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An E3-like Factor that Promotes SUMO Conjugation to the Yeast Septins

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Summary

Covalent attachment of the ubiquitin-related protein SUMO to other proteins participates in many processes including signal transduction, transcriptional regulation, and growth control. We report the characterization of Siz1 as an E3-like factor in the SUMO pathway. Siz1 is required for SUMO attachment to the *S. cerevisiae* septins in vivo and strongly stimulates septin sumoylation in vitro. Siz1 and the related protein Siz2 promote SUMO conjugation to different substrates at different stages of the cell cycle and, together, are required for most SUMO conjugation in yeast. Siz1, Siz2, and the PIAS (protein inhibitor of activated STAT) proteins form a conserved family defined by an unusual RING-related motif. Our results suggest that this family functions by promoting SUMO conjugation to specific substrates.

Introduction

SUMO is a conserved ubiquitin-related protein that functions by being covalently attached to other proteins as a posttranslational modification. Substrates for SUMO attachment include proteins participating in transcriptional regulation, signal transduction, inflammation, and control of cell growth (Melchior, 2000). The set of known sumoylated proteins, which includes p53 and c-*jun*, is growing rapidly, suggesting that sumoylation may be a widespread mechanism for controlling protein activity.

SUMO is 18% identical to ubiquitin (Ub), a highly conserved, 76 residue protein that targets proteins for proteasome-dependent proteolysis, participates in endocytosis, and serves several other functions (Hershko and Ciechanover, 1998). Ub usually becomes attached to its substrates via an isopeptide bond between the C terminus of Ub and the ϵ-amino group of an internal Lys residue in the substrate. Proteins to be degraded by the proteasome are linked to a multiubiquitin chain, in which successive copies of Ub are attached to an internal Lys in the previous Ub in the chain. SUMO is also attached to substrates at internal Lys residues, but it does not target proteins for proteasome-dependent proteolysis. Instead, SUMO appears to modulate the ability of substrates to interact with other proteins. For example, SUMO attachment targets RanGAP1 to the nuclear pore complex by promoting binding to RanBP2/Nup358 (Mahajan et al., 1997). In contrast, SUMO blocks ubiquitylation and degradation of the inflammatory response regulatory protein IκBα by being attached at its major ubiquitylation site (Desterro et al., 1998).

S. cerevisiae SUMO was first isolated as a dosage suppressor of mutants in *mif2*, which encodes a centromere binding protein (Meluh and Koshland, 1995). SUMO is conjugated to many different *S. cerevisiae* proteins, most of which are unknown, and the pattern of conjugates changes during the cell cycle and under different growth conditions (Li and Hochstrasser, 1999). *S. cerevisiae* cells incapable of SUMO conjugation are inviable, and conditional mutants arrest in the cell cycle in G_2/M, with fully replicated DNA but an undivided nucleus (Li and Hochstrasser, 1999; Seufert et al., 1995).

The most abundant SUMO conjugates in G_2/M-arrested *S. cerevisiae* cells are members of the septin family of cytoskeletal proteins (Johnson and Blobel, 1999; Takahashi et al., 1999). Septins are the central components of a belt of 10 nm filaments encircling the *S. cerevisiae* bud neck and are required for normal bud growth and for cytokinesis (Longtine et al., 1996). Septins are present at the bud neck throughout the cell cycle: a septin-containing ring forms in the mother cell during G_1, and as the bud emerges, the ring extends into the bud, forming a continuous hourglass-shaped structure. Yeast express five septins during vegetative growth: Cdc3, Cdc10, Cdc11, Cdc12, and Sep7/Shs1. The soluble form of the septins is a heteromultimer with an approximate stoichiometry of two each of the Cdc3, Cdc10, and Cdc12 polypeptides and one Cdc11 polypeptide (Frazier et al., 1998). Sep7 is not tightly associated. Purified septins assemble into linear filaments in vitro, and the present model for the structure of the neck ring is that it consists of parallel septin filaments traversing the bud neck parallel to the mother-bud axis (Field et al., 1996).

There are four major sumoylation-site Lys residues in Cdc3, one in Cdc11, and two in Sep7, all within the consensus sequence (IVL)KX(ED) (Johnson and Blobel, 1999), which is also found at SUMO attachment sites in mammalian proteins. A mutant lacking all these Lys residues accumulates septin rings from previous cell cycles, but it grows well, indicating that the cell cycle arrests in SUMO conjugation mutants do not arise from a deficiency in septin sumoylation. SUMO attachment to septins is intricately regulated, occurring only on the mother cell side of the bud neck and exclusively during mitosis, with conjugates appearing shortly before anaphase onset and disappearing suddenly at cytokinesis.

The SUMO and Ub conjugation pathways are distinct but share multiple similarities. Ub conjugation is carried out in three enzymatic steps, catalyzed by a Ub-activating enzyme (E1), one of a family of Ub-conjugating enzymes (E2s), and a Ub-protein ligase (E3) (Hershko and Ciechanover, 1998). The Ub-substrate isopeptide bond can also be cleaved by a family of deubiquitylating enzymes (Dubs). The E1 for SUMO is a heterodimer of Aos1 and Uba2, proteins with sequence similarity to the N and C termini, respectively, of Ub E1s (Johnson et al., 1997; Okuma et al., 1999). The SUMO pathway E2 is Ubc9, a protein with strong sequence similarity to Ub

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E2s (Desterro et al., 1997; Johnson and Blobel, 1997). There are also specific proteases that cleave SUMO-substrate isopeptide bonds, which are encoded by the yeast *ULP1* and *ULP2/SMT4* genes (Li and Hochstrasser, 1999, 2000; Schwienhorst et al., 2000). *UBA2*, *AOS1*, *UBC9*, and *ULP1* are all essential genes, and conditional alleles display cell cycle defects.

The mechanism of regulation of SUMO conjugation has not been described for any substrate. In the Ub pathway, E3s provide substrate specificity, and it is the interaction between the substrate and the E3 that is regulated. Ubiquitylation E3s do not form a single family of similar proteins and include a wide variety of monomeric proteins as well as large multiprotein complexes. Some examples of E3s are the HECT domain proteins (Huibregtse et al., 1995), N-recognin (Kwon et al., 1998), SCF complexes (Deshaies, 1999), and the anaphase promoting complex (APC) or cyclosome (Page and Hieter, 1999). Many E3s contain, or include a subunit containing, a Zn^{2+} binding domain known as a RING domain (Joazeiro and Weissman, 2000).

We were interested in identifying proteins responsible for substrate specificity and regulation in septin sumoylation, and found Siz1, which plays an E3-like role in the SUMO pathway.

