Chapter 12

Isolation of Ubiquitylated Proteins Using Tandem Ubiquitin-Binding Entities

Fabienne Aillet, Fernando Lopitz-Otsoa, Roland Hjerpe, Mónica Torres-Ramos, Valérie Lang, and Manuel S. Rodríguez

Abstract

Studying postubiquitylation events has always been a difficult task due to the labile nature of these post-translational modifications. When utilized in tandem, ubiquitin-binding entities (TUBEs) not only increase up to thousand times the affinity for poly-ubiquitin chains but also protect ubiquitylated proteins from the action of the proteasome and de-ubiquitylating enzymes.

Key words: TUBEs, Ubiquitylation, Isolation, Purification, Analysis

1. Introduction

Purification and enrichment of ubiquitylated proteins is hampered by their inherent instability, stemming both from proteasomal degradation and de-conjugation by de-ubiquitylating enzymes (DUBs) (1, 2). To prevent loss of modified protein, traditional methods rely on the use of tagged ubiquitin, proteasome and DUB-inhibitors, and affinity purification under denaturing conditions (3, 4). While such strategies are powerful methods for pull down of ubiquitylated proteins, artifacts related to over expression of tagged ubiquitin or proteasomal inhibition cannot be excluded (5, 6).

The exploitation of ubiquitin-binding domains (UBDs) as agarose conjugates for ubiquitin affinity capture circumvents the requirement of tagged ubiquitin (6). However, the generally low affinity of most UBDs for ubiquitin and poly-ubiquitin is limiting and the need for DUB and proteasome inhibitors remains. The tandem ubiquitin-binding domains (TUBEs) were developed to overcome limitations of current tools and methods (7, 8). They consist of tandem UBA domain repeats amino-terminally fused to

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GST. The tandem UBA arrangement leads to up to a 1000-fold higher affinity for poly-ubiquitin chains (7). Further, the GST tag allows purification of captured poly-ubiquitin conjugates using well-established methods. In addition to drastically increasing the affinity of UBA domains for poly-ubiquitin, the TUBEs also protect poly-ubiquitylated proteins from de-conjugation and proteasomal degradation in cell lysates (7), thus obviating the strict requirement for DUB and proteasomal inhibitors. Cell or tissue lysis in the direct presence of TUBEs immediately shield conjugated proteins from degradation/deconjugation, and ubiquitin purification can then be done by conventional GST pull down.

Since purification takes place under native conditions, one can also easily identify factors specifically interacting with ubiquitylated species of a particular protein, a process which previously required tagging of the protein of interest directly or de-convolution of complicated mass spectrometry data. This may be achieved by a TUBE-IP double purification, where ubiquitylated proteins in a first step are captured using TUBEs, followed by elution with glutathione. The eluate can then be applied to antibody-coupled resins for IP analysis (7, 9). The ease with which a protein of interest can be assayed for ubiquitylation using the TUBEs, as well as the protective properties of these on ubiquitin conjugates, makes it an attractive novel tool for researchers. Below follow protocols detailing the use of the TUBEs for purification of ubiquitin conjugates.

2. Materials

Prepare all solutions using ultrapure water (8 MΩ cm at 25°C) and analytical grade reagents. Prepare and store all reagents at 4°C (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. We do not add sodium azide to the reagents.

2.1. Buffers

1. Lysis buffer 1: 20 mM Tris–HCl, pH 8, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT. Add extemporary 1 mM pefabloc SC and one mini-tablet of complete protease inhibitor cocktail (Roche).
2. Lysis buffer 2: 50 mM Tris–HCl, pH 8.5, 150 mM NaCl, 5 mM EDTA, 1% Igepal (Sigma), 1 mM pefabloc SC, and one mini-tablet of complete protease inhibitor cocktail.
3. Lysis buffer 3: 50 mM NaF (Sigma), 5 mM tetra-sodium pyrophosphate (Sigma), 10 mM β-glyceropyrophosphate (Sigma), 0.2% Igepal, 2 mM EDTA, 20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 1 mM Pefabloc SC, and one mini-tablet of complete protease inhibitor cocktail.
4. Washing buffer: PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, 1.76 mM KH_2PO_4, pH 7.4) with 0.05% Tween 20 (Sigma).

