

# 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<sup>Slimb</sup> 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.

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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 F-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 I $\kappa$ B $\alpha$  and p27<sup>Kip1</sup> (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 (Pozo 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,  $\beta$ -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).

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Among them, the *Drosophila* F-box protein Slimb and its mammalian homolog  $\beta$ -TrCP are well characterized for their target specificity (for review, see Maniatis 1999). The specific targets for Slimb/ $\beta$ -TrCP are pIkB $\alpha$  in the Dorsal/NF $\kappa$ B pathway, Arm/ $\beta$ -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 Smoothed (Smo). In the absence of Hh, Ptc inhibits Smo activity, and the effector 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 (Ci<sup>FL</sup>) 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 margin 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 Ci<sup>FL</sup>, 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 Ci<sup>FL</sup> stability, we found that there is mechanistic difference in controlling Ci<sup>FL</sup> stability between anterior and posterior cells separated by the MF. Whereas the Cul1-based SCF<sup>Slimb</sup> complex controls Ci<sup>FL</sup> stability in anterior cells, a Cul3-dependent protein degradation mechanism controls Ci<sup>FL</sup> 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*

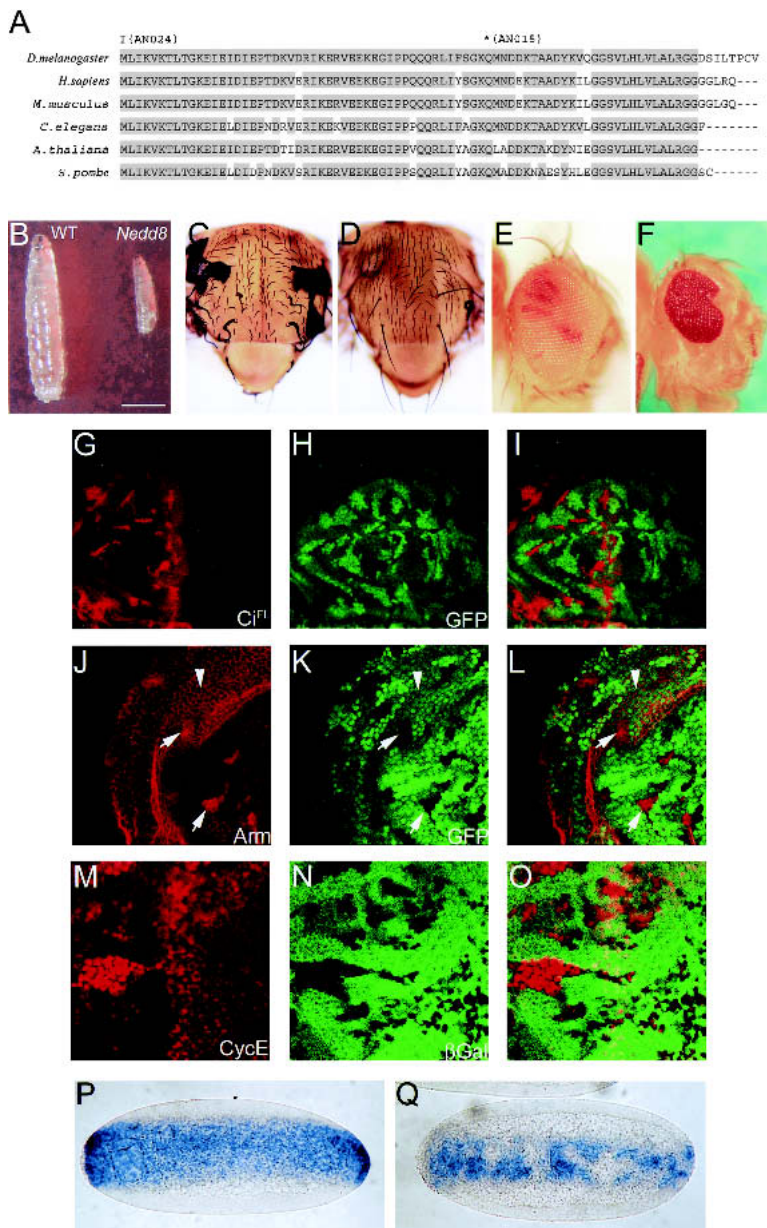
*Nedd8* is highly conserved from yeast to mammals (Fig. 1A). We identified several *Nedd8* alleles in *Drosophila*, including two null alleles *Nedd8*<sup>AN015</sup> and *Nedd8*<sup>AN024</sup> that were used in the present study (see Materials and Methods). The *Nedd8* null mutants were growth-arrested 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*<sup>AN015</sup> mutant cells (Fig. 1D,F), whereas in control experiments, large *Nedd8*<sup>+</sup> 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 (Ci<sup>FL</sup>) 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 NF $\kappa$ B homolog Dorsal is required for patterning the dorsoventral identity. Accumulation of pIkB $\alpha$ /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*<sup>AN015</sup>/*Nedd8*<sup>203</sup> females in which *Nedd8*<sup>203</sup> 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 Ci<sup>FL</sup> 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

