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An RNA interference screen identifies *Inhibitor of Apoptosis Protein 2* as a regulator of innate immune signalling in *Drosophila*

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Innate immunity in vertebrates and invertebrates is of central importance as a biological programme for host defence against pathogenic challenges. To find novel components of the *Drosophila* immune deficiency (IMD) pathway in cultured haemocyte-like cells, we screened an RNA interference library for modifiers of a pathway-specific reporter. Selected modifiers were further characterized using an independent reporter assay and placed into the pathway in relation to known pathway components. Interestingly, the screen identified the *Inhibitor of Apoptosis Protein 2* (*IAP2*) as being required for IMD signalling. Whereas loss of *DIAP1*, the other member of the IAP protein family in *Drosophila*, leads to apoptosis, we show that *IAP2* is dispensable for cell viability in haemocyte-like cells. Cell-based epistasis experiments show that *IAP2* acts at the level of Tak1 (transforming growth factor-β-activated kinase 1). Our results indicate that IAP gene family members may have acquired other functions, such as the regulation of the tumour necrosis factor-like IMD pathway during innate immune responses.

Keywords: innate immune responses; signalling; *Drosophila*; RNAi; functional genomics; apoptosis
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INTRODUCTION

Innate immunity is essential as a first-line defence mechanism against pathogenic challenges in most metazoans. During recent years, it has become clear that the molecular mechanisms that control and execute innate immune responses in humans have well-conserved counterparts in genetically tractable organisms (reviewed by Hoffmann & Reichhart, 2002). In particular, the analysis of signalling pathways in model organisms such as *Drosophila* has opened new avenues with which to understand and genetically dissect cellular processes that initiate innate immune responses.

Drosophila immunity, which is devoid of an adaptive response, relies mainly on two nuclear factor kappa-B (NF-κB) signalling pathways, commonly referred to as Toll and immune deficiency (IMD) pathways. Following microbial challenge, these pathways regulate the production of antimicrobial peptides by the fat body (an equivalent of the mammalian liver) and blood cells. The induced peptides are subsequently secreted in the haemolymph (blood; Hoffmann, 2003). Additionally, as in mammals, microbial challenges activate Jun amino-terminal kinase (JNK) and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathways, but their role in insect immune response is less understood (Boutros *et al*, 2002; Agaisse *et al*, 2003). The JAK–STAT pathway has also recently been implicated in antiviral responses (Dostert *et al*, 2005).

The IMD pathway in *Drosophila* is related to the mammalian tumour necrosis factor (TNF) signalling pathways, as it uses structurally and functionally similar components (Hoffmann & Reichhart, 2002). IMD signalling is mainly activated by Gram-negative bacteria, and loss-of-function mutants are susceptible to Gram-negative bacterial infection (for reviews, see Tzou *et al*, 2002; Hoffmann, 2003; Brennan & Anderson, 2004, and references therein). Bacterial patterns are recognized by the transmembrane receptor, peptidoglycan recognition protein-LC (PGRP-LC). Subsequently, activation of PGRP-LC initiates a signalling cascade that leads to the processing and nuclear translocation of the NF-κB protein Relish. Three proteins that contain the death domain (DD) are involved in Relish activation: the *Drosophila* homologue of Fas-associated death protein (FADD), the homologue of RIP adaptor proteins IMD and the caspase 8 homologue DREDD. Downstream of IMD, the *Drosophila* homologue of transforming growth factor-β-activated kinase 1 (Tak1) activates the signalosome equivalent consisting of IRD5 (IKKβ; immune response-deficient 5) and Kenny (IKKγ). Relish is phosphorylated by the active IKK complex and proteolytically cleaved. Activated Relish translocates to the nucleus and promotes transcription of a distinct set of antibacterial peptides genes, including *Diptericin* (*Dipt*), *Cecropin* (*CecA1* and *A2*), *Attacin* (*AttA*, *AttB* and *AttD*) and *Metchnikowin* (*Mtk*).

