D-Cbl Binding to Drk Leads to Dose-Dependent Down-Regulation of EGFR Signaling and Increases Receptor-Ligand Endocytosis

Pei-Yu Wang, Li-Mei Pai

Abstract

Proper control of Epidermal Growth Factor Receptor (EGFR) signaling is critical for normal development and regulated cell behaviors. Abnormal EGFR signaling is associated with tumorigenic process of various cancers. Complicated feedback networks control EGFR signaling through ligand production, and internalization-mediated destruction of ligand-receptor complexes. Previously, we found that two isoforms of D-Cbl, D-CblS and D-CblL, regulate EGFR signaling through distinct mechanisms. While D-CblL plays a crucial role in dose-dependent down-regulation of EGFR signaling, D-CblS acts in normal restriction of EGFR signaling and does not display dosage effect. Here, we determined the underlying molecular mechanism, and found that Drk facilitates the dose-dependent regulation of EGFR signaling through binding to the proline-rich motif of D-CblL, PR. Furthermore, the RING finger domain of D-CblL is essential for promoting endocytosis of the ligand-receptor complex. Interestingly, a fusion protein of the two essential domains of D-CblL, RING-PR, is sufficient to down-regulate EGFR signal in a dose-dependent manner by promoting internalization of the ligand, Gurken. Besides, RING-SH2Drk, a fusion protein of the RING finger domain of D-Cbl and the SH2 domain of Drk, also effectively down-regulates EGFR signaling in Drosophila follicle cells, and suppresses the effects of constitutively activated EGFR. The RING-SH2Drk suppresses EGFR signaling by promoting the endosomal trafficking of ligand-receptor complexes, suggesting that Drk plays a negative role in EGFR signaling by enhancing receptor endocytosis through cooperating with the RING domain of D-Cbl. Interfering the recruitment of signal transducer, Drk, to the receptor by the RING-SH2Drk might further reduces EGFR signaling. The fusion proteins we developed may provide alternative strategies for therapy of cancers caused by hyper-activation of EGFR signaling.

Introduction

Ubiquitination occurs via sequential activation and conjugation of ubiquitin to target proteins by ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) [1]. Aside from protein degradation, ubiquitination represents a crucial signal for the endocytosis of signaling molecules such as EGFR. The attenuation of EGFR signaling by endocytosis serves to properly control cell growth, differentiation, and normal developmental processes [2,3,4,5]. Consistent with an intimate role in signaling regulation, as well as in other cellular processes, emerging evidence has shown that derailed endocytosis disrupts developmental processes and leads to cancer formation [6,7].

A critical E3 ubiquitin ligase mediating the ubiquitination-dependent receptor endocytosis is the proto-oncogene Cstias B-lineage lymphoma (Cbl), which was first identified as the cellular homolog of v-cbl, which induces pre-B-cell lymphomas and myeloid tumors [8,9]. Cbl is involved in multiple signaling pathways, and plays a negative role in EGFR signaling that is conserved among many species [10]. In Drosophila, D-Cbl negatively regulates EGFR signaling in dorsoventral patterning during oogenesis [11], in eye development [12,13,14], and in border cell migration [15]. The Cbl recognizes receptor tyrosine kinase (RTK, such as EGFR) and non-receptor tyrosine kinases through a phosphotyrosine-binding (PTB) domain [10,16]. Internalizing EGFR requires c-Cbl and Lys63-linked polyubiquitin chain modification mediated by c-Cbl is essential for sorting activated receptors to the lysosomal degradation compartment [17,18,19,20]. The RING finger domain in Cbl is highly conserved during evolution, particularly for critical amino acids related to E3 ligase activity [16,20]. The C-terminal proline-rich (PR) domain for protein-protein interaction and the ubiquitin association (UBA) domain are found only in some members of the family [21]. In Drosophila, two major isoforms, D-CblL and D-CblS are generated from the single D-Cbl gene [22]; the shorter isoform D-CblS lacks the proline-rich and UBA domains.

Studies in mammals have shown that the endocytosis of EGFR involves multiple pathways, depending on EGFR concentration and exhibits cell-based specificities [2,3,24]. Therefore, redundancy of multiple pathway of endocytosis made it difficult to dissect
which molecule the process requires. The *Drosophila* eggshell patterning has served as a sensitive and simple system to read out the levels of EGFR signaling [25,26], thus representing an ideal model of mechanistic studies. The advantage of this in vivo system is that it provides physiological conditions with a gradient of ligand concentration to induce different levels of EGFR activation that is reflecting through the D/V patterning of eggshell and embryo. The Gurken, a TGF-β homolog, is produced by the oocyte and activates EGFR in follicle cells to specify the dorsal cell fates, followed by attenuation of EGFR signaling via negative regulators, such as sprouty, and lekkon, which together determine the area of the follicle epithelium where the dorsal appendages (DA) form [27,28]. Importantly, examining the Gurken distribution in loss of D-Cbl and D-CblL over-expression conditions has revealed that D-CblL promotes endocytosis of ligand-receptor to control the amount of available ligand. We demonstrated that D-CblL facilitates the activated receptor to traffic through the endocytic pathway for terminating signaling at lysosomal degradation compartment [29]. Therefore, over-expression of D-CblL at different levels resulted in different degrees of ventralization, corresponding to phenotypes resulting from different severities of *gurken* mutant alleles [30]. This dose-dependent, negative effect on EGFR signaling is specific to D-CblL and is not produced by over-expression of D-CblS.

