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RESEARCH ARTICLE

Overexpression of SUMO perturbs the growth and development of *Caenorhabditis elegans*

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Abstract Small ubiquitin-related modifiers (SUMOs) are important regulator proteins. *Caenorhabditis elegans* contains a single SUMO ortholog, SMO-1, necessary for the reproduction of *C. elegans*. In this study, we constructed transgenic *C. elegans* strains expressing human SUMO-1 under the control of pan-neuronal (*aex-3*) or pan-muscular (*myo-4*) promoter and SUMO-2 under the control of *myo-4* promoter. Interestingly, muscular overexpression of SUMO-1 or -2 resulted in morphological changes of the posterior part of the nematode. Movement, reproduction and aging of *C. elegans* were perturbed by the overexpression of SUMO-1 or -2. Genome-wide expression analyses revealed that several genes encoding components of SUMOylation pathway and ubiquitin-proteasome system were upregulated in SUMO-overexpressing nematodes. Since muscular overexpression of SMO-1 also brought up reproductive and mobility perturbations, our results imply that the phenotypes were largely due to an excess of SUMO, suggesting that a tight control of SUMO levels is important for the normal development of multicellular organisms.

Keywords SUMO (small ubiquitin-related modifier) · Ubiquitin · Cell stress · Monoubiquitylated histone 2B · Development · Gene expression · Muscle · *C. elegans*

Abbreviations

SUMO	Small ubiquitin-like modifier
SMO	*Caenorhabditis elegans* SUMO
UBC9	SUMO E2 conjugase
H2Bub1	Monoubiquitylated histone 2B

Introduction

Small ubiquitin-like modifiers (SUMOs) are important post-translational modifiers involved in the regulation of various processes in eukaryotic cells. The SUMOs are ~100-amino-acids-long proteins that can be covalently attached to specific lysines often, but not always, found within a minimal consensus motif ψKxE (Ψ is a large hydrophobic residue, x is any residue) [1, 2]. Mammals ubiquitously express SUMO-1, SUMO-2, and SUMO-3, which can be conjugated to proteins. SUMO-4 seems to have a limited expression profile, and it is currently unclear whether it can be conjugated to proteins in vivo [3, 4]. The conjugated form of SUMO-1 is only ~50% identical with that of SUMO-2 or -3, whereas SUMO-2 and -3 are nearly (97%) identical. The SUMOs share a similar three-dimensional structure with the ubiquitin, but their amino acid sequences and surface charge distributions differ considerably. In contrast to ubiquitin, SUMO-1 does not form polymers. Even though SUMO-2 and -3 are capable

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of mediating polymer formation, none of the SUMO isoforms in itself directly targets substrate proteins for proteasomal degradation [5]. However, recently identified SUMO-targeted ubiquitin ligases (STUbLs), such as RNF4, are capable of detecting poly-SUMO chain-modified proteins and mediating their ubiquitylation, leading to proteasomal degradation [6]. The STUbLs recognize poly-SUMO chains through their SUMO-interacting motifs (SIMs) that bind SUMOs in a non-covalent fashion [7, 8]. The SIMs, that are composed of short hydrophobic patches often flanked by acidic residues, are not restricted to the STUbLs, but are also found in several other proteins involved in the SUMOylation process as well as in other regulatory, especially nuclear, proteins [9].

SUMOylation is a well-conserved and essential regulatory process in eukaryotes. SUMOs are first activated in an ATP-dependent fashion by the SAE-1 and -2 dimer and subsequently conjugated by UBC9. PIAS (protein inhibitor of activated STAT) proteins and nucleoporin RanBP2 are established SUMO E3 ligases, but also some other proteins, such as polycomb protein 2 (PC2) and topoisomerase I-binding, arginine/serine-rich (TOPORS), are able to enhance SUMOylation in an E3 activity-like manner [10]. SUMOylation is not a static modification, but it is dynamically adjusted by forward and reverse reactions. The reverse reaction is catalyzed by the isopeptidase activity of a family of SUMO-specific proteases (SENP1–3, 5–7) [11, 12]. The endopeptidase activity of SENPs is also responsible for removing the short C-terminal extension (2–11 amino acids) from immature pro-SUMOs, which exposes the C-terminal Gly–Gly motif for the isopeptide bond formation. SUMOylation can regulate the target protein's function by altering its subcellular or subnuclear localization or its interaction with other proteins [1, 13].

Caenorhabditis elegans has a single SUMO gene encoding SMO-1. Interestingly, the SMO-1 lacks the N-terminal consensus site that mediates the polymerization of mammalian SUMO-2/3. Over 200 SUMO target candidates were recently identified in *C. elegans* using a proteomics approach [14]. However, the functional consequences of the modification have been addressed only on a few SUMO targets in *C. elegans* [14–17]. *C. elegans* harbors several ubiquitin-like proteases (ULP-1–5) that seem to correspond to mammalian SENPs. In contrast, only one E3 ligase, GEI-17, has thus far been identified in *C. elegans* [18]. The *smo-1* is essential for the survival of the nematode [19]. Homozygous deletion of the *smo-1* results in sterility with severe abnormalities in the gonad, germ line, and vulva. In addition to vulval effects, loss of *ubc-9* function results in abnormal development of the pharyngeal muscle in nematodes [20]. Similar to the nematode, *Smt3*, the single SUMO ortholog of *Drosophila melanogaster* is necessary for normal development [21]. In mice, disruption of *Ubc9* results in embryonic lethality with dramatic problems in the nuclear architecture and chromosome structure [22]. On the other hand, mere removal of the *Sumo-1* in mice does not lead to any major defects [23]. The lack of apparent phenotype is most likely due to compensation by the *Sumo-2* and *-3* genes. This notion is supported by recent data from zebrafish showing that SUMO paralogs are dispensable but functionally redundant during early development of the organism [24]. Conversely, injection of mRNA encoding Sumo1 or Ubc9 to zebrafish embryos, however, did not interfere with their early development [25].

In this study, we have investigated how overexpression of SUMO-1 or -2 influences the physiology of *C. elegans*. Our original aim was to create transgenic nematode strains for isolation of SUMO-modified muscular and neuronal proteins in vivo. Surprisingly, however, overexpression of SUMO-1 or -2 in the nematode dramatically perturbed its growth and development. Since muscular overexpression of SMO-1 also resulted in reproductive and mobility perturbations, our results imply that a long-lasting imbalance in SUMO conjugation system can disturb the development and homeostasis of a multicellular organism.

Materials and methods

Strains

Caenorhabditis elegans strains were maintained in standard conditions. The wild-type *C. elegans* used in this study is Bristol N2 strain from which all the different transgenic strains originate from *dat-1::*GFP reporter construct that expresses Green Fluorescent Protein under the control of dopamine transporter (*dat-1*) promoter used as a selection marker in transgenic animals. The reporter construct was provided by Drs. R. Nass and R. Blakely. The *smo-1* knockout strain VC186 (*smo-1*(*ok359*)/szT1[*lon-2*(*e678*)]) [19] was provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). The constructs *aex-3*::SUMO-1, *myo-4*::SUMO-1 and *myo-4*::SUMO-2 were integrated into the *C. elegans* genome. Integration of extrachromosomal arrays was achieved by X-ray irradiation followed by selection as described [26]. Following selection, integrated strains were outcrossed ten times. The integrated lines used were Is62[*aex-3*::His-SUMO-1; *dat-1*::GFP], Is63[*myo-4*::His-SUMO-1; *dat-1*::GFP], and Is65[*myo-4*::His-HA-SUMO-2; *dat-1*::GFP]. The extrachromosomal arrays used for the studies of conjugation negative SUMOs were Ex84[*aex-3*::His-SUMO-1GA; *dat-1*::GFP], Ex85[*aex-3*::His-HA-SUMO-2GA; *dat-1*::GFP], Ex86[*myo-4*::His-SUMO-1GA; *dat-1*::GFP], and Ex87[*myo-4*::His-HA-SUMO-2GA; *dat-1*::GFP] (as controls Ex62[*aex-3*::His-SUMO-1; *dat-1*::

GFP], Ex63[*myo-4*::His-SUMO-1; *dat-1*::GFP], Ex64[*aex3*::His-HA-SUMO-2; *dat-1*::GFP] and Ex65[*myo-4*::His-HA-SUMO-2; *dat-1*::GFP]). In addition, Ex95[*myo-4*::Myc-SMO-1; *dat-1*::GFP] was used. The extrachromosomal arrays for rescue experiments were VC186 strain Ex89 [*smo-1*::His-HA-SUMO-2; *dat-1*::GFP] and Ex88[*smo-1*::His-SUMO-1; *dat-1*::GFP]. The transgene was injected into *smo-1* heterozygotes and GFP-positive progeny were selected, cloned, and F2 generations then tested by PCR for homozygosity.

