Small Ubiquitin-like Modifier (SUMO) Conjugation Impedes Transcriptional Silencing by the Polycomb Group Repressor Sex Comb on Midleg*

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The Drosophila protein Sex Comb on Midleg (Scm) is a member of the Polycomb group (PcG), a set of transcriptional repressors that maintain silencing of homeotic genes during development. Recent findings have identified PcG proteins both as targets for modification by the small ubiquitin-like modifier (SUMO) protein and as catalytic components of the SUMO conjugation pathway. We have found that the SUMO-conjugating enzyme Ubc9 binds to Scm and that this interaction, which requires the Scm C-terminal sterile α motif (SAM) domain, is crucial for the efficient sumoylation of Scm. Scm is associated with the major Polycomb response element (PRE) of the homeotic gene Ultrabithorax (Ubx), and efficient PRE recruitment requires an intact Scm SAM domain. Global reduction of sumoylation augments binding of Scm to the PRE. This is likely to be a direct effect of Scm sumoylation because mutations in the SUMO acceptor sites in Scm enhance its recruitment to the PRE, whereas translational fusion of SUMO to the Scm N terminus interferes with this recruitment. In the metametathorax, Ubx expression promotes haltere formation and suppresses wing development. When SUMO levels are reduced, we observe decreased expression of Ubx and partial haltere-to-wing transformation phenotypes. These observations suggest that SUMO negatively regulates Scm function by impeding its recruitment to the Ubx major PRE.

Hox genes, which specify segment identity, are expressed in spatially restricted patterns throughout metazoan development. In the Drosophila embryo, the early pattern of homeotic gene expression relies on the transient action of the transcriptional activators and repressors encoded by the segmentation genes. Subsequently, the Polycomb group (PcG)1 and Trithorax group (TrxG) proteins maintain the transcriptional states established by the segmentation proteins in each cell lineage through many rounds of cell division (1). The PcG proteins maintain the repressed state in cell lineages in which a homeotic gene is initially repressed by the segmentation factors, whereas the TrxG proteins maintain the active state in cell lineages in which a homeotic gene is initially activated by the segmentation factors.

At least three separate PcG protein complexes, termed Pleiohomo- gene Regressive Complex (PhoRC), Polycomb Repressive Complex 1 (PRC1), and Polycomb Repressive Complex 2 (PRC2), work in concert to maintain the repressed state. PhoRC is comprised of the DNA-binding factor Pleiohomeotic (Pho) and the mono- and dimethyl-H3K9 and H4K20-binding factor Scm-related gene containing four MBT domains (Sfmbt) (2). PhoRC is thought to recruit PRC2, a complex containing the PcG proteins Enhancer of zeste (E(z)), Suppressor of zeste 12 (Su(z)12), and Extra sexcombs (Esc), to chromatin. PRC2 is a histone methytransferase responsible for methylating lysine 27 of histone H3 (3, 4). The resulting trimethylated form of H3K27 is believed to serve as a docking site for the chromodomain of Polycomb (Pc), a core component of PRC1. Pc and the three other core components of PRC1, Posterior sex combs (Psc), Polyhomeotic (Ph), and Sex combs extra (Sce, also known as Ring) copurify with multiple TATA box-binding protein (TBP)-associated factors (TAFs), suggesting a direct interaction between PRC1 and promoter complexes that modulate transcription (5). Characterized functions of PRC1 family complexes include ubiquitylation of histone H2A (6) and compaction of polycondes (7). Individual components of each complex are crucial for proper function in vivo as loss of any of the PcG genes results in ectopic expression of homeotic genes. PcG protein complexes are directed to target genes through recruitment to specific DNA sequences called Polycomb response elements (PREs). Although this recruitment to PREs is required to prevent expression from target promoters, the exact mechanism of repression remains unclear.

Small ubiquitin-like modifier (SUMO), a ubiquitin family protein, is covalently conjugated to lysine side chains in a large number of target proteins. The attachment of SUMO to its many substrates is known to regulate a variety of important cellular functions including subcellular targeting, protein stability, and transcription factor activity (8). Multiple connections have been observed between PcG protein function and the SUMO conjugation pathway. The human homolog of Polycomb, hPc2 (also known as Cbx4), can function as an E3-type SUMO ligase by promoting sumoylation of C-terminal binding protein (CtBP) and homeodomain-interacting protein kinase 2.
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(HIPK2). CtBP, HIPK2, and the SUMO-conjugating enzyme, Ubc9, colocalize with hPc2 in discrete intranuclear foci called polycomb bodies (9). Recent studies suggest that SUMO modification of mouse Pc2 increases its affinity for H3K27me3 nucleosomes, leading to enhanced promoter association and Hox gene repression (10). Pho was identified as a SUMO conjugation target in a proteomic screen for Drosophila targets that either are modified by or interact with SUMO (11). Sumoylation of the Pho homolog Yin Yang 1 (YY1) appears to modulate its capacity to activate or repress transcription (12). Sumoylation of the Caenorhabditis elegans PcG protein SOP-2 regulates its localization to PcG nuclear bodies and is required to prevent ectopic expression of homeotic genes (13). SOP-2 is related to the Drosophila PcG proteins Ph and Sex Comb on Midleg (Scm) by virtue of a shared sterile α motif (SAM) protein-protein interaction domain. Although Ph is a core subunit of PRC1 (7, 14), Scm appears only peripherally associated with PRC1 (15) but nonetheless is also essential for PcG-mediated silencing (16).

