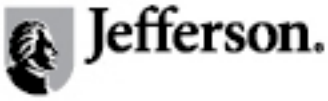

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Multiple domains in Siz SUMO ligases contribute to substrate selectivity.

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Multiple domains in Siz SUMO ligases contribute to substrate selectivity

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Summary

Saccharomyces cerevisiae **contains two Siz/PIAS SUMO E3 ligases, Siz1 and Siz2/Nfi1, and one other known ligase, Mms21. Although ubiquitin ligases are highly substrate-specific, the degree to which SUMO ligases target distinct sets of substrates is unknown. Here we show that although Siz1 and Siz2 each have unique substrates in vivo, sumoylation of many substrates can be stimulated by either protein. Furthermore, in the absence of both Siz proteins, many of the same substrates are still sumoylated at low levels. Some of this residual sumoylation depends on *MMS21*. Siz1 targets its unique substrates through at least two distinct domains. Sumoylation of PCNA (proliferating cell nuclear antigen) and the splicing factor Prp45 requires part of the N-terminal region of Siz1, the 'PINIT' domain, whereas sumoylation of the bud neck-associated septin proteins Cdc3, Cdc11 and Shs1/Sep7 requires the C-terminal domain of Siz1, which is also sufficient for cell cycle-dependent localization of Siz1 to the bud neck. Remarkably, the non-sumoylated septins Cdc10 and Cdc12 also undergo Siz1-dependent sumoylation if they are fused to the short ΨKXE SUMO attachment-site sequence. Collectively, these results suggest that local concentration of the E3, rather than a single direct interaction with the substrate polypeptide, is the major factor in substrate selectivity by Siz proteins.**

Supplementary material available online at
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Key words: Smt3, Ubc9, PIAS, SP-RING, Protein modification

Introduction

The ubiquitin (Ub)-related protein SUMO functions by becoming covalently attached to other proteins as a post-translational modification, and SUMO conjugation is essential for viability of *Saccharomyces cerevisiae* and of most other eukaryotic cells (Gill, 2004; Hay, 2005; Johnson, 2004; Muller et al., 2004). Proteomic approaches have identified 300-400 proteins in *S. cerevisiae* that are sumoylated, which suggests that 5-10% of all yeast proteins are modified by SUMO to some extent (Denison et al., 2004; Hannich et al., 2005; Panse et al., 2004; Wohlschlegel et al., 2004; Wykoff and O'Shea, 2005; Zhou et al., 2004). However, the essential function is unknown.

Like Ub, SUMO is attached to lysine residues in substrates through an amide bond between the C terminus of SUMO and the ϵ-amino group of the lysine residue. SUMO conjugation is carried out by a three-step enzyme pathway, analogous to the Ub pathway, that consists of a heterodimeric activating enzyme (E1), a single conjugating enzyme (E2) called Ubc9, and a growing collection of SUMO ligases (E3s) (Gill, 2004; Johnson, 2004; Muller et al., 2004). In the Ub pathway, E3s are the primary substrate specificity factors (Hershko and Ciechanover, 1998; Pickart and Eddins, 2004), but it remains to be determined whether SUMO E3s act similarly. Unlike E2s for Ub, Ubc9 binds directly to the ΨKXE consensus site for SUMO attachment (where Ψ is a large hydrophobic residue and K is the modified lysine), and in vitro Ubc9 can promote site-specific sumoylation of many physiological targets in the absence of an E3. However, in vivo the vast majority of sumoylation is E3-dependent, at least in yeast.

S. cerevisiae contains members of two of the four known families of SUMO E3s, including two Siz/PIAS (protein inhibitor of activated STAT) proteins, called Siz1 and Siz2/Nfi1, as well as a distinct E3 called Mms21 (Johnson and Gupta, 2001; Takahashi et al., 2001a; Zhao and Blobel, 2005). Deletion of *SIZ1* and *SIZ2* results in loss of >90% of all SUMO conjugates, indicating that most SUMO conjugation in yeast is Siz dependent. *MMS21* is an essential gene, but *mms21* mutants in which the SUMO ligase activity has been inactivated are viable (Zhao and Blobel, 2005). Siz/PIAS E3s are found in all eukaryotes and share a conserved ~400 amino acid N-terminal region that contains several distinct domains. One of these is the SP-RING (Siz/PIAS-RING), which is related to the RING Zn^{2+}-binding domains present in many E3s for Ub and which is required for the sumoylation reaction (Sachdev et al., 2001; Takahashi et al., 2001a). Immediately N-terminal to the SP-RING is a conserved region called the 'PINIT' domain that has also been implicated in the sumoylation reaction (Takahashi and Kikuchi, 2005). Near their N termini, Siz/PIAS proteins contain a sequence called a SAP (scaffold attachment factor, acinus, PIAS) domain. SAP domains, including those from PIAS proteins, bind DNA (Okubo et al., 2004; Tan et al., 2002). Siz/PIAS proteins also contain unique C-terminal domains, which share little sequence similarity with each other or with other proteins. The functions of the various parts

of Siz/PIAS proteins have only been partially characterized, and it is not known how Siz/PIAS proteins interact with their substrates.

In fact, it is not clear to what extent each SUMO E3 has distinct substrates. *SIZ1* is required for sumoylation of the bud neck-associated septin proteins Cdc3, Cdc11 and Shs1/Sep7 and of the PCNA (proliferating cell nuclear antigen) ortholog Pol30 at its major sumoylation site, whereas *SIZ2* is required for sumoylation of several small proteins that have not been identified (Hoege et al., 2002; Johnson and Gupta, 2001; Takahashi et al., 2001b). By contrast, either Siz1 or Siz2 can stimulate sumoylation of Flp or Top2 (Chen et al., 2005; Takahashi et al., 2005). It is not known which of these situations is more common. This issue has been complicated by the apparent promiscuity of Siz/PIAS-dependent sumoylation in vitro. For example, *SIZ1* is absolutely required for septin sumoylation in vivo, but in vitro Siz1 and Siz2 stimulate septin sumoylation at comparable rates (Takahashi et al., 2003), suggesting that in vitro experiments do not necessarily reproduce in vivo substrate specificity. Genetic data suggest that *SIZ1* and *SIZ2* have some overlapping functions. One such function involves controlling the copy number of the endogenous yeast plasmid the 2 micron circle (2 μm) (Chen et al., 2005). High copy number of 2 μm is responsible for many of the conspicuous growth defects of the *siz1 siz2* double mutant. Knockout mice have been made for three of the four mouse PIAS genes, but no defect in SUMO conjugation has been detected in the single mutants, consistent with the possibility that PIAS proteins also have overlapping functions (Liu et al., 2004; Roth et al., 2004; Santti et al., 2005; Wong et al., 2004).

