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Corresponding Author:	Malu Martínez-Chantar Center for Cooperative Research in Biosciences Derio, Bizkaia SPAIN
Corresponding Author's Institution:	Center for Cooperative Research in Biosciences
Corresponding Author E-Mail:	mlmartinez@cicbiogune.es
Order of Authors:	Fernando Lopitz-Otsoa
	Teresa C Delgado
	Sofía Lachiondo-Ortega
	Mikel Azkargorta
	Felix Elortza
	Manuel S Rodríguez
	Malu Martínez-Chantar
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Cover Letter

Derio, 4th April 2019

Editors and Academic Editors of *JOVE*,

Please find enclosed the original research article entitled "**SUMO-binding entities (SUBEs) are useful tools for the enrichment, isolation, identification and characterization of the SUMO proteome in liver cancer** " by Lopitz-Otsoa *et al* to be considered for publication for *JOVE*. We believe that this protocol will be of the interest for many researchers interested in the role of pot translational modifications of SUMOylation in liver disease.

Best regards,

Maria L Martinez Chantar, PhD
Group Leader, Metabolomics Unit
Associate professor, University of the Basque Country
CIC bioGUNE
CIBERehd – Spanish Carlos III Health Institute
Bizkaia technology park, 801 building
48160, Derio Spain
e-mail: mlmartinez@cicbiogune.es
Tel Office: (+34)944061318
Tel Lab: (+34)944061304
Fax: (+34)944061301
http://www.cicbiogune.es

TITLE:

SUMO-Binding Entities (SUBEs) as Tools for the Enrichment, Isolation, Identification, and Characterization of the SUMO Proteome in Liver Cancer

AUTHORS AND AFFILIATION:

Fernando Lopitz-Otsoa[1*], Teresa C Delgado[1*], Sofía Lachiondo-Ortega[1], Mikel Azkargorta[2], Felix Elortza[2], Manuel S Rodríguez[3,*], María Luz Martínez-Chantar[1,]

[1]Liver Disease and Liver Metabolism Lab, CIC bioGUNE, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), 48160 Derio, Bizkaia, Spain.
[2]Proteomics Platforms, CIC bioGUNE. Carlos III Networked Proteomics Platform (ProteoRed-ISCIII).
[3]UbiCARE, Advanced Technology Institute in Life Sciences (ITAV)-CNRS-IPBS, 31106 Toulouse, France.

*These authors contributed equally.

Corresponding authors:

Manuel S Rodríguez (manuel.rodriguez@itav.fr)
María Luz Martínez-Chantar (mlmartinez@cicbiogune.es)

Email Addresses of Co-authors:

Fernando Lopitz-Otsoa (flopitz@cicbiogune.es)
Teresa Cardoso Delgado (tcardoso@cicbiogune.es)
Sofia Lachiondo-Ortega (slachiondo@cicbiogune.es)
Mikel Azkargorta (mazkargorta@cicbiogune.es)
Felix Elortza (felortza@cicbiogune.es)

KEYWORDS:

SUMO, SUBEs, Liver cancer, Hepatocellular Carcinoma, hepatoma, Mass-Spectrometry

SUMMARY

Here, we present a protocol to enrich, isolate, identify, and characterize proteins modified by SUMO in vivo both from human hepatoma cells and liver tumors obtained from mouse models of hepatocellular carcinoma by using SUMO-binding entities (SUBEs).

ABSTRACT

Post-translational modification is a key mechanism regulating protein homeostasis and function in eukaryotic cells. Among all ubiquitin-like proteins in liver cancer, the modification by SUMO (Small Ubiquitin MOdifier) has been given the most attention. Isolation of endogenous SUMOylated proteins in vivo is challenging due to the presence of active SUMO-specific proteases. Initial studies of SUMOylation in vivo were based on the molecular detection of specific SUMOylated proteins (e.g., by western blot). However, in many cases, antibodies, generally made with non-modified recombinant protein, did not immunoprecipitate SUMOylated forms of the protein of interest. Nickel chromatography has been the other approach to study SUMOylation by capturing histidine-tagged versions of SUMO molecules. This approach is mainly used in cells stably expressing or transiently

transfected with His-SUMO molecules. To overcome these limitations, SUMO-binding entities (SUBEs) were developed to isolate endogenous SUMOylated proteins. Herein, we describe all the steps required for the enrichment, isolation, and identification of SUMOylated substrates from human hepatoma cells and hepatic tissues from a liver cancer mouse model by using SUBEs. Firstly, we describe methods involved in the preparation and lysis of the human hepatoma cells and liver tumor tissue samples. Then, a thorough explanation of the preparation of SUBEs and controls is detailed along with the protocol for the protein pull-down assays. Finally, some examples are provided regarding the options available for the identification and characterization of the SUMOylated proteome, namely the use of western-blot analysis for the detection of a specific SUMOylated substrate from liver tumors or the use of proteomics by mass spectrometry for high-throughput characterization of the SUMOylated proteome and interactome in hepatoma cells.

INTRODUCTION

Liver cancer is the sixth most common cancer worldwide and the second cause of cancer-associated deaths[1]. Hepatocellular Carcinoma (HCC) is the most prevailing form of primary liver cancer. Historically, common risk factors for the development of HCC included chronic hepatitis B or C infection and abusive alcohol consumption. In the last decades, the metabolic syndrome, Type 2 diabetes non-alcoholic fatty liver disease (NAFLD) has emerged as risk factors for the development of HCC[2]. HCC is very heterogeneous, both phenotypically and genetically, wherein a complex network of signaling pathways are disrupted. In the last years, even though there has been an increase in our knowledge about the molecular pathways implicated in the pathogenesis of HCC, there are still no effective therapeutic approaches for HCC management. Many pathways are activated in HCC and inhibiting one generally drives the compensation by other pathways[3]. This has been one of the main difficulties when treating HCC. Thus, a more global approach may provide a potential therapeutic approach for the clinical management of liver cancer e.g., targeting post-translational modifications (PTMs), as multiple signaling pathways can be simultaneously regulated by PTMs of proteins.

Post-translational modifications are considered as key mechanisms regulating protein homeostasis and functions[4]. Structural and functional changes are introduced by PTMs, thereby, increasing proteome diversity. The most common PTMs include phosphorylation, methylation, acetylation, glycosylation, ubiquitination, and conjugation of ubiquitin-like proteins (UbLs). Among all UbLs, protein modification by SUMO (Small Ubiquitin MOdifier) has attracted attention in association with its critical role in a variety of cellular processes, including transcription, cellular localization, DNA repair, and cell cycle progression[5]. Recently, SUMOylation was shown to be altered in liver cancer[6-9], and changes in the SUMOylation of specific proteins has been described to play a role in the progression of cancer-related diseases[9].

In mammals, there are five SUMO paralogues, SUMO-1 to SUMO-5. To date, no experimental evidence is available about the existence of endogenous SUMO-4 and endogenous SUMO-5 conjugation reactions at the protein level[10-12]. SUMOylation in mammals is carried out by an enzymatic thiol-ester cascade involving three enzymes, the heterodimeric SUMO activating enzyme (SAE1/SAE2) or E1, the SUMO conjugating enzyme (Ubc9) or E2 and a SUMO-E3-ligase specific for each target protein. The action of several

families of SUMO E3s appears to be in a dynamic equilibrium with SUMO-specific proteases (SUSPs or SENPs)[13] making the SUMOylation reaction highly reversible. Moreover, only a small fraction of the SUMOylated protein versus non-SUMOylated total protein is present. Thereby, isolating endogenous SUMOylated proteins in vivo is rather challenging[13].