To understand how D-CblL controls EGFR signaling at the molecular level, this study investigates which molecular interaction with D-CblL is sufficient to facilitate the endocytosis of the ligand-EGFR complex in *Drosophila* egg chambers. This work first demonstrates that the factor Downstream of receptor kinase (Drk) plays a major role in D-CblL mediated down-regulation of EGFR signaling in a dose-dependent fashion. In addition, E3 ligase activity is required for D-CblL activity, because over-expression of Δ70Z-D-CblL, an E3 defective mutant, blocked ligand-receptor internalization and produced a dominant-negative effect. We generated the RING-SH2Drk chimeric protein, containing two functional domains of D-Cbl and Drk, and found that this chimeric protein not only attenuated EGFR signaling, but also down-regulated constitutively activated EGFR, λ-top. We further demonstrated that RING-SH2Drk suppresses EGFR signaling by enhancing the endosomal trafficking of the ligand-receptor complex and interfering with the recruitment of the endogenous Drk.

**Results**

Drk plays a major role in D-CblL mediated down-regulation of EGFR signaling

In this study, we set out to elucidate the molecular mechanism by which D-CblL promotes the endocytosis of the ligand-receptor complex. Since this effect of D-CblL was not observed for D-CblS even when expressed at a similar level [30], we suspected that D-CblL may mediate the internalization by its extra C-terminus that is distinct from D-CblS. In mammals, Grb2 (Growth factor receptor binding protein 2), Eps15 and the CIN85-Endophilin complex are involved in Cbl-mediated down-regulation of EGFR signaling [31,32,33]. We then tested for their involvement in D-CblL mediated down-regulation of EGFR signaling in *Drosophila* oogenesis by a sensitive genetic assay. While *Drosophila* Eps15, endophilin A and endophilin B had no or minor effects (Table S1), the *Drosophila* Grb2 homolog *dk* exhibits a strong link to D-CblL activity described below.

Mammalian studies demonstrated that c-Cbl is recruited to EGFR through directly binding the Y1045 residue of EGFR or indirectly interacting with Grb2 [34,35,36,37]. The Tyr1068/1086 of EGFR is the direct docking site for the SH2 domain of Grb2 [38]. The SH3 domain of Grb2 binds to the proline-rich region of D-CblL, which is absent in D-CblS. We used the *dk*R74A mutant, which loses binding to EGFR caused by mutation in the SH2 domain [39], for a genetic interaction assay. The ventralized effect by *EQ1-Gal4*-driven over-expression of D-CblL in the follicle cells was significantly reduced in the heterozygous *dk*R74A mutant background (Table 1 and Figure 1A–D). *EQ1-Gal4* is mainly expressed in the follicle cells [30]. We reasoned that if Drk was required for the effect of D-CblL over-expression, the interruption of interaction between Drk and D-CblL, would block the D-CblL over-expression effects. To address this issue, we found one consensus sequence PPLPR of the Grb2/Drk binding motif on D-CblL (named PR), and generated a mutant in this motif by replacing the first and fifth prolines with alamines (mPR) (Figure 1E). The results from the yeast two hybrid system showed that the wild-type PR, but not the mPR, interacted with full-length Drk (Figure S2). Consistently, in the anti-D-Cbl immunoprecipitation assay, much less Drk was pulled down in the D-CblL-mPR complex than in the wild-type D-CblL complex, even though D-CblL-mPR was expressed at a higher level compared to that of D-CblL (Figure 1F). We then over-expressed D-CblL-mPR or D-CblL in follicle cells using *EQ1-Gal4*. At comparable levels (Figure S1A), the over-expression effects of D-CblL-mPR on EGFR signaling were much weaker than that of D-CblL. Furthermore, the effect of D-CblL-mPR over-expression was not suppressed in the heterozygous *dk*R74A mutant background, suggesting that PR of D-CblL might be a critical binding domain for Drk (Table 1).

