SUMO Pathway Modulation of Regulatory Protein Binding at the Ribosomal DNA Locus in *Saccharomyces cerevisiae*

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ABSTRACT In this report, we identify cellular targets of Ulp2, one of two *Saccharomyces cerevisiae* small ubiquitin-related modifier (SUMO) proteases, and investigate the function of SUMO modification of these proteins. PolySUMO conjugates from *ulp2Δ* and *ulp2Δ slx5Δ* cells were isolated using an engineered affinity reagent containing the four SUMO-interacting motifs (SIMs) of Slx5, a component of the Slx5/Slx8 SUMO-targeted ubiquitin ligase (STUBL). Two proteins identified, Net1 and Tof2, regulate ribosomal DNA (rDNA) silencing and were found to be hypersumoylated in *ulp2Δ*, *slx5Δ*, and *ulp2Δ slx5Δ* cells. The increase in sumoylation of Net1 and Tof2 in *ulp2Δ*, but not *ulp1ts* cells, indicates that these nucleolar proteins are specific substrates of Ulp2. Based on quantitative chromatin-immunoprecipitation assays, both Net1 and Tof2 lose binding to their rDNA sites in *ulp2Δ* cells and both factors largely regain this association in *ulp2Δ slx5Δ*. A parsimonious interpretation of these results is that hypersumoylation of these proteins causes them to be ubiquitylated by Slx5/Slx8, impairing their association with rDNA. Fob1, a protein that anchors both Net1 and Tof2 to the replication-fork barrier (RFB) in the rDNA repeats, is sumoylated in wild-type cells, and its modification levels increase specifically in *ulp2Δ* cells. Fob1 experiences a 50% reduction in rDNA binding in *ulp2Δ* cells, which is also rescued by elimination of Slx5. Additionally, overexpression of Sir2, another RFB-associated factor, suppresses the growth defect of *ulp2Δ* cells. Our data suggest that regulation of rDNA regulatory proteins by Ulp2 and the Slx5/Slx8 STUBL may be the cause of multiple *ulp2Δ* cellular defects.

KEYWORDS SUMO; Ulp2; rDNA; RENT complex; Fob1

POST-TRANSLATIONAL protein modifications provide a common mechanism for regulating protein–protein interactions, protein localization, and protein degradation. The small ubiquitin-related modifier (SUMO) protein family modifies target proteins through covalent attachment to lysine side chains (Johnson 2004). The function of substrate “sumoylation” varies with the protein that is sumoylated, but

a common role is to modulate the assembly of large protein complexes either by creating additional binding sites or by blocking existing sites (Kerscher 2007).

Protein sumoylation occurs through an enzymatic cascade similar to ubiquitylation (Johnson 2004). In *Saccharomyces cerevisiae*, a single gene, *SMT3*, codes for SUMO, and as in most species, SUMO ligation is essential for viability. Removal of SUMO from target proteins is also an important regulatory step. *S. cerevisiae* has two SUMO proteases, Ulp1 and Ulp2 (Hickey *et al.* 2012). Most Ulp1 is bound to the nuclear pore complex (NPC), and the enzyme is essential for cell-cycle progression. NPC tethering regulates Ulp1 activity by restricting its access to sumoylated proteins (Li and Hochstrasser 2003; Panse *et al.* 2003). Ulp1 also processes the SUMO precursor by removing its last three residues, thereby exposing the C-terminal Gly-Gly motif required for SUMO conjugation.

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doi: 10.1534/genetics.116.187252

Manuscript received July 30, 2015; accepted for publication January 20, 2016; published Early Online January 28, 2016.

Supporting information is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.187252/-/DC1.

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Ulp1 is essential for growth even if cells are provided with mature SUMO, indicating a critical function for its protein desumoylation activity.

Ulp2 is less active than Ulp1 *in vitro* and is not required for viability, although cells lacking Ulp2 are growth defective and sensitive to a wide range of stresses. Ulp2 localizes throughout the nucleus, with a slight concentration in the nucleolus (Srikumar *et al.* 2013b). It has long, poorly conserved regions flanking the catalytic domain, which are of ill-defined function other than a pair of nuclear-localization signals (NLSs) in the N-terminal domain (Kroetz *et al.* 2009). Large amounts of high molecular weight (HMW) SUMO conjugates, which accumulate in the stacker of SDS-PAGE gels, are observed in *ulp2Δ* cells. Correspondingly, Ulp2 has a preference *in vitro* for cleaving between SUMO monomers in polySUMO chains (Hickey *et al.* 2012; Eckhoff and Dohmen 2015). The identities of most of the HMW-SUMO conjugates are unknown, as is their contribution to the growth defects of *ulp2Δ* cells (Bylebyl *et al.* 2003).

Slx5/Slx8 is a heterodimeric ubiquitin ligase (E3) that can recognize SUMO-protein conjugates and is therefore referred to as a SUMO-targeted ubiquitin ligase (STUbL) (Prudden *et al.* 2007; Sun *et al.* 2007; Uzunova *et al.* 2007; Xie *et al.* 2010). The Slx5 subunit interacts directly with SUMO and polySUMO chains through four tandem SUMO-interacting motifs (SIMs). Slx8 has a single apparent SIM (Uzunova *et al.* 2007; Xie *et al.* 2007; Mullen and Brill 2008). Deleting the *SLX5* gene suppresses *ulp2Δ* defects but exacerbates those of the *ulp1ts* mutant, consistent with distinct cellular roles of the two SUMO proteases (Xie *et al.* 2007; Mullen *et al.* 2011). Slx5/Slx8 has multiple functions and targets [not all requiring prior SUMO ligation (Xie *et al.* 2010)], including roles in DNA replication and repair (Burgess *et al.* 2007). The STUbL localizes at various sites within the nucleus as well, such as DNA replication forks and the NPC (Torres-Rosell *et al.* 2007; Nagai *et al.* 2008; Cook *et al.* 2009).

The SUMO pathway has been linked to the nucleolus, the cellular hub for the initial stages of ribosome assembly (Strunnikov *et al.* 2001; D'Amours *et al.* 2004; Panse *et al.* 2006; Takahashi *et al.* 2008; Srikumar *et al.* 2013b). To maintain robust ribosome production, cells have numerous ribosomal DNA (rDNA) copies, even though repeated DNA is prone to aberrant homologous recombination. *S. cerevisiae* has between 100 and 200 copies of the rDNA repeat, which consists of the 35S precursor rRNA gene, the 5S rRNA gene, and two nontranscribed spacers (Straight *et al.* 1999). Excessive homologous recombination of the rDNA leads to an increased number of rDNA repeats, which can produce extrachromosomal rDNA circles, potentially promoting senescence (Sinclair and Guarente 1997). Suppression of homologous rDNA recombination relies on a variety of proteins, and many post-translational modifications of nucleolar proteins have been implicated in rDNA silencing, including acetylation, phosphorylation, methylation, ubiquitylation, and sumoylation (Straight *et al.* 1999; Shou *et al.* 2002; Queralt *et al.* 2006; Ide *et al.* 2013; Ryu and Ahn 2014).

In this study, we have identified Net1 and Tof2 as proteins that become highly sumoylated in *ulp2Δ* cells. These paralogous scaffolding proteins are part of a complex rDNA regulatory system (see Figure 8). Net1 along with Sir2 and Cdc14 form the regulator of nucleolar silencing and telophase exit (RENT) complex (Straight *et al.* 1999). Nucleolar silencing by the RENT complex prevents homologous recombination of the rDNA repeats and represses RNA polymerase II transcription within the region. Deleting *NET1* causes nucleolar proteins, such as Nop1, to mislocalize throughout the nucleus (Straight *et al.* 1999; Shou *et al.* 2001). Sir2 is a histone deacetylase that deacetylates histones H3 and H4 in the silent genomic regions in yeast, which include rDNA (Imai *et al.* 2000). Sir2 localizes to the rDNA by binding Net1, which also activates Sir2. Cdc14, the final member of the RENT complex, is a phosphatase responsible for dephosphorylating specific cell-cycle regulators in a coordinated fashion (Shou *et al.* 2001; Yoshida *et al.* 2002). Cdc14 is sequestered in the nucleolus and its activity is suppressed when bound to Net1 in the cell-cycle stages leading up to early anaphase (Waples *et al.* 2009). Cdc14 is released from the nucleolus in a two-step process during mitosis, and once released, it dephosphorylates Cdk1 targets, leading to exit from mitosis (Waples *et al.* 2009). Net1 localizes to the rDNA through binding to Fob1 and RNA polymerase I (RNAPI) (Huang and Moazed 2003). Fob1 is a replication fork-blocking protein that binds specifically to the replication-fork barrier (RFB) sequence in the nontranscribed regions of rDNA repeats (Kobayashi and Horiuchi 1996).

Tof2 also binds to Fob1 and Cdc14, but not to Sir2 or RNAPI (Huang and Moazed 2003). Furthermore, Tof2 interacts with the cohibin complex, Lrs4-Csm1, which in turn binds to and localizes cohesin and condensin complexes (Huang *et al.* 2006; Johzuka and Horiuchi 2009). Cohibin additionally associates with Src1-Nur1, thereby tethering the rDNA to the inner nuclear membrane (Huang and Moazed 2003; Graumann *et al.* 2004). Particularly germane to the present work, cohibin also binds to and localizes Ulp2 to the nucleolus (Srikumar *et al.* 2013b), placing Ulp2 physically adjacent to Net1 and Tof2, consistent with previous chromatin immunoprecipitation (ChIP) analysis of Ulp2 within the rDNA repeats (Strunnikov *et al.* 2001). Tof2 has been shown to be sumoylated and the level of the sumoylation increases when cells are treated with MMS, a DNA-damaging agent (Wohlschlegel *et al.* 2004; Denison *et al.* 2005; Cremona *et al.* 2012; Albuquerque *et al.* 2013; Srikumar *et al.* 2013b).

