Hyd ubiquitinates the NF-κB co-factor Akirin to activate an effective immune response in *Drosophila*

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One Sentence Summary

E3-ubiquitin ligase Hyd mediated ubiquitination of Akirin is required for its binding to NF-κB and the activation of a subset of target genes mediating the immune response against Gram-negative bacteria infection in *Drosophila*.

ABSTRACT

Activation of inflammatory response is a tightly controlled process that is mediated by NF-κB pathways. Ubiquitinations are known to modulate the mammalian inflammatory response at all levels of the NF-κB pathways, but the mechanism involved in this ubiquitin-mediated modulation of the inflammation is still not fully understood. Here, we used *Drosophila* genetics to identify E3-ubiquitin ligases involved in the innate immune response and explore their conservation in mammals. To this aim, we conducted an *ex-vivo* RNA interference screen of the E3 ubiquitin-ligases encoded by the fruit fly genome downstream of the *Drosophila* IMD pathway. This screen identified Hyperplastic Discs "Hyd" as acting genetically at the level of the NF-κB cofactor Akirin. *Drosophila* lacking Hyd failed to express the full set of anti-microbial peptides coding genes and succumbed to immune challenges. Remarkably, HyD-mediated K63-polyubiquitination of Akirin is required for the efficient binding of Akirin to the NF-κB transcription factor Relish. This Hyd-mediated interaction triggers the activation of a subset of Relish target genes, required *in-vivo* to survive immune challenges. We show further that Urb5, the mammalian homolog of Hyd, is also required in the NF-κB pathway. 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 in mammals points to *Drosophila* as a relevant model to explore the immune response (Maeda & Omata, 2008, Vidal & Cagan, 2006). In *Drosophila*, the defense against microbes is ensured mainly by the massive production of antimicrobial peptides (AMP) (Ferrandon, Imler et al., 2007). Their expression is under the control of two transcription factors belonging to the NF-κB 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. Even though NF-κB factors act primarily in activating the inflammatory response, they also have a role in the termination of inflammation through establishing a negative feed-forward regulatory loop (Chen, Egan et al., 2003, Lawrence, Gilroy et al., 2001).

We have previously identified Akirin, a conserved protein from insects to humans (Goto, Matsushita et al., 2008). Akirin occupies a critical position in the IMD molecular cascade since in its absence the immune response is inefficient. Akirin is a NF-κB co-factor selectively required for the expression of a subgroup of NF-κB target genes, mostly composed of effectors (Bonnay, Nguyen et al., 2014, Goto et al., 2008, Tartey, Matsushita et al., 2014).

Post-transcriptional regulation by ubiquitin pathway is key for proper immune response, albeit not fully understood (Park, Jin et al., 2014). 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 (Terzic, Marinovic-Terzic et al., 2007). In both mammals and *Drosophila*, ubiquitination is involved at various levels of the NF-κB pathways (Thevenon, Engel et al., 2009). Furthermore, deregulation of Ubiquitin-ligases is implicated in inflammatory pathologies (Aksentijevich & Zhou, 2017, Kattah, Malynn et al., 2017) and tumor progression (Gallo, Ko et al., 2017).

To deepen our understanding of NF-κB pathway regulation by the ubiquitin system, we focused on identifying *Drosophila* new ubiquitin-ligases required for the activity of the IMD pathway. In *Drosophila*, IAP2 is the only E3-ubiquitin ligase identified so far as a component of the IMD pathway (Gesellchen, Kuttenkeuler et al., 2005, Kleino, Valanne et al., 2005). This protein is involved in the formation and activity of upstream protein complexes around the IMD protein and the IKK kinases. We designed a RNAi-based screen in *Drosophila* S2 cells that identified the HECT E3-ubiquitin ligase Hyperplastic Discs "Hyd" (Lee, Amanai et al., 2002, Mansfield, Hersperger et al., 1994) as a key IMD pathway regulator.

Several E3-ubiquitin ligases emerged as positive or negative regulators of the IMD pathway. We decided to focus on Hyd as it is the unique HECT E3-ubiquitin ligase involved in the IMD pathway. We could show that Hyd is required *in-vivo* to survive an immune challenge with Gram-negative bacteria. Epistasic analysis revealed that Hyd acts at the level of the NF-κB co-factor Akirin, which is known to orchestrate the activation of a subset of NF-κB target genes in combination with the SWI/SNF chromatin remodeling complex (Bonnay et al., 2014). This is consistent with the described localization of Hyd within the nucleus (Lee et al., 2002, Mansfield et al., 1994).

We could show that Hyd decorates Akirin with K63-polyUb chain, which is required for Akirin binding to the NF-κB factor Relish. Furthermore, we observed that Ubr5 (also known as EDD1), the human ortholog of Hyd (Callaghan, Russell et al., 1998), has a

conserved function in human HeLa cell line. Similarly to hAkirin2, Ubr5 is required for the activation of only a subset of NF-κB target genes. We demonstrate here that upon immune challenge, ubiquitin chains are instrumental to bridge NF-κB and its co-factor Akirin to activate an effective immune response.

RESULTS

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

To uncover new 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 (Gramates, Marygold et al., 2017). 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 (Tauszig, Jouanguy et al., 2000). 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 (Kleino & Silverman, 2014). 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 this 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-κB factor *relish,* its cofactor *akirin, hyd* or some of the other E3-ubiquitin ligases of the screen. We challenged S2 cells with HKE and endogenous *Attacin-A* mRNA level was monitored by RT-qPCR. Interfering with *relish*, *akirin* or *hyd* expression significantly decreased *Attacin-A* induction by HKE, compared to control (*dsGFP*) (Fig 1B, Fig S1). The RING-domain E3-ubiquitin ligases Bon, CG5334, m-cup, Mkrn1 and Mura are modulators of *Attacin-A* expression (Fig 1B), and the HECT E3-ubiquitin ligase Hyd is a *bona-fide* positive regulator of the IMD pathway 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) (Goto et al., 2008). Measurement of *Attacin-A* expression by RT-qPCR assessed activation of the IMD pathway. We could show that Hyd is required genetically at the level or downstream of Relish (Fig 1C) to exert its positive regulation on IMD pathway activation.

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). Upon immune challenge in S2 cells, using RT-qPCR, we could show that depletion of Hyd recapitulates the transcriptional phenotype of cells depleted for

Akirin (Fig 1D). Consequently, Hyd is acting on Akirin-dependent NF-κB transcriptional selectivity *ex-vivo*.

We next investigated if Akirin and Hyd were similarly required for NF-κB transcriptional selectivity *in-vivo*. As *Drosophila* embryonic development is impaired in absence of Akirin, we used the *C564-Gal4* transgene (McGuire, Roman et al., 2004) to express RNAi constructs targeting *akirin*, *hyd* and *relish* in the adult fat body, the main immune organ of *Drosophila* (Ferrandon et al., 2007). 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 1E).

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 1F, Fig S2).

Our results demonstrate that Hyd is required genetically 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 physically interacts with Akirin to trigger K63-polyubiquitination

We next investigated if Akirin could be a *bona-fide* target for the E3 ubiquitin-ligase Hyd. In S2 cells, we could show that V5-tagged Hyd (*Hyd-V5*) (Wang, Tang et al., 2014) binds to endogenous Akirin (Fig 2A). By contrast V5-tagged HydCS (*HydCS-V5*), which displays a mutated HECT domain by conversion of the catalytic cysteine at position 2854 to serine (Wang et al., 2014), is unable to co-immunoprecipitate with Akirin (Fig 2A). As a control, we confirmed that IAP2, the E3-ubiquitin ligase acting upstream of Akirin in the IMD

signaling cascade (Gesellchen et al., 2005, Kleino et al., 2005) 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 showed that Akirin is K63-polyubiquitinalyted 1h and 3h after immune challenge with HKE (Fig 2B). This immune-induced post-translational modification of Akirin is abolished upon knockdown of Hyd (Fig 2C). Collectively, this shows that upon immune challenge, Hyd physically interacts with Akirin through its catalytic HECT domain to decorate Akirin with K63-polyUb chains.

