMP synthesis (Imler and Hoffmann, 2001). However, Toll-5 and Toll-9 may also play a role in the Toll pathway activation since their over-expression is sufficient to induce the *drosomycin* and *metchnikowin* target genes (Bettencourt et al., 2004b; Imler et al., 2000; Luo et al., 2001; Ooi et al., 2002; Tauszig et al., 2000). In addition, Toll-5, also named Tehao, was also demonstrated to interact with the downstream members of Toll pathway, Myd88 and Pelle (detailed below), further suggesting its implication in the Toll pathway activation (Luo et al., 2001).

Following Spz binding, Toll recruits the adaptor protein Myeloid differentiation primary response gene (88) (MyD88) through their common TIR domains (Horng and Medzhitov, 2001; Sun et al., 2002; Tauszig-Delamasure et al., 2002). Then, Myd88 functions as a platform to recruit a secondary adaptor, Tube, through its Death-domain (DD) (Sun et al., 2002; Xiao et al., 1999). Afterwards, Tube recruits the Pelle kinase, an homolog of mammalian Interleukin-1 receptor associated kinase 1 (IRAK1) through their common DD and form, together with Myd88 a tripartite complex at the origin of the activation of the NF- $\kappa$ B factors Dorsal and/or DIF (Moncrieffe et al., 2008). Of note, Pellino, a RING-domain containing protein has been shown to be required for Toll pathway activation, possibly by promoting a signaling poly-ubiquitinylation of Pelle (Haghighyeghi et al., 2010).

At the top of the intracellular Toll pathway are the NF- $\kappa$ B factors Dorsal and DIF. These factors are close homologs of the mammalian c-Rel, Rel-A and Rel-B proteins, and, unlike Relish, are sequestered in the cytoplasmic by an Ankyrin-repeats containing protein, Cactus (Wu and Anderson, 1998). Cactus is an I $\kappa$ B $\alpha$ -like protein and its phosphorylation and degradation by the proteasome releases Dorsal

and DIF, which then translocate to the nucleus. Cactus is phosphorylated by Pelle and the subsequent degradation of polyubiquitinated Cactus is mediated by a member of the  $\beta$ -TrCP ubiquitin ligase family, Slimb (Daigneault et al., 2013). Additionally, a genome-wide RNAi screen in S2 cells highlighted the role of G protein-coupled receptor kinase 2 (Gprk2) in Toll signaling pathway at the level of Cactus (Valanne et al., 2010). This new component of the signaling pathway binds Cactus directly but is not involved in its degradation. Gprk2 is conserved from flies to mammals, where it also plays a role in the NF- $\kappa$ B pathways.

Similarly to the Relish-68 N-terminal fragment, Dorsal and DIF bear a Rel-homology domain (RHD) responsible for their transcription factor activity. The Dorsal and DIF RHDs are functional in homo-dimers (Dorsal/Dorsal, DIF/DIF) or in heterodimers (Dorsal/DIF) (Tanji et al., 2010b). Importantly, while Dorsal is effective for both Toll-dependent embryonic patterning and immune response in larvae and embryonic S2 cells, DIF is only required for immunity and is the only Toll-pathway-dependent NF- $\kappa$ B factor required in adult flies (Manfruegli et al., 1999; Rutschmann et al., 2000). In the nucleus, DIF and Dorsal bind to their cognate  $\kappa$ B response element, GGGAAA(A/T/G)YCC to trans-activate the transcription of hundreds of target genes, notably the anti-fungal peptide Drosomycin (Lemaitre and Hoffmann, 2007). Of note, two members of the GATA transcription factors family (which are characterized by their ability to bind GATA sequences), Pannier and U-shaped, were found to positively influence Dorsal target genes transcription in an S2 cells Toll pathway activation assay (Valanne et al., 2010).

### 1.2.3 Toll pathway effectors

Similarly to the IMD pathway, the most well characterized effectors of Toll pathway activation are anti-microbial peptides. The antifungal Drosomycins and Metchnikowin and the anti-Gram-positive bacterial Defensin peptides are the principal induced targets of systemic Toll pathway activation, although Toll also activates the *diptericin-A* gene (De Gregorio et al., 2002a). Nevertheless, since the effectiveness of the anti-fungal Drosomycin peptide is questioned by both *in vitro* and

*in vivo* studies (Tzou et al., 2002), other potent Toll pathway effectors might also play a role especially in the anti-fungal defense (Ferrandon et al., 2007).

#### **1.2.4 Toll pathway negative regulation**

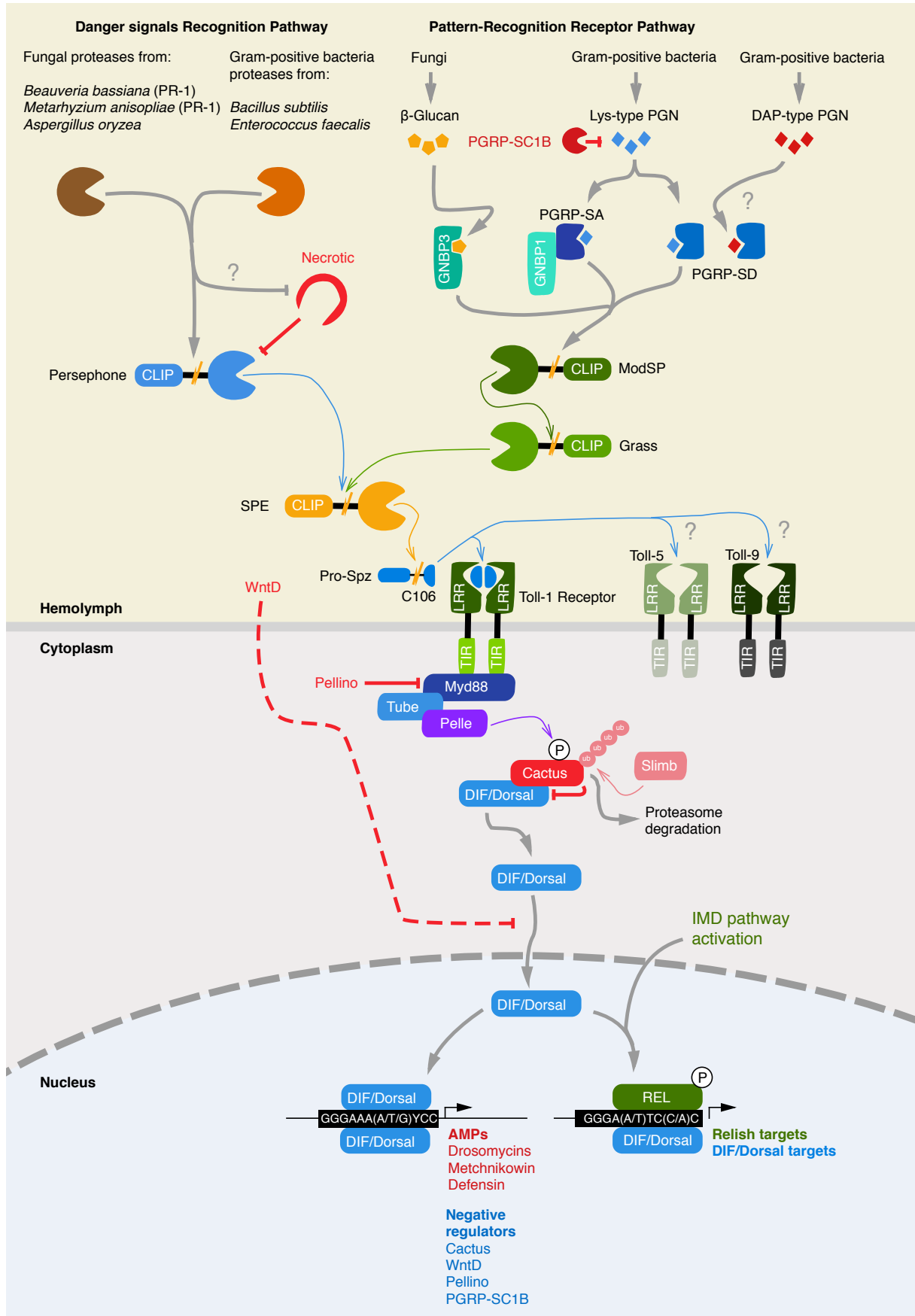
Like the IMD pathway, Toll pathway activation must be tightly controlled. This must be the case during embryonic development, to allow the dorso-ventral axis to be established, and later in the adult immune response pathway to prevent unnecessary and potentially harmful activations. Negative regulatory factors interact all along the Toll pathway to fine-tune its activation.

First, during pattern-recognition events, Lys-type PGN may be scavenged by the soluble PGRP-SC1B amidase activity (Mellroth et al., 2003) prior to PRR recognition, therefore preventing the activation of PRR recognition pathway. Alternatively, the Serine protease inhibitor (Serpine) Serpin43Ac (referred to as Necrotic) is a constitutive inhibitor of Psh, preventing danger signal recognition pathway activation (Levashina et al., 1999; Ligoxygakis et al., 2002). Deficiency in Necrotic constitutively activates the Toll pathway and is highly detrimental to flies, leading to general melanization and early death of young adult flies in a Psh-dependent manner (Ligoxygakis et al., 2002). These studies also suggest that Necrotic degradation would be a mandatory step for the activation of Toll pathway by exogenous proteases, although the precise molecular mechanism of such degradation has not been described.

Further in the pathway, a negative role of Pellino in the regulation of Myd88 protein stability has been described (Ji et al., 2014). This work contradicts a previous study showing Pellino's requirement for Toll signaling (Haghighyeghi et al., 2010) (previously described). Ji et al demonstrated that Pellino was induced on Toll pathway activation and accumulated close to the cytoplasmic membrane, in combination with Myd88. This interaction led to the poly-ubiquitination of Myd88 and its targeting to the proteasome. Overall, this work suggests that Pellino might work in a feedback regulatory loop preventing excessive Toll pathway activation (Ji et al., 2014).

As Cactus is transcriptionally induced during Toll pathway activation, Cactus also acts as a dynamic negative regulator of the pathway. At later stages of Toll pathway activation, newly-synthesized Cactus may overcome the phosphorylation and degradation signals, and further shut down the pathway, by sequestering Dorsal and DIF in the cytoplasm (Nicolas et al., 1998). Finally, Wnt inhibitor of Dorsal (WntD), a member of *Drosophila* Wnt family induced by the activation of the Toll pathway also exerts a negative retro-control on the pathway (Gordon et al., 2005). WntD is a secreted protein that would block the Toll pathway activation at the level of Cactus and upstream of DIF and Dorsal. However, mechanisms of such negative regulation would require a yet-unknown signaling cascade preventing Dorsal and DIF nuclear translocation. Intriguingly, although displaying an over-expression of AMPs, WntD deficient flies are more susceptible to lethal bacterial infections by *Listeria monocytogenes*, further demonstrating that unbalanced immune pathways activation have a strong fitness cost in flies and therefore influenced the evolution of such negative regulation feedbacks (Gordon et al., 2005, 2008).

# Chapter 1: Innate immune responses in *Drosophila melanogaster*





### Figure 3 The Toll pathway of *Drosophila melanogaster* in immunity.

The Toll pathway is initiated by the recognition of microbe-associated molecular patterns (MAMPs) or danger signals through two parallel extra-cellular molecular cascade, Pattern Recognition Receptor (PRR) and Danger signals recognition pathways respectively. In the PRR pathway, GNB3 recognizes  $\beta$ -glucan from fungi while PGRP-SA and GNB1 recognizes Lysine-type (Lys-type) PGN from Gram-positive bacteria. Alternatively, PGRP-SD also recognizes Lys-type PGN and may also bind to Gram-negative bacterial-derived DAP-type PGN. GNB3, PGRP-SA, GNB1 and PGRP-SD further activate a CLIP-domain-serine-protease cascade involving Modular serine protease (ModSP) and Gram-positive specific serine protease (Grass), ultimately activating the Spätzle processing enzyme (SPE). Alternatively, in the Danger signal recognition pathway, SPE can be activated by Persephone, another CLIP-domain serine protease that can be activated in the presence of protease from entomopathogenic Gram-positive bacteria and fungi. Activated SPE releases the C-terminal portion of the cytokine Pro-Spätzle, C106, which binds as homodimers to the extracellular Leucine-rich repeats of Toll-1 receptors.

Experimental evidences suggest that Toll-5 and -9 could also activate the Toll pathway. Once dimerized, the Toll Interleukin-1 Receptor (TIR) intracytoplasmic domain of Toll receptors recruit the adaptor proteins Myeloid differentiation primary response gene (88) (Myd88), Tube and the Interleukin-1 receptor associated kinase 1 (IRAK1)-like Pelle kinase. Pelle further phosphorylates the I $\kappa$ B-like protein Cactus. Phosphorylated Cactus is then poly-ubiquitinated by Slimb and addressed to the proteasome for degradation. Degradation of Cactus releases the NF- $\kappa$ B factors Dorsal and Dorsal-related immunity factor (DIF) that enters to the nucleus as homo or heterodimers. Alternatively, DIF and Dorsal can heterodimerize with Relish-68 upon both Toll and Immune deficiency (IMD) pathways activation. DIF/Dorsal dimers bind to their cognate  $\kappa$ B response element, the consensus sequence 5'-GGGAAA(A/T/G)YCC-3' and activate Toll-pathway target genes. DIF/Dorsal target genes include the antimicrobial peptides from the Drosomycins family, Metchnikowin and Defensin, and negative regulators retro-controlling the activation of the pathway. Rel68/DIF or Rel68/Dorsal bind to the  $\kappa$ B Response element 5'-GGGA(A/T)TC(C/A)C-3' and are able to activate both IMD and Toll pathways target genes. Negative regulators of Toll pathway are highlighted in red and are described more in detail in the main text.

## 2. NF- $\kappa$ B-independent immune pathways in *Drosophila*: The JNK and JAK/STAT pathways

### 2.1 The JNK pathway

The Jun N-terminal Kinase (JNK) pathway also referred to as the Stress-activated protein kinase pathway (SAPK) is an ancient evolutionary conserved eukaryotic signaling pathway. JNK is one of the three *Drosophila* members of the mitogen activated protein kinases (MAPK) signaling pathways family (Ríos-Barrera and Riesgo-Escovar, 2013), also including the extracellular regulated kinase (ERK), and the p38 MAPK pathways. The JNK pathway is well conserved from yeast to humans, and plays a fundamental role in developmental processes in *Drosophila* such as embryonic dorsal closure (Rousset et al., 2010), thorax closure (Martin-Blanco et al., 2000), follicle cell morphogenesis (Dobens et al., 2001) and male genitalia disc closure (Macías et al., 2004) by regulating cell elongation (Agnès et al., 1999).

Additionally, JNK pathway is one of the most crucial pathways in the stress response in adult animals and can be activated by a wide variety of stimuli such as UV irradiation, reactive oxygen species, DNA damage, heat, infections and inflammation (Biteau et al., 2011). Depending on the tissular context, JNK pathway is able to influence apoptosis, autophagy, resistance to oxidative damages (cytoprotection), metabolism and growth, cell proliferation, regeneration and tissue repair (Biteau et al., 2011).

The activation of JNK pathway is initiated by the activation of a JNK Kinase Kinase (JNKKK) stress stimuli mentioned above. Several known JNKKK are involved in this process in *Drosophila* including (but not restricted to) the Mixed Lineage Kinase 2 (MLK2, also named Slipper) required for JNK-dependent dorsal closure (Stronach and Perrimon, 2002), the Apoptotic Signal-regulating Kinase 1 (ASK1), required for Reaper-dependent cell death regulation (Kuranaga et al., 2002), MEK Kinase 1 (MEKK1) in response to toxic metal (Sodium arsenite, Cadmium) exposure (Ryabinina et al., 2006), and Transforming growth factor beta (TGF- $\beta$ )-activated kinase 1 (TAK1), upon IMD pathway activation (previously described in **1.1.1b**) (Silverman et al., 2003).

Once activated, JNKKK may phosphorylate and activate two JNK Kinases (JNKK), Hemipterous (Hep) (Glise et al., 1995), mediating the majority of JNK signaling effects in *Drosophila* and dMKK4 (Sathyanarayana et al., 2003), acting in parallel of Hemipterous especially during immune responses (Geuking et al., 2009). JNKK then phosphorylate the final Jun N-terminal Kinase (JNK) Basket (Kockel et al., 1997). Basket is able to target numerous proteins in the cytoplasm prominently

including the transcription factors of *Drosophila* activator protein 1 (AP-1) family, Jun and Fos (Riesgo-Escovar and Hafen, 1997a, 1997b) and the Forkhead box O (FOXO) transcription factor (Wang et al., 2005).

Once in the nucleus, AP-1 and FOXO transcription factors activate numerous target genes causing a range of highly tissue- and context-specific cellular responses, ranging from apoptosis, morphogenesis, cell migration, metabolism, cytoprotection and cell proliferation. Of note, one of AP-1 transcriptional targets, Puckered is a Basket-specific phosphatase preventing its activation and therefore restricts JNK pathway activation in a negative feedback loop (Martin-Blanco et al., 1998). A schematic view of the JNK pathway is provided in **Figure 4A**.

## 2.2 The JAK/STAT pathway

### 2.2.1 Biological relevance of the JAK/STAT pathway

The Janus Kinase / Signal Transducers and Activators of Transcription (JAK/STAT) pathway was first described in the human innate immune system, where it plays a crucial role in the antiviral and anti-mycobacterial defense by allowing the production of cytokines of the Interferons (IFN) family (Stark and Darnell, 2012). The *Drosophila* JAK/STAT pathway controls several key embryonic and adult biological processes such as embryonic patterning (Zeidler et al., 2000), wing and eye formation (Yan et al., 1996a; Zeidler et al., 1999) and maintenance of stem cells (Kiger et al., 2001; Wang et al., 2011), among which intestinal stem cells (Jiang et al., 2009).

Additionally, the JAK/STAT pathway directly contributes to immune and stress responses in *Drosophila* by activating several infection-induced genes such as thioester-containing proteins (TEP), opsonization molecules involved in phagocytosis, and Turandot (Tot) family peptides, putative effectors of the stress response (Agaisse and Perrimon, 2004). The JAK/STAT pathway is also involved in *Drosophila* hematopoiesis by influencing the commitment of larval hemocytes commitment towards lamellocytes during parasitoid wasp egg encapsulation response (Sorrentino et al., 2004). In addition, the pathway is required in the antiviral response against the picorna-like Dicistroviridae family members *Drosophila* C virus

(DCV) and Cricket paralysis virus (CrPV) (Kemp et al., 2013). However, the mechanism of viral detection by the JAK/STAT pathway and the mode of action of its anti-viral effectors have not been clarified (Kemp et al., 2013).

Additionally, the JAK/STAT pathway is involved in the anti-microbial defense of the gut, by inducing a discrete subset of anti-microbial peptides including Drosomycin-like 3 (Drsl-3) during intestinal infections. In this case, JAK/STAT activation probably occurs indirectly, following epithelial damage by the pathogen (Buchon et al., 2009b). Finally, JAK/STAT might be required, together with PGRP-LE PRR to recognize and fight the intracellular bacteria *Listeria*, through the production of the anti-microbial peptide Listericin (Goto et al., 2010).

### **2.2.2 Signaling events of the *Drosophila* JAK/STAT pathway**

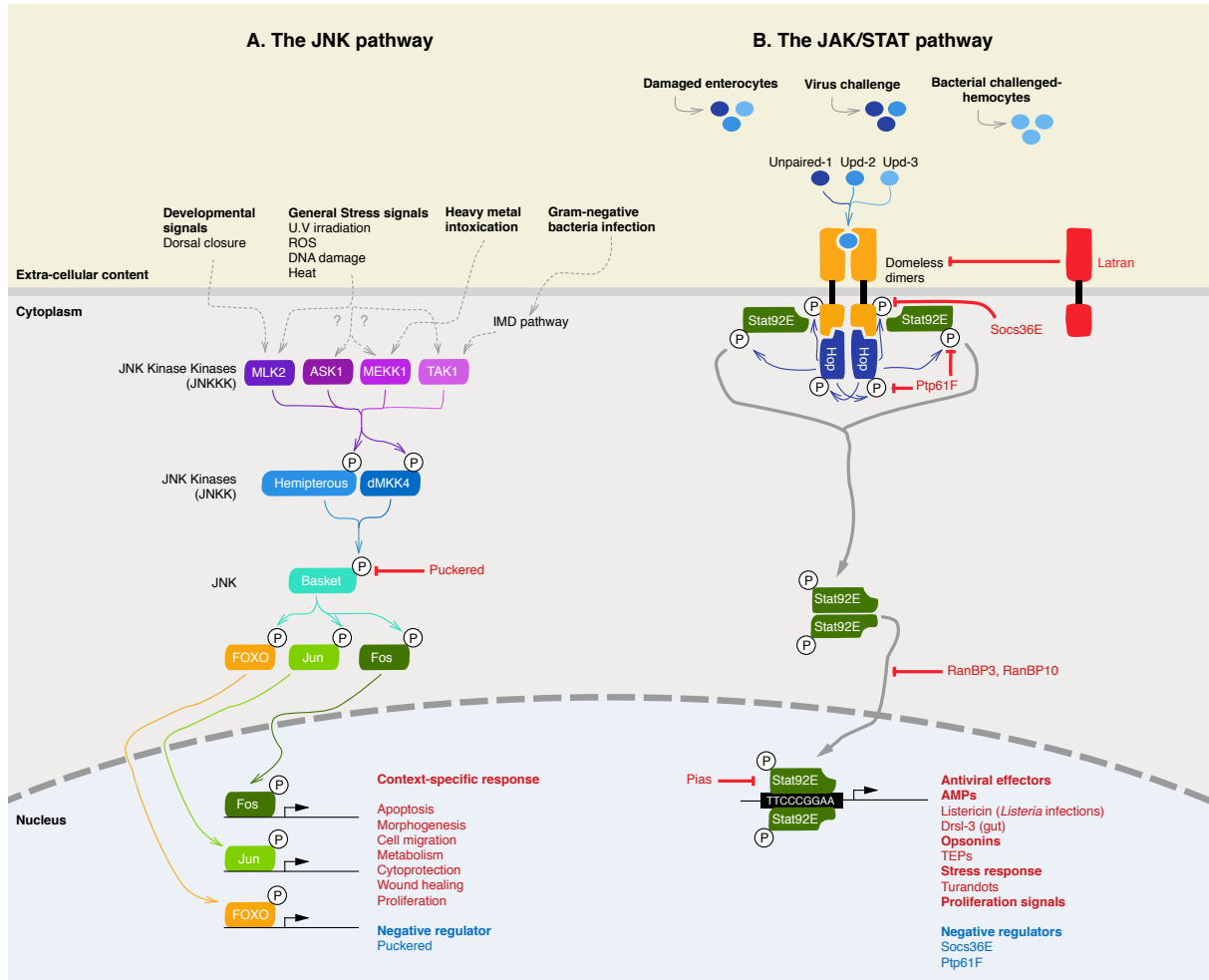
In *Drosophila*, the JAK/STAT pathway is initiated by the three cytokine-like protein ligands: Unpaired (Upd) (Harrison et al., 1998), Upd2 (Hombria et al., 2005) and Upd3 (Wright et al., 2011). Of note, this family of ligands seems to be specific to *Drosophila* species but shares some homology with leptins, a family of hormones regulating fat storage in mammals (Harrison et al., 1998). Of note, Upd3 transcriptional activation is specifically induced in hemocytes following a bacterial challenge (Agaisse et al., 2003) while Upd and Upd2 are specifically induced during viral infections, overall suggesting the presence of multiple regulatory elements controlling their expression (Myllymäki and Rämetsä, 2014).

Upd-family ligands bind to the receptor Domeless (Dome), a transmembrane receptor sharing functional and sequence similarities with the Interleukin-6 Receptor (IL-6R) (Chen et al., 2002). This binding provokes receptor homo-dimerization and activates the *Drosophila* Janus Kinase (JAK) Hopscotch (Binari and Perrimon, 1994). JAK is constitutively associated with the cytoplasmic portion of the Domeless receptor. Activated Hopscotch on each Domeless monomer phosphorylate each other as well as specific tyrosine residues of Domeless cytoplasmic portion, enabling the formation of docking sites for the binding of Stat92E transcription factors (Myllymäki and Rämetsä, 2014). Following their binding to Domeless, Stat92Es are phosphorylated by Hopscotch on Tyr<sub>704</sub> residue allowing them to dimerize and

translocate to the nucleus, where they bind their cognate DNA response-element, the consensus motif TTCCCGGAA (Brown et al., 2003; Yan et al., 1996b).

Several factors have been shown to perform a strict negative control of JAK/STAT activation. First, Eye Transformer (ET, also called Latran), a *Drosophila* homolog of mammalian gp130 protein was shown to down-regulate JAK/STAT pathway signaling possibly by forming inactive Domeless-ET heterodimers (Makki et al., 2010). Additionally, Suppressor of Cytokine Signaling 36E (Socs36E) and Protein Tyrosine Phosphatase 61F (Ptp61F), two transcription targets of Stat92E were shown to act as negative feedback loops. Firstly, Socs36E can reduce Domeless stability by addressing it to lysosomal degradation (Vidal et al., 2010). Second, Protein tyrosine phosphatase 61F (Ptp61F) could remove phosphate groups from Hopscotch, and possibly also from Stat36E, thus preventing the activation of the pathway (Baeg et al., 2005).

Additionally, nuclear localization and DNA-binding capability of Stat92E are also tightly regulated. The Ras-like guanine nucleotide-binding protein 3 (RanBP3) and RanBP10 proteins would block JAK/STAT signaling by inhibiting Stat92E nuclear import (Baeg et al., 2005). Finally, Protein inhibitor of activated STAT (Pias) was shown to interact with Stat92E and negatively regulate its transcription (Betz et al., 2001), possibly by blocking Stat92E interaction with DNA as it is the case for their mammalian homologs (Chung et al., 1997). A schematic view of the JAK/STAT pathway is provided in **Figure 4B**.



**Figure 4 The Jun N-terminal kinase (JNK) and JAK/STAT pathways in *Drosophila melanogaster*.**

**A.** The JNK pathway is activated by a wide range of signals including developmental and stress signals, heavy metal intoxication and Gram-negative bacterial recognition by the IMD pathway. These upstream signals lead to the activation by phosphorylation of four potential JNK kinase kinases (JNKKK): MLK2, ASK1, MEKK1 and TAK1. These four JNKKK then activate two JNK kinases (JNKK): Hemipterous and dMEKK4. These two JNKK finally activate the sole Jun N-terminal kinase (JNK) Basket. Activated Basket mediate the phosphorylation of several cytoplasmic proteins including the transcription factors from the Activating Protein 1 (AP-1) family Fos and Jun, and Foxo. Once activated, these three transcription factors mediate highly context-specific events that include apoptosis, morphogenesis, cell migration, metabolism, cytoprotection, wound healing and proliferation. One known negative regulator of JNK pathway, Puckered, is also induced and repress the pathway at the level of Basket phosphorylation.

**B.** The Janus Kinase / Signal Transducers and Activators of Transcription (JAK/STAT) pathway is activated subsequently to the binding of cytokines from the Unpaired family (Upd1, 2 and 3) to

Domeless receptors. Unpaired molecules are released, during the innate immune response, by bacterial-challenged hemocytes, damaged enterocytes and in the course of viral infections. Once Domeless receptors dimerized after Upd binding, each of the Domeless-associated JAK Hopscotch (Hop) phosphorylates each other and the Domeless receptor. Phosphorylated Domeless is able to recruit the transcription factor Stat92E, which will be consequently phosphorylated by Hop. Phosphorylated Stat92E further translocate to the nucleus as homodimers and bind to their cognate response element, the consensus sequence 5'-TTCCCGGAA-3' to activate their target genes. Stat92E-target genes include yet unidentified antiviral effectors, anti-microbial peptides (AMPs), opsonins, candidate stress-response molecules and cell-proliferation signals, and two of the negative regulators of the pathway. All the negative regulators of JAK/STAT pathway are highlighted in red and are described more in detail in the main text.

### **3. Local immune responses**

To reach the body cavity of the fly, microbes have to cross first an epithelium generally shielded by chitin barriers. The most targeted epithelium lines the gastrointestinal tract, where the microorganisms from outside (beneficial, commensal or pathogenic) are constantly present. This organ has developed complex and powerful defense mechanisms to prevent pathogen proliferation while tolerating beneficial or commensal bacteria. These mechanisms are detailed below (3.1.). Secondly, the trachea and the male genital plates have also been reported to mount specific epithelial responses detailed further (3.2.).

#### **3.1 Intestinal immune responses**

##### **3.1.1 *Drosophila* gut physiological properties**

###### **(a) Anatomical and functional regions of *Drosophila* gut**

The *Drosophila* gut is composed of a tubular monolayer of epithelial cells, surrounded by visceral muscles, nerves and trachea. Those epithelial cells have distinct embryonic origins and therefore harbor distinct functions depending on their

position along the digestive tract. Three main regions of *Drosophila* gut can be distinguished: foregut, midgut and hindgut (Murakami et al., 1999).

The foregut, developed from the ectoderm, is composed of proboscis –a feeding and drinking appendage-, pharynx and esophagus (both passing the food through the foregut), crop -a food storage organ- and proventriculus, a pear-shaped organ regulating the passage of food to the midgut.

The midgut, developed from the endoderm, is mainly dedicated to digestive and metabolic functions. This region is divided into the anterior midgut, the copper cells and the posterior midgut. A recent study, however, distinguished six anatomically distinct regions (anteriorly to posteriorly named R0 to R5), that remain stable from young to old adult flies, associated with distinct metabolic and digestive activities (Buchon et al., 2013a) (**Figure 5A**). In this system, R0 corresponds to the endodermal part of the proventriculus and R5, the first region joining the hindgut. One remarkable region of the midgut is the R3 region, at the median of the midgut, which contains highly differentiated cells named the copper cells. These cells secrete  $H^+$  in exchange of Adenosine Triphosphate (ATP). As a result copper-cells regions is the most acidic section of the gut (pH < 4.0). Similarly to mammalian stomach, the acidity of this region causes proteins denaturation and provides an optimal pH for the activity of some digestive proteases (Dubreuil, 2004).

Unlike other gut regions, the adult *Drosophila* midgut is constantly self-renewing through divisions of intestinal stem cells (ISCs) (Jiang and Edgar, 2011). These multipotent stem cells provide the gut epithelium with two types of differentiated cells, enterocytes (ECs) and entero-endocrine cells (EECs) (**Figure 5B**). ECs and EECs are specialized through a differentiation process involving a progenitor intermediate state, namely enteroblasts (EBs). On one hand, ECs are large polyploid cells numerally dominating the midgut epithelium. Their main functions are to secrete digestive enzymes and absorb nutrients. The EC commitment is mediated by a strong activation of the Notch pathway in enteroblasts (Micchelli and Perrimon, 2006). By contrast, a lower activation of Notch pathway in EB further mediates the EEC commitment (Ohlstein and Spradling, 2007). These Notch pathway activation-dependent differentiation processes of EB are initiated through the local release of Notch Receptor ligand, Delta, by ISCs. Interestingly, Notch pathway-independent signals may account for EEC commitment, as Notch or



Delta depletion in ISC clones is not preventing the differentiation of the EEC lineage (Ohlstein and Spradling, 2007).

EECs are small diploid cells that interdigitate with the larger EC cells. It is thought that EECs carry out neural-like functions in regulating intestinal physiology and reporting nutritional states to the other organs (Cognigni et al., 2011). Additionally, a recent study showed that EECs indirectly influence ISC proliferation by secreting the neuroendocrine hormone Bursicon (Scopelliti et al., 2014). This hormonal signal is sensed in a paracrine fashion by the visceral muscles cells (VMCs) underlying midgut epithelium. Consequently, VMCs stimulate ISCs proliferation through the release of EGF ligands (further detailed in **3.1.2b**).

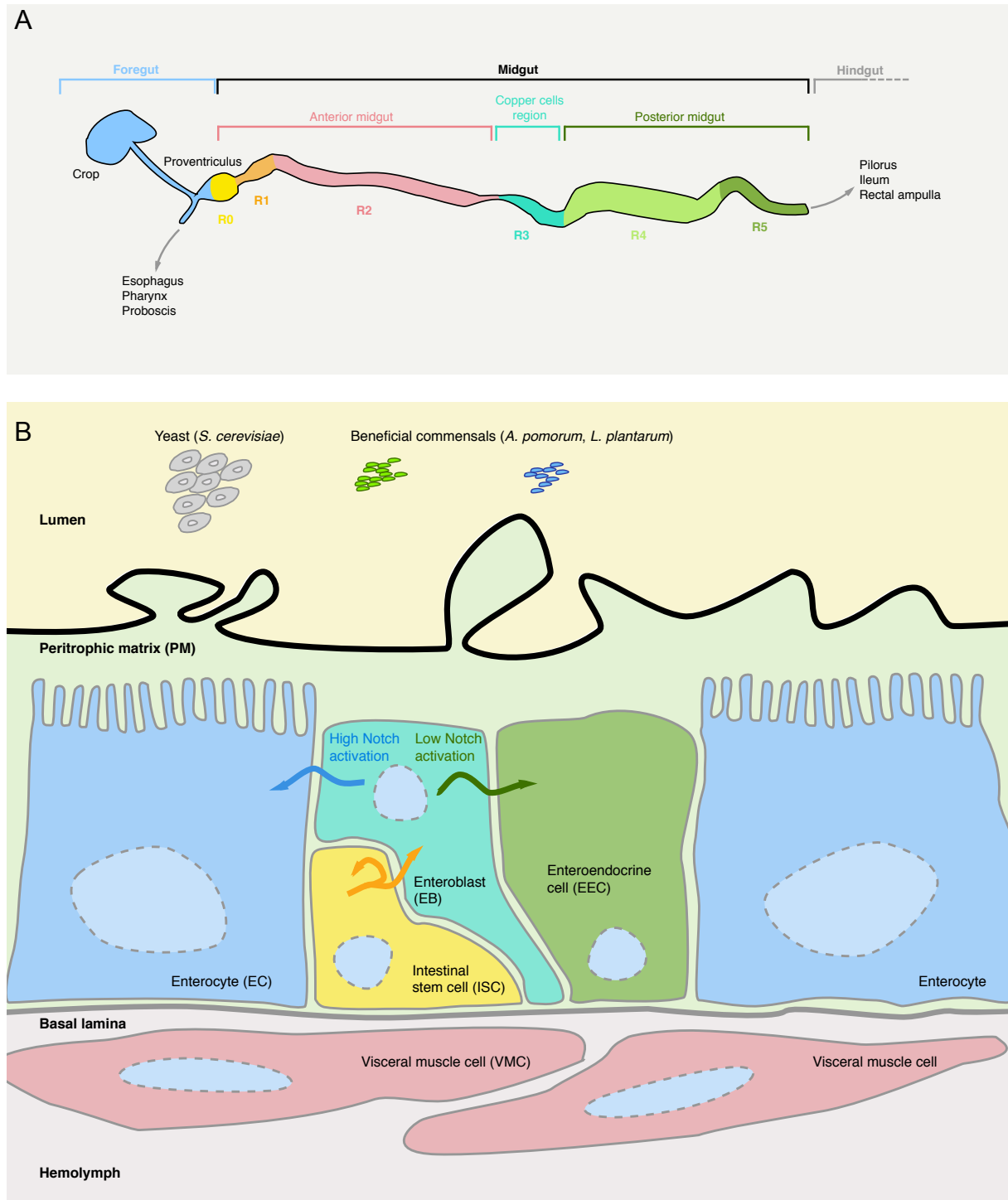
The most posterior part of *Drosophila* intestine is the hindgut, which derives from the ectoderm. The main functions of the hindgut are i) to excrete metabolized nutrients and ii) to regulate salt balance. The hindgut is subdivided into three main sections (anteriorly to posteriorly): pylorus, ileum and rectal ampulla. The pylorus is able to constrict sphincter muscles and regulate the passage of gut contents; it is also the location where Malpighian tubules, the functional analogues of mammalian kidneys connect to the digestive tract (Demerec, 1950). The ileum is responsible for ion and water exchanges and finally, the rectal ampulla voids the gut contents.

### **(b) Passive defense mechanisms of *Drosophila* gut**

A specific characteristic of foregut and hindgut epithelia is the presence of an impermeable cuticle preventing digestive functions. In contrast, the midgut is surrounded by a semi-permeable chitin and glycoproteins matrix called the peritrophic matrix (PM) that allows digestive enzymes to reach the bolus (Hegedus et al., 2009). *Drosophila* PMs are secreted by the proventriculus in the foregut as multiple layers. They are further compressed by muscular contraction of muscle cells of the proventriculus to finally form two layers as they enter the midgut (King, 1988). This so-called type II PM (Lehane, 1997) constitutes the first defense layer of the fly intestine against pathogenic bacteria as it is passively impenetrable by components larger than 10nm, or proteins larger than 200 kDa, as estimated from the maximal diameter of PM pores (Lehane, 1997). The protective role of the PM is confirmed by the susceptibility of Drosocrystalin (Dcy) mutants to the *Pseudomonas entomophila*

and *Serratia marcescens* pathogens (Kuraishi et al., 2011a). Dcy is a chitin-binding-domain protein, and the mutant flies show a reduction in PM width. This study also shows that Dcy expression is positively regulated in the presence of pathogenic bacteria, suggesting that PM is dynamically involved in the gut immune system.

A common metazoan feature is the presence of an intestinal microbiota that actively participates to the metabolism of nutrients. In mammals, a dense and diverse microbiota, composed of bacteria, archaea, viruses and unicellular eukaryotes resides inside the gut lumen. Human intestinal microbiota is estimated at  $10^{14}$  cells comprising over 50 bacterial phyla (Schloss and Handelsman, 2004), dominated by the Firmicutes and Bacteroidetes phyla (Eckburg et al., 2005). In *Drosophila*, the gut microbiota is quantitatively and qualitatively reduced. 16S rRNA sequencing-based studies on wild flies and laboratory stocks identified up to 30 bacterial species in the fly gut, mostly members of the *Lactobacillus* and *Acetobacter* genera (Chandler et al., 2011; Corby-Harris et al., 2007; Cox and Gilmore, 2007; Ren et al., 2007; Ryu et al., 2008; Wong et al., 2011). Nonetheless, two independent mono-association studies on *Lactobacillus plantarum* and *Acetobacter pomorum* have demonstrated the importance of *Drosophila* microbiota for post-natal growth in larvae. The presence of *L. plantarum* in the gut modulates the target of rapamycin (Tor) pathway, which in turns induces the production of *Drosophila* insulin-like peptides (Dilps) increasing larval growth rate (Storelli et al., 2011). In the case of *A. pomorum*, the positive influence of on larval growth is mediated by its pyrroloquinoline quinone-dependent alcohol dehydrogenase activity, inducing insulin signaling (Shin et al., 2011). Interestingly, no specific mechanisms of recognition dedicated to such beneficial bacteria have been identified so far. However, the intensity of the immune responses as well as the damages eventually produced by these responses on the gut epithelium are much lower in the case of the microbiota than pathogenic bacteria (Buchon et al., 2009b). Nonetheless, whether the natural flora of the adult gut provides any sort of protection against pathogenic infections is still an open question.



**Figure 5** Histological and cellular organization of *Drosophila melanogaster* midgut.

**A.** The *Drosophila* gut is separated in three main portions that have distinct embryonic origins: the foregut (light blue) and the hindgut (grey) arose from ectodermal tissues while the midgut (black) developed from the endoderm. The midgut is probably the most complex and studied portion of the intestine and is further divided into six anatomically distinct regions: R0, R1 and R2 being part of so-

called anterior midgut; R3, the copper cells regions; R4 and R5 being part of so-called posterior midgut.

**B.** Midgut tissues are composed of a monolayer of epithelial cells covered on its apical side by a semi-permeable glycoproteic layer called peritrophic matrix. On the basal pole, the epithelium is separated from visceral muscles cells by a basal lamina. Midgut epithelia are capable of constant regeneration thanks to the asymmetric divisions of intestinal stem cells (ISCs). Mitosis of these cells give rise to another ISC and a differentiating progenitor called Enteroblast (EB). This diploid enteroblast differentiate into polyploid enterocytes (EC) or diploid entero-endocrine cells (EEC). In physiological conditions, the large majority of midgut epithelial cells is composed of ECs.

These schemes are based on the work of Nicolas Buchon and Bruno Lemaitre (Buchon et al., 2013a; Lemaitre and Miguel-Aliaga, 2013).

### **3.1.2 Active defense mechanisms**

Beyond the PM, the intestinal epithelium can respond to a pathogenic threat through three main mechanisms: an oxidative burst provoked by the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Dual Oxidase (Duox) enzyme **(a)**, the secretion of Anti-microbial peptides by the NF- $\kappa$ B immune deficiency (IMD) pathway **(b)** and the maintenance of gut homeostasis through the regulation of stem cells division **(c)**.

#### **(a) The oxidative burst**

The production of microbicidal Reactive Oxygen Species (ROS) is a key component of eukaryotic immune system as it provides a broad defense against all types of microorganisms (Medzhitov and Janeway, 1997). ROS include free radicals (superoxide anion ( $O_2^{\cdot-}$ ), hydroxyl radical ( $\cdot OH$ )) and non-radical molecules (hydrogen peroxide ( $H_2O_2$ ), singlet oxygen ( $^1O_2$ )). ROS damage proteins, lipids and nucleic acids and therefore do not target specifically microbial structures. However, anti-oxidant systems are mobilized to prevent damages on the host cell such as the extracellular immune-regulated catalase (IRC) in *Drosophila* gut (Ha et al., 2005a). These past ten years, the group of Won-Jae Lee in particular and others, have demonstrated that ROS production by enterocytes is one of the most crucial defense mechanisms in the gut. ROS are produced in the gut by epithelial cells through the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Dual Oxidase (Duox)

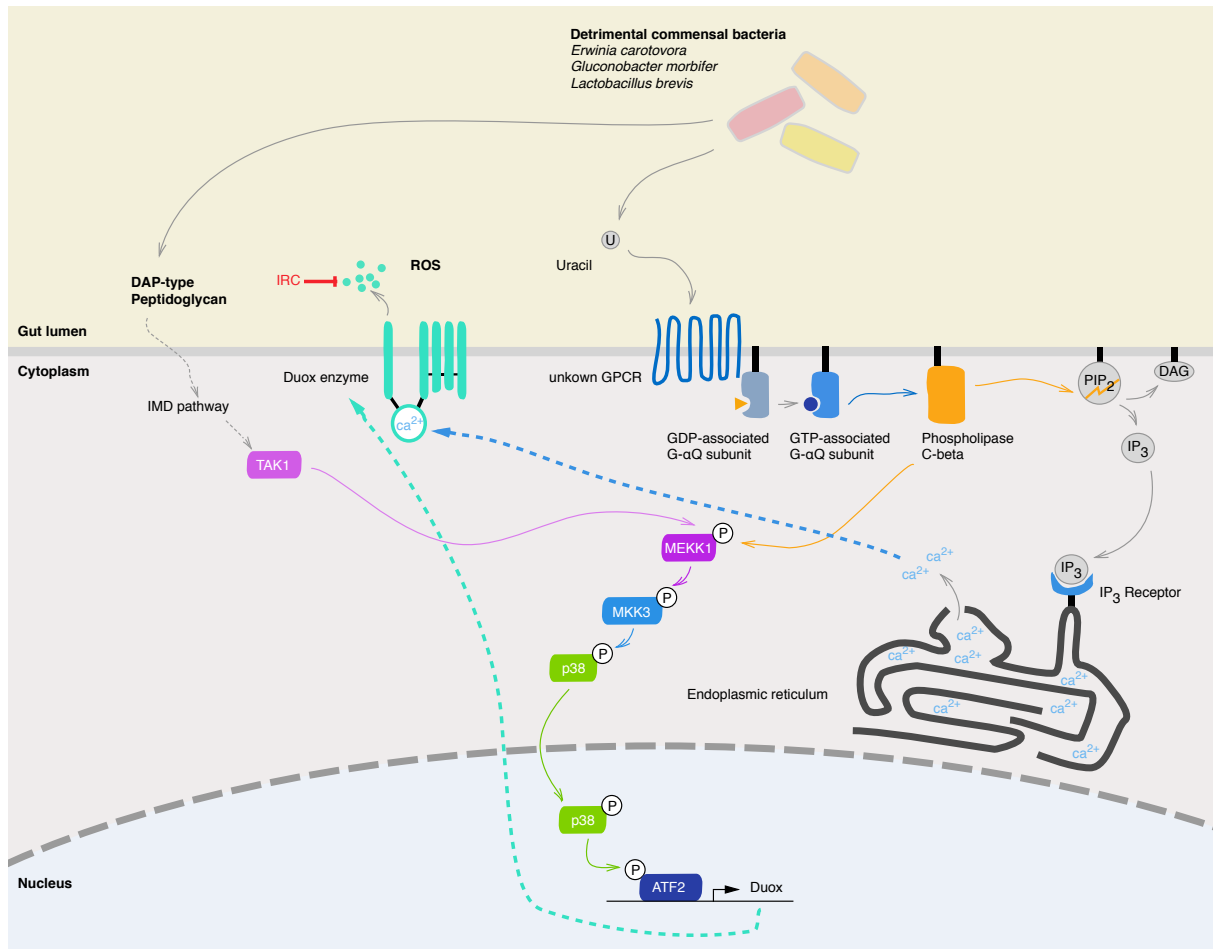
enzyme. Duox is located at the apical surface of the gut epithelium and preferentially expressed in the foregut and the hindgut (Ha et al., 2005b). In healthy feeding conditions, this enzyme produces basal levels of ROS, maintaining the population of commensal bacteria. In contrast, intestinal bacterial infections provoke an upregulation of Duox mRNA and ROS production (Ha et al., 2009a). This upregulated ROS production is required to contend the infection since genetic ablation of Duox by conditional knock-down (KD) in the gut significantly decreases the survival rate to intestinal infection with *Erwinia carotovora* Ecc15 (Ha et al., 2005b). However, ROS over-production can be deleterious to the host despite the presence of IRC, by inducing enterocytes cell death notably in the presence of *Pseudomonas entomophila* infection (Chakrabarti et al., 2012).

Known mechanisms of regulation of ROS production directly target the Duox enzyme (**Figure 6**). A first regulatory mechanism of regulation, the “Duox activation pathway” directly impacts Duox enzymatic activity through the G protein  $\alpha$  q sub-unit (G $\alpha$ q) - Phospholipase C- $\beta$  (PLC- $\beta$ ) signaling pathway (Ha et al., 2009b). This pathway is initiated by the recognition of micro-organisms by an unknown G protein-coupled Receptor (GPCR). Upon activation, this GPCR replaces a G $\alpha$ q-associated Guanosine Diphosphate (GDP) with a Guanosine Triphosphate (GTP) through an uncharacterized mechanism. Associated with GTP, G $\alpha$ q is able to activate membrane-bound PLC- $\beta$ , which then hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP<sub>2</sub>) into membrane-free inositol 1,4,5-triphosphate (IP<sub>3</sub>) and membrane-bound diacylglycerol (DAG) (Rhee, 2001). Membrane-free IP<sub>3</sub> reach IP<sub>3</sub> receptor (IP<sub>3</sub>R) on the endoplasmic reticulum (ER). This receptor-ligand interaction then mediates the release of ER-contained Ca<sup>2+</sup> to the cytoplasm. Released Ca<sup>2+</sup> further positively regulates Duox enzyme activity through its Ca<sup>2+</sup>-binding EF hand domain and ultimately supports microbicidal ROS production.

