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Comparative Analysis of Yeast PIAS-Type SUMO Ligases *In Vivo* and *In Vitro*

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SUMO/Smt3, a ubiquitin-like modifier, is known to conjugate other proteins and modulate their functions in various processes. Recently, Ull1/Siz1 was discovered as a novel PIAS-type E3 required for septin sumoylation in yeast. We demonstrate here that the second PIAS-type Nfi1/Siz2 is also a SUMO ligase. It interacted with Smt3, SUMO/Smt3 conjugating enzyme Ubc9 and a septin component Cdc3 in the two-hybrid system. The region containing the RING-like domain of Nfi1/Siz2 bound directly to Ubc9 and Cdc3, but not to Smt3. Nfi1/Siz2 stimulated Smt3 conjugation to Cdc3 *in vitro*. In this *in vitro* system, Smt3 formed polymeric chains in the presence of higher concentrations of E1 and E2 enzymes. When the lysine[15] residue of Smt3 was substituted with arginine, Smt3 chain-polymerization was abolished. Using this polysumoylation-deficient mutant Smt3, we found that Cdc3 and Nfi1/Siz2 were modified with Smt3 at multiple sites. Finally we found that the C-terminal truncated form of Ull1/Siz1 was mis-localized *in vivo*, but retained its SUMO ligase activity *in vitro*. We discuss the regulation of these SUMO ligases *in vivo* and *in vitro*.

Key words: auto-sumoylation, *in vitro* conjugation system, polymerization, Smt3/SUMO.

Abbreviations: SUMO, small ubiquitin-like modifier; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HA, hemagglutinin; PIAS, protein inhibitor of activated STAT (signal transducers and activators of transcription); PML, promyelocytic leukemia protein; *SMT3*, suppressor of *mif2*; *ULL1*, ubiquitin-like protein ligase 1; E1, activating enzyme; E2, conjugating enzyme, E3 protein ligase; GAD, Gal4 activation domain.

SUMO (small ubiquitin-like modifier)/Smt3 is a member of a growing family of ubiquitin-related proteins and is known to be conjugated to RanGAP1, PML, IκBα, p53, yeast septin components, *etc.* (*1–6*). In budding yeast, Smt3 is the only member of the SUMO family, and the Smt3-conjugation system is essential for mitotic growth (*7*). The lethality of the *smt3* deletion mutant can be suppressed by expressing human SUMO-1, suggesting that SUMO-1 is a functional homologue of yeast Smt3 (*8*).

For the modification of all SUMO family members, common E1 and E2 enzymes are required. The E1 enzymes are heterodimers known as Uba2/Aos1 for yeast Smt3 and SAE1/SAE2 for mammalian SUMO systems, and form a thioester bond between the C-terminal glycine of SUMO and a cysteine residue of Uba2 through utilizing ATP (*9–11*). Then, SUMO transfers to an E2 enzyme, known as Ubc9 conjugating enzyme (*12*, *13*). Recently, it was discovered that SUMO1/Smt3 ligases (E3s) are involved in this conjugation pathway. In budding yeast there are two PIAS family proteins, Ull1/Siz1 and Nfi1/Siz2. Ull1/Siz1 was identified as an E3 factor specific for septin components (*14–16*). PIAS1 was also identified as a SUMO-1 ligase for p53 (*17*). The PIAS family members contain a zinc-binding RING-like domain with an octet of ordered cysteine and histidine residues, as in the case of certain ubiquitin-ligase components, such as Apc11 of the anaphase promoting complex and Rbx1 of the SCF-ubiquitin ligase complex (*18–21*). Thus, not only are the amino acid sequences and three-dimensional structures similar between SUMO/Smt3 and ubiquitin, but their conjugation systems and the enzymes involved are highly related (*7*, *9*, *10*, *12*, *13*). The other PIAS-type protein, Nfi1/Siz2 remains to be elucidated.

SUMO-2 and SUMO-3, but not SUMO-1, can form polymers *in vitro* with the aid of E1 and E2 enzymes (*22*). Also, yeast Smt3 has been reported to conjugate to Smt3, thus forming a polymeric chain of Smt3 *in vitro* (*15*). These results may reasonably be explained by the fact that SUMO-2, SUMO-3 and yeast Smt3 contain consensus acceptor lysine residues (ψKXE: where ψ is a large hydrophobic residue and X is any amino acid residue) for sumoylation, but SUMO-1 does not (*22*, *23*).

In this paper we have improved our *in vitro* system and determined the poly-sumoylation site of Smt3. Using a polysumoylation-deficient *smt3* mutant, we were able to distinguish between the Smt3 polymeric chain conjugation at a single site of target proteins and Smt3 conjugation to multiple sites of target proteins. Furthermore, we demonstrate here that Nfi1/Siz2 stimulates sumoylation of a test substrate and is multiply auto-sumoylated *in vitro*. Finally we have found that the C-terminal truncated form of Ull1/Siz1 is mis-localized *in vivo*, but retains its SUMO ligase activity *in vitro*. We discuss the regulation of these SUMO ligases.

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EXPERIMENTAL PROCEDURES

Strains and Genetic Manipulations—*Escherichia coli* strain DH5α was used for plasmid propagation, and strains BL21 (DE3) and M15 (pREP4) for protein purification. Strains of *Saccharomyces cerevisiae* W303–1A (*MATa ade2 ura3 trp1 leu2 his3 can1 ssd-d2*) and its isogenic T-20 (*ull1::cgHIS3 CDC3HA-TRP1*) were described previously (*16*). PJ69–4A (*MATa ura3 trp1 leu2 his3 gal4 gal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) was used for the two-hybrid system (*24*). Media and genetic techniques for yeast were described previously by Kaiser *et al*. (*25*).