K63-polyUb chains on Akirin are instrumental to link Relish

We previously showed that Akirin physically bridges the NF-κB factor Relish and BAP60, a core member of the SWI/SNF chromatin-remodeling complex (Bonnay et al., 2014). 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 2D). As previously reported (Bonnay et al., 2014), Akirin-V5 co-precipitated either with the active form of the NF-κB factor Relish (Rel68-HA) or with BAP60 (BAP60-HA) (Fig 2D). However, in the absence of Hyd, the interaction between Akirin-V5 and Rel68-HA is weakened (Fig 2D). Of note the interaction between Akirin-V5 and BAP60-HA is independent of Hyd (Fig 2D). These results demonstrate that Hyd is required to deposit K63-polyUb chains on Akirin for subsequent binding to the NF-κB factor Relish.

We investigated within Akirin the position of the specific lysine residues targeted by Hyd. The highly conserved N-terminal and C-Terminal part of Akirin are known to be important for its function (Macqueen & Johnston, 2009). We substituted into arginine residues

one by one all lysine residues of the N-terminal (K7 and K21) as well as C-terminal (K139, K145, K156, K173 and K183) conserved regions and one lysine residue (K81) in the non-conserved region of a V5-tagged Akirin (Fig S4). Subsequently, these single lysine-to-arginine mutants were analyzed regarding their ability to activate the IMD pathway. The mutated forms of *Akirin-V5* were correctly translated and addressed to the nucleus of S2 cells, where Akirin exerts its function, as shown by immunofluorescence using antibodies targeting the V5 tag (Fig 3A, Fig S5).

We then evaluated the functionality of the mutated version of Akirin-V5 proteins. We performed a rescue experiment using dsRNA targeting the Akirin 5' untranslated region (UTR) in order to silence endogenous *akirin* in S2 cells. When the coding sequence of wild-type *Akirin-V5* devoid of its 5' UTR was co-expressed in the same cells, *AttacinA-Luciferase* expression was high in PGRP-LC transfected cells (Fig 3B). The dsRNA was shown to suppress activation of the IMD pathway (Fig 3B). We rescued this phenotype with all *Akirin-V5* constructs, except with *V5-Akirin*^{K7R} and *V5-Akirin*^{K21R}, indicating that K7 and K21 are functionally required for IMD pathway activation (Fig 3B).

We next investigated whether ubiquitin conjugation takes place on these residues. *Drosophila* S2 cells were transiently transfected with *V5-Akirin*, *V5-Akirin*^{K7R} or *V5-Akirin*^{K21R} and immune stimulated. Protein complexes were isolated by immuno-precipitation using anti-V5 antibodies linked to agarose beads. The formation of poly-ubiquitin chains on Akirin was detected using *V5-Akirin* and *V5-Akirin*^{K7R} constructs, but was poorly detectable in cells expressing *V5-Akirin*^{K21R} (Fig 3C). Importantly, the active form of Relish, RelishΔS29-S45 (Stoven, Silverman et al., 2003), co-immunoprecipitated with both *V5-Akirin* and *V5-Akirin*^{K7R} but failed to interact with *V5-Akirin*^{K21R} (Fig 3C). These results indicate that Hyd mediated polyUb decoration of Akirin on its lysine 21 is required to interact with Relish.

Ubr5 - the human ortholog of Hyd - is required for the NF-κB transcriptional selectivity during the inflammatory response

The Akirin-dependent molecular mechanism underlying the selective activation of NF-κB target genes is well conserved from *Drosophila* to mammals (Bonnay et al., 2014, Goto et al., 2008, Tartey et al., 2014). Therefore, we addressed the potential requirement of Ubr5 (the ortholog of the *Drosophila* E3-ubiquitin ligase Hyd) in NF-κB selective transcriptional response mediated by hAkirin2 during the human inflammatory response. We depleted HeLa cells for either NF-κB1, hAkirin2 or Ubr5 by siRNA (using scrambled siRNA as controls). We monitored, upon stimulation by IL1β, the expression levels of NF-κB target genes that are dependent of hAkirin2 (such as *IL6*) or independent ones (such as *IL8*) (Tartey et al., 2014). As expected, lacking NFkB1 in HeLa cells impaired both *IL6* and *IL8* activation upon IL1β stimulation (Tartey et al., 2014). However, the activation of *IL6* and *IL8* is uncoupled in HeLa cells depleted for hAK2 or Ubr5 (Fig 4A, FigS6). This result suggests a conserved function of Ubr5 in the selective transcription of NF-κB 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-κB pathway regulation in *Drosophila* and mammals. In fruit fly, Hyd deposits K63-polyUb chains on the lysine 21 of Akirin and these ubiquitin marks are required to bridge Akirin and the NF-κB factor Relish. This interaction is necessary for the transcription of an essential NF-κB target genes subset, downstream of the IMD pathway (Fig 4B).

DISCUSSION

The transduction of a signal downstream of a receptor often requires the formation of protein complexes. K63-polyubiquitination is one of the ways to form such complexes (Sun & Chen, 2004). 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-κB transcription factor Relish. It is now established that both the Akirin-dependent and the Akirin-independent genes of the IMD pathway are required for *Drosophila* survival upon Gram-negative bacterial infection (Bonnay et al., 2014), highlighting the biological importance of Hyd. Of note, the K63 poly-ubiquitination of Akirin by Hyd is performed only after immune challenge. This suggests that an immune-triggered signal governs this event and remains to be explore. Additionally, 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 (Scheffner & Kumar, 2014). Here using *Drosophila*, we describe for the first time a function for the HECT E3-ubiquitin ligase Hyd in the innate immune response. We found that the mammalian ortholog of Hyd, Ubr5 (Callaghan et al., 1998) is involved in NF-κB transcriptional selective response in human cell line as well. This suggests a conserved role for Hyd/Ubr5 on human-Akirin2, even though we do not know if hAkirin2 is ubiquitinated. A dedicated study of Ubr5 role in NF-κB pathway is needed to completely assess it. It is known that Ubr5 inhibits the TNF receptor associated factor 3 (Traf3) (Cho, Kim et al., 2017) an inhibitor of the NF-κB pathway (He, Saha et al., 2007). Thus, the role of Ubr5 might be indirect. Uncontrolled activation of NF-κB due to deregulation of ubiquitin-ligases has been reported in many diseases (Iwai, 2014). When Hyd/Ubr5 is attenuated, only a subset of NF-κB genes is expressed, diminishing the intensity of the innate immune response in *Drosophila* and inflammatory response in mammals,

similarly to the inactivation of Akirin (Bonnay et al., 2014, Tartey et al., 2014). The HECT E3-ubiquitin ligase Ubr5 is therefore an interesting drug target for the development of small inhibitory compounds. They could be used to modulate NF-κB signaling, control the development of inflammatory diseases and potentially improve treatments of cancer (Taniguchi & Karin, 2018).

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 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.

E3 ligase 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 (Gramates et al., 2017). Based on this list, a *Drosophila* E3 ligase dsRNA library was generated in Michael Boutros's laboratory as previously described (Boutros, Kiger et al., 2004). The screen experiments were performed using 1F3 cells stably expressing AttA firefly luciferase (Fukuyama, Verdier et al., 2013). 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 x 10^4 cells per well. Cells were then transfected with 3 µg of each dsRNA in the *Drosophila* E3-ubiquitin ligase dsRNA library in triplicate by bathing method as previously described (Bonnay et al., 2014). 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 (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, Pelz et al., 2013) 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 (Bonnay et al., 2014).

Plasmid Constructs

pAC-Akirin, pAC-Akirin-V5, pAC-PGRP-LC, pAC-IMD, pMT-Rel-HA and pMT-Bap-HA constructs were described previously (Bonnay et al., 2014, Goto et al., 2008). Site-direct mutation of pAC-Akirin-V5 construct on K7R, K21R, K81R, K139R, K145R, K156R, K173R and K183R were performed by QuikChange Mutagenesis Kit (Stratagene) using appropriate primers.

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). Primers used for q-RT-PCR are listed in Supplementary Table 3.

