The eta7/csn3-3 Auxin Response Mutant of Arabidopsis Defines a Novel Function for the CSN3 Subunit of the COP9 Signalosome

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Abstract

The COP9 signalosome (CSN) is an eight subunit protein complex conserved in all higher eukaryotes. In Arabidopsis thaliana, the CSN regulates auxin response by removing the ubiquitin-like protein NEDD8/RUB1 from the CUL1 subunit of the SCF\textsuperscript{TIR1/AFB} ubiquitin-ligase (deneddylation). Previously described null mutations in any CSN subunit result in the pleiotropic cop/det/fus phenotype and cause seedling lethality, hampering the study of CSN functions in plant development. In a genetic screen to identify enhancers of the auxin response defects conferred by the tir1-1 mutation, we identified a viable csn mutant of subunit 3 (CSN3), designated eta7/csn3-3. In addition to enhancing tir1-1 mutant phenotypes, the csn3-3 mutation alone confers several phenotypes indicative of impaired auxin signaling including auxin resistant root growth and diminished auxin responsive gene expression. Unexpectedly however, csn3-3 plants are not defective in either the CSN-mediated deneddylation of CUL1 or in SCF\textsuperscript{TIR1}-mediated degradation of Aux/IAA proteins. These findings suggest that csn3-3 is an atypical csn mutant that defines a novel CSN or CSN3-specific function. Consistent with this possibility, we observe dramatic differences in double mutant interactions between csn3-3 and other auxin signaling mutants compared to another weak csn mutant, csnn1-10. Lastly, unlike other csn mutants, assembly of the CSN holocomplex is unaffected in csn3-3 plants. We hypothesize that in addition to its role in the CSN as a cullin demeddylyase, CSN3 functions in a distinct protein complex that is required for proper auxin signaling.

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Introduction

The phytohormone auxin (indole-3-acetic acid or IAA) regulates numerous plant developmental processes, by modulating the expression of auxin responsive genes to control cell division, expansion and differentiation [1]. Two large families of transcriptional regulators play essential roles in auxin mediated gene expression: short-lived Aux/IAA proteins and Auxin Response Factor (ARF) transcription factors. In the absence of an auxin signal, Aux/IAAs heterodimerize with ARFs to repress the transcriptional activation of auxin responsive genes [2,3]. To activate auxin responsive gene expression, Aux/IAA repression must be removed, which is accomplished by the SCF\textsuperscript{TIR1/AFB} E3 ubiquitin ligase and the 26S proteasome mediated proteolysis of Aux/IAAs in an auxin-dependent manner [4–6].

Ubiquitin is a small conserved protein, which can be covalently conjugated to specific substrate proteins through the activity of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-ligase (E3) [7]. Proteins with a polyubiquitin chain can then be recognized and degraded by the 26S proteasome. A major class of E3 ubiquitin ligases are the cullin-RING ligases (CRLs), among which the SCF (for Skp1-Cullin-F-box) sub-type has been well-studied [8]. SCF ligases consist of four subunits: the cullin protein, CUL1, binds the RING box protein RBX1 with its C-terminus, while the SKP1 adaptor protein (ASK1 in Arabidopsis) recruits any of many F-box proteins (FBPs) to the N-terminus of CUL1 [9,10]. The TIR1, AFB1, AFB2, and AFB3 F-box proteins of the SCF\textsuperscript{TIR1/AFB} complex are auxin receptors, targeting Aux/IAAs for ubiquitination and degradation upon binding auxin [11–13]. Mutations in SCF\textsuperscript{TIR1/AFB} subunits, such as axr6/cul1, ask1 and tir1, cause the stabilization of Aux/IAAs and a variety of auxin response defects including auxin resistant root growth and reduced lateral root development [4,14,15]. The proper function of SCF ligases, as well as other CRLs, also requires a process called neddylation, in which the ubiquitin-like protein RUB/NEDD8 is covalently conjugated to the cullin subunit [16–18]. The AXR1, ECR1, and RCE1 enzymes mediate cullin neddylation, and mutations in these factors result in a reduction in the level of neddylated cullin and pleiotropic growth defects, including diminished auxin signaling [16,19]. The NEDD8 modification of CUL1 is a highly dynamic process [20,21]. Cleavage of NEDD8 from cullins (deneddylation) is promoted by a protein complex called the COP9 signalosome.
(CSN), as the CSN complex purified from porcine spleen deneddylates Cull in *vivo* [21,22]. Consistently, studies in various organisms have shown that deneddylated cullin proteins accumulate in *csn* mutants [22–25].

