Detection of protein SUMOylation *in vivo*

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The small ubiquitin-like modifiers (SUMOs) are posttranslationally conjugated to eukaryotic cellular proteins with generally unpredictable consequences. SUMO substrates are found in many cellular systems, and functional analysis has revealed that substrate SUMOylation often has an important role in their regulation. Here we describe a cell-based protocol which can be used to detect the SUMOylation of a protein that relies on the enrichment of SUMO conjugates by purification of 6His-SUMO under denaturing conditions, followed by western blotting for the protein of interest. By purifying under denaturing conditions this method not only reduces the risk of false-positive identifications by non-covalent interactions, but also preserves SUMO-substrate conjugates by inhibiting SUMO-specific proteases—two caveats that may complicate other less stringent purification methods. In preliminary form, this protocol takes 4–5 d to perform, and it can be further elaborated to provide a multi-angled approach to investigate protein conjugation by SUMO.

INTRODUCTION

The covalent linkage of molecular adducts to other proteins hugely expands the functional repertoire of the 20 different amino acids that can be strung together to form proteins. The small ubiquitin-like modifiers (SUMOs) are a sub-family of three post-translational modifiers (PTMs) that share sequence and structural similarity with ubiquitin[1,2], and genetic studies have shown that SUMO conjugation is required for cell viability[3–6]. These so-called ubiquitin-like modifiers (Ubls) are each capable of forming an isopeptide bond via their C-terminal carboxyl group to the ε-amino group of a lysine side-chain in a target protein[7]. For the targets of SUMOylation (proteins that undergo SUMO conjugation) this lysine is commonly found within a SUMO consensus conjugation motif ψKxD/E, where ψ is a hydrophobic amino acid, K the target lysine, x any residue and D/E an acidic residue[8,9]. SUMO-2 and SUMO-3 differ from each other in sequence by only three amino acids and have very similar conjugation profiles *in vivo* (**Fig. 1**), whereas SUMO-1 shares only ~45% sequence identity with them and has a significantly different substrate proteome[10]. Specifically, the constitutively modified protein RanGAP1 is largely conjugated to SUMO-1, with only a small fraction conjugated to SUMO-2/-3 (ref. 11). The downstream effects of SUMOylation are commonly mediated by the recruitment of effector proteins containing a SUMO interaction motif (SIM)[12–15]. Owing to the fact that lysine side-chains are potentially capable of accepting many PTMs in an exclusive manner, competition for modification sites may be important for some SUMO substrates[16,17].

If a protein is suspected of being a novel SUMO substrate, three important questions need to be answered: (i) is the protein linked covalently to SUMO? (ii) at which lysine acceptor residue(s) does the conjugation occur? and (iii) what is the functional consequence of SUMO conjugation?

When a protein is a suspected SUMO substrate before attempting experiments to verify the PTM, a good first point of reference is the published literature. Hundreds of SUMO substrates have been described previously, and a simple search of the literature should indicate whether the protein of interest is among them. Furthermore, proteomic studies of SUMO conjugates have expanded rapidly, and collectively describe in the region of 1,000 putative substrates from higher eukaryotes. Although the early studies are relatively small, for the larger ones[18–21] lists of 100s of putative conjugates can simply be downloaded and searched, potentially providing a useful lead in the search for evidence.

At a practical level a number of different experimental approaches can be taken to answer these three questions. In regard to the first, with the exception of a SUMO to substrate branched-peptide being identified by mass spectrometry from an

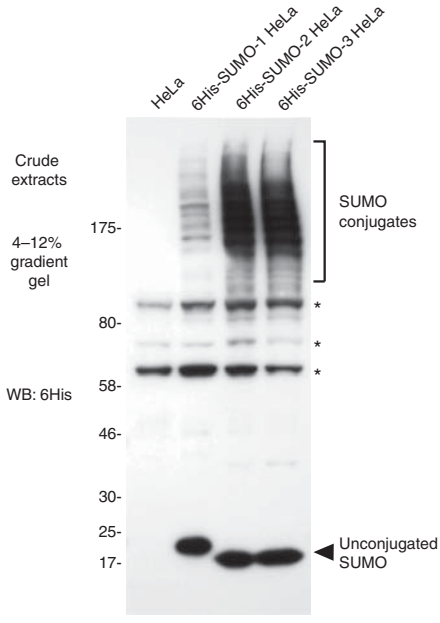

Figure 1 | Stable expression of the three 6His-small ubiquitin-like modifier (SUMO) paralogues in HeLa cells. Parent HeLa cells (HeLa) and the three HeLa cell lines stably expressing 6His-SUMO-1, 6His-SUMO-2 and 6His-SUMO-3 (as indicated) were lysed under denaturing conditions and fractionated on a 4–12% gradient gel. Western blot analysis using anti-6His antibody shows the clear expression of all three SUMO proteins reactive to the 6His antibody in both conjugated and unconjugated forms. Molecular weight markers (kDa) are shown, and non-specific bands are indicated by asterisks.

