DEN1 deneddylates non-cullin proteins in vivo

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Summary

The ubiquitin-like protein Nedd8/Rub1 covalently modifies and activates cullin ubiquitin ligases. However, the repertoire of Nedd8-modified proteins and the regulation of protein neddylation status are not clear. The cysteine protease DEN1/NEDP1 specifically processes the Nedd8 precursor and has been suggested to deconjugate Nedd8 from cullin proteins. By characterizing the Drosophila DEN1 protein and DEN1 null mutants, we provide in vitro and in vivo evidence that DEN1, in addition to processing Nedd8, deneddylates many cellular proteins. Although purified DEN1 protein efficiently deneddylates the Nedd8-conjugated cullin proteins Cul1 and Cul3, neddylation Cul1 and Cul3 protein levels are not enhanced in DEN1null. Strikingly, many cellular proteins are highly neddylated in DEN1 mutants and are deneddylated by purified DEN1 protein. DEN1 deneddylation activity is distinct from that of the cullin-deneddylating CSN. Genetic analyses indicate that a balance between neddylation and deneddylation maintained by DEN1 is crucial for animal viability.

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Introduction

Conjugation by the ubiquitin (Ub)-like protein (Ubl) modulates protein activity in diverse cellular processes (Kerscher et al., 2006). Ubls are synthesized as precursors and are proteolytically processed to expose the C-terminal Gly that forms an isopeptide bond with Lys in target proteins. This conjugation process is catalyzed sequentially by E1 activating enzymes, E2 conjugating enzymes and, in many cases, E3 ligases that recognize target proteins. Conjugation of SUMO (small Ub-like modifier, also known as Sentrin) regulates the activities of a large array of cellular proteins (Johnson, 2004). The conjugated SUMO moiety can be removed by Sentrin-specific proteases (SENP s) that also process SUMO precursors (Mukhopadhyay and Dasso, 2007). The other Ubl, Nedd8, modifies and activates cullin family proteins that function as scaffold to organize cullin-RING Ub ligase (CRLs) complexes (Hori et al., 1999; Pan et al., 2004; Wu et al., 2006). The process of neddylation has also been shown to regulate several cellular proteins and among these are the tumor suppressor protein p53 (Xiromidas et al., 2004), the breast cancer-associated protein BCA3 (Gao et al., 2006) and ribosomal proteins (Xiromidas et al., 2008). However, the repertoire of neddylated proteins and the regulation of their neddylation are not known.

The neddylation state of cullin proteins can be reversed to the unnededdylation state by the COP9 signalosome (CSN), which contains eight subunits (CSN1-8) (Cope et al., 2002; Zhou et al., 2001). The CSN5/Jab1 subunit includes the MPN/JAMM domain (Tran et al., 2003) that is proposed to catalyze Nedd8 deconjugation. In CSN mutants, neddylated cullin proteins are not recycled to unnededdylation states, leading to neddylation-dependent destruction of CRL activity, the degradation of cullin proteins and substrate receptors of CRLs (Wu et al., 2003; Wu et al., 2006; Wu et al., 2005). Therefore, both neddylation and deneddylation are essential to maintain optimal CRL activity in vivo.

The human deneddylase 1 (DEN1) (Gan-Erdene et al., 2003; Wu et al., 2003), also named Nedd8-specific protease (NEDP1) (Mendoza et al., 2003), is a member of the SENP family and was initially named SENP8 (Mukhopadhyay and Dasso, 2007). The human DEN1 has been shown specifically to process the Nedd8 precursor (pNedd8) and to deconjugate Nedd8 from cullin proteins. It has been suggested that DEN1 is more efficient at deneddylating hyperneddylated than mononeddylated Cul1 but is less efficient in removing Nedd8 from mononeddylated Cul1 (Wu et al., 2003). However, the existence of hyperneddylated Cul1 in vivo is not yet known. DEN1 is highly conserved throughout evolution and its family members can be found in yeast, insects, plants and mammals. In Schizosaccharomyces pombe, the DEN1 homolog Npl1 can deneddylate cullin proteins in vitro, but in vivo cullin deneddylation activity was not detected (Zhou and Watts, 2005), raising the possibility that DEN1 may not function to deneddylate cullin proteins in vivo.

