High-stringency tandem affinity purification of proteins conjugated to ubiquitin-like moieties

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Published online 15 April 2010; doi:10.1038/nprot.2010.40

The post-translational modification of proteins with ubiquitin and ubiquitin-like proteins (Ubl) is vital to many cellular functions, and thus the identification of Ubl targets is key to understanding their function. In most cases, only a small proportion of the cellular pool of proteins is found conjugated to a particular Ubl, making identification of Ubl targets technically challenging. For the purposes of proteomic analyses, we have developed a protocol for the large-scale purification of Ubl-linked proteins that minimizes sample contamination with noncovalent interactors and prevents the cleavage of Ubl–substrate bonds catalyzed by Ubl-specific proteases. This is achieved by introducing a denaturing lysis step (in the presence of sodium dodecyl sulfate and alkylating agents that irreversibly inhibit Ubl proteases) before TAP (tandem affinity purification) that allows for efficient purification of putative Ubl-specific substrates in a form suitable for proteomic analysis. The timescale from cell lysis to purified protein sample is 5–6 d.

INTRODUCTION

Ubiquitin and ubiquitin-like proteins (Ubls) form a superfamily of post-translational modifiers. They share many similarities within sequence and structure, but the biological consequences of the modification cover broad and often distinct physiological effects[1–8]. Members of this family also share a common mechanism of conjugation, in which they are processed through a cascade of specific enzymes to be finally attached covalently onto the target protein[9,10]. This bond is formed between carboxy terminal glycine of ubiquitin or the Ubl and the acceptor amino acid (usually lysine) on a target protein. The covalent nature of this link to the substrate is resistant to denaturing conditions, which allows it to be differentiated from other noncovalent interactions[11]. The most widely known and studied members of this family are ubiquitin and the small ubiquitin-like modifiers (SUMO-1, SUMO-2 and SUMO-3)[12–14]. For practical reasons the acronym 'Ubl' will be used throughout this paper to define both ubiquitin and Ubls.

Proteomic approaches are rapidly becoming the methods of choice to provide systems-wide information on the post-translational conjugation of proteins with Ubls[15–18]. A number of approaches for identification of Ubl attachment sites, and thus validation of the data, have been developed. These methods, however, are not able to identify large numbers of Ubl conjugates in complex *in vivo*-generated peptide mixtures[18–21]. Taking only the SUMO subfamily of Ubl as an example, many different approaches have been taken, including immunoprecipitation of exogenously expressed Ubl[22,23] and nickel affinity purification of stably expressed histidine-tagged SUMOs[24]. However, low-stringency purifications or ineffective protease inhibition generally result in poor substrate to contaminant ratios, hence limiting the number of putative targets identified in a single experiment. Thus, there is requirement for a robust purification procedure that highly enriches Ubl-modified proteins from eukaryotic sources while limiting sample contamination with noncovalently bound species.

This paper details a large-scale protocol for enrichment of putative Ubl conjugates that is based on the highly stringent TAP method[25–27]. A number of different tandem purification affinity tags have been developed, but a systematic comparison revealed the Protein A and calmodulin-binding protein (CBP) combination to be most effective in the purification of low-concentration proteins[27]. TAP protocols were originally developed for the purification of noncovalent interactors, but our objective was to eliminate noncovalent interactions while taking advantage of the highly efficient purification strategy. We therefore modified the original procedure to allow cell lysis under denaturing conditions in the presence of alkylating agents that irreversibly inhibit Ubl proteases. The common chemistry of Ubl–substrate bonds means that this technique is broadly applicable across the Ubl superfamily. The proof of principle has been shown with the purification of SUMO-2 covalent conjugates from cultured human cells[15]. Here, HeLa cells stably expressing TAP-tagged SUMO-2 at close to endogenous levels were harvested in a buffer containing 2% (wt/vol) sodium dodecyl sulfate (SDS) and 10 mM iodoacetamide (IAA). SDS creates a denaturing environment, which dissociates proteins that noncovalently associate with TAP-SUMO-2, whereas IAA irreversibly inhibits SUMO-specific proteases by alkylation of their active-site cysteine residues, thereby minimizing SUMO deconjugation[28]. As these conditions are incompatible with the standard TAP protocols, the SDS concentration is reduced to 0.1% (wt/vol) by dilution and the purification is then carried out in the presence of 0.75 M NaCl to limit the association of proteins that refold during dilution. The resultant covalent conjugates are then purified from the refolded crude extracts using the two-step TAP protocol[26] by IgG affinity with TEV (Tobacco Etch virus) protease cleavage and then calmodulin affinity chromatography. After purification, owing to low protein concentrations, proteins are precipitated with trichloroacetic acid (TCA). From this point, samples can be analyzed as per user preference, although currently we use in-gel tryptic digestion of proteins fractionated by polyacrylamide gel electrophoresis (PAGE)[29], as this provides useful information on the observed molecular weights of identified proteins. See **Box 1** for details of downstream processing. The high stringency of the purification can be seen when comparing purifications from TAP-SUMO-2 and TAP-only purification (panel **d** of **Fig. 1**).

BOX 1 | APPLYING THE DENATURING TAP PURIFICATION PROTOCOL TO A PROTEOMIC STUDY

Independently of the denaturing TAP purification protocol described here, the individual user can decide on their own preferred methods of cell preparation, cell culture, protein digestion, MS analysis, data processing and bioinformatic analyses. However, it is important to briefly describe these steps in the framework of the entire procedure, which work together to form a single proteomic study. See **Figure 2a** for a schematic representation of how these additional steps (sections highlighted in yellow) from **Boxes 1** and **2** integrate with the TAP procedure described in this paper. Examples of the techniques that can be applied and the issues that might be considered are shown below:

(A) Cell preparation

1. Nonquantitative experiment—this can be done as a preliminary small-scale experiment or when the comparative quantitation is not necessary.
2. Quantitative proteomics (refs. 40,41 and also see **Box 2**)—to effectively discriminate contaminants from putative Ubl substrates, quantitative proteomic analysis is required. Techniques such as SILAC, iTRAQ, ICAT[42] or label-free[39] can be applied for quantitation.