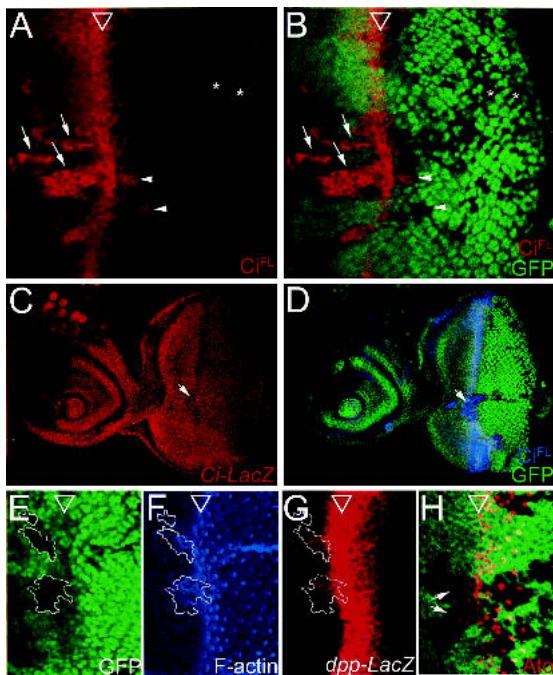


**Figure 1.** Conservation of Nedd8 in evolution and *Drosophila* Nedd8 mutant phenotypes. (A) Sequence comparison of Nedd8. *Drosophila* Nedd8 shares 88%–98% identity to other Nedd8 from yeast to mammals. Also indicated are the point mutations in *Nedd8*<sup>AN024</sup> and *Nedd8*<sup>AN015</sup> alleles. Conceptually, *Nedd8*<sup>AN015</sup> encodes a C-terminus-truncated protein by a nonsense mutation at aa 49. Thus, Gly76, the essential residue required for conjugation to the substrate protein (Wada et al. 1998), was missing in this mutant. In *Nedd8*<sup>AN024</sup>, the translation start codon was replaced by a missense mutation. (B) The 4-d-old larvae of wild-type (left) and *Nedd8*<sup>AN024</sup> (right). Note growth of the *Nedd8*<sup>AN024</sup> larva was arrested in the first instar stage. Bar, 0.5mm. (C–F) The growth and survival defect of *Nedd8* mutant cells. (C,D) The adult nota of *w f hs-FLP*; + *FRT*<sup>40A</sup>/*M2(Z)*<sup>f+30b</sup> *FRT*<sup>40A</sup> (C) and *w f hs-FLP*; *Nedd8*<sup>AN015</sup> *FRT*<sup>40A</sup>/*M2(Z)*<sup>f+30b</sup> *FRT*<sup>40A</sup> (D). (C) Almost all cells in the adult notum are marked with *forked* (note curly *forked* bristles) compared to D, in which no *Nedd8*<sup>AN015</sup> mutant clones can be observed. (E,F) The adult eyes of *w ey-FLP*; + *FRT*<sup>40A</sup>/*cl2L3* *p[w+30C]* *FRT*<sup>40A</sup> (E) and *w ey-FLP*; *Nedd8*<sup>AN015</sup> *FRT*<sup>40A</sup>/*cl2L3* *p[w+30C]* *FRT*<sup>40A</sup> (F). The wild-type clones containing white ommatidia occupy almost the whole eye in E, compared to F in which *Nedd8*<sup>AN015</sup> clones (marked by white ommatidia) are absent, leaving a defective eye that contains only *Nedd8*<sup>AN015/+</sup> cells in red ommatidia. (G–Q) Accumulation of Ci<sup>FL</sup>, Arm, and CycE, and disruption of *twist* mRNA expression in *Nedd8* mutant cells. The wing (G–L) or eye discs (M–O) carrying *Nedd8* mutant clones marked by the lack of GFP (H,K) or  $\beta$ -galactosidase expression (N). Accumulation of Ci<sup>FL</sup> (red in G,I) appears only in mutant clones located in the anterior compartment of the wing disc (for imaginal discs in this and following figures, anterior is to the left). Accumulation of Arm (red in J,L) appears in the cytosol of *Nedd8* mutant cells (arrows), in contrast to the cell surface-associated Arm in wild-type cells (arrowheads). (M, O) Accumulation of CycE is also detected in the *Nedd8* mutant clones in the eye disc. (P,Q) In situ hybridization of *twist* mRNA in wild-type embryos (P) and *Nedd8* mutant embryos laid by *Nedd8*<sup>AN015</sup>/*Nedd8*<sup>203</sup> females (Q).

types of cells. Thus, we can observe *Nedd8* 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 Nedd8 regulates the Ci<sup>FL</sup> level in the Hh pathway and the effects of Ci upregulation on eye development. We found that in the *Nedd8* clones that located anterior to the MF, Ci<sup>FL</sup> accumulated to a level identical to that in the MF cells that transduce the Hh signaling pathway (Fig. 2A,B, arrows). Accumulation of Ci<sup>FL</sup> also existed in posterior mutant cells that located proximally (Fig. 2A,B, arrowheads) but not distally (Fig. 2A,B, asterisks) to the MF. Ci<sup>FL</sup> accumulation in *Nedd8* mutant cells was not caused by an increase in the *ci* transcription level, be-

cause expression of *ci-lacZ* that recapitulates endogenous *ci* expression (Jiang and Struhl 1998) remained constant in *Nedd8* mutant cells (Fig. 2C,D), indicating that posttranscriptional defects resulted in Ci<sup>FL</sup> accumulation.

Elevated Ci<sup>FL</sup> levels caused anterior *Nedd8* mutant cells to adopt MF fate precociously. *Nedd8* 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 phenotypes were observed only in mutant cells abutting the MF anteriorly, suggesting that accumulated Ci<sup>FL</sup> in *Nedd8* mutant cells was able to respond to Hh signaling.



