**Drosophila Smt3 negatively regulates JNK signaling through sequestering Hipk in the nucleus**

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**SUMMARY**
Post-translational modification by the small ubiquitin-related modifier (SUMO) is important for a variety of cellular and developmental processes. However, the precise mechanism(s) that connects sumoylation to specific developmental signaling pathways remains relatively less clear. Here, we show that Smt3 knockdown in *Drosophila* wing discs causes phenotypes resembling JNK gain of function, including ectopic apoptosis and apoptosis-induced compensatory growth. Smt3 depletion leads to an increased expression of JNK target genes *Mmp1* and *pucker*. We show that, although knockdown of the homeodomain-interacting protein kinase (Hipk) suppresses Smt3 depletion-induced activation of JNK, Hipk overexpression synergistically enhances this type of JNK activation. We further demonstrate that Hipk is sumoylated in vivo, and its nuclear localization is dependent on the sumoylation pathway. Our results thus establish a mechanistic connection between the sumoylation pathway and the JNK pathway through the action of Hipk. We propose that the sumoylation-controlled balance between cytoplasmic and nuclear Hipk plays a crucial role in regulating JNK signaling.

**KEY WORDS: Drosophila, Smt3, JNK, Hipk, Sumoylation**

**INTRODUCTION**
Small ubiquitin-related modifier (SUMO) is a polypeptide that is covalently, but reversibly, conjugated to substrate proteins. This post-translational modification, termed sumoylation, plays important physiological roles by regulating various cellular activities. Extensive studies have revealed that sumoylation plays important roles in a variety of cellular processes such as transcriptional regulation, nuclear-cyttoplasmic transportation, nuclear organization and DNA repair (Chen and Qi, 2010; Dou et al., 2010; Heun, 2007; Lin et al., 2003; Rui et al., 2002). Similar to the ubiquitilation process, sumoylation is achieved through sequential enzymatic reactions. It is initiated by an E1 activating enzyme (SAE1/SAE2, SUMO1-activating enzyme) that activates the SUMO molecule at its C terminus, which is subsequently linked to the E2-conjugating enzyme Ubc9 (ubiquitin-conjugating enzyme (SAE1/SAE2, SUMO1-activating enzyme) that activates the SUMO molecule at its C terminus, which is subsequently linked to the E2-conjugating enzyme Ubc9 (ubiquitin-conjugating enzyme). The SUMO activation is followed by E3 ligase-mediated transfer to a specific substrate protein (Geiss-Friedlander and Melchior, 2007).

The gene that encodes SUMO was initially identified in *Saccharomyces cerevisiae* (Meluh and Kosherland, 1995). In mammalian cells, there are three SUMO genes, whereas only a single gene, *smt3*, exists in *Drosophila* (Huang et al., 1998; Johnson et al., 1997; Su and Li, 2002), making *Drosophila* a useful experimental system in which to study the biological functions of sumoylation. Several studies in *Drosophila* have suggested different functions of sumoylation, including in the regulation of cell signaling during development and ecdysteroid biosynthesis, but their underlying mechanisms remain largely unclear (Miles et al., 2008; Nie et al., 2009; Talamilo et al., 2008).

The c-Jun N-terminal kinase (JNK) signaling is an evolutionarily conserved pathway, which is activated in response to environmental stress, apoptotic signals and proinflammatory cytokine tumor necrosis factor (TNF) (Liu et al., 1996; Moreno et al., 2000; Ryoo et al., 2004; Xia et al., 1995). In *Drosophila*, JNK is encoded by the gene *basket* (*bsk*). The upstream regulators of Bsk include a series of kinases that form a signaling cascade (Stanch and Perrimon, 2002; Takatsu et al., 2000; Tateno et al., 2000; Xue et al., 2007). MSN, a MAPK kinase kinase kinase (MAPKKKK) receives signals from cell surface receptors and initiates this signaling cascade (Liu et al., 1999; Xue et al., 2007). While the main signaling pathway transmits from MSN to JNK hierarchically, other factors connected to this main pathway also function in fine-tuning of the signaling, especially in activating or repressing the JNK activity (Chen et al., 2002; Neisch et al., 2010; Stanley et al., 2001; Yang et al., 1997).