In the Drosophila genome, CG8493 has the highest homology to human DEN1, with 36% identical and 57% similar residues in the protein sequence. In the BioGRID interaction database (http://www.thebiogrid.org), Nedd8 is indicated as a DEN1-interacting protein. In this report, we show that the Drosophila DEN1 protein, which is encoded by CG8493, can process the C-terminal fragment of pNedd8 and remove Nedd8 from neddylated Cul1 and Cul3 in vitro assays. We generated DEN1 mutants, and show that processing a Nedd8-CFP fusion protein is defective in DEN1 mutants. However, the protein levels of neddylated Cul1 and Cul3 are not enhanced in DEN1 mutants, suggesting that DEN1 does not function in Cul1 and Cul3 deneddylation in vivo.
using anti-Nedd8 antibodies to detect endogenous neddylated proteins, ectopic neddylated proteins appeared in DEN1 mutants, and can be deneddylated by purified DEN1 protein in an in vitro assay. We further explore the activity of DEN1 in vivo by combining genetic and biochemical analyses in this report.

Results

Processing of pNedd8 by Drosophila DEN1 in vitro

We first demonstrated the specific interaction between Drosophila DEN1 and Nedd8. Purified GST-DEN1 and GST-DEN1CA with the catalytic residue Cys mutated to Ala (supplementary material Fig. S1A) were incubated with His-tagged pNedd8, which includes the intact di-Gly motif at the processing site. Both fusion proteins, but not the GST control, pulled down His-pNedd8GG (supplementary material Fig. S1B), suggesting a direct interaction between DEN1 and Nedd8. The binding to Nedd8 was specific as GST-DEN1 pulled down His-mNedd8 (the mature form of Nedd8) but not His-UB and His-SUMO (supplementary material Fig. S1C).

We tested the Nedd8 processing activity of DEN1 towards the substrate His-pNedd8GG, which bears the intact di-Gly motif. As shown in Fig. 1A, incubation with purified DEN1 led to the production of a cleaved form of Nedd8 (arrow) in a concentration-dependent manner (lanes 1-5). This cleaved fragment was equivalent in electrophoretic mobility to the purified mature Nedd8 (His-mNedd8, lane 6). Peptide sequencing by mass spectrometry confirmed that the cleavage occurred immediately after the second Gly of the Gly\textsuperscript{75}-Gly\textsuperscript{76} processing site (supplementary material Fig. S2). A non-specific signal with a faster mobility than His-mNedd8 appeared in the preparation of His-pNedd8GG (indicated by asterisk, lanes 1-5). The di-Gly motif was essential for processing as its replacement with Ala-Ala in His-pNedd8AA rendered DEN1 ineffective in generating the mature form of Nedd8 (lane 7). Purified DEN1CA failed to generate any mature Nedd8 from His-pNedd8GG and His-pNedd8AA in the same set of experiments (Fig. 1B), suggesting that the processing requires the cysteine protease activity of DEN1.

Processing of Nedd8-CFP fusion proteins by DEN1 in vivo

To characterize Nedd8 processing activity of DEN1 in vivo, we generated DEN1 mutants by imprecise excision of P-elements inserted in the CG8493 locus (supplementary material Fig. S3A). Three independent alleles all deleted the coding exon 2 and generated DEN1null locus (supplementary material Fig. S3A).

