Ubiquitination and Degradation

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ABSTRACT/SUMMARY

Modification by ubiquitin (Ub) and ubiquitin-like proteins (UBLs) is involved in the regulation of numerous cellular processes, and has therefore become an important subject of research in various areas of biomedicine. Being more “classical” biochemical than molecular biological in its nature, studies of the system have encountered several major difficulties. First and foremost, we lack information on the identity but mostly on mechanisms of action for many of its basic components. For example, our knowledge on modes of recognition of target substrates by ligases and consensus ubiquitination sites is sparse. In addition, lack of basic tools such as antibodies directed against specific internal polyubiquitin chain linkages or analytical methods to decipher the structure of intact chains and their formation, made experimental approaches difficult if not impossible. Here we describe selected basic procedures that allow one to become acquainted with this rapidly evolving field, realizing that one cannot provide a comprehensive coverage of all or even a small part of the methodologies related to this research area. We provide information on how to set a cell free system for ubiquitination - a powerful tool that enables researchers to reconstitute the modification from purified components - and how to identify ubiquitin adducts in cells. Next, we describe methods to follow stability (degradation) of proteins in cell free systems and in cells. Last, we describe experimental approaches to identify “non-canonical” sites of ubiquitination, such as N-terminal modification and tagging of amino acid residues other than lysine.

INTRODUCTION

Covalent modification of proteins by ubiquitin (Ub) and ubiquitin-like proteins (UbL) is involved in the regulation of numerous cellular pathways. Among them are cell cycle and division, growth and differentiation, apoptosis, maintenance of the cell's quality control and response to stress. The ability of the ubiquitin system to regulate such a broad array of processes is due to the fact that the tagging generates an extremely diverse form of a protein-protein interaction module that, according to its structure, enables it to associate with numerous different downstream effectors. Furthermore, the same target protein can be modified by different modules under distinct pathophysiological conditions, thus being targeted to disparate fates. In many cases, the modification is followed by targeting of the tagged proteins to proteasomal or lysosomal degradation, thus terminating their function[1-4]. Yet, in many other processes, the modification does not lead to destruction of the target, and is therefore reversible. Thus, mono- oligo- and polyubiquitination, generation of chains based on linkages to different internal lysines within the ubiquitin molecule, along with mono- oligo- and poly-modifications by SUMO and other UbLs, have converted this novel mode of post-translational modification into an immense regulatory platform. Thus, ubiquitin and UBLs can be regarded as modules that mediate interaction of the substrates to which they are conjugated with other downstream effectors. In the case of degradation, this downstream effector is the 26S proteasome complex. With the myriad of targeted substrates and numerous diverse processes regulated, it has not been surprising to find that aberrations in the system are implicated in the pathogenesis of many diseases – among them malignancies, inflammatory and immune disorders, and neurodegeneration. This has consequently led to efforts to develop mechanism- and

processes-based drugs; one successful anti-cancer drug is already in use. The present protocols describe methods to monitor protein ubiquitination and degradation both in cell free reconstituted systems and in intact cells.

The nature of the bond between Ub and its substrate has been well characterized: the Ub C-terminal Gly^{76} carboxyl group forms in most cases an isopeptide bond with the ε-amino group of an internal lysine residue in the target substrate. As for specificity of the targeted lysines, for some proteolytic substrates, IκBα for example, the modified lysines are highly specific[5], whereas for others, e.g. cyclin B[6] and the ζ chain of the T cell receptor[7], any single lysine, whether native or artificially inserted, can serve as a ubiquitin anchor.

Recent findings indicate that for several proteins, the first ubiquitin moiety is fused linearly to the α-NH_2 group of the N-terminal residue[8] or to residues other than lysine - cysteine[9], serine, and threonine[10,11]. We describe methods that allow the researcher to distinguish between the different sites of ubiquitination.

When the protein is marked for degradation, a polyubiquitin chain is generated where each moiety is typically bound via an isopeptide bond between Gly^{76} of one moiety and the ε-NH_2 group of internal lysine 48 of the previously conjugated moiety[12]. Recent studies report a role for all other internal lysine residues in targeting proteins for proteasomal degradation. Thus, Ubc6 synthesizes preferentially K11-based chains that function in the ERAD (ER-associated degradation) pathway[13]. Heterogeneous mixed chains based on lysines 11, 48 and 63 that target cyclin B1 for proteasomal degradation have been recently described[14]. Interestingly, a similar result was obtained when the chains lacked lysine 48-based linkages[14], though a polyubiquitin chain that contains

Lys^{48} linkages is still regarded as the hallmark of the proteasomal recognition signal. Conjugation of substrates of the ubiquitin fusion degradation (UFD) pathway requires the presence of internal lysines 29 and 48 in the ubiquitin moiety[15].

As we noted, other chains that serve as non-proteolytic signals have been described. Thus, K63-based[16] and linear, head to tail polyubiquitin chains[17,18] have been shown to be involved in NF-κB activation. In another case it has been shown that the autoubiquitinating activity of Ring1B generates an atypical, multiply branched mixed K6-K27-, and K48-based polyubiquitin chain[19] that serves to activate the Ring1B ligase activity towards its natural substrate - Histone H2A - that is monoubiquitinated. In that context it should be noted that monoubiquitination not only serves to epigenetically modulate transcriptional activity by altering nucleosomal structure[20], but also has other functions such as routing proteins and endocytic vesicles to their proper subcellular destinations[21,22]. Similarly, oligoubiquitination may also serve to route endocytic vesicles[23]. The present protocols describe ways to distinguish between different types of ubiquitin chains.

Experimental design

Preparation of cell extract and its sub-fractions for monitoring of conjugation and degradation

Conjugation and degradation can be monitored in crude extract (prepared according to Step 2). It should be noted that commercial reticulocyte lysate preparations used for *in vitro* translation of specific mRNAs should not be used for monitoring degradation. For one, they are expensive and packed in small aliquots that make fractionation difficult, and the obtained fractions small and sufficient for a few reactions only. Also, and probably more important, they contain hemin that is added to stimulate translation. Hemin is a potent inhibitor of deubiquitinating enzymes, which can make it also an inhibitor of proteolysis[24]. The extract prepared according to the protocol can be used also to monitor ATP dependence of degradation, as ATP can be depleted from the cells prior to their disruption by using inhibitors of anaerobic and aerobic respiration that are later removed during dialysis. The extract can be re-supplemented with ATP (and ATP-regenerating system to counteract the activity of ATPases). ATP can be also depleted directly from the crude extract, though here, because respiration does not occur, one needs to use a trap composed of hexokinase and 2-Deoxy-D-glucose. ATP depletion from cells will be also important if one plans to fractionate the extract in order to monitor ubiquitin dependence of degradation, or to follow the fate of exogenously added (e.g. tagged) ubiquitin. Typically, the cell extract is fractionated over the anion-exchange resin diethylaminoethyl (DEAE)-cellulose, where ubiquitin is eluted in Fraction I, the unabsorbed, flow-through material that contains also certain E2 enzymes. Fraction II, the high salt eluate, contains E1, the remaining E2s, all the E3s, and the 26S

proteasome, but not free ubiquitin. Depletion of ATP from cells inhibits ubiquitination, whereas de-ubiquitination continues. This leads to release of ubiquitin from all conjugated substrates and resolution of the free ubiquitin in Fraction I. If ATP is not depleted, ubiquitin-conjugated proteins are resolved in Fraction II. The ubiquitin moiety will be released upon incubation in the studied extract and will be conjugated to substrates, among them the test substrate. Consequently, it will be impossible to monitor ubiquitin dependence of conjugation and degradation in Fraction II.

Labeling of proteolytic substrates

In many cases, monitoring the conjugation and/or degradation of a specific protein substrate requires its labeling (Step 14). The fate of an unlabeled protein can be also followed after blotting to a nitrocellulose membrane, and its detection using a specific antibody.

Biosynthetic labeling of proteins is a frequently used procedure to label substrates and follow their fate *in vitro*. In principle, it is preferred to use wheat germ extract as a source for the protein biosynthetic machinery. This extract lacks many, although not all, of the mammalian E3 enzymes. Therefore, in most cases, a protein synthesized in this extract can be used in experiments in which a cell-free system is reconstituted from purified enzymes, and in particular, when the role of a specific E3 is tested. A protein synthesized in reticulocyte lysate may be “contaminated” in many cases with endogenous E2 and/or E3 enzyme(s) derived from the lysate. The enzymes, which are being carried to the reconstituted conjugation/degradation system, may interfere with the examination of the role of an exogenously added E2 or E3 in these processes. Yet, at times, one must use the reticulocyte lysate, as the translation efficiency in the wheat

germ extract can be extremely low. In that case, if needed, the “contaminating” E2 or E3 in the lysate can be inactivated after translation by N-ethylmaleimide (NEM; 10 min incubation at room temperature in a final concentration of 10 mM of freshly prepared solution). Because E1, all known E2s, and some of the E3s (HECT domain-containing) have an essential –SH group, the alkylating agent inactivates them. The NEM is then neutralized by the addition of DTT (final concentration of 7.5 mM). It should be noted that this procedure can also denature/inactivate the substrate. In most cases, however, the substrate can still be utilized and its behavior mimics faithfully that of the native substrate.

Conjugation of proteolytic substrates in a cell free system

To demonstrate that the degradation of a certain protein proceeds in a ubiquitin-dependent manner, it is essential to demonstrate the intermediates in the process, ubiquitin–protein adducts (Steps 16-19). Typically, incubation of the labeled protein in a complete cell extract in the presence of ATP will lead to the formation of high molecular mass adducts that can be detected following resolution of the mixture in SDS-PAGE. To increase the amount of the adducts generated, one can use two approaches. The nonhydrolyzable ATP analog, adenosine-5'-O-(3-thiotriphosphate) (ATPγS) can be used instead of ATP[25]. The ubiquitin-activating enzyme, E1, can catalyze activation of ubiquitin in the presence of the analog, as it utilizes the α high-energy bond of the nucleotide that is cleavable also in this derivative. In contrast, assembly and activity of the 26S proteasome complex requires the γ of ATP bond that cannot be cleaved in the analog. Caution should be exercised, however when utilizing the ATP analog. Often, phosphorylation of the target protein is required in order for the ubiquitin ligase to

recognize it and conjugate it with ubiquitin[26]. In these cases, the analog cannot substitute the hydrolyzable native ATP. An additional approach to increase the amount of generated conjugates in a cell-free system is to use ubiquitin aldehyde (UbAl), a specific inhibitor of certain ubiquitin C-terminal hydrolases, isopeptidases[27].

Degradation of proteolytic substrates in a cell free system

As noted earlier, for accumulation of ubiquitin adducts in cell free systems, it is possible to inhibit the activity of the proteasome by utilization of the nonhydrolyzable analog ATPγS (see above). Stabilization of a protein under such conditions strongly suggests that the protein is indeed degraded by the 26S proteasome. To demonstrate more directly the involvement of the 26S proteasome in protein degradation, it is recommended to use inhibitors of the 20S proteasome. Inhibition of the proteasome in a cell free system sometimes requires higher concentrations of the inhibitor (two- to fivefold) compared to the concentrations used to inhibit the enzyme in cultured cells.