The COP9 signalosome (CSN) is an evolutionarily conserved protein complex of eight subunits [26]. It was originally identified in Arabidopsis through genetic screens for mutants exhibiting a constitutive photomorphogenic/deciliated (cop/del) phenotype, and was subsequently biochemically purified from both plant and animal protein extracts [26,27]. The CSN is structurally related to the 19S lid of the 26S proteasome and eukaryotic translation initiation factor 3 (eIF3), and is composed of six PCI (for Proteasome, COP9, eIF3) domain-containing subunits (CSN1-4, 7, and 8) and two MPN (for Mpr1p, Pdc1p N-terminal) domain-containing subunits (CSN5 and CSN6) [27]. It has been established that the CSN regulates SCF activity by deneddylating the CUL1 subunit of the SCF [22,28]. Besides CUL1, the CSN also deneddylates other cullin proteins and broadly regulates many CRLs, including those containing CUL2, CUL3 and CUL4 [21,23,29,30]. In plants, the CSN has been implicated in a variety of processes, including auxin, jasmonate (JA), and gibberellin (GA) signaling, flower development, and light signaling via its interaction with many CRLs [29,31,32]. SCN-mediated cullin deneddylation is catalyzed by the JAMM metalloenzyme motif within the CSN5 subunit [22,32]. However, CSN5 can only provide this isopeptidase activity after incorporation into the CSN holocomplex, as deneddylation activity is defective in all previously characterized CSN subunit mutants [23,34,35].

Neddylation plays a positive role in regulating SCF activity, by promoting a conformational change in CUL1 that shortens the distance between FBP-bound substrates and the E2 ubiquitin-conjugating enzyme [36–39]. Although in *vivo* biochemical studies indicated that the CSN negatively regulates E3 ubiquitin ligase activity [21,33], genetic evidence suggests otherwise [28,39,40]. Studies in yeast and *Drosophila* suggested that although the CSN returns CRL activities to basal levels, it can also facilitate CRL activities by either maintaining the stability of labile substrate adapters or by recycling unstable, deneddylated cullins into the more stable deneddylated isoform [41,42]. In Arabidopsis, the CSN is also required for the proper functions of SCF*FUS11*, as well as SCF*NPL1*, in the degradation of Aux/IAA proteins or the DELLA proteins, respectively [28,31,43]. Together, these findings suggest that cullin neddylation/deneddylation is a highly dynamic process essential for maintaining proper CRL function [44,45]. In addition to regulating SCF activity by deneddylating cullins, recent biochemical findings indicate that the CSN also inhibits SCF complexes by a noncatalytic mechanism [46,47]. Following deneddylation, the CSN remains stably associated with the SCF, sterically hindering both the re-neddylation of CUL1 and SCF interactions with the E2 enzyme.

Arabidopsis *null* mutants of any CSN subunit are phenotypically indistinguishable and exhibit the pleiotropic *cop/del/fus* phenotype, which is characterized by short hypocotyl and open cotyledons of dark-grown seedlings, accumulation of anthocyanin and seedling lethality [23,27,40,49]. Severe deneddylation defects have been found in previously described null mutants of each of the CSN subunits [23,40], but the associated seedling lethality hampers the further analyses of CSN functions. Recently however, a few weak, viable Arabidopsis *csn* mutants have been described. These include mutants lacking one of the two copies of CSN5 and CSN6 encoded in the Arabidopsis genome [23,50], as well as hypomorphic missense alleles of CSN1 and CSN2 [40,43]. Importantly however, these viable *csn* mutants still exhibit diminished deneddylation activity, resulting in the accumulation of neddylated CUL1 and reduced SCF activity [23,29,34,40,43].

In a previously described genetic screen for enhancers of *tir1-1* [15,51–54], we identified two weak *csn* subunit mutants, designated as *eta6/csn1-10* [43] and *eta7/csn3-3*. Our phenotypic characterization of these *csn* mutants, together with expression studies with auxin regulated reporters, demonstrate that *csn1-10* and *csn3-3* confer very similar reductions in auxin response. However, unlike *csn1-10*, which is a typical *csn* mutant with defects in CSN-mediated deneddylation and Aux/IAA protein degradation [43], neither of these defects were observed in the *csn3-3* mutant. Furthermore, genetic interactions between these *csn* mutants and additional auxin signaling mutants also distinguish *csn3-3* from other *csn* mutants. Our studies suggest that *csn3-3* is a unique *csn* mutant that defines a novel functional activity for the CSN3 subunit of the COP9 signalosome in the regulation of auxin signaling.

