

Methods in
Molecular Biology 1475

Springer Protocols



Manuel S. Rodriguez *Editor*

SUMO

Methods and Protocols

 Humana Press

Tools to Study SUMO Conjugation in *Caenorhabditis elegans*

Federico Pelisch and Ronald T. Hay

Abstract

The cell biology of sumoylation has mostly been studied using transformed cultured cells and yeast. In recent years, genetic analysis has demonstrated important roles for sumoylation in the biology of *C. elegans*. Here, we expand the existing set of tools making it possible to address the role of sumoylation in the nematode *C. elegans* using a combination of genetics, imaging, and biochemistry. Most importantly, the dynamics of SUMO conjugation and deconjugation can be followed very precisely both in space and time within living worms. Additionally, the biochemistry of SUMO conjugation and deconjugation can be addressed using recombinant purified components of the *C. elegans* sumoylation machinery, including E3 ligases and SUMO proteases. These tools and reagents will be useful to gain insights into the biological role of SUMO in the context of a multicellular organism.

Key words SUMO, *Caenorhabditis elegans*, Live imaging, Cell division, Chromosomes

1 Introduction

Small ubiquitin-related modifier (SUMO) conjugation is essential for development in mammals [1, 2] and in the nematode *Caenorhabditis elegans* (*C. elegans*) [3–6]. SUMO is conjugated to substrate proteins through the action of an E1-activating enzyme (the Sae1/Sae2 heterodimer in humans), an E2-conjugating enzyme (Ubc9 in humans), and SUMO-specific E3 ligases [7, 8]. The most studied type of SUMO E3 ligase is the SP-RING E3 ligase family, which includes PIAS proteins in vertebrates and their yeast homologs Siz1 and Siz2 [9, 10] and the related protein *mms21* [11]. Regulation is also achieved at the level of desumoylation by SUMO-specific isopeptidases: SENP1, 2, 3, 5, 6, and 7 in vertebrates [12, 13]. While mammals contain three different SUMO proteins [7], in *C. elegans* there is one SUMO ortholog, SMO-1 (hereafter CeSUMO). The Ubc9 ortholog is UBC-9, while the PIAS and *mms21* orthologs are GEI-17 [14] and ZK1248.11.1 (hereafter

Table 1
Comparison of sumoylation pathway components between the yeast *Saccharomyces cerevisiae*, human, and the nematode *C. elegans*

| Protein | <i>Saccharomyces cerevisiae</i> | <i>Homo sapiens</i> | <i>Caenorhabditis elegans</i> |
|-------------------------|---------------------------------|---------------------|-------------------------------|
| Modifier | Smt3p | SUMO1, 2, 3 | SMO-1 (CeSUMO) |
| E1 (activating enzyme) | Aos1p/Uba2p | SAE1/SAE2 | AOS-1/UBA-2 |
| E2 (conjugating enzyme) | Ubc9p | UBC9 | UBC-9 |
| SP-RING SUMO ligases | Siz1p, Siz2p | PIAS1, 2, 3, 4 | GEI-17 |
| | Mms21p | NSE2 | MMS-21 |
| SUMO proteases | Ulp1p | SEN1 | ULP-1 |
| | | SEN2 | |
| | | SEN3 and SEN5 | ULP-5 |
| | | SEN6 and SEN7 | ULP-2 and ULP-4 |
| | Ulp2p | | |

MMS-21), respectively (Table 1). Four SUMO proteases (ubiquitin-like proteases, ULPs), ULP-1, ULP-2, ULP-4, and ULP-5, have been recognized in *C. elegans* (Table 1) while ULP-3 is the putative Nedd8 protease (NEDP1) ortholog. SUMO has been shown to play many roles in *C. elegans* including gonadal and vulval development [15, 16], regulation of translesion synthesis DNA polymerase POLH-1 stability [17], cytoplasmic intermediate filament assembly [18], and Hox gene expression [19]. In the case of Hox gene regulation, a SUMO protease is also involved [19] and other examples have shown that SUMO proteases play important roles in *C. elegans*. The SUMO protease ULP-4 regulates mitotic cell cycle progression in *C. elegans* embryos, affecting chromosome structure, congression, and segregation [20]. Additionally, HMGS-1, the ortholog of human HMGS1 enzyme that mediates the first committed step of the mevalonate pathway, undergoes an age-dependent sumoylation that is temporally balanced by the activity of ULP-4 [21]. More recently, a role for the SUMO protease ULP-2 has been described in the regulation of adherens junction assembly and dysregulation of ULP-2 activity impairs epidermal morphogenesis [22]. Altogether, *C. elegans* is emerging as an extremely useful system to address the biological function of sumoylation and desumoylation. Nevertheless, the field could benefit from the development of novel reagents and techniques. We provide here a set of tools and reagents to study the role of sumoylation in *C. elegans* both in vivo and in vitro.

2 Materials

2.1 Strains

2.1.1 *C. elegans* Strains

The strains used so far have been generated by the bombardment method, as described in [23] (Table 2). Even though current strains are being generated by either transposon-mediated single-site insertions (MosSCI [24]) or genome editing through CRISPR/Cas9 [25, 26], the strains described in Table 2 are established and reproduce the localization pattern of the endogenous SUMO. *C. elegans* N2 Bristol is used as wild type and the strain HT1593 [*unc-119(ed3)*] III is used for bombardment. Worms are grown in NGM plates (0.3% (w/v) NaCl, 2% agar (w/v), 0.25% (w/v) peptone, 5 mg/l cholesterol, 1 mM CaCl₂, 1 mM MgSO₄, 25 mM potassium phosphate, pH 6).

2.1.2 Bacterial Strains

The bacterial strains used for feeding worms are the *E. coli* OP50 and BH101 strains, available from the Caenorhabditis Genetics Center (CGC). For RNAi, we use the Ahringer library [27]. HT115(DE3) bacteria are used, allowing for RNA induction upon IPTG addition. RNAi clones related to the SUMO pathway are depicted in Table 3.

Table 2
Strains expressing different versions of CeSUMO

| Strain | Strain | Genotype |
|----------------------------------|--------|--|
| mCherry-CeSUMO(GG) | FGP1 | <i>fjgIs20[pAA64; Ppie-1 mCherry::smo-1(GG) unc-119(+)]</i> , <i>unc-119(ed3)</i> |
| mCherry-CeSUMO(GA) | FGP2 | <i>fjgIs21[pAA64; Ppie-1 mCherry::smo-1(GA) unc-119(+)]</i> , <i>unc-119(ed3)</i> |
| GFP-CeSUMO(GG) | FGP3 | <i>fjgIs23[pIC26; pie-1/GFP-TEV-S-Tag::smo-1(GG) unc-119(+)]</i> , <i>unc-119(ed3)</i> |
| GFP-CeSUMO(GA) | FGP4 | <i>fjgIs24[pIC26; pie-1/GFP-TEV-S-Tag::smo-1(GA) unc-119(+)]</i> , <i>unc-119(ed3)</i> |
| mCherry-CeSUMO(GG)/GFP-AIR-2 | FGP5 | <i>ltIs14[pASM05; pie-1::GFP-TEV-STag::air-2+ unc-19(+)]</i> , <i>fjgIs20[pAA64; Ppie-1 mCherry::smo-1(GG) unc-119(+)]</i> , <i>unc-119(ed3)</i> |
| mCherry-CeSUMO(GG)/GFP-β-tubulin | FGP7 | <i>ruIs57[pie-1::GFP::tubulin+ unc-119(+)]</i> , <i>fjgIs20[pAA64; Ppie-1 mCherry::smo-1(GG) unc-119(+)]</i> , <i>unc-119(ed3)</i> |
| mCherry-CeSUMO(GG)/GFP-H2B | FGP8 | <i>ruIs32[pie-1::GFP::H2B+ unc-119(+)]</i> , <i>fjgIs20[pAA64; Ppie-1 mCherry::smo-1(GG) unc-119(+)]</i> , <i>unc-119(ed3)</i> |
| 6×His-CeSUMO(GG) | FGP14 | <i>fjgIs35[pRH21; Psmo-1::6xHis::smo-1(GG)::smo-1 3' UTR unc-119(+)]</i> , <i>unc-119(ed3)</i> |

Table 3
RNAi clones used to knock down components of the sumoylation pathway in *C. elegans*

| GenePairs name | SourceBioscience location | Gene |
|----------------|---------------------------|---------------|
| W02A11.4 | I-6B15 | <i>uba-2</i> |
| K12C11.2 | I-1O13 | <i>smo-1</i> |
| F29B9.6 | IV-2K06 | <i>ubc-9</i> |
| W10D5.3 | I-4D09 | <i>gei-17</i> |
| T10F2.3 | III-2N21 | <i>ulp-1</i> |
| Y38A8.3 | II-4K17 | <i>ulp-2</i> |
| C41C4.6 | II-6A07 | <i>ulp-4</i> |
| K02F2.4 | I-3I02 | <i>ulp-5</i> |

2.2 Plasmids

The vector pHISTEV30a is used to express 6×His-tagged proteins in bacteria [28] (Fig. 1). The primers used for cloning are detailed below. The protein sequences shown correspond to the recombinant product after TEV protease cleavage, with amino acids coming from the polylinker and/or TEV cleavage site underlined.