Immunoprecipitation and Western blot

The experiments were realized according to (Bonnay et al., 2014). Immunoprecipitations were performed with rabbit polyclonal anti-Akirin (Bonnay et al., 2014) 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.

Cell staining

Immunofluorescence and histology experiments were realized as previously described (Bonnay et al., 2014). Monoclonal mouse anti-V5 tag GFP antibody was used (Invitrogen; 1/500). Cells were stained in a solution of Vectashield/DAPI (Vector Laboratories) to visualize nuclei and examined with a Zeiss LSM780.

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^{ts} system (McGuire et al., 2004). 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) (Reichhart, Gubb 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 (Bonnay et al., 2014, 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).

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. Distribution of Lysines present on Akirin

Fig. S5. Cellular localization of Akirin mutants (K156R, K173R and K183R)

Fig. S6. 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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FIGURE LEGENDS

Figure 1. Hyd is involved in IMD pathway activation

(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.

(D) 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.

(E) 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.

(F) 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 ± standard deviation of three independent experiments realized on 5×10^5 cells (B-D) 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 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 3. Akirin is ubiquitinated on the Lys21

(A) Cellular localization of Akirin modified constructs. S2 cells were transiently transfected with pAC-Akirin-V5 constructs modified on K7R, K21R, K81R, K139R, K145R. Immunolocalization and DAPI staining was done 4h after immune challenge by HKE. The

cells were visualized by DIC. Images are representative of at least 3 samples. Scale bar: 10μm.

(B) Level of *Attacin-A* after induction of the IMD pathway in S2 cells expressing modified Akirin constructs. S2 cells were co-transfected with the AttA-Luc reporter, dsRNA against 5'UTR Akirin, and pAC-Akirin-V5 or pAC-Akirin-V5 modified constructs on K7R, K21R, K81R, K139R, K145R, K156R, K173R and K183R. Cells were co-transfected with renilla luciferase expression plasmid for normalization and PGRP-LC for stimulation. Data are represented as mean ± standard deviation of three independent experiments realized on 5×10^5 cells per sample. Statistical significance was established by comparing values from Wt to other conditions. *P-value < 0.05; **P-value < 0.01; ***P-value < 0.001.

(C) Co-immunoprecipitation assay between modified Akirin constructs and Relish in S2 cells. The cells were transiently transfected with pAC-Akirin-V5, pAC-AkirinK7R-V5, pAC-AkirinK21R-V5 and/or RelishΔS29-S45. Cell lysates were immunoprecipitated with anti-V5 coupled agarose beads. Immunoprecipitates were analyzed by Western blotting with anti-Ubiquitin or anti-Relish antibodies. Data are representative of 2 independent experiments.

Figure 4. Hyd/Ubr5 is necessary for NF-κB 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 NFkB1, hAkirin2 (positive controls), and Ubr5. The cells were stimulated with recombinant human IL1β (10 ng/ml) for 4h. Data are represented as mean ± standard deviation of three independent experiments realized on 5×10^5 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 1

A

% induction: 50 | 75 | 100 | 150 | 250

bon		CG9934		nopo		CG3356		gol		CG4325	
hyd		CG1909		CG10761		ari-2		CG5071		Sce	
Iap2		Pex10		CG14435		godzilla		Cnot4		CG6179	
CG5334		Fancl		CG7747		tn		CG12099		SkpF	
CG13344		mib2		CG42797		CG1317		Nse1		Roc1a	
stc		CG32850		Trim9		Roc2		elgi		CG17019	
CG5087		FBXO11		Trc8		Topors		CG11982		CG2617	
CG17991		Pex12		Hakai		Cul5		Roc1b		dor	
CG17717		CG13442		CG2681		CG32369		CG32350		mei-P26	
HERC2		CG8419		CG8974		CG5604		Mi-2		CG17721	
CG17048		hiw		CG14983		CG15141		Rbpn-5		CG33552	
d4		CG3909		CG6923		Pex2		CG8141		Cul2	
Su(z)2		mdlc		unk		CG34375		CG10144		poe	
CG13025		Ube3a		ImgA		CG12477		CHIP		CG2218	
msl-2		CG15800		CG13481		CG33144		CG11360		Prp19	
CG32581		sinah		CG5382		CG8786		park		ago	
Ubr3		CG34308		CG16947		Cbl		dx		CG9014	
CG31053		Su(dx)		CG7694		Cul1		CG11070		morgue	
snama		mib1		l(3)73Ah		CG17260		CG6966		Cul3	
CG16781		Cdc16		dnr1		CG17329		elfless		Psc	
roq		CG32486		sina		CG9855		Traf4		trx	
Vhl		rdx		CG5347		CG15814		CG13605		Ubr1	
CG15011		CG5555		l(3)76BDr		Mul1		CG32847		CG11414	
ari-1		ctrip		snky		Ppa		Traf6		m-cup	
CG34289		It		CG7376		neur		CG8910		Mkrn1	
qin		Smurf		Nedd4		CG4080		CG8184		CG2926	
CG10916		POSH		CG9941		CG4238		CG4813		CG31807	
CG12362		CG2991		CG6752		CG11321		CG1815		mura	
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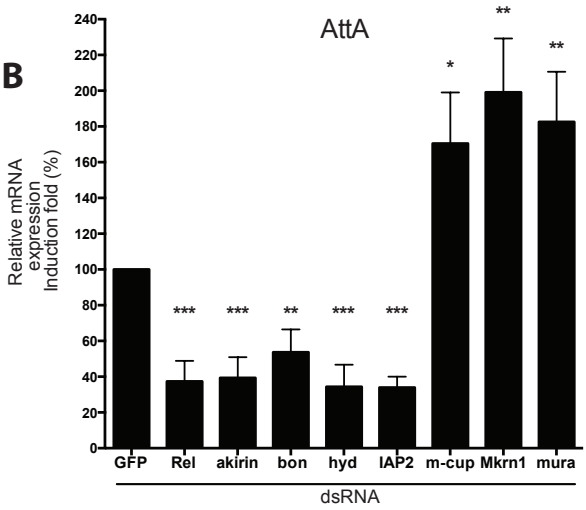
B



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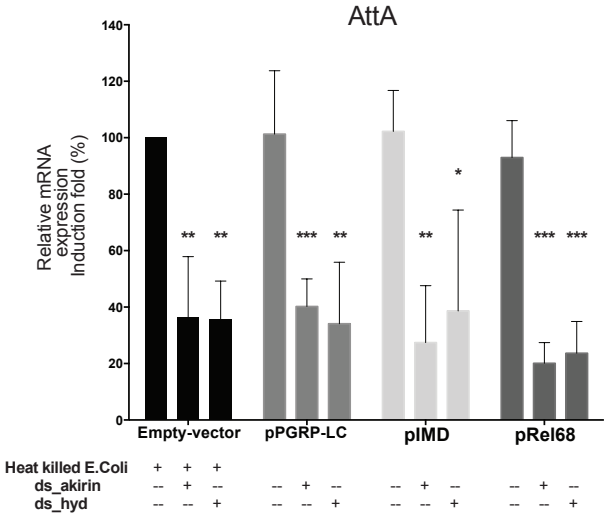

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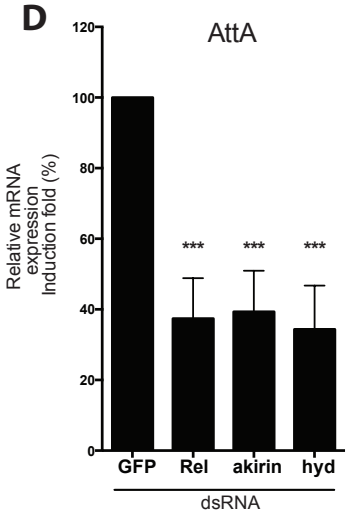

