UBXN1 modulates Translation via the ATF-4/GADD34 Axis

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Abstract

To ensure the folding and maturation of one-third of the cellular proteome, protein homeostasis, or proteostasis, within the endoplasmic reticulum (ER), is essential. A build-up of misfolded proteins in the ER can be caused by cellular stress or mutations in ER proteins that affect folding. When abnormal proteins build up, ER stress develops, and this leads to the unfolded protein response (UPR) being activated. Our lab previously found that UBXN1 knockout (KO) cells exhibited an enrichment of ER-localized proteins, according to preliminary proteomic studies. We find here that UBXN1 modulates protein translation via the PERK Pathway. Subsequent analysis revealed that UBXN1 KO cells exhibited a remarkable increase in translation, even in the presence of thapsigargin-induced ER stress. Our findings indicate that the PERK arm of the integrated stress response (ISR) is activated mechanistically when UBXN1 deficiency exists, as demonstrated by increased expression of ATF4 protein, and its downstream transcriptional target, GADD34 protein. The increase of ATF4 target genes involved in translation and protein synthesis in UBXN1 KO cells was verified by qPCR. This increase in ATF4 and GADD34 was restored by pharmacological suppression of eIF2α phosphorylation by ISRIB, indicating the role of eIF2α phosphorylation in UBXN1-mediated translational control. Puromycin incorporation experiments demonstrated that UBXN1 KO cells experienced an earlier translation resumption following ER stress. All things considered, these results identified UBXN1 as a translation regulator under ER stress, operating to postpone translation resumption through the ATF4/GADD34 Axis.

Dedication

This Thesis is dedicated to my Parents.

Acknowledgment

I first wish to express sincere gratitude and appreciation to Dr. Malavika Raman. This endeavor would have been impossible without your guidance, patience, and reassurance. I attribute so much of my growth as both a scientist and person over the last 1.5 years to your steadfast encouragement. I'll always be thankful that I chose such a wonderful mentor. I would also like to thank Dr. Peter Juo for being the reader of my thesis.

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Words cannot possibly express the gratitude I have for my family who showed interest in my work and supported me throughout my time at Tufts. You never allowed me to quit or doubt myself. Your belief in me provided me the motivation I needed to persevere through graduate school to which I will be eternally grateful. I could never repay you, for the breadth of unwavering support, faith, and love you showed me throughout my Master's.

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List of Abbreviations.

AD: Alzheimer's Disease
ALS: amyotrophic lateral sclerosis
ATF4: activating transcription factor 4
ATF6: activating transcription factor 6
BAG6: BCL2-associated athanogene 6
BiP: binding immunoglobulin protein (glucose-regulated protein 78 kDa, GRP78)
BRCA1: Breast cancer type 1 susceptibility protein
BTZ: Bortezomib
bZIP: basic leucine zipper
C. elegans: Caenorhabditis elegans
CHOP: C/EBP homologous protein
CHX: cycloheximide
CYC1: Cytochrome C1
DR5: death receptor 5
DTT: dithiothreitol
DUB: deubiquitinase
eIF: eukaryotic initiation factor
eiF5: eukaryotic initiation factor 5
eiFC3: Eukaryotic translation initiation factor 3 subunit C
eIF2α: eukaryotic initiation factor 2α
ER: Endoplasmic reticulum
ERAD: Endoplasmic reticulum-associated degradation
ESRE: ER stress response element
E1: ubiquitin-activating enzyme
E2: ubiquitin-conjugating enzyme
E3: ubiquitin-ligase
GADD34: growth arrest and DNA damage-inducible protein (also known as Protein phosphatase 1 regulatory subunit 15A (PPP1R15A))
G3BP1: Ras GTPase-activating protein-binding protein 1
HD: Huntington's Disease
Hsp: Heat-shock protein
IBMPFD: inclusion of body myopathy with Paget's disease of the bone with frontotemporal dementia
IRE1α: Inositol requiring enzyme 1 α xv
ISRIB: Integrated Stress Response inhibitor
LARS: Leucine-tRNA synthetase
MiNA: Mitochondrial Network Analysis
MPN: MPR1/PAD1-N-terminal
MSP-1: multisystem proteinopathy 1
MTOC: microtubule organizing center
NBD: nucleotide-binding domain
NPL4: nuclear protein localization homolog 4
OP-PURO: O-propargyl-puromycin
PERK: protein kinase R (PKR)-like ER kinase

PQC: Protein Quality Control
Proteostasis: Protein homeostasis
PP1: protein phosphatase-1
PrP: Prion protein
PUB: peptide: N-glycanase/UBA or UBX
PUL: PLAP, Ufd3p, Lub1p
REDD1: Regulated in development and DNA damage-response 1
rpl26: 60S ribosomal protein L26
RIDD: regulated IRE1-dependent decay
RIP1: receptor-interacting protein 1
RLRs: RIG-I-like receptors
ROS: reactive oxygen species
SAPK: stress-activated protein kinase
SBD: substrate binding domain
siRNA: small interfering RNA SRP: signal recognition particle
Tg: thapsigargin
TMT: tandem mass tag
Tu: tunicamycin
UBA: ubiquitin-associated domain
UBD: ubiquitin-binding domain
UBL: ubiquitin-like protein
UBX: ubiquitin regulatory X domain
UBXN1: UBX domain protein 1 (SAKS1)
UBXN1 KO: UBX domain protein 1 knockout
UBXD8: ubiquitin-X domain adaptor 8 (Fas Associated Factor Family Member 2 (FAF2))
UFD1: ubiquitin fusion degradation 1
UFM1: ubiquitin fold modifier 1
uORF: upstream open reading frame
UPR: unfolded protein response
UPS: ubiquitin-proteasome system
UTR: untranslated region
VBM: VCP-binding motif
VCP: valosin containing protein/p97 in mammals, Cdc48p in yeast
VIM: VCP-interacting motif
WARS: Tryptophanyl-tRNA synthetase
xbp1: x-box binding protein-1
xbp1s: x-box binding protein-1 spliced fo

Chapter 1: Introduction

1.1 Co-translational Translocation and Protein Processing in the ER

The ER is a crucial compartment for protein synthesis and maturation as it processes over one-third of the cellular proteome for traffic through the secretory pathway. Ribosomes associate with the cytosolic surface of rough ER sheets to facilitate the co-translational translocation of proteins into the ER[1]. When ribosomes translate an mRNA encoding a protein with a transmembrane domain or a short N-terminal hydrophobic signal sequence, translation is paused [2]. As soon as the signal sequence enters the cytoplasm, the ribosome-associated signal recognition particle (SRP) detects it and promptly directs the mRNA-ribosome complex to the endoplasmic reticulum (ER), where it docks at the SRP receptor (SR). The ribosome nascent chain complex is then guided to the polypeptide conducting channel Sec61[3] by SR. After GTP hydrolysis between SR and SRP, the nascent polypeptide chain inserts into the Sec61 channel and SRP separates from the ribosome and SR [4, 5], triggering the restart of protein synthesis. Membrane proteins are translated directly into the ER bilayer from the Sec61 complex by moving through the SRP lateral gate. On the other hand, soluble luminal proteins utilize the ER luminal chaperone proteins BiP/Grp78 for unidirectional passage into the ER lumen [6-8]. J domains of ER transmembrane proteins, like Sec63, mediate a direct interaction between these chaperones and the precursor polypeptides during the transit [9, 10]. ATP hydrolysis is necessary for the function of ER luminal BiP, which is facilitated by the nucleotide-exchange factors Sil1 and GRP170 [11]. The signal peptidase complex cleaves off the signal sequence during or after the translocation of the protein is finished. This protein is then folded and subjected to covalent modifications like N glycosylation.

Three transmembrane domains make up the Sec63 protein. A J-domain is in the ER luminal loop, which interacts with BiP to enable the unidirectional translocation of precursor proteins through the Sec61 translocation pore. Furthermore, Sec63 has a precursor-specific function in the early stage of co-translational protein transport, working with BiP. Proteins like pERj3, PrP, and PPCECA depend on Sec63 for an effective initial insertion into the Sec61 channel. While the vast majority of ER clients utilize the co-translational method for E import, 3-5% of ER clients that contain a C-terminal transmembrane domain use a posttranslational method involving a distinct set of targeting factors. This mechanism includes cytosolic and membrane proteins for targeting and integration of the membrane [12].

1.2 ER protein misfolding triggers adaptive responses.

The ER is the biggest organelle in cells and performs a variety of tasks, such as detoxification, lipid and steroid production, protein folding, processing, and transport [13]. The ER is constantly being repaired to preserve its integrity and functionality. The ER serves as a quality-control checkpoint for the proteins in the secretory pathway, allowing mature folded proteins to depart to the Golgi apparatus [14]. A disruption in ER proteostasis can lead to conditions such as cardiovascular, neurological, oncological, and metabolic diseases[15]. When misfolded proteins accumulate in the ER, they trigger the unfolded protein response (UPR) and ER-associated degradation (ERAD) [16].

1.2.1 The unfolded protein response (UPR)

The UPR is an adaptive response to the buildup of these misfolded proteins that can lead to ER stress. Activation of the UPR results in the suppression of protein synthesis, transcriptional activation of ER folding proteins [17, 18], and regulation of apoptosis [19] Misfolded proteins in the ER are sensed by three ER membrane sensors—double-stranded RNA-activated protein kinase R (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). These sensors are responsible for both initiating and controlling the UPR. In unstressed cells, these sensors are dormant due to binding to the chaperone BiP. As unfolded proteins accumulate they compete with the UPR sensors for BiP binding. Hence, when there's an accumulation of unfolded proteins, BiP titrates away, activating the UPR sensors[20]. UPR's transcriptional target genes are associated with various tissue-specific metabolic processes, oxidative stress, autophagy, ERAD, protein folding, and mitochondrial failure[21, 22].

The IRE1 pathway. IRE1 is the most evolutionarily conserved of the three UPR branches. IRE1 belongs to the subset of transmembrane proteins that are single-spanning and have both dual protein kinase and ribonuclease activities[23, 24]. Once activated, IRE1 dimerizes or oligomerizes, which causes the protein kinase domain's positive regulatory sites, the activation group, the linker group, and the RNAase domain to be transphosphorylated. This triggers the conversion of IRE1 to IRE1p. These modifications necessitate the presence of adenosine nucleotides (ATP/ADP) as cofactors for IRE1 to display nuclease activity. Phosphorylation of IRE1a activates its RNAse activity leading

to an unconventional splicing event that excises a 26-nucleotide intron from the mRNA that codes for the UPR-specific transcription factor *x-box binding protein 1 (xbp1)*. This results in the formation of spliced *xbp1 (xbp1s*) from the unspliced *xbp1* (*xbp1u*). IRE1a activation also has another effect under ER stress conditions known as Regulated IRE1 a dependent decay (RIDD)[25], where IRE1 a degrades the mRNAs targeted towards the ER while they are undergoing translation at the SEC61 translocon, hence decreasing mRNA abundance[26, 27].

The ATF-6 pathway: ATF-6 (Full Length), when activated, translocates from the ER to the Golgi Apparatus in vesicles. At the Golgi Apparatus, it is cleaved by Site 2 and Site 1 proteases[28]. Once cleaved, it gives rise to an N terminal cytosolic ATF-6 which which is a transcription factor that activates genes to reset ER proteostasis. ATF6 also directly regulates the transcription of *xbp1* mRNA[29].

