The novel HECT-type ubiquitin-protein ligase Pub2p shares partially overlapping function with Pub1p in *Schizosaccharomyces pombe*

Katsuyuki K. Tamai and Chikashi Shimoda*

Department of Biology, Graduate School of Science, Osaka City University, Sugimoto 3-3-138, Sumiyoshi-ku, Osaka 558-8585, Japan
*Author for correspondence (e-mail: shimoda@sci.osaka-cu.ac.jp)

Accepted 16 February 2002
Journal of Cell Science 115, 1847-1857 (2002) © The Company of Biologists Ltd

Summary

The fission yeast *Schizosaccharomyces pombe* has three putative ubiquitin-protein ligases of the Nedd4/Rsp5 family, named Pub1p, Pub2p and Pub3p. Pub1p has been reported to be involved in cell cycle regulation and proliferation under acidic pH conditions. Here we characterize Pub2p, which contains a conserved HECT domain and a WW domain but lacks a C2 domain. Transcription of the *pub2*$^+$ gene was constitutive and further enhanced by nitrogen starvation. A *pub2*-null mutation gave no remarkable phenotypes, but intensified temperature sensitivity in a *pub1Δ* background. Moderately overexpressed *pub2*$^+$ suppressed the temperature sensitivity of *pub1Δ* cells, which suggests that the function of Pub2p overlaps with that of Pub1p. Overexpression of *pub2*$^+$ by a strong *nmt1* promoter in wild-type strains caused growth arrest and cell elongation, probably owing to defects in G2 progression or the G2/M transition. Unlike Pub1p, however, overexpression of Pub2p did not reduce the levels of Cdc25p. Pub2-GFP was found throughout the cell, especially at the cell surface in the polar regions. Pub2p contains a conserved cysteine residue (Cys639) in its putative catalytic HECT domain that can be thiol-ubiquitinated. Substitution of Cys639 by alanine (Pub2CA) caused a functional defect, because growth arrest and cell elongation were not induced by overexpression of Pub2CA. A chimeric Pub1 protein, in which the HECT domain was replaced by the Pub2 HECT domain, completely suppressed the temperature sensitivity of *pub1Δ* cells, suggesting that the HECT domain of Pub2p has the catalytic activity of a ubiquitin ligase. We conclude that Pub2p is a HECT-type ubiquitin-protein ligase that shares partially overlapping function with Pub1p.

Key words: C2 domain, HECT domain, G2 arrest, Ubiquitin ligase

Introduction

The ubiquitin-proteasome system controls crucial steps in a wide variety of biological processes such as cell cycle regulation, the immune response and transcriptional regulation. The ubiquitination of proteins acts as a tag for their degradation by proteasomes in an ATP-dependent manner. As ubiquitination is highly specific, the ubiquitin-proteasome system may regulate protein degradation both temporally and spatially. Because protein degradation is an irreversible reaction, the ubiquitin-proteasome system guarantees unidirectional stepwise progression in a multi-step sequence such as the cell division cycle.

The ubiquitination of proteins is catalyzed by three enzymes, E1, E2 and E3. The ubiquitin-activating enzyme (E1) binds to and activates free ubiquitin (Ub) molecules using ATP; the activated Ub is subsequently transferred to a ubiquitin-conjugating enzyme (E2). In many cases, ubiquitin-protein ligases (E3) are necessary for substrate-specific multi-ubiquitination (for reviews, see Varshavsky, 1997; Hershko and Ciechanover, 1998). Ubiquitin-protein ligases are classified as either a RING finger or a HECT type (for a review, see Jackson et al., 2000). Typical RING-finger-type ubiquitin ligases include the anaphase promoting complex (APC) and the SCF (Skp1/Cullin/F-box) complex, both of which are involved in controlling cell cycle progression. HECT-type ubiquitin ligases contain a HECT (homologous to the E6-AP carboxyl terminus) domain, which is the ubiquitin-protein ligase catalytic domain (Huibregtse et al., 1995). HECT-type ubiquitin ligases form a thioester intermediate with Ub via a conserved cysteine (Cys) residue located in the HECT domain (Scheffner et al., 1995). Mammalian Nedd4p and *S. cerevisiae* Rsp5p constitute a subfamily of HECT-type ubiquitin ligases. Nedd4/Rsp5 proteins are characterized by a C2 domain and several WW domains, in addition to the catalytic HECT domain (for a review, see Harvey and Kumar, 1999). The C2 domain acts as a Ca^{2+}-dependent phospholipid-binding site. Indeed, the N-terminal C2 domain of mammalian Nedd4p is required for its localization to the plasma membrane (Plant et al., 1997). The WW domain binds to several proline-rich motifs such as the PY, PPLP and PGM motifs (Bedford, 2000) (for a review, see Kay et al., 2000). Compared with APC and SCF, little is known about the biological functions of HECT-type ubiquitin ligases; however, mammalian Nedd4p and budding yeast Rsp5p have been analyzed extensively and their in vivo substrates have been identified. Human Nedd4p downregulates epithelial Na^+ channel proteins by ubiquitin-mediated proteolysis (Staub et al., 1996), whereas Rsp5p, an essential HECT-type ubiquitin ligase in *Saccharomyces cerevisiae*, ubiquitinates the large subunit of RNA polymerase II (Rpb1p) (Huibregtse et al., 1997), uracil permease (Fur4p) (Galan et al., 1996), a general

amino-acid permease (Gap1p) (Hein et al., 1995) and a receptor protein for α-mating pheromone (Ste2p) (Hicke et al., 1998). Rsp5p also promotes the endocytosis of plasma-membrane-integrated proteins, such as Ste2p and Fur4p, by ubiquitination (for a review, see Rotin et al., 2000).

In the fission yeast *Schizosaccharomyces pombe*, Pub1p (E6-AP-like protein ubiquitin ligase), which is encoded by the *pub1*$^+$ gene, has been reported to be a HECT-type ubiquitin ligase (Nefsky and Beach, 1996). Pub1p has been proposed to regulate G2/M transition via ubiquitination of Cdc25p, which dephosphorylates the phosphotyrosine of Cdc2p (Russell and Nurse, 1986; Gould and Nurse, 1989; Dunphy and Kumagai, 1991; Gautier et al., 1991; Lundgren et al., 1991; Millar et al., 1992). Disruption of the *pub1*$^+$ gene markedly reduces the level of ubiquitinated Cdc25p. In addition to cell cycle regulation, Pub1p is involved both in cell viability in low pH medium and in leucine transport (Saleki et al., 1997; Karagiannis et al., 1999). Pub1p ubiquitin ligase belongs to the Nedd4/Rsp5 subfamily, because it is composed of a highly conserved HECT domain as well as a single C2 domain and three WW domains. We have identified two more genes encoding ubiquitin ligases of this subfamily in the *S. pombe* genome sequence database (The Sanger Centre, UK), which we designated *pub2*$^+$ and *pub3*$^+$. In this article, we report that the *pub2*$^+$ gene product has ubiquitin-protein ligase activity in vivo and shares a partially overlapping function with Pub1p.

Materials and Methods

S. pombe strains, media and culture conditions

The *S. pombe* strains used in this study are listed in Table 1. Cells were grown in YEA complete medium or in EMM2 minimal medium supplemented with 100 μg/ml adenine sulfate, uracil or leucine when necessary. These media have been described by Gutz et al. (Gutz et al., 1974) and Moreno et al. (Moreno et al., 1991). The pH of EMM2 medium was adjusted as described in Saleki et al. (Saleki et al., 1997). EMM2 medium lacking NH_4Cl (EMM2-N) was used for mating and sporulation. To repress the *nmt1*, *nmt41* or *nmt81* promoter activity, 20 μM thiamine was added to the medium (Maundrell, 1993). Cells were usually grown at 30°C.

Synchronous culture

Mitotic cell cycles were synchronized using the *cdc25-22* temperature-sensitive mutation. *cdc25-22* cells were grown in EMM2 until mid-log phase at 25°C and shifted to 36.5°C to arrest the cell cycle in late G2 phase. After a 4 hour incubation at 36.5°C, the culture was shifted to 25°C to restart the cell cycle. Synchrony was monitored by scoring septated cells (Alfa et al., 1993).

Flow cytometric analysis

Cells were fixed with cold 70% ethanol, resuspended in 50 mM Na^+-citrate buffer and treated with 0.1 mg/ml RNase A for 2 hours at 37°C. After washing with Na^+-citrate buffer, cells were stained with propidium iodide (PI) at a final concentration of 10 μg/ml. Stained cells were analyzed by a flow cytometer (FACScaliber, Becton Dickinson).

Isolation of *pub2*$^+$ and *pub3*$^+$

A 13 kb genomic DNA fragment containing *pub2*$^+$ was fortuitously cloned from an *S. pombe* genomic DNA library (Ikemoto et al., 2000) during a previous screening for other genes. A 4.0 kb *Cla*I/*Pst*I fragment, containing the *pub2*$^+$ ORF, was subcloned into a pBluescript II-KS(+) plasmid (Stratagene, La Jolla, CA). We also isolated the *pub2*$^+$ cDNA clone from an *S. pombe* cDNA library pTN-RC5 (Nakamura et al., 2001). The nucleotide sequences of the genomic and cDNA clones were determined. The *pub2*$^+$ gene is split by three introns, and the exonic sequence potentially encodes a Pub2 protein comprising 671 amino acids. *pub2*$^+$ proved to be identical to SPAC1805.15c (The Sanger Centre Genome Sequence Database).

