Genetic Analysis Connects SLX5 and SLX8 to the SUMO Pathway in Saccharomyces cerevisiae

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ABSTRACT

MOT1 encodes an essential ATPase that functions as a general transcriptional regulator in vivo by modulating TATA-binding protein (TBP) DNA-binding activity. Although MOT1 was originally identified both biochemically and in several genetic screens as a transcriptional repressor, a combination of subsequent genetic, chromatin immunoprecipitation, and microarray analysis suggested that MOT1 might also have an additional role in vivo as a transcriptional activator. To better understand the role(s) of MOT1 in vivo, we selected for genomic suppressors of a mot1 temperature-sensitive mutation. This selection identified mutations in SPT15 (TBP) and BUR6, both of which are clearly linked with MOT1 at the functional level. The vast majority of the suppressor mutations, however, unexpectedly occurred in six genes that encode known components of the SUMO pathway and in two other genes with unknown functions, SLX5 and SLX8. Additional results presented here, including extensive synthetic lethality observed between slx5Δ and slx8Δ and SUMO pathway mutations, suggest that SLX5 and SLX8 are new components or regulators of the SUMO pathway and that SUMO modification might have a general role in transcriptional regulation as part of the TBP regulatory network.

The binding of TATA-binding protein (TBP) to the TATA box is the first step in promoter recognition, resulting in the recruitment of the other general transcription factors and pol II (Davison et al. 1983; Sawadogo and Roeder 1985). As the first step in promoter-specific transcription, the recruitment of TBP is a frequent target for regulation. Several proteins that regulate TBP activity have been identified, including Mot1 (Auble and Hahn 1993), NC2 (Meisterernst and Roeder 1991), the TBP-associated factors (Dylamacht et al. 1991), and TFIIA (Reinberg et al. 1987). A greater understanding of these proteins, their biochemical activities, their potential links to regulatory pathways, and their functional overlap with each other is important for understanding the mechanisms responsible for regulating TBP in vivo.

Mot1 was identified in multiple genetic selections in yeast as a general inhibitor of transcription (Davis et al. 1992; Piatii et al. 1992; Prelich 1997) and biochemically as an ATP-dependent inhibitor of TBP (Auble and Hahn 1993; Auble et al. 1994), suggesting that it has a broad inhibitory role on transcription in vivo, mediated through effects on TBP. Indeed, MOT1 encodes a large ATPase that uses ATP hydrolysis to remove TBP from the TATA box (Auble et al. 1994), and a TBP mutant that is defective for binding Mot1 increases transcription from a basal reporter (Cang et al. 1999). Mot1’s ATP-dependent inhibitory activity appears specific to TBP, as three other site-specific DNA-binding proteins were unaffected by Mot1 in vitro. On the basis of its initial characterization as a TBP inhibitor, it was somewhat surprising that mot1 mutations reduce activation of some genes in vivo (Madison and Winston 1997; Prelich 1997; Lemaire et al. 2000; Andraud et al. 2002; Dasgupta et al. 2002; Geisberg et al. 2002), suggesting that it also has a positive role at some promoters. The positive role of Mot1 was proposed to be direct, since chromatin immunoprecipitation assays revealed that Mot1 was present at actively transcribed MOT1-stimulated genes and was recruited under inducing conditions (Andraud et al. 2002; Dasgupta et al. 2002; Geisberg et al. 2002). Thus, although the initial biochemical characterization of Mot1 in isolation led to straightforward conclusions, unexpected complexity has emerged. Many questions still remain about the direct and indirect roles of Mot1, including why it affects only a subset of promoters, what distinguishes whether it activates or represses a promoter, and how it overlaps at the functional level with other TBP regulators.