(B) Protein separation

This might or might not be required, depending on the aims of the study and desired method of protein digestion (see point C).

1. SDS-PAGE fractionation—this is valuable when monitoring the molecular mass changes of Ubl substrates is important.
2. Off-gel separation[36]—allows for separation based on the isoelectric point of intact proteins in solution.

(C) Protein digestion

1. Choice of enzyme (trypsin, chymotrypsin, LysC, etc.)
2. In-gel (e.g., trypsin) digestion[29]—required when SDS-PAGE separation has been performed.
3. In-solution—this technique can be considered for better yields (but often compromised purity) than in-gel digestion and when advantages of SDS-PAGE fractionation are not important. Attention should be paid here to compatibility issues between trypsin and detergents (such as NP-40).
4. Filter-aided sample preparation (FASP)—this method should combine advantages of in-gel (good purity) and in-solution (good yields) digestions, but it has not been tested yet in combination with our proteomic analyses.

(D) MS sample preparation and analysis

1. STAGE (stop and go extraction) tip purification[43]—this can be an alternative method for efficient and simple desalting of small samples for MS analysis.
2. High-performance liquid chromatography system—the choice of the right column and running time may significantly influence the resolution and intensity of peaks, which in turn can affect the amount and quality of data.
3. Mass spectrometer—choice of equipment and settings will influence the final results. Instruments such as the LTQ Orbitrap (Thermo Scientific) provide the high sensitivity and accuracy required for such an analysis.

(E) MS data processing

1. MaxQuant[44]—this software suite has been well tested in proteomic analyses[15,45] and is appropriate for high-throughput quantitative analysis.
2. MS Quant—simpler but more labor-intensive predecessor of MaxQuant
3. Census[46]—has been successfully tested in combination with SILAC method by its developers but never for our TAP-Ubl analyses.

(F) Bioinformatic analysis

Many bioinformatic tools are currently available; hence, advice or collaboration with persons knowledgeable in the subject is recommended. Examples of such tools include AmiGO (http://amigo.geneontology.org), Panther or Ingenuity Pathway Analysis (IPA). For some examples of how data can be graphically represented, see Golebiowski *et al.*[15].

In combination with stable isotope labeling of amino acids in cell culture (SILAC), as a quantitative tool to separate putative substrates from purification contaminants[30–32] (also see **Box 2** and **Fig. 2**), we have successfully used this protocol to identify over 750 substrates of SUMO-2 (ref. 15). This represented a sixfold greater depth than studies using nickel purifications of 6His-SUMO[33], in spite of the fact that similar mass spectrometry (MS) equipment and data processing applications were used. Furthermore, the protocol has also been applied to the study of SUMO-1 (unpublished data), and the distantly related Ubl NEDD8 (ref. 34), further validating the technique and showing its potential application to ubiquitin and other members of the Ubl superfamily. This protocol assumes that some kind of quantitative methodology, such as SILAC, will be employed. SILAC, however, is not included here as a part of the major protocol, but only explained briefly and suggestions of how it can be integrated with TAP procedure are pointed out (see **Boxes 1** and **2** and TAP procedure for more details). A full description of the SILAC method is available and well described in literature[30–32].

A limitation to this technique is the requirement for cell lines stably expressing the TAP-tagged Ubl of choice, which, if not already available, will take some investment of time to generate. Another drawback is the relatively large quantities of starting material. This protocol begins with ~800 mg of cellular protein in crude extracts and ultimately yields ~20–30 μg of purified proteins, with an overall yield of free SUMO at around 20% of its initial pre-purification levels (**Fig. 2c** for more details). Additional methods of sample analysis such as FASP method[35], off-gel fractionation[36] and further advances in MS-related techniques might significantly drive down the required amount of starting material for these studies.

Analysis of Ubl substrates

Once the steps in this procedure have been followed, the results can be analyzed in various ways. A logical approach to the

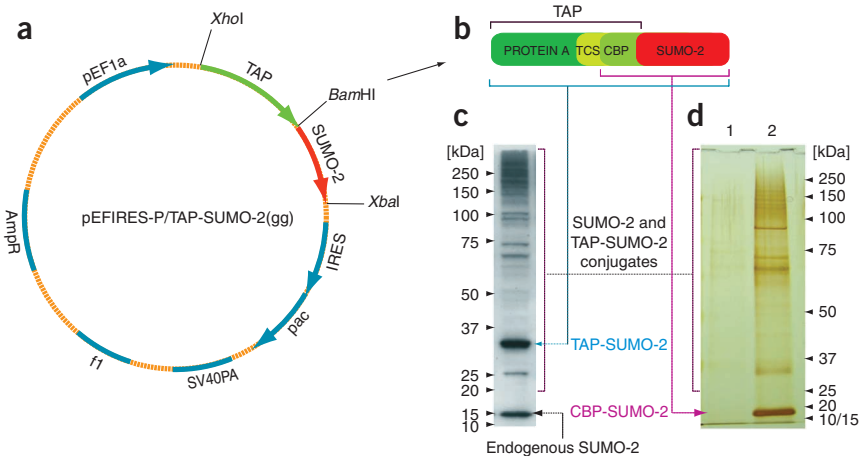

Figure 1 | The pEFIRES-P/TAP-SUMO-2 vector and its expression products. (**a**) Map of the vector pEFIRES-P encoding TAP-SUMO-2 fusion protein. (**b**) Schematic representation of the TAP-tag, in which TCS indicates TEV cleavage site and CBP, calmodulin-binding protein. (**c**) Western blot of the crude lysate of HeLa/TAP-SUMO-2 cells probed for SUMO-2 shows endogenous as well as TAP-tagged forms of SUMO-2 and respective conjugates above. Only clones expressing TAP-tagged version of SUMO-2 at levels comparable with its endogenous form were chosen for experimentation, as indicated. (**d**) Silver staining comparison between TAP-only (lane 1) versus TAP-SUMO-2 (lane 2) purifications. Note the high stringency of the purification protocol comparing lanes 1 and 2, although a substantial number of contaminants from lane 1 are still ultimately detected by mass spectrometry. This problem can be solved at the stage of data filtering, which is addressed in **Boxes 1** and **2**.