5. Elution buffer: 50 mM Tris–HCl containing 10 mM glutathione (Sigma), pH 9.
6. Dialysis buffer: 50 mM Tris–HCl, pH 8.5, 150 mM NaCl, 5 mM EDTA, 0.1% Igepal.
7. Immunoprecipitation (IP) buffer: 50 mM Tris–HCl, pH 8.5, 150 mM NaCl, 5 mM EDTA, 1% Igepal. Add extemporaneously 1 mM Pefabloc SC.
8. Boiling buffer: 50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, Bromophenol Blue, 10% β-mercaptoethanol.

2.2. Affinity Chromatography

1. Preparation of glutathione beads: Reconstitute 10 mL lyophilized glutathione-agarose beads (Sigma) in de-ionized water overnight at 4°C (or minimally for 30 min at room temperature). After swelling, the agarose has to be washed thoroughly with ten volumes of de-ionized water or PBS three times by centrifugation at 300 × *g* during 5 min to remove lactose or ethanol (present in the lyophilized powder to preserve agarose beads). At the end, the beads are suspended in PBS to obtain 50% (v/v) slurry.
2. Immunoprecipitation: Ubiquitylated proteins captured by TUBEs is performed with specific antibodies (e.g., IκBα or p53) cross-linked to Protein A or Protein G magnetic beads (Invitrogen) (7).

2.3. Protein Concentration and Purification

1. TUBEs are concentrated using Amicon Ultra Centrifugal Filter, nominal molecular weight cut off of 3 kDa (Millipore).
2. Before immunoprecipitation, glutathione eluted polyubiquitylated proteins are dialyzed using Slide-A-Lyzer-7kD (Thermo Scientific).

3. Methods

Carry out all procedures at 4°C unless otherwise specified. A diagram integrating all steps and alternative procedures of this protocol is illustrated in Fig. 1.

3.1. Preparation of Recombinant TUBEs

TUBEs can be purchased through Life-Sensors Inc. (Malvern, PA, USA) or produced in *Escherichia coli* (C41-DE3) using a standard protocol for the production of recombinant proteins as follows:

1. Shake 1 L of culture bacteria in LB at 37°C in the presence of 0.01% antifoam (Sigma) to reach an OD_{600} of approximately 0.5. Induce expression of TUBEs by addition of 1 mM *I*so*p*ropyl β-D-1-*t*hio*g*alactopyranoside (IPTG) and grow bacteria for 6 h at 20°C.