Although Scm can associate with PRC1 through an interaction with Ph (15), a recent study suggests that targeting of Scm to chromatin can occur independently of the three identified PcG complexes (17). Scm contains two MBT repeats that are also found in the tumor suppressor lethal (3), malignant brain tumor (l(3)mbt) (18), and the PhoRC component Sfmbt (2). The Scm MBT repeats bind preferentially to monomethylated lysine residues, an activity that is required for repression of homeotic genes (19). Although Scm does not directly bind DNA, it contains two non-traditional zinc finger domains at its N terminus, in addition to the C-terminal SAM protein-protein interaction domain. The Scm SAM domain allows self-interaction and mediates the binding of Scm to Ph (20, 21). The importance of the Scm SAM domain is demonstrated by Scm mutants that disrupt or alter the domain and behave as genetic nulls (15, 18, 22). In addition, tissue-specific overexpression of the isolated Scm SAM domain alone induces homeotic transformation phenotypes, suggesting a dominant-negative effect on PcG-mediated repression (15).

In this study, we identify Scm as a substrate for SUMO modification. Chromatin immunoprecipitation (ChIP) experiments are used to track Scm association with the bxd PRE, located upstream of the Hox gene Ubx, where it participates in Ubx silencing. Increased recruitment of Scm to the PRE is observed under conditions that decrease Scm sumoylation. SUMO pathway-dependent changes in PRE association of Scm correlate with changes in Ubx expression and homeotic transformations. These findings suggest that the SUMO conjugation pathway is an important regulator of Scm and PcG-mediated repression.

EXPERIMENTAL PROCEDURES

Embryo Extracts and Anti-FLAG Immunoaffinity Enrichment—Drosophila embryos were harvested from a transgenic line that expresses FLAG-Scm from the normal genomic promoter (15), and nuclear extracts were prepared as described (23). For affinity enrichment, 50–100-mg aliquots of nuclear proteins were supplemented with Tween 20 to 0.1%, fresh DTT to 0.5 mM, and fresh protease inhibitors as follows: 0.5 mM PMSE, 1.0 μg/ml aprotinin, 1.0 μg/ml leupeptin, 1.0 μg/ml chymostatin, 1.0 μg/ml pepstatin A, 1.0 μg/ml antipain, 50 μg/ml 1-chloro-3-tosylamido-7-amino-2-heptanone. Protein aliquots were incubated with anti-FLAG M2-agarose beads (Sigma) overnight at 4°C with rotation, using 1.0 μl of bead slurry/1.0 mg of nuclear extract. Beads were then pelleted by brief low speed centrifugation and washed in 1.0 ml of Buffer C (20 mM Hapes, pH 7.6, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol plus protease inhibitors as above) plus 0.5 mM NaCl for 15 min at room temperature. After two additional washes in Buffer C plus 0.15 M NaCl, FLAG-Scm was eluted in 100 μl of Buffer C plus 0.15 M NaCl containing 0.4 mg/ml FLAG peptide (DYKDDDDK) with rotation at room temperature for 30 min. Samples prepared in the presence of N-ethylmaleimide (NEM) included 20 mM NEM throughout nuclear extraction and anti-FLAG affinity enrichment.

Stable Transfections and RNAi—Drosophila S2 cells were cultured at 24°C in Schneider’s insect medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences). Transfections were performed on cells growing in 6-well plates using Effectene (Qiagen). To generate the FLAG-Scm stably transformed cell line, the pMT-FLAG-Scm vector was cotransfected with pCoHygro (Invitrogen) in S2 cells, and transformed cells were selected for using medium containing 300 μg/ml hygromycin (Invitrogen) as described previously (24). The pMT-FLAG-Scm construct was a gift kindly provided by Chongwoo Kim. GFP, SUMO, and Ulp1 double-stranded RNA (dsRNA) was generated using the MEGAscript RNAi kit (Ambion) using PCR-derived templates containing flanking T7 promoters (11, 25). RNA interference was performed as described previously (26).

Scm Sumoylation Assays—The pPAC-FLAG-Scm (WT) plasmid was created by amplifying the Scm cDNA from pBS-FLAG-Scm using PCR primers containing KpnI sites and ligating into the KpnI site of pPAC-FLAG (27). The Scm lysine-to-arginine point mutations at positions 210, 574, and 624 were created using pBS-pro-FLAG-Scm as a template, and mutagenesis was carried out using the QuikChange kit (Stratagene) according to the manufacturer’s protocol. Each of the resulting pBS-pro-FLAG-Scm mutants was PCR-amplified and cloned into the KpnI site of pPAC-FLAG, generating the pPAC-FLAG-Scm single mutants K210R, K574R, K624R, or the triple mutant (K210R/K574R/K624R). The pPAC-FLAG-Scm (ΔSAM) construct was made by PCR-amplifying the Scm cDNA sequence encoding amino acids 1–797 and cloning the resulting fragment into the KpnI site of pPAC-FLAG. To evaluate sumoylation of Scm, 1 μg of pPAC-FLAG-Scm was transiently transfected in 529SU cells (a cell line stably transformed with copper-inducible HA-Ubc9 and FLAG-SUMO) alone or with increasing amounts of pPAC-FLAG-Ulp1 (1, 5, 25, 100, or 500 ng). The cells were left untreated or were induced with 500 μM CuSO4 24 h after transfection and were harvested 48 h after induction for anti-Scm Western blot.

