RESEARCH ARTICLE

The role of ubiquitin-conjugating enzyme Ube2j1 phosphorylation and its degradation by proteasome during endoplasmic stress recovery

Muthukumar Elangovan¹ · Hae Kwan Chong¹ · Jin Hee Park¹ · Eui Ju Yeo¹ · Yung Joon Yoo¹

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Abstract The human Ube2j1 and Ube2j2 are the only ubiquitin-conjugating enzymes (E2s) that are localized to endoplasmic reticulum (ER) through its C-terminal transmembrane domains. Ube2j1 is a known substrate of MAPK signalling pathway and it is phosphorylated at serine-184 during ER stress. Here, we demonstrate that Ube2j1, not Ube2j2 is essential for the recovery of cells from transient ER stress. The ectopic expression of wild-type Ube2j1 and phospho-mimic mutant, Ube2j1^{S184D} but not phospho-mutant Ube2j1^{S184A} can recover cells from ER stress. We also found that ubiquitin-ligase (E3), c-IAP1 preferentially interacts with phosphorylated Ube2j1. Moreover, we noticed that phosphorylated Ube2j1 is rapidly degraded by the proteasome during ER stress cell recovery. Taken together, these data suggest that Ube2j1 and its phosphorylation is important for transient ER stress cell recovery and the phosphorylated Ube2j1 is degraded by the proteasome.

Keywords ER stress · MAPK signaling · Ube2j1 · Ube2j2 · Ubiquitin-conjugating enzyme · Ubiquitin-proteasome system

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✉ Muthukumar Elangovan
muthu@gist.ac.kr

✉ Yung Joon Yoo
yjyoo@gist.ac.kr

¹ School of Life Sciences, Gwangju Institute of Science & Technology (GIST), 123 Cheomdangwagi-ro, Buk-gu, Gwangju 61005, Republic of Korea

Abbreviations

CHX	Cycloheximide
ER stress	Endoplasmic reticulum stress
ERAD	Endoplasmic reticulum-associated degradation
MAPK	Mitogen-activated protein kinase

Introduction

The modification of target proteins with ubiquitin and degradation by the proteasome is achieved by an enzymatic cascade, involving an E1 (ubiquitin-activating) enzyme, an E2 (ubiquitin-conjugating) enzyme, and an E3 (ubiquitin ligase) enzyme (Glickman and Ciechanover 2002; Pickart 2004). First, E1 catalyzes the formation of a thioester bond between its active site cysteine and the C-terminus of ubiquitin in an ATP-dependent manner. E2s catalyzes the transfer of ubiquitin from E1 to the active site cysteine of the E2. The final step in the ubiquitination is catalyzed by E3 which determines the substrate selectivity, can act directly (HECT E3s) or indirectly (RING E3s) through the transfer of ubiquitin from E2 to the target protein (Glickman and Ciechanover 2002; Pickart 2004).

Ubiquitin-mediated protein degradation is involved in many cellular processes, including the degradation of unfolded or misfolded proteins in ER through the pathway called endoplasmic reticulum-associated protein degradation (ERAD) (Bonifacino and Weissman 1998; Meusser et al. 2005). To be degraded in proteasomes, first ERAD substrates must be retro-translocated to the cytosol with the involvement of chaperones and components of the protein translocon machinery (Pilon and Schekman 1997; Plemper et al. 1997). Ubiquitination enzymes that are localized at the cytosolic face of the ER membrane then target ERAD substrate for

proteasomal degradation (Biederer et al. 1997; Hampton et al. 1996). There are only three E2s: Ube2j1, Ube2j2, and Ube2G2 that are implicated in the degradation of ERAD substrates (Christianson and Ye 2014). Ube2j1 and Ube2j2 are the only ER resident E2s which has a short luminal region, a single transmembrane domain and catalytic domain that is oriented toward the cytosol. Ube2G2 lacks both luminal and transmembrane domain and it functions in ERAD from the cytosol (Christianson and Ye 2014).

