Sumoylation of the Yeast Gcn5 Protein†

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ABSTRACT: Sumoylation, the process by which the ubiquitin-related SUMO protein is covalently attached to lysine side chains in other proteins, is involved in numerous processes in the eukaryotic cell, including transcriptional repression. In this study, we identify Gcn5, the histone-modifying subunit of the transcriptional regulatory complex SAGA, as a sumoylation substrate in yeast. In vitro, multiple sumoylation of recombinant Gcn5 alone or as a trimer with its interacting proteins Ada2 and Ada3 did not affect Gcn5's histone acetyltransferase (HAT) activity, suggesting that modification of Gcn5 with yeast SUMO (Smt3) may not directly regulate its HAT function. Through site-directed mutagenesis, the primary in vivo sumoylation site was identified as lysine-25, although an unsumoylatable K-to-R mutation of this residue led to no obvious in vivo effects. However, fusion of SUMO to the N-terminus of Gcn5 to mimic constitutive sumoylation resulted in defective growth on 3-aminotriazole media and reduced basal and activated transcription of the SAGA-dependent gene *TRP3*. Taken together with recent identification of multiple additional subunits of SAGA as sumoylated proteins in vivo, these data suggest that Gcn5 sumoylation may have an inhibitory role in transcriptional regulation.

Gcn5 is a yeast transcriptional regulatory protein involved in the activation of numerous genes (*22*, *27*). It is a component of the coactivator complex SAGA[1] (*13*, *28*, *39*) and possesses histone acetyltransferase (HAT) enzymatic activity (*5*), which can acetylate histones in repressive chromatin structure and contribute to increased gene expression. Much is known about Gcn5 function and its relationship to other subunits within SAGA and related complexes. For example, the C-terminal bromodomain of Gcn5 can potentiate nucleosome acetylation by SAGA (*36*) and is a putative interaction site with acetyllysine on histone substrate (*11*, *44*). Also, the SANT domain of the Ada2 protein interacts directly with Gcn5 and affects its HAT activity (*4*, *37*).

However, another potential level of regulation of Gcn5 and SAGA function is covalent modification of Gcn5 itself, and this possibility has not previously been investigated in detail. Although histones within chromatin are the best known substrates for covalent modifications (*16*) [such as acetylation, methylation, phosphorylation, ubiquitylation (*29*), sumoylation (*34*)] that affect transcription, there are also numerous examples of transcriptional regulatory proteins that are modified in similar ways to modulate their functions. One example is the mammalian tumor suppressor p53, which is known to be acetylated, phosphorylated, ubiquitylated, and methylated (*8*) on certain residues as part of its activator function (*3*). Most recently, p53, like histones, was found to be sumoylated (*12*, *30*).

SUMO is a ubiquitin-related protein that is conjugated to various proteins in eukaryotic cells (*17*). Through the C-terminus of its mature form, SUMO is covalently linked to particular lysine side chains, resulting in a variety of in vivo effects on diverse physiological processes in the cell. Besides histones and p53, among the many proteins known to be modified by SUMO are various other transcription factors (*41*), nuclear receptors (*26*), nuclear transport proteins such as RanGAP (*24*, *25*), and DNA replication/repair factors such as PCNA (*15*). Functions that SUMO is known to affect or mediate include signal transduction, intracellular localization, and response to DNA damage. In addition, there is evidence that sumoylation, like ubiquitin in some cases (*9*, *14*, *20*, *38*), can be involved in transcriptional regulation, both positive and negative (*41*), but this aspect of sumoylation has not been fully investigated. Sumoylation tends to be involved in gene repression, although there are exceptions to this general finding.

In this study, we find that the Gcn5 protein is a substrate for sumoylation in vivo in yeast, and we have identified a single lysine residue as the primary site of sumoylation. Gcn5 can also be multiply sumoylated in vitro by recombinant forms of yeast SUMO (Smt3) and SUMO pathway enzymes. Although this sumoylation does not affect Gcn5's HAT activity in vitro, we find that a SUMO-Gcn5 fusion does reduce transcription of a SAGA-dependent gene, indicating a possible regulatory role of Gcn5 sumoylation.

MATERIALS AND METHODS

Construction of Gcn5 Plasmids. Plasmids were purified from *Escherichia coli* cells and constructed by standard

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[1] Abbreviations: SAGA, Spt-Ada-Gcn5 acetyltansferase; HAT, histone acetyltransferase; SUMO, small ubiquitin-related modifier; ATP, adenosine triphosphate; IP, immunoprecipitation; PCR, polymerase chain reaction.

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recombinant techniques (*33*). To produce a yeast integrative plasmid with FLAG-tagged Gcn5, QuikChange mutagenesis (Stratagene) was used to insert a single FLAG epitope (DYKDDDDK) at the N-terminal end of Gcn5 in pRS306-P_{GCN5}-yGCN5 (*42*). To create a SUMO-Gcn5 fusion plasmid, *Nco*I and *Nde*I restriction sites were introduced at the N-terminus of Gcn5 in pPC87-yGCN5 (*7*) by QuikChange mutagenesis, and a recombinant fragment of the yeast SUMO gene *SMT3* (a kind gift of P. Meluh) lacking the codons for the Gly-Gly C-terminal motif was subcloned as an *Nco*I/*Nde*I fragment. In addition, an N-terminally, singly FLAG-tagged version of pPC87-SUMO-Gcn5 was produced by QuikChange mutagenesis.