identification of Ubl substrates by MS is the detection of characteristic C-terminal fragments that remain covalently linked to the substrate after tryptic digestion of a Ubl-substrate conjugate (so-called branched peptides). Unfortunately, tryptic digestions do not always generate unambiguous branched peptides, as, e.g., ubiquitin, NEDD8 and ISG15, when cleaved by trypsin will leave an identical diglycine motif (GG) on the ε-amino group of the target lysine. Although diglycine-modified lysines can be readily searched for in MS data sets using sequence database search programs such as MASCOT or Sequest, they cannot definitively identify the modifier from which the residues originated. Furthermore, all three SUMOs leave extended C-terminal fragments from tryptic digestions, which are not amenable to high-throughput database searching because of the complex MS/MS fragmentation spectra. Therefore, until techniques are developed to circumvent these issues while still maintaining the integrity of the modification system, proteomic approaches aimed at identifying Ubl substrates will rely on the identification of proteins from within affinity-purified mixtures rather than the tryptic remnant of the isopeptide adduct. It should be kept in mind that diglycine artefacts can also be generated in certain conditions because of the action of IAA, but this can be resolved by replacing it with chloroacetamide; this issue is discussed in Nielsen *et al.*[37] In such a case, however, the efficiency of chloroacetamide against Ubl proteases should be carefully examined. IAA artefacts are not a concern in this protocol as free amino groups of Tris buffer used throughout our protocol prevent artificial formation of these adducts. Moreover, the major aim of the analysis described here is not searching for branched, but for just linear peptides.

Experimental design

In this section, we will describe considerations for the steps described in the procedure. It should be pointed out that the user can decide on their own preferred methods of cell preparation and culture, protein digestion, MS analysis, data processing and bioinformatic analyses. These are described in **Box 1**.

Developing stable cell lines expressing TAP-Ubl. This protocol relies on the availability of a cell line expressing a TAP-tagged version of the Ubl of interest. We have used a eukaryotic expression vector pEFIRES-P[38] that contains encephalomyocarditis virus (EMCV) and internal ribosome entry site (IRES), which directly

BOX 2 | QUANTITATIVE PROTEOMICS

Quantitative proteomics using stable isotope labeling of amino acids in cell culture (SILAC) is a means by which the relative amounts of a protein from different cell cultures can be determined. This can be used to resolve the issue of separating contaminants from genuine substrates in Ubl proteomic studies. Two or three different pools of cultured cells are grown in different batches of the medium containing stable isotopic forms of specific amino acids (e.g., arginine and lysine), thereby effectively creating culture-specific forms of all proteins, such that the origin of a protein from within a final mixture can be determined by the mass of the peptides detected[30,32]. In terms of TAP-Ubl proteomics, this has the advantage of allowing a direct comparison between cells expressing TAP-only and those expressing TAP-Ubl. At a practical level, for TAP-purified Ubl proteomic studies, two or three different pools of cells are grown in the order of 50 culture dishes of 15-cm diameter per SILAC condition. A good first SILAC experiment would be the comparison between untreated TAP-only cells, untreated TAP-Ubl cells and TAP-Ubl cells treated in some way known to affect the spectrum of Ubl conjugates. With such data, quantitative comparison of TAP-only with untreated TAP-Ubl allows the distinction between 'contaminants' and 'substrates,' whereas comparison between untreated and treated TAP-Ubl allows the determination of the change in the conjugation status of specific substrates under that treatment. For an example of the use of SILAC in a Ubl proteomic study, see Glebiowski *et al.*[15] and **Figure 2**.

It is important to note that when using SILAC, small amount of crude lysate (Step 9 of the PROCEDURE) separately from each batch of 50 plates before mixing should be taken for further analysis. These would serve firstly as a control for incorporation of SILAC-specific amino acids and secondly for determination of the changes in abundance of total protein levels and correct mixing of the three pools of lysates[15].

