DISSERTATION

DETECTION AND MEASUREMENTS OF FREE UBIQUITIN IN FIXED CELLS AND CHARACTERIZATION OF OTUB1 CONTRIBUTION TO UBIQUITIN HOMEOSTASIS

Submitted by

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ABSTRACT

DETECTION AND MEASUREMENTS OF FREE UBIQUITIN IN FIXED CELLS AND CHARACTERIZATION OF OTUB1 CONTRIBUTION TO UBIQUITIN HOMEOSTASIS

Post-translational modifications with Ubiquitin (Ub) have been found to participate in a wide range of cell functions, including protein degradation, endocytosis, regulation of gene expression and cell cycle progression. Therefore, regulation of free Ub levels is essential to ensure that enough Ub is available for conjugation, while excess Ub does not compete in the large number of processes that depend on binding to ubiquitinated proteins or polyUb. Not surprisingly, changes in Ub pool dynamics can affect the cell functions, and perturbations of free Ub levels have been reported to cause neurological and developmental disorders. Although there are techniques to measure Ub pools *in vitro*, visualization and quantification of free Ub inside individual cells has not been possible.

One way to regulate the intracellular concentration of free Ub, is by means of Deubiquitinating enzymes (DUBs), however specific details about the regulatory mechanism are, in large part, unknown. Most studies about DUBs have focused on enzymatic activity and regulation *in vitro*, with only few reports on the regulation of Ub homeostasis *in vivo*. The role of OTUB1 in Ub homeostasis has been hypothesized because its catalytic activity is affected by the ratio of [Ub~E2] to [E2] in response to free Ub concentration. Interaction between OTUB1 and a subset of E2s can stimulate

OTUB1 isopeptidase activity, whereas interactions with Ub~E2s can inhibit the ubiquitin transfer from the thioester Ub~E2 adduct.

This dissertation describes the successful development of a technique to detect and quantify changes in free Ub levels in fixed cells using a high affinity binding protein. The method was used to quantify changes in Ub levels after proteasome and E1 inhibition and to establish the free Ub distribution in hippocampal neurons. It was shown also that OTUB1 activity is not directly involved in the regulation of free Ub levels under stress conditions. However, a new mechanism for regulation of UBE2D expression levels dependent on OTUB1 was identified. This mechanism is independent of proteasomal degradation and could possibly involve translational regulation.

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CHAPTER 1: INTRODUCTION

1.1 Ubiquitin homeostasis and regulation of ubiquitin cellular levels

Ubiquitin (Ub) is a conserved protein that modifies a wide variety of proteins post-translationally. Conjugation of Ub is catalyzed by the sequential actions of three different enzymes: Ub-activating enzyme (E1), Ub-conjugating enzyme (E2) and Ub-ligase (E3). Ub is activated by an E1 enzyme in an ATP-dependent manner through the formation of a high-energy intermediate. Ub is then transferred to an E2 enzyme and finally conjugated to its substrate by an E3 enzyme, which confers substrate specificity (Figure 1.1a). Ub attached to E1, E2 and some E3 enzymes via a thioester bond forms the activated Ub pool. Active Ub is linked to the N-terminus or ε-amino group of lysine residues on target proteins. The bond between the Ub C-terminus and the lysine ε-amino group on a target protein is an isopeptide bond. Additionally, Ub can form chains with another Ub through any of its seven internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) or its N-terminal α-amino group (Figure 1.1b). This allows the formation of a variety of polyUb signals that differ by linkage[1,2].

Regulation of the Ub pathway is important because Ub participates in a variety of cellular processes such as endocytosis, cell cycle regulation, gene expression, DNA repair, and degradation of proteins by the Ub proteasome system and lysosomes[3]. The conjugation of Ub to proteins is regulated by E3 enzymes and deubiquitinating enzymes (DUBs). After Ub is transferred to E2s, the Ub~E2 thioester complexes work in conjunction with the E3 enzymes to catalyze the transfer of Ub to specific substrates. Ub transfer to a substrate can be mediated by different types of E3s of either the RING

or HECT ligases. The RING E3 ligases comprise the biggest family of ubiquitin ligases; they are responsible for mediating the direct transfer of Ub from E2 enzymes to substrates. Unlike the RING E3 ligases, HECT E3 ligases form a thioester intermediate with Ub before the Ub is transferred to the substrate[4].

The activities of the ubiquitin activating enzymes are counteracted by the activities of deubiquitylating enzymes (DUBs). DUBs cleave the peptide or isopeptide bonds between Ub and other protein molecules, releasing Ub from Ub gene products and polyUb chains on target proteins[5,6]. The human genome encodes ~100 DUBs that are classified into seven subfamilies according to their sequences and the configuration of the catalytic residues in the active site: Ub-specific proteases (USPs), Ub carboxy-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Machado–Joseph disease enzymes (MJDs), MINDYs and ZUP1 are classified as cysteine proteases, whereas the JAMM/MPN+ metalloenzymes (JAMM) family consists of zinc-dependent metalloproteinases[7].

In light of the process that adds Ub to different target proteins, one can envision three different Ub pools (free Ub, activated Ub and conjugated Ub) that are in dynamic equilibrium (Figure 1.2). Regulation of free Ub levels is essential to ensure Ub availability for Ub conjugation. Different studies have shown that overexpression and depletion of Ub is associated with disorders during mice embryonic development, cell cycle[8], meiotic progression[9] and neuronal survival and function[10].

1.2 Mechanisms that regulate ubiquitin levels

There are different mechanisms to ensure the availability of free Ub inside the cells and to regulate those levels under normal or stress conditions. The mechanisms involved in the regulation of Ub homeostasis are: Ub biosynthesis, DUB activity, Ub degradation (Figure 1.3) and redistribution of Ub from histones.

1.2.1 Ubiquitin gene expression and polyprotein processing

Ub levels can be regulated through the expression of Ub genes. Ub is a highly conserved protein; in mammalian cells Ub is encoded by four Ub genes. *UBA52* and *UBA80* express single Ub fused to the 40S ribosomal protein L40 and 60S ribosomal protein S27a, respectively. *UBB* and *UBC* are polyUb genes expressed under stress conditions; they encode 3-4 and 9-10 Ubs in tandem, respectively[11,12]. In higher eukaryotes and plants all the Ub genes encode the same protein sequence[13].

The accumulation of misfolded proteins during stress conditions increases the free Ub demand. Higher free Ub demand inside cells takes place when the cells encounter different stress conditions that affect proteins stability such as starvation, heat shock, proteotoxic and oxidative stress[14]. Although all Ub genes are expressed under normal cellular conditions, there is up-regulation in the expression levels of polyubiquitin genes during proteasome inhibition, oxidative stress, and heat shock[14-16]. The increased expression of the *UBC* gene during stress conditions is mediated by heat shock elements in the promoter region of the gene that are recognized by transcription factors from the heat shock factor family[16].

The functional role of Ub genes during development and their effects in different cellular functions have been evaluated. The *UBA52* gene has been showed to be essential during embryonic development and a regulator of the ribosomal protein complex[17]. Other studies performed with polyUb genes revealed that the loss of *UBC* leads to embryonic death possibly due to compromised cell proliferation during liver development. At the same time, mouse embryonic fibroblasts (MEFs) isolated from *UBC*$^{-/-}$ embryos showed reduce proliferation, probably due to problems in cell-cycle progression. Also, those cells cannot restore Ub levels in response to cellular stresses such as proteasome inhibitors and heat shock[8]. While *UBB* knockout mice are viable, they exhibit infertility due to failed meiotic progression as well as neuronal loss within the hypothalamus[9,10]. Likewise, mice overexpressing Ub 2 to 3-fold had structural abnormalities at neuromuscular junctions[18]. These results show that maintaining free Ub levels is essential in different cellular processes.

1.2.2 Ubiquitin regulation by DUBs

The cleavage of polyubiquitin and Ub conjugates by DUBs is also important to maintain Ub homeostasis. To regulate free Ub levels inside the cells, DUBs have different functions. These functions include the co-translational processing of nascent polyubiquitin and Ub-linked to ribosomal subunit proteins, editing and removing Ub signals from protein conjugates, and recycling Ub from substrates targeted to degradation by the proteasome and lysosome.

A variety of DUBs are involved in the *de novo* generation of free Ub from Ub precursors. Enzymes such as UCHL3, USP9X and USP7 assist in the processing of

single Ub fused to ribosomal subunits[19,20]. In contrast, the DUBs involved in the processing of polyUb products remain unknown[19]. Generation of free Ub from proubiquitin precursors by DUB enzymes is a clear example of the redundancy of some DUB activities and the role of these enzymes in the regulation of Ub homeostasis.

In neurons, changes in the levels of some DUBs assist in the regulation of free Ub levels. Regulating Ub homeostasis in neurons has been shown to be essential for different neuronal functions and is consistent with the unusually high abundance of free Ub in neuronal tissues. Measurements of Ub pools in mouse brains have shown that there is approximately 60% free Ub compared with 40% conjugated Ub. In contrast, HEK293 and MEF cells contain 23% free Ub compared with 77% conjugated Ub[21]. Deregulation in the levels of DUBs such as UCHL1 and USP14 has been directly associated with disruption of free Ub levels in neurons. Specifically, immunoblots of brain extracts of mice with loss of expression of UCHL1 or USP14 have showed 20 and 30% decreases in the levels of free Ub respectively[18,22].

UCHL1 is mainly expressed in neurons, testis and different types of cancer[20,23]. As with other UCH family of DUBs, entrance of substrates into the active site of UCHL1 is restricted by a flexible loop[24]. Although *in vitro* studies have shown that UCHL1 cleaves small peptide adducts (~ 10 residues) from the C terminus of Ub, the function that it plays *in vivo* it is not clear[20]. However, it has been reported that UCHL1 has a high affinity for Ub in neurons (Kd = 385 nM)[25] and that perturbation of the enzyme substrate interaction could affect the enzymatic activity and probably Ub stability[26,27].

Different animal models have been used to evaluate the role of UCHL1 in the nervous system. Expression of a truncated version of UCHL1 causes gracile axonal

dystrophy, which is characterized by axonal degeneration and ataxia at different stages of development and has been associated with accumulation of ubiquitinated proteins[28]. At the same time, deletion of UCHL1 in mice has been involved in neurodegeneration characterized by an increase in proteasomal activity in the first weeks of development[29]. In humans, mutations or changes in expression levels of UCHL1 have been associated with different neurodegenerative diseases such as Parkinson's and Alzheimer's diseases[24,26,30,31].

Similarly, USP14 (Ubp6 in yeast) activity in neurons has been linked with the regulation of free Ub levels. Along with PN11 and UCH37, USP14 is one of the three DUBs associated with the proteasome[32,33]. The catalytic activity of USP14 is regulated in different ways. Upon association with the 19S regulatory particle of the proteasome, the catalytic activity of the enzyme increases and Ub binding to the enzyme induces a conformational change that allows access of the substrate to the active site[33,34]. In yeast and mammals, it has been reported that Ubp6/USP14 delays the degradation of ubiquitinated substrates independently of its catalytic activity; when the enzymes are depleted from proteasomes, this results in rapid decrease of free Ub levels with concomitant increases in Ub conjugates[35-37]. In addition, it has been shown that USP14 preferentially removes Ub chains from substrates ubiquitinated at multiple sites, leaving the remaining Ub chain to be processed by the other DUBs associated with the proteasome[37].

Compared with UCHL1, USP14 depletion results in a greater decrease in free Ub levels. USP14 deficiency has been studied in the ataxia mice (ax^J), where there is a 40% decrease in free Ub and a two-fold increase in *UBC* and *UBB* transcription[18,38].

Different studies have shown that USP14 activity is required for the synapse formation and development, specifically at the neuromuscular junction[39,40]. Those changes correlate with the decrease in free Ub levels measured in synaptosomes prepared from ax^J mice, where free Ub pool plays an essential role in receptors recycling and signaling[39,41].

An additional way to regulate the intracellular levels of free Ub is via the endocytic pathway. Besides proteasomal degradation of polyubiquitinated proteins (typically, conjugated with K48-linked polyUb), proteins conjugated with monoubiquitin or K63-linked polyubiquitin chains can be degraded in the lysosome[5]. In yeast, Doa4 is an endosome-associated DUB that cleaves conjugated Ub from proteins associated with lysosomal membranes before they enter into multivesicular bodies[42]. Deletion of Doa4 causes accumulation of conjugated Ub, an increased rate of Ub degradation, and decreased cell viability[43]. Rfu1 and Ub phosphorylated at serine 57 can regulate Doa4 activity. For example, under normal conditions Rfu1 represses Doa4 activity ensuring the balance between free Ub and conjugated Ub. In the presence of stress conditions, Rfu1 is downregulated; therefore, an increase in Doa4 activity favors the generation of free Ub[44]. In the case of Ub phosphorylation at serine 57, expression of a phosphomimic increased endocytic trafficking and Ub degradation, suggesting its function as a possible regulator of Doa4 activity[45]. A mammalian homologue for Doa4 is not known yet.

1.2.3 Ubiquitin degradation

Ub degradation has been postulated as a mechanism to regulate free Ub levels. Although it has been shown that Ub is a very stable protein, studies using radiolabeled or fluorescent forms of Ub have suggested that Ub is degraded and that its the half-life depends upon the cell line evaluated[46,47]. Different intracellular mechanisms have been proposed for Ub degradation. First, as previously discussed, the functions of the DUBs plays an important role in Ub recycling and preventing its degradation together with the substrates during proteasomal and lysosomal degradation[35,44]. It has been shown that conjugated Ub can be degraded by the proteasome in a "piggyback" mechanism[48,49]. *In vitro* experiments using radio-labeled Ub and cells extracts without Ub, but containing the other components of the Ub proteasome pathway, showed that Ub degradation is ATP- and proteasome-dependent[48]. These experiments suggested that distal Ub is removed by the DUBs while the most proximal Ub is degraded with the substrate[48]. Second, *in vitro* studies have shown that when Ub contains at least 20 amino acids in a C- terminal extension it can reach into the catalytic core of the 20S proteasome core particle and be degraded[49]. Although there is only one report about expression of an aberrant form of Ub with additional 19 amino acids that results from a frameshift ($UBB^{+)}$ in some patients with Alzheimer's disease and Down syndrome, it has been hypothesized that Ub conjugated to peptides could be generated by reaction of activated Ub with endogenous short peptides[49,50].

1.2.4 Redistribution of Ub from histones

Histones are targets to several post-translational modifications to modulate gene expression inside the cells. Ubiquitination plays an important role in transcriptional activity, chromatin remodeling and DNA repair[51,52]. H2A and H2B are mainly monoubiquitinated: around ~ 5-15 % and ~ 1-1.5 % of total H2A and H2B respectively are ubiquitinated[52]. Representing an important Ub supply for the cells.

Several studies have shown that during proteotoxic stress there is accumulation of polyubiquitinated substrates[21,53]. This phenomenon disturbs the equilibrium between Ub pools, leading to a decrease in the free Ub levels. Expression of wild-type Ub with an N-terminal GFP (GFP-Ub) was used to study the dynamic between Ub pools[53]. After proteasome inhibition, a decrease in the limiting free Ub pool was observed. At the same time, deubiquitination of histone H2A and chromatin remodeling[53,54] was detected. Histones deubiquitination has been reported after heat shock as well, another form of proteotoxic stress[53,55,56]. The cells incubated with proteasome inhibitor did not present a change in the rate of deubiquitination compared with control cells, it was suggested that the redistribution of Ub conjugated to histones to the cytoplasm was due to the limiting amount of free Ub available[53,54].

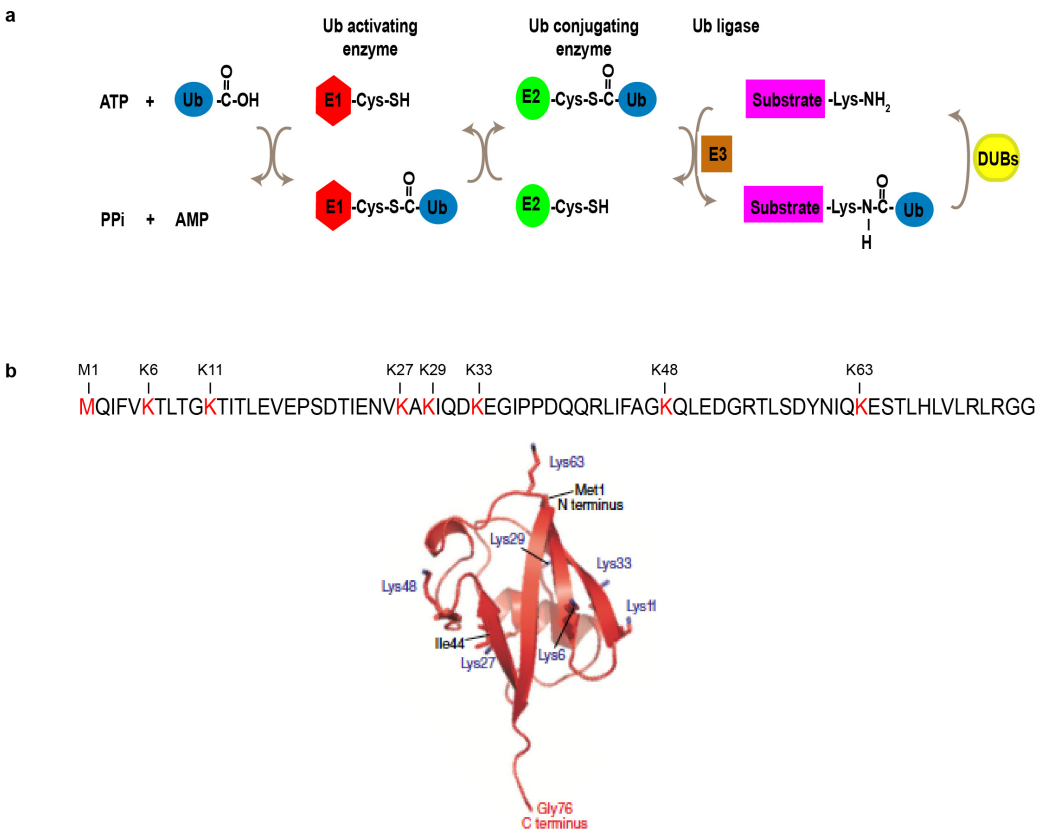


Figure. 1.1. Steps in the Ub pathway and Ub sequence. a) Steps in the ubiquitination pathway. Figure adapted from Hanpude *et al* (2015). B) Amino acid sequence for Ub. In red the 7 lysine residues and the N-terminus involved in formation of Ub chains are indicated. In the bottom there is a ribbon Ub structure showing the lysine residues. Figure adapted from Traub *et al* (2007).