**Figure 2.** *Nedd8* affects transduction of the Hh pathway in *Drosophila* eye development. (A–D) The Ci<sup>FL</sup> protein, but not *ci* expression, is upregulated in the *Nedd8* mutant cells (revealed by the lack of GFP expression, in green). (A,B) A late third-instar eye disc containing *Nedd8* mutant clones and stained by anti-Ci<sup>FL</sup> antibody (2A1) in red. The open arrowheads mark the position of the MF in this and following figures. In the mutant cells anterior to the MF, Ci<sup>FL</sup> accumulation (arrows) reaches a level identical to that observed in the MF where Hh signaling is fully induced. In the posterior mutant cells, Ci<sup>FL</sup> accumulation is persistent at a lower level abutting the MF (arrowheads) and not detected in more posterior cells (asterisks). Note that the anterior clones are obviously larger than the posterior clones, suggesting a survival defect in the *Nedd8* mutant cells. (C,D) A late third-instar eye disc containing *Nedd8* mutant clones and double stained for *ci-LacZ* expression in red (C) and Ci<sup>FL</sup> in blue (D). (C) The *ci-LacZ* expression level is ubiquitous throughout the whole eye disc, and remains unchanged in the *Nedd8* mutant clones (C, arrow), in which the Ci<sup>FL</sup> protein accumulates (D, arrow). (E–H) Accumulated Ci<sup>FL</sup> responds to Hh signaling to induce MF fate in *Nedd8* mutant clones. (E–G) A late third-instar eye disc containing *Nedd8* mutant clones was double stained for F-actin in blue (F) and *dpp-LacZ* in red (G). (E) The *Nedd8* mutant clones are marked by the lack of GFP expression and outlined by a white line in E–G. (F) F-actin staining (blue) by phalloidin shows that the *Nedd8* mutant cells constrict precociously. (G) Expression of *dpp-lacZ* (red) is induced in the mutant cells abutting the MF anteriorly. (H) In the *Nedd8* mutant clones, as shown by lack of *arm-lacZ* expression (green), expression of the proneural protein Atonal (red) is induced (arrows).

#### *Nedd8*-mediated Ci<sup>FL</sup> processing is downstream of *Smo* signaling and PKA phosphorylation

Although Ci<sup>FL</sup> accumulation in *Nedd8* cells could result from a defect in the machinery controlling Ci<sup>FL</sup> protein processing, it was equally possible that Ci<sup>FL</sup> accumulation could be caused by the activation of Hh signaling. For example, *smo* has been shown to be under posttran-

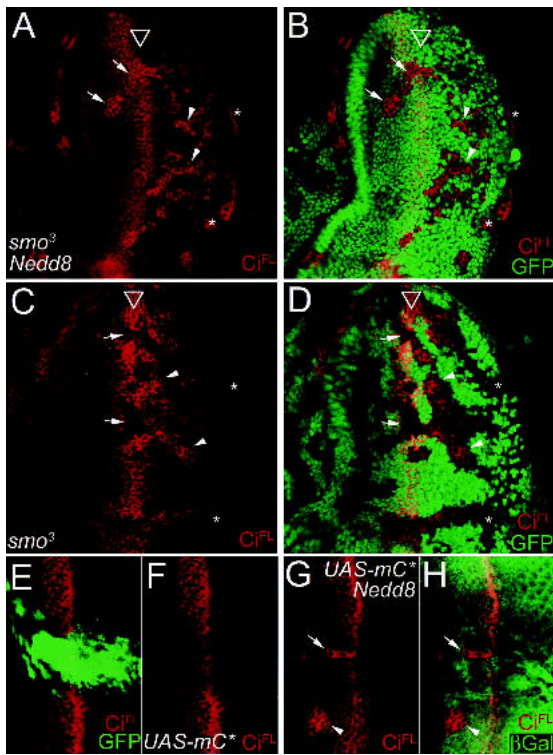
scriptional regulation by Hh signaling (Alcedo et al. 2000; Deneff et al. 2000; Ingham et al. 2000). Therefore, Nedd8 activity could have affected the protein stability of Smo, leading to signal activation and Ci<sup>FL</sup> accumulation. To test whether the effect of *Nedd8* on Ci<sup>FL</sup> processing is dependent on *smo*, we generated *smo*<sup>3</sup> and *Nedd8* double mutant clones in the eye disc. When the double mutant clones were located anterior to the MF, the level of Ci<sup>FL</sup> accumulation was indistinguishable from that in the *Nedd8* mutant clones (cf. arrows in Figs. 2A and 3A). As a control, anterior *smo*<sup>3</sup> clones failed to enhance Ci<sup>FL</sup> accumulation (Fig. 3C,D). This result indicated that *Nedd8*-mediated Ci<sup>FL</sup> processing was independent of *smo*. (Ci<sup>FL</sup> accumulation in posterior cells is described below.)

Ci protein processing is known to depend on the phosphorylation status of Ci<sup>FL</sup> by PKA (Chen et al. 1998; Price and Kalderon 1999). The level of Ci<sup>FL</sup> 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 Ci<sup>FL</sup> 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, Ci<sup>FL</sup> 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 Ci<sup>FL</sup> downregulation by PKA activity requires Nedd8 activity, and the effect of the Nedd8 pathway on Ci<sup>FL</sup> processing is unlikely to be mediated through modulation of PKA activity.

#### *Nedd8* modifies *Cull1* and modulates *Cull1* stability in *Drosophila*

The genetic evidence described above suggested that Nedd8 could be directly required for Ci<sup>FL</sup> proteolytic processing, consistent with the hypothesis that neddylation affects Ci<sup>FL</sup> proteolysis through regulating SCF<sup>Slimb</sup> 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, Cull1 is involved in the formation of SCF complexes that function as E3 ligase. We generated a null *Cull1* allele, *Cull1*<sup>EX</sup> (see Materials and Methods). In *Cull1*<sup>EX</sup> homozygous larvae in the first instar stage, the Cull1 signal detected by  $\alpha$ Cull1 antibodies was almost gone (Fig. 4A, right lane). The residual Cull1 protein in *Cull1*<sup>EX</sup> larvae was probably maternally contributed.

