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Covalent conjugation of Groucho with SUMO-1 modulates its corepressor activity

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ABSTRACT

Groucho is a corepressor that forms a macromolecular complex for its corepressor activity, in which HDAC1 is an essential component for the modulation of chromatin structure and transcriptional repression of target genes. Here, we show that Groucho is covalently conjugated with small ubiquitin-related modifier-1 (SUMO-1) *in vitro* and *in vivo*. SUMO conjugations of Groucho occur at four different lysine residues. Substitutions of all these residues abolished sumoylation of Groucho and inhibited its corepressor activity. In addition, Groucho corepressor activity was reduced by inhibition of SUMO-1 conjugation via Ubc9 knockdown through expression of short-hairpin RNA against Ubc9. Furthermore, interactions between Groucho and HDAC1 are enhanced by sumoylation of Groucho, which is mediated by the SUMO-interaction motif of HDAC1. Taken together, these findings indicate that Groucho sumoylation increases its corepressor activity by enhancing the recruitment of HDAC1 to Groucho corepressor complex.

A corepressor is defined as a protein that participates in transcriptional repression via interaction with a DNA-binding transcription factor because of its lack of DNA-binding activity. Groucho is the founding member of the Gro/TLE corepressor family, and is involved in a wide array of developmental processes, including neurogenesis, segmentation, and sex determination in *Drosophila* [1]. Groucho family members function as important regulators of several signaling pathway such as Wingless/Wnt, Notch, and Dpp/TGFβ, as well as regulation of a variety of developmental and cellular differentiation processes [2].

Groucho is recruited by a specific set of transcriptional repressors via its short peptide motif, which includes either the WRPW or Eh1 motif [2]. Domain analysis of Groucho shows that the N-terminal Q domain is responsible for tetramerization and transcriptional repression and that the CcN and SP domains are required for nuclear localization and regulation by several nuclear protein kinases [3–5]. Carboxyl-terminal WD40 repeats of Groucho form a propeller structure containing a series of seven blades which mediates its association with cellular binding partners. Groucho forms a high molecular weight complex to repress transcription of target genes involving HDAC1 which modulates chromatin structure [6,7].

SUMO modification of a target protein modulates its function and acts in a variety of biological processes, including transcriptional regulation, DNA repair, protein trafficking, and mitotic cell division [8]. Small ubiquitin-like modifier-1 (SUMO-1) is expressed as a precursor protein, and is processed by a SUMO-specific protease to expose C-terminal di-glycine for transesterification to E1 enzyme [9,10]. The SUMO moiety conjugated to E1 enzyme is transferred to the Ubc9, an E2-conjugating enzyme, and is finally attached to a target substrate by Ubc9 itself or with the aid of E3 SUMO ligases. Many SUMO-modified proteins harbor a SUMO-interaction motif (SIM), which binds to the SUMO moiety of sumoylated proteins. SIM is a short peptide motif that consists of a short stretch of hydrophobic amino acids followed by serine and acidic amino acids. Noncovalent interactions between the SUMO moieties of SUMO-modified proteins and SIM of other proteins affect the assembly of macromolecular structures such as PML nuclear bodies [11]. In addition, SUMO-interaction motif-mediated SUMO modification was recently reported [12,13]. SIM-dependent SUMO modification allows the sumoylation of a target protein to distinguish the SUMO paralogs that are to be covalently modified [14,15].

Here, we show that Groucho corepressor is covalently conjugated with SUMO-1 at multiple lysine residues. In addition, we provide evidence that HDAC1 has a SUMO-interaction motif similar to the SIMs found in other sumoylated proteins, and this SIM efficiently recognized sumoylated Groucho. Thus, SUMO

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modifications of Groucho enhances its binding to HDAC1, thereby promoting Groucho corepressor activity.

Materials and methods

Cell culture and transfection. COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For immunoblot analysis, COS-7 cells were transfected with Fugene6 reagent (Roche Molecular Biochemicals) in six-well plates.