We further analyzed the function of the D-CblL-mPR mutant using a constitutive expression promoter, HS83 [40], which was also used in rescue assays by D-CblL and D-CblS. Two transgenic lines, hs83-3-D-cblL-mPR-7 and hs83-3-D-cblL-mPR-4, rescued the lethality of the *drkEOA* mutant background, suggesting that PR of D-CblL might be a critical binding domain for Drk (Table 1).

| Progenies | % Eggshell phenotype* |
|-----------|----------------------|
| V3⁰ | V2⁰ | V1⁰ | Wt⁰ | N |
| dk*R74A/+ | 0 | 0 | 3 | 97 | 421 |
| L-A10 | 1 | 40 | 38 | 21 | 294 |
| L-A10 in dk*R74A/+ | 0 | 29 | 56 | 15 | 247 |
| L-A12 | 5.2 | 66.2 | 26.3 | 2.3 | 311 |
| L-A12 in dk*R74A/+ | 0 | 27 | 63 | 10 | 176 |
| mPR-GB | 1 | 25 | 75 | 212 |
| mPR-GB in dk*R74A/+ | 0 | 25 | 75 | 320 |
| mPR-GB | 0 | 47 | 49 | 4 | 250 |
| mPR-GB in dk*R74A/+ | 0 | 50 | 50 | 0 | 362 |

*The number shows percentage in each phenotype.

The Egfr activity is correlated with morphology of dorsal appendage, two dorsal appendages indicate normal activity of Egfr (Wt), two dorsal appendages fused at the base indicate low level of Egfr activity (V1), one fused dorsal appendage indicates the lower level of Egfr activity (V2), and no appendage indicates the lowest level of Egfr activity (V3).

*EQ1-Gal4*-driven over-expression of each D-CblL or D-CblL-mPR transgenic line was either in the wild-type background or in the heterozygous *dk*R74A mutant background.

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D-CblL-mPR showed no pattern defect in wing or the eggs they laid (data not shown), although the expression level of hs83-D-cblL-mPR was similar to that of hs83-D-cblL (Figure S1B). These data indicate that the interaction between D-CblL and Drk underlies the functional difference between the D-CblS and D-CblL, and provide the basis for the dose-dependent, negative effects of D-CblL on EGFR signaling. However, this interaction is not essential for D-CblL function in terms of the normal restriction of EGFR signaling.

The E3 ligase activity of D-CblL is essential for its negative role in EGFR signaling

A screen for D-Cbl loss-of-function alleles has identified mutations in the RING domain [13]. Indeed, mouse fibroblasts that express h83-D-cblL-mPR was similar to that of h83-D-cblL (Figure S1B). These data indicate that the interaction between D-CblL and Drk underlies the functional difference between the D-CblS and D-CblL, and provide the basis for the dose-dependent, negative effects of D-CblL on EGFR signaling. However, this interaction is not essential for D-CblL function in terms of the normal restriction of EGFR signaling.

The E3 ligase activity of D-CblL is essential for its negative role in EGFR signaling

A screen for D-Cbl loss-of-function alleles has identified mutations in the RING domain [13]. Indeed, mouse fibroblasts that express Δ70Z-Cbl, an E3 defective mutant with a deletion of 17 amino acids prior to the RING finger domain, show increased EGFR activation upon ligand stimulation [41,42]. In addition, the D-cbl and D-cblS-mco (D-CblS-Δ70Z) act as dominant negative mutants in the Drosophila eye and wing, which is presumably resulted from competing with wild-type D-Cbl for binding to the EGFR [43]. However, one proposed inhibitory function of Cbl in EGFR signaling is acting as a competitor with Sos (Son of sevenless) by binding to Grb2, thereby blocking signaling through the Ras-MAPK pathway [21]. We decided to test whether the E3 activity is essential for D-CblL function in the dose-dependent EGFR regulation. First we tested the involvement of ubiquitination in D-CblL-mediated regulation using the E2-conjugase mutant, eff8, which has been shown to be involved in D-cbl function [43,44,45]. The effect of D-CblL over-expression was reduced in the eff8 heterozygous mutant (Table S2), indicating the attribution of ubiquitination in D-CblL effects. A deletion mutant similar to D-CblS-Δ70Z, which should be E3 defective, was generated for D-CblL, and notably dominant negative effects on EGFR signaling were observed upon its over-expression in the wing, eye and follicle cells (Figure 2). Significantly, the dominant effect of D-CblL-Δ70Z could be suppressed in drrkEOA heterozygous mutant background (Figure 2I), indicating that the interaction between Drk and D-CblL is required for the dose-dependent effect.

