NXP-2 association with SUMO-2 depends on lysines required for transcriptional repression

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Small ubiquitin-like modifier (SUMO) modification of transcription factors is generally associated with repression. Reverse genetic analysis of SUMO-1, and -2 conserved residues emphasized the importance of dual charge reversals in abrogating the critical role of SUMO-2 K33, K35, and K42 in repression. GST-SUMO-2-affinity chromatography followed by liquid chromatography (LC)-MS analysis identified proteins that appeared to bind preferentially to WT SUMO-2 versus SUMO-2 K33E and K35E. LSD1, NXP-2, KIAA0809 (ARIP4), SAE2, RanGAP1, PELP1, and SETDB1 bound to SUMO-2 and not to SUMO-2 K33E, K42E, or K35E and K42E. Although LSD1 is a histone lysine demethylase, and histone H3K4 was demethylated at a SUMO-2-repressed promoter, neither overexpression of a dominant-negative LSD1 nor LSD1 depletion with RNA interference affected SUMO-2-mediated repression, indicating that LSD1 is not essential for repression, in this context. When tethered to a promoter by fusion to Gal4, NXP-2 repressed transcription, consistent with a role for NXP-2 in SUMO-mediated repression. SUMO-2-associated proteins identified in this study may contribute to SUMO-dependent regulation of transcription or other processes.

regulation | repressor | ubiquitin-like

Small ubiquitin-like modifier (SUMO) is a ubiquitin-like protein that is reversibly covalently attached to lysines in target proteins, resulting in altered protein function, localization, or stability. SUMO modification of corepressors, coactivators, histones, histone-modifying enzymes, or sequence-specific transcription factors usually down-regulates transcription. For example, mutation so as to prevent SUMO modification increases transcription-factor activity associated with Elk-1, Sp3, c-myb, c-jun, AP2, or the p300 N terminus (1–5). In contrast to SUMO, ubiquitin modification is more frequently associated with proteasome-dependent degradation or activation of transcription. In fact, activation of transcription by the VP16 activation domain, in yeast, requires an E3 ubiquitin ligase (6). Addition of a SUMO consensus acceptor site to the Gal4-VP16 activation domain reduces transcription 10-fold (7). Thus, ubiquitin and SUMO modifications can have opposite effects on transcription.

Despite these differences, SUMO-1, -2, and -3 are ubiquitin-like proteins and have ubiquitin-like structures. SUMO-2 and -3 are 95% identical and are 46% and 48%, respectively, identical to SUMO-1. In contrast, SUMO-1, -2, and -3 share only 18% identity with ubiquitin (Fig. 1). The differences in primary sequence and their role in the opposing transcriptional properties of SUMO and ubiquitin are being elucidated. SUMO interaction with histone deacetylases has been proposed as one mechanism of repression (1, 8). However, an unbiased directed screen to identify candidate SUMO-associated repressors has not been undertaken.

The objective of our experiments was to identify proteins whose association with SUMO-2 depends on SUMO-2 residues required for repression. Such proteins would be candidate SUMO corepressors. We have used two different linear fusions of SUMO-2 or -1 to the Gal4 DNA-binding domain to extend the recent comprehensive evaluation of SUMO-2 residues that are critical for repression (9). Paired charge-reversal mutations of SUMO-2 K33, K35, and K42 substantially decreased repression and were, therefore, compared with WT SUMO-2 in differential protein-affinity chromatography. Proteins that bound to WT SUMO-2 but not to repression-deficient SUMO-2 mutants were identified by liquid chromatography (LC)-MS peptide analyses. The repressive affect of the SUMO-2-associated proteins was further evaluated by individual fusion to the Gal4 DNA-binding domain.

Results

Identification of SUMO-2 Residues Required for Transcriptional Repression by Fusion to Gal4 or Gal4-Sp3(K539R). Reverse genetic analyses of SUMO-2 repression were based on the hypothesis that surface residues conserved among SUMO-1, -2, and -3, but not in other ubiquitin-like proteins (Fig. 1, asterisks), are more likely to mediate SUMO-specific functions, whereas residues common among ubiquitin-like proteins would more likely be necessary for their common secondary structure. One set of mutations was in the β-1/2 surface and the neighboring α-helix: K21 (β1), S28 (β2), K33 (β2), K35 (β2), and K42 (α1). The K33, K35, and K42 side chains are on the same side of the β-1/2 surface and are separated by $<$4.8 Å (10). The second set was in β5: 80DED82-AAA and 82DTID85-ATIA. These residues are oriented opposite to K33, K35, and K42. K33, K35, K42, DED80–82, and D85 are not conserved in Ubiquitin or Nedd8 (Fig. 1).

