Physical and Genetic Interactions Between Uls1 and the Slx5–Slx8 SUMO-Targeted Ubiquitin Ligase

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ABSTRACT The Slx5–Slx8 complex is a ubiquitin ligase that preferentially ubiquitylates SUMOylated substrates, targeting them for proteolysis. Mutations in SLX5, SLX8, and other SUMO pathway genes were previously identified in our laboratory as genomic suppressors of a point mutation (mot1-301) in the transcriptional regulator MOT1. To further understand the links between the SUMO and ubiquitin pathways, a screen was performed for high-copy suppressors of mot1-301, yielding three genes (MOT3, MIT1, and ULS1). MOT3 and MIT1 have characteristics of prions, and ULS1 is believed to encode another SUMO-targeted ubiquitin ligase (STUbL) that functionally overlaps with Slx5-Slx8. Here we focus on ULS1, obtaining results suggesting that the relationship between ULS1 and SLX5 is more complex than expected. Uls1 interacted with Slx5 physically in yeast two-hybrid and co-immunoprecipitation assays, a uls1 mutation that blocked the interaction between Uls1 and Slx5 interfered with ULS1 function, and genetic analyses indicated an antagonistic relationship between ULS1 and SLX5. Combined, our results challenge the assumption that Uls1 and Slx5 are simply partially overlapping STUbLs and begin to illuminate a regulatory relationship between these two proteins.

The ubiquitin family consists of a group of approximately 10 structurally related but functionally distinct proteins that are conjugated to substrates as part of regulatory signal transduction pathways. As the founding member, ubiquitin is by far the best understood member of the family, with the principles and techniques that emerge from studying ubiquitin helping to guide studies of the remaining family members (Kerscher et al. 2006). Like ubiquitin itself, the ubiquitin family member Small Ubiquitin-like MODifier (SUMO) has broad biological importance: the SUMO pathway is essential for viability in most eukaryotes, the components are highly conserved from yeast to humans, and more than 500 of the ~5800 yeast proteins are posttranslationally modified by SUMO, affecting 15 major biological pathways (Bettermann et al. 2012; Bossis and Melchior 2006; Denison et al. 2005; Makhnevych et al. 2009; Rosonina et al. 2010; Shin et al. 2005; Wohlschlegel et al. 2004). The core components of the SUMO pathway responsible for the maturation, conjugation, and removal of SUMO from substrates have been extensively characterized (Desterro et al. 1999; Geiss-Friedlander and Melchior 2007; Johnson and Blobel 1997; Johnson et al. 1997; Kerscher et al. 2006; Li and Hochstrasser 2000), and X-ray crystallographic structures are available for most of these proteins (Capili and Lima 2007; Lois and Lima 2005; Yunus and Lima 2009), revealing details of their catalytic mechanisms.

In contrast to the extensive progress studying SUMO conjugation and de-conjugation enzymes, less is known about regulators and downstream effectors of SUMOylation. SUMOylation can have different effects on its target proteins, mediated by disrupting or creating protein-protein interactions. These altered interactions result in different biological outcomes for different substrates, including changes in cellular localization and blocking or stimulating proteolytic degradation (Huang et al. 2003; Lallemand-Breitenbach et al. 2008; Lin et al. 2006). The differential downstream effects are very likely mediated by recognition of the SUMOylated substrate by different SUMO-binding effector proteins. One such SUMO-binding effector that begins to account for the differential effects of SUMO is Slx5-Slx8. Slx5-Slx8 is a heterodimeric ubiquitin E3 ligase that preferentially targets selective SUMO conjugates for ubiquitylation (Mullen and Brill 2008; Prudden et al. 2007; Tatham et al. 2008; Uzunova et al. 2007; Xie et al. 2007); Slx8 is the active ubiquitin E3 ligase, and it is recruited...
to SUMOylated substrates by its Sxl5 partner, which possesses several SUMO-interacting motifs (SIMs). Because the Sxl5-Sxl8 complex and its Schizosaccharomyces pombe, Drosophila, and human orthologs preferentially ubiquitylate SUMOylated substrates, they have been termed SUMO-Targeted Ubiquitin Ligases (STUbLs). This finding was unexpected because SUMO was proposed to compete with ubiquitin for some substrates (Desterro et al. 1998), but the existence of STUbLs demonstrated that SUMOylation can actually stimulate ubiquitylation of some proteins. Sxl5-Sxl8 also can target substrates via a SUMO-independent mechanism (Xie et al. 2010), but because of the notorious difficulty in identifying E3 substrates, it is currently unclear how many of its substrates are SUMO-dependent vs. SUMO-independent.