**Results**

*eta7* is a weak allele of COP9 signalosome subunit 3 (CSN3)

We have previously reported the identification of several auxin response mutants isolated from a genetic screen for enhancers of the *tir1-1* auxin response defect (designated as *eta* mutants), including *eta1/avr6-3* [15], *eta2-1/csn1* [52], *eta3/gtb1* [53], *eta4/pdr9-1* [54], *eta5/aur4* [51] and *eta6/csn1-10* [43]. The *eta7* mutant was also identified in this screen. Map based cloning positioned the *eta7* mutation within a 330 kb interval on chromosome five. This interval contained 102 predicted genes, including *CSN3/FUS11*, which encodes subunit 3 of the COP9 signalosome (CSN) [55]. Given that *csn* subunit mutations are known to confer diminished auxin response phenotypes, we sequenced the *CSN3* locus from *eta7* plants and identified a single mutation (**Figure 1A**). This mutation results in a G293E missense mutation within the highly conserved PCI domain of CSN3. This domain is important for subunit interaction and CSN complex assembly [56]. Primary sequence alignment among the PCI domains of several CSN3 orthologs revealed that Gly293 is extremely highly conserved throughout eukaryotes (**Figure 1B**). To confirm that this mutation was responsible for the *eta7* auxin response defect, we conducted complementation tests by transforming a genomic *CSN3* construct into *eta7* mutant plants. The *CSN3* transgene fully restored auxin sensitivity to *eta7* seedlings when tested in root growth assays (**Figure 1C**). We therefore renamed *eta7* as *csn3-3*.

**Characterization of *csn3-3* phenotypes**

The CSN plays a well-established role in auxin signaling, acting as a deneddylase to regulate SCF*FUS11* activity. Mutants in any CSN subunit exhibit auxin related phenotypes, such as auxin resistant root growth and reduced lateral root formation [28,34,43]. In a dose-response assay measuring the auxin inhibition of root elongation, we found *csn3-3* was mildly resistant to exogenous auxins. After transfer to media supplemented with 0.05 μM 2,4-D, root growth of wild-type (Col) seedlings was nearly completely inhibited (**Figure 2A**). However, *csn3-3* seedlings were resistant to this inhibition, displaying only 47% inhibition of root growth. A similar degree of auxin resistant root growth was observed with the *csn1-10* and *tir1-1* mutants. Additionally, both *csn1-10* and *csn3-3* enhanced the weak auxin resistance phenotype of *tir1-1*, with *csn1-10 tir1-1* and *csn3-3 tir1-1* double mutants exhibiting comparable dose-response profiles in root growth inhibition assays (**Figure 2A**). Similar assays using IAA-supplemented media also found that *csn3-3* exhibited auxin resistance...
and enhanced the tir1-1 phenotype, demonstrating that the auxin response defect of csn3-3 is not 2,4-D specific (Figure 2B).

Previous reports have shown that plants expressing a CSN5 antisense construct develop fewer lateral roots and reduced root hair elongation compared to wild-type controls [28]. Consistent with this finding, the csn3-3 and the csn1-10 mutations enhanced the weak lateral root defect of tir1-1 seedlings, with both double mutants developing fewer than 50% of the number of lateral roots observed in tir1-1 seedlings (Figure 2C). Furthermore, while lateral root numbers in csn3-3 and csn1-10 single mutants were comparable to wild-type controls, csn3 csn1-10 double mutants developed significantly fewer lateral roots, suggesting that auxin response is more severely impaired in the double mutant background.

To further compare the auxin response defects of csn3-3 and csn1-10 mutants, we introduced the BAK1:GUS and DR5:GUS auxin responsive reporters [3,57] to examine auxin mediated gene expression. Treatment of Col [BAK1:GUS] control seedlings with 2,4-D or IAA triggered a strong GUS signal in the root elongation zone. In contrast, only a slight induction of BA3:GUS activity was observed in csn3-3 and csn1-10 mutants (Figures 2D and S1). Like the auxin-resistant root growth phenotype of csn3-3, the diminished BAK1:GUS expression was complemented by a genomic CSN3 transgene (Figure 2E). Similar findings were obtained with csn3-3 and csn1-10 seedlings carrying the DR5:GUS reporter (Figure 2D). Together, our analysis of root growth inhibition, lateral root development, and auxin mediated gene expression suggest that the csn3-3 and csn1-10 mutations confer a very similar reduction in auxin sensitivity.

In Arabidopsis, loss of any of the eight CSN subunits results in an identical suite of phenotypes, including constitutive photomorphogenesis, anthocyanin accumulation, and seedling lethality [23,35,58]. We observed similar phenotypes upon examining the csn2-2 null mutant (Figure 3A). Previously described viable csn mutants include single mutants of the two MPN domain subunits, which are each encoded by two highly homologous genes (CSN5A/CSN5B [59] and CSN6A/CSN6B [50]), and the weak csn1-10 [43] and csn2-5 [40] missense mutants. Unlike previously described csn3 alleles, [23,35,55], csn3-3 is viable throughout development and does not exhibit a constitutive photomorphogenic (cop) phenotype (Figure 3A-C). Likewise, the csn1-10 and csn2-5 mutants also do not exhibit an obvious cop phenotype, although both mutants are mildly dwarfed compared to wild-type and csn3-3 adult plants [40,43].