The PERK pathway: Autophosphorylation of the kinase domain of PERK causes PERK to phosphorylate the initiation factor eIF2α at Ser51 (eIF2α-P), which momentarily stops the initiation of mRNA translation, thereby decreasing the protein-folding load on the ER. eIF2α-P paradoxically increases the translation of some mRNAs, including *atf4* mRNA, to boost the capacity for protein transport in the ER. The coding open reading frame (ORF) of ATF4 is overlapped by inhibitory upstream ORFs (uORFs) in the mammalian *atf4* mRNA[30]. The elongating ribosome cannot access the main ATF4 ORF due to the presence of inhibitory uORFs when levels of the eIF2 ternary complex are high. The inhibitory uORFs can be bypassed when eIF2 ternary complex

concentrations are lower (as in the case of UPR activation), enabling translation start for ATF4 from the coding ORF. Hence, ATF4 protein is increased when eIF2α is phosphorylated and eIF2 ternary complex quantities are decreased. Following translation, ATF4 translocates to the nucleus and activates UPR genes, which code for proteins essential for amino acid transport and biosynthesis as well as the antioxidant response. Additionally, ATF4 stimulates the transcription of CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP), which combines with ATF4, forming heterodimers that increase the transcription of genes involved in autophagy, mRNA translation, and the UPR[31]. ATF4 and CHOP trigger the transcription of growth arrest and DNA-damage-inducible protein 34 (*gadd34*) which directly dephosphorylates eIF2α-P to reinitiate global mRNA translation after ER protein folding equilibrium is restored[32].

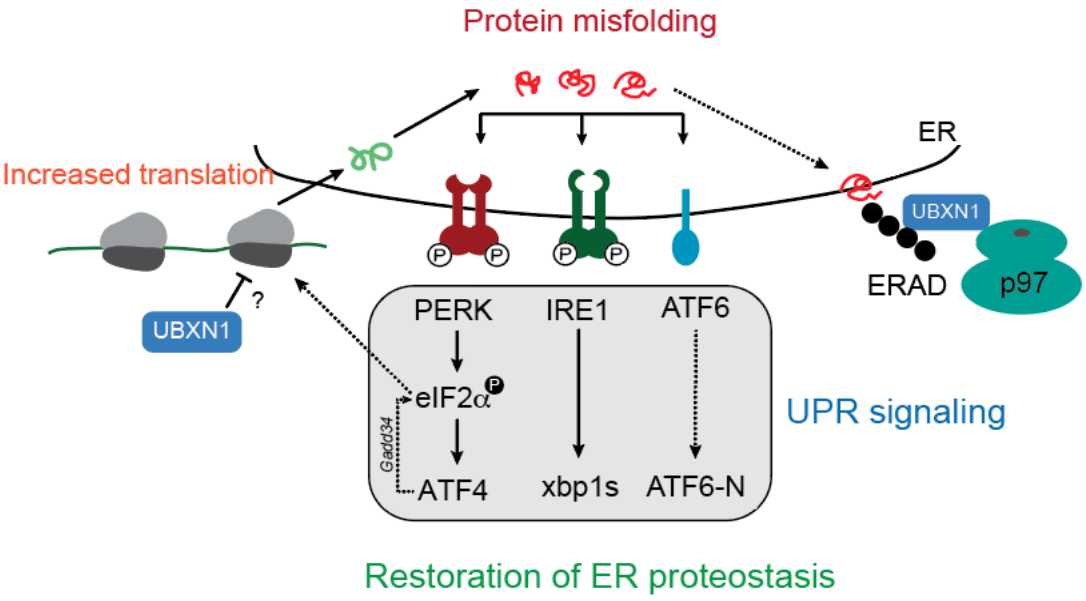


Figure 1.1: The Unfolded Protein Response. Translation initiation and the eiF2 complex. Reprinted from [33]. UBXN1 maintains ER proteostasis and represses UPR activation by modulating translation and is licensed under CC-BY-4.0.

1.2.2. Translation initiation and the eiF2 complex.

A multistep process involving at least nine eukaryotic initiation factors (eIFs) is used by eukaryotic ribosomes to start translation [34, 35]. The eIF2 ternary complex, which consists of eIF2-GTP-Met-tRNAi, first attaches to the 40S subunit of the ribosome and recruits Met-tRNA to start the assembly of a 43S preinitiation complex[35]. With the aid of many cap-binding proteins, the 43S complex is placed onto the 5' untranslated region (UTR) of mRNA and scans down the mRNA transcript in the 5' to 3' direction until it hits the AUG initiation codon[36, 37]. The 48S initiation complex is formed by the codon-anticodon base pairing of the initiator methionine with the AUG start codon, which is established by GTP hydrolysis in the eIF2 complex [37-39]. This event allows the eIFs to disassociate allowing the 60S subunit to combine with the 40S subunit to form the active 80S complex. The polypeptide chain is constructed by elongation cycles of codon recognition, peptidyl transfer, and tRNA-mRNA translocation mediated by GTP hydrolysis and other elongation factors [40].

The eIF2 complex must be loaded with GTP to associate with the tRNA loaded with the initiator methionine (tRNAiMet) [41]. After a successful translation initiation round, eIF2 is liberated from the remaining translation machinery and bound to GDP. To support a subsequent round of tRNAiMet loading, it must be recycled to GTP which is accomplished by the guanine nucleotide exchange factor (GEF) eIF2B. eIF2B first extracts eIF5 from eIF2, which is still bound after the ribosome separates [42]. Then, eIF2B helps eIF2 exchange GDP for GTP, which enables treatment to attach to eIF2 and

reassemble the eIF2 ternary complex [43]. The 80S subunit separates and the ribosome is recycled for another translation cycle when the ribosome encounters a stop codon [40].

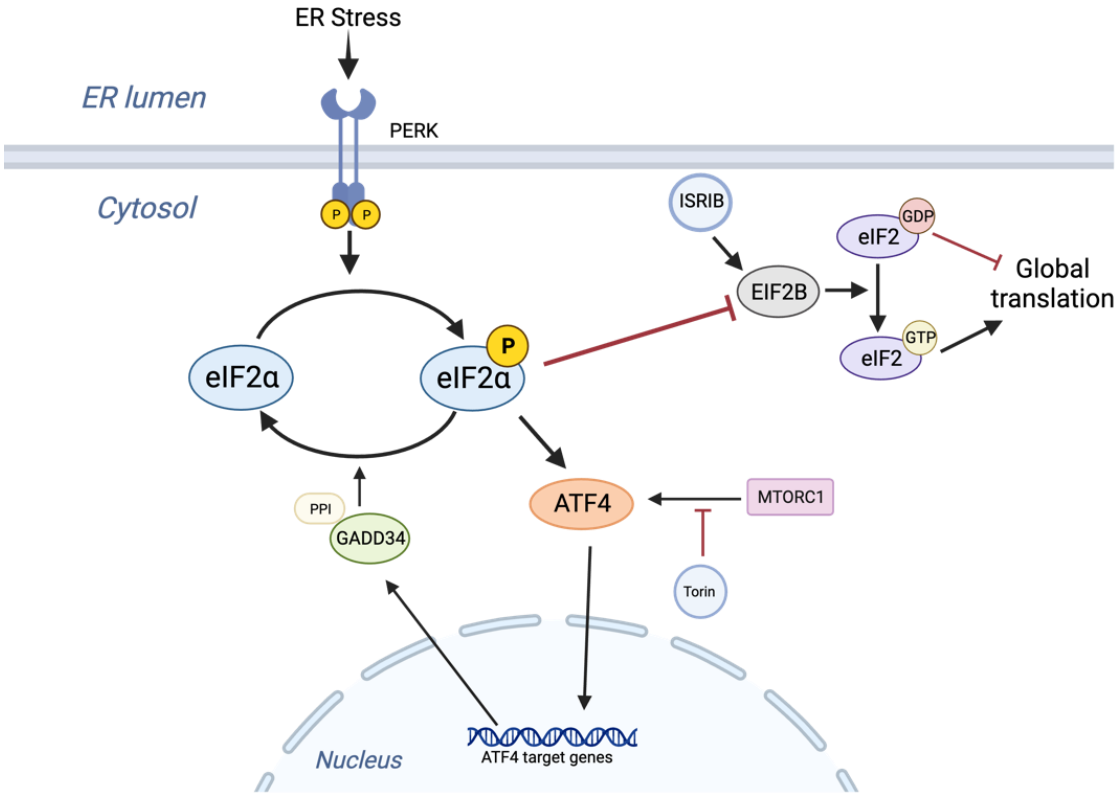


Figure 1.2: The PERK pathway. Created with BioRender.com

1.2.3 ER-associated degradation by VCP removes misfolded proteins from the ER.

ERAD is a component of the ER-mediated protein quality control system, which regulates the removal of aberrant proteins from the ER. Four phases make up the ERAD degradation mechanism: substrate identification by lectin and chaperones, displacement across the ER membrane triggered by VCP/p97, polyubiquitination by E3 ligases, and destruction by the 26S proteasome[44].

1.3 The p97 AAA-ATPase is an essential chaperone for a variety of ubiquitin-dependent protein quality control pathways.

Valosin -Valosin-containing protein (VCP/p97), identifies ubiquitylated proteins and is essential for mediating their degradation by the proteasome. p97 has been implicated in numerous processes that touch on nearly every aspect of cellular physiology. Based on its crucial function in the ubiquitin-proteasome system (UPS), it is now evident that p97 controls several cellular stress responses and plays a major role in protecting cells from proteotoxic stress. It is therefore no surprise that p97 is linked to illness. Dominant mutations in regulatory regions induce late-onset multisystem proteinopathy (MSP-1), also known as Inclusion Body Myopathy with Paget Disease of Bone and/or Frontotemporal Dementia (IBMPFD), while p97 homozygous knockout in mice is fatal[45]. The primary characteristics of MSP1 include amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), Paget's disease of bone (PDB), and inclusion body myopathy (IBM). On the other hand, p97 is overexpressed in some forms of cancer, likely as an adaptation to the higher load of protein degradation [46]. VCP recognizes ubiquitylated substrates through a variety of specialized adaptor proteins, and it unfolds substrates by passing them through a central pore in the hexamer in an ATP-dependent manner.

1.3.1 Structure of p97

The primary mode of action for VCP is as a homo-hexamer, where each monomer has two ATPase domains (D1 and D2) after an N-terminal domain [45]. The N-domain

displays either a co-axial confirmation (also known as "up") or a co-planar posture (also known as "down") about the D1–D2 ring, depending on the nucleotide bound state of the hexamer. Although the D1 and D2 ATPase domains are both functional, the D1 domain's ATP binding increases the stability of the hexamer, while the D2 domain's ATP binding and hydrolysis produce the force required for unfolding [47]. p97 can separate proteins from complexes or cellular structures for activation or removal. It may also pull misfolded or damaged proteins out of membranes and unravel fully folded proteins for proteasomal breakdown. By drawing substrates into the hexamer's central channel, VCP can unfold them through sequential ATP hydrolysis by each monomer. Unfoldase activity and ATPase activity are linked, which permits the unfolding of the substrate in a nucleotide-dependent fashion [48]. Proteosome engagement and its degradation of a substrate depend on a flexible, unstructured initiation region, which is why p77 facilitated unfolding is essential for those substrates that lack an unstructured region [49-51]. The Ufd1-Npl4 dimeric adaptor is essential for identifying ubiquitylated substrates and mediating unfolding reactions [49, 52].

