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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 *puckered*. 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 sumolylated 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-cytoplasmic 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 ubiquitylation 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 9), 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 Koshland, 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; Talamillo 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., 2002; 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 (Stronach 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; Shanley 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.

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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 Smt3 depletion-induced phenotype in the wing, we identified Hipk. We show that Hipk knockdown suppresses Smt3 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 $sumo^{04493}$ in this study, which harbors a *P*-element insertion in the upstream of the transcriptional start site (5′-UTR) of *smt3* gene that impairs 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) (Cell Signaling), Wingless antibodies (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 by 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. DNA template for RNA production was amplified with primers containing T7 promoter. The pair of primers for Smt3 is 5′-TAATACGACTCACTATAGGGGGCGTGTAGCTGTAGCAGAAGC-3′ and 5′-CCCTATAGTGAGTCGTATTACTTATGGAGCGCCACCAGTCTG-3′. The primers for GFP are 5′-TAATACGACTCACTATAGGGAGATCTATGGTGAGCAAGGG-3′ and 5′-CCCTATAGTGAGTCGTATTACTTGTACAGCTCGTCCATGC-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*, $smt3^{04493}$, which 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 now 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

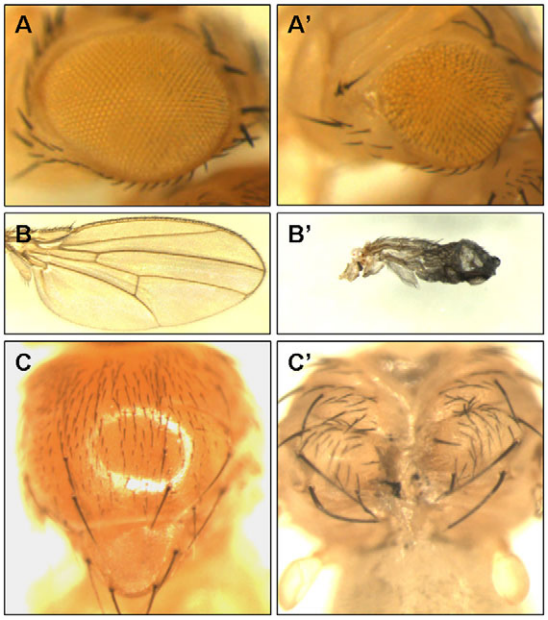

Fig. 1. Smt3 knockdown flies display developmental defects in various contexts. (**A-A′**) Eye development is compromised upon Smt3 knockdown specifically in the eyes [compare A (*ey-Gal4/+*) with A′ (*ey>smt3-IR*)]. (**B-B′**) Wing development is defective when Smt3 is depleted under the control of *A9-Gal4*. Adult wings of *A9-Gal4/+* (B) and *A9>smt3-IR* (B′) flies are shown. (**C-C′**) Light microscopy images showing adult thoraxes of control (C, *pnr-Gal4/+*) and Smt3 knockdown flies (C′, *pnr>smt3-IR*). Note the midline defects in the notum and missing scutellum for the Smt3 knockdown flies.

