

Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast

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A ubiquitin-like modifier, NEDD8, is covalently attached to cullin-family proteins, but its physiological role is poorly understood. Here we report that the NEDD8-modifying pathway is essential for cell viability and function of Pcu1 (cullin-1 orthologue) in fission yeast. Pcu1 assembled on SCF ubiquitin-ligase was completely modified by NEDD8. Pcu1^{K713R} defective for NEDD8 conjugation lost the ability to complement lethality due to *pcu1* deletion. Forced expression of Pcu1^{K713R} or depletion of NEDD8 in cells resulted in impaired cell proliferation and marked stabilization of the cyclin-dependent kinase inhibitor Rum1, which is a substrate of the SCF complex. Based on these findings, we propose that covalent modification of cullin-1 by the NEDD8 system plays an essential role in the function of SCF in fission yeast.

Keywords: cullin/NEDD8/Rub1/SCF/ubiquitin

Introduction

Ubiquitin (Ub), an 8.6 kDa highly conserved protein, is distributed widely in all eukaryotic cells and has become a landmark molecule in a post-translational protein-modifying system that plays a central role in intracellular protein breakdown (Hochstrasser, 1996; Hershko and Ciechanover, 1998; Kornitzer and Ciechanover, 2000). Ub is covalently attached to target proteins via an isopeptide linkage between the C-terminal glycine of Ub and the ϵ -NH₂ group of the lysine residue of the acceptor substrate. This protein ubiquitylation is mediated by a cascade of three enzymes, termed E1 (Ub-activating), E2 (Ub-conjugating) and E3 (Ub-ligating) enzymes, to form a poly-Ub chain, which serves as the degradation signal for

proteolytic attack by the 26S proteasome, a eukaryotic ATP-dependent protease complex (Coux *et al.*, 1996; Baumeister *et al.*, 1998; Bochtler *et al.*, 1999; Voges *et al.*, 1999).

The progress in the study of the Ub system has revealed the existence of multiple molecules that are structurally related to Ub (reviewed by Hochstrasser, 1998). Of these, the gene encoding NEDD8 (a mammalian orthologue of the budding yeast Rub1, a modifier related to Ub 1) was identified as one of the neural precursor cell-expressed developmentally down-regulated genes in mice (Kumar *et al.*, 1993). NEDD8 has the highest homology to Ub, displaying an overall identity of 57%, among an expanding family of Ub-like proteins in eukaryotes. Recently, we found that human NEDD8 was covalently linked to target proteins via the C-terminal glycine residue in a manner analogous to ubiquitylation, where the reaction is catalysed by the APP-BP1-hUba3 heterodimer complex and hUbc12 as the E1- and E2-like enzymes, respectively (Osaka *et al.*, 1998; Gong and Yeh, 1999). A similar Ub-related conjugation pathway for yeast Rub1 is known to operate in *Saccharomyces cerevisiae* (Liakopoulos *et al.*, 1998), indicating that the NEDD8/Rub1-modifying system has been widely conserved during evolution.

Yeast Cdc53 is structurally related to Cul-1, one member of human cullin (abbreviated Cul)-family proteins, and was first found to be modified by Rub1 (Lammer *et al.*, 1998). To date, it has become clear that Cdc53/Cul-1 functions as a common subunit of the growing family of Ub-protein ligases termed SCF (Skp1, Cul-1 or Cdc53, F-box protein), consisting of the core subunits Skp1, Cul-1/Cdc53 and Roc1/Rbx1/Hrt1, and substrate recognition adaptors called F-box proteins (Kamura *et al.*, 1999a; Ohta *et al.*, 1999; Seol *et al.*, 1999; Skowrya *et al.*, 1999; Tan *et al.*, 1999), which are responsible for ubiquitylation of a variety of regulatory factors involved in the cell cycle and signal transduction (reviewed by Hershko and Ciechanover, 1998; Patton *et al.*, 1998a; Deshaies, 1999; Harper and Elledge, 1999). Recently, it was reported that NEDD8 attaches to human Cul-2, which assembles with the von Hippel-Lindau tumour suppressor protein pVHL and elongin B/C, forming an SCF-like protein complex, CBC^{VHL} (Liakopoulos *et al.*, 1999; Wada *et al.*, 1999a). Quite recently, the Rbx1 subunit of SCF was found to activate Rub1 modification of cullins Cdc53 and Cul-2 (Kamura *et al.*, 1999b). We reported covalent modification of Cul-4A by NEDD8 in rabbit reticulocyte lysates (Osaka *et al.*, 1998) and subsequently found that other human cullins, Cul-1, Cul-2, Cul-3, Cul-4B and Cul-5, are targets of the NEDD8-ligating pathway (Hori *et al.*, 1999). It is notable that mutation of the Rub1-ligating system resulted in impairment of the auxin response in *Arabidopsis* (Pozo *et al.*, 1998) and cell cycle progression in budding yeast

(Lammer *et al.*, 1998), although it is not essential in the latter organisms (Lammer *et al.*, 1998; Liakopoulos *et al.*, 1998). These observations imply a fundamentally important role for the modification of Cul-family proteins by NEDD8, which perhaps functions as a new modulator of SCF Ub-protein ligases. However, the role of the NEDD8/Rub1 conjugation pathway at the physiological level remains obscure.

We report here that disruptions of the genes encoding NEDD8 and the ligating E1 and E2 enzymes had a lethal effect in *Schizosaccharomyces pombe*. Moreover, we show that NEDD8 modification plays an indispensable role in the functions of Cul-family proteins Pcu1 and Pcu4 [Cul-1 and Cul-4A or Cul-4B orthologues, respectively (Kominami *et al.*, 1998)] in fission yeast, based on the findings that Pcu1^{K713R} and Pcu4^{K680R} (Lys713 and Lys680 in Pcu1 and Pcu4, respectively, replaced by arginine), which are defective for modification by NEDD8, have completely lost their functions *in vivo*.

Results

The NEDD8 system is essential in fission yeast

Computer-assisted homology analysis revealed the existence of three genes, *ned8*⁺, *uba3*⁺ and *ubc12*⁺ encoding SpNEDD8, SpUba3 and SpUbc12, respectively. These genes are thought to be actual components of the NEDD8-ligating pathway in *S.pombe*, judging from their high overall similarities to their human homologues (Figure 1A). SpNEDD8, SpUba3 and SpUbc12 display 81, 40 and 55% identity to their human counterparts, respectively. SpUba3 and SpUbc12 have a presumptive active cysteine residue that forms a thioester linkage with NEDD8, which resembles a conserved cysteine residue in E1 and E2 of the Ub pathway.

To explore the function of the NEDD8-ligating pathway, null alleles of the *ned8*⁺, *uba3*⁺ or *ubc12*⁺ genes were created by replacing the majority of their open reading frames (ORFs) with the *ura4*⁺ marker (Figure 1B). Tetrad analyses of all these strains resulted in only two viable Ura⁻ colonies from each ascus (data not shown), indicating that *ned8*⁺, *uba3*⁺ and *ubc12*⁺ are essential for cell viability. The lethality of $\Delta ubc12$ was complemented by human Ubc12 (data not shown). Microscopic observation showed that $\Delta ned8$ spores germinated and stopped growing after two or three cycles of cell division with an elongated cell shape (Figure 1C, upper panel). $\Delta uba3$ and $\Delta ubc12$ spores germinated, divided several times into a microcolony and ceased division after forming a number of elongated cells. 4',6-Diamidino-2-phenylindole (DAPI) staining revealed that most of the elongated cells in $\Delta ned8$, $\Delta uba3$ or $\Delta ubc12$ strains had single nuclei and decondensed chromosomes (Figure 1C, lower panel). These results strongly indicate that the NEDD8-modifying system in fission yeast is essential for cell cycle progression.