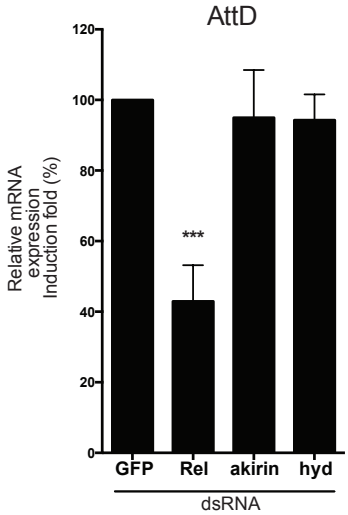

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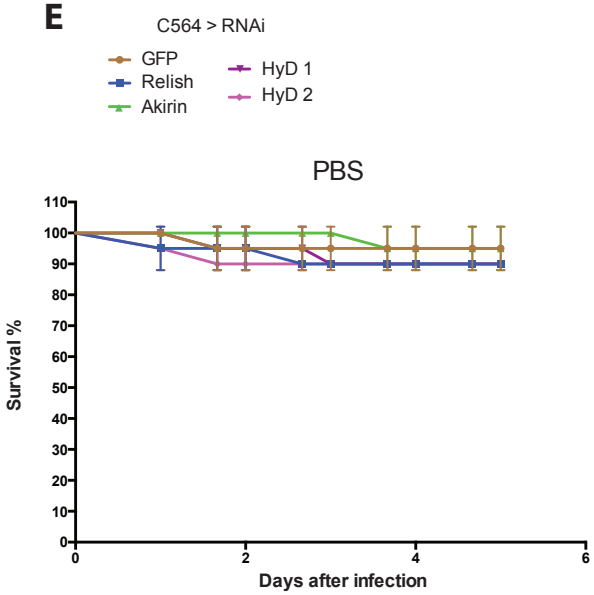

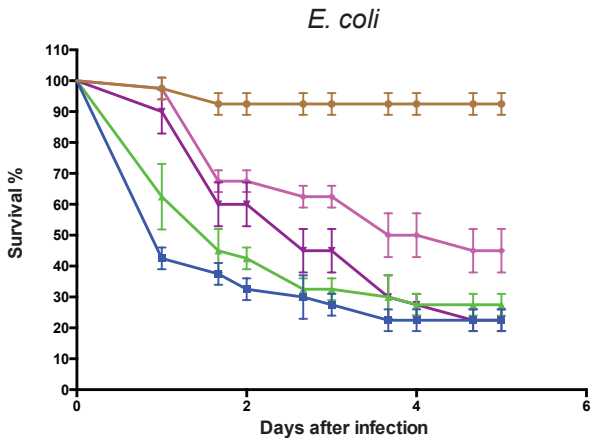

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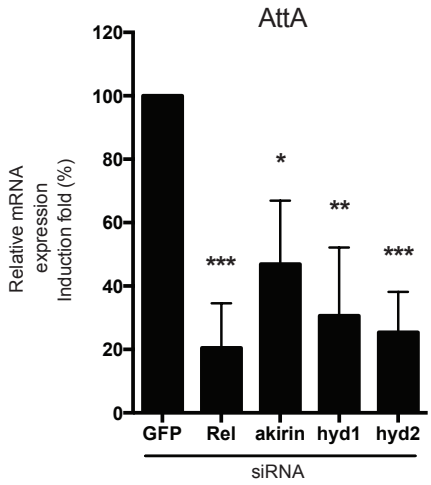


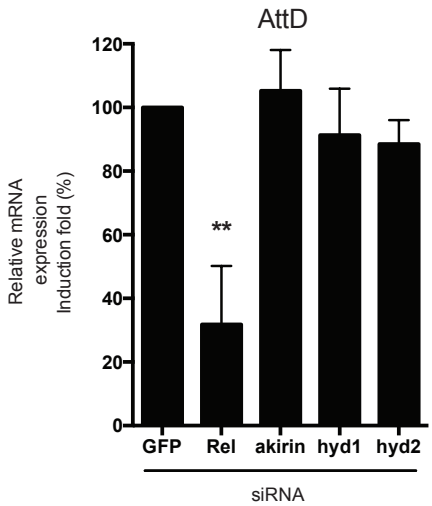
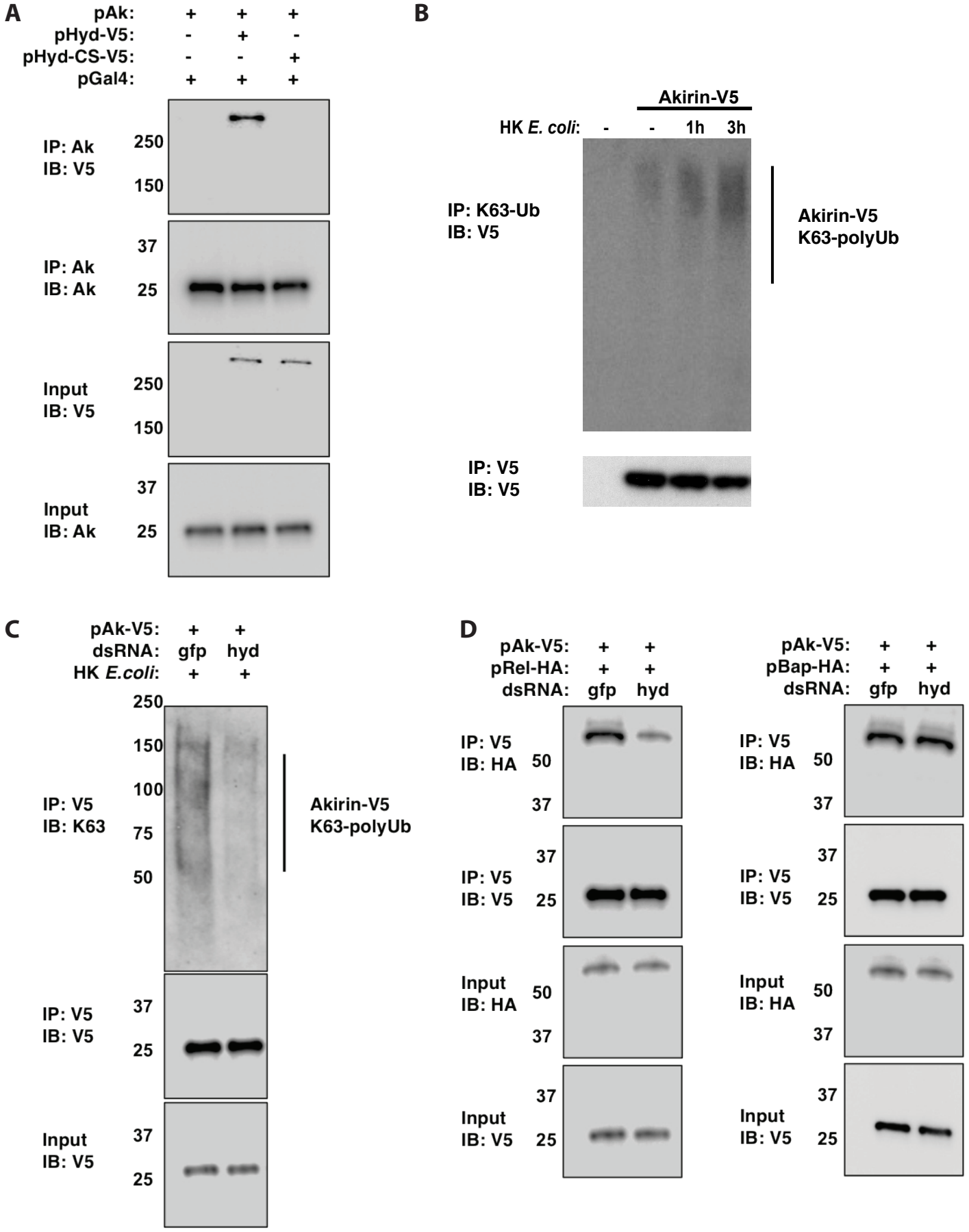