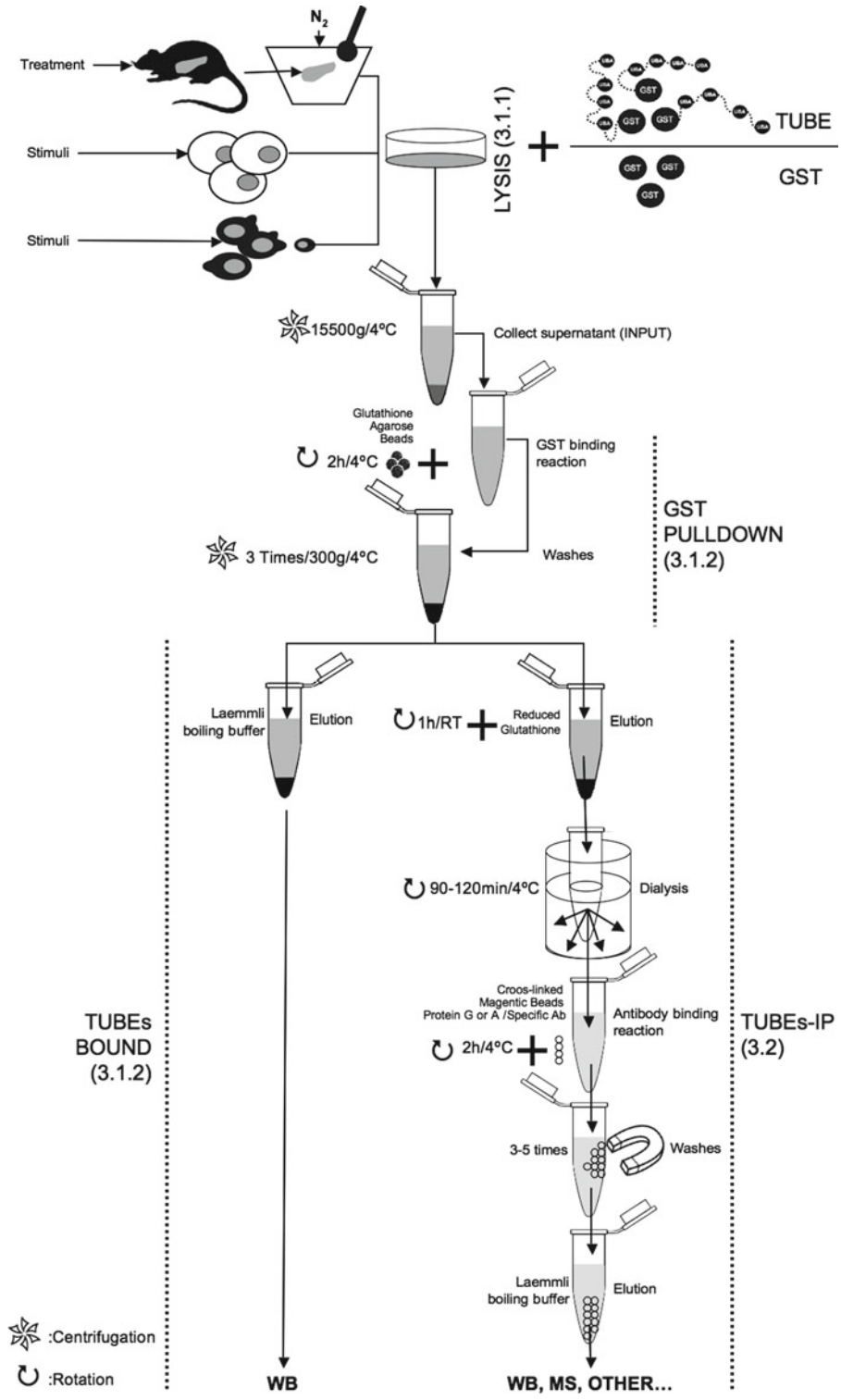


Fig. 1. Purification of total and specific ubiquitylated proteins using TUBEs. Integrative diagram of all steps as described in Subheadings 3.2 and 3.3.

2. Pellet bacteria by centrifugation at 6,693 × *g* for 30 min at 4°C. Wash pellet twice with cold PBS and resuspend in 10 mL of cold PBS supplemented with 2 mM benzamidine.
3. Lyse cells on ice by sonication at 10 µm (six pulses of 30 s, 1 min incubation on ice between each pulse).
4. Supplement lysates with Triton X-100 to a final concentration of 1% (v/v) and clarify by ultracentrifugation for 1 h at 48,384 × *g* and 4°C.
5. Incubate the clarified lysate with 1 ml of glutathione-agarose beads in a 50-mL tube for 2 h at 4°C.
6. Load the agarose beads into a column and rinse five times with one column volume of PBS + 1% Triton X-100.
7. Elute TUBEs with 1 mL of 10 mM reduced glutathione diluted in 50 mM Tris–HCl, pH 9.5. Repeat this step at least five times. Check the protein peak by Bradford assay.
8. Exchange buffer and concentrate in PBS by using Amicon Ultra Centrifugal Filter with a nominal molecular weight cut off of 3 kDa. Store TUBEs in PBS (for surface plasmon resonance analysis) or Tris–HCl, pH 8, containing 10% glycerol (see Note 1).
9. Estimate protein concentration by UV absorbance at 280 nm using the extinction coefficient ε (Rad23): 66,900 and MW: 58.2 kDa; ε (GST): 43,110 and MW: 30 kDa.