Plasmids. Expression plasmids for GFP-SUMO-1, HA-SENP1, wild-type, and deletion mutants of Groucho were described previously [5,16]. Plasmids encoding GAL4-NK3 and G5-SVEn-luc reporter plasmids were described previously [6,17]. The SUMO-Groucho plasmid encoding amino acids 1–719 of Groucho fused to SUMO-1 in its N-terminus was constructed by the insertion of a PCR-amplified DNA fragment for SUMO-1 (aa 1–95) into EcoRI site of pEntr-Groucho. Point mutants of Groucho for sumoylation sites were generated by using the QuickChange mutagenesis kit (Stratagene) according to the recommendations of the manufacturer. Mutations were verified by DNA sequencing. Mutagenesis was done on the pEntr-derived Groucho plasmid, and Myc-tagged expression plasmids for mutant Groucho were generated using Gateway Technology (Invitrogen).

Luciferase reporter assays. For the Luciferase reporter assays, COS-7 cells seeded onto 12-well plates were transfected with the expression plasmid encoding GAL4-NK3, G5-SVEn-Luc reporter plasmid, which harbored the luciferase gene under the control of an SV40 enhancer and five copies of GAL4-binding sites, together with either wild-type Groucho or Groucho (4KR) mutant. The total amounts of plasmids were adjusted using empty vectors. Forty-eight hours after transfection, luciferase activity was measured using the Luciferase Reporter Assay System (Promega) and a Genios luminometer (TECAN, Austria). Each experiment was repeated at least three times.

In vitro sumoylation assays. GST-SAE1/SAE2 and GST-SUMO-1(GG) fusion proteins were expressed in *Escherichia coli* BL21(DE3), and were purified via affinity chromatography using glutathione–Sepharose according to the protocols of the manufacturer (Amersham Biosciences). *In vitro* sumoylation assays were performed as described previously [16]. In brief, *in vitro*-translated Myc-tagged Groucho that had been created using the TNT-coupled Reticulocyte Lysate System (Promega) was mixed with 500 ng of purified E1(GST-SAE1/SAE2), 400 ng of His-Ubc9, and 2 μg of GST-SUMO-1(GG), and was then incubated at 37 °C for 60 min. Reaction products were visualized by Western blotting with anti-Myc antibody (Roche Molecular Biochemicals).

In vitro pull-down assays. Myc-tagged HDAC1 constructs were subjected to *in vitro* translation using a TNT-coupled Reticulocyte Lysate System (Promega). Pull-down assays were performed by incubating equal amounts of GST or GST-Groucho (1–333) fusion proteins immobilized onto glutathione–Sepharose beads with *in vitro*-translated HDAC1. The mixture was placed onto a rocking platform for 2 h, and was then washed five times with a buffer containing 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.05% Nonidet P-40. Bound proteins were eluted and separated by 8% SDS–polyacrylamide gel electrophoresis.

Co-immunoprecipitation and Western blotting. Co-immunoprecipitation was performed after the lysis of 2×10^7 cells with lysis buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 1 mM EDTA). After incubation on ice for 10 min and centrifugation for 10 min at 4 °C, equal volumes of protein were incubated overnight with antibody and protein A/G–Sepharose beads at 4 °C on a rotating wheel. The beads were washed three times with lysis buffer. The whole cell lysates and immunoprecipitates were separated by SDS–PAGE, and were then transferred onto polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-Myc (Invitrogen) or anti-HA antibody (Invitrogen).

Protein expression and affinity purification. BL21(DE3) cells containing pT-E1E2S1, which encodes E1 and E2 enzymes of SUMO conjugation as well as an active form of SUMO-1 [18], were transformed with an expression plasmid encoding GST-Gro (aa 1–333), and were grown at 37 °C in LB medium containing ampicillin and chloramphenicol. Cultures ($OD^{600} = 0.55$) were induced with 1 mM IPTG for 10 h at 25 °C. After cells were centrifuged, the pellet was resuspended in PBT (PBS with 1% Triton X-100), and was then lysed with sonication. The supernatant of the lysates was incubated with glutathione–Sepharose beads for 1 h at 4 °C. The beads were washed three times with PBT, and proteins were eluted three times with 10 mM reduced glutathione, 10 mM Tris–Cl (pH 7.4).