immunoprecipitation of an endogenous protein, no single experiment is sufficient to prove that a protein is SUMO modified. The next-best approach for the detection of a SUMO modified substrate is immunoprecipitation of the protein followed by western blot for SUMO(s), using only endogenous proteins. However, for most substrates it appears that only a low proportion of the cellular pool is modified by SUMO, making the detection of a SUMOylated protein without over-expression of SUMO or the substrate, technically very challenging (see ref. 10 for examples). In response to this, many techniques and methods have been developed to investigate substrate SUMOylation, which individually are not definitive, but together can generate sufficient evidence to make it reasonable to suppose that a test protein is genuinely a target for SUMO conjugation. Significantly, as many of the proteins that facilitate SUMO conjugation or deconjugation have been expressed in and purified from bacteria, this has allowed the development of biochemical assays to aid the identification of SUMO substrates[22,23]. Although these are powerful tools, the non-physiological conditions applied limit their biological relevance. As such, *in vitro* approaches alone cannot be regarded as proof of SUMOylation. *In vivo* evidence is essential.

This paper details a protocol that uses 6His-SUMO constructs stably expressed in HeLa cells, the 6His-SUMO proteins can be purified from extracts using nickel affinity chromatography under denaturing conditions. Any protein that has a covalent 6His-SUMO modification should be purified from the extract. These purifications can then be analyzed by western blot for any protein whose SUMOylation is suspected. We have used this technique for the enrichment of SUMO conjugates in a system where SUMO is expressed at levels similar to the endogenous protein[10], which limits the risk of over-expression-related artifacts. The protocol uses 6 M guanidine-HCl or 8 M urea to maintain denaturing conditions throughout the purification procedure, which provides at least two advantages over other methods to detect SUMOylation *in vivo*. First, false positives are minimized, as non-covalent interactions are almost completely eliminated and second, false negatives are less likely, as the SUMO proteases that rapidly deconjugate SUMO from substrates are denatured during the lysis and purification stages, so preserving the SUMO to substrate isopeptide bonds. As the basic chemistry of conjugation and deconjugation is common to ubiquitin and other Ubls, the protocol described here is widely applicable with little variation (see ref. 24 for examples).

This method has been previously used to identify a wide variety of proteins modified by SUMO and has also been adapted for proteomic studies of SUMO conjugates (see refs. 10,15,21,25,26 for examples). Therefore, in theory this procedure is applicable for the identification of SUMO conjugation to any cellular target, although in practice the success of the basic approach relies on a number of factors. Namely that the putative SUMO substrate (the test protein) is expressed in the 6His-SUMO HeLa cells, it is modified by SUMO under the conditions of the experiment, and that the degree of modification is high enough to be detectable by the technique. In most cases these limitations can be overcome by a number of variations to the method, such as transient expression of 6His-SUMO constructs in different cell types, the treatment of cells with agents that increase net SUMOylation (e.g., MG132 or heat-shock), or the over-expression of substrates or components of the SUMO pathway. However, it must be stressed that the results of such experiments should be considered carefully, due to the increased risk of false-positives. Therefore, perhaps the most important point to remember when investigating SUMO conjugation is that in the absence of definitive proof of conjugation, the more evidence gathered to support the SUMOylation of the test protein, the more confident the identification. A multi-angled approach incorporating a range of *in vitro* and *in vivo* studies is more favorable than any single method alone. Specifically, immunoprecipitations of a test protein under native conditions followed by western blotting for SUMO or an epitope-tagged form of transfected SUMO is also a common approach to further validating SUMO targets *in vivo* (see refs. 10 and 27 for examples).

Once a SUMO modified form of the substrate has been detected, this protocol can be modified using exogenous expression of mutant forms of the test protein, to identify the site(s) of SUMOylation. Although this assay cannot readily be adapted to answer the third question relating to the role of SUMOylation in the function of a particular protein, some experimental design considerations are largely substrate and assay-independent (see **Box 1** for details).

Experimental design

Cell lines. This protocol describes the use of HeLa cells stably expressing 6His-SUMO-1 (1–97) (accession number P62165), 6His-SUMO-2, (1–92) (accession number CAG46970) or 6His-SUMO-3 (1–93) (accession number CAA67897) at levels similar to the endogenous proteins[10]. The stable cell lines should be maintained under 2 μM puromycin selection, which can be omitted when cells are used for experimentation. Should it be desired that the 6His-SUMOs be introduced to a different cell type, plasmids for the transient expression of the same SUMO sequences (pCDNA3-6His-SUMO-1, SUMO-2 or SUMO-3), are available[28]. In these experiments caution should be taken to keep over-expression of the protein as close to physiological levels as possible. This can be done by titration of the amounts of transfected DNA introduced into cells followed by western blot of cell lysates, to determine suitable expression levels in comparison with the endogenous SUMOs from that particular cell line. Furthermore, preferential purification of RanGAP1 modified with SUMO-1 over SUMO-2 may also be a good indicator of close to physiological expression levels. Further adaptations to the protocol may also be required to accommodate the characteristics of the cells under analysis, such as the types of growth medium used, the transfection method and the cell harvest protocol. If, for example, there was concern that scraping cells into suspension risked excessive cell lysis, then trypsinization could be carried out before phosphate buffered saline (PBS) washes, or alternatively, cells could be left adhered to the plate, washed with PBS, then lysed *in situ*. This latter method requires further plates to be seeded for crude cell lysate analyses (see **Fig. 2**).