DNA constructs

Human SUMO-1 (immature) fused to an N-terminal hexahistidine tag and SUMO-2 (mature) containing N-terminal hexahistidine and hemagglutinin (HA) tag, or their conjugation-deficient forms SUMO-1-GA, SUMO-2-GA [27], and Myc-SMO-1 were cloned into pPD49.26 vector with the *aex-3* or *myo-4* promoter (*aex-3*::His-SUMO-1, *aex-3*::His-HA-SUMO-2, *myo-4*::His-SUMO-1, *myo-4*::His-HA-SUMO-2, *myo-4*::Myc-SMO-1). The promoter of *smo-1* (ID K12C11.2) was obtained from Geneservice [28], and subsequently cloned into pPD49.26 vector to control the expression of mammalian SUMO-1 and SUMO-2. The nematode *smo-1* was cloned from N2 cDNA into mammalian expression vector pCMV-Myc (Clontech). pCMV-Myc-SUMO-1 and pCMV-Myc-SUMO-2 (both expressing mature SUMO forms with the C-terminally exposed diglycines) were from D. Owerbach.

Phenotypic characterization

Egg-laying

Young adult animals with a row of visible eggs were selected and three animals per plate were transferred to fresh NGM agar. Worms were allowed to lay eggs for 4 h and then removed. Experiments were performed with ten replicated plates for each strain. Eggs were counted under transmitted light microscopy.

Movement

L4 animals were selected onto new NGM agar plates with a fresh lawn of *E. coli*. After 30 s for acclimatization, the number of times the worm's head crossed its body axis within 30 s was counted as a body bend. Data were collected from 30 worms.

Aging

L4 worms from each strain were selected onto new NGM agar plates containing 60 μg/ml of FuDR; ten worms per plate (five plates per strain). During the assay, worms were kept in a 20°C and counted every second day by gentle prodding with a platinum wire. Worms that did not respond were considered dead. The wild-type strain N2 served as a control in these experiments.

Microscopy and brood size

Differential-interface contrast (Nomarski) microscopy was used to visualize the phenotype. Nematodes were mounted on M9-buffer to pads of 2% agarose containing 0.2% sodium azide to inhibit movement. The differential-interface contrast images were obtained using a DMRB (Leica) microscope. Brood size for rescue experiments were conducted by transferring single animals, beginning at the L4 stage, to new plates for five consecutive days. Viable progeny were measured 24–48 h following egg-laying.

Immunoblotting, Ni^{2+}-NTA pull-down, and immunoprecipitation

Synchronization of worms was prepared as described previously [29]. Nematodes were collected with M9-buffer containing 20 mM *N*-ethylmaleimide (NEM). For the enrichment of histidine-tagged proteins, the pelleted worms were lysed in 1 ml of Buffer A (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris–Cl, 0.05% Tween-20, pH 8) containing 5 mM imidazole. After a freeze–thaw, they were incubated at room temperature for 15 min in rotation. To enhance the lysis, the samples were sonicated 2 × 20 s. After removal of the cell debris, Ni^{2+}-NTA magnetic agarose beads (Qiagen) were added and incubated for 1 h at room temperature. The beads were washed in the following sequence: 0.5 ml of Buffer A, 1 ml of Buffer B (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.05% Tween-20, pH 6.3), 0.5 ml Buffer A, 1 ml of 1:1 Buffer A:Buffer C (50 mM Na_2HPO_4/NaH_2PO_4, 100 mM NaCl, 20% glycerol, 0.2% Nonidet P-40, 0.05% Tween-20, pH 8), 1 ml 1:3 Buffer A:Buffer C, 1 ml of Buffer C, 0.5 ml of Buffer C containing 20 mM imidazole. The Ni-NTA-isolated proteins were eluted in 2× SDS sample buffer and incubated for 1 min; H_2O and β-mercaptoethanol were added, and the mixture was heated for 5 min at 95°C. For direct immunoblotting, the worm pellets were suspended in SDS sample buffer containing 10 mM NEM with 1:200 protease inhibitor mixture (PIC; Sigma-Aldrich) and lysed by sonication 2 × 10 s. Samples were heated for 5 min at 95°C and separated on 15% SDS-PAGE or NuPAGE 4–12% Bis–Tris gradient gels (Invitrogen). Proteins were transferred onto nitrocellulose membranes and visualized by indicated primary antibody and horseradish peroxidase-conjugated secondary antibody using the enhanced chemiluminescence detection reagents

according to the manufacturer's instructions (Pierce). For samples analyzed with a Li-COR Odyssey infrared imaging system (LI-COR), DyLight™ 680 and DyLight™ 800 fluorescent dye-conjugated secondary antibodies were used according to the manufacturer's instructions. Conditions for SUMOylation assays in COS-1 cells have been described [30]. Primary antibodies used in this study: mouse monoclonal anti-SUMO-1 (GMP-1) (33-2400) from Zymed Laboratories (Invitrogen), mouse monoclonal anti-SUMO-2/3 (M114-3) from MBL International, mouse monoclonal anti-tubulin (T6199) from Sigma-Aldrich, mouse monoclonal anti-HA.11 (MMS-101) from Nordic Biosite, rabbit polyclonal anti-histone H2B (07-371) from Upstate, mouse monoclonal anti-ubiquitylated histone H2B (MM-0029) from Médimabs, rabbit polyclonal anti-histone H3 (ab1791) from Abcam, rabbit anti-serum against androgen receptor [31], mouse monoclonal c-Myc (9E10; sc-40), mouse monoclonal anti-ubiquitin (P4D1; sc-8017), and mouse monoclonal anti-tubulin (B-7; sc-5286) (for detection of mammalian tubulin) from Santa Cruz Biotechnology.

Microarray analysis

Synchronized L1 *C. elegans* were collected with M9-buffer, and excess bacteria were washed with M9-buffer. Total RNA for microarray was isolated by RiboPure (Ambion). Total RNA was labeled and hybridized using One Cycle cDNA synthesis followed by Biotin RNA synthesis (Affymetrix, Palo Alto, CA, USA). Affymetrix *C. elegans* genome arrays in three independent biological replicates per strain were hybridized, washed, and scanned according to the manufacturer's recommendations at the Biomedicum Biochip Center (Helsinki, Finland). Analysis of microarray data was performed using R statistical software version 2.11 [32] with associated libraries from Bioconductor project version 2.6 [33]. Data were normalized using the Robust Multiarray Average (RMA) method [34]. Normalized data were filtered in order to remove probe sets with small variance across samples (50% of probe sets with the least variance). Probe sets that were not linked to any known or predicted *C. elegans* gene were also filtered out. The Linear Models for Microarray Data (Limma) package [35] using linear model fitting for statistical testing with empirical Bayes variance smoothing procedure were applied to detection of differentially expressed genes in *aex-3*::His-SUMO-1 and *myo-4*::His-SUMO-1 nematodes when compared to N2. The obtained p values were corrected for multiple testing using Benjamini and Hochberg false discovery rate procedure [36]. For downstream analysis, genes with FDR corrected $p < 0.001$ were used including 1,805 probe sets from *aex-3*::His-SUMO-1 versus N2 comparison (899 under- and 906 overexpressed) and 1,342 probe sets from *myo-4*::His-SUMO-1 versus N2 comparison (774 under and 568 overexpressed). Enrichment analysis was performed for differentially expressed genes in *aex-3*::His-SUMO-1 and *myo-4*::His-SUMO-1 using the DAVID Functional Annotation Chart tool [37, 38]. In DAVID, Gene Ontology (GO) biological process terms from December 2009 [39] at the level 5 of GO hierarchy were tested for enrichment. The complete dataset, including raw data files, has been deposited to the Gene expression omnibus (GEO) database public repository (accession number GSE19972).