Ubiquitination and degradation of proteolytic substrates in cells

It appears that the vast majority of the proteolytic substrates of the ubiquitin-proteasome system (UPS) is degraded by the 26S proteasome following generation of a covalently conjugated polyubiquitin chain. One established exception is ornithine decarboxylase, ODC[28]. This enzyme is degraded by the 26S complex without prior ubiquitination. A non-covalent association with another protein, antizyme, renders ODC susceptible to degradation by the proteasome. It appears that the cell cycle inhibitor p21 is degraded via both ubiquitination-dependent and independent modes[29-31].

The core catalytic subunit of the 26S enzyme is the 20S proteasome complex, and inhibition of its active sites inhibits all proteolytic activities of the 26S proteasome. It

appears that the 20S proteasome by itself is not involved in targeting proteins in the intact cell, despite being the core catalytic active complex, and all the activity - which is regulated - is mediated by the 26S enzyme. To test whether a certain protein is degraded by the 26S proteasome *in vivo*, the 20S complex should be inhibited. This leads to accumulation of the intermediate ubiquitin adducts of the test protein that are hard to detect when the proteasome is active. Demonstration of the intermediate adducts serves as a strong evidence that the protein is degraded by the 26S proteasome complex following tagging by ubiquitin. A complementary approach to the utilization of proteasome inhibitors that results in stabilization of the substrate, is the use of cells that harbor a temperature sensitive mutation in the ubiquitin-activating enzyme E1, the first enzyme in the ubiquitin proteolytic cascade. At the non-permissive temperature, the cells fail to conjugate the target proteins which are consequently stabilized. Such cells can be, for example, cells derived from mouse mammary carcinoma cell line (FM3A-wild type), ts85 (mutant)[32], or the mouse embryonic fibroblast cells BALB/3T3 (wild type) and ts20 (mutant)[33]. When using these cells, the experimental approach can be either pulse-chase labeling and immunoprecipitation or cycloheximide chase. It should be mentioned however that the mutation is “leaky”, and it is hard to inhibit the enzyme completely. For each experiment, the level of inhibition should be monitored by one of several methods, and it should be at least 20% or less of the level in the untreated cells.

MATERIALS

REAGENTS

- Adenosine 5'-[γ-thio]triphosphate tetralithium salt (ATPγS; Sigma, cat. no. A1388)
- Adenosine 5'-triphosphate disodium salt (ATP; Sigma, cat. no. A7699)
- Albumin from bovine serum (BSA; Sigma, cat. no. A9430)
- Ammonium sulfate [$(NH_4)_2SO_4$; Sigma, cat. no. A4418]
- Antibodies against the test protein or tag [mouse anti-Flag (M2; Sigma, cat. no. F3165), mouse anti-Flag affinity agarose gel (M2; Sigma, cat. no. A2220), mouse anti-HA (16B12; Covance, cat no. BIOT-101L), anti-Myc (9E10; Santa Cruz Biotechnology, Inc., cat. no. sc-40), anti-Mdm2 antibody (a mixture of 4B2 and 2A9 monoclonal antibodies, was kindly provided by Dr. Moshe Oren, Weizmann Institute, Israel)]
- Chloramine-T hydrate ($CH_3C_6H_4SO_2N(Cl)Na$ x $2H_2O$; Sigma, cat. no. C9887) **CAUTION** Corrosive
- Chloroform ($CHCl_3$; Sigma, cat. no. C2432) **CAUTION** Harmful
- *clasto*-Lactacystin β-lactone(Enzo® Life Sciences, cat. no. PI108-0100, Boston Biochem Inc., cat. no. I-100) or MG 132 (Z-Leu-Leu-Leu-CHO; Enzo® Life Sciences, cat. no. PI102-0005, Boston Biochem Inc., cat. no. I-130), or epoxomicin (Enzo® Life Sciences, cat. no. PI127-0100, Boston Biochem Inc., cat. no. I-110), or Z-Leu-Leu-Leu-vinyl sulfone (Enzo® Life Sciences, cat. no. ZW9170-0500), or MG 262 (Z-Leu-Leu-Leu-$B(OH)_2$; Enzo® Life Sciences, cat. no. PI109-0100, Boston Biochem Inc., cat.no. I-120)
- Cultured cells in a monolayer or in suspension (tested for the absence of mycoplasma contamination)
- Coupled transcription–translation systems [TNT® Reticulocyte Lysate system with SP6 (Promega, cat. no. L4600), T7 (Promega, cat. no. L4610) or T3 (Promega, cat. no. L4950) polymerase promoters or TNT® Wheat Germ Extract system with SP6 (Promega, cat. no. L4130), T7 (Promega, cat. no. L4140) or T3 (Promega, cat. no. L4120) polymerase promoters and S30 for bacterially derived extract (Promega, cat. no. L1130)].
- Cycloheximide (Calbiochem, cat. no. 239764) **CAUTION** Toxic, dangerous for the environment
- 2-Deoxy-D-glucose ($C_6H_{12}O_5$; Sigma, cat. no. D8375)
- Dialyzed serum (the same serum in which cells are grown)
- Diethylaminoethyl cellulose (DEAE cellulose, DE-52; Whatman, cat no. 4057910)
- 2,4-dinitrophenol [$(O_2N)_2C_6H_3OH$; Sigma, cat. no. D198501]
- 1, 4-Dithiothreitol (DTT, $C_4H_{10}O_2S_2$; Sigma, cat. no. 43817) **CAUTION** Harmful

- Ethylenediaminetetraacetic acid [EDTA, $HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$; Sigma, cat. no. 431788] **CAUTION** Harmful
- E2: UbcH5c (Enzo® Life Sciences, cat. no. BML-UW9070), UbcH7 (Enzo® Life Sciences, cat. no. BML-UW9080), UbcH8 (Enzo® Life Sciences, cat. no. BML-UW9135)
- H_2O, double-distilled (ddH_2O)
- Hexokinase (HK, suspension in 3.2 M ammonium sulfate solution, pH~6.5; Roche, cat. no. 11426362001)
- *In vitro* translation–transcription coupled kit [TNT® reticulocyte lysate system with SP6 (Promega, cat. no. L4600), T7 (Promega, cat. no. L4610) or T3 (Promega, cat. no. L4950) polymerase promoters or TNT® wheat germ extract system with SP6 (Promega, cat. no. L4130), T7 (Promega, cat. no. L4140) or T3 (Promega, cat. no. L4120) polymerase promoters or S30 for bacterially derived extract with T7 polymerase promoter (Promega, cat. no. L1130)]
- Iodoacetamide (ICH_2CONH_2; Sigma, cat. no. I1149) **CAUTION** Toxic
- Isoamyl alcohol [$(CH_3)_2CHCH_2CH_2OH$; Sigma, cat. no. 320021] **CAUTION** Harmful
- L-[^{35}S]Methionine – for *in vitro* translation (specific activity 1000 Ci/mmol at 43.3 mCi/ml; PerkinElmer®, cat. no. NEG009005MC) **CAUTION** When handling radioactive materials, appropriate safety precautions must be followed
- L-[^{35}S]Methionine – for pulse-chase labeling (EasyTag; specific activity 1000 Ci/mmol at 10.2 mCi/ml; PerkinElmer®, cat. no. NEG709A005MC) **CAUTION** When handling radioactive materials, appropriate safety precautions must be followed
- L-Methionine (Sigma, cat. no. M9625)
- MAb to Polyubiquitin (K^{63}-linkage-specific, HWA4C4; Enzo® Life Sciences, cat. no. BML-PW0600)
- Magnesium chloride ($MgCl_2$; Sigma, cat. no. M8266)
- Methionine-free medium (the same medium in which the cells are growing but that lacks methionine and used for labeling). Dulbecco's modified Eagle medium (DMEM), minimum essential medium (MEM), or Richter's Improved MEM Insulin (RPMI) medium are the media that are most frequently used.
- Methylated ubiquitin (MeUb; Enzo® Life Sciences, cat. no. BML-UW855-0001, Boston Biochem Inc., cat. no. U-502)
- Methylene blue ($C_{16}H_{18}ClN_3S$ x $3H_2O$; Sigma, cat. no. M9140) **CAUTION** Harmful
- N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), pH 7.5 (HEPES; Sigma, cat. no. H3537)
- *N*-Ethylmaleimide (NEM, $C_6H_7NO_2$; Sigma, cat. no. E-3876) **CAUTION** Toxic

- New Zealand white rabbits (preferably females) of approx 2 kg body wt (2–3 months old) for preparation of reticulocyte lysate. **CAUTION** Experiments involving live animals must be according to international, national and institutional regulations
- Nuclease-free water (Promega, cat. no. P1193)
- Phenol (C_6H_5OH; Sigma, cat. no. 77613) **CAUTION** Toxic
- Phenylhydrazine ($C_6H_5NHNH_2$; Sigma, cat. no. 26252) **CAUTION** Toxic**,** dangerous for the environment
- Phosphate buffered saline (PBS; Sigma, cat. no. P5368)
- Phosphocreatine ($C_4H_8N_3O_5PNa_2$; Sigma, cat. no. P7936)
- Phosphocreatine kinase (Sigma, cat. no. C3755)
- Potassium chloride (KCl; Sigma, cat. no. P9541)
- Potassium phosphate dibasic (K_2HPO_4; Sigma, cat. no. P2222) **CAUTION** Harmful
- Potassium phosphate monobasic (KH_2PO_4; Sigma, cat. no. P5655)
- Protease Inhibitor Cocktail Set1 (Calbiochem, cat. no. 53913)
- Proteins A and G (immobilized; Roche, cat. nos. 11134515001 and 11243233001, respectively)
- Purified protein substrates (see main text)
- Rabbit Reticulocyte Lysate for *in vitro* translation (Promega, cat. no. L4960)
- Restriction enzymes
- RiboMAX™ Express Systems for generation of mRNA from linearized cDNA (RiboMAX™ Large Scale RNA Production System—SP6; Promega, cat. no. P1280 or RiboMAX™ Large Scale RNA Production System T7; Promega, cat. no. P1300)
- Ribonuclease Inhibitor (RNasin®; Promega, cat. no. N2111)
- Sodium ^{125}I (Na[^{125}I]; specific activity of 100–350 mCi/ml, PerkinElmer®, cat. no. NEZ033A025MC) **CAUTION** When handling radioactive materials, appropriate safety precautions must be followed
- Sodium azide (NaN_3; Sigma, cat. no. S8032) **CAUTION** Toxic, dangerous for the environment
- Sodium bicarbonate ($NaHCO_3$; Sigma, cat. no. S6297)
- Sodium chloride (NaCl; Sigma, cat. no. S7653)
- Sodium citrate [$HOC(COONa)(CH_2COONa)_2 \times 2H_2O$; Sigma, cat. no. S1804]
- Sodium dodecyl sulfate (SDS, $CH_3(CH_2)_{11}OS)_3Na$; Sigma, cat. no. L4390) **CAUTION** Harmful, flammable
- Sodium fluoride (NaF; Sigma, cat. no. S7920) **CAUTION** Toxic
- Sodium iodide (NaI; Sigma, cat. no. 383112) **CAUTION** Harmful
- Sodium metabisulfite ($Na_2S_2O_5$; Sigma, cat. no. 255556) **CAUTION** Harmful

- Sodium phosphate dibasic (Na_2HPO_4; Sigma, cat. no. S7907)
- Sodium phosphate monobasic (NaH_2PO_4; Sigma, cat. no. S8282)
- Trichloroacetic acid (TCA, Cl_3CCOOH; Sigma, cat. no. T0324) **CAUTION** Corrosive**,** dangerous for the environment
- Triton® X-100 (Sigma, cat. no. T9284) **CAUTION** Harmful**,** dangerous for the environment
- Trizma® base (Tris, $C_4H_{11}NO_3$; Sigma, cat. no. T1503)
- Ubiquitin (Sigma, cat. no. U6253)
- Ubiquitin aldehyde (UbAl; Enzo® Life Sciences, cat. no. UW8450-0050 or Boston Biochem Inc., cat. no. U-201)
- Ubiquitin with all its internal lysines substituted with arginines (UbK0; Boston Biochem Inc., cat. no. UM-NOK)
- Wheat Germ Extract Plus for *in vitro* translation (Promega, cat. no. L3250)
- Wheat Germ Flexi® Vectors for independent mRNA-driven *in vitro* translation (Promega, cat. nos. L5671, L5681)

EQUIPMENT (disposable)

- Centrifugation filtration device (GE Healthcare, cat. no. 289322)
- Desalting column PD MiniTrap G-25 (GE Healthcare, cat. no. 28918007)
- Dialysis tubing

EQUIPMENT (permanent)

- FPLC with a MonoQ column (GE Healthcare)
- Luminescence Spectrometer LS 50S (PerkinElmer®)
- Nitrogen Cavitation Bomb in a volume of 45 ml (Parr Instrument Company, cat. no. 4639, 211 53rd Street, Moline, IL 61265- 9984. Tel: 1-800-872-7720 or 1-309-762-7716; Fax: 309-762-9453)
- PhosphorImager (Fuji, Japan)
- β-scintillation counter
- γ-counter

REAGENT SETUP

Buffer A. KPi pH 7.0 (3 mM), DTT (1 mM).