One of the factors suggested to have a role in activating JNK is the homeodomain-interacting protein kinases (Hipks) (Hofmann et al., 2003; Lan et al., 2007; Li et al., 2005). Hipks are a family of serine/threonine kinases that are initially identified as the regulators of transcriptional co-repressors (Choi et al., 2005; Kim et al., 1998; Sung et al., 2005; Zhang et al., 2003). Although there are four members of Hipk proteins in vertebrates, *Drosophila* has only one ortholog: Hipk. The *Drosophila* Hipk shares the highest homology with mammalian Hipk2 (Choi et al., 2005; Link et al., 2007). Hipk functions in a variety of biological processes, some of which are in common with the JNK pathway, such as apoptosis and morphogenesis (Inoue et al., 2010; Isono et al., 2006; Link et al., 2007; McEwen et al., 2000; Zhang et al., 2003). However, an operational connection between Hipk and JNK at a mechanistic level has not been well established.
In this study, we show that knockdown of the SUMO gene (smt3) leads to an upregulation of the JNK signaling pathway in *Drosophila*. In a genetic screen for suppressors of Sm3 depletion-induced phenotype in the wing, we identified Hipk. We show that Hipk knockdown suppresses Sm3 depletion-induced JNK signaling upregulation. We further show that *Drosophila* Hipk is a target of sumoylation and its proper nuclear localization is dependent on the sumoylation pathway. Our results suggest a model, in which the sumoylation pathway normally keeps Hipk inside the nucleus; but downregulation of this pathway causes a translocation of Hipk to the cytoplasm, leading to an activation of JNK signaling. Our study thus provides a mechanistic connection between the subcellular localization of Hipk, a process regulated by sumoylation and JNK signaling.

**MATERIALS AND METHODS**

**Drosophila strains**

Flies were reared on a cornmeal and agar medium at 25°C according to standard protocols. The RNAi lines of *smt3* described previously (Talamillo et al., 2008) were kindly provided by Dr Rosa Barrio (CIC bioGUNE, Bizkaia, Spain). The *smt3* mutant allele referred to as *sumo* in this study, which harbors a P-element insertion in the upstream of the transcriptional start site (5′-UTR) of *smt3* gene that impair the transcription of *smt3* was obtained from the Bloomington Stock Center. The *hipk*-RNAi allele and *UAS-hipk* have been described previously (Lee et al., 2009a; Lee et al., 2009b). The *UAS-smt3* flies have been described previously (Nie et al., 2009; Takanaka and Courey, 2005). The RNAi stocks of *smt3* and *hipk* are available from the Vienna Drosophila RNAi Center (VDRC) and Fly Stocks of National Institute of Genetics (NIG-FLY).

**Immunohistochemistry and microscopy**

Wandering third instar larvae with correct genotypes were collected and dissected in cold phosphate-buffered saline (PBS). Imaginal discs were fixed in 4% paraformaldehyde. After proper washes, the discs were blocked in 10% goat serum, and stained with different primary antibodies (see below). Subsequently, corresponding fluorescent secondary antibodies (1:100, Jackson ImmunoResearch) were used for signal detection. The images were photographed with Leica confocal microscope SP5. The primary antibodies and their dilutions used for immunohistochemistry are as follows: antibodies against cleaved Caspase 3 (1:100, Jackson ImmunoResearch), Wingless (1:100) (Developmental Studies Hybridoma Bank, DSHB), mouse anti-Mmp1 (1:50) (DSHB 3A6B4/5H7B11/3B8D12) and anti-HA antibodies (1:100) (Roche).

**Western blot and immunoprecipitation**

The extracts were prepared as previously described (Huang et al., 2010). Adult heads were cut from newly enclosed flies and homogenized in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF] in the presence of a protease inhibitor cocktail. After incubation on ice for 15 minutes, the lysates were spun down at a maximum speed. The supernatants were used either for immunoblot or for co-immunoprecipitation assays. The samples were mixed with 2× SDS buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 0.1% Bromophenol Blue, 20% 2-mercaptoethanol], boiled for 5 minutes and centrifuged at the maximum speed at room temperature for 5 minutes. The supernatants were then applied to SDS-polyacrylamide gel and transferred to a PVDF membrane. For western blotting, the membranes were blocked for 1 hour at room temperature and probed with anti-HA antibody (Roche), anti-SUMO antibody (Abgent) and anti-Actin antibody (Santa Cruz), followed by horseradish-peroxidase linked secondary antibody. The signals were detected using SuperSignal West Pico Trial Kit (Thermo Scientific).