We also identified another ORF that is homologous to Pub1p in the database. This ORF, SPBC16E9.11c, was designated *pub3*$^+$. The genomic *pub3*$^+$ gene was isolated by a PCR-based method (see below).

Gene disruption of *pub1*$^+$ and *pub2*$^+$

The *pub2*$^+$ gene was disrupted by one-step gene replacement (Rothstein, 1983). The 1.2 kb *Hin*dIII fragment carrying about 60% of the Pub2 ORF was replaced by a 1.8 kb *Hin*dIII fragment containing *ura4*$^+$ (Grimm et al., 1988). After digestion with *Eco*RI/*Pst*I, the linearized DNA fragment harboring the *pub2::ura4*$^+$ allele was introduced into the diploid strain C525 using the lithium acetate method (Okazaki et al., 1990). Stable Ura^+ transformants were subjected to genomic Southern blotting to confirm that they carried one copy of *pub2::ura4*$^+$ and one copy of *pub2*$^+$ (data not shown).

The *pub1*$^+$ gene was disrupted as follows. The genomic DNA fragment carrying *pub1*$^+$ in pBluescript II was digested with *Nsp*V and blunt-ended with T4 DNA polymerase. It was further digested with *Bgl*II and ligated to a *Bam*HI/*Hin*cII fragment carrying a *ura4*$^+$ cassette. The *pub1::ura4*$^+$ fragment was amplified with primers

Table 1. Strain list

Strain	Genotype	Source
L968	*h*^{90}	U. Leupold
L972	*h*$^-$	U. Leupold
C525	*h*^{90}*ade6-M216 leu1-32 ura4-D18/h*^{90} *ade6-M210 leu1-32 ura4-D18*	Lab Collection
MM71-6B	*h*$^-$ *ade6-M210 leu1-32*	Lab Collection
MM72-1D	*h*$^-$ *leu1-32 ura4-D18*	Lab Collection
MM72-11C	*h*$^-$ *leu1-32*	Lab Collection
K164-9	*h*$^-$ *cdc25-22*	K. Tanaka
KJ33-1A	*h*^{90} *ste11::ura4*$^+$ *ade6-M216 leu1-32 ura4-D18*	K. Kitamura
OM1715	*h*$^+$ *cdc25:6HA(ura4*$^+$*) leu1-32 ura4-D18*	P. Russell
KKT39-7B	*h*^{90} *pub2::ura4*$^+$ *ura4-D18*	This work
KKT81-3A	*h*$^-$ *pub1::ura4*$^+$ *ade6-M210 leu1-32 ura4-D18*	This work
KKT82-2D	*h*$^-$ *pub1::ura4*$^+$ *pub2::ura4*$^+$ *ura4-D18*	This work
KKT83-9A	*h*$^-$ *pub1::ura4*$^+$ *ura4-D18*	This work
KKT85-8A	*h*$^-$ *pub2::ura4*$^+$ *ade6-M210 leu1-32 ura4-D18*	This work
KKT87	*h*$^-$ *pub2:3HA (pIL) leu1-32 ura4-D18*	This work

designed to amplify *pub1*$^+$ genomic DNA and introduced into C525. Verification of *pub1Δ* was conducted by genomic Southern blotting. Genomic DNA was digested with *Cla*I. The *Xho*I/*Bgl*II fragment cut out from *pub1*$^+$ in pBluescript II was used as the probe for Southern blotting.

Plasmid construction

The plasmid pREP2-Myc was constructed by inserting three copies of Myc into the *Not*I site of pREP2. The plasmid pREP81-GFP was constructed by inserting the mutant version of GFP (GFP^{S65T}) into the *Not*I site of pREP81 (Nakamura et al., 2001). To overexpress the *pub2*$^+$ gene, plasmid pREP1-*pub2*$^+$ was constructed. *pub2*$^+$ cDNA was amplified from the cDNA library, pTN-RC5 (Nakamura et al., 2001), by PCR with the following primers: 5′-CCAGATCTCATATG(*Nde*I)GAAAATATTCGCTTG-3′ and 5′-CCGCGGCCGC(*Not*I)-CCTCCGTACCAAATCC-3′. The amplified DNA was digested with *Nde*I and *Not*I and ligated into pREP1 to generate pREP1-*pub2*$^+$. The same *Nde*I/*Not*I fragment was inserted into the plasmid pREP81-GFP. In vitro mutagenesis for the substitution of Pub2 Cys639 to Ala (Pub2CA) was done by using the above primers and the following primers: 5′-CATACTGCCTTCAATCG-3′ and 5′-AAACGATTGA-AGGCAGTATG-3′. To amplify the Pub2 HECT domain, the following primers were used: 5′-CCCCATATGCAGCTAAAGGT-TAGCAGAG-3′, 5′-CCGCGGCCGCCCTCCGTACCAAATCC-3′. The Pub1-HECT^{Pub2} chimeric protein-encoding gene was constructed with the primers: 5′-ATCCCCGTGAATACTTCTATATTTTGTCTC-ATGC-3′, 5′-GCATGAGACAAAATATAGAAGTATTCACGGGA-3′.

The ubiquitin-encoding gene was amplified in the same way but with the following primers: 5′-GGGCGGCCGC(*Not*I)ATGCAGA-TTTTTGTC-3′, 5′-GGAGATCT(*Bgl*II)TACTTAAGCTTCTTCTTA-GG-3′. The amplified DNA fragment was inserted into pREP1-GST after *Not*I/*Bgl*II digestion. For amplification of genomic DNA containing *pub1*$^+$, the following primers were used: 5′-GTGGA-TAGCAAATAGTCTTTATGACCAGCC-3′, 5′-AGGATTGTTTTAC-AAGGCTATTGTGGTTGG-3′. The amplified DNA fragment was blunt-ended with T4 DNA polymerase and ligated into the *Eco*RV site of pBluescript II KS(+). *pub1*$^+$ cDNA was amplified with the primers 5′-GGCATATG(*Nde*I)TCAAACTCAGCTCAATCTCG-3′ and 5′-CCGCGGCCGC(*Not*I)CCTCCTGACCAAAACCAATCG-3′. The amplified fragment was inserted into pREP41 after digestion with *Nde*I/*Not*I.

Southern and northern blotting

Genomic DNA was prepared according to Hereford et al. (Hereford et al., 1979). DNA was restricted, fractionated on 1% agarose gels and then transferred onto nylon membranes (Biodyne B, Pall Co.). Non-radioactive probes were prepared by using the DIG-DNA Labeling Kit (Boehringer Mannheim, Mannheim). Hybridization was carried out according to the manufacturer's instructions using the DIG-Hybridization Kit (Boehringer Mannheim, Mannheim).

Northern blotting was carried out as follows. Total RNA was prepared from *S. pombe* cultures by the method of Jensen et al. (Jensen et al., 1983). 10 μg of total RNA was fractionated on 1.5% agarose gels and then transferred onto nylon membranes (Biodyne B, Pall Co.) (Thomas, 1980). *pub1*$^+$ and *pub2*$^+$ cDNAs were labeled with [α-^{32}P]ATP by the random primer method (Feinberg et al., 1983) and used as hybridization probes.

Immunological detection of Pub2-ubiquitin conjugation

Both pREP1-GST:Ub and pREP2-HECT^{pub2}:Myc plasmids were introduced into the haploid strain MM72-1D (*h*$^-$ *leu1-32 ura4-D18*). The transformants were grown to mid-log phase in EMM2 containing 20 μM thiamine to repress the *nmt1* promoter. After washing twice with sterilized H_2O, the cells were transferred into EMM2 without thiamine to switch on the *nmt1* promoter and incubated for 20 hours. Cells were disintegrated by glass beads in ice-cold lysis buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing 50 mM *N*-ethylmaleimide and protease inhibitors; 5 μg/ml aprotinin, 3 μg/ml leupeptin and 1 mM PMSF. The protein concentration of total cell lysates was determined by the Lowry method. Whole-cell extracts containing 5 μg of protein were incubated with 20 μl of Glutathione-Sepharose 4B (Pharmacia Biotech) with rotation at 4°C for 1 hour. Glutathione beads were washed at least four times with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors used in lysis buffer. After washing, the samples were divided into two portions. One was incubated for 30 minutes at 60°C in SDS-PAGE sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol) containing 100 mM dithiothreitol (DTT). The other was treated in the same way except that 4 M urea was added instead of 100 mM DTT (Scheffner, 1995). After SDS-PAGE on 10% gels, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore Co.). Western blotting was performed with a 9E10 anti-c-Myc antibody (1:1000 dilution) (Sigma) and HRP-conjugated anti-mouse IgG (1:1000) (Promega).

Localization of Pub1-GFP and Pub2-GFP

Plasmids pREP81-*pub1*$^+$*-GFP*, pREP81-*pub2*$^+$*-GFP* and pREP81-*GFP* were introduced into MM72-11C (*h*$^-$ *leu1-32*) cells. The transformed cells were grown to mid-log phase in EMM2 containing 20 μM thiamine and then transferred to EMM2 without thiamine. Cells were observed without fixation under a fluorescence microscope (Model BX50, Olympus Co.).