Buffer B. KPi pH 7.0 (3 mM), KCl (20 mM), DTT (1 mM).

Buffer C. Tris-HCl pH 7.2 (20 mM), KCl (500 mM), DTT (1 mM).

Buffer D. Tris-HCl pH 7.2 (20 mM), DTT (1 mM).

Chloramine-T. 10 mg/ml freshly dissolved in NaPi pH 7.5 (50 mM).

Chloroform:isoamyl alcohol. Mix the reagents in a 24:1 ratio, respectively.

Cycloheximide. Dissolve in ethanol (100 mg/ml) or H_2O (20 mg/ml). Final concentration in cultured cell medium is 20–100 μg/ml. **CRITICAL STEP** Freshly dissolved solution.

2-Deoxy-D-glucose. Dissolve in water. Stock solution is 1 M.

HEPES-saline. HEPES pH 7.5 (25 mM), NaCl (124 mM), KCl (4 mM), $MgSO_4$ (1.2 mM), and $CaCl_2$ (1 mM).

Hexokinase. Centrifuge the ammonium sulfate slurry and resuspend to the original volume and concentration (10 mg/ml) in Tris-HCl buffer pH 7.6 (20 mM). Dilute in the same buffer. Stock solution in the buffer can be stored at 4°C for at least 4 weeks.

"Hot" lysis buffer I. SDS (1%), EDTA (1 mM) in PBS.

"Hot" lysis buffer II. Triton® X-100 (2%), sodium deoxycholate (DOC) (0.5%), BSA (1%), EDTA (1 mM), and protease inhibitors mixture (diluted according to the manufacturer's instructions) in PBS. **CRITICAL STEP** Protease inhibitors mixture should be added to the lysis buffer just before use.

"Hot" lysis buffer III. Triton® X-100 (1%), SDS (1%), sodium deoxycholate (DOC) (0.5%), BSA (1%), and EDTA (1 mM) in PBS.

Krebs-Ringer phosphate buffer (KRP). NaCl (130 mM), KCl (5 mM), $CaCl_2$ (1.3 mM), $MgSO_4$ (1.3 mM), and NaPi pH 7.4 (10 mM).

KRP with ATP depleting reagents. KRP that contains also 2-Deoxy-D-glucose (20 mM diluted out of 1 M stock solution) and 2,4-dinitrophenol (2,4-DNP; 0.2 mM diluted out of 20 mM stock solution. $NaHCO_3$ should be added to allow dissolution of the 2,4-DNP in the stock solution).

Lysis buffer with iodoacetamide and N-ethylmaleimide (NEM). CRITICAL STEP Iodoacetamide and NEM (final concentration of 5 mM each) should be freshly dissolved (0.1 M) and added to the lysis buffer just before use.

Na-metabisulfite. 20 mg/ml freshly dissolved in NaPi pH 7.5 (50 mM).

Phenol:chloroform:isoamyl alcohol. Mix the reagents in a 25:24:1 ratio, respectively, centrifuge for 5 min at 4,000 rpm, and use the lower phase.

Phosphocreatine kinase. Dissolve in Tris-HCl pH 7.6 (50 mM). Stock solution is 10 mg/ml.

Phosphocreatine. Dissolve in H_2O. Stock solution is 1 M.

Potassium phosphate buffer (KPi) pH 7.0 (300 mM). Titrate K_2HPO_4 (300 mM) with KH_2PO_4 (300 mM) to pH 7.0.

Proteasome inhibitors. Dissolve in DMSO. Stock solutions are of approximately 10 mM and final concentration in cultured cells is approximately 10–20 µM. Please refer to the catalogs of the suppliers.

RIPA buffer. NaCl (150 mM), sodium deoxycholate (0.5%), Tris-HCl pH 8 (50 mM), SDS (0.1%), NP-40 (1%), and protease inhibitors mixture. **CRITICAL STEP** Protease inhibitors mixture should be added to the lysis buffer just before use.

Sample buffer x 2. Tris-HCl pH 6.8 (125 mM), SDS (4%), glycerol (20%), and β_2-mercaptoethanol (1.4 M).

Sodium acetate. pH 4.5 (3 M). Titrate acetic acid (stock solution is 17.5 M) to the desired pH using NaOH, and bring to the desired volume using ddH_2O.

Sodium phosphate (NaPi) pH 7.5 (100 mM). Titrate Na_2HPO_4 (100 mM) with NaH_2PO_4 (100 mM) to pH 7.5.

Staining solution. Methylene blue (1 g), sodium citrate (0.4 g), sodium chloride (0.85 g), distilled water to 100 ml. Dissolve first sodium citrate in normal saline and then the dye. Filter it and store at 4°C.

Tris-hydroxymethyl aminomethane (Trizma® base; Tris buffer). 1 M stock solution brought to the desired pH with concentrated HCl. Please refer to the supplier's catalog for change in pH between 4, 25, and 37°C.
As a rule for all procedures carried out in the cold, one should use a Tris buffer prepared at 25°C at pH 7.2. For reactions carried out at 37°C, one should use a Tris buffer prepared at 25°C at pH 7.6.

PROCEDURES

1 For ubiquitination/degradation of proteolytic substrates in a cell free system, proceed according to Steps 2-20 (see flow chart, shown in Figure 1); for ubiquitination/degradation of proteolytic substrates in cells, proceed according to Steps 21-28 (see flow charts, shown in Figure 2 and Figure 3).

Preparation of crude cell extracts for monitoring conjugation and degradation of protein substrates in a cell free system

2 Rabbit reticulocyte lysate (Method A) contains all the enzymes required for conjugation and degradation of most protein targets, and is therefore useful for a starting experiment. Reticulocytes contain a relatively small number of proteins and do not have lysosomes from which proteases can leak during preparation of the extract. Unlike cultured cells extract, one can obtain reticulocyte lysate in a relatively large amount. Also, the lack of requirement for tissue culture media and sera make this lysate significantly less expensive than its nucleated cultured cells counterpart. Yet, nucleated cell extract (Method B) can be useful in certain cases with specific requirements. Thus, under certain conditions, ubiquitin system elements and/or exogenous modifying enzymes have to be activated/modified (during NF-κB activation or β-catenin degradation where certain proteins have to be phosphorylated or modified by the UBL NEDD8), or certain proteins have to be present (like the human papilloma virus E6 protein that is present in HeLa cells and is necessary for E6-AP-dependent degradation of p53). In other cases one would like to use a cell extract where a specific component(s) was inactivated (e.g. siRNA).

A. Preparation of reticulocyte lysate.

i. Inject rabbits subcutaneously with 10 mg/kg of phenylhydrazine (dissolved in phosphate-buffered saline [PBS]) on days 1, 2, 4, and 6.

ii. Bleed the rabbits from the ear artery or vein, or from the heart (following anesthesia) on day 8.

iii. Determine the % of reticulocytes in the peripheral blood (should be >90%). [Incubate 0.3 ml of blood with 0.2 ml of staining solution (described under REAGENT SETUP) for 15 min at 37°C. Prepare a thin smear of the stained blood specimen by using a spreader slide. Air dry the smear. Count reticulocytes under immersion objective in about 10 fields. Reticulocytes are identified by fine deep violet filaments and granules arranged in a network].

TIMING (i-iii) - 8 days

iv. Wash the cells (centrifuge at 1,000 x g for 10 min) three times with ice-cold PBS. **CRITICAL STEP** Aspirate carefully the thin layer of white blood cells ("buffy coat") that overlays the pelleted red blood cells.

v. If it is necessary to deplete ATP ("Experimental design"), proceed as described under this section, otherwise, move to vi. Resuspend the cells in one volume of Krebs-Ringer phosphate buffer containing 2-Deoxy-D-glucose (20 mM) and 2,4-dinitrophenol (0.2 mM). Following incubation accompanied by gentle shaking for 90 min at 37°C, wash the cells twice in ice cold PBS.

vi. Break cells open in 1.6 volumes (of pelleted cells volume) of ice-cold H_2O containing DTT (1 mM).

vii. Centrifuge at 80,000 x g for 1 hr at 4°C to remove particulate material.

viii. Collect the supernatant and freeze in aliquots at –70° C. **PAUSE POINT** Reticulocyte lysate is stable at –70° C for several years.

TIMING (for iv-viii) – 5 hr

B. Preparation of extract from cultured cells

i. Seed cells and grow them to almost confluency (in case of adhering cells) or to a density of 10^5-10^6 cells/ml (in case of cells in suspension)

TIMING for i 2-3 days

All procedures from here on are carried out at 4°C/on ice.

ii. Wash cells three times in HEPES pH 7.5 (20 mM) saline buffer, and resuspend to a concentration of 10^7–10^8 cells/ml in HEPES pH 7.5 (20 mM), that contains DTT (1 mM).

iii. If it is necessary to deplete ATP, proceed as described under this section, otherwise, move to iv. Resuspend the cells in Krebs-Ringer phosphate buffer (to a density of 10^7 cells/ml in the presence of 2-Deoxy-D-glucose, 2,4-dinitrophenol (as described earlier), NaF (20 mM), and NaN_3 (10 mM). In case of adhering cells, add to the plate the same solution. Following incubation for 60 min at 37° C, wash the cells twice in HEPES–saline and resuspend in HEPES–DTT (1 mM).

iv. Cavitate the cells in a high-pressure nitrogen chamber. For HeLa cells, the best conditions are 1,000 psi for 30 min. However, these conditions

can be different for different cells. Alternatively, cells can be frozen (in liquid N_2) and thawed several times. **CRITICAL STEP** Make sure that most of the cells are disrupted by visualizing the suspension in a light microscope before and after cavitation. Following disruption, one should observe mostly intact nuclei and cell debris.

v. Centrifuge the homogenate successively at 3,000 x g and 10,000 x g for 15 min (each speed), and then at 80,000 x g for 60 min. The supernatant is collected and frozen at –70° C. Extract should contain 5-10 mg/ml of protein. **PAUSE POINT** Extract is stable at –70° C for several years.