The finding that Sxl5-Sxl8 and its orthologs are STUbLs has raised the issue of whether other STUbLs exist. Rad18 targets PCNA for ubiquitylation through its intrinsic SUMO-binding activity and, thus, the Ubc4 ubiquitin E2 in pull-down assays (Uzunova et al. 2007), but the existence of other STUbLs has not been reported for Uls1 to date, and thus, other possibilities need to be considered. Importantly, ubiquitin E3 activity has not been reported for Uls1, and so, other papers have argued that Uls1 might be another STUbL with some functional overlap with Sxl5-Sxl8, although their specific relationship remains unknown. Importantly, ubiquitin E3 activity has not been reported for Uls1 to date, and thus, other possibilities need to be considered.

Our laboratory has been using a genetic approach to investigate the SUMO pathway. We previously found that a mutation in MOT1, which encodes an essential transcriptional regulator that removes TATA-binding protein from DNA (Auble et al. 1994), is extremely sensitive to perturbation of the SUMO pathway. Ninety-seven percent of mutations that suppressed MOT1-301 expression were in genes that encode components of the SUMO pathway (Wang et al. 2006), and mutations in every step of the SUMO pathway suppressed mot1-301. This selection, thus, is highly sensitive and extremely selective to defects in SUMOylation. Mot1-301 is an unstable protein due to its SUMO-, ubiquitin-, and proteosome-dependent degradation, and mutations in the SUMO pathway, the Sxl5-Sxl8 STUbL, the ubiquitin E2 Ubc4, or in K101 and K109, the presumed SUMOylation sites of Mot1-301, dramatically stabilize the protein, accounting for the suppression phenotype (Wang and Prelich 2009). Here we continue to take advantage of this system, using an overexpression strategy in an attempt to uncover additional components or regulators of the SUMO pathway. We report physical interactions and additional genetic interactions between Uls1 and Sxl5-Sxl8. Furthermore, we show that this interaction is important for Uls1 function, as loss of this interaction results in physiological deficiencies. Additionally, we report that Sxl5 is SUMOylated and that its SUMOylation is reduced by uls1Δ. These data are most consistent with a regulatory relationship between these two proteins rather than the current model in which they act as semi-redundant STUbLs.

### MATERIALS AND METHODS

#### Strains, plasmids, and media

Saccharomyces cerevisiae strains used in this study are listed in Table 1. All plasmids used in this study are listed in Table 2. All media used, including rich medium (YPD) and synthetic complete drop-out medium (for example, SC-Ura) were made as described previously (Rose et al. 1990). SC+Gal plates were synthetic complete (SC) medium containing 2% galactose and 1 µg/ml antimycin A. Standard genetic methods for mating, sporulation, transformation, and tetrad analysis were used throughout this study (Rose et al. 1990).

#### Screening for high-copy suppressors

To screen the systematic YGPM library (Jones et al. 2008) (pool and 96-well plates), a 100-ml GY2150 (mot1-301/mtot1-301 diploid) culture was grown at 30°C to 2 × 10^7 cells/ml. Cells were harvested and resuspended in water to a density of 4 × 10^8 cells/ml. Aliquots containing approximately 4 × 10^7 cells were placed into each well of a 96-well plate, pelleted at 3500 rpm for 10 min, and resuspended in 50 µl of transformation buffer (0.3 M LiOAc, 0.8 mg/ml salmon sperm carrier DNA). Ninety-six nanograms of each library plasmid DNA were added, cells were mixed with a multiplicity vortexer for 2 min, and 100 µl of 50% PEG (product code P3640-500G; Sigma) was added to each well. Cells were mixed for an additional 5 min. After a 2-hr incubation at 42°C, cells were pelleted, resuspended in water, and spotted to SC-leucine plates. Transformants were resuspended in water, plated on selective plates, and grown at 30°C for 2–4 days, and then selected SC+Gal-Leu plates at 37°C. Three plasmids were obtained, and by subcloning the responsible genes, we identified MOT1, ULS1, and MIT1. Because the known high-copy suppressors UBA2, UBC9, SMT3, and ULP2 did not emerge from this screen, a random genomic library (Yoshihisa and Anraku 1989) was transformed into ZY142 (mot1-301 haploid), screening for T° and Gal° phenotypes. From 9000 transformants examined, MOT1 (1×), MOT3 (2×), and ULS1 (6×) were identified as high-copy suppressors.