We further investigated whether Nedd8 conjugates to Cull1 in *Drosophila*. Cell extracts of eye-antennal discs and brain lobes isolated from wild-type third instar lar-



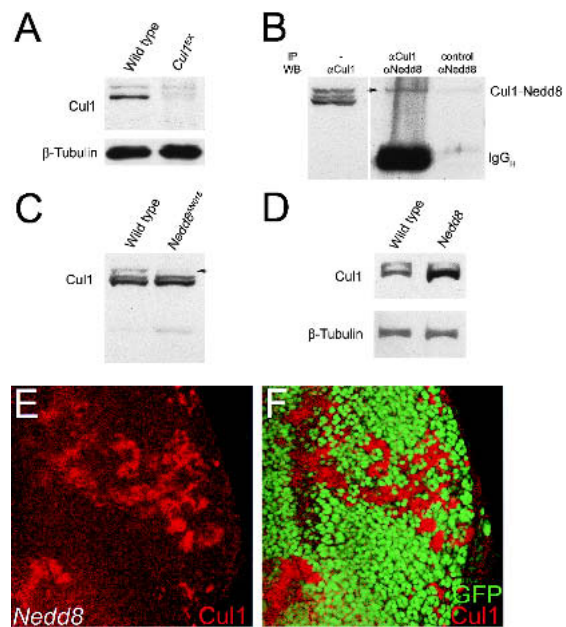
**Figure 3.** The Nedd8 pathway on Ci<sup>FL</sup> proteolytic processing is downstream of the Hh receptor Smo and PKA activity. (A–D) Ci<sup>FL</sup> accumulation in *Nedd8* mutant cells is independent of *smo* activity. (A,B) A late third-instar eye disc carrying *smo* and *Nedd8* double mutant clones and stained for Ci<sup>FL</sup> in red. Ci<sup>FL</sup> 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 Ci<sup>FL</sup> in red. In the *smo* mutant cells marked by the lack of GFP expression (green in D), Ci<sup>FL</sup> expression is missing in clones anterior to and in the MF (arrows). However, Ci<sup>FL</sup> accumulation is detected in mutant cells in the posterior clones (arrowheads) except near the posterior margin (asterisks). (E–H) *Nedd8* is required for PKA activity in promoting Ci<sup>FL</sup> proteolytic processing. (E,F) 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 Ci<sup>FL</sup> 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 *Nedd8* mutant clones. Ci<sup>FL</sup> accumulation (red) persists in the *Nedd8* mutant clones even when PKA is constitutively activated in the mutant cells (arrows). The accumulated Ci<sup>FL</sup> level is similar to that in the mutant cells without mC\* expression (arrowheads). The *Nedd8* 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 Ci<sup>FL</sup> level in MF cells near the *Nedd8* clone.

vae were immunoprecipitated by  $\alpha$ Cul1 antibodies and immunoblotted by  $\alpha$ Nedd8 antibodies (Fig. 4B, middle lane). The Nedd8-positive signal, indicated by the arrow in Figure 4B, suggests that Cul1 was modified by Nedd8 in *Drosophila*. Consistently, the Nedd8-modified Cul1 signal disappeared in cell extracts prepared from the first instar *Nedd8* mutant larvae (Fig. 4C, arrow).

In addition, we found that unmodified Cul1 accumulated in the *Nedd8* hypomorphic mutants *Nedd8<sup>EP(2)2063/AN015</sup>* (Fig. 4D). This effect of Cul1 accumulation was also observed in *Nedd8* null mutant clones in third-instar eye discs (Fig. 4E,F). These data suggested that Nedd8 modification of Cul1 might modulate Cul1 stability.

#### *Cul1* functions in the same pathway as *Nedd8* in modulating protein stability

To test whether Nedd8 activity in controlling protein stability is mediated through Cul1, we examined whether depleting Cul1 would exhibit the same pheno-



**Figure 4.** Nedd8 modifies Cul1 and controls its stability. (A) Western blot by  $\alpha$ Cul1 antibodies. Cell extracts were prepared from first-instar larvae of wild-type (left lane) and *Cul1<sup>EX</sup>* mutant (right lane). The levels of Cul1 proteins of 90 kD are greatly reduced in *Cul1<sup>EX</sup>* cell extract. (B) Conjugation of Nedd8 to Cul1. Western blot of cell extract prepared from the eye discs and brain lobes of third-instar larvae by  $\alpha$ Cul1 antibodies (left lane). Immunocomplex of Cul1 from precipitation by  $\alpha$ Cul1 antibodies is blotted by  $\alpha$ Nedd8 antibodies (middle lane). An arrow indicates the putative Nedd8-modified Cul1 form. In the control experiment without the addition of  $\alpha$ Cul1 antibodies in precipitation, no Nedd8 positive signal can be detected (right lane). (C) Immunoblotting of cell extract prepared from first-instar larvae of wild-type (left lane) and *Nedd8<sup>AN015</sup>* (right lane) by  $\alpha$ Cul1 antibodies. The Nedd8-modified form of Cul1 is missing in *Nedd8* larvae (arrow). (D) In the absence of Nedd8 modification, the Cul1 level is increased. Western blot analysis of cell extract from third-instar eye discs and brain lobes by  $\alpha$ Cul1 antibodies indicates that the Cul1 protein is accumulated in *Nedd8<sup>AN015</sup>/Nedd8<sup>EP(2)2063</sup>* (right lane). (E,F) A late third-instar eye disc containing *Nedd8* mutant clones and stained with  $\alpha$ Cul1 antibodies (red). Cul1 accumulates in the *Nedd8* mutant clones (red in E) that are marked by the lack of GFP expression (green in F).

types as depleting Nedd8. *Cul1<sup>EX</sup>* clones were generated in developing wing discs, and the protein levels of Ci<sup>FL</sup> and Arm were examined. Accumulations of Ci<sup>FL</sup> and Arm were found in *Cul1<sup>EX</sup>* 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 Cull1 function together for controlling the protein stability of Ci and Arm in the wing disc and CycE in the eye disc.