Sumoylation of both septins and PCNA shows striking cell cycle dependence, with PCNA being modified during S phase, whereas septins are modified during mitosis (Hoege et al., 2002; Johnson and Blobel, 1999). PCNA is also heavily sumoylated upon treatment of cells with high levels of the DNA-damaging agent methyl methane sulfonate (MMS), but MMS does not induce sumoylation of septins. This distinct regulation is particularly striking given the fact that both septins and the major sumoylation site of PCNA absolutely require Siz1 for their sumoylation. Thus, Siz1 functions as part of two separately regulated sumoylation systems.

Here we address two questions related to how substrates are selected for sumoylation. One is whether different SUMO E3s target distinct sets of substrates, similar to E3s in the Ub pathway, or whether they generally target overlapping sets of substrates. The second question focuses on what features of Siz1 are responsible for selecting Siz1-dependent substrates, such as septins and PCNA, for sumoylation.

Results

More than one E3 stimulates sumoylation of many SUMO substrates

It is difficult to determine from anti-SUMO immunoblots of whole cell lysates, which contain thousands of bands, whether most substrates are targeted specifically by one or the other Siz protein. It is also not known whether deleting both *SIZ* genes completely eliminates sumoylation of the proteins that undergo Siz-stimulated sumoylation. To answer these questions, we surveyed a number of SUMO substrates for Siz dependence. C-terminal His_8- and HA-epitope tags were fused to substrate genes at their chromosomal loci, generating strains that expressed native levels of tagged proteins. These strains had no growth defects, demonstrating that the tagged proteins are at least partially functional (not shown). Tagged proteins were isolated by Ni^{2+} chromatography and analyzed by immunoblotting with antibodies against either HA or SUMO (Fig. 1). This experiment identified one new protein, the splicing factor Prp45, whose sumoylation is stimulated only by *SIZ1*. However, sumoylation of most substrates could be stimulated by either *SIZ1* or *SIZ2*. For some proteins, such as Top2 (Fig. 1) and Flp1 (Chen et al., 2005), sumoylation took place at approximately wild-type levels in both the *siz1* and *siz2* single mutants. Sumoylation of other substrates, including Spt7, Abf1, Rsc2, Gcn5, Ysh1 and Top1 occurred at near wild-type levels in the *siz2* mutant, but was reduced approx. two to fivefold in the *siz1* mutant. Sumoylation of all substrates shown in Fig. 1 was dramatically reduced in the double mutant, although several substrates, most notably Top1, but also Rsc2, Gcn5 and Top2, were still detectably sumoylated (Fig. 1). These experiments showed that sumoylation of many substrates can be stimulated by either Siz protein and that many Siz substrates are still sumoylated, albeit at dramatically reduced levels, in the absence of both *SIZ1* and *SIZ2*.

Mms21, the recently identified third *S. cerevisiae* SUMO E3, was an obvious candidate to contribute to Siz-independent

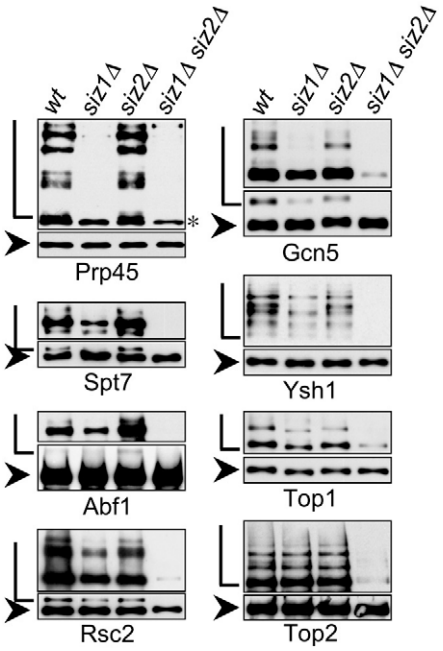

Fig. 1. Both Siz1 and Siz2 stimulate sumoylation of many proteins. Lysates from cells in which the indicated proteins had been tagged with the HA epitope and a His_8 tag were subjected to denaturing Ni-NTA affinity chromatography. Eluates were analyzed by SDS-PAGE and immunoblotting with antibodies against HA (lower panels) or Smt3 (top panels). *SIZ* genotypes are indicated over the lanes. Bands corresponding to unmodified proteins are indicated with arrowheads. Open brackets indicate sumoylated species. For Spt7, Abf1, Rsc2 and Gcn5, sumoylated species were detected with the HA Ab on a short exposure and are shown. Other substrates required long exposures of the HA blot to detect sumoylated species (not shown). A cross-reacting band is designated with an asterisk.

sumoylation. We wanted to determine whether Siz proteins and Mms21 function redundantly, by examining sumoylation in strains that both lack *SIZ* genes and contain mutations that eliminate the sumoylating activity of *MMS21*. However, Zhao and Blobel reported that *mms21 siz1* and *mms21 siz2* double mutants, as well as the *siz1 siz2 mms21* triple mutant, are all inviable (Zhao and Blobel, 2005). Because the inviability of these mutants might have involved hyper-amplification of 2 μm, which causes severe growth defects, we repeated this experiment in a cir° strain, which lacks 2 μm. We also used a different allele of *mms21*. Instead of a C-terminal truncation mutant, we used a mutant that contained two point mutations in the SP-RING of Mms21 (*mms21-sp*), which eliminates the sumoylation stimulating activity of the *S. pombe* ortholog of *MMS21* (Andrews et al., 2005). In our experiment, both double mutants *siz1 mms21-sp* and *siz2 mms21-sp* were viable, although they grew more slowly (doubling time of 2.7 and 2.5 hours, respectively) than either the *siz1 siz2* double mutant (1.8 hours) or the *mms21-sp* single mutant (2.0 hours; Fig. 2A). The *siz1 siz2 mms21-sp* triple mutant was dead in our experiment (not shown), as found by Zhao and Blobel (Zhao and Blobel, 2005). These results indicate that *SIZ1*, *SIZ2* and *MMS21* are each capable of carrying out a shared function that is required for yeast viability. It is most likely that the relevant difference between our results and those in Zhao and Blobel is the absence of 2 μm, but we have not ruled out the possibility that the *mms21* allele or a difference in the strain background is involved. Partial functional redundancy of Siz proteins and Mms21 was also supported by the observation that sumoylation of the nucleolar protein Net1 was more strongly reduced in the *siz1 siz2* and *mms21-sp siz1* double mutants than in any of the three single mutants (Fig. 2B). Thus, *SIZ1*, *SIZ2*, and *MMS21* can all promote sumoylation of certain shared substrates.

