Distinct protein degradation mechanisms mediated by Cul1 and Cul3 controlling Ci stability in Drosophila eye development

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The ubiquitin-like protein, Nedd8, covalently modifies members of the Cullin family. Cullins are the major components of a series of ubiquitin ligases that control the degradation of a broad range of proteins. We found that Nedd8 modifies Cul1 in Drosophila. In Drosophila Nedd8 and Cul1 mutants, protein levels of the signal transduction effectors, Cubitus interruptus (Ci) and Armadillo (Arm), and the cell cycle regulator, Cyclin E (CycE), are highly accumulated, suggesting that the Cul1-based SCF complex requires Nedd8 modification for the degradation processes of Ci, Arm, and CycE in vivo. We further show that two distinct degradation mechanisms modulating Ci stability in the developing eye disc are separated by the morphogenetic furrow (MF) in which retinal differentiation is initiated. In cells anterior to the MF, Ci proteolytic processing promoted by PKA requires the activity of the Nedd8-modified Cul1-based SCF\textsuperscript{limb} complex. In posterior cells, Ci degradation is controlled by a mechanism that requires the activity of Cul3, another member of the Cullin family. This posterior Ci degradation mechanism, which partially requires Nedd8 modification, is activated by Hedgehog (Hh) signaling and is PKA-independent.

Keywords: Nedd8; Cullins; SCF; Ci; protein degradation; eye development

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Ubiquitin-mediated protein degradation mechanisms control the stability of various proteins that are essential for cellular function. Nedd8 is a ubiquitin-like small protein modifier. The Nedd8 conjugation process, called neddylation, is similar to ubiquitination. Neddylation utilizes the E1 activating-enzyme complex composed of two subunits, APP-BP1 and UBA3, and the E2 conjugating-enzyme, UBC12 (Yeh et al. 2000). The only known substrates of neddylation are Cullin family proteins, Cul1, Cul2, Cul3, Cul4A, Cul4B, and Cul5, which have been shown to be modified by Nedd8 in mammalian cells [Osaka et al. 1998; Hori et al. 1999]. Cullins directly interact with Roc1, a Ring finger protein, and the Cullin-Roc1 complex comprises the core module of a series of ubiquitin E3 ligases, which confer substrate specificity and therefore regulate the degradation process [Kamura et al. 1999b; Ohta et al. 1999; Seol et al. 1999; Skowyra et al. 1999]. Among Cullins, many studies focused on Cul1, an essential component of the SCF complex which functions as ubiquitin E3 ligase. The SCF complex consists of core subunits: Cul1/Cdc53, Skp1, Roc1/Hrt/Rbx1, and a substrate-recognition E-box protein. Cul1 functions as a scaffold protein within the SCF complex; the N-terminal domain of Cul1 interacts with the adaptor protein Skp1 that links with the F-box protein, and the C-terminal domain interacts with Roc1 and the ubiquitin E2 enzyme.

In vitro, neddylation of Cul1 is required for ubiquitination of IκBα and p27\textsuperscript{kip1} [Morimoto et al. 2000; Podust et al. 2000; Read et al. 2000]. In addition, neddylation enhances E2-ubiquitin recruitment to SCF [Kawakami et al. 2001]. In fission yeast, Nedd8 is essential for the SCF-mediated degradation of Rum-1, a cyclin-dependent kinase inhibitor [Osaka et al. 2000]. In Arabidopsis thaliana, the Nedd8 pathway is required for SCF-mediated Auxin response [Pozol et al. 1998; Schwechheimer et al. 2001]. In mice deficient for UBA3, a subunit of the E1 enzyme in neddylation, embryonic development is aberrant, with accumulation of two putative SCF substrates, β-catenin and cyclin E (CycE; Tateishi et al. 2001).

In the SCF complex, F-box proteins convey substrate specificity by direct interaction with substrates for degradation. Many F-box proteins have been characterized in metazoans, and increasing numbers of specific targets for F-box proteins are being found [Deshaies 1999].
Among them, the *Drosophila* F-box protein Slimb and its mammalian homolog β-TrCP are well characterized for their target specificity [for review, see Maniatis 1999]. The specific targets for Slimb/β-TrCP are plkBα in the Dorsal/NFκB pathway, Arm/β-catenin in the Wg/Wnt pathway, and Ci/Gli in the Hedgehog (Hh) pathway [Jiang and Struhl 1998; Yaron et al. 1998; Spencer et al. 1999, Winston et al. 1999].

The Hh pathway controls growth and pattern formation in many developmental processes in both vertebrates and invertebrates (for review, see Ingham and McMahon 2001). The Hh signal is transmitted through a receptor complex consisting of Patched (Ptc) and Smoothened (Smo). In the absence of Hh, Ptc inhibits Smo activity, and the effecter Cubitus interruptus (Ci) is phosphorylated by PKA, leading to the proteolysis of Ci, which is converted into Ci75 with the C terminus truncated. Ci75 functions as a transcriptional repressor in the Hh signaling pathway. Upon binding to Ptc, Hh relieves Smo from its repression state. Activated Smo mediates signaling to prohibit proteolytic processing of Ci. The intact full-length Ci (CiFL) functions as a transcriptional activator for expression of target genes of the Hh pathway.

