

The RING-finger scaffold protein Plenty of SH3s targets TAK1 to control immunity signalling in *Drosophila*

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Imd-mediated innate immunity is activated in response to infection by Gram-negative bacteria and leads to the activation of Jun amino-terminal kinase (JNK) and Relish, a nuclear factor- κ B transcription factor responsible for the expression of antimicrobial peptides. Plenty of SH3s (POSH) has been shown to function as a scaffold protein for JNK activation, leading to apoptosis in mammals. Here, we report that POSH controls Imd-mediated immunity signalling in *Drosophila*. In POSH-deficient flies, JNK activation and Relish induction were delayed and sustained, which indicated that POSH is required for properly timed activation and termination of the cascade. The RING finger of POSH, possessing ubiquitin-ligase activity, was essential for termination of JNK activation. We show that POSH binds to and degrades TAK1, a crucial activator of both the JNK and the Relish signalling pathways. These results establish a novel role for POSH in the *Drosophila* immune system.

Keywords: JNK; POSH; Relish; TAK1; Imd

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INTRODUCTION

Innate immunity is an evolutionarily conserved defence mechanism against infectious microorganisms (Hoffmann *et al*, 1999; Akira, 2001). Genetic approaches in *Drosophila* have uncovered two distinct pathways that are mediated by Toll and Imd (Hoffmann, 2003; Vodovar *et al*, 2004). The Toll pathway is primarily activated in response to infection by fungi and Gram-positive bacteria, whereas the Imd pathway is predominantly activated by Gram-negative bacteria, and in turn activates the Jun amino-terminal kinase (JNK) signalling cascade and a nuclear factor- κ B (NF- κ B) transcription factor, Relish, which induces the

expression of a battery of genes encoding antimicrobial peptides (Lemaitre *et al*, 1995).

Among the components of the Imd pathway, TAK1 plays a crucial role as an activator of both the JNK and the Relish pathways (Vidal *et al*, 2001; Silverman *et al*, 2003; Park *et al*, 2004). Flies that are deficient in TAK1 do not produce antibacterial peptides and are therefore highly susceptible to Gram-negative bacterial infection (Vidal *et al*, 2001). In *Drosophila* S2 cells, TAK1 has been shown to be required for the peptidoglycan-induced activation of I κ B kinase (IKK) and JNK (Leulier *et al*, 2003; Silverman *et al*, 2003; Kaneko *et al*, 2004). TAK1 is also important for limiting the duration of JNK activation, thereby resulting in a transient JNK-dependent response that precedes the sustained induction of the Relish-dependent genes.

Although it has been shown that proteasomal degradation of TAK1 leads to the rapid termination of JNK signalling (Park *et al*, 2004), the degradation mechanism has yet to be entirely clarified, and a ubiquitin ligase that promotes TAK1 degradation has not been reported. Here, we identify Plenty of SH3s (POSH) as a crucial component that controls the termination of immunity signalling in *Drosophila*. POSH, which was initially isolated as a Rac1-interacting protein, consists of a RING-finger domain and four SH3 domains, and has been shown to function as a scaffold protein for JNK signalling components leading to apoptosis in mammals (Tapon *et al*, 1998; Xu *et al*, 2003). In *Drosophila*, overexpression of POSH in imaginal discs activates JNK, which results in various defects in adult morphology (Seong *et al*, 2001). Using flies that are deficient in *POSH*, we provide evidence that POSH is required for the properly timed activation and termination of Imd-mediated immune responses, and that the RING finger of POSH, which shows ubiquitin-ligase activity, is essential for this regulation.