SUMOylation in vivo was initially studied by western blot using antibodies against the protein of interest[14]. Immunoprecipitation of the protein was performed with specific antibodies and then PAGE-western blot was carried out with anti-SUMO antibodies. The main problem with this strategy is that antibodies generated against a non-modified recombinant protein are not always able to immunoprecipitate the SUMOylated form of a protein. Alternatively, nickel chromatography after the transient expression of histidine tagged (His6) versions of SUMO molecules and the protein of interest has been used to study SUMOylation in cells. On this basis, it will be more convenient to detect SUMO-modified forms from cells stably expressing His6-SUMO[15]. For in vivo studies, tandem-SUMO-interacting motifs (SIM) based enrichment was demonstrated for the purification of polySUMO conjugates[16]. Other groups have been using epitope-tagged antibody SUMO approaches providing a feasible tool to investigate endogenous SUMOylation in primary cells, tissues, and organs[17,18]. And more recently, Nielsen and colleagues have used antibody-based enrichment to identify endogenous and site-specific SUMO in cells and tissues[19].

In order to provide complementary information on the role of SUMOylation in vivo, SUMO-binding entities (SUBEs), also known as SUMO traps, were developed[20]. Of relevance, tandem ubiquitin binding entities (TUBEs) are considered the conceptual precursors of SUBEs and are commercially available tools for the detection and isolation of polyubiquitylated proteins[21]. SUBEs are recombinant proteins that comprise tandem repeats of SIMs thereby recognizing SUMO molecules on modified proteins with an increase in the overall affinity for SUMO substrates. SUMO-traps were engineered by introducing an E3 ubiquitin-protein ligase RNF4-derived SIM2 and SIM3 motifs in tandem, into a vector containing glutathione S-transferase (GST), a heterologous carrier protein[20]. Although SUBEs cannot be used properly to identify mono-SUMOylated target proteins, this method provides a tool to facilitate the purification and identification of poly-SUMO target proteins in vivo. Herein, we describe the application of SUBEs to isolate SUMOylated proteins both in human hepatoma cells and in mouse liver biopsies, an important tool for the study of liver cancer. An overall scheme of the protocol described in this manuscript is shown in **Figure 1**.

PROTOCOL

All experiments were approved by the CIC bioGUNE institutional committees for animal care and handling. All efforts were made to minimize animal suffering and to reduce the number of animals used. 3-months old male glycine *N*-methyltransferase (*Gnmt)* deficient ($Gnmt^{-/-}$) and its wild type littermates ($Gnmt^{+/+}$) were used.

1. Cell Preparation and Lysis

NOTE: Herein, Huh-7 (human hepatoma cell line) and THLE2 (human hepatic cell line) were used.

1.1. Maintain cells in P100 plates in standard growth media at 37 °C in a humidified atmosphere of 5% CO_2-95% humidity.

1.2. Grow cells in P100 plates plating at a density of 1.2–1.5 x 10^6 cells per dish by counting the cells using a Neubauer haemocytometry counting chamber.

1.2.1. Culture Huh-7 in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin-amphotericin B (PSA) and 1% glutamine.

1.2.2. Culture THLE2 cells in culture dishes pre-coated with 0.01 mg/mL fibronectin, 0.01 mg/mL bovine serum albumin (BSA) and 0.03 mg/mL collagen type I dissolved in growth medium which consists of bronchial epithelial cell growth basal medium (BEGM) supplemented with growth factors (0.4% BPE, 0.1% insulin, 0.1% hydrocortisone, 0.1% retinoic acid, 0.1% transferrin, 0.1% triiodothyronine as well as 10% FBS, 1% PSA, 5 ng/mL epidermal growth factor (EGF) and 70 ng/mL phosphoethanolamine.

1.3. At the end point of the experiment, aspirate the media from the plates and wash cells with 5 mL of sterile 1x phosphate-buffered saline (PBS). Lyse cells directly on the plate placed on ice using 500 µL of lysis buffer (50 mM Tris pH 8.5, 150 mM NaCl, 5 mM EDTA, 1% nonidet P-40 (NP40), supplemented with complete EDTA-free protease inhibitor cocktail and 50 µM PR-619 for each P100 dish. Using a cell scraper, gently scrape the cells off the bottom of the plate into the Lysis medium.

NOTE: Check that all the cells have detached from the plate by visually inspecting the plate base after the treatment.

1.4. Alternatively, harvest cells by trypsinization by aspirating cell media and add 1 mL of 1x (0.05%) trypsin-EDTA to the plate, enough to cover the cells and placing the plate in the incubator set at 37 °C, 5% CO_2, and 95% humidity for ~5 min ensuring all cells have detached from the plate. Add 2 mL of pre-warmed growth medium in order to stop trypsinization. Centrifuge at 150 g for 10 min and aspirate the supernatant. Wash with 1x PBS and centrifuge at 150 *x g* for 10 min. After aspirating the supernatant, add 500 µL of lysis buffer (50 mM Tris pH 8.5, 150 mM NaCl, 5 mM EDTA, 1% NP40, supplemented with complete EDTA-free protease inhibitor cocktail and 50 µM PR-619 for each P100 dish.

NOTE: The addition of the PR-619 is critical.

1.5. Centrifuge at 15,500 *x g* and 4°C for 10 min. Transfer the supernatant to another tube and discard the pellet.

NOTE: The protocol can be paused here, and samples stored at -80 °C until further analysis.

2. Tissue Preparation and Lysis

2.1. Upon animal sacrifice, collect mouse livers, wash with cold PBS, and snap freeze immediately in liquid nitrogen. Store the samples −80 °C until further analysis.

2.2. Homogenize 75 mg fragments from snap-frozen/or fresh livers in 1 mL of ice-cold lysis buffer (50 mM Tris pH 8.5, 150 mM NaCl, 5 mM EDTA, 1% NP40, supplemented with complete EDTA-free protease inhibitor cocktail and 50 µM PR-619). Run the homogenizer at 6500 x rpm, 2 x 60 s each, with a 30 s pause (see **Table of Materials**).

2.3. Centrifuge the samples in a microfuge at 15, 500 *x g* and 4 °C for 10 min. Transfer the supernatant to another tube and discard the pellet.

2.4. Alternatively, triturate 75 mg of frozen tissues in liquid nitrogen. Then recover the tissue in 1 mL of lysis buffer.

2.5. Centrifuge the sample in a microfuge at 15, 500 *x g* and 4 °C for 10 min. Transfer the supernatant to another tube and discard the pellet.

NOTE: The protocol can be paused here, and samples stored at -80 °C until further analysis.

3. Binding of GST-SUBEs or GST Control to Glutathione-Agarose Beads

NOTE: The synthesis of the GST-SUBEs or GST control are outside the scope of this manuscript and can be reviewed in previously published literature[20]. Alternatively, GST- and Control SUBEs are commercially available (e.g., SignalChem).

3.1. Preparation of glutathione beads

3.1.1. Add 1 mL of de-ionized water to 70 mg of lyophilized glutathione-agarose beads. Reconstitute the beads overnight at 4 °C (or for at least 30 min at room temperature).

3.1.2. Wash the beads thoroughly after swelling (to remove lactose and ethanol that are usually present in the lyophilized powder agarose beads). To do so, first wash with 10 mL of de-ionized water or PBS followed by centrifugation at 300 *x g* for 5 min at room temperature. Perform this thrice.