Results

SIZ1 and *SIZ2* Are Required for Most SUMO Conjugation in Yeast

To identify additional proteins involved in septin sumoylation, we examined known septin interacting proteins. One candidate, called Nfi1 (*n*eck *f*ilament *i*nteracting) had been found in a 2-hybrid screen with *CDC12* and contained a region resembling a RING domain. *NFI1* has recently been renamed *SIZ2* (*S*AP and M*iz*1 domains) (Strunnikov et al., 2001). A *siz2*Δ strain contained wild-type levels of SUMO-septin conjugates (Figure 1A) but lacked an unidentified ~45 kDa SUMO conjugate, prompting us to delete a related gene, *SIZ1* (Strunnikov et al., 2001). Deleting *SIZ1* eliminated SUMO-septin conjugates as well as virtually all SUMO conjugates in nocodazole-arrested cells. The *siz1*Δ strain also contained dramatically reduced levels of SUMO conjugates in logarithmically growing cells. The remaining conjugates were mostly *SIZ2*-dependent, as deletion of both *SIZ1* and *SIZ2* resulted in further reduction of SUMO conjugation. However, *siz1*Δ *siz2*Δ cells did contain low levels of SUMO conjugates, indicating that not all yeast SUMO conjugation is *SIZ1* and *SIZ2* dependent.

Siz1 and Siz2 are 904 and 726 amino acid proteins, respectively, and are 42% identical to each other over an ~470 residue N-terminal domain (Figure 1B). Both proteins have C-terminal extensions with no similarity to each other or to other known proteins. There are no other similar genes in *S. cerevisiae*, but other eukaryotes contain related proteins. Many of these are members of the PIAS (protein inhibitor of activated STAT) family, which were identified based on their ability to bind STAT transcription factors and prevent STAT-activated transcription (Shuai, 2000). One striking feature of this family is a conserved domain related to a C3HC4 RING domain (Figure 1C), which shows substantial similarity to genuine RING domains (~38% identity to the BRCA1 RING), but which lacks two of the absolutely conserved cysteine residues involved in Zn^{2+} chelation. There is also significant similarity between the Sizs and PIASs outside the RING-related domain (~25% identity over ~400 residues), extending through most of the N-terminal region of Siz1 and Siz2. The PIAS proteins contain an "SXS" motif, which promotes binding to SUMO in the yeast 2-hybrid assay (Minty et al., 2000), and the Sizs contain a related motif at a similar position. The Sizs and most PIASs also contain regions that conform to the consensus sequence for the SAP domain, a motif implicated in chromatin organization (Aravind and Koonin, 2000).

Despite the striking reduction in SUMO conjugation, the *siz1*Δ *siz2*Δ mutant was viable, in contrast to mutants in other genes of the SUMO pathway. *SMT3* (the gene encoding SUMO) and *UBA2* were still essential in the *siz1*Δ *siz2*Δ mutant (not shown), indicating that the residual conjugation in this mutant carries out SUMO's essential functions. When the *siz1*Δ, *siz2*Δ, and *siz1*Δ *siz2*Δ mutants were assessed for sensitivity to various stress conditions, the only notable effect was that the *siz1*Δ *siz2*Δ strain showed reduced plating efficiency, most strikingly at lower temperatures (Figure 2A). Its plating efficiency was 51% at 37°C, 15% at 30°C, and 8% at 20°C, compared to 86%, 81%, and 76% for wt. With this exception and taking into account the consistent growth defect in the double mutant, none of these strains were hypersensitive to either high or low temperatures, to DNA damaging agents (UV light or methyl methane sulfonate), to hydroxyurea, to caffeine, or to the microtubule destabilizing drug thiabendazole (not shown). They mated efficiently, and the single mutants sporulated with the same efficiency as wt to produce viable segregants; the double mutant was not tested for sporulation (not shown).

Microscopic examination revealed that *siz1*Δ *siz2*Δ cultures contained many enlarged large-budded cells with nuclei in the early stages of nuclear division (Figure 2B), much like conditional mutants in the essential components of the SUMO pathway. To determine whether this resulted from a cell cycle delay in all cells or from an arrest or prolonged delay in a subpopulation, we examined cell cycle progression in α-factor synchronized cultures (Figure 2C). After 3 hr in α-factor, the vast majority of wt cells arrested in G_1. Sixty minutes after release from α-factor arrest, almost all of these cells had completed DNA replication. At this point, α-factor was readded to prevent a second cell cycle. By 90 min, many cells had undergone cytokinesis, and over the next 60–90 min, virtually all cells divided and rearrested in G_1. In contrast, the *siz1*Δ *siz2*Δ mutant contained a large population of cells with 2C DNA content even after 3 hr in α-factor, although other cells arrested and formed mating projections as wt (not shown). Like wt, by 60 min, virtually all G_1-arrested mutant cells had completed replication. Strikingly, many of these cells underwent cytokinesis and progressed to G_1 at the same rate as wt, while other cells remained arrested with 2C DNA content. When large-budded *siz1*Δ *siz2*Δ cells from an α-factor-arrested culture were picked with a micromanipulator and incubated at 36°C, 85% divided at least once, and 30% formed viable colonies (not shown), indicating that most of these cells are competent to com-

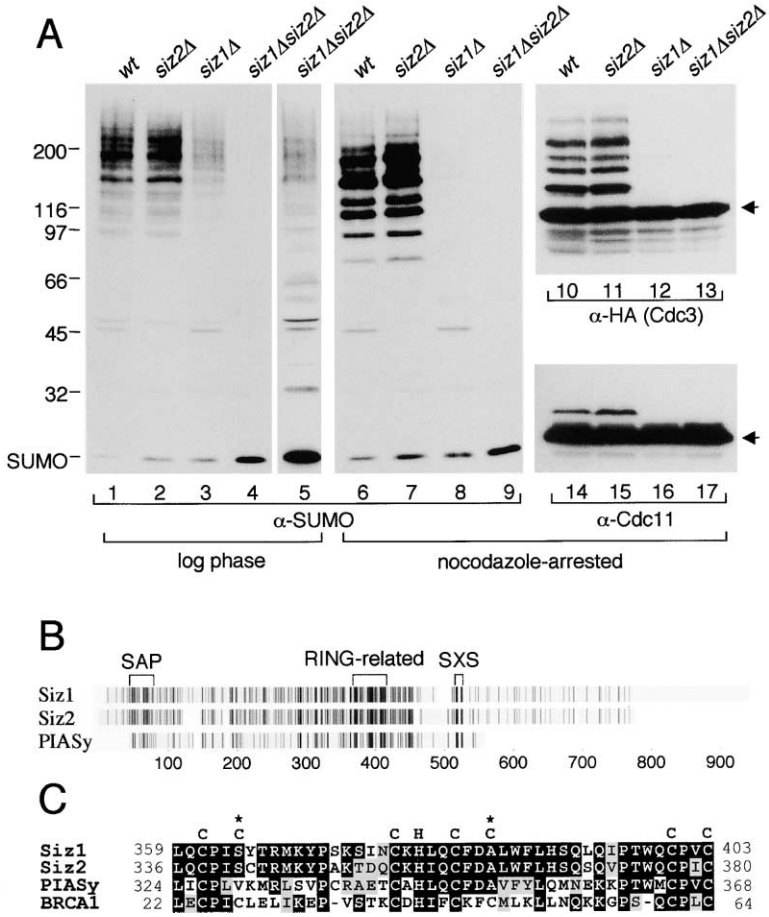

Figure 1. *SIZ1* and *SIZ2* Promote SUMO Conjugation In Vivo

(A) Whole-cell lysates from log phase (lanes 1–5) or nocodazole-arrested (lanes 6–17) cells expressing Cdc3-HA were analyzed by SDS-PAGE and immunoblotting with Abs against SUMO (lanes 1–9), the HA epitope (lanes 10–13), or Cdc11 (lanes 14–17). Genotypes are indicated over the lanes. Lane 5 is an overexposure of lane 4. Arrows indicate unmodified Cdc3-HA (lanes 10–13) and Cdc11 (lanes 14–17).