Results and discussion

Covalent conjugation of Groucho with small ubiquitin-like modifier-1

A variety of transcription factors are known to be covalently conjugated with SUMO-1, and these modifications are associated with their transcriptional activities [19]. Covalent conjugation of a substrate with SUMO-1 requires both Ubc9 binding and an acceptor lysine residue, the consensus sequence of which can be represented as ψ KXE (where ψ is hydrophobic amino acid and X is any amino acid). Sequence analysis of Groucho revealed that four potential sumoylation target sequences were present in the repression domain (K32 of IKEE, K46 of IKLE), SP domain (K279 of IKQE), and WD40 repeats (K516 of IKAE). Furthermore, Groucho strongly bound to Ubc9 *in vitro* (data not shown).

To determine whether Groucho could be conjugated with SUMO-1, *in vitro*-translated Myc-tagged Groucho was subjected to an *in vitro* sumoylation assay using affinity-purified SAE1/2, Ubc9, and SUMO-1. Multiple slowly migrating bands of Groucho were detected only when all of the components necessary for sumoylation were included in the reaction, thus suggesting that multiple lysine residues of Groucho were conjugated with SUMO-1 (Fig. 1A). To address sumoylation of Groucho in cultured cells, a SUMO-1 or GFP-SUMO-1 expression plasmid was transfected into COS-7 cells together with expression plasmids encoding Groucho, followed by Western blotting with anti-Myc antibody (Fig. 1B). A slower migrating band of different molecular weight was detected depending on the use of the SUMO-1 or GFP-fused SUMO-1 (lane 2 vs lane 3). This band disappeared upon co-expression of an increasing amount of SENP1, a SUMO-1-specific deconjugating enzyme (lanes 4–6). These results indicate that Groucho could be a target for covalent SUMO-1 conjugation *in vitro* and *in vivo*.

Determination of Groucho sumoylation sites

To explore the role of covalent modification of Groucho with SUMO-1, we determined the acceptor lysine residues for SUMO-1 conjugation *in vitro*. To this end, deletion mutants of Groucho were generated, and each mutant was subjected to an *in vitro* sumoylation assays (Fig. 2A). Amino-terminal Groucho (aa 1–398) was as efficiently modified with SUMO-1 as full-length Groucho, whereas C-terminal Groucho (aa 399–719) was not. Further analysis of N-terminal Groucho deletion mutants revealed that both of the Groucho deletion mutants (aa 1–198 and aa 199–398) were modified with SUMO-1 (Fig. 2A). Candi-

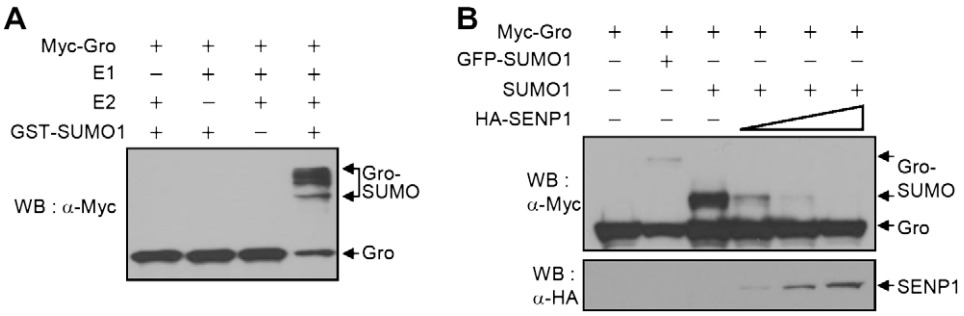

Fig. 1. Covalent conjugation of Groucho with SUMO-1 *in vitro* and *in vivo*. (A) *In vitro*-translated Myc-tagged Groucho was subjected to an *in vitro* sumoylation assay. Affinity-purified GST-SAE1/SAE2 heterodimer (E1), GST-Ubc9 (E2), and GST-SUMO-1 were mixed as indicated in the figure, and reaction mixtures were analyzed by Western blotting using anti-Myc antibody. (B) COS-7 cells were transfected with expression plasmids encoding Myc-Groucho and either GFP-SUMO-1 or SUMO-1 expression plasmids. To determine the deconjugation of sumoylated Groucho by SUMO-1-specific protease, increasing amounts of HA-SENP1 expression plasmids were co-transfected (lanes 4–6).