RESULTS AND DISCUSSION

To assess the role of POSH *in vivo*, we created a null allele of *POSH* by the excision of a flanking *P*-element in *EP(2)1026*. We obtained a mutant bearing a deletion that included the start codon and extended 1,282 nucleotide bases into the coding region of *POSH* (supplementary Fig 1A,B online). Northern blot analysis

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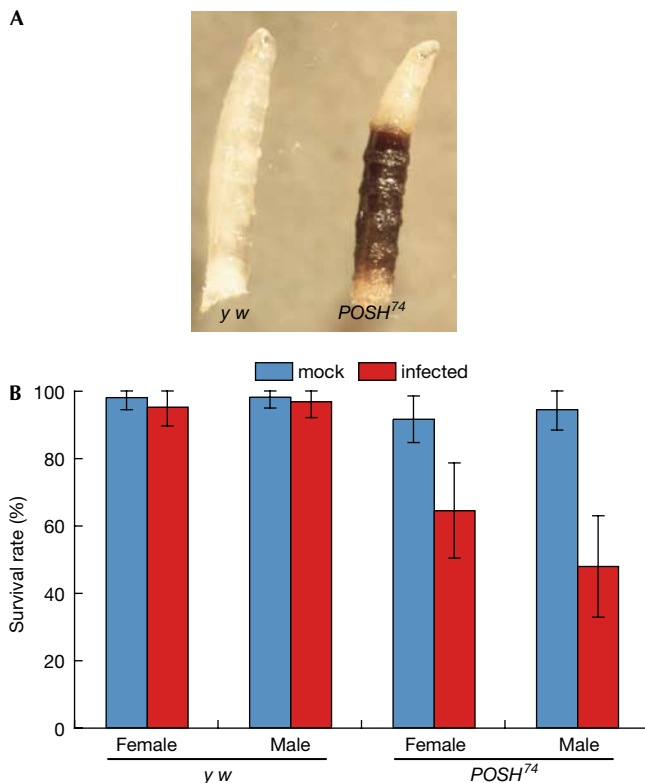


Fig 1 | Plenty of SH3s-deficient flies show defects in the innate immune system. (A) Melanized larvae observed in *POSH*⁷⁴ stocks. (B) Viability of flies infected with Gram-negative bacteria. Wild-type and *POSH* mutant adult flies were injected with a needle dipped in *Escherichia coli* culture. Viability was determined at 3 days after infection.

indicated that *POSH* transcripts were not detectable in *POSH*⁷⁴ homozygous animals (supplementary Fig 1C online). Most *POSH*⁷⁴ homozygous individuals survived embryogenesis, and the JNK cascade operating during dorsal closure remained intact (data not shown; Harden, 2002). Furthermore, *POSH*⁷⁴ showed no genetic interaction with the JNK pathway components in embryos (data not shown). Taken together, these results indicate that not every instance of JNK signalling requires POSH as a scaffold. Although mutants showed no obvious developmental defects, we observed that adult flies did not live as long as control flies, and that older cultures often contained dead larvae and pupae that were heavily melanized (Fig 1A). In addition, the viability of *POSH*-deficient flies after injection with *Escherichia coli* was significantly lower than that of wild type (Fig 1B). A possible cause for such phenotypes is defective immune function, and we therefore searched for a role for POSH in the immune response.

We tested whether the TAK1-mediated immune response was compromised by the *POSH*⁷⁴ mutation. Infection with *E. coli* activates TAK1, which consequently phosphorylates JNK and induces the expression of Relish/NF- κ B, resulting in the subsequent induction of a battery of genes encoding antimicrobial peptides, such as Diptericin (Park et al, 2004). Northern blot analysis indicated that the induction of *relish* and *diphtericin* (*dpt*) messenger RNAs after *E. coli* infection was significantly delayed in *POSH*⁷⁴ flies compared with that in controls (Fig 2A). Interest-

ingly, we also observed that expression of *relish* was sustained for a longer period after infection in *POSH*⁷⁴ flies.