| Table 1 | S. cerevisiae strains used in this study |
|-----------------|------------------------------------|
| **Strain** | **Genotype** |
| GY285 | MATa leu2Δ1 ura3-52 |
| GY2280 | MATa his4-912b lys2-1286 succ2Δuas(-1900/-390) ura3-52 trp1Δ63 leu2Δ1 uls1Δ::KANMX |
| GY2296 | MATa his4-912b lys2-1286 succ2Δuas(-1900/-390) ura3-52 trp1Δ63 leu2Δ1 ura1Δ::TRP1 mot1-301 |
| OY844 | MATa his4-912b succ2Δuas(-1900/-390) trp1Δ63 leu2Δ1 ura3-52 mot1-301 his10Δ::KAN |
| OY843 | MATa his4-912b his3Δ200 trp1Δ63 leu2Δ1 ura3-52 mot1-301 his10Δ::KAN |
| ZY48 | MATa his4-912b lys2-1286 succ2Δuas(-1900/-390) ura3-52 leu2Δ1 sllΔ::URA3 mot1-301 |
| ZY142 | MATa his4-912b lys2-1286 succ2Δuas(-1900/-390) trp1Δ63 mot1-301 |
| ZY356 | MATa his4-912b lys2-1286 succ2Δuas(-1900/-390) trp1Δ63 leu2Δ1 ade8 mot1-301 |
| ZY528 | MATa his4-912b lys2-1286 succ2Δuas(-1900/-390) ura3-52 trp1Δ63 leu2Δ1 KI::TRP1::SLX5p-TAP-SLX5 |
| ZY616 | MATa his4-912b lys2-1286 succ2Δuas(-1900/-390) ura3-52 trp1Δ63 leu2Δ1 mot1-301-HA::KAN |
Table 2 Plasmids used in this study

| Plasmid | Genotype |
|---------|----------|
| pTW10   | AMP 2µ LEU2 MOT3 |
| pTW11   | AMP 2µ LEU2 ULS1 |
| pTW13   | AMP 2µ URA3 MOT3 |
| pTW24   | AMP 2µ LEU2 MIT1 |
| pTW32   | AMP 2µ LEU2 GAL4-AD-ULS1 |
| pTW48   | AMP 2µ LEU2 GAL4-AD-uls1ΔS31-897 |
| pTW6   | AMP CEN LEU2 HA-ULS1 |
| pTW78   | AMP 2µ LEU2 HA-uls1Δ370-373 |
| pTW94   | AMP 2µ LEU2 HA-uls1-D1108A,E1109A |
| pTW96   | AMP 2µ leuls1-C1385S |
| pTW104  | AMP 2µ leuls1-D1108A,E1109A |
| pTW105  | AMP 2µ leuls1-C1385S |
| pTW118  | AMP CEN LEU2 3HA-SLX5 |
| pTW120  | AMP 2µ leuls3HA-SLX5 |
| pTW125  | AMP CEN LEU2 3HA-SLX5-K31R |
| pTW129  | AMP CEN LEU2 3HA-SLX5-K465R,K473R |
| pTW131  | AMP 2µ leuls3HA-SLX5-K465R,K473R |
| pTW133  | AMP CEN LEU2 SLX5-GFP |
| pTW135  | AMP CEN LEU2 3HA-SLX5-K31R,K465R,K473R |
| pTW137  | AMP 2µ leuls3HA-SLX5-K31R,K465R,K473R |
| pTW143  | AMP 2µ leuls1-ΔRING(1328-1386) |
| pTW145  | AMP 2µ leuls1-ΔRING(1328-1386) |
| pTW147  | AMP 2µ leuls1-GAL4-AD-uls1(531-897) |
| pTW149  | AMP 2µ leuls1-GAL4-AD-uls1(554-955) |
| pTW151  | AMP CEN LEU2 HA-uls1A370-373 |
| pTW153  | AMP CEN LEU2 3HA-SLX5-ΔRING(1328-1386) |
| pTW155  | AMP CEN LEU2 HA-uls1-C1385S |
| pTW163  | AMP CEN LEU2 HA-uls1A531-897 |
| pTW167  | AMP CEN LEU2 HA-uls1-D1108A,E1109A |

Yeast two-hybrid assays

pGBKT7- and pGADT7-based plasmids containing Gal4BD or Gal4AD fused to SLX5, SLX8, UBC9, and SMT3 were described previously (Li et al. 2007). ULS1 and its derivatives were cloned into pGADT7 or pGBKT7 by standard PCR-based cloning. Combinations of plasmids were transformed into the yeast two-hybrid reporter strain PJ69-4A (James et al. 1996) and selected on SC plates lacking leucine and tryptophan. Positive interactions were detected on SC plates lacking leucine, tryptophan, and adenine.

Co-immunoprecipitation

For immunoprecipitation and Western analyses Uls1 was tagged at the N terminus with a single HA tag. HA-Uls1 was functional in all three times, and loaded onto an SDS polyacrylamide gel for detection by Western blotting.