(B) Alignment of Siz1, Siz2, and human PIASy. Gray lines indicate identities between at least two proteins and black lines among all three. The SAP domain, RING-related domain, and SXS-like motif are indicated.

(C) Alignment of the RING-related motifs in Siz1, Siz2, and PIASy to RING domain from BRCA1. Residues identical in at least two proteins are shaded black, and conserved residues gray. The conserved Zn^{2+}-chelating residues are indicated. Asterisks mark residues missing in the Siz family.

plete mitosis and undergo further cell divisions. Thus, some *siz1*Δ *siz2*Δ cells proceed through the cell cycle at wild-type rates, while others enter a prolonged delay or arrest. Another indication of the heterogeneity of *siz1*Δ *siz2*Δ cells was that the colonies formed at 20°C varied dramatically, with some nearly wt-sized colonies containing mostly normal-looking cells but with sectors of abnormal cells, and other very small but visible colonies containing a few hundred enlarged, inviable cells (Figure 2A). This pattern of growth, along with the plating efficiency data, suggested that certain cells contained a heritable defect such that if they were grown at 20°C, their lineage would grow for a while, but ultimately die out; whereas if they were grown at 36°C, the defect

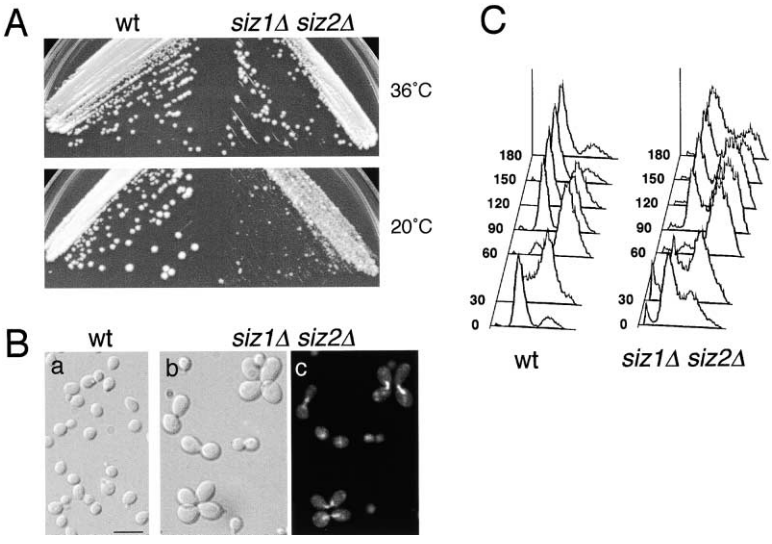

Figure 2. *siz1*Δ *siz2*Δ Mutants Are Cold Sensitive and Accumulate in G_2/M

(A) wt and *siz1*Δ *siz2*Δ cells were incubated on YPD plates at 20°C for 4 days or 36°C for 2 days.

(B) Microscopy of wt (a) or *siz1*Δ *siz2*Δ (b and c) cells grown at 30°C using Nomarski optics (a and b) or DAPI staining (c). Bar, 10 μm.

(C) Flow cytometry of propidium iodide stained cells from a timecourse after release from α-factor arrest of wt or *siz1*Δ *siz2*Δ strains. Cells were preincubated in α-factor for three hours at 30°C. At 0 min, α-factor was washed out, and incubation at 30°C continued with time points taken every 30 min. At 60 min, α-factor was readded to prevent a second cell cycle.

would be repaired and the lineage would continue. One possible source of such an effect could be a defect in telomere maintenance, as is seen in *S. pombe* SUMO mutants (Tanaka et al., 1999). However, telomeres in the *siz1*Δ *siz2*Δ strain were the same length as in wt cells (not shown).

We have previously observed that cells lacking the SUMO attachment sites on their septins accumulate remnants of septin rings from previous cell divisions (Johnson and Blobel, 1999). However, we were unable to observe old septin rings in *siz1*Δ cells by immunofluorescence microscopy against either HA-tagged Cdc3 or Cdc11. The *cdc3* attachment site mutant is also synthetically lethal with a *cdc12-1* mutant, while the *siz1*Δ *cdc12-1* strain was viable. These results suggest that the Lys to Arg mutations at the SUMO attachment sites in the septins also affect some process other than SUMO conjugation.

Cell Cycle-Dependent Bud Neck Localization and Phosphorylation of Siz1

To determine Siz1's subcellular localization, Siz1 was C-terminally tagged with enhanced green fluorescence protein (GFP). This fusion was functional and restored septin sumoylation in a *siz1*Δ strain. Siz1-GFP localized both to the bud neck and to the nucleus, where its distribution was somewhat punctate (Figure 3A). Bud neck localized Siz1-GFP was observed exclusively in large-budded cells, never in unbudded or small-budded cells. Nocodazole-arrested cells all displayed bud neck localized Siz1-GFP, usually as a single ring colocalizing with the mother cell side of the bud neck (Figures 3B and 3C), although sometimes Siz1-GFP was present on both sides of the septin ring. These results were consistent with our previous observation that SUMO is attached only on the mother cell side of the bud neck in mitotic cells of log phase cultures but is frequently on both sides in nocodazole-arrested cells (Johnson and Blobel, 1999). Bud neck localization of Siz1 depended on the septins, as Siz1-GFP disappeared from the bud neck when nocodazole-arrested *cdc10-1* cells were shifted to the nonpermissive temperature, which results in disassembly of the septin ring (Figure 3C; not shown). However, bud neck localization of Siz1-GFP did not require Ubc9 or sumoylated septins, as it was still present in a *ubc9-Pro69Ser* mutant at the nonpermissive temperature, where the mutant Ubc9 protein is rapidly degraded (Betting and Seufert, 1996) and SUMO-septin conjugates disappeared (Figure 3C).