Ube2j1 and Ube2j2 are the two mammalian homologs of yeast Ubc6 (Lester et al. 2000). Ube2j1 is less conserved than Ube2j2 and possess a long divergent C-terminal segment (Oh et al. 2006). The expression of Ube2j1 and Ube2j2 are increased during ER stress (Oh et al. 2006). It was demonstrated that Ube2j1 is the E2 for the degradation of ERAD substrates such as α -TCR (α -T-cell receptor), TRAF2 (TNF-receptor-associated factor 2) and MHC I (Wu et al. 2005; Burr et al. 2011; Menon et al. 2013). Ube2j1 has been shown to associate with different E3s including Derlin-1, RMA1 and c-IAP1 (Wu et al. 2005; Younger et al. 2006). Apart from the role of Ube2j1 as one of the E2s for ERAD substrates, the post-translational modification of Ube2j1 has also been demonstrated (Oh et al. 2006; Menon et al. 2013). ER stress induces the phosphorylation of Ube2j1, and not Ube2j2 at serine 184 residue by p38 MAPK signalling pathway (Menon et al. 2013). But, the function of the phosphorylated Ube2j1 is not clear. Phosphorylation of Ube2j1 did not affect the ubiquitination of ERAD substrate, α -TCR (Menon et al. 2013). In this study, we addressed the functional role of Ube2j1 phosphorylation during ER stress recovery and found that the phosphorylation of Ube2j1 is important for ER stress recovery and the degradation of phosphorylated Ube2j1 by proteasome during ER stress recovery.

Materials and methods

Cell culture, transfection and reagents

HeLa cells were cultured in modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY, USA) and maintained as described previously (Elangovan et al. 2010). siRNA experiments were performed using commercial siRNA against Ube2j1 (Cat No. 10620318 and 10,620,319, Stealth™ siRNA, Invitrogen) and Ube2j2 (ON-Targetplus Human Ube2J2 (118424) siRNA-SMARTpool, Thermo Scientific). As a control, non-targeting siRNA (Invitrogen, Carlsbad, CA, USA) was used. siRNA transfection was performed using Lipofectamine™ RNAimax and plasmids were transfected using Lipofectamine 2000™ Reagent. Briefly, 3.5×10^5 cells were plated in 6-well plates, cultured for 24 h

and transfected with 50 nM of indicated siRNAs or 2 μ g of indicated plasmids in figure captions. Cells were collected by trypsin treatment at indicated time-points for lysis as described previously (Elangovan et al. 2010). Tunicamycin (Sigma Aldrich, St. Louis, Missouri, United States) treatment was done as previously described (Oh et al. 2006).

Plasmids and cloning

The human Ube2j1 coding region was PCR-amplified from total HeLa cDNA and cloned into the pEGFP-C1 (Invitrogen) using standard cloning protocol. For His-tag Ube2j1, full-length Ube2j1 was cloned into pEF1/V5-His (kindly provided by Dr. Chang-duk Jun, Gwangju Institute of Science and Technology, South Korea). The GFP-tagged Ube2j1 mutants S184A and S184D were generated by standard protocol using QuikChange site-directed mutagenesis (Stratagene). All the PCR-generated and coding segments used in this study were verified by DNA sequencing.

Electrophoresis and immunoblotting

The harvested cells were washed twice with PBS and resuspended in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 20 mM epoxomicin, and 10 mM IAA) containing 1X protease inhibitor cocktail (Roche Biochemical, Indianapolis, IN, USA) and briefly sonicated. The soluble proteins were recovered after centrifugation at 10,000 g at 4 °C for 20 min and the protein concentration was determined by the Bradford method. Proteins (30 μ g) were separated by SDS-PAGE in 4–12% precast gels (KOMA, Seoul, South Korea) and Immunoblots were performed as described previously (Elangovan et al. 2010). The membranes were blocked with 5% non-fat milk in TBS, and incubated with primary antibodies anti-Ube2j1 antibody, anti-ube2j2 antibody, anti- β -actin antibody, anti-Grp78 antibody, anti-GFP antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-cleaved caspase-3 and anti-cleaved PARP antibody (Cell Signalling Technology, Beverly, MA, USA). The immunoreactive proteins were visualized using ECL reagent (GE Healthcare/Amersham Bioscience, Piscataway, NJ, USA).

Immunoprecipitation and his-pull down assay

Cells were resuspended in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10 mM IAA, 20 mM epoxomicin, 0.1% Triton X-100) containing 1X protease inhibitor cocktail and briefly sonicated. The soluble proteins were recovered after centrifugation at 10,000 g at 4 °C for 20 min and 500 μ g protein lysate in 500 μ L lysis buffer was precleared with protein A/G agarose beads (Santa Cruz

Biotechnology Inc.), for 30 min at 4 °C and cleared lysates were subjected to immunoprecipitation with indicated antibody at 4 °C. After 4 h, protein A/G agarose beads were added for another 1 h at 4 °C. Beads were washed thrice with ice-cold lysis buffer and bound proteins were eluted with sample buffer. His-pull down assay was performed by using Ni-NTA resin (Qiagen, Valencia, CA).