CUL1 deneddylation and Aux/IAA stability are not affected in csn3-3 seedlings

The CSN cleaves the RUB/NEDD8 peptide from the cullin ubiquitin-ligases. All previously described csn mutants, including the weak csn1-10 and csn2-5 alleles, result in an increase in the CUL1-NEDD8 to unmodified CUL1 ratio [40,43]. We therefore examined if the csn3-3 mutation affected CUL1 modification. We included csn1-10 for comparison with csn3-3, since both mutants are viable csn mutants and display similar auxin response defects (Figure 2). While a clear increase in CUL1-NEDD8:CUL1 was seen in extracts prepared from csn1-10 seedlings, no change from wild-type was detected in csn3-3 seedlings, no change from wild-type was detected in csn1-10 seedlings (Figure 2D). Once again however, the csn3-3 mutation did not increase the CUL1-NEDD8 to unmodified CUL1 ratio [40,43].

Since the csn3-3 mutation unexpectedly did not affect the CUL1-NEDD8:CUL1 ratio, we examined double mutants with csn1-10 to test the possibility that csn3-3 might enhance the weak deneddylation phenotype of csn1-10. Once again however, the csn3-3 mutation did not increase the CUL1-NEDD8:CUL1 ratio (Figure 4A). Since the CSN also regulates other cullin-based E3 ubiquitin-ligases by deneddylating their cullin subunit, we applied a similar immunoblotting assay to examine CUL4 modification in csn3-3 seedlings. Similar to our findings with CUL1, we observed no accumulation of the CUL4-NEDD8 isofrom in csn3-3 extracts, whereas neddylated CUL4 was readily detectable in csn1-10 and csn3-2 extracts (Figure 4A), as reported in previous studies [35]. Since the csn3-3 mutation unexpectedly did not affect the CUL1-NEDD8:CUL1 ratio, we examined double mutants with csn1-10 to test the possibility that csn3-3 might enhance the weak deneddylation phenotype of csn1-10. Once again however, the csn3-3 mutation did not increase the CUL1-NEDD8:CUL1 ratio (Figure 4A). Since the CSN also regulates other cullin-based E3 ubiquitin-ligases by deneddylating their cullin subunit, we applied a similar immunoblotting assay to examine CUL4 modification in csn3-3 seedlings. Similar to our findings with CUL1, we observed no accumulation of the CUL4-NEDD8 isofrom in csn3-3 extracts, whereas neddylated CUL4 was readily detectable in csn1-10 and csn3-2 extracts (Figure 4A). Together, our analyses of CUL1 and CUL4 suggest that the csn3-3 mutation does not affect the deneddylation activity of the CSN.

The reduced deneddylation activity of several previously characterized csn mutants has been found to result in diminished SCFTRI/AFB activity, resulting in the stabilization of Aux/IAA proteins [28,40]. Since our finding that csn3-3 plants exhibited no change in cullin deneddylation was quite surprising, we examined SCFTRI/AFB activity by monitoring Aux/IAA stability using the previously described HS:AXR3NT-GUS reporter protein [4]. csn1-10 seedlings were again included for comparison. 6-d.o. Col, csn1-10 and csn3-3 seedlings, all of which carried the HS:AXR3NT-
The GUS construct, were heat-shocked at 37°C for 2 hours to induce expression, followed by return to ambient temperature and treatment with auxin. AXR3NT-GUS activity was examined both qualitatively and quantitatively at 20 min intervals during the treatment to monitor the remaining levels of AXR3NT-GUS fusion protein (Figure 4C, D). Following the 2h heat-shock...
induction, similar AXR3NT-GUS levels were observed in wild-type, csn3-3 and csn1-10 seedlings (Figure 4C). During the auxin treatment, AXR3NT-GUS activity in csn3-3 and wild-type seedlings diminished with comparable kinetics (Figure 4D), suggesting that SCFTIR1/AFB activity is unaffected in csn3-3 plants.

In contrast, csn1-10 seedlings exhibited substantially slower AXR3NT-GUS degradation (Figures 4C, D). To further confirm that csn3 had no effect on SCFTIR1/AFB-mediated proteolysis, we examined another Aux/IAA reporter, IAA28-myc [60]. The IAA28-myc construct was crossed into csn3-3 and csn1-10 backgrounds. Abundance of the IAA28-myc protein was then examined by treating seedlings with IAA and immunoblotting root protein extracts. As previously reported [60], IAA28-myc was rapidly degraded in wild-type seedlings and was nearly undetectable after a 10 minute auxin treatment (Figure S2). While IAA28-myc was clearly more stable in csn1-10 seedlings, it was rapidly degraded in the csn3-3 background (Figure S2). Combined with our HS:AXR3NT-GUS degradation data, this strongly suggests that SCFTIR1/AFB activity is unaltered by the csn3-3 mutation.