To further evaluate sumoylation of Scm, the FLAG-Scm stable line was treated with either Ulp1 or SUMO dsRNA (5 μg/well), and after 48 h, the cells were treated with 500 μM CuSO4. After a 48-h induction, samples were either lysed directly by boiling in SDS loading buffer and analyzed by SDS-PAGE and anti-FLAG immunoblot or resuspended in lysis buffer (50 mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM...
EDTA, and 1% Triton X-100), subjected to immunoprecipitation using anti-FLAG M2 beads (Sigma), and analyzed by anti-SUMO Western blot. To evaluate the sumoylation state of Scm lacking a SAM domain or with potential SUMO acceptor lysines mutated to arginine, S2 cells alone or treated with 5 μg of Ulp1 dsRNA for 24 h were transiently transfected with a constitutively active expression vector encoding FLAG-Scm WT, ΔSAM, or K210R/K574R/K624R. Cells expressing FLAG-Scm were harvested 48 h after transfection, lysed by boiling in sample buffer, and analyzed by anti-FLAG Western blot. In vitro sumoylation assays using 35S-labeled Scm and purified SUMO pathway components were performed as described previously (24).

In Vitro Protein Interaction Assays—In vitro-translated 35S-labeled luciferase, Scm (WT), and Scm (ΔSAM) were prepared using the TstT T7 Quick coupled system (Promega). The pGem-Scm (WT) and pGem-Scm (ΔSAM) templates were created by PCR-amplifying either the full-length Scm ORF or the Scm ORF encoding amino acids 1–797 using primers containing KpnI sites and inserting each into the KpnI site of pGem-3Zf (Promega). The GST-tagged expression vectors pGEX-Ubc9 (27), and pGEX4T1 were expressed in Escherichia coli BL21 and purified on glutathione-agarose beads according to the manufacturer’s instructions (Amersham Biosciences). GST pulldown assays were performed as described previously (28). Approximately 5 μg of each glutathione bead-immobilized GST fusion protein was incubated with the indicated 35S-labeled protein. After extensive washing bound, radiolabeled proteins were eluted in SDS sample buffer, subjected to SDS-PAGE, and analyzed by autoradiography.

Chromatin Immunoprecipitation and RT-PCR—Formaldehyde cross-linked chromatin was prepared from fly S2 cells, and immunoprecipitations were performed as described (29) (30) using 5–10 μl of antiserum against Pc (30), Ph (23), or Scm (22) per ChIP. A negative control immunoprecipitation (“mock”) was performed in parallel using rabbit preimmune sera plus protein A-agarose beads. Amplification of Ubx PRE fragments and a control RpL1140 fragment by end point PCR was performed as described (29, 30).

To evaluate the effects of sumoylation on recruitment of Scm to the bxd PRE, S2 cells were transfected with 1 μg/well of pPAC-FLAG-Scm (WT), pPAC-FLAG-Scm (ΔSAM), pPAC-FLAG-Scm (K210R/K574R/K624R), pPAC-FLAG-SUMO-Scm (WT), or pPAC-FLAG-SUMO-Scm (ΔSAM) as indicated and harvested 48 h later for anti-FLAG ChIP and anti-FLAG Western blot to verify equivalent expression levels. The FLAG-SUMO-Scm fusion constructs were created by inserting PCR-generated fragments encoding Scm (WT or ΔSAM) into the NotI site of pPAC-FLAG-SUMO (ΔGG). The pPAC-FLAG-SUMO (ΔGG) vector was created by inserting a PCR product encoding the first 86 residues of SUMO followed by an NRLN linker into the KpnI site of pPAC-FLAG. For the RNAi ChIP experiment, the FLAG-Scm stable line was treated with 5 μg/well of GFP, Ulp1, or SUMO dsRNA, and 500 μM CuSO4 was added 4 days later. After 24 h of induction, the cells were harvested for anti-FLAG ChIP, anti-SUMO Western blot, and anti-FLAG Western blot. Anti-FLAG ChIP was performed as described previously (31).

Quantitative real-time PCR was performed with FastStart SYBR Green mix (Roche Applied Science) using a DNA Engine Opticon 2 system (MJ Research). ChIP experiments were performed in duplicate, and data were graphed with each bar representing the mean (± S.D.) (see Figs. 3 and 4). To determine the effect of SUMO or Ulp1 depletion on Ubx expression, S2 cells were treated with 5 μg of GFP, SUMO, or Ulp1 dsRNA for 5 days. Total RNA was extracted using TRIzol reagent according to the manufacturer’s instructions (Invitrogen). After residual genomic DNA was degraded using RQ1 RNase-free DNase (Promega), the RNA was re-extracted, reverse transcription was performed using the Omniscript RT kit (Qiagen), and transcript abundance was determined using real-time PCR with primers specific to Ubx or Actin 5C (Act 5C). The data were presented as -fold change in Ubx mRNA abundance normalized to the Act 5C internal control, relative to the GFP dsRNA-treated control sample.