Plasmids—pGBDU and pGAD vectors for the two-hybrid system were gifts from E. Craig (*24*). pT-8 (pGBDU-C1-*ULP1*) (*26*) and pT-23 (pTS910CU-*ULL1-GFP*) (*16*) were described previously. pT-43 (pQE30-*SMT3gg*) contains the same *Bam*HI-*Sal*I fragment of pT-35 (pGEX-KG-*SMT3gg*) (*16*) on the pQE30 vector. To construct pT-44 (pQE30-*SMT3*gg^{K11R}), pT-45 (pQE30-*SMT3gg*^{K15R}) and pT-46 (pQE30-*SMT3gg*^{K19R}), the appropriate mutation was introduced by polymerase chain reaction-based site-directed mutagenesis. For the mutations of K11R, K15R, and K19R, the following primer pairs were used, respectively: SMT3-K11R-SS (CAAGAAGCTAGGCCAGAGGTC) and SMT3-K11R-AS (GACCTCTGGCCTAGCTTCTTG), SMT3-K15R-SS (CCAGAGGTCAGGCCAGAAGTC) and SMT3-K15R-AS (GACTTCTGGCCTGACCTCTGG), SMT3-K19R-SS (CCAGAAGTCAGGCCTGAGACT), and SMT3-K19R-AS (AGTCTCAGGCCTGACTTCTGG) (mutant nucleotides are underlined). To construct pT-47 (pQE30-*SMT3gg*^{3KR}), the following primers were used: SMT3-K11R, K15R, K19R-SS: (GCGCGGATCCATGTCGGACTCAGAAGTCAATCAAGAAGCTAGGCCAGAGGTCAGGCCAGAAGTCAGGCCTGAGACTCACATCAATTTAAAGGTGTCCGAT) (mutant nucleotides are underlined; 3KR stands for the three K11R, K15R, K19R substitutions) and SMT3-C-GG (*16*). To construct pT-58 (pGEX-KG-*UBA2*) and pT-72 (pGBDU-C1-*UBA2*), a DNA fragment carrying the *UBA2* open reading frame was amplified by polymerase chain reaction, cut with *Bam*HI and *Sal*I, and cloned into pGEX-KG and pGBDU-C1 vectors, respectively. To construct pT-59 (pGEX-KG-*AOS1*), a DNA fragment carrying the *AOS1* open reading frame was amplified by polymerase chain reaction, cut with *Sal*I, and cloned into the *Xho*I site of pGEX-KG. pT-60 (pGEX-KG-*UBC9*) and pT-71 (pGBDU-C1-*UBC9*) contain the same *Bam*HI–*Sal*I fragment of pT-42 (pET21a-*UBC9*) (*16*) on pGEX-KG and pGBDU-C1 vectors, respectively. To construct pT-61 (pET21b-*NFI1*), a DNA fragment carrying the *NFI1* open reading frame was amplified by polymerase chain reaction using the following primer pairs: NFI1-N-EcoRI (CCGGAATTCCATGGCAAGTGTCATGTCAAAT) and NFI1-C-Sal1 (GCGCGTCGACATCTGATGTTAAATCGACCACT), and genomic DNA of W303–1A as template, cut with *Eco*RI and *Sal*I, and inserted into pET21b vector. pT-63 (pET21a-*SMT3*) and pMK09 (pGBDU-C1-*SMT3*) contain the same fragment of pMK02 (*8*). pT-64 (pGEX-KG-*RING*^{Nfi1}) was constructed by cloning a DNA fragment carrying the RING-like domain sequence from the 334th to 446th amino acid of Nfi1, and amplified by polymerase chain reaction. pT-65 (pGBDU-C1-CDC3) was constructed by cloning a DNA fragment carrying the *CDC3* open reading frame amplified by polymerase chain reaction. To construct pT-73 (pGBDU-C1-*SMT4*), a DNA fragment carrying the *SMT4* open reading frame was amplified by polymerase chain reaction using the following primers: SMT4-N-Sal1 (GCGCGTCGACATGTCTGCCAGAAAACGCAA) and SMT4-C-Pst1 (GCAACTGCAGTCAAGGGTCTTCATCTTCCA), cut with *Sal*I and *Pst*I, and inserted into pGBDU-C1. pT-74 (pGAD-*NFI1ΔDN*) was isolated from the pGAD bank in the two-hybrid screening. To construct pT-77 (pET21b-*ull1*$^{\Delta C440}$), the 1,395 bp DNA fragment from the N-terminus of the *ULL1* open reading frame was amplified by polymerase chain reaction using the following primer pair: ULL1-N-*Eco*RI (CCGGAATTCCATGATAAATTTAGAGGATTACTG) and ULL1-ΔC440-Sal1 (GCGCGTCGACAGTACCTTTTTCTGGGCTTCT), cut with *Eco*RI and *Sal*I, and inserted into pET21b vector. pT-80 (pTS910CU-*ull1*^{C377S}-GFP) contains the same fragment of pT-18 (*14*). pT-81 (pTS910CU-*ull1*$^{\Delta C440}$-*GFP*) contains a DNA fragment amplified by polymerase chain reaction from the –1 kb promoter region to 465th amino acid of Ull1 (a C-terminal truncated Ull1) on pTS910CU (*27*).

Purification of Proteins for In Vitro Conjugation Assay—For the preparation of His-tagged recombinant proteins, cultures of M15 (pREP4) carrying pT-43 (pQE30-*SMT3gg*), pT-44 (pQE30-*SMT3gg*^{K11R}), pT-45 (pQE30-*SMT3gg*^{K15R}), pT-46 (pQE30-*SMT3gg*^{K19R}) or pT-47 (pQE30-*SMT3gg*^{3KR}) and cultures of BL21 (DE3) carrying pT-77 (pET21b-*ull1*$^{\Delta C440}$) or pT-61 (pET21b-*NFI1*) were grown to mid-log phase, and each recombinant protein was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside at 37°C for 1.5 h. Cells were collected, resuspended in lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% Nonidet P-40, 1 mM PMSF] and disrupted by sonication. The lysates were centrifuged at 15,000 ×*g* for 15 min and supernatant fractions containing His-tagged proteins were incubated with Ni-NTA agarose (QIAGEN) as instructed by the manufacturer. GST-Uba2, GST-Aos1 and GST-Ubc9 were expressed from plasmid pT-58, pT-59, and pT-60, respectively, in *E. coli* BL21 (DE3). Each cell lysate was mixed and incubated with glutathione-Sepharose 4B (Amersham Pharmacia) at 4°C for 2 h. After the beads were washed three times with lysis buffer, each protein was eluted with 10 mM glutathione in 50 mM Tris-HCl (pH 8.0), and the samples were dialyzed against 10 mM Tris-HCl (pH 7.5) buffer containing 3 mM $MgCl_2$. For the preparation of the untagged mature forms of Smt3 and Ubc9, GST-Smt3gg and GST-Ubc9 bound to beads were treated with thrombin and the supernatants were dialyzed to remove glutathione. GST-Smt3gg and T7-Cdc3-His were described previously (*16*).