Covalent attachment of NEDD8 to Pcu1 and Pcu4

It is interesting to determine why disruption of the NEDD8 pathway in fission yeast is lethal. Presumably, modification of target protein(s) by NEDD8 (abbreviated NEDD8-ylation) may play an indispensable role in maintaining cell viability. Recently, we found that NEDD8 is covalently attached to all six known members

of a family of human cullins *in vitro* (Osaka *et al.*, 1998; Hori *et al.*, 1999), suggesting that NEDD8-ylation of cullins plays a universal role in cells. Here, we first investigated whether or not *S.pombe* cullins are modified by NEDD8. For this purpose, Myc-NEDD8 and haemagglutinin (HA)-Pcu1 under the control of the thiamine-repressible promoter (*nmt1* promoter) were co-expressed in cells. As shown in Figure 2B, when HA-Pcu1 was immunoprecipitated and used for western blotting, two Pcu1 bands were observed by anti-HA antibody staining, whereas only the upper band was stained with anti-Myc antibody, indicating that the HA-Pcu1 with a slow electrophoretic mobility is actually modified by Myc-NEDD8 *in vivo*.

To ascertain the role of NEDD8-ylation in cullins, we next determined the site undergoing NEDD8-ylation in Pcu1 and Pcu4, which are *S.pombe* homologues of a human Cul-1 and Cul-4A or Cul-4B, respectively (Kominami *et al.*, 1998). Previously, we found that the 171 C-terminal amino acids of human Cul-4A were sufficient for ligation by NEDD8 *in vitro* (Osaka *et al.*, 1998) and, accordingly, we predicted that the NEDD8-ylation site exists in the C-terminal region of cullins. Comparing amino acid sequences among various cullins, we found two lysine residues within 69–71 C-terminal amino acids that have been conserved in all species (Figure 2A). To determine which lysine residue is modified by NEDD8, we synthesized two Pcu4 variants, FLAG-Pcu4^{K680R} (Lys680 in Pcu4 was replaced by arginine) or FLAG-Pcu4^{K710R}, in rabbit reticulocyte lysate (Figure 2A). [³⁵S]FLAG-Pcu4 formed a linkage with GST-NEDD8, but not with GST-Ub or GST-NEDD8^{AG76}, in which Gly76 had been deleted (Figure 2C). However, [³⁵S]FLAG-Pcu4^{K680R} did not form a linkage with GST-NEDD8, although [³⁵S]FLAG-Pcu4^{K710R} did, like wild-type Pcu4 (Figure 2C), indicating that Lys680 in Pcu4 is a presumptive acceptor site for covalent attachment to NEDD8 *in vitro*.

Subsequently, we investigated whether or not FLAG-Pcu4^{K680R} and Myc-Pcu1^{K713R} (note that Lys713 in Pcu1 corresponds to Lys680 in Pcu4; see Figure 2A) can form a linkage with NEDD8 *in vivo*. When the epitope-tagged Myc-Pcu1 or FLAG-Pcu4 under the control of the *nmt1* promoter were overexpressed, two close bands with different electrophoretic mobilities for Myc-Pcu1 and FLAG-Pcu4 were detected by western blot analysis (Figure 2D). However, when the mutated cullins, Myc-Pcu1^{K713R} or FLAG-Pcu4^{K680R}, were overexpressed instead of their wild types, both upper bands disappeared completely, indicating that the larger forms were FLAG-Pcu4 and Myc-Pcu1 ligated by NEDD8. From these *in vitro* and *in vivo* experiments, it was concluded that the conserved lysine residue at positions 713 or 680 near the C-terminal region of Pcu1 or Pcu4, respectively, is the site required for NEDD8-ylation. Recently, consistent with these findings, Lys689 of human Cul-2 (Wada *et al.*, 1999b) and Lys720 of human Cul-1 (Read *et al.*, 2000) were reported to be the sites that undergo NEDD8-ylation.

Modification of the SCF complex by NEDD8 *in vivo*

We next attempted to clarify whether Pcu1 complexed with Pop1, an *S.pombe* F-box protein (Kominami and

A

NEDD8

Sp 1 MLIKVKTLTGKEI~~LD~~TD~~PN~~DKVSR~~IK~~ERVEEKEGIPP~~SOORLI~~YAGKOMAD~~DKNA~~ESYH
 Hs 1 MLIKVKTLTGKEI~~ID~~TE~~PT~~DKVER~~IK~~ERVEEKEGIPP~~QOORLI~~YS~~GKOM~~N~~DEK~~TAADYK

Sp 61 LE~~GG~~SVLH~~LV~~LALRGGSC
 Hs 61 IL~~GG~~SVLH~~LV~~LALRGGGLRQ
 ↑

Uba3

Sp 1 M~~P~~SSDV~~C~~KAGSHRHS~~GW~~--IQS--~~L~~KK~~P~~GP~~F~~NLDAP~~E~~NPE~~E~~TLKSAF--SS~~K~~IL~~I~~IGAGGLG
 Hs 1 M~~A~~VDGG~~C~~GDTGDWEGR~~W~~NHVKK~~F~~ERS~~G~~PFTHPDF~~E~~PST~~E~~SLQFLLDTC~~K~~V~~L~~VIGAGGLG

Sp 57 CE~~I~~L~~K~~D~~L~~ALS~~G~~FR~~D~~LS~~V~~ID~~M~~DT~~I~~DIT~~N~~L~~N~~ROFL~~F~~NES~~N~~IDE~~P~~KAN~~V~~AAS~~M~~IM~~K~~RIP~~S~~TVV
 Hs 61 CE~~L~~L~~K~~N~~L~~ALS~~G~~FR~~Q~~I~~H~~V~~I~~DM~~D~~T~~I~~DV~~S~~N~~L~~N~~R~~OFL~~R~~PK~~D~~I~~G~~R~~P~~KA~~V~~AAE~~F~~L~~N~~DR~~V~~PN~~C~~NV

Sp 117 TP~~F~~Y~~G~~K~~I~~OD~~K~~TI~~E~~F~~Y~~KE~~F~~KL~~T~~I~~C~~GL~~D~~SV~~E~~ARR~~W~~IN~~S~~T~~L~~V~~A~~-----IAKT~~G~~DL~~I~~PL~~V~~DG
 Hs 121 VP~~H~~FN~~K~~I~~O~~DF~~N~~DT~~F~~Y~~R~~Q~~H~~I~~I~~V~~C~~GL~~D~~S~~I~~I~~A~~RR~~W~~IN~~G~~M~~L~~IS~~L~~LN~~Y~~ED~~G~~V~~L~~DP~~S~~S~~I~~V~~L~~IDG

Sp 170 G~~S~~E~~G~~L~~K~~Q~~A~~R~~V~~I~~P~~T~~I~~S~~G~~Y~~E~~C~~S~~D~~M~~L~~T~~P~~K~~I~~S~~Y~~P~~I~~C~~T~~L~~A~~N~~T~~P~~R~~L~~P~~E~~H~~C~~V~~E~~W~~A~~Y~~L~~L~~E~~W~~P~~R~~V~~
 Hs 181 G~~T~~E~~G~~F~~K~~G~~N~~A~~R~~V~~I~~L~~P~~G~~M~~I~~A~~G~~I~~E~~C~~T~~L~~E~~L~~Y~~P~~D~~Q~~V~~N~~F~~D~~M~~C~~T~~I~~A~~S~~M~~P~~R~~L~~P~~E~~H~~C~~I~~E~~Y~~V~~R~~M~~L~~Q~~W~~P~~--

Sp 230 FL~~N~~AS~~V~~DS~~F~~S~~F~~K~~Q~~EV~~F~~E~~P~~LD~~G~~K~~N~~S~~N~~F~~E~~P~~D~~N~~I~~R~~H~~I~~D~~W~~L~~V~~K~~R~~S~~I~~E~~R~~A~~N~~K~~F~~Q~~I~~P~~SS~~S~~IN~~R~~FF~~V~~O
 Hs 239 -----K~~E~~Q~~P~~F~~G~~--E~~G~~V--P~~L~~D~~G~~D~~D~~P~~E~~H~~I~~Q~~W~~I~~F~~K~~S~~L~~E~~R~~A~~S~~Q~~N~~I~~R~~G~~V~~T~~Y--R--L~~T~~O