To investigate the effects of the *POSH* mutation more quantitatively, we carried out real-time PCR analysis of immune response genes (Fig 2B,C). Consistent with northern blot analysis, the induction of *dpt* was delayed, and *relish* induction was sustained, with a maximum expression at 2 h after infection. We also analysed the expression pattern of *attacin A* (*attA*), which is dependent on Relish (Fig 2D). Induction of *attA* was almost normal, but unlike in the control flies, its expression level continued to increase up to 4 h after infection. The maximum level of expression was twice that of control flies, consistent with Relish activity being elevated in *POSH* mutant flies. We also analysed the expression profile of *puckered* (*puc*), a target gene of the JNK pathway (Fig 2E). Induction of *puc* was slightly delayed, with the highest level of expression at 1 h after infection, and expression was sustained for a longer period in *POSH*⁷⁴ flies compared with that in controls. These results indicate that POSH is required for properly timed activation and rapid termination of both JNK- and Relish-mediated immune response pathways.

TAK1 has been shown to be required for the maintenance of *relish* mRNA expression, and its proteasomal degradation is responsible for the rapid termination of Relish and JNK signalling (Park et al, 2004). POSH contains a CH4C4-type RING-finger domain near its N terminus. It has been shown that the RING-finger domain of mammalian POSH possesses E3 ubiquitin-ligase activity and regulates the level of POSH protein through the proteasomal pathway (Xu et al, 2003). We confirmed that *Drosophila* POSH also had E3 ubiquitin-ligase activity, by demonstrating its auto-ubiquitination (Fig 3A). To assess the functional importance of the RING domain, we constructed *POSH*^{mmR} bearing point mutations in the RING domain, and *POSH*^{AR} lacking the RING-finger domain altogether. Neither of these proteins was able to auto-ubiquitinate after expression in cultured *Drosophila* cells (S2). It is conceivable, therefore, that POSH is responsible for the feedback regulation of the immune response through TAK1 ubiquitination and its subsequent degradation.

To test this hypothesis, we carried out a glutathione S-transferase (GST) pull-down assay to investigate whether POSH was able to form a complex with TAK1. A bacterially expressed GST-POSH fusion protein pulled down TAK1 protein generated by *in vitro* transcription/translation (Fig 3B). We also tested the interaction between POSH and TAK1 in S2 cells by immunoprecipitation assay (Fig 3C). Lysate from cells co-transfected with POSH and haemagglutinin (HA)-tagged TAK1 (HA-TAK1) was immunoprecipitated with an anti-POSH antibody and then subjected to immunoblot analysis with an anti-HA antibody. HA-TAK1 was precipitated with an anti-POSH antibody, but not from the lysate of cells lacking the POSH expression construct (Fig 3C). These results indicate that POSH is able to bind to TAK1. To determine whether POSH could ubiquitinate TAK1, we transfected S2 cells with HA-TAK1, ubiquitin and either POSH or RING-defective POSH. HA-TAK1 was immunoprecipitated with an anti-HA antibody and then subjected to immunoblot analysis with an anti-ubiquitin antibody. This assay showed that the RING finger of POSH was able to ubiquitinate TAK1 (Fig 3D). Finally, we examined the effect of POSH overexpression on TAK1 stability using a pulse-chase experiment (Fig 3E). HA-tagged TAK1 was