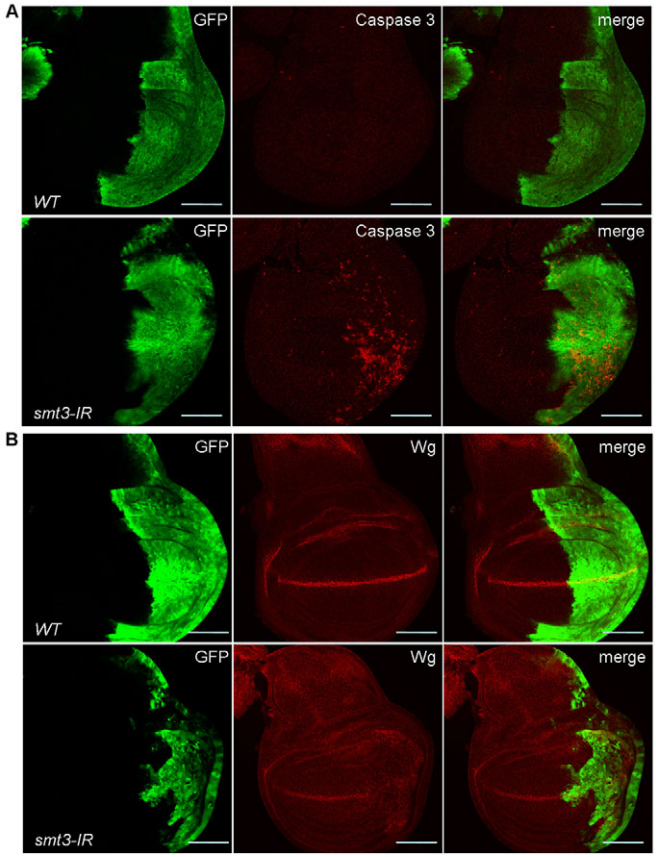

Fig. 2. RNAi depletion of Smt3 induces apoptosis and ectopic Wg expression. (**A**) Immunostaining images showing apoptotic cells detected by Caspase 3 signals (middle panels) in wing discs. Left panels mark posterior wing compartments with *en-Gal4* driven GFP expression. (**B**) Wingless expression pattern in wild-type (*en-Gal4/+*, upper panels) and Smt3 knockdown (*en>smt3-IR*, lower panels) wing imaginal discs. Scale bars: 75 μm.

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

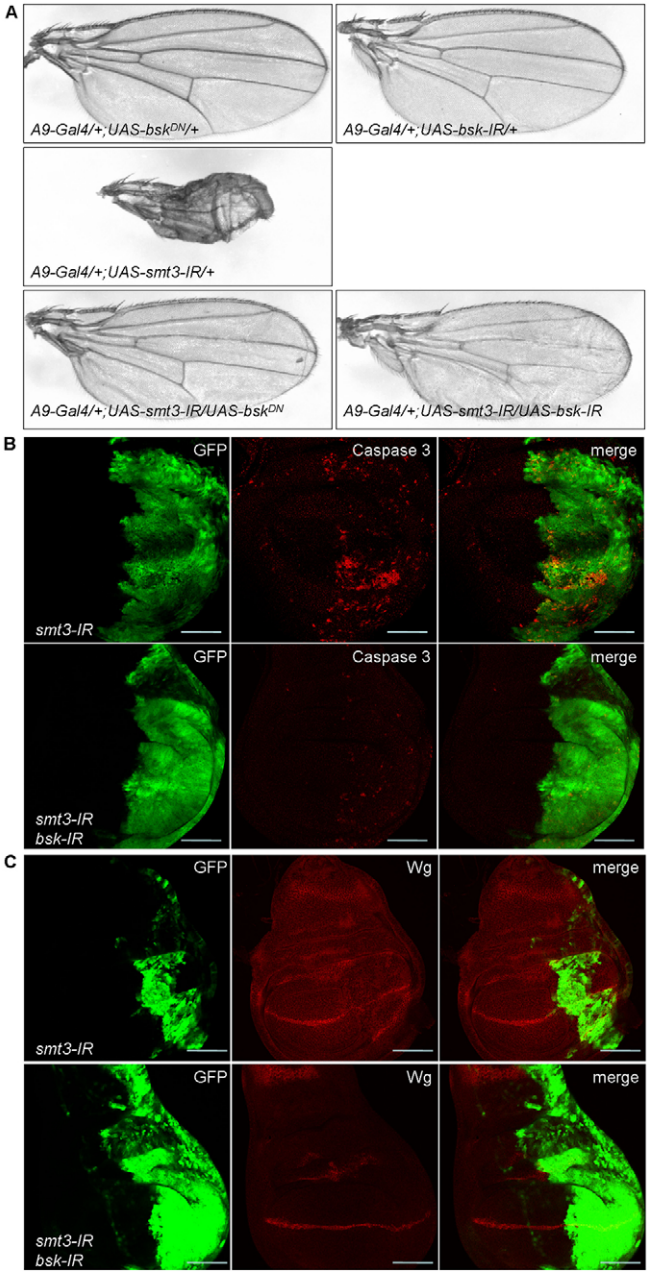

Fig. 3. The Smt3 depletion-induced phenotype is dependent on *Drosophila* JNK. (**A**) Blocking JNK signaling rescues wing growth defects in *A9>smt3-IR* flies. Genotypes: upper panels are *A9-Gal4/+; UAS-bsk^{DN}/+* and *A9-Gal4/+; UAS-bsk-IR/+*. Middle panel is *A9>smt3-IR* fly (*A9-Gal4/+; UAS-smt3-IR/+*).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