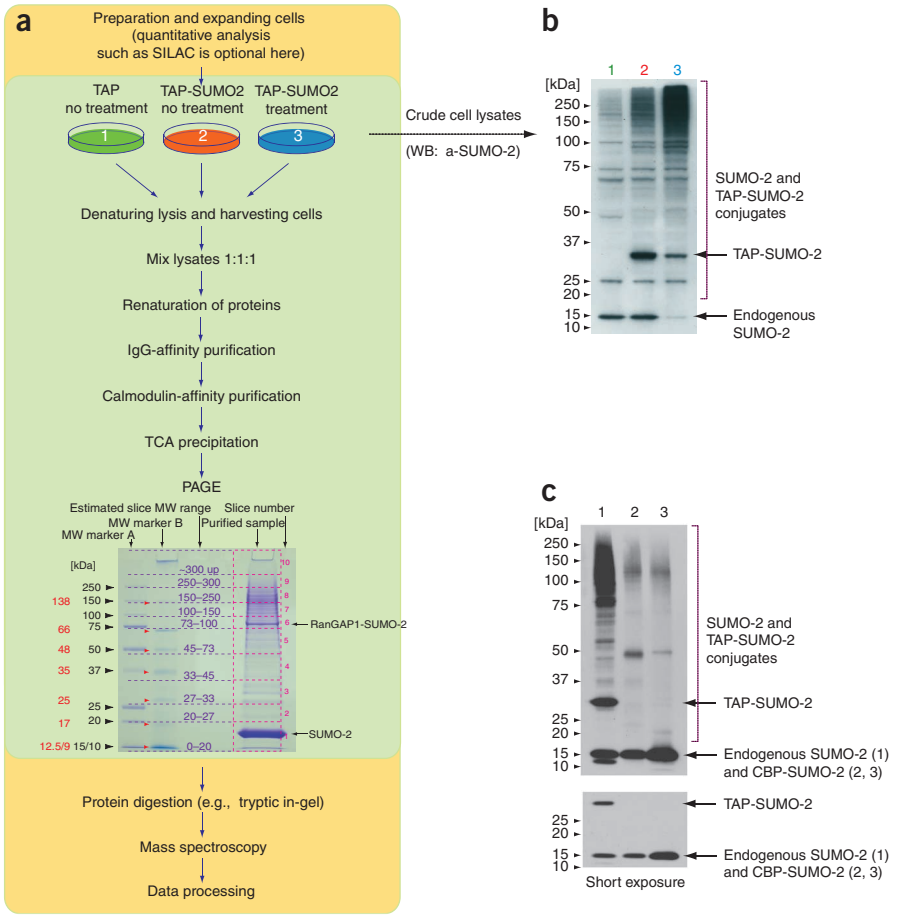

Figure 2 | Overview of the TAP procedure for SUMO-2. (**a**) TAP procedure workflow as described in this protocol. Green area: actual procedure of TAP purification including additional steps such as maintenance and lysis of the cells, denaturation and renaturation of the proteins, TCA precipitation and SDS-PAGE. This is presented in the context of the other steps required for a complete study. Yellow area: optional (SILAC or any other method necessary for quantitative analysis) or mandatory steps (bottom part), which complement the procedure indicated in the green area (See **Boxes 1** and **2** for more details). (**b**) Result of the heat shock experiment, in which crude extracts from HeLa/TAP (lane 1, 37 °C) and HeLa/TAP-SUMO-2 (lane 2, 37 °C and lane 3, 43 °C for 30 min) were analyzed by western blot probed against SUMO-2. Lanes 1, 2 and 3 represent the three cell culture conditions, as shown in the top part of the green area. In this example, treatment of TAP-SUMO-2 cells with heat shock (lane 3, 30 min at 43 °C) causes apparent increase of SUMO-2 and TAP-SUMO-2 conjugates when compared with untreated control maintained as normal at 37 °C (lane 2). At the same time, levels of free forms of SUMO-2 and TAP-SUMO-2 are being significantly reduced, whereas the ratio between the two remains approximately the same. This suggests comparable levels of incorporation of both SUMO-2 forms into conjugates; hence, the results of TAP-SUMO-2 purification should closely reflect the endogenous response. (**c**) Overview of the major steps of purification as evaluated by western blotting against SUMO-2. Lane 1, crude cell lysate (Steps 3–7 of the PROCEDURE), lane 2, supernatant after TEV digestion (Step 20 of the PROCEDURE) and lane 3, final sample after TCA precipitation (Steps 38 and 39 of the PROCEDURE). Total amount of starting protein material is around 800 mg (lane 1) and final sample yield is about 20–30 mg (lane 3). Ratios of equivalent protein amounts in lanes 1, 2 and 3 are 1, 10 and 50, respectively. Our estimation, as judged by western blot analyses, suggests that in this procedure, about 20% of free CBP-SUMO-2 can be recovered as compared with the input crude lysate.

links the expression of the gene of interest with that for puromycin resistance. In conjunction with pEF-1a promoter, this allows for expression of the desired protein. The mature form of SUMO-2 (residues 1–93) was subcloned into this vector, producing an N-terminally TAP-tagged fusion protein (**Fig. 1a**) with the C-terminal diglycine residue exposed. Importantly, as Ubls are covalently conjugated to their targets through this C-terminal motif, only N-terminal tag fusions can be considered. In addition, a vector expressing TAP-tag only was created to serve as a control. After stable transfection into HeLa cells, only clones expressing TAP-SUMO-2 at levels comparable with that of endogenous SUMO-2 were selected for further analysis (see **Fig. 1c**). Most eukaryotic expression vectors as well as cell lines should be applicable here. Such transfected HeLa cells are resistant to around 10 μM puromycin, although we find 2 μM is appropriate for selection.

Proteomic environment. While working with protein samples for MS analysis, great care should be taken to avoid keratin contamination. Although this is difficult to eliminate completely, it should be minimized, as excessive amounts of keratins in MS sample may significantly reduce numbers and quality of genuine target proteins identified. To prevent this, dedicated equipment used solely for proteomic analysis should be employed in a 'clean' environment using minimally handled reagents. When handling reagents dedicated to proteomic projects, fresh gloves and a lab coat should be worn, especially during the final stages of the PROCEDURE (Steps 38–41), wherein a laminar flow hood or similar is required.

Preliminary experiments. Bearing in mind the scale of proteomic experiments involving TAP-Ubl purification, it is worthwhile carrying out a few preliminary experiments to optimize experimental conditions such as treatment time points, drug concentrations etc. This might be achieved by western blotting of crude extracts in different experimental conditions (see **Fig. 2b**). In such a case, prepare each sample by lysing cells with standard Laemmli PAGE sample buffer (without reducing agents such as dithiothreitol (DTT) and β-mercaptoethanol, which should be added just before heating the sample for PAGE) supplemented with 10 mM IAA and measure the protein concentration to allow for even gel loading. If the analysis of crude extracts is not conclusive, then small-scale TAP purifications can be performed (see **Fig. 1d** and Steps 1–40 of the protocol). Comparison between TAP-Ubl and TAP-only samples, which can be done without employing SILAC and without mixing samples (see Step 10 of the protocol and **Figs. 1d** and **2b** for examples), could also be useful. In previous analysis performed by us[15], about 50% of proteins identified by MS have been validated during the data filtering as probable SUMO-2 conjugates and the remaining half as contaminants. Analysis of quantitative MS data

reveals that in terms of total abundance (not number of individual proteins), contaminants constitute only about 15% of the total purified sample.

