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Addenda

Autophagy and Neurodegeneration

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Addendum to:

Loss of Autophagy in the Central Nervous System Causes Neurodegeneration in Mice

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ABSTRACT

The proteasome and lysosome are sophisticated apparatuses capable of shredding unnecessary proteins in eukaryotic cells. The proteasome and its partner ubiquitin (which functions as a destination signal for proteolysis) play crucial roles in selective breakdown of not only short-lived regulatory proteins but also abnormal proteins that need to be rapidly eliminated from the cells. It is generally accepted that deficits of the proteasome-ubiquitin system are associated with various neurodegenerative diseases, since ubiquitin-positive inclusions frequently appear in neurons of patients and mice models of neurodegenerative diseases. However, investigators working in the field of neuronal diseases have focused their attention in recent years on autophagy (Greek for "the eating of oneself") following the recent discovery that ablation of autophagy leads to accumulation of ubiquitin-positive inclusions, which are the pathological hallmark of neurodegenerative diseases. Here we discuss the consequences of autophagy deficiency in neurons.

Autophagy is the bulk protein degradation pathway associated with marked membrane dynamics. In response to various stimuli, such as starvation and glucagon, an isolation membrane appears in the cytosol, where it gradually elongates to sequestrate cytoplasmic constituents. Subsequently, the edges of the membrane fuse together to form vesicles, which represent double-membrane structures termed autophagosomes. Autophagosomes rapidly fuse with lysosomes, and their contents together with the inner membrane are degraded by a variety of lysosomal digestive hydrolases.[1] The most important role of autophagy is the supply of amino acids under nutrient-poor environmental conditions in yeast[2] and during starvation in neonate mice (see Fig. 1).[3,4] In addition, autophagy plays important roles in cellular remodeling during differentiation and development of multicellular organisms, such as fly, worm and slime mold.[5] It also is responsible for cellular defense against invading bacteria.[6,7] There are growing lines of evidence for the importance of autophagy that occurs constitutively even in nutrient-rich conditions and indications that it plays a key role in global turnover of cellular components including organelles.

We recently generated the first liver-specific autophagy-deficient mouse, and found that the mutant mice exhibited progressive hepatomegaly together with an unusual accumulation of aberrant organelles and heavily-ubiquitinated proteins, resulting in severe liver dysfunction.[4] These results suggest that autophagy is involved in homeostasis of hepatocytes by removal of waste material deleterious to the survival of these cells. Subsequently, we investigated the pathophysiological roles of autophagy in the brain. For this purpose, we generated neuron-specific autophagy-deficient mice ($Atg7^{F/F}$:Nes mice) by crossing *Atg7*-conditional knockout mice ($Atg7^{F/F}$) with transgenic mice expressing the Cre recombinase under the control of the neuron-specific Nestin (Nes) promoter, Nes-*Cre*. We found that mice lacking Atg7 (an E1-like enzyme for Atg-conjugation systems) in the central nervous system exhibited various behavioral deficits, such as abnormal limb-clasping reflexes and reduction of coordinated movement, and died within 28 weeks after birth. Histological analysis showed *Atg7*-deficiency caused neuronal loss in the cerebral and cerebellar cortices. Intriguingly, polyubiquitinated proteins accumulated abundantly in autophagy-deficient neurons and appeared as inclusion bodies whose size and number increased with aging, whereas the function of the proteasome, whose impairment is generally known to cause an abnormality of ubiquitin-mediated proteolysis, was not affected by the autophagy defect.[8] Other investigators also reported that almost all these phenotypes, if not all, were observed in neural-specific mice deficient in *Atg5*, another autophagy-essential gene.[9] Thus, many critical symptoms in neural-specific autophagy-deficient mice are similar to those of patients with neurodegenerative disorders.

