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# Regulation of NF-KB pathways during the innate immune response

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**ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ**

**UPR9022 CNRS - Modèles insectes d'immunité innée**

**THÈSE** présentée par

**Alexandre CAMMARATA-MOUCHTOURIS**

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Pour obtenir le grade de : **Docteur de l'Université de Strasbourg**  
Discipline/S spécialité : Aspects cellulaire et moléculaire de la biologie

**Régulation des voies NF- $\kappa$ B  
au cours de la réponse immunitaire innée**

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## Abbreviations

3'UTR: 3' Untranslated region

ACF: ATP-utilizing chromatin assembly and remodeling factor

Ago1: Argonaute 1

AMP: Anti-microbial peptide

ATP: Adenosine triphosphate

Att: Attacin

BAFs: Brahma-associated factors

BAP complex: OSA-associated Brahma complex

Bap60: Brahma-associated protein 60kDa

Bcl3: B-cell lymphoma 3

Brg-1: Brahma-related gene 1

Brm: Brahma

C/EBP $\delta$ : CCAAT/enhancer-binding protein- $\delta$

CBP: CREB-binding protein

Chd: Chromodomain-helicase DNA binding

ChIP: Chromatin Immunoprecipitation

CHRAC: chromatin accessibility complex

CREB1: cAMP-responsive-element-binding protein 1

CRISPR/Cas: Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9

DAP-type: meso-diaminopymelic-type

Dcr-1: Dicer-1

DD: Death domain

DIAP2: Drosophila inhibitor of apoptosis 2

DIF: Dorsal-related immunity factor

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

Drs: Drosomycin

dsRNA: double-stranded RNA

*E. coli*: *Escherichia coli*

*Ef*: *Enterococcus faecalis*

EGF: Epidermal growth factor

ELAM-1: endothelial-leukocyte adhesion molecule 1

H3K27: Histone 3 Lysine 27

H3K4: Histone 3 Lysine residue 4

H3K4ac: H3K4 acetylation

H3K4me3: H3K4 tri-methylation

H3K9: Histone 3 Lysine 9

HAT: Histone acetyl transferase

HDAC: Histone deacetylase

HECT: Homologous to the E6-AP Carboxyl Terminus

HKE: Heat-killed *Escherichia coli*

Hyd: hyperplastic discs

IAP: Inhibitor of apoptosis

|  |  |
|--|--|
| IBM: IAP2 binding motif                          | Lys-PGN: Lys-type peptidoglycan  |
| ICAM-1: Intercellular adhesion molecule 1        | Mad: Mothers against Dpp   |
| IFN: Interferon                                  | MAMP: Microbial-associated molecular pattern                                   |
| IKK: Inhibitor of NF- $\kappa$ B Kinase          | MAP: Mitogen associated protein  |
| IL-1R: Interleukin-1 Receptor                    | MAPK: MAP kinase   |
| IL: Interleukin                                  | MAPKK: MAPK kinase   |
| IMD: Immune deficiency                           | MAPKKK: MAPKK kinase   |
| Ino80: Inositol auxotroph 80                     | MCP-1: Monocyte chemotactic protein 1  |
| IP-10: Interferon-inducible protein 10           | miRNAs: microRNAs  |
| IRAK1: Interleukin-1 receptor associated kinase1 | <i>Ml: Micrococcus luteus</i>  |
| Ird5: Immune-response deficient 5                | Myd88: Myeloid differentiation primary response gene 88                        |
| IRF: IFN-regulatory factors                      | NCoR: Nuclear receptor corepressor   |
| Iswi: Imitation switch                           | ncRNAs: non-coding RNAs  |
| I $\kappa$ B: Inhibitor of NF- $\kappa$ B        | NEMO: NF- $\kappa$ B essential modulator                                       |
| JMJC: Jumonji C                                  | NF- $\kappa$ B: Nuclear factor kappa-light-chain-enhancer of activated B cells |
| K48: Lysine 48                                   | NLS: Nuclear localization signal   |
| K63: Lysine 63                                   | NURD: Nucleosome remodeling and deacetylase                                    |
| KD: Knock-down                                   | NURF: Nucleosome remodeling factor   |
| Key: Kenny                                       | PAMP: Pathogen-associated molecular pattern                                    |
| KO: Knock-out                                    | PGN: Peptidoglycan   |
| lncRNAs: long non-coding RNAs                    | PGRP: peptidoglycan-recognition protein  |
| Loqs: Loquacious                                 | Pirk: Poor immune response upon knock-in                                       |
| LPRG: Late primary response genes                | PolyUb: polymer of ubiquitin   |
| LPS: Lipopolysaccharide                          | PRG: Primary response genes  |
| LSD1: Lysine-specific demethylase 1              |  |

PRR: Pattern-recognition receptor

Psh: Persephone

RANTES: Regulated upon activation, normal T-Cell expressed, and presumably secreted

RHD: Rel-homology domain

RING: Really interesting new gene

RNAi: RNA-interference

RuNX1: runt-related transcription factor 1

Ruvbl2: RuvB Like AAA ATPase 2

SET: suppressor of variegation-enhancer of zeste-trithorax

siRNA: short-interfering RNA

SmarcA1: SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin A1

SmarcD2: SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin D2

SMRT: silencing mediator of retinoic acid and thyroid hormone receptors

Spz: Spätzle

SRG: Secondary response genes

SWI/SNF: Switching / Sucrose -fermentable

Syt7: Synaptotagmin7

TAB2: TAK1-associated binding protein 2

TAD: Transactivation domain

TAK1: TGF- $\beta$ -activated kinase 1

TGF: Transforming growth factor

TIR: Toll/interleukin-1 receptor

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TNFR: Tumor-necrosis factor receptor

TRAF3: TNF receptor associated factor 3

TRIF: TIR domain containing adapter inducing interferon- $\beta$

TSS: Transcription start site

UBD: Ubiquitin binding domain

Ubr5: Ubiquitin protein ligase E3 component *n*-recognin 5

Uev1a: Ubiquitin-conjugating variant 1a enzyme

VCAM-1: Vascular cell adhesion molecule 1

# Regulation of NF- $\kappa$ B pathways during the innate immune response

*Every moved subject is necessarily moved by a moving one*

Aristotle, Physics, VII, 1, 241 b

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## I. Introduction: behind the notion of dynamic regulation

The term biology is formed by combining the Greek words *bios* (life) and *logos* (word/science). Jean-Baptiste Lamarck was one of the first to use it (*Hydrogéologie*, 1802). However, an idea of a *science of nature* wasn't new. Heraclitus of Ephesus (535-475 BC) developed the concept that a dynamic force was at play behind every living matter. This dynamic notion was best illustrated by his famous affirmation: *No man ever steps in the same river twice, for it's not the same river and he's not the same man*. For him, *contradiction* was the driving force of nature. There are interactions of different kinds that affect the living subjects, sometimes simultaneously, but always in a dynamic way. Studying them was the aim of the philosopher. This notion of contradiction also applied to the scientific approach, as the questions one might ask comes from the difference between what is known/understood and what is observed, the *science of nature* being inherently empirical. Contradiction becomes the engine of thought and logic the mean to understand. The logical thinking predominates and will then evolve into technical interest, based on the research focusing on the problems of knowledge. From the thought of contradiction comes the logical reasoning, from which can be deduced the technical tools needed to generate the *logos*, thus allowing more understanding (and more visible contradictions along the way).

Aristotle (384-322 BC) refers to the branches of learning as *epistêmai* (knowledge), best regarded as organized bodies of understanding rather than as ongoing records of empirical researches. He pursued the idea that movement is the basis of life. To understand it, it is necessary to study the characteristics of the subject and those of its environment (which allows this movement). For life to exist, underlying dynamics are needed. Understanding biological empirical observations amounts to understand the *kinesis* (transformative movements). Dynamics exist as potentials for the subject moved, which can transform into *energia* (accomplished actions). Dynamic movements correspond to the transition process



from potential to an act and the results of these movements are transformative actions. The construction of dynamics can not exist without time but also produces a time. Potentiality expresses the ability of the moved but does not perform the movement alone. This principle is found in the process, in the configuration of the stimulus-response movement, where there is a reception mechanism: that is the concept of dynamic interaction. Understanding dynamic movement in the form of temporal dimension allows to break with a mechanical reading of the biological phenomena, allowing the study of complex physiological phenomena.

Claude Bernard (1813-1878) made this idea of dynamic interaction his own and actually searched for applications of this concept. In 1865, Bernard made a presentation based on his researches to the Académie des Sciences where he stated that: *The functions of man and of higher animals seem to us independent of the physico-chemical conditions of the external environment, because its actual stimuli are found in an inner, organic, liquid environment* (Cooper, 2008). The internal environment of a living organism serves not only to protect and nurture the functions of living tissues but also constitutes the source of the stimuli that regulate the physiological phenomena that actually allows the tissues to function. It is the challenge to physiology to describe and explain these inner causes studied, during experimental investigations. Bernard believed that complex phenomena in living beings were built up from simpler phenomena, which could be associated together for a common final purpose: *The physiologist's prime object is to determine the elementary conditions of physiological phenomena and to grasp their natural subordination, so as to understand and then to follow the different combinations in the varied mechanism of animal organisms. Nevertheless, if we break up a living organism by isolating its different parts, it is only for the sake of ease in experimental analysis, and by no means in order to conceive them separately* (Cooper, 2008). Bernard was well aware of the issue of how the mechanisms identified by analysis could combine and work together to achieve highly integrated operations across

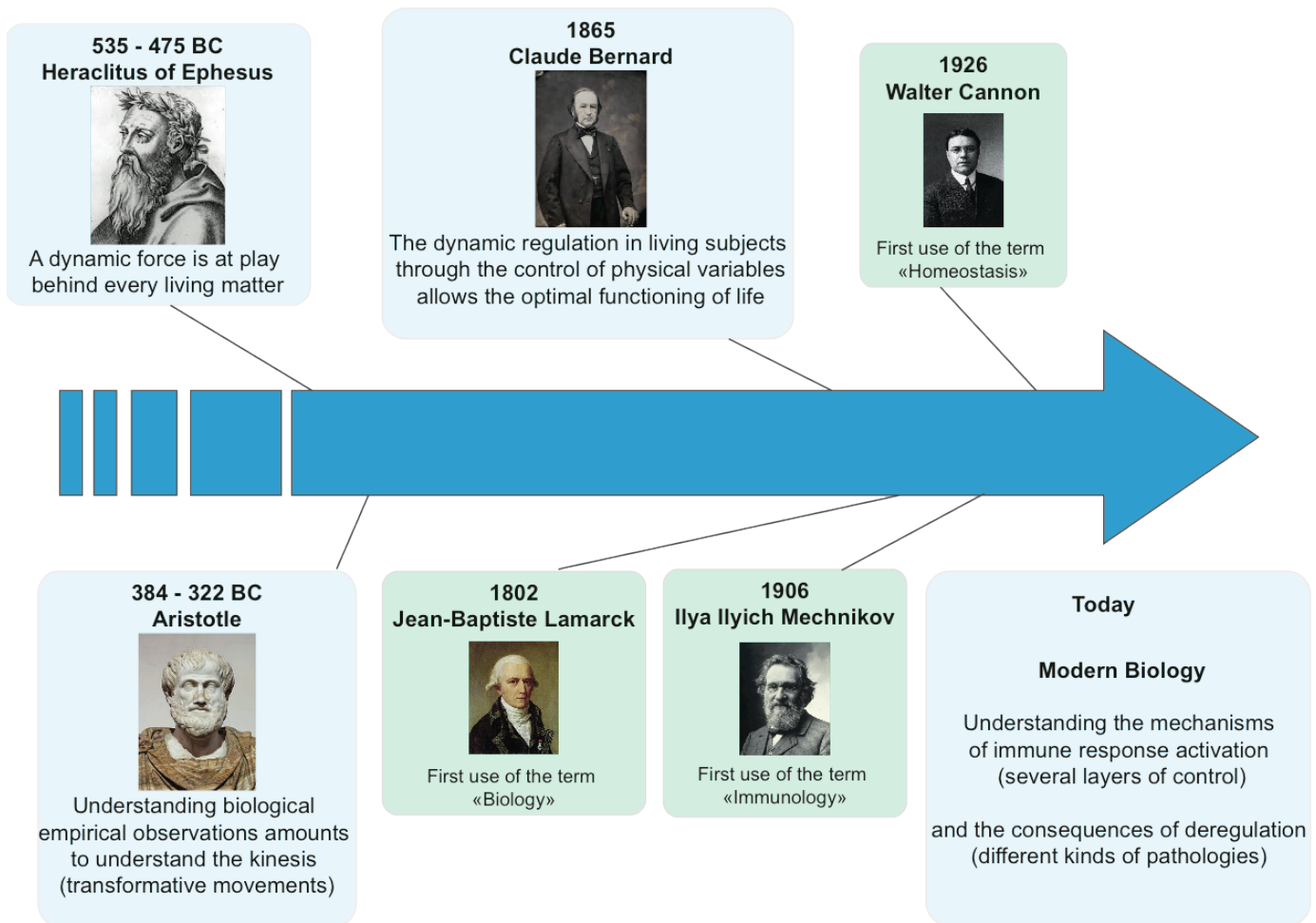
multiple tissues. Synthesis could not merely consist in the summation of simple phenomena uncovered in analytical experiments. Bernard used the example of water to illustrate that point: the separate properties of hydrogen and oxygen cannot predict their properties in combination as water. The dynamic regulation in living subjects through the control of physical variables allows the optimal functioning of life. Walter Cannon is an American physiologist that continued Bernard's work in the beginning of the 20<sup>th</sup> century. He defined and used the neologism *homeostasis* to qualify and explain the concept of inner regulation (Young, 2001). The term comes from the Greek words *homeo* (similar to) and *stasis* (standing still). Living beings are open systems continually in contact with the outer environment and prone to large fluctuations whenever environmental conditions are disturbed. However, through the agency of automatic, internal compensating adjustments, higher organisms are able to keep internal fluctuations within acceptable limits. Thus the variation of physiological variables is kept within a small range of values. Physiological negative feedback was the mechanism thought at that time to make homeostasis work. Feedback signals can be used to maintain stability against externally imposed fluctuations. Negative feedback can be used to approach a stable condition and once achieved, be maintained. A critic here would be that the term *homeostasis* by itself excluded a part of the notion of dynamic interactions established by Claude Bernard. For example, it does not account for the transformation of the subject over-time, as the organism itself is a physical variable. In any case, the concept of *homeostasis* made its way to all the branches of biology and even more. In particular, immunology made this concept the basis of its understanding.

Immunology comes from the latin *immunis* (exempt), meaning exempt of pathological conditions. The term was coined by Russian biologist Ilya Ilyich Mechnikov in 1906 and designates the science that studies the immune system. This system is a host defense system comprising many biological structures and processes within an organism that protects against

disease. Metazoans have established an 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. A localized protective response elicited by an injurious agent and the injured tissue was named inflammation. It evolved as a rapid and highly beneficial response to microbial infection, tissue injury, and other insults (Janeway and Medzhitov, 2002; Medzhitov, 2008; Nathan, 2002). When host cells capable of innate immune activation, such as tissue macrophages, encounter a microbe or another foreign or host irritant, the inflammatory response initiates within minutes (Smale, 2010). The host cells first recognize the stimulus through a wide variety of sensing mechanisms, often involving transmembrane receptors. These interactions transmit signals to the nucleus, resulting in the activation of numerous genes via both transcriptional and posttranscriptional mechanisms (Akira et al., 2006; Beutler, 2009; Ishii et al., 2008; Medzhitov, 2007). The products of these genes carry out diverse physiological functions. Some inducible gene products, such as antimicrobial peptides and complement factors, directly target infectious microorganisms. Others, including pro-inflammatory cytokines and chemokines, activate the recruitment of cells of both the innate and adaptive immune systems to the site of infection. When abnormally regulated however, innate immune responses contribute to the development of pathologies including autoimmune diseases, chronic inflammation and cancer (Karin et al., 2006; 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 notion of intrinsically dynamic regulation proves then to be essential in the understanding of the innate immune system.

Studying a biological system requires working with an adequate model. *Drosophila melanogaster* is a small fly that has been widely used 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) contributed to its success as a model organism. *Drosophila* and humans share many genes and molecular pathways with similar functions (Rubin and Lewis, 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. *Drosophila* and humans share many molecular pathways underlying the activation of their innate immune systems (Hoffmann and Reichhart, 2002) and studies demonstrated the relevance of this model to study innate immunity (Leulier and Lemaitre, 2008).

The focus of my PhD is to understand the subtlety of the dynamic systems involved in the regulation of innate immune pathways using *Drosophila melanogaster* as a model. In the next part, I will introduce the main innate immune pathway in flies and mammals and highlight the different players involved in its dynamic, in the format of a review article. I will then present my work on several aspects of this dynamic in *Drosophila* and mammals.



Timeline: the idea of regulation

The foundations of the idea of dynamic interactions date to a time when biology didn't have its modern meaning. The logical vision precedes the techniques, which come from the temporal context.

## II. Innate immune pathways dynamic regulation - review

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 pathway 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 counteract microbial challenges directly (e.g. Anti-microbial Peptides (AMPs), Reactive Oxygen Species (ROS)) or indirectly (e.g. Cytokines, Fever). In addition, regulators, activated or not by the pathway, will be present to allow a strong response or shutdown the activation.

This review aims at understanding the common points of innate immune pathways (namely the NF- $\kappa$ B ones) from *Drosophila* to humans. Consequently, the figures are highlighting the common features of the pathways, in a *Matryoshka* way. Like with the way those Russian dolls are made, more complexity layers will be uncovered at each part.

### A. NF- $\kappa$ B pathways are crucial for innate immune response

#### a) NF- $\kappa$ B: definition

Nuclear factor kappa B (NF- $\kappa$ B) was discovered biochemically as a DNA-binding activity protein in activated B cells, with affinity for the transcriptional enhancer of the immunoglobulin  $\kappa$  light-chain gene (Sen and Baltimore, 1986). NF- $\kappa$ B is a nuclear factor that gave its name to different pathways that can activate it in the same general manner (Figure 1).

#### b) Overview of the NF- $\kappa$ B pathways in *Drosophila*

There are two NF- $\kappa$ B pathways in *Drosophila* that play a fundamental role in the defense against invasive microbes by triggering the massive release of anti-microbial peptides. The Immune deficiency (IMD) and the Toll pathways are able to recognize,

discriminate and fight three main pathogen families: Gram-negative bacteria for IMD, Gram-positive bacteria and fungi for Toll.

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 and PGRP-LE (Neyen et al., 2012). The 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). In *Drosophila*, six members of PGRP family possess a PGRP domain bearing a functional peptidoglycan (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., 2011; 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., 2005; Gendrin et al., 2013; Kaneko et al., 2006; Maillet et al., 2008). Following proteolytic cleavage of its C-terminal Inhibitor of NF- $\kappa$ B (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 (Gilmore, 2006).

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”. Flies deficient in this pathway succumb more

rapidly when infected (Lemaitre et al., 1996). The term “danger-signal” 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. Alternatively, danger signals, in this case proteases produced by fungi, Gram-positive bacteria and possibly some Gram-negative bacteria, are sensed by the proteolytically activable serine protease Persephone (Psh), engaging the “danger-signal” pathway (Chamy et al., 2008; Gottar et al., 2006; Issa et al., 2018). 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. 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., 2004; Imler et al., 2000; Ooi et al., 2002). The NF- $\kappa$ B factors Dorsal and Dorsal-related immunity factor (DIF) are sequestered in the cytoplasm 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 or DIF, which then translocates to the nucleus and exerts DNA-binding activity (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989).

The *Drosophila* genome encodes three NF- $\kappa$ B factors: Relish, Dorsal and DIF. 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). 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 animals and plants against microorganisms (Toke, 2005). In addition to AMPs, the IMD pathway induces a few hundreds 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 or production of reactive oxygen species (Ferrandon et al., 2007).

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 GGGAAATCCC 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., 2010). Another study identified the response element of such heterodimers as GGGA(A/T)TC(C/A)C (Senger et al., 2004). Similarly to the IMD pathway, the most well characterized effectors of Toll pathway activation are AMPs. 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., 2002).

### c) Overview of the NF- $\kappa$ B pathways in humans

Hundreds of substances and physiological conditions are known to activate human NF- $\kappa$ B. These include (i) bacterial, fungal, or viral products that are often recognized by the Toll-like receptor (TLR) pathways; (ii) intercellular signaling mediators often recognized by members of the Tumor necrosis factor (TNF) receptor superfamily; (iii) immunoglobulin domain-containing receptors that regulate the adaptive immune response; and (iv) metabolic

or genotoxic stress conditions (Hoffmann and Baltimore, 2006). Signaling to NF- $\kappa$ B mediates multiple aspects of innate and adaptive immunity (Bonizzi and Karin, 2004; Ghosh et al., 1998; Silverman and Maniatis, 2001). NF- $\kappa$ B plays an essential role in early events of innate immune responses. Activation of NF- $\kappa$ B results in the upregulation of adhesion molecules and chemokines by cells within the tissue. This then leads to the recruitment and activation of effector cells, initially neutrophils and then macrophages and other leukocytes. NF- $\kappa$ B is also crucial for the production of antimicrobial effector molecules and for the survival of leukocytes in an inflammatory milieu. In fact, NF- $\kappa$ B has a fundamental role in mediating all of the classical attributes of inflammation - *rubor*, *calor*, *dolor* and *tumor* - by regulating transcriptional programs in tissue epithelial and stromal cells, vascular endothelial cells and haematopoietic cells (Ghosh and Hayden, 2008).

Two major signaling pathways lead to translocation of NF- $\kappa$ B dimers from the cytoplasm to the nucleus: the classical and the alternative pathway. The classical NF- $\kappa$ B pathway is activated by a variety of inflammatory signals, resulting in coordinate expression of multiple inflammatory and innate immune genes. The alternative pathway plays a central role in the expression of genes involved in development and maintenance of secondary lymphoid organs. The biochemical characteristics of these pathways echo this functional distinction: the canonical pathway is fast acting (responds within minutes) and is reversible due to the presence of multiple negative feedback mechanisms, whereas the non-canonical pathway responds more slowly (over hours and days), providing long-lasting nuclear NF- $\kappa$ B activity (Hoffmann and Baltimore, 2006). The classical way is the one of interest for us. In this pathway, proinflammatory cytokines and pathogen-associated molecular patterns (PAMPs), working through different receptors belonging to the tumor necrosis factor (TNF) receptor (TNFR) and Toll-like receptor (TLR)-interleukin-1 (IL-1) receptor (IL-1R) superfamilies. Toll-like receptors (TLR) are evolutionarily conserved Pattern recognition

receptors (PRRs) that recognize conserved Microbial-associated molecular patterns (MAMPs) (Janeway and Medzhitov, 2002; Kopp and Medzhitov, 2003). The role of TLRs as arbitrators of the self–non-self decision means that they play a central role in innate immunity as well as in the initiation of the adaptive immune responses. To date, 11 mammalian TLRs have been described, and each of them signals to NF- $\kappa$ B. These receptors have varied tissue distributions and recognize many different MAMPs including lipopolysaccharide (LPS), double-stranded RNA (dsRNA), nonmethylated CpG DNA, and flagellin. Some members of the TLR family are also capable of heterodimerization, thereby further expanding the repertoire of molecules that are recognized. The intracellular domain of Toll-like receptors bears strong homology with the intracellular domain of the IL-1R, and it is this shared Toll-IL-1R (TIR) domain that mediates interaction with down-stream signaling adapters that lead to activation of NF- $\kappa$ B (Hayden and Ghosh, 2004). The nature of the protein complexes that mediate signaling, and the way in which signals are regulated and integrated at the level of allosteric assembly determines whether the signaling output leads to a protective immune response or to serious pathologies such as sepsis (Gay et al., 2014).

NF- $\kappa$ B pathway stimulation starts with the internalization of specific receptors, triggering the activation of the I $\kappa$ B kinase (IKK) complex. The most common form of this complex consists of the IKK $\alpha$  and IKK $\beta$  catalytic subunits and the IKK $\gamma$  regulatory subunit (also called NEMO for ‘NF- $\kappa$ B essential modulator’). Hypomorphic NEMO deficiency is associated with susceptibility to various bacteria, fungi and viruses, responsible of a wide range of infectious phenotypes observed (Picard et al., 2011). Like NF- $\kappa$ B proteins, IKK $\alpha$  and IKK $\beta$  undergo homo- and hetero-dimerization. The activated IKK complex, predominantly acting through IKK $\beta$  in an IKK $\gamma$ -dependent manner, catalyzes the phosphorylation of I $\kappa$ Bs, polyubiquitination and subsequent degradation by the 26S proteasome. The freed NF- $\kappa$ B

dimers translocate to the nucleus, where they bind to specific sequences in the promoter or enhancer regions of target genes (Hayden and Ghosh, 2004).

The NF- $\kappa$ B family consists of five members: NF- $\kappa$ B1 (p105/p50), NF- $\kappa$ B2 (p100/p52), RelA (p65), RelB and c-Rel. NF- $\kappa$ B1 and NF- $\kappa$ B2 are synthesized as large precursors, p105 and p100, that are post-translationally processed to the DNA-binding subunits p50 and p52, respectively (Bonizzi and Karin, 2004). The subunits p50 and p52 carry the Rel-homology domain (RHD), which is a common feature of all NF- $\kappa$ B proteins; the RHD contains a nuclear localization sequence (NLS) and is involved in dimerization and sequence-specific DNA binding (Ghosh et al., 1998). Although p50 and p52 lack a transcription activation domain, such a domain is present in RelA, RelB,  $\nu$ -Rel and c-Rel. The NF- $\kappa$ B proteins form numerous homo- and hetero-dimers that are associated with specific biological responses that stem from their ability to regulate target gene transcription differentially. For instance, p50 and p52 homodimers function as repressors, whereas dimers that contain RelA or c-Rel are transcriptional activators. RelB exhibits a greater regulatory flexibility and can be both an activator (Ryseck et al., 1992) and a repressor (Ruben et al., 1992). RelB does not homodimerize but it forms stable heterodimers with either p50 or p52. NF- $\kappa$ B dimers are retained in the cytoplasm by I $\kappa$ Bs, which are specific inhibitors that bind to the RHD and interfere with its NLS function. Five homologous proteins are known to play functionally inhibitory roles on the DNA-binding activity of NF- $\kappa$ B and have been termed I $\kappa$ B proteins. This family includes the canonical I $\kappa$ Bs I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ , as well as the precursor proteins p105 and p100, whose C-terminal portions have also been termed I $\kappa$ B $\gamma$  and I $\kappa$ B $\delta$ , respectively, and whose N-terminal portions encode p50 and p52 (Hoffmann and Baltimore, 2006). Collectively, NF- $\kappa$ B transcription factor dimers bind to 9–10 base pair DNA sites ( $\kappa$ B sites), which have a great amount of variability (GGGRNWYYCC; R, A or G; N, any nucleotide; W, A or T; Y, C or T). All vertebrate NF- $\kappa$ B family proteins can form

homodimers or heterodimers *in vivo*, except for RelB, which only forms heterodimers *in vivo*. This combinatorial diversity contributes to the regulation of distinct, but over-lapping, sets of genes for at least three reasons: because the individual dimers have distinct DNA-binding site specificities for a collection of related  $\kappa$ B sites, because of the different protein–protein interactions the individual dimers make at target promoters and because of the gene activation profile of different dimers under specific physiological conditions. An example of the action of combinatorial diversity can be observed after TNF $\alpha$  stimulation. The latter leads to the translocation of the RelA/p50 and RelB/p50 dimers, but also of the RelA/RelB heterodimer that does not bind to  $\kappa$ B sites. That gives RelA a major regulatory role through the dampening of RelB activity (Jacque et al., 2005).

The NF- $\kappa$ B pathway mediates a wide variety extracellular and intracellular signals to control a diverse set of cellular responses (Hoffmann and Baltimore, 2006). Molecular biological studies have led to identification of functional  $\kappa$ B sites in about a hundred genes whose induction correlates with NF- $\kappa$ B activation. These are reflective of NF- $\kappa$ B's functions in regulating the communication between cells, regulating cell survival, and regulating proliferation. Genes encoding inflammatory mediators that control cell activation and chemotaxis, such as the pro-inflammatory cytokines TNF, IL-1, and IL-12 and chemokines such as monocyte chemotactic protein (MCP)-1, interferon (IFN)-inducible protein (IP)-10, and Regulated upon activation, normal T-cell expressed, and presumably secreted (RANTES), are prominent examples whose NF- $\kappa$ B responsiveness was established long ago. Similarly, NF- $\kappa$ B's control of the expression of many of the cognate receptors is reflective of its major role in tuning the sensitivity of cells to such extracellular messengers.

The activation and nuclear translocation of classical NF- $\kappa$ B dimers is also associated with increased transcription of genes encoding chemokines, cytokines, adhesion molecules [intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1)

and endothelial-leukocyte adhesion molecule 1 (ELAM-1)], enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis. These molecules are important components of the innate immune response to invading microorganisms and are required for migration of inflammatory and phagocytic cells to tissues where NF- $\kappa$ B has been activated in response to infection or injury (Bonizzi and Karin, 2004).