In *Drosophila*, Hh signaling functions in patterning the A/P compartments in developing tissues such as embryonic segments and wing and leg imaginal discs. In development of the eye imaginal disc, Hh signaling is a major driving force of the retinal differentiation wave, the morphogenetic furrow (MF), which is caused by transient constriction in cell apical surface [Heberlein et al. 1993, Ma et al. 1993]. The MF progresses anteriorly from the posterior region of the eye disc during the third instar larval and early pupal stages [Ready et al. 1976]. Anterior to the advancing MF, cells are proliferating, whereas posterior cells differentiate sequentially into photoreceptor, cone, or pigment cells which produce and secrete Hh proteins. Transduction of Hh signaling in the MF is revealed by the accumulation of CiFL, which activates expression of target genes such as *dpp* and *atonal* in the MF [Heberlein et al. 1993; Dominguez and Hafen 1997; Greenwood and Struhl 1999]. The induced MF cells soon differentiate and produce Hh proteins for further induction of more anterior cells, thus making the MF move forward.

The effect of neddylation on a broad spectrum of E3 ligases remains largely unknown. To investigate the role of neddylation in protein degradation control during developmental processes, we identified and analyzed *Nedd8* and *Cul1* mutants in *Drosophila*. Our results suggest that neddylation is required for Cul1-mediated protein downregulation of the signaling pathway effectors Ci and Armadillo (Arm) and the cell cycle regulator CycE. Using the developing eye disc as a model system to study the regulation of CiFL stability, we found that there is mechanistic difference in controlling CiFL stability between anterior and posterior cells separated by the MF. Whereas the Cul1-based SCF\(^{\text{Slimb}}\) complex controls CiFL stability in anterior cells, a Cul3-dependent protein degradation mechanism controls CiFL stability in posterior cells. We further investigated the differences between these two protein degradation mechanisms.

**Results**

**Effects of Nedd8 mutations on growth and protein stability**

Nedd8 is highly conserved from yeast to mammals [Fig. 1A]. We identified several *Nedd8* alleles in *Drosophila*, including two null alleles *Nedd8\(^{AN015}\)* and *Nedd8\(^{AN004}\)* that were used in the present study [see Materials and Methods]. The *Nedd8* null mutants were growth-arrested in the first-instar larval stage and died within several days without further growth [Fig. 1B]. We generated mutant clones to analyze *Nedd8* loss-of-function phenotypes, and observed in the adult flies very few *Nedd8\(^{AN015}\)* mutant cells [Fig. 1D,F], whereas in control experiments, large *Nedd8* clones were frequently recovered [Fig. 1C,E]. *Nedd8* mutant clones of small size, however, were present in the developing discs, suggesting that *Nedd8* mutant cells were defective in proliferation and survival.

To study the relationship between *Nedd8* and the F-box protein Slimb-mediated protein degradation, we examined the protein stability for substrates of Slimb in *Nedd8* mutant cells. As shown in Figure 1G–I and J–L, respectively, *Nedd8* mutant cells in developing wing discs accumulated high levels of full-length Ci (CiFL) and Arm proteins, phenotypes identical to those observed in the *slimb* mutants [Jiang and Struhl 1998]. In *Drosophila* embryonic development, the signaling pathway mediated by the NFκB homolog Dorsal is required for patterning the dorsoventral identity. Accumulation of plkBα/Cactus inhibits Dorsal activation, leading to repression of the downstream target gene, *twist*, an effect that has been observed in *slimb* mutants [Spencer et al. 1999]. We examined *twist* expression in embryos laid by *Nedd8\(^{AN015}\)/Nedd8\(^{2001}\)* females in which *Nedd8\(^{2001}\)* is a hypomorphic allele [see Materials and Methods]. In such embryos, the *twist* expression domain was reduced along the dorsoventral axis and often found missing in many cells [Fig. 1Q], revealing a requirement for *Nedd8* in *Dorsal* signaling.

We further tested whether *Nedd8* affects the protein level of CycE that is regulated by the F-box protein, Archipelago (*Ago*, Moberg et al. 2001). As shown in Figure 1M–O, CycE accumulated in *Nedd8* mutant cells in the eye disc. Our results suggest that *Nedd8* might affect the stability of a broad range of proteins through F-box proteins in flies.

**Consequence of CiFL accumulation in Nedd8 mutant cells in the developing eye disc and its response to Hh signaling**

The *Drosophila* eye imaginal disc is an excellent model system for developmental study. Cells are undifferentiated and dividing randomly anterior to the MF, and cells posterior to the MF are differentiating into different
Caused by an increase in the CiFL accumulation in heads) but not distally (Fig. 2A, B, asterisks) to the MF. Thus, we can observe Ned8 phenotypes in cells of different differentiation states in a single eye disc. The Hh pathway is the major signaling pathway in eye development, and the protein level of its effector Ci is tightly regulated in Drosophila (Ingham and McMahon 2001). We focused our studies on how Ned8 regulates the CiFL level in the Hh pathway and the effects of Ci upregulation on eye development. We found that in the Ned8 clones that located anterior to the MF, CiFL accumulated to a level identical to that in the MF cells that transduce the Hh signaling pathway (Fig. 2A, B, arrows). Accumulation of CiFL also existed in posterior mutant cells that located proximally (Fig. 2A, B, arrowheads) but not distally [Fig. 2A, B, asterisks] to the MF. CiFL accumulation in Ned8 mutant cells was not caused by an increase in the ci transcription level, because expression of ci-lacZ that recapitulates endogenous ci expression (Jiang and Struhl 1998) remained constant in Ned8 mutant cells [Fig. 2C, D], indicating that posttranscriptional defects resulted in CiFL accumulation.