Subcelluar fractionation by differential centrifugation

Preparation of cell lysates and their fractionation by differential centrifugation were done as described by Dunn and Hicke (Dunn and Hicke, 2001). Spheroplasts were prepared by treating cells by Zymolyase 100T (SEIKAGAKU Co., Tokyo) in YE medium containing 1.2 M sorbitol and then mechanically broken in lysis buffer (20 mM MES pH6.5, 0.1 M NaCl, 5 mM $MgCl_2$, and protease inhibitors). The lysates were centrifuged first at 13,000 ***g*** for 30 minutes to separate precipitate (P13) and supernatant. The latter fraction was subjected to a second centrifugation at 100,000 ***g*** for 30 minutes, resulting in pellet (P100) and supernatant (S100) fractions. P13 mainly contains large membrane compartments such as plasma membranes and vacuoles. The lighter membrane components such as endoplasmic reticulum, Golgi apparatus and endosomes were recovered in P100. S100 is a cytosolic fraction (Dunn and Hicke, 2001). The quantity of Pub2-HA in each fraction was analyzed by western blotting with anti-HA antibody (3F10).

Results

Novel *S. pombe* Nedd4/Rsp5 homologues Pub2p and Pub3p

To understand further the function of Nedd4/Rsp5 subfamily ubiquitin-protein ligases, we first identified the homologues in *S. pombe*. In the *S. pombe* genome sequence database (The Sanger Centre, UK), there are seven ORFs encoding putative HECT-type ubiquitin ligases. We found that three of them highly resemble the Nedd4/Rsp5 family proteins (Fig. 1). One of them, designated *pub1*$^+$, has been reported already (Nefsky and Beach, 1996; Saleki et al., 1997; Karagiannis et al., 1999).

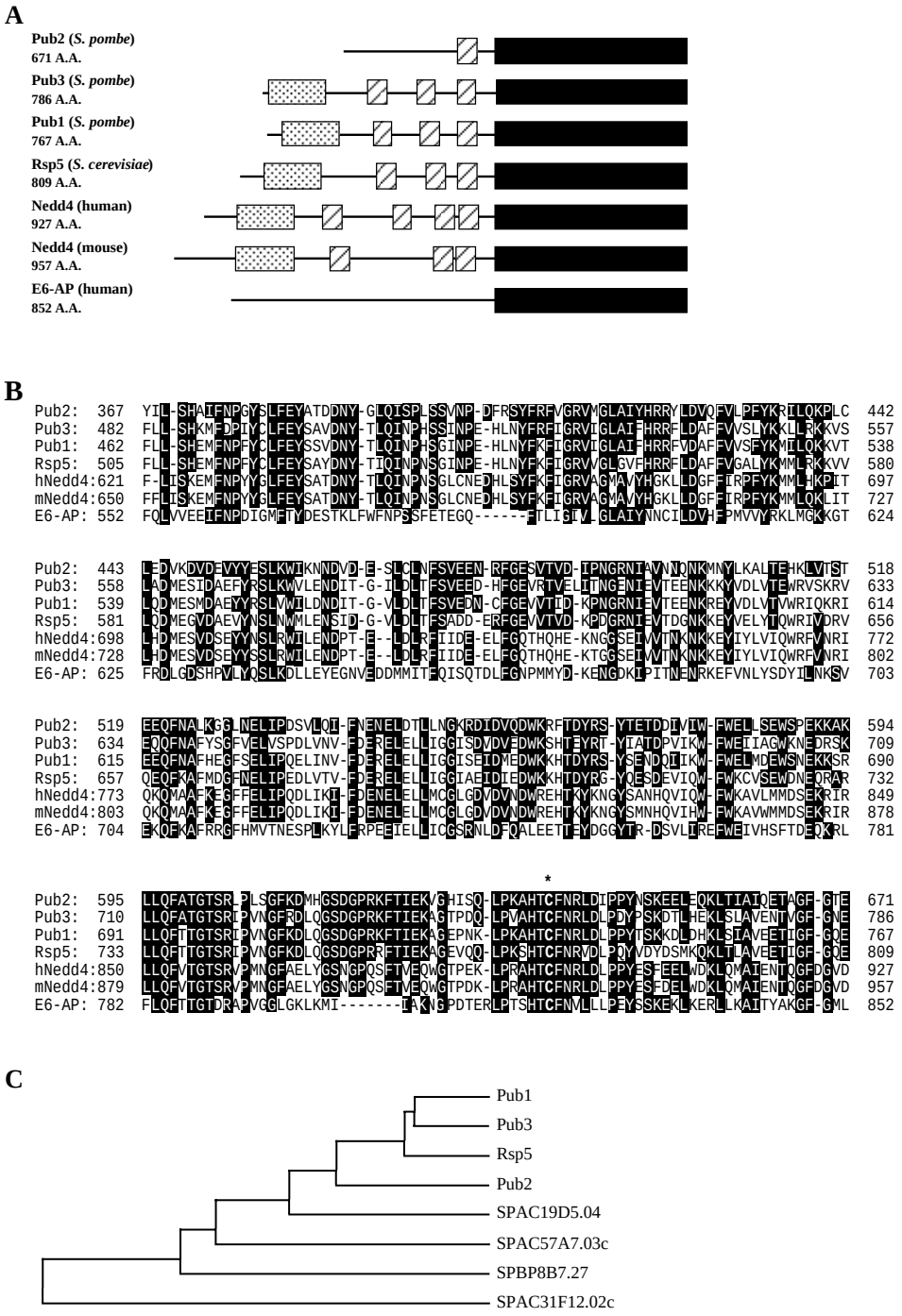

Fig. 1. Structural comparison of Nedd4/Rsp5 family proteins. (A) Schematic representation of the domain structures. The C2, WW and HECT domains are indicated by shaded, hatched and black boxes, respectively. A.A., amino acids. (B) Amino-acid sequence alignment of the HECT domains. Amino acids that are identical in Pub2p are indicated by dark shading. The asterisk indicates the conserved cysteine residue (Cys639 for Pub2p), which is required for the thioester bond formation with the C-terminal glycine residue of ubiquitin. hNedd4 and mNedd4 indicate human and mouse Nedd4p, respectively. E6-AP is a prototype HECT-type ubiquitin-protein ligase from human. (C) A phylogenetic tree of HECT domains, constructed by the UPGMA method, of seven HECT-type ubiquitin ligases of *S. pombe* and Rsp5p of *S. cerevisiae*.

We therefore designated the other two genes *pub2*$^+$ (SPAC1805.15c; Accession number, AL117390) and *pub3*$^+$ (SPBC16E9.11c; Accession number, Z99759).

The *pub2*$^+$ gene encodes a 77 kDa protein (Pub2p) comprising 671 amino acids and containing a single WW domain and a HECT domain but no C2 domain (Fig. 1A). The *pub3*$^+$ gene product (Pub3p) consists of 786 amino acids (89 kDa) and has a C2 domain in its N-terminus, three WW domains in its central region and a HECT domain in its C-terminus (Fig. 1A). Pub3p resembles Pub1p in size and primary structure, with an amino acid identity of 66% (74% in the HECT domain) (Fig. 1B). It therefore seems likely that *pub1*$^+$ and *pub3*$^+$ are redundant genes. In support of this, a *pub1*Δ *pub3Δ* double disruptant was non-viable (K.K.T. and C.S., unpublished data).

By contrast, the amino-acid sequence similarity between Pub2p and Pub1p is relatively low (39% identity), and the domain structure is different. Compared with other members of the Nedd4/Rsp5 family, the most prominent feature of Pub2p is its lack of C2 domain. Despite the overall differences between Pub2p and Pub1p/Pub3p, their HECT domains are highly conserved (Fig. 1B,C).

Transcription of *pub2⁺*

Although Pub1p has been proposed to negatively regulate the G2/M transition, the fluctuation of transcript level of *pub1⁺* is not known. We examined transcription of *pub1⁺* and *pub2⁺* in the cell cycle. Synchronous cell division was attained by the *cdc25-22* temperature-sensitive mutation. Cell cycle progression of *cdc25-22* cells was blocked in late G2 phase at 36.5°C for 4 hours, and the cell cycle was restarted by a shift to permissive temperature, 24°C. RNA samples were taken every 20 minutes for 5 hours and then subjected to northern analysis with *pub1*- or *pub2*-specific probes. As shown in Fig. 2A, the mRNA level of *pub2⁺* was low relative to that of *pub1⁺*. The abundance of *pub2⁺* mRNA did not fluctuate throughout the cell cycle.

We next examined whether the transcription of *pub2⁺* mRNA was affected by stress, such as nutrient starvation, heat shock or osmotic stress. We measured the abundance of *pub1⁺* and *pub2⁺* mRNA after nitrogen starvation (Fig. 2B). Homothallic (h^{90}) cells were grown in nitrogen-rich medium until mid-log phase and then transferred to nitrogen-free medium. Notably, the *pub2⁺* mRNA level was significantly enhanced 2 hours after nitrogen starvation, and this elevated expression level persisted (Fig. 2B). By contrast, the expression of *pub1⁺* was not affected by nutritional state. Upregulation of *pub2⁺* mRNA was also observed in a heterothallic haploid strain (data not shown), suggesting that *pub2⁺* transcription is stimulated by the stress of nitrogen starvation, independent of the mating reaction.