Genetic interactions distinguish csn3-3 and csn1-10
The CSN regulates auxin signaling by deneddylating CUL1 to modulate SCFTIR1/AFB activity [22,28,44]. Our findings with the csn1-10 mutant are consistent with this model. The csn3-3 mutation on the other hand, affects neither CUL1 deneddylation nor SCFTIR1/AFB-mediated Aux/IAA degradation, yet confers auxin response defects virtually identical in severity to csn1-10. These findings strongly suggest that the CSN, or at least the CSN3

Figure 3. csn3-3 does not exhibit characteristic csn mutant phenotypes. (A) Seedling phenotypes of csn3-2 and csn3-3 mutants. (B) Phenotypes of Col, csn1-10 and csn3-3 adult (30-d.o.) plants. (C) 5-d.o. Col and csn3-3 etiolated seedlings. Size bars = 1 mm. doi:10.1371/journal.pone.0066578.g003

Figure 4. csn3-3 affects auxin response independent of SCFTIR1/AFB. (A) CUL1 western blot analysis of protein extracts prepared from Col and csn mutant seedlings. The upper band indicates the modified (neddylated) CUL1. RPT5 is shown as a loading control. Numbers below the blot indicate the ratio of CUL1-NEDD8:CUL1 ± SD from three experiments. (B) Western blot analysis of CUL4 neddylation status in Col and csn mutant seedling extracts. (C) Col, csn1-10 and csn3-3 carrying the HS:AXR3NT-GUS transgene were heat shocked for 2 h and stained immediately or following incubation with 1 μM 2,4-D for 20 min. (D) Quantitative measurement of the β-glucuronidase activity of the HS:AXR3NT-GUS reporter in Col, csn1-10, and csn3-3 seedlings. Seedlings were heat-shocked for 2 h, and then returned to room temperature and treated with 1 μM 2,4-D for 20, 40, or 60 min. β-glucuronidase activity is presented as the fraction remaining compared to the 0 min time point. Values shown depict the mean ± SD of 6 technical replicates. Similar results were obtained in two additional biological replicates. doi:10.1371/journal.pone.0066578.g004
subunit, plays a second role in the regulation of auxin signaling in addition to deneddylating CUL1. If so, we reasoned that csn3-3 and csn1-10 may exhibit distinct genetic interactions when combined with other mutations affecting auxin signaling.

We therefore crossed axr1-12 plants with csn1-10 and csn3-3 to generate double mutants. The axr1-12 mutation affects a subunit of the NEDD8 activating enzyme. This mutation confers a reduction in CUL1 neddylation and strong auxin response defects [16,17,61]. The interaction between axr1-12 and csn1-10 appeared additive, as double mutants exhibited a moderately more severe dwarf phenotype than the single mutant parents (Figure 5A). In sharp contrast, csn3-3 axr1-12 doubles exhibited a seedling-lethal phenotype (Figure 5B), indicating that axr1-12 and csn3-3 interact synergistically. Double mutant seedlings exhibited cotyledon sharp contrast, additive, as double mutants exhibited a moderately more severe

Discussion

In plants, animals, and fungi, the CSN has a well-established role as a cullin deneddylase that regulates CRL ubiquitin-ligase activity [44]. Prior reports in Arabidopsis have demonstrated that reduced deneddylase activity in various csn mutants affects SCF^TIR1/AFB^ ubiquitin-ligase activity and consequently results in impaired auxin signaling [28,35,40]. In this study, we identified the csn3-3 missense mutation as an enhancer of the auxin resistant root growth phenotype conferred by tir1-1. Consistent with diminished auxin signaling, csn3-3 also enhanced the tir1-1 lateral root development defect and conferred diminished auxin mediated expression of the DR5:GUS and B3:GUS reporters. While these findings were hardly surprising given the aforementioned studies in Arabidopsis Csn subunit mutants, we quite unexpectedly found
that neither the deneddylation activity of the CSN nor the SCF\(^{TIR1/AFB}\) mediated regulation of Aux/IAA protein stability was affected by the \(csn3-3\) mutation. These findings clearly distinguish \(csn3-3\) from all previously characterized \(csn\) mutants.

It could be argued that \(csn3-3\) is simply a weak mutation that does not confer sufficient defects to be detected in assays examining neddylation status of cullins or the SCF\(^{TIR1/AFB}\) activity. However, our comparison of \(csn3-3\) phenotypes to those of a second weak CSN subunit mutant, \(csn1-10\), strongly argues against this possibility. \(csn3-3\) and \(csn1-10\) confer virtually identical dose-response curves in assays examining auxin-mediated inhibition of root growth, with both mutants exhibiting 50% growth inhibition at \(\sim 50\) nM 2,4-D. Likewise, both mutants enhance the \(tir1-1\) auxin resistant root growth and reduced lateral root development phenotypes to a similar extent. Furthermore, both \(csn3-3\) and \(csn1-10\) confer comparable reductions in auxin mediated expression of the \(B:3\) and \(DR5:GUS\) reporters. Together, these findings indicate that \(csn3-3\) and \(csn1-10\) impair auxin signaling to a similar extent. However, whereas cullin deneddylation and Aux/IAA degradation were unaffected in \(csn3-3\) seedlings, both of these molecular defects were clearly apparent in \(csn1-10\) mutants. Prior studies have clearly demonstrated that the CSN3 subunit is required for CSN deneddylase activity [35], which we have confirmed with the \(csn3-2\) null allele. The \(csn3-3\) missense mutation however, confers a reduction in auxin response without affecting cullin deneddylation, suggesting that CSN3 plays a second role in auxin signaling in addition to its role in regulating the SCF\(^{TIR1/AFB}\) ubiquitin ligase (Figure 7).