RT PCR assays

To isolate RNA, 10 ml of cell culture (1 x 10^7 cells/ml) was centrifuged, washed with 1 ml of H2O, and resuspended in 0.2 ml of RNA breaking buffer (0.5 M NaCl, 0.2 M Tris-HCl [pH 7.6], 0.01 M EDTA, 1% SDS). Two hundred fifty microliters of washed glass beads and 0.2 ml of phenol:chloroform were added. After samples were vortexed for 2 min, 0.3 ml more RNA breaking buffer and 0.3 ml more phenol:chloroform were added. The aqueous phase was collected after centrifugation and extracted with 0.3 ml of phenol:chloroform. The aqueous phase was collected and mixed with ether. After 0.5 hr at −70°C, the pellet was harvested by centrifugation, washed with 70% ethanol, and dried in a Speed Vac. The final RNA product was dissolved in 100 µl of sterile water. Two micrograms of RNA was used to quantify the transcription level of the targeted gene with SYBR Green RT-PCR reagent kit (catalog no. 4310179; Life Technologies) and optimized primer concentrations. Primers used to detect MOT1 were GO1952 (5’TCTCTTCTCG ACCCCGATAACG) and GO1953 (5’TGCTTGGAATCCGCAATT), and GPD6 was detected using GO1954 (5’GATGTCCCACACC GTCTCITCCG) and GO1955 (5’GGCCACCGTCAAAAAACG).

Assays of protein half-life

Yeast cultures were grown overnight at 30°C to log phase in selective medium to maintain plasmids carrying the 3HA-tagged mot1-301 alleles. To start the chase, 1 ml of culture was first collected at time zero in an Eppendorf tube pre-loaded with 10 µl of 10% sodium azide. Cells were then pelleted and frozen on dry ice. Cycloheximide (catalog no. C7698; Sigma) was added to the remainder of the culture to a final concentration of 0.5 mg/ml, and 1-ml samples were collected every 10 or 20 min in tubes containing sodium azide and frozen on dry ice. Crude extracts were prepared by the postalkaline extraction method (Kushnirov 2000). Ten microliters of supernatant were loaded for SDS-PAGE, followed by Western blotting analysis using anti-HA antibody (code SC-7392; Santa Cruz Biotechnology) to detect Mot1 or anti-G6PDH (code A9521; Sigma) to detect G6PDH as a loading control.

RESULTS

Identification of high-copy suppressors of mot1-301

We previously found that selecting for mutations that suppress mot1-301 is a remarkably sensitive method to identify mutations in the SUMO pathway (Wang et al. 2006). Mutations that affect the SUMO pathway, the Slx5-Slx8 STUbL, or the Ubc4 ubiquitin E2 stabilize the mot1-301 alleles. To start the chase, 1 ml of culture was first collected at time zero in a Eppendorf tube pre-loaded with 10 µl of 10% sodium azide. Cells were then pelleted and frozen on dry ice. Cycloheximide (catalog no. C7698; Sigma) was added to the remainder of the culture to a final concentration of 0.5 mg/ml, and 1-ml samples were collected every 10 or 20 min in tubes containing sodium azide and frozen on dry ice. Crude extracts were prepared by the postalkaline extraction method (Kushnirov 2000). Ten microliters of supernatant were loaded for SDS-PAGE, followed by Western blotting analysis using anti-HA antibody (code SC-7392; Santa Cruz Biotechnology) to detect Mot1 or anti-G6PDH (code A9521; Sigma) to detect G6PDH as a loading control.

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Insights Into the suppression mechanism of 2µ ULS1

We were intrigued by the ability of 2µ ULS1 to suppress mot1-301 because although ULS1 has been functionally linked with the SUMO pathway, it is proposed to be another STUbL that functionally overlaps with Slx5-Slx8. We therefore tested whether deletion of ULS1 suppressed mot1-301. Although slx5Δ and slx8Δ did not, and conversely, 2µ ULS1 suppressed mot1-301 but 2µ SLX5 did not (Figure 3A). Thus, ULS1 and SLX5 displayed opposing patterns of suppression. The mechanism by which SUMO pathway mutations suppress mot1-301 is understood: the Mot1-301 protein becomes SUMOylated, presumably as part of a quality control surveillance mechanism (Wang and Prelich 2009), which results in recruitment of the Slx5-Slx8 STUbL, followed by ubiquitylation and proteosome-mediated degradation of Mot1-301. SUMO pathway defects thereby increase Mot1-301 stability and steady-state protein levels, with SUMOylated Mot1-301 accumulating in slx5Δ strains. We examined whether overexpression of ULS1 had the same effects. As expected, 2µ ULS1 increased the Mot1-301 protein level (Figure 3B), and no significant change in mot1-301 transcription was detected by RT-PCR (Figure 3C). The stability of Mot1-301 was examined using a cycloheximide chase protocol, revealing an increase in Mot1-301 protein stability when ULS1 was overexpressed (Figure 3D). Finally, 2µ ULS1 increased the level of Mot1-301 SUMOylation similar to that of the slx5Δ control (Figure 3E). Thus, by these criteria, the overexpression of ULS1 mimics effects caused by deletion of SLX5, with 2µ ULS1 causing slightly weaker effects than slx5Δ.