Conventional *Drosophila* diets contain yeasts, usually growing on decaying food and representing an essential nutrients source (Phaff and Knapp, 1956). Of note, the Duox activation pathway is activated by pathogenic bacteria as much as by dietary yeast. As demonstrated by the decreased lifespan of Duox, PLC- $\beta$  and G $\alpha$ q knock-down flies reared on live yeast-containing medium, this pathway is crucial for regulating the commensal gut flora and therefore directly affects nutrition and health (Ha et al., 2009b). Finally, bacterially-secreted uracil is a danger signal for activation

of the Duox activation pathway (Lee et al., 2013). This study provides an attractive model of danger signal sensing in the gut since detrimental bacteria (e.g. *E. carotovora*, *Gluconobacter morbifer*, *Lactobacillus brevis*) secrete significant amounts of uracil, which the beneficial bacteria (*L. plantarum*, *A. pomorum*) do not.

Additionally, ROS production can also be impacted by the regulation of the expression level of *duox* gene. This so-called “Duox expression pathway” is under the control of the mitogen-activated protein (MAP) kinase p38 pathway involving MEKK1, MKK3 and p38 kinases and the Activating Transcription Factor 2 (ATF2) transcription factor. 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 (Ha et al., 2009a). The first (previously described) would join the p38 pathway at the level of PLC-β activation. The latter implies the recognition of bacterial meso-diaminopymelic-type peptidoglycan (DAP-type PGN) by the peptidoglycan-recognition protein-LC (PGRP-LC) receptors. After recognition, these receptors oligomerize and recruit the adaptor molecules immune deficiency (IMD) and FAS associated death domain (FADD), further engaging the molecular pathway activating the NF-κB factor Relish and culminating in the production of anti-microbial peptides and negative regulators of the pathway (Kaneko and Silverman, 2005) (further detailed in the section 1.1.). Additionally, at least in the case of Duox regulation in the gut, the IMD pathway would merge with the p38 pathway possibly through the activation of Transforming growth factor beta (TGF-β)-activated kinase 1 (TAK1) and its subsequent phosphorylation of MEKK1. The biological relevance of the Duox expression pathway seems limited to intestinal bacterial infections, as the conditional KD of members of the pathway (MEKK1, p38, ATF2) in the gut does not impact the lifespan of conventionally raised flies but significantly sensitizes flies to pathogenic oral infections (Ha et al., 2009a).



**Figure 6 ROS synthesis through the Duox activation and expression pathways in *Drosophila melanogaster***

Detrimental commensal bacteria produce significant amounts of uracil that is sensed by enterocytes through an unknown G protein-coupled receptor (GPCR). Once activated, this GPCR converts G- $\alpha$ Q subunit-associated GDP into GTP. GTP-associated G- $\alpha$ Q activates the phospholipase C  $\beta$  (PLC- $\beta$ ), further hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into membrane-free inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and membrane-bound diacylglycerol (DAG). Membrane-free IP<sub>3</sub> reach IP<sub>3</sub> receptor (IP<sub>3</sub>R) on the endoplasmic reticulum (ER). This receptor-ligand interaction then mediates the release of ER-contained Ca<sup>2+</sup> to the cytoplasm. Released Ca<sup>2+</sup> further positively regulates Duox enzyme activity through its Ca<sup>2+</sup>-binding EF hand domain and ultimately supports microbicidal ROS production.

ROS production can also be sustained by increasing the Duox enzyme mRNA levels. This so-called Duox expression pathway is triggered by TAK1, following DAP-type PGN recognition through the IMD pathway or by PLC- $\beta$ . These signals lead to the phosphorylation and activation of MEKK1, further engaging a kinase cascade involving MKK3 and p38 kinases. Ultimately, p38 activates by phosphorylation the transcription factor Activating Transcription Factor 2 (ATF2) to induce the

expression of Duox. The production of ROS may be deleterious for the host. *Drosophila* enterocytes therefore express the extracellular immune-regulated catalase (IRC), an anti-oxidant system to prevent damages on the epithelium.

### **(b) Local production of anti-microbial peptides (AMPs)**

Another aspect of the *Drosophila* gut immune response is the local production of AMPs. Gut AMP synthesis relies mostly on the activation of the NF- $\kappa$ B IMD pathway (Tzou et al., 2000) (see above, 1.1.1). Although all regions of the gut are capable of activating the IMD pathway following bacterial challenge, only discrete regions of the gut show a visible AMPs response. This localized response correlates with a clear regionalization of the expression of PGRP receptors and their negative regulators (Bosco-Drayon et al., 2012a; Buchon et al., 2009b). Such precise regulation allows tolerance of the beneficial microbiota under normal physiological conditions, together with an induced response to pathogenic immune challenge (Ferrandon, 2013).

In the absence of local AMP production in the gut, flies are more susceptible to Gram-negative bacterial pathogenic infection such as *Erwinia carotovora* Ecc15 (Basset et al., 2000; Buchon et al., 2009b), *Pseudomonas entomophila* (Liehl et al., 2006), *Pseudomonas aeruginosa* PA14 (Limmer et al., 2011a) and *Serratia marcescens* DB11 (Nehme et al., 2007). Additionally, the over-expression of only one AMP -Diptericin-, in an IMD pathway deficient background, is sufficient to restore a wild-type resistance to such infections (Liehl et al., 2006; Nehme et al., 2007; Ryu et al., 2006).

On the other hand, prolonged IMD pathway activation at high levels in the gut reduces viability (Ryu et al., 2008), which necessitates multiple repressors. From our current knowledge, described mechanisms of negative regulation of the IMD pathway in the gut involve the proteins PGRP-LB, PGRP-SC, PGRP-LE, Pirk and Caudal (previously described in 1.1.1c). Of note, the up-regulation of NF- $\kappa$ B pathways upon intestinal infection is similarly observed in mammals (Buchon et al., 2009b). Like in *Drosophila*, over-activated intestinal NF- $\kappa$ B responses in mammals are pathologic and are linked with Inflammatory Bowel Diseases (IBD), including ulcerative colitis



and Crohn's disease (Karin et al., 2006; Maeda et al., 2005; Salzman et al., 2007; Xavier and Podolsky, 2007; Xiao et al., 2007; Zaph et al., 2007).

### (c) Maintenance of gut homeostasis

Intestinal infections may cause epithelial damages to the gut. These damages may originate from the pathogen directly or be provoked by the epithelial immune responses. The integrity of the intestinal epithelium must be kept to prevent systemic infections by external microbes. The most exposed portion of the digestive tract is the midgut. The midgut tissues are composed of epithelial cells and muscles. Even in basal conditions, young flies fed on conventional food, a continuous renewal of epithelial cells is occurring. This homeostasis is maintained through compensatory asymmetrical divisions of intestinal stem cells (ISCs) in response to the cell death of enterocytes (EC). In aged flies, more apoptotic epithelial cells are observed, consequently the number of dividing ISCs increases and miss-differentiation events may occur (Biteau et al., 2010). Finally, in infectious conditions, for example by *Erwinia carotovora* or *Pseudomonas entomophila*, or in flies treated with damaging chemicals such as Dextran Sodium Sulfate (DSS) or Paraquat, ISCs divisions are strongly sustained to compensate cell loss (Buchon et al., 2009b).

The regulation of ISC division involves three main factors: (i) the ISC itself is able to self-regulate its cell cycle through autocrine signals, (ii) the stem cell niche composed of the Enteroblasts, which are daughters of the ISCs and precursors of enterocytes and entero-endocrine cells, enterocytes and visceral muscles cells at the basal pole, emitting paracrine signals and (iii) long-range signals from other organs, notably from the brain, transiting by the circulatory system (Ferrandon, 2013). Signaling pathways and molecular events underlying these different routes of ISC proliferative abilities regulation are detailed below and summarized in **Figure 7**.

(i) First, the Platelet-derived Growth Factor (PDGF)-Vascular Endothelial Growth Factor (VEGF) Receptor-related (Pvr) pathway was shown to control ISC proliferation in an autocrine fashion in the anterior part of the midgut (Bond and Foley, 2012). This study showed that the Pvr receptor and its ligand PDGF- and VEGF-related factor 2 (Pvf2) were co-expressed by ISCs in physiological conditions.

Accordingly, Pvf/Pvr defective flies showed defective proliferation and differentiation of ISCs while hyperactivation of the Pvr pathway drove overproliferation of ISCs and formation of intestinal pseudo-tumors. However, ISC-proliferation induced consequently to pathogenic *Pseudomonas entomophila* intestinal damages was still present in dysfunctional Pvr pathway mutant flies, suggesting that this pathway is not essential for intestinal repair following pathogen-induced damage.

Another study reported the implication of Hippo signaling in cell-autonomous ISC proliferation during intestinal infections (Karpowicz et al., 2010). The Hippo signaling pathway, also known as the Salvador-Warts-Hippo (SWH) pathway is responsible for tissue growth regulation in a large variety of metazoan, including humans (Harvey and Hariharan, 2012). The pathway is initiated by the repression of a core complex of two Serine/Threonine kinases, Hippo (HPO) and Warts (WTS) and two additional partners, Salvador (SAV) and Mob as Tumor Suppressor (MATS), overall called the Hippo kinase cassette. The Hippo kinase cassette constitutively phosphorylates the transcriptional co-activator Yorkie (Yki), resulting in the negative regulation of its activity (Huang et al., 2005). The activation of Hippo pathway therefore first requires the inhibition of Hippo kinase cassette. This inhibition is initiated by many upstream signaling components. One of these is the absence of contact between the Fat (FT) receptor and its Dachshous (DCHS) ligand, both being large trans-membrane proto-cadherins. Devoid of FT - DCHS cell to cell contacts, this upstream signaling pathway inhibits the Hippo kinase cassette (Sharma and McNeill, 2013). When de-repressed, Yki functions as a cell proliferation promoting factor together with Mothers against Dpp (Mad) (Kagey et al., 2012), the transcriptional enhancer Scalloped (Sd) (Wu et al., 2008), Homothorax (Peng et al., 2009) and WW-binding protein 2 (Wbp2) transcription factors (Zhang et al., 2011). Yki-dependent cell cycle promotion is mediated through the transcriptional activation of *Drosophila* inhibitor of Apoptosis 1 (Diap1) and Cyclin E (Huang et al., 2005).

Following intestinal infection by *P. entomophila* or intestinal injury by Dextran Sodium Sulfate (DSS) ingestion, Yki expression levels and its targeted genes are increased in midgut ISCs. This Hippo pathway activation is required for ISC proliferation since a Yki intestine-restricted knocked-down reduced the amount of dividing ISCs following these challenges (Shaw et al., 2010). Finally, Yki-dependent ISC proliferation would additionally require JAK/STAT pathway activation mediated

through a Yki-dependent induction of Upd cytokines acting in an autocrine fashion in ISCs (Karpowicz et al., 2010).

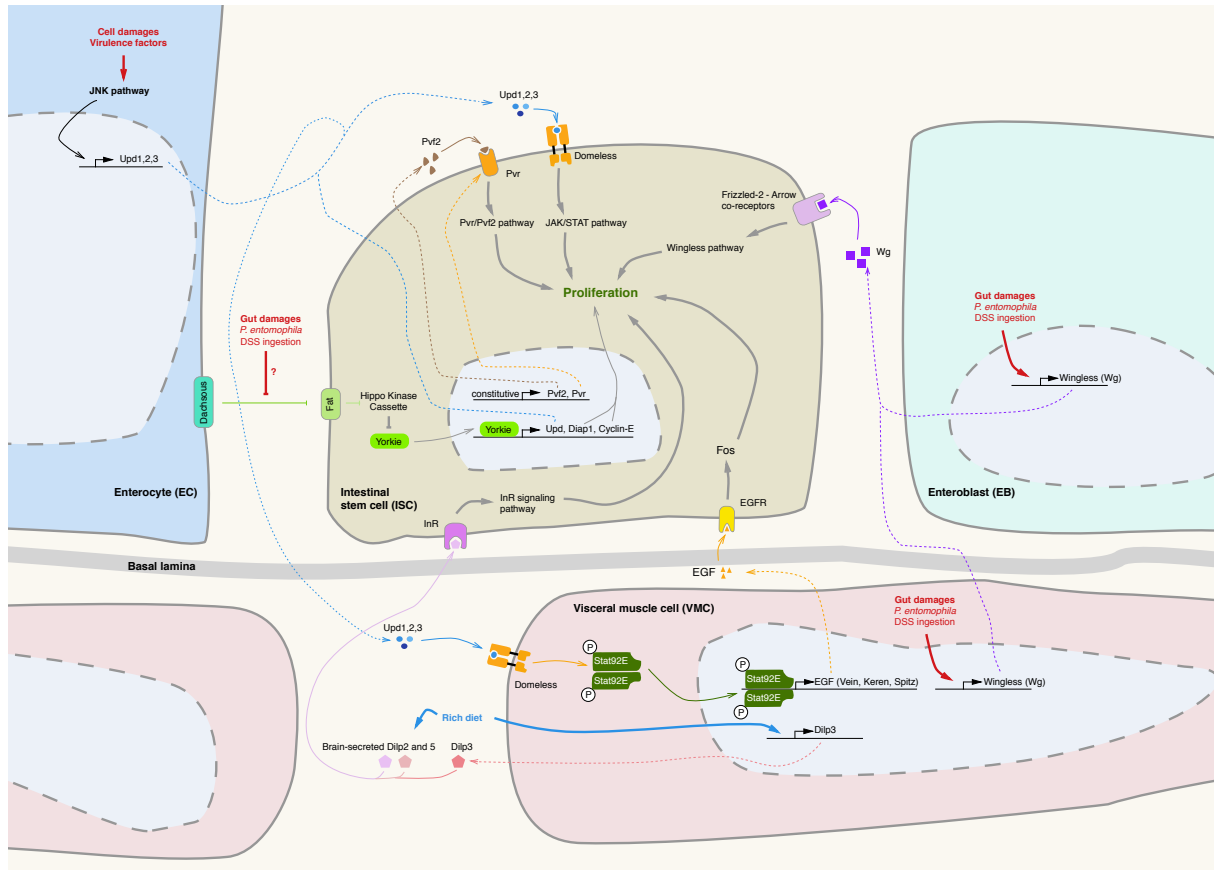
(ii), (iii) Secondly, paracrine or endocrine signals are sent to the ISC by its niche and by the brain to promote its proliferation. These signals include JAK/STAT pathway ligands (Upd1, 2 and 3), Insulin Receptor (InR) pathway ligands (Dilp2, 3 and 5), Epidermal Growth Factor-Receptor (EGFR) pathway ligands (Vein, Keren and Spitz) and Wingless/ $\beta$ -Catenin pathway ligands (Wingless (Wg)).

First, JAK/STAT pathway ligands Upd1, 2 and 3 are produced by enterocytes damaged by ROS or bacterial virulence factors, at least partially in a JNK-pathway dependent-manner and provoke ISC proliferation (Jiang et al., 2009). Alternatively, these ligands may as well be recognized by visceral muscles cells (VMCs). JAK/STAT pathway-activated VMCs would subsequently express and secrete the EGFR ligand Vein to sustain ISC proliferation (Biteau and Jasper, 2011; Buchon et al., 2010; Xu et al., 2011). Two other EGF ligands, Keren and Spitz would also be induced by VMCs and be required for ISC proliferation following pathogenic intestinal infections by *P. entomophila* (Buchon et al., 2009b, 2010). This activated EGFR pathway in ISC would lead to their increased proliferation in a Fos- (AP-1 transcription factor family) dependent manner (Biteau and Jasper, 2011).

Additionally, the insulin signaling pathway has also been shown to affect ISC proliferation in both a paracrine and endocrine fashion but so far, in response to metabolic triggers but not infectious conditions (O'Brien et al., 2011; Shim et al., 2013). During this response, VMCs secrete *Drosophila* Insulin-like Peptides (Dilp)-3 and brain cells secrete Dilp-2 and -5 in response to a rich diet, which results in Insulin Receptor-dependent ISC proliferation. Following Dilp (-2, -3 or -5) binding, the Insulin receptor phosphorylates its substrate, Chico (Böhni et al., 1999). In turn, phosphorylated Chico activates a phosphorylation cascade including the Phosphatidylinositol 3 Kinase (PI3K) complex (Leevers et al., 1996), Phosphatase and tensin homolog (PTEN) (Goberdhan et al., 1999), Phosphatidylinositol-dependent kinase 1 (Pdk1) (Rintelen et al., 2001) and the Akt kinase (Verdu et al., 1999). Once activated, Akt promotes cell proliferation, by inhibiting either FOXO, a transcription factor involved in the repression of growth (Puig et al., 2003), either the

Tuberous sclerosis 1 (TSC1) / TSC2 complex (Schleich and Teleman, 2009), further engaging the Target Of Rapamycin (TOR) pathway.

Finally, a third paracrine signaling molecule, Wingless (Wg), is produced by VMCs and enteroblasts, and activates the Wingless/ $\beta$ -Catenin pathway in ISC to induce their proliferation, notably in response to intestine-damaging infections (Cordero et al., 2012; Lin et al., 2008). In *Drosophila*, Wingless signaling is initiated by the binding of the Wg ligand to its co-receptors, Frizzled 2 (Fz-2) (Tomlinson et al., 1997) and Arrow (Wehrli et al., 2000). Once activated, these receptors initiate a cytoplasmic signaling cascade leading to the Dishevelled (Dsh)-mediated (Noordermeer et al., 1994) inactivation of the protein destruction complex containing Axin (Nakamura et al., 1998), Adenomatosis polyposis coli (APC) (Rubinfeld et al., 1996), protein phosphatase 2A (PP2A) (Bajpai et al., 2004), Glycogen synthase kinase 3 (GSK3) (Rubinfeld et al., 1996) and Casein kinase 1 $\alpha$  (CK1 $\alpha$ ) (Legent et al., 2012) and sequestering  $\beta$ -Catenin (also called Armadillo) on the cytoplasmic membrane. Finally, stabilized  $\beta$ -Catenins translocate to the nucleus to activate their target genes together with T-Cell factor (Tcf) transcription factor (van de Wetering et al., 1997). Wingless production is clearly activated in ISCs and enteroblast upon intestinal damages produced by *P. entomophila* infection or DSS treatment (Cordero et al., 2012). Furthermore, this study showed that Wg signaling in ISC and enteroblast is required for ISCs compensatory renewal following these epithelial damages.



**Figure 7 Signaling pathways governing intestinal stem cells (ISC) proliferation in the *Drosophila* midgut epithelium**

Midgut ISC proliferation is constitutively maintained by the autocrine Pvf2/Pvr pathway. Additionally, ISC proliferation can be induced following bacterial infections or chemical treatments (such as DSS)-induced cell damages by its surrounding environment. Damaged enterocytes (EC) secrete Unpaired (Upd) 1,2 and 3 cytokines that activate JAK/STAT signaling pathway in ISC to promote proliferation, or in visceral muscle cells (VMCs) to promote EGF (Vein, Keren, Spitz) ligands secretion. VMCs-secreted EGFs activate EGFR signaling pathway to promote ISC proliferation in a Fos-dependent manner. Upon intestinal damages, VMCs and Enteroblasts (EBs) are also able to secrete Wingless molecules that are recognized by Frizzled-2 and Arrow co-receptors on ISCs. Frizzled-2 and Arrow then activate the Wingless pathway that ultimately sustains ISC proliferation. Finally, rich diets provoke brain and VMCs-mediated secretion of *Drosophila* insulin-like peptides (Dilps) 2, 3 and 5. Dilps activate the Insulin Receptor (InR) on ISC that promotes ISC proliferation. This scheme was based on a recent review of Dominique Ferrandon (Ferrandon, 2013).

### 3.2 Other local immune responses: trachea and male genital plates

*Drosophila* respiratory organs are composed of numerous tracheal tubes bringing external oxygen to internal tissues. These epithelial structures are able to mount a local immune response to fight microbial threats (Ferrandon, 2013). First, to access to the internal cavity of trachea, microbes must penetrate through elaborate spiracles protecting the entry of tracheal networks (Uv et al., 2003). Inside tracheal cavities, microbes are further physically separated from the tracheal epithelium by a secreted cuticle layer on their apical side. If any micro-organism is able to get through these barriers, an IMD-dependent AMP-based immune response is triggered (Ferrandon et al., 1998; Tzou et al., 2000; Wagner et al., 2009). This IMD-dependent response was shown to involve PGRP-LC and -LE -dependent DAP-type PGN recognition and PGRP-LA positive regulation (Gendrin et al., 2013; Takehana et al., 2004; Wagner et al., 2008). Additionally, a negative control of tracheal IMD pathway activation is performed and involve PGRP-LF and Tollo (previously described in 1.1.4.), both acting at the level of PGRP-LC receptors (Akhouayri et al., 2011b; Basbous et al., 2011). However, the physiological relevance of these immune mechanisms remain to be elucidated as no relevant tracheal pathogenic infection models was described so far (Ferrandon, 2013).

Additionally, male genital plates may also be a potential route for bacterial infections in *Drosophila* (Gendrin et al., 2009). This works showed that the Gram-negative bacteria *Erwinia carotovora* Ecc15 is able to persist in the genital plates after deposition and subsequently provoke both a local and systemic immune response from the fly. The local defense response of genitalia to such infections seems to rely mostly on the induction of Defensin, but not Diptericin AMP following Gram-negative DAP-type PGN recognition. Of note, the CecA1 gene was shown to be constitutively activated in this organ. This AMP response is likely to be triggered by the IMD pathway since Relish mutants die of bacteremia following bacterial deposition on genital plates. In addition to a local response, genital infections by *E. carotovora* also induced a potent systemic IMD-dependent immune response mediated by the infiltration of TCT in the hemolymph. Of note, *Drosophila* female genitalia do not show a local immune response to bacterial challenge, but are immune-stimulated in response to accessory gland sex-peptides (SP) contained in male seminal fluid, which activate both Toll and IMD pathways (Peng et al., 2005).

## 4. Systemic immune responses

Systemic immune responses can be triggered by a direct penetration of pathogens in the hemocoel by pricking the thorax of flies with a sharp needle. This procedure is a simple and efficient way of reproducibly infecting flies and has been widely used to understand the main molecular pathways of *Drosophila* immune system. Systemic immune responses are based on two components: cellular and humoral immune responses. These responses mostly rely on two immune tissues, the hemocytes and the fat-body, respectively. So far, systemic immune responses were shown to be mandatory for the defense of flies against Gram-negative bacterial, Gram-positive bacterial, viral and fungal infections and parasitoid wasp infestations (Braun et al., 1998; Carton and Boulétreau, 1985; Kemp et al., 2013; Lemaitre et al., 1995a, 1996).

### 4.1 Cellular immune responses

The ingress of pathogens inside *Drosophila* internal cavity immediately triggers a cellular response. *Drosophila* mounts a variety of cellular responses to invading bacteria and other parasites, all of which rely on mesoderm-derived hemocytes. Hemocytes may circulate in the hemolymph or be associated with internal tissues (sessile). Three distinct populations of hemocytes have been identified: plasmatocytes, crystal cells and lamellocytes (Meister, 2004a). All three hemocyte populations are present in larvae, while embryos lack lamellocytes, and adults have only plasmatocytes. The production and differentiation of *Drosophila* hemocytes are described below (4.1.1.) together with the four cellular immune processes they mediate: phagocytosis (4.1.2.), encapsulation (4.1.3.), melanisation (4.1.4.) and coagulation (4.1.5.).

#### 4.1.1 *Drosophila* hematopoiesis

Two waves of hemocytes release occur during development: the first during embryogenesis and a second during late larval stages (Holz et al., 2003).

The first embryonic hemocytes are generated from the procephalic mesoderm (Tepass et al., 1994). Once differentiated, this plasmatocyte lineage migrates throughout the whole embryo and plays major functions in phagocytosing apoptotic cells (Franc et al., 1999). A second population of hemocytes gives rise to crystal cells, which differentiate and remain localized close to the anterior section of the gut (Lebestky et al., 2000). The second wave of hematopoiesis occurs at the end of embryogenesis, with the formation of lymph gland precursors in the lateral mesoderm and their subsequent migration to dorsal embryonic regions (Rugendorff et al., 1994).

At late larval stages, lymph glands consist of two to seven paired lobes distributed along the dorsal vessel (Evans et al., 2003). These lobes are the center of the second wave of hematopoiesis (Lanot et al., 2001). Posterior lobes are mostly composed of undifferentiated cells while anterior lobes contain differentiated hemocytes (Lanot et al., 2001). Larval lymph glands are able to produce the three hemocytes lineages. Under normal physiological conditions, plasmatocytes represent the predominant population of circulating hemocytes (95%) while crystal cells only represent 5% and lamellocytes are almost absent (Lanot et al., 2001).

Plasmatocytes are 10  $\mu\text{m}$  spherical phagocytic cells also playing important roles in encapsulation and coagulation (Lanot et al., 2001). Besides their role in the cellular immune responses, plasmatocytes are also involved in the humoral response in the secretion of extracellular matrix proteins and AMPs following microbial infections (Dimarcq et al., 1997; Fessler et al., 1994). Additionally, plasmatocytes secrete signals which inform distant tissues of an infection (Agaisse et al., 2003; Brennan et al., 2007; Irving et al., 2005). The crystal cells contain crystalline inclusions composed of prophenoloxdase (PPO), a zymogen that is released and required during the melanisation and encapsulation processes (RIZKI and RIZKI, 1959). The lamellocytes are large flat cells, which encapsulate pathogens that are too large to be phagocytosed by plasmatocytes (Carton and Boulétreau, 1985). Although almost absent in the absence of immune challenge, lamellocytes differentiate in massive amounts from the lymph gland after parasitization by hymenopteran wasp larvae (Lanot et al., 2001).



All differentiated hemocytes arise from a common pro-hemocyte precursor, whose identity is defined and maintained by the GATA transcription factor *Serpent* (Tokusumi et al., 2010). The proliferation state of pro-hemocytes is controlled by the Platelet-derived Growth Factor (PDGF)-Vascular Endothelial Growth Factor (VEGF) Receptor-related (*Pvr*) pathway (Munier et al., 2002), the ribosomal protein S6 Kinase (*Ras*)/Extracellular signal-regulated kinase (ERK) pathway (Asha et al., 2003), the JAK/STAT pathway (Luo et al., 1995) and possibly also the Toll pathway (Qiu et al., 1998). Commitment of pro-hemocytes towards plasmatocytes requires the activity of Glial cell missing (*Gcm*) 1 and *Gcm*2 transcription factors (Lebestky et al., 2000) while crystal cells differentiation is achieved through the activation of the Notch pathway, and subsequent transcriptional induction of *Lozenge*, a member of the *Runx* family transcription factors (Duvic et al., 2002; Lebestky et al., 2000). Finally, the lamellocytes differentiation process would require the activation of JAK/STAT and Dorsal-dependent Toll pathways (Sorrentino et al., 2004).

During metamorphosis from larval to pupal stages, a 20-hydroxyecdysone pulse provokes a strong increase of lymph glands activity. At this stage, lymph glands produce numerous phagocytic pupal macrophages (Lanot et al., 2001). These cells are essential components of the tissue remodeling occurring during metamorphosis. Additionally, this event also provokes the irreversible dispersal of the lymph gland. At the end, the release of pupal macrophages coincides with dissolution of the lymph gland. The only hemocytes that remain in adult flies are plasmatocytes of both larval and embryonic origin. (Holz et al., 2003).

#### **4.1.2 Phagocytosis**

*Drosophila* plasmatocytes are able to recognize and engulf invading microbes, small particles and apoptotic cells. Phagocytosis relies on cell-surface receptors that mediate recognition and engulfment. So far, three such receptors have been shown to mediate the phagocytosis of bacteria: *Eater* (Chung and Kocks, 2011; Kocks et al., 2005), *Nimrod C1* (*NimC1*) (Estévez-Lao and Hillyer, 2014; Kurucz et al., 2007), *Draper* (Fujita et al., 2012; Manaka et al., 2004), *Drosophila* Scavenger receptor *Cl* (*dSR-Cl*) (Rämet et al., 2001), *PGRP-SC1a* (Garver et al., 2006) and *PGRP-LC*

(Rämet et al., 2002). Eater, NimC1 and Draper receptors are characterized by the presence of Epidermal Growth Factor (EGF) repeats called NIM repeats located immediately after a CCXG(Y/W) amino acid motif (Somogyi et al., 2008). The molecular function of such motifs is probably linked with recognition of an unidentified MAMP.

Eater, NimC1 and dSR-CI are required for plasmatocyte response to Gram-positive (*Staphylococcus aureus*) and Gram-negative bacterial infections (*Escherichia coli*, *Serratia marcescens* and *Pseudomonas aeruginosa* in the case of Eater, *Escherichia coli* only in the case of NimC1 and dSR-CI) (Chung and Kocks, 2011; Kocks et al., 2005; Kurucz et al., 2007; Rämet et al., 2001). Draper on the other hand, initiates phagocytosis of *S. aureus* by recognizing lipoteichoic acid (Hashimoto et al., 2009). Finally, PGRP-SC1a and PGRP-LC were shown to be involved specifically in the phagocytic response against Gram-positive and Gram-negative bacteria, respectively (Garver et al., 2006; Rämet et al., 2002).

Thioester-containing proteins (TEP) may also play a role in phagocytosing pathogens. These TEP proteins are close homologs of vertebrate complement factors C3/C4/C5 and the  $\alpha$ 2-macroglobulin family of protease inhibitors. *Drosophila* has six TEP genes (TEP1 to 6). Three of these TEP proteins (TEP1, TEP2 and TEP4) are up-regulated following immune challenge with a mixture of Gram-negative and Gram-positive bacteria (Bou Aoun et al., 2011). Although no immune function has been assigned for the TEPs in *Drosophila*, in *Anopheles* mosquitoes this family of proteins has a major role in the control of the *Plasmodium falciparum* parasite (Blandin et al., 2004, 2009).

Plasmatocytes can also engulf apoptotic cells via scavenger receptors. The characterized receptors include Draper (Manaka et al., 2004) and Croquemort (Franc et al., 1999). Following particle, bacteria or apoptotic corpses binding to their cognate receptors, the cellular membrane of phagocytic cells invaginates in a vesicle that excises from the cytoplasmic membrane called phagosome (Kinchen and Ravichandran, 2008). This phagosome then moves and matures within the cell through several stages, leading to its progressive acidification and fusion to lysosomes to complete the degradation of its content. The phagocytic activity of

*Drosophila* hemocytes can be blocked by injecting a suspension of 0,3µm latex beads in the thorax of adult flies (Haller et al., 2014).

### 4.1.3 Encapsulation

Encapsulation is a cellular response that has only been observed in invertebrates (Meister, 2004a). In *Drosophila*, encapsulation is achieved by the concerted action of the three hemocytes lineages: plasmatocytes, lamellocytes and crystal cells. This cellular response is triggered following parasitization by hymenopteran wasps (Carton and Boulétreau, 1985). The female wasp injects its eggs directly into the hemocoel of *Drosophila* larvae. Unless blocked by the host immune response, the eggs will hatch into larvae, which feed on host tissues. This process ultimately kills the fly.

To block parasitic larval growth, the wasp egg is first recognized by *Drosophila* plasmatocytes that attach to the egg chorion (Russo et al., 1996). A few hours later, massive differentiation of hemocytes is triggered in the lymph gland, resulting in the release of lamellocytes and crystal cells (Lanot et al., 2001). Lamellocytes form a multilayered capsule around the egg. This capsule is then melanized through the release of PPO by crystal cells. The parasite egg is eventually killed inside of the capsule either by asphyxiation or by the local release of cytotoxic superoxide anions (Nappi et al., 1995) and nitric oxides (Nappi et al., 2000).

### 4.1.4 Melanisation

Melanisation is a cellular immune response shared by many invertebrates that is characterized by the deposition of black-pigmented melanin on foreign bodies (Söderhäll and Cerenius, 1998). Melanisation is required for wound healing, encapsulation, and more generally in the defense against microorganisms. Besides the physical properties of melanin to encapsulate or clot a wound, the melanisation reaction also releases intermediate cytotoxic metabolites that can act on microbes (Meister, 2004b).

The melanisation response is initiated by a complex upstream signaling pathway leading to the activation of the Pro-phenoloxidase enzyme (PPO), the main enzyme responsible for the formation of melanin. First, the recognition of a wound-derived or an infectious component is thought to be mediated by two class of sensing molecules: the pattern-recognition receptors PGRP-LC, PGRP-LE and GGBP3 and the Serine proteases Hyan, Melanization Protease 1 (MP1) and MP2 (Castillejo-López and Häcker, 2005; Leclerc et al., 2006; Nam et al., 2012; Tang et al., 2006). As expected, PGRP-LC and –LE were shown to mediate melanisation in response to Gram-negative bacteria while GGBP3-dependent melanisation was observed during fungal infections (Matskevich et al., 2010; Schmidt et al., 2008; Takehana et al., 2004). These upstream components activate the Pro-phenoloxydase activating enzyme (pPA), which subsequently activates the Pro-phenoloxidase enzyme (PPO). Of note, two Serine protease inhibitors (Serpins), Spn27A (De Gregorio et al., 2002b) and Spn28Dc (Scherfer et al., 2008) have been shown to negatively regulate the PPO activation pathway after an infection (Spn27A) or wounding (Spn28D).

Three PPO are encoded by *Drosophila melanogaster* (PPO-1, -2 and -3), and produced by crystal cells (PPO-1 and -2) (Rizki et al., 1985) and lamellocytes (PPO-3) (Irving et al., 2005). In larvae, PPO is released following the rupture of crystal cells upon injury or infection. Interestingly, crystal cell rupture is dependent of JNK pathway activation (Bidla et al., 2007). However, melanisation can still occur in adults, despite their lack of crystal cells.

Once activated, Phenoloxydases (PO) are able to catalyze the oxidation of Tyrosine residues into 3,4-dihydroxyphenylalanine (DOPA) first, and to convert DOPA into dopaquinone (Nappi and Seymour, 1991). Alternatively, DOPA can be converted into Dopamine by the DOPA decarboxylase enzyme (DDC) (Hirsh and Davidson, 1981). Dopaquinone is then converted into Dopachrome (5,6-dihydroxyindole) by the Dopachrome conversion enzyme (DCE) (Li and Nappi, 1991). Dopachrome and Dopamine are ultimately converted into melanin polymers after several enzymatic reactions involving PO (Söderhäll and Cerenius, 1998).

#### **4.1.5 Coagulation**

Coagulation is a process initiated by larval plasmatocytes and crystal cells to limit the loss of hemolymph after an injury. This cellular response occurs in two phases. First, at a very early stage, a soft clot made of transglutaminase-crosslinked aggregated proteins is formed at the site of the wound (Theopold et al., 2004). The establishment of this clot was shown to require soluble coagulogens secreted by the fat-body (among which Fondue (Scherfer et al., 2006), lipophorin, larval serum proteins and fat-body protein 1 (Karlsson et al., 2004)) and the plasmatocyte-secreted coagulogen Hemolectin (Lesch et al., 2007). In a second phase, crystal-cell released PPO provokes a melanisation reaction at the site of the wound, further crosslinking and hardening the initial clot (Galko and Krasnow, 2004).

## 4.2 Humoral immune response

The humoral response consists of soluble immune effectors secreted by internal tissues to fight a potential threat. In *Drosophila*, the humoral response is essential to fight almost all class of pathogens including Gram-negative bacteria, Gram-positive bacteria, fungi and viruses. As far as we know, *Drosophila* humoral response mostly relies on the systemic secretion of anti-microbial peptides (AMPs) (previously described in 1.1.3.) by the fat body and to a lower extent by hemocytes. Upon infection, these AMPs can reach a range of concentration from 0,5  $\mu\text{M}$  (Diptericin) to 100  $\mu\text{M}$  (Drosomycin) in the hemolymph and may persist several weeks after the initial challenge (Imler and Bulet, 2005; Uttenweiler-Joseph et al., 1998). Systemic production of AMPs in response to infection is almost entirely dependent of the activation of the NF- $\kappa\text{B}$  Toll and IMD pathway (previously described in 1.).

One exception is the production of Listericin in response to intracellular *Listeria* infections, which requires both IMD and JAK/STAT pathway molecular components (See above, 2.2.1.). Additionally, the group of Michael Hoch characterized an evolutionarily conserved NF- $\kappa\text{B}$ -independent pathway inducing the transcription of AMPs upon starvation relying on the transcription factor FOXO (Becker et al., 2010). This pathway would require the starvation-induced repression of the cytohesin Steppkle, a previously well-characterized component of Insulin

signaling in *Drosophila*. Starvation-induced reduction of Insulin signaling provokes the derepression of FOXO transcription factors, which then enters in the nucleus. The study revealed that nuclear FOXO induced the expression of *Attacin-A*, *Drosomycin*, *Diptericin-A*, *Drosocin* and *Metchnikowin* but not *Cecropin-A1* or *Cecropin-C* AMPs. This specificity may be partially explained by the relative presence of FOXO/Forkhead consensus binding sites (TTGTTTAC) (Furuyama et al., 2000) on AMPs promoter. Finally, the biological relevance of this FOXO-dependent AMP production was shown in a *Mycobacterium marinorum* larval infection assay, in which FOXO mutants are susceptible (Clark et al., 2013; Dionne et al., 2006). Overall, these study suggest that metabolic changes provoked by pathogens are able to trigger a potent humoral immune response independently of microbes' recognition. This metabolism to immunity switch represents an additional arm of *Drosophila* immunity sensing machinery.

## 5. Intrinsic immune response against viruses

In the wild, *Drosophila melanogaster* can be naturally infected by a large number of viruses (*Drosophila* A, C, F, P and X viruses (DAV, DCV, DFV, DPV, DXV), Sigma virus (SIGMAV), *Gypsy* retrovirus and Nora virus) (Bucheton, 1995; Dobos et al., 1979; Fleuriet, 1981; Habayeb et al., 2006; Jousset et al., 1972; Plus et al., 1975, 1976) and therefore has developed an efficient anti-viral immunity. These past ten years, *Drosophila* was used as a model of choice to characterize anti-viral genes. Unlike in mammals, in which the anti-viral immunity is profoundly dependent of the systemic release of Interferon cytokines, the most potent known *Drosophila* antiviral defense takes place intrinsically in individual cells, by fighting the molecular steps of virus replication. This intrinsic defense, required against all types of viral infections in flies, relies mostly on the short-interfering RNA (siRNA) pathway (Kemp et al., 2013).

In insect cells, the siRNA pathway is triggered by the recognition of cytosolic dsRNAs. dsRNA is not common cellular component under normal physiological conditions. Elevated levels of dsRNA may arise from viral genome of dsRNA viruses (e.g., DXV), viral replication intermediates of single-stranded RNA (ssRNA) viruses of

positive (e.g., DCV) or negative polarity (e.g., SIGMAV) or transcripts from endogenous transposons. The recent observation that DNA viruses also trigger a potent siRNA response suggests that secondary structures of ssRNA may also serve as a recognition motif (Bronkhorst et al., 2012; Kemp et al., 2013). Viral dsRNAs are sensed and cleaved by the Ribonuclease (RNAse) III enzyme Dicer-2 (Dcr-2) into 21-nt long siRNA duplexes bearing 5' monophosphates and 2nt 3' hydroxyl overhangs (Okamura and Lai, 2008; van Rij and Berezikov, 2009). These viral siRNA (vsiRNA) are further loaded onto Argonaute-2 (Ago-2) proteins within the RNA induced silencing complex (RISC) with the help of the dsRNA-binding protein R2D2 (Marques et al., 2013).

Once loaded into the RISC complex, one strand (the passenger strand) is eliminated by the concerted action of Ago2 and the endonuclease complex Component 3 promoter of RISC (C3PO). The other strand (the guide strand) is maintained and O-methylated at its 3' end by the RNA methyltransferase DmHen1. This last event terminates the maturation of the RISC complex in which Ago-2 mediates a sequence-specific cleavage of viral RNAs, preventing formation of new viruses. This defense system was shown to be crucial for flies' survival and virus load control in several systemic viral infections models such as the single-stranded RNA viruses DCV, Cricket paralysis virus (CrPV), Vesicular stomatitis virus (VSV), Sindbis virus (SINV), Flock-house virus (FHV) and the DNA virus Invertebrate iridescent virus 6 (IIV6) infections (Bronkhorst et al., 2012; Kemp et al., 2013).

## 6. Objectives and aims of the PhD work

The general aim of my PhD was to study the molecular network underlying the activation of NF- $\kappa$ B using *Drosophila melanogaster* as a model in two biological contexts: the local immune response of the intestine and the systemic immune system. Similarly to mammals, *Drosophila* needs to fine-tune the levels of immune response activation. In *Drosophila* intestine for example, the local immune response relies on two molecular pathways: the synthesis of AMPs by the NF- $\kappa$ B IMD pathway and the synthesis of ROS by the Duox pathway. When misregulated, these defense mechanisms are detrimental to the flies. Regulatory mechanisms underlying the

activation or the repression of the immune system are therefore crucial to maintain the fitness and the homeostasis of *Drosophila*.

Prior to my arrival in the lab, the team of Prof. Jean-Marc Reichhart and Dr. Nicolas Matt identified big-bang, a gene involved in the intestinal defense against Gram-negative bacterial oral infections. This gene had been previously studied by the team of Prof. Gabrielle Boulianne in the embryonic and larval development of *Drosophila* and was found to be expressed in the gut (Kim et al., 2006b). Interestingly, mutants for this gene also showed a decreased lifespan and an abnormal activation of the NF- $\kappa$ B IMD pathway in the midgut. Based on these initial observations, I joined a collaborative work together with a former PhD student, Eva Berros-Cohen aiming at better understanding the molecular function of big-bang in the regulation of gut immune responses (see below, **Chapter 2**), which led to a recent publication (Bonney et al., 2013).

In the second part of my PhD, I focused my work on the molecular characterization of another gene, akirin, in the systemic activation of NF- $\kappa$ B IMD pathway in *Drosophila*. Akirins were identified for the first time in 2008 in a collaborative study between our laboratory and the team of Prof. Shizuo Akira. They were described as nuclear factors required for the activation of NF- $\kappa$ B pathways in insects and mammals (Goto et al., 2008). Interestingly, the mammalian homolog of Akirin, Akirin-2 had been shown to regulate only a subset of NF- $\kappa$ B target genes, suggesting that Akirins manipulated NF- $\kappa$ B responses in a selective way. This work physically and genetically localized Akirins' function in the nuclear compartment but did not explore their mode of action. Indeed, understanding the molecular basis of NF- $\kappa$ B selectivity is a key focus in the field. Therefore, we used Akirin to re-explore the NF- $\kappa$ B pathway aiming at understanding the mechanisms underlying the NF- $\kappa$ B transcriptional selectivity. In order to better understand the molecular function of Akirins, my work, together with a post-doctoral researcher (Dr. Xuan-Hung Nguyen) aimed first, to explore the subsets of NF- $\kappa$ B target genes *Drosophila* that Akirin is influencing and second, to identify and characterize Akirin's molecular partners (see below, **Chapter 3**). This work led to recent back-to-back publications, together with a manuscript from Prof. Osamu Takeuchi's team, which deciphered the role of Akirins during NF- $\kappa$ B-dependent transcription in *Drosophila* and in mice (Bonney et al., 2014; Tartey et al., 2014).



## VII. Chapter 2: big-bang gene modulates gut immune response

### 1. Scientific context of the study

#### 1.1 The big-bang gene

Big-bang (bbg) was first characterized by the team of Gabrielle Boulianne in a study of *Drosophila* development (Kim et al., 2006b). bbg was initially discovered using a Gal4 enhancer trap expression pattern screen for developmental genes with a UAS-LacZ read-out and referred to as C94-Gal4 (Gustafson and Boulianne, 1996). Among other trapped sequences, C94-Gal4 showed a particularly specific pattern of expression in a broad stripe overlapping the presumptive wing margin in the late third instar larvae wing imaginal disc, suggesting the isolation of a novel gene (Gustafson and Boulianne, 1996). This result prompted the team to further investigate the expression pattern of this sequence in other tissues at other developmental stages.

They could further demonstrate, using the same C94-Gal4 / UAS-LacZ reporter line, that the gene expression pattern of this construct was expressed in other imaginal discs (halteres, eye-antennal but not leg discs), the developing peripheral and central nervous systems, sensory organs at various locations (abdominal segments, posterior spiracles and telson (terminal tail structure of *Drosophila* larvae), muscles cells of the developing pharynx, salivary glands (as many other genes) and the ventral nerve cord (Kim et al., 2006b). Following *Drosophila* genome release, C94-Gal4 trapped DNA sequence was analyzed and matched to the 3' end of the gene CG9598, further referred to as big-bang (bbg). The name big-bang was attributed to this gene based on the fact that this gene is large (90 kb) and bbg-deficient flies appeared to be mildly bang-sensitive (sensitivity to loud and sudden noises). This latter phenotype is likely due to a nervous system defect (Kim et al., 2006b).

Bbg encodes five distinct isoforms through alternative splicing (referred to as Bbg-RC,K (288 kDa), -RE (119 kDa), -RF,G,H (112 kDa), -RI (118 kDa) and -RJ

(201 kDa)). All these isoforms share their C-terminal domain that contains two PSD-95, Discs-Large, ZO-1 (PDZ) domains, which are protein-protein interaction domains found in cytoplasmic membrane-associated proteins (Brône and Eggermont, 2005). In situ hybridization and immuno-staining targeting all BBG isoforms revealed additional expression patterns that were not visualized in the C94-Gal4 reporter, particularly during gut development. The different isoforms were shown to be sequentially expressed at different stages of embryonic development and at different locations in the developing gut (Kim et al., 2006b).

The short Bbg-RI isoform (118 kDa) was the first expressed isoform and localizes in the midgut starting from embryonic stage 5. Bbg-RF,G,H isoform (112 kDa) was further expressed between stage 7 and 9 in the posterior midgut and the hindgut. Both of these isoforms were expressed in the anterior midgut and the foregut at stage 13 and further localized also in the posterior midgut and hindgut until late embryogenesis. Bbg-RC,K (288 kDa) and –RJ (201 kDa) started to be expressed only starting from stage 13 in the foregut and hindgut but not in the midgut. Finally, the expression of all Bbg isoforms decreased and disappeared at very late stages of embryogenesis. However, this study did not investigate post-larval tissues bbg expression.

A mutant for all Bbg isoforms with a deletion in the third exon (*bbg*<sup>B211</sup>) was generated by the team of Prof. Boulianne (Kim et al., 2006b) and sent to our laboratory. Intriguingly, this mutant displayed a reduced adult lifespan compared to wild-type flies and was difficult to maintain in our livestock, suggesting that this gene may have an immune function. Based on these observations, preliminary work prior to my arrival showed that *bbg*<sup>B211</sup> adults were susceptible to *Serratia marcescens* Db11 oral infections. These results prompted us to decipher the function of bbg in the gut immune response, with a particular emphasis on the midgut, which is the region where the most described immune responses occur.

## 1.2 Midgut immune responses and homeostasis

The midgut is a crucial organ for *Drosophila* physiology. It is the location of most of the digestive functions thanks to its permeability, its absorptive properties and the expression of digestive enzymes. The midgut is composed of a monolayer of

epithelial cells protected on their apical side by the peritrophic matrix, a semi-permeable barrier allowing monomeric proteins to penetrate into the lumen. Unlike the foregut and the hindgut, which are protected by an impermeable cuticle layer, the midgut is permeable to small molecules and more exposed to pathogens and immunogenic stimuli. Consequently, the midgut must fine-tune the activation and the repression of its immune system. On one hand, defense mechanisms (AMPs, ROS, stem cells proliferation) must be activated in the presence of entomopathogens (*P. entomophila*, *S. marcescens*) to protect the integrity of the whole body. On the other hand immune repressors are crucial to promote the survival of beneficial endogenous bacteria (*A. pomorum*, *L. plantarum*) and to reduce unnecessary metabolic expenditure.