RT-quantitative PCR

Synchronized L1 *C. elegans* were collected with M9-buffer, and excess bacteria were washed with M9-buffer. Total RNA was isolated and purified using TRIZOL® Reagent (Invitrogen) and converted to cDNA using Transcriptor First Strand cDNA Synthesis kit (Roche) following the manufacturer's instructions. The synthesized cDNA was quantified in real-time PCR using Mx3000P (Stratagene), with FastStart SYBR Green Master (Roche) and specific primers for *ubc-9* (5′-GAA TTG CTG CAG GAC GCC TC and 5′-CTT CTG GAC GAG TTC GGC AG), *ulp-5* (5′-GCT CAC TCC AAA ATG CGA AGC and 5′-CTT GAC AAT CGT CTA CTC TTC AC), *smo-1* (5′-GTT TCA GAG ACT CCC GCT ATA AAC and 5′-GAG ACA GAG AAA CCG AGT ATC TC), *hda-3* (5′-GTC CAC ATT CAC AGA TCA AAT GGC and 5′-CAT TGG AGC ACA ACT GCT TCT GG), *hmg-4* (5′-GTT GAA GAT GTA GGT CAT TTG GCG and 5′-GAA TCT CAT CTC CAT GAG CTG AAC), *met-2* (5′-CAA CTC TCG TGC AAT AAT GGA GAC and 5′-GAA GCA ACT TCA TAA CCT GGT TCA C), *nhr-180* (5′-CTT CTA CTG AGA TAT CAC ATA AAA C and 5′-GTG ATT CAG GCA CGT GGC AAC), *nhr-35* (5′-GGA ACA TAC AAC GGT GAC AGC and 5′-GTT GCT GTT CGG TCT GTG TGG), *SUMO-1* (5′-CTG ACC AGG AGG CAA AAC CTT C and 5′-CTT CAA TCA CAT CTT CTT CCT CC), and *SUMO-2* (5′-CCA AGG AAG GAG TCA AGA CTG AG and 5′-CCG TCT GCT GTT GGA ACA CAT C). For normalization of the mRNA levels between samples, *act-1* (5′-TCG GTA TGG GAC AGA AGG AC and 5′-CAT CCC AGT TGG TGA CGA TA) was used. Fold changes were calculated using the 2-(ΔΔCt) method [40].

Protein modeling

For comparative modeling of SMO-1, the NMR structure of SUMO-1 (PDB code 1A5R, model 1) [41] and the X-ray structure of SUMO-2 determined at 1.6-Å resolution (1WM2) [42] were used as templates. In the model building, the rotamers of the substituted side chains were built

and minimized using the Prime module of the Maestro program [43]. Due to the high sequence similarities between SMO-1 and the templates, model building was straightforward and expected to provide models suitable for comparison of surface properties of the proteins. Electrostatic potentials were calculated using DelPhi [44] and protein surfaces were colored according to the calculated potentials using the Pymol program [45].

Results

Expression of SUMO-1 and -2 in *C. elegans*

Mammalian SUMO-1 was integrated into the *C. elegans* genome under the control of pan-neuronal (*aex-3*) or pan-muscular (*myo-4,* also known as *unc-54*) promoter and SUMO-2 was integrated under the control of *myo-4* promoter (Fig. 1a). Expression of the SUMOs was verified by immunoblotting using SUMO-1 and SUMO-2/3-specific antibodies (Fig. 1b). The pan-neuronal expression of SUMO-1 was weaker than that in the muscles. The difference in the expression level of SUMO-1 between the neuronal cells and the muscular cells may derive from a weaker transcriptional activity of the pan-neuronal promoter driving the expression. The histidine tags of SUMOs enabled isolation of both free and SUMO-conjugated proteins from the transgenic nematodes using Ni^{2+}-NTA chromatography. Immunoblotting of the Ni^{2+}-NTA-enriched proteins with SUMO-specific antibodies revealed in addition to abundant free SUMO, a ladder of SUMO-modified proteins of which the amount in relation to the amount of free SUMO was more pronounced in the SUMO-1-expressing nematodes than in the SUMO-2-expressing *C. elegans* (Fig. 1c). In all transgenic *C. elegans* strains, the amount of the unconjugated SUMO exceeded that of the SUMOs conjugated to any single protein species. Since there are no reliable antibodies against the SMO-1 protein, we compared the levels of overexpressed SUMOs to SMO-1 indirectly by analyzing their mRNA levels using RT-QPCR (Fig. 1d). Interestingly, the level of endogenous SMO-1 mRNA was increased in the transgenic *C. elegans* strains. However, the expression levels of SUMO-1 and -2 mRNAs were markedly stronger than that of SMO-1.

To further evaluate the functionality of different SUMOs across species boundaries, the SMO-1 cDNA was cloned into a mammalian expression vector containing a c-Myc tag (pCMV-Myc) and expressed in parallel with Myc-tagged SUMO-1 and SUMO-2 in COS-1 cells. Immunoblotting of the cell extracts with anti-Myc antibody showed that the level and the pattern of conjugation of SMO-1 to mammalian proteins resemble more those of SUMO-2 than SUMO-1 (Fig. 1e). A well-characterized SUMO target androgen receptor was also modified by SMO-1 in COS-1 cells in a fashion that was comparable to that with SUMO-2. These results indicate that the SMO-1 can conjugate to proteins in mammalian cells and conversely SUMO-1 and -2 in *C. elegans*.

Surface properties of SMO-1 protein

The sequence identity between SMO-1 and SUMO-1 is 62.8% and that between SMO-1 and SUMO-2 46.2% for the sequences of the protein main body shown in Fig. 2. UBC-9 is somewhat better conserved than the SMO-1, being 72% identical with the human E2 conjugase. Even though the primary sequence of SMO-1 is more similar to that of mammalian SUMO-1 than SUMO-2, the above comparison of these proteins' conjugation properties in mammalian cells suggests that other molecular properties of SMO-1 and SUMO-2 might be more alike. To test this notion, we built molecular surface models for SMO-1 based on the existing three-dimensional structure of SUMO-1 or that of SUMO-2. In keeping with the close relationship between these two mammalian SUMOs and the SMO-1, the surface charge distributions of all three proteins are highly alike and similarly unevenly distributed (Fig. 2). Interestingly. however, the surface potential of SMO-1 appears to more closely resemble that of SUMO-2 than SUMO-1.