#### The SCF<sup>Slimb</sup> complex controls Ci<sup>FL</sup> processing exclusively in cells anterior to the MF of the eye disc

We then tested whether the Cull1-based SCF complex is involved in controlling Ci<sup>FL</sup> stability in the eye disc. When located anterior to the MF, *Cul1<sup>EX</sup>* cells accumulated high levels of Ci<sup>FL</sup> (Fig. 6A,B), a phenotype similar to *Nedd8* mutant cells. However, *Cul1<sup>EX</sup>* cells that located posterior to the MF only expressed a basal level of Ci<sup>FL</sup>, suggesting that the Cull1-based SCF complex may not control Ci stability in the posterior cells.

In addition to the Nedd8-Cull1 core component, the SCF complex also includes a substrate-specific F-box protein. To investigate whether SCF activity in Ci<sup>FL</sup> processing is limited to the anterior cells of the eye disc, we examined the mutant phenotype of *slimb* that is re-

quired for Ci<sup>FL</sup> proteolytic processing in tissues such as wing and leg discs (Jiang and Struhl 1998). When *slimb<sup>1</sup>* mutant clones were generated in eye discs, high levels of Ci<sup>FL</sup> accumulation were detected exclusively in clones located anterior to the MF. No accumulation of Ci<sup>FL</sup> could be detected in posterior *slimb<sup>1</sup>* clones (Fig. 6C,D). Suppression of Ci<sup>FL</sup> accumulation in the posterior cells was not due to possible residual activity present in hypomorphic *slimb<sup>1</sup>*, because we observed identical results of Ci<sup>FL</sup> accumulation in the strong hypomorphic allele *slimb<sup>2</sup>* and the null allele *slimb<sup>P</sup>* (data not shown).

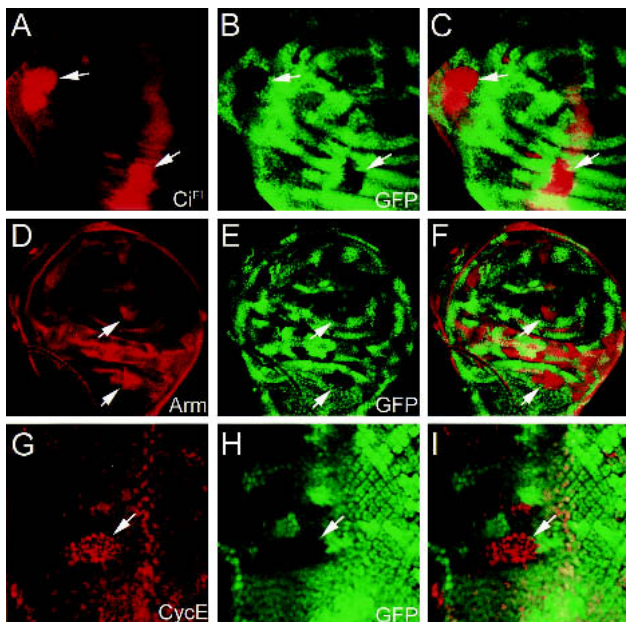
In summary, our results strongly suggested that in vivo, the Nedd8-modified, Cull1-based SCF<sup>Slimb</sup> complex controls Ci<sup>FL</sup> proteolysis in anterior cells. Following the sweep of the MF, Ci<sup>FL</sup> stability in the posterior cells is controlled by an SCF<sup>Slimb</sup>-independent mechanism.

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

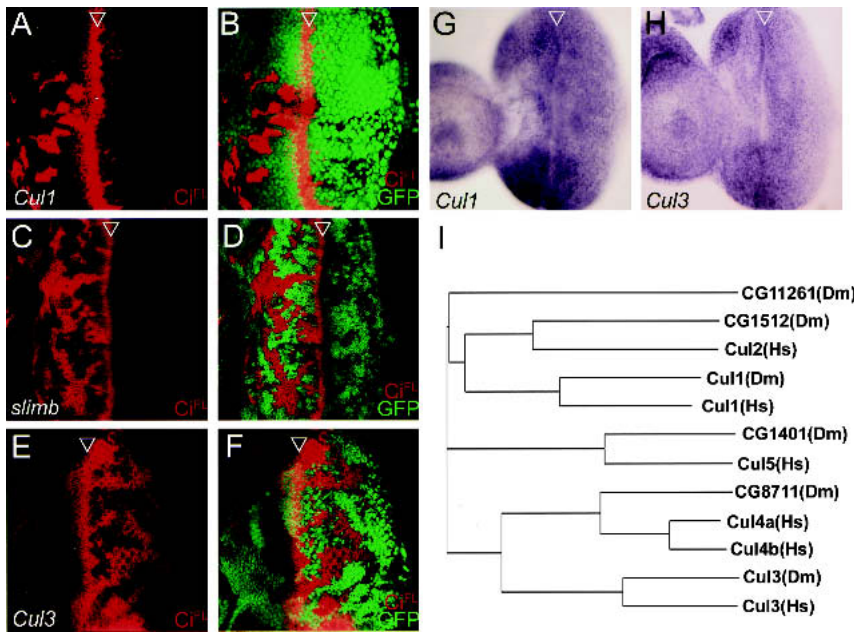
The finding that Ci<sup>FL</sup> accumulated in posterior *smo<sup>3</sup>* clones (Fig. 3C,D, arrowheads; Dominguez 1999) indicated that Smo signaling contributes to the downregulation of Ci<sup>FL</sup> in the posterior cells of the eye disc. This effect is in contrast to the *smo* role in the MF,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