Cell culture choices. Those interested in the substrate subproteome of a Ubl could simply follow the protocol described here for ~100 to 200 15-cm diameter cell culture dishes of their TAP-Ubl cells, and analyze the results by MS. However, because not every identified protein will be a true substrate, this creates the issue of filtering the real target proteins from the 'purification contaminants'. A number of techniques are available for the comparative quantitation of proteins, including iTRAQ (Applied Biosystems, Foster City, CA, USA) and label-free methods[39], but it is the metabolic labeling technique known as SILAC (see **Box 2**) that is most commonly used in the Ubl field. By quantitatively comparing a control data set, such as TAP-only purifications, with the test set (TAP-Ubl), an informed judgement can be made on which of the hundreds of identified proteins are most likely to be Ubl conjugates based on the difference in abundance of specific proteins (see Golebiowski *et al.*[15] for an example)[30].

Purification monitoring. It can be informative to collect small samples of elutions and resins at different stages of the purification, e.g., in Steps 7, 12, 14, 18, 20, 29 and 39. This allows assessment of the efficiency of particular steps by silver staining or western blotting.

Timing. To produce a purified protein sample ready for analysis from cultured cells takes around 5–6 d to complete. However, it typically takes 6–8 d to culture cells prior to protein extraction and purification, and 1–2 d to extract peptides from polyacrylamide gels ready for MS analysis. Beyond MS data collection, downstream processing can take much longer than sample preparation (see **Box 1**).

MATERIALS

REAGENTS

- *Materials related to cell culture:*
  - HeLa/TAP, HeLa/TAP-SUMO-2 or -1 cells (RT Hay laboratory)
  - Dulbecco's modified Eagle's medium (Gibco, cat. no. 61965)
  - Trypsin EDTA (Gibco, cat. no. 25300)
  - Fetal bovine serum (Biosera, cat. no. S1830-500)
  - Antibiotics as desired (such as penicillin and streptomycin)
  - For materials related to SILAC cell culture and labeling see refs.15,30,32 and **Box 2**.
- IAA (Sigma, cat. no. I-6125) **! CAUTION** IAA is toxic; hence, avoid direct exposure.
- β-mercaptoethanol (Sigma, cat. no. M-7154) **! CAUTION** β-mercaptoethanol is toxic; hence, avoid direct exposure.
- NP-40 (Calbiochem, cat. no. 492016)
- SDS (Melford, cat. no. B2008)
- Imidazole (Sigma, cat. no. I-0125)
- EDTA (Sigma, cat. no. E-5134)
- EGTA (Fluka, cat. no. 03777)
- Tris base (Sigma, cat. no. T-6066)
- Complete, EDTA-free protease inhibitor (Roche, cat. no. 11873580001)
- Glycerol (BDH, cat. no. 101186M)
- Bromophenol blue (BDH, cat. no. 443053A)
- DTT (Formedium, cat. no. DTT010)
- Sodium chloride (Sigma, cat. no. S-7653)
- Magnesium acetate (Sigma, cat. no. M-2545)
- Calcium chloride (Fisher Scientific, cat. no. C/1500/53)
- Sodium hydroxide (VWR, cat. no. 28244.295)
- TCA (BDH, cat. no. 304894L)
- Deoxycholate (DOC; Calbiochem, cat. no. 264101)
- LDS sample buffer (4×; Invitrogen, cat. no. B31010)
- Reducing agent for LDS sample buffer (10×; Invitrogen, cat. no. NP0004)
- Coomassie R-250 (BDH, cat. no. 443283M)
- MOPS buffer (Invitrogen, cat. no. NP001)
- IgG–sepharose 6 Fast Flow (GE Healthcare, cat. no. 17-0969-01)
- Calmodulin–sepharose 4B (GE Healthcare, cat. no. 17-0529-01)
- TEV protease (~1 U/μl; made in-house; unit definition/activity has been established by comparison with ProTEV protease, Promega, cat. no. V6051—see manufacturer's description for details)

EQUIPMENT

- Flat-ended pipette tips (Starlab, cat. no. I1022-2600)
- Flat-ended tips minimize bead loss during supernatant aspiration steps.
- Cell scrapers (TPP, cat. no. 99003)
- Bench-top centrifuge (Heraeus Biofuge—Thermo Scientific)
- Ultra-speed centrifuge (Beckman-Coulter, Avanti J-25 and rotor type 70 Ti)
- Liquid aspirator set-up (Vacuum gas pump; VWR, cat. no. PM 20405-86)
- 'Falcon' plastic sample tubes (15 and 50 ml; Greiner, cat. nos. 188271 and 210261, respectively)
- Screw-capped sample tubes (1.5 ml; Alphalabs, cat. no. CP5515)
- 'Lo-Bind' tubes (1.5 ml; 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 ~30 ml samples (Branson digital sonifier 450; Branson)
- PAGE gels and equipment. **▲ CRITICAL** These can be used as per user preference, but use of 10% (wt/vol) precast gels, such as Invitrogen, cat. no. NP0301, are suggested; in case of electrophoresis setup from different manufacturer, appropriate running and sample buffers should be used instead of the ones mentioned in this protocol.
- 5 and 10 ml plastic disposable syringes
- Needles (~19 G)
- Minisart single-use filter units (0.2 and 0.45 μm; Sartorius Stedim Biotech, cat. no. 16534 and 16555, respectively)
- Miscellaneous items necessary to set up gravity-flow purification system, such as plastic disposable columns, tubes, taps, bottles, rack, etc. Alternatively, one may wish to use his/her own pump-assisted system such as fast protein liquid chromatography, but we find in-house-assembled gravity system as the safest option.
- Hematological roller mixer Stuart SRT-9 (Stuart Scientific)
- Sterile scalpels

REAGENT SETUP

pEFIRES-P vectors expressing TAP, TAP-SUMO-1 and TAP-SUMO-2 proteins, HeLa cells with stable expression of these proteins and restriction maps are available from us on request. For details regarding pEFIRES-P vector, see **Figure 1a,b** and Hobbs *et al.*[38].