Protein complex formation on the rDNA is hierarchical (see Figure 8). Fob1 is required for both Net1 and Tof2 to bind to the RFB (Huang and Moazed 2003). RNAPI localizes Net1 to the transcription initiation region (TIR) (Huang and Moazed 2003). Net1 is in turn necessary for Sir2 and Cdc14 nucleolar localization (Huang and Moazed 2003). Proper Tof2 localization is required for the proper placement of the cohibin complex (Huang *et al.* 2006), which ultimately is needed for cohesin and condensin localization (Johzuka and Horiuchi 2009). The combination of Tof2 and Fob1 is required for DNA helicases and topoisomerases to bind to the rDNA (Weitao

et al. 2003; Krawczyk *et al.* 2014). Therefore, the regulation/binding of *Fob1* can have a large impact on multiple rDNA-binding proteins.

The composition of rDNA chromatin changes through the cell cycle. During S phase, *Fob1* functions as a unidirectional, physical block to the DNA replication machinery (Kobayashi and Horiuchi 1996). Paradoxically, *Fob1* both is required for and acts to prevent homologous rDNA recombination (Kobayashi *et al.* 1998). Stalled replication forks create torsional strain in the replicating DNA, and *Fob1* recruits topoisomerase I and DNA helicases to the stalled fork to relieve the strain (Weitao *et al.* 2003; Krawczyk *et al.* 2014). Conversely, the RFB is downstream of the 35S transcription region and prevents the DNA polymerase machinery from proceeding into the RNAPII-transcribed region. The resulting stalled replication fork is responsible for the high rates of DNA double-stranded breaks and homologous recombination that characterize the rDNA locus (Kobayashi and Horiuchi 1996).

After S phase, *Fob1* primarily functions to link the RENT complex with cohesin and condensin (Huang *et al.* 2006). The levels of *Tof2* at the rDNA locus might also be cyclical, with *TOF2* mRNA expression peaking in S/G₂ phase (Spellman *et al.* 1998; Pramila *et al.* 2006). In early anaphase, *Net1* is phosphorylated by *Cdc5* and releases both *Cdc14* and *Sir2*, the other two RENT complex components (Shou *et al.* 1999; Straight *et al.* 1999). As cells enter anaphase, condensin redistributes from throughout the nucleus to the nucleolus and telomeres. In anaphase, *Cdc14* is fully released from the nucleolus by the mitotic exit network (Waples *et al.* 2009), as is the cohibin complex (Brito *et al.* 2010). *Ycs4*, a component of condensin, is polysumoylated in anaphase. Both the modification of *Ycs4* and the redistribution of condensin are dependent on *Ulp2* (D'Amours *et al.* 2004).

In this article, we investigate the role of sumoylation at the rDNA locus with a particular focus on *Net1*, *Tof2*, and *Fob1*. *Net1* and *Tof2* were isolated from a purification of HMW-SUMO conjugates from *ulp2Δ* cells. All three proteins are sumoylated and are *Ulp2* targets. We find that binding of these proteins to rDNA is altered in various SUMO pathway mutants, in particular *ulp2Δ* cells. Importantly, loss of the *Slx5/Slx8* STUbL in *ulp2Δ* cells substantially reverses these rDNA-binding defects. Loss or overexpression of these proteins and other RENT complex components also shows genetic interactions with *ulp2Δ*. Most notably, the overexpression of *Sir2* suppresses the temperature-sensitive growth defect of *ulp2Δ* cells and this suppression depends on *Sir2* localization to the rDNA. Taken together, these results highlight the importance of the SUMO pathway in controlling rDNA chromatin composition.

Materials and Methods

Bacterial and yeast growth and manipulation

Yeast strains used in this study are listed in Supporting Information, Table S1. Standard techniques were used for growing and manipulating yeast and *Escherichia coli*

(Guthrie and Fink 1991). Deletion strains in the BY4741 genetic background were retrieved from the *Saccharomyces* Gene Deletion Project collection (Open Biosystems) (Winzeler *et al.* 1999). TAP-tagged strains were from the Yeast TAP-Tag ORF library (GE) (Ghaemmaghami *et al.* 2003).

Plasmid construction

Plasmids containing either the *His10-GST-SLX5_{4xSIM}* or the *His10-GST-slx5_{4xmsim}* insert were created by cloning the *SLX5* fragments into a pGEX-4T vector (GE), after which the His10 sequence was inserted using Quikchange (Agilent) mutagenesis. Plasmid pRS316-*TOF2*-TAP was created by amplifying the *TOF2*-TAP ORF plus 500 bp upstream and 200 bp downstream of the ORF from genomic DNA isolated from the TAP-TAG library. The plasmid pRS425-*NET1* was created by amplifying the *NET1*-TAP ORF plus 500 bp upstream and 200 bp downstream of the ORF from genomic DNA isolated from the TAP-TAG library. The amplified product was digested with *NotI* (at a restriction site introduced in the PCR primer), *HindIII* (at a naturally occurring site within *NET1*), and *PstI* (at a site in the promoter of the *HIS3MX6* cassette). The two DNA pieces (1–2886 and 2887–5900) were individually cloned into pBlueScript vectors and confirmed by sequencing. They were then subcloned sequentially into pRS425.

A plasmid bearing four tandem *SMT3* ORFs, the first three lacking stop codons and each lacking sequence for the GGATY motif required for SUMO protease cleavage while encoding a TEV protease cleavage site between the second and third *SMT3*, was constructed by PCR. Two fragments, each bearing two tandem *SMT3* ORFs, were amplified and cloned into the p414GPD plasmid (Mumberg *et al.* 1995). The resulting 4xSUMO fragment from this plasmid was then subcloned to pET28a, using *BamHI* and *SalI* cleavage. The resulting expressed protein bore an N-terminal 6His-T7 tag.

A plasmid containing the *SIR2* gene was created by amplifying the *SIR2* ORF plus 500 bp upstream and 260 bp downstream from genomic DNA isolated from MHY500 yeast. The amplified product was digested with *XbaI* and *XhoI* (at sites introduced in the primers) and ligated into pRS425. The *sir2* mutants were generated by Quikchange mutagenesis.

Creation of GST-SIM and GST-msim affinity resins

Rosetta DE3* *E. coli* cells (Novagen) were used to express H₁₀GST-Slx5_{4xSIM} (GST-SIM) and H₁₀GST-Slx5_{4xmsim} fusion proteins (GST-msim). Expression was induced with 1 mM IPTG when cells had reached an OD₆₀₀ of 0.6. Induced cultures (2 liters) were incubated at 30° for 2 hr and then moved to 16° and incubated overnight. Cell pellets were resuspended in 100 ml of lysis buffer AE [50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 0.1% Tween, 10% glycerol] with protease inhibitors (two Roche Complete tablets), 100 mg lysozyme, and 20 mg DNase and incubated for 15 min at 16°. Cells were then sonicated for a total of 100 sec in 10-sec intervals, with 10-sec rests between, all on ice. Lysates were clarified by centrifugation at 26,700 × g for 20 min and were then added to 3 ml of packed HisPur Cobalt

resin (Thermo Scientific) that had been equilibrated in lysis buffer AE. The resin–cell lysate mixture was rotated for 1 hr at 4° before being moved to a 25-ml Bio-Rad (Hercules, CA) disposable column. The resin was washed with 25 ml lysis buffer AE and 50 ml wash buffer [50 mM HEPES-NaOH (pH 7.5), 250 mM NaCl, 5 mM MgCl₂, 1% Tween, 5 mM imidazole]. Proteins were eluted stepwise by successive addition of 1-ml aliquots of elution buffer, which is buffer A [50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 0.1% Tween] plus 200 mM imidazole, pooling the relevant eluates. Eluted proteins were added to the equilibrated glutathione resin in a 15-ml conical tube. Buffer A was added to bring the mixture up to 15 ml. The resin–protein mixture was rotated at 4° for 12 hr and was then poured into a 15-ml Bio-Rad disposable column. The protein solution was allowed to drain through the column. The resin was washed with 50 ml PBS supplemented with an additional 250 mM NaCl and then with 10 ml PBS. Protein was eluted stepwise by addition of 1-ml elution buffer aliquots (PBS + 20 mM reduced glutathione, pH 7.5). Eluates were collected, and fractions containing either the GST-SIM or GST-msim proteins were combined. Proteins were concentrated using 10-kDa cutoff centrifugal concentrators (Millipore, Bedford, MA). Samples were passed through Pierce (Pierce Chemical, Rockford, IL) Zeba desalting columns to remove the glutathione.

The purified fusion proteins were examined by PAGE to determine purity and protein concentration, using a Syngene G-box for quantification. Either 20 mg of the GST-SIM fusion protein or 40 mg of the GST-msim fusion protein was cross-linked to 2 and 4 ml, respectively, of Thermo Scientific AminoLINK Coupling Resin following the manufacturer's protocol. Resins were stored at 4° in PBS + 0.5% sodium azide and used within 1 week.

Linear polySUMO pulldown by GST-SIM or GST-msim

The 4xSUMO protein was expressed in Rosetta2(DE3) pLysS cells. Cells were harvested by centrifugation, resuspended in 4S lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol) containing protease inhibitors (Roche complete EDTA), and then frozen in liquid nitrogen. The cells were thawed, DNase (10 µg/ml) was added, and the cells were sonicated on ice four times for 20 sec each. The extract was centrifuged at 30,000 × g for 20 min, and the clarified extract was incubated with TALON resin (Clontech). The resin was washed with 10 column volumes of 4S lysis buffer followed by 25 column volumes of 4S lysis buffer containing 10 mM imidazole. Bound protein was eluted with 4S lysis buffer containing 150 mM imidazole. Eluates were desalted using Zeba spin columns and frozen in aliquots in liquid nitrogen. The 2xSUMO was created by cleaving the 4xSUMO with recombinant TEV protease; the 4xSUMO protein (30 µl at 2.8 mg/ml) was incubated with 1 µg TEV protease in buffer A at 4° overnight. The 1xSUMO was created by standard expression and purification of His6-Smt3 from *E. coli* BL21 (DE3*) cells as in Li and Hochstrasser (2000).