Preparation of Yeast Cell Lysates and Immunoblot Analysis—Cell lysates were prepared and immunoblotting was performed as described previously (*14*). Antibodies used in this study were 2 μg/ml anti-HA (16B12; BabCO), anti-T7 (Novagen) and anti-His (Amersham Pharmacia) mouse monoclonal antibodies, 0.2 μg/ml anti-PSTAIRE (*sc-53*; Santa Cruz Biotechnology), anti-Cdc3 (provided by J. Pringle), anti-Smt3 (provided by M. Hochstrasser) and anti-GST rabbit polyclonal antibodies, and

0.2 μg/ml anti-Ubc9 (*yC-19*; Santa Cruz Biotechnology) goat polyclonal antibody. As the secondary antibody, 1.5 μl of goat anti-mouse, goat anti-rabbit or donkey anti-goat IgG conjugated to horseradish peroxidase (Promega) was used for 1 h incubation at room temperature. Bands were detected with detection reagent (New Life Science).

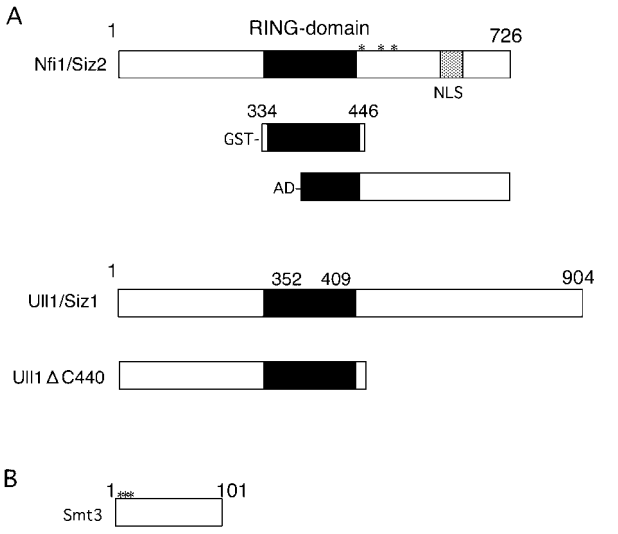

Fig. 1. **Schematic structures of Nfi1, Ull1, their derivatives, and Smt3.** A, the RING-like domains of Nfi1 (from the 334th to 446th a.a.) and Ull1 (from the 352th to 409th a.a.) are shown as black *boxes*. The position of NLS is shown in gray. B, structure of Smt3. Consensus sequences (ψKXE) for sumoylation acceptor sites are indicated by asterisks.

In Vitro Binding Assay—Cell lysates from transformants of BL21 (DE3) with pT-64 (pGEX-KG-*RING*^{Nfi1}), pGEX-KG, pT-63 (pET21a-*SMT3*), pT-42 (pET21a-*UBC9*), or pT-36 (pET21b-*CDC3*) were prepared as described previously (*16*). Using GST-RING^{Nfi1} or GST, with T7-Smt3, T7-Ubc9, or T7-Cdc3, the *in vitro* binding assay was performed as described previously (*16*).

Fluorescence Microscopy—Cells of the *ull1* disruptant (T-20) expressing Ull1-GFP (pT-23), Ull1^{C377S}-GFP (pT-80), or Ull1^{ΔC440}-GFP (pT-81) were fixed with 5% formaldehyde and samples were examined under a fluorescence microscope.

RESULTS AND DISCUSSION

The Nfi1 RING-Like Domain Interacts with Ubc9 and Cdc3—As described previously (*14*), we isolated *NFI1* whose C-terminal half (from the 372th to 726th a.a.) was fused to the *GAL4* activation domain using Smt3 as bait in the two-hybrid system (Fig. 1A, 2A). This C-terminal half of Nfi1 also interacted with Ubc9 and Cdc3, but not with Uba2, Smt4 or Ulp1 in the two-hybrid system (Fig. 2A).

Since the RING-like domain of Ull1 (from the 327th to 465th a.a.) interacted with Ubc9 and Cdc3 in the two-hybrid system and the *in vitro* binding assay (*16*), we examined whether the Nfi1 RING-like domain (from the 334th to 446th a.a.) also interacted directly with E2 or a tentative substrate by an *in vitro* binding assay. A GST-tagged RING-like domain of Nfi1 (GST-RING) or GST was expressed in *E. coli* and bound to glutathione-Sepharose 4B beads. T7-tagged Smt3, T7-Ubc9, and T7-Cdc3