To prepare potential sumoylation-site mutant constructs of Gcn5, QuikChange mutagenesis was used on pRS306-P_{GCN5}-FLAG-yGCN5 to mutate *GCN5* codons 25, 76, or 380 from lysine to arginine (K25R, K76R, and K380R mutations). In addition, K25R versions of untagged pRS306-P_{GCN5}-yGCN5 and pPC87-yGCN5 were similarly produced for use in phenotype studies and quantitation of transcription, respectively.

Yeast Strains, Media, and Growth Conditions. HA-tagged strains used for initial testing of Gcn5 sumoylation were YKH012 (*14*), containing a triple HA tag at the C-terminus of Gcn5, and EJY301, with a single HA tag at the C-terminus of Cdc3 (*18*). Strains with N-terminally FLAG-tagged Gcn5 (wild-type, K25R, K76R, or K380R) or untagged Gcn5 (wild-type or K25R) were produced by linearizing the appropriate pRS306-P_{GCN5}-yGCN5 plasmid with *Nsi*I and transforming into *gcn5*Δ strain FY1370 (*28*) for genomic integration at the *URA3* locus. In addition, the untagged wild-type and K25R constructs were integrated into the *gcn5*Δ *ADA2-2FLAG* strain SB349 (*37*) for complex immunoprecipitation. Strains with His_8 and HA-tagged SAGA subunit genes were produced by attaching a sequence encoding GHHHHHHHHGYPYDVPDYAAFL to the C-terminal ends of the *GCN5*, *ADA3*, and *SPT7* genes by transforming strains with the products of assembly PCR reactions as described (*18*). Also, strains containing 13Myc tags at the C-termini of *GCN5* or *ADA2* at their genomic loci were made as previously described (*23*, *35*). For studies involving the SUMO-Gcn5 fusion and/or real-time PCR-based RNA analysis, versions of pPC87-yGCN5 encoding wild-type Gcn5, Gcn5 K25R, SUMO-Gcn5, FLAG-Gcn5, or FLAG-SUMO-Gcn5 were transformed into *gcn5*Δ *trp1*Δ strain SB303 (*40*). For the sporulation experiment, a plasmid containing the *HO* gene was transformed into haploid *GCN5* wild-type and K25R integrant cells to diploidize, and selected diploids were sporulated by standard protocols (*1*, *31*),

Yeast strains were grown in the appropriate rich (YPD or YPRaff, containing 2% glucose or raffinose as a carbon source) or selective media (SC minus tryptophan) as described (*1*, *31*) at a temperature of 30 °C unless otherwise noted. IP experiments typically used 50 mL cultures grown to an OD_{600} of 0.5. For carbon source changes in Figure 2B, FLAG-Gcn5 integrant cells were grown to an OD_{600} of 0.5 and then treated as described in the figure legend. For *GAL1* induction and RNA analysis, pPC87-yGCN5-containing cells were grown in SC-Trp to an OD_{600} of 0.3, harvested, grown in YPRaff to an OD_{600} of 0.5, and induced by the addition of galactose to 2%. For plate growth studies, overnight cultures were diluted with sterile water to an OD_{600} of 0.4, and 5 μL of 5-fold serial dilutions was spotted on SC-Trp or SC-Trp + 50 mM 3-aminotriazole plates, which were incubated for 2 days at 30 or 33 °C.

Immunoprecipitation and Western Analysis. For analysis of sumoylated Gcn5 and other proteins from yeast cells, immunoprecipitation of SDS-resolubilized proteins from TCA-precipitated extracts was performed as described by Robzyk et al. (*29*), but with 100 μL extract in approximately 500 μL volume containing 30 μL of the appropriate antibody/beads. Immunoprecipitated FLAG-tagged proteins were recovered by peptide elution as described, while HA- and Myc-tagged proteins were recovered by boiling the beads in SDS sample buffer. Typically, 5 μL of final elution sample was used for a lane on a 10% SDS−PAGE gel, with Western transfer to nitrocellulose. Ni-NTA pulldowns in Figure 1B were performed under denaturing conditions as described (*43*). Immunoprecipitations of Ada2- and Gcn5-containing complexes for Figure 3C were performed under nondenaturing conditions with anti-FLAG M2 antibody as described previously (*37*). Primary antibodies used for Western blots were affinity-purified anti-SUMO (*18*), anti-FLAG M2 (1:1000 dilution; Sigma), anti-HA (HA.11; 1:10000; Covance), anti-Gcn5 (1:4000 dilution) (*13*), and anti-Ada3 (1:5000 dilution; a kind gift of L. Guarente). Blots were visualized with HRP-conjugated secondary antibodies (Bio-Rad) and ECL (Amersham Biosciences), and MagicMark (Invitrogen) was used as a molecular weight marker.