1. pHISTEV30a-CeSUMO(GG).

Primers: CeSUMONcofwd: 5'-ACTGGCCATGGCC
 GATGATGCAGCTCAAG-3'

CeSUMOHindrev: 5'-ACTGAAGCTTTCATCCG
 CCCAGCTGCTC-3'

Protein sequence: GAMADDAAQAGDNAEYIKIKVV
 GQDSNEVHFRVKYGTSMAKLKKSYADRTGVAVNSL
 RFLFDGRRINDDDDTPKTLEMEDDDVIEVYQEQLGG

2. pHISTEV30a-YFP-CeSUMO(GG): In this case, the CeSUMO(GG) cDNA was cloned into pHISTEV30a-YFP [29].

Primers: YFPCeSUMOBamfwd: 5'-ATCGGGATCCG
 CCGATGATGCAGCTCAAG-3'

YFPCeSUMONotrev: 5'-ATCGGCGGCCGCTCA
 TCCGCCAGCTGCTC-3'

Protein sequence: GAMAMVSKGEELFTGVVPIVELDG
 DVNGHKFSVSGEGEGDATYGKLTCLKICTTGKLPVPW
 PTLVTTTLGYGLQCFARYPDHMKQHDFFKSAMPEG
 YVQERTIFFKDDGNYKTRAEVKFE GDTLVNR
 IELKGI DFKEDGNILGHKLEYNYNSH
 NVYITADKQKNGIKANFKIRHNIEDGGVQL
 ADHYQQNTPIGDGPVLLPDNHYSYQSALS KDPN
 EKRDHMVLLFEVTAAGITLGMDELYKGSADDAQAG

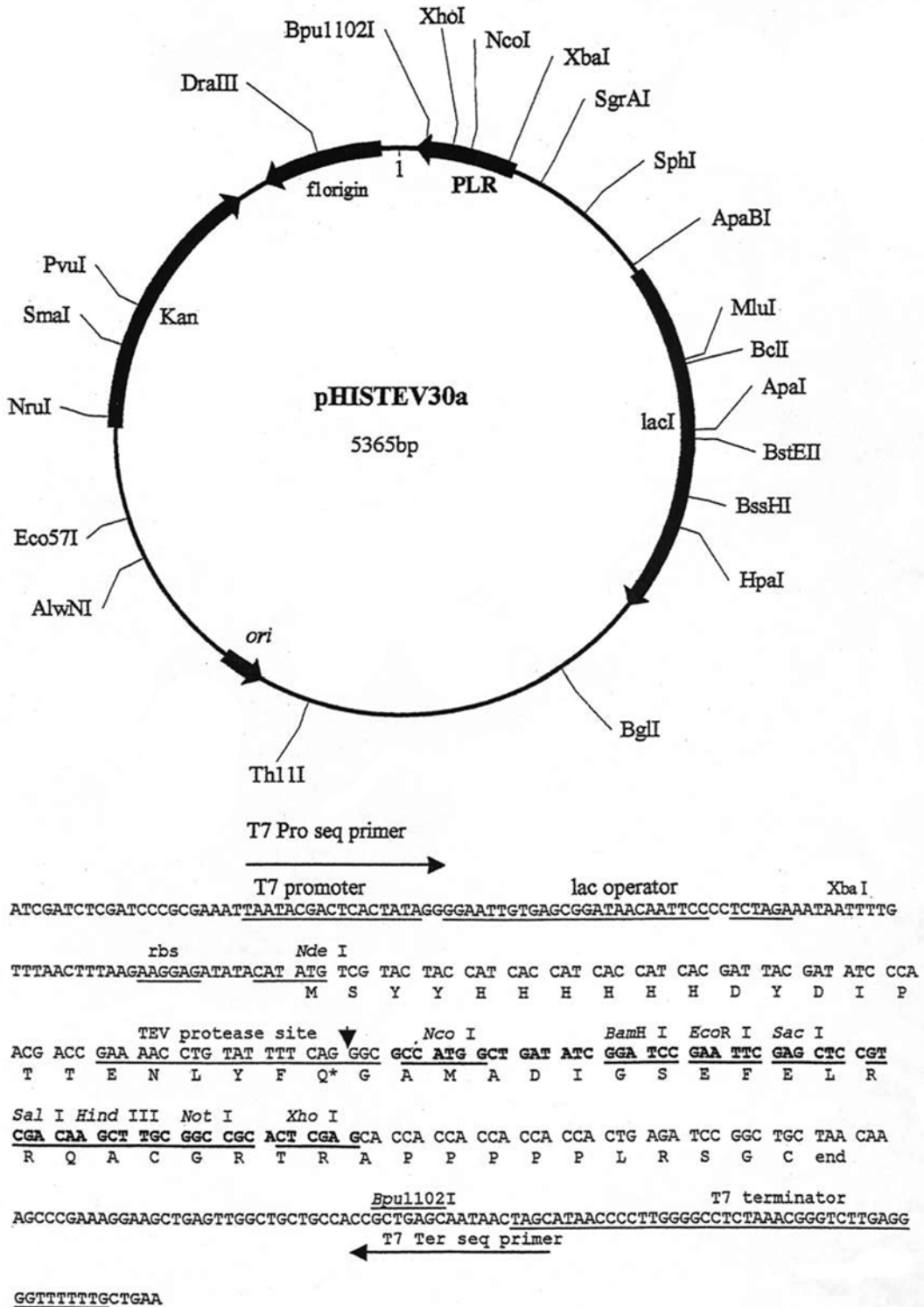


Fig. 1 Map of the pHISTEV30a vector is depicted with the detailed sequence of the multiple cloning site (MCS) below

DNAEYIKIKVVGQDSNEVHFRVKYGTSMAKLKKSYAD
 RTGVAVNSLRFLFDGRRINDDDTPKTLLEM
 EDDDVIEVYQEQLGG

3. pHISTEV30a-CeSUMO-HA.

Primers: CeSUMONcofwd: 5'-ACTGGCCATGGCCGATG
 ATGCAGCTCAAG-3'

CeSUMOLGG-HAHindrev: 5'-ATCGAAGCTTCTAAGCGT
 AATCTGGAAACATCGTATGGGTAA
 AATCCGCCAGCTGCTCTTG-3'

Protein sequence: GAMADDAAQAGDNAEYIKIKVVGQ
 DSNEVHFRVKYGTSMAKLKKSYADRTGVAVNSLRFL
 FDGRRINDDDTPKTLLEMEDDDVIEVYQEQL
 LGGFYPYDVPDYA

4. pHISTEV30a-UBC-9.

Primers: C.e. ubc-9 for: 5'-ATCGCCATGGGATCG
 GGAATTGCTGCAGGAC-3'

C.e. ubc-9 rev: 5'-ATCGAAGCTTCTACTCGAGCATTT
 GCTTC-3'

Protein sequence: GAMGSGIAAGRLAERKHW
 RKDHPFGFIKPVKNADGTLNLFNWECAIPGRKDTIW
 EGGLYRIRMLFKDDFPSTPPKCKFEPPLFHPNVYPSGTV
 CLSLLDENKDWKPSISIKQLLIGIQDLLNHPNI
 EDPAQAEAYQIYCQNRAEYEKRVKKEAVKYAAELVQ
 KQMLE

5. pHISTEV30a-GEI-17 (133–509 in isoform f,
 NP_001021678.3)

Primers: gei-17133Ncofwd: 5'-ACTGCCATGGGACA
 ACAAATGATGGCGTCAC-3'

gei-17509Notrev: 5'-ACTGGCGGCCGCTA
 AAGAGAGTTCATTATACCTC-3'

These primers are predicted to amplify other isoforms as well.
 From N2 worms, we have been able to clone isoforms c and f,
 and the protein sequence below corresponds to the isoform f.