Preliminary experiment. The most logical first experiment is to attempt to detect SUMO-modified forms of the test protein from nickel affinity purifications from 6His-SUMO expressing cells. This can be done by simply carrying out the purification protocol from the different cell lines simultaneously; parent HeLa cells (SUMOylation negative control), 6His-SUMO-1 cells, and 6His-SUMO-2 (and 6His-SUMO-3 if required) cells, followed by western blotting for the test protein and the constitutively modified protein RanGAP1. RanGAP1 is preferentially modified by SUMO-1 although it is still detectably modified by SUMO-2/3, and so should give a very

BOX 1 | TESTING THE FUNCTIONAL SIGNIFICANCE OF SUMO CONJUGATION

Many of the principles applied to the validation of small ubiquitin-like modifiers (SUMO) conjugation *in vivo*, are also broadly applicable to the study of the functional consequences of SUMO modification, and have been successfully incorporated into many technically unrelated functional analyses. A good example is the gene reporter assay, which is used for the functional analysis of transcription factor (TF) or transcription cofactor (TcF) activity *in vivo*. A large proportion of SUMO substrates are involved in gene transcription[20,31,32], and so the role of SUMO in TF and TcF function *in vivo* has been relatively well documented. Transfection of a reporter plasmid in combination with a plasmid expressing wild type or a mutant form of the TF or TcF that can not be conjugated to SUMO, allows inference of the influence of SUMO on TF or TcF function by comparing reporter output activity among the different constructs. As with SUMO conjugation site identification, simply comparing wild-type to a lysine to arginine mutant is not conclusive, as the observed functional change may actually be a consequence of SUMOylation at another site (which is influenced by the mutation), or the conjugation of another PTM at the same site. Therefore, where appropriate, the analysis of mutants not only to the target lysine(s), but also the other residues within the ψKxD/E SUMO conjugation consensus motif[8,9] permits more confident association of any affects of mutation to SUMO conjugation at that particular site. Ψ and D/E mutants should give similar, albeit more moderate effects to that of the target lysine, while the 'x' residue should have little or no effect.

The use of SUMO enzyme over-expression or RNAi mediated inhibition of SUMO metabolizing proteins can further increase confidence in any conclusions of SUMO involvement in a protein function, especially where the target lysine is not found within a consensus motif. However, the potentially pleiotropic consequences of altered SUMO enzyme activity in a cell environment have to be considered when interpreting data from these experiments. A good example of the challenges in investigating the role of SUMO conjugation in protein function is that of the transcription factor p53 for which SUMO conjugation at lysine 386 has been reported to have both repressive and activating functions (see ref. 33 for a summary). Such issues reinforce the need for a multi-disciplinary approach to *in vivo* SUMO studies. A good example of the employment of many different experimental methods to investigate the modification of a protein with SUMO and its functional consequences can be found in reference 15 for the transcription cofactor p300.

clear positive response in a successful purification. A positive result for the test protein will show as an antibody-reactive band for the test protein in purifications from 6His-SUMO cells, but not from cells lacking 6His-SUMO. Also for a single modification, this form of the protein should have an apparent molecular weight ~10–20 kDa heavier than the unmodified protein. Higher molecular weight forms may also be visible, possibly representing multiply modified forms of the protein.

The design of this experiment depends on the availability of antibodies specific for the test protein. If an antibody suitable for western blotting from crude cell lysates is unavailable then epitope tagged forms of the protein can be introduced into the cells, using a transfection method preferred by the user. It is advisable to carry out titrations of the DNA encoding the tagged substrate followed by 6His-SUMO purifications to gauge the extent of over-expression required for a positive result, if obtained.

6His-tagged proteins are purified from protein mixtures by nickel affinity chromatography using nickel-nitrilotriacetic acid (NTA) agarose. The polyHis tag binds the matrix with micromolar affinity by the formation of a Ni^{2+} chelation complex between two histidine imidazole rings and a single NTA group covalently linked to the agarose. Imidazole elutes polyHis-tagged proteins by competing with histidines for nickel binding, although divalent cation chelating chemicals, low pH or reducing agents, can also be used to elute bound proteins. Cobalt affinity resins may also be applied to this technique although comparisons with nickel equivalents for purification efficiency have not been carried out to our knowledge.

The protocol is divided into two parts such that crude extracts are prepared from the same cells used for the nickel purification (**Fig. 2**). These can then be processed simultaneously or used for troubleshooting analysis later. For each dish of cells a single 200-µl crude cell lysate sample and a single 50-µl nickel affinity purification sample will be produced. Both samples are then analyzed by western blot using the same antibodies (see **Fig. 2**).