SUMO-1 and SUMO-2 are able to partially rescue the lack of SMO-1 in reproduction

Disruption of the *smo-1* in *C. elegans* is deleterious. However, homozygous worms survive, but they have an abnormal vulva with reproductive problems [19]. We wanted to test if the mammalian SUMOs can substitute the lack of *smo-1* and hence rescue the phenotype. To that end, human SUMO-1 and SUMO-2 were linked to the *smo-1* gene promoter and the constructs were microinjected to the *smo-1* knock-out strain VC186. Positive transgenic animals were then cloned and F2 progeny were tested for *smo-1* mutant hetero- or homozygosity by PCR. SUMO-1 and SUMO-2 transgenic animals with a *smo-1* null mutant background were viable and produced progeny. Brood size assay determined that SUMO-1-expressing animals in a *smo-1* null mutant background could produce nearly the same number of progeny as the N2 wild-type animals (226 ± 72, $n = 14$ vs 256 ± 64, $n = 5$; mean $\pm$ SD). However, the rescue was not completely penetrant as there were also transgenic positive sterile notched animals. SUMO-2 transgenic animals produced close to half the progeny as N2 wild-type (97 ± 40 vs 236 ± 74; mean $\pm$ SD, $n = 15$), while non transgenic *smo-1* null mutant

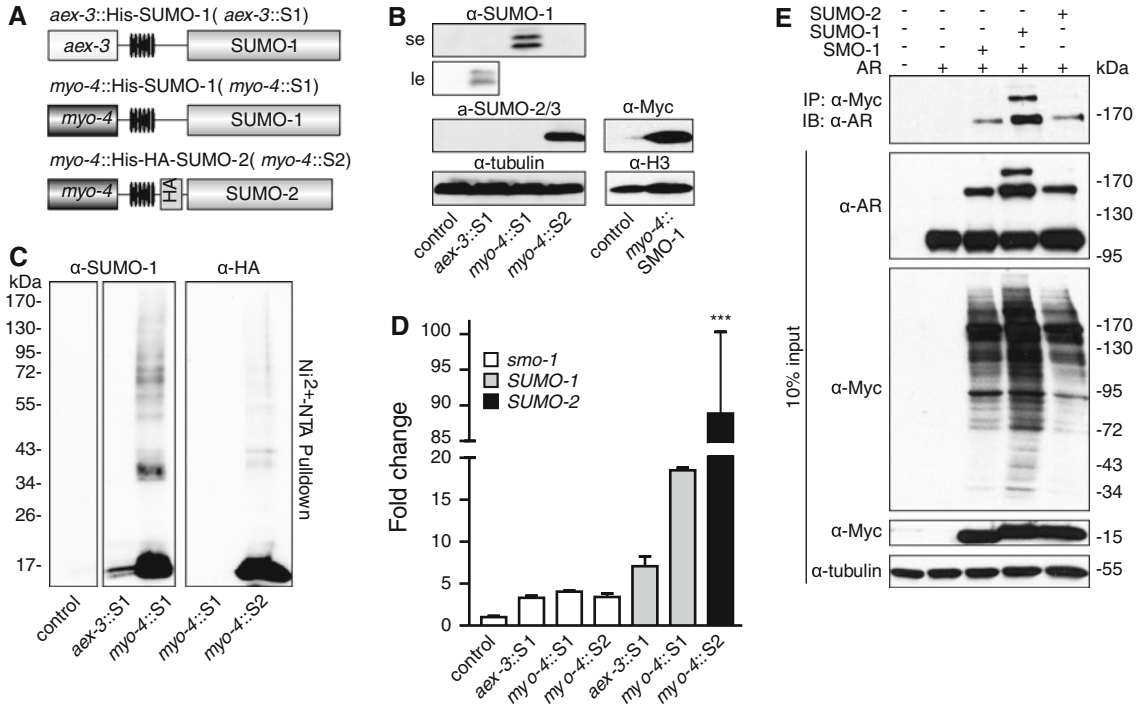

Fig. 1 Mammalian and *C. elegans* SUMOs are essentially compatible. **a** Structure of the constructs used in this study. Histidine-tagged SUMO-1 is expressed with a pan-neuronal (*aex-3*) and a pan-muscular (*myo-4*) promoter. Hexahistidine- and hemagglutinin-tagged SUMO-2 was expressed with the pan-muscular *myo-4* promoter. The constructs were cloned into pPD49.26 and subsequently integrated into the *C. elegans* genome. **b** The expression levels of the proteins were checked by immunoblotting. *C. elegans* were lysed and the SUMO levels were analyzed using anti-SUMO-1, anti-SUMO-2/3, and anti-c-Myc antibodies. Immunoblotting with anti-tubulin and anti-histone H3 were used as loading controls. A short (*se*) and a longer exposure (*le*) of the anti-SUMO-1 immunoblot are shown to visualize the expression of SUMO-1 in the *aex-3*::His-SUMO-1 nematodes. The reason for the doublet of immunoreactive bands in the SUMO-1-expressing nematodes is not known, but it is likely to derive from a post-synthetic modification in SUMO-1. **c** The mammalian SUMO is conjugated to *C. elegans* proteins. Nematodes were collected, lysed and a magnetic Ni^{2+}-NTA resin was used to enrich the histidine-tagged proteins. The proteins were then detected with anti-SUMO-1 (*aex-3*::His-SUMO-1, *myo-4*::His-SUMO-1) and anti-HA (*myo-4*::His-HA-SUMO-2, *myo-4*::His-SUMO-1 as a control) antibodies. **d** The relative mRNA levels of *smo-1*, *SUMO-1* and *SUMO-2*. The levels of mRNA were quantified by RT-QPCR as described in "Materials and methods". The amount of *smo-1* mRNA in control *dat-1*::GFP *C. elegans* was set to 1. For statistical analyses, a one-way analysis of variance followed by a Tukey's post test was used. $***p < 0.001$. **e** The SMO-1 is conjugated to mammalian targets. COS-1 cells were transfected with pCMV-Myc-SMO-1, -SUMO-1 or -SUMO-2 together with pcDNA-FLAG-AR (androgen receptor) as indicated. Twenty-four hours after transfection, cells were treated with 100 nM of testosterone. Cell lysates were immunoprecipitated (*IP*) with anti-c-Myc antibody and immunoblotted (*IB*) with anti-AR antibody. The input samples were immunoblotted with anti-c-Myc (*upper panel* 7.5% SDS-PAGE for detection of SUMO-conjugates; *lower panel* 15% SDS-PAGE for detection of free SUMO), anti-AR, and anti-tubulin antibodies as indicated

animals were completely sterile (0 progeny, $n = 15$). The progeny calculations only included rescued animals. The SUMO-1-rescued animals appeared superficially normal, while SUMO-2-rescued animals had a somewhat abnormal vulva displayed as a small notch (M. Lakso, unpublished observations), but this did not prevent egg-laying and progeny were hatched outside the mother. These results provide further evidence for the functionality and complementarity of the mammalian SUMO in *C. elegans*.

An excess of SUMO is harmful to the growth of *C. elegans*

Inspection of the nematodes revealed that muscular expression of either SUMO-1 or -2 resulted in a morphological change in the posterior part of the *C. elegans*, whereas the neuronal expression of SUMO-1 did not affect the morphology. The *myo-4*::SUMO-1 and -2 nematodes had a thicker and shorter tail part compared to the *dat-1*::GFP control nematodes (Fig. 3a). In line with the malformation of the tails, movement of the *myo-4*::SUMO-1 and -2 nematodes was significantly impaired (Fig. 3c). Also, the egg-laying capacity was diminished in the *C. elegans* expressing SUMOs in their muscles (Fig. 3b). A similar, but less pronounced, tendency was seen with the *aex-3*::SUMO-1 nematodes. Moreover, all SUMO-1 or -2 transgenic nematodes had shortened life spans in the order *aex-3*::SUMO-1 > *myo-4*::SUMO-1 > *myo-4*::SUMO-2. Notably, the half-life of *myo-4*::SUMO-2 *C. elegans* was only ~50% of that of the control nematode. In general,