The role of specific SUMO residues in repression was evaluated in the context of Gal4-SUMO-2 fusion-protein effects on transcription activated by Sp1 sites in pGL2-Gal4-TK-Luc (Table 1) and, in the context of SUMO-1 or -2 rescue of repression by in-frame fusion with the SUMOylation-defective Sp3 K539R mutant, (Gal4-S1 or S2-Sp3KR; Fig. 2) by using the G5Luc reporter (10). In these assays, Gal4-SUMO-2 expression repressed 90–95% of the of the luciferase reporter activity observed after expression of the Gal4 DNA-binding domain alone (data not shown). Gal4-SUMO-2 expression had little or no affect on control pGK-β-galactosidase activity (data not shown). Similarly, expression of Gal4-S2-Sp3KR and Gal4-S1-Sp3KR repressed 98% and 96% of Gal4-Sp3K539R activity, respectively (Fig. 2), whereas Gal4-S2-Sp3KR and Gal4-S1-Sp3KR had no effect on control pRL-tk reporter activity, which lacks upstream Gal4-binding sites (10).

The repressive effects on luciferase reporter activity by Gal4-SUMO-2 mutants were directly compared with those of Gal4-SUMO-2, which was set to 100% (Table 1). In this context, single amino acid substitutions of SUMO-2 K33, K35, or K42 with R, A, or E (Fig. 1) had very small effects on WT SUMO-2

Conflict of interest statement: No conflicts declared.

Abbreviation: SUMO, small ubiquitin-like modifier.

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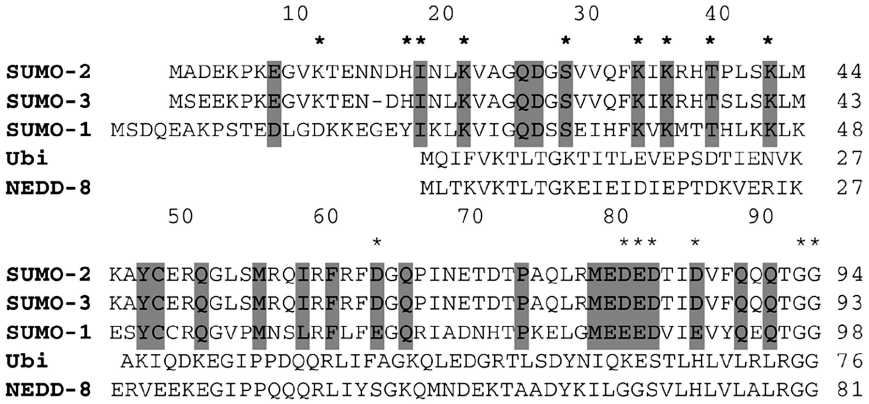

Fig. 1. Alignment of human SUMO-1, -2, and -3 proteins with ubiquitin and Nedd8. Asterisks indicate residues selected for mutagenesis and reporter assays (Table 1). Residues conserved or charge-conserved among SUMO proteins but not conserved between SUMO and ubiquitin or Nedd8 are shaded.

repression levels. Although the K33E mutant had the largest effect, K33E still repressed at 68% of WT SUMO-2 levels (Table 1). Also, substitution of K21 with A or E or of S28 with A had almost no effect on SUMO-2 repression levels (Table 1). Furthermore, in the context of Gal4-S1-Sp3KR fusions, SUMO-1 K25A or K46A, which align with SUMO-2 K21A and K42A, repressed less than did WT SUMO-1 but still had very substantial repressive effects (Fig. 2). Similarly, the SUMO-1 double mutant Gal4-S1 K37A,K39A-Sp3KR and the homologous SUMO-2 double mutant Gal4-S2 K33A,K35A-Sp3KR repressed less than WT SUMO-1 and -2 but still had very substantial repressive effects (Fig. 2). In contrast, the double charge-reversal mutants K33E and K35E (EE1), K33E and K42E (EE2), or K35E and K42E (EE3) were severely impaired and repressed at only 7%, 4%, and 33%, respectively, of WT SUMO-2 levels, in the context of Gal4-SUMO-2 (Table 1). In the context of transcription activated by a SUMOylation-deficient mutant glucocorticoid receptor, SUMO-2 K33A, K35A, or K42A mutants repress at 20–60% of WT SUMO-2 levels, SUMO-2 K33E, K35E, or K42E lack more of WT SUMO-2 repressive activity, and the SUMO-2 K33E,K42E or homologous SUMO-1 K37E,K46E double mutants lack virtually all repressive activity (9). Thus, in different contexts, dual charge-reversal mutations of SUMO-2 K33, K35, and K42 most consistently abrogate SUMO repressive effects.