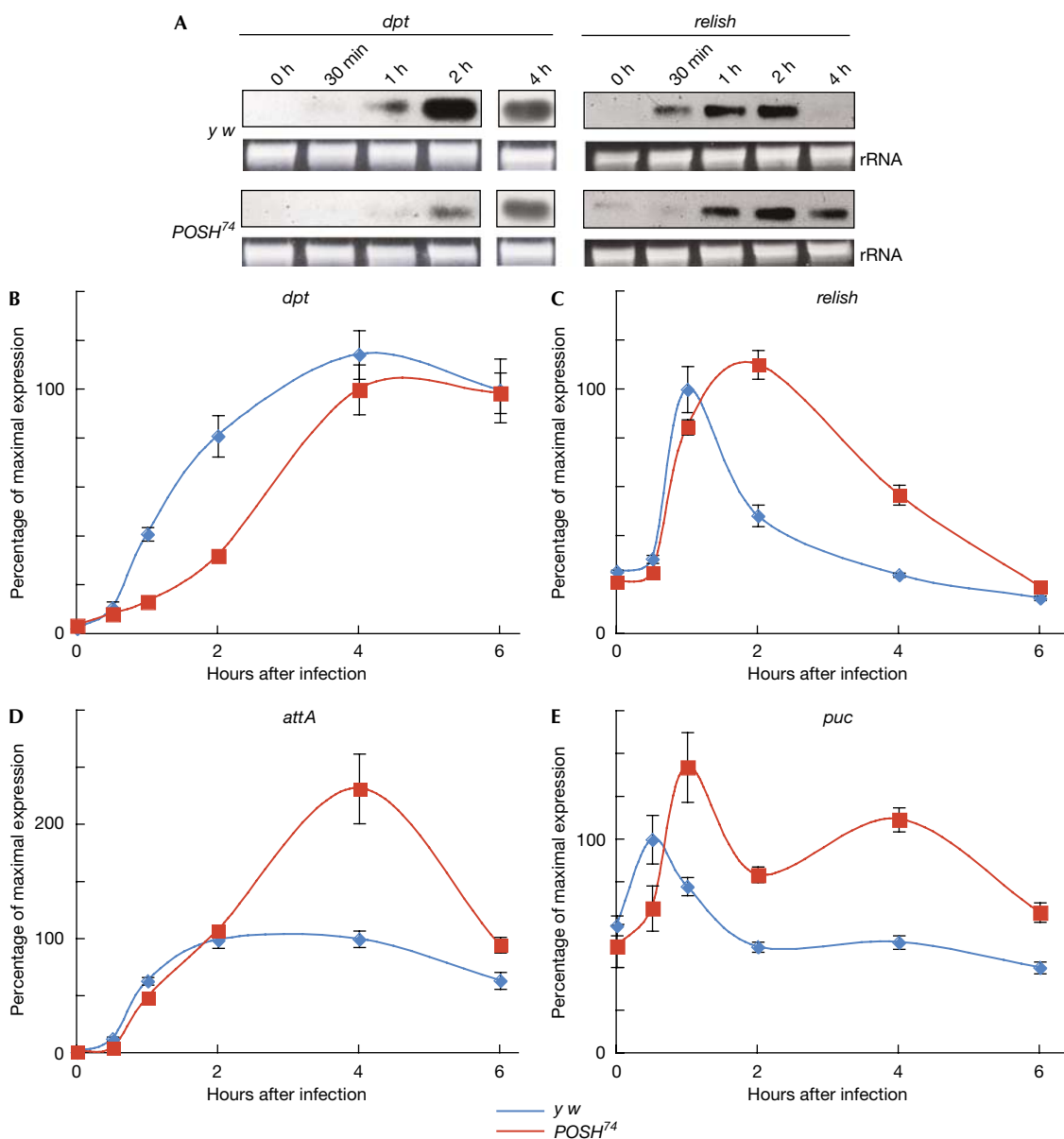


Fig 2 | The immune response induced by bacterial infection is compromised in *POSH⁷⁴* mutants. (A) Northern blot analysis of immune response genes, *dipericin* (*dpt*) and *relish*. Total RNA was isolated at an indicated time point after *Escherichia coli* infection and subjected to northern blot analysis. The inductions of both *dpt* and *relish* are significantly delayed compared with those in control flies. Expression of *relish* is sustained for a longer period in *POSH⁷⁴*. rRNA, ribosomal RNA. (B–E) Real-time PCR analysis of immune response genes, *dpt*, *relish*, *attacin A* (*attA*) and *puckered* (*puc*). In *POSH⁷⁴*, induction of *dpt* is delayed (B), *relish* expression is sustained for a longer period (C) and induction of *attA*, the expression of which depends on Relish, is slightly delayed, but its expression level at 4 h after infection is higher than that of the control (D). Expression of *puc*, a Jun amino-terminal kinase target gene, is delayed and sustained in *POSH⁷⁴* (E).

pulse labelled with [³⁵S]methionine for 1 h and then chased under various conditions. Pulse-labelled HA-TAK1 was detected in S2 cells after 45 min of chasing, but not in POSH-overexpressing S2 cells at the same time point, which suggests that POSH promotes TAK1 degradation. In contrast, HA-TAK1 was exceptionally stable in S2 cells expressing *POSH^{mR}*, a mutant form lacking ubiquitin-ligase activity. Overexpression of *POSH^{mR}* might exert a dominant-negative effect on interactions between any endogenous POSH

and TAK1, and thereby inhibit the degradation of TAK1. These results indicate that the RING-finger function of POSH is essential for the degradation of TAK1.