TIMING for ii-v 4 hr

Fractionation of cell extract to Fraction I and Fraction II[34] TIMING 24 h

All procedures are carried out at 4°C.

3 Swell the resin (DEAE cellulose; DE-52) in 0.3 M potassium phosphate pH 7.0, for several hours (can be prepared a day before fractionation and kept at 4°C). Use enough resin to absorb proteins in the extract. As a rule, use 0.6 resin volume per volume of reticulocyte lysate or 1 ml resin/~5 mg of protein of nucleated cell extract [in principle, one can use also a chromatographic system such as fast protein liquid chromatography (FPLC; GE Healthcare) with an anion exchange resin-loaded column (MonoQ or Q Sepharose), though, for resolution of large quantities, the DEAE resin procedure is advantageous].

4 Load the resin onto a column and wash with 9 column volumes of buffer A without DTT (can be done a day before fractionation if the column is kept at 4°C).

5 Wash the resin with 1 column volume of buffer A + 1 mM DTT.

6 Load the extract. Once all the material is loaded, elute Fraction I with buffer A. **CRITICAL STEP** When resolving reticulocyte lysate, collect only the dark red fraction. When resolving cell extract, collect only the fractions with the highest absorption at 280 nm. Freeze Fraction I in aliquots at –70ºC.

7 Wash the column extensively with buffer B. **CRITICAL STEP** When resolving reticulocyte lysate, make sure all the hemoglobin is eluted. When resolving nucleated cell extract, wash until the absorbance at 280 nm returns to baseline.

8 Elute Fraction II with 2.5 column volumes of a buffer C. **CRITICAL STEP** Collect Fraction II into a flask immersed in ice.

9 Add ammonium sulfate to saturation (~70 g/l of solution) and swirl on ice for 30 min.

10 Centrifuge at 15,000 rpm for 20 min.

11 Resuspend pellet in buffer D using 0.2–0.3 volume of the original extract. **CRITICAL STEP** At times, it will be impossible to dissolve all the proteins. This is not essential. Collect however the slurry of the pelleted proteins into the dialysis tubing; it will be dissolved during dialysis.

12 Dialyze overnight against 100-500 volumes of buffer D (change buffer in the morning for additional several hours).

13 Remove particulate material by centrifugation at 15,000 rpm for 15 min. Freeze in aliquots at –70°C. **PAUSE POINT** Fraction I and Fraction II are stable at –70°C for several years.

Labeling of proteolytic substrates

14 Two methods of labeling have proved to be useful, iodination (Method A) and biosynthetic incorporation of L-[^{35}S]methionine (Methods B and/or C). Iodination is

utilized mostly when a purified recombinant or a pure commercial protein are available. The main advantage of the method is the high specific radioactivity that can be obtained and the low cost. The disadvantage of the method is that one needs a pure protein. Also, during iodination, unless it is carried out using the Bolton–Hunter reagent, the protein can be damaged from chloramine T, the reagent used to oxidize the iodide anion converting it to a free radical. In addition, during storage, the labeled substrate may be subjected to radiochemical damage from the isotope. A different method of labeling utilizes incorporation of ^{35}S-labeled methionine to a protein that is synthesized in a cell-free system from its corresponding mRNA (and cDNA). The generated protein is native; however, the specific activity obtained is relatively low. Also, the labeled protein is contained in the crude extract in which it is synthesized, and therefore it is not pure. This extract contains, among other proteins, enzymes of the ubiquitin system that may interfere in the reconstitution of a cell-free system from purified components.

A. Radioiodination of proteins **TIMING 1 hr**

i. Add the reagents in the following order to 1.5 ml microcentrifuge tube. The volume of the reaction mixture can vary from 20 to 100 µl.

a) NaPi pH 7.5 (100 mM).

b) Protein substrate, 10–500 µg dissolved in water or buffer **CRITICAL STEP** Make sure that the buffer does not contain free amino or hydroxyl groups, Tris-HCl, for example, as this may result in iodination of these groups. As the buffer is in large molar excess over the protein, the protein will not be labeled.

c) Unlabeled NaI (50 nmol).

d) Radiolabeled $Na^{125}I$ (0.1–2.0 mCi).

e) 10–50 µg of chloramine-T solution in NaPi pH 7.5 (50 mM). Mix gently and incubate for 1–2 min at room temperature.

g) 20–100 µg of Na-metabisulfite (double the amount of the chloramine T used) solution in NaPi pH 7.5 (50 mM). Mix gently.

ii. To remove unreacted radioactive iodine, resolve the mixture over a desalting column equilibrated with Tris-HCl pH 7.6 (10 mM), and NaCl (150 mM). **CRITICAL STEP** In case the amount of the iodinated protein is less than 10 µg, it is recommended to add to the resolving buffer a protein carrier to protect the labeled protein from absorbance to the tube and from radio-damage. One can use 1 mg/ml of cytochrome C or ovalbumin. These proteins are not ubiquitinated and are not degraded by the UPS, and therefore do not compete with the labeled substrate on components of the system.

iii. Collect fractions (in a fraction collector or manually), each of approx 10% of the column volume. The radioactive protein is typically eluted in fraction 4 (void volume of the column which is ~35% of the column's total volume).

iv. Store in aliquots at –18°C. **PAUSE POINT** Labeled proteins are stable at –20°C for several weeks.

B. Biosynthetic labeling of proteins **TIMING ~4 hr**

i. Clone the cDNA of the target protein into a vector that contains T7, T3, or SP6 RNA polymerase promoters. To increase translation efficiency in

wheat germ extract, it is recommended to use Wheat Germ Flexi® Vectors (Promega, cat. nos. L5671, L5681; these vectors have T7 and SP6 promoters but lack T3).

ii. Linearize the DNA template by restriction digestion. **CRITICAL STEP** Supercoiled DNA templates can be used for transcription, although translation efficiency from the resulting RNAs may be lower. **TIMING for ii 1 hr**

iii. Transcribe the linear cDNA using systems that generate sufficient amount of mRNA (typically 6-12 µg of mRNA is required for a standard translation reaction), such as RiboMAX™ Large Scale RNA Production System SP6 (Promega, cat. no. P1280) or RiboMAX™ Large Scale RNA Production System T7 (Promega, cat. no. P1300). **TIMING for iii 30 min**

iv. Purify the mRNA from the transcription reaction using either gel filtration [for example PD MiniTrap G-25 columns (GE Healthcare, cat. no. 28918007)], **TIMING 4 min,** or phenol:chloroform extraction, **TIMING 40 min**. **CRITICAL STEP** Ethanol precipitation alone is not recommended.

Phenol:chloroform extraction:

- Extract RNA with 1 volume of sodium acetate pH 4.5 (3 M)-saturated phenol:chloroform:isoamyl alcohol (25:24:1). Vortex for 1 min and then spin the sample at top speed in a microcentrifuge for 2 min.
- Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol (24:1). Vortex for 1 min and centrifuge as described in the previous step.

- Transfer the upper, aqueous phase to a fresh tube. Any transferred chloroform can be removed by a quick spin (10 seconds) in a microcentrifuge followed by removal of the bottom phase with a micropipette. Add 0.1 volume of sodium acetate pH 4.5 (3 M), and 1 volume of isopropanol or 2.5 volumes of 95% ethanol. Mix and place on ice for 2-5 min. Spin at top speed in a microcentrifuge for 10 min.
- Carefully pour off or aspirate the supernatant and wash the pellet carefully with 1 ml of 70% ethanol. Aspirate the ethanol, dry the pellet under vacuum and dissolve the RNA in nuclease-free water to a volume identical to that of the transcription reaction. Store in aliquots at -70°C.

v. To translate the protein add the following reagents to 1.5 ml microcentrifuge tube :

a) G-25-purified or phenol:chloroform-extracted mRNA template in nuclease-free water – 6-12 µg.

b) [^{35}S]methionine (1,000 Ci/mmol at 43.3 mCi/ml, PerkinElmer®) – ~40 µCi.

c) Wheat Germ Extract Plus (Promega, cat. no. L3250) / Rabbit Reticulocyte Lysate (Promega, cat. no. L4960) – 30 µl.

d) Nuclease-free water to the final volume of 50 µl.

vi. Incubate at 30°C for 1 hr.

TIMING for v-vi ~ 70 min

C. Biosynthetic labeling of proteins using coupled transcription–translation kit

TIMING ~70 min

Use a coupled transcription–translation cell-free extract that synthesizes the mRNA from its cognate cDNA and translates it in a coupled manner. Such systems require only the addition of the cDNA and the labeled amino acid, and are available commercially [TNT® Reticulocyte Lysate system with SP6 (Promega, cat. no. L4600), T7 (Promega, cat. no. L4610) or T3 (Promega, cat. no. L4950) polymerase promoters or TNT® Wheat Germ Extract system with SP6 (Promega, cat. no. L4130), T7 (Promega, cat. no. L4140) or T3 (Promega, cat. no. L4120) polymerase promoters and S30 for bacterially derived extract (Promega, cat. no. L1130)]. Biosynthesis is carried out basically according to the manufacturer's instructions. The kit must be based on an RNA polymerase promoter (T7, T3, or SP6) identical to that present in the cDNA.

TROUBLESHOOTING

Conjugation and degradation of proteolytic substrates in a cell free system

15 For conjugation of protein substrates, proceed with Steps 16-19. For degradation proceed with Step 20.

Conjugation of proteolytic substrates in a cell free system TIMING 2 hr

16 This section describes: (A) conjugation in crude extract using WT ubiquitin; (B) conjugation in Fraction II which lacks ubiquitin and which allows inclusion of different species of ubiquitin involved in formation of different chains; and (C) conjugation using purified recombinant components.

A. The volume of the reaction mixture can vary from 10 to 1000 μl dependent on the need. For demonstration of conjugates, the volume is typically on the low range, while for analytical purposes, such as mass spectrometry, the volume is larger. Addition of all the reagents is carried out on ice. Described is a typical reaction mixture of 10.0 μl.

   a. Tris-HCl pH 7.6 (50 mM).

   b. $MgCl_2$ (5 mM).

   c. DTT (2 mM).

   d. Reticulocyte lysate 2-4 μl or complete cell extract 50-100 μg.

   e. Ubiquitin (5 μg).

   f. UbAl (0.5–1.0 μg) - add only for conjugation assay.

   g. ATPγS (2 mM). For monitoring of ATP-dependence, ATPγS is not added, and residual ATP in the extract is depleted using 2-Deoxy-D-glucose (10 mM) and hexokinase (0.5 μg). If phosphorylation is required for substrate recognition, ATP (0.5 mM) and ATP regenerating system [phosphocreatine (10 mM) and phosphocreatine kinase (0.5 μg)] are substituting for ATPγS.

   h. Substrate. Use either a labeled protein (25,000–100,000 cpm) or an unlabeled substrate in an amount that is sufficient for detection by Western blot analysis (50–1,000 ng).

B. Conjugation using Fraction II.

   Add all the reagents as described under Method A, but instead of reticulocyte lysate or complete cell extract, add Fraction II (25-50 μg) derived from reticulocyte lysate or Fraction II (50-100 μg) derived from nucleated cell extract.