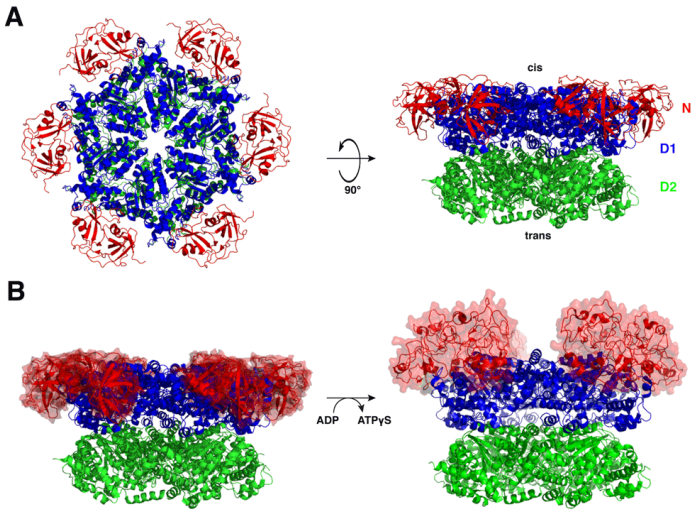


Figure 1.3: **p97 structure**. A. Cdc48 is a homohexamer, and each monomer comprises an N-terminal (N) domain (red) and two AAA ATPase domains: D1 (blue) and D2 (green). The N-terminal (D1) side of the central pore is referred to as the cis side, and the C-terminal (D2) side is the trans side. B. ATP binding produces an upward rotation of the N domains into a so-called “up conformation”, in which they are positioned above the plane of the D1 ring. Left, ADP-bound state (PDB code 5FTK); right, ATPγS-bound state (PDB code 5FTN). PDB,

Protein Data Bank. Reprinted from: Bodnar N and Rapoport T. Toward an understanding of Cdc48/p97 ATPase [version 1]. F1000Research 2017, 6:1318. Toward an understanding of the Cdc48/p97 ATPase is licensed under CC-BY-4.0.

1.3.2 UBX/UBA-domain containing adaptors provide p97 with substrate specificity.

To identify ubiquitinated substrates, p97 interfaces with a variety of adaptor proteins because it has a low affinity for ubiquitin by itself [53]. p97 adaptor proteins with Ubiquitin Regulatory X (UBX) and UBX-like domains make up the biggest family of these proteins [54-56]. While there is a weak similarity in the amino acid sequence homology between the UBX domain and ubiquitin, they adopt the same 3D fold. A highly conserved Phe-Pro-Arg motif is present in a hydrophobic pocket through which the UBX domain interacts with the amino terminus of the N-domain of p97. Most of these adapters containing UBX domains also contain a Ubiquitin Binding A (UBA) domain which primarily recognizes polyubiquitin on substrate proteins. In addition, UBLs and linear peptide motifs such as the VCP-interacting motif (VIM), VCP-binding motif (VBM), and the SHP box have been identified on adapters with UBX domain that interact with the N-terminus of p97 [57]. The peptide: N-glycanase/UBA or UBX (PUB) [58, 59] and PLAP, Ufd3p, Lub1p (PUL) domains [60] are two more p97 interacting domains that have been shown to bind at the C-terminus of p97.

Although each p97 molecule has 6 potential N-domain binding sites, research has shown that not all of these sites are accessible for binding. It has been shown that the adaptors adhere to a binding hierarchy based on the adaptor class. By binding to p97 in a mutually exclusive way, the first class distinguishes between regulatory roles such as homotypic membrane fusion (p47) and protein degradation (UFD1-NPL4) [61, 62]. Both p47 and UFD1-NPL4 interact with p97 via a mutually exclusive, bipartite mechanism, which

prevents the other from binding. This is followed by a second class of UBX Domain containing adaptors which allows co-adapters to bind as it acts as a substrate [54]. Thus adaptors facilitate substrate binding and spatial control of p97.

1.3.3 UBXN1

UBXN1 or SAKS1 is an adaptor of p97 containing both the N-terminal UBA domain and the C-terminal UBX domain. It facilitates protein degradation by binding to ubiquitin and p97 through its UBA and UBX domains, respectively [63]. Furthermore, UBXN1 specifically prevents ERAD substrate degradation f[64]. In some instances, it has been shown to shield polyubiquitin chains from deubiquitination [65]. The BAG6/BCL2-associated athanogene 6 chaperone complex uses the VCP-UBXN1 interaction to target ubiquitinated proteins for proteasomal clearance before ER translocation[66]. Studies have shown that UBXN1 has been involved in attenuating BRCA1 enzymatic activity[67, 68] and in regulating the innate antiviral response to infections [69]. Furthermore, some of the p97-dependent roles of UBXN1 include degradation of ubiquitylated BAG-6 clients and regulation of aggregosome formation.

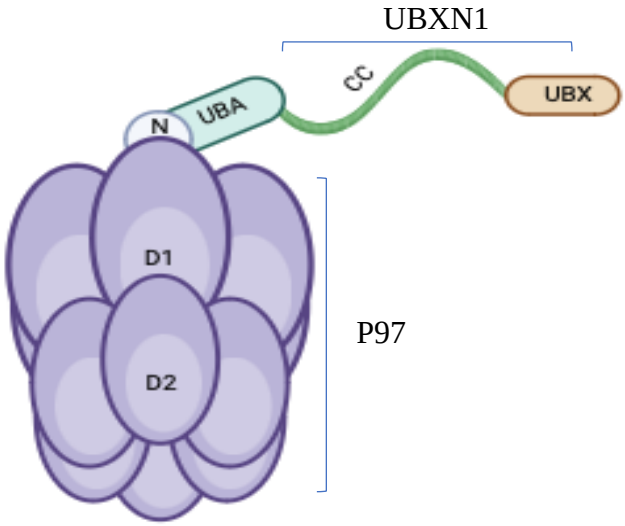


Figure 1.4: p97 depicted in association with its adaptor UBXN1.

UBXN1 associates with the N-domain of p97 through its UBX domain. The UBX domain of UBXN1 is connected to the ubiquitin-binding UBA domain via a flexible coiled-coiled (cc) linker. Created with BioRender.com.

1.3.3.1 UBXN1 Acts as a Guardian Against ER Stress and Cellular Dysfunction

A recent study from our research group showed that UBXN1 has a novel role in maintaining balance within the endoplasmic reticulum (ER), a crucial cellular compartment responsible for protein folding and maturation. The research team focused on understanding how UBXN1 contributes to ER-proteostasis, the delicate process of ensuring proper protein folding within the ER. Disruptions in ER-proteostasis trigger ER stress, a cellular alarm system that, when chronically activated, can lead to various diseases.

The importance of UBXN1 in repressing ER stress and the unfolded protein response (UPR) was a major takeaway. The lab utilized CRISPR-Cas9 gene editing and siRNA knockdown techniques to eliminate UBXN1 in cells which resulted in robust activation of all three major UPR signaling pathways – PERK, IRE1α, and ATF6. This activation was evidenced by increased expression of specific proteins downstream of each pathway, including BiP, phosphorylated eIF2α, cleaved ATF6 (ATF6p50), and spliced XBP1s. Further analysis revealed that UBXN1 deficiency translated into increased expression of genes encoding protein folding chaperones and ER-associated degradation (ERAD) components. This suggests that the activated UPR attempts to compensate for the disrupted ER-proteostasis caused by UBXN1 loss. Proteomic analysis confirmed this, demonstrating an enrichment of proteins involved in protein folding, translocation into the ER, and ER-quality control in UBXN1-deficient cells.

Interestingly, while the UPR activation in UBXN1-deficient cells might initially serve as a protective mechanism to restore ER balance, it ultimately renders these cells more

susceptible to further ER stress. This was demonstrated by the cells' inability to recover effectively when exposed to external ER stressors, leading to increased expression of the pro-apoptotic protein CHOP.

Understanding the role of UBXN1 can pave the way for future research on therapeutic strategies that target ER stress and associated pathologies [33].

1.3.3.2 UBXN1 regulates Protein synthesis, Independent of p97.

The study from our lab also employed puromycin incorporation and ribosome profiling techniques to demonstrate a surprising finding that UBXN1 acts as a repressor of protein translation. This means UBXN1 helps keep protein production in check, both in healthy cells and those experiencing ER stress. Reintroducing wildtype UBXN1 into UBXN1-deficient cells restored this translational control [33].

But UBXN1 exerts this control independently of p97, its known partner protein. Previously, UBXN1 and p97 functioned together in tasks like aggresome formation and protein degradation. However, UBXN1 appears to manage protein translation as a solo act, a finding supported by its other p97-independent roles discussed earlier. These additional functions, such as regulating immune signaling and BRCA1 activity, also involve a suppressive effect, mirroring the translational repression observed here.

This discovery raises a fascinating possibility – other p97 adaptor proteins might possess independent functions, independent of p97. This opens a new avenue for future research, prompting scientists to explore the potential solo acts within the p97 adaptor family.

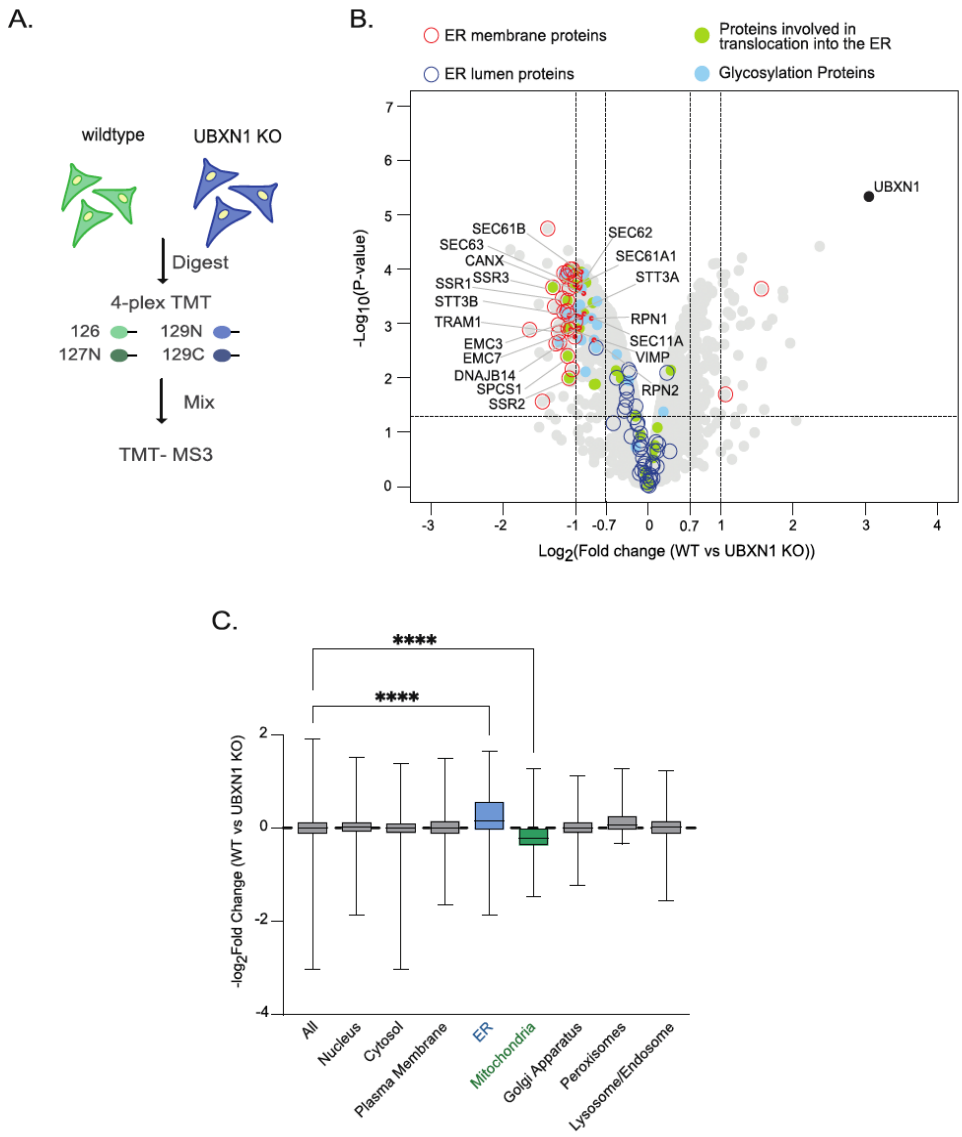


Figure 1.5: Loss of UBXN1 increases the abundance of ER proteins. A. Schematic workflow of quantitative tandem mass tag (TMT) proteomics in HFT wildtype and UBXN1 KO cells. B. Volcano plot of the (−log10-transformed P value versus the log2-transformed ratio of wildtype/UBXN1 KO) proteins identified by TMT proteomics in wildtype and UBXN1 KO cells. A negative log2 fold change indicates proteins that are increased in abundance in UBXN1 KO cells. Proteins with a log2 fold change ≥ |0.5| are considered significant. ER, membrane, and luminal proteins are outlined by a red and blue circle respectively. Proteins involved in ER translocation and glycosylation are colored in green and blue respectively (n = 2 biologically independent samples for each genotype. C. Proteins identified in the quantitative proteomics study were categorized by organellar compartment. Data are means ± SEM (**** where P < 0.0001). One-way ANOVA with Brown-Forsythe and Welch ANOVA tests of -logFC **C**. Reprinted from [33]. UBXN1 maintains ER proteostasis and represses UPR activation by modulating translation and is licensed under CC-BY-4.0.