Figure 1. D-CblL directly interacts with Drk through its PR motif. (A and A’), (B and B’), (C and C’), (D and D’). Two dorsal appendages fused at the base indicate low level of EGFR activity (V1), whereas (C and C’) one fused dorsal appendage indicates a even lower level of EGFR activity (V2). (D and D’) No appendage indicates the lowest level of EGFR activity (V3). (E) The schematic structure shows the wild-type D-CblL, which contains the TKB domain in its N-terminus (red), the RING finger domain (yellow) and the proline-rich domain in the C-terminus. The predicated binding motif of Drk on D-CblL is PPLPPR (shown in purple). The first and fifth prolines of the PR motif on D-CblL-mPR mutant were replaced by alanines (shown in Box). (F) The D-CblL was immunoprecipitated from Ore (wild-type) or two hs83-D-cblL-mPR; cblF165 rescued fly lines (line 4 and line 7) by the anti-D-CblL antibody (rE6). The immunoprecipitates (IP-10 μl out of 30 μl) were separated by the SDS-PAGE, and detected by the anti-D-CblL antibody (8C4) and the anti-Drk antibody. Supernatants (Sup.) were produced after immunoprecipitating, indicating the ability of immunoprecipitation in this experiment. 30 μl out of 400 μl total lysate was analyzed by western blotting in each lane. The Drk signal is near 24KD (arrow).

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of D-CblL on EGFR signaling. Furthermore, the HS83-D-CblL-A70Z could not rescue the cb1F165 null mutant (data not shown), reinforcing the dependence of the negative role of D-CblL in EGFR signaling depends on the E3 activity.

We had previously shown that there is an increase of the endocytic Gurken (HRP-Gurken) in follicle cells after D-CblL over-expression, implying that D-CblL promotes the endocytosis of the Gurken-EGFR complex [29]. To clarify the requirement of ubiquitination in D-CblL-mediated ligand-receptor endocytosis, the distribution of HRP-Gurken in follicle cells was examined. The HRP-Gurken signal was abolished in follicle cells with D-CblL in follicle cells was examined (Figure 5C, C'), whereas the signal was clearly detected in wild-type or D-CblL expressing follicle cells (Figure 3A and B). Taken together, our results showed that the E3 activity of D-CblL plays an essential role in promoting ligand-receptor endocytosis.

The D-CblL fusion proteins down-regulate EGFR signaling

Based on the functional implications of the D-CblL's interaction with Drk and E3 ligase activity, we therefore aimed to test next whether these two functional domains are sufficient to effectively down-regulate EGFR. A fusion protein containing the RING finger domain and the PR motif of D-CblL was generated (Figure 4A). We expected that the fusion protein could interact with EGFR through binding to Drk. In line with this notion, we also generated a chimeric protein that contained the RING finger domain of D-CblL and the SH2 domain of Drk (Figure 4A). This RING-SH2Drk chimera should be able to bind to the activated EGFR on pY1068 or pY1086 that can be recognized by the SH2 domain of Drk.

To test the effects of these fusion proteins on EGFR signaling, ectopic expression of UAS-Flag-RING-SH2Drk and of UAS-RING-PR were induced in follicle cells by GR1-Gal4 at 29°C and 32°C, respectively (Figure S1). At 25°C, only low levels of D-Cbl fusion proteins were expressed, resulting in a slight defect on the eggshell morphology (data not shown). High-level expression of RING-SH2Drk at 29°C resulted in significantly reduced EGFR signaling, and about half of the eggshells showed an intermediate ventralization phenotype indicated by the fusion of two dorsal appendages (Table 3 and Figure 1C). The effects were correlated with expression levels of the fusion protein (Figure S1). Even though expression of RING-PR had a weaker effect than that caused by expression of RING-SH2Drk, high level expression of RING-PR, induced by hsGα4 (Figure S1D), also effectively down-regulated EGFR signaling and caused about 1/3 of the eggshells to display the intermediate ventralization phenotype (Table S3, Figure 1C). Consistent with our previous observations [29], Gurken distribution outside of the follicle cells was also reduced when RING-SH2Drk or RING-PR were over-expressed in follicle cells, similar to the effects caused by D-CblL over-expression (Figure 4). These results showed that these small fusion proteins, as well as full length D-CblL, could down-regulate EGFR signaling in a dose-dependent manner. Furthermore, RING-SH2Drk and RING-PR can promote the internalization of ligand-receptor complexes and lead to a reduction of extracellular Gurken distribution.