3.1.3. After 3 washes, resuspend the beads in 1 mL of PBS to obtain a 50% (v/v) slurry.

NOTE: This volume is suitable for the analysis of 10 samples.

3.2. For each sample, add 100 µg of GST-SUBEs or GST control (see reference[20]) to 100 µL of the glutathione beads slurry and 500 µL of PBS.

NOTE: The relative abundance of the SUMOylated proteins of interest determines the amount of GST-SUBEs used for pull-downs. For each new experimental model, analyze the condition prior to the actual experiment by western blotting the input, bound, and flow-through (FT) material using anti-SUMO2/3 antibodies or against proteins of interest (Liver Kinase B1 (LKB1).

3.3. Incubate all the GST-SUBEs or GST control with beads prepared in 3.2., slowly rotating in rotator or mini roller (see **Table of Materials**) at 4 °C for at least 2 h (slow binding

reaction).

NOTE: Adding 1 mM dithiothreitol (DTT) improves GST binding to the glutathione beads.

3.4. Recover the agarose beads by centrifugation at 300 *x g* for 5 min at room temperature. At the end, resuspended the beads in PBS to obtain 50% (v/v) slurry.

NOTE: The protocol can be paused here, and samples stored at -80 ˚C until further analysis.

4. GST Pull Down Assay

4.1. After step 1.5, 2.3 or 2.5, take 1/10 of total volume (e.g. 50 µL) and dilute in the same volume of 3x boiling buffer (250 mM Tris-HCl pH 6.8, 500 mM β-mercaptoethanol, 50% glycerol, 10% SDS, bromophenol blue). This fraction is considered as INPUT.

4.2. Add 450 µL of clarified lysate from steps 1.5, 2.3 or 2.5 to 100 µL glutathione beads slurry. Incubate the lysate with beads, slowly rotating at 4 °C for at least 2 h.

NOTE: Alternatively, 100-200 µg of total protein from steps 1.5, 2.3 or 2.5 (quantified with the Bradford assay) in a total volume of 450 µL can be used.

4.3. Spin down the beads in a microfuge at 300 *x g* for 5 min and collect the supernatant for the analysis. Transfer 1/10 of the total volume (e.g., 50 µL) in a separate tube and dilute in an equal volume of 3x boiling buffer. This fraction is the flow-through (**FT**) fraction.

4.4. Wash the remaining sample three times with 1 mL ice-cold PBS, 0.05% Tween 20, spin down at 4 °C and 300 *x g* for 1 min. Carefully aspirate ensuring no liquid remains. The beads correspond to SUBEs BOUND (SB) fraction.

4.5. Elute the sample with 15 µL of 3x boiling buffer and 15 µL of the lysis buffer. This is called the BOUND Fraction.

5. Identification and Characterization of SUMO Targets by Western Blot analysis

5.1. Perform western blot analysis using an anti-SUMO2/3 antibody or any other specific antibody of choice as described previously[22].

5. Identification and Characterization of SUMO Targets by Western Blot analysis

5.1. Perform western blot analysis using an anti-SUMO2/3 antibody or the specific antibody of the choice (**Figure 2b**).

6. Identification and Characterization of the SUMOylated Proteome by Mass Spectrometry

NOTE: In the case of Mass-Spectrometry (MS) analysis, samples were processed using the Filter-Aided Sample Preparation (FASP) method described by Wisniewski et al.[23].

6.1. Desalt the peptides by using stage-tip C18 microcolumns and resuspend them in 0.1% formic acid (FA) prior to MS analysis.

6.2. Load the samples onto a LC-MS system (see **Table of Materials**) and analyze them in triplicate (technical replicates) (**Figure 2b**).

6.3. Carry on with the protein identification and abundance calculation by using an associated software.

6.4. For statistical analysis and heatmap generation, load the data onto the Perseus platform (http://www.perseus.tufts.edu/hopper/). Apply a permutation-based false discovery rate (FDR)-corrected t-test for the comparison of the abundances. Proteins with a $q < 0.05$ and a SUBEs/GST ratio greater than 2 were considered as enriched[24].

NOTE: Proteins identified with at least two different peptides are considered in the final analysis.

REPRESENTATIVE RESULTS

Identification of a Specific SUMOylation Substrate in Liver Tumor Biopsies by Western Blot Analysis

Liver Kinase B1 (LKB1) SUMOylation has been recently shown to be an important oncogenic driver in liver cancer[9,25]. Mice deficient in glycine *N*-methyltransferase (*Gnmt)*, often referred to as *Gnmt*$^{-/-}$, is a model that spontaneously develops hepatocellular carcinoma (HCC), the most common type of primary liver cancer. SUBEs were used to enrich and isolate the SUMOylated proteins both in *Gnmt*$^{-/-}$ mice with liver cancer mice and its wild type littermates (*Gnmt*$^{+/+}$*)*. In **Figure 2a**, Ponceau S staining of the three different fractions (input, FT and BOUND) obtained in the SUBEs pull-down assay are included. A Ponceau S stain is useful to control a possible deleterious effect on the loading of blotted proteins to be evaluated by Western blots. In **Figure 2b** a Western blot analysis of LKB1 by using SUBEs to capture endogenous SUMOylated LKB1 is shown. The levels of LKB1 SUMOylation are augmented in liver tumors. In the case of Western blot analysis, equal loads and transferred proteins were observed by Ponceau staining of the input fraction and were not significantly altered after washes (flow through fraction). The amount of protein captured with SUBEs was significantly higher, particularly in the tumors. Alternatively, staining a duplicate gel with Coomassie blue can provide similar information. Sticky proteins such as p53 or SUMOylated forms of some proteins might bind to the GST control. To remove the background, use low-density agarose beads, perform a coating with BSA, or incorporate additional washes. However, this could affect applications such as Mass Spectrometry and might result in loss of low affinity interacting proteins.

Characterization of the SUMO Interactome in Human Hepatoma Cells by Mass Spectrometry Analysis

To investigate the capacity of the SUMO-trap to interact with naturally SUMOylated proteins, Huh-7 (human hepatoma) and non-transformed liver epithelial human THLE2 cell lines were used. The first step is the visualization of the total material captured with SUBEs and using GST as a negative control. For this purpose, we can use conventional protein staining protocols as shown in **Figure 3a**. Then, we performed mass spectrometry analysis.

An average of 2268 proteins were identified in the Huh7 GST samples (2339, 2297 and 2168 for each load, respectively), whereas 2812 proteins were identified on average in the Huh7 SUBEs sample (2815, 2817 and 2806). After the subtraction, 742 proteins were enriched in the SUBEs. On the other hand, an average of 2497 proteins were identified in the THLE2 GST samples (2476, 2520 and 2495, respectively) and 2763 in the SUBEs (2823, 2783 and 2684). Of these, 577 were considered to be enriched in the SUBEs samples. Analysis of technical replicates retrieves the heatmap shown in **Figure 3b**, which was calculated using the default settings available (Euclidean distance, average linkage and pre-processed with k-means). The heatmap depicts the distribution of the 100 most significantly and exclusively enriched proteins in each cell line.

FIGURE AND TABLE LEGENDS

Figure 1: Schematic diagram of the protocol flow chart used for the enrichment, isolation and identification and characterization of the SUMOylated proteome in vivo for the study of liver cancer.

Figure 2: Modification of LKB1 by SUMO-2 in mouse models of Hepatocellular Carcinoma. (**a**) Ponceau S staining of the three different fractions (input, Flow through (FT) and BOUND) obtained in the SUBEs pull-down assay. (**b**) Western blot analysis of LKB1 by using SUMO binding entities (SUBEs) to capture endogenous SUMOylated LKB1; GAPDH is used as a loading control.