Elevated CiFL levels caused anterior Ned8 mutant cells to adopt MF fate precociously. Ned8 mutant cells constricted in the apical surface, as revealed by the intensified phalloidin staining (Fig. 2F), and expressed the Hh-target gene, dpp, as detected by the expression of dpp-lacZ reporter gene (Fig. 2G; Blackman et al. 1991). Furthermore, the early photoreceptor marker, Atonal, was induced [Fig. 2H, arrows]. These phenomena were observed only in mutant cells abutting the MF anteriorly, suggesting that accumulated CiFL in Ned8 mutant cells was able to respond to Hh signaling.
Nedd8-mediated CiFL processing is downstream of Smo signaling and PKA phosphorylation

Although CiFL accumulation in Nedd8 cells could result from a defect in the machinery controlling CiFL protein processing, it was equally possible that CiFL accumulation could be caused by the activation of Hh signaling. For example, smo has been shown to be under posttranscriptional regulation by Hh signaling (Alcedo et al. 2000; Denef et al. 2000; Ingham et al. 2000). Therefore, Nedd8 activity could have affected the protein stability of Smo, leading to signal activation and CiFL accumulation. To test whether the effect of Nedd8 on CiFL processing is dependent on smo, we generated smo mutants and Nedd8 double mutant clones in the eye disc. When the double mutant clones were located anterior to the MF, the level of CiFL accumulation was indistinguishable from that in the Nedd8 mutant clones (cf. arrows in Figs. 2A and 3A). As a control, anterior smo clones failed to enhance CiFL accumulation (Fig. 3C,D). This result indicated that Nedd8-mediated CiFL processing was independent of smo. (CiFL accumulation in posterior cells is described below.)

Ci protein processing is known to depend on the phosphorylation status of CiFL by PKA [Chen et al. 1998; Price and Kalderon 1999]. The level of CiFL is downregulated when PKA is constitutively activated by the expression of its catalytic subunit (UAS-C*; Li et al. 1995). Therefore, we examined the functional relationship between PKA activity and Nedd8 modification. When the UAS-C* transgene was driven by eq-GAL4 for misexpression in the equator region of the eye disc, as visualized by the coexpressed GFP (green in Fig. 3E), the level of CiFL in the equator region was reduced (Fig. 3F), consistent with the observations that PKA phosphorylates Ci and promotes Ci proteolysis. We then generated Nedd8 mutant clones in the equator region where PKA is constitutively activated. In Nedd8 clones that overlapped the eq-GAL4 expression domain, CiFL accumulated to a high level [Fig. 3G, arrow], identical to the level in the Nedd8 clone located externally to the eq-GAL4 expression domain [Fig. 3G, arrowhead]. These results indicated that CiFL downregulation by PKA activity requires Nedd8 activity, and the effect of the Nedd8 pathway on CiFL processing is unlikely to be mediated through modulation of PKA activity.

Nedd8 modifies Cul1 and modulates Cul1 stability in Drosophila

The genetic evidence described above suggested that Nedd8 could be directly required for CiFL proteolytic processing, consistent with the hypothesis that neddylation affects CiFL proteolysis through regulating SCF

activity. Cullin proteins are the identified targets for Nedd8 modification. In the Drosophila genome, six Cullin proteins are identified, each corresponding to a mammalian homolog [Fig. 6I, below]. Among them, Cul1 is involved in the formation of SCF complexes that function as E3 ligases. We generated a null Cul1 allele, Cul1EX (see Materials and Methods). In Cul1EX homozygous larvae in the first instar stage, the Cul1 signal detected by αCul1 antibodies was almost gone [Fig. 4A, right lane]. The residual Cul1 protein in Cul1EX larvae was probably maternally contributed.

We further investigated whether Nedd8 conjugates to Cul1 in Drosophila. Cell extracts of eye-antennal discs and brain lobes isolated from wild-type third instar lar-
regulated CiFL level in MF cells near the pressed PKA catalytic subunit can be observed by the down-regulation of arm-lacZ (arrowheads). The signal is similar to that in the mutant cells without mC* expression (Fig. 3). CiFL accumulation in clones anterior to and in the MF (arrows). However, CiFL accumulation is detected in mutant cells in the posterior clones (arrowheads) except near the posterior margin (asterisks). (E–H) A late third-instar eye disc expressing smo mutant cells marked by the lack of GFP expression (green in D), CiFL expression is missing in clones anterior to and in the MF (arrows). However, CiFL accumulation is detected in mutant cells in the posterior clones (arrowheads) except near the posterior margin (asterisks). (E–H) A late third-instar eye disc expressing smo mutant cells marked by the lack of GFP expression (green in D), CiFL expression is missing in clones anterior to and in the MF (arrows). However, CiFL accumulation is detected in mutant cells in the posterior clones (arrowheads) except near the posterior margin (asterisks). (E–H) A late third-instar eye disc expressing smo mutant cells marked by the lack of GFP expression (green in D), CiFL expression is missing in clones anterior to and in the MF (arrows). However, CiFL accumulation is detected in mutant cells in the posterior clones (arrowheads) except near the posterior margin (asterisks). (E–H) A late third-instar eye disc expressing smo mutant cells marked by the lack of GFP expression (green in D), CiFL expression is missing in clones anterior to and in the MF (arrows). However, CiFL accumulation is detected in mutant cells in the posterior clones (arrowheads) except near the posterior margin (asterisks). (E–H) A late third-instar eye disc expressing smo mutant cells marked by the lack of GFP expression (green in D), CiFL expression is missing in clones anterior to and in the MF (arrows). However, CiFL accumulation is detected in mutant cells in the posterior clones (arrowheads) except near the posterior margin (asterisks).