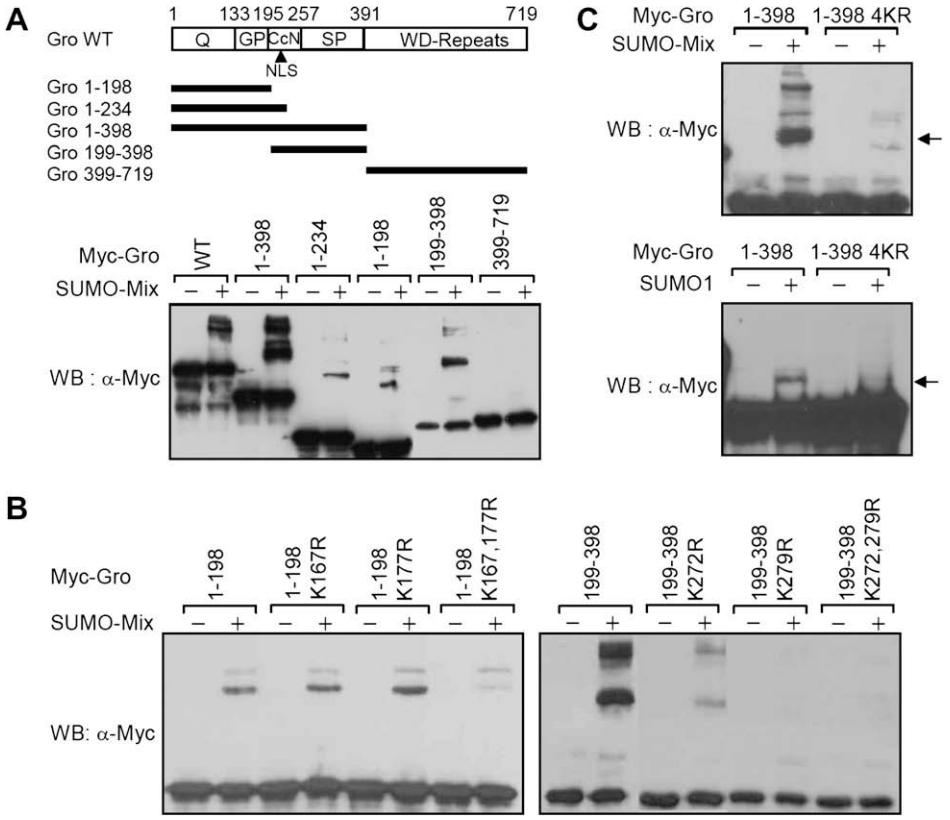

Fig. 2. Determination of Groucho sumoylation sites *in vitro* and *in vivo*. (A) *In vitro*-translated Myc-Groucho deletion mutants were subjected to *in vitro* sumoylation assays. Reactions were terminated by the addition of sample buffer, followed by Western blotting using anti-Myc antibody. Schematics of Groucho deletion mutants are depicted at the top of the figure. (B) SUMO modifications of Groucho point mutants were determined using an *in vitro* sumoylation assay as described above. (C) Expression plasmid encoding either Myc-Groucho (1–398) or Myc-Groucho (1–398) 4KR mutant (K167, 177, 272, and 279R) was transfected into COS-7 cells together with the SUMO-1 expression plasmid, followed by Western blotting using anti-Myc antibody (lower panel). The same plasmids were subjected to an *in vitro* sumoylation assay using GST-SUMO-1 (upper panel). The difference in the migration of sumoylated Groucho (arrows) depends on the size of expressed SUMO-1 protein (untagged SUMO-1 vs GST-SUMO-1).