For co-immunoprecipitations, antibodies as well as control IgG, were coupled to Dynabeads Protein A/G (Invitrogen). The extracts were incubated with the beads for 6 hours at 4°C and eluted with SDS-loading buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 0.05% Bromophenol Blue, 10% 2-mercaptoethanol] before SDS-PAGE for immunoblotting.

**Fractionation assay**

Fly heads from newly enclosed flies were collected. The nuclear and cytoplasmic fractions were separated by the NER-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) following the manufacturer’s instructions. The samples were mixed with 2× SDS buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 0.1% bromophenol blue, 20% 2-mercaptoethanol], boiled and applied for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before western analysis.

**TUNEL assay**

The wing imaginal discs of proper genotypes were dissected in ice-cold PBS and fixed in 4% formaldehyde before being permeabilized in 1% Triton X-100 for 30 minutes. After sufficient washes, samples were incubated in the mixture of Enzyme and Label solutions (Beyotime Kit) at 37°C for 1.5 hours. The rest of the experiment was carried out following the manufacturer’s instructions.

**RNA interference and immunostaining of cultured S2 cells**

S2 cells were maintained in Schneider’s insect medium with 10% fetal bovine serum and antibiotics at 25°C, following the standard protocol. RNAi template for RNA production was amplified with primers containing T7 promoter. The pair of primers for Smt3 is 5′-TAATACGACTCACTATAGGGGCGGTCGACTGTACGACAAGC-3′ and 5′-CCCTATAGTGAGTCGATATTACTTATGAGCAGCGCCACAGTCG-3′. The primers for GFP are 5′-TAATACGACTCACTATAGGGGAGATCTATGGTGAGCAAGGG-3′ and 5′-CCCTATAGTGAGTCGATATTACTTATGAGCAGCGCCACAGTCG-3′. The DNA templates were in vitro transcribed into dsRNAs using the RiboMAX Large Scale RNA production System-T7 (Promega). S2 cells were seeded on polylysine-treated coverslips in dishes. dsRNA was introduced into cultured S2 cells, using standard calcium phosphate transfection method 3 days before immunostaining. pAc5.1A-HA-Hipk expression plasmids were introduced into the S2 cell 36 hours prior to immunostaining. The transfected cells were fixed in 4% paraformaldehyde. After primary antibodies and fluorescent secondary antibodies incubation, the images were obtained with a Leica confocal microscope SP5.

**RESULTS**

*Drosophila Smt3 is essential for development and tissue growth**

In *Drosophila*, *smt3* encodes the SUMO molecule that is ubiquitously expressed and predominantly distributed in the nucleus (Lehembre et al., 2000; Nie et al., 2009; Talamillo et al., 2008). A recent proteomic study has identified over 100 *Drosophila* proteins as substrates of sumoylation, proteins that play important roles in early embryonic development (Nie et al., 2009). Talamillo and colleagues reported that Smt3 knockdown produces developmental arrest and alters the ecdysteroid synthesis that is essential for metamorphosis (Talamillo et al., 2008). To further investigate the biological functions of Smt3 during development, we analyzed a mutant allele of *smt3, smt3* that harbors a P-element insertion in the upstream of the transcription start site (Nie et al., 2009). The mutant animals fail to survive beyond the second instar larval stage, and ubiquitous knockdown of *smt3* causes developmental arrest at the pupal stage (data not shown). These results are consistent with those described recently (Talamillo et al., 2008) and further demonstrate that Smt3 is essential for development. To gain a better understanding of the functional role of Smt3 during development, we used *Gal4* lines to deplete Smt3 in a tissue-specific manner. Our results show that either *ey-Gal4*-driven or *A9-Gal4*-driven expression of an *smt3*-RNAi construct
severely reduced the sizes of the eye or the wing, respectively (Fig. 1A, B; see Fig. 1A, B for wild-type controls). The specificity of the smt3-RNAi construct was validated by a genetic rescue experiment with UAS-smt3 transgene (see Fig. S1 in the supplementary material). In addition, depletion of Smt3 in the notum and scutellum under the control of pnr-Gal4 caused a defect in the midline of the notum and a loss of scutellum (Fig. 1C; see Fig. 1C for wild-type control). These results demonstrate a crucial role of Drosophila smt3 in development, and its tissue-specific disruption leads to corresponding tissue losses.