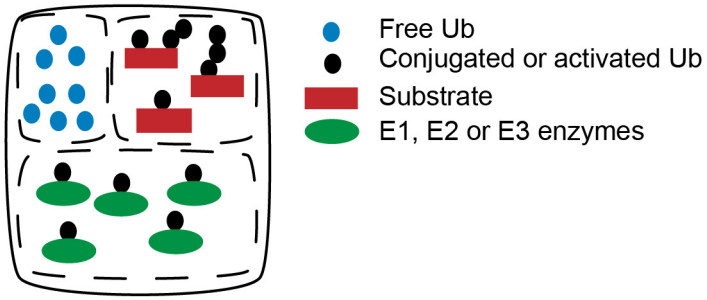


Figure. 1.2. Graphic representation of Ub pools found inside cells Figure adapted from Choi *et al* (2019).

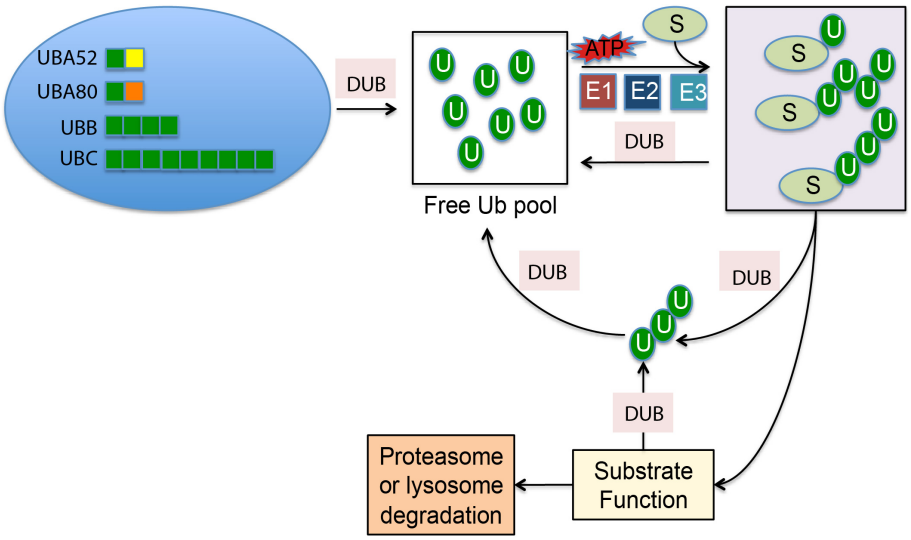


Figure 1.3. Ways to regulate Ub homeostasis in cells. In cells there are different ways to regulate the Ub levels. In mammals four Ub genes (*UBA52, UBA80, UBB, UBC*) are expressed; after processing by DUBs free Ub is generated. Free Ub is conjugated to different substrates through the sequential action of different enzymes (E1, E2, E3). Free Ub can be generated by DUBs from Ub conjugates directly or before degradation of the substrate by proteasome or lysosome. In some cases Ub can also be degradated with the substrate. U: Ubiquitin, S: Substrate. Figure adapted from Park and Ryu (2014).

CHAPTER 2: DETECTION OF FREE UBIQUITIN IN FIXED CELLS

2.1 Introduction

Although there are several biochemical techniques available to measure free Ub pools, visualization of free Ub inside cells has been so far a challenge. Quantification and visualization of endogenous free Ub has not been possible through direct staining using immunofluorescence because the antibodies available typically recognize more than one Ub form[3]. Other approaches have used fluorescent-labeled Ub to visualize free Ub and its dynamics under different cellular conditions. One approach has been the expression of genetically-encoded GFP-Ub or or GFP-$Ub^{K0,G76V}$. $Ub^{K0,G76V}$ is an Ub variant without internal lysine residues and with the C-terminal glycine residue changed to valine, that mimics the localization of endogenous free Ub inside cells because it does not have lysine residues available for the formation of Ub chains[53]. Another study used a similar approach but a small fluorophore; TAMRA-Ub introduction in live cells by electroporation showed the same distribution for GFP-$Ub^{K0,G76V}$ with the advantage of having a smaller size compared with GFP[57]. However, TAMRA-Ub and GFP-$Ub^{K0,G76V}$ have N-terminus unavailable to form Ub chains. Although these approaches have provided valuable insights into Ub dynamics and distribution of the different Ub pools, they are based on the ectopic expression of Ub. In addition, the increase in molecular weight and the addition of a tag may change protein properties, especially regarding the ability of chains formation.

Here, we describe a method to detect free Ub (unconjugated monoUb and polyUb chains with a free C terminus) in fixed cells using a high affinity and selective

free Ub binding protein. The binding protein, tUI, was formed by fusion of two non-overlapping Ub binding domains (UBDs) derived from proteins known previously to bind Ub. Specifically, tUI is formed by the ubiquilin-1 associated UBA ($UQ1^{UBA}$) domain linked by two amino acids to the N-terminus of $IsoT^{Buz}$ domain (Figure 2.1a). The $UQ1^{UBA}$ domain recognizes the hydrophobic patch of Ub while the $IsoT^{Buz}$ domain gives selectivity for free Ub through the recognition of the its unconjugated C-terminus[58,59]. Although the affinity of each individual domain for free Ub is in the micromolar range ($UQ1^{UBA}$, K_d =22 μM[60]; $IsoT^{BUZ}$, K_d = 3 μM[58]), when these domains were linked to form tUI the affinity remarkably increased (K_d = 66 pM)[61]. The tUI binding protein prefers free Ub with >10^5-fold higher affinity compared with tUI-GB1, a mimic of conjugated Ub (Figure 2.1b)[61]. Taking advantage of the tUI sensor properties, we wanted to develop a technique to visualize endogenous free Ub by fluorescence microscopy in fixed cells and to quantify the changes in free Ub levels upon induction of stress conditions.

2.2 Experimental procedures

2.2.1 Binding protein purification and labeling

To generate HA-tUI, the influenza hemagglutinin A tag (HA) was inserted using In-Fusion cloning (TakaRa). The HA tag was introduced at the C-terminus of the $IsoT^{BUZ}$ domain. The mutations in the $IsoT^{BUZ}$ domain of the binding protein (W209A or R221G) were introduced using site-directed mutagenesis. HA-tUI was cloned into the pET28a vector. For protein expression, the plasmid was transformed into BL21-CodonPlus (DE3) *Escherichia coli* cells. Protein expression was induced for 20 hours at 18 °C by addition of 0.2 mM IPTG after the cells reached an optimal density ($OD_{600\ nm}$) of 0.6.

Then the cells were harvested and resuspended in cold buffer A (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, 10 mM imidazole and 10 mM β-mercaptoethanol). Cell lysates were obtained by sonication. The binding protein was purified from lysates using a Histrap HP column (GE Healthcare). Samples were eluted with a gradient of buffer A supplemented with 500 mM imidazole. Fractions collected from Histrap were purified through gel filtration using a Superdex 75 column (GE Healthcare). Samples were eluted with PBS and supplemented with 1 mM TCEP. Purity of the fractions collected was assessed by SDS-PAGE.

Binding protein labeling. HA-tUI and HA-tUI^{R221G} were labeled with Atto532 (ATTO-TEC) via cysteine-maleimide chemistry. For the labeling reaction a 1.5 molar excess of the dye was incubated with 150 µM sensor in 50 mM HEPES, pH 7.4, 100 mM NaCl for 2 hours at 25 °C. The free dye was removed by incubation of the labeled sensor with a Ni-NTA resin, following manufacturer recommendation for the amount of resin used (Thermo Fisher). The labeled sensor was eluted with PBS supplemented with 250 mM imidazole and 0.5 mM DTT. Labeling efficiency and removal of free dye were confirmed by SDS-PAGE. To detect the fluorescence, the gel was scanned using a Typhoon FLA 9500 (GE Healthcare). Degree of labeling and protein concentration were calculated as previously described[61].

Binding assays. For the binding assay using HA-tI^{R221G}, PBS buffer supplemented with 0.05 % Brij35, 0.2 mg/mL ovalbumin and 1 mM DTT was used. The assay was performed with 30 nM HA-tUI^{R221G} and up to 200 µM Ub. To measure the fluorescence intensity a FluoroMax-4 spectrofluorimeter (HORIBA) was used.

2.2.2 Cell fixation and staining

HeLa, U2OS and Retinal pigment epithelium (RPE)1 (tert) were obtained from the ATCC, while the immortalized MEF cell line was from Averil Ma (The University of California, San Francisco) and was described in Pasupala *et al* (2018). HeLa, U2OS, and MEF cells were maintained in DMEM (Gibco), while Retinal pigment epithelium (RPE)1 cells were cultured in DMEM/Ham's F-12, 50/50 mix (Corning). The media was supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. For the induction of the stress conditions, cells were incubated for 1 hour with 1 µM Bortezomib (BTZ, Ubiquitin-Proteasome Biotechnologies), 10 µM E1 inhibitor (Compound 1, Takeda Oncology) or vehicle alone (0.1% DMSO). Each experiment was performed a minimum of two times.

Cells grown to a confluency <80% on 10 mm glass coverslips (0117500; Marienfeld) were fixed with 4% paraformaldehyde in PBS for 30 minutes at 37 °C. After fixation, the cells were permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature. Then, the cells were blocked with 5% BSA and 0.5% Tween-20 in PBS. HA-tUI or HA-tUI^{R221G} were diluted in blocking solution at a concentration of 100 nM and incubated with the fixed cells for 30 minutes at room temperature. For the competition experiments, 100 nM HA-tUI was preincubated with 100 µM Ub for 5 minutes at room temperature before addition to the cells. After washing with PBS to remove unbound sensor, the cells were incubated overnight at 4 °C with different primary antibodies. The next day, the cells were washed with PBS and incubated with the secondary antibody for 1 hour at room temperature. Finally, the cells were incubated with DAPI to stain the

nuclei and mounted on slides using ProLong Diamond Antifade medium (ThermoFisher).

The primary antibodies used during the staining process were: anti-HA diluted at 1:1,000 (clone HA-7, mouse monoclonal; Sigma-Aldrich) or anti-HA diluted at 1:2,000 (clone C29F4, rabbit monoclonal; Cell Signaling), anti-Ub diluted at 1:1,000 (clone FK2, mouse monoclonal; Enzo Life Sciences), or anti-K48 Ub diluted at 1:200 (clone Apu2, rabbit monoclonal; Millipore). The secondary antibodies used were: Alexa Fluor 568 diluted at 1:500 (donkey anti-mouse IgG (H+L); ThermoFisher) or Alexa Fluor 488 diluted at 1:400 (goat anti-rabbit IgG (H+L); ThermoFisher).

2.2.3 Culture of neuronal cells and staining

Dissociated rat hippocampal neurons isolated from embryonic day 18 (E18) rat embryos were obtained from Jim Bamburg's lab (CSU). Cells were plated at a density of 396 cells/mm^2 on poly-D-lysine (SIGMA) coated glass-bottom petri dishes. Neurons were maintained in a humidified atmosphere at 37 °C and 5% CO_2. Neurons were maintained in homemade Neurobasal medium containing 2.5 mM Glucosa, 122 mM NaCl and 175 µM L-Cysteine. The medium was supplemented with N21-MAX, 500 µM Glutamax I and 25 U/mL penicillin and streptomycin. The entire medium was changed every other day. At the third day after plating, 1 µM 5-Fluoro-2'-deoxyuridine (FdU) was added to the cells for 24 hours. FdU is and anti-mitotic drug used to avoid proliferating cells[62]. The cells were maintained in culture for 21 days in vitro (DIV) to ensure synaptic maturation.

For staining of the plasma membrane MemBright-488 (Idylle)[63] was used. After completing the staining protocol, the cells were washed with PBS and incubated with 800 nM of the dye diluted in PBS for 1 hour at 37 °C. Then the cells were washed and mounted on coverslips.

For neurons, the primary antibodies used were: anti-MAP2 diluted at 1:800 (chicken polyclonal; Abcam) and the Ub antibodies previously described for immunoblotting. For F-actin detection Alexa Fluor™ 488 Phalloidin diluted at 1:400 (ThermoFisher) was used. The secondary antibodies used were: Alexa Fluor 647-conjugated goat anti-chicken IgG (ThermoFisher; 1:500 dilution) or Alexa Fluor 488-conjugated goat anti-chicken IgG (ThermoFisher; 1:500 dilution) were used.

2.2.4 Microscopy and image analysis

Cells images were acquired using a Zeiss LSM 880 confocal microscope with C-Apochromat 40x/1.20 W, Plan-Apochromat 63x/1.40 Oil DIC M27 or alpha Plan-Apochromat 100x/1.46 Oil DIC M27 objectives. The images were acquired with ZEN Black software (v.14.0.9.201). For the quantification of free Ub signal (i.e., staining of HA-tUI), the maximum projections of the images were generated and measured using ImageJ 1.51 h (National Institute of Health, NIH). The cell contours were drawn using HCS CellMask dye as a reference. Autofluorescence intensities recorded from unstained cells were subtracted from the cells incubated with HA-tUI. Three-dimensional reconstructions of RPE1 cells were obtained with the Imaris software (v.9.1.1, Bitplane) from z-sections acquired every 0.242 µm. For the neurons, the super resolution (SR) images were acquired with the Airyscan mode. The planes from the Z-

stacks were collected every 0.17 µm. Image processing and alignment were performed using ZEN Black software (v.14.0.9.201). The 3D reconstructions for neurons were done with ZEN Blue 2.3.

2.3 Results

2.3.1 Optimization of free ubiquitin detection in fixed cells using tUI

Due to the very high affinity of Atto532-tUI for free Ub, the initial approach was to directly use this sensor as a tool to detect endogenous free Ub in fixed cells. However, the main problem when we used this sensor (or the same binding protein labeled with Atto594, Atto490LS, Atto550 and CF™633) was the high background signal (data not shown). As an alternative, we decided to express the hemagglutinin A-tagged tUI (HA-tUI); incubation of the binding protein with fixed cells was followed by detection with anti-HA antibody.

The specificity of the binding protein for free Ub was tested by two different ways. First, after the cells were fixed, the binding protein was preincubated with a 1,000-fold excess of free Ub. Second, a mutation in the binding protein IsoT^{BUZ} domain was introduced. Based on the IsoT^{BUZ} affinity for the free C-terminus, residues directly involved in the recognition of Ub C-terminal diglycine were mutated. Characterization of the IsoT^{BUZ} domain had identified mutations that affect its free Ub recognition and the catalytic activity of Isopeptidase T[58]. We tried two mutation sites: W209A and R221G. Only the second mutation yielded a protein that was soluble and could be purified successfully. Compared with the original binding protein, Atto532-HA-tUI^{R221G} showed more than a 29,000-fold decrease in the affinity for free Ub (K_d = 1.96 µM) (Figure 2.2a).

When the sensors were used to stain U2OS cells, we detected fluorescence signal only for the cells that were directly incubated with HA-tUI (Figure 2.2b). On the other hand, the measured fluorescence intensities for cells incubated with either HA-tUI plus excess free Ub or with HA-tUI^{R221G} were much lower. In addition, quantification of the signal intensities in HeLa cells stained using either HA-tUI or HA-tUI plus free Ub showed that the competition with free Ub reduced the signal ~3-fold (Figure 2.2c). Free Ub staining in HeLa, U2OS, MEF and RPE1 cells showed that free Ub was diffuse throughout the nuclear and cytoplasmic regions (Figure 2.3a). This was expected based on the small molecular weight of Ub (8.6 kDa) and also from experiments where cells expressed GFP-Ub^{K0,G76V} [53]. These results suggest free Ub staining using HA-tUI is specific and that free Ub localizes evenly through the cells.