1.4 Thesis Research goals and overview.

This thesis project aims to dissect the molecular mechanism by which UBXN1 negatively regulates protein translation, both under basal conditions and during ER stress. Our previous work demonstrated elevated levels of eIF2α in UBXN1-depleted cells, suggesting a potential role for the ATF4/GADD34 axis in this phenomenon.

Specific Objectives:

1.2.1 Investigate the role of the ATF4/GADD34 pathway:

- Quantify and compare the expression levels of ATF4 and GADD34 in wild-type and UBXN1-depleted cells using quantitative real-time PCR (qRT-PCR).
- Analyze the expression profiles of ATF4- and CHOP-dependent target genes, including aminoacyl tRNA synthetases, ribosomal proteins, and translation initiation factors.
- Determine if UBXN1-deficient cells exhibit earlier and more sustained peak expression of these targets, potentially contributing to increased translation.

1.4.2 Dissect the upstream signaling pathway:

- Employ Integrated Stress Response Inhibitor (ISRIB) to assess the involvement of the PERK-eIF2α pathway in ATF4 upregulation.
- Measure ATF4 levels in ISRIB-treated cells.
- Utilize Torin, a pan-mTOR inhibitor, to dissect the potential contribution of the mTORC pathway to ATF4 activation.

1.4.3 Functional validation of the ATF4/GADD34 axis:

- Employ puromycin incorporation assays to evaluate the impact of ATF4 depletion on translation reinitiation in UBXN1-depleted cells.
- Correlate the observed changes in translation with the expression profiles of ATF4 and GADD34 in the same samples.
- Establish if the accelerated translation restart observed in UBXN1-deficient cells can be attributed to enhanced GADD34 expression.

By achieving these objectives, we aim to elucidate the precise mechanism by which UBXN1 regulates protein translation and identify the specific signaling pathways involved in this process. This knowledge will significantly advance our understanding of the role of UBXN1 in cellular homeostasis and potentially uncover novel therapeutic targets for diseases associated with dysregulated protein synthesis.

CHAPTER 2: MATERIALS AND METHODS

2.1 siRNA, Antibodies and Reagents.

UBXN1 (16135-1-AP 1:7000 dilution), GADD-34 (10449-1-AP 1:2000 dilution) antibodies were purchased from Proteintech Inc; rabbit BiP (3177 dilution) antibody was purchased from Cell Signaling Technology; mouse ATF4 (sc-390063 1:500 dilution), Beta-Actin (sc-69879 1:3000 dilution), and GAPDH (sc-47724 1:3000 dilution) were purchased from Santa Cruz Biotechnology. Anti-Puromycin, clone 12D10 (MABE343 1:3000 dilution) antibody was purchased from EMD Millipore. Secondary antibodies, HRP-conjugated anti-mouse (W4021 1:10,000) and anti-rabbit (W4011 1:10,000) were purchased from Promega. siRNA against BAG6 (s15467) was purchased from Ambion (Thermo Fisher Scientific).

siRNAs against UBXN1-2 (D-008652-02), UBXN1-3 (D-008652-03), and UBXN1-4 (D-008652-04) were purchased from Dharmacon. siControl (SIC001) was purchased from Millipore Sigma. Tunicamycin (ICN15002801) and Thapsigargin (5860051MG) were purchased from Fisher Scientific. Puromycin (P-600-100) and Dithiothreitol (DTT25) were purchased from GoldBio. Triton X-100 (97063-864) and was purchased from VWR. ISRIB (S1013) and Torin1 (S2827) were purchased from Selleckchem.

2.2 Cell culture, transfections, immunoblot.

HeLa Kyoto cells were grown in a humidified, 5 % CO2 atmosphere at 37 C. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 100 U/mL penicillin and streptomycin. For siRNA transfections,

cells were either reverse or forward transfected in 6-well plates with 20 nM siUBXN1 or siControl utilizing Lipofectamine RNAiMax (Invitrogen). After 8 or 24 hours depending on the experiment, cells were split into 12-well plates for treatment. 48 hours post-transfection, cells were treated with the indicated drugs for the indicated time points before harvest for immunoblot or Reverse Transcriptase Polymerase Chain Reaction.

For UPR induction experiments (Figures 3.2,3.3,3.4,3.5, and 3.7), cell pellets were resuspended in 1 % SDS lysis buffer (1 % SDS, 150 mM NaCl, 50 mM Tris-Cl (pH 7.2), 0.5 % NP-40, sodium vanadate, sodium fluoride, and HALT protease inhibitors (Pierce PI-78425)), vortexed briefly, boiled for 10 minutes at 95C, and vortexed briefly. Lysates were incubated at 65C for 5 minutes and then centrifuged for 15 minutes at 14,000 rpm at room temperature. The supernatant was saved, and protein concentration was assessed using the DCA protein assay kit (Biorad). SDS-PAGE and immunoblot was used to analyze protein expression.

For puromycin immunoblots (Figures 3.1 and 3.6), cell pellets were resuspended in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-Cl (pH 7.2), 1 % sodium deoxycholate, 150 mM NaCl, 0.1 % SDS, 1 % NP-40, sodium fluoride, sodium vanadate, and HALT protease inhibitors). Cell pellets were resuspended in RIPA buffer and incubated on ice with a 10-second vortex pulse performed every 5 minutes for 25 minutes total. Lysates were centrifuged at 4C for 15 minutes at 14,000 rpm.

2.3 Quantitative Real-Time PCR

Cells were cultured in either 6- or 12-well dishes for quantitative real-time PCR analysis. Total RNA was isolated using the Quick-RNA Miniprep Kit (Zymo Research cat. no.

R1055) for all real-time PCR studies. RNA purity and concentration were determined by NanoDrop. cDNA was generated utilizing the iScript cDNA synthesis kit (Biorad cat. no. 1708890) with an equivalent amount (1 μg) of RNA for each sample. Gene expression was analyzed on an Applied Biosystems StepOnePlus real-time PCR system utilizing PowerUp SYBR Green Master Mix (Applied Biosystems cat. no. A25741). GAPDH was used as a housekeeping gene to normalize gene expression across samples and the 2-DDCt method was used to determine fold changes between wildtype and UBXN1 KO cells.

Eifs2

Forward primer - 5'ACACATACGAGGAGCTGCTGAATC-3'

Reverse primer - 5'AG CTTGGTTCCTACTCGGACGACTTG-3'

RPL26

Forward primer - 5′ CGA TCC ATG CCC ATC CGA AA 3′

Reverse primer - 5′ TGC CTA CGT GGA CAG TTG TG 3′

GADD34

Forward primer 5′ CTGATAAGAACCCAGGGGAGGA3′

Reverse primer 5′ ATCCTGGAGACAAGGCAGAAGTA3′

EIFC3

Forward primer - 5′CCAAGGCCATGAAGATGGGT3′

Reverse primer - 5′ATGATGGAGTGCACAGTGGG3′

EIF5

Forward primer - 5′ GTGCAATGTCAGGACCTCCA3′

Reverse primer - 5′ GGCCTGGGACAGAATC3′

WARS1

Forward primer - 5′ATGCCCCCTCCCAAAAAGAA3′

Reverse primer - 5′TGCAGGACAGAGGGTCTAGG3′

LARS1

Forward primer – 5’CTCGACGAGTTCCTGTCCTG3’

Reverse primer – 5’GGTCCAGCCTAAGGTTCTGA3’

REDD1 (DDIT4)

Forward primer – 5’GTGCCCTCCAAGACAGAGAC3’

Reverse primer – 5’CACCCGCACACAACTCAATG3’

2.4 Puromycilation Translation Assay

The non-radioactive SUNSET assay was used to measure puromycin incorporation into newly synthesized proteins as a proxy for protein translation [70]. Hela Kyoto wildtype and UBXN1 KO cells were seeded at a density of 400 k cells /well in a 6-well plate to achieve 70-80 % confluence at the time of puromycin addition. The next day, cells were pre-incubated with 1 μM thapsigargin for 1 hour before culture with 1 μM puromycin for

30 minutes. At the end of the incubation period, cells were washed twice with ice-cold PBS and the plate was placed immediately on ice. Ice-cold RIPA buffer was added to the wells and cells were gently scraped with a cell scraper into the RIPA buffer. Lysates were incubated on ice for 30 minutes with a brief vortex every 5 minutes for 10 seconds. Lysates were centrifuged at 12,000 rpm for 10 minutes at 4C. Protein concentration was estimated using the DC assay (Bio-Rad). Puromycin incorporation was determined by SDS-PAGE (~20 μg per sample) and immunoblot with the anti-puromycin antibody, clone 12D10.

2.5 Quantification and Statistical Analysis

Immunoblots were quantified using densitometry on FIJI and protein levels were normalized to the loading control for that experiment (GAPDH or Beta-actin). Fold changes, SEM, and statistical significance was calculated using GraphPad Prism (version 9.4). Statistical tests performed are indicated in figure legends and include unpaired two-tailed Student's t-test or one-way ANOVA with post-hoc analysis indicated in figure legends.

Chapter 3: Results

3.1 UBXN1 negatively regulates translation levels in cells both basally and under ER stress.

According to the previous research in our lab, the deletion of UBXN1 enhanced the abundance of ER proteins. We investigated how UBXN1 depletion affected this phenotype. Puromycin incorporation into newly synthesized proteins was measured using puromycin incorporation. Puromycin can occupy the acceptor (A) site on a ribosome for inclusion into the C-terminus of elongating polypeptides because it shares structural similarities with the 3' end of tyrosine-tRNAs. Puromycin incorporation prevents the polypeptide from lengthening any further, causing puromycilated proteins to prematurely exit the ribosome. Accordingly, the rate of global translation is proportional to the amount of puromycilated protein. We used a monoclonal puromycin antibody to measure the quantity of puromycilated proteins in wildtype and UBXN1 KO cells after they had been incubated with puromycin for 30 minutes. When UBXN1 KO cells were compared to wild type, we found a significant increase in puromycilated proteins, suggesting that there was increased basal translation in these cells (Fig 3.1 a and b). Treatment with thapsigargin (inhibits the endoplasmic reticulum Ca2+ ATPase) significantly reduced protein synthesis in WT cells, as seen by a marked decrease in puromycin staining. While in UBXN1 KO cells, translation was reduced but to a lesser extent than WT cells on thapsigargin treatment (Fig 3.1 a and b). These results show that UBXN1 repressed translation basally and with ER stress.