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Another effector arm of IMD signalling, branching off the IMD pathway at the level of Tak1, links the detection of Gram-negative bacteria to JNK signalling and leads to a rapid upregulation of cytoskeletal genes (Boutros *et al*, 2002).

Although most of the known components were identified by genetic screens or candidate gene approaches, significant gaps remain in the understanding of innate immune signalling pathways. A key advance in recent years has been the discovery and use of RNA interference (RNAi), which allows the silencing of genes through introduction of short, double-stranded RNAs (dsRNAs) homologous to endogenous messenger RNAs (Fire *et al*, 1998). RNAi has been successfully used to study gene function in invertebrate and mammalian cell culture (reviewed by Hannon & Rossi, 2004).

To identify new components of the IMD pathway, we performed an RNAi screen in cultured *Drosophila* haemocyte-like cells. We identified putative new regulators that were required to induce Rel-dependent reporter genes after *Escherichia coli* stimulation and mapped them in relation to known pathway components by cell-based epistasis analysis. We further characterized the function of the positive pathway regulator, Inhibitor of Apoptosis Protein 2 (IAP2). Surprisingly, our results show that IAP2, in contrast to other inhibitors of apoptosis protein family members, is not involved in apoptosis, but is required for the expression of NF-κB and JNK pathway-dependent target genes during innate immune responses.

RESULTS AND DISCUSSION

Identification of new IMD pathway components

The exposure of *Drosophila* SL2 cells to Gram-negative bacteria leads to an upregulation of immune-responsive transcripts, including antimicrobial peptides (Samakovlis *et al*, 1992; Boutros *et al*, 2002). To monitor IMD pathway activity, we fused approximately 350 bp promoter sequence of *Metchnikowin*, which contains Relish-specific binding sites (Senger *et al*, 2004), to a firefly luciferase gene (supplementary Fig 1A online). On transfection of the *mtk luc* reporter into SL2 cells and treatment with heat-inactivated *E. coli*, we observed an approximately 22-fold induction of firefly reporter gene activity (supplementary Fig 1B online), whereas a *Renilla* co-reporter gene constitutively expressed under the control of the viral IZ promoter (Invitrogen) was not significantly induced. While monitoring *mtk luc* induction, we screened an RNAi library (Hild *et al*, 2003; Boutros *et al*, 2004) and identified all previously known IMD pathway modifiers, as well as putative new regulators of IMD–Rel pathway activity. To confirm the requirement of selected candidate genes, we resynthesized dsRNAs and re-tested them using a second reporter system derived from the IMD signalling-responsive *attacinA* enhancer (Tauszig *et al*, 2000; Fig 1A,B). Confirmed candidates included the GTPase-activating protein GAP1, which reduces the IMD pathway reporter activity to 35%, a level similar to that observed with dsRNAs targeting *Key* or *Tak1*. GAP1 has been previously implicated in early embryonic development as a regulator of small GTPase pathways (Gaul *et al*, 1992). Another signalling factor identified is CG7417/ORF1, a gene homologous to a family of mammalian Tak1-binding proteins (TAB), which are required for Tak1-mediated NF-κB activation (Shibuya *et al*, 1996; Takaesu *et al*, 2000). CG7417 could function as an activator or an adaptor for Tak1 in the IMD pathway; however its role still remains to be characterized *in vivo* (Fig 1; supplementary Table 1 online).