A plausible genetic interpretation for the similarities between slx5Δ and 2µ ULS1 was that overexpression of ULS1 counteracted or interfered with the function of the Slx5-Slx8 STUbL. If this model was true, then overexpressing SLX5 or SLX8 might reverse the 2µ ULS1 phenotype. A mot1-301 strain containing a 2µ ULS1 plasmid therefore was transformed with 2µ (high-copy number) or CEN (low-copy number) plasmids containing SLX5 or SLX8. Indeed, the 2µ ULS1 phenotype was reversed by 2µ SLX5 but not by 2µ SLX8, and surprisingly, even a CEN SLX5 plasmid reversed the 2µ ULS1 phenotype (Figure 4A). This result supported the idea that 2µ ULS1 might suppress mot1-301 by inhibiting or interfering with SLX5. Because Uls1 and Slx5 both interact physically with the ubiquitin E2 Ubc4 (Uzunova et al. 2007), we tested whether overexpression of UBC4 reversed the suppression of mot1-301 by 2µ ULS1. The suppression caused by 2µ ULS1 was not abolished (data not shown), suggesting that 2µ ULS1 does not suppress mot1-301 by titrating Ubc4 away from Slx5. To determine whether suppression by 2µ ULS1 is mediated via Slx5, we tested whether deletion of SLX5 counteracted the suppression caused by 2µ ULS1. Thus, deletion of SLX5 did not reverse the suppression caused by 2µ ULS1 (Figure 4B). Thus, deprivation of Slx5-Slx8 is required for the high-copy phenotype of MOT3 and MIT1 but not for SUMO pathway-mediated suppression of mot1-301. (A) The indicated 2µ plasmids including empty prs425 vector were introduced into YZ142 (mot1-301 HSP104Δ) and OY844 (mot1-301 hsp104Δ) strains, and dilutions of transformants were spotted to test the Ts and Gal phenotypes. (B) Known SUMO pathway genes that suppress mot1-301 were overexpressed in OY844 (mot1-301 hsp104Δ), and the indicated phenotypes were tested by spotting. Control refers to SC-Leu plates at 30°C.
ULS1. (D) Mot1-301 stability was examined in ZY142 (mot1-301), ZY613 (mot1-301 slx5Δ), and ZY142 transformed with 2μ ULS1 by Western blotting during a cycloheximide chase. (E) The levels of Mot1-301 SUMOylation were assayed in ZY142 (mot1-301), ZY613 (mot1-301 slx5Δ), and ZY142 transformed with 2μ ULS1. Proteins were subjected to immunoprecipitation (IP) and Western blotting (WB) using the indicated antibodies. The α-HA blot on the left shows the level of Mot1-301 protein in crude extracts, and Ponceau staining of the filter shows equivalent transfer of proteins across the membrane.

SLX5 was specific for ULS1 or instead was a more general phenomenon, we tested whether overexpression of SLX5 was able to reverse the phenotypes of other high-copy suppressors of mot1-301. 2μ SLX5 reversed the 'Ts' and Gal' phenotypes caused by 2μ ULS1 but not those of 2μ SMT3, UBA2, UBC9, ULP2, MOT3, or MIT1 (Figure 4B). In another test of specificity, the SUMO pathway genes whose overexpression does not suppress mot1-301 were tested for the ability to reverse the 2μ ULS1 phenotype. Overexpression of those SUMO pathway genes reversed the 2μ ULS1 phenotype to various extents, with 2μ SLX5, SIZ1, and SIZ2 having the strongest effect (Figure 4C). Together these results suggest that ULS1 opposes or antagonizes SLX5.

Physical interaction between Uls1 and Slx5

Prompted by the genetic interactions between Sli5 and Uls1, we next determined whether we could detect any physical interactions between these two proteins. We first assayed for physical interactions between Uls1 and Sli5-Slx8 by using the yeast two-hybrid system. Gal4AD-Uls1 was positive with Gal4BD-Slx5 in the two-hybrid system but not with empty Gal4BD or Gal4BD-Ubc9 controls (Figure 5A). A much weaker interaction between AD-Uls1 and BD-Slx8 also was observed. Co-immunoprecipitation (Co-IP) assays were performed to further test for physical interactions between Uls1 and Slx5. Tagged versions of Uls1 and Slx5 co-immunoprecipitated regardless of which protein was immunoprecipitated (Figure 5B), whereas no Uls1-Slx8 interaction was detectable by co-IP (data not shown). These results indicated that a physical interaction occurs between Uls1 and Slx5, although from these assays, we cannot distinguish whether the interaction is direct or requires intermediates.