Lysis buffer 50 mM Tris-HCl pH 8.0, 2% (wt/vol) SDS, 10 mM IAA, 1 mM EDTA, Roche Complete Protease Inhibitor (EDTA-free). Prepare 150 ml by mixing the following: 7.5 ml 1 M Tris/HCl pH 8.0, 15 ml 20% (wt/vol) SDS, 7.5 ml 200 mM IAA, 300 μl 500 mM EDTA pH 8.0, Roche Complete Protease Inhibitor tablets as recommended by manufacturer (usually three big tablets for 150 ml), water up to 150 ml. Store (without IAA and Roche protease inhibitors) for up to a month in room temperature (RT). **▲ CRITICAL** IAA for all the buffers should be prepared best as 200 mM solution in water and stored at −20 °C protected from light. Add IAA and Complete protease inhibitor just before use and store at room temperature. **! CAUTION** IAA is toxic; avoid exposure.

TAP base buffer (TBB) Composition of 2× solution: 100 mM Tris pH 8.0, 1.5 M NaCl. This buffer serves as a basic buffer for making up other buffers: renaturation buffer, TEV buffer, calmodulin elution buffer and calmodulin binding buffer. Prepare 2.3 l by mixing the following: 230 ml 1 M Tris pH 8.0, 202 g NaCl, water up to 2.3 l. Store for up to a month in 4 °C.

Renaturation buffer (RB) 50 mM Tris-HCl pH 8.0, 0.75 M NaCl, 1% NP-40, 2 mM IAA, 0.5 mM EDTA. Prepare 4 l by mixing the following: 2 l TAP base buffer, 40 ml 100% NP-40, 40 ml, 200 mM IAA, 4 ml 0.5 M EDTA, water up to 4 l. **▲ CRITICAL** Prepare on the day fresh and store at 4 °C until required.

TEV buffer 50 mM Tris-HCl pH 8.0, 0.75 M NaCl, 1% (vol/vol) NP-40, 1 mM DTT, 0.5 mM EDTA. Prepare 200 ml by mixing the following: 100 ml TAP base buffer, 10 ml 20% (vol/vol) NP-40, 200 µl 0.5M EDTA, 200 µl 1M DTT, water up to 200 ml. ▲ **CRITICAL** Prepare on the day fresh and store at 4 °C until required.

Calmodulin elution buffer (CELB) 50 mM Tris-HCl pH 8.0, 0.75 M NaCl, 1 mM DTT, 1 mM Mg Acetate, 1 mM imidazole, 1% (vol/vol) NP-40, 10 mM EGTA. Prepare 40 ml by mixing the following: 20 ml TAP base buffer, 40 µl 1M DTT, 40 µl 1 M MgAc, 40 µl 1 M imidazole, 2 ml 20% (vol/vol) NP-40, 800 µl 0.5 M EGTA, water up to 200 ml. ▲ **CRITICAL** Prepare fresh on the day and store at 4 °C until required.

Calmodulin binding buffer (CBB) 50 mM Tris-HCl pH 8.0, 0.75 M NaCl, 1 mM DTT, 1 mM Mg Acetate, 1 mM imidazole, 1% (vol/vol) NP-40, 2 mM $CaCl_2$. Prepare 200 ml by mixing the following: 100 ml TAP base buffer, 200 µl 1M DTT, 200 µl 1 M MgAc, 200 µl 1 M imidazole, 10 ml 20% NP-40, 400 µl 1M $CaCl_2$, water up to 200 ml. ▲ **CRITICAL** Prepare fresh on the day and store at 4 °C until required.

LDS sample buffer Prepare 300 µl of 1.2× solution by mixing the following: 90 µl 4× LDS buffer, 36 µl 10× reducing agent, 175 µl of water. ▲ **CRITICAL** Prepare fresh on the day and store at RT until required.

TCA/DOC solution Prepare 5 ml of 100% (wt/vol) solution by dissolving 5 g TCA in water to achieve final volume of 5 ml and add 25 µl of 2% (wt/vol) DOC (deoxycholate). Mix thoroughly and store at RT for up to a month.

Stain/desatain solutions for PAGE These should be made up as normal according to common laboratory recipies for Coomassie-based staining but using exclusively 'proteomics-only' clean chemicals.

BCA protein assay kit Prepare and use as described by manufacturer (Thermo Scientific, cat. no. 23225).

PROCEDURE

▲ **CRITICAL** This protocol describes a 150-dish (15 cm) experiment that could be used for a 3 × 50-Petri-dish SILAC study. Alternatively, this protocol can be applied to unlabeled cell study of 150 Petri dishes. All buffer volumes, timing and other parameters in this protocol are calculated with the assumption of processing 150 Petri dishes in one experiment, but this can be scaled up or down if desired. Also see 'Experimental design' and **Box 2** for more information.

Preparation and processing of cell lysates ● TIMING after cell growth: harvesting of the cells (Steps 2–7) usually 6–9 h and lysate processing (Steps 8–12) 4–6 h

1| Grow cells to 80–90% confluency in 15-cm diameter Petri dishes containing Dulbecco's modified Eagle's medium, 10% (vol/vol) fetal calf serum and appropriate antibiotics.
? TROUBLESHOOTING

2| Aspirate the medium from the first plate and wash twice with PBS, then remove PBS by aspiration.
▲ **CRITICAL STEP** This step is performed in RT to avoid chilling the dishes, which may cause SDS precipitation during the lysis step.

3| Add 3 ml of fresh lysis buffer to the first Petri dish, scrape cells into lysis buffer using a cell scraper and remove the (gloopy) solution with syringe through a ~19 G needle at RT.

4| Repeat Step 3 for another three Petri dishes, transferring the lysate with the syringe from one dish to another at RT.