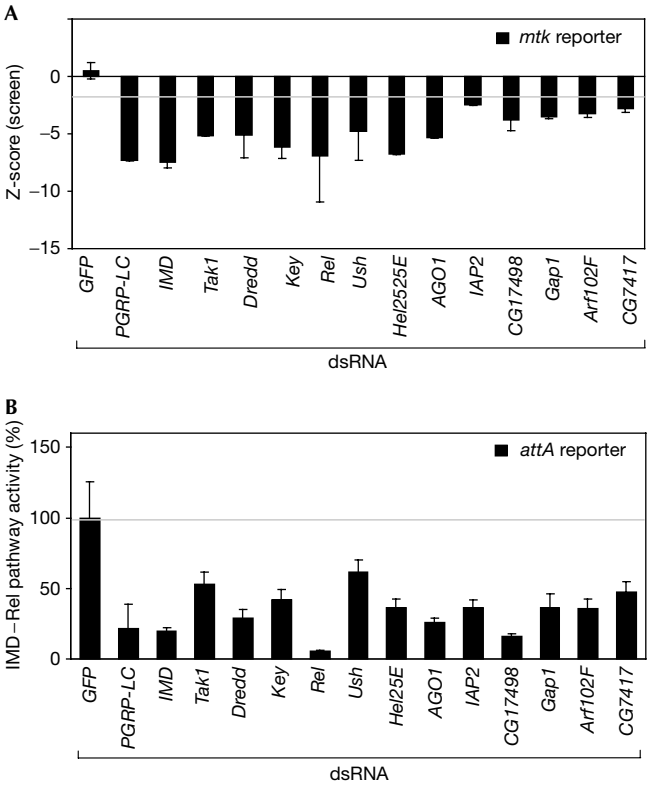

Fig 1 | Identification of new immune deficiency pathway components. (**A**) Effect of depletion of candidate transcript on *mtk* reporter induction. Depletion of known and new immune deficiency (IMD) pathway components by RNA interference (RNAi) leads to a significant reduction of IMD–Rel pathway activity (black bars). *Z*-scores were calculated as the number of median-adjusted standard deviations that a particular RNAi experiment differed from the median of all RNAi experiments in a 96-well plate. (**B**) Depletion of known and newly identified components leads to a reduction of the IMD pathway-dependent *attacineA* (*attA*) reporter. The percentage reduction of pathway activity after *Escherichia coli* stimulation as compared with a control double-stranded RNA (dsRNA; green fluorescent protein (GFP)) is shown. The pathway-specific reporter is normalized against a constitutive co-reporter. Error bars represent the standard deviation of three experiments.

We further analysed candidates by mapping their position within the IMD signalling cascade. We performed a cell-based epistasis analysis, activating the pathway ectopically by overexpression of IMD (Georgel *et al*, 2001) or constitutively active Rel (RelΔPEST; Stoven *et al*, 2003), whereas IMD pathway components and candidate genes were depleted by RNAi. Signalling activity was monitored with the *mtk luc* reporter. This approach correctly predicted the position of already known pathway components (Fig 2A,B). We found that the homologue of the mammalian Friend of GATA (FOG) protein U-shaped (Ush) acts downstream of IMD and at the same level as *Relish* (Fig 2A,B). Ush has been implicated in haemocyte differentiation (Fossett *et al*, 2001). Its depletion could lead to a misdifferentiation of the cells, which would then be unable to mount an immune response.