3.2. Purification of Total Ubiquitylated Proteins

3.2.1. Lysis of Organs and Tissues

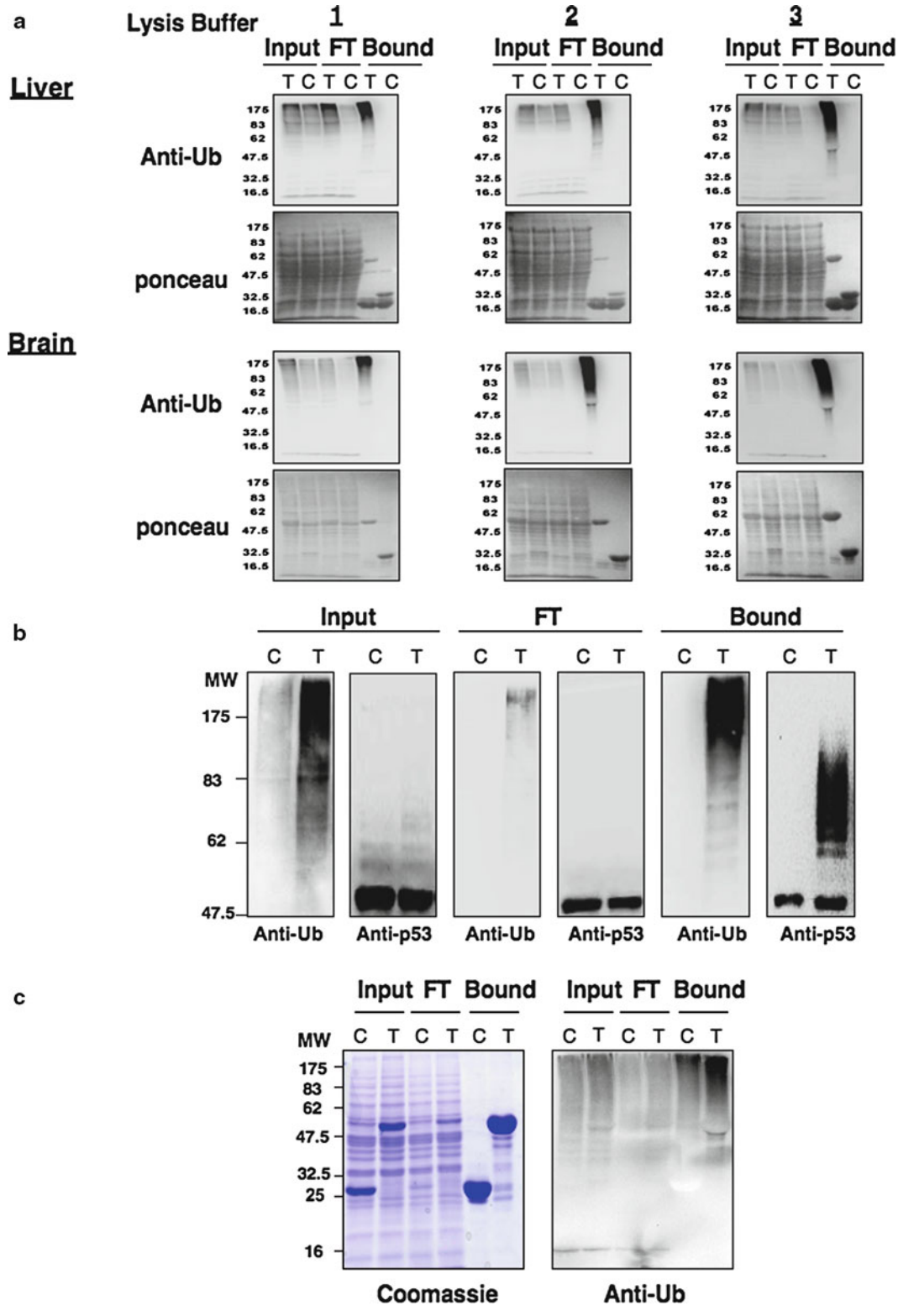
1. Deeply anesthetize adult male Sprague–Dawley rats (250–300 g) with chloral hydrate (see Note 2). Extract tissues and organs, wash with cold PBS, and freeze immediately in liquid nitrogen. All samples are stored at −80°C.
2. Triturate frozen tissues in liquid nitrogen and recover in 1 mL of lysis buffer 1, 2, or 3 per 75 mg frozen tissue, including 100 µg of TUBEs (see Notes 3 and 4) (Fig. 2a).