Sp 290 G~~I~~V~~K~~R~~I~~P~~A~~V~~A~~ST~~N~~A~~I~~I~~A~~AS~~C~~C~~N~~E~~A~~L~~K~~I~~L~~T~~E~~SN~~P~~F~~L~~D~~N~~Y~~M~~M~~V~~Y~~G~~E~~D~~G~~A~~Y~~T~~Y~~T~~F~~N~~L~~E~~K~~R~~S~~D~~
 Hs 283 G~~V~~V~~K~~R~~I~~P~~A~~V~~A~~ST~~N~~A~~V~~I~~A~~AV~~C~~A~~T~~E~~V~~F~~K~~I~~A~~I~~S~~A~~Y~~I~~P~~L~~N~~N~~Y~~L~~V~~F~~N~~D~~V~~D~~G~~L~~Y~~T~~Y~~T~~F~~E~~A~~R~~K~~E~~N~~

Sp 350 C~~P~~V~~C~~G~~V~~I~~S~~E~~V~~Y~~D~~I~~S~~A~~S~~S~~T~~V~~T~~L~~K~~D~~I~~L~~N~~H~~Y~~S~~K~~S~~Y~~N~~L~~Q--N~~P~~SV~~S~~T~~A~~---A~~G~~T~~P~~L~~Y~~L~~A~~S~~P~~P~~A~~L
 Hs 343 C~~P~~A~~C~~S~~Q~~L~~P~~Q--N~~L~~Q~~F~~S~~P~~S~~A~~K~~L~~Q~~E~~V~~L~~D~~Y~~L~~T~~N~~S~~A~~S~~L~~O~~M~~K~~S~~P~~A~~I~~T~~A~~T~~L~~E~~G~~K~~N~~R~~T~~L~~Y~~L~~Q~~S~~V~~T~~S~~I

Sp 405 Q~~V~~A~~T~~S~~K~~N~~L~~S~~Q~~P~~I~~L~~S~~I~~T~~S~~V~~D~~V~~-N~~L~~V~~I~~T~~D~~K~~N~~L~~S~~T~~S~~L~~S~~V~~Q~~L~~R~~E~~C~~
 Hs 401 E~~E~~R~~T~~R~~P~~N~~L~~S~~K~~T~~L~~K~~E~~L~~G~~L~~V~~D~~G~~Q~~E~~L~~A~~V~~A~~D~~V~~T~~T~~P~~Q~~T~~V~~L~~F~~K~~L~~H~~F~~T~~S~~

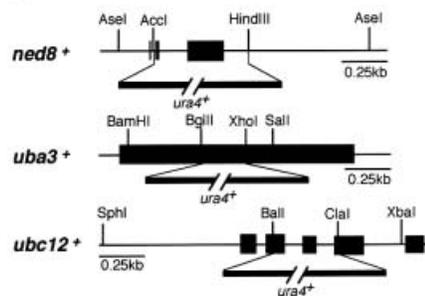
Ubc12

Sp 1 M~~R~~K~~I~~W~~E~~L~~K~~--K~~K~~E--~~A~~Q~~K~~E~~K~~N~~A~~S--G~~I~~S~~P~~A~~Q~~I~~R~~I~~O~~K~~D~~V~~T~~D~~L~~E~~I~~P~~S~~I~~M~~S~~T~~S~~W~~P~~D~~P~~I~~K~~N~~V~~L~~
 Hs 1 M~~I~~K~~L~~F~~S~~L~~K~~Q~~Q~~K~~K~~E~~E~~S~~A~~G~~G~~T~~K~~G~~S~~S~~K~~K~~A~~S~~A~~A~~Q~~L~~R~~I~~O~~K~~D~~I~~N~~E~~L~~N~~L~~P~~K~~I~~C~~D~~I~~S~~F~~S~~D~~P~~D~~D~~L~~N~~F~~

Sp 55 H~~L~~E~~I~~R~~P~~D~~E~~G~~Y~~Y~~K~~G~~G~~K~~F~~K~~F~~R~~I~~Q~~I~~D~~D~~N~~Y~~P~~H~~D~~P~~P~~K~~V~~K~~C~~L~~N~~K~~I~~Y~~H~~P~~N~~I~~D~~L~~E~~G~~N~~V~~C~~L~~N~~I~~L~~R~~Q~~D~~W~~N~~
 Hs 61 K~~L~~V~~I~~C~~P~~D~~E~~G~~F~~Y~~K~~S~~G~~K~~F~~V~~S~~F~~K~~V~~G~~Q~~G~~Y~~P~~H~~D~~P~~P~~K~~V~~K~~E~~T~~M~~V~~Y~~H~~P~~N~~I~~D~~L~~E~~G~~N~~V~~C~~L~~N~~I~~L~~R~~E~~D~~W~~K~~

Sp 115 P~~V~~L~~N~~L~~N~~S~~I~~L~~V~~G~~L~~O~~F~~L~~F~~L~~S~~P~~N~~A~~E~~D~~P~~L~~N~~K~~E~~A~~A~~A~~D~~L~~H~~K~~D~~P~~Q~~G~~F~~A~~S~~R~~V~~R~~T~~A~~M~~K~~G~~G~~L~~V~~N~~G~~I~~S~~F~~D~~N~~V~~M~~A
 Hs 121 P~~V~~L~~T~~I~~N~~S~~T~~I~~Y~~G~~L~~O~~Y~~L~~F~~L~~E~~P~~N~~P~~E~~D~~P~~L~~N~~K~~E~~A~~A~~E~~V~~L~~Q~~N~~N~~R~~R~~L~~E~~Q~~N~~V~~Q~~R~~S~~M~~R~~G~~G~~Y~~I~~G~~S~~T~~Y~~E~~R~~C~~L~~K

B



C

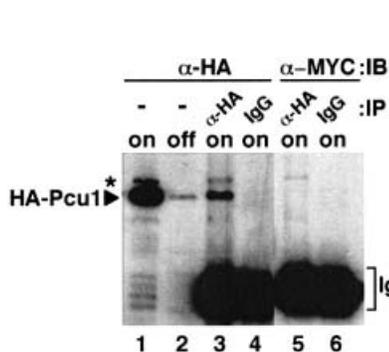


Fig. 1. Functional analysis of the NEDD8-ligating system in fission yeast. (A) Amino acid comparison of *S.pombe* (Sp) NEDD8 (DDBJ/EMBL/GenBank accession No. AJ003818), Uba3 (SWISS-PROT accession No. Q09765) and Ubc12 (DDBJ/EMBL/GenBank accession No. AL031532, SPC777.10C) with their human homologues (Hs). The identical amino acids are boxed in black. The asterisks in Uba3 and Ubc12 indicate the putative active cysteine residues. The arrow in NEDD8 indicates the processing site. (B) Disruption of *ned8*⁺, *uba3*⁺ and *ubc12*⁺ genes. The coding sequences in exons of the *ned8*⁺, *uba3*⁺ and *ubc12*⁺ genes are shown by solid boxes. For gene disruption, the indicated DNA fragments were replaced by a PCR-generated fragment containing the *ura4*⁺ marker gene. (C) After tetrad analysis of diploids heterozygous for *ned8*, *uba3* and *ubc12*, inviable Ura⁺ cells that had germinated from each of the spores were observed under a phase-contrast microscope (upper panel). Note that in SpUba3 and SpUbc12, a part of the cells derived from a microcolony is shown. To observe the defective phenotype by DAPI staining, heterozygous diploids were sporulated and treated with glusulase to kill non-sporulating cells. Spores were grown in minimal medium lacking uracil at 30°C for 24 h then stained with DAPI (lower panel). Bar, 35 μm (upper panels) and 10 μm (lower panels).

