

# The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer

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**SMT3 is an essential *Saccharomyces cerevisiae* gene encoding a 11.5 kDa protein similar to the mammalian ubiquitin-like protein SUMO-1. We have found that Smt3p, like SUMO-1 and ubiquitin, can be attached to other proteins post-translationally and have characterized the processes leading to the activation of the Smt3p C-terminus for conjugation. First, the SMT3 translation product is cleaved endoproteolytically to expose Gly98, the mature C-terminus. The presence of Gly98 is critical for Smt3p's abilities to be conjugated to protein substrates and to complement the lethality of a *smt3Δ* strain. Smt3p undergoes ATP-dependent activation by a novel heterodimeric enzyme consisting of Uba2p, a previously identified 71 kDa protein similar to the C-terminus of ubiquitin-activating enzymes (E1s), and Aos1p (activation of Smt3p), a 40 kDa protein similar to the N-terminus of E1s. Experiments with conditional *uba2* mutants showed that Uba2p is required for Smt3p conjugation *in vivo*. Furthermore, UBA2 and AOS1 are both essential genes, providing additional evidence that they act in a distinct pathway whose role in cell viability is to conjugate Smt3p to other proteins.**

**Keywords:** Aos1p/post-translational modification/Uba2p/ubiquitin-activating enzyme/yeast

## Introduction

*SMT3* is an essential *Saccharomyces cerevisiae* gene encoding a member of a recently discovered family of ubiquitin-like proteins that includes the mammalian protein SUMO-1 [also called GMP1 (Matunis *et al.*, 1996), PIC1 (Boddy *et al.*, 1996), UBL1 (Shen *et al.*, 1996) or sentrin (Okura *et al.*, 1996)]. SUMO-1 was identified as a protein found covalently linked to the Ran GTPase-activating protein (RanGAP1) (Matunis *et al.*, 1996; Mahajan *et al.*, 1997) and is one of very few proteins other than ubiquitin (Ub) shown to be attached to other proteins post-translationally. Attachment of SUMO-1 targets the otherwise cytosolic RanGAP1 to the nuclear pore complex by promoting binding to Nup358/RanBP2 (Mahajan *et al.*, 1997). *SMT3* was originally isolated as a high-copy suppressor of mutations in *MIF2*, which encodes a centro-

mere-binding protein (Meluh and Koshland, 1995). Smt3p is 48% identical to SUMO-1 and 17% identical to Ub.

The 76-residue protein Ub is highly conserved, with only three conservative changes between yeast and man. All known functions of Ub, many of which involve proteasome-dependent proteolysis, are mediated by its conjugation to other proteins (reviewed by Hochstrasser, 1996; King *et al.*, 1996). A number of proteins including Smt3p and SUMO-1 contain Ub-like domains with much less sequence similarity to Ub than among genuine Ub homologs. Most of these, including the *S.cerevisiae* nucleotide excision repair protein Rad23p (Watkins *et al.*, 1993) and Dsk2p, involved in spindle pole body duplication (Biggins *et al.*, 1996), do not become conjugated to other proteins. The only Ub-like proteins other than SUMO-1 shown to form conjugates are the interferon-inducible protein UCRP (Loeb and Haas, 1992) and a baculovirus Ub variant (Haas *et al.*, 1996). One distinguishing feature of Ub-like proteins capable of conjugation is likely to be a diglycine sequence corresponding to the C-terminal Gly75 and Gly76 of Ub, which is critical for interactions between Ub and Ub-pathway enzymes. Like SUMO-1, UCRP and the baculovirus Ub variant, Smt3p contains these glycine residues.

Post-translational attachment of Ub to other proteins is carried out by a multi-step pathway culminating in formation of an isopeptide bond between the C-terminal carboxyl group of Ub and the ε-amino group of a lysine side-chain in an acceptor protein. All Ub genes encode natural fusion proteins in which Ub's C-terminus is linked to another copy of Ub, a small ribosomal protein or an oligopeptide (Özkaynak *et al.*, 1987; Jentsch *et al.*, 1991). Ub fusions are rapidly cleaved by Ub-specific endoproteases to expose Gly76, the mature Ub C-terminus (Bachmair *et al.*, 1986; Tobias and Varshavsky, 1991). Ub conjugation proceeds through a series of covalent intermediates containing high-energy thioester bonds between Ub's C-terminal carboxyl group and cysteine side-chains in pathway enzymes (reviewed in Pickart, 1988). In the initial step, Ub-activating enzyme (E1) utilizes ATP to adenylate the Ub C-terminus, which is then transferred to a conserved Cys residue in the E1, yielding the E1–Ub thioester, AMP and pyrophosphate (Ciechanover *et al.*, 1982; Haas and Rose, 1982). Ub is transferred from the E1 to a Cys residue in a Ub-conjugating enzyme (E2) (Hershko *et al.*, 1983). Cells contain multiple E2s (~13 in yeast) which are involved in ubiquitinating different proteins. In some cases Ub can be transferred directly from the E2 to the acceptor protein, but more often Ub–isopeptide bond formation is facilitated by a third heterogeneous class of proteins termed Ub–protein ligases or recognins (E3) (Hershko *et al.*, 1983; Bartel *et al.*, 1990; Scheffner *et al.*, 1993; Peters *et al.*, 1996; Zachariae *et al.*, 1996). At least some E3s also



**Fig. 1.** C-terminal proteolytic processing of Smt3p. (A) Sequence comparison of Ub and *SMT3* translation product C-termini. Identical residues are boxed. An arrowhead indicates the site of the proteolytic cleavage producing the mature Ub C-terminus. An asterisk designates a stop codon. (B) Diagram of Smt3p-βgal fusion proteins. The sequence of the Smt3p-βgal junction is shown in single letter code. (C) Immunoblot analysis, using a monoclonal antibody against βgal, of whole cell lysates from exponential cultures of wild-type *S. cerevisiae* strain BBY48 containing plasmids expressing the fusion proteins depicted in (B) (see Materials and methods). Lanes are numbered according to the construct numbers in (B). Bands corresponding to uncleaved Smt3p-βgal and to the βgal-containing cleavage product are indicated.

contain an essential conserved Cys residue that mediates an E3-Ub thioester (Scheffner *et al.*, 1995).

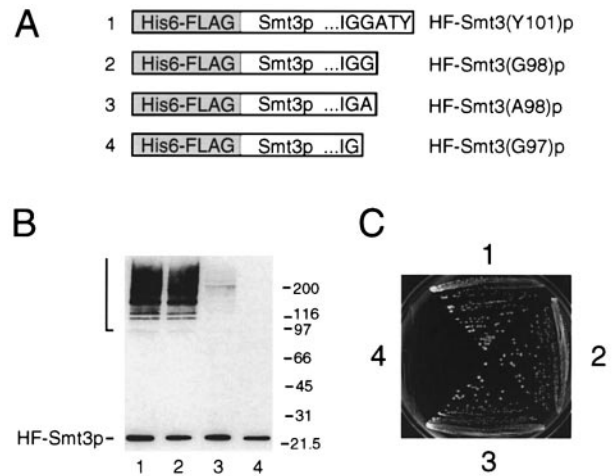
*Saccharomyces cerevisiae* contains a single 114 kDa E1 enzyme encoded by the *UBA1* gene, an essential gene with extensive sequence similarity to the E1 genes in other organisms including plants and mammals (McGrath *et al.*, 1991). However, another essential gene termed *UBA2*, encoding a 71 kDa protein, also contains regions of similarity to E1s over nearly its entire length, including a cysteine residue corresponding to the active site residue involved in the E1-Ub thioester (Dohmen *et al.*, 1995). *uba2* mutants lacking this cysteine residue are unable to carry out the essential biological function of *UBA2*, suggesting that Uba2p activity also involves thioester formation, but it has not been possible to detect Uba2p-Ub thioesters. Thus, Uba2p is a good candidate to be the activating enzyme for a different Ub-like protein.

We have found that Smt3p can be attached to other proteins, and report the characterization of the processes involved in activating its C-terminus for conjugation, including the isolation of a novel heterodimeric Smt3p-activating enzyme.