In addition, we found that unmodified Cul1 accumulated in the Ned8 hypomorphic mutants Ned8EP(2)2063/AN015 (Fig. 4D). This effect of Cul1 accumulation was also observed in Ned8 null mutant clones in third-instar eye discs (Fig. 4E,F). These data suggested that Ned8 modification of Cul1 might modulate Cul1 stability.

Cul1 functions in the same pathway as Ned8 in modulating protein stability

To test whether Ned8 activity in controlling protein stability is mediated through Cul1, we examined whether depleting Cul1 would exhibit the same phenomenon.

Figure 3. The Ned8 pathway on CiFL proteolytic processing is downstream of the Hh receptor Smo and PKA activity. (A–D) CiFL accumulation in Ned8 mutant cells is independent of smo activity. (A,B) A late third-instar eye disc carrying smo and Ned8 double mutant clones and stained for CiFL in red. CiFL accumulates in all double mutant clones (marked by the lack of GFP staining in B) regardless of their locations in the eye disc. (C,D) A late third-instar eye disc carrying smo clones and stained for CiFL in red. In the smo mutant cells marked by the lack of GFP expression (green in D), CiFL expression is missing in clones anterior to and in the MF (arrows). However, CiFL accumulation is detected in mutant cells in the posterior clones (arrowheads) except near the posterior margin (asterisks). [E–H] Ned8 is required for PKA activity in promoting CiFL proteolytic processing. (E) A late third-instar eye disc expressing PKA catalytic domain (UAS-mC*) driven by eq-GAL4 in the equator region, as visualized by the coexpressed GFP (green in E). Constitutive activation of PKA suppresses CiFL accumulation in MF (red in F). (G,H) A late third-instar eye disc expressing UAS-mC* under the control of eq-GAL4 and containing Ned8 mutant clones. CiFL accumulation (red) persists in the Ned8 mutant clones even when PKA is constitutively activated in the mutant cells (arrows). The accumulated CiFL level is similar to that in the mutant cells without mC* expression (arrowheads). The Ned8 mutant clones are marked by the lack of arm-lacZ expression (green in H), and the effect of misexpressed PKA catalytic subunit can be observed by the down-regulated CiFL level in MF cells near the Ned8 clone.

Figure 4. Ned8 modifies Cul1 and controls its stability. (A) Western blot by αCul1 antibodies. Cell extracts were prepared from first-instar larvae of wild-type (left lane) and Cul1125 mutant (right lane). The levels of Cul1 proteins of 90 kD are greatly reduced in Cul1125 cell extract. (B) Conjugation of Ned8 to Cul1. Western blot of cell extract prepared from the eye discs and brain lobes of third-instar larvae by αCul1 antibodies (left lane). Immunocomplex of Cul1 from precipitation by αCul1 antibodies is blotted by αNed8 antibodies (middle lane). An arrow indicates the putative Ned8-modified Cul1 form. In the control experiment without the addition of αCul1 antibodies in precipitation, no Ned8 positive signal can be detected (right lane). [C] Immunoblotting of cell extract prepared from first-instar larvae of wild-type (left lane) and Ned8AN015 (right lane) by αCul1 antibodies. The Ned8-modified form of Cul1 is missing in Ned8 AN015 cell extract (right lane, arrow). (D) In the absence of Ned8 modification, the Cul1 level is increased. Western blot analysis of cell extract from third-instar eye discs and brain lobes by αCul1 antibodies indicates that the Cul1 protein is accumulated in Ned8AN015 (Ned8EP(2)2063) (right lane). (E,F) A late third-instar eye disc containing Ned8 mutant clones and stained with αCul1 antibodies (red). Cul1 accumulates in the Ned8 mutant clones (red in F) that are marked by the lack of GFP expression (green in F).
types as depleting Nedd8. *Cul1EX* clones were generated in developing wing discs, and the protein levels of CiFL and Arm were examined. Accumulations of CiFL and Arm were found in *Cul1EX* mutant cells (Fig. 5A–C and D–F, respectively), identical to the phenotypes observed in *Nedd8* mutant clones. In the eye disc, CycE accumulated in *Cul1* mutant cells (Fig. 5 G–I). These results suggest that Nedd8 and Cul1 function together for controlling the protein stability of Ci and Arm in the wing disc and CycE in the eye disc.

The SCF\textsuperscript{slimb} complex controls CiFL processing exclusively in cells anterior to the MF of the eye disc

We then tested whether the Cul1-based SCF complex is involved in controlling CiFL stability in the eye disc. When located anterior to the MF, *Cul1EX* cells accumulated high levels of CiFL (Fig. 6A,B), a phenotype similar to *Nedd8* mutant cells. However, *Cul1EX* cells that located posterior to the MF only expressed a basal level of CiFL, suggesting that the Cul1-based SCF complex may not control Ci stability in the posterior cells.

In addition to the Nedd8-Cul1 core component, the SCF complex also includes a substrate-specific F-box protein. To investigate whether SCF activity in CiFL processing is limited to the anterior cells of the eye disc, we examined the mutant phenotype of *slimb* that is required for CiFL proteolytic processing in tissues such as wing and leg discs (Jiang and Struhl 1998). When *slimb* mutant clones were generated in eye discs, high levels of CiFL accumulation were detected exclusively in clones located anterior to the MF. No accumulation of CiFL could be detected in posterior *slimb* clones (Fig. 6C,D). Suppression of CiFL accumulation in the posterior cells was not due to possible residual activity present in hypomorphic *slimb*, because we observed identical results of CiFL accumulation in the strong hypomorphic allele *slimb* and the null allele *slimb* (data not shown).