Table 1. Repression phenotype of Gal4-SUMO-2 mutants

Mutation	Repression, %	SE, %
WT (GG → AA)	100	0
K11R	96	2
17HI18 → AA	95	3
K21A	112	<1
K21E	112	<1
S28A	95	1
K33R, A, E	91, 95, 68	1, 1, 12
K35R, A, E	92, 86, 83	1, 6, 10
K42A, E	89, 85	8, 1
K33E, K35E (EE1)	7	<1
K33E, K42E (EE2)	4	<1
K35E, K42E (EE3)	33	18
T38V	96	9
D63A	75	2
Δ1–64	−59	2
Δ1–80	−67	39
80DED82 → AAA	84	1
82DTID85 → ATIA	75	16

Although many SUMO mutations did not significantly disrupt repression, mutations that affected the SUMO fold were substantially impaired in repression. Mutations in the negatively charged β5 surface, 80DED82-AAA and 82DTID85-ATIA, had only minor consequences and repressed at 84% and 75% of Gal4-WT SUMO-2 levels, respectively (Table 1). Mutation of SUMO-2 K11, which is a potential site for successive SUMO modifications (11), to R resulted in WT SUMO-2 repression (Table 1), excluding a role for K11 mono- or polySUMOylation in SUMO-2 repression, as recently reported (12). In fact, SUMO-1 amino acids 1–20 were not at all required for Gal4-S1-Sp3KR repression (Fig. 2 and ref. 12). I18 in β1 is hydrogen bonded to I34 in β2, and 17HI18 mutation to AA did not affect

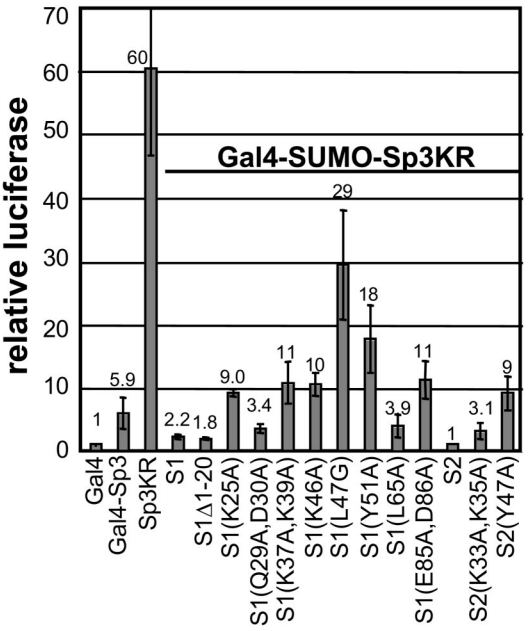

Fig. 2. Residues in SUMO-1 and -2 required for maximal repression of Sp3-dependent transcription. WT and mutant SUMO-1 and -2 were assayed for repression in the context of Gal4-SUMO-Sp3K539R fusions containing Gal4 amino acids 1–147, mature SUMO-1 (1–96) or SUMO-2 (1–92), both terminating in AA, and either WT or SUMOylation-deficient (K539R) Sp3 amino acids 74–605. Note that the Sp3K539R mutation in the Sp3 SUMO acceptor lysine dramatically increases transcription, and repression was restored by fusion of SUMO-1 or -2 to Sp3. Plasmids expressing the indicated Gal4 fusions were cotransfected with the G5Luc reporter into HeLa cells. Relative luciferase activity and standard deviation are shown above each data bar.

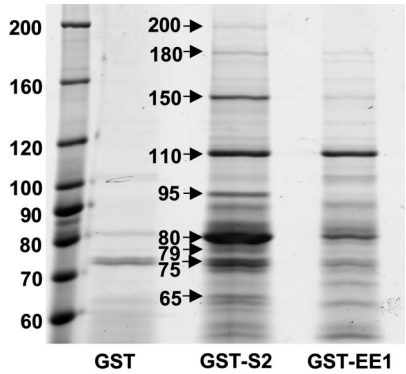

Fig. 3. Identification of SUMO-2-interacting proteins by affinity chromatography. Cell lysates were affinity-purified by using GST, GST-SUMO-2 (GST-S2), or GST-SUMO-2 K33E,K35E (GST-EE1). Bound proteins were eluted in Laemmli buffer, resolved by SDS/PAGE, and visualized by SYPRO ruby staining. Nine gel slices, containing proteins with enhanced binding to GST-SUMO versus GST-EE1 and ranging in size from 200 to 65 kDa, were excised and examined by liquid chromatography-MS.

Gal4-SUMO-2 repression (Table 1). Similarly, T38 in α1 is hydrogen bonded to K35 in β2, and a T38V mutant was WT for Gal4-SUMO-2 repression (Table 1 and ref. 10). Importantly, Gal4-SUMO-2 deleted for amino acids 1–64 or 1–80 failed to repress (Table 1), and a SUMO-1 L47G point mutant was severely defective in repression in the Gal4-S1-Sp3KR fusion (Fig. 2), indicating that repression depends on an intact SUMO fold. Western blot using Gal4-specific antibody showed that all Gal4-SUMO-2 fusions were expressed at similar levels (data not shown).