Figure 3: Differences between the SUMOylated proteome between the tumoral Huh-7 and non-transformed liver epithelial THLE2 human cell lines. (**a**) Sypro staining of captured protein material, with GST (Negative control) and SUBEs. (**c**) Heatmap depicting the differentially enriched proteins in Huh-7 and THLE2 SUBE samples.

DISCUSSION

Herein, we have provided a complete and detailed description of the methodology reporting the use of SUBEs for the enrichment, isolation and identification and characterization of the SUMOylated proteins in in vivo models of liver cancer. Both in mouse liver tumors and human hepatoma cells, we were able to correctly isolate and identify SUMOylated proteins of interest and to perform a high-throughput characterization of the SUMOylated proteome and interactome. Even though the synthesis of SUBEs is outside the scope of this manuscript, for further information the following references should be looked at[26]. The protocol described is fast and very sensitive and the critical step of the protocol includes the use of SENPs inhibitors (PR-619). In alternative, chemical isopeptidase inhibitors such as NEM (*N*-Ethylmaleimide) and IAA (2-Iodoacetamide) in the lysis buffer can be used, however, previous reports have shown that for the SUBEs protocol, the use of PR-619 is advantageous as the other inhibitors interfere with GST binding to the glutathione beads[20].

SUBEs are recombinant proteins that comprise tandem repeats of SIMs thereby recognizing SUMO molecules on modified proteins with an increase in the overall affinity for SUMO substrates. Due to its high specificity and sensitivity, the use of SUBEs for the isolation of the SUMOylated proteome is advantageous relative to other approaches in the literature such as the detection by western-blot of specific SUMOylated proteins using antibodies against

the protein of interest or the nickel chromatography using the different histidine-tagged versions of SUMO molecules. However, it must be taken into consideration that as the SUBEs protocol is performed under non-denaturing conditions, the interaction between SUMOylated proteins and other interacting proteins is maintained. Therefore, we obtain information about the SUMO interactome rather than only a list of SUMOylated target proteins. Thus, further experiments are necessary to confirm if the identified protein is a SUMO target or an interacting factor. Other limitation of the SUBEs is the fact that control GST traps used are able to capture many background proteins related to oxidative stress. This issue is especially relevant during the MS analysis due to the high sensitivity of the technique. In order to overcome these limitations, biotinylated SUMO-traps (bioSUBEs) have been developed[26]. Another limitation of SUBEs resides in the fact that we are only able to capture proteins modified by SUMO 2 and SUMO 3 whereas SUMO 1-modified proteins cannot be isolated.

Other concern of the use of SUBEs is related to the amount of starting material necessary for the procedure. The starting material used to capture SUMOylated proteins should consider the different experimental conditions explored. While basal SUMOylation has been reported in various cellular contexts, SUMOylation is a process that is strongly induced after multiple stress conditions/stimuli. If comparing untreated versus treated samples, one has to be sure that the column is not saturated, and differences can be observed between those conditions. In the case of the mouse phenotypes we are analyzing, no treatments have been used and basal SUMOylation levels are low. For this reason, high amounts of proteins were used. The background level should be controlled by using GST and if the unspecific binding is high, the amount of starting material or the binding time should be reduced. The analysis of the FT fraction can be indicative of the capture efficiency even if these traps prefer poly-SUMOylated proteins and a total depletion should not be expected, a reduction of total SUMOylation is in general well observed when the capture efficiency is optimal.

Finally, other application of the SUBEs technology includes the combination of SUBEs technology with Real-time Surface Plasmon Resonance (SPR) allowing the real-time interactions with SUMOylated proteins from cell extracts[27]. Also, more recently, biotinylated SUMO-traps (bioSUBEs) have been developed with the advantage to reduce the background associated to bigger tags, e.g., during mass spectrometry analysis[26]. In addition, the bioSUBE version can be used to detect SUMOylated proteins in living cells by fluorescence by using streptavidin-labelled with distinct fluorescent dyes taking advantage of the streptavidin binding to biotin. Also, methods for detection and quantification of SUMOylated proteins can be considered with both GST and bioSUBEs versions such as it was done with the tandem ubiquitin binding entities (TUBEs)[21].

Overall, the use of SUBEs for the isolation and characterization of the SUMOylated proteome relevant in liver cancer is a fast and sensitive method providing vast information on the still rather unknown role of the SUMOylation pathway in liver cancer.

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DISCLOSURES

Dr. Martínez-Chantar advises for Mitotherapeutix LLC.

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Figure1

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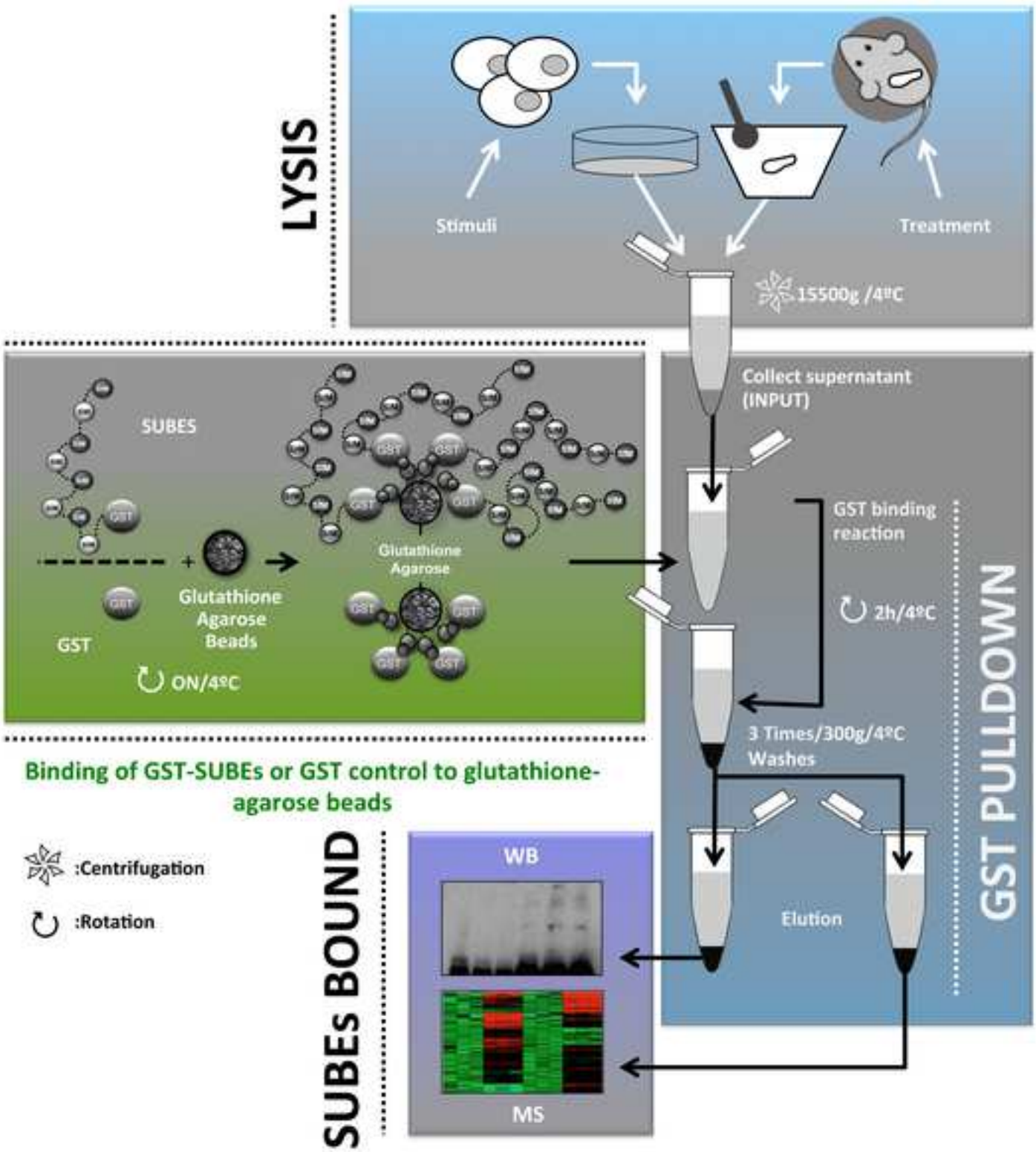