As Ste11p is a key transcription factor for many genes whose expression is upregulated by starvation (Sugimoto et al., 1991), we investigated whether the increase in *pub2⁺* mRNA is regulated by Ste11p. We performed northern blot analysis using h^{90} *ste11Δ* cells. As shown in Fig. 2B, the expression of *pub2⁺* was repressed in *ste11Δ* cells; however, there was still an increase in mRNA levels after starvation. The Ste11-binding site, called the 'TR box', was not present in the putative promoter region of *pub2⁺*. Together, these results suggest that the *pub2⁺* transcription is affected by the *ste11Δ* mutation but that the Ste11 transcription factor might have only an indirect effect on transcription of *pub2⁺*.

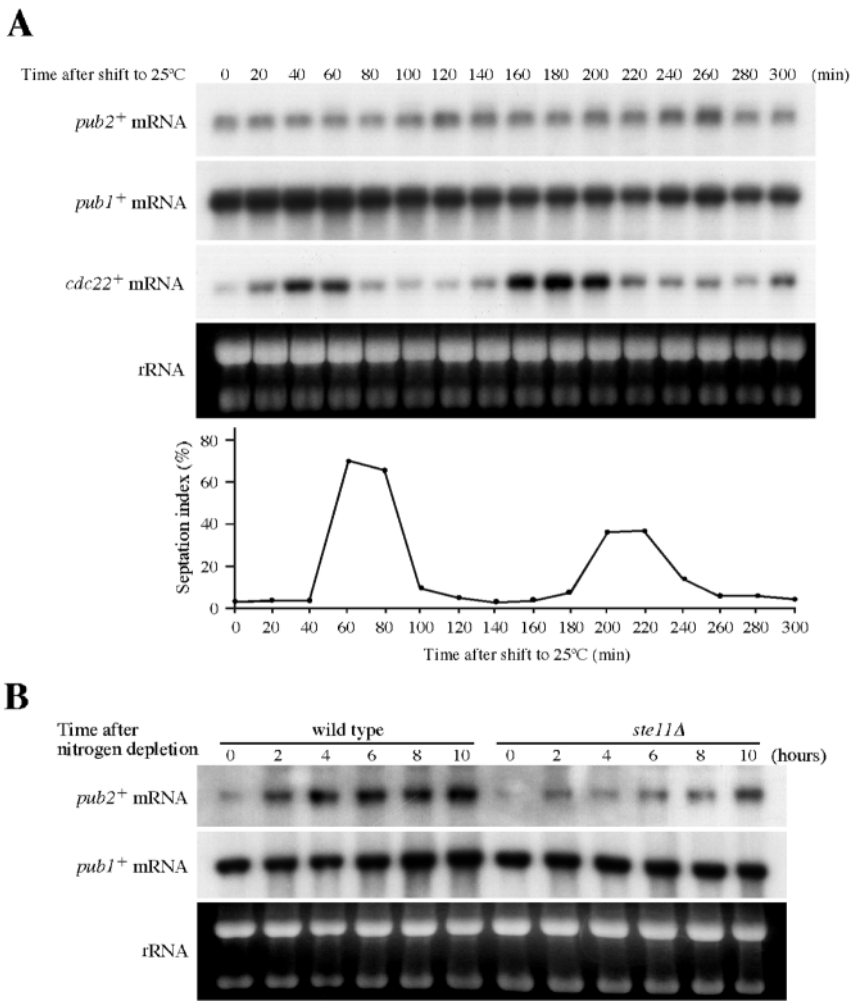

Fig. 2. Transcriptional regulation of *pub1⁺* and *pub2⁺*. (A) Northern analysis of *pub1⁺* and *pub2⁺* mRNA in mitotically growing cells. Synchronous cultures were attained by using the *cdc25-22* temperature-sensitive mutant K164-9 (see the Materials and Methods). A 2.3 kb fragment containing *pub1* and a 2.0 kb fragment containing *pub2* were used as hybridization probes. The *cdc22⁺* mRNA was also traced as an example whose levels fluctuate during cell cycle, peaking at the G1/S boundary (Hofmann et al., 1994). A 2.7 kb fragment containing *cdc22* was used as a probe. The septation index, which reaches a peak in G1/S phase, was determined in order to monitor progression of the cell cycle. The agarose gels were stained with ethidium bromide, and ribosomal RNAs were used as loading controls and size markers. (B) Northern analysis of *pub1⁺* and *pub2⁺* mRNA after nitrogen starvation. h^{90} wild-type (L968) and *ste11Δ* (KJ33-1A) strains were cultured in EMM2-N liquid sporulation medium. The same probes as in (A) were used.

Phenotypes of *pub2Δ*

To investigate functions of Pub2p, we disrupted the chromosomal *pub2⁺* gene by replacing a substantial part of the *pub2⁺* ORF with the *ura4⁺* cassette. Tetrad analysis of the heterozygous *pub2Δ* diploid cells revealed that the *pub2⁺* gene is not essential for vegetative growth. The size and shape of *pub2Δ* cells were not distinguishable from those of isogenic wild-type cells (data not shown). As *pub2⁺* transcription was stimulated by nitrogen depletion, we thought that *pub2Δ* might be defective in mating and/or sporulation. h^{90} *pub2Δ* cells were incubated in liquid sporulation medium (EMM2-N) to induce mating and sporulation. The frequency of zygotes and asci was scored after 20 and 40 hours in EMM2-N. The efficiency of conjugation and sporulation of *pub2Δ* cells was indistinguishable from that of wild-type cells (data not shown). Finally, we found that the germination efficiency of *pub2Δ* spores was also normal (data not shown). Thus, we conclude that *pub2⁺* is not required for sexual development.

We also examined the sensitivity of the *pub2Δ* mutant to other environmental stresses. The *pub2Δ* cells were exposed to

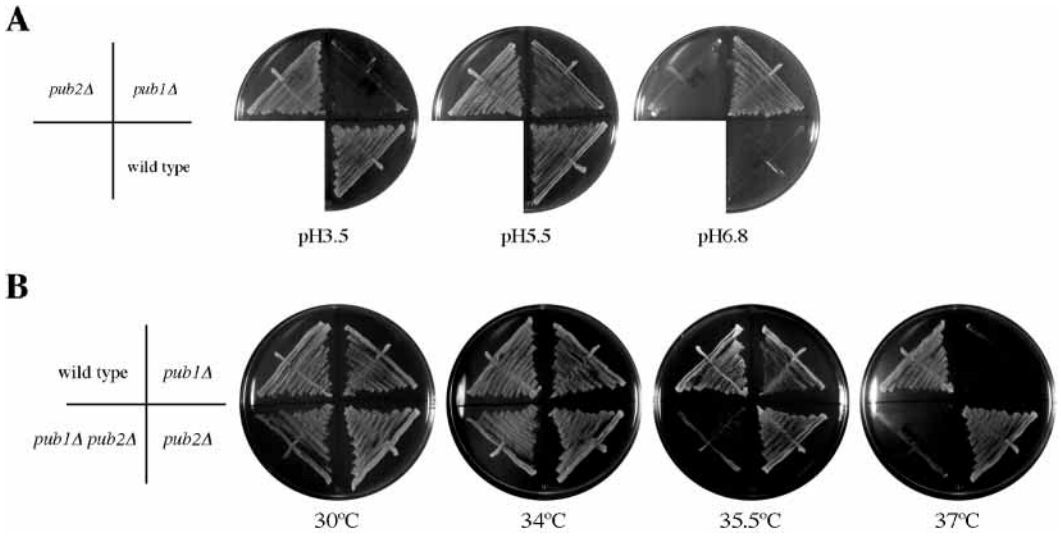

Fig. 3. Growth behavior of *pub1* and *pub2* disruptants. (A) pH sensitivity of leucine auxotrophic *pub*$^+$ (MM71-6B), *pub1Δ* (KKT81-3A) and *pub2Δ* (KKT85-8A) strains. These strains were grown for 5 days on EMM2 media at the indicated pH. (B) Colony formation at different incubation temperatures. L972 (wildtype), KKT83-9A (*pub1Δ*), KKT39-7C (*pub2Δ*) and KKT82-2D (*pub1Δ pub2Δ*) were incubated on YEA medium at 30°C, 34°C, 35.5°C or 37°C.

high and low incubation temperature (18-37°C), high osmolarity (1.2 M sorbitol) and high concentrations of salt solutions (1.2 M NaCl and 0.5 M $CaCl_2$). The growth of *pub2Δ* cells under these conditions was not different from that of wild-type cells. Saleki et al. (Saleki et al., 1997) have shown that the *pub1Δ* mutant is sensitive to low pH. Some auxotrophic strains are not able to grow at pH6.8, and *pub1Δ* can tolerate high pH (Karagiannis et al., 1999). We therefore examined whether *pub2Δ* cells showed similar traits. As shown in Fig. 3A, *pub2Δ* formed colonies on EMM2 minimal medium at pH 3.5, but not at pH 6.8, as do wild-type cells.

