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DENEDDYLASE1 Deconjugates NEDD8 from Non-Cullin Protein Substrates in *Arabidopsis thaliana*

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The evolutionarily conserved 8-kD protein NEDD8 (NEURAL PRECURSOR CELL EXPRESSED, DEVELOPMENTALLY DOWN-REGULATED8) belongs to the family of ubiquitin-like modifiers. Like ubiquitin, NEDD8 is conjugated to and deconjugated from target proteins. Many targets and functions of ubiquitylation have been described; by contrast, few targets of NEDD8 have been identified. In plants as well as in non-plant organisms, the cullin subunits of cullin-RING E3 ligases are NEDD8 conjugates with a demonstrated functional role for the NEDD8 modification. The existence of other non-cullin NEDD8 targets has generally been questioned. NEDD8 is translated as a precursor protein and proteolytic processing exposes a C-terminal glycine required for NEDD8 conjugation. In animals and yeast, DENEDDYLASE1 (DEN1) processes NEDD8. Here, we show that mutants of a *DEN1* homolog from *Arabidopsis thaliana* have no detectable defects in NEDD8 processing but do accumulate a broad range of NEDD8 conjugates; this provides direct evidence for the existence of non-cullin NEDD8 conjugates. We further identify AUXIN RESISTANT1 (AXR1), a subunit of the heterodimeric NEDD8 E1 activating enzyme, as a NEDD8-modified protein in *den1* mutants and wild type and provide evidence that AXR1 function may be compromised in the absence of DEN1 activity. Thus, in plants, neddylation may serve as a regulatory mechanism for cullin and non-cullin proteins.

INTRODUCTION

NEDD8 (NEURAL PRECURSOR CELL EXPRESSED, DEVELOPMENTALLY DOWN-REGULATED8), in *Arabidopsis thaliana* also known as RUB (RELATED TO UBIQUITIN), is an evolutionarily conserved 8-kD protein closely related to ubiquitin (Rao-Naik et al., 1998; Hochstrasser, 2009). Like ubiquitin, NEDD8 is conjugated to substrate proteins through an enzymatic cascade that includes the E1 NEDD8 activating enzyme (NAE); in Arabidopsis, NAE is a heterodimer of AXR1 (AUXIN RESISTANT1) or AXL (AXR1-LIKE) and ECR1 (E1 C-TERMINAL RELATED1). The NEDD8-conjugating cascade also includes an E2 conjugating enzyme; in Arabidopsis, this is RUB1 CONJUGATING ENZYME1 (RCE1; Pozo et al., 1998; del Pozo and Estelle, 1999; del Pozo et al., 2002; Dharmasiri et al., 2007; Woodward et al., 2007). NEDD8 is ultimately conjugated to its protein substrate with the help of E3 NEDD8 ligases like RBX1 (RING BOX1), a constitutive subunit of cullin-RING E3 ubiquitin ligases (CRLs), and DEFECTIVE IN CULLIN NEDDYLATION (DCN; Gray et al., 2002; Duda et al., 2008; Kurz et al., 2008).

The cullin subunits of CRLs are the best-characterized substrates for NEDD8 conjugation (neddylation) (Duda et al., 2008; Huang et al., 2008). Cullin neddylation is promoted by the CRL core subunit RBX1 and required for the assembly of functional CRL complexes that ubiquitylate their cognate substrate proteins to target them for degradation by the 26S proteasome (Gray et al., 2002; Duda et al., 2008). CRL function and protein complex assembly are antagonized by cullin deneddylation through the COP9 signalosome (CSN) (Schwechheimer et al., 2001; Wei et al., 2008; Schwechheimer and Isono, 2010; Lingaraju et al., 2014). Arabidopsis mutants for all eight CSN subunits have been described, including mutants for the paralogous proteins CSN5A and CSN5B, which are the deneddylating subunits of CSN (Gusmaroli et al., 2004, 2007; Dohmann et al., 2005). Whereas *csn* loss-of-function mutants display the strong characteristic constitutively photomorphogenic (cop) phenotype and accumulate cullins in their NEDD8-modified form, mutants partially impaired in CSN function, such as *csn5a* and *csn5b*, have comparatively mild phenotypes and are only partially defective in cullin deneddylation (Gusmaroli et al., 2004, 2007; Dohmann et al., 2005).

Proper neddylation is essential for plant development. Arabidopsis mutants lacking two of the three *RUB* genes (Bostick et al., 2004) or mutants defective in both paralogous subunits of the NAE, *AXR1* and *AXL*, have severe developmental defects beginning during embryogenesis (Leyser et al., 1993; Dharmasiri et al., 2007; Hotton et al., 2011). Weaker mutants, such as *axr1* single mutants, undergo largely normal embryo differentiation but have substantial growth defects, including a strong insensitivity to the phytohormone auxin when grown on medium containing auxin concentrations that inhibit root growth in the wild type (Lincoln et al., 1990; Leyser et al., 1993; Schwechheimer et al., 2002). The auxin insensitivity of the *axr1* mutants can be explained by impaired functionality of their cognate E3 ligase SCF^{TIR1} and related CRLs and, consequently, an inability to degrade the auxin-labile AUX/IAA repressor proteins such as AXR2 and AXR3 (Gray et al., 2001). This auxin insensitivity can also be observed when wild-type seedlings are treated with the NAE inhibitor MLN4924, which blocks NEDD8 conjugation in an MLN4924 concentration-dependent manner (Brownell et al., 2010; Hakenjos et al., 2011). Auxin-insensitive root growth is thus an indicator for defects in

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neddylation and SCF^{TIR1} function. Importantly, weak mutants of CSN such as *csn5a* and *csn5b* mutants also display this phenotype, suggesting that an adequate balance of neddylation and deneddylation is required for proper CRL and SCF^{TIR1} function (Schwechheimer et al., 2001; Gusmaroli et al., 2004, 2007; Dohmann et al., 2005).

Ubiquitin and ubiquitin-like modifiers such as SMALL UBIQUITIN-LIKE MODIFIER (SUMO) modify hundreds of distinct target proteins and thereby affect protein activity or fate (Miller and Vierstra, 2011; Vierstra, 2012; Kim et al., 2013). Therefore, it is surprising that, to date, only cullins have been recognized as bona fide NEDD8 modification substrates. For NEDD8, a number of non-cullin NEDD8-modified proteins have previously been identified, mainly in animal systems (Xirodimas, 2008; Mergner and Schwechheimer, 2014; Enchev et al., 2015), but recent observations of nonspecific crosstalk between the ubiquitin and the NEDD8 conjugation machineries suggests that at least some of these neddylation substrates may in fact be ubiquitylation substrates that have become neddylated rather than ubiquitylated as a consequence of NEDD8 overexpression (Hjerpe et al., 2012a, 2012b). It has thus been questioned whether other biologically relevant non-cullin neddylation substrates exist in eukaryotes (Enchev et al., 2015). In plants, to date, only one non-cullin substrate, ML3, has been biochemically validated, although the existence of others has been proposed (Hakenjos et al., 2011, 2013; Hotton et al., 2012). However, the biological significance of ML3 neddylation remains to be elucidated since the function of ML3 itself is unknown (Hakenjos et al., 2013).

The Arabidopsis genome encodes three distinct NEDD8-encoding *RUB* genes belonging to two structurally distinct families (Rao-Naik et al., 1998). Whereas Arabidopsis *RUB3* encodes a NEDD8 precursor with a C-terminal extension, *RUB1* and *RUB2* additionally bear an N-terminal extension with full-length ubiquitin (Rao-Naik et al., 1998). Mature NEDD8 is derived from these precursors through the N- and C-terminal processing of these extensions. DEN1/NEDP1/SENP8 (DENEDDYLASE1/NEDD8-SPECIFIC PROTEASE1/ SENTRIN-SPECIFIC PROTEASE8; hitherto DEN1) was originally described from *Drosophila melanogaster* and mammals as a NEDD8-specific processing enzyme (Gan-Erdene et al., 2003; Mendoza et al., 2003; Wu et al., 2003; Shen et al., 2005; Chan et al., 2008; Shin et al., 2011). In addition, several ubiquitin C-terminal hydrolases from animals and yeasts were shown to possess a dual specificity for ubiquitin and NEDD8 processing (Wada et al., 1998; Johnston et al., 1999; Linghu et al., 2002; Hemelaar et al., 2004; Frickel et al., 2007; Yu et al., 2007). Although NEDD8 and neddylation are essential in many organisms, none of the mutants described to date from any organism for these NEDD8 processing enzymes are inviable or have an apparent defect in NEDD8 processing. It has therefore been suggested that the different enzymes may act in a functionally redundant manner (Enchev et al., 2015).