**Figure 1**

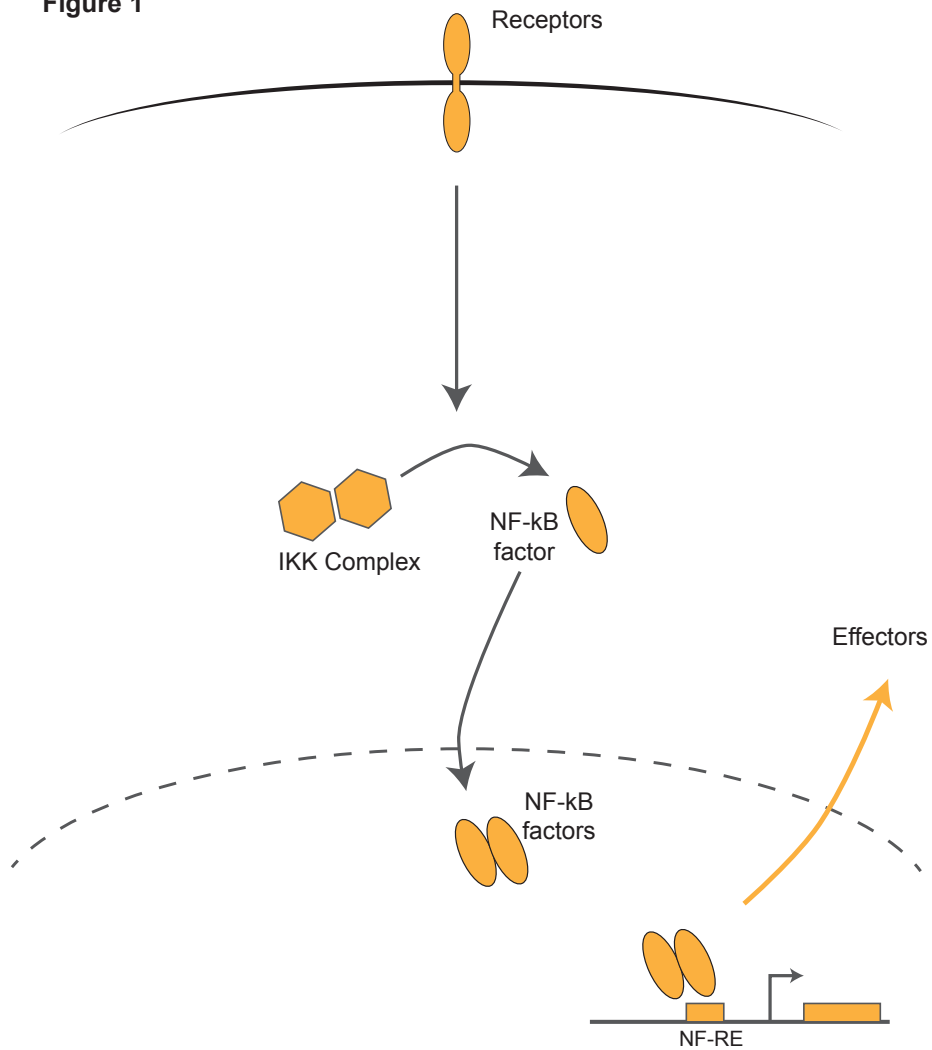


Figure 1: Core of a NF-κB pathway

Taken to its essentials, the activation logic in NF-κB pathways in *Drosophila* and mammals is centered around the axis: Receptors - IκB proteins - NF-κB factors. After activation of the receptors, IKK complexes will be processed. This regulatory step will allow the translocation of the NF-κB factors to the nucleus followed by the expression of hundreds of genes.

## **B. Signal transduction in NF- $\kappa$ B pathways and their regulation**

The transduction of a signal along a pathway necessitates a series of events controlled by different molecules (Figure 2).

### a) Classes of regulators

NF- $\kappa$ B regulators fall into two main categories: signal-specific regulators and gene-specific regulators. The first category consists of regulators that inhibit signal transduction. Although these proteins inhibit inflammatory signaling through various mechanisms, they all function proximal to the receptor, and so are expected to block gene induction by that receptor in a global manner. The second category includes transcriptional repressors or other negative regulators that function to modulate gene expression. There are sub-two types of transcriptional negative regulator: basal repressors and inducible repressors. Basal repressors are constitutively expressed and are important for the basal repression of many inflammatory genes (Medzhitov and Horng, 2009).

### b) *Drosophila* NF- $\kappa$ B pathways signal transduction regulation

In *Drosophila* IMD pathway, to establish the formation of a signaling complex, the first interaction occurs between PGRP-LC or -LE and IMD, a protein adaptor. The cleaved N-terminal of IMD exposes an Inhibitor of apoptosis 2 (IAP2) binding motif (IBM) that allows the recruitment of the tetrameric DIAP2, Ubiquitin-conjugating variant 1a enzyme (Uev1a), Bendless and Effete complex (Zhou et al., 2005). This complex will add Lysine 63 (K63)-linked ubiquitin chains on cleaved IMD, which will serve as a scaffold to recruit the Mitogen-associated protein (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 the Inhibitor of NF- $\kappa$ B Kinase (IKK) complex (Silverman et al., 2003; Vidal et al., 2001). *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, which is mandatory for its activation (Ertürk-Hasdemir et al., 2009).

Upregulated IMD pathway activations in the gut, which is in constant contact with microorganisms can lead to a premature death. 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 (Figure 2) (Guo et al., 2014; Lhocine et al., 2008; Maillet et al., 2008; Paredes et al., 2011).

In the *Drosophila* Toll pathway, following Spätzle (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., 2001). 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). Cactus is phosphorylated by Pelle and the subsequent degradation of poly-ubiquitinated Cactus leads to the translocation of the NF- $\kappa$ B factors to the nucleus (Daigneault et al., 2013).

Like the IMD pathway, Toll pathway activation must be tightly controlled. This must be the case in the adult immune response pathway to prevent unnecessary and potentially harmful activations. However, not much is known about negative regulation of the pathway. Only one negative regulator has been identified up until now: Pellino, which regulates Myd88 protein stability (Ji et al., 2014). The authors 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. While this work is in contradiction with a previous study that showed Pellino's requirement for Toll signaling (Haghighyeghi et al., 2010), this protein is part of the only feedback regulatory loop described in the Toll pathway that prevents excessive activation.

c) Human NF- $\kappa$ B pathway signal transduction regulation

In the classical NF- $\kappa$ B pathway, the binding of a ligand to a cell surface receptor (TNF-R or TLR) recruits adaptors to the cytoplasmic domain of the receptor that all share the TIR domain (Dunne and O'Neill, 2003). MyD88 for example is crucial for normal NF- $\kappa$ B induction in response to IL-1, IL-18, and LPS (Adachi et al., 1998; Kawai et al., 1999). In turn, these adaptors recruit an IKK complex, which consists of catalytic kinase subunits (IKK $\alpha$  and/or IKK $\beta$ ) and a scaffold, sensing protein called NEMO. This clustering of molecules at the receptor activates the IKK complex. IKK then phosphorylates I $\kappa$ B at two serine residues, which leads to its K48 ubiquitination and degradation by the proteasome. NF- $\kappa$ B then enters the nucleus to turn on target genes. There is an auto-regulatory aspect of the pathway, wherein NF- $\kappa$ B activates expression of the I $\kappa$ B $\alpha$  gene that leads to re-sequestration of the complex in the cytoplasm by the newly synthesized I $\kappa$ B protein. Mutations enhancing the inhibitory activity of I $\kappa$ B $\alpha$  underlie a severe and syndromic immunodeficiency, making it an important regulatory checkpoint for the NF- $\kappa$ B activation (Boisson et al., 2017). In most cases, the latter is transient and cyclical in the presence of continual inducer (Gilmore, 2006). Even if up-stream signaling components are in large part receptor-specific, the principles of signaling are similar, involving the recruitment of specific adaptor proteins and the activation of kinase cascades in which protein-protein interactions are controlled by poly-ubiquitination (Ramirez-Carrozzi et al., 2006).

The NF- $\kappa$ B factors RelB, c-Rel, and p65 contain a transactivation domain (TAD) located toward the C terminus that is necessary for transactivation by these proteins. Homodimers of p52 and p50 lack TADs and hence have no intrinsic ability to drive transcription. In fact, binding of p52 or p50 homodimers to  $\kappa$ B sites of resting cells leads to repression of gene expression (Zhong et al., 2002). The repressive function of p50 or p52 homodimers may provide a threshold for NF- $\kappa$ B transactivation that can be regulated through the expression and processing of the p100 and p105 precursors. The TADs on p65, c-Rel, and RelB promote transcription by facilitating the recruitment of coactivators and the displacement of repressors. The function of TADs is enhanced through direct modifications of NF- $\kappa$ B including phosphorylation and represents another layer of regulation of the NF- $\kappa$ B-mediated transcriptional response (Chen and Greene, 2004; Hayden and Ghosh, 2004). Post-translational modifications can play also an important role in the regulation of NF- $\kappa$ B factors themselves. For example, in the alternative NF- $\kappa$ B pathway, post-translational modifications of RelB affect its regulation (Baud and Collares, 2016).

Gene-specific expression can also be influenced by upstream signaling steps. For example, TLR4 signaling through MyD88 and TRIF - a second signaling adaptor protein TIR domain-containing adaptor-inducing interferon-beta - initiates translocation of NF- $\kappa$ B into the nucleus. Signaling through MyD88 is the primary driver for LPS-dependent NF- $\kappa$ B translocation to the nucleus, with TRIF-dependent signaling playing also an important role in the transcription of the cytokine TNF $\alpha$  (Sakai et al., 2017).

d) Role of nuclear I $\kappa$  proteins in the regulation of NF- $\kappa$ B pathways

The NF- $\kappa$ B pathways can be activated in many different cell types and from different stimuli. The fact that NF- $\kappa$ B comprises a family of homologous but different transcription factors suggests that specificity in the pathway may be achieved through their promoter-specific functions. In humans, it is known that this specificity in transcriptional responses can,

in part, be provided through the combinatorial effects of transcription factors. For NF- $\kappa$ B, the complement of heterodimers and homodimers of the five family members that are selectively regulated by I $\kappa$ B proteins, as previously discussed, is a primary source of transcriptional specificity (Ghosh and Hayden, 2008). However, attempts to elucidate an overall precise specificity code for NF- $\kappa$ B dimers compared to  $\kappa$ B-site sequences have failed so far, both biochemically and genetically. In comprehensive knockout studies, the gene-specific requirement for specific NF- $\kappa$ B dimers did not neatly correlate with the specific sequence of the  $\kappa$ B site (Hoffmann et al., 2003), suggesting that other factors play a role in defining the specific NF- $\kappa$ B dimer requirement.

Members of the I $\kappa$ B family are noteworthy examples of inducible negative regulators (Medzhitov and Horng, 2009). Negative feedback control mediated by I $\kappa$ B $\alpha$  and I $\kappa$ B $\epsilon$  allows for dynamic regulation of NF- $\kappa$ B. I $\kappa$ BNS and B cell lymphoma 3 (BCL-3) also limit inflammation in a gene-specific manner (Bates and Miyamoto, 2004; Kuwata et al., 2006; Wessells et al., 2004). The signaling module receives input signals in the form of IKK activity profiles. These are stimulus specifically regulated via negative feedback mechanisms and autocrine loops to result in specific dynamics. Each IKK dynamic profile is transduced by the signaling module to generate a stimulus-specific NF- $\kappa$ B activity profile critical for stimulus-specific gene expression. In addition to sequestering NF- $\kappa$ B dimers in the cytoplasm, I $\kappa$ B family members have also been suggested to stabilize nuclear and DNA-bound dimers.

In *Drosophila*, Pickle is a nuclear I $\kappa$ B that integrates signaling inputs from both the Imd and Toll pathways by skewing the transcriptional output of the NF- $\kappa$ B dimer repertoire. Pickle interacts with the NF- $\kappa$ B protein Relish, selectively repressing Relish homodimers while leaving other NF- $\kappa$ B dimer combinations unscathed. Although loss of Pickle results in hyper-induction of Relish target genes and improved host resistance to pathogenic bacteria in

the short term, chronic inactivation of Pickle causes loss of immune tolerance and shortened lifespan (Morris et al., 2016).

In mammals, the I $\kappa$ B protein family comprises three functional groups: the typical I $\kappa$ B proteins I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$ , which are present in the cytoplasm of unstimulated cells and undergo stimulus-induced degradation and resynthesis; the precursor proteins p100 and p105, which can be processed to form the NF- $\kappa$ B family members p52 and p50, respectively, or can be degraded; and the atypical I $\kappa$ B proteins I $\kappa$ B $\zeta$  (encoded by *NFKBIZ*), BCL-3 and I $\kappa$ BNS (encoded by *NFKBID*), which are generally not expressed in unstimulated cells, but are induced following activation and mediate their effects in the nucleus (Ghosh and Hayden, 2008).

e) Involvement of non coding RNAs (ncRNAs) in the regulation of NF- $\kappa$ B response

The magnitude of induction of specific target genes is also influenced by intrinsic differences in mRNA stability and by the active regulation of mRNA stability and translation, by microRNAs (miRNAs) and proteins that bind 3'-untranslated regions (Anderson, 2010; Hao and Baltimore, 2009; O'Connell et al., 2010). Current estimates indicate that only 1%–2% of the genome has protein coding potential whereas 85% of the genome is transcribed (Hangauer et al., 2013). miRNAs are small ncRNAs molecules controlled by the RNA interference (RNAi) pathway proteins DICER1/Argonaute1/Loquacious that regulate gene expression at the post-transcriptional level through base-pairing predominantly with a 3'-untranslated region of target mRNA, followed by mRNA degradation or translational repression. The role of miRNAs in regulating inflammatory gene expression is well known (Baltimore et al., 2008), as post-transcriptional mechanisms to fine-tune the inflammatory gene expression in innate immunity (Carpenter et al., 2014). Disruption of this regulation may lead to the development of various pathological conditions, including autoimmune inflammation (Baulina et al., 2016; Philippe et al., 2013). Additionally, the DICER1-

dependent biogenesis of microRNAs might not be the only way in which the RNAi pathway is involved in regulation as reduced DICER1 expression contributes to the initiation and/or progression of autoimmune and autoinflammatory diseases (De Cauwer et al., 2018).

Studies have also provided evidence of a role for long non-coding RNAs (lncRNAs) (Guttman et al., 2009; Lefevre et al., 2008). One of the largest groups of RNA transcribed from the genome is lncRNAs. Thousands of these transcripts have been discovered in diverse cell types (Guttman and Rinn, 2012; Guttman et al., 2009, 2010; Mortazavi et al., 2008; Rinn and Chang, 2012). LncRNAs are emerging as major regulators of chromatin remodeling, transcription and post-transcriptional regulation of gene expression in diverse biological contexts, like the regulation of gene expression within the immune system (Carpenter and Fitzgerald, 2015; Rinn and Chang, 2012).

**Figure 2**

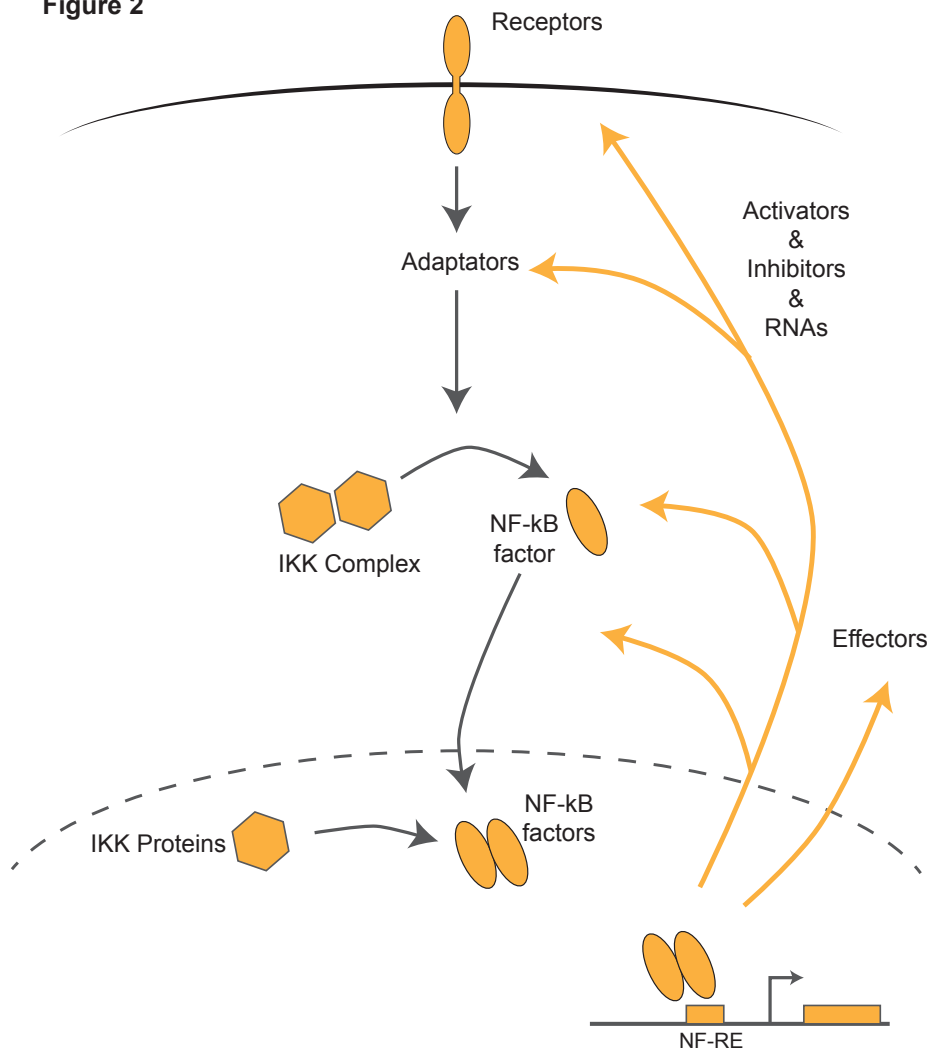


Figure 2: Signal transduction and genes expression of NF-κB pathway

After activation of receptors, the signal is transduced through the actions of adaptors. The controlled expression of hundreds of genes lead to positive - and negative - regulation of the pathways. ncRNAs have taken an important role as well in regulating those genes.

### C. Dynamic of NF- $\kappa$ B pathways genes expression

Studies in mammals show that the components of the pathway act in a dynamic manner to regulate it in a more complex pattern than just linearly (Figure 3).

#### a) Kinetic aspect of NF- $\kappa$ B response

Steady-state NF- $\kappa$ B activity in unstimulated cells (basal activity) is not zero, but must be kept at a low level, as NF- $\kappa$ B is a powerful transcriptional activator of inflammatory effectors including secreted cytokines and chemokines that initiate and amplify an adaptive immune response, metalloproteases and other proteins that can affect tissue remodeling and damage, as well as survival and proliferation regulators. Indeed, many inflammatory diseases and cancer types are characterized by elevated basal NF- $\kappa$ B activity (Nathan and Ding, 2010).

Initial activation of canonical NF- $\kappa$ B activity is typically rapid and does not require *de novo* protein synthesis (Hoffmann and Baltimore, 2006). Upon cell stimulation, increases in nuclear NF- $\kappa$ B activity can be detected within 10 minutes. One such early responding promoter is that of I $\kappa$ B $\alpha$ , which mediates a powerful negative feedback mechanism that is responsible for post-induction repression of NF- $\kappa$ B activity upon stimulus removal (Hoffmann et al., 2002; Scott et al., 1993) and may result in oscillatory NF- $\kappa$ B activity during chronic stimulation (Hoffmann et al., 2002; Nelson et al., 2004). However, the oscillatory propensity in the signaling system caused by I $\kappa$ B $\alpha$  is counteracted by a second negative feedback mechanism mediated by I $\kappa$ B $\epsilon$ , which is delayed and functions in anti-phase to I $\kappa$ B $\alpha$  (Kearns et al., 2006). These insights indicate that cells have the capacity to intricately modulate the temporal activity profile of NF- $\kappa$ B (Werner et al., 2005).

Several specificity mechanisms are occurring during the NF- $\kappa$ B activation process, and others are occurring on gene promoters. The formers are determined by the receptors that activate specific signaling networks, and the latters are encoded in the regulatory code of each NF- $\kappa$ B target gene. Because NF- $\kappa$ B is constitutively present in resting cells in a latent form,



the stimulus-responsive activation mechanisms that render it capable of binding DNA and activating genes comprise the first biochemical steps able to mediate stimulus-specific gene expression. Interestingly, NF- $\kappa$ B activation occurs with a stimulus-specific temporal profile. Even very short transient TNF stimulations generate a complete hour of NF- $\kappa$ B activity, which is sufficient to drive the expression of many genes; however, longer stimulation was required for sustained activity, which is responsible for driving the expression of a second set of genes (Hoffmann and Baltimore, 2006).

Dynamic control of NF- $\kappa$ B is stimulus specific. The stimulus may be described in terms of its chemical identity as well as the concentration dose and duration dose. The latter two may be related when the half-life of the stimulus is short, as low doses decay quickly below the level of effective concentration. In short, the dynamic control of NF- $\kappa$ B is a function of the concentration dose, and exposure duration, and importantly the identity of the stimulus. The dynamic control of NF- $\kappa$ B activity determines the NF- $\kappa$ B-mediated gene expression program. These observations led to the hypothesis of a Temporal Signaling Code (Hoffmann and Baltimore, 2006), which states that information about the stimulus is encoded in the temporal profile or dynamics of NF- $\kappa$ B activity to specify which genes are to be expressed (Basak et al., 2012).

An inflammatory response is initiated by the temporally controlled activation of genes encoding a broad range of regulatory and effector proteins. A central goal of therapeutic research is to devise strategies for the selective modulation of proinflammatory gene transcription, to allow the suppression of genes responsible for inflammation-associated pathologies while maintaining a robust host response to microbial infection. Toward this goal, studies have revealed an unexpected level of diversity in the mechanisms by which chromatin structure and individual transcription factors contribute to the selective regulation of inflammatory genes (Smale, 2010).

From a transcription perspective, the many genes activated in response to an inflammatory stimulus can be divided at their most fundamental level into two classes: primary and secondary response genes. Primary response genes (PRGs) are usually activated most rapidly and are formally defined as those genes that can be induced without *de novo* protein synthesis (Herschman, 1991; Yamamoto and Alberts, 1976). In other words, the transcription factors required for activation of these genes must be expressed in the unstimulated cell and must be either constitutively active or activated via posttranslational mechanisms after cell stimulation. Secondary response genes (SRGs) are generally induced more slowly and require new protein synthesis. The transcription of SRGs can depend on the *de novo* synthesis of transcription factors, signaling molecules needed for the activation of transcription factors or cytokines that can act in an autocrine fashion to activate additional signaling pathways and transcription factors. Although SRGs require newly synthesized proteins, the factors responsible for the activation of PRGs can also contribute directly to their transcription. Inducible repressors block the expression of SRGs, whereas basal repressors inhibit the expression of PRGs. These PRGs might require repression in the basal state to prevent low-level constitutive expression. Therefore, it is probable that different modes of transcriptional repression can operate to inhibit PRGs and SRGs (Medzhitov and Horng, 2009).

b) Timing dichotomy of NF- $\kappa$ B response in humans depends on epigenetic regulation

To better address this question of temporal selectivity, another layer of regulation has to be considered: the epigenetic level. Chromosomal biology involves a dynamic balance between genome packaging and access. Chromatin remodelers are complexes of proteins that use the energy of Adenosine Triphosphate (ATP) hydrolysis to change the packaging state of chromatin by moving, ejecting, or restructuring the nucleosome, which is the primary repeating unit of chromatin structure (Becker and Hörz, 2002; Saha et al., 2006). Remodelers

work with other chromatin factors to control packaging and unpacking as the DNA elements that control chromosomal processes (enhancers, promoters, replication origins) must be exposed in a regulated manner to properly execute various processes, such as gene transcription, DNA replication, DNA repair, and DNA recombination. Therefore, chromatin structure not only provides a packaging solution, but also an opportunity for regulation. There are currently four different families of chromatin remodeling complexes. Although all remodeler catalytic subunits share a conserved ATPase domain, each family member bears unique flanking domains, allowing their separation into four distinct families (Flaus et al., 2006). Individual families are conserved from yeast to human with ortholog complexes often conserving key domains, suggesting that conserved domains reflect conserved functions:

- The Switching / Sucrose non fermentable (SWI/SNF) family remodelers. This family has many activities through the sliding and ejecting of nucleosomes at many loci.
- The Imitation switch (ISWI) family remodelers. ISWI family complexes optimize nucleosome spacing to promote chromatin assembly and activation of transcription.
- The chromodomain, helicase, DNA binding (CHD) family remodelers. Certain CHD remodelers slide or eject nucleosomes to repress transcription.
- The Inositol auxotroph 80 (INO80) family remodelers. INO80 has diverse functions, including promoting transcriptional activation and DNA repair.

For transcriptional regulation, specialized remodelers can have antagonistic functions: those that organize chromatin and restrict access to DNA (promoting repression) and those that disorganize/eject nucleosomes (promoting activation). For example, cells alter the subunit composition of their remodelers to activate genes that promote tissue-specific differentiation and to repress genes that reinforce self-renewal (Carpenter and Fitzgerald, 2015; Clapier and Cairns, 2009).

The genes that are induced most rapidly, the PRGs, are regulated directly by transcription factors acting alone or in combination (Carpenter and Fitzgerald, 2015). Many of these PGRs are induced within minutes, followed by the induction of SRGs and the initiation of autocrine and paracrine feedback loops (Amit et al., 2009). PRGs mostly contain CpG islands within their promoters (Fowler et al., 2011; Smale, 2010; Yamamoto and Alberts, 1976). Such genes are associated with poised RNA polymerase II even in unstimulated cells (Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009; Sims et al., 2004; Suzuki and Bird, 2008). They have open chromatin and do not require chromatin remodeling by the SWI-SNF remodeling complex (Fowler et al., 2011; Ramirez-Carrozzi et al., 2009; Sims et al., 2004). PRGs are permissive for very rapid induction (Fowler et al., 2011; Ghisletti et al., 2010). A group of intermediately expressed genes known as the late primary response genes (LPRGs) are induced later in the absence of new protein synthesis. Unlike classical PRGs they do require some degree of chromatin remodeling (Ramirez-Carrozzi et al., 2006, 2009; Saccani et al., 2001). A third of the PRGs require key components of the SWI-SNF remodeler family complexes for their activation (Ramirez-Carrozzi et al., 2006, 2009; Saccani et al., 2001). Following the first wave of induction, a second wave of gene expression, the so-called SRGs, requires *de novo* protein synthesis and comprehensive chromatin remodeling and activity of enhancers for the activation of transcription to occur. PRGs include cytokines and chemokines that in turn amplify gene regulation in autocrine and paracrine manners (Ramirez-Carrozzi et al., 2006, 2009; Saccani et al., 2001; Smale, 2012). Requirements for SWI/SNF complexes were observed at secondary response genes and primary response genes induced with delayed kinetics but not at rapidly induced primary response genes. Furthermore, a strong and consistent antagonism in the recruitment between SWI/SNF and CHD complexes was observed at the promoter sites of those genes, revealing the CHD role in the regulation of pro-inflammatory genes (Ramirez-Carrozzi et al., 2006).

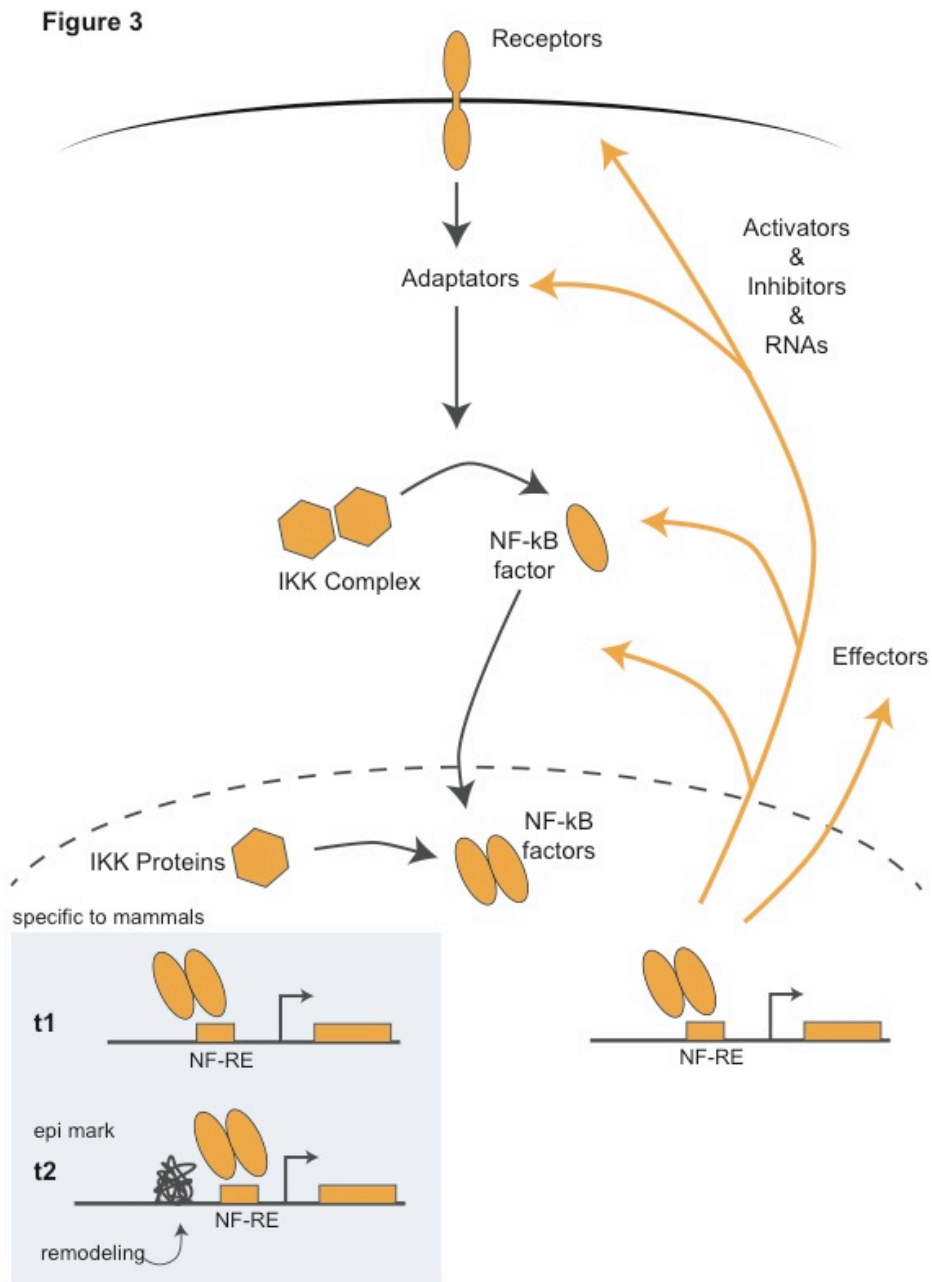


Figure 3: Temporal control of NF-κB target genes expression

The regulatory actions of different proteins of the pathways lead to a temporally controlled expression of genes. In mammals, subsets of genes have been classified in two expression waves - t1 and t2 - based on their requirements to get expressed (remodeling).