To ask whether Siz1 is posttranslationally modified during the cell cycle, the genomic copy of *SIZ1* was tagged with the influenza virus hemagglutinin (HA) epitope tag (Field et al., 1988). The HA-tagged version was functional, and septin sumoylation took place as in wt. When these cells were arrested with nocodazole, Siz1 shifted quantitatively into a higher molecular weight form, while cells arrested in late anaphase/telophase by a ts mutation in the mitotic exit gene *CDC15* contained approximately equal amounts of the lower and higher forms of Siz1 (Figure 4A). Treatment of Siz1 isolated from nocodazole-arrested cells with lambda protein phosphatase shifted the higher form into the lower, indicating that the higher molecular weight species was

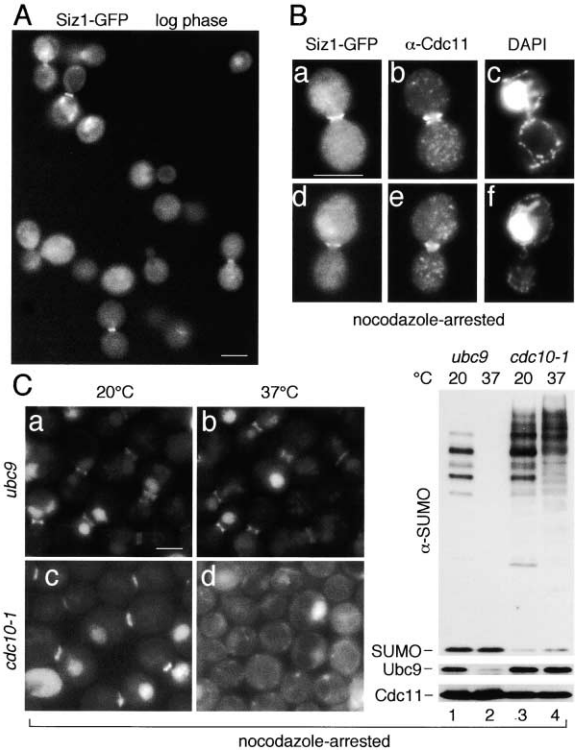

Figure 3. Cell Cycle- and Septin-Dependent Bud Neck Localization of Siz1

(A) Siz1-GFP expressed from p416-Siz1-GFP in log phase EJY333 was detected by fluorescence microscopy. Bar, 5 μm.

(B) EJY333 cells overexpressing Siz1-GFP from pSiz1-GFP were prepared for immunofluorescence microscopy and analyzed for Siz1-GFP (a and d); with an Ab against Cdc11 (b and e) or by DAPI staining (c and f). (a), (b), and (c) and (d), (e), and (f) are two different cells. Bar, 5 μm.

(C) *ubc9* ts (a and b; lanes 1 and 2) or *cdc10-1* ts (c and d; lanes 3 and 4) strains expressing Siz1-GFP from p416-Siz1-GFP were transferred from SD to YPD, grown at 20°C for 2 hr, nocodazole arrested for 3 hr at 20°C, and either held at 20°C for an additional1 hr (a and c; lanes 1 and 3) or shifted to 37°C for 1 hr (b and d; lanes 2 and 4). Siz1-GFP localization was observed by fluorescence microscopy (a–d). Bar, 5 μm. Whole-cell lysates were analyzed by SDS-PAGE and immunoblotting with Abs against SUMO (top panel), Ubc9 (middle panel), or Cdc11 (bottom panel). SUMO-Cdc11 is visible in lanes 1 and 3 on a longer exposure.

phosphorylated (Figure 4B). Thus, Siz1 phosphorylation coincided with the period during the cell cycle when Siz1 localized to the bud neck and when septin sumoylation takes place.

Reconstitution of Septin Sumoylation In Vitro

To determine whether Siz1 plays a direct role in SUMO conjugation, we reconstituted septin sumoylation in vitro using purified proteins. This task was complicated by the facts that crude yeast lysate did not conjugate SUMO to septins and that Siz1 stimulation of sumoylation was inhibited by very low concentrations of the nonionic detergent Triton X-100 or by buffering concentrations of Tris. The in vitro reaction was originally established using *E. coli*-produced SUMO, Uba2/Aos1 (E1), and Ubc9 (E2), but with tagged septins affinity purified from log phase yeast and tagged Siz1 overexpressed

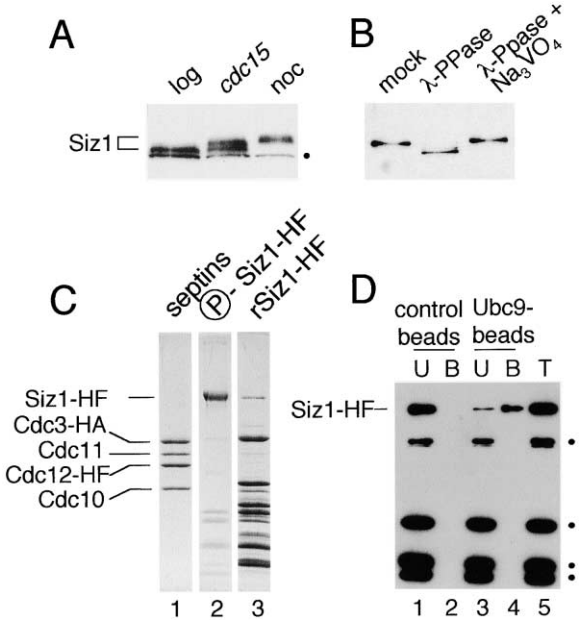

Figure 4. Siz1 Undergoes Cell Cycle-Dependent Phosphorylation and Binds Ubc9

(A) Whole-cell lysates of log phase, *cdc15*-arrested or nocodazole-arrested cells expressing HA-tagged Siz1 were analyzed by SDS-PAGE and immunoblotting with a mAb against the HA epitope. Bands containing Siz1-HA are indicated. A dot designates a band that cross-reacts with the anti-HA antibody.

(B) His_6- and FLAG-tagged Siz1 isolated from nocodazole-arrested yeast was mock treated, treated with lambda protein phosphatase (λ-PPase), or treated with λ-PPase in the presence of the phosphatase inhibitor Na_3VO_4. Products were detected by immunobloting with an Ab against the FLAG epitope.

(C) Preparations of septins (lane 1), phosphorylated His_6- and FLAG-tagged Siz1 from nocodazole-arrested yeast (lane 2), and *E. coli*-produced His_6- and FLAG-tagged Siz1 (lane 3) were analyzed by SDS-PAGE and staining with Coomassie brilliant blue. Full-length Siz1 is indicated.

(D) Whole-cell lysate from *E. coli* expressing Siz1-HF was incubated with ethanolamine-blocked Affi-gel 10 beads (lanes 1 and 2) or with Ubc9-coupled Affi-gel 10 beads (lanes 3 and 4). Unbound (lanes 1 and 3) and bound (lanes 2 and 4) Siz1-HF-derived proteins were detected by immunobloting with an Ab against the FLAG epitope. Lane 5, total protein. Full-length Siz1-HF is indicated. Dots indicate C-terminal fragments of Siz1-HF.

in and affinity purified from nocodazole-arrested yeast (Figure 4C). This Siz1 was quantitatively phosphorylated (Figure 4B). We had previously determined that no proteins copurified in stoichiometric quantities with tagged Siz1 expressed from its own promoter, indicating that it is not part of a stable complex (not shown). Ubc9 does bind Siz1 in vitro (Figure 4D), but was probably removed in the wash in this experiment.