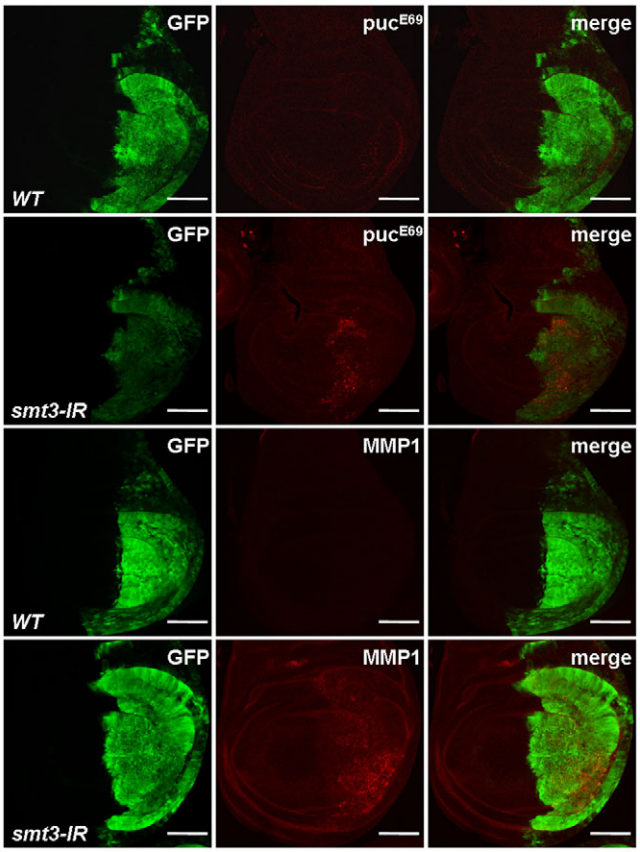

Fig. 4. Depletion of Smt3 upregulates JNK signaling. Immunofluorescent images show localization of β-galactosidase in *en/+* and *en>smt3-IR* larval wing discs of heterozygous *puc^{E69}* flies. Wing discs of *en/+* and *en>smt3-IR* larvae were immunostained with Mmp1 antibody to indicate the activity of the JNK pathway in the posterior region. Scale bars: 75 μm.

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* (*puc^{E69}*). *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, puc^{E69}) and increased Mmp1 signals (Fig. 4, Mmp1), when compared with the