Protein sequence: GAMGQQMMASHHSHLQQQHPS
 TPKKMYADNFEPLPLPFYDVISVLLKPVELHSSDSPT
 LKQTKQLQFPFLLEHISKISYRADVT
 PLPRYELQLRFFNLTEPVQGPQKDDFPLNICYARV
 D D S V V Q L P N V I P T N K T N A E P K R P
 S R P V N I T S N M N R Y K K E H T V A V E W L A D K R V
 W A A G V Y F V H R V N S D I L F K R L N Q N V S R H R S L E V T K Q
 E V I K K L S G G E D D I A M D R L N I S L L D P L C K T R M T T P
 S R C Q D C T H L Q C F D L L S Y L M M N E K K P T W Q C P V C S S
 N C P Y D R L I V D D Y F L D M L A K V D K N T T E V E L K E

D G S Y D V I K E E A F C I S D D D D D D V V P A T V N G
T A S C S S T N G N G L A N E A A K K K P A D D D I I T
L S D D D D E E L N R G I M N S L

6. pHISTEV30a-ULP-4 (145–333)

Primers: ulp-4catNcofor 5'-ATCGCCATGGATCTTCTC
AACGATACGATG-3'

ulp-4catHindrev 5'-ATCGAAGCTTTTAAAAGTCGAAGT
TGTCGAGATC-3'

Protein sequence: GAMDLLNDTMIDFYLNHI
VEHVLPDSNGSNVTVLPSIFWHNLSLRQHAFDSEDE
KMMSDEQKMDLKFGLHDFVADFDLQDFDYIV
VPVNEWEHWSLAVICHFPFTAQARTVIFDSQLTA
DLNNLQNMATLIESFMKYSYEKRTGNAMPFPL
P C I L P Q R M P Q Q T N N F D C G I F I A E F A
RRFLLSPPKDLDNFDF

7. pHISTEV30a-ULP-4 (145–382)

Primers: ulp-4catNcofor 5'-ATCGCCATGGATCTTCT
CAACGATACGATG-3'

ulp-4Hindrev 5'-ATCGAAGCTTTTAAAAGTG
CACGATGAGGTG-3'

Protein sequence: GAMDLLNDTMIDFYLNHIVEHVLP
DSNGSNVTVLPSIFWHNLSLRQHAFDSEDEKMS
DEQKMDLKFGLHDFVADFDLQDFDYIVVPVNE
WEHWSLAVICHFPFTAQARTVIFDSQLTADLN
NLQNMATLIESFMKYSYEKRTGNAMPFPLPC
I L P Q R M P Q Q T N N F D C G I F I A E F A R R F L L S P P
K D L D N F D F A R E Y P D F S T A T K R T E M Q R V V L S L
S T N R A R W R P L V E L L N G Y S T A A P H R A L

2.3 Antibody Methods

1. The complete list of antibodies is described in Table 4. Please *see* Note 1.
2. Binding buffer: 0.02 M Sodium phosphate, pH 7.0.
3. Elution buffer: 0.1 M Glycine-HCl, pH 2.5.
4. Neutralising buffer: 1 M Tris-HCl, pH 9.0.
5. *N*-hydroxy-succinimide (NHS) beads.
6. Coupling buffer: 0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3.
7. NHS buffer A: 0.5 M NaCl, 0.5 M ethanolamine, pH 8.3.
8. NHS buffer B: 0.5 M NaCl, 0.1 M sodium acetate, pH 4.

2.4 Recombinant Proteins

1. Lysis buffer: 50 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole, complete protease inhibitor cocktail tablet, EDTA-free (Roche, 1 tablet per 50 ml), pH 7.5.

Table 4
List of antigens used for the generation of antibodies against components of the *C. elegans* sumoylation pathway

| Antigen | Species | Antibody clonality |
|--------------------------------------|-------------|--------------------|
| CeSUMO (full length) | Mouse (6F2) | Monoclonal |
| CeSUMO (full length) | Sheep | Polyclonal |
| UBC-9 (full length) | Sheep | Polyclonal |
| GEI-17 (aa 133–509, isoform f) | Rabbit | Polyclonal |
| ULP-1 “1.1” (SDSRSEKSFISPTPD-Cys) | Rabbit | Polyclonal |
| ULP-1 “1.2” (Cys-ASRRITTPRFTQKNM) | Rabbit | Polyclonal |
| ULP-2 “2.1” (Cys-CKNLKMPKINSEPNM) | Rabbit | Polyclonal |
| ULP-2 “2.2” (Cys-MPITRLVRRRLRIPE) | Rabbit | Polyclonal |
| ULP-4 “4.1” (Cys-PKDLDNFDFAREYP) | Rabbit | Polyclonal |
| ULP-4 “4.2” (YGSFQDSDVSMRED-Cys) | Rabbit | Polyclonal |
| ULP-4 (aa 145–382) | Rabbit | Polyclonal |

2. Binding buffer: 50 mM Tris–HCl, 500 mM NaCl, 10 mM imidazole, pH 7.5.
3. Washing buffer: 50 mM Tris–HCl, 500 mM NaCl, 30 mM imidazole, pH 7.5.
4. Elution buffer: 50 mM Tris–HCl, 150 mM NaCl, 150 mM imidazole, 0.5 mM TCEP, pH 7.5.
5. Dialysis buffer: 50 mM Tris–HCl, 150 mM NaCl, 0.5 mM TCEP, pH 7.5 (*see Note 2*).
6. Guanidine cleaning buffer: 6 M Guanidine hydrochloride, 0.2 M acetic acid.
7. Ni²⁺-charged agarose beads: We use Ni-NTA beads from QIAGEN but beads from other providers should work as well.
8. 6×His-TEV protease is produced in-house by expressing MBP-TCS-6×His-TEV in BL21(DE3) bacteria, where TCS stands for TEV cleavage site. After induction with 1 mM IPTG, the target protein 6×His-TEV is cleaved from MBP in vivo owing to the presence of the TCS between MBP and 6×His. On 4–12% Novex gels, the cleavage products are as follows: 6×His-TEV (~24 kDa) and MBP (~42 kDa).

2.5 Live Imaging

2.5.1 In Utero Live Imaging

1. M9 buffer: 20 mM KH_2PO_4 , 40 mM Na_2HPO_4 , 80 mM NaCl, 1 mM MgSO_4 . Anesthetic solution: M9 buffer containing tricaine (0.1%) and tetramisole (0.01%) [30].
2. Standard microscope slides and cover slips.
3. 2% Agarose in M9 buffer.
4. Petroleum jelly.
5. Heat block set at 75 °C.
6. Toothpick with an eyelash attached to an end.
7. While in our hands spinning disk confocal microscope is the best suited for in utero imaging, standard wide-field/deconvolution microscopy is good enough for many strains.

2.5.2 Ex Utero Live Imaging

1. Blastomere culture medium: 60% L-15 medium, 25 mM HEPES pH 7.4, 0.5 mg/ml inulin, and 20% heat-inactivated FCS. This medium is used for meiotic recording. M9 buffer is suitable for mitotic recordings.
2. Standard microscope slides and cover slips.
3. 2% Agarose in M9 buffer.
4. Petroleum jelly.
5. Heat block set at 75 °C.
6. Toothpick with an eyelash attached to an end.
7. Confocal or wide-field microscopes work well for ex utero imaging.

2.6 Proximity Ligation Assay

Proximity ligation assays [31] and labeling of primary antibodies are performed with commercially available kits (OLINK Biosciences). We describe here its adaptation for use in *C. elegans*.

1. PLA wash buffer I: PBS + 0.5% Triton X-100.
2. PLA wash buffer II: PBS + 0.05% Tween-20.
3. Hoechst solution: Hoechst 33258 at 1.5 µg/ml in PBS + 0.1% Tween-20.
4. Mounting medium: 4% *n*-Propyl-gallate, 90% glycerol, in PBS. Commercial, anti-fade reagents are also a good choice.

2.7 Purification of 6xHis-CeSUMO Conjugates

1. GuHCl lysis buffer (pH 8): 6 M GuHCl, 10 mM Tris-HCl 10 mM, 100 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 5 mM imidazole, 10 mM IAA.
2. Urea I (pH 8): 8 M Urea, 10 mM Tris-HCl 10 mM, 100 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 5 mM imidazole, 10 mM IAA.
3. Urea II (pH 6.3): 8 M Urea, 10 mM Tris-HCl 10 mM, 100 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 0.2% Triton X-100, 5 mM imidazole, 10 mM IAA.