Identification of SUMO-2-Interacting Proteins by Using GST-SUMO-2-Affinity Chromatography. Because the SUMO-2 K33, K35, and K42 dual charge-reversal mutations were most impaired in repression, we used these mutations to discriminate among candidate repressor proteins that bound to WT SUMO-2. Denaturing PAGE of large-scale pull downs with GST-SUMO-2 WT (GST-S2), GST-SUMO-2 K33E, K35E (GST-EE1), or GST identified proteins that bound to GST-SUMO-2 and bound less to GST-EE1 or GST (Fig. 3). These proteins were excised and digested with trypsin. Their peptides were identified by liquid chromatography-MS analyses. A 110-kDa band present in both GST-S2 and EE2 lanes was also selected. Protein names, gene identifiers, calculated molecular mass, sizes at point(s) in gel where identified, and estimated peptide abundances are indicated in Table 2.

The identified proteins include SAE2, PIASxβ, and arkadia-like protein 1, which are involved in SUMO/ubiquitin conjugation, SETDB1 and LSD1, which are a histone lysine methyltransferase and a histone lysine demethylase, respectively, PELP1, ARIP4, and MTA-2, which are coactivators or repressors, p53BP1, zinc-finger protein 198 (ZNF198), the nuclear matrix protein NXP-2, and HSP70 (13–16). Because WT SUMO-2 was used in these experiments, proteins could have been retained by GST-SUMO-2 affinity resins because of either binding or covalent linkage to GST-SUMO-2. Covalent linkage would result in a 50-kDa increase in size. As shown in Table 2, most SUMO-2-associated proteins migrated close to their predicted size and, in some instances, also at multiple sizes consistent with successive SUMOylations. However, RanGAP1 was also identified to be an abundant protein at 110 kDa, which would be consistent with covalent attachment to GST-SUMO-2 (Fig. 3 and Table 2).

PELP1, SetDB1, LSD1, RanGAP, and NXP-2 Bind Preferentially to WT but Not SUMO-2 K33E,K42E (EE2) or K35E,K42E (EE3). To determine whether the proteins that appeared to bind to WT SUMO-2 but not to SUMO-2 K33E,K35E (EE1) could bind to SUMO-2 K33E,K42E (EE2) or to SUMO-2 K35E,K42E (EE3), the proteins were assayed for binding to GST-SUMO-2 versus GST-EE2, GST-EE3, and GST (Fig. 4). Ubc9 was included as a control, because Ubc9 makes no contacts with SUMO-2 K33, K35, and K42 (reviewed in ref. 17). SAE2, SETDB1, LSD1, RanGAP1, and PELP1 bound well to GST-SUMO-2 and very poorly to GST-EE2, GST-EE3, or GST. Although only a small fraction of 293T cell RanGAP1 was at the expected size of 65 kDa and more was at 80 kDa, consistent with SUMO modification, 65-kDa RanGAP1 preferentially bound to GST-SUMO-2 (Fig. 4*A*) (18). Hemagglutinin-tagged NXP-2, transiently expressed in 293T cells, also bound well to GST-SUMO-2 but not to GST-EE2, GST-EE3, or GST. In a similar experiment, ARIP4 bound to GST-SUMO-2, but not to GST-EE1 (data not shown). By contrast, His-tagged Ubc9 expressed in bacteria bound to GST-SUMO-2, GST-EE1, GST-EE2, and GST-EE3 with equal efficiency (Fig. 4*B*), indicating that the EE1, EE2, and

Table 2. SUMO-2-interacting proteins identified by LC/MS

Name	Gene ID	Molecular mass, kDa	Protein size in gel, kDa	Abundance at each size(s) detected
p53BP1	5032189	214	200 and 180	222 and 473
ZNF198	37574606	155	180	1,088
ARIP4	24307995	151	180, 150 and 95	2,988, 3,505, and 743
SETDB1	41281393	143	180	528
PELP1	24415383	137	180 and 79	1,047 and 2,208
NXP-2	28872812	107	200 and 150	2,257 and 6,831
LSD1	58761546	93	110	601
Nucleolin	55956788	77	80	2,940
MTA2	14141170	75	79	783
SAE2	4885649	72	95	61,090
Hsp70	16507237	72	80, 79, 75, and 65	39,186, 9,429, 114,449, and 3,988
PIASxβ	56699458	69	75	1,129
Calnexin	10716563	68	79	783
Malic enzyme	4505145	66	65	37,963
RanGAP1	4506411	64	180, 110, 95, 79, and 65	11,510, 319,079, 6,141, 4,995, and 4,501
Chaperonin	31542947	61	65	1,628
Arkadia-like	56549656	37	79	2,550

