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Protein phosphatase 4 negatively regulates the Immune Deficiency-NF-κB pathway during the *Drosophila* immune response

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Abstract

The evolutionarily conserved immune deficiency (IMD) signaling pathway shields *Drosophila* against bacterial infections. It regulates the expression of antimicrobial peptides encoding genes through the activation of the NF-κB transcription factor Relish. Tight regulation of the signaling cascade ensures a balanced immune response, which is otherwise highly harmful. Several phosphorylation events mediate intracellular progression of the IMD pathway. However, signal termination by dephosphorylation remains largely elusive. Here, we identify the highly conserved protein phosphatase 4 (PP4) complex as a *bona fide* negative regulator of the IMD pathway. RNA interference-mediated gene silencing of *PP4-19c*, *PP4R2*, and *Falafel*, which encode the catalytic and regulatory subunits of the phosphatase complex, respectively, caused a marked upregulation of bacterial-induced antimicrobial peptide gene expression in both *Drosophila melanogaster* S2 cells and adult flies. Deregulated IMD signaling is associated with reduced lifespan of *PP4*-deficient flies in the absence of any infection. In contrast, flies overexpressing this phosphatase are highly sensitive to bacterial infections. Altogether, our results highlight an evolutionarily conserved function of PP4c in the regulation of NF-κB signaling from *Drosophila* to mammals.

Introduction

Since the discovery of NF-κB transcription factors in 1986 (1), concerted research activities have provided considerable progress in elucidating the triggers and the components of their signaling cascades, as well as in characterizing their functions. A particular interest for the characterization of NF-κB signaling stems from their central role in the regulation of inflammation and innate immune reactions. Indeed, NF-κB factors control the expression of genes encoding effector and costimulatory molecules, as well as inflammatory cytokines that are essential for the onset of an efficient immune response against invading microorganisms.

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However, besides their beneficial effects in controlling the infections, exacerbated NF-κB signaling is highly detrimental. Accordingly, the intensity and duration of their signals are tightly controlled, and their deregulation is frequently associated with chronic inflammatory diseases, tissue damage, and autoimmune diseases, as well as the development and progression of tumors (2–6). In this context, the characterization of the regulatory processes that ensure the proper modulation of NF-κB signaling profiles attracts a particular interest.

NF-κB pathways are highly conserved, and the *Drosophila* model has provided a prominent insight into their role in the regulation of the innate immune response in metazoans (7, 8). Two NF-κB pathways, the Toll and the immune deficiency (IMD) pathways, play a crucial role in controlling the *Drosophila* immune response. More precisely, these pathways regulate the expression of antimicrobial peptide encoding genes, which constitute the principal effectors of the humoral response (9–12). Both pathways share considerable similarities with NF-κB cascades controlling innate immunity and inflammation in mammals. Notably, the Toll receptor is the founding member of the Toll-like receptors family in mammals, and its downstream signaling cascade is analogous to the Myeloid differentiation factor 88-dependent TLR signaling cascade (7, 13). The IMD pathway is akin to the TNF receptor signaling pathway and also resembles the TIR-domain-containing adapter inducing IFN-β-dependent TLR signaling in mammals (7, 14–16).

The IMD pathway is activated upon the sensing of diaminopimelic acid-type peptidoglycan by the peptidoglycan recognition proteins (PGRP)-LC and PGRP-LE on the cell membrane or in the cytosol, respectively (17–27). Ligand binding triggers receptor multimerization and proto-amyloid formation through the conversion of cryptic receptor-interacting protein (RIP) homotypic interaction motif (cRHIM). This receptor agglomeration in turn seeds fibrils formation of the adaptor protein IMD, whose sequence also carries cRHIM motifs (28, 29). Via its death domain, which is homologous to that of the mammalian Receptor Interacting Protein (RIP1), IMD further recruits a signaling complex, including the *Drosophila* Fas associated death domain adaptor (FADD) and the caspase-8 homologue, death related ced-3/Nedd2-like protein (DREDD) (30–33). On its ubiquitinylation by the E3 ligase *Drosophila* inhibitor of apoptosis 2 (IAP2), the latter cleaves IMD at its N terminus, thus exposing an evolutionarily conserved IAP binding motif (34–39). Consequently, with the concerted activity of the E2 conjugating enzymes Bendless (Ubc13), Uev1a, and Effete (Ubc5), IAP2 further targets IMD for K63-linked ubiquitin chains (39). These connect IMD to the TGF-β activated protein kinase 1 (TAK1), via its associated protein TAK1-binding protein 2 (TAB2), and to the IκB kinase (IKK) signalosome, which includes a regulatory subunit (Kenny) and a catalytic subunit (immune response deficient 5 (ird5)), both homologous to mammalian IKKγ and IKKβ, respectively (37, 40–44). The establishment of this ubiquitin-dependent signaling platform is presumed to activate TAK1, which, in turn, phosphorylates IKKβ, itself required for the phosphorylation of the *Drosophila* NF-κB transcription factor Relish on serine residues in its Rel homology domain (28, 45). Like its mammalian counterparts p100 and p105, Relish is also characterized by a C-terminal ankyrin-repeat IκB domain. This domain is cleaved by DREDD in an IKK-dependent fashion (45–47). Whereas the phosphorylation of Relish is not required for its cleavage and nuclear translocation, this modification is crucial for the optimal expression of Relish-dependent antimicrobial peptide-encoding genes such as *Attacin* (45, 47, 48).

Several signal terminators have been shown to negatively regulate the IMD pathway by acting at different levels and through different mechanisms (16, 49–51). These include catalytic PGRPs, which degrade peptidoglycan into small entities of low immunostimulatory potential. In addition, the nonamidase membrane-associated PGRP-LF receptor and the alternatively spliced regulatory isoforms of PGRP-LC (rPGRP-LC), which lack the intracellular cRHIM domain, likely act as decoy receptors preventing the intracellular progression of the signaling cascade (52–64). At the intracellular level, Pirk, also known as Rudra or PIMs, most likely interrupts the IMD amyloid fibrils signaling platform (29, 65–67). Finally, several ubiquitinating and deubiquitinating enzymes were described to promote K48-linked ubiquitination and subsequent proteasomal degradation of IMD pathway signaling intermediates or to interrupt the formation of K63-linked ubiquitin chains that are required for signal transmission (68–77). In particular, IMD is targeted to proteasomal degradation on its phosphorylation by TAK1, a process that likely triggers both the removal and the addition of K-63 and K-48 polyubiquitin chains respectively (42). Despite the identification of several protein kinases in the IMD-NF-κB cascades, far less is known about negative regulators operating signal termination by dephosphorylation.

In this article, we report the characterization of the protein phosphatase 4 (PP4) holoenzyme as a new negative regulator of the IMD pathway. We show that PP4c and its two regulatory subunits, PP4R2 and PP4R3, also known as Falafel (Flfl), are required for the proper downregulation of the IMD pathway after an immune stimulus. Moreover, our results indicate that flies deficient for the expression of *PP4c* exhibit an inflammatory-like state that is marked by a progressive activation of IMD-NF-κB signaling with aging and a reduced lifespan in the absence of any infection. Finally, we show that PP4 specifically interacts with the IKK complex for the inhibition of Relish activation. Overall, our results highlight an evolutionarily conserved function of PP4c in the regulation of NF-κB signaling from *Drosophila* to mammals.

Materials and Methods

Plasmid constructs

A cDNA clone for PP4-19c (FMO03839) was obtained from the *Drosophila* Genomics Resource Center. This clone contains a metallothionein promoter and FLAG-hemagglutinin (FLAG-HA) tag C-terminal fusion. Metallothionein promoter expression plasmids encoding FLAG-tagged PP4-19c, wild-type and phosphatase dead mutant described by Lipinszki et al. (78) were a kind gift from Dr Zoltan Lipinszki. pAC-PGRP-LC, pAC-IMD and pAC-Rel (S29-S45) constructs were described previously (79, 80). A methallothionein expression plasmid encoding wild-type FLAG-tagged TAK1 was a kind gift from Dr Neal Silverman (42). Metallothionein expression plasmids encoding wild type HA-tagged IKKβ (ird5) or HA-tagged IKKγ were a kind gift from Dr Hidehiro Fukuyama.

