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Identification of a 26S Proteasome-Associated UCH in Fission Yeast

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We have identified a 26S proteasome-associated ubiquitin carboxyl-terminal hydrolase (UCH) in *Schizosaccharomyces pombe.* The gene (designated *uch2*$^+$) encodes a protein containing a UCH catalytic domain at its N-terminus and a short extension at its C-terminus. *uch2*$^+$ is nonessential as the *uch2* null mutant strain showed no significant difference from the wild-type strain. The GFP-tagged Uch2p is localized predominantly to the nuclear periphery, which is similar to the 26S proteasome localization. Deletion of the C-terminal extension of Uch2p resulted in a drastic change of its subcellular localization: it showed a generally diffused distribution instead of a perinuclear pattern. Glycerol gradient centrifugation analysis and coimmunoprecipitation studies of fission yeast extracts using anti-Mts4p antiserum suggest that Uch2p is associated with the 26S proteasome and the association of Uch2p with the 26S proteasome is mediated by its C-terminal extension.

Key Words: **ubiquitin; ubiquitin C-terminal hydrolase; 26S proteasome; fission yeast.**

The covalent attachment of ubiquitin to a variety of cellular proteins is thought to regulate cellular processes such as elimination of abnormal proteins, regulation of chromosome structure and segregation, cell-cycle progression, receptor-mediated signal transduction, gene expression, protein localization, antigen presentation, and the stress response (1, 2).

Polyubiquitinated cellular proteins are degraded by the 26S proteasome, a ~2000-kDa proteolytic complex (3). The 26S proteasome contains a barrel-shaped proteolytic core complex (the 20S proteasome) and the 19S regulatory complex (also known as PA700) attached to one or both ends of the 20S proteasome (4). The structure and function of the 20S proteasome have been studied extensively. The proteolytic activity of the 20S proteasome is regulated by the 19S regulatory complex (5). Dan Finley and co-workers found that the 19S regulatory complex could be dissociated into two subcomplexes in *S. cerevisiae:* the base and the lid (6). Genetic and biochemical studies on *Saccharomyces cerevisiae, Drosophila,* and mammalian cells have identified ~20 subunits in the 19S regulatory complex. It contains 6 distinct ATPases and ~14 non-ATPase subunits (7). A major challenge of research on the 19S regulatory complex is to define the exact roles of each of its subunits.

Ubiquitination of proteins is reversible. Modified proteins are deubiquitinated by deubiquitinating enzymes (DUBs). These proteases specifically hydrolyze peptide bonds or isopeptide bonds after the Gly76 of ubiquitin. They may be involved in the processing of primary ubiquitin gene products, salvage of ubiquitin that has been trapped by reaction with small cellular nucleophiles, editing of ubiquitinated proteins and disassembly of the polyubiquitin degradation signal (8). Two classes of DUBs are known. First, ubiquitin carboxyl-terminal hydrolases (UCHs), are relatively small (<40 kDa) proteases which cleave ubiquitin with short (<20 amino acids) C-terminal extension (8). Second, ubiquitin-specific processing proteases (UBPs), constitute a large family of proteases with molecular sizes ranging from 50 to 250 kDa. UBPs cleave ubiquitin that are attached to larger peptides or proteins or attached to proteins via isopeptide bonds (9). The UCH family exhibits no apparent homology to the UBP family. More than 90 DUBs have been identified. These DUBs have been shown to be involved in many biologically important processes including growth, differentiation, oncogenesis and chromosome structure regulation (10). A novel protease (BAP1) with a UCH domain and a number of additional motifs was identified recently. BAP1 binds to the RING finger domain of the Breast/Ovarian Cancer Susceptibility Gene 1 product

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(BRCA1) and enhances BRCA1-mediated inhibition of breast cancer cell growth (11).

Recently, *S. cerevisiae* Doa4p/Ubp4p was found to partially copurify with the 26S proteasome and the association of Doa4p with the 26S proteasome is important for its function (12). Doa4p functions late in the ubiquitin-26S proteasome pathway, possibly by deubiquitinating polyubiquitin-substrate intermediates associated with the 26S proteasome. However, no integral DUB in the 26S proteasome was reported in *S. cerevisiae*. In this paper we report that the *S. pombe* Uch2p is closely associated with the 26S proteasome and probably is an integral subunits in the 26S proteasome. The association of *S. pombe* Uch2p with the 26S proteasome is mediated by its short C-terminal extension.