RNA isolation and RT-PCR

Total RNA was isolated from harvested cells using Trizol (Molecular Research Centre Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Reverse transcription of the RNA was performed as described previously [16]. The diluted RT-generated DNA (2 µl) was used for the amplification of target genes using the following primers: Ube2j1 5'-GCGATTCAAGCCAAGCTGAC-3' and 5'-TCTG GAGTCCGTACGATGT-3'; Ube2j2 5'-GAAA GACCCGGTGCCCTTACA-3' and 5'-CACA AACAAGTTCGCCAGGG-3'; Hsp70 5'-CAAG ATCACCATACCAACG-3' and 5'-GCTCAAACCTCGTCC TTCTCG-3'; Grp78 5'-TAGCGTATGGTGCTGCTGTC-3' and 5'-TTTGTCAAGGGTCTTTCACC-3'; Actin 5'-TGTC GCATCCACGAACTAC-3' and 5'-GGAGCAATGATCTT GATCTTCA-3'.

Cell proliferation and death assay

The relative cell proliferation was determined by MTT assay as described previously (Oh et al. 2013). Cell death assay was performed by collecting both floating cells and the adherent cells by dissociating from the plate by trypsin treatment. Both floating and adherent cells were mixed together and incubated with 0.02% solution of Trypan blue. The percentage of dead cells were calculated by counting (under the microscope) the number of stained cells by the number of unstained cells. The counting was duplicated to get an average and the expressed result is from two independent experiments.

Results

Ube2j1, and not Ube2j2 is essential for cells to recover from ER stress

Previous studies have shown that the increase in the expression level of Ube2j1 and its phosphorylation during ER stress but its role and the fate of phosphorylated Ube2j1 during transient ER stress recovery were not well-studied. First, we checked the expression level of Ube2j1 and its homolog, Ube2j2 in HeLa cells during ER stress by tunicamycin. The expression level of both Ube2j1 and Ube2j2 were analyzed by RT-PCR using specific primers and are increased time-

dependently (Fig. 1a). The expression level of ER stress markers, Grp78 was increased at 4 h. But, the expression level of the cytosolic marker, Hsp70 was not affected by the treatment of tunicamycin (Fig. 1a). We further validated the induced expression level and phosphorylation of Ube2j1 and Ube2j2 by immunoblots in tunicamycin-treated HeLa cells. The phosphorylated Ube2j1 was confirmed by the treatment of alkaline phosphatase (AP) (Fig. 1b). Consistent with RT-PCR result, the amount of Ube2j1 was 2.1 fold increased whereas the amount of Ube2j2 was increased by 2.2 fold after treatment with tunicamycin (Fig. 1b). Ube2j2 was not phosphorylated by the treatment of tunicamycin.

We next investigated whether Ube2j1 and Ube2j2 are essential for the cells during ER stress recovery. For this purpose, HeLa cells were transfected with Ube2j1 and Ube2j2 siRNA followed by the treatment of tunicamycin and cells were harvested at every 12 h. Inhibition of both Ube2j1 and Ube2j2 expression levels by their specific siRNAs was observed by immunoblots with anti-Ube2j1 and anti-Ube2j2 antibodies whereas scrambled control siRNA did not suppress the expression level of Ube2j1 and Ube2j2 (Fig. 1d, f). Treatment of tunicamycin for 6 h in control siRNA transfected cells induced the phosphorylation of Ube2j1 and this time-point was considered as 0 h. The level of phosphorylation of Ube2j1 was reduced at 12 h and completely abrogated from 24 h after the withdrawal of tunicamycin (Fig. 1d). As observed by immunoblot with anti-Grp78 antibodies, the expression level of ER stress marker, Grp78 was increased at 0 h and the expression level was reduced after the withdrawal of tunicamycin from 24 h in control siRNA transfected cells. By contrast, the expression level of Grp78 continued to increase after the withdrawal of tunicamycin in Ube2j1 but not Ube2j2 siRNA-transfected cells (Fig. 1d, f). When the percentage of dead cells were calculated by trypan blue staining, about $37.7 \pm 1.5\%$ dead cells were observed at 48 h after tunicamycin withdrawal in Ube2j1 siRNA transfected cells whereas only $10 \pm 2.2\%$ of dead cells were observed in Ube2j2 siRNA transfected cells (Fig. 1c, e). This difference in the cell death was further confirmed by the PARP cleavage in Ube2j1 but not in Ube2j2 siRNA transfected cells (Fig. 1d, f). These results suggest that Ube2j1, and not Ube2j2 is essential for the cells to recover from ER stress. The difference in cell death between Ube2j1 and Ube2j2 siRNA transfected cell could be due to the fact that only Ube2j1, and not Ube2j2 is known to be phosphorylated during ER stress.