Fly strains

Stocks were raised on standard cornmeal-yeast-agar medium at 25°C with 60% humidity. *relish*^{E20} (46) and *Dif*^{1} (81) flies were used as mutant deficient for the IMD and Toll pathways, respectively. Flies carrying an upstream activating sequence-RNA interference

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(RNAi) against *Pp4-19c* (25317) (UAS-RNAi PP4-1), *R2* (1053999), *Flfl* (103793) were obtained from the Vienna *Drosophila* Resource Center (VDRC) (http://stockcenter.vdrc.at/control/main). Flies carrying an UAS-PP4-19c construct (F001063) (82) were obtained from FlyORF (http://flyorf.ch/index.php). Flies carrying a UAS-RNAi transgene against GFP (397-05) or a UAS-GFP construct (BL#5431), obtained from the *Drosophila* Genetic Resource Center (Kyoto, Japan; http://www.dgrc.kit.ac.jp/index.html) and the Bloomington *Drosophila* stock center (Bloomington, IN; http://flystocks.bio.indiana.edu/), were used as wild-type control in knockdown experiments using the VDRC lines and overexpression experiments, respectively. A fly strain carrying an UAS-RNAi against *Pp4-19c* (BL #27726) (UAS-RNAi PP4-2) and the control line carrying an UAS-RNAi targeting mCherry (BL#35785) were obtained from Bloomington *Drosophila* Stock Center. Flies carrying Yolk-Gal4 (33) or C564-Gal4 (6982) (83) drivers were used to drive the expression of UAS constructs in the fat body. The C564-Gal4 fly line was obtained from Bloomington *Drosophila* Stock Center and combined with Tub-Gal80^{ts} (84). For assaying IMD and Toll pathway signaling, Gal4-driven RNAi and open reading frame expression were enhanced by incubating 2- to 3-d-old female flies for 6 d at 29°C before their infection.

Microbial strains and infections

We used *Escherichia coli* strain ATCC 23724, *Enterobacter cloacae*, *Enterococcus faecalis* (OG1RF), and *Micrococcus luteus* (ATCC 4698) bacteria for septic injuries (85). Bacteria were grown in Luria broth (*E. coli*, *E. cloacae*) or brain-heart infusion broth (*M. luteus, E. faecalis*) at 30°C (*E. cloacae, M. luteus*) or 37°C (*E. coli, E. faecalis*). A bacterial suspension with OD at 600 nm (OD_{600}) was prepared for septic infections with *E. faecalis*. All other infections were performed using a concentrated preparation of an overnight bacterial culture. Survival experiments were performed on 15-25 females infected with *E. cloacae* or *E. faecalis* by septic injury at 29°C three independent times. Survival assays were performed at 29°C to maintain optimal functioning of the UAS-Gal4 system and to ensure continuous efficient knockdown or overexpression of target genes during the experiments. Control survival experiments were made by sterile injury (85). Reverse transcription-quantitative PCR (RT-qPCR) experiments were performed on 10-20 females (9 d old) not infected and infected with *E. coli* for 4, 16, and 24 h or *M. luteus* for 24 h, by septic injury at 29°C, three times independently.

Cell culture, transfection and luciferase reporter assay

S2 cells were cultured at 25°C in Schneider's medium (Biowest) supplemented with 10% FCS (lot RUF35205; Thermo Scientific), 8mM penicillin/streptomycin (Life Technologies) and 100 U/ml L-glutamine (Life Technologies). For transient transfection, S2 cells were seeded in a 24-well plate at 0.5×10^6/ml. Transfection was performed by the calcium phosphate coprecipitation method or using the Effectene transfection kit (Qiagen), according to the manufacturer’s instructions. Each plate was transfected with 10 μg of indicated plasmids. After 12-16 h, the cells were washed with PBS and incubated in fresh medium. For expression of recombinant proteins by expression vectors containing a metallothionein promoter, CuSO4 was added. Forty-eight hours later, cells were infected with heat-killed *E. coli* (HKE) for IMD activation. Luciferase reporter assays were performed for IMD pathway activation measurements. In brief, S2 cells were transfected with Attacin A-firefly luciferase

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reporter (86) and Actin5C-Renilla luciferase, and the pathway was induced with HKE 60 h after transfection. Twenty-four hours later, S2 cells were harvested by centrifugation and lysed in Passive Lysis Buffer (Promega). Firefly and Renilla luciferases' activities were measured using standard procedures.

Immunofluorescence

Cells were seeded on eight-well Lab-Tek Chamber Slide, rinsed with PBS 1x, and fixed with 2% paraformaldehyde. Cells were then permeabilized with 0.1% Triton X-100, saturated with 3% BSA, and incubated 1 h with HA mouse Ab ab18181 (Abcam) and then with Cy3 goat anti-mouse secondary Ab A10521 (Life Technologies). Slides were mounted in a solution of Vectashield/DAPI and samples were observed using a Zeiss LSM780 confocal microscope. Images were processed using ImageJ and Adobe Photoshop.

Immunoprecipitation and Western Blot

The cells were harvested 72 h after transfection, washed in PBS, and lysed in 500 µl of buffer containing 30 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM MgAc, 2 mM DTT, 1% Nonidet P-40, and complete protease inhibitor mixture (Roche). Immunoprecipitations were performed overnight with rotation at 4°C, using mouse monoclonal anti-FLAG or anti-HA Abs coupled with agarose beads (Sigma). Immunoprecipitates and proteins from total cell lysates (10% IP) were resolved by SDS-PAGE and detected by Western blotting using rabbit anti-FLAG (Abcam), rabbit anti-HA (Abcam) or rat anti-HA-HRP linked (Roche), mouse anti-actin (Millipore), rabbit anti-PP4R2, and rat anti-Flfl (78) (gift from Zoltan Lipinszki). The secondary Abs used are mouse-HRP linked (NA931; GE Healthcare), rabbit-HRP linked (NA934; GE Healthcare), or rabbit-HRP (W401B; Promega) and rat-HRP linked (A9037; Sigma).

Gene knock down in S2 cells

dsRNA preparation—DNA templates for dsRNA preparation were PCR-derived fragments flanked by two T7 promoter sequences (TTAATACGACTCACTATAGG). Fragment for *GFP* is nt 35-736; GenBank accession no. L29345. The other fragments were generated from genomic DNA templates using oligonucleotides designed for use with DKFZ Genome-RNAi libraries. The corresponding references are HFA21251 and BKN23059 for *PP4-19c*, DRSC27825 and BKN60140 for *PP4-R2*, 64410 and AMB30767 for *PP4-R3*, and DRSC37194 for *Relish*. Primers for IKKβ dsRNAs were ACTGGAATGGACGAAAAGGAACTGT-Forward and CTTGTTAGCTGATCATAGGCAAAGG-Reverse, and for IKKγ were CACTCGTTTGAGTTCGTACCAG-Forward and CTCCTCTCGCAAATTGCTTCTG-Reverse. ssRNAs were synthesized with the MEGAscript T7 transcription kit (Ambion). Annealed dsRNAs were ethanol precipitated and dissolved in sterile deionized water.

dsRNA bathing—Cultured S2 cells were pelleted and washed once in PBS to remove FCS supplemented Schneider's medium and resuspended in serum-free Schneider's medium (Biowest) supplemented with 8 mM penicillin/streptomycin (Life Technologies) and 100 U/ml L-glutamine (Life Technologies) at 1.5×10^6 cells/ml. A total of 30 µl of this cell suspension (45×10^3 cells) was added to 10 µl of dsRNA (500 ng/µl) and incubated at 24°C

for 1 h in a U shaped 96-well plate. A total of 160 μl of FCS-supplemented Schneider's medium was then added and cells were incubated for 6 days at 24°C. Cells were stimulated with HKE for 2, 4, 8, 12, 16, or 24 h and frozen before RNA extraction.