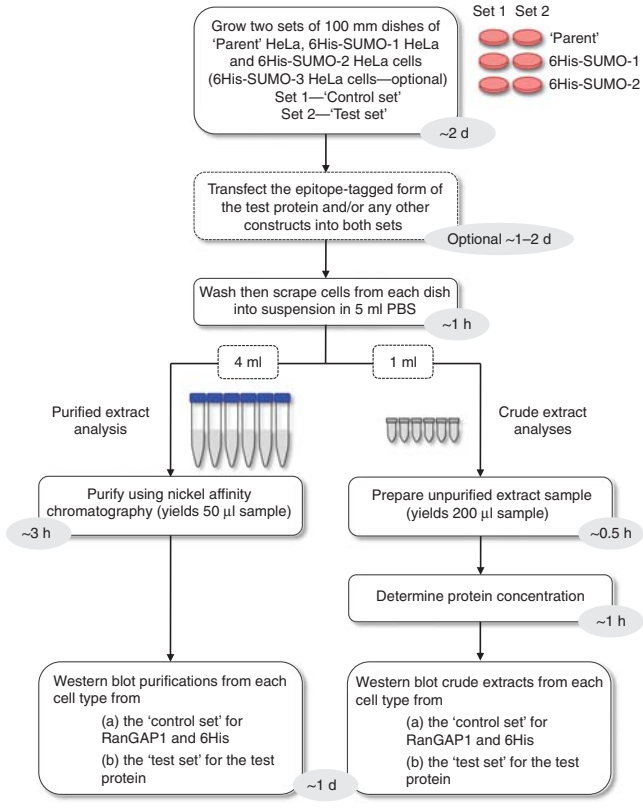

Figure 2 | Workflow of a typical *in vivo* analysis for protein SUMOylation for one test protein. The protocol is divided into two subsections, where crude cell extracts are prepared in parallel to the nickel affinity purifications from the same cell cultures. Western blots from the purified cell extracts are used to test for the enrichment of 6His-small ubiquitin-like modifiers (SUMOs), and SUMO modified forms of RanGAP1 and the test protein. The crude extract analyses allow the determination of the expression levels in the cells of 6His-SUMOs, RanGAP1 and the test protein. To study more than one test protein, more sets of cells (see top) are used.

The protocol described here is designed for the analysis of a single test protein. In this instance two sets of the 6His-SUMO-1, 6His-SUMO-2 and parent HeLa cells are processed in parallel. Extracts and purifications from one set of cells ('control set') are western blotted for RanGAP1 and 6His-SUMO to verify purification efficiency, and the other set ('test set') are blotted for the test protein (see **Fig. 2**). If more than one test protein is to be analyzed then the number of test sets is increased accordingly, although only one 'control set' is needed.

Regulating SUMO enzyme expression to confirm substrate SUMOylation. For a number of reasons initial studies investigating the SUMO conjugation of a particular protein may not be conclusive. As such variations to the basic assays can be adopted that rely on the transient over-expression or siRNA mediated suppression of components of the SUMO conjugation and deconjugation systems, with the view to altering the intensity of conjugation bands in western blots. Examples of over-expressed proteins include the substrate itself, SUMOs, wild-type Ubc9, dominant negative Ubc9 (C93S), E3 ligases, SUMO proteases and dominant negative SUMO proteases (active site-cysteine mutant). Expression of these proteins should affect the result of a nickel NTA purification in a logical way. Also, it may be possible to increase endogenous SUMOylation of a particular substrate by treatment of the cells with proteasome inhibitors, such as MG132[29], by heat-shock[20] or by any stimulus the user suspects may be an influence. However, caution should be applied when interpreting experiments that relied on a cell treatment or protein over-expression to produce a positive result when under untreated conditions there was none. Counter-intuitive responses or false positives substrate identifications may be much more likely under these conditions.

Identifying SUMO conjugation sites by mutational analysis. After a positive identification of a SUMOylated protein, the assays can be modified to identify the site of *in vivo* modification. Commonly a transient expression approach is taken, whereby cells are transfected with wild type or mutant variants of epitope-tagged forms of the test protein, followed by sample processing in a similar way to the initial studies. As mutation to arginine of a target lysine abrogates SUMOylation at that site, the disappearance of the conjugated band for a particular lysine–arginine mutant is a strong indicator as to the site of modification. However, it is important to note that if mutation of a lysine causes the disappearance of a SUMO-substrate conjugate, this is not necessarily proof that this lysine is conjugated by SUMO. This is merely an indication that this lysine *is required* for SUMO conjugation (potentially influencing modification at another site). Mutation to the hydrophobic and acidic residues of the SUMO conjugation consensus motif ψKxD/E is known to reduce conjugation efficiency at the consensus lysine[8,9]. As such, mutation to these residues but not the 'x' residue, within a suspected conjugation site, should affect conjugation in a similar way, although often to a lesser degree to mutation of the actual target lysine. This type of consensus mutational analysis is more rigorous and strongly implicates that a specific lysine is the target for SUMOylation.