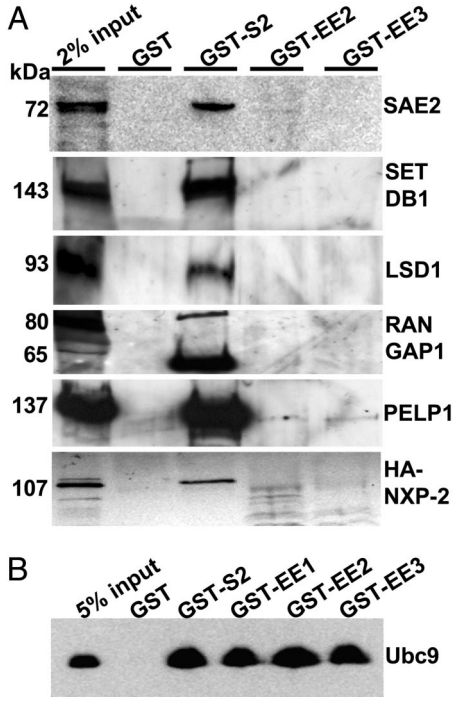

Fig. 4. SUMO-2-bound protein dependence on K33 and K42 or K35 and K42. (*A*) GST pull downs using GST, GST-SUMO-2 (GST-S2), GST-S2 K33E,K42E (GST-EE2), or GST-S2 K35E,K42E (GST-EE3) with whole-cell Nonidet P-40 extracts from 293T cells, followed by SDS elution and immunoblotting with antibodies specific for the indicated proteins. Two percent of the lysate input is indicated for reference. For NXP-2 analyses, lysates were made from 293T cells that were transfected with a hemagglutinin (HA)-tagged NXP-2 expression vector. Input and bound NXP-2 were detected by immunoblotting with anti-HA antibody. Eighty-kilodalton RanGAP was predominant in input lysate, whereas 65-kDa RanGAP preferentially bound GST-S2. (*B*) His- and X-press-tagged Ubc9 was expressed in bacteria and incubated either with GST or with GST fused to S2, EE1, EE2, or EE3. Bound Ubc9 was detected by immunoblotting using anti-X-press antibody.

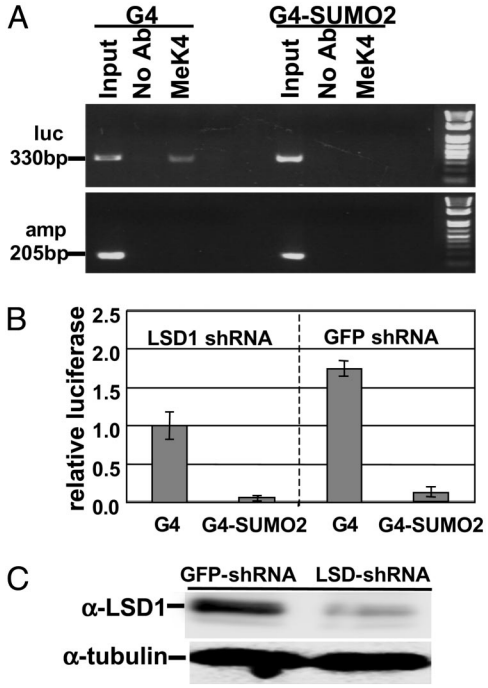

Fig. 5. Recruitment of Gal4-SUMO-2 to reporter DNA results in demethylation of H3K4, but LSD1 is not required for repression. (*A*) The 293T cells were transfected with pGL2-Gal4-TK-Luc and a plasmid encoding either Gal4 alone or Gal4 fused to SUMO-2. DNA–protein complexes were immunoprecipitated with antibody specific for dimethyl H3K4 (MeK4, *Upper*), and a 330-bp DNA fragment was amplified as previously described. Control PCR amplified 205 bp from input but not immunoprecipitation samples (*Lower*). (*B*) Reporter assay in LSD1-specific short hairpin RNA (shRNA), HeLa stable cell lines. Two hundred nanograms of pcDNA3-Gal4 or pcDNA3-Gal4-SUMO-2 was transfected into HeLa cells that stably express shRNAs targeting either GFP or LSD1. (*C*) LSD1 levels in GFP or LSD1 shRNA stable cell lines were compared by immunoblotting against LSD1 (*Upper*) and α-tubulin (*Lower*) as a loading control.

EE3 mutations do not disrupt other SUMO-2 interactions. These data suggest that SETDB1, LSD1, PELP1, and ARIP4, which are transcriptional regulators, and the nuclear matrix protein NXP-2 are potential corepressors for SUMO-2.