date lysine residues within these two fragments were substituted with arginines, and the sumoylation of the resulting point mutants was analyzed by *in vitro* sumoylation assays (Fig. 2B). Substitution of either Lys167 or Lys177 with arginine did not affect sumoylation of the Groucho (aa 1–198) fragment. However, substitutions of both Lys167 and Lys177 with arginines abolished SUMO-1 modification of the Groucho (1–198) fragment. Substitution of Lys272 or Lys279 with arginine reduced sumoylation of the Groucho (199–398) fragment, and substitutions of both Lys 272 and Lys 279 completely abolished its SUMO modification. These results indicate that Lys167, Lys177, Lys 272, and Lys279 are SUMO-1 acceptor sequences of Groucho. To verify the identified sumoylation sites of Groucho, all four lysine residues were substituted with arginines (4KR), and the mutants were used for *in vitro* and *in vivo* sumoylation assays (Fig. 2C). In these assays, the Groucho 4KR mutant was no longer modified

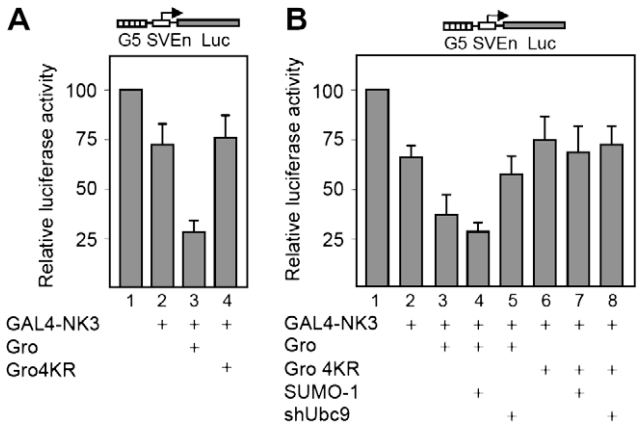

Fig. 3. Corepressor activities of wild-type Groucho and the sumoylation-deficient Groucho mutant. (A) G5-SVEn-luc reporter plasmid and GAL4-NK3 expression plasmids were transfected into COS-7 cells together with either wild-type Groucho or Groucho 4KR, a sumoylation-deficient mutant. Thirty-six hours after transfection, luciferase activity was measured using the Luciferase Reporter assay system (Promega). Transfection efficiency was normalized by the expression of β-galactosidase. These experiments were repeated at least three times. Error bars indicate the error of the means of three independent experiments. (B) GAL4-NK3 expression plasmid and G5-SVEn-luc reporter plasmid were transfected into COS-7 cells together with either wild-type Groucho or Groucho 4KR expression plasmid in combination with SUMO-1 expression plasmid or shUbc9 plasmid expressing short-hairpin RNA against Ubc9 E2-conjugating enzyme.

with SUMO-1 *in vitro* (upper panel), and the same was observed in the Western blots of cell lysates from the cells transfected with a plasmid encoding the Groucho 4KR (aa 1–398) mutant (lower panel). Taken together, these findings indicate that Groucho could be SUMO-modified at multiple lysine residues present in the GP and SP domains of Groucho.

Enhancement of Groucho corepressor activity via SUMO modification

Next, we addressed whether SUMO modification of Groucho affects its corepressor activity. COS-7 cells were transfected with G5-SVEn-luciferase reporter plasmid and GAL4-NK3 expression plasmid together with either the wild-type Groucho or sumoylation-deficient Groucho 4KR expression plasmid, followed by a luciferase assay to determine the corepressor activity of Groucho. Wild-type Groucho showed corepressor activity on the GAL4-NK3-mediated transcriptional repression on the synthetic promoter under the control of GAL4-binding sites and SV40 enhancer, whereas the sumoylation-deficient Groucho 4KR mutant did not (Fig. 3A). To further characterize the corepressor activity of Groucho depending on its SUMO modification, SUMO-1 expression plasmid or short-hairpin Ubc9 (shUbc9) expression plasmid was transfected together with wild-type Groucho or Groucho 4KR mutant. Forced expression of SUMO-1 further increased the corepressor activity of wild-type Groucho on GAL4-NK3-mediated transcription repression, whereas knockdown of endogenous Ubc9 decreased Groucho corepressor activity. These effects were not observed in the same experiment with sumoylation-deficient Groucho 4KR mutant (Fig. 3B). These results indicate that SUMO modifications of Groucho are associated with its corepressor activity on the target protein.