In Vitro Sumoylation and HAT Assays of Gcn5. For in vitro sumoylation experiments, recombinant His_6-Gcn5 was expressed in *E. coli* strain BL21(DE3) from the pRSETA-yGCN5 plasmid and purified with Ni^{2+}-NTA agarose (Qiagen) affinity chromatography as previously described (*6*). Gcn5/Ada2/Ada3 trimer, provided by S. Tan (The Pennsylvania State University), was expressed from a polycistronic plasmid in *E. coli* and purified as described in Balasubramanian et al. (*2*). Recombinant yeast SUMO, Uba2, Aos1, and Ubc9 were expressed in and purified from *E. coli* and used in sumoylation reactions with or without ATP as previously described (*19*). Reactions contained either no Gcn5 or a 1× or 2× amount of His_6-Gcn5 or trimer, and after incubation the samples were split into two parts. One part was boiled with SDS sample buffer and run on 10% SDS−PAGE gels, which were subsequently stained with Coomassie blue to visualize protein bands; the other part was used for in vitro free histone or nucleosomal histone acetyltransferase (HAT) assays with tritium-labeled acetyl-CoA as previously described (*13*, *36*) and quantitated by scintillation counting.

Quantitation of Transcription by Real-Time PCR. GAL1 and *TRP3* transcriptional analysis was performed by isolating total RNA from cells (RNeasy kit; Qiagen), performing reverse transcription with random primers (TaqMan reverse transcriptase kit; Applied Biosystems), and analyzing on a real-time quantitative PCR machine (ABI Prism 700 sequence detection system; Applied Biosystems) with appropriate primers (*GAL1*, *TRP3*, and *ACT1* or 18S rDNA primers for normalization) as described previously (*14*). For *TRP3* induction, cells (SB303 strain containing pPC87 plasmids with wild-type GCN5, K25R mutant, or no GCN5) were grown in SC without tryptophan media to mid-log phase. Half of the sample was treated with 40 mM 3-aminotriazole (3-AT) and half was grown as an uninduced

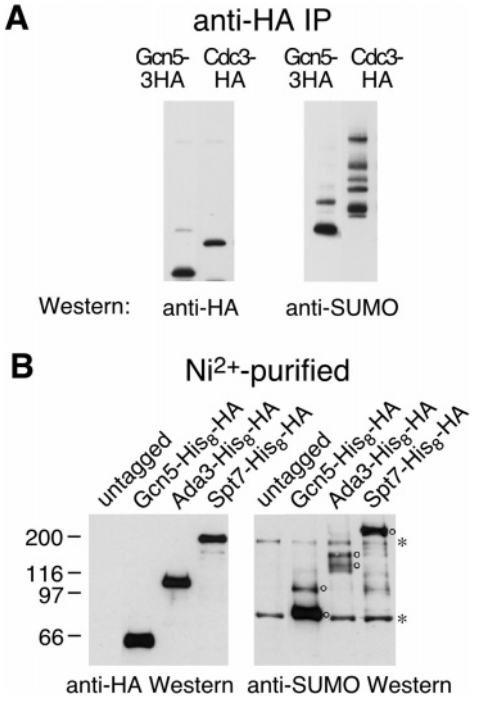

FIGURE 1: Yeast Gcn5 protein is sumoylated in vivo. Extracts of cells containing epitope-tagged versions of Gcn5 were prepared in TCA to denature and precipitate proteins, which were subsequently resolubilized in SDS. Immunoprecipitations were then performed with the appropriate anti-tag antibodies and used for Western blot analysis to visualize total Gcn5 (anti-tag) and sumoylated Gcn5 (anti-SUMO). (A) Sumoylated forms of HA-tagged Gcn5 are observed in IP's from TCA extracts (left). Cells for extracts were from log-phase YP/2% raffinose cultures. At right are samples from a parallel experiment using a strain containing HA-tagged Cdc3 (a known multiply sumoylated yeast protein) as a positive control. (B) Gcn5 and other SAGA subunits are sumoylated. C-terminally His_8-HA epitope-tagged Gcn5, Ada3, or Spt7 were pulled down with Ni^{2+}-NTA agarose and visualized by Western analysis. Open circles denote apparent sumoylated forms of the proteins; asterisks indicate two extract proteins that copurified as background in all samples.

control. Samples were harvested 1.5 h after induction, and RNA was prepared and quantitated.

RESULTS

Identification of Yeast Gcn5 as a Sumoylated Protein in Vivo. Gcn5 was one of the proteins identified as a SUMO substrate in a previously published experiment using a yeast strain that expressed a His_6- and FLAG-tagged version of yeast SUMO (ref *18* and unpublished results). SUMO-modified proteins were purified by affinity chromatography on Ni-NTA and anti-FLAG antibody columns, separated by SDS−PAGE gels, and analyzed by mass spectrometry. One of the proteins identified by this analysis was Gcn5 (data not shown).