Gal4-SUMO-2 Binding to Reporter DNA Is Associated with H3K4 Demethylation. Because LSD1 effects H3K4 demethylation and transcriptional repression, we determined whether Gal4-SUMO-2 repression is associated with decreased H3K4 dimethylation by using a chromatin immunoprecipitation (ChIP) assay with H3 dimethyl K4-specific antisera. Dimethylated H3K4-specific antibody immunoprecipitated promoter DNA from protein and DNA cross-linked lysates of Gal4 control-transfected cells but did not immunoprecipitate promoter DNA from lysates of Gal4-SUMO-2-transfected cells (Fig. 5*A*). Similar levels of promoter DNA were detected in the input lanes before immunoprecipitation (Fig. 5*A*). These data indicate that Gal4-SUMO-2-mediated repression is associated with less H3K4 dimethylation at Gal4-DNA-binding sites, consistent with a role for LSD1 in Gal4-SUMO-2-mediated H3K4 demethylation and promoter repression.

To specifically evaluate the role of LSD1 in Gal4-SUMO-2-mediated repression, the effect of RNA interference (RNAi)-mediated LSD1 depletion and of a dominant-negative demethylase-deficient LSD1 mutant on Gal4-SUMO-2-mediated repression were assessed (19). Gal4-SUMO-2 repressed reporter activity similarly in HeLa cells deficient in LSD1 as a consequence of LSD1 RNAi expression and in control HeLa cells with WT levels of LSD1 (Fig. 5 *B* and *C*). Furthermore, overexpression of a dominant-negative demethylase-deficient LSD1, which has a point mutation in the FAD-binding domain, also had no effect on Gal4-SUMO-2 repression (data not shown).

NXP-2 Represses Transcription When Tethered to a Promoter. To determine which of the SUMO-2-bound proteins have repressive effects on a nearby promoter, the proteins were assayed after in-frame fusion to the 3′ end of the Gal4 DNA-binding domain, by using pGL2-Gal4-TK-Luc. As expected from LSD1's potent H3K4 demethylase activity, Gal-4 LSD1 repressed reporter activity (data not shown and ref. 19). SETDB1, PELP1, and RanGAP1 neither repressed nor activated the Gal-4-dependent promoter (data not shown). In contrast, Gal4-NXP-2 repressed transcription at levels comparable to Gal4-SUMO-2 (Fig. 6). Gal4-NXP-2 expression resulted in <25% of Gal4-dependent reporter activity after transfection of 30 or 100 ng of expression vector (Fig. 6). Thus, NXP-2 is a candidate SUMO-bound repressor.

Discussion

These experiments further implicate SUMO-2 K33, K35, and K42 as key residues that are likely to engage protein mediators of transcriptional repression (20). Dual charge-reversal mutations had uniformly substantial effects in abrogating SUMO-2-mediated repression. Proteins that associated with SUMO-2, but not with the dual charge-reversal mutants, included SETDB1,

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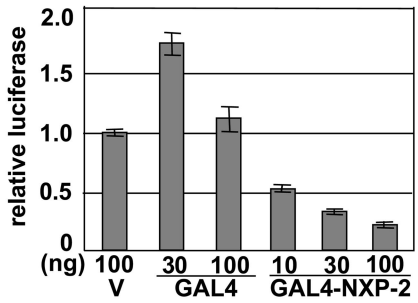

Fig. 6. NXP-2 represses transcription when tethered to a promoter by Gal4 fusion. Relative luciferase activities in 293T cells transfected with pGL2-Gal4-TK-Luc reporter plasmid and vector (100 ng), Gal4 (30 and 100 ng), or Gal4-NXP-2 (10, 30, and 100 ng) expression plasmids. Results are expressed relative to luciferase activities observed with vector alone, which is set to 1.0.

LSD1, PELP1, and ARIP4, which are regulators of transcription, and the nuclear matrix protein NXP-2. The potential role of LSD1 and NXP-2 were investigated. LSD1 was not required for SUMO-2-mediated repression of transcription at a promoter in HeLa cells. NXP-2 was discovered to have repressive effects when targeted to a Gal4-dependent reporter by gene fusion.

The previous finding that single mutations in SUMO-2 K33, K35, or K42 substantially interfere with repression appears to be assay-specific. The repressive effects of WT and mutant SUMO-1 and -2 had been assayed in the context of a SUMOylation-deficient glucocorticoid receptor in CV-1 cells (9), whereas we used a promoter and reporter regulated by upstream binding sites for Sp1 in 293T cells or by in-frame fusion to Sp3KR in HeLa cells. Consequently, the observed differences could be due to differences in transcription activation by the SUMOylation-deficient glucocorticoid receptor, by Sp1, or by a SUMOylation-deficient Sp3, or to differences in transcription-factor abundances in CV-1, 293T, and HeLa cells.