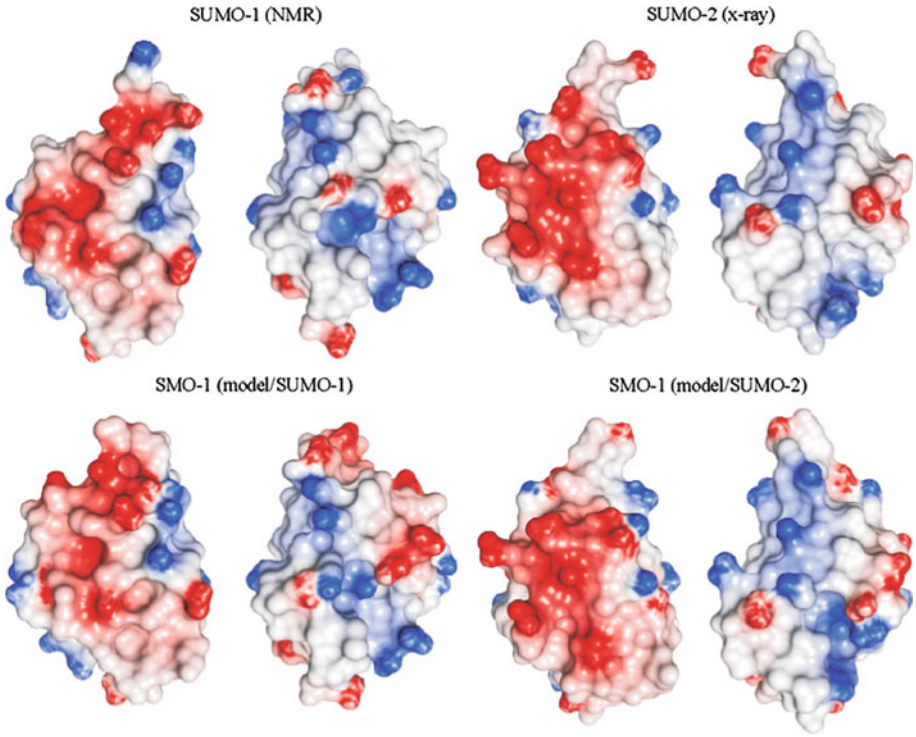

Fig. 2 Surface properties of SMO-1, SUMO-1 and SUMO-2. The molecular surfaces of the NMR structure of SUMO-1 [*SUMO1 (NMR)*], crystal structure of SUMO-2 *[SUMO-2 (X-ray)*], and *SMO-1 models* build using SUMO1 (NMR) or SUMO-2 (X-ray) as a template. Surfaces are colored according to the electrostatic potential with a range from −8 kT (*red*, negatively charged) to 8 kT (*blue*, positively charged). Two opposite surface views are shown for each protein generated by 180° rotation about the vertical axis

these transgenic nematodes expressing SUMO-1 or -2 in their muscles grew and reproduced more slowly than the *aex-3*::SUMO-1 transgenic or the control *C. elegans*. The observed phenotypes were not simply a result of the expression of foreign SUMO proteins in the worms, since overexpression of the nematode's own SMO-1 using an extrachromosomal *myo-4*::SMO-1 array resulted in a similar phenotype. The egg-laying and movement were affected with the increased amount of SMO-1 in muscles (Fig. 3b, c). The tail abnormality was also similar to the worms with human SUMO (own unpublished observations). It is of note that this is not a general feature of transgenic nematodes expressing heterologous proteins in their muscles, as for example expression of human adenosine receptors (A1, A2a, A2b, or A3) or cystatin B under the control of *myo-4* promoter does not result in the above mentioned phenotypic changes (our unpublished observations). Moreover, we also obtained an integrated transgenic animal overexpressing β-galactosidase (PJ1145) from the Caenorhabditis Genetics Center and we did not observe any morphological changes in these animals.

To differentiate whether the phenotype was due to the high amount of free, unconjugated SUMOs or abnormal conjugation of *C. elegans* proteins by SUMOs, we examined how the conjugation negative SUMO-1GA and SUMO-2GA, where the glycine needed for conjugation is converted to alanine, affected the nematodes. The expression levels of the conjugation negative SUMO forms were comparable to those of their wild-type counterparts (M. Rytinki and J. J. Palvimo, unpublished observations). The morphological changes of nematodes expressing SUMO-1GA or -2GA in their muscles were similar to those with the conjugation-competent SUMOs, albeit these changes occurred much less frequently. The penetrance of the phenotype was assessed from the extrachromosomal arrays by calculating the percentage of worms with the tail phenotype of all the worms having the positive selection marker: 68% (*myo-4*::SUMO-1) and 66% (*myo-4*::SUMO-2) had the abnormal tail morphology, whereas only 6% of both of the conjugation-negative (*myo-4*::SUMO-1GA, *myo-4*::SUMO-2GA) SUMO-expressing worms had the abnormal tail phenotype. These results indicate that the changes in the SUMO-1 and -2 transgenic nematodes are largely, but not entirely, due to an excess of free, unconjugated SUMO in the nematodes.

Augmented expression of ubiquitin and SUMO pathway genes in SUMO-overexpressing nematodes

To investigate the consequences of SUMO-1 overexpression on gene expression in *C. elegans*, RNA was isolated from L1 larvae of the *aex-3*::SUMO-1 and *myo-4*::SUMO-1 strains and genome-wide expression analyses were performed using Affymetrix *C. elegans* genome arrays. Interestingly, analysis of the gene expression data revealed that a large group of genes of which expression was significantly ($p < 0.001$) altered in the transgenic nematodes encoded proteins involved in the nematode development,