Based on sequence similarities, we and others have recently identified four proteins as candidates for NEDD8 processing enzymes from Arabidopsis: a DEN1 homologous protein belonging to the C48 peptidase family (Colby et al., 2006; Mergner and Schwechheimer, 2014) as well as three C12 family peptidases UCH1 (UBIQUITIN CARBOXYL-TERMINAL HYDROLASE1), UCH2, and UCH3 (Yang et al., 2007). Here, we examine the DEN1 homologous protein as well as *den1* mutants from Arabidopsis. We show that DEN1 is not essential for NEDD8 processing but required for protein deneddylation since *den1* mutants accumulate a broad range of NEDD8 conjugates. Furthermore, we show that AXR1 is one abundant NEDD8 conjugate in *den1* mutant plants and provide evidence that AXR1 function may be compromised in these mutants. We thus conclude that many non-cullin NEDD8-modified proteins exist in plants and that neddylation and deneddylation may function as regulatory mechanisms of non-cullin proteins in plant development.

RESULTS

Arabidopsis *den1* Mutants Accumulate NEDD8 Conjugates

Human DEN1 was originally identified as a protein capable of processing the C terminus of NEDD8 propeptides. To understand NEDD8 precursor processing in Arabidopsis, we analyzed AT5G60190, the closest homolog of human and Drosophila DEN1 from Arabidopsis (Supplemental Figure 1). DEN1 belongs to the family of cysteine proteases, and Arabidopsis AT5G60190 is a predicted active protease based on the conservation of the residues critical for this biochemical activity (Figure 1A). Due to the overall sequence conservation, the conservation of the active site as well as the biochemical activities of AT5G60190 described in due course, we designated this protein DEN1. We further isolated two *den1* mutant alleles, *den1-1* and *den1-2*, from the GABI-Kat (Rosso et al., 2003) and the SAIL (Sessions et al., 2002) T-DNA insertion mutant collections, respectively (Figure 1A). Both mutant alleles carry insertions in close proximity to the catalytic center of the cysteine protease, and the insertions would therefore be predicted to strongly impair protein function.

In Arabidopsis, mature NEDD8 is the product of the proteolytic processing of the precursors derived from *RUB1*, *RUB2*, and *RUB3* (Rao-Naik et al., 1998). Whereas *RUB3* encodes an unfused NEDD8 that only requires C-terminal processing, *RUB1* and *RUB2* encode UB (ubiquitin)-NEDD8 chains that are processed at the UB and NEDD8 C termini, respectively. When we probed total protein extracts of the wild type and *den1* mutants with an anti-NEDD8 antibody that recognizes all three Arabidopsis NEDD8 isoforms (Hakenjos et al., 2011), we observed that NEDD8 processing was not detectably defective in the *den1* mutants since the levels of mature unconjugated NEDD8 and NEDD8-conjugated cullins were indistinguishable between the mutant and the wild type (Figure 1B; Supplemental Figure 2). Surprisingly, however, *den1* mutants accumulated NEDD8-modified proteins of a broad molecular mass range (Figure 1B; Supplemental Figure 2). Particularly prominent were two abundant NEDD8-modified proteins with an apparent molecular mass of 72 and 130 kD, which we designated p72 and p130 (Figure 1B; Supplemental Figure 2). Since the accumulation of the non-cullin as well as the cullin NEDD8 conjugates was reduced in the *den1* mutant following treatment with the NAE inhibitor MLN4924, we concluded that not only the cullins but also the other non-cullin NEDD8-modified proteins were substrates downstream of NAE (Figure 1C). Despite the fact that the *den1* mutants had a prominent molecular phenotype, we did not observe any apparent growth defects when examining the mutants in standard growth conditions or following

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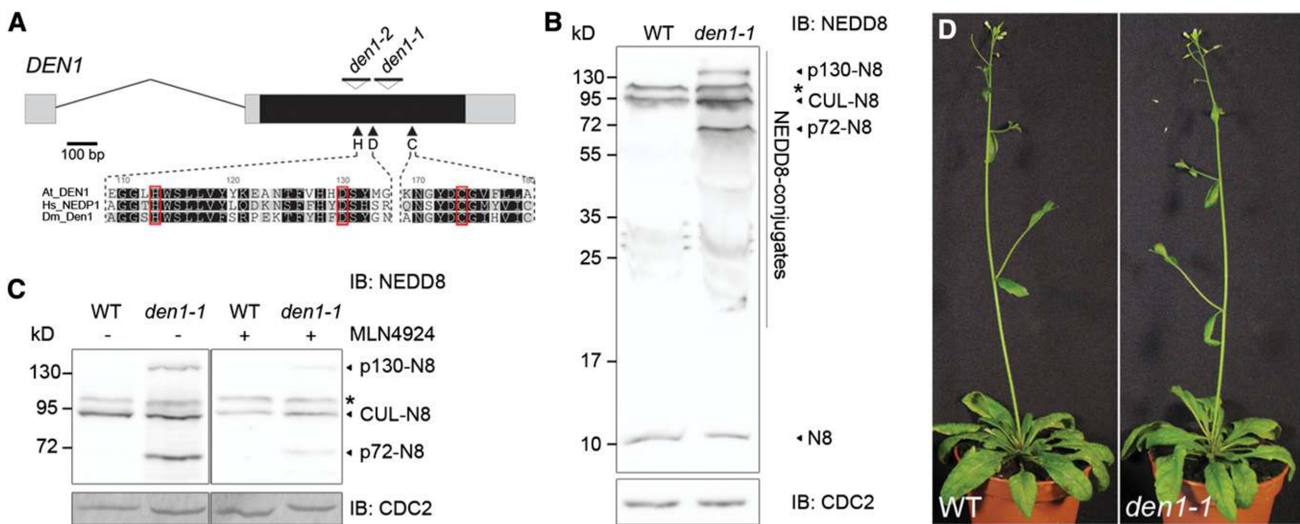

Figure 1. *den1* Mutants Accumulate NEDD8 Conjugates.

(A) Scheme of the *DEN1* gene and positions of the respective T-DNA insertions in *den1-1* and *den1-2* mutants. The open reading frame is shown as a block box, untranslated regions as gray boxes, and the single intron as a line. Arrowheads mark the position of the histidine (H), aspartic acid (D), and cysteine (C) codons of the catalytic triad. The amino acids surrounding the catalytic triad are highly conserved among eukaryotes as shown by the alignment of the corresponding protein sequences from Arabidopsis DEN1 (At_DEN1), human DEN1 (Hs_NEDP1), and Drosophila DEN1 (Dm_Den1).

(B) Immunoblot (IB) with an anti-NEDD8 antibody of total protein extracts (150 µg) from 7-d-old light-grown seedlings. NEDD8 (N8) and N8 conjugates are marked on the right; an asterisk indicates a background cross-reactive band unrelated to N8. For a phenotypic analysis of the *den1-2* mutant allele, see Supplemental Figure 2.

(C) Immunoblot with anti-NEDD8 antibody of total protein extracts (60 µg) from 7-d-old seedlings grown on GM or GM supplemented with 10 µM MLN4924. The two parts of the immunoblot come from the same experiment and same exposure but were spliced together. An immunoblot with an anti-CDC2 antibody serves as loading control. Based on its insensitivity to MLN4924 treatment, we consider the band at ~100 kD, marked with an asterisk, as a cross-reaction of the NEDD8 antibody.

(D) Phenotype of 4-week-old wild-type and *den1-1* mutant plants grown under constant light. The *den1-1* mutants have no apparent phenotypes.

a diverse set of treatments (Figure 1D). In summary, we concluded that the loss of *DEN1* as a candidate C-terminal NEDD8 hydrolase was not sufficient to detectably impair NEDD8 processing in planta but resulted in the accumulation of a broad range of NEDD8 conjugates, presumably DEN1 deneddylation targets. Since the two available *den1* mutant alleles had identical molecular phenotypes, we performed all subsequent analyses with *den1-1*.