For pulldown experiments, 20 µl of glutathione beads, 1 mM purified fusion protein (either SIM or msim), 1 mM SUMO (4x, 2x, or 1x), and 0.5 µg of BSA were rotated end over end for 2 hr at 4°. The resin was centrifuged at 1000 × g for 30 sec at 4° and the supernatant was removed. The resin was washed twice with binding buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM MgCl₂] and then centrifuged at 1000 × g for 30 sec. Twenty microliters of 1× SDS sample buffer was added, and the samples were boiled for 10 min, resolved in an 8% polyacrylamide gel, and analyzed by anti-SUMO immunoblotting.

Large-scale SUMO-conjugate purification from yeast lysates

A total of 2 liters of *ulp2Δ* or *ulp2Δ slx5Δ* cells was grown in YPD to an OD₆₀₀ of between 1.5 and 1.8. Cells were lysed by spheroplasting with lyticase (Scott and Schekman 1980) followed by manual grinding in liquid nitrogen. The resulting cell powder was resuspended in 50 mL lysis buffer YA (50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 0.1% Tween-20, 5 mM EDTA, Roche protease inhibitors, 40 mM N-ethylmaleimide [NEM]). The lysates were clarified by centrifugation first at 26,700 × g at 4° for 15 min and then at 90,000 × g at 4° for 1 hr. Final protein concentrations were calculated using the bicinchoninic acid (BCA) assay (Pierce).

To remove nonspecific binding proteins, 300 mg of protein from each lysate was incubated with GST-msim resin for 2.5 hr at 4°. The flow-through from the column was collected, and half was added to GST-SIM resin and the other half to GST-msim resin in 15-ml Bio-Rad columns and brought up to 8 ml total. The columns were rotated end over end overnight at 4° and were then allowed to settle. Each flow-through was collected and passed through the column two more times. The resin was washed with 50 ml wash buffer (50 mM HEPES-NaOH, pH 7.5, 250 mM NaCl, 5 mM MgCl₂, 0.1% Tween-20) and then with 25 ml buffer A. The resin was moved to a 1.2-ml Bio-Rad disposable column, and 800 µl of 1 µg/ml SIM peptide [VDVIDLTIEEDE; derived from PIAS-x (Bruderer *et al.* 2011)] in buffer A was added. The SIM peptide was synthesized and HPLC purified at the Keck Biotechnology Resource Center (Yale School of Medicine). Each column was rotated for 1 hr at 30°, and the eluates were collected and precipitated by adding 200 µl 100% TCA (4°) and incubating at –20° overnight. The precipitated protein mixture was then spun at 21,000 × g for 10 min at 4°. The protein pellet was washed with 900 µl of 100% acetone that had been chilled to –20°. After recentrifuging, the protein pellet was dried in a Speedvac, and the pellet was redissolved in 30 µl 1× Sample Buffer (SB) and boiled for 10 min. Three microliters of each purified sample was checked by anti-SUMO immunoblotting (Li and Hochstrasser 1999).

Protein identification by liquid chromatography–tandem mass spectrometry

The remainder of the undiluted purified protein samples was resolved on two 0.75-mm gels (10% polyacrylamide resolving gel with a 6% stacking gel). After the gels were washed in

filtered deionized water and stained with GelCode Blue, gel slices were excised from the gel.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was carried out on an optimized platform (Xu *et al.* 2009). Briefly, after gel excision and trypsin digestion, the resulting peptides were injected into a reverse-phase C₁₈ capillary column coupled with a hybrid LTQ Orbitrap Velos MS (Thermo Fisher Scientific) with one MS survey scan and up to 10 data-dependent MS/MS scans. The collected MS/MS spectra were matched to a yeast Uniprot database by the Sequest algorithm (Eng *et al.* 1994). Searching parameters included mass tolerance of precursor ions (± 10 ppm) and product ion (± 0.5 Da), tryptic restriction, dynamic mass shifts for oxidized Met (+15.9949), two maximal modification sites, and two maximal missed cleavages, as well as only *b* and *y* ions counted. To evaluate the false discovery rate during spectrum–peptide matching, all original protein sequences were reversed to generate a decoy database that was concatenated to the original database (Peng *et al.* 2003). Assigned peptides were filtered to reduce the protein false discovery rate to $<1\%$. The spectrum–peptide matching number (also termed spectral counting) of individual proteins was used as a semiquantitative index for comparing protein abundance in different samples (Liu *et al.* 2004).

Sumoylation analysis by denaturing immunoprecipitation and immunoblotting

Twenty-five milliliters of log-phase cells ($OD_{600} = 1.5\text{--}1.8$) grown in YPD were harvested. The cells were lysed in either of two ways. Cells expressing *Net1*-TAP or *Tof2*-TAP were lysed by resuspending the cell pellets in 500 μ l 2 M NaOH/0.75% β -mercaptoethanol and incubating on ice for 5 min. A total of 500 μ l of 50% TCA was then added and the lysate was vortexed briefly, incubated on ice for 5 min, and centrifuged at $21,000 \times g$ for 10 min at 4° . The pellets were washed with 900 μ l acetone (-20°) and centrifuged, and the resulting pellets were air dried. The pellets were then resuspended in 130 μ l resuspension buffer [0.5 M Tris-HCl, pH 8.8, 6.5% SDS, 12% glycerol, 100 mM dithiothreitol (DTT)]. Samples were incubated at 65° for 20 min with vortexing every 5 min. After centrifuging at $21,000 \times g$ for 15 min at room temperature, the supernatants (~ 90 μ l) were diluted into 1.1 ml of RIPA buffer [50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton-X100, Roche protease inhibitors (complete EDTA), and 40 mM NEM]. The second method of lysis, used for the strains expressing *Fob1*-TAP, began by resuspending cell pellets in resuspension buffer and heating them for 20 min at 100° . After removing cell debris by centrifugation, lysates were diluted 1:10 in RIPA buffer and clarified by a $26,700 \times g$ spin for 20 min at 4° . Clarified supernatants were concentrated to 1.5 ml, using Millipore 10-kDa cutoff centrifugal concentrators.

Protein concentrations were determined using the BCA assay. Roughly 2 mg of protein was added to 50 μ l RIPA buffer-equilibrated IgG resin (50% slurry). The mixture was rotated for 3 hr at 4° . The resin was washed five times with 1 ml RIPA buffer. Samples were resolved by SDS–PAGE and analyzed by

anti-SUMO immunoblotting. The membranes were washed with PBS + 0.5% sodium azide and immunoblotted again with peroxidase–anti-peroxidase (PAP).

Cell-cycle analysis

One milliliter of log-phase yeast cells in YPD (OD_{600} between 1.5 and 1.8) was fixed in 1% formaldehyde for 2 hr at room temperature. After being washed twice in water, cells were resuspended in 1 ml 70% ethanol and incubated at room temperature for 30 min. Cells resuspended in water were stained with DAPI (45 μ g/ml; Molecular Probes, Eugene, OR) and imaged using a Plan-Apochromat 100 $\times$ NA1.4 objective lens on a Zeiss Axioskop microscope (Carl Zeiss Microimaging, Thornwood, NY). For DAPI visualization, the XF 100-2 filter set was used (Omega Optical, Brattleboro, VT). Pictures were taken on a Zeiss AxioCam camera, using a Uniblitz shutter driver (model VMM-D1; Vincent Associates, Rochester, NY) and Axiovision software. Between 250 and 500 cells were counted for each strain. The cells were divided into subgroups of 50 cells and the average of each subgroup was taken to determine the percentage in each cell-cycle stage. The error bars in the graphs represent the standard deviations between the subgroups. Significance was calculated by chi-square tests.

ChIP Assays

ChIP assays were performed exactly as described in Huang and Moazed (2003) with the exception that quantitative PCR (qPCR) with SYBR Green Master Mix (MM) (Bio-Rad) was performed on a Roche LightCycler 480. The immunoprecipitated samples were diluted 1:8 in molecular grade water. The inputs were diluted either 1:2000 or 1:20,000 in water. Primer pairs used were as described in Huang and Moazed (2003). Individual reactions were 10 μ l and consisted of either 5 μ l MM, 1 μ l diluted DNA, and 4 μ l 2 mM primer pair or 5 μ l MM, 4 μ l diluted DNA, and 1 μ l 8 mM primer pair.

The relative fold enrichment was calculated by averaging quadruplicates of each reaction. Relative amounts of the DNA were calculated using serial dilutions of a 320-ng/mL genomic DNA sample from MHY606 [wild type (WT)] cells. The relative amount of the rDNA ChIP assay reaction was then divided by the relative amount of the *CUP1* ChIP assay (Huang *et al.* 2006). This total was then divided by the rDNA levels in the input over the *CUP1* level in the input. The error bars indicate the error implicit in the qPCR.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Isolation of HMW-SUMO conjugates by tandem SIM pulldown

Our initial goal was to develop a method for isolating yeast SUMO-protein conjugates, specifically HMW polySUMO

conjugates, without tagging SUMO or overexpressing SUMO pathway proteins. Adding an affinity tag to SUMO has been useful, but it results in a strong reduction in SUMO conjugation *in vivo* (Hannich *et al.* 2005). Furthermore, no antibodies capable of efficiently immunoprecipitating untagged yeast SUMO are currently available. To get around these problems, we sought to develop an alternative affinity reagent that binds preferentially to polySUMO-modified yeast proteins.