RING-SH2Drk down-regulates EGFR signaling through endosomal sorting and competition with Drk

We previously demonstrated that D-CblL promotes the internalization of the Grk/EGFR complex via the Rab5/Rab7 endocytic pathway [29]. To determine the route in endosomal trafficking of RING-SH2Drk-mediated endocytosis, we assayed the HRP-Grk/EGFR complex using the anti-Hrp antibody in follicle cells expressing the RING-SH2Drk chimera protein. More HRP-Grk signals were co-localized with Rab5-GFP and Rab7-GFP in follicle cells expressing either D-CblL (Figure 5E, E', F, K, L and O) or RING-SH2Drk (Figure 5C, C', D, I, J and K), compared to those observed in wild-type cells (Figure 5A, A', B, G, H and I). The frequencies of detecting co-localization signals of HRP-Grk and Rab5-GFP/Rab7-GFP were 17%/15% (n = 14/n = 22) and 38%/23% (n = 16/n = 14) in cells expressing D-CblL and RING-SH2Drk, respectively. Furthermore, the signals of exogenous D-CblL and RING-SH2Drk proteins detected by anti-Flag antibody were trapped near the cell cortex in follicle cells expressing dominant negative Rab5S43N (data not shown). We then further examined the multivesicular body (MVB) sorting of D-CblL and RING-SH2Drk-mediated trafficking using the anti-Hrs antibody that labels sorting endosomes. 22% (n = 12) and 29% (n = 11) of HRP-Grk signals were co-localized with Hrs signal in follicle cells respectively expressing D-CblL (Figure 5Q Q', Q, Q' and R) or RING-SH2Drk (Figure 5O O, O' and P), whereas only 16% (n = 12) HRP-Grk/Hrs co-localization signal were observed in wild-type egg chambers (Figure 5M M' and N). This observation suggests that RING-SH2Drk-mediated endocytosis shares the same trafficking route with D-CblL through the early endosome, late endosome and MVB to lysosome, and all steps of endocytosis are increased in the D-CblL or RING-SH2Drk expressing cells.

Because the SH2 domain of RING-SH2Drk was derived from Drk, we assumed that the docking site for RING-SH2Drk on EGFR is the same as that for Drk. Therefore, this chimeric protein might compete with endogenous Drk for binding to EGFR. This possibility was tested by immunoprecipitation using anti-EGFR antibodies to determine the amount of Drk in the receptor complex. 40% of Drk in the EGFR complex was reduced in egg chambers expressing RING-SH2Drk or full length D-CblL, compared to the wild-type egg chambers (Figure 6). This result indicates that RING-SH2Drk interferes with the interaction between endogenous Drk and EGFR, which may lead to reduced signal transduction. Taken together, the chimeric protein RING-SH2Drk may down-regulate EGFR signaling through promoting the endosomal trafficking of the EGFR complex and reducing the recruitment of Drk/Sos in signal transduction.

**Discussion**

To dissect the molecular machinery for EGFR endocytosis, we studied the mechanism by which D-CblL promotes EGFR...
Figure 2. Dominant negative effects of D-CblL-Δ70Z. (A) This picture is a dorsal view of the wild-type eggshell. The dorsal midline is the region between the two dorsal appendages (indicated by yellow arrow). (B) Expression of D-CblL-Δ70Z in follicle cells driven by EQ1-Gal4 resulted in a dorsalized eggshell characterized by an expanded dorsal midline (yellow arrow). (C) In the wild-type embryo, the eight ventral denticle belts represent the ventral structure. (D) Expression of D-CblL-Δ70Z in the follicle cells driven by GR1-Gal4 resulted in a dorsalized embryo that lost the anterior ventral structure. (E) Wild-type adult eye. (F) Ubiquitous expression of D-CblL-Δ70Z in eyes driven by GMR-Gal4 generated a rough eye phenotype. (G) Wild-type adult wing. (H) Expression of D-CblL-Δ70Z in the wing by MS1096-Gal4 caused an extra vein phenotype. (I) The effect of UAS-D-cblL-Δ70Z expression could be suppressed in drk^{ECD/4} background. The percentage of the wild-type and dorsalized eggshell are indicated in the blue and red column, respectively.

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endocytosis in Drosophila follicle cells. We then applied our results to generate a chimeric protein, containing the RING finger domain of Cbl fused to the SH2 domain of Drk, which can very effectively down-regulate EGFR signaling.