4. Urea III (pH 6.3): 8 M Urea, 10 mM Tris-HCl 10 mM, 100 mM Na₂HPO₄/NaH₂PO₄, 0.1% Triton X-100, 5 mM imidazole, 10 mM IAA.
5. Elution buffer: 50 mM Tris-HCl pH 6.8, 30% glycerol, 2% SDS, 100 mM DTT, 200 mM imidazole, 0.01% bromophenol blue.

3 Methods

3.1 Polyclonal Antibody Preparation

The basic protocol consists of affinity purifying the antibodies from a serum of choice. Dot blotting is used to determine the best-responding serum for each antibody. Afterwards, the serum is depleted of bacterial-reacting antibodies by passage through an HT115 lysate column. Finally, specific antibodies are affinity-purified. In the case of CeSUMO antibodies, a conjugation reaction is first performed to increase immunogenicity. This way, an extra step is added later on to deplete the serum from substrate-recognizing antibodies. Figure 2 details the protocol as used for anti-CeSUMO antibodies generated in sheep, but can be used for other protein antigens (*see* Table 4 for all the antigens used).

Recombinant CeSUMO needs to be conjugated *in vitro* to murine IRF2 (Fig. 2b).

3.1.1 Test Sera with Specific and Nonspecific Antigens in Dot-Blot Assays

1. Draw with a pencil as many rows of circles on a piece of nitrocellulose as required and spot the desired amount of recombinant protein using the circles as guide (we typically use between 1 and ~200 ng of protein) (*see* Note 3).
2. Block the membrane in 5% nonfat dry milk for 1 h at room temperature.
3. As a starting point, dilute the sera 1:1000 and incubate membranes for 1 h at room temperature or overnight at 4 °C.
4. Wash 3× with TBS + 0.1% Tween-20 and incubate membranes with secondary antibody at the appropriate concentration for 1 h at room temperature.
5. Wash 3× with TBS + 0.1% Tween-20 and develop using enhanced chemiluminescence.

3.1.2 Coupling of Proteins/Peptides/ Bacterial Lysate to NHS Beads

1. Dialyze 40 mg of protein/peptide at 1–2 mg/ml against coupling buffer (*see* Note 4).
2. Wash 5 ml of NHS beads on a sintered glass funnel with 50 ml of ice-cold 1 mM HCl.
3. Wash beads with 100 ml of ice-cold coupling buffer.
4. Transfer NHS beads to a 15 ml Falcon tube.
5. Measure the protein concentration of the dialyzed protein and add to NHS beads overnight at 4 °C.

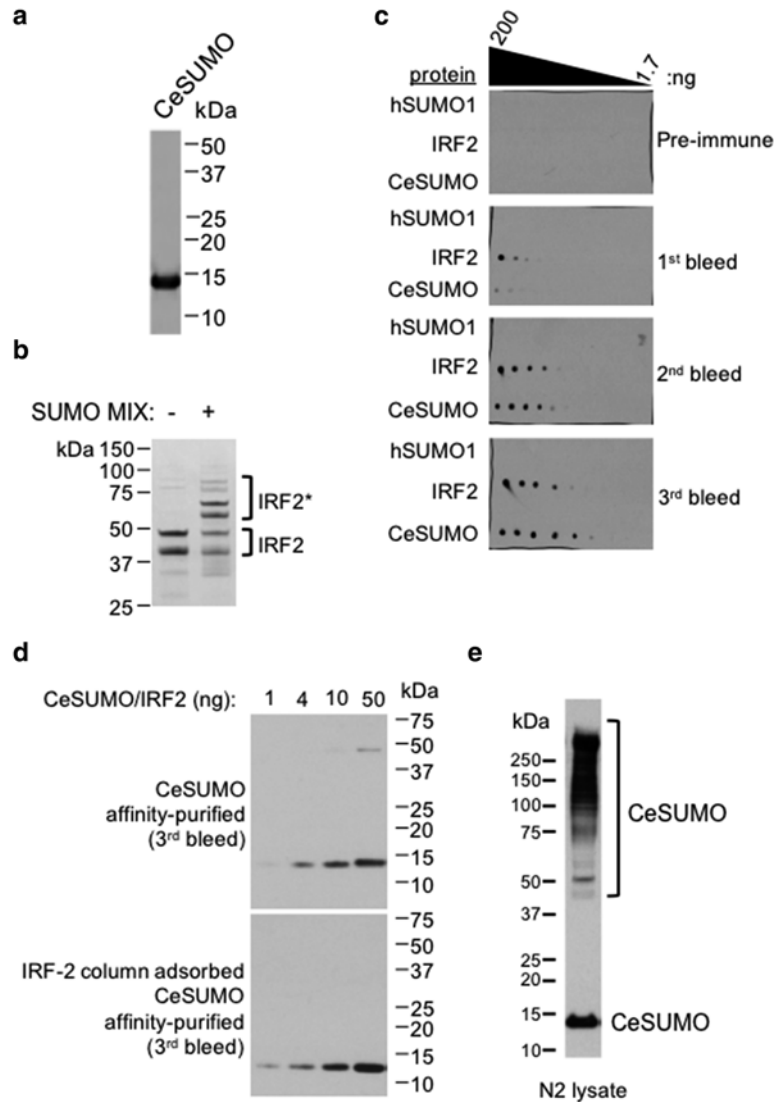


Fig. 2 Basic steps during polyclonal anti-CeSUMO antibody purification and testing. (a) CeSUMO was first expressed in bacteria and purified as described in this chapter. Subsequently, CeSUMO was conjugated to murine IRF2 in vitro and the resulting product was used to immunize sheep (b). (c) The different bleeds were tested for reactivity and specificity in dot blot assays using recombinant purified human SUMO-1 (hSUMO1), IRF2, and CeSUMO (200, 100, 50, 25, 12.5, 6.25, 3.13, and 1.7 ng). (d) The efficiency of the anti-IRF2 antibody depletion was tested in western blot assays using the indicated amounts of CeSUMO and IRF2. (e) The final product, anti-IRF2-depleted, affinity-purified, sheep anti-CeSUMO, recognizes both free and conjugated CeSUMO from worm lysate

6. Take an aliquot of the sample, pellet NHS beads, and measure protein concentration of the supernatant.
7. If >85 % of the protein is bound, continue with the protocol.
8. Pour protein/NHS bead mix on a column and wash with 25 ml of coupling buffer.
9. Wash with 50 ml of NHS buffer A.
10. Wash with 50 ml of NHS buffer B.
11. Add 50 ml of NHS buffer A and let stand for 30 min.
12. Wash with 50 ml of NHS buffer B.
13. Wash with 50 ml of NHS buffer A.
14. Wash with 50 ml of NHS buffer B.
15. Store column in PBS, 0.5 M NaCl, and 0.1 % sodium azide.
16. Adsorb the sera with HT1115 bacterial lysate-coupled NHS beads and use the flow-through for the following steps (*see Note 5*).

3.1.3 Affinity Purification

1. Pass 50–100 ml coupling buffer over column.
2. Dilute 15 ml of serum with 135 ml PBS and pass through a 0.2 μ M filter.
3. Pass diluted serum over column two times (keep diluted serum in case of problem).
4. Wash column with 50–100 ml 10 mM Tris–HCl pH 7.5, and 0.5 M NaCl (keep wash in case of problem).
5. Elute with 0.1 M glycine pH 2.25, collecting 20 1 ml fractions into 1.5 ml disposable tubes containing 100 μ l 1 M Tris–HCl pH 8.
6. Mix immediately and pool antibody-containing fractions.
7. Add glycerol to 10 %, aliquot, and store at -80°C .

Figure 2d highlights the efficiency of the anti-IRF2 antibody depletion procedure used for CeSUMO antibodies. The resulting antibody was tested by detecting SUMO conjugates from an N2 lysate (Fig. 2e).

3.2 Monoclonal Antibody Preparation

CeSUMO was conjugated in vitro to mIRF2 and the mixture was used to immunize mice. After selection of CeSUMO reactive sera by ELISA and dot blot, five different lines are isolated, re-cloned, and characterized (Table 5).