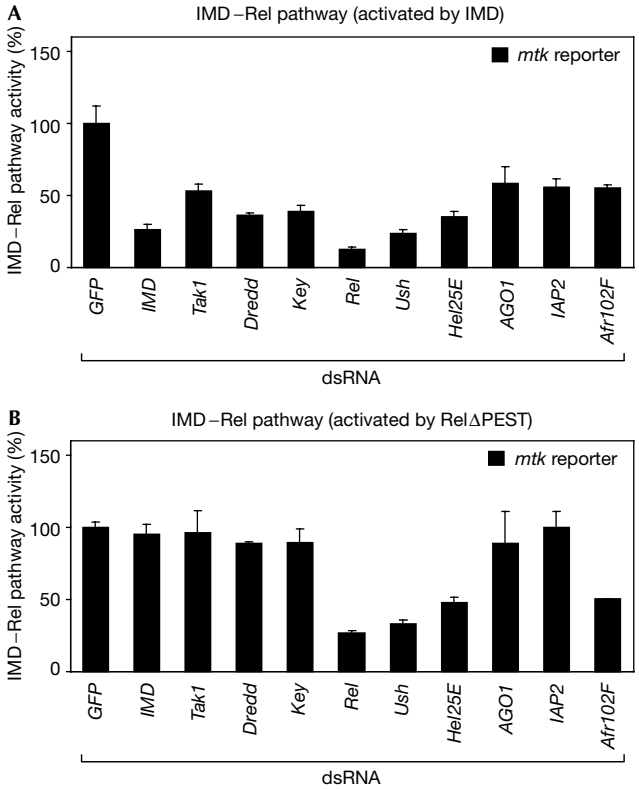

Fig 2 | Epistasis analysis mapping the position of candidates in the immune deficiency pathway. Immune deficiency (IMD)/Rel signalling is activated by expression of IMD (**A**) or a dominant active Relish protein (RelΔPEST) (**B**). Depletion of IMD–Rel cascade members downstream of *IMD* and upstream of *Relish* can block reporter induction by IMD but not by RelΔPEST (*IMD*, *Tak1*, *Dredd*, *Key*; *mtk* reporter). Double-stranded RNAs (dsRNAs) against Ush, Hel25E and Afr102F block the activation of the *mtk luc* reporter induced by IMD and Relish, indicating that they act at the level of Relish. IAP2 and Argonaute1 (AGO1) act between IMD and Relish, as their knockdown results in the loss of reporter induction by IMD. Error bars represent the standard deviation of four experiments.

Furthermore, Argonaute1 (AGO1), a component of the eukaryotic translation initiation factor 2 complex implicated in microRNA processing (Okamura *et al*, 2004), was identified as a modifier of the IMD signalling pathway, reducing its activity to 25% as compared with dsRNA against *GFP* (Fig 1). Its requirement in cultured haemocyte cells seems to be IMD pathway specific, as it was not identified as a factor for cell viability or other signalling pathways from screens (our unpublished data). Epistasis experiments place its action between IMD and Rel (Fig 2). Other factors that were identified are shown in Fig 2 and supplementary Table 1 online.

IAP2 is specifically required for IMD signalling

We further evaluated *IAP2* because of its domain composition, which is suggestive of a role as an anti-apoptotic factor (Hay *et al*, 1995). *IAP2* and *DIAP1* are members of a two-gene family in *Drosophila*, with highly conserved homologues in insects, mice and humans (Fig 3A; reviewed by Vaux & Silke, 2005). *DIAP1* is essential for cell survival and was shown to bind to, and thereby inhibit, effector caspases (Meier *et al*, 2000; Muro *et al*, 2002).

To confirm that *IAP2* is a positive regulator of the IMD pathway, we tested the effect of *IAP2* depletion on the induction of endogenous target genes by quantitative real-time reverse transcription–PCR (qPCR). SL2 cells were treated with dsRNA against *GFP* as a negative control, *IMD* as a positive control or *IAP2*. We then monitored expression levels of the IMD–Rel target genes *cec* and *mtk* in immune-stimulated and unstimulated cells. As shown in Fig 3B,C, RNAi against *IAP2* significantly reduced the levels of both target genes similarly to RNAi against *IMD* (Fig 3D,E).

In addition to the IMD pathway, *Drosophila* immunity relies on the Toll and the JAK–STAT signalling pathways. We tested for a putative implication of *IAP2* in Toll or JAK–STAT signalling using luciferase reporter assays (Tauszig *et al*, 2000; Muller *et al*, 2005; see the supplementary information online). Knockdown of known Toll or JAK–STAT pathway components showed a significant reduction of reporter induction, whereas dsRNA directed against *IAP2* or *GFP*, as a control, did not influence Toll or JAK–STAT pathway activity (Fig 3F,G). These results indicate that *IAP2* is specifically involved in the IMD signalling pathway but no other known immune-responsive pathways.

As IAPs have been widely implicated as regulators of cell death (Deveraux & Reed, 1999), we investigated whether IAP2 functions as a regulator of cell viability. Therefore, we quantified cell proliferation after 5 days of RNAi against an unrelated factor (*GFP*), *DIAP1* and *IAP2*. As shown in Fig 4A, RNAi targeting *DIAP1* led to a decrease of cell numbers from 0.5 million seeded cells to less than 20,000 live cells after 5 days. This is consistent with previous reports, which showed the essential role of *DIAP1* in cell viability (Muro *et al*, 2002). In contrast, *IAP2*-depleted samples, similarly to *GFP* RNAi, showed an increase in cell numbers from 0.5 to 2.2 million. This indicates that IAP2, in contrast to DIAP1, is dispensable for cell viability. These results were confirmed by propidium iodide (PI) staining using fluorescence-activated cell sorting (FACS) analysis (Fig 4B) and viability assays monitoring intracellular ATP levels (supplementary Fig 2 online). Taken together, these results show that *IAP2* is a regulator of IMD signalling and is not required for cell growth or survival.