With lower concentrations of E1 and E2 in the reaction, conjugation of SUMO to both Cdc3 and Cdc11 was strongly dependent on addition of Siz1, and increasing the concentration of Siz1 increased the proportion of the septins that were sumoylated (Figure 5A). SUMO conjugation took place primarily on the major in vivo attachment site Lys residues in Cdc3 and Cdc11 (Figure 5B), and was specific for genuine SUMO substrates, as Cdc12 was not modified, nor was BSA present as a carrier. Furthermore, formation of any SUMO conjugates depended strongly on inclusion of septins as substrates (Figure 6). However, when 10-fold more E1 and E2 were present, substantial septin sumoylation occurred in the absence of Siz1 (Figure 5A). Adding Siz1 greatly increased SUMO conjugation under these conditions as well, as almost all of the vast excess of SUMO was incorporated into higher molecular weight species (Figures 5A and 6). Surprisingly, Siz1's effect did not depend on its phosphorylation, as recombinant Siz1 isolated from *E. coli* had a similar effect. Noting the limits of this semiquantitative assay, recombinant Siz1 appeared to be approximately equally active as the yeast-expressed phosphorylated Siz1 (Figure 5A).

To prove definitively that no uncharacterized yeast proteins copurifying with the septins were affecting this reaction, it would have been preferable to replace the yeast septins with recombinant septins. Cdc3, Cdc10, Cdc11, and Cdc12 were produced in *E. coli*, but the weak E1- and E2-induced sumoylation of recombinant Cdc3 and Cdc11 was not stimulated by Siz1, even when the other septins were included in the reaction (not shown). Possible explanations are that these did not assemble correctly into the multimer capable of polymerizing or that other modifications of the septins are required for Siz1-dependent sumoylation. However, we believe it is unlikely that proteins copurifying with the yeast septins were required for stimulating sumoylation, because this septin preparation was affinity purified under very stringent conditions, including washes with buffers containing 1M NaCl or 1% Triton X-100 and 0.1% SDS. Any protein that remained bound probably would not have an off rate high enough to act catalytically in the sumoylation of more than half the Cdc3 and Cdc11 present in the reaction.

Siz1 also promoted formation of chains containing SUMO-SUMO isopeptide bonds. In reactions containing only SUMO, E1, and E2, a band of an appropriate size to be di-SUMO formed in the presence of low concentrations of E1 and E2 (Figure 6). When 10-fold more E1 and E2 were added, a ladder of bands formed. If the SUMO was reductively methylated (me-SUMO), blocking Lys residues to isopeptide bond formation, the ladder was eliminated. Instead, a single SUMO-containing product formed, which was probably an adduct of SUMO and Ubc9. Addition of recombinant Siz1 to these reactions stimulated formation of the higher molecular weight forms, especially in the presence of high E1 and E2, where virtually all of the SUMO was incorporated into higher molecular weight species, even when no septins were present. me-SUMO dramatically reduced formation of these species: the major low molecular weight bands that are likely to be di-SUMO, tri-SUMO, etc. were completely eliminated, and only a small fraction of the methylated SUMO was incorporated into high molecular weight conjugates. However, there were many high molecular weight species in these reactions that could not be explained. Addition of me-SUMO to the septin reactions also eliminated the extremely high molecular weight forms of Cdc3 and Cdc11 (Figure 5B). Cdc3 appeared to have approximately 4–7 copies of SUMO attached, close to the genuine number of SUMO attachment sites on Cdc3; Cdc11 also showed lower molecular weight conjugates, although many of these were much

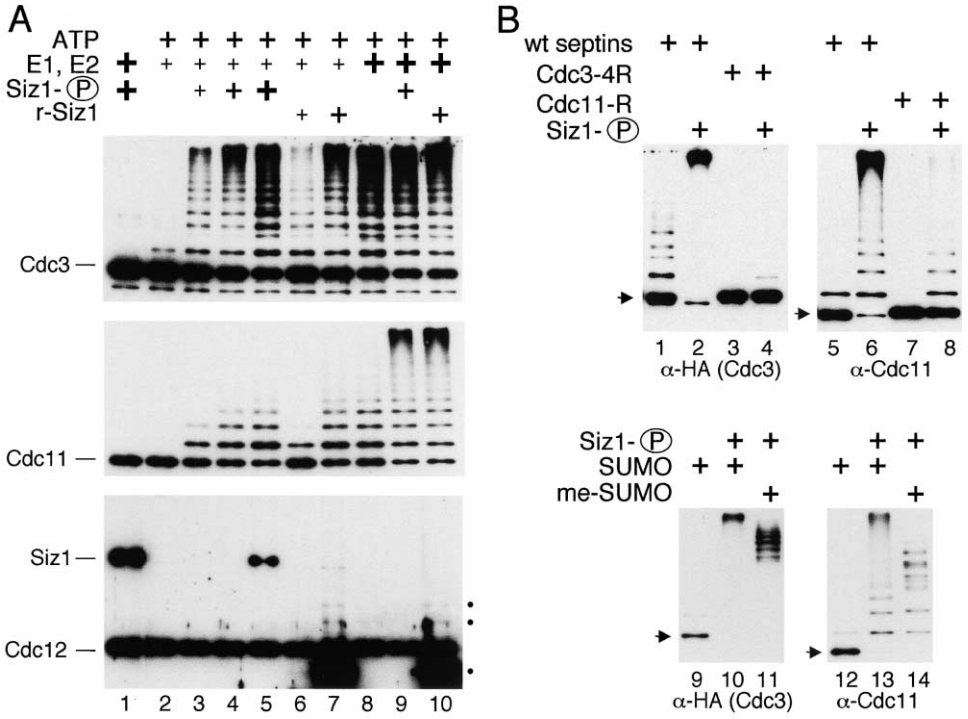

Figure 5. Reconstitution of SUMO Conjugation to the Septins In Vitro

(A) Septins and SUMO were incubated for 90 min at 30°C with 5 mM ATP (lanes 2–10), recombinant Uba2/Aos1 and Ubc9 (E1 and E2; 10-fold more in lanes 1 and 8–10 than in lanes 2–7), and varying amounts of phosphorylated Siz1 (1× in lane 3, 5× in lanes 4 and 9, and 25× in lanes 1 and 5) or recombinant Siz1 (1× in lane 6 and 5× in lanes 7 and 10). Septins and Siz1 were present in the proportions shown in Figure 4C in lanes 1 and 5 for phosphorylated Siz1 and lanes 7 and 10 for recombinant Siz1. Septins were detected by immunoblotting with Abs against HA for Cdc3-HA (top panel), against Cdc11 (middle panel), or against the FLAG epitope (lower panel). Proteins detected are indicated. Dots designate bands that contain C-terminal fragments of recombinant Siz1. Note that Cdc12 and Siz1 both bear the FLAG epitope tag. Siz1 rapidly becomes undetectable in these reactions, probably because of aggregation.