To verify this finding and further characterize the in vivo sumoylation of Gcn5 (suGcn5), we prepared extracts from yeast strains containing epitope-tagged versions of Gcn5 and used them for immunoprecipitation and analysis by Western blotting with anti-tag and anti-SUMO antibodies. As shown in Figure 1A, Gcn5 from cells grown in rich media containing raffinose has at least one and possibly multiple SUMO modifications, as does a known sumoylation substrate, the septin Cdc3.

Since Gcn5 is part of multisubunit complexes such as SAGA, the question arose as to whether other subunits within SAGA are sumoylated as well. To investigate this, tagged versions of Gcn5 and other subunits (Ada3 and Spt7) within SAGA were purified from yeast extracts and tested for the presence of SUMO by Western blotting (Figure 1B). Interestingly, each of these subunits appears to have one or more sumoylated forms. These results partially overlap with those described in a study by Wohlschlegel et al. (*43*), in which a proteomics technique (mass spectrometry of a total purification of His_8-SUMO proteins from extract) identified Gcn5 as well as Ada2, Ada3, Spt7, Spt8, and Sgf73 as sumoylated SAGA substrates. It is noteworthy that Spt7 is also ubiquitylated (*32*), indicating possible regulation through alternative ubiquitylation/sumoylation. In addition, although multiple SAGA subunits are sumoylated, Gcn5 may be a particularly strong target, as evidenced by the intensity of its apparent single and double sumoylation bands in Figure 1B and by an immunoprecipitation experiment showing much greater sumoylation of 13Myc-tagged Gcn5 than several other similarly tagged SAGA subunits (data not shown).

In Vitro Sumoylation of Gcn5. To further characterize Gcn5 sumoylation and its possible functional effects, we tested Gcn5 in an in vitro sumoylation system. This system contained bacterially expressed and purified SUMO and components of the yeast sumoylation pathway (Aos1, Uba2, and Ubc9). As shown in Figure 2A, recombinant His_6-Gcn5 is multiply modified by SUMO in an ATP-dependent manner, leading to nearly quantitative sumoylation and a ladder of bands visible on both the anti-Gcn5 and anti-SUMO Western blots. This extensive sumoylation, however, does not significantly affect the HAT activity of Gcn5, since HAT assays with sumoylated and mock-sumoylated (no ATP) Gcn5 give very similar results (Figure 2A, lower).

Although free histone HAT activity is not affected by sumoylation of recombinant Gcn5, the question remained as to whether SUMO may modulate Gcn5 HAT activity in the context of multisubunit complexes and/or on the more physiological substrate, nucleosomes. To investigate this, we used highly purified native SAGA and ADA complexes for in vitro sumoylation reactions but found them to be poor substrates for sumoylation, with only a small fraction receiving SUMO modification (data not shown). So in order to test Gcn5 in a complex, we used a trimer consisting of Gcn5/Ada2/Ada3. Previous studies have shown that this trimer, unlike solitary recombinant Gcn5, is able to acetylate nucleosomes in addition to free histones (*2*). The trimer is partially sumoylated in vitro (perhaps about half), but neither free histone nor nucleosomal HAT activities are affected (Figure 2B), showing that SUMO on Gcn5 does not interfere with or promote the acetylation reaction in this in vitro system.

Mapping of the Gcn5 Sumoylation Site. We next sought to determine the lysine residue or residues that are sumoylated within Gcn5. As shown in Figure 3A, yeast Gcn5 contains three sumoylation consensus sequences [(I/V/L)KXE], two in the N-terminal region (K25 and K76) and one in the C-terminal bromodomain (K380). To examine whether any of these was a major site of sumoylation, we mutated each of the three lysines to arginine, to preserve the charge of the residue but yield it incapable of being sumoylated, and performed anti-FLAG immunoprecipita-