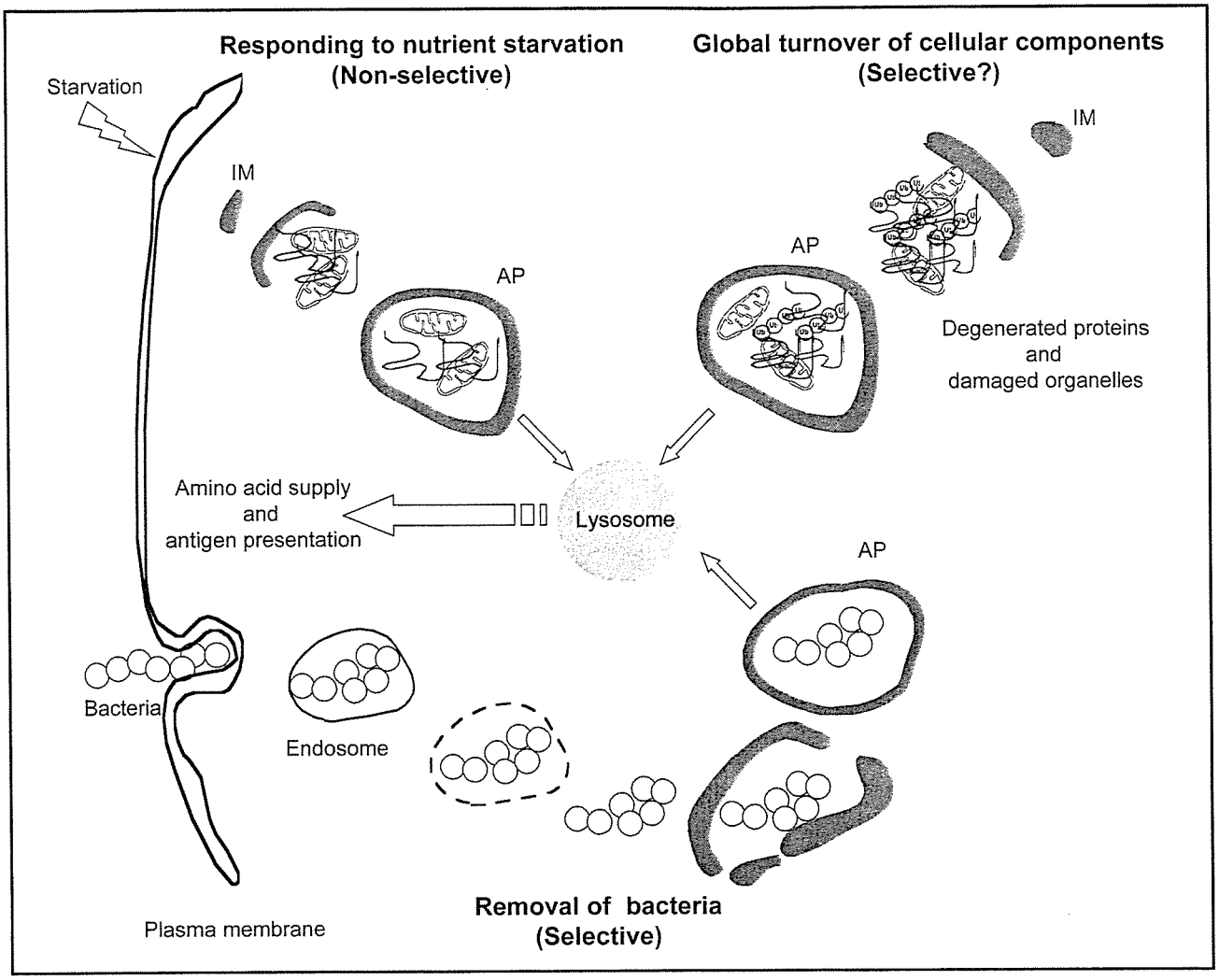

Figure 1. Schematic representation of the physiological functions of autophagy. Autophagy is induced in response to emergency states such as nutrient-starvation and bacterial infection, and results in the degradation of cytoplasmic components for amino acid supply (non-selective process) and the killing of bacteria that invade the cytoplasm (selective process). Autophagy occurs constitutively even under a nutrient-rich state and contributes to global turnover of cellular components. It is an essential cell process that maintains homeostasis in quiescent cells (e.g., hepatocytes and neurons). Future studies should focus on whether autophagy can degrade ubiquitinated proteins selectively or engulfs them simultaneously with nonubiquitinated proteins. IM, isolation membrane; AP, autophagosome; Ub, ubiquitin.