## Results

### Identification of the mature Smt3p C-terminus

To test whether Smt3p is cleaved at its C-terminus as predicted by analogy with Ub (Figure 1A), Smt3p fusions to *Escherichia coli* β-galactosidase (βgal) containing small deletions in the Smt3p C-terminal region (Figure 1B) were expressed in *S. cerevisiae*. Smt3(Y101)p-βgal, containing full-length Smt3p, was efficiently cleaved near the Smt3p/βgal junction as was Smt3(G98)p-βgal, in which the three



**Fig. 2.** Deletion analysis of *SMT3* C-terminus. (A) Diagram of *SMT3* C-terminal variants bearing an N-terminal His6 tag and a FLAG epitope tag. The sequence of the constructs' C-termini is shown. (B) Immunoblot analysis, using a monoclonal antibody against the FLAG epitope, of whole cell lysates from wild-type yeast strain DF5 bearing plasmids expressing the Smt3p variants depicted in A (see Materials and methods). Lanes are numbered according to the construct numbers in (A). The band corresponding to free HF-Smt3p is indicated. A half-open square bracket designates high molecular weight HF-Smt3p conjugates. (C) *S. cerevisiae* strain EJY251-11b (*smt3Δ::HIS3*) bearing a *URA3*-marked *CEN* plasmid expressing HF-Smt3(Y101)p from *P<sub>SMT3</sub>* and a *LEU2*-marked *CEN* plasmid expressing one of the HF-Smt3p C-terminal variants depicted in (A) from *P<sub>GAL10</sub>* were pregrown on an SG plate lacking leucine and streaked onto an SG 5-FOA plate containing 5-FOA. The SG 5-FOA plate was photographed after 5 days of growth at 30°C. Sector numbers correspond to construct numbers in (A). See Materials and methods for full description of strains and plasmids.

C-terminal residues of Smt3p were deleted (Figure 1C, lanes 1 and 2). However, if one more residue, Gly98, was also deleted, the fusion remained uncleaved (Figure 1C, lane 3). Even if Gly98 was changed to an Ala, another small residue, no proteolytic processing took place (Figure 1C, lane 4). Therefore, the Smt3p sequence up to Gly98 is sufficient, and Gly98 absolutely required for cleavage to take place, consistent with the possibility that Smt3p is cleaved after Gly98.

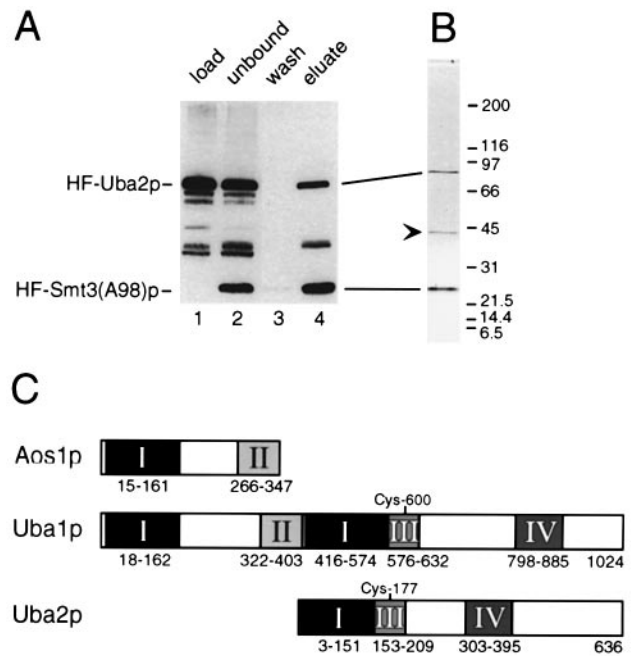
To obtain further evidence that the active C-terminus of Smt3p is Gly98, Smt3p C-terminal variants (Figure 2A) were expressed in yeast to determine whether they could be incorporated into higher molecular weight conjugates. When His6- (Hoffmann and Roeder, 1991) and FLAG (Brizzard *et al.* 1994) epitope-tagged full-length Smt3p [HF-Smt3(Y101)p] was expressed from the galactose-inducible promoter *P<sub>GAL10</sub>* in wild-type (*SMT3*) yeast, several anti-FLAG-reactive HF-Smt3p-containing bands over 100 kDa were observed (Figure 2B, lane 1). The band pattern is consistent with Smt3p being attached to 5–10 different proteins, whose identities are unknown. This construct was also tested for its ability to substitute for wild-type *SMT3* *in vivo* by expressing it in a *smt3Δ* strain bearing *SMT3* on a *URA3*-marked plasmid. This strain formed colonies when transferred to a plate containing galactose and 5-fluoro-orotic acid (5-FOA) (Boeke *et al.*, 1984; Figure 2C), which selects for cells that have lost the *URA3*-containing plasmid, indicating that His6- and FLAG-tagged Smt3p can carry out *SMT3*'s essential functions. HF-Smt3(G98)p, in which the three C-terminal

residues of Smt3p were deleted, was indistinguishable from full-length in its abilities to be incorporated into conjugates and to complement the lethality of the *smt3Δ* strain (Figure 2B, lane 2 and C). In contrast, HF-Smt3(A98)p, an otherwise identical construct in which Gly98 was replaced with an Ala, was incorporated very inefficiently, with a greater proportion of conjugates 200 kDa and larger instead of the prominent ~140 kDa band seen with HF-Smt3(G98)p (Figure 2B, lane 3). No incorporation of HF-Smt3(G97)p, lacking the C-terminal four residues of Smt3p, was detected (Figure 2B, lane 4). HF-Smt3(G97)p expression also did not permit growth of the *smt3Δ* strain, but surprisingly the *smt3Δ* strain expressing HF-Smt3(A98)p grew well (Figure 2C) (see Discussion). This parallel between a construct's ability to complement the null allele's lethality and its incorporation into conjugates suggests that at least one essential function of *SMT3* involves its attachment to another protein. These results also demonstrate that the three C-terminal residues of the *SMT3* gene product are dispensable for Smt3p conjugation and biological activity, whereas alterations at Gly98 have severe effects on one or both functions. We conclude that Gly98 is the mature C-terminus of Smt3p. While this manuscript was being reviewed, Kamitani *et al.* (1997) reached an analogous conclusion for SUMO-1.

#### Identification of the Smt3p-activating enzyme

A highly effective method for purifying Ub-activating enzymes (*E1s*) and Ub-conjugating enzymes (*E2s*) takes advantage of their enzyme mechanisms, which involve formation of covalent intermediates with Ub (see Introduction; Ciechanover *et al.*, 1982). A protein mixture containing these enzymes is supplemented with ATP and incubated with an affinity column to which Ub has been coupled. *E1s* and *E2s* form thioester bonds between their active site cysteine residues and the column-bound Ub. After washing they can be gently eluted in buffer containing a thiol reducing agent, such as dithiothreitol (DTT), which breaks the thioester bonds.

We attempted an analogous approach, applying an ATP-supplemented whole yeast cell lysate to an HF-Smt3(G98)p-agarose column. The resulting DTT eluate contained an activity that when incubated with ATP and HF-Smt3(G98)p formed a ladder of HF-Smt3(G98)p-containing bands, possibly HF-Smt3(G98)p homopolymers (data not shown). While this result was promising, the protein yield in the DTT eluate was so low that we decided to test directly whether Uba2p, a good candidate to be the Smt3p-activating enzyme (see Introduction), could bind the Smt3p column. In a pilot experiment, Uba2p tagged C-terminally with His6 and FLAG tags (HF-Uba2p) was expressed in yeast and partially purified via the His6 tag on Ni-NTA-agarose. This fraction was incubated with ATP and an HF-Smt3(A98)p-agarose column, which was then washed with a high-salt buffer and eluted in a high-salt, high pH, DTT-containing buffer (Figure 3A). While the DTT eluate did contain HF-Uba2p (Figure 3A, lane 4), suggesting that Uba2p is involved in Smt3p activation, only 10% or less of the total HF-Uba2p in the preparation had bound (Figure 3A, lane 2 versus lane 4). One of several possible explanations for this result was that there was another protein required for HF-Uba2p to bind the column and that this protein was present in