To better understand the role of MOT1 and to find genes that are functionally related to MOT1 in vivo, we performed genomic suppressor analysis of a mot1 temperature-sensitive mutation. This selection unexpectedly identified nearly the entire known small ubiquitin-like modifier (SUMO) pathway. SUMO is a 101-amino-acid protein in the ubiquitin family (Matunis et al. 1996; Gill 2004; Johnson 2004). Ubiquitin and SUMO are
clearly related at the structural level (Bayer et al. 1998), but differ both functionally and at the primary amino acid level, where they share only 18% identity. Like ubiquitin, SUMO is covalently attached to lysine residues of target proteins by a series of steps that requires maturation by a protease (Li and Hochstrasser 1999, 2000) and E1 (Johnson et al. 1997), E2 (Johnson and Blobel 1997), and E3 (Johnson and Gupta 2001; Strunnikov et al. 2001; Takahashi et al. 2001; Zhao et al. 2004) proteins that are analogous to those employed by the ubiquitin pathway. The protease, E1, E2, and E3 proteins of the SUMO pathway are specific for SUMO modification, as they do not catalyze conjugation of ubiquitin to target substrates. Unlike ubiquitin, whose role in proteosome-mediated protein degradation has been extensively characterized, the function of SUMO in vivo is not understood as well (Gill 2004; Johnson 2004; Hay 2005). SUMO modification has been proposed to affect target proteins via three mechanisms: (1) stabilizing proteins by counteracting ubiquitin-dependent degradation, (2) affecting protein localization, and (3) modulating protein-protein interactions. The SUMO pathway is conserved from yeast to humans, and in yeast most of the genes that encode SUMO pathway components are essential for viability (Dohmen et al. 1995; Seufert et al. 1995; Johnson et al. 1997; Li and Hochstrasser 1999). Much of what is known about the biochemistry of SUMOylation has been drawn from analogy to the ubiquitin pathway (Hershko et al. 2000), while genetic analysis has been hampered by the essential nature of the SUMO pathway for viability and the lack of many classical genetic selections that link the SUMO pathway to other biological processes (Meluh and Koshland 1995; Biggins et al. 2001; Soutelle et al. 2004). The genetic selection that is described here is particularly sensitive to perturbation of the SUMO pathway, resulting in the identification of two new SUMO pathway components.

Materials and Methods

Yeast strains, media, and genetic methods: *S. cerevisiae* strains used in this study are listed in Table 1. All media used, including rich media (YPD), sucrose media (YPSuc), synthetic complete drop-out media (for example, SC-Ura), and sporulation media were made as described (Rose et al. 1990). SCGal plates were synthetic complete (SC) media containing 2% galactose and 1 µg/ml antiamycin A. Standard genetic methods for mating, sporulation, transformation, and tetrad analysis were used throughout this study (Rose et al. 1990).

Plasmids: pPM218 is a pRS316-based *URA3*-marked CEN plasmid containing wild-type *UBC9*, p153 is a pRS314-based *TRPI*-marked CEN plasmid containing wild-type *UBA2* (Johnson et al. 1997). pF371, pF372, pF374, pF375, and pF376 are pRS315-based LEU2-marked CEN plasmids containing wild-type *MOT1*, mot1-14, mot1-42, mot1-71, and mot1-81, respectively (Darst et al. 2003). Plasmids containing wild-type *SMT3*, *MFC–SMT3*, and *GFP–SMT3* for the “SUMO fingerprint” assays (Pansé et al. 2004) were provided by Ed Hurt.

Cloning the suppressor genes: The suppressor genes from complementation group A (SLX5), group C (*AOS1*), group G (*BUR6*), group H (*ULPI*), group I (*SPT15*), and group J (*ULP2*) were cloned by transforming the recessive *mot1-301 sup* mutant strains with a CEN *URA3* genomic library (Rose et al. 1987) and selecting for His<sup>+</sup> colonies. Plasmid DNA was recovered from Suc<sup>+</sup>, His<sup>+</sup>, Gal<sup>+</sup>, and Ts<sup>+</sup> candidates (Hoffman and Winston 1987) and transformed back into the suppressor strain to confirm the complementation phenotypes. The mutant gene was confirmed by direct DNA sequencing of PCR-amplified products from the suppressor strain. The suppressor genes from complementation group B (*UBA2*), group D (*SLX9*), group E (*UBC9*), and group F (*SMT3*) were identified by transformation with those candidate genes on CEN plasmids and confirmed by direct DNA sequencing of PCR-amplified products from the suppressor strain.

Western analysis: Extracts were prepared by growing 30 ml of yeast to a concentration of 2–3 × 10<sup>7</sup> cells/ml. Cells were harvested by centrifugation at 2000 rpm for 5 min, resuspended in 400 µl of breaking buffer [50 mM Tris (pH 7.5), 10% glycerol, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 100 mM NaCl, 1 mM PMSF, 10 mM NEM, and protease inhibitors], and incubated on ice for 30 min before lysis. Cells were disrupted by vortexing with glass beads, and extracts were clarified by centrifugation at 16,000 × g for 15 min. Fifty micrograms of protein from each extract were separated by SDS–PAGE. Proteins were transferred to Immobilon P (Millipore, Bedford, MA), and the filter was probed with anti-SUMO (gift from Pamela Meluh), anti-G6-PDH (Sigma, St. Louis), or anti-Flag antibody (Sigma). Primary antibody was washed with phosphate-buffered saline plus 0.1% Tween 20, and the filter was then probed with horseradish-peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Amersham, Buckinghamshire, UK). Final detection was done with a chemiluminescent kit (SuperSignal, Pierce, Rockford, IL) according to the manufacturer’s instructions. For the “SUMO fingerprint” assay (Panse et al. 2004), the genomic *TUP1* locus was FLAG tagged at the 3′-end in an *smt3Δ::TRPI* shuffle strain.