In summary, our results strongly suggested that in vivo, the Nedd8-modified, Cul1-based SCF\textsuperscript{slimb} complex controls CiFL proteolysis in anterior cells. Following the sweep of the MF, CiFL stability in the posterior cells is controlled by an SCF\textsuperscript{slimb}-independent mechanism.

Ci downregulation in the posterior cells of the eye disc requires Smo signaling and Nedd8 modification activity

The finding that CiFL accumulated in posterior *smo* clones (Fig. 3C,D, arrowheads; Dominguez 1999) indicated that Smo signaling contributes to the downregulation of CiFL in the posterior cells of the eye disc. This effect is in contrast to the smo role in the MF, where smo is required for CiFL activation (Fig. 3C,D, arrows). CiFL accumulation was also observed in the posterior *Nedd8* mutant clones located proximally to the MF (Fig. 2A,B, arrowheads). In the *smo3* *Nedd8* double mutant clones, the level of CiFL was further enhanced (Fig. 3A,B, arrowheads), even in clones located distally to the MF (Fig. 3A,B, asterisks), whereas no CiFL accumulation was detected in *Nedd8* (Fig. 2A,B, asterisks) or *smo* clones (Fig. 3C,D, asterisks), suggesting that Nedd8 and Smo function partially redundantly to downregulate Ci stability in the posterior cells of the eye disc.

CiFL degradation in the posterior cells of the eye disc is mediated by a Cul3-dependent mechanism

The involvement of Nedd8 in controlling CiFL levels in the posterior cells of the eye disc suggests that Cullin proteases other than Cul1 may be involved in the posterior mechanism to control Ci stability. Among the mammalian Cullin family, Cul3 shares with the Cul1-based SCF complex the substrate CycE (Singer et al. 1999). To test whether Cul3 affects CiFL degradation in the eye disc, we analyzed the available *Drosophila* Cul3 mutants (see Materials and Methods). We found that CiFL accumulated in Cul3 mutant clones located posterior to the MF (Fig. 6E,F), with a higher level in nondifferentiating cells that surround differentiating photoreceptor clusters. In contrast, no CiFL accumulation was detected in anterior Cul3 mutant clones, indicating that Cul3 controls CiFL protein stability only in the posterior cells of the eye disc. Ci accumulation in posterior Cul3 mutant cells was controlled at the posttranscriptional level because *ci* expression was normal, as revealed by in situ

Figure 5. Accumulation of CiFL, Arm, and CycE in *Cul1* mutant cells. *Cul1EX* mutant clones are marked by the lack of GFP expression in wing discs (green in B,E) and eye disc (green in H). (A–C) CiFL (red in A,C) accumulates in the *Cul1EX* mutant clones (indicated by arrows) in the anterior compartment of the wing discs. (D–F) In *Cul1EX* mutant cells, Arm is upregulated (red in D,F) in cytosol (arrows) of wing disc cells. (G–I) Enhancement of CycE protein level (red in G,I) is observed in *Cul1EX* mutant clones (arrows) in the eye disc.
CiFL degradation in the posterior cells of the eye disc is constitutive and PKA-independent

PKA phosphorylation promotes CiFL processing, and plays a role in the Hh signaling pathway for Ci activation. We therefore examined the requirement of PKA in CiFL degradation in the posterior cells of the eye disc. We generated PKA-deficient cells by overexpressing the regulatory subunit of PKA [UAS-R*] that functions to sequester the catalytic subunit and, therefore, to inhibit PKA activity [Li et al. 1995]. The UAS-R* transgene was expressed in random clones of cells labeled with GFP (see Materials and Methods). Consistent with the requirement of PKA activity in CiFL processing, CiFL accumulation was inhibited when PKA activity was inhibited in GFP-positive cells located anterior to the MF [Fig. 7A,B, arrows]. In contrast, no CiFL accumulation could be detected in GFP-positive cells located posterior to the MF [Fig. 7A,B, arrows], indicating that CiFL downregulation is not regulated by PKA activity. PKA-independent CiFL downregulation in posterior cells of the eye disc could cause either proteolytic processing of CiFL to the short form, Ci75, or complete Ci degradation. To address this question, we expressed the UAS-HACiU−3P transgene in which an HA tag was fused to the N terminus of Ci and the three PKA phosphorylation sites required for Ci processing were mutated [Wang et al. 1999]. Expression of the phosphorylation-defective form of CiFL was driven by eq-GAL4 in the equator region of the eye disc and monitored by the coexpressed GFP [Fig. 7C]. While the CiFL antibody detected a high level of CiFL in the anterior equator of the eye disc where PKA phosphorylation is required for CiFL processing, expression of CiFL was completely diminished in the posterior cells in which the GFP signal was strongly detected [Fig. 7D]. The aHA antibody that could recognize the full-length Ci [Ci155] and the short form of Ci [Ci75] also failed to detect any signal in the posterior region [Fig. 7E], suggesting that instead of processing to Ci75, CiFL was completely degraded. We also tested another Ci mutant in which, in addition to the phosphorylation sites, the proteolytic processing site of Ci was also mutated [Wang et al. 1999]. Expression of the uncleavable and unphosphorylated CiFL [UAS-HACiU−3P] by eq-GAL4 in the equator region revealed no detectable Ci

Figure 6. SCFSlimb complex and a Cul3-based degradation machinery to control the CiFL stability in the anterior and posterior cells of the eye disc, respectively. (A–D) The Cul1-based, SCFSlimb complex functions in the anterior cells to promote CiFL proteolytic processing. In the Cul1EX (A) and slimb1 (C) mutant clones located anterior to the MF, CiFL accumulation is detected (red), whereas in the posterior clones, no accumulation can be observed. The mutant clones of Cul1EX (B) and slimb1 (D) are marked by the lack of GFP expression. (E,F) In the Cul3EX mutant clones, marked by the lack of GFP expression, only posterior clones accumulate CiFL (red). (G,H) The ubiquitous expression patterns of Cul1 (G) and Cul3 (H) in the eye discs as revealed by in situ hybridization with antisense probes of Cul1 and Cul3, respectively.