## **D. NF- $\kappa$ B pathways: selectivity of gene expression**

In *Drosophila* and mammals, dynamic regulation of the NF- $\kappa$ B pathways involves nuclear co-activators and epigenetic processes that allow a fine-tuned expression of genes (Figure 4).

### a) NF- $\kappa$ B transcriptional selectivity in *Drosophila* and humans

The study of the molecular cascade of the IMD pathway in *Drosophila* led to the identification of the nuclear protein Akirin by our laboratory. This evolutionarily conserved player in the NF- $\kappa$ B pathway is required for IMD target gene expression by the Relish transcription factor (Goto et al., 2007). In particular, we showed that in *Drosophila* Akirin is mostly required for the transcription of genes that are effectors of the pathway (Bonnay et al., 2014). NF- $\kappa$ B-dependent genes contributing to the down-regulation of inflammation were mostly characterized as Akirin independent. Mechanistically speaking, a large-scale unbiased proteomic analysis revealed that Akirin orchestrates NF- $\kappa$ B transcriptional selectivity through the recruitment of the SWI/SNF chromatin-remodeling complex. Furthermore, this subset of genes was characterized by the presence of a Histone 3 Lysine residue 4 acetylation (H3K4ac) epigenetic mark: the deposition of an acetyl group on the 4<sup>th</sup> lysine of the 3<sup>rd</sup> histone, one of the proteins that package the genetic material. This epigenetic signature is associated with transcriptionally active transcription start sites (TSS) in human and yeast (Guillemette et al., 2011). This mark was specifically enriched upon IMD pathway activation on Akirin-dependent promoters in an Akirin, SWI/SNF and Relish-dependent manner. These new findings link chromatin remodeling to epigenetic control of NF- $\kappa$ B target genes selectivity. Removing Akirin or SWI/SNF lead to an impaired expression of several AMP-coding genes, resulting in a weakened innate immune defense of *Drosophila* against Gram-negative bacteria and a worse survival after infection. This observation suggests that the full set of IMD-induced AMPs is required to efficiently contend Gram- negative bacterial infections.

Akirin is highly conserved and the two mouse genes (akirin-1 and akirin-2) encoding Akirin proteins, have been identified. A study showed that, as in *Drosophila*, Akirin-2 acts downstream of the TLR, TNFR and IL-1R signaling pathways (Tartey et al., 2014). Most interestingly, Akirin-2 is required in mammals for the regulation of only a subset of LPS and IL-1 inducible genes with mainly pro-inflammatory activity. Moreover, like in *Drosophila*, Akirin-2 bridges the NF- $\kappa$ B factor and the chromatin remodeling SWI/SNF complex. Interestingly, mouse Akirin-2 appeared to participate to the innate immune response through its interaction with the nuclear I $\kappa$ B protein I $\kappa$ B- $\zeta$ , an atypical member of the I $\kappa$ B proteins family (Tartey et al., 2014). Additionally, it was suggested that I $\kappa$ B- $\zeta$  may influence the regulation of histone modification through selective H3K4 tri-methylation of TLR-induced promoters (Hildebrand et al., 2013). Increasing number of studies report that I $\kappa$ B- $\zeta$  regulates the activity of the canonical NF- $\kappa$ B p50 transcription (Kannan et al., 2011; Kohda et al., 2014; Yamamoto et al., 2004). A conditional knockout of *akirin-2* in macrophages compromised the immune response of mice against *Listeria monocytogenes* intra-peritoneal infections *in vivo*. Together with another study in the tick showing an NF- $\kappa$ B-dependent immune function of Akirin against Gram-negative bacterial infections (Naranjo et al., 2013), these results argue for a conserved role of Akirins in the innate immune response of metazoan. Devoid of known predicted functional domains in their sequence, Akirins may act as scaffolds gathering chromatin-remodeling complexes with sequence-specific targeting transcription factors.

b) Epigenetic code of the immune response

The fact that several chromatin remodelers are being found as associated with NF- $\kappa$ B differential transcription highlights the importance of epigenetic regulation. Histones are subject to a large number of covalent modifications, including lysine and arginine methylation, lysine acetylation, serine phosphorylation and lysine ubiquitylation (Kouzarides, 2007). The counteracting activities of histone acetyltransferases (HATs) and histone

deacetylases (HDACs) establish the levels of histone acetylation, whereas lysine methylation is regulated by suppressor of variegation-enhancer of zeste-trithorax (SET) domain family proteins (for methylation) and lysine-specific demethylase 1 (LSD1) and Jumonji C (JMJC) domain-containing proteins (for demethylation) (Kouzarides, 2007). Strahl and Allis first proposed that histone modifications are found in non-random patterns in the genome to form a 'histone code', with distinct combinations of modifications specifying unique states of gene expression (Strahl and Allis, 2000). In the past years, genome-wide maps of histone modifications coupled with transcriptional profiling have identified many histone modifications as being either 'active' or 'inactive'. Histone 3 lysine 4 trimethylation (H3K4me3), for example, is associated with transcriptionally active or poised loci, whereas Histone 3 lysine 27 (H3K27) and/or Histone 3 lysine 9 (H3K9) trimethylation correlate with gene silencing (Kouzarides, 2007). The transcriptional activity of inflammatory cytokine genes can be divided into three different states: poised, activated and silenced (McCall et al., 2010). Whereas some histone modifications seem to associate strongly with a particular state of gene expression, others seem to mark much smaller subsets of active or inactive genes (Medzhitov and Horng, 2009; Vermeulen et al., 2007).

Having epigenetic marks deposited on histone proteins means that histone-modifying enzymes are at play. Histone-modifying enzymes (so-called writers, erasers, and readers) modify histones and in so doing create access to binding sites for transcription factors. In addition, histone reader enzymes that dock to modified histones through defined protein domains are essential for the recruitment of additional components of the transcriptional machinery (Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009; Sims et al., 2004; Suzuki and Bird, 2008). These dynamic modifications include acetylation, methylation, phosphorylation, sumoylation, citrullination, and ubiquitination and occur in characteristic temporal and spatial patterns that are associated with different transcriptional activities



(Struhl, 1999). The histone code or epigenetic landscape surrounding genes determines the transcriptional output that occurs following a given signal (Jenuwein and Allis, 2001). The fate of gene expression through chromatin structure is largely established within cells during development. Chromatin modifications act as a rate-limiting step in the activation of gene expression during infection. For example, the balance of histone acetylation is maintained by the interplay between HAT and HDAC. An HAT is a protein that acetylates core histones on lysine residues, which has important regulatory effects on chromatin structure and assembly, and on gene transcription. In general, increased levels of histone acetylation are associated with activation of gene expression. A HDAC is a protein that removes the acetyl groups from lysine residues that are located at the amino termini of histones. In general, decreased levels of histone acetylation are associated with the repression of gene expression. Both can have direct influence on gene transcription. Indeed, gene expression can be controlled through transcriptional corepressors, which regulate transcription but do not by themselves bind DNA. Nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) associate with a broad array of inflammatory gene promoters at basal levels in macrophages and their repressor functions are mediated through recruitment of HDACs (Ghisletti et al., 2009). Subsets of inflammatory genes are regulated by NCoR or SMRT and a select group of genes can be regulated by both corepressors. The dismissal of NCoR and SMRT are essential to allow signal-induced transcription of inflammatory genes (Ghisletti et al., 2009). Conversely transcriptional co-activators, a protein complex that associates with the ligand-binding domain of transcription factors, reorganize chromatin templates and recruit the basal transcriptional machinery to the promoter region. CREB-binding protein (CBP) and p300 are known transcriptional co-activators that interact with many transcription factors to promote recruitment of the RNA polymerase holoenzyme and other transcriptional regulators, thereby allowing transcriptional induction. In addition, p300

and CBP have HAT activity, such that these proteins can influence chromatin activity by modulating nucleosomal histones (Bhatt and Ghosh, 2014; Ghosh and Hayden, 2008; Medzhitov and Horng, 2009).

c) Immune pathways cross-talk

Inflammation is a multicomponent response to tissue stress, injury and infection, and a crucial point of its control is at the level of gene transcription. The inducible inflammatory gene expression program - such as that triggered by TLR signaling in macrophages - is comprised of several coordinately regulated sets of genes that encode key functional programs (Hotamisligil, 2006; Medzhitov, 2008; Nathan, 2002). The pro-inflammatory transcriptional programs that are regulated by NF- $\kappa$ B organize and execute the inflammatory response. Although some of these differences arise from epigenetic modifications that are associated with different genes in different cell types, there is little doubt that a significant degree of diversity results from the complexity of the NF- $\kappa$ B system. Indeed, even repeated stimulation of the same cells with the same stimuli can result in a markedly altered transcriptional output. For example, it has been shown that in LPS tolerance, only the NF- $\kappa$ B-regulated pro-inflammatory genes - that could be harmful if continually expressed - are hyporesponsive to repeated LPS stimulation and are therefore tolerizable genes (Ghosh and Hayden, 2008). By contrast, other NF- $\kappa$ B-regulated genes, such as AMPs, are non-tolerizable (Foster et al., 2007), as their continued expression is necessary for protection against infection.

Even though we focused this work on NF- $\kappa$ B signaling, stimuli that activate NF- $\kappa$ B also activate other immune signaling pathways. This activation may modify the signal processing characteristics of the signaling module or may coordinately regulate the activity of other transcription factors to effect stimulus-specific gene expression and cellular responses. These transcription factors can be divided into three categories on the basis of their mode of activation and function (Medzhitov and Horng, 2009). The first category (class I) consists of

transcription factors that are constitutively expressed by many cell types and that are activated by signal-dependent post-translational modifications (e.g. NF- $\kappa$ B, IFN-regulatory factors (IRFs)). In most cases, these transcription factors are retained in the cytoplasm in the basal state and their signal-dependent activation involves their nuclear translocation. The genes that are induced most rapidly by LPS stimulation (the PRGs) are regulated by these transcription factors. Positive feed-forward mechanisms might ensure the sustained activation of these transcription factors and their participation in subsequent waves of gene induction. For example, the production of TNF triggered by LPS stimulation seems to be crucial for autocrine signaling and induction of a second wave of NF- $\kappa$ B activation (Covert et al., 2005; Werner et al., 2005). The second category of transcription factors (class II) are synthesized *de novo* after LPS stimulation (e.g. CCAAT/enhancer-binding protein- $\delta$  (C/EBP $\delta$ )). These transcription factors regulate subsequent waves of gene expression after the PRGs, and they can do so over a prolonged period of time (Ravasi et al., 2007). The activity of these transcription factors is often subject to positive feedback control, and because these proteins are transcriptionally upregulated, a general principle here seems to be transcriptional autoregulation. For example, the amplification of the LPS-induced transcriptional response by C/EBP $\delta$  requires its autoinduction (Litvak et al., 2009). The third category of transcription factors (class III) consists of lineage-specific transcriptional regulators, the expression of which is turned on during cell differentiation. Notable member of this group includes Pu.1 (also known as SPI1) in macrophages (Friedman, 2007; Valledor et al., 1998). The complex transcriptional program induced in macrophages after LPS stimulation is in fact a product of the coordinated action of the three categories of transcription factors described.

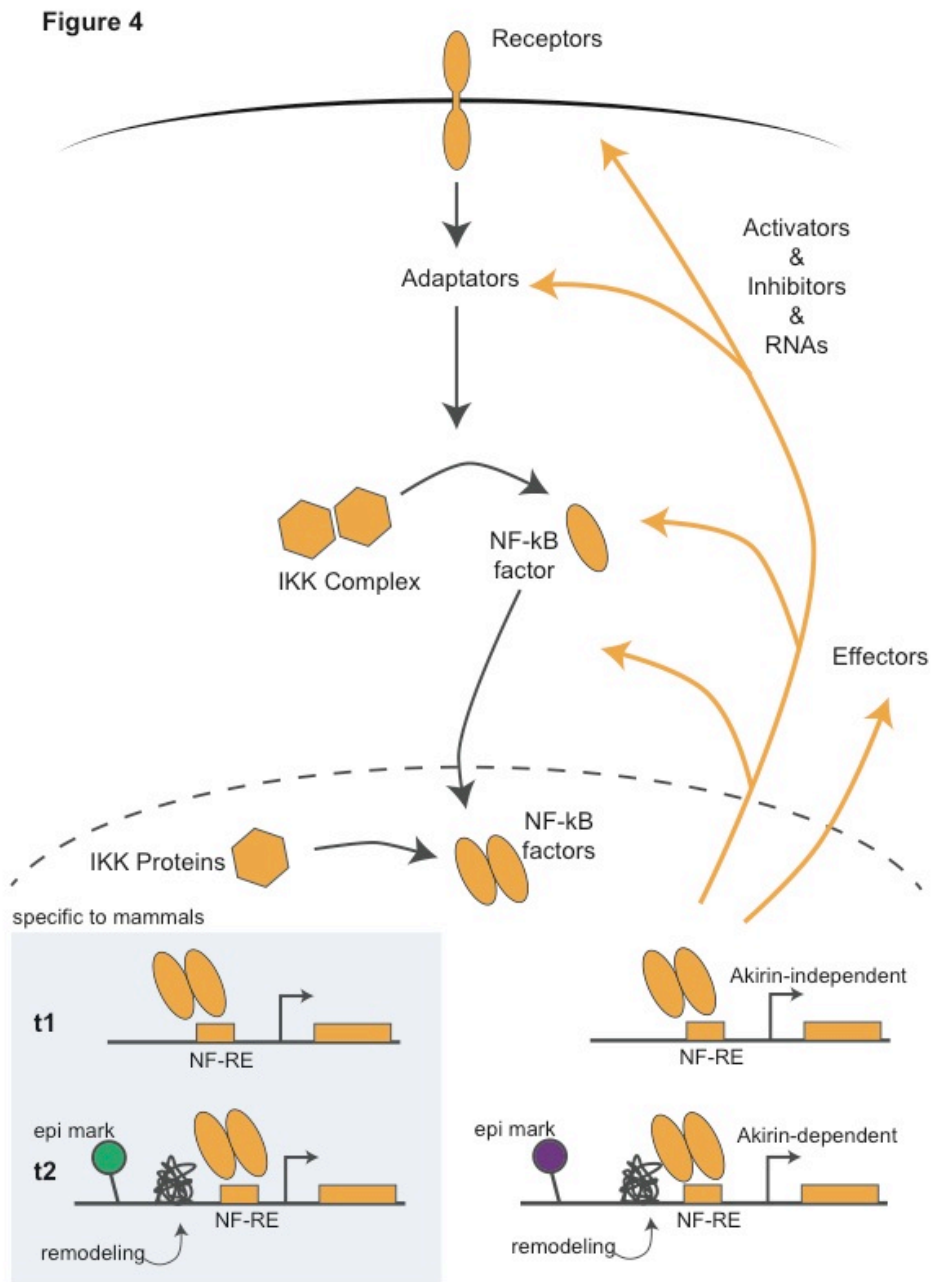


Figure 4: Fine-tuning of gene transcription characterizes NF-κB pathways expression

NF-κB target genes are activated by subsets through the action of co-activators (like Akirin) that allow and the recruitment of chromatin remodeling complexes at specific epigenetic mark.

## **Conclusion**

On a physiological level, inflammation is a fundamental adaptation to the loss of cellular and tissue homeostasis with many physiological roles, including host defense, tissue remodeling and repair, and the regulation of metabolism. The complexity of the inflammatory response requires that its many functional programs be controlled coordinately in some situations but independently in others. This is achieved through multiple mechanisms that operate at different levels, including regulation of signaling pathways and control at the level of gene expression. So, the mechanisms that regulate inflammatory responses can be divided into cell-specific, signal-specific and gene-specific mechanisms. Cell-specific mechanisms operate at the level of different cell types, and include regulation of their recruitment and activation. Signal-specific mechanisms operate at the level of signaling pathways. Finally, gene-specific mechanisms operate at the level of individual genes and gene subsets through mechanisms particularly well suited to provide functional specificity in an inflammatory response. Only a cohesive view will allow us to understand the dynamic of inflammation.

Concerning NF- $\kappa$ B pathways in particular, the initial view that the selective activation of an inducible gene is dictated primarily by the combinatorial binding of a specific set of transcription factors has been replaced by models with several additional regulatory layers implicated in a dynamic pattern of several waves of gene expressions. Those newly identified layers of regulation raise questions, not only mechanistically but also functionally and physiologically. The key to understand the activation (and resolution) of the innate immune response seems linked with the characterization of the dynamics that are behind its regulation. Discovering novel layers where regulation could operate is one thing, assessing all the dynamic movements in them is another challenge altogether.

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### **III. Innate immune dynamic response: three practical cases of regulation**

#### **A. Toll pathway delayed regulation**

The induction of an inflammatory response is essential for host defense during infection. Timely resolution is also important to limit the detrimental effects of inflammation, particularly when it is inappropriately sustained or increased. Fine-tuning regulating inflammation is key for survival. After an immune challenge, the shutdown of inflammatory pathways is necessary to avoid development of autoimmune diseases or cancer. For this reason, acute inflammation leads to the up-regulation of expression of negative regulators. In *Drosophila*, upon recognition of microbial threats, the IMD and the Toll pathways activate the innate immune response. The IMD pathway is negatively controlled by a wide-set of negative regulators acting at all levels of the pathway but beside the I $\kappa$ B protein Cactus, mechanisms controlling the negative regulation of the Toll pathway remain obscure.

The first project I will present describes the identification of new immune-induced negative regulators acting at various level of the Toll pathway. Upon immune challenge, the expression of these negative regulators is buffered by constitutively expressed miRNAs. Later, this buffering becomes insufficient, leading to a shutdown of the Toll pathway. Consequently, inactivation of Ago1 prevents Toll pathway activation upon immune challenge, whereas knockdown of our newly discovered negative regulators lead to over-activation of the Toll pathway upon immune stimulation. Altogether, these results uncover a time-dependent regulation model for an inflammatory pathway by miRNAs.

This subject was started by a post-doctoral researcher in our team, Aleksandra Krupa (nicknamed Ola), on the ERC Advanced grant IMMUDROSO obtained in 2010 by Pr. Jean-Marc Reichhart. After she unfortunately passed away, we took up the project. I have organized it as a report article, with missing experiments going to be completed to three replicates or finished to be analyzed in the coming months.

## **Delayed inactivation of immune response through miRNA buffering of negative regulators expression**

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### **Abstract**

After an immune challenge, the shutdown of inflammatory pathways is necessary to avoid development of autoimmune diseases or cancer. By the use of high throughput techniques, we analyzed the regulation of *Drosophila* Toll pathway, the ortholog of mammalian interleukin 1 receptor (IL-1R) inflammatory pathway. Toll activation leads to the expression of Synaptotagmin7 (Syt7). This protein allows the Toll receptor protein to return to the membrane, leading to the pathway inactivation. We show that *syt7* mRNA is targeted by constitutively expressed miR310-313. Toll pathway activation and subsequent *syt7* mRNA expression is required to override the miRNA inhibition and initiate the shutdown of the pathway. Taken together, our results uncover a time-dependent regulation model for an immune pathway.

### **Report article**

The optimal lifespan of organisms depends on various levels of regulation, which includes the coupling of molecular signaling pathways activation with their subsequent inactivation. Inflammation is a good example of such paradigm, where inappropriate or excessive responses can result in septic choc or chronic inflammation (Maeda and Omata,



2008). The expression of inhibitors in the same time frame as effectors creates a negative feed-forward loop that results in the shutdown of immune pathways (Mitchell et al., 2016). However, the timing of the response should be tightly controlled in order to give time to the pathway to reach a sufficient level of activity before the inhibition takes place. This important point is rarely addressed, with the notable exception of circadian rhythms studies (Man et al., 2016). We use *Drosophila melanogaster* immunity as a model to better understand this phenomenon (Alarco et al., 2004; Hoffmann et al., 2003). The Immune deficiency (IMD) and the Toll pathways are activated after infection respectively by Gram-negative bacteria and Gram-positive bacteria or fungi (Hoffmann et al., 2003; Hultmark, 2003). Whereas several protein inhibitors of the IMD pathway have been identified (Aparicio et al., 2013; Fernando et al., 2014; Shibata et al., 2013; Thevenon et al., 2009), only one has been described for the Toll pathway: Pellino. It targets Myeloid differentiation primary response gene 88 (MyD88), a key component of the pathway, for ubiquitination and degradation (Ji et al., 2014). In addition to proteins, non-coding regulatory RNAs such as microRNAs have also been characterized as modulators of signaling pathways. miRNAs are a class of small non-coding RNAs (~22 nucleotides) that mainly regulate expression of specific genes (Anderson, 2010; Hao and Baltimore, 2009; O'Connell et al., 2010). These RNAs play crucial roles in key biological processes, such as cell proliferation, cell fate, differentiation, apoptosis and immunity (Baltimore et al., 2008; Baulina et al., 2016; Bejarano et al., 2010; Morante et al., 2013).

A recent study showed that some miRNAs could act on the control of the Toll pathway but their exact mechanism of action is elusive (Atilano et al., 2017). The authors observed a lack of Toll pathway activation after infection in Argonaute 1 knock down (*Ago1<sup>KD</sup>*) flies, which express short interfering RNA against *Ago1* in the fat body (the main immune organ of *Drosophila*), using the UAS-Gal4 system. To avoid possible unspecific effects of Gal4 on susceptibility of the flies to infection, we directly expressed the RNAi hairpin construct under

the control of the *YPI* promoter driving expression in the fat body. After infection with the Gram-positive bacteria *Micrococcus luteus* (*MI*), *Ago1<sup>KD</sup>* flies show an impairment of the expression of *drosomycin* (*drs*), a Toll dependent anti-microbial peptide (AMP) coding gene (Fig. 1A). The *Ago1<sup>KD</sup>* flies activate the Toll pathway in the same way as control flies for the first 6h, as measured by the expression of *drs* mRNA. However, whereas the expression of *drs* reaches a maximum around 24h post infection in control flies, *drs* expression starts to go down after 12h in mutant flies (Fig. 1B). This result is confirmed when two other components of the miRNA pathway, Dicer-1 (*Dcr1*) or Loquacious (Loqs), are depleted (Fig. S1A). We hypothesized that some miRNAs might be required to keep the Toll pathway active, meaning that they are not inhibitors of the pathway but could act by targeting inhibitors. Surprisingly, we did not detect significant changes of any miRNA expression by small RNA-sequencing, after infection with several pathogens capable of activating the Toll pathway (Fig. S1B).

To identify the inhibitors targeted by the miRNAs, we performed a microarray experiment on flies depleted for Ago1 4 hours after an infection. We selected genes that were induced at least 2 fold after an infection in control flies and whose expressions were at least 2 times higher after infection of Ago1 depleted flies. We identified 36 genes that fitted these criteria (Fig. 2A). In order to identify among these genes which ones could be inhibitors of the pathway, we knockdown each of them in *Drosophila* S2 cells in which a chimeric Epidermal growth factor (EGF)-receptor extracellular domain was fused to the Toll transmembrane and cytosolic domain (ERT-S2) (Sun et al. 2004). As EGF binding to the chimeric receptor can then activate the Toll pathway, this *ex-vivo* construct allows us to monitor its activation timing. In this system, the knockdown of *Ago1* almost completely abolished the expression of *drs* after EGF stimulation, as expected from the *in vivo* experiments (figure to be added). The knockdown of 7 out of 36 genes showed an increase of Toll pathway activation at 16h and 24h post induction suggesting that they are *bona fide* Toll pathway negative regulators (Fig.

S2A-B). We decided to focus on *Synaptotagmin7* (*Syt7*) (Fig. 2B), since it is part of a list of genes whose inactivation leads to an increased resistance to fungi infection (Lu et al. 2015). *In vivo*, flies inactivated for *Syt7* through RNA interference survive better after *Enterococcus faecalis* (*Ef*) infection (Fig. 2C). Furthermore, they express a higher level of *drs* after *MI* challenge, than in control flies (Fig. 2D).

We reasoned that if such a complex system has been selected, it might protect the flies against deleterious activation of Toll pathway overtime. Indeed, the lifespan of *Syt7* null-mutants flies was significantly reduced compared to wild-type flies maintained in a medium with antibiotics (Fig. 3A). We could correlate this weakness with the precocious expression of *drs* in *Syt7* null-mutant 20 day-old flies, while this expression is only detectable two to three weeks later in control flies (Fig. 3B). Therefore the *Syt7* negative regulator seems to be required to slow down Toll pathway activation during aging and delay the death of flies due to this physiological inflammation-like process. To better characterize the function of *Syt7* in the regulation of the Toll pathway, we over-expressed *Syt7* in stimulated ERT-S2 cells (Fig. 3C). While it partially blocked the expression of the reporter gene *drs*, the effect was suppressed by the over-expression of Myd88, Pelle or Dorsal-related immunity factor (DIF), showing that *Syt7* acts above Myd88 in the pathway, most probably at the Toll protein level (Fig. 3C). *Syt7* is a member of a family of proteins involved in the control of membrane proteins trafficking through the regulation of exocytosis (Martinez et al., 2000). Since the endocytosis of the Toll receptor after binding of its ligand Spätzle is crucial for the signal transduction to the nucleus (Huang et al., 2010), we reasoned that *Syt7* could be involved in Toll trafficking after stimulation. We performed immunolocalisation of Toll in non-permeabilised stimulated ERT-S2 cells and saw Toll localization at the cell surface is strongly reduced in *Syt7* knockdown cells compared to control cells (Fig. 3D). Since this is associated with an increased and sustained Toll signaling, those results strongly suggest that *Syt7* regulates the Toll pathway

through the return of the receptor to the cell surface, halting the signal transduction. Of note, the level of Toll receptor protein expression in the *Syt7* knockdown cells was found to be the same than in control cells (figure to be added).

*Syt7* expression is induced by the Toll pathway and increased in *Ago1<sup>KD</sup>* flies or cells, suggesting that some miRNAs may target its mRNA. After *in silico* analysis, several miRNA candidates emerged as putatively capable to target *Syt7* (Fig. S4A). We tested them using miR knock out (KO) mutant flies developed by the Cohen lab (Chen et al., 2014) (Fig. 4A and supplementary figure to be added). We could observe that only the deletion of the cluster *miR310-313* led to an increased susceptibility to infections, reduced Toll pathway activation and increased *Syt7* mRNA expression (Fig. 4A and supplementary figure to be added). To strengthen our understanding of the link between *Syt7* and the *miR310-313*, we deleted the *miR310-313* target sequence in the 3'untranslated region (3'UTR) of *Syt7* gene through Crispr-Cas9 technology (supplementary figure to be added). After infection, the survival of the mutant line is impaired compared to control flies, Toll pathway activation is reduced and *Syt7* mRNA expression is higher 12 hours after infection (Fig. 4B). This phenotype is similar to the one of *Ago1<sup>KD</sup>* flies (Fig. 4B).

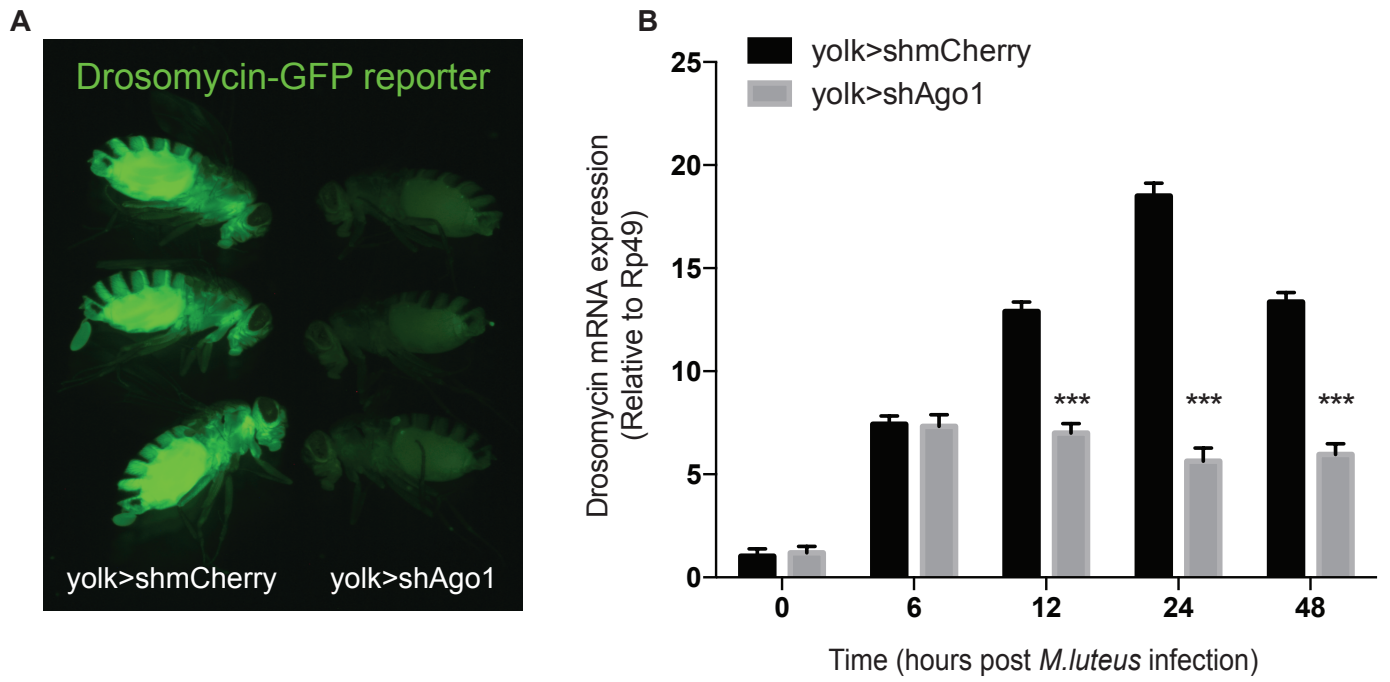
The involvement of miRNA in immune pathway regulation has been the focus of many studies (Momen-Heravi and Bala, 2018; Singh et al., 2013). Here we identify a new mode of regulation by miRNAs that buffer and delay the expressions of some target genes until a sufficient amount of pathway activation is reached. We propose a model where after an infection, the activation of the Toll pathway leads to the expression of AMPs as well as negative regulators (among them *Syt7*). After activation of the Toll pathway, the regulator proteins are not produced until their mRNA expression can overcome the miRNA inhibition (Fig. 4C). This system of regulation of a pathway allows a two steps process: anti-microbial effectors are expressed in an early phase to counteract the infection before negative regulators

shutdown the pathway and prevent an uncontrolled inflammatory-like response, which is as dangerous for the organism as an uncontrolled infection.