2.3.2 Alterations in free ubiquitin levels can be detected by staining fixed mammalian cells

After validating the binding protein's specificity, we decided to check if the HA-tUI could be used as a tool to quantify fluctuations in free Ub in mammalian cells. Different assays performed with whole cells lysates have shown that free Ub levels can be affected by perturbations in components of the Ub pathway[21,53,61]. Localization and detection of changes in free Ub will help us to understand the mechanisms involved in the regulation of Ub homeostasis. HA-tUI was used to detect changes in free Ub levels after proteasome inhibition, which causes accumulation of Ub conjugates[21,53], and inhibition of the E1 activating enzymes, which prevents Ub activation[64]. Those conditions were evaluated in HeLa, U2OS, MEF and RPE1 cells (Figure 2.3a). As

expected, the two treatments resulted in perturbations of the free Ub pool in the four cell lines tested. After one-hour incubation with proteasome inhibitor, BTZ, we observed for all the cell lines a global decrease in the free Ub levels in all the cell lines, but without detectable perturbations in the cellular distribution free Ub distribution. Relative to the control, there was a 1.3–1.5-fold decrease in free Ub after proteasome inhibition (Figure 2.3a-c). On the contrary, E1 inhibition resulted in increased free Ub levels although with a higher cell-to-cell variability. In MEF and RPE1 cells, there were 2-fold increases in the signal, whereas in HeLa and U2OS cells the signal was 3-fold higher compared to the control cells (Figure 2.3a-c). Taken together, these results indicate that free Ub staining using HA-tUI can be used to quantify fluctuations in intracellular free Ub levels induced by different stress conditions.

During the evaluation of free Ub by staining the fixed cells, an increase in fluorescence was observed for cells going through mitosis. Measurements of the total fluorescence signal in interphase (23.7 ± 2.5 (n = 5)) and mitotic RPE1 cells (38.4 ± 3.5 (n = 3)) showed that in mitotic cells the average signal intensity for free Ub staining was 1.6-fold higher compared to cells in interphase (Figure 2.3d). These results suggest that there are differences in the free Ub levels that depend on the phase of the cell cycle.

Finally, we wanted to compare the localization of free Ub with K48-linked Ub and mono- and polyubiquitinated conjugates. For HeLa cells, we observed that free Ub was evenly distributed through the whole cell while K48-linked polyUb and conjugated Ub were enriched at different cellular compartments. K48-linked polyUb accumulated mainly in the cytoplasm while Ub conjugates were especially enriched in the nuclei (Figure 2.4a, b). The enrichment of Ub conjugates in the nuclear region is consistent

with the high content of monoubiquitinated histones. In mammalian cells, between 5-15% and 1-1.5% of histones H2A and H2B, respectively, have been reported as ubiquitinated[52]. These results confirm that specific Ub pools (i.e., free versus conjugated) have distinct distributions inside cells.

2.3.3 Staining of free ubiquitin in neurons

After determining that HA-tUI was specific for free Ub staining, we explored its use to localize free Ub in neurons. Inside neurons, as in other mammalian cells, a variety of cellular processes have been linked to the activity of the Ub proteasome pathway, including the degradation and recycling of synaptic proteins that are necessary for proper synaptic protein homeostasis[65]. Moreover, a variety of neurological disorders have been associated with disruption of Ub homeostasis[18]. Staining for free Ub in hippocampal neurons at 21 DIV showed a granular distribution throughout the cells with accumulation in foci with 0.5 ± 0.2 μm diameter (Figure 2.5a, b). The controls for the staining showed a significantly decreased signal and no visible foci arising from non-specific binding (Figure 2.5a). This confirms that HA-tUI is specific for the free Ub in neuronal cells and that free Ub probably is accumulating at distinct sites in the neurons

Based on the diameter of the free Ub foci and their close localization to the MAP2 staining (Figure 2.5 a), we hypothesized that some of these foci could be located in dendritic spines. To test this, we stained the neuron's plasma membrane and used phalloidin to stain F-actin. These approaches helped to identify spines through the visualization of their morphology. In addition, staining with the FK2 monoclonal anti-Ub antibody to detect mono and polyubiquitinated conjugates showed that, although some

conjugates co-localized with the free Ub foci, most appeared to be distinct from the areas enriched in free Ub (Figure 2.5 c). The absence of high concentrations of free Ub from the majority of the spines was confirmed after staining of the plasma membranes (Figure 2.5 d). These results suggest that free Ub could accumulate inside some possible spines. However, most of the free Ub foci do not co-localize with any of the antibodies used to determine free Ub foci localization.

2.4 Discussion

The free Ub staining procedure described here detects unconjugated monoUb and polyUb chains with a free C terminus. This method offers several advantages and opportunities compared with previous methods to visualize and quantify free Ub changes inside cells. First, endogenous free Ub is detected specifically via the use of a high binding protein. This was confirmed by two methods, one that used HA-tUI with addition of excess free Ub, and another that used a version of tUI, HA-tUI^{R221G}, that binds Ub with 29,000-fold weaker affinity as a negative control for non-specific binding in the staining procedure (Figure 2.2b). Second, it could be potentially used in immunofluorescence protocols for tissue sections. Using free Ub staining in tissues will help to understand the free Ub dynamics beyond a single cell, allowing the evaluation that adjacent cells could have in the regulation of free Ub levels. Third, it is a fast and easy-to-use technique that does not require transformation or introduction of exogenous probes to the cells to be evaluated. Finally, sensitivity of the technique is sufficient to quantify a 30% decrease and 200% increase in endogenous free Ub levels after induction of stress conditions (Figure 2.3a-c). However, cell-to-cell variability in free Ub

levels, probably due to difference in the phase of the cell cycle, should be considered. To account for this variability, it is required to quantify a large number of cells to obtain representatives measurements of the cells evaluated.

One potential limitation of the detection method is the possible hydrolysis of activated and conjugated Ub that could help to increase the levels of free Ub detected. Hydrolysis of the thioesters could take place during the fixation procedure. This problem could be approached in future studies by testing more effective fixation reagents at crosslinking. A mixture of 3.9% paraformaldehyde and 0.1% glutaraldehyde was used to fix neurons, but the subsequent incubation with sodium borohydride or glycine did not quench the unreacted groups. As a result HA-tUI bound non-specifically (data not shown). Another limitation is that it is not possible to use the binding protein in live cells because the high affinity of the sensor for free Ub could interfere with the amount of free Ub available to be conjugated and the binding with proteins that interact with free Ub. To avoid perturbing cellular physiology, a sensor with a K_d in the range of intracellular free Ub concentration (i.e., low-micromolar) will be required for measurements of free Ub in live cells.

Evaluation of different conditions that perturb the free Ub levels inside cells was used to test the sensitivity of the staining procedure to changes in free Ub. Induction of proteotoxic stress by proteasome inhibition results in a decrease in free Ub in some of the evaluated cells[21,53]. In our study, evaluation by staining with HA-tUI of four different cell lines (HeLa, U2OS, MEF and RPE1) showed that after 1 hour of proteasome inhibition there is a decrease in the free Ub pool compared with control cells (Figure 2.3a-c). The 1.4-fold decrease of free Ub in HeLa cells is consistent with measurements

reported for the same cells using solution assays[61]. However, the free Ub levels in MEF cells were ~ 25 % lower after proteasome inhibition in fixed cells compared with the in solution measurements[61]. This difference could be due to hydrolysis of the activated Ub during the fixation process. The amount of activated Ub detected in MEF cells using in solution measurements[61] can account for the differences observed in free Ub levels after proteasome inhibition. Staining with HA-tUI of the same cell lines after E1 inhibition showed, as expected, an increase in free Ub levels compared with the control cells. Although free Ub appeared to be evenly distributed in most cells under normal conditions and after proteasome inhibition, some of the cells presented a higher accumulation of free Ub close to the nucleus after E1 inhibition (Figure 2.3a). Additional studies using cell cycle markers will be required to establish if this treatment has a particular effect on the cell cycle progression and histone ubiquitination. Overall, the results showed that the free Ub staining in fixed cells detects changes in free Ub levels and localization after stress conditions.

Higher levels of free Ub were observed in all the cell lines evaluated during mitosis in comparison with cells in interphase (Figure 2.3d). Although the reasons for these differences in free Ub levels were not explored, the increase during mitosis could be due to the previously reported increase in the deubiquitinating activity of USP16 over ubiquitinated histone H2A in mitotic cells[66]. Joo *et al* (2007) showed that this process is necessary for the cell cycle progression. Further studies using free Ub staining simultaneously with different cell cycle markers could give additional information about regulation of free Ub levels along the cell cycle.

Because free Ub is a protein of small size that can distributed evenly through the

cells as previously shown in this study for a group of mammalian cells, a specific free Ub distribution was not expected in neurons due to the cell size and compartmentalization. Using the free Ub staining technique described in this work, free Ub in cultured primary hippocampal neurons showed a diffuse localization through the soma, dendrites and axons (Figure 2.5a). However, unexpectedly, foci of intense staining by HA-tUI were observed in regions close to the dendrites (Figure 2.5a, d). Control experiments to check for HA-tUI specificity confirmed that those foci represented sites of free Ub accumulation (Figure 2.5a). Moreover, the controls showed that the HA-tUI probe was not binding to Nedd8, a protein with ~80% homology to Ub and the most abundant Ub-like protein in mouse neurons[67]. tUI prefers free Ub with 120-fold higher affinity compared with Nedd8[61].

Neuronal spines have been identified as sites where different components of the Ub proteasome pathway are compartmentalized[68]. Use of acting staining by phalloidin or a plasma membrane dye to identify possible filopodia and spines showed that, at the time of fixation, free and conjugated Ub were not located inside all the filopodia and spines (only in approximately 5% of the spines). Accumulation of conjugated Ub was also observed in some of the identified spines, and those foci did not always co-localize with free Ub. The absence of conjugated Ub in some of the spines agrees with a previous report where only 40.7 ± 2.3% of the puncta observed for conjugated Ub overlapped with synaptophysin[69]. Together, these results show that although free and conjugated Ub do not colocalize they could share a compartmentalization pattern as other components of the Ub proteasome pathway.

The distributions of some components of the Ub proteasome pathway inside the

neurons suggest that their location could be modulated by neuronal activity. After KCl-induced depolarization, redistribution of Ub conjugates and proteasomes from the dendritic shaft into the spines has been observed in mature hippocampal neurons (21 DIV)[70]. Specifically, 10 minutes after depolarization with 60 mM KCl there is ~67% increase in conjugated Ub in the dendritic shaft and the spines. After 20 minutes there is a decrease in conjugated Ub, that coincides with a ~90% increase in fluorescent signal for the proteasomes (Rpt1-GFP) in the spines[70]. The absence of free and conjugated Ub (Figure 2.5c, d) from most of the spines could be explained by the previously reported redistribution of conjugated Ub upon synaptic stimulation[70]. Also, our preliminary observation showed that free Ub does not localize in all the mature spines identified by staining with anti-PSD-95 antibody (data not shown). Note that in this experiment the amount of mature spines based on phalloidin staining was low compared with the amount of filopodia; therefore, additional experiments with neurons that have a higher density of spines are needed. Also, evaluation of free Ub redistribution after depolarization (e.g., induced with KCl) will be required to test if free Ub localization changes upon neuronal depolarization. To increase the density of mature synapses, an approach to consider for future experiments is to culture the neurons together with glia cells.

In conclusion, staining of free Ub using HA-tUI allows visualization and quantification of free Ub and its localization in different cell types. HeLa, U2OS, MEF and RPE1 cells showed changes in free Ub levels after proteasome and E1 inhibition. In neurons, free Ub staining revealed an unexpected compartmentalization of free Ub. The accumulation of free Ub in only some of the observed spines could be a free Ub deposit

available for the turnover of synaptic proteins. However, additional experiments will be required to confirm this observation and to identify the location of most of the free Ub foci observed.

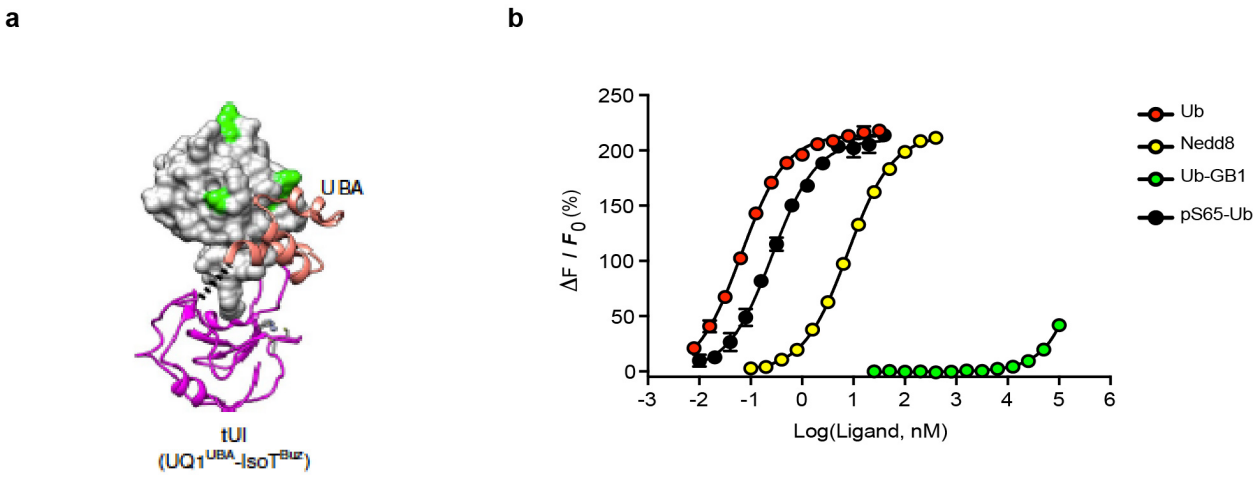


Figure 2.1. Representation of tUI and its affinity for Ub. a) Surface and ribbon representation representing of Ub in complex with the UBDs forming tUI. The UBDs are shown as ribbon representation. In the Ub surface lysine and M1 side chains are shown in green. The black dotted lines indicate the linker used to connect UBDs. Ub complexes with tUI was modeled from composites of individual UBD-Ub complex structures. b) Atto532-tUI binding affinity for free Ub. Figure adapted from Choi *et al* (2019).

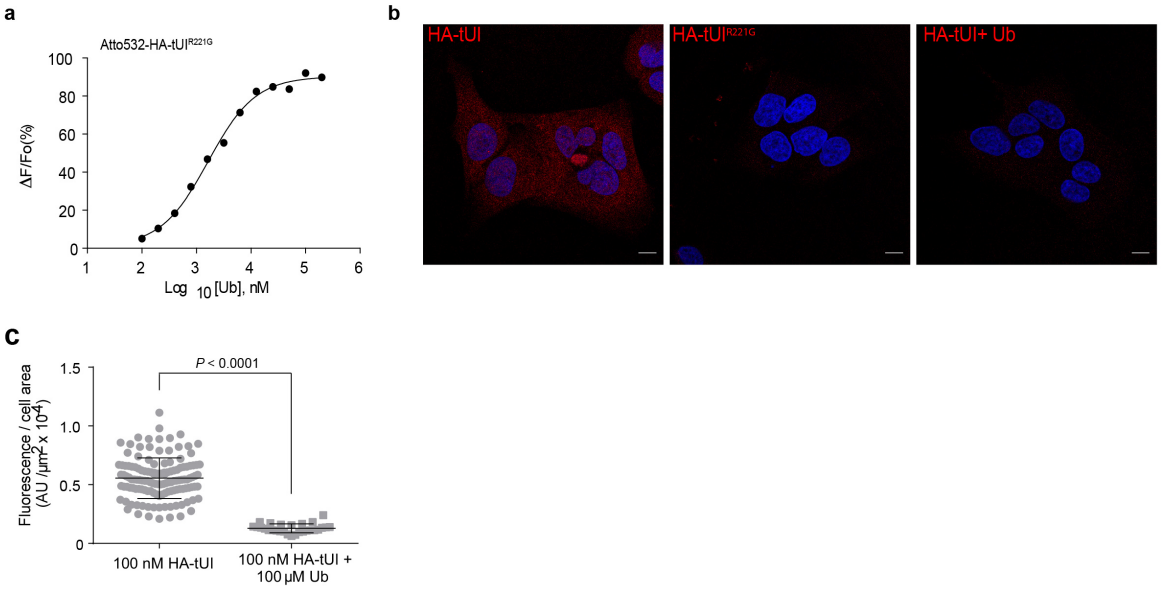
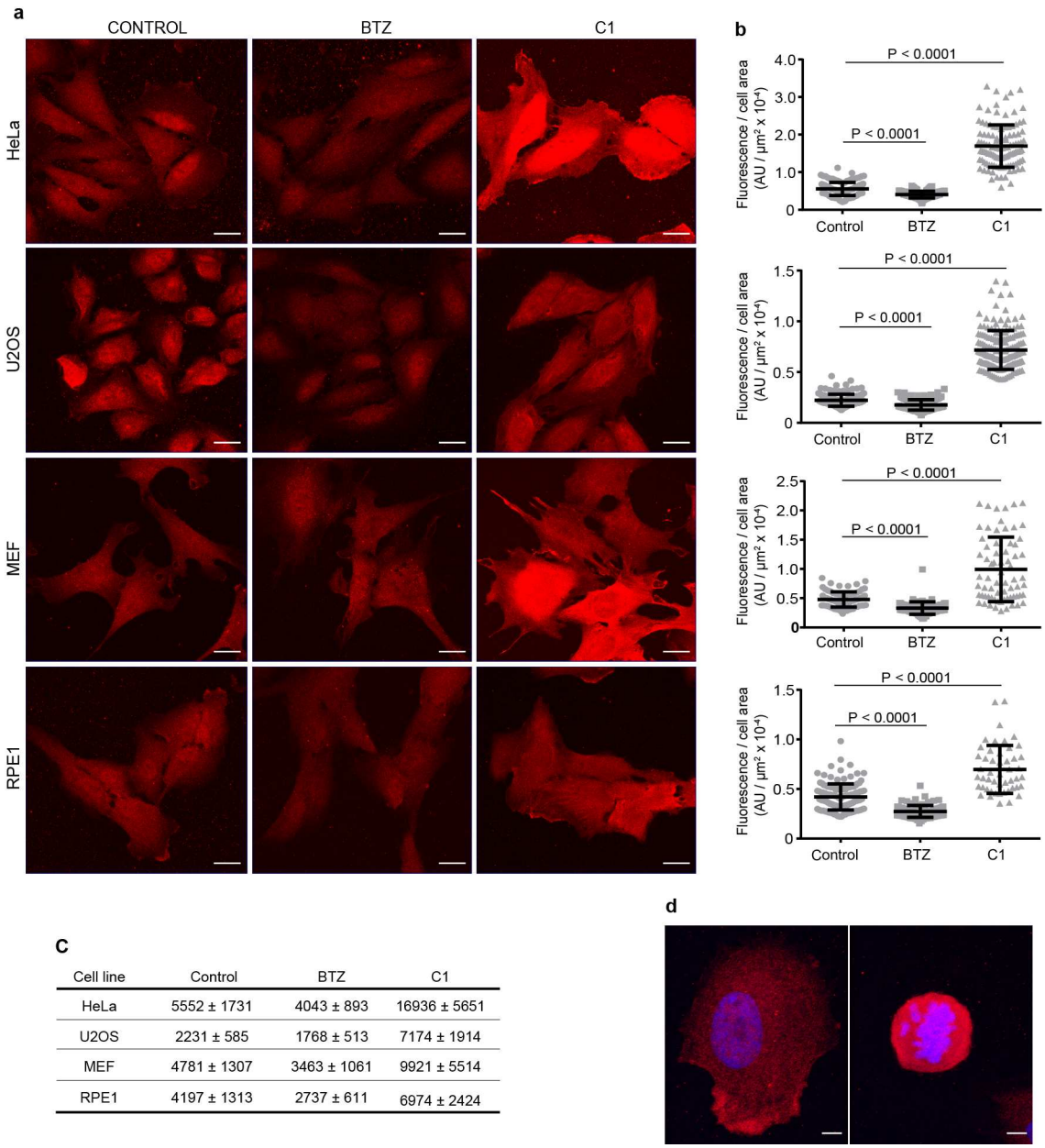