ER stress recovery by Ube2j1 phosphorylation-mimic mutant (S184D)

Given that Ube2j1 is essential during ER stress recovery and also the fact that it is phosphorylated during ER stress, we analyzed the role of phosphorylation of Ube2j1 during ER stress recovery. For this purpose, HeLa cells were first

◀ **Fig. 1** Ube2j1 is essential for cells to recover from ER stress. **a** HeLa cells were treated with 20 $\mu\text{g/ml}$ tunicamycin and harvested at 0 h, 1 h, 2 h, and 4 h. The expressed RNA levels of Ube2j1 and Ube2j2 were increased time-dependently. Grp78 was used as positive control for ER stress was increased. Hsp70, cytoplasmic stress marker was not increased. **b** HeLa cells were treated with 20 $\mu\text{g/ml}$ of tunicamycin and harvested at indicated time points. Ube2j1 and Ube2j2 were analyzed by immunoblotting against anti-Ube2j1 and anti-Ube2j2 antibodies after SDS-PAGE. The phosphorylated Ube2j1, which was confirmed by the treatment of 10 units of alkaline phosphatase (AP) was increased with time. The total amount of Ube2j1 was increased by 2.1 fold based on the densitometric quantification after dephosphorylation with AP. The Ube2j2 was increased by 2.2 fold at 12 h. **c** HeLa cells were transfected with control siRNA or Ube2j1 siRNA. After 12 h, cells were replated in a 12-well plate and treated with tunicamycin (20 $\mu\text{g/mL}$). After 6 h, media was replaced with fresh media and this time point was considered as '0' and cells were harvested at 0, 12, 24, 36 and 48 h time point and the percentage of dead cells were calculated by trypan blue exclusion staining. The percentage of dead cells was increased to 37.71 ± 1.51 at 48 h in Ube2j1 siRNA transfected cells compared to 10.09 ± 2.21 at 48 h in control siRNA transfected cells. **d** Cells were harvested, lysed and immunoblot was performed using indicated antibodies. The expression of ER stress marker, GRP78 was increased and an apoptotic marker, cleaved PARP was observed in Ube2j1 siRNA transfected cells but not in control siRNA transfected cells. **e** HeLa cells were transfected with control siRNA or Ube2j2 siRNA and the percentage of dead cells was calculated by trypan blue exclusion staining as described in **c**. The percentage of dead cells was 15.8 ± 1.81 and 8.41 ± 0.60 at 48 h in Ube2j2 and control siRNA transfected cells respectively. **f** Cells were harvested and immunoblot was performed using indicated antibodies. There is no difference in the level of Grp78 between Ube2j2 and control siRNA transfected cells. The cleaved PARP was not observed in control and Ube2j2 siRNA transfected cells. The expression level of actin was used as a loading control. The data are shown as the mean $\pm$ SD, *, $p < 0.01$; **, $p < 0.05$

transfected with control siRNA or Ube2j1 siRNA followed by the ectopic expression of GFP, GFP-Ube2j1/wt, GFP-Ube2j1/S184A (phosphorylation mutant) and GFP-Ube2j1/S184D (phosphorylation mimic mutant) and the expression of all these vectors were confirmed by GFP immunoblot using anti-GFP antibody (Fig. 2b). When cells were stained with trypan blue and dead cells were calculated 36 h after the withdrawal of tunicamycin, there is no significant effect on the cell death in control siRNA-transfected cells (Fig. 2a, 1–6); whereas in Ube2j1 siRNA transfected cells about $31.1 \pm 1\%$ dead cells were observed. Percentage of dead cells were $32.8 \pm 2.7\%$ and $31.9 \pm 1.3\%$ were observed by the ectopic expression of GFP or GFP-Ube2j1/S184A (phosphorylation mutant) (Fig. 2a, 9 and 11) respectively, as these could not recover cells from ER stress. By contrast, only $13.1 \pm 1.9\%$ and $23.5 \pm 2.3\%$ of dead cells were observed by the ectopic expression of GFP-Ube2j1/wt or GFP-Ube2j1/S184D (phosphorylation mimic mutant) respectively (Fig. 2a, 10 and 12). These results clearly demonstrated that the phosphorylation of Ube2j1 plays a role during ER stress recovery. We also repeated the same experiment with a different method by employing MTT assay instead of trypan blue staining to calculate the cell

