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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

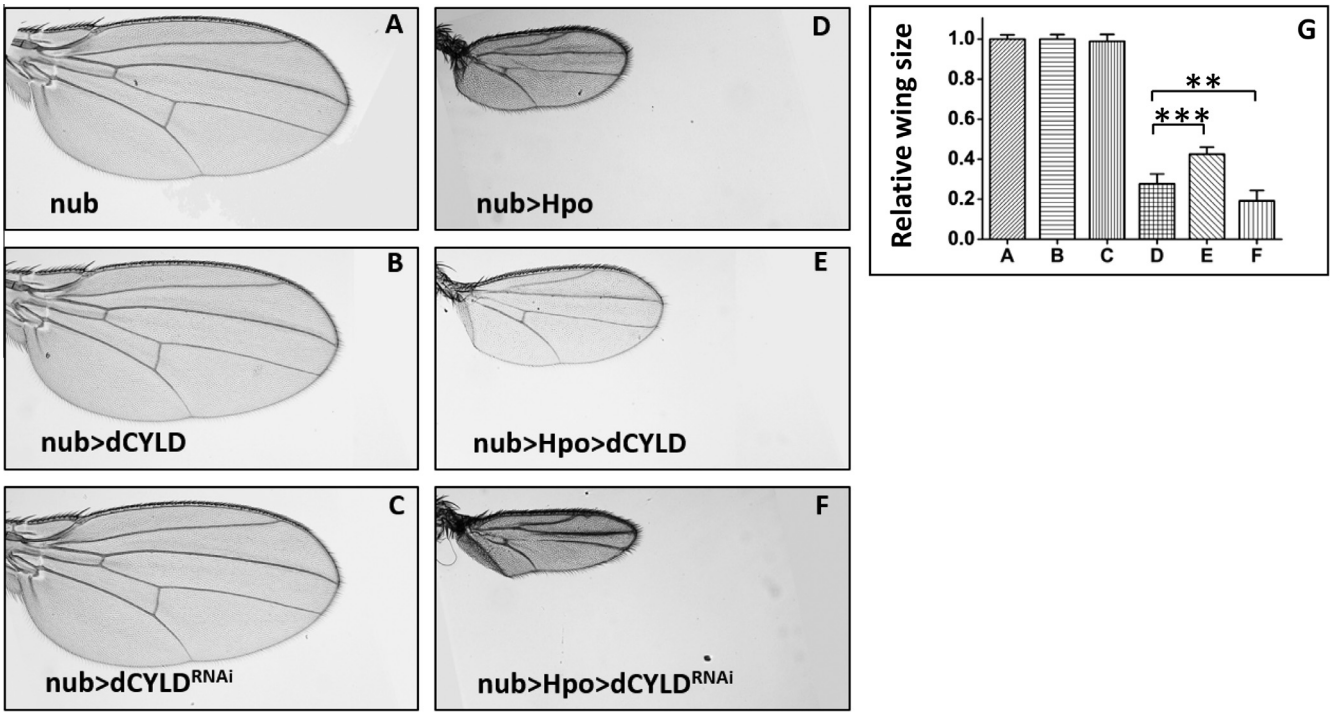

Fig. 1. dCYLD negatively regulates Hpo in vivo. (A–F) Dorsal view of adult male wings; anterior is to the left. Wings express the follow transgenes: (A) *nub-Gla4*, (B) *nub-Gal4* and U*AS-dCYLD*, (C) *nub-Gal4* and *UAS-dCYLD*^{RNAi}, (D) *nub-Gal4* and *UAS-Hpo*, (E) *nub-Gal4, UAS-Hpo* and *UAS-dCYLD*, and (F) *nub-Gal4*, *UAS-Hpo* and *UAS-dCYLD*^{RNAi}. (G) The relative wing size was quantified using the unpaired *t*-test.