SUMO-2 K33, K35, and K42 or the equivalent SUMO-1 K37, K39, and K46 have been implicated in protein interactions. SUMO-1 K39 and K46 show large chemical shifts in complexes with peptides derived from PIASx, PML, and SAE2 (21). Furthermore, the cocrystal structure of thymidine DNA glycosylase with SUMO-1 shows that SUMO-1 K39 and K37 make contact with a 307DVQEV311 motif in thymidine DNA glycosylase (22). Moreover, the cocrystal of SUMO-1 with RanGAP1, RanBP2/NUP358, and UBC-9 reveals that RanBP2 D2631 is hydrogen bonded to SUMO-1 K39, with RanBP2-SUMO-1 contacts spanning a 2631DVLIV2635 motif in RanBP2 (23). Taken together, these data suggest that the SUMO-binding motifs of diverse proteins can interact directly with the positive lysine cluster. SUMO interaction motifs consisting of key hydrophobic and acidic residues such as K–X3–5- I/V- I/L-I/L-X3- D/E/Q/N- D/E- D/E have been proposed (21, 24). The Epstein–Barr virus nuclear protein EBNA3C 507DDDVIEV513 is also critical for SUMO interaction (25). ARIP4 and a closely related SNF-2-domain-containing protein, SRCAP, have similar sequences (15, 26), and ARIP4 has now been identified to bind to SUMO-2, with dependence on K33, K35, and K42. These acidic SUMO-interacting amino acids may bind directly to the SUMO lysine-rich repressor patch. However, the substantially greater effect of mutations to glutamic acid over alanine and the effects of mutations in surrounding residues (9) are consistent with a broader SUMO site for intermolecular interactions relevant to repression.

Proteins with known roles in transcription or chromatin regulation bound preferentially to WT SUMO-2. LSD1 is an FAD-dependent lysine amine oxidase, which is part of a multiprotein CtBP and CoREST complex (27, 28), and ZNF198 is also a component of this complex, although its function is undefined. LSD1 specifically converts dimethylated H3K4 to mono- and unmethylated lysine (19). SETDB1 is an H3K9 methylating enzyme. H3K4 demethylation and H3K9 methylation are associated with transcriptional repression. Thus, SUMO binding of LSD1, ZNF198, and SETDB1 would result in concerted H3K4 demethylation and H3K9 methylation, potentially with additive or synergistic gene silencing. Indeed, Gal4 tethering of SUMO-2 to reporter DNA decreased H3K4 dimethylation, consistent with SUMO-mediated recruitment of LSD1. However, neither expression of an LSD1 dominant-negative mutant nor RNA interference experiments yielded any evidence of an essential role for LSD1 in SUMO-mediated repression in the transient assays system used here. Surprisingly, Gal4 fusions with SETDB1 were completely inert in our system, despite the fact that recombinant SetDB1 increases H3K9 methylation in *in vitro* assays (14). The level of H3K9 at the SUMO-2-repressed promoter has not been determined, but increased methylation may be anticipated, because HP1, an H3 methyl K9 binding protein, is recruited to a Gal4-dependent promoter by Gal4-Ubc9 (29)

Of the SUMO-2-interacting proteins assayed (RanGAP1, LSD1, SETDB1, PELP1, and NXP-2), only NXP-2 and LSD1 repressed when tethered to a promoter as fusions to the Gal4 DNA-binding domain. NXP-2, was identified in an immunoscreen of a cDNA library for nuclear matrix proteins, interacts with SUMO-1 by yeast two-hybrid (30), and is SUMO modified (31).

SUMOylation of transcription factors such as SATB2 may function to repress transcription by sequestration to the nuclear matrix (32). Further studies of the epigenetic changes affected by SUMO-2 through interaction with nuclear matrix and chromatin-associated proteins may reveal new aspects of SUMO-mediated transcriptional repression.

Materials and Methods

Plasmids. DNA encoding Gal4 DNA-binding domain, residues 1–147, was ligated into pcDNA3 with or without in-frame fusion to the 5′ end of the SUMO-2 ORF. SUMO-2 point mutants were made by using QuikChange site-directed mutagenesis (Stratagene). The C terminus of SUMO-2 was mutated from G93;G94 to A93;A94 to prevent SUMO-2 conjugation to SUMOylated substrates. SUMO-2 effects would then depend on Gal4-SUMO-2 attraction of repressive protein(s) to Gal4 DNA-binding sites and not on protein conjugation to Gal4-SUMO-2. Truncation mutants were made by insertion of a stop codon immediately C-terminal to the indicated amino acid. To generate Gal4 fusions, RanGAP1, NXP-2/KIAA0136, or SETDB1 cDNAs were cloned in-frame into pcDNA-3 3′ to the Gal4 DNA-binding domain.