Different ubiquitin species (typically mutated in all, in all but one, or in a single lysine residue, or methylated ubiquitin in which also the N-terminal residue is blocked) should be added (2.5-5.0 μg). In addition, the reaction mixture should be supplemented with E1 (0.25 μg) and E2 (~0.5 μg). In most cases, UbcH5c will be sufficient, but certain reactions will require UbcH7 or UbcH8.

C. Conjugation using purified enzymes.

This reaction mixture contains instead of the lysate, crude cell extract or Fraction II, purified E1 (~1 μg), E2 (0.5 μg) and E3 (should be titrated). In case of monitoring self ubiquitination, no substrate is needed, and the labeled E3 can be added.

17 Incubate the mixture for 30 min at 37°C, and resolve via SDS-PAGE (7.5–10% acrylamide). **CRITICAL STEP** For detection of the high molecular mass ubiquitin conjugates, do not remove the stacking gel.

18 Detect conjugates using a PhosphorImager (labeled proteins), or via enhanced chemiluminescence (ECL) following Western blot (for unlabeled substrates) using a specific primary antibody against the test protein and a secondary tagged antibody.

TROUBLESHOOTING

19 See also boxes 1, 2 and 3 for confirmation that the products of the reaction are indeed ubiquitin conjugates, and for identification of ubiquitination sites and types.

Degradation of proteolytic substrates in a cell free system

20 For monitoring of degradation in a cell free system one can use – with a few modifications - a similar assay to that used for monitoring of their conjugation (Method A). For proteins that are degraded inefficiently or slowly, it is difficult to

follow the reduction in the density of a protein band in gel analysis. In this case it is recommended to monitor the appearance of radioactivity in trichloroacetic acid (TCA)-soluble fraction (Method B). Control reactions are complete mixtures that have been incubated on ice, or mixtures that were incubated at 37°C in the absence of ATP (ATP should be depleted) or ubiquitin (Fraction II is not completely depleted of ubiquitin. Therefore some proteolytic activity - the level of which is dependent on the substrate - can still be observed even in mixtures to which ubiquitin was not added).

A. Monitoring of disappearance of the substrate **TIMING 2-3 hr**

i. Follow procedure 16A except for steps 16Af and 16Ag (addition of UbAl and ATPγS). Instead of ATPγS use as a source of energy ATP (0.5 mM) that its concentration is maintained constant by ATP-regenerating system [phosphocreatine (10 mM) and phosphocreatine kinase (0.5 μg)]. For depletion of endogenous ATP, the system should contain, instead of ATP and the ATP-regenerating system, 2-Deoxy-D-glucose (10 mM) and hexokinase (0.5 μg).

ii. Incubate the reaction mixture for 2–3 hr at 37°C and resolve it via SDS-PAGE.

iii. Monitor the disappearance of the substrate either by using a PhosphorImager (in case the protein substrate is radioactively labeled), or via Western blot analysis (in case of unlabeled substrate). **TROUBLESHOOTING**

B. Monitoring of the appearance of acid-soluble radioactivity **TIMING 3-4 hr**

i. Label the proteolytic substrate as described under 14A, 14B, or 14C.

ii. In case the protein was labeled synthetically [during translation of its mRNA (14B) or in a coupled reaction (14C)], one needs to remove the excess of unincorporated label as well as free ubiquitin (if this was required). To remove the labeling amino acid, extensive dialysis against a buffer that contains also 1 mM of the corresponding unlabeled amino acids is sufficient [Tris HCl pH 7.2 (20 mM), DTT (1 mM)]. To remove both the labeling amino acid and ubiquitin, fractionation on DEAE is most efficient (steps 3-13). Typically, for a translation mixture of 50 μl, a 200 μl column of DE-52 is sufficient (poured into 1 ml insulin syringe plugged with glass wool). Wash the column after loading of the translation mix with 50 column volumes (10 ml) of buffer B. Elute the labeled protein in 3 column volumes of buffer C, dialyze it extensively against a buffer D and concentrate it in a centrifugation filtration device (GE Healthcare, cat. no. 289322) of a molecular weight cutoff (MWCO) lower by at least 3-fold than that of the labeled protein. Instead of dialyzing, one can remove the eluting salt by repeated concentration-dilution cycles in this device.

iii. For setting the degradation reaction mixture, follow procedure 20Ai-ii.

iv. At the end of the incubation, add a carrier protein (10–25 μl of 100 mg/ml solution of bovine serum albumin, BSA).

v. Add 0.6 ml of ice-cold TCA (20%).

v. Mix the reaction and incubate on ice for 10 min.

vi. Centrifuge for 5 min at 15,000 x g.

vii. Collect 0.5 ml of the supernatant.

viii. Count the radioactivity in either γ-counter (for iodine-labeled substrates; 14A) or a β-scintillation counter (for methionine-labeled substrates; 14B/C).

Ubiquitination/Degradation in cells

21 For ubiquitination of proteolytic substrates in cells, proceed according to Steps 22-26, and for degradation in cells, proceed with Steps 27-28.

Ubiquitination of proteolytic substrates in cells. TIMING 72 hr

22 Transform the cells with cDNA coding for the studied substrate alone or with tagged ubiquitin (HA-, Flag-, or Myc-). If you examine the ubiquitination of an endogenous protein, you may use only tagged ubiquitin. **TIMING 24-48 hr**

23 After 24-48 hr, add the proteasome inhibitor: MG132 (20 μM) or *clasto*-Lactacystin β-lactone (20 μM) or epoxomicin (20 μM) for 2-3 hr. **TIMING 2-3 hr**

24 Lyse the cells. To avoid deubiquitination, use the "hot" lysis (Method A) or add to the lysis buffer inhibitors of deubiquitinating enzymes (Method B).

A. "Hot" lysis of cells. **TIMING 15 hr**

i. Aspirate medium from the dishes.

ii. Wash cells with ice cold PBS.

iii. Add "hot" lysis buffer I to cells (250 μl/6 cm plate).

iv. Scrape cells into a microcentrifuge tube.

v. Boil for 5 min at 100°C; seal the tube (use a cap locker or cap locked tubes).

vi. Shear lysate with 25G needle (3-5 times).

vii. Boil for 3 min at 100°C.

viii. Mix and centrifuge the sample for 5 min at room temperature.

ix. Transfer the supernatant to a clean tube.

x. Add 1 volume of “hot” lysis buffer II and antibody against the target protein (using antibodies directed against the substrate or against its fused tag). Alternatively, you can use an antibody against the tag fused to ubiquitin.

xi. Shake gently (rotate) overnight.

xii. Add ~20 μl of a mixture of equal amounts of immobilized proteins A and G suspended (50% beads/volume) in “hot” lysis buffer II.

xiii. Shake gently (rotate) for 1 hr.

xiv. Spin beads at 3,000 rpm for 1 min.

xv. Aspirate supernatant with a 30G needle.

xvi. Wash beads twice with “hot” lysis buffer III.

xvii. Wash beads twice with PBS.

xviii. Boil beads for 5 min at 100°C with 15-20 μl of sample buffer.

PAUSE POINT The samples are stable at –20° C for several years.

B. Lysis with inhibitors of deubiquitinating enzymes. **TIMING 15 hr**

i. Lyse the cells with a RIPA buffer containing freshly dissolved iodoacetamide and N-ethylmaleimide (5 mM each) to inhibit deubiquitinating enzymes.

ii. Immunoprecipitate the tested protein (using antibodies directed against the substrate or against its fused tag). Alternatively, you can use an antibody against the tag fused to ubiquitin.

iii. Shake gently (rotate) overnight.

iv. Add ~20 μl of a mixture of equal amounts of immobilized proteins A and G suspended (50% beads/volume) in RIPA buffer.

v. Shake gently (rotate) for 1 hr.

vi. Centrifuge the samples at 3,000 rpm for 1 min.

vii. Aspirate the supernatant. **CRITICAL STEP** Make sure not to aspirate the beads.

viii. Add 1 ml of RIPA buffer.

ix. Centrifuge the samples at 3,000 rpm for 1 min.

x. Aspirate the supernatant. **CRITICAL STEP** Make sure not to aspirate the beads.

xi. Repeat Steps viii-x 5-6 times to thoroughly wash the precipitated protein.

xii. Boil the samples for 5 min at 100°C with 15-20 μl of sample buffer.

PAUSE POINT The samples are stable at –20° C for several years.

25 Resolve the proteins via SDS-PAGE followed by blotting onto nitrocellulose membrane. **CRITICAL STEP** In order to visualize conjugates in the entire range of molecular weights, do not remove the stacking gel while transferring proteins to the nitrocellulose membrane. **TIMING 3.5 hr**

26 In case proteins were precipitated with an anti-substrate antibody, conjugates can be visualized using antibodies against the substrate or ubiquitin or against the tag fused to either of them. In case anti-ubiquitin tag antibodies were used for immunoprecipitation, conjugates can be visualized only by using antibodies directed against the substrate or its fused tag. **TIMING 4.5 hr**

TROUBLESHOOTING

Degradation of proteolytic substrates in cells

27 Transform cells with cDNA coding for the substrate. If you examine the endogenous protein, this step is not necessary. **TIMING** 24-48 hr

28 To monitor the degradation of a protein substrate, one can use either cycloheximide chase, Method A, or pulse-chase labeling and immunoprecipitation, Method B. The advantage of Method A is that it does not necessitate the use of radioactive material and immunoprecipitation, and one can resolve a whole cell extract via SDS-PAGE. The disadvantage is the potential interference of a drug in the proteolytic process. Thus, if the drug inhibits the synthesis of a short-lived ubiquitin ligase (E3), an inhibitor or an activator involved in the process, the test protein can be stabilized or further destabilized, dependent on the role of the component affected.

A. Cycloheximide chase. **TIMING 60 hr**

   i. In case of cDNA (coding for a substrate)-transfected cells (Step 27), incubate the cells for 24-48 hr before adding cycloheximide (20-100 μM). Otherwise [testing the stability of an endogenous protein(s)], add the cycloheximide when the cells reach the desired confluency.

   ii. Add proteasome inhibitor for 2-4 hr (as a control for stabilization).

   iii. Harvest the cells at the desired times after addition of cycloheximide.

   iv. Monitor degradation/stabilization of the target protein via Western blot analysis.

B. Pulse-chase labeling and immunoprecipitation. **TIMING 60 hr**

i. In case of cDNA (coding for a substrate)-transfected cells (Step 27), incubate the cells for 24-48 hr before labeling. Otherwise [testing the stability of an endogenous protein(s)], add the label when the cells reach the desired confluency.

ii. Wash the cells twice in a methionine-free medium at 4°C.

iii. Add methionine-free medium that contains dialyzed serum (serum is added in the concentration used for growing the cells).

iv. Incubate for 1 hr (to remove endogenous methionine), remove the medium (by aspiration for adhering cells and following centrifugation at 800 x g for 10 min for cells in suspension), and add fresh methionine-free medium with dialyzed serum. To save on labeled methionine, for adherent cells add medium to barely cover the cells' layer (1–1.5 ml for a 60 mm dish; cells can be rocked in the incubator. For non-adhering cells, resuspend them to 2×10^6/ml).

v. Add labeled methionine (50–250 µCi/ml), and continue the incubation for 0.5-1.0 hr (pulse).

vi. Add the proteasome inhibitor to the control dish (as described under Aii). The inhibitor should be added for the last ~30 min of the labeling period and should remain throughout the entire experiment.

vii. Remove the labeling medium.

viii. Add ice-cold complete medium that contains, in addition to the inhibitor (as needed), also 2 mM of unlabeled methionine, and wash the cells twice in the same medium.

ix. Add pre-warmed complete medium [that contains the inhibitor (as needed) and 2 mM of unlabeled methionine], and continue the incubation for the desired times (chase).

x. Withdraw samples at various time points and monitor degradation/stabilization of the target protein(s) by immunoprecipitation followed by SDS-PAGE and PhosphorImage analysis.