Figure 2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pHyd-V5: - + -
pHyd-CS-V5: - - +
pGal4: + + +
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IB: V5
250
150
IP: Ak
IB: Ak
37
25
Input
IB: V5
250
150
Input
IB: Ak
37
25
B
Akirin-V5
HK E. coli: - - 1h 3h
IP: K63-Ub
IB: V5
Akirin-V5
K63-polyUb
IP: V5
IB: V5
C
pAk-V5: + +
dsRNA: gfp hyd
HK E.coli: + +
250
150
100
75
50
IP: V5
IB: K63
Akirin-V5
K63-polyUb
IP: V5
IB: V5
37
25
Input
IB: V5
37
25
D
pAk-V5: + +
pRel-HA: + +
dsRNA: gfp hyd
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IP: V5
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IB: HA
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37
Input
IB: V5
37
25
pAk-V5: + +
pBap-HA: + +
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Input
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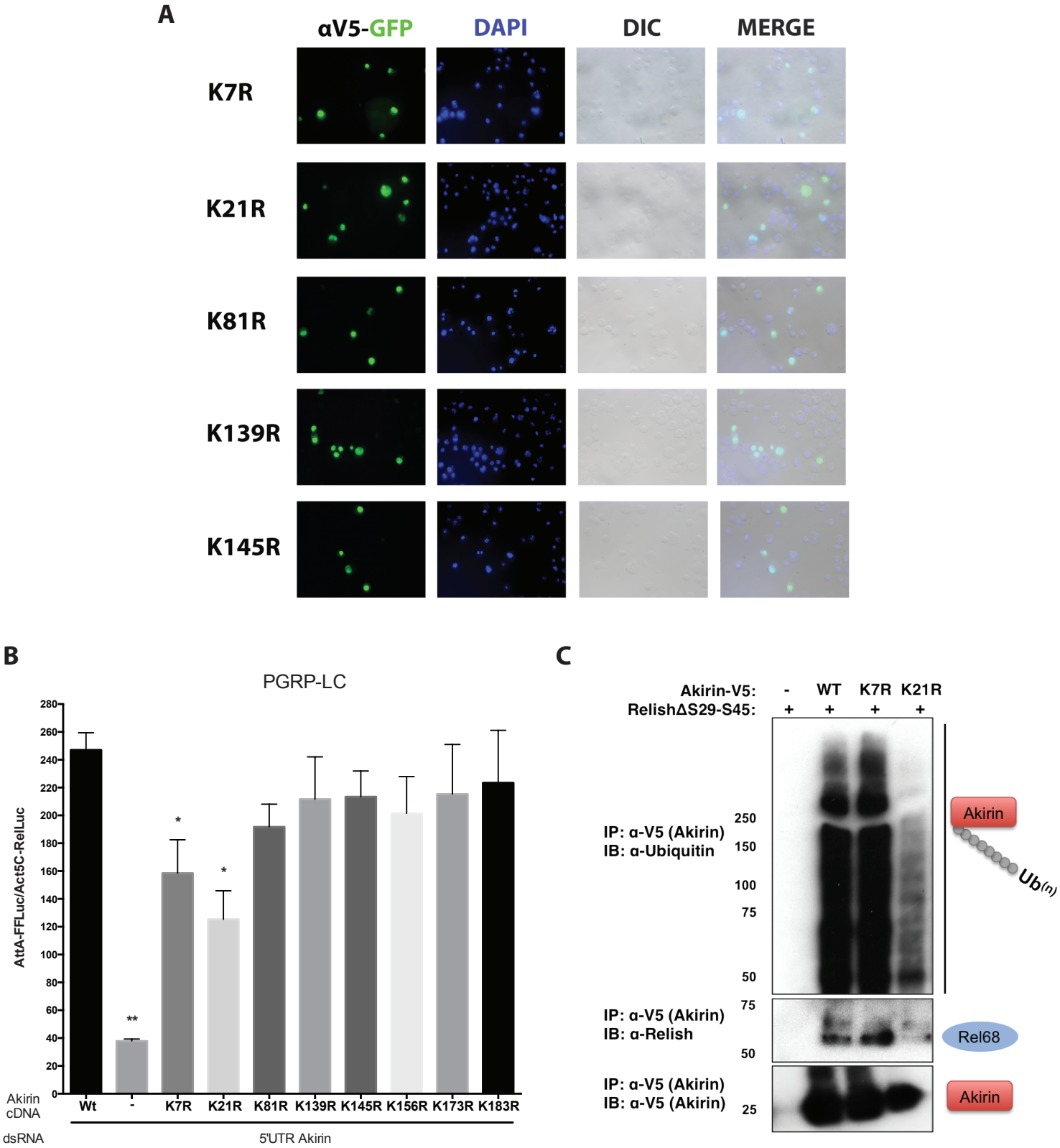
Figure 3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DAPI
DIC
MERGE
K7R
K21R
K81R
K139R
K145R
B
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AttA-FFLuc/Act5C-RelLuc
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
*
*
**
Akirin cDNA
Wt
-
K7R
K21R
K81R
K139R
K145R
K156R
K173R
K183R
dsRNA
5'UTR Akirin
C
Akirin-V5:
RelishΔS29-S45:
-
WT
K7R
K21R
+
+
+
+
IP: α-V5 (Akirin)
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150
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50
Akirin
Ub(n)
IP: α-V5 (Akirin)
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Rel68
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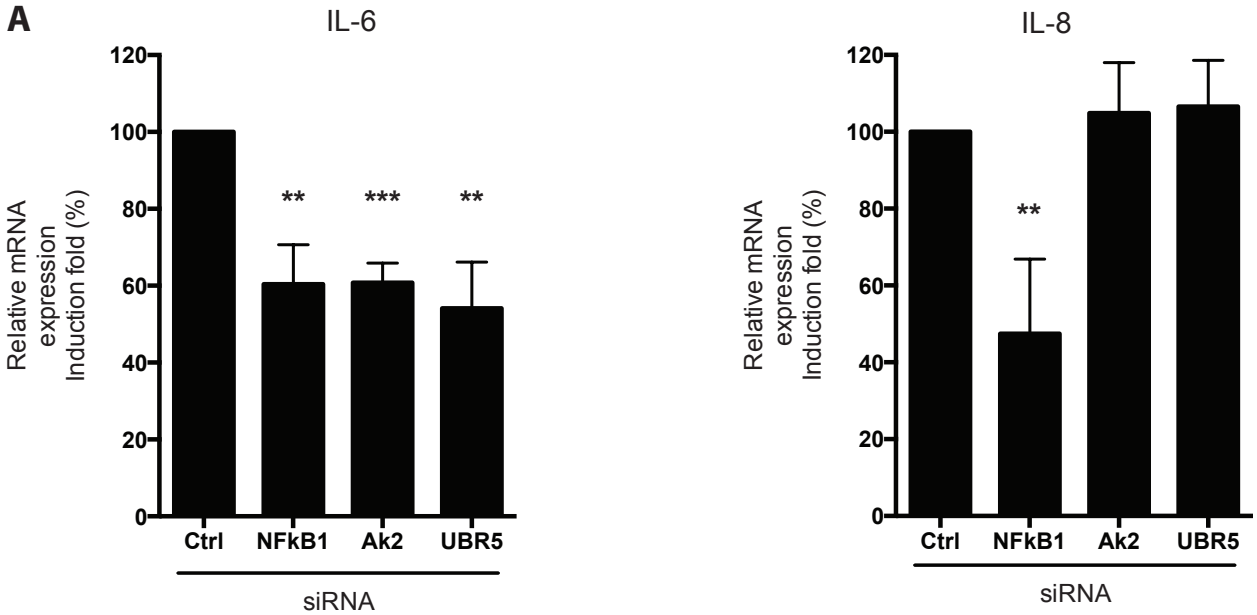
IB: α-V5 (Akirin)
25
Akirin