RNA analysis: Cells were grown to 1–2 × 10<sup>7</sup> cells/ml, and RNA was isolated as previously described (Carlson and Botstein 1982). RNA was separated in a 1% formaldehyde agarose gel. Blotting and hybridization to DNA probes were performed as previously described (Swanson et al. 1991). The probes used were a 2.4-kb BamHI–EcoRI fragment of pDE32-1 (*SPT13*) and 1575, 523, 591, and 945-bp PCR products of the *INO1*, *BNA1*, *HSP26*, and *URA1* ORFs, respectively. RNA was crosslinked to GeneScreen (New England Nuclear, Boston) with the auto crosslink mode in a Stratalinker 1800 (Stratagene, La Jolla, CA). Probes were radio-labeled with <sup>32</sup>P using a nick translation kit from Roche according to the manufacturer’s instructions.

Results

Isolation and cloning of genomic suppressors of *mot1-301*: Mot1 has been studied extensively at the biochemical level, yet much remains to be learned about its functions in vivo. To gain insight into the role of *MOT1*, in particular its relationship with TBP and the other TBP regulators in vivo, we selected for genomic mutations that suppress the temperature-sensitive *mot1-301* mutation. This allele (originally designated *bur3-1), which arose from our original Bur selection for mutations that increase transcription from a UAS-less *SUC2* promoter (Prelich and Winston 1993), causes temperature-sensitive growth,
## TABLE 1

### S. cerevisiae strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>GY481</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 leu2Δ1 trp1Δ63</td>
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<tr>
<td>GY485</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 leu2Δ1 mot1-301</td>
</tr>
<tr>
<td>GY514</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 leu2Δ1 mot1-301 slx5-103</td>
</tr>
<tr>
<td>GY515</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 ade8 mot1-301 slx5-104</td>
</tr>
<tr>
<td>GY529</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 ade8 mot1-301 aos1-101</td>
</tr>
<tr>
<td>GY527</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 leu2Δ1 mot1-301 slx8-101</td>
</tr>
<tr>
<td>GY528</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 leu2Δ1 mot1-301 slx8-102</td>
</tr>
<tr>
<td>GY529</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 leu2Δ1 mot1-301 smt3-101</td>
</tr>
<tr>
<td>GY530</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 ade8 mot1-301 slx8-103</td>
</tr>
<tr>
<td>GY532</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 leu2Δ1 mot1-301 suc2-9101</td>
</tr>
<tr>
<td>GY533</td>
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</tr>
<tr>
<td>GY549</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 ade8 mot1-301 slx8-105</td>
</tr>
<tr>
<td>YY34</td>
<td>MATa his4-9176 lys2-173R2 sue2ΔUAS(-1900/-390) ura3-52 leu2Δ1 trp1Δ63 spt15Δ102::LEU2 [pYC1(spt15-601 TRP1 CEN AMP)]</td>
</tr>
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<td>YY38</td>
<td>MATa his4-9176 lys2-173R2 sue2ΔUAS(-1900/-390) ura3-52 leu2Δ1 trp1Δ63 spt15Δ102::LEU2 [pYC10(spt15-610 TRP1 CEN AMP)]</td>
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<td>OY167</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 leu2Δ1 trp1Δ63 mot1Δ::KAN [pMR13(MOT1 URA3 CEN AMP)] [slx8Δ::TRP1]</td>
</tr>
<tr>
<td>OY168</td>
<td>MATa his4-9126 lys2-1286 sue2ΔUAS(-1900/-390) ura3-52 leu2Δ1 trp1Δ63 mot1Δ::KAN [pMR13(MOT1 URA3 CEN AMP)]</td>
</tr>
<tr>
<td>OY173</td>
<td>MATa hisΔ200 lys2-801 ura3-52 leu2Δ1 trp1Δ63 ubc9Δ::TRP1 ubc9Δ::LEU2::LEU2</td>
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increased transcription from the UAS-less su2Δ̃uas(-1900/-390) reporter (Bur− phenotype), altered transcription from the his4-912 allele (Spt− phenotype), and poor growth on galactose-containing medium. The mot1-301 mutation results in a glutamate-to-lysine change at the last HEAT repeat and the C-terminal ATPase domain of Mot1. By selecting for revertants that grow at the non-permissive temperature, 95 spontaneous Ts− suppressors of mot1-301 were isolated; 11 mutations were dominant, and 84 suppressors were recessive, with the recessive mutations comprising 11 complementation groups. An example of the mot1-301 and mot1-301 sup− phenotypes is provided in Figure 1A. Similar to the representative mutant shown here, the majority of the suppressors reversed all of the mot1-301 mutant phenotypes that were tested. Using standard complementation-based cloning with a genomic CEN plasmid library, combined with plasmid complementation using several previously cloned candidate genes, we have identified the gene responsible for 10 of the complementation groups. An example of cloning the gene responsible for complementation group E by plasmid-based complementation with a CEN library plasmid is provided in Figure 1B, and a summary of the cloning of the suppressor genes is provided in Table 2. For each gene listed in Table 2 we have confirmed that the correct gene has been cloned by linkage analysis, by subcloning the individual gene on a CEN plasmid, and by DNA sequencing of one or more mutant alleles.