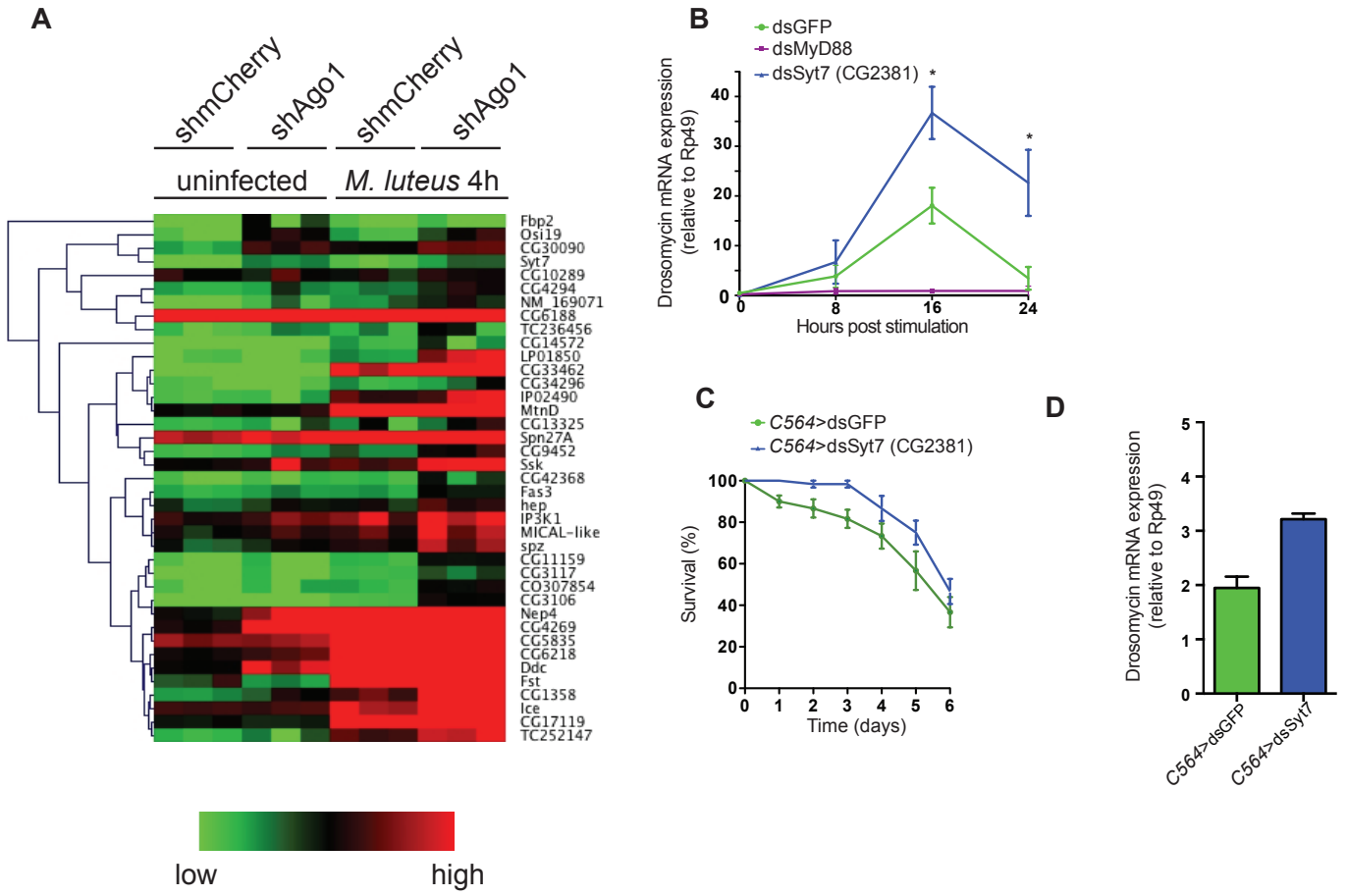
We identified *Syt7* as a negative regulator of the Toll pathway acting on the return of the receptor to the cell membrane, even though its precise mode of action is out of the scope of this study. This is only the second negative regulator of the Toll pathway identified yet with Pellino, but whose precise timing of action has not been analyzed (Ji et al., 2014). The *Drosophila* S2 cells with a chimeric EGF-Toll-receptor (ERT-S2) (Sun et al., 2004) that we used allowed us to consider a more physiological approach considering the regulation timing of the Toll pathway. As several other putative negative regulators were identified in this study, future work might shed light on their role.

In our model, the regulator mRNAs are first targeted by constitutively expressed miRNAs, as observed with the inhibition of *Syt7* by *miR310-313*. Several other miRNAs that are required for a full immune response have been identified by (Atilano et al., 2017). They could potentially target other negative regulators. Of note, as *miR310-313* is constitutively expressed, it might have another function apart from this immune regulation role. Indeed these miRNAs are managing homeostasis upon dietary fluctuations (Çiçek et al., 2016). There has been an increased amount of evidence linking aging and over-expression of NF-κB pathways in *Drosophila* and mammals (Bonney et al., 2013; Franceschi et al., 2007; Garschall and Flatt, 2018; Kounatidis et al., 2017). Overall, our results in *Drosophila* link a time-based regulation model of an immune pathway with the “inflammaging process”.

Figure 1

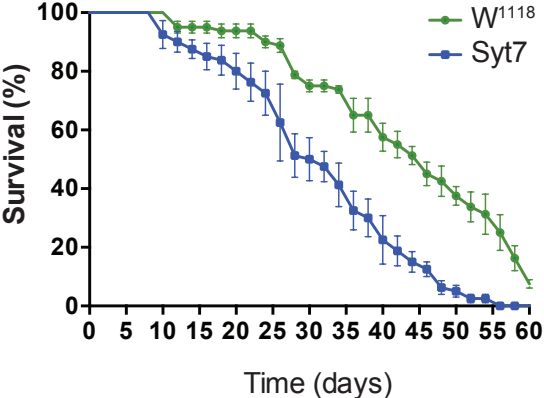


**Figure 2**

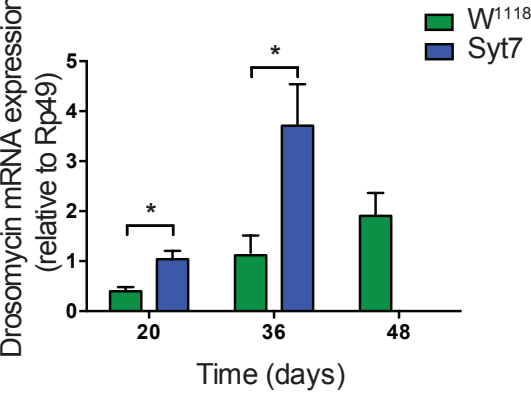


**Figure 3**

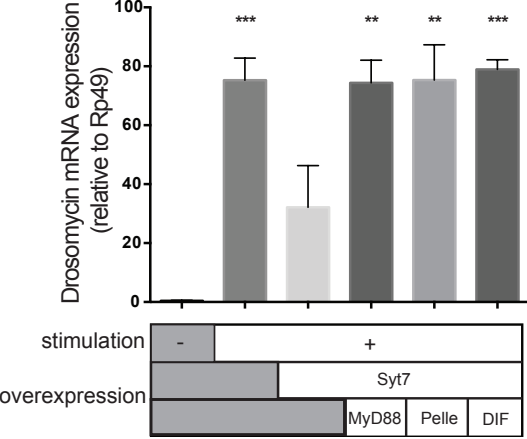
**A**



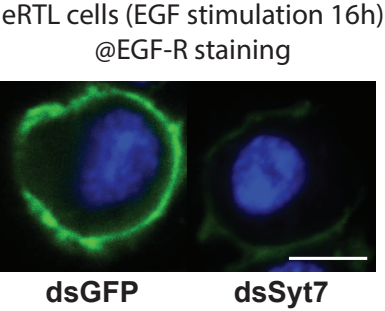
**B**



**C**



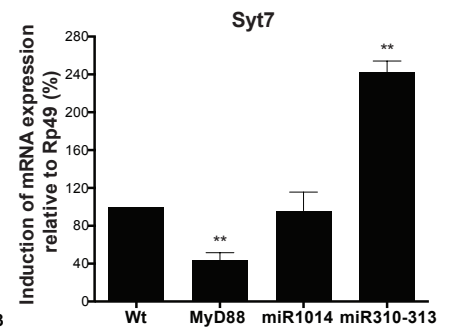
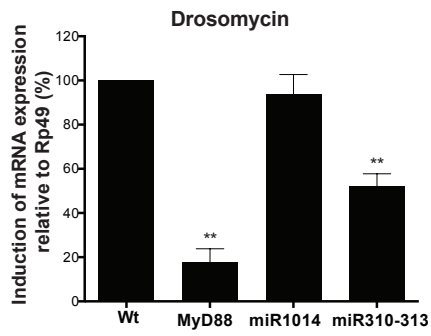
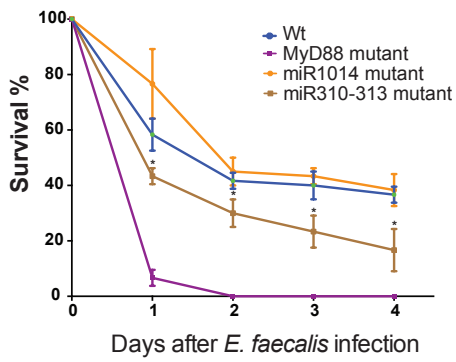
**D**



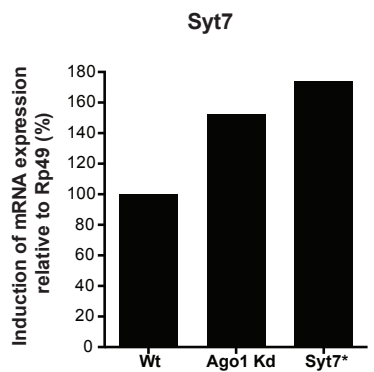
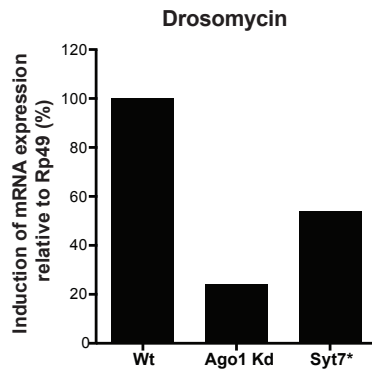
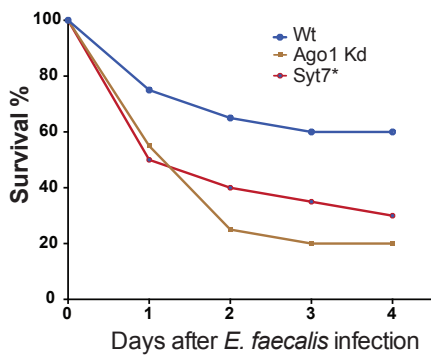


**Figure 4**

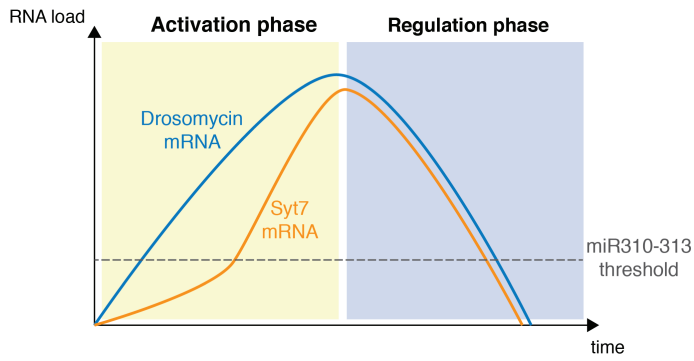
**A**



**B**



**C**



## Figures legends

### *Figure 1: miRNAs are constitutively involved in the Toll pathway regulation*

(A) Impairing miRNAs synthesis attenuates Toll pathway activation in *Drosophila*. Inactivation of Ago1 by short hairpin in adult fat body (yolk>shmAgo1) or control yolk>shmCherry was induced in *Drosophila* expressing Drosomycin-GFP reporter.. Epifluorescence pictures were taken 4 hours post-*M.luteus* infection.

(B) Drosomycin is reduced in Ago1 depleted flies. Quantitative RT-PCR experiments performed on batches of 10 nine-day-old females yolk>shmAgo1 infected by *M.luteus* by septic injury. *Drosophila* expressing yolk>shmCherry were used as control.

Data are represented as mean  $\pm$  standard deviation of three independent experiments. Statistical significance was established by t-test comparing values from yolk>shmAgo1 with yolk>shmCherry control.

\*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

### *Figure 2: Identification of new regulators in the Toll pathway*

(A) Heatmap representation of microarray analysis. Identification of Toll activated-genes dampen by miRNAs in shmCherry and shAgo1 flies, infected by *M.luteus* for 4 hours or uninfected. Genes showing a twofold augmentation or reduction of their expression, upon infection and knockdown of Ago1, are respectively indicated in red or green.

(B) Toll pathway inhibitors *ex-vivo* mini screen. Quantitative RT-PCR of *Drosomycin* mRNA from ERT-S2 cells transfected with dsRNA against GFP (negative control), MyD88 (positive control) and Syt7, followed by EGF stimulation for 16 hours.

Statistical significance was established by t-test comparing values from genes knockdown with GFP dsRNA control.

\*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

(C) Toll pathway regulators screen. *In-vivo* survival experiments performed on batches of 20 nine-day-old females expressing dsGFP or dsSyt7 under the control of the C564 promoter. Flies were infected by *B.bassiana* by septic injury. The experiment was performed three independent times.

(D) Toll pathway regulators screen. Quantitative RT-PCR experiment performed on batches of 10 nine-day-old females expressing dsGFP or dsSyt7 under the control of the C564 promoter. Flies were infected by *M.luteus* by septic injury for 48h. The experiment was performed three independent times.

*Figure 3: Syt7 function as a Toll pathway regulator*

(A) Non-infected female flies (wild-type w<sup>1118</sup> and Syt7 mutant #23394) were raised in a medium with antibiotics, at 25°C and survival was monitored each day. Experiments were performed on batches of 20 female flies, three independent times.

(B) Non-infected female flies (wild-type w<sup>1118</sup> and Syt7 mutant #23394) were raised in a medium with antibiotics, at 25°C. Quantitative RT-PCR experiment was performed on batches of 20 females 20, 36 and 48 days old.

Data are represented as mean ± standard deviation of three independent experiments. Statistical significance was established by t-test comparing values from genes knockdown with wild-type flies as control.

\*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

(C) Epistasis analysis of Syt7 position within the Toll pathway. ERT-S2 cells expressing Syt7, MyD88, Pelle and/or Dif plasmids were stimulated or not with EGF for 16

hours. Subsequent activation of the Toll pathway was assessed by quantitative RT-PCR on the *Drosomycin* gene .

Data are represented as mean  $\pm$  standard deviation of three independent experiments realized on  $5 \times 10^5$  cells per sample. Statistical significance was established by t-test comparing values from the different stimulated conditions with untreated cells expressing an empty vector.

\*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

(D) Immunofluorescence of non-permeabilised ERT-S2 cells after 16 hours of EGF stimulation. Staining was performed using an anti-EGFr antibody and DAPI.

Images are representative of at least 3 cells samples. Scale bars (all panels): 2  $\mu$ m.

*Figure 4: miR310-313 regulates the Toll pathway by directly targeting Syt7*

(A) Identification of the miRNAs targeting *Syt7*. miRNA Knock-Out flies were infected by *E. faecalis*. Survival was monitored for 4 days and quantitative RT-PCR of *Drosomycin* and *Synaptotagmin7* (*Syt7*) was performed on batches of 20 nine-day-old females. The experiment was performed three independent times.

Data are represented as mean  $\pm$  standard deviation of three independent experiments. Statistical significance was established by t-test comparing values from stimulated with unstimulated conditions (PBS control) and genes knockdown with wild-type flies as control.

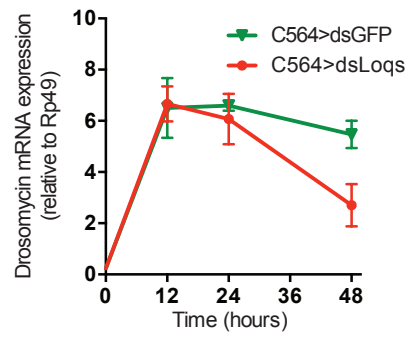
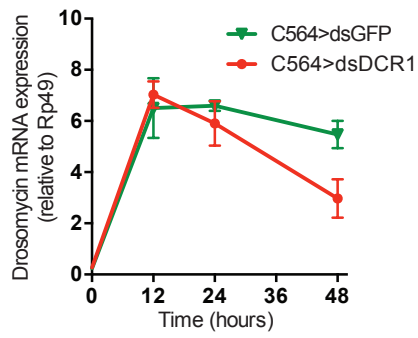
\*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

(B) Invalidation of the miR310-313 target site on 3'UTR of *Syt7* by Crispr-Cas9. Survival of wild-type (Wt), Ago1 Kd and *Syt7*-modified (*Syt7\**) flies was monitored after infection by *E. faecalis*. Quantitative RT-PCR of *Drosomycin* and *Synaptotagmin7* (*Syt7*) was performed on batches of 20 nine-day-old females.

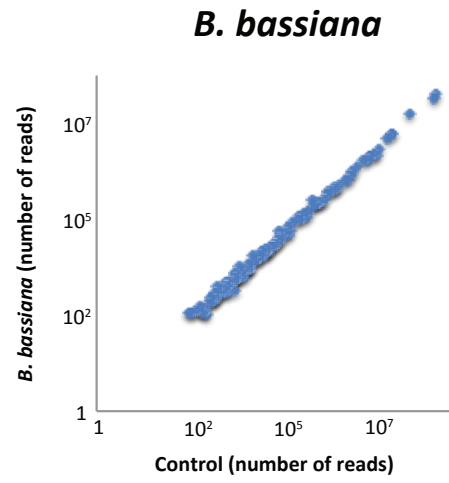
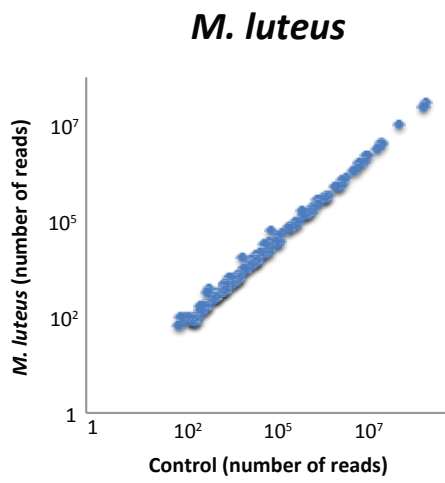
(C) Model of the Toll pathway activation: the inhibitors are first buffered by constitutively expressed miRNAs, before the load of mRNAs become sufficient to trigger the regulation phase.

**Figure S1**

**A**



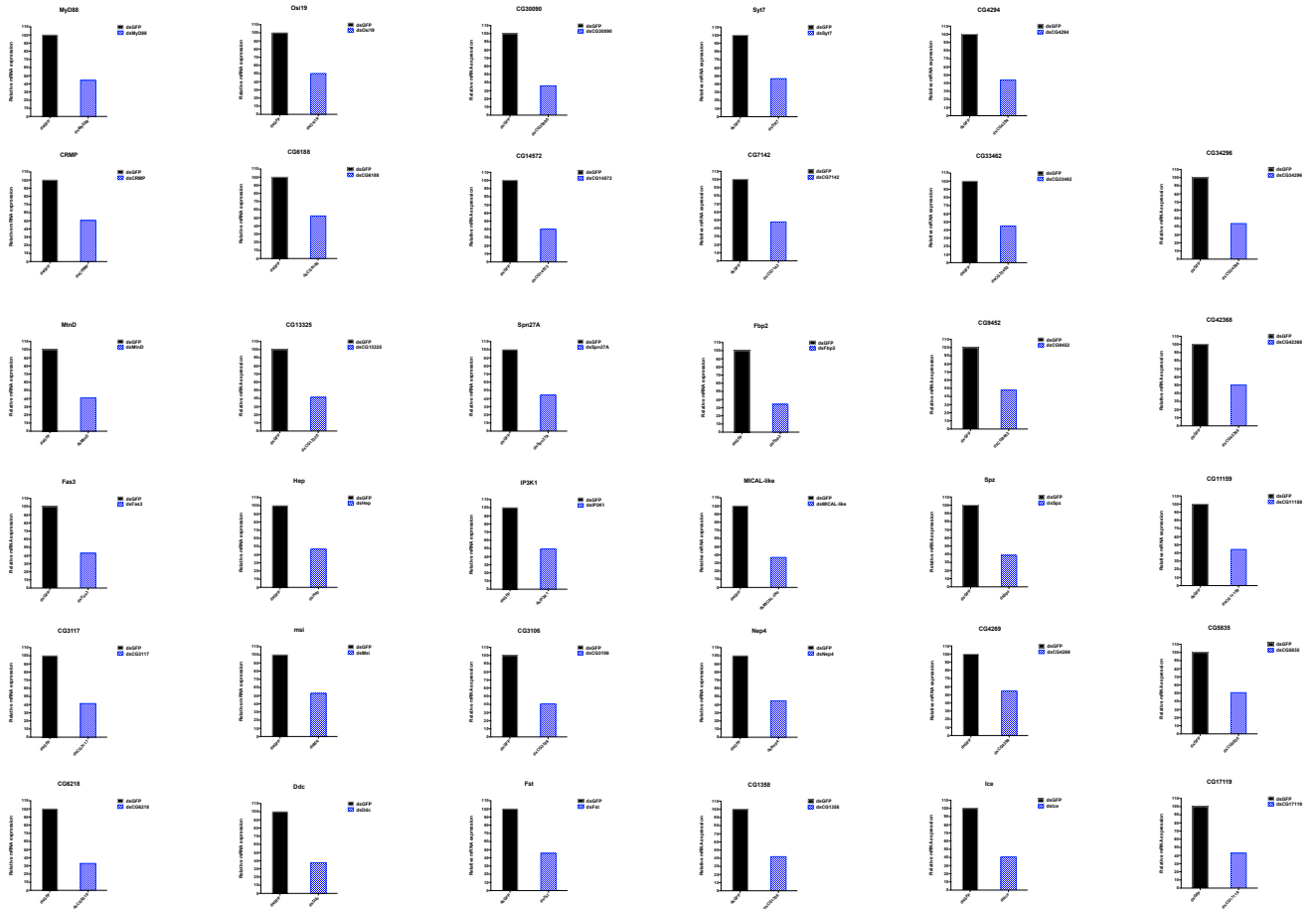
**B**





# Figure S2

## B





**Figure S3**

|                    | <b>Bloomington<br/>Fly line</b> |
|--------------------|---------------------------------|
| <b>miR-10</b>      | 58880                           |
| <b>miR-92a</b>     | 58937                           |
| <b>miR-92b</b>     | 58938                           |
| <b>miR-263a</b>    | 58902                           |
| <b>miR-274</b>     | 58904                           |
| <b>miR-275</b>     | 58905                           |
| <b>miR-283</b>     | 58912                           |
| <b>miR-305</b>     | 58905                           |
| <b>miR-310-313</b> | 58923                           |
| <b>miR-970</b>     | 58951                           |
| <b>miR-971</b>     | 58952                           |
| <b>miR-994</b>     | 58964                           |
| <b>miR-1006</b>    | 58884                           |
| <b>miR-1014</b>    | 58888                           |

## Supplementary Data

### *Figure S1: miRNAs pathway is involved in Toll pathway regulation*

- (A) *In vivo* survival experiments performed on batches of 20 nine-day-old females expressing dsGFP, ds Dcr1 or dsLoqs under the control of the C564 promoter. Flies were infected by *B.bassiana* by septic injury. The experiment was performed three independent times.
- (B) Scatterplot showing the number of read for a small RNAseq in fat-bodies of adult flies infected with the Gram positive bacteria *M. luteus* or the fungi *B. bassiana* .

### *Figure S2: Mini-screen ex-vivo of new regulators in the Toll pathway*

- (A) *Ex vivo* mini-screen of the 34 genes that are at least 2-fold up-regulated after *M. luteus* infection and at least 2-fold higher in Ago1-depleted flies in the micro-array experiment. Quantitative RT-PCR of *Drosomycin* was performed in ERT-S2 cells, stimulated by EGF for 16 hours. The experiment was performed once.
- (B) In the same way as in (A), knockdown efficiency of the 34 genes was assessed by quantitative RT-PCR. The experiment was performed once.

### *Figure S3: Putative miRNAs-KO flies list*

List of Bloomington knockout flies from the *in-silico* analysis of miRNAs that were identified as putatively able to target Syt7.

## **Material and Methods**

*As the project is still ongoing and several experiments are missing, please note that this part is under progress.*

### Cell culture

eRTL 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) 2 mM glutamax and 0,05% de puromycin.

### RNA interference

The double-strand RNAs for the knockdown experiments in *Drosophila* cells were prepared according to (Bonnay et al., 2014). Fragments for the different genes were generated from genomic DNA templates using oligonucleotides designed for use with Genome-RNAi libraries (Schmidt et al., 2013).

### Plasmid Constructs

pMT-Syt7-HA, pMT-MyD88-HA, pMT-Pelle-HA and pMT-Dif-HA constructs were ordered at the Drosophila Genomics Resource Center (DGRC).

### Cell transfection

*Drosophila* S2 cells were transfected with double-strand RNAs using the bathing method described in (Bonnay et al., 2014) or with plasmids using the Effectene transfection kit (Qiagen).

### RNA extraction and quantification

For the *ex-vivo* experiments, RNA was extracted from cells and treated with DNase, using RNA Spin kit (Macherey Nagel). For the *in-vivo* experiments, the procedure was done accordingly to (Bonnay et al., 2014). Similarly, reverse-transcription and quantitative real-time PCR were performed as indicated in (Bonnay et al., 2014).

### Immunofluorescence

Mouse EGFR Monoclonal Antibody (Thermofisher) primary antibody was used at a concentration of 5µg/mL in PBS containing 1% (wt/vol) bovine serum albumin. Goat anti-mouse Alexa488 secondary antibody (Invitrogen) was used at 1/1,000 in PBS. Stimulated eRTL 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. Slides were mounted in a solution of Vectashield/DAPI (Vector Laboratories) and observed using a Zeiss LSM780 confocal microscope.

### Fly strains

Stocks were raised on standard cornmeal-yeast-agar medium at 25°C with 60% humidity. To generate conditional knockdown in adult flies, we used the GAL4 system (McGuire et al., 2004). Fly lines carrying UAS-RNAi transgenes were obtained from the Vienna Drosophila RNAi Center (<http://stockcenter.vdrc.at/control/main>). Fly line carrying a UAS-RNAi transgene against GFP (397-05) was obtained from the Drosophila Genetic Resource Center (Kyoto, Japan; <http://www.dgrc.kit.ac.jp/index.html>). UAS-RNAi flies were crossed with C564-GAL4/CyO flies at 18°C. Emerged adult flies were then transferred to 29°C to activate the UAS-GAL4 system for 6-7 days. Syt7 null mutant and the miRNA knockout lines were purchased at Bloomington (<https://bdsc.indiana.edu>).

### Immune challenge

eRTL cells were stimulated with EGF (40:1) (Sun et al., 2004). Microbial challenges were performed by pricking adult flies with a sharpened tungsten needle dipped into either PBS or concentrated of either *E.faecalis* or *M.luteus* (Reichhart et al., 2011).

### Statistical analysis

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

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## **B. NF- $\kappa$ B transcriptional selectivity in *Drosophila* and mammals**

In both *Drosophila* and mammals, inflammatory stimuli induce gene expression programs that are almost entirely NF- $\kappa$ B dependent (Ghosh and Hayden, 2012). On the other hand, aberrant regulation of NF- $\kappa$ B signaling is strongly suspected in numerous cancers, inflammatory and autoimmune diseases (Maeda and Omata, 2008). The similarity between the immune pathways in flies and mammals makes *Drosophila melanogaster* an excellent model to study the innate response (Vidal and Cagan, 2006). In *Drosophila* the IMD pathway is activated upon sensing of Gram-negative bacteria, triggering the nuclear translocation of the NF- $\kappa$ B transcription factor Relish. A genome-wide RNA-interference screen in *Drosophila melanogaster* cell culture (S2 cells) performed in our laboratory and aiming at finding new modulators of the IMD pathway, led to the discovery of a new NF- $\kappa$ B modulator called Akirin (Goto et al., 2007). We showed that this protein, which has a strict nuclear localization, acts at the level of the NF- $\kappa$ B factor Relish. Akirin is highly conserved and the two mouse genes (*akirin1* and *akirin2*) encoding Akirin proteins have been identified and knocked-out. Our results showed that Akirin2 acts downstream of the Toll-like receptor (TLR), Tumor necrosis factor receptor (TNFR) and interleukin 1 receptor (IL-1R) signaling pathways. Most interestingly, Akirin2 is required in mammals for the regulation of only a subset of lipopolysaccharides (LPS) and IL-1 inducible genes mostly with pro-inflammatory activity. In contrast, Akirin2 does not significantly target genes involved in the resolution of the inflammatory process. Similarly, in *Drosophila*, Akirin is required upon immune challenge for the transcription of a subset of immune effector genes, including Attacin-A, but dispensable for the transcription of genes that are negative regulators. Therefore, Akirin acts as molecular selector specifying the choice between subsets of NF- $\kappa$ B target genes in both flies and mammals (Bonnay et al., 2014; Goto et al., 2007; Tartey et al., 2014).



a) Activation of Akirin by an E3-ubiquitin ligase

Akirin being deprived of known structural domains, we still wonder how it is recruited to a subset of NF- $\kappa$ B promoters upon immune challenge. In both mammals and *Drosophila*, NF- $\kappa$ B dependent signaling pathways are among the best-known examples of the role of ubiquitin linkage to target proteins in signal transduction. During a pilot study, we have observed that upon immune challenge, Akirin is decorated with poly-ubiquitin chains. Therefore, E3 ubiquitin ligases triggering ubiquitin linkage on Akirin following an immune challenge may contribute to Akirin function and to the Akirin-based NF- $\kappa$ B selectivity. To test this hypothesis, we performed in *Drosophila* S2 cells a RNAi screen of the ubiquitin-ligases encoded by the *Drosophila* genome. This screen uncovered the E3 ligase Hyperplastic Discs (Hyd) as important for NF- $\kappa$ B activation and selectivity upon immune challenge. Therefore these post-translational modifications of Akirin are required for the selective activation of NF- $\kappa$ B target genes.