SURVIVAL OF NEURAL-SPECIFIC AUTOPHAGY-DEFICIENT MICE DURING NEONATAL STARVATION

Autophagy-deficient mice such as *Atg5*- and *Atg7*-knockout mice die within 1 day after birth.[3,4] The cause of death is considered to be mainly due to low production of amino acids via autophagy. In addition, the mutant mice show defective suckling, suggestive of abnormality of the central nervous system. However, $Atg7^{F/F}$:Nes mice (i.e., those with neuron-specific defects) suckled and avoided death during the neonatal starvation period that was experienced by autophagy-null mice. It is plausible that such an outcome is due to deletion of the Atg7 protein only late embryogenesis in $Atg7^{F/F}$:Nes mice. In fact, the Atg7 protein remained until embryonic day 15.5 (E15.5), and the activity, as examined by conversion of LC3 (a covalent modifier activated by Atg7) to the lipidation form, remained unchanged until E15.5. Alternatively, autophagy in other tissues such as the liver, cardiac muscle and lung, which is known to be aggressively active during the neonate starvation period,[3] might be important for suckling. Further analysis using tissue specific *Atg*-knockout mice is needed to clarify the cause of defective suckling in autophagy-deficient mice.

INTERPLAY BETWEEN NEURONAL DEGENERATION AND UBIQUITIN-POSITIVE AGGREGATION IN AUTOPHAGY-DEFICIENT MICE

Histological analyses of the brains of $Atg7^{F/F}$:Nes mice revealed loss of specific neurons, such as pyramidal neurons in the cerebral cortex and hippocampus, and Purkinje cells in the cerebellum. Unexpectedly, immunohistological analysis using anti-ubiquitin antibody identified ubiquitin-positive proteinaceous aggregates throughout the brain, although the staining intensity varied from one region to another. Few ubiquitin-positive inclusions were recognized in brain regions with evident neuronal loss, whereas many ubiquitin-inclusions were noted in areas with barely any neuronal loss such as the hypothalamus. Although we could not determine whether neuronal death is due to accumulation and subsequent inclusion-formation of ubiquitinated proteins, neurons with large

inclusions survived. Conversely, large pyramidal neurons and Purkinje cells seem vulnerable to ubiquitinated proteins and die before the formation of large inclusions. Whether the formation of inclusion bodies in neurons is protective or toxic is under debate and further studies are needed.

DIFFERENTIAL ROLES OF AUTOPHAGY IN GROWING AND QUIESCENT CELLS

While accumulation of ubiquitinated proteins and cell death were noted in autophagy-deficient hepatocytes[4] and neurons,[8] such phenotypes were not observed in growing cells such as mouse embryonic fibroblasts (MEFs) and astroglial cells, irrespective of autophagy deficiency (data not shown). Thus, it seems that autophagy is not required in rapidly dividing cells, at least with respect to their multiplication. These results might also reflect the difference in autophagic activity among cell types. Indeed, degradation of long-lived proteins through autophagy constitutes more than 30% of starvation-induced protein degradation in hepatocytes.[4] In contrast, autophagy-dependent proteolysis in proliferating MEFs is low, irrespective of the presence or absence of nutrient stress (data not shown). It is possible that the cell division cycle results in dilution of ubiquitinated proteins in autophagy-deficient MEFs, preventing their accumulation. Alternatively, other degradation pathways, such as chaperone-mediated autophagy, could contribute to degradation of long-lived proteins in growing MEFs. Considered together, it is clear that macroautophagy (the massive autophagy pathway discussed here) plays important roles in proteolysis in quiescent cells.

SELECTIVITY OF UBIQUITINATED PROTEINS BY AUTOPHAGY

Autophagy-deficiency is considered to result in delays of global turnover of cytoplasmic components, resulting in accumulation of misfolded and/or unfolded proteins followed by formation of inclusion bodies. However, a recent report showed that p62/SQSTM1 harboring a ubiquitin binding domain (UBA), interacted with LC3 and was degraded via autophagy,[10] suggesting that ubiquitinated proteins are selectively sequestered into autophagosomes. In an independent study, we observed gross accumulation of p62/SQSTM1 and p62/SQSTM1-positive inclusions in autophagy-deficient neurons and hepatocytes (manuscript in preparation). These results imply the existence of selective autophagy for ubiquitinated proteins and indicate that p62 (which retains its shuttling ability of ubiquitinated proteins) is a specific substrate for autophagy. In either case (nonselective or selective degradation of ubiquitinated proteins by autophagy), our results indicate that autophagy operates not only as a supplier of amino acids under nutrient-poor conditions but also as a house cleaner of damaged proteins under nutrient-rich conditions (Fig. 1).