proliferation and we could observe that Ube2j1/wt or GFP-Ube2j1/S184D (phosphorylation mimic mutant) could indeed recover cells from ER stress in Ube2j1 siRNA-transfected cells (Supplemental Fig. 1a). The recovery of ER stress by the ectopic expression of Ube2j1/wt or GFP-Ube2j1/S184D (phosphorylation mimic mutant) was further confirmed by the reduced expression of Grp78 and the absence/reduced PARP cleavage in Ube2j1 siRNA transfected cells (Fig. 2b, lane 10 and 12), (Supplemental Fig. 1b, lane 4 and 6). The cell protective role of phosphorylated Ube2j1 during ER stress recovery could be due to higher binding affinity to its partners such as E3 ligase. Increased affinity of phosphorylated Ube2j1 with E3 ligase could accelerate the rate of degradation of misfolded proteins during ER stress recovery. Taken together, these results clearly demonstrated that the phosphorylation of Ube2j1 is important for the cells to recover from transient ER stress.

Phosphorylated Ube2j1 preferentially interacts with c-IAP1

To further analyze the role of phosphorylated Ube2j1 and to examine whether it can interact with E3, we selected E3 ligase, c-IAP1 which was already shown to interact with Ube2j1 (Wu et al. 2005). We cloned and expressed GFP-c-IAP1 in HeLa cells and immunoprecipated with GFP antibody. GFP immunoprecipitant was analyzed by Ube2j1 immunoblot using the Ube2j1 antibody which clearly showed that Ube2j1 interacts with GFP-c-IAP1 but not GFP (Fig. 3a). To check whether phosphorylated Ube2j1 can interact with c-IAP1, we induced the phosphorylation of Ube2j1 by tunicamycin treatment in HeLa cells ectopically expressing GFP-c-IAP1 and performed GFP immunoprecipitation. When Ube2j1 is present in both phosphorylated and unphosphorylated form, GFP-c-IAP1 preferentially enriched the phosphorylated Ube2j1 (Fig. 3b). When His-tagged cIAP1 was co-expressed with GFP, GFP-Ube2j1/wt, GFP-Ube2j1/S184A and GFP-Ube2j1/S184D in HeLa cells and His pull-down was done by Ni-NTA, the enrichment of GFP-Ube2j1/S184D was more followed by GFP-Ube2j1/wt and Ube2j1/S184A (Fig. 3c). These data suggest that Ube2j1 is phosphorylated during ER stress and the phosphorylated Ube2j1 can preferentially interact with E3 ligase, c-IAP1.

Proteasomal degradation of Ube2j1

Given the fact that the expression of Ube2j1 is induced and it is phosphorylated during ER stress, we investigated the fate of induced Ube2j1 and whether it is degraded via proteasome during transient ER stress recovery. For this purpose, we chased the level of Ube2j1 after the withdrawal of

Fig. 2 Recovery of cells from ER stress by phosphorylation mimic Ube2j1 (S184D) mutant. **a** HeLa cells were transfected with control siRNA (1–6) or Ube2j1 siRNA (7–12) for 12 h. Cells were re-plated in 12-well plate and transfected with GFP (3 and 9), GFP-Ube2j1 (Wt) (4 and 10), GFP-Ube2j1(S184A) (5 and 11), GFP-Ube2j1(S184A) and GFP-Ube2j1(S184D) (6 and 12). At 24 h posttransfection, cells were treated with tunicamycin (20 μ g/mL) (2–6 and 8–12) for 6 h followed by media replacement with fresh media. After 36 h, the percentage of dead cells was calculated by trypan blue exclusion staining. The percentage of dead cells were increased in Ube2j1 siRNA transfected cells (8) and ectopic expression of GFP-Ube2j1 (Wt) (10) and