Young adult flies display a moderated IMD pathway-based AMPs immune response restricted to the very anterior (RO-R1) and posterior part (R4-R5) of the midgut, under normal culture conditions (Buchon et al., 2013b). Given that microorganisms can be found all along the midgut, this low level activation of the NF- $\kappa$ B IMD pathway is regulated by the joint action of several repressors, including the amidase PGRPs (PGRP-LB, -SC1 and -SC2), Pirk and Caudal (previously described in 1.1.4.). Perturbing one of those negative regulators drastically increases the IMD pathway activation in response to endogenous bacteria in the midgut and severely reduces the longevity of flies.

Interestingly, as *Drosophila* ages under normal culture conditions, the gut microbial content and the strength of local innate immune responses in the midgut also increases (Buchon et al., 2013b). These immune responses include a stronger IMD pathway-dependent production of AMPs, sustained ROS secretions and increased self-renewal divisions of intestinal stem cells (ISCs) (Biteau et al., 2008; Choi et al., 2008). Increased viability is associated with moderate rates of ISC divisions (Biteau et al., 2010), further pointing out the need of an equilibrated immune reaction in the midgut. Fly age-related gut pathologies (sustained inflammatory responses, abnormal cell division rates) are reminiscent of mammalian intestinal chronic inflammatory diseases and colorectal cancers (Garrett et al., 2010). Functional assays have been developed to study such pathologies in flies by the ingestion of pathogenic bacteria (*P. entomophila*, *S. marcescens*), detrimental commensals (*E. carotovora*) or chemical compounds (DSS, Bleomycin) (Amcheslavsky et al., 2009; Buchon et al., 2009b). These models have elucidated

the complex regulatory network controlling ISC division and differentiation (See above, **Chapter 1, 3.1.2.**).

Based on the initial observations that *big-bang* deficient flies were susceptible to oral infections, we investigated the function of big-bang gene as a potential regulator of immune responses in the adult midgut. Our work first determined that BBG was expressed all along the midgut epithelium, starting from the proventriculus to the posterior midgut regions, further strengthening the hypothesis of a midgut-related immune function of BBG. Interestingly, *bbg* deficient flies (*bbg*<sup>B211</sup>) constitutively over-activated the IMD pathway in the anterior part of the midgut. We could also demonstrate that *bbg*<sup>B211</sup> flies died prematurely in the presence of the endogenous gut flora. This pathology was correlated with an increased number of ISCs divisions, further pointing out a role of BBG in the immune tolerance of the midgut. Co-immuno-localisation with the septate junctions (SJ)-associated protein Coracle and transmission electron microscopy analyses showed that Bbg was an essential component of SJs, a functional equivalent to mammalian tight junctions. Finally, we showed that both Bbg and Coracle midgut-epithelium-restricted deficiency led to faster lethality and increased permeability towards invasive *Pseudomonas aeruginosa* PA14 infections. Collectively, our results showed that BBG is an important component of *Drosophila* midgut SJs and highlighted the role of SJs in the midgut epithelium for immune tolerance and immune defense against pathogens.

## 2. Manuscript

# big bang gene modulates gut immune tolerance in *Drosophila*

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**Chronic inflammation of the intestine is detrimental to mammals. Similarly, constant activation of the immune response in the gut by the endogenous flora is suspected to be harmful to *Drosophila*. Therefore, the innate immune response in the gut of *Drosophila melanogaster* is tightly balanced to simultaneously prevent infections by pathogenic microorganisms and tolerate the endogenous flora. Here we describe the role of the *big bang* (*bbg*) gene, encoding multiple membrane-associated PDZ (PSD-95, Discs-large, ZO-1) domain-containing protein isoforms, in the modulation of the gut immune response. We show that in the adult *Drosophila* midgut, BBG is present at the level of the septate junctions, on the apical side of the enterocytes. In the absence of BBG, these junctions become loose, enabling the intestinal flora to trigger a constitutive activation of the anterior midgut immune response. This chronic epithelial inflammation leads to a reduced lifespan of *bbg* mutant flies. Clearing the commensal flora by antibiotics prevents the abnormal activation of the gut immune response and restores a normal lifespan. We now provide genetic evidence that *Drosophila* septate junctions are part of the gut immune barrier, a function that is evolutionarily conserved in mammals. Collectively, our data suggest that septate junctions are required to maintain the subtle balance between immune tolerance and immune response in the *Drosophila* gut, which represents a powerful model to study inflammatory bowel diseases.**

epithelial immunity | gut homeostasis

In contact with many types of microorganisms, metazoans have developed strategies to defend themselves against pathogenic threats, but have also taken advantage of these microorganisms to achieve complex biological processes such as digestion, implying a selective immune tolerance to the intestinal flora (1).

In the wild, the fruit fly *Drosophila melanogaster* thrives on rotten fruits, a diet that is rich in various microbes, including entomopathogenic bacteria, and that requires a powerful epithelial immune response to prevent oral infections (2, 3). In the gut of *Drosophila*, the immune response mainly relies on the local production of microbicidal reactive oxygen species (ROS) (4) and release of antimicrobial peptides (AMPs) (for review see ref. 5). ROS synthesis is proposed to be triggered by yet to be identified pathogen-associated molecular patterns (PAMPs) (6). In contrast, the secretion of AMPs depends on the direct recognition of microbial meso-diaminopymelic acid type peptidoglycan (DAP-type PGN) of Gram-negative [Gram (–) bacteria] (reviewed in ref. 5). This is sensed by the transmembrane peptidoglycan recognition protein LC (PGRP-LC) receptors, which trigger the immune deficiency (IMD) pathway that culminates in the nuclear translocation of the NF- $\kappa$ B transcription factor Relish. In the nucleus, Relish activates the transcription of its target genes, including the AMP coding genes, that will participate in the clearance of bacteria (reviewed in ref. 7).

Aside from being activated by invasive bacteria, the IMD pathway is also triggered by endogenous commensal bacteria naturally present in the gut lumen. Constant activation of the *Drosophila* gut

innate immune response is likely to be detrimental to the flies as in the case of chronic inflammation in mammals. Accordingly, the innate immune response is tightly regulated in the *Drosophila* gut to maintain gut homeostasis, simultaneously preventing infections by pathogenic microorganisms and allowing tolerance to the endogenous flora. Recent studies have demonstrated that the IMD pathway can be modulated in the gut either by (i) a PGRP-LC interacting inhibitor of Imd signaling (PIMS) (8–10), (ii) peptidoglycan amidases responsible for PAMP degradation [peptidoglycan recognition protein LB (PGRP-LB) and PGRP-SC1/2] (11–13), and (iii) the transcriptional regulator Caudal that specifically represses transcription of Relish-dependent AMP coding genes (14). Collectively, these mechanisms account for both the tolerance toward the endogenous flora and for the resolution of the immune response.

In *Drosophila*, the gut immune barrier is based on the peritrophic matrix, a chitinous multilayered structure that isolates the bolus from the gut epithelial cells (15), and on the inducible local response described above. When bacteria escape this barrier and enter the body cavity, flies rely on a powerful systemic immune response involving the massive release of AMPs by the fat body (an equivalent of the mammalian liver) for their defense. In the fat body, the expression of AMPs is under the control of two pathways, namely the IMD pathway, triggered by Gram (–) bacteria as in the gut, and the Toll pathway, activated in response to fungi and Gram-positive bacteria [Gram (+) bacteria] (reviewed in ref. 5).

To identify genes specifically involved in the molecular mechanism underlying the gut immune barrier function, we undertook a pilot genetic screen. This screen identified *big-bang* (*bbg*; CG42230) that encodes multiple-PSD-95, Discs-large, ZO-1 (PDZ) domain-containing protein isoforms associated with the membrane and expressed in various epithelia during larval stages (16). We demonstrate here that *bbg* null mutant flies display a reduced lifespan and a chronic inflammation of the anterior midgut epithelium. Removing the endogenous gut flora by antibiotic treatment rescues both phenotypes. We further establish that BBG is localized in the gut epithelial septate junctions and that these junctions are disorganized in the absence of BBG, which may account for the sensitivity of *bbg* mutant flies to oral infection. Collectively, our data suggest that BBG and gut septate

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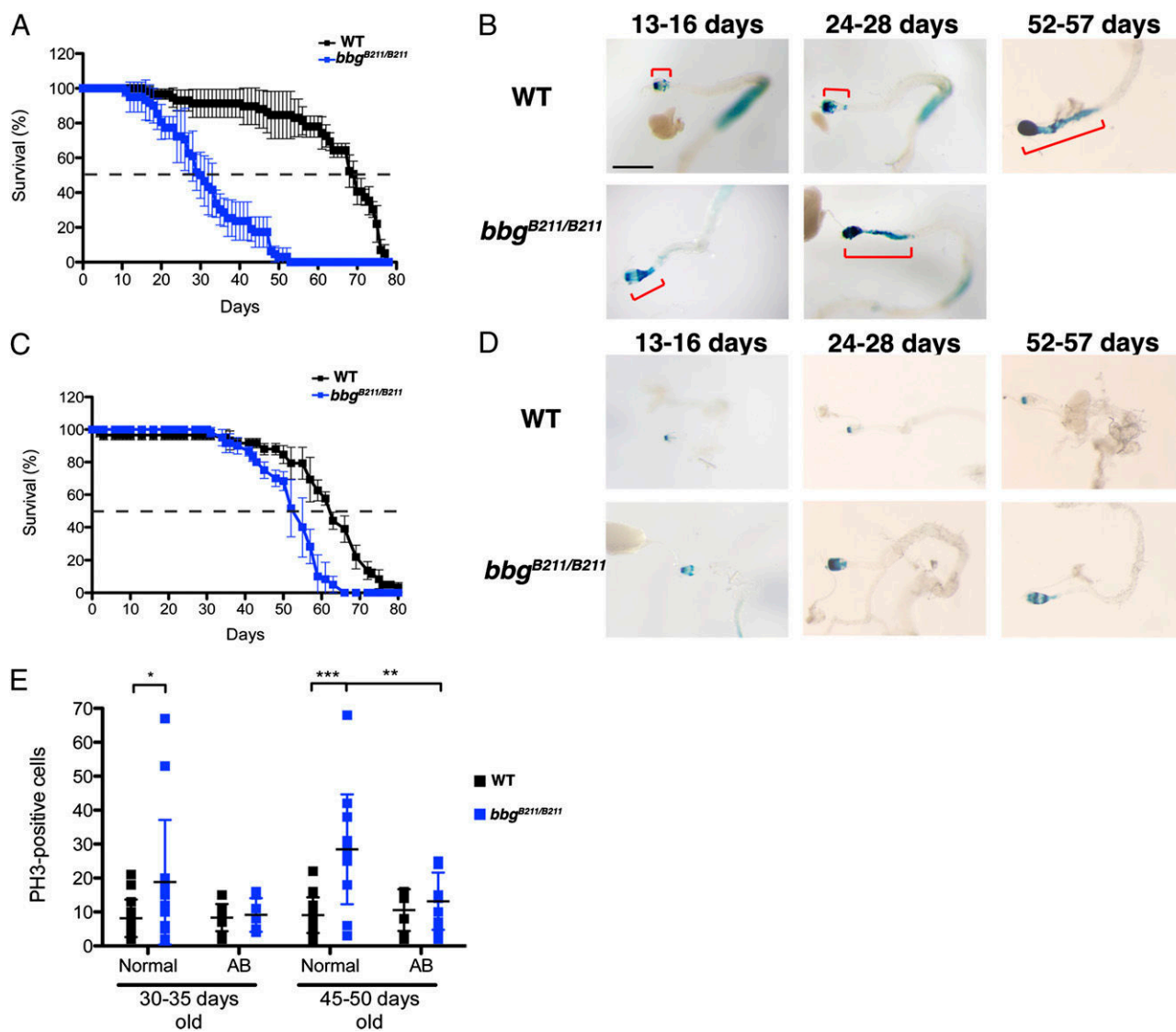
junctions are required for maintaining a tight balance between immune response and immune tolerance in the gut.

## Results

**BBG Promotes Immune Tolerance in the Gut and Is Required for Normal Lifespan.** To analyze the immune response of flies mutant for *bbg*, we took advantage of a null mutant allele of the *bbg* gene (*bbg*<sup>B211</sup>) (16). We noted that on regular cornmeal medium, the LT<sub>50</sub> (time for half of the fly population to die) of control wild-type flies *w*<sup>A5001</sup> (WT) flies was 70 d, whereas that of *bbg*<sup>B211/B211</sup> flies was reduced by ~40 d (Fig. 1A), demonstrating that BBG was required for the normal lifespan of *Drosophila*. Because constitutive activation of the *Drosophila* immune response is known to reduce lifespan (9, 14, 17) the reduction in LT<sub>50</sub> noted above could reflect a stronger constitutive immune

response. Analysis of the IMD pathway *diptericin-LacZ* reporter staining indeed revealed a much higher constitutive activation of the pathway in the anterior midgut of *bbg*<sup>B211/B211</sup> flies than in WT flies (Fig. 1B). This constitutive activation of the IMD pathway increases and extends posteriorly during aging in both WT and *bbg*<sup>B211/B211</sup> flies (Fig. 1B) (18), although with much faster kinetics in *bbg*<sup>B211/B211</sup> flies (Fig. 1B).

To determine if the enhanced mortality and IMD pathway activation were of microbial origin, we ablated the endogenous gut flora in WT and *bbg*<sup>B211/B211</sup> mutant flies by feeding them with a mixture of antibiotics (19). First, we ascertained that these antibiotics did not interfere with IMD pathway activation by monitoring the transcription of the *diptericin* gene in S2 cells upon stimulation with heat-killed *Escherichia coli* in the presence of the antibiotics (Fig. S1). As previously reported (19),



**Fig. 1.** BBG is required for normal lifespan and immune tolerance in the gut. (A and C) Lifespan experiments on regular cornmeal medium (A) and on medium containing antibiotics (C). In normal conditions, the lifespan of *bbg*<sup>B211/B211</sup> flies is reduced compared with WT flies (A). Premature death of mutant flies is rescued by a treatment with antibiotics (C). Dashed line indicates the LT<sub>50</sub>. Each curve represents the mean of three independent experiments with three groups of 20 flies. Error bars are SD. (B and D) *Diptericin-lacZ* reporter activity in absence of infection with (D) or without (B) antibiotic treatment. IMD pathway activation increases with time on normal cornmeal medium but remains stronger in *bbg*<sup>B211/B211</sup> mutant flies (B). Antibiotic treatment abolishes IMD pathway activation observed in B (D). Age of the flies is indicated. Representative image from an experimental sample of 15 guts. (Scale bar, 300  $\mu$ m.) (E) Mitotic count in gut epithelial cells from WT or *bbg*<sup>B211/B211</sup> mutant flies raised on regular (normal) or antibiotic-treated cornmeal medium (AB) for either 30–35 d or 45–50 d after hatching. The mean of PH3-positive cells per condition is represented by a black line. A minimum of 15 guts per condition were analyzed. \**P* value < 0.05; \*\**P* value < 0.01; \*\*\**P* value < 0.001.

the antibiotic treatment was not toxic to flies, but we observed a slightly diminished lifespan in WT flies upon treatment (Fig. 1C). The *dipteracin-LacZ* reporter activity was strongly reduced in the anterior midgut of germ-free-like (GFL) WT and *bbg*<sup>B211/B211</sup> flies (Fig. 1D). Importantly, the lifespan of GFL *bbg*<sup>B211/B211</sup> flies (LT<sub>50</sub>: 53 d) was extended, nearly matching that of GFL WT flies (LT<sub>50</sub>: 62 d) (Fig. 1C), indicating that the absence of BBG results in high susceptibility of the flies to their endogenous gut flora.

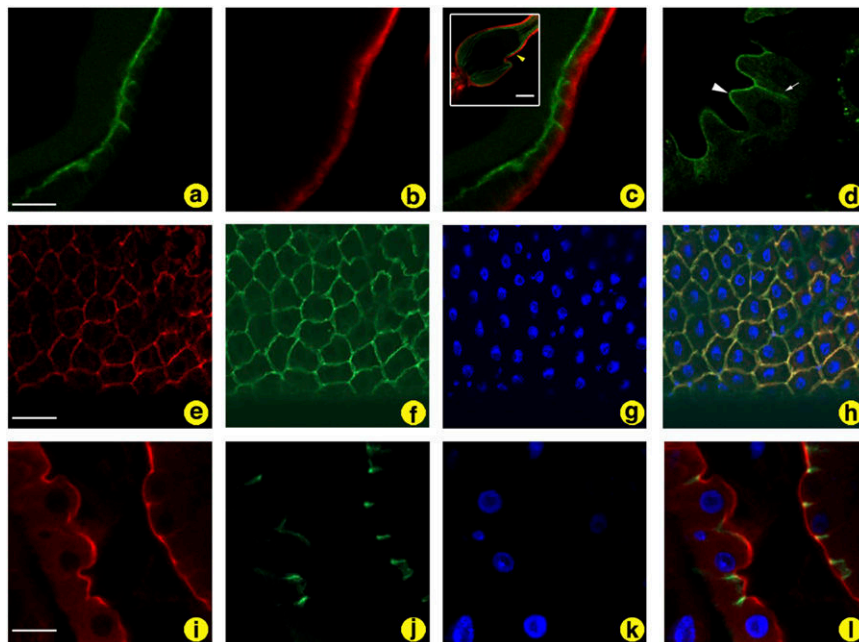
High activation of the IMD pathway in the gut has recently been linked to impaired lifespan and increased proliferative activities in *Drosophila* intestinal stem cells (11, 20). We stained *bbg*<sup>B211/B211</sup> mutant and WT fly guts with an antiphosphohistone H3 (anti-PH3) antibody that marks dividing stem cells. A low number of PH3-positive cells was detected in the midgut of WT flies, whereas the number of mitotic cells was markedly increased in the gut of *bbg*<sup>B211/B211</sup> mutant flies, indicative of a high epithelium renewal that increased with aging (Fig. 1E). Strikingly, addition of antibiotics in the feeding medium fully suppressed the higher mitotic count observed in *bbg*<sup>B211/B211</sup> mutant fly guts (Fig. 1E).

Collectively, these data demonstrate that the absence of BBG results in a strong activation of the IMD pathway in the anterior midgut and an abnormally high proliferation rate of gut stem cells in response to endogenous microbiota. This leads to the shortening of lifespan in *bbg*<sup>B211/B211</sup> mutant flies and indicates that BBG is required for immune tolerance toward the endogenous gut flora.

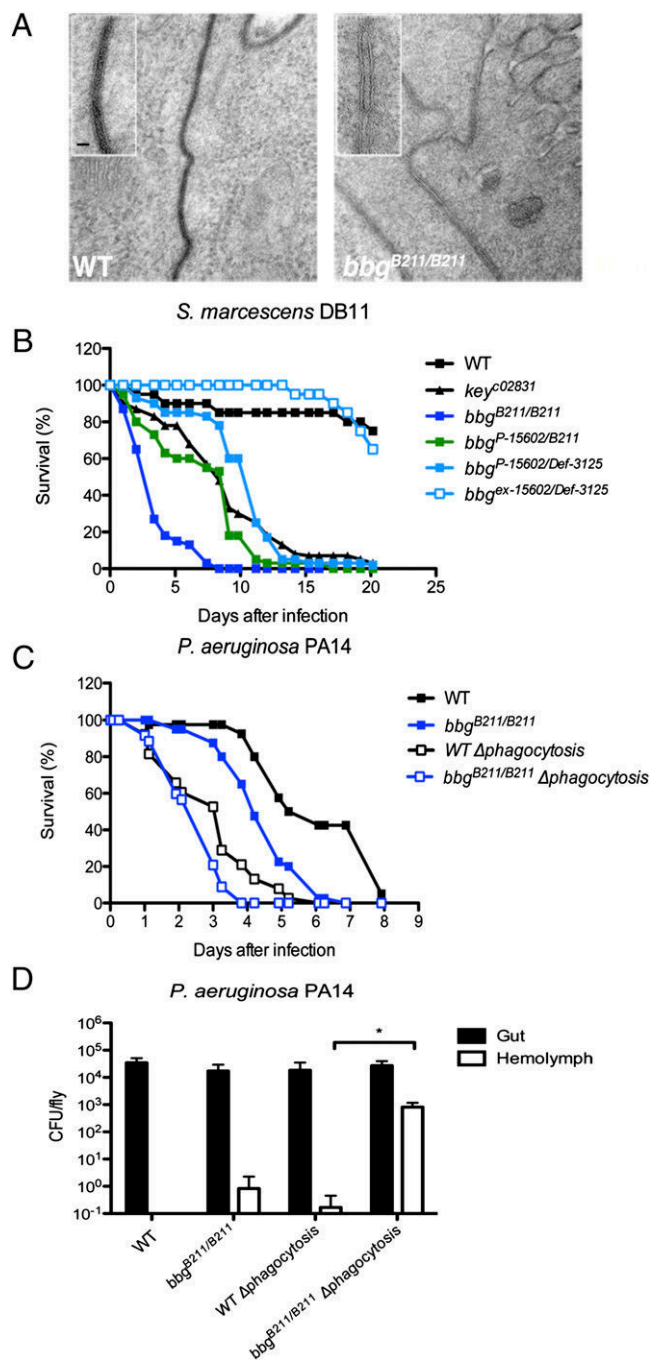
***bbg* Gene Is Expressed in the Midgut.** We used an antibody raised against the C-terminal part of the protein, recognizing all BBG isoforms and detecting the protein in the embryonic gut primordium (16) to ascertain the distribution of BBG in adult flies. We detected BBG only in the digestive tract, including the proventriculus [a pear-shaped structure formed by the folding of the esophagus and anterior midgut epithelia and secreting the

peritrophic membrane (21)] (Fig. 2A, C, D, E, H, I, and L). In the gut epithelium, BBG was present at the apical side of the cells (Fig. 2A, C, and D) and was excluded from the distribution domain of Adducin (Fig. 2B and C), a marker of the basolateral side in midgut epithelial cells. BBG displayed the classical subcellular distribution of proteins involved in cellular junctions. The protein was clearly visible on histological sections along the lateral surface of contact between enterocytes (Fig. 2D, arrow) and as an apical ring around epithelial cells (Fig. 2E–H). As in other invertebrates, the *Drosophila* paracellular epithelial midgut barrier is mediated by lateral membrane structures, the septate junctions (SJs) named smooth SJs (SSJs) in the case of endodermal derivatives (22, 23). BBG colocalized with Coracle, which belongs to the 4.1 superfamily of plasma membrane-associated cytoplasmic proteins known to participate in SJs (24–26) at the lateral side of enterocytes in the proventriculus and the midgut (Fig. 2E–L). The lack of BBG in SSJs did not impair the enterocyte apicobasal polarity, as assessed by the normal distribution of both Coracle and Adducin in *bbg*<sup>B211/B211</sup> mutant flies (Fig. S2). Q-PCR analysis on whole gut showed that four isoforms (*bbg-RF*, *-RG*, *-RH*, and *-RJ*, as referred by [www.flybase.org](http://www.flybase.org)) were strongly expressed whereas *bbg-RC*, *-RI*, and *-RE* expression were barely detectable (Fig. S3). Lacking a transmembrane domain (16), BBG is a PDZ-domain cytoplasmic protein, which is associated with the membrane both at the apical and the lateral sides of the digestive tract epithelial cells, where its distribution matches that of septate junctions.

**BBG Strengthens Septate Junctions and Restrains Invasive Entomopathogenic Bacteria to the Gut Lumen.** Transmission electronic microscopy (TEM) observation of WT *Drosophila* midgut sections showed that the 20-nm paracellular space at the level of the SSJs was decorated with an electronic dense structure (Fig. 3A), whereas the SSJs in the midgut of *bbg*<sup>B211/B211</sup> mutant flies did not present this electronic dense structure and showed a gap of 30 nm between the two plasma membranes (Fig. 3A). Coracle is a central protein of SJs (23, 25) and most of the *Drosophila*



**Fig. 2.** BBG is localized at the apical and lateral sides of gut epithelial cells. BBG is localized at the apical (arrowhead) and lateral (arrow) sides of the proventriculus (A–C) and of the midgut (D–L) epithelial cells. BBG is distributed as an apical ring in these cells (E) and matches the localization of the septate junction-associated protein Coracle (H and L, merge). (A–C) Immunolocalization of BBG (green) and Adducin (red) on whole gut or (D) on paraffin section. (C) Square, lower magnification of proventriculus; arrowhead, region of A–C. (E–L) Immunolocalization of BBG (red) and Coracle (green) on whole gut. (G and K) DAPI staining. (H) Merge of E–G. (L) Merge of I–K. Representative image from an experimental sample of 10 guts. [Scale bars (A–D) 20  $\mu$ m; (C, square) 50  $\mu$ m; (E–H) 30  $\mu$ m; (I–L) 15  $\mu$ m.]



**Fig. 3.** Lack of BBG results in septate junctions and permeability defects in the gut. (A) BBG participates in septate junctions structure. TEM micrographs of transversal sections through the anterior midgut of WT or  $bbg^{B211/B211}$ . In WT fly midgut, the paracellular space at the level of the septate junctions spans 20 nm, whereas it reaches 30 nm in flies defective for  $bbg$ . Magnification, 120,000 $\times$ . Squares are numeric magnifications of original pictures. Representative image from an experimental sample of five guts. (Scale bar, 20 nm.) (B and C) Survival curves for WT,  $key^{C02831}$  ( $key^{C02831}$ ),  $bbg$  null mutants ( $bbg^{B211/B211}$ ,  $bbg^{P-15602/B211}$ ),  $bbg^{P-15602/Def-3125}$  or  $bbg^{ex-15602/Def-3125}$  flies fed on (C) *P. aeruginosa* PA14 (OD<sub>600</sub> = 0.25), or (B) *S. marcescens* DB11 (OD<sub>600</sub> = 1) at 25 °C. Each curve is representative of three independent experiments with three groups of 20 flies; statistics are detailed in Fig. S6. (D) Gut permeability toward *P. aeruginosa* PA14. Flies were preinjected ( $\Delta$ phagocytosis) or not with latex beads and fed 1 d later on a solution of *P. aeruginosa* PA14 (OD<sub>600</sub> = 0.25) at 25 °C. Bacterial counts from hemolymph or dissected guts of the same genotypes were evaluated 2 d after oral infection. Data are representative of three independent experiments performed with three groups of 10 flies for each condition. \**P* value < 0.05.

*coracle* mutant alleles are either embryonic or larval homozygous lethal (25). We knocked down *coracle* in adults by driving an RNAi transgenic construct targeting *coracle* in the midgut. The efficiency of the knockdown was monitored by immunodetection of Coracle (Fig. 4A). The SSJs lacking Coracle displayed an enlargement of the space between the plasma membrane similar to that observed in  $bbg^{B211/B211}$  mutant flies (e.g., 30 nm; Fig. 4B), demonstrating that absence of either Coracle or BBG resulted in a slackening of SSJs. By feeding WT and  $bbg^{B211/B211}$  mutant flies with Dextran Blue, an inert dye, we could not detect any difference in the gut permeability to large molecules, indicating that the slackening of SSJs observed in the gut of  $bbg^{B211/B211}$  mutant flies did not change its permeability toward large-sized inert compounds (Fig. S4).

However,  $bbg^{B211/B211}$  mutant flies died faster than WT or IMD pathway-deficient flies ( $kenny^{C02831}$ ) (27) after an oral infection with bacteria able to cross the intestinal barrier, such as *Serratia marcescens* DB11 or *Pseudomonas aeruginosa* PA14 (27, 28) (Fig. 3B and C). The same results were obtained when we used several allelic or hemizygous combinations of  $bbg$  mutations (Fig. 3B). Importantly, the excision of the P-element inserted in the  $bbg$  gene restored a WT phenotype to the flies, demonstrating that the impaired survival was indeed due to the disruption of  $bbg$  (Fig. 3B). *P. aeruginosa* PA14 is known to kill *Drosophila* by crossing the gut epithelium and escaping the phagocytic activity of hemocytes (27). Accordingly, inactivating hemocyte phagocytosis by microinjecting latex beads before oral challenge led to a much faster death rate for both WT and mutant flies (Fig. 3C), with  $bbg^{B211/B211}$  mutant flies still more susceptible to the bacteria than WT flies. This sensitivity toward *P. aeruginosa* PA14 upon impaired phagocytosis correlated with the increased bacterial load that we observed in the hemolymph of  $bbg^{B211/B211}$  mutant flies compared with WT flies 24 h after an oral challenge (Fig. 3D). Importantly, silencing *coracle* in the midgut recapitulated the survival phenotypes that we observed in  $bbg^{B211/B211}$  mutant flies following oral infection by Gram (–) bacteria (Fig. 4C). Taken together, these data showed that BBG and Coracle were required in the *Drosophila* gut against oral infection, demonstrating the overall role of septate junction integrity as part of epithelial defense mechanisms.

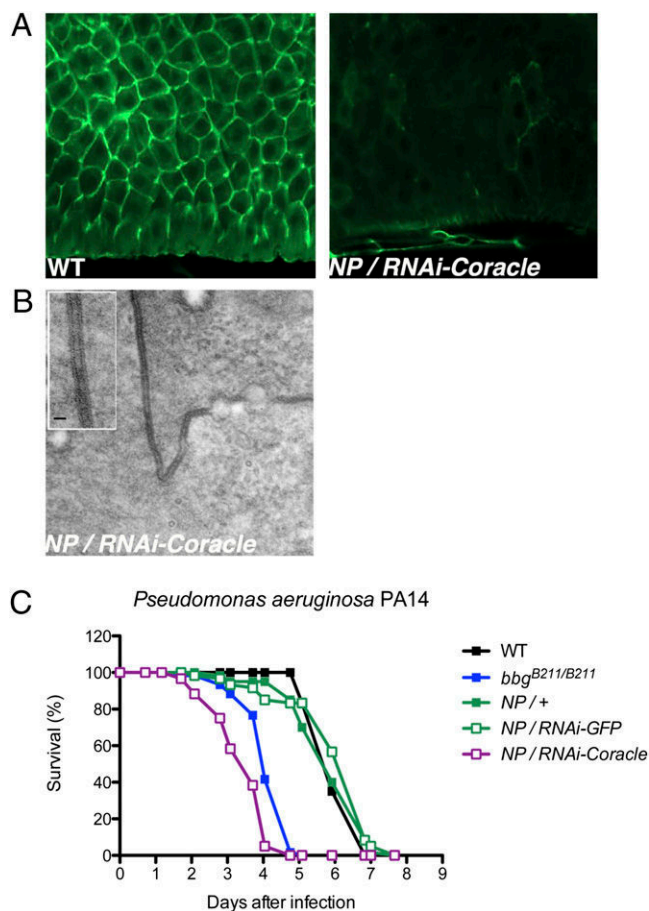
Another defense mechanism of the *Drosophila* gut against oral infections is provided by the local synthesis of AMPs such as Diptericin through the activation of the IMD pathway. When fed on *S. marcescens* DB11 or *P. aeruginosa* PA14, which are known to trigger a potent AMP response (27, 28), the diptericin expression levels measured by Q-RT PCR in the gut of  $bbg^{B211/B211}$  and WT flies were not different, indicating that absence of BBG did not impair inducible AMPs synthesis in the gut epithelium (Fig. S5). Additionally, when conventionally challenged by septic injury using Gram (–) or Gram (+) bacteria, WT and  $bbg^{B211/B211}$  mutant flies exhibited no differences in the fat-body-dependent transcription of AMPs or ability to survive *Agrobacterium tumefaciens* or *Enterococcus faecalis* infections (Fig. S5). This indicates that BBG is not required for the humoral response against bacteria.

In conclusion, the absence of BBG results in a defect of the SSJs that weakens the gut epithelial barrier and leads to an increased permeability of the gut to invasive entomopathogenic bacteria such as *P. aeruginosa* PA14.

## Discussion

In the fly gut, the host defense relies on active defense mechanisms, such as the well-documented inducible synthesis of AMPs and ROS upon pathogen challenge, and on passive structural barriers, an issue not addressed experimentally until recently. The peritrophic matrix, a chitinous multilayered structure that isolates the bolus from the gut epithelial cells represents one of these barriers and was shown to specifically shield the *Drosophila* gut against pore-forming toxins secreted by bacteria (15). The function





**Fig. 4.** SJs are required to prevent oral infection by invasive bacteria. (A) *coracle* knockdown. Immunolocalization of Coracle (Green) in *Drosophila* anterior midgut isolated from WT or *NP<sup>3084</sup>-Gal4/UAS-RNAi-coracle* (*NP/RNAi coracle*) flies. (Scale bar, 20  $\mu$ m.) (B) Coracle participates in septate junction structure. TEM micrograph of transversal section through the anterior midgut of flies knocked down for *coracle* in the midgut (*NP/RNAi coracle*). In WT flies midgut the paracellular space at the level of the septate junctions spans 20 nm, whereas it reaches 30 nm in flies defective for Coracle (*NP/RNAi coracle*). Magnification, 120,000 $\times$ . Square is numeric magnification of original picture. Representative image from an experimental sample of five guts. (Scale bar, 20 nm.) (C) Knockdown of Coracle in the gut impairs survival to *P.aeruginosa*. WT, *bbg<sup>B211/B211</sup>*, *NP<sup>3084</sup>+*, *NP<sup>3084</sup>-Gal4/UAS-RNAi-gfp* (*NP/RNAi-GFP*), *NP<sup>3084</sup>-Gal4/UAS-RNAi-coracle* (*NP/RNAi-Coracle*), flies were challenged at 25  $^{\circ}$ C with *P.aeruginosa* PA14. Data are representative of three independent experiments performed with three groups of 20 flies; statistics are detailed in Fig. S6.

of the peritrophic matrix can be compared with that of the mammalian gut mucus layer, which regulates the spatial relationships between microbiota and host (29). In *Drosophila*, septate junctions are functionally related to mammalian tight junctions and are known to participate in epithelial barrier function (23). To our knowledge, no genetic evidence supports a role for gut junctions in the immune barrier in insects. Here we show that BBG is an integral gut protein localized at the apical and lateral sides of gut epithelial cells, and required for the integrity of septate junctions. Disorganized septate junctions result in acute susceptibility to invasive enteric pathogens such as *P.aeruginosa* or *S.marcescens*, highlighting the role for septate junctions in host defense. Septate junctions are not conserved in mammals, but it is tempting to speculate that the PDZ proteins [such as Zonula Occludens-1 (ZO-1)] associated with the mammalian tight junctions, and the PDZ-domain protein BBG described here, share similar immune barrier functions.

We demonstrate here that BBG and septate junction cohesion are required to dampen the continuous activation of the IMD pathway by the endogenous flora in the anterior midgut. The constitutive strong activation of the IMD pathway when gut integrity is lost, is reminiscent of the chronic inflammation observed in mammalian inflammatory bowel diseases (IBDs). There are two main clinical forms of IBDs, Crohn disease (CD) affecting any part of the intestine, and ulcerative colitis, which is restricted to the colon (30). The etiology of IBDs is not fully understood but it seems that CD arises from a combination of different factors, such as environment, genetic susceptibility, microbial flora, and altered immune responses (31). However, the chronic inflammatory response observed in CD is mainly thought to originate from a breach of the intestinal mucosal barrier that exposes lamina propria immune cells to the continuous presence of resident luminal bacteria, bacterial products, or dietary antigens (32). Consistent with a bacteria-linked mechanism for IBDs, treatment with antibiotics (e.g., rifamycin, as in our case) can induce a remission in mammalian models of CD and even prevent relapse (33). Similarly, we could rescue the chronic inflammation observed in the gut of *bbg<sup>B211/B211</sup>* mutant flies and restore their lifespan to WT levels by depleting the gut flora using antibiotic treatment. The vertebrate gut flora, defined as an “organ within an organ” (34) is known to be critically required for host homeostasis. In *Drosophila*, the role of the endogenous flora in fly fitness and longevity is still controversial (18, 35). In our hands, WT flies fed on antibiotic-containing medium displayed a slightly shortened lifespan compared with WT flies fed on regular medium, arguing in favor of a beneficial role for the endogenous flora toward longevity in insects.

Additionally, in mammalian models of CD, the space between epithelial cells shows increased permeability due to the malfunction of tight junctions, which are essential for sealing this paracellular space (36). The defect in tight junctions appears before the first signs of inflammation, even in the absence of endogenous flora (37). Similarly, the paracellular space enlargement observed in the SSJs of *bbg<sup>B211/B211</sup>* mutant flies is present before the onset of the chronic IMD pathway activation in the gut epithelium.

The mechanism by which disrupted septate junctions lead to a constitutive IMD pathway activation in response to the endogenous flora remains unclear. The Toll-like receptors (TLRs) are a family of evolutionary conserved receptors able to activate the innate immune response upon recognition of microbial patterns (for review see ref. 38). In mouse, the bacterial flagellin sensor TLR5, located at the basal surface of the enterocytes, cannot detect flagellin originating from the apically located gut luminal flora (39). However, TLR5 triggers a potent inflammatory response against invasive bacteria (such as *Salmonella*) able to reach the basal side of the enterocytes (39). Moreover, any breach of the gut mucosal barrier results in basal exposure to TLR5 of flagellin from the endogenous flora, which leads to TLR5 activation and subsequent chronic gut inflammation (40). Similarly, we could speculate that enhanced paracellular space in *bbg<sup>B211/B211</sup>* mutant flies would facilitate the access of endogenous flora-derived bacterial PAMPs to the laterobasal side of the *Drosophila* gut enterocytes, thus facilitating access to the PGRP-LC immune receptors.

The BBG protein is uniformly distributed along the gut, but *bbg<sup>B211/B211</sup>* mutant flies display constitutive IMD pathway activation only in the anterior midgut. Other control mechanisms of the IMD pathway may explain this restriction. *Caudal*, a transcriptional repressor acting directly on AMP promoters has been shown to participate specifically in posterior midgut immune tolerance (14). Genetic ablation of PGRP-LB, an amidase that negatively regulates the IMD pathway, results in endogenous flora-dependent AMP expression only in the middle and posterior midgut (11). Moreover, a null mutation in PIMS, an inhibitor that sequesters the PGRP-LC receptors upstream of the IMD pathway, does not affect the pathway in the middle part of

the gut. This regional distribution of functionally different and overlapping inhibitors of the IMD pathway further highlights the necessity to keep the endogenous flora under tight control to avoid chronic immune stimulation.

The endogenous flora and the balance between gut immune and metabolic functions establish a tripartite relationship, which is critical for fly fitness. As stated by Maloy and Powrie, “deciphering how the immune response in the gut impacts the composition of the flora, how members of this flora interact within different regions in the gut and how we could stably shape the gut microbiota will be key issues if we want to understand and cure IBDs” (30). Given the similarities in both etiology and symptoms between mammalian IBDs and *bbg*<sup>B211/B211</sup> mutation-dependent chronic immune stimulation of the gut, we propose the impaired gut permeability mutants of *Drosophila* as simple and powerful models to study the mechanisms of IBDs.

## Materials and Methods

Stocks were raised on standard cornmeal-yeast agar medium at 25 °C. Antibiotic treatment and lifespan experiments were performed as already described (19). w<sup>A5001</sup> mutant flies were used as control. *kenny*<sup>C02831</sup> (41) and

*Myd88* or *Dif*<sup>1</sup> (42, 43) were used as mutant deficient for the IMD and Toll pathway, respectively. The *bbg*<sup>B211</sup> null mutant allele was generated by imprecise excision of a P-element, P[GawB]bbgC96, inserted within a doc element located 1.86 kb upstream of the 3' exons of *bbg* (16). The *bbg*<sup>15602</sup> mutant allele carrying a P element [EY02818] (P-15602) inserted in the third exon of the *bbg* gene (16) and the deficiency Df(3L)z-GS1a, P[wA<sup>R</sup>]66E/TM3, Sb<sup>1</sup> (Def-3125) covering the *bbg* gene were obtained from the Bloomington *Drosophila* Stock Center. We precisely excised the P element [EY02818] to create the *bbg*<sup>Ex-15602</sup> allele. Further description of materials and methods are found in *SI Materials and Methods*.

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# Supporting Information

Bonnay et al. 10.1073/pnas.1221910110

## SI Materials and Methods

**Fly Strains.** The *dipteracin-GFP* (1) and the *dipteracin-LacZ* (2) reporter lines were described previously. The flies carrying a UAS-RNAi against Coracle were obtained from the Vienna-*Drosophila* RNAi Center. The Gal4 driver NP3084 was selected for its strong and specific expression in the midgut of adult flies in a screen of enhancer trap Gal4 lines expressed in embryonic and/or larval gut tissues and was obtained from the *Drosophila* Genetic Resource at the National Institute of Genetics (Shizuoka, Japan; [www.shigen.nig.ac.jp/fly/nigfly/](http://www.shigen.nig.ac.jp/fly/nigfly/)).

**Bacterial Strains, Infections, and Counts in the Hemolymph.** We used *Agrobacterium tumefaciens*, *Enterococcus faecalis*, *Escherichia coli* strain DH5 $\alpha$ GFP, and *Micrococcus luteus* (CIP A270) bacteria for septic injury and *Serratia marcescens* strain DB11 (3) and *Pseudomonas aeruginosa* strain PA14 (4) for oral infections. The *E. coli* strain DH5 $\alpha$ GFP was generated in our laboratory. Bacteria were grown in Luria broth (LB) (*E. coli*, *A. tumefaciens*, *S. marcescens* DB11) or brain–heart infusion broth (BHB) (*M. luteus*, *E. faecalis*, *P. aeruginosa* PA14) at 29 °C (*A. tumefaciens*) or 37 °C (*E. coli*, *M. luteus*, *P. aeruginosa* PA14, *E. faecalis*, *S. marcescens* DB11). When required, antibiotics were added at 100  $\mu$ g/mL. Infection experiments and bacterial counts were performed as previously described (5, 6).

(whole gut). The mouse monoclonal anti-Adducin antibody (Developmental Studies Hybridoma Bank, clone 1B1) was used at a dilution of 1/20 and the guinea pig polyclonal anti-Coracle antibody (9) or the antiphosphohistone H3 (Millipore) at 1/2,000. For immunostaining dissected guts were fixed in PBS containing 4% (wt/vol) paraformaldehyde for 30 min and denatured with EDTA 1 mM/urea 300 g/L at 95 °C for 10 min and then processed (10). For paraffin sections, flies were dissected in PBS containing 4% (wt/vol) paraformaldehyde, fixed overnight at 4 °C, and embedded in paraffin. Seven-micrometer sections were dropped on SuperFrost slides. After rehydration, slides were permeabilized in PBS-Tween 20 0.05% and blocked in normal goat serum 5%/PBS-Tween 20 0.05% for 30 min. Samples were incubated overnight at 4 °C with the primary antibody, washed three times for 15 min with PBS-Tween 20 0.05%, and labeled overnight with the secondary antibody diluted in normal goat serum at 5%/PBS-Tween 20 0.05% (vol/vol) at 4 °C. Slides were mounted in a solution of Vectashield/DAPI (Vector Laboratories) and observed using a Zeiss LSM510 confocal microscope. X-gal staining was as previously described (6). Ultrathin sectioning and transmission electronic microscopy (TEM) were performed as previously described (10). Images were processed using Adobe Photoshop.

**Quantitative Reverse-Transcription PCR.** Primers used for real-time PCR were as follows:

mRNA isoform	Forward	Reverse
Bbg-RC	5'-AAGAGAACCAGGCTCAGTTGCTCA-3'	5'-AGGAGTAATTGGAGCCCACGAAA-3'
Bbg-RE	5'-GCGGGTGTAGCTGAAAGTGGAAA-3'	5'-AAGCAGTTCGTCTCTGTAGGCGAT-3'
Bbg-RF,G,H	5'-ACCTTCGAGTGCAAACAGAAAGCA-3'	5'-TTCTCTCTAACCGCTGATCCGCTT-3'
Bbg-J	5'-TCGGAACGTGATCGAACCTGTCTCT-3'	5'-GTCCACCGGCGTTTACTTCCATTT-3'
Bbg-I	5'-TGCTCAGAAATTAATCGCTACAGGG-3'	5'-GCGTTAATGCCGCTAATGCTGCTT-3'
Bbg-total	5'-TTCCACCCAATTTTCAGCGAACCCAC-3'	5'-TAACTGGGTGCTGGCTCTACGTT-3'

**Blue Dextran Feeding.** Flies were fed with a solution containing 1% of Blue Dextran 2000 (Pharmacia) in 50 mM sucrose for 24 h. Midguts were dissected in PBS, mounted in DAPI-containing Vectashield (Vector) and observed using a binocular microscope (Leica MZFL3).

**Injection of Latex Beads.** A total of 69 nL of fourfold concentrated Surfactant-Free Red CML latex beads (0.30  $\mu$ m-diameter polystyrene beads; Interfacial Dynamics) were injected into recipient flies to block phagocytosis, as previously described (7).

**Immunostaining and Histology.** Rabbit polyclonal anti-BBG antibody (8) was used at a dilution of 1/800 (sections) or 1/2,000

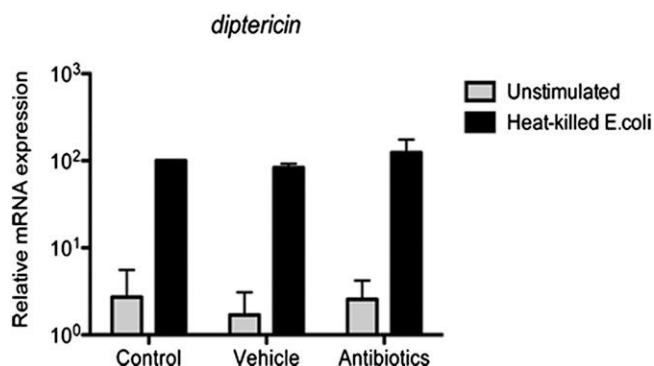
Results were normalized using expression of *ribosomal protein 49* (*rp49*). *Diptericin* expression was detected with the primers previously described (11).

**Antibiotic Treatment of S2 Cells.** Antibiotics medium was prepared by freshly adding 1 mL of a 100 $\times$  stock of antibiotics in 50% ethanol per 100 mL of complete Schneider medium to a final concentration of 500  $\mu$ g/mL ampicillin, 50  $\mu$ g/mL tetracycline, and 200  $\mu$ g/mL rifamycin. Cells were stimulated with 100 heat-killed *E. coli* per cell. The vehicle was used as a control.