5| Transfer the lysate from the set of four processed Petri dishes into a 50 ml falcon tube placed on dry ice.
▲ **CRITICAL STEP** This allows for rapid freezing of lysates thereby avoiding SDS precipitation.

6| Repeat Steps 2–5 for the next four Petri dishes and continue until all 50 are harvested.

7| Repeat Steps 2–6 for the other two pools of 50 Petri dishes to get three separate lysates (usually ~50 to 70 ml each).
■ **PAUSE POINT** Cell lysates obtained from Step 7 can be stored at −80 °C for at least several months.

8| If frozen, thaw cell lysates from Step 7 thoroughly by agitation in water bath at RT.

9| Sonicate the individual lysates thoroughly (~5 × 1 min, large probe, 10–20% power) until viscosity is significantly reduced. *Optional*: After this point, about 0.5 ml of each lysate may be saved separately for further control analyses. For more information, see Box 2 and Ong SE *et al.*[30].
▲ **CRITICAL STEP** Avoid sample overheating or cooling to the point of SDS precipitation—temperature range of 20–35 °C should be safe.

10| Measure the protein concentration in each lysate using the BCA assay (as described by the manufacturer) and combine them in 1:1:1 ratio (wt/wt/wt) followed by two further bursts of sonication. (Store lysates at RT in the meantime.)

11| Spin lysates for 1 h, at 100,000–180,000*g* at RT. After centrifugation, a small glassy pellet should be visible. A clear middle layer will also be apparent with a lipid-containing cloudy upper layer. While waiting for centrifugation step to complete, IgG affinity column can be prepared as described in Step 13.
? TROUBLESHOOTING

12| Carefully remove only the middle clear layer. This should constitute about 70–90% of the sample. Clarify the remaining cloudy liquid by repeatedly filtering through 0.2–0.45 µm syringe-fitted filters until clear and then add to the rest of the sample. Filter the entire lysate pool once more at RT.

Purification—stage I (IgG affinity and TEV cleavage) ● TIMING ~2 d (usually, IgG affinity purification overnight takes 12–17 h and TEV digestion another ~36 to 42 h)

13| Prepare an IgG affinity column by loading IgG–sepharose bead slurry into a new, disposable 6 ml plastic column until about 3.5 ml of beads are packed and equilibrate with 60 ml of RB at RT.

14| Refold the denatured proteins by mixing the lysate supernatant with 25 volumes of RB in a large clean glass bottle—it should be around 3.5–4 liter. Stirr RB quickly and inject the lysate supernatant slowly into the center of the vortex (~20 ml min^{-1}) and mix gently for further 5–60 min at 4 °C.
▲ **CRITICAL STEP** Make sure that IAA is freshly prepared and protected from light at this step.

15| Pass the refolded protein solution over the IgG–sepharose column at a rate of ~200 to 300 ml h^{-1} at 4 °C. Store the flow-through at 4 °C in case of problems.
▲ **CRITICAL STEP** If using pump-assisted flow system, it must be made sure that the beads are not allowed to dry out. If using gravity-flow system, check the flow rate and remember that normally, it slows down over time slightly.

16| Wash the column with 50 ml RB at 4 °C.

17| Wash the column with 150 ml TEV buffer at 4 °C.

18| Transfer beads to 15-ml falcon tube and wash with 3 × 10 ml TEV buffer (centrifuge at slow speed to pellet the beads), then adjust to a final volume (beads + TEV buffer) to 7 ml. Remaining TEV buffer (at least 5 ml) can be saved for Step 21 at 4 °C.

19| Add 500 U of TEV protease and rotate for 12–16 h on a roller. Add a further 200 U of TEV protease and incubate for ~8 h and then add a further 200 U of TEV protease and incubate for a further 12–16 h—around 36–42 h in total at 4 °C. See 'REAGENTS' for unit definition.

Purification—stage II (calmodulin affinity) ● TIMING 5–6 h

20| Take IgG bead suspension from Step 19 (containing TEV protease and cleaved-off proteins) and load onto a new disposable 6-ml column and collect flow-through into a new 50-ml tube at 4 °C.

21| Wash the column with a further 3 × 1 ml TEV buffer to elute the remaining proteins and collect the flow-through to give a total of ~7 ml at 4 °C.

22| Wash the IgG bead column with 35 ml CBB and collect the flow-through.

23| Mix ~7 ml TEV eluate (from Step 21) with 35 ml CBB flow-through (from Step 22) and add 15 µl 1M $CaCl_2$ (~42 ml in total) at 4 °C.

24| Prepare the calmodulin–sepharose affinity resin by washing ~1.2 ml of calmodulin–sepharose with 2 × 13 ml CELB in a 15 ml falcon tube at 4 °C.

25| Equilibrate the resin by washing with 3 × 13 ml CBB and transfer it to a 50 ml falcon tube at 4 °C.

26| Combine ~42 ml of sample (from Step 23) with ~1.2 ml of pre-washed calmodulin beads in a 50 ml tube and rotate for ~2 to 3 h on a roller at 4 °C.

27| Load the calmodulin beads onto a new 6-ml disposable column and collect the flow-through. Recirculate this flow-through over the column. Keep the final flow-through in case of problems.

28| Wash the column with 6 × 5 ml CBB at 4 °C.

29| Elute the calmodulin-binding proteins by adding 7–10 × 1 ml CELB to the column and collecting 7–10 1 ml fractions in Lo-Bind eppendorf tubes at RT or 4 °C.
■ **PAUSE POINT** Fractions from Step 29 can be snap-frozen and stored at −80 °C for at least several months or used directly in Step 30.

TCA precipitation ● TIMING 2–3 h
30| Take the samples from Step 29 and add 330 µl of TCA/DOC per ml of protein solution and incubate on ice for 30 min.

31| Spin the tubes for 15 min at 16,000*g* at 4 °C. The result of this step is usually a bi-phasic solution with a glassy liquid pellet at the bottom of the tube and (often hardly visible) foggy layer on top of it.