GST-SUMO-2 was a gift from F. A. Grasser (IMH, Hamburg/Saar, Germany). GST-SUMO-2 mutants were generated by using QuikChange mutagenesis. Plasmids encoding LSD1 and LSD1-M2 (19), Gal4-Sp3, Gal4-Sp3K539R, and Gal4-SUMO-1-Sp3K539R fusions (2) have been described. Gal4-SUMO-2-Sp3K539R has the SUMO-2 ORF in place of SUMO-1.

G5Luc (2) contains five Gal4-binding sites, and pGL2-Gal4-TK-Luc contains five Gal4-binding sites upstream of, and two Sp1 sites within, a TK promoter (nucleotides −105 to +52). Both reporter plasmids contain a 3′ firefly luciferase gene (2, 29). PELP1 cDNA was a gift from Rakesh Kumar (M. D. Anderson Cancer Center, Houston). RanGAP1 cDNA was a gift from Yoshihiro Yoneda (Osaka University, Osaka). NXP-2 cDNA was a gift from Takahiro Nagase (Kazusa DNA Institute, Chiba, Japan). SETDB1 cDNA was purchased from Open Biosystems (Huntsville, AL).

Reporter Assays. One microgram of cDNA encoding each Gal4 fusion protein, 0.5 μg of pGK β-galactosidase, and 0.5 μg of pGL2-Gal4-TK-Luc reporter construct were transfected into

293T cells by using Superfect (Qiagen). Cells were harvested at 24 h for luciferase and β-galactosidase assays (33). Data in Table 1 are expressed as percentages of mean repression relative to WT SUMO-2, which was set at 100%. Repression by WT SUMO-2 was calculated relative to reporter alone. For Gal4-Sp3 experiments, 50 ng of Gal4 effector plasmid, 500 ng of G5Luc reporter vector, and 25 ng of pTKRL *Renilla* control reporter vector were transfected into HeLa cells by using Lipofectamine (Invitrogen). Firefly and *Renilla* luciferase activities were determined by using Dual Luciferase (Promega). The results are presented as average luciferase activity relative to Gal4 alone, which was set at 1. Assays were done in triplicate and in multiple independent experiments.

GST Pull Down and Western Blotting. GST fusion proteins used the GST gene-fusion system (Amersham Pharmacia). Pellets from 5 liters of 293T cells were lysed in 30 ml of Nonidet P-40 buffer (150 mM NaCl/0.5% Nonidet P-40/50 mM Tris, pH 7.40) with protease inhibitors. Lysates were homogenized, cleared by centrifugation, precleared with GST beads, and rocked with agarose linked to GST, GST SUMO-2, or GST-SUMO-2 mutants overnight at 4°C. After washing, proteins were recovered by boiling in Laemmli buffer. SYPRO ruby-stained proteins were excised and analyzed by nanospray liquid chromatography-MS at the Partners Center for Genetics and Genomics at Harvard University. Proteins were identified based on at least three independent peptides with X-correlation coefficients >1.8, 1+; 2.5, 2+; and 3.0, 3+ (34). The relative abundance of proteins in each gel slice was estimated from the sum of peptide areas (34). Proteins eluted from small-scale GST pull downs were detected by Western blotting with the following antibodies: SETDB1, (BL540; Bethyl Laboratories, Montgomery, TX), PELP1 (BL751; Bethyl Laboratories), SAE2 (IMG5111A, Imgenex, San Diego), RanGAP1 (Zymed catalog no. 33-0800), anti-hemagglutinin (Upstate Biotechnology, Lake Placid, NY), and anti-Xpress (Invitrogen).

Chromatin Immunoprecipitation Assays. Chromatin immunoprecipitation assays followed the Upstate Biotechnology protocol. Triplicate 293T cell dishes were seeded at 1×10^6 cells per 10-cm dish the day before transfection with 1 μg of pGL2-Gal4-TK-Luc and 10 μg of pcDNA-Gal4 or pcDNA-Gal4-SUMO2 by using Superfect (Qiagen). After immunoprecipitation of protein–DNA complexes and reversal of cross-linking, a 330-bp sequence beginning 100 bp downstream of the Gal4 binding sites was amplified by using primers 5′-GACGCCAAAAACATAAAGAAAGGCC-3′ and 5′-TTCACGTTCATTATAATGTCGTTC-3′. A 205-nt control PCR product from the ampicillin-resistance cassette in pGL2-Gal4-TK-Luc was generated for each reaction by using the forward primer (F) (5′-CAGTGAGGCACCTATCTCAGCGATC-3′) and reverse primer (R) (5′-GCAGGACCACTTCTGCGCTCGGGCCC-3′) with 25 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min.

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