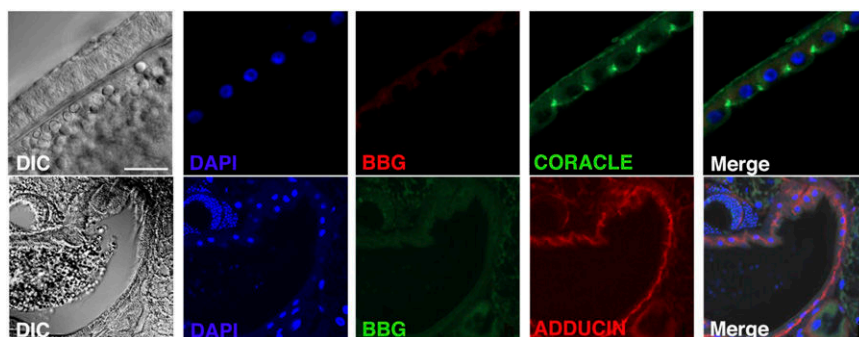
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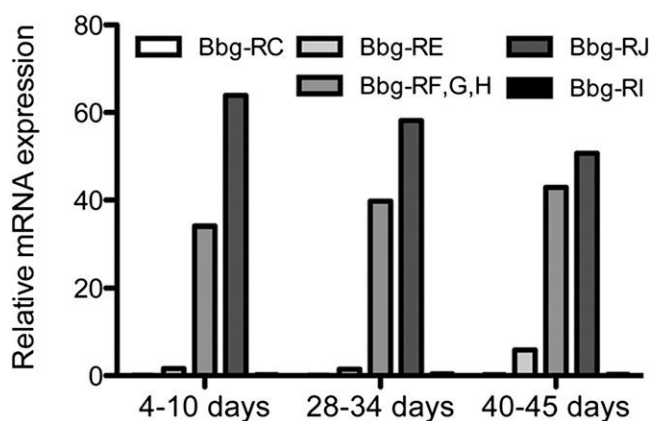
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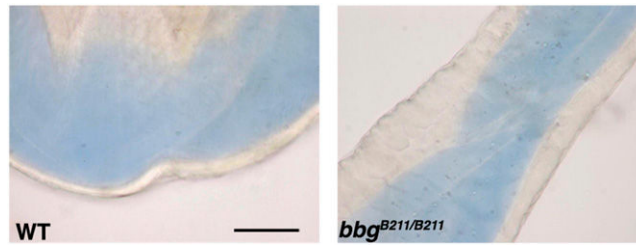
**Fig. S1.** Antibiotics do not impair the IMD pathway. Quantitative RT-PCR of *diptericin* mRNA, normalized by *RP49* mRNA, from *Drosophila* S2 cells cultured in Schneider medium (control), Schneider medium supplemented with ethanol (vehicle), or Schneider medium supplemented with antibiotics (antibiotics). S2 cells were stimulated during 24 h with heat-killed *E. coli* or left unstimulated. Control heat-killed *E. coli* stimulated S2 cells *diptericin* mRNA level was set at 100. Data are representative of three independent experiments performed with  $2 \cdot 10^6$  S2 cells.



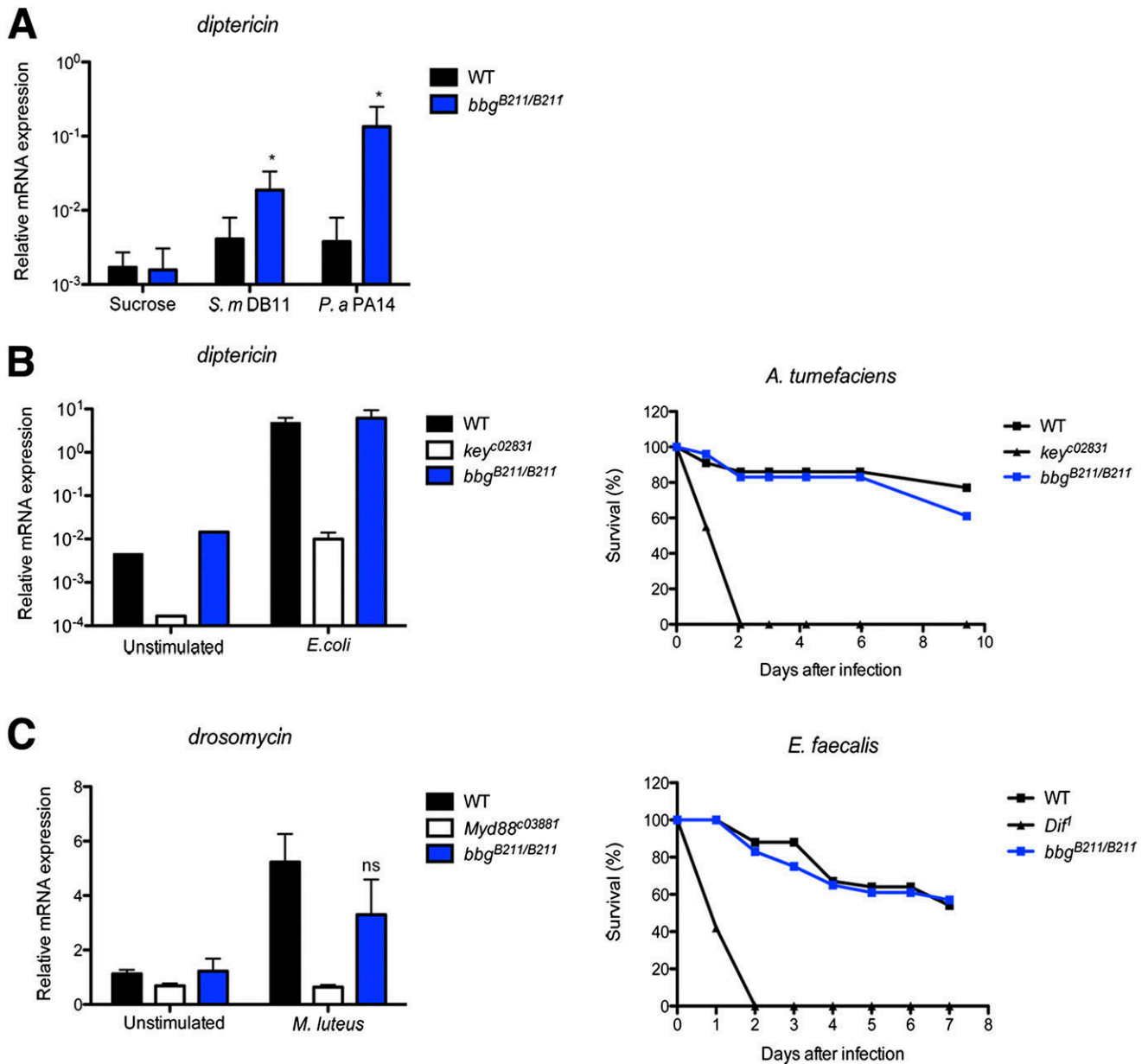
**Fig. S2.** Bbg is not required for enterocyte polarity maintenance. Immunolocalization on a *bbg<sup>B211/B211</sup>* fly midgut. Nuclei were stained with DAPI. Bbg is not detected as expected. Coracle is localized at the apicolateral sides and Adducin at the basal sides of the enterocytes. Representative image from an experimental sample of 10 guts. (Scale bar, 10  $\mu$ m.)



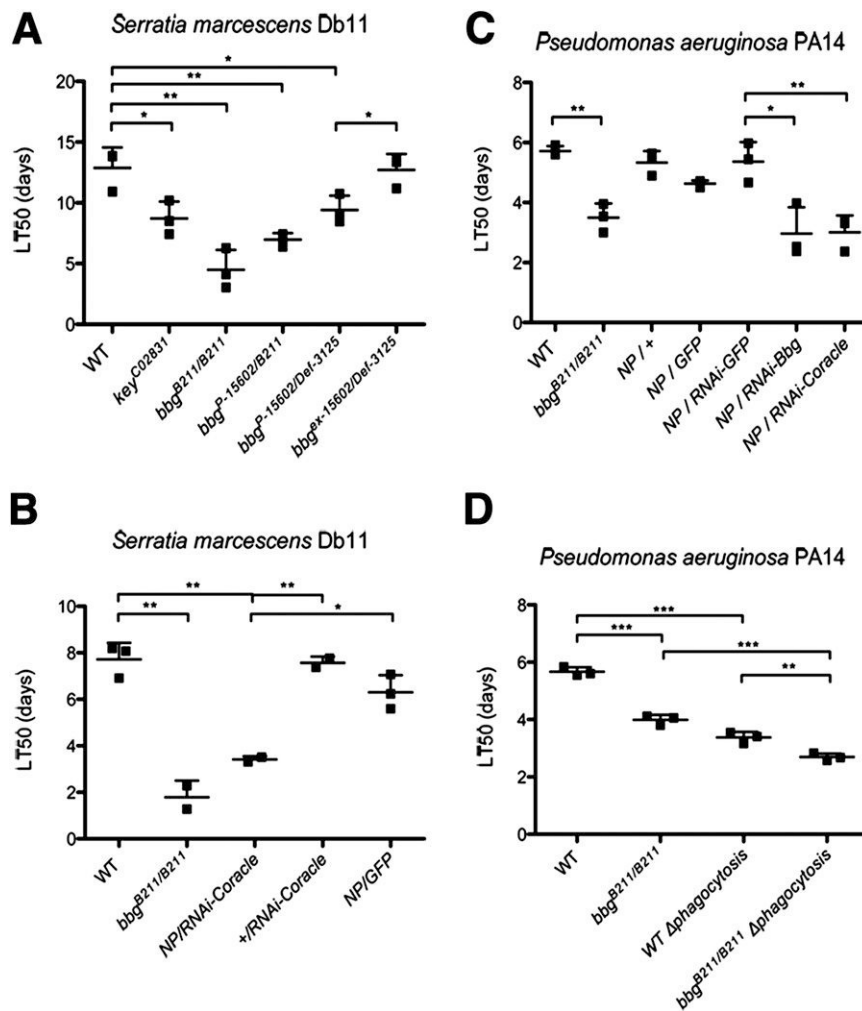
**Fig. S3.** Expression levels by quantitative RT-PCR of the different *bbg* isoforms (*bbg-RC*, *-RE*, *-RF-RG-RH* "RF, G, H"), *-RJ* and *-RI*) in WT guts. Adult flies were collected 4–10, 28–34, or 40–45 d after hatching. mRNA levels were expressed as percentage of the total *bbg* mRNA level. Data are representative of five guts in each condition.



**Fig. 54.** Permeability of *Drosophila* midgut toward chemical compounds. Anterior midgut from WT or *bbg*<sup>B211/B211</sup> flies fed on Dextran Blue. Representative image from an experimental sample of 10 guts. (Scale bar, 30  $\mu$ m.)



**Fig. 55.** BBG is not required for epithelial- or humoral-induced innate immune response. (A) Quantification by RT-PCR of *dipteracin* mRNA, normalized by *RP49* mRNA after feeding with either sucrose or bacteria. Flies were fed on sucrose or *P. aeruginosa* PA14 (OD<sub>600</sub> = 0.25) for 24 h and *S. marcescens* DB11 (OD<sub>600</sub> = 1) for 48 h. (B and C) Survival curves for WT, *bbg*<sup>B211/B211</sup>, *kenny*<sup>C02831</sup> (*key*<sup>C02831</sup>), and *Dif*<sup>1</sup> flies following *A. tumefaciens* (B) or *E. faecalis* (C) systemic immune challenge. Each curve is representative of three independent experiments with a group of 20 flies. Quantification by RT-PCR of *dipteracin* or *drosomycin* mRNA normalized by *RP49* mRNA after systemic immune challenge using the Gram (–) bacteria *E. coli* or the Gram (+) bacteria *M. luteus*. RNA were extracted from whole WT, *bbg*<sup>B211/B211</sup>, *kenny*<sup>C02831</sup> (*key*<sup>C02831</sup>), or *Myd88* flies, 6 h after challenge for *dipteracin* (B) and 24 h after challenge for *drosomycin* (C). Results are representative of three independent experiments. \**P* value < 0.05; \*\**P* value < 0.01; \*\*\**P* value < 0.001.



**Fig. S6.** Statistical analysis of survival curves. Survival experiments described in this study were performed at least three times independently. Because log-rank analysis can only compare two survival curves at a time in the same experiment, we decided, as previously reported (6), to compute the median lethal time 50 ( $LT_{50}$ ) and perform statistical analysis on  $LT_{50}$  using Student's  $t$  test. Oral infection experiments were performed with *S. marcescens* DB11 (A and B) or with *P. aeruginosa* PA14 (C and D). \*  $P$  value < 0.05; \*\*  $P$  value < 0.01; \*\*\*  $P$  value < 0.001.

## VIII. Chapter 3: Akirin specifies NF- $\kappa$ B selectivity of *Drosophila* innate immune response via chromatin remodeling

### 1. Scientific context of the study

#### 1.1 The *akirin* gene family

The *akirin* gene first emerged during the formation of primitive eukaryotes, around two billion years ago (Hedges et al., 2004; Macqueen and Johnston, 2009). Protists from Alveolata (e.g. *Guillardia theta*) and Heterolobosea (e.g. *Naegleria gruberi*) phyla are considered as the most ancient organisms with an *akirin* gene. Although these observations place the origin of *akirin* prior to the split between animals, plants and fungi, no *akirin* orthologue has yet been identified in plant or fungal genomes. Furthermore, as *akirin* was identified only in a few non-metazoan species and mostly predicted as an inactive pseudogene, its presence in non-metazoan eukaryotes seems to be an exception rather than a rule (Macqueen and Johnston, 2009). By contrast, *akirin* is found in almost all metazoan genomes, including its most primitive phylum, Placozoa (e.g. *Trichoplax adherens*). As far as known, sponges are the only animals that do not have an *akirin* gene. The *akirin* gene has duplicated at the emergence of primitive jawless fishes. A single *akirin-1* (also called *Mighty*) and *akirin-2* (also called *FBI1*, and closest homolog of invertebrate *akirin*) gene was identified in almost every sequenced vertebrate species from the marine lamprey to humans except in avian species (e.g. chicken, zebra finch and turkey) that likely have lost *akirin-1* (Macqueen and Johnston, 2009).

#### 1.2 Known functions of Akirins in the immune responses of metazoa

The function of Akirin proteins was described for the first time in a collaborative study between fly and mouse geneticists (Goto et al., 2008). *akirin* was initially highlighted during a genome-wide screen in *Drosophila* S2 cells investigating

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new mechanisms of the IMD pathway activation. Knocking-down *akirin* in S2 cells abolished the activation of a Relish-dependent *Attacin-A*-Luciferase reporter. To identify which step of the IMD pathway activation was controlled by *akirin*, genetic activations of the IMD pathway by the over-expression of PGRP-LC, IMD and Relish $\Delta$ S<sub>29</sub>-S<sub>45</sub> constructs were performed. The knock-down of *akirin* abolished all these activating signals, indicating that Akirin played a role at the level of Relish transcription factor. Along the same line, genetic ablation of mouse *akirin-2* prevented the expression of a subset of target genes of the NF- $\kappa$ B-dependent Toll-like receptors (TLRs), Interleukin-1 receptor (IL1-R) and Tumor necrosis factor alpha receptor (TNF-R) pathways including Interleukin-6 (IL-6), B-cell lymphoma 3 (Bcl3), RANTES and interferon protein 10 (IP10). Intriguingly, the absence of *akirin-2* did not affect NF- $\kappa$ B-dependent stimulation of keratinocyte-derived cytokine (KC, mouse homolog of the CXCL1 chemokine), Inhibitor of NF- $\kappa$ B  $\alpha$  (I $\kappa$ B- $\alpha$ ), Inhibitor of NF- $\kappa$ B  $\zeta$  (I $\kappa$ B- $\zeta$ ) genes. These results suggested that Akirins would act in a specific way together with NF- $\kappa$ B, to activate the transcription of genes.

*Drosophila* Akirin is a 201 aminoacids (AA) protein that shares 39,4% of identity with mouse (201 AA) and human (203 AA) Akirin-2. *Drosophila* Akirin (*DmAkirin*) and human Akirin-2 (*HsAkirin-2*) are functionally very close as the immune-deficiency provoked by the absence of *DmAkirin* in flies can be rescued by over-expressing *HsAkirin-2*. Akirin-1 however, does not seem to have an immune function, at least in mice, as knocked-out *akirin-1* mice cells induced the full set of NF- $\kappa$ B target genes upon Lipopolysaccharide (LPS, an agonist of TLRs) or TNF stimulation. Interestingly, *Drosophila* Akirin is also very close (69,4% identical) to *Anopheles gambiae* Akirin (*AgAkirin*). Although the question has not been addressed, it would be tempting to believe that *AgAkirin* may as well play a role in the NF- $\kappa$ B-dependent immune response against *Plasmodium* parasites. Finally, note that the tick (*Ixodes scapularis*, vector of Lyme disease) ortholog of *akirin*, known as *subolesin* was shown to participate to the NF- $\kappa$ B-dependent innate immune response against *Anaplasma phagocytophylum*, one of the Gram-negative bacterium pathogens responsible for the Lyme disease (Naranjo et al., 2013). Intriguingly, Subolesin and *IsRelish*-like NF- $\kappa$ B seem to promote each other's transcription upon *A. phagocytophylum* immune challenge. However, this positive feedback loop was observed neither in *Drosophila* nor in mice immune responses and could have evolved specifically in arachnids.



### 1.3 Known functions of Akirins in the development of metazoa

Besides their role in the innate immunity, *Drosophila* and mammalian Akirins are required for embryonic development. *akirin-2* knocked-out mice die at embryonic day 9,5 and *akirin*-deficient flies die at early to mid stages of embryogenesis. By contrast, *akirin-1* knocked-out mice are viable and fertile (Goto et al., 2008). A couple of studies addressed the function of Akirins in developmental processes more in details (Chen et al., 2013; Clemons et al., 2013; Mobley et al., 2014; Moreno-Cid et al., 2010; Nowak et al., 2012).

First, Akirin was shown to be required for meiosis during *C. elegans* oogenesis (Clemons et al., 2013). At the prophase I stage, *akirin* deficient oocytes were unable to disassembly the synaptonemal complex (SC) mediating homologous chromosomes association before crossovers formation. Consequently, *akirin* deficient oocytes delayed meiosis and exhibited aberrant chromosome condensation. Whether CeAkirin would act directly or indirectly (through an Akirin-dependent transcriptional program) in this process is still an open question. Along the same line, another study reported that the inactivation of Akirin ortholog proteins of *Aedes albopictus* (but not *Aedes aegypti*) and *Phlebotomus perniciosus* (the sand fly) by blood meal-delivered anti-Akirin neutralizing antibodies reduced their reproductive abilities (Moreno-Cid et al., 2010).

In *Drosophila*, a recent report showed that *akirin* is required for embryonic lateral transverse muscles development by promoting the activation of the targets of Twist, an important transcriptional regulator of *Drosophila* mesodermal fates (Nowak et al., 2012). Additionally, the genetic approach of this study explored a putative molecular mechanism of *Drosophila* Akirin that will be further described in the 1.4. section of this chapter. The role of Akirin in muscle development was also supported by a couple of studies describing the positive influence of *akirin-1* and -2 in the skeletal myogenesis of vertebrates. Note that the role of Akirins in myogenesis has gained a particular interest in economically impacting animal models such as salmon and Japanese black beef (Macqueen et al., 2010a, 2010b; Marshall et al., 2008; Salerno et al., 2009; Sasaki et al., 2009).

#### 1.4 What are the molecular functions of Akirins?

Akirins are strictly nuclear factors in *Drosophila* and mammals. Bio-informatic prediction and genetic manipulation of *Drosophila akirin* and human *akirin-2* characterized a conserved nuclear localization signal (NLS) in the N-terminal portion of the proteins between aminoacids 20 and 30 (Goto et al., 2008). However, the molecular function of the rest of the protein is more enigmatic, as no functional domain can be predicted. Note that the predicted secondary structure of *Drosophila* Akirin shows two  $\alpha$ -helixes, spanning from P<sub>80</sub> to Q<sub>99</sub>, and from F<sub>160</sub> to Y<sub>195</sub>.

Genetic and proteomic evidences from *Drosophila* studies (Giot et al., 2003; Nowak et al., 2012) have strongly suggested that Akirins are physically and functionally connected to the mating-type Switching / Sucrose non-fermentable (SWI/SNF) chromatin remodeling complex Brahma-associated protein 60kDa (Bap60). More specifically, Nowak et al showed that Akirin and Brahma complex members co-occupied a significant subset of their DNA-binding in embryos and larval salivary glands. Moreover, they observed that trans-heterozygous embryos lacking one copy of *akirin* and *bap60* recapitulate the defective patterns of lateral transversal muscles observed in *akirin*-mutant embryos, further strengthening the hypothesis of a functional link between these two genes.

#### 1.5 SWI/SNF chromatin remodeling

SWI/SNF complexes such as the Brahma complex are part of a larger superfamily of SWI-like ATP-dependent chromatin remodeling complex. This superfamily comprises four distinct families, based on the sequence of the ATPase subunit responsible for nucleosome remodeling: SWI/SNF, Imitation switch (Iswi), Chromodomain-Helicase-DNA binding (Chd) and Inositol auxotroph 80 (Ino80) complexes (Ho and Crabtree, 2010). Together with DNA methylation and histone modifications, chromatin-remodeling complexes control the assembly and regulation of eukaryotic chromatin. Despite their genetic variability, all chromatin-remodeling complexes have a similar molecular function: increase the mobility of nucleosome, the basic unit of chromatin assembly (Côté et al., 1994). Chromatin remodelers use the energy of ATP hydrolysis to change the packaging state of chromatin by moving,

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ejecting or restructuring the nucleosome (Becker and Hörz, 2002; Saha et al., 2006). The most described output of this feature is the control of gene expression (Clapier and Cairns, 2009). Nonetheless, these four families of chromatin remodelers were also shown to be involved in other epigenetic events.

For example, the Iswi complex was implicated in the maintenance of chromatin structure on the *Drosophila* male X chromosome (Deuring et al., 2000). Alternatively, the Ino80 complex was shown to be required in the regulation of telomere structure and function (Yu et al., 2007), in the segregation of chromosomes during cell divisions (Krogan et al., 2004; Ogiwara et al., 2007) and in the control of DNA replication (Vincent et al., 2008) and DNA repair (van Attikum et al., 2004; Kusch et al., 2004). Moreover, even within their role in transcription regulation, chromatin remodelers do not function in a consistent manner. The SWI/SNF Brahma-like complexes in particular, can act either as transcriptional activators or repressors and can even switch between those two modes of action at the same gene, as illustrated in the development of T lymphocytes or in EGFR signaling during *Drosophila* wing development (Chi et al., 2003; Rendina et al., 2010a; Terriente-Félix and de Celis, 2009).

## **1.6 The Brahma complex**

Unlike more complex metazoan (e.g. vertebrates), *Drosophila* SWI/SNF chromatin-remodeling complexes are associated with a single ATPase subunit: Brahma (Elfring et al., 1994; Tamkun et al., 1992). The contribution of Brahma in the nucleosome remodeling of flies is very broad as its absence leads to nucleosome occupancy changes throughout the whole genome (Shi et al., 2014). As far as known, Brahma is part of a multimeric complex composed of six core members: Brahma, Moira, SNF5-related-1 (Snr1) Brahma-associated protein 55kDa (Bap55), Brahma-associated protein 60kDa (Bap60) and actin.

Brahma was the first identified member of this complex. The initial indication that *brahma* was involved in transcriptional regulation came from genetic interactions observed between *brahma* and *polycomb* genes in the determination of body segment identity of flies (Kennison and Tamkun, 1988). Polycomb acts as a

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repressor of the homeotic Antennapedia complex (ANT-C) and Bithorax complex (BX-C) genes (Wedeen et al., 1986). Consequently, loss-of-function mutations of *polycomb* cause homeotic transformations. One of them is the appearance of first legs identity structures (sex combs) at the location of second or third legs caused by the derepression of the *sex-comb reduced* (Scr) gene, one member of ANT-C complex gene in the second and third legs imaginal discs (Pattatucci and Kaufman, 1991). The loss of Polycomb further alters the fates of antenna to legs (because of *antennapedia* derepression), wings to halteres (*ultrabithorax* derepression) and fourth abdominal segment to more posterior identity (*abdominal-A* and *-B* derepression) (Wedeen et al., 1986).

Heterozygous mutations in *brahma* suppressed all these homeotic transformations, therefore showing that Brahma acts as an activator of ANT-C and BX-C homeotic genes. The study further showed that *brahma* genetically interacted with *trithorax* (Trx), another activator of ANT-C and BX-C genes (Tamkun et al., 1992). As a consequence, Brahma and its functional partners are considered as members of the Trithorax Group (trxG).

Brahma is a 1638 residues protein that is structurally related to the Swi2 protein, the core member of the SWI/SNF complex in yeast (Tamkun et al., 1992). Genetic screens for SWI/SNF suppressor mutants in yeast have established that SWI/SNF complex-transcriptional activity was linked with histones and other chromatin-related proteins (Bortvin and Winston, 1996; Kruger et al., 1995). Finally, biochemical studies on Brahma described two main conserved domains responsible for its chromatin-remodeling activity. First, a DNA-dependent ATPase domain, serves as a DNA-translocating motor to break histone-DNA contacts and is fundamentally required for nucleosome remodeling. Second, a C-terminal bromodomain, recognizes acetylated Lysines of histones (and other proteins) and may impact remodeler targeting, remodeling efficiency or both (Clapier and Cairns, 2009; Tamkun, 1995).

Maira, another subunit of Drosophila SWI/SNF complex, is a 170kDa protein that is able to bind to itself thanks to a Leucine zipper motif and to interact with Brahma, possibly with the help of its Swi3, Ada2, N-CoR, TFIIB (SANT) domain, a sequence that is predicted to bind histones (Boyer et al., 2004; Crosby et al., 1999). Another Brahma complex subunit, Bap60 was characterized as essential for SWI/SNF-mediated transcriptional activation or repression (Möller et al., 2005).

### *Chapter 3: Akirin specifies NF- $\kappa$ B selectivity of Drosophila innate immune response via chromatin remodeling*

Interestingly, the study could demonstrate that Bap60 physically bound DNA and contextual transcription factors (Sisterless-A and Scute) during *Drosophila* development and suggested a role of this subunit in determining site-specificity binding of the Brahma complex. The Snr1 subunit was shown to act as a regulatory subunit to restrict Brahma complex-dependent chromatin remodeling (Zrally et al., 2004). Finally, not much is known about the molecular function of the Bap55 subunit. This protein was described as an actin-related protein (a functionally diverse group of proteins that share 17-64% of sequence identity with actin) that is present at stoichiometric levels in Brahma purified complexes (Papoulas et al., 1998).

Additionally, the Brahma core complex is able to associate with two signature subsets of proteins to form distinct specialized complexes. On one hand, the subunit Osa associates with Brahma complex to establish the BAP complex (Vázquez et al., 1999). On the other hand, the subunits Polybromo, Bap170 and Supporter of activation of Yellow protein (Sayp) associate with the Brahma complex to form the PBAP complex (Mohrmann et al., 2004). Those two specialized complexes target mutually exclusive genes in *Drosophila*. They were shown to execute similar, independent or antagonistic functions in transcriptional regulation but appeared to direct mostly distinct biological processes (Moshkin et al., 2007).

The *osa* gene (also named eyelid) encodes a 2713 aminoacids protein with one known functional domain: an AT-rich interaction (ARID) domain (from aminoacid 993 to 1087) (Vázquez et al., 1999). *Drosophila* Osa-associated BAP complex has been implicated in numerous developmental processes: temporal patterning of larval neural stem cells (neuroblasts), commitment of adult intestinal stem cells and eye and wing imaginal discs development (Baig et al., 2010; Eroglu et al., 2014; Milán et al., 2004; Terriente-Félix and de Celis, 2009; Zeng et al., 2013). On the other hand, *Drosophila* PBAP complex was shown to be required in the maintenance of ovarian germline stem cells and wing imaginal discs development (He et al., 2014; Rendina et al., 2010b). Hence, both BAP and PBAP complexes were involved in the development of the wing imaginal discs by regulating the targets of the EGFR pathway. Nonetheless, BAP and PBAP act antagonistically in this process. The BAP complex is required for cell growth, survival and subsequent tissue patterning within wing imaginal discs by promoting the expression of the EGFR targets *delta*, *rhuboid* and *argos* (Terriente-Félix and de Celis, 2009). Conversely, loss of

Bap170, a member of the PBAP complex, causes EGFR pathway over-activation by up-regulating *rhomboid* and down-regulating *argos*, a negative regulator of the pathway (Rendina et al., 2010a). Thus, PBAP would repress the activation of EGFR in the wing imaginal disc.

## 1.7 SWI/SNF complexes in mammals

Two SWI/SNF-like complexes have been described in mammals, based on distinct ATPase catalytic subunits-encoding genes: Brahma-related gene 1 (Brg-1) and Brahma complexes. The complexity of these complexes is further increased by the multiple isoforms of each sub-unit they may assemble: *Drosophila* Moira protein has two mammalian orthologs, Brahma-associated factor 155 (BAF155) and BAF170; Bap60 has three of them: BAF60A, B and C; Bap55 has two of them: BAF53A and B; Osa has four of them: BAF200, BAF250A, B and C; Sayp has four of them: BAF45A, B, C and D (Ho and Crabtree, 2010).

All these combinations of mammalian SWI/SNF complexes seem to have distinguishable functions. For example, while *brg-1* knock-out mice die at early stages of embryogenesis because of multiple developmental defects (in zygotic genome activation, neurons, lymphocytes, adipose tissue, heart tissue differentiation and erythropoiesis), *brahma* knock-out mice are normal and viable, and only show a greater body mass (Bultman et al., 2000, 2005; Lessard et al., 2007; Lickert et al., 2004; Pedersen et al., 2001). *BAF250* family members (mammalian counterparts of *Drosophila* Osa) also display distinct properties. *BAF250a* is required for embryonic development as *BAF25a* knock-out mice die at embryonic day 6,5, and *BAF250a* knock-out embryonic stem cells (ESCs) show defective self-renewal and mesodermal differentiation properties (Gao et al., 2008; Yan et al., 2008). By contrast, *BAF250b* is required for the maintenance of pluripotency as *BAF250b* knock-out ESCs spontaneously differentiate in culture (Yan et al., 2008). Another distinguishable function can be attributed to the *BAF53A* and *B* genes (mammalian counterparts of *Drosophila* Bap55). *BAF53A* is required for proliferation of neural stem cells (Lessard et al., 2007), while *BAF53B* is required later, in a neuron-specific manner, for activity-dependent dendritic outgrowth in mice (Wu et al., 2007). Finally, among the *BAF45* family members, *BAF45A* is required for neuronal progenitor proliferation while

*BAF45C* is required for heart and muscle development (Lange et al., 2008; Lessard et al., 2007).

Besides their roles in developmental processes, a couple of studies observed that SWI/SNF complexes influence the immune responses of mammals, especially in the T- and B-lymphocytes lineages. As short as 10min of T-cell receptor (TCR) stimulation by phorbol 12-myristate 13 acetate (PMA) leads to a drastic shift of Brg1-associated proteins from soluble nuclear extracts to chromatin-bound insoluble extracts (Zhao et al., 1998). This work also pointed out the role of actin and actin-related proteins (BAF53) in physically escorting SWI/SNF complexes to their target regulatory sequences. Furthermore, chromatin-IP experiments showed the recruitment of Brg1, together with transcriptional enhancers on the NF- $\kappa$ B-targeted apoptosis inhibitor Bcl2-related gene expressed in fetal liver 1 (*Bfl-1*) promoter upon TCR activation (Edelstein et al., 2003).

Another study demonstrated that Brg1 and BAF155 (an ortholog of *Drosophila moira*) were required for peripheral CD4<sup>+</sup> T lymphocytes activation, proliferation and cytokines (Interferon (IFN)- $\gamma$ , IL-2, IL-4 and IL-17) production following PMA-mediated TCR signaling. Brg1-mediated activation of T cells occurred first indirectly, thanks to its binding on AP-1 and NF- $\kappa$ B immune-activating transcription factors promoter to sustain their expression levels, or more directly by promoting cytokines promoter activation (Jeong et al., 2010). Additionally, conditional knockout experiments in mice showed that Brg1 was required at all stages of T-Cell lineage development, likely thanks to its influence on TCR and Wnt signaling pathways (Chi et al., 2003). Finally, a recent study supported a role for Brg1 complex in B-lymphocytes activation (Holley et al., 2014). Brg1 depleted B-cells were able to properly develop and differentiate into plasmacytes upon but failed to undergo hypertrophy and secrete IgM following LPS treatments. Microarray analyses of *Brg-1* depleted B-cell identified several impaired immune-related signaling pathways: The Toll-like Receptor, MAPK and JAK/STAT signaling pathways. Consequently, B-cell Brg1-depleted mice were susceptible to opportunistic infections (notably conjunctivitis and pus-filled uterine masses associated with *Pasteurella pneumotropica*,) further demonstrating the essential role of Brg1 in B-cell mediated immune responses.

## **2. Manuscript**



# Akirin specifies NF- $\kappa$ B selectivity of *Drosophila* innate immune response via chromatin remodeling

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## Abstract

The network of NF- $\kappa$ B-dependent transcription that activates both pro- and anti-inflammatory genes in mammals is still unclear. As NF- $\kappa$ B factors are evolutionarily conserved, we used *Drosophila* to understand this network. The NF- $\kappa$ B transcription factor Relish activates effector gene expression following Gram-negative bacterial immune challenge. Here, we show, using a genome-wide approach, that the conserved nuclear protein Akirin is a NF- $\kappa$ B co-factor required for the activation of a subset of Relish-dependent genes correlating with the presence of H3K4ac epigenetic marks. A large-scale unbiased proteomic analysis revealed that Akirin orchestrates NF- $\kappa$ B transcriptional selectivity through the recruitment of the Osa-containing-SWI/SNF-like Brahma complex (BAP). Immune challenge in *Drosophila* shows that Akirin is required for the transcription of a subset of effector genes, but dispensable for the transcription of genes that are negative regulators of the innate immune response. Therefore, Akirins act as molecular selectors specifying the choice between subsets of NF- $\kappa$ B target genes. The discovery of this mechanism, conserved in mammals, paves the way for the establishment of more specific and less toxic anti-inflammatory drugs targeting pro-inflammatory genes.

**Keywords** Chromatin remodeling; *Drosophila*; Innate immune response; NF- $\kappa$ B

**Subject Categories** Chromatin, Epigenetics, Genomics & Functional Genomics; Immunology; Signal Transduction

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See also: **S Tartey et al**

## Introduction

In mammals, the NF- $\kappa$ B family is composed of five related transcription factors, namely p50, p52, p65, REL, and RELB, which regulate gene expression following various stimuli. NF- $\kappa$ B factors are

conserved among metazoans, and the *Drosophila* NF- $\kappa$ B transcription factors, DIF and Relish, are homologous to human REL and p52/p50, respectively (Hetru & Hoffmann, 2009). Inflammatory stimuli induce gene expression programs that are almost entirely NF- $\kappa$ B dependent (Ghosh & Hayden, 2012). Aberrant regulation of NF- $\kappa$ B signaling is strongly suspected in numerous cancers, inflammatory, and autoimmune diseases (Maeda & Omata, 2008). Moreover, activation of NF- $\kappa$ B signaling in response to commensal bacteria in the gut has been shown to be required for optimal intestinal homeostasis (Mukherji *et al*, 2013). Massive efforts in drug development have been aimed at targeting NF- $\kappa$ B signaling during inflammatory diseases. However, interfering with the NF- $\kappa$ B pathway can potentially lead to numerous adverse effects. Commonly used anti-inflammatory agents act through inhibition of the NF- $\kappa$ B pathway to exert both therapeutic and adverse side effects (Oeckinghaus *et al*, 2011; Hayden & Ghosh, 2012). NF- $\kappa$ B factors act mainly to trigger inflammation, but recent studies suggest that they also function during the resolution of inflammation (Lawrence *et al*, 2001; Hayden & Ghosh, 2012), emphasizing the need for the development of specific drugs switching on, or off, particular subsets of NF- $\kappa$ B target genes. Identifying this new generation of drug targets requires a comprehensive, large-scale dissection of NF- $\kappa$ B-regulated pathways to identify factors able to restrict the range of NF- $\kappa$ B target activities. It has been proposed that the selective activation of NF- $\kappa$ B target genes depends on chromatin remodeling factors (Kawahara *et al*, 2009; Smale, 2010). These selector molecules represent a ‘missing link’ in our understanding of both the complexity and selectivity of NF- $\kappa$ B signaling.

In *Drosophila*, the NF- $\kappa$ B transcription factors Relish and DIF (dorsal-related immunity factor) are activated upon an immune challenge downstream of the immune deficiency (IMD) and Toll pathways, respectively. Direct recognition of Gram-negative bacterial DAP-type peptidoglycan, by the peptidoglycan recognition protein-LC (PGRP-LC), occurs at the cell surface to activate the IMD pathway. Gram-positive or fungal microbial patterns, however, are recognized by circulating proteins, which trigger the activation of the Toll pathway (Ferrandon *et al*, 2007). Both pathways culminate

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with the nuclear translocation of an NF- $\kappa$ B transcription factor and activate the expression of anti-microbial peptide (AMP) coding genes in the fat body (a functional equivalent of the mammalian liver). The Toll pathway shares significant similarities with the signaling cascades downstream of the mammalian Toll-like receptors (TLRs) and the interleukin-1 receptor (IL-1R), highlighting a common ancestry for these immune mechanisms. The IMD pathway is akin to the tumor necrosis factor receptor (TNFR) pathway in vertebrates (Hoffmann & Reichhart, 2002).

A genome-wide RNA-mediated interference screen in *Drosophila melanogaster* identified Akirin as new NF- $\kappa$ B modulators in the IMD pathway (Goto et al, 2008). Akirins have a strict nuclear localization and were shown in flies to act at the level of the NF- $\kappa$ B factor Relish, but to be dispensable for activation of DIF target genes. Akirin was therefore identified as a new component of the IMD pathway driving the innate immune response after an immune challenge with Gram-negative bacteria (Ferrandon et al, 2007; Goto et al, 2008). Akirins are highly conserved, and the two mouse genes (*akirin-1* and *akirin-2*) have been identified and knocked out. Analysis of Akirin-2 deficient mouse embryonic fibroblasts showed that Akirin-2 acts downstream of the TLR, TNFR, and IL-1R signaling pathways. However, Akirin-2 was required for the regulation of only a specific subset of LPS and IL-1 inducible genes (Goto et al, 2008), although the molecular basis for this specificity remained unclear.

We provide here a comprehensive view of Akirin function in NF- $\kappa$ B transcriptional selectivity during the innate immune response, using *Drosophila melanogaster* as a model. We performed a two-hybrid screen aimed at identifying Akirin partners. We found that BAP60, a component of the Brahma (SWI/SNF) ATP-dependent chromatin-remodeling complex, binds to Akirin upon immune challenge. In *Drosophila*, the Brahma complex forms the BAP complex when associated with Osa, whereas an association with both Polybromo and BAP170 defines the PBAP complex. Each complex targets a mutually exclusive subset of Brahma-dependent genes (Mohrman et al, 2004; Moshkin et al, 2007). We show that the BAP, but not PBAP, complex is required *in vivo* for efficient anti-microbial peptide synthesis and for the survival of flies following Gram-negative bacterial infection. Upon immune challenge, Akirin is able to bind Relish, forming a link between this transcription factor and the BAP complex on the promoter of a subset of NF- $\kappa$ B target genes. Relish-dependent genes thus fall into two groups, either relying on Akirin and the BAP complex (and encoding mostly AMPs), or expressing most of the negative regulators of the IMD pathway and AMPs independently of Akirin.

We demonstrate here that NF- $\kappa$ B transcriptional selectivity relies on a tripartite relationship between Relish, Akirin, and the BAP complex, following immune stimulation in *Drosophila*. These components form an active transcription complex on promoter regions decorated with H3K4ac epigenetic marks.

## Results

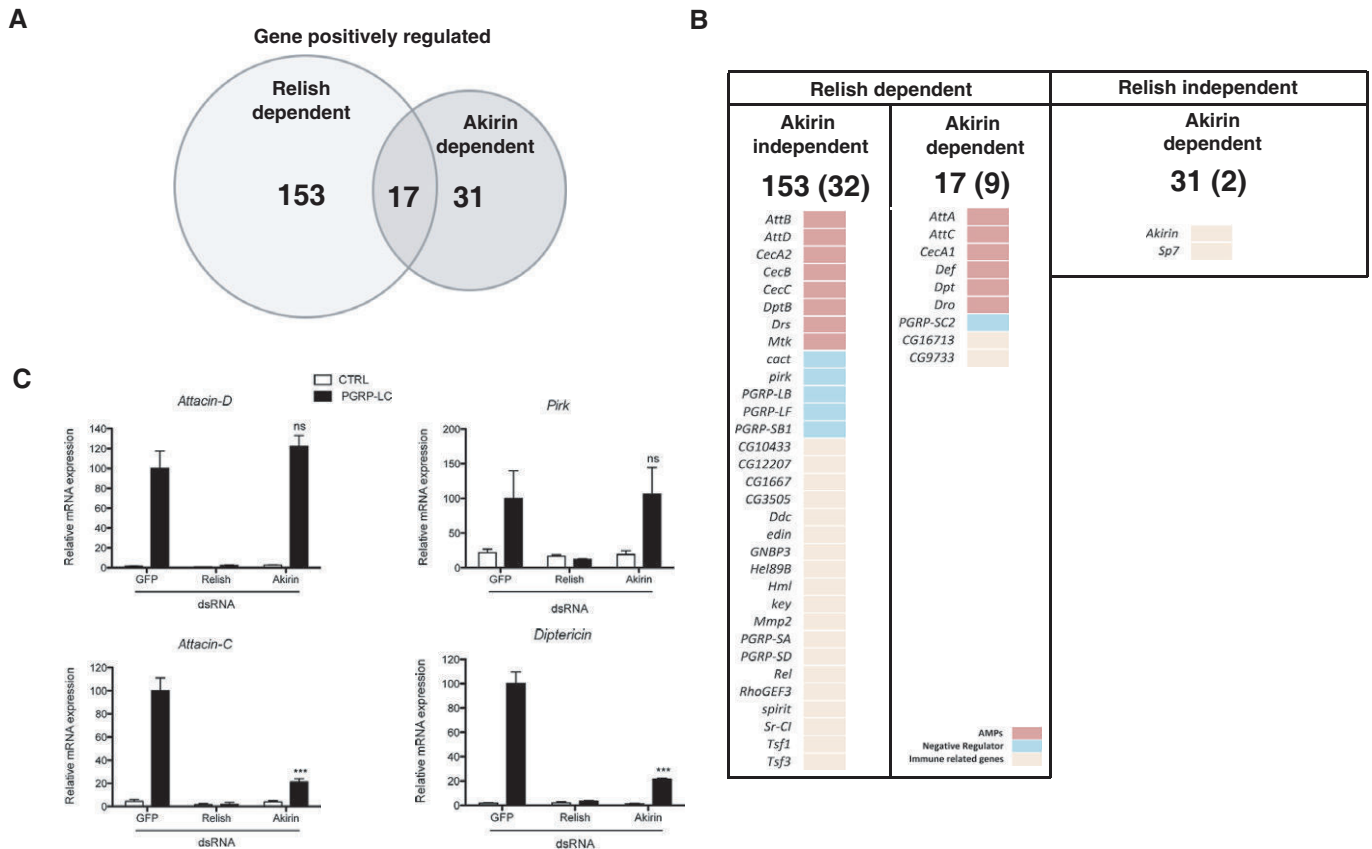
### Akirin is required for the activation of a subset of Relish-dependent genes

*Drosophila* Akirin had been genetically shown to be required at the level of the NF- $\kappa$ B factor Relish to activate two IMD pathway

effectors, the antimicrobial peptide (AMP) coding genes *attacin* and *diptericin* (Goto et al, 2008). We conducted a genome-wide analysis using Agilent DNA microarrays in *Drosophila* S2 cells to explore the impact of Akirin on the expression of the Relish-dependent transcriptome. *Drosophila* S2 cells were treated by dsRNA against *akirin*, *relish*, or *GFP* as a control, and the IMD pathway was activated by expressing a truncated form of Peptidoglycan receptor protein-Long Chain a (PGRP-LCa) (Goto et al, 2008). Total RNA was extracted from FACS-sorted transfected cells to evaluate gene expression (Supplementary Fig S1A). Microarray analysis revealed that Relish is required for the transcriptional activation of 170 genes upon challenge. The expression level of these genes showed at least a twofold reduction in the absence of Relish when compared to control *Drosophila* S2 cells. Among these 170 genes, 17 were also dependent on Akirin for their expression (Fig 1A), demonstrating that Akirin is required for the activation of only a restricted subset of Relish target genes. Upon immune challenge, Akirin *per se* is required for the activation of 31 genes independently of Relish (Fig 1A).

To understand the role of Akirin in this restricted activation, we first focused on genes encoding proteins with known immune functions (Fig 1B). In agreement with previous microarray data, Relish was required for the activation of 41 of these immune-related (IR) genes, pointing to Relish as a major immune transcription factor (Irving et al, 2001; De Gregorio et al, 2002; Pal et al, 2008). Akirin was only required for the activation of 9, among the 41 Relish-dependent, IR genes (Fig 1B). Among the 32 Relish-dependent, but Akirin-independent, IR genes, we found 8 genes encoding AMP effectors of the innate immune response, (*Attacin-B*, *Attacin-D*, *Cecropin-A2*, *Cecropin-B*, *Cecropin-C*, *Diptericin-B*, *Drosomycin*, and *Metchnikowin*) with either anti-bacterial (*Attacin-B*, *Attacin-D*, *Cecropin-A2*, *Cecropin-C*, *Diptericin-B*) or anti-fungal (*Drosomycin*, *Metchnikowin*) activities (Imler & Bulet, 2005). An additional group of 5 genes were shown to encode negative regulators of the IMD (*Pirk*, *PGRP-LB*, *PGRP-LF*, *PGRP-SB1*) or the Toll pathways (*Cactus*). The 19 remaining genes were involved in immune signal transduction (*Kenny*, *Relish*); chitin, nucleic acid, or peptidoglycan binding (*Sr-CI*, *Helicase89B*, *Gnbp3*, *PGRP-SA*, *PGRP-SD*); iron metabolism (*Tsf1*, *Tsf3*), or were suspected AMP (*Edin*). In contrast, we found that Akirin is almost exclusively required for the activation of genes encoding peptides with anti-bacterial activities (*Attacin-A*, *Attacin-C*, *Cecropin-A1*, *Defensin*, *Diptericin-A*, and *Drosocin*) (Imler & Bulet, 2005).

We validated the genome-wide analysis by monitoring the transcription of several IR genes upon immune challenge in S2 cells using RT-PCR (Fig 1C and Supplementary Fig S1C) and confirmed that *Pirk* and *Attacin-D* expression is Relish dependent but Akirin independent. In contrast, *Attacin-C* and *Diptericin-A* rely on both Relish and Akirin for their expression (Fig 1C). Of note, we found 8 genes that, after stimulation, had a twofold higher expression level compared to control when Relish was absent, and similarly, loss of Akirin results in the overexpression of 205 genes (Supplementary Fig S1B). Among these genes, 203 are not induced in control conditions (*dsGFP*) upon PGRP-LC stimulation, indicating a genuine derepression in absence of Akirin. As previously reported, upon immune challenge, Relish is not involved in gene repression (De Gregorio et al, 2002). Conversely, Akirin could function as a potent gene activator or repressor. Collectively, these data suggest that Akirin is



**Figure 1. Akirin influences the expression of only a subset of Relish target genes.**

A, B Venn diagram (A) and table representation of microarray analysis (B). Genes in PGRP-LC-stimulated S2 cells showing a twofold reduction of their expression upon knockdown of *relish* or *akirin* compared to control (dsRNA against *GFP*). Numbers in brackets correspond to genes with GO terms matching immune function. Red corresponds to anti-microbial peptides, blue to negative regulators of NF-κB pathways, and beige to other immune-related functions.

C Quantitative RT-PCR of *Pirik*, *Attacin-D*, *Attacin-C*, and *Diptericin-A* mRNA from sorted *Drosophila* S2 cells co-transfected with dsRNA against *GFP*, *relish* or *akirin*, and a PGRP-LCa overexpressing vector to stimulate the IMD pathway.

Data information: Data are represented as mean  $\pm$  standard deviation of three independent experiments performed with  $1-5 \times 10^5$  S2 cells. \**P*-value < 0.05; \*\**P*-value < 0.01; \*\*\**P*-value < 0.001.