#### Ci<sup>FL</sup> degradation in the posterior cells of the eye disc is mediated by a Cul3-dependent mechanism

The involvement of Nedd8 in controlling Ci<sup>FL</sup> levels in the posterior cells of the eye disc suggests that Cullin proteins other than Cull1 may be involved in the posterior mechanism to control Ci stability. Among the mammalian Cullin family, Cul3 shares with the Cull1-based SCF complex the substrate CycE (Singer et al. 1999). To test whether Cul3 affects Ci<sup>FL</sup> degradation in the eye disc, we analyzed the available *Drosophila* Cul3 mutants (see Materials and Methods). We found that Ci<sup>FL</sup> 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 Ci<sup>FL</sup> accumulation was detected in anterior *Cul3* mutant clones, indicating that Cul3 controls Ci<sup>FL</sup> 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 Ci<sup>FL</sup>, Arm, and CycE in *Cul1* mutant cells. *Cul1<sup>EX</sup>* 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) Ci<sup>FL</sup> (red in A,C) accumulates in the *Cul1<sup>EX</sup>* mutant clones (indicated by arrows) in the anterior compartment of the wing discs. (D–F) In *Cul1<sup>EX</sup>* 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 *Cul1<sup>EX</sup>* mutant clones (arrows) in the eye disc.



**Figure 6.** SCF<sup>Slimb</sup> complex and a Cul3-based degradation machinery to control the Ci<sup>FL</sup> stability in the anterior and posterior cells of the eye discs, respectively. [A–D] The Cul1-based, SCF<sup>Slimb</sup> complex functions in the anterior cells to promote Ci<sup>FL</sup> proteolytic processing. In the *Cul1<sup>EX</sup>* (A) and *slimb<sup>1</sup>* (C) mutant clones located anterior to the MF, Ci<sup>FL</sup> accumulation is detected (red), whereas in the posterior clones, no accumulation can be observed. The mutant clones of *Cul1<sup>EX</sup>* (B) and *slimb<sup>1</sup>* (D) are marked by the lack of GFP expression. [E,F] In the *Cul3<sup>gt2</sup>* mutant clones, marked by the lack of GFP expression (green), only posterior clones accumulate Ci<sup>FL</sup> (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. In the control experiments, the sense probes gave no signals (data not shown). [I] The phylogenetic tree of the *Drosophila* Cullin family (denoted by Dm) to their human counterparts (Hs).

hybridization (data not shown). These results showed that the Ci<sup>FL</sup> degradation machinery in the posterior cells of the eye disc requires a Cul-3-mediated degradation mechanism. Ci accumulation was also detected in *Cul3* mutant cells located in the A/P boundary of the wing disc (data not shown). The level of Arm in *Cul3* mutant clones in wing discs and the level of CycE in *Cul3* mutant clones in eye discs remained constant (data not shown), suggesting that Cul3 activity was specific to Ci.

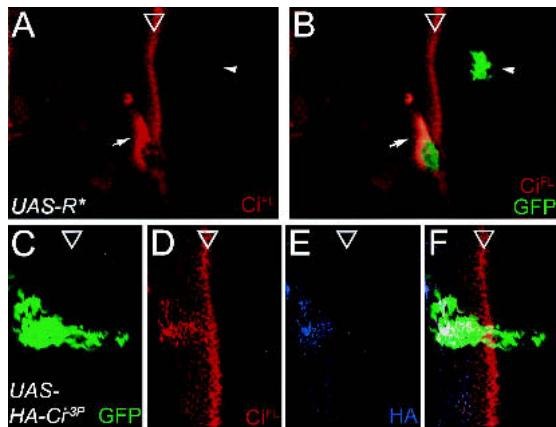
In contrast to the Cul1-based SCF<sup>Slimb</sup> complex that controls Ci<sup>FL</sup> processing only in the anterior cells of the eye disc, the Cul3-mediated Ci degradation mechanism is specific to the posterior cells. These specific activities in controlling Ci protein stability were not caused by differential gene expression of *Cul1* and *Cul3* in the eye disc. We detected ubiquitous mRNA expression patterns of both *Cul1* (Fig. 6G) and *Cul3* (Fig. 6H), and ubiquitous Cul1 protein expression all along the eye disc (Fig. 4E), suggesting that control of specificity is mediated by mechanisms other than regulation of *Cul1* and *Cul3* expression.

#### *Ci<sup>FL</sup> degradation in the posterior cells of the eye disc is constitutive and PKA-independent*

PKA phosphorylation promotes Ci<sup>FL</sup> processing, and plays a role in the Hh signaling pathway for Ci activation. We therefore examined the requirement of PKA in Ci<sup>FL</sup> 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 Ci<sup>FL</sup> processing, Ci<sup>FL</sup> accumulated when PKA activity was inhibited in GFP-positive cells located anterior to the MF (Fig. 7A,B, arrows). In contrast, no Ci<sup>FL</sup> accumulation could be detected in GFP-positive cells located posterior to the MF (Fig. 7A,B, arrowheads), indicating that Ci<sup>FL</sup> downregulation is not regulated by PKA activity.