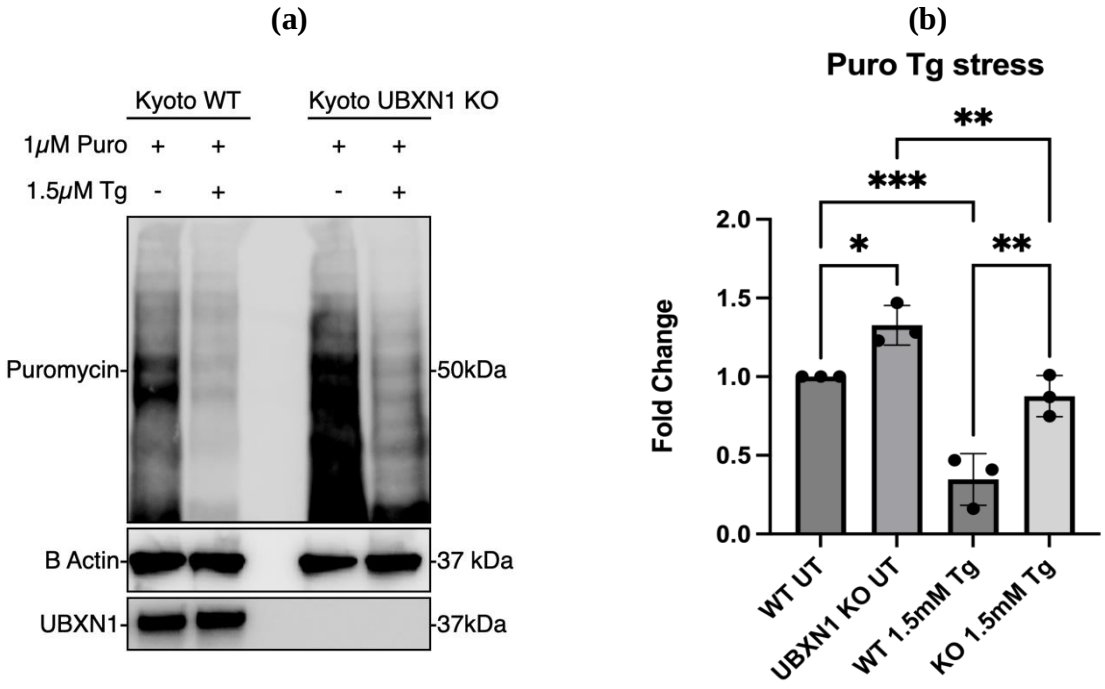


Figure 3.1: UBXN1 represses protein translation. (a) Immunoblot of puromycin incorporation into HeLa Kyoto wildtype and UBXN1 KO cells. Cells were pulsed with 1 μM puromycin for 30 minutes after pre-treatment with 1.5 μM thapsigargin for 1 hour. **(b)** Quantifications of the entire lanes corresponding to **3.1a.** (n ≥ three biologically independent experiments). Data are means ± SEM (*, **, ***, and **** where $P < 0.05$, 0.001, 0.001, and 0.0001, respectively.). One-Way ANOVA

3.2: UBXN1 represses the expression of ATF4 and GADD34 under ER stress.

Previous studies from our lab had found that loss of UBXN1 causes significantly higher expression of p-eif2A which leads to translation attenuation and triggers selective translation of ATF4 mRNA [71]. GADD34 is a transcription factor activated by ATF4, which functions as a scaffolding protein to bring protein phosphatase 1 (PP1) close to p-eif2A and hence causing its dephosphorylation. UBXN1 was depleted from Kyoto wild-type cells for 48 hours and then treated with dithiothreitol (DTT) for a time course of 0-8 hours to induce ER stress. Lysates were resolved by SDS-PAGE and the expression levels of BiP, ATF4, and GADD34 were evaluated by immunoblot. A time-dependent

increase in each protein was observed in wildtype cells and siUBXN1 cells after stress, however, the increase in siUBXN1 cells was more robust at the 2hr and 4hr timepoint (Figure 3.2 a-c). Taken together, these results show that UBXN1 depletion robustly activates ATF4, which is a downstream target of PERK activation, and GADD34, which is a downstream target of ATF4.

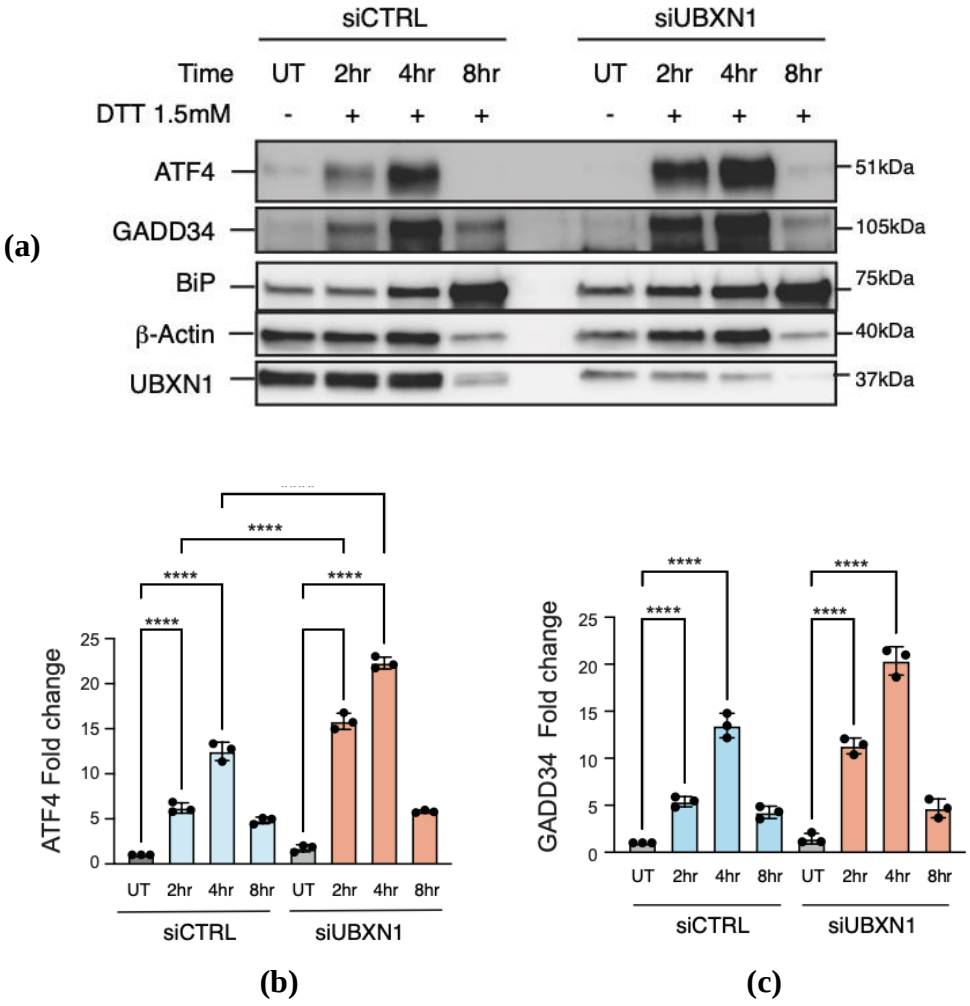


Figure 3.2: UBXN1 represses the expression of ATF4 and GADD34 under ER stress. UBXN1 was depleted by siRNA transfection from Kyoto wild-type cells for 48 hours for all experiments. **a.** Cells were treated with 1.5 mM DTT for the indicated time points (0-8 hours). **b. and c.** Ratio (siUBXN1/siCtrl) of the fold change of ATF4 and GADD34 Untreated cells and at 2, 4, and 8-hour timepoint corresponding to **3.3c**. (n = three biologically independent samples). Data are means ± SEM (*, **, *** and **** where P < 0.05, 0.001, 0.001, and 0.0001, respectively.). One-way ANOVA with Tukey's multiple comparisons test

3.3: UBXN1 depletion upregulates ATF4 targets involved in translation initiation.

RNA-seq studies have revealed that ATF4 and CHOP co-activate a host of genes involved in translation and protein synthesis including amino-acyl tRNA synthetases, translation initiation factors, and amino acid transporters[31]. We wanted to determine whether UBXN1 deletion leads to an increase in protein synthesis through the ATF4 pathway. Hence, we performed quantitative real-time PCR (qPCR) to measure relevant ATF4-CHOP targets [61] including aminoacyl tRNA synthetases (*wars, lars*), *gadd34,* ribosomal proteins, *ul24* (Rpl26) and initiation factors (*Eifs2, Eifc3, and Eif5*) and determine whether their levels peak earlier and persist longer in UBXN1 depleted cells. Leucine-tRNA synthetase (*lars*) and tryptophanyl-tRNA synthetase (*wars*) catalyze the attachment of a specific amino acid to the 3' end of its corresponding tRNA, which is a crucial step in the synthesis of proteins. They do this by converting the corresponding amino acid into an energetically dense aminoacyl-adenylate intermediate that transfers the amino acid to the tRNA [71]. eiFs2 forms a ternary complex with GTP and initiator tRNA and hence helps in the translation initiation. EiF5 acts as a GTPase Activator Protein (GAP), hence facilitating the hydrolysis of GTP by eiF2 once the AUG codon is recognized [72]. UBXN1 was depleted from Kyoto wildtype cells for 48 hours and then cells were treated with DTT for 4 hours. Interestingly, we saw a 5-fold increase in RPL-26 expression in the ER-stressed UBXN1 depleted cells as compared to the control (Fig 3.3a). We also saw a 3-fold and a 2.5-fold increase in eiFS2 and GADD34 expression respectively (Fig 3.3 b and c). Transcript levels of WARS and LARS also increased by 2 and 2.5-fold respectively under ER Stress (Fig 3.3 e and f). These results suggest that

UBXN1 might be involved in prolonging the restart of translation as it represses the synthesis of ATF4, and the downstream transcriptional targets evaluated here.