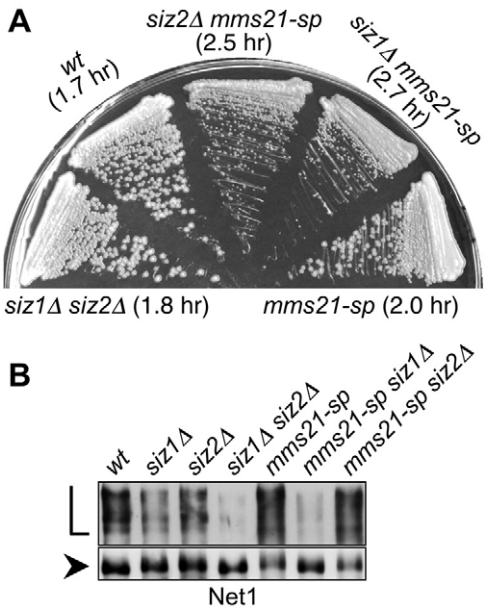

Fig. 2. Siz proteins and Mms21 have overlapping functions. (A) Strains of the indicated genotypes were incubated on a YPD plate for 2 days at 30°C. Doubling times of mutants are indicated in parentheses. (B) Yeast strains of the indicated genotypes containing Net-His_8 HA were analyzed as in Fig. 1, with immunoblotting with anti-Smt3 (top panel) or anti-HA (bottom panel). Designations are as in Fig. 1.

Different domains in Siz1 are required for sumoylation of different proteins

Siz1 and Siz2 are large proteins with several conserved domains. To determine which domains are required for targeting various substrates for sumoylation, we constructed chimeras between Siz1 and Siz2, as well as versions of Siz1 containing short deletions. These Siz constructs were expressed at similar levels to wild-type Siz1 except for construct 4 (Fig. 3A), which was present in at least 20-fold higher levels (Fig. 3B). This probably results from the presence of the C-terminal domain of Siz1, which targets Siz1 for proteolysis (Takahashi and Kikuchi, 2005), in all constructs except this one. Further evidence for the correct folding and activity of these Siz variants was provided by the fact that they all promoted wild-type levels of SUMO conjugation to at least one substrate (see below).

Plasmids expressing these Siz variants were introduced into *siz1 siz2* strains containing tagged versions of substrate proteins, and sumoylation of these substrates was examined. Several conclusions can be drawn from these experiments. One is that the C-terminal domain, which is divergent between Siz1 and Siz2, is necessary and sufficient for the Siz1-specificity of septin sumoylation. The septin Cdc3 was sumoylated efficiently by construct 3, which contained the C terminus of Siz1 together with the N-terminal region from Siz2, but not by construct 4, which contained part of the N terminus of Siz1 and the C-terminal region from Siz2 (Fig. 3D). Consistent with a role for the non-conserved C-terminal domains of Siz/PIAS proteins in substrate specificity, the C-terminal domain of Siz2 was required for formation of three uncharacterized *SIZ2*-dependent SUMO conjugates detected between 40 and 55 kDa on anti-SUMO immunoblots of whole cell lysates (Fig. 3C). Thus, the C-terminal domains of both Siz1 and Siz2 confer specificity for certain substrates.

However, the C-terminal domains are not responsible for all differences in substrate specificity between Siz1 and Siz2. Remarkably, the Siz1-dependence of SUMO attachment to PCNA (Pol30) and Prp45 involved sequences in the N-terminal region of Siz1 (Fig. 3D). Sumoylation of these proteins was not stimulated by construct 3, which promoted septin sumoylation, but instead was promoted by construct 4, which contained only the N-terminal 309 amino acids of Siz1.

The SAP domain of Siz1 was also required for sumoylation of PCNA and for the Siz-dependent fraction of SUMO attachment to most other proteins tested, also required the SAP domain of Siz1. Deleting the SAP domain had no effect on sumoylation of septins, and only slightly reduced sumoylation of Prp45, indicating that the SAP domain is not essential for the ability of Siz1 to stimulate sumoylation. The requirement for the SAP domain could indicate that DNA binding or a protein interaction involving the SAP domain is required for Siz proteins to stimulate SUMO attachment to certain proteins. However, the SAPΔ effect might result, at least partially, from the dramatically reduced nuclear localization of this mutant, which can be detected with Siz1-ΔSAP-GFP fusions (Takahashi and Kikuchi, 2005) (data not shown). Siz1 normally localizes primarily to the nucleus throughout the cell