To characterize further the role of POSH in the regulation of JNK signalling, we used immunocompetent S2 cells in an *in vitro* model system used to study the immune response triggered by peptidoglycan in a commercial lipopolysaccharide preparation (Leulier et al, 2003; Silverman et al, 2003; Kaneko et al, 2004).

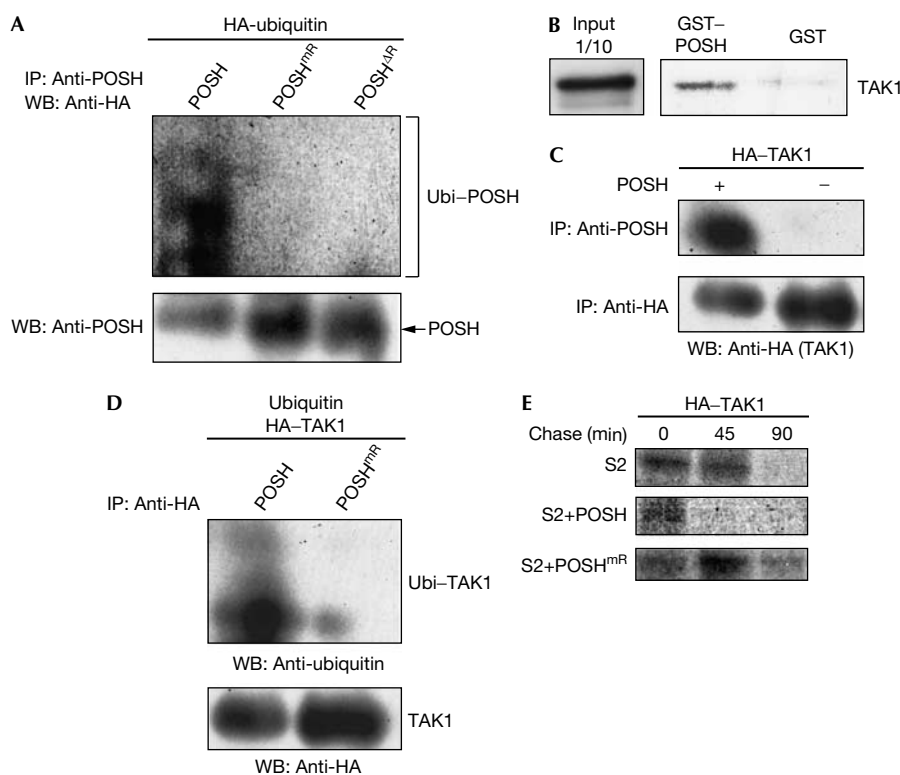


Fig 3 | Plenty of SH3s binds to and affects TAK1 stability by proteasomal degradation. (A) Auto-ubiquitination occurs with wild-type Plenty of SH3s (POSH), but not with POSH^{mR} harbouring point mutations in the RING domain or with POSH^{ΔR} lacking the RING-finger domain altogether. HA, haemagglutinin; IP, immunoprecipitation; WB, western blotting; Ubi, ubiquitin (B) Glutathione *S*-transferase (GST) pull-down assay showing that POSH binds to *in vitro* transcribed/translated TAK1. (C) Interaction between POSH and TAK1 in S2 cells. TAK1 and POSH were transfected into S2 cells, and co-immunoprecipitated with an anti-POSH antibody. (D) Ubiquitination of TAK1 by POSH, but not by POSH^{mR}. S2 cells were transfected with ubiquitin, HA-TAK1 and either POSH or POSH^{mR}. Lysates were prepared and incubated for 1 h at 4 °C with an anti-HA antibody. Immunoprecipitates were subjected to western blot analysis with either an anti-ubiquitin or an anti-HA antibody. (E) Pulse-chase experiments showing POSH-dependent degradation of TAK1. Cells were pulse labelled for 1 h in 500 μl of PBS containing 100 μCi [³⁵S]methionine and then chased with Schneider's medium supplemented with 10% fetal bovine serum. The cells were then lysed, and HA-TAK1 protein was immunoprecipitated with an anti-HA antibody and analysed by SDS-polyacrylamide gel electrophoresis, followed by fluorography. HA-TAK1 is rapidly degraded in cells transfected with POSH, but not with POSH^{mR}.