PKA-independent Ci<sup>FL</sup> downregulation in posterior cells of the eye disc could cause either proteolytic processing of Ci<sup>FL</sup> to the short form, Ci75, or complete Ci degradation. To address this question, we expressed the *UAS-HACi<sup>-3P</sup>* 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 Ci<sup>FL</sup> was driven by *eq-GAL4* in the equator region of the eye disc and monitored by the co-expressed GFP (Fig. 7C). While the  $\alpha$ Ci<sup>FL</sup> antibody detected a high level of Ci<sup>FL</sup> in the anterior equator of the eye disc where PKA phosphorylation is required for Ci<sup>FL</sup> processing, expression of Ci<sup>FL</sup> was completely diminished in the posterior cells in which the GFP signal was strongly detected (Fig. 7D). The  $\alpha$ HA 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, Ci<sup>FL</sup> 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 Ci<sup>FL</sup> (*UAS-HACi<sup>U-3P</sup>*) by *eq-GAL4* in the equator region revealed no detectable Ci



**Figure 7.** Regulation of  $Ci^{FL}$  degradation in posterior cells of the eye disc. (A,B) PKA activity is not required for  $Ci^{FL}$  degradation in the posterior cells. To inhibit PKA activity, the dominant negative form of PKA regulatory domain is expressed in random clones marked by GFP expression (see Materials and Methods). The protein level of  $Ci^{FL}$  (red) is upregulated in anterior (arrow) but not posterior clones (arrowhead). (C–F)  $Ci^{FL}$  degradation is independent of PKA phosphorylation. The PKA phosphorylation site-mutated form of Ci,  $Ci^{-3P}$ , is expressed by *eq-GAL4* in the equator, as shown by the coexpressed GFP (C). (D) Misexpressed  $Ci^{-3P}$  is refractory to PKA-mediated proteolytic processing in the anterior cells, as shown by antibody staining for  $Ci^{FL}$ . However, no  $Ci^{-3P}$  expression can be detected in the posterior cells. (E) The  $Ci^{-3P}$  is fused with an HA tag in the N terminus, and no signal can be detected with an antibody against HA in the posterior cells, suggesting that both the full-length and the short form of Ci are degraded completely. (F) The merged image of C,D,E.

level in the posterior equator region (data not shown), similar to the result obtained from expression of *UAS-HACi<sup>-3P</sup>*. This result suggested that proteolytic processing of  $Ci^{FL}$  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  $Ci^{FL}$  to the short form  $Ci75$  in the anterior cells. To sum up, our results suggested that in the posterior cells of the eye disc,  $Ci^{FL}$  is degraded constitutively, and this degradation process is independent of PKA phosphorylation.

## Discussion

### *Nedd8-modified Cull1 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,  $Ci^{FL}$ , and CycE (Figs. 1,5). In *Nedd8* mutant clones, the presence of Cull1, although at a higher level (Fig. 4E), is unable to downregulate the protein stability. In addition, we showed that in *Drosophila* Nedd8 modifies Cull1 in biochemical assays (Fig. 4B,C). These results indicate that neddylation of Cull1 is essential for Cull1 activity in vivo.

Accumulation of Arm and  $Ci^{FL}$  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 Cull1*

We found that in biochemical assay, the unmodified Cull1 accumulated with the reduction of *Nedd8* activity (Fig. 4D). In *Nedd8* mutant cells in the developing eye disc, Cull1 also accumulated to a high level (Fig. 4E,F), raising the possibility that neddylation activates Cull1 and at the same time turns the modified Cull1 into a labile state for protein degradation. In this way, Cull1 activity could be tightly controlled in signaling pathways during developmental processes. Although the mechanism by which Nedd8-modified Cull1 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<sup>slimb</sup> in Ci proteolytic processing*

In anterior cells of developing discs, we show that  $Ci^{FL}$  proteolytic processing requires the activity of the Nedd8-modified, Cull1-based SCF<sup>slimb</sup> complex. This  $Ci^{FL}$  proteolytic processing is inhibited by Smo signaling and promoted by PKA phosphorylation on  $Ci^{FL}$ . The mechanism by which  $Ci^{FL}$  is proteolyzed from  $Ci^{FL}$  to  $Ci75$  is not clear. We propose that Nedd8 modifies and activates SCF<sup>slimb</sup> for Ci ubiquitination and then proteolysis, because of Cull1 modification by Nedd8 and  $Ci^{FL}$  accumulation in *Nedd8*, *Cul1*, and *slimb* mutants. Consistently, proteolysis of  $Ci^{FL}$  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  $Ci^{FL}$  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  $Ci^{FL}$  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 Ci<sup>FL</sup> 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<sup>Slimb</sup>-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 Ci<sup>FL</sup> 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 Ci<sup>FL</sup> 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. Ci<sup>FL</sup> 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 Ci<sup>FL</sup>.

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

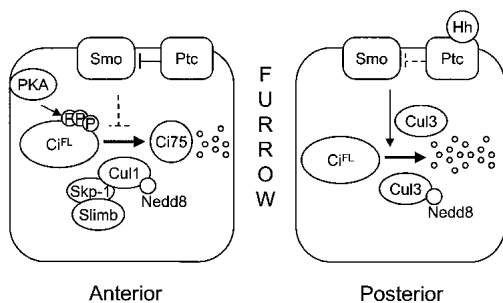
Nedd8 pathway, activates a Cul3-based ubiquitin ligase to degrade Ci in a PKA-independent mechanism in posterior cells of the eye disc.