DEN1 Does Not Deneddylate Cullins in Planta

The CSN subunit CSN5 deneddylates the cullin subunits of cullin-RING ligases, such as CULLIN1 and CULLIN4. At the molecular level, *csn5a* single as well as *csn5a csn5b* double mutants accumulate NEDD8-conjugated cullins but not other NEDD8-modified proteins (Figure 2A) (Dohmann et al., 2005). In other systems, DEN1 homologs were reported to also deneddylate cullins (Mendoza et al., 2003). In contrast, we found that the pattern of neddylated protein accumulation in the *den1* mutant was distinct from the pattern detected in the *csn5a* mutant, suggesting that DEN1 and CSN5 may have distinct protein substrate ranges (Figure 2A). Since we occasionally observed slight variations in cullin neddylation between different protein samples when we detected cullin neddylation with the NEDD8 antibody, we also compared the cullin neddylation patterns with anti-CULLIN1 and anti-CULLIN4 antibodies (Figure 2B). However, these analyses also led us to the conclusion that DEN1 does not deneddylate cullins in planta.

While the loss of both *CSN5* isoforms in the *csn5a csn5b* mutant resulted in seedling growth arrest, single mutants of *CSN5A* or *CSN5B* are viable and only *csn5a* mutants have clearly apparent growth phenotypes (Gusmaroli et al., 2004; Dohmann et al., 2005). To examine the possibility of a genetic interaction between *DEN1* and *CSN5*, we introduced the *den1* mutation into *csn5* single and double mutants. However, the phenotypic analyses indicated that the *csn5a den1* double mutants, as well as the *csn5a csn5b den1* triple mutants, had the phenotypes of the respective *csn5* mutants (Figure 2C). Furthermore, at the molecular level, *csn5a den1* double mutants had the combined deneddylation defects of the respective single mutants (Figure 2A). We thus concluded that DEN1 and CSN5 interact in an additive manner in Arabidopsis and may have a differential set of deneddylation substrates in planta.

DEN1 Is a Deneddylating Enzyme

We next tested the ability of recombinant DEN1 to deneddylate NEDD8 conjugates. To this end, we added purified glutathione *S*-transferase (GST)-tagged wild-type DEN1 and DEN1C166A, a predicted inactive DEN1 variant with an alanine replacement mutation of the catalytically important Cys-166, to total protein extracts from *csn5a den1* double mutants. In these experiments,

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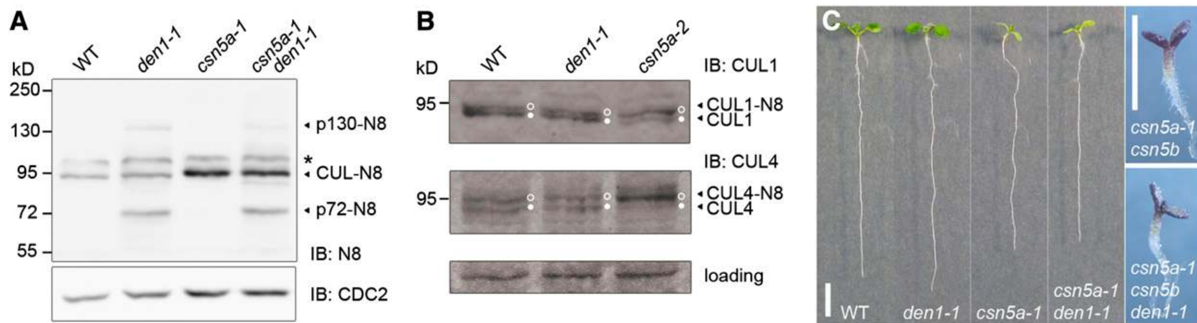

Figure 2. DEN1 and CSN5 Have Different Deneddylation Substrates in Vivo.

(A) Immunoblot (IB) with an anti-NEDD8 antibody of total protein extracts (60 µg) from 7-d-old seedlings. NEDD8 (N8) conjugates are indicated on the right. An asterisk denotes a background cross-reactive band unrelated to N8.

(B) Immunoblot analyses of total protein extracts (30 µg) prepared from 7-d-old seedlings and probed with anti-CULLIN1 (CUL1) and anti-CULLIN4 (CUL4) antibodies. Open circles mark the NEDD8 (N8)-conjugated and filled circles the unconjugated forms of the cullins. A cross-reacting band of the anti-CUL1 blot serves as loading control.

(C) Representative photographs of 7-d-old light-grown seedlings of the wild-type and homozygous mutant seedlings as specified in the figure. Bars = 5 mm.

we observed that non-cullin but also cullin NEDD8 conjugates were hydrolyzed by catalytically active but not by catalytically inactive DEN1 (Figure 3A). We thus concluded that DEN1 has the ability to deconjugate cullin and non-cullin NEDD8 conjugates in vitro. Since our analyses of *den1* mutants had suggested that DEN1 cannot deconjugate cullins in planta, we introduced transgenes for the overexpression of untagged and FLAG epitope-tagged DEN1 into the *den1* mutant. In line with our previous results, the expression of *DEN1* in the *den1* background did not affect cullin (de)neddylation, even when DEN1 was strongly overexpressed from the 35S:FLAG:DEN1 transgene, but did result in the disappearance of the non-cullin NEDD8 conjugates (Figure 3B). We thus confirmed our previous observations that DEN1 does not hydrolyze neddylated cullins in planta.

The differences between the in vivo and in vitro deneddylation activity of DEN1 may be explained by the differential protein localization of DEN1 and the predominantly nuclear cullins (Figures 3A and 3B) (del Pozo et al., 2002). We therefore examined the nucleo-cytoplasmic partitioning of DEN1 by differential centrifugation of total protein extracts prepared from the 35S:FLAG:DEN1 transgenic line. Here, we found that DEN1 accumulated in the cytoplasmic and in the nuclear fraction, whereas the vast majority of NEDD8 conjugates accumulated in the nuclear fraction (Figure 3C). This nucleo-cytoplasmic partitioning of the DEN1 protein was also confirmed when we examined the cellular distribution of a YELLOW FLUORESCENT PROTEIN (YFP)-tagged DEN1:YFP:HA using confocal microscopy in a stably transformed line expressing a 35S:DEN1:YFP:HA transgene (Figure 3D). Like the other transgenes used in this study, 35S:DEN1:YFP:HA fully complemented the molecular phenotype of *den1* mutants (Supplemental Figure 3). Unfortunately, the sensitivity of the anti-DEN1 antibody only allowed detection of the protein in the overexpression lines, and it can therefore not be stated with absolute certainty that the distribution of the DEN1 protein as observed in the overexpression lines correctly reflects the intracellular distribution of endogenous DEN1 in planta.

Since at least one report has so far described a physical interaction between DEN1 and CSN5 or the CSN complex (Christmann et al., 2013), we also examined a possible interaction between DEN1 and CSN5 in Arabidopsis. However, following immunoprecipitation of FLAG:DEN1 from plants, we did not detect CSN5 after probing DEN1 immunoprecipitates with a CSN5 antibody (Figure 3E). Along the same lines, our analysis of DEN1 and CSN5 distribution by size exclusion chromatography indicated that DEN1 was a predominantly monomeric protein that elutes with a profile that is distinct from that of CSN5, which elutes as a subunit of the CSN protein complex and as a CSN5 monomer (Supplemental Figure 4). From these experiments, we concluded that DEN1 may be present in the cytoplasm and in the nucleus, that it deneddylates nuclear non-cullin NEDD8 conjugates, and that it acts independently from CSN5 in vivo.

DEN1 Can Process RUB1 in Vitro

DEN1 was originally identified as an enzyme required for NEDD8 precursor processing (Gan-Erdene et al., 2003; Mendoza et al., 2003; Wuetal, 2003; Shen et al., 2005; Chan et al., 2008; Shin et al., 2011). Since the processing of the Arabidopsis NEDD8 RUB precursors was not detectably affected in the *den1* mutant, we tested the enzymatic activity of DEN1 toward an artificial recombinant substrate, UB:NEDD8:His. UB:NEDD8:His encodes a His-tagged variant of the endogenous UB (ubiquitin)-NEDD8 fusion protein as found in Arabidopsis RUB1 and RUB2. Incubation with purified wild-type DEN1 but not with the catalytically inactive DEN1C166A led to the release of a cleaved protein corresponding to the C-terminally processed UB-NEDD8 (Figure 4A). This indicated that Arabidopsis DEN1, just like its mammalian counterparts, was able to process the C terminus of NEDD8 but not the C terminus of ubiquitin in vitro. We then exchanged the UB and NEDD8 moieties to generate NEDD8:UB:His to examine whether DEN1 was able to process NEDD8 also in the context of a longer C-terminal extension and, conversely, whether DEN1