RT-qPCR—For quantitative analysis of *Attacin A*, *PP4-19c*, *R2*, *R3* and *rp49* (ribosomal protein-49), RNA from cells was extracted and treated with DNAse, using Total RNA isolation NucleoSpin 96 RNA (Macherey-Nagel). RNA extraction from flies and dissected fat bodies was performed with TRI Reagent RT (Molecular Research Center) and BAN (4-bromoanisole) (Molecular Research Center) after mechanical lysis by 1.4-mm ceramic beads using a Precellys24 tissue homogenizer. cDNAs were synthesized using the Bio-Rad iScript cDNA Synthesis kit, and quantitative PCR was performed using BioRad iQ SYBR Green. Real-time PCR was performed in 384-well plates using CFX384 Touch Real-Time PCR Detection System (Bio-Rad). The amount of mRNA was normalized to control *rp49* mRNA values. Primers used for qPCR are for *Attacin A* (GGCCCATGCCAATTTATTCA-Forward and AGCAAAGACCTTGGCATCCA-Reverse), *Attacin D* (TTTATGGAGCGGTCAACGCCAATG-Forward and TGCAAATTGAGTCCTCCGCCAAAC-Reverse), *Diptericin A* (GCTGCGCAATCGCTTCTACT-Forward and TGGTGGAGTGGGCTTCATG-Reverse), *Drosomycin* (CGTGAGAACCTTTTCCAATATGATG-Forward and TCCCAGGACCACCAGCAT-Reverse), *Rp49* (GACGTTCAAGGGACAGTATCTG-Forward and AAACGCGGTTCTGCATGAG-Reverse), *PP4-19c* (CCTTCACCTCGTTCTCCTTG-Forward and ATGTCCGACTACAGCGACCT-Reverse) or (AAACCCGGTTGGCAAAAACG-Forward and TTCACCAAATCAGCGCAGTG-Reverse) *PP4-R2* (CGGTAACGCCGATGAGGGCT-Forward and CATTGTCGTCCGAACGCGGG-Reverse for RNA extracted from S2 cells and CGATCCTCGGAAGCAGTA-Forward and GATCGATTGTGCTAACCACTA-Reverse for RNA extracted from flies), *R3* (ACAACAATGTCATGAAATCCGT-Forward and TGTGTGGCGGAGAGGAT-Reverse) and *Relish* (CCACCAATATGCCATTGTGTGCCA-Forward and TTCCTCGACACAATTACGCTCCGT-Reverse). The expression level of the gene of interest was normalized to that of the RNA coding for rp49 determined in each sample and compared with the control sample (*GFP*) set as 1.

Results

PP4c negatively regulates the IMD pathway

To identify new regulators of the IMD pathway, we previously conducted a high-throughput RNAi screen in *Drosophila* S2 hemocyte-like cells (79). This screen identified Akirin that we characterized as a nuclear protein driving the selectivity of Relish transcriptional activity (79, 80). In this study, we re-explored the results of this screen and focused on genes inducing an overactivation of the IMD pathway when silenced by RNAi. We thus selected *CG32505*, which encodes the catalytic subunit of the phospho-serine-threonine phosphatase 4 (PP4-19c) (87). This gene was of particular interest because no previous studies have explored the role of phosphatases in the negative regulation of IMD signaling despite the established knowledge that several of its intracellular components require phosphorylation. To confirm this result, we used two nonoverlapping dsRNA constructs, *dsPP4-1* and

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dsPP4-2, and monitored the IMD pathway activation profile by quantifying the expression of three of its target genes, *Attacin A*, *Diptericin A* (whose expression is Akirin dependent), and *Attacin D* (whose expression is Akirin independent), in S2 cells. We first confirmed that both constructs efficiently silence the *PP4-19c* transcript as compared with the *dsGFP* control (Supplemental Fig. 1A). *PP4-19c* knockdown leads to a constitutive activation of the IMD pathway in S2 cells (Fig. 1A-C). Moreover, *dsPP4-19c*-treated cells exhibit an enhanced and prolonged activation of the IMD pathway on their stimulation with HKE as compared with *dsGFP* control cells (Fig. 1A-C). Conversely, the overexpression of the wild-type *PP4-19c* construct significantly inhibits IMD pathway activation after HKE induction (Fig. 1D and Supplemental Fig. 1B). This phenotype is strictly dependent on its catalytic activity because the overexpression of a phosphatase dead-mutant (*PP4-PD*) construct does not alter *Attacin A* expression in immune induced cells. Altogether, these results indicated that PP4-19c is a negative regulator of the IMD pathway in *Drosophila* S2 cells and prompted us to investigate its role in the control of the immune response of adult flies.

PP4c is essential for centrosome maturation in the *Drosophila* embryo, and loss-of-function mutants exhibit a high lethality rate (88). Therefore, we took advantage of the yeast UAS-Gal4 system to selectively drive a restricted expression of a dsRNA targeting the *PP4-19c* transcript in the fat body of adult flies, the main immune organ of *Drosophila*, using either the *c564-Gal4* (combined to Tub-Gal80^{ts}) (83, 84) or the *yolk-Gal4* transgenes (33). Two different transgenic fly strains carrying hairpin constructs targeting the *PP4* transcripts, designated here by UAS-RNAi-PP4-1 and UAS-RNAi-PP4-2, were used. In all cases, the expression of the *PP4-19c* transcript was significantly reduced in the fat body of adult female flies (Supplemental Fig. 1C, 1D). Compared with *dsGFP* and *dsmCherry* control flies, ds*PP4-19c* flies show a constitutive, as well as an enhanced and prolonged, expression of *Attacin A* at 4, 16, and 24 h after their infection with *E. coli* (Fig. 2A-C). Impairing the expression of IMD pathway negative regulators is known to result in a shortening of *Drosophila* lifespan (55, 68, 89). This is reminiscent of NF-κB dependent chronic inflammatory diseases in mammals. We show that *dsPP4-19c* flies recapitulate this shortened-lifespan phenotype (Fig. 2D) that correlates with an exacerbated activation of the IMD pathway in the aging flies (Fig. 2E). In a complementary approach, we overexpressed *PP4-19C* in the fat body of adult flies using the *yolk-Gal4* driver (Supplemental Fig. 1E) and checked for the IMD pathway activation 4 h after their infection with *E. coli*. Compared with control flies overexpressing *GFP*, the *Attacin A* expression is significantly reduced in flies overexpressing *PP4-19c* (Fig. 2F). This impaired IMD pathway activation is most probably accounting for the susceptibility of yolk-Gal4 > UAS:PP4-19c flies to an infection with the Gram-negative bacterium *E. cloacae*, as is the case for the IMD pathway mutant *Rel*^{E20} (Fig. 2G).

In contrast with IMD, the Toll pathway is not altered in flies deficient for or overexpressing *PP4-19c*, as shown by the quantification of the *Drosomycin* transcript, a conventional readout of the Toll pathway, 24 h after the infection of flies with *M. luteus* (Fig. 2H, 2I). In keeping with these results, flies overexpressing PP4 do not show an enhanced susceptibility to an infection by *E. faecalis*, as is the case for the Toll pathway mutant *Spaetzle* (*Spz*) (Fig.

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2J). In summary, these results indicate that PP4-19c is specifically involved in the negative regulation of the IMD-NF-κB pathway in *Drosophila*.