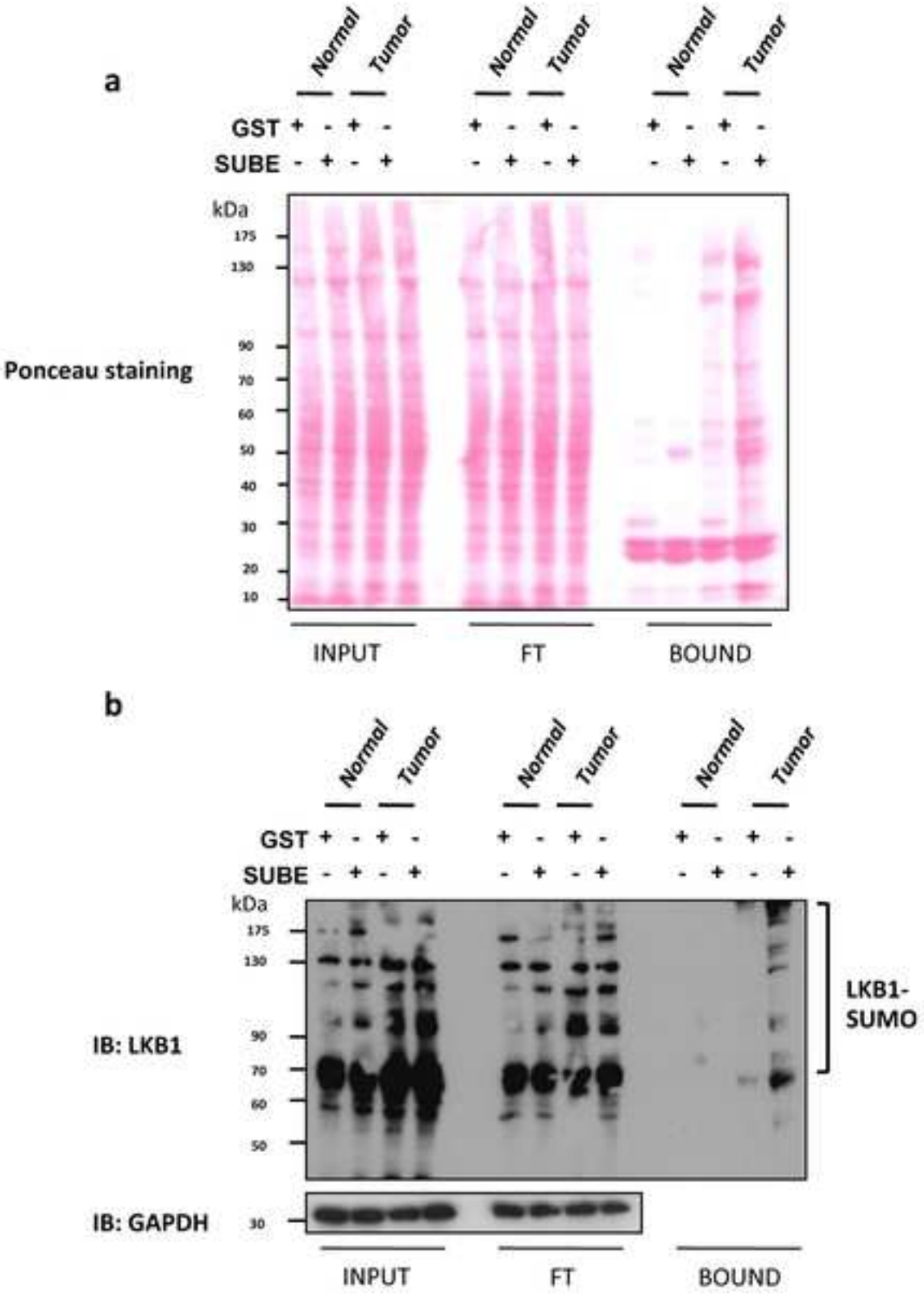
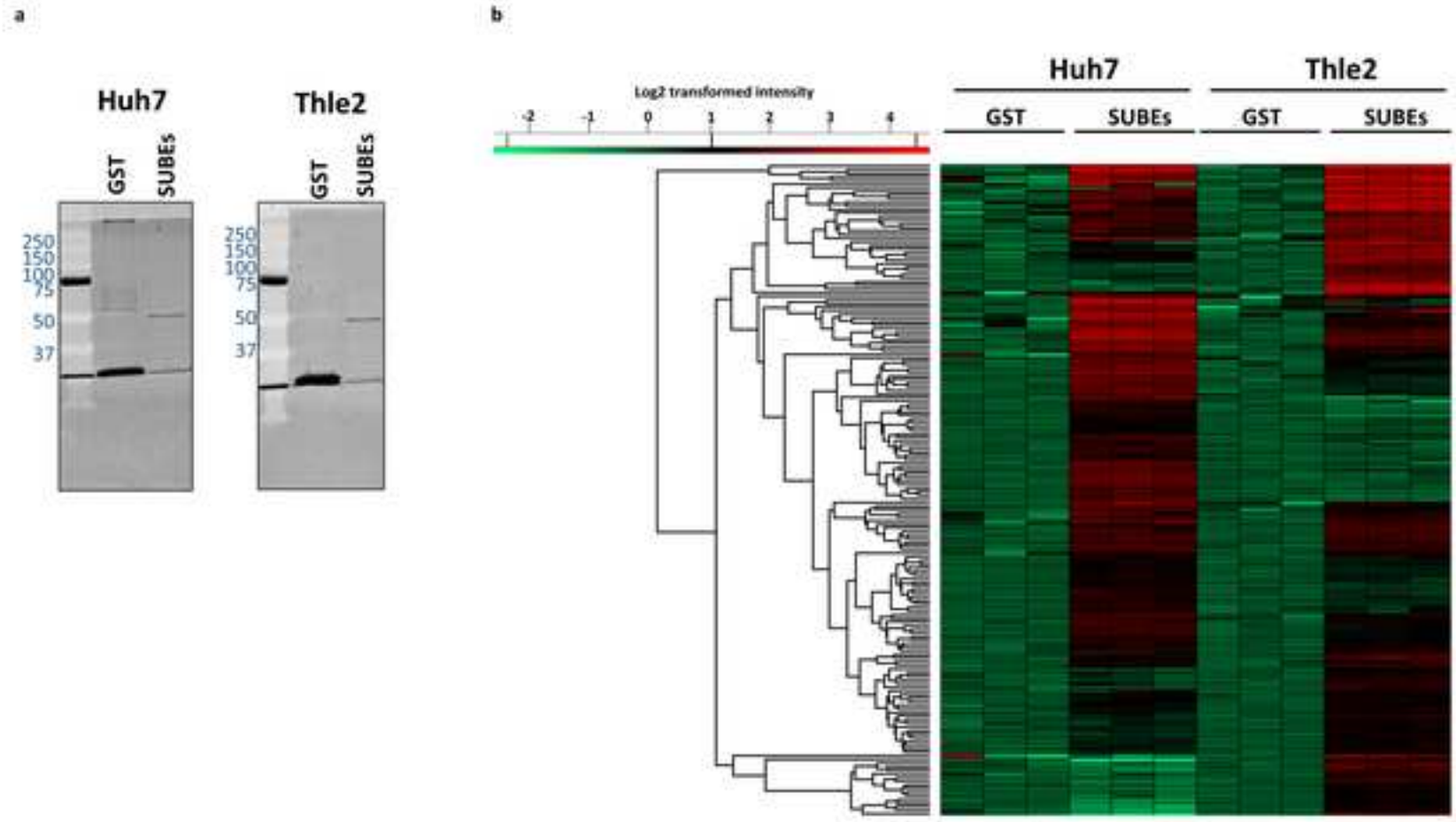