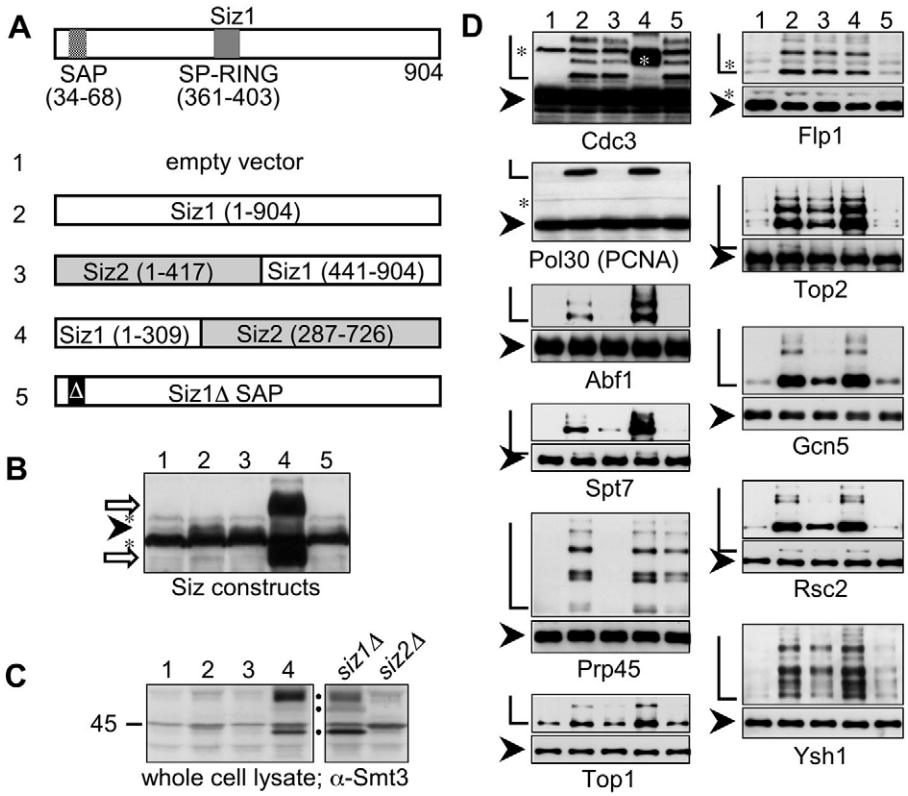

Fig. 3. Different domains in Siz1 participate in sumoylation of different substrates. (A) Diagram of the Siz variants used in experiments in B-D. Portions corresponding to Siz2 sequence are shaded grey. All variants also include a C-terminal HA epitope tag. (B) Lysates from *siz1 siz2* cells expressing the indicated HA-tagged Siz variants were analyzed by SDS-PAGE and immunoblotting with an antibody against the HA epitope. The band corresponding to Siz1-HA, as well as constructs 3 and 5 is indicated with an arrowhead. Open arrows indicate two bands corresponding to construct 4 [the upper band is sumoylated (data not shown)]. Asterisks designate bands that cross-react with the Ab. (C) (Left) Lysates from *siz1 siz2* cells that lacked the major sumoylation sites in all three septins (EJY411) and contained the indicated constructs were analyzed by SDS-PAGE and immunoblotting with an Ab against Smt3. (Right) Lysates from *siz1* or *siz2* strains that lacked the major septin sumoylation sites were analyzed by immunoblotting with an Ab against Smt3. Dots between panels indicate position of*SIZ2*-dependent SUMO conjugates. (D) *siz1 siz2* cells contained Siz variants as indicated over the lanes and tagged substrates as indicated below each pair of panels. Cdc3 and Pol30 were tagged with only HA and were analyzed by subjecting whole cell lysates to SDS-PAGE and immunoblotting with an Ab against HA. The Cdc3-HA cultures were arrested with nocodazole, and the Pol30-HA cultures were treated for 2 hours with 0.2 % MMS. The sumoylated form of Pol30 indicated is the mono-sumoylated Lys164 conjugate. Other substrates bore HA and His_8 tags and were analyzed by denaturing Ni-NTA affinity chromatography followed by SDS-PAGE and immunoblotting with antibodies against HA (lower panels) or Smt3 (top panels). Designations are as in Fig. 1. The white asterisk in lane 4 of the Cdc3 panel indicates the band corresponding to the construct 4 fusion protein, which is visible in this experiment because a whole cell lysate was analyzed.

cycle (Johnson and Gupta, 2001; Takahashi et al., 2001b). This effect could result from a role for the SAP domain in either nuclear import or nuclear retention (see Discussion). All substrates tested other than the septins localize to the nucleus and would be expected to be sumoylated in the nucleus.

Fig. 3 showed that the determinants required for Siz1-dependent sumoylation of PCNA and Prp45 are in the N-terminal region of Siz1. To analyze this region further, two additional chimeras were made containing either the N-terminal or C-terminal half of this region. This experiment showed that amino acids 180-309 of Siz1 were sufficient to account for the differential substrate specificity between Siz1 and Siz2 for PCNA and Prp45 (Fig. 4B). This sequence is part of the PINIT domain and is quite well conserved between Siz1 and Siz2, with 41% sequence identity and 63% similarity. This similarity suggested that further characterization of this sequence was unlikely to identify an entire domain that would be sufficient for targeting Siz1 to PCNA and Prp45, but rather would probably identify a fairly small sequence difference that prevents Siz2 from promoting sumoylation of these proteins. This experiment (Fig. 4B) was done using cells treated with 0.2% MMS, and the chimera supported an equal level of PCNA sumoylation as full-length Siz1, indicating that the mechanism for induction of PCNA sumoylation upon DNA damage is intact when only these 130 amino acids of Siz1 are present. We also tested whether this chimera still promoted correct cell-cycle-dependent sumoylation of PCNA by doing alpha factor release experiments. (Fig. 4C). In both the wild-type and chimera samples, PCNA sumoylation peaked 30 min after release from alpha factor block, consistent with previous results in wild-type cells (Hoege et al., 2002). This result demonstrated that no Siz1 sequences outside amino acids 180-309 of Siz1 are required for cell-cycle regulation of PCNA sumoylation.

We also further characterized the C-terminal domain of Siz1. This sequence has been shown previously to be required for localization of a portion of Siz1 to the bud neck (Takahashi et al., 2003), and we also found that only those chimeras containing it localized to the bud neck (data not shown). To identify the sequences involved, segments of this domain were fused to GFP and tested for bud neck localization (Fig. 5B). At least two sections of this C-terminal domain localized to the bud neck independently: one containing the extreme C terminus of the protein (residues 836-904), and the other a larger region upstream of this (residues 556-836). Deleting from amino acid 836 in either direction resulted in a gradual reduction of bud neck localization, supporting a model where multiple sequences within the C-terminal domain contribute additively to its ability to bind the bud neck. The C-terminal domain, starting from residue 556, contains very high