(B) Reactions contained Uba2/Aos1, Ubc9, ATP, phosphorylated Siz1 (lanes 2, 4, 6, 8, 10, 11, 13, and 14), wt septins (lanes 1, 2, 5, 6, and 9–14), septins from EJY334 expressing Cdc3-R4,11,30,63-HA (lanes 3 and 4), septins from EJY335 expressing Cdc11-R412-HA (lanes 7 and 8), SUMO (lanes 1–10, 12, and 13), or reductively methylated SUMO (me-SUMO; lanes 11 and 14). Cdc3-HA and Cdc11 were detected by immunoblotting. Bands containing unmodified Cdc3 and Cdc11 are indicated by arrows.

larger than expected given that only one to two copies are attached in vivo. These bands are also unexplained. The quantitative incorporation of Cdc3 and Cdc11 into modified forms suggests that me-SUMO acts primarily by preventing SUMO from acting as a substrate rather than by inhibiting the activation pathway. These data indicate that SUMO can be covalently attached to itself, that Siz1 stimulates this activity even in the absence of any yeast-derived proteins, and that SUMO polymerization can take place either on another protein or as a chain containing only SUMO.

Discussion

Based on our results, we propose that Siz1 plays a role analogous to that of E3s in the Ub pathway. "E3s" are defined as having three characteristics: they bind the substrate (either directly or indirectly); they bind the E2; and, most importantly, they stimulate transfer of Ub from the E2-Ub thiolester intermediate to an amide bond with either the substrate or with another Ub moiety (Hershko and Ciechanover, 1998). Siz1 did bind the E2 Ubc9, and it also clearly met this third criterion, as it stimulated transfer of SUMO both to septins and to SUMO itself. This reaction did not take place if ATP, the E1, or the E2 were absent or if the thiolester-forming Cys in Ubc9 was mutated to a Ser (not shown), demonstrating dependence on the E2 intermediate. The bonds formed were amide bonds, as in vitro septin sumoylation required the previously defined SUMO attachment site Lys residues in Cdc3 and Cdc11, and as SUMO polymerization was dramatically inhibited by reductive methylation of SUMO.

Siz1 also interacted with the septins, at least indirectly. Siz1 showed septin-dependent localization to the bud neck, and this localization coincided exactly with the timing and location of SUMO attachment. Siz1 also showed the genetic hallmark of an E3 with respect to substrate specificity: it was required for sumoylation of some substrates (the septins), but not of others (the 45 kDa Siz2-dependent conjugate). We were unable to demonstrate direct binding between Siz1 and the septin complex, in part because neither purified septins nor purified Siz1 was stable enough in solution to be used in the soluble phase. However, the in vivo interaction between Siz1 and the septins is likely to involve other factors, acting either positively or negatively, as Siz1 localized preferentially only to one side of the bud neck,

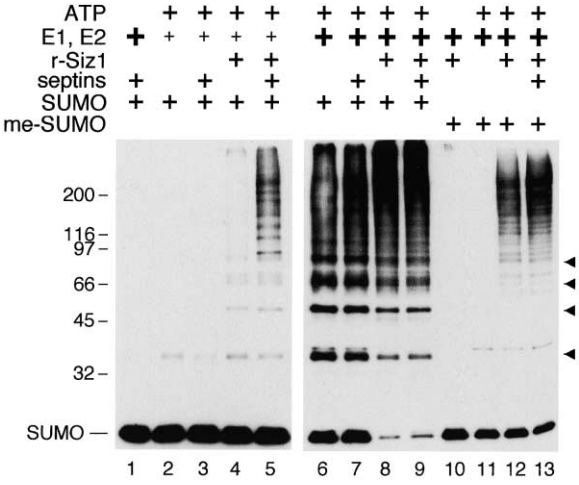

Figure 6. Siz1 Promotes Polymerization of SUMO

Reactions contained SUMO (lanes 1–9) or reductively methylated SUMO (lanes 10–13), varying amounts of Uba2/Aos2 and Ubc9 (E1 and E2; 10-fold more in lanes 1 and 6–13 than in lanes 2–5), phosphorylated Siz1 (lane 1), recombinant Siz1 (lanes 4, 5, 8–10, 12, and 13), septins (lanes 1, 3, 5, 7, 9, and 13) and 5 mM ATP (lanes 2–13). SUMO was detected by immunoblotting. Reactions in lanes 1, 3, 5, 7, and 9 are the same as in Figure 5A, lanes 1, 2, 7, 8, and 10, respectively. The band containing free SUMO is indicated. Arrows designate bands that are likely to be di-SUMO, tri-SUMO, etc.

while septins, obviously, were present on both. Also, only very low levels of Siz1 appeared to bind to the bud neck. A highly overexpressed Siz1-GFP fusion was quite difficult to detect at the bud neck (Figure 3B), while Cdc12-GFP expressed from the same promoter in the same plasmid is very bright (Johnson and Blobel, 1999). Overexpression of Siz1 also did not increase the proportion of septins sumoylated in vivo, even when SUMO was cooverexpressed, and despite the fact that Siz1 was quantitatively phosphorylated (not shown). This may be because another enzyme of the SUMO pathway was limiting, because overexpressed Siz1 formed aggregates, or it could be because some other factor limits the amount of Siz1 that localizes at the bud neck. Many proteins show septin-dependent bud neck localization, some only to one side or the other of the bud neck, and one of these could either prevent or promote Siz1 binding in vivo.

Thus, Siz1 fulfills all three aspects of this definition of an E3. However, an E3-like activity for SUMO is somewhat different from that for Ub because the E2 Ubc9 binds directly to at least some substrates, in interactions involving the sumoylation site consensus sequence (Sampson et al., 2001) or other sequences in the substrate (Buschmann et al., 2001). Purified E1 and Ubc9 alone attach SUMO to RanGAP1 (Okuma et al., 1999; Sampson et al., 2001), IκBα (Desterro et al., 1999), and to the septins (Figure 5) in vitro, and these reactions are specific for the correct sumoylation sites. In contrast, no consensus attachment sites have been identified for ubiquitylation, which can often take place on any one of a number of Lys residues in the substrate. There is also no evidence that Ub E2s engage in E3-independent binding or ubiquitylation of substrates in vivo. However, some Ub E2s can attach Ub to other proteins in vitro in the absence of E3s, and some are also capable of E3-independent formation of Ub chains with branch sites at specific Lys residues (Chen et al., 1991). Thus, this difference between the SUMO and Ub pathways appears to be more in the degree of specificity provided by the E2 rather than a fundamental difference in E2 activity. Siz1 would only differ fundamentally from Ub E3s if Ubc9 provided all the substrate specificity, leaving Siz1 to stimulate transfer of SUMO to any substrate selected by Ubc9. This seems unlikely, as Siz1's localization to the bud neck is independent of Ubc9 and as Siz1 stimulates SUMO attachment to some, not all, substrates in vivo.