The dramatically different double mutant phenotypes exhibited when the \(csn1-10\) and \(csn3-3\) mutations were combined with \(axr6-3\), \(axr1-12\), or \(eta2-1\) also indicate that \(csn1-10\) and \(csn3-3\) affect distinct aspects of auxin signaling. While \(csn3-3\) confers seedling lethality when combined with \(axr6-3\) or \(axr1-12\), the \(csn1-10\) mutation does not. In contrast, \(csn1-10\), but not \(csn3-3\), confers a seedling arrest phenotype in the \(eta2-1\) background. Given that SCF\(^{TIR1/AFB}\) activity appears unaffected in \(csn3-3\) plants, it seems unlikely that the lethality of \(csn3-3\) \(axr1-12\) and \(csn3-3\) \(axr6-3\) seedlings is due to a further reduction in SCF\(^{TIR1/AFB}\) activity. Instead, we speculate that \(csn3-3\) specifically affects auxin signaling downstream or perhaps independently of SCF\(^{TIR1/AFB}\) (Figure 7), such that the combination of \(csn3-3\) with \(axr6-3\) or \(axr1-12\) causes auxin sensitivity to fall below the threshold required for early seedling development. Although it is unclear what these differential genetic interactions might mean mechanistically, it is interesting to note that both \(axr6-3\) and \(axr1-12\) result in a reduction in neddylated CUL1 [15,16,19,37]. On the other hand, the \(eta2-1\) mutation has no effect on CUL1 neddylation status [52]. Rather, the \(eta2-1\) mutation abolishes the CUL1 binding activity of CAND1, resulting in the disruption of CAND1-mediated cycling of SCF complexes [43].

Figure 5. \(csn3-3\) and \(csn1-10\) exhibit distinct double mutant interactions. (A) Adult \(csn1-10 axr1-12\) double mutant exhibit a slightly more severe dwarf phenotype than either of the single mutants. Size bar = 4 cm. (B) \(csn3-3\) interacts with \(axr1-12\) synergistically, resulting in the seedling lethality. Right panel shows the seedling-lethal phenotype of heterozygous \(csn3-3/CSN3\) in the \(axr1-12\) background. Size bar = 1 mm. (C–D) While \(csn1-10\) \(axr6-3\) plants are viable and complete the life cycle, \(csn3-3\) interacts with \(axr6-3\) synergistically, with double mutants exhibiting developmental arrest at the seedling stage. Size bars = 4 cm (C) and 1 mm (D), respectively. (E–F) \(csn1-10\) exhibits a stronger interaction with \(eta2-1\) than does \(csn3-3\). As previously reported [43], \(csn1-10 eta2-1\) seedlings fail to develop past the early seedling stage. Size bars = 1 mm (E) and 4 cm (F), respectively.

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Consistent with the hypothesis that an SCF^{TIR1/AFB}-independent pathway may regulate auxin signaling to control gene expression, like csn3-3, the previously described ibr5 mutants of Arabidopsis also exhibit diminished auxin response without inhibiting SCF^{TIR1/AFB}-mediated degradation of Aux/IAA proteins [60]. IBR5 encodes a putative dual-specificity protein phosphatase. However, although Aux/IAA proteins are highly unstable in both csn3-3 and ibr5 mutants, ibr5 seedlings exhibited reduced steady-state levels of the AXR3NT-GUS and IAA28-myc reporter proteins. In our analysis of csn3-3 mutants, however, both of these reporter proteins were present at levels comparable to wild-type controls. Furthermore, unlike csn3-3, ibr5 does not interact with arf1 in a synergistic manner [60]. Together, these findings suggest that it is unlikely that csn3-3 and ibr5 share a common auxin signaling defect.

The fact that the csn3-3 mutation did not diminish CSN deneddylation activity, yet conferred reduced auxin response phenotypes, suggests that this mutation defines a novel function for CSN3. Therefore, a major question is whether csn3-3 defines a new role in auxin signaling for the CSN holocomplex or a distinct CSN3-containing complex. While deneddylation is the only known biochemical activity of the CSN itself, additional activities including deubiquitylating and protein kinase activities have been reported to be associated with the CSN [44,65,66]. Furthermore, in animal systems some CSN subunits have been found to be DNA associated and suggested to regulate transcription [67,68]. Also, whether or not all CSN subunits function solely within the CSN holocomplex is unclear. On one hand, null mutations in any of the eight Arabidopsis subunits confer identical seedling-lethal phenotypes [23] and transcription profiles [35], suggesting that each subunit only functions within the CSN.
However, it is possible that CSN subcomplexes or individual subunits have additional functions that are masked by the early seedling lethality of these null mutants. Consistent with this possibility, fission yeast csn1 and csn2 mutations confer DNA replication defects whereas other subunit mutations do not [25]. Similarly, while both Drosophila csn4null and csn5null mutants are embryo-lethal, these two mutants exhibit distinct developmental arrest phenotypes [63,69] and differentially affect gene expression [70].