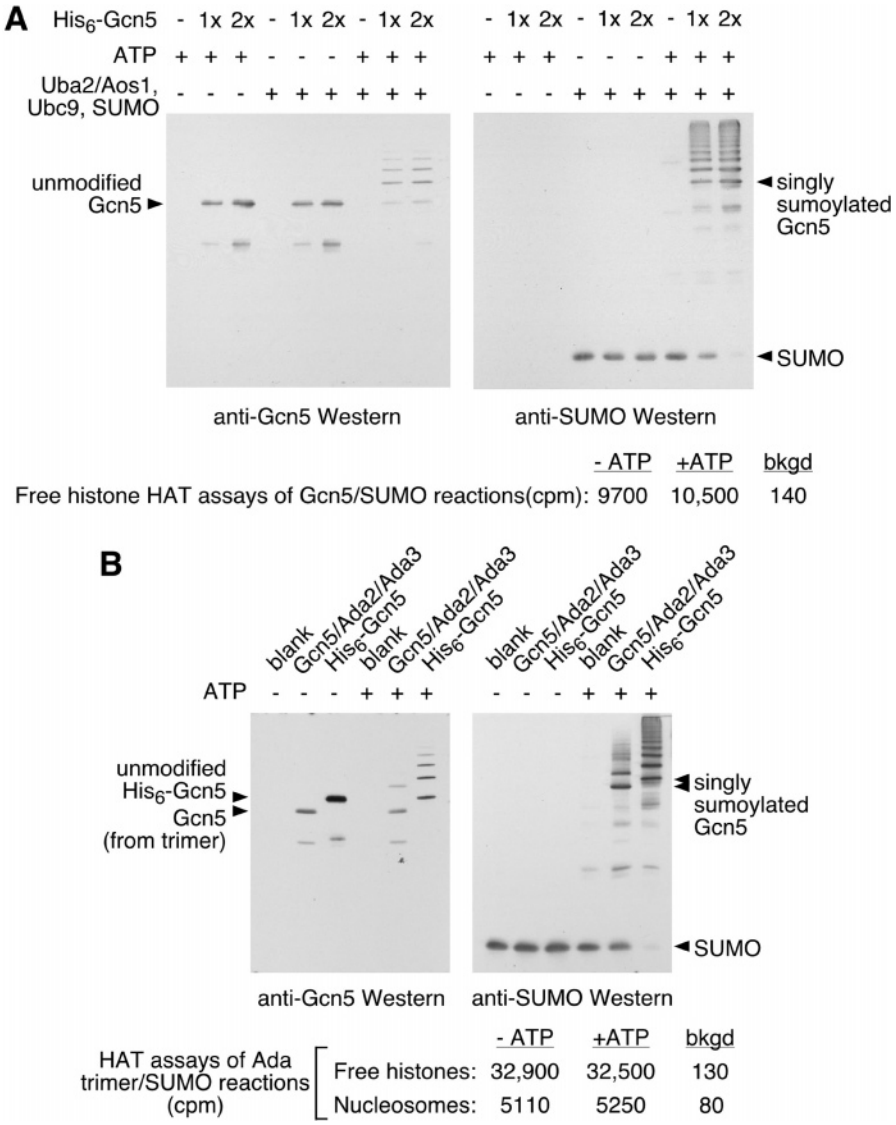

FIGURE 2: Sumoylation of Gcn5 in vitro in a reconstituted system. ATP-dependent sumoylation reactions were performed with *E. coli* expressed and purified versions of yeast sumoylation pathway components and Gcn5. (A) Gcn5 is multiply sumoylated in vitro. Reactions were performed with the components indicated, along with no (−), low (1×), or high (2×) amounts of recombinant Gcn5 substrate. Reactions were performed and run on SDS−PAGE and Western blotted for visualization by anti-Gcn5 (left) or anti-SUMO (right) antibodies. Liquid HAT assays were also performed with free histone substrate and unsumoylated (−ATP) or sumoylated (+ATP) reaction samples and quantitated by scintillation counting (bottom). (B) Gcn5 is partially sumoylated in a Gcn5/Ada2/Ada3 adaptor trimer. Samples were prepared and analyzed as described above. Gcn5 from the trimer runs at a faster SDS−PAGE mobility than solo Gcn5 due to its lack of an N-terminal tag. HAT assays were performed with both free histone and nucleosomal substrates.

tions. While the amount of each substituted protein is similar to wild type as judged by anti-FLAG Western analysis, anti-SUMO blotting shows that the K25R substitution mutation abolishes suGcn5, whereas K76R or K380R results in no significant change in sumoylation level (Figure 3B). Therefore, lysine-25 is identified as the major, and possibly sole, sumoylation site of Gcn5 in vivo.

We then used this apparently unsumoylatable Gcn5 mutant in an attempt to investigate possible roles of suGcn5 in vivo. Wide-ranging phenotype tests were performed in which wild-type and K25R mutant strains were streaked on various media and compared, but little or no growth defect was observed for the K25R mutant (data not shown). To test possible effects on meiosis, wild-type and K25R cells were diploidized and sporulated, but the rates of tetrad formation were very similar (data not shown). Another question we sought to answer was whether sumoylation could have a role, either positive or negative, in Gcn5 participation in SAGA and other complexes, so we performed immunoprecipitations of native complexes from extracts of cells containing FLAG-tagged Ada2 or Gcn5 and analyzed them by Western blotting. As shown in Figure 3C, there is no significant difference in the level of Gcn5 in the immunoprecipitated complex when Gcn5 is wild-type or K25R, indicating that lack of suGcn5 does not affect Gcn5 entry into or maintenance within the complexes.

We wondered if sumoylation has a role in transcription at SAGA-dependent genes, such as the induction of the *GAL1* gene in galactose media. To test this, we performed galactose induction of wild-type and Gcn5 K25R cells and analyzed *GAL1* transcription by way of real-time quantitative PCR (Figure 4A). The results indicate that there is no significant difference in *GAL1* derepression between wild-type and K25R cells.