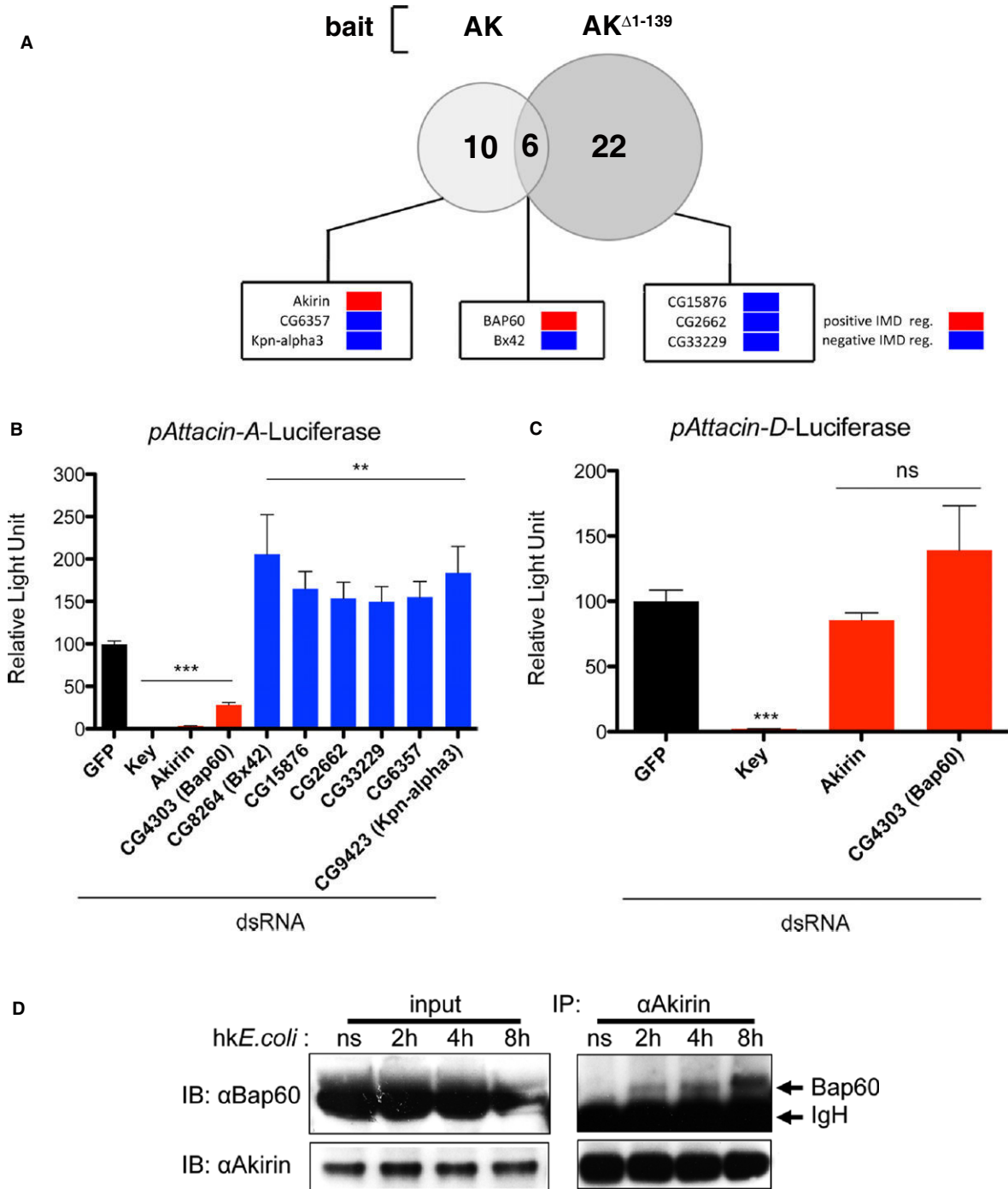
required for activation of a subset of Relish target genes mainly coding for IMD pathway effectors (AMPs) with anti-bacterial properties, whereas negative regulators of the pathway are mostly independent of Akirin.

### The Brahma complex member BAP60 interacts with Akirin

To identify the molecular partners that might account for the mode of action of Akirin, we undertook a yeast two-hybrid screen. We screened a *Drosophila* embryonic cDNA library using as baits a construct corresponding to the full-length Akirin (AK) or to the highly conserved C-terminal part of the protein encompassing residues 140–201 (AK<sup>Δ1–139</sup>), suspected to be important for Akirin function (Macqueen & Johnston, 2009) (Supplementary Fig S2A). These baits were not toxic for yeast and unable to drive expression of the *HIS3* reporter (Supplementary Fig S2B). Out of 200 million clones, we isolated 211 cDNAs corresponding to 38 proteins, 10 of which interacted with AK, 22 with the truncated form AK<sup>Δ1–139</sup> only, and 6 with both (Fig 2A). Unexpectedly, we observed an increased number of protein interactions with AK<sup>Δ1–139</sup> compared to AK,

indicating that the N-terminal part of Akirin restricts protein binding to Akirin. We also found that Akirin was able to interact with itself.

We used *Drosophila* S2 cells to verify these 38 proteins as genuine partners of Akirin. First, we transfected S2 cells with *attacin-A-luciferase*, a reporter of the IMD pathway known to be strongly induced upon immune challenge with heat-killed *Escherichia coli* (HKE) (Tauszig et al, 2000). *Drosophila kenny* (*key*) is essential for IMD pathway activation (Rutschmann et al, 2000) and is a homolog of mammalian IKKγ. Addition of dsRNA targeting either *key* or *akirin* to the culture medium strongly reduced *attacin-A-luciferase* expression, compared to control (*GFP*) dsRNA knockdown (Fig 2B). We then evaluated the ability of dsRNAs targeting individually each of the 38 putative Akirin partners to interfere with *attacin-A-luciferase* expression in response to HKE (Supplementary Fig S2C). Using this method, we showed that 30 putative partners of Akirin were not involved in IMD pathway activation; by contrast, we found that the knockdown of *bx42*, *CG2662*, *CG15876*, *CG33229*, *CG6357*, or *kpn-α3* resulted in a significant increased *attacin-A-luciferase* response to HKE (Fig 2B). Neither the unconfirmed nor the negative regulators of the IMD pathway were analyzed further. However,



**Figure 2. Akirin interacts with Bap60 upon immune challenge and activates selected Relish target gene promoters.**

**A** Schematic representation of two-hybrid results and subsequent functional assay. Proteins interacting with full-length (AK) or N-terminally truncated (AK<sup>Δ1-139</sup>) Akirin in two-hybrid assay. Proteins interacting with bait constructs encompassing full-length, or AK<sup>Δ1-139</sup> were tested for their ability to modulate the IMD pathway. Genes leading to increased IMD pathway activation when knocked down were identified as IMD negative regulators. Genes leading to decreased IMD pathway activation when knocked down were identified as positive IMD regulators.

**B, C** Dual luciferase assay from S2 cell co-transfected with *attacin-A-* (B) or *attacin-D-luciferase* (C) reporter plasmids and dsRNAs targeting *GFP*, *kenny* (*key*), *akirin*, and Akirin's putative partners extracts following 48 h of heat-killed *E. coli* stimulation. Data, normalized to dsRNA *GFP* controls, were from three independent experiments performed with  $5 \times 10^5$  S2 cells.

**D** Whole-cell lysates from S2 cells stimulated with heat-killed *E. coli* at indicated time points were immunoprecipitated with anti-Bap60 or anti-Akirin antibodies. Whole-cell lysate (input, left panel) and immunoprecipitated samples (right panel) were immunoblotted and probed with antibodies against Bap60 and Akirin.

Data information: Data are represented as mean  $\pm$  standard deviation from three independent experiments. \**P*-value < 0.05; \*\**P*-value < 0.01; \*\*\**P*-value < 0.001.

attenuation of *bap60*, or *akirin*, significantly reduced *attacin-A-luciferase* expression upon HKE stimulation (Fig 2B). Thus, BAP60, a core member of the *Drosophila* Brahma SWI/SNF-like ATP-dependent chromatin-remodeling complex (Moller *et al*, 2005), is a new positive regulator of the IMD pathway.

As *attacin-D* expression required Relish, but not Akirin, we constructed an *attacin-D-luciferase* reporter, which was strongly expressed upon HKE stimulation in S2 cells (Fig 2C). Addition to the culture medium of dsRNA targeting *kenny* (*key*), but not *akirin*, strongly reduced *attacin-D-luciferase* expression when compared to control (Fig 2C), indicating that the *attacin-D-luciferase* reporter recapitulated the behavior of endogenous *attacin-D* (Fig 1C). Similarly to *akirin* knockdown, silencing of *bap60* did not affect challenge-induced expression of the *attacin-D-luciferase* reporter when compared to control (dsGFP, Fig 2C), suggesting that BAP60 and Akirin cooperate to regulate the transcription of a subset of Relish target genes, including *attacin-A* but excluding *attacin-D*.

To explore the ability of BAP60 and Akirin to physically interact, we performed immunoprecipitation (IP) experiments in S2 cells. Protein extracts from cells transfected with tagged versions of BAP60 (BAP60-Flag) and Akirin (Akirin-V5) were immunoprecipitated with an anti-Flag antibody. The corresponding blot, revealed with an anti-V5 antibody, indicated that Akirin associated with BAP60 (Supplementary Fig S2D). We then immunoprecipitated endogenous Akirin from *Drosophila* S2 cells using an anti-Akirin polyclonal antibody (Supplementary Fig S2E). We indeed detected BAP60 on blots from samples prepared 2, 4, and 8 h after challenge with HKE (Fig 2D), but we could never detect a robust endogenous interaction between Akirin and BAP60 at early time points (Supplementary Fig S3) or in the absence of an immune stimulation (Fig 2D).

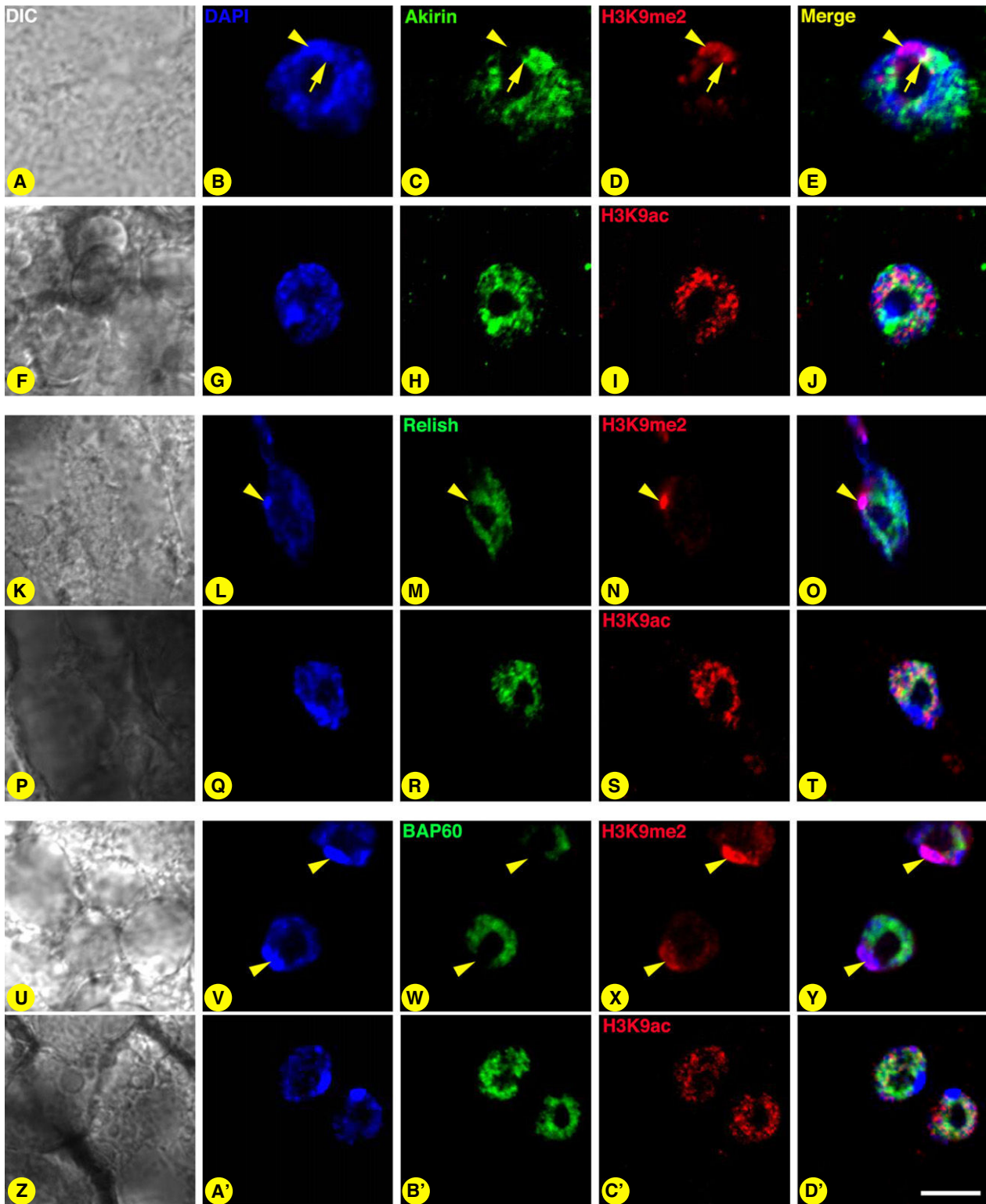
Immunolocalization, both in S2 cell culture (Supplementary Fig S4) and in adult *Drosophila* (Fig 3A–J and Supplementary Fig S5), showed that Akirin is ubiquitous, thus confirming the microarray data in Flybase (Crosby *et al*, 2007). We found that endogenous Akirin is strictly localized in the nucleus (Fig 3A–J and Supplementary Figs S4 and S5) as inferred from previous overexpression experiments (Goto *et al*, 2008). Interestingly, Akirin seems excluded from heterochromatic and transcriptionally inert regions, labeled by DAPI or an anti-H3K9 di-methyl (H3K9me2) antibody (Fig 3A–J and Supplementary Fig S4, see arrowhead in Fig 3E). However, as we observed a small overlap between heterochromatin and Akirin labeling (see arrow in Fig 3E), we cannot exclude that Akirin may also be involved in gene repression. In contrast, Akirin distribution within the nucleus matched H3S10 phosphorylation (H3S10p) and partially H3K9 acetylation marks (H3K9ac) (see Fig 3J and Supplementary Figs S4 and S6), indicating a pre-eminent role in active gene transcription. The NF- $\kappa$ B factor Relish is a 110-kD protein localized in the cytoplasm, cleaved upon immune challenge into 49 kD (Rel-49) and 68 kD (Rel-68) peptide, the latter being relocated to the nucleus and activating gene transcription (Stoven *et al*, 2000). Rel-68 as well as BAP60 sub-nuclear distributions (Fig 3K–T and Supplementary Fig S4) partially overlapped H3K9ac labeling and was excluded from heterochromatic regions (Fig 3U–D' and Supplementary Fig S4). Collectively, these results suggested a dynamic contribution of Akirin and BAP60 to the Brahma complex during immune challenge.

### Akirin, Relish, and the Brahma complex are recruited to the vicinity of IMD target genes

Although Akirin has been shown to function downstream, or at the level of, the NF- $\kappa$ B transcription factor Relish, yeast two-hybrid assays, failed to identify their interaction. Using S2 cells transiently transfected with V5-tagged Akirin and a Flag-tagged constitutively active form of Relish (Relish $\Delta$ S29–S45; Stoven *et al*, 2003), we immunoprecipitated Akirin with Relish $\Delta$ S29–S45 (Fig 4) and reciprocally. In addition, we established a stable S2 cell line that expressed V5-tagged Akirin under the control of the copper-inducible metallothionein promoter and immunoprecipitated V5-tagged Akirin from the lysate of these cells. We detected a faint band corresponding to endogenous Rel-68 in the blot of the Akirin precipitate, but the association between Rel-68 and Akirin was significantly enhanced upon HKE stimulation (Fig 4C). Additionally, we could immunoprecipitate *in vitro* His-tagged Akirin prepared in bacteria with Flag-tagged Relish  $\Delta$ S29–S45 purified from S2 cells suggesting their direct interaction (Supplementary Fig S7). Taken together, these data indicate that the interaction between Akirin and the NF- $\kappa$ B factor Relish depends on immune challenge.

A recent large-scale screen to isolate new interacting partners of IMD pathway core members identified the SWI/SNF Brahma complex BAP55 subunit as a putative partner of dIAP2, dTAK1, and IMD suggesting an involvement of BAP55 in the direct regulation of the IMD pathway (Fukuyama *et al*, 2013). We immunoprecipitated Flag-tagged BAP55 from transfected S2 cells and observed an interaction between Flag-BAP55 and Akirin-V5 (Supplementary Fig S2D), suggesting that the recruitment of the Brahma complex onto Akirin-dependent promoters is not triggered by a direct physical interaction with Relish.

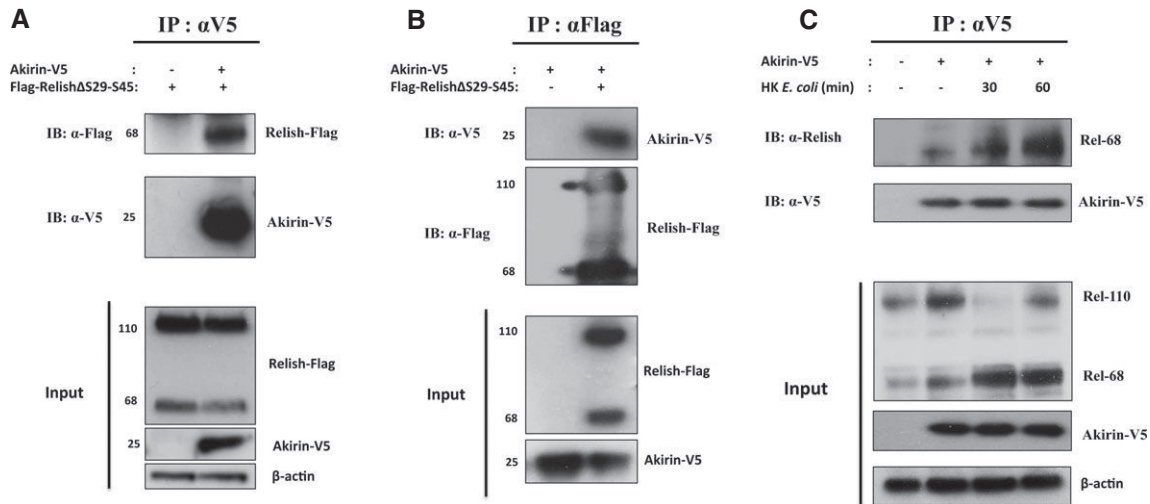
To determine if Akirin and the Brahma (SWI/SNF) remodeling complex were physically present on the promoter of Relish target genes, we immunoprecipitated sheared cross-linked chromatin prepared from *Drosophila* S2 cells stimulated by HKE at different time points, using anti-Relish, anti-phospho-serine 5 of the RNA Pol II carboxy-terminal domain (CTD), anti-Akirin, or anti-BAP60 antibodies (Fig 5A–F and Supplementary Fig S8). Chromatin IP (ChIP) of initiating RNA Pol II (anti-RNA Pol II S5p) (Corden, 1990) showed that Pol II was gradually recruited on *attacin-A* and *attacin-D* promoter sites (Supplementary Fig S8A). Additional ChIP experiments demonstrated that Relish, Akirin, and BAP60 were recruited simultaneously to the same site on Akirin-dependent proximal promoters (*p-attacin-A*, *p-drosocin*, *p-cecropin-A1*) following immune challenge (Fig 5A, C and D). In contrast, we found that Relish, but not BAP60 or Akirin, was recruited to the promoter of Akirin-independent proximal promoters (*p-attacin-D*, *p-metchnikowin*) upon HKE stimulation (Fig 5E and F). None or weak recruitment of Relish, Akirin, or Bap60 was observed on the *attacin-A* coding sequence or on the immune-unrelated hunchback promoter (Fig 5B and Supplementary Fig S8B). We found also that H3K4ac, an epigenetic mark of active gene transcription (Guillemette *et al*, 2011), was selectively enriched on Akirin-dependent, but not on Akirin-independent promoters (Fig 5A–F). Most importantly, during an immune challenge, the removal of either Akirin or Bap60 impaired the recruitment of Relish to Akirin-dependent promoter, preventing both H3K4 acetylation (Fig 5G–J) and subsequent gene transcription. Collectively, these data demonstrate that the presence of Akirin,



**Figure 3. Akirin, Relish, and Bap60 overlap non-condensed DNA regions in fat body cells.**

(A–D') Fat body cells from adult *Drosophila* were visualized by DIC (A, F, K, P, U, Z). Immunolocalization of Akirin (C and H), Relish (M and R), Bap60 (W and B'), H3K9me2 (D, N, X), the active chromatin marker H3K9ac (I, S, C'), and DAPI staining (B, G, I, Q, V, A') in whole fat body, 6 h after an immune challenge with *E. coli*. Akirin, Relish, and Bap60 sub-nuclear localizations were mostly excluded from DAPI-rich regions but partially overlapped H3K9ac regions (arrowheads) (E, J, O, T, Y, D'). In addition, Akirin systematically overlapped a small region in H3K9me2 distribution (arrows).

Data information: Images are representative of at least 3 fat body samples. Scale bars (all panels): 5  $\mu$ m.



**Figure 4. Heat-killed *E. coli* challenge stabilizes the interaction between Akirin and Relish.**

A, B Reciprocal co-immunoprecipitation assays between ectopic Akirin and Relish in S2 cells. Wild-type S2 cells were transiently transfected with V5-tagged Akirin and Flag-tagged Relish $\Delta$ S29-S45. Cell lysates were immunoprecipitated with (A) anti-FLAG coupled or (B) anti-V5 coupled agarose beads. Immunoprecipitates were analyzed by Western blotting with anti-V5 or anti-Flag antibodies.

C Heat-killed *E. coli* (HKE) promote the interaction of Akirin with Rel-68. S2 cells stably expressing V5-tagged Akirin were treated with heat-killed *E. coli* at the indicated time points. Cell lysates were immunoprecipitated using anti-V5 coupled agarose beads. Endogenous Relish was detected in Akirin immunoprecipitates using anti-Relish antibody.

Data information: Data are representative of 3 independent experiments.

BAP60, and Relish is required at the same level of the proximal promoter for an efficient transcription of Akirin-dependent genes.

### Promoter regions of Akirin-dependent genes

To understand the bases of Akirin specificity, we used bioinformatics to compare Akirin-dependent and Akirin-independent promoters. First, we evaluated if specific transcription factors would account for this specificity. Using MatInspector (<http://www.genomatix.de/>), we compared the DNA sequences of *attacin-A* and *attacin-D* promoters and identified the transcription factor binding sites specific for *attacin-A* and absent on the *attacin-D* promoter (Supplementary Fig S9A). The knockdown of these transcription factors by RNAi in S2 cells did not decrease *attacin-A-luciferase* induction upon immune challenge (Supplementary Fig S9B), ruling out a possible role for these transcription factors in Akirin-dependent transcription.

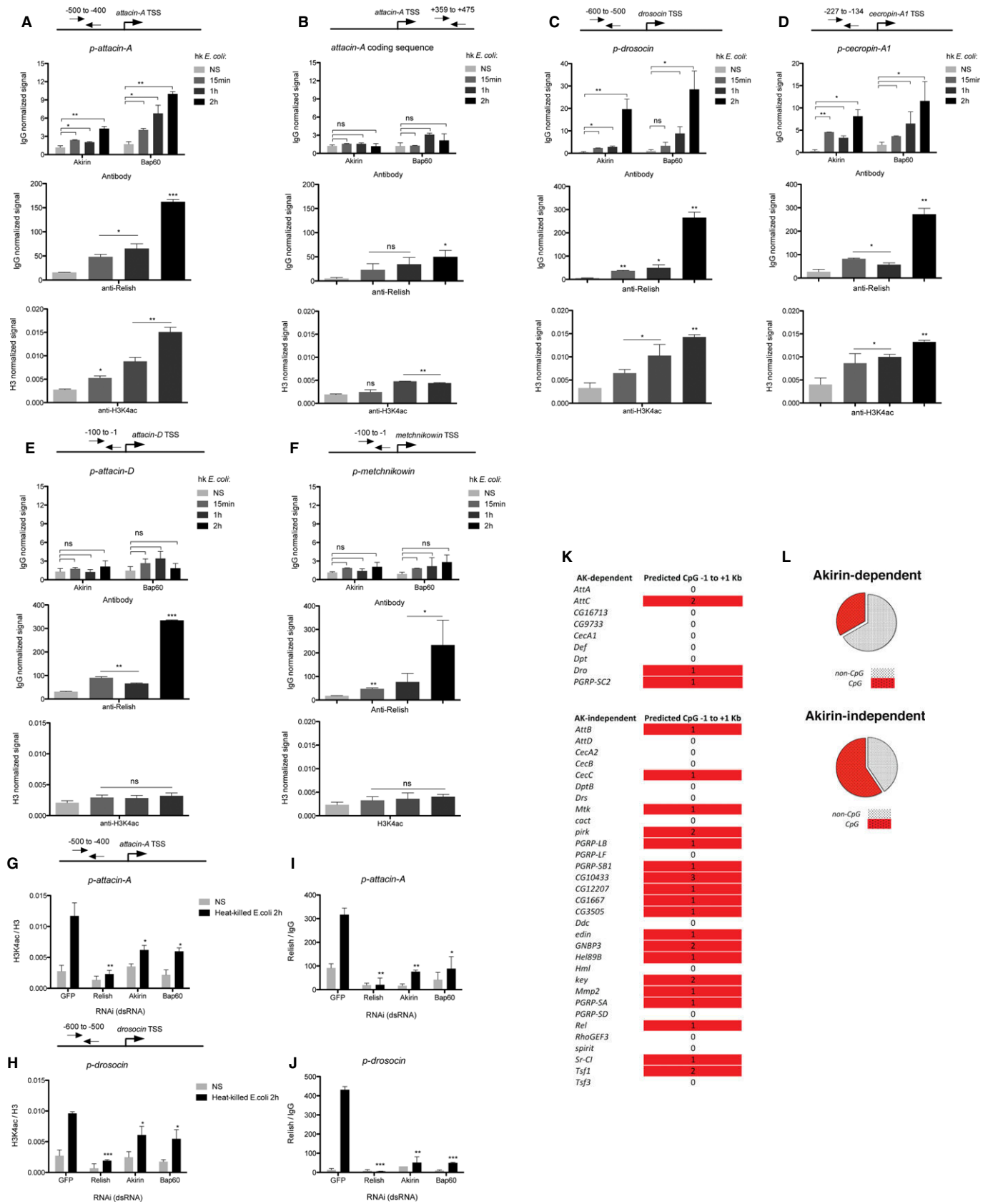
In mammalian cells, SWI/SNF-mediated nucleosome remodeling has been described to be required for the activation of immune gene promoters without CpG islands. In opposition, promoters located within CpG islands are frequently activated in a SWI/SNF-independent manner (Hargreaves *et al*, 2009; Ramirez-Carrozzi *et al*, 2009). The group of Prof. Osamu Takeuchi found that mammalian Akirin-2 interacts with the SWI/SNF complex upon immune challenge and activates preferentially target genes with low CpG content. Conversely, mammalian Akirin-2-independent genes were enriched in CpG islands (Tartey *et al*, 2014). Along the same line, we used a bioinformatics prediction tool (Cpplot; EMBOSS); we listed the CpG-rich regions predicted to be present in the vicinity (-1 kb to +1 kb) of the *Drosophila* IMD-dependent transcription start sites (Fig 5K). We observed that most Akirin-independent promoters were CpG-enriched (Fig 5L) and that Akirin-dependent promoters displayed a

low CpG content, suggesting that CpG-rich regions in *Drosophila* would somehow mimic mammalian CpG islands.

### The BAP complex fine-tunes the IMD-dependent innate immune response in *Drosophila*

As in human and yeast, distinct SWI/SNF-type ATP-dependent chromatin remodelers target two non-overlapping sets of genes in *Drosophila*, namely the BAP and the PBAP complexes (Wang, 2003; Mohrmann *et al*, 2004). In the fly, we find that RNAi-mediated silencing of the Osa-associated BAP complex genes, *bap55*, *bap60*, *brm*, *moira*, *osa*, and *snr-1*, reduced *attacin-A-luciferase* expression after HKE treatment to levels similar to those observed following *kenny* or *akirin* knockdown (Fig 6A). In contrast, with respect to the Polybromo-associated PBAP complex, we find that *polybromo* knockdown led to a significant increase of reporter expression. Importantly, we show that neither the BAP nor the PBAP complex was required for *attacin-D-luciferase* expression upon HKE treatment in S2 cells (Fig 6B). Collectively, these results established that the BAP complex, but not PBAP, was required for activation of the Akirin-dependent subset of Relish target genes.

*Drosophila* S2 cells, transfected with a constitutively active Toll receptor (Toll<sup>ALRR</sup>), showed strong activation of a *drosomycin-luciferase* reporter, fully blocked by the knockdown of *myd88*, a critical Toll receptor adaptor (Fig 6C) (Tauszig *et al*, 2000) (Tauszig-Delamasure *et al*, 2002). These basal levels of *drosomycin-luciferase* reporter expression were strongly enhanced upon Brahma complex component knockdown (*bap55*, *bap60*, *brahma*, *moira*, *osa*, *snr-1*, or *polybromo*), demonstrating that the SWI/SNF complex negatively regulated expression of Toll pathway target genes in *Drosophila* S2 cells as previously observed (Kuttenkeuler *et al*, 2010).





**Figure 5. Akirin, Bap60, and Relish bind on Akirin-dependent immune gene promoters.**

- A–F Chromatin IP with anti-Akirin, anti-Bap60, anti-Relish, and anti-H3K4ac antibodies on sheared chromatin from S2 cells following heat-killed *E. coli* stimulation at indicated time points. The graphs show recruitment of Akirin, Bap60, and Relish, relative to the values obtained with rabbit control IgG, or of H3K4ac relative to the values obtained with anti-H3 antibody on two Akirin-dependent (A, *p-attacin-A*; C, *p-drosocin*; D, *p-cecropin-A1*), Akirin-independent (E, *p-attacin-D*; F, *p-metchnikowin*) genes proximal promoter, or on *attacin-A* coding sequence (B) as an internal control.
- G–J Chromatin IP with anti-Relish and anti-H3K4ac antibodies on sheared chromatin from S2 cells knocked down for *GFP*, *relish*, *akirin*, or *bap60* following heat-killed *E. coli* stimulation at indicated time points. The graphs show recruitment of Relish (I, J) relative to the values obtained with rabbit control IgG, or of H3K4ac (G, H) relative to the values obtained with anti-H3 antibody on two Akirin-dependent (G, I, *p-attacin-A*; H, J, *p-drosocin*) proximal promoters.
- K Bioinformatical CpG-rich region analysis of Akirin-dependent and Akirin-independent promoters. Predicted CpG-rich regions were counted on the genomic regions –1 kb to +1 kb relative to the transcription start site for Akirin-dependent and Akirin-independent genes with CpGplot (EMBOSS). Red squares annotate genes containing at least one CpG-rich region within its promoter.
- L Pie chart representation of CpG-rich region analysis of Akirin-dependent and Akirin-independent promoters. Red areas annotate genes containing at least one CpG-rich region within its promoter.

Data information: Data are represented as mean  $\pm$  standard deviation of three independent experiments performed on  $1.5 \times 10^6$  (A–F) or  $5 \times 10^5$  cells (G–J) per IP. Hk *E. coli*: heat-killed *E. coli*. TSS: transcriptional start site. Statistical significance was established by comparing values from stimulated (15 min, 1 h, 2 h of hk *E. coli*) with unstimulated conditions (NS) (A–F) or comparing Relish, Akirin, and Bap60 knockdown with GFP dsRNA control in stimulated (2 h hk *E. coli*) conditions (G–J). \**P*-value < 0.05; \*\**P*-value < 0.01; \*\*\**P*-value < 0.001.

We next investigated if Akirin and the Brahma complex were similarly required for transcriptional selectivity *in vivo*. As *Drosophila* embryonic development is impaired in absence of Akirin or functional Brahma complex, we used the *C564-Gal4* (Hrdlicka *et al*, 2002) or *Hml-Gal4* (Goto *et al*, 2001) transgenes to express RNAi constructs targeting *akirin*, *brahma*, *moira*, *relish*, and *polybromo*, respectively, in the adult fat body (Supplementary Fig S10A) or in larval hemocytes (Supplementary Fig S11A and B). Of note, even restricted to the fat body, the knockdown of *osa* was lethal to the flies. Following *E. coli* immune challenge, expression of *Attacin-A*, *Attacin-C*, and *Diptericin-A* was significantly reduced in the absence of Akirin or a functional Brahma complex, when compared to *Attacin-D*, *Cecropin-A2*, *Cecropin-B*, and *Pirk* or control (*RNAi-GFP*, Fig 6D–I, Supplementary Fig S11C and D). However, all these IMD pathway effector genes were dependent on Relish, but independent of Polybromo (Fig 6D–I).

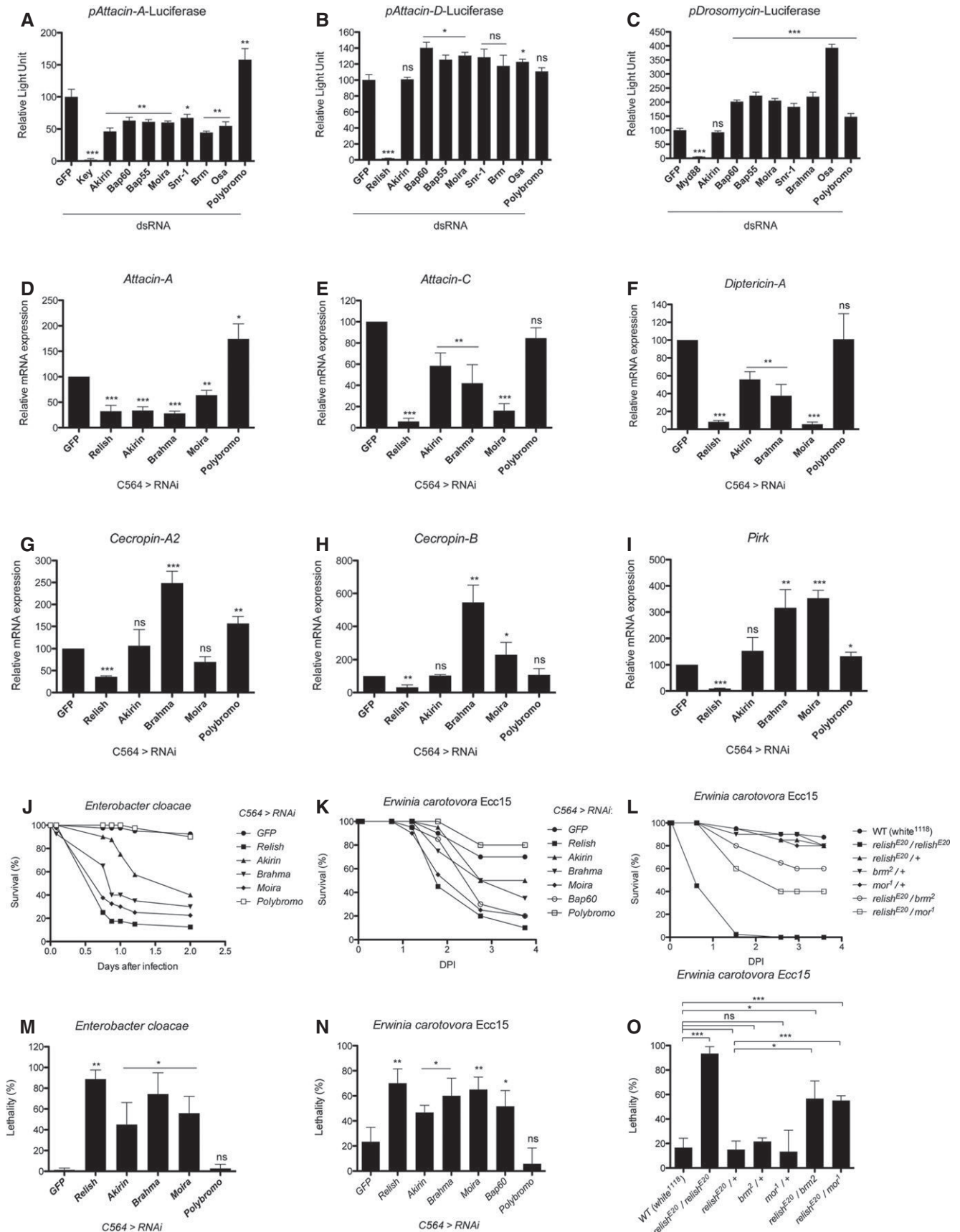
Flies depleted of Akirin (*C564 > RNAi-akirin*), Relish (*C564 > RNAi-relish*), or members of the Brahma complex (*C564 > RNAi-brahma* or *C564 > RNAi-moira*) had a significant decrease in survival following *Enterobacter cloacae* (*E. cloacae*) (Bou Aoun *et al*, 2011) or *Erwinia carotovora* Ecc15 (*E. carotovora* Ecc15) (Vidal *et al*, 2001) infections when compared to control flies (*C564 > RNAi-GFP*) or flies lacking a functional PBAP complex (*C564 > RNAi-polybromo*) (Fig 6J, K, M and N). Flies carrying a single functional copy of *relish* and *brahma* or *relish* and *moira* also showed a significant decrease in survival after *E. carotovora* Ecc15 infection (Fig 6L and O). In addition, flies lacking Akirin, Relish, or components of the Brahma complex were not susceptible to the entomopathogenic fungus *Beauveria bassiana* (*B. bassiana*), a classical agonist of the Toll pathway (Supplementary Fig S10C–E) (Lemaitre *et al*, 1997). Taken together, our results demonstrate that Akirin and the BAP complex dynamically interact to selectively activate a subset of Relish target genes during the immune response, allowing *Drosophila* to survive a Gram-negative bacterial challenge.

## Discussion

The IMD pathway in *Drosophila* regulates the systemic immune response against Gram-negative bacteria, and the molecular cascade

from the PGRP-LC receptor down to the activation of the NF-κB factor Relish has been extensively studied. The Akirin molecule is required for IMD target gene activation by the Relish transcription factor (Goto *et al*, 2008), and this finding suggests that IMD effector gene transcription might depend on additional factors that remained to be identified. In order to further elucidate NF-κB-dependent gene activation, we re-explore the IMD pathway using Akirin as a starting point. We undertook an unbiased two-hybrid screen that identified BAP60 as an Akirin transcriptional partner during the innate immune response, confirming the data of the protein-interaction map of the fly proteome (Giot *et al*, 2003). Additionally, we show that BAP55, an Actin-related component of the SWI/SNF Brahma complex (Papoulas *et al*, 1998; Armstrong *et al*, 2002), engages Akirin upon immune challenge, as does the NF-κB factor Relish itself.

BAP60 is a core component of the SWI/SNF-like BAP complex, conferring site-specific anchoring properties at specific promoter sites, via direct binding to transcription factors such as SisterlessA or Scute (Moller *et al*, 2005). Although BAP60, Relish and Akirin are part of the same complex (Figs 2 and 4, and Supplementary Figs S2D and S3), we could detect a direct interaction between Akirin and BAP60, probably between Akirin and Relish, but not between BAP60 and Relish. We speculate that Akirin might act as a bridge between Relish and BAP60 in order to recruit the SWI/SNF complex to the vicinity of Relish target genes. Alternatively, we cannot exclude that Akirin and the SWI/SNF complex are recruited on the promoter of Relish target genes independently of Relish itself. Consistent with this SWI/SNF-dependent chromatin remodeling process, it was recently suggested that DNA-methyltransferase associated protein 1 (DMAP1), also known to interact with BAP55 (Guruharsha *et al*, 2012), would associate with Akirin (Goto *et al*, 2014). The possibility that methyl groups on H3K4 are replaced by acetyl groups to allow full transcription would fit with our finding that H3K4ac is a hallmark of active Akirin-dependent promoters. It has been shown that Akirin links BAP60 to the transcription factor Twist during *Drosophila* myogenesis (Nowak *et al*, 2012). Thus, Akirin might act as a molecular bridge between BAP60 and several other transcription factors. Notably, this interaction between BAP60 and Akirin is conserved during evolution as mouse Akirin-2 binds all three BAF60s, the mammalian homologs of *Drosophila* BAP60 (Prof. Osamu Takeuchi personal communication).



**Figure 6. The Brahma BAP complex is required for Akirin-dependent immune response against Gram-negative bacteria.**

- A, B Dual luciferase assay from S2 cell extracts co-transfected with *attacin-A*- (A) or *attacin-D*-luciferase (B) reporter plasmids and dsRNAs against *GFP*, *kenny* (*key*), *akirin*, or Brahma complex members following 48 h of heat-killed *E. coli* stimulation.
- C Dual luciferase assay from S2 cell extracts co-transfected with *drosomycin-luciferase*, Toll<sup>ALRR</sup> *pActin5C* expressing vector, and dsRNAs against *GFP*, *kenny* (*key*), *akirin*, or Brahma complex members. Results were normalized to the dsRNA *GFP* controls.
- D–I Quantitative RT–PCR of *Attacin-A*, *Attacin-C*, *Diptericin-A*, *Cecropin-A2*, *Cecropin-B*, and *Pirk* mRNA on *C564-gal4/UAS-RNAi* flies following an *E. coli* 6-h challenge.
- J, K Survival assays following *E. cloacae* (J) or *E. carotovora* Ecc15 (K) septic infection of *C564-gal4/UAS-RNAi* flies.
- L Survival assay from *E. carotovora* Ecc15 septic infection of *relish*<sup>E20</sup>, *brahma*<sup>2</sup>, and *moira*<sup>2</sup> heterozygous or trans-heterozygous mutant flies.
- M, N Lethality calculations following *E. cloacae* (M) or *E. carotovora* Ecc15 (N) septic infection of *C564-gal4/UAS-RNAi* flies.
- O Lethality calculation from *E. carotovora* Ecc15 septic infection of *relish*<sup>E20</sup>, *brahma*<sup>2</sup>, and *moira*<sup>2</sup> heterozygous or trans-heterozygous mutant flies.

Data information: Data are represented as mean  $\pm$  standard deviation of three independent experiments performed with three batches of 15–20 flies. \**P*-value < 0.05; \*\**P*-value < 0.01; \*\*\**P*-value < 0.001.

Unlike the Polybromo/BAP170 containing SWI/SNF complex (PBAP), the BAP complex is required during the immune response against Gram-negative bacterial infections, to coordinate the transcription of IMD pathway effector genes. In contrast, during embryonic myogenesis, Akirin interacts genetically with both the BAP and PBAP complexes (Nowak *et al*, 2012). In addition, both the PBAB and BAP complexes are involved in the negative regulation of the Toll pathway (Fig 6C), suggesting that the specificity of Akirin toward BAP, or PBAP, is transcription-factor dependent.

In murine macrophages depleted of functional SWI/SNF complexes, LPS stimulation results in the activation of only a subset of TLR4 target genes (Ramirez-Carrozzi *et al*, 2006). This SWI/SNF-based selectivity was recently suggested to be dependent on the differential CpG island context of NF- $\kappa$ B target gene promoters. Absence of CpG island results in stable nucleosome assembly at promoter sites, requiring both chromatin remodeling and transcription factors to activate gene transcription. In contrast, CpG islands appear to be responsible for unstable nucleosome assembly at promoter sites, thus explaining their SWI/SNF independence (Ramirez-Carrozzi *et al*, 2009). The genome of *D. melanogaster* is unmethylated and lacks classical CpG islands (Deaton & Bird, 2011). Even though *Drosophila* does not display CpG islands or methylation (Nanty *et al*, 2011), we undertook a bioinformatic analysis (EMBOSS, CpG plot) and identified an enrichment of the CpG content in the sequences spanning the NF- $\kappa$ B target genes that are independent of Akirin and the SWI/SNF complex. In contrast, the promoters of Akirin and SWI/SNF-dependent genes are depleted of CpG-rich regions. However, these data cannot be generalized as we have only analyzed immune genes. Work from our collaborators (Prof. Osamu Takeuchi personal communication) suggests similarly that mouse Akirin-2-dependent gene promoters show a low frequency of CpG island association compared to Akirin-2-independent promoters. It is tempting to speculate that, like CpG islands in vertebrates, CpG-rich sequences in *Drosophila* would establish regions of nucleosomal instability precluding any need of Akirin and the SWI/SNF complex for the control of gene transcription. However, additional factors such as H3K4ac marks must account for the observed Akirin selectivity.

In *Drosophila*, exposure to microbial cell wall proteoglycans or danger signals leads to the activation of the IMD or Toll pathways resulting in the nuclear translocation of their respective NF- $\kappa$ B factors and activation of the transcription of target genes (Ferrandon *et al*, 2007). These effector genes encode not only AMPs, but also molecules that feed back to regulate these pathways and dampen their response. Similarly to mammals, activation and resolution of

the *Drosophila* innate immune response have to be tightly controlled in order to prevent adverse side effects (Ryu *et al*, 2008; Paredes *et al*, 2011; Bonnay *et al*, 2013). Here, we have identified Akirin as an NF- $\kappa$ B co-factor required for the selective transcription of a subset of direct immune effectors, that is AMPs, but dispensable for the expression of genes encoding negative regulators of the IMD pathway (except *PGRP-SC2*).

Removing Akirin or Brahma lead to an impaired expression of several antimicrobial peptide-coding genes, resulting in a weakened innate immune defense of *Drosophila* against Gram-negative bacteria. This observation suggests that the full cocktail of IMD-induced anti-microbial peptides is required to efficiently contend Gram-negative bacterial infections. The evolutionary reason why two distinct groups of AMPs coding genes, sharing similar bactericidal features, are under the transcriptional control of either Relish alone or in combination with Akirin is still an open question. As mammalian Akirin-2 similarly displays pro-inflammatory properties (Prof. Osamu Takeuchi personal communication and (Goto *et al*, 2008), Akirins represent putative therapeutic targets for small chemicals able to block the inflammatory response without interfering with the expression of genes involved in the resolution of inflammation.

## Materials and Methods

### Fly strains

Stocks were raised on standard cornmeal–yeast–agar medium at 25°C with 60% humidity. *w*<sup>1118</sup> mutant flies were used as control. *relish*<sup>E20</sup> (Hedengren *et al*, 1999) and *Myd88*<sup>c03881</sup> (Tauszig-Delamasure *et al*, 2002) flies were used as mutant deficient for the IMD and Toll pathway, respectively. Flies carrying an *UAS-RNAi* transgene targeting *relish* (108469), *akirin* (109671), *brahma* (37720), *moira* (6969), *bap60* (12675) *osa* (7810), and *polybromo* (108418) were obtained from the Vienna *Drosophila* RNAi Center (<http://stockcenter.vdrc.at/control/main>). Flies carrying a *UAS-RNAi* transgene against *GFP* (397-05) were obtained from the *Drosophila* Genetic Resource Center (Kyoto, Japan; <http://www.dgrc.kit.ac.jp/index.html>). *moira*<sup>1</sup> (3615) and *brahma*<sup>2</sup> (3622) mutants and flies carrying Gal4 driver *C564* (6982) used to express *UAS* constructs in the fat body (Hrdlicka *et al*, 2002) were obtained from Bloomington *Drosophila* Stock Center (Bloomington, USA; <http://flystocks.bio.indiana.edu/>). Gal4-driven *RNAi* expression was enhanced by incubating 3-day-old flies for six further days at 29°C.