MATERIALS

REAGENTS

- All materials required to culture and manipulate HeLa cells
- DMEM (Gibco, cat. no. 61965)
- Trypsin EDTA (Gibco, cat. no. 25300)
- Foetal bovine serum (Biosera, cat. no. S1830-500)
- Antibiotics as desired (such as penicillin and streptomycin)
- Puromycin dihydrochloride (Melford, cat. no. P0121)
- 6His-SUMO-1, 6His-SUMO-2 and 6His-SUMO-3 cells and parent HeLa cells (available upon request from the laboratory of RT Hay)
- Guanidinium-HCl (Merck, cat. no. 1.04220.5000) ! CAUTION Guanidinium-HCl is harmful, avoid exposure.
- Tris-(hydroxymethyl) aminomethane (VWR, cat. no. 103157P)
- $Na_2HPO_4.2H_2O$ (BDH, cat. no. 103834G)
- $NaH_2PO_4.2H_2O$ (BDH, cat. no. 301324Q)
- Urea (BDH, cat. no. 102908D)
- Triton X-100 (Fischer Scientific, cat. no. BP151-100)
- β-mercaptoethanol (Sigma, cat. no. M7154-100ML) ! CAUTION β-mercaptoethanol is toxic, avoid exposure.
- Imidazole (Sigma, cat. no. I0125-500G)
- Sodium dodecyl sulfate (Melford, cat. no. B2008)
- Glycerol (BDH, cat. no. 101186M)
- Bromophenol blue (BDH, cat. no. 443053A)
- Nickel-NTA agarose resin (Qiagen, cat. no. 1018244)
- Antibody specific for the protein of interest or an epitope tag if a tagged form of the protein is over-expressed.
- Anti-RanGAP-1 antibody (Zymed, cat. no. 19C7)
- Anti-6His antibody (Clontech, cat. no. 631212)
- PBS (Gibco, cat. no. 14190)
- BCA protein concentration assay kit (Thermo Scientific, cat. nos. 23228 and 23224)

EQUIPMENT

- Flat-ended pipette tips (Starlab, cat. no. I1022-2600) ▲ CRITICAL Flat ended tips minimize bead loss during supernatant aspiration steps.
- Cell scrapers (TPP, cat. no. 99003)
- Bench-top centrifuges (Heraeus Biofuge, Thermo)
- Liquid aspirator setup (Vacuum gas pump, VWR, cat. no. PM 20405-86)
- 15 ml plastic sample tubes (Greiner, cat. no. 188271)
- 1.5 ml screw-capped sample tubes (Alphalabs, cat. no. CP5515)
- 1.5 ml 'Lo-Bind' tubes (Eppendorf, cat. no. 022431081) ▲ CRITICAL It is important to use tubes with low protein-binding capacity to avoid proteins in wash buffers contaminating the final elution samples.
- Sonicator with probe appropriate for 200 µl and 5 ml samples (Branson digital sonifier 450)
- Polyacrylamide gel electrophoresis gels and equipment (As per user preference)
- Western-blotting materials and transfer equipment (As per user preference)
- Film processing equipment (As per user preference)

REAGENT SETUP

Sodium phosphate buffer For the desired pH, mix the indicated volumes of each 0.2 M phosphate stock solution, to give 50 ml of 0.2 M sodium phosphate buffer. This is then diluted 1:1 for use in the final solutions in the protocol (100 mM). pH can be checked before use and titrated as necessary.

pH (at 25 °C)	Volume 0.2 M Na_2HPO_4 (ml)	Volume 0.2 M NaH_2PO_4 (ml)
6.3	11.25	38.75
8.0	47.35	2.65

Cell lysis buffer (prepare fresh on the day of use) 6 M Guanidinium-HCl, 10 mM Tris, 100 mM sodium phosphate buffer pH 8.0. ! CAUTION Guanidinium-HCl is harmful, avoid exposure. ▲ CRITICAL Divalent cation chelating agents and reducing agents can strip the nickel ions from the resin. EDTA is safe up to 1 mM, DTT up to 5 mM and β-mercaptoethanol up to 20 mM in lysis and wash buffers. ! CAUTION β-mercaptoethanol is toxic, avoid exposure.

pH 8.0 wash buffer (prepare fresh on the day of use) 8 M Urea, 10 mM Tris, 100 mM sodium phosphate buffer pH 8.0, 0.1% (vol/vol) Triton X-100, 5 mM β-mercaptoethanol.
pH 6.3 wash buffer (prepare fresh on the day of use) 8 M Urea, 10 mM Tris, 100 mM sodium phosphate buffer pH 6.3, 0.1% (vol/vol) Triton X-100, 5 mM and β-mercaptoethanol. ▲ CRITICAL It is important to ensure that pH 6.3 wash buffer is not below pH 6.0. Protonation of histidines will cause dissociation from the nickel resin. Owing to the high osmolarity of the solution this is best checked with pH indicator paper rather than a pH electrode meter.