For the full text, please refer to the original paper.
level in the posterior equator region [data not shown], similar to the result obtained from expression of UAS-HACI\textsuperscript{3P}. This result suggested that proteolytic processing of CiFL to the short form Ci75 is not a prerequisite for complete degradation in the posterior cells, in contrast to the proteolytic processing of the phosphorylated CiFL to the short form Ci75 in the anterior cells. To sum up, our results suggested that in the posterior cells of the eye disc, CiFL is degraded constitutively, and this degradation is independent of PKA phosphorylation.

Discussion

**Nedd8-modified Cul1 is required for the function of different SCF complexes in vivo**

In this work, we first isolated and characterized *Drosophila* Nedd8 and Cul1 mutants. Immunostaining of the developing discs showed that Nedd8 and Cul1 are required for controlling the protein stabilities of Arm, CiFL, and CycE (Figs. 1, 5). In Nedd8 mutant clones, the presence of Cul1, although at a higher level [Fig. 4E], is unable to downregulate the protein stability. In addition, we showed that in *Drosophila* Nedd8 modifies Cul1 in biochemical assays [Fig. 4B,C]. These results indicate that neddylation of Cul1 is essential for Cul1 activity in vivo.

Accumulation of Arm and CiFL has also been reported for *slimb* mutants [Jiang and Struhl 1998]. The identified F-box protein Ago has been shown to control CycE degradation in vivo [Moberg et al. 2001]. These results are consistent with the idea that individual SCF targets specific substrates for protein degradation via a recognition process mediated by a specific F-box protein, such as Slimb for Arm and Ci, and Ago for CycE in flies. Since Nedd8 affects substrate stability that depends on either Slimb or Ago, we infer that Nedd8 modification regulates SCF activity, but not substrate specificity.

**Nedd8 modification regulates both activity and stability of Cul1**

We found that in biochemical assay, the unmodified Cul1 accumulated with the reduction of Nedd8 activity [Fig. 4D]. In Nedd8 mutant cells in the developing eye disc, Cul1 also accumulated to a high level [Fig. 4E,F], raising the possibility that neddylation activates Cul1 and at the same time turns the modified Cul1 into a labile state for protein degradation. In this way, Cul1 activity could be tightly controlled in signaling pathways during developmental processes. Although the mechanism by which Nedd8-modified Cul1 is targeted for protein degradation is not clear, it has been shown that some unknown proteins with Nedd8 conjugation are targeted for proteasome degradation by the Nedd8-interacting protein NUB1 [Kamitani et al. 2001].

**Possible roles of Nedd8 modification and SCF\textsubscript{slimb} in Ci proteolytic processing**

In anterior cells of developing discs, we show that CiFL proteolytic processing requires the activity of the Nedd8-modified, Cul1-based SCF\textsubscript{slimb} complex. This CiFL proteolytic processing is inhibited by Smo signaling and promoted by PKA phosphorylation on CiFL. The mechanism by which CiFL is proteolyzed from CiFL to Ci75 is not clear. We propose that Nedd8 modifies and activates SCF\textsubscript{slimb} for Ci ubiquitination and then proteolysis, because of Cul1 modification by Nedd8 and CiFL accumulation in *Nedd8, Cul1, and slimb* mutants. Consistently, proteolysis of CiFL depends on 26S proteasome activity [Ingham 1998]. However, ubiquitinated Ci is not detected in cells treated with 26S proteasome inhibitors [Chen et al. 1999]. We could not exclude the possibility that Nedd8 directly modifies Ci through the SCF complex, because it has been shown that Nedd8 might direct its conjugates to proteasome-dependent protein degradation through NUB1 [Kamitani et al. 2001]. In this case, SCF could function as the E3 ligase for Nedd8 modification on its substrate [Kamura et al. 1999a].

In the Hh signaling pathway, it is not clear how Smo signaling prevents CiFL from proteolysis. According to our double mutant analysis [Fig. 3], Nedd8 could be downstream or parallel to Smo and PKA signaling. Thus, it is possible that Hh signaling prevents CiFL from proteolysis through downregulating the level of Nedd8-
modified Cul1. However, we failed to detect a change in the level of Nedd8-modified Cul1 in cell extracts prepared from the eye discs with ectopic Hh expression [data not shown]. We infer that Hh may affect CiFL proteolysis through a Nedd8-independent mechanism.

A distinct protein degradation mechanism in controlling Ci stability in posterior cells of the eye disc

We propose two modes of Ci downregulation in Drosophila eye development [Fig. 8]. In the undifferentiated cells anterior to the MF, Ci is phosphorylated by PKA constantly and processed by an SCF{Slimb}-dependent mechanism to generate the repressor form of Ci75. Upon binding to Hh, cells in the MF transduce Smo signaling to prevent this proteolytic processing. Thus, the transcriptional activator CiFL is preserved for activation of downstream genes in the MF.