Previously, we showed that full-length *Slx5* contains four SIMs capable of binding to SUMO (Xie *et al.* 2007). To determine whether the four SIMs were sufficient for HMW SUMO-conjugate binding, a fragment containing the first 160 N-terminal residues of *Slx5* protein, which included all four SIMs, was fused to the C terminus of a His₁₀-GST protein and purified from *E. coli* by metal-chelate affinity chromatography (Figure 1A). The resulting GST-SIM fusion protein was used in GST-based pulldown assays with free yeast SUMO or linear chains with two or four SUMO units (Figure 1B). As a negative control, all four SIMs were replaced with previously characterized mutated versions (Xie *et al.* 2007) (GST-msim, Figure 1A). GST-SIM efficiently pulled down the 4xSUMO tandem fusion but brought down 2xSUMO and free SUMO proteins far less well (Figure 1B), indicating that the *Slx5* fragment binds preferentially to polySUMO. The mutant GST-msim fusion, by contrast, showed far weaker polySUMO interaction, although weak residual binding was still detected.

These data indicated that GST-SIM might be useful as an affinity reagent for isolating polySUMO-modified proteins in yeast. To test this, we used lysates from two yeast strains, *ulp2Δ* and *ulp2Δ slx5Δ*, which accumulate high levels of HMW-SUMO conjugates. Such conjugates are present at much lower levels in WT and *slx5Δ* cells (Figure 1D). Comparing the identities of the HMW-SUMO conjugates isolated from the two lysates could help identify *in vivo* substrates of *Ulp2* and potentially *Slx5*. At present, very few such targets are known. While similar levels of HMW-SUMO conjugates accumulated in *ulp2Δ* and *ulp2Δ slx5Δ* cells, combining *slx5Δ* with *ulp2Δ* strongly suppressed the growth defects observed in the *ulp2Δ* single mutant (Figure 1, C and D) (Mullen *et al.* 2011). The *ulp2Δ* and *ulp2Δ slx5Δ* cells also both showed comparable depletion of free SUMO (Figure 1D). Hence, neither the presence of hypersumoylated proteins *per se* nor the reduction in free SUMO can by itself account for the growth differences between the two strains.

HMW conjugates from both *ulp2Δ* and *ulp2Δ slx5Δ* cells were isolated from yeast extracts, using an affinity matrix created by cross-linking GST-SIM to beads (see *Materials and Methods*). A large amount of HMW-SUMO conjugates from *ulp2Δ* cells were isolated on the matrix as revealed by anti-SUMO immunoblotting (Figure 1E). By contrast, the control GST-msim resin bound to only a small fraction of the conjugates seen with the WT SIM fusion. Notably, only about half the amount of HMW-SUMO conjugates isolated from *ulp2Δ* cells on the GST-SIM column were seen when

equal amounts of protein from *ulp2Δ slx5Δ* cells were used for purification (Figure 1E, lane 1 vs. lane 3). This contrasted with the comparable levels of HMW-SUMO conjugates noted when denatured extracts from the two strains were analyzed directly by immunoblotting (Figure 1D and Figure S1). This finding was reproducible and suggested that while both strains contained similar quantities of HMW-SUMO conjugates, either the conjugates were qualitatively different or their subcellular localization was different. HMW-SUMO conjugates in *ulp2Δ slx5Δ* cells cannot be ubiquitinated by *Slx5/Slx8*, and one possibility is that this might reduce their extractability under the mild conditions used for affinity purification. Together, these results indicate that the GST-SIM affinity resin is an effective reagent for the specific isolation of HMW-SUMO conjugates from yeast.

Identification of *Net1* and *Tof2* as potential *Ulp2* substrates

HMW SUMO conjugates were isolated under nondenaturing conditions from *ulp2Δ* and *ulp2Δ slx5Δ* cells, using the GST-SIM affinity resin and GST-msim control. The conjugates were eluted by competition with excess SIM peptide (Figure 1E). The HMW-SUMO species were seen as a smear stretching from the top of the resolving gel to the top of the stacking gel, as revealed by anti-SUMO immunoblotting. The remainder of the eluates were resolved on a preparative SDS-PAGE gel and stained with GelCode Blue (Figure S1). Three gel slices, corresponding to the top of the stacking gel, the area between the stacking and resolving gels, and the resolving gel from ≥ 75 kDa, were cut from each lane and subjected to LC-MS/MS to identify the eluted yeast proteins.

Table S2 lists the proteins identified from the purifications. Proteins were sorted into three groups: those with significant yields (1) from both *ulp2Δ* and *ulp2Δ slx5Δ* lysates, (2) from *ulp2Δ* lysates only, and (3) from *ulp2Δ slx5Δ* only. Previously characterized sumoylated substrates, such as the septin *Cdc3* and the ATPase *Cdc48*, were identified, confirming that the procedure successfully identified sumoylated proteins. Because of the mild, nondenaturing conditions used for the purifications, some of the proteins identified might be bound to sumoylated proteins rather than being sumoylated themselves. The molecular chaperones *Sse1*, *Ssa1*, and *Hsp82* might be examples of this, although we did not pursue them further.

Net1 and *Tof2*, both chromatin-associated scaffolding proteins in the nucleolus, were highly abundant in the SUMO-conjugate purification from the *ulp2Δ* strain but were completely absent in the *ulp2Δ slx5Δ* purification. This suggested the hypothesis that these hypersumoylated proteins contribute to the poor growth of *ulp2Δ* cells, which is substantially alleviated in the *ulp2Δ slx5Δ* double mutant. Conversely, several proteins were isolated in greater amounts from *ulp2Δ slx5Δ* compared to *ulp2Δ* cells; interestingly, these included multiple ribosomal proteins as well as *Snf1*, a known substrate of the *Slx5/Slx8* STUbL (Simpson-Lavy and Johnston 2013).

A Full-Length Slx5

GST-SIM protein

GST-msim protein

B

C

D

E

Figure 1 A novel affinity matrix for purifying polySUMO-protein conjugates from yeast. (A) Schematic of full-length Slx5 and the 160-residue WT SIM₄ and mutant msim₄ Slx5-derived peptides, both fused to His₁₀-GST (called GST-SIM and GST-msim, respectively). (B) GST-SIM and GST-msim fusion proteins were mixed with purified, recombinant yeast SUMO or linear noncleavable SUMO chains containing either two or four tandem SUMO moieties. Glutathione-Sepharose pulldowns were evaluated by anti-SUMO immunoblotting. (C) Serial (fivefold) dilution and growth analysis of WT, *ulp1ts*, *ulp2Δ*, *ulp2Δ slx5Δ* and *slx5Δ* strains. Plates were incubated as indicated for 4 days. (D) Anti-SUMO immunoblot analysis of denatured proteins derived from the indicated strains. The longer exposure (bottom) allowed visualization of free SUMO. Anti-PGK blotting was used as a loading control. Molecular

Net1 and Tof2 are hypersumoylated in *ulp2Δ*, *slx5Δ*, and *ulp2Δ slx5Δ* cells

We decided to focus on the related *Net1* and *Tof2* proteins, given their uniquely strong recovery from the *ulp2Δ* single mutant. To verify direct sumoylation of these proteins, we performed denaturing immunoprecipitations (IPs) of TAP-tagged versions of the proteins expressed from their normal chromosomal loci and analyzed them by anti-SUMO immunoblotting (Figure 2). Only covalently attached SUMO should be detected under these conditions. The TAP-tagged alleles were introduced into WT, *ulp1ts*, *ulp2Δ*, *slx5Δ*, and *ulp2Δ slx5Δ* backgrounds. To test for a potential nonenzymatic function for *Ulp2* in causing the accumulation of these sumoylated proteins, a strain expressing a catalytically inactive form of *Ulp2*, *ulp2-C624A-13myc*, was also created. The protein-A segment in the TAP tag binds to an IgG resin, which is used for IP, and it is also recognized by the PAP antibody complex used for immunoblotting. If a TAP-tagged protein is sumoylated, the signal from the modified protein species detected with SUMO-specific antibodies will be brighter than when blotting with the PAP antibody (Cremona *et al.* 2012).

Sumoylated forms of *Net1* were not visible in WT cells, but were readily detected in *ulp2Δ*, *ulp2-C624A-13myc*, *slx5Δ*, and *ulp2Δ slx5Δ* cells as a smear above the unmodified *Net1*-TAP protein (Figure 2A). *Net1* sumoylation was not detected in *ulp1ts* cells, suggesting that *Net1* is an *Ulp2*-specific substrate. In contrast to *Net1*, singly sumoylated forms of *Tof2* were occasionally detected in both WT and *ulp1ts* cells (Cremona *et al.* 2012); however, a significant fraction of *Tof2* is more heavily sumoylated in *ulp2Δ*, *ulp2-C624A-13myc*, *slx5Δ*, and *ulp2Δ slx5Δ* cells (Figure 2B). Again, the increased sumoylation of *Tof2* in the *ulp2Δ* strains suggested that it is primarily a target of *Ulp2*.

Taken together, these results demonstrate that both *Net1* and *Tof2* are sumoylated and that they are desumoylated primarily by *Ulp2* and not *Ulp1*. Surprisingly, deleting *SLX5*, either alone or in combination with *ULP2* deletion, also resulted in enhanced polysumoylation of *Net1* and *Tof2*; this was unexpected because neither protein was isolated from *ulp2Δ slx5Δ* extracts on the SIM-fusion resin. On the one hand, the similar levels of *Net1* and *Tof2* sumoylation in *ulp2Δ* and *ulp2Δ slx5Δ* appear to argue against our earlier hypothesis that these hypermodified scaffold proteins contribute to the slow growth of *ulp2Δ* cells since their levels should have decreased in the faster growing *ulp2Δ slx5Δ* strain. Conversely, hypersumoylation of the nucleolar *Net1* and *Tof2* proteins in *slx5Δ* cells points to a potential role for the *Slx5/Slx8* STUbL in the regulation of rDNA.