Figure 2.2. Cell staining with HA-tUI and controls to check HA-tUI specificity. a) Atto532-tUIR221G binding affinity for free Ub. b) U2OS cells stained with 100 nM HA-tUI, 100 nM HA-tUIR221G and100 nM HA-tUI plus 100 uM free Ub (red). The nuclei were stained with DAPI (blue). Scale 10 μm. c) Quantification of the fluorescence signal in HeLa cells after staining with 100 nM HA-tUI (n = 131) and 100 nM HA-tUI plus 100 uM free Ub (n = 28). AU, arbitrary units; error bars indicate mean ± s.d. Statistical analysis used two-tailed unpaired Student's *t*-test with Welch's correction. Graph in (c) from Choi *et al* (2019).



c

Cell line	Control	BTZ	C1
HeLa	5552 ± 1731	4043 ± 893	16936 ± 5651
U2OS	2231 ± 585	1768 ± 513	7174 ± 1914
MEF	4781 ± 1307	3463 ± 1061	9921 ± 5514
RPE1	4197 ± 1313	2737 ± 611	6974 ± 2424

Figure 2.3. Staining free Ub in HeLa, U2OS, MEF and RPE1 cells. a) Images maximum projection of cells after incubation for 1 hour with 1 µM BTZ or 10 µM E1 inhibitor. Scale bars, 20 µm. b) Graphics showing the measurements from HeLa, U2OS, MEF and RPE1 cells fluorescence after the different treatments. AU, arbitrary units. Cells analyzed per condition: HeLa, control n = 131, BTZ n = 125, C1 n = 116; U2OS, control n = 161, BTZ n = 170, C1 n = 175; MEF, control n = 80, BTZ n = 79, C1 n = 69; RPE1, control n = 133, BTZ n = 87, C1 n = 49. Bars show mean ± s.d. Statistical analyses used two-tailed unpaired Student's t-test with Welch's correction where appropriate. c) Table showing the fluorescence staining (mean fluorescence ± s.d.) of the cells evaluated for each treatment. d) Representative image of free Ub staining (red) in RPE1 Cells during interphase (left panel) and mitotic (right panel) phase. Nuclei were stained with DAPI (blue). Scale bars, 5 µm. Figure from Choi *et al* (2019).

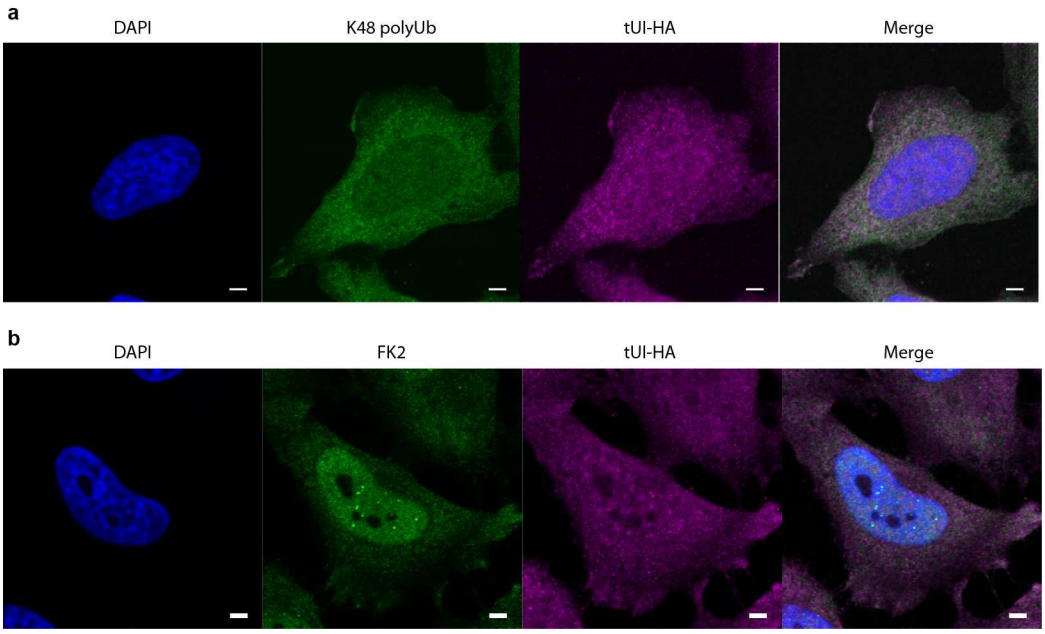


Figure 2.4. Staining free Ub, K48-linked polyUb or total conjugated Ub in HeLa cells. Single plane images from a) cells were fixed and stained for K48-linked polyUb (green) or b) conjugated Ub (green). Nuclei were stained with DAPI (blue) and free Ub with HA-tUI (magenta). Scale 5 µm. Figure from Choi *et al* (2019).

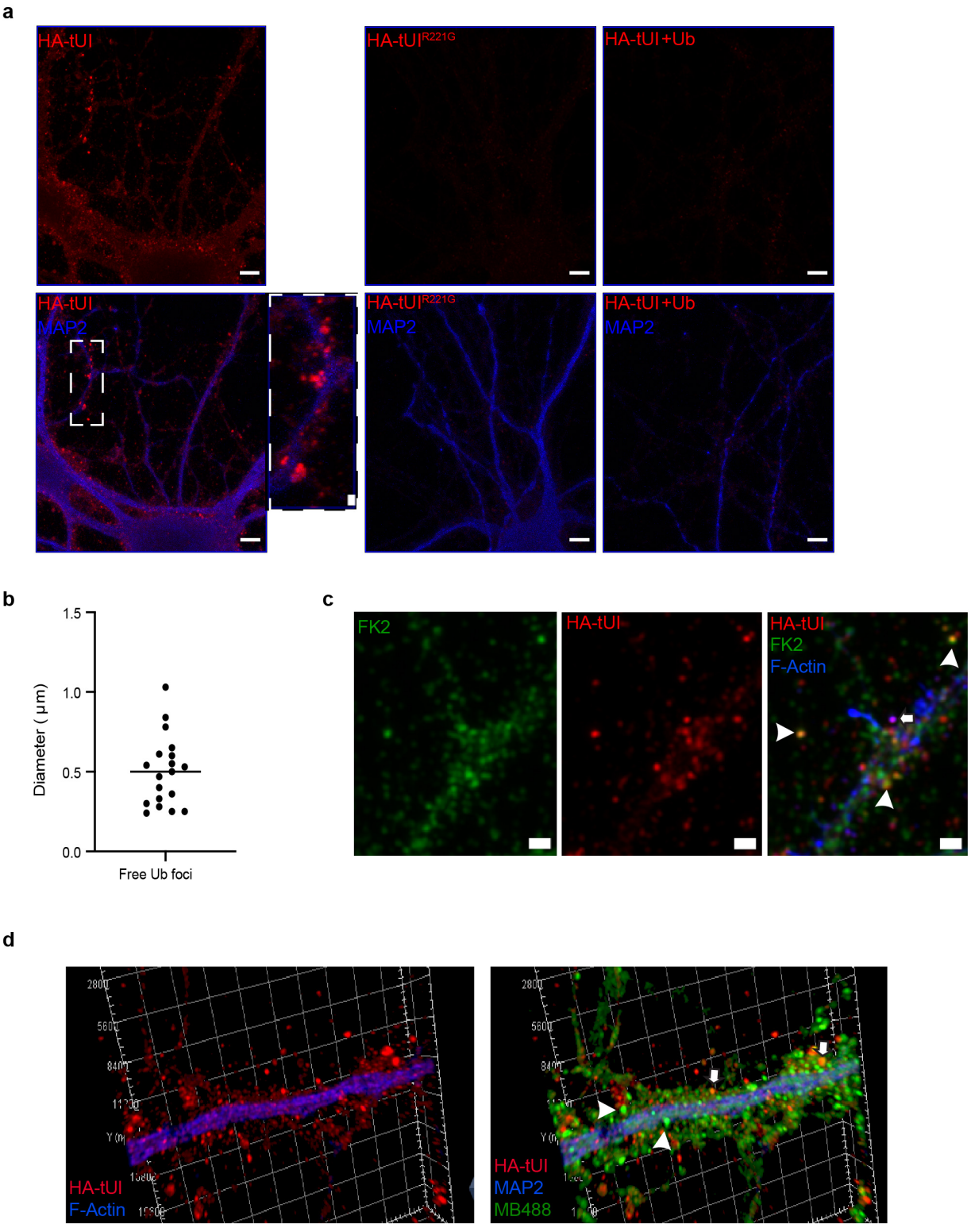


Figure 2.5. Hippocampal neurons at 21 DIV stained with HA-tUI. a) Maximum projection images showing free Ub staining (HA-tUI) of hippocampal neurons at 21 DIV. Enlargement of a dendritic region is shown in the dashed square. Controls for non-specific staining used HA-tUI^{R221G} or HA-tUI incubated with excess free Ub, as described in the text. Dendrites are stained with anti-MAP2 antibody (blue). Scale bar,

5 μm. In the zoomed image, the scale bar represents 0.5 μm. b) Graph showing the sizes of the foci detected by free Ub staining with HA-tUI (number of foci evaluated = 19, in two independent experiments). c) Images of maximum projections of a neuron process using SR. F-actin was stained using Alexa-488 phalloidin (blue). Co-localization of conjugated Ub (FK2 anti-Ub monoclonal antibody) and free Ub (HA-tUI) is shown (arrowheads). Co-localization of free Ub with phalloidin (blue) is indicated (arrow). d) Three-dimensional reconstruction of SR images from neurons stained for free Ub (HA-tUI; red), MAP2 (blue) and plasma membranes (Membryght-488[71]; green). Possible spines without free Ub staining are showed (arrowhead). Accumulation of free Ub inside possible spines formations is shown (arrow).

CHAPTER 3: ROLE OF THE OTUB1 DEUBIQUITINATING ENZYME IN UBIQUITIN HOMEOSTASIS

3.1 Introduction

OTUB1 is a deubiquitinating enzyme that belongs to the ovarian tumor protease family of DUBs. Its deubiquitinating activity is highly specific for cleavage of K48-linked polyUb chains[72,73]. The catalytic activity of OTUB1 is involved in a variety of cellular processes that include immune cell response for the induction of virus-triggered type I interferon pathways[74,75], regulation of transcription factors levels[76,77], signaling in the NF-κB and MAPK pathways[78], and induction of T-cell anergy in lymphocytes[79]. In addition, different *in vitro* and *in vivo* studies have shown that OTUB1 also has non-catalytic functions that are independent of its isopeptidase activity[80,81].

Structural studies have shown that two sites for Ub binding in OTUB1, the distal and proximal sites Ub sites, mediate its polyUb substrate specificity[72,80]. The proximal site includes N- terminal residues that are disordered in the apoenzyme[79,80] (Figure 3.1a, b). These binding sites play an important role in the catalytic activity of OTUB1 and also in its interaction with E2 enzymes. An E2 or Ub~E2 can interact with OTUB1 through the OTU domain and stabilization of the N-terminal residues. Interaction between OTUB1 and a subset of E2s can stimulate OTUB1 isopeptidase activity[82] (Figure 3.1c), whereas interactions with Ub~E2s can inhibit the ubiquitin transfer from the thioester Ub~E2 adduct[82,83] (Figure 3.1d). This last interaction is regulated allosterically by free Ub binding to the distal site of OTUB1 through the stabilization of an alpha helix in the N-terminal residues. By binding of free Ub at the distal site and

donor Ub of Ub~E2 at the proximal site, the complex mimics the conformation of bound K48-diUb and the OTUB1 catalytic activity is inhibited[80] (Figure 3.1d).

Evidence of OTUB1 interaction with different E2s has been collected. Mass spectrometry studies have reported E2 interaction partners for OTUB1 that include UBE2D1, UBE2D2, UBE2D3, UBE2E1, UBE2E2, UBE2E3, and UBE2N[81,84]. Some of those E2s (UBE2D and UBE2E1) have been shown to increase the catalytic activity of OTUB1 towards diUb K-48 *in vitro*[82]. Although UBE2N interacts with OTUB1 *in vivo,* it does not affect the catalytic activity of the enzyme[82]. At the same time, this study showed that the enzyme UBE2W increases OTUB1 catalytic activity.

In addition to the mass spectrometry and *in vitro* studies, for UBE2N, UBE2D and UBE2E1 there are reports of *in vivo* interactions with OTUB1. It has been shown that OTUB1 interacts with E2s preventing the formation of different Ub chains where those enzymes are involved. This interaction does not involve OTUB1 catalytic activity. The first case of OTUB1 interaction with an E2 was reported in the DNA double-strand break damage response, where formation of K63-polyUb chains on chromatin is prevented by OTUB1 interacting with UBE2N[80,81]. Interactions of OTUB1 with UBE2D interfere with p53[85] and SMAD2/3[86] ubiquitination. Lastly, for UBE2E1 it was reported recently than interaction with OTUB1 is necessary to avoid UBE2E1 auto-ubiquitination and degradation by the proteasome[87].

The ability of OTUB1 to interact with different partners that modulate its activity suggests that OTUB1 could have an *in vivo* role in Ub homeostasis. Due to the high intracellular concentration of OTUB1 (1.1 μM), together with Kds of its effectors (i.e., Ub, E2, and Ub~E2) in the low micromolar range, OTUB1 could sense the ratio of [Ub~E2]to

[E2] in response to free Ub concentration. [80,82,88]Based on these observations we hypothesized that OTUB1 was able to detect changes in the concentration of its effectors (i.e., Ub, E2, and Ub~E2) *in vivo* and modulate their concentration through OTUB1 catalytic and non-catalytic activities. To test this hypothesis, we evaluated the effects of OTUB1 on intracellular Ub pools under normal and stress conditions. No differences in the free Ub levels where found under stress conditions in *OTUB1*-knockout U2OS cells compared with WT. However, decreases in the levels of several E2s, including UBE2D and UBE2E1, were observed in *OTUB1*-knockout U2OS cells and *Otub1*$^{-/-}$ null MEF cells relative to *OTUB1* wild-type controls. Further experiments indicate that OTUB1 is involved in the production and biosynthesis of UBE2D, probably through post-transcriptional regulation. As previously reported, UBE2E1 is modulated by OTUB1 to prevent proteasomal degradation.

3.2 Experimental procedures

The following cell lines used in this work were provided and characterized by our collaborators[87].

- *Otub1* wild-type (WT) and *Otub1*$^{-/-}$ null MEF cells. Cells were provided by Averil Ma (The University of California, San Francisco).
- *OTUB1* WT and *OTUB1* knockout U2OS cells. Cells were provided by C. Wolberger (Johns Hopkins Medical Institute, Baltimore, MD).

3.2.1 Quantification of ubiquitin pools

To quantify the different ubiquitin pools in cell lysates we used in-solution assays that are based on the recognition of free Ub by high affinity sensors[61]. Briefly, the cells were resuspended and sonicated in a lysis buffer containing 100 mM MOPS, pH 6.0, 8 M urea, 20 mM N-Ethylmaleimide (NEM) and protease inhibitor (P8340 SIGMA). The lysates were sonicated and cleared. After protein quantification using the bicinchoninic acid assay the samples were divided into three fractions to be treated independently:

- Usp2cc treated samples: the samples were diluted to reduce the urea concentration to less than 2 M using a buffer containing: 25 mM HEPES (pH 7.5), 140 mM NaCl and 10 mM DTT. Usp2cc was added in a ratio 1:10 (Usp2cc : total protein) Usp2cc was added and the sample incubated at 37 °C for 1 hour.
- β-mercaptoethanol treated samples: the samples were incubated for 1 hour at 37 °C with a buffer with 100 mM CHES (pH 9), 150 mM β-mercaptoethanol.
- Hydrazine treated samples: the samples were incubated for 1 hour at 37 °C with hydrazine-HCl 200 mM (pH 8.5).