In the posterior cells that are undergoing differentiation, a novel mechanism controls Ci degradation. Our mutant analyses suggest that this mechanism is comprised of Smo signaling, Nedd8 modification, and Cul3 activity. The effect of Smo signaling in promoting Ci degradation in the posterior cells is in contrast to its effect on the anterior cells, in which Smo signaling prohibits CiFL processing. In addition to Smo signaling, Nedd8 modification activity also participates in this posterior Ci degradation. Further Cul1 mutant analysis suggests that Cullin proteins other than Cul1 are likely involved in this posterior degradation mechanism. This hypothesis has led to the identification of Cul3 as one candidate functioning in Ci degradation. More surprisingly, Cul3 activity is very restricted; Cul3 controls Ci degradation in the posterior, but not anterior, cells of the eye disc. CiFL accumulation may have an impact on proper differentiation of the posterior cells. In Cul3 mutants, cone cell differentiation is affected [C.-Y. Ou and C.-T. Chien, unpubl.], probably due to the accumulation of CiFL.

Furthermore, the Ci degradation process is also distinct in posterior cells; Ci degradation is independent of PKA phosphorylation and proteolytic processing to the short form Ci-75 [Fig. 7]. Based on these results, we propose that Smo signaling, acting in concert with the Nedd8 pathway, activates a Cul3-based ubiquitin ligase to degrade Ci in a PKA-independent mechanism in posterior cells of the eye disc.

It is not clear how Nedd8 modifies Cul3 in flies. We observed strong genetic interaction between Nedd8 and Cul3 during eye and antennal development [Y.-F. Lin and C.-T. Chien, unpubl.], suggesting that Nedd8 may also regulate Cul3. However, depletion of Nedd8 activity only affects posterior cells abutting the MF [Fig. 2A], in contrast to depletion of Cul3 activity, which increases the CiFL level in all posterior clones [Fig. 6E], indicating that some Cul3 activity is Nedd8-independent. It is possible that a basal Cul3 activity for Ci degradation is further enhanced by Nedd8 modification near the MF in which accumulated Ci may require efficient degradation for cells to enter proper differentiation.

Switch of Ci degradation mechanisms

Different protein-protein interactions may result in a switch between two Ci degradation mechanisms in eye discs. Ci is known to interact with Cos2, Fu, and Su(fu) to comprise a protein complex [for review, see Ingham and McMahon 2001] that promotes Ci degradation. Cos2, a motor-like protein with a kinesin motif, is required for tethering Ci in the cytosolic compartment and Ci proteolytic processing in the Drosophila developing wing. Similarly, Fu, a serine/threonine kinase, is also required for Ci processing. However, in Su(fu) mutants, levels of both long and short forms of Ci are reduced [Ohlmeyer and Kalderon 1998; Lefers et al. 2001], suggesting that Su(fu) plays an additional role in Ci protein stability. Interestingly, the role of Su(fu) in controlling Ci stability seems modulated by Hh signaling. Our results indicate that, in contrast to the effect of Hh signaling in the anterior cells, Hh signaling downregulates the Ci level in the posterior cells of the eye disc. It is possible that the Ci protein complex is modulated by the sweep of the MF, and this change requires Hh signaling to expose Ci to the Cul3-based protein degradation machinery. Alternatively, additional factors may be activated by the sweeping of the MF and be required for Hh signaling to induce Cul3 activity that leads to constitutive Ci degradation.

Similar to Ci, CycE is degraded by two different mechanisms in mammalian cells. The Cul1-based SCF complex recognizes the phosphorylated form of Cdk2-bound CycE for ubiquitination [Deal et al. 1999; Skowyra et al. 1999; Koepp et al. 2001; Strohmaier et al. 2001; Yeh et al. 2001], and Cul3 targets unbound CycE for ubiquitination, which is independent of protein phosphorylation [Singer et al. 1999]. β-catenin is also degraded by two different mechanisms in mammalian cells [Polakis 2001]. One mechanism involves the SCF{βTrCP} complex (βTrCP is the mammalian homolog of Slimb) that recognizes phosphorylated β-catenin. The other mechanism involves the Ebi complex comprised of Ebi, Skp1, SIP, and Siah-1, which targets β-catenin in a phosphorylation-independent manner.

Figure 8. A proposed model for Ci degradation in Drosophila eye development (see Discussion for details).
Degradation of the Gli proteins in vertebrates

In vertebrates, the three Ci-related proteins Gli1, Gli2, and Gli3 transduce Hh signaling in different developmental processes [for review, see Ingham and McMahon 2001]. Ectopic expression of the Gli proteins in Drosophila showed that Gli2 and Gli3, but not Gli1, are proteolytically generated to repressor forms. Although the proteolytic cleavage of Gli3 is under the regulation of Hh signaling, Gli2 proteolysis is independent of Hh (Azab-Blanc et al. 2000). Consistently, proteolytic processing of Gli3, but not Gli1, has been observed in mouse embryos (Dai et al. 1999). In cultured cells, Gli3 processing is dependent on Hh signaling and PKA activity, in contrast to Gli1 and Gli2. [Dai et al. 1999; Ruiz i Altaba 1999]. Apparently, the Gli proteins are controlled by different protein downregulation mechanisms. It will be interesting to investigate whether Nedd8, Cul1, Cul3, and perhaps other Cullins are differentially involved in protein degradation of the Gli proteins.

Materials and methods

Genetics

The Drosophila Nedd8 alleles, Nedd8\textsuperscript{A005} and Nedd8\textsuperscript{A004} (Fig. 1A), were recovered in an EMS screen for lethal mutations that failed to complement Df(2L)TW3 (36F7–9, 37B2–7). The insertion site of EP(2)2063 is located 91 bp upstream of the translation start site of Nedd8. Imprecise excision of the EP element generated a null allele Nedd8\textsuperscript{A004} that included a deletion of the N-terminal half of the Nedd8 protein (aa 1–45), and a weak allele Nedd8\textsuperscript{A005} that included a small insertion of 45 bp in the EP insertion site.