Figure 4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IL-8
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40
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**
***
**
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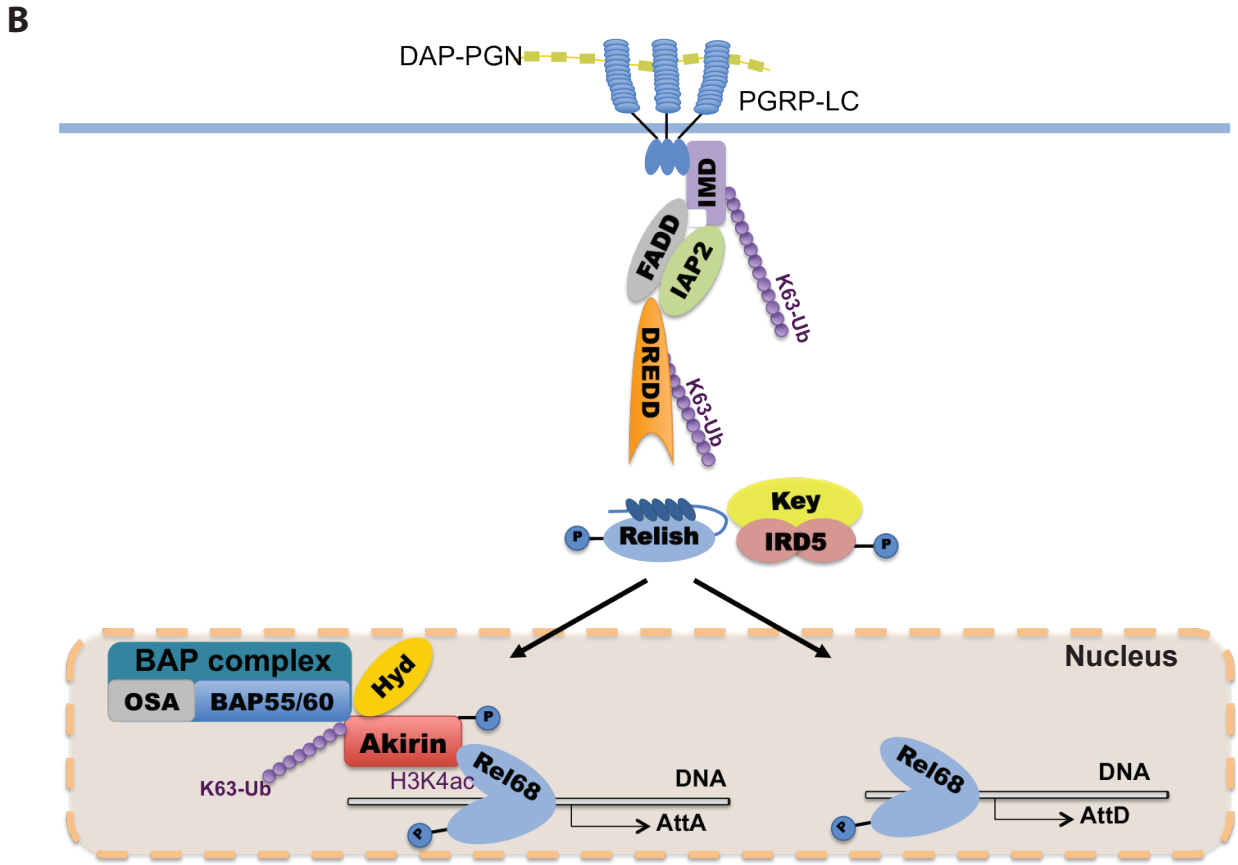
Ak2
UBR5
siRNA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BAP complex
OSA
BAP55/60
Hyd
Akirin
K63-Ub
H3K4ac
Rel68
DNA
AttA
Rel68
DNA
AttD

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. Distribution of Lysines present on Akirin

Illustration of the different lysine residues on protein sequence of Akirin, divided into the variable and conserved regions.

Fig. S5. Cellular localization of Akirin mutants (K156R, K173R and K183R)

Cellular localization of Akirin modified constructs. S2 cells were transiently transfected with pAC-Akirin-V5 constructs modified on K156R, K173R and K183R. Immunolocalization and

DAPI staining was done 4h after immune challenge by HKE. The cells were visualized by DIC. Images are representative of at least 3 samples. Scale bar: 10µm.

Fig. S6. 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 ± 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**
Relish	DRSC37194	TGCCATGTGGAGTGCATTAT	TGCCATGTGGAGTGCATTAT
Akirin	DRSC26196	ATCTTCCATCTGCAGCATCC	ACGGACTAGGTTCGGTGCTA
Hyd	DRSC28294	GCGACCGAATAAGTCCAGAG	GCCACACGACCAGAGGTTAT
bon	DRSC38123	AGCCAGAAGTCGAAGGTGAA	TTGCTCAGACTCAGCGAAGA
IAP2	DRSC38402	AAATCCATGTGATCTGCGGT	CCAGTGTAGCCAATTGTCCC
m-cup	DRSC28310	GCGACCGAATAAGTCCAGAG	GCCACACGACCAGAGGTTAT

Mkrn1	BKN24610	GATTGGTGTGTGCGTTTCAC	ATCGGCGAGATTATCATTGG
mura	DRSC26645	ATCTGGGTTTTGAGTGACCG	ATGAGTGATCGGGACAGAGG

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

Gene	**UniGene ID**	**siRNA ID**
Negative Control	-	AM4611
NFkB1	Hs.618430	s9504
Ak2	Hs.485915	s30221
UBR5	Hs.492445	s224201

Table S3. Oligonucleotides used for quantitative real-time PCR

For *Drosophila* S2 cells and adult flies

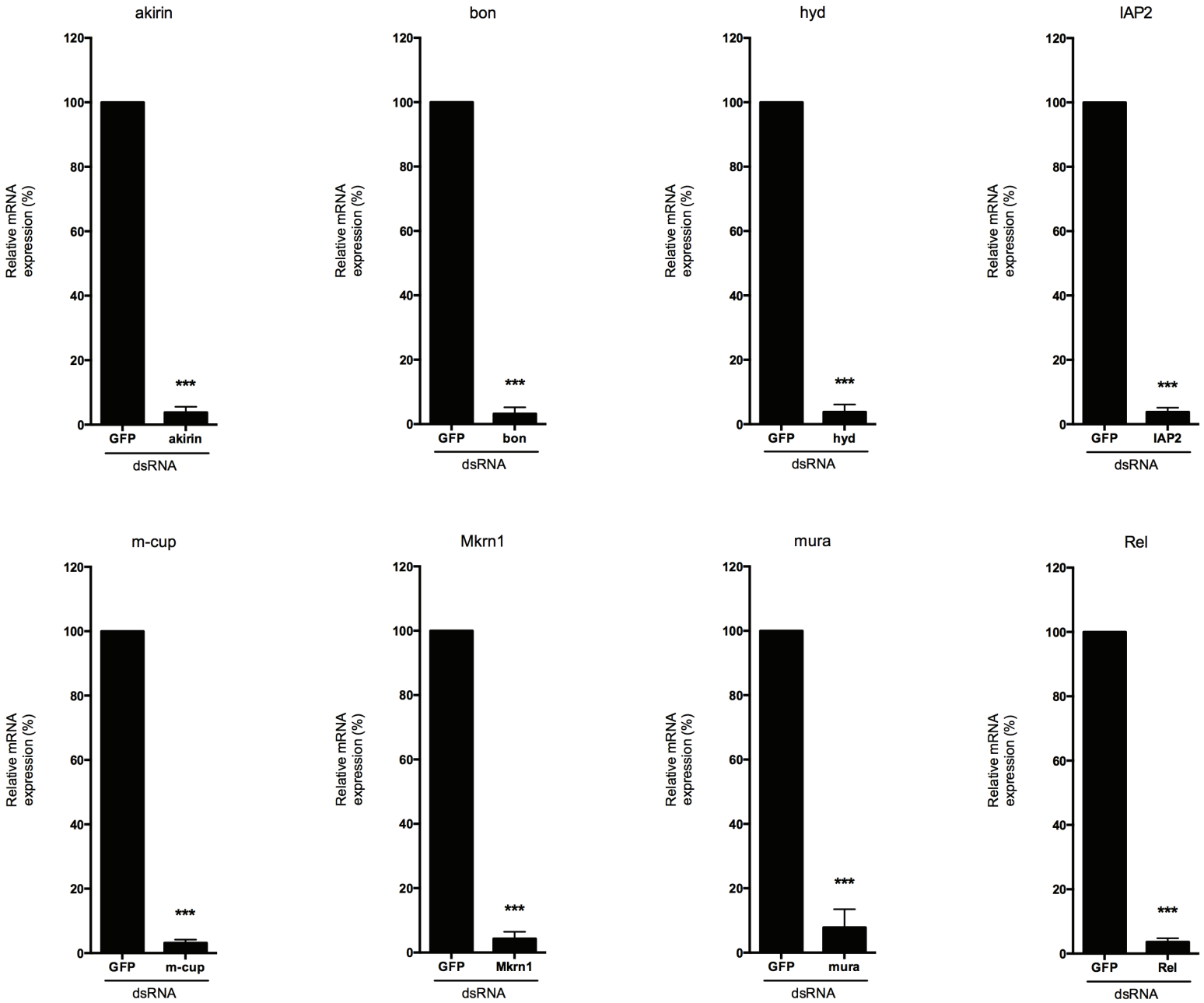
Gene	**Forward**	**Reverse**
Attacin-A	GGCCCATGCCAATTTATTCA	AGCAAAGACCTTGGCATCCA
Attacin-D	TTTATGGAGCGGTCAACGCCAATG	TGCAAATTGAGTCCTCCGCCAAAC
rp49	GACGCTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG
Relish	GGTGATAGTGCCCTGCATGT	CCATACCCAGCAAAGGTCGT
Akirin	CCGAACCTAGTCCGTTCAGTG	CTTGTGCAGTCTCTTGATCTCAT
HyD	GAGGTGGTTCTACAGGGCAAG	ATAAGGTCTTCGGGCACGTAA
Bon	AAAGGTCGGAGTCAAACTCTTCG	AAGGCATTCTAACAGCTTGGG
IAP2	CTCTTGTCCCGATCTCTTGTTG	GGTAGTAGAAACCTGCCTTTGC
m-cup	ACAAAGCTCAGTCACGACCTG	GACGAGAATCGCGGGGTAG