Consistent with *puc* expression results in *POSH* mutant flies, the reduction of POSH by RNA interference (RNAi) significantly decreased the level of JNK activation, but it was sustained for a longer period than in control cells (Fig 4A). In contrast, overexpression of POSH induced JNK activation at a high level, but then inactivated it rapidly. When POSH^{mR} was transfected into S2 cells, phosphorylated JNK persisted for a longer period than in control cells. We also assessed the effects of POSH overexpression and the role of the RING-finger function *in vivo* using *armadillo-GAL4* (*arm-GAL4*) as a ubiquitous expression driver (Fig 4B). Following bacterial infection, the expression level of *puc*, a target of JNK signalling, rapidly declined in flies overexpressing POSH (*arm > POSH*). In contrast, *puc* expression was sustained in flies overexpressing POSH^{mR} (*arm > POSH^{mR}*), which suggests that POSH^{mR} has a dominant-negative effect on JNK signalling. Together, these results indicate that POSH affects both activation and inactivation of JNK signalling induced by microbial infection, and that the RING-finger domain of POSH is essential for the negative feedback regulation of JNK signalling.

The profile of *puc* expression in *POSH*⁷⁴ flies is remarkably similar to that seen in *relish* flies (Park et al, 2004), which supports the idea that POSH mediates Relish-dependent inactivation of JNK in the immune system.

Our results show that POSH modulates the TAK1-mediated innate immune response. Following microbial infection, POSH facilitates rapid JNK activation and the induction of *relish*/NF-κB expression, both of which are mediated by TAK1 (Silverman et al, 2003). Conversely, POSH is also required for the rapid termination of both JNK activation and *relish* transcription through the E3 ubiquitin-ligase activity of the RING-finger domain. Our results indicate that POSH is involved in a mechanism of negative feedback regulation of JNK and NF-κB signalling. The *Drosophila* TAK1-mediated innate immune response is similar to the tumour necrosis factor (TNF) signalling cascade in mammals (Leulier et al, 2002). Imd protein contains a death domain with homology to that of mammalian TNF-receptor-interacting protein (RIP; Georgel et al, 2001). Most of the pathway components—such as FADD, TAK1—and *Drosophila* homologues of IKKγ and IKKβ are