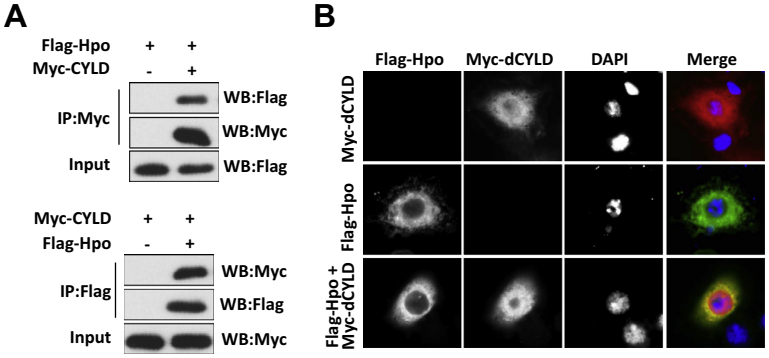

Fig. 2. dCYLD associates and colocalizes with Hpo in the cytoplasm. (A) Myc-dCYLD and Flag-Hpo coimmunoprecipitated with each other. The indicated constructs were transfected into S2 cells. Flag-Hpo or Myc-dCYLD was immunoprecipitated with anti-Flag or anti-Myc antibody, respectively. Western blotting was done to detect specific proteins as indicated to the right of each panel. (B) Myc-CYLD and Flag-Hpo colocalized in the cytoplasm. S2 cells were transfected with indicated constructs and stained with anti-FLAG or anti-Myc antibody. Nuclei were stained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

represses Wts activity in vivo. Since Yki is negatively regulated by Wts, it is reasonable to predict that dCYLD would positively modulate Yki activity by inhibiting Hpo and then Wts activation. Consistent with our expectation, the enlarged eyes induced by *GMR > Yki* were slightly but repeatedly enhanced by overexpression and suppressed by knock-down of *dCYLD*, respectively (Fig. 4G–I).

Yki-S168 phosphorylation by Wts is widely used as readout for judging the activation level of Hippo signaling. We further examined whether the level of Yki-S168 phosphorylation is modulated by dCYLD in S2R+ cells. Our results showed that dCYLD have no obvious effect on Yki-S168 phosphorylation when dCYLD and Yki were coexpressed (Fig. 4J). However, Yki-S168 phosphorylation was drastically promoted by Hpo and this phosphorylation was then significantly reduced by dCYLD (Fig. 4J), suggesting that dCYLD inhibits Hpo activity and then activates Yki through repressing its phosphorylation. Taken together, we proposed that Hippo signaling is negatively regulated by dCYLD-binding to Hpo and in turn inhibits Wts activity, ultimately leading to Yki-S168 hypophosphorylation and Yki activation.

4. Discussion

Previous studies have revealed that CYLD plays critical roles in many biological processes including immune response, apoptosis, mitosis, cell migration and tumorigenesis in both *Drosophila* and vertebrates [36]. However, the physiological functions of CYLD in animal development and tumorigenesis remain poorly understood. Based on genetic and biochemical analysis, our results indicated that a new function of dCYLD is regulating tissue growth through suppressing the Hippo pathway in *Drosophila* wings and eyes.

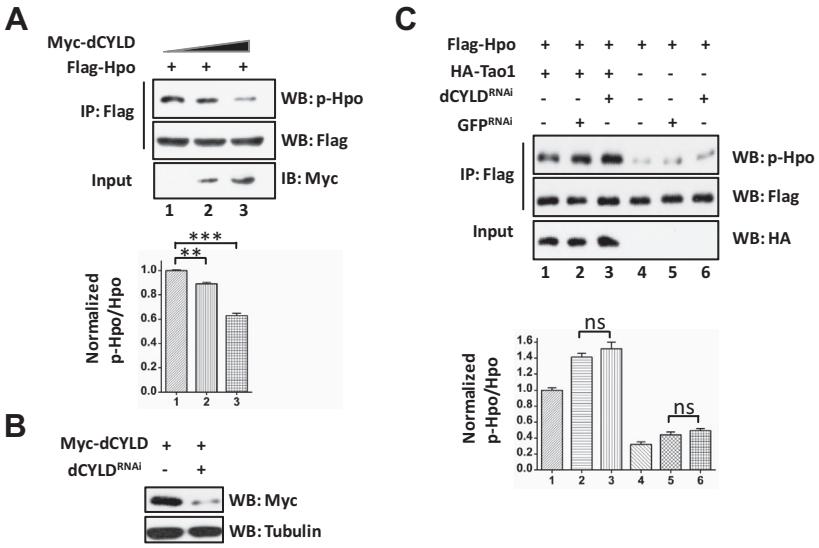

Fig. 3. dCYLD decreases Hpo phosphorylation level in *Drosophila* S2R+ cells. (A) dCYLD decreased Hpo phosphorylation level in a dosage-dependent manner. Myc-dCYLD and Flag-Hpo were transient expressed in S2R+ cells and Hpo phosphorylation was measured using a commercially available phospho-MST1/2 antibody. (B) Knockdown of *dCYLD* caused remarkable decreased level of dCYLD. (C) Knockdown of *dCYLD* mildly affected Hpo phosphorylation. S2R+ cells were transfected with dsRNA targeting *dCYLD*. Knockdown of this gene had negligible effect on Hpo phosphorylation, but this effect was enhanced when cells were cotransfected with Tao-1.