TABLE 2

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of isolates</th>
<th>Gene</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>SPT15</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>G</td>
<td>2</td>
<td>BUR6</td>
<td>Regulator of TBP</td>
</tr>
<tr>
<td>F</td>
<td>4</td>
<td>SMT3</td>
<td>SUMO</td>
</tr>
<tr>
<td>B</td>
<td>7</td>
<td>UBA2</td>
<td>SUMO E1 subunit</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>AOS1</td>
<td>SUMO E1 subunit</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>UBC9</td>
<td>SUMO E2</td>
</tr>
<tr>
<td>H</td>
<td>3</td>
<td>ULP1</td>
<td>SUMO protease</td>
</tr>
<tr>
<td>J</td>
<td>3</td>
<td>ULP2</td>
<td>SUMO protease</td>
</tr>
<tr>
<td>A</td>
<td>42</td>
<td>SLX5</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>12</td>
<td>SLX8</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>1</td>
<td>Uncloned</td>
<td></td>
</tr>
</tbody>
</table>

The suppressor genes can be placed into three groups. The first group contains SPT15 and BUR6, which encode TBP and the α-subunit of negative cofactor 2 (NC2). These genes are clearly functionally related to MOT1, since both Mot1 and Bur6 regulate TBP, validating the underlying purpose for the selection. The suppressor mutations cause an Arg98-to-Cys change in TBP and a Pro76-to-Leu change in Bur6. On the basis of the TBP–NC2–DNA crystal structure (Kamada et al., 2001), these residues lie on the N-terminal stirrup of TBP and on helix 2 of the histone fold domain of Bur6. To more completely identify the amino acid positions of TBP that are important for suppression, a plasmid shuffle strategy using a library of randomly mutagenized SPT15 plasmids yielded an additional 19 spt15 missense mutations at nine amino acid positions that suppress mot1-301 (Figure 2). These mutations do not cluster tightly on any single surface of TBP, but occur largely at positions that are known or likely to reduce TBP DNA-binding or NC2-binding activity (Nikolov et al., 1992, 1996; Bryant et al., 1996; Cang et al., 1999; Kamada et al., 2001). These suppressors contribute to understanding the mechanism of suppression, indicating that reduction of TBP DNA-binding activity suppresses the mot1-301 defect and, by extension, that TBP is indeed more active in the mot1-301 strains.
were the SUMO E3's \textit{SIZ1}, \textit{SIZ2/NFI1}, and \textit{MMS21} (Johnson and Gupta 2001; Zhao et al. 2004).

The third group of suppressor genes consists of \textit{SLX5} and \textit{SLX8}. These two genes were identified together previously in a screen for mutations that cause a synthetic lethal phenotype when combined with a deletion of \textit{SGS1} or \textit{TOP3} (Mullen et al. 2001). \textit{SGS1} and \textit{TOP3} have roles in maintaining genome stability, so the reason why \textit{SLX5} and \textit{SLX8} were also identified in our \textit{mot1-301} suppressor hunt is not immediately obvious. \textit{SLX5} and \textit{SLX8} each encode RING finger domain proteins that interact with each other as revealed in co-immunoprecipitation assays (Mullen et al. 2001). Although the specific roles of \textit{SLX5} and \textit{SLX8} were unknown, it was proposed that they function together as part of a complex or pathway on the basis of their physical interaction and similar mutant phenotypes.