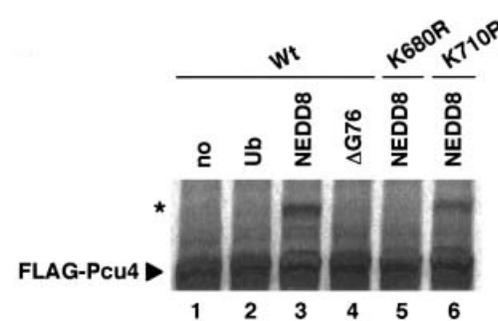
A

Hs-CUL1	KLLIQAAIVRIMKMRKVLKHKQQLLGEVLTQLSSRFKPRVPVTKKCTIDILIEKEYLERVDGEKDTYSYLA	752
Hs-CUL2	KMYLQAAIVRIMKARKVLRHNAALIQEIVISQSRARFNPSISMIKKCTEVLIDKOYIERSQASADEYSYVA	745
Hs-CUL3	KHEIEAAIVRIMKSRKKMQHNVLVAEVTQQLKARFLPSPVVIKKRIEGLIEREYLARTPEDRKVYTYVA	768
Hs-CUL4A	QYQIDAAIVRIMKMRKTLGHNLVSELYNQLK..FPVKPGDLKKRIEGLIDRDYMERDKDNPQYHYVA	659
Hs-CUL4B	QYQIDAAIVRIMKMRKTLSHNLVSELYNQLK..FPVKPADLKKRIEGLIDRDYMERDKENPNQYNYIA	717
Hs-CUL5	ILRTQEAIIQIMKMRKKISNAQLQTELVETLKNMFLPQKKMTKEQTEWLIHKKYIRDESINTFFIYMA	780
Cdc53	QIFLEACIVRIMKAKRNLPHTTLVNECIAQSHORFNAKVSVMVKRAIDS LIQKGYLQRGDDG.ESYAYLA	815
Pcu1	KLLLSAIVRIMKARRTLKHHVVLKETIDQIKSRFTPKVSDIKQCTDMLIEKEYLER..QGRDEYIYLA	767
Pcu3	KHQADACIVRIMKDRKVCHEHNQLMAEVTROLNPRFHPSPMMIKRRIEALIEREYLQRQADNGRIYIYLA	798
Pcu4	QFELQASIVRVMKQKEKMKHDDLQYVYINNVKDRGIPLVSDVKTATEKLLLEKEYLER..EDN.DIYTYVT	734
Pcu1 ^{K713R}	-----R-----	767
Pcu4 ^{K680R}	-----R-----	734
Pcu4 ^{K710R}	-----R-----	734

B



C



D

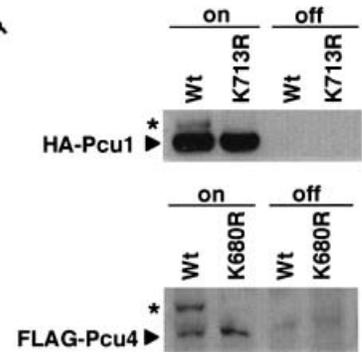


Fig. 2. Identification of a lysine residue modified by NEDD8 in Pcu1 and Pcu4 in *S.pombe*. (A) Amino acid sequences of the C-terminal region of six members of the human cullin family, Hs-Cul-1, -2, -3, -4A, -4B and -5, three members of the fission yeast cullin family, Pcu1, Pcu3 and Pcu4, and the budding yeast Cdc53 are shown. The numbers shown are the last residue numbers of the peptides in the respective proteins. Gaps (shown by dots) are inserted to maximize sequence homologies. Amino acids conserved in half or more of these cullins are boxed in black. The arrowheads indicate two lysine residues conserved in all members of the cullin family proteins. Pcu1^{K713R}, Pcu4^{K680R} and Pcu4^{K710R} indicate that lysine at positions 713, 680 and 710 in Pcu1 and Pcu4, respectively, was replaced by arginine. (B) Wild-type cells containing pREP41-*HA-pcu1*⁺ and pREP81-*Myc-ned8*⁺ were grown in minimal medium without (de-repressed, lanes 1 and 3–6) or with (repressed, lane 2) thiamine. Cell extracts were immunoprecipitated with anti-HA antibody or non-immune mouse IgG. Cell extracts (lanes 1 and 2) and immunoprecipitates (lanes 3–6) were separated by SDS-PAGE, followed by immunoblotting using anti-HA or anti-Myc antibody. An asterisk indicates HA-Pcu1 ligated by Myc-NEDD8. (C) After [³⁵S]FLAG-Pcu4 was synthesized in a reticulocyte lysate transcription/translation system in the presence or absence of unlabeled GST-Ub, GST-NEDD8 or GST-NEDD8^{ΔG76} (ΔG76) lacking the C-terminal glycine residue, samples of the resultant translational products were subjected directly to SDS-PAGE in the presence of DTT and autoradiographed (lanes 1–4). Two Pcu4 variants, FLAG-Pcu4^{K680R} and FLAG-Pcu4^{K710R}, were synthesized *in vitro* in the presence of GST-NEDD8 and analysed by the same method (lanes 5 and 6). An asterisk indicates [³⁵S]FLAG-Pcu4 ligated by GST-NEDD8. (D) Wild-type cells containing pREP41-*HA-pcu1*⁺ (Wt) or pREP41-*HA-pcu1*^{K713R} (K713R) (upper panel), or pREP81-*FLAG-pcu4*⁺ (Wt) or pREP81-*FLAG-pcu4*^{K680R} (K680R) (lower panel) were grown in minimal medium with or without thiamine. Proteins were detected by immunoblotting using anti-HA or anti-FLAG antibody. Asterisks indicate FLAG-Pcu4 or HA-Pcu1 ligated by NEDD8. Note that the *mtt1* promoter on pREP41 is ~12-fold more active than that on pREP81 and that the expressed levels of the mutants FLAG-Pcu4^{K680R} and Myc-Pcu1^{K713R}, which did not form a linkage to NEDD8, were roughly similar to those of their wild types.

Toda, 1997), is modified by NEDD8 *in vivo*. To investigate the NEDD8-ylation of Pcu1 in the SCF^{Pop1} complex under physiological conditions, we replaced the chromosomal *pcu1*⁺ and *pop1*⁺ genes by their epitope-tagged genes, *pcu1*⁺-*Myc*₁₃ and *pop1*⁺-*HA*₃, respectively. Next, SCF^{Pop1}-HA was immunoprecipitated by anti-HA antibody from the resultant extracts, and subjected to western blotting with anti-HA and anti-Myc antibodies. As previously reported (Kominami *et al.*, 1998), Pcu1-Myc₁₃ was co-immunoprecipitated with Pop1-HA₃ (Figure 3A, lane 8). Western blotting with anti-Myc antibody revealed two Pcu1 bands in the cell extracts (Figure 3A, lane 3 or 7), whereas only the upper band was immunoprecipitated by Pop1-HA₃ (Figure 3A, lane 8). These results indicate that Pcu1 assembled into the SCF^{Pop1} complex is modified preferentially by NEDD8.

To test whether NEDD8-ylation of Pcu1 is needed for assembly of the SCF^{Pop1} complex, the same immunoprecipitation with anti-HA antibody was performed for the lysates of cells containing pREP41-*Myc-pcu1*^{K713R}, in which chromosomal *pop1*⁺ had been replaced by *pop1*⁺-*HA*₃. When the immunoblotting with anti-Myc antibody was carried out for the Pop1-HA₃ immunoprecipitates, Pcu1^{K713R} was found to be incorporated into the SCF^{Pop1} complex (Figure 3B), indicating that NEDD8-ylation of Pcu1 is not essential for the formation of the SCF^{Pop1} complex. Furthermore, both Pcu1^{K713R} and wild-type Pcu1 modified with or without NEDD8 were fractionated similarly at ~400 kDa by molecular sieve chromatography, indicating that NEDD8 modification does not have an apparent effect on complex formation nor oligomerization of SCF (data not shown).