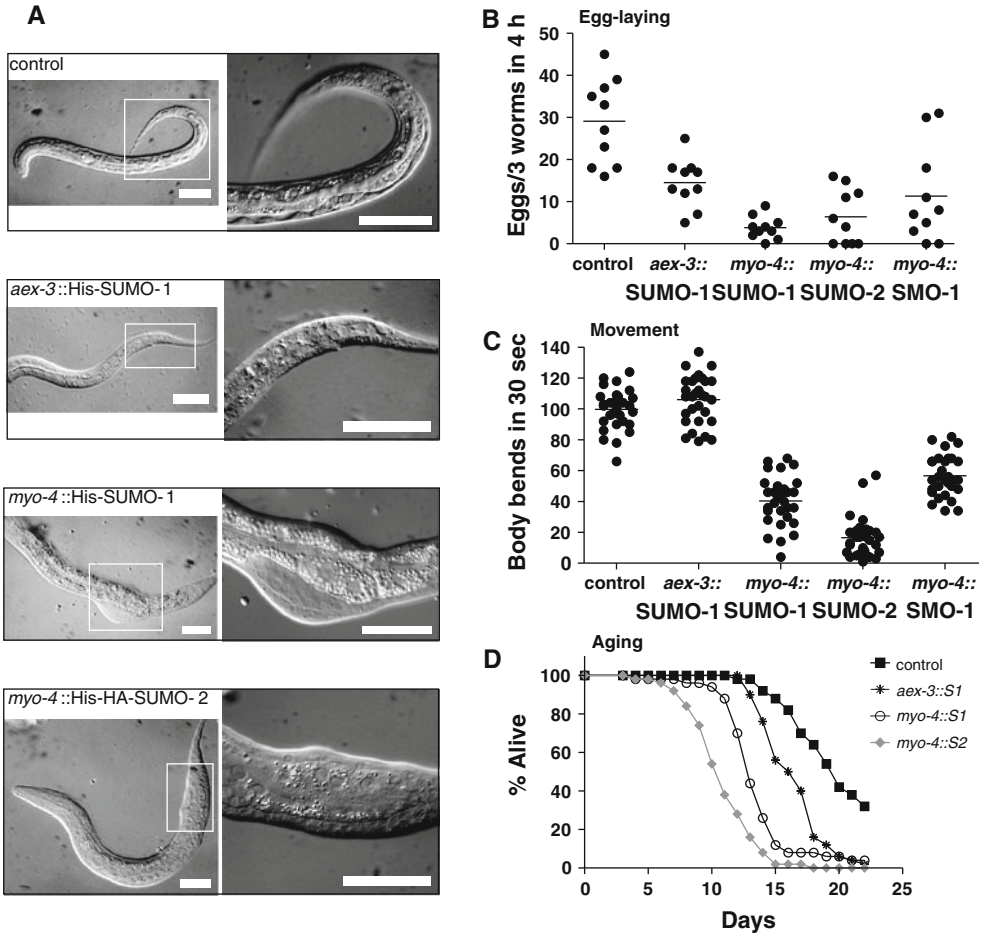

Fig. 3 Overexpression of SUMO-1 or -2 in muscles causes phenotypic changes in *C. elegans*. **a** SUMO-overexpression results in weakening and malformation of the muscles. The transgenic *C. elegans* were visualized by differential-interface contrast (Nomarski) microscope. Images were taken of young (L1/L2 stage) *C. elegans*. (*Scale bar* 10 μM.) **b** Egg-laying deficiency in SUMO expressing *C. elegans*. Every *dot* in the scatter plot represents the number of eggs from three worms laid within 4 h. For statistical analyses, a one-way analysis of variance followed by a Tukey's post test was used. The egg-laying capacity of the intrachromosomal arrays *aex-3*::SUMO-1 ($p < 0.01$), *myo-4*::SUMO-1 ($p < 0.001$), and *myo-4*::SUMO-2 ($p < 0.001$), and that of the extrachromosomal array *myo-4*::SMO-1 ($p < 0.001$) were significantly different from the control worms. **c** Movement is reduced in the *C. elegans* expressing SUMOs in muscles. Every *dot* in the scatter blot represents the number of body bends made by one worm in 30 s. The movement of the intrachromosomal arrays *myo-4*::SUMO-1, *myo-4*::SUMO-2, and that of the extrachromosomal array *myo-4*::SMO-1 were significantly ($p < 0.001$) different from the control worms. **d** Life span of SUMO-expressing *C. elegans* is reduced. The percentage of live *C. elegans* plotted versus time in days. The half-lives of *aex-3*::SUMO-1, *myo-4*::SUMO-1 and of *myo-4*::SUMO-2 were significantly ($p < 0.001$) different from the control worms

regulation of cell cycle, DNA/RNA metabolism and chromosome structure (Fig. 4a). For example, many nuclear pore complex proteins, some non-histone chromatin protein genes, such as *hmg-12*, and centromere protein-related genes *hcp-3* and *-4* were up-regulated (Table 1). In addition, a cluster of genes encoding components of ubiquitin-proteasome pathway, and interestingly, those of SUMOylation pathway were up-regulated in the transgenic worms (Table 2). Interestingly, the list of down-regulated genes is enriched in nuclear hormone receptor genes that encode transcription factors (Table 3). The latter list also contains collagen and intermediate filament genes. We validated by RT-QPCR analysis the up-regulation of the SUMO pathway genes, *ubc-9, ulp-5* and *smo-1*, and that of the histone/chromatin-associated genes, *hda-3, hmg-4* and *met-2*, and confirmed the down-regulation of nuclear hormone receptor genes *nhr-180* and *nhr-35* (Fig. 4b).

The up-regulated ubiquitylation system could represent a general cell stress-related phenomenon [46], but, for example, the well-known stress responsive gene *hsp-16.2* was not found to be significantly up-regulated [47, 48]. On the other hand, *dna-9*, *dnaj-11* and *dnaj-23* that encode HSP40/DNaJ family members were up-regulated both in

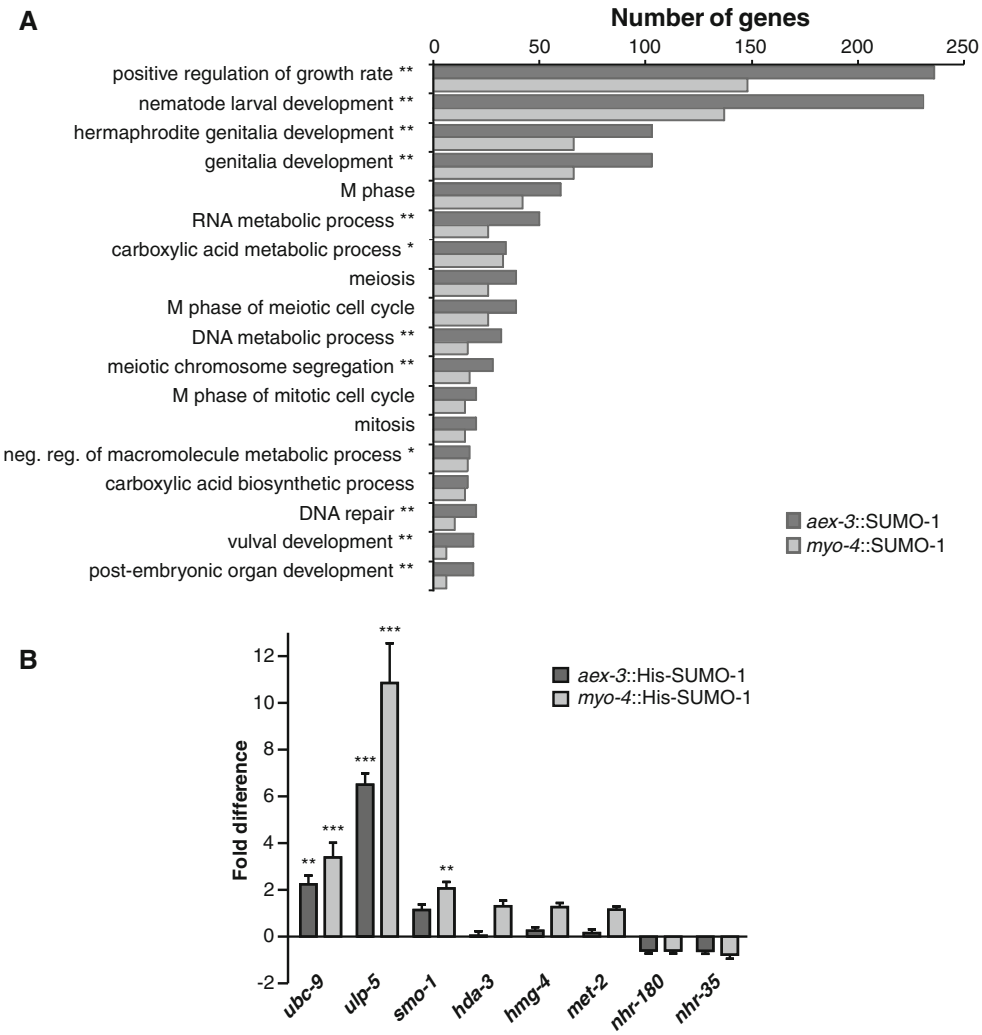

Fig. 4 Genome-wide analysis of gene expression in transgenic *C. elegans* strains. **a** *Bars* represent numbers or genes that fall into each statistically significant (Benjamini-Hochberg FDR corrected $p < 0.01$) GO term obtained from the enrichment analysis of differentially expressed genes in *aex-3*::His-SUMO-1 and *myo-4*::His-SUMO-1 versus N2 (Benjamini-Hochberg corrected $p < 0.01$; GO terms with ≥15 genes shown). Terms with *(*aex-3*) or **(*myo-4*) were statistically significant for only one of the two nematode strains with the limits used. **b** Confirmation of the microarray data for *ubc-9*, *ulp-5*, *smo-1*, *hda-3*, *hmg-4*, *met-2*, *nhr-180* and *nhr-35* by RT-QPCR. The fold differences in the expression of the selected genes in *aex-3*::His-SUMO-1 and *myo-4*::His-SUMO-1 strains are shown in relation to their expression in *dat-1*::GFP *C. elegans* and presented as the mean ± SD values from three samples. For statistical analyses, a two-way analysis of variance followed by a Bonferroni's post test was used. ***$p < 0.001$; **$p < 0.01$

aex-3::His-SUMO-1 and *myo-4*::His-SUMO-1 strains ($p < 0.001$). These proteins act as co-chaperones binding to specific target proteins and transporting them to HSP70 proteins [49]. SUMO overexpression thus seems to lead to an increase in the need of co-chaperones, but it does not induce a massive stress response in these nematodes [50].

Stress-induced protein ubiquitylation in the transgenic nematodes

We next analyzed whether the cellular protein ubiquitylation is altered in the transgenic *C. elegans* by immunoblotting analysis using an anti-ubiquitin antibody that also recognizes the *C. elegans* ubiquitin. The overall protein ubiquitylation patterns did not dramatically differ between the control and the transgenic strains, albeit there were minor differences between the groups (Fig. 5a, b). The effect of cell stress on the protein ubiquitylation was next evaluated by exposing the worms to 34°C for 2 h [48]. Heat treatment induced a marked increase in the ubiquitylated protein species in *C. elegans*, which was less pronounced in the *myo-4*::SUMO-1 and -2 nematodes than in the control or *aex-3*::SUMO-1 nematodes.