To gain insight into the physical interaction between Uls1 and Slx5, we next examined the domains of Uls1 that were required for this interaction. In particular, because both Uls1 and Slx5 contain SIMs and SUMO, we wanted to determine whether the interaction was mediated by the SIMs and SUMO. Different ULS1 fragments were constructed into a Gal4-AD yeast two-hybrid vector (Figure 6A, bottom) and tested for interaction with BD-Slx5. In the context of full-length AD-Uls1, an internal deletion of amino acids 531–897 was unable to interact with Slx5 (Figure 6B). This defect was not simply the result of an expression or general folding problem, because Uls1Δ531–897 maintained the interaction with BD-Smt3 (SUMO) in the yeast two-hybrid system (Figure 6B) and was expressed well of double transformants were tested. Relative to the empty vector transformants, overexpression of SLX5 only reversed the phenotypes of 2μ ULS1 not that of the other high-copy suppressors. (C) Empty vector or 2μ ULS1 was transformed into strain ZY356 (mot1-301) overexpressing the indicated genes, and the phenotypes of double transformants were tested. A range of suppression is observed, with 2μ SLX5 and 2μ SIZ2 showing the strongest effect.
Uls1-Slx5 interaction did not require binding of Uls1 or Slx5 to the two-hybrid system. Both the AD-Uls1531 SIM at amino acids 543–551 was sufficient for interacting with Slx5, the Uls1531–551 fragment and a par- tially overlapping fragment (Uls1554–953) that lacks the purported SIM at amino acids 543–551 (Uzunova et al. 2007) were tested in the two-hybrid system. Both the AD-Uls1531–554 and AD-Uls1554–955 fragments were sufficient for interaction with BD-Slx5 (Figure 6B), and neither interacted with SUMO in the two-hybrid system. Mutations of the Slx5 SIMs that abolish binding to SUMO also had no effect on the Uls1-Slx5 two-hybrid interaction (see Supporting Information, Figure S1). Combined, these results indicated that a re- gion located between the Uls1 SIMs and ATPase domain was required and sufficient to interact with Slx5 in vivo and that the Uls1-Slx5 interaction did not require binding of Uls1 or Slx5 to SUMO.

Domains required for ULS1 function

Having identified the region responsible for the interaction with Slx5, we next used two assays to determine the domains of Uls1 that were required for its function. We first tested which of the Uls1 domains was required for its high-copy suppression of mot1-301. Missense mutations or deletions (Figure 6A, upper) were generated in the SIMs (Shirai and Mizuta 2008), ATPase, RING, and Slx5-interacting domains that are predicted to inactivate their functions, and those derivatives were tested for their effects on the 2μ ULS1 plate phenotype (Figure 7A). Mutations in the ATPase, Slx5-interacting domain, and SIMs greatly reduced the ULS1 high-copy phenotypes (Figure 7A, lanes 3, 4, and 7), with some residual activity observed in the SIM mutant upon further incubation, likely because additional SIM-like motifs have been described for Uls1 (Uzunova et al. 2007). The RING domain mutations were less informative because although the uls1ΔRING mutation abolishes the suppression phenotype of 2μ ULS1 (Figure 7A, lane 5), its expression was greatly reduced. The expression of the uls1ΔC138SS RING missense mutation also was re- duced compared to that of wild-type ULS1 but did not abolish the suppression phenotype (Figure 7A, lane 6). Because we and others have been unable to detect E3 activity for Uls1, it remains unknown whether the C138SS mutation abolishes potential E3 activity of Uls1. Taken together, overexpression of ULS1 suppresses mot1-301 through a mechanism that minimally requires the Uls1 ATPase and SUMO-binding activities and its interaction with Slx5.

Because a recent large-scale study suggested that uls1Δ is sensitive to cycloheximide (Alamgir et al. 2010), we tested whether the uls1Δ domain mutations complemented the uls1Δ cycloheximide-sensitive phenotype when present on a low-copy CEN plasmid. Similar to results obtained for the ULS1 high-copy phenotype in suppressing mot1-301, the Uls1 ΔATPase domain and the Slx5-interacting region were required to complement the uls1Δ cycloheximide-sensitive pheno- type (Figure 7B, lanes 4 and 7). The SIM mutation had no effect (Figure 7B, lane 3), and the requirement for the RING domain is difficult to assess (Figure 7B, lanes 5 and 6) for the reasons listed above. These results demonstrated that the ATPase domain and the interaction with Slx5 were important for Uls1 function, both in the context of the ULS1 overexpression phenotype and in a low-copy number complementation context.