Fly Crosses and Cuticle Analysis—Flies were maintained on standard medium at 25 °C, unless otherwise noted. Cuticle structures were imaged using a Zeiss Axioskop microscope. To generate random SUMO dsRNA-expressing clones, hs-FLPase; vgQ-LacZ2; actin > CD2 > Gal4 females were crossed with UAS-SUMORNAi/CyO, ActGFP (11) males, and first instar and second instar larval progeny were heat-shocked at 35 °C for 40 min. Tissue-specific SUMO RNAi was performed by crossing A9 (Gal4) females with UAS-SUMORNAi/CyO, ActGFP males. The adult halteres were dissected, mounted on a glass slide in a 1:1 mix of lactic acid/Hoyer’s medium, and allowed to clear by incubation at 50 °C overnight. Depletion of SUMO or Ulp1 RNA during embryogenesis was accomplished by crossing Arm-Gal4 females with UAS-SUMORNAi/CyO, ActGFP, or UAS-Ulp1RNAi (NIG-Fly) males. Dechorionated and devitellinized embryos were mounted in lactic acid/Hoyer’s medium, and embryonic cuticles were imaged using dark field optics (24).

RESULTS

Scm Is a SUMO Conjugation Target in Drosophila Embryos and S2 Cells—In a previous study, the C. elegans PcG protein SOP-2 was shown to be sumoylated (13). Given the similarity of Drosophila Scm to worm SOP-2, along with the observation that multiple components of the PcG group in Drosophila and humans are SUMO conjugation targets, we tested the possibility that Scm might also be a target for SUMO modification. Embryonic extracts were prepared in the presence or absence of the SUMO protease inhibitor NEM from a fly line expressing FLAG-tagged Scm under the control of the Scm promoter and regulatory region. In previous studies, this FLAG-Scm construct was found to fully rescue the Scm mutant phenotype (15). In samples prepared by anti-FLAG immunofluorescence enrichment of untreated embryo extracts, anti-Scm Western blots revealed a single band with an apparent molecular mass of ~100 kDa (Fig. 1A). This finding is consistent with the Scm predicted molecular mass of 94 kDa and previous Scm detection by Western blot (22). Extracts prepared in the presence of NEM contain an additional, slower migrating form of Scm that runs ~20 kDa larger (Fig. 1A). This 20-kDa shift is consistent with the observed change in SDS-PAGE migration of proteins singly modified by SUMO (27). An anti-SUMO Western blot detects
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A

anti-FLAG IP

NEM - +

kDa 148 -

98 -

western anti-Scm

anti-FLAG IP

NEM + +

SUMO-FLAG-Scm

FLAG-Scm

western: anti-Scm anti-SUMO

B

FLAG-Ulp1 - - - -

Ubc9/SUMO - - + + + + + +

FLAG-Scm - + + + + + +

kDa 150 -

100 -

SUMO-FLAG-Scm

FLAG-Scm

western: anti-Scm

C

dsRNA - - Ulp1 SUMO

FLAG-Scm - + + +

kDa 150 -

100 -

SUMO-FLAG-Scm

FLAG-Scm

western: anti-FLAG

D

anti-FLAG IP

dsRNA - - Ulp1 SUMO

FLAG-Scm - + + +

kDa 250 -

150 -

100 -

SUMO-FLAG-Scm

western: anti-SUMO

E

SUMO mix Scm - + + +

SUMO-Scm

Scm

F

FLAG-Scm - WT KR3

Ulp1 dsRNA - - + - +

kDa 100 -

SUMO-FLAG-Scm

FLAG-Scm

western: anti-FLAG
the higher molecular weight band, suggesting that this is a Scm-SUMO conjugate (Fig. 1A, right panel).

SUMOylation is a reversible process, and multiple SUMO proteases exist that can catalyze deconjugation (32). Drosophila Ulp1 is a SUMO protease that has been shown to promote the removal of SUMO from a range of targets including Dorsal and glutamyl-prolyl-tRNA synthetase (EPRS) (25). To test whether Scm is a target for Ulp1-mediated deconjugation, we transiently transfected S295U cells (S2 cells stably transfected with copper-inducible FLAG-SUMO and HA-Ubc9) with expression constructs encoding FLAG-Scm, with or without increasing amounts of FLAG-Ulp1 (Fig. 1B). Lysates evaluated by anti-Scm Western blot reveal a major band of unmodified FLAG-Scm and detect additional higher molecular weight bands that appear more abundant in samples overexpressing Ubc9 and SUMO. Mono-sumoylated Scm appears to be the most abundant form of modified Scm. However, longer exposures reveal bands that are consistent with di- and trisumoylated Scm (data not shown). Overexpression of FLAG-Ulp1 causes a dosage-dependent decrease in the accumulation of these upper bands, suggesting that Ulp1 is capable of deconjugating SUMO from Scm. To confirm this, we examined Scm sumoylation in S2 cells depleted of Ulp1 or SUMO by RNA interference (RNAi). The addition of Ulp1 dsRNA significantly increases the level of the FLAG-Scm-SUMO conjugate, whereas SUMO RNAi is effective in preventing its formation (Fig. 1C). An anti-FLAG immunoprecipitation followed by anti-SUMO Western blot reveals a discrete band at ~120 kDa likely to be mono-sumoylated Scm and additional high molecular weight sumoylated proteins (Fig. 1D). We have also demonstrated Scm sumoylation in vitro with a conjugation assay that includes 35S-labeled Scm and purified recombinant HA-SAE1, FLAG-SAE2, GST-Ubc9, and SUMO (Fig. 1E).