**Fig. 3.** Identification and domain structure of Aos1p. (A) Whole cell lysate from *S. cerevisiae* strain JD90-1A (*uba2Δ::HIS3*) bearing pJD417, which expresses His6-FLAG-Uba2p (HF-Uba2p) was fractionated on Ni-NTA-agarose. HF-Uba2p-enriched Ni-NTA column eluate was supplemented with ATP and fractionated at 25°C on a HF-Smt3(A98)p-agarose column (see Materials and methods). Equivalent amounts of the Ni-NTA column eluate (lane 1) and the Smt3p column fractions, the flow-through (lane 2), the final wash (lane 3) and the DTT eluate (lane 4) were analyzed by SDS-PAGE and immunoblotting with a monoclonal antibody against the FLAG epitope. Bands corresponding to HF-Uba2p and HF-Smt3(A98)p, which leaches off the column, are indicated. (B) Smt3p column eluate was further purified on an anti-FLAG antibody column (see Materials and methods), and the resulting fraction analyzed by SDS-PAGE and Coomassie Blue staining. An arrowhead indicates the band that proved to be Aos1p. (C) Sequence similarity domains in Aos1p, Uba1p and Uba2p were defined as described in Materials and methods. Domain I includes a potential nucleotide-binding motif and is found in a number of proteins, such as *E. coli* thiF and chINB, not involved in activation of Ub-like proteins. In *E1s*, domain III contains the active site cysteine residue (Cys600 in Uba1p) which forms a thioester with the C-terminus of Ub. Uba2p shares this domain including the conserved cysteine residue (Cys177). Domains II and IV are each found in several other proteins in addition to *E1s*, but nothing is known about their functions. Residues included in each domain are numbered.

substoichiometric amounts relative to the overexpressed HF-Uba2p. The Ni-NTA and Smt3p column fractionations were repeated on a larger scale, followed by further purification on an anti-FLAG antibody column. Coomassie Blue staining of the resulting fraction confirmed the presence of equimolar amounts of a 40 kDa protein copurifying with HF-Uba2p (Figure 3B). This protein was identified by direct mass spectrometric analysis of a protease digestion mixture (see Materials and methods) as the product of *S. cerevisiae* open reading frame (ORF) YPR180W, now called *AOS1* (activation of Smt3p).

The predicted protein sequence of Aos1p is 29% identical to the N-terminal region of the yeast *E1* Uba1p, with most of the sequence similarity concentrated in two separate regions at the N- and C-termini of Aos1p (domains I and II in Figure 3C). Uba2p is similar to the C-terminal region of Uba1p, also with two regions of greater similarity (domain I/domain III and domain IV in Figure 3C),

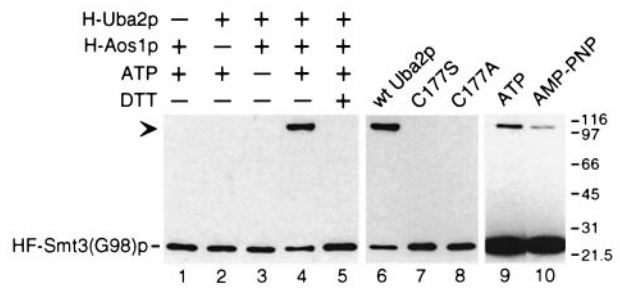
suggesting that Aosl1p and Uba2p combine to carry out functions analogous to those performed by the single protein Uba1p. Uba1p and the numerous other *E1s* whose genes have been sequenced are extremely similar to each other (53% identity between Uba1p and a human *E1*), and their most striking features are two copies of an ~150-residue domain (Figure 3C, domain I) that contains a potential nucleotide-binding motif and is also found in several other proteins, such as *E.coli* thiF (Vander Horn *et al.*, 1993) and chlN (Pitterle and Rajagopalan, 1989), not involved in activation of Ub-like proteins. The second copy of this domain in *E1s* is followed directly by a region containing the active site Cys residue (Cys600 in Uba1p) that forms the thioester with Ub (domain III in Figure 3C) (Hatfield and Vierstra, 1992). Uba2p also contains this domain including a Cys residue (Cys177) corresponding to the *E1* active site. *E1s* and Uba2p share another region of high sequence similarity (domain IV) whose function is unknown.

Several proteins other than Aosl1p have been identified that show sequence similarity only to the N-terminal region of *E1s*, including the human amyloid precursor protein-binding protein (APP-BP) (Chow *et al.*, 1996), *Schizosaccharomyces pombe* RAD31 (Shayeghi *et al.*, 1997), *S.cerevisiae* ORF YPL003W and AXR1, an *Arabidopsis thaliana* protein whose mutants are auxin resistant (Leyser *et al.*, 1993). No enzymatic activity has been identified for any of these proteins, but they all contain domains I and II and lack domains III and IV, suggesting that they too may act in activation of Ub or a Ub-like protein by interacting with a second protein analogous to Uba2p. APP-BP, AXR1 and YPL003W are all larger than Aosl1p and considerably more similar in sequence to each other than to Aosl1p or RAD31. The complete yeast genome sequence also contains one other ORF, YPR066W, with one copy of domain I plus domains III and IV, which may be analogous to Uba2p.

A diploid *S.cerevisiae* strain in which one of the two copies of the *AOS1* coding region was replaced by the *HIS3* gene was generated by homologous recombination (Rothstein, 1991). When this strain was sporulated and the tetrads dissected, no more than two viable colonies per tetrad were produced, and all viable colonies were unable to grow on plates lacking histidine (data not shown), indicating that the inviable segregants contained *aos1Δ::HIS3*. Microscopic examination of the inviable segregants revealed that *aos1Δ::HIS3* cells grew normally for several generations, forming colonies of ~100 cells, but the cells became increasingly enlarged and ultimately lysed. This behavior is likely to result from dilution of *AOS1* product present in the spore until there are no longer sufficient amounts to fulfill *AOS1*'s essential functions in vegetative growth. The *AOS1* deletion phenotype is similar to those seen for the *SMT3* and *UBA2* deletion strains (data not shown; Dohmen *et al.*, 1995).

#### **Smt3p thioester formation with Aosl1p/Uba2p**

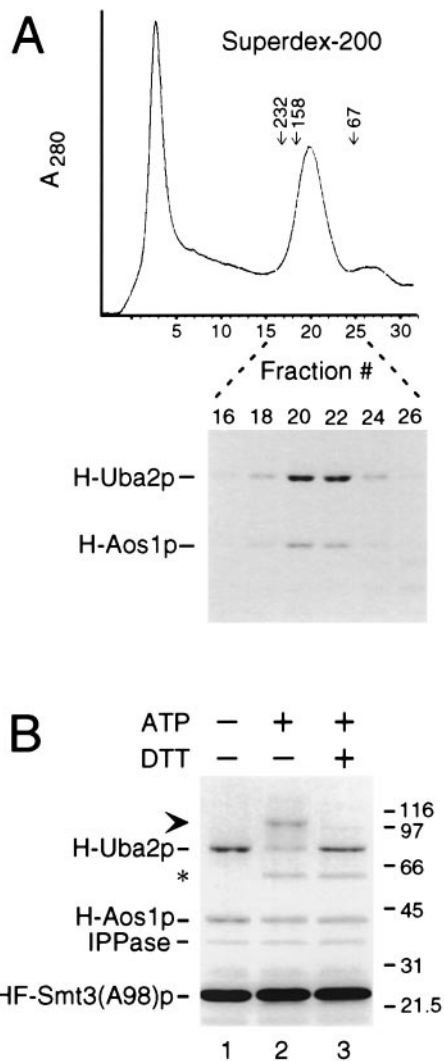
To confirm that Aosl1p and Uba2p are necessary and sufficient for Smt3p activation *in vitro*, thioester formation assays were performed using recombinant proteins expressed in *E.coli*. As *E.coli* does not have Ub-like systems, any contaminating proteins are unlikely to affect the reaction. His6-tagged Uba2p and Aosl1p (H-Uba2p



**Fig. 4.** Both Aosl1p and Uba2p are required for Smt3p thioester formation. Purified recombinant HF-Smt3(G98)p was incubated for 30 min at 25°C with some of the following: partially purified recombinant His6-tagged Uba2p (H-Uba2p) (lanes 2–6), partially purified recombinant His6-tagged Aosl1p (H-Aosl1p) (lanes 1 and 3–8) and 5 mM ATP (lanes 1, 2 and 4–8). Lane 6, same as lane 4. Lane 7, same as lane 6, except with H-Uba2p(C177S) instead of H-Uba2p. Lane 8, same as lane 6, except with H-Uba2p(C177A) instead of H-Uba2p. Lane 9, different preparations of H-Uba2p and H-Aosl1p were incubated for 10 min at 25°C with HF-Smt3(G98)p and 10 nM ATP. Lane 10, same as lane 9, except with 10 nM AMP-PNP instead of ATP. Reactions were then incubated with SDS-loading buffer lacking reducing agent (lanes 1–4 and 6–10) or containing 100 mM DTT (lane 5) at 37°C for 10 min, followed by SDS-PAGE and immunoblotting with the anti-FLAG antibody (see Materials and methods). Presence or absence of various components is indicated above the lanes. The band corresponding to free HF-Smt3(G98)p is indicated. An arrowhead designates the putative H-Uba2p-HF-Smt3(G98)p thioester product.