Drk plays two roles in EGFR signaling in Drosophila oogenesis

Mammalian studies have reported that c-Cbl regulates EGFR signaling through its interacting molecules, such as Grb2, CIN85-Endophilin complex and Eps15 [21]. This study investigated the role of these molecules in D-CblL mediated regulation, and found that elimination of Drk interaction resulted in significant reduction of the effect of D-CblL over-expression (Table 1). In consideration of our data and results from previous studies, we conclude that the Grb2/Drk has dual roles in EGFR signaling both in mammals and Drosophila. Acting as a signaling transducer, Grb2 binds to the proline rich motif of RasGEF/Sos through the SH3 domain, leading to Ras activation and activation of MAPK cascade [46]. Similarly, loss of function of a drk mutant in Drosophila caused a ventralization of the egg, a phenotype representing hypo-activation of EGFR signaling [47]. In contrast, a recombinant SH2 domain of Grb2 inhibited EGFR endocytosis, indicating the requirement of Grb2 in EGFR endocytosis [48]. Here, we demonstrated that interaction between D-CblL and Drk is crucial for promoting EGFR endocytosis by D-CblL. Our protein interaction data in this study (Figure 1F and Figure S2) agree with previous finding that Drk/Grb2 interacts with the proline-rich motif of Cbl [49,50]. In cell culture studies, the indirect binding of EGFR to Cbl through Grb2 is necessary for receptor internalization, whereas endosomal sorting requires direct binding to Cbl [31,35]. Our findings showed that the chimeric protein is sufficient to down-regulate EGFR signaling and suppresses the effect of l-top over-expression in Drosophila ovaries (Table 3 and 4). Expression of RING-SH2Dxx facilitates the endosomal trafficking of ligand-receptor complex through Rab5 (early) and Rab7 (late) endosomes, and MVBs (Figure 5). This result argues that recruiting the RING domain of Cbl to EGFR by the SH2 domain of Drk can promote the trafficking of EGFR to degradation compartments, such as MVBs. Furthermore, expression of RING-SH2Dxx led to reduced Drk binding on EGFR (Figure 6). This observation suggests that RING-SH2Dxx chimeric protein not only promotes the trafficking of EGFR to lysosomal degradation pathway, but also competes away the endogenous Drk, which may also contribute to reducing EGFR signaling. Interestingly, over-expression of D-CblL also reduced the binding of Drk to EGFR, suggesting that D-CblL might sequester Drk from binding to the receptor for signaling.

Importantly, the lethal effect of D-CblL was significantly reduced when the Drk binding motif was mutated in D-CblL (Table 2). This indicates that D-CblL efficiently down-regulates EGFR signaling through Drk. Large amounts of D-CblL in the cell may lead to comprised EGFR signaling levels that are too low for survival. However, even when we eliminated the interaction with Drk in the D-CblL-mPR mutant, this protein could still down-regulate l-top (Table S3). This result further demonstrated that D-CblL down-regulates EGFR through multiple mechanisms, and other D-CblL interacting molecules besides Drk might play important roles in down-regulating EGFR signaling even when Drk is absent.

The role of the RING finger domain in Cbl-mediated down-regulation of EGFR signaling

Our previous study demonstrated that D-cbl is required for down-regulation of EGFR signaling during DV patterning of the eggshell and embryo. Here we further showed that the RING finger domain of D-CblL is essential for its negative effect on EGFR, since the D-CblL mutant protein lacking this domain (Δ70Z-D-CblL) exhibited a dominant negative effect (Figure 2). In addition, the Δ70Z-D-CblL mutant also failed to rescue c6P163 mutant, indicating that RING finger domain activity plays a major role in D-CblL function. These data are consistent with results from studies on the D-Cbl loss-of-function alleles by Wang et al. [13] and from research reports in mammals [42,51,52]. Ubiquitination has been considered as a signal to mediate EGFR
endocytosis at two critical steps: receptor internalization and endosomal sorting [17,31,35,53,54]. D-CblL promotes EGFR endocytosis in a dose-dependent manner, but the endocytosis of the ligand-receptor complex was significantly reduced in D70Z-D-CblL over-expressing follicle cells. Interestingly, a cell culture system that expressed an EGFR mutant with a reduced ubiquitination level (to only 1%) still displayed normal internalization [53]. Therefore, one possibility is that the endocytic signal is not ubiquitination of EGFR itself [53], and that other molecules might be involved and ubiquitinated by Cbl. Cbl can directly bind

![Diagram of RING domain fusion proteins altering Grk distribution](image)

**Figure 4. The RING domain fusion protein alters the Grk distribution.** (A) The schematic structure shows the constructions of E3 fusion proteins. RING-SH2<sup>Drk</sup>, the RING finger domain of D-CblL (red orthogon) fused with the SH2 domain of Drk (yellow orthogon). RING-PR, the RING finger domain was fused with PR motif (amino acid 638 to 643; PPLPPR; gray orthogon). RING was composed with the RING finger domain and the linker (blue orthogon) of D-CblL. The RING, RING-PR and RING-SH2<sup>Drk</sup> were in-frame fused with a Flag-tag (red circle). (B-F) Gurken is asymmetrically expressed (green) in the dorsal-anterior corner of the middle-stage wild-type egg chamber (B). The normal Gurken expression pattern spreads about 8 to 10 follicle cells (indicated by white line in B). Expression of D-CblL (C), RING-PR (E), or RING-SH2<sup>Drk</sup> (F) led to a reduced Grk distribution (white lines shown in C, E, F), but no significant effect was observed in the egg chamber expressing RING (D). (G) Quantitative data of B, C, D, E, and F, and the percentages indicated middle stage egg chambers with a normal pattern (*p*<0.05; **p**<0.001).

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the Y1045 residue of EGFR through its SH2 domain. However, the binding-deficient Y1045F EGFR mutant is internalized almost as efficiently as the wild-type EGFR, indicating that direct binding of Cbl to EGFR is not necessary for EGFR endocytosis [55].