The fusion protein Myc-Nedd8GG-CFP with the di-Gly processing motif in between Nedd8 and CFP was used as the in vivo substrate. Using the GAL4/UAS system (Duffy, 2002), the fusion protein was expressed by the ubiquitous tubP-GAL4 driver in larvae. When the larval extracts were subjected to western blots using anti-Myc antibodies, Myc-Nedd8GG-CFP was expressed as a ~40 kDa protein (arrow in Fig. 2, lane 3). Consistent with DEN1 being a Nedd8 processing enzyme, the full-length Myc-Nedd8GG-CFP protein was expressed at a highly elevated level in DEN1null larvae (lane 4). The Myc-Nedd8AA-CFP fusion with di-Ala in replacing di-Gly displayed identical levels of the proteins at 40 kDa in wild type and DEN1null (lanes 5, 6), and their levels were similar to that of Myc-Nedd8GG-CFP in DEN1null (lane 4), indicating that Myc-Nedd8AA-CFP was refractory to DEN1 processing. In addition, an 11 kDa Myc-positive signal from the processing of Myc-Nedd8GG-CFP (lane 3) appeared with a mobility identical to Myc-mNedd8 (lane 2, arrowhead on left), indicating that this 11 kDa Myc-positive protein in lane 3 represents a mature form of Nedd8. When the fusion proteins could not be processed by DEN1 or DEN1 was absent, many proteolytic fragments were detected (lanes 4, 5, 6), including one (indicated by asterisk on right) that appears to have a faster mobility than Myc-mNedd8 (lane 2). The identity of this proteolytic product is not clear. The processing of the Myc-Nedd8GG-CFP fusion protein by DEN1 was also evident when probed with anti-CFP antibodies that also recognize the fused CFP (supplementary material Fig. S4). These results suggest that efficiently processing C-terminal CFP from Nedd8 depends on the di-Gly motif and the DEN1 activity in vivo.

Effects of DEN1 mutations on Cul1 and Cul3 neddylation

We then studied the DEN1 deneddylation activity by first examining whether DEN1 was capable of removing the Nedd8 moiety from neddylated cullin proteins. The Myc-mNedd8 and Flag-Cul1 or Flag-Cul3 constructs were transfected into S2 cells, and Cul1 and Cul3 proteins were immunoprecipitated by the anti-Flag antibody. When the Flag immunoprecipitates were incubated with purified DEN1, the neddylated levels of Cul1 and Cul3 were reduced, when compared with those without DEN1 treatment or treated with catalytic inactive DEN1CA (Fig. 3A,B). This demonstrated that DEN1 indeed has the deneddylation activity.

We then addressed whether Cul1 and Cul3 are the in vivo substrate for deneddylation by DEN1. If DEN1 functions to deneddylate cullin proteins in vivo, the protein levels of neddylated cullin proteins would increase in DEN1null. Unexpectedly, the protein levels of neddylated Cul1 and Cul3 were instead reduced in the larval lysates prepared from DEN1null, when compared with wild type (Fig. 3C,D). The lack of increase in the neddylated forms was not stage specific because the neddylated Cul1 level was not altered by the DEN1null mutation in adult flies (supplementary material Fig. S5).
The decrease in Cul1 and Cul3 neddylation might be caused by inefficient processing of pNedd8 in DEN1null mutants, leading to a limited supply of mature Nedd8 for conjugation. To bypass the requirement of precursor processing, the Myc-mNedd8 protein was overexpressed by tubP-GALA4 and the larval lysates were immunoprecipitated by Cul1 or Cul3 antibodies. The immunocomplexes were analyzed by western blots using antibodies against Myc. The protein level of Myc-Nedd8GG-CFP at 40 kDa is more reduced in wild type compared with DEN1null (lanes 3, 4, arrow on left). However, Myc-Nedd8AA-CFP is expressed at identical levels in both wild type and DEN1null (lanes 5, 6). The product from the processing of Myc-Nedd8GG-CFP runs at 11 kDa with a mobility identical to Myc-mNedd8 (lane 2, arrowhead), but slightly slower than a non-specific proteolytic product in lanes 4-6 (asterisk). Lane 1 is wild-type control without transgene expression and the bottom panel shows α-Tub expression.