Our finding of both genes together in a second selection is consistent with this proposal. We have directed the bulk of our subsequent efforts in this suppression hunt toward understanding the roles of \textit{SLX5} and \textit{SLX8} and how mutations in these genes suppress \textit{mot1-301}.

Evidence indicating a role for \textit{SLX5} and \textit{SLX8} in the SUMO pathway: Multiple recessive \textit{slx5} and \textit{slx8} alleles were isolated in our selection, suggesting that suppression of \textit{mot1-301} was due to loss of function in these genes. To test that idea, precise \textit{slx5} \textit{D} \textit{URA3} and \textit{slx8} \textit{D} \textit{TRP1} deletions were created and tested for suppression of \textit{mot1-301}. As shown in Figure 3A, the \textit{slx5} \textit{D} and \textit{slx8} \textit{D} deletions efficiently suppressed \textit{mot1-301}. Furthermore, \textit{slx5} \textit{D} \textit{slx8} \textit{D} double mutants exhibited growth defects and suppression activity identical to the individual deletions, with no apparent additive or combinatorial effects. We conclude that suppression is due to loss of \textit{SLX5} and \textit{SLX8} function and that \textit{SLX5} and \textit{SLX8} have nonredundant functions as part of a pathway necessary for the \textit{mot1-301} phenotypes.

It was quite interesting that our selection identified every step of the SUMO pathway with the conspicuous absence of the known yeast SUMO E3 genes (\textit{SIZ1}, \textit{SIZ2}, and \textit{MMS21}). The absence of E3 genes from our selection could be due to redundancy of the known E3 genes or because an undiscovered E3 might be required for targeting a substrate that is important for the \textit{mot1-301} suppression phenotype. To test if mutations in any of the known SUMO E3 genes can suppress \textit{mot1-301}, \textit{SIZ1} and \textit{SIZ2} were deleted both individually and in combination. \textit{SIZ1} and \textit{SIZ2} deletions individually resulted in partial suppression of \textit{mot1-301}, whereas the \textit{siz1} \textit{D} \textit{siz2} \textit{D} double mutant completely reversed all of...
played no combinatorial effect, demonstrating the specificity of the double-mutant phenotypes.

Figure 4.—Analysis of double-mutant phenotypes. (A) slx5Δ is synthetically lethal with a uba2 mutation. Tetrads from a diploid heterozygous for slx5Δ and uba2-101 show the expected pattern of double-mutant lethality. Dissection of the same diploid transformed with a UBA2 CEN plasmid reverses the double-mutant lethal phenotype. The dead spores were slx5Δ uba2-101 double mutants. (B) A table of the double-mutant combinations. Both slx5Δ and slx8Δ were synthetically lethal with mutations in genes that encode the E3 (SIZ2) or SUMO (SMT3). Double mutants of slx5Δ or slx8Δ with suppressor alleles in SPT15 (TBP) or BUR6 (NC2 subunit) displayed no combinatorial effect, demonstrating the specificity of the double-mutant phenotypes.

The isolation of slx5 and slx8 mutations in the same selection as SUMO pathway components suggested that these genes might also have a role in SUMOylation. Alternatively, SLX5 and SLX8 might be more directly related to SPT15 (TBP) and BUR6, the other genes identified in the selection. The notion that SLX5 and SLX8 might function in the SUMO pathway is supported by an interaction detected between Slx5 and Smt3 (SUMO) in a high-throughput two-hybrid study (Uetz et al. 2000). To test whether SLX5 and SLX8 are more closely related to the SUMO pathway or to TBP and BUR6, an extensive series of crosses was undertaken to examine double-mutant phenotypes. slx5Δ and slx8Δ deletion strains were crossed with strains containing representative suppressor alleles in AOS1 (E1 subunit), UBA2 (E1 subunit), UBC9 (E2), and SMT3 (SUMO) (each of which are essential genes) and deletions of SIZ1 and SIZ2. The SUMO pathway mutants used in this analysis were healthy and caused no detectable phenotypes in a MOT1+ background, and therefore any combinatorial effects detected were likely to be meaningful and not due to simple additive growth defects. Interestingly, both slx5Δ and slx8Δ displayed synthetic lethal or synthetic sickness phenotypes with every SUMO pathway mutant tested, with the strongest effects occurring in combination with the E1 or E2 mutations. A representative example of tetrads from a double-mutant cross is in Figure 4A, and a summary of all the crosses is in Figure 4B. The double-mutant phenotypes observed with slx5Δ or slx8Δ were stronger than those that were observed when siz2Δ was crossed with the E1 and E2 mutants (Figure 4B). Importantly, these double-mutant phenotypes were specific, since no combinatorial phenotypes were observed when slx5Δ or slx8Δ strains were crossed with the suppressing mutations in SPT15 (TBP) or BUR6. On the basis of their isolation in the same suppressor hunt and these double-mutant phenotypes, we conclude that SLX5 and SLX8 are functionally related to the SUMO pathway.

Insights into the mechanism of suppression: The identification of suppressor mutations is important because it establishes a functional link between two or more genes; however, fully understanding the molecular basis for suppression can be a daunting task. We have obtained several results that begin to address the mechanism resulting in suppression of the mot1-301 phenotypes. First, as described above, we identified a series of TBP mutations that suppress mot1-301 (Figure 2), with most of the affected residues previously shown to affect TBP DNA-binding activity. This suggests that the underlying cause of the mot1-301 phenotypes is a more active population of TBP, which is suppressed by compensatory SPT15 (TBP) or BUR6 mutations that reduce TBP binding. Second, suppression could simply result from over-expression of the mot1-301 protein in suppressor strains. Western blots, however, revealed no change in mot1-301 levels in wild-type vs. slx5Δ strains (data not shown), suggesting that proteins levels are unaffected by the suppressors.

MOT1 has been proposed to have direct roles as both an activator and a repressor of transcription. To determine whether our suppressing mutations affect both MOT1-mediated repression and activation, or whether they specifically affect only one class of MOT1-regulated genes, Northern analysis was performed. RNA prepared from a mot1-301 strain and from mot1-301 strains containing
suppressors. Strains with the indicated genotypes were grown in rich YPD media at 30°C and harvested. RNA was isolated and probed for the indicated transcripts. mot1-301 increases transcription of HSP26 and INO1 and reduces transcription of BNA1 and URA1. Both the activation and the repression defects were suppressed by the SUMO pathway mutations slx8Δ and slx5Δ. The same blots were probed with SPT15 as a loading control.

Figure 5.—Northern analysis of mot1-301 and its suppressors. Strains with the indicated genotypes were grown in rich YPD media at 30°C and harvested. RNA was isolated and probed for the indicated transcripts. mot1-301 increases transcription of HSP26 and INO1 and reduces transcription of BNA1 and URA1. Both the activation and the repression defects were suppressed by the SUMO pathway mutations slx8Δ and slx5Δ. The same blots were probed with SPT15 as a loading control.

Figure 6.—Specificity of suppression by slx5Δ and slx8Δ. (A) Allele specificity. slx8Δ was tested for suppression of mot1-301 and four other mot1 mutations using a plasmid shuffle strategy. slx8Δ suppressed mot1-301 completely, but only partially suppressed any of the other mot1 alleles. The mot1-301 strain shown here contains mot1-301 on a CEN plasmid, which produces weaker Ts+ phenotypes than genomic mot1-301, as compared with Figure 1. (B) Gene specificity. slx5Δ specifically suppressed the Bur+ phenotype of spt15-610 (defective for binding Mot1), but not of spt15-601 (defective for binding NC2). Photos of Gal plates on day 2 (D2) and day 3 (D3) are shown. slx5Δ did not suppress the Gal− phenotype of either spt15 allele and caused a tighter Gal− phenotype when combined with spt15-601.
only obvious motif is a C3HC4 RING domain near the carboxy-terminus of both proteins (Figure 7A). RING domains and RING-related domains are found in some ubiquitin and SUMO E3’s, suggesting that SLX5 and SLX8 might encode new E3’s that target Mot1 or some Mot1-related protein for SUMOylation. To determine whether the RING domains are required for activity, we sequenced nine of our suppressor alleles, and each of these mutations directly or indirectly affect the RING domains. Two \textit{slx5} alleles and one \textit{slx8} allele truncate the proteins before the RING domain, while the remaining five mutations all are located within the RING domain (Figure 7A). In particular, the \textit{slx5}-104, \textit{slx8}-101, and \textit{slx8}-103 mutations cause amino acid changes of conserved cysteine residues within the Slx5 and Slx8 RING domains. These RING domain missense mutations cause indistinguishable phenotypes when compared to \textit{slx5Δ} or \textit{slx8Δ}.

On the basis of the requirement for the RING domain and the synthetic phenotypes with SUMO pathway mutations, we anticipated that Slx5 and Slx8 might have SUMO E3 activity. Several candidate substrates that are part of the Mot1/TBP regulatory system contain consensus SUMOylation sites (ΨKXE), including Mot1, TBP, and both the Bur6 and Ydr1 subunits of NC2. Despite extensive effort, we have been unable to detect SUMOylation of any of these proteins \textit{in vivo}. In the absence of a confirmed Slx5- or Slx8-dependent \textit{in vivo} substrate, we asked whether \textit{slx5Δ} and \textit{slx8Δ} deletions broadly affect SUMOylation by probing a Western blot of proteins in a crude cell extract with an anti-SUMO antibody, expecting to observe reduction of SUMOylation. Contrary to our expectation, the intensity of bands observed after probing with an anti-SUMO antibody increased in the \textit{slx5Δ} strain, indicated by the asterisks (*).
increases in SUMOylation (data not shown). Probing the same filter with an anti-G6-PDH control antibody demonstrated that equal levels of protein were present in each lane. Since the identity of the SUMOylated proteins in the crude extract are unknown, we were not able to distinguish whether the amount of the SUMOylation of the target proteins was affected. It was therefore important to test whether slx5Δ or slx8Δ increased SUMOylation of a known substrate. To achieve this goal and to assay SUMOylation independently of the SUMO antibody, we next examined SUMOylation of Tup1 using a SUMO “fingerprint” assay (Panse et al. 2004) in which in vivo expression of SMT3 and two epitope-tagged versions of SMT3 generate a ladder of any SUMOylated substrates, detectable by their differential mobility in SDS-polyacrylamide gels. The expected ladder of modified Tup1–Flag is observed in both SLX5+ and slx5Δ strains, but in agreement with the pattern observed in the crude extract, the intensity of each of the SUMOylated Tup1 bands is increased in the slx5Δ strain (compare lanes 1 and 4, 2 and 5, and 3 and 6 in Figure 8B). This is not due to a simple increase in the amount of the Tup1 protein, since the level of unmodified Tup1 was unchanged. Thus, slx5 and slx8 mutations increased SUMOylation of the major substrates detectable in a crude extract and at least one specific substrate in a different assay.

**DISCUSSION**

The major goal of any suppressor hunt is to establish a genetic link between genes or groups of genes that were previously unsuspected of being functionally linked. Our selection for genomic suppressors of mot1-301 has yielded two genes (SPT15 and BUR6) that were known to be related to MOT1, confirming that the selection is achieving its goal and suggesting that the other genes arising from the selection are also functionally relevant to MOT1. In addition, we identified six genes (SMT3, AOS1, UBA2, UBC9, ULP1, and ULP2) that compose nearly the entire known SUMO pathway and two other genes (SLX5 and SLX8) that were previously implicated as having a role in maintaining genome stability. This selection therefore establishes two important functional connections: linking the SUMO pathway to MOT1 and, by extension, to regulation of TBP, and linking SLX5 and SLX8 to the SUMO pathway.

**Insights into the mechanism of suppression:** Establishing links among the SUMO pathway, MOT1, and SLX5-SLX8 is an important first step, yet ultimately we would like to understand the molecular basis for those links. To achieve this goal, two questions need to be addressed. First, What is the biochemical defect of the Mot1-301 protein, and second, How do the suppressors compensate for that defect? Although definitive answers to these questions have proven elusive, progress has been made, and at least a broad model can be derived from the results reported here. We do not know the specific biochemical defect of the Mot1-301 protein; it is expressed at normal levels and co-immunoprecipitates with TBP from yeast extracts equivalently to wild-type Mot1 (data not shown). At a functional level, however, we can safely infer that Mot1-301 is defective for regulating TBP, and not for some uncharacterized TBP-independent role, since mot1-301 is synthetically lethal with an spt15 mutant (spt15-610), that is defective for binding Mot1 (Cang et al. 1999), slx5Δ suppresses both mot1-301 and this spt15-610 allele (Figure 6B), and an extensive collection of spt15 mutations suppress mot1-301 (Figure 2). Because the majority of the TBP residues that suppress mot1-301 have been shown to reduce TBP DNA-binding or NC2-binding activity, we further infer that the mot1-301 defect results in a more active TBP population.

How, then, do the suppressor mutations compensate for this mot1 defect? When considering the proposed dual role of MOT1 in activating and repressing specific genes, one possibility is that the suppressors selectively affect only one of these roles. This possibility was especially relevant for the SUMO pathway mutations, since SUMOylation is required for repression activity but not for the activation function of some dual-function site-specific DNA-binding proteins such as the androgen receptor and Sp3 (Poukka et al. 1999; Ross et al. 2002), and it was therefore reasonable to speculate that SUMOylation might affect only Mot1-dependent repression. Alternatively, they could affect a more general aspect of Mot1 biology and thereby reverse all mot1 defects. All the suppressor mutations that we tested reversed both the activation and the repression defects of mot1-301, suggesting that a fundamental aspect of MOT1 has been affected.

The major barrier for understanding the suppression mechanism at the molecular level is knowing the identity of the relevant SUMOylated substrate. This will be a particularly challenging question to resolve because typically only a small percentage of the relevant substrate is SUMOylated in vivo, for example, the substrate responsible for the initial genetic identification of SMT3 (SUMO) as a suppressor of a mif2 mutation (Meluh and Koshland 1995) still remains unknown. We have tested a small number of candidates that are functionally related to Mot1 and that have a consensus SUMOylation motif (PKXE), including Mot1 itself, TBP, and the Bur6 and Ydr1 subunits of NC2, to determine whether they are SUMOylated in vivo, and if so, whether that SUMOylation is SLX5 and SLX8 dependent, but the results thus far are negative. Because the mot1-301 mutation introduces a lysine residue at position 1226 within a reasonable SUMO consensus motif, we addressed the possibility that this lysine residue of Mot1-301 might become aberrantly SUMOylated, thereby partially inactivating the protein. By this model, mutations in the SUMO pathway would affect Mot1-301 through K1226,
but might have no effect on wild-type Mot1. We have been unable to detect SUMOylation of either Mot1* or Mot1-301, however, and mutation of E1226 to arginine, which would abolish the suspected SUMOylation event at that site, caused a phenotype identical to that of K1226 and was suppressible by slx8Δ. We therefore conclude that the SUMO pathway does not function through K1226 of Mot1-301. A related issue is whether increased or decreased SUMOylation is responsible for suppression of mot1-301. The definitive answer to this issue awaits identification of the substrate, as the results obtained thus far are inconclusive. For example, slx5Δ, slx8Δ, ubc9-101, and the sizeΔ sizeΔ double mutant suppress mot1-301, but slx5Δ, slx8Δ, and ubc9-101 appear to increase SUMOylation, while sizeΔ sizeΔ decreases SUMOylation. Further attempts to identify the relevant substrate through both biochemical and genetic methods are in progress.

Sls5 and Slx8 as a part of the SUMO pathway in yeast: Regardless of the specific mechanism underlying the role of the SUMO pathway in suppression of mot1-301, an important contribution from this study is the genetic connection of SLX5 and SLX8 with this pathway. SLX5 and SLX8 were originally identified in a screen for mutations that were synthetically lethal with mutations in the SGS1 DNA helicase, and thus their only functional link was to maintenance of genome stability (Mullen et al. 2001). Their relationship with SGS1 at the molecular level remains elusive to date. Sixty-four percent (54/84) of our identified suppressor mutations were in these two genes, and 26 of the remaining 29 identified mutations (90%) were in known SUMO pathway components; this was our first indication that SLX5 and SLX8 were functionally linked to SUMO. Furthermore, we observed extensive synthetic double-mutant phenotypes when slx5Δ and slx8Δ null alleles were crossed with mutations in known SUMO pathway genes, but not with the spt15 and bur6 suppressor mutations, and the double-mutant phenotypes were more severe than those observed when sizeΔ was crossed with the same SUMO pathway mutants. The identification of each step of the pathway except for E3 mutants in our original suppressor collection, combined with RING domains that are required for SLX5 and SLX8 function and the detection of an Slx5–Smt3 interaction in a high-throughput two-hybrid screen (Uetz et al. 2000), suggested that Slk5 and Slk8 might be new SUMO E3 proteins. Surprisingly, however, deletions of the SIZ1 and SIZ2 E3’s individually partially suppressed mot1-301, and sizeΔ sizeΔ double mutants completely suppressed mot1-301, indicating that they are partially redundant E3’s for the relevant substrate. Thus, it remains an open issue whether Siz1–Siz2 and Slx5–Slx8 constitute overlapping E3’s or whether Slx5 and Slx8 have a new role in the SUMO pathway.

Combined, these results are most consistent with a broad model in which the mot1-301 mutation results in a more active population of TBP that can be suppressed by either spt15 (TBP) or bur6 mutations that reduce TBP DNA-binding activity or by altering the SUMO pathway. SUMO pathway defects presumably alter a fundamental property of the Mot1-301 protein or TBP to restore Mot1-mediated repression and activation, with Slx5 and Slx8 functioning as new components or regulators of the pathway. Although this model is by necessity quite broad at this time, we look forward to future experiments that will elaborate the details, with the key questions being the specific roles of Slx5 and Slx8 and identification of the substrate for SUMO-mediated suppression that ultimately impacts on TBP activity.

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