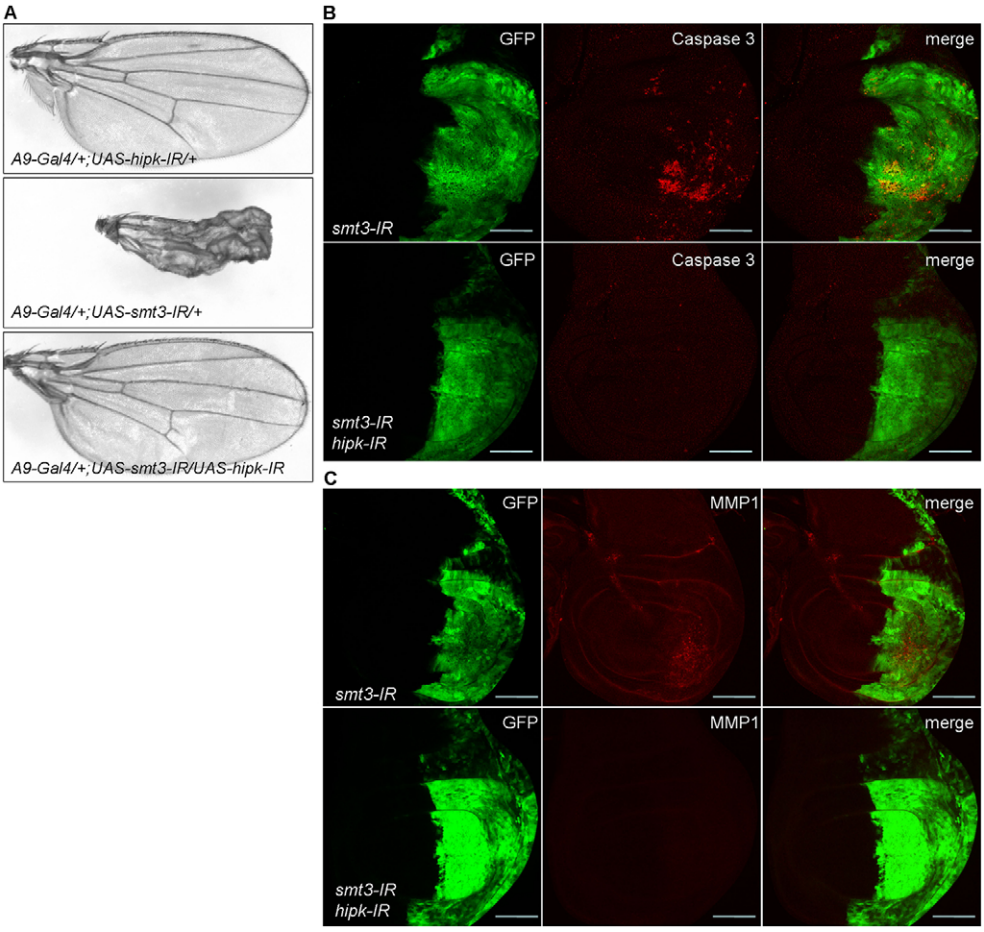

Fig. 5. The JNK signaling activation induced by Smt3 depletion depends on the action of Hipk. (**A**) Knockdown of Hipk rescues the wing growth defects in *A9>smt3-IR* flies. Genotypes: upper panel is *A9-Gal4/+; UAS-hipk-IR/+*. Middle panel is *A9-Gal4/+; UAS-smt3-IR/+*. Bottom panel is *A9-Gal4/+; 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.

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 developmental defects of *A9>smt3-IR* flies. Both Smt3-depletion-induced apoptosis (monitored by Caspase 3 cleavage) and transcriptional activation of Mmp1 were attenuated by the Hipk depletion (Fig. 5B,C). Genetic experiments suggest that Hipk is required for full activation of JNK (see Fig. S3 in the supplementary material). In addition, *pnr-Gal4*-driven overexpression of Hipk caused thorax and scutellum defects that resemble the JNK gain-of-function phenotype and Smt3-depletion phenotype (see Fig. S4 in the supplementary material). These results suggest that Hipk plays an important role in the JNK activation induced by Smt3 depletion.

To determine whether overexpression of Hipk alone is sufficient to induce JNK pathway activation, we artificially expressed Hipk under the control of *en-Gal4* in the posterior region of the wing discs. Our results show that overexpression of Hipk resulted in only a slight increase of Caspase 3-positive cells and a mild elevation of Mmp1 expression (Fig. 6), indicating that Hipk overexpression alone activates JNK pathway only weakly, and is insufficient to induce strong JNK signaling. To further investigate the relationship between Hipk action and the Smt3 status for their roles in JNK activation, we used the sensitive eye phenotype for our investigation. As shown previously, the severity of rough eyes reflects the strength of JNK-dependent apoptosis (Igaki et al., 2002; Moreno et al., 2002; Tiwari and Roy, 2009; Xue et al., 2007). Here, we simultaneously expressed *UAS-hipk* and *smt3*-RNAi constructs under the control of *GMR-Gal4*. Our results show that, when compared with the wild-type ommatidia (Fig. 7A), Smt3 knockdown driven by *GMR-Gal4* caused a slight irregular ommatidia pattern (Fig. 7B; see Fig. S5 in the supplementary material). *GMR-Gal4*-driven expression of Hipk did not lead to any obvious abnormality (Fig. 7C), which is consistent with the observation that overexpression of Hipk alone activates only mild apoptosis (Fig. 6). However, simultaneous expression of Hipk and *smt3* knockdown led to significantly enhanced rough eyes with reduced size and fused ommatidia (Fig. 7D; see Fig. S5 in the supplementary material). At the molecular level, overexpression of Hipk synergistically promoted the expression of JNK target gene *Mmp1* and ectopic apoptosis caused by Smt3 knockdown (see Fig. S6A,B in the supplementary material). These results demonstrate a synergy between Hipk overexpression and Smt3 removal in JNK activation, suggesting that the role of Hipk on JNK is dependent on the status of the