FURTHER ISSUES

Accumulating evidence indicates that the formation of ubiquitin-positive inclusion bodies (representing the pathological hallmark of various neurodegenerative diseases) is the direct consequence of a dysfunctional proteasome degrading machinery.[11] On the other hand, the accumulation of autophagosomes owing to impairment of fusion with lysosomes is observed in various disorders, including Alzheimer's disease,[12-14] and it has been proposed that autophagy functions to degrade toxic proteins in familial neurodegenerative diseases.[15-17] However, it is still not clear whether the two proteolytic systems (autophagy and proteasomes) work independently or cooperatively to maintain protein homeostasis in the cell. Furthermore, we do not know whether both autophagy and the proteasome degrade a similar set of normal and/or misfolded proteins. While selective autophagy for excess organelles and bacteria invading the cytosol is already reported,[6,18] it is still not clear whether autophagy can engulf ubiquitinated proteins in a selective fashion in order to deliver them to the lysosome. If ubiquitinated proteins are selectively enwrapped by autophagy, p62 may be a possible candidate involved in this mechanism, based on its ability to interact with both LC3 and the ubiquitin-chain. Resolving these unanswered questions should help in the design of new strategies for treatment of neurodegenerative diseases.

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Direct interactions between NEDD8 and ubiquitin E2 conjugating enzymes upregulate cullin-based E3 ligase activity

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Although cullin-1 neddylation is crucial for the activation of SCF ubiquitin E3 ligases, the underlying mechanisms for NEDD8-mediated activation of SCF remain unclear. Here we demonstrate by NMR and mutational studies that NEDD8 binds the ubiquitin E2 (UBC4), but not NEDD8 E2 (UBC12). Our data imply that NEDD8 forms an active platform on the SCF complex for selective recruitment of ubiquitin-charged E2s in collaboration with RBX1, and thereby upregulates the E3 activity.

The SKP1/cullin-1/F-box protein (SCF) complex is a multisubunit ubiquitin E3 ligase that promotes ubiquitination of many important regulatory proteins of diverse cellular pathways (see recent review[1]). Cullin-1, together with the RING-finger protein RBX1 (also called ROC1), forms the catalytic core of the SCF complex. The E3 activity of the SCF complex is modulated by the covalent attachment of the ubiquitin-like molecule NEDD8 to cullin-1 (refs. 2–5). This 'neddylation' pathway is considered essential for cell viability in various organisms, though not in budding yeast[3]. In the neddylation process, the APP-BP1–UBA3 heterodimer (NEDD8 E1) and UBC12 (NEDD8 E2) catalyze the formation of an isopeptide bond between the C-terminal glycine residue of NEDD8 and a lysine residue in the cullin homology domain, whereas the COP9 signalosome catalyzes deneddylation[6]. *In vitro* experiments indicate that cullin-1 neddylation upregulates the E3 activity of the SCF complex and thereby enhances protein ubiquitination[2–5]. Furthermore, it has been shown that this modification is important for the recruitment of E2 to the SCF complex[7,8]. However, the underlying mechanisms of the activation of the SCF complex, through the enhancement of E2 recruitment upon neddylation of cullin-1, remain to be understood. We examined the possible interaction of human NEDD8 with human UBC4, which is an E2 enzyme that catalyzes the formation of polyubiquitin chains upon neddylation of cullin-1 (ref. 7).