Ring finger protein 1 (*rfp-1*) expression was increased in the SUMO transgenic nematodes (Table 2). Its yeast and human orthologs, BRE1A/B and RNF20/40, respectively, act as E3 ligases in the ubiquitylation of histone H2B at lysine 120 (K123 in yeast) [51–54]. Since the amino acid region ubiquitylated in mammalian H2B is conserved in *C. elegans*, it was possible to use a monoclonal antibody specific for the monoubiquitylated H2B (H2Bub1) for *C. elegans* samples. Immunoblotting analyses interestingly revealed that the level of H2Bub1 in relation to the total H2B was modestly increased in the SUMO-overexpressing worms (Fig. 5c, d). We also checked the effect of heat stress on the H2Bub1. In contrast to the increase in the amount of high-molecular-mass ubiquitylated protein species (Fig. 5a), heat treatment dramatically diminished the amount of H2Bub1 in *C. elegans*. However, a similar relative decrease in the level of H2Bub1 was seen both in the

Table 1 Selection of nucleus- and chromatin-associated genes showing >1.5-fold up-regulated expression ($p < 0.001$) both in *aex-3*::His-SUMO-1 and *myo-4*::His-SUMO-1 *C. elegans*

Gene	Description	Human ortholog	*aex-3*::His-SUMO-1		*myo-4*::His-SUMO-1	
			p value	Fold change	*p* value	Fold change
npp-5	Nuclear pore complex protein	*NUP107*	0.0001	1.972	0.0006	1.719
sgo-1	chromosome segregation protein homolog	–	0.0001	1.889	0.0004	1.772
hcp-3	Centromere protein (CENP)-A homolog	–	0.0001	2.003	0.0006	1.769
set-32	SET (trithorax/polycomb) domain containing	*SETDB1*	0.0002	1.757	0.0003	1.731
set-17	SET (trithorax/polycomb) domain containing	*PRDM9*	0.0003	1.682	0.0009	1.624
csc-1	Chromosome segregation and cytokinesis defective	–	0.0003	1.682	0.0008	1.593
npp-2	Nuclear pore complex protein	*NUP85*	0.0003	1.663	0.0009	1.565
npp-19	Nuclear pore complex protein	*NUP53*	0.0003	1.724	0.0006	1.673
npp-17	Nuclear pore complex protein	*RAE1*	0.0004	1.670	0.0004	1.693
met-2	histone methyltransferase-like	*SETDB1*	0.0005	1.614	0.0007	1.622
hcp-4	Centromere protein (CENP)-C homolog	*NEFH*	0.0006	1.651	0.0005	1.631
set-14	SET (trithorax/polycomb) domain containing	*SMYD3*	0.0007	1.580	0.0004	1.646
hmg-12	Structure-specific DNA recognition protein	*HMGA2*	0.0009	1.619	0.0005	1.734

Table 2 Selection of SUMO and ubiquitin/proteasome pathway genes showing >1.5-fold up-regulated expression ($p < 0.001$) both in *aex-3*::His-SUMO-1 and *myo-4*::His-SUMO-1 *C. elegans*

Gene	Description	Human ortholog	*aex-3*::His-SUMO-1		*myo-4*::His-SUMO-1	
			p value	Fold change	*p* value	Fold change
skr-15	Ubiquitin ligase complex component	*SKP1*	0.0001	2.559	0.0006	1.919
skr-7	Ubiquitin ligase complex component	*SKP1*	0.0001	2.183	0.0001	1.897
ubc-9	SUMO conjugating enzyme	*UBC-9*	0.0002	1.931	0.0002	1.865
smo-1	SUMO (ubiquitin-related) homolog	*SUMO*	0.0002	1.848	0.0007	1.712
ulp-5	Ubiquitin-like protease	*SENP7*	0.0002	1.920	0.0004	1.773
pas-5	Proteasome alpha subunit	*PSMA5*	0.0002	1.816	0.0006	1.671
rfp-1	Ring finger protein, UBC associated	*RNF20/40*	0.0003	1.718	0.0003	1.721
gei-17	GEX interacting protein	*PIAS1*	0.0005	1.854	0.0010	1.772
ulp-1	Ubiquitin-like protease	*SENP1*	0.0006	1.694	0.0001	1.876
duo-1	Ubiquitin specific protease	*USP29*	0.0006	1.585	0.0010	1.600
ufd-3	Ubiquitin fusion degradation	*PLA2P*	0.0009	1.531	0.0010	1.532

SUMO-expressing and the control nematodes. Since heat stress has previously been shown to attenuate H2B ubiquitylation merely in cultured human tumor cells [55], our data from a multicellular organism lend strong support to the notion that the phenomenon is biologically relevant.

Discussion

In this study, we have described *C. elegans* models expressing human SUMO-1 and SUMO-2 under the control of pan-neuronal/-muscular promoters. The overexpression of SUMO resulted in a phenotypic change in the nematode. The SUMOs were shown to be capable of conjugating to target proteins in *C. elegans*, and, conversely, the nematode SMO-1 was functional in mammalian cells. Furthermore, in the rescue experiments, the *smo-1* knockout phenotype was partially saved by the addition of mammalian SUMO-1 or SUMO-2 under the control of the *smo-1* promoter. The muscularly expressed SUMOs caused the posterior part of the nematode to be abnormal. The tail part was thicker and shorter than that of the wild-type nematode. On top of the phenotypic changes, the movement, lifespan and reproduction of the nematode were also negatively affected. All these observations are connected to the function of muscles. The down-regulated collagen and intermediate filament gene expression may have contributed to these phenotypes. Weak muscles may affect the egg-laying and movement, also secondarily affecting the eating and defecation processes. For the *myo-4*::SUMO-2, the phenotype was more

Table 3 Selection of genes showing <0.5-fold down-regulated expression ($p < 0.001$) both in *aex-3*::His-SUMO-1 or *myo-4*::His-SUMO-1 *C. elegans*

Gene	Description	Human ortholog	*aex-3*::His-SUMO-1		*myo-4*::His-SUMO-1	
			p value	Fold change	*p* value	Fold change
col-48	Collagen	*COL9A2*	0.0001	0.301	0.0001	0.478
col-120	Collagen	*COL10A1*	0.0001	0.296	0.0001	0.426
col-175	Collagen	*COL23A1*	0.0001	0.387	0.0001	0.440
ifd-1	Intermediate filament, D	*LMNA*	0.0001	0.412	0.0001	0.497
ifc-1	Intermediate filament, C	*KRT3*	0.0001	0.353	0.0002	0.406
ifb-2	Intermediate filament, B	*LMNA*	0.0001	0.419	0.0001	0.444
nhr-99	Nuclear hormone receptor family	*RARB*	0.0001	0.308	0.0001	0.323
nhr-35	Nuclear hormone receptor family	*HNF4G*	0.0001	0.392	0.0001	0.384
nhr-55	Nuclear hormone receptor family	*NR2C2*	0.0001	0.492	0.0004	0.554
cki-1	CKI family (cyclin-dependent kinase inhibitor)	*CDKN1B*	0.0001	0.408	0.0001	0.447
fkh-9	ForKHead transcription factor family	*FOXL1*	0.0001	0.445	0.0001	0.429
fkh-7	ForKHead transcription factor family	*FOXP4*	0.0001	0.444	0.0001	0.432
nhr-122	Nuclear hormone receptor family	*HNF4A*	0.0002	0.493	0.0001	0.448
nhr-64	Nuclear hormone receptor family	*HNF4A*	0.0002	0.481	0.0001	0.435
nhr-180	Nuclear hormone receptor family	*NR1I3*	0.0002	0.508	0.0001	0.457
nhr-42	Nuclear hormone receptor family	*ESRRG*	0.0002	0.435	0.0001	0.364
ets-4	ETS class transcription factor	*SPDEF*	0.0002	0.523	0.0001	0.493
nhr-134	Nuclear hormone receptor family	*PPARG*	0.0003	0.518	0.0003	0.492
nhr-106	Nuclear hormone receptor family	*NR1I3*	0.0005	0.478	0.0003	0.488
nhr-18	Nuclear hormone receptor family	*NR2C2*	0.0005	0.383	0.0001	0.272
nhr-32	Nuclear hormone receptor family	*HNF4A*	0.0008	0.494	0.0003	0.441