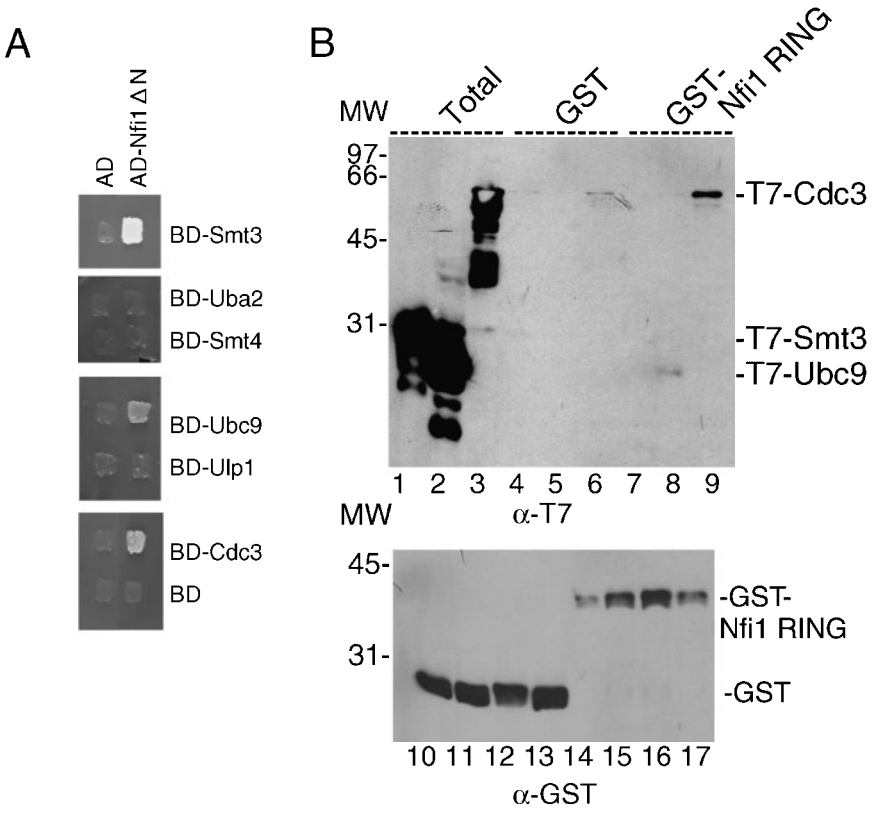

Fig. 2. **The RING-like domain of Nfi1 interacts with Ubc9 and Cdc3.** A, two-hybrid interaction. Yeast strain PJ69–4A was co-transformed with pMK09 (pGBDU-*SMT3*), pT-72 (pGBDU-*UBA2*), pT-73 (pGBDU-*SMT4*), pT-71 (pGBDU-*UBC9*), pT-8 (pGBDU-*ULP1*), pT-65 (pGBDU-*CDC3*), or pGBDU-vector, together with pGAD vector or pT-74 (pGAD-*NFI1ΔN*). The transformants were streaked on a minimal plate lacking histidine, uracil and leucine, and incubated at 25°C. B, *in vitro* binding assay. GST or the GST-tagged RING-like domain of Nfi1 was bound to glutathione-Sepharose 4B beads. An *E. coli* lysate containing T7-Smt3, T7-Ubc9, or T7-Cdc3 was incubated with the beads. After washing, bound proteins were subjected to immunoblotting. Smt3, Ubc9 and Cdc3 were probed with anti-T7 (lanes 1–9). GST and the GST-RING-like domain were probed with anti-GST (lanes 10–17). Lane *1*, total lysate of T7-Smt3; *l*ane 2, total lysate of T7-Ubc9; lane 3, total lysate of T7-Cdc3; lanes 4 and 11, GST beads plus T7-Smt3 lysate; lanes 5 and 12, GST beads plus T7-Ubc9 lysate; lanes 6 and 13, GST beads plus T7-Cdc3 lysate, lanes 7 and 15, GST-RING beads plus T7-Smt3 lysate; lanes 8 and 16, GST-RING beads plus T7-Ubc9 lysate; lanes 9 and 17, GST-RING beads plus T7-Cdc3 lysate; lane 10, total lysate of GST; lane 14, total lysate of GST-RING.