Figure3



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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
(Gnmt−/−)/ (Gnmt+/+) mice	CIC bioGUNE		
0.5% Trypsin-EDTA	Life Technologies	15400-054	
BEBM	Lonza/Clonetics Corporation	cc-3171	
BEGM Bullet Kit	Lonza/Clonetics Corporation	CC3170	
Bromophenol blue	Sigma	115-39-9	
BSA	Sigma	A4503	
C18 microcolumns	Millipore	Z720070	
Collagen type I	Santa Cruz Biotechnology	sc-136157	
Complete tablets EDTA-free	Roche	4693132001	
DMEM	Life Technologies	A14431-01	
DTT	Sigma	43815	
EDTA	Sigma	E6758	
EGF	Sigma	e9644	
FBS	Life Technologies	10270	
Fibronectin	Life Technologies	33010018	
Glutamine	Life Technologies	25030-024	
Glutathione agarose beads	Sigma	G4510	
Glycerol	Sigma	G5516	
GST-Control	SignalChem	G52-30H	
GST-SUBEs	SignalChem	S291-340G	
Huh7	CLS (Cell Lines Service)	300156	https://clsgmbh.de/
IAA (2-Iodoacetamide)	Merck	L58046844	
LKB1 antibody	Santa Cruz Biotechnology	sc-32245	
Mini LabRoller Rotator	LABNET	H5500	https://www.labnetinternational.com
NaCl	Merck	1.06404E+11	
nanoElute	BRUKER		https://www.bruker.com/
NEM (N-Ethylmaleimide)	Sigma	E3876	
NP40	Fluka	74385	
PBS	Life Technologies	14190-094	
Peaks software	Bioinformatics Solutions Inc.		http://www.bioinfor.com/
Phosphoetanolamine	Sigma	P0503	
Ponceau S solution	Sigma	P7170	
PR-619	Merck	662141	
Precellys 24	Bertin Technologies	P000669-PR240-A	
PSA	Life Technologies	151-40-122	
PSG	Life Technologies	10378-016	
SDS	Sigma	L3771	
SUMO2/3 antibody	Abcam	Ab3742	
THLE-2	ATCC	ATCC CRL-2706	http://www.lgcstandards-atcc.org

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Title of Article:	SUMO-Binding Entities (SUBEs) are Useful Tools for the Enrichment, Isolation, Identification and Characterization of the SUMO Proteome in Liver Cancer
Author(s):	Fernando Lopitz-Otsoa, Teresa C Delgado, Sofía Lachiondo-Ortega, Mikel Azkargorta, Felix Elortza, Manuel S Rodríguez, María Luz Martínez-Chantar

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CORRESPONDING AUTHOR

Name:	María Luz Martínez-Chantar
Department:	Liver Disease and Liver Metabolism Lab
Institution:	CIC bioGUNE, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd)
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Dr. Vineeta Bajaj, Ph.D.
Review Editor JoVE

Dear Dr. Vineeta, in order to improve the quality of the manuscript we have written a new version of the manuscript and tried to address all the Reviewers main concerns.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have carefully read the MS in order to correct some typos that we found.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points throughout.

This was done in the new version of the MS.

3. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 56-66, 68-75, 81-84, 94-97, 152-156, 159-161, 209-213

The sections were drawn from our own publications. However, we have rewritten them in order to avoid overlap.

4. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

This was done in the new version of the MS.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

This was done in the new version of the MS.

6. The Protocol should contain only action items that direct the reader to do something.

This was done in the new version of the MS.

7. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

This was done in the new version of the MS.

8. The Protocol should be made up almost entirely of discrete steps without large

paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
This was done in the new version of the MS.

9. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
This was done in the new version of the MS.

10. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
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Not applicable to this MS.

12. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
This is correctly done

Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

Manuscript Summary:
The authors propose a method for the enrichment of SUMO post translational modified protein substrates using GST-SUMO-binding entity (SUBE). Additionally, Lopitz-Otsoa et al. show the use of the SUBE with samples from human hepatoma cells and liver tumors.

Major Concerns:
The use of SIM-based peptide slurries to isolate is well-accepted (and SIM-peptide agarose is commercially available). The major concern for this method manuscript is

that the authors do not clearly show that SUMOylated proteins are enriched in the pull-down lysates.
The methodology and specificity of the SUBEs has been previously demonstrated and validated as highlighted in the Introduction. The novelty of this manuscript is the fact that we are able to identify SUMOylated proteomes or the SUMO proteome in liver cancer cells and tissue.

First, only ponceau staining is shown to indicate pull-down efficiency. Instead the elute should be probed with anti-SUMO1 and anti-SUMO2/3 antibodies to prove that indeed SUMOylated proteins are present. Second, it is difficult to conclude from the Fig. 2b that an enrichment of SUMOylated LKB1 is achieved; the authors do not clearly indicate the bands on the LKB1 blot that correspond to the SUMO-modified form.
As mentioned above, the validation of the SUBEs has been previously done. The bands corresponding to the SUMOylated LKB1 are shown in the new version of the figure.

Third, additional controls are lacking and clearly required. For example, an additional pulldown should include a positive control (i.e. E2-Ubc9 overexpression) and negative control (i.e. SUMO inhibitors ginkgolic acid or small molecule inhibitor). Address these major concerns will clearly increase the impact of this relevant proposal.
As mentioned earlier, these protocol is in order to obtain the endogenous SUMOylated proteome and therefore the overexpression of Ubc9 is out of the scope of this MS. The validation of the SUBEs has been previously done in literature.

Minor Concerns:

1. It is unclear how the authors prevent changes in the SUMOylome during isolation of the lysate. Are SUMO protease inhibitors like NEM included in the lysate?
No, in our case we have added PR-619 as inhibitor of SUMO protases.

2. The mass spectrometric analysis should be elaborated on.
This was added.

3. Provide information on how statistical analysis can be performed on the presented data.
This was added.

Reviewer #2:

Manuscript Summary:
The analysis of proteins modified by SUMO is rather challenging. SUMO-Binding Entities (SUBEs) enable the enrichment of SUMO-modified proteins from cell and tissue lysates. Subsequently, these modified proteins can be detected by immunoblotting as well as by mass spectrometry. This protocol paper will enable the reader to exploit SUBEs in an efficient manner to study SUMO signal transduction. The paper carefully lists advantages and disadvantages of the methodology and pitfalls including the co-purification of interacting proteins due to the mild nature of the methodology.