Mkrn1	AGACCATCTGCCGCTACTAC	TGCTGCTTGTACTAGGCTTCG
mura	ACTTGAACAACCCGTCCTCAT	GTTCGGAGTTTCCAAAGTGGTTA

For mammalian HeLa cells

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

Figure S1


akirin
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IAP2
m-cup
Mkrn1
mura
Rel
Relative mRNA expression (%)
120
100
80
60
40
20
0
***
GFP
dsRNA

Figure S2

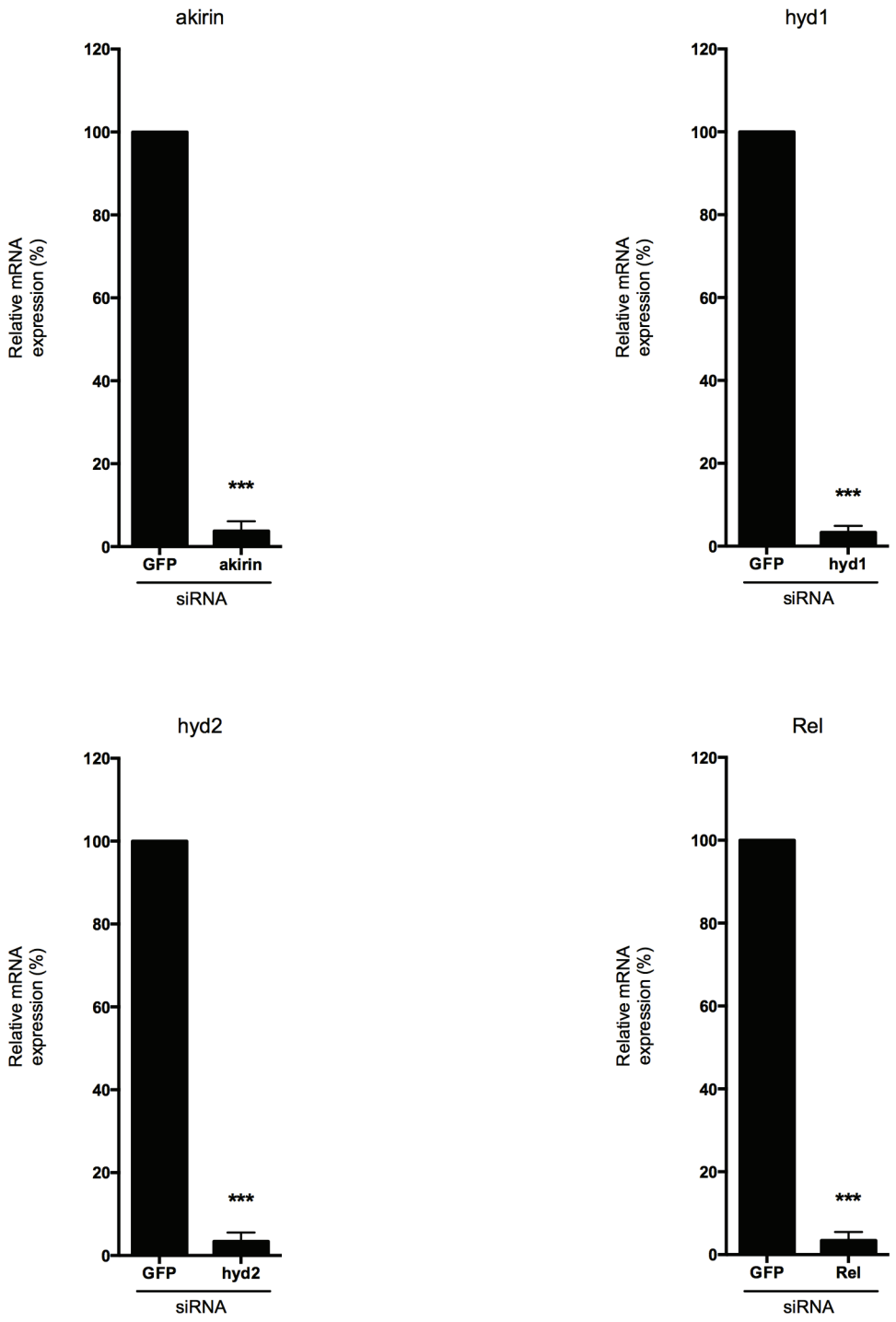

akirin
hyd1
hyd2
Rel
Relative mRNA expression (%)
120
100
80
60
40
20
0
***
GFP
akirin
hyd1
hyd2
Rel
siRNA

Figure S3

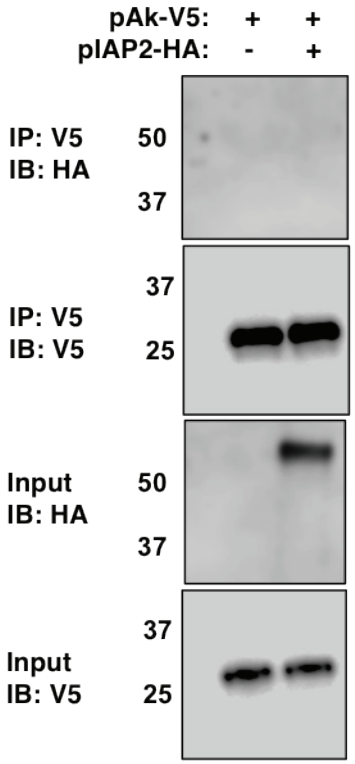
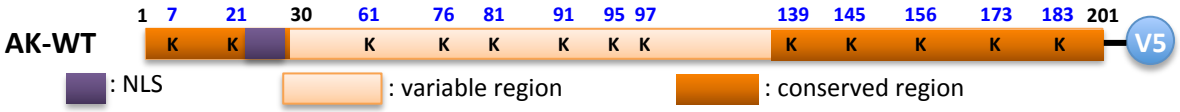

pAk-V5: + +
pIAP2-HA: - +
IP: V5
IB: HA
50
37
37
IP: V5
IB: V5
25
Input
IB: HA
50
37
37
Input
IB: V5
25