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 Ci<sup>FL</sup> 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 (Dealy et al. 1999; Skowrya et al. 1999; Koepf 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).  $\beta$ -catenin is also degraded by two different mechanisms in mammalian cells (Polakis 2001). One mechanism involves the SCF <sup>$\beta$ TrCP</sup> complex ( $\beta$ TrCP is the mammalian homolog of Slimb) that recognizes phosphorylated  $\beta$ -catenin. The other mechanism involves the Ebi complex comprised of Ebi, Skp1, SIP, and Siah-1, which targets  $\beta$ -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 proteolyzed to generate repressor forms. Although the proteolytic cleavage of Gli3 is under the regulation of Hh signaling, Gli2 proteolysis is independent of Hh (Aza-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<sup>AN015</sup>* and *Nedd8<sup>AN024</sup>* (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<sup>172</sup>* that included a deletion of the N-terminal half of the Nedd8 protein (aa 1–45), and a weak allele *Nedd8<sup>203</sup>* 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* homozygotes 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 *Cul1* null allele, *Cul1<sup>EX</sup>*, suggested that *lin-19* is a hypomorphic allele of *Cul1*. We performed imprecise excision of the P-element to isolate a null allele *Cul1<sup>EX</sup>*, which includes a deletion of aa 1–90 of the Cul1 protein. When homozygous, or in *trans* to the deficiency *Df(2R)CA53* (43E7–18;44B6) that uncovers *Cul1*, *Cul1<sup>EX</sup>* caused lethality in the second-instar larval stage, suggesting that *Cul1<sup>EX</sup>* is a null allele. *Cul3<sup>06430</sup>* is a P-element insertion line, and *Cul3<sup>gt2</sup>* is an EMS allele (both from Bloomington Stock Center), and both function like genetic null and exhibit identical phenotypes described. To identify homozygotes of *Nedd8*, *Cul1*, or *Cul3*, the green balancer *CyO Kr-GAL4 UAS-GFP* was used.

Other fly stocks used were *f<sup>36a</sup>*, *M(2L)24F<sup>f+30b</sup>* (Cifuentes and Garcia-Bellido 1997), *ey-FLP*, *cl2L3* (Newsome et al. 2000), *ubi-nlsGFP* (gift from S. Luschmig), *arm-LacZ FRT<sup>40A</sup>* (gift from J. Treisman), *ci-LacZ* (Eaton and Kornberg 1990), *dpp-LacZ* (Blackman et al. 1991), *smo<sup>3</sup>* (Chen and Struhl 1996; van den Heuvel and Ingham 1996), *Act-y+<Gal4 UAS-GFP<sup>S65T</sup>* (Ito et al. 1997), *eq-GAL4* (gift from H. Sun), *UAS-mC\**, *UAS-R\** (Li et al. 1995), *UAS-HACi<sup>-3P</sup>* (Wang et al. 1999), *slimb<sup>1</sup>*, *slimb<sup>2</sup>*, and *slimb<sup>P1493</sup>* (Jiang and Struhl 1998). The following flies were used to generate *Nedd8* clones: *hs-FLP<sup>122</sup>*, *Nedd8<sup>AN015</sup> FRT<sup>40A</sup>/cl2L3 P[ubi-nlsGFP] FRT<sup>40A</sup>* (Fig. 1G–L), *w ey-FLP; Nedd8<sup>AN015</sup>*

*FRT<sup>40A</sup>/cl2L3 P[arm-LacZ] FRT<sup>40A</sup>* (Figs. 1M–O, 2H), and *w ey-FLP; Nedd8<sup>AN015</sup> FRT<sup>40A</sup>/cl2L3 P[ubi-nlsGFP] FRT<sup>40A</sup>* (Figs. 2A–G and 4E,F). We used *w ey-FLP; smo<sup>3</sup> FRT<sup>40A</sup>/cl2L3 P[ubi-nlsGFP] FRT<sup>40A</sup>* flies for generation of *smo* clones, and *w ey-FLP; smo<sup>3</sup> Nedd8<sup>AN015</sup> FRT<sup>40A</sup>/cl2L3 P[ubi-nlsGFP] FRT<sup>40A</sup>* for *Nedd8* and *smo* double mutant clones. To generate *Nedd8* clones in mC\* expression domain in eye discs, *UAS-mC\* Nedd8<sup>AN015</sup> FRT<sup>40A</sup>/cl2L3 P[ubi-nlsGFP] FRT<sup>40</sup>*; *eq-Gal4 UAS-GFP UAS-Flp* flies were used. To generate clones expressing R\*, *hs-Flp*, *Act-y+<Gal4 UAS-GFP<sup>S65T</sup>/UAS-R\** flies were used. To generate *Cul1* and *Cul3* clones, we used *w ey-FLP* (or *hs-FLP*); *FRT<sup>42</sup> Cul1<sup>EX</sup>/FRT<sup>42</sup> P[ubi-nlsGFP]* and *w ey-FLP; Cul3<sup>gt2</sup> FRT<sup>40A</sup>/cl2L3 P[ubi-nlsGFP] FRT<sup>40A</sup>* 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  $\alpha$ Ci (1:1; Motzny and Holmgren 1995), rabbit  $\alpha$ Ato (1:1000; Jarman et al. 1994), rabbit  $\alpha$  $\beta$ -galactosidase (1:1000; Cappel), rat  $\alpha$ Elav (1:125; Developmental Studies Hybridoma Bank), mouse  $\alpha$ Arm N27A1 (1:100; Peifer et al. 1994), mouse  $\alpha$ CycE (1:3; Moberg et al. 2001), mouse  $\alpha$ HA (1:100; Roche), and rabbit  $\alpha$ Myc (9E10, 1:250; Santa Cruz).

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

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