and H-Aosl1p) were partially purified on Ni-NTA-agarose. When these were mixed and incubated with HF-Smt3(G98)p and ATP, a HF-Smt3(G98)p-containing band was formed at ~105 kDa (Figure 4A, lane 4). This product could be destroyed by the reducing agent DTT (Figure 4A, lane 5), and it did not form in reactions lacking any of these components (Figure 4A, lanes 1–3 and data not shown). Apart from the analogy with Ub and *E1s*, several lines of evidence suggested that the DTT-sensitive product contained HF-Smt3p and H-Uba2p linked by a thioester bond between the Smt3p C-terminal carboxyl group and Cys177 of Uba2p. The DTT-sensitive bond could not be a disulfide bond, as HF-Smt3p does not contain a cysteine residue. Uba2p was confirmed to be the other component by Coomassie Blue staining of thioester reactions containing HF-Smt3(A98)p and a purified preparation of H-Aosl1p/H-Uba2p heterodimer (see below). These showed that the H-Uba2p band disappeared almost quantitatively upon ATP incubation and formation of the 105 kDa band, and reappeared upon addition of DTT (Figure 5B). Furthermore, Uba2p mutants in which Cys177 was replaced with Ser or Ala were inactive in this reaction (Figure 4, lanes 7 and 8). While the effect of the Smt3p C-terminus on thioester formation was not studied in detail, it was clear that the kinetics were altered in reactions containing mutant HF-Smt3p having Ala rather than Gly at position 98. For example, reactions including an excess of HF-Smt3(A98)p reached a steady-state in which Uba2p was almost quantitatively incorporated into the thioester complex (Figure 5B), whereas those including HF-Smt3(G98)p did not (data not shown).

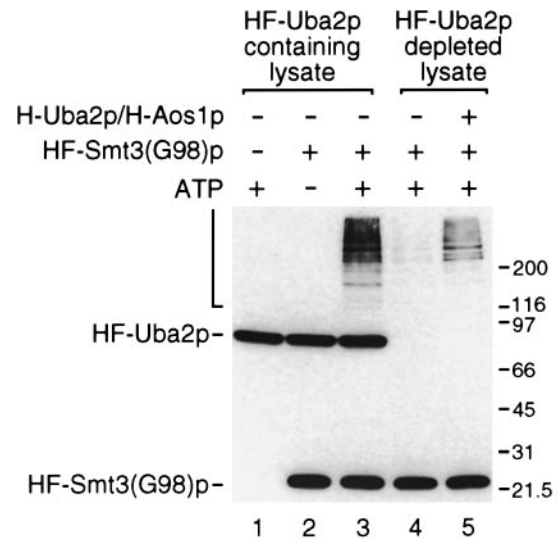
One salient feature of the *E1* mechanism is the formation of enzyme-bound Ub-adenylate prior to thioester formation. Because this reaction cleaves the bond between the  $\alpha$ - and  $\beta$ -phosphoryl groups of ATP,  $\beta$ - $\gamma$  non-hydrolyzable ATP analogs such as AMP-PNP can substitute for ATP in



**Fig. 5.** Aos1p and Uba2p form an active heterodimer. **(A)** The DTT eluate from an HF-Smt3(G98)p-agarose column that had been loaded with a mixture of partially purified recombinant His6-tagged Uba2p (H-Uba2p) and His6-tagged Aos1p (H-Aos1p) was analyzed by gel filtration chromatography on a Superdex 200 column (see Materials and methods). Shown are the elution profile and analysis of peak fractions by SDS-PAGE and Coomassie Blue staining. Sizing column standards shown are catalase, aldolase and bovine serum albumin. **(B)** Peak fractions from **(A)** were pooled and incubated for 90 min at 25°C with purified recombinant HF-Smt3(A98)p and inorganic pyrophosphatase without ATP (lane 1) or with 5 mM ATP (lanes 2 and 3). Samples were then incubated for 10 min at 37°C with SDS-loading buffer lacking reducing agent (lanes 1 and 2) or containing 10 mM DTT (lane 3), followed by SDS-PAGE and Coomassie Blue staining. Bands corresponding to H-Uba2p, H-Aos1p, HF-Smt3(A98)p and inorganic pyrophosphatase (IPPase) are indicated. An arrowhead indicates the putative HF-Smt3(A98)p-Uba2p thioester product. An asterisk designates an HF-Smt3p-containing reducing agent-stable band that forms in the presence of ATP and HF-Smt3(A98)p. Note reduced level of H-Uba2p in lane 2 relative to lanes 1 and 3.

Ub activation (Haas *et al.*, 1983; Johnston and Cohen, 1991). When the ATP in Smt3p thioester formation assays was replaced with AMP-PNP, the DTT-sensitive product still formed (Figure 4, lane 10), suggesting that Smt3p activation also proceeds through an Smt3p-adenylate intermediate.

To determine the composition of the active Uba2p/Aos1p complex, recombinant H-Uba2p and H-Aos1p that

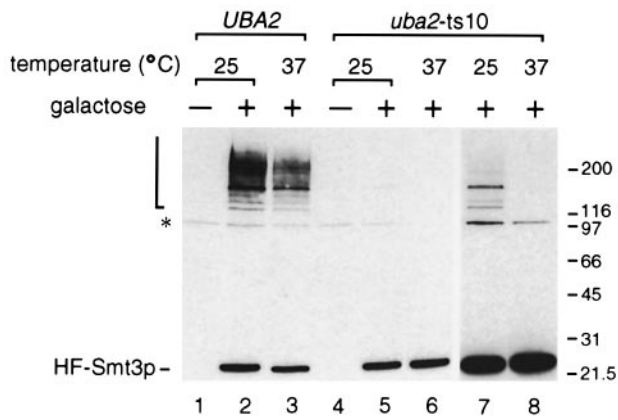


**Fig. 6.** Uba2p promotes Smt3p conjugation *in vitro*. Whole cell lysate from JD90-1A (*uba2Δ::HIS3*) containing pJD417 expressing HF-Uba2p was prepared and an aliquot was depleted of HF-Uba2p by passage over Ni-NTA-agarose and anti-FLAG antibody-agarose. Undepleted (lanes 1–3) or depleted (lanes 4 and 5) lysates were incubated for 90 min at 25°C with some of the following: HF-Smt3(G98)p (lanes 2–5), 5 mM ATP (lanes 1 and 3–5) and recombinant H-Uba2p/H-Aos1p heterodimer purified by HF-Smt3(G98) affinity chromatography (lane 5) (see Materials and methods). Samples were heated at 100°C in β-mercaptoethanol-containing SDS sample buffer, followed by SDS-PAGE and immunoblotting using the monoclonal antibody against the FLAG epitope. Note that the FLAG epitope is present on both the HF-Uba2p in the undepleted yeast lysate and the recombinant HF-Smt3(G98)p added to the reactions. Bands corresponding to HF-Uba2p and HF-Smt3(G98)p are indicated. A half-open square bracket designates high molecular weight HF-Smt3p conjugates.

had been isolated by Smt3p affinity chromatography were analyzed by gel filtration chromatography. H-Aos1p and H-Uba2p co-eluted at ~128 kDa (Figure 5A), close to the calculated molecular weight of of 113 kDa for a H-Uba2p/H-Aos1p heterodimer. The native complex isolated from yeast eluted at a similar position (data not shown). The peak material was highly active, with almost all of the Uba2p capable of being incorporated into the HF-Smt3p thioester (Figure 5B), suggesting that the active form of the Smt3p-activating enzyme is an Aos1p/Uba2p heterodimer.