MATERIALS AND METHODS

Strains, media, and genetic techniques. All *S. pombe* strains were derived from the wild-type heterothalic strains *972h*$^-$ and *975h*$^+$. Standard genetic manipulation and media were as described (13). Yeast transformation was carried out by electroporation (14). Cultures were grown at 30°C except when indicated.

uch2$^+$ *gene disruption.* For *uch2*$^+$ gene disruption, the entire ~1 kb coding region of *uch2*$^+$ was replaced with the 1.8 kb *S. pombe ura4*$^+$ gene. A linearized DNA cassette consisting of the *uch2::ura4*$^+$ allele, retaining approximately 0.5 kb of flanking sequence to provide homology on both sides, was transformed into the wild type diploid, MBY257 (*h*$^-$, *his3-D1, ade6-210, ura4D-18, leu1-32*)/MBY266 (*h*$^+$, *his3-D1, ade6-216, ura4-D18, leu1-32*) for integration into the *uch2*$^+$ locus by homologous recombination. A diploid strain (LTY001) carrying a replacement of one of the copies of *uch2*$^+$ by the *uch::ura4*$^+$ allele, was identified by screening uracil prototrophic colonies by polymerase chain reaction (PCR). The diploid strain was sporulated and tetrads were dissected on rich media. The stable null mutant strain (*uch2-1*) with uracil prototrophy was further confirmed by PCR analysis.

Generation of gfp-tagged constructs. The entire coding region of the uch2$^+$ gene was amplified from wild-type genomic DNA by PCR and cloned into a modified pJK210 vector (15) that incorporated a green fluorescent protein (GFP) cDNA (*gfp*) in its cloning sites (N. Naqvi and M. K. Balasubramanian, unpublished). This resulted in the entire *uch2*$^+$ coding region linking in-frame with the *gfp* gene at its 3′ end in the pJK210 vector. The construct was linearized with *Eco*RI which cut at a unique site within the *uch2*$^+$ coding sequence and transformed into MBY192 (*h*$^-$, *ura4-D18, leu1-32*). Transformants that were *ura4*$^+$ were selected. Integration of the *uch2*$^+$*-gfp* at the *uch2*$^+$ locus was confirmed by colony PCR. The stable *uch2*$^+$*-gfp* strain was referred to as *uch2-2*. Another strain (*uch2-3*) was constructed likewise. The nucleotide sequence corresponding to the amino acid residues 222-300 of Uch2p (as shown in Fig. 1) was deleted. This replaced *uch2*$^+$ with *uch2*Δ*C-gfp*.

Fluorescence microscopy. Fluorescence microscopy was carried out essentially as described (16). For GFP fluorescence experiments, cells were permeabilized without fixation and mounted in DAPI mounting medium. Cells were viewed using a Leica DMLB microscope and digital images were captured using an Optronix DEI-470T cooled charge-coupled device camera.

Glycerol gradient centrifugation. Glycerol gradient centrifugation was carried out essentially as described (17). In brief, 1 liter of cells of each wild type, *uch2-2* or *uch2-3* strain were grown to an A_{595} of 0.8 and harvested by centrifugation. The cell pellet was lysed in the lysis buffer: 50 mM Tris-HCl (pH 7.5) buffer containing 10% glycerol, 2 mM ATP, 5 mM $MgCl_2$, 5 μg/ml pepstatin A, 5 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysis of the cells was by the use of glass beads. 30 mg of each protein extract were layered on top of 30 ml 10–40% of glycerol gradient and spun for 24 h at 27,000 rpm at 4°C using a Beckman SW28 rotor. 2 ml fractions were collected from the bottom and 10 μg of proteins from alternate fractions were analyzed by Western blotting.

Coimmunoprecipitation. 2 mg protein extracts from each of wild-type, *uch2-2* or *uch2-3* strain were incubated with 4 μl of anti-Mts4p antiserum in a total volume of 1ml lysis buffer for 2 h at 4°C with gentle rotation. 100 μl of 50% slurry of protein A-agarose (Sigma) was added and incubated for another 2 h at 4°C with gentle rotation. After 5 washes in lysis buffer containing 150 mM NaCl, the proteins bound to the antibodies were released by boiling in SDS loading buffer and subjected to Western blotting.