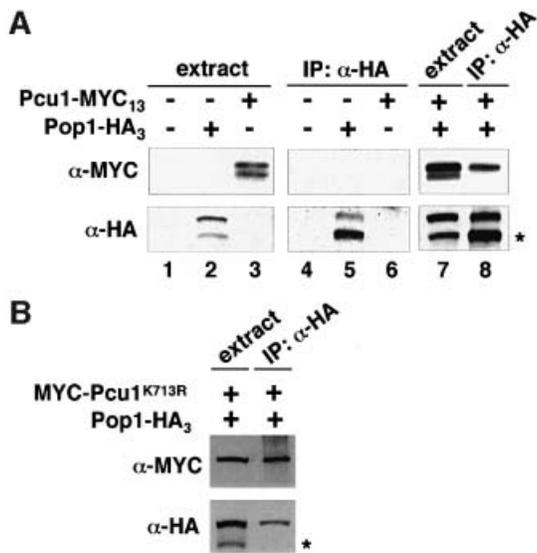


Fig. 3. Preferential modification of Pcu1 by NEDD8 in the SCF^{Pop1} complex. (A) Wild-type cells (lanes 1 and 4) or cells in which chromosomal *pcu1*⁺ and *pop1*⁺ had been replaced by the epitope-tagged genes *pop1*⁺-HA₃ (lanes 2 and 5), *pcu1*⁺-Myc₁₃ (lanes 3 and 6) or *pop1*⁺-HA₃ and *pcu1*⁺-Myc₁₃ (lanes 7 and 8) were grown in YE. Cell extracts were immunoprecipitated (IP) with anti-HA antibody. The cell extracts (lanes 1–3 and 7) and IP-proteins (lanes 4–6 and 8) were separated by SDS-PAGE followed by immunoblotting with anti-HA, anti-Myc antibody. (B) Fission yeast cells containing the integrated *pop1*⁺-HA₃ and pREP41-Myc-Pcu1^{K713R} were grown in minimal medium without thiamine for 20 h at 32°C. Cell extracts were immunoprecipitated (IP) with anti-HA antibody. The cell extracts and immunoprecipitated proteins were separated by SDS-PAGE and followed by immunoblotting with anti-HA, anti-Myc antibody. Asterisks indicate truncated Pop1, which may be produced by proteolytic digestion or synthesized from a different translation initiation site.

Impairment of SCF function by Pcu1^{K713R}

Since *S.pombe* *pcu1*⁺ is essential for cell viability (Kominami *et al.*, 1998), we examined whether or not the NEDD8-ylation-defective mutant *Myc-pcu1*^{K713R} can complement the loss of *pcu1*. As shown in Figure 4A (lower panel), the lethality of Δ *pcu1* cells was not complemented by overexpression of *Myc-pcu1*^{K713R} from the *nmt1* promoter, unlike the wild-type *Myc-pcu1*⁺, indicating that modification of Pcu1 by NEDD8 plays a pivotal role in the proliferation of *S.pombe* cells. Intriguingly, the weak expression of *Myc-pcu1*⁺, but not *Myc-pcu1*^{K713R} under repression of the *nmt1* promoter, was also able to complement the lethality of Δ *pcu1* cells (Figure 4A, upper panel). It is noteworthy that this weak expression of *Myc-pcu1*^{K713R} did not cause any defective phenotypes in wild-type cells, unlike the growth-suppressive effect caused by overexpression of *Myc-pcu1*^{K713R} (see Figure 5). Accordingly, it is unlikely that the lack of complementation by *Myc-pcu1*^{K713R} is the toxic effect due to its overexpression (Figure 4A, upper panel). Furthermore, Δ *pcu1* spores expressing Pcu1^{K713R} weakly from the repressed *nmt1* promoter were germinated and stopped growing after two or three cycles of cell division, similarly to the Δ *pcu1* spores (Figure 4B).

We next tested the effect of forced expression of the *Myc-pcu1*^{K713R} mutant in yeast cells. At 18 h after

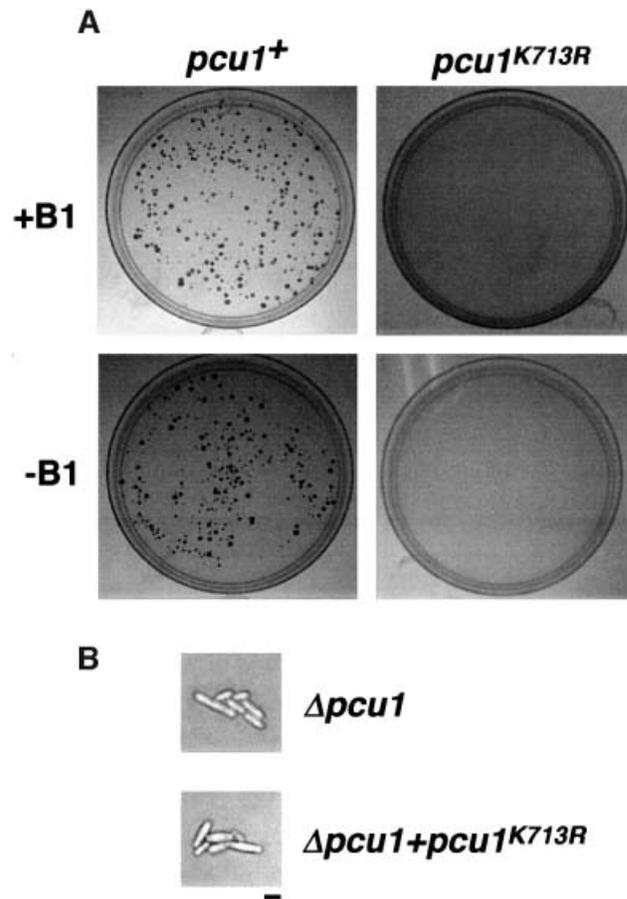


Fig. 4. *pcu1*^{K713R} loses the ability to complement the lethality of Δ *pcu1* cells. (A) Spores (5×10^3) derived from the IO100 (*pcu1::ura4*⁺/*pcu1*⁺) diploid strain containing [pREP41-Myc-*pcu1*⁺ LEU2] or [pREP41-Myc-*pcu1*^{K713R} LEU2] were germinated and grown on minimal medium plates lacking uracil and leucine in the presence (upper panel, repressed) or absence (lower panel, de-repressed) of thiamine (B1). (B) Δ *pcu1* cells and Δ *pcu1* cells expressing Myc-Pcu1^{K713R}, germinated from spores, are shown. Bar, 10 μ m.

induction of *Myc-pcu1*^{K713R}, a number of cells started to elongate (Figure 5A and B) and cell division was severely inhibited (data not shown). The most elongated cells had a 2C DNA content, which is characteristic of haploid cells in the G₂/M phase (Figure 5A), and decondensed chromosomes (Figure 5B), suggesting that cell cycle progression had been largely delayed at the G₂ phase.

This effect may be due to dysfunction of SCF mediated by lack of NEDD8-ylation of Pcu1. It is notable that Pcu1 is complexed with two related F-box/WD40-repeat proteins, Pop1 and Pop2, forming SCF^{Pop1} and/or SCF^{Pop2}, which ubiquitinyrate the CDK (cyclin-dependent kinase) inhibitor Rum1 and the S phase regulator Cdc18 (Kominami and Toda, 1997; Jallepalli *et al.*, 1998; Kominami *et al.*, 1998; Maekawa *et al.*, 1998). To ascertain the role of NEDD8-ylation of Pcu1 in SCF function, we measured the level of Rum1 (Moreno and Nurse, 1994) in the *Myc-Pcu1*^{K713R}-overexpressing cells. As shown in Figure 5C, Rum1 accumulated after induction of the *Myc-Pcu1*^{K713R}, which is consistent with the appearance of abnormality in cell morphology, although the level of Rum1 protein in exponentially growing wild-type cells was extremely low and barely detectable, as