3.2.1 Hybridoma Supernatant Preparation

Hybridoma cells are maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum and glutamine. High-titer tissue culture supernatants are obtained with the CELLline CL 1000 Bioreactor (Sartorius) as per the manufacturer's instructions (*see Note 6*). This high-concentration supernatant (usually ranging between 1 and 3 mg/ml) is ready to use for western blotting,

Table 5
List of hybridoma clones expressing different anti-CeSUMO antibodies

| Parental | Re-cloning | In-house name | Isotype |
|----------|------------|---------------|---------|
| 6F2 | D1 | SUMO1.1 | IgG1k |
| | D6 | SUMO1.2 | |
| 6A4 | A8 | SUMO2.1 | IgG1k |
| | C9 | SUMO2.2 | |
| 5A3 | B10 | SUMO3.1 | IgMk |
| | A4 | SUMO3.2 | |
| 8A1 | D10 | SUMO4.1 | IgG1k |
| | D11 | SUMO4.2 | |

immunofluorescence, and immunoprecipitation. Add sodium azide to 0.1 % and keep at 4 °C (*see Note 7*).

The monoclonal antibodies were tested against recombinant CeSUMO (Fig. 3a) or worm lysate (Fig. 3b) in western blot assays. For immunoprecipitation tests, we used YFP-CeSUMO and performed either WB (Fig. 3c) or just irradiated the beads with UV light (Fig. 3d). Monoclonal antibodies are available at DSHB <http://dshb.biology.uiowa.edu/SUMO-6F2>.

3.2.2 Protein G Purification

For labeling the antibody either with PLA probes or fluorescent dyes, antibody purification from the supernatant is recommended.

1. Dialyze antibody solution overnight against wash/binding buffer with at least two buffer exchanges. Remove any particulate matter from the sample by centrifugation or filtration through a 0.45 µm filter.
2. Pour protein G slurry into column and allow column to flow by gravity to pack the column bed.
3. Equilibrate the packed affinity resin with 10 column volumes (CV) of the wash/binding buffer.
4. Gently apply sample to the column by layering onto the top of the resin. Be careful not to disturb the bed surface.
5. Wash column with 10 CV of the 1× wash/binding buffer, or until the absorbance of eluate at 280 nm approaches the background level.
6. Before beginning the elution step, set up enough tubes to collect the entire elution volume as 1 ml fractions (5 CV will be used to elute the antibody). To each collection tube add 250 µl

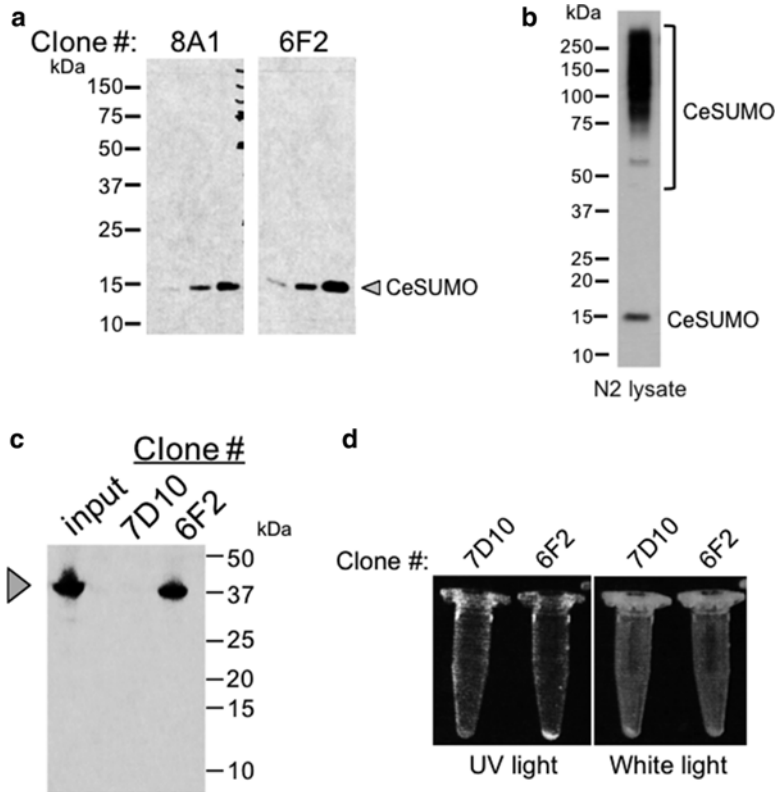


Fig. 3 Monoclonal antibody testing. **(a)** Two different clones (8A1 and 6F2) were tested on western blot assays with recombinant CeSUMO. Supernatants from each hybridoma cell line were used at 1/500 dilution. **(b)** The clone 6F2 was also tested in western blot assays using N2 lysate. Free CeSUMO can be observed at ~15 kDa while a strong smear corresponding to CeSUMO conjugates can be seen above 75 kDa. **(c)** Two supernatants were tested in immunoprecipitation assays against recombinant purified YFP-CeSUMO. While clone 6F2 immunoprecipitated YFP-CeSUMO, clone 7D10 did not. This can also be tested by irradiating beads with UV light, as seen in **(d)**

of 1 M Tris-HCl pH 9. To elute the antibody, gently add 1 ml of 1× elution buffer to the top of the resin, collecting the eluate in a prepared collection tube. Repeat until the entire volume has been collected, up to 4 column volumes.

7. Pool antibody-containing fractions, dialyze against the desired buffer, aliquot, and store at -80°C .

3.3 Ex Utero Live Imaging

3.3.1 Preparing the Worms

1. Worms at the L4 stage are plated on RNAi plates for 24–36 h before imaging. Bacterial (HT115) clones expressing dsRNA for feeding strains are obtained from the Ahringer library [27].
2. Bacteria are grown at 37°C to OD_{600} of 0.8, shifted to 20°C , supplemented with 1 mM IPTG, and further incubated for 2 h.

3. Then, they are spread on 6 cm NGM plates supplemented with 1 mM IPTG and incubated for 12 h at 20 °C.
4. L4 worms are then added to plates and fed for 24–36 h before analysis.

3.3.2 Meiotic Recording

1. Worms are dissected in 4 μ l L-15 blastomere culture medium on a 22 \times 22 mm cover slip.
2. A ring of petroleum jelly is deposited around the drop of medium to serve as a spacer and prevent compression of the embryos.
3. A slide is placed on top to seal the chamber and prevent evaporation during filming.
4. Live imaging is conducted at 20–23 °C using a spinning-disk confocal microscope (MAG Biosystems) mounted on a microscope (IX81; Olympus) with a 100 \times /1.45 Plan Achromat oil immersion lens (Olympus), a camera (Cascade II; Photometrics), spinning-disk head (CSU-X1; Yokogawa Electric Corporation), and MetaMorph software (Molecular Devices).

3.3.3 Mitotic Recording

Recording mitotic divisions is easier due to the presence of the eggshell. Worms are dissected in M9 buffer and dissected embryos can be placed on a 2% agarose pad; gently position a cover slip on top and image. Figure 4a shows still images from a time-lapse of embryos expressing GFP-tubulin and mCherry-CeSUMO (FGP7) acquired using a spinning disk confocal microscope. Images start prior to nuclear envelope breakdown and continue until late anaphase. The accumulation of CeSUMO on metaphase is conjugation dependent as it is abolished by the knockdown of GEI-17 (Fig. 4b) [20].

3.4 In Utero Live Imaging

1. Place healthy worms with a single row of embryos in anesthetic solution for 30 min.
2. Pipet worms onto a 2% agarose pad and covered with a cover slip.
3. You may seal the specimen with petroleum jelly.
4. Image worms immediately.

In our case, live imaging is conducted at 20–23 °C using a spinning-disk confocal microscope (MAG Biosystems) mounted on a microscope (IX81; Olympus) with a 100 \times /1.45 Plan Achromat oil immersion lens (Olympus), a camera (Cascade II; Photometrics), spinning-disk head (CSU-X1; Yokogawa Electric Corporation), and MetaMorph software (Molecular Devices). As a standard, image stacks are obtained at 1 μ m z-steps at 20-s intervals using 2 \times 2 binning (*see Note 8*). Worms expressing GFP-H2B and mCherry-CeSUMO (FGP8) are imaged as described above and the green channel is shown on the left panel of Fig. 4c. The embryo going through metaphase of mitosis is enlarged and the red channel (CeSUMO) shown on the right.