^{1}H-^{15}N HSQC spectral analyses of isotopically labeled NEDD8 in the presence and absence of unlabeled UBC4 showed chemical shift perturbations of amide resonances for Thr7, Leu8, Ile44, Ser46, Gly47, Lys48, Gln49, Met50, Val70, Leu71 and Leu73, indicating that NEDD8 interacts with UBC4 through its Ile44 hydrophobic patch (**Fig. 1a**, **Supplementary Fig. 1** and **Supplementary Methods** online). In similar titration experiments using UBC12 instead of UBC4, no

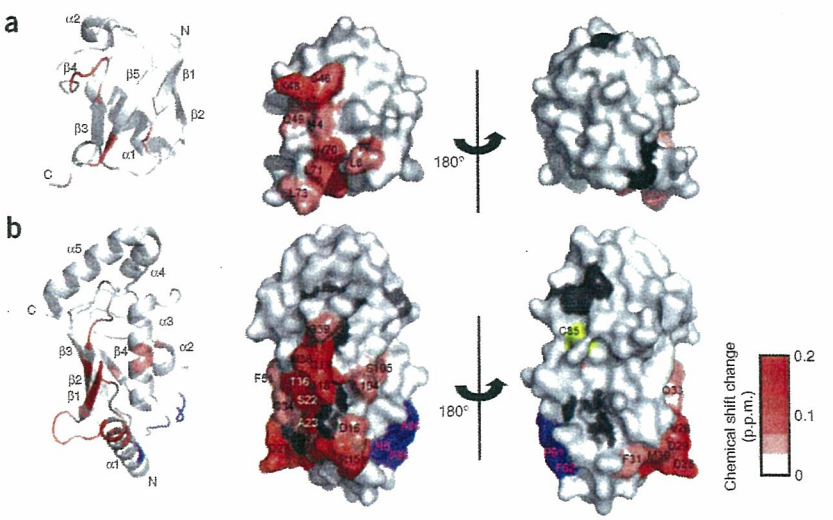

Figure 1 Identification of the binding sites on NEDD8 and UBC4. (**a**,**b**) Mapping of the perturbed residues of NEDD8 (**a**) and UBC4 (**b**) upon binding to each other. Residues are highlighted in red on the crystal structures of NEDD8 (PDB 1NDD) and UBC4 (PDB 2ESK). Red gradient indicates the strength of the perturbation. Blue, residues involved in the interaction with the RING-finger domain in the crystal structure of c-Cbl (PDB 1FBV)[10]; gray, prolines; yellow, catalytic cysteine (C85). This figure was prepared with PyMOL (http://pymol.sourceforge.net).

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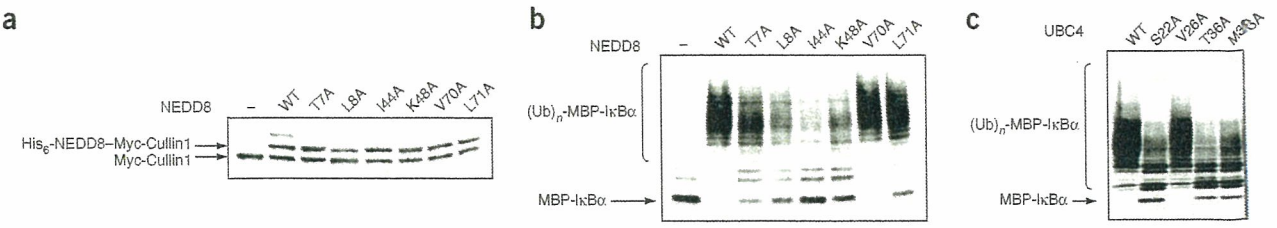

Figure 2 Mutations affecting the NEDD8 and UBC4 interaction compromise upregulation of the E3 activity of $SCF^{\beta\text{-}TrCP1}$ in the ubiquitination of IκBα. (**a**) Effects of NEDD8 mutations on *in vitro* neddylation of cullin-1. (**b**,**c**) Effects of mutations of NEDD8 (**b**) and UBC4 (**c**) on ubiquitination of phosphorylated IκBα. Ubiquitination (symbolized by $(UB)_n$, where *n* represents number of ubiquitins) of a maltose-binding protein (MBP)-IκBα fusion construct by $SCF^{\beta\text{-}TrCP1}$ was examined in the presence or absence of NEDD8 or UBC4 mutants. MBP-IκBα prephosphorylated by IKKβ was used as a substrate.

specific interaction was detected between NEDD8 and UBC12 (**Supplementary Fig. 1**). Next, we identified the NEDD8-binding surface on UBC4. Binding of NEDD8 induced chemical shift perturbations of NMR signals for α1 helix (Leu10, Asp12, Ala14 and Arg15), α1-β1 loop (Asp16, Ala19 and Gln20), β1 strand (Cys20–Ala23), β1-β2 loop (Val26–Phe31) and β2 strand (Trp33–Thr36, Met38 and Gly39) of UBC4 (**Fig. 1b** and **Supplementary Fig. 2** online).

