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CYLD negatively regulates Hippo signaling by limiting Hpo phosphorylation in *Drosophila*

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ABSTRACT

Cylindromatosis (CYLD), a deubiquitinase and regulator of microtubule dynamics, has important roles in the regulation of inflammation, immune response, apoptosis, mitosis, cell migration and tumorigenesis. Although great progress has been made in the biochemical and cellular functions of CYLD, its role in animal development remains elusive. In this study, we identified *Drosophila* CYLD (dCYLD) as a negative regulator of the Hippo pathway in vivo. dCYLD associates and colocalizes with Hpo, a core component of the Hippo pathway, in the cytoplasm, and decreases Hpo activity through limiting its phosphorylation at T195. We also showed that dCYLD limits Hippo signal transduction as evidenced by decreasing phosphorylation and thereby increasing activity of Yki, the key downstream effector of the Hippo pathway. These findings uncover dCYLD as a negative regulator of the Hippo pathway and provide new insights into the physiological function of dCYLD in animal development.

1. Introduction

The evolutionarily conserved Hippo signaling pathway plays critical roles in organ size control through coordinated regulation of cell proliferation and cell survival, and its dysfunction contributes to the development of human cancer [1–3]. Central to this pathway is a kinase cascade formed by two kinase complexes of Hippo (Hpo)/Salvador (Sav) and Warts (Wts)/Mob as tumor suppressor (Mats) that ultimately phosphorylates and inactivates the primary downstream effector Yki [4–9]. Thus, elucidating the molecular mechanisms underlying the regulation of the Hippo kinase cascade is important for understanding the role of Hippo pathway in animal development and human diseases.

CYLD is well established as a tumor suppressor in the development of familial cylindromatosis [10] as well as multiple human cancers [11]. CYLD belongs to the ubiquitin-specific protease (USP) subfamily of the deubiquitinase (DUB) family [12]. CYLD predominantly functions as a negative regulator of the nuclear factor-κB (NF-κB) signaling pathway by removing the lysine 63-linked polyubiquitin chains from various target proteins such as NEMO, TRAF2/6 and Bcl3 [13–16]. In addition, CYLD has been implicated in many other signaling pathways including JNK signaling [17,18], TCR signaling [19], Wnt/beta-catenin signaling [20], and TGF-β signaling [21,22]. It is worthy to note that dCYLD deubiquinates dTRAF2 and positively regulates TNFα-induced apoptosis through JNK signaling rather than NF-κB inactivation in *Drosophila* [18,23].

In addition to the USP deubiquitinase domain, which is also named as UCH domain in *Drosophila* [18], CYLD contains three conserved cytoskeleton-associated protein glycine-rich (CAP-Gly) domains in mammals and one such domain in *Drosophila*. Thus, CYLD may exert its functions through the interaction with the cytoskeleton. In addition to its binding to NEMO [24,25], CAP-Gly domains of CYLD have indeed been implicated in association with tubulin and enhancing tubulin polymerization into microtubules [26,27]. Most recently, CYLD was demonstrated to stabilize the astral microtubules and regulate spindle orientation via its dual functions as a microtubule-associated protein and deubiquitinase [28]. Given that the stability and polymerization–depolymerization dynamics of microtubules are important for cell migration, cell cycle progression, cell growth, and cell shape maintenance, the interaction of CYLD with tubulin may be important for its regulation of microtubule assembly in these biological processes. In this study, we provide the first evidence that *Drosophila* dCYLD negatively regulates Hippo signaling through limiting Hpo phosphorylation at T195. Our findings uncover dCYLD as a new regulator of the Hippo pathway and provide new insights into understanding CYLD function in physiological and pathological conditions.

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2. Materials and methods

2.1. Plasmid construction

Full-length of d*CYLD* tagged with Myc was amplified from the cDNAs of S2R+ cells by PCR and cloned into pAc-V5-HisB vector (Invitrogen) at KpnI/XhoI site, then verified by sequencing. The plasmid attB-UAS-Flag Hpo was gifted by Dr. Lei Zhang [29].

2.2. Cell culture and transient transfection

S2R+ cells were cultured in Schneider's Medium (Gibco) with 10% FBS (Hyclone) at room temperature without CO_2. Transient transfection was performed using Lipofectamine (GE), according to the manufacturer's instructions.

2.3. Immunoprecipitation and Western blotting analysis

S2R+ cells were transfected with corresponding plasmids and cultured for 36 h, then lysed using Nonidet P-40 lysis buffer containing protease inhibitors. Immunoprecipitations and Western blotting were performed as previous reports [30]. Antibodies used in this study are as follows: rabbit anti-Flag (Sigma, 1:5000), mouse anti-Flag (Sigma 1:5000), mouse anti-Myc (Abmart, 1:5000), mouse anti-HA (Abmart 1:5000), anti-phospho-MST1/MST2/Hpo antibody (Cell Signaling Technology), and anti-phospho-Yki antibody as described previously [9].

2.4. Immunofluorescence

Cells were transfected with indicated plasmids after being seeded on round slide coverslips in 24-well plates. 36 h after transfection, cells were fixed in 4% formaldehyde and permeabilized with 0.05% Triton X-100 at room temperature. Cells were then blocked for 30 min in 10% BSA (BBI), and incubated with primary antibody for 2 h and secondary antibody for another 1 h at room temperature. Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) was used to detect Flag-Hpo. Alexa Fluor Cy3 goat anti-Rabbit IgG (Invitrogen) were used to detect Myc-dCYLD. Cells were examined by Zeiss microscopy under at 63× magnifications.