This project led to an article submitted and in review process in *Science Signaling* (pre-publication on bioRxiv: <http://dx.doi.org/10.1101/323170>).

## **Hyd ubiquitinates the NF- $\kappa$ B co-factor Akirin to activate an effective immune response in *Drosophila***

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### **One Sentence Summary**

Upon microbial infection in *Drosophila*, the E3-ubiquitin ligase Hyd ubiquitinylates the NF- $\kappa$ B co-factor Akirin for its efficient binding to the NF- $\kappa$ B factor Relish and subsequent activation of immune effectors genes.

## ABSTRACT

The *Drosophila* IMD pathway is activated upon microbial challenge with Gram-negative bacteria to trigger the innate immune response. In order to decipher this NF- $\kappa$ B signaling pathway, we undertook an *ex-vivo* RNAi screen targeting specifically E3 ubiquitin ligases and identified the HECT E3 ubiquitin ligase Hyperplastic Discs “Hyd” as a new actor of the IMD pathway. We showed that Hyd targets the NF- $\kappa$ B cofactor of Akirin. The K63-polyubiquitination chains deposited by Hyd decorate Akirin for its efficient binding to the NF- $\kappa$ B transcription factor Relish. We showed that this Hyd-mediated interaction is critical to activate immune-induced genes that depend on both Relish and Akirin, but is dispensable for those that depend solely on Relish. Therefore Hyd is key in operating a NF- $\kappa$ B transcriptional selectivity downstream of the IMD pathway. *Drosophila* depleted for Hyd or Akirin failed to express the full set of immune-induced anti-microbial peptide coding genes and succumbed to immune challenges. We showed further that Ubr5, the mammalian homolog of Hyd, is also required downstream of the NF- $\kappa$ B pathway for the IL1 $\beta$ -mediated *IL6* activation. This study links the action of a E3-ubiquitin ligase to the activation of immune effector genes, deepening our understanding of the involvement of ubiquitination in inflammation and identifying a potential target for the control of inflammatory diseases.

## INTRODUCTION

During evolution, metazoans developed strategies to effectively protect themselves from microbial threats. The similarity between the molecular pathways mediating the innate immune response in insects and mammals points to *Drosophila* as a relevant model to explore the immune response ([1](#), [2](#)). In *Drosophila*, the defense against microbes is ensured mainly by the massive production of antimicrobial peptides (AMP) ([3](#)). Their expression is under the control of two transcription factors belonging to the NF- $\kappa$ B family: Dorsal-related Immunity Factor (DIF) and Relish, acting downstream of Toll and IMD pathways respectively. They are the homologues of mammalian RelB and p50 transcription factors.

Post-translational regulation of proteins by ubiquitin pathway is key for proper immune response, albeit not fully understood ([4](#)). Conjugation of ubiquitin polymers to proteins by an ubiquitin-ligase is a key mechanism for controlling their activity or stability. Lysine (Lys) residues of proteins can be modified by a polymer of ubiquitin (polyubiquitin) linked through Lys 48 (K48) or Lys63 (K63) of the ubiquitin molecule. Whereas K48-linked polyubiquitin mainly triggers degradation of proteins by the proteasome, K63-linked polyubiquitin regulates, mainly through modification of interactions, the activity and the subcellular localization of proteins ([5](#)). In both mammals and *Drosophila*, ubiquitination is involved at various levels of the NF- $\kappa$ B pathways ([6](#)). Furthermore, deregulation of ubiquitin-ligases is implicated in inflammatory pathologies ([7](#), [8](#)) and tumor progression ([9](#)).

In *Drosophila*, IAP2 is the only E3-ubiquitin ligase identified so far as a positive regulator of the IMD pathway ([10](#), [11](#)). This protein is involved in the formation and activity of upstream protein complexes formed around the IMD protein and the IKK kinase. To deepen our understanding of NF- $\kappa$ B pathway regulation by the ubiquitination system, we focused on identifying *Drosophila* new ubiquitin-ligases required for the activity of the IMD pathway through a RNAi-based screen in *Drosophila* S2 cells.

Several E3-ubiquitin ligases emerged from this screen as positive or negative regulators of the IMD pathway. We decided to focus on Hyd as i) it is the unique HECT E3-ubiquitin ligase potentially involved in the IMD pathway and ii) it has also emerged as a potential IMD pathway regulator in a parallel pilot screen undertaken in our laboratory (unpublished data from Dr. Akira Goto and ([12](#)). Our data showed that Hyd is required *in-vivo* to survive an immune challenge with Gram-negative bacteria. Epistatic analysis revealed that Hyd acts at the level of the NF- $\kappa$ B co-factor Akirin, which is known to orchestrate the activation of a subset of NF- $\kappa$ B target genes in combination with the SWI/SNF chromatin remodeling complex ([13-15](#)). This is consistent with the described localization of Hyd within the nucleus ([16, 17](#)).

We showed that Hyd decorates Akirin with K63-polyUb chain, which is required for Akirin binding to the NF- $\kappa$ B factor Relish. Furthermore, we observed that Ubr5 (also known as EDD1), the human ortholog of Hyd ([18](#)), has a conserved function in NF- $\kappa$ B signaling in human HeLa cell line. Similarly to human-Akirin2, Ubr5 is required for the activation of only a subset of NF- $\kappa$ B target genes. We demonstrate here that upon immune challenge, ubiquitin chains are instrumental to bridge NF- $\kappa$ B and its co-factor Akirin to activate an effective immune response.

## RESULTS

### **Hyd is an E3-ubiquitin ligase required for the activation of the IMD pathway**

To uncover novel E3-ubiquitin ligases that modulate the IMD pathway, we screened a library of 174 double strand RNA (dsRNA) targeting putative E3-ubiquitin ligases encoded in the *Drosophila* genome as described in Flybase ([19](#)). We used stably transfected *Drosophila* S2 cells expressing the *Attacin-A-luciferase* gene, a reporter of the activation of the IMD pathway upon immune challenge with Gram-negative bacteria ([20](#)). We evaluated the ability

of dsRNA targeting individually each of the 174 putative E3 ubiquitin-ligases to interfere with the IMD reporter upon stimulation by heat-killed *Escherichia coli* (HKE), a regular IMD pathway agonist.

*IAP2* is an E3-ubiquitin ligase that positively regulates the pathway by targeting IMD and DREDD (21). The knockdown of *IAP2* resulted in a strong decrease of the *Attacin-A-luciferase* reporter induction upon immune stimulation regarding to *dsGFP* control (Fig 1A), providing proof of concept for the screen. Knockdown of six E3-ubiquitin ligase-coding genes (*m-cup*, *Mkrn1*, *CG2926*, *CG31807*, *mura* and *CG12200*) resulted in a strong increase in *Attacin-A-luciferase* activity upon immune stimulation. Therefore these E3-ubiquitin ligases behave as negative regulators of the IMD pathway. Conversely, the knockdown of three genes encoding either two Really Interesting New Gene (RING) domain E3-ubiquitin ligases *bon* and *CG5334*, or HECT domain E3-ubiquitin ligase *hyd*, resulted in a significant decrease of *Attacin-A-luciferase* activity (Fig 1A). This suggests that Bon, CG5334 and Hyd are new positive regulators of the IMD pathway. We decided to focus on the exploration of Hyd, as it is the unique HECT domain E3-ubiquitin ligase involved in the *Drosophila* IMD pathway.

To validate reporter-assay experiments, *Drosophila* S2 cells were transfected with *dsRNA* targeting either the NF- $\kappa$ B factor *relish*, its cofactor *akirin*, *hyd* or some of the other E3-ubiquitin ligases of the screen. We challenged S2 cells with HKE and monitored endogenous *Attacin-A* mRNA level by RT-qPCR. Interfering with *relish*, *akirin* or *hyd* expression significantly decreased HKE-mediated *Attacin-A* induction, compared to control (*dsGFP*) (Fig 1B, Fig S1). We observed that the RING-domain E3-ubiquitin ligases Bon, CG5334, *m-cup*, *Mkrn1* and *Mura* are required for the normal activation of *Attacin-A* expression and that the HECT E3-ubiquitin ligase Hyd acts as a positive regulator of *Attacin-A* expression in *Drosophila* S2 cells (Fig 1B).

In order to identify at which level of the IMD pathway Hyd is required, we undertook an epistasis analysis. *Drosophila* S2 cells were treated by dsRNA targeting *hyd* or *akirin* as a control and the IMD pathway was activated at different levels by transfecting either a truncated form of Peptidoglycan Receptor Protein-Long Chain a (PGRP-LCa), IMD or the 68kD active-form of Relish (Rel68) ([13](#)). Measurement of *Attacin-A* expression by RT-qPCR assessed activation of the IMD pathway. We could show that Hyd is required at the same level or downstream of Relish (Fig 1C) to exert its positive regulation on IMD pathway activation.

### **Hyd acts at the level of Akirin to trigger full activation of the IMD pathway**

Downstream of the IMD pathway, Relish target genes are divided in two subsets: genes that depend only on Relish for their expression (including *Attacin-D* and the majority of negative regulators) and ones requiring Akirin in addition to Relish (including *Attacin-A* and the majority of effectors) ([Bonnay, Nguyen et al., 2014](#)). Upon immune challenge in S2 cells, using RT-qPCR, we observed that Hyd depletion recapitulates the immune phenotype of cells depleted for Akirin (Fig 2A). Consequently, Hyd is acting on Akirin-dependent NF- $\kappa$ B transcriptional selectivity *ex-vivo*.

We next investigated if Akirin and Hyd were similarly required for NF- $\kappa$ B transcriptional selectivity *in-vivo*. As *Drosophila* embryonic development is impaired in absence of Akirin, we used the *C564-Gal4* transgene ([22](#)) to express RNAi constructs targeting *akirin*, *hyd* and *relish* in the adult fat body, the main immune organ of *Drosophila* ([3](#)). Flies depleted of Akirin (*C564 > RNAi-akirin*), Relish (*C564 > RNAi-relish*) or Hyd (*C564 > RNAi-hyd1* or *C564 > RNAi-hyd2*) displayed an impaired survival following *E. coli* infections when compared to control flies (*C564 > RNAi-GFP*) or following PBS pricking (Fig 2B).

Following immune challenge by *E. coli*, expression of *Attacin-A*, but not of *Attacin-D*, was reduced in the absence of Akirin or Hyd, when compared to control flies (*C564 > RNAi-GFP*) (Fig 2C, Fig S2).

Our results indicate that Hyd is required at the level of Relish to activate the Akirin-dependent subset of Relish target genes during the immune response, allowing *Drosophila* to survive a Gram-negative bacterial challenge.

### **Hyd mediated K63-polyubiquitination of Akirin is instrumental for its link to Relish**

We next investigated if Akirin could be a *bona-fide* target for the E3 ubiquitin-ligase Hyd. Co-immunoprecipitation assay in S2 cells showed that V5-tagged Hyd (*Hyd-V5*) (23) binds to endogenous Akirin (Fig 3A). By contrast V5-tagged HydCS (*HydCS-V5*), which displays a mutated HECT domain by conversion of the catalytic cysteine at position 2854 to serine (23), is unable to bind to Akirin (Fig 3A). As a control we confirmed that IAP2, the E3-ubiquitin ligase acting upstream of Akirin in the IMD signaling cascade (10, 11), does not interact with Akirin (Fig S3).

Protein extracts from cells transfected with a tagged version of Akirin (*Akirin-V5*) were immunoprecipitated with an anti-V5 antibody. Western-blot experiments with antibodies targeting K63-polyUb chains suggested that Akirin is K63-polyubiquitinated 1h and 3h after immune challenge with HKE (Fig 3B). This immune-induced post-translational modification of Akirin is indeed attenuated upon knockdown of Hyd (Fig 3C). Collectively, these data indicate that upon immune challenge, Hyd physically interacts with Akirin through its catalytic HECT domain to decorate Akirin with K63-polyUb chains. We previously published that Akirin physically bridges the NF- $\kappa$ B factor Relish and BAP60, a core member of the SWI/SNF chromatin-remodeling complex (14). To understand whether Akirin K63-polyubiquitination is instrumental for the interaction of Akirin with Relish or BAP60, we



performed co-immunoprecipitation experiments in S2 cells depleted for Hyd and transfected with *Akirin-V5* and *Rel68-HA* or *BAP60-HA* (Fig 3D). As previously reported ([14](#)), Akirin-V5 co-precipitated either with the active form of the NF- $\kappa$ B factor Relish (Rel68-HA) or with BAP60 (BAP60-HA) (Fig 3D). However, in the absence of Hyd, the interaction between Akirin-V5 and Rel68-HA is weakened (Fig 3D). Of note the interaction between Akirin-V5 and BAP60-HA is independent of Hyd (Fig 3D). These results indicate that Hyd is required to deposit K63-polyUb chains on Akirin for subsequent binding to the NF- $\kappa$ B factor Relish.

### **Ubr5 - the human ortholog of Hyd - is required for the NF- $\kappa$ B transcriptional selectivity during the inflammatory response**

The Akirin-dependent molecular mechanism underlying the selective activation of NF- $\kappa$ B target genes is well conserved from *Drosophila* to mammals ([13-15](#)). Therefore, we addressed the potential requirement of Ubr5 (the ortholog of the *Drosophila* E3-ubiquitin ligase Hyd) in NF- $\kappa$ B selective transcriptional response mediated by hAkirin2 during the human inflammatory response. We depleted HeLa cells for either NF- $\kappa$ B1, hAkirin2 or Ubr5 by siRNA (using scrambled siRNA as controls). We monitored, upon stimulation by IL1 $\beta$ , the expression levels of NF- $\kappa$ B target genes that are dependent of hAkirin2 (such as *IL6*) or independent (such as *IL8*) ([15](#)). As expected ([15](#)), lacking NF $\kappa$ B1 in HeLa cells impaired both *IL6* and *IL8* activation upon IL1 $\beta$  stimulation. However, the activation of *IL6* and *IL8* is uncoupled in HeLa cells depleted for hAK2 or Ubr5 (Fig 4A, Fig S4). This result suggests a conserved function of Ubr5 in the selective transcription of NF- $\kappa$ B target genes mediated by hAkirin2 that remains to be functionally explored.

Taken altogether, our results show that Hyd/Ubr5 is a HECT E3-ubiquitin ligase involved in NF- $\kappa$ B pathway regulation in *Drosophila* and mammals. In fruit fly, Hyd deposits K63-polyUb chains on Akirin and these ubiquitin marks are required to bridge Akirin and the

NF- $\kappa$ B factor Relish. This interaction is necessary for the transcription of an essential NF- $\kappa$ B target genes subset, downstream of the IMD pathway (Fig 4B).

## DISCUSSION

Using *Drosophila* genetics, we describe here for the first time a function for the HECT E3-ubiquitin ligase Hyd in the innate immune response. We could also show using HeLa cells that this function of Hyd downstream of the NF- $\kappa$ B pathway is conserved in humans.

In both humans and *Drosophila*, NF- $\kappa$ B dependent signaling pathways are among the best-known examples of the role of ubiquitin linkage to target proteins in signal transduction ([4](#), [24](#)), ubiquitination being involved at every level of the NF- $\kappa$ B pathway, from membrane receptors to chromatin-associated proteins. In order to identify new E3-ubiquitin ligases involved in the *Drosophila* innate immune response, we conducted a RNAi-based screen. We showed, in addition to IAP2 already known to be a bona-fide member of the IMD pathway ([10](#), [11](#)), that other RING-domain E3 ubiquitin ligases (CG5334, bon) were involved in the activation of the IMD pathway in *Drosophila* S2 cells after immune challenge. In addition, our results indicate that other RING-domain E3 ubiquitin ligases such as m-cup, Mkrin1 and mura down-regulate IMD pathway target genes activation. Interestingly, this screen also indicates that a HECT E3-ubiquitin ligase, namely Hyd, is involved in the innate immune response.

In *Drosophila*, Hyd was reported to be located in the nuclear and in the cytoplasmic fraction of cells to participate in various phenomena during development such as cellular proliferation ([17](#)). More precisely, Hyd shapes hedgehog signaling by differentially restraining the transcriptional activity of Cubitus interruptus via selective association with respective promoters ([23](#)). And more recently, Hyd and its mammalian orthologue Ubr5 were reported to act at the level of Wnt signaling target genes promoter to enable gene transcription

(25). Here we identified the HECT E3 ubiquitin-ligase Hyd in *Drosophila* as responsible for the ubiquitination of Akirin and its subsequent binding to the NF- $\kappa$ B transcription factor Relish. Altogether these results point to a conserved function of the HECT E3-ubiquitin ligase Hyd/Ubr5 as a nuclear selector for gene activation.

Downstream of the IMD pathway, the NF- $\kappa$ B transcription factor relish target genes could be divided in two subgroups, Akirin-dependent and Akirin-independent genes (14). Targeting Hyd by RNAi in *Drosophila* S2 cells impaired the activation of Akirin-dependent genes upon immune stimulation. Depleting Hyd from *Drosophila* fat-body prevents fly survival to immune challenge with the Gram-negative bacteria *E.coli*, demonstrating the biological relevancy of its function. Co-immunoprecipitation experiments showed that Hyd interacts with Akirin through its catalytic domain to deposit K63-polyUb chains. Of note, the K63-polyubiquitination of Akirin by Hyd is performed only after immune challenge, suggesting that an immune-triggered signal governs this event and remains to be explore.

It is still unclear how the K63-polyubiquitin chains on Akirin physically interact with Relish to set a bridge, as no Ubiquitin Binding Domain (UBD) have been described for Relish. The HECT Ubiquitin ligase family is known in mammals and *Drosophila* to regulate many biological phenomenon (26). We found that the mammalian ortholog of Hyd, Ubr5 (18) is involved in NF- $\kappa$ B transcriptional selective response in human cell line as well. This suggests a conserved role for Hyd/Ubr5 on hAkirin2, even though we do not know if hAkirin2 is ultimately ubiquitinated. A dedicated study of Ubr5 role in NF- $\kappa$ B pathway is needed to completely assess it. It is known that Ubr5 inhibits the TNF receptor associated factor 3 (Traf3) (27) an inhibitor of the NF- $\kappa$ B pathway (28). Thus, the role of Ubr5 might be indirect.

When Hyd/Ubr5 is attenuated, only a subset of NF- $\kappa$ B genes is expressed, diminishing the intensity of the innate immune response in *Drosophila* and inflammatory response in

mammals, similarly to the inactivation of Akirin ([14](#), [15](#)). The link between excessive activation of NF- $\kappa$ B signaling pathway during e.g chronic inflammation and cancer progression or appearance is now on the spotlight ([29](#)). Uncontrolled activation of NF- $\kappa$ B due to deregulation of ubiquitin-ligases has been reported in many diseases ([30](#)) and Ubr5 involved in several types of cancer in human ([31](#)). Our findings point to the HECT E3-ubiquitin ligase Ubr5 as an interesting drug target to modulate NF- $\kappa$ B signaling, control the development of inflammatory diseases and potentially improve treatments of cancer.

## **MATERIAL AND METHODS**

### **Cell culture**

S2 cells were cultured at 25°C in Schneider's medium (Biowest) supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin (50  $\mu$ g/ml of each) and 2 mM glutamax. HeLa cell line was cultured and maintained in DMEM containing 10% (vol/vol) FCS, 40  $\mu$ g/mL gentamycin. Recombinant human IL1 $\beta$  was purchased from Invitrogen.

### **E3-ubiquitin ligases screening methods**

A comprehensive list containing 174 E3 ubiquitin ligases in the *Drosophila* genome, consisting predominantly of HECT, RING, and U-box proteins was curated manually by GO- and protein domain-term search in Flybase FB2012\_06 Dmel Release 5.48 ([19](#)). Based on this list, a *Drosophila* E3 ligase dsRNA library was generated in Michael Boutros's laboratory as previously described ([32](#)). The screen experiments were performed using 1F3 cells stably expressing AttA firefly luciferase ([12](#)). Two days after transfection with an Actin renilla luciferase construct, cells were collected and distributed into 96-well screening plates at a density of  $4.5 \times 10^4$  cells per well. Cells were then transfected with 3  $\mu$ g of each dsRNA in the *Drosophila* E3-ubiquitin ligase dsRNA library in triplicate by bathing method as previously

described ([14](#)). At day 5 post-transfection, cells were stimulated with heat-killed *E. coli* (40:1) before determining both firefly and renilla luciferase activities.

### **RNA interference**

The double-strand RNAs for the knockdown experiments in *Drosophila* cells were prepared according to ([14](#)). Fragments for the different genes were generated from genomic DNA templates using oligonucleotides designed for use with Genome-RNAi libraries ([33](#)) and are listed in Supplementary Table 1. The small interfering RNAs used for the knockdown experiment in HeLA cells were purchased from Ambion (Supplementary Table S2).

### **Luciferase assay**

The luciferase assay was realized accordingly to ([14](#)).

### **Plasmid Constructs**

pAC-Akirin, pAC-Akirin-V5, pAC-PGRP-LC, pAC-IMD, pMT-Rel-HA and pMT-Bap-HA constructs were described previously ([13](#), [14](#)).

### **Cell transfection**

*Drosophila* S2 cells were transfected with double-strand RNAs using the bathing method described in ([14](#)) or with plasmids using the Effectene transfection kit (Qiagen). HeLa cells were transfected with siRNA using Lipofectamine RNAiMax (Invitrogen).

### **RNA extraction and quantification**

For the *ex-vivo* experiments, RNA was extracted from cells and treated with DNase, using RNA Spin kit (Macherey Nagel). For the *in-vivo* experiments, the procedure was done

accordingly to (14). Similarly, reverse-transcription and q-RT-PCR were performed as indicated in (14). Primers used for q-RT-PCR are listed in Supplementary Table 3.

### **Immunoprecipitation and Western blot**

The experiments were realized according to (14). Immunoprecipitations were performed with rabbit polyclonal anti-Akirin (14) and anti-ubiquitin Lys63 specific antibodies (Millipore 05-1308) coupled with Dynabeads Protein G (Invitrogen) and anti-V5 antibodies coupled to agarose beads (Sigma). Proteins were detected by Western blotting using anti-Akirin, anti-ubiquitin Lys63 specific, anti-ubiquitin (Santa cruz biotechnology SC-8017), anti-V5 (Invitrogen r96025), anti-HA (Abcam ab9110) and anti-Relish (gift from Tony Ip) antibodies.

### **Fly strains**

Stocks were raised on standard cornmeal-yeast-agar medium at 25°C with 60% humidity. To generate conditional knockdown in adult flies, we used the GAL4-GAL80<sup>ts</sup> system (22). Fly lines carrying a UAS-RNAi transgene targeting relish (108469), akirin (109671), and hyd (44675, 44676) were obtained from the Vienna Drosophila RNAi Center (<http://stockcenter.vdrc.at/control/main>). Fly line carrying a UAS-RNAi transgene against GFP (397-05) was obtained from the Drosophila Genetic Resource Center (Kyoto, Japan; <http://www.dgrc.kit.ac.jp/index.html>). UAS-RNAi flies were crossed with Actin-GAL4/CyO; Tub-GAL80ts flies at 18°C. Emerged adult flies were then transferred to 29°C to activate the UAS-GAL4 system for 6-7 days.

### **Immune challenge**

Cells were stimulated with heat-killed *E. coli* (40:1) (34). Microbial challenges were performed by pricking adult flies with a sharpened tungsten needle dipped into either PBS or

concentrated *Escherichia coli* strain DH5aGFP bacteria solution ([14](#), [34](#)). Bacteria were grown in Luria broth (LB) at 29°C.

### **Statistical analysis**

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

### **SUPPLEMENTARY MATERIALS**

Fig. S1. Knockdown efficiency of the double strand RNA used in *Drosophila* S2 cells

Fig. S2. Knockdown efficiency of the Gal4-UAS system used in adult flies

Fig. S3. Interaction between IAP2 and Akirin

Fig. S4. Knockdown efficiency of the small interfering RNA used in HeLa cells

Table S1. Oligonucleotides used to generate double strand RNA in *Drosophila* S2 cells

Table S2. Oligonucleotides used to generate small interfering RNA in mammalian HeLa cells

Table S3. Oligonucleotides used for quantitative real-time PCR

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**Author contributions:** N.M., X.-H.N., M.-O.F., A.O. and M.B. designed the experiments. A.C.-M., X.-H.N., A.G. and F.B. performed the experiments. A.C.-M., J.-M.R. and N.M. wrote the manuscript. J.-M.R. and N.M. supervised the study. **Competing interests:** The authors declare that they have no competing interests.

## FIGURE LEGENDS

### Figure 1. E3-ubiquitin ligases screen identified *ex-vivo* Hyd as involved in IMD pathway

(A) E3 ubiquitin-ligases screen in *Drosophila* S2 cells realized by luciferase assay. The different genes were knocked down by dsRNA. Induction of IMD pathway was done by 48h HKE stimulation and assessed by measure of *Attacin-A* and put on percentage compared to control (dsGFP).

(B) Quantitative RT-PCR of *Attacin-A* mRNA from S2 cells transfected with dsRNA against GFP (negative control), relish, akirin (positive controls) and some E3-ubiquitin ligases from the screen, following 4h of HKE stimulation.

(C) Epistasis analysis of Hyd position within the IMD pathway. The IMD pathway was induced by either HKE stimulation or the transfection of S2 cells with PGRP-LC, IMD-V5 or Rel-HA plasmids. Cells treated with vector alone serve as a control. Cells were also transfected with dsRNA targeting akirin or hyd.

Data information: Data are represented as mean  $\pm$  standard deviation of three independent experiments realized on  $5 \times 10^5$  cells (B-C) per sample. Statistical significance was established by comparing values from stimulated with unstimulated conditions and genes knockdown with GFP dsRNA control. \*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

**Figure 2. Hyd is required for the full activation of IMD response**

(A) Quantitative RT-PCR of *Attacin-A* and *Attacin-D* mRNA from S2 cells transfected with dsRNA against GFP (negative control), relish, akirin (positive controls), and hyd, following 4h of HKE stimulation.

(B) *In-vivo* survival experiments performed on batches of 20 nine-day-old females infected by *E. coli* septic injury (with PBS pricking as control), at 25°C three independent times.

(C) Quantitative RT-PCR of *Attacin-A* and *Attacin-D* mRNA, from three batches of 10 nine-day-old males infected with *E. coli* for 6h by septic injury at 25°C, three times independently.

Data information: Data are represented as mean  $\pm$  standard deviation of three independent experiments realized on  $5 \times 10^5$  cells (A and C) per sample. Statistical significance was established by comparing values from stimulated with unstimulated conditions and genes knockdown with GFP dsRNA control. \*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

**Figure 3. Hyd mediated-ubiquitination of Akirin is necessary for interaction with Relish**

(A) Co-immunoprecipitation assay between over-expressed Akirin and Hyd in S2 cells. The cells were transiently transfected with pAC-Akirin, pGal4 and/or pUAS-Hyd-V5 and pUAS-Hyd-CS-V5. Cell lysates were immunoprecipitated with anti-Akirin coupled agarose beads. Immunoprecipitates were analyzed by Western blotting with anti-V5 or anti-Akirin antibodies.

(B) Immunoprecipitation assay of K63-polyUb chains on Akirin before and after immune challenge (1h and 3h HKE). S2 cells were transiently transfected with pAC-Akirin-V5. Cell lysates were immunoprecipitated with anti-K63-polyUb coupled agarose beads. Immunoprecipitates were analyzed by Western blotting with anti-V5 antibodies.

(C) Immunoprecipitation assay of Akirin after immune challenge (4h HKE). S2 cells were transiently transfected with pAC-Akirin-V5 and dsRNA targeting GFP or hyd. Cell lysates were immunoprecipitated with anti-V5 coupled agarose beads. Immunoprecipitates were analyzed by Western blotting with anti-K63-polyUb and anti-V5 antibodies.

(D) Co-immunoprecipitation assays between over-expressed Akirin and Relish or Bap in S2 cells. The cells were transiently transfected with pAC-Akirin-V5 and pMT-Rel-HA or pMT-Bap-HA; and dsRNA targeting GFP or hyd. Cell lysates were immunoprecipitated with anti-V5 coupled agarose beads. Immunoprecipitates were analyzed by Western blotting with anti-HA or anti-V5 antibodies.

Data information: Data are representative of 2 independent experiments.