severe than for the other transgenic worms. These nematodes appeared to have spasms in the muscles. SUMOylation was also recently shown to regulate the assembly and function of cytoplasmic intermediate filament protein IFB-1 in *C. elegans* [14]. The IFB-1 is necessary for the normal development and attachment of muscles to the cuticle. Moreover, the levels of SUMOs are decreased during myogenesis of mammalian C2C12 myoblasts; also, the localization and amount of Ubc9 is important for the normal development of the myoblasts [56]. It is also possible that the structural weakening of the muscles affects the development and proper localization/shape of other organs, leading to additional problems in these worms. Taken together, these data suggest that the SUMOylation pathway has an important role in the normal development of muscles. SUMO pathway may also participate in the function of the neuromuscular junctions.

SUMOylation, especially by SUMO-2, has been connected to cellular stress responses [57, 58]. However, the overexpression of human SUMOs in the nematode does not appear to simply induce an extensive proteotoxic stress, as the stress responsive *hsp-16* genes, for instance *hsp-16.2*, were not up-regulated in the *myo-4*::SUMO-1 and -2 nematodes [47, 48]. On the other hand, some *HSP40/DNaJ* genes were up-regulated, suggesting an increased demand of co-chaperones in the transgenic nematodes. Our gene expression studies nevertheless revealed that the overexpression of SUMO up-regulates both the SUMOylation and the ubiquitin-proteasome pathway genes. Although, the overall pattern of ubiquitylated proteins in the SUMO-overexpressing nematodes did not markedly differ from that in the control worms, at the level of H2B monoubiquitylation, SUMO-overexpressing nematodes showed increased ubiquitylation. The latter result may at least in part be due to increased expression of *rfp-1*, an ortholog of the human H2B ubiquitin E3 ligase RNF20/40 [53, 54], in the transgenic nematodes. Moreover, our results, showing to the best of our knowledge for the first time in the context of a whole organism that the H2Bub1 responds to cell stress, support the physiological relevance of similar data from cell culture studies [55]. In mammalian cells, H2Bub1 has recently been associated with transcribed regions of highly expressed genes [59]. The observed dramatic decrease in the H2Bub1 in response to cell stress may thus be linked to general attenuation of transcription under adverse growth conditions. The H2Bub1 is also a prerequisite for histone H3 methylation at K4 and K79 [60]. In keeping with the increased H2Bub1 levels, the H3K4

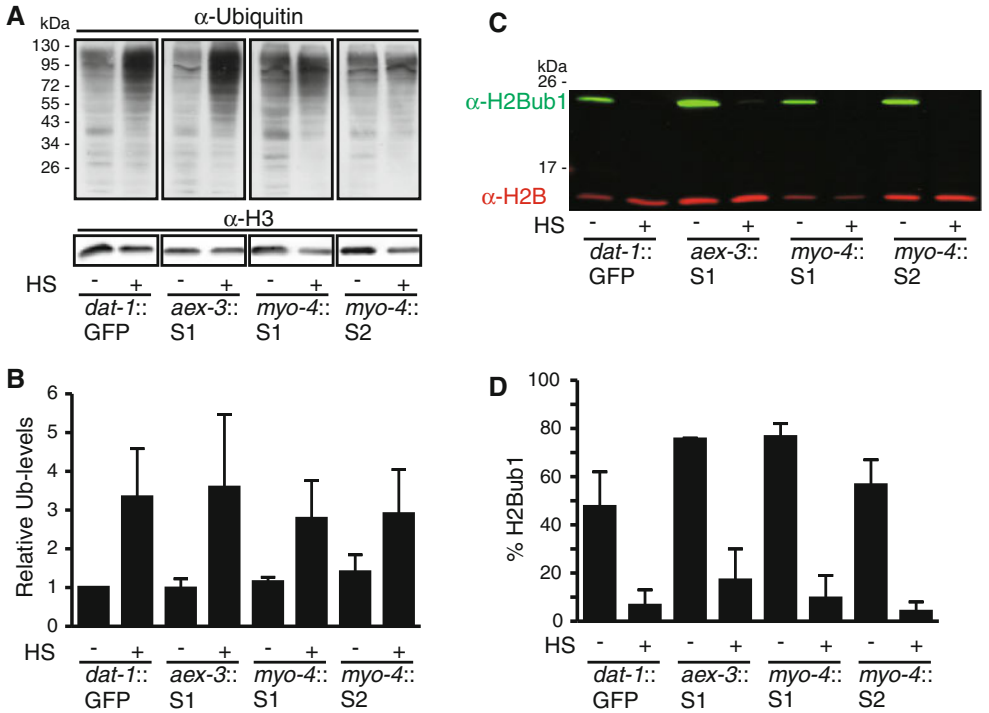

Fig. 5 Effect of heat stress on protein ubiquitylation in transgenic *C. elegans* strains. **a** Protein lysates from the indicated *C. elegans* strains were separated on SDS-PAGE and immunoblotted with anti-ubiquitin and anti-histone H3 antibodies. Heat stress samples were exposed to 34°C for 2 h prior to sample preparation. A representative immunoblot is shown. **b** Quantification of the ladder of ubiquitylated proteins relative to histone H3 levels is shown. Data are presented as the mean ± SD values from three samples. For statistical analyses, a two-way analysis of variance followed by a Bonferroni's post test was used; $p > 0.05$. **c** L4 stage larvae were treated as in (**a**), and the samples were immunoblotted with anti-ubiquitylated H2B (H2Bub1) and anti-H2B antibodies. A representative immunoblot is shown. **d** Immunoreactive H2Bub1 and H2B bands were quantified by Odyssey infrared imaging system, and the percentages of H2Bub1 of the total H2B in different *C. elegans* strains are shown. Data are presented as the mean ± SD values from two samples. For statistical analyses, a two-way analysis of variance followed by a Bonferroni's post test was used; $p > 0.05$

trimethylation levels were also elevated in the SUMO transgenic nematodes (M. Rytinki and J. J. Palvimo, unpublished observation).

Interestingly, the posterior part of the *C. elegans* seems to be affected by the different modifications on the SUMO/ubiquitin conjugation pathway. Jones et al. [61] reported recently that knocking-out various ubiquitin-protein conjugases, including *ubc-9*, in *C. elegans* results in a variety of tail abnormalities. However, these phenotypes are not identical with the ones seen in our SUMO-overexpressing nematodes. The common feature in these and our studies is that the level of free versus conjugated SUMO and/or ubiquitin is potentially altered. The higher level of free versus protein-conjugated SUMO in the *myo-4*::SUMO-2 than in *myo-4*::SUMO-1 was consistent with the more severe phenotype in the SUMO-2-expressing *C. elegans*, suggesting that an excess of SUMO was contributing to the phenotype in these nematodes. It is also interesting that the surface properties of SMO-1 and SUMO-2 are highly similar to each other and that the amino acids critical for the non-covalent association with the SUMO-interacting motifs are conserved in the SMO-1 [41, 62, 63]. Therefore, the mechanism by which increased levels of SUMOs could perturb the cellular homeostasis may involve squelching or sequestration-based blockage of the function of components of ubiquitin or other ubiquitin-like conjugation pathways. Taken together, these data suggest that the SUMOylation pathway is interconnected to ubiquitin pathways in the regulation of versatile eukaryotic processes. SUMO levels need to be properly regulated for the normal development of multicellular organisms, such as *C. elegans*.

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