Elution buffer (store in aliquots at −20 °C for at least 1 year) 200 mM Imidazole, 5% (wt/vol) SDS, 150 mM Tris-HCl pH 6.7, 30% (vol/vol) glycerol, 720 mM β-mercaptoethanol and 0.0025% (wt/vol) bromophenol blue. ▲ CRITICAL SDS and reducing agent are present to keep as many proteins as possible soluble during the elution step.
2× Laemmli sample buffer (can be stored at room temperature (RT) (~15–25 °C) for at least 1 year) 5% (wt/vol) SDS, 25% (vol/vol) glycerol, 150 mM Tris-HCl pH 6.8, 0.01% (wt/vol) bromophenol blue and 0.7 M β-mercaptoethanol—added after protein concentration assay. ▲ CRITICAL β-mercaptoethanol interferes with the BCA protein assay. Add to the lysates after protein estimation and before western blot analysis.

PROCEDURE

Cell lysate prepraration ● TIMING ~2 h after cell growth

1| Grow cells in 100-mm diameter dishes such that you have two dishes per cell type (two for parent HeLas and two for each 6His-SUMO HeLas) to ~80–90% confluency in DMEM and 10% FCS (vol/vol). These are the 'test set' and 'control set' of plates (**Fig. 2**).

2| Remove medium and wash twice with 10 ml of PBS. You can use ice-cold PBS throughout if desired, but it is not required.

3| Add 5 ml of PBS and scrape cells into suspension using a cell scraper.

4| Transfer the cell suspension to a 15-ml tube.

5| Take 1 ml of each suspension and transfer to a new 1.5-ml tube. This gives you two tubes per 100-mm diameter cell-culture dish, one containing 4 ml and the other 1 ml of cell suspension.
▲ CRITICAL STEP When taking this sample make sure the cells are well suspended and trim the end of a 1,000 µl pipette tip to ensure easy passage of cells.

6| Spin the 4 ml cell suspension from step 5 at 3,000*g* for 5 min at RT. Remove the supernatant and lyse the cells by re-suspending in 5 ml of *'Cell lysis buffer'*. Proceed to step 11 for this sample.
! CAUTION Guanidinium-HCl in the cell lysis buffer is harmful, avoid exposure
■ PAUSE POINT Cell lysates from step 6 can be stored at −20 °C for up to 1 month.

7| Spin 1 ml of the cell suspension from Step 5 for 2 min, at ~1,000*g* at RT. Remove the supernatant and re-suspend the pellet in 200 µl of 2× Laemmli sample buffer.
■ PAUSE POINT Cell extracts from step 7 can be stored at −20 °C for at least 1 year.

8| Boil the samples from Step 7 for 5 min then disrupt each mechanically either by sonication for 10 s on low power or passing through a 19G needle 20 times using a 1-ml syringe.
▲ CRITICAL STEP Clean the sonicator probe between samples to avoid cross-contamination.

9| Determine protein concentration using the BCA assay (as described by the manufacturer).

10| Add β-mercaptoethanol to 0.75 M and store the samples for western blot analysis.
! CAUTION β-mercaptoethanol is toxic, avoid exposure.
■ PAUSE POINT Samples from step 10 can be stored at −20 or −80 °C for at least 1 year.

Nickel affinity chromatography to purify covalent SUMO conjugates ● TIMING ~5 h

11| Take the 5 ml cell lysates from Step 6 and add β-mercaptoethanol to 5 mM and imidazole to 5 mM then sonicate briefly (~30 s at medium power with a small probe).
▲ CRITICAL STEP Clean the sonicator probe between samples to avoid cross-contamination.

12| Centrifuge the lysates for 15 min, at 3,000*g*, at RT to sediment any large particulate material, and remove the supernatant carefully from the pellet.
▲ CRITICAL STEP Solid material may stick to the beads or be carried with beads during washes, contaminating the purifications.

13| Add the supernatant to 50 µl of a packed volume of new Ni^{2+} NTA sepharose or agarose beads that have been pre-washed three times with ten bead volumes of *Cell lysis buffer*.

14| Gently mix the beads with the lysates for 2–3 h at RT or 4 °C overnight.

TABLE 1 | Guide for fractionation of proteins on SDS-polyacrylamide gels.