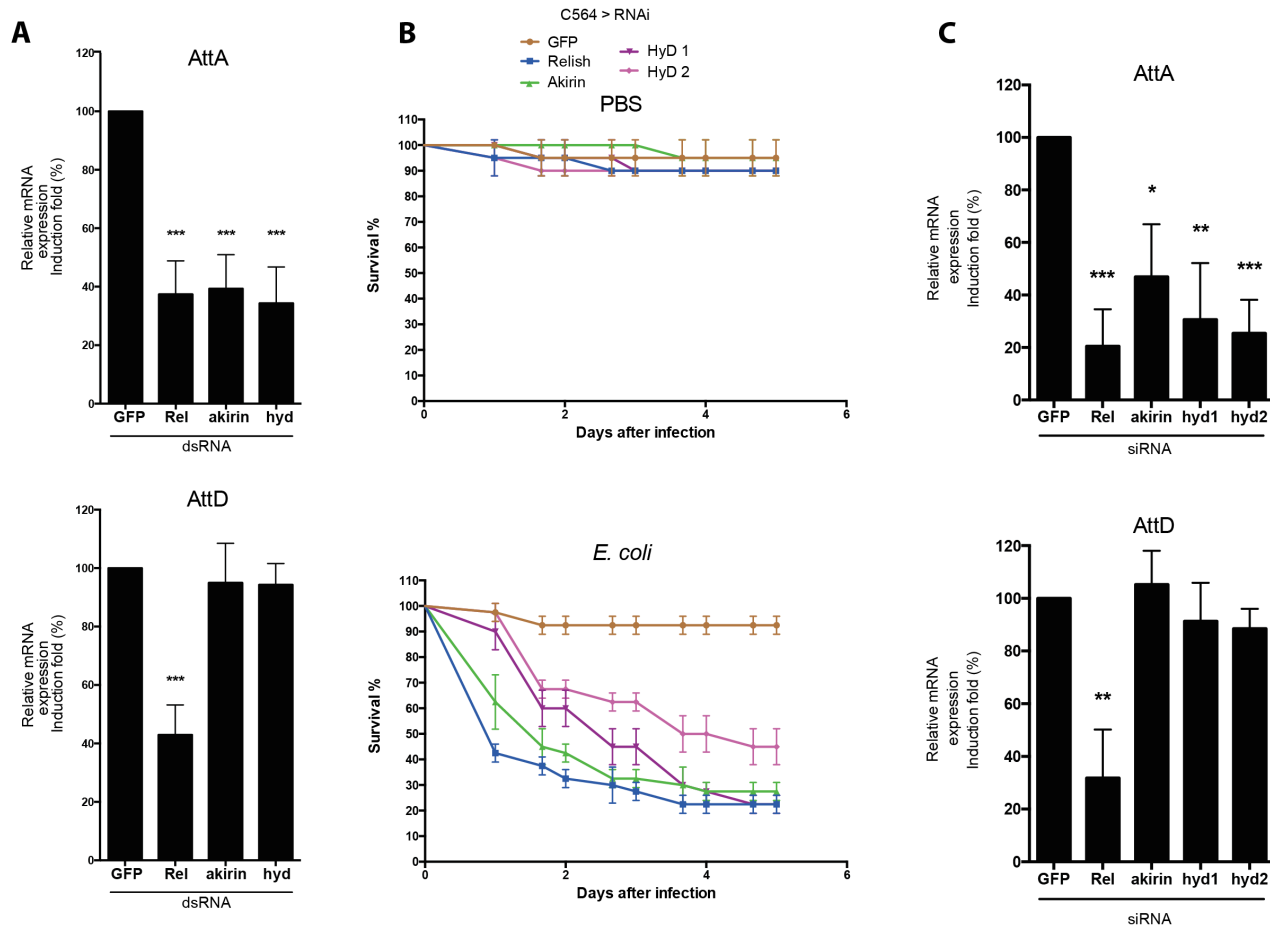
**Figure 4. Hyd/Ubr5 is necessary for NF- $\kappa$ B target genes activation**

(A) Quantitative RT-PCR of *IL-6* and *IL-8* mRNA from HeLa cells. They were transfected with scrambled siRNA (negative control) or siRNA targeting NF $\kappa$ B1, hAkirin2 (positive controls), and Ubr5. The cells were stimulated with recombinant human IL1 $\beta$  (10 ng/ml) for 4h. Data are represented as mean  $\pm$  standard deviation of three independent experiments realized on  $5 \times 10^5$  cells per sample. Statistical significance was established by comparing values from stimulated with unstimulated conditions and genes knockdown with scrambled siRNA control. \*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

(B) Model showing the role of Hyd in the expression of the Akirin-dependent genes in the IMD pathway. After activation of the pathway, allowed by the K63-polyUb chains deposition on the complexes IMD and DREDD by the E3-ubiquitin ligase Iap2, Relish is translocated. The K63-polyUb of Akirin by Hyd allows the protein to link to Relish. This interaction is crucial for the expression of Akirin-dependent genes, necessary for an adequate innate immune response.

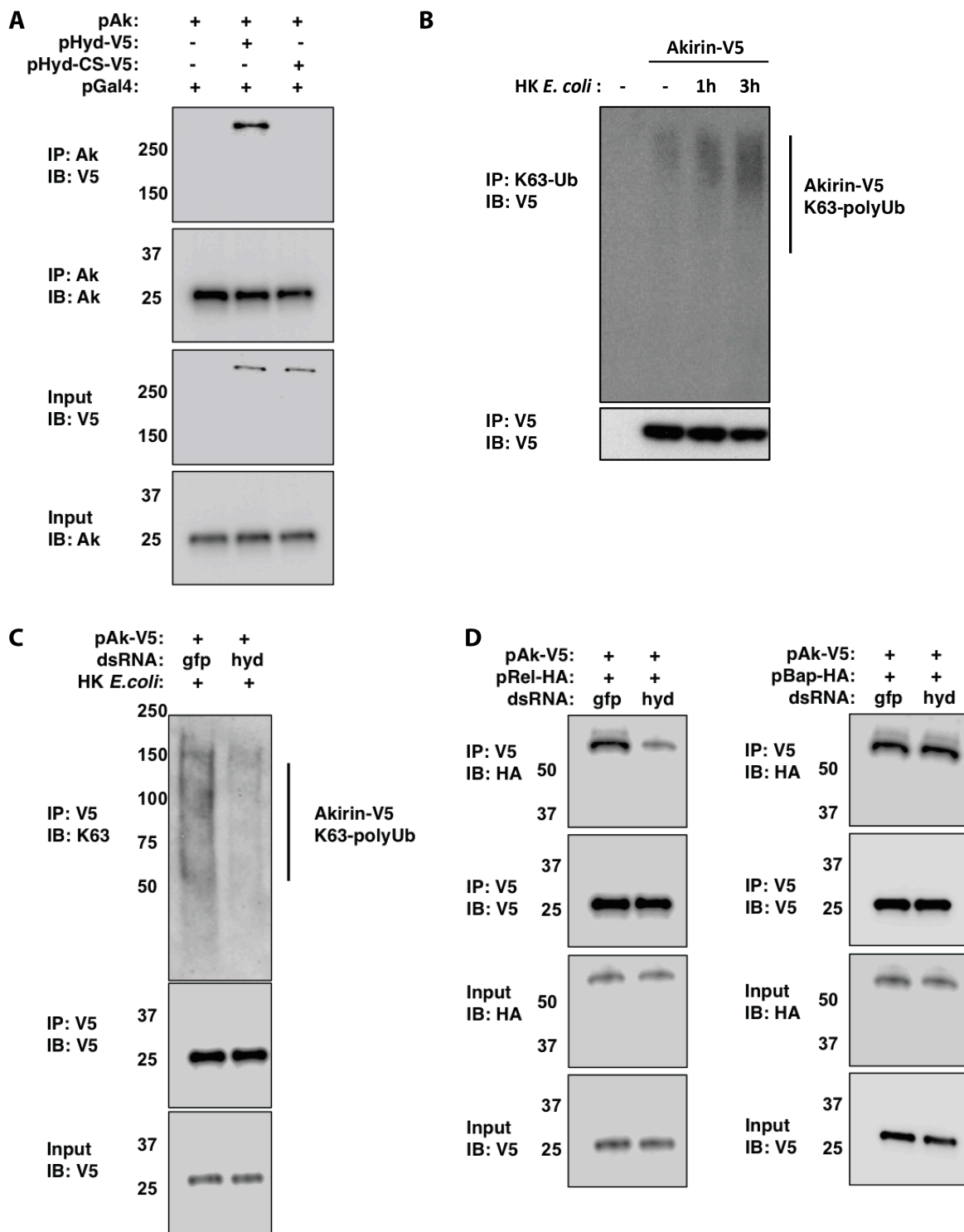


**Figure 2**

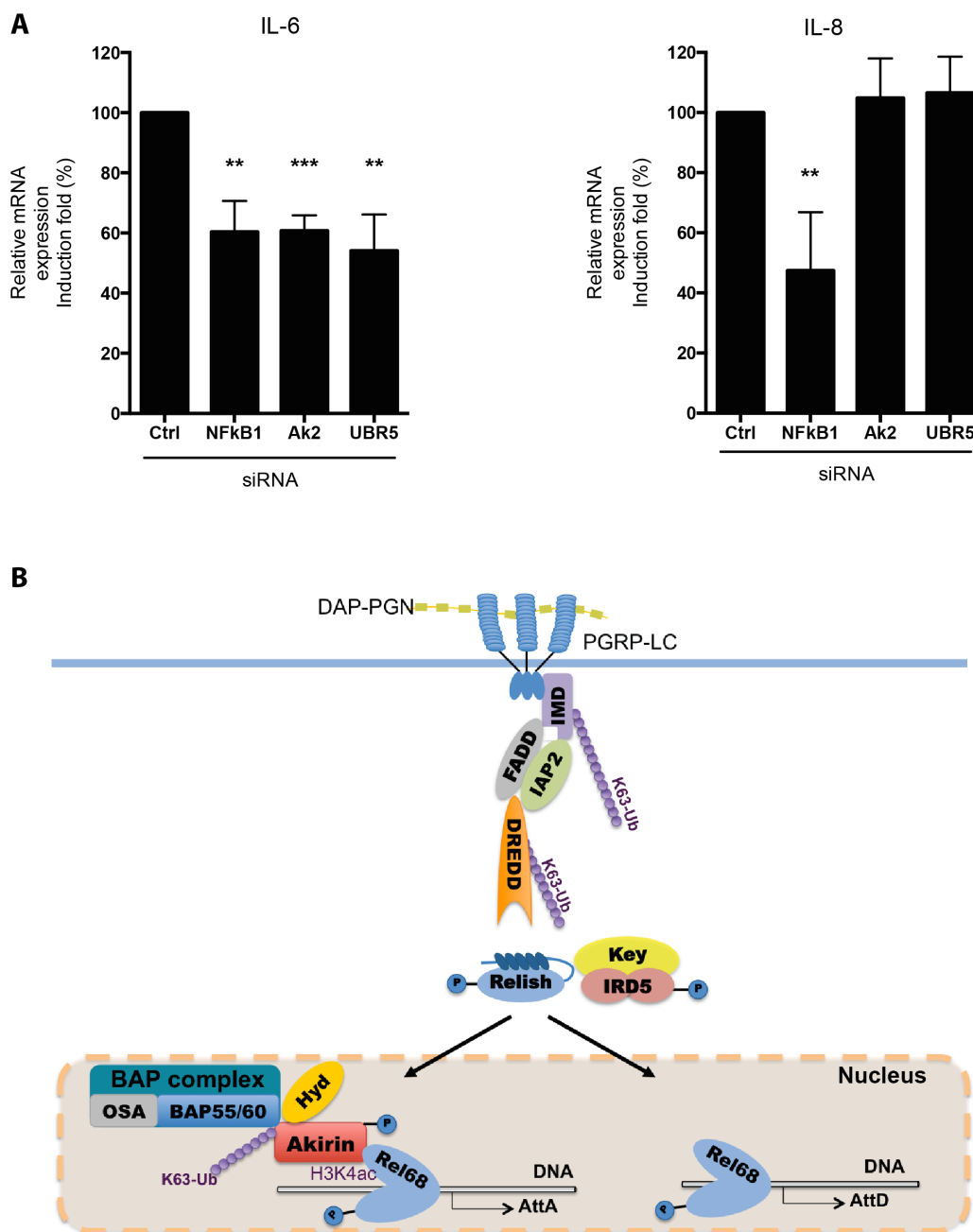




**Figure 3**



**Figure 4**



## SUPPLEMENTARY MATERIALS

### **Fig. S1. Knockdown efficiency of the double strand RNA used in *Drosophila* S2 cells**

Quantitative RT-PCR of akirin, bon, hyd, IAP2, m-cup, Mkrn1, mura and relish mRNA from S2 cells transfected with dsRNA against GFP and the respective genes.

### **Fig. S2. Knockdown efficiency of the Gal4-UAS system used in adult flies**

Quantitative RT-PCR of akirin, hyd and relish mRNA from the adult fly lines in which the Gal4-UAS system was used to knockdown the respective genes (two lines for hyd).

### **Fig. S3. Interaction between IAP2 and Akirin**

Co-immunoprecipitation assay between over-expressed IAP2 and Akirin in S2 cells. The cells were transiently transfected with pAC-Akirin-V5 and/or pMT-IAP2-HA. Cell lysates were immunoprecipitated with anti-V5 coupled agarose beads. Immunoprecipitates were analyzed by Western blotting with anti-HA or anti-V5 antibodies. Data are representative of 2 independent experiments.

### **Fig. S4. Knockdown efficiency of the small interfering RNA used in HeLa cells**

Quantitative RT-PCR of hAkirin2, NFkB1 and Ubr5 mRNA from HeLa cells transfected with scrambled siRNA or targeting the respective genes.

Data information: Data for Fig. S1-2 and 6 are represented as mean  $\pm$  standard deviation of three independent experiments. Statistical significance was established by comparing values from stimulated with unstimulated conditions and genes knockdown with GFP dsRNA or scrambled siRNA control. \*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

**Table S1. Oligonucleotides used to generate double strand RNA in *Drosophila* S2 cells**

Are indicated: gene reference, dsRNA reference (<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.

| Gene          | dsRNA reference | Forward              | Reverse               |
|---------------|-----------------|----------------------|-----------------------|
| <i>Relish</i> | DRSC37194       | TGCCATGTGGAGTGCATTAT | TGCCATGTGGAGTGCATTAT  |
| <i>Akirin</i> | DRSC26196       | ATCTTCCATCTGCAGCATCC | ACGGACTAGGTTTCGGTGCTA |
| <i>Hyd</i>    | DRSC28294       | GCGACCGAATAAGTCCAGAG | GCCACACGACCAGAGGTTAT  |
| <i>bon</i>    | DRSC38123       | AGCCAGAAGTCGAAGGTGAA | TTGCTCAGACTCAGCGAAGA  |
| <i>IAP2</i>   | DRSC38402       | AAATCCATGTGATCTGCGGT | CCAGTG TAGCCAATTGTCCC |
| <i>m-cup</i>  | DRSC28310       | GCGACCGAATAAGTCCAGAG | GCCACACGACCAGAGGTTAT  |
| <i>Mkrn1</i>  | BKN24610        | GATTGGTGTGTGCGTTTCAC | ATCGGCGAGATTATCATTGG  |
| <i>mura</i>   | DRSC26645       | ATCTGGGTTTTGAGTGACCG | ATGAGTGATCGGGACAGAGG  |

**Table S2. Oligonucleotides used to generate small interfering RNA in mammalian HeLa cells (Ambion)**

| Gene                    | UniGene ID | siRNA ID |
|-------------------------|------------|----------|
| <i>Negative Control</i> | -          | AM4611   |
| <i>NFkB1</i>            | Hs.618430  | s9504    |
| <i>Ak2</i>              | Hs.485915  | s30221   |
| <i>UBR5</i>             | Hs.492445  | s224201  |

**Table S3. Oligonucleotides used for quantitative real-time PCR**

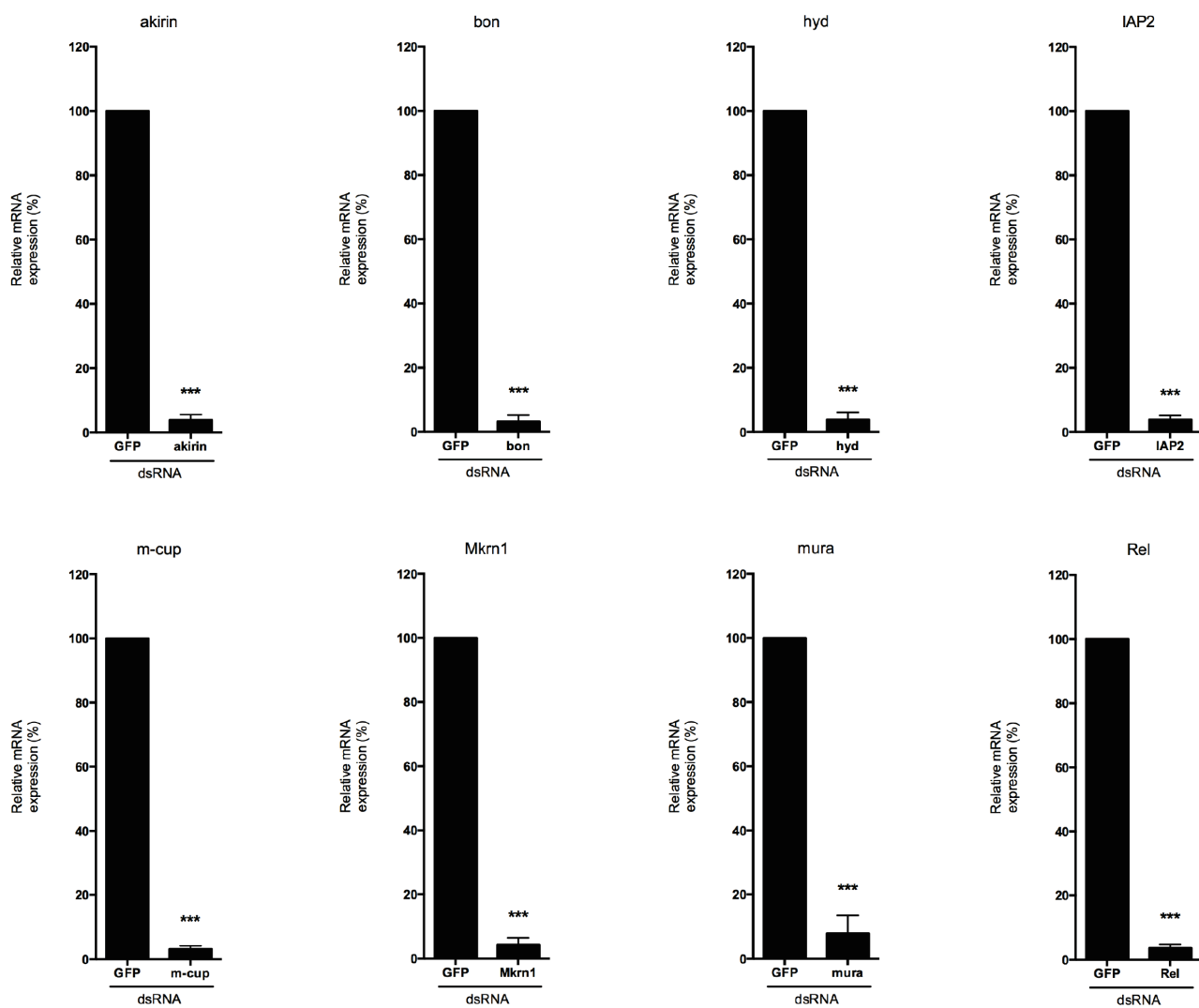
For *Drosophila* S2 cells and adult flies

| Gene             | Forward                   | Reverse                  |
|------------------|---------------------------|--------------------------|
| <i>Attacin-A</i> | GGCCCATGCCAATTTATTCA      | AGCAAAGACCTTGGCATCCA     |
| <i>Attacin-D</i> | TTTATGGAGCGGTCAACGCCAATG  | TGCAAATTGAGTCCTCCGCCAAAC |
| <i>rp49</i>      | GACGCTTCAAGGGACAGTATCTG   | AAACGCGGTTCTGCATGAG      |
| <i>Relish</i>    | GGTGATAGTGCCTGCATGT       | CCATACCCAGCAAAGGTCGT     |
| <i>Akirin</i>    | CCGAACCTAGTCCGTTTCAGTG    | CTTGTGCAGTCTCTTGATCTCAT  |
| <i>HyD</i>       | GAGGTGGTTCTACAGGGCAAG     | ATAAGGTCTTCGGGCACGTAA    |
| <i>Bon</i>       | AAAGGTTCGGAGTCAAACCTCTTCG | AAGGCATTCTAACAGCTTGGG    |
| <i>IAP2</i>      | CTCTTGTCCTCGATCTCTTGTTG   | GGTAGTAGAAACCTGCCTTTGC   |
| <i>m-cup</i>     | ACAAAGCTCAGTCACGACCTG     | GACGAGAATCGCGGGGTAG      |
| <i>Mkrnl</i>     | AGACCATCTGCCGCTACTAC      | TGCTGCTTGTACTAGGCTTCG    |
| <i>mura</i>      | ACTTGAACAACCCGTCCTCAT     | GTTCGGAGTTTCCAAAGTGGTTA  |

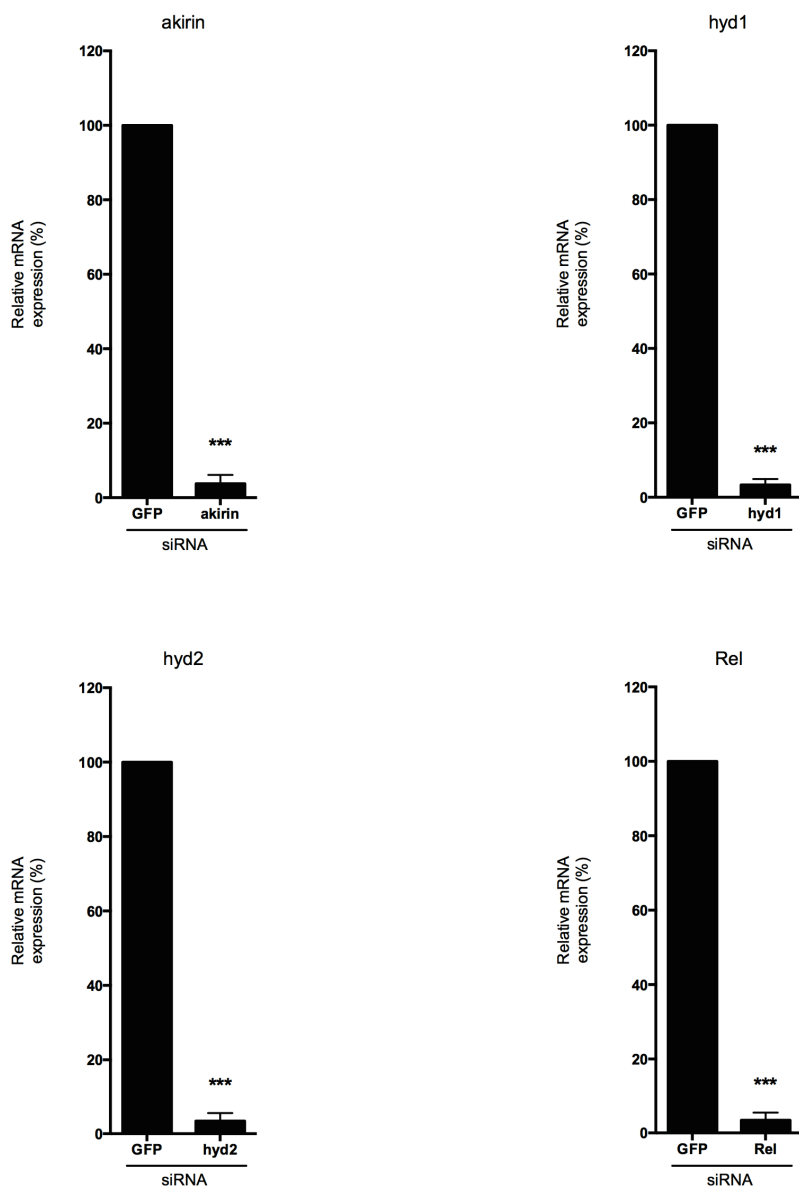
For mammalian HeLa cells

| Gene         | Reference (PrimePCR™ SYBR® Green Assay BioRad) |
|--------------|--|
| <i>IL-6</i>  | qHsaCID0020314                                 |
| <i>IL-8</i>  | qHsaCED0046633                                 |
| <i>GAPDH</i> | qHsaCED0038674                                 |
| <i>NFkB1</i> | qHsaCED0002379                                 |
| <i>Ak2</i>   | qHsaCID0011447                                 |
| <i>Ubr5</i>  | qHsaCID0014740                                 |

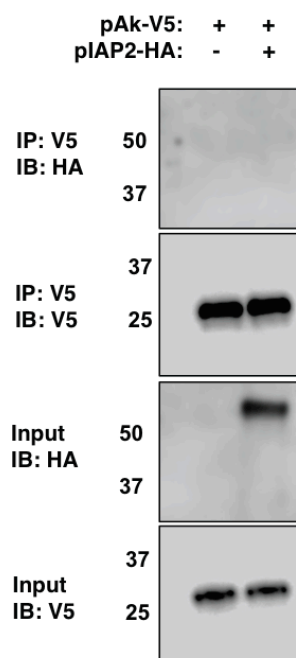
**Figure S1**



**Figure S2**

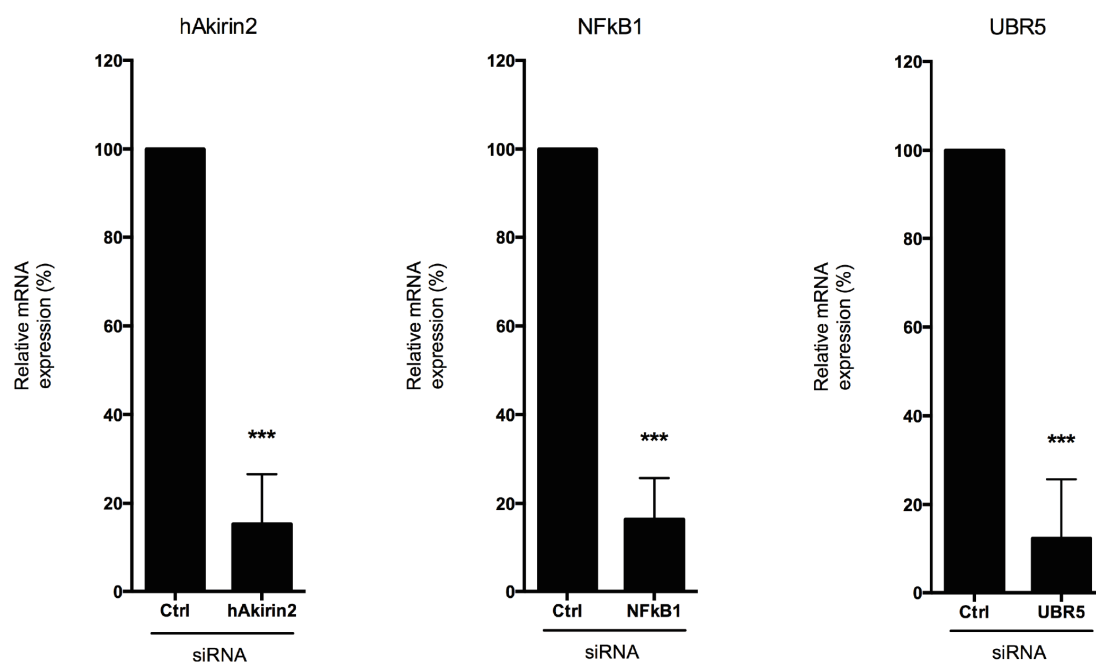


**Figure S3**





**Figure S4**



b) Akirin is a molecular timer for NF- $\kappa$ B target genes expression

To explore further the association of Akirin with other proteins during the innate immune response, we undertook a mass-spectrometry-based proteomic in *Drosophila* S2 cells. This led to the identification of members of other chromatin remodeling families potentially involved in the modulation of the NF- $\kappa$ B pathway. In order to confirm those results, we undertook a two-steps mini-screen (knockdown *ex-vivo* and *in-vivo*). We also performed Chromatin immunoprecipitation experiments at different time points to establish the dynamic recruitment of the different complexes on Akirin-dependent genes at their promoter sites. This differential recruitment affects the kinetic of transcription of Akirin-dependent versus independent NF- $\kappa$ B genes. This work will bring new insights about the mechanism that governs NF- $\kappa$ B transcriptional selectivity.

This work is written in article form but will require several months of work to complete the experiments and controls.

## **Akirin2 is a molecular timer that controls the sequential activation of innate immune genes**

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### **Introduction**

Metazoans have established an immune system to optimally survive, notably against pathogenic invading microorganisms. There are two main types of defense systems: innate and adaptive. The innate immune system is at the forefront of the defense response, being able to activate in a controlled manner the expression of anti-microbial peptides (AMPs), the inflammation process and the adaptive immune system (Medzhitov, 2008; Nathan, 2002; Sen and Smale, 2010). When abnormally regulated, innate immune responses contribute to the development of pathologies including autoimmune diseases, chronic inflammation and cancer (Karin et al., 2006; Maeda and Omata, 2008).

In *Drosophila*, the defense against microbes is ensured mainly by the massive production of AMPs (Ferrandon et al., 2007). Their expression is under the control of three transcription factors belonging to the NF- $\kappa$ B family: Dorsal and Dorsal-related Immunity Factor (DIF), acting downstream of the Toll pathway and Relish, downstream of the immune deficiency (IMD) pathway. They are the homologues of mammalian RelA, RelB and NF- $\kappa$ B1 transcription factors respectively. The *Drosophila* Toll pathway shares significant similarities with the signaling cascade downstream of the mammalian Interleukin-1 Receptor (IL-1R) and

the Toll-like receptors (TLRs), highlighting a common ancestry of these immune mechanisms. The IMD pathway is similar to the Tumor Necrosis Factor (TNF) Receptor (TNFR) pathway in vertebrates (Hoffmann and Reichhart, 2002). In both *Drosophila* and mammals, inflammatory stimuli induce gene expression programs that are almost entirely NF- $\kappa$ B dependent (Ghosh and Hayden, 2012). The similarity between the immune pathways in flies and mammals makes *Drosophila melanogaster* an excellent model to study the innate response. The fruit fly is already proven to be a powerful tool to decipher a variety of human diseases (Bilen and Bonini, 2005; Vidal and Cagan, 2006).