Sumoylated Groucho efficiently recruits HDAC1 through its SUMO-interaction motif

Groucho recruits several proteins to form a corepressor complex, in which HDAC1 is an essential component to modulate the acetylation status of histone [6,20]. To explore the molecular mechanism underlying the regulation of Groucho corepressor activity via sumoylation, we determined whether binding of HDAC1 to Groucho depends on Groucho sumoylation. The SUMO-interaction motif (SIM) of a protein is known to participate in binding to a sumoylated protein [12]. Analysis of HDAC1 amino acid sequence revealed a potential SUMO-interaction motif with a consensus sequences containing hydrophobic amino acids followed by acidic amino acids (Fig. 4A). Site-directed mutagenesis was carried out to generate an SIM mutant of HDAC1, and the ability of this mutant to bind to sumoylated Groucho was analyzed with a GST pull-down assay. Sumoylated Groucho was produced in *E. coli* harboring SUMO-1, SAE1/2, Ubc9, and the Groucho expression plasmid (Fig. 4B). Binding of wild-type HDAC1 to sumoylated Groucho was markedly increased, whereas binding of HDAC1 SIM mutant was not (Fig. 4C). Next, we attempted to verify the efficient binding of HDAC1 to Groucho in a SIM-dependent manner in mammalian cells. To this end, an expression plasmid encoding SUMO-Groucho fusion protein was generated (Fig. 4D). COS-7 cells were transfected with either a plasmid encoding wild-type Groucho or SUMO-Groucho fusion protein in combination with either wild-type HDAC1 or a SIM mutant HDAC1 expression plasmids. The cell lysates were immunoprecipitated with anti-HA antibody, which recognizes HA-Groucho, followed by Western blotting using anti-Myc antibody. As shown in Fig. 4E, wild-type HDAC1 bound more strongly to the SUMO-Groucho fusion protein than to the wild-type Groucho, whereas HDAC1 SIM mutant did not. These results demonstrated that the SUMO-interaction motif of HDAC1 is required for efficient binding to SUMO-modified Groucho.

Covalent conjugations of proteins with small ubiquitin-like modifier modulate the cellular functions of proteins in a variety of ways, including protein–protein interaction, cellular compartmentalization, alteration of enzymatic activity, and polysumoylation-dependent degradation [8,21,22]. In particular, SUMO modification participates in the formation of high molecular weight complexes or macromolecular structures such as PML nuclear bodies, nuclear pore complexes, and nucleolar structures [19,23,24]. We demonstrated that Groucho could be sumoylated at multiple lysine residues and, in turn, its corepressor activity is enhanced via efficient recruitment of HDAC1 through its SUMO-interaction motif. It has been shown that HDAC1 is also modified with SUMO-1 at Lys444 and Lys476, and that this modification affects enzymatic activity [25]. In this study, we presented evidence that HDAC1 harbors a SUMO-interaction motif consisting of hydrophobic amino acids followed by serine and acidic amino acids (Fig. 4A). Affinity-purified sumoylated Groucho bound to HDAC1 in a SIM-dependent manner, which suggested that the SIM of HDAC1 contributes to its function in transcriptional repression. Given that SUMO modifications are closely associated with repression of transcription [26,27], HDAC1 could be efficiently recruited by transcriptional repressors and other components of corepressor complexes, which are covalently conjugated with SUMO-1 or SUMO-2. Therefore, both covalent modification of HDAC1 at specific lysine residues and noncovalent interaction with other sumoylated proteins through its SIM might contribute to its functions in transcriptional repression. We and others have provided evidence that the corepressor activity of Groucho could be regulated by phosphorylation, which is crucial for Groucho-mediated regulation of cellular differentiation and a variety of developmental processes [2,5,28]. The data presented here add more complexity to our understanding of the regulation of Groucho corepressor activity, and provide a possibility that diverse stimuli participate in the fine tuning of transcriptional activity exerted by Groucho corepressor.