## Microbial strains and infections

We used *Escherichia coli* strain DH5 $\alpha$ GFP, *Enterobacter cloacae*, *Erwinia carotovora* Ecc15, and *Micrococcus luteus* (CIPA270) bacteria for septic injuries (Reichhart et al, 2011). Natural *B. bassiana* infections were performed as previously described (Lemaitre et al, 1997). The *E. coli* strain DH5 $\alpha$ GFP was generated in our laboratory. Bacteria were grown in Luria broth (LB) (*E. coli*, *E. cloacae*, *E. carotovora* Ecc15) or brain–heart infusion broth (BHB) (*M. luteus*) at 29°C (*E. cloacae*, *E. carotovora* Ecc15) or 37°C (*E. coli*, *M. luteus*). Survival experiments were performed on two batches of 15–20 nine-day-old females infected by *E. cloacae* or *E. carotovora* Ecc15 septic injury or *B. bassiana* natural infection at 25°C three independent times. Control survival experiments (Supplementary Fig S10B) were made by sterile injury (Reichhart et al, 2011). qRT–PCR experiments were performed on three batches of 10–20 nine-day-old males infected with *E. coli* for 6 h, or *M. luteus* for 24 h, by septic injury at 25°C, three times independently. Immunostaining experiments were performed on 3-day-old control (*w<sup>1118</sup>*) females infected with *E. coli* for 6 h.

## Cell sorting and microarray analysis

To perform microarray,  $2 \times 10^6$  S2 cells ( $10^6$ /ml) were transfected in 6-well plates by calcium phosphate precipitation with 1  $\mu$ g of *p-actin5C-tomato*, 1  $\mu$ g of *p-actin5C-PGRP-LCa* (or empty *p-actin5C* vector), and 5  $\mu$ g of dsRNAs against *GFP*, *relish*, or *akirin*. After 12–16 h, the cells were washed with PBS and incubated in fresh complete Schneider's medium for 48 h. Cells were rinsed with PBS and re-suspended in serum-free Schneider's *Drosophila* Medium (Biowest) before sorting.  $10^5$  to  $5 \times 10^5$  transfected Tomato-positive S2 cells were sorted in serum-free medium with the help of to the flow cytometry facility at Institut de Génétique et de Biologie Moléculaire et Cellulaire (Illkirch, France; <http://www.igbmc.fr/technologies/6/team/64/>). RNA was extracted and treated with DNase, using RNA Spin kit (Macherey Nagel). RNA quality was checked by Eukaryote Total RNA Pico assay (Agilent) and validated with a RIN > 6.5. 200 ng of RNA were used to perform microarray (Agilent DNA microarrays *Drosophila*) at the GeneCore Genomics facility of EMBL (<http://genecore3.genecore.embl.de/genecore3/>). Total RNA was quantified on Invitrogen Qubit 2.0 Fluorometer (Q32866) and quality-checked on the Agilent Bioanalyzer 2100 (G2940CA). Samples were normalized to 100 ng in 1.5  $\mu$ l working volume for the labeling reaction and were one-color Cy3-labeled using Agilent LowInput QuickAmp Labeling Kit (5190-2331). The resulting Cy3-labeled cRNAs were then hybridized onto the 4x44k *Drosophila* V2 microarray using Agilent GeneExpression Hyb Kit (5188–5242) for 20 h at 65°C. The microarray was scanned using Agilent Microarray Scanner (G2565CA), and data extracted with Feature Extraction Software v10.7.2. *relish* and *akirin* were reported, respectively, as Relish and Akirin-dependent genes in our assay, validating their knockdown. The GEO accession number for the microarray data is GSE54915.

## Two-hybrid

Two-hybrid screens and assays were carried out using a LexA-based system (Vojtek et al, 1993) and yeast strains L40 $\Delta$ GAL4 (Fromont-

Racine et al, 1997) (kind gift of Drs. P. Legrain and M. Fromont-Racine) and Y187 (Clontech). A 0–24 h *Drosophila* embryo cDNA library was a generous gift of Dr. S. Elledge. Standard yeast handling techniques were used.

## Immunoprecipitation and Western blot

Cells were treated for the indicated times with heat-killed *E. coli* (40:1) at 25°C. The cells were harvested, washed in PBS, and lysed in 500  $\mu$ l of buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM DTT, 1% NP-40, phosphatase inhibitor cocktail (Roche), and complete protease inhibitor cocktail (Roche). Immunoprecipitations were performed overnight at 4°C with rabbit polyclonal anti-Akirin antibody coupled with Dynabeads Protein G (Invitrogen), anti-V5 agarose (Sigma), or anti-Flag agarose (Sigma). Proteins from total cell lysates and immunoprecipitates were resolved by SDS-PAGE and detected by Western blotting using anti-V5 HRP (Invitrogen), anti-Akirin, anti-Flag HRP (Sigma), anti-Bap60 (gift from Susumu Hirose), anti-Relish (gift from Tony Ip), and anti- $\beta$ -actin antibodies (BD Transduction Laboratories).

## Chromatin immunoprecipitation

ChIP was carried out as previously described (Batsche et al, 2006). S2 cells were cross-linked in phosphate-buffered saline (PBS) containing 1% formaldehyde (Sigma) for 10 min at room temperature. The crosslinking reaction was quenched with PBS containing 125 mM glycine. The chromatin was fragmented by sonication to produce average DNA lengths of 0.5 kb. 2  $\mu$ g of rabbit polyclonal anti-Akirin, anti-BAP60, anti-Relish, anti-H3 (Abcam, ab1791), anti-H3K4ac (Abcam, ab113672), anti-RNA Pol II CTD repeat YSPTSPS (phospho S5) (Abcam, ab5131), and rabbit control IgG (Abcam, ab46540) were used for IP. After ChIP, the eluted DNA was detected by quantitative PCR using the primers listed in Supplementary Table S1. Levels of Akirin, BAP60, and Relish are expressed relatively to the signal obtained for ChIP using rabbit control IgG. The level of H3K4ac is expressed relatively to the signal obtained for ChIP using anti-H3 antibody. Values are averaged from three independent experiments.

## Bioinformatical analysis

Predicted CpG-rich regions were counted on the genomic regions –1 kb to +1 kb relative to the transcription start site for Akirin-dependent and Akirin-independent genes with CpGplot (EMBOSS).

Predicted transcription factors' binding sites were analyzed with MatInspector ([www.genomatix.de/](http://www.genomatix.de/)) from the proximal 1 kb sequence of *attacin-A* or *attacin-D* 5'-promoter.

More methods are available in the Supplementary Methods section.

Supplementary information for this article is available online: <http://emboj.embopress.org>

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### Author contributions

FB, X-HN, J-MR, and NM designed research. FB, X-HN, EC-B, EB, and NM performed research. FB, XHN, EC-B, EB, LT, JC, J-MR, and NM analyzed data. OT shared data; J-MR and NM wrote the paper.

### Conflict of interest

The authors declare that they have no conflict of interest.

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**Akirin specifies NF- $\kappa$ B selectivity of *Drosophila* innate immune response via chromatin remodeling**

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## SUPPLEMENTARY METHODS

### Cell culture and transfection

S2 cells were cultured at 25°C in Schneider's medium (Biowest) supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin (50 µg/ml of each) and 2 mM glutamax. For transient transfection, S2 cells were seeded in 100-mm-diameter tissue culture plates at  $10 \times 10^6$ /plate 16 h prior to transfection. Transfection was performed by the calcium phosphate co-precipitation method. Each plate was transfected with 30 µg of indicated plasmids. After 12–16 h, the cells were washed with PBS and incubated in fresh medium. The cells were harvested 48 h later, and whole-cell lysates were prepared for immunoprecipitation or western blot.

### dsRNA preparation

DNA Templates for dsRNA preparation were PCR-derived fragments flanked by two T7 promoter sequences (TTAATACGACTCACTATAGG). Fragments for *GFP*, *kenny* and *akirin* were as follows: *GFP* (nucleotides 35–736, GenBank accession L29345), *kenny* (nucleotides 222–744, NCBI accession NM\_079132), *akirin* (nucleotides 100–600; GenBank accession number AY095189).

Fragments for putative Akirin partners and the Brahma complex were generated from genomic DNA templates using oligonucleotides designed for use with DKFZ Genome-RNAi libraries and are listed in Supplementary Table 2.

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



### **dsRNA bathing**

Cultured S2 cells were pelleted and washed once in PBS to remove fetal calf serum (FCS) supplemented Schneider's medium and resuspended in serum-free Schneider's medium (Biowest) supplemented with penicillin (50 µg/ml of each) and 2 mM glutamax, at  $1,5 \times 10^6$  cells/ml. 30 µl of this cell suspension ( $45 \times 10^3$  cells) was added to 10 µl of dsRNA (500 ng/µl) and incubated at 23 °C for one hour in a U-shape 96-wells plate. 160 µl of FCS-supplemented Schneider's medium was then added and cells were incubated for six days at 23 °C. Cells were detached from the U-shape 96-wells plate by pipetting up and down, counted and plated into a 24-wells plate at  $5 \times 10^5$  cells / well. Cells were stimulated with heat-killed *E. coli* (40:1) for 2 or 4 hours, washed once in PBS, and frozen prior to RNA extraction. RNA was extracted and treated with DNase, using RNA Spin kit (Macherey Nagel). Reverse-transcription and quantitative real-time PCR were performed as indicated below.

### **Immunofluorescence and Histology**

Rabbit polyclonal anti-Akirin (1/100), anti-Relish (courtesy of Tony Ip; 1/500), anti-Bap60 (courtesy of Susumu Hirose; 1/500), anti-phospho-Histone H3 (Ser10) (H3S10p) (Millipore, 05-1336; 1/200) and mouse monoclonal anti-H3K9me2 (Abcam, ab1220; 1/500) primary antibodies were used at the indicated (v/v) dilutions in PBS containing 0,1% Triton X-100 and 1% (wt/vol) bovine serum albumin (PBS-TA). Goat anti-rabbit Alexa Fluor®488 and goat anti-mouse Cy3 secondary antibodies (Invitrogen) were used at 1/1,000 in PBS-TA. Fat-bodies from *E. coli* infected females flies were dissected in PBS, fixed in PBS containing 4% (wt/vol) paraformaldehyde for 20 min at RT. Heat-killed *E. coli* stimulated S2 cells were seeded on eight-wells Lab-Tek®II Chamber Slide™, fixed in PBS containing 2% (wt/vol) paraformaldehyde for 15 min at RT and saturated in PBS-TA. Slides were mounted in a solution of Vectashield/DAPI (Vector Laboratories) and observed using a Zeiss LSM780

confocal microscope. Images were processed using Adobe Photoshop.

### **Luciferase assays**

*Drosophila* S2 cells were transfected with 50 ng of *p-attacin-A-firefly luciferase* (*attacin-A-luciferase*), *p-attacin-D-firefly luciferase* (*attacin-D-luciferase*) or *p-drosomycin-firefly luciferase* (*drosomycin-luciferase*) reporter vectors, 12,5 ng of a *p-actin5C-renilla luciferase* transfection control vector and 1,25 µg of dsRNAs. Cells were stimulated with heat-killed *E.coli* (40:1) for 48 h, lysed and luciferase activity was quantified in a luminometer (Mithras LB 940, Berthold technologies) immediately after addition of the substrate (Dual luciferase assay kit, Promega). All experiments were done more than three times independently with duplicate wells.

### **RNA extraction and quantification**

Total RNAs from whole flies and dissected fat-bodies were extracted with Trizol Reagent® RT (Molecular Research Center) after mechanical lysis by 1,4 mm ceramic beads using a Precellys®24 tissue homogenizer (Berthin technologies). RNA was reverse-transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad). 1-10 ng of reverse-transcribed RNA was used as a template for quantitative real-time PCR (Q-RT-PCR). Q-RT-PCR reactions were set up using iQ™ Custom SYBR® Green Supermix (Bio-Rad) and in a 1/50,000–1/75,000 final concentration of SYBRGreen. Real-time PCR was then performed in 384-well plates using a CFX384 system (Bio-Rad). The level of expression of the gene of interest was then normalized against the measured level of the RNA coding for ribosomal protein-49 determined in each sample. Primers used for Q-RT-PCR are listed in Supplementary Table 1.

### **shRNA-expressing stable cell-lines generation**

Copper-inducible *pMetallothionein* shRNA-Akirin stably transfected S2 cells were generated by CaPO<sub>4</sub> co-transfection of 1 µg vector containing a metallothionein promoter (pMT-V5) expressing *Drosophila* miR-1-based short-hairpin RNA directed against *akirin* (designed after Transgenic RNAi project database; <http://www.flyrnai.org/TRiP-HOME.html>; sense sequence: TTGCTAAGAAGCGAGACGAAA) and 100 ng of puromycin resistance selection vector (pJL1) in 3 x 10<sup>6</sup> S2 cells. Transfected cells were seeded in 96-well plates at 10<sup>5</sup> cells/ml in complete Schneider's medium containing 1 µg/mL puromycin for selection. Positive clones were selected and amplified. RNAi was expressed by adding 0,5 mM CuSO<sub>4</sub> for 4 days to the culture medium.

### ***In vitro* binding assay**

His-tagged Akirin was expressed from the pDEST17 (Invitrogen) plasmid in the *Escherichia coli* strain Rosetta-gami B (DE3) pLysS (Novagen). Expression was induced with 0.1 mM IPTG at 20°C for 16 h. Bacterial cells were lysed in lysis buffer (25 mM Hepes pH 7.5, 300 mM KCl, 5 mM imidazole, 0.1 mM PMSF, 5 mM DTT, protease inhibitor cocktail) by sonication, and the resulting lysate was cleared by centrifugation at 15,000 rpm for 60 min at 4°C. His-tagged Akirin was purified with His GraviTrap column (GR Healthcare) and eluted with a buffer containing 20 mM sodium phosphate, 500 mM NaCl and 500 mM imidazole, pH 7.4. Flag-tagged Relish $\Delta$ S29-S45 was expressed in S2 cells and pulled down using anti-Flag M2 agarose affinity gel (Sigma). The beads were washed extensively in lysis buffer. After a washing step, purified His-tagged Akirin was added to the beads, and the mixture was incubated at 4°C overnight. The beads were washed four times in lysis buffer and then boiled. Proteins were detected by Western blotting with anti-Flag (Sigma) and anti-His antibodies (Invitrogen).

## Statistical analysis

All P values were calculated using the two-tailed unpaired Student t test (Graph-Pad Prism).

## SUPPLEMENTARY FIGURE LEGENDS

### Figure S1. Experimental strategy for microarray analysis.

**A.** S2 cells were co-transfected with Tomato *p-actin5C*, PGRP-LCa *p-actin5C* or empty *p-actin5C* vectors and dsRNAs against *GFP*, *relish* or *akirin*. Transfected Tomato-positive cells were sorted and processed to perform mRNA analysis by Q-RT-PCR or *Drosophila* microarrays.

**B.** Venn diagram and table representation of microarray analysis. Upon PGRP-LC stimulation, genes showing a two-fold increase in expression from S2 cells knocked-down for *relish*, or simultaneously for *relish* and *akirin*, compared to our control (dsRNA against *GFP*) are listed. The numbers in brackets corresponds to genes with GO-terms matching immune function. These immune-related genes are listed with a color code, red corresponding to anti-microbial peptides, blue to negative regulators of NF- $\kappa$ B pathways and beige to others immune related functions.

**C.** Quantitative RT-PCR of *Attacin-D*, *Pgrp-lb*, *Attacin-C*, *Diptericin-A*, *Akirin* and *Relish* mRNA from S2 cells bathed in dsRNA against *GFP*, *relish* or *akirin* and stimulated at indicated time points with heat-killed *E. coli*. Data are represented as mean +/- standard deviation of three independent experiments. \**P* value < 0.05; \*\**P* value < 0.01; \*\*\**P* value < 0.001.

### Figure S2. Yeast two-hybrid screen and co-immunoprecipitation between Akirin and Brahma complex members.

**A.** Schematic representation of the bait constructs used in two-hybrid experiments. LexA DNA binding domain (DBD), NLS and Akirin sequences are annotated. The numbers represent Akirin amino-acids used in bait constructs.

**B.** Histidine prototrophy tests. AK, AK<sup>Δ1-139</sup> and Ras were expressed in the L40 yeast two-hybrid reporter strain as fusions to LexA DBD, together with Raf fused to the GAL4 activation domain used here as positive control. A plasmid expressing only the GAL4 activation domain was also used as a negative control. Growth on medium lacking Tryptophan (Trp(-)); Leucine (Leu(-)) and Histidine (His(-)), indicates a positive two-hybrid interaction.

**C.** Identified partners of Akirin and their function in the IMD pathway. The two first columns (“Gene” and “interaction”) list genes encoding proteins interacting with full-length (AK) or N-terminally truncated (AK<sup>Δ1-139</sup>) Akirin protein. The third column (“IMD”) indicates the effect of the knock-down of each gene on *attacin-A-luciferase* activity in S2 cells following 48h heat-killed *E. coli* stimulation. The effect on the IMD pathway is considered as “negative”, if the knock-down leads to a significant (*P* value at least < 0.05) increase of more than 50% over the control sample in *attacin-A-luciferase* activity; “positive”, if the knock-down leads to a significant decrease of more than 30% below control sample in *attacin-A-luciferase* activity; “no”, if the knock-down leads to no change. Results are representative of at least three independent experiments.

**D.** Akirin associates with BAP60 and BAP55. WT S2 cells were transiently transfected with Flag-tagged BAP55 or BAP60 and V5-tagged Akirin as indicated. Cell lysates were immunoprecipitated using anti-Flag coupled agarose beads followed by immunoblotting with anti-V5, anti-Flag antibodies. Representative data from 3 independent experiments are shown.

**E.** Whole cell lysates from wild-type S2 cells (first lane) and copper-inducible *pMetallothionein* shRNA-Akirin stably transfected S2 cells (second and third lane) with (third

lane) or without (first and second lane) CuSO<sub>4</sub> treatment were immuno-blotted with Akirin antibody to assess antibody specificity.  $\beta$ -actin was used as loading control.

Data are representative of 3 experiments.

**Figure S3. Akirin and Bap60 dynamically interact starting from 2hours post-*E. coli* stimulation.**

**A.** WT S2 cells were stimulated with heat-killed *E. coli* for 15 or 120 min. Cell lysates were immunoprecipitated and immunoblots revealed with anti-Bap60 or anti-Akirin antibodies.

**B.** Quantification by band intensity measurement (Image J) of Bap60 proteins bound to Akirin relative loading.

Representative data from 3 independent experiments are shown. WCL: Whole-cell lysate; IP: Akirin: Immuno-precipitation with anti-Akirin antibody; IP: Bap60: Immuno-precipitation with anti-Bap60 antibody.

Data are represented as mean +/- standard deviation of three independent experiments. \**P* value < 0.05; \*\**P* value < 0.01; \*\*\**P* value < 0.001.

**Figure S4. Akirin, Relish and Bap60 are excluded from silenced chromatin and match active chromatin in S2 cells.**

S2 cells are visualized by DIC in (A, A', F, F', K, K'). Immuno-localization of endogenous Akirin (C, C'), Relish (H, H'), Bap60 (M, M'), H3K9me2 (D, I, N), H3K9ac (D', I', N') in S2 cells after an immune challenge. S2 cells were stained with DAPI (B, B', G, G', L, L'). Akirin, Relish and Bap60 sub-nuclear localizations were mostly excluded from the silenced chromatin marker H3K9me2 and DAPI-rich regions (E, J, O, see arrowheads) and mostly matched the active chromatin marker H3K9ac (E', J', O'). Scale bars (all panels): 5 micrometers.

**Figure S5. Akirin is ubiquitously expressed in *Drosophila*.**

**A.** mRNA signal of Akirin in various fly tissues retrieved from <http://flyatlas.org/>.

**B-G.** Merged images from immuno-localization of endogenous Akirin (red) in the following adult *Drosophila* tissues: carcass (**B**), fat-body (**C**) oenocytes (**D**), trachea and hemocytes (**E**), midgut (**F**) and Malpighian tubules (**G**). Tissues were visualized by DIC and stained with DAPI (blue).

**Figure S6. Akirin, Relish and Bap60 localize to H3S10p positive, transcriptionally active chromatin in fat-body cells.**

Fat-body cells are visualized by DIC in (**A, F, K**) and stained with DAPI (**B, G, L**). Immuno-localization of endogenous Akirin (**C**), Relish (**M**), Bap60 (**H**), H3S10p (**D, I, N**) in fat-body cells after an immune challenge. Akirin, Relish and Bap60 sub-nuclear localization matched with the transcriptionally active chromatin marker H3S10p (**E, J, O**). Scale bars (all panels): 5 micrometers.

**Figure S7. Confirmation of the physical interaction between Akirin and Relish by *in vitro* binding assay.**

His-tagged Akirin was expressed in bacteria and purified with His GraviTrap column (GR Healthcare). Flag-tagged Relish $\Delta$ S29-S45 was expressed in S2 cells and purified with anti-Flag M2 agarose affinity gel (Sigma). The beads with purified Flag-tagged proteins were incubated with His-tagged Akirin. Beads were then washed and the eluate analyzed by Western blotting with anti-Flag and anti-His antibodies.

**Figure S8. ChIP control of Relish target gene transcriptional activation.**

**A.** Chromatin IP with anti-RNA-Pol II S5p antibody compared to IgG control on sheared chromatin from S2 cells following heat-killed *E. coli* stimulation at indicated time points. The graph shows recruitment of RNA Pol II S5p on Akirin-dependent *p-attacin-A*, Akirin independent *p-attacin-D* or immune-unrelated *p-hunchback* proximal promoters.

**B.** Chromatin IP with anti-Akirin, BAP60, Relish antibodies compared to IgG control, or antiH3K4ac antibodies compared to H3 control, on sheared chromatin from S2 cells following heat-killed *E. coli* stimulation at indicated time points. The graph shows recruitment of detected proteins on immune-unrelated *p-hunchback* proximal promoter.

Data are represented as mean +/- standard deviation of three independent experiments performed on  $1,5 \cdot 10^6$  cells per IP. \**P* value < 0.05; \*\**P* value < 0.01; \*\*\**P* value < 0.001.

**Figure S9. Transcription factors binding sites analysis on *Attacin-A* promoter.**

**A.** Putative transcription factor binding sites, present in *Attacin-A* but absent in *Attacin-D*, were analyzed up to 1kb upstream of the transcriptional start site (TSS) by MatInspector ([www.genomatix.de](http://www.genomatix.de)).

**B.** Dual luciferase assay from S2 cell extracts co-transfected with an *attacin-A-luciferase* reporter plasmid and dsRNAs against *GFP*, *key*, *akirin* and putative *pAttacin-A*-specific transcription factors following 48h of heat-killed *E. coli* stimulation.

Data are presented as mean +/- standard deviation of three independent experiments.

**Figure S10. The Brahma complex is not required for Toll pathway activation and survival to sterile injury.**

**A.** Evaluation of knockdown efficiency *in vivo*. Quantitative RT-PCR of *relish*, *akirin*, *brahma* and *polybromo* mRNAs from dissected *Drosophila* fat-bodies of *C564-gal4 / UAS-*



*RNAi-akirin*, *UAS-RNAi-Brahma* or *UAS-RNAi-Polybromo* (red columns) compared to *C564-gal4 / UAS-RNAi-GFP* (black columns).

**B-C.** Survival of *C564-gal4 / UAS-RNAi* flies following sterile injury (**B**) or *Beauveria bassiana* natural infection (**C**).

**D.** Statistical analysis of survival assays following *B. bassiana* infection. Because log-rank analysis can only compare two survival curves at one time in the same experiment, we computed the median lethal time 50 (LT50) and performed statistical analysis on LT50 using Student's t test.

**E.** Quantitative RT-PCR of *drosomycin* mRNA from *C564-gal4 / UAS-RNAi* flies following a 24 h *M. luteus* challenge.

Data are presented as mean +/- standard deviation of three independent experiments. \**P* value < 0.05; \*\**P* value < 0.01; \*\*\**P* value < 0.001.

**Figure S11. The Brahma complex is required for Akirin-dependent immune response in larval hemocytes.**

**A.** Schematic representation of the experimental procedure. 15-20 wandering L3 larvae are challenged with a needle previously dipped in an *E. coli* pellet or in sterile PBS solution and incubated for two hours at 29°C to induce the immune response. Larvae are carefully opened with sharp tweezers into 100µL of sterile PBS to recover hemocytes. Hemocyte suspension is then immediately frozen on dry ice and kept at -80°C before RNA extraction and mRNA quantification.

**B.** Validation of the purity of larval hemocytes. Bleeds from L3 *Hml-gal4 / UAS-GFP* larvae were fixed, stained with DAPI and observed with an epifluorescence microscope. All visualized cells were GFP<sup>+</sup>, showing the purity of the extracted tissue.

**C-D.** Quantitative RT-PCR of (**C**) Akirin-dependent *Attacin-A*, *Attacin-C* and *Diptericin-A*

and **(D)** Akirin-independent *Attacin-D*, *Pirk* and *Cecropin-A2* mRNAs from hemocytes of *Hml-gal4 / UAS-RNAi-GFP*, *UAS-RNAi-Relish*, *UAS-RNAi-Akirin* or *UAS-RNAi-Brahma*, L3 larvae two hours following *E. coli* challenge or control (PBS).

Data are presented as mean +/- standard deviation of three independent experiments. \**P* value < 0.05; \*\**P* value < 0.01; \*\*\**P* value < 0.001.

## SUPPLEMENTARY TABLES

### Table S1

Gene	Forward	Reverse
<i>attacin-A</i>	GGCCCATGCCAATTTATTCA	AGCAAAGACCTTGGCATCCA
<i>attacin-C</i>	AAGGCATTTGCCTCGCAGAATCAG	AGCTCCATGACCTTTGATGTGGGA
<i>attacin-D</i>	TTTATGGAGCGGTCAACGCCAATG	TGCAAATTGAGTCCTCCGCCAAC
<i>cecropin-A2</i>	CATTCTGGCCATCACCATTGGACA	GTGTGCTGACCAACACGTTTCGATT
<i>cecropin-B</i>	TTCGTCTTTGTGGCACTCATCCTG	GGTATGCTGACCAATGCGTTTCGAT
<i>diptericin-A</i>	GCTGCGCAATCGTTCTACT	TGGTGGAGTGGGCTTCATG
<i>pgrp-lb</i>	CATTGACCCCTGCCTACAAGC	GCCTTCGGTGTCTGTTTATGT
<i>pirk</i>	AGCAGCGAAAGAAACGATA	GCTCTTTCTGGCAAGTGGAG
<i>drosomycin</i>	CGTGAGAACCTTTTCCAATATGATG	TCCAGGACCACCAGCAT
<i>akirin</i>	AAGAGACTGCACAAGCGCAAACAG	ATCATGCTCTCGCAAATGAGCTGC
<i>relish</i>	CCACCAATATGCCATTGTGTGCCA	TTCCTCGACACAATTACGCTCCGT
<i>brahma</i>	CAAGCCCAATCGCATTACAACGGT	GCAACTCCTGCATGCGCAATGATA
<i>moira</i>	TTAAGGATGAGGTGCCCGCTACAA	TTCCGGTTCCTGCGATTCCACTA
<i>osa</i>	CTACATCCGCTCTGACAAGAAG	CGTCTGTTCTCGGTAATTC
<i>polybromo</i>	CCTCACACTGTTACGACTATTT	CTTCGCCAATCTCATCGTACTC
<i>rp49</i>	GACGCTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG
ChIP <i>p-attacin-A</i>	CGGCTGAAACTTCACTCAAATC	TTTGCCTGGAGGGATTATTCT
ChIP <i>p-attacin-D</i>	GGAAATCACCGAAGTTGCGTA	GACTGCATATTTCCGACGGT
ChIP <i>p-hunchback</i>	TCCGGCTGCTCTCTCATTTTCGATT	ATCTCCTCACTGCTCCTCACAAACA
ChIP <i>p-drosocin</i>	GTGTGCTTGTGTGTGTATG	TTCGCTCTCTTTGAAGTCTG
ChIP <i>p-cecropin-A1</i>	ATTGGCCCAGAACCCTTTA	TCTGCACATCTAGGGAACAATC
ChIP <i>p-metchnikowin</i>	AATCTGCGACTCGTTTGTCTGGGA	GGTGGCGGAATTGATTGATGCTT
ChIP <i>attacin-A</i> coding sequence	CTGGTCATGGTGCCTCTTT	AGACCTTGGCATCCAGATTG

### Table S2

Gene	dsRNA reference	Forward	Reverse	Size
<i>CG4882</i>	DRSC29963	TTGCTTGACCAAGACACTGG	AGACAATTGACAATGCTGCG	324
<i>CG6841</i>	AMB18892	GGAATCGGAGACCAAAGCTA	CTGGCGATCAGTGGGAATAT	250
<i>CG8264 (Bx42)</i>	DRSC32296	CTTGCTGGGTTCGGTAGATGT	GCGTGACATTTCTGAGCAAA	199
<i>CG9423 (kpn-α3)</i>	DRSC34268	TGCTGTGTGGTGGACAAAAT	GACTAACATGGCACCAGCT	258
<i>CG3445</i>	BKN23640	TGAGTTCTTCGGGGATTACG	GGCACGTCCCTAACATCCTA	395
<i>CG18446</i>	DRSC35017	GCAAAGATGTACCCGCAAAT	ACAAAGGGTGTATCGTCGG	281
<i>CG5893</i>	DRSC25367	GGCATTCCAGCTATTTTGA	GGGCGAACAATAAACCAG	482
<i>CG6920</i>	DRSC35880	CAGCAGCCTGTGTACATCGT	TGGACTATTTGGGGAGCAC	296
<i>CG14213</i>	BKN29239	GCTGCTCAAGAACCTGGAAC	TCATCGATGGTTGGACGTTA	129
<i>CG1913</i>	DRSC30933	ACTAAGCGTCACGCCACTTC	CACTGAATCTGGCCGATTTT	247
<i>CG4800</i>	DRSC29100	ACAAGGCCATGAAGGACATC	TAGGTTCCGTTTTGTTGGG	204
<i>CG10489</i>	MRC107_D10	CACTTTGAATGCGAACTTGGACTTG	CACAAGGAATCATCATGAACTGAGG	541
<i>CG4303 (Bap60)</i>	DRSC32657	GTTGCGACATCTTTGCTACG	CAGTCGACCACCACCAGTAA	180
<i>CG17446</i>	DRSC32679	GAAGCAATTCTTCTTTGGCG	GCGAATGGAGGGAAACAATA	199
<i>CG6686</i>	DRSC30666	GGGTGTGTCTGTGCAACTCA	GCCCAATGTCAAGTTGGATT	243

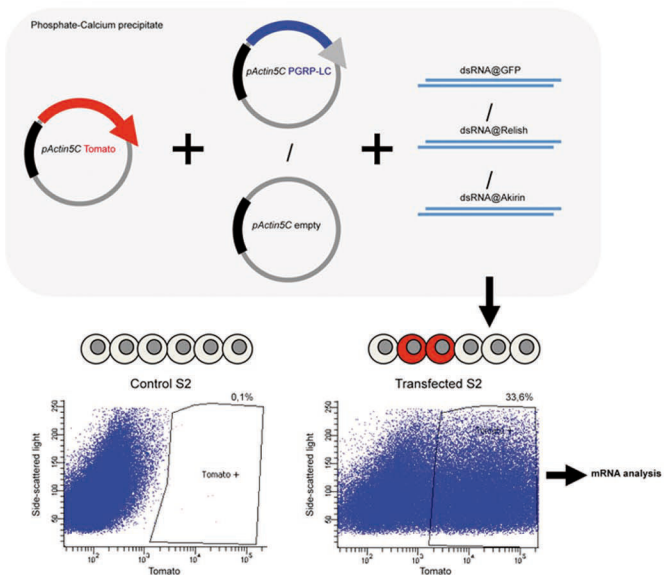
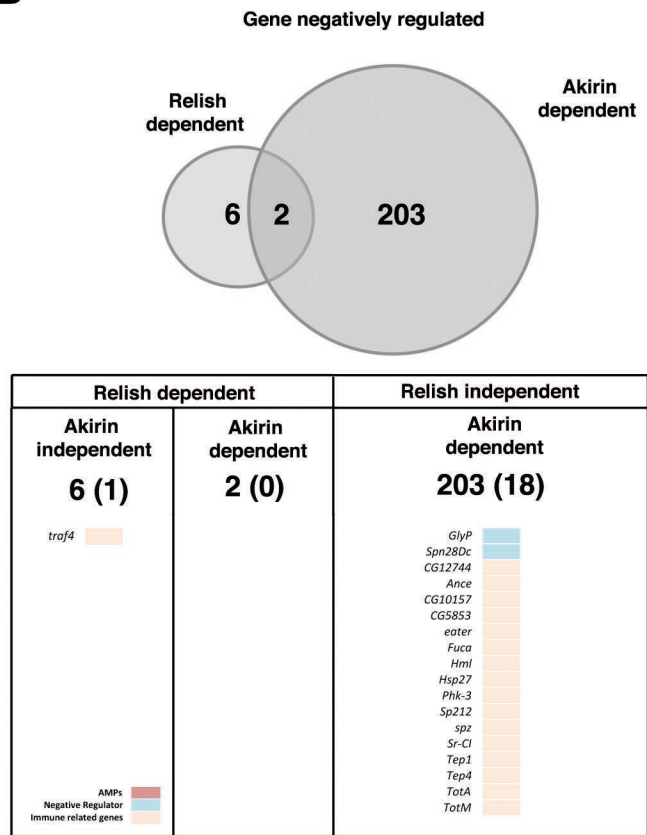
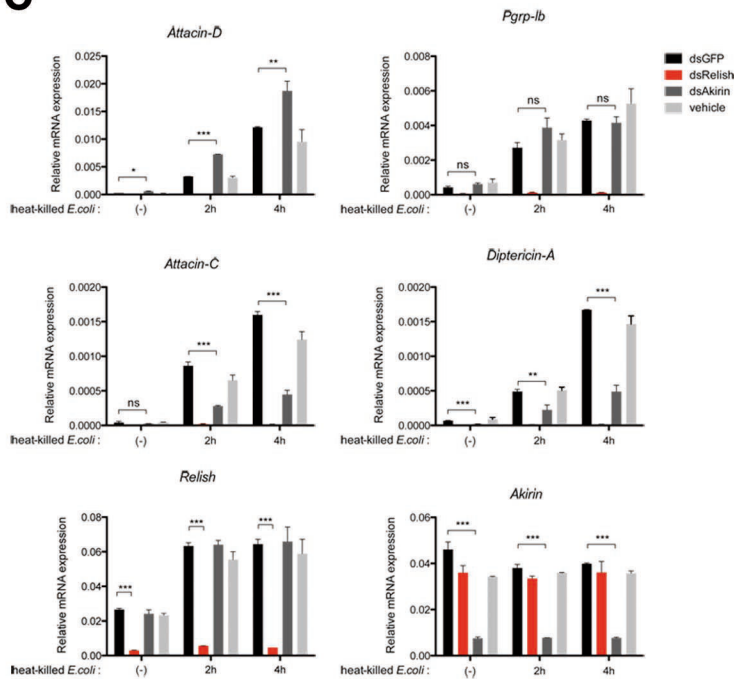
<i>CG8616</i>	DRSC36284	GTGCTGAACAAGCAGACCAA	CTGTGCGGTAATCGGTTGTA	243
<i>CG10279</i>	AMB31576	CGAAGCCCATGTCTAACATG	GGGAGCTGGCCCAACAGA	246
<i>CG10123</i>	67481	ACCCAACAAAAAGACTAGCAGC	CAGTACTGCTGGAGTTTGATCG	779
<i>CG9045</i>	VDRC04472	AGCCCTCCAGCCCATCCAGT	CGATTCTCCTTTCTGCCCTCGTC	352
<i>CG5924</i>	62418	AGAGCTTTTGC GTTAAAGTTGG	CTGTTCAAAGA AACTGTGCGCC	651
<i>CG5942 (Brm)</i>	AMB31683	ATACGTTCTTTTCGATGCG	AGAGGCAGGGCCTGCGGGAG	253
<i>CG6546 (Bap55)</i>	AMB33383	TGTATACCGGCGACAGATCA	CCGAGCTTATGTTGAGAAAG	255
<i>CG18740 (Moirai)</i>	DRSC32753	GATTCTCCGGAATGTGATCG	GGACAATCGACGGGTAGAGA	206
<i>CG1064 (Snr-1)</i>	AMB22422	AGGGCCAATCAGTCAATTTG	ATCCGTGTCGTCGAAACAC	255
<i>CG7467 (Osa)</i>	DRSC33078	AGGCGGTTGGTGTCTATGTC	AGAATATGGATCGCAGTGGC	247
<i>CG11375 (Pb)</i>	AMB26594	TCCATTCTGCTCCCAAATCT	TGTGTTTAAGGAGCGTTTGG	253
<i>brain-specific homeobox</i>	DRSC25413	ATCCACGGACTTGTAGGTGC	ACGGAGATCAGATTTTCGCAG	218
<i>abdominal A</i>	BKN45974	CAGGGATACCTGGGCAGA	AGCTACCAGTCGATGAGCGT	218
<i>homothorax</i>	AMB28154	CCATTGCGCAAATTATATTCAA	TTGAAATGCAAATTTTTATGTTCAA	252
<i>rainy head</i>	AMB18490	CCAAACGTTTTTACTGCCCA	AATGCCAGCTGACCTACCT	250
<i>runt</i>	BKN45422	CAGCTCCACACCAGATCTCA	GTGGTGCAGTTCTCAGCTC	327
<i>Big brother</i>	AMB19953	GCTTCCATCGCTACGTTTTG	CAATAATCCCTGGAACCTCGG	247
<i>pleiohomeotic</i>	AMB20338	ACCCTTATGTGGGCAAGCTA	ATGCTTTGCCAGCTCAGT	249
<i>pleiohomeotic like</i>	AMB23337	TGAGTTGCCTCTTTGTCACCT	TTGATCTATGCGGAAAACCA	253
<i>buttonless</i>	AMB27600	TAGCAGAACTCCGCTTCCAG	GAACCAAACGGAGGGCTATA	252
<i>twist</i>	DRSC23185	CAAAGTTTCGAGGGCTACGA	TTGAGGGTCTGGATCTTGCT	398
<i>knirps</i>	BKN46266	CTGATGATGGTGGTGCAAAT	TGCAGGAGCACGAACAGG	158
<i>paired</i>	BKN29894	TTGATACCACACTGGGCAAA	GGAGTACAAGCGCAGTAGCC	572
<i>zerknüllt</i>	AMB23112	ACGGTTGCTTAGCTCCAACA	TGGTCCAAGTCTAAATCCGC	254
<i>tramtrack</i>	AMB18912	TAAAAGCGATGAACCCGATC	ATCTCTGTGAGGTGTTCCGC	249
<i>jumeau</i>	BKN20412	GGTTAAGTCACCAGGCGGTA	GCTCTACATCCGAGTCTGCC	713
<i>Eip74EF</i>	DRSC23794	CCGCGCTGGTAGTAGTACCT	CCCAGAGTGTTATCCAACCG	355
<i>slow border cells</i>	AMB20077	GTAGCCGTTGTACAGGCCTG	CAGACCCTGCGGAACCG	252
<i>tinman</i>	BKN46496	AGCAACTGCATACCAACAC	GCTCCTTAGGGAGGACGAGT	476
<i>Suppressor of Hairless</i>	AMB19826	AAGAATGCCGATCTGTGCAT	CGTTACGGAGCACACCAG	243
<i>achintya</i>	AMB34190	CGAATGGGTGCCACTATCAT	CGCCACTAATTTGTTGTTTGG	255

## Supplementary Tables legends

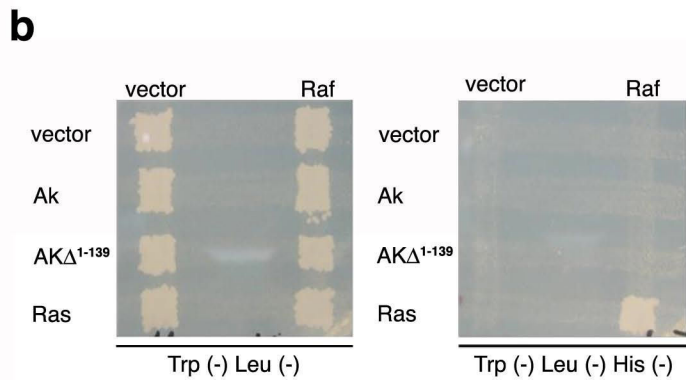
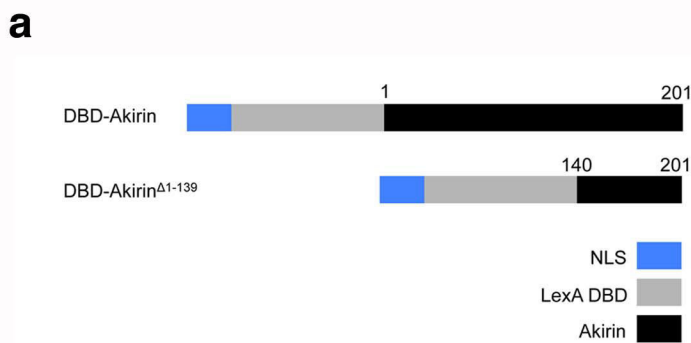
**Table S1. List of oligonucleotides used for quantitative real-time PCR.**

**Table S2. List of oligonucleotides used to generate dsRNA for the functional RNAi screen in S2 cells**

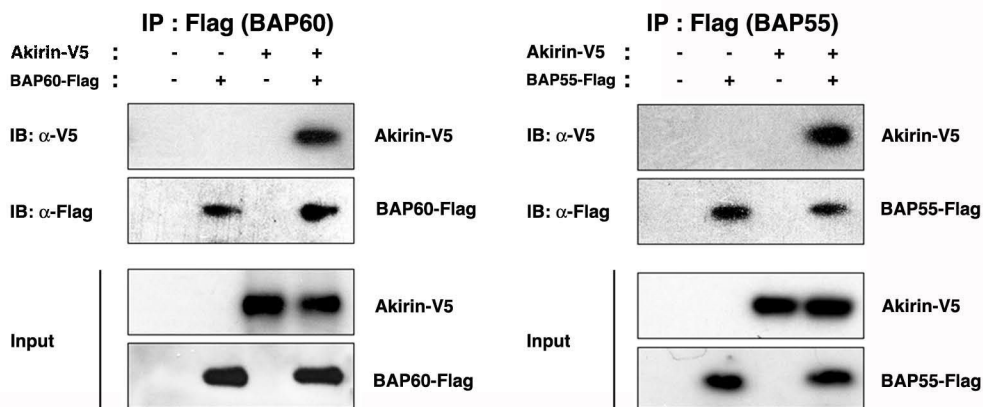
Are indicated : gene reference, dsRNA reference (from <http://www.genomernai.org/GenomeRNAi>), forward and reverse primers (without T7 promoter sequence TTAATACGACTCACTATAGG) used to produce T7 DNA matrix PCR product and PCR product size.