A genome-wide RNA-interference screen in *Drosophila melanogaster* cell culture (S2 cells) performed in our laboratory and aiming at finding new modulators of the IMD pathway, led to the discovery of a NF- $\kappa$ B modulator called Akirin. We showed that this protein, which has a strict nuclear localization, acts at the level of the NF- $\kappa$ B factor Relish. Akirin is highly conserved and the two mouse genes (*akirin-1* and *akirin-2*) encoding Akirin proteins, have been identified and knocked-out. Inactivation of *akirin-1* does not show any obvious phenotype, whereas *akirin-2* knockout results in early embryonic lethality. Akirin2 acts downstream of the TLR, TNFR and IL-1R signaling pathways. Most interestingly, Akirin2 is required in mammals for the regulation of only a subset of lipopolysaccharide (LPS) and IL-1 inducible genes with mainly pro-inflammatory activity. In contrast, Akirin does not significantly target genes involved in the resolution of the inflammatory process (Goto et al., 2007). Similarly, in *Drosophila*, Akirin is required upon immune challenge for the transcription of a subset composed of mostly immune effector genes, including Attacin-A, but dispensable for the transcription of most genes that are negative regulators of the innate immune response. Therefore, Akirin acts as molecular selector specifying the choice between subsets of NF- $\kappa$ B target genes in both flies and mammals (Bonnay et al., 2014; Goto et al., 2007; Tartey et al., 2014).

A large-scale unbiased proteomic analysis revealed that Akirin orchestrates NF- $\kappa$ B transcriptional selectivity through the recruitment of the Switching / Sucrose-fermentable (SWI/SNF) chromatin-remodeling complex. Removing Akirin or the SWI/SNF complex OSA-associated Brahma complex (BAP) leads to an impaired expression of several AMP coding genes, resulting in a weakened innate immune defense and an impaired survival of *Drosophila* against Gram-negative bacteria. This observation suggests that the full cocktail of IMD-induced AMPs is required to efficiently contend Gram-negative bacterial infections (Bonney et al., 2014). Similarly, mammalian Akirin2 is directly recruited to its target gene promoters and was found to control chromatin remodeling by recruiting Brahma-associated factors 60 (BAF60) proteins, components of the SWI/SNF remodeling family. Mice lacking Akirin2 in macrophages show impaired cytokine production in response to *Listeria* infection and clearance of infecting bacteria *in-vivo*. Overall, it suggests that NF- $\kappa$ B and Akirins have evolved in metazoan species to selectively control the transcription of NF- $\kappa$ B target genes through SWI/SNF chromatin remodeler recruitment.

In humans, NF- $\kappa$ B genes are already divided into two subsets, this time depending on their timing of expression (Carpenter and Fitzgerald, 2015). The first wave of genes is composed by the most rapidly expressed, the primary response genes (PRGs), induced directly after immune stimulation. PRGs have open chromatin and are induced in the absence of new protein synthesis. The second wave is constituted by a group of intermediately expressed genes known as the late primary response genes (LPRGs), induced later in the absence of new protein synthesis. Unlike classical PRGs, they do require some degree of chromatin remodeling for their activation. The second wave is also constituted by genes called secondary response genes (SRGs), which require *de novo* protein synthesis and comprehensive chromatin remodeling for the activation of transcription. The recruitment of the complex Brahma (BRM) from the chromatin remodeling family SWI/SNF is needed for

the expression of the genes from the second wave (LPRGs and SRGs). Furthermore, a strong and consistent antagonism in the recruitment between BRM and Mi-2 - from the family Chromodomain-helicase DNA binding (CHD) - complexes was observed at the promoter sites of those genes. This result showed the involvement of Mi-2 in the regulation of pro-inflammatory genes (Fowler et al., 2011; Ramirez-Carrozzi et al., 2006, 2009; Saccani et al., 2001; Sen and Smale, 2010).

Here we propose to use *Drosophila* as a model to unravel the complex mechanisms underlying this NF- $\kappa$ B transcriptional response selectivity. Our results show that in *Drosophila*, Akirin is responsible for the recruitment of three remodeling complexes in total, two of them needed for the activation (BAP from SWI/SNF family and Nucleosome remodelling factor (NURF) from Immitation switch (ISWI) family) and one for the regulation of this subset of NF- $\kappa$ B genes (Nucleosome remodelling and deacetylase (NuRD) from CHD family). We could also observe that the complexes are recruited at a different timing. Because the complexes proved to be conserved, we performed a bio-informatic analysis on previously published data in humans (Tartey et al., 2014; Tong et al., 2016). This allowed us to infer that the Akirin-based selectivity and the temporal-selectivity established in humans are the same process. Akirin's role as a "molecular timer" would thus be to control the expression of the second wave of inflammatory genes through the timely recruitment of chromatin remodeling complexes.

## Results

### Involvement of several remodeling complexes in NF- $\kappa$ B genes expression selectivity

From yeast to human, chromatin remodelers are divided into 4 families (SWI/SNF, ISWI, CHD and Inositol auxotroph 80 (INO80)), each of them including several functional complexes (Clapier and Cairns, 2009). Chromatin remodelers use the energy of ATP hydrolysis to regulate nucleosome dynamics (reposition, eviction) and hence gate access to the underlying DNA for transcription. We previously showed that the BAP complex from the SWI/SNF family acts together with Akirin for selective gene transcription upon immune challenge, both in *Drosophila* and humans (Bonnay et al., 2014; Tartey et al., 2014). To explore further the association of Akirin with others proteins during the innate immune response, we undertook a mass-spectrometry-based proteomic assay in *Drosophila* S2 cells. This led to the identification of 169 candidates genes potentially involved in the modulation of the NF- $\kappa$ B pathway in *Drosophila*. Among these candidate genes, our analysis identified ISWI and CHD, but not INO80 as chromatin-remodeling complexes also potentially involved in the Akirin-dependent regulation of NF- $\kappa$ B factor Relish target genes (figure to be added).

Based on these preliminary data, we performed a two-steps mini-screen in *Drosophila* (*ex-vivo* and *in-vivo*). First, we knockdown every remodeling complexes in *Drosophila* (based on Clapier and Cairns, 2009) in S2 cells using dsRNA (Fig 1.A and S1A-2-3A). We could see that the NURF complex (ISWI family) was involved in the activation and the NuRD complex (CHD family) in the regulation of Akirin-dependent genes. We evaluated if the remodeling complexes are involved *in-vivo* in the modulation of Akirin-dependent NF- $\kappa$ B target genes transcription. We drove UAS-RNAi targeting the remodeling complexes in adult fat-bodies by using the C564-Gal4 transgene (Hrdlicka et al., 2002; McGuire et al., 2004) and monitored the survival of the flies upon immune challenge with the Gram-negative bacteria *Escherichia coli*, killing only IMD pathway deficient flies (Bonnay et al., 2014). We first impaired the

BAP complex, as a proof of concept from (Bonnay et al., 2014), and *Drosophila* succumbed then to the infection (Fig S1.B). After this validation step, we impaired the complexes ISWI and INO80 and we could see that ISWI knockdown gave the same phenotype than for the BAP complex (Fig 1.B and S3.B). Similarly, the NuRD complex will be disrupted by using UAS-RNAi fly lines targeting dMi-2 (figure to be added). We will evaluate also the defect in NF- $\kappa$ B target genes expression *in-vivo* by RT-qPCR through the measure of induction of Akirin-dependent (Attacin-A) versus Akirin-independent (Attacin-D) immune induced genes (figure to be added). These results show that three chromatin remodeling complexes (Fig 1.C) are involved for the activation and regulation of the Akirin-dependent genes, controlling innate immune genes expression.

### **Remodeling dynamic controls NF- $\kappa$ B genes expression selectivity**

In order to ascertain the link between the chromatin remodeler complexes and Akirin, we performed a co-immunoprecipitation assay. *Drosophila* S2 cells were transfected with a tagged version of Akirin (Akirin-V5) and with components of complexes from the four chromatin remodeling families (Bap60-HA, Nurf38-HA, Mi2-HA and Reptin-HA). Protein extracts were immunoprecipitated with an anti-HA antibody. Western-blot experiments with antibodies targeting V5-tag suggested that after immune stimulation, Akirin is able to bind the three remodeling complexes BAP, NURF and NuRD (Fig 2.A). This result is according to a previous publication of our team, showing that Akirin physically bridges the NF- $\kappa$ B factor Relish and the BAP complex (Bonnay et al., 2014), and to the previous results from the mini-screen.

Using *Drosophila* S2 cells, we could observe that upon immune challenge, the BAP complex is recruited to the promoters' sites of the Akirin-dependent genes by Chromatin immunoprecipitation (ChIP) (Bonnay et al., 2014). Aiming at deciphering the chromatin



remodelers' ballet leading to the normal expression of Akirin-dependent immune-induced genes, we evaluated the recruitment of BAP, NURF, NuRD and INO80 at the Akirin-dependent promoter site of a NF- $\kappa$ B target gene (Attacin-A) upon immune challenge over time (Fig 2.B). We could observe that the three remodeling complexes identified above are recruited at a different timing: the BAP and the NURF complexes after 2 hours and the NuRD complex more after 4 hours of stimulation. As controls, we evaluated the presence of Relish and the chromatin remodelers (BAP, NURF, NuRD, INO80) away from the promoter of an Akirin-immune induced gene (Attacin-A) and at the promoter site of an Akirin-independent immune induced gene (Attacin-D) (Fig 2.B).

### **Conservation of the NF- $\kappa$ B genes expression control**

The Akirin-dependent molecular mechanism underlying the selective activation of NF- $\kappa$ B target genes is well conserved from *Drosophila* to humans (Goto et al., 2007; Tartey et al., 2014). Therefore, we addressed the potential requirement of the chromatin remodelers in NF- $\kappa$ B selective transcriptional response mediated by hAkirin2 during the human inflammatory response. We depleted HeLa cells by small interfering RNA (siRNA) for either hAkirin2 (ortholog of Akirin), SWI/SNF related matrix associated actine dependent regulator of chromatin D2 (SmarcD2, ortholog of Bap60, BAP complex), SmarcA1 (ortholog of Iswi, NURF complex), CHD3 (ortholog of Mi2, NuRD complex) or RuvB like AAA ATPase 2 (Ruvbl2, ortholog of Reptin, INO80 complex), using scrambled siRNA as controls (Fig 3.A). We monitored, upon stimulation by IL1 $\beta$ , the expression levels of NF- $\kappa$ B target genes that are dependent of hAkirin2 (such as IL6 and IL12 $\beta$ ) or independent (such as IL8 and TNF) (Tartey et al., 2014). As expected, the activation of IL6 and IL12 $\beta$  is impaired in HeLa cells depleted for hAkirin2. The activation is similarly affected in cells depleted for SmarcD2 and SmarcA1. However in cells depleted for CHD3, the expression of Akirin-dependent genes rised (Fig

3.A). This result suggests a conserved function for the remodeling complexes BAP and NURF as involved in the activation and NuRD in the regulation of NF- $\kappa$ B target genes mediated by hAkirin2 from *Drosophila* to humans.

The Stephen Smale laboratory showed in humans that the chromatin remodeling complexes BRM and Mi-2 were involved in an antagonist manner in the regulation of secondary response genes (Ramirez-Carrozzi et al., 2006), the first one involved in the activation and the latter in the regulation of those genes. BRM and Mi-2 being the orthologs of *Drosophila* complexes BAP and NuRD, this led to the hypothesis that the Akirin-based selectivity might be linked with the temporal selectivity known in humans. In order to assert that on a global scale, we took the micro-array data published in Tartey et al., 2014, that determines which genes are hAkirin2-dependent in humans, and we compared it to a RNA-seq published in Tong et al., 2016, that determines which genes are part of the first or the second wave of NF- $\kappa$ B genes. A first version of this bio-informatic analysis showed us that 91% of genes that are hAkirin2-independent correspond to PRGs, and that 87% of genes that are hAkirin2-dependent correspond to SRGs (Fig 3.B). This preliminary analysis showed a clear link between both types of selectivities. We checked in HeLa cells the expression timing of some Akirin-dependent genes (IL6 and IL12 $\beta$ ) compared to -independent (IL8 and TNF) and confirmed the pattern showed by the analysis: Akirin-dependent genes are expressed at a later time (Fig 3.C).

### **NF- $\kappa$ B selective transcription controlled by a molecular timer: Akirin**

Taken altogether, our results show that Akirin is a nuclear NF- $\kappa$ B co-factor involved in the selective timely controlled expression of innate immune genes (Fig 4). In *Drosophila*, upon immune challenge, Akirin will be responsible for the recruitment of three chromatin remodeling complexes at a differential timing. The recruitment of BAP and NURF is

necessary for the expression of mostly effector genes of the pathway and NuRD for their subsequent regulation. In humans, hAkirin2 and the conserved chromatin remodeling complexes play the same role. Additionally, hAkirin2-independent genes are expressed faster and constitute the primary response wave (PRGs), with hAkirin2-dependent genes corresponding to the secondary response wave of immune genes expressed (LPRGs and SRGs).

## **Discussion**

We showed that the evolutionary conserved protein Akirin plays a central role in immune gene expression in insects and mammals. The nuclear co-factor is involved in the selective transcription of a subset of NF- $\kappa$ B target genes in the innate immune response of *Drosophila* and mammals, through the differential recruitment of chromatin remodeling complexes. The absence of this process is critical enough to make flies susceptible to Gram-negative infections (Bonnay et al., 2014, the present work) and mice to *Listeria monocytogenes* infections (Tartey et al., 2014).

Saccani et al. (2001) found that NF- $\kappa$ B associates with its target genes in LPS-stimulated macrophages with variable kinetics, leading to the discovery of PRGs, LPRGs and SRGs. A subsequent study revealed variable requirements for BRM (SWI/SNF family) and Mi-2 (CHD family) nucleosome remodeling complexes at genes induced by LPS in mouse macrophages, in an antagonist manner (Ramirez-Carrozzi et al., 2006). The study showed that most PRGs are induced by LPS in a BRM-independent manner, with almost all SRGs exhibiting BRM dependence. BRM dependence was also observed at a subset of PRGs that were generally induced with delayed kinetics. The promoters of representative BRM-independent genes were found to be accessible to nuclease cleavage; in contrast, the promoters of BRM-dependent genes exhibited low nuclease accessibility prior to cell

stimulation, with increased accessibility following stimulation, suggestive of inducible nucleosome remodeling (Ramirez- Carrozzi et al., 2006). Mi-2 showed was equally necessary, but for an opposite effect, as a regulator of these genes. The results of Osamu Takeuchi's laboratory (Tartey et al., 2014) and our own results show that hAkirin2 is able to recruit the complexes BRM, NURF and Mi-2, the three being orthologs of the complexes identified in *Drosophila*. Those complexes act in a similar fashion than in (Ramirez-Carrozzi et al., 2006) and our bio-informatic analysis, combining (Tartey et al., 2014; Tong et al., 2016), allowed us to associate the akirin-dependent-genes with the secondary waves. This points to a synchronicity between the remodeling temporality of remodeling and the timely-controlled expression of pro-inflammatory genes.

Additionally to this recruitment of epigenetic machinery to explain the NF- $\kappa$ B genes differential expression, it is known that *Nf $\kappa$ ibz*, which encodes the nuclear I $\kappa$ B protein I $\kappa$ B $\zeta$ , is activated at the transcriptional level during the primary response to LPS and other inflammatory stimuli. I $\kappa$ B $\zeta$  is subsequently required for the activation of a select subset of SRGs (Yamamoto and Takeda, 2008; Yamamoto et al., 2004). Interestingly, mouse Akirin-2 appeared to participate to the innate immune response through its interaction with I $\kappa$ B $\zeta$  (Tartey et al., 2014). Indeed, the data suggested that I $\kappa$ B $\zeta$  is recruited to the LPS-inducible gene promoters together with Akirin2 and BRM complex, adding another proof to the crucial role of Akirin to the controlled expression of NF- $\kappa$ B target genes.

The mechanism behind this control lies in the differential recruitment of chromatin complexes to Akirin-dependent genes promoters. How are Akirin and the remodellers recruited specifically to Akirin dependent genes? The answer might lie on the dynamic of epigenetic marks. Chromatin remodeling processes are thought to be recruited through the recognition of post-translational modification of histones by specific protein-protein interaction domains such as bromodomains (Clapier and Cairns, 2009). Previous work of our

team in *Drosophila* (Bonnay et al., 2014) revealed that a Histone 3 Lysine 4 (H3K4) acetylation mark was specifically enriched on Akirin-dependent promoters in an Akirin and BAP complex dependent manner. This epigenetic signature is associated with transcriptionally active transcription start sites (TSS) in human and yeast (Guillemette et al., 2011). In humans, what is known is that at the PRGs promoters sites, chromatin structures consist of high histone acetylation and H3K4 trimethylation levels, with the reverse being observed at the SRGs promoters sites (Kayama et al., 2008). Additionally, it was suggested that I $\kappa$ B $\zeta$  may influence the regulation of histone modification through selective H3K4 trimethylation of TLR-induced promoters (Hildebrand et al., 2013). It would therefore be interesting to connect the temporality of the deposition/removal of methylation/acetylation groups on the H3K4 site, with the timing of the chromatin remodeling complexes recruitment depending on Akirin. This would help precise what constitutes the epigenetic code of inflammation regulation.

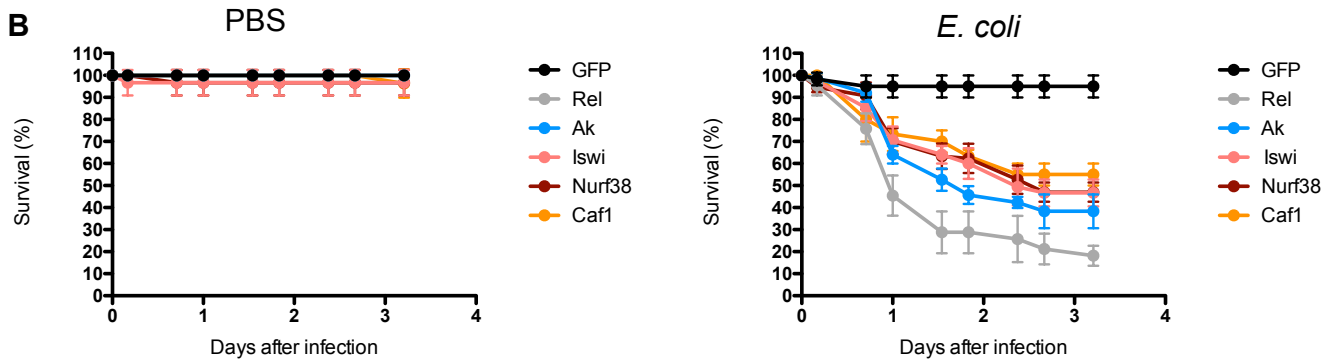
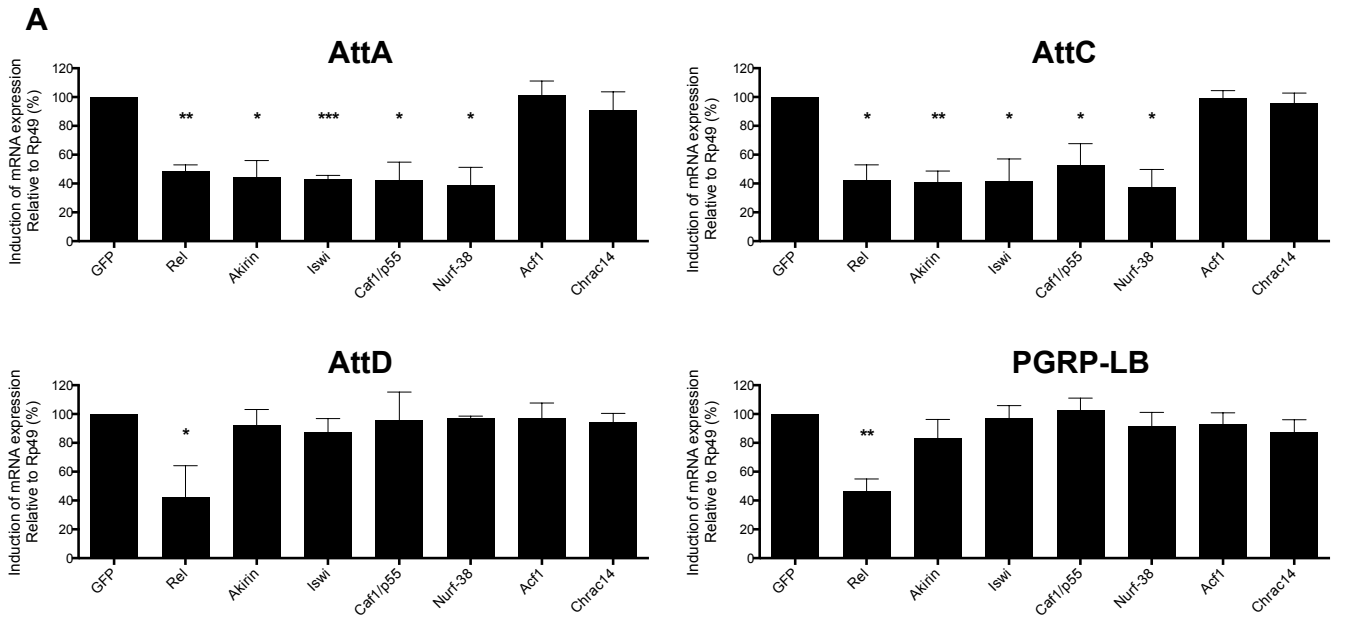
Our study links the concept of epigenetic regulation to the control of the activation of pro-inflammatory genes through the prism of the nuclear co-factor Akirin. However, since the subtleties of the NF- $\kappa$ B response are known to sometimes be cell-specific and stimulus-specific, the role of Akirin in other contexts must be studied. Nevertheless, there is a high functional conservation of Akirin and this dynamic regulatory mechanism. Akirin is highly conserved among metazoa including *Drosophila*, mice, teleosts, and humans (Macqueen and Johnston, 2009). To its detailed importance in *Drosophila*, mice and humans (Bonnay et al., 2014; Goto et al., 2007; Tartey et al., 2014) should be added the fact that Akirin is indispensable for the expression of defense genes in the epidermis of *Caenorhabditis elegans* following natural fungal infection, through the recruitment of the remodeling complex NuRD (Polanowska et al., 2018). Another study in the tick shows a NF- $\kappa$ B-dependent immune

function of Akirin against Gram-negative bacterial infections (Naranjo et al., 2013). These results argue for a conserved role of Akirin in the innate immune response of metazoan.

In the context of understanding how the innate immune genes activation and regulation operate (Tong et al., 2016), our work places the Akirin as a focus point to assess this dynamic. Indeed, Akirin is able to control the differential recruitment of the chromatin remodelers allowing the expression of the secondary response wave. Akirin should thus be considered as a molecular timer, linking epigenetic dynamics and innate immune/inflammation expression. This co-factor, or its partners, could be therefore considered as putative drug targets for future autoimmune diseases or cancer treatments.

This project being still ongoing, additional experiments will be added like a Chromatin accessibility assay to prove that remodeling is indeed occurring/required at the promoters sites of Akirin-dependent genes and big scale analysis in *Drosophila* like Chip-seq to confirm the hypothesis at a larger scale.

**Figure 1**

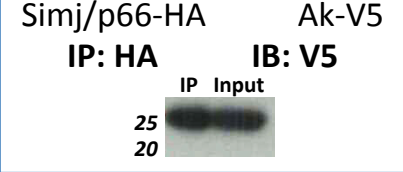
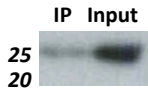
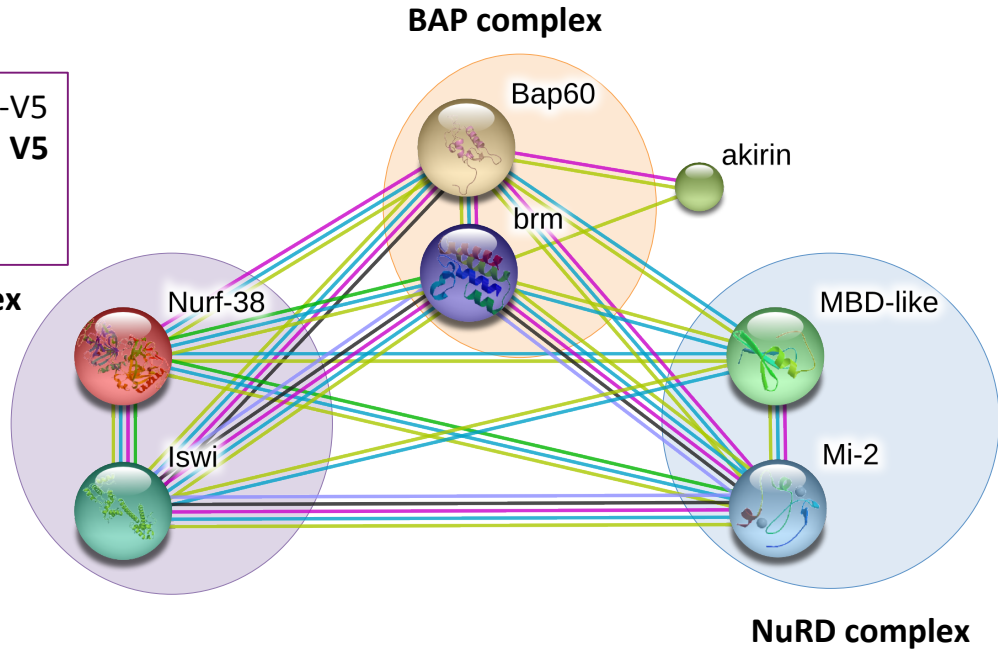
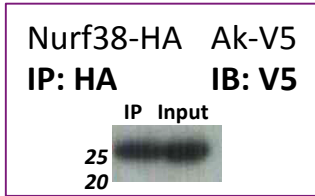


**C**

|                |               | involvement |
|----------------|---------------|-------------|
| SWI/SNF Family | BAP Complex   | ✓           |
|                | PBAP Complex  | ✗           |
| ISWI Family    | NURF Complex  | ✓           |
|                | CHRAC Complex | ✗           |
|                | ACF Complex   | ✗           |
| CHD Family     | CHD1 Complex  | ✗           |
|                | NuRD Complex  | ✓           |
| INO80 Family   | Pho Complex   | ✗           |
|                | Trp Complex   | ✗           |

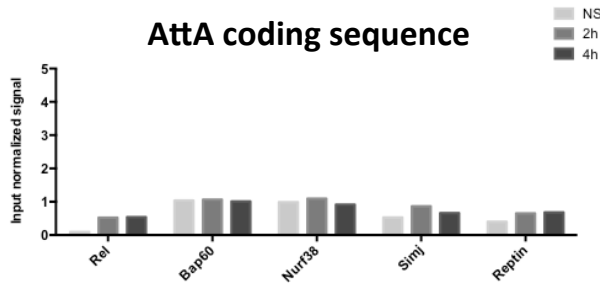
**Figure 2**

**A**

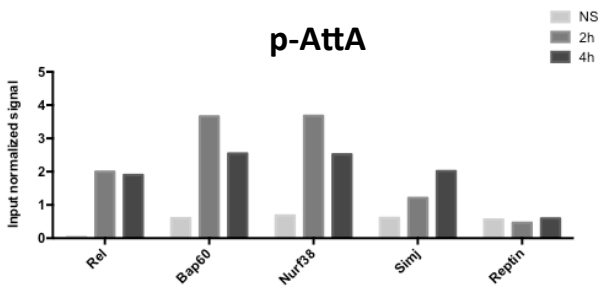


**B**

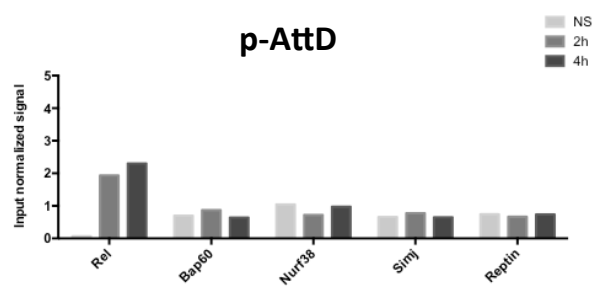
**Atta coding sequence**



**p-AtTA**



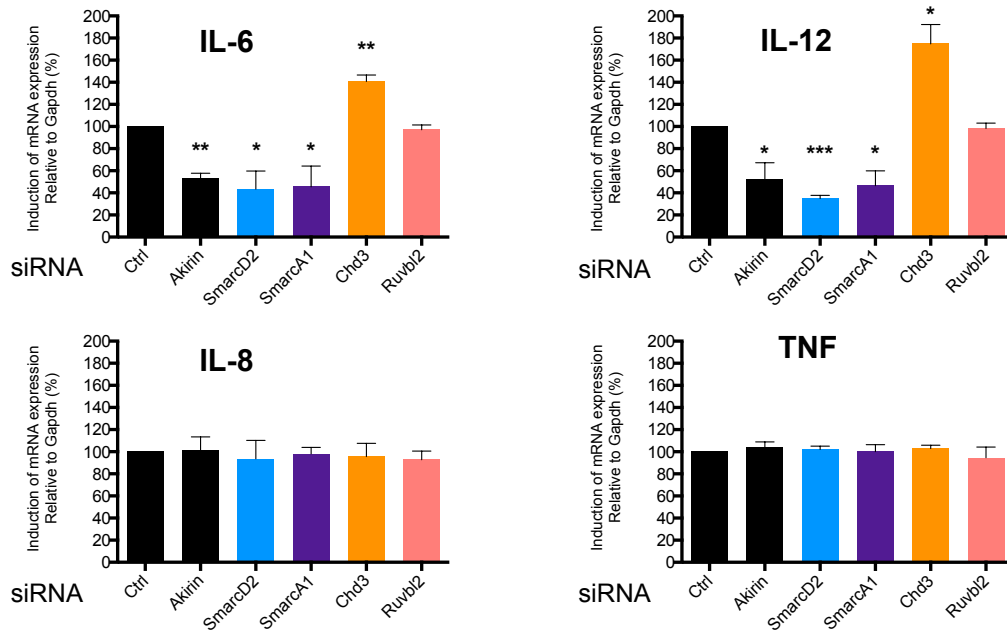
**p-AtTD**





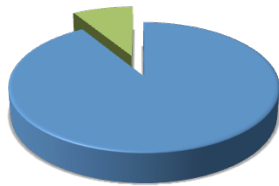
**Figure 3**

**A**



**B**

**hAk-2 independent genes**  
(Tartey 2014 micro-array)



■ Primary Response Genes (PRG)