Enhanced neddylation on cellular proteins in DEN1 mutants

The reduction in Cul1 neddylation in DEN1null could be explained by enhanced neddylation of other cellular substrates that are not deneddylated in the absence of DEN1. To test whether DEN1 has the deneddylation activity on other targets, we first examined the level of Nedd8 in the absence of DEN1 activity by clonal analysis. Myc-Nedd8GG-CFP was expressed by the GAL4 driver ms1096 in the wing pouch (circled by white-dotted outlines in Fig. 4A-C) and mutant clones homozygous for DEN1null were induced concomitantly. In the DEN1null clones (GFP-negative cells, indicated by arrows), Nedd8 proteins detected by the anti-Myc antibody accumulated to high levels, when compared with neighboring wild-type GFP-positive cells (Fig. 4A). The accumulation of Nedd8 proteins depended on the di-Gly motif as the levels of Myc-mNedd8 were probed with the anti-Myc antibody. Although the Myc-positive signals were rarely detected in wild type, a strong smear pattern was detected in DEN1null (0, 2.5, 5 μg in lanes 1, 2, 3, respectively) and DEN1CA (5 μg in lane 4). Western blots against Cul1 (A) or Cul3 (B) show that the protein levels of neddylated Cul1 and Cul3 are reduced in the presence of DEN1 but not DEN1CA. (C,D) Protein extracts prepared from wild-type and DEN1null larvae were analyzed by western blots for Cul1 (C) and Cul3 (D) expression. The levels of neddylated Cul1 and Cul3, denoted as Cul1Nedd8 and Cul3Nedd8, are reduced in DEN1null mutants. (E,F) UAS-myc-mNedd8 was expressed by tubP-GALA4 in wild-type and DEN1null, and larval lysates were immunoprecipitated by antibodies against Cul1 (E) or Cul3 (F). The immunocomplexes were analyzed using anti-Cul1 (left in E), anti-Cul3 (left in F) and anti-Myc antibodies (right panels) in western blots. Cul1Nedd8 levels are dramatically reduced, whereas the Cul3Nedd8 levels are only slightly reduced.
mNedd8 in larval lysates (supplementary material Fig. S6). In the wild-type lysates, a few discrete bands were detected by the Nedd8 antibodies (Fig. 5B, lane 1). APP-BP1 encodes the regulatory subunit of the E1 activating enzyme that is required in protein neddylation (Kim et al., 2007). Most of these Nedd8-positive signals detected in the wild-type lysates were probably background signals, as they were still present in the lysates prepared from the null APP-BP1<sup>Ex192</sup> mutant (lane 3). In DEN1<sup>null</sup> lysates, however, many ectopic Nedd8-positive signals were detected (lane 2). In addition, a smearing background that was not detected in wild-type also appeared in DEN1<sup>null</sup> lysates. To test whether these Nedd8 signals in DEN1<sup>null</sup> mutants depended on the E1 activating enzyme for neddylation, the lysates of the double mutant for APP-BP1<sup>Ex192</sup> and DEN1<sup>null</sup> were immunoblotted with the Nedd8 antibodies. Strikingly, absence of the E1 activity suppressed the appearance of Nedd8-positive signals in DEN1<sup>null</sup> mutants (lane 4). This result strongly suggests that many cellular proteins are highly transiently neddylated in vivo and they are efficiently deneddylated in the presence of DEN1.

These Nedd8-positive signals in DEN1<sup>null</sup> were distinct from neddylated cellular proteins whose neddylation status is controlled by the CSN (Lyapina et al., 2001; Zhou et al., 2001). In CSN5<sup>null</sup> mutants that lack the cullin deneddylation activity (Cope et al., 2002; Oron et al., 2002), neddylated proteins accumulated in the range of 90-100 kDa (Fig. 5, lane 3), probably representing neddylated cellular proteins. The accumulated signals in CSN5<sup>null</sup> mutants were strikingly distinct from those appeared in DEN1<sup>null</sup> mutants (lane 2), and both types of signals appeared additively in DEN1<sup>null</sup>:CSN5<sup>null</sup> double mutants (lane 4), suggesting that CSN and DEN1 control different sets of target proteins for their neddylation status in vivo. In addition, the neddylated levels of Cul1 and Cul3 proteins were no different in CSN5<sup>null</sup> single and DEN1<sup>null</sup>:CSN5<sup>null</sup> double mutants (supplementary material Fig. S7), suggesting that DEN1 has no effect on CSN deneddylation activity in regulating these cellular proteins.