Knockdown of smt3 leads to apoptosis and activates wg expression in the wing discs

The tissue loss caused by Smt3 depletion can be attributed to several events, including cell apoptosis. To test whether apoptosis is induced upon Smt3 depletion, we stained for the cleaved Caspase 3 that marks cells undergoing apoptosis. en-Gal4 was used to specifically knockdown smt3 in the posterior compartment of the wing disc. As shown in Fig. 2A, the GFP signals mark the territory where en-Gal4 is expressed. When compared with the anterior compartment where relatively few apoptotic cells were observed, the GFP-positive posterior region exhibited a significantly increased population of Caspase 3-positive cells. As shown in Fig. S2 in the supplementary material, the apoptotic cells were also detected in the TUNEL assay. Together, these results suggest that Smt3 depletion promotes apoptosis.

The imaginal discs that develop into adult appendages can recover from damages caused by physical injury or apoptosis through regenerative growth (McEwen and Peifer, 2005; Smith-Bolton et al., 2009; Wang et al., 2009). The expression of the Wingless (Wg) morphogen in surviving cells is required for this regenerative repair (Ryoo et al., 2004; Smith-Bolton et al., 2009). We sought to determine whether the Smt3 depletion-induced apoptosis may trigger regenerative growth by examining wg expression. In the control discs, Wg forms a stripe at the dorsal-ventral boundary (Fig. 2B, upper panels). Upon Smt3 depletion under the control of en-Gal4 in the posterior region, the Wg morphogen expression became obscure at the D/V boundary (Fig. 2B, lower panels). In addition, ectopic expression of Wg was induced in the surviving cells of the entire posterior wing pouch, suggesting that regenerative growth takes place in this part of the disc (Fig. 2B, lower panels). Taken together, our results suggest that depletion of Smt3 causes apoptosis and induces Wg morphogen ectopic expression.

Reduction of Smt3 promotes JNK signaling activity

Both apoptosis and apoptosis-induced compensatory proliferation are governed by the JNK signaling pathway (Igaki et al., 2002; Moreno et al., 2002; Perez-Garijo et al., 2009). To determine whether JNK is required for Smt3 depletion-induced phenotypes, we blocked JNK activity simultaneously in the Smt3 knockdown
tissues through the use of RNAi against the *Drosophila* JNK (*bsk*) or the use of a dominant-negative form of Bsk (Fig. 3A-C). Three lines of evidence show that both apoptosis and ectopic *wg* expression induced by Smt3 knockdown are dependent on JNK activity. First, either depletion of *Drosophila* JNK by *bsk*-RNAi or expression of a dominant-negative form *bsk*DN rescued the small wing phenotype induced by Smt3 knockdown (Fig. 3A). Second, JNK abrogation substantially reduced the apoptosis in the *en>smt3-IR* discs as shown in Fig. 3B. Finally, the ectopic expression of *wg* no longer occurs in the Smt3 knockdown area when JNK is inactivated through *bsk*-RNAi; instead, these experimental discs exhibit a *wg* expression pattern similar to that of wild-type control (Fig. 3C). These observations demonstrate that JNK activity is required to manifest the effects of Smt3 depletion in establishing the observed wing phenotypes.