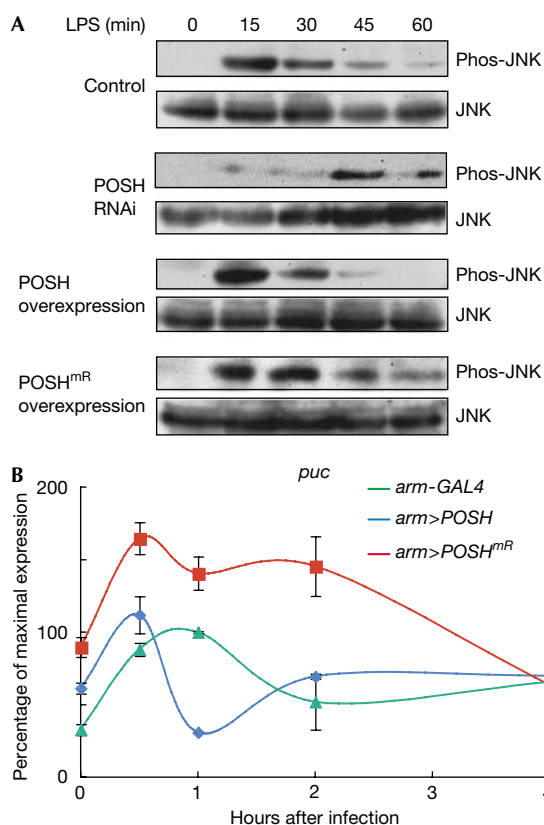


Fig 4 | Regulation of Jun amino-terminal kinase signalling by Plenty of SH3s. (A) Time-course analysis of Jun amino-terminal kinase (JNK) phosphorylation after peptidoglycan stimulation of S2 cells transfected with Plenty of SH3s double-stranded RNA (POSH dsRNA), POSH or POSH^{mR} DNA. Peptidoglycan-induced JNK activation is delayed and sustained in cells treated with POSH dsRNA. In contrast, JNK activation is rapidly terminated when wild-type POSH is overexpressed, whereas the activated JNK persists for a longer time period when POSH^{mR} is overexpressed. LPS, lipopolysaccharide; phos-JNK, phosphorylated JNK. (B) Real-time PCR analysis showing the time-course analysis of *puckered* (*puc*) expression. Expression level of *puc*, a target of the JNK pathway, is rapidly reduced in flies overexpressing POSH, but is sustained in those overexpressing POSH^{mR}, which suggests that RING-defective POSH has a dominant-negative effect on JNK signalling activated by infection.

conserved in mammalian TNF signalling. The activation of Relish involves its phosphorylation and cleavage by DREDD, the *Drosophila* homologue of caspase 8 (Silverman et al, 2000; Stoven et al, 2000). Eiger, a *Drosophila* homologue of the TNF- α superfamily ligand, has recently been identified (Igaki et al, 2002; Moreno et al, 2002; Kauppila et al, 2003). Overexpression of Eiger induces cell death by activating the JNK signalling pathway, as in mammals. Although the Eiger signalling pathway has not been fully elucidated in *Drosophila*, it has been clearly shown that TAK1 is essential to transduce the cell-death signal. It is possible that POSH is involved in Eiger signalling and modulates TAK-mediated JNK signalling as in the immune system. It will be of interest to determine whether POSH functions in mammalian TNF signalling.

METHODS

Fly stocks. *y w (y w^{67c23}Df(1)w^{67c23})* was used as a control strain. *EP(2)1206* (Rorth et al, 1998) and *arm-GAL4* were obtained from Szeged and the Kyoto Stock Center, respectively.

DNA constructs. Mutant POSH complementary DNAs were constructed using PCR mutagenesis and standard molecular biology techniques. POSH^{mR} has the point mutations C28S, C33S and C36S, and POSH^{AR} lacks the N-terminal 107 residues containing the entire RING domain.