**A****B****C**

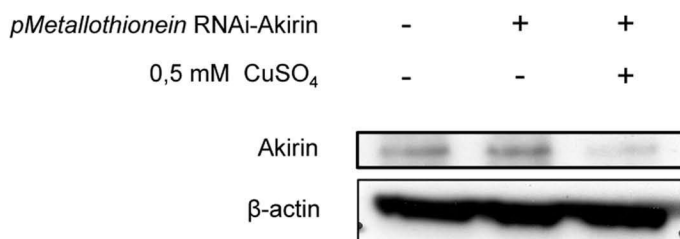
Supplementary figure 1



**d**

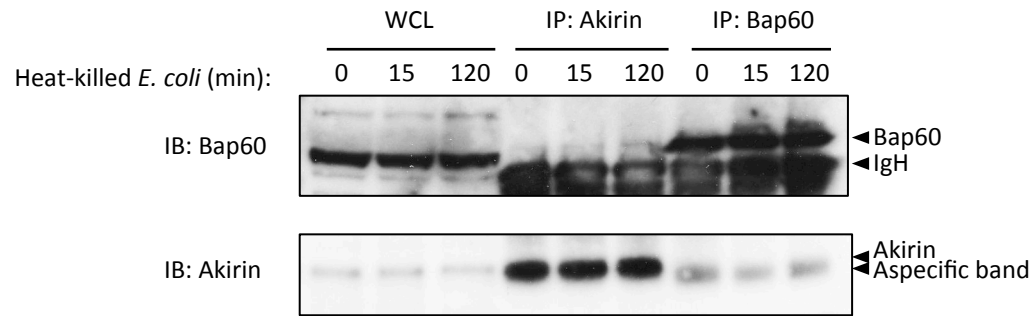
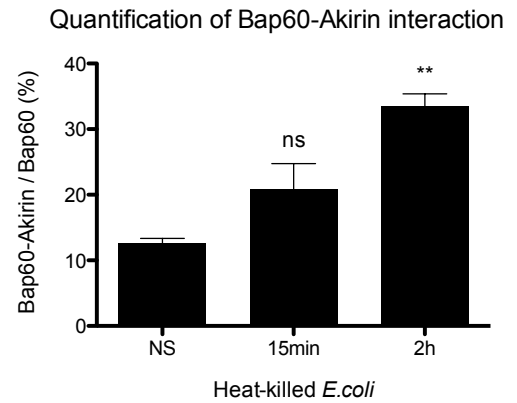


**e**

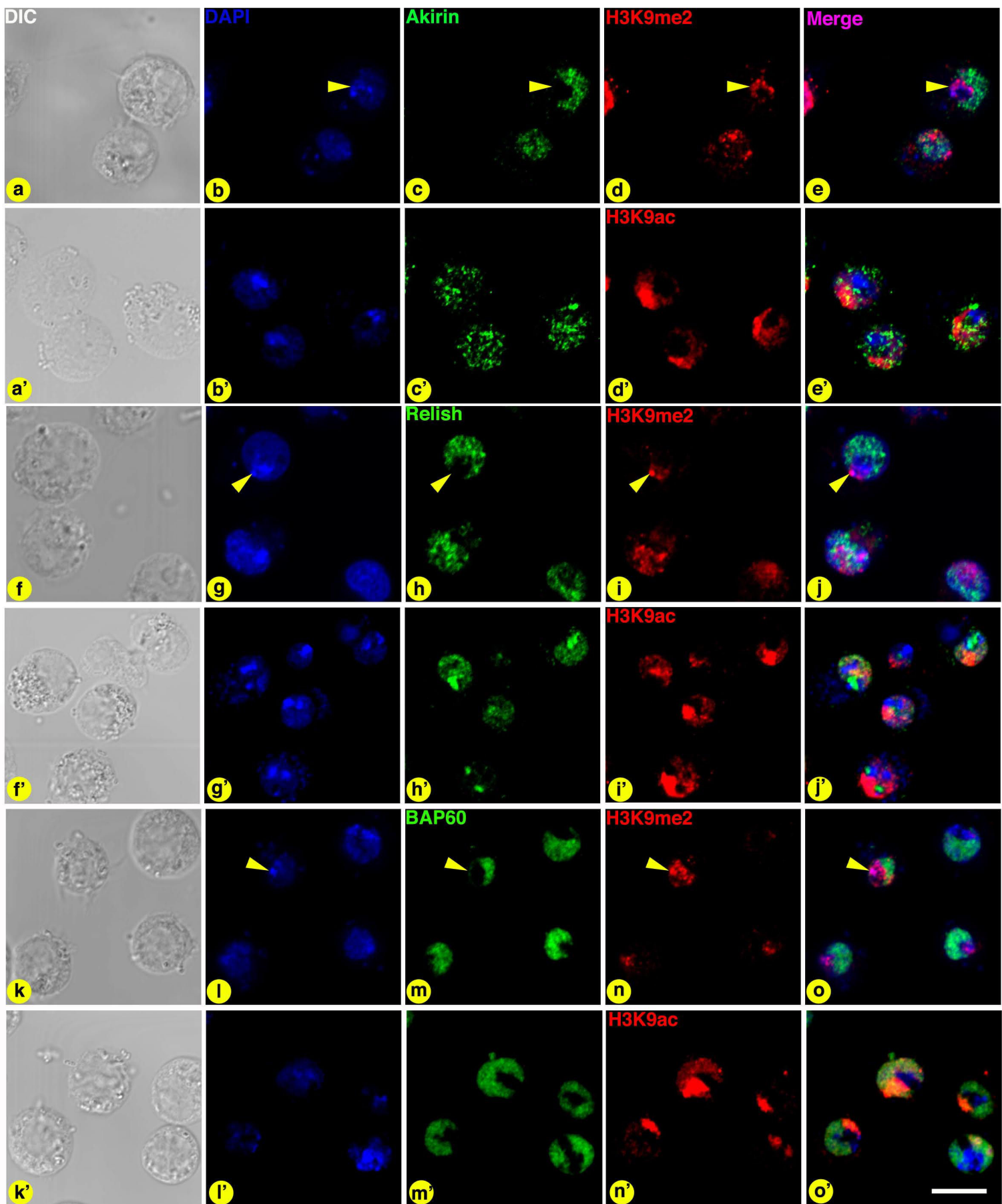


**c**

gene	interaction	IMD
kap-alpha3	AK	negative
Myb	AK	no
akirin	AK	positive
CG8223	AK	no
CG6357	AK	negative
Cand1	AK	no
seq	AK	no
ttk	AK	no
Pole2	AK	no
Rm62	AK	no
Blm	AK $\Delta$ 1-139	no
D	AK $\Delta$ 1-139	no
Prosalpha6	AK $\Delta$ 1-139	no
kni	AK $\Delta$ 1-139	no
east	AK $\Delta$ 1-139	no
His4r	AK $\Delta$ 1-139	no
pho	AK $\Delta$ 1-139	no
Cfp1	AK $\Delta$ 1-139	no
CG15876	AK $\Delta$ 1-139	negative
bbx	AK $\Delta$ 1-139	no
SNCF	AK $\Delta$ 1-139	no
Ran	AK $\Delta$ 1-139	no
cid	AK $\Delta$ 1-139	no
Aos1	AK $\Delta$ 1-139	no
lic	AK $\Delta$ 1-139	no
CG12170	AK $\Delta$ 1-139	no
lola	AK $\Delta$ 1-139	no
CG43143	AK $\Delta$ 1-139	no
Uev1A	AK $\Delta$ 1-139	no
Topalpha	AK $\Delta$ 1-139	no
CG2662	AK $\Delta$ 1-139	negative
CG33229	AK $\Delta$ 1-139	negative
Bx42	both	negative
CG6841	both	no
CG4882	both	no
Bap60	both	positive
pho1	both	no
EcR	both	no

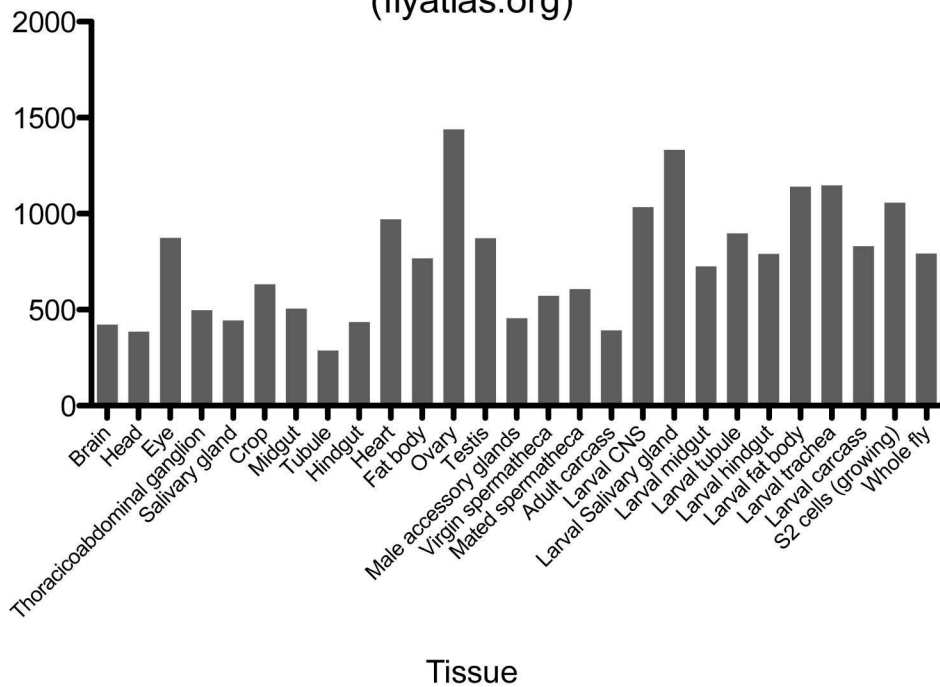
**A****B****Supplementary figure 3**



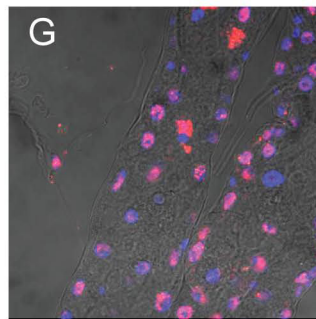
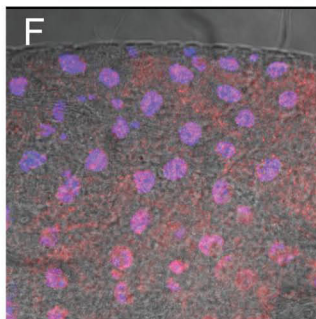
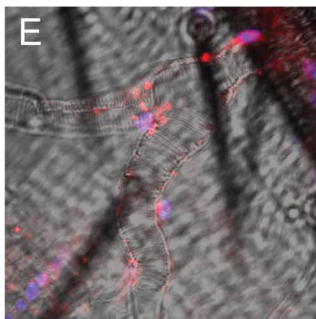
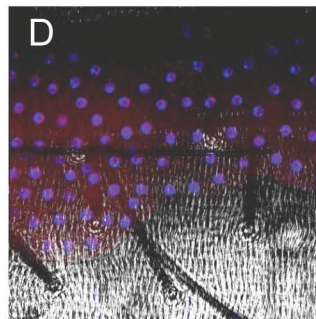
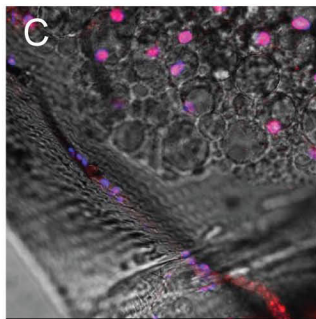
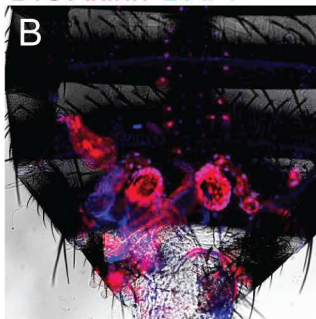


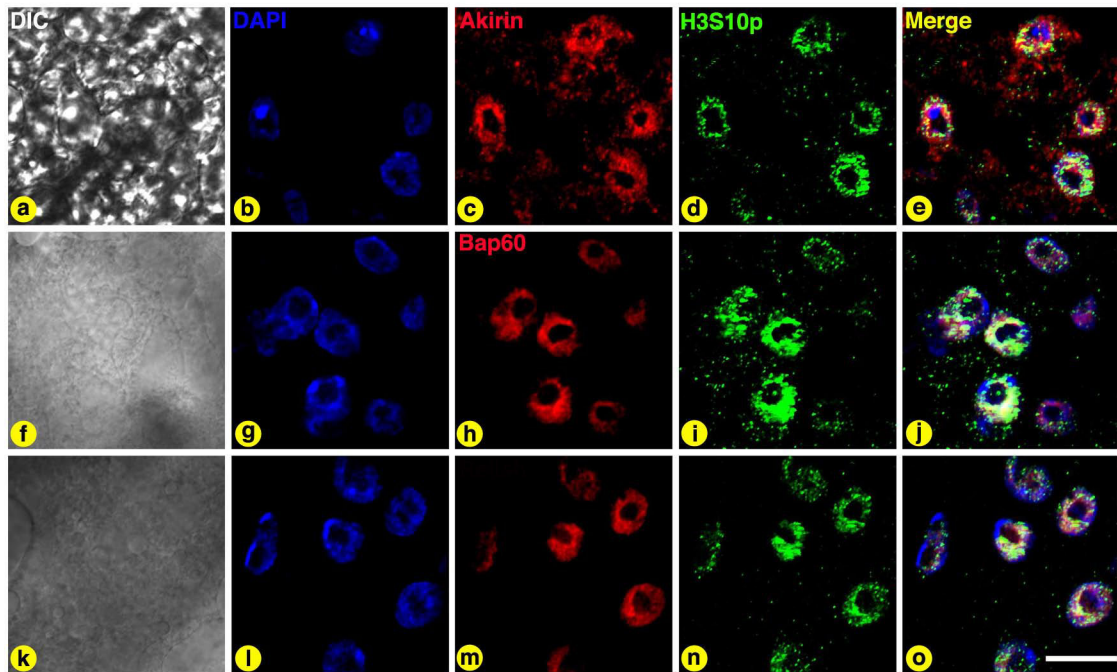
Supplementary figure 4

**A** *akirin* gene expression profile  
(flyatlas.org)



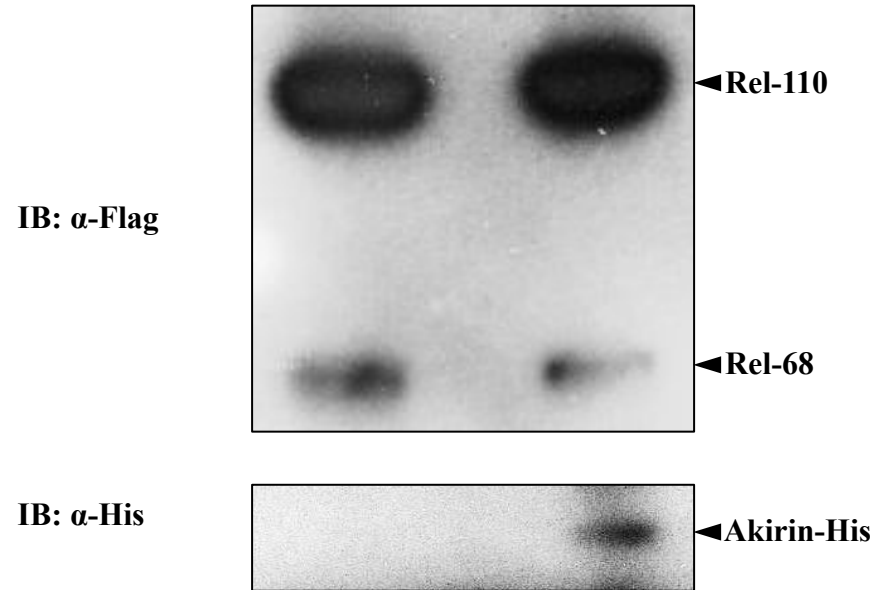
DIC Akirin DAPI



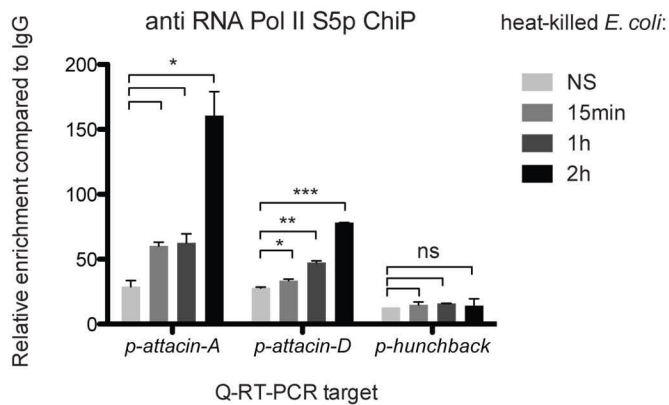
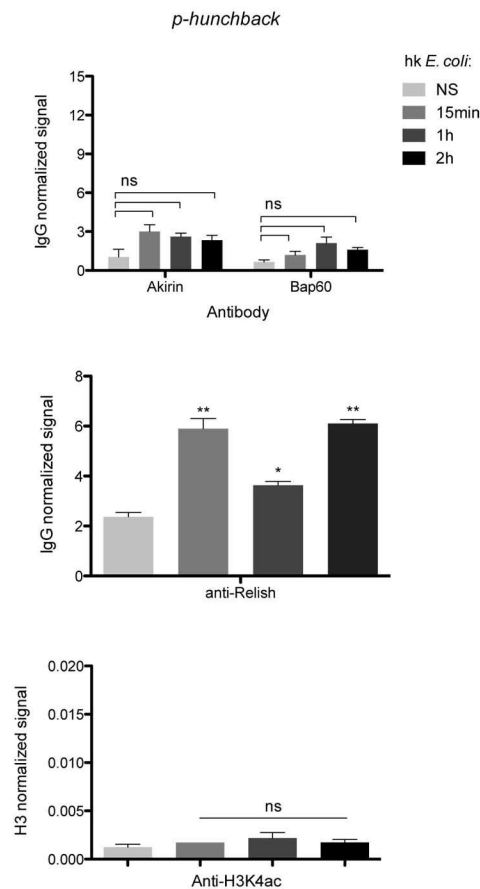


## IP : Flag (Relish)

Akirin-His	:	-	+	+
Flag-Relish $\Delta$ S29-S45:		+	-	+

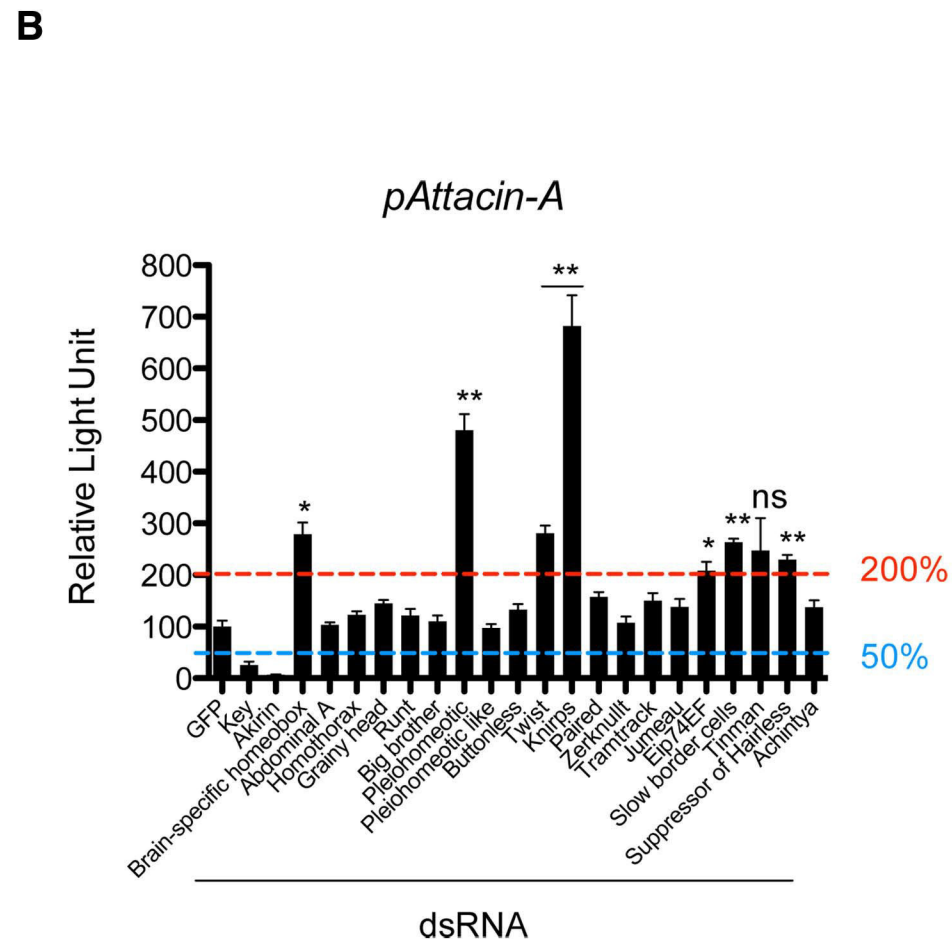


Supplementary figure 7

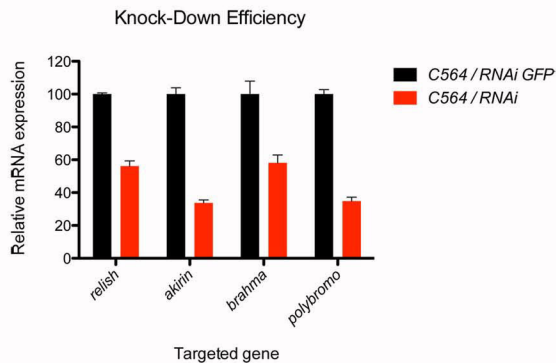
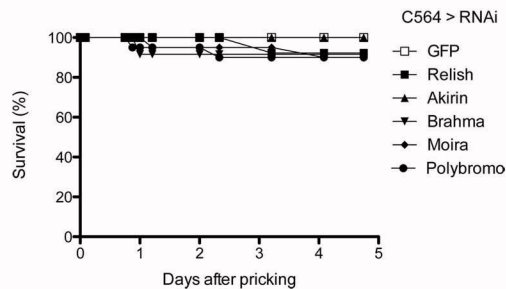
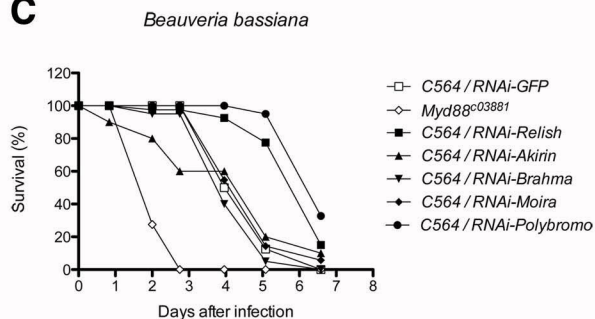
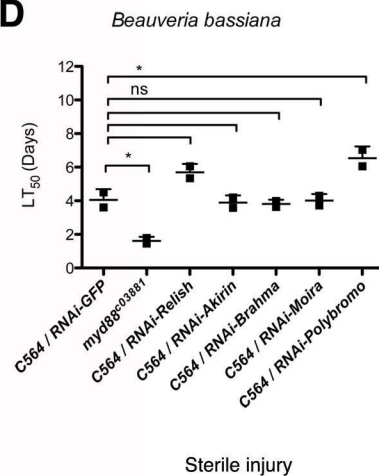
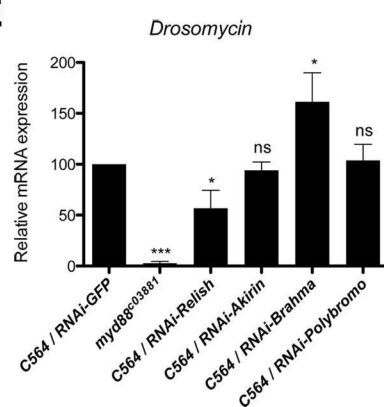
**A****B****Supplementary Figure 8**

**A**

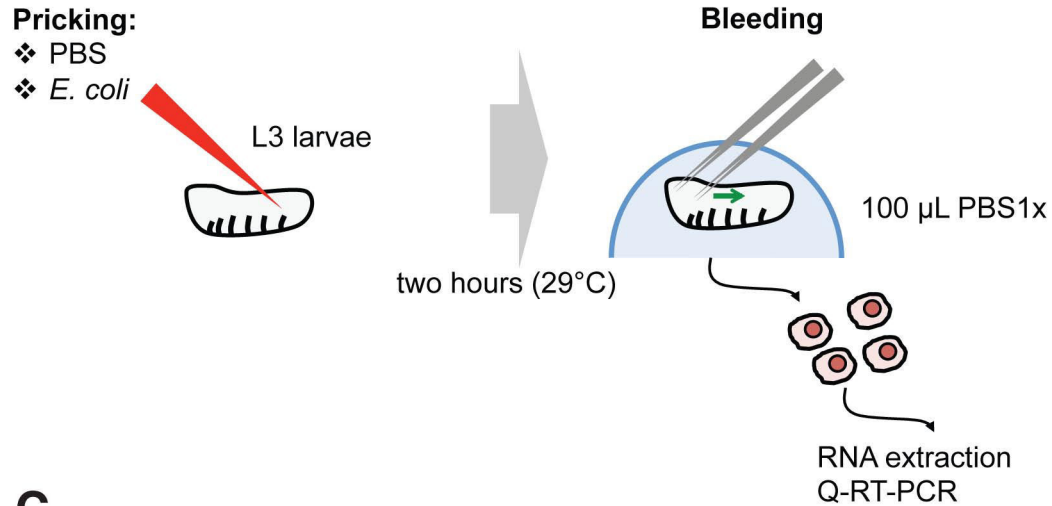
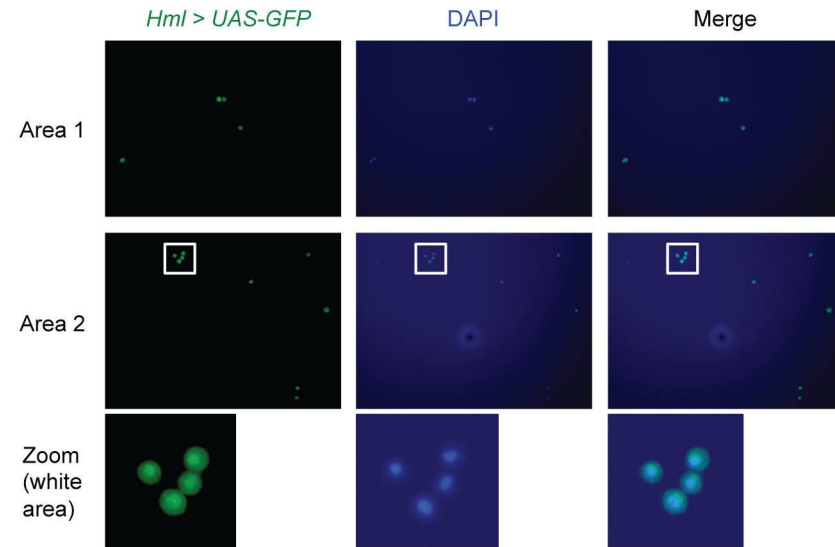
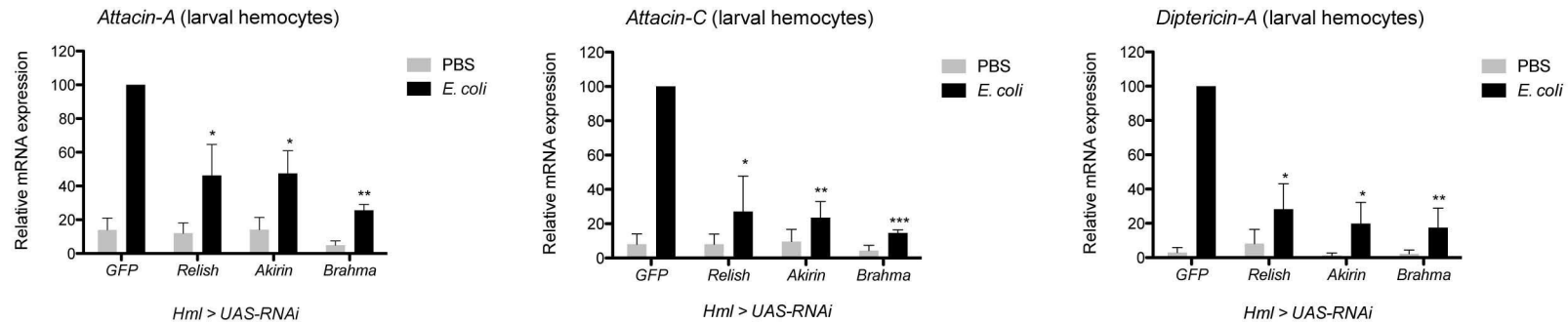
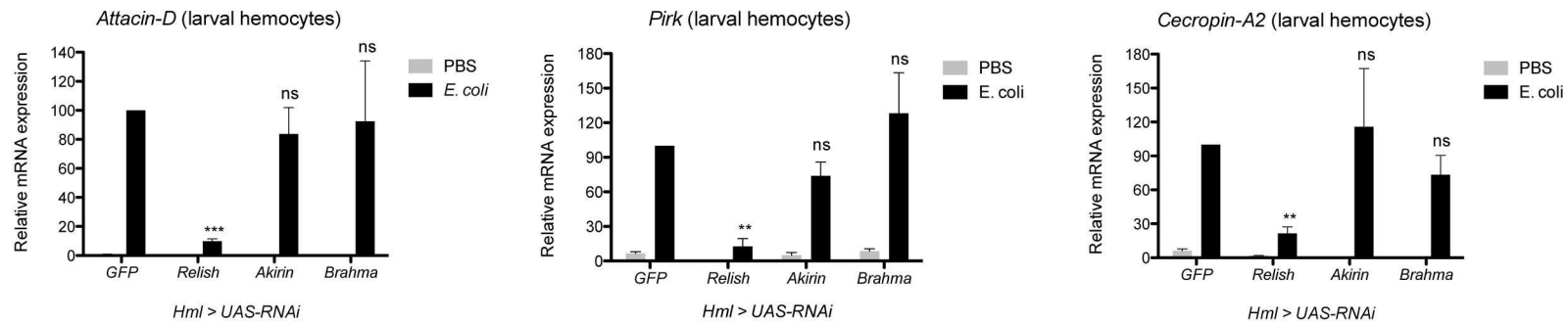
<b>pAttacin-A-specific transcription factors binding sites</b>	<b>position relative to +1 TSS</b>
Grainy head	-56; -970; -694; -901
Achintya	-368
Suppressor of Hairless	-399
Tinman	-403
Slow border cells	-418
Eip74EF	-461
Jumeau	-474
Paired	-494
Brain-specific homeobox	-537; -775
Homothorax	-538; -656; -658
Tramtrack	-553
Zerknult	-655
Knirps	-729
Twist	-750
Abdominal A	-780
Buttonless	-838
Pleiohomeotic	-853
Pleiohomeotic like	-853
Big brother	-967
Runt	-967



**Supplementary figure 9**

**A****B****C****D****E**

Supplementary figure 10

**A****B****C****D**

**Supplementary Figure 11**



## IX. References

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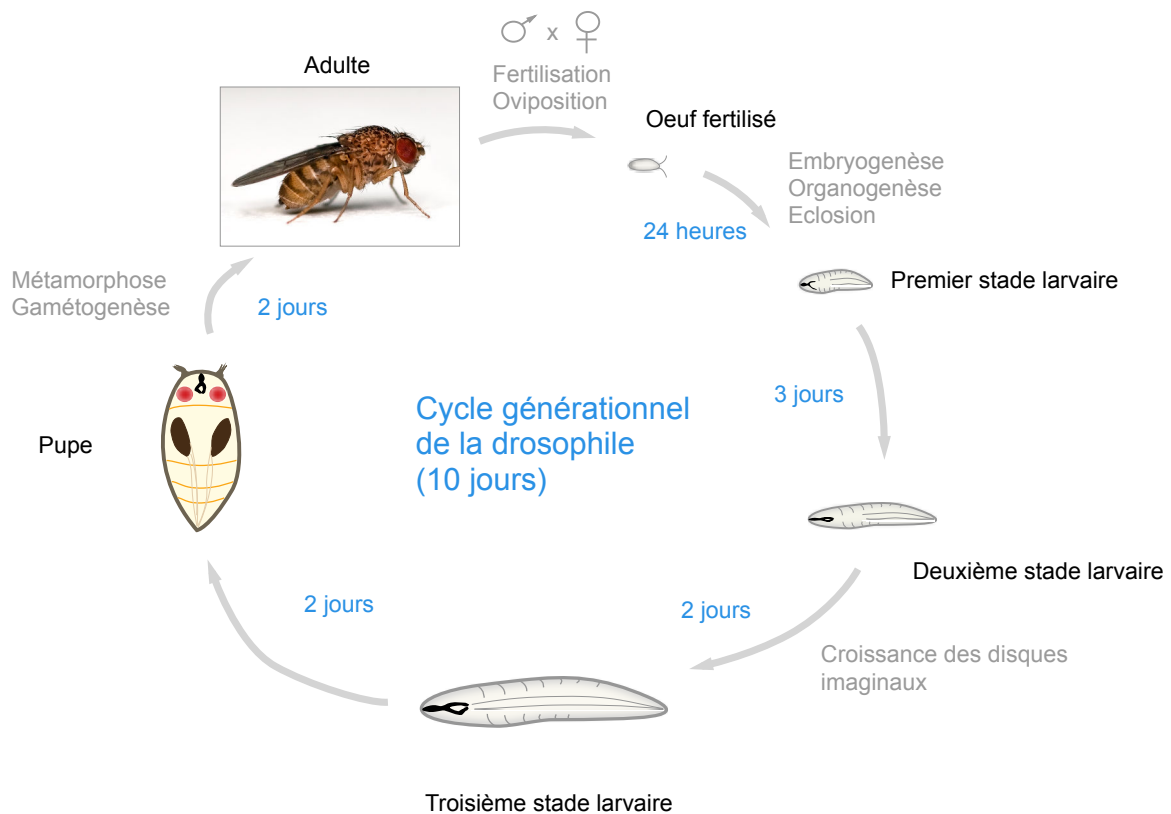
# **Annexe : Résumé approfondi de la thèse en français**

## **Introduction générale et objectifs de la thèse**

Au cours de l'évolution, les métazoaires ont établi un système immunitaire puissant leur permettant de se défendre contre les micro-organismes pathogènes. Deux sous-ensembles de systèmes immunitaires ont été formés : l'immunité innée et l'immunité acquise.

L'immunité innée constitue un ensemble de mécanismes de défense, apparu il y a plus d'un milliard d'année au sein du règne animal et conservé parmi l'ensemble des métazoaires. Ce système met en jeu des acteurs cellulaires de diverses origines, des voies d'activation moléculaires et des effecteurs, qui tous ensemble procurent à l'hôte une réponse immunitaire efficace et immédiate. Pour être fonctionnel, un mécanisme de l'immunité innée nécessite le concours de trois catégories de molécules. Premièrement, des senseurs capables d'une part de discriminer et de détecter des motifs microbiens ou des signaux de dangers, et d'autre part, d'engager une voie de signalisation en aval. Deuxièmement, des molécules adaptatrices formant les voies moléculaires capables de relayer le signal de reconnaissance jusqu'à la production d'effecteurs. Enfin, des molécules effectrices, pouvant agir de façon directe (telles que les peptides antimicrobiens (PAMs) ou les espèces oxygénées réactives (ROS)) ou indirecte (telles que les cytokines ou la fièvre) afin de contrer l'attaque de pathogènes.

L'immunité adaptative est apparue plus récemment dans l'évolution, il y a 650 millions d'années environ, lors de l'apparition des premiers poissons cartilagineux et est partagée par l'ensemble des vertébrés à mâchoire. Le système immunitaire adaptatif est basé sur la reconnaissance spécifique d'antigènes et permet de maintenir une mémoire immunitaire. La découverte d'une mémoire immunitaire acquise chez les mammifères a notamment permis le développement des vaccins. Ceux-ci constituent avec la découverte des antibiotiques, l'un des accomplissements les plus marquants de la recherche biomédicale contemporaine. Néanmoins, il faut noter qu'une pleine activation du système immunitaire adaptatif requiert l'activation concomitante du système immunitaire inné.



**Figure A1 : Le cycle de vie de *Drosophila melanogaster***

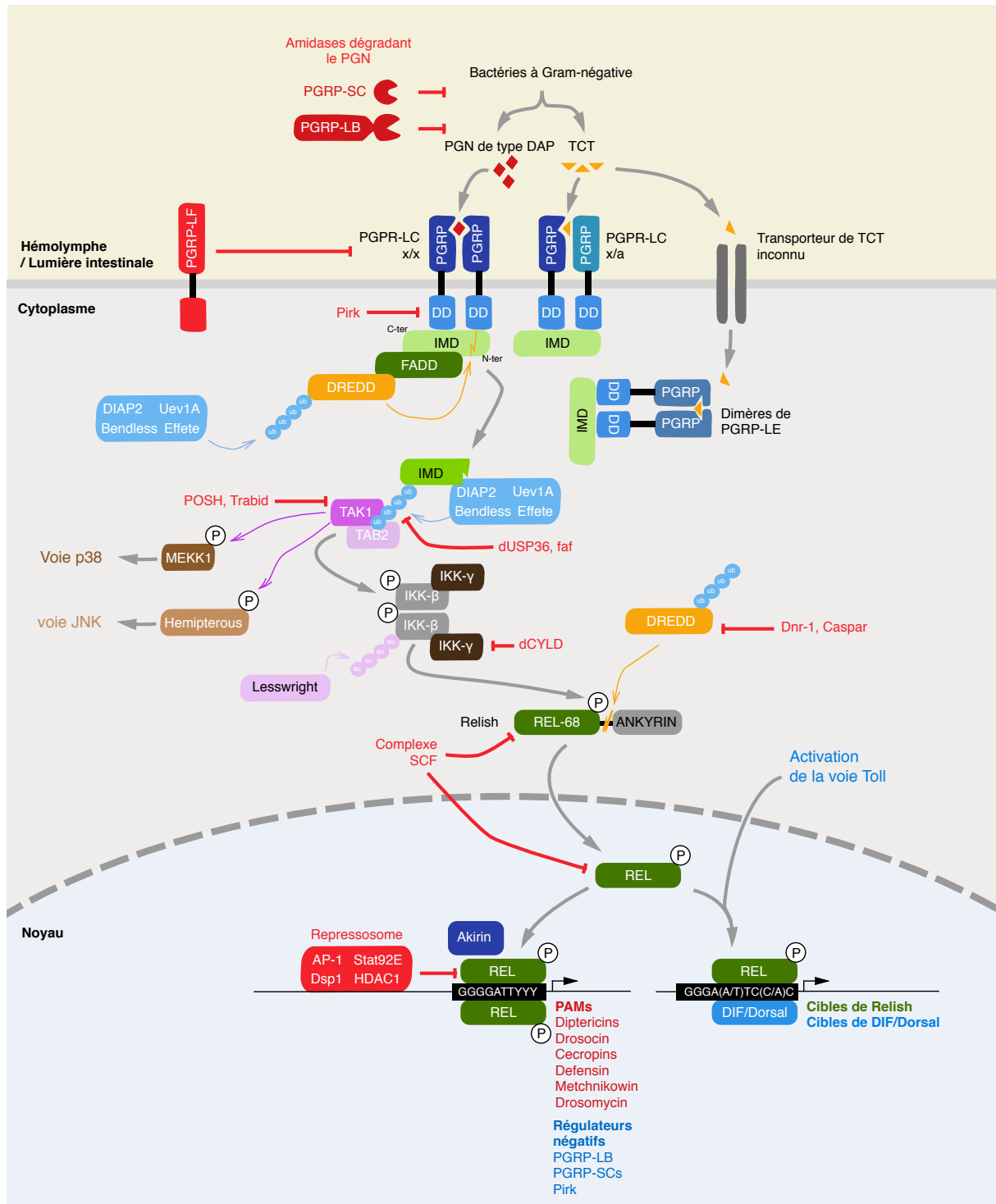
Le contexte scientifique de ma thèse a été l'exploration des mécanismes d'activation de l'immunité innée. Chez l'Homme, le système immunitaire est avant tout requis pour se défendre contre les agents infectieux. Néanmoins, l'activation du système immunitaire inné peut également être délétère lorsqu'elle n'est pas maîtrisée et peut être à l'origine d'un ensemble de pathologies telles que les maladies auto-immunes, l'inflammation chronique et le cancer. Une activation chronique de l'inflammation est notamment associée à l'athérosclérose, les diabètes de type II ou les pathologies inflammatoires de l'intestin ou du colon (colite ulcéreuse, maladie de Crohn). Ces pathologies sont particulièrement difficiles à traiter avec les molécules thérapeutiques anti-inflammatoires actuelles et sont devenues un problème de santé majeur. Une meilleure compréhension des mécanismes de régulation ainsi qu'une analyse complète des voies moléculaires sous-tendant l'activation de l'immunité innée seront cruciales pour l'élaboration d'une nouvelle génération de molécules anti-inflammatoires.



*Drosophila melanogaster* est un petit diptère dont l'utilisation a été déterminante ces cent dernières années pour aborder des sujets de recherche complexes en génétique et en biologie du développement notamment. La présence d'outils génétiques puissants et le temps de génération court (huit à dix jours) (**Figure A1**) de la drosophile ont largement contribué à son succès en tant qu'organisme modèle. Dans la nature, le cycle de vie des drosophiles s'orchestre principalement autour de fruits en décomposition ce qui place cette mouche au contact direct de nombreux micro-organismes. Pour envahir la cavité générale de l'insecte, ces micro-organismes doivent tout d'abord surmonter deux barrières physiques mises en place dans l'appareil digestif : un tissu épithélial monostratifié recouvrant l'ensemble du tube digestif et la matrice péritrophique. Cette dernière consiste en une membrane formée de chitine et de glycoprotéines protégeant l'épithélium de l'intestin moyen.

Certaines espèces bactériennes, telles qu'*Acetobacter pomorum* et *Lactobacillus plantarum* sont nécessaires au métabolisme de digestion et forment la flore intestinale naturelle de la drosophile. En revanche, des espèces bactériennes pathogènes telles que *Pseudomonas aeruginosa* PA14 ou *Serratia marcescens* DB11 peuvent franchir cette double barrière et provoquer une bactériémie létale pour la mouche.

Flore commensale, comme organismes pathogènes provoquent une réponse immunitaire locale de l'épithélium intestinal, notamment au niveau de l'intestin moyen. C'est l'intensité de cette réponse qui va varier en fonction de la capacité invasive de la flore microbienne intestinale. Ainsi dans l'intestin moyen, cette réponse va se traduire par la sécrétion de peptides antimicrobiens (PAMs), comme la Diptéricine-A, et d'espèces oxygénées réactives (ROS). Plus précisément, les PAMs sont produits par les entérocytes intestinaux grâce à l'activation de la voie NF- $\kappa$ B dite IMD (IMMune Deficiency) , suite à la reconnaissance du peptidoglycane (PGN) de type DAP (DAP-type PGN) contenu dans les bactéries à Gram (-) et certaines bactéries à Gram (+). Des régulateurs négatifs (PIRK, PGRP-SC et PGRP-LB) sont également produits suite à l'activation d'IMD et inhibent la voie afin de maintenir un faible niveau d'activation basal en présence de bactéries commensales non prolifératives (**Figure A2**).



**Figure A2 : Activation et régulation de la voie IMD chez *Drosophila melanogaster***

Lorsque l'épithélium intestinal rompt, ou lors d'une blessure, la présence de bactéries ou de champignons est détectée dans la cavité générale par l'intermédiaire des hémocytes et du corps-gras, les deux principaux tissus acteurs de la réponse

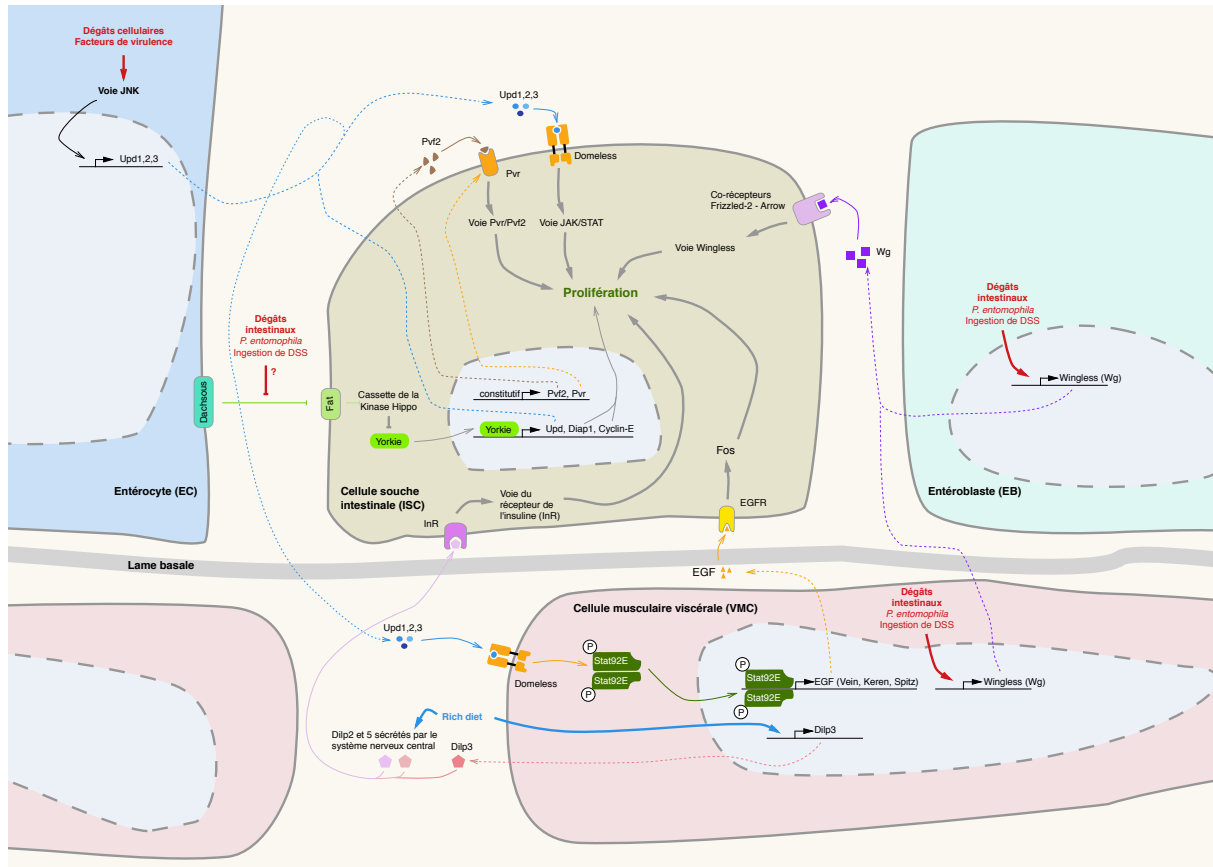
immunitaire systémique. Cette détection est permise par l'activation de la voie IMD ou de la voie Toll, deuxième voie NF- $\kappa$ B activée en réponse à des infections de bactéries à Gram (+) et fongiques. Suite à cela, le corps-gras libère une quantité massive de PAMs pour maîtriser et détruire l'invasion microbienne.

L'étude de ces deux systèmes, local et systémique, nous a permis d'identifier deux gènes requis pour le bon fonctionnement de la réponse immunitaire innée chez la drosophile : big-bang et akirin. L'objectif de ma thèse a été de caractériser la fonction de ces deux gènes.

## **II. Le gène big-bang module la tolérance immunitaire intestinale chez la drosophile**

L'intestin moyen est un organe crucial dans la physiologie des drosophiles. C'est précisément dans ce segment de l'intestin que se déroule la plus grande partie des activités digestives. En effet, l'intestin moyen, contrairement à l'intestin antérieur ou postérieur est protégé par une matrice semi-perméable, dite matrice péritrophique, composée de chitine et de glycoprotéines laissant circuler les enzymes digestives. Cette matrice protège un épithélium monostratifié principalement composé d'entérocytes capables de produire des PAMs et des ROS en cas d'infection. Lors du vieillissement de drosophiles élevées en condition standard, le contenu microbien de l'intestin ainsi que la force de l'activation de ces réponses immunitaires locales croissent, provoquant une augmentation du nombre de divisions régénératives des cellules souches intestinales (ISCs). Une étude a révélé qu'une viabilité optimale des drosophiles est associée à un taux modéré de division des ISCs (Biteau et al., 2010), renforçant l'idée qu'un équilibre immunitaire dans l'intestin moyen est crucial pour la physiologie de la drosophile. De façon étonnante, les pathologies liées à l'âge observées chez la drosophile présentent certaines similarités (une augmentation des réponses inflammatoires, une division anormale des cellules souches) avec les maladies intestinales chroniques chez l'Homme. Des tests fonctionnels ont été développés chez la drosophile afin d'approfondir la compréhension de ce type de pathologies, par exemple, par ingestion de bactéries pathogènes dégradant l'intestin (*Pseudomonas entomophila*, *Serratia marcescens*), de commensaux délétères (*Erwinia carotovora*) ou de

composés chimiques toxiques (DSS, Bléomycine). Ces modèles d'étude ont notamment permis de mettre en évidence le réseau de régulation complexe contrôlant la différenciation et la division des ISCs (**Figure A3**).

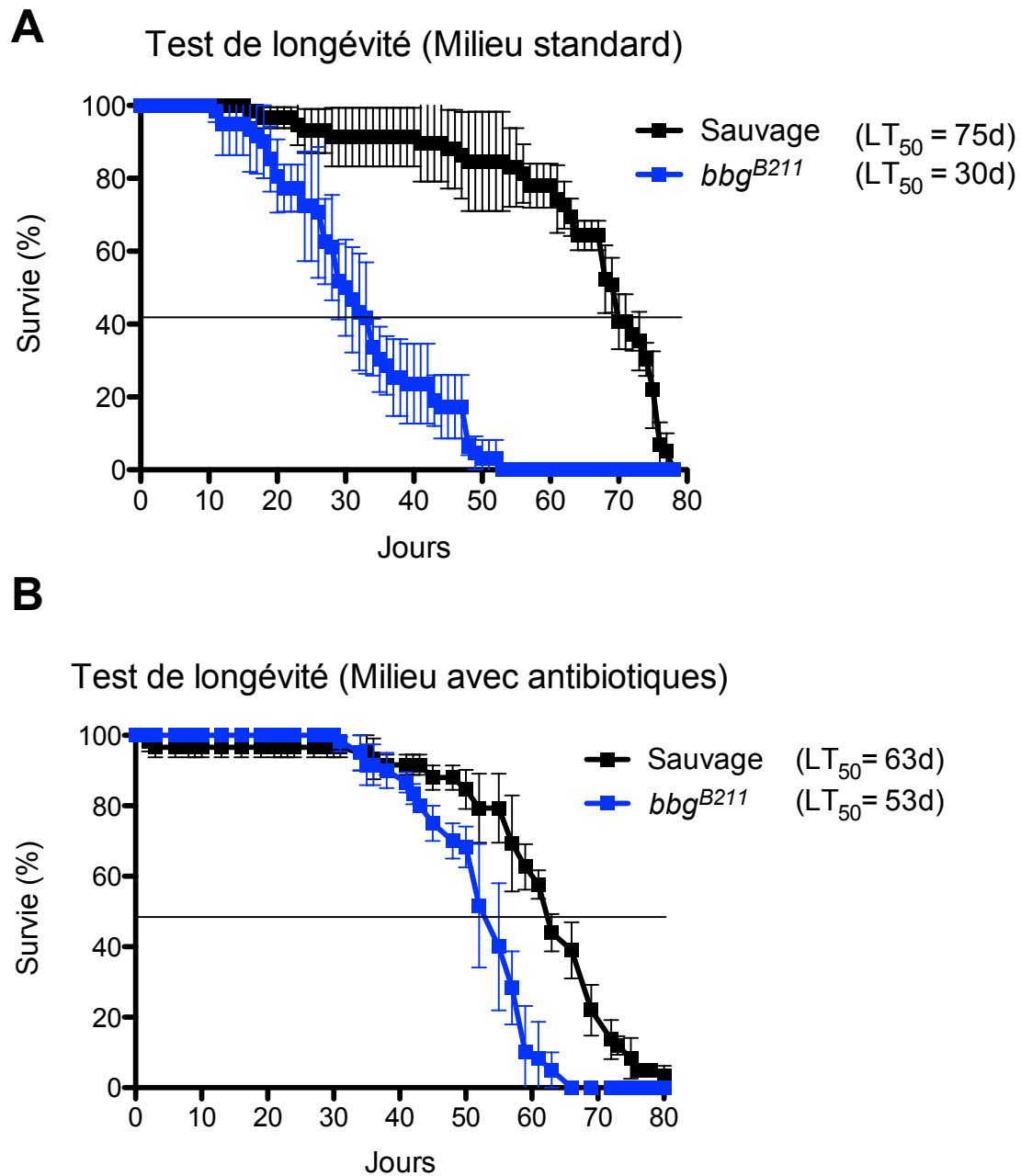


**Figure A3 : Voies de signalisation contrôlant la prolifération des cellules souches intestinales dans l'épithélium de l'intestin moyen de drosophile**

Un crible génétique préalablement réalisé au laboratoire avait identifié big-bang (CG42230, *bbg*) comme requis dans la défense locale de la drosophile contre des infections intestinales. Basé sur ces observations initiales, nous avons envisagé que big-bang pourrait agir comme un régulateur des voies immunitaires dans l'intestin moyen de drosophile.