IAP2 acts upstream or at the same level as Tak1

Our results indicated that *IAP2* is required for the IMD–Rel branch and cell-based epistasis mapped it downstream of *IMD* and upstream of *Relish* (Figs 1,2). As the IMD pathway branches at the same level or downstream of *Tak1* into Rel- and JNK-dependent signalling, we examined whether *IAP2* is also required for activation of the JNK branch. Thus, we monitored the expression of the IMD/JNK-specific target genes *Puckered* and *Matrix metalloproteinase 1* (*Mmp-1*; Boutros *et al*, 2002) by qPCR. As shown in Fig 4C and supplementary Fig 3 online, depletion of *IAP2* by RNAi disrupts *Mmp-1* and *puc* induction after innate immune stimuli to a level similar to that of knockdown of known factors specific for the IMD–JNK branch (*Mkk4/hep*). These experiments, together with the epistasis experiments, support a model whereby IAP2 acts, similarly to Tak1, downstream of IMD and upstream or at the level of the branching point of the IMD–Rel and IMD–JNK signalling arms (Fig 4D).

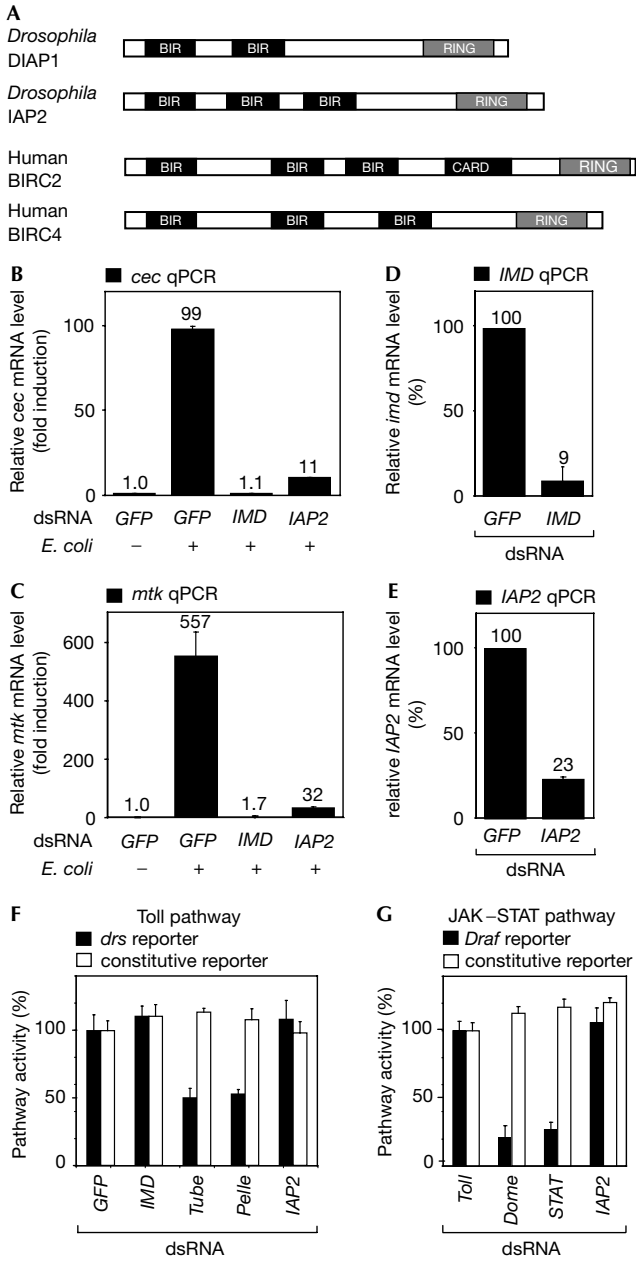

Fig 3 | Inhibitor of Apoptosis Protein 2 is specifically required for immune deficiency signalling. (**A**) Domain structure of Inhibitor of Apoptosis Protein 2 (IAP2). *Drosophila* proteins DIAP1 and IAP2 and two human IAPs, BIRC2 (BIR-containing protein 2) and BIRC4 are depicted. Two or three BIR motifs located close to the amino terminus are characteristic features of all IAPs. (**B**,**C**) Quantitative real-time reverse transcription–PCR (qPCR) experiments monitoring the effect of *IAP2* RNA interference (RNAi) on endogenous immune deficiency (IMD)–Rel target gene induction after an immune stimulus. The messenger RNA levels of the IMD–Rel target genes *cecropinA2* (*cec*) and *Metchnikowin* (*Mtk*) are induced 99- and 557-fold in response to bacterial induction, respectively (*GFP*, +). Depletion of IMD by RNAi inhibits induction of *cec* and *mtk* expression (*IMD*, +). Similarly, *IAP2* RNAi significantly reduces expression of both peptides in response to a bacterial challenge (*IAP2*, +). (**D**,**E**) *IMD* and *IAP2* levels are strongly reduced by the corresponding double-stranded RNA (dsRNA). (**F**) Test for requirement of IAP2 in the Toll pathway in SL2 cells. Toll signalling is reduced when components of the Toll pathway are knocked down by RNAi (*Tube*, *Pelle*). Depletion of unrelated factors (*GFP*), components of the IMD pathway (*IMD*) or *IAP2* does not affect Toll signalling activity (black bars, *Drosomycin* reporter). (**G**) Test of IAP2 activity in the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway in S2R$^+$ cells. JAK–STAT signalling is disturbed when components of the JAK–STAT cascade are depleted by RNAi (*Dome*, *STAT92E*; black bars, *2x6xDraf* luc reporter). Signalling is not blocked when unrelated factors (*Toll*) or IAP2 are knocked down by RNAi (*IAP2*). Error bars represent standard deviation of three independent replicates.

In conclusion, we identified several new components of the IMD innate immune pathway. Our experiments implicate several signalling factors in the control of IMD-dependent responses in haemocyte-like cells, including a GTPase-activating protein, a homologue of the mammalian Tak1-binding protein, and several proteins involved in RNA binding and processing. Their role in *Drosophila* immune response *in vivo* remains to be characterized. Strikingly, the screen identifies *IAP2*, a member of the Inhibitor of Apoptosis Protein family, as being required for *Drosophila* innate immune signalling. We show that IAP2 is specifically involved in the IMD signalling pathway, as it disrupts the induction of the IMD–Rel and IMD–JNK pathway target genes and is not required for other immune-induced pathways, such as Toll and JAK–STAT. Cell-based epistasis analysis and qPCR experiments monitoring the IMD–JNK branch suggest a function of IAP2 downstream of IMD and upstream or at the same level as Tak1. Although most previously characterized IAPs were shown to act as inhibitors of caspases (Deveraux & Reed, 1999), it is unlikely that the role of IAP2 is to inhibit DREDD, the caspase implicated in IMD signalling. If this were correct, depletion of IAP2 should lead to an enhancement of pathway activity after immune stimulus or to a constitutive expression of target genes without an immune stimulus, which is not the case. As human Tak1 has been shown to be activated by polyubiquitination (Wang *et al*, 2001), and it has recently been shown that ubiquitination is required for the activation of Tak1 and the IKK complex in *Drosophila* (Zhou *et al*, 2005), we might speculate that IAP2 may have a role in Tak1 ubiquitination through its RING domain. Whether mammalian IAPs have a role in innate immune responses remains to be established.

METHODS

Cell culture. *Drosophila* SL2 and S2R$^+$ cells were cultured in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (PAA) and 1% penicillin–streptomycin (Invitrogen) at 25 °C.