#### **Uba2p is required for Smt3p conjugation**

To determine whether the Aos1p/Uba2p activity is required for the ultimate conjugation of Smt3p to other proteins, we developed an *in vitro* Smt3p conjugation assay. When HF-Smt3(G98)p and ATP were incubated with whole yeast cell lysate, a series of DTT-resistant high molecular weight HF-Smt3(G98)p-containing bands formed (Figure 6, lane 3). While the FLAG epitope, used for detection in Figure 6, was present on both HF-Smt3(G98)p and on HF-Uba2p, present in the yeast lysate as the only source of Uba2p, it is likely that all of the bands >100 kDa formed upon ATP addition contained HF-Smt3(G98)p rather than HF-Uba2p. In support of this conclusion, these bands were not detected in the absence of added HF-Smt3(G98)p (Figure 6, lane 1), and they were detected when HF-Uba2p was replaced with untagged Uba2p (Figure 6, lane 5). The pattern of Smt3p-containing bands



**Fig. 7.** *UBA2* is required for Smt3p attachment *in vivo*. Transformants of *S.cerevisiae* strain JD90-1A (*uba2Δ::HIS3*) bearing a 2  $\mu$ -based *URA3*-marked plasmid expressing HF-Smt3(Y101) from *P<sub>GAL10</sub>* and one of two *TRP1*-marked *CEN* plasmids, pIS3 containing wild-type *UBA2* (lanes 1–3) or pIS2-*ts10* containing *uba2-ts10* (lanes 4–8), were grown to exponential phase at 25°C in synthetic medium containing raffinose and lacking tryptophan and uracil. Cultures were supplemented with 2% galactose and sampled immediately (lanes 1 and 4) and then split and growth continued for 2 h either at 25°C (lanes 2, 5 and 7) or at 37°C (lanes 3, 6 and 8) (see Materials and methods). Whole cell lysates were analyzed by SDS–PAGE and immunoblotting with the monoclonal antibody against the FLAG epitope. Lanes 7 and 8 are overexposures of lanes 5 and 6 respectively. The band produced by HF-Smt3(Y101)p expression is indicated (HF-Smt3p). The half-open square bracket designates high molecular weight HF-Smt3p conjugates. An asterisk indicates a yeast protein that sometimes cross-reacts with the anti-FLAG antibody.

was similar but not identical to that seen *in vivo* (Figure 2B and Figure 7), with a greater proportion of HF-Smt3(G98)p found in very high molecular weight bands rather than in the prominent *in vivo* band at ~140 kDa.

When yeast lysate that had been depleted of HF-Uba2p and associated proteins by passage over Ni-NTA-agarose and an anti-FLAG antibody column was used in the same reaction, the amount of conjugates was decreased by >10-fold, although some were still detectable (Figure 6, lane 4). Re-addition of recombinant H-Uba2p/H-Aos1p restored most of the Smt3p conjugation activity (Figure 6, lane 5). These results show that Uba2p is involved in at least the vast majority of HF-Smt3p conjugation in these lysates and that the Aos1p/Uba2p heterodimer can contribute to this activity. The residual conjugation activity after HF-Uba2p depletion may result from the presence of a distinct enzyme with Smt3p-activating activity, or from incomplete removal of Uba2p.

To determine whether Uba2p is required for Smt3p conjugation *in vivo*, HF-Smt3(Y101)p was expressed in cells containing either wild-type *UBA2* or a temperature-sensitive mutant *uba2-ts10*. *uba2-ts10* cells grew at near wild-type rates at 25°C but failed to form colonies at 37°C (data not shown). Cultures of *UBA2* or *uba2-ts10* strains bearing plasmids expressing HF-Smt3(Y101)p from the galactose-inducible promoter *P<sub>GAL10</sub>* were pregrown at 25°C in non-inducing medium and then supplemented with galactose and either kept at 25°C or shifted to 37°C. After 2 h, cells from all four cultures had synthesized similar amounts of HF-Smt3p, suggesting that their transcriptional and translational apparatuses were equally functional (Figure 7, lanes 2, 3, 5 and 6). However, the *UBA2* strain incubated at either temperature had formed

significant amounts of HF-Smt3p conjugates (Figure 7, lanes 2 and 3), while the *uba2-ts10* strain incubated at 25°C formed much lower levels of conjugates (Figure 7, lane 5) which were more apparent on a longer exposure (Figure 7, lane 7). The *uba2-ts10* strain incubated at 37°C did not form any detectable HF-Smt3p conjugates (Figure 7, lanes 6 and 8). Therefore, *UBA2* is required for high levels of Smt3p conjugation *in vivo*. The marked difference between the *UBA2* and *uba2-ts10* strains even at the permissive temperature where both strains grew at similar rates can be explained by noting that endogenous levels of Smt3p are many fold lower than those produced here by *GAL*-induced overexpression (data not shown). *uba2-ts10* may be able to support the level of Smt3p conjugation required for wild-type growth rates and yet have significantly reduced Smt3p-activating activity relative to *UBA2*. A similar argument can be used to explain the HF-Smt3(A98)p result in Figure 2B and C.

## Discussion

We have characterized the processes involved in activating the Ub-like protein Smt3p for conjugation to other proteins. Smt3p could be incorporated both *in vivo* and *in vitro* into high molecular weight conjugates, making Smt3p one of the few proteins shown to be capable of post-translational attachment to other proteins. Smt3p's ability to form conjugates depended on the presence of Gly98, the C-terminal residue of mature Smt3p, produced from the *SMT3* translation product by endoproteolytic cleavage. In *SMT3* C-terminal mutants, the ability to form conjugates correlated with ability to complement the lethality of a *smt3Δ* strain, providing evidence that at least one of *SMT3*'s essential functions is mediated by its attachment to another protein. We have also isolated a novel enzyme that catalyzes the ATP-dependent activation of Smt3p, the first step in the conjugation pathway. This Smt3p-activating enzyme is a heterodimer of Uba2p, described previously, and Aos1p, identified in this work, both of which share significant sequence similarity with Ub-activating enzymes. When the Aos1p/Uba2p heterodimer was incubated with ATP and Smt3(G98)p, a reducing agent-sensitive covalent adduct formed between Smt3p and Uba2p. This adduct is likely to be an activated intermediate containing a thioester bond between the C-terminal carboxyl group of Smt3p and the active site Cys177 of Uba2p. *In vivo* experiments with a temperature-sensitive *uba2* mutant showed that Smt3p conjugation in yeast requires the activity of *UBA2*, indicating that Uba2p/Aos1p constitutes the only Smt3p-activating activity in *S.cerevisiae*. Furthermore, deletions of the *SMT3*, *UBA2* and *AOS1* genes are all lethal, consistent with the possibility that their unique essential functions all involve Smt3p conjugation.

Smt3p processing and activation parallel the Ub pathway, yet Smt3p attachment is likely to be functionally distinct from ubiquitinylation and regulated independently. Several features are conserved in the two pathways: synthesis as a precursor, endoproteolytic processing of the C-terminus, ATP-dependent activation by related enzymes with similar mechanisms and finally covalent attachment to other proteins. However, the high degree of sequence divergence between Ub and Smt3p makes it unlikely that Ub-interacting proteins, either enzymes of the Ub pathway

or factors that bind Ub–protein conjugates, would interact with Smt3p. The activation steps of the two pathways are clearly distinct, as we were unable to detect formation of thioesters between Smt3p and Uba1p or between Ub and Uba2p (Dohmen *et al.*, 1995) under conditions where Smt3p–Uba2p and Ub–Uba1p thioesters formed (data not shown). Ubiquitylation primarily targets its substrates for proteasome-dependent proteolysis, although non-proteolytic roles have been described (Hochstrasser, 1996). The evolutionary conservation of Ub probably reflects the proteolytic pathway's requirements for Ub to interact with numerous other proteins, including E1, E2s, E3s, Ub-isopeptidases, proteasome subunits and possibly the proteolytic substrate. As Smt3p is only 17% identical to Ub and several residues involved in Ub's interaction with the proteasome (Beal *et al.*, 1996) are not conserved, it is unlikely that Smt3p targets proteins to the proteasome. The function of Smt3p conjugation is unknown, but attachment of the mammalian Smt3p-related protein SUMO-1 modulates protein–protein interactions (Mahajan *et al.*, 1997), and Smt3p may function similarly.

The Ran GTPase-activating protein RanGAP1 is the major substrate for SUMO-1 modification in mammalian cells (Matunis *et al.*, 1996). The composition of the Smt3p conjugates remains to be characterized, but conspicuously absent was a band of an appropriate size to be Smt3p attached to Rna1p, the yeast RanGAP1 homolog, which would be expected at ~70 kDa (46 kDa for Rna1p plus ~24 kDa, the apparent molecular weight of the 14 kDa HF-Smt3p). Immunoblotting with antibodies against Rna1p also does not detect modified forms (Corbett *et al.*, 1995), indicating that Rna1p is unlikely to be a substrate of the Smt3p conjugation pathway. The pattern of Smt3p conjugates was consistent with Smt3p being attached to only 5–10 proteins of ~100–200 kDa, although there was some smearing in the very high molecular weight range. The most prominent band was at ~140 kDa, suggesting a substrate of ~115 kDa. Smt3p was highly overexpressed in the experiments shown in Figures 2B and 7, and the endogenous *SMT3* product was present. However, the pattern of conjugates was similar, although dramatically reduced in intensity, when HF-Smt3p was expressed from the  $P_{SMT3}$  promoter on a *CEN*-based plasmid in the absence of endogenous *SMT3* (data not shown). Under these conditions cells did not grow as rapidly as wild-type and were enlarged, consistent with a deficiency of active Smt3p resulting either from the N-terminal tags or inadequate promoter sequence. However, the band at ~140 kDa was still the most prominent, with a second major band at ~180 kDa, indicating that these are likely to represent natural Smt3p conjugates, not overexpression artifacts. The increased intensity of these same bands during Smt3p overexpression suggests that under native expression levels only a small fraction of potential Smt3p substrates actually become Smt3p-conjugated.