TROUBLESHOOTING

TIMING

Step 2, preparation of cell extracts: preparation of reticulocyte lysate (Method A) 9 days; preparation of extract from cultured cells (Method B): 3 days

Steps 3-13, fractionation of cell extract: 24 hr

Step 14, labeling of proteolytic substrates: radioiodination of proteins (Method A): 1 hr; biosynthetic labeling of proteins (Method B): 4 hr; biosynthetic labeling of proteins using coupled transcription–translation kit (Method C): 70 min

Steps 16-18, conjugation of proteolytic substrates in a cell free system: 2 hr

Step 20, monitoring of degradation of proteolytic substrates in a cell free system: monitoring of disappearance of substrates (Method A): 2-3 hr; monitoring of appearance of acid-soluble radioactivity (Method B): 3-4 hr

Step 22, transformation of cells: 24-48 hr

Step 23, incubation with a proteasome inhibitor: 2-3 hr

Step 24, lysis of the cells and immunoprecipitation: “hot” lysis of cells (Method A): 15 hr; lysis with ubiquitin hydrolases inhibitors (Method B): 15 hr

Steps 25-26, SDS-PAGE and Western blot analysis: 8 hr

Steps 27-28, degradation of proteolytic substrates *in vivo*: cycloheximide chase (Method A): 60 hr; pulse-chase labeling and immunoprecipitation (Method B): 60 hr.

Box 1

How to determine that the generated high molecular mass adducts are indeed ubiquitin derivatives of the test protein.

- Adducts should not be generated in an ATP-depleted cell free system (or in cells from which ATP was depleted).
- The adducts should be stable to denaturing agents (e.g. SDS) and to prolonged heating (60°C) in the presence of high concentrations of β_2-mercaptoethanol[35] or at pH 12 (see however Box 2)
- Generation of the conjugates of the specific substrate in a cell free system should be inhibited reversibly by the addition of increasing concentrations of methylated ubiquitin (MeUb[36]) or ubiquitin K0 (in which all internal lysines were substituted with arginines). This reductively methylated or lysine-less derivative of ubiquitin lacks free amino groups that can be targeted by ubiquitin, and therefore cannot polymerize to generate polyubiquitin chains. They serve therefore as chain terminators in the polyubiquitination reaction, and consequently as inhibitors in this reaction. Their inhibition can be abrogated by the addition of excess WT ubiquitin. For experiments in intact cells, expression of K0 ubiquitin should also inhibit generation of high molecular mass adducts. Use of ubiquitin K48R for inhibiting generation of high molecular mass adducts can be misleading. That is because while the Lys^{48}-based polyubiquitin chain is the archetypical proteasomal degradation signal, chains based on other lysines can also be recognized by the proteasome[13], but also serve many non-proteolytic functions. Such chains can be formed using ubiquitin K48R[14,19].
- Adducts can be precipitated from the reaction mixture with an antibody directed

against the test protein, and following SDS-PAGE, can be detected with an anti-ubiquitin antibody (available from Enzo® Life Sciences, Boston Biochem Inc., Millipore, Sigma and several other suppliers). Alternatively, the reaction (or cell transfection) can be carried out in the presence of HA-, Myc-, His-, or Flag-tagged ubiquitin, and the gel-resolved immunoprecipitate can be analyzed with one of the corresponding antibodies.

- A cell-free system can be reconstituted from purified or isolated components of the ubiquitin system, and the formation of conjugates should be dependent on the addition of certain critical components. Rather than using a complete cell extract, it is possible to use Fraction II, derived from ATP-depleted cells (see above, step 16) and free or tagged ubiquitin (see above, step 16). Since Fraction II is lacking ubiquitin, formation of conjugates that is dependent on the addition of exogenous ubiquitin will strongly suggest that the high molecular mass derivatives generated are indeed ubiquitin adducts of the test substrate. Similarly, when using recombinant enzymes, conjugation should be dependent on the addition of E1, E2, and E3.

Box 2

Distinction between sites of ubiquitination.

N-terminal ubiquitination

One should suspect that a ubiquitinated protein is modified at the N-terminal residue if: (i) it does not contain any internal lysine residues, or (ii) all internal lysine residues have been mutated (with Arg, for example). However these should not

always be the only considerations, as proteins with internal lysines can also undergo N-terminal ubiquitination.

Several lines of experimental evidence support the assumption that a target substrate is modified at the N-terminal residue:

- Mass spectrometric analysis of tryptic digest of isolated adducts. Conjugates should be preferably synthesized using non-polymerizable species of ubiquitin. In a cell free system, one should use MeUB, while in cells, a tagged target substrate should be expressed along with tagged K0 ubiquitin; the protein should be precipitated and the adduct should be identified by Western blot or staining. Utilization of a ubiquitin species that cannot polymerize allows synthesis of a large amount of distinct and easy to identify monoubiquitin derivative. In contrast, the WT protein will generate a broad array of adducts scattered along a wide range of molecular masses. In case of N-terminal ubiquitination, it is expected that tryptic digestion will generate a fusion peptide between the C-terminal diGly of ubiquitin (-aa-aa-aa-aa-74R-↓-75G-76G-COOH) and the N-terminal domain of the target protein[37]. The example described above relates to trypsin digest. If a tryptic site resides 2-4 residues downstream to the N-terminal residue of the substrate, isolation and characterization of the tetra- or hexapeptide may be difficult. In this case, digestion with a different proteolytic enzyme should be considered, or the tryptic site in the substrate can be mutated, making the further downstream site to become now the first cleavage site, thus generating a longer peptide that can be easily identified.
- Fusion of a tag to the N-terminal residue. Fusion of 6 x Myc to the N-terminal

residue but not to the C-terminal residue of the protein, will prevent the formation of ubiquitin adducts. It should be noted that while Myc contains lysine, it does not seem to be ubiquitinated.

- Selective modification of the N-terminal domain/residue of the protein. This can be done in two ways. First, the N-terminal domain can be altered so the N-terminal residue will be recognized by one of several N-terminal acetyl transferases (NATs)[38-40]. This will typically require substitution of 1-3 of the first four N-terminal residues. For experiments in a cell free system one can use the same mutant synthesized in an *in vitro* translation system (where acetylation will also occur) or a substrate in which the N-terminal residue was modified specifically by carbamylation at low pH. This modification blocks selectively the αNH_2 group. Carbamylation is carried out in potassium phosphate pH 6.0 (0.2 M), urea (6 M) and potassium cyanate (50 mM). Following incubation for 8 hr at 37°C, the reaction is stopped by the addition of Gly-Gly to a final concentration of 150 mM. The pH is adjusted to 8.1 with 30% (w/v) K_2HPO_4 pH 11, and the mixture is incubated for an additional 1 hr at 37°C. The latter treatment releases carbamyl groups bound to non-amine residues within the protein. Reagents are removed by dialysis against water.
- Site directed mutagenesis or selective modification of all internal lysines. Ubiquitination in the absence of internal lysine residues suggests that the protein is modified at the N-terminal residue (see however below for modification of residues other than lysines). All internal lysine residues can be modified by substitution (site-directed mutagenesis) with arginines. Also, one can modify chemically the

internal lysines by guanidination. This modification selectively blocks the ε-NH_2 groups that are typically targeted by ubiquitin. Guanidination is carried out in *O*-methylisourea (0.3 M) for 2 days at 4°C. The modifying reagent is removed by dialysis for 2 days against four changes of KPi pH 7.0 (50 mM), and two changes of ddH_20.

The degree of modification of amino groups can be determined by the Fluorescamine method[41] , which monitors the content of free, primary amine groups in proteins. Fluorescence measurements (Luminescence Spectrometer) should be performed using an excitation wavelength of 390 nm and an emission wavelength of 475 nm. The results can be normalized to equal concentrations of WT and MeUb (Enzo® Life Sciences or Boston Biochem Inc.), the first having 8 free amino groups (7 internal lysines and one free N-terminal group), and the later is supposed to have none.

- If N-terminal ubiquitination is involved in targeting the substrate for degradation, the modifications at the N-terminal residue/domain described above (rendering the substrate susceptible to NATs activity and carbamylation at low pH) should result in stabilization of the tested protein.
- Truncation of the N-terminal segment of the test protein (first 15–30 residues) may prevent its ubiquitination and degradation.

Identification of internal lysines targeted by ubiquitin

- It appears that for many substrates, the ubiquitination sites are not specific, and any single lysine can be targeted, even if inserted randomly[6,7]. For a smaller group of proteins, IκBα, p53 and Pax3, for example, specific lysines have been

identified, though the degree of specificity is varying. Thus for IκBα, ubiquitination must occur on lysines 21 and/or 22[42], while for p53, it is a cluster of 6 lysines in the C-terminal domain that were reported to be targeted[43]. For a third group of proteins, the p105 precursor of NF-κB for example, multiple rather than a single of a few lysines must be ubiquitinated, regardless of their specific sites[44,45]. It is possible that part of the "promiscuity" that we observe is due to the utilization of cell free systems, where proteins are tested away from their natural cellular environment and under conditions that are not physiologically relevant. One way to identify a specific lysine(s) that serve as a ubiquitination anchor(s) is to mutate systematically and sequentially and increasing number of lysines in the substrate.

Ubiquitination on the non- lysine residues

It has recently been demonstrated that cysteine, threonine and serine, can also be targeted by ubiquitin[9-11].

- Cysteine. Incubation of the adducts in the presence of β_2-mercaptoethanol (1.4 M) at pH 11, or treatment with DTT (100 mM) under denaturing conditions should cleave the thiol-ester bond between ubiquitin and cysteine, but not the isopeptide bond between ubiquitin and the ε–NH_2 group of the internal lysine.

  β_2-mercaptoethanol treatment. Add one volume of: Tris-HCl pH 11 (100 mM), SDS (4%), glycerol (20%), β_2-mercaptoethanol (1.4 M) and boil at 95° for 10 min.

  DTT treatment. Add one volume of Tris-HCl pH 6.8 (100 mM), SDS (4%), glycerol (20%), urea (8 M) along with DTT (100 mM). Boil samples at 95°C for 10 min.

- Serine/threonine. Treatment of the adducts at basic pH should cleave the ester bond between ubiquitin and serine/threonine.

Boil adducts in 0.5% SDS for 3 min followed by incubation in either sodium hydroxylamine pH 9 (1 M) for 4 hr at 37°C, or sodium hydroxide (0.1 M) for 2 hr at 37°C[9,46]. Prior to SDS-PAGE, dialyze the sodium hydroxylamine-treated samples against PBS overnight at 4°C using low molecular weight cut-off mini dialysis units (Pierce Chemical Co.).

Box 3

Distinction between different types of ubiquitin chains.

Several methods are available by which one can initially identify the type of chains generated.