We examined the level of suGcn5 in different carbon sources, either glucose (repressing for *GAL1*), raffinose

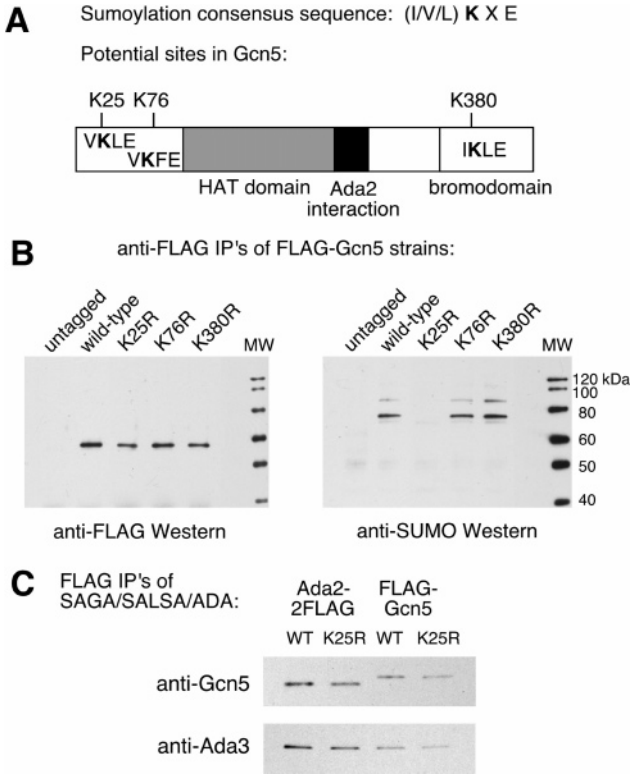

FIGURE 3: Mapping of the Gcn5 sumoylation site. (A) Diagram of sumoylation consensus sites within the Gcn5 protein. Functional domains of Gcn5 and the sequences of the consensus sites are indicated. (B) Lysine-25 is the primary sumoylation site on Gcn5 in vivo. Unsumoylatable lysine-to-arginine substitution mutants of the three consensus sites were prepared and analyzed by immunoprecipitation and Western blot analysis. (C) Participation of Gcn5 in native complexes is not affected by loss of sumoylation. Complexes including SAGA, SALSA, and ADA were immunoprecipitated via a FLAG tag on Gcn5 (right) or another adaptor subunit Ada2 (left), and Western blots were visualized with antibodies against Gcn5 (top) or another adaptor subunit Ada3 (bottom) in order to compare amounts of Gcn5-containing vs total adaptor complexes.

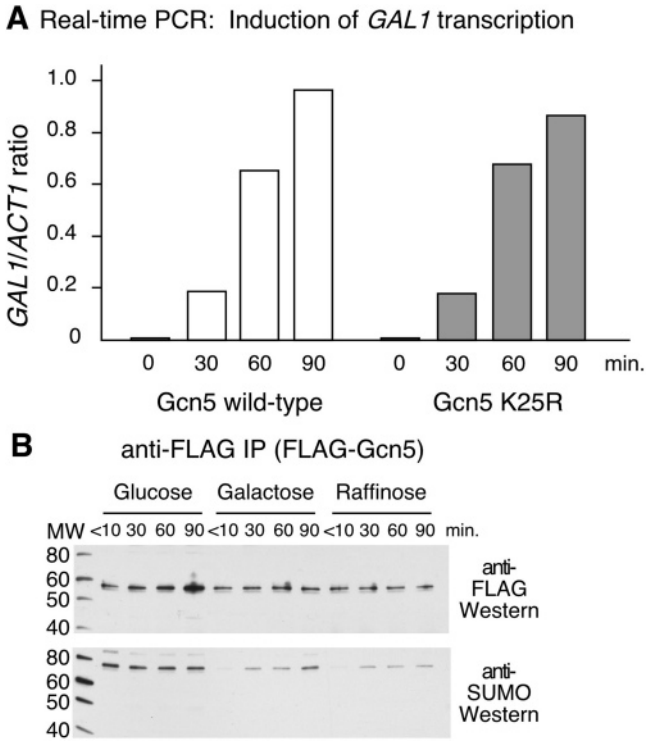

FIGURE 4: Analysis of Gcn5 sumoylation levels in glucose-to-galactose changes. (A) Induction of *GAL1* transcription is not affected by the K25R mutant. Real-time quantitative PCR was performed on cDNA generated from total RNA extracted from different time points after addition of 2% galactose to yeast cells. (B) Rapid loss of SUMO from Gcn5 when shifting from glucose media to galactose or raffinose media. Cells containing FLAG-Gcn5 were grown in YPD (glucose) media and washed with and resuspended in YP (no carbon source), to which glucose, galactose, or raffinose was then added to 2%. Time points were taken immediately after addition of the carbon source (with less than 10 min subsequent harvesting/processing time) and at 30 min, 1 h, and 1.5 h. The first samples took several minutes to harvest and process at 4 °C, and even in this brief time frame the Gal and Raff cells experienced dramatic loss of Gcn5 sumoylation.

