



# Ubiquitin E3 ligase dSmurf is essential for Wts protein turnover and Hippo signaling



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## ABSTRACT

The Hippo pathway has been implicated in controlling organ size and tumorigenesis and the underlying molecular mechanisms have attracted intensive attentions. In this work, we identified dSmurf as a new regulator of Wts, a core component of the Hippo pathway, in *Drosophila*. Our data revealed that Wts and dSmurf colocalize to cytoplasm and physically form an immunoprecipitated complex in S2 cells. Sufficient knock-down of dSmurf increases the protein abundance of Wts and thus increases phosphorylation level at S168 of Yki, the key downstream target of Wts in the Hippo pathway. Genetic epistasis assays showed that halving dosage of dSmurf dominantly enhances the phenotype caused by overexpression of Wts and restrains Yki activity in *Drosophila* eyes. Our works defines a novel role of dSmurf in animal development through modulating Wts turnover and thereby Hippo signal transduction, implying that targeting dSmurf may be a promising therapeutic strategy to manipulate the Hippo pathway in pathological conditions.

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## 1. Introduction

The Hippo signaling pathway was first discovered in *Drosophila melanogaster*, and then proved to be a highly conserved regulator of organ size control and tumorigenesis in mammals [1–5]. Central to the Hippo pathway is a kinase cascade in which Hippo (Hpo) phosphorylates and activates Warts (Wts) [6–10], and in turn phosphorylates and inactivates the activity of transcriptional co-activator Yorkie (Yki), the key downstream effector of Wts, to transcriptionally regulate target genes involved in cell proliferation and survival [11,12]. Large tumor suppressor 1/2 (Lats1/2), the mammalian homologs of Wts, is often down-regulated or mutated in a variety of human cancers including breast tumor [13], astrocytoma [14] and sarcomas [15]. Given the critical roles in cancer development, studies on Lats/Wts may have important implications in our understanding of tumorigenesis.

Smad ubiquitination regulatory factor 1 and 2 (Smurf1 and 2) are two members of the HECT-type of E3 ubiquitin ligase. Smurf1/2 has been initially implicated to play crucial roles in embryonic development and diverse cellular processes responsive to TGFβ/BMP signaling pathway by promoting ubiquitination and degradation of SMADs [16–18]. In addition to TGFβ/BMP pathway,

the substrates of Smurfs are involved in broad biological processes such as cell cycle progression, metastasis and cell apoptosis [19]. Smurf1/2 as well as WWP1/2, NEDDL1/2, NEDD4, NEDD4L and ITCH comprise an E3 ubiquitin ligase group called the NEDD4-like family. The functions of these family members have been implicated in regulation of ubiquitin-mediated trafficking, and lysosome- or proteasome-mediated protein degradation [20,21]. Recent studies have revealed that ITCH, WWP1 or NEDD4 physically associate with Lats1 and functionally regulate Lats1 protein stability through their E3 ligase activities in mammalian cells [22–25]. However, the regulation of Lats/Wts turnover in animal development remains largely unknown. Here, we identified that the E3 ligase dSmurf associates with Wts and is essential for Wts turnover and function in *Drosophila*. Our work may provide new insight to understanding Smurfs in tumorigenesis by linking them to the Hippo pathway.

## 2. Materials and methods

Primary antibodies used in this work: rabbit anti-HSV (1:5000, Abcam) and anti-p-Yki, and mouse anti-Flag (1:5000, Abcam).

### 2.1. Plasmid construction

For HSV-wts construction, full-length of wts was amplified from cDNA clone SD26853 (BDGP) by PCR and ligated into the

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expression vector of pAc5.1/V5-HisB. The insert was then verified by sequencing. *Flag-dSmurf* and *Flag-dSmurf*<sup>C1029A</sup> plasmids were gifted by Dr. Dahua Chen [26].

## 2.2. Cell culture and cell based assays

*Drosophila* S2 cells were cultured in *Drosophila* cell culture medium (Thermo, SH30278.02) supplemented with 10% fetal bovine serum at 25 °C. X-tremeGENE HP DNA Transfection Reagent (Roche) was used for transient transfection of S2 cells. After 36 h transfection, cells were harvested and lysed in 2× loading buffer (0.25 M Tris-HCl pH 6.8, DTT 78 mg/mL, SDS 100 mg/mL, 50% Glycerine, and 5 mg/mL bromophenol blue) for determining the level of protein expression by Western blotting.

For co-immunoprecipitation, cells were lysed in NP-40 buffer (150 mM NaCl, 1% Triton X-100, 10 mM Tris pH 7.4, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 0.5% NP-40, and 1 mM PMSF) at 4 °C for 30 min. The supernatants of the samples after centrifugation were incubated with the indicated antibodies at 4 °C for 30 min. Samples were combined with 20 µl Protein A/G agarose (GE, 10080881) for 2 h at 4 °C. Beads were washed three times with NP-40 buffer, followed by Western blotting.

For the luciferase reporter assay, cells were seeded into 24-well plate. Cells were transfected with the indicated plasmids and *pRenilla* as internal control. After 36 h, luciferase activity was assessed using the Dual-Glo Luciferase Assay System (Promega, E2940) according to the manufacturer's protocol.