To address the functional relevance of the interaction observed between NEDD8 and UBC4, we mutated the amino acid residues located in the UBC4-binding site of NEDD8 (T7A, L8A, I44A, K48A, V70A and L71A). Whereas these NEDD8 mutants as well as the wild-type NEDD8 were conjugated to cullin-1 to similar extents by *in vitro* neddylation reactions (**Fig. 2a**), an *in vitro* ubiquitination assay revealed differential modulation of their abilities to upregulate the E3 activity of $SCF^{\beta\text{-}TrCP1}$ (substrate-recognition subunit indicated by superscript text) in ubiquitination of IκBα. Notably, the I44A mutant completely lacked the ability to activate this ligase (**Fig. 2b**). An NMR titration experiment confirmed that amino acid substitution of Ile44 with alanine in NEDD8 resulted in loss of its affinity for UBC4 (**Supplementary Fig. 2**). Reciprocally, certain UBC4 mutants with a single amino acid substitution in the NEDD8-binding site (typified by S22A) could not interact with NEDD8 (**Supplementary Fig. 1**) or promote the E3 activity of the neddylated $SCF^{\beta\text{-}TrCP1}$ for ubiquitination of IκBα (**Fig. 2c**), even though these UBC4 mutants had E2 activities comparable to the wild-type UBC4 in *in vitro* ubiquitination reactions with the RMA1 (RING-type) and RSP5 (HECT-type) E3 ligases (**Supplementary Fig. 3** online).

A previous X-ray crystallographic study of SCF^{Skp2} has shown that the neddylation site is in close spatial proximity to the RING-finger domain of RBX1 (ref. 9). The present NMR data suggest that the NEDD8-binding site is distinct from but adjoins the putative RING-binding site on UBC4 (**Fig. 1b**)[10]. On the basis of these data, we propose that NEDD8 provides a hydrophobic surface area for its interaction with the E2 surface area remote from Cys85, the catalytic cysteine, and thereby enhances recruitment of the ubiquitin-charged E2 in collaboration with RBX1. Furthermore, we found that the I44A mutant of NEDD8 did not facilitate the reaction whereby VBC-CUL2 ligase (together with UBCH5c) ubiquitinates its native substrate, indicating that NEDD8-ubiquitin E2 interactions contribute to upregulation of not only cullin-1–based but also cullin-2–based E3 ligase activities (**Supplementary Fig. 4** online).

RBX1 could induce neddylation as well as ubiquitination by allowing alternative binding of different E2s[11]. Our data demonstrate that NEDD8 interacts with the ubiquitin E2, UBC4, but not with the NEDD8 E2, UBC12. The NEDD8-binding sequence in UBC4 identified on the basis of the present NMR data is poorly conserved in UBC12, which explains why UBC12 did not bind NEDD8 (**Supplementary Fig. 5** online). Obviously, ubiquitin and NEDD8 have distinct roles in the proteasome-dependent protein degradation system, despite the fact that these modifiers share 57% amino acid sequence identity. The present study suggests that once NEDD8 is attached onto the cullin subunit, it forms the active platform for selective recruitment of ubiquitin-charged E2 in collaboration with RBX1, excluding the UBC12–NEDD8 complex (**Supplementary Fig. 6** online), and thereby induces substrate ubiquitination. Indeed, cullin-1 neddylation enhances polyubiquitin chain elongation (**Fig. 2b**,**c**) as well as initial ubiquitin conjugation (**Supplementary Fig. 7** online). The E2-specific interaction of NEDD8 described here could be the mechanism that prevents poly-NEDD8 formation on cullins and concomitantly promotes polyubiquitination of substrates.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

K.K. contributed to overall guidance of the project. E.S., Y.Y. and K.K. contributed to the design and execution of the NMR study. E.S., K.I., T.C. and K.T. contributed to the design of the mutational studies. E.S., Y.M., Y.S. and N.M. contributed to the execution of the mutational studies. E.S. and K.K. wrote the manuscript. K.I., T.C. and K.T. commented on the manuscript. All authors edited and approved the final version of the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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