Overexpression of *pub2*$^+$

To gain insight into the function of Pub2p in vegetative cells, we overexpressed the epitope-tagged Pub2 (Pub2-HA) in wild-type cells. Overexpression was controlled by a strong *nmt1* promoter, which is thiamine repressible. After 16 hours of thiamine removal, Pub2-HA had accumulated as revealed by western blotting using an anti-HA monoclonal antibody 12CA5 (Fig. 4A, lane 1, 3). Cells overexpressing *pub2*$^+$ become elongated (Fig. 4Ba,c), and cell proliferation was repressed (Fig. 4C). Flow cytometric analysis revealed that most of the *pub2*$^+$-overexpressing haploid cells contained 2C DNA (Fig. 4D). Seemingly, substantial proportions of the culture showed DNA content higher than 2C. This might occur because of the remarkable elongation of *pub2*$^+$-overexpressing cells. Immunostaining with an anti-tubulin antibody (TAT-1) showed that the cells contained only cytoplasmic microtubules, indicating that they were in interphase (data not shown). Together, these results strongly suggest that the *pub2*$^+$-overexpressing cells arrested in the G2 phase of the cell cycle.

Nefsky and Beach (Nefsky and Beach, 1996) have demonstrated that Pub1p is involved in ubiquitination of Cdc25p, which dephosphorylates the phospho-Tyr^{15} of Cdc2p. It is possible that Pub2p may also ubiquitinate Cdc25p, as this would explain the Pub2-induced delay of G2 progression. If this is the case, levels of Cdc25p should be reduced in *pub2*$^+$-overexpressing cells. We incubated strain OM1715 harboring pREP1-*pub2*$^+$, in which Cdc25-6HA is integrated at the chromosomal *cdc25*$^+$ locus, in medium lacking thiamine. During the incubation, protein samples were removed and analyzed for Cdc25-6HA by an anti-HA monoclonal antibody (12CA5). Unexpectedly, levels of Cdc25-6HA increased when Pub2p was overproduced (Fig. 4E). Therefore, Pub2p is not likely to be involved in the degradation of Cdc25p. The accumulation of Cdc25p in *pub2*$^+$-overexpressing cells might be due to a secondary effect of cell cycle arrest in G2 phase. Accumulation of Cdc25p in G2 arrest cells has been reported (Moreno et al., 1990).

To address the mechanism of G2 arrest caused by Pub2 overproduction, we overexpressed the *pub2*$^+$ gene in *wee1-50* or *cdc25-22* temperature-sensitive mutants. Wee1p is a kinase that inhibits G2/M transition by phosphorylating Cdc2p. Therefore, the *wee1* mutation accelerates the commitment to M phase, resulting in a small cell size. Pub2p was overproduced in the *wee1-50* cells. At both restrictive and permissive temperatures, *wee1-50* cells failed to grow when *pub2*$^+$ was overexpressed (data not shown). Cdc25p regulates the onset of M phase by dephosphorylating Cdc2p. If Pub2-induced G2 arrest is mediated by Cdc25p, the *cdc25-22* temperature-sensitive mutation might strengthen the G2 arrest phenotype. However, growth inhibition and cell elongation were not observed in *cdc25-22* cells at 26 or 30°C unless *pub2*$^+$ on a multicopy expression vector, pREP1, was overexpressed (data not shown). These results suggest that the G2 arrest phenotype arising from *pub2*$^+$ overexpression is caused by a mechanism that is not related to Wee1 kinase and Cdc25 protein phosphatase.

Another possibility is that Pub2p overproduction activates checkpoint machinery, leading to cell cycle arrest at G2 phase. If so, the G2 arrest phenotype caused by Pub2 overproduction might depend on the checkpoint genes. To inspect this possibility, we overexpressed *pub2*$^+$ in *rad1Δ*, *rad3Δ*, *cds1Δ*

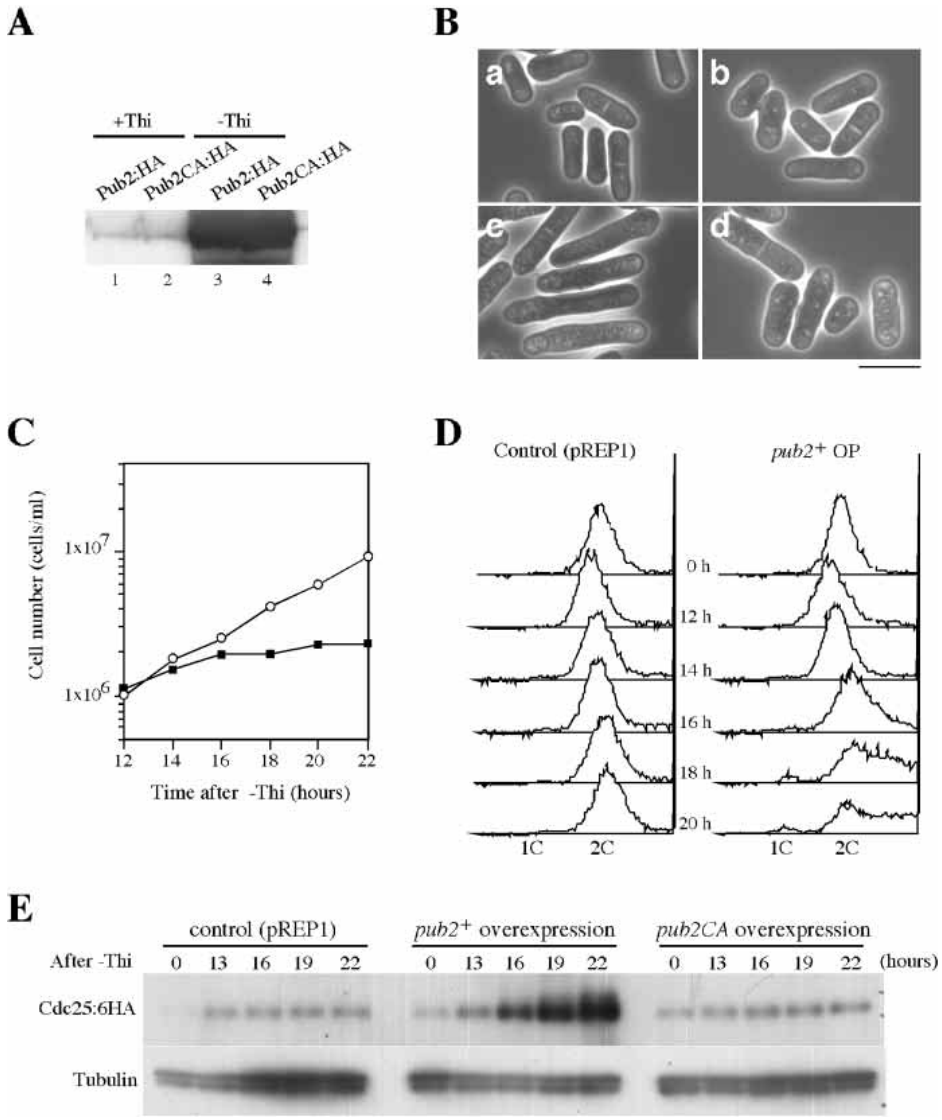

Fig. 4. Effects of overexpression of *pub2*$^+$. (A) h^- wild-type cells (MM72-11C) bearing pREP1-*pub2-HA* or pREP1-*pub2CA-HA* were grown up to mid-log phase in EMM2 containing 20 μM thiamine (lane 1, 2) and transferred to thiamine-free EMM2 to overexpress Pub2-HA and Pub2CA-HA (lane 3, 4). Western blotting was conducted with the anti-HA antibody (12CA5). (B) High levels of overexpression of *pub2*$^+$ induces cell elongation. The MM72-11C strain was transformed with plasmid pREP1-*pub2-HA* (a and c) or pREP1-*pub2CA-HA* (b and d). Cells were grown up to mid-log phase in EMM2 containing 20 μM thiamine (a and b) and transferred to thiamine-free EMM2 (c and d). After incubation for 18 hours, cell size was observed under phase-contrast optics. Bar, 10 μm. (C) Cell multiplication after thiamine removal. MM72-11C cells transformed with either pREP1 (control) or pREP1-*pub2*$^+$, were grown in EMM2 medium supplemented with 20 μM thiamine to mid-log phase and then transferred to EMM2 without thiamine to induce expression of *pub2*$^+$. *pub2*$^+$-overexpressing cells (closed squares) stopped growing after 16 hours, whereas control cells (open circles) continued to multiply. (D) Flow cytometric analysis for *pub2*$^+$-overexpressing cells. Cells harboring pREP1-*pub2*$^+$ or pREP1 were grown up to mid-log phase in EMM2 containing 20 μM thiamine and transferred to EMM2 without thiamine. Samples were withdrawn at the indicated time points. (E) Accumulation of Cdc25-6HA in *pub2*$^+$-overexpressing cells. The OM1715 strain carrying either pREP1 (an empty vector), pREP1-*pub2*$^+$ or pREP1-*pub2CA* was incubated in EMM2 without thiamine. Western blotting with anti-HA antibody (12CA5) revealed Cdc25-6HA. Tubulin was detected by anti-α-tubulin antibody (TAT-1) and was used as an internal reference marker.

and *chk1Δ*. The cell elongation phenotype was observed in either disruptant (data not shown), indicating that *pub2*$^+$ overexpression activates neither DNA replication checkpoint nor DNA damage checkpoint. These observations imply that *pub2*$^+$ overexpression affects G2/M transition by an unknown mechanism.