Loss of *Net1* and *Tof2* binding to rDNA in *ulp1ts* and *ulp2Δ* cells

Sumoylation can regulate macromolecular interactions in protein complexes by either creating or blocking protein-binding sites (Johnson 2004). *Net1* and *Tof2* both interact with specific chromatin sites within the rDNA locus (Huang and Moazed 2003). To determine whether changes in sumoylation of these proteins correlated with altered association with rDNA chromatin, we used quantitative ChIP assays to measure rDNA binding in various SUMO pathway mutants. ChIP assays were performed with *Net1*-TAP and *Tof2*-TAP in WT, *ulp1ts*, *ulp2Δ*, *ulp2-C624A-13myc*, *slx5Δ*, and *ulp2Δ slx5Δ* cells. Briefly, sheared DNA was isolated from cross-linked and immunoprecipitated samples, and levels of *Net1* and *Tof2* binding across the rDNA intergenic region were measured by qPCR, using previously designed primer pairs (Huang *et al.* 2006) (Figure 3A).

In agreement with published data (Huang *et al.* 2006), *Net1*-TAP ChIP assays with WT cells revealed two strong rDNA-binding peaks: one at the RFB (primer pair 15) and the other at the RNA polymerase I TIR (primer pair 23) (Figure 3, B–D). Also as expected, *Tof2*-TAP ChIP assays performed with WT cells showed a single substantial rDNA-binding peak centered on the RFB (Figure 3, E–G). Strikingly, in both *ulp2Δ* and *ulp2-C624A-13myc* cells, *Net1*-TAP binding to the rDNA locus was sharply curtailed (Figure 3B). Similarly, *Tof2*-TAP lost much of its association with the RFB in *ulp2Δ* and *ulp2-C624A-13myc* cells (Figure 3E).

Unexpectedly, rDNA binding by both *Net1* and *Tof2* was also strongly reduced in *ulp1ts* cells (Figure 3, C and F). These results were unanticipated because sumoylation of these two nucleolar proteins was not obviously altered in the *ulp1ts* mutant (Figure 2, A and B). Moreover, *Ulp1* is concentrated at NPCs that are largely excluded from the nucleolar region (Hannich *et al.* 2005). These data suggest either that the hypersumoylation of *Net1* and *Tof2* is not by itself responsible for their loss of rDNA binding in *ulp2Δ* cells or that loss of rDNA binding in the two SUMO protease mutants has distinct causes (see below). Related to this, *Net1* and *Tof2* in *slx5Δ* cells bound to the rDNA at levels similar to those in WT cells (Figure 3, C and F) even though the proteins are hypersumoylated in *slx5Δ* but not WT cells.

Strikingly, *Net1* and *Tof2* regained a substantial fraction of their association with rDNA chromatin when *SLX5* was deleted in the *ulp2Δ* strains (Figure 3, D and G). This is consistent with a function for *Slx5/Slx8* at the rDNA locus even though *slx5Δ* by itself led to no obvious alteration in rDNA binding by these two proteins. Potentially, loss of the *Slx5/Slx8* STUbL suppresses the growth defects of *ulp2Δ* cells, at

mass markers (in kDa) are indicated at left. (E) Anti-SUMO immunoblot analysis of aliquots from large-scale purifications from *ulp2Δ* and *ulp2Δ slx5Δ* strains, using affinity resins made with the GST-SIM or GST-msim fusion proteins cross-linked to Sepharose. Proteins were eluted from the columns with excess SIM peptide (SUMO conjugate profiles obtained by elution with Laemmli SDS buffer were indistinguishable from those with SIM peptide; data not shown).

Figure 2 Net1 and Tof2 proteins are hypersumoylated in SUMO-pathway mutants. (A) Immunoprecipitation (IP) using IgG-Sepharose of Net1-TAP from denatured yeast extracts from the indicated strains followed by immunoblot analysis with anti-SUMO antibodies and peroxidase-anti-peroxidase (PAP) complex. Net1-TAP was expressed from the *NET1* chromosomal locus. Anti-PGK was used as a loading control for protein inputs. The asterisk indicates the unmodified TAP-tagged protein. (B) Analysis of Tof2-TAP sumoylation was done as in A except the strains carried the *TOF2-TAP* allele.

least in part, by reestablishing a state permissive for binding of *Net1* and/or *Tof2* (and potentially other sumoylated proteins) to the rDNA. Taken together, these results demonstrate that multiple enzymes of the SUMO pathway are involved in controlling the chromatin composition of key sites within the rDNA locus.

***Fob1* is regulated by the SUMO pathway**

As noted earlier, both *Net1* and *Tof2* require *Fob1* to bind to the RFB (Figure 4A) (Huang *et al.* 2006). *Net1* and *Tof2* binding to the rDNA is strongly affected by mutation of components of the SUMO system (Figure 3), yet the extent of their modification by SUMO does not correlate well with their rDNA-binding capacity. This led us to ask whether *Fob1* might be more directly linked to SUMO-dependent rDNA regulation. Specifically, we sought to determine whether *Fob1* is sumoylated and whether mutations in the SUMO pathway also affect rDNA binding by *Fob1*.

To test whether *Fob1* is modified by SUMO, we immunoprecipitated *Fob1*-TAP from denatured extracts of various yeast SUMO-pathway mutants and analyzed the precipitated protein by anti-SUMO immunoblotting (Figure 4B). A ladder of HMW species that reacted with the anti-SUMO antibody

were observed in WT cells. The SUMO modification levels of *Fob1*-TAP were slightly but reproducibly reduced in *ulp1ts* cells relative to WT (Figure 4B). In contrast, *Fob1*-TAP polysumoylation was strongly enhanced in *ulp2Δ* cells, but this increase in *Fob1* modification was reversed in the *ulp2Δ slz5Δ* double mutant. Interestingly, *Fob1* was not identified in our LC-MS/MS analysis of *ulp2Δ* cells, possibly due to low extractability under the mild conditions used.

To examine rDNA binding of *Fob1*, quantitative ChIP assays were performed using primers for the RFB and the TIR (primer sets 15 and 23). We found that *Fob1*-TAP bound to the rDNA at similar levels in WT, *ulp1ts*, and *slz5Δ* cells (Figure 4C). *Fob1*-TAP displayed an ~50% loss of rDNA binding in *ulp2Δ* cells. Paralleling the reversal of excess *Fob1* sumoylation seen in the *ulp2Δ slz5Δ* cells (Figure 4B), RFB binding by *Fob1* was also fully restored in the double mutant. Overall, the correlation of growth defects, reduced rDNA binding, and excess SUMO modification is much stronger for *Fob1* than for *Net1* or *Tof2*. This suggests that sumoylation of the core *Fob1* scaffolding factor might be more closely linked to the control of rDNA function than that of the latter two proteins.

Figure 3 Quantitative ChIP analysis of Net1 and Tof2 binding to rDNA. (A) Schematic of the rDNA nontranscribed spacer sequences showing positions of PCR fragments generated from each indicated primer pair. Primer pair 15 is centered on the RFB and primer pair 23 on the TIR. Ribosomal 5S and 35S RNA transcription units are indicated. ARS, autonomously replicating sequence; RFB, replication fork barrier; TIR, transcription initiation region for RNA polymerase I. (B) Quantitative Net1-TAP ChIP assays using primer pairs 9–25 and the indicated strains that also carried the *NET1-TAP* allele. Net1-TAP was precipitated using IgG-Sepharose beads. The relative fold enrichment was equal to $([rDNA \text{ ChIP}]/[CUP1 \text{ ChIP}])/([rDNA \text{ input}]/[CUP1 \text{ input}])$ where bracketed information equals the relative DNA concentration. (C) Quantitative Net1-TAP ChIP assays as in B with the indicated primer pairs and strains. (D) Quantitative Net1-TAP ChIP assays as in B with the indicated primer pairs and strains. (E) Quantitative Tof2-TAP ChIP assays with the indicated primer pairs and strains, which also carried the *TOF2-TAP* allele. Tof2-TAP was precipitated using IgG-Sepharose beads. (F) Quantitative Tof2-TAP ChIP assays as in E with the indicated primer pairs and strains. (G) Quantitative Tof2-TAP ChIP assays as in E with the indicated primer pairs and strains.

Figure 4 Altered Fob1 sumoylation and rDNA binding in SUMO-pathway mutants. (A) Model showing the binding of Fob1, Net1, and Tof2 to the RFB of the rDNA repeat. Net1 and Tof2 require Fob1 for their association with the RFB. (B) IgG-Sepharose IP of Fob1-TAP from denatured extracts of the indicated strains followed by immunoblotting with anti-SUMO and PAP antibodies. Molecular size markers are indicated on the right (in kDa). Anti-glucose-6-phosphate dehydrogenase (G-6-PDH) immunoblotting was used as a loading control for inputs. (C) Quantitative Fob1-TAP ChIP assays using primer pairs 15 and 23 (see Figure 3A). Fob1-TAP was pulled down from cross-linked extracts derived from the indicated strains, which also carried the *FOB1-TAP* allele.

Cell-cycle arrest cannot explain the loss of rDNA binding of Net1, Tof2, or Fob1 in *ulp2Δ*

A potential explanation for the loss of Net1 and Tof2 binding to the rDNA in *ulp1ts* and *ulp2Δ* cells is that both *ulp2Δ* and

ulp1ts cells are stalled at a similar cell-cycle stage and both Net1 and Tof2 do not bind to the rDNA at this stage. Fluorescence-activated cell sorting (FACS) and microscopic analysis have indicated that both of these mutants accumulate

Figure 5 Cell-cycle distribution of SUMO-pathway mutants. Asynchronous, log-phase cells were fixed in 1% formaldehyde and stained with DAPI in the mounting medium. Between 200 and 300 cells were counted and binned into the following categories: cell with no bud, budded cell with one nucleus, large-budded cell with one elongating nucleus in the bud neck, and large-budded cell with two nuclei. These stages correspond to G₁ (G₀), S/G₂, mitosis/anaphase, and late anaphase, respectively. Images of representative cells for each cell-cycle stage are displayed below the graphs. Chi-square tests were used to determine significance. All mutant strains had statistically significant differences from the WT cell cycle. The cell-cycle profiles of *ulp1ts* and *ulp2Δ* cells were also significantly different. *** $P < 0.0005$.

after S phase at some point in the G₂/M phase of the cell cycle (Li and Hochstrasser 1999; Strunnikov *et al.* 2001). The above ChIP assays were performed on asynchronous populations, so if stalling after S phase is what causes the loss in rDNA binding of *Net1* and *Tof2* in *ulp1ts* and *ulp2Δ* cells, then both mutants must accumulate in significantly different cell-cycle stages than WT and *slx5Δ* cells.