**Protein deneddylation by DEN1**

To demonstrate the deneddylase activity of DEN1 in vitro, the DEN1<sup>null</sup> larval lysates were incubated with purified GST, GST-DEN1 and GST-DEN1CA proteins. When the DEN1<sup>null</sup> larval lysates were incubated with GST alone, the pattern of Nedd8-positive signals was largely similar to that without any treatment (Fig. 6, lanes 2, 3). Strikingly, incubation with GST-DEN1 eliminated these ectopic Nedd8-positive signals and the smearing background that appeared in DEN1<sup>null</sup> lysates (lane 4). This deneddylation activity was not observed for the catalytically inactive GST-DEN1CA that failed to erase the Nedd8-positive signals in the DEN1<sup>null</sup> lysates (lane 5). These results suggest that DEN1 is capable of removing Nedd8 moieties conjugated on many cellular proteins.

**Suppression of Nedd8 mutant lethality by DEN1**

We then tested whether the DEN1 activity is important in the Nedd8 pathway by carrying out genetic analyses. The transheterozygous hypomorphic mutants Nedd8<sup>22063</sup>/Nedd8<sup>4015</sup> and Nedd8<sup>22063</sup>/Nedd8<sup>40124</sup> died at the pupal stage. Surprisingly, when one wild-type allele of DEN1 was removed from the Nedd8 hypomorphic
In this study, we show that the Discussion neddylation in Drosophila can be explained by the lethality that causes a deficiency in Nedd8P2063DEN1null/Nedd8AN015 Nedd8P2063DEN1null/Nedd8AN024 without any treatment (lane 2). (lane 3) or GST-DEN1CA (lane 5), the neddylation pattern is similar to that in wild-type lysates (lane 2). The pupal lethality was rescued to the adult stage (adult viability, 0% for both Nedd8P2063/Nedd8AN024 and Nedd8P2063/DEN1null/Nedd8AN024, n=200 for each; 42% for Nedd8P2063/DEN1null/Nedd8AN015, n=126; and 10% for Nedd8P2063/DEN1null/Nedd8AN024, n=163). This suppression effect can be explained by the lethality that causes a deficiency in neddylation in Nedd8 hypomorphs being counteracted by lowering the DEN1 deneddylation activity.

Discussion
In this study, we show that the Drosophila DEN1 protease specifically interacts with Nedd8 and can cleave off the Nedd8 C-terminal tail, thus promoting the maturation of Nedd8. The DEN1 protease is also able to remove the Nedd8 moiety from conjugated cullin proteins in an in vitro assay. However, this deneddylation activity is not prominent when assayed in DEN1 mutant extracts. Instead, we found the accumulation of several Nedd8-positive proteins of unknown identity in the DEN1 mutants. Accumulation of these proteins requires the Nedd8-E1 activation enzyme, indicating the involvement of the neddylation process. We further show that these neddylated proteins are distinct from neddylated cullin proteins that appear in CSN mutants, suggesting that DEN1 and CSN activities are largely non-overlapping in vivo. The purified DEN1 protein but not the enzymatic-dead DEN1 mutant can deplete the accumulation of these neddylated proteins, supporting the activity of DEN1 in clipping the Nedd8 moiety off non-cullin proteins. Finally, genetic suppression of Nedd8 mutant lethality by the DEN1 mutation implicates neddylation of non-cullin proteins as being important to animal viability.

The cysteine protease DEN1, in addition to process pNedd8, functions to deneddylate cellular proteins efficiently. A recent study has suggested the neddylation of many ribosomal proteins, which is important in regulating their protein stability (Xirodimas et al., 2008). The deneddylation activity of DEN1 is distinct from the JAMM domain-catalyzed deneddylation activity of the CSN. It is not clear why in vivo neddylated cullin proteins are not optimal substrates for DEN1 and neddylated non-cullin proteins are not deneddylated by the CSN. Specific Nedd8 binding provides the substrate specificity for DEN1 (Reverter et al., 2005) and the Nedd8 moiety on these non-cullin proteins can be easily accessible to DEN1 for efficient deneddylation. This can also account for the low abundance of non-cullin proteins in the presence of DEN1. The Nedd8 moiety on cullin proteins is proposed to provide a hydrophobic surface to recruit Nedd8-conjugated E2, thus facilitating CRL ubiquitination activity (Pan et al., 2004). One possibility is that the Nedd8 moiety is sequestered in protein complexes and is not accessible to DEN1. The CSN complex physically associates with CRLs in vivo, which may facilitate the deconjugation of Nedd8 by the CSN (Fig. 7).