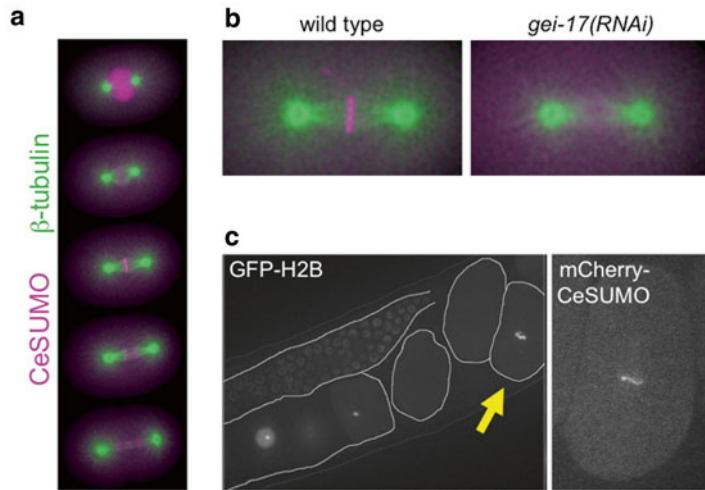


Fig. 4 Time-lapse microscopy using fluorescently labeled CeSUMO strains. **(a)** Ex utero live imaging of the first embryonic mitotic division of an embryo expressing mCherry-CeSUMO and GFP-tubulin. Images were acquired using a spinning disk confocal microscope. **(b)** Same as in **(a)**, but comparing embryos fed control RNAi (“wild type”) with others fed *gei-17(RNAi)*. **(c)** In utero image from a time-lapse movie using a strain expressing mCherry-CeSUMO and GFP-H2B. On the *left* panel, under the green channel, the pachytene region can be seen, three oocytes below it, and three embryos. The third embryo, indicated with a *yellow arrow*, is going through metaphase of the first mitotic cycle. An enlarged image of the red channel corresponding to the indicated embryo is shown on the *right*

3.5 Proximity Ligation Assay

Duolink® in situ Proximity Ligation Assays (PLA®) are performed using primary antibodies directly coupled to the PLA probes or using secondary antibody PLA probes (Sigma-Aldrich). When using the secondary antibody approach the theoretical maximum distance between the epitopes in the two target proteins is 30–40 nm to be able to create a signal [31]. Resolution could be taken down by a few nanometers if the assay is performed using directly labeled primary antibodies.

3.5.1 Making PLA Probes with Duolink® In Situ Probemaker

Using only primary antibodies for PLA assays is useful, as it will shorten the distance required for the probes to become ligated and amplified. Thus, the antigens will need to be in closer proximity than with the indirect method. Coupling of primary antibodies to PLA probes is carried out exactly as described by the manufacturer.

3.5.2 PLA

The protocol below has been optimized for detection of the interaction between CeSUMO and AIR-2 [20], but has since been used for other proteins.

1. 35 worms are placed on a drop of 4 μ l of M9 worm buffer in a poly-D-lysine-coated slide and a cover slip is gently laid on top.
2. Once the worms extruded the embryos, slides are placed on a metal block on dry ice for 10 min.
3. The cover slip is taken off with a scalpel blade, and the samples are fixed in methanol at -20 °C for 30 min (optimal fixation time will depend on each antibody).
4. Three sequential washes of 5 min each are performed with PLA wash buffer I, PLA wash buffer II, and PBS.
5. Slides are incubated with monoclonal α -CeSUMO (6F2/D1, 10 μ g/ml) and α -AIR-2 (10 μ g/ml), both previously coupled to the PLA oligonucleotide arms using the Duolink® in situ Probemaker overnight at 4 °C. For indirect PLA, the same primary antibodies are used (unlabeled, at 1 μ g/ml) and after an overnight incubation at 4 °C, slides are incubated with anti-mouse and anti-rabbit secondary antibodies coupled to the PLA oligonucleotide probes.
6. Ligation and amplification are performed for 30 min and 90 min, respectively, at 37 °C in a humid chamber.
7. Wash with PLA wash buffer I for 5 min.
8. Slides are incubated in Hoechst solution for 5 min.
9. Slides are mounted in mounting medium and imaged (*see Note 9*).

3.6 Purification of 6 \times His-CeSUMO Conjugates

For this procedure, we use a strain expressing a 6 \times His-tagged version of CeSUMO driven by its own promoter and 3'UTR (FGP14) (*see Note 10*).

1. If necessary, synchronize worms by bleaching and plate at \sim 4000 worms/9 cm plate. If this is not necessary, go to **step 7**.
2. Wash worms from plates with 6.5 ml M9 buffer and add 1 ml sodium hypochlorite solution (6–14%) and 2.5 ml 1 M NaOH (10 ml total).
3. Let stand for 4 min mixing from time to time.
4. Centrifuge for 1 min at 1,000 $\times g$.
5. Wash two times with M9 and let hatch overnight.
6. Grow 6 \times His-CeSUMO worms at 20–25 °C until the desired stage.
7. Wash worms with M9 + 10 mM iodoacetamide (*see Note 11*).
8. Centrifuge for 1 min at 1,000 $\times g$ and discard the supernatant (leaving 100 μ l).
9. Take 10 μ l as input and add 1 ml GuHCl lysis buffer to the remainder.

10. Heat samples at 90 °C for 10 min and sonicate the GuHCl lysate 6 × 30" with 30" rest at MAX setting using a Bioruptor water bath sonicator (Diagenode).
11. Centrifuge for 30 min at 13,000 × *g* at 4 °C and transfer supernatant to a new tube.
12. Add 20 µl of Ni-NTA beads (~50%, equilibrated in GuHCl lysis buffer) and incubate at room temperature for 2 h at room temperature or overnight at 4 °C.
13. Wash with: 1 × GuHCl lysis buffer, 1 × urea pH 8, and 1 × urea pH 6.3 (1 ml each).
14. Add 30 µl of Ni-NTA loading buffer and incubate for 10 min shaking at RT.
15. Incubate at 100 °C for 10 min, spin down beads, and load supernatant on a gel.

3.7 Protein Purification

3.7.1 *CeSUMO*, *UBC-9*, *GEI-17*, and *ULP-4*

The purification of recombinant full-length or fragment *CeSUMO*, *ubc-9*, *gei-17*, and *ulp-4* cDNAs (as described above) is expressed from pHISTEV30a vector as N-terminal 6×His-tagged protein with a TEV protease site between the tag and the ORF. *E. coli* strain BL21(DE3) Rosetta is used for protein expression.

1. 20 ml of LB medium supplemented with kanamycin (50 µg/ml) and chloramphenicol (35 µg/ml) is inoculated with a single colony from a freshly streaked LB+kanamycin plate and the bacterial culture is incubated overnight at 37 °C with shaking at 220 rpm.
2. The following day, 5 ml of the overnight culture is used to inoculate 500 ml of LB medium with kanamycin (50 µg/ml) in a 2 l flask and cells are grown at 37 °C with shaking at 220 rpm until OD₆₀₀ reaches ~0.6–0.8.
3. The cell culture is then cooled down in ice-cold water for 10–15 min.
4. 100 µM IPTG (final concentration) is added to induce protein expression and the cell culture is incubated at 20 °C with shaking at 220 rpm overnight (15–19 h) (*see Note 12*).
5. Bacteria are harvested by centrifugation (6200 × *g* for 20 min at 4 °C) and the cell pellet is resuspended in 35 ml of lysis buffer supplemented with protease inhibitors (Roche).
6. Purification is carried out immediately. Alternatively, the cell suspension can be flash-frozen in liquid nitrogen and stored at –80 °C until further use.
7. Bacteria are lysed by sonication (Digital Sonifier, Branson): big tip, 4 × 20" pulses at 50% amplitude, with a 20" cooling period between pulses. Samples are kept on ice during sonication to prevent heating.