- Analysis of isolated conjugates by mass spectrometry[14,47]. Here one can obtain the percentage of each linkage in the chain, but not its localization and specific order.
- Utilization of two sets of ubiquitin molecules[19,48] in which different lysine residues were mutated. In the first set, all but a single lysine residue were substituted with arginines (designated KX mutants, where X denotes the position of the singly remained lysine). In the second set, single lysine residues were substituted with an arginine moiety (designated RX, where X denotes the position of the singly mutated lysine). When used in different combinations, these sets of mutant ubiquitins can provide useful information on the type of chains that are generated on different substrates by different ligases.

One should bear in mind however several difficulties that limit the usefulness of such ubiquitin species: (i) inclusion in a reaction mixture of a single ubiquitin species (in particular of the KX type) may not enable formation of mixed chains,

and (ii) similarly, such ubiquitin species may not enable formation of doubly (or more) branched chains[19].

- Use of a yeast strain in which all endogenous ubiquitin genes were inactivated, and, in a short time window, expression of a single mutant species can be activated[44,49]. One can also express distinct ubiquitin species in mammalian cells. However, in such cells, it is impossible to inhibit expression of the endogenous WT ubiquitin which may participate in chain formation, in addition to the expressed ubiquitin, giving rise to mixed chains.
- Employment of antibodies directed against specific internal linkages in the polyubiquitin chain. For now, only specific anti-K63 antibody is available[50] (Enzo® Life Sciences, cat. no. BML-PW0600)

TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

TABLE 1. Troubleshooting table.

Problem	Possible reason	Solution
Inefficient labeling of protein	Poor quality of DNA	Use midi or maxi kit for DNA preparation
	Low methionine-containing substrate	Try to use a mixture of ^{35}S-Methionine and ^{35}S-Cysteine that is needed for metabolic labeling in cells. It does work in *in vitro* labeling in most cases Try to change to a different RNA polymerase promoter. SP6 is usually the most efficient, followed by T3 and then T7
There is no ubiquitination/ degradation in complete cell lysate	Lysate does not contain the appropriate E3 or a certain component is not activated	Change the cell line from which lysate is prepared or use extract derived from appropriately signaled cells
	Low protein concentration of the extract	Use a more concentrated extract
There is no ubiquitination/ degradation in Fraction II	Fraction II does not contain appropriate E2	Try different E2 enzymes or add Fraction I (which contains also WT ubiquitin)
After “hot” lysis ubiquitin conjugates are not well resolved (smeared)	You did not shear the DNA	Use sonication instead of needle to shear the DNA
There is no degradation *in vivo*	The cells are not suitable for your experiment	Monitor the half life in several cell lines (as it may vary)
	The cycloheximide does not work	Use freshly dissolved cycloheximide. As a control use known short-lived proteins (e.g. c-Myc, Mdm2, MyoD): see Figure 7
	The tested protein has longer half life then you expect	Change the time points

ANTICIPATED RESULTS

Typical ubiquitin conjugates generated in a cell free system are shown in Figure 4. It should be noted that when using ATP, the conjugates are of somewhat lower molecular mass compared to those generated using ATPγS (compare lane 3 to lane 2). That because in the presence of ATPγS, the proteasome is inactive. The use of MeUB generates multiply monoubiquitinated rather than polyubiquitinated substrate (lane 6). Similarly, conjugates generated in cells in the presence of K0 ubiquitin are of lower molecular mass than those generated in the presence of WT ubiquitin (Figure 5). When monitoring degradation in a cell free system in the presence of WT ubiquitin, one can see decline in the amount of conjugates along time, reflecting their degradation (compare lanes 6 to lane 5 in Figure 6). That in contrast to time-dependent accumulation of conjugates observed when MeUb is used (compare lane 9 to lane 8). Please note that in monitoring the degradation of a protein using the radiolabeling pulse-chase and immunoprecipitation method, the radioactive protein disappears (Figure 8i) but there is no effect on the total amount of protein that is in steady state (Figure 8ii).

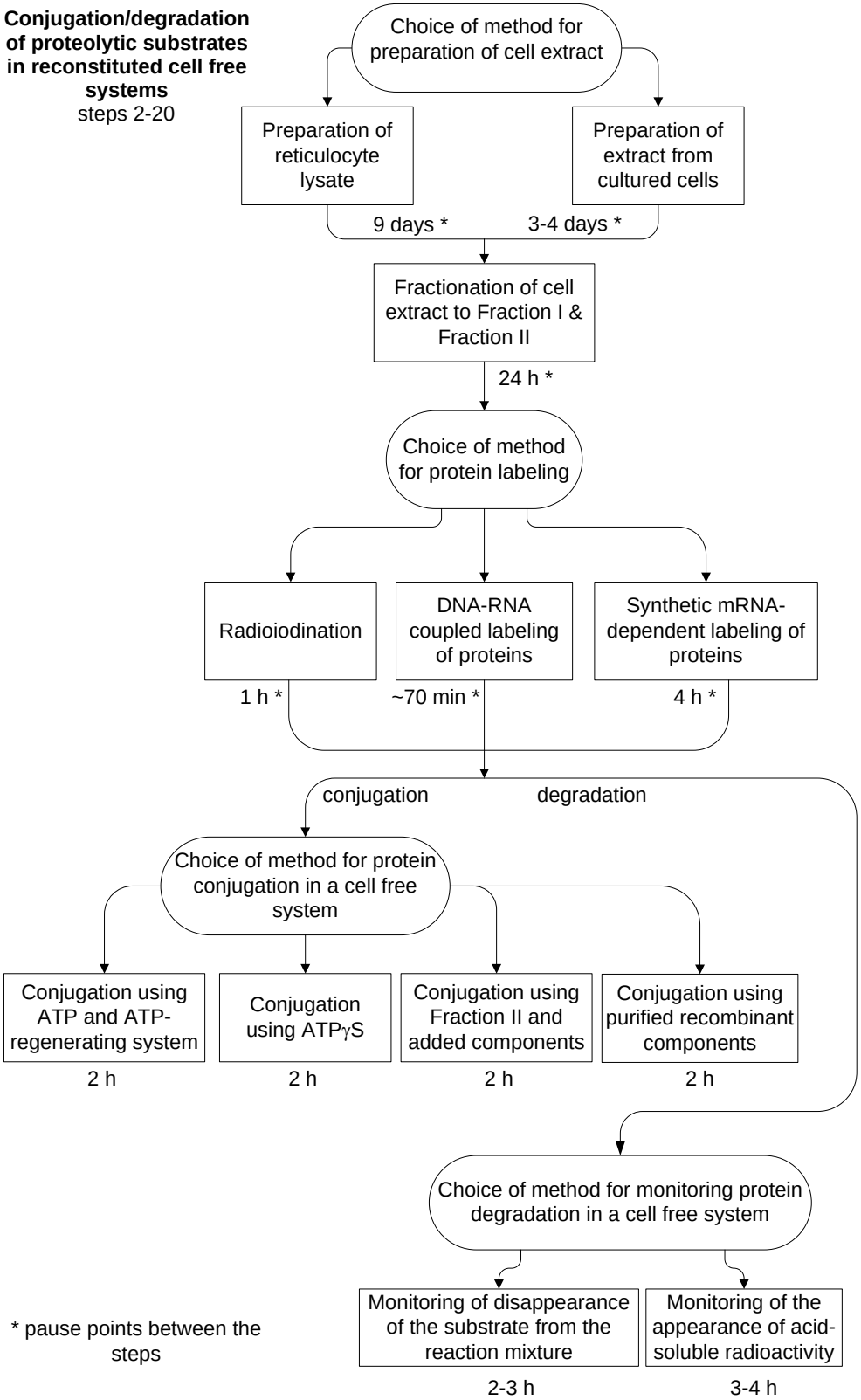

Figure 1. Summary of methods for detection of conjugation or degradation of proteolytic substrates in reconstituted cell free system.

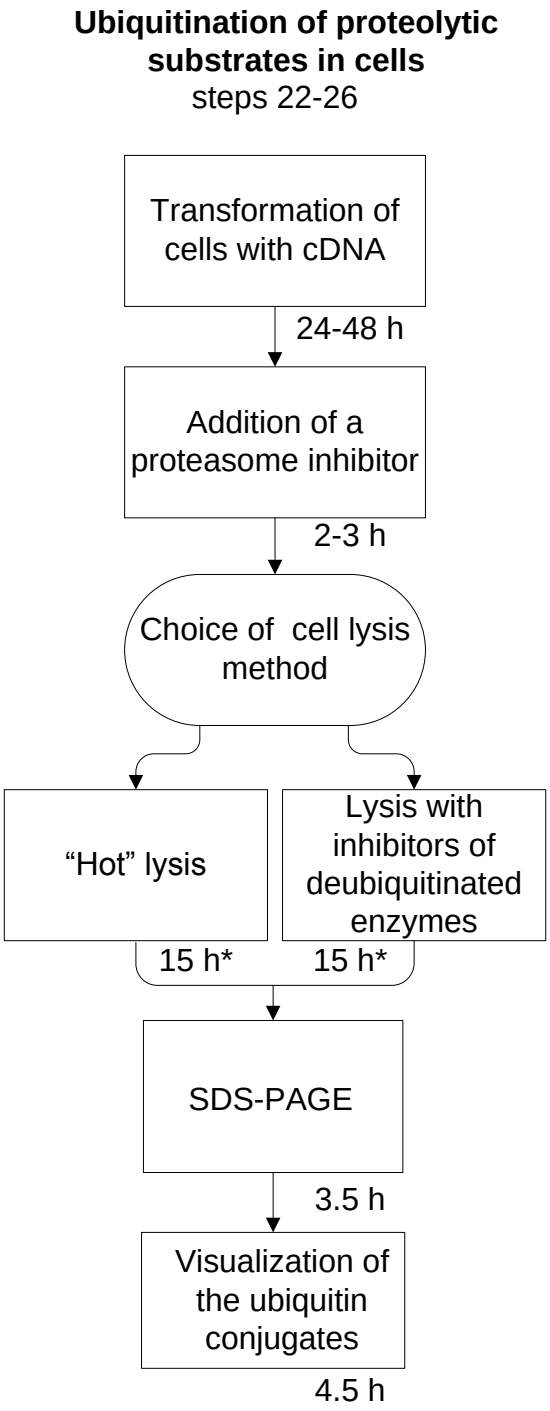

Figure 2. The strategy for identification of ubiquitination of proteolytic substrates in cells.