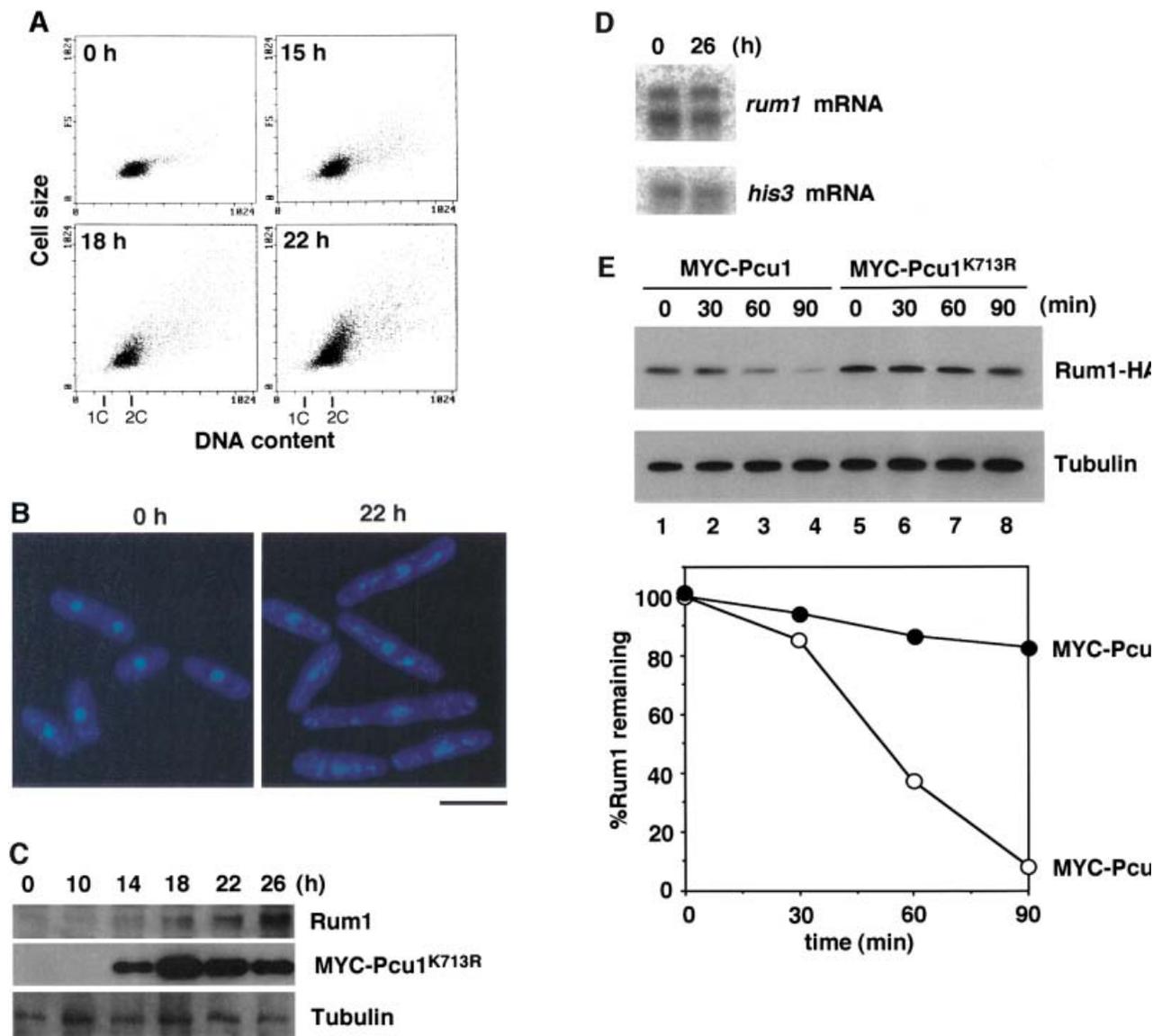


Fig. 5.

Stabilization of Rum1 in the *Pcu1*^{K713R}-overexpressed cells. Fission yeast cells (SKP401) containing pREP41-*Myc-pcu1*^{K713R} were grown in minimal medium without thiamine for the indicated time at 32°C. (A) DNA content and cell size were measured by a flow cytometer. (B) Cells were stained with DAPI. Bar, 10 µm. (C) Cell extracts were separated by SDS-PAGE, followed by immunoblotting using anti-Myc, anti-Rum1 or anti-tubulin antibody. (D) Northern blot analysis was carried out using *rum1* or *his3* cDNA as a probe. (E) Wild-type cells containing pREP42-*rum1*⁺-HA/His6 and pREP41-*Myc-pcu1*⁺ (lanes 1–4), or pREP42-*rum1*⁺-HA/His6 and pREP41-*Myc-pcu1*^{K713R} (lanes 5–8) were grown in minimal medium without thiamine for 22 h. At time 0, thiamine was added to repress the *mtl1* promoter. After the indicated times, cell extracts were prepared, separated by SDS-PAGE, then subjected to immunoblotting using anti-HA or anti-tubulin antibody (upper panels). The levels of Rum1 were quantified densitometrically, and the relative amounts of remaining Rum1 are shown (lower panel).

previously reported (Kominami *et al.*, 1998). Accumulation of *Myc-Pcu1*^{K713R} preceded that of Rum1, whereas no appreciable change in the tubulin level was observed. Northern blot analysis revealed no obvious change in the mRNA levels of Rum1 (Figure 5D), indicating that the increase in Rum1 is not due to the elevated level of transcription, but rather is due to the defective degradation of Rum1. Finally, we examined the stability of Rum1 in the cells. For this, HA/His₆-tagged Rum1 was ectopically expressed from the *mtl1* promoter, then the disappearance of Rum1-HA/His₆ was monitored by immunoblotting after the addition of thiamine to repress the promoter. As shown in Figure 5E, Rum1 was degraded rapidly in the presence of wild-type *Pcu1* after a short lag time, whereas it was stabilized greatly by overexpression of *Pcu1*^{K713R}

(upper panel). Their decay curves reveal that the apparent half-life of Rum1 was <45 min under the expression of the wild-type *Pcu1*, which is in accordance with the previously reported value (Benito *et al.*, 1998), but no significant degradation of Rum1 was observed in the presence of excess NEDD8-ylation-defective *Pcu1* mutant (Figure 5E, lower panel).

Since overexpression of Rum1 has been shown to induce various cell defects, including an increment of genome ploidy (Moreno and Nurse, 1994), it is likely that the deletion of *rum1*⁺ would cancel cellular impairments induced by *Pcu1*^{K713R}. However, when the *rum1*⁺-deleted cells (PN1012) containing pREP41-*Myc-pcu1*^{K713R} were grown in minimal medium in the absence of thiamine for 24 h at 32°C, the delay in cell cycle progression and

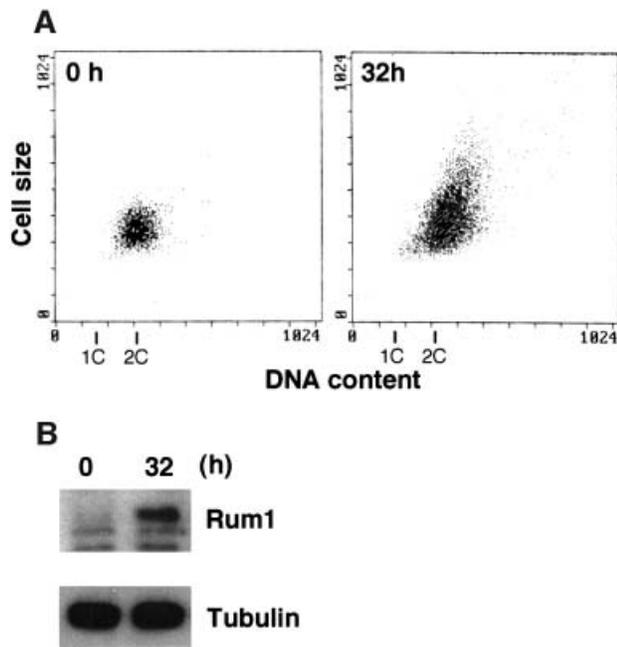


Fig. 6. Accumulation of Rum1 in NEDD8-depleted cells. $\Delta ned8^+$ cells containing pREP81- $ned8^+$ were grown in minimal medium with thiamine for the indicated time at 32°C. (A) DNA content and cell size were measured by a flow cytometer. (B) Cell extracts were separated by SDS-PAGE, followed by immunoblotting using anti-Rum1 or anti-tubulin antibody.

abnormal morphology induced by Pcu1^{K713R} were observed to the same extent as in *rum1*⁺ cells (data not shown), suggesting that accumulation of Rum1 caused by overexpression of Pcu1^{K713R} was not directly responsible for the inhibition of cell cycle progression and that there is another unknown target(s) of SCF involved in normal cell proliferation (see Discussion).