The observation that Siz1 localized to the mother cell side of the bud neck exclusively during mitosis, coinciding with the place and time that septins are sumoylated, suggested that Siz1 may participate in the cell cycle and mother-bud regulation of this modification. One model for how Siz1 could participate is that the mitosis-specific phosphorylation might promote Siz1's association with some feature of the mother cell side of the bud neck, thereby stimulating SUMO conjugation only in this zone at this point in the cell cycle.

Another issue is whether chains of SUMO, analogous to the chains formed by Ub, form in vivo as they did in vitro. Formation of these chains depended on the concentrations of SUMO, E1, and E2, and when any of these were limited, especially SUMO, chain formation was dramatically reduced (not shown). In vivo, there is very little free unconjugated SUMO (Johnson et al., 1997), which would probably limit the formation of chains. A SUMO-SUMO branch site has recently been identified in the N-terminal domain of mammalian SUMO-2 (Tatham et al., 2001), and there is a repeat with a related sequence in yeast SUMO. Identifying and mutating the branch site should clarify the physiological role of SUMO polymerization.

The phenotype of the *siz1*Δ mutant was consistent with that of the mutant lacking sumoylation sites on the septins, insofar as both grew well and had few obvious phenotypes. The *siz2*Δ strain was also without striking phenotypes, but the *siz1*Δ *siz2*Δ strain grew poorly, suggesting that *SIZ1* and *SIZ2* act redundantly with regard to the substrate(s) involved in the growth defect. A recent paper states that the *siz1*Δ *siz2*Δ mutant is inviable (Strunnikov et al., 2001), but it is likely that this resulted from incubating tetrad dissections at 23°C, where most *siz1*Δ *siz2*Δ cells do not form viable colonies. It is also possible that a strain difference is responsible.

Another question raised by our results is whether there are other E3-like factors for SUMO in yeast that are unrelated to Siz1 and Siz2, or whether Ubc9 carries out a low level of sumoylation unassisted by an E3 in vivo. While there is no way to prove or disprove the existence of another E3 except by identifying it, the ability of Ubc9 to bind directly to some substrates suggests Ubc9 may carry out E3-independent sumoylation. However, in yeast, the majority of SUMO conjugation required *SIZ1* or *SIZ2*, and dependence on other factors is likely to be common in vivo in other organisms as well. The sequence similarity between the Sizs and proteins of the PIAS family suggests that this whole family may be involved in SUMO conjugation. The PIAS pro-

Table 1. *S. cerevisiae* Strains

Name	Relevant genotype	Source
YWO102	*MATα ubc9Δ::TRP1 leu2::ubc9Pro-Ser::LEU2*	Seufert et al., 1995
JD52	*MATa trp1-Δ1 ura3-52 his3-Δ200 leu2-3,112 lys2-801*	J. Dohmen
EJY324	*MATa siz2Δ::TRP1*	This study
EJY325	*MATa siz1Δ::LEU2*	This study
EJY326	*MATa siz1Δ::LEU2 siz2Δ::TRP1*	This study
EJY301	*MATa CDC3-HA::HIS3*	Johnson and Blobel, 1999
EJY327	*MATa CDC3-HA::HIS3 siz2Δ::TRP1*	This study
EJY328	*MATa CDC3-HA::HIS3 siz1Δ::LEU2*	This study
EJY329	*MATa CDC3-HA::HIS3 siz1Δ::LEU2 siz2Δ::TRP1*	This study
EJY330	*MATa SIZ1-HA::HIS3*	This study
EJY331	*MATa SIZ1-HA::HIS3 cdc15-2::URA3*	This study
EJY332	*MATa CDC3-HA::HIS3 CDC12-HF::TRP1*	This study
EJY333	*MATa/MATα siz1Δ::TRP1/siz1Δ::TRP1*	This study
EJY334	*MATa cdc3-R4,11,30,63-HA::TRP1 CDC12-FLAG::LEU2*	This study
EJY335	*MATa cdc11-R412-HA::HIS3 CDC12-FLAG::LEU2*	This study
EJY336	*MATα cdc10-1::TRP1*	This study

teins were isolated via their ability to bind to STAT transcriptional activators, but there is no evidence that STAT proteins are sumoylated. However, one member of this family, ARIP3, was isolated through its interaction with the androgen receptor (Moilanen et al., 1999), which is sumoylated (Poukka et al., 2000). The role of the PIAS family in mammalian SUMO conjugation is an intriguing area for future research.

Experimental Procedures

Media and Genetic Techniques

Standard techniques were used (Ausubel et al., 2000). Rich yeast media, containing either 2% glucose (YPD) or 2% raffinose (YPR), and synthetic yeast media (SD) were prepared as described (Sherman et al., 1986). Cell cycle arrests were induced by incubating for 3 hr with 10 μM α-factor (Sigma) or 15 μg/ml nocodazole (Acros).

Plasmids and Yeast Strain Constructions

Plasmids for expressing His_6-tagged Uba2 and Ubc9 in *E. coli* have been described (Johnson and Blobel, 1997; Johnson et al., 1997). His_6-tagged *AOS1* was transferred to pET28a (Novagen), so that Uba2 and Aos1 could be coexpressed. A pET21b-based plasmid expressed His_6-tagged Smt3 (SUMO) bearing the N-terminal extension MASMHHHHHH. A pET21a-based plasmid expressed Siz1 bearing the FLAG epitope and His_6-containing C-terminal extension GDYKDDDDKRGSHHHHHHHH. p424GAL-Siz1-HF was a p424GAL1-based (Mumberg et al., 1994) plasmid for galactose-inducible expression of the same tagged version of Siz1 in yeast. pSiz1-GFP is a pYX242-GFP-based (Rosenblum et al., 1998) plasmid expressing Siz1 with a C-terminal enhanced GFP tag from the *TPI1* promoter. p416-Siz1-GFP expressed the same fusion from the *SIZ1* promoter. Construction details available on request.

S. cerevisiae strains used are listed in Table 1. All strains are derivatives of JD51 (Dohmen et al., 1995) except for YWO102, derived from DF5 (Seufert et al., 1995), and EJY333, which was made from the W303-derived (Thomas and Rothstein, 1989) *ade3* mutant strains W1536-5B and W1536-8B, generous gifts of Dr. Rodney Rothstein. Strains were constructed as described (Johnson and Blobel, 1999). Construction details and oligonucleotide sequences are available on request. The HA epitope-containing sequence on *CDC3* and *SIZ1* encodes the C-terminal extension GYPYDVPDYAAFL. The FLAG and His_8-containing sequence on Cdc12 (*CDC12-HF*) is the same as on Siz1-HF. The FLAG sequence on Cdc12 in EJY334 and EJY335 encodes DYKDDDDK.

Antibodies and Immunoblot Analyses

Yeast whole-cell lysates were prepared as in Yaffe and Schatz (1984) and subjected to immunoblotting followed by chemiluminescent detection (Johnson and Blobel, 1999). Antibodies were an affinity-purified rabbit polyclonal Ab against Smt3 (SUMO) (Johnson and Blobel, 1999), the 16B12 mAb against the HA epitope (BAbCO), the M2 mAB against the FLAG epitope (IBI/Kodak), a rabbit polyclonal Ab against Cdc11 (Santa Cruz Biotechnology), and a goat polyclonal Ab against Ubc9 (yC-19) (Santa Cruz Biotechnology).