Dilutions of the samples with PBS supplemented with 0.2 mg/mL ovalbumin were performed to ensure the concentration of Ub was in the linear range of detection and to reduce the concentrations of urea (<0.2 M), β-mercaptoethanol (<20 mM) and hydrazine (<20 mM).

3.2.2 Quantification of activated E2s by western blot

To evaluate the thioesterified forms of different E2s using immunoblotting a method previously reported was followed[89]. The cells were lysed in a buffer containing:

150mM MES (pH 3–5), 150mM NaCl, 0.2% Nonidet P40, 20 mM NEM and protease inhibitor (P8340 SIGMA), and incubated for 30 minutes at 4 °C. The low pH buffer decreases the rate of thioester hydrolysis. Then the samples were cleared by centrifugation at 13,000 rpm for 10 minutes at 4 °C. The cell extracts were loaded onto 8-16 % SurePAGE Gels (GenScript) using 1x Tris-MOPS-SDS buffer without the presence of any reducing agent. The gels were run at 4 °C. To confirm that bands corresponded to thioesterified forms of the E2s, portions of the samples also were incubated 10 minutes at 70 °C with 200 mM DTT. The gels were transferred to nitrocellulose membranes (0.2 µm) using 1x Towbin buffer with 20 % methanol for 1 hour at 250 milliamperes at 4 °C.

The membranes were incubated overnight at 4 °C with different types of antibodies according to the E2s evaluated: anti-UBE2D diluted at 1:500 (polyclonal antibody, rabbit; Prointech), anti-UBE2E1 (polyclonal antibody, rabbit; Abcam), anti-UBE2N (monoclonal antibody, mouse; Thermofisher), anti-UBE2S (monoclonal, mouse; Santa Cruz), anti-UBE2C (monoclonal, mouse; Santa Cruz), anti-UBE2W (polyclonal, rabbit; SIGMA) and anti-OTUB1 (polyclonal, rabbit; Bethyl) were all diluted at 1: 1,000. Also, anti-FLAG diluted at 1:5,000 (monoclonal, mouse; Wako) was used. For loading controls, the membranes were stained with REVERT™ 700 total protein staining (LI-COR) or anti-tubulin antibody DM1A diluted at 1:100,000 (monoclonal, mouse; SIGMA).

3.2.3 Digestion of Ub-protein conjugates by Usp2cc

To visualize the possible ubiquitination of UBE2D, the lysates were evaluated by western blotting after incubation with the non-specific DUB Usp2cc[90]. For this purpose,

the whole-cell lysate was made in a buffer containing 25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA pH 8, 10 % glycerol, 0.2 % Triton X-100, 20 mM NEM and protease inhibitor (P8340 SIGMA). After 30 min at 4 °C, the NEM was neutralized by adding DTT at 1:1 molar ratio, the samples were incubated 20 minutes at 4 °C. Usp2cc was added and the sample incubated at 37 °C for 1 hour.

3.2.4 OTUB1 knockdown by siRNA

Transfections in U2OS cells were performed using Lipofectamine RNAimax (Invitrogen). The following protocol was used: In 3.5 cm dishes, 30 pmol siRNA (Dharmacon) was diluted in 500 µL Opti-MEM. After mixing gently, 4 µL RNAimax was added and incubated at room temperature for 20 minutes. Finally, cells (2.5×10^5) were added in 2.5 mL of medium. The final siRNA concentration was 10 nM. The sequences of SMARTpool siRNA for OTUB1 (catalog number M-021061-01-0005) were obtained from Dharmacon™. After 72 hours, the cells were harvested and the lysates were evaluated by immunoblotting under reducing conditions.

3.2.5 Cycloheximide chase

Cycloheximide (CHX) was added to a final concentration of 50 ug/mL at different time points. In one of the experiments, proteasome inhibitor (10 µM MG-132 in DMSO) or DMSO alone (control) was added 2 hours before incubation with (CHX). After incubation of the cells at different times, the cells were harvested and lysed. The whole-cell lysates were evaluated by immunoblotting as previously described.

3.2.6 qRT-PCR

OTUB1 knockout and WT U2OS cells were incubated with DMSO or 1 μM Bortezomib (BTZ) for 4 hours or 10 μM MG-132 for 2 hours. For each treatment, three replicates were performed. After each treatment the cells were harvested and total RNA was extracted using Aurum Total RNA mini kit (Bio-Rad). For the synthesis of cDNA, 5x iScript Reverse transcriptase supermix (Bio-Rad) was used with 500 ng RNA. RT-PCR reactions contained 2 μL of diluted cDNA, 500 nM forward and reverse primers, and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). All the reactions were run on a CFX96 qPCR system (Bio-Rad). Relative mRNA values for each gene were quantified using the Ct values normalized by the Ct values for GAPDH using 2 or 3 experimental replicates. Primers used for RT-PCR were: 5'-AGATGTTATCGCCTTTGGGA-3' and 5'-TCCAAACTCCTCTCCACCAG-3' for UBE2E1[87]; 5'-GAGTAATTTGGGGTTTGTCTTGG-3' and 5'-CCTTTCTTTTGGATGGGTGAT-3' UBE2D1[91]: 5'-TTGTCCATCTGTTCTCTGTTGTG-3' and 5'-TCCATTCCCGAGCTATTCTGT-3' UBE2D2[91]; 5'-AAGATCACAGTGGTC GCC TG-3' and 5'-CCGTGCAATCTCTGGCACTA-3' for UBE2D3; 5'-TGGTCTCCAGCGTTGACTG-3' and 5'-GGCCTTGTAGGTGTGTGCTATCTC-3' UBE2D4[91] and for GAPDH; 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3'

3.2.7 Generation of stable cell lines

For the generation of stable cell lines, the *OTUB1* knockout U2OS cells and the plasmids (pCDNA 3.1 3xFLAG_OTUB1^{WT} and pCDNA 3.1 3xFLAG_OTUB1^{C91S}) were provided by C. Wolberger. The pCDNA 3.1 3xFLAG_OTUB1^{WT} plasmid was modified

by site-directed mutagenesis or In-Fusion cloning (TakaRa); the mutations selected were reported[82,83] to interfere with E2 interaction (pCDNA 3.1 3xFLAG_OTUB1^{T134R} and pCDNA 3.1 3xFLAG_OTUB1$^{E28A\ D35A}$). Initially the cells were transfected with the indicated plasmids (pCDNA 3.1 3xFLAG_OTUB1^{WT}, pCDNA 3.1 3xFLAG_OTUB1^{T134R} and pCDNA 3.1 3xFLAG_OTUB1$^{E28A\ D35A}$). Selection of the polyclonal cells started 48 hours after transfection, cells were diluted (1:10 and 1:20) and 400 µg/mL Neomycin was added. The media was changed every 4 days. Monoclonal populations were isolated by limiting dilution using the Addgene protocol (https://www.addgene.org). Briefly, cells with confluences between 40 and 70% were trypsinized pippeting up and down to avoid the formation of clumps. Then the cells were passed through a 0.40 µm cell strainer mesh before counting. In all cases, the cell densities were $<10^6$ cells/mL. Finally, 100 µL of a 5 cell/mL dilution was pipetted in each well of 96-well plates. The single colonies that grew in the wells were transferred after trypsination to 24-well plates. All the colonies were selected and confirmed using immunofluorescence and immunoblotting.

3.3 Results

3.3.1 Effect of OTUB1 on ubiquitin pools

OTUB1 has been postulated as a regulator of ubiquitin homeostasis. The high intracellular concentration of the enzyme (1.1 µM)[88] and the low-micromolar concentrations of its different effector proteins (i.e., free Ub, Ub~E2 and E2) suggest that OTUB1 may be involved in the regulation of free Ub levels. Initially, to gain insight into the function of OTUB1 in Ub homeostasis, we measured the Ub pools under

proteotoxic stress conditions in *OTUB1* knockout and WT U2OS cells treated with either DMSO (control) or the proteasome inhibitor (BTZ) for 1 hour. Although we hypothesized that the absence of OTUB1 would affect the free Ub pool under proteasome inhibition, no differences in the levels of free Ub were observed (Figure 3.2). That the proteasome was inhibited by the BTZ treatment as expected was apparent from the increase in conjugated Ub (Figure 3.2). These results suggest that the presence of OTUB1 is not necessary to regulate free Ub levels under stress from proteasome inhibition.

3.3.2 OTUB1 regulation of Ub~E2 and E2 levels

Based on the variability observed in measurements for the activated Ub pool in *OTUB1* WT and knockout U2OS cells (data not shown), we decided to compare steady-state levels of specific E2 and Ub~UBE2D thioesters (e.g., Ub~UBE2D and UBE2D) in cell lysates under non-reducing conditions using immunoblotting. UBE2D was selected for the initial screening because it has been reported as one of the most abundant E2 ubiquitin-conjugating enzymes in MEF cells (1.7 μM)[88]. After finding that there was a 60% decrease in the total levels of UBE2D in *OTUB1* knockout U2OS cells compared with WT (Figure 3.3a), we decided to evaluate whether this was observed with other E2s. A decrease in the levels of different E2s including UBE2D had been reported using the same cell lines[87]. In the light of this information and previous reports about OTUB1 interactions with E2s *in vivo* and *in vitro,* we evaluated by Western blot the levels of the following E2s: UBE2D, UBE2E1, UBE2N, UBE2W, UBE2C and UBE2S. Among these E2s, only UBE2D, UBE2E1 and UBE2N have been reported as OTUB1 interaction partners *in vivo*[81,86,87,92]. UBE2W has been shown to be an E2 that stimulates OTUB1

catalytic activity *in vitro* [82]. Lastly, although for UBE2C and UBE2S there are no reports of interactions with OTUB1 *in vivo* or *in vitro*, the levels of these E2s are low in OTUB1 KO cells compared with WT [87] (Table 3.1).

Evaluation of the total levels of the selected E2s using reducing conditions in the lysates (DTT) showed that in OTUB1 knockout U2OS cells there is a decrease in most of the enzymes evaluated compared with WT cells. For UBE2D, UBE2E1 and UBE2C there was a decrease of more than 50%, while 80% reduction was observed for UBE2W (Figure 3.3a-d). As previously reported[87], no changes in the levels of UBE2N between these cell lines were detected (Figure3.3a). This is consistent with the decrease in E2s levels reported in *OTUB1* KO U2OS cells[87].

To test whether the E2s with lower levels were decreased because in the absence of OTUB1 they were targeted more efficiently for proteasomal degradation, the cells were incubated for 4 hours with proteasome inhibitor (BTZ). UBE2D and UBE2E1 levels in *OTUB1*-knockout U2OS cells were not affected by the inhibition of the proteasome (Figure 3.3a, b). The levels for total UBE2C, UBE2S and UBE2W increased after proteasome inhibition in *OTUB1*-knockout cells (Figure 3.3c, d).

Test for effects of OTUB1 depletion on E2s levels were extended to *Otub1*$^{-/-}$ null and WT MEF cells (Figure 3.4). Analysis of these cells showed similar effects to those observed in U2OS cells, although a less drastic decrease in the levels of UBE2D (~ 30%) in *Otub1*$^{-/-}$ null MEF cells compared with WT cells was observed (Figures 3.4a). At the same time, decrease, in UBE2C, UBE2W and UBE2E1 were observed (Figure 3.4b-d). No changes in the levels of UBE2N and UBE2S were detected between *Otub1*$^{-/-}$ null and WT MEF cells (Figure 3.4a, b). These results are consistent with the decrease in

the levels of UBE2D, UBE1E1, UBE2C and UBE2S reported in *Otub1*$^{-/-}$ null MEF cells[87]. After proteasome inhibition, an increase in the levels of UBE2S and UBE2W in *Otub1*$^{-/-}$ null MEF cells was observed (Figure 3.4b). All these results together suggest that OTUB1 is playing an important role in the regulation of multiple E2s. Among these, stabilization after proteasome inhibition for only a subset of the E2s suggests that the regulation by OTUB1 use mechanisms in addition to preventing proteasomal degradation.

To confirm the direct effect of OTUB1 on E2s levels, endogenous OTUB1 was knocked down by siRNA (Figure 3.5). These experiments showed an 80 % decrease in OTUB1 levels compared with cells transfected with non-silencing control siRNA. Simultaneously, a decrease in the levels of UBE2E1 and UBE2W was observed, but UBE2D levels were unaffected (Figure 3.5a, b). Levels of UBE2E1 were previously shown to be sensitive to OTUB1 expression levels[87]. These results suggest that the mechanism regulating UBE2D levels is different from the mechanism regulating UBE2E1 and UBE2W.

Immunoblot analysis of the lysates under non-reducing conditions allowed the detection of Ub-thioesterified forms of the E2s. Results from these analyses showed that there is a 50% reduction in the thioestherified fraction of UBE2D and UBE2E1 relative to the total amount of each E2 in the *OTUB1*-knockout U2OS cells compared with WT cells (Figure 3.3a, b). For UBE2S, UBE2C and UBE2N, there were no differences in the fraction of thioesterified E2s between cell lines (Figure 3.3a, c, d).

Ub~UBE2W was not detected by immunoblotting, possibly because its levels were below the detection sensitivity (Figure 3.3d). In contrast, there were no decreases in any of the thioesterified fractions of E2s evaluated in the *Otub1*$^{-/-}$ *null* MEF cells compared with WT MEF cells (Figure 3.4). Taken together, our results suggest that OTUB1 is involved in the regulation of thioesteridied E2s levels.

3.3.3 UBE2D is not targeted to proteasomal degradation upon OTUB1 depletion

After finding that UBE2D levels were not stabilized by proteasome inhibition in the OTUB1 knockout cells, we decided to check if the apparent decrease in this E2 was due to accumulation of higher molecular mass forms of the protein, possibly because of polyubiquitination, that shifted material away from the main SDS-PAGE band. To evaluate this possibility, cell extracts were incubated with Usp2cc. The results showed no difference in UBE2D levels after incubation of the lysates with Usp2cc (Figure 3.6). This suggests that the difference in UBE2D levels between OTUB1 WT and knockout U2OS cells is not due to accumulation of ubiquitinated forms of UBE2D in *OTUB1* knockout cells.

To evaluate if OTUB1 was directly involved in targeting UBE2D to degradation by the proteasome, we measured the half-life of the protein in *OTUB1* WT and knockout U2OS cells (Figure 3.7a). In accord with previous results, an increase in UBE2E1 turnover in *OTUB1* knockout U2OS cells compared with the WT was observed when the cells where treated with CHX[87]. In contrast, no difference in UBE2D half-life between cell lines was detected. CHX activity was confirmed in cells pre-incubated with

proteasome inhibitor; there was stabilization in UBE2E1 and UBE2D levels after CHX addition (Figure 3.7b). These results suggest UBE2D regulation by OTUB1 is not mediated by proteasomal degradation.

3.3.4 OTUB1 depletion does not affect the levels of mRNA for UBE2D

After finding that OTUB1 was not regulating UBE2D levels by changing the rate of its proteasomal degradation, we decided to check if the lower UBE2D levels were due to differences at the transcriptional level of the mRNA between *OTUB1* knockout and WT U2OS cells. Measurements of the relative expression levels of the different mRNA for all the UBE2D isoforms (UBE2D1, UBE2D2, UBE2D3 and UBE2D4) and UBE2E1 showed that after *OTUB1* knockout in U2OS cells, there was no decrease in the levels of transcription compared with WT cells. A 25-45 % increase in UBE2D1 and UBE2D4 mRNA levels was detected in *OTUB1*-knockout cells compared with WT (Figure 3.8). In addition, the effects of the proteasome inhibitors on the transcript levels were variable between the mRNAs evaluated (Figure 3.8). This indicates that OTUB1 does not regulate either UBE2D or UBE1E1 transcription.

3.3.5 Effects of OTUB1 rescue over E2s levels and Ub~E2s stability

After detection of lower levels of total and thioesterified UBE2D and UBE2E1 in *OTUB1*-knockout in U2OS cells, we wanted to test if the effect could be rescued by expression of *OTUB1* mutants reported to affect OTUB1–E2 interactions. Mutations in the catalytic OTU domain (T134R) and the N-terminus of OTUB1 (E28A and D35A) known to disrupt the interaction with E2s[82,83] were evaluated. The OTUB1^{T134R} mutant

had been shown to have two important effects on the interaction with UBE2D2. First, mutation T134R in the OTU domain introduces steric clashes that compromised the ability to inhibit E2 dependent Ub chains synthesis[83]. Second, OTUB1^{T134R} catalytic activity is not stimulated by incubation with UBE2D like OUTB1 WT incubation of the mutant with UBE2D2 does not increase the isopeptidase activity of the enzyme[82]. The OTUB1^{E28AD35A} mutant presented a decrease stimulation of the isopeptidase activity after incubation with UBE2D compared with OTUB1 WT[82]. After rescue with OTUB1^{WT} and the mutants, total (UBE2D and UBE2E1) and thioesterified (UB~UBE2E1) levels were evaluated. The expression levels of the various OTUB1 proteins in all the cell lines were evaluated and found to be similar to the endogenous expression levels in WT cells. Quantification of total UBE2D and UBE2E1 levels after rescue with the OTUB1 variants showed complete rescue for all the cells lines evaluated (Figure 3.9a). These results suggest that direct interaction of OTUB1 with the E2 proteins is not required for their stabilization.