Antibodies and Western blotting. The samples were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were probed with anti-Mts4p antiserum (18), diluted 1 in 5,000, or anti-Mts2p antiserum (AFFINITI), diluted 1 in 500, or anti-GFP antiserum (Living colors peptide antibody-HRP conjugate, Clontech), diluted 1 in 200. Subsequently, the membranes were incubated with goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugate secondary antibody (Bio-Rad). No secondary antibody was needed for the membranes probed with anti-GFP antibody–HRP conjugate. The membranes were visualized by enhanced chemiluminescence (SuperSignal West Pico, Pierce).

RESULTS

Identification of a homolog of mouse UCH-L5 in S. pombe. We have cloned a novel mouse UCH (designated UCH-L5) cDNA which encodes a protein containing an ~100 amino acid C-terminal extension in addition to the N-terminal UCH domain. Both UCH-L5 and truncated UCH-L5 (without the 100 amino acid extension) cleaved substrate proteins specifically between ubiquitin G76 and small peptide extensions but not α-linked ubiquitin dimers (T. Li and T. S. Teo, unpublished data), indicating that UCH-L5 belongs to the UCH family of DUB enzymes. In an effort to study the function of this gene, the mouse UCH-L5 protein sequence (GenBank Accession No. AAD50311) was used as query to search both the *S. cerevisiae* and the *S. pombe* genome sequence databases. In the *S. cerevisiae* genome sequence database, only a previously identified gene, *Yuh1,* was found to encode a protein, Yuh1p, which shared high sequence similarity with mouse UCH-L5 at the UCH domain. However, Yuh1p, which is thought to be the homolog of UCH-L3, possesses only the UCH domain and does not have a C-terminal extension. Two predicted open reading frames (ORF) with significant amino acid sequence homology with UCH-L5 at the UCH domain were identified in the *S. pombe* genome sequence database. The first gene (designated *uch1*$^+$) encodes a protein homologous to UCH-L3 and Yuh1p. The second, *uch2*$^+$, encodes a protein homologous to mouse UCH-L5 at both the UCH domain and the C-terminal extension. *uch2*$^+$ resides on cosmid c409 on chromosome II. As shown in Fig. 1, *uch2*$^+$ predicts a 300 amino acid protein which is 37%

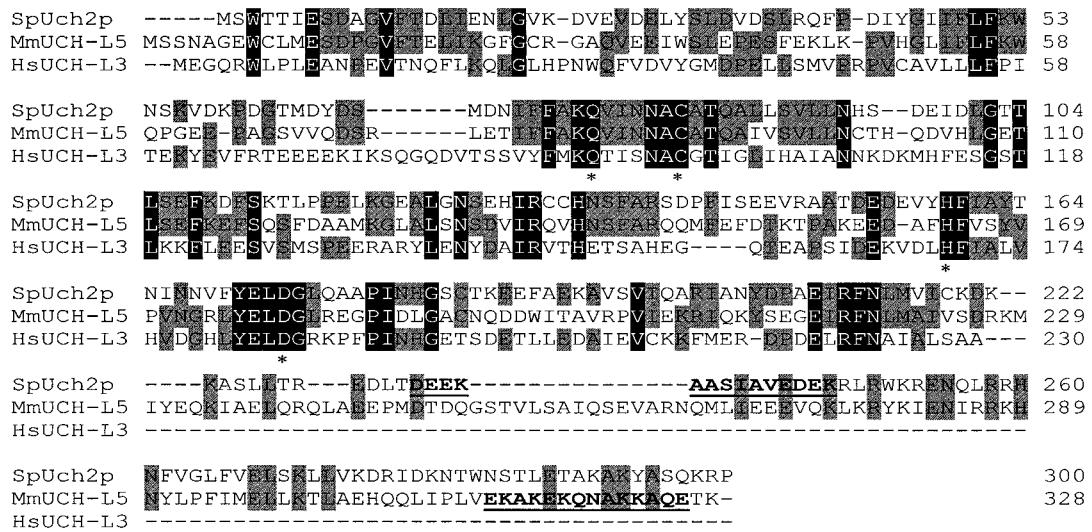

FIG. 1. Alignment of the amino acid sequence of fission yeast Uch2p (CAB52608) with those of mouse UCH-L5 (AAD50311) and human UCH-L3 (P15374). Identical amino acids in all three UCHs are in black and identical amino acids in any two UCHs are in gray. The putative UCH catalytic sites are marked in asterisks. KEKE-like motifs are underlined and in boldface. Hyphens indicate the absence of amino acid residues.