Affinity Purification of Epitope-Tagged Proteins

His_6-SUMO, Ubc9-His_6, and His_6-Aos1/Uba2-His_6 were expressed in *E. coli* and purified by Ni-NTA affinity chromatography as described (Johnson and Blobel, 1997; Johnson et al., 1997), except cells were lysed with Y-PER (Pierce) and Aos1 and Uba2 were coexpressed in the same cells. SUMO and Ubc9 preparations were >99% pure and the Uba2/Aos1 preparation >90%. His_6-SUMO was reductively methylated as described (White and Rayment, 1993).

Septins were purified from log phase EJY332, EJY334, or EJY335 cells in a variation of the method in Frazier et al. (1998). Cells from an $A_{600} \approx 1$ culture were harvested by centrifugation, washed in water, frozen dropwise in liquid N_2, ground with a mortar and pestle under liquid N_2 to a fine powder, and extracted at 4°C with 10–20 volumes of buffer A (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, and 0.2% Triton X-100) plus 50 mM Tris (pH 8.0), 2 μg/ml of the protease inhibitors antipain, aprotinin, chymostatin, leupeptin, and pepstatin, plus 5 mM NaF and 200 μM Na_3VO_4. Subsequent steps were done at 4°C. Cell debris was pelleted at 27,000 × g_{max}, and the resulting supernatant bound in batch for 2–3 hr to anti-FLAG Ab agarose (IBI/Kodak). Beads were loaded into a column and washed with 50–100 column volumes of: (1) buffer A, (2) TBS-T (25 mM Tris [pH 7.5], 150 mM NaCl, and 0.1% Tween-20), (3) RIPA (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100) with 0.1% SDS, and (4) buffer B (50 mM Tris, 1 M NaCl, and 1 mM $MgCl_2$) plus 0.2% Triton X-100, and eluted with 75 μg/ml FLAG peptide (Sigma) in buffer B plus 0.2% Triton X-100. Eluate was bound for 2 hr in batch to Ni-NTA agarose, washed with buffer B plus 0.2% Triton X-100, followed by buffer B, and eluted in buffer B containing 200 mM imidazole.

Phosphorylated Siz1-His_6-FLAG was purified from EJY325 cells containing p424GAL-Siz1-HF, that were grown to $A_{600} \approx 0.8$ in SD − Trp containing 2% raffinose, harvested, resuspended to $A_{600} \approx 0.8$ in YPR, and incubated at 30°C for 1 hr before addition of 15 μg/ml nocodazole, followed by incubation at 30°C for 2 hr. Galactose was then added to 2% and incubation continued for 3 hr. Cells were harvested and Siz1-HF isolated exactly as the septins except that the wash of the anti-FLAG column with RIPA buffer plus 0.1% SDS was omitted, and that 0.1 mM $ZnCl_2$ was included in Ni-NTA column steps and in elution buffer. Recombinant Siz1-HF was expressed approximately as SUMO, Ubc9, and Uba2/Aos1 except using BL21 CodonPlus (DE3)-RIL cells (Stratagene) as the host and inducing with 0.1 mM IPTG for 4 hr at 20°C in LB amp supplemented with 10 μM $ZnCl_2$. Cells were extracted with Y-PER supplemented with 1 mM PMSF and protease inhibitors as for the yeast septin preparation; cell debris was pelleted, and supernatant was diluted 10-fold

with buffer A plus 50 mM Tris and protease inhibitors as for yeast lysates. Subsequent steps were as for purification of Siz1 from yeast lysate.

Ubc9 binding assay was performed using Ubc9-His_6 coupled to Affi-gel 10 (Bio-Rad) according to the manufacturer's instructions (~15 μg Ubc9 / μl beads) or uncoupled Affi-gel 10 beads blocked with ethanolamine. *E. coli* expressing Siz1-HF were extracted with Y-PER, the supernatant diluted 5-fold with buffer C (50 mM HEPES (pH 7.0), 150 mM NaCl, and 0.2% Triton X-100), and 100 μg total protein rotated with 10 μl beads for 1 hr at 4°C. Supernatant was retained as unbound fraction; beads were washed 3 times with buffer C, and the bound fraction removed by boiling in SDS loading buffer.

In Vitro Sumoylation Assay

In vitro reactions were performed immediately after elution of Siz1 and septin preparations. Reactions in Figure 5A and Figure 6 were done at the same time using the septin and Siz1 preparations shown in Figure 4C. Reactions were performed in siliconized tubes and contained 50 mM HEPES (pH 7.0), 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 20 μg/ml bovine serum albumin (Fraction V; Sigma), 5 mM Tris, 20 mM imidazole, 5 μM $ZnCl_2$ (NaCl, Tris, imidazole, and $ZnCl_2$ were added as part of the septin and Siz1 fractions or separately in the controls), and the following as noted: 5 mM ATP; 40 μg/ml SUMO or reductively methylated SUMO; ~2.5 μg/ml septin preparation; either 22 μg/ml Uba2/Aos1 and 10 μg/ml Ubc9 or 2.2 μg/ml Uba2/Aos1 and 1 μg/ml Ubc9; either ~2.5 μg/ml, 0.5 μg/ml, or 0.1 μg/ml of phosphorylated Siz1; or ~13.5 μg/ml or 2.7 μg/ml of the recombinant Siz1 preparation, which contained primarily C-terminal fragments of Siz1 and *E. coli* proteins. Visual inspection of Coomassie stained gels loaded with phosphorylated and recombinant Siz1 preparations suggested that there were approximately equal amounts of full-length Siz1 in the reactions containing 0.5 μg/ml phosphorylated Siz1 and 13.5 μg/ml of the recombinant preparation. Reactions in Figure 5B were performed with different preparations of septins and Siz1 that had not been subjected to Ni-NTA chromatography. They contained the same buffer, except lacking imidazole and $ZnCl_2$, plus 5 mM ATP, 10 μg/ml Uba2/Aos1, 23 μg/ml Ubc9, 23 μg/ml SUMO or 45 μg/ml reductively methylated SUMO, ~0.5 μg/ml septins, and ~2 μg/ml phosphorylated Siz1. Lanes 9–14 also lacked BSA.

Fluorescence Microscopy

Yeast cells were prepared for indirect immunofluorescence microscopy essentially as described (Pringle et al., 1991). Cdc11 antibody was detected with Cy3-conjugated donkey anti-rabbit IgG (Jackson Labs). Cells were mounted in 4′, 6-diamidino-2-phenylindole (DAPI)-containing medium to visualize DNA. Cells subjected to DAPI-staining alone were fixed in cold 70% ethanol rather than formaldehyde. All micrographs were taken using a 63× oil objective on a Leica DM.RXA microscope with a Cool SNAP fx digital camera (Roper Scientific) and IP Lab software (Scanlytics).

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