We next tested whether or not Rum1 is stabilized in cells lacking the NEDD8 system by producing an *S.pombe* strain whose chromosomal *ned8*⁺ was deleted by *ura4*⁺ and NEDD8 expression can be controlled conditionally by the *nmt1* promoter. At 24 h after repression of NEDD8, a number of cells started to elongate and cell growth was inhibited (data not shown). The most elongated cells had a 2C DNA content (Figure 6A) and decondensed chromosomes like those of $\Delta ned8$ cells (data not shown). Western blotting revealed that the depletion of NEDD8 caused accumulation of Rum1 (Figure 6B). Based on these results, it was concluded that covalent modification of Pcu1 by NEDD8 is essential for the function of SCF Ub-ligase in fission yeast. It is noteworthy that the NEDD8-modified levels of both Pcu1 and Pcu4 did not change significantly during the cell cycle progression of the fission yeast (data not shown).

NEDD8-ylation is essential for function of Pcu4

It is of interest to determine whether the modification of NEDD8 influences the functions of other cullins. As we first found that mammalian Cul-4A was covalently ligated by NEDD8 in reticulocyte lysates (Osaka *et al.*, 1998), we analysed the role of NEDD8 modification of Pcu4, its

possible counterpart in *S.pombe*. To examine the loss-of-function phenotypes of the *pcu4*⁺ gene, we replaced almost the entire ORF of the *pcu4*⁺ gene with the *ura4*⁺ marker (Figure 7A). Tetrad manipulation of a heterozygous diploid dissected out two normal sized and two tiny colonies (Figure 7B). In all cases, small colonies were Ura⁺, indicating that *pcu4*⁺ is important for normal growth, but not essential for cell viability. Microscopic observation showed that a number of $\Delta pcu4$ cells were extremely elongated with decondensed chromosomes (Figure 7C). Interestingly, all of the defective phenotypes observed in $\Delta pcu4$ cells, such as abnormal morphology (Figure 7D) and slow growth rate (Figure 7E), were complemented by *FLAG-pcu4*⁺, but not by *FLAG-pcu4*^{K680R}. Overexpression of Pcu4^{K680} from the *nmt1* promoter of pREP81 vector in the wild-type cells, however, did not show any significant abnormality, differing from that of Pcu1^{K713R} (data not shown). This finding strongly indicates that *FLAG-Pcu4*^{K680R}, which cannot be modified by NEDD8, completely loses the function of Pcu4 *in vivo* and that modification of Pcu4 by NEDD8 plays a pivotal role in the function of Pcu4.

Discussion

In the present study, we showed that the NEDD8-ligating pathway is essential in fission yeast, presumably due to the indispensable role of NEDD8-ylation of Pcu1 in the function of *S.pombe* SCF Ub-ligase. However, it may also affect pleiotropically various cellular functions, because NEDD8 modifies all Cul proteins (Osaka *et al.*, 1998; Hori *et al.*, 1999; Liakopoulos *et al.*, 1999; Wada *et al.*, 1999a,b) and possibly other as yet unknown target(s). Intriguingly, overexpression of the NEDD8-ylation-defective mutant Myc-Pcu1^{K713R} resulted in inhibition of cell division at the G₂ phase concomitant with abnormal accumulation of Rum1 capable of inhibiting the activity of Cdc2 mitotic kinase (Correa-Bordes and Nurse, 1995; Martin-Castellanos *et al.*, 1996). Details of the mechanism by which forced expression of Pcu1^{K713R} induces the G₂ arrest remain elusive, but it is noteworthy that accumulation of Rum1 is not a major cause of induction of cell cycle abnormality, because disruption of the *rum1*⁺ gene did not cancel the cell abnormalities caused by overexpression of Pcu1^{K713R}. Consistent with this result, we found that *pcu1* deletion is still lethal in a *rum1* deletion background (T.Toda and S.Katayama, unpublished result). Quite recently, it was reported that Cul-1, perhaps existing as an SCF complex, is present abundantly in centrosomes and that the centrosome-associated Cul-1 is modified mostly by NEDD8 (Freed *et al.*, 1999). The NEDD8-ylation, therefore, may play a pivotal role in the centrosome function(s), which somehow influences the G₂-M transition of the cell cycle, finally causing a cell cycle delay at the G₂ phase.

It is notable that ectopic expression of Rum1 or *pop1* mutation causes a high ploidy phenotype of the cell (Kominami *et al.*, 1997), but here we found that overexpression of Pcu1^{K713R} did not have a serious influence on induction of polyploidy, irrespective of Rum1 accumulation. This difference is presumably due to the fact that overexpression of Pcu1^{K713R} may result in dysfunction of all species of SCF complexes, which cause

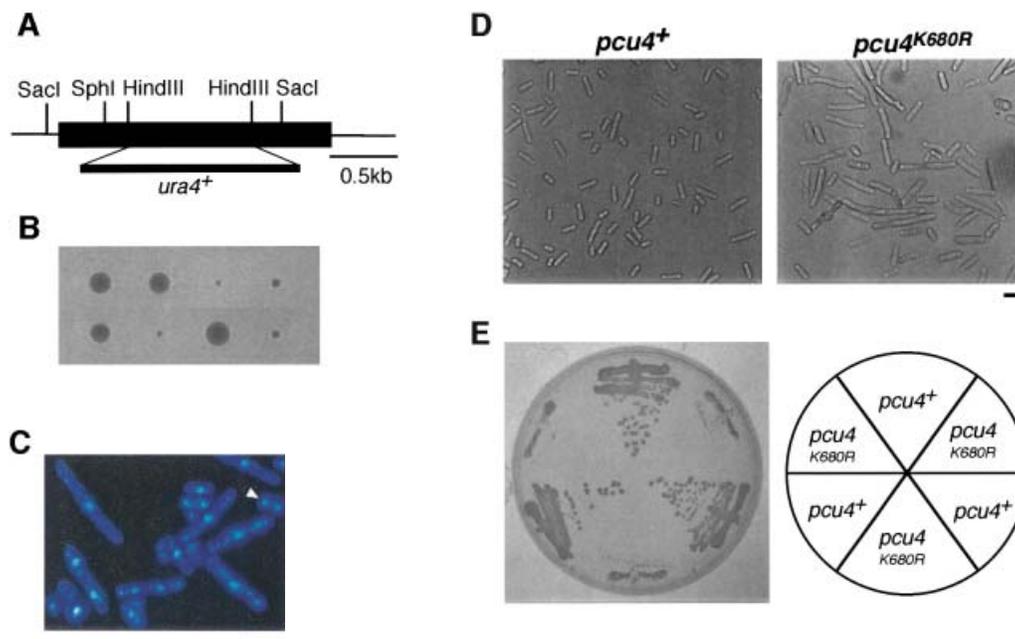


Fig. 7. *pcu4*^{K680R} loses the ability to complement the defective phenotypes of $\Delta pcu4$ cells. (A) Disruption of the *pcu4*⁺ gene. Almost the entire ORF of the *pcu4*⁺ gene (DDBJ/EMBL/GenBank accession No. Z99260, SPC3A11.08) was deleted by use of a PCR-generated fragment containing the *ura4*⁺ marker. (B) Tetrad analysis of diploids heterozygous for *pcu4*. (C) $\Delta pcu4$ cells were stained by DAPI as described in Figure 1C. Ungerminated spores are marked with an arrow. Bar, 10 μ m. (D) $\Delta pcu4$ cells containing pREP81-FLAG-*pcu4*⁺ or pREP81-FLAG-*pcu4*^{K680R} were grown in minimal medium without thiamine, then observed under a phase contrast microscope. Bar, 10 μ m. (E) $\Delta pcu4$ cells containing pREP81-FLAG-*pcu4*⁺ or pREP81-FLAG-*pcu4*^{K680R} were grown on a minimal plate without thiamine for 4 days at 30°C.