Ub exists in a dynamic equilibrium between free and conjugated forms that is determined by the relative rates of conjugation, deubiquitylation by Ub-specific isopeptidases and Ub-dependent proteolysis, which releases free Ub. Under most conditions, a large fraction (20–70%) of Ub is unconjugated (Haas, 1988). However, at least for His6- and FLAG-tagged Smt3p, virtually all of the Smt3p was present in conjugates, unless high expression was

recently induced as in Figures 2B and 7 (data not shown). Even in cells expressing HF-Smt3p from  $P_{GAL10}$  on high copy plasmids, almost no free HF-Smt3p could be detected after 36 h of continuous growth under inducing conditions (data not shown). Thus, the steady-state for Smt3p seems to be shifted almost completely to the conjugated forms. The situation is similar with SUMO-1, for which very low levels of the free unconjugated protein are detected *in vivo* (Matunis *et al.*, 1996). This may explain the otherwise surprising result that HF-Smt3(A98)p, having an Ala rather than Gly at its C-terminal position 98, was able to substitute for wild-type Smt3p in allowing yeast growth. The analogous Ub mutant Ub(A76) can be attached to other proteins, but when it is overexpressed, wild-type (Ub<sup>+</sup>) cells exhibit phenotypes of Ub deficiency (Hodgins *et al.*, 1992), suggesting that this mutant cannot carry out the functions of Ub. This inability has been attributed to the fact that the isopeptide bond between Ub(A76) and its substrate is resistant to cleavage by Ub isopeptidases. If Smt3(A98)p isopeptide bonds are also resistant to cleavage, as Smt3(A98)p peptide bonds are (Figure 1C), this result suggests that Smt3p deconjugation is not essential to Smt3p function, at least when Smt3p is overexpressed.

Assessing the number of different proteins attached to Smt3p based on the band pattern depends on whether multiple Smt3p moieties can be ligated to a single substrate polypeptide, forming a ladder of Smt3p conjugates. Multiple copies of Smt3p could be linked either to several Lys residues of the substrate or, like Ub, as a polymer containing Smt3p–Smt3p linkages, although none of the Lys residues of Ub involved in Ub–Ub linkages (Chau *et al.*, 1989; Arnason and Ellison, 1994; Baboshina and Haas, 1996) are conserved in Smt3p. When HF-Smt3(A98)p, which may form deconjugation-resistant conjugates, was overexpressed, the band pattern of HF-Smt3p conjugates shifted dramatically toward higher molecular weight adducts compared with the pattern with HF-Smt3(G98)p (Figure 2B, lanes 2 and 3; see also Figure 7, lanes 2 and 3). This may indicate that multiple copies of Smt3p can be attached to the same substrate molecule, with the steady-state shifted toward multiply-Smt3p-conjugated forms when Smt3p cannot be removed. However, it is also possible that the mutant altered the kinetics of conjugation, biasing substrate selection in favor of different proteins.

The heterodimeric structure of the Smt3p-activating enzyme adds potential levels of regulational and functional variety to the Smt3p conjugation system. When Uba2p was isolated by Smt3p affinity chromatography, which depends on the enzymatic activity of the activating enzyme, only Aos1p co-purified. However, co-immunoprecipitation experiments with Uba2p have yielded several other proteins that remain to be identified (Dohmen *et al.*, 1995). At least some of these proteins appeared to be present in the fraction that was loaded on the Smt3p column (data not shown). These proteins may have failed to co-purify on the Smt3p column because they were removed in the column wash. Alternatively, Uba2p may participate in distinct multi-protein complexes of varying composition, so that Uba2p binding by Aos1p and by the other proteins are mutually exclusive. The other Uba2p-binding proteins could serve to regulate the level of Smt3p



activation, perhaps by competing with Aosl1p for Uba2p binding, or the other Uba2p-containing complexes could have their own unique activities.

ATP-dependent activation of Smt3p by Aosl1p/Uba2p is the first step in the Smt3p conjugation pathway, which has at least one more step. Our initial experiments fractionating whole yeast lysates by Smt3p affinity chromatography yielded a fraction capable of forming a ladder of DTT-resistant Smt3p-containing bands, possibly a Smt3p homopolymer, upon ATP addition. This fraction contained Aosl1p/Uba2p, but purified Aosl1p/Uba2p does not have this activity. Consequently there must be an *E2*-like and possibly an *E3*-like activity in this fraction. As such a ladder was never observed either *in vivo* or in Smt3p conjugation by unfractionated yeast lysate, it is likely that there are yet other regulatory factors in this system. One candidate for an *E2*-like enzyme in Smt3p conjugation is Ubc9p (Seufert *et al.*, 1995), whose *Xenopus* homolog co-immunoprecipitates with a complex including SUMO-1-conjugated RanGAP1 (Saitoh *et al.*, 1997) and whose human homolog interacts in a two-hybrid screen with SUMO-1 (Shen *et al.*, 1996). Reconstitution of Smt3p conjugation awaits identification of the natural substrates.

## Materials and methods

### Media, genetic techniques and strains

Standard techniques were used (Ausubel *et al.*, 1994). Rich (YPD) and synthetic yeast media were prepared according to Sherman *et al.* (1986) with synthetic media containing either 2% glucose (SD), 2% raffinose (SR) or 2% raffinose and 2% galactose (SRG). Strains were cured of *URA3*-containing plasmids using 5-FOA (Boeke *et al.*, 1984). *S. cerevisiae* strains used were DF5 (*MATa/MAT $\alpha$  trp1-1/trp1-1 ura3-52/ura3-52 his3- $\Delta$ 200/his3- $\Delta$ 200 leu2-3,112/leu2-3,112 lys2-801/lys2-801*) (Finley *et al.*, 1987), JD90-1A (*MAT $\alpha$  uba2 $\Delta$ ::HIS3 trp1- $\Delta$ 63 ura3-52 his3- $\Delta$ 200 leu2-3,112 lys2-801*) (Dohmen *et al.*, 1995), and the DF5-derived strains BBY48 (*MAT $\alpha$* ) (Bartel *et al.*, 1990), EJY251 (*MATa/MAT $\alpha$  SMT3/smt3 $\Delta$ ::HIS3*), EJY251-11b (*MAT $\alpha$  smt3 $\Delta$ ::HIS3*) and EJY260 (*MATa/MAT $\alpha$  AOS1/aos1 $\Delta$ ::HIS3*), described below. EJY251 was produced from DF5 using one-step gene transplacement (Rothstein, 1991). Three PCR fragments containing the 5' flanking region of *SMT3* (~1000 bp), the *HIS3* gene and the *SMT3* 3' flanking region (~1000 bp) were produced with *Bgl*I site-containing primers so that these fragments could be digested with *Bgl*I and ligated together in the above order. The ligation reaction was the template in a second PCR reaction with the outside primers from the *SMT3* 5' and 3' flanks, yielding a deletion allele in which the *SMT3* coding sequence was replaced with *HIS3*. This PCR product was transformed into DF5, and His<sup>+</sup> transformants were selected. The deletion was verified by PCR analysis of yeast DNA isolated from the transformants. EJY251-11b was one haploid segregant produced when EJY251 was transformed with pHFSMT3-1 (described below), sporulated and the tetrads dissected. EJY260 was produced similarly to EJY251, by transforming a PCR product comprising the *HIS3* gene flanked by ~1000 bp of the 5' and 3' flanking sequences from the *AOS1* locus, into DF5.