8. Samples are centrifuged ($30,000\times g$ for 45 min at 4 °C) to remove any insoluble material.
9. The supernatant is loaded onto a column with 6 ml Ni-NTA agarose beads (packed volume, QIAGEN), washed with water, and pre-equilibrated with binding buffer (*see* **Note 13**).
10. The column is washed successively with binding buffer (~10 column volumes) and washing buffer (~10 column volumes) and the fusion protein is then eluted with elution buffer. Keep fractions for SDS-PAGE analysis.
11. Fractions containing 6×His-tagged protein are pooled and quantified with the Bradford reagent by measuring absorbance at 595 nm.
12. TEV protease is added (1 mg of TEV protease per 100 mg of the fusion protein) and samples are dialyzed overnight at 4 °C against 50 mM Tris-HCl, 150 mM NaCl, and 0.5 mM TCEP, pH 7.5.
13. In the meantime, the Ni-NTA column is cleaned using guanidine cleaning buffer (~7 column volumes), followed by extensive washing with water.
14. Once most of the fusion protein is cleaved (usually ~16 h at 4 °C, check by SDS-PAGE and Coomassie staining), imidazole is added to the final concentration of 10 mM (30 mM for GEI-17).
15. Samples are centrifuged ($3900\times g$ for 15 min at 4 °C) to remove any precipitated material and the supernatant is then passed through the Ni-NTA agarose column pre-equilibrated with 50 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole (30 mM for GEI-17), and 0.5 mM TCEP, pH 7.5.
16. A flow-through fraction is collected. This step removes free 6×His-tag, any uncleaved 6×His-tagged protein, and the 6×His-TEV protease.
17. The flow-through fraction is dialyzed overnight at 4 °C against 50 mM Tris-HCl, 150 mM NaCl, and 0.5 mM TCEP, pH 7.5.
18. Samples are concentrated using a centrifugal concentrator (Sartorius) with a molecular weight cutoff of 10,000 for GEI-17, 5000 for UBC-9 and ULP-4, and 3000 for CeSUMO.
19. Purified proteins are aliquoted, flash-frozen in liquid nitrogen, and stored at -80 °C.
20. This protocol yields high amounts of >90% pure protein. If further purification is needed, proteins can be subject to size-exclusion chromatography (Superdex 75 for CeSUMO, UBC-9, and ULP-4 CD; Superdex 200 for GEI-17). Alternatively, CeSUMO, GEI-17, and ULP-4 can be further purified using anion exchange (monoQ), while UBC-9 may be further purified through cation exchange (SP).
21. In Fig. 5, an aliquot of all the steps was taken from a UBC-9 preparation.

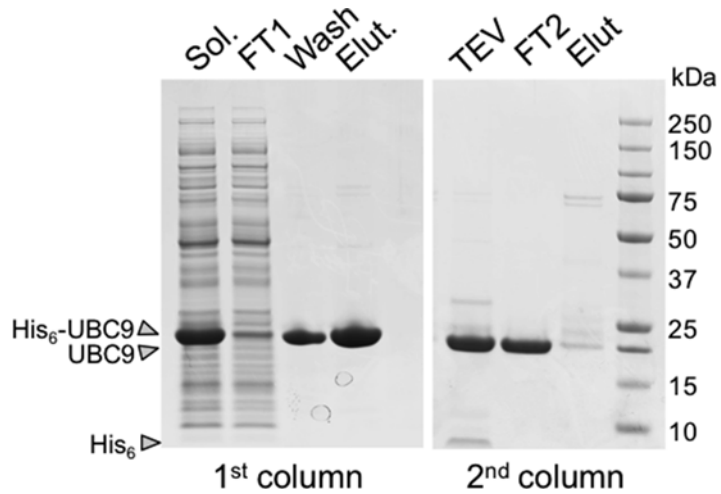


Fig. 5 Basic protocol for recombinant protein purification using the pHISTEV30a vector. After induction with IPTG soluble material (Sol.) was obtained and an aliquot loaded on a gel. An aliquot of the flow-through (FT1) from the Ni-NTA column was loaded on the gel. After washing with buffer containing 30 mM imidazole (wash), the protein was eluted with 200 mM imidazole (Elut.). After treatment with 6×His-TEV protease (TEV), a second flow-through (FT2) was taken consisting of the untagged purified recombinant protein

3.7.2 Expression and Purification of TEV Protease

1. BL21(DE3) cells containing pRK793 are grown at 37 °C in LB containing 100 µg/ml ampicillin and 25–30 µg/ml chloramphenicol.
2. Induce production of the fusion protein (MBP-TEV) by adding IPTG to a final concentration of 1 mM at $A_{600}=0.8$ (after chilling cultures in ice water for 10 min). At the same time, lower the temperature to 25 °C unless using a bench-top shaker for induction step.
3. Harvest the cells by centrifugation after a minimum 4 h of induction (overnight expression is fine).
4. Resuspend the cell pellet in 5–10 ml of 50 mM sodium phosphate buffer pH 8, 150 mM NaCl, 10% glycerol, and 25 mM imidazole (lysis buffer) per 1 g of wet cell paste. **Add protease inhibitor** (we use complete EDTA-free, Roche) (*see Note 14*).
5. Lyse the cells (sonication) and remove the cell debris by centrifugation (20,000 rpm, 30 min). Filter the supernatant (0.2 µm filter).
6. Load the sample onto an appropriately sized Ni-NTA column (expect 30 mg TEV/L cell culture) equilibrated with lysis buffer.
7. Wash the column with 20 column volumes of lysis buffer; elute the TEV protease in successive ½ column volume fractions of

50 mM sodium phosphate pH 8, 150 mM NaCl, and 300 mM imidazole; and analyze fractions by SDS-PAGE.

8. Pool the appropriate fractions and desalt/dialyze against 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA, and 1 mM DTT. You may notice some precipitation here, but do not worry; just spin down and filter before proceeding.
9. Concentrate the protease to 1–2 mg/ml. Add glycerol to 50% and snap-freeze and store in 1 ml aliquots at –80 °C. Once raised from –80 °C, store at –20 °C.

3.8 SUMO Conjugation

Conjugation assays contained 50 mM Tris-HCl pH 7.5, 5 mM dithiothreitol, 5 mM MgCl₂, 2 mM ATP, 100 ng of SAE1/SAE2, 1 μM UBC-9 (reduced to 100 nM for E3-dependent conjugation), ~1 μg of substrate protein, and 5 μg of SUMO and are incubated at 37 °C for 4 h. Figure 6a shows a typical time course of an *in vitro* conjugation using RanGAP1 as a test substrate.

3.9 Processing of SUMO and Chain Editing

CeSUMO chains are assembled on GEI-17 and purified by size-exclusion chromatography (SEC) using a Superdex 200 column (Fig. 6b). SUMO processing assays contained 150 mM NaCl, 0.5 mM TCEP, 50 μM SUMO, and 100 nM SENP1 or ULP-4 recombinant catalytic domains (CD), and reactions are incubated at 30 °C for 60 min. Chain editing assays are performed by adding 0.5, 1, and 4.5 μM of the catalytic domain of ULP-4 (aa 145–333 in NP_495703.2) for the indicated times, whereas CeSUMO processing is performed for 2 h at 37 °C using 1 μM ULP-4 CD- and CeSUMO-modified GEI-17. The effect of pH and salt on ULP-4 activity is shown in Fig. 6c, d.

4 Notes

1. Water and chemicals used for buffer preparation should be of high purity. Filter buffers through a 0.45 μm filter before use.
2. Salt concentration and pH will need to be optimized for other proteins.
3. Make sure to let the protein(s) dry on the membrane before proceeding to the blocking step.
4. The procedure can be scaled down as required, provided that small amounts of antibody are to be purified.
5. In the case of the CeSUMO antibody, deplete serum of anti-IRF2 antibodies by passing through a column of IRF2 coupled to NHS beads and use the flow-through for the following steps. The same strategy is used if a tagged protein is used as antigen, to deplete of anti-tag antibodies.

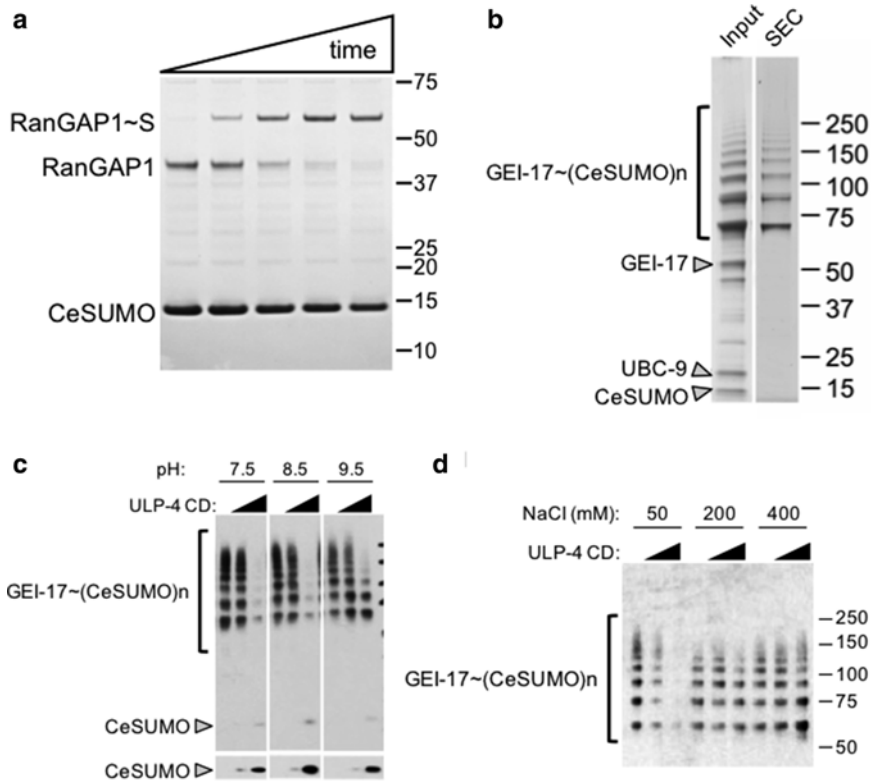


Fig. 6 In vitro conjugation and protease activity assays. **(a)** RanGAP1 was used as a substrate in conjugation reactions and aliquots were taken at 0, 2, 5, 15, and 30 min. Samples were analyzed by SDS-PAGE and Coomassie staining. **(b)** An in vitro conjugation reaction was performed using GEI-17 as the substrate. After 4 h, the reaction was diluted and fractionated over a Superdex 200 SEC column. Fractions containing SUMO-modified GEI-17 were pooled and concentrated. SUMO protease activity of ULP-4 was tested under different pH conditions **(c)** and salt concentrations **(d)**