Figure 6 Identifying the region of Uls1 that interacts with Slx5. (A) A schematic illustration of the Uls1 domain architecture is displayed on top, with mutations in the SIM, ATPase, and RING domains shown below the protein. Uls1-AD two-hybrid derivatives are on the bottom. (B) Interactions of different Uls1-AD fragments were tested in the yeast two-hybrid system. Full-length Gal4AD-Uls1 (1–1619) and its indicated derivatives were retransformed into the yeast two-hybrid reporter strain PJ69-4A expressing binding domain (BD) only, BD-Slx5, or BD-Smt3 (SUMO). Transforms were selected and replica plated to test their His and Ade phenotypes, which are indicative of activation of the two-hybrid reporters. C-His plates contained 10 mM 3AT to reduce background signal. (C) Gal4AD-Uls1 and its derivatives were assayed for their expression level by Western blotting. The asterisks indicate migration of the respective fusion proteins.
tested whether Slx5 was SUMOylated and, if so, whether its modification was affected by ULS1. 3HA-tagged Slx5 was immunoprecipitated with HA beads and Western blotted to assess whether it was SUMOylated. When we probed with an anti-SUMO antibody, discrete bands and a high-molecular-weight smear were detected, both of which increased in intensity in a 2μ 3FLAG-SMT3 strain that overexpresses SUMO (Figure 9A), indicating that Slx5 was SUMOylated. Probing the same samples with an anti-FLAG antibody revealed a band that migrated more slowly than Slx5 when 3FLAG-SMT3 was expressed, confirming that Slx5 was SUMOylated, although the 3FLAG-SUMO fusion was less efficient than untagged SUMO for forming higher molecular weight conjugates. We then examined whether SUMOylation of 3HA-Slx5 was affected by ULS1. As shown in Figure 9B, SUMOylation of Slx5 was reduced in the uls1Δ strain but not when ULS1 was overexpressed, indicating that Uls1 affected the SUMOylation of Slx5. To test whether this effect was specific, the SUMOylation of Toa1, a known SUMO substrate, was examined in the uls1Δ and 2μ ULS1 strains (Figure 9C). The Toa1 SUMOylation level remained unchanged regardless of the ULS1 genotype, indicating that the effect of uls1Δ on Slx5 SUMOylation displayed some specificity.

**DISCUSSION**

The results presented here reveal unexpected connections between ULS1 and SLX5 and provide new insights into their relationship. Previous results suggested that Uls1 and Slx5-Slx8 are independent STUbLs that have overlapping roles in targeting SUMOylated substrates for degradation, based on the presence of SIMs and a RING domain in Uls1, the accumulation of high-molecular-weight SUMO conjugates in a uls1Δ strain, the combinatorial phenotypes in uls1Δ slx5Δ double mutants, and the physical interactions with ubiquitin E2 Ubc4 detected for both Uls1 and Slx5 (Uzunova et al. 2007). Other results, however, suggested that a re-evaluation of the proposed role of Uls1 as a redundant STUbL with Slx5 was warranted. First, no ubiquitin E3 activity has been reported for Uls1 to date. Second, not every RING protein possesses E3 activity; RING proteins such as Slx5, Bard1, Tfb3, and Far1 (Deshaies and Joazeiro 2009) for example, do not have intrinsic ubiquitin E3 activity, although Slx5 and Bard1 associate directly with RING-containing E3s. Third, deletion of genes that do not encode E3s, such as SGS1, SRS2, and ULP2, leads to accumulation of high-molecular-weight SUMO conjugates similar to
ulslΔ (Mullen and Brill 2008), so the presence of high-molecular-weight conjugates does not necessarily imply loss of a STUbL. Fourth, multiple mutations result in combinatorial growth defects with slx5Δ (Pan et al. 2006; Wang et al. 2006), but many of these genes do not encode ubiquitin E3s (AOS1, SMT3, CCR4, and many others). This is not surprising, as synthetic sick or lethal combinations can be used to infer a functional link, but the mechanistic basis for that link often remains obscure. Finally, it is not known whether the physical interactions of Uls1 and Slx5 with Ubc4 are direct. Thus, in our view the dual issues of whether Uls1 is a STUbL and its relationship with the Slx5-Slx8 STUbL remain open questions.

Because of the inability to express recombinant Uls1, our results do not directly address the issue of whether Uls1 is a STUbL, but they do imply that the relationship between ULS1 and SLX5 is more complex than their being STUbLs with partially overlapping functions. First, ULS1 and SLX5 displayed opposite patterns of suppression of mot1-301; slx5Δ strongly suppressed mot1-301, but uls1Δ had no effect on mot1-301, and conversely, overexpression of ULS1 suppressed mot1-301, but overexpression of SLX5, SLX8, or SLX5 and SLX8 did not. These results are most consistent with Uls1 and Slx5-Slx8 having opposing, not overlapping, roles in vivo; and because slx5Δ but not uls1Δ stabilizes Mot1-301, they are not redundant for targeting the ubiquitylation and destruction of Mot1-301. The lack of known substrates is clearly hindering progress with understanding these proteins, but we are not aware of any substrates that are redundantly targeted by Slx5 and Uls1. As additional substrates of Slx5-Slx8 become identified, it will be interesting to test whether the antagonistic relationship that we detect here for Slx5 and Uls1 toward Mot1-301 is also applicable to those substrates. Second, we detected a strong interaction between Slx5 and Uls1 in yeast two-hybrid and co-immunoprecipitation assays, and an internal ULS1 deletion that specifically abolished the interaction with Slx5 reduced the function of ULS1 (Figure 7), suggesting that the two proteins function together or that one protein regulates the other. A physical interaction between these two proteins would not be expected if they were acting simply as independent STUbLs. At this point, we cannot distinguish whether the interaction between Uls1 and Slx5 is direct or mediated by another protein that interacts with both Slx5 and Uls1, such as Elg1 (Parnas et al. 2011) or Ubc4 (Uzunova et al. 2007). Third, increasing expression of SLX5 reversed the 2μ ULS1 phenotype (Figure 6A). The simplest interpretation of these combined results is that Uls1 negatively affects the function of Slx5. We expect that Slx5 would be hyperactive in a uls1Δ strain, but we cannot assess that prediction due to the absence of any known hypermorphic SLX5 phenotype.