3.2.2. Lysis of Mammalian Cells

1. Grow cells in 100 mm dishes to a density of approximately $1.2–1.5 \times 10^6$ (see Note 5) cells per time point.
2. Lyse cells directly on the plate (which is placed on ice). For one 100 mm dish, use 500 µL lysis buffer 3 containing either 100 µg of GST-TUBEs or GST control. Importantly, the fact that the TUBEs are present during the lysis likely results in a higher recovery of ubiquitylated proteins due to an immediate protection from DUBs and proteasome activity (Fig. 2b). Alternatively, cells may be harvested by trypsination and pelleted in order to reduce the volume of lysis buffer and amount of TUBEs.

3.2.3. Lysis of Yeast

1. Grow exponential culture until OD = 1–2 when using YPD medium or 0.5–1 for selective medium.
2. Wash 1.5 ODs of cells with PBS or 50 mM Tris–HCl, pH 7.5, before lysis and spin down to remove supernatant. Resuspend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cell pellet in 250 µL of lysis buffer 2 supplemented with 100 µg of GST-TUBES or GST control. Sonicate cells twice 30 s at 10 µm (see Note 6) in 1.5-mL Eppendorf tubes. Maintain samples on ice during the cell disruption procedure. Due to their capacity to protect from DUBs and proteasome activities, TUBEs must be present during lysis step to guarantee maximum recovery of ubiquitylated proteins (Fig. 2c).

3.2.4. GST Pull Down

1. Clarify the lysate by centrifugation in a microfuge at 15500 ×*g* and 4°C for 10 min.
2. Collect the supernatant. Take 1/10 of total volume (e.g., 50 µL) and dilute in equal volume of 3× boiling buffer. This fraction is considered as input (Fig. 1).
3. Add 450 µL of clarified lysate to 100 µL glutathione beads slurry. Adding 1 mM DTT improves GST binding to glutathione column. Incubate lysate with beads, slowly rotating at 4°C for at least 2 h (slow binding reaction) (see Note 7).
4. Spin down beads in a microfuge (300 ×*g* for 5 min) and collect supernatant for analysis.
5. Wash three times with 1 mL ice-cold PBS, 0.05% Tween 20, spin down in a microfuge at 4°C and 300 ×*g* for 1 min. Aspirate carefully until no liquid remains. The beads correspond to TUBEs BOUND (Fig. 1).
6. If you do not wish to continue with an IP, elute the sample with 30 µL 1:1 3× boiling buffer and lysis buffer 3. Perform Western blot analysis using an antiubiquitin antibody or the specific antibody of your choice (Fig. 2) (see Notes 8 and 9).

3.3. Purification of Specific Ubiquitylated Proteins (TUBEs-IP)

1. Continued from step 5 above (Subheading 3.2.4). Elute with 200 µL elution buffer (see Note 10). Use an Eppendorf thermo mixer set to 300×*g* for 45–60 min, or a rotator.
2. Centrifuge in a microfuge at 300 ×*g* for 5 min.
3. Recover supernatant with a long pipette tip avoiding taking beads.

Fig. 2. Analysis of total and specific ubiquitylated proteins from tissues, cell lines, and yeast using direct TUBE-capture protocol. (**a**) Total ubiquitylated proteins are extracted from rat liver and brain using three different lysis buffers. The efficiency of protein extraction can be observed by membrane staining. To avoid the cleavage of TUBEs by the action of liver proteases, chemical inhibitors should be included and buffer 3 should be preferred. Total ubiquitin chains are analyzed in samples either containing GST-control proteins (C) or TUBEs (T) by Western blot using an antiubiquitin antibody (e.g., Clone P4D1, Santa Cruz). (**b**) One million of MCF-7 cells are plated 24 h before lysis with buffer 3, either containing GST-control proteins (C) or TUBEs (T). Pull-down material is analyzed by Western blot using antiubiquitin antibody and anti-p53 antibody (clone DO1, Santa Cruz). (**c**) Extraction of ubiquitylated proteins from yeast. Total ubiquitylated proteins are analyzed by Western blot using antiubiquitin and all fractions are also stained by Coomassie blue. *FT* Flow through obtained after GST pull down, *Bound* corresponds to TUBE-captured material.