Given that csn3-3 confers no apparent defects in cullin deneddylase, SCF^{TRI1/AFB} activity, or CSN holocomplex assembly, but does specifically abolish the ~130 kD sCSN3c complex, we hypothesize that a defective sCSN3c may be the basis of the auxin signaling defects displayed by csn3-3 mutant plants (Figure 7). Consistent with this possibility, expression of a P_{CSN2:CSN3} transgene in csn3-3 mutant plants restored both the auxin response defects (Figures 1C, 2E) and the sCSN3c complex (Figures 6C). Prior studies have reported CSN subunits in complexes smaller than the CSN holocomplex. While some support has been presented for CSN3 functioning autonomously of other CSN subunits [71,72], these smaller CSN complexes have generally been proposed to be mini-CSN complexes containing several, but not all subunits [64]. Whether these represent intermediates in CSN holocomplex assembly or functionally distinct complexes is uncertain. Interestingly, one study examining these mini-CSN complexes from animal cells by non-denaturing polyacrylamide electrophoresis detected CSN3-containing complexes that appeared to lack other CSN subunits [71]. In Arabidopsis, Rubio et al. [73] also detected, but did not discuss or characterize, small CSN3-containing complexes.

Recent analyses of CSN subunit interactions using in vitro reconstituted human CSN subunits suggest that the CSN consists of two symmetrical modules; CSN1/2/3/8 and CSN4/5/6/7 [64]. With this subunit topology, CSN3 would directly interact with CSN1/8 and possibly CSN4, which is consistent with prior two-hybrid studies [74]. However, our gel filtration analysis indicates that neither CSN1/4/8 nor CSN5 are components of the sCSN3c complex. While we cannot eliminate the possibility that CSN2/6/7 are sCSN3c components, this seems unlikely given these prior findings. Thus, together with our finding that CSN holocomplex assembly is unaffected by csn3-3, we hypothesize that sCSN3c represents a novel complex rather than a mini-CSN complex. Identification of the other components within sCSN3c may provide crucial information into what role this complex might play in auxin signaling. Furthermore, since the residue affected by the csn3-3 missense mutation is extremely highly conserved across eukaryotes, it seems likely that sCSN3c function may be similarly conserved.

Materials and Methods

Plant Materials and Growth Conditions

All Arabidopsis lines used in this study are in the Col-0 ecotype. Seeds were sterilized by 30% bleach + 0.1% Triton-X100 for 10 min and were stratified at 4°C for 1-4 days. Seedlings were grown under sterile conditions on vertically oriented ATS nutrient medium [61] under long-day conditions. Adult plants were grown in soil under long-day conditions at 20°C. The TIR1-1, csn1-10, csn3-2 (SALK_106465), eta2-1, axr6-3, and axr1-12 mutants have been described previously [35,43,75]. The B3:GUS [37], DR5:GUS [5], HS:AXR3NT-GUS [4], and P_{AA229-IAA29-myc} [60] transgenes were introduced into the csn3-3 and csn1-10 backgrounds by crossing. For construction of double mutant and reporter lines, the csn3-3 mutation was genotyped by using a CAPS marker for PCR products generated with primers Ex7F (5’-CAACGAGGGAAGCTTGGTGG-3’) and Ex8R (5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCTCTTGCAT-3’). When digested with Sp I, the 289 bp CSN3 PCR product is cleaved into 163 and 126 bp fragments, whereas the csn3-3 mutation abolishes the Sp I recognition site. The eta2-1, axr1-12, and axr6-3 mutations were confirmed by sequencing PCR products spanning the mutation sites.

For root growth assays, 5-d.o. seedlings were transferred to ATS medium supplemented with various concentrations of 2,4-D, and root growth was measured after an additional 4 days. Percent inhibition was calculated by dividing the average growth on auxin media by the average growth on ATS control media and subtracting this ratio from 100%. For measuring IAA-induced root growth inhibition, 6-d.o. seedlings were transferred to freshly made IAA plates and were grown under long-day illumination through yellow long-pass filters to slow indolic compound breakdown. Protein extractions for gel filtration and western experiments were made from 7-10-d.o. seedlings grown in liquid ATS medium on a shaker at 20°C.