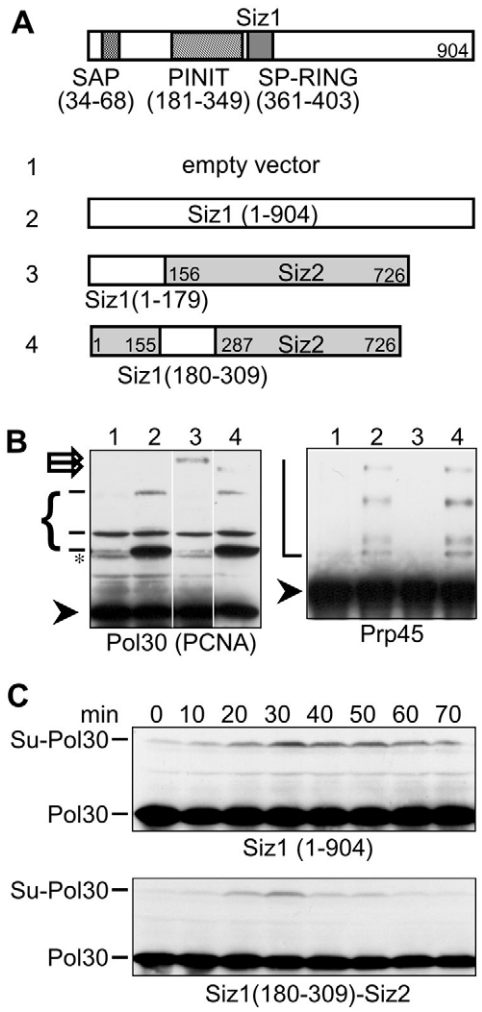

Fig. 4. Siz1 specificity for Pol30 and Prp45 involves part of the conserved PINIT domain. (A) Diagram of Siz variants used in B and C. Portions corresponding to Siz2 sequence are in grey. (B) Lysates prepared from *siz1* cells containing the indicated Siz variants and either Pol30-HA or Prp45-HA-His_8 were either analyzed directly (Pol30) or subjected to Ni-NTA affinity chromatography (Prp45), followed by SDS-PAGE and immunoblotting with an Ab against HA. Pol30-HA cultures were treated with 0.2% MMS. Designations are as in Fig. 1. A bracket indicates the three SUMO-Pol30 bands (the middle one is monosumoylated at Lys127, which is not Siz1 dependent). Open arrows indicate the bands corresponding to the HA-tagged Siz1-Siz2 fusions in lanes 3 and 4 of the Pol30 blot. Full-length Siz1 is not visible here. All lanes of the Pol30 panel were from the same exposure of the same blot, but lanes 3 and 4 were spliced to remove irrelevant lanes. (C) *siz1* Pol30-HA cells containing the indicated Siz variants were arrested with alpha factor and released into fresh medium. Whole cell lysates made from samples taken at the indicated timepoints were analyzed as in B. Bands corresponding to unmodified Pol30 and the Lys164-linked SUMO conjugate are indicated.

proportions of Ser/Thr (25%), Pro (11%) and Asn (11%) residues, but there are no obvious repeated motifs that could account for these observations.

Fig. 5B shows nocodazole-arrested cells, but similar experiments with log phase cells showed that bud neck localization of these truncations occurred only during mitosis, with the same timing as full-length Siz1 (not shown). This result demonstrated that the cell cycle-dependent localization is retained by these truncations. We also tested whether the C terminus of Siz1 contains all sequences necessary for the cell-cycle-dependent sumoylation of septins by doing alpha factor release experiments (Fig. 5D). This experiment showed that the Siz2(1-417)-Siz1(441-904) fusion (construct 3 in Fig. 3) supported wild-type cell-cycle-dependent Cdc3 sumoylation. Thus, no Siz1-specific sequences outside the C terminus are required for cell cycle regulation of septin sumoylation.

Adding sumoylation consensus sequences to Cdc10 and Cdc12 allows Siz1-dependent sumoylation

The above results led us to ask whether co-localization of Siz1 and the substrate may be the primary factor in preferential stimulation of SUMO attachment to certain substrates by Siz proteins. To address this question we tested whether the septins Cdc10 and Cdc12, which are present in septin complexes at the bud neck but which are not normally sumoylated at high levels, would be sumoylated if the SUMO attachment site sequence ΨKXE were added to them. This was done by adding the tetrapeptide LKEE to the C terminus of Cdc10 and the dipeptide EE to the C terminus of Cdc12, generating the sequence LKEE. These sequences should introduce binding sites for Ubc9, but would not be expected to create specific binding sites for Siz1. Both fusions also contained His_6 and HA-epitope tags C-terminal to the sumoylation site. These fusions, Cdc10-S and Cdc12-S, both now underwent Siz1-dependent sumoylation, which, like sumoylation of Cdc3, Cdc11 and Shs1, was stimulated by G_2/M arrest with nocodazole and required the C terminus of Siz1 (Fig. 6). Whereas Cdc12 was sumoylated at a relatively low level, Cdc10 was sumoylated robustly, to a level comparable to that of Cdc11 (Fig. 6B, lower panel, lane 3). We do not know why introduction of one sumoylation site in Cdc10 resulted in formation of two sumoylated species of Cdc10, one of which reacted poorly with the anti-HA antibody (Fig. 6A). These results suggest that the same Siz1-bud neck interactions that occur in wild-type cells allow Siz1 to stimulate SUMO attachment to suitable lysine residues in neighboring proteins.

Discussion

We found that there are four classes of Siz-dependent SUMO substrates: those that require Siz1 (such as PCNA, Prp45 and septins), those that require Siz2 (several unidentified conjugates), those whose sumoylation can be promoted by either Siz1 or Siz2 (many substrates), and those that can be sumoylated with the assistance of any of Siz1, Siz2 or Mms21 (Net1). Two extreme models could explain how SUMO E3s stimulate sumoylation of overlapping sets of substrates. One is that different E3s specifically interact with the same substrates. In the ubiquitin system different E3s sometimes target the same substrates, but often under different conditions (Hershko and Ciechanover, 1998; Muratani et al., 2005). A second model is that SUMO E3s may not interact directly with the substrate at all, but merely stimulate SUMO attachment to lysine residues that are selected by Ubc9. Although this model contrasts with what is known about ubiquitin E3s, Ubc9 is clearly different from Ub E2s in its ability to bind directly to the attachment site peptide (Bernier-Villamor et al., 2002).