Indeed, the chimeric protein (RING-SH2Drk) we generated promoted ligand-receptors endocytosis as well (Figure 4 and Figure 5). Therefore, we conclude that direct interaction between Cbl and EGFR may not be necessary for ligand-receptor endocytosis in vertebrate and invertebrate cells. Considering that Cbl acts as an adaptor in many signaling pathways [21], we were surprised to find that the RING finger domain, while recruited specifically to EGFR, is sufficient to down-regulate EGFR signaling. This finding implies the possibility of using the RING finger domain as a therapeutic tool in human diseases treatment.

### Materials and Methods

**Fly strains**

The strains used include: wild-type (Oregon-R, OreR; DrkE0A/CyO [39]; Eps15<sup>B</sup> and Eps15<sup>B2</sup> [56]; ena<sup>B</sup>/CyO (Pai unpublished research); UAS-CblL-A9; UAS-CblL-A10; UAS-CblL-A12; UAS-CblL-A18; H33-CblL-6 and H33-CblS-2 [30]; EQ1 and GR1 Gal4 lines [57]; cblF<sup>T</sup>/TM3 [11]; UAS-λ-top [57]; UAS-GFP-Rab5 [58]; UAS-GFP-Rab7 [59]; HRP-grk-5 [29].

### Table 3. RING-PR and RING-SH2<sup>Drk</sup> down-regulate Egfr signaling in a dose dependent manner.

| Genotypes       | V3 | V2 | V1 | Wt | N |
|-----------------|----|----|----|----|---|
| At 25°C         |    |    |    |    |   |
| flag-D-CblL-1   | 18 | 80 | 1  | 1  | 364 |
| flag-D-CblL-6   | 4  | 93 | 3  | 0  | 243 |
| At 29°C (32°C)  |    |    |    |    |   |
| RING-A<sup>+</sup> | 0  | 0  | 3  | 1  | 99 (85) 271 (367) |
| RING-B<sup>+</sup> | 0  | 0  | 5  | 0  | 100 (89) 233 (333) |
| RING-SH2<sup>Drk</sup>-2<sup>+</sup> | 2  | 40 | 26 | 48 | 72 (12) 334 (503) |
| RING-SH2<sup>Drk</sup>-3<sup>+</sup> | 0  | 19 | 36 | 36 | 57 (6) 376 (430) |
| RING-PR-A<sup>+</sup> | 0  | 8  | 2  | 22 | 67 (76) 220 (374) |
| RING-PR-D<sup>+</sup> | 0  | 0  | 12 | 7  | 88 (93) 154 (164) |
| RING-PR-A; RING-PR-D<sup>+</sup> | 34 | 23 | 43 | 23 | 253 |

The egg collection was done at 25°C, and expressions were driven by EQ1-Gal4.

The egg collection was done at two temperatures, 29°C and 32°C, and expressions were driven by GR1-Gal4.

Two copies of transgene driven by hsGal4 were induced at 37°C for 1 hour, and then eggs were collected at 25°C.

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### Figure 6. The amount of Drk in the EGFR complex. The EGFR complex were immunoprecipitated by mouse monoclonal anti-EGFR anti-bodies from ovariant lysates extracted from wild-type females or females expressing D-CblL, or RING-SH2<sup>Drk</sup> in their follicle cells. The immunoprecipitate was separated by SDS-PAGE, and analyzed by rabbit polyclonal anti-EGFR (upper panel) or anti-Drk antibodies (lower panel). The EGFR signal appeared near 170kD (arrowhead in upper panel), and the Drk signal was near 24kD (arrowhead in lower panel). DOI: 10.1371/journal.pone.0017097.g006

Cbl acts as an adaptor in many signaling pathways [21], we were surprised to find that the RING finger domain, while recruited specifically to EGFR, is sufficient to down-regulate EGFR signaling. This finding implies the possibility of using the RING finger domain as a therapeutic tool in human diseases treatment.
DNA constructs