Evaluation of the effects of the same mutants on the fraction of thioesterified UBE2E1 was performed. Expression of the different OTUB1 versions showed that expression of FLAG-OTUB1^{T134R} cannot restore the fraction of thioesterified UBE2E1 in *OTUB1* knockout U2OS cells (Figure 3.9b). In contrast, a larger fraction of UBE2E1 as the Ub~UBE2E1 thioester was observed in cells expressing FLAG-OTUB1^{E28AD35A}; this could be explained by the higher expression of this mutant OTUB1 compared with cells expressing endogenous wild-type OTUB1 (Figure 3.9b). These observations suggest that interaction of OTUB1 with E2 through the residue T134 in OTUB1 is involved in the stabilization of Ub~UBE2E1.

3.4 Discussion

An important role in the regulation of free Ub has been hypothesized for OTUB1 due to its ability to its ability to interact with different partners (E2 and Ub~E2) that can modulate OTUB1's activities[80,82]. It has been hypothesized that free Ub concentration inside the cells directly affects the ratio of [Ub~E2] to [E2], regulating the formation of complexes of OTUB1-E2 (by an increase OTUB1 isopeptidase activity) and OTUB1-Ub-Ub~E2 (OTUB1 is catalytically inactive and blocks transfer of donor Ub from Ub~E2)[82,83,93]. It was expected that after proteasome inhibition, which induces the accumulation of Ub chains and a decrease in free Ub in Hela cells[61], the ratio of [Ub~E2] to [E2] would decrease. OTUB1 increased catalytic activity due to stimulation by increasing E2s levels. Based on this information, we anticipated lower free Ub levels and a decrease of [Ub~E2] to [E2] ratio in *OTUB1*-knockout U2OS cells compared with WT cells after proteasome inhibition. However, no differences in the levels of free Ub were found in comparisons between *OTUB1*-knockout and WT U2OS cells after proteasome inhibition (Figure 3.2). Additionally, a ~ 30% increase in conjugated Ub and 10% increase in total Ub was observed in both cell lines. Non-change in free Ub levels after proteasome inhibition has been observed as well in MEF cells, where the increase in total Ub levels has been attributed to over expression of Ub genes[61]. During proteasome inhibition Ub supplied by histones[53] and overexpression of Ub genes[14] can help to compensate free Ub levels. Quantification of Ub pools in *OTUB1*-knockout and WT U2OS cells after proteasome inhibition showed that OTUB1 activity is not directly involved in the regulation of free Ub levels under stress conditions.

Different studies have shown that OTUB1 is able to directly interact with a variety of E2 enzymes. Those interactions can affect the isopeptidase activity of OTUB1 *in vitro* or could prevent the ubiquitination of different substrates when the E2s are thioesterified[80,81,86,92]. The inhibition of the transfer of donor Ub from Ub~E2s to different substrates takes place through OTUB1 interaction with the E3 binding surface on the E2. At the same time, this results in blocking of the thioester bond to be attacked by an acceptor Ub. A recent report described that OTUB1 modulate UBE2E1 levels by inhibiting E2 auto-ubiquitination and therefore prevent proteasomal degradation[87]. In this study, we show that OTUB1 could be involved in the stabilization of a different E2, UBE2D (also known as Ubch5), through a mechanism that is independent of proteasomal degradation.

Evaluation of protein levels using tandem mass tag mass spectrometry to compare WT and *Otub1*$^{-/-}$ *null* MEF cells showed that the loss of OTUB1 caused a decrease in UBE2E1, UBE2E2, UBE2C, UBE2S, and UBE2D3 levels compared with WT cells[87]. Moreover, these results were confirmed in *OTUB1* knockout U2OS and *Otub1*$^{-/-}$ null MEF cells through immunoblot analysis.

In this study using immunoblotting of cell lysates, a decrease in the levels of the same previously identified E2s was observed. In addition to the E2s originally reported, a decrease in the amount of UBE2W was detected. For this E2 there is only *in vitro* evidence reported for interaction with OTUB1 in which UBE2W increased the OTUB1 isopeptidase activity[82]. Quantification of the total E2s levels from different cell lysates by immunoblotting showed that the decrease was only statistically significant for UBE2E1 and UBE2W in *OTUB1* knockout U2OS cells (Figure 3.3a-d). For UBE2D, the P value

(P= 0.0544) was slightly higher than the 0.05 cut-off. These results confirm the previously observed effect of OTUB1 on the intracellular levels of different E2s.

The interaction between OTUB1 and UBE2E1 suppress UBE2E1 auto-ubiquitination and degradation by the proteasome[87]. Incubation of the *OTUB1*-knockout U2OS cells for 2 hours with 10 μM MG-132 stabilized the E2[87]. At the same time, experiments to check the half-life of the protein using CHX to block new translation showed a decrease in half-life of the protein in *OTUB1*-knockout U2OS cells compared with WT[87]. Although our results did not show stabilization in the UBE2E1 levels after proteasome inhibition using 1 μM BTZ for 4 hours (Figure 3.3b), the stabilization was observed when the cells were incubated with MG-132 (Figure 3.7b). Both inhibitors block the chymotrypsin-like activity of the proteasome 20S proteasome[94], and so why they had different effects in the stabilization of UBE2E1 is unclear.

Unlike with UBE2E1, UBE2D stabilization after proteasome inhibition and differences in the protein half-life between *OTUB1*-knockout and WT U2OS cells were not observed (Figure 3.3a; Figure 3.7a). Taken together, these results suggest that compared with UBE2E1, UBE2D1 regulation by OTUB1 is not dependent of proteasomal degradation.

The *OTUB1* knockdown experiments performed in U2OS cells achieved a significant decrease in UBE2E1 and UBE2W levels (Figure 3.5a, b). These results for UBE2E1 were consistent with past results, showing that OTUB1 levels directly affect the protein levels of UBE2E1[87]. On the other hand, no effect was observed in UBE2D levels, giving additional evidence for a different mechanism of regulation by OTUB1. The decrease in UBE2W levels after *OTUB1* knockdown and the previously mentioned

stabilization after proteasome inhibition support the idea that UBE2W could be regulated like UBE2E1. *In vitro* studies have shown that UBE2W could also be auto-ubiquitinated *in vitro*[95].

Quantification of the relative expression of mRNA levels for UBE2E1 and UBE2D showed that there is no decrease in the levels of mRNA in *OTUB1*-knockout U2OS cells compared with WT. Interestingly, although the level of UBE2D4 mRNA was significantly higher in *OTUB1*-knockout compared with WT cells, the increase in mRNA levels for this isoform did show an increase in UBE2D protein levels. Although this suggests the cells are trying to compensate the decrease in UBE2D by increasing mRNA levels for some of the UBE2D isoforms (i.e., UBE2D1 and UBE2D4), increase at the protein level was not observed. One possible explanation is that the most abundant UBE2D isoform is UBE2D3[88], and for that reason, changes in the mRNA for UBE2D4 are probably not going to have a major effect. The results suggest that the changes of UBE2D and UBE1E1 amounts in response to OTUB1 depletion are not regulated through transcription. Rather, these observations suggest that UBE2D levels could be regulated at the translational level.

In OTUB1, mutations to make the T134R and E28AD35A protein variants have identified those residues as important for the interaction between OTUB1 and E2s[82,83]. Expression of FLAG-OTUB1^{T134R} or FLAG-OTUB^{1E28AD35A} in *OTUB1* knockout U2OS cells restored the total levels of UBE2D and UBE2E1 to wild-type levels (Figure 3.9a, b). Although for UBE2E1, the OTUB1 residue T134 was necessary for the interaction to prevent auto-ubiquitination of UBE2E1 and its degradation by the proteasome, we observed a complete rescue when FLAG-OTUB1^{T134R} was expressed (Figure 3.9b). The

difference in our observed results from Pasupala *et al* (2018) could be explained by our use of *OTUB1*-knockout U2OS cells instead of OTUB1 knockdown. The generation of stable knockout cell lines could allow the cells to adapt and, for that reason, the UBE2E1 levels are different when compared with the transiently-transfected cells after siRNA-mediated OTUB1 knockdown. To check the role of OTUB1-UBE2E1 interaction over UBE2E1 protein levels, it will be necessary to transiently transfect *OTUB1*-knockout U2OS cells with OTUB1^{T134R}.

In addition to the decrease in total E2s levels, a decrease in the fraction of Ub-thioesterified forms of the E2s was observed for UBE2E1 and UBE2D in *OTUB1*-knockout U2OS cells compared with WT cells. However, these decreases in the fractions of thioesterified UBE2E1 and UBE2D were not detected in *Otub1*$^{-/-}$ null MEF cells compared with WT MEF cells. Although differences in the fractions of thioesterified E2s between cells lines had been previously reported[82], the reasons for this observation have not been explored. With UBE2E1 and UBE2D, regulation of the thioesterified forms may depend on not only OTUB1. Evaluation of the rescue by the two OTUB1 mutants (T134R and E28AD35A) on the amount of Ub~UBE2E1 showed that interaction with OTUB1 through T134 is required (Figure 3.9b). This is consistent with the predicted steric clashes on the OTUB1-E2 interaction generated by the T134R mutation, compromising OTUB1 ability to interfere with access to the Ub-thioesterified E2 [83]. Although it is reasonable that binding by OTUB1 could stabilize ubiquitin-thioesterified E2s, further studies will be required to establish that and to determine the differences in binding affinities between the OTUB1 variants and Ub~E2s. Furthermore, evaluation of the catalytic mutant OTUB1^{C91S} will be necessary to confirm the effect over stabilization

of Ub~E2s levels because the OTUB1^{T134R} and OTUB1^{E28AD35A} still are catalytically active. The *OTUB1*-knockout U2OS cells rescue with OTUB1^{C91S} will show if stabilization of Ub~E2s is independent of OTUB1 catalytic activity.

Previous analysis of UBE2D and UBE2N in U2OS cell lysates under non-reducing conditions showed a low fraction of thioesterified E2s[82]. Nevertheless, our immunoblot analyses of these E2s, UBE2D and UBE2N, in U2OS cells showed that more around 50% are thioesterified (Figure 3.3 and 3.4). The difference in these observations could be due to experimental conditions use for gel electrophoresis. We used a Bis-Tris (pH = 6.7) acrylamide gel buffer and Tris-MOPS-SDS electrophoresis running buffer (pH = 7.7) that are less basic than the buffers used with conventional SDS-PAGE gels. The more neutral pH conditions relative to previous studies can help to avoid hydrolysis of the protein thioesters.

In conclusion, we have shown that OTUB1 is not necessary to regulate free Ub homeostasis after proteotoxic stress. Nevertheless, OTUB1 is involved in the regulation of a subset of E2 and Ub~E2s levels. We found that UBE2D levels are regulated post-transcriptionally by OTUB1 using a mechanism that is independent of proteasome degradation and that probably involves translation regulation. Although additional experiments to evaluate the translation rates for UBE2D in *OTUB1*-knockout and WT U2OS cells will be necessary to test this hypothesis, a recent study showed that UBE2D interacts differently with catalytically-inactive OTUB1 *in vitro* than does UBE2E1[93]. OTUB1^{C91S} prevents UBE2E1 auto-ubiquitination, but under the same parameters UBE2D is unaffected[93]. This supports our hypothesis about a new mechanism of regulation for E2 total levels by OTUB1 through a post-transcriptional mechanism.

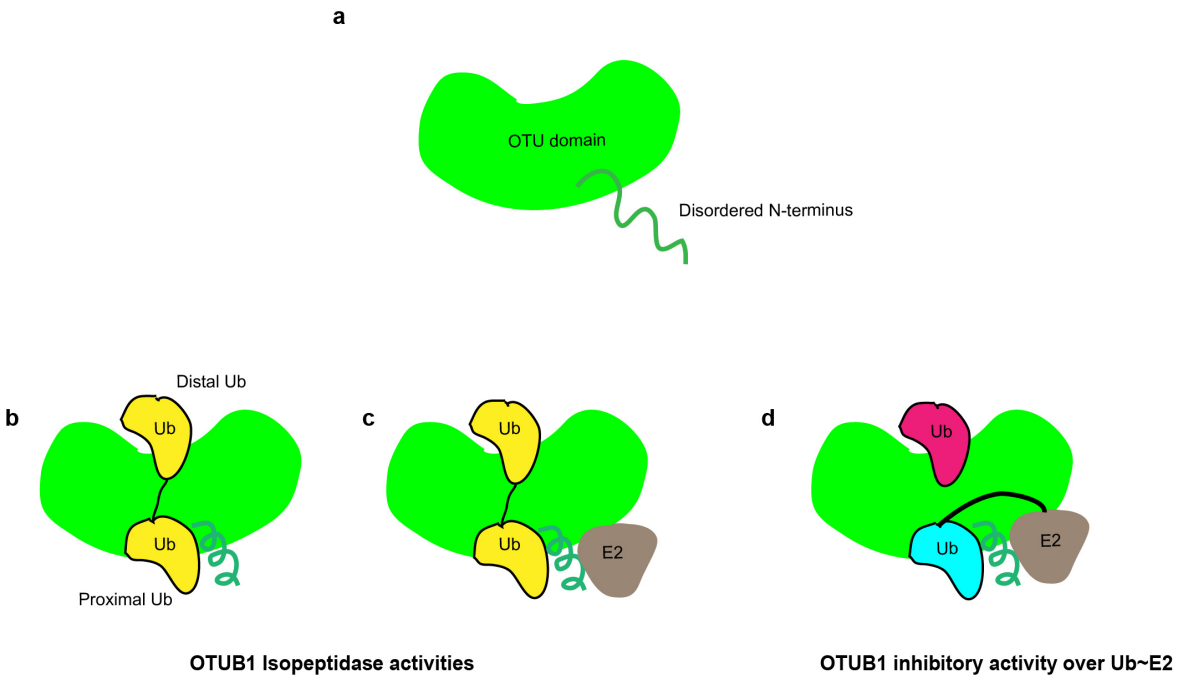


Figure 3.1. Diagrams showing OTUB1 structure and effects in activity after interactions with E2s. a) Schematic showing the OTUB1 domains in the apoenzyme. b) Schematics showing when OTUB1 has active isopeptidase activity and (c) how interaction with an E2 can increase the isopeptidase activity. d) Schematic of OTUB1 showing how interaction with Ub~E2 and free Ub can interfere with the isopeptidase activity of the enzyme and block the transfer of the donor Ub to substrates. Figure adapted from Lauren *et al* (2020).

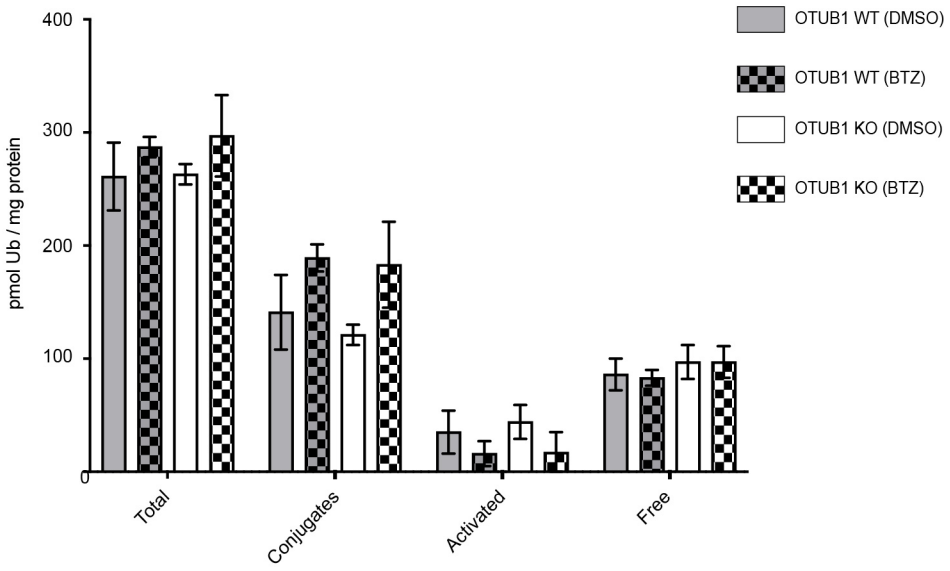
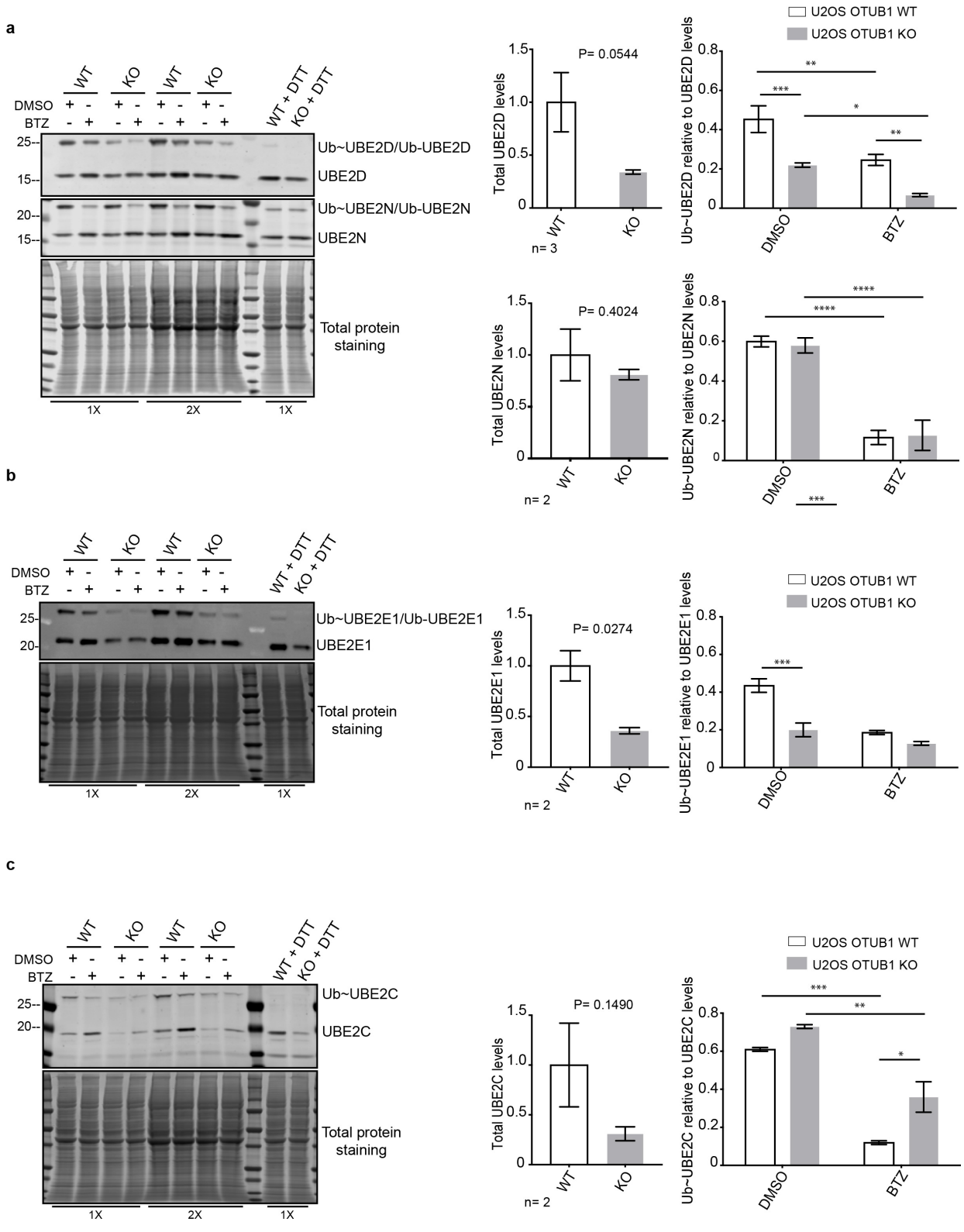