Our results are most consistent with an intermediate model,

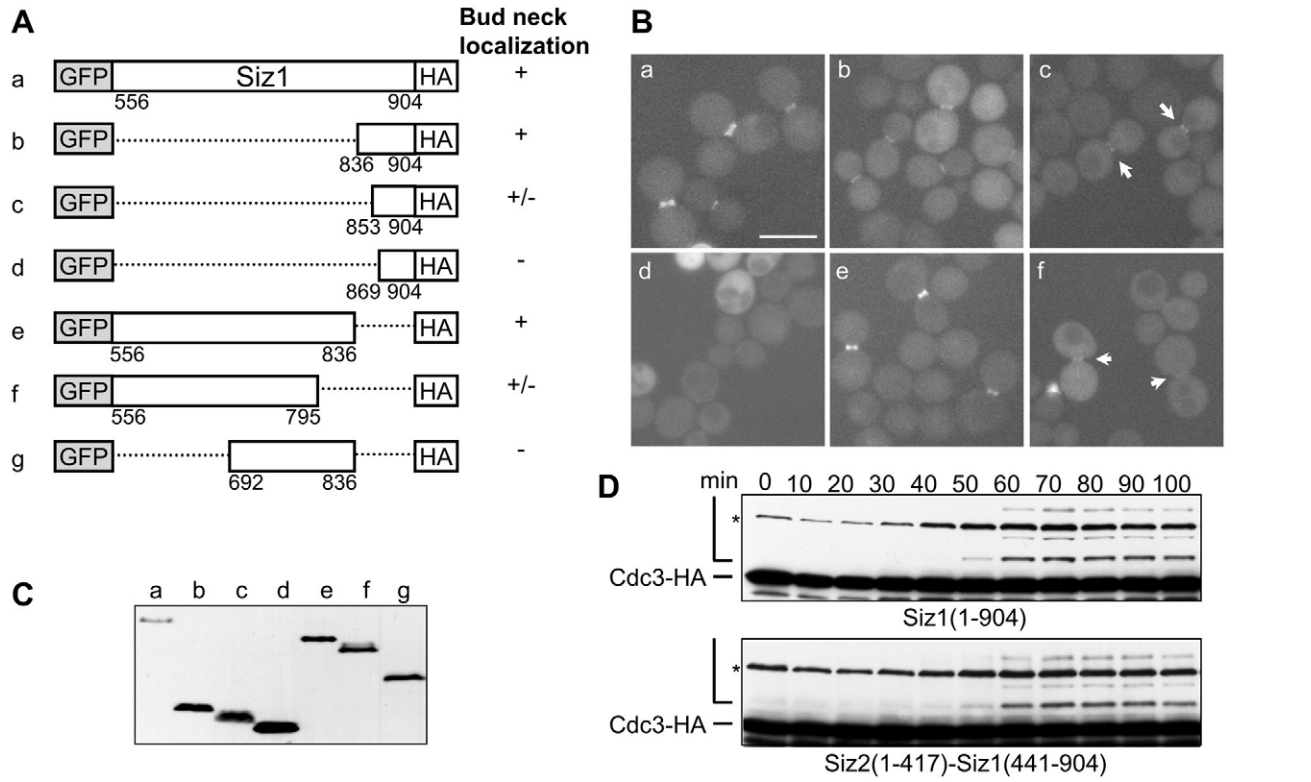

Fig. 5. The C-terminal domain of Siz1 is sufficient for cell-cycle-dependent binding to the bud neck. (A) Diagram of GFP fusions containing parts of the C-terminal domain of Siz1. Ability to localize to the bud neck is indicated. (B) Fluorescence microscopy of nocodazole-arrested *siz1* cells containing GFP fusions as illustrated in A. Bar, 5 μm. (C) Lysates of *siz1* cells containing GFP fusions were analyzed by SDS-PAGE and immunoblotting with an antibody against HA. Note that fusions that fail to localize to the bud neck are present in quantities comparable to those that do. Lane g was from the same exposure of the same blot, but was spliced to remove extraneous lanes. (D) *siz1* Cdc3-HA cells containing the indicated Siz variants were analyzed as in Fig. 4C. Unmodified Cdc3-HA is indicated, and SUMO-Cdc3 species are indicated by open square brackets. An asterisk indicates a cross-reacting band.

in which the likelihood of a Siz E3 stimulating SUMO attachment to a particular substrate is determined by the local concentration of the E3. This could be affected by several factors including overall subcellular localization of the E3 and substrate, interactions between the E3 and features of the substrate's environment, and direct E3-substrate interactions. This model would explain the continuum of substrate specificities that is observed among SUMO E3s. Sumoylation of substrates would be strongly stimulated by E3s that interact with nearby features, but might also be enhanced at some level by other E3s present at lower concentrations. This would also explain how Cdc10 and Cdc12 could be converted into Siz1-dependent SUMO substrates merely by adding two or four amino acids to generate the sumoylation consensus sequence: Siz1 binds to the bud neck, resulting in a high local concentration in the vicinity of the introduced consensus sequences. We are not implying that Siz1 may not interact directly with Cdc10 and/or Cdc12, only that any Siz1 interactions that occur with Cdc10-S and Cdc12-S probably occur equally with wild-type Cdc10 and Cdc12. In wild-type cells, Siz1 interactions with Cdc10 or Cdc12 may stabilize the Siz1-bud neck interaction, thereby promoting SUMO conjugation to the other septins. This model is also consistent with the observation that a non-sumoylated protein can be targeted for sumoylation merely by adding a sumoylation consensus sequence and a nuclear localization sequence (Rodriguez et al., 2001). The E3-dependence of this phenomenon is unknown, but the known E3s are concentrated either in the nucleus or at the nuclear pore complex.

This model would also explain the results where Siz/PIAS E3s appear to show only modest substrate specificity in vitro, where the E3s are present at relatively high concentrations. In cells, Siz1 has been reported to be present at only ~150 molecules/cell (Ghaemmaghami et al., 2003). This model might also account for the observation that often many subunits of a particular multisubunit complex are modified by SUMO (Denison et al., 2004; Hannich et al., 2005; Panse et al., 2004; Sterner et al., 2006; Wohlschlegel et al., 2004). Binding of a Siz protein to a complex might target multiple subunits for sumoylation simultaneously. Consistent with this possibility, both the septins and the SAGA subunits Spt7 and Gcn5 showed similar patterns of E3 dependency (Figs 1, 6) (Johnson and Blobel, 1999). The biological significance of such a mode of substrate selection is unclear, but it is conceivable that in some situations attaching SUMO to any of several different lysine residue in a particular locale may be sufficient for SUMO's function. The ability of SUMO to be attached to multiple subunits of various multiprotein complexes raises the disheartening possibility that SUMO attachment to these subunits may be functionally redundant.