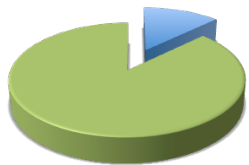
104 genes

■ Secondary Response Genes (SRG)

10 genes

Tong 2016 RNA-seq

**hAk-2 dependent genes**  
(Tartey 2014 micro-array)



■ Primary Response Genes (PRG)

6 genes

■ Secondary Response Genes (SRG)

41 genes

Tong 2016 RNA-seq

**C**

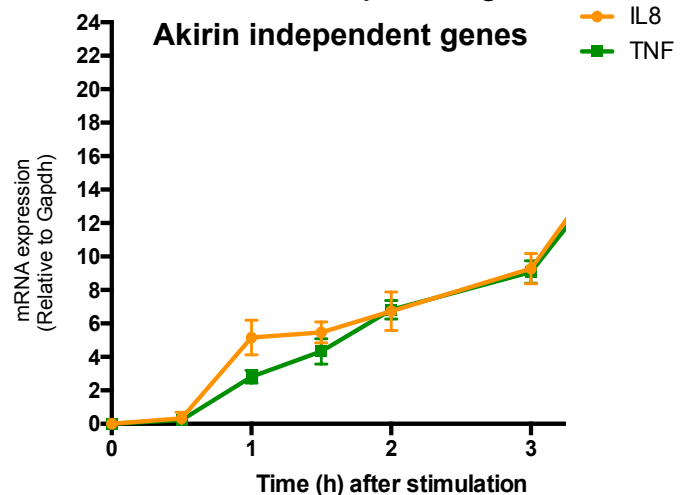
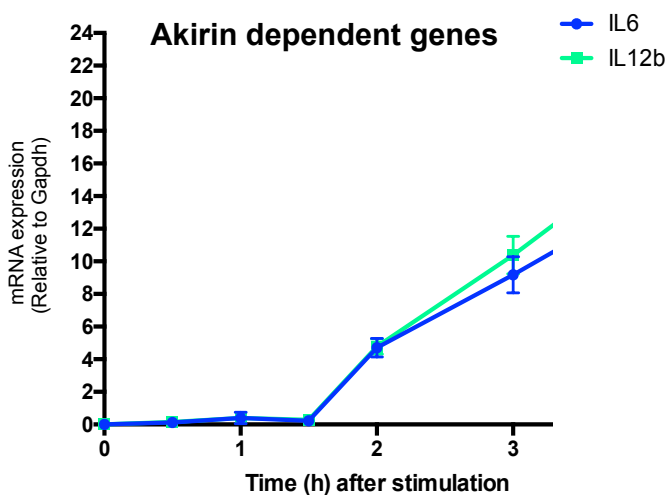
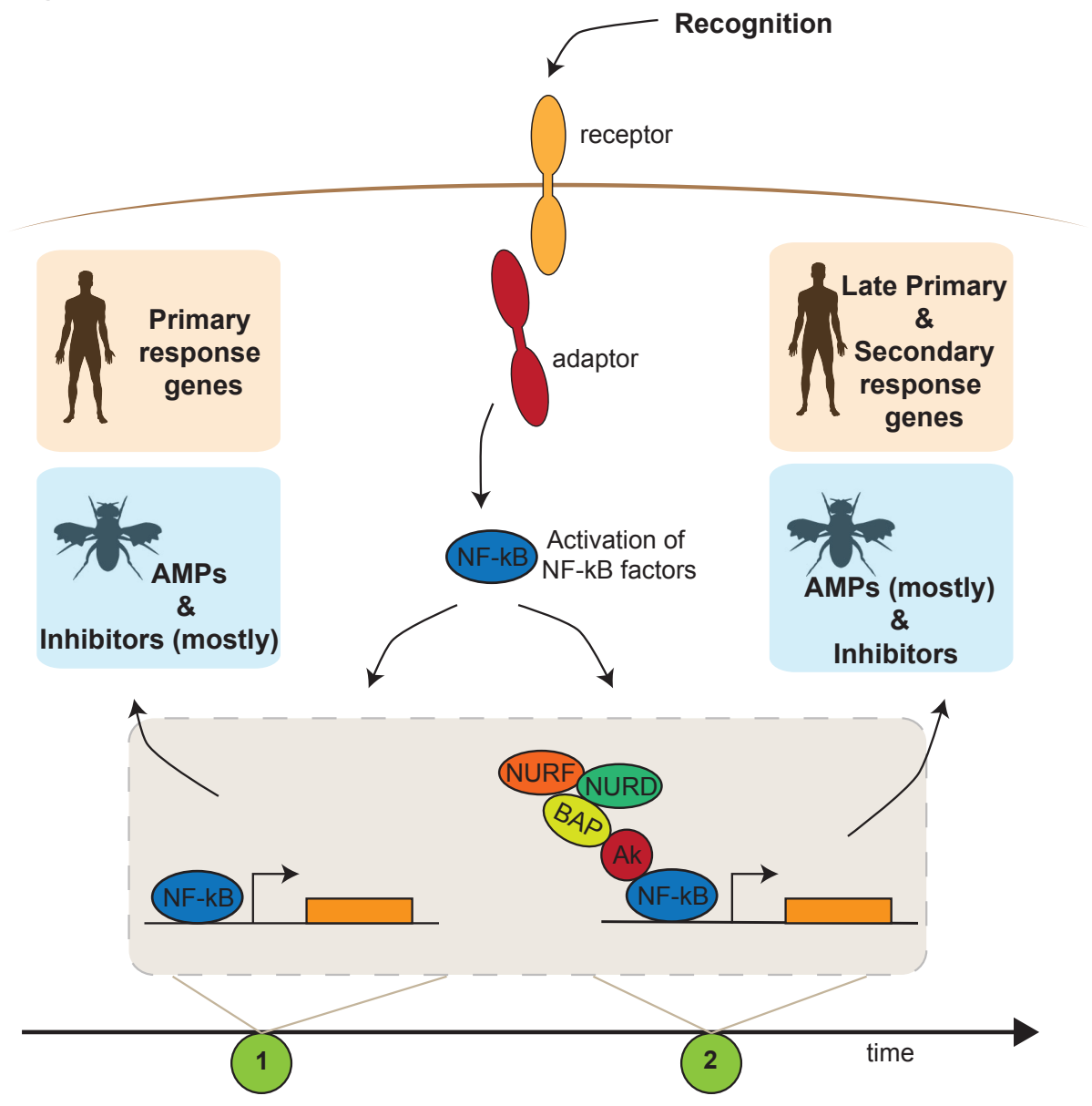


Figure 4



## Figures legends

### *Figure 1: Chromatin remodelers mini-screen - ISWI family*

A. Quantitative RT-PCR of *Attacin-A*, *Attacin-C*, *Attacin-D* and *PGRP-LB* mRNA from S2 cells transfected with dsRNA against GFP (negative control), relish, akirin (positive controls), Iswi, Caf1/p55, Nurf-38, Acf1 and Chrac14, following 4h of HKE stimulation.

Data are represented as mean  $\pm$  standard deviation of three independent experiments. Statistical significance was established by t-test comparing values from stimulated with unstimulated conditions and genes knockdown with GFP dsRNA control.

\*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

B. *In-vivo* survival experiments performed on batches of 20 nine-day-old females expressing dsRNA against GFP, Relish (Rel), Akirin (Ak), Iswi, Nurf38 and Caf1 in the fat body through the C564 promoter. Flies were infected by *E.coli* septic injury (with PBS pricking as control), at 25°C three independent times.

C. Table of the four chromatin remodeling families and their complexes. The BAP, NURF and NuRD complex were the only complexes involved in Akirin-dependent NF- $\kappa$ B transcriptional selectivity.

### *Figure 2: Akirin-dependent dynamic remodeling*

A. Scheme showing the different complexes involved with Akirin using the software String (Szklarczyk et al., 2017). The bonds between the proteins represent the links known in the literature. Also showing, the co-immunoprecipitation assays between over-expressed Akirin and Bap60 (BAP complex), Nurf38 (NURF complex), Simj/p66 (NuRD complex) or Reptin (INO80 family) in S2 cells. The cells were transiently transfected with pAC-Akirin-V5 and pMT-Bap60-HA, pMT-Nurf38-HA, pMT-Simj-

HA or pMT-Reptin-HA. Cell lysates were immunoprecipitated with anti-HA coupled to agarose beads. Immunoprecipitates were analyzed by Western blotting with anti-V5 antibodies. Data are representative of 1 experiment.

B. Chromatin-ImmunoPrecipitation experiment realized in S2 cells, following transient transfection of pMT-Rel-HA, pMT-Bap60-HA, pMT-Nurf38-HA, pMT-Simj-HA or pMT-Reptin-HA. Assay realized with anti-HA antibodies on sheared chromatin from S2 cells following heat-killed *E.coli* stimulation at different time points (non stimulated NS, 2h and 4h). The graphs show recruitment of Relish, Bap60, Nurf38, Simj and Reptin relative to the input on Akirin-dependent (p-Attacin-A), Akirin-independent (p-Attacin-D) genes proximal promoter, or on Attacin-A coding sequence as an internal control. Data are representative of 1 experiment.

*Figure 3: Role of Akirin as a molecular timer in humans*

A. Quantitative RT-PCR of *IL-6*, *IL-12 $\beta$* , *IL-8* and *TNF* mRNA from HeLa cells. They were transfected with scrambled siRNA (negative control) or siRNA targeting hAkirin2 (positive control), SmarCD2, SmarCA1, Chd3 and Ruvbl2. The cells were stimulated with recombinant human IL1 $\beta$  (10 ng/ml) for 4h.

Data are represented as mean  $\pm$  standard deviation of three independent experiments. Statistical significance was established by t-test comparing values from stimulated with unstimulated conditions and genes knockdown with scrambled siRNA control.

\*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

B. Bio-informatics analysis: comparison of the micro-array data published in (Tartey et al., 2014) and the RNA-seq published in (Tong et al., 2016). The pie charts show the proportion of similarity between the genes that are Akirin-independent/dependent and the ones that PRGs/SRGs.

C. Quantitative RT-PCR of *IL-6*, *IL12 $\beta$* , *IL-8* and *TNF* mRNA from HeLa cells. The cells were stimulated with recombinant human IL1 $\beta$  (10 ng/ml) at different time points (0, 30min, 1h, 1h30min, 2h, 3h).

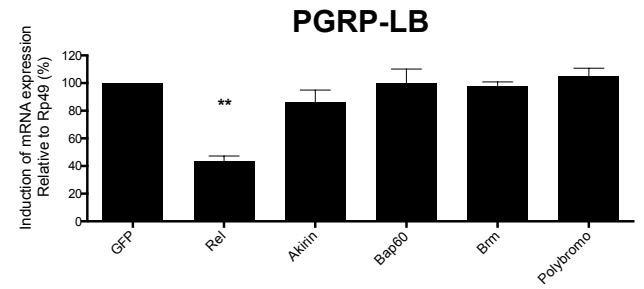
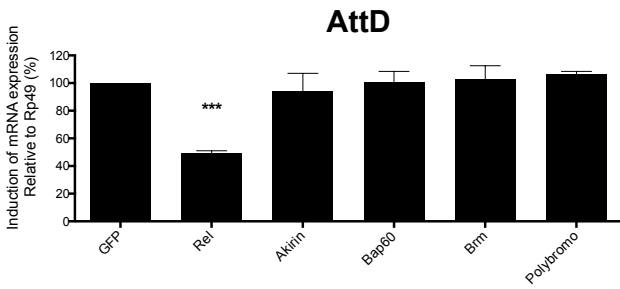
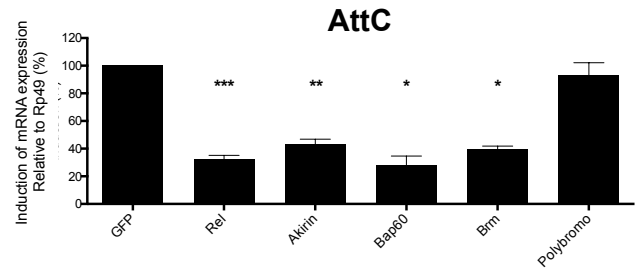
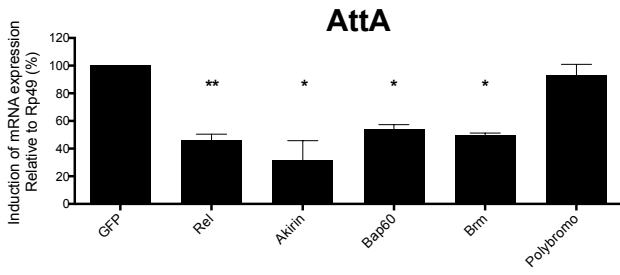
Data are represented as mean  $\pm$  standard deviation of three independent experiments.

*Figure 4: Model showing the role of Akirin in Drosophila and humans*

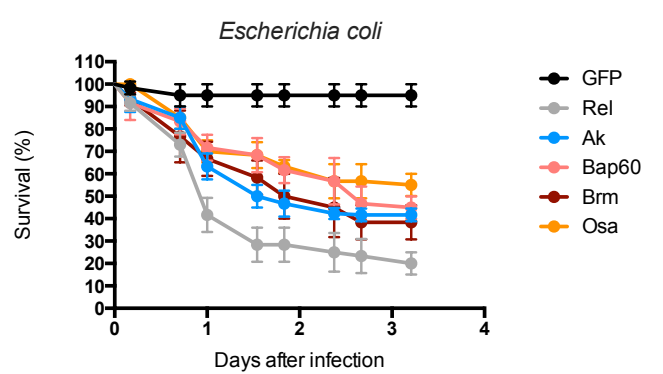
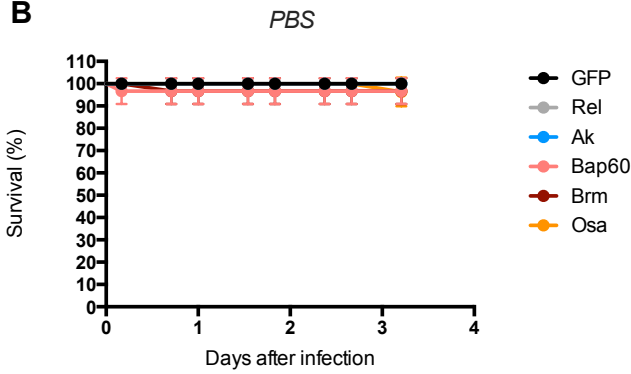
Upon immune challenge, the activation of the NF- $\kappa$ B pathway leads to the translocation of the nuclear factors. First, the subset of genes that do not need chromatin remodeling will get transcribed (1). Second, another subset will require the binding of Akirin at their promoter sites, leading to the differential recruitment of several chromatin remodelers (2). This mechanistic dichotomy controls the selective transcription of NF- $\kappa$ B target genes.

# Figure S1

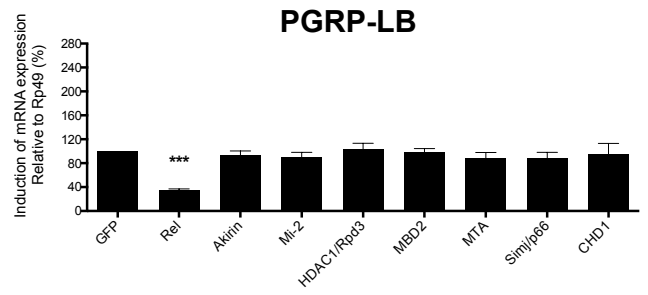
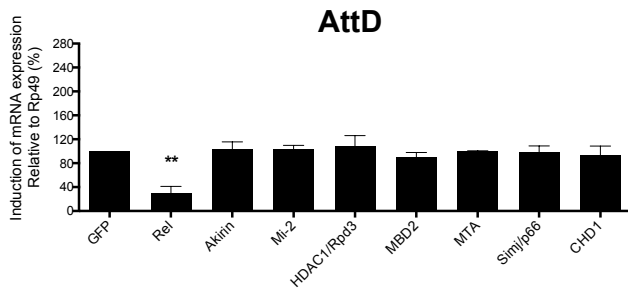
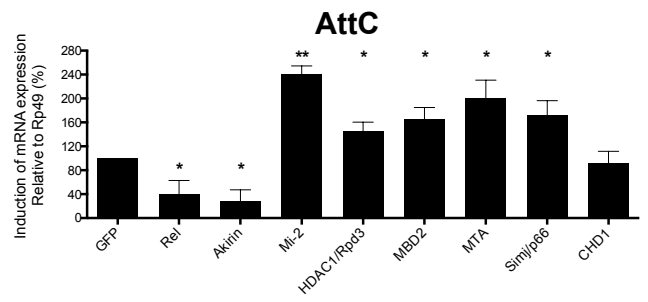
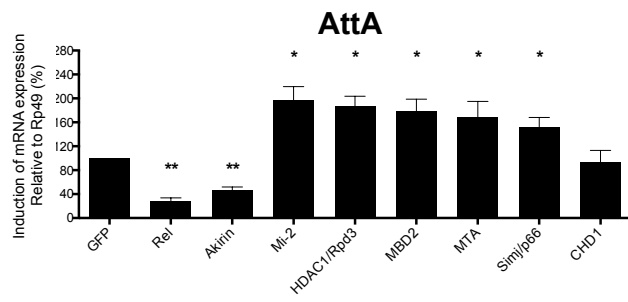
**A**



**B**

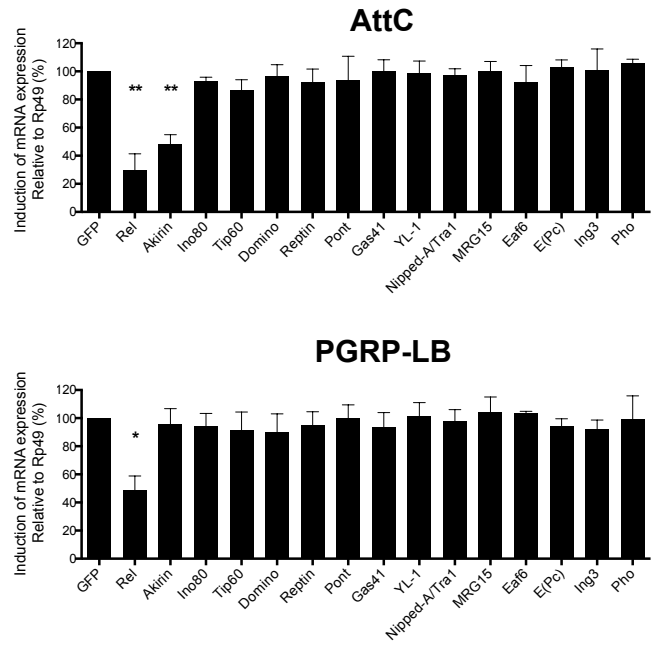
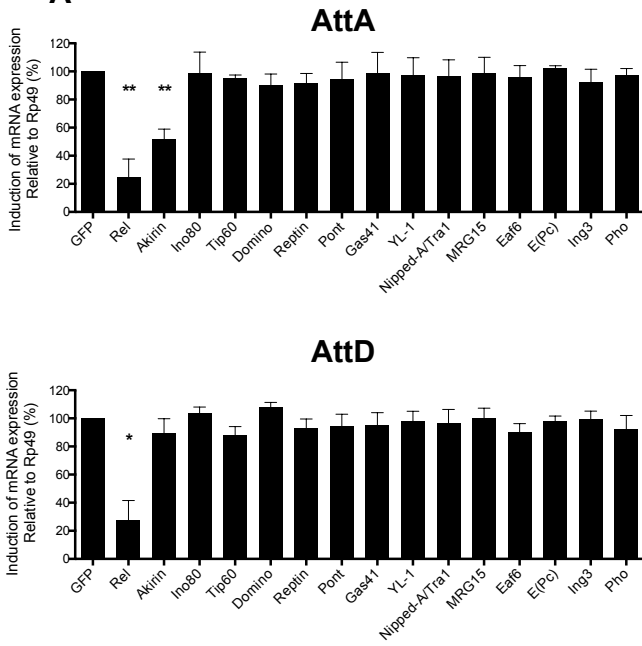


**Figure S2**

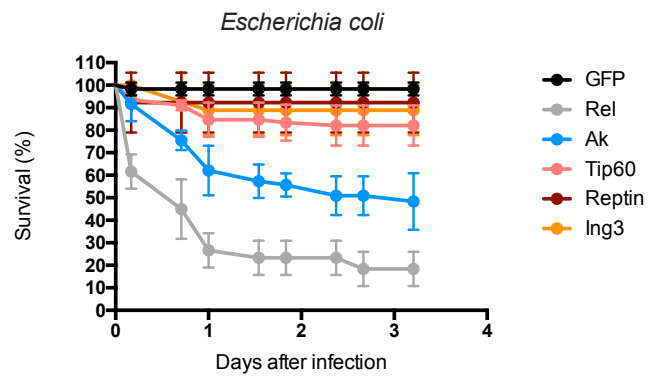
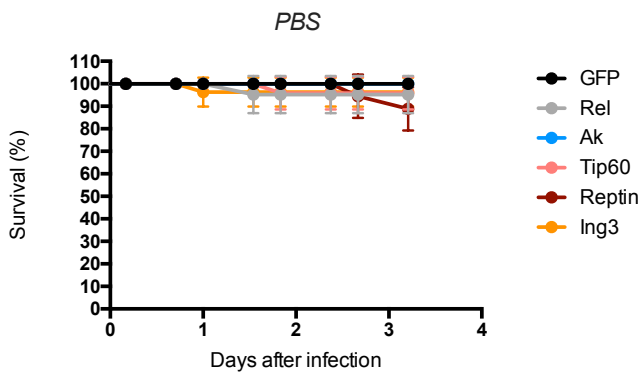


# Figure S3

**A**



**B**





## Supplementary data

### *Figure S1: Chromatin remodelers mini-screen - SWI/SNF family*

A. Quantitative RT-PCR of *Attacin-A*, *Attacin-C*, *Attacin-D* and *PGRP-LB* mRNA from S2 cells transfected with dsRNA against GFP (negative control), relish, akirin (positive controls), Bap60, Brm and Polybromo following 4 hours of HKE stimulation.

Data are represented as mean  $\pm$  standard deviation of three independent experiments. Statistical significance was established by t-test comparing values from stimulated with unstimulated conditions and genes knockdown with GFP dsRNA control.

\*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

B. *In-vivo* survival experiments were performed on batches of 20 nine-day-old females expressing dsRNA against GFP, Relish (Rel), Akirin (Ak), Bap60, Brm or Osa under the fat body specific promoter C564. Flies were infected by *E.coli* septic injury (with PBS pricking as control), at 25°C three independent times.

### *Figure S2: Chromatin remodelers mini-screen - CHD family*

Quantitative RT-PCR of *Attacin-A*, *Attacin-C*, *Attacin-D* and *PGRP-LB* mRNA from S2 cells transfected with dsRNA against GFP (negative control), relish, akirin (positive controls), Mi-2, HDAC1/Rpd3, MBD2, Simj/p66 and CHD1, following 4 hours of HKE stimulation.

Data are represented as mean  $\pm$  standard deviation of three independent experiments. Statistical significance was established by t-test comparing values from stimulated with unstimulated conditions and genes knockdown with GFP dsRNA control.

\*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

*Figure S3: Chromatin remodelers mini-screen - INO80 family*

A. Quantitative RT-PCR of *Attacin-A*, *Attacin-C*, *Attacin-D* and *PGRP-LB* mRNA from S2 cells transfected with dsRNA against GFP (negative control), relish, akirin (positive controls), Ino80, Tip60, Domino, Reptin, Pont, Gas41, YL-1, Nipped-A/Tra1, MRG15, Eaf6, E(Pc), Ing3 and Pho following 4 hours of HKE stimulation.

Data are represented as mean  $\pm$  standard deviation of three independent experiments. Statistical significance was established by t-test comparing values from stimulated with unstimulated conditions and genes knockdown with GFP dsRNA control.

\*P-value < 0.05; \*\*P-value < 0.01; \*\*\*P-value < 0.001.

B. *In-vivo* survival experiments were performed on batches of 20 nine-day-old females expressing dsRNA against GFP, Relish (Rel), Akirin (Ak), Tip60, Reptin or Ing3 under the fat body specific promoter C564. Flies were infected by *E.coli* septic injury (with PBS pricking as control), at 25°C three independent times.

## **Material and Methods**

*As the project is still ongoing and several experiments are missing, please note that this part is under progress.*

### Cell culture

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. HeLa cell line was cultured and maintained in DMEM containing 10% (vol/vol) FCS, 40 µg/mL gentamycin. Recombinant human IL1β was purchased from Invitrogen.

### RNA interference

The double-strand RNAs for the knockdown experiments in *Drosophila* cells were prepared according to (Bonnay et al., 2014). Fragments for the different genes were generated from genomic DNA templates using oligonucleotides designed for use with Genome-RNAi libraries (Schmidt et al., 2013). The small interfering RNAs used for the knockdown experiment in HeLa cells were purchased from Ambion.

### Plasmid Constructs

pAC-Akirin-V5 and pMT-Rel-HA constructs were described previously (Bonnay et al., 2014; Goto et al., 2007) and constructs pMT-Bap60-HA, pMT-Nurf38-HA, pMT-Simj-HA and pMT-Reptin-HA were purchased at the *Drosophila* Genomics Resource Center (DGRC).

### Cell transfection

*Drosophila* S2 cells were transfected with double-strand RNAs using the bathing method described in (Bonnay et al., 2014) or with plasmids using the Effectene transfection kit

(Qiagen). HeLa cells were transfected with siRNA using Lipofectamine RNAiMax (Invitrogen).

#### RNA extraction and quantification

For the *ex-vivo* experiments, RNA was extracted from cells and treated with DNase, using RNA Spin kit (Macherey Nagel). For the *in-vivo* experiments, the procedure was done accordingly to (Bonnay et al., 2014). Similarly, reverse-transcription and quantitative real-time PCR were performed as indicated in (Bonnay et al., 2014).

#### Co-immunoprecipitation

The experiments were realized according to (Bonnay et al., 2014). Immunoprecipitations were performed with anti-HA antibodies coupled to agarose beads (Sigma). Proteins were detected by Western blotting using anti-V5 (Invitrogen) antibody.

#### Fly strains

Stocks were raised on standard cornmeal-yeast-agar medium at 25°C with 60% humidity. To generate conditional knockdown in adult flies, we used the GAL4-GAL80<sup>ts</sup> system (McGuire et al., 2004). Fly lines carrying a UAS-RNAi transgene targeting relish (108469), akirin (109671), and other lines were obtained from the Vienna Drosophila RNAi Center (<http://stockcenter.vdrc.at/control/main>). Fly line carrying a UAS-RNAi transgene against GFP (397-05) was obtained from the Drosophila Genetic Resource Center (Kyoto, Japan; <http://www.dgrc.kit.ac.jp/index.html>). UAS-RNAi flies were crossed with C564-GAL4/CyO; Tub-GAL80ts flies at 18°C. Emerged adult flies were then transferred to 29°C to activate the UAS-GAL4 system for 6-7 days.

### Immune challenge

Cells were stimulated with heat-killed *E. coli* (40:1) (Reichhart et al., 2011). Microbial challenges were performed by pricking adult flies with a sharpened tungsten needle dipped into either PBS or concentrated *Escherichia coli* strain DH5aGFP bacteria solution (Reichhart et al., 2011). Bacteria were grown in Luria broth (LB) at 29°C.

### Statistical analysis

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

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## Régulation des voies NF- $\kappa$ B au cours de la réponse immunitaire innée

### Résumé

Le système immunitaire inné est un mécanisme de défense commun à tous les métazoaires. 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. La similarité entre les voies moléculaires - comme la voie NF- $\kappa$ B - relayant la réponse immunitaire innée chez les insectes et les mammifères fait de la drosophile un excellent modèle pour explorer la réponse immune.

Après une stimulation immunitaire, l'arrêt des voies moléculaires de l'immunité est nécessaire pour éviter le développement de maladies auto-immunes ou du cancer. Mon premier projet s'est attaché à comprendre un mode de régulation original dépendant du temps, dans une des voies NF- $\kappa$ B de la drosophile.

Mon deuxième projet concerne l'activation de la réponse immunitaire. Une protéine nucléaire contrôle l'implication de machinerie épigénétique dans le contrôle de l'expression d'une des voies NF- $\kappa$ B de la drosophile. Le tout permet de mieux saisir la dynamique de régulation de la réponse innée.

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

### Résumé en anglais

The innate immune system is a defense mechanism common to all metazoans. Its activation can be deleterious when it is uncontrolled. The study of the mechanisms underlying this balance between the activation or not of the innate immune response is the basis of my thesis work. The similarity of the molecular pathways - such as the NF- $\kappa$ B pathway - relaying the innate immune response in insects and mammals makes *Drosophila* an excellent model for exploring the immune response.

After immune stimulation, stopping the molecular pathways of immunity is necessary to prevent the development of autoimmune diseases or cancer. My first project focused on understanding a time-dependent mode of regulation in one of *Drosophila*'s NF- $\kappa$ B pathways.

My second project concerns the activation of the immune response. A nuclear protein controls the involvement of epigenetic machinery in controlling the expression of one of *Drosophila*'s NF- $\kappa$ B pathways. All this makes it possible to better grasp the dynamics of regulation of the innate response.

Key words: Innate immunity, NF- $\kappa$ B, Inflammation, *Drosophila*, Humans