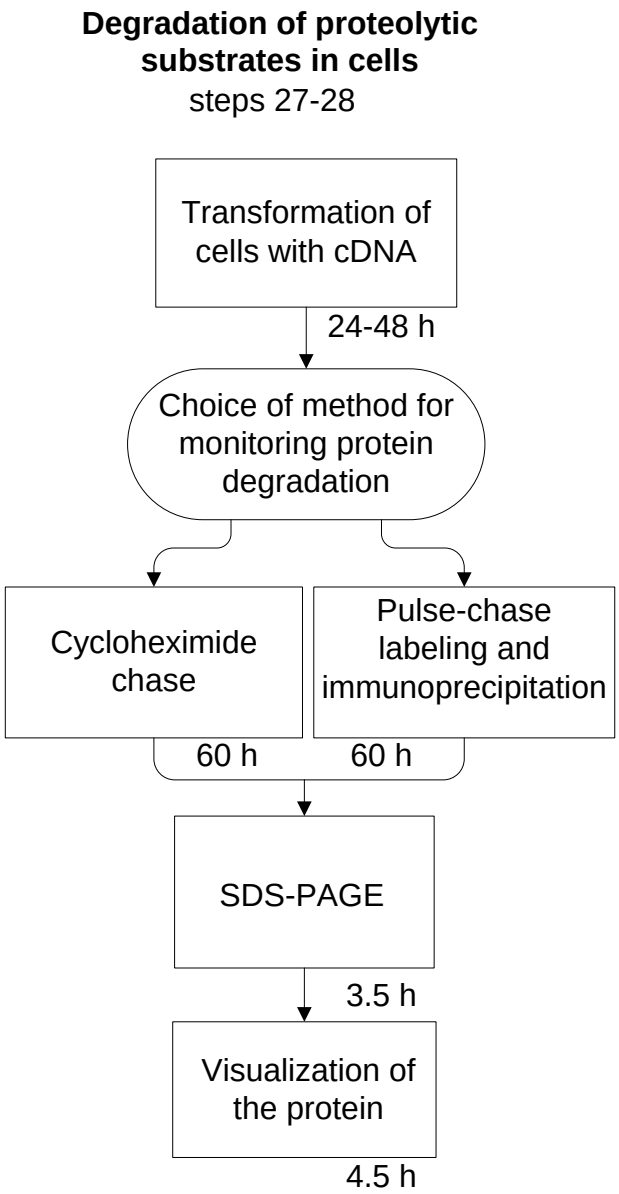

Figure 3. Summary of methods for detection of degradation of proteolytic substrates in cells.

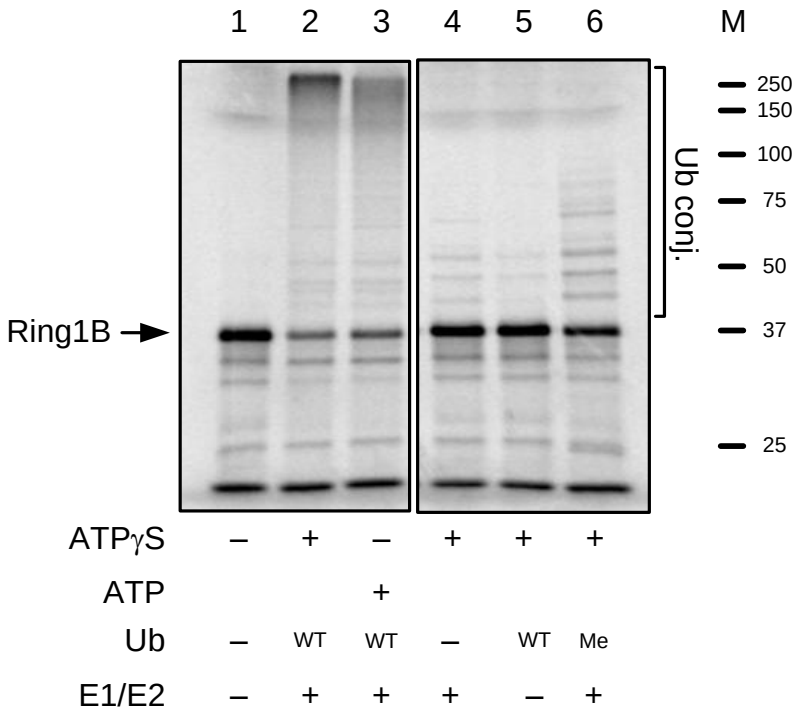

Figure 4. Ubiquitination of Ring1B in a cell free system.

In vitro-translated and ^{35}S-labeled Ring1B was self ubiquitinated without addition of ATP, Ub and E1/E2 (lane 1), in the presence of ATPγS (lane 2), or ATP and ATP-regenerating system (lane 3), in the absence of Ub (lane 4), in the absence of E1/E2 (lane 5), or in the presence of methylated ubiquitin (lane 6). "ATP +" denotes ATP and ATP-regenerating system. MeUb denotes methylated ubiquitin, and Ub conj. denotes ubiquitin conjugates.

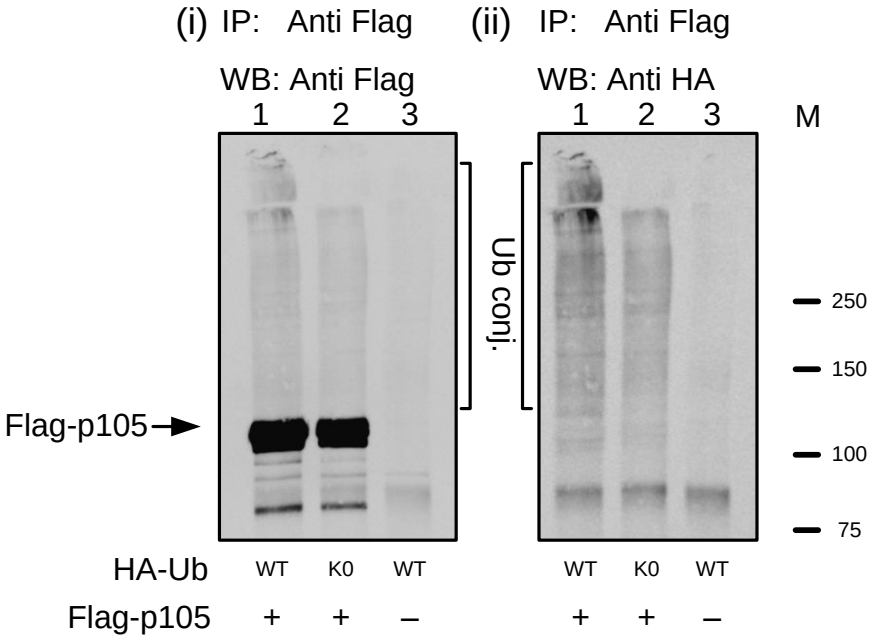

Figure 5. Ubiquitination of p105 in HEK 293 cells.

HEK 293 cells were transfected with cDNAs coding for Flag-p105 (lanes 1-2), and HA-WT Ub (lane 1 and 3) or HA-UbK0 (lane 2). p105 and its conjugates were immunoprecipitated using anti-Flag, and detected by either anti-Flag (i) or anti-HA (ii).

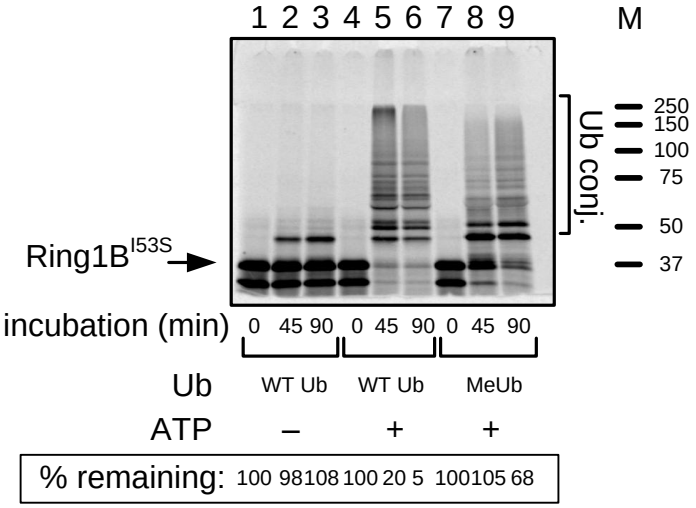

Figure 6. Degradation of Ring1B^{I53S} in a cell free system.

In vitro-translated and ^{35}S-labeled Ring1B^{I53S} was degraded in a cell free system in the presence of WT or methylated ubiquitins, in the presence or absence of ATP (as described under Figure 1) as indicated. Degradation was calculated based on the radioactivity remained in the lane along time relative to time 0.

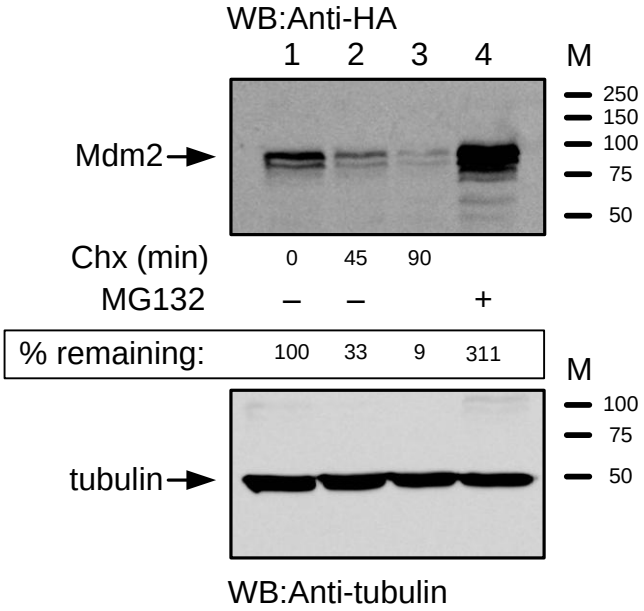

Figure 7. Degradation of Mdm2 in HEK 293 cells using cycloheximide chase.

HEK 293 cells were grown to desired 75% confluency. Degradation of endogenous Mdm2 was monitored following the addition of cycloheximide (lanes 1-3). Degradation of Mdm2 was inhibited and the enzyme was accumulated following inhibition of the proteasome by MG132 added for 2 hr (lane 4).

Tubulin was used as a marker to ascertain equal protein loading.

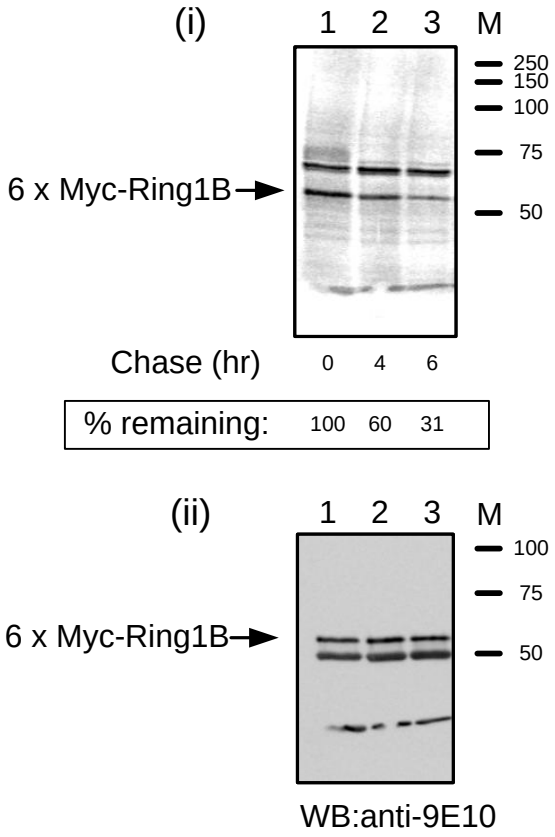

Figure 8. Monitoring of degradation of Myc-Ring1B using pulse-chase labeling and immunoprecipitation.

Cos7 cells were transfected with a cDNA coding for Myc-Ring1B (lanes 1-3). Stability of the protein was monitored in a pulse-chase labeling and immunoprecipitation experiment. Immunoprecipitation was carried out using anti-Myc antibody, and the proteins were visualized following SDS-PAGE, blotting into nitrocellulose membrane. Radioactive Ring1B was detected using PhosphorImaging (i) and total Ring1B in cells was detected using anti-Myc antibody (ii).

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