To generate Flag-D-CblL, the D-ChiL 328-5333 cDNA fragment was cloned into pUAST-Flag vector with BglII/KpnI sites. D-ChiL-D7OZ was generated by site-directed mutagenesis, which was designed according to the previously reported method [20]. To generate the D-ChiLmPR[323] mutant, we replaced the Pro[323] and Pro[324] with Ala using site-directed mutagenesis. The primers used included: Cbl-mut-2270-F 5′-gtggtggtgttcctgctgagcagc-3′ and Cbl-mut-2270-R 5′-ggagggctggtggctc-3′. To generate the D-ChiLmPR, the RING finger domain of D-ChiL was amplified by PCR using the following primers: Cbl-mut-2270-F 5′-gtggtggtgttcctgctgagcagc-3′ and Cbl-mut-2270-R 5′-ggagggctggtggctc-3′. The RING fragment was cloned into pUAST-Flag using KpnI and EcoRI sites and was in-frame fused to 3′ of the Flag-tag. To generate pUAST-Flag-RING-SH2[283], the SH2 domain of Drk (amino acid 53 to 160) was amplified by PCR using the following primers: Cbl-mut-2270-F 5′-gtggtggtgttcctgctgagcagc-3′ and Cbl-mut-2270-R 5′-ggagggctggtggctc-3′. The SH2[283] fragment was cloned into pUAST-Flag using XhoI and XbaI sites and was in-frame fused to 3′ of the RING finger domain. To generate the pUAST-Flag-RING-PR, the following two polynucleotides PR-F 5′-aattctgccctgctcagggtg-3′ and PR-R 5′-ctagatccgggggaggggaggggaggggaggggaggggaggggaggggagggg-3′ were synthesized in vitro, and they were ligated into pUAST-Flag vector with BglII and XhoI sites. To generate Flag-D-CblL, the D-CblL 328-3533 cDNA fragment was cloned into pUAST-Flag vector using XbaI and EcoRI sites, and it was used as a positive control. The proline-rich domain of D-CblL was amplified by PCR and cloned into the yeast two-hybrid system at 29°C. The Samples 10 μl of immunoprecipitates or 30 μl of total lysate were separated by SDS-PAGE and detected with anti-Drk [39], 8C4 [30], rabbit anti-EGFR [29] antibodies.

Supporting Information

Figure S1 The expression levels of transgenes. (A) To compare the expression levels between D-ChiL and D-ChiLmPR transgenic lines, UAS-D-chiL (A10 and A12) and UAS-D-chiL-mPR mutants (AE and GB) were expressed in follicle cells driven by EQ1-Gal4 at 25°C. The endogenous D-ChiL level was detected in one ovary extract. LE indicated long exposure, and SE indicated short exposure. (B) To compare the expression level of each hs83-D-chiL transgenic line, the protein samples were extracted from ovarioles carrying the hs83-D-chiL or hs83-D-chiL-mPR mutant gene. The D-ChiL and D-ChiLmPR were detected by a mouse anti-D-ChiL and Flag-RING-SH2[283] line, the full-length D-ChiL and chimeras’ expression levels were analyzed by the anti-Flag anti-body. The transgenic lines used are as follows: Flag-L1, Flag-L6, Flag-RING-SH2[283]-2 and Flag-RING-SH2[283]-5. (D) To compare the expression level of each Flag-D-ChiL and Flag-RING-SH2[283] line, the full-length D-ChiL and chimeras’ expression levels were analyzed by the anti-Flag anti-body. The transgenic lines used are as follows: Flag-L1, Flag-L6, Flag-RING-SH2[283]-2 and Flag-RING-SH2[283]-5. (E) To compare the expression level of each Flag-D-ChiL and Flag-RING-SH2[283] line, the full-length D-ChiL and chimeras’ expression levels were analyzed by the anti-Flag anti-body. The transgenic lines used are as follows: Flag-L1, Flag-L6, Flag-RING-SH2[283]-2 and Flag-RING-SH2[283]-5. (D) To compare the expression level of each Flag-D-ChiL and Flag-RING-SH2[283] line, the full-length D-ChiL and chimeras’ expression levels were analyzed by the anti-Flag anti-body. The transgenic lines used are as follows: Flag-L1, Flag-L6, Flag-RING-SH2[283]-2 and Flag-RING-SH2[283]-5.
Flag-RING-PR is about 14kD, and Flag-RING-SH2\textsuperscript{Dcr} is about 25kD.

**Figure S2** Drk interacts with the PR motif of D-CblL. (A) The interaction between Drk and D-CblL was analyzed by a yeast-two hybrid system. Yeasts are co-transformed with pGBKT7-Drk-FL and pCL1 (as a positive control), pGADT7 vector (as a negative control), wild-type or mPR proline-rich domain. (B) On the G2 selection plate, the duplicated experiments show the successful transformation of each line. (C) On the G3 selection plate, yeast containing the PR mutant or the pGADT7 vector could not grow, whereas yeast containing the wild-type or pCL1 plasmid could grow.

**Table S1** Over-expression of D-CblL has little genetic interaction with D-eps15 and D-endophilin B.

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**Table S3** D-CblL-mPR partially suppresses \( \lambda \)-top effects.

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**Author Contributions**

Conceived and designed the experiments: L-MP P-YW. Performed the experiments: P-YW. Analyzed the data: L-MP P-YW. Contributed reagents/materials/analysis tools: L-MP. Wrote the paper: L-MP P-YW. Designed experiments: L-MP P-YW. Made the application of grants: L-MP.
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