SUMO acceptor lysines usually fall within a consensus motif ΨKX(E/D) (Ψ is any hydrophobic amino acid, and X is any amino acid). We utilized SUMOsp, a web server-based sumoylation site prediction program (33), to identify potential SUMO attachment sites in Scm. Three high probability sites were identified, each matching the SUMO consensus motif, and these lysines are at positions 210 (MKLE), 574 (IKQE), and 624 (IKSE). Mutation of these sites individually did not significantly diminish Scm sumoylation (data now shown), and therefore we created a triple mutation (K210R/K574R/K624R) in which all three lysines were mutated to arginine to determine whether they collectively account for all or some of the sumoylated Scm that forms in cultured cells. Upon evaluation of Ulp1 RNAi-treated lysates, we found that the triple mutation significantly reduced formation of the SUMO conjugate (Fig. 1F). Comparison by immunoblot of dilution series (data not shown) of the extracts expressing wild-type Scm but not Scm lacking its SUMO domain, GST or GST-Ubc9 was expressed in E. coli and purified on glutathione-agarose beads. The immobilized proteins were incubated with in vitro-translated 35S-labeled luciferase (Luc), full-length Scm (WT), or Scm lacking its C-terminal SAM domain (ΔSAM), and after extensive washing, samples were analyzed by SDS-PAGE and autoradiography. B, the Scm SAM domain is essential for efficient sumoylation of Scm. S2 cells were transiently transfected with expression vectors encoding FLAG-Scm (WT) or FLAG-Scm (ΔSAM) in the presence or absence of Ulp1 dsRNA. Lysates were subjected to SDS-PAGE and assessed by anti-SUMO immunoblot.

**FIGURE 1.** Scm is a SUMO conjugation target in Drosophila embryos and S2 cells. A, detection of the Scm-SUMO conjugate in embryo extracts. FLAG-Scm-expressing embryo extracts were prepared in the presence or absence of the SUMO protease inhibitor NEM and subjected to anti-FLAG immunoblotting with anti-FLAG or anti-SUMO antibody, IP, immunoprecipitation. B, Scm is a target for Ulp1-mediated desumoylation. S295U cells (S2 cells stably transfected with expression constructs encoding HA-tagged Ubc9 and FLAG-tagged SUMO under a copper-inducible promoter) were left untreated or treated with CuSO4, and transiently transfected with a FLAG-Scm expression construct and increasing amounts of a FLAG-Ulp1 vector, as indicated. Lysates were analyzed by anti-Scm Western blot. C and D, perturbing sumoylation of Scm by Ulp1 or SUMO RNAi. FLAG-Scm was expressed in untreated S2 cells or cells that were depleted of Ulp1 or SUMO by RNAi, and lysates were analyzed by anti-FLAG Western blot (C) or immunoprecipitated with the FLAG antibody and analyzed by anti-SUMO Western blot (D). E, in vitro sumoylation of Scm. In vitro-translated 35S-labeled Scm was incubated in buffer alone or with purified recombinant HA-SAE1, FLAG-SAE2, GST-Ubc9, and mature SUMO (SUMO mix), and samples were analyzed by SDS-PAGE and autoradiography. F, Scm lysines 210, 574, and 624 may serve as SUMO attachment sites. S2 cells were transiently transfected with expression vectors encoding FLAG-Scm (WT) or with a triple lysine-to-arginine mutant (positions 210, 574, and 624 (K210R/K574R/K624R (KR3))) in the presence or absence of Ulp1 dsRNA. The sumoylation state of each Scm mutant was assessed by anti-FLAG Western blot.
were analyzed by SDS-PAGE and autoradiography. GST-Ubc9 shows a robust interaction with WT Scm but fails to significantly pull down Scm ΔSAM (Fig. 2A). This suggests that Ubc9 directly interacts with Scm via its SAM domain or, alternatively, that Ubc9 preferentially interacts with an oligomerized form of Scm. A direct Ubc9-SAM domain interaction appears unlikely given that Ubc9 fails to interact with the isolated Scm SAM domain in vitro (data not shown). Recognition of the target by the conjugating enzyme is known to be crucial for efficient SUMO modification of the target; thus, we sought to evaluate sumoylation of Scm ΔSAM in S2 cells. FLAG-Scm expression constructs encoding WT or ΔSAM Scm were transfected into S2 cells or in cells depleted of Ulp1 using RNAi. An anti-FLAG immunoblot reveals that the ΔSAM form of Scm is much less efficiently sumoylated than full-length Scm (Fig. 2B), arguing that the Scm-Ubc9 interaction is crucial for the efficient sumoylation of Scm in vivo.