Figure. 3.2. *OTUB1*-knockout does not affect free Ub levels in U2OS after proteasome inhibition. Quantification of Ub pools in *OTUB1*-Knockout and WT U2OS cells treated with either DMSO (control) or the proteasome inhibitor (BTZ; 1uM) for 1 hour. Error bars, ± s.d. (*OTUB1* WT (DMSO) n=3; *OTUB1* WT (BTZ) n=3; *OTUB1* KO (DMSO) n=3; *OTUB1* KO (BTZ) n=2). Values are in picomoles ubiquitin per milligram total protein. Statistical analysis was performed by one-way analysis of variance using Tukey's multiple comparisons.


a
WT
KO
WT
KO
WT + DTT
KO + DTT
DMSO
BTZ
25--
15--
20--
15--
Ub~UBE2D/Ub-UBE2D
UBE2D
Ub~UBE2N/Ub-UBE2N
UBE2N
Total protein staining
1X
2X
1X
Total UBE2D levels
P= 0.0544
n= 3
WT
KO
U2OS OTUB1 WT
U2OS OTUB1 KO
Ub~UBE2D relative to UBE2D levels
DMSO
BTZ
Total UBE2N levels
P= 0.4024
n= 2
Ub~UBE2N relative to UBE2N levels
b
Ub~UBE2E1/Ub-UBE2E1
UBE2E1
Total protein staining
Total UBE2E1 levels
P= 0.0274
n= 2
Ub~UBE2E1 relative to UBE2E1 levels
c
Ub~UBE2C
UBE2C
Total protein staining
Total UBE2C levels
P= 0.1490
n= 2
Ub~UBE2C relative to UBE2C levels

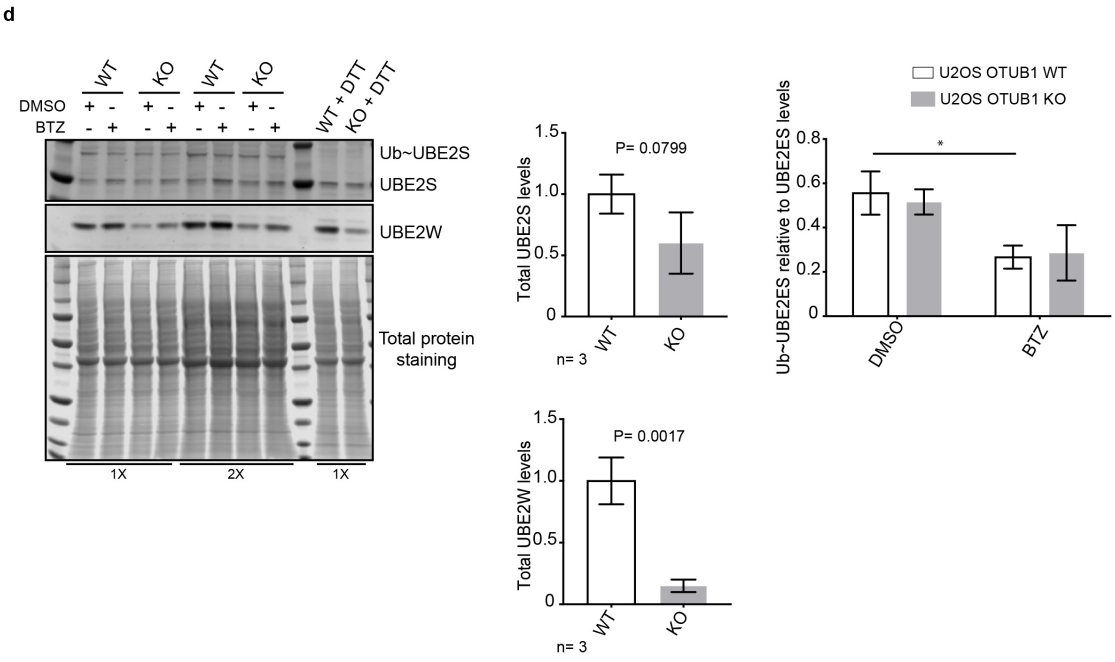


Figure 3.3. *OTUB1*-knockout affects the levels of some E2s and Ub~E2s in U2OS cells. Images of representative western blot analysis with quantification of total levels of different E2s and fraction of Ub~E2 relative to the total E2 levels in whole cell lysates. a) UBE2D and UBE2N. b) UBE2E1. c) UBE2C. d) UBE2W and UBE2S. Lysates were evaluated under non-reducing conditions to be able to detect Ub~E2. DTT (200 mM) was added to check for the presence of thioesterified form of the E2s. The thioester linkage was denoted as "~". Total protein staining was used as loading control. The statistical analysis used to compare the total levels of E2s was unpaired two-tailed t-tests. To compare the fraction of thioesterified E2s one-way analysis of variance followed by Tukey's multiple comparisons was used. * indicates P ≤ 0.05; ** indicates P ≤ 0.01; *** indicates P ≤ 0.001; **** indicates P ≤ 0.0001.

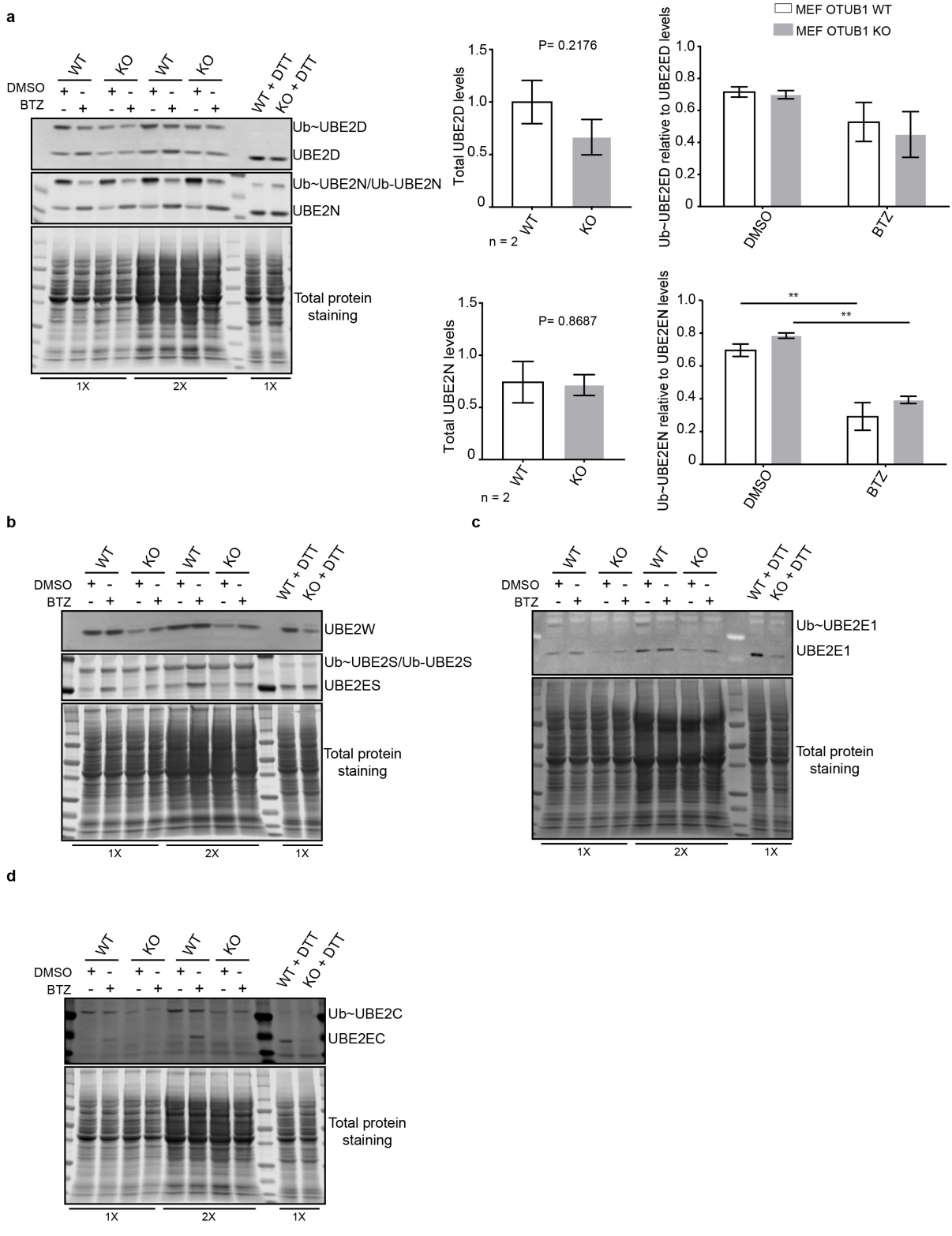

a
WT
KO
WT
KO
WT + DTT
KO + DTT
DMSO
BTZ
Ub~UBE2D
UBE2D
Ub~UBE2N/Ub-UBE2N
UBE2N
Total protein staining
1X
2X
1X
P= 0.2176
Total UBE2D levels
n = 2
P= 0.8687
Total UBE2N levels
MEF OTUB1 WT
MEF OTUB1 KO
Ub~UBE2ED relative to UBE2ED levels
Ub~UBE2EN relative to UBE2EN levels
DMSO
BTZ
**
**
b
UBE2W
Ub~UBE2S/Ub-UBE2S
UBE2ES
c
Ub~UBE2E1
UBE2E1
d
Ub~UBE2C
UBE2EC

Figure 3.4. *Otub1*$^{-/-}$ null affect the levels of some E2s and Ub~E2s in MEF cells. Images of representative western blot analysis with quantification of total levels of different E2s and fraction of Ub~E2 relative to the total E2 levels. a) UBE2D and UBE2N. b) UBE2W and UBE2s. c) UBE2E1. d) UBE2C. Lysates were evaluated under non-reducing conditions to be able to detect Ub~E2. DTT (200 mM) was added to check for the presence of thioesterified form of the E2s. The thioester linkage was denoted as "~". Total protein staining was used as loading control. The statistical analysis used to compare the total levels of E2s was unpaired two-tailed t-tests. To compare the fraction of thioesterified E2s one-way analysis of variance followed by Tukey's multiple comparisons was used. ** indicates $P \leq 0.01$.

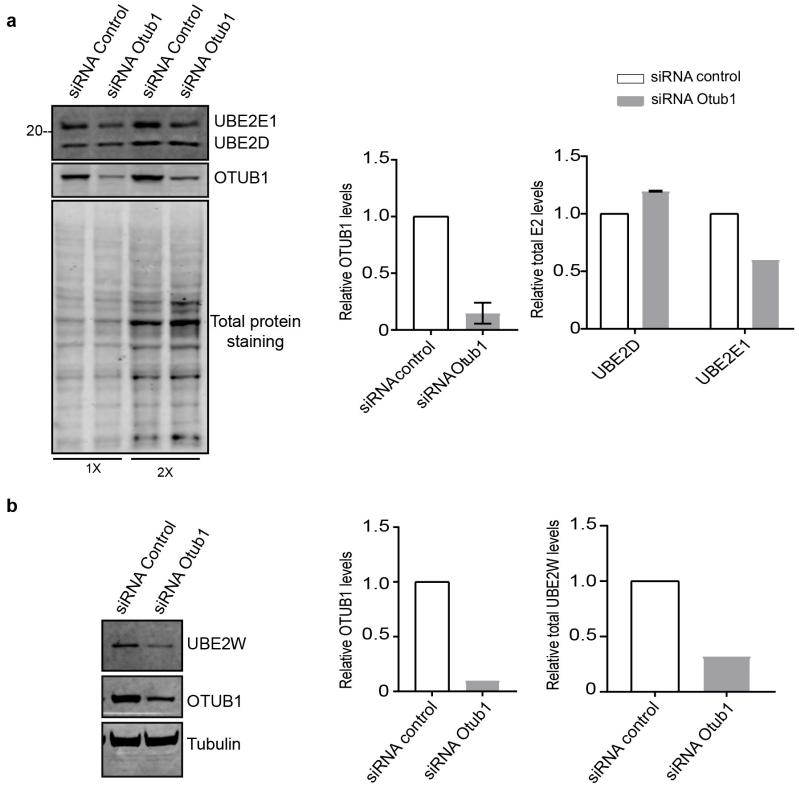


Figure 3.5. Effects of OTUB1 knockdown over E2s levels suggest that there is one more mechanism of E2s regulation. Western blot images and quantification for E2s in whole cell lysates after 48 hours knockdown using siRNA control or siRNA Otub1. a) UBE2D and UBE2E1. b) UBE2W. Values for OTUB1 and the E2s were normalized to 1.

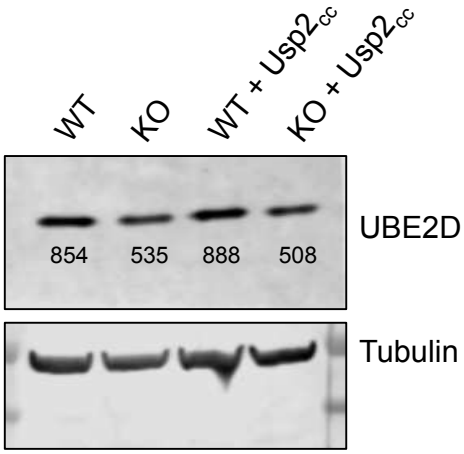


Figure 3.6. *OTUB1*-Knockout does not involved accumulation of ubiquitinated forms of UBE2D. Immunoblot detection of UBE2D after incubation of U2OS cell lysates with and without $Usp2_{cc}$. Tubulin was used as loading control. Quantification of the UBE2D band intensity is showed below the bands.