Cell-cycle analysis to determine exactly when *ulp2Δ* cells stall is problematic because *ulp2Δ* cells are difficult to synchronize (Li and Hochstrasser 2000; Hannich *et al.* 2005). To determine whether *ulp2Δ* or *ulp1ts* cells accumulate at the same stage(s) and to see whether these distributions are different from those of WT or the other mutants, cell-cycle distributions of asynchronous cells were determined and quantified by fixing the cells and staining with the DNA dye DAPI. The cells were counted and binned into four categories: no bud (G₀, G₁), budded with one nucleus (S, G₂), large budded with nucleus elongating at neck between the mother and daughter cells (mitosis/anaphase), and large budded with two nuclei (late anaphase) (Figure 5).

Wild-type BY4741 cells had a typical WT cell-cycle profile: a majority of cells were not budded, 30% of cells had small or large buds with a single nucleus, 2% of cells had elongated DNA staining at the bud neck, and 14% of cells had large buds with separated daughter nuclei (Figure 5). When tested at the semipermissive temperature of 30°, *ulp1ts* cells differed significantly from WT and had reduced numbers of unbudded cells and increased numbers of budded cells with a single nucleus. The *ulp2Δ* distribution was quite different from that of *ulp1ts* but differed only slightly from WT, with slightly more large budded cells and essentially none in mitosis/anaphase. The difference between the *ulp2Δ* cell cycle and the *ulp1ts* cell cycle was statistically significant. Notably, the *ulp2Δ slx5Δ* and *slx5Δ* distributions were closest to that of *ulp1ts* cells: a reduction in unbudded cells and an increase in large-budded cells with a single nucleus or in mitosis/anaphase (Figure 5). The similarity in cell-cycle profiles of

ulp2Δ and WT cells and the dissimilarity of *ulp2Δ* and *ulp1ts* mutant profiles imply that stalling of the mutants, at least *ulp2Δ*, at specific cell-cycle stages was not the cause of the loss of rDNA binding by *Net1* and *Tof2* (or *Fob1*).

Genetic interactions of *ulp2Δ* with rDNA regulatory factor mutants

The *ulp2Δ* mutant is sensitive to many stress conditions, including heat shock and DNA-damaging agents such as hydroxyurea (HU) (Li and Hochstrasser 2000; Bylebyl *et al.* 2003; Schwartz *et al.* 2007). Since factors linked to rDNA silencing and condensation have been found to be substrates of *Ulp2*, we asked whether increased levels of these factors or related proteins could overcome or exacerbate the *ulp2Δ* temperature-sensitive growth defects. Specifically, we overexpressed components of the RENT complex (*Net1*, *Cdc14*, and *Sir2*) and *Tof2* and tested growth at various temperatures. We also tested whether overexpression of *Sir2* can suppress the HU sensitivity of *ulp2Δ* cells (Figure 6 and Figure S2).

Overproduction of *Net1* showed only weak suppression at most, and overexpressing another RENT complex component, the cell-cycle-dependent phosphatase *Cdc14*, reduced the fitness of *ulp2Δ* cells even at 30° (Figure S3A). Overexpressing *Cdc14* in WT or *ulp1ts* cells had no obvious deleterious effects (not shown), indicating that excess *Cdc14* is specifically harmful in *ulp2Δ* cells. Increased dosage of *TOF2* by expression from a low-copy vector was also mildly deleterious only in *ulp2Δ* cells, whereas its deletion in an *ulp2Δ* background did not alter growth further (Figure S3B and not shown).

When *ulp2Δ* cells were provided with a high-copy plasmid expressing *SIR2* from its own promoter, they were able to grow at a nonpermissive temperature (Figure 6A, 36°). This suppression depended on the deacetylase activity of *Sir2*; overexpression of an inactive *Sir2* mutant, *sir2-N345A*, did not suppress the temperature sensitivity of *ulp2Δ* cells (Figure 6, A and B). Importantly, overproducing *sir2-L159S* or *sir2-L220Q*, mutant

Figure 6 Genetic interactions between *ulp2Δ* and Sir2. (A) Growth analysis of *ulp2Δ* cells transformed with the indicated gene sequences cloned into the high-copy pRS425 (*LEU2*) plasmid. Cells were spotted in fivefold serial dilution on SD-Leu plates. Plates were incubated for 4 days under the indicated conditions. (B) Anti-Sir2 immunoblot analysis of denatured proteins derived from the indicated strains. Anti-PGK blotting was used as a loading control. (C) Growth analysis of *ulp2Δ* cells transformed with the indicated alleles cloned into pRS425 and analyzed as in A. (D) Immunoblot analysis performed as in B.

proteins that no longer localize to the nucleolus (Cuperus *et al.* 2000), showed minimal suppression of the temperature-sensitive growth defect (Figure 6, C and D), indicating that the suppression by overproduction of Sir2 requires its nucleolar localization. Overexpression of *sir2* mutants that specifically disrupt Sir2 function at telomeres and the silent mating-type loci, *sir2-D223G* and *sir2-C233R*, still suppressed the temperature sensitivity of *ulp2Δ* cells, implying that only the rDNA function of Sir2 is required for *ulp2Δ* suppression. Sir2 overexpression also suppressed the HU sensitivity of *ulp2Δ* and *slx5Δ* cells (Figure S2 and Figure 7). Unlike in *ulp2Δ* cells, however, overexpressing *sir2-L159S* in *slx5Δ* suppressed the

HU sensitivity just as well as high-copy WT *SIR2*. This suggests that rDNA localization of Sir2 is not required for suppression of the HU sensitivity of *slx5Δ* cells (Figure S2B). Additionally, overexpression of *SIR2* suppressed the HU sensitivity of a *ulp2Δ slx5Δ* double mutant (Figure 7B), indicating that *ulp2Δ* suppression by overexpressed Sir2 does not require Slx5.

Since Sir2 and Fob1 often have antagonistic and epistatic genetic interactions (Feser *et al.* 2010; Saka *et al.* 2013; Yoshida *et al.* 2014; Choudhury *et al.* 2015), we also checked for genetic interactions between *ulp2Δ* and *fob1Δ* with the expectation that if Sir2 overproduction suppresses *ulp2Δ* defects, then the deletion of Fob1 might also suppress them. Surprisingly, *ulp2Δ*

Figure 7 Epistasis analyses of rDNA interacting factors. (A) Growth analysis of WT, *ulp2Δ*, *slx5Δ*, and *ulp2Δ slx5Δ* cells transformed with either an empty pRS425 high-copy (*LEU2*) plasmid or pRS425-SIR2, as indicated. Cells were spotted in fivefold serial dilution on SD-Leu or SD-Leu + 100 mM HU plates. Plates without HU were incubated for 2 days and plates containing HU were incubated for 4 days. (B) Growth analysis of the indicated strains transformed with empty pRS425 plasmid or pRS425-SIR2. Cells were spotted in fivefold serial dilution on SD-Leu plates. Plates were incubated for 4 days under the indicated conditions.

fob1Δ cells grew slightly worse than the *ulp2Δ* single mutant (Figure 7B, 34°C). The double mutant could also be rescued by the overproduction of *Sir2* (Figure 7B, 34°C). These results show that the suppression caused by the overproduction of *Sir2* does not depend on *Fob1*.

Altogether, these genetic interactions indicate that the RENT complex, especially *Sir2*, has close functional links to *Ulp2* and *Slx5*.

Discussion

In this study, we isolated and identified HMW-SUMO conjugates from *ulp2Δ* mutant cells, using a novel polySUMO-binding affinity reagent and LC-MS/MS, and demonstrated that the rDNA-associated proteins *Net1* and *Tof2* are substrates of the *Ulp2* SUMO protease. *Ulp2* was found to regulate the rDNA chromatin binding by *Net1* and *Tof2*. Remarkably, the defects in rDNA binding by these two proteins in *ulp2Δ* are strongly suppressed by inactivating the *Slx5/Slx8* STUbL, as are the growth defects of the *ulp2Δ* mutant, suggesting that the STUbL might convert these hypersumoylated proteins into more deleterious species. An additional factor, *Fob1*, which recruits both *Net1* and *Tof2* to the RFB in the rDNA locus, also becomes hypersumoylated in *ulp2Δ* cells. *Fob1* partially loses binding to the rDNA in *ulp2Δ* cells, and like *Net1* and *Tof2*, this binding is restored in *ulp2Δ slx5Δ* cells. Unlike *Net1* and *Tof2*, however,

Fob1 retains binding to the rDNA in *ulp1ts* cells, which suggests that specific *Fob1* desumoylation by *Ulp2* might be an important component of the SUMO protease's regulation of the rDNA locus (Figure 8).

Protein sumoylation is essential for viability in *S. cerevisiae*, as it is in mammals, but polysumoylation is not required based on the continued growth of yeast cells that express a version of SUMO lacking all chain-forming lysine residues (Bylebyl *et al.* 2003; Srikumar *et al.* 2013a). Nevertheless, polysumoylation is important for specific processes, such as meiosis (Cheng *et al.* 2006; Klug *et al.* 2013) and maintenance of higher-order chromatin structure (Srikumar *et al.* 2013a). A difficulty in studying aspects of polySUMO function in yeast has been the lack of effective affinity reagents for isolating polySUMO-modified proteins. Available antibodies to yeast SUMO (*Smt3*) are not effective for immunopurification of conjugates, and epitope tagging *Smt3*, while useful, leads to a strong reduction in SUMO conjugation efficiency (Wohlschlegel *et al.* 2004; Hannich *et al.* 2005; Felberbaum *et al.* 2012). We show in the present work that a 160-residue fragment of the *Slx5* STUbL, which bears four tandem SIMs, has high affinity for polySUMO and yeast HMW-SUMO conjugates. A similar affinity approach has been successful in polySUMO conjugate purification from human cells, using a tandem-SIM fragment from the human STUbL RNF4 (Bruderer *et al.* 2011; Da Silva-Ferrada *et al.* 2013).