32| Remove as much of the upper layer as possible from each tube but without taking any of the lower protein-containing layers. Keep the supernatant at 4 °C in case of problems.

33| Resuspend the pellet from one tube into 0.5 ml acetone and use this solution to sequentially pool all the pellets into a single tube. Note that acetone does not need to be cold (RT or 4 °C).

34| Take another 0.5 ml of acetone and wash each tube sequentially with it at RT or 4 °C. This will give about 1.2 ml of solution of combined pellets in acetone in a single tube.

35| Spin this tube for 15 min at 16,000*g* at 4 °C.

36| Remove the supernatant. Note that white pellet may be visible here.
? TROUBLESHOOTING

37| Wash the surface of the pellet gently three times with acetone (spin for 5 min at 16,000*g*), without disturbing the pellet at RT or 4 °C.

38| Air-dry the pellet (laminar hood or speed-vac) and process as desired. See **Box 1** (points B and C) for alternatives regarding further processing of the sample.

SDS-PAGE and processing the gel at RT ● TIMING 12–24 h including overnight staining/destaining of the gel
39| Fractionate the sample by SDS-PAGE as desired. We use 10% (wt/vol) NuPage Bis-Tris precast gels and LDS sample buffer from Invitrogen and run the sample one-half to three-fourth way down the gel, but it can be run further if a very fine separation is needed. Remember to leave at least two lanes free (no markers there) on each side of the sample and apply protein weight marker gently, so that nothing contaminates the sample in the analyzed lane. Any other precast gel system with appropriate sample and running buffers is also applicable here.

40| Stain and destain the gel by ones preferred method (such as coomassie-based staining) and take a photograph.
? TROUBLESHOOTING

41| Cut the sample lane into the desired number of slices and then chop them into very small ~1 mm^3 pieces, using a new scalpel for each slice to prevent cross-contamination. For further procedures, see **Box 1**.
■ **PAUSE POINT** Samples (gel pieces) can now be stored at −80 °C for at least several months.

● TIMING
Steps 1–7, Harvesting of the cells: 6–9 h
Steps 8–12, Lysate processing: 4–6 h
Steps 13–19, Purification—stage I (IgG affinity and TEV cleavage): ~2 d
Steps 20–29, Purification—stage II (calmodulin affinity): 5–6 h
Steps 30–38, TCA precipitation: 2–3 h
Steps 39–41, SDS-PAGE and processing the gel at RT: 12–24 h

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	Cells are not growing well or are dying	One or more of the medium components is missing, expired or supplemented inaccurately	Always double-check if all components of the medium are added—amino acids including glutamine, serum, antibiotics, etc. This applies especially to the SILAC medium
11	After centrifugation, all or most of the sample is cloudy	Usually not sufficient centrifugation speed	Aspirate off all supernatant without dividing into clear and cloudy part and filter several times until a clear solution is obtained as described in Step 12
36	No visible pellet	Unknown	There seems to be no correlation between the size of the pellet and the amount of protein present. Do not worry about this and proceed with the protocol
40	Low yields of purified proteins, as seen on coomassie-stained gel	IAA can be inactive because of extensive and prolonged light exposure or because of other factors. This is most important in Steps 3 and 14	Make sure IAA powder is not expired and has been stored correctly. Stock solution should be prepared as described in reagents section for lysis buffer and RB buffer. Make sure it is properly dissolved before use
		TEV digestion could be inefficient	Make sure that TEV protease is active. Take the samples of beads before and after TEV digestion (Steps 18–20) and test with western blot, coomassie or silver staining for cleavage efficiency. Keep IgG–sepharose beads after digestion in case an additional cycle of digestion is required

ANTICIPATED RESULTS

In the majority of cases, only a small fraction of the total cellular pool of a SUMO target protein is actually modified at a given time. Owing to the dynamic and highly labile nature of Ubl conjugates, it is not easy to preserve and recover proteins modified in such a way. Our protocol requires a relatively large amount of initial material (~3 × 10^9 cells) when compared with procedures such as western blot. This produces ~500–800 mg of protein in the crude lysates and ultimately yields between 20 and 30 µg of protein in the purified sample. A typical coomassie-stained gel of one such purification is shown in **Figure 2a**. When analyzed as described in Golebiowski *et al.*[15], a SUMO-2 purification of this scale should identify ~1,000–2,000 proteins. In the example presented in **Figure 2a**, two pronounced single bands visible by coomassie staining are SUMO-2 alone at the very bottom and RanGAP1-SUMO-2 conjugate in the middle/upper part of the gel. All the other recovered proteins create less defined and less intense bands due to low abundance. For in-gel digestion and further analysis, the gel can be cut into as many slices as desired. We recommend cutting the gel into 5–15 slices with the higher number being applied if the later analysis is going to focus on the migration of species throughout the gel. Such analyses can be used to estimate the extent of Ubl-modification of a substrate, bearing in mind that each SUMO-2 moiety increases apparent molecular weights by 15 kDa on average. In fact, however, the SUMO-2 moiety purified during our experiments is still fused to the CBP fragment of the TAP-tag (**Fig. 1b,d**). This adds another 5.5 kDa to the mass of SUMO-2, although the mass difference between this and endogenous SUMO-2 is not always apparent on SDS-PAGE gels. For some examples of bioinformatic analyses and data presentation, refer to **Box 1** and Golebiowski *et al.*[15].

ACKNOWLEDGMENTS F.G. is supported by the Marie Curie Fellowship and Biotechnology and Biological Sciences Research Council. M.H.T. is supported by a Cancer Research UK program grant.

AUTHOR CONTRIBUTIONS F.G. and M.H.T. were responsible for optimization of the protocol's efficiency and specificity, as well as for its integration with quantitative proteomic applications. A.N. originally applied and modified the standard TAP procedure to facilitate purification of Ubl covalent conjugates. R.T.H. provided valuable advice and discussion.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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