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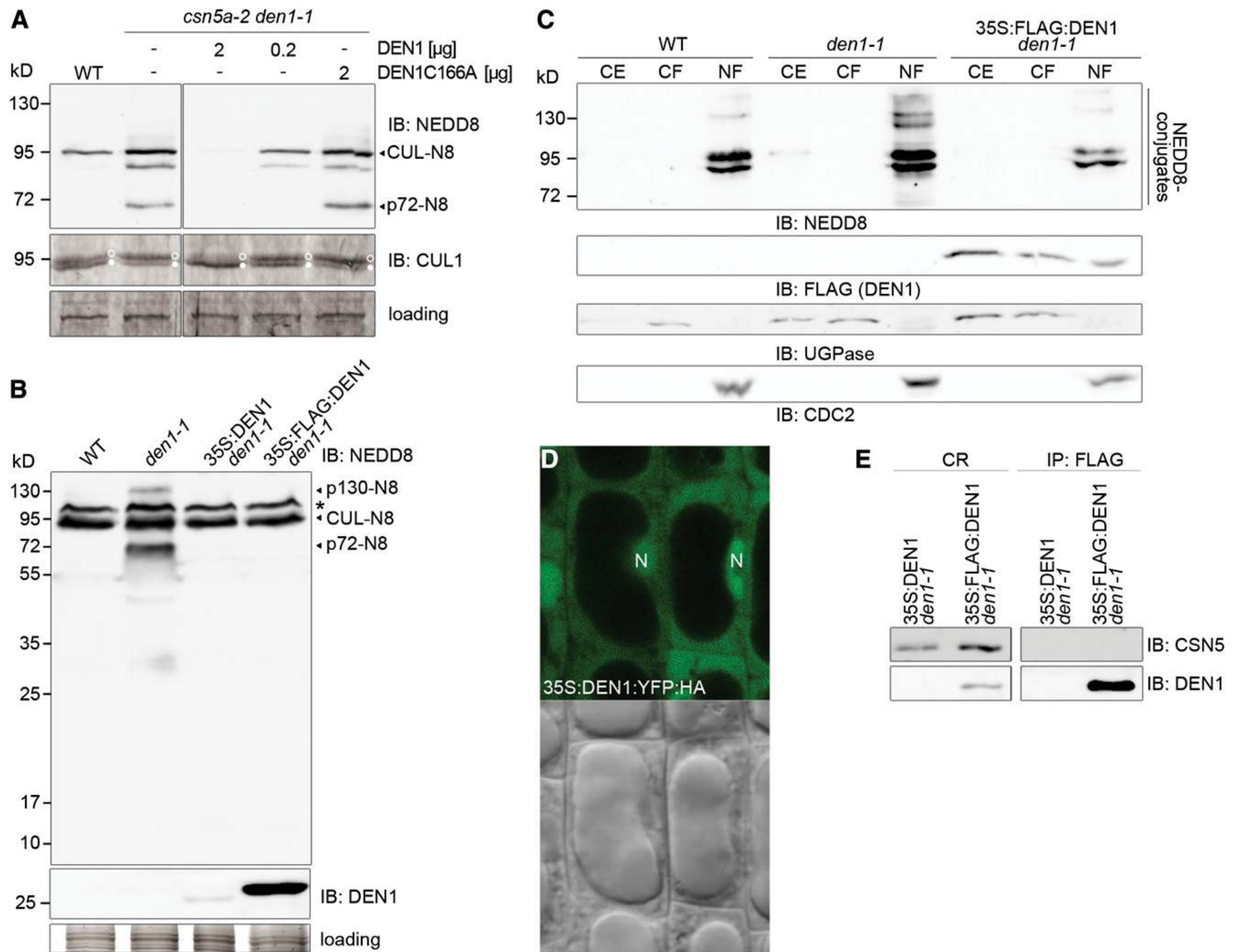

Figure 3. DEN1 Deneddylates Nuclear Non-Cullin NEDD8 Conjugates in Planta.

(A) Immunoblots (IB) of total protein extracts (52 µg) prepared from 7-d-old seedlings probed with anti-NEDD8 and anti-CULLIN1 (CUL1) antibodies. Prior to gel loading, the protein extracts were incubated with purified recombinant DEN1 or the enzymatically inactive DEN1C166A as specified in the figure. Filled and open circles indicate free CUL1 and CUL1-N8 conjugates, respectively. A cross-reacting band of the CUL1 antibody serves as loading control.

(B) Immunoblot analysis of crude protein (60 µg) extracts prepared from 7-d-old seedlings. Blots were probed with antibodies against NEDD8 (N8) and DEN1. N8 conjugates are marked on the right; an asterisk indicates a background cross-reactive band unrelated to N8.

(C) Immunoblots of total protein extracts (CE), cytoplasm (CF), and nuclear fractions (NF) obtained from protein extracts prepared from 14-d-old seedlings. Blots were probed with anti-NEDD8, anti-flag (DEN1), anti-UGPase, and anti-CDC2 antibodies.

(D) Confocal microscopy image of root epidermis cells from 6-d-old seedlings expressing DEN1:YFP:HA.

(E) Immunoprecipitation (IP) of FLAG:DEN1 from 7-d-old seedlings with anti-flag agarose. Blots were probed with anti-CSN5 and anti-DEN1. CR, crude input control.

was able to process ubiquitin with a shorter C-terminal extension. However, DEN1 could neither cleave NEDD8 when present in the NEDD8:UB arrangement nor process the ubiquitin C terminus of NEDD8:UB:His, indicating that it was specific for the C-terminal processing of the NEDD8 moiety of the Arabidopsis RUB propeptides (Figure 4B).

At the same time, we also examined the processing activity of the related cysteine protease UCH3, whose counterpart from the mammalian system has a dual specificity for ubiquitin and NEDD8 processing (Wada et al., 1998; Mergner and Schwechheimer, 2014). In our experiments, UCH3 efficiently cleaved the His C terminus of UB:NEDD8:His as well as the NEDD8:UB:His substrate, indicating that Arabidopsis UCH3 also possessed, at least in vitro, a dual specificity for NEDD8 and ubiquitin C-terminal processing (Figures 4A and 4B). However, just like DEN1, UCH3 was also unable to cleave after NEDD8 or UB when these proteins represented the N-terminal moieties of the fusion proteins. To further examine the DEN1 and UCH3 enzyme activities, we incubated recombinant DEN1 and UCH3 with total protein extracts from plants expressing an N-terminally HA-tagged NEDD8, HA:

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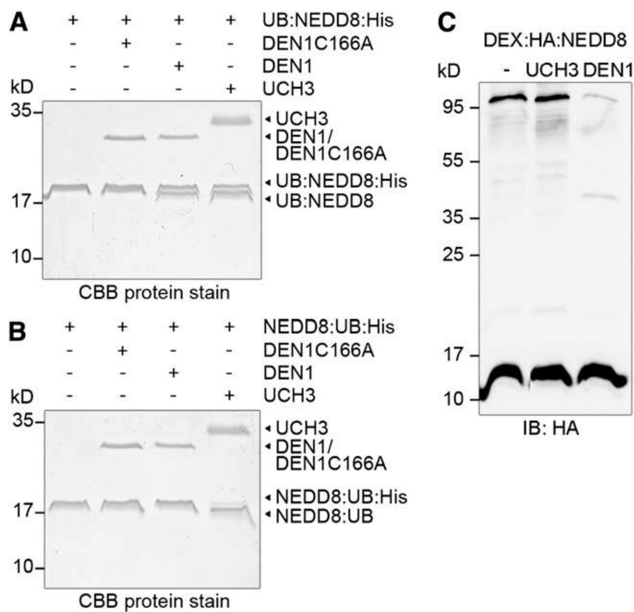

Figure 4. In Vitro Activity of DEN1.

(A) and **(B)** Processing of UB:NEDD8:His **(A)** or NEDD8:UB:His **(B)** with purified DEN1, DEN1C166A, and UCH3 and analyses by SDS-PAGE using Coomassie blue (CBB) staining. Purified enzymes and the unprocessed and processed form of UB:NEDD8:His or NEDD8:UB:His are marked by arrowheads.

(C) Immunoblot with anti-HA antibody of crude protein extract from 7-d-old HSN seedlings following induction with dexamethasone and incubation with purified DEN1 or UCH3. The HA:NEDD8 monomer is indicated by a an arrowhead.