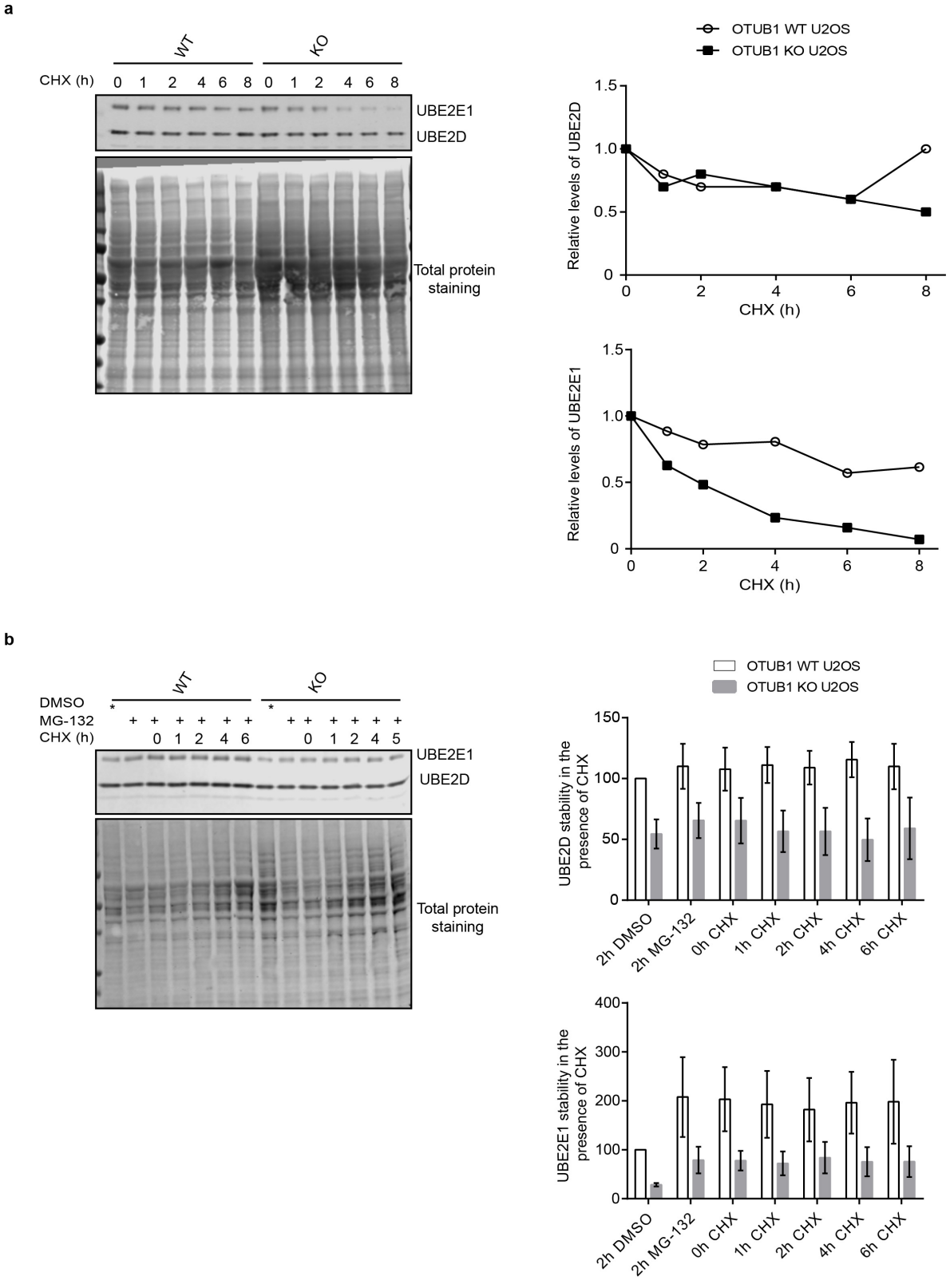

a
WT
KO
CHX (h)
0 1 2 4 6 8 0 1 2 4 6 8
UBE2E1
UBE2D
Total protein staining
OTUB1 WT U2OS
OTUB1 KO U2OS
Relative levels of UBE2D
CHX (h)
Relative levels of UBE2E1
CHX (h)
b
WT
KO
DMSO
MG-132
CHX (h)
0 1 2 4 6 0 1 2 4 5
UBE2E1
UBE2D
Total protein staining
OTUB1 WT U2OS
OTUB1 KO U2OS
UBE2D stability in the presence of CHX
UBE2E1 stability in the presence of CHX
2h DMSO
2h MG-132
0h CHX
1h CHX
2h CHX
4h CHX
6h CHX

Figure 3.7. Regulation of UBE2D by OTUB1 does not involve proteasomal degradation. a) Western blot images after incubation with cycloheximide (CHX; 50 ug/mL) to evaluate UBE2D and EBE2E1 half-life at different time points in of *OTUB1*-knockout and WT U2OS cells. UBE2D and UBE2E1 levels were normalized against total protein staining and plotted on the right side. b) Representative western blot image (n=2) of UBE2D and UBE2E1 levels in U2OS cells incubated with 10 uM MG-132 prior to incubation with CHX for the indicated time. Values in *OTUB1* WT samples incubated with DMSO were normalized to 100.

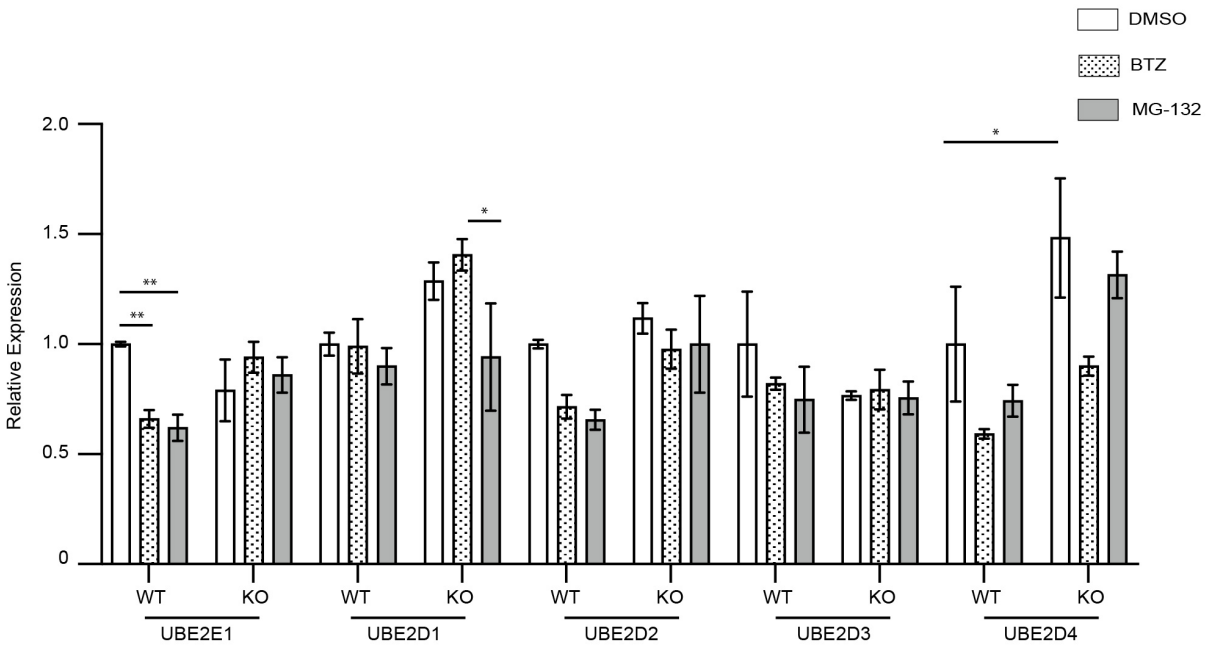


Figure 3.8. The decrease in UBE2D protein levels is not regulated at transcriptional level. qRT-PCR analysis of UBE2E1 ad UBE2D isoforms in U2OS *OTUB1*-knockout and WT U2OS cells. The statistical analysis was performed using one-way analysis of variance followed followed by multiple comparisons. * indicates $P \leq 0.05$; ** indicates $P \leq 0.01$.

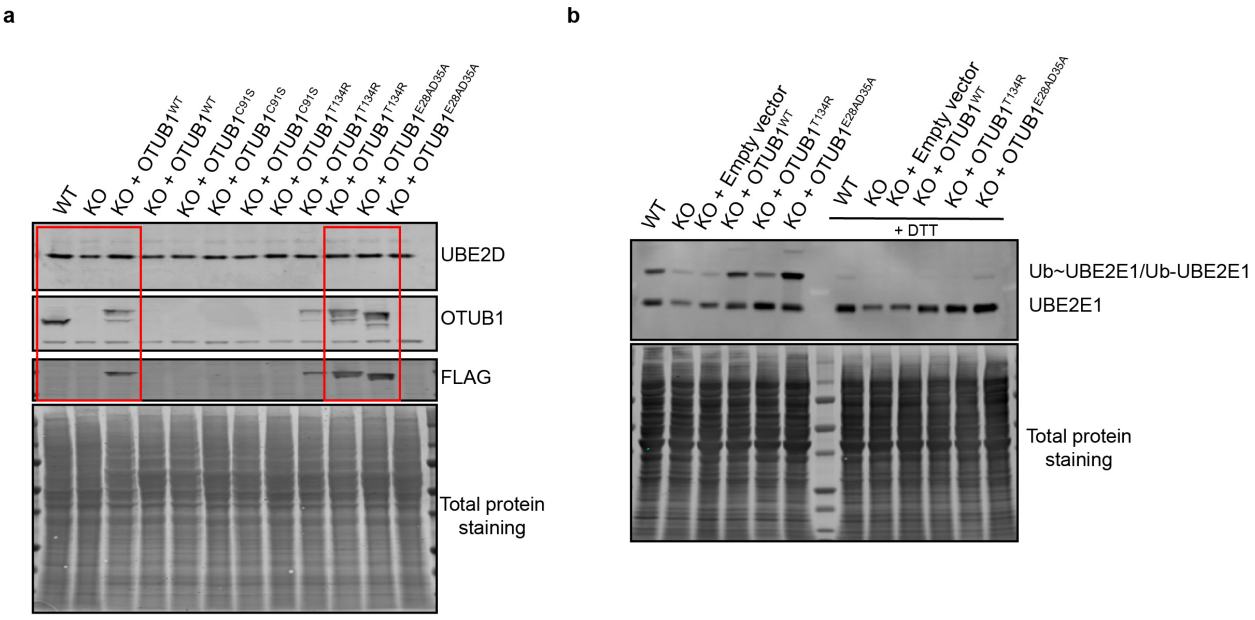


Figure 3.9. Interaction with OTUB1 is required to rescue the fraction of Ub~UBE2E1 but not to rescue the total levels of UBE2D and UBE2E1 in U2OS cells. a) Western blot image for the selection of monoclonal cells expressing OTUB1 constructs. Inside the red box are showed the cells selected for later analysis. b) Western blot image of evaluation of the Ub~UBE2E1 and UBE2E1 levels after rescue with the different constructs. Total protein staining was used as loading control.

Table 3.1. E2 enzymes evaluated in this study. The E2 enzymes were selected based on reports of interactions with OTUB1. The E2 effect over the OTUB1 isopeptidase activity was tested through *in vitro* cleavage of a fluorescent K48 diUb ((+)= strongly stimulated cleavage; (+/-)= mildly stimulated cleavage)[82]. The detection of interaction partners was performed using mass spectrometry in HEK293 cells[81,84]. Decrease levels after OTUB1 knockout were detected in U2OS and MEF cells[87].

E2s evaluated	Increase OTUB1 isopeptidase activity *in vitro*[82]	Interact with OTUB1[81,84]	Interact with *OTUB1 in vivo*[81,85-87]	Decrease levels after *OTUB1* knockout[87]
UBE2D	+	✓	✓	✓
UBE2N	+/−	✓	✓	
UBE2W	+			
UBE2E1	+	✓	✓	✓
UBE2C				✓
UBE2S				✓

CHAPTER 4: DISCUSSION AND PERSPECTIVES

4.1 Detection of free ubiquitin to evaluate the effect of different environmental conditions in single cells

Inside cells, a variety of things can perturb Ub homeostasis. Perhaps best documented are the effects of exposure to different stressors such as drugs that affect the Ub–proteasome pathway (e.g., proteasome and E1 inhibitors), and heat and oxidative stress[14,21,61]. Also, different animal and cellular models for neurological disorders like amyotrophic lateral sclerosis[96], spinal muscular atrophy, Huntington's disease and motor end plate diseases have been clearly shown to have phenotypes associated with disruption of Ub homeostasis[97,98].

An example of Ub homeostasis perturbation is the loss of Usp14 that results in motor and neurological defects in ataxia mice (ax^J)[38]. Using immunoblotting a 40% decrease in free Ub levels in all tissues of these mice compared with WT mice was observed, the most drastic decrease in free Ub levels was at the synaptic terminals, with a 60% decrease in free Ub levels[40]. The transgenic expression of Ub in ax^J mice had shown a rescue in free Ub levels as well as defects in motor endplates and muscles[40]. These experiments supported the role of Usp14 in Ub recycling and synaptic function[39,40]. Using the free Ub staining described in this work in neurons Usp14 knockout can be used to visualize changes in free Ub distribution and localization compared with WT neurons. We predict these experiments will give important information about the availability of free Ub required to participate in the recycling and degradation of synaptic proteins.

The staining procedure using HA-tUI described in this work offers a sensitive and specific way to detect free Ub in fixed cells. We anticipate that the use of this method with additional cell probes (e.g., specific antibodies) will be useful to understand more about the mechanisms regulating Ub homeostasis in different cells models. Free Ub detection with tUI will provide information not only about localization, but also about changes in free Ub generated under specific cellular conditions.

4.2 OTUB1 is a regulator of ubiquitin availability and E2 activities

E2 enzymes play a central role in the enzymatic cascade for Ub conjugation due to their ability to interact with the Ub-thioesterified E1 enzyme, and with at least one of the several hundred E3 Ub-protein ligases, or directly with ubiquitination protein substrates[99]. This makes the E2s important targets for regulation of the Ub pathway. Different regulatory mechanisms affecting E2 activity and levels have been described. For example, E2s activity can be regulated through non-covalent interactions with other molecules such as Ub and E3s. Most of the times those interactions take place through the backside surface, opposite face from the active site, of the E2 and can enhance or repress the E2 catalytic activity[99]. Additional ways to regulate E2s are transcription[100], translation[101] and post-translational modifications such as ubiquitination[87,102-104] and phosphorylation[99] (table 4.1).

OTUB1 has been described to have regulatory interactions with specific E2s in which that in some cases can increase OTUB1 catalytic activity or, in interactions with Ub-thioesterified E2s in the presence of free Ub can inhibit the transfer of Ub needed to form conjugates or polyUb chains[80,82,83]. In addition, it has been reported that specific

interaction of OTUB1 with UBE2E1 prevents the E2 auto-ubiquitination and degradation by the proteasome[87]. Here we showed that OTUB1 could be regulating the levels of UBE2D using a new mechanism, independent of proteasomal degradation and possibly involving regulation of translation. Further evaluation of the rates of translation for UBE2D in *OTUB1*-knockout and WT U2OS cells will be required to test this hypothesis. One approach that we started exploring was the detection of newly synthesized UBE2D using the non-canonical amino acid azidohomoalanine [74,105]. Using click chemistry the proteins that incorporate AHA should be detected through biotin immunoblotting after UBE2D immunoprecipitation.

In addition, initial results discussed in Chapter 3 showed that interaction of OTUB1 with Ub~E2 could be involved in stabilization of the thioesterified E2s. This is consistent with structural information about the interaction between OTUB1 and Ub~E2, where it has been shown that the interaction blocks the access to the thioester bond[82,83]. The rescue assays performed in this study with OTUB1^{T134R} showed that this residue is required for the stabilization of Ub~UBE2E1 levels. However, structural studies have shown that OTUB1 interacts through different contact points with the donor Ub and the E2[83]. Future experiments will have to evaluate the relevance of those interactions in rescue experiments to understand the role of OTUB1 in the stabilization of Ub~E2s. In addition, binding studies will be required to see how different mutations affect the affinity between OTUB1 and Ub~E2s. Furthermore, *in vivo* studies expressing E2s with mutations in the residues involved in OTUB1 interactions will be necessary to understand why OTUB1 only affects the stability of Ub~UBE2D and Ub~UBE2E1 in U2OS cells.

4.3 Factors involved in the regulation of UBE2D activity

UBE2D is an abundant, promiscuous E2 that participates in the formation of a variety of Ub linkages *in vitro* and *in vivo*[106]. Different properties of UBE2D appear to contribute to its ability to form a variety of Ub–Ub chain linkages. First, compared with other E2s, structural studies have shown that Ub covalently attached to UBE2D is relatively exposed and able to interact with Ub binding proteins[106]. Second, UBE2D also interacts non-covalently with Ub[99,106]. Putting together these two features, it has been hypothesized that the formation of a variety of linkages is facilitated by the increase local concentration of Ub~UBE2D interacting with free Ub or Ub attached to UBE2D[99,106].

OTUB1 has been reported to act as an additional factor in ubiquitination reactions mediated by UBE2D. Interaction of OTUB1 with UBE2D has been shown to inhibit p53[85] and SMAD2/3[86] ubiquitination. The results described in chapter 3 showed that OTUB1 affects the total levels of UBE2D and the stability of Ub~UBE2D, introducing a new way of regulation of UBE2D activity. This opens questions about the possible effects that OTUB1 could have over the substrates that are ubiquitinated by UBE2D. Further studies will be required to shed light on the effect of OTUB1 over UBE2D activity *in vivo*.

4.4 UBE2D as an example of regulation by OTUB1

Different structural studies have shown how the catalytic activity of OTUB1 can be stimulated by interaction with E2[82,93]. At the same time, this interaction increases the affinity for K48-linked diUb *in vitro*, lowering its Km from 102.5 uM to 6.6 uM[82,93]. Based

on the estimated intracellular concentration of K48-linked polyUb chains (~10 μM[21,107]), it has been hypothesized that OTUB1 interacts with endogenous intracellular E2s to increase the affinity for K48-linked polyUb substrates[93].

Binding and thermodynamic measurements have shown that UBED3 is a strong candidate to affect the activity of OTUB1 inside the cells due to its high intracellular concentration (1.7 μM in MEF cells)[88], which is similar to the effective concentration of UBE2D3 needed to stimulate OTUB1 catalytic activity (EC_{50} = 1.6 μM)[93]. Although the effects of UBE2D on OTUB1 catalytic activity *in vivo* were not explored in our study, the effect of OTUB1 on UBE2D protein levels could have implications for regulation of its catalytic activity.

Table 4.1. Examples of E2 enzymes where abundance are known to be subject to regulation.
In this table the E2s included are human, otherwise it is indicated.

Type of regulation	E2
Transcriptional	UBE2L3[100]
Post-transcriptional	BIRC6[101]
Post-translational modification and regulated degradation	UBE2E1[87] UBE2C[104] Ubc7 (yeast)[103]

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