phosphorylation mimic mutant, GFP-Ube2j1 (S184D) (12) recover ER-stress induced cell death but not by the expression of phosphorylation site mutant, GFP-Ube2j1 (S184A) (11) or GFP (9) alone. **b** Cells were also lysed and immunoblot was performed using indicated antibodies. The expression of ER stress marker, Grp78, and apoptotic markers such as cleaved caspase-3 and cleaved PARP (lane 10 and 12) were increased and decreased, respectively by the ectopic expression of GFP-Ube2j1 (Wt) and GFP-Ube2j1 (S184D) but not by GFP-Ube2j1 (S184A) and GFP (lane 9 and 11). The expression level of actin was used as a loading control. The data are shown as the mean $\pm$ SD, *, $p < 0.01$; **, $p < 0.05$

tunicamycin but with the presence of CHX (cycloheximide) alone or the co-treatment of both CHX and MG132, we clearly observed by Ube2j1 immunoblot that the phosphorylated and unphosphorylated Ube2j1 was accumulated from 12 h by the co-treatment of both CHX and MG132 (Fig. 4a). This accumulation was not detected by the treatment of CHX alone. These effects could not be attributed to the induced expression of Ube2j1 even after the withdrawal of tunicamycin since CHX inhibits the synthesis of Ube2j1. Taken together, these results indicate that under ER stress both induced and phosphorylated Ube2j1 can be degraded by proteasome during ER stress recovery, presumably via preferential interaction of phosphorylated Ube2j1 with E3 ligase.

Rapid degradation of phosphorylation-mimic mutant Ube2j1/S184D

Since Ube2j1 is phosphorylated under ER stress and it is degraded in proteasome during ER stress recovery, we examined whether phosphorylation has any role on the rate of

degradation of Ube2j1. For that purpose, we overexpressed GFP, GFP-Ube2j1/wt, GFP-Ube2j1/S184A and GFP-Ube2j1/S184D in HeLa cells. GFP-Ube2j1/wt, GFP-Ube2j1/S184A, and GFP-Ube2j1/S184D but not GFP stabilized by the treatment of proteasome inhibitor, MG132 which suggest that all the overexpressed versions of Ube2j1 are degraded by the proteasome (Fig. 4b). To further analyze the role of phosphorylation on the rate of degradation of Ube2j1, we chased the degradation of GFP-Ube2j1/wt, GFP-Ube2j1/S184A, and GFP-Ube2j1/S184D in cells treated with CHX (Fig. 4c). The level of GFP-Ube2j1/wt was decreased by 30% whereas the level of phosphorylation mimic mutant, GFP-Ube2j1/S184D was decreased by 80% after 8 h CHX treatment. The phosphorylation mutant, GFP-Ube2j1/S184A was decreased by 20% at 8 h and it is consistently degraded slower than the phosphorylation mimic mutant and wild type (Fig. 4c, d). Once ER stress is relieved during recovery, the phosphorylated Ube2j1 that has the higher affinity for E3 ligase, c-IAP1 (Fig. 3b), itself could be the target for ubiquitination and it is degraded rapidly than phospho-mutant, GFP-Ube2j1/S184A or GFP-Ube2j1/wt. But, further experiments are warranted at

Fig. 3 Preferential interaction of phosphorylated Ube2j1 with cIAP1. **a** HeLa cells were transfected with GFP or GFP-cIAP1. After 36 h, cells were harvested and lysed. Lysates were subjected to immunoprecipitation with GFP antibody and immunoblotted with the Ube2j1 antibody. The arrow indicates the interaction between GFP-cIAP1 and Ube2j1. **b** HeLa cells were transfected with GFP or GFP-cIAP1. After 36 h, cells were treated with tunicamycin (20 μ g/mL) for 12 h. Cells were harvested and lysed. Lysates were subjected to immunoprecipitation with GFP antibody and immunoblotted with the Ube2j1 antibody. The interaction between GFP-cIAP1 and phosphorylated Ube2j1 upon tunicamycin treatment is shown by an arrow. **c** HeLa cells were co-transfected with His₆-IAP1 and GFP or GFP-Ube2j1/wt, GFP-Ube2j1/S184A, GFP-Ube2j1/S184D for 24 h. Cells were harvested, lysed and His₆-IAP1 pull-down by Ni-NTA was performed and it was confirmed by anti-His immunoblot and the interacting protein was analyzed by immunoblotting with the anti-GFP antibody. The expression level of actin was used as a loading control

this juncture to identify the ubiquitination components during the degradation of Ube2j1 by the proteasome. Taken together, these results suggest that Ube2j1 is phosphorylated under ER stress and phosphorylation can affect the rate of degradation of Ube2j1 during ER stress recovery.