Major Concerns:
none

Minor Concerns:
1. Line 35. "Post-translational modification is a key mechanism" instead of "Post-translational modifications are key mechanisms".
Corrected
2. Line 37. "Isolation of" instead of "The isolating".
Corrected
3. Line 80. The lack of evidence of existence of endogenous SUMO-4 and endogenous SUMO-5 at the protein level need to be stated.
Corrected
4. Line 213. "loss of low affinity interacting proteins" instead of "loss of information".
Corrected
5. Line 270. "Another limitation" instead of "Other minor limitation".
Corrected
6. Line 276. "dyes" instead of "dies".
Corrected

Reviewer #3:

Manuscript Summary:
In their manuscript, the authors describe a protocol to isolate SUMOylated proteins using SUBEs, a relatively new tool. They apply their protocol to both cultured liver cells and liver cancer tissue samples, and analyze the results by either immunoblot or MS/MS.

Major Concerns:

Paragraph 152-156. The whole procedure of bead reconstitution is very unclear to me. Volumes need to be more clearly stated.

Paragraph 186-193. In my opinion, point 5 should be included as part of representative results. Or else, if the authors want to keep it as part of the protocol, they should give many more details, so it can be reproduced by others.
We have added detail on the Mass Spectrometry analysis and not the Western blot because is a standard routine technique.

Paragraph 217-228. This whole paragraph is very unclear and, in my view, it needs to be fully rewritten. I think they are using MS, but this is not even indicated.
We have rewritten the paragraph.

Minor Concerns:

I enclose below some specific suggestions that, in my opinión could make the text more clear:

L.42. Use histidine-tagged, rather than histidinylated.
Corrected
L.64-66. A mechanism is not an approach. May be targeting/inhibiting the mechanism is what the authors mean?.
Corrected
L 72. ubiquitination and CONJUGATION OF ubiquitin like proteins (UbLs).

Corrected
L 84. Define abbreviations.
Added.
L 86. highly REVERSIBLE.
Corrected
Paragraph 88-92. Include a comment on the small fraction of SUMOylated vs non-SUMOylated versions of a protein as additional problem?
Added.
L.95. Use histidine-tagged, rather than histidinylated.
Corrected
Paragraph 94-106. Mention TUBEs as "conceptual" precursors of SUBEs?
Added.
L 103. into a vector with a GST tag.
Corrected
L 114. define abbreviations, e.g. PSA?
Added.
L 120. cell density should be per sqcm or per dish.
Corrected
L.123. Does this step need to be carried out in the sterile hood?
Corrected
L 134. What is next, after deactivating the trypsin?
Better explained
L 145. Lysis buffer is the same as above? Specify.
Yes, this was added
L 146. I guess there is a centrifugation step before transferring the supernatant. Describe.
This was added.
L 149. How are the samples stored when the protocol is paused? This should be described every time they indicate a pause in the protocol.
This was added.
L 152. Step 3.1 is unclear. Is 10 ml the volume of lyophilized beads?
This was corrected and better explained in the new version.
L 154. ten volumes is 100 ml?
This was wrong in the last version of the paper and was corrected
L 164. They mention a column that has not previously been described. Clarify.
This was wrong in the last version of the paper and was corrected
L 165. temperature of the centrifugation step?
This was added.
L 175. Instead of what?
This was wrong in the last version of the paper and was corrected
L 179. FT is the same as unbound fraction in L 161? Keep consistency.
FT is used in all the MS
L183. Unclear.
This was corrected
L 200 and 203. What is the meaning of "liver cancer mice" or "hepatocellular carcinoma mice". Do these mice develop cancer as a result of GNMT deficiency? If so, state more clearly
This was better explained
L 204 and 207. Do they mean LOAD instead of charge?

Yes, load is the correct term.
L215. I believe authors mean either SUMO interactome or SUMOylated proteome. Clarify.
This was corrected
L240. The title is not representative of the figure, it only refers to part b.
This was corrected
L 255. IAA not mentioned in the protocol.
It was not used in the protocol. It as an alternative option that we discuss.
L262. Use histidine-tagged, rather than histidinylated.
This was corrected
L276 streptavidin-labelled WHAT?. DYES instead of dies?
This was corrected

Reviewer #4:

Manuscript Summary:

Lopitz-Otsoa et al. describe a method for the enrichment of proteins modified by SUMO2/3 using SUBEs, which are recombinant constructs with multiple SUMO interacting motifs in order to non-covalently trap SUMOylated proteins. The study of SUMOylated proteins outside the context of epitope-tagged mutant constructs and genetic engineering is important for the field and our overall understanding of SUMO in the biology of health and disease. Several other methods exist for studying SUMO in this context, but nonetheless additional methods are always welcome to provide complementary insight.

The SUBEs method has been demonstrated to work in the past (Da Silva-Ferrada et al., 2013), as also referenced to by the authors. The technical novelty of the current method is not entirely clear to this reviewer, although the application in liver cells and tissue is a step forward. However, there are some major issues with the manuscript in its current state, mainly relating to lack of proper referencing to other methods that already exist, lack of experimental and technical detail, and lack of supporting evidence for efficacy of the method. To avoid confusion of potential readers, and in light of several other well-documented methods already being available, I cannot recommend publication of this work in its current state.

Concerns:

Introduction:
- Antibody-based enrichments and His6-tagged SUMO methods are presented as the only alternative methods, which is simplifying matters a bit too much. There is a plethora of methods out there using differently tagged forms of SUMO in order to enrich substrates, with their primary advantages being very high affinity (enabling rigorous washing procedures), and ability to identify mono-SUMOylated proteins. The authors also state that such tagged versions of SUMO cannot be used outside of cell culture, which is not true - there are various labs using mice with epitope-tagged SUMO.
- Tandem-SIM based enrichment was shown in the Hay lab (Bruderer et al., 2009), where they demonstrated the affinity for poly-SUMOylation and used an in-gel based size cut-off of at least two SUMO shifts to weed out false positives. Antibody-based enrichment of SUMO has been successfully applied in the Melchior lab (Becker et al.,

2013; Barysch et al., 2014), also with application in liver. More recently, the Nielsen lab (Hendriks et al., 2018) applied an antibody-based enrichment to identify endogenous and site-specific SUMO in cells and some mouse organs, including liver. None of these works are referenced, and they absolutely should be.
This was added to the new version of the manuscript

- One of the main drawbacks of approaches like SUBEs is that they cannot properly identify mono-SUMOylated target proteins, because multiple SUMO-SIM interactions are required to stabilize binding. Antibody approaches do not suffer from this limitation, and can otherwise be employed in exactly the same setting. Whereas this does not invalidate usage of SUBEs, this limitation should be pointed out.
This was added to the new version of the manuscript in the Introduction and was already in the Discussion.

Protocol:
- The authors describe usage of two specific cell lines, and a (for SUMO study) modest number of cells required - with an added warning that using more material could result in only purifying the abundant SUMO species. This suggests a robustness issue, and this would be better described by suggesting how much SUBEs should be used per number of cells (or amount of starting protein material) used. WB is discussed as a way of checking how much SUBEs may be required, which suggests a trial-and-error approach required for each different cell line and/or treatment
We have changes this section of the manuscript.