Genetic interaction between *pub1*$^+$ and *pub2*$^+$

The *pub2*-null mutant exhibited no apparent defects in vegetative growth. The expression level of *pub1*$^+$ was higher than that of *pub2*$^+$ in vegetative cells (Fig. 2A). We therefore speculated that Pub1p and Pub2p might share an overlapping function. To examine this possible redundancy between Pub1p and Pub2p, we investigated the growth properties of *pub1Δ pub2Δ* cells. We found that this double disruptant was viable at 30°C (Fig. 3B), and its morphology was not different from wild-type cells. Nefsky and Beach (Nefsky and Beach, 1996) have reported that cells bearing *pub1Δ* divide at a noticeably smaller cell size but form colonies even at 37°C. We constructed a *pub1*-null mutant, *pub1::ura4*$^+$, in which virtually the whole sequence of the *pub1* ORF was deleted, and examined the temperature sensitivity of this null mutant. This *pub1Δ* mutant was unable to form colonies on complete medium at 37°C (Fig. 3B). We next carefully examined the effect of incubation temperature on the growth of single and double disruptant strains. As shown in Fig. 3B, the *pub1Δ pub2Δ* double mutant did not form colonies, even at 35.5°C, whereas both *pub1Δ* and *pub2Δ* single mutants grew well. This observation indicates that, at least in vegetative cells, Pub1p and Pub2p carry out a similar function.

To study further the genetic relationship between *pub1*$^+$ and *pub2*$^+$, we tested whether overexpression of *pub2*$^+$ can override the temperature sensitivity of *pub1Δ* cells. As mentioned above, strong overexpression of Pub2p is toxic to cells, the *pub2*$^+$ gene was moderately expressed by a weaker *nmt41* promoter. Neither cell elongation nor growth arrest was detected when Pub2p was expressed from *nmt41* promoter in

a wild-type background. The *pub1Δ* cells, which were transformed with pREP41-*pub2*$^+$, were incubated at 37°C for 3 days. As shown in Fig. 7B, cells moderately overexpressing *pub2*$^+$ grew, in contrast to control *pub1Δ* cells bearing the empty vector pREP41. This result confirms that Pub2p must share some overlapping functions with Pub1p.

Localization of Pub2p

It has been shown that Nedd4p is localized to the plasma membrane via its C2 domain (Plant et al., 1997; Plant et al., 2000). However, a C2-domain-truncated variant of Rsp5p still associates with the membrane fraction (Dunn and Hicke, 2001); therefore, the exact role of the C2 domain in the Nedd4/Rsp5 family of ubiquitin ligases remains controversial. Notably, Pub2p is unique among the other family members in that it lacks a C2 domain.

To define the cellular localization of Pub2p, a Pub2-GFP fusion protein was expressed by the weakest version of the *nmt* promoter, *nmt81*. A multicopy plasmid containing the *nmt81-pub2*$^+$*-GFP* fusion gene was introduced into wild-type strain MM72-11C. As a negative control, pREP81-GFP was also introduced into the same strain. As the localization of Pub1p has not been documented so far, we thus also observed the fluorescent signals in the cells expressing the *nmt81-pub1*$^+$*-GFP* fusion gene. Like the authentic Pub2 protein, overexpression of Pub2-GFP induced growth arrest and cell elongation (data not shown). The *nmt41-pub1*$^+$*-GFP* fusion gene complemented the temperature sensitivity of *pub1Δ* (data not shown). These results indicated that both Pub1-GFP and Pub2-GFP were functional. As Fig. 5A shows, Pub1-GFP localized to the plasma membrane and to unidentified cytoplasmic bodies. The latter structures might be endosomes, because a similar staining image was seen for GFP-fused membrane-bound proteins targeted to vacuoles (Katzmann et al., 2001; Morishita et al., 2002). Basically the same staining pattern was observed, when Pub1-GFP was expressed by the authentic promoter. On the other hand, Pub2-GFP was present both in the nuclei and in the cytoplasm. Furthermore, we noted that Pub2-GFP localized to the cell surface in the polar regions (Fig. 5Ac). This observation suggests that Pub2p localizes to the plasma membrane independently of a C2 domain. It is plausible that the cell-surface localization of other members of the Nedd4/Rsp5 family may be attained in a manner both dependent on, and independent of, the C2 domain.

Localization of Pub2-GFP was observed with cells expressing the fusion gene by the *nmt81* promoter because the expression by the native *pub2* promoter was too low to visualize any fluorescent signals. Therefore, the subcellular distribution of Pub2p was explored by subcellular fractionation using a Pub2-HA integrant strain (KKT87). As shown in Fig. 5B, Pub2-HA was detected not only in the soluble fraction (S100) but also in the low (P13) and high speed (P100) pellet fractions. Treatment of lysates with a high concentration of salts (e.g., 0.5 M NaCl) increased the proportion of Pub2-HA in S100, although treatment with 1% Triton X-100 showed no such consequence (data not shown). These results strongly suggested that a portion of Pub2-HA was associated with cellular structures, supporting the microscopic observation that Pub2-GFP was present at the plasma membrane in the polar regions.

Pub2 is thiol-ubiquitinated in vivo

HECT-type ubiquitin ligases directly bind to a ubiquitin molecule through the conserved cysteine residue located in the HECT domain (Scheffner et al., 1995). In Pub2p, this residue corresponds to cysteine 639 (Fig. 1B). To confirm the significance of this residue in the function of Pub2p, we generated an alanine mutation at Cys639 (Pub2CA). To test the function of Pub2CA, we noted the fact that the high overexpression of *pub2*$^+$ is toxic. We overexpressed Pub2CA in *h*$^-$ wild-type cells under the control of the strong *nmt1* promoter. The accumulation of Pub2CA was confirmed by western blotting (Fig. 4A, lane 2, 4). Overexpression of Pub2CA had little influence on cell elongation (Fig. 4Bb,d) and cell proliferation (data not shown). Consistent with this observation, no accumulation of Cdc25-6HA was found in the *pub2CA*-overexpressing cells (Fig. 4E). These observations strongly suggest that Pub2p requires the conserved Cys639 residue for correct function and so acts as a HECT-type ubiquitin-protein ligase.

Next, we examined whether Pub2p forms a thioester linkage with ubiquitin via Cys639. As we predicted that putative Pub2p-Ub intermediates would be highly unstable, we fused only the HECT domain, which lacks a putative substrate-binding site, to an Myc epitope (HECT^{Pub2}-Myc) instead of generating a full-length Pub2-Myc fusion protein. The HECT^{Pub2}-Myc fusion protein was co-expressed with a GST-Ub fusion protein in wild-type cells. GST-Ub was pulled down from cell lysates by glutathione beads, and the precipitate was

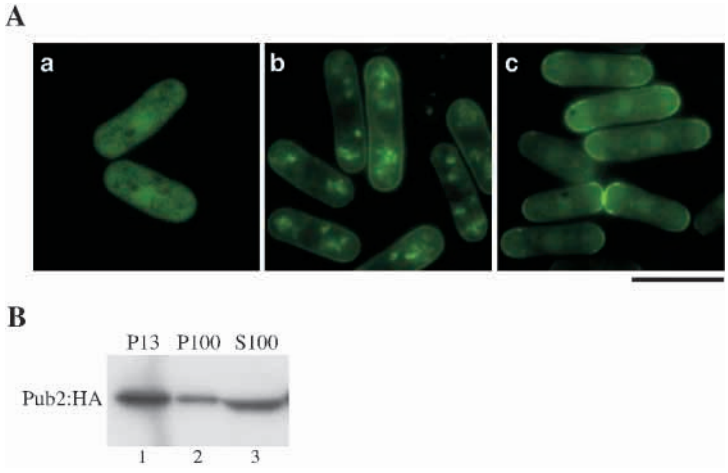

Fig. 5. Localization of Pub2p. (A) Localization of Pub1-GFP and Pub2-GFP. pREP81-based plasmids carrying GFP, Pub1-GFP or Pub2-GFP fusion constructs were introduced into the wild-type strain MM72-11C. After a 12 hour incubation on thiamine-free EMM2, cells were observed under a fluorescence microscope. (a) GFP, (b) Pub1-GFP and (c) Pub2-GFP. Scale bar, 10 μm. (B) Fractionation of cell lysates. Cell lysates were prepared from the Pub2-HA integrant (KKT87). P13 is a precipitate after centrifugation at 13,000 ***g***, and P100 and S100 fractions are the sediment and the supernatant, respectively, after the second centrifugation at 100,000 ***g***. Pub2-HA was detected by western blotting with anti-HA antibody 3F10.

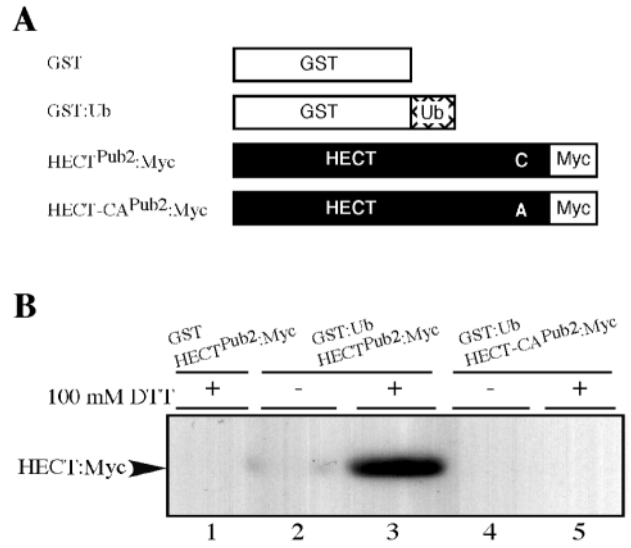

Fig. 6. Conjugation of Pub2p with ubiquitin. (A) A schematic representation of the fusion constructs. Ubiquitin (Ub) was fused to the C-terminus of glutathione-S-transferase (GST). A Myc epitope tag was fused to the C-terminus of the HECT domain of wild-type Pub2p (HECT^{Pub2}-Myc) or to that of Cys639Ala mutant HECT domain (HECT CA^{Pub2}:Myc). (B) Association of the Pub2 HECT domain with ubiquitin. GST-Ub and HECT-Myc fusion proteins were co-expressed in wild-type cells (MM72-1D), which were incubated in thiamine-free EMM2 for 20 hours. GST-fusion protein was pulled down from crude cell-free extracts by glutathione beads. Pulled-down proteins were resolved by SDS-PAGE on 10% gels after treatment (+) or mock treatment (–) with 100 mM DTT. Western blots were probed with an anti-Myc antibody (9E10).