Cell set	Sample	Cell type	Amount loaded on gel	Antibody	Reason for sample analysis
Control	Crude extract	Parent HeLa	20 μg		
		6His-SUMO-1 HeLa	20 μg	6His	Determines the expression levels of 6His-SUMOs
		6His-SUMO-2 HeLa	20 μg		
Control	Crude extract	Parent HeLa	20 μg		
		6His-SUMO-1 HeLa	20 μg	RanGAP1	Determines the expression levels of RanGAP1
		6His-SUMO-2 HeLa	20 μg		
Test	Crude extract	Parent HeLa	20 μg		
		6His-SUMO-1 HeLa	20 μg	Test protein	Determines the expression levels of the test protein
		6His-SUMO-2 HeLa	20 μg		
Control	Purified extract	Parent HeLa	2–3 μl		
		6His-SUMO-1 HeLa	2–3 μl	6His	Determines the efficiency of purification of 6His-SUMOs
		6His-SUMO-2 HeLa	2–3 μl		
Control	Purified extract	Parent HeLa	25 μl		
		6His-SUMO-1 HeLa	25 μl	RanGAP1	Confirms whether SUMO-modified proteins were purified
		6His-SUMO-2 HeLa	25 μl		
Test	Purified extract	Parent HeLa	25 μl		
		6His-SUMO-1 HeLa	25 μl	Test protein	Confirms whether the test protein was detectably SUMO-modified
		6His-SUMO-2 HeLa	25 μl		

SUMO, small ubiquitin-like modifier.

15| Spin the bead suspensions for 2 min at 750*g*, at RT and carefully remove the supernatant by pipetting.
▲ **CRITICAL STEP** It is not necessary to aspirate the beads dry. A small meniscus of 20–50 μl of liquid can be left on the beads.

16| Carry out the following washes by resuspending beads in the indicated volume of buffer followed by centrifugation for 2 min at 750*g*, at RT before removing the supernatant: Wash the beads with 4 ml of *Cell lysis buffer* containing 5 mM β-mercaptoethanol and 0.1% Triton X-100 (vol/vol). Wash with 4 ml of pH 8.0 wash buffer. Finally, wash the beads three times with 4 ml of pH 6.3 wash buffer.
▲ **CRITICAL STEP** It is not necessary to aspirate the beads dry. A small meniscus of 20–50 μl of liquid can be left on the beads.

17| Resuspend the beads in 1.5 ml of pH 6.3 wash buffer and transfer to a fresh 1.5 ml Eppendorf 'LoBind' tube, then centrifuge for 1 min at 6,000*g*, at RT and remove the supernatant using an aspirator fitted with a flat-ended pipette tip.
▲ **CRITICAL STEP** At this stage it is important to leave as little liquid as possible with the beads, but it is not necessary to aspirate the beads until dry. Remove liquid until no visible meniscus is above the beads and avoid loss of beads as much as possible.

18| Add 50 μl of elution buffer to each sample and flick to mix. Incubate for 20 min at RT to elute the proteins.
▲ **CRITICAL STEP** The samples can be boiled for 1–2 min if desired but the proteins should elute at RT.
■ **PAUSE POINT** The samples can be stored at −20 or −80 °C for at least 1 year.

Western blot analysis ● **TIMING** ~**1 d**

19| If frozen, thaw out samples from steps 10 and 18 thoroughly.

20| Centrifuge samples 5 min at 17,000*g*, RT.

21| Analyze the samples by western blot using your preferred SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting methods. Samples should be fractionated on SDS-PAGE as described in **Table 1**, or in a similar way appropriate to the experimental design.
? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Possible solution
21	Neither RanGAP1 nor the test protein was detected in purified extracts	Poor 6His-SUMO purification	Check to see if 6His-SUMOs were clearly enriched in the elutions from the purification from those cells (see **Fig. 3** for example). If not, then repeat the method carefully Make sure all buffers are at the correct pH
		Inactive antibodies	Did the antibodies react to the proteins in the crude cell extracts? If not, they may need replacing
21	RanGAP1 gave the expected pattern in the purified extracts but the test protein was not detected in them	Western blot exposure too short	Make sure blots are exposed until the background is observed
		The test protein is not expressed in HeLa cells or levels of expression are low	Was the test protein seen in crude cell extracts? If endogenous expression is low, try over-expression of the protein or an epitope tagged form of it. Or consider purifying transiently expressed 6His-SUMO conjugates from a different cell line that expresses the protein well
		The test protein is not detectably modified under these conditions	If the test protein seems to be abundant in the crude cell lysates, then consider over-expression of Ubc9 or stimulation of the cells with some treatment that you suspect may regulate the protein's sumoylation (i.e., heat shock or MG132)
		Inactive antibody	If the antibody gives no signal in crude or purified samples, then maybe it is no longer active. Try a new aliquot
21	The test protein or a non-specific contaminant was detected equally in all purified extracts including that from the parent HeLa cells	Non-specific binding of unmodified test protein or an antibody cross-reactant to the nickel beads	The stringency of the purification can be increased by any of the following methods: Adding or increasing imidazole in the lysis and wash buffers. This will need to be tested empirically from 5–50 mM imidazole Preincubating nickel resin with 0.01–0.05% BSA (wt/vol) in PBS. (Wash away BSA before equilibration with *Cell lysis buffer*) Adding 0.1% SDS (wt/vol) to pH 6.3 wash buffer for three of the four washes (the last wash being without SDS) Increasing the number or volume of washes. Try adding four more pH 6.3 wash buffer washes between Steps 16 and 17 Removing SDS and β-mercaptoethanol from the elution buffer will produce a more specific elution, although be aware that many proteins may not be soluble in this buffer and may precipitate on the beads Spinning the cell lysates from Step 11 at $>50{,}000g$ for 30 min at RT rather than the $3{,}000g$ for 15 min as described in Step 12, will remove smaller protein aggregates