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

The major form of PP4, conserved from yeast to mammals comprises PP4c and two regulatory subunits: a core protein, PP4R2, and a regulatory protein, PP4R3. Nevertheless, several other proteins were shown to bind PP4c, and additional mutually exclusive complexes have been described in metazoans (90–93). In *Drosophila*, PP4R2 and PP4R3, also known as *Flfl*, are requisite for PP4c function in the regulation of developmental signaling pathways, such as hedgehog, JNK, and wingless, during centrosome maturation and neuroblast asymmetric division, as well as for the coordination of glial cell recruitment and phagocytosis of degenerating axons from the CNS (78, 94–99). To check whether these PP4c regulatory subunits are also required for modulating the IMD pathway activation profile, we used specific dsRNA constructs to silence their expression in S2 cells and further checked for the *Attacin A* expression 4 and 16 h after their stimulation with HKE. We first confirmed that two pairs of nonoverlapping dsRNA constructs targeting each of the *PP4-R2* (*dsPP4-R2 1* and *dsPP4-R2 2*) and *Flfl* (*dsFlfl1 and dsFlfl2*) transcripts efficiently reduced their expression (Supplemental Fig 1F). Similarly to the attenuation of *PP4-19c* by ds*PP4-1*, impairing the expression of *PP4R2* and of *Flfl* leads to a significant increase in *Attacin A* expression in HKE induced S2 cells (Fig. 3A). Collectively, these results indicate that both PP4R2 and Flfl are required with PP4-19c for the negative regulation of the IMD pathway in *Drosophila S2* cells. We further validated these results in transgenic adult flies in which the RNAi-mediated knockdown of *PP4R2* and *Flfl* using either the *c564-Gal4* or the *yolk-Gal4* drivers, leads to a systemic overactivation of the IMD pathway after an infection with *E. coli* (Fig. 3B, 3C).

PP4c interacts with the IKK complex in the IMD pathway

In an attempt to identify the cellular target of PP4-19c in the IMD pathway, we first undertook an epistasis analysis. Therefore, we overexpressed either PGRP-LC, IMD or a constitutively active form of Relish (with a short internal truncation Relish S29-S45) (100) in S2 cells with or without a concomitant overexpression of the full-length catalytically active form of PP4-19c and monitored IMD pathway activation using an *Attacin A-Luciferase* reporter. As previously described, the overexpression of PGPR-LC and the IMD adaptor protein triggers a constitutive activation of the IMD pathway (65, 79) (Fig. 4A-C). The activity of the *Attacin A* reporter is significantly reduced in cells cotransfected with the *PP4* expression plasmid (Fig. 4A, 4B). Conversely, the induction of the *Attacin A - Luciferase* reporter by the cells overexpressing *Relish S29-S45* is not affected regardless of whether they are cotransfected with PP4-19c (Fig. 4C). These observations indicate that PP4c acts downstream of IMD and upstream of Relish. In addition, confocal microscopy analysis indicated a strictly cytoplasmic localization of the tagged PP4c-FLAG-HA in S2 cells that is not changed regardless of whether the cells are stimulated by the IMD pathway agonist (Fig. 4D). Altogether, these data suggest that PP4 targets a cytoplasmic component of the IMD pathway, acting downstream of IMD and upstream of Relish. In this context, we reasoned that TAK1 and the IKK complex both represent possible candidates for the

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presumed target. Indeed, these proteins are phosphorylated during the progression of the IMD intracellular cascade and thus their dephosphorylation is an appropriate mechanism for fine-tuning the duration and intensity of the signaling (40, 51). In keeping with this hypothesis, we checked whether PP4 interacts with these potential targets. Hence we performed immunoprecipitation experiments on protein extracts from S2 cells cotransfected with the tagged *HA-FLAG-PP4-19c* and *FLAG-PP4-19c* and *FLAG-TAK1, HA-IKKβ* or *HA-IKKγ*. Transfections were performed with or without a PGRP-LC expression vector to analyze protein interactions in the presence or absence of an IMD activating stimulus respectively. As shown in Fig. 5A, no interaction between the tagged versions of TAK1 and PP4-19c recombinant proteins is observed. In contrast, our results revealed both components of the IKK complex as coimmunoprecipitates with the PP4-19c recombinant protein (Fig. 5B, 5C). Using an anti-R2 Ab, we also detected the endogenous PP4R2 protein in the PP4-IKK coimmunoprecipitated complexes (Fig. 5B, 5C). These results indicate an interaction between PP4 and the IKK complex and suggest that PP4 inhibitory mechanism could potentially operate through the targeting of the IKK complex. However, our attempts to analyze IKK dephosphorylation by PP4 have been unsuccessful so far (data not shown). Therefore, to further support the connection between PP4 and the signalosome, we checked whether the constitutive activation of the IMD pathway triggered by the silencing of PP4 in S2 cells would be reverted by the depletion of either constituent of this signaling complex (Supplemental Fig. 2). This was confirmed as revealed by the comparative analysis, shown in Fig. 5D, of the *Attacin A* expression levels detected in S2 cells double knocked down for the expression of *PP4-19c* together with each of the transcripts encoding the IKK subunits and the cells that were treated with only *dsPP4*. Altogether, our experimental evidence so far put forward the hypothesis that the PP4 phosphatase negatively regulates the IMD pathway in *Drosophila* likely through an interaction with the IKK signalosome.

Discussion

NF-κB transcription factors are key regulators of innate immunity and inflammation from insects to mammals. However, their activation comes with a significant cost on fitness, tissue homeostasis, and lifespan (55, 68, 89, 101, 102). Therefore, the intensity and duration of NF-κB signaling are tightly regulated in physiological conditions. Several negative regulators of the *Drosophila* IMD-NF-κB pathway have been previously identified acting at multiple levels and by different means. These include the interruption of the initial signaling trigger and receptor activation, the disruption of supramolecular signaling complexes, and the proteasomal degradation of signaling intermediates on K48-linked ubiquitination (28). In this study, we introduce the PP4 phosphatase as a new regulator of the IMD pathway and provide, to our knowledge, the first evidence for the negative regulation of this pathway through the interruption of protein phosphorylation that is essential for the regulation of NF-κB-Relish transcriptional activity. Our results show that RNAi mediated knockdown of *PP4-19c*, *PP4R2*, and *Flfl*, which encode the catalytic, scaffold, and regulatory subunits of the PP4 complex, respectively, lead to an enhanced and prolonged activation of the IMD pathway both in S2 cells and in adult flies. In a complementary approach, overexpression of PP4-19c significantly limits the IMD pathway activation in S2 cells following their induction by HKE. This phenotype is strictly dependent on its catalytic activity. Similarly

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to the IMD pathway mutants, flies overexpressing *PP4-19c* display a compromised Relish-dependent antimicrobial peptide gene expression and are susceptible to infections by Gram-negative bacteria. Moreover, as is the case for IMD pathway negative regulators, RNAi-mediated silencing of *PP4-19c* leads to a shortened lifespan of adult flies that correlates with a progressively intensified activation of the IMD pathway in the aging flies compared with wild-type flies. Altogether, these results provide evidence of an important role of PP4 in the modulation of the IMD pathway signaling. Genetic analysis placed PP4-19c downstream of the IMD adaptor protein and upstream of the IKK signalosome. Using an immunoprecipitation approach, we show that PP4-19c and its PP4R2 regulatory subunit specifically interact with both the regulatory and catalytic subunits of this complex. With the identification of PP4 as a key player in the modulation of the IMD pathway, these data put forward the IKK signalosome as a hub for different negative regulators acting by different means for the fine-tuning of Relish-NF-κB signaling. These include deubiquitination, autophagy and now protein dephosphorylation (73, 101). Further analysis is required for the clarification of how these processes are regulated and coordinated on immune challenge. Whether PP4 directly targets the IKK complex or any of its regulatory annexes remains an open question. In all cases, the costly effects of exacerbated IMD signaling are attested by a loss of tissue homeostasis and/or reduced lifespan of the flies.