RNA synthesis, RNA interference and luciferase assay. dsRNA synthesis, RNAi treatment and luciferase experiments were performed, as described before (Muller *et al*, 2005). Complete primer and amplicon sequence information can be found at http://rnai.dkfz.de. Details on used reporter constructs, dsRNA concentration and cells can be found in the supplementary information online. SL2 cells were transiently transfected with specific reporter and expression constructs using Effectene or

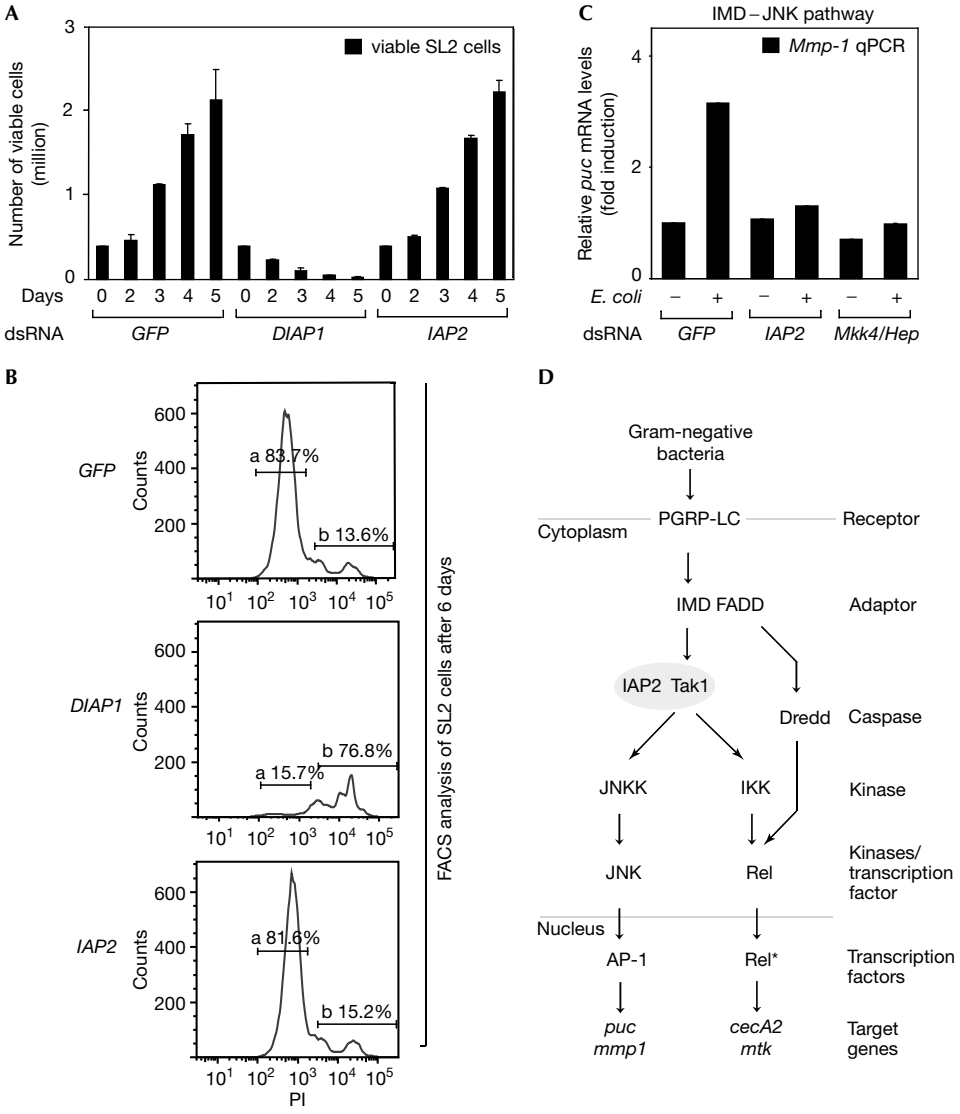

Fig 4 | Inhibitor of Apoptosis Protein 2 is dispensable for cell viability and required for immune deficiency/Jun amino-terminal kinase branch. (**A**) Haemocytometer count monitoring the growth of SL2 cells treated with *GFP*, *DIAP1* and *IAP2* double-stranded RNA (dsRNA) from 0 to 5 days. RNA interference (RNAi) targeting a factor not involved in maintenance of viability (*GFP*) does not affect cell growth, whereas depletion of *DIAP1* by RNAi causes severe growth defects. Knockdown of *IAP2* by RNAi does not influence cell growth. (**B**) Fluorescence-activated cell sorting (FACS) assays comparing the effects of *GFP*, *DIAP1* and *IAP2* RNAi on SL2 cell viability. At 6 days after RNAi treatment, cells were stained with propidium iodide (PI) and analysed by FACS. About 84% of cells were viable when treated with an unrelated control dsRNA (*GFP*; gate a, top panel). Only 16% of cells treated with dsRNA depleting *DIAP1* were viable on day 6 (gate a, middle panel), in contrast to 82% living cells in *IAP2* dsRNA-treated samples (gate a, bottom panel). Gate a: PI-negative events, viable cells; gate b: PI-positive events, dead cells and debris. (**C**) Quantitative real-time reverse transcription–PCR (qPCR) analysis of Inhibitor of Apoptosis Protein 2 (IAP2) function in JNK signalling in SL2 cells. Messenger RNA levels of the JNK target gene *Matrix metalloproteinase 1* (*Mmp-1*) increase after stimulation with heat-inactivated *Escherichia coli* for 1 h (*GFP*, +). Knockdown of JNK components (*Mkk4/hep*) blocks induction of the IMD–JNK cascade (*Mkk4/hep*, +). Similarly to *Mkk4/hep* RNAi, *IAP2* knockdown suppresses induction of IMD–JNK signalling (*IAP2*, +). Error bars represent standard deviation of two replicates. If not indicated otherwise, error bars represent standard deviation of four replicates. (**D**) Model placing IAP2 function at the level of Tak1 (transforming growth factor-β-activated kinase 1) in the IMD signalling pathway.