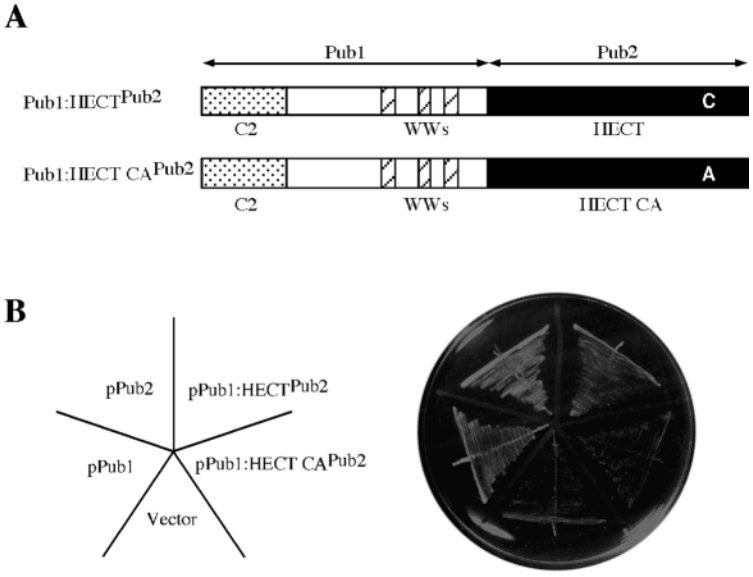

Fig. 7. The in vivo functional assay of a putative catalytic HECT domain of Pub2p. (A) Schematic representation of chimeric proteins of the Pub2 HECT domain fused with the N-terminal portion of Pub1p. 'HECT^{Pub2}' and 'HECT CA^{Pub2}' indicate the HECT domain of wild-type and the Cys639Ala mutant Pub2p, respectively. (B) Complementation of the temperature-sensitive growth of *pub1Δ* by the chimeric proteins. pREP41-based plasmids carrying each chimeric construct were transformed into the KKT81-3A strain, which was grown on thiamine-free EMM2 plates at 37°C for 3 days.

treated with 100 mM DTT to cleave any thioester bond that might have been formed between HECT^{Pub2}-Myc and GST-Ub. The presence of HECT^{Pub2}-Myc protein was detected by western analysis with an anti c-Myc antibody (9E10). When HECT^{Pub2}-Myc and GST-Ub were co-expressed, detection of the HECT^{Pub2}-Myc signal was dependent on addition of DTT (Fig. 6, lane 2, 3). However, when HECT^{Pub2}-Myc and GST alone were co-expressed, the HECT^{Pub2}-Myc signal was not detected at all (Fig. 6, lane 1). Next, we tested whether the alanine mutant HECT tagged with Myc (HECT-CA^{Pub2}-Myc) binds to GST-Ub. As shown in Fig. 6 (lane 4, 5), the alanine mutant HECT did not associate with ubiquitin proteins. Together, these results support the notion that Cys639 of Pub2p forms a thioester bond with ubiquitin.

HECT^{Pub2} might serve as a catalytic domain

Finally, we addressed the question of whether or not Pub2p has ubiquitin-protein ligase activity. It has been established that Pub1p is a HECT-type ubiquitin-protein ligase. We reasoned that if the HECT domain of Pub2p (HECT^{Pub2}) has catalytic activity, then a protein chimera of Pub1p, whose HECT domain is replaced with HECT^{Pub2}, would be functional. This chimeric protein (Pub1-HECT^{Pub2}; Fig. 7A) was overexpressed from the attenuated *nmt41* promoter in temperature-sensitive *pub1Δ* cells. As shown in Fig. 7B, Pub1-HECT^{Pub2} suppressed the temperature sensitivity of *pub1Δ* cells. By contrast, Pub1-HECT-CA^{Pub2} did not complement the temperature sensitivity. These experiments suggest that the wild-type HECT domain of Pub2p can act as the catalytic domain of Pub1 ubiquitin ligase and that the Cys639 residue is essential for this catalytic activity.

Discussion

Protein-ubiquitin ligases classified as belonging to the Nedd4/Rsp5 subfamily contain a highly conserved HECT domain. Proteins of this subfamily are also characterized by their domain structure. Most of the Nedd4/Rsp5-related proteins contain a C2 domain at the N-terminus, several WW domains in the central region and a HECT domain at the C-terminus (reviewed by Harvey and Kumar, 1999; Rotin et al., 2000). The budding yeast *S. cerevisiae* has a single essential gene, *RSP5*, that represents this family of ubiquitin-protein ligases. By contrast, at least seven genes encoding Nedd4/Rsp5 family proteins have been identified in the human genome. As reported here, there are three Nedd4/Rsp5-related genes in the genome of the fission yeast *S. pombe*.

We have presented evidence to show that Pub2p is thiol-ubiquitinated (Fig. 6). In terms of domain structure, Pub2p is unique among the Nedd4/Rsp5 protein family, as it lacks a C2 domain and contains only one WW domain (Fig. 1A). We found that a single knock-out of any of the *pub* genes does not render the cells non-viable; however, a *pub1Δ pub3Δ* double knock-out mutant is non-viable, indicating that Pub1p and Pub3p have redundant functions (K.K.T. and C.S., unpublished). By contrast, our results suggested that Pub2p shares only partially overlapping function with

Pub1p. The *pub1*$^+$ gene is constitutively transcribed in vegetative cells at relatively high levels, whereas the *pub2*$^+$ gene is transcribed at low levels. Interestingly, the transcription of *pub2*$^+$ is greatly enhanced by nitrogen starvation (Fig. 2). The abundance of *pub3*$^+$ mRNA is extremely low in vegetative cells and is not increased by nitrogen starvation (K.K.T. and C.S., unpublished). Such different control of gene expression suggests that the Nedd4/Rsp5-related proteins of *S. pombe* have different cellular functions during vegetative growth under various environmental conditions. Moderate overexpression of the *pub2*$^+$ gene suppressed the temperature sensitivity of *pub1Δ* cells (Fig. 7B). Conversely, disruption of *pub2*$^+$ intensified the temperature-sensitivity phenotype of *pub1Δ* cells (Fig. 3B). These findings suggest that *pub1* and *pub2* may have partly overlapping functions.

On the basis of the following observations, Pub1p has been assumed to have a role in controlling the cell cycle. *Pub1Δ* cells prematurely traverse the G2/M boundary. A *pub1Δ wee1-50*^{ts} double mutant is non-viable at the restrictive temperature, probably owing to mitotic catastrophe. Pub1 ubiquitin ligase ubiquitinates Cdc25 phosphatase, a crucial inducer of M phase, and the level of Cdc25p is elevated in *pub1Δ* cells (Nefsky and Beach, 1996). Together, these facts support the idea that Pub1p is a negative regulator of G2/M transition. We therefore addressed whether Pub2p is also involved in cell cycle control. Although *pub2* disruptants were normal in their progression through the cell cycle, high overexpression of *pub2*$^+$ caused a marked cell elongation and the repression of cell multiplication (Fig. 4A-D). Therefore, Pub2p, in conjunction with Pub1p, possibly regulates the progression of G2 or the start of M-phase. However, as overexpression of *pub2*$^+$ did not reduce levels of Cdc25p (Fig. 4E), it is unlikely that Pub2p performs precisely the same functions as Pub1p in cell cycle regulation.

It should be noted that the phenotype of *pub1* mutants is pleiotropic. They are sensitive to acidic pH (pH 3.5) and have reduced repression of leucine uptake in the presence of ammonium ions (Saleki et al., 1997; Karagiannis et al., 1999). These phenotypes imply that Pub1p is involved in ubiquitination and degradation of leucine-specific permease in the plasma membrane (Karagiannis et al., 1999). This notion is strongly supported by our observation in this study that Pub1-GFP is localized to the cell membrane (Fig. 5Ab). In budding yeast, Rsp5p is also necessary for adaptation to nutrient limitation. When exogenous amino acids are limited, *S. cerevisiae* cells repress the pathway that regulates the uptake of specific amino acids. Under starvation conditions, several amino-acid-specific permeases such as tryptophan permease (Tat2p) and histidine permease (Hip1p) are subjected to Rsp5-mediated ubiquitination, targeted to vacuoles and degraded therein. Conversely, the broad-range amino-acid permease Gap1p is targeted to the plasma membrane and stabilized (Hein et al., 1995; Beck et al., 1999). For fission yeast, there are no data concerning the regulation of amino-acid permeases in nitrogen-limited medium. It is possible that Pub2p is responsible for regulating the ubiquitination of membrane proteins such as amino acid permeases. Our present results showing that Pub2p appears to be located in the plasma membrane and that *pub2*$^+$ is preferentially expressed under starvation conditions favor such a possibility. Future studies should examine whether Pub2p is implicated in the ubiquitination of membrane proteins, especially under starvation conditions.