To monitor the JNK pathway activity directly, we analyzed two reporters for their dependence on Smt3. The first reporter is *puc-lacZ* (*pucE69*). *puckered* (*puc*) is a transcriptional target of JNK and is activated in the proximal peripodial cells of the wild-type wing discs (Agnes et al., 1999; McEwen et al., 2000; Miotto et al., 2006; Zeitlinger and Bohmann, 1999). The second reporter is the *Matrix metalloproteinase 1* (*Mmp1*) gene, another downstream transcriptional target of JNK (Rodahl et al., 2009; Uhlirova and Bohmann, 2006). Our results show that Smt3 depletion, under the control of *en-Gal4* and marked by the GFP-positive cells, led to both ectopic *puc-lacZ* reporter activity (Fig. 4, *pucE69*) and increased Mmp1 signals (Fig. 4, Mmp1), when compared with the

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**Fig. 3.** The Smt3 depletion-induced phenotype is dependent on *Drosophila* JNK. (A) Blocking JNK signaling rescues wing growth defects in *A9>smt3-IR* flies. Genotypes: upper panels are *A9-Gal4/+; UAS-bskDN/+* and *A9-Gal4/+; UAS-bsk-IR/+*. Middle panel is *A9>smt3-IR* fly (*A9-Gal4/+; UAS-smt3-IR/+*). Bottom panels are *A9-Gal4/+; UAS-smt3-IR/UAS-bskDN* and *A9-Gal4/+; UAS-smt3-IR/UAS-bsk-IR*. 

(B) Downregulation of JNK signaling rescues the apoptosis resulting from Smt3 depletion in *en>smt3-IR* Wing discs were immunostained with Caspase 3 antibody (middle panels). 

(C) Downregulation of JNK signaling suppresses ectopic *Wg* expression and restores the D/V boundary *Wg* pattern in *en>smt3-IR* animals. Red shows *Wg* signal. Scale bar: 75 μm.

**Fig. 4.** Depletion of Smt3 upregulates JNK signaling. Immunofluorescent images show localization of β-galactosidase in *en/+* and *en>smt3-IR* larval wing discs of heterozygous *pucE69* flies. Wing discs of *en/+* and *en>smt3-IR* larvae were immunostained with Mmp1 antibody to indicate the activity of the JNK pathway in the posterior region. Scale bars: 75 μm.
GFP-negative and Smt3-positive cells in the anterior region. Taken together, these results provide direct evidence that Smt3 depletion promotes the JNK activity in vivo.

**Hipk is required for JNK signaling activation induced by Smt3 depletion**

The JNK signaling pathway is regulated by multiple factors (Huang et al., 2009; Minden and Karin, 1997). To identify possible effectors through which Smt3 regulates JNK activity, we took advantage of the small-wing phenotype in A9>smt3-IR flies as a tool to screen for genetic suppressor(s). We identified the homeodomain interacting protein kinase (Hipk) as one such suppressor. Fig. 5A shows that knockdown of Hipk was sufficient to rescue the wing growth defects in A9>smt3-IR flies. Genotypes: upper panel is A9-Gal4+; UAS-hipk-IR+. Middle panel is A9-Gal4+; UAS-smt3-IR+. Bottom panel is A9-Gal4+; UAS-smt3-IR/UAS-hipk-IR. (B) Apoptosis induced by Smt3 depletion is reversed when Hipk is knocked down. Apoptotic cells were visualized using Caspase 3 antibody (middle panels). (C) RNAi ablation of Hipk offsets the upregulation of JNK activity (as indicated by the Mmp1 signals) induced by Smt3 knockdown. Wing discs were stained with Mmp1 antibody (middle panels). Scale bars: 75 µm.

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sumoylation pathway. We will further discuss the implications of these findings regarding the operational relationship between Hipk and Smt3 on JNK activation (see below and Discussion).

**Hipk is sumoylated in vivo in the presence of Smt3**

As Hipk regulates JNK signaling in a manner that is dependent on sumoylation perturbation (i.e. Smt3 depletion), it is possible that Hipk may itself be a target of sumoylation. To test this possibility directly, we performed an immunoprecipitation assay using protein extracts from the heads of the flies that express HA-tagged Hipk transgene under the control of GMR-Gal4 (GMR> Hipk-HA). In our analysis, anti-HA antibody was used for immunoprecipitation, followed by immunoblot using an anti-SUMO antibody. A Hipk-HA band was detected in this experiment by the anti-SUMO antibody (Fig. 8A, lane 2). Importantly, the amount of this band is reduced by Smt3 knockdown (Fig. 8A, lane 3). In a reciprocal experiment, Hipk was detected in the products immunoprecipitated by the anti-SUMO antibody (Fig. 8B). Together, these results provide evidence that Hipk is sumoylated in vivo.