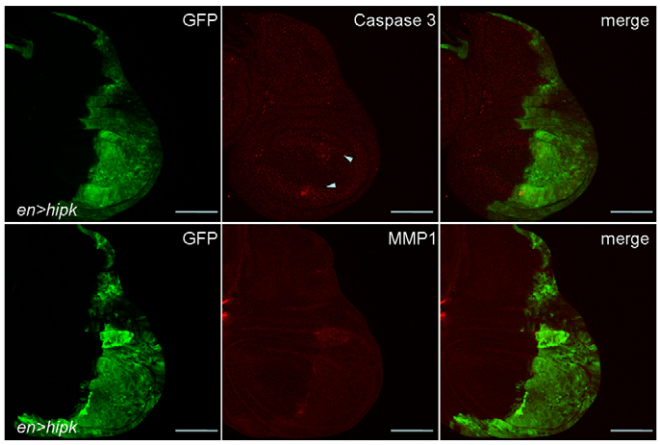

Fig. 6. Overexpression of Hipk triggers a mild increase of JNK signaling. Detectable apoptosis indicated by Caspase 3 signals (upper panels, arrowheads) and slight Mmp1 upregulation (lower panels) in the posterior region of the wing discs were induced by overexpression of Hipk (*en>hipk*). Scale bars: 75 μm.

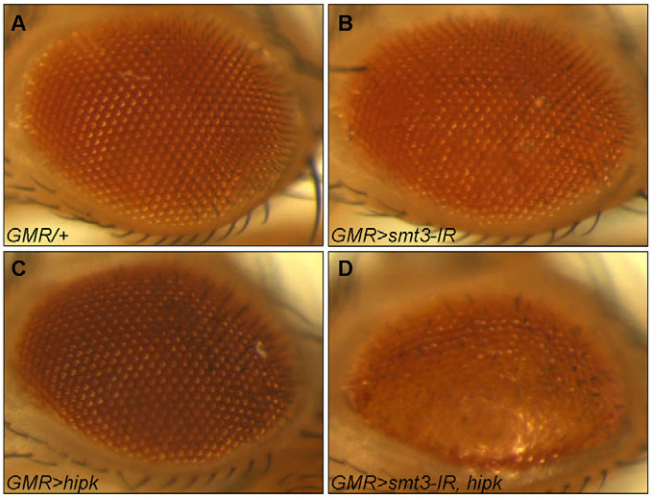

Fig. 7. Overexpression of Hipk and knockdown of *smt3* synergistically induce developmental defects in the eye. Light microscopic photographs showing *Drosophila* adult eyes. Genotypes: (**A**) *GMR-Gal4/+*, (**B**) *GMR-Gal4/+; UAS-smt3-IR/+*, (**C**) *GMR-Gal4/+; UAS-hipk/+* and (**D**) *GMR-Gal4/+; UAS-smt3-IR/UAS-hipk*.

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

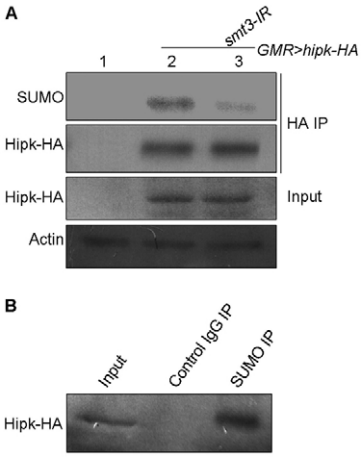

Fig. 8. Hipk is sumoylated in vivo. (**A**) Extracts were prepared from the heads of adult wild-type (*GMR-Gal4/+*, lane 1), *GMR>hipk-HA* (*GMR-Gal4/+; UAS-hipk-HA/+*, lane 2) and *GMR>hipk-HA, smt3-IR* (*GMR-Gal4/+; UAS-hipk-HA/UAS-smt3-IR*, lane 3) flies. The proteins were pulled down with anti-HA 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.