### Plasmid constructs

413-Gal4-ER-VP16, a *HIS3*-marked plasmid expressing a fusion protein including domains from Gal4p, the human estrogen receptor and viral protein 16 (Louvion *et al.*, 1993), was a gift from Dr K. Madura (Piscataway, NJ). pJD417 is a YEplac181-derived (Gietz and Sugino, 1988) *LEU2*-marked 2  $\mu$  plasmid expressing HF-Uba2p [Uba2p tagged at its C-terminus with a His6 tag (Hoffmann and Roeder, 1991) and a FLAG tag (Brizzard *et al.*, 1994)] from the *P<sub>CUP1</sub>* promoter (Dohmen *et al.*, 1995). pJD397, pJD312 and pJD313 are *E. coli* expression plasmids derived from pET11a (Novagen) expressing C-terminally His6-tagged wild-type Uba2p (H-Uba2p), Uba2(C177A)p containing Ala in place of Cys177 or Uba2(C177S)p containing Ser in place of Cys177 respectively. pIS3 is a pRS314-based (Sikorski and Hieter, 1989) *TRP1*-marked *CEN* plasmid containing the 3.6 kb fragment of the *UBA2* locus extending from a *Sau*3A site 0.6 kb upstream of the ATG start codon to a *Sall* site

1.1 kb downstream of the TAA stop codon. pIS2-ts10 is the same as pIS3 but contains the *uba2-ts10* allele (isolation described below). pET-AOS1 is a pET21b-based *E. coli* expression plasmid for expressing Aosl1p bearing the His6-containing N-terminal extension MASMHHHHH-HMSS.

2  $\mu$ -based *URA3*-marked plasmids expressing Smt3p fusions to *E. coli*  $\beta$ -galactosidase ( $\beta$ gal) from *P<sub>GAL10</sub>* were constructed by inserting *Bam*HI-digested PCR fragments encoding Smt3p and its C-terminal variants into *Bam*HI-digested pLGS5 (Guarente *et al.*, 1982). The Smt3p variants encoded by these plasmids all bear the N-terminal extension MTGS. Amino acid sequences of Smt3p/ $\beta$ gal junctions up to the *Bam*HI site-encoded GS are shown in Figure 1B. *URA3*- and *leu2-d*-marked 2  $\mu$  plasmids for *P<sub>GAL10</sub>*-driven expression of Smt3p C-terminal variants were produced by inserting PCR-derived fragments into *Sac*I- and *Bam*HI-digested pEMBLyex4 (Cesareni and Murray, 1987). These constructs bear the N-terminal extension MTSHHHHHHHMHDYKDDDDKMGMS containing His6- and FLAG-tags. Amino acid sequences of their C-termini are shown in Figure 2A. *LEU2*-marked *CEN* plasmids expressing the same Smt3p variants from *P<sub>GAL10</sub>* were constructed by inserting the *Apa*I-*Bam*HI fragments containing *P<sub>GAL10</sub>* and the *SMT3* sequence from the pEMBLyex4-derived plasmids into pRS315 (Sikorski and Hieter, 1989). pHFSMT3-1, a *URA3*-marked *CEN* plasmid expressing His6- and FLAG-tagged full-length Smt3p from the *SMT3* promoter, was produced by a three-fragment ligation joining an ~1000 bp *Sac*I/*Spe*I-digested PCR product containing the *SMT3* 5' flanking sequence, a *Spe*I/*Bam*HI-digested PCR product containing tagged *SMT3*, and *Sac*I/*Spe*I-digested pRS316 (Sikorski and Hieter, 1989). pET-HFSmt3(G98) and pET-HFSmt3(A98) are pET21b-derived (Novagen) *E. coli* expression plasmids for expressing His6- and FLAG-tagged Smt3p C-terminal variants. pET-HFSmt3(G98) expresses HF-Smt3(G98)p, which has Gly98 at its C-terminus and bears the N-terminal extension MASMHHHHH-HHMHDYKDDDDKMGMS. The Smt3p variant expressed by pET-HFSmt3(A98) lacks the Met residue preceding the His6 tag in the N-terminal extension, and contains an Ala residue in place of Gly98 at its C-terminus. Junctions were verified by DNA sequencing, and in most cases the entire *SMT3* coding region was sequenced.

### Immunoblot analyses of whole yeast lysates

Yeast strain BBY48 bearing pLGS5-derived plasmids expressing Smt3p- $\beta$ gal fusions were grown to mid-exponential phase ( $A_{600}$  ~1.0) in SRG lacking uracil at 30°C. Culture aliquots containing equal numbers of cells, determined by  $A_{600}$ , were collected by centrifugation, washed once in water, and the yeast pellet frozen in liquid N<sub>2</sub>. Proteins were extracted as described by Yaffe and Schatz (1984) followed by resuspension in SDS- and DTT-containing loading buffer, SDS-6% PAGE and electroblotting to nitrocellulose (Schleicher and Schuell). Approximately 20  $\mu$ g of protein was loaded per lane. Filters were stained with amidoblack to confirm equal amounts of protein in each lane, followed by incubation with a monoclonal antibody against  $\beta$ gal (Promega) and chemiluminescent detection (Pierce).

Transformants of yeast strain DF5 that contained 413-Gal4-ER-VP16 and one of the pEMBLyex4-derived plasmids expressing a His6-FLAG-tagged Smt3p C-terminal variant were grown to exponential phase ( $A_{600}$  of ~0.6) in SD lacking histidine and uracil at 30°C. Cultures were supplemented with  $\beta$ -estradiol (final concentration 0.2  $\mu$ M), and incubation continued at 30°C for 7 h (final  $A_{600}$  of ~2.5). Samples were processed as described above, except with fractionation by SDS-PAGE on a 6–15% acrylamide gradient and FLAG epitope detection with the M2 monoclonal antibody (IBI/Kodak).

Transformants of yeast strain JD90-1A bearing the pEMBLyex4-derived plasmid expressing His6-FLAG-tagged full-length Smt3p [HF-Smt3(Y101)] and either pIS3 (containing *SMT3*) or pIS-ts10 (containing *smt3-ts10*) were grown to exponential phase ( $A_{600}$  of ~0.8) at 25°C in SR lacking uracil and tryptophan. Cultures were supplemented to 2% galactose (Bio-101), split, and incubation continued at either 25°C or 37°C for 2 h. The cultures containing *SMT3* at 25°C and 37°C and *smt3-ts10* at 25°C and 37°C grew (measured by  $A_{600}$ ) by 58%, 65%, 42% and 46% respectively, during this incubation. Samples taken immediately after galactose addition or after 2 h were processed as described above, using a 6–15% acrylamide gradient for SDS-PAGE and the M2 monoclonal antibody against the FLAG-epitope.

### Smt3p affinity chromatography and identification of Aosl1p

Two millilitres of a saturated culture of *E. coli* BL21(DE3) bearing pET-HFSmt3(G98) or pET-HFSmt3(A98) were diluted into 1 l of Luria broth containing 50  $\mu$ g/ml ampicillin, grown at 37°C to an  $A_{600}$  of ~0.6, supplemented with isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) to



0.2 mM, and incubation at 37°C continued for 4 h. Subsequent steps took place at 4°C. Cells were harvested by centrifugation, resuspended in 50 ml 50 mM Na-phosphate (pH 8.0), 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and lysed in a French Pressure Cell at 14000 p.s.i. Lysates were clarified by centrifugation at 27 000  $g_{max}$ , and the supernatant bound in batch for 1 h to 0.8 ml of Ni-NTA-agarose (Qiagen). The Ni-NTA-agarose was loaded into a column, washed with 50 ml of 50 mM Na-phosphate (pH 6.0), 300 mM NaCl, 10% glycerol and eluted with phosphate-buffered saline containing 200 mM imidazole. The HF-Smt3p-containing eluate was dialyzed against 20 mM K-HEPES (pH 7.5). Twenty milligrams of HF-Smt3(G98)p or 15 mg of HF-Smt3(A98)p were coupled to 1 ml of Affi-gel-15 (Bio-Rad) according to the manufacturer's instructions. In both cases >75% of the added protein appeared to bind.

Yeast strain JD90-1A bearing pJD417 (expressing HF-Uba2p) was disrupted in liquid nitrogen in a variation on the method described in Ausubel *et al.* (1994). A 4 l culture was grown in YPD at 30°C to an  $A_{600}$  of ~6. Subsequent steps took place at 4°C. Cells were harvested by centrifugation, washed 1× in distilled H<sub>2</sub>O, and resuspended in one cell volume of 50 mM Tris (pH 8.0), 1 mM MgCl<sub>2</sub>, 5 mM β-mercaptoethanol (βME) and 2 μg/ml of each of the protease inhibitors aprotinin, chymostatin, leupeptin and pepstatin (Sigma). Cell slurries were frozen dropwise in liquid N<sub>2</sub>, ground to a fine powder under liquid N<sub>2</sub> using a mortar and pestle, melted and brought to a final concentration of 300 mM NaCl. Cell debris was pelleted at 27 000  $g_{max}$ , and the resulting supernatant subjected to further centrifugation at 200 000  $g_{av}$  for 90 min. The high-speed supernatant was diluted with one volume of 50 mM Tris pH 8.0, 300 mM NaCl and fractionated on Ni-NTA-agarose as above except that the wash buffer also contained 1 mM MgCl<sub>2</sub> and 5 mM βME, and the elution buffer contained 200 mM imidazole in 50 mM BisTris pH 6.5, 100 mM NaCl, 1 mM MgCl<sub>2</sub> and 1 mM βME.