The Drosophila lin-19 allele (from Bloomington Stock Center) contains a P-element insertion in the first exon, 990 bp upstream of the start ATG codon. lin-19 homoyzogotes died in the third-instar larval stage. Excision of the P-element rescued the larval lethality, suggesting that the P-element insertion caused the lethality. Immunoblotting lin-19 cell extracts for Cul1, and complementation test with a null allele, suggested that Cul1 is an EMS allele (both from Bloomington Stock Center) that uncovered Cul1, Cul1 null allele, Cul1\textsuperscript{A004}, suggested that lin-19 is a hypomorphic allele of Cul1. We performed imprecise excision of the P-element to isolate a null allele Cul1\textsuperscript{EX}, which includes a deletion of aa 1–90 of the Cul1 protein. When homozygous, or in trans to the deficiency Df(2R)CA53 (43E7–8;44G6) that uncovers Cul1, Cul1\textsuperscript{EX} caused lethality in the second-instar larval stage, suggesting that Cul1\textsuperscript{EX} is a null allele. Cul3\textsuperscript{A004} is a P-element insertion line, and Cul3\textsuperscript{A004} is an EMS allele [both from Bloomington Stock Center], and both function like genetic null and exhibit identical phenotypes. To identify homoyzogotes of Nedd8, Cul1, or Cul3, the green balancer CyO Kr-GAL4 UAS-GFP was used.

Other fly stocks used were F\textsuperscript{0}a, M(2L)24F F\textsuperscript{30B} (Cifuentes and Garcia-Bellido 1997), ey-FLP, cl2L3 (Newsome et al., 2000), ubi-nlsGFP (gift from S. Luschnig), arm-LacZ FRT\textsuperscript{A00}A (gift from J. Treisman), ci-LacZ [Eaton and Kornberg 1990], dpp-LacZ [Blackman et al. 1991], smo\textsuperscript{A} [Chen and Struhl 1996; van den Heuvel and Ingham 1996], Act\textsuperscript{A}+\textsuperscript{Gal4} UAS-GFP (Ito et al. 1997), eq-GAL4 (gift from H. Sun), UAS-myc, UAS-R\textsuperscript{A} [Li et al. 1995], UAS-HAC1-3P [Wang et al. 1999], slab\textsuperscript{A}, slab\textsuperscript{B}, and slab\textsuperscript{P}\textsuperscript{A} [Ito et al. 1997; Jiang and Struhl 1998]. The following flies were used to generate Nedd8 clones: hs-FLP\textsuperscript{z22}, Nedd8\textsuperscript{A005} FRT\textsuperscript{A00}A cl2L3 P[ubi-nlsGFP] FRT\textsuperscript{A00}A (Figs. 1–4) and ey-FLP, Nedd8\textsuperscript{A005} FRT\textsuperscript{A00}A cl2L3 P[ubi-nlsGFP] FRT\textsuperscript{A00}A (Figs. 2A–G and 4E,F). We used w ey-FLP; smo\textsuperscript{A} FRT\textsuperscript{A00}A cl2L3 P[ubi-nlsGFP] FRT\textsuperscript{A00}A flies for generation of smo clones, and w ey-FLP; smo\textsuperscript{A} Nedd8\textsuperscript{A005} FRT\textsuperscript{A00}A cl2L3 P[ubi-nlsGFP] FRT\textsuperscript{A00}A for Nedd8 and smo double mutant clones. To generate Nedd8 clones in mC\textsuperscript{A} expression domain in eye discs, UAS-mc\textsuperscript{A} Nedd8\textsuperscript{A005} FRT\textsuperscript{A00}A cl2L3 P[ubi-nlsGFP] FRT\textsuperscript{A00}A eq-Gal4 UAS-FP flies were used. To generate clones expressing R\textsuperscript{*}, hs-FLP, Act\textsuperscript{A}+\textsuperscript{Gal4} UAS-GFP;FRT\textsuperscript{A00}A cl2L3 P[ubi-nlsGFP] and w ey-FLP, Cul3\textsuperscript{EX} cl2L3 P[ubi-nlsGFP] FRT\textsuperscript{A00}A flies, respectively.

Immunohistochemistry, Western blotting, and immunoprecipitation

The imaginal discs were fixed and stained as described previously [Chen and Chien 1999]. The primary antibodies used were rat α-C11/1; Mortzny and Holmgren 1995], rabbit α-Ato (1:1000; Harman et al. 1994), rabbit α-β-galactosidase (1:1000; Cappel), rat α-Elav (1:125; Developmental Studies Hybridoma Bank), mouse α-Arm N27A1 (1:100; Peifer et al. 1994), mouse α-CyE (1:3; Moberg et al. 2001), mouse α-HA (1:100; Roche), and rabbit α-Myc (9E10, 1:250; Santa Cruz).

The Western blotting and immunoprecipitation were performed as in standard protocol. The primary antibodies used were rabbit α-Cul1 (1:250; Zymed) and rabbit α-Nedd8 (1:500; Alexis). For immunoprecipitation of Cul1, 200-μL cell extracts from eye imaginal discs and brains of 50 third-instar larvae were added with 5 μg of rabbit α-Cul1 antibodies. In the control experiment, the rabbit α-Cul1 antibodies were not added.

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References