Many of the previously identified IMD negative regulators act in a negative feedback loop (55–57, 65–67). Our preliminary data show that the expression of the genes encoding *PP4-19c* and its regulatory subunits are not induced on *E. coli* infection (Supplemental Fig. 3). Further experiments will be needed to decipher the complete process that leads to the activation and recruitment of the PP4 complex on IMD pathway activation. Another question pertains to the molecular mechanisms underlying the interaction of PP4 with its target in the IMD cascade. As is the case for all phosphoprotein phosphatases, it is generally accepted that the PP4 functional profile can be diversified by the combinatorial association of its catalytic subunit with distinct scaffold and regulatory subunits driving its activity toward different cellular targets (78, 90, 103–105). Although our results provide the first evidence of PP4 function in the *Drosophila* IMD pathway, its role in the regulation of cell division as well as many developmental processes in *Drosophila* is well documented. The Flfl regulatory subunit was previously shown to be required for PP4 function in the regulation of cell-cycle progression, asymmetric neuroblast division, proper glial responses to nerve injury in the adult brain and the regulation of Wingless and Notch pathways in the wing imaginal disc (78, 88, 94, 95, 98, 99, 106–109). Flfl belongs to the highly conserved family of PP4R3 orthologs that is characterized by a well-defined domain organization (78, 90, 91). This comprises the succession of an N-terminal Pleckstrin Homology superfamily-like domain and a Smk-1/DUF625 domain followed by a variable number of ARM (armadillo/HEAT repeats), and finally a C-terminal unstructured low-complexity region (78, 91, 98). In a recent study, Lipinszki *et al* showed that Flfl directly binds the key Centromeric Protein C via its EVH1 domain (which belongs to Pleckstrin Homology-like domains), thus recruiting PP4-19c to centromeres and that this interaction is critical for regulating the integrity of the mitotic centromeres (78). The EVH1 domain of Flfl was also shown to bind Mira for the regulation of neuroblast asymmetric division (98). Our attempts to detect an interaction between R3 and the IKK complex have been unsuccessful so far. Our current data also

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preclude the requirement of Flfl for the interaction between recombinant PP4 and the tagged versions of the IKK subunits (Supplemental Fig 4). Also, the depletion of PP4R2 did not prohibit the interaction of recombinant Flag-PP4-19c with HA-IKKβ or HA-IKKγ. Presently, we cannot exclude the possibility that other ancillary proteins may be required for the interaction between PP4 and the IKK complex, or that this phosphatase may be acting at different levels for the tweaking of IMD signaling.

Several research activities have lately indicated a role of PP4, in the regulation of NF-κB signaling in mammals (110–113). A decline in PP4 expression is associated with aberrant NF-κB, sustained malignancy, and enhanced metastasis of T cell lymphomas and lung cancer cells (110, 114). In addition, the PP4R1 subunit in mammals is targeted by the Merkel polyomavirus to subvert the NF-κB-dependent antiviral response (90, 114). These studies attest of the central role of PP4 in the regulation of NF-κB-mediated immune responses in mammals. Notably, PP4 was shown to interact with several components of the NF-κB cascades including members of the NF-κB transcription factors, the E3 ubiquitin ligases TRAF2 and TRAF6 (PP4R1), as well as the IKK signalosome (110, 111, 113, 115). The pleiotropic functions of PP4 might be explained by the coaction of different regulatory subunits. Remarkably, the PP4/PP4R1 complex was shown to target the IKK complex for the suppression of NF-κB signaling in Jurkat T cells and primary T lymphocytes (110). In line with these data, our current study reveals an evolutionarily conserved function of PP4 for the modulation of NF-κB signaling from insects to mammals. SMEK, the human homologue of Flfl, was shown to be required for targeting Par3 dephosphorylation by PP4 during neuronal differentiation (116). However, no immune function of SMEK has been reported to date. Given the high conservation of NF-κB signaling from insects to mammals, it is tempting to speculate a similar immunological role for SMEK. The innate immune response, conserved among metazoan, is the unique line of defense for invertebrates against pathogens. Although highly potent to counterstrike or prevent microbial infections, deregulation of NF-κB signaling could be considered as a shared evolutionary threat. The paradox between the necessity of these pathways and the danger implied by their deregulation underlies their tight regulation by conserved factors, such as PP4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

- The PP4 complex modulates the IMD-NF-κB dependent immune response in *Drosophila*.
- Loss of PP4 leads to exacerbated IMD-NF-κB signaling and a reduced lifespan
- Genetic and Co-IP data propose an interaction between PP4 and the IKK complex

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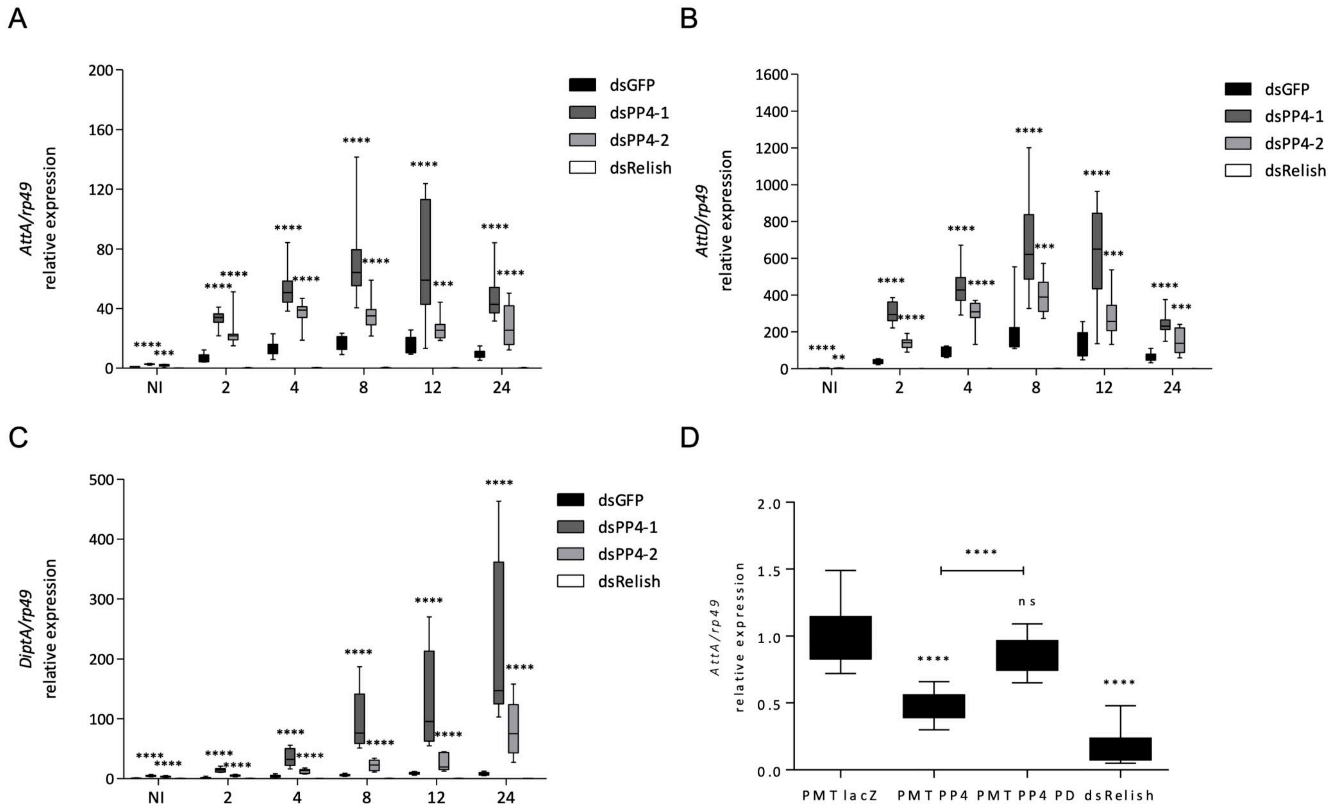

Figure 1. PP4-19c negatively regulates the IMD pathway in *Drosophila* S2 cells.