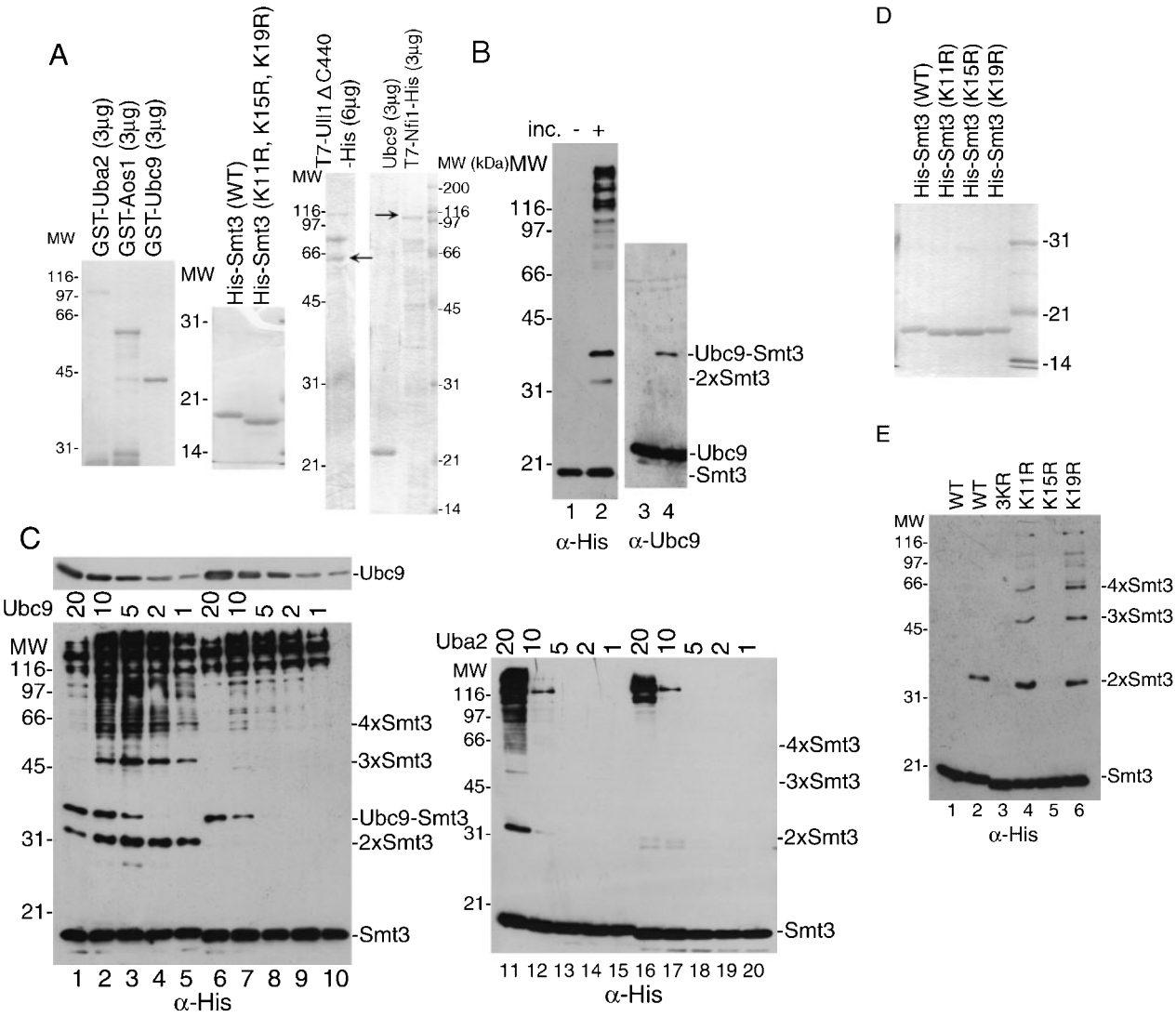

Fig. 3. **Smt3 conjugation *in vitro*.** A, preparation of recombinant proteins for *in vitro* assay. Purified proteins used for the *in vitro* system were subjected to SDS–polyacrylamide gel electrophoresis, and gels were stained with Coomassie Brilliant Blue. Loaded samples are 3 μg GST-Uba2, 3 μg GST-Aos1, 3 μg GST-Ubc9, 3 μg of His-Smt3, 3 μg of His-Smt3^{3KR}, 6μg of T7-Ull1ΔC440-His (marked by an arrow), 3 μg Ubc9, and 3 μg of T7-Nfi1-His (marked by an arrow). B, *in vitro* conjugation system. Reaction mixtures of 7.2 μg GST-Uba2, 7.6 μg GST-Aos1, 3.4 μg Ubc9, and 3 μg of His-Smt3 were incubated in the presence of 10 mM ATP at 37°C for 0 min (inc. −) or 90 min (inc. +), and subjected to immunoblotting. Smt3 and Ubc9 were probed with anti-His and anti-Ubc9, respectively. C, conjugation efficiency depends on substrate. Left panel: 3.6 μg GST-Uba2, 1.9 μg GST-Aos1, and 3 μg of His-Smt3 (lanes 1–5) or His-Smt3^{3KR} (lanes 6–10) were incubated with various amounts of Ubc9 in the presence of 10 mM ATP at 37°C for 90 min, and the reaction mixtures were subjected to immunoblotting. Smt3 and Ubc9 were probed with anti-His and anti-Ubc9, respectively. Lanes 1 and 6, 3.4 μg Ubc9; lanes 2 and 7, 1.7 μg Ubc9; lanes 3 and 8, 0.85 μg Ubc9; lanes 4 and 9, 0.34 μg Ubc9; lanes 5 and 10, 0.17 μg Ubc9. Numbers indicate the amount of Ubc9 in arbitrary units. Right panel: Reaction mixtures of 0.16 μg Ubc9 and 3 μg His-Smt3 (lanes11–15) or His-Smt3^{3KR} (lanes 16–20) were incubated with various amounts of GST-Uba2 and GST-Aos1 in the presence of 10 mM ATP at 37°C for 60 min (lanes11–15) or 90 min (lanes 16–20), and subjected to immunoblotting. Smt3 was probed with anti-His. Lanes 11 and 16, 3.6 μg GST-Uba2, 2.5 μg GST-Aos1; lanes 12 and 17, 1.8 μg GST-Uba2, 1.3 μg GST-Aos1; lanes 13 and 18, 0.72 μg GST-Uba2, 0.5 μg GST-Aos1; lanes 14 and 19, 0.36 μg GST-Uba2, 0.25 μg GST-Aos1; lanes 15 and 20, 0.18 μg GST-Uba2, 0.13 μg GST-Aos1. Numbers indicate the amount of Uba2/Aos1 in arbitrary units. D, preparation of Smt3 mutant proteins. 3 μg of the purified proteins of His-Smt3 (WT), His-Smt3 ^{K11R}, His-Smt3 ^{K15R}, or His-Smt3 ^{K19R} were loaded on an SDS-polyacrylamide gel, as in A. E, Reaction mixtures of 2.2 μg GST-Uba2, 1.3 μg GST-Aos1, 0.16 μg Ubc9, together with 3μg His-Smt3 (lanes 1–2), His-Smt3 ^{3KR} (lane 3), His-Smt3 ^{K11R} (lane 4), His-Smt3 ^{K15R} (lane 5), or His-Smt3 ^{K19R} (lane 6), were incubated in the presence of 10 mM ATP at 37°C for 0 min (lane 1) or 90 min (lanes 2–6), and subjected to immunoblotting. Smt3 was probed with anti-His.

were separately expressed in *E. coli,* and each cell lysate was mixed with beads bound with GST-Nfi1-RING or GST. After washing, the bound proteins were subjected to immunoblotting. As shown in Fig. 2B, Cdc3 bound to GST-Nfi1-RING (*lane 9*) and Ubc9 bound to it weakly (*lane 8*). In contrast, Smt3 did not interact with GST-Nfi1-RING (*lane 7*). None of these compounds interacted with GST (*lanes 4–6*). Thus, both Ubc9 and Cdc3 directly and specifically interacts with the region containing the RING-like domain of Nfi1. Although Nfi1 is not an E3 for