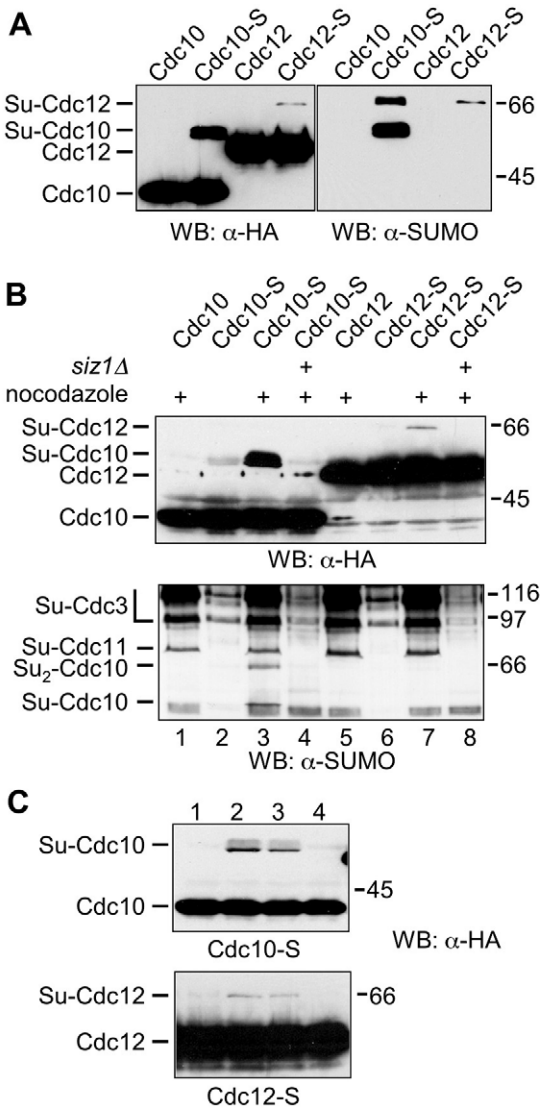

Fig. 6. Addition of a sumoylation consensus sequence converts Cdc10 and Cdc12 into Siz1 substrates. (A) Lysates from strains expressing Cdc10-His_8-HA (Cdc10), Cdc12-His_8-HA (Cdc12), or versions of these with a sumoylation site consensus sequence between the C terminus of the protein and the tag (Cdc10-S and Cdc12-S) were analyzed by denaturing Ni-NTA chromatography, SDS-PAGE, and immunobloting with antibodies against HA (left panel) or Smt3 (right panel). Bands corresponding to unmodified and sumoylated (Su) proteins are indicated. The second SUMO-containing species in the Cdc10-S sample (right panel) is visible on a much darker exposure of the HA blot. (B) Whole cell lysates from strains expressing the indicated forms of Cdc10 and Cdc12 were analyzed by SDS-PAGE and immunobloting with antibodies against HA (top panel) or Smt3 (bottom panel). Strains lacked *SIZ1* or had been arrested with nocodazole as indicated. Bands corresponding to unmodified and sumoylated septins are indicated. Su_2-Cdc10 indicates the second sumoylated form of Cdc10, but is not necessarily a di-sumoylated species. Positions of sumoylated Cdc11 and Cdc3 were determined based on previous results (Johnson and Blobel, 1999). (C) Whole cell lysates prepared from *siz1Δ* cells expressing Cdc10-S (top panels) or Cdc12-S (bottom panels) and Siz1 variants shown in Fig. 3A were analyzed by SDS-PAGE and immunoblotting with an antibody against HA. Lanes are numbered as in Fig. 3D. Bands corresponding to unmodified and sumoylated septins are indicated.

Our results do not address the ongoing debate in the ubiquitin/SUMO field regarding the mechanism of non-HECT E3s, such as RING and SP-RING E3s, which have been proposed to act either by 'allosteric' activation of the E2-Ubl (ubiquitin-like protein) thiolester intermediate or as relatively inert 'bridging factors' that bring together the substrate and thiolester intermediate. The main difference between these mechanisms involves the functional interaction between the E3 and E2-Ubl thiolester, not the interaction between the E3 and the substrate. Either mechanism is consistent with a model where the E3 stimulates sumoylation in the absence of a strong direct interaction with the substrate, as long as the E3 binds the E2. The only prediction from these mechanisms regarding substrate interactions is that if an E3 plays an active role in stimulating Ubl transfer, it might increase Ubl transfer in vitro without interacting with the substrate at all. The E3 domain of the mammalian SUMO E3 RanBP2/Nup358 has this activity and appears to act by positioning Ubc9 and SUMO in an optimal orientation for SUMO transfer (Pichler et al., 2004; Reverter and Lima, 2005). However, RanBP2 is a stoichiometric subunit of the nuclear pore complex, and it seems likely that in vivo its subcellular localization has a major impact on which substrates are targeted for sumoylation.

We show that the C terminus of Siz1 is necessary and sufficient for bud neck localization, in contrast to published reports indicating that it is necessary but not sufficient (Takahashi and Kikuchi, 2005; Takahashi et al., 2003). The reason for this difference is unclear, but we have generated a series of GFP fusions, some of them non-overlapping, that all localize to the bud neck, making it unlikely that our results are an artifact. It has been suggested that the function of bud neck localization of Siz1 during mitosis may be to sequester Siz1 away from the nucleus, thereby reducing nuclear sumoylation (Takahashi and Kikuchi, 2005). Our results suggest that such competition for Siz1 is not responsible for regulation of PCNA sumoylation: a chimera that fails to localize to the bud neck supports the same cell cycle regulation of PCNA sumoylation as does full-length Siz1 (Fig. 4C). We are not able to conclude from our results what part of Siz1 is sufficient for the interactions that result in sumoylation of PCNA and Prp45. We showed that the relevant difference between Siz1 and Siz2 that makes sumoylation of these substrates dependent on Siz1 is residues 180-309 of Siz1. However, in this chimera, Siz2-derived sequences, such as the SAP domain, could be contributing to the interaction by functionally substituting for the corresponding sections of Siz1.