**Depletion of Smt3 leads to translocation of Hipk from the nucleus to the cytoplasm**

Our experiments described thus far suggest that sumoylation suppresses the ability of Hipk to activate the JNK signaling pathway (Fig. 5A-C). They also show that Hipk itself is sumoylated (Fig. 8), suggesting that the sumoylation status of Hipk may be crucial for its role in regulating JNK activation. It has been reported that sumoylation can alter protein localization and/or change protein conformation (Geiss-Friedlander and Melchior, 2007; Girdwood et al., 2004; Lin et al., 2003; Sanchez et al., 2010; Zhong et al., 2000). To investigate the role of sumoylation on Hipk subcellular localization, we separated the nuclear and cytosolic fractions of the extracts from Hipk-expressing adult fly heads with or without smt3-RNAi expression. Western blot with anti-SUMO antibody detected sumoylated Hipk primarily in the nuclear fraction (Fig. 9A, SUMO). Although Smt3 knockdown did not alter the total amount of Hipk (Fig. 8A, Hipk-HA, compare lanes 2 and 3), its nuclear abundance was decreased upon the reduction of Smt3 (Fig. 9A, nuclear fraction of Hipk-HA). Concomitantly, the level of Hipk was increased in the cytosolic fraction (Fig. 9A, cytosolic fraction of Hipk-HA). These results suggest a translocation of Hipk from the nucleus to the cytoplasm when the sumoylation pathway is compromised by Smt3 depletion.

To monitor directly the effects of sumoylation on the dynamic localization of Hipk, we performed immunostaining experiments using an HA antibody that detects an HA-tagged Hipk protein. Fig. 9B (upper panels) shows that Hipk is ubiquitously expressed with a primary localization in the nucleus, a pattern resembling the subcellular localization of Smt3 (Talamillo et al., 2008). The nuclear accumulation of Hipk is in agreement with its reported activity of interacting with other transcriptional regulators (Choi et al., 2005; Zhang et al., 2003). To further evaluate the effect of sumoylation on Hipk localization, we conducted immunostaining of HA-tagged Hipk proteins on endogenous Drosophila tissues and in vitro cultured Drosophila S2 cells. In the case of Smt3 knockdown, the amount of nuclear Hipk was significantly reduced when compared with the wild-type control (Fig. 9B, Hipk-HA, lower panels; also see Fig. 9A, nuclear fraction). Meanwhile, a noticeable increase of the Hipk signals was detected in the cytoplasmic fraction upon the perturbation of sumoylation pathway by Smt3 depletion (Fig. 9B, Hipk-HA, lower panels; also see Fig. 9A, cytosolic fraction). The nucleus-to-cytoplasm translocation of Hipk induced by the impairment of the sumoylation pathway was further confirmed by experiments in Drosophila S2 cells (see Fig. S7 in the supplementary material). Together, our fractionation and immunostaining results suggest a role of the sumoylation pathway in regulating the subcellular localization of Hipk.

**DISCUSSION**

Sumoylation is a post-translational modification that regulates multiple biological activities by modifying a variety of different substrates. In this study, we show that tissue-specific perturbation of the sumoylation pathway activates the JNK signaling pathway. In particular, knockdown of the Drosophila SUMO gene smt3 recapitulates several key gain-of-function features of the JNK
pathway, including apoptosis and wg ectopic expression. These results suggest that sumoylation plays a crucial role in regulating JNK signaling. Further experiments demonstrate that Hipk is responsible for Smt3 depletion-induced JNK activation. Our experiments show that Hipk itself is sumoylated (Fig. 8) and that its nuclear localization is dependent on the sumoylation pathway (Fig. 9). Based on these findings, we propose a model in which Hipk is normally kept in the nucleus, but a compromised sumoylation pathway (such as that produced by depletion of Smt3) allows some Hipk molecules to translocate to the cytoplasm and activate the JNK signaling pathway.