NEDD8, expressed from a dexamethasone-inducible vector system and examined NEDD8 conjugate abundance by immunoblotting. Already after 30 min, DEN1 had almost completely deneddylated all NEDD8 conjugates from these extracts, whereas UCH3 did not affect NEDD8 conjugate abundance (Figure 4C). Thus, whereas UCH3 can process C-terminal extensions from NEDD8 as well as ubiquitin, it is unable to process NEDD8-protein conjugates. Conversely, DEN1 is specific for the C-terminal processing of NEDD8 precursors, at least in vitro, but can also hydrolyze NEDD8 protein conjugates.

DEN1 Is a Ubiquitin-Modified Protein

To gain an understanding of possible molecular associations of DEN1 with other proteins, we also analyzed immunoprecipitates of FLAG:DEN1 by mass spectrometry. In line with our findings that DEN1 was seemingly a monomeric protein (Supplemental Figure 4), we did not detect any stoichiometric protein interactors when analyzing FLAG:DEN1 immunoprecipitates (Figure 5; Supplemental Data Set 1). Interestingly, however, we obtained an indication that DEN1 was modified by either ubiquitin or NEDD8 (Supplemental Figures 5A, 5B, and 6). Since, following trypsin digestion, both modifiers, ubiquitin or NEDD8, leave an identical mass footprint corresponding to two glycines on their substrate proteins, it is not possible to distinguish between these two modifications solely based on these mass spectrometry results. We therefore probed the FLAG:DEN1 immunoprecipitates with antibodies directed against NEDD8 and ubiquitin. Whereas we did not obtain a signal with the NEDD8 antibody, we detected at least two high molecular mass forms of DEN1 with the ubiquitin antibody (Figure 5). Since the mass spectrometric analysis suggested that the ubiquitin modification resided at one of two adjacent lysine residues, Lys-217 and Lys-218, at the DEN1 C terminus (Supplemental Figures 5A and 5B), we also generated transgenic lines for the expression of DEN1 mutant variants where both lysines were replaced by arginine (Supplemental Figure 5C). Indeed, mutagenesis of these residues abolished the ubiquitylation of DEN1, suggesting that one of these two lysines or both lysine residues are ubiquitylation sites in DEN1 in planta (Supplemental Figure 5C).

We also tested whether the ubiquitin conjugation of DEN1 was required for its biochemical activity as a deneddylase and to this end examined the neddylation patterns of *den1* mutants in the absence and presence of wild-type and mutant *DEN1* transgenes. However, we found that the activity of DEN1 was not compromised by the mutation of the ubiquitylation site (Supplemental Figure 5D). Finally, since ubiquitin conjugation may target the protein for proteasomal degradation, we also examined the stability of DEN1 following treatment of plants for up to 8 h with the protein biosynthesis inhibitor cycloheximide (Supplemental Figure 5E). Since the protein abundance of DEN1 was unaltered when we compared the wild type and the mutant DEN1 protein, we concluded that DEN1 ubiquitylation affected neither its deneddylation function nor its protein stability.

AXR1 Corresponds to the NEDD8-Modified Protein p72

In order to identify NEDD8 conjugates that accumulated in the *den1* mutant, we performed a two-step purification of a His- and StrepII-tagged NEDD8 expressed from a 2-kb *RUB1* promoter fragment. Samples were analyzed by mass spectrometry after the first native purification step using the StrepII-tag and after a second denaturing purification step using the His-tag (Supplemental Figure 7 and Supplemental Data Set 2). One of the proteins that attracted our attention was the AXR1 subunit of the NAE. AXR1 was strongly enriched after both purification steps, and AXR1 was thereby distinguishable from its interaction partner ECR1, which was only enriched after the first nondenaturing purification step (Figure 6A). We took this as an indication that AXR1 itself might be a neddylated protein and introduced the *axr1* mutant and a previously published AXR1$_{pro}$:10myc:AXR1 (myc:AXR1) transgene into the *den1* mutant background (Hotton et al., 2011). When we analyzed NEDD8 conjugate formation in the *den1 axr1* background, we detected a decrease in the intensity of the p72 band specific for *den1* in line with the hypothesis that p72 corresponded to a NEDD8-modified form of AXR1 (Figure 6C). Although the p72 band was not completely abolished in the *den1 axr1* mutant, we reasoned that AXL, the functional ortholog of AXR1, might be responsible for the residual staining at the molecular mass corresponding of p72. In further support of AXR1 being a NEDD8-modified protein in *den1* mutants, we detected a novel high molecular mass form of AXR1 that was present in the *den1* mutant but not in the wild-type background when probing

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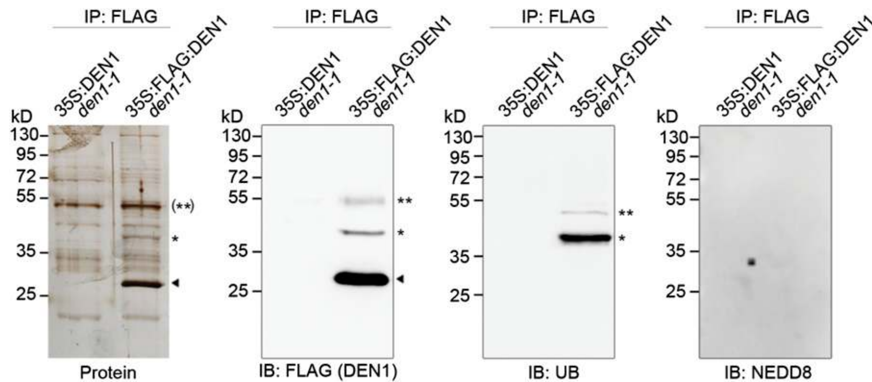

Figure 5. DEN1 Is Ubiquitinated.

Immunoprecipitation of FLAG:DEN1 from 7-d-old 35S:DEN1 and 35S:FLAG:DEN1 seedlings with anti-flag agarose. Left panel, silver stain of total protein. The three blots on the right were probed with anti-flag, anti-ubiquitin, and anti-NEDD8 antibodies. Triangles indicate FLAG:DEN1, and asterisks indicate FLAG:DEN1 carrying mono- and diubiquitin modification. In the silver-stained gel (left panel), the diubiquitin-modified DEN1 is obscured by a background band and can therefore only be presumed at the indicated position based on its migration in the immunoblot. IP, immunoprecipitation; IB, immunoblot.

extracts of myc:AXR1 plants with an anti-myc antibody (Figures 6B and 6C). Since this putatively neddylated form of myc:AXR1 comigrated with the neddylated forms of the cullins, we could not confirm the neddylation of myc:AXR1 using the NEDD8 antibody (Figure 6B). Instead, we tested the sensitivity of this protein to deneddylation by recombinant DEN1 and confirmed in this way the accumulation of myc:AXR1 as a neddylated protein in *den1* mutants (Figure 6C). We also tested using immunoprecipitations of myc:AXR1 from *axr1-30* mutant seedlings complemented with the *myc:AXR1* transgene whether neddylated AXR1 was also detectable in the absence of the *den1* mutation. Indeed, we were able to readily detect neddylated AXR1 from this background, indicating that AXR1 neddylation is a common posttranslational modification of this NAE protein (Figure 6D). We thus concluded that p72 corresponds to neddylated AXR1, and possibly also to a neddylated form of the closely related AXL, that AXR1 is neddylated in planta also in the presence of DEN1, and that DEN1 is required for the removal of this posttranslational AXR1 modification. Unfortunately, the mass spectrometric results did not reveal a good candidate for the second strongly accumulated neddylated protein in *den1* mutants, p130, and neither could we gain insights from this analysis into the identity of the NEDD8-conjugated lysine in AXR1 (Supplemental Data Set 2).

Neddylated AXR1 May Be Functionally Impaired in *den1* Mutants

Auxin insensitive root growth is a hallmark phenotype of *axr1* mutants (Lincoln et al., 1990). Since AXR1 was neddylated in *den1* mutants, we wanted to examine whether AXR1 neddylation had an influence on the auxin sensitivity in roots of *den1* mutants as readout for AXR1 functionality. Whereas we did not detect significant differences in the response to root growth inhibitory concentrations of the synthetic auxin 2,4-D when comparing the wild type and the *den1* mutant, we found that *den1* mutant roots were less sensitive to 2,4-D than the wild type when the seedlings were treated with the neddylation inhibitor MLN4924 (Figures 7A and 7B). MLN4924 inhibits ECR1, which forms the NEDD8 E1 together with AXR1. Consequently, MLN4924 treatments lead to a misregulation of cullin neddylation and, thus, the slight but statistically significant increase in 2,4-D insensitivity as observed in the MLN4924-treated *den1* mutant. This finding would thus be in line with a further reduction in E1 activity in addition to the partial impairment of E1 function through AXR1 neddylation in *den1*. A further indication for an impairment of AXR1 function in the *den1* mutant background came from our observation that *den1 axr1* double mutants expressing myc:AXR1 were less sensitive to 2,4-D than the *axr1* single mutant expressing myc:AXR1 (Figures 7C and 7D). Since it had previously been noted that this particular myc:AXR1 transgene cannot fully complement the *axr1* mutant defect (Hotton et al., 2011), expression of myc:AXR1 may be seen as a sensitized mutant background for such analyses. In summary, our data provide evidence for a partial impairment of E1 function in the *den1* mutants due to the accumulation of neddylated AXR1.