In a pilot experiment, Ni-NTA column eluate containing 500 μg of protein (at 0.3 mg/ml) was diluted with one volume of 50 mM BisTris (pH 6.5), 100 mM NaCl, 19 mM MgCl<sub>2</sub>, 0.1 mM DTT and 4 mM ATP and loaded onto the HF-Smt3(A98)p-Affigel-15 column, which had been equilibrated with 50 mM BisTris (pH 6.5), 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.1 mM DTT and 2 mM ATP (column buffer). The flow-through was re-applied to the column twice, the second time after the final ATP concentration was adjusted to 5 mM. The column was washed three times with 10 ml of column buffer containing 500 mM NaCl and lacking DTT and then eluted in 10 mM DTT, 50 mM Tris (pH 9.0), 1 M NaCl and 1 mM MgCl<sub>2</sub>.

For a larger scale preparation, Ni-NTA column eluate containing 6 mg protein (derived from high-speed supernatant containing ~800 mg) was applied to the HF-Smt3(A98)p-Affigel-15 column and the same procedure followed. The DTT eluate was exchanged into 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 0.1 mM DTT (TBS+) using a Biomax-10 ultrafiltration unit (Millipore) and then applied to a 0.5 ml anti-FLAG M2-affinity gel (IBI/Kodak) column. The column was washed with TBS+ and eluted with TBS+ containing 75 μg/ml FLAG peptide (IBI/Kodak). Anti-FLAG column eluate was fractionated by SDS-PAGE and electroblotted to Immobilon-PS<sup>4</sup> polyvinylidene difluoride membrane (Millipore). The ~40 kDa band, containing ~2 μg of protein, was excised from the amidoblack-stained membrane and identified by direct analysis of a Lys-C endoproteinase digest by MALDI-TOF mass spectrometry by the Rockefeller University Protein/DNA Technology Center as described (Gharahdaghi *et al.*, 1996). Nine major peaks with measured masses of 1170.56, 1542.18, 1823.77, 1929.79, 2001.64, 2258.88, 2437.39, 2699.6 and 2741.39 were within one mass unit of the peptide masses predicted by a theoretical digest of the YPR180W product, as determined by the MS-Fit program on the WWW server of the UCSF Mass Spectrometry Facility.

Domains of sequence similarity between Aos1p, Uba2p and other proteins were initially identified using the FASTA algorithm on the WWW server of the *Saccharomyces* Genome Database and were refined by multiple sequence alignments using the program MEGALIGN (DNASTAR).

#### Smt3p thioester formation assays

Recombinant H-Aos1p (tagged N-terminally with His6), H-Uba2p, H-Uba2(C177A)p and H-Uba1(C177S)p (all tagged C-terminally with His6) were produced as described above for HF-Smt3p, except that expression was induced with 0.02 mM IPTG; the subsequent 4 h incubation took place at 20°C; the lysis and wash buffers also contained 1 mM MgCl<sub>2</sub> and 5 mM βME; clarified lysates were supplemented with 20 mM imidazole prior to Ni-NTA-agarose addition; and the elution

buffer contained 200 mM imidazole in 50 mM BisTris pH 6.5, 100 mM NaCl, 1 mM MgCl<sub>2</sub> and 1 mM βME. Eluates were concentrated and exchanged into this same buffer, except lacking imidazole and containing 0.1 mM DTT in place of the βME, using Biomax-10 ultrafiltration units. The resulting H-Uba2p, H-Uba2(C177A)p and H-Uba2(C177S)p preparations all contained ~70% H-Uba2p, as estimated by Coomassie Blue staining, with the balance composed of ~10 major *E.coli*-derived proteins. The H-Aos1p contained ~5% H-Aos1p with a similar array of contaminants. Most thioester formation reactions contained 50 mM BisTris (pH 6.5), 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.1 mM DTT (reaction buffer), 5 U/ml inorganic pyrophosphatase (Sigma) and some of the following: 5 mM ATP, 15 μg/ml HF-Smt3(G98)p, 200 μg/ml H-Uba2p preparation and 750 μg/ml H-Aos1p preparation. Reactions were incubated for 30 min at 25°C and stopped by 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 (6–15% acrylamide gradient) and immunoblotting using the anti-FLAG antibody. In the experiment testing whether AMP-PNP (adenylyl-imidodiphosphate) could substitute for ATP, a similarly derived but less pure mixture of recombinant H-Aos1 and H-Uba2 was incubated for 10 min at 25°C in reaction buffer with 15 μg/ml HF-Smt3(G98)p and either 10 nM ATP or 10 nM AMP-PNP (Boehringer Mannheim).

A mixture of Ni-NTA-purified recombinant H-Uba2p and H-Aos1p was fractionated on HF-Smt3(G98)p-Affigel-15 as described above. The DTT eluate was fractionated using the Pharmacia FPLC system on a Superdex-200 column (Pharmacia) in 20 mM Na-HEPES (pH 7.0), 150 mM NaCl, 1 mM MgCl<sub>2</sub>. Peak fractions were used in thioester formation reactions as described above except containing 25 μg/ml of Aos1p/Uba2p complex and 250 μg/ml HF-Uba2(A98)p and were incubated for 90 min.

#### Depletion of HF-Uba2p

The high-speed supernatant from a whole cell lysate of JD90-1A bearing pJD417 was fractionated on Ni-NTA-agarose as described above, and the unbound fraction was passed through an additional 1 ml Ni-NTA column. This flow-through was exchanged into 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM MgCl<sub>2</sub> and 0.1 mM DTT using a Sephadex G-25 column. Of this, 1.4 ml (9.5 mg/ml protein) was incubated with 100 μl anti-FLAG M2-affinity gel for 2 h at 4°C, and the unbound fraction was used as the 'depleted' lysate. 'Undepleted' lysate was high-speed supernatant exchanged into the same buffer using the G-25 column. Reactions were incubated for 90 min at 25°C and contained 25 mM Tris (pH 7.5), 75 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.1 mM DTT, 4.6 mg/ml whole yeast lysate (either 'undepleted' or 'depleted') and some of the following: 5 mM ATP, 30 μg/ml HF-Smt3(G98)p and 4 μg/ml Aos1p/Uba2p complex.

#### Isolation of a *ts UBA2* allele

Mutations were introduced into a DNA fragment extending from position -580 with respect to the start codon to position 740 downstream of the stop codon of the *UBA2* ORF by error-prone PCR according to Spee *et al.* (1993). The fragment was amplified 30 cycles with 3.25 mM MgCl<sub>2</sub>, 200 μM dITP/dGTP/dTTP/dCTP, 30 μM dATP and *Taq* polymerase using pJDA315 (Dohmen *et al.*, 1995) as a template. The resulting mixture of PCR products was co-transformed with *Bam*HI-linearized pIS1 into yeast strain JD62-6A (Dohmen *et al.*, 1995). The *UBA2* gene of this strain is under control of the glucose-repressible  $P_{GAL1}$  promoter. Plasmid pIS1 is based on the *LEU2*-marked *CEN* plasmid pRS315 (Sikorski and Hieter, 1989) and contains a *uba2Δ* allele (a *Bam*HI site in place of the *UBA2* ORF) constructed as described (Dohmen *et al.*, 1995). Yeast transformants carrying plasmids resulting from *in vivo* recombination between pIS1 and PCR products were selected at 25°C on SD plates ( $P_{GAL1}$  is off) lacking leucine. Transformants that had obtained plasmids carrying *ts UBA2* alleles were identified by replica plating and incubation at 37°C. After isolation of one of these plasmids, pIS1-ts10, it was confirmed by mapping analysis that the *ts* phenotype was due to a mutation within the *UBA2* ORF. The *uba2-ts10* allele was moved into the *TRP1/CEN6*-based plasmid pRS314 yielding pIS2-ts10.

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