For immunofluorescence, cells were seeded on coverslips in 24-well plates. 36 h posttransfection, cells were fixed in 4% PBS-buffered formaldehyde, permeabilized with 0.05% Triton X-100 for 20 min at room temperature. Cells were then incubated for 30 min in 3% BSA (Sangon), with primary antibody in 3% BSA for 2 h. Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) and Alexa Fluor cy3 goat anti-Rabbit IgG (Invitrogen) were used to detect primary antibodies. Cells were examined by Zeiss microscopy under 60× magnification.

## 2.3. Double-stranded RNA synthesis for knock-down of *dSmurf* in S2 cells

Two independent double-strand RNAs were designed to target different exon regions of *dSmurf* with the primer pairs as follow.

*dSmurf*1F: 5'-GAATTAATACGACTCACTATAGGGAGATGTCGCCG-GATGACGATGAG-3'  
*dSmurf* 1R: 5'-GAATTAATACGACTCACTATAGGGAGAGCGTGTG-TGCCGTTGCTG-3'  
*dSmurf* 2F: 5'-GAATTAATACGACTCACTATAGGGAGAGTACGGA-CAACGGTCGAG-3'  
*dSmurf* 2R: 5'-GAATTAATACGACTCACTATAGGGAGATGCAGAG-GGTGTCACTGAAG-3'

Then mMESSAGE mMACHINE®Kit (Life technologies, AM1344) was used for reverse transcription according to the technical manual. For knock-down and transfection treatment, S2 cells were seeded into 6-well plate with 8 µg of double strand RNA in the medium. Transient transfection was taken 12 h post seeding. After 36 h transfection, cells were harvested for co-IP or Western blotting.

## 2.4. *Drosophila* stocks

*Drosophila* stocks used in this research were all maintained at 25 °C. *UAS-yki*, *UAS-wts* and *dSmurf*<sup>Δ5C</sup>/CyO were described previously [6,26–28].

## 3. Results

### 3.1. *Wts* is a *dSmurf*-associating protein

Recent studies have implicated that the NEDD4-like family members including ITCH, WWP1 and NEDD4 individually regulate Lats1 stability in cultured mammalian cells in a manner dependent on their E3 ligase activity [22–25]. Given the entire family members share a similar structure, we wondered whether *Smurf1* and 2 regulate Lats1/2 stability in a similar fashion. To this end, we chose *Drosophila* as the assay system to test this possibility, because of no data to date showing NEDD4-like family members, including *dSmurf* (the *Drosophila* homolog of *Smurf1/2*), regulate the turnover of *Wts* (the *Drosophila* homologs of *Lats1/2*). Firstly, we found that *Wts* is a *dSmurf*-associating protein in our immunoprecipitation assays when Flag-tagged *dSmurf* and HSV-tagged *Wts* were co-expressed in S2 cells (Fig. 1A). We next examined the subcellular localization of *dSmurf* and *Wts*, and found that they exclusively co-localized in the cytoplasm of S2 cells (Fig. 1B), suggesting *dSmurf* and *Wts* may functionally associate with each other in the cytoplasmic compartment.

### 3.2. *dSmurf* is essential for *Wts* turnover

Previous studies revealed that the NEDD4-like family members ITCH, WWP1 and NEDD4 directly bind to and target Lats1 for ubiquitination-mediated degradation. It is reasonable to expect that *dSmurf* may also promote *Wts* degradation in a similar manner. To our surprise, the stability of exogenously expressed HSV-*Wts* was kept constant when co-expressed with either wild-type of Flag-*dSmurf* (Fig. 2A) or ligase activity-dead mutant of Flag-*dSmurf*<sup>C1029A</sup> (Fig. 2B) in a gradually increasing amount, suggesting overexpression of *dSmurf* is not sufficient to modulate *Wts* stability. However, when we knocked down endogenous *dSmurf* by double-strand RNA mediated RNA interference (RNAi), the accumulation of HSV-*Wts* was significantly increased (Fig. 2C and D), indicating that *dSmurf* is an essential factor for *Wts* turnover in S2 cells.

### 3.3. Knock-down of *dSmurf* increases *YkiS168* phosphorylation and inhibits *Yki* activity

As a primary phosphorylation site by *Wts*, *YkiS168* phosphorylation has been generally used to evaluate the activation level of Hippo signaling [27,29–31]. Since knock-down of *dSmurf* stabilizes *Wts* abundance, we thus asked whether knock-down of *dSmurf* modulates the phosphorylation level of *YkiS168*. To this end, an equal amount of Flag-*Yki* was transfected into S2 cells in which *dSmurf* is knocked down or not by double-strand RNAs targeted respectively to *dSmurf* or GFP control. Cell lysates were immunoprecipitated by anti-Flag antibody for detection of phosphorylated and total *Yki* by Western blotting. As anticipated, *YkiS168* phosphorylation was significantly increased while *dSmurf* was knocked down (Fig. 3A). As *YkiS168* phosphorylation is an inhibitory indicator for *Yki* transcriptional activity [27,29–31], we then conducted a HRE (Hippo Responsive Element for Sd-binding)-luciferase assay in S2 cells to investigate the effect of *dSmurf* on *Yki*-Sd transcriptional activity. As previously shown, a transcription activation of HRE reporter was significantly induced by co-expressing *Yki* and Sd [11]. Notably, knock-down of *dSmurf* evidently reduced the transcription activity of the *Yki*/Sd complex although overexpression of *dSmurf* had little effect (Fig. 3B). These results suggest that *dSmurf* is essential for modulating the phosphorylation level of *Yki* and thereby its activity in transcriptional regulation of S2 cells.

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