Complementation

The CSN3 genomic DNA construct was composed of the CSN3 coding region together with a 1.3 kb fragment upstream of the start codon plus a 600bp fragment downstream of the stop codon. The whole sequence was amplified from BAC clone F18022 (5’-GGGGGACACAGTTGGATACAAAAAACAGCGCTGCTTGGATGGCCCATGTTG-3’ and 5’-GGGGACACATTGTGTAACAGGAACGCTTGATGCTGTTG-3’). The generated PCR product was then digested with Nde I and Bam H1 and ligated into pBA3:GUS [76]. The genomic DNA construct was then transformed into 1011 L4 transcomplementation strain GV3101 according to standard procedures [77]. Two independent T3 homozygous lines (L2 and L4) were used for the root growth and GUS assays to assess complementation.
supplemented with 1 M medium for 15 min. Seedlings were then moved into medium
from 7- to 10-d.o. seedlings (or seedling roots for IAA28-myc
Covance and used as recommended. For IAA28-myc, cullins and
a
Sciences. Monoclonal
antibodies were purchased from BIOMOL Int'l, L.P./Enzo Life

GUS Histochemical Staining
IAA28-myc degradation assay
Experiments were done as described previously [60] with modifications. 7-d.o. light-grown seedlings of Col, csn1-10 and
csn3-3 carrying the IAA28-myc construct were removed from ATS plates and floated in liquid ATS supplemented with 0.5 mM IAA. At the indicated time points, roots were excised and homogenized for protein extraction.

Antibodies and Immunoblot Analysis
The CUL1 antibody has been described previously [9] and the CUL4 antibody was obtained from Dr. Xing Wang Deng (Yale U.). The quantification of CUL1-NEDD8:CUL1 ratios was performed in Image J. Antibodies against the CSN1, CSN3, and CSN8 subunits were raised by immunizing New Zealand white rabbits (Cocalico Biological, Reamstown, PA) with recombinant 6xHis-CSNx protein purified from E. coli using standard protocols [9]. Crude sera were subsequently affinity purified against nitrocellulose-bound antigens [78]. The CSN4, CSN5 and Rpt5 antibodies were purchased from BIOMOL Int’l, L.P./Enzo Life Sciences. Monoclonal α-myc 9E10 antibody was purchased from Covance and used as recommended. For IAA28-myc, cullins and CSN subunit immunoblotting, protein extracts were prepared from 7- to 10-d.o. seedlings (or seedling roots for IAA28-myc assay) in protein extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1X Protease Inhibitor Cocktail Kit (Thermo)). 50 μg of protein were loaded in each lane and separated by 10% SDS-PAGE (cullins and CSN subunits) or 4–12% NuPAGE® Bis-Tris Gel (Invitrogen). For gel filtration, proteins of each fraction were concentrated with StrataClean™ Resin (Stratagene) and separated on 4–12% NuPAGE® Bis-Tris Gel (Invitrogen), blotted, and used for western detection.

Gel Filtration Chromatography
Gels were conducted as described previously [43]. In brief, 7-d.o. seedlings grown in liquid ATS medium were homogenized in extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 10 mM MgCl2, 0.5 mM NaN3, 1 mM DTT, 1 mM PMSF, and 1X Protease Inhibitor Cocktail Kit (Thermo)). Homogenates were centrifuged twice for 10 min at 4°C to remove debris. Supernatants were then filtered though a 0.45 μm HT Tuffryn® Membrane ( Pall). 600 μg of total protein was fractionated through a Superdex 200 10/300 GL column (Amersham/GE Healthcare). After loading the sample, proteins were eluted in filtered and degassed extraction buffer at a flow rate of 0.2 mL/min. 0.5 mL fractions were collected after the 6 mL void volume was reached. All procedures were carried out at 4°C.

Supporting Information
Figure S1 IAA induced BA3:GUS expression is reduced by the csn1-10 and csn3-3 mutations to a similar extent. 6-d.o. transgenic Col, csn1-10 and csn3-3 seedlings carrying the BA3:GUS reporter were treated with 1 μM IAA for 3 h before histochemical staining for β-glucuronidase activity. (TIF)

Figure S2 α-myc western detection of the IAA28-myc fusion protein. The PIAA28:IAA28-myc reporter was introduced into the csn1-10 and csn3-3 backgrounds by crossing. Protein extracts were made from 7-d.o. seedling roots treated with IAA for the indicated time. RPT5 was used as a loading control. (TIF)

Figure S3 sCSN3c is not a breakdown product of the CSN holocomplex during the in vitro fractionation. Fractions (#5–9) of the first gel filtration run using Col seedling protein extracts were isolated and injected into the column for a second round of gel filtration. CSN3 western detection was conducted using fractions from the 2nd gel filtration. No CSN3 was detected in the sCSN3c fractions. (TIF)

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Author Contributions
Conceived and designed the experiments: HH MQ WMG. Performed the experiments: HH MQ WMG. Analyzed the data: HH MQ WMG. Wrote the paper: HH WMG.

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