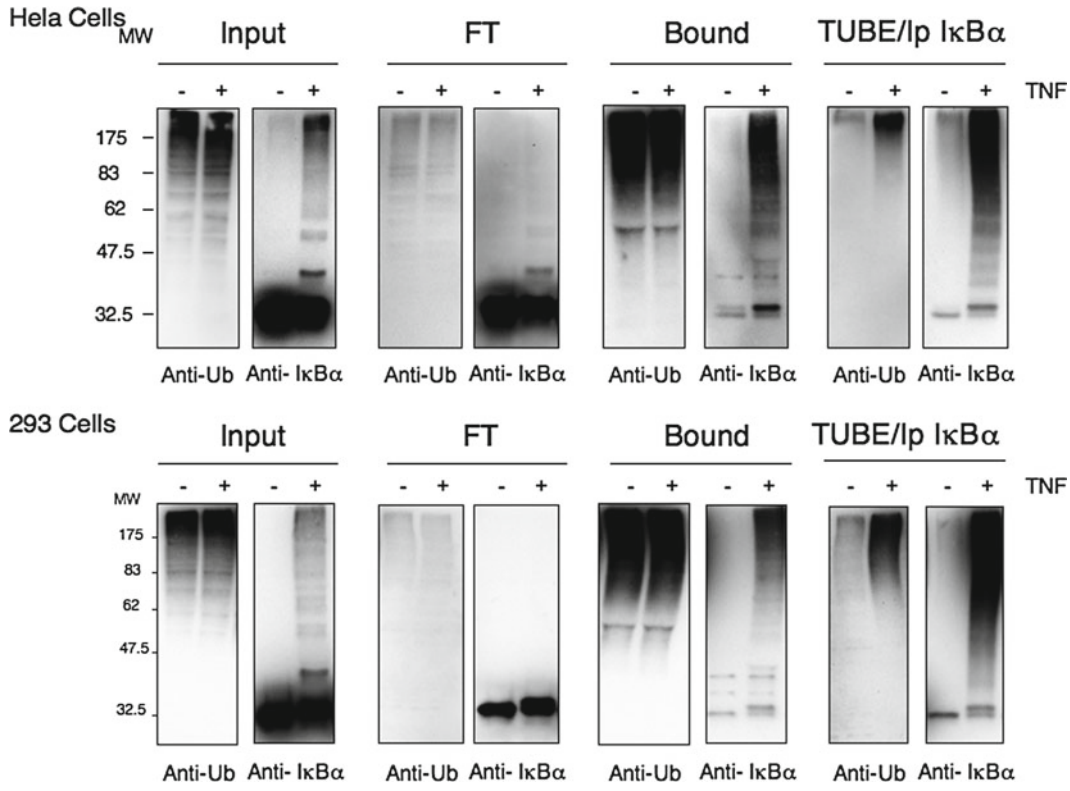

Fig. 3. Analysis of specific ubiquitylated proteins using TUBE-IP procedure (e.g., IκBα). HeLa (0.4×10^6 cells/point) and HEK293 (1.2×10^6 cells/point) cells are plated and after 24 h treated for 1 h with 20 μM of the proteasome inhibitor MG132 and stimulated for 20 min with 10 ng/mL of TNF-α (R&D Systems). Cells are lysed in lysis buffer 3 containing TUBEs, centrifuged as indicated in Fig. 1 and incubated overnight with glutathione beads. GST-captured material is eluted and submitted to IκBα immunoprecipitation. *FT* Flow through obtained after the GST pull down, *Bound* corresponds to part of the GST-captured material.