Cellfectin according to the manufacturer's instructions. When applicable, immune stimulation of cells was performed by adding heat-inactivated *E. coli* (DSM498) to a final concentration of 20 μg/ml. At 16 h after stimulation, luciferase activity was measured.

RNA extraction and quantitative real-time reverse transcription–PCR. Total RNA from SL2 cells was isolated using Qiagen Shredder and RNeasy Mini columns or Trizol (Invitrogen) according to the manufacturer's description. A 5 μg portion of total RNA was treated with DNase I (Fermentas, St Leon-Rot,

Germany) for 30 min before reverse transcription with Superscript II (Invitrogen) and oligo(dT)$_{12–18}$ (Invitrogen). qPCR was performed using LightCycler 1.0 and LightCycler 480 instruments, TaqMan Master Kit, and the *Drosophila* Universal ProbeLibrary (Roche Applied Science, Mannheim, Germany). *Rp49* levels were used to normalize the data. Protocols for RNAi treatment and induction, as well as primer sequences and probe information, are provided in the supplementary information online.

Fluorescence-activated cell sorting and cell viability analysis. Flow cytometry analysis was performed in SL2 cells after RNAi treatment in triplicate in 96-well tissue culture plates (Falcon, BD Biosciences, Heidelberg, Germany; as described in the supplementary information online). Cells were incubated for 6 days at 25 °C. Cells were carefully resuspended in 100 µl complete medium and diluted 1:1 with staining solution (PBS, 4 µg/ml PI) to a final volume of 200 µl. Samples were analysed by flow cytometry (FACSArray, BD Biosciences), sampling 40 µl with a maximum of 20,000 ungated events per probe. To determine total PI-negative and PI-positive cell numbers, events were gated using FlowJo (TreeStar, Ashland, OR, USA) software.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

Note in proof. Kleino *et al* (2005) Inhibitor of apoptosis 2 and TAK1-binding protein are components of the *Drosophila* Imd pathway. *EMBO J* (in press; doi:10.1038/sj.emboj.7600807).

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