Sumoylation regulates the biological activities of its substrates through several distinct mechanisms. These mechanisms include altering subcellular localization of its substrate proteins and/or molecular shuttling between the nucleus and the cytoplasm (Ishov et al., 1999; Li et al., 2005), mediating protein-protein interactions (Muller et al., 1998), locking its substrates in a particular conformational state (i.e. active or inactive) (Girdwood et al., 2004) or altering protein stability and clearance (Zhang et al., 2003). Our study highlights the importance of sumoylation-dependent subcellular localization of Hipk in regulating its biological activities. We propose that sumoylation normally restricts Hipk to the nucleus and facilitates the execution of its nuclear functions, such as interaction with and phosphorylation of transcriptional corepressors (Choi et al., 2005; Ecsedy et al., 2003; Zhang et al., 2003). However, unsumoylated or desumoylated Hipk becomes accessible to the cytoplasm for executing its cytoplasmic function(s). As shown in our study, one such cytoplasmic function of Hipk is to modulate the JNK signaling pathway.

In the reverse experiment, immunoprecipitates were collected with anti-SUMO antibody, and detected by antibodies against HA. Control IgG serves as a negative control.

**Fig. 8.** Hipk is sumoylated in vivo. (A) Extracts were prepared from the heads of adult wild-type (GMR-Gal4/+; lane 1), GMR>hipk-HA (GMR-Gal4/+; UAS-hipk-HA, lane 2) and GMR>hipk-HA, smt3-IR (GMR-Gal4/+; UAS-hipk-HA/UAS-smt3-IR, lane 3) flies. The proteins were pulled down with anti-HA antibodies, separated by SDS-PAGE, and immunoblotted with anti-SUMO and anti-HA antibodies independently. (B) In the reverse experiment, immunoprecipitates were collected with anti-SUMO antibody, and detected by antibodies against HA. Control IgG serves as a negative control.
we report for the first time that *Drosophila* Hipk potentiates JNK signaling through a sumoylation-dependent regulation of its subcellular localization. Our study and the work from Verheyen’s laboratory underscore the roles of *Drosophila* Hipk both inside and outside of the nucleus in fine-tuning signaling pathways. It remains to be determined precisely how Hipk regulates the JNK pathway and whether it involves a direct mechanism such as phosphorylating relevant components of this pathway.

The subcellular localization of Hipk represents an important mechanism in defining its functional specificity. In particular, Hipk controls the degradation of transcriptional co-repressor CtBP inside the nucleus (Zhang et al., 2003), while the cytoplasmic Hipk interacts with the nonhistone chromosomal factor Hmgal (high-mobility group A1) to inhibit cell growth (Pierantoni et al., 2001). Hipk has also been shown to, within the speckled subnuclear structures, interact with p53 to promote its phosphorylation (Gostissa et al., 2003; Moller et al., 2003). Our results presented in this report show that Hipk is normally sequestered in the nucleus but gains access to the cytoplasm, upon sumoylation perturbation, to activate the JNK signaling pathway. The idea that the subcellular localization of Hipk is crucial for its functional specificity also explains why upregulating Hipk alone did not result in a robust activation of JNK (Fig. 6). We suggest that, without sumoylation perturbation, the majority of transgene-expressed Hipk is, like the endogenously expressed Hipk, sumoylated and kept in the nucleus, making it inaccessible to activating JNK.

The JNK signaling pathway is composed of stepwise actions of kinases (Geuking et al., 2005; Geuking et al., 2009). The canonical JNK pathway receives signals from death stimuli, such as tumor necrosis factor (TNF) and oxidative stresses. In addition to the JNK pathway, other factors such as Hipk proteins are also stimulated by a variety of stresses. For example, the human HIPK1 responds to the stimulation of TNFα to relocate itself from the nucleus to the cytoplasm (Li et al., 2005). In addition, the mammalian Hipk2 phosphorylates p53 in response to UV irradiation (D’Orazi et al., 2002; Hofmann et al., 2002; Zhang et al., 2003) and phosphorylates cAMP response element-binding protein (CREB) to cope with genotoxic stress (Sakamoto et al., 2010). We propose that stress signals such as TNF may activate not only the canonical JNK pathway but also the Hipk-dependent JNK activation mechanism(s). The idea that Hipk acts downstream of TNF is consistent with our genetic evidence that RNAi ablation of Hipk partially rescues the perturbation, the majority of transgene-expressed Hipk is, like the nuclear structure when modified by SUMO-1.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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