identical with mouse UCH-L5. The deduced amino acid sequence of *uch2*$^+$ shows high similarity in many areas with mouse UCH-L5 and human UCH-L3, most importantly at position 89-97 (containing the catalytic sites Q89 and C95) and 169-185 (containing catalytic sites H169, D184 and the YELDG motif) (19). The numbering system used here is based on the human UCH-L3. The sequence similarities indicate that Uch2p probably has UCH activity. Interestingly, there are KEKE-like motifs at the C-termini of mouse UCH-L5 and fission yeast Uch2p (Fig. 1). This motif might mediate protein-protein interactions with the 26S proteasome (20), implying that Uch2p may be associated with the 26S proteasome.

uch2$^+$ *is not essential for cell viability.* To study the effect of a complete loss of function of *uch2*$^+$ in *S. pombe*, we made a construct in which the ~1-kb coding region of *uch2*$^+$ was replaced with the selectable marker gene *ura4*$^+$. Linearized DNA containing this *uch2::ura4*$^+$ allele was used to transform a uracil auxotrophic diploid (MBY257/266). Uracil prototrophs were screened for successful replacement of one of the wild-type copies of *uch2*$^+$ by the *uch2::ura4*$^+$ allele by PCR and one strain of the genotype *uch2::ura4*$^+$/*uch2*$^+$ was identified. This strain was sporulated and tetrads were dissected on rich media. Under these conditions, all the four spores from each tetrad formed colonies. Further examination showed that only two of the four colonies of most tetrads were *ura4*$^+$, and all the *ura4*$^+$ colonies were confirmed to bear the genotype *uch2::ura4*$^+$ by PCR, indicating that the *uch2*$^+$ gene is not essential for cell viability. The growth rate of the *uch2* null mutant strain (*uch2-1*) showed no significant difference with the wild type strain both at 30°C and 37°C (data not shown).

Uch2p is associated with the 26S proteasome. To determine the subcellular localization of the Uch2p in *S. pombe*, both the *uch2*$^+$ and *uch2*Δ*C* (a truncated *uch2* minus its C-terminal extension sequence) were amplified by PCR and fused in-frame with the *gfp* gene at their 3′ ends. The constructs were transformed into the MBY192 strain. Using colony PCR to screen the *ura4*$^+$ colonies, *uch2*$^+$*-gfp* strain (*uch2-2*) and *uch2*Δ*C-gfp* strain (*uch2-3*) were identified. The GFP-tagged proteins were detected using anti-GFP antibody (Fig. 3A). A ~61 kDa band was observed in the *uch2-2* strain and a ~51 kDa band in the *uch2-3* strain. The bands corresponded to the predicted molecular weights of Uch2p-GFP and Uch2pΔC-GFP respectively. No band was detected in the wild-type strain. These findings suggest that the GFP-tagged proteins were expressed correctly under the control of the *uch2*$^+$ endogenous promoter.

Fluorescence microscopy revealed that the GFP signal of *uch2-2* strain (which expressed the Uch2p-GFP) exhibited a perinuclear localization during interphase (Figs. 2A and 2C) and during mitosis (Figs. 2B and 2D). The subcellular localization of Uch2p-GFP is very similar to that of the 26S proteasome in *S. pombe* (17). Interestingly, the GFP signal of the *uch2-3* strain (which expressed the Uch2pΔC-GFP) exhibited a generally diffused distribution in the cells (Fig. 2E), indicating that the C-terminal extension containing a KEKE-like motif may mediate the association with the nuclear periphery. No GFP signal was detected in wild type strain (Fig. 2F). Considering the subcellular local-

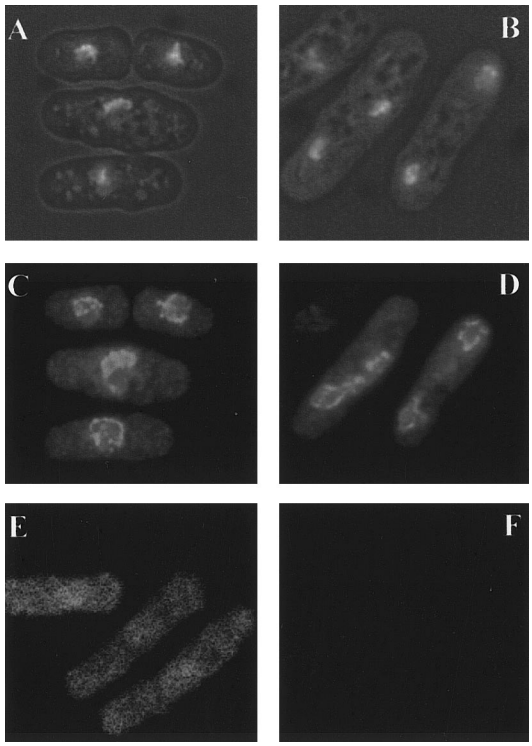

FIG. 2. Fluorescence microscopy of *uch2-2* and *uch2-3* strains. Micrographs are fluorescence images of cells of the *uch2-2* strain at interphase (A, C) and at mitosis (B, D). Cells in A and B were stained with DAPI showing their DNA and cells in C and D showed their Uch2p-GFP subcellular localization. Micrograph E shows the subcellular localization of Uch2pΔC-GFP in cells of the *uch2-3* strain. Micrograph F shows the fluorescence image of the wild type strain as a control.

ization and the role of the C-terminal extension, we postulate that Uch2p is associated with the 26S proteasome.

By the analysis of fractions from glycerol gradient centrifugation we are able to confirm that Uch2p is associated with the 26S proteasome. Protein extracts of fission yeast strain expressing Uch2p-GFP were subjected to glycerol gradient centrifugation and fractions were analyzed for the GFP-tag signal by Western blotting. Uch2p-GFP was found predominantly in fractions 2-6 corresponding to the more rapidly sedimenting end of the gradient (Fig. 3B). This gradient distribution of Uch2p-GFP is very similar to those of two identified 26S proteasome subunits, Mts4p and Mts2p in fission yeast (18, 21). On the other hand, Uch2pΔC-GFP showed quite a different sedimentation pattern. The Uch2pΔC-GFP appeared in fractions 8-14 (Fig. 3C), indicating that the C-terminal extension plays an important role in the association of Uch2p with the 26S proteasome. Results of our coimmunoprecipitation experiment provided further supporting evidence that Uch2p was indeed associated with the 26S proteasome. As shown in Fig. 4, Uch2p-GFP was coimmunoprecipitated with the 26S proteasome by the use of antiserum against Mts4p, but when preimmune serum was used, both Mts4p and Uch2p-GFP could not be detected. However, Uch2pΔC-GFP failed to be coimmunoprecipitated with 26S proteasome by anti-Mts4p antiserum (Fig. 4). Taken together, we suggest that Uch2p is associated with the 26S proteasome and may be one of its subunits in fission yeast.

DISCUSSION

Recently Xu *et al.* (1999) deposited in GenBank the nucleotide sequence of a human UCH37 (Accession Number: AF147717). The deduced amino acid sequence of UCH37 is 96% identical to mouse UCH-L5 and 37% identical to fission yeast Uch2p. Xu *et al.* suggested that UCH37 is a subunit of PA700. However, there is no publication of evidence that UCH37 is a subunit of or associated with PA700. A previously re-

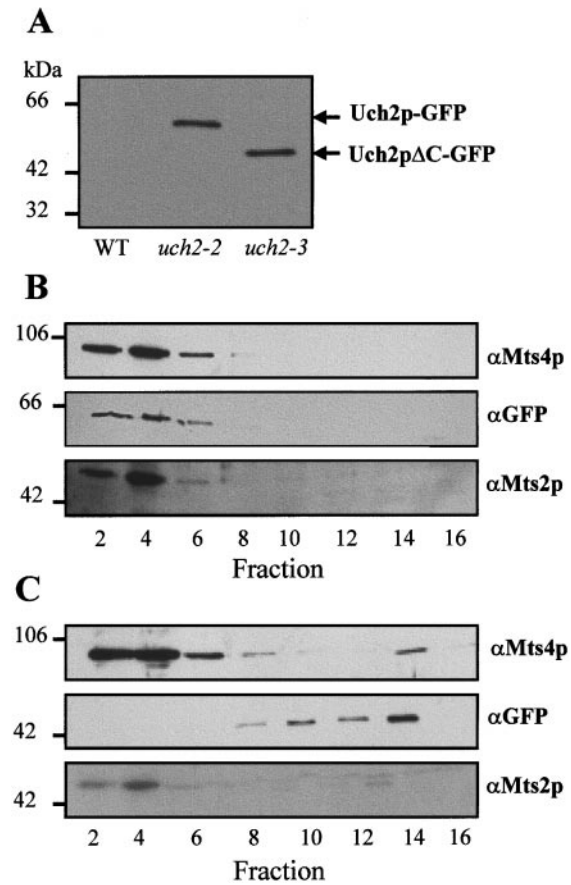

FIG. 3. Western blot analysis of protein extracts from wild-type strain (lane 1), *uch2-2* strain (lane 2) and *uch2-3* strain (lane 3). The protein extracts were probed with anti-GFP antibody (A). Western blot analysis of fractions from glycerol gradient centrifugation of protein extracts from *uch2-2* strain (B) and *uch2-3* strain (C). The fractions were probed with anti-Mts4p antibody (top panel), anti-GFP antibody (middle panel) or anti-Mts2p antibody (bottom panel). Protein loading was 10 μg of protein/lane. Molecular mass markers are shown in kilodaltons (kDa).

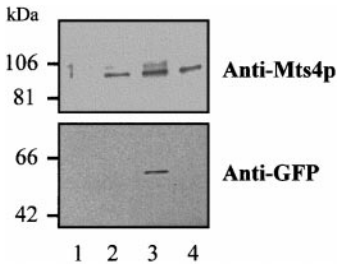

FIG. 4. Coimmunoprecipitation of GFP-tagged Uch2p with Mts4p. Protein extracts from wild-type strain (lane 2), *uch2-2* strain which expresses Uch2p-GFP (lane 3) and *uch2-3* strain which expresses Uch2pΔC-GFP (lane 4) were immunoprecipitated using anti-Mts4p antiserum. Preimmune serum was used as a control to incubate with the protein extracts from *uch2-2* strain (lane 1). The Western blots were probed with either anti-Mts4p antiserum or anti-GFP antiserum.

ported PA700-associated isopeptidase had been shown to selectively rescue poorly ubiquitinated or slowly degraded ubiquitin-protein conjugates from proteolysis by dissembling the polyubiquitin chains from the distal end (22). This enzyme has an apparent molecular weight of 37 kDa, which is similar to that of the mouse UCH-L5 (37.488 kDa). This isopeptidase was reported to be unable to cleave α-linked ubiquitin dimers (23), suggesting its relation with the UCH family. It will be of interest to determine whether the UCH37 possesses the specific isopeptidase activity and whether it is associated with PA700. In this paper we provide evidence suggesting that Uch2p, the *S. pombe* homolog of UCH37, is probably a subunit of the 19S regulatory complex.

Surprisingly, *uch2*$^+$ is a nonessential gene in *S. pombe* as the *uch2* null mutant strain shows similar growth rate to the wild-type strain. This finding is quite different from the phenotypes of many 26S proteasome subunit mutants in *S. pombe.* Four subunits of *S. pombe* 26S proteasome (Mts2p, Mts3p, Mts4p and Pad1p) were identified by a genetic screen for mutants that are resistant to the microtubule destabilizing drug methyl 2-benzimidazolecarbamate (MRC) and are also temperature sensitive (ts) for growth (18, 21, 24, 25). At the restrictive temperature these mutant cells stop growing and become arrested in metaphase. In order to determine whether Uch2p is also involved in cell cycle regulation, flow cytometry was used to compare the DNA contents of exponentially growing *S. pombe* haploid cells of the *uch2* null mutant strain and the wild-type strain. No significant difference was observed (data not shown). This indicates that Uch2p may not play a pivotal role in cell cycle regulation or that there is redundancy.

Substrate selection of 26S proteasome is thought to be mediated by interaction of specific components of the 19S regulatory complex with the multiubiquitin chains conjugated to targeted proteins. One non-ATPase subunit, S5a, has been shown to bind to polyubiquitin chain *in vitro* (26, 27). However, disruption of its homolog, Mcb1p, in *S. cerevisiae* showed little change in growth (28). This implies that there is redundancy with respect to this ubiquitin-recognizing component in the S26 proteasome. The Uch2p possesses the ability to bind to the ubiquitin due to the UCH domain, but the preference of binding multiubiquitin chain is unclear. Analysis of double deletions of *uch2*$^+$ and the gene encoding the homolog of S5a in *S. pombe* might provide insight into the substrate specificity of the 26S proteasome.

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