4. Remove glutathione by dialysis using small filters Slide-A-Lyzer-7kD. Dialyse twice against 1 L of dialysis buffer during 45–60 min (2 L total dialysis buffer) (see Note 11).
5. After dialysis, transfer sample to a new 1.5-mL tube. Add 300 μL of lysis buffer 2 to reach 500 μL final volume.
6. Add to these 500 μL, 30 μL of protein A or protein G magnetic beads previously cross-linked to specific antibodies. For example, p53 or IκBα antibodies are cross-linked using a dimethylpimelimidate based protocol (see Note 12). Incubate 90–120 min by rotating at 4°C.
7. Separate beads and supernatant with a magnetic separator. Keep 1/10 (flow through).
8. Wash beads three times with 1 mL lysis buffer 2 without inhibitors (protease inhibitors are optional in this step).
9. Resuspend beads in 60 μL (30 μL IP buffer plus 30 μL boiling buffer).

10. Boil 5 min at 95°C and load 10 µL on 8–15% polyacrylamide gel (according to the molecular size of the protein of interest) for Western blot analysis (Fig. 3).

4. Notes

1. Do not freeze–thaw TUBEs more than 3–4 times. Prepare small aliquots and store at −80 or −20°C. TUBEs are highly hydrophobic proteins tending to aggregate in conditions without detergents. For this reason, long-term storage buffers should contain at least 10% of glycerol.
2. Rats were kept on a 12/12 h light/dark cycle with constant ambient temperature and humidity. Food and water were available ad libitum. All experiments were approved by the respective institutional committees for animal care and handling. All efforts were made to minimize animal suffering and to reduce the number of animals used.
3. The amount of TUBEs used depends on the relative abundance of the ubiquitylated protein of interest. Very abundant poly-ubiquitylated proteins are easily captured compared to low abundant monoubiquitylated ones. It is recommended to set up conditions analysing by Western blot input, bound, and unbound material using antiubiquitin antibodies and specific antibodies against the protein of interest.
4. Lysis conditions should be selected according to the protein of interest. Tris-, PBS-, or Hepes-based buffers work very well even in the absence of detergents as soon as the protein of interest is released from the cell compartments. Highly denaturing buffers containing guanidinium or urea should be avoided.
5. Do not use too many cells otherwise you can saturate the TUBES and recover only highly abundant ubiquitylated proteins (and loose the low abundant ones). Ideally, TUBEs should be able to pull down 85–95% of total ubiquitylated proteins.
6. Alternative yeast lysis buffers and procedures (e.g., cryolysis) can be considered as long as they do not denature the TUBEs. It is important to verify that used lysis buffers are compatible with an efficient GST capture.
7. This is a slow binding reaction, therefore the incubation can be extended from few hours until overnight to increase recovery without de-conjugation of substrates such as IκBα or p53. The stability of modified forms for other protein substrates should be individually tested when setting up conditions. Beware that ubiquitylated proteins from your GST control will not be protected and signals detected by Western blot will always be lower compared to the one obtained with TUBEs.

8. Ponceau staining of membranes provides useful information about equal charge and transfer of proteins, efficiency of washes, and TUBEs-capture efficacy (Fig. 2a). In tissues such as liver or kidney (data not shown), the cleavage of GST and TUBEs can be significantly reduced by adding higher concentrations of protease inhibitors. Alternatively Coomassie staining of a duplicated gel can provide some of this information (Fig. 2c).
9. Sticky proteins such as p53, IκBα, or ubiquitylated forms of some proteins might bind to the GST control. To remove background, low-density agarose beads, BSA coating, or additional washes can be considered. However, this could affect applications such as mass spectrometry and might result in loss of information.
10. To optimize elution, pH must be between 8.8 and 9.5. Often elution with a single wash is not sufficient. If ubiquitylated proteins are retained on beads, a second wash improves recovery.
11. Dialysis buffer must be prepared in advance and stored at 4°C until use but dialysis can be performed at room temperature if a 2 h protocol is used.
12. Protein G or protein A-agarose or, alternatively, cross-linking protocols can be used according to requirements of experimentation.

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