DISCUSSION

We genetically and biochemically analyzed DEN1 from Arabidopsis. The founding members of the *DEN1* protein family were originally described as enzymes specific for the processing of NEDD8 precursors in mammalian systems, and subsequent studies had implicated DEN1 from animals, yeasts, and fungi also in the deneddylation of, e.g., cullins as bona fide NEDD8-conjugated proteins (Gan-Erdene et al., 2003; Wu et al., 2003; Reverter et al., 2005; Christmann et al., 2013). Our analysis of Arabidopsis DEN1 revealed that DEN1, although capable of processing the Arabidopsis NEDD8 precursors in vitro, is not solely responsible for precursor processing in planta since *den1* loss-of-function mutants do not have any detectable defects in processing the Arabidopsis RUB precursors. This is in agreement with observations from other non-plant systems where the loss of any hitherto described NEDD8 processing enzyme including the NEDD8-specific enzyme DEN1 but also hydrolases with dual specificity for NEDD8 and ubiquitin

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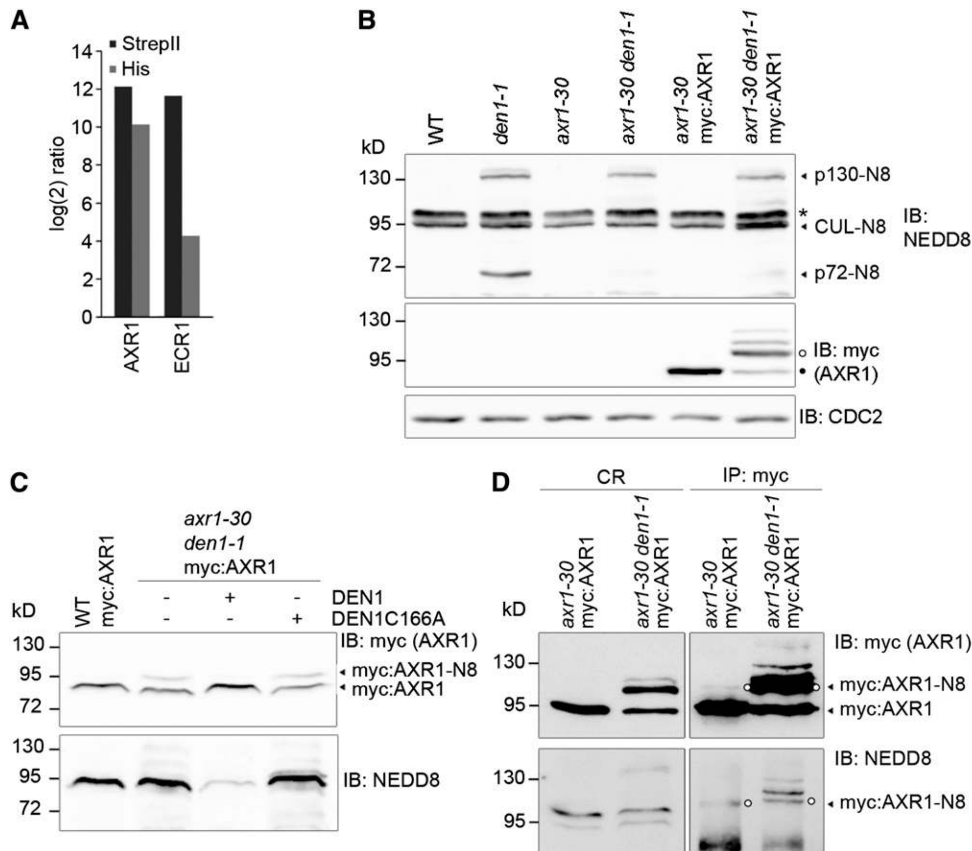

Figure 6. AXR1 Is a Neddylated Protein.

(A) Graph of the log_2 ratio of relative (sample/control) raw abundance of peptides for AXR1 and ECR1 after mass spectrometric analysis following two consecutive purification steps using nondenaturing (StrepII) and denaturing (His) conditions to purify His:StrepII:NEDD8 conjugates.

(B) Immunoblots (IB) of crude protein extracts from 7-d-old seedlings probed with anti-NEDD8, anti-myc, and anti-CDC2 antibodies. Filled and open circles mark the position of the unmodified and modified form of myc:AXR1 in the anti-myc blot.

(C) Immunoblots with anti-myc and anti-NEDD8 antibodies of crude protein extracts from 7-d-old *axr1-30 den1-1* myc:AXR1 seedlings incubated with purified DEN1 or DEN1C166A. Crude protein extract from myc:AXR1 expressed in the wild-type background serves as a negative control. The arrowheads indicate free myc:AXR1 and myc:AXR1-NEDD8 conjugates, respectively.

(D) Immunoblots of crude extracts (CR) and immunoprecipitations (IP) with anti-myc probed with anti-myc and anti-NEDD8 from the genotypes indicated in the figure. The position of myc:AXR1-N8 is indicated by open circles.

does not lead to biochemically detectable processing defects or developmental defects indicative for defects in NEDD8 precursor processing (Mergner and Schwechheimer, 2014; Enchev et al., 2015). Besides DEN1, the Arabidopsis genome encodes three further candidate NEDD8 processing enzymes that have also an additional predicted or tested specificity for ubiquitin processing, UCH1, UCH2, and UCH3 (Mergner and Schwechheimer, 2014). Previous work had shown that the defects in the related *UCH1* and *UCH2* genes would not lead to biochemically discernable ubiquitin-processing defects, again arguing for a functional redundancy among the different processing enzymes (Yang et al., 2007). Here, we show that UCH3 has the predicted dual ubiquitin- and NEDD8-processing activity when tested in vitro and we thus propose that DEN1, UCH3, and possibly also UCH1 and UCH2, which we and others have been unable to recover as biochemically active recombinant proteins when purified from bacteria (Yang et al., 2007), act in a functionally redundant manner in NEDD8 precursor processing.

Our data show that DEN1 is essential for the deconjugation of NEDD8 from a broad range of neddylated proteins that accumulate in the Arabidopsis *den1* mutants. Previous studies on DEN1 had indicated that this protein may also deneddylate cullins (Wu et al., 2003; Christmann et al., 2013). In contrast, our *den1* mutant and DEN1 protein analysis reveal that DEN1, although possessing cullin deneddylating activity in vitro, is not required for cullin deneddylation in planta, also not in the absence of the cullin deneddylating CSN subunit CSN5. The differential substrate range of the deneddylating subunit CSN5 of CSN and DEN1 had previously been attributed to the inaccessibility of the cullin NEDD8 modification in the CRL E3 ligases (Reverter et al., 2005; Duda et al., 2008). A similar argument may also be used to explain the differences between the in vitro and in vivo activities of Arabidopsis DEN1, under