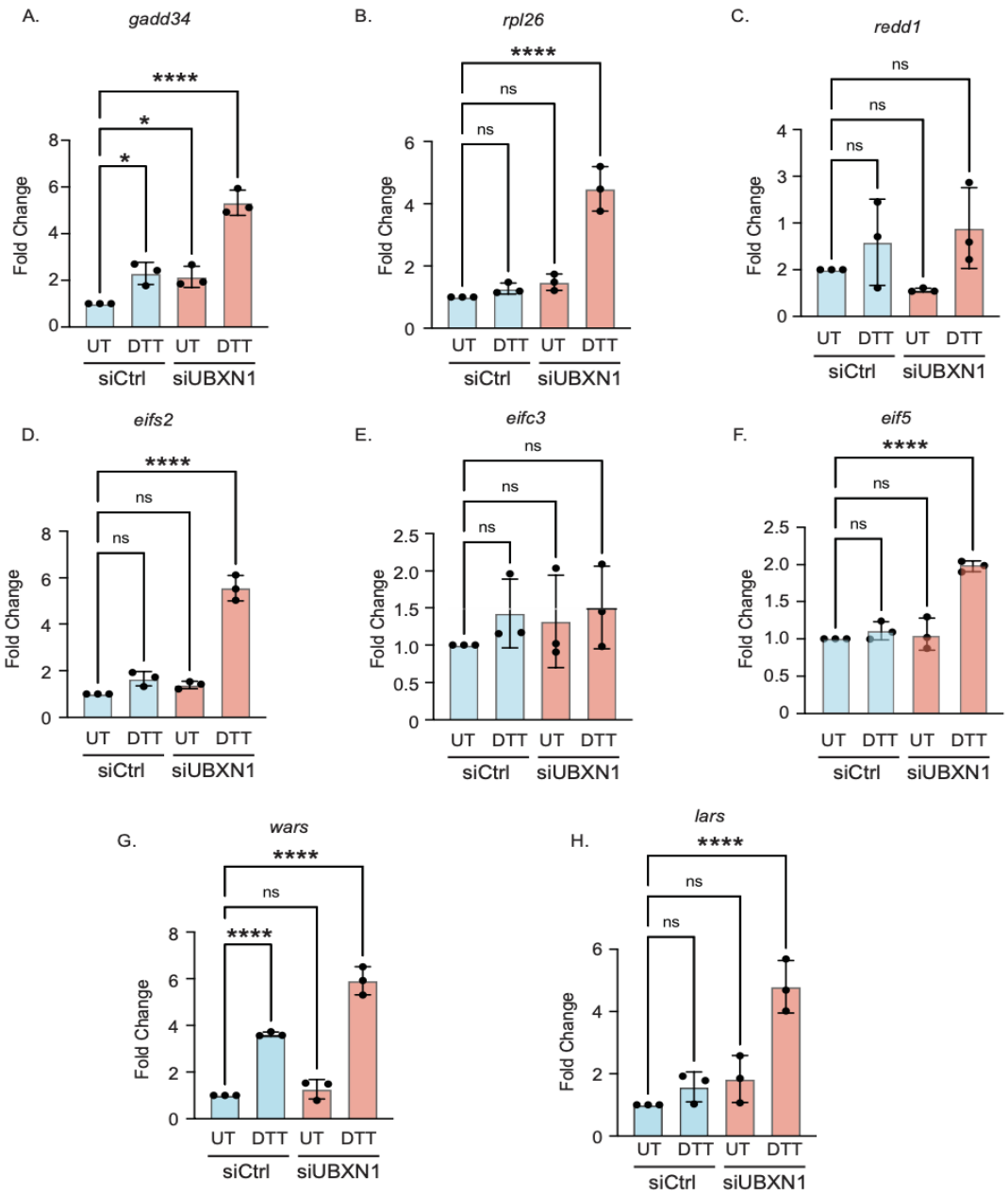


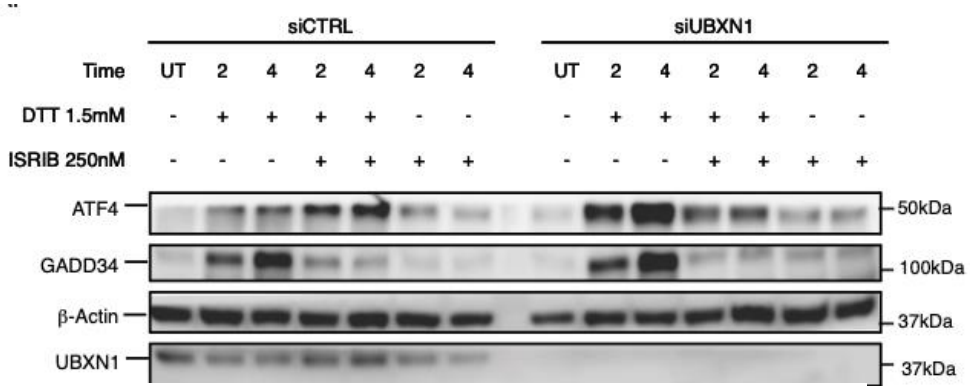
Figure 3.3: UBXN1 depletion upregulates ATF4 targets involved in translation initiation. UBXN1 was depleted by siRNA transfection from Kyoto wild-type cells for 48 hours for all experiments. Transcript levels of genes **(a)** *gadd34* **(b)** *rpl26* **(c)** *redd1* **(d)** *eifs2* **(e)** *eifc3* **(f)** *eif5* **(g)** *wars* **(h)** *lars* were measured in siCTRL and siUBXN1 cells with or without a 4-hr. treatment with 1.5mM DTT (n = three biologically independent samples). Data are means ± SEM (*, **, ***, **** where P < 0.05, 0.01, and 0.001, 0.0001 respectively.) **(a - h)** One-way ANOVA with Tukey's multiple comparisons test.

3.4: Small Molecule ISRIB rescues ATF4 activation under ER Stress.

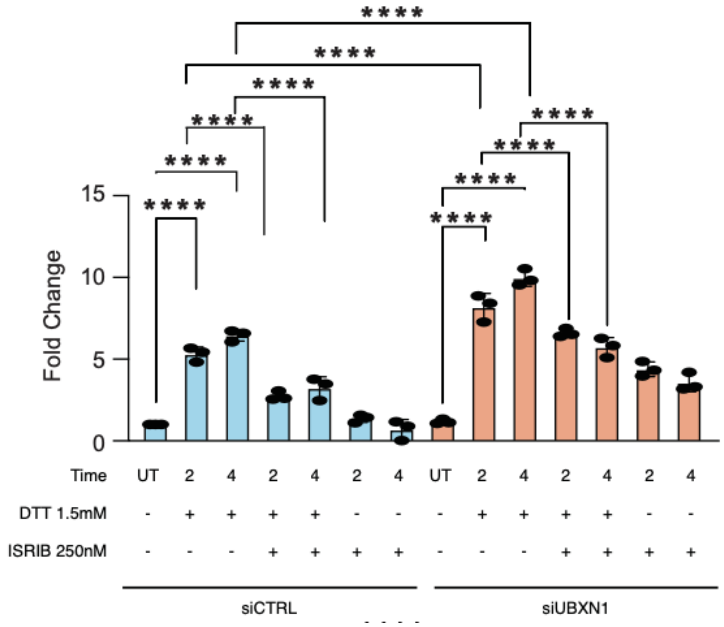
So far, we have established that UBXN1 represses the expression of ATF4, GADD34, and other transcriptional targets of ATF4. Next, we wanted to see if blocking the phosphorylation of eif2A rescued the levels of ATF-4 and GADD34 in UBXN1-depleted cells. We used a small-molecule ISR inhibitor, ISRIB for our studies. P-eIF2α inhibits the guanine nucleotide exchange factor eIF2B, which converts inactive eIF2•GDP to active eIF2•GTP. ISRIB rescues translation while eif2A is phosphorylated by facilitating the assembly of more eiF2B, hence, overriding phospho-eiF2a inhibition and enabling translation even with ER stress [73]. We evaluated ATF4 activation in response to acute depletion of UBXN1 with siRNA and treatment with ISRIB to ascertain if blocking eiF2A phosphorylation would rescue ATF4 levels. UBXN1 was depleted from Kyoto wildtype cells for 48 hours and cells were treated with dithiothreitol (DTT), ISRIB, and a combination of DTT and ISRIB for a time course of 0-4 hours. Lysates were resolved by SDS-PAGE and immunoblotted to measure the expression levels of ATF4, and induction of GADD34.

We observed that the levels of ATF4 and GADD34 were reduced by ISRIB in the presence of DTT. This suggests that the elevated levels of ATF4 translation are due to the PERK-eiF2a pathway (Fig 3.4 a,b, and c). We see that the levels of ATF4 and GADD34 were turned back over by ISRIB under ER stress at both 2-hr. and 4-hr. treatments in the presence of DTT. This ascertains that the elevated levels of ATF4 translation are due to the PERK-eiF2a pathway.

(a)


siCTRL
siUBXN1
Time UT 2 4 2 4 2 4 UT 2 4 2 4 2 4
DTT 1.5mM
ISRIB 250nM
ATF4
GADD34
β-Actin
UBXN1
50kDa
100kDa
37kDa
37kDa

(b)


Fold Change
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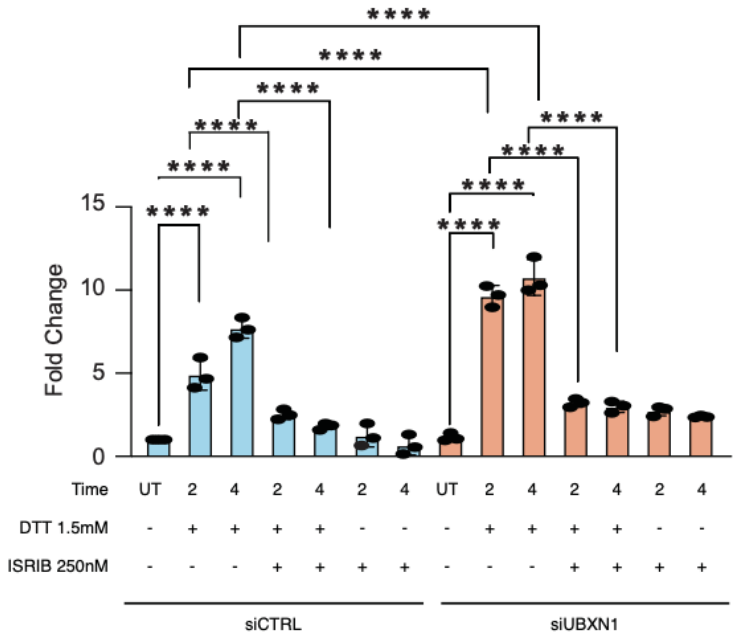
15
****
Time UT 2 4 2 4 2 4 UT 2 4 2 4 2 4
DTT 1.5mM
ISRIB 250nM
siCTRL
siUBXN1

(c)


Fold Change
0
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Time UT 2 4 2 4 2 4 UT 2 4 2 4 2 4
DTT 1.5mM
ISRIB 250nM
siCTRL
siUBXN1

Figure 3.4: Small Molecule ISRIB rescues ATF4 activation under ER Stress. UBXN1 was depleted by siRNA transfection from Kyoto wild-type cells for 48 hours for all experiments. **(A).** Cells were treated with 1.5 mM DTT, with or without 250nM ISRIB and IRSIB alone for the indicated time points (0-4 hours). **(b).** Ratio (siUBXN1/siCtrl) of the fold change of **GADD34** of Untreated cells and treated cells at 2 and 4-hr. timepoint corresponding to **3.4a**. **(c)** Ratio (siUBXN1/siCtrl) of the fold change of **ATF-4** of Untreated cells and treated cells at 2 and 4-hr. timepoint corresponding to **3.4A** (n = three biologically independent samples). Data are means ± SEM (*, **, **** where $P < 0.05$, 0.001, and 0.001, respectively.). One-way ANOVA with Tukey's multiple comparisons.

3.5: UBXN1 represses ATF4 translation under ER stress independent of the mTOR pathway.

ATF4 can also be induced by activation of the mTOR pathway. Under nutrient-rich conditions, the mTOR complex 1 (mTORC1) is active and phosphorylates 4E-BPs (4E-binding proteins). When phosphorylated, 4E-BPs are unable to bind to the translation initiation factor eIF4E. Free eIF4E can then form a complex with other proteins to initiate the translation of ATF4 mRNA [74]. We wanted to evaluate if the mTOR pathway was also involved in this increased expression of ATF4 in UBXN1-depleted cells.

To understand this, we used Torin1, which is a potent ATP competitive inhibitor of both mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [75, 76]. Kyoto WT and UBXN1 KO cells were treated with dithiothreitol (DTT), a combination of DTT and ISRIB, and a combination of DTT and Torin1 for a time course of 4 hours. Lysates were resolved by SDS-PAGE and immunoblot to measure the expression levels of ATF4, and induction of GADD34 in response to ER stress (Fig 3.5a). As expected, ISRIB decreased ATF4 levels under ER stress, however, Torin1 failed to produce a similar effect (Fig 3.5a-c). This suggests that UBXN1 represses ATF4 translation under ER stress independent of the mTOR pathway.