6. These type of commercially available bioreactor chambers are very useful as the concentration of antibody in the supernatant is, in our experience, always >1 mg/ml.
7. Some applications may require or benefit from a cleaner antibody preparation. In those cases, which include the labeling for PLA assays, protein G purification from the hybridoma supernatant is performed.
8. Parameters such as amount and depth of z-stacks, exposure time, binning, and Δt should be determined for each strain with extra care to avoid phototoxicity.
9. We have found for all of our PLA assays that the channel corresponding to the PLA signal SHOULD NOT be deconvolved. Deconvolution leads to “dirtier” images.

10. In this protocol, sumoylated proteins are enriched through a denaturing purification using Ni-NTA beads. This is crucial to make sure that you are not detecting non-covalent SUMO interaction as opposed to conjugation (covalent).
11. Iodoacetamide should be handled with care and preferably prepared fresh. Small 0.4 M aliquots can be stored at -20°C .
12. IPTG concentration, induction temperature, and length should be optimized for each protein. We use 0.1 M IPTG at 20°C for 16 h as the standard protocol.
13. Filtering the supernatant through a $0.45\ \mu\text{m}$ filter prior to binding is optional but might help in keeping a good flow by avoiding clumps to get stuck in the column.
14. All handling post-lysis should be done at 4°C and the protease should be frozen as soon as possible.

Acknowledgments

This work was supported by the Wellcome Trust (grant 098391/Z/12/7), Cancer Research UK (grant C434/A13067), BBSRC (grant BB/J015199/1), and a Marie Curie Fellowship (grant 297881). Some nematode strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). R.T.H. is a Senior Investigator of the Wellcome Trust. Wellcome Trust grant 097045/B/11/Z provided infrastructure support. The author(s) would like to acknowledge networking support by the Proteostasis COST action (BM1307).

References

1. Nacerddine K, Lehembre F, Bhaumik M et al (2005) The SUMO pathway is essential for nuclear integrity and chromosome segregation in mice. *Dev Cell* 9(6):769–779
2. Wang L, Wansleben C, Zhao S et al (2014) SUMO2 is essential while SUMO3 is dispensable for mouse embryonic development. *EMBO Rep* 15(8):878–885
3. Fernandez AG, Gunsalus KC, Huang J et al (2005) New genes with roles in the *C. elegans* embryo revealed using RNAi of ovary-enriched ORFeome clones. *Genome Res* 15(2):250–259
4. Jones D, Crowe E, Stevens TA et al (2002) Functional and phylogenetic analysis of the ubiquitylation system in *Caenorhabditis elegans*: ubiquitin-conjugating enzymes, ubiquitin-activating enzymes, and ubiquitin-like proteins. *Genome Biol* 3(1):research0002–research0002.0015
5. Kamath RS, Fraser AG, Dong Y et al (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421(6920):231–237
6. Rual JF, Ceron J, Koreth J et al (2004) Toward improving *Caenorhabditis elegans* phenome mapping with an ORFeome-based RNAi library. *Genome Res* 14(10B):2162–2168
7. Flotho A, Melchior F (2013) Sumoylation: a regulatory protein modification in health and disease. *Annu Rev Biochem* 82:357–385
8. Gareau JR, Lima CD (2010) The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat Rev Mol Cell Biol* 11(12):861–871
9. Yunus AA, Lima CD (2009) Structure of the Siz/PIAS SUMO E3 ligase Siz1 and determinants required for SUMO modification of PCNA. *Mol Cell* 35(5):669–682
10. Johnson ES, Gupta AA (2001) An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell* 106(6):735–744

11. Zhao X, Blobel G (2005) A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. *Proc Natl Acad Sci U S A* 102(13):4777–4782
12. Hay RT (2007) SUMO-specific proteases: a twist in the tail. *Trends Cell Biol* 17(8):370–376
13. Mukhopadhyay D, Dasso M (2007) Modification in reverse: the SUMO proteases. *Trends Biochem Sci* 32(6):286–295
14. Holway AH, Kim SH, La Volpe A et al (2006) Checkpoint silencing during the DNA damage response in *Caenorhabditis elegans* embryos. *J Cell Biol* 172(7):999–1008
15. Ward JD, Bojanala N, Bernal T et al (2013) Sumoylated NHR-25/NR5A regulates cell fate during *C. elegans* vulval development. *PLoS Genet* 9(12):e1003992
16. Broday L, Kolotuev I, Didier C et al (2004) The small ubiquitin-like modifier (SUMO) is required for gonadal and uterine-vulval morphogenesis in *Caenorhabditis elegans*. *Genes Dev* 18(19):2380–2391
17. Kim SH, Michael WM (2008) Regulated proteolysis of DNA polymerase eta during the DNA-damage response in *C. elegans*. *Mol Cell* 32(6):757–766
18. Kaminsky R, Denison C, Bening-Abu-Shach U et al (2009) SUMO regulates the assembly and function of a cytoplasmic intermediate filament protein in *C. elegans*. *Dev Cell* 17(5):724–735
19. Zhang H, Smolen GA, Palmer R et al (2004) SUMO modification is required for in vivo Hox gene regulation by the *Caenorhabditis elegans* Polycomb group protein SOP-2. *Nat Genet* 36(5):507–511
20. Pelisch F, Sonnevile R, Pourkarimi E et al (2014) Dynamic SUMO modification regulates mitotic chromosome assembly and cell cycle progression in *Caenorhabditis elegans*. *Nat Commun* 5:5485. doi:[10.1038/ncomms6485](https://doi.org/10.1038/ncomms6485)
21. Sapir A, Tsur A, Koorman T et al (2014) Controlled sumoylation of the mevalonate pathway enzyme HMGS-1 regulates metabolism during aging. *Proc Natl Acad Sci U S A* 111(37):E3880–E3889
22. Tsur A, Bening Abu-Shach U, Broday L (2015) ULP-2 SUMO protease regulates E-cadherin recruitment to adherens junctions. *Dev Cell* 35(1):63–77
23. Green RA, Audhya A, Pozniakovsky A et al (2008) Expression and imaging of fluorescent proteins in the *C. elegans* gonad and early embryo. *Methods Cell Biol* 85:179–218
24. Frokjaer-Jensen C, Davis MW, Hopkins CE et al (2008) Single-copy insertion of transgenes in *Caenorhabditis elegans*. *Nat Genet* 40(11):1375–1383
25. Dickinson DJ, Pani AM, Heppert JK et al (2015) Streamlined genome engineering with a self-excising drug selection cassette. *Genetics* 200(4):1035–1049
26. Ward JD (2015) Rapid and precise engineering of the *Caenorhabditis elegans* genome with lethal mutation co-conversion and inactivation of NHEJ repair. *Genetics* 199(2):363–377
27. Kamath RS, Martinez-Campos M, Zipperlen P et al (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol* 2(1):research0002.0001–research0002.0010
28. Shen L, Tatham MH, Dong C et al (2006) SUMO protease SENP1 induces isomerization of the scissile peptide bond. *Nat Struct Mol Biol* 13(12):1069–1077
29. Tatham MH, Hay RT (2009) FRET-based in vitro assays for the analysis of SUMO protease activities. *Methods Mol Biol* 497:253–268
30. McCarter J, Bartlett B, Dang T et al (1999) On the control of oocyte meiotic maturation and ovulation in *Caenorhabditis elegans*. *Dev Biol* 205(1):111–128
31. Soderberg O, Gullberg M, Jarvius M et al (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods* 3(12):995–1000