Discussion

In the present study, we have demonstrated that Ube2j1, and not Ube2j2 is essential for ER stress recovery and the phosphorylation of Ube2j1 is also important for ER stress recovery. Moreover, phosphorylated Ube2j1 is degraded rapidly by the proteasome. During ER stress, accumulated misfolded proteins can be cleared by the induction of ERAD components (Bonifacino and Weissman 1998; Meusser et al. 2005). Although both Ube2j1 and Ube2j2 are induced during ER stress, we found Ube2j1, and not Ube2j2 is essential

for cells to recover from transient ER stress. This could be attributed to the phosphorylation of Ube2j1 and the fact from our data which suggest that phosphorylated Ube2j1 has more affinity for E3 ligase. During ER stress, accumulated misfolded proteins should be cleared from the ER and retro-translocated to the cytosol. The retro-translocated proteins will then be delivered to the 26S proteasome for degradation. To cope with the accumulated misfolded proteins, expression of ERAD components including E2s such as Ube2j1 and Ube2j2 are induced. This increase in the expression level of Ube2j1 and Ube2j2 accelerates the rate of ubiquitination of ERAD substrates.

During ER stress, not only the rate of ubiquitination by E2s but also the rate of transfer of ubiquitin by E3s to the accumulated ERAD substrates is also important for the rapid degradation. It has been shown that S184A mutation of Ube2j1 did not affect the ubiquitination of the ERAD substrate, α TCR (Menon et al. 2013). In this study, we found that phosphorylated Ube2j1

Fig. 4 Proteasomal degradation of Ube2j1. **a** HeLa cells were treated with DMSO or 20 μ M tunicamycin. After 12 h, media was replaced with fresh media containing 50 μ M cycloheximide (CHX) or co-treatment of both CHX and 20 μ M MG132. Cells were harvested at indicated time points. Accumulation of phosphorylated Ube2j1 was observed at 12 and 24 h by the co-treatment of CHX and MG132, not by CHX alone. The expression level of actin was used as a loading control. **b** HeLa cells were transfected with GFP, GFP-Ube2j1/wt, GFP-Ube2j1/S184A and GFP-Ube2j1/S184D for 24 h and cultured with or without MG132. After 12 h, cells were harvested, lysed, immunoblotted with indicated antibodies. GFP-Ube2j1/wt, GFP-Ube2j1/S184A, and

GFP-Ube2j1/S184D but not GFP are accumulated by the treatment of MG132. **c** HeLa cells were transfected with GFP-Ube2j1/wt, GFP-Ube2j1/S184A and GFP-Ube2j1/S184D for 24 h and cultured with CHX. Cells were harvested at 0, 4, 8 and 12 h and lysed, immunoblotted with the anti-GFP antibody. The expression level of actin was used as a loading control. **d** Graph showing the degradation rate of GFP-Ube2j1/wt, GFP-Ube2j1/S184A and GFP-Ube2j1/S184D from optical densities of protein bands in immunoblot (c) using densitometer and normalized with actin. The data are shown as the mean $\pm$ SD, **, $p < 0.05$

has more affinity for E3, c-IAP1. Thus, the rate of transfer of ubiquitin to substrates via E3 is efficient by phosphorylated Ube2j1 and this explains the importance of Ube2j1 phosphorylation during transient ER stress cell recovery. Although the interaction between c-IAP1 and Ube2j1 was shown in this study, there are more than 13 different E3 ubiquitin ligases known to be involved in ERAD (Christianson and Ye 2014). Hence, further study is needed at this juncture to determine whether Ube2j1 has multiple E3 partners.

Another important finding of this study is that Ube2j1 itself can be degraded by the proteasome during ER stress recovery. This suggests that once ER stress is relieved in the cell, the fate of induced ERAD components including Ube2j1 to be

degraded by the proteasome. The phosphorylated Ube2j1 is degraded rapidly when compared to S184A mutant and wild type. This is in agreement with our observation that phosphorylated Ube2j1 has more affinity for E3 and hence its rapid degradation. In conclusion, our results demonstrate the important role of Ube2j1 phosphorylation and degradation during ER stress cell recovery.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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