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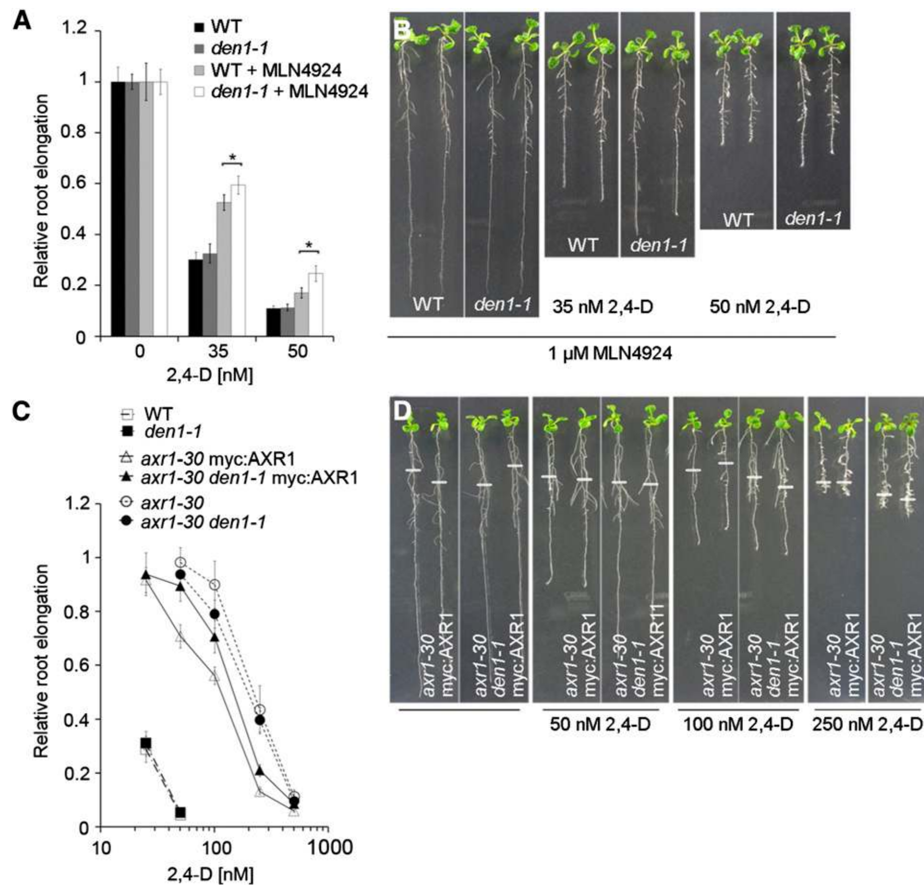

Figure 7. AXR1 Neddylation Impairs 2,4-D-Responsive Growth.

(A) and **(B)** Quantitative analysis of root growth of 5-d-old wild-type seedlings and *den1-1* mutants transferred from unsupplemented GM to GM containing MLN4924 and 2,4-D for an additional 5 d.

(A) The relative root elongation was measured using ImageJ and normalized to the growth of the untreated roots. Average and SE are shown ($n \geq 12$), and asterisks indicate a significant difference (Student's *t* test).

(B) Representative photographs of 10-d-old wild-type and *den1-1* seedlings.

(C) and **(D)** Root growth assay with wild-type, *den1-1*, and different *axr1* mutant lines. Five-day-old GM-grown seedlings were transferred to GM containing 2,4-D (25, 50, 100, 250, and 500 nM) for 5 d.

(C) Root length was measured using ImageJ and is shown as relative to the growth of the untreated roots. Average and SE values are shown ($n \geq 10$).

(D) Representative photographs of 10-d-old seedlings transferred to media containing 50, 100, and 250 nM, respectively.

the premise that CRLs become instable and consequently neddylated cullins accessible to DEN1 in the protein extracts prepared for the respective deneddylation experiments.

Regardless of the functional interplay between DEN1 and CSN5, which may be different between Arabidopsis, where we did not detect an interaction, and *Aspergillus nidulans* or mammalian cells, where such an interaction had been reported (Christmann et al., 2013), our data clearly indicate the presence of NEDD8-modified proteins other than cullins in planta. Although previous reports about a nonspecificity of the NEDD8 and ubiquitin conjugation machineries in certain experimental conditions had put into question the existence of non-cullin NEDD8 conjugates, our observation of an accumulation of such non-cullin NEDD8 conjugates in the *den1* mutant clearly proves their existence. In that regard, our observation is comparable to similar observations with *den1* mutants from *A. nidulans* and Drosophila where NEDD8 conjugate accumulations had also been reported but have remained biochemically unexplored (Chan et al., 2008; Christmann et al., 2013). Taken together, these observations clearly demonstrate that protein neddylation is a common posttranslational modification of many substrate proteins in the respective organisms.

Furthermore, our results identify AXR1 as a novel NEDD8-modified protein that can be detected in this posttranslationally modified form in a genotype comparable to the wild type (an *axr1* mutant complemented with an myc:AXR1 transgene under control of the *AXR1* promoter), and this form accumulates in the *den1* mutant. Our observation that AXR1 in the absence of the deneddylating activity of DEN1 seems partially impaired in protein function suggests that AXR1 neddylation serves to negatively control protein activity and thus indicates that also other as yet unidentified neddylated proteins may be regulated by NEDD8 modification in plants.

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The uncertainty about the biological validity of previously reported protein neddylation events as a consequence of the above mentioned risk of them being an artifact of specific biochemical conditions has recently resulted in the compilation of a set of criteria that must be fulfilled for a given protein to be considered a bona fide NEDD8-modified protein (Enchev et al., 2015). According to these criteria, the neddylation target should be conjugated via a C-terminal glycine residue to its protein target. The DEN1 sensitivity of the NEDD8 modification of AXR1 and the stability of the modification under reducing conditions indirectly demonstrate that AXR1 is modified in this manner. Second, genuine NEDD8-modified proteins should be detectable as such under homeostatic conditions in the presence of normal NEDD8 and substrate protein availability. Since we can detect AXR1 in its neddylated form from *axr1-30* myc:AXR1 where myc:AXR1 is expressed from the *AXR1* promoter, we also see this criterion as being fulfilled. Third, neddylation should be sensitive to treatments with the NAE inhibitor MLN4924, and in agreement with this, we found AXR1 neddylation to be reduced after MLN4924 treatment. Additionally, we suggest that AXR1 neddylation impairs NAE function as concluded from genetic and physiological analyses, and thereby we fulfill the further criterion that protein neddylation has a consequence for the function of the neddylated protein. In conclusion, we judge that AXR1 is a bona fide NEDD8-modified protein from Arabidopsis. Future research will have to elucidate the identity and control of further NEDD8 conjugates, on the one side, and the mechanisms and signals that control neddylation and its substrate specificity, on the other.

METHODS

Biological Material

All experiments were performed in the *Arabidopsis thaliana* ecotype Columbia. *den1-1* (GK381A12) and *den1-2* (SAIL_588_H02) were obtained from the Nottingham Arabidopsis Stock Centre and selected for homozygosity by PCR-based genotyping. Other previously reported mutant lines were *csn5a-1* (SALK_063436), *csn5a-2* (SALK_027705), *csn5b-1* (SALK_077134), and *axr1-30* (SAIL_904_E06). *den1-1* was crossed into the *csn5a-2*, *csn5a-1 csn5b* double mutant, and myc:AXR1 *axr1-30* mutant background, and homozygous double and triple mutants were identified by PCR-based genotyping. Primer sequences for genotyping are listed in Supplemental Table 1. The transgenic $AXR1_{pro}$:myc:AXR1 (myc:AXR1) line was generously provided by Judy Callis (University of California, Davis, CA). The transgenic line for the dexamethasone-inducible expression of HA:STREP:NEDD8 was previously described (Hakenjos et al., 2011).

Cloning Procedures

To generate the *DEN1* overexpression constructs 35S:FLAG:DEN1, 35S:DEN1, and 35S:DEN1:YFP:HA, the *DEN1* coding region was PCR amplified from cDNA with primers 14 and 15 or 14 and 16 and cloned using Gateway technology (Invitrogen) into pEarleyGate202 and pEarleyGate101, respectively. Mutagenesis for DEN1K217218R was performed using a nested PCR reaction with the primers 14 and 17 as well as 14 and 15 and subsequent cloning of the fragment into pEarelyGate202. These transgenes were introduced into the *den1-1* mutant by the *Agrobacterium tumefaciens*-mediated floral dip method (Clough and Bent, 1998).

To generate GST-tagged versions of DEN1 and UCH3, the respective open reading frames were PCR amplified from cDNA using primer pairs with additional *Bam*HI (*DEN1*) or *Eco*RI (*UCH3*) restriction sites before and *Not*I restriction sites after the translational start and stop codons, respectively. After digestion, the PCR fragments were ligated into the pGEX6p-1 vector (GE Healthcare). Mutagenesis of GST:DEN1 to obtain GST:DEN1C166A was performed using *Dpn*I digestion-based site-directed mutagenesis with the primers 22 and 23. UB:NEDD8:His was obtained by generating a PCR fragment of AtRUB1 from Arabidopsis cDNA with primers 24 and 25 and ligating the PCR fragment into pET21a (Novagen). The NEDD8:UB:His construct was obtained by overlap extension PCR with the primers 26 to 29, and the fusion product was cloned into pET21a. To generate a NEDD8 construct with an N-terminal His:StrepII tag expressed under control of the At*RUB1* promoter, $RUB1_{pro}$:His:StrepII:NEDD8, we first used primers 30 and 31 to substitute the HA-tag by a His-tag in the previously described HSN construct (Hakenjos et al., 2011). The construct was subcloned into pTA7002 (Aoyama and Chua, 1997). To obtain the *RUB1* promoter sequence, we used the primers 32 and 33 to amplify a 2-kb region upstream of the start codon of Arabidopsis *RUB1* using genomic DNA as a template. The fragments were digested with restriction enzymes and cloned into pGreen0179 (Hellens et al., 2000). Primer sequences are listed in Supplemental Table 1.