[A-C] Relative expression of [A] *Attacin A* [*AttA*], [*B*] *Attacin D* [*AttD*] *and* [*C*] *Diptericin A* [*DiptA*] transcripts in total RNA extracts of S2 cells soaked with two different dsRNA constructs [ds*PP4-1* and ds*PP4-2*] targeting *PP4-19c* mRNA. Cells soaked with *GFP* dsRNA and *Relish* dsRNA were used as positive and negative controls, respectively. The IMD pathway was induced 6 d after soaking by adding HKE. Transcripts' expression was quantified by RT-qPCR in noninduced condition [NI] and at 2, 4, 8, 12, and 24 h post-induction. *rp49* transcript was used as a reference gene. Transcripts levels are compared with those detected in NI *dsGFP* controls.

[D] Relative expression of *AttA* in total RNA extracts from S2 cells transiently transfected with a metallothionein promoter-driven transgene expressing wild type *PP4-19c* [PMT PP4] and a phosphatase-dead mutant allele [PMT PP4 PD]. CuSO4 was added for 48 h, and then IMD pathway activation was stimulated with HKE for 4 h. Relative expression of *AttA* to *rp49* transcripts was compared with that triggered in cells transfected with *lacZ* expression vector [PMT lacZ] as controls. Data obtained from three independent experiments are combined in a single value [mean ± SD]. Statistical tests were performed using the Mann-Whitney *U* test within Prism software [$^{ns}p > 0.05$;0.001< $^{**}p < 0.01$; 0.0001 < $^{***}p < 0.001$; $^{****}p < 0.0001$].

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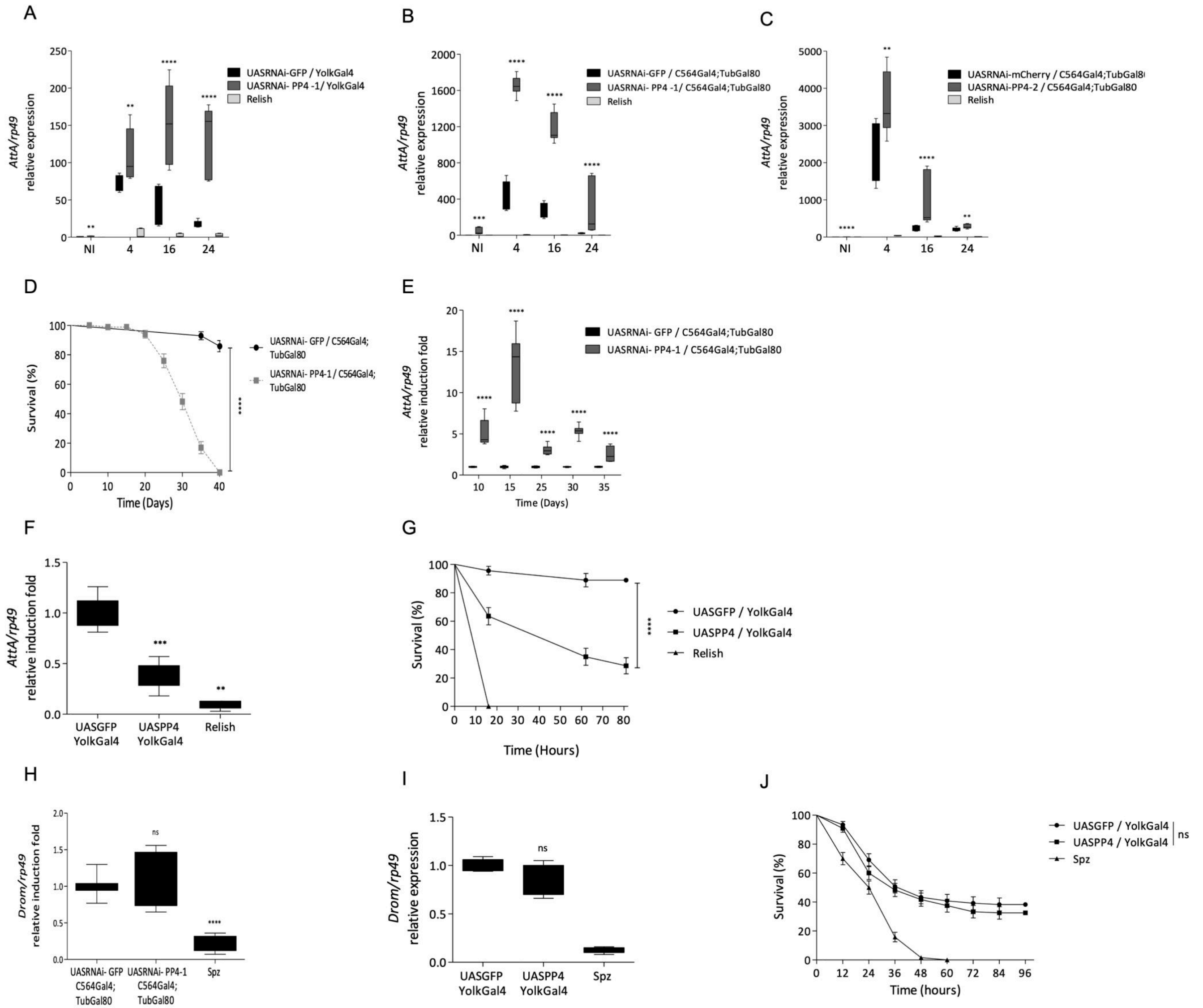

Figure 2. PP4-19c is required for the regulation of adult flies' immune response

[A-C] Relative expression of *AttA* transcripts in total RNA extracts from the offsprings of two *PP4-19c* RNAi lines, UAS-RNAi-PP4-1 [A and B] and UAS-RNAi-PP4-2 [C] crossed with *yolk-Gal4* [A] or *c564-Gal4* [B and C] drivers. A fly line expressing RNAi *GFP* [A and B] or RNAi *mCherry* [C], and Relish-deficient flies were used as positive and negative controls, respectively. The transcript expression was quantified by RT-qPCR in noninfected condition [NI] or 4, 16, and 24 h postinfection, with *E. coli*. *rp49* transcript was used as a reference gene. Transcripts levels are compared with those detected in NI RNAi *GFP* control.

[D] Survival of flies expressing UAS-RNAi PP4-1 under the control of *c564-Gal4*. Flies were counted every 5 d, for a period of 40 d.

[E] Relative expression of *AttA* to *rp49* transcripts in total RNA extracts of surviving flies was compared with that detected in RNAi *GFP*-expressing flies used as wild type controls.

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[F] Relative expression of *AttA* transcripts in flies overexpressing *PP4-19c* [UASPP4] under the control of *yolk-Gal4* driver. Normalized expression of *AttA* was measured 4 h postinfection with *E. coli* and compared with that triggered in flies overexpressing *GFP* [UASGFP] used as control.

[G] Survival of flies overexpressing *PP4-19c* [UASPP4] under the control of *yolk-Gal4* driver after an infection with *Enterobacter cloacae*. Flies overexpressing *GFP* and *Relish* mutants were used as positive and negative controls, respectively. Infected flies were incubated at 29°C, and the number of surviving flies was counted every 24 h.

[H and I] Relative expression of *Drosomycin* [*Drom*] transcript in total RNA extracts of flies expressing UAS-RNAi PP4-1 under the control of *c564-Gal4* driver [H] or UAS *PP4* under the control of *yolk-Gal4* driver [I] infected with *M. luteus* for 24 h to activate the Toll pathway. Relative expression of *Drom* transcripts to *rp49* transcripts was compared with that triggered in flies expressing RNAi *GFP* [H] *or* UAS *GFP* [I] used as wild-type controls. *Spz* mutants were used as negative controls.