We are grateful to Kenji Kitamura of Hiroshima University, Koichi Tanaka of the University of Tokyo and Taro Nakamura of Osaka City University for valuable discussion during the course of this work. We thank Paul Russell of the Scripps Research Institute for a gift of *S. pombe* strain, OM1715 and K. Gull of University of Manchester for antibodies. We also acknowledge members of this laboratory for helpful discussion. This study was supported by Grant-in-Aid for Scientific Research on Priority Areas (C) 'Genome Biology' from the Ministry of Education, Science, Sports and Culture of Japan to C.S.

References

Alfa, C., Fantes, P., Hyams, J., McLeod, M. and Warbrick, E. (eds) (1993). *Experiments with Fission Yeast: A Laboratory Course Manual*, pp. 48-51. New York: Cold Spring Harbor Laboratory Press.

Beck, T., Schmidt, A. and Hall, M. N. (1999). Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. *J. Cell Biol.* **146**, 1227-1237.

Bedford, M. T., Sarbassova, D., Xu, J., Leder, P. and Yaffe, M. B. (2000). A novel Pro-Arg motif recognized by WW domains. *J. Biol. Chem.* **275**, 10359-10369.

Dunn, R. and Hicke, L. (2001). Domains of the Rsp5 ubiquitin-protein ligase required for receptor-mediated and fluid-phase endocytosis. *Mol. Biol. Cell* **12**, 421-435.

Dunphy, W. G. and Kumagai, A. (1991). The cdc25 protein contains an intrinsic phosphatase activity. *Cell* **67**, 189-196.

Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13.

Galan, J. M., Moreau, V., André, B., Volland, C. and Haguenauer-Tsapis, R. (1996). Ubiquitination mediated by the Npi1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. *J. Biol. Chem.* **271**, 10946-10952.

Gautier, J., Solomon, M. J., Booher, R. N., Fernando Bazan, J. and Kirschner, M. W. (1991). cdc25 is a specific tyrosine phosphatase that directly activates p34^{cdc2}. *Cell* **67**, 197-211.

Gould, K. L. and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast *cdc2*$^+$ protein kinase regulates entry into mitosis. *Nature* **342**, 39-45.

Grimm, C., Kohli, J., Murray, J. and Maundrell, K. (1988). Genetic engineering of *Schizosaccharomyces pombe*: A system for gene disruption and replacement using the *ura4* gene as a selectable marker. *Mol. Gen. Genet.* **215**, 81-86.

Gutz, H., Heslot, H., Leupold, U. and Loprieno, N. (1974). *Schizosaccharomyces pombe*. In *Handbook of Genetics*, vol. 1 (ed. R. D. King), pp. 395-446. New York: Plenum Press.

Harvey, K. F. and Kumar, S. (1999). Nedd4-like proteins: an emerging family of ubiquitin-protein ligases implicated in diverse cellular functions. *Trends Cell Biol.* **9**, 166-169.

Hein, C., Springael, J.-Y., Volland, C., Haguenauer-Tsapis, R. and André, B. (1995). *NPI1*, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. *Mol. Microbiol.* **18**, 77-87.

Hereford, L., Fahrner, K., Woolford, J., Jr, Rosbash, M. and Kaback, D. B. (1979). Isolation of yeast histone genes H2A and H2B. *Cell* **18**, 1261-1271.

Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425-479.

Hofmann, J. F. X. and Beach, D. (1994). cdt1 is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis. *EMBO J.* **13**, 425-434.

Hicke, L., Zanolari, B. and Reizman, H. (1998). Cytoplasmic tail phosphorylation of the α-factor receptor is required for its ubiquitination and internalization. *J. Cell Biol.* **141**, 349-358.

Huibregtse, J. M., Scheffner, M., Beaudenon, S. and Howley, P. M. (1995). A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc. Natl. Acad. Sci. USA* **92**, 2563-2567.

Huibregtse, J. M., Yang, J. C. and Beaudenon, S. L. (1997). The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase. *Proc. Natl. Acad. Sci. USA* **94**, 3656-3661.

Ikemoto, S., Nakamura, T., Kubo, M. and Shimoda, C. (2000). *S. pombe* sporulation-specific coiled-coil protein Spo15p is localized to the spindle pole body and is essential for its modification. *J. Cell Sci.* **113**, 545-554.

Jackson, P. K., Eldridge, A. G., Freed, E., Furstenthal, L., Hsu, J. Y., Kaiser, B. K. and Reimann, J. D. R. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. *Trends Cell. Biol.* **10**, 429-439.

Jensen, R., Sprague, G. F., Jr and Herskowitz, I. (1983). Regulation of yeast mating-type interconversion: feedback control of HO gene expression by the mating-type locus. *Proc. Natl. Acad. Sci. USA* **80**, 3035-3039.

Karagiannis, J., Saleki, R. and Young, P. G. (1999). The *pub1* E3 ubiquitin ligase negatively regulates leucine uptake in response to NH_4^+ in fission yeast. *Curr. Genet.* **35**, 593-601.

Katzmann, D. J., Babst, M. and Emr, S. D. (2001). Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell* **106**, 145-155.

Kay, B. K., Williamson, M. P. and Sudol, M. (2000). The importance of being proline: the interaction of proline-rich motifs signaling proteins with their cognate domains. *FASEB J.* **14**, 231-241.

Lundgren, K., Walworth, N., Booher, R., Dembski, M., Kirschner, M. and Beach, D. (1991). Mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. *Cell* **64**, 1111-1122.

Maundrell, K. (1993). Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene* **123**, 127-130.

Millar, J. B. A., Lenaers, G. and Russell, P. (1992). Pyp3 PTPase acts as a mitotic inducer in fission yeast. *EMBO J.* **11**, 4933-4941.

Moreno, S., Nurse, P. and Russell, P. (1990). Regulation of mitosis by cyclic accumulation of $p80^{cdc25}$ mitotic inducer in fission yeast. *Nature* **344**, 549-552.

Moreno, S., Klar, A. and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* **194**, 795-823.

Morishita, M., Morimoto, F., Kitamura, K., Koga, T., Fukui, Y., Maekawa, H., Yamashita, I. and Shimoda, C. (2002). Phosphatidylinositol 3-phosphate 5-kinase is required for the cellular response to nutritional starvation and mating pheromone signals in *Schizosaccharomyces pombe*. *Genes. Cells* **7**, 199-216.

Nakamura, T., Nakamura-Kubo, M., Hirata, A. and Shimoda, C. (2001). The *Schizosaccharomyces pombe spo3*$^+$ gene is required for assembly of the forespore membrane and genetically interacts with *psy1*$^+$-encoding syntaxin-like protein. *Mol. Biol. Cell* **12**, 3955-3972.

Nefsky, B. and Beach, D. (1996). Pub1 acts as an E6-AP-like protein ubiquitin ligase in the degradation of cdc25. *EMBO J.* **15**, 1301-1312.

Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K. and Okayama, H. (1990). High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **18**, 6485-6489.

Plant, P. J., Yeger, H., Staub, O., Howard, P. and Rotin, D. (1997). The C2 domain of the ubiquitin protein ligase Nedd4 mediates Ca^{2+}-dependent plasma membrane localization. *J. Biol. Chem.* **272**, 32329-32336.

Plant, P. J., Lafont, F., Lecat, S., Verkade, P., Simons, K. and Rotin, D. (2000). Apical membrane targeting of Nedd4 is mediated by an sssociation of its C2 domain with Annexin XIIIb. *J. Cell Biol.* **149**, 1473-1483.

Rothstein, R. (1983). One-step gene disruption in yeast. *Methods Enzymol.* **101**, 202-211.

Rotin, D., Staub, O. and Haguenauer-Tsapis, R. (2000). Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. *J. Membr. Biol.* **176**, 1-17.

Russell, P. and Nurse, P. (1986). *cdc25*$^+$ functions as an inducer in the mitotic control of fission yeast. *Cell* **45**, 145-153.

Saleki, R., Jia, Z., Karagiannis, J. and Young, P. G. (1997). Tolerance of low pH in *Schizosaccharomyces pombe* requires a functioning *pub1* ubiquitin ligase. *Mol. Gen. Genet.* **254**, 520-528.

Scheffner, M., Nuber, U. and Huibregtse, J. M. (1995). Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373**, 81-83.

Staub, O., Dho, S., Henry, P. C., Correa, J., Ishikawa, T., McGlade, J. and Rotin, D. (1996). WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na^+ channel deleted in Liddle's syndrome. *EMBO J.* **15**, 2371-2380.

Sugimoto, A., Iino, Y., Maeda, T., Watanabe, Y. and Yamamoto, M. (1991). *Schizosaccharomyces pombe ste11*$^+$ encodes a transcription factor with an HMG motif that is a critical regulator of sexual development. *Genes Dev.* **5**, 1990-1999.

Thomas, P. S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* **77**, 5201-5205.

Varshavsky, A. (1997). The ubiquitin system. *Trends Biochem. Sci.* **22**, 383-387.