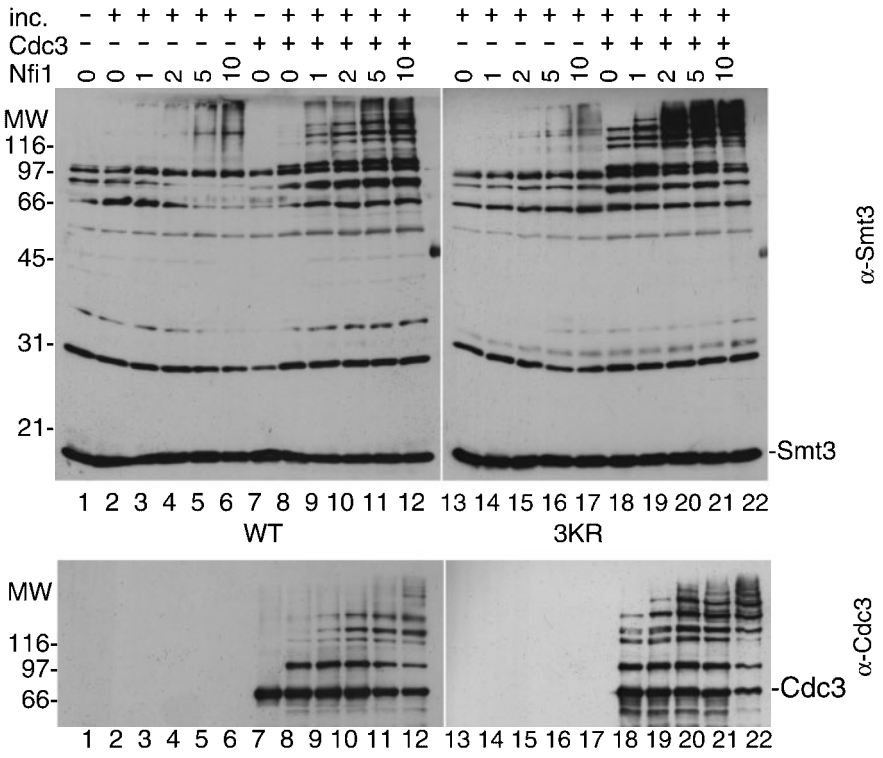

Fig. 4. **Nfi1 stimulates Smt3 conjugation to Cdc3.** Reaction mixtures of 1.8 μg GST-Uba2, 0.85 μg GST-Aos1, 0.17 μg Ubc9 and either 3 μg of His-Smt3 (lanes 1–12) or His-Smt3^{3KR} (lanes 13–22), and various amounts of Nfi1 were incubated with (lanes 7–12 and 18–22) or without (lanes 1–6 and 13–17) 2.4 μg Cdc3 in the presence of 10 mM ATP at 37°C for 0 min (inc. –; lanes 1 and 7) or 90 min (inc. +; lanes 2–6, 8–12). Smt3 and Cdc3 were probed with anti-Smt3 and anti-Cdc3, respectively. Lanes 1, 2, 7, 8, 13 and18, no Nfi1; lanes 3, 9, 14 and 19, 0.28 μg Nfi1; lanes 4, 10, 15 and 20, 0.56 μg Nfi1; lanes 5, 11, 16 and 21, 1.4 μg Nfi1; lanes 6, 12, 17 and 22, 2.8 μg Nfi1. Numbers indicate the amount of Nfi1 in arbitrary units.

Cdc3 sumoylation *in vivo*, as described previously (*14*, *15*), it binds to Cdc3 *in vitro*, as in the case of Ull1.

Formation of High Molecular Weight Conjugates of Smt3 In Vitro—First, we improved our *in vitro* system and evaluated the Smt3 conjugation system. We purified recombinant proteins of His-Smt3, GST-Uba2, GST-Aos1 and GST-Ubc9 from *E. coli* extracts. Each component was subjected to SDS-polyacrylamide gel electrophoresis and shown to be highly purified, as judged by Coomassie Brilliant Blue staining (Fig. 3A). In the presence of E1 (GST-Uba2 + GST-Aos1), E2 (Ubc9) and His-Smt3, various high molecular mass forms of His-Smt3 were detected after 90 min incubation, even in the absence of added substrates (Fig. 3B). Among these high molecular mass forms of His-Smt3, the band at 38 kDa should correspond to an Smt3-conjugate of Ubc9 (Ubc9-Smt3) because it was stained by both Smt3 and Ubc9 antibodies (Fig. 3B) and estimated by its molecular size. It should be noted that this was not a Ubc9-Smt3 intermediate formed through thioester bond formation because the samples were boiled in the presence of a reducing agent (715 mM β-mercaptoethanol). Since Ubc9 has no consensus acceptor site for sumoylation, this suggests that the substrate specificity in sumoylation is missing under certain conditions of this *in vitro* system.

The band at 34 kDa should correspond to a dimer of Smt3 (2×Smt3) because it was stained only with the anti-His antibody, and its molecular size corresponds to the size of a dimer of Smt3. As shown in Fig. 3C lanes 1–5, decreasing the amount of Ubc9 resulted in the disappearance of the Ubc9-Smt3 conjugate. In contrast, at least the dimer of Smt3 (2×Smt3) and trimer of Smt3 (3×Smt3) were still detected when Ubc9 was limiting (Fig. 3C lanes 4–5). Thus, Smt3 forms polymeric chains, as reported by others (*15*).

Identification of Polymerization Sites of Smt3—Smt3 contains three potential consensus acceptor lysine residues for sumoylation, AK^{11}PE, VK^{15}PE, and VK^{19}PE, located in the N-terminal region (Fig. 1B). To examine whether the polymeric chain formation of Smt3 occurs through these putative acceptor sites, we constructed *SMT3^{3KR}* by changing each of the three lysine residues to arginine residues and prepared His- Smt3^{3KR} (Fig. 3A). As shown in Fig. 3C lanes 6–10, no polymers of Smt3^{3KR} (2×Smt3, 3×Smt3, 4×Smt3) were detected, even under conditions where the Ubc9-Smt3 conjugate was detected (Fig. 3C lanes 6–7). It should be pointed out that Smt3^{3KR} is functional for conjugation to other targets, since Smt3^{3KR} is conjugated to Cdc3 and to Nfi1 as described below (Fig. 4 lanes 18–22 and Fig. 5 lane 6, respectively).

Next we decreased the quantity of Uba2/Aos1 starting from the reaction conditions of *lane 5* in Fig. 3C, using Smt3 (lanes 11–15) or Smt3^{3KR} (lanes 16–20). Decreasing the quantity of Uba2/Aos1 led to the disappearance of Smt3 polymers and unidentified high molecular weight conjugates (Fig. 3C, lanes11–20). Those high molecular weight bands detected in lanes 16 and 17 should correspond to certain Smt3^{3KR}-conjugates to other proteins, because Smt3^{3KR} does not polymerize. Some of these may be Smt3-conjugates of Uba2, since Uba2 is a high molecular weight protein and has consensus sites for sumoylation.

To investigate which lysine residue of Smt3 is an acceptor site for polymerization, we constructed single mutants (*SMT3^{K11R}*, *SMT3^{K15R}*, *SMT3^{K19R}*) and purified their recombinant proteins from *E. coli* lysates (Fig. 3D). As shown in Fig. 3E, Smt3^{K11R} (lane 4) and Smt3^{K19R} (lane

6) mutant proteins formed polymeric chains *in vitro*. In contrast, similar to Smt3^{3KR} (lane 3), Smt3^{K15R} *(lane 5)* did not form chains, indicating that lysine residue-15 of Smt3 is the acceptor site for polymerization.

Polymeric chain formation of Smt3 may be necessary under certain restricted conditions, since mammalian SUMO-2 and SUMO-3 form high molecular weight conjugates upon certain stresses (*28*). However, we have not yet found any conditions under which the *SMT3^{3KR}* mutant shows growth defects (data not shown).

Nfi1 Has SUMO Ligase Activity—Although it has been suggested that Nfi1 is involved in the sumoylation pathway *in vivo* (*14*, *15*), it has not been characterized in detail. To examine whether Nfi1 has SUMO ligase activity *in vitro*, we purified the T7-, His-tagged Nfi1 protein (Fig. 3A). As a test substrate, we used the same preparation of purified T7-Cdc3, as described previously (*16*). As shown in Fig. 4, increasing the amount of Nfi1 increased the amount of Smt3-conjugates of Cdc3 (Fig. 4 *lanes 8–12*). Furthermore, in the absence of Cdc3, high molecular weight bands were detected with increasing Nfi1 concentration (Fig. 4 lanes 1–6). Probably, they are Smt3-conjugates of Nfi1, as described below (Fig. 5). These results demonstrate that Nfi1 is the second SUMO ligase in budding yeast.