[J] Survival of adult flies overexpressing *PP4-19c* under the control of *yolk-Gal4* to an infection with *Enterococcus faecalis* monitored at 29°C. Flies overexpressing *GFP* and *Spz* mutants were used as positive and negative controls, respectively.

Data obtained from three independent experiments are combined in a single value [mean ± SD]. Log rank test for the survival assays and Mann-Whitney *U* test for the RT-qPCR data within Prism software [$^{ns}p > 0.05$;0.001< $^{**}p < 0.01$; 0.0001< $^{***}p < 0.001$; $^{****}p < 0.0001$].

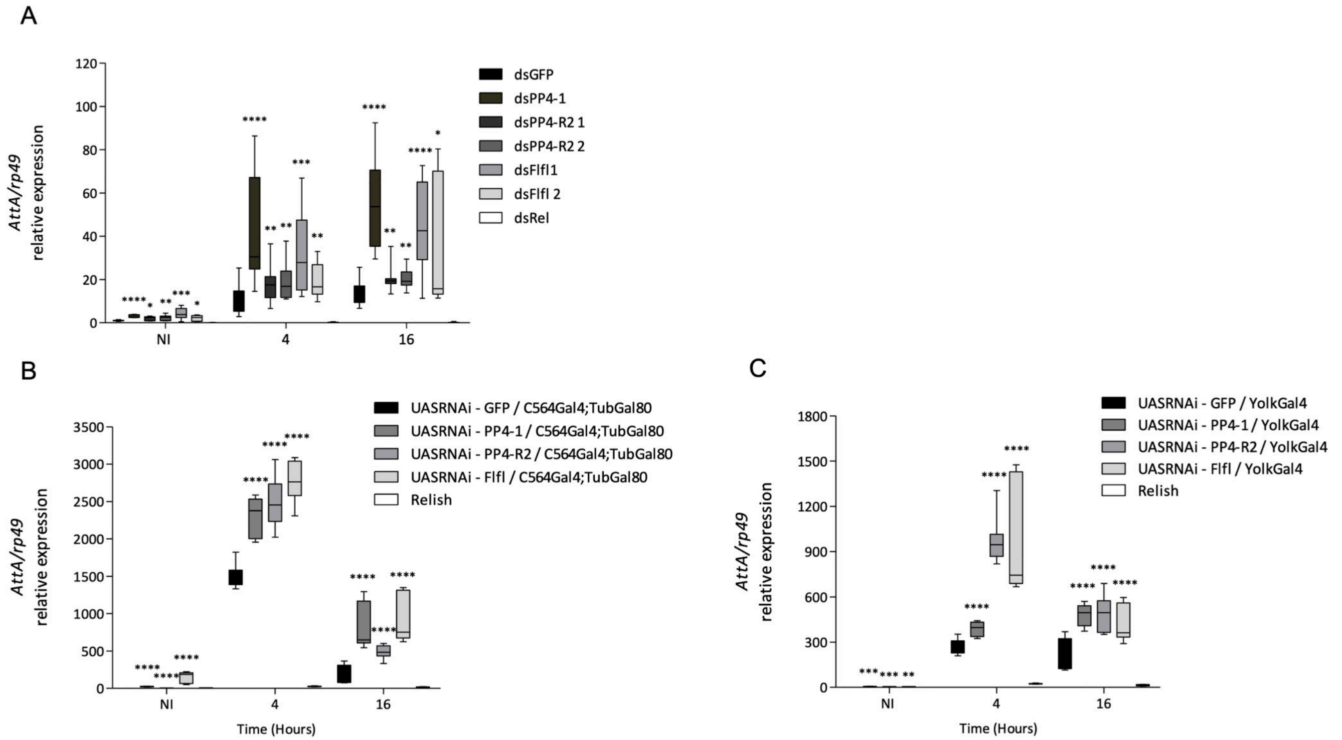

Figure 3. The R2 and Flfl regulatory subunits of PP4 are required for IMD pathway negative regulation in S2 cells and in adult flies

[A] Relative expression of *AttA* transcripts in total RNA extracts from S2 cells soaked with two dsRNA constructs targeting specifically *PP4-R2* and *Flfl* transcripts. Cells soaked with *GFP* dsRNA and *Relish* dsRNA were used as negative and positive controls, respectively. The IMD pathway was induced 6 d after soaking by adding HKE. Transcripts' expression was quantified by RT-qPCR in noninduced condition [NI] and 4 and 16 h postinduction. *rp49* transcript was used as reference gene. Transcripts levels are compared with those detected in NI *dsGFP* control.

[B and C] Relative expression of *AttA* transcripts in total RNA extracts of flies expressing UAS-RNAi *PP4-R2* and UAS-RNAi *Flfl* under the control of *c564-Gal4* [B] or *yolk-Gal4* [C] drivers. Flies expressing UAS-RNAi *GFP* and Relish mutants were used as positive and negative controls, respectively. Transcripts' expression was quantified by RT-qPCR in NI and at 4 and 16 h postinfection with *E. coli*. *rp49* transcript was used as a reference gene. Transcripts levels are compared with those detected in NI condition of UAS-RNAi GFP control flies.

Data obtained from three independent experiments are combined in a single value [mean ± SD]. Statistical tests were performed using the Mann-Whitney *U* test within Prism software [$^{ns}p > 0.05$; $0.01 < {*}p < 0.05$; $0.001 < {**}p < 0.01$; $0.0001 < {***}p < 0.001$; ${****}p < 0.0001$].

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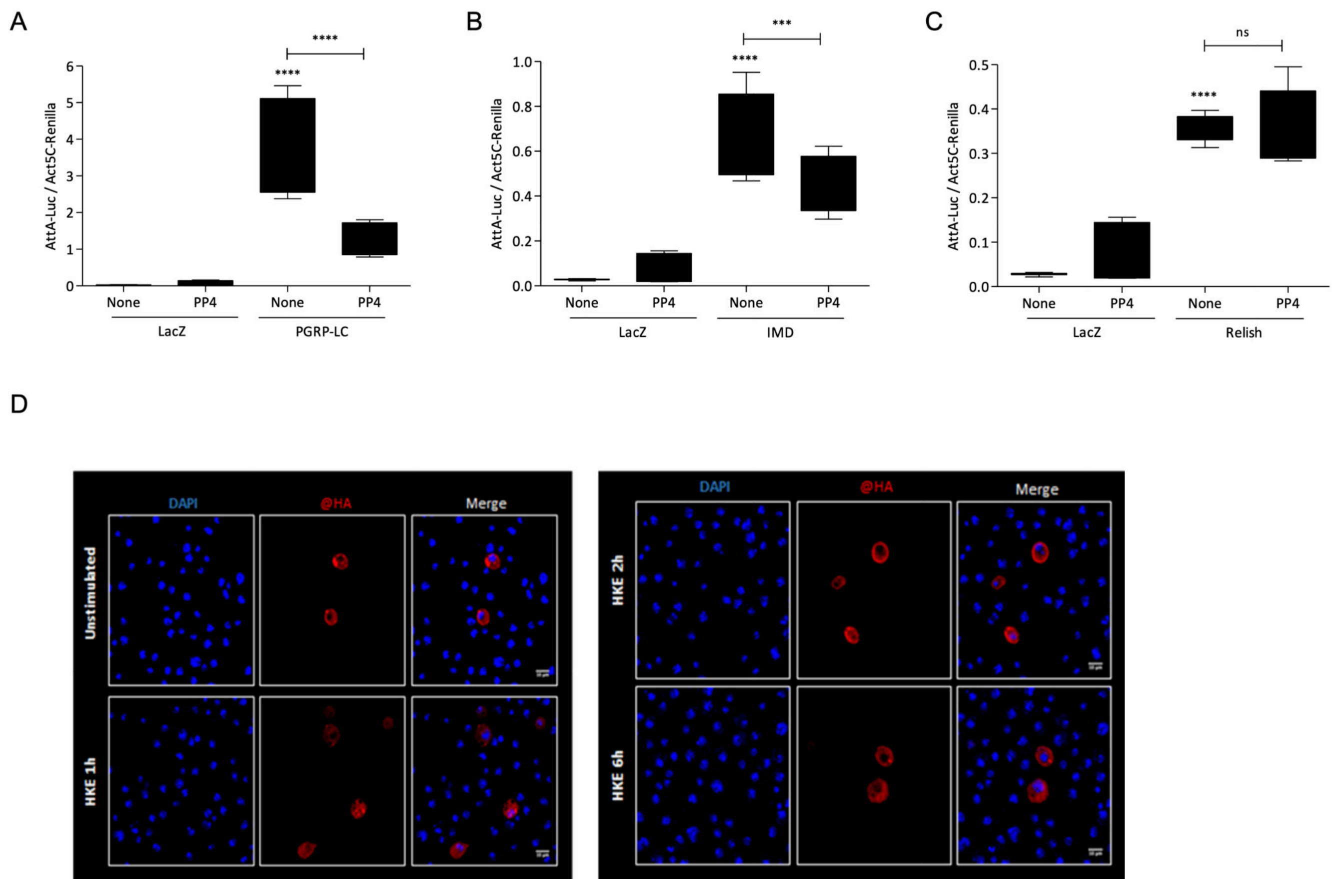