Sumoylation of many substrates depended on the SAP domain in Siz1, but it remains to be determined whether the SAP domain has a direct role in substrate selection. SAP domains bind DNA, and NMR analysis of the PIAS1 SAP domain identified specific residues that are perturbed by DNA binding, suggesting that they are contact sites for DNA (Okubo et al., 2004). The idea that interactions between Siz proteins and DNA contribute to determining which proteins are sumoylated is appealing, especially in the case of proteins such as PCNA, the sumoylation of which during S phase and in response to DNA damage suggests that a specific DNA-protein

structure might be the target for sumoylation. However, the failure of Siz1 mutants lacking the SAP domain to localize to the nucleus complicates interpretation of these results. The SAP domain could promote either nuclear import or nuclear retention. If the ΔSAP mutant is not imported into the nucleus, its inability to sumoylate nuclear proteins is uninformative. Our attempt to distinguish between these possibilities by examining Siz1-ΔSAP-GFP localization in the presence of the nuclear export inhibitor leptomycin B (Neville and Rosbash, 1999) was inconclusive (not shown). The SAP domain does have two properties that favor a role in nuclear retention: it does not contain a classic nuclear localization sequence, and it apparently binds DNA. Furthermore, Duval and colleagues found that point mutations in the SAP domain, the PINIT domain, and the SP-RING of PIAS3L all showed similar effects on its localization: the mutant proteins localized to both the nucleus and the cytoplasm, unlike wild-type PIAS3L, which is strictly nuclear (Duval et al., 2003). The SP-RING and PINIT mutations both probably eliminate the sumoylation activity of this protein (Sachdev et al., 2001; Takahashi and Kikuchi, 2005), suggesting that sumoylation activity toward certain substrates may be involved in PIAS3L nuclear retention. We also observed that a Siz1 mutant with an SP-RING mutation was defective in localizing to the nucleus (not shown). Thus, it is possible that the inability of Siz1-ΔSAP to sumoylate certain proteins may be the cause, rather than the effect, of its failure to localize to the nucleus. Further work will be required to determine how the SAP domain participates in sumoylation.

Materials and Methods

Media and genetic techniques

Standard techniques were used (Ausubel et al., 2000). Rich yeast medium, containing either 2% glucose (YPD) or 2% raffinose (YPR), and synthetic yeast media (SD) were prepared as described previously (Sherman et al., 1986). galactose (2%) was used to induce expression from GAL promoters. Cell cycle arrests were induced by incubating with 10 μM α-factor (Sigma) or 15 μg/ml nocodazole (Acros, Fisher Scientific).

Plasmids and yeast strain constructions

pRS416-based plasmids expressing Siz1-HA, Siz1-Siz2 chimeras and the Siz1-ΔSAP mutant contained the *SIZ1* promoter starting at nucleotide –477, and a C-terminal HA tag consisting of the sequence GYPYDVPDYAAFL. Chimera 3 in Fig. 3 contained bp 1-1251 of *SIZ2*, encoding residues 1-417, and bp 1321-2712 of *SIZ1*, encoding residues 441-904. Chimera 4 in Fig. 3 contained bp 1-927 of *SIZ1*, encoding residues 1-309, and bp 858-2178 of *SIZ2*, encoding residues 287-726. Chimera 3 in Fig. 4 contained bp 1-537 of *SIZ1*, encoding residues 1-179, and bp 466-2178 of *SIZ2*, encoding residues 156-726. Chimera 4 in Fig. 4 contained bp 1-465 of *SIZ2*, encoding residues 1-155, bp 538-927 of *SIZ1*, encoding residues 180-309, and bp 859-2178 of *SIZ2*, encoding residues 287-726. The Siz1-ΔSAP construct contains a deletions of bp 139-180 of *SIZ1*, which encodes residues 47-60. A larger deletion lacking residues 34-68 had a similar effect. GFP fusion plasmids (Fig. 5) contained the SIZ1 promoter starting at nucleotide –1457, an N-terminal enhanced GFP derived from pYX242-GFP (Rosenblum et al., 1998), a C-terminal HA tag (GYPYDVPDYAAFL) and sections of *SIZ1* as follows: (a) bp 1666-2712 (aa 556-904), (b) 2506-2712 (aa 836-904), (c) 2557-2712 (aa 853-904), (d) 2608-2712 (aa 869-904), (e) 1666-2508 (aa 556-836), (f) 1666-2385 (aa 556-795) and (g) 2077-2508 (aa 692-836). Construction details are available on request. Relevant portions of plasmids were sequenced in most cases. Alternatively, independent plasmid isolates were tested to make sure they had similar effects.

S. cerevisiae strains used are listed in Table S1 in supplementary material. All strains are derivatives of JD51 (Dohmen et al., 1995). The sequence of the C-terminal HA tag on Cdc3 and Pol30 was GYPYDVPDYAAFL. The C-terminal HA-His_8 tag on Flp, Top1, Top2, Abf1, Prp45, Rsc2 and Ysh1 was GYPYDVPDYAAF-LHHHHHHHH, and the His_8-HA tag on Spt7, Gcn5, Net1, Cdc10 and Cdc12 was GHHHHHHHHGYPYDVPDYAAFL. The strain expressing Cdc10-S contained sequence encoding LKEE between the C-terminus of Cdc10 and the tag, whereas Cdc12-S contained the sequence EE (Fig. 6). Strains were constructed by an assembly PCR-based approach (Johnson and Blobel, 1999). *mms21-sp::URA3* contained C200S and H202A mutations and was made by a similar approach. Alleles of septin genes lacking sumoylation sites have been described (Johnson and Blobel, 1999). Some strains were cured of 2 μm as described previously (Tsalik and Gartenberg, 1998). Construction details and oligonucleotide sequences are available on request.

Antibodies and immunoblot analyses

Yeast whole cell lysates were prepared as described by Yaffe and Schatz (Yaffe and Schatz, 1984) and subjected to SDS-PAGE and immunoblotting followed by chemiluminescent detection (Supersignal, Pierce) as described previously (Johnson and Blobel, 1999). Antibodies were, an affinity-purified rabbit polyclonal Ab against Smt3 (SUMO) (Johnson and Blobel, 1999) and the 16B12 mAb against the HA epitope (BAbCO). HA and His_8-tagged proteins were purified from yeast using Ni^{2+}-nitriloacetic-acid affinity chromatography under denaturing conditions (6 M guanidine-HCl) as described by Chen et al. (Chen et al., 2005). Protein assays (Coomassie Plus, Pierce) were performed so that equal amounts of protein were added for each lane of a particular experiment.

Fluorescence microscopy

Live yeast cells expressing GFP fusions were imaged 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, BD Biosciences Bioimaging, Rockville, MA).

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