**Sumoylation-resistant Forms of Scm Are More Efficiently Recruited to the Ubx Major PRE in Drosophila S2 Cells**—The proper binding of PcG proteins to the Ubx major PRE, called PRE$_D$ (Fig. 3A), is necessary to maintain silencing of Ubx (30). Recent studies have shown that Scm associates with this PRE in fly S2 cells and in larval imaginal discs (17, 34), along with sub-units of PRC1 and PRC2. Thus, we used ChIP to test whether alterations in Scm sumoylation status impact its chromatin binding at PRE$_D$. In the following ChIP experiments, PCR amplicons were produced that correspond to the regions illustrated in Fig. 3A. ChIP assays were performed on S2 cells using antibodies against Pc, Ph, and Scm, and PRE$_D$ interactions were initially evaluated by semiquantitative end point PCR. We find that Scm is significantly associated with this PRE region (Fig. 3A) with a distribution that mirrors the chromatin binding of Ph and Pc.

To extend these findings, we analyzed conjugation-defective Scm mutants and constitutively sumoylated mimic SUMO-Scm fusions by performing anti-FLAG ChIP and quantitative real-time PCR on S2 cells transiently transfected with the indicated FLAG-Scm expression constructs (Fig. 3, B–D). In comparison with the untransfected control cells, we find that wild-type FLAG-Scm is present at high levels at the PRE and that FLAG-ScmΔSAM is associated with the Ubx PRE at levels substantially lower than observed for wild-type Scm (Fig. 3B). This is consistent with previous studies showing that the Scm SAM domain is required for PcG-mediated repression (15, 22). To directly test the effect of Scm sumoylation on its recruitment to the Ubx major PRE, we performed anti-FLAG ChIP on S2 cells transiently expressing a conjugation-defective form of FLAG-Scm. Although forms of Scm containing a single lysine to arginine mutation are predicted to be SUMO attachment sites (K210R/K574R/K624R) (Fig. 3A), are more efficiently recruited to the Ubx major PRE (Fig. 3C).

In an attempt to mimic the SUMO-conjugated form of Scm, we fused SUMO (residues 1–86) to the N terminus of Scm and assessed PRE targeting by ChIP. Although it is unlikely that a SUMO-Scm fusion behaves identically to a true SUMO-Scm conjugate, the SUMO fusion technique has been used to accurately recapitulate conjugate activity in a number of cases (10, 35, 36). When FLAG-SUMO is fused to full-length Scm, a significant reduction in PRE occupancy is observed (as compared with FLAG-Scm WT) (Fig. 3D). Consistent with this finding, the low level of PRE recruitment seen with FLAG-Scm ΔSAM is further reduced by the fusion of SUMO to the Scm deletion mutant lacking the SAM domain.

**Global Changes in Sumoylation Alter Targeting of Scm to the PRE**—To further investigate the connection between SUMO and Scm, we evaluated the recruitment of Scm to the PRE after perturbing sumoylation by RNAi. A copper-inducible FLAG-Scm stable S2 cell line was treated with dsRNA against GFP, SUMO, or Ulp1, and the Scm sumoylation state was assessed by anti-FLAG Western blot (Fig. 4A). As expected, SUMO RNAi prevents formation of the Scm-SUMO conjugate, and depletion of Ulp1 increases Scm sumoylation. These RNAi conditions are known to cause global changes in the spectrum of SUMO conjugates (25). An anti-SUMO immunoblot shows that treating cells with SUMO dsRNA can effectively prevent conjugate formation and that Ulp1 RNAi increases the appearance of sumoylated proteins (Fig. 4B). These samples were analyzed by anti-FLAG ChIP, and depletion of SUMO was found to augment the Scm-PRE association, whereas Ulp1 RNAi may cause a slight reduction in the recruitment of Scm to the PRE (Fig. 4C). These findings suggest that sumoylation of Scm reduces the efficiency of its recruitment to the Ubx PRE.

**Reduced Sumoylation Leads to Diminished Ubx Expression and Can Induce Haltere-to-Wing Transformation Phenotypes**—Recruitment of Scm to PRE$_D$ is predicted to be a requirement in preventing the inappropriate expression of Ubx. Because our previous results suggested that sumoylation of Scm regulates its association with the Ubx major PRE, we chose to evaluate potential changes in Ubx expression levels following global perturbation of SUMO conjugation. S2 cells were transfected with dsRNA targeting GFP (control), SUMO, or Ulp1, and Ubx expression was evaluated by quantitative RT-PCR (Fig. 5A). As compared with the control, Ubx expression is significantly lower in cells that have reduced levels of SUMO. In contrast, enhancing sumoylation of Scm by knocking down Ulp1 results in higher than normal Ubx expression levels. Ubx is crucial for...
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A

\[ \text{dsRNA} \rightarrow \text{- GFP SUMO Ulp1} \]

\[ \text{FLAG-Scm} \]

\[ \text{kDa} \]

\[
\begin{align*}
150 & \quad 100 & \quad 75 \\
\text{SUMO-FLAG-Scm} & \quad \text{FLAG-Scm} \\
\text{western: anti-FLAG} & \quad \text{SUMO-FLAG-Scm} \\
\end{align*}
\]