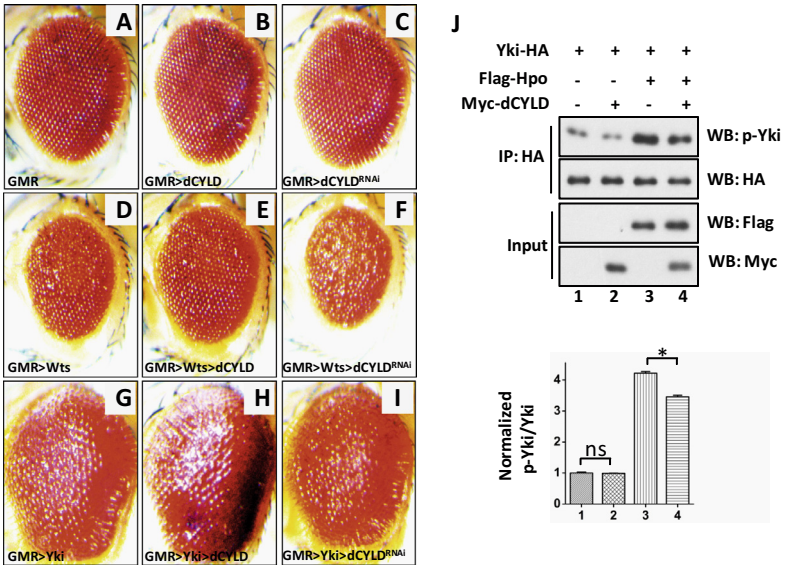

Fig. 4. dCYLD regulates Wts and Yki activity. (A) dCYLD regulates core Hippo pathway members Wts and Yki in vivo. Adult male *Drosophila* eyes are shown, and anterior is to the left. The *Drosophila* genotypes are indicated as follow: (A) *GMR-Gla4*, (B) *GMR-Gal4* and *UAS-dCYLD*, (C) *GMR-Gal4* and *UAS-dCYLD*^{RNAi}, (D) *GMR-Gal4* and *UAS-Wts*, (E) *GMR-Gal4*, *UAS-Hpo* and *UAS-dCYLD*, (F) *GMR-Gal4*, *UAS-Hpo* and *UAS-dCYLD*^{RNAi}, (G) *GMR-Gal4* and *UAS-Yki*, (H) *GMR-Gal4*, *UAS-Yki* and *UAS-dCYLD*, (I) *GMR-Gal4*, *UAS-Yki* and *UAS-dCYLD*^{RNAi}. (J) dCYLD regulates Hpo-mediated phosphorylation of Yki. The indicated constructs were transfected into S2R+ cells, and Yki phosphorylation was detected using a phospho-specific antibody [9].

dCYLD, to our knowledge, is the first described deubiquitinase implicated in the regulation of Hippo pathway. The following evidence has led us to propose dCYLD as a new regulator of Hippo pathway: (1) dCYLD could modify the alteration of tissue growth caused by altered Hippo pathway activity; (2) dCYLD overexpression suppresses the core Hippo pathway kinase cassette.

Previous reports indicate that CYLD functions as a tumor suppressor. In this study we found that dCYLD modulates Hippo pathway activity just like a growth-promoting factor. Moreover, dCYLD seemed more sensitive to Hippo pathway in eyes than in wings (data not shown), even though overexpression or knockdown of dCYLD alone does not affect tissue growth in *Drosophila*. One likely explanation for this is that the role of dCYLD may be restricted to specific cell lineages and stimulatory conditions under physiological conditions.

Many intriguing questions remain unanswered. For example, it remains to be determined whether the deubiquitinase activity of dCYLD is essential for limiting Hpo phosphorylation. In addition, the mechanisms for how dCYLD leads to the decreased phosphorylation of Hpo need to be further investigated. One possibility is that dCYLD recruits phosphatases, such as PP2A, to Hpo to dephosphorylate Hpo. Previous studies indicate that CYLD associates with PP2A [37], which has been reported to interact with and dephosphorylate Hpo [38]. It is also possible that dCYLD deubiquitinates some proteins which repress Hpo activity, taking Par-1 as an example [39]. Another possibility is that dCYLD regulates Hippo path-

way through stabilizing microtubules which are known to participate in Hippo pathway regulation [40]. Elucidation of these issues is essential for better understanding of how dCYLD regulates Hippo pathway.

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