Figure 8 Hierarchical protein binding at the rDNA locus and intersections with SUMO. Fob1 binds to the replication fork barrier (RFB) and is responsible for localizing Net1, Tof2, and Top1 to this site (Huang *et al.* 2006; Krawczyk *et al.* 2014). Tof2 increases the interaction of Fob1 with the rDNA (thin arrow). Net1 is also localized to the transcription initiation region through binding to RNAPI. Sir2 depends on Net1 for localization to the rDNA (Huang and Moazed 2003). Tof2 can also bind Cdc14 and the cohibin complex (Huang *et al.* 2006; Geil *et al.* 2008; Mekhail *et al.* 2008; Waples *et al.* 2009). The cohibin complex binds to and localizes cohesin, condensin, the Src1-Nur1 complex, and Ulp2 (Wysocka *et al.* 2004; Mekhail *et al.* 2008; Johzuka and Horiuchi 2009; Srikumar *et al.* 2013b). Experimentally determined sumoylation of individual proteins is indicated by an encircled "S" next to the protein. RNAPI contains 14 subunits; 12 of these subunits are sumoylated, including Rpa190, which is the specific subunit bound by Net1 (Shou *et al.* 2001; Huang and Moazed 2003). ARS, autonomously replicating sequence.

We had initially expected that our GST-SIM resin would precipitate higher levels of HMW-SUMO conjugates from *slx5Δ* and *ulp2Δ slx5Δ* compared to *ulp2Δ* single mutants based on the assumption that deleting *Slx5* would prevent ubiquitylation and degradation of polySUMO-modified proteins (Uzunova *et al.* 2007). The resin did enrich polysumoylated proteins from *slx5Δ* extracts, but at a significantly lower efficiency than from *ulp2Δ* cells (data not shown). The *ulp2Δ slx5Δ* cells had levels of HMW-SUMO conjugates similar to those in *ulp2Δ* cells based on immunoblotting, but the SIM-fusion resin pulled down only roughly half of these HMW-SUMO conjugates. One explanation is that the HMW-SUMO conjugates are qualitatively different in *ulp2Δ* and *ulp2Δ slx5Δ*. These differences may reflect SUMO-SUMO linkages, differential ubiquitin conjugation to these chains, HMW-SUMO conjugates being multisumoylated (single SUMOs on different substrate lysines) vs. polysumoylated, or distinct protein populations modified by SUMO in the two strains. Localization or solubility of the conjugates might also be different.

Most sumoylated yeast proteins are desumoylated by Ulp1 rather than by Ulp2 (Hickey *et al.* 2012 and our unpublished data). However, *ulp2Δ* cells have severe and diverse growth defects, including temperature sensitivity, sporulation defects, hypersensitivity to DNA damage, and hypersensitivity to ER stress, implying multiple substrates linked to growth regulation and stress resistance (Li and Hochstrasser 1999, 2000;

Strunnikov *et al.* 2001; Kroetz *et al.* 2009; Felberbaum *et al.* 2012). Several recent studies, including the present work, have identified novel Ulp2 substrates (Cremona *et al.* 2012; Srikumar *et al.* 2013b), which will allow more precise analyses of the molecular basis for the defects associated with loss of Ulp2.

For Net1, Tof2, and Fob1—all proteins that associate with specific sites within the rDNA repeats—loss of Ulp2 leads both to their hypersumoylation and to reduction of rDNA binding (Figure 3 and Figure 4). Strikingly, loss of *Slx5*, which by itself has no major effect on the rDNA binding of these proteins, allows a substantial restoration of binding in *ulp2Δ*. Since *Slx5* binds preferentially to SUMO chains (Figure 1B), these results suggest that *Slx5/Slx8* modifies polysumoylated forms of Net1, Tof2, and Fob1 and interferes with their ability to bind at the rDNA locus either directly, through alteration of their structure, or indirectly, by causing their degradation by the proteasome. That the timely removal of SUMO chains from these particular proteins by Ulp2 is important for growth regulation in general, and rDNA function in particular, is supported by the genetic data in Figure 6 and Figure 7.

The genetic interactions between Sir2, *Slx5*, and Ulp2 also raise interesting questions about the roles of *Slx5* and Ulp2 in chromatin-mediated silencing. Sir2 is responsible for transcriptional silencing at the cryptic mating-type loci, telomeres, and the rDNA locus. Previously, *Slx5* and Sir2 were found to interact physically based on yeast two-hybrid and

in vitro assays (Darst *et al.* 2008). Darst *et al.* (2008) showed that *slx5Δ* cells have reduced rDNA and telomeric silencing. Our work shows that the suppression of *slx5Δ* sensitivity to HU by high-copy *Sir2* is not dependent on the rDNA localization of the overexpressed *Sir2* protein (Figure S2). This suggests that *Slx5* interaction with *Sir2* at telomeres might underlie the suppression of HU sensitivity. On the other hand, *Sir2* rDNA localization is required for its dosage suppression of the temperature-sensitive *ulp2Δ* growth defect. Therefore, excess *Sir2* likely suppresses the *slx5Δ* and *ulp2Δ* defects by different mechanisms.

Sumoylation could contribute to nucleolar function in multiple capacities: during S phase, it could promote replication fork arrest at the RFB; it might facilitate double-strand break repair at the RFB by homologous recombination; and during anaphase, it could regulate association of condensin and other proteins (Strunnikov *et al.* 2001; D'Amours *et al.* 2004) (Figure 8). Because *Ulp1* is usually excluded from the nucleolus except under certain stress conditions (Sydorskyy *et al.* 2010), *Ulp2* is probably the major SUMO protease operating to coordinate these nucleolar functions of SUMO. *Ulp2* activity is inhibited by the *Cdc5* Polo kinase in mitosis (Baldwin *et al.* 2009). Prior to this, *Ulp2* might desumoylate proteins such as *Fob1*, *Net1*, and *Tof2* at stalled replication forks and might also function to keep cohesin and condensin desumoylated until mitosis (Strunnikov *et al.* 2001; D'Amours *et al.* 2004; D'Ambrosio and Lavoie 2014). In our HMW-SUMO conjugate purification from *ulp2Δ* cells, we identified the cohesin subunits *Smc1* and *Smc3* (Table S2). Detailed cell-cycle analysis of the sumoylation of these various proteins will be needed to clarify the functions of *Ulp2* in replication and segregation of the rDNA and potentially in ribosome biogenesis as well.

Acknowledgments

We thank Nicole Wilson for helpful comments on the manuscript and Robert Tomko for sharing purified TEV protease. This work was supported by a National Institutes of Health (NIH) grant (R01 GM053756) (to M.H.) and in part by NIH training grant T32GM7223 (to J.G.).

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Communicating editor: S. Biggins

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Supporting Information

www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.187252/-/DC1

SUMO Pathway Modulation of Regulatory Protein Binding at the Ribosomal DNA Locus in *Saccharomyces cerevisiae*

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Figure S1. Characterization of proteins from the large-scale SIM/msim purifications. A Analysis of *ulp2* Δ cell proteins by SDS-PAGE followed by GelCode Blue staining to visualize total protein elutions from the SIM and msim resins. B. Analysis of protein eluates as in (A) except using proteins purified from *ulp2* Δ *slx5* Δ cells. C. Anti-SUMO immunoblot analysis of the input samples from *ulp2* Δ and *ulp2* Δ *slx5* Δ strains that were used for the large-scale SUMO conjugate purifications on GST-SIM and GST-msim affinity columns. D. Anti-SUMO immunoblot analysis of aliquots from large-scale purifications from *ulp2* Δ and *ulp2* Δ *slx5* Δ strains using affinity resins made with the GST-SIM or GST-msim fusion proteins cross-linked to Sepharose. Proteins were eluted from the columns with excess SIM peptide. This blot is a darker exposure of the immunoblot in Figure 1E.

A. **B.**

Figure S2. Overexpression of *SIR2* suppresses both *ulp2Δ* and *slx5Δ* HU-sensitivity. A Growth analysis of *ulp2Δ* cells transformed with the indicated gene sequences cloned into the high-copy pRS425 (*LEU2*) plasmid. Cells were spotted in 5-fold serial dilution on SD-Leu and SD-Leu + 25 mM HU plates. Plates without HU were incubated for 2 d and plates containing HU were incubated for 5 d, all at 30°C. B. Growth analysis of *slx5Δ* cells transformed with the indicated alleles cloned into pRS425. Cells were spotted in 5-fold serial dilution and incubated under the indicated conditions. Plates without HU were incubated for 4 d and plates containing HU were incubated for 6 d.

Figure S3. Overexpression of RENT components and Tof2 can suppress or enhance *ulp2Δ* growth defects.
 A. Growth analysis of *ulp2Δ* cells transformed with the indicated gene sequences cloned into pRS425, with the exception of *CDC14*, which was cloned into the CEN vector pRS315. Cells were spotted in 5-fold serial dilution on SD-Leu plates, and incubated for 4 d at the indicated temperatures. B. Growth analysis of WT and *ulp2Δ* cells transformed with the indicated gene sequences cloned into pRS425. Cells were streaked onto SD-Leu plates, and incubated for 4 d.

Table S1. Yeast Strains used in this study.

Strain	Genotype	Source
MHY500	<i>MATa his3Δ-200 leu2-3,211 ura3-52 lys2-801 trp1-1</i>	Chen <i>et al.</i> 1993
MHY501	<i>MATα his3Δ-200 leu2-3,211 ura3-52 lys2-801 trp1-1</i>	Chen <i>et al.</i> 1993
MHY606	a/α (MHY500 x MHY501)	Chen <i>et al.</i> 1993
MHY1488	<i>MATa LEU2::ulp1ts(3-33) ura3-52 lys2-801 trp1-1 his3Δ200 ulp1Δ::HIS3</i>	Li <i>et al.</i> 2003
MHY1381	<i>MATa his3200 leu2-3,112 ura3-52 lys2-801 trp1-1 ulp2Δ::HIS3</i>	Li <i>et al.</i> 2000
MHY7200	<i>MATα leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 ulp2Δ::HIS3 slx5Δ::kanMX4</i>	This study
MHY3857	<i>MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2 slx5Δ::kanMX4</i>	Xie <i>et al.</i> 2007
MHY7901	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 NET1-TAP::HIS3MX6</i>	TAP-tag library
MHY9116	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 NET1-TAP::HIS3MX6 ulp1Δ::kanMX4 YCplac22-ulp1ts/NAT</i>	This study
MHY7910	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 NET1-TAP::HIS3MX6 ulp2Δ::kanMX4</i>	This study
MHY7921	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 NET1-TAP::HIS3MX6 slx5Δ::kanMX4</i>	This study
MHY9117	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 NET1-TAP::HIS3MX6 slx5Δ::kanMX4 ulp2Δ::kanMX4</i>	This study
MHY9128	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 NET1-TAP::HIS3MX6 ulp2::kanMX6 ulp2C624A-13MYC:LEU2</i>	This study
MHY7902	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TOF2-TAP::HIS3MX6</i>	TAP-tag library
MHY9118	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TOF2-TAP::HIS3MX6 ulp1Δ::kanMX6 YCPlac22-ulp1ts/NAT</i>	This study
MHY7914	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TOF2-TAP::HIS3MX6 ulp2Δ::kanMX4</i>	This study
MHY7925	<i>MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TOF2-TAP::HIS3MX6 slx5Δ::kanMX4 ulp2Δ::kanMX4</i>	This study
MHY9119	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TOF2-TAP::HIS3MX6 slx5Δ::kanMX4 ulp2Δ::kanMX4</i>	This study
MHY9129	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 NET1-TAP::HIS3MX6 ulp2Δ::kanMX4 ulp2C624A-13MYC:LEU2</i>	This study

MHY9120	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FOB1-TAP::HIS3MX6</i>	TAP-tag library
MHY9121	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FOB1-TAP::HIS3MX6 ulp1Δ YCPlac22-ulp1ts/NAT</i>	This study
MHY9122	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FOB1-TAP::HIS3MX6 ulp2Δ::kanMX4</i>	This study
MHY9123	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FOB1-TAP::HIS3MX6 slx5Δ::kanMX4</i>	This study
MHY9124	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FOB1-TAP::HIS3MX6 slx5Δ::kanMX4 ulp2Δ::NATMX4</i>	This study
MHY9183	<i>MATa his3Δ-200 leu2-3,211 ura3-52 lys2-801 trp1-1 fob1Δ::hphMX4</i>	This study
MHY9192	<i>MATa his3200 leu2-3,112 ura3-52 lys2-801 trp1-1 ulp2Δ::HIS3 fob1Δ::hphMX4</i>	This study
MHY5285	<i>MATa his3Δ-200 leu2-3,211 ura3-52 lys2-801 trp1-1 sir2Δ::kanMX6</i>	This study

Table S2. Proteins isolated on a SIM-fusion affinity resin and identified by LC-MS/MS.

	<i>ulp2Δ</i>			<i>ulp2Δ slx5Δ</i>				Mass (kD)
	SIM	m		SIM	m			
Protein	SC	SC	p-value	SC	SC	p-value	Description	
Smt3	134	27	1.3E-18	66	17	2.7E-08	Yeast SUMO	12
Sse1	40	8	1.4E-06	55	0	2.7E-18	Heat shock protein homolog	77
Acs2	51	10	4E-08	33	0	1.5E-11	Acetyl-coenzyme A synthetase 2	75
Cdc3	19	0	3.1E-07	3	0	0.04447	Septin ring component	60
Ssa1	111	57	2.6E-05	91	36	7E-07	Heat shock protein	70
Hsp82	26	6	0.00024	22	1	1.2E-06	Cytoplasmic chaperone of Hsp90 family	81
Eft1	108	77	0.02234	104	59	0.00039	Elongation factor 2	93
Met6	14	2	0.00146	7	0	0.00198	methionine synthase	86
Def1	6	0	0.00423	4	0	0.01992	RNA polymerase II degradation factor 1	84
Ssc1	6	0	0.00423	5	0	0.00911	Heat shock protein SSC1, mitochondrial	71
Ape2	4	0	0.01992	3	0	0.04447	Aminopeptidase 2, mitochondrial	106
Net1	170	39	4.9E-21	0	0	1	Nucleolar scaffolding protein	128
Tof2	24	0	8.7E-09	0	0	1	Topoisomerase 1-associated factor 2	86
Cdc48	35	9	5.1E-05	17	8	0.0687	Cell division control protein 48	92
Pgk	17	4	0.00325	11	6	0.22182	Phosphoglycerate kinase	45
Smc3	6	0	0.00423	0	0	1	Structural maintenance of chromosomes	141
Sec23	6	0	0.00423	2	0	0.10287	Protein transport protein	85
Rpa190	22	7	0.00431	6	3	0.31266	RNA polymerase I subunit	186
Ade3	14	4	0.01528	11	4	0.06532	C-1-tetrahydrofolate synthase, cytoplasmic	102
Shs1	4	0	0.01992	0	0	1	Seventh homolog of septin 1	63
Rps31	20	9	0.03859	6	2	0.14798	Ubiquitin-40S ribosomal protein S31	17
Tdh3	12	4	0.04076	11	8	0.49039	Glyceraldehyde-3-phosphate dehydrogenase	36
Tup1	3	0	0.04447	2	0	0.10287	General transcriptional corepressor	78

Smc1	3	0	0.04447	0	0	1	Structural maintenance of chromosomes	141
Smi1	3	0	0.04447	0	0	1	Cell wall assembly regulator	57
Gln4	3	0	0.04447	0	0	1	Glutaminyl-tRNA synthetase	93
Tup1	3	0	0.04447	2	0	0.10287	General transcriptional corepressor	78
Aco1	3	0	0.04447	1	0	0.25643	Aconitate hydratase, mitochondrial	85
Prt1	70	77	0.56363	45	5	1.3E-09	Eukaryotic translation initiation factor 3 subunit B	88
Ade5	9	5	0.28169	16	0	2.7E-06	Bifunctional purine biosynthetic protein	86
Fas1	64	52	0.26478	83	36	1.3E-05	Fatty acid synthase subunit beta	229
Fas2	65	48	0.1091	72	29	1.4E-05	Fatty acid synthase subunit alpha	207
Gln4	9	11	0.65445	11	0	0.0001	Glutamyl-tRNA synthetase	81
Acc1	47	33	0.11658	39	13	0.00023	Acetyl-CoA carboxylase	250
Sec26	59	42	0.08996	79	41	0.00047	Coatomer subunit beta	99
Ths1	7	6	0.78141	8	0	0.00094	Threonyl-tRNA synthetase, cytoplasmic	84
Rpn2	9	3	0.07642	7	0	0.00198	26S proteasome regulatory subunit	104
Rsp	0	0	1	7	0	0.00198	40S ribosomal protein S6-B	27
Ura1	33	67	0.00059	46	22	0.00326	protein required for uracil synthesis	245
Abp1	3	1	0.30632	6	0	0.00423	Actin-binding protein	66
Snf1	2	1	0.55995	6	0	0.00423	Carbon catabolite-derepressing protein kinase	72
Rps8b	0	0	1	6	0	0.00423	40S ribosomal protein S8-B	22
Pfk2	28	21	0.31648	27	10	0.0044	6-phosphofructokinase subunit alpha	108
Pyc2	8	6	0.59235	14	3	0.00545	Pyruvate carboxylase isoform 2	130
Ala1	16	10	0.2372	9	1	0.00666	Alanyl-tRNA synthetase, mitochondrial	110
Leu1	15	9	0.21822	11	2	0.00882	3-isopropylmalate dehydratase	86
Vas1	5	1	0.08798	5	0	0.00911	Valyl-tRNA synthetase, mitochondrial	126

Nip1	54	90	0.00256	15	4	0.0092	Eukaryotic translation initiation factor 3 subunit C	93
His2	23	25	0.7728	31	14	0.01029	Histidine biosynthesis trifunctional protein	88
Tps2	15	9	0.21822	14	4	0.01528	Trehalose-phosphatase	103
Kgd1	0	0	1	4	0	0.01992	2-oxoglutarate dehydrogenase, mitochondrial	114
Rpl28	0	0	1	4	0	0.01992	60S ribosomal protein L28	17
Kap123	11	10	0.82723	16	6	0.02987	Importin subunit beta-4	123
Rpg1	95	122	0.06647	26	13	0.03557	Eukaryotic translation initiation factor 3 subunit A	110
Cdc60	20	22	0.75758	24	12	0.04346	Leucyl-tRNA synthetase, cytoplasmic	124
Nop3	0	8	0.00094	3	0	0.04447	Nucleolar protein 3	45
Ecm33	2	0	0.10287	3	0	0.04447	Cell wall protein	44
Crn1	1	0	0.25643	3	0	0.04447	Coronin-like protein	73
Rpl4B	1	0	0.25643	3	0	0.04447	60S ribosomal protein L4-B	39
Rpl18b	0	1	0.26	3	0	0.04447	60S ribosomal protein L18-B	21
Rsp5	4	2	0.41	3	0	0.04447	E3 ubiquitin-protein ligase	92
Sec24	3	2	0.65	3	0	0.04447	Component CopII vesicle coat	104
Rpl17B	0	0	1.00	3	0	0.04447	60S ribosomal protein L17-B	21