the cell cycle arrest at multiple stages and a variety of abnormal phenotypes, whereas *pop1* mutation causes the defect of the SCF^{Pop1} ligase leading to abnormal accumulation of Rum1. Intriguingly, we also observed a slight, but significant accumulation of Cdc18, which is known to be another target of SCF^{Pop1/2} in fission yeast (Kominami and Toda, 1997; Jallepalli *et al.*, 1998; Kominami *et al.*, 1998; Maekawa *et al.*, 1998), which was consistent with the observation that deletions of *pop1*⁺ and/or *pop2*⁺ resulted in a relatively low accumulation of Cdc18 compared with the marked accumulation of Rum1 (Kominami *et al.*, 1998). It remains to be discovered, however, why Myc-Pcu1^{K713R} unmodified by NEDD8 completely loses SCF^{Pop1/2} function *in vivo*. We found here that NEDD8-ylation of Pcu1 is not required for formation of the SCF complex, because Pcu1^{K713R}, like wild-type Pcu1, was incorporated into the SCF^{Pop1} complex. The observation that Pcu1^{K713R} is capable of forming an SCF complex with an apparently normal size indicates that Pcu1^{K713R} possesses considerable, if not total, functional integrity compared with the wild-type Pcu1, except for the linkage with NEDD8, although Pcu1^{K713R} may not be structurally equivalent to the wild-type Pcu1. Therefore, although unlikely, modification of Pcu1 by NEDD8 may be necessary for interaction with Roc1/Rbx1/Hrt1, which has recently been identified as the fourth essential subunit of the SCF (Kamura *et al.*, 1999a; Ohta *et al.*, 1999; Seol *et al.*, 1999; Skowyra *et al.*, 1999; Tan *et al.*, 1999), or for recruitment of the E2 enzyme. Alternatively, the possibility that NEDD8 is related to the stability or localization of the SCF complex cannot be ruled out. Further study is required to clarify this issue.

Similarly, a deletion of the *S.cerevisiae* *ENR2* (equivalent to *APP-BP1*) is synthetic lethal in temperature-sensitive alleles of *cdc34* and enhances the phenotypes of *cdc4*, *cdc53* and *skp1*. This suggests that modification of Cdc53 by Rub1/NEDD8 affects the optimal assembly or functions of the SCF complex (Lammer *et al.*, 1998). Accordingly, the Rub1/NEDD8 ligation pathway is closely linked to cell cycle regulation, although this pathway and the modification of Cdc53 by Rub1 do not seem to be strictly essential for the function of SCF^{CDC4} in budding yeast (Lammer *et al.*, 1998; Liakopoulos *et al.*, 1998), unlike in fission yeast. In *Arabidopsis*, AXR1 (APP-BP1 homologue) has been identified as one of the genes required for a normal auxin response (Leyser *et al.*, 1993). In the *axr1* mutant, it is known that ECR1 (Uba3 homologue) cannot form an efficient thioester linkage with Rub1/NEDD8 (Poza *et al.*, 1998). Interestingly, an F-box protein mutant, *tir1*, identified from genetic analysis of auxin-response mutants, has a synergistic interaction with *axr1*, implying that modification of the SCF^{tir} function by the NEDD8 ligation system is required for the normal auxin response (Ruegger *et al.*, 1998; Gray *et al.*, 1999). These observations are consistent with our conclusion that the NEDD8 ligation system regulates the function of the SCF complex. Taken together, these findings suggest that the NEDD8/Rub1-ligating pathway may play a critical role in various functions of the SCF complex in a variety of eukaryotic cells.

Budding yeast Cdc53 functions as a common component of distinct SCF Ub-ligases that regulate multiple cellular functions, such as the cell cycle (Feldman *et al.*, 1997; Skowyra *et al.*, 1997), gene expression (Li and Johnston, 1997) and methionine biosynthesis (Patton *et al.*,

1998b), by replacing the appropriate F-box proteins. Consistent with this notion, the mutation of hamster *SMC*, encoding a protein nearly identical to APP-BP1, is responsible for cell cycle defects in the hamster ts41 cell line (Handeli and Weintraub, 1992; Chen *et al.*, 2000). Moreover, mammalian SCF^{βTrCP} has recently been found to act as a Ub-ligase for IκBα (Yaron *et al.*, 1998; Suzuki *et al.*, 1999, 2000; Winston *et al.*, 1999), and β-catenin (Kitagawa *et al.*, 1999; Maniatis, 1999; Winston *et al.*, 1999), suggesting its involvement in signal transduction. Recently, it was also suggested that SCF^{SKP2} catalyses ubiquitylation of the CDK inhibitor p27^{Kip1} (Carrano *et al.*, 1999; Tsvetkov *et al.*, 1999) and E2F-1 (Marti *et al.*, 1999). It was found recently that Cul-1 associated with phosphorylated IκBα and p27^{Kip1} was modified preferentially by NEDD8 in mammalian cells (Podust *et al.*, 2000; Read *et al.*, 2000; T.Kawakami, T.Chiba and K.Tanaka, in preparation), which seems to be in agreement with the present finding that Pcu1 linked to SCF^{Pop1} was almost fully modified by NEDD8. Interestingly, the formation of NEDD8–Cul-2 conjugates is stimulated by pVHL, but not by a tumorigenic pVHL variant, suggesting that ligation of NEDD8 to Cul-2 presumably is linked to the pVHL tumour suppression function via CBC^{pVHL} (Liakopoulos *et al.*, 1999). Here we also provided evidence that modification of NEDD8 by Pcu4 plays a critical role in the function of Pcu4, although whether or not Pcu4 is incorporated into the SCF-like complex remains unknown. Quite recently, it was reported that the CBC^{pVHL} complex containing Cul-2 functions as a Ub–protein ligase (Iwai *et al.*, 1999; Lisztwan *et al.*, 1999) and Cul-3 targets cyclin E for ubiquitylation (Singer *et al.*, 1999), implying that all Cul-family proteins may act as components of the Ub–protein ligase complex. Thus, it is likely that NEDD8-ylation is a common and essential modification required not only for Cul-1 of the SCF Ub-ligase complex, but also for function(s) of other Cul-family proteins.

Materials and methods

Strains, media and genetic methods

In this study, the following strains were used: TP4-5A (*h-leu1ura4ade6-M210*), TP4-1D (*h⁺leu1ura4his2ade6-M216*), IO100 (*h⁺leu1/leu1-ura4/ura4his7/his7ade6-M210/ade6-M216pcu1::ura4⁺/pcu1⁺*), SKP401 (*h⁺leu1pop2⁺-3HA-kan*), SKP414-17 (*h⁺leu1ura4pcu1⁺-13Myc-kan*), SKP424-17 (*h⁺leu1pcu1⁺-13Myc-kan1*, *pop1⁺-3HA-kan*), SKP408-4 (*h⁺leu1ura4pop1⁺-3HA-kan*) and PN1012 (*h⁹⁰ leu1 ade6 ura4 rum1::ura4⁺*). Cells were grown in YE medium or minimal medium supplemented with amino acids, uracil and/or adenine (Moreno *et al.*, 1991). To regulate the expression of genes under the thiamine-repressible promoter (*nmt1* promoter), cells were grown in minimal medium with or without 10 μM thiamine (Maundrell, 1990). For gene disruption, a diploid strain crossed between TP4-5A and TP4-1D was used. Correct disruption of all heterozygous diploids was confirmed by both genomic Southern blotting and PCR analyses.

Immunological analyses

Cells were suspended in ESB buffer [2% SDS, 80 mM Tris pH 6.8, 10% glycerol, 1.5% dithiothreitol (DTT) and 0.1 mg/ml bromophenol blue], boiled for 3 min, followed by treatment with glass beads. Cell extracts were separated by SDS–PAGE, followed by immunoblotting using anti-Myc antibody (9E10 Boehringer Mannheim), anti-FLAG M2 antibody (IB13010 Kodak), anti-HA antibody (16B12 BAbCO), anti-Rum1 antibody (provided by S.Moreno) or anti-tubulin antibody (T5168 Sigma or YOL1/34 Harlan). For immunoprecipitation, cell extracts (2 mg) were prepared as described (Moreno *et al.*, 1991) and mixed with anti-HA antibody, followed by AffiPrep Protein A (Bio-Rad). After

washing with HB buffer (Moreno *et al.*, 1991), the absorbed proteins were eluted by heating for 3 min in SDS–PAGE sample buffer.

Flow cytometry

Cells were fixed with ethanol, treated with DNase-free RNase A (100 μg/ml) and stained with 2.5 μg/ml propidium iodide. Samples of 30 000 cells were analysed with a flow cytometer (EPICS XL Beckman Coulter).

Acknowledgements

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