Figure 4. PP4 acts upstream of Relish in the IMD cytoplasmic cascade

S2 cells were transfected with [A] PGRP-LC [TM+Intra], [B] IMD, and [C] Rel [S29-S45] expression vectors alone or together with a *PP4-19c* expression vector. Cells transfected with a LacZ-expressing vector were used as control. IMD pathway activation was monitored with the *AttA*-Firefly Luciferase reporter gene. *Actin*-5C-Renilla Luciferase activity was measured to normalize transfection efficiency.

Data obtained from three independent experiments are combined in a single value [mean ± SD]. Statistical tests were performed using the Mann-Whitney U test within Prism software [$^{ns}p > 0.05$; 0.0001< $***p < 0.001$; $****p < 0.0001$].

[D] Confocal microscopy of S2 cells showing the cellular localization of recombinant PP4-FLAG-HA at 1, 2 and 6 h after cell stimulation with HKE. For cell staining, nuclei were visualized using DAPI [blue]. Scale bars, 10 μm.

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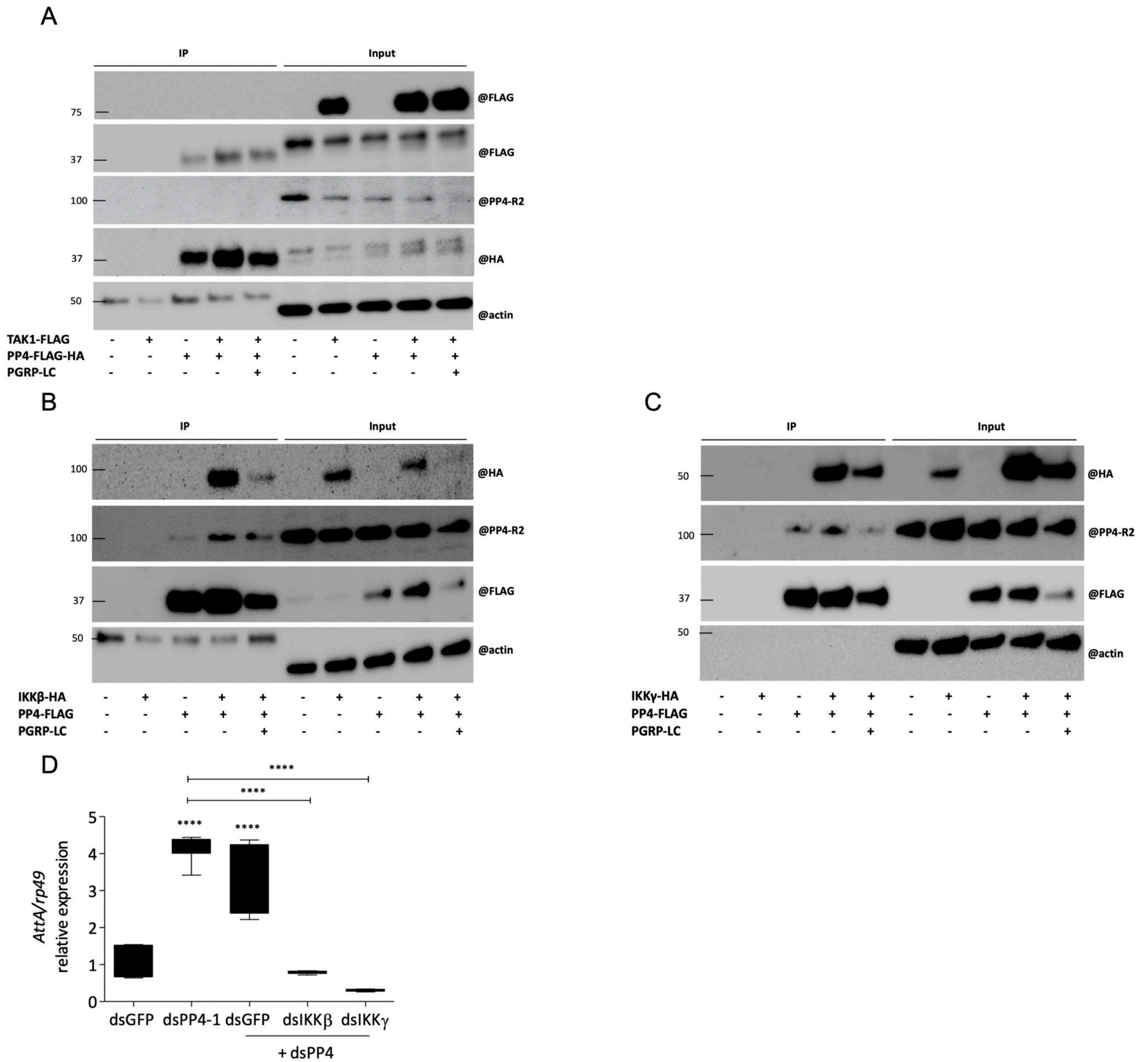

Figure 5. PP4-19c interacts with the IKK signalosome in the *Drosophila* IMD pathway.

[A] Immunoprecipitation [IP] was performed using anti-HA Ab coupled to agarose beads and immunoblotting [IB] with anti-FLAG, anti-HA, and anti-actin Abs. Total protein extracts were obtained from S2 cells transiently transfected with metallothionein promoter expression plasmids encoding PP4-FLAG-HA and TAK1-FLAG. Data were obtained from three independent experiments.

[B and C] Immunoprecipitation [IP] was performed using anti-FLAG Ab coupled to agarose beads, and immunoblotting [IB] with anti-HA, anti-FLAG, anti-PP4R2, and anti-actin Abs. Lysates were obtained from S2 cells transiently transfected with PP4-FLAG, IKK-β-HA, [B] and IKK-γ-HA [C] expression plasmids. Data were obtained from three independent experiments.

[D] Relative expression of *Attacin A* [*AttA]* transcripts in total RNA extracts of S2 cells soaked with dsRNA constructs targeting *PP4-19c* only, or together with dsRNAs targeting *GFP, IKKβ or IKKγ*. Cells soaked with *GFP* dsRNA were used as controls. Transcripts' expression was quantified by RT-qPCR in noninduced condition. *rp49* transcript was used as a reference gene. Transcripts levels are compared with those detected in *dsGFP* controls. Data obtained from three independent experiments are combined in a single value [mean ± SD]. Statistical tests were performed using the Mann-Whitney *U* test within Prism software [****$p < 0.0001$].

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