- PR619 is used as the SUMO protease inhibitor - how effective is this inhibitor when only added in the lysis buffer? Previous works have demonstrated high efficiency of NEM (which the authors mention in the discussion only), by simply blotting for SUMO in samples lysed in the same buffer plus and minus NEM. Evidence should be provided (either directly or in reference) that PR619 in the lysis buffer prevents removal of SUMO, and that its absence would allow all SUMO to be cleaved.
The optimization of the inhibitors to use with SUBEs is out of the scope of this publication.
However in previous references (Da Silva-Ferrada 2013 Scientific Reports) this was discussed and this was added to the discussion of this manuscript.

- It should be highlighted that addition of such a SUMO protease inhibitor is absolutely critical to the protocol, as standard-range protease inhibitors and non-ionic detergents used for standard pulldown do nothing to slow down SUMO proteases.
This part was added to the new manuscript.

- Homogenization of mouse tissue is usually trickier than cultured cells, and evidence should be provided that PR619 is sufficient to prevent removal of SUMO when homogenizing mouse organs.
The use of PR619 in tissues has been previously described.

Deubiquitinase inhibitor PR-619 reduces Smad4 expression and **suppresses renal fibrosis** in **mice** with **unilateral ureteral obstruction**. Soji K, Doi S, Nakashima A, Sasaki K, Doi T, Masaki T. PLoS One. 2018 Aug 16;13(8):e0202409. doi: 10.1371/journal.pone.0202409. eCollection 2018.

Highly Multiplexed Quantitative Mass Spectrometry Analysis of **Ubiquitylomes**. Rose CM, Isasa M, Ordureau A, Prado MA, Beausoleil SA, Jedrychowski MP, Finley DJ, Harper JW, Gygi SP. Cell Syst. 2016 Oct 26;3(4):395-403.e4. doi: 10.1016/j.cels.2016.08.009. Epub 2016 Sep 22.

Uncovering global SUMOylation signaling networks in a site-specific manner. Hendriks IA, D'Souza RC, Yang B, Verlaan-de Vries M, Mann M, Vertegaal AC. Nat Struct Mol Biol. 2014 Oct;21(10):927-36. doi: 10.1038/nsmb.2890. Epub 2014 Sep 14.

- For a protocol that depends entirely on GST-SUBEs, the absence of how to acquire and/or produce these is somewhat jarring.

We understand that but this part is out of the scope of this manuscript and the references where you can find this part was added to the protocol as well as the reference of the product that is now commercially available.

- There is a considerable amount of starting material, 75 mg in the case of mouse organs, yet only 100-200 µg of total protein is recommended to be used in the pulldown. Considering 100 µg of SUBEs is bound to the beads matched to that sample amount, the 1:1 ratio seems excessive. Usually, a trap-sample ratio of 1:1,000 is more routine, and a 1:1 ratio suggests either highly inefficient binding of SUMO, or a large amount of background binding.

Concerning the amount of starting material used to capture SUMOylated proteins. We have included the following sentence in the main text.

The starting material used to capture SUMOylated proteins should consider the different experimental conditions explored. While basal SUMOylation has been reported in various cellular context, SUMOylation is a process that is strongly induced after multiple stress conditions/stimuli. If comparing untreated versus treated samples, one has to be sure that the column is not saturated and differences can be observed between those conditions. In the case of the mouse phenotypes we are analyzing, no treatments have been used and basal SUMOylation levels are low. For this reason high amounts of proteins were used. The background level should be controlled using GST and if the unspecific binding is high, the amount of starting material or the binding time should be reduced. The analysis of the FT can be indicative of the capture efficiency even if these traps prefer poly-SUMOylated proteins and a total depletion should not be expected, a reduction of total SUMOylation is in general well observed when the capture efficiency is optimal.

- Detail regarding follow-up analysis by WB or mass spectrometry is lacking.

We have added detain regarding the mass spectrometry analysis however we have omitted further data regarding the Western blot technique, which is more routinely performed in many laboratories, according to the JOVE author instructions.

Data and Figures:

- Figure 1 is blurry to the point where it is not possible to read any of the smaller text.

We have improved Figure 1.

- Figure 2 is meant to demonstrate successful enrichment of a SUMO target. The Ponceau stain shows a much higher presence of total protein in the tumor lane, but also shows more signal enriched from the tumor when only GST is used. Contrarily, it doesn't show any signal coming from the normal liver when SUBEs are used. This seems odd - this reviewer would expect both GST lanes to appear (mostly) empty, and then with more signal in the SUBEs lanes.

Even though we see an increase in the Ponceau staining of proteins in the BOUND fraction only with the GST is present in the western blot against LKB1 we clearly see that modified version of LKB1 are not increased.

- The WB for LKB1 shows a multitude of bands, and there aren't any visual indicators as to which band could be LKB1. Also, with so many bands visible, some controls should be done (e.g. a knockout experiment) to demonstrate the antibody is specific. Further, none of the bands appear to this reviewer as obvious SUMO bands. Usually, a clean blot would show an input band at one certain height, and then a SUMOylated band (or smear) higher up (usually by 15 kDa or more) in the enriched lane. In this case, the visible signal merely correlates with the Ponceau, and likely is just cross-reaction of the antibodies to background proteins. Considering this appears to be n=1, it could simply be technical variance related to the purification procedure.

This analysis is part of a bigger study published recently (Zubiete-Franco et al. 2019 eBiomedicine) where we present in vivo triplicates for the SUBEs protocol and we detect SUMOylated LKB1.

- A more appropriate control would be to blot for total SUMO across these samples, as it should demonstrate enrichment of SUMO when SUBEs are used and absence when GST is used. That is the core application of the protocol, which should be demonstrated, rather than a putative SUMO target protein.

This analysis is part of a bigger study published recently (Zubiete-Franco et al. 2019 eBiomedicine) where we present in vivo triplicates for the SUBEs protocol and we detect SUMOylated LKB1.

- Figure 3a has an unlabeled blot, and the authors also do not describe the protein that is being blotted for.

This was added in the new version of the manuscript

- Extensive data filtering was done on figure 3b, excluding non-enriched proteins. For the sake of technical representation of the method (rather than biological significance), the full heatmap should be shown, as this would demonstrate consistency between replicates and potential prevalence of non-related proteins.

Since we believe that heatmaps should preferably display differences between conditions, our choice is to keep fig3 showing only those proteins that meet the criteria to be considered differential. However, to satisfy the reviewer's requirements we also provide full data on the number of proteins identified and substracted from the final selection.

- Figure 3b appears to be n=3, but replicate numbers and types aren't documented anywhere in the manuscript. Are these biological replicates (i.e. different batches of cell culture) or technical replicates (i.e. multiple injections of the exact same sample)?

Single biological replicates were acquired in triplicate in the mass spectrometer. Biological replicates are being performed and are out of the scope of this manuscript.

- Figure 3b has a scale that is too blurry to read.
The scale for that figure has been augmented.

Discussion:
- Some of my concerns with the discussion were described for the introduction - the lack of mentioning of other methods that can be used to endogenously study SUMOylation (also in liver).
This was improved in the new version of the manuscript.

- The authors accurately highlight some main limitations of the SUBEs method, but should include the bias for enrichment of poly- or multi-SUMOylated proteins.
This was added.

- Proteomic analysis was performed on some samples, but no numbers are provided. How many proteins were identified as putative SUMO target proteins? What about the sample yield and purity in terms of SUMO enrichment? What could the average user expect to see if they were to use SUBEs in a proteomic context?
Single biological replicates were acquired in triplicate in the mass spectrometer. Full data on the total number of proteins identified and substracted is provided in the revised text.