Chemical Treatments

To examine the effect of MLN4924 (Millenium Pharmaceuticals) on protein neddylation, wild-type and mutant seeds were grown for 4 d on standard growth medium (GM) and then transferred to GM containing 10 µM MLN4924 for additional 4 d. For the induction of HSN expression, HSN transgenic seedlings were grown on GM for 7 d and then incubated in liquid GM supplemented with 30 mM dexamethasone for 16 h. For 2,4-D response assays, seedlings were grown 5 d on GM and subsequently transferred to MLN4924- and 2,4-D-containing media for 5 d.

Immunobiological Analyses and Size Exclusion Chromatography

For immunoblots and gel filtration analysis, total protein extracts were prepared from 7-d-old Arabidopsis seedlings in protein extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0,5% Triton X-100, 5 µM *N*-ethylmaleimide, and plant protease inhibitor cocktail [Sigma-Aldrich]) or buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 5 µM *N*-ethylmaleimide, and protease inhibitor cocktail). Subcellular fractionation was performed as previously published (Folta and Kaufman, 2006). Immunoblots were performed according to standard protocols. The following antibodies were used for protein detection: anti-NEDD8 (1:1000; Hakenjos et al., 2011), anti-CULLIN1 (1:1000; Schwechheimer et al., 2002), anti-CULLIN4 (1:1000; Dohmann et al., 2005), anti-CSN5 (1:1000; Kwok et al., 1998), anti-CDC2 (1:5000; Santa Cruz Biotechnology), anti-HA-peroxidase (1:1000; Roche), anti-flag (1: 2000; Sigma-Aldrich), anti-c-Myc (1:3000; Sigma-Aldrich), anti-RGA (1:1000; (Willige et al., 2007), anti-UGPase (1:2500; Agrisera), and anti-Ubiquitin P4D1 (1:2000; Santa Cruz Biotechnology). The anti-DEN1 (1:1000) antibody was raised in rabbits against purified recombinant full-length DEN1 at Eurogentec. Anti-flag M2 affinity gel (Sigma-Aldrich) and anti-c-Myc agarose (Sigma-Aldrich) were used for immunoprecipitations using protein extracts prepared from 0.5 mg (fresh weight) 7-d-old seedlings. Size exclusion chromatography of 1 mg of total plant protein extract was performed using a Superose 6 column (GE Healthcare) as described previously (Schwechheimer et al., 2002).

Mass Spectrometry

For mass spectrometry, proteins were purified from 10 g of 7-d-old $RUB1_{pro}$:His:StrepII:NEDD8 seedlings using a StrepTactin affinity column (Sigma-Aldrich) as described previously (Hakenjos et al., 2011). Part of the sample (sample I) was supplemented with Laemmli buffer and boiled for 5 min. The other part (sample II) was subjected to a second purification step under denaturing conditions using the Ni-NTA system (Thermo Scientific) as described previously (Miller et al., 2010). For tryptic digestions,

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samples I and II were reduced and alkylated by 50 mM DTT and 10 mg/mL chloroacetamide, respectively. Tryptic in-gel digestion was performed according to standard procedures. Nanoflow liquid chromatography-tandem mass spectrometry was performed by coupling an Eksigent nanoLC-Ultra 1D+ (Eksigent) to a LTQ-Orbitrap XL ETD (Thermo Scientific). Peptides were delivered to a trap column (100 μm × 2 cm, packed in house with Reprosil-Pur C_{18}-AQ 5 μm resin; Dr. Maisch) at a flow rate of 5 μL/min in 100% solvent A (0.1% formic acid in HPLC-grade water). After 10 min of loading and washing, peptides were transferred to an analytical column (75 μm × 40 cm, packed in house with Reprosil-Pur C_{18}-GOLD, 3 μm resin; Dr. Maisch) and separated using a 225-min gradient from 4 to 32% of solvent B (0.1% formic acid and 5% DMSO in acetonitrile; solvent A: 0.1% formic acid and 5% DMSO in water) at 300 nL/min flow rate. The LTQ Orbitrap XL was operated in data-dependent mode, automatically switching between MS and MS2. Full-scan mass spectra were acquired in the Orbitrap at 60,000 (*m/z* 400) resolution after accumulation to a target value of 1,000,000. Tandem mass spectra were generated for up to eight peptide precursors in the linear ion trap using collision-induced dissociation at a normalized collision energy of 35% after accumulation to a target value of 5000 for max 100 ms. Intensity-based label-free quantification was performed using Progenesis (version 4.2; Nonlinear Dynamics). The generated peak list was then searched using Mascot (version 2.4.1) against the NCBI protein sequence databases (download October 26, 2011, 15.8 Mio sequences) and SwissProt (version 57, 0.5 Mio sequences) for protein identification. The variable modification of K (GlyGly) was considered in the database search in order to identify NEDD8- or ubiquitin-modified peptides.

Enzyme Activity Assay

Processing of UB:NEDD8:His and NEDD8:UB:His was performed with DEN1, DEN1C166A, and UCH3 proteins that were cleaved from the purified GST fusion proteins using PreScission Protease (GE Healthcare). Glutathione-Sepharose 4B (GE Healthcare) and Talon Metal Affinity resin (Clontech) were used for protein purification from *Escherichia coli* [rosetta (DE3)pLysS]. For processing reactions, 0.2 μg UB:NEDD8:His or NEDD8:UB:His was incubated with 0.2 μg purified DEN1, DEN1C166A, or UCH3 in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10 mM DTT at 20°C for 60 min. The deconjugation assay was performed with protein extract from 7-d-old HSN seedlings containing 15 μg total protein incubated with 0.5 μg purified DEN1 or UCH3 in 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8, 5% glycerol, and 10 mM DTT at 20°C for 30 min. For the in vitro deneddylation assays, crude protein extracts from 7-d-old seedlings containing 350 μg (cullin deneddylation) and 500 μg (myc:AXR1 deneddylation) total protein were incubated with 0.2 or 2 μg purified DEN1 or DEN1C166A in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% Triton X-100 at 20°C for 25 min.

Phylogenetic Analysis

Protein sequences for DEN1 orthologous proteins from human (NEDP1, ENSG00000166192) and *Drosophila melanogaster* (Den1; FBgn0033716) were retrieved from the ensemble genome browser database (http://www.ensembl.org). Alignments were generated using the ClustalW software (BLOSUM, gap open penalty 10 and gap extension penalty 0.1).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *RUB1* (AT1G31340), *AXR1* (AT1G05180), *CSN5A* (AT1G22920), *CSN5B* (AT1G71230), *CULLIN1* (AT4G02570), *CULLIN4* (AT5G46210), *UCH3* (AT4G17510), and *DEN1* (AT5G60190). GenBank accession numbers of human and fruitfly genes mentioned in this work are *NEDD8* (NP_006147), *NEDP1* (AAG21828), and *Den1* (NM_136919).

Supplemental Data

Supplemental Figure 1. DEN1 alignment.

Supplemental Figure 2. *den1-1* and *den1-2* have indistinguishable molecular phenotypes.

Supplemental Figure 3. 35S:DEN1:YFP:HA complements the *den1-1* phenotype.

Supplemental Figure 4. FLAG:DEN1 elutes largely as a monomer after gel filtration.

Supplemental Figure 5. DEN1 is a ubiquitin-modified but stable protein.

Supplemental Figure 6. Result of the mass spectrometric analysis of DEN1.

Supplemental Figure 7. Two-step purification of neddylated proteins.

Supplemental Table 1. List of primers used in this study.

Supplemental Data Set 1. Results of the mass spectrometric analysis following DEN1 immunoprecipitation.

Supplemental Data Set 2. Results of the mass spectrometric analysis following two-step purification of neddylated proteins.

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AUTHOR CONTRIBUTIONS

J.M., B.K., and C.S. designed the experiments. J.M. performed all experiments apart from mass spectrometry. S.H. performed mass spectrometry. S.H., J.M., and B.K. analyzed the mass spectrometric data. J.M. and C.S. wrote the article.

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