Figure S4



WT-AK	--K--K----------K----K---K-----K--K--K----------K----K----K------K---K--
K7R-AK	--R--K----------K----K---K-----K--K--K----------K----K----K------K---K--
K21R-AK	--K--R----------K----K---K-----K--K--K----------K----K----K------K---K--
K7/21R-AK	--R--R----------K----K---K-----K--K--K----------K----K----K------K---K--
K81R-AK	--K--K----------K----K---R-----K--K--K----------K----K----K------K---K--
K139R-AK	--K--K----------K----K---K-----K--K--K----------R----K----K------K---K--
K145R-AK	--K--K----------K----K---K-----K--K--K----------K----R----K------K---K--
K156R-AK	--K--K----------K----K---K-----K--K--K----------K----K----R------K---K--
K173R-AK	--K--K----------K----K---K-----K--K--K----------K----K----K------R---K--
K183R-AK	--K--K----------K----K---K-----K--K--K----------K----K----K------K---R--

Figure S5

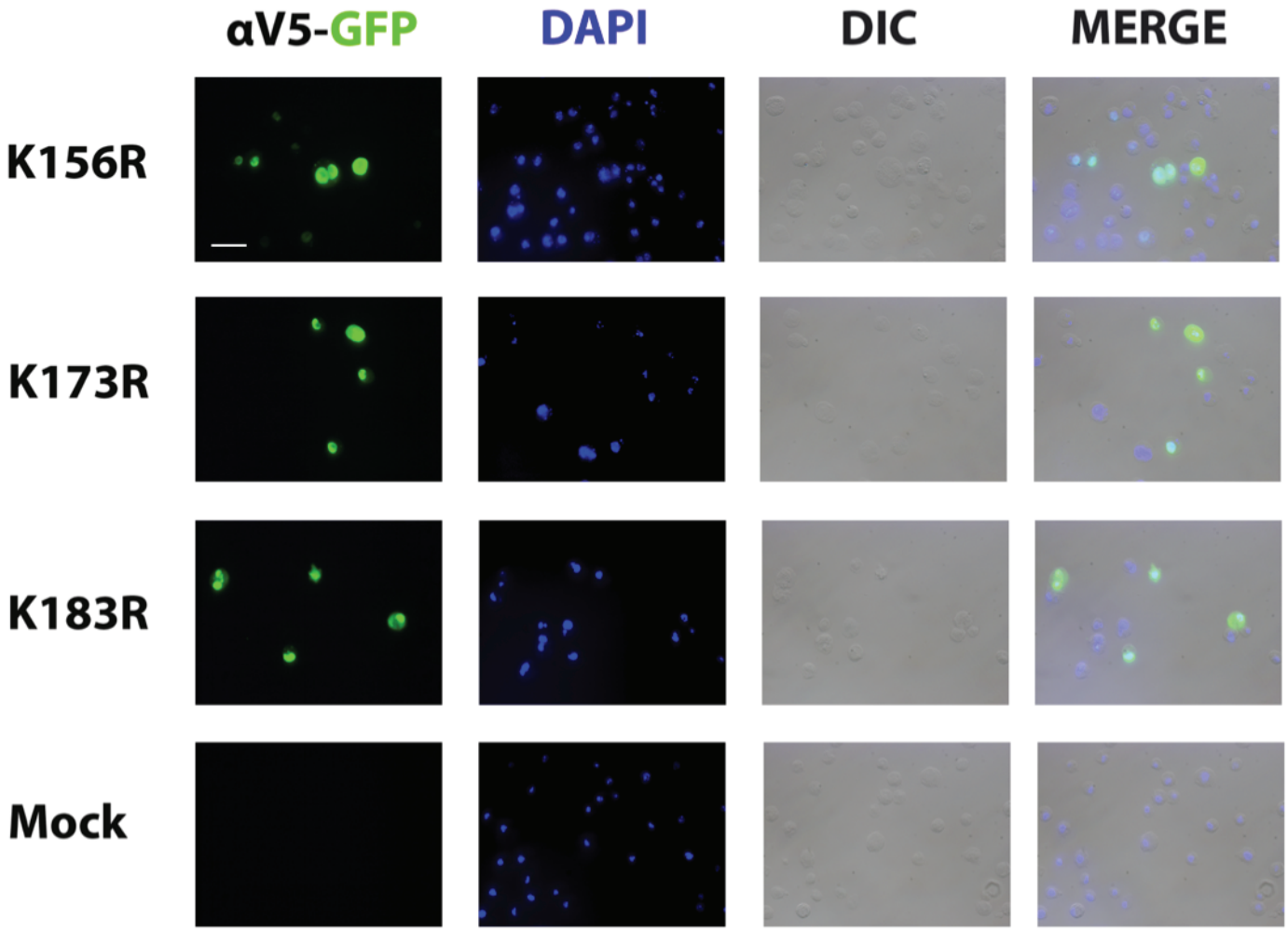
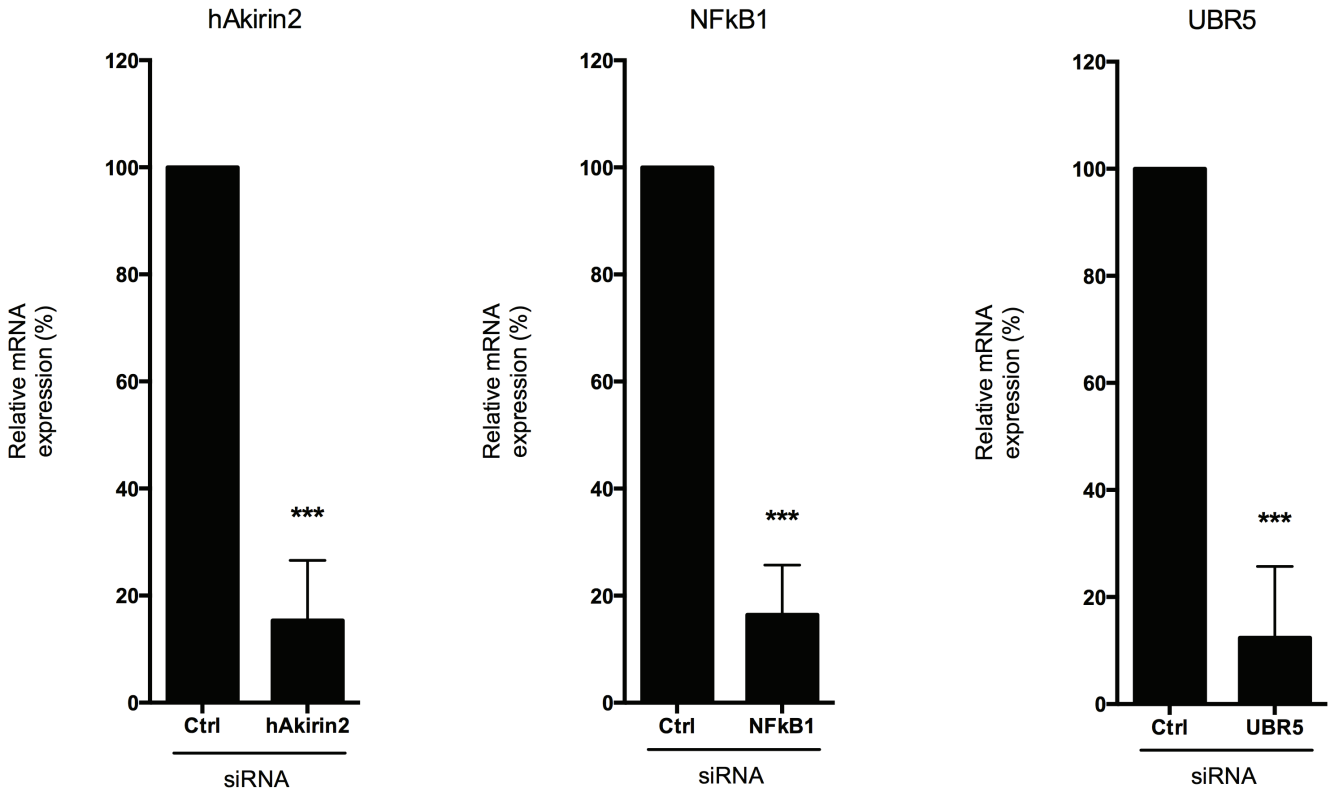


Figure S6


hAkirin2
NFkB1
UBR5
Relative mRNA expression (%)
120
100
80
60
40
20
0
***
Ctrl
hAkirin2
NFkB1
UBR5
siRNA