In the experiment shown in Fig. 4, we used conditions under which polysumoylation rarely occurs (similar conditions to those of lane 12 of Fig. 3C), but Cdc3 was efficiently modified. Furthermore, time course experiments revealed that the sumoylation of Cdc3 started earlier than polysumoylation (data not shown). Thus, the polymerization of Smt3 is kinetically slower than the conjugation to primary substrates.

As described above, the Smt3^{3KR} protein is useful for distinguishing whether multiply modified bands of Cdc3 represent multiple site modifications of Cdc3, or poly-Smt3 chain formation at a single site of Cdc3, since Smt3^{3KR} does not polymerize. As shown in Fig. 4, Cdc3 was modified with 5 to 7 molecules of Smt3^{3KR} *in vitro* (lanes 20–22), indicating that Cdc3 has at least 5 to 7 sites for sumoylation. This result is consistent with the fact that Cdc3 contains 7 potential consensus acceptor lysine residues for sumoylation (K-4, K-11, K-30, K-63, K-287, K-426, K-437).

Nfi1 Is Sumoylated In Vitro—Current studies suggest that PIAS stimulates sumoylation of target proteins and is also a good substrate for sumoylation *in vitro* (*29*). In fact, T7-Nfi1 was sumoylated *in vitro*, without additional substrate proteins (Fig. 5). After incubation, a new band corresponding to Nfi1 modified with Smt3 was detected in the complete reaction mixture (Fig. 5, lane 3). When GST-Smt3 was used in place of untagged Smt3, new bands of larger size were detected in lane 4. The size difference between these major bands corresponds to the size of GST. This indicates that Nfi1 not only stimulates the transfer of Smt3 to target proteins, but also to itself. Furthermore, when the His-Smt3^{3KR} mutant protein was added instead of His-Smt3 (lanes 5 and 6), the multiple bands did not disappear, suggesting that Nfi1 has multiple sites for sumoylation. This is consistent with the fact that Nfi1 possesses at least three consensus acceptor sequences for sumoylation (Fig. 1A).

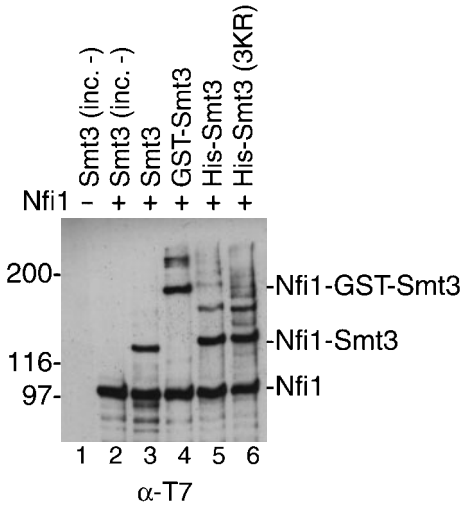

Fig. 5. **Nfi1 is modified with Smt3.** Reaction mixtures of 2.2 μg GST-Uba2, 1.3 μg GST-Aos1, 0.16 μg Ubc9 and 2.8 μg Nfi1, together with 3 μg Smt3 (lanes 1–3), His-Smt3 (lane 5), His-Smt3^{3KR} (lane 6), or 6μg of GST-Smt3 (lane 4) were incubated in the presence of 10 mM ATP at 37°C for 0 min (lanes 1–2), or 90 min (lanes 3–6), and subjected to immunoblotting. Nfi1 was probed with anti-T7.

The C-Terminal Domain of Ull1 Is Required for Bud-Neck Localization—We previously showed that Ull1 has an E3 activity for Cdc3 sumoylation *in vivo* and *in vitro* (*14*, *16*), and the *ull1^{C377S}* mutant, which contains a mutation in the RING-like domain, does not stimulate the sumoylation of Cdc3 *in vivo* [(*14*) and Fig. 6B]. However, this GFP-fusion protein of Ull1^{C377S} lacking the E3 activity was still able to localize at the bud-neck, although the frequency was lower than that of the wild-type protein (Fig. 6A), suggesting that its inability to promote the sumoylation of Cdc3 *in vivo* is not due to a loss of bud-neck localization. In contrast, we unexpectedly found that another type of *ull1* mutant, *ull1$^{\Delta 440}$*-GFP lacking the C-terminal 440 a.a., did not promote the sumoylation of Cdc3 *in vivo* (Fig. 6B), and was localized in the nucleus instead of the bud-neck (Fig. 6A). When we fused the C-terminal half of the Ull1 (466th to 904th) lacking the RING-like domain to GFP and expressed from its own or even *GAL1* promoter, the GFP-Ull1-C-terminus was not detected at the bud neck (data not shown). These results suggest that the C-terminal half of Ull1 is required, but not sufficient for bud neck localization.

To examine whether the C-terminal truncated form of Ull1 promotes the sumoylation of Cdc3 *in vitro*, we purified the mutant protein, T7-, His-Ull1$^{\Delta C440}$ (Fig. 3A). The addition of Ull1$^{\Delta C440}$ increased the amount of Cdc3 that was sumoylated (Fig. 6C), indicating that Ull1$^{\Delta C440}$ has a SUMO ligase activity. Thus the inability of Ull1$^{\Delta C440}$ to promote the sumoylation of Cdc3 *in vivo* may be due to its loss of bud-neck localization.

Regulation of SUMO Ligases—Ull1 localizes to the nucleus at an early phase of the cell cycle, but is mainly localized at the bud-neck in M-phase. In contrast, Nfi1 is always localized in the nucleus (data not shown). Thus, the specific binding of these SUMO ligases to substrates must be restricted by their localization.