● TIMING

Steps 1–10, Cell lysate prepraration: ~2 h after cell growth
Steps 11–18, Nickel affinity chromatography to purify covalent SUMO conjugates: ~5 h
Steps 19–21, SDS-PAGE and western blot analysis: 1 d

ANTICIPATED RESULTS

A positive result should look similar to the data shown in **Figure 3**. In this experiment nickel column purified samples were prepared from 6His-SUMO-1, 6His-SUMO-2 cells and non-6His expressing parent HeLa. Western blots from the nickel affinity purifications for 6His (**Fig. 3a**), RanGAP1 (**Fig. 3b**) and the test protein (**Fig. 3c**) show the enrichment of SUMO-modified proteins, and confirmed that RanGAP1 is a preferential SUMO-1 target, whereas the test protein is modified with both SUMOs, but preferentially by SUMO-2. Non-specific bands are those bands that appear in the control HeLa parental cells as well as the 6His-SUMO 1 and 2 HeLa cells. Neither RanGAP1 nor the test protein gave a signal in the purifications from control HeLa not expressing a 6His-SUMO construct. In most cases the blots for SUMO substrates yield single species as shown for RanGAP1 and the test protein here, however, it is not uncommon to see poly-SUMOylated proteins giving multiple species (see SART1 in ref. 10). Also, some unmodified proteins can be purified non-specifically by the nickel affinity chromatography (see p53 in ref. 25). As such it is important to accurately estimate the apparent molecular weight of the protein detected in the purified extracts and compare this with that of the unmodified protein in the crude cell extract, to clearly identify the SUMO modified species. One SUMO moiety typically causes a molecular weight shift of approximately 10–20 kDa to a modified substrate, although because of electrophoretic artifacts, the position of the target lysine within the substrate can affect the apparent molecular weight shift of a SUMO molecule (see PCNA in ref. 30 for a good example). In the example shown here (**Fig. 3**) both RanGAP1 (63.5 kDa) and the test protein (41.8 kDa) appear to have single copies of SUMO attached. The test protein antibody-reactive species in the crude extracts (**Fig. 3d**) shows a molecular weight approximately that expected of the unmodified protein, and shows comparable amounts of the test protein in each of the three cell lines. It is also common, as shown here (compare **Fig. 3c,d**), to find that SUMO-modified forms of some proteins are undetectable in crude cell lysates, due to very low proportions of the cellular pool of many substrates being modified by SUMO at any time.

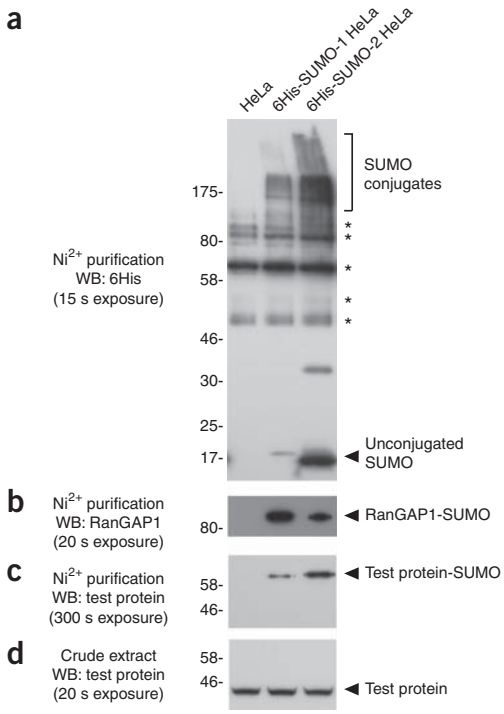

Figure 3 | Results of an experiment to determine if a test protein is modified by small ubiquitin-like modifier-1 (SUMO-1) or SUMO-2 *in vivo*. Anti-6His (**a**), RanGAP1 (**b**) and test protein (**c**) immunoblots from nickel affinity purifications from HeLa cells as indicated. The high molecular weight species reactive to the 6His antibody in **a** are proteins covalently conjugated to 6His-SUMO. Western blot for the target protein from crude cell lysates (**d**). Note the apparent absence of a modified form of the protein in spite of its purification from the same cells as shown in **c**. Molecular weight markers are shown (kDa), and non-specific bands are indicated by asterisks.

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AUTHOR CONTRIBUTIONS M.H.T. was involved in the development of the protocol and prepared the manuscript. M.S.R. created the 6His-SUMO cell lines, devised and refined the purification technique and edited the manuscript. D.P.X. was involved in developing and refining the technique and edited the manuscript. R.T.H. is the principal investigator that supervised the work and edited the manuscript.

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