B

\[ \text{dsRNA} \rightarrow \text{- GFP SUMO Ulp1} \]

\[ \text{kDa} \]

\[
\begin{align*}
250 & \quad 150 & \quad 100 & \quad 75 \\
\text{SUMO-FLAG-Scm} & \quad \text{FLAG-Scm} \\
\text{western: anti-SUMO} & \quad \text{SUMO-FLAG-Scm} \\
\end{align*}
\]

C

\[
\begin{align*}
\text{percent input} \\
\text{GFP dsRNA SUMO dsRNA Ulp1 dsRNA} \\
\end{align*}
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FIGURE 4. Globally perturbing sumoylation alters the association of Scm with the bxd PRE. A and B, SUMO and Ulp1 RNAi each significantly change the spectrum of sumoylated proteins including the conjugation state of Scm. FLAG-Scm-expressing cells were treated with GFP, SUMO, or Ulp1 dsRNA and then subjected to anti-FLAG and anti-SUMO Western blot. C, a global reduction in sumoylation augments the association of Scm with PRE. Cells were treated as in A. were analyzed by anti-FLAG ChIP (amplicons shown in Fig. 3A) (** = p < 0.05, *** = p < 0.10).

development of the haltere. A slight reduction in \textit{Ubx} expression, such as the \( \sim 2 \)-fold decline in \textit{Ubx} heterozygotes, produces varying mild haltere-to-wing transformation phenotypes consisting of one or more ectopic bristles and an increase in haltere volume. Stronger \textit{Ubx} loss of function is characterized by the appearance of ectopic wing tissue and can result in a complete haltere-to-wing transformation. To further evaluate the role of SUMO in regulating \textit{Ubx} expression, we generated SUMO RNAi clones during larval development to identify potential effects on adult haltere morphology. Consistent with an established role in numerous developmental processes, adults lacking SUMO in clonal patches exhibited a range of defects that included reduced wings and malformed legs (data not shown). Haltere transformations were also observed, ranging from the appearance of ectopic bristles to the significant outgrowth of wing tissue (Fig. 5B). To confirm this result, we used a Gal4 driver (A9-Gal4) to express SUMO dsRNA in the haltere. These halteres had multiple defects similar to those observed for \textit{Ubx}-null heterozygotes including ectopic bristle formation and increased size (Fig. 5C).

The absence of an individual PcG gene expressed during embryogenesis can produce a cuticle phenotype in which all denticle belts are transformed into copies of one segment identity. For example, when maternal and zygotic Scm is removed, all denticle belts are converted to that of the eighth abdominal segment (16). To evaluate the possibility that SUMO may regulate Scm function during embryogenesis, we assessed cuticle phenotypes from embryos expressing arm-Gal4-driven SUMO or Ulp1 dsRNA. Although wild-type cuticles exhibit eight distinct abdominal denticle bands, the SUMO- and Ulp1-depleted embryos display a range of defects that include missing and fused abdominal denticle bands (Fig. 5D). Interestingly, the defects observed in each RNAi background did not resemble the posteriorized cuticle phenotype typically seen in strong PcG mutants. Instead the RNAi phenotypes resemble those previously observed in mutant embryos doubly heterozygous for \textit{ph} and various other PcG alleles (including \textit{Scm}) (37). These phenotypes presumably reflect PcG control of segmentation genes, which has been revealed by both genetic and molecular studies (16, 37–40). The observed cuticle phenotypes are also consistent with the idea that multiple PcG proteins are regulated by sumoylation or act as effectors of the SUMO conjugation system.

DISCUSSION

Sumoylation Blocks Recruitment of Scm to the PRE and Repression of PcG Targets—In this study, we demonstrate that the Drosophila Polycomb group protein Scm is a SUMO conjugation target, and our results suggest that sumoylation is an important regulator of Scm function. In particular, we observe that a form of Scm lacking the major SUMO acceptor sites is more efficiently recruited to the \textit{Ubx} major PRE than is wild-type Scm (Fig. 3C). The simplest interpretation of this finding is that sumoylation of Scm blocks its function and that eliminating the SUMO acceptor lysines therefore enhances Scm-mediated silencing. However, it is possible that mutating either the conjugation sites or the covalent attachment of SUMO to these sites both result in the reduced interaction of Scm with a negative regulator of Scm function, as has been suggested for transcription factor conjugation substrates that contain sumoylation sites within a synergy control motif (24). To differentiate between these two possibilities, we assessed PRE targeting of SUMO-Scm fusions and the effects of globally perturbing sumoylation \textit{in vivo} by depleting the SUMO protease Ulp1 or SUMO itself using RNA interference. SUMO-Scm fusions have a reduced capacity to accumulate at PRE, (Fig. 3D). Inhibiting sumoylation of Scm augments its PRE association, whereas increasing the formation of the SUMO-Scm conjugate decreases the amount of PRE-bound Scm (Fig. 4C). These RNAi-induced changes in the PRE association of Scm directly correlate in the expected manner with changes in Scm-mediated repression of \textit{Ubx} in cultured cells (Fig. 5A). In addition, adult phenotypic analysis reveals that conditions of decreased sumoylation can produce homeotic transformations consistent
with increased PcG silencing (Fig. 5, B and C). Taken together, these results argue that sumoylation of Scm inhibits the ability of Scm to repress its target genes.