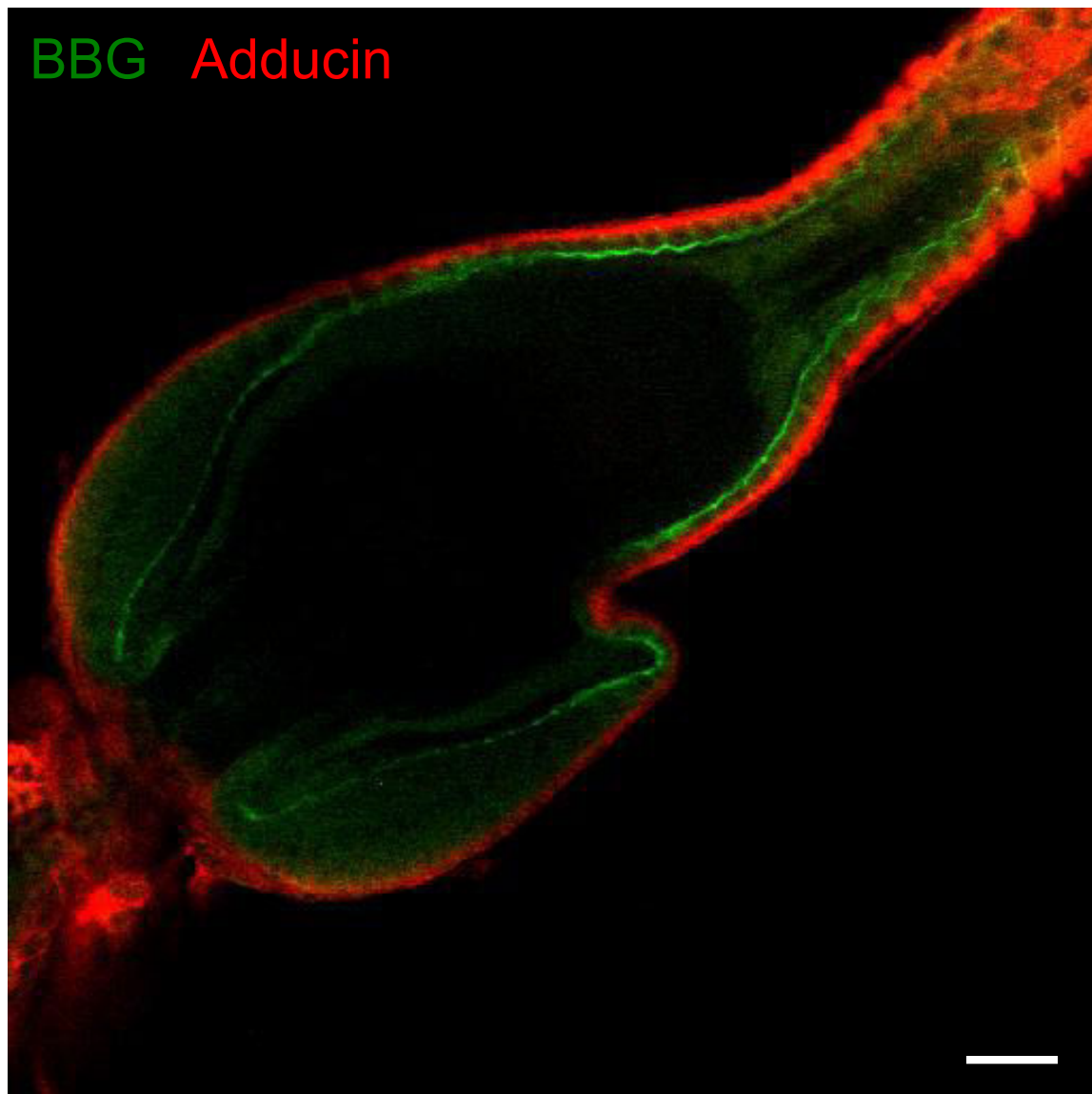
Le gène *bbg* code pour une protéine à multiples domaines PSD-95, Disc-large, ZO-1 (PDZ) associée à la membrane cytoplasmique. Pour étudier *bbg* au cours de la réponse immunitaire, nous avons utilisé le mutant nul *bbg*<sup>B211</sup> généré précédemment par nos collaborateurs.

La durée de vie moyenne des mouches adultes mutantes *bbg*<sup>B211</sup> est de 30 jours, en revanche, cette durée est de 70 jours pour des mouches sauvages (**Figure A4A**). Chez l'adulte j'ai pu mettre en évidence une localisation de BBG dans l'intestin moyen de drosophile (**Figure A5**). Or il est connu qu'une sur-activation des voies NF-κB dans l'intestin (inflammation intestinale) entraîne une létalité précoce. Afin d'évaluer si l'absence de BBG peut entraîner une inflammation anormale de l'intestin et expliquer le phénotype de mortalité précoce, j'ai mesuré le niveau d'activation de la voie IMD dans l'intestin moyen de mouches sauvages ou *bbg*<sup>B211</sup>, placées sur un milieu nutritif standard. Pour ce faire, j'ai mesuré le niveau d'activation d'un gène rapporteur de la voie IMD où l'expression de la b-galactosidase est placée sous la dépendance du promoteur de la diptéricine-A (*dpt-lacZ*).



**Figure A4 : Big-bang est requise pour la longévité des drosophiles adultes.**

Tests de longévité réalisés sur un milieu standard (A) et sur milieu contenant des antibiotiques (B). En conditions standard, la longévité des mutants *bbg*<sup>B211</sup> est réduite comparée à celle des mouches sauvages (A). La mort prématurée des mutants *bbg*<sup>B211</sup> est empêchée par l'ajout d'un traitement par antibiotiques (B). Les lignes interposées sur le graphique indiquent le temps de létalité à 50% ( $LT_{50}$ ). Chaque courbe représente la moyenne de trois expériences indépendantes réalisées avec trois groupes de 20 mouches adultes. Les barres d'erreurs sont des écart-types.

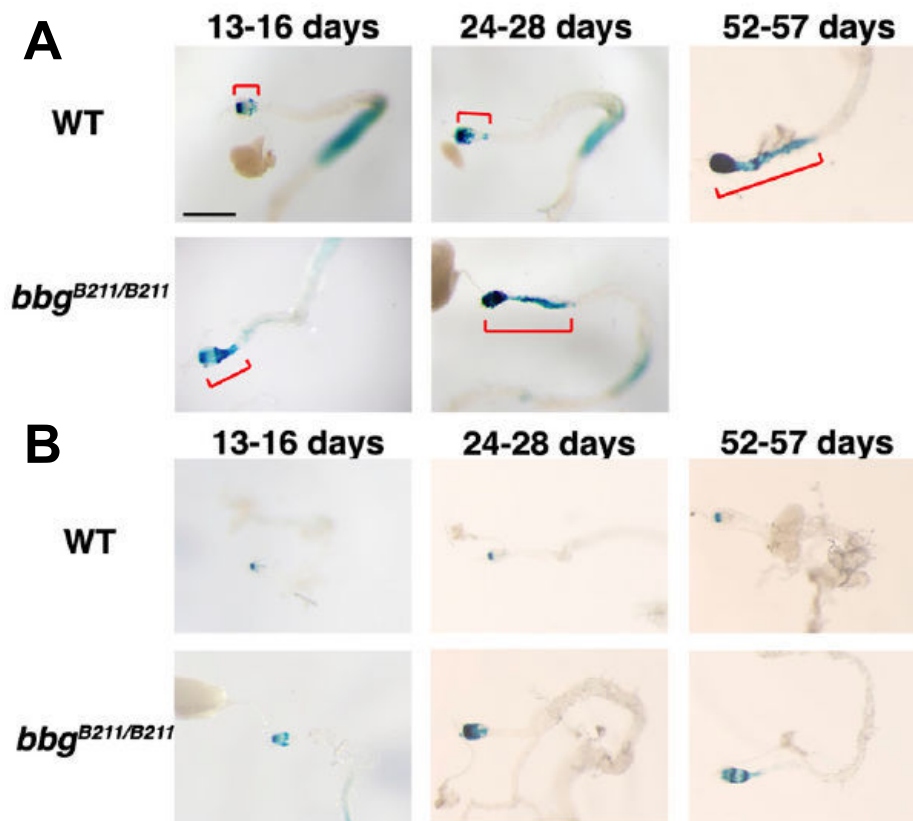


**Figure A5 : Big-bang est localisée au pôle apical des cellules épithéliales de l'intestin moyen.**

Immunolocalisation de Big-bang (BBG, vert) et de l'Adducin (marqueur du pôle basal de l'épithélium, rouge) sur intestin entier. Image représentative d'un échantillon expérimental de 10 intestins. Echelle : 60µm.

J'ai ainsi pu mettre en évidence une sur-activation de la voie IMD en absence de BBG (**Figure A6A**). Chez la drosophile la présence d'une sur-activation de cette voie a déjà été associée à un phénotype de longévité réduite dans le cas de gènes contrôlant négativement l'activation d'IMD (pour exemple : PIRK). Afin de comprendre si la sur-activation de la voie IMD observée est due à un rôle de BBG dans la régulation négative de la voie, ou à une surexposition de la flore endogène,

j'ai élevé des mouches sauvages et *bbg*<sup>B211</sup> en conditions semi-stériles. En éliminant la flore microbienne, à l'aide d'un cocktail d'antibiotiques, les mutants *bbg*<sup>B211</sup> ont retrouvé une durée de vie moyenne similaire à celle de mouches sauvages (**Figure A4B, A6B**). Ainsi l'absence de *bbg* favorise l'exposition des motifs microbiens de la flore résidente dans l'intestin au système immunitaire.

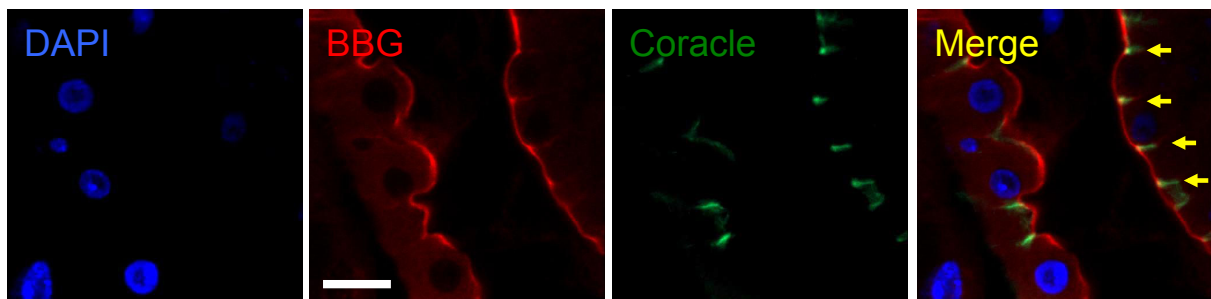


**Figure A6 : Big-bang est requise pour la tolérance immunitaire intestinale dans l'intestin moyen antérieur.**

Mesure de l'activité du rapporteur *Diptericin-LacZ* sur l'intestin moyen de drosophiles élevées sur un milieu standard (**A**) et sur milieu contenant des antibiotiques (**B**). L'activation locale de la voie IMD dans l'intestin moyen antérieur augmente avec l'âge des drosophiles sauvages élevées sur un milieu standard mais est plus élevée chez les mutants *bbg*<sup>B211</sup> (**A**). Le traitement par ajout d'antibiotiques abolit la sur-activation de la voie IMD observée en A (**B**). L'âge des drosophiles utilisées est indiqué pour chaque condition. Chaque image est représentative d'un échantillon expérimental de 15 intestins. Echelle : 300µm

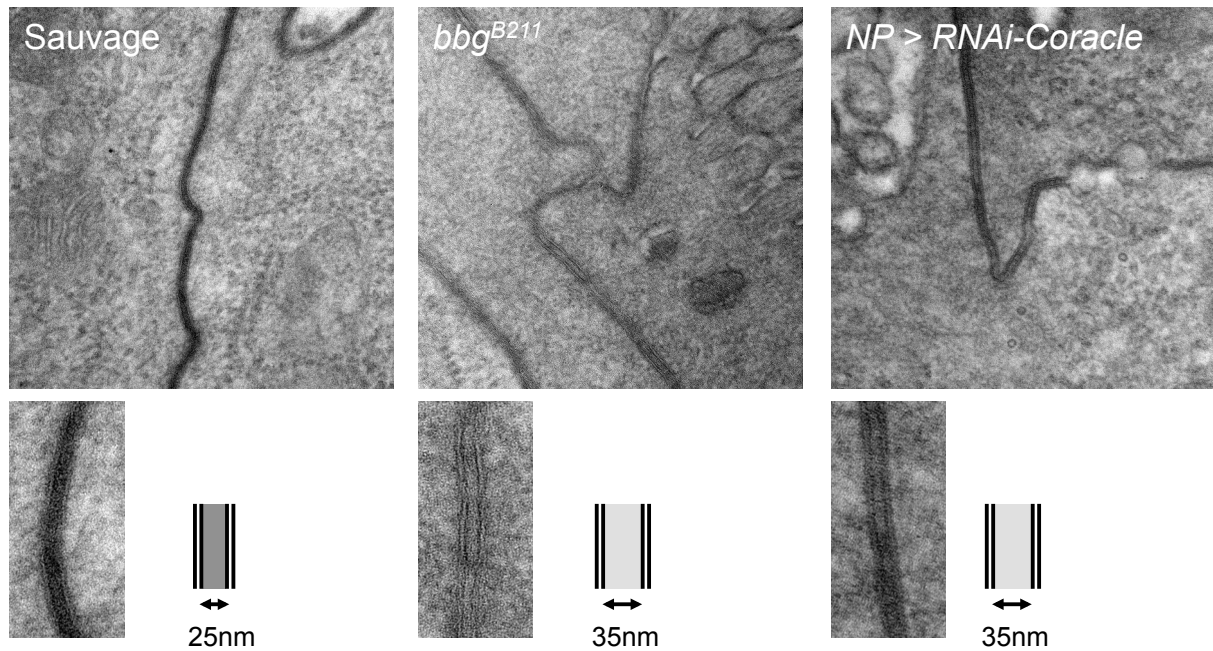


Pour comprendre ce phénomène j'ai réalisé un immuno-marquage de BBG sur des coupes d'intestin moyen. Ceci m'a permis de localiser BBG par immunofluorescence au pôle apical des cellules épithéliales, notamment au niveau des jonctions septées, analogues des jonctions serrées des mammifères (**Figure A7**). Illustrée par microscopie électronique à transmission, l'absence de bbg provoque l'élargissement de l'espace inter-membranaire et la perte des densifications de la jonction entre les cellules épithéliales intestinales (**Figure A8**). Par ailleurs, j'ai pu démontrer que cette désorganisation des jonctions septées, en l'absence de bbg, provoque une susceptibilité des drosophiles aux infections orales par la bactérie *Pseudomonas aeruginosa* (**Figure A9**). Cette dernière franchit plus rapidement la barrière épithéliale de l'intestin et envahissent l'hémolymphe, liquide circulant de la cavité générale. J'ai également pu démontrer que l'absence de Coracle, protéine structurante des jonctions septées, aboutit aux mêmes résultats (**Figure A8 et A9**).



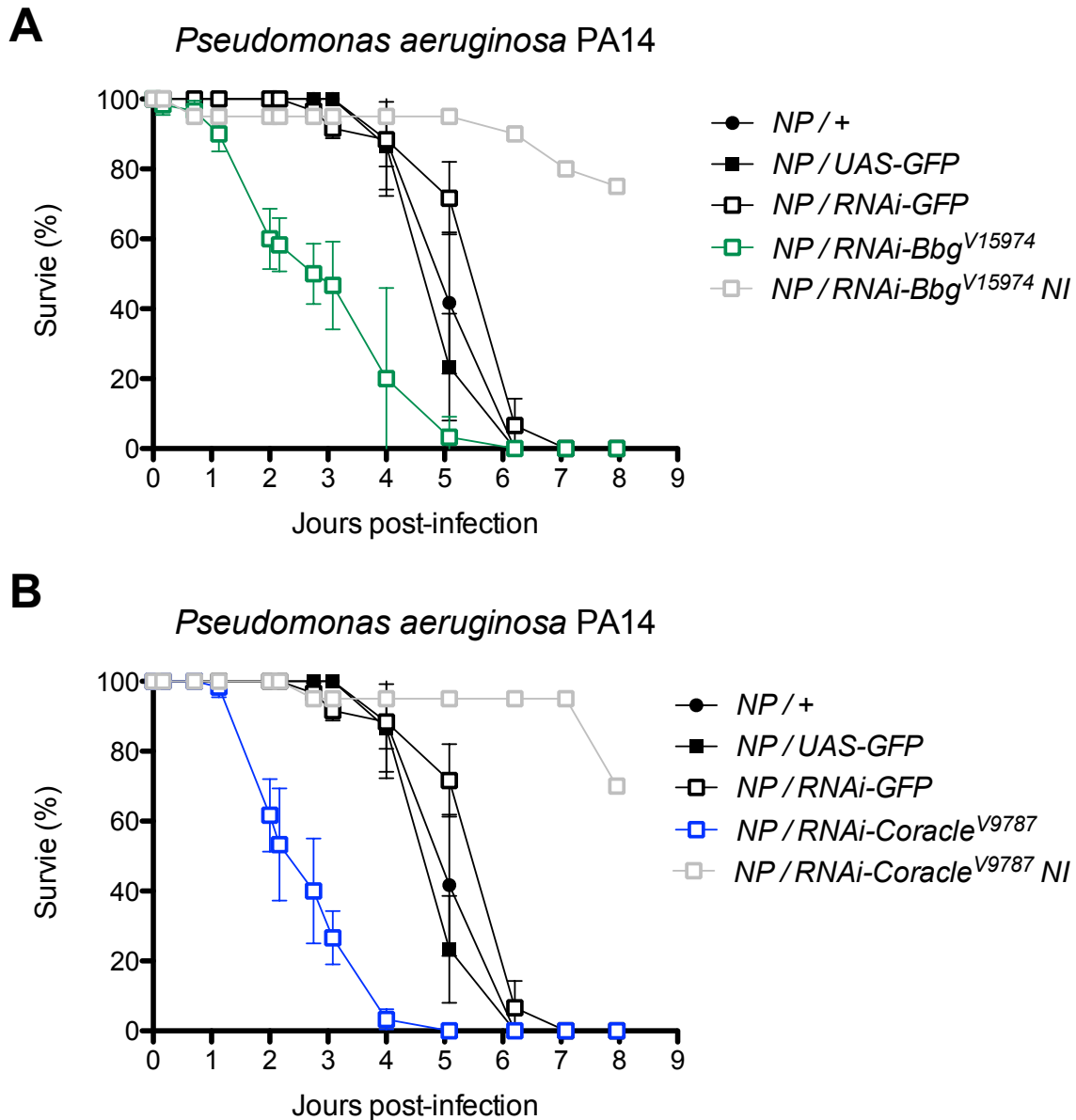
**Figure A7 : Big-bang est localisée aux côtés apical et latéral des entérocytes de l'intestin moyen, notamment au niveau des jonctions septées.**

Immunolocalisation de Big-bang (rouge) et de Coracle (vert) sur l'intestin entier (marquage nucléaire par DAPI (bleu)). Big-bang (BBG, rouge) est distribuée à la façon d'un anneau apical autour dans les entérocytes intestinaux. La localisation de BBG coïncide avec celle de Coracle (vert), une protéine associée aux jonctions septées. Images représentatives d'un échantillon expérimental de 10 intestins. Echelle : 30µm.



**Figure A8 : L'absence de Big-bang provoque une déstructuration des jonctions septées de l'intestin moyen.**

Micrographes par microscopie électronique à transmission de coupes transversales à travers l'intestin moyen antérieur de drosophile sauvage, *bbg<sup>B211</sup>* ou déficientes en Coracle par ARN-interférence générée dans l'intestin moyen (*NP > RNAi-Coracle*). Dans l'intestin moyen de drosophiles sauvages, l'espace paracellulaire au niveau des jonctions septées est limité à 25nm, tandis qu'il atteint 35nm dans des mutants déficients pour Big-bang ou Coracle. Grossissement : 120,000x. Les images rectangulaires constituent un grossissement des images originales au niveau de la jonction septée. Images représentatives d'un échantillon expérimental de cinq intestins.



**Figure A9 : Les jonctions septées sont requises pour prévenir l'infection intestinale par la bactérie invasive *Pseudomonas aeruginosa*.**

Une déficience en Big-bang (A) ou Coracle (B) dans l'intestin moyen des drosophiles amoindrit la survie des mouches infectées par *P. aeruginosa*. Des drosophiles contrôle (*NP / +*), (*NP / UAS-GFP*) (*NP / RNAi-GFP*), déficientes pour Big-bang (*NP / RNAi-Bbg<sup>V15974</sup>*) ou pour Coracle (*NP / RNAi-Coracle<sup>V9787</sup>*) ont été infectées par ingestion de *P. aeruginosa* à 25°C ou maintenues sur une solution de saccharose contrôle sans pathogène (mentionné par *NI*). Ces données sont représentatives de trois expériences indépendantes réalisées avec trois groupes de 20 drosophiles.

Collectivement, mes résultats indiquent que bbg et les jonctions septées jouent un rôle essentiel pour la tolérance immunitaire de l'épithélium intestinal envers

les bactéries de la flore endogène. En outre, ces jonctions sont requises, dans le cas d'infections intestinales par des bactéries entomopathogènes telles que *P. aeruginosa* pour ralentir le passage des bactéries vers la circulation générale et permettre vraisemblablement au système immunitaire inné de contenir l'infection.

Il reste désormais à explorer le rôle moléculaire de BBG au sein du complexe de jonction septée, notamment via l'identification de ses partenaires protéiques. Sa fonction au pôle apical de l'entérocyte, au delà des jonctions septées est également inconnue. La protéine BBG n'est conservée qu'au sein des *Drosophilidae*, il reste donc à découvrir si d'autres protéines à domaine PDZ telles que Zonula-Ocludens-1 (ZO-1) peuvent jouer un rôle protectif chez les mammifères au cours de pathologies inflammatoires chroniques analogues au phénotype observé dans l'intestin des mouches *bbg* telles que la maladie de Crohn.

### **III. L'Akirine spécifie la sélectivité des gènes cibles de NF-κB par le remodelage chromatinien au cours de la réponse immunitaire innée de la drosophile**

Au cours d'une réponse immunitaire innée, les facteurs de transcription NF-κB activent simultanément des gènes à activité pro-inflammatoire et anti-inflammatoire, afin de combattre une éventuelle infection et de limiter la période inflammatoire dans le temps. En 2008, notre laboratoire a identifié l'Akirine, une protéine strictement nucléaire conservée chez les mammifères (Akirine-2) agissant au niveau de NF-κB pour sélectivement activer la transcription de gènes à pro-inflammatoire. Cependant le mécanisme d'action des Akirines et les bases moléculaires de cette spectaculaire sélectivité transcriptionnelle étaient inconnus, ce qui a fait l'objet d'une partie significative de mon travail de thèse.

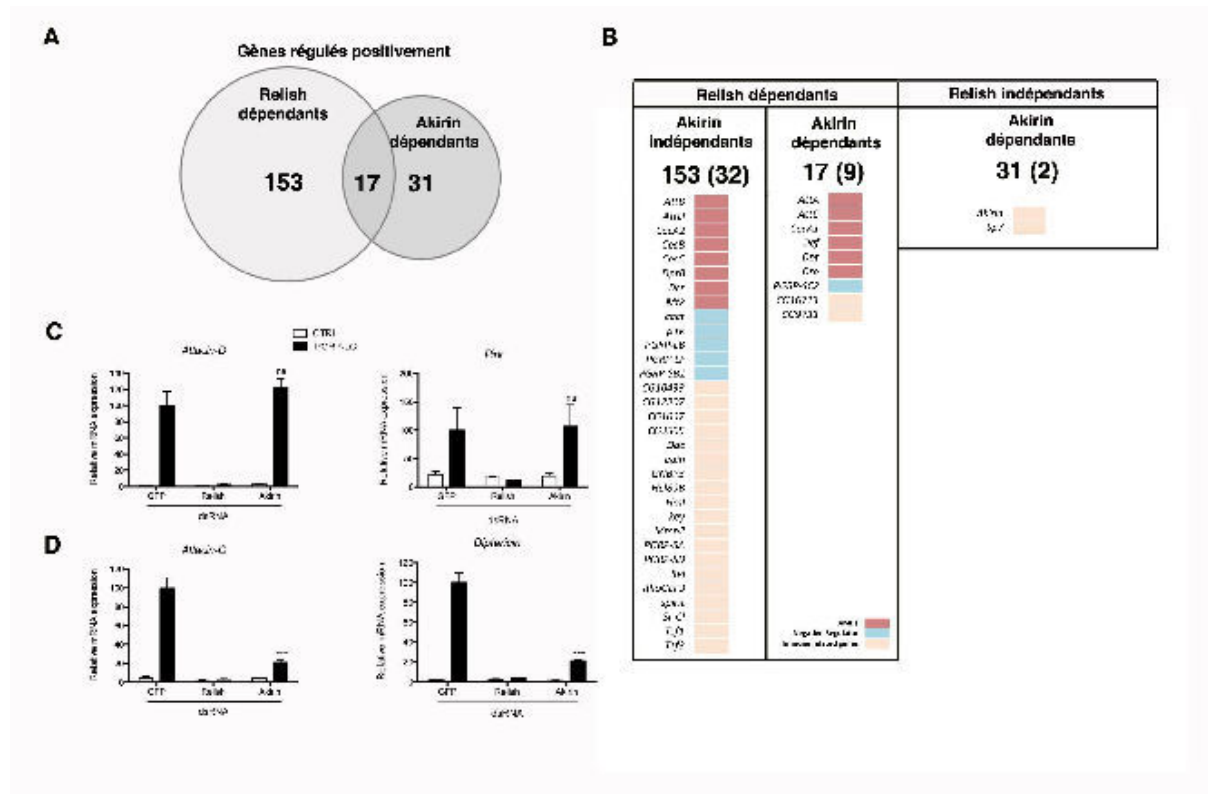
Le gène *akirin* est apparu lors de la formation des premiers eukaryotes, il y a environ deux milliards d'année. Les protistes provenant des phyla Alveolata (par exemple, *Guilardia theta*) et Heterolobosea (par exemple, *Naegleria gruberi*) sont considérés comme les organismes les plus primitifs possédant un gène *akirin*. Bien que ces observations placent l'origine du gène *akirin* en amont de la séparation des règnes des animaux, végétaux et des champignons, aucun gène orthologue *akirin* n'a pu être identifié jusqu'à présent dans un génome végétal ou fongique. De plus,

seules quelques espèces unicellulaires possèdent le gène *akirin*, et pour la plupart d'entre elles, ce gène est prédit comme étant un pseudogène inactif. En revanche, le gène *akirin* est présent chez l'ensemble des métaozaires, notamment parmi ses espèces les plus primitives telles que les placozoaires (par exemple, *Trichoplax adherens*), à l'exception des éponges qui sembleraient l'avoir perdu. Le gène *akirin* s'est dupliqué avec l'apparition des premiers poissons cartilagineux. La quasi-totalité des espèces vertébrées, à l'exception notables des espèces aviaires possèdent donc les gènes *akirin-1* (également appelé *Mighty*) et *akirin-2* (également appelé *FBI1*, et correspondant à l'homologue le plus proche du gène *akirin* invertébré).

L'Akirine de drosophile est une protéine de 201 acide aminés (AA) partageant 39,4% d'identité avec l'Akirine-2 de souris (201 AA) et humaine (203 AA). L'Akirine de drosophile (*DmAkirin*) et l'Akirine-2 humaine (*HsAkirin-2*) sont très proches fonctionnellement dans l'immunité. En effet, la déficience immunitaire provoquée par l'absence de *DmAkirin* peut être abolie en sur-exprimant *HsAkirin-2*. L'Akirine-1 quant à elle, ne semble pas reliée à une fonction immunitaire, du moins chez la souris. En effet, des cellules murines déficientes knock-out *akirin-1* induisent la totalité des gènes cibles de NF- $\kappa$ B suite à une stimulation au Lipopolysaccharide (LPS), un agoniste des Toll-like receptors (TLRs) ou au TNF. En outre, l'Akirine de drosophile est très proche (69,4% d'identité) de l'Akirine du moustique *Anopheles gambiae* (*AgAkirin*), le principal vecteur de la malaria. Même si cette question n'a pas encore été clairement adressée, il serait tentant de spéculer que l'*AgAkirin* pourrait également jouer un rôle dans la réponse immunitaire NF- $\kappa$ B-dépendante contre les parasites *Plasmodium*. Enfin, il faut souligner que l'orthologue de *akirin* chez la tique (*Ixodes scapularis*, vecteur de la maladie de Lyme), également appelé *subolesin* participe également à la réponse immunitaire NF- $\kappa$ B-dépendante dirigée contre *Anaplasma phagocytophylum*, l'une des bactéries à Gram-négative responsables de la maladie de Lyme. Il semblerait en outre que la *Subolesin* et le facteur *Relish-like* de la tique promouvrait leur expression transcriptionnelle réciproque suite à un challenge bactérien par *A. phagocytophylum*. Cette boucle de régulation positive n'a pour l'instant pas été observée chez la drosophile ou chez la souris et pourrait provenir d'une évolution spécifiques aux arachnides.

Afin d'établir l'étendue de la sélectivité induite par l'Akirine chez la drosophile, j'ai réalisé un micro-array, dont l'analyse a mis en évidence parmi les gènes immunitaires cibles du facteur NF- $\kappa$ B *Relish*, 10 gènes définis comme « Akirine-

dépendants », et 32 autres comme « Akirine-indépendants » (**Figure A10**). Parallèlement, un double crible protéomique, destiné à identifier des protéines partenaires de l'Akirine dans la réponse immunitaire chez la drosophile, a mis en évidence la protéine Brahma-associated Protein 60kDa (Bap60). Mes résultats indiquent qu'après une stimulation immunitaire, Bap60 est effectivement requis pour l'activation des gènes Akirine-dépendants, mais pas pour celle des gènes Akirine-indépendants (**Figure A11**). Afin d'explorer la dynamique de cette interaction au niveau moléculaire, j'ai utilisé une approche d'immuno-précipitation sur chromatine. Ceci a mis en évidence le recrutement d'Akirine et de Bap60 au promoteur proximal de gènes Akirine-dépendants (attacine-A) et non de gènes Akirine-indépendants (attacine-D) de façon croissante 15min, 30min, 1h et 2h après stimulation immunitaire (**Figure A12**). De plus, les interactions endogènes entre Akirine, Bap60 et Relish ont été observées par immuno-précipitation au cours de la réponse immunitaire, renforçant l'hypothèse d'un complexe tripartite se formant à proximité du promoteur des gènes Akirine-dépendants.

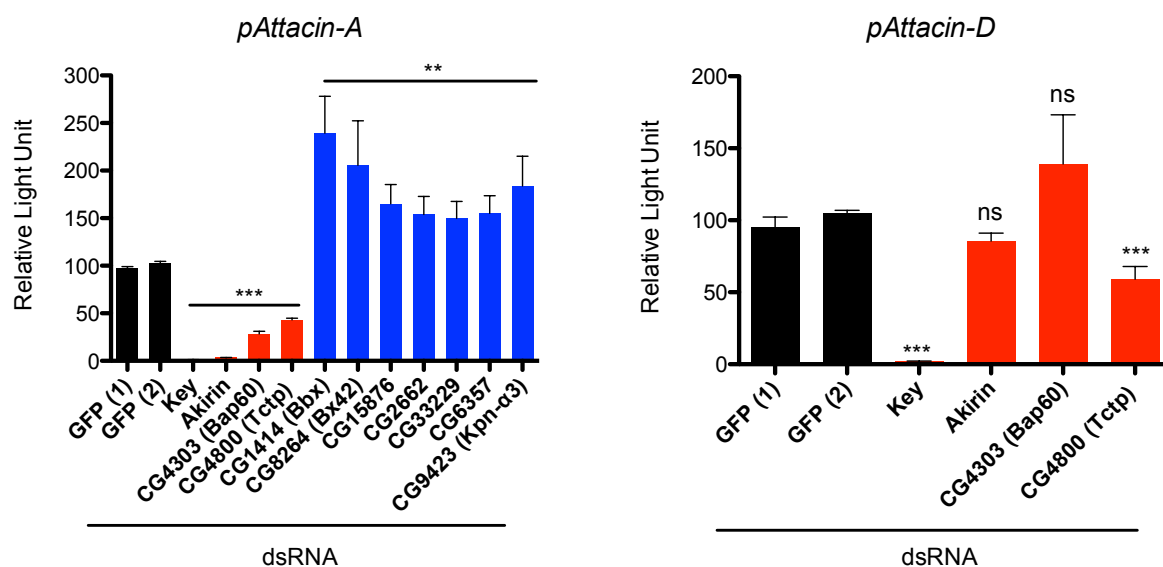


**Figure A10 : L'Akirine influence l'expression seule d'un sous-ensemble de gènes cibles de Relish.**

**A-B** : Diagramme de Venn (**A**) et représentation en tableau (**B**) de l'analyse du micro-array. Ces représentations indiquent les gènes stimulés en cellules S2 stimulées par une surexpression de PGRP-LC montrant une réduction de leur expression d'au moins deux fois en l'absence de relish ou d'akirin (RNAi-Relish, RNAi-Akirin) par rapport à une situation contrôle (RNAi-GFP). Les nombres entre parenthèse correspondent aux gènes dont le GO (ontologie du gène) correspond à une fonction reliée à l'immunité.

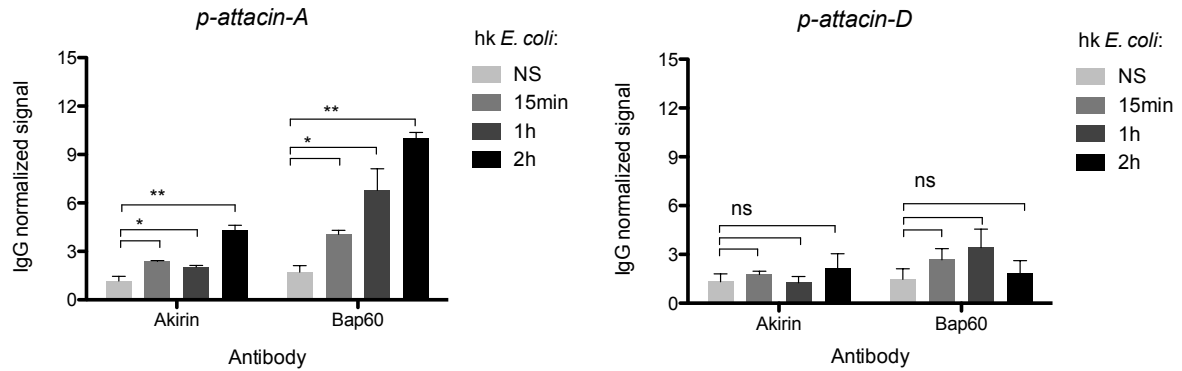
**C-D** : PCR quantitative mesurant le niveau d'expression en ARNm des gènes akirin-indépendents (**C**) *Pirk*, *Attacin-D* et des gènes akirin-dépendents (**D**) *Attacin-C* et *Diptericin-A* réalisée sur des cellules S2 triées transfectées par des ARN double-brin (ARNdb) dirigés contre *GFP*, *relish* ou *akirin* et un vecteur sur-exprimant PGRP-LCa pour stimuler la voie IMD.

Ces données représentent la moyenne +/- l'écart type de trois expériences indépendantes réalisées avec  $1.5 \times 10^6$  cellules S2. Student t test : \* P-value < 0.05 ; \*\* P-value < 0.01 ; \*\*\* P-value < 0.001.



**Figure A11 : Bap60 est un partenaire fonctionnel de l'Akirine dans l'activation sélective d'un sous-ensemble de gènes cibles de Relish.**

Essai luciférase réalisé sur des cellules S2 co-transfectées avec le plasmide rapporteur *attacin-A* (*pAttacin-A*) ou *attacin-D* (*pAttacin-D*) luciférase, l'ARNdb ciblant *GFP*, *kenny* (*key*), *akirin* et les partenaires de l'Akirine identifiés par un crible protéomique double-hybride, après 48h de stimulation par des bactéries *Escherichia coli* tuées à la chaleur (HK *E. coli*). Les données, normalisées rapport au contrôles dsRNA *GFP*, proviennent de trois expériences indépendantes réalisées avec  $5 \times 10^5$  cellules S2. Ces données représentent la moyenne +/- l'écart type de trois expériences indépendantes. Student t test : \* P-value < 0.05 ; \*\* P-value < 0.01 ; \*\*\* P-value < 0.001.

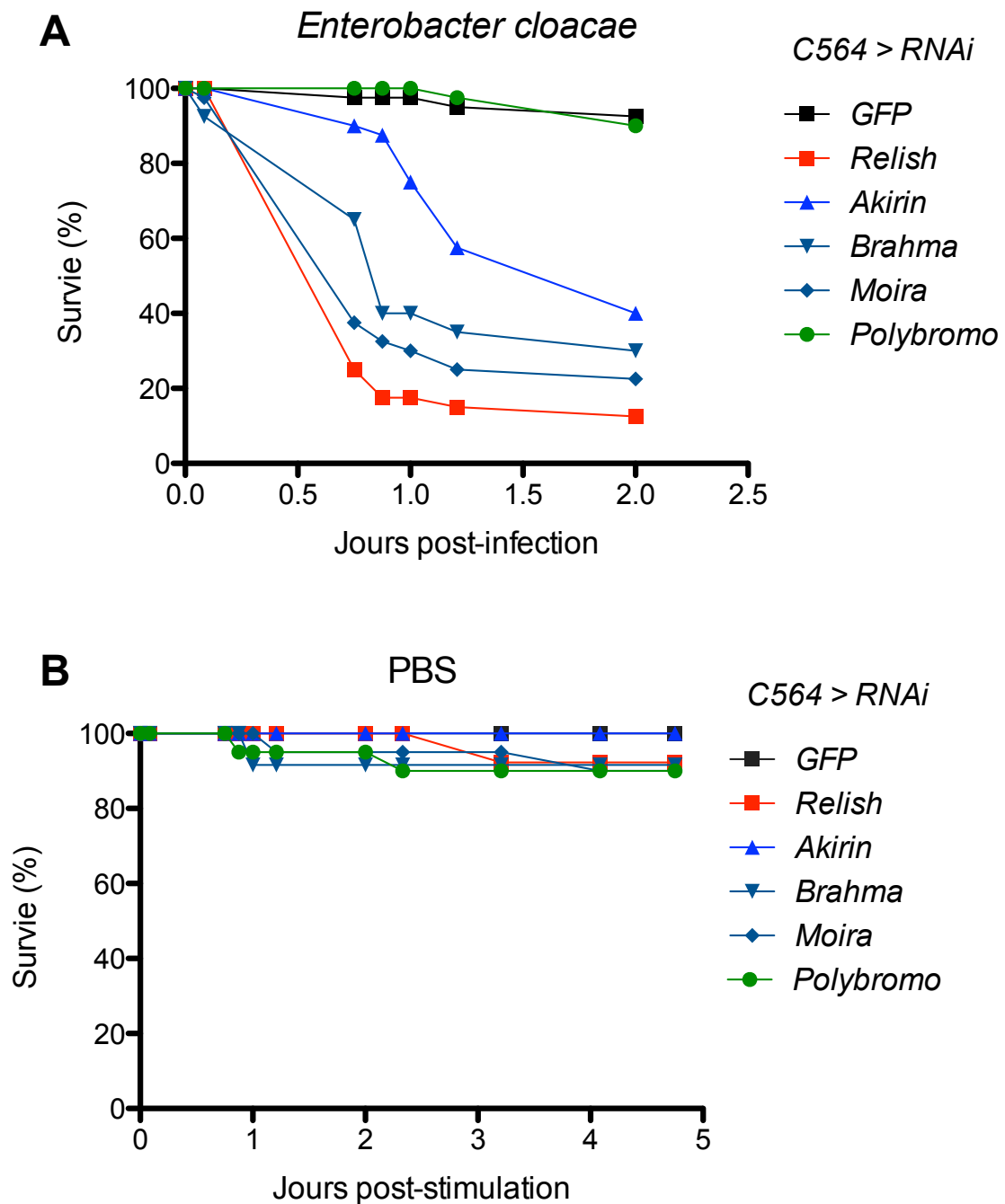


**Figure A12 : Akirine et Bap60 se lient aux promoteurs de gènes immunitaires Akirine-dépendants.**

Immunoprecipitation sur chromatine réalisée avec un anticorps anti-Akirin et anti-Bap60 sur une chromatine soniquée de cellules S2 stimulées par des bactéries *E. coli* tuées à la chaleur (hk *E. coli*) aux temps indiqués. Les graphiques montrent le recrutement de l'Akirine et Bap60 relatif aux valeurs obtenues avec l'anticorps contrôle isotypique de lapin, sur le promoteur proximal du gène akirin-dépendant *Attacin-A* (*p-attacin-A*) et du gène akirin-indépendant *Attacin-D* (*p-attacin-D*). Ces données représentent la moyenne +/- l'écart type de trois expériences indépendantes réalisées avec  $1,5 \times 10^6$  cellules S2 par IP. La signification statistique a été établie en comparant les valeurs des conditions stimulées (15min, 1h, 2h hk *E. coli*) avec les valeurs des conditions non-stimulées (NS). Student t test : \* P-value < 0.05 ; \*\* P-value < 0.01 ; \*\*\* P-value < 0.001.

Bap60 est un constituant essentiel du complexe de remodelage chromatinien SWI/SNF Brahma de *Drosophila melanogaster*, composé également de quatre autres sous-unités obligatoires (Brahma, Bap55, Moira et Snr1) et d'une à deux sous-unités additionnelles (Osa ou Polybromo et BAP170). Associé à Osa (complexe BAP) ou à Polybromo et BAP170 (complexe PBAP), le complexe Brahma cible des sous-ensembles de gènes mutuellement exclusifs. L'inactivation de chaque membre du complexe BAP (Bap60, Brahma, Bap55, Moira, Snr1 et Osa) par ARNi inactive la réponse immunitaire Akirine-dépendante (PAMs) sans affecter les gènes Akirine-indépendants. Cette déficience est néanmoins suffisante pour entraîner la mort des mouches suite à une infection systémique par la bactérie à Gram (-) *Enterobacter cloacae* (Figure A13). L'inactivation de Polybromo, spécifique au complexe PBAP, n'entraîne en revanche aucune déficience immunitaire des drosophiles adultes (Figure A13).





**Figure A13 : Le complexe Brahma BAP est requis pour lutter contre les infections aux bactéries à Gram-négative.**

Tests de survie après infection septique par *Enterobacter cloacae* ou piqûre au PBS stérile de drosophiles exprimant un construit RNAi dirigé contre la *GFP* (contrôle), *Relish*, *Akirin*, des membres du complexe Brahma (*Brahma*, *Moira*) ou du complexe PBAP (*Polybromo*). Ces données sont représentatives de trois expériences indépendantes réalisées avec trois groupes de 15 à 20 drosophiles adultes. Student t test : \* P-value < 0.05 ; \*\* P-value < 0.01 ; \*\*\* P-value < 0.001.

Collectivement, ces résultats suggèrent que l'Akirine, via son interaction avec le complexe BAP, agit comme un sélecteur des gènes cibles de NF- $\kappa$ B au cours de la réponse immunitaire innée. Soutenant cette conclusion, l'analyse bio-informatique des promoteurs Akirine-dépendants révèle un faible pourcentage de séquences CpG (30%) par rapport aux promoteurs Akirine-indépendants (65%), une caractéristique biochimique des promoteurs nécessitant un remodelage chromatinien par SWI/SNF pour leur activation. Plus largement, comprendre comment les Akirines orientent sélectivement la transcription des gènes à propriété pro-inflammatoire permettrait d'établir des stratégies thérapeutiques anti-inflammatoires plus spécifiques et provoquant moins d'effets secondaires pour traiter les inflammations chroniques telles que la polyarthrite rhumatoïde, le lupus ou la maladie de Crohn.

## Caractérisation des mécanismes de régulation de la voie IMD au cours de la réponse immunitaire chez *Drosophila melanogaster*

### Résumé

Le système immunitaire inné est un mécanisme de défense commun à tous les métazoaires. Chez l'Homme comme chez la drosophile, son activation peut être délétère lorsqu'elle est incontrôlée. L'étude des mécanismes qui sous-tendent cet équilibre entre l'activation ou non de la réponse immunitaire innée est à la base de mes travaux de thèse.

En utilisant le modèle *Drosophila melanogaster*, j'ai caractérisé la protéine Big-bang comme un acteur important de la balance immunitaire intestinale. Mes résultats démontrent que Big-bang est un constituant des jonctions obturantes de l'épithélium intestinal. Son absence provoque une rupture de tolérance immunitaire envers la flore bactérienne endogène et d'autre part une sensibilité accrue aux pathogènes invasifs. Mes travaux de thèse ont également permis de caractériser Akirine, une protéine nucléaire qui agit au niveau des facteurs NF- $\kappa$ B de la drosophile à l'Homme. Mes résultats démontrent qu'Akirine est un sélecteur qui agit de concert avec le complexe de remodelage de la chromatine SWI/SNF et NF- $\kappa$ B pour transcrire un sous-ensemble de gènes pro-inflammatoires.

**Mots clefs** : Immunité innée, NF- $\kappa$ B, Inflammation, Tolérance, Drosophile

### Résumé en anglais

The innate immune is required by all metazoan to defend themselves against microorganisms. When abnormally activated however, innate immune responses cause deleterious chronic inflammation. The study of the fragile equilibrium between immune responses and tolerance has fundamentally shaped the projects of my PhD work.

First, using *Drosophila melangoaster* as a model, I characterized Big-bang as a major player of the immune balance in the gut. I could show that Big-bang is a *bona fide* component of midgut epithelium septate junctions. Consequently, big-bang deficient flies have an impaired tolerance against commensal microorganisms and are susceptible to invasive gut pathogens, ultimately leading to a premature death of flies. I focused the second part of my PhD work on the characterization of Akirin, a nuclear protein required for the activation of NF- $\kappa$ B response from *Drosophila* to humans. My results showed that Akirin is a selector molecule, acting together with NF- $\kappa$ B and the SWI/SNF chromatin-remodeling complex to sustain the transcription of a subset of pro-inflammatory genes. **Key words**: Innate immunity, NF- $\kappa$ B, Inflammation, Tolerance, *Drosophila*