This study confirms the existence of many neddylated non-cullin proteins in vivo. SUMO conjugation modulates cellular activities of numerous target proteins. It is proposed that transient SUMO modification could have a long-lasting effect on target proteins (Hay, 2005). In the absence of the DEN1 activity, the accumulation of neddylated proteins suggests that many cellular proteins are indeed neddylated; these proteins may exist transiently or in a small fraction in wild-type cells. As inferred from our genetic analyses, balanced neddylation and deneddylation by DEN1 on proteins play an essential role in animal viability. The spectrum of neddylated proteins whose Nedd8 moieties are efficiently removed by the evolutionarily conserved DEN1 has probably been underestimated and the effects of neddylation on target proteins await further exploration.

Materials and Methods

Mutant strains
Three DEN1 deletion alleles were isolated in this study (supplementary material Fig. S3A). Nedd8P2063, Nedd8AN015, Nedd8AN024 (Ou et al., 2002), APP-BP1E532 (Kim et al., 2007) and CSN5null (Oron et al., 2002) have been described previously; transgenic strains UAS-flag-Cul1 and UAS-flag-Cul3 have been described previously (Wu et al.,
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2005). UAS-myc-Nedd8GG-CFP, UAS-myc-Nedd8AA-CFP and UAS-myc-mNedd8 were generated in this work. To express the mNedd8, a Nedd8 cDNA with an engineered stop codon immediately after the di-Gly motif was subcloned to generate the UAS-myc-mNedd8 transgene and the His-mNedd8 construct.

Clonal analysis
To generate mitotic clones for DEN1null in wing pouches expressing different myc-Nedd8-CFP transgenes, larvae of the genotype ms(1)096 hsFLP; FRT2 DE1null/FRT2[pUAS-mi-GFP] carrying either UAS-myc-Nedd8GG-CFP, UAS-myc-Nedd8AA-CFP or UAS-myc-mNedd8 transgene were heat-treated at 37°C for 30 minutes 48-72 hours after egg laying. Wing discs were dissected from late third instar larvae for immunostaining using the mouse anti-Myc antibody (9E10, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) following the procedure described previously (Wu et al., 2005).

Antibodies
Primary antibodies were used against GST (rabbit, 1:1000, Pharmacia), His (rabbit, 1:1000, Cell Signaling), Flag (M2, Sigma), Myc (9E10, 1:1000), α-Tub (mouse, 1:300,000, Sigma), Cul1 (rabbit, 1:1000; Zymed), Cul3 (mouse, 1:1000; BD Transduction Laboratories) and Nedd8 (rabbit, 1:1000, generated in this work).

Biochemistry
Processing of His-pNedd8 was performed with DEN1 and DEN1CA proteins that were cleaved from GST fusion proteins by PreScission Protease (Amersham Biosciences). The pNedd8 processing reaction was in a total volume of 20 μl containing 40 mM Tris-Cl (pH 7.4), 1 mM DTT, 150 ng of His-pNedd8GG or His-pNedd8AA at 37°C for 2 hours. Deneddylation assay for neddylated Cul1 and Cul3 was performed with the purified DEN1 or DEN1CA in 20 μl reaction containing 0.8 mM MgCl2, 1 nM ATP, 5 mM Tris-Cl (pH 7.5), 2 mM DTT and 5 μl of the Flag immunoprecipitated complexes for 2 hours at 37°C. For the deneddylase assay in Fig. 5, the glutathione sepharose-bound GST, GST-DEN1 and GST-DEN1CA fusion proteins were incubated with 150 μg wild-type or DEN1null larval proteins in the buffer of 40 mM Tris (pH 7.4), 1 mM DTT, 150 ng of His-pNedd8GG or His-pNedd8AA-CFP and 1 μg GST-DEN1 null enzyme.

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References