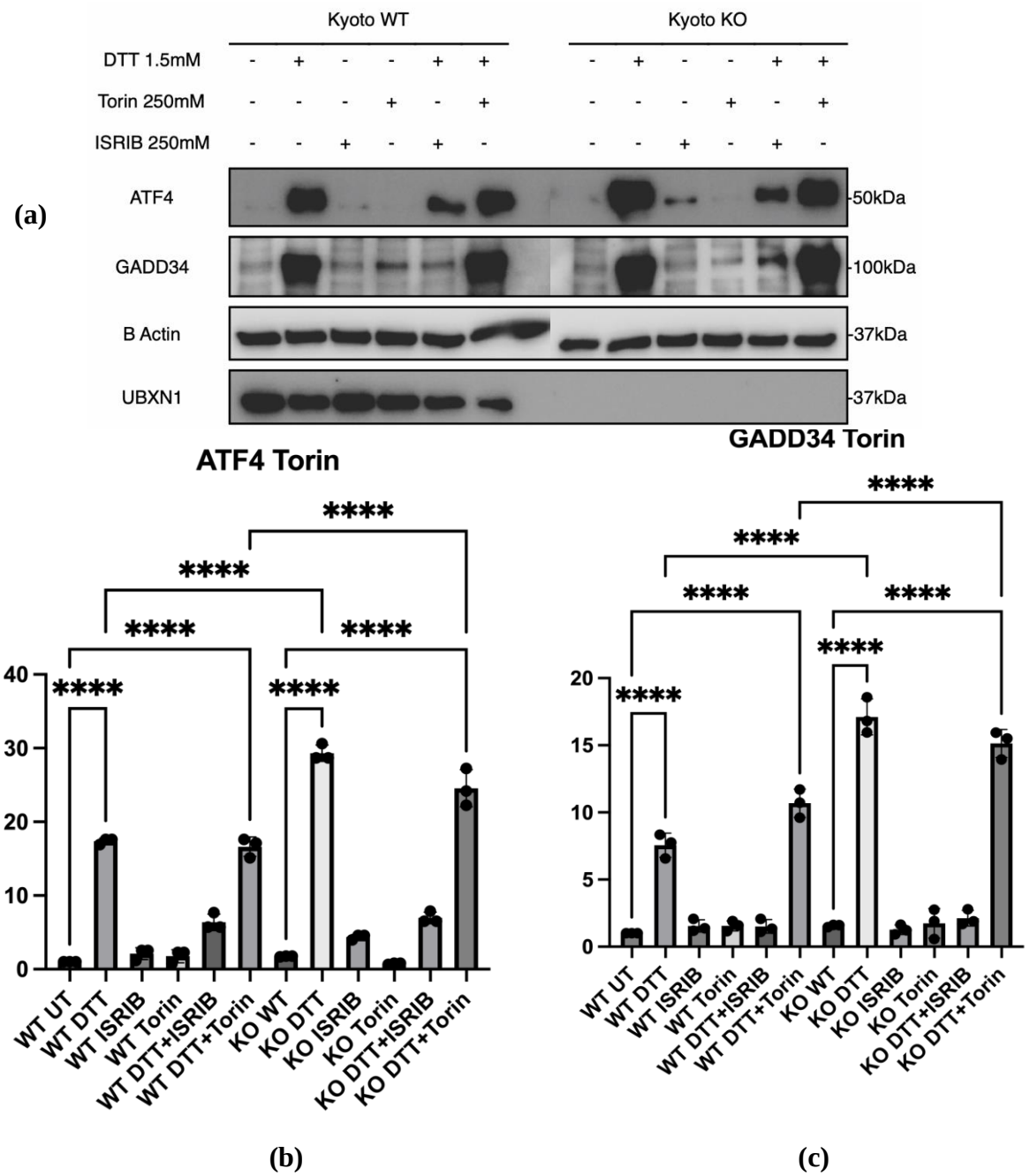
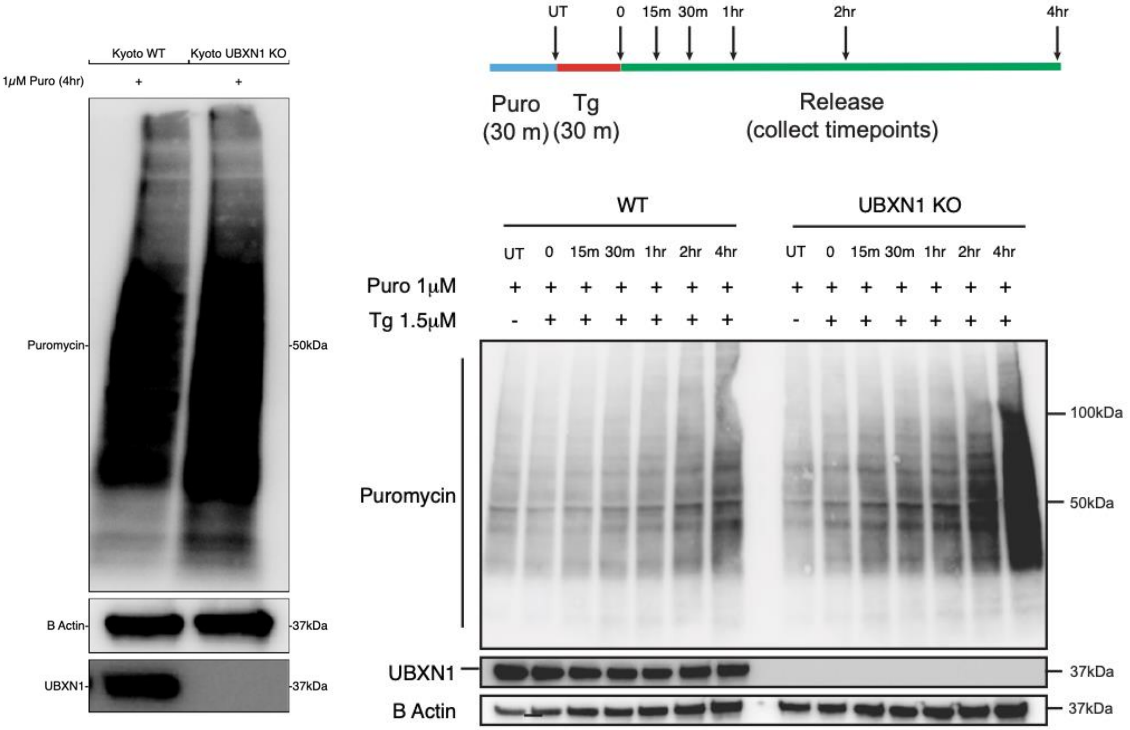


Figure 3.5: UBXN1 represses ATF4 translation under ER stress independent of the mTOR pathway. (a) **I**mmunoblot of Kyoto wildtype and UBXN1 KO cells treated with 1.5 mM dithiothreitol (DTT), 250nM ISRIB, and 250mM Torin alone or DTT in combination with ISRIB and Torin for 4-hrs. (b) and (c) Band intensity quantifications of ATF4, and GADD34 for Untreated and 4-hour treatment timepoint corresponding to 3.5A. ratio of the fold change (KO/WT) is reported. (n = three biologically independent samples) Data are means ± SEM (**** where $P < 0.001$.). One-way ANOVA with Tukey's multiple comparisons.

3.6: UBXN1 prolongs translation restart initiation after ER stress.

Since we saw an increase in the transcript levels of tRNA synthetases and other translation initiation factors like *eifs2* and *eif5*, we wanted to evaluate if the deletion of UBXN1 caused an early restart of translation in cells. Kyoto WT and UBXN1 KO cells were pretreated with thapsigargin and then washed with Phosphate Buffered Saline (PBS) and incubated with Puromycin for a time course of (30mins to 4 hrs.). We used a monoclonal puromycin antibody to measure the quantity of puromycilated proteins. Interestingly, we observe in UBXN1 KO cells, translation restart occurs earlier than in wild-type cells (at 15 min) (Fig 3.6b). These results taken together indicate that UBXN1 depletion causes an early translation restart in cells.

(a) **(b)**



(c)

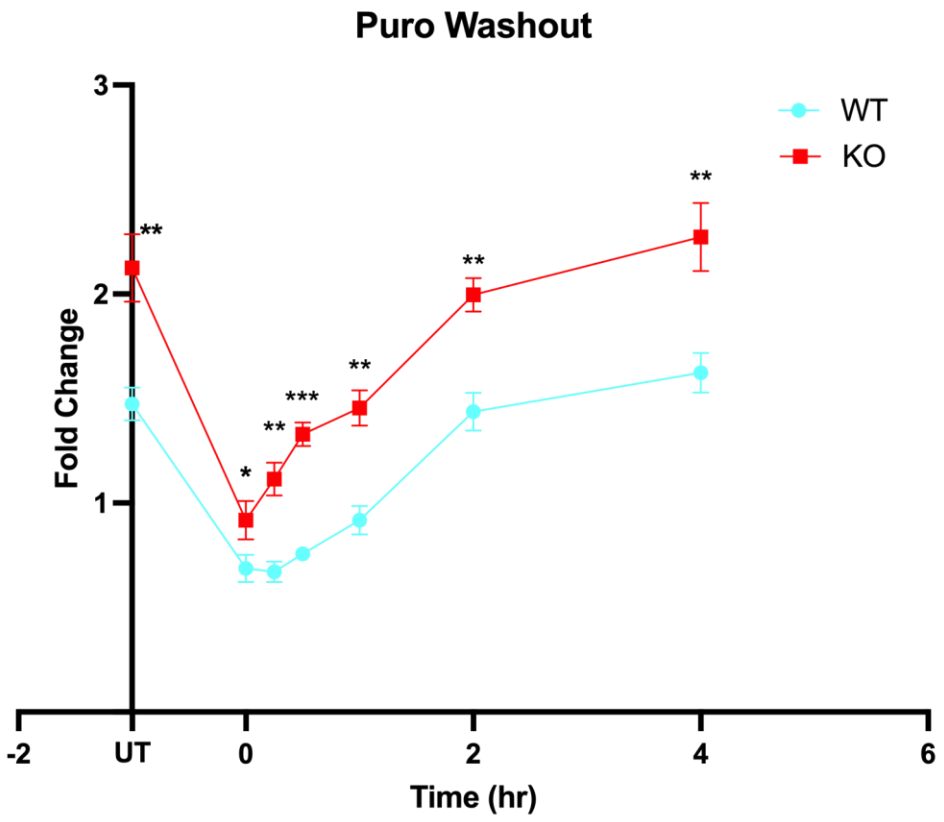
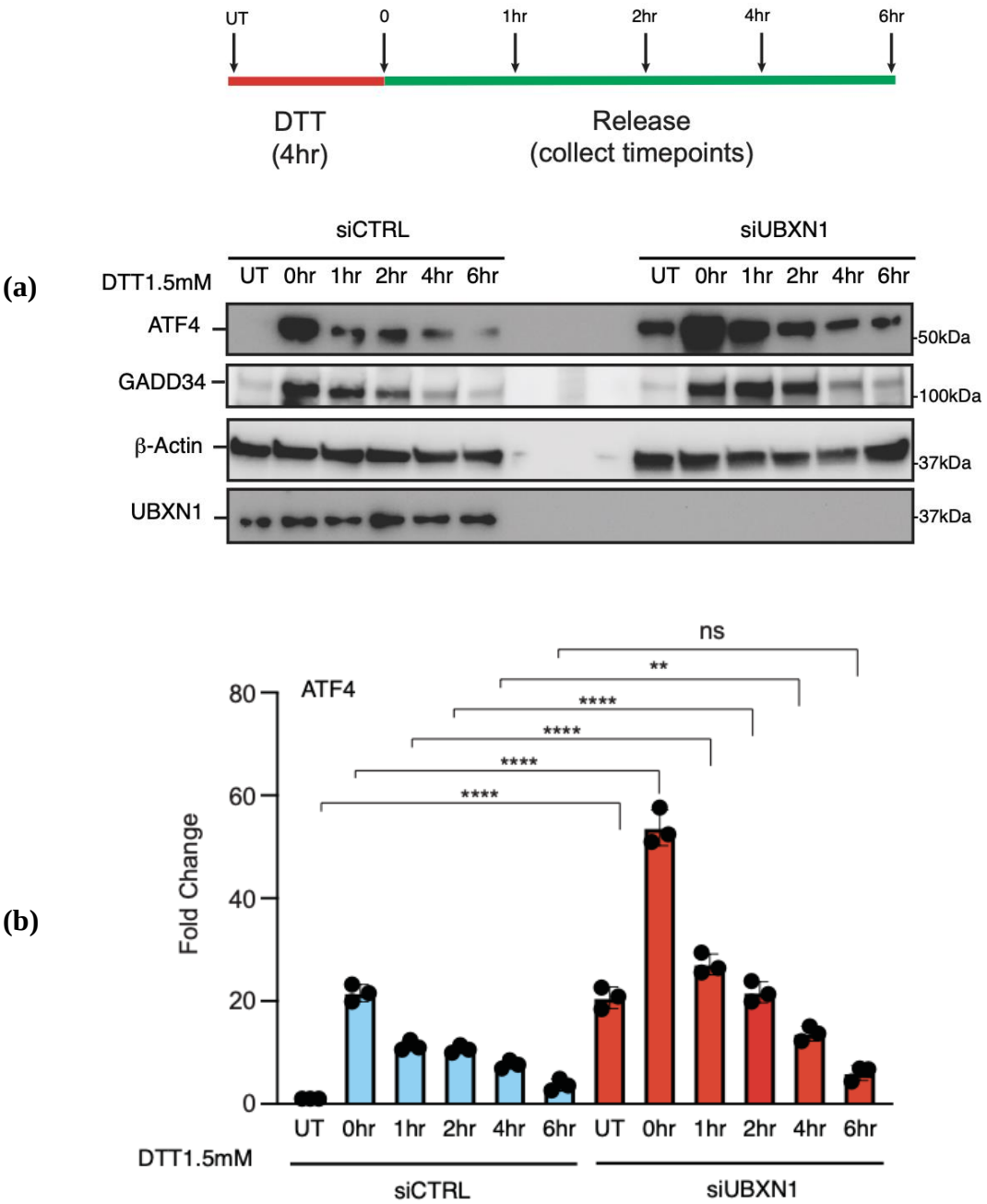


