THE INVESTIGATION OF SMALL HEAT SHOCK PROTEIN EXPRESSION UNDER ENDOPLASMIC RETICULUM STRESS TREATED C2C12 MYOTUBES

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ABSTRACT

Skeletal muscle function declines with age and is associated with reduced health span. Dysregulated proteostasis is a key hallmark of ageing, characterised by an imbalance between protein synthesis, breakdown, and folding within the cell. To counteract this, chaperones are expressed to prevent the accumulation of misfolded protein species. The endoplasmic reticulum (ER) is crucial for maintaining protein and ensuring the newly synthesized proteins are folded correctly to achieve their functional form, which can be disrupted under stressful conditions. Research, including our lab preliminary data, revealed that ER stress-related and unfolded protein response (UPR) signaling pathways proteins are altered in aged mouse skeletal muscle. Furthermore, another group of proteins, small heat shock protein (sHSP), which performs holdase function under general protein aggregation, also recorded an increase in the data. We hypothesized that ER stresses stimulate the expression of sHSP to prevent the formation of protein aggregates. Herein, this thesis used two different pharmacological ER stress inducers, Tunicamycin and Thapsigargin, in C2C12 myotubes to investigate the regulation of sHSP under ER stress. Our results show that HSPB1 and HSPB6 decrease throughout ER stress whilst HSPB5 and HSPB7 remain unchanged. The results show that ER stress regulates sHSP expressions throughout two types of ER stress inducers. Altogether, this thesis improves the understanding of sHSP regulation under various types of unfolded protein responses in skeletal muscle.

KAEYWORDS: skeletal muscle, endoplasmic reticulum, small heat shock protein, proteostasis, chaperone, unfolded protein response

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ABBREVIATION

ACD	α-crystallin domain
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
ATP	adenosine triphosphate
APP	amyloid precursor protein
BAX	BCL-2 associated X protein
BAK	BCL-2 homologues antagonist/killer
BCL-2	B cell lymphoma 2
BiP	immunoglobulin heavy chain-binding protein
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CTR	C-terminal region
°C	degrees Celsius
CO2	carbon dioxide
CHOP	C/EBP Homologous Protein
CReP	constitutive repressor of eIF2a phosphorylation
CTRL	control
Cyt-C	cytochrome c
cvHSP	cardio-vascular heat shock protein
DM1	myotonic dystrophy type 1
DMEM	Dulbecco's modified eagles' medium
DMSO	dimethyl sulfoxide
eIF2α	eukaryotic translation initiation factor 2 alpha
ER	endoplasmic reticulum

ERAD	ER-associated degradation
ERCC	endoplasmic Reticulum quality control
ERK1	extracellular signal-regulated kinase 1
ERQC	ER quality control
Filamin C	FLNC
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GADD34	growth arrest and DNA damage-inducible protein 34
GEF	guanine nucleotide exchange factor
GDP	guanosine diphosphate
GTP	guanosine-5'-triphosphate
h	hours
HSPs	heat shock proteins
IRE1	inositol-requiring enzyme 1
kDa	kilodalton
mM	millimtre
mg	milligram
mL	milliliter
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
MHC	major histocompatibility complex
NTR	N-terminal region
RIDD	regulated Ire1-dependent decay
sHSP	small heat shock protein
PAGE	polyacrylamide gel electrophoresis
PDI	protein Disulfide Isomerases
PERK	PRKP-like ER kinase

pH	potential of hydrogen
PTM	post-translational modifications
PVDF	polyvinylidene fluoride
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
P-PERK	phosphorylated PERK/phosphorylated PERK
P-eIF2α	phosphorylation of eIF2α/phosphorylated eIF2α
PP1	protein phosphatase 1
PN	proteostasis network
Ser	serine
SDS	sodium dodecyl-sulfae
S1P	site-2 protease
S2P	site-1 protease
s-IBM	sporadic inclusion-body myositis
SR	sarcoplasmic reticulum
TBS-T	Tris-buffered saline Tween-20
Thr	threonine
tRNA	transfer ribonucleic acid
µL	microliter
UPR	unfolded protein response
UPS	ubiquitin-proteasome system
UPRE	unfolded protein response element
X-BP1	X-box binding protein-1

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction of skeletal muscle

Skeletal muscle is one of the integral tissues within the human body, which accounts for ~40% of our body mass (Janssen *et al.*, 2000). Skeletal muscles function as a dynamic tissue and perform multiple roles: Skeletal muscles a peculiar as they are essential for posture and locomotion (Bottinelli and Reggiani, 2000). Besides supporting basic body functional movement, skeletal muscle also plays a role in energy metabolism: skeletal muscle stores over 80% of carbohydrates and it is responsible for glucose uptake and regulation of blood glucose concentrations via gluconeogenesis in the post-absorptive state (Felig, 1973; DeFronzo *et al.*, 1985).

Due to the large proportion content and the crucial role that skeletal muscle functioning in the body, it is not surprising that failure of skeletal muscle maintenance results in deleterious consequences. The decline of skeletal muscle has been shown associated with type 2 diabetes (Srikanthan and Karlamangla, 2011), cancer (Caan *et al.*, 2018), and obesity (Wolfe, 2006), It has also been reported that loss of muscle mass is a reliable index to predict the longevity in older adults (Srikanthan and Karlamangla, 2014).

Besides hurting an individual's quality of life, impaired skeletal muscle also results in extra economic burdens to healthcare systems, over £2.5 billion in UK healthcare costs were costed by muscle wasting annually (Pinedo-Villanueva *et al.*, 2019).

1.2 Role of Proteostasis, chaperone family and the effect of ageing

1.2.1 Introduction of proteostasis and chaperone family

More than 11,000 different proteins exist in human cells at any given time and proteins in polypeptide chain structures are required to fold into well-defined 3D structures to allow a proper of cellular functions to cooperate throughout its lifetime (Klaips, Jayaraj and Hartl, 2018). A proteostasis network (PN) is a precise control mechanism that manipulates proteins required within a mammalian cell to be folded (Hipp, Kasturi and Hartl, 2019). For the maintenance of healthy cellular proteostasis, a complicated and precise surveillance network of cellular mechanisms is working and inspecting protein biology from synthesis and folding to trafficking and clearance (Calamini and Morimoto, 2012).

A group of proteins, namely chaperone, is one of the primary arsenal for the proteostasis network and related to the majority of the interaction within the proteostasis network. The chaperone family ensures the protein is in correct folding before sending to its final destination, and also ensures the protein maintains within a correct folding/functional mode throughout its cellular lifetime (Cuervo and Wong, 2014). Moreover, if this arm of PN fails, the secondary arsenal will take over and degrade the proteins that are damaged, unfolded, and aggregated within the proteome. The degradation network includes the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system (Vilchez, Saez and Dillin, 2014).

Upon failure of the two-surveillance system mentioned above, loss of proteostasis begins to occur and causes detrimental effects on overall cellular physiology and life. It is therefore not surprise to notice that loss of proteostasis is considered one of the hallmarks of ageing (López-Otín *et al.*, 2013). and indeed the loss is robustly linked to the onset and progression of age-related morbidities (López-Otín *et al.*, 2013)especially age-related neurodegenerative diseases like Alzheimer's disease, Parkinson's disease (Hartl, Bracher and Hayer-Hartl, 2011) and some eye-related disease like cataract (López-Otín *et al.*, 2013).

1.2.2 Muscle-related disease related to UPR

Recently, the collapse of proteostasis in ageing is also reported to cause muscle-related diseases such as sarcopenia (Jiao and Demontis, 2017)and myopathy disease (Iannibelli *et al.*, 2023). Chaperones (i.e. BiP) have shown an increase under myositis patients and a mouse myositis model (i.e major histocompatibility complex (MHC) class I -transgenic mice) (Nagaraju *et al.*, 2005). A separate study also reported myositis induced by overexpression of MHC-I mice model cross with immunodeficient Rag2-/-has an upregulation of chaperones and proteostasis-related protein (i.e. ATF6 and BiP) respectively (Fréret *et al.*, 2013).

Sporadic inclusion-body myositis (s-IBM) is a progressive age-associated myopathy that happens in adults primarily over the age of 50. s-IBM is characterized by chronic muscle inflammation and muscle weakness in the proximal muscle and distal muscles (Askanas and Engel, 2001). Literature has reported that proteostasis stress markers in the skeletal muscle (i.e. ATF4, CHOP, ATF6, BiP, and XBP1) had increased in s-IBM patients (Nogalska *et al.*,

2015). A separate study also reported that chaperone protein like BiP has an increase in s-IBM patients when compare to control muscle biopsies (Vattemi *et al.*, 2004).

1.2.3 Current Understanding of UPR in Skeletal Muscle

Dysfunction of the proteostasis network throughout ageing and result in causing diseases, understanding of proteostasis in other organisms is better known when comparing to skeletal muscle. Although studies report that the upregulation of ER-related chaperones has been recorded in skeletal disease, the relationship between UPR and skeletal muscle remains unclear.

A recent study reported that chaperone-like BiP has a higher regulation in older adults in their skeletal muscle and is negatively associated with their muscle strength and power (J. Max Michel, Joshua S. Godwin, Daniel L. Plotkin, Mason C. McIntosh *et al.*, 2024). Still, more research is needed to have a better understanding of how UPR is regulated in skeletal muscle.

1.3 Endoplasmic Reticulum and its role in protein quality control

1.3.1 General background about ER

The endoplasmic reticulum (ER) is called as sarcoplasmic reticulum (SR) in skeletal muscle, which is responsible for regulating proteostasis and calcium homeostasis (Bohnert, McMillan and Kumar, 2018).ER was discovered and named “ergastoplasm” at the end of the 19th century by Garnier in 1897 and was named ER in 1945 (Porter, Makuck and Rivkees, 2002). ER is a membrane-bound organelle that is responsible for protein synthesis, protein folding, modification, and synthesis (Ashby and Tepikin, 2001; Fagone

and Jackowski, 2009). ER nearly synthesised two-thirds of the protein before proteins are sent to their final destination in general cell tissues (Hipp, Kasturi and Hartl, 2019).

Two types of ER membranes have been characterized, namely rough ER and smooth ER. Smooth ER is mainly involved in lipid synthesis, detoxification, and calcium storage. Meanwhile, rough ER assists in proteostasis regulation since bounded ribosomes are attached to the cytoplasmic surface of the rough ER. Ribosomes are involved in synthesizing proteins that are destined for secretory (i.e. hormones/antibodies), membrane, organ, and lysosomal protein mainly (Bruce Alberts, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, 2002).

1.3.2 Function of ER: Manipulation of protein quality control system

ER manipulates a protein quality control system and ensures the proteostasis within the cell; (1) decides the fate of protein by either allowing further folding cycles ;(2) promotes the degradation of the misfolded protein by ER-associated degradation (ERAD) (Olzmann, Kopito and Christianson, 2013).

Proteins are often required to maintain a degree of structural flexibility or preserve unstructured regions for undergoing protein functioning. Because of that, this might cause the risk of protein misfolding and aggregations over time (Chiti and Dobson, 2017). Stable folded proteins might also face the risk of unfolding and aggregations under stress conditions (i.e. elevation of temperature and oxidative stress) (López-Otín *et al.*, 2013)

ER, homeostasis is the key regulator that manipulates the process of protein synthesis and folding by the assist of chaperone (Read and Schröder, 2021). When the process of protein synthesis and folding are interfered with by homeostasis changes such as Ca2+ depletion, oxidative stress energy depletion, etc (Yan *et al.*, 2002; Cao and Kaufman, 2012). This will result in the accumulation of improperly unfolded protein within ER, which is known as ER stress, or called unfolded protein response (UPR) (Schröder and Kaufman, 2005).

1.3.3.1 Initial activation of ER stress: Activation of chaperone

Chaperones surround the ER under normal proteostasis conditions, chaperones including Immunoglobulin heavy chain-binding protein (BiP) and protein Disulfide Isomerases (PDI) (Ellgaard and Helenius, 2003). Act as the role of chaperones to fold proteins that are required for ER quality control (ERQC) in the ER, which can ensure proper protein folding and conformational maintenance and cooperate with the degradation mechanism (Hipp, Kasturi and Hartl, 2019). Each chaperone functions differently and they change their regulations when unfolded proteins are accumulated upon ER. BiP and PDI are the most studied chaperones within the literature that are well-used as a marker to indicate ER stress, their regulation and activation are mentioned below.

1.3.3.2 Immunoglobulin heavy chain-binding protein (BiP)

BiP is a chaperon that belongs to the Heat Shock Protein 70 family, it plays a crucial role and is a central and essential chaperon as it performs multiple functions upon UPR: involving in

ERQC and protein import, guiding polypeptide folding, assembling of protein complexes, and channeling misfolded polypeptides to degradation (Preissler and Ron, 2019).

In the simplest scenario, BiP is bounded in an inactive form of PERK and IRE1, which are UPR transmembrane stress sensors. Accumulation of unfolded clients will compete for BiP and BiP will be released from the UPR sensors to cope with unfolded clients. Dissociation of BiP with PERK and IRE1a will result in dimerization and activation of the UPR pathway and eventually enhance the correct protein folding (Ibrahim, Abdelmalek and Elfiky, 2019; Preissler and Ron, 2019).

BiP is also responsible for maintaining ER's permeability barrier during protein translocation by sealing the luminal end of the translocon pore before and early in translocation (Hamman, Hendershot and Johnson, 1998). BiP helps the proteasome to perform protein degradation by targeting protein aggregates for retrograde translocation (Pobre, Poet and Hendershot, 2019).

1.3.3.3 Protein disulfide isomerase (PDI)

PDI is a group of multifunctional enzymes, which consists of 21 members in a great variety in enzymatic activity, domain architecture, and tissue specifically (Rahman *et al.*, 2022). PDI is classified as a group of the thioredoxin superfamily and its general function is to act as a folding catalyst to preserve the native conformation and stability of other proteins by oxidation, reduction, and isomerization of disulfide (S-S) bonds (Galligan and Petersen, 2012). PDI plays a crucial role in maintaining proteostasis since the loss of PDI activity has

been associated with the pathogenesis of numerous diseases, including Alzheimer's disease, liver disease, and type-2 diabetes (Galligan and Petersen, 2012).

Under ER stress/ UPR, BiP will preferentially bind to the accumulation of misfolded proteins and dissociate form the three independent UPR pathway proteins (Bohnert, McMillan and Kumar, 2018). Following that, the three pathways would activate for adapting response, feedback control, and cell fate regulation (Oslowski and Urano, 2011). Namely, PRKP-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) (Schröder and Kaufman, 2005)

1.3.4 Downstream of UPR/ER stress pathway

1 3.4.1 Inositol requiring enzyme (IRE1)

IRE1a is a type 1 ER transmembrane protein that provides a platform for ER to control cell fate during stress and it is one of the three main signaling branches of ER stress (Bashir *et al.*, 2021)

In normal conditions, BiP binds the IRE1 luminal domain and maintains an inactive form of monomeric IRE1 (Coelho and Domingos, 2014). Under ER stress conditions, IRE1 is firstly activated by the dissociation of BiP since BiP is recruited to cope with the misfolded protein (Coelho and Domingos, 2014). IRE1 will bind with the accumulating unfolded protein and undergo dimerization or oligomerization in ER and trans-autophosphorylation to the cytosol. Then the phosphorylated IRE1 will activate, catalyse, and splice the nonconventional XBP-1

(X-box binding protein-1) mRNA and result in generating functional transcriptional factor of XBP1 (Preissler and Ron, 2019; Bashir *et al.*, 2021).

Spliced XBP1 will then translocate to the nucleus, bind to the unfolded protein response element (UPRE), and increase the expression of responsible genes that manipulate the fate of ER homeostasis. XBP1 protein induces the expression of genes involved in ER protein quality control (ERQC) and ER-associated degradation (ERAD).

Additionally, activated oligomeric IRE1 may also degrade ER-related mRNAs through regulated IRE1-dependent decay (RIDD) (Preissler and Ron, 2019; Bashir *et al.*, 2021). RIDD is a process that degrades the ER-targeted protein mRNA so that the incoming load of protein decreases during ER stress. It was first reported by Hollien and Weissman that a subset of mRNA is degraded during ER stress by a mechanism that is dependent on IRE1 but not XBP1 (Hollien and Weissman, 2006) .RIDD has been reported as an additional challenge to the ER folding machinery under stress, RIDD mostly encodes proteins with signal peptides or transmembrane (Coelho and Domingos, 2014).

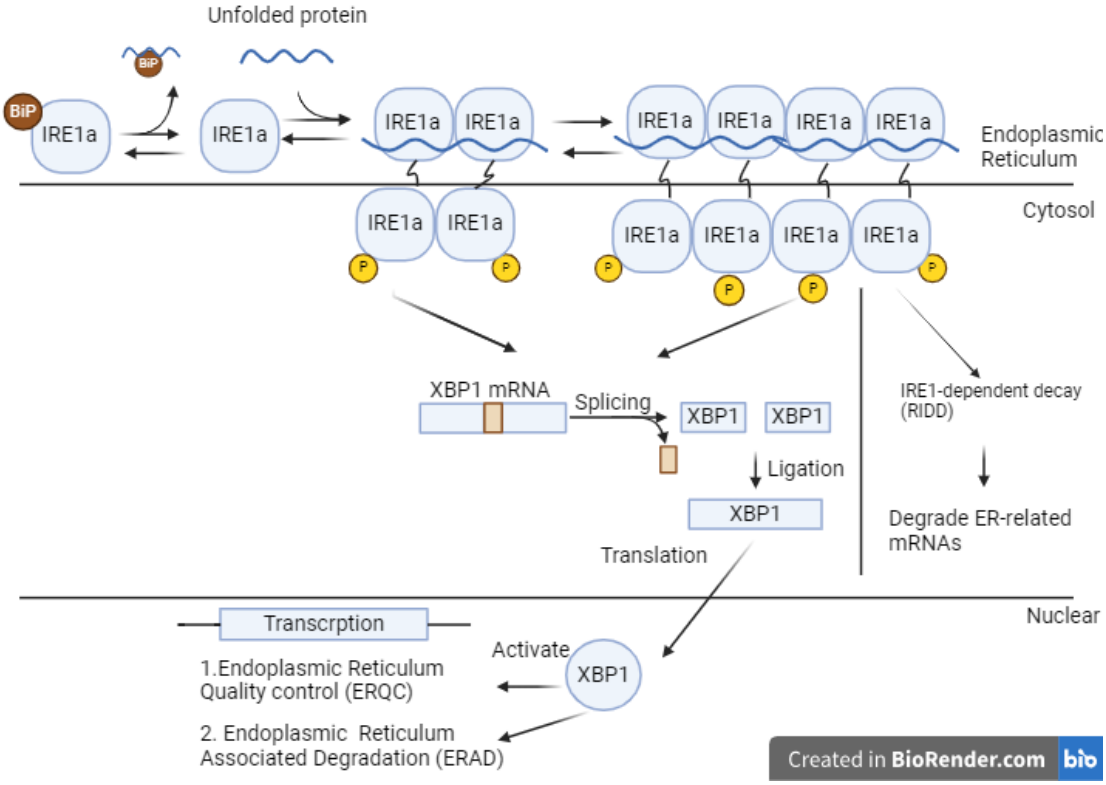


Figure 1.1: IRE1 pathway. Created with BioRender.com

1.3.4.2 PRK-like endoplasmic reticulum kinase (PERK)

PERK is one of the branches of ER stress that responds to the accumulated unfolded aggregates. Initiation of PERK signaling will halt general protein production, increase the efficiency of ER quality control, and maintain proteostasis.

In the initiation of ER stress, BiP chaperone will dissociate from PERK to bind with aggregated proteins and PERK will auto-phosphorylated itself to activate its downstream

pathway protein. Phosphorylated PERK (P-PERK) will reduce the over-frequency of mRNA translation initiation. Meanwhile, increase some of the protein mRNA by phosphorylating eukaryotic translation initiation factor 2 alpha (eIF2α) on serine residue 51(Harding, Zhang and Ron, 1999; Hetz, Zhang and Kaufman, 2020).

Phosphorylation of eIF2a (P-eIF2a) can attenuate the general protein translation in multiple ways. Phosphorylated eIF2a firstly inhibits eIF2B, eIF2B is a guanine nucleotide exchange factor (GEF) and its role is to recycle eIF2 from its inactive guanosine diphosphate (GDP) bound to its active its active guanosine-5'-triphosphate-bound (GTP-bound) form. Inhibition of eIF2B results in a decline in the availability of eIF2·GTP·tRNA ternary complexes and lower availability in the global translation of mRNA (Preissler and Ron, 2019).

Consistently, p-eIF2a also boosts the synthesis of specific transcription factors, including activating transcription factor 4 (ATF4), which is a stress-inducible transcription factor and involved in ER protein folding, autophagy, anti-oxidative response, and amino acid metabolism (Hetz, Zhang and Kaufman, 2020). ATF4 increases the two important transcription genes, GADD34 and CHOP.

Growth arrest and DNA damage-inducible protein 34 (GADD34) is a regulatory subunit of PP1 and its main role is to dephosphorylate eIF2a. During ER stress, GADD34 forms a complex with PP1 to dephosphorylate eIF2a (Novoa *et al.*, 2001). Meanwhile, a constitutive repressor of eIF2a phosphorylation (CReP), serves as a cofactor of PP1 and helps to phosphorylate eIF2a during ER stress (Harding *et al.*, 2009). ATF4 enhances the expression

linked with eIF2a under a negative feedback loop to dephosphorylate eIF2a to restore protein synthesis through regulating protein phosphatase 1 (PP1) regulatory subunit GADD34. C/EBP Homologous Protein (CHOP) is a transcription factor that controls genes involved in apoptosis, chronical expression of CHOP will result in the promotion of cell apoptosis by directing cell to apoptosis and restoring the overloaded proteostasis network (Almeida *et al.*, 2022).

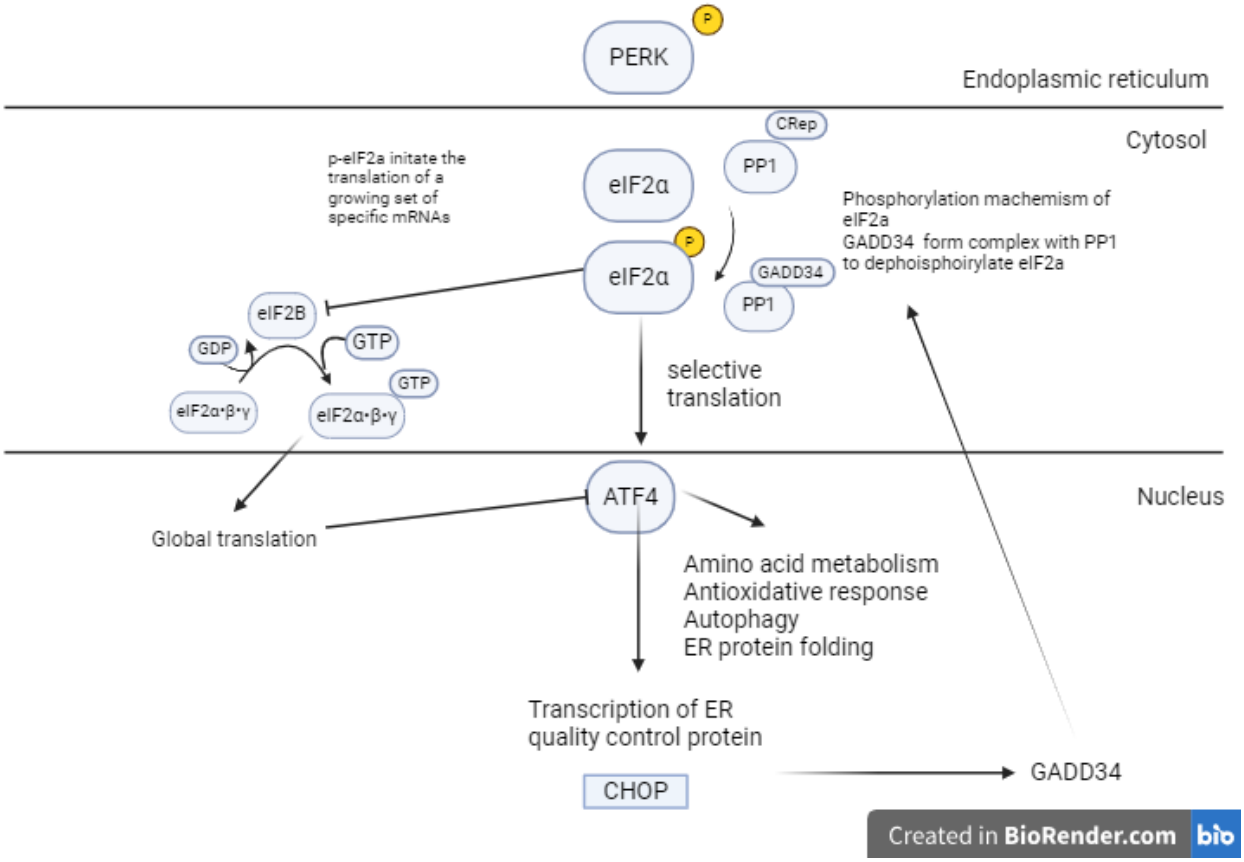


Figure 1.2: PERK pathway. Created with BioRender.com

1.3.4.3 Activating transcription factor 6 (ATF6)

ATF6 is a transcription factor that syntheses as an ER-resident transmembrane protein and bears a large ER-luminal domain (Walter and Ron, 2011). When there is an accumulation of unfolded proteins, ATF6 is packed into transport vesicles, pinch off the ER, and delivered into the Golgi apparatus (Schindler and Schekman, 2009).

Within the Golgi apparatus, ATF6 encounters two proteases, S1P and S2P (site-1 and site-2 protease), which remove the lumen domain and the transmembrane anchor in order (Haze *et al.*, 1999). The cleavage of ATF6 then generates a N-terminal cytosolic fragment, named ATF6(N), ATF6(N) moves into the nucleus and activates gene transcription, prominently UPR target genes involved in the protein process, such as BiP, PDI, and GRP94 (HSP90B1, a chaperone of the HSP90 family) (Walter and Ron, 2011).

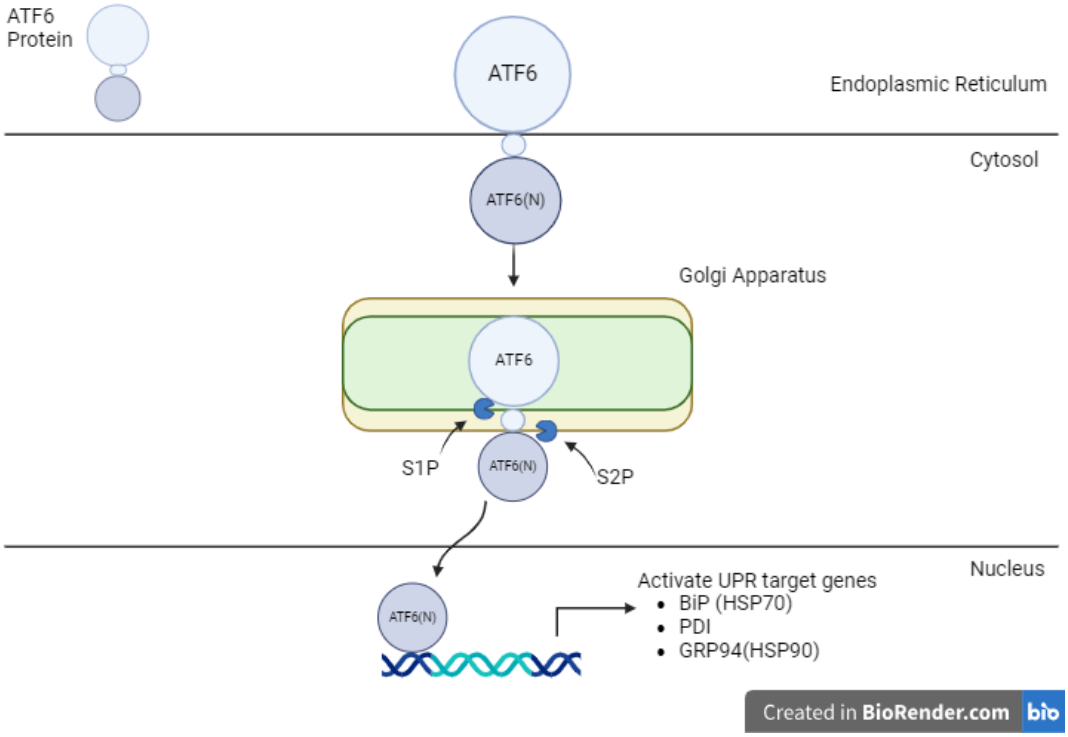


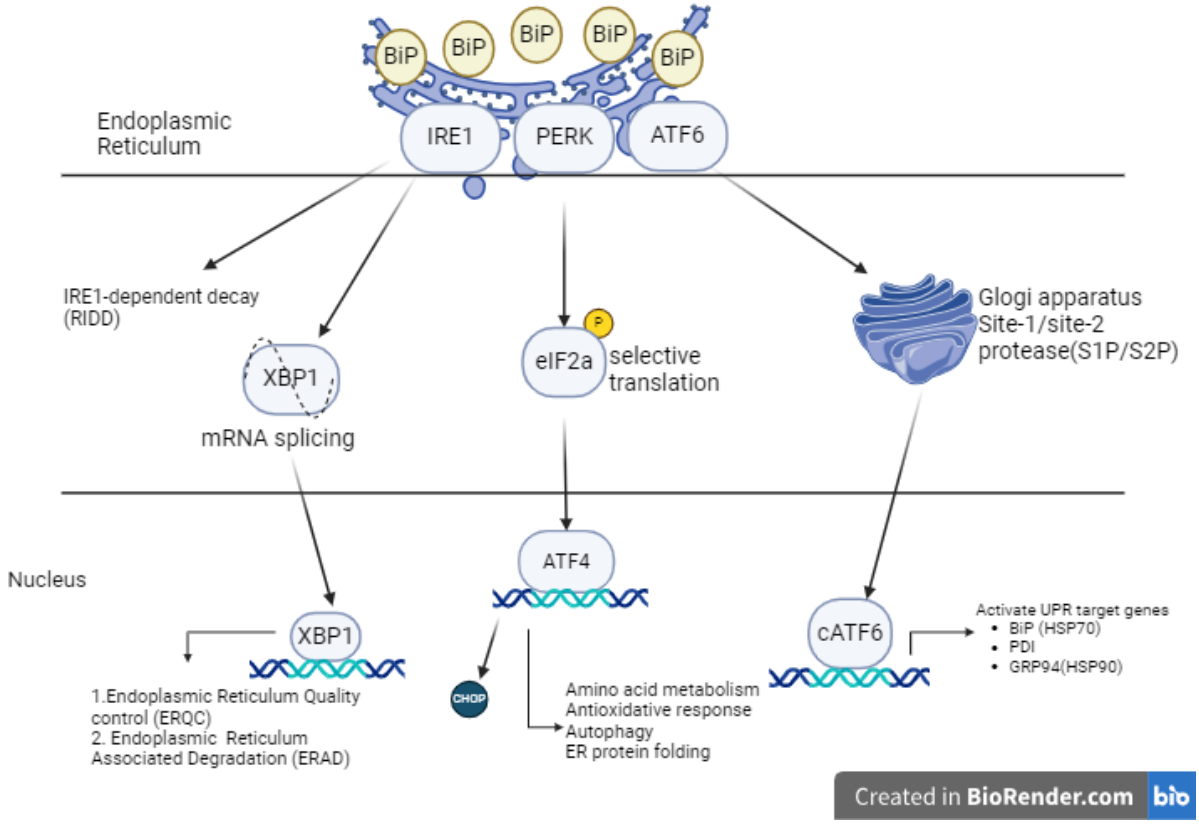
Figure 1.3: ATF6 pathway. Created with BioRender.com

1.3.5 Results of UPR: ERAD: Activation of cell apoptosis

Under normal physiological processes, a high rate of protein synthesis and secretion sustains the initiation of UPR without triggering the cell death pathways. Moreover, when the capacity of the UPR to sustain proteostasis is overwhelmed, unresolved ER stress results in apoptosis (Hetz, 2012; Hetz, Zhang and Kaufman, 2020). B cell lymphoma 2 (BCL-2) protein family regulate the cell death under ER stress, BCL-2 family members at the mitochondria, namely BAX and BAK, follow with the assembly of the apoptosome and the activation of executor caspase 3 under the activation of the canonical apoptosis pathway (Tait and Green, 2010). Prolonged activation of IRE1a has been shown to initiate the IRE1-TRAF2-JNK pro-apoptotic pathway and results in apoptosis (Zhang *et al.*, 2005).

C/EBP homologous protein (CHOP) is a protein that is mainly regulated by the three UPR branches (Oyadomari and Mori, 2004). Under ER stress, CHOP either functions as a transcriptional activator or repressor to regulate the level of apoptotic cell death in cells (Hu *et al.*, 2019). CHOP inhibits the BCL2-family proteins which are mainly anti-apoptotic proteins and proapoptotic proteins (Hata, Engelman and Faber, 2015).

CHOP also up-regulates BIM and increases the BAK and BAX expression and form oligomerization. The oligomerization of BAK-BAX can induce the release of cytochrome c (Cyt-C) and apoptosis-inducing factor (AIF) through mitochondria permeabilization, and eventually lead to cell death (Iurlaro and Muñoz-Pinedo, 2016).



Simplify Figure 1.4: Simplified version of UPR pathway. Created with BioRender.com

1.4 Introduction of small Heat Shock Protein family

1.4.1 Chaperones: Critical protein to regulate UPR

Molecular chaperones and folding proteins that are required for ERQC of protein are abundant in the ER, which can ensure proper protein folding and conformational maintenance and cooperate with the degradation mechanism (Hipp, Kasturi and Hartl, 2019). Although from the literature, proteins are encoded by their amino acid sequence to form their native conformation structure (Anfinsen, 1973), the majority of proteins require the assistance by the chaperone protein and other external factor to finalize their protein structure at a biologically relevant time scale (Balchin, Hayer-Hartl and Hartl, 2016).

In ER, chaperones help to promote the maturation of proteins and turn proteins into native and functional conformations. Unfolded proteins are generally functional and semi-folded proteins result in aggregation (Jahn and Radford, 2008). Chaperone supports the progression of folding intermediates toward the native state and inhibits pathway reactions that might lead to the accumulation of misfolded or aggregated species (Hipp, Kasturi and Hartl, 2019).

Chaperones are classified into different groups based on sequence homology (Kim *et al.*, 2013). Many of them are stress proteins or heat shock proteins (HSPs) since they occur under stress conditions. Each chaperone family performs in distance function, for example: assistance in macromolecular complex assembly, protein transport, and degradation (Kim *et al.*, 2013).

Chaperones are classified into two big categories: ATP-independent chaperones and ATP-dependent chaperones, the latter requires ATP hydrolysis to initiate their reaction (Mitra *et al.*, 2022). ATP-dependent chaperone for using ATP to drive catalytic cycles or regulate allosteric changes (Kim *et al.*, 2013). A great understanding has been known about the mechanism of ATP-dependent chaperones when compared with ATP-independent so far.

Table 1.1: Main chaperone families and their functions in eukaryotes

Molecular Chaperone Family	Characteristics	Function
TRiC	-1MDa -ATF-dependent	Perform as a chaperonins, which is a large, multi-subunit chaperone complexes. It provides a protected environment for protein folding within their central cavities.
HSP110	-100 kDA	Nucleotide exchange factor for HSP70. Important in disaggregating proteins in metazoans. Mainly cooperate

		with HSP70 for protein folding and degrading misfolded proteins.
HSP100	-100 kDa -ATF- dependent	Chaperone family found in fungi, bacteria and plants.HSP100 composed with hexameric rings. HSP100 family cooperate with HSP70 & HSP40 for mediating protein disaggregation in yeast and fungi. *HSP100 is not exist in human genome
HSP90	-90 kDa -ATF-dependent	Target proteins for folding and conforming protein's regulation based on their function and structural. Regulate as a homodimer and major substrate are kinase, steroid receptor molecules and other signalling proteins.

HSP70	-70 kDa -ATF-dependent	One of the major chaperone family which at least consist 8 homolougous chaperone proteins. Perform on preventing protein aggregation, folding newly synthesis protein and conformational maintenance. HSP70 cooperate with HSP40 & HSP110 in protein disaggregation.
HSP60	-60 kDa -ATP-dependent	Chaperonin within mitochondria and requires HSP10 to act as a cofactor for folding mitochondria protein
HSP40(J proteins)	-40 kDa	HSP40 contains the HSP70-interacting J domain. Function as a regulator of HSP70 ATPase cycle of protein substrate binding and release. HSP40 serve as a co-chaperone of HSP70 and

		recruit HSP70 to different cellular locations.
Small heat shock protein(sHSP)	-12-45 kDa -ATF-independent	sHSP contain a conserved α-crystallin domain, packed with β-sheet. sHSP involved in oligomerization and form a large heterogeneous oligomers (-1 MDa). sHSP perform holdase function by binding to non-native states protein to prevent aggregation. sHSP also sequestrate misfolded proteins to avoid toxic aggregates.

Information extracted from (Hipp, Kasturi and Hartl, 2019)

1.4.2 Small heat shock protein (sHSP) family

Small heat shock proteins (sHSP) are a group of ATP-independent chaperones that weigh between 15 and 42 kDa (Horwitz, 1992; Jakob *et al.*, 1993). HSP family are widespread throughout all the kingdoms of life, counting from a few in bacteria and yeast, 4 in Drosophila nelanogaster,19 in a plant like Arabidopsis thaliana, and 11 in human (Haslbeck *et al.*, 2004, 2005; Iyer *et al.*, 2021).

11 sHSPs exist in humans and four out of eleven (HSPB1/5/6/8) ubiquitously exist in the human body, the rest of the seven sHSPs are tissue-specific. For example, HSPB4 only existed in eye lens protein while HSPB9 is expressed only in the testes (Smulders *et al.*, 1996; Gu, Fan and Yu, 2023).

Gene name	Alias	Tissue profile
HSPB1	HSP25, HSP27	Ubiquitous
HSPB2	Hs. 78846, MKBP, DMPK-binding protein	Cardiac and skeletal muscle
HSPB3	HSPL27, HSP17, DHMN2C	Cardiac and skeletal muscle
HSPB4	Crystallin alpha A, CRYA1, CRYAA	Eye lens
HSPB5	Crystallin alpha B, CRYA2, CRYAB	Ubiquitous
HSPB6	HSP20,PPP1R91,FLJ32389, HSP20	Ubiquitous
HSPB7	cvHSP	Cardiac and skeletal muscle, cardiovascular and insulin-sensitive tissues
HSPB8	HSP22, CRYAC, E2IG1, PP1629, CMT2L	Ubiquitous

HSPB9	CT51	Testis
HSPB10	ODF1, CT133, ODF27, ODFPG, PDFP	Testis and kidney
HSPB11	IFT25	Testis(male), fallopian(female)

Table 1.2: sHSP family exist in human, extracted and summarise from lyer et al., 2021, Gu, Fan and Yu., 2023 and protein atlas (https://www.proteinatlas.org/)

1.4.3 Structure of sHSP

All sHSP in Humans share the same domain architecture: The architecture contains a highly variable N-terminal region (NTR), a flexible polar C-terminal region (CTR), and an α-crystallin domain (ACD). The three domains share the same amino acid content and only the α-crystallin domain ACD is a negative folded region that forms an IgG-like β-sandwich fold (Janowska *et al.*, 2019).

The α-crystallin domain (ACD) are length of approximately 90 amino acids and it is rich in histidine. ACD is the only negative folded region in the α-crystallin domain and it is a shared feature among all the sHSP and is sometimes present in duplicate (Caspers, Leunissen and de Jong, 1995). In addition, sHSP is rich in β-strands that form an IgG-like β-sandwich structure and flanked by NTR and CTR within the sHSP. ACD can modulate sHSP activity by

giving the ability to respond the changes in pH and metal ion (Janowska *et al.*, 2019; Gu, Fan and Yu, 2023).

Meanwhile, CTR is enriched in polar and charged residues and thus CTR becomes highly disordered. CTR serves, as a solubility tag that enables the sHSP to be found in multiple tissues expressing under extremely high concentrations while remaining soluble, one example will be sHSP in eye lens (Smulders et al., 1996).

N-terminal regions (NTR) are disordered as they contain numerous hydrophobic residues (Bloemendal, 1977). Two sHSP subunits will form a dimer through their ACD structure via antiparallel alignment of the long β6 +7 strands (Janowska *et al.*, 2019). sHSP has shown the ability to form a variety of range of dimers, up to 12 subunits are needed for forming oligomers. For formatting oligomers, sHSP is required to undergo via dimers, following subsequent assembly of tetrameric or hexameric, and finalize into polymerization (Gu, Fan and Yu, 2023). The formation of sHSP oligomerization is mainly manipulated by ACD, the CTR I-X/V motif, and the NTR (Strauch and Haslbeck, 2016).

1.4.4 Post-translation modification of sHSP

Post-translational modifications (PTM) are essential to activate the function of proteins by altering the spatial structure and charge properties. Phosphorylation is one of the most well-studied PTM in literature and it has been shown to be involved in the activation of sHSP.

HSPB1, one of the most studied sHSP, is activated by phosphorylation and it has a few phosphorylation sites: Three in humans (Ser15, Ser78, Ser82) and two in mice and rats

(Ser15, Ser82, which Ser82 is the homologous to human Ser82) (Kostenko and Moens, 2009; Miskiewicz *et al.*, 2023). Phosphorylation of HSPB1 is related to several pathways: p38/MK2, PI3/AKT and ERK1/2 pathways (Kostenko and Moens, 2009).

HSPB4 phosphorylate at Thr148 and modulated neuroinflammation in the retina via inflammatory pathways (Nath *et al.*, 2021).

HSPB5 has also been shown to have three phosphorylation sides (Ser19, Ser45 and Ser59) and phosphorylated via the p38/MK2 pathway (Hoover *et al.*, 2000; Piao *et al.*, 2005). HSPB6 is involved in cAMP/PKA and cGMP/PKG pathway and phosphorylated in Ser16 (Li *et al.*, 2017).

In addition, HSPB8 can phosphorylated by cAMP-dependent protein kinase at Ser24 and Ser57 (Shemetov, Seit-Nebi and Gusev, 2011), phosphorylated by ERK1 at Ser24, Ser27, and Thr87 (Shemetov *et al.*, 2008)and interact with PKC by phosphorylated at Ser14 and Thr63 (Benndorf *et al.*, 2001). The above sHSP mentioned are relatively better studied in comparison to other sHSP families.

1.4.5 Function of sHSP: Regulate protein aggregates under stress

Unlike the ATP-dependent chaperones, the main function of the sHSP family is to act as the first line of defense against misfolded polypeptides that appear upon the protein aggregation and wait until the ATP-dependent chaperones do the real work of protein folding (Dubińska-Magiera *et al.*, 2014; Mitra *et al.*, 2022). sHSP mainly shields and helps

the aggregates to maintain in their soluble state and prevent precipitation which potentially causes toxicity (Dubińska-Magiera *et al.*, 2014).

sHSP can sequester proteins in non-native conformations to inhibit the formation of aggregates when the client association rate exceeds the aggregation and refolding rates. They either slowly release their clients or transfer them to ATP-dependent chaperones to facilitate folding. sHSP tends to become kinetic traps that prevent protein aggregation and protein folding in some circumstances (Mitra *et al.*, 2022). The binding of sHSP to aggregates can lead to a better interaction between the ATP-dependent chaperones and the aggregates by decreasing their molecular mass and increasing the surface-to-volume ratio, and thus more binding sites are available for the ATP-dependent chaperone to interact with the aggregates upon UPR (Mitra *et al.*, 2022).

In addition, sHSP also can facilitate the disassembly process once they have performed the holdase function on unfolded proteins. They can diminish strong interactions between non-native proteins and sequestering proteins in their near-native conformations (Florian Stengel *et al*., 2012; Ungelenk *et al.*, 2016).

Under stress conditions, the sHSP oligomer will disaggregate into smaller species of sHSP and co-aggregate with the unfolded proteins. The co-aggregate of sHSP and unfolded proteins is more flexible and smaller compared to protein aggregates without sHSP. Once the sHSP co-aggregate is formed, the ATP-dependent chaperones (i.e.HSP70 and HSP40) will be activated and help to restore the proteins to their native structure through ATP-

dependent chaperon pathway and recycle sHSP from the co-aggregates (Gu, Fan and Yu, 2023).

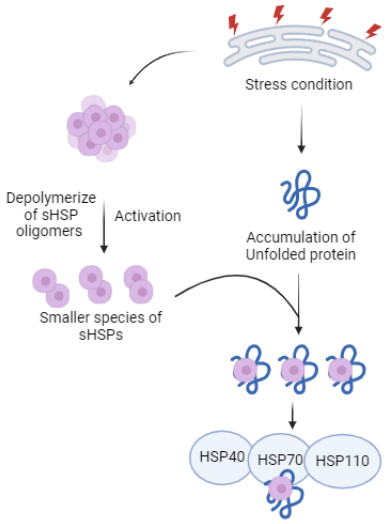


Figure 1.5: sHSP regulate and response to aggregate proteins. Created with BioRender.com

1.4.6 Functions of sHSP in muscle

Other than chaperone activity, sHSP indicates different responses respectively. For example, HSPB1 is a negative regulator of the NF-kappaB, and overexpression of HSPB1 can inhibit the atrophy markers, MuRF1, and atrogin-1 in mRNA level under muscle atrophy conditions (Dodd *et al.*, 2009).

HSPB5 is better studied in cardiac muscle compared to skeletal muscle in the literature. Literature has reported that HSPB5 performs a protective function in the cytoskeleton. Phosphorylation of HSPB5 (phosphorylation side Ser59) is activated under stress conditions in the C2C12 cell line through the p38/MAPKAP2 kinase pathway and results in protecting

the three major components of the cytoskeleton (i.e. actin microfilaments, microtubules and intermediate filaments) (Launay *et al.*, 2006).

HSPB6 is reported as a key mediator of cardioprotection in the heart (Fan and Kranias, 2011) and overexpressed HSPB6 can provide a protective effect against β-agonist-induced apoptosis (Fan *et al.*, 2005).

HSPB6 transgenic mouse in cardiac-specific overexpression also showed 6-fold improved functional recovery of contractile performance throughout the whole reperfusion period when compared with normal mouse heart, indicating HSPB6 can improve recovery of cardiac function and reduced infarction (Fan *et al*., 2005).

HSPB7, also known as cardio-vascular heat shock protein (cvHSP), is highly expressed and plays a critical role in cardiac muscle. HSPB7 is involved in the direct modulation of actin thin filament length and knockout of HSPB7 results in lingering the thin filaments in heart (Wu *et al.*, 2017). In addition, HSPB7 is reported as an interactive partner of dimerized filamin c (FLNC), and the absence of HSPB7 diaphragm muscle results in muscle fibrosis, sarcomere disarray, and sarcolemma integrity loss (Juo *et al.*, 2016)

1.4.7 Groups of sHSP oligomerization in muscle

Under stress conditions, sHSP phosphorylates, activates, and disassembles from oligomers to smaller species to facilitate its chaperone function (Sugiyama *et al.*, 2000). sHSP has been shown to form heterooligomeric complexes with different sHSP among literature (Sugiyama *et al.*, 2000; Mymrikov *et al.*, 2017, 2020)

According to the literature, there are a total of 11 sHSP exist within the human and sever sHSP is highly expressed in different types of muscle tissues (Zhu and Reiser, 2018), including HSPB1, HSPB2, HSPB3, HSPB5, HSPB6, HPSB7, and HSPB8 (Sugiyama *et al.*, 2000; Kappé *et al.*, 2001).

Different sHSP represent different functions and they form specific forms of complex respectively, according to Sugiyama et al., 2000, there are two main chaperone systems that exist within the mammalian sHSP family : Which are HSPB1/HSPB5/HSPB6 system and HSPB2/HSPB3 system. HSPB1/HSPB5/HSPB6 stimulated in a large amount and form co-oligomers. Furthermore, HSPB2/HPSB3 form small oligomers and participate in stress response in smooth muscle cells (Sugiyama *et al.*, 2000; den Engelsman *et al.*, 2009). Recent study reports HSPB7 can also interact with HSPB6 and HSPB8 individually (Muranova, Shatov, Slushchev, *et al*>, 2021)

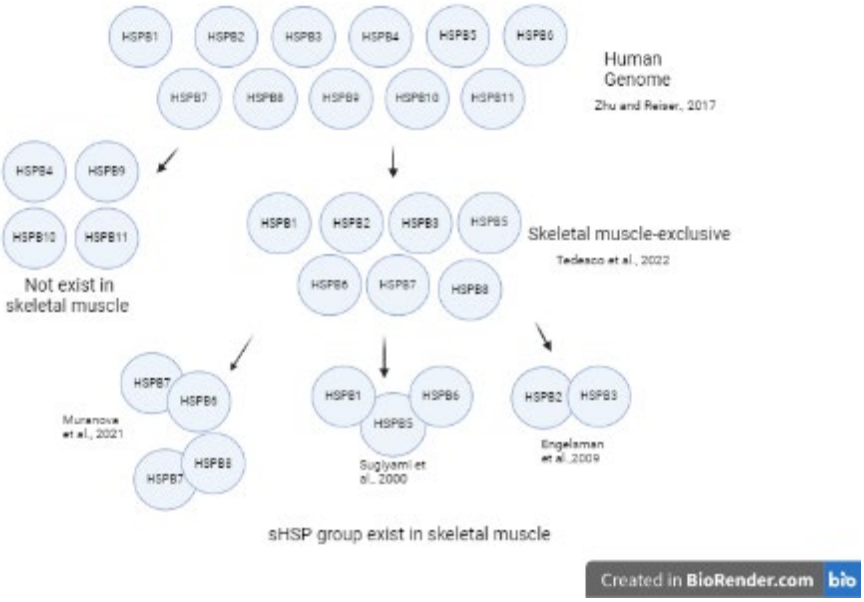


Figure 1.6: Classification of sHSP. Created with BioRender.com

1.5 Gap within the literature

1.5.1 sHSP increase during ageing muscle proteomics

From the literature and our lab's pioneer data, small heat shock families have shown an increase in ageing human muscle (Gueugneau *et al.*, 2021). Including HSPB1, HSPB5, and HSPB6. Doran et al. 2007 also demonstrated that HSPB5 and HSPB7 are drastically increase in aged rat skeletal muscle.

Table 3: Articles reported that sHSP increase in aged skeletal muscle

Age-related changes of sHSP	Model	Author/Reference
HSPB1	Norway Rat (Male, 16 month vs 29 month)	(Chung and Ng, 2006)
HSPB1	Chicken (28, 50, 250, 500 and 700 days)	(Ueda *et al.*, 2015)
HSPB5	Wistar Rat (Gastrocnemius muscle, 3 month vs 30 month)	(Connell *et al.*, 2007)
HSPB1, HSPB5	Human	(Yamaguchi *et al.*, 2007)
HSPB1, HSPB5	Human (Male)	(Beltran Valls *et al.*, 2015)
HSPB5, HSPB7	Wistar Rat (Gastrocnemius muscle, 3 month vs 30 month)	(Doran *et al.*, 2007)

sHSP were higher in the insoluble fraction of aged muscles

Table 1.4: Articles reported that ER-related chaperones increase in aged skeletal muscle

Age-related changes of UPR protein	Model	Author/Reference
BiP, CHOP	Mice (3M vs 24M with old-sedentary, 24M with old-exercise)	(Belaya *et al.*, 2018)

1.5.2 Relationship between Unfolded Protein Response and muscle ageing/ related disease

Dysfunction of proteostasis is a hallmark of ageing which is encountered in numerous age-related diseases and result in the accumulation of protein aggregates (López-Otín et al., 2013). Proteostasis mechanisms are required for protein stabilization and to ensure the proteins can be folded and function properly (López-Otín et al., 2013).

Skeletal muscle is an essential organ, which represents 30% to 40% of human body mass (Janssen *et al.*, 2000). Muscle atrophy is defined as loss of skeletal muscle mass and it has shown an association with several chronic diseases such as diabetes, diabetes, and ageing (Bonaldo and Sandri, 2013). Currently, the molecular mechanisms behind muscle atrophy remain enigmatic for therapeutic engagement to counteract several pathologies (Gallot and Bohnert, 2021).

Recently, there has been reported that the loss of proteostasis is linked with ageing or aging-related disease (Powers *et al.*, 2009). More specifically, there is a potential relationship linked between unfolded protein response and muscle-related disease; An ER stress chaperone, BiP was significantly increased in the human myositis patients and the transgenic mouse model of myositis (Nagaraju *et al.*, 2005). BiP, spliced XBP1, and other ER-related proteins also increased expression at the western level in myotonic dystrophy type 1(DM1) patients' muscles (Ikezoe *et al.*, 2007). Immunohistochemistry of ER-related proteins (BiP, P-PERK, P-eIF2-a, and Calnexin) is also increased in DM1 patients' biopsies (Ikezoe *et al.*, 2007).

1.5.3 Linkage between unfolded protein response and disease

Compared to the UPR study within skeletal muscle, more studies have been done on another aspect, especially within neurodegenerative disease, UPR shows it links to relative diseases. Moreno et al. 2012 have reported that inhibition of eIF2a phosphorylation can Rescue synaptic deficits and neuronal loss in prion-infected mice. In contrast, increased eIF2a phosphorylation levels exacerbate neurotoxicity and reduce survival in prion-diseased mice (Moreno *et al.*, 2012). Duran-Aniotz et al also reported that IRE1 deletion reduces the expression of amyloid precursor protein (APP) at the molecular level and strengthens the learning and memory capacity of Alzheimer's disease mice (Duran-Aniotz *et al.*, 2017).

1.5.4 sHSP work has been done on skeletal muscle

Thought sHSP has been relatively well studied on brain tissue and cardiac tissue. Still, there is limited information to show the study on the skeletal muscle. Recently literature has reported that sHSP has a role in myogenesis in C2C12 myoblast (Thakur *et al.*, 2019) cell proliferation and differentiation in skeletal muscle.

1.5.5 Current literature about sHSP/ER and sHSP/UPR

Although there have numerous literature reported about the relationship between sHSP and UPR, same as ER stress leading to UPR. Not much literature has been reported about the relationship between sHSP and ER stress. A study in plants revealed that overexpressed sHSP alleviates the ER stress induced by tunicamycin in tomatoes (Zhao *et al.*, 2006). ER-located sHSP has also reported that it can improve stress tolerance in tomatoes during salt stress conditions (Fu *et al.*, 2016).

1.6 Hypothesis

Based on the literature and our lab preliminary data, both ER-related proteins and sHSP increase in aged skeletal muscle. This led to the hypothesis that the endoplasmic reticulum stress stimulate the expression of the main sHSP species to hold misfolded protein and prevent protein aggregation in skeletal muscle

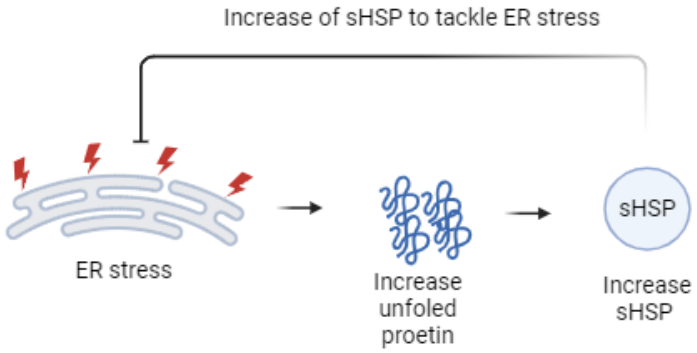


Figure 1.7: Hypothesis of the relationship between sHSP and ER stress. Created with BioRender.com

1.7 Aim

This present study aims to:

Compare sHSP expression by using ER stress under two chemical inductions.

Namely Tunicamycin, and Thapsigargin.

Chapter One: Validation of Antibodies

Firstly, we selected sHSP antibodies according to our lab's preliminary data that showed a significant increase in aged mouse muscle. Then we chose some of the general ER stress chaperones and markers for UPR pathway-specific antibodies that upon frequently in the literature. We have validated all the antibodies under C2C12 myotubes under ER stress. conditions by using Thapsigargin and Tunicamycin, which are ER stress inducer and well proved to cause ER stress. For validating sHSP, heat stress condition was used on C2C12 myotubes to validate the antibodies.

Chapter Two: Optimise ER Stress and test sHSP under two ER stress conditions

Once we had validated the antibodies, we then moved to observe how the ER stress markers regulate under ER stress in all three types of conditions (Thapsigargin, Tunicamycin, and Heat stress). We created a three-detailed experiment to study how the cells are influenced by the ER stress under different time costs/dosages.

According to the literature, there are common pharma logical ways to induce ER stress in cells. Tunicamycin is a well-known ER stress inducer since it can inhibit N-glycosylation, an important process for the proper folding and function of glycoproteins. Tunicamycin inhibits the addition of N-linked glycans to nascent proteins and results in causing accumulation of misfolded proteins within the ER, causing ER stress (Oslowski and Urano, 2011).

Thapsigargin is also another well-studied ER stress inducer, it can inhibit Ca2+-ATPase pumps, which are responsible for transporting calcium ions from cytosol into the ER and maintaining high calcium concentration in the ER. When thapsigargin inhibits the Ca2+ pump and decreases the level of Ca2+ within the ER. Since calcium is important since it is essential to act as a cofactor for various chaperone proteins, a decrease in the level of Ca2+ leads to the accumulation of misfolded protein and triggers UPR within the ER (Oslowski and Urano, 2011).

As a result, ER stress is created by treating either Tunicamycin or Thapsigargin in cells for studying the response of sHSP under ER stress conditions.

Table 1.5: Literature used tunicamycin or thapsigargin to induce ER stress response on C2C12 myotubes

Tunicamycin (Dosage used)	**Thapsigargin (Dosage used)**
(Eo and Valentine, 2021)(0.5 ug/ml)	(Takahashi *et al.*, 2017)(0.1/0.3.1.0 uM)
(Takahashi *et al.*, 2017) (0.3/1.0/3.0 ug/ml)	(Wei *et al.*, 2016)(0.1 uM)
(Quan *et al.*, 2015)(0.5 ug/ml)	(Arai *et al.*, 2017)(0.1 uM)
(Gu *et al.*, 2015)(0.5 ug/ml)	(Welc, Judge and Clanton, 2013)(0.1uM)
(Diane *et al.*, 2020)(0.5 ug/ml)	(Gutierrez-Martin, Martin-Romero and Henao, 2005)(1uM)
(Hu *et al.*, 2014)(0.5 ug/ml)	(Chen *et al.*, 2024)(0.01/0.1/0.5 uM)
(Welc, Judge and Clanton, 2013)(0.1/1/10 ug/ml)	(Koh *et al.*, 2013)(1.3 ug/ml^{-1})
(Koh *et al.*, 2013) (1 ug/ml^{-1})	(Porter, Makuck and Rivkees, 2002)(0.1 uM,0.1/0.01nM)
(Jung *et al.*, 2018)(2uM)	(Kowaltowski *et al.*, 2019)(2 uM)

Table 1.6: Literature reported sHSP response by using tunicamycin or thapsigargin in other cell line

Literature	Cell line	Detected sHSP
(Ito *et al.*, 2005)	U373 MG , U251 MG	HSPB1, HSPB5

CHAPTER 2

GENERAL METHODS

2.1 Cell lines and culture

Mouse skeletal muscle C2C12 myoblast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). C2C12 were seeded on 10cm dishes (Greiner Culture dish TC treated 100mm deep base 20mm) (Scientific Laboratory Supplies (SLS), UK, G664160)

C2C12 myoblasts cells were cultured in Dulbecco's Modified Eagle Medium (Gluta DMEM; Thermo Fisher Scientific, Loughborough, UK, 31966021) containing 25 mM of glucose and 1 mM of sodium pyruvate, supplemented with 10% (v/v) Hyclone fetal bovine serum (FBS, Fisher Scientific, Loughborough, UK, 31966021) and 1% (v/v) Penicillin-Streptomycin (10 000 Units/mL-ug/mL, Thermo Fisher Scientific, Loughborough, UK).

Medium (10mL) was replaced every 48 hours and cultures were maintained in a humidified incubator at 37°C with an atmosphere of 5% CO2 and 95% air. When the cell confluence was attained around 90% in the 10cm dish, 2mL of trypsin (Cibco Trypsin EDTA 0.25%; Fisher Scientific UK Limited, 2520056) was added into the dish, cells were then put back into the incubator for 3 minutes before resuspended and mixed by 8ml of Gluta DMEM. Cells were collected into a 15mL of falcon tube and centrifuged for 1.0 RCF for 3 minutes. The supernatant was aspirated and cells were resuspended again by 10ml of Gluta DMEM. Cells were either reseeded on a 10cm dish for the remaining culture or a 6-well plate (6-Well CytoOneRPlate, TC-treated; Starlab, UK, CC7682-7506) for differentiation.

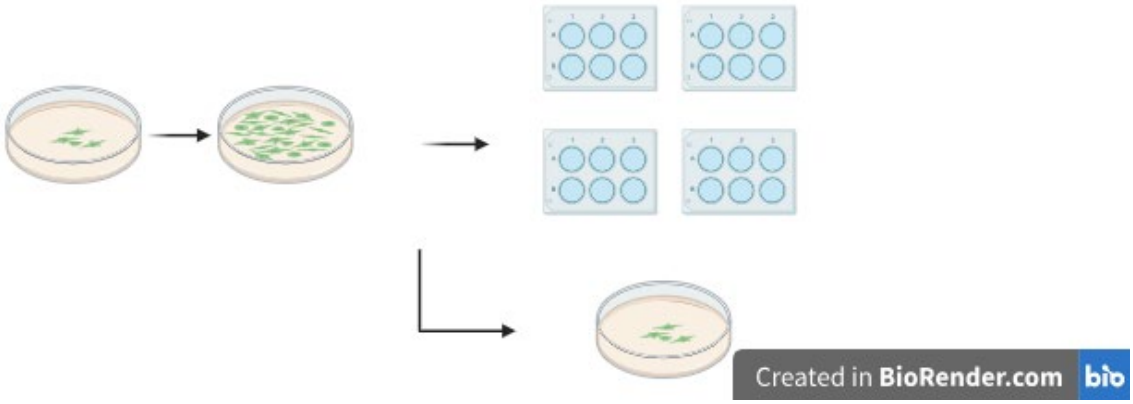


Figure 2.1: C2C12 cell culture and differentiation method. Created with BioRender.com

2.2 Cell differentiation

Once C2C12 myoblasts were culture in a 6-well dish and attained around 90% in the 6-well dish, then differentiation media were added and differentiated for 5-7 days. Differentiation media in DMEM GlutaMAX supplemented with 25Mm glucose and 1mM of sodium pyruvate, supplemented with 2%(v/v) of horse serum (Sigma-Aldrich, Cambridgeshire, UK, H1270) and 1% (v/v) of Penicillin-Streptomycin (10000 Units/Ml-UG/Ml). The media was changed every 48 hours and cells were kept culturing in a humidified incubator at 37°C with an atmosphere of 5% CO2 and 95% air until myotubes were fully formed and experimental procedures began.

2.3 Drug reconstitution and cell treatment

Tunicamycin(Merck Life Science, UK, T7765) and Thapsigargin (Merck Life Science, UK, T9033) were reconstituted in DMSO and treatment conditions were described in the figure.

legend. Drugs stock in dimethyl sulfoxide ≥99.9% ACS (DMSO; VWR International, 0231-500ml) respectively.

2.4 Cell lysis

Cells were lysed in ice and used Lai's lab standard 1x lysis buffer and the 1x lysis buffer containing: 250 mM of sucrose, 50 mM of Tris-base (pH 7.5), 50 mM of sodium fluoride, 10 mM of sodium β-Glycerolphosphate, 5 mM of sodium pyrophosphate, 1 mM of EDTA, 1 mM of EGTA, 1 mM of benzamidine, 1 mM of sodium orthovanodate, 1 x complete Mini EDTA-free protease inhibitor cocktail (Roche), 1% of Triton X-100 and 100 mM of 2-chloroacetamide. Cell lysates were centrifuged at 4°C for 15 min at 13,000 rpm and the supernatant was collected for protein measurement once being lysed.

2.5 Protein assay

Analysis of total protein was made using the Bradford protein assay (ThermoFisher Scientific, Leicestershire, UK, 23200). Succinctly, 5 µL of cell lysate was diluted in 45 µL ddH2O.Diluted samples were loaded in triplicate into a 96-well microplate containing 300 µL Bradford protein reagent. The absorbance of the samples was measured at 595 nm using the FLUOstar OMEGA microplate reader. Protein in each sample was quantified from BSA standards, used as a standard curve, and ranging from 1-150 µg to define their protein concentration.

2.6 Sample preparation

To ensure the same amount of protein concentration for each sample was prepared, sample preparation and calculation were following the completion of the protein assay.

Cell lysates were prepared in lysis buffer and 4x NuPAGE LDS sample buffer (Invitrogen, NP0008) containing 2-mercaptoethanol (final concentration 5%). Samples were mixed by vortex and centrifuge before being left to denature overnight at room temperature.

2.7 Western Blot

2.7.1 Gel Electrophoresis

Prepared cell lysates were then loaded into 10% BIS-Tris gels before SDS-PAGE (30-45µg of total protein). Gels were run in 1x MOPS buffer (ThermoFisher Scientific, Leicestershire, UK, 10747573) for 30 minutes at 80V, followed by around 60 minutes at 120V.

2.7.2 Transfer and Blocking

Proteins were transferred onto PVDF membranes (GE Healthcare Life Sciences; 10600021) via wet transfer for 1h at 100V and transfer tanks were covered with ice and ice packs. Each membrane was blocked in either 5% Milk or 3% BSA diluted in Tris-buffered saline Tween-20 (TBS-T): 137 mM of sodium chloride, 20 mM of Tris-base (7.5 pH), 0.1%(v/v) Tween-20, for 1 hour and then washed in TBS-T (3x5 min) before being incubated overnight at 4°C.

2.7.3 Primary Antibody

Primary antibodies were diluted in 3% of BSA made up in 1xTBST. The list of primary antibodies used is described below. Membranes were left to incubate overnight in the primary antibody at 4°C for around 16-20 hours.

Table 2.1: List of antibodies used

Antibody	**Source**	**Catalogue Number**	**Concentration**
HSPB1	Cell Signalling Technology	2442S	1:1500
HSPB5	Proteintech	15808-1-AP	1:1000
HSPB6	Abcam	AB184161-1001	1:1000
HSPB7	Proteintech	Proteintech	1:1000
P-PERK	Cell Signalling Technology	3179S	1:500
P-eIF2a	Cell Signalling Technology	3398S	1:1000
ATF4	Cell Signalling Technology	11815S	1:500
ATF6	Cell Signalling Technology	65880S	1:500
IRE1α	Cell Signalling Technology	3294S	1:1000
CHOP	Cell Signalling Technology	2895S	1:1000

BiP	Cell Signalling Technology	3177S	1:1000
PDI	Cell Signalling Technology	3501S	1:5000
GAPDH	Cell Signalling Technology	5174S	1:25000

2.7.4 Secondary Antibody

After incubating with the primary antibody overnight, membranes were then washed 3 times for 5 minutes in TBS-T before incubation in horseradish peroxidase-conjugated rabbit or mouse secondary antibodies (1:5000 or 1:10,000) at room temperature for 1 hour under darkness. Membranes were washed again a further 3 times for 5 minutes before imaging.

2.7.5 Imaging

For capturing antibody binding, an enhanced chemiluminescence horseradish peroxidase substrate detection kit was used (Merck Life Science, UK; WBKLS0500). Images were undertaken using a G: BOX Chemi-XR5 (Syngene, Cambridgeshire, UK).

2.8 Analysis

Manual band quantification was performed using ImageJ/Fiji (National Institutes of Health, USA). Corrections were performed by removing the effects of the background signal.

Proteins of interest were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading controls before measuring the fold change of the protein.

2.9 Statistical analysis

All statistical analyses were performed using GraphPad Software Inc Prism version 10.2.3. For time course and dose-response experiments, a one-way analysis of variance (ANOVA) was performed with Dunnett's post-hoc test compared to control (CON). Values of $P < 0.05$ (*) were considered statistically significant. Data were presented as mean ± SEM. All the experiments were repeated at least three times.

CHAPTER 3

RESULTS

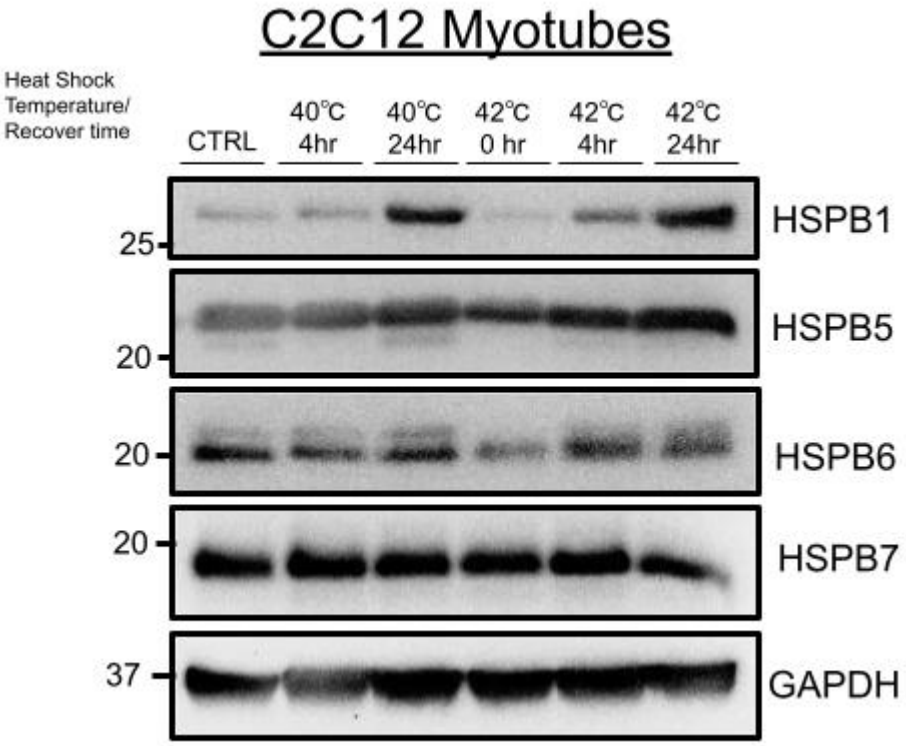
3.1 Validation of the antibodies

3.1.1 Background

As aforementioned, sHSP and ER-related protein increased in aged skeletal muscle throughout the literature, together with our lab's preliminary data. Therefore, we have decided to induce ER stress under the skeletal muscle cell line to observe the regulation of sHSP under ER stress-treated C2C12 myotubes. Before doing that, antibodies need to be validated and optimised to ensure they are appropriate for detecting target proteins by using Western Blot.

3.1.2 Validations of the antibodies in heat shock and drug-treated conditions

sHSP Antibodies were validated under the heat shock treatment **(Fig. 3.1a)** and ER stress antibodies were validated under the ER stress treatment **(Fig. 3.1b & 3.1c)**. The blots showed the indicated antibodies were ideal for detecting endogenous sHSP and ER stress markers as the blots indicated robust results.



a)

Figure 3.1a: Validation of sHSP antibodies on Heat shock experiment.

C2C12 myotubes were either placed in the standard condition (CTRL placed under 37°C incubator), placed under 40°C or 42°C for two hours to induce heat shock (40°C and 42°C lanes respectively). Cells were then replaced to the standard condition (37°C) either lyse immediately (0 h) or indicated durations (4 h, 24 h) once treated with heat shock. Lysates were collected, prepared, and analyzed by SDS-PAGE and western blotting with indicated antibodies. (a) Representative images for sHSP antibody validations for Heat shock experiment.

Around 20 ug of sHSP were loaded into 10% Bis/Tris gels from running western blot, followed by the procedures listed above. All the sHSP used 5% non-fat milk for blotting on

the day 1 western blot before incubated with the concentration of the primary antibody listed above.

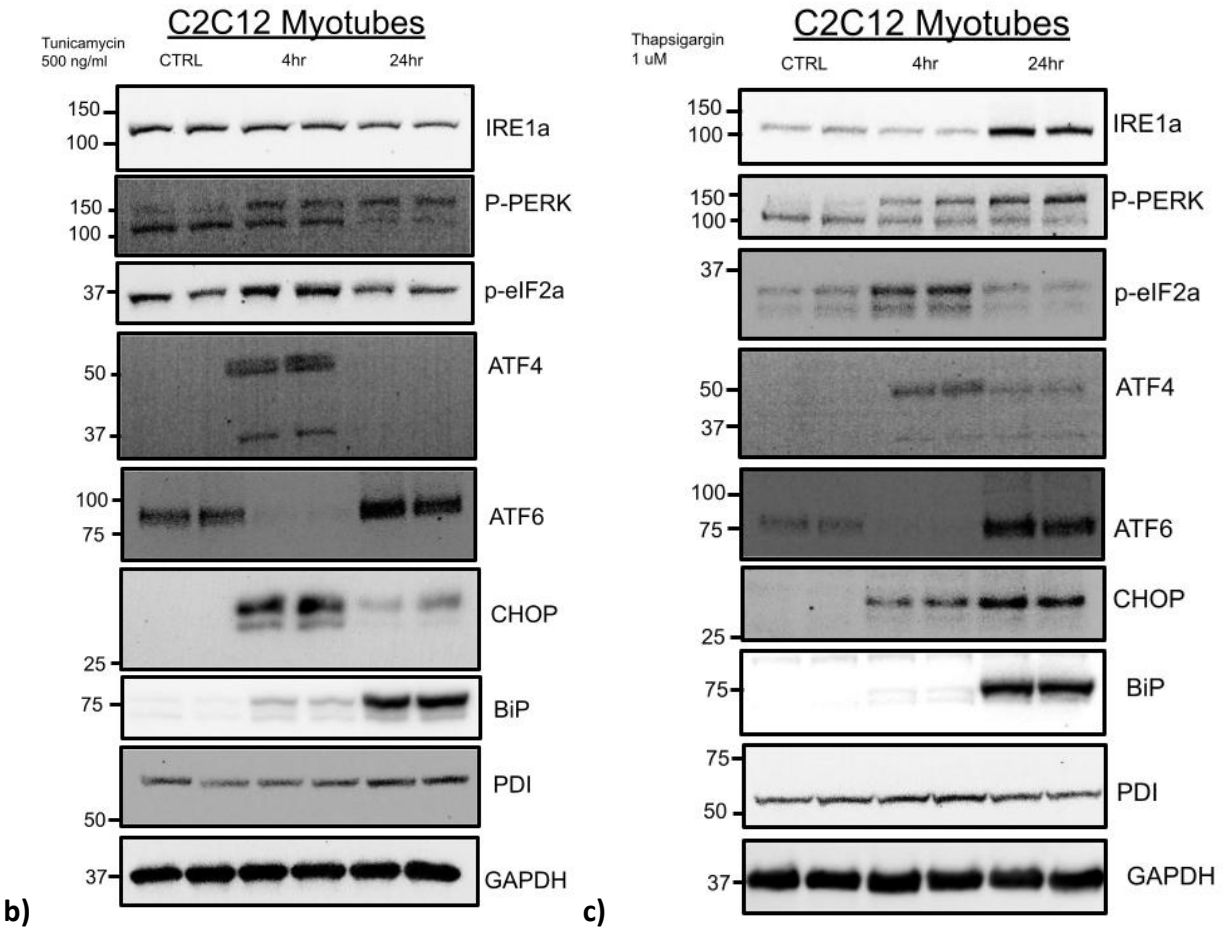


Figure 3.1b and 3.1c Validation of antibodies on Tunicamycin and Thapsigargin

C2C12 myotubes were treated sterile either DMSO (0.1%, 24 h) as vehicle control (CON) or with drug (i.e. 500 ng/ml of Tunicamycin or 1 uM of Thapsigargin) for 4 h or 24 h. Lysates were collected, prepared, and analyzed by SDS-PAGE and western blotting with indicated antibodies. (b) Representative images for antibody validations for 500 ng/ml Tunicamycin. (c) Representative images for antibody validations for 1.0 µM Thapsigargin.

Around 35 - 40 ug of proteins were loaded into 10% Bis/Tris gels from running western blot, followed by the procedures listed above. Except IRE1α, P-PERK, and p-eIF2a were blotted by 3% BSA, the rest of the proteins were used 5% non-fat milk for blotting procedure on Day 1 western blot before being incubated with the listed concentration of the primary antibody.

3.2 Investigation of sHSP under Tunicamycin-treated C2C12 myotubes

3.2.1 Background

Once we ensured that all the antibodies were working consistently for detection. We then used Tunicamycin, an ER stress inducer to stimulate ER stress under C2C12 myotubes. Based on the literature and the previous validation data (Zhang et al., 2022). ER stress markers are changed through 24 h tunicamycin. Therefore a more detailed 24-hour time course treatment was completed as to investigate sHSP response under ER stress-treated C2C12. In addition, we tried to use a 10-fold lower dosage of Tunicamycin and aimed to see whether ER stress and sHSP protein content would be affected by the dosage content.

3.2.2Evidence of Tunicamycin Inducing ER stress pathway

In line with a previous study (Zhang et al., 2022), P-eIF2α, ATF4, ATF6, CHOP, and BiP were increased significantly over the course of treatment with either 50 ng/ml or 500 ng/ml Tunicamycin. Under 50 ng/ml Tunicamycin, ATF4 has a significant increase in 6 and 12 h treatment **(Fig. 3.3i)**. CHOP also has a significant increase in 6 h and 12h **(Fig. 3.3m)** and BiP has a significant increase under 16 h and 24 h treatment **(Fig. 3.3o)**.

Similarly ,when the cell were treated under 500 ng/ml Tunicamycin, ATF4 protein content has a significantly increased at 3 h and 6 h treatment **(Fig. 3.3j)**. ATF6 has an increase under 6 h **(Fig. 3.3l)** and both CHOP and BiP have a significant increase in 16 h 500 ng/ml tunicamycin **(Fig. 3.3n and 3.3p)**.

As anticipated, both 50 ng/ml and 500 ng/ml of Tunicamycin treatment were able to stimulate ER stress response under C2C12 myotubes within 24 hours of treatment.

3.2.3 Results of sHSP regulation under Tunicamycin-induced ER stress in C2C12 myotubes

HSPB1, but not HSPB5, HSPB6 and HSPB7, protein content is decreasd by Tunicamycin-induced ER stress in C2C12 myotubes

Tunicamycin stimulated ER stress as expected. But instead of increasing sHSP protein content decreased under ER stress induction. Using ER stress inducer, Tunicamycin, we showed that HSPB1 protein content was significantly decreased at 16 h and 24 h after the treatment of 50 ng/ml Tunicamycin**(Fig. 3.2c)** and a significant increase at 6 h, 12 h, 16 h, and 24 h after 500 ng/ml treatment of Tunicamycin **(Fig. 3.2d).**

3.3 Investigation of sHSP under Thapsigargin-treated C2C12 myotubes

3.3.1 Background

Similarly, Thapsigargin is another well-known ER stress inducer that can induce ER stress under C2C12 myotubes (Chen et al., 2022). Together with the previous validation data and references from the literature (Takahashi et al., 2017). We have completed a detailed time course treatment to investigate sHSP response under ER stress-treated C2C12. In

addition, we tried to use a 10-fold less dosage of Thapsigargin (0.1 μM) and aim to see whether ER stress and sHSP protein content would be affect by the dosage content .

3.3.2 Evidence of Thapsigargin inducing ER stress

In line with the literature, IRE1α, CHOP, and BiP are significantly increased under Thapsigargin treatment (Takahashi et al., 2017). Under 0.1 μM Thapsigargint treatment, IRE1α had increased under 12, 16, and 24h of treatment **(Fig. 3.5c)**, CHOP had significantly increased under 6 h after Thapsigargin treatment **(Fig. 3.5m)** and BiP had an obvious increase at 12 h, 16 h and 24 h of treatment **(Fig. 3.5o)**. Additionally, ATF4 and ATF6 also recorded a significant increase in the result for under 6 h and 24 h of treatment respectively **(Fig. 3.5i and 3.5k)**.

Furthermore, the protein content of p-eIF2α, ATF4, ATF6, and BiP were significantly increased under 1.0 μM of Thapsigargin treatment. p-eIF2α has an obvious increase under 12 h, 16 h, and 24 h under treatment **(Fig. 3.5)**, ATF4 had a significant increase under 6 h of treatment **(Fig. 3.5j)** and ATF6 was increased during 16 h and 24 h treatment **(Fig. 3.5l).** BiP was increased in 12 h, 16 hand 24 h of treatment **(Fig. 3.5p)**.

As anticipated, both 0.1μM and 1.0 μM dosage Thapsigargin treatment were able to stimulate ER stress response under C2C12 myotubes under 24 hours of treatment.

3.3.3 Results of sHSP regulation under Thapsigargin-induced C2C12 myotubes

HSPB1 and HSPB6, but not HSPB5 and HSPB7 decreased by the Thapsigargin-induced ER stress in C2C12 myotubes

Thapsigargin stimulated ER stress as expectedly. But instead of increase, sHSP protein content decreased under ER stress induction. Using the ER stress inducer, Thapsigargin, we showed that HSPB1 protein content was significantly decreased at 24 h after the treatment of 0.1 μM Thapsigargin **(Fig. 3.4c)** and significantly increased at 12 h and 24 h after being treated with 1.0μM Thapsigargin **(Fig. 3.5d)**. HSPB6 has recorded a significant increase in 24 h in both 0.1 μM and 1.0μM Thapsigargin respectively **(Fig. 3.4g and 3.4h)**.

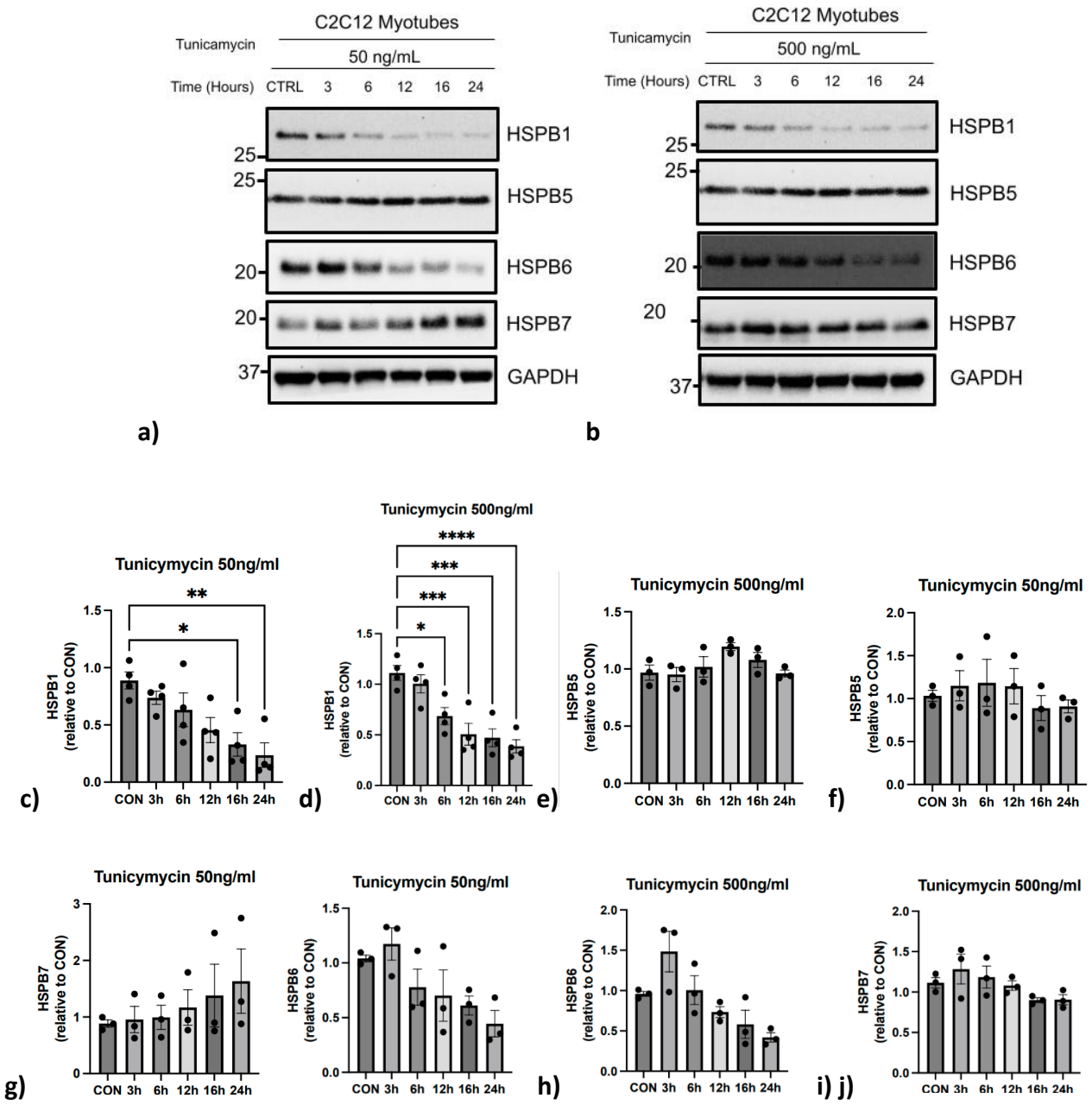
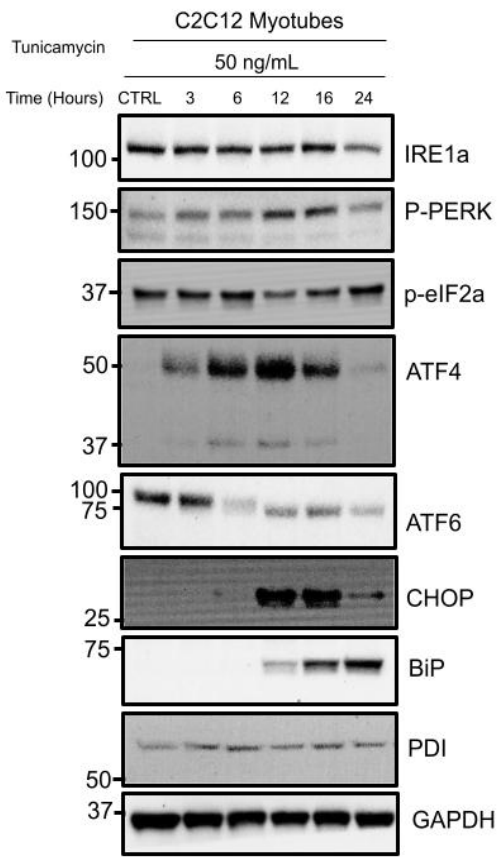


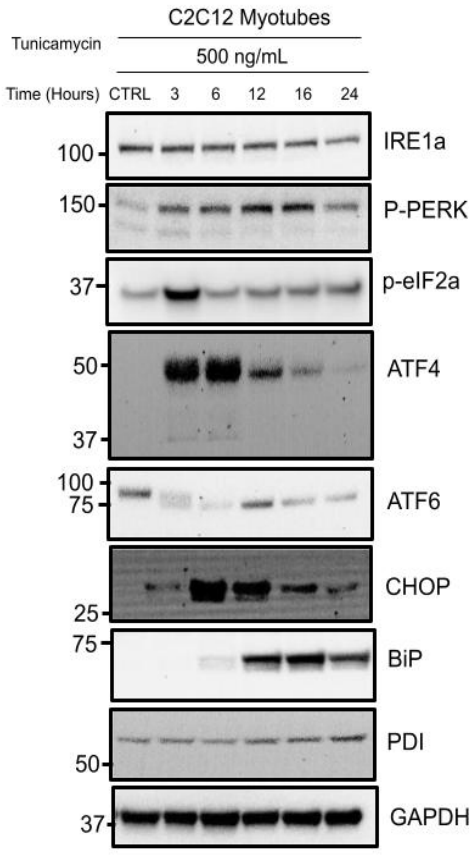
Figure 3.2 Tunicamycin treatment decreased HSPB1, but not other sHSP

C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON) or Tunicamycin (50ng/ml or 500ng/ml) for 3, 6, 12, 16, or 24h. Lysates were collected, prepared, and analyzed by SDS-PAGE and western blotting with indicated antibodies. (a)

Representative images from one of three independent experiments for 50ng/ml Tunicamycin treatment. (b) Representative images from one of three independent experiments for 500ng/ml Tunicamycin treatment. (c-j) Quantification of HSPB1, HSPB5, HPSB6 and HSPB7. Data are expressed as mean ± SEM (n = 3) fold changes relative to control. One-way ANOVA with Dunnett's post-hoc test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 compared to CON.



a)



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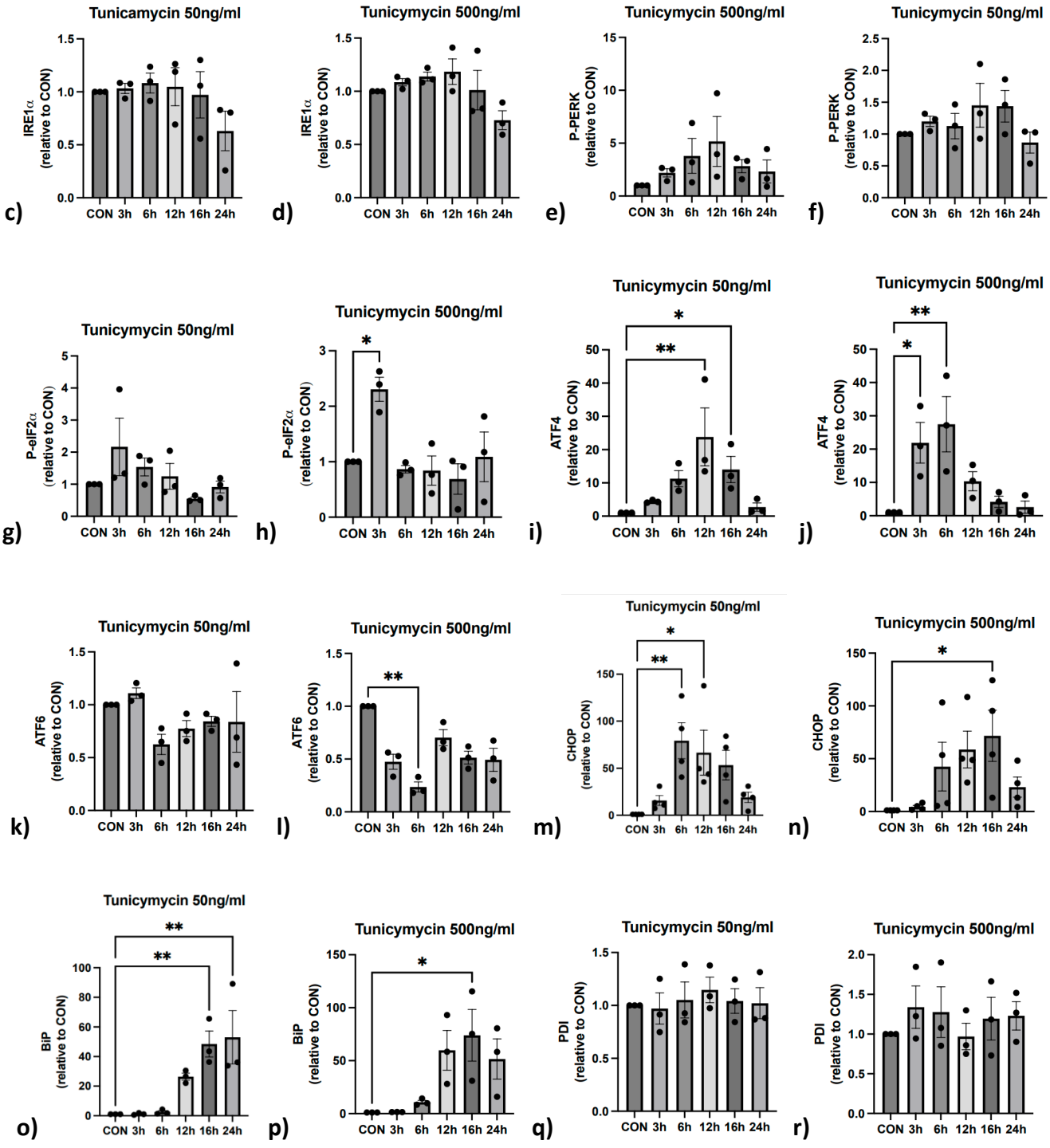
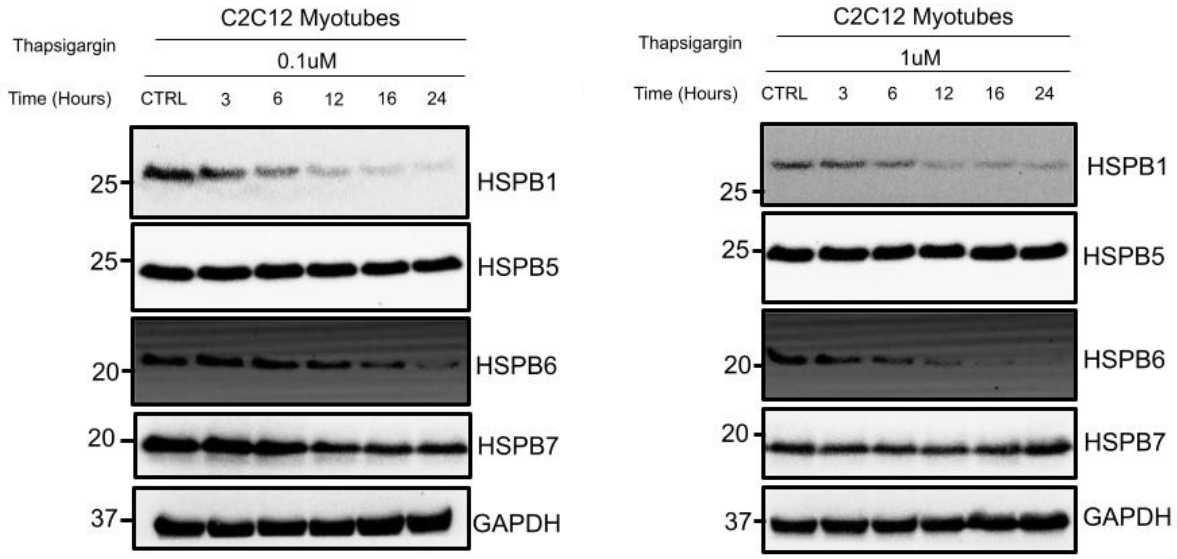

Tunicamycin 50ng/ml
IRE1α (relative to CON)
c)
Tunicymycin 500ng/ml
IRE1α (relative to CON)
d)
Tunicymycin 500ng/ml
P-PERK (relative to CON)
e)
Tunicymycin 50ng/ml
P-PERK (relative to CON)
f)
Tunicymycin 50ng/ml
P-eIF2α (relative to CON)
g)
Tunicymycin 500ng/ml
P-eIF2α (relative to CON)
h)
Tunicymycin 50ng/ml
ATF4 (relative to CON)
i)
Tunicymycin 500ng/ml
ATF4 (relative to CON)
j)
Tunicymycin 50ng/ml
ATF6 (relative to CON)
k)
Tunicymycin 500ng/ml
ATF6 (relative to CON)
l)
Tunicymycin 50ng/ml
CHOP (relative to CON)
m)
Tunicymycin 500ng/ml
CHOP (relative to CON)
n)
Tunicymycin 50ng/ml
BiP (relative to CON)
o)
Tunicymycin 500ng/ml
BiP (relative to CON)
p)
Tunicymycin 50ng/ml
PDI (relative to CON)
q)
Tunicymycin 500ng/ml
PDI (relative to CON)
r)
CON 3h 6h 12h 16h 24h

Figure 3.3 Tunicamycin induced ER stress through P-eIF2α/ATF4, ATF6 pathway and stimulated BiP and CHOP, but not through IRE1α protein contents in C2C12 myotubes

C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON) or Tunicamycin (50ng/ml or 500ng/ml) for 3, 6, 12, 16, or 24h. Lysates were collected, prepared, and analyzed by SDS-PAGE and western blotting with indicated antibodies. (a) Representative images from one of three independent experiments for 50ng/ml Tunicamycin treatment. (b) Representative images from one of three independent experiments for 500ng/ml Tunicamycin treatment. (c-r) Quantification of IRE1α, P-PERK, P-eIF2α, ATF4, ATF6, CHOP, BiP, and PDI. Data are expressed as mean ± SEM (n = 3) fold changes relative to control. One-way ANOVA with Dunnett's post-hoc test, $*P < 0.05$, $**P < 0.01$ compared to CON.


C2C12 Myotubes
Thapsigargin
0.1uM
Time (Hours)
CTRL 3 6 12 16 24
25
HSPB1
25
HSPB5
20
HSPB6
20
HSPB7
37
GAPDH
a)
C2C12 Myotubes
Thapsigargin
1uM
Time (Hours)
CTRL 3 6 12 16 24
25
HSPB1
25
HSPB5
20
HSPB6
20
HSPB7
37
GAPDH
b)

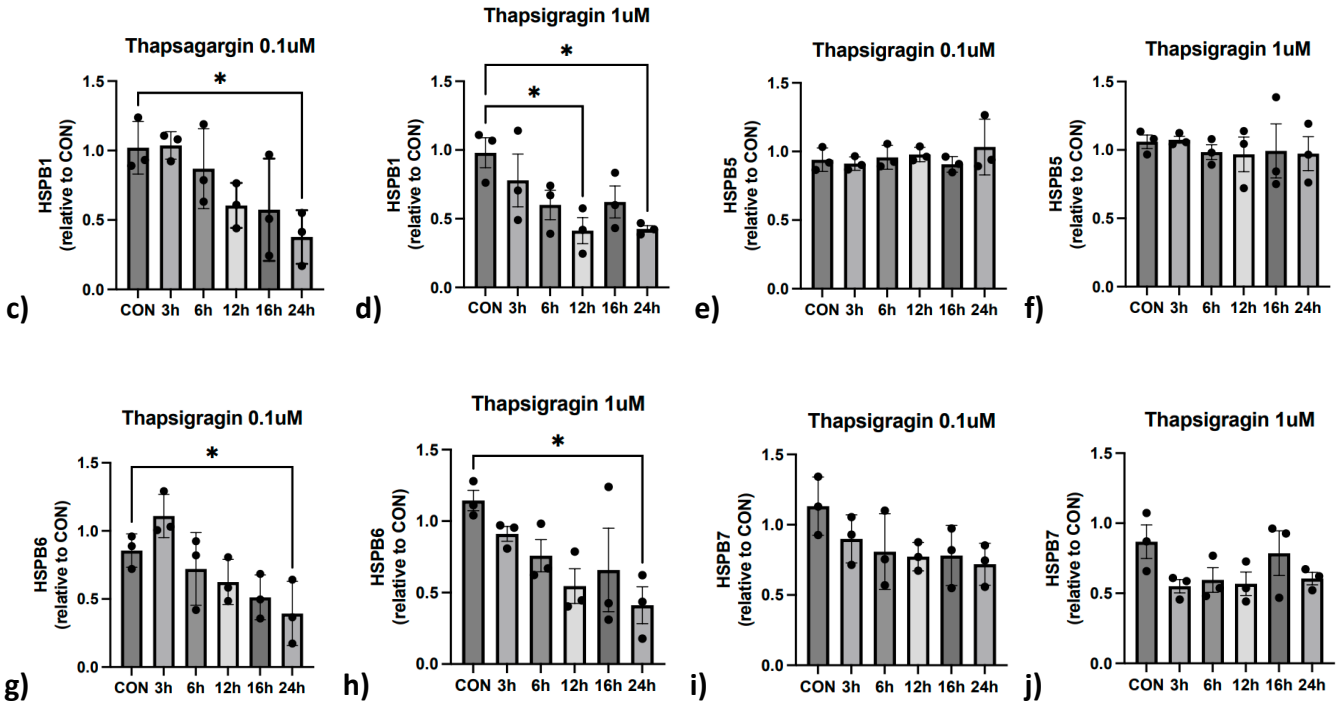
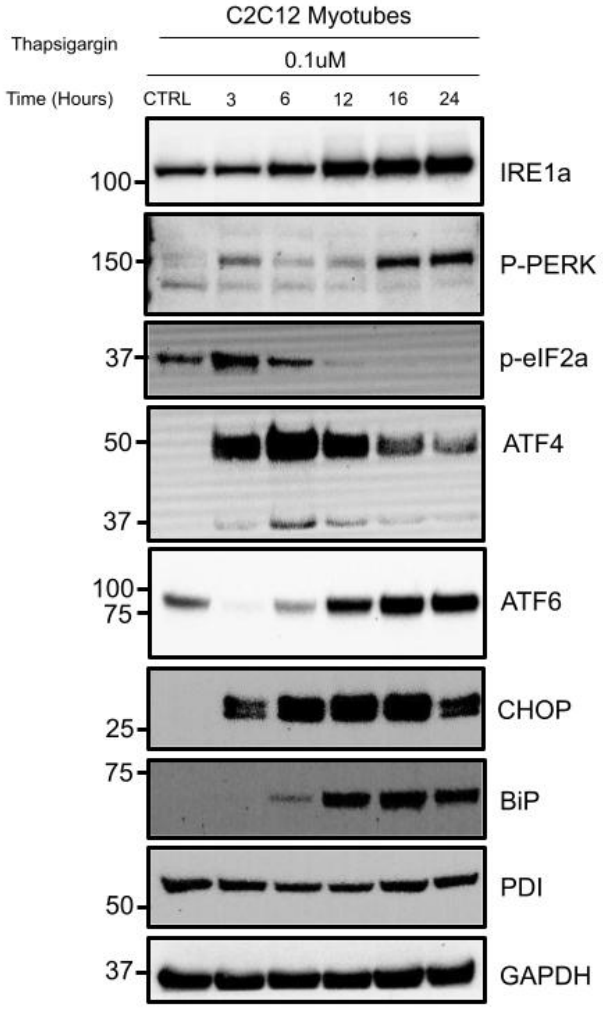
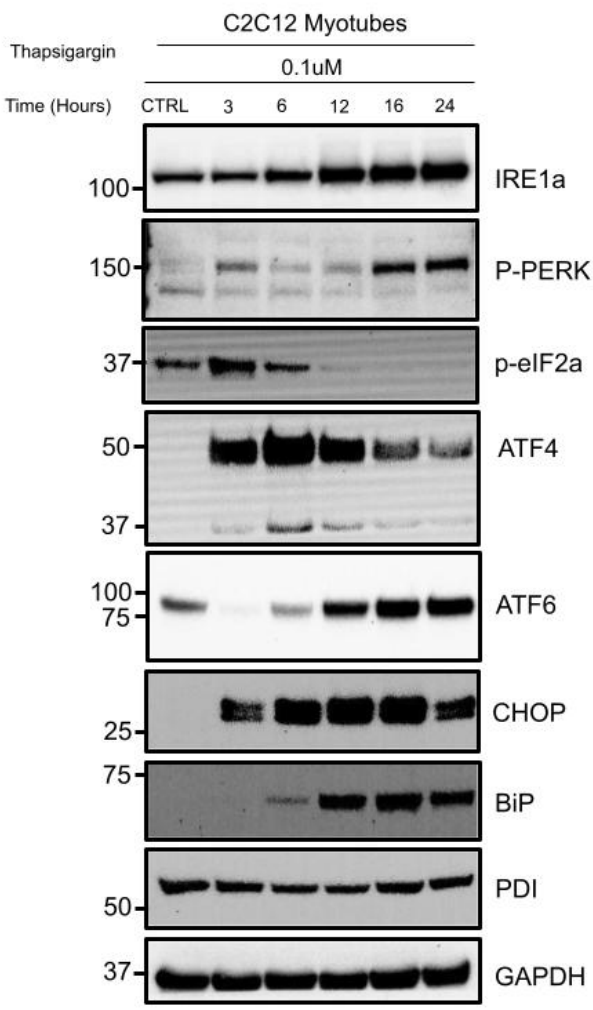

Thapsagargin 0.1uM
HSPB1 (relative to CON)
CON 3h 6h 12h 16h 24h
c)
Thapsigragin 1uM
HSPB1 (relative to CON)
CON 3h 6h 12h 16h 24h
d)
Thapsigragin 0.1uM
HSPB5 (relative to CON)
CON 3h 6h 12h 16h 24h
e)
Thapsigragin 1uM
HSPB5 (relative to CON)
CON 3h 6h 12h 16h 24h
f)
Thapsigragin 0.1uM
HSPB6 (relative to CON)
CON 3h 6h 12h 16h 24h
g)
Thapsigragin 1uM
HSPB6 (relative to CON)
CON 3h 6h 12h 16h 24h
h)
Thapsigragin 0.1uM
HSPB7 (relative to CON)
CON 3h 6h 12h 16h 24h
i)
Thapsigragin 1uM
HSPB7 (relative to CON)
CON 3h 6h 12h 16h 24h
j)

Figure 3.4 Thapsigargin treatment decreased HSPB1 and HSPB6 , but not HSPB5 and HSPB7

C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON) or Thapsigargin (0.1μM or 1.0μM) for 3, 6, 12, 16, or 24h. Lysates were collected, prepared, and analyzed by SDS-PAGE and western blotting with indicated antibodies. (a) Representative images from one of three independent experiments for 0.1 μM Thapsigargin treatment. (b) Representative images from one of three independent experiments for 1.0 μM Thapsigargin treatment. (c-j) Quantification of HSPB1, HSPB5, HPSB6 and HSPB7. Data are expressed as mean ± SEM (n = 3) fold changes relative to control. One-way ANOVA with Dunnett's post-hoc test, $*P < 0.05$, compared to CON.


C2C12 Myotubes
Thapsigargin
0.1uM
Time (Hours)
CTRL
3
6
12
16
24
100
IRE1a
150
P-PERK
37
p-eIF2a
50
ATF4
37
100
75
ATF6
25
CHOP
75
BiP
50
PDI
37
GAPDH

a)


C2C12 Myotubes
Thapsigargin
0.1uM
Time (Hours)
CTRL
3
6
12
16
24
100
IRE1a
150
P-PERK
37
p-eIF2a
50
ATF4
37
100
75
ATF6
25
CHOP
75
BiP
50
PDI
37
GAPDH

b)

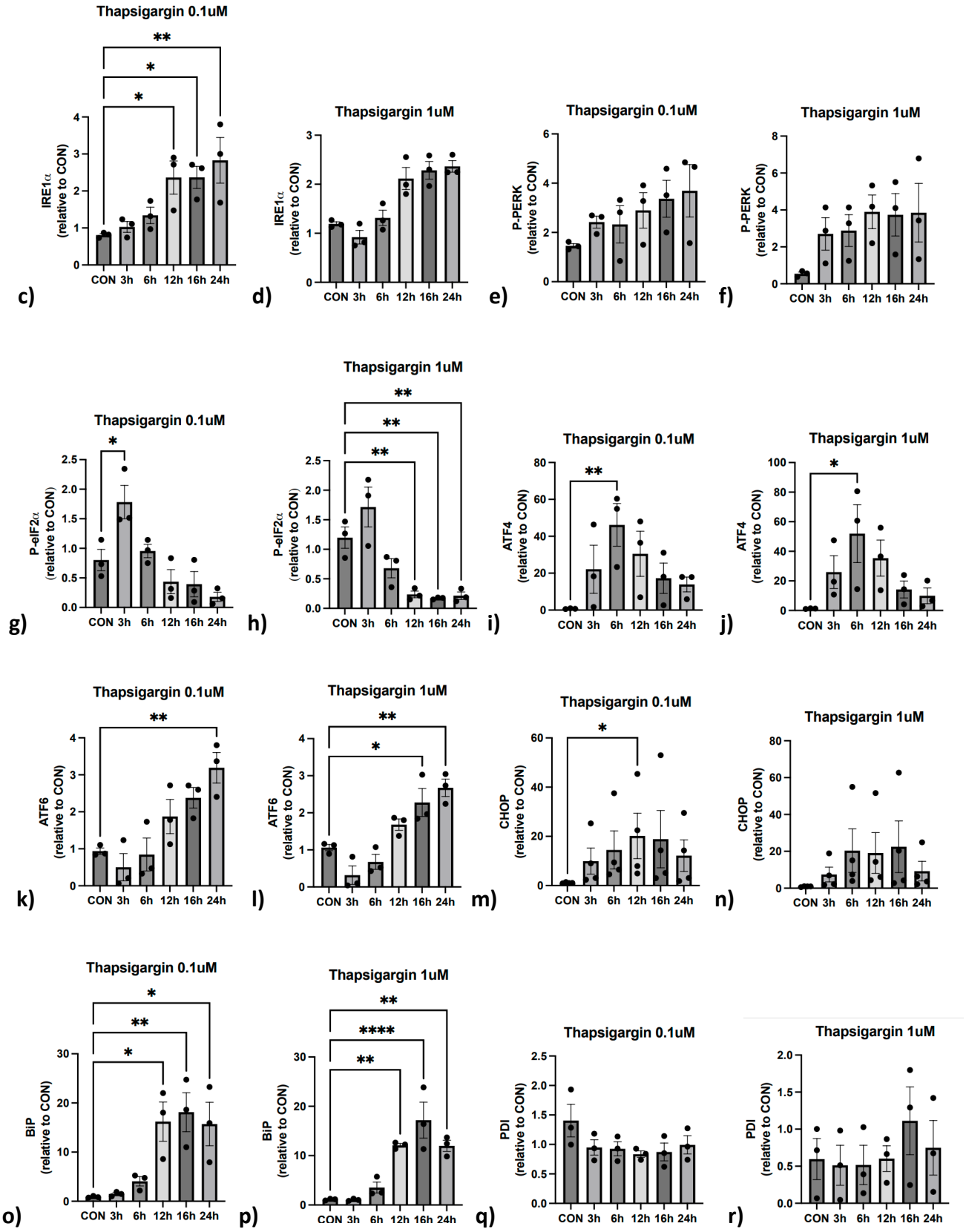

Thapsigargin 0.1uM
IRE1α (relative to CON)
c)
Thapsigargin 1uM
IRE1α (relative to CON)
d)
Thapsigargin 0.1uM
P-PERK (relative to CON)
e)
Thapsigargin 1uM
P-PERK (relative to CON)
f)
Thapsigargin 0.1uM
P-eIF2α (relative to CON)
g)
Thapsigargin 1uM
P-eIF2α (relative to CON)
h)
Thapsigargin 0.1uM
ATF4 (relative to CON)
i)
Thapsigargin 1uM
ATF4 (relative to CON)
j)
Thapsigargin 0.1uM
ATF6 (relative to CON)
k)
Thapsigargin 1uM
ATF6 (relative to CON)
l)
Thapsigargin 0.1uM
CHOP (relative to CON)
m)
Thapsigargin 1uM
CHOP (relative to CON)
n)
Thapsigargin 0.1uM
BiP (relative to CON)
o)
Thapsigargin 1uM
BiP (relative to CON)
p)
Thapsigargin 0.1uM
PDI (relative to CON)
q)
Thapsigargin 1uM
PDI (relative to CON)
r)
CON 3h 6h 12h 16h 24h

Figure 3.5 Thapsigargin induced ER stress through IRE1α, P-eIF2α/ATF4 and ATF6 pathway and stimulate CHOP and BiP protein contents in C2C12 myotubes

C2C12 myotubes were treated with DMSO (0.1%,9 h) as a vehicle control (CON) or Thapsigargin (0.1μM or 1.0μM) for 3, 6, 12, 16, or 24h. Lysates were collected, prepared, and analyzed by SDS-PAGE and western blotting with indicated antibodies. (a) Representative images from one of three independent experiments for 0.1μM Thapsigargin treatment. (b) Representative images from one of three independent experiments for 1.0μM Thapsigargin treatment. (c-r) Quantification of IRE1α, P-PERK, P-eIF2α, ATF4, ATF6, CHOP, BiP, and PDI. Data are expressed as mean ± SEM (n=3) fold changes relative to control. One-way ANOVA with Dunnett's post-hoc test, **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 compared to CON.

CHAPERTER 4

DISCUSSION

4.1 Conclusion of the Findings

Knowledge of the sHSP regulation upon The rationale of this study inspired from the lack of current concerning the molecular mechanisms underpin the response of sHSP under specific ER stress treated in skeletal muscle. sHSP and ER related proteins have been shown well to increase under aged skeletal muscle (Chung and Ng, 2006; Connel *et al*., 2007; Doran.*et al*., 2007; Huang *et al*., 2007; Yamaguchi *et al*., 2007; Beltran Valls *et al*., 2015; Ueda *et al*., 2015). From the literature, we know that both sHSP and ER-related proteins increased under aged skeletal muscle. Still, there relationship between sHSP and ER stress in skeletal muscle remains ambiguous. Therefore, the main objective of this investigation was to observe the response of sHSP under ER stress treated skeletal muscle. The key questions we sought to answer were: (a) How sHSP response under ER stress ;(b) Does sHSP response differently under different types of ER stress inducer; (c) Whether the amount of dosage affect the response of sHSP under ER stress treated skeletal muscle. In addition, (d) Are all the sHSP have the same regulation trend. Our results demonstrate that sHSP decrease under ER stress treated C2C12 myotubes, which is opposite with the original hypothesis.

4.2 Analysis of the Findings

All the sHSP we tested, except HSPB5 and HSPB7, HSPB1 and HSPB6 decrease throughout ER stress conditions(Figure 3.2c, 3.2d, 3.4c, 3.4d, 3.4g, 3.4h). The results are opposite toward our original hypothesis and it might due to the sHSP activation binds to the misfolded created by ER stress and precipitate in pallet which was not detectable in this study.

As sHSP are well known for being a first line responders upon cellular stress that delay the irreversible protein aggregation (Janowska *et al.*, 2019). It is reasonable to record in a decrease of sHSP once induced ER stress into the cell if sHSP perform holdase function, bind to the aggregates and produce precipitates. sHSP might disassemble and response to unfolded proteins before and during stress such that results in a decrease of sHSP under ER stress.

As sHSP cannot rescue the aggregations of protein aggregates stimulated by either Tunicamycin or Thapsigargin, unfolded protein accumulate and ER stress response occur. This eventually lead to the dysfunction of proteostasis within the ER and the cell.

HSPB1, HSPB5 and HSPB6 are reported to form an oligomer during stress and perform (Sugiyama *et al.*, 2000), thus it is reasonable that both HSPB1 and HSPB6 record a decrease under ER stress. Compare to HSPB1 and HSPB6, as HSPB5 is highly exist in skeletal muscle and has a major role in protecting microfilaments, microtubules and intermediate filament components (Suraj P. Bhat and N.Nagineni, 1989; Dimauro and Caporossi, 2022). Hence, instead of binding to proteins aggregates, HSPB5 might has a higher priority to maintain its maintenance function under ER stress. Other than HSPB5, HSPB7 also have a partly diverge in function and this may lead to the different regulation response under ER stress. HSPB7 has reported to have a role for interacting with the contractile and cytoskeleton proteins (Muranova, Shatov, Bukach, *et al.*, 2021).

When compare to HSPB5 and HSPB7, HSPB1 and HSPB6 do not perform the function of structural maintenance (Dreiza *et al.*, 2010; Shan *et al.*, 2022). HSPB1 is more related to apoptosis and autophagy activity (Charette and Landry, 2000; Shen *et al.*, 2016). Meanwhile HSPB6 performs multiple roles in the muscle contractile process (Dreiza *et al.*, 2010). Although all sHSP perform chaperone function in general, their individual function within the cell might affect their protein expression changed under ER stress.

4.3 Implication of the Findings

A greater understanding of the mechanisms of sHSP chaperone activity may able to help in the development of novel therapeutic strategies to modulate the protein aggregates in muscle-related disease. In addition, understand the mechanism of sHSP oligomerization and regulation under stress may able to provide create new therapeutic strategies for pharmaceutical to encounter the aggregation accumulated in other non-muscle related disease (neurodenerative disease like Parkinson's disease etc).

4.4 Further perspectives

Insoluble pallet

Although our findings clearly demonstrate the induction of ER stress lead to a decrease in HSPB1 and HSPB5 in C2C12 myotubes, we do not know whether sHSP response differently within the insoluble pallet. Hence, we suggest that the further experiment can consider using a robust detergent buffer to breakdown the insoluble pallet as to detect the sHSP response in the insoluble fraction during ER stress.

Additional recovery period after inducing ER stress

Also, we suggest to create a washout period after the cell treated in ER stress inducer to observe the response of sHSP after the stress conditions. sHSP function as first line defender of stress response and it is possible to result in a sharp increase in sHSP after adding a wash out period in cell since stress are removed and sHSP will dissociate with protein aggregates and result in a great amount of sHSP in protein level.

Localization of sHSP

As literature have outlined that sHSP regulate protein aggregates under stress but not particularly in ER stress, we suggest that the further study can also look at the sHSP localization under ER stress. sHSP is located in tubulin, microtubules (Hino et al., 2000) and mainly in plasma membrane, mitochondria and cytosol but not endoplasmic reticulum (The Human Protein Atlas. (2024). Available at: https://www.proteinatlas.org/). Thus, it will be interesting to see whether sHSP translocate to endoplasmic reticulum during ER stress as to deal with the protein aggregates since aggregates need to send to ER for refolding by ATP-dependent chaperones.

Oligomerization pattern of sHSP

sHSP perform several types of oligomerzation under non-stress condition and disassemble into small pieces of sHSP and binds to protein aggregates (Gu, Fan and Yu., 2023). Therefore we suggest that further study can look at the sHSP oligomerization pattern under ER stress by using Immunoprecipipation, sHSP might co-immunoprecipipate with each other and

change their ratio of oligomerization group under ER stress, since the response of sHSP are different from our findings.

Measurement the ER stress level

In addition, we suggest using other method to measure ER stress in cell. Methods including immunofluorescence of ER chaperons (CHOP/BiP) or observe the morphology of endoplasmic reticulum or even the measure the 26-S proteasome protein turnover level will provide a better understanding on how sHSP helps to resolve ER stress and how do sHSP response throughout different level of ER stress. Since we do not exactly know at which time point, the stress will be the most severe and causing sHSP change its regulation.

Rest of the sHSP in skeletal muscle

Although sHSP perform chaperone activity in general, more study can be done to observe the rest of the three sHSP that are reported to exist in skeletal muscle but detect in this study(i.e HSPB2, HSPB3 and HSPB8).As sHSP are a chaperone family and perform different oligomerization complexes in non-stress condition. Their response upon ER stress might be different with each other. Thus understanding their expression individually can provide a more clear outline of their specific role in ER stress.

4.5 Limitation

This thesis is only observing sHSP regulation under protein, but not in the gene level. As a result, we do not know whether sHSP decrease its gene expression or not under stimulated

ER stress in C2C12 myotubes. sHSP gene level might be increase if they are only binded in the insoluble fraction during ER stress.

Furthermore, this study does not test some of the important phosphorylation proteins, as some of the sHSