structure of C132A was determined by the molecular replacement technique using AMoRe[32] with the refined model of SBD. Structure refinement of the mutant SBD was guided by referring to the structure of the wild type. Refinement statistics of both structures are summarized in **Table 1**.

Binding assay. Neuro2a cells were transfected with Flag-tagged murine Fbs1 ΔN-2(95–297) and its derivatives that lack the region from the N terminus to the F-box domain[7] by lipofection (Lipofectamine Plus; Gibco BRL). At 48 h after transfection, the whole-cell extracts (WCEs), solubilized with TBS containing 0.5% (v/v) NP-40, were used for immunoprecipitation using mouse monoclonal anti-Flag (M2; Sigma), or pull-down assay using RNase B–immobilized resin, as described[7]. The (co-)immunoprecipitated proteins were detected by immunoblotting using mouse monoclonal antibodies anti-Flag (M2) and anti-integrin β1 (Transduction Laboratories).

***In vitro* ubiquitination assay.** Recombinant His-tagged human Ubc4 was produced in *E. coli*. Recombinant His-tagged mouse E1 (Uba1), His-tagged rat Fbs1 (ΔN-2) and each SCF^{Fbs1} (Flag-tagged human Skp1, human Cul1-HA/His-tagged rat Fbs1 derivatives, T7-tagged human Roc1) were produced by baculovirus-infected HiFive insect cells. Each SCF^{Fbs1} complex was obtained by simultaneously infecting four baculoviruses. These proteins were affinity-purified by a HiTrap HP column (Amersham Pharmacia Biotech), as described[7]. Each 1 μg of GTF was incubated in 50 μl of the reaction mixture containing ATP-regenerating system, 0.5 μg E1, 1 μg Ubc4 (E2), 2 μg SCF^{Fbs1}, 6.5 μg recombinant GST-ubiquitin and NEDD8 system[33] at 30 °C. After the reaction was terminated by adding 25 μl of 3× SDS-PAGE sample buffer, the proteins in 8 μl of the boiled supernatants were separated with 5–20% (w/v) SDS-PAGE, and the high-molecular-mass ubiquitinated proteins were detected by immunoblotting with anti-fetuin (Chemicon International).

NMR spectroscopy. The DNA fragment encoding residues 97–297 of Fbs1was inserted into the pGEX-6P-1 plasmid vector (Amersham Biosciences) with a N-terminal GST moiety. The protein was expressed in *E. coli* BL21(DE3) CodonPlus strain (Stratagene) in M9 minimal medium with appropriate $[^{15}N]NH_4Cl$ and $[^{13}C_6]$glucose. GST-fusion protein was purified from cell lysates on a glutathione-Sepharose column. The fusion protein was cleaved by incubation with PreScission protease (Amersham Biosciences), and GST was removed by loading a second glutathione-Sepharose column. The protein was further purified using a Superose12 gel filtration column.

NMR samples contained 0.1–1.0 mM SBD(97–297) in 10 mM NaH_2PO_4/Na_2HPO_4, pH 6.5, 50 mM KCl and 10 mM DTT. For chemical shift perturbation experiments, a ten-fold molar excess of chitobiose, ten-fold molar excess of Manα1′3(Manα1′6)Man (Calbiochem) or one molar equivalent of $Man_3GlcNAc_2$ (Sigma) was added to the protein solution. NMR spectra were acquired at 30 °C on Bruker DMX500 and DRX800 spectrometers. The ^1H, ^{15}N and ^{13}C resonances of the backbone were assigned using a standard set of double- and triple-resonance experiments[34].

Coordinates. The atomic coordinates of the SBD and C132A SBD–chitobiose complex have been deposited in the Protein Data Bank (accession codes 1UMH and 1UMI, respectively).

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

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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A novel protein-conjugating system for Ufm1, a ubiquitin-fold modifier

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Several studies have addressed the importance of various ubiquitin-like (UBL) post-translational modifiers. These UBLs are covalently linked to most, if not all, target protein(s) through an enzymatic cascade analogous to ubiquitylation, consisting of E1 (activating), E2 (conjugating), and E3 (ligating) enzymes. In this report, we describe the identification of a novel ubiquitin-fold modifier 1 (Ufm1) with a molecular mass of 9.1 kDa, displaying apparently similar tertiary structure, although lacking obvious sequence identity, to ubiquitin. Ufm1 is first cleaved at the C-terminus to expose its conserved Gly residue. This Gly residue is essential for its subsequent conjugating reactions. The C-terminally processed Ufm1 is activated by a novel E1-like enzyme, Uba5, by forming a high-energy thioester bond. Activated Ufm1 is then transferred to its cognate E2-like enzyme, Ufc1, in a similar thioester linkage. Ufm1 forms several complexes in HEK293 cells and mouse tissues, revealing that it conjugates to the target proteins. Ufm1, Uba5, and Ufc1 are all conserved in metazoa and plants but not in yeast, suggesting its potential roles in various multicellular organisms.
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Introduction

Protein modification plays a pivotal role in the regulation and expansion of genetic information. In the past two decades, a new type of post-translational protein-modifying system has been identified whose uniqueness is that protein(s) is used as a ligand, that is, modification of protein, by protein, and for protein. A typical system is the ubiquitylation, a modification system in which a single or multiple ubiquitin molecules are attached to a protein, which serves as a signaling player that controls a variety of cellular functions (Hershko and Ciechanover, 1998; Pickart, 2001). Protein ubiquitylation is catalyzed by an elaborate system highly regulated in the cells, which is catalyzed by a sequential reaction of multiple enzymes consisting of activating (E1), conjugating (E2), and ligating (E3) enzymes. E1, which initiates the reaction, forms a high-energy thiolester bond with ubiquitin via adenylation in an ATP-dependent manner. The E1-activated ubiquitin is then transferred to E2 in a thioester linkage. In some cases, E2 can directly transfer the ubiquitin to substrate proteins in an isopeptide linkage; however, E2s mostly requires the participation of E3 to achieve substrate-specific ubiquitylation reaction in the cells. E3s are defined as enzymes required for recognition of specific substrates for ubiquitylation, other than E1 and E2 (Varshavsky, 1997; Bonifacino and Weissman, 1998; Glickman and Ciechanover, 2002).

A set of novel molecules called ubiquitin-like proteins (UBLs) that have structural similarities to ubiquitin has been recently identified (Jentsch and Pyrowolakis, 2000). They are divided into two subclasses: type-1 UBLs, which ligate to target proteins in a manner similar, but not identical, to the ubiquitylation pathway, such as SUMO, NEDD8, and UCRP/ISG15, and type-2 UBLs (also called UDPs, ubiquitin-domain proteins), which contain ubiquitin-like structure embedded in a variety of different classes of large proteins with apparently distinct functions, such as Rad23, Elongin B, Scythe, Parkin, and HOIL-1 (Tanaka *et al*, 1998; Jentsch and Pyrowolakis, 2000; Yeh *et al*, 2000; Schwartz and Hochstrasser, 2003).

In this report, we describe a unique human UBL-type modifier named ubiquitin-fold modifier 1 (Ufm1) that is synthesized in a precursor form consisting of 85 amino-acid residues. We also identified the human activating (Uba5) and conjugating (Ufc1) enzymes for Ufm1. Prior to activation by Uba5, the extra two amino acids at the C-terminal region of the human proUfm1 protein are removed to expose Gly whose residue is necessary for conjugation to target molecule(s). Lastly, we show that the mature Ufm1 is conjugated to yet unidentified endogenous proteins, forming ~28, 38, 47, and 70 kDa complexes in human HEK293 cells and various mouse tissues.

Results

Identification of a novel protein-activating enzyme, Uba5

Our initial plan was to identify the molecule(s) that interacts with human Atg8p homolog GATE16, a type-1 UBL modifier required for autophagy (Klionsky and Emr, 2000; Ohsumi, 2001), using a yeast two-hybrid screening. Please note that the nomenclature of the autophagy-related genes was recently unified as *ATG* (Klionsky *et al*, 2003). Among several

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positive clones, we identified fragments of FLJ23251 (Figure 1A), which encodes a 404-amino-acid protein highly conserved in various multicellular organisms, such as *Homo sapiens*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Arabidopsis thaliana*, but absent in yeasts (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) (Figure 1B). The sequence of FLJ23251 in the region containing residues 72–229 is highly homologous to the corresponding regions in Uba1 (i.e., E1 for ubiquitin) and other E1-like proteins for UBLs including the ATP-binding motif (GXGXXG) (Figure 1A and B). We named this protein Uba5, because it is a member of the E1-like enzyme family. Uba5 also has a metal-binding motif conserved in other E1-like enzymes such as Uba2, Uba3, Uba4, and Atg7. Most of E1-like enzymes have an active site Cys residue within the conserved 10–20 amino-acid residues downstream from the metal-binding

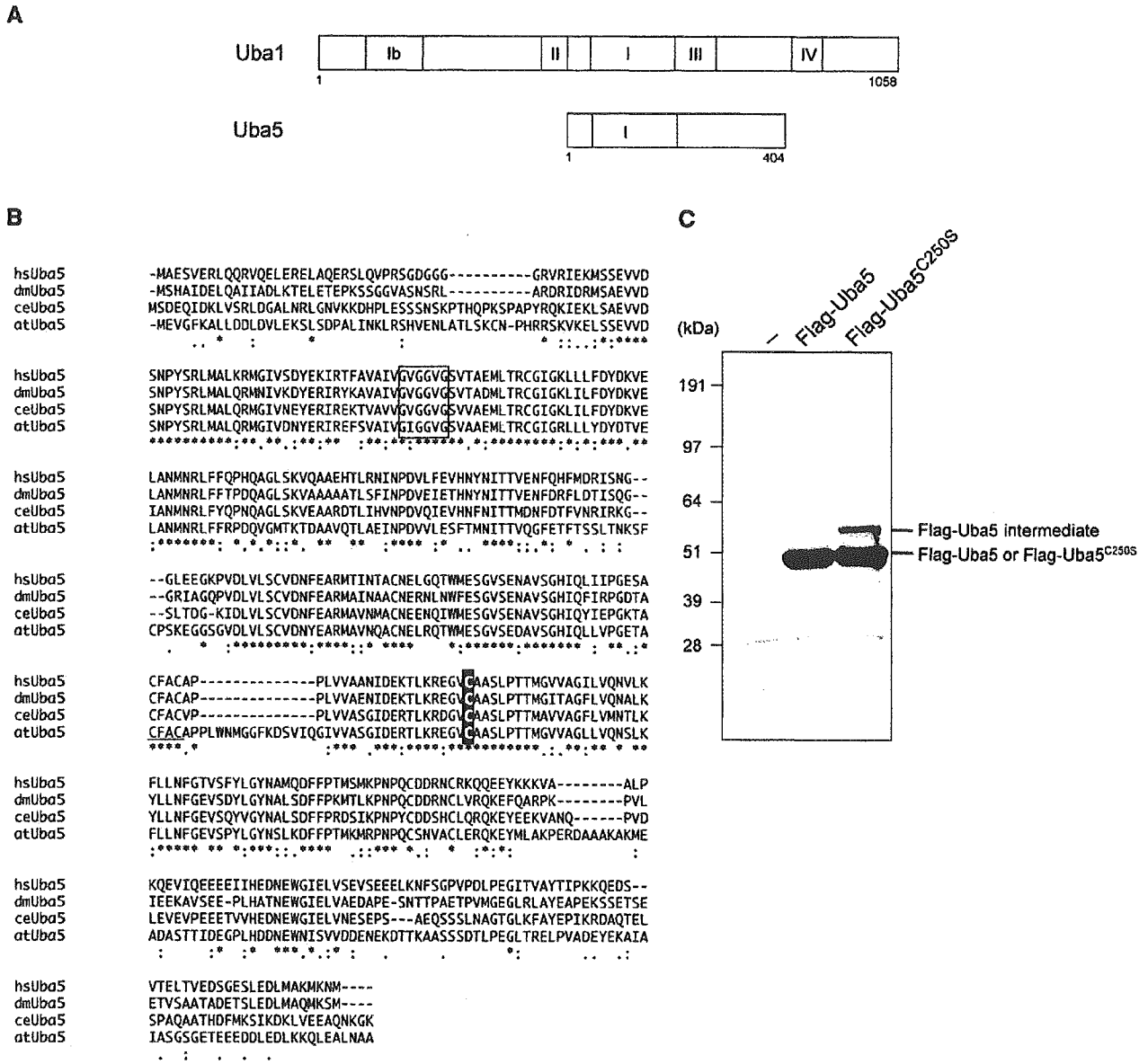

Figure 1 Uba5, a novel E1-like enzyme. (**A**) Schematic representation of Uba1 and Uba5 in *H. sapiens*. Uba1 is divided into several domains, including I, Ib, II, III, and IV boxes, which are conserved in other E1-like enzymes, and other regions without obvious similarity, described previously (Komatsu *et al*, 2001). Note that Uba5 is of a relatively small size and includes the box I and two other parts. The box I region of Uba1 (amino acids 459–611) has 48.4% similarity and 22.3% identity to amino acids 72–229 of Uba5, which includes the conserved ATP-binding motif (GXGXXG). The sequence of Uba5 is available from GenBank™ under the accession number AK026904. hs, *H. sapiens*; ce, *C. elegans*; dm, *D. melanogaster*; at, *A. thaliana*. (**B**) Sequence alignment of hsUba5 and its homologs of other species (dm, NM_132494; ce, NM_058847; at, NM_100414). The amino-acid sequence of hsUba5 is compared by the ClustalW program. *Asterisks*, identical amino acids; *single* and *double dots*, weakly and strongly similar amino acids, respectively, determined by the criteria of ClustalW program. *Open box* indicates an ATP-binding motif. The putative active site Cys residue is boxed in black. The metal-binding motif is underlined. (**C**) Identification of the intermediate linked to Uba5 in HEK293 cells. Both Uba5 and Uba5^{C250S}, in which the predicted active site Cys positioned at 250 was changed to Ser by site-directed mutagenesis, were tagged with Flag peptide at N-terminus, resulting in Flag-Uba5 and Flag-Uba5^{C250S}, respectively. Each Flag-Uba5 and Flag-Uba5^{C250S} was expressed in HEK293 cells. The cell lysates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-Flag antibody.

motif. In the case of Uba5, the Cys^{250} seems to be the most possible active site Cys residue (Figure 1B). If an active site Cys residue within an E1 and E1-like enzymes is changed to Ser, an O-ester bond instead of a thioester bond is formed with its respective modifier protein and the intermediates become stable even under reducing conditions. Therefore, we mutated Cys^{250} within Uba5 to Ser and expressed it as a Flag-fused $Uba5^{C250S}$ (Flag-$Uba5^{C250S}$) or Flag-Uba5 as control in HEK293 cells. As shown in Figure 1C, both Flag-Uba5 and Flag-$Uba5^{C250S}$ were expressed as ~50 kDa proteins in HEK293 cells. When Flag-$Uba5^{C250S}$ was expressed, an additional band with a higher molecular mass of ~60 kDa was clearly observed, indicating that Flag-$Uba5^{C250S}$ forms an intermediate complex with an endogenous protein. These results suggest that Uba5 is indeed a novel protein-activating enzyme for a presumptive modifier (see below).

Identification of a novel ubiquitin-fold molecule, Ufm1

Because Uba5 was identified as GATE-16-binding protein, we initially assumed that Uba5 is another GATE-16-activating enzyme, in addition to Atg7. To test this possibility, we examined whether $Uba5^{C250S}$ (the presumptive active site Cys at position 250 was replaced by Ser) forms an intermediate complex with GATE-16 or not. Unexpectedly, we could not identify a stable complex between $Uba5^{C250S}$ and GATE-16 (data not shown). Therefore, we attempted to identify a protein(s) that physically associates with Uba5 in the cells. To do this, Flag-Uba5 was expressed in HEK293 cells, then immunoprecipitated by anti-Flag antibody. The immunoprecipitates were eluted with a Flag peptide, then digested with Lys-C endopeptides (*Achromobacter* protease I) and the cleaved fragments were directly analyzed using a highly sensitive 'direct nano-flow LC–MS/MS' system as described in Materials and methods. Following database search, a total of 28 peptides were assigned to MS/MS spectra obtained from four nano-LC–MS/MS analyses for the Flag-Uba5-associated complexes. These peptide data identified three proteins as Uba5-associated components: GATE-16, and hypothetical proteins BM-002 and CGI-126 (excluding the bait protein Uba5 and the background proteins, such as HSP70 and keratins).

One of these identified proteins, BM-002, is an 85-amino-acid protein with a predicted molecular mass of ~9.1 kDa. This protein is conserved in multicellular organisms, but not in yeasts, like Uba5 (Figure 2A). The human BM-002 has high identity over the species in the central region but has elongated sequences at both N- and C-terminal regions in some species. Although the protein shows no clear overall sequence identity to ubiquitin or other modifiers (Figure 2B), the tertiary structure of BM-002 displays a striking resemblance to human ubiquitin (Figure 2C). The human structure of BM-002 was constructed by a computer-assisted modeling, based on the structure of its *C. elegans* homolog that has been analyzed previously, as a protein possessing 'ubiquitin-like fold' with secondary structure elements ordered β–β–α–β–β–α (α-helix and β-sheet) along the sequence (Cort *et al*, 2002). Thus, we named human BM-002 as Ufm1.

Ubiquitin is synthesized in a precursor form that must be processed by de-ubiquitylating enzymes (DUBs) to generate a Gly–Gly sequence at the C-terminus. Similarly, Ufm1 has a single Gly residue conserved across species at the C-terminal region, although the length and sequences of amino acids extending from this Gly residue vary among species. To test whether the C-terminus of Ufm1 is post-translationally cleaved, we constructed an expression vector for Ufm1 tagged at both the N- and C-ends, that is, a Flag epitope at the N-terminus and an HA epitope at the C-terminus (Flag-Ufm1-HA) (Figure 2D). After transfection of Flag-Ufm1-HA into HEK293 cells, the cell lysate was subjected to SDS–PAGE, and Flag-Ufm1-HA was detected by immunoblotting. A 10-kDa protein corresponding to Ufm1 was recognized with anti-Flag antibody, while no appreciable protein was observed with anti-HA antibody (Figure 2E, lanes 2 and 7). The mobility on SDS–PAGE was similar to that of Flag-Ufm1ΔC2 (equivalent to mature $Ufm1^{1-83}$ protein) lacking the C-terminal Ser^{84} and Cys^{85} of proUfm1 (Figure 2E, lane 4). These results suggested that the C-terminus of Ufm1 is post-translationally cleaved in the cells, producing mature Ufm1 with the C-terminal Gly^{83} residue. It is known that the replacement of C-terminal Gly residue of Ub and other UBLs with an Ala residue inhibits the C-terminal processing (Kabeya *et al*, 2000; Tanida *et al*, 2003). To examine whether Gly^{83} of Ufm1 is essential for the cleavage, Gly^{83} of Flag-Ufm1-HA was mutated to Ala, and expressed in HEK293 cells (Figure 2D, Flag-$Ufm1^{G83A}$-HA). The mobility of most Flag-$Ufm1^{G83A}$-HA on SDS–PAGE was apparently slower than that of Flag-Ufm1-HA (Figure 2E, lane 3). This mutant was recognized by immunoblotting with anti-HA antibody as well as anti-Flag antibody, suggesting that mutation Gly^{83} to Ala confers resistance to its C-terminal cleavage.

Uba5 is an Ufm1-activating enzyme

We next investigated whether Uba5 forms an intermediate complex with Ufm1. We expressed Flag-Uba5 or Flag-$Uba5^{C250S}$ with Myc-tagged Ufm1 (Myc-Ufm1) in HEK293 cells. Myc-tagged Ufm1ΔC3 lacking the C-terminal Gly^{83} of mature Ufm1 (Myc-Ufm1ΔC3; i.e., deletion form of three residues from precursor $Ufm1^{1-85}$ protein) was used as control. Each cell lysate was prepared and analyzed by immunoblotting with anti-Flag antibody. Flag-$Uba5^{C250S}$ formed an intermediate with an endogenous protein as shown in Figure 1 (Figure 3A, lane 7). When Flag-$Uba5^{C250S}$ was coexpressed with Myc-Ufm1, the intermediate shifted to higher molecular weight (Figure 3A, lane 8). The higher band was not detected when Myc-Ufm1ΔC3 was coexpressed (Figure 3A, lane 9). To verify that the intermediate is indeed the Uba5–Ufm1 complex, Flag-$Uba5^{C250S}$ was immunoprecipitated and blotted with anti-Flag and anti-Myc antibody. Consistent with the above data, a higher sized intermediate was observed when Flag-$Uba5^{C250S}$ was coexpressed with Myc-Ufm1 (Figure 3B, top panel, lane 5), but not alone or with Myc-Ufm1ΔC3 (Figure 3B, top panel, lanes 4 and 6). The intermediate was also recognized by anti-Myc antibody (Figure 3B, lower panel, lane 5), indicating the existence of the Flag-$Uba5^{C250S}$–Myc-Ufm1 complex. Note that the small-sized intermediate is presumably a complex with an endogenous Ufm1, as mentioned. These results indicate that Uba5 forms an intermediate with Ufm1 and the Gly^{83} residue of Ufm1 is essential for the formation of the intermediate with Uba5 *in vivo*.

We subsequently tested whether Uba5 can activate Ufm1 *in vitro*. The thioester formation assay was performed using recombinant proteins expressed in *Escherichia coli*. Recombinant GST-tagged Uba5 and mature Ufm1

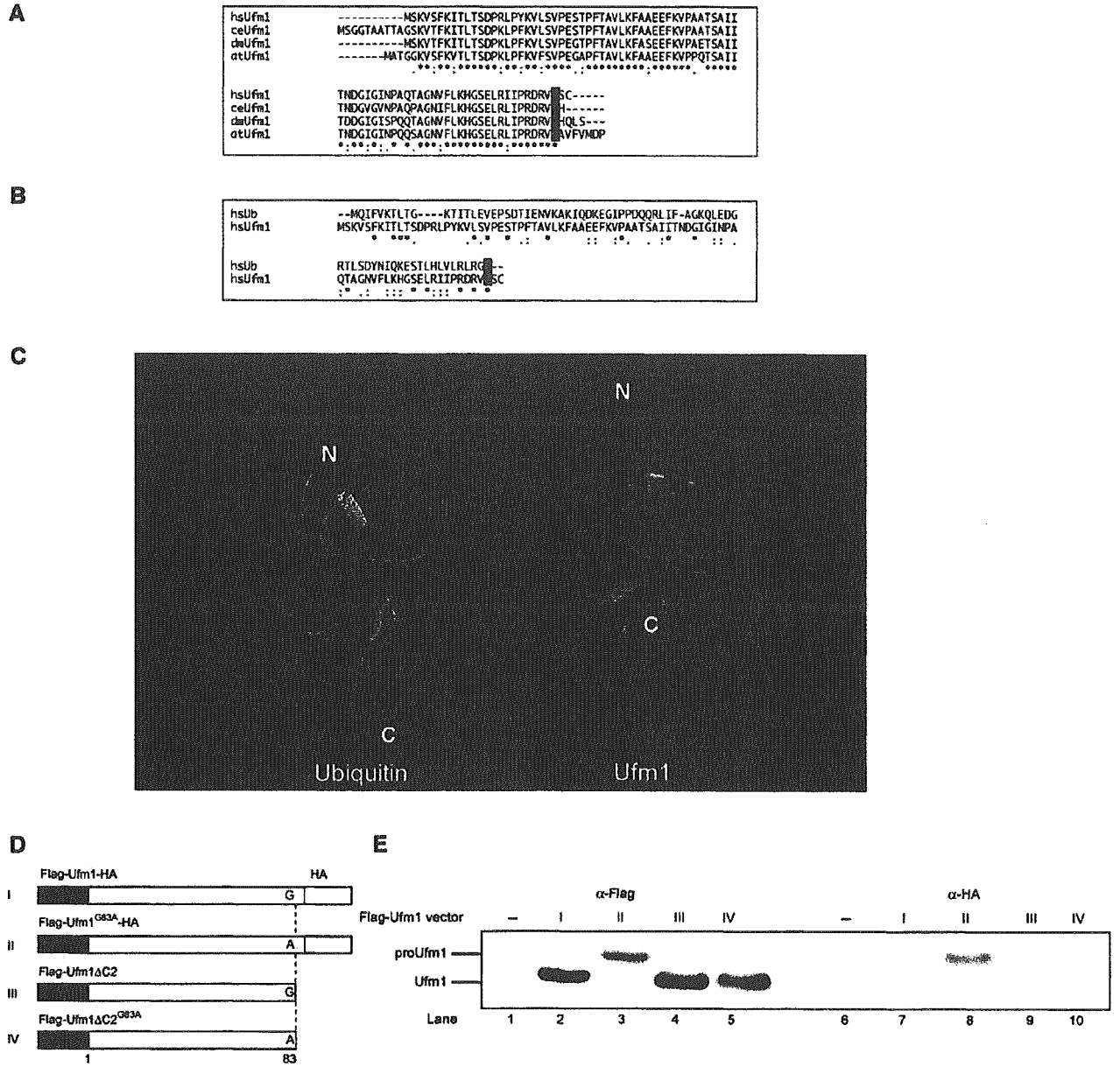

Figure 2 Ufm1, a novel ubiquitin-fold molecule. (**A**) Sequence alignment of hsUfm1 and its homologs. The sequence of hsUfm1 is available from GenBank™ under the accession number BC005193 (dm, a coding region of dmUfm1 was found from *D. melanogaster* genomic sequence; ce, NM_066304; at, NM_106420). The homology analysis was performed as described in Figure 1B. The C-terminal conserved Gly residue is boxed in black. (**B**) Sequence alignment of hsUbiquitin with hsUfm1. The homology analysis was performed as described in Figure 1B. The C-terminal conserved Gly residue is boxed in black. (**C**) Structural ribbon of hsUbiquitin and predicted structural ribbon of hsUfm1. α-Helices and β-strands are shown in green and yellow, respectively. The homology model of hsUfm1 was created from the *C. elegans* Ufm1 structure (Cort *et al*, 2002) by using MOE program (2003.02; Chemical Computing Group Inc., Montreal, Quebec, Canada). (**D**) Schematic representation of mammalian expression plasmids for Ufm1 and the derivative mutants. Flag epitope tags at the N-terminus, HA epitope tags at the C-terminus, and putative cleavage site Gly^{83} residue (vertical dotted lines) are indicated. To construct $Ufm1^{G83A}$, a single point mutation was introduced into Ufm1, which led to an amino-acid substitution from Gly to Ala at position 83. To construct Ufm1ΔC2, the two C-terminal residues were deleted by PCR. $Ufm1\Delta C2^{G83A}$ was also produced by site-directed mutagenesis of Ufm1ΔC2. The ΔC2 mutants were tagged with the Flag epitopes at N-terminus. (**E**) ProUfm1 processing. HEK293 cells were transfected with Flag-Ufm1-HA, Flag-$Ufm1^{G83A}$-HA, Flag-Ufm1ΔC2, or Flag-$Ufm1\Delta C2^{G83A}$. The cell lysates were subjected to SDS–PAGE and analyzed by immunoblots with anti-Flag and anti-HA antibodies. ProUfm1 and mature Ufm1 are indicated on the left. The numbers at the top from I to IV are similar to those in (D).

(Ufm1ΔC2) with exposed C-terminal Gly^{83} residue were purified, mixed and incubated in the presence of ATP and then subjected to SDS–PAGE at either reducing or nonreducing conditions. GST-Ufm1ΔC3 was used as control. An ~100 kDa band corresponding to the GST-Ufm1ΔC2–GST-Uba5 intermediate complex was clearly observed when the mixture was applied at nonreducing conditions (Figure 3C, lane 3). This intermediate was not observed when ATP or GST-Uba5 was excluded from the mixture (Figure 3C, lanes 1 and 2), or when the mixture was loaded in the presence of

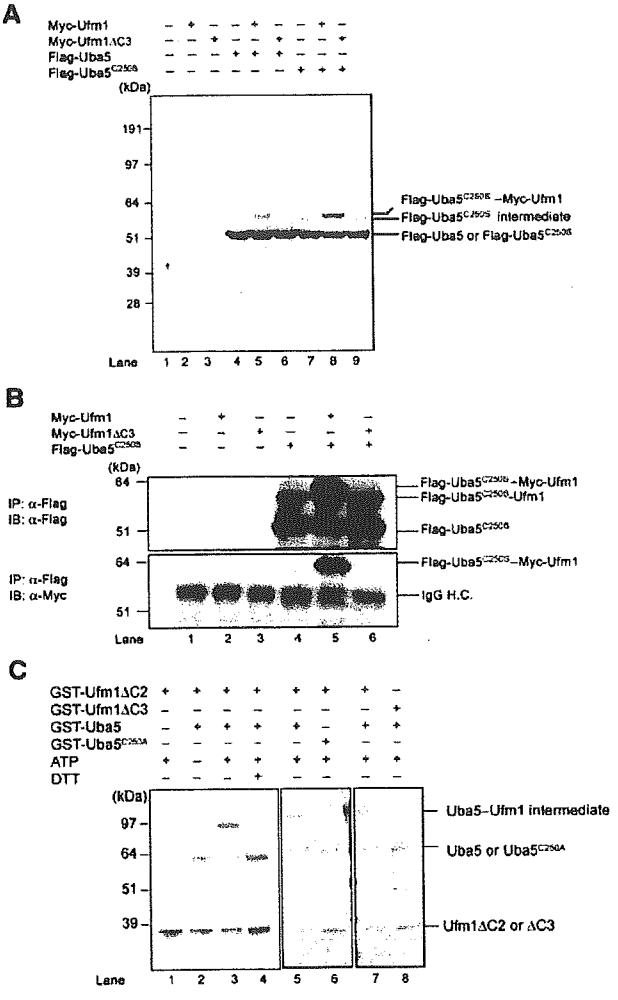

Figure 3 Demonstration that Uba5 is an Ufm1-activating enzyme. (**A**) Immunoblotting analysis. Each Myc-tagged Ufm1 (Myc-Ufm1) and Myc-Ufm1ΔC3 was expressed alone (lanes 2 and 3, respectively), and coexpressed with Flag-Uba5 (lanes 5 and 6, respectively) or Flag-Uba5^{C250S} (lanes 8 and 9, respectively). Each Flag-Uba5 and Flag-Uba5^{C250S} was also expressed alone (lanes 4 and 7, respectively). The cell lysates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-Flag antibody. The bands corresponding to Flag-Uba5, Flag-Uba5^{C250S}, and Flag-Uba5^{C250S} intermediates are indicated on the right. (**B**) Immunoblotting analysis after immunoprecipitation. Each Myc-Ufm1 and Myc-Ufm1ΔC3 was expressed alone (lanes 2 and 3, respectively), and coexpressed with Flag-Uba5^{C250S} (lanes 5 and 6, respectively). Flag-Uba5^{C250S} was also expressed alone (lane 4). The cell lysates were immunoprecipitated with anti-Flag antibody. The resulting immunoprecipitates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-Flag and anti-Myc antibodies. The bands corresponding to Flag-Uba5^{C250S}, Flag-Uba5^{C250S}-endogenous Ufm1, and Flag-Uba5^{C250S}–Myc-Ufm1 intermediates are indicated. (**C**) *In vitro* activating assay of Ufm1 by Uba5. Purified recombinant GST-Ufm1ΔC2 (2 μg) (lanes 1–7) was incubated for 30 min at 25°C with some of the following: 2 μg of purified recombinant GST-Uba5 (lanes 2–5, 7, and 8), GST-Uba5^{C250A} (lane 6), and 5 mM ATP (lanes 1 and 3–8). Lane 8 was conducted similar to lane 7, except that GST-Ufm1ΔC3 was used instead of GST-Ufm1ΔC2. Reactions were then incubated with SDS loading buffer lacking reducing agent (lanes 1–3 and 5–8) or containing 100 mM DTT (lane 4). The presence or absence of various components is indicated above the lanes. The bands corresponding to free GST-Uba5, GST-Uba5^{C250A}, GST-Ufm1ΔC2 (mature Ufm1), GST-Ufm1ΔC3, and GST-Uba5–GST-Ufm1ΔC2 thioester product are indicated on the right.

a reducing agent dithiothreitol (DTT) (Figure 3C, lane 4). Furthermore, GST-tagged Uba5^{C250A} mutant, a presumptive active site Cys mutant, could not form the intermediate even at nonreducing conditions (Figure 3C, lane 6). GST-tagged Ufm1ΔC3 was also incapable of forming the intermediate in this reaction (Figure 3C, lane 8). Taken together, we concluded that Uba5 is an Ufm1-activating enzyme and has the active site in Cys^{250}.

Identification of a novel protein-conjugating enzyme, Ufc1

The LC–MS/MS analysis revealed CGI-126 protein as another Uba5 interacting protein. CGI-126 is a protein of 167-amino-acid residues with a predicted molecular mass of 19.4 kDa. This protein is also conserved in multicellular organisms, like Uba5 and Ufm1 (Figure 4A). The C-terminal half of human CGI-126 has a high identity across species as shown in Figure 4A. CGI-126 has a highly conserved region, for example, residues 113–126, with limited similarity to the region of Ubc's that encodes an active site Cys residue capable of forming a thioester bond (Figure 4A). We assumed that this protein may be an E2-like conjugating enzyme for Ufm1 and thus named it Ufm1-conjugating enzyme 1 (Ufc1). If Ufc1 is an authentic E2 enzyme for Ufm1, it is expected to form an intermediate complex with Ufm1 via a thioester linkage. To test this possibility in the same way as Uba5, we mutated the predicted active site Cys residue within Ufc1 (Figure 4A, Cys^{116}) to Ser. We expressed Flag-Ufc1 or Flag-Ufc1^{C116S} (a presumptive active site Cys at position 116 was replaced by Ser) in combination with Myc-Ufm1 or Myc-Ufm1ΔC3 in HEK293 cells. Flag-Ufc1^{C116S} formed a stable intermediate band when coexpressed with Myc-Ufm1 (Figure 4B, lane 8), but not alone or with Myc-Ufm1ΔC3 (Figure 4B, lanes 7 and 9). To ascertain that this is the Flag-Ufc1^{C116S}–Myc-Ufm1 intermediate, Flag-Ufc1^{C116S} was immunoprecipitated and blotted with anti-Myc antibody (Figure 4C). Indeed, Myc-Ufm1, but not Myc-Ufm1ΔC3, formed a complex with Flag-Ufc1^{C116S} (Figure 4C, lanes 5 and 6, top and bottom panels). Note that Flag-Ufc1^{C116S} intermediate with a faster electrophoretic mobility than the Flag-Ufc1^{C116S}–Myc-Ufm1 complex is presumably the intermediate with the endogenous Ufm1 (Figure 4C, lanes 4–6, upper panel). These results indicate that Ufc1 forms an intermediate with Ufm1 *in vivo*.

To confirm that Ufc1 is indeed an E2-like enzyme that conjugates with Ufm1 *via* a thioester linkage, we conducted an *in vitro* Ufm1 conjugation assay. Recombinant GST-Uba5, GST-Ufc1, and GST-Ufm1ΔC2 were mixed and incubated in the presence of ATP. GST-Ufc1^{C116A} mutant and GST-Ufm1ΔC3 were used as negative controls. Under nonreducing conditions, an ~70 kDa band corresponding to GST-Ufm1ΔC2–GST-Ufc1 intermediate was observed (Figure 4D, lane 4). This product was not formed at reducing conditions, or when any of the components was omitted from the reaction (Figure 4D, lanes 1–3 and 5). GST-tagged Ufc1^{C116A} mutant could not form the intermediate, suggesting that Cys116 is indeed the active site (Figure 4D, lane 7). GST-Ufm1ΔC3 was again unable to form the intermediate complex in this reaction (Figure 4D, lane 9). Taken together, we concluded that Ufc1 functions as an Ufm1-conjugating enzyme and has the active site in Cys^{116}.

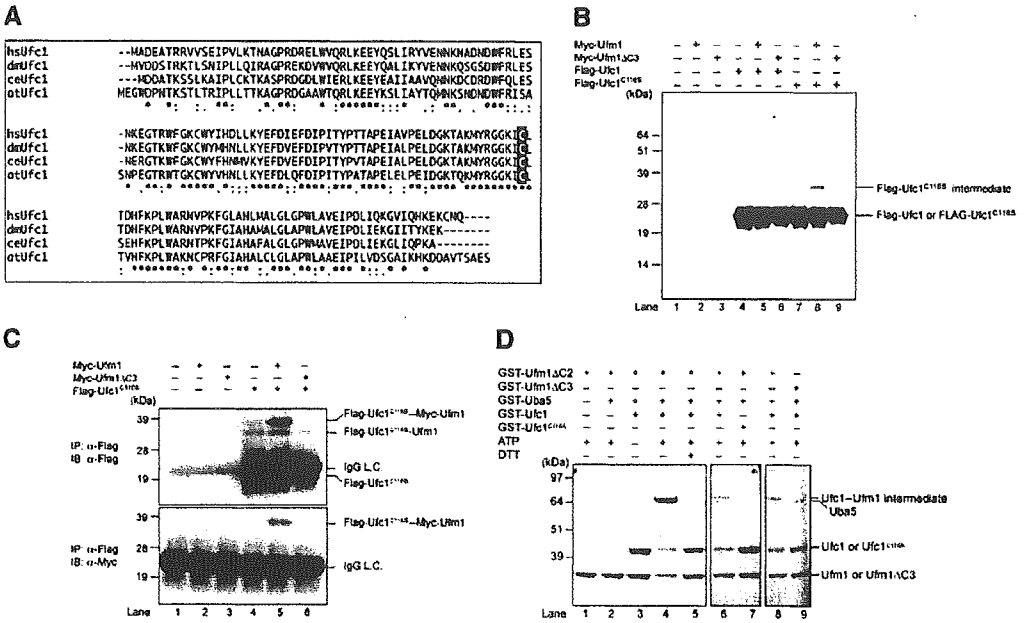

Figure 4 Ufc1, a novel E2-like enzyme. (**A**) Sequence alignment of hsUfc1 and its homologs. The sequence of Ufc1 is available from GenBanK™ under the accession number BC005187 (dm, NM_137230; ce, NM_066654; at, BT001180). The homology analysis was performed as described in Figure 1B. The putative active site Cys residue is boxed in black. (**B**) Immunoblotting analysis. Each Myc-tagged Ufm1 (Myc-Ufm1) and Myc-Ufm1ΔC3 was expressed alone (lanes 2 and 3, respectively), and coexpressed with Flag-Ufc1 (lanes 5 and 6, respectively) or Flag-Ufc1^{C116S} (lanes 8 and 9, respectively). Each Flag-Ufc1 and Flag-Ufc1^{C116S} was also expressed alone (lanes 4 and 7, respectively). The cell lysates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-Flag antibody. The bands corresponding to Flag-Ufc1, Flag-Ufc1^{C116S}, and Flag-Ufc1^{C116S} intermediates are indicated on the right. (**C**) Immunoblotting analysis after immunoprecipitation. Each Myc-Ufm1 and Myc-Ufm1ΔC3 was expressed alone (lanes 2 and 3, respectively), and coexpressed with Flag-Ufc1^{C116S} (lanes 5 and 6, respectively). Flag-Ufc1^{C116S} was also expressed alone (lane 4). The cell lysates were immunoprecipitated with anti-Flag antibody. The resulting immunoprecipitates were subjected to SDS–PAGE and analyzed by immunoblots with anti-Flag and anti-Myc antibodies. The bands corresponding to Flag-Ufc1^{C116S}, Flag-Ufc1^{C116S}–endogenous Ufm1, and Flag-Ufc1^{C116S}–Myc-Ufm1 intermediates are indicated. (**D**) *In vitro* thioester bond formation assay of Ufm1 by Ufc1. Purified recombinant GST-Ufm1ΔC2 (2 μg) (lanes 1–8) was incubated for 30 min at 25°C with the following: purified recombinant GST-Uba5 (0.2 μg) (lanes 2–9), GST-Ufc1 (2 μg) (lanes 3–6, 8, and 9), GST-Ufc1^{C116S} (2 μg) (lane 7), and 5 mM ATP (lanes 1, 2, and 4–9). Lane 9 was conducted similar to lane 8, except that GST-Ufm1ΔC3 was used instead of GST-Ufm1ΔC2. Reactions were then incubated with SDS loading buffer lacking reducing agent (lanes 1–4 and 6–9) or containing 100 mM DTT (lane 5). The presence or absence of various components is indicated above the lanes. The bands corresponding to free GST-Ufm1ΔC2 (mature Ufm1), GST-Ufm1ΔC3, GST-Uba5, GST-Ufc1, GST-Ufc1^{C116S}, and GST-Ufc1–GST-Ufm1ΔC2 thioester product are indicated on the right.

Conjugation of Ufm1 to cellular protein(s)

We next examined whether Ufm1 conjugates to the target protein(s) in cells. To this end, we expressed Flag- and 6xHis-tagged Ufm1 constructs in HEK293 cells, and purified them under denaturing conditions by Ni^{2+} beads. The resulting precipitates were then analyzed by immunoblotting with anti-Flag antibody. When FlagHis-Ufm1-HA (proUfm1) or FlagHis-Ufm1ΔC2 (mature form) was expressed, several proteins with sizes of about 28, 38, and 47 kDa were detected, in addition to the 10 kDa corresponding to free FlagHis-Ufm1ΔC2 (Figure 5A, lanes 2 and 4). These bands were not detected by FlagHis-Ufm1^{G83A}-HA and FlagHis-Ufm1ΔC3, suggesting that both C-terminal cleavage and C-terminal Gly residue are required for the conjugation reaction (Figure 5A, lanes 3 and 6). Moreover, these protein bands were resistant to reducing agents, such as DTT and β-mercaptoethanol. These results indicate that Ufm1 is covalently attached to some target proteins, probably through an isopeptide bond between the C-terminal Gly^{83} of Ufm1 and a Lys residue in the cellular proteins. It is of note that FlagHis-Ufm1^{G83A} mutant with exposed C-terminal Ala instead of Gly can conjugate to target proteins (Figure 5A, lane 5), consistent with the previous report on ubiquitin and SUMO (Hodgins *et al*, 1992; Kamitani *et al*, 1997). Since C-terminal Gly to Ala mutation confers resistance to the Ufm1 processing, the conjugates with FlagHis-Ufm1^{G83A} mutant may be more stable than those with FlagHis-Ufm1ΔC2 (Figure 5A, compare lanes 4 and 5). These results suggest that the Ufm1 conjugation is also a reversible reaction.

We further investigated the expression of Ufm1 and its conjugated proteins in mouse tissues using anti-Ufm1 serum. Ufm1 was widely expressed in all tissues examined, such as brain, heart, lung, liver, and kidney (Figure 5B, left panel). In addition, several bands with striking similarity to proteins detected in HEK293 cells were observed. These bands were not detected by preimmune or preabsorbed antisera (Figure 5B, right panel), suggesting that they are likely the Ufm1 conjugates. Although the intensity of each band varied among tissues and HEK293 cells, 28 and 38 kDa proteins were commonly detected. The 70-kDa band observed in all tissues was also detected faintly in HEK293 cells (Figure 5A, lane 5). The 47-kDa band observed in HEK293 cells was not clear. These protein bands were resistant to reducing agents, such as DTT and β-mercaptoethanol, indicating that Ufm1 covalently attaches to cellular proteins like other Ubl proteins. The targets of Ufm1 appeared to be common in a variety

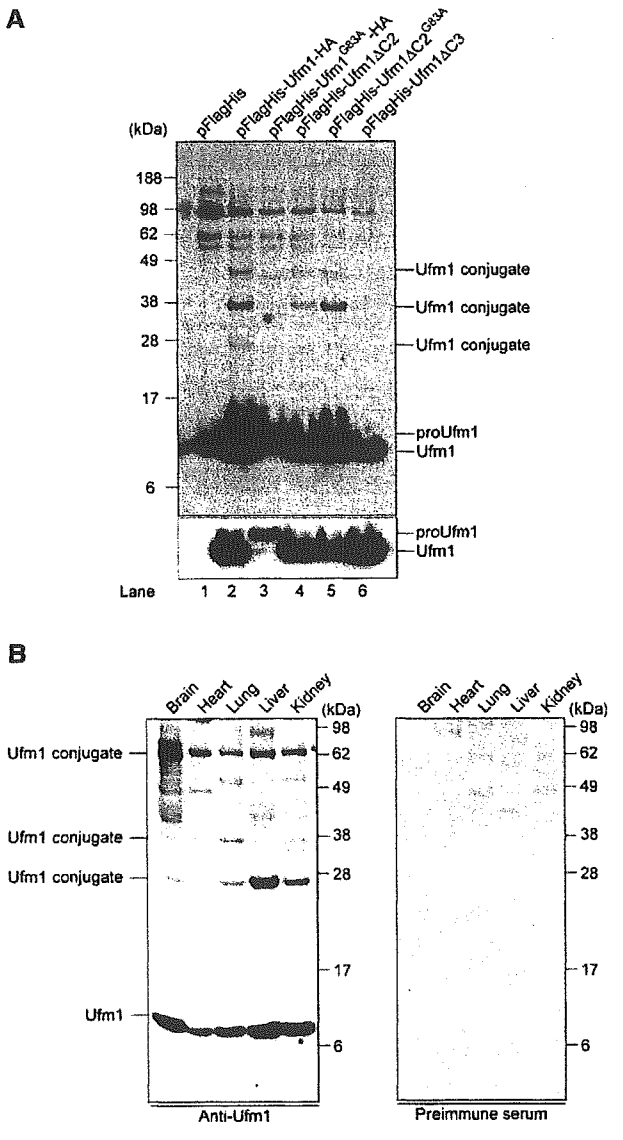

Figure 5 Formation of a covalent protein conjugate(s) with Ufm1 in HEK293 cells and mouse tissues. (**A**) Ufm1 conjugates in human HEK293 cells. HEK293 cells were transfected with FlagHis-Ufm1-HA, FlagHis-Ufm1^{G83A}-HA, FlagHis-Ufm1ΔC2, FlagHis-Ufm1ΔC2^{G83A}, or FlagHis-Ufm1ΔC3 expression plasmids. These cells were lysed under denaturing conditions, and the lysates were precipitated with Ni^{2+} beads. The precipitates were subjected to SDS–PAGE and analyzed by immunoblotting with anti-Flag antibody. The bottom panel shows the short exposure of the upper panel. The bands corresponding to mature Ufm1, proUfm, and Ufm1 conjugates are indicated on the right. (**B**) Ufm1 conjugates in various mouse tissues. Homogenates from mouse tissues as indicated were prepared and subjected to SDS–PAGE and analyzed by immunoblotting with anti-Ufm1 serum (left panel) or preimmune serum (right panel). The bands corresponding to Ufm1 and conjugates between Ufm1 and target proteins are indicated on the left.

of tissues. These results suggest the universal roles of Ufm1 in the regulation of cellular function in multicellular organisms.

Subcellular localization of Ufm1 in HeLa cells

We finally examined the subcellular distribution of Ufm1 in HeLa cells. Immunocytochemical analysis using anti-Ufm1

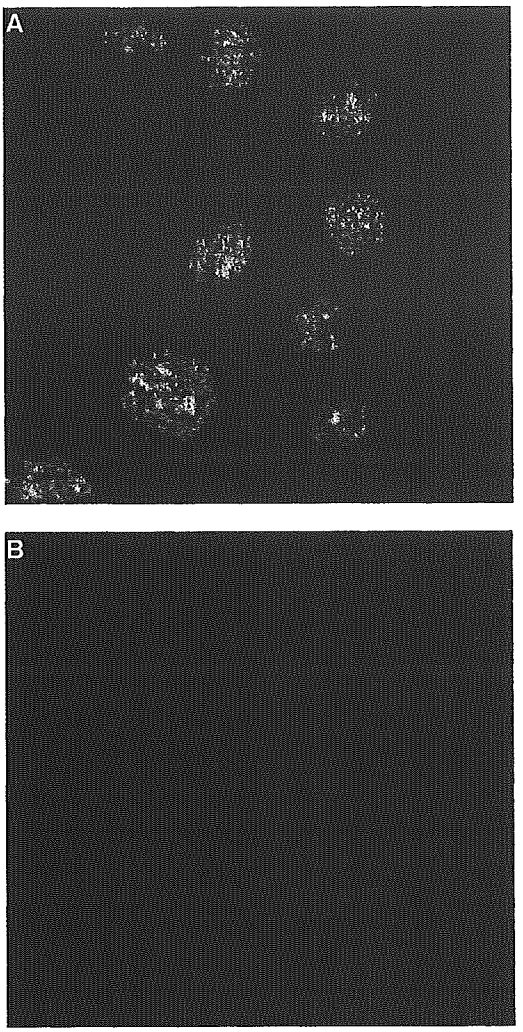

Figure 6 Intracellular distribution of Ufm1 in HeLa cells. (**A**) HeLa cells were seeded on coverslips 24 h before fixation for immunostaining. Ufm1 was detected with anti-Ufm1 serum and visualized with Alexa 488 nm anti-rabbit antibody. (**B**) Immunocytochemical analysis was conducted as for (A), except that preimmune serum was used. Cells were observed using a fluorescence microscope. Magnification, × 400.

serum revealed that Ufm1 was predominantly localized in the nucleus and diffusely in the cytoplasm (Figure 6A). These staining patterns were not observed when anti-Ufm1 serum had been preadsorbed with excess amounts of recombinant Ufm1 protein or preimmune serum was used instead of anti-Ufm1 serum (Figure 6B). Moreover, Ufm1 localization in the cytoplasm and nucleus was similar to the localization of exogenously expressed GFP-tagged Ufm1 in HeLa cells (data not shown). In the nucleus, strong immunoreactivity to anti-Ufm1 serum was observed as a dot-like structure. Although such dots-like structures were detected by preimmune serum, those intensities were weak. Thus, some of these dot-like structures may represent conjugates of Ufm1.

Discussion

In the present study, we reported that Ufm1 acts as a new post-translational UBL modifier, based on the following

criteria: (1) It is a small protein of 9.1 kDa with a ubiquitin-fold structure. (2) Ufm1 is synthesized in a precursor form, and the extra amino-acid residues at the C-terminal side need to be processed to expose the Gly residue. (3) The C-terminal processing and exposure of glycine residue are essential to the formation of Ufm conjugates in the cells. (4) Ufm1 has specific E1-like (Uba5) and E2-like (Ufc1) enzymes for activation and conjugation, respectively. Intriguingly, many UBL modifiers are evolutionarily conserved from yeast to human, except interferon-inducible UBL modifiers, such as UCRP/ISG15, Fat10, and Fau1/MNSFβ (Nakamura *et al*, 1995; D'Cunha *et al*, 1996; Liu *et al*, 1999). Ufm1, Uba5, and Ufc1 found in the present study are conserved in various multicellular organisms (Figures 1B, 2A, and 4A), but not in both budding and fission yeasts, suggesting that they all have been generated by coevolution.

We identified Uba5 as an E1 enzyme for Ufm1. This enzyme is relatively small compared to Uba1, that is, an E1 for ubiquitin (Figure 1A). In the *in vitro* assay, the recombinant Uba5 protein formed a thioester linkage with recombinant Ufm1 (Figure 3C) and transferred the activated Ufm1 to recombinant Ufc1 (an E2 enzyme) (Figure 4D), indicating that Uba5 can activate Ufm1 as a single molecule. This is in marked contrast to other E1s such as Uba2 and Uba3, which retain obvious similarities to the C-terminal half of Uba1 but require the formation of heterodimer complexes with respective partner molecules, AOS1 and APP-BP1, respectively, with similarities to the N-terminal half of Uba1 (Johnson *et al*, 1997; Liakopoulos *et al*, 1998; Osaka *et al*, 1998). Another E1-like enzyme, Uba4 that activates Urm1, is of similar size to Uba5 (Furukawa *et al*, 2000), but it remains unknown whether Uba4 acts as a single molecule or needs a partner subunit. The homology of Uba5 to Uba1 is less than those of Uba2 and Uba3, except their ThiF domain conserved in E1s, and thus it is likely that Uba5 may uniquely activate Ufm1, differing from other E1s such as Uba1, Uba2/AOS1, and Uba3/APP-BP1. Thus, although the structure of APP-BP1/Uba3 heterodimer is determined and the mechanism by which E1s activate their cognate UBLs was proposed (Walden *et al*, 2003a, b), the weak homology of Uba5 with other E1s hampered the computer-assisted structural analysis. To clarify this issue, structural analysis of Uba5 is required. This issue is currently under investigation in our laboratories. So far, most E1-like enzymes activate single species of UBL protein, although Atg7 is exception, which can activate both Atg8 and Atg12 (Mizushima *et al*, 1998; Tanida *et al*, 1999; Ichimura *et al*, 2000). A total of 10 E1-like enzymes can be identified in the human genome by computer analysis. Considering the limited number of E1-like proteins, it is possible that some E1-like proteins can activate a distinct set of UBL proteins. Whether or not Uba5 is capable of activating proteins other than Ufm1 remains to be clarified.

There are more than a dozen of E2 family genes in human genomes. In the budding yeast, 13 different E2s, namely Ubc1–Ubc13, have been documented and functionally characterized. Functionally, most of them catalyze the conjugation of ubiquitin, except that Ubc9 and Ubc12 are for SUMO and NEDD8/Rub1, respectively (Johnson and Blobel, 1997; Lammer *et al*, 1998; Osaka *et al*, 1998). In addition, in the autophagic pathway, Atg3 and Atg10 are both E2 enzymes for Atg8 and Atg12, respectively, but they do not have obvious sequence similarities to known Ubc's, except for a short region encompassing an active Cys residue (Shintani *et al*, 1999; Ichimura *et al*, 2000). Similarly, Ufc1 is a unique E2-like enzyme with no obvious sequence homology with other E2s, except approximately 10 amino-acid residues encompassing the active site Cys residue.

In assessing the biological roles of the Ufm1-modifying system, characterization of the target molecule(s) is of particular importance. Regarding this issue, we identified several putative proteins that are conjugated with Ufm1 in human HEK293 cells and various mouse tissues. It is noteworthy that the sizes of these bands (28, 38, 47 kDa) increase by 10 kDa, which is consistent with the size of Ufm1. Considering that several Ubl modifiers can attach to target proteins as a polymer, it is possible that these bands correspond to multi- or poly-Ufm1 conjugates. In fact, Ufm1 has six Lys residues. Whether Ufm1 is conjugated to several distinct proteins or multiple Lys residues in a single target or polymerized in a single Lys residue awaits future study. Unfortunately, we could not identify the protein, and detailed analysis of the cellular function of Ufm1 conjugation awaits future study. It was recently reported that Uba5 is induced by certain reagents that induce stress in the endoplasmic reticulum (ER), a so-called 'unfolded protein response' (Harding *et al*, 2003). However, we could not observe the induction of Uba5, Ufc1, and Ufm1 by treatment with various compounds known to induce ER stress in mammalian cells (data not shown). In addition, exposure to other stresses including high temperature or heavy metals also did not induce the appearance of obvious new conjugation band(s) of Ufm1, by immunoblot analysis. Further studies on the biological roles of the Ufm1 conjugation pathway are under investigation in our laboratories.

Materials and methods

DNA construction

The cDNA encoding human Uba5 was obtained by PCR from human liver cDNA with the Uba5-s5′ primer (5′-CGGAGGGATCCC CATGGCGGAGTCTGTGGAG-3′) and the Uba5-r3′ primer (5′-CAGTCCTCGAGCTACATATTCTTCATTTT-3′). It was then subcloned into pcDNA3 vector (Invitrogen, San Diego, CA). A point mutation for Cys at position 250 to Ser or Ala was generated by PCR-based site-directed mutagenesis. The Flag tag was introduced at the N-terminus of Uba5 or Uba5^{C250S}. Similarly, cDNA encoding human Ufm1 was amplified by PCR from human liver cDNA with the Ufm1-s5′ primer (5′-TTCCGGGATCCCCATGTCGAAGGTTTCCTTT-3′) and the Ufm1-r3′ primer (5′-AGTAGCTCGAGTTAACAACTTCCAA CACGAT-3′), and subcloned into pcDNA3 vector. The Flag, FlagHis, or Myc tags were introduced at the N-terminus of Ufm1. The HA tag was introduced at the C-terminus of Ufm1. The C-terminal deletion mutants of Ufm1 named Ufm1ΔC2 and Ufm1ΔC3, encoding amino acids 1–83 and 1–82, respectively, were generated by PCR. A point mutation for Gly at position 83 to Ala of Ufm1 and Ufm1ΔC2 (Ufm1^{G83A} and Ufm1ΔC2^{G83A}, respectively) was generated by PCR-based site-directed mutagenesis. The cDNA encoding human Ufc1 was obtained by PCR from human liver cDNA with the Ufc1-s5′ primer (5′-GCCCTGGATCCAGATGGCGGATGAAGCCACG-3′) and the Ufc1-r3′ primer (5′-TTCTCGAGTCATTGGTTGCATTTCTCTT-3′). It was then subcloned into pcDNA3 vector. A point mutation for Cys at position 116 to Ser or Ala was generated by PCR-based site-directed mutagenesis. The Flag tag was introduced at the N-terminus of Ufc1 and Ufc1^{C116S}. To express GST-fused Ufm1ΔC2, Ufm1ΔC3, Uba5, Uba5^{C250A}, Ufc1, and Ufc1^{C116A} in *E. coli*, these cDNAs were subcloned into pGEX-6p vector (Amersham Biosciences). All mutations mentioned above were confirmed by DNA sequencing.

Cell culture and transfection

Media and reagents for cell culture were purchased from Life Technologies (Grand Island, NY). HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 5 U/ml penicillin, and 50 μg/ml streptomycin. HEK293 cells at subconfluence were transfected with the indicated plasmids using Fugene 6 reagent (Roche Molecular Biochemicals, Mannheim, Germany). Cells were analyzed at 20–24 h after transfection.

Immunological analysis

For immunoblot analysis, cells were lysed with ice-cold TNE buffer (10 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitors) and the lysates were separated by SDS–PAGE (12% gel or 4–12% gradient gel) and transferred to a polyvinylidene difluoride (PVDF) membrane. Mouse monoclonal anti-Flag antibody (M2; Sigma Chemical Co., St Louis, MO), anti-HA antibody (F7; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal anti-Myc antibody (N14; Santa Cruz) were used for immunodetection. Development was performed by the Western lighting detection methods.

For immunoprecipitation analysis, cells were lysed by 200 μl of TNE, and the lysate was then centrifuged at 10 000 *g* for 10 min at 4°C to remove debris. In the next step, 800 μl of TNE and 30 μl of M2-agarose (Sigma) were added to the lysate, and the mixture was mixed under constant rotation for 12 h at 4°C. The immunoprecipitates were washed five times with ice-cold TNE. The complex was boiled for 10 min in SDS sample buffer in the presence of β-mercaptoethanol to elute proteins and centrifuged at 10 000 *g* for 10 min at 4°C. The supernatant was subjected to SDS–PAGE, transferred to PVDF membrane, and analyzed by immunoblots with anti-Flag (M2) or anti-Myc (N14) antibody.

For purification of 6xHis-tagged proteins under denaturing conditions, cells were lysed by 1 ml of denaturing lysis buffer (8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris–HCl, pH 8.0) in the presence of 20 mM *N*-ethylmaleimide as an inhibitor of isopeptidases, and the lysate was sonicated briefly and then centrifuged at 10 000 *g* for 10 min at room temperature to remove debris. Then, 30 μl of Ni-NTA Superflow (QIAGEN) was added to the lysate, and the mixture was shaken under constant rotation for 30 min at room temperature. The precipitates were washed five times with denaturing wash buffer (8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris–HCl, pH 5.9). To elute proteins, elution buffer (8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris–HCl, pH 4.5) was added to the complex, and the mixture was centrifuged at 10 000 *g* for 10 min at room temperature. The resulting supernatant was subjected to SDS–PAGE, transferred to PVDF membrane, and analyzed by immunoblots with anti-Flag (M2).

Freshly isolated tissues from mice were homogenized in lysis buffer (50 mM Tris–HCl, pH 7.5, 1% SDS, 5 mM EDTA, and 10 mM β-mercaptoethanol) using potter-Elvehjem homogenizer. The homogenate was centrifuged at 10 000 *g* for 10 min to remove debris. The resulting supernatant was subjected to SDS–PAGE, transferred to PVDF membrane, and analyzed by immunoblotting with anti-Ufm1 or preimmune serum. The anti-Ufm1 polyclonal antibody was raised in rabbits using the recombinant protein produced in *E. coli* as an antigen.

In vitro thioester formation assay

Recombinant GST-Ufm1ΔC2, GST-Ufm1ΔC3, GST-Uba5, GST-Uba5^{C250A}, GST-Ufc1, and GST-Ufc1^{C116A} (tagged N-terminally with GST) were produced in *E. coli* and recombinant proteins were purified by chromatography on glutathione sepharose 4B (Amersham Biosciences). After elution of proteins from the beads, the preparations were dialyzed against 50 mM BisTris (pH 6.5), 100 mM NaCl, 10 mM $MgCl_2$, and 0.1 mM DTT (reaction buffer). Most thioester formation reactions contained reaction buffer with 4 μg GST-Ufm1ΔC2 or GST-Ufm1ΔC3 and some of the following: 5 mM ATP, 2 or 0.2 μg GST-Uba5 or GST-Uba5^{C250A}, and 4 μg GST-Ufc1 or GST-Ufc1^{C116A}. Reactions were incubated for 30 min at 25°C and stopped by the addition of SDS-containing loading buffer either lacking reducing agent or containing 100 mM DTT, followed by a 10 min incubation at 37°C, SDS–PAGE (4–12% acrylamide gradient) and Coomassie brilliant blue staining.

Protein identification by LC–MS/MS analysis

The Uba5-associated complexes were digested with *Achromobacter* protease I and the resulting peptides were analyzed using a nanoscale LC–MS/MS system as described previously (Natsume *et al*, 2002). The peptide mixture was applied to a Mightysil-PR-18 (1 μm particle, Kanto Chemical) frit-less column (45 mm × 0.150 mm ID) and separated using a 0–40% gradient of acetonitrile containing 0.1% formic acid over 30 min at a flow rate of 50 nl/min. Eluted peptides were sprayed directly into a quadropole time-of-flight hybrid mass spectrometer (Q-Tof *Ultima*, Micromass, Manchester, UK). MS and MS/MS spectra were obtained in a data-dependent mode. Up to four precursor ions above an intensity threshold of 10 counts/s were selected for MS/MS analyses from each survey scan. All MS/MS spectra were searched against protein sequences of Swiss Prot and RefSeq (NCBI) using batch processes of Mascot software package (Matrix Science, London, UK). The criteria for match acceptance were the following: (1) When the match score was 10 over each threshold, identification was accepted without further consideration. (2) When the difference of score and threshold was lower than 10, or when proteins were identified based on a single matched MS/MS spectrum, we manually confirmed the raw data prior to acceptance. (3) Peptides assigned by less than three y series ions and peptides with +4 charge state were all eliminated regardless of their scores.

Immunofluorescence

HeLa cells grown on glass coverslips were fixed in 4% paraformaldehyde (PFA) in PBS for 15 min, and permeabilized with 0.2% (vol/vol) Triton X-100 in PBS for 30 min. After permeabilization, the cells were blocked for 30 min with 5% (vol/vol) normal goat serum in PBS, incubated for 1 h at 37°C with anti-Ufm1 serum or preimmune serum, washed with PBS, and incubated for 30 min with Alexa 488 nm anti-rabbit antibodies (Molecular Probes). The coverslips were washed and mounted on slides. Fluorescence images were obtained using a fluorescence microscope (DMIRE2; Leica) equipped with a cooled charge-coupled device camera (CTR MIC; Leica). Pictures were taken using Leica Qfluoro software (Leica).

Acknowledgements

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Hub1 is an essential ubiquitin-like protein without functioning as a typical modifier in fission yeast

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Hub1 exhibits 23% sequence identity to ubiquitin. However, Hub1 lacks the C-terminal Gly, which is essential for covalent attachment to target protein(s) of ubiquitin and other ubiquitin-like (UBL) modifiers. Instead, Hub1 proteins in all eukaryotes retain the di-Tyr just before a single variable residue at the C-terminus, so one intriguing question is whether Hub1 could be linked to substrate through the conserved Tyr or not. Here we studied Hub1 in *Schizosaccharomyces pombe*. Gene disruption experiment revealed that *hub1*$^+$ is essential. Remarkably, the mutant cells harbouring Hub1 lacking the di-Tyr could grow similar to wild-type cells, indicating that the di-Tyr is dispensable for the essential function of Hub1. Moreover, we could not observe cleavage of Flag-tag fused with C-terminus of Hub1. It suggests that the processing for conjugation via conserved Tyr is not likely to occur in Hub1, and Hub1 is a novel class of the UBL protein family. Finally, we isolated a temperature-sensitive allele, *hub1-1*. This temperature sensitivity could be suppressed by overproduction of Rpb10 or Snu66, the former of which is one of the common subunits of the RNA polymerases and the other is the component of the spliceosome. We also observed that pre-mRNA splicing was impaired in *hub1-1*.

Introduction

Ubiquitin consists of 76 amino acid residues and is a highly conserved protein across species in eukaryotes. This small protein functions characteristically; i.e. it is covalently attached to the substrate proteins via an isopeptide linkage between the C-terminal Gly of ubiquitin and the ε-NH_2 group of Lys of the acceptor substrate. A polyubiquitin chain is formed by repeated reactions through which another ubiquitin links a Lys residue at position 48 within one ubiquitin associated with the target protein, which becomes a marker for proteasome degradation (Glickman & Ciechanover 2002; Pickart 2004). In addition to aberrant proteins, many regulatory proteins in a diverse array of biologically important processes, such as mitotic cell cycle, signal transduction, or developmental programs, are known to be substrates of this protein modifying system. In addition, it has also become clear that ubiquitylation has various cellular roles other than proteolysis, such as vesicular transport and gene silencing (Glickman & Ciechanover 2002; Pickart 2004).

The conjugation of ubiquitin to the substrates is carried out by three types of enzymes; E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugation enzyme) and E3 (ubiquitin ligating enzyme). E1 catalyses the formation of a high-energy thioester bond between the C-terminal Gly residue of ubiquitin and a specific Cys residue in itself. E2 receives the activated ubiquitin molecules from E1, forming a thioester linkage like E1. E3 recognizes the substrates, and attaches ubiquitin transferred by E2 to the substrates or helps E2 to transfer ubiquitin to the substrates (Glickman & Ciechanover 2002; Pickart 2004). E3 plays a critical role in the selection of target proteins, because each distinct E3 usually binds a protein substrate with a degree of selectivity for ubiquitylation in a temporally and spatially regulated fashion.

Deubiquitinating enzymes (DUBs) or ubiquitin-specific processing proteases are required to generate functional ubiquitin monomers, because ubiquitin is translated as fusion proteins with certain ribosomal proteins or a polyubiquitin in which several ubiquitin moieties link tandemly. DUBs process these ubiquitin precursors at the C-terminus of the ubiquitin segment(s). After this processing, Gly76 appears at the C-terminus of ubiquitin. Since the conjugation of ubiquitin is formed via this Gly76, exposure of Gly76 is essential for the protein-modifying function of ubiquitin (Fischer 2003; Kim *et al.* 2003).

Various ubiquitin-like (UBL) modifiers have been identified so far, including SUMO, NEDD8 and UCRP/ISG15, which are structurally similar to ubiquitin (Tanaka

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et al. 1998; Jentsch & Pyrowolakis 2000; Schwartz & Hochstrasser 2003). They are covalently linked to target proteins via the C-terminal Gly residue in a manner analogous to ubiquitylation, where the reaction is catalysed by the E1-, E2- and E3-like enzymes. It is of note that most DUB-like enzymes are also necessary to remove the C-terminal extension for their conjugation except UBL(s) synthesized with Gly in the C-terminal end (Li & Hochstrasser 1999; Gan-Erdene *et al.* 2003; Mendoza *et al.* 2003; Wu *et al.* 2003; Hemelaar *et al.* 2004).

Recent studies described a novel UBL protein, Hub1 (Dittmar *et al.* 2002; Lüders *et al.* 2003; Ramelot *et al.* 2003). Hub1 is a UBL protein with apparently similar tertiary structure (Ramelot *et al.* 2003) despite the low (23%) residue identity to ubiquitin. However, whether Hub1 is a modifier protein is a matter of controversy. There is a characteristic difference between Hub1 and ubiquitin; i.e. Gly is not evolutionarily conserved at the C-terminus, but the di-Tyr sequence is conserved at the C-terminus, followed by one more extra non-conserved residue. Dittmar *et al.* (2002) reported that Hub1 in *Saccharomyces cerevisiae* was processed at the C-terminus similar to ubiquitin, and functioned as a modifier using the conserved Tyr residue, but Lüders *et al.* (2003) raised an objection to this conclusion using the same budding yeast. Hub1 is not essential for viability in *S. cerevisiae*, but deletion mutant shows the defect in cell polarization during the formation of mating projections (Dittmar *et al.* 2002), and slow growth phenotype in a specific strain background (Lüders *et al.* 2003). Hub1 is also identified as Ubl5 or Beacon in other organisms (Friedman *et al.* 2001; Brailoiu *et al.* 2003; Kantham *et al.* 2003; McNally *et al.* 2003), but it remains unresolved whether Ubl5/Beacon is a modifier protein. In the present study, we address this issue by working on Hub1 in *Schizosaccharomyces pombe*. We found that Hub1 is essential and functions without processing of its C-terminus to expose the conserved residue unlike other UBL modifiers. In addition, we discuss the biological function of Hub1 by isolating and examining *hub1-1* mutation and its multicopy suppressor genes.

Results

Hub1 is essential for *S. pombe*

From the database, we identified a new UBL protein in *S. pombe*. This protein is highly conserved among eukaryotes, displaying 64–75% amino acid identity ranging from yeast to human (Fig. 1A). In *S. cerevisiae*, this gene is named *HUB1* (Dittmar *et al.* 2002; Lüders *et al.* 2003), so we followed this nomenclature and named *S. pombe* gene *hub1*$^+$. Though Hub1 is a UBL protein, there is a characteristic difference between Hub1 and ubiquitin. It has no conserved Gly residue at the C-terminus, which is essential for the covalent attachment of ubiquitin and other known UBL modifiers to other proteins. On the other hand, di-Tyr sequence is conserved among all Hub1 homologues, and it seems to be equivalent to Gly for ubiquitin (Fig. 1A).

The tertiary structure of *S. pombe* Hub1 displayed a striking resemblance to human ubiquitin (Fig. 1B). The structure of *S. pombe* Hub1 was constructed by a computer-assisted modelling, based on the structure of its *S. cerevisiae* homologue that has been analysed previously (Ramelot *et al.* 2003).

To explore the function of Hub1 in *S. pombe*, we disrupted the *hub1*$^+$ gene by replacing the entire ORF with the Aureobasidine A resistant gene (*aur1*^{R}) in diploid strain (Fig. 1C, left panel). Heterozygous diploids were sporulated and dissected. From each tetrad, only two viable spore clones could be obtained (Fig. 1C, right panel), and all of them had Aureobasidine A sensitive phenotype. Thus, it is clear that *hub1*$^+$ is essential for mitotic growth in *S. pombe*. Microscopic analysis revealed that *hub1*$^+$ deleted cells ceased their growth with an elongated cell shape after 2 or 3 cycles of cell division (data not shown). To confirm the effect of lack of Hub1, we constructed the strain whose growth is dependent of *hub1*$^+$ under the thiamine repressive promoter. At 12 h after repression of Hub1 by addition of thiamine, cells started to elongate, and to slightly enlarge in size (Fig. 1D).

Localization of Hub1

To determine the localization of Hub1 in living cells, GFP-Hub1 was constructed and introduced into the Δ*hub1* strain. The fusion gene complemented the deletion strain, so the essential function of Hub1 was not compromised by fusion to GFP. As shown in Fig. 2, GFP-Hub1 was observed in both the nucleus and the cytoplasm.

Di-Tyr is dispensable for Hub1

We examined the importance of the conserved Tyr residues in Hub1. Our initial working hypothesis was that Hub1 is an essential modifier and the conserved Tyr is used for its modification. If this model is correct, we can expect the Tyr residue to be essential for Hub1 function and growth of *S. pombe*. A Δ*hub1::aur1*^{R}/*hub1*$^+$ heterozygous diploid strain (YHY22P) was transformed with pHY58 (pUR19 (*ura4*$^+$)-*hub1*$^+$) and a Δ*hub1* haploid strain whose growth depended on pHY58 was obtained.

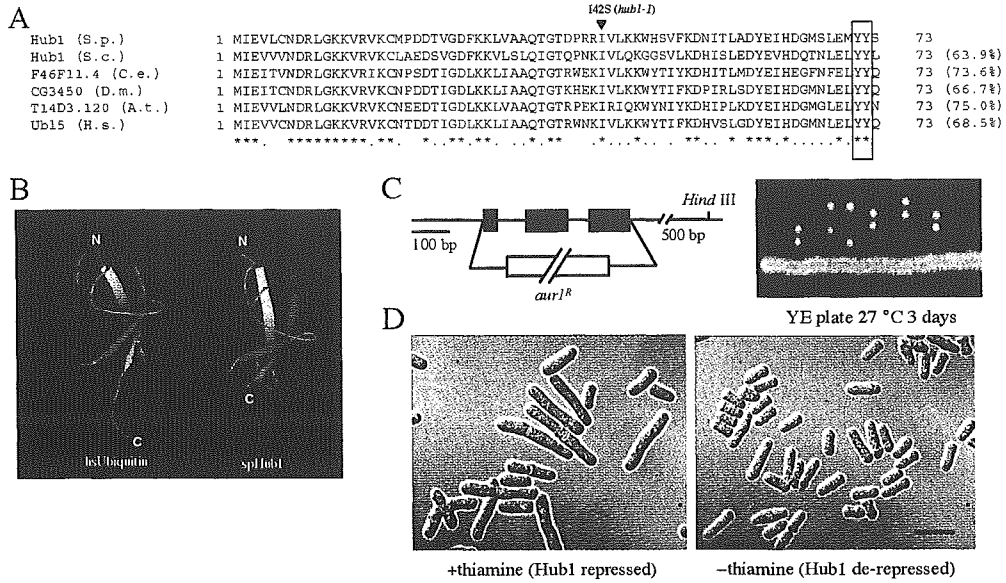

Figure 1 Hub1 is an essential ubiquitin-like protein in *S. pombe*. (A) Sequence alignment of the Hub1 proteins. *S. c.*; *Saccharomyces cerevisiae, S. p.*; *Schizosaccharomyces pombe, H. s.*; *Homo sapiens, C. e.*; *Caenorhabditis elegans, D. m.*; *Drosophila melanogaster, A. t.*; *Arabidopsis thaliana*. The amino acid sequences of the Hub1 proteins of various species are compared by the GENETYX-Mac ver. 11 program (Software Development Co., Tokyo, Japan). *Asterisks*, identical amino acids; *dots*, similar amino acids, determined by the criteria of GENETYX-Mac. Di-Tyr residues conserved at the C-terminus among every species are boxed. Identities with spHub1 (Hub1 of *S. pombe*) are described at the right side of each protein. Arrowhead indicates the mutation site of *hub1-1*. (B) Structural ribbon of human ubiquitin and predicted structural ribbon of SpHub1. α-helices and β-strands are shown in green and yellow, respectively. The homology model of SpHub1 was created from the ScHub1 structure (Ramelot *et al.* 2003) by using MOE program (2003.02; Chemical Computing Group Inc., Montreal, Quebec, Canada). (C) Disruption of the *hub1*$^+$ gene (left panel). Predicted chromosomal structure of the *hub1*$^+$ gene containing the inserted *aur1*^{R} marker gene. Solid boxes show the coding sequence in exons of the *hub1*$^+$ gene. An entire region of *hub1*$^+$ was deleted and replaced by the Aureobasidin A resistant gene, *aur1*^{R}. *Hin*dIII site at the 500 bp downstream of *hub1*$^+$ ORF was used for constructing *hub1-1*. Tetrad analysis (right panel). A Δ*hub1::aur1*^{R}/*hub1*$^+$ heterozygous diploid strain (YHY22P) was sporulated for tetrad dissection on aYE plate and incubated at 27 °C for 3 days. (D) Δ*hub1* with pHY56 (pREP81-Flag-His$_6$-*hub1*$^+$ ORF) was grown in EMM-Leu media with (repressed) or without (de-repressed) thiamine at 32 °C for 12 h. Both cells were fixed with 70% ethanol, and then observed under the microscope. Scale bar = 10 μm.

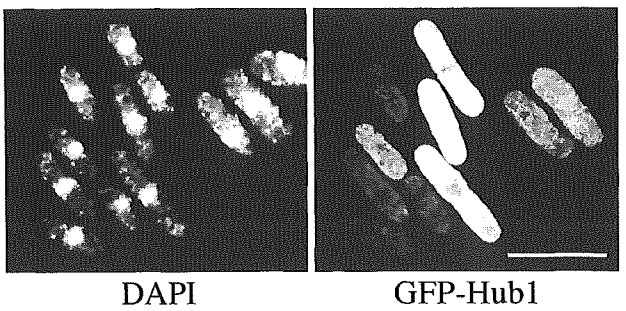

Figure 2 Localization of Hub1. YHY26P (Δ*hub1::aur1*^{R} with pREP81-GFP-Hub1) cells were grown at 26 °C in EMM-Leu media, stained with DAPI, and monitored by direct fluorescence. GFP-Hub1 was observed in both the nucleus and the cytoplasm. Scale bar = 10 μm.

This strain (YHY23P) was further transformed with pALSK (*LEU2*), pHY59 (pALSK-*hub1*$^+$) or pHY60 (pALSK-*hub1*ΔYY) in which conserved di-Tyr was deleted. Each transformant was streaked on a SD-Leu plate containing 0.5 mg/mL 5-FOA. 5-FOA is a toxic reagent for uracil autotrophic cells, so cells should dispense with pHY58 (pUR19-*hub1*$^+$) to grow on 5-FOA-containing media. Of the three types of transformants, only the transformant with pALSK failed to grow on this medium while the transformant with pHY60 (pALSK-*hub1*ΔYY) could grow as well as one with pHY59 (pALSK-*hub1*$^+$) (Fig. 3). Thus, pHY60 (pALSK-*hub1*ΔYY) could replace pHY58 (pUR19-*hub1*$^+$), indicating that the di-Tyr motif is not essential for the Hub1 function and for the growth of *S. pombe*. Moreover, cells bearing the Hub1ΔYY showed no growth defect even at higher temperature (data not shown).

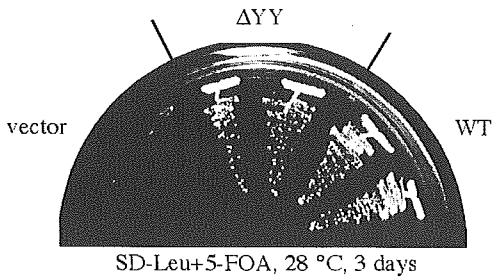

Figure 3 Di-Tyr is dispensable for the Hub1 function. YHY23P (*Δhub1::aur1*^{R} pUR19 (*ura4*$^+$)-*hub1*$^+$) was further transformed with pALSK (*LEU2*), pHY59 (pALSK-*hub1*$^+$) or pHY60 (pALSK-*hub1*ΔYY) in which conserved di-Tyr at the C-terminus of Hub1 was deleted. Each transformant was streaked on a SD-Leu plate containing 0.5 mg/mL 5-FOA and incubated at 28 °C for 3 days.

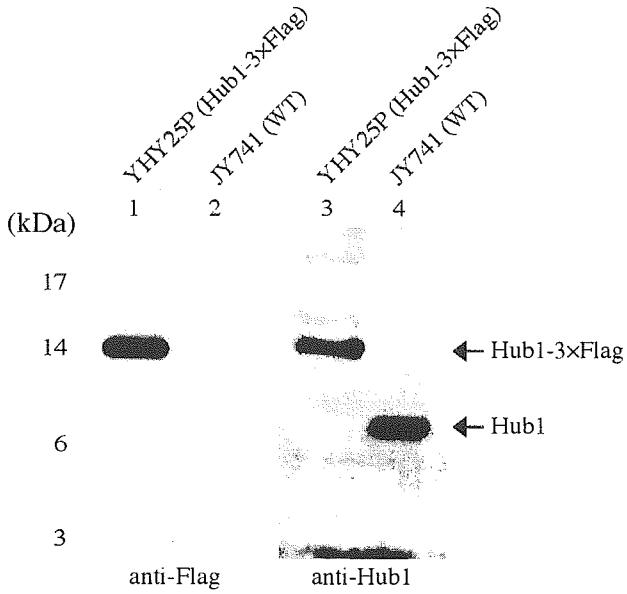

Figure 4 Hub1 is not processed at the C-terminus. WT (JY741) or C-terminally 3 × Flag tagged strain (YHY25P) was cultured in 4 mL YE medium at 26 °C until the OD_{600nm} reached around 1.0. Cells were harvested and processed according to the method described in 'Experimental procedures'. Hub1 protein was detected by Western blotting analysis using anti-Flag (left panel) or anti-Hub1 antibody (right panel). Numbers at the left side indicate the molecular size (kDa).

Processing of Hub1 at the C-terminus

Ubiquitin and other UBL modifiers, such as NEDD8 or SUMO are processed to have conserved Gly exposed at the C-terminus, which is used for their covalent attachment to their substrate proteins. Alternatively, in Hub1, Tyr is conserved just before the last non-conserved residue. It is also possible that one of the other conserved residues located the 5′ upside of Tyr is used for its conjugation. In either case, if Hub1 acts as a modifier protein, it should be processed to remove its non-conserved residue(s) at the C-terminus, because it is unlikely that non-conserved residue is used for its conjugation. To examine whether processing of Hub1 occur, a strain expressing Hub1–3 × Flag was obtained by chromosomal tagging of the *hub1*$^+$ gene in JY741. It is known that the C-terminal β-galactosidase fused with ubiquitin and the natural C-terminal tripeptide Ala-Thr-Tyr of SUMO are removed co-translationally and their pre-processed forms are barely observed (Bachmair *et al.* 1986; Li & Hochstrasser 2003). However, in the case of Hub1, we could easily detect Hub1 with C-terminal Flag tag using anti-Flag antibody (Fig. 4). On the contrary, we could not detect the Hub1 molecules without C-terminal Flag tag, judging from the Western blot analyses with anti-Flag and anti-Hub1 antibodies (Fig. 4). This result indicated that the processing at the C-terminus of Hub1 did not occur. In comparison with ubiquitin and other UBL modifiers, this feature is unique to Hub1.

Multicopy suppressor of *hub1-1* temperature sensitive mutant

As a useful tool for characterization of essential genes, we generated one temperature-sensitive allele of *hub1*$^+$ by random mutagenesis. Sequence analysis revealed that Ile42 changed to Ser42. Ile42 is conserved among Hub1 proteins (Fig. 1A), and it is located as a first residue of the third strands (Ramelot *et al.* 2003), so Ile42 is expected to be important for the maintenance of the UBL structure. Under restrictive temperature, *hub1-1* cells were elongated similar to the Hub1-depleted cells. DAPI staining showed that cytokinesis might be defective in *hub1-1* (Fig. 5A).

We searched another phenotype of *hub1-1*, and found that high osmolarity suppressed temperature sensitivity. Both non-ionic (1 M sorbitol) and ionic (0.75 M KCl) high osmolarity worked well (Fig. 5B). Hyperosmotic conditions could also diminish the cell elongation phenotype of *hub1-1* (Fig. 5C), indicating that high osmolarity suppressed the primary defect of *hub1-1*.

Next, we tried to isolate the multicopy suppressors of *hub1-1*. The *hub1-1* cells were transformed with a multicopy pALSK vector based *S. pombe* genomic library and plated at 34 °C. Plasmids were rescued from transformants that grew at this temperature. Among 77 positive clones excluding *hub1*$^+$ itself, 53 clones included *rpb10*$^+$. Rpb10 is a common subunit in all three forms of the RNA polymerase (Lalo *et al.* 1993; Gadal *et al.* 1999;

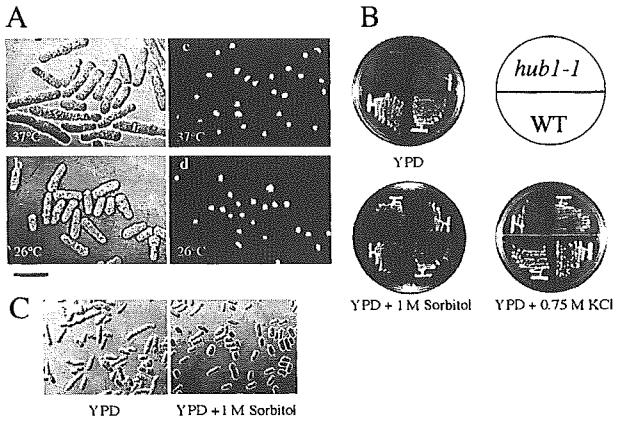

Figure 5 Osmotic stabilizer suppressed the *hub1-1* phenotype. (A) Cell morphology of *hub1-1* cells was observed under the microscope. Cells were cultured at 37 °C for 8 h or at 26 °C, and fixed with 70% ethanol. DNA was stained with DAPI. Scale bar = 10 μm (B) WT or *hub1-1* cells were streaked on a YPD plate or YPD plus osmotic stabilizer plates, and incubated at the restricted temperature. (1) YPD incubated at 36 °C for 3 days; (2) YPD + 1 M sorbitol incubated at 36 °C for 3 days; (3) YPD + 0.75 M KCl incubated at 36 °C for 5 days. (C) *hub1-1* cells incubated at 36 °C for 2 days on a YPD or YPD + 1 M sorbitol plate were observed under the microscope.

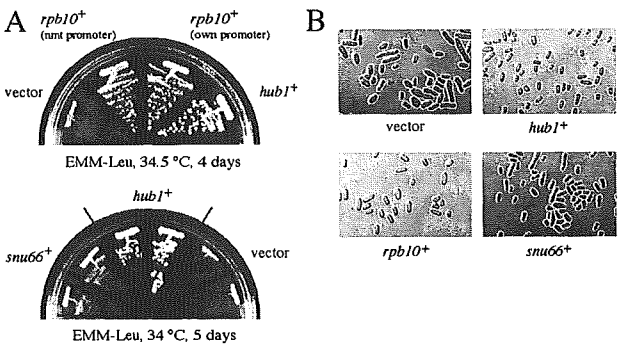

Figure 6 Multicopy suppressors of *hub1-1*. (A) *hub1-1* cells transformed with pREP1, pREP81-*rpb10*$^+$ ORF, pALSK-*rpb10*$^+$, pREP1-*snu66*$^+$ ORF or pALSK-*hub1*$^+$ were streaked on a EMM-Leu plate, and incubated at 34.5 °C for 4 days (top) or at 34 °C for 5 days (bottom). (B) Cell morphology of *hub1-1* cells transformed with the multicopy suppressors under the restrictive temperature was observed under the microscope. Each transformant was grown on an EMM-Leu plate at 34 °C for 2 days.

Kimura *et al.* 2001). To confirm that *rpb10*$^+$ is a suppressor gene of *hub1-1*, only *rpb10*$^+$ ORF region was subcloned into the expression vector, pREP81. *hub1-1* transformed with pHY63 (pREP81-*rpb10*$^+$ ORF) could grow at the restricted temperature as those with the original clones (Fig. 6A, upper), so we could confirm that *rpb10*$^+$ was truly a multicopy suppressor of *hub1-1*.

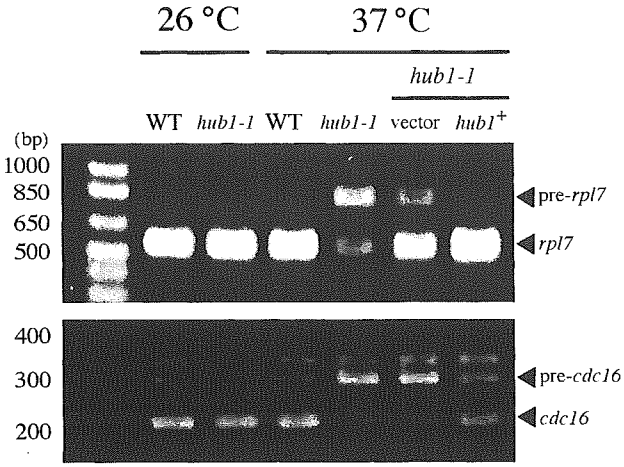

Figure 7 Hub1 is involved in premRNA splicing. RT-PCR splicing assays on *rpl7*$^+$ and *cdc16*$^+$ in *hub1-1*. Total RNA was isolated from WT or *hub1-1* cells at the permissive (26 °C) or after 3 h incubation at the restrictive (37 °C) temperature, and from *hub1-1* cells transformed with pREP1 or pALSK-*hub1*$^+$ after 3 h incubation at 37 °C. Unspliced and processed mRNAs are indicated. Numbers at the left side indicate the molecular size (bp).

Recently, it was reported that Hub1 in *S. cerevisiae* interacts Snu66 in the two-hybrid system (Hazbun *et al.* 2003). Snu66 is one of the components in the spliceosome (Jurica & Moore 2003; Kaufer & Potashkin 2000). We examined whether there is a genetic interaction between Hub1 and Snu66 in *S. pombe*. For this purpose, *snu66*$^+$ was cloned into the expression vector, pREP1, and introduced into *hub1-1*. Transformed cells could grow under the restricted temperature (Fig. 6A, lower). Overproduciton of Rpb10 or Snu66 could suppress elongated cell morphology in proportion to the suppression activity on the media (Fig. 6B).

The genetic interaction between Snu66 and Hub1 suggested that *hub1*$^+$ might be involved in pre-mRNA splicing. To verify whether or not Hub1 is required for splicing in *S. pombe*, we used RT-PCR to measure the steady state levels of premRNA and mRNA of *rpl7*$^+$ or *cdc16*$^+$ in *hub1-1* cells. After 3 h incubation at 37 °C, significant accumulation of premRNA of both genes was observed in the *hub1-1* mutant (Fig. 7). From this result, we conclude that Hub1 plays a role for the efficient splicing of premRNA.

Discussion

Hub1 is one of the highly conserved UBL proteins in eukaryotes. In addition, Dittmar *et al.* (2002) showed that in *S. cerevisiae*, Hub1 acts as a modifier protein like

ubiquitin. They also showed that Tyr72 is essential for its conjugation. However, we showed in this study that Tyr72 is dispensable for the essential function of Hub1 in *S. pombe* (Fig. 3). It is therefore possible that Hub1 has two distinct functions; one is an essential function, which is independent of the conjugation, and another is a nonessential function, which is fulfilled by conjugation to other proteins. However, we doubt the function of Hub1 as a typical modifier based on another reason described below.

It is highly improbable that the conjugation sites vary from species to species, but it is possible that there are some other conserved conjugation sites in addition to Tyr72. In either case, the C-terminal processing of Hub1 to remove the non-conserved residue(s) at the C-terminus should be required to have Tyr72 or another hypothetical conjugation site exposed at the C-terminus. We constructed the Hub1 tagged with 3 × Flag C-terminally and examined whether the processing of Hub1 occurred at the C-terminus. However, we could not detect cleavage of the Flag tag, namely the C-terminal processing of Hub1 (Fig. 4), though we could not exclude the possibility that very little amount of Hub1 is processed. Considering that ubiquitin and other modifiers like NEDD8 and SUMO are processed co-translationally (Bachmair *et al.* 1986; Gan-Erdene *et al.* 2003; Li & Hochstrasser 2003; Mendoza *et al.* 2003; Wu *et al.* 2003; Hemelaar *et al.* 2004), the processing of Hub1 is not likely to happen. This means that Hub1 has a native C-terminal residue encoded by genome, which is different in different species (Fig. 1A). This result is consistent with the finding of Lüders *et al.* (2003), in which C-terminally tagged Hub1 existed stably. Thus, all our results indicate that Hub1 is unlikely to be a typical modifier protein like ubiquitin and other UBL proteins.

To date, a set of novel molecules called ubiquitin-like proteins (UBLs) that have structural similarity to ubiquitin, has been identified (Jentsch & Pyrowolakis 2000). Currently, these molecules are divided into two subclasses; type-1 UBLs, which ligate to target proteins in a manner similar, but not identical, to the ubiquitylation pathway, such as SUMO, NEDD8 and UCRP/ISG15, and type-2 UBLs (or called as UDPs, ubiquitin-domain proteins) that contain UBL structure embedded in a variety of different classes of large proteins with apparently distinct functions, such as Rad23, Elongin B, Scythe, Parkin and HOIL-1 (Tanaka *et al.* 1998; Jentsch & Pyrowolakis 2000; Yeh *et al.* 2000; Schwartz & Hochstrasser 2003). However, Hub1 obviously differs from these two-types of UBL family, based on the following two reasons. One is that the size of Hub1 is similar to that of type-1 UBLs, but it is not likely to be a covalent modifier protein. The other is that Hub1 does not form the domain structure in the large protein unlike type-2 UBL(s). Considered together, we propose that Hub1 is defined as a novel or third subgroup of UBL family proteins.

Though the precise mechanism of the biological function of Hub1 remains to be elucidated, we speculate that Hub1 is involved in the RNA metabolism, because we isolated *rpb10*$^+$ and *snu66*$^+$ as multicopy suppressors of the *hub1-1* mutation (Fig. 6), and *hub1-1* showed the pre-mRNA splicing defect (Fig. 7).

Rpb10 is one of the shared subunits among three forms of the RNA polymerase. In *S. pombe*, the RNA polymerase II is composed of 12 different subunits. Among them, Rpb5, 6, 8, 10 and Rpb12 are common subunits of RNA polymerases (Kimura *et al.* 2001). All these subunits are essential for growth, but the function of each subunit is still totally unknown. Furthermore, each subunit may have unknown functions independent of RNA polymerases, because the presence of intracellular pools of unassembled subunits are indicated (Kimura *et al.* 2001). To date, there is no report about *rpb10*$^+$ as a multicopy suppressor gene in *S. pombe*, and in *S. cerevisiae*, the only known fact is that overproduction of Rpb10 can suppress assembly mutants of Rpc19 and Rpc40, both of which are components of RNA polymerase I and III (Gadal *et al.* 1999). We tried to find some genetic interactions between *hub1*$^+$ and the homologues of these genes in *S. pombe*, but at least, overproduction of the counterparts of Rpc19 and Rpc40 in *S. pombe* could not work as multicopy suppressors (data not shown). We also checked the sensitivity of *hub1-1* to 6-Azauracil (6-AU), but *hub1-1* grew normally on 6-AU containing media (data not shown). Sensitivity to 6-AU often correlates with defects in the elongation phase of transcription by RNA polymerase II (Hampsey 1997; Ishiguro *et al.* 2000), so Hub1 is unlikely to be involved in the elongation phase of transcription by the RNA polymerase II.

Another suppressor gene product, Snu66 is one of the components of the 25S [U4/U6·U5] tri-snRNP (small nuclear ribonucleoprotein), which is a subcomplex of the spliceosome (Kaufer & Potashkin 2000; Jurica & Moore 2003). Over 100 spliceosomal protein components have been identified so far, but the function of many of these proteins remains unknown. Snu66 was shown to play an important role in pre-mRNA splicing *in vitro* (Gottschalk *et al.* 1999), but *SNU66* is not an essential gene in *S. cerevisiae*, and its *in vivo* function is not still apparent. Hub1 has not been reported as a component of the spliceosome and the localization of Hub1 was not restricted to the nucleus (Fig. 2), so Hub1 is not likely to be the core and stable component of the

spliceosome. However, pre-mRNA splicing was impaired in *hub1-1* (Fig. 7), and it is possible that Hub1 has some regulatory functions to pre-mRNA splicing. It is conceivable that a defect in making proper mRNAs causes various phenotypes of *hub1-1*. We may also explain the phenotypic difference of the deletion mutants between *S. cerevisiae* and *S. pombe*; the former has no obvious phenotype, and the latter is lethal, because there are few genes that require premRNA splicing in *S. cerevisiae*.

Along with multicopy suppressors, osmotic stabilizer could suppress the *hub1-1* mutation (Fig. 5B,C). In *S. pombe*, it is known that Spc1/Sty1 MAP kinase cascade is activated by various stress signals, including hyperosmotic shock (Hohmann 2002). Hub1 may work in cooperation with this MAP kinase cascade.

Experimental procedures

Strains, media and genetic manipulations

Escherichia coli strain DH5α was used for propagating plasmids, and BL21 (DE3) was used for expression and purification of recombinant proteins. The following yeast strains were used in this study: JY741 (*h⁻ leu1 ura4-D18 ade6-M216*), JY746 (*h⁺ leu1 ura4-D18 ade6-M210*), YHY22P (*h⁻/h⁺ leu1/leu1 ura4-D18/ura4-D18 ade6-M216/ade6-M210 Δhub1::aur1^R/hub1⁺*), YHY23P (*Δhub1::aur1^R* pHY58), YHY24P (*h⁻ leu1 ura4-D18 ade6-M216 hub1-1-ura4⁺*), YHY25P (*h⁻ leu1 ura4-D18 ade6-M216 hub1–3 × Flag-kanMX6*), and YHY26P (*Δhub1::aur1^R* pHY57) YHY25P was made by using pHY68. Media and methods for mating, sporulation, tetrad analysis, and transformation were previously described (Moreno *et al.* 1991; Alfa *et al.* 1993; Guthrie & Fink 2002). 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. For gene disruption, a diploid strain crossed between JY741 and JY746 was used. Correct disruption was confirmed by polymerase chain reaction (PCR) analysis.

Plasmids

Plasmids pREP1, pREP81 and pUR19, were previously described (Moreno *et al.* 1991; Barbet *et al.* 1992). Multicopy vector for *S. pombe*, pALSK (*LEU2*) and pRSC81 (pREP81-GFP) were kind gifts from Dr A. Toh-e (The University of Tokyo) and Dr A. Matsuyama (Chemical Genetics Laboratory, Riken), respectively. The constructed plasmids were as follows: pHY56 (pREP81-Flag-His_6-*hub1⁺* ORF), pHY57 (pREP81-GFP- Flag-His_6-*hub1⁺* ORF), pHY58 (pUR19-*hub1⁺*), pHY59 (pALSK-*hub1⁺*), pHY60 (pALSK-*hub1*ΔYY), pHY63 (pREP81-*rpb10⁺* ORF), pHY64 (pREP1-*snu66⁺* ORF), pHY67 (pET15b-*hub1c*) and pHY68 (pBluescript II KS–PFT cassette-*kanMX6*). PFT cassette was from pYS419 (Saeki *et al.* 2002). Gene name followed by open reading frame (ORF) means the DNA fragment from the ATG codon to the terminal codon of its gene. Gene name only means the DNA fragment with the 5′ and 3′ regions of its gene. *hub1c* means cDNA of *hub1⁺* gene. For pHY56, *hub1⁺* ORF was amplified by PCR using primers tagubl4N (5′-GGAATTCCATATGGACTACAAGGACGACGATGACAAGCATCATCATCATCATCACATGATCGAAGTTTTATGGTAT-3′) and ubl4wt c (5′-GCGCGGATCCTTAAGAATAATACATCTC-3′), and cloned into *NdeI*/*Bam*HI sites of pREP81. For pHY57, DNA fragments containing Flag-His_6-*hub1⁺* ORF were excised from pHY56 with *NdeI* and *Bam*HI, and cloned into *NdeI*/*Bam*HI sites of pRSC81. *hub1⁺* DNA fragments with the 5′ and 3′ regions were obtained by PCR with the primers ubl5 A (5′-GGCCCTGCAGGGCGAAGGACGACGCTTC-3′) and ubl5 D (5′-GGCCCTGCAGGTAACCAATACATATCTG-3′), and genomic DNA as a template. Amplified fragments were digested with *Pst*I and cloned into pUR19 for pHY58 and pALSK for pHY59. pHY60 was constructed from the pHY59 by using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the primers, ubl4sens (5′-GGAATGAGCTTGGAGATGTCTTAAAAAGCGAAAC-3′) and ubl4anti (5′-GTTTCGCTTTTTAAGACATCTCCAAGCTCATTCC-3′). Hub1 cDNA fragments were amplified from the *S. pombe* cDNA library using two primers, cUBL4N (5′-GCGCGGATCCTATGATCGAAGTTTTATGTAACGAT-3′) and cUbl4 sC (5′-GCGCAGATCTTTAAGAATAATACATCTCCAAGCTC-3′). Amplified fragments were cloned into *Bam*HI site of pET15b (Novagen, Madison, WI, USA) for pHY67. pHY62 (pALSK-*rpb10⁺*) was isolated from the *S. pombe* genomic library.

Immunoblot analysis

For Fig. 4, cells were suspended in lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-KOH, pH 7.6, 100 mM β-glycerolphosphate, 50 mM NaF, 1 mM $MgCl_2$, 1 mM ethylene glycol bis (beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 5% glycerol, 0.25% Triton X-100 containing complete protease inhibitors ethylenediaminetetraacetic acid (EDTA) free (Roche)), broken by vortexing with glass beads at 4 °C for 5 min, and the cleared lysates were prepared by centrifugation at 20 000 *g* at 4 °C for 10 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were performed with the NuPAGE system (Invitrogen, San Diego, CA, USA) as per instructions provided by the manufacturer. As primary antibodies, anti-Flag M2 antibody (Sigma Chemical Co., St. Louis, MO, USA) or anti-Hub1 antibody were used, and anti-mouse or anti-rabbit IgG HRP conjugate (Promega, Madison, WI, USA) were used as secondary antibodies.

Purification of recombinant Hub1 protein, and antibody preparation

pHY67 was expressed in BL21 (DE3) at 37 °C. The cell pellet from a two little culture was suspended in 40 mL of sonication buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol (DTT)). Then, 10% Triton X-100 was added to the crude lysate to a final concentration of 0.5%. The crude lysate was

centrifuged at 13 000 *g* for 20 min at 4 °C. The pellet was resuspended in 30 mL sonication buffer with 4% Triton X-100. Hub1 inclusion bodies were pelleted by centrifugation at 13 000 *g* for 20 min. This wash step was repeated twice. After that, inclusion bodies were washed with distilled water twice. The purified inclusion bodies were suspended in denature buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 6 M guanidine hydrochloride (GuHCl), pH 8.0). The suspension was held at room temperature for 1 h. Any precipitate was removed by centrifugation at 35 000 *g* for 20 min. Chelating Sepharose fast flow beads (Amersham Biosciences) charged with Ni were added to the supernatant and rotated for 30 min at 4 °C. Refolding was performed on the Ni beads using a linear 6 M – 0 M GuHCl gradient in 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0 by Äkta FPLC equipment (Amersham Biosciences). After renaturation, proteins were eluted by the addition of 250 mM imidazole. Anti-Hub1 polyclonal antibody was prepared using purified recombinant His_6-Hub1 as antigen.

Microscopic analysis

Living cells or fixed cells were soaked into 0.5 μg/mL DAPI solution for observing chromatin. Cell fixation was done with 70% ethanol. For observation of GFP-Hub1, YHY22P was transformed with pHY57, and then sporulated. The resultant tetrads were dissected, and haploid cells (YHY26P) containing both the Δ*hub1* and pHY57 were selected. YHY26P was cultured in EMM-Leu media at 26 °C. Photographs were taken by a cooled CCD camera using IP lab software equipped with model AX70 Olympus microscope using the UPlanApo ×100 objective lens.

Isolation of temperature-sensitive alleles of *hub1*$^+$

We used the GeneMorph kit (Stratagene) for introducing mutations into *hub1*$^+$ ORF. Random mutagenized *hub1*$^+$ ORFs were cloned into pREP81. This mutagenized *hub1* library was used for transformation of YHY23P (Δ*hub1::aur1*^{R} pUR19-*hub1*$^+$), and transformed cells were spread on to the EMM-Leu +5-FOA media. The growth rate of the obtained 264 colonies was checked at 26 °C or 37.2 °C. Cells that could not grow at 37.2 °C were selected, and from those cells, plasmids were retrieved and the mutation sites were determined by sequence analysis. The same mutations were introduced into pHY59 (pALSK-*hub1*$^+$) with the QuickChange site-directed mutagenesis kit (Stratagene), and then the *ura4*$^+$ maker excised from pUR19 was inserted into the *Hin*dIII site located to the 3′ region of *hub1*$^+$ ORF (Fig. 1C, left panel). The wild-type strain, JY741, was transformed with the excised DNA fragments containing the mutagenized *hub1*$^+$ ORF and *ura4*$^+$. The growth of transformants was checked for whether it was temperature-sensitive or not. One of the transformants showed clear temperature-sensitive growth, so we designated this allele *hub1-1*.

RT-PCR analysis

RT-PCR analysis was carried out as previously described (Oltra *et al.* 2004; Webb & Wise 2004). Total RNA was isolated using RNeasy Mini Kit (Qiagen). RNA was converted to cDNA using the SuperScript™ II Reverse Transcriptase (Invitrogen). Primers used for RT-PCR amplification were 5′-GAAGACTAAGGCTCAGAAACAATC-3′ and 5′-GGACCAACAGTGTAAATTTCATG-3′ (for *rpl7* intron) or 5′-TTATTGGATAAATGCCTTGAAGTCC-3′ and 5′-TCGGAAAATAAACATTTGTGCAATC-3′ (for *cdc16* intron 2). PCR products were resolved on 1% or 4% agarose gels and stained with ethidium bromide.

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