This study provides the first experimental tests to define the functional domains of Uls1. Mutational analysis confirmed the functional importance of both the ATPase and SIM motifs, and in addition, we were able to identify a domain in Uls1 located between the RING and ATPase domains that was both required and sufficient for interaction with Slx5. Importantly, the interaction-defective mutant uls1ΔS31-897 was defective for the ULS1 high-copy phenotype and was unable to fully complement the uls1Δ phenotype when present on a CEN plasmid, suggesting that the Uls1-Slx5 interaction is functionally relevant. In agreement with the requirement for a separate domain, the SIMs of Uls1-Slx5 are not redundant for interaction with Slx5. In addition, we were able to identify a domain in Uls1 located between the RING and ATPase domains that was both required and sufficient for interaction with Slx5. Importantly, the interaction-defective mutant uls1ΔS31-897 was defective for the ULS1 high-copy phenotype and was unable to fully complement the uls1Δ phenotype when present on a CEN plasmid, suggesting that the Uls1-Slx5 interaction is functionally relevant. In agreement with the requirement for a separate domain, the SIMs of Uls1 and Slx5 were not required for the interaction between the two proteins (Figure 6B and Figure S1), excluding the possibility that the interaction is mediated by SUMO or SUMOylated substrates. We cannot rule out the possibility, however, that the SIMs of Slx5 or Uls1 can regulate the interaction.

The genetic links established between ULS1 and SLX5 imply that Uls1 inhibits Slx5 and raise the issue of how this might occur mechanistically. Uls1 did not affect the steady-state protein level or cellular localization of Slx5. We therefore suspect that it affects the function of Slx5 at some other level such as its ability to recognize SUMOylated substrates or its interaction with Slx5 or other proteins or by affecting the E3 activity of the Slx5-Slx8 complex. We were able to detect that Slx5 was SUMOylated and that the SUMOylation of Slx5 was reduced by uls1Δ (Figure 9B). We attempted to create a SUMOylation-deficient slx5Δ mutant to assess its functional significance, but as has been observed for other SUMO substrates (Psakhye and Jentsch 2012), nonsense mutations at three predicted SUMOylation sites of Slx5 (K31, K465, and K473) either alone or in combination did not abolish the SUMOylation of Slx5 (data not shown). Determining the relevance of SUMOylation on Slx5 function thus will require a more complete study to map and mutate the SUMOylation sites.
Although this report focuses on the relationship between UlS1 and Slx5, two other results from our library screen should prove to be interesting subjects for further study. First, although directed overexpression of SMT3, UBA2, UBC9, and ULP2, all of which are known components of the SUMO conjugation and de-conjugation pathway, suppress mot1-301, overexpression of the other pathway components AOS1, SIZ1, SIZ2, ULP1, and UBC4 did not suppress. It is unclear whether this is due to a trivial explanation such as the extent of overexpression, or whether it reveals more about the regulation or roles of these genes. Second, the two other high-copy suppressors identified in our screen suppress mot1-301 by an unanticipated mechanism that is distinct from ULS1 and the previously identified genomic suppressors (Wang et al. 2006). MOT3 encodes a transcription regulator (Hongay et al. 2002; Madison et al. 1998) that can exist in a prion state, mediated by its glutamine-rich and asparagine-rich repeats (Alberti et al. 2009). MTT1 also encodes a transcription regulator (Cain et al. 2012) with an asparagine-rich domain, and it also possesses prion-like properties (Alberti et al. 2009). Interestingly, the suppression of mot1-301 by high-copy MOT3 and MTT1, but not by high-copy ULS1, is dependent on HSP104, which is required for prion formation and propagation (Chernoff et al. 1995; Satpute-Krishnan et al. 2007; Wegrzyn et al. 2001). This result indicates that the overexpression of MOT3 and MTT1 increased the level of the Mot1-301 protein by a different mechanism from overexpression of ULS1 and that the formation of prions was responsible for their high-copy suppression phenotype. Interestingly, protein aggregates and prions have been reported to inhibit the proteosome (Bence et al. 2001; Deriziotis and Tabrizi 2008; Kristiansen et al. 2007), and inhibition of proteosomal degradation by MOT3 and MTT1 prions provides a satisfying model to explain their effects on stabilization of Mot1-301.

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