2.5. Drosophila stocks

All fly stocks were maintained at 25 °C. The *ISO4* strain was used as the host for all P element-mediated transformations. UAS-d$CYLD^{RNAi}$ (15340) strains were obtained from the Vienna *Drosophila* Resource Center (VDRC).

3. Results

3.1. dCYLD negatively regulates Hpo activity in vivo

CYLD and the core components of Hippo signaling (e.g., *Hpo*, *Wts*, *Sav* and *Mats*) share some common features: (1) they are all tumor suppressors; (2) they are all implicated in the regulation of cell survival, cell cycle, and cell migration; (3) they are all involved in the regulation of the cytoskeleton [31–33]. In order to decipher the functional link between *CYLD* and the Hippo pathway, the genetic epistatic analysis was conducted to determine whether the overexpression phenotype of *Hpo* or *Wts* is modulated by co-expressing *dCYLD*. To this end, we generated transgenic flies of *UAS-dCYLD* which could induce *dCYLD* overexpression in specific tissues by appropriate Gal4 drivers. Overexpression of *dCYLD* with wing-specific *Nub-Gal4* (*Nub* > *dCYLD*) resulted in *Drosophila* wings similar to that of *Nub-Gal4* control (Fig. 1A, B and G). Intriguingly, when it was crossed to the transgenic flies of *UAS-Hpo* and *UAS-Wts* individually, co-overexpression of *dCYLD* significantly suppressed the small wing phenotype caused by *Nub* > *Hpo* but very mildly effect on *Nub* > *Wts* (Fig. 1D, E and G and data not shown). To further confirm the effects of *CYLD* overexpression on the activity of *Nub* > *Hpo* and *Nub-Wts*, we utilized RNA interference of *dCYLD* ($dCYLD^{RNAi}$) to evaluate its effect on wing development by *Nub* > *Hpo* and *Nub-Wts*. In contrast to *dCYLD* overexpression, the small wings of *Nub* > *Hpo*, but not of *Nub-Wts*, were enhanced by *dCYLD* depletion, although no obvious wing phenotype resulted from *Nub* > $dCYLD^{RNAi}$ (Fig. 1C, D, F and G and data not shown). Similar regulation of *dCYLD* on *Hpo* overexpression with *GMR-Gal4* was observed in *Drosophila* eyes (data not shown). Taken together, these results suggested that *dCYLD* negatively regulates *Hpo* activity in *Drosophila*.

3.2. dCYLD associates and colocalizes with Hpo in the cytoplasm

The above genetic interaction between *dCYLD* and *Hpo* prompted us to test whether dCYLD associates with Hpo in a protein complex. To examine this possibility, we performed co-immunoprecipitation (Co-IP) assays to test the interaction between dCYLD and Hpo. We transiently transfected S2R+ cells with *Flag-Hpo* and *Myc-dCYLD* and found that the proteins dCYLD and Hpo indeed reciprocally associated with each other (Fig. 2A). These results indicated that dCYLD may antagonize Hpo activity in a common complex. Our immunostaining assays further revealed that Myc-dCYLD colocalizes with Flag-Hpo in the cytoplasm, although dCYLD widely distributes in the cytoplasm and the nucleus (Fig. 2B).

3.3. dCYLD limits Hpo phosphorylation

As the upstream kinase in the core kinase cascade of Hippo signaling, the activity of Hpo is regulated by multiple inputs, and Hpo phosphorylation at T195 has been used as an important indicator for evaluating its activity. Consistent with dCYLD-induced down-regulation of Hpo activity in flies, Hpo-T195 phosphorylation level detected by a specific phosphorylation antibody was dramatically decreased in a dCYLD dosage-dependent manner in S2R+ cells (Fig. 3A). Based on the finding that Hpo activity was enhanced by $dCYLD^{RNAi}$ in *Drosophila* wings (Fig. 1D, F and G), we expected that the phosphorylated Hpo would be increased by knock-down of dCYLD in cultured cells. However, Hpo-T195 phosphorylation was only mildly increased by efficient depletion of dCYLD regardless of presence or absence of Tao-1, a promoting factor of Hpo-T195 phosphorylation and Hpo activity (Fig. 3B and C) [34,35]. Taken together, these data suggest that dCYLD suppresses Hpo activity by limiting its phosphorylation level at T195.

3.4. dCYLD represses activity of the core kinase cascade of Hippo signaling

Hpo phosphorylates and activates Wts, and in turn phosphorylates and inactivates Yki [9]. We thus asked whether dCYLD further modulates Hippo signal transduction from Hpo to Wts and thereby Yki in flies. Since our data revealed that dCYLD has little effect on Wts activity in *Drosophila* wings but obviously represses Hpo activity in both wings and eyes, we thus expected that the development of *Drosophila* eyes might be more sensitive for dCYLD regulation on Wts. As anticipated, small and rough eyes resulted from overexpression of *Wts* by eye-specific *GMR-Gal4* driver (*GMR* > *Wts*) were obviously released by simultaneous overexpression of *dCYLD* (Fig. 4A, B, D and E). Conversely, the eye smallness and roughness caused by *GMR* > *Wts* were enhanced by knock-down of *dCYLD* (Fig. 4C and F). Thence, these findings suggested that dCYLD