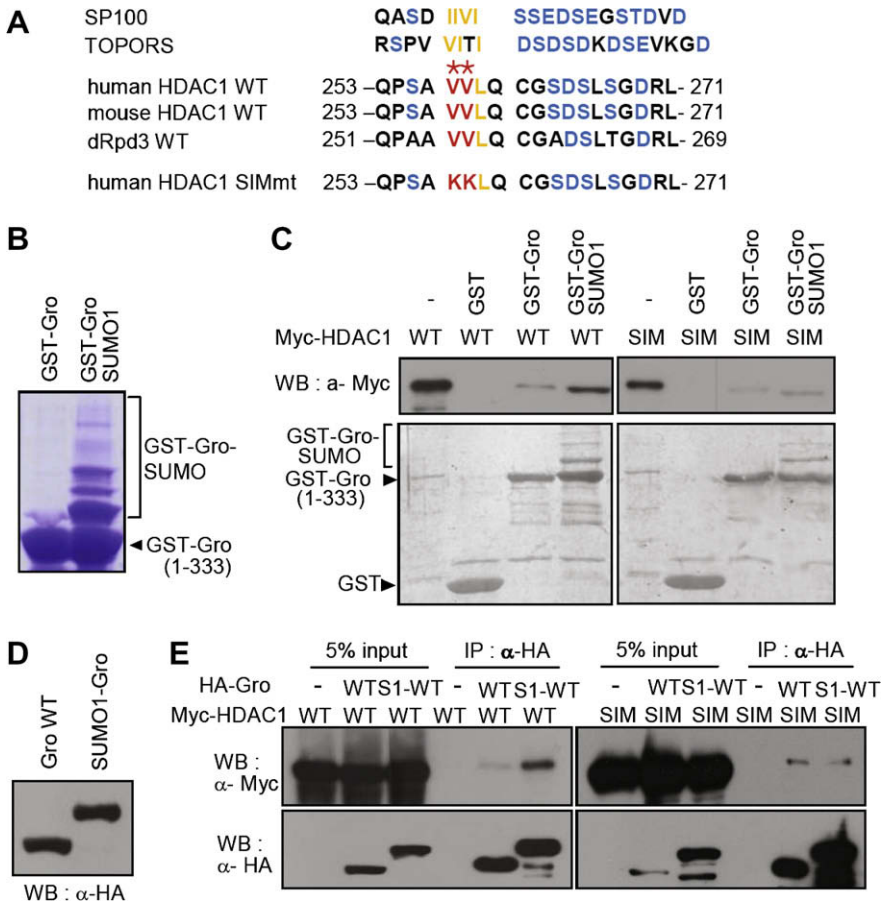

Fig. 4. SUMO modification of Groucho promotes its binding to HDAC1 through a SUMO-interaction motif (SIM). (A) The SIM of HDAC1 consists of a short stretch of hydrophobic amino acids followed by serine and acidic amino acids such as aspartic acid. SIM of HDAC1 is well-conserved in humans, mice, and *Drosophila*. The amino acids of HDAC1 SIM that were mutated are indicated with asterisks. (B) Amino-terminal Groucho (1–333) fused to GST was conjugated with SUMO-1 in *E. coli*. An *E. coli* strain harboring pT-E1E2S1 expression plasmid encoding SAE1/SAE2, Ubc9, and SUMO-1 was transformed with an expression plasmid encoding GST-Gro (1–333). Induction of bacterial cells with IPTG produced SUMO-modified Groucho. Affinity-purified sumoylated GST-Groucho was utilized in the GST pull-down assay to determine the role of HDAC1 SIM. (C) Wild-type HDAC1 and HDAC1 SIM mutants (V257K and V258K) were *in vitro*-translated using rabbit reticulocyte lysates, and were then subjected to a GST pull-down assay using affinity-purified GST-Gro (1–333) or sumoylated GST-Gro (1–333). Bound proteins were eluted with sample buffer, followed by Western blotting using anti-Myc antibody (upper panel). The PVDF membrane was stained with Coomassie blue to show GST-fusion protein utilized in the GST pull-down assay (lower panel). (D) A mammalian expression plasmid encoding SUMO-Groucho fusion protein was generated, and its expression was verified by Western blotting. (E) COS-7 cells were transfected with either HA-Groucho (WT) or HA-Groucho fused to SUMO-1 (S1-WT) together with either wild-type Myc-HDAC1 or Myc-HDAC1 SIM mutant (SIM, V257K, and V258K) expression plasmids. Cells were lysed and immunoprecipitated with anti-HA antibody, followed by Western blotting using anti-Myc antibody.

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