Immune response assays. An overnight culture of *E. coli* was resuspended in PBS and injected into adult flies using a microinjection needle. Injected flies were collected at an appropriate time and were subjected to northern blot analysis. Total RNA was isolated from adult flies using an RNeasy Total RNA kit (QIAGEN, Tokyo, Japan). Northern blot analysis was carried out with digoxigenin-UTP-labelled RNA probes (Roche, Tokyo, Japan) prepared using *dpt* or *relish* cDNA clone, and signals were visualized by the CDP-star system (Roche). For real-time PCR, cDNAs were synthesized using the Superscript II reverse transcriptase system (Invitrogen, Carlsbad, CA, USA). Real-time PCR was carried out using SYBER Premix Ex Taq (Takara, Shiga, Japan) and a Chromo 4 Detector (MJ Research, Hercules, CA, USA). Results were normalized to the *rp49* mRNA level. All the experiments were repeated at least three times. Primer sequences are available on request. The peptidoglycan-induced immune response was examined using S2 cells, as described previously (Park et al, 2004). Cells were collected at an appropriate time after induction and were subjected to western blot analysis with an anti-phospho-JNK antibody G9 (Cell Signaling Technology, Beverly, MA, USA). Primary and secondary antibodies were removed by treating with stripping buffer (10 mM Tris-HCl (pH 6.7), 100 mM 2- β -mercaptoethanol and 20% SDS) at 50 °C for 30 min and were subjected to western blot analysis with an anti-JNK antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Ubiquitination analysis. To analyse the self-ubiquitination of POSH, S2 cells were transfected with pUAS-HA-ubiquitin (a gift from S. Hatakeyama, Kyusyu University), pWAGAL4 (a gift from Y. Hiromi, National Institute of Genetics), and pUAS-POSH, pUAS-POSH^{mR} or pUAS-POSH^{AR}. At 48 h after transfection, cells were treated with 5 μ M lactacystin for 3 h. Cell lysates were incubated for 1 h at 4 °C with a rabbit anti-POSH antibody (raised against a GST fusion protein of amino acids 479–781 of POSH) immobilized using an ImmunoPure Plus IgG Orientation Kit (Pierce, Rockford, IL, USA). The immunoprecipitates were analysed by western blot with either an anti-HA antibody 12CA5 (Sigma, St Louis, MO, USA) or an anti-POSH antibody. To detect the ubiquitination of TAK1, cells were transfected with pWAGAL4, pUAS-ubiquitin, pUAS-HA-TAK1 and either pUAS-POSH or pUAS-POSH^{mR}. Cells were treated with lactacystin and lysed. The lysates were incubated for 1 h at 4 °C with an anti-HA antibody Y-11 (Santa Cruz Biotech.). Protein G beads were added to solution for 1 h at 4 °C, followed by centrifugation. The immunoprecipitates were washed five times with lysis buffer and subjected to western blot analysis with either an anti-ubiquitin antibody 6C1 (Sigma) or an anti-HA antibody 12CA5.

Glutathione S-transferase pull-down assay and immunoprecipitation assay. To make GST fusion proteins, the protein-coding regions of POSH cDNAs were cloned into pGEX-41 (Amersham

Pharmacia Biotech, Piscataway, NJ, USA). Bacterially expressed fusion proteins were immobilized onto glutathione–Sepharose 4B beads according to the manufacturer's instructions (Amersham Pharmacia Biotech). Proteins labelled with [³⁵S]methionine were produced by the *in vitro* transcription and translation system (Promega, Madison, WI, USA). A GST–POSH fusion protein was incubated with labelled TAK1 protein, and then analysed by SDS–polyacrylamide gel electrophoresis, followed by fluorography. For immunoprecipitation assay, cells were transfected with pWAGAL4 and pUAS–HA–TAK1 with or without pUAS–POSH. At 48 h after transfection, cell lysates were incubated for 1 h at 4 °C with an immobilized anti-POSH antibody. The immunoprecipitates were subjected to western blot analysis with either an anti-HA antibody 12CA5 (Sigma) or an anti-POSH antibody.

RNA interference experiments. A 500 bp fragment of POSH cDNA flanked by a T7 promoter at both ends was generated by PCR using appropriate primers. Double-stranded RNA (dsRNA) was produced using a MEGAscript RNAi Kit (Ambion, Austin, TX, USA). S2 cells were incubated with serum-free medium containing 25 µg/ml dsRNA for 4 h. The RNAi-treated cells were grown in serum-containing medium for 72 h before immune response experiments.

Pulse–chase experiment. Cells were pulse labelled for 1 h in 500 µl of PBS containing 100 µCi [³⁵S]methionine and then chased with Schneider's medium supplemented with 10% fetal bovine serum. The cells were then lysed with 250 µl of lysis buffer (100 mM potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM dithiothreitol and protease inhibitor cocktail tablets (Roche)). HA-tagged TAK1 protein was immunoprecipitated from the lysates with an anti-HA antibody 12CA5 (Sigma) and analysed by SDS–polyacrylamide gel electrophoresis, followed by fluorography.

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

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