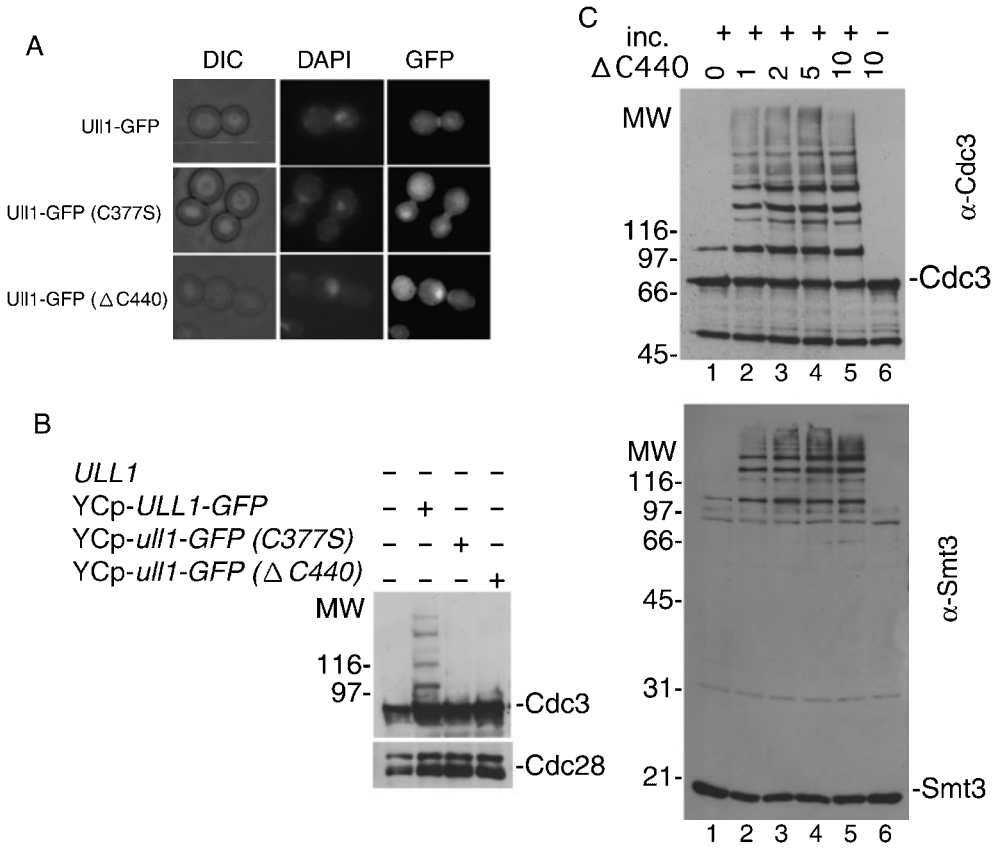

Fig. 6. **The C-terminal truncated form of Ull1 retains its SUMO ligase activity *in vitro*.** A, the C-terminal truncated form of Ull1 localizes in the nucleus. Cells expressing Ull1-GFP, Ull1^{C377S}-GFP or Ull1$^{\Delta C440}$-GFP were fixed with 5% formaldehyde, and samples were examined under a fluorescence microscope for GFP. Coincident DAPI staining is shown. B, the C-terminal 440 a.a. of Ull1 are required for the sumoylation of Cdc3 *in vivo*. The *ull1* disruptant (T-20) expressing HA-tagged Cdc3 was transformed with a low-copy plasmid vector pTS910CU, pT-23 (*ULL1-GFP*), pT-80 (*ull1^{C377S}-GFP*) or pT-81 (*ull1$^{\Delta C440}$-GFP*). Cell extracts of those transformants were prepared and subjected to immunoblotting. HA-Cdc3 was detected with anti-HA. Equal loading of each sample was confirmed by anti-PSTAIRE staining (lower panel). C, *in vitro* conjugation activity of the C-terminal truncated form of Ull1. Reaction mixtures of 2.2 μg GST-Uba2, 1.3 μg GST-Aos1, 0.16 μg Ubc9, 2.4 μg Cdc3 and 3μg His-Smt3 were incubated with various amounts of Ull1$^{\Delta C440}$ in the presence of 10 mM ATP at 37°C for 90 min (lanes 1–5) or 0 min (*lane 6*), and subjected to immunoblotting. Smt3 and Cdc3 were probed with anti-Smt3 and anti-Cdc3, respectively. Lane 1, no Ull1$^{\Delta C440}$; lane 2, 0.25 μg Ull1$^{\Delta C440}$; lane 3, 0.5 μg Ull1$^{\Delta C440}$; lane 4, 1.2 μg Ull1$^{\Delta C440}$; lanes 5 and 6, 2.5 μg Ull1$^{\Delta C440}$. Numbers indicate the amount of Ull1$^{\Delta C440}$ in arbitrary units.

Even though Nfi1 does not promote the sumoylation of Cdc3 *in vivo* (*14*), Nfi1 bound to both Cdc3 and Ubc9 in the two-hybrid assay (Fig. 2A), the RING-like domain of Nfi1 interacted directly with Cdc3, as well as Ubc9 (Fig. 2B), and Nfi1 stimulated the sumoylation of Cdc3 *in vitro* (Fig. 4). Furthermore, Ull1$^{\Delta C440}$ could catalyze the sumoylation of Cdc3 *in vitro*, even though Ull1$^{\Delta C440}$ and Cdc3 do not interact with each other *in vivo* (Fig. 6). Taken together, these results suggest that the RING-like domains of both Ull1 and Nfi1 are core elements as SUMO ligases in the interaction with both Ubc9 and Cdc3. Furthermore, these results suggest that the budding yeast PIAS-type SUMO ligases are regulated by some additional factors that do not solely regulate the conjugation activity.

Among the proteins in the *in vitro* system, Cdc3 seems to be sumoylated most efficiently. Nfi1 was autosumoylated and Smt3 polymerized *in vitro*. In contrast, it was difficult to detect Nfi1 modified with Smt3 or Smt3-polymers *in vivo*. These results suggest that free molecules of Smt3 and/or enzymes in the sumoylation pathway are limiting *in vivo*, or that the substrate specificity of sumoylation is low in the *in vitro* system.

Sumoylation Versus Ubiquitination—Several findings have suggested that there are similarities between ubiquitination and sumoylation. On the other hand, some important aspects are very specific to sumoylation. For example, the typical acceptor sites for sumoylation are specific consensus sequences (ψKXE). In contrast, no consensus attachment sites have been identified for ubiquitination. For example, the anaphase promoting complex specifically recognizes the destruction box of its substrate, but its acceptor lysine residues are not known. Furthermore, *in vitro* sumoylation occurs without SUMO ligases when higher concentration of Ubc9 are used, suggesting that Ubc9 has an intrinsic activity for substrate recognition. These results support a notion that PIAS-type proteins stimulate sumoylation as bridging factors between Ubc9 and its consensus acceptor sites on target proteins.

Further studies of which domains in each factor are important for specificity or regulation and what new factors are involved in this conjugation system will lead to an understandings of the precise mechanism of this system in the future.

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