(derepressing for *GAL1*), or galactose (activating for *GAL1*). In these experiments, cells were grown in glucose medium (YPD) and then washed and shifted back to glucose or to raffinose or galactose. A subsequent time course reveals that the levels of total Gcn5 are not changed. However, although the glucose-to-glucose shift caused no significant change in suGcn5 levels, both the raffinose and galactose cells have very rapid loss of suGcn5, followed by a gradual increase to levels similar to those of the glucose samples (Figure 4B).

Effect of SUMO-Gcn5 Fusion in Vivo. Since an unsumoylatable Gcn5 mutant led to no obvious in vivo effects, but the level of suGcn5 is transiently lowered in conditions known to require Gcn5 enzymatic activity, we wondered if use of an opposite type of mutant, quantitatively and constitutively sumoylated, might be an effective method of revealing potential effects of suGcn5. To mimic a state of quantitative sumoylation, we created strains containing a SUMO-Gcn5 fusion; this method of N-terminal fusion of a SUMO protein to a substrate has been used successfully in previous studies to examine effects of sumoylation of other proteins in vivo (*34*). Importantly, our construct lacked the C-terminal Gly-Gly residues of SUMO (the natural cleavage site), so that the overall SUMO structure should be formed yet uncleavable by endogenous SUMO proteases in yeast. Western blots of immunoprecipitated extracts from a FLAG-tagged SUMO-Gcn5 strain showed that the fusion is in fact largely intact (Figure 5A).

Plating experiments demonstrated that a SUMO-Gcn5 strain does not exhibit obvious growth defects at 30 °C compared to wild-type cells on minimal, synthetic complete (SC/glucose), SC/raffinose, or SC/galactose media (data not shown). However, when spotted on SC/glucose media containing 3-aminotriazole (3-AT), a significant defect in growth is observed. 3-AT is an inhibitor of the histidine biosynthetic gene product His3, and it is known to cause derepression of multiple amino acid biosynthetic genes, including *HIS3* and *TRP3*, both of which are known to have SAGA-dependent transcription. Furthermore, *gcn5*Δ strains display a significant growth phenotype on 3-AT media. Interestingly, SUMO-Gcn5 also displays a phenotype on 50 mM 3-AT (Figure 5B, lower left), and this effect is magnified at elevated temperature (33 °C; Figure 5B, lower right). The Gcn5 K25R mutant, in contrast, shows no phenotype on 3-AT media (data not shown).

To investigate whether the 3-AT phenotype of SUMO-Gcn5 cells could be an indication of a transcriptional defect, we examined *TRP3* RNA levels in 3-AT induced cells by way of real-time quantitative PCR. As shown in Figure 5C,