Only one of the three identified Scm sumoylation sites resides within a characterized functional domain; lysine 210 is within the first of two methyl-lysine-binding MBT repeats. Previous studies have shown that mutations in the MBT repeats that inhibit methyl-lysine binding also prevent Scm from effectively repressing Ubx expression in wing imaginal discs (19). We speculate that the attachment of SUMO to this lysine residue may interfere with methyl-lysine binding activity and thus prevent Scm from repressing its targets. Another possible explanation for the inhibitory effect of SUMO on Scm is that attachment of the bulky SUMO moiety could prevent functionally important interactions with other PcG components, including PRC1.

The Scm SAM domain appears to play multiple roles in regulating Scm function. It has been shown that artificially tethered forms of Scm lacking the SAM domain lose the ability to repress reporter genes, suggesting a direct role for the SAM domain in transcriptional silencing independent of a role in PRE targeting (41). Here we find that the Scm SAM domain participates in binding the SUMO-conjugating enzyme Ubc9 and is required for efficient Scm sumoylation (Fig. 2, A and B). Although our overall findings suggest that preventing sumoylation of Scm should augment its association with PREs, we instead found that a form of Scm lacking its SAM domain shows reduced PRE binding (Fig. 3B). This result could reflect additional roles of the SAM domain, aside from its contribution to sumoylation, such as promoting Scm oligomerization, interactions with the PRC1 subunit Ph, or interactions with other components in PcG silencing (15, 20, 21).

Multiple Connections between SUMO and PcG/TrxG Function—Many connections between the SUMO pathway and the PcG/TrxG proteins are evident. The human homolog of Pc (hPc2 or Cbx4) functions as an E3 SUMO ligase for CtBP, HIPK2, CCCTC-binding factor (CTCF), and cystathionine β-synthase (CBS) (9, 42). Furthermore, the PcG protein Pho and the TrxG protein Osa were both recently identified in a proteomic screen to identify novel Drosophila SUMO conjugation targets (11). The human PRC2 components SUZ12 and EZH2 can be modified by SUMO (43). MEL-18, a human homolog of the PRC1 component Psc, functions as an anti-E3 SUMO ligase by inhibiting sumoylation of HSF2 and RanGAP1 (44).

Perhaps most interestingly with respect to the findings reported here, the SP-RING finger domain containing TrxG protein Tonalli (Tna) is a predicted SUMO ligase, and tna mutations result in multiple homeotic transformation phenotypes including partial haltere-to-wing transformations indicative of reduced Ubx activity (45). We observe similar haltere-to-wing transformations when SUMO levels are reduced in the developing haltere using RNAi (Fig. 5, B and C). Both results may be caused, in part, by loss of Scm sumoylation and thus a

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**FIGURE 5.** Global changes in sumoylation cause altered expression of PcG target genes and produce homeotic transformation phenotypes. A, in cultured Drosophila cells, sumoylation inhibits repression of Ubx. S2 cells were treated with GFP, SUMO, or Ulp1 dsRNA, and Ubx expression levels were determined by quantitative RT-PCR. The Act 5C transcript was used to normalize Ubx expression levels, and the -fold change was determined by comparison with the GFP control. B, haltere-to-wing transformation phenotypes occur when SUMO RNAi clones are produced during development. Random clones that produce SUMO dsRNA were induced during larval development, and halteres with extensive outgrowth of wing tissue were dissected for microscopic analysis. C, mild haltere-to-wing phenotypes are produced when A9-Gal4 drives expression of SUMO dsRNA in the haltere. A9-driven SUMO RNAi results in halteres that are larger than normal and have one or more ectopic bristles. D, arm-Gal4-driven knockdown of either Ulp1 or SUMO produces embryonic segmentation defects. Ulp1 or SUMO dsRNA was expressed in the developing embryo through the action of the arm-Gal4 driver, and the embryonic cuticles showed similar fused or missing denticle bands.
SUMO-modified Scm inhibits PcG-mediated repression.

SUMO Blocks Scm-mediated Repression

In summary, we have identified Scm as a SUMO conjugation target and show that sumoylation is an important regulator of PcG-mediated repression. We determine that sumoylation of Scm decreases repression of its target genes, at least in part by inhibiting an association with PREs. Our analysis supports the idea that the Scm SAM domain contributes to Scm recruitment to the PRE in addition to possibly serving as a docking platform for Ubc9, which allows efficient sumoylation of Scm. Given the apparent inhibitory effects of sumoylation on Scm function, we speculate that changing the accessibility of the Scm SAM domain to its binding partners may play a role in regulating PRE recruitment and repression. Additional regulatory roles for SUMO in regulating homeotic gene expression are likely to be observed in future studies given the large number of PcG/TrxG proteins that are either sumoylation targets or effectors of SUMO modification.

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