Figure 3.6: UBXN1 prolongs translation restart initiation after ER stress. Immunoblot of puromycin incorporation into Kyoto wildtype and UBXN1 KO cells. Cells were pulsed with 1 µM puromycin for **(a)** 4-hr **(b)** 30 min, 45min, 1hr, 1.5hr, 2.5hr, and 4-hr after pre-treatment with 1.5 µM thapsigargin for 30 minutes. **(c)** Quantifications of the entire lanes corresponding to 3.6a and 3.6b ($n \geq$ three biologically independent experiments). Data are means ± SEM (*, ** and *** where $P < 0.001$ and 0.0001, respectively.) Unpaired T-Test (Each timepoint is compared between WT and KO).

3.7: UBXN1 represses retention of GADD34 after ER stress.

We have established that UBXN1 delays the restart of translation via the ATF4 - GADD34 axis. We next evaluated GADD34 levels after ER stress to determine whether

their levels persist longer in UBXN1 KO cells. We pre-treated Kyoto WT and UBXN1 KO cells with DTT for 30mins washed with PBS and then cultured with complete media for a time course of 0 to 6 hours. We found that both ATF4 and GADD34 persisted longer time in UBXN1 KO cells (Fig 3.7 a, b, and c).



(c)

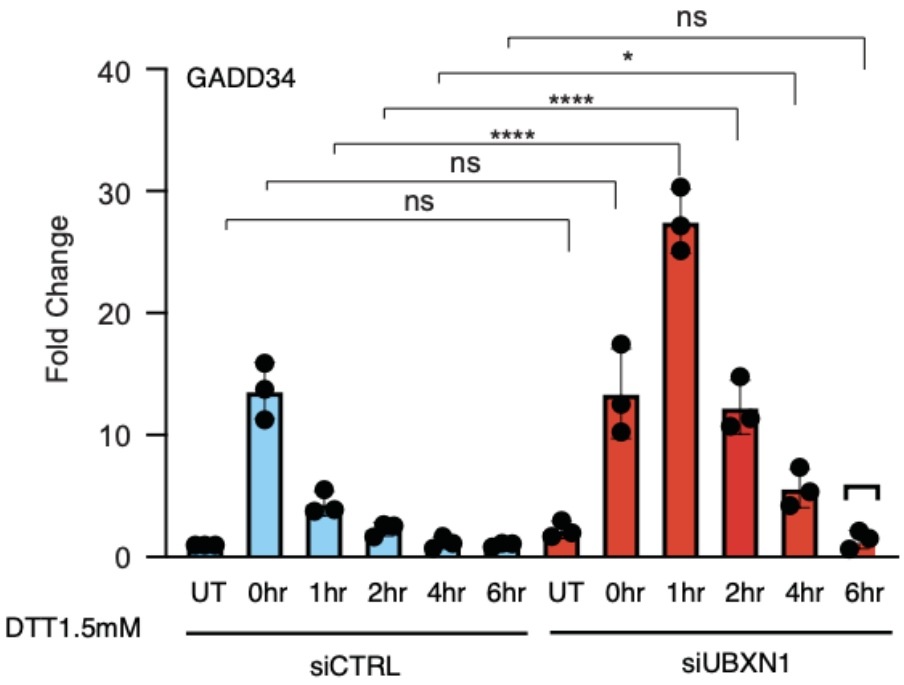


Figure 3.7: UBXN1 represses retention of GADD34 after ER stress. (a) Immunoblot of Kyoto Wildtype and UBXN1 KO cells untreated and with 1.5mM DTT pre-treatment, washed with PBS, and released with complete media. Cells were harvested at the indicated time points from 0-6hrs. **(b) and (c)** Band intensity quantifications of ATF4, and GADD34 for Untreated and 6-hour treatment timepoint corresponding to 3.5A. ratio of the fold change (KO/WT) is reported. (n = three biologically independent samples) Data are means ± SEM (*, ****where P < 0.05, and 0.001, respectively.). One-way ANOVA with Tukey's multiple comparisons.

Chapter 4: Discussion.

4.1: UBXN1: A Novel Regulator of Translation during ER Stress.

This work has demonstrated a novel role for UBXN1, an adapter protein for the p97 AAA-ATPase, in controlling the translation of proteins during ER stress. Notably, these results indicate a p97- p97-independent role for UBXN1 in suppressing the resumption of translation after ER stress. The surprising discovery contributes to our expanding understanding of the complex roles of UBXN1 in maintaining cellular proteostasis.

4.2: Impact of UBXN1 Deficiency on Protein Homeostasis.

A possible connection between UBXN1 and ER function was suggested by the preliminary TMT proteomics results, which revealed a higher abundance of ER-localized proteins in UBXN1 KO cells [71]. Puromycin incorporation experiments in UBXN1 KO cells revealed upregulated translation, which may provide a plausible reason for the elevated protein abundance. Additionally, the fact that UBXN1 KO cells continued to exhibit increased translation even in the face of ER stress indicates that UBXN1 is essential for controlling the cellular response to protein misfolding (**Figure 3.1a & b**). The delicate balance between the synthesis and degradation of proteins may be disrupted by this dysregulation of protein synthesis, which might result in a buildup of misfolded proteins and jeopardize cellular proteostasis [79].

4.3: Dissecting the UBXN1-PERK Signaling Axis.

Our lab's earlier research revealed that UBXN1 deletion results in noticeably increased p-eif2A expression, which inhibits translation and induces selective translation of ATF4 mRNA. These studies effectively demonstrate that UBXN1 deficiency leads to the activation of the PERK arm of the integrated stress response (ISR) as we see increased expression of ATF4 and its transcriptional target, GADD34 (**Figure 3.2a - c**). It has been demonstrated in earlier research that forced over-expression of ATF4 expression in combination with ER stress overrides translation arrest, leading to the resumption of protein synthesis, which ultimately results in cell death[31, 77].

This shows that ATF4 could function as a timer, controlling the amount and duration of translation resumption following ER stress. Carefully adjusting ATF4 activation is necessary to guarantee that translation only starts again when ER stress is reduced; otherwise, cell death occurs because of the ATF4 pro-apoptotic target CHOP. Therefore, increased ATF4 levels in UBXN1 KO cells could make it possible for protein synthesis to proceed even in the presence of ER stress, worsening the UPR. The observed upregulation of PERK phosphorylation, ATF4 protein levels, and GADD34 protein levels in UBXN1 KO cells suggests that UBXN1 might function upstream of the PERK-ATF4 pathway to modulate its activation. This finding aligns with previous studies demonstrating the essential role of PERK-ATF4 signaling in regulating protein synthesis during ER stress [33,80].

4.4: Functional Implications: Early Translation Restart and Proteostasis.

The puromycin washout experiment (**Figure 3.6b & c**) suggests that the loss of UBXN1 causes the early translation restart seen in UBXN1 KO cells after ER stress. This is likely due to an ATF4-mediated increase in tRNA synthetases, translation initiation factors as well as GADD34, which causes the dephosphorylation of eif2A (**Figure 3.2**). Under physiological settings, cells can prioritize ER stress resolution by lowering the amount of protein in the ER through a temporary halt of translation [80, 81]. In UBXN1 KO cells, translation may resume too soon, which might cause misfolded protein buildup and compromise cellular proteostasis [71]. The downstream effects of UBXN1 loss on protein quality control systems and cellular health require more investigation. This can involve analyzing protein aggregation or assessing the survival of the UBXN1 KO cells under extended ER stress conditions.

4.5: UBXN1 and the mTOR Pathway.

The mTOR pathway is a known regulator of translation initiation, and its activation can also lead to ATF4 upregulation through a distinct mechanism (9). Our results suggest that UBXN1 does not regulate translation through the mTOR pathway because ISRIB decreased ATF4 levels under ER stress, however Torin1 failed to produce a similar effect (Fig 3.5a-c). However, further investigation is still needed to definitively rule out a potential interplay between these two pathways in UBXN1's function. For instance, studies could explore the phosphorylation status of key mTORC1 targets such as S6K1 in UBXN1 KO cells under ER stress conditions [78].

We still need to determine how exactly UBXN1 suppresses translation. The observation of a delayed decline in ATF4 and GADD34 protein levels in UBXN1 KO cells following ER stress resolution implies a possible function of UBXN1 in controlling the turnover or stability of ATF4 and GADD34 proteins. These factors and UBXN1 may interact through protein-protein interactions, or UBXN1 may affect their mRNA stability through unknown processes. Further investigation focusing on mRNA decay may be able to clarify these possible relationships.

4.6: Clinical Implications and Future Directions

Protein misfolding and ER stress are implicated in various human diseases, including neurodegenerative disorders and cystic fibrosis [10, 11]. Our study on the role of UBXN1 in regulating translation during ER stress opens new avenues for exploring potential therapeutic strategies. Understanding the mechanisms by which UBXN1 modulates the PERK-ATF4 pathway could lead to the development of drugs that target this pathway for the treatment of diseases associated with ER stress.

We discovered that UBXN1 regulates the quantity of ER proteins via repressing translation. However, a crucial question to consider is if UBXN1 directly controls the translation of ER proteins and if that is the case, which ones? Although the exact mechanism of ER stress regulation in UBXN1-null cells remains unknown, it is plausible that UBXN1 specifically represses the translation of ER proteins, and that the increased translation of ER proteins in these cells upsets the equilibrium of ER proteostasis and

triggers the UPR. This would confirm UBXN1's significance as a preventive ER quality control element. In the future, we will use mass spectrometry on puromycilated proteins from both wildtype and UBXN1 KO cells to identify the proteins that are translated in UBXN1 KO cells. The puromycin analog O-propargyl-puromycin (OP-puro), which has an alkyne group at the end, will be used. Copper(I)-catalyzed azide-alkyne cycloaddition (click chemistry) can be used to react nascent polypeptide-OP-puro conjugates with biotin-azide for affinity isolation[79]. These peptides would then be labeled with TMT and further analyzed using LC-MS. This would help us interrogate the translatome of UBXN1 KO cells.

Next, as previous research from our lab has found UBXN1 interacting with polysomes [33], identifying the ribosomal proteins that interact with UBXN1 can help us get more insight into the mechanism by which UBXN1 suppresses translation. To evaluate this, we plan to use TURBO-ID, coupled with proximal proteomics [80].

Chapter 5: Bibliography

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