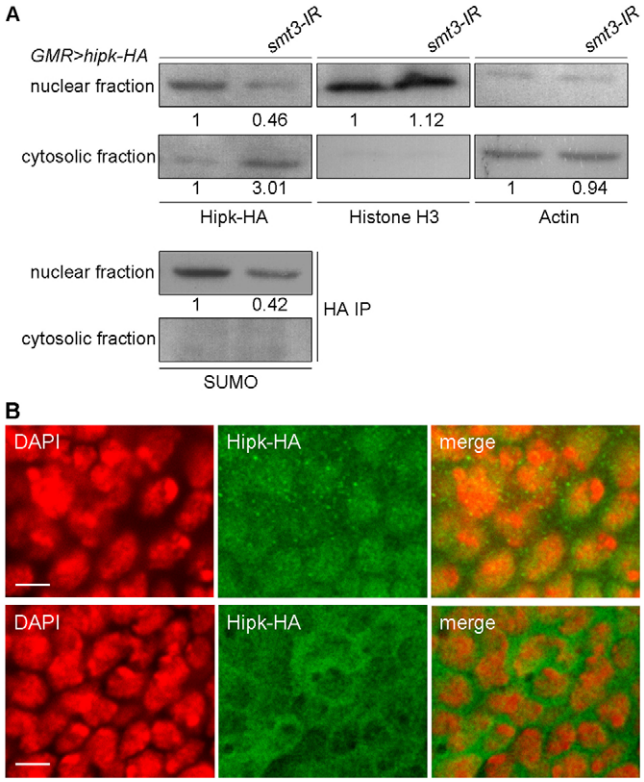

Fig. 9. Depletion of Smt3 translocates Hipk from the nucleus to the cytoplasm. (**A**) Western blot analyses of the cytosolic and the nuclear extracts from heads of the *GMR>hipk-HA* (*GMR-Gal4/+; UAS-hipk-HA/+*) and *GMR>hipk-HA* fly lines with compromised sumoylation pathway (*GMR-Gal4/+; UAS-hipk-HA/UAS-smt3-IR*). Anti-HA antibody is used to measure the nuclear and cytoplasmic abundance of Hipk. Anti-SUMO antibody was used to detect sumoylated Hipk-HA pulled down by anti-HA antibody. Histone H3 and Actin antibodies are used for loading controls. The quantification of the western blot results is shown below each lane. The ratios (numbers below the bands of Smt3-IR samples) represent the relative intensity of the signals in the absence of Smt3 (*GMR-Gal4/+; UAS-hipk-HA/UAS-smt3-IR*) compared with that in the presence of Smt3 (*GMR-Gal4/+; UAS-hipk-HA/+*) that were normalized to 1. (**B**) Wing imaginal discs of third instar larvae expressing HA tagged Hipk (*A9>hipk-HA*) in the presence of Smt3 (upper panels) and in the absence of Smt3 (lower panels) were stained with anti-HA antibodies (green channels). Note the reduction of Hipk signals in the nuclei when Smt3 is depleted. Nuclei were visualized using DAPI staining (red channels). Scale bars: 4 μm.

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 co-repressors (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.

Hipk family members play roles in different biological processes, such as cell cycle progression, p53-dependent apoptosis, and transcriptional regulation (D'Orazi et al., 2002; Pierantoni et al., 2001; Zhang et al., 2003). The mammalian cells contain four Hipk proteins that perform overlapping, but distinct functions. For example, Hipk1 and Hipk2 have functionally redundant roles in mediating cell proliferation and apoptosis during development (Inoue et al., 2010; Isono et al., 2006). Hipk1 interacts with transcription factor c-Myb (Matre et al., 2009), while Hipk2 phosphorylates transcriptional co-repressor Groucho (Choi et al., 2005), suggesting their distinct roles in transcription regulation. Therefore, it would be interesting to elucidate whether *Drosophila* Hipk executes the functions of all the mammalian counterparts, although *Drosophila* Hipk shares most homology with Hipk2. This all-in-one mode of Hipk function requires different strategies to regulate its functions. Previous studies have shown that *Drosophila* Hipk promotes various signaling pathways such as the Wnt pathway through stabilizing Armadillo, and the Notch pathway through inhibiting the global co-repressor Groucho (Lee et al., 2009a; Lee et al., 2009b). In this work,

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 Hmga1 (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 overexpressing 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 cyclic AMP 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 *Drosophila* TNF (Egr)-induced phenotype in the eye (see Fig. S3A in the supplementary material). A major finding of our current study is that it establishes a cross-regulation between the sumoylation and the JNK pathways through the action of Hipk. We currently do not know whether TNF or even JNK itself may regulate the sumoylation pathway, but it remains an interesting possibility that will require further investigation. We note that the relationship between sumoylation and JNK pathways is likely to be more complex than Hipk-mediated action described in our current work. It has been shown that sumoylation is required for Axin-mediated JNK activation (Rui et al., 2002). Thus, it is possible that sumoylation may have different, even opposite, effects on the JNK pathway through distinct sumoylation targets. The robust increase of the JNK activity detected in Smt3-depleted cells in our study demonstrates an overall negative role of the sumoylation pathway in JNK signaling.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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