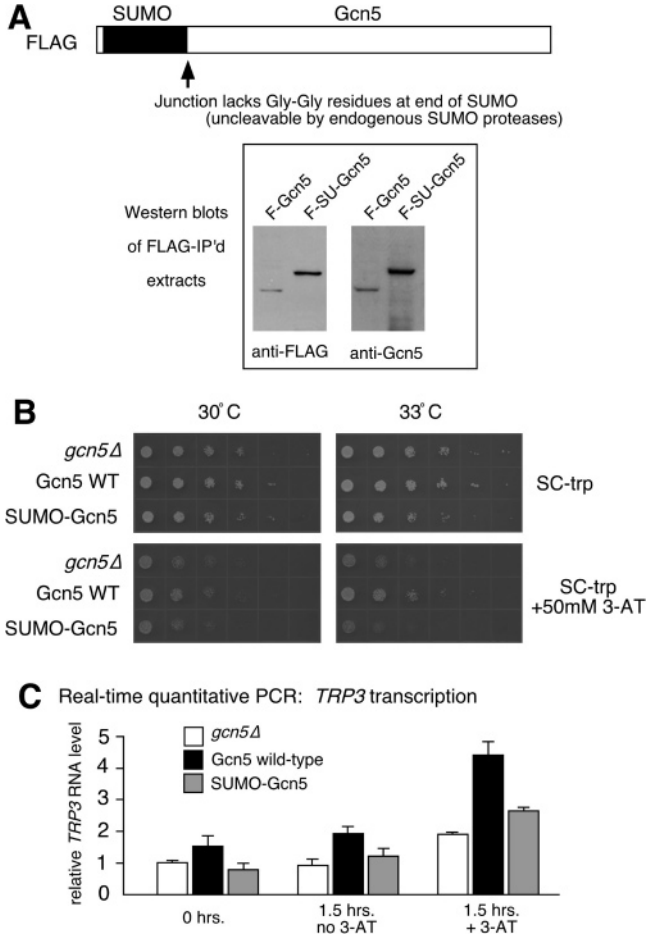

FIGURE 5: Effects of a SUMO-Gcn5 fusion on growth and transcription in vivo. (A) Construction of a yeast strain containing SUMO-Gcn5 fusion. At the top is a diagram of the fusion, where the glycine−glycine residues at the end of SUMO have been mutated to render it uncleavable. FLAG-tagged and untagged constructs were prepared in the vector pPC87 and transformed into *gcn5*Δ yeast, using Trp selection. Below are anti-FLAG and anti-Gcn5 Western blots of immunoprecipitations of tagged SUMO-Gcn5 and unfused Gcn5 from a parallel strain, demonstrating that SUMO-Gcn5 is largely intact in the cell. (B) Growth defect of SUMO-Gcn5 cells on 3-aminotriazole (3-AT) media. Serial 1:5 dilutions of *gcn5*Δ, untagged Gcn5 wild type, and untagged SUMO-Gcn5 cells were spotted on SC-Trp media containing 50 mM 3-AT. (C) 3-AT-induced *TRP3* transcription is partially inhibited in a SUMO-Gcn5 strain. Real-time quantitative PCR was performed on cDNA generated from total RNA extracted from cells at a zero time point or at 1.5 h after the addition of 3-AT. Data represent the average of several independent experiments.

there is a reduction in levels of *TRP3* transcript from SUMO-Gcn5 cells compared to wild-type, suggesting that the SUMO moiety could be inhibitory in the context of Gcn5 and SAGA/SALSA. This effect parallels SUMO's role in repression observed in certain other transcriptional systems: SUMO attachment to histone H4 and transcription factors such as Elk1, Sp3, c-Myb, and c-Jun has been previously implicated in repression.

DISCUSSION

In recent years the number of known sumoylated proteins has increased dramatically, and our results verify that yeast Gcn5 can be added to this list; it is clearly sumoylated in vivo, primarily at its lysine-25 residue. In recent years, the number of known in vivo effects of specific sumoylation events has grown, and the most common role of sumoylation in mammalian cells is in transcriptional repression. Indeed, the consequences of Gcn5 sumoylation also may be related to transcriptional repression. Although we did not detect an effect of unsumoylatable K25R mutant, we did observe an inhibitory effect on *TRP3* transcription when SUMO is fused to Gcn5 and a consistent significant defect in growth on 3-AT, indicating impairment of the amino acid biosynthetic pathway. In addition, we detected a profound transient loss of Gcn5 sumoylation when the carbon source was altered, conditions that require SAGA for expression of genes leading to biosynthesis of sugar metabolism enzymes.

Thus, in vivo, SUMO may interfere in some way with the chromatin modification and/or coactivator functions of Gcn5. The location of the sumoylation site in Gcn5 is near the amino-terminal border of the acetylation domain of Gcn5. While in vitro sumoylation experiments with Gcn5 did not show effects on HAT activity, it should be noted that these experiments were not performed in the context of a full complex (SAGA and ADA were poor substrates in vitro) and may not reflect activity in the cell. Also, histone acetylation enzymes, including Gcn5 homologues in higher eukaryotes, have many additional acetylation substrates relevant to transcription. Thus it may be that sumoylation of Gcn5 alters acetylation of yet unknown target substrates in the amino acid biosynthetic or carbon source metabolism pathways.

Gcn5 and its complexes, including SAGA and SALSA, regulate or are present at many yeast genes (*27*), as well as being particularly critical for genes transcribed during the G2/M stage of the cell cycle (*21*). Thus, it is possible that sumoylation may be involved in the regulation of a small subset of genes which was not revealed by the limited number of genes and conditions investigated. Consistent with this idea was the finding that the SUMO-Gcn5 fusion showed specific effects on growth on 3-AT and not other conditions tested. In any case, only a small percentage of Gcn5 was found to be sumoylated, so it is not surprising that effects might be difficult to uncover. It may be that specialized conditions, or perhaps particular stages in the cell cycle, could result in higher amounts of sumoylation and more dramatic transcriptional effects. Again, the fact that changes in the carbon source lead to sudden, dramatic changes in Gcn5 sumoylation levels (Figure 4B) suggests that its sumoylation state can be variable, depending on conditions.

Another possible explanation for the subtle effect of the K25R nonsumoylatable mutant is known redundancy of sumoylation in Gcn5-containing complexes. One possibility relates to previous findings that regulatory and chromatin effects of SAGA are clearly synergistic, involving the bromo and SANT domains, TBP recruitment, and regulation of the ubiquitylation state of H2B. Thus, stronger effects of loss of sumoylation may manifest if in combination with defects in these other mechanisms of SAGA, which we did not test in this study. Further, our work and other recent reports identified Gcn5 as well as five other SAGA subunits as in vivo targets for sumoylation, including the two other proteins in the adaptor subgroup, Ada2 and Ada3, as well as Spt7 (*10*, *43*). A conceivable scenario is that transcriptional inhibition or other regulatory effects could be redundantly mediated by SUMO modifications on various SAGA subunits instead of solely Gcn5. Perhaps if Gcn5 sumoylation

is blocked (such as by the K25R mutation), sumoylation of other subunits will increase to compensate; another possibility is that Gcn5 is only a minor component of multiple-subunit SAGA sumoylation, and these modifications all similarly contribute to regulation in an additive manner. Future studies of sumoylation of the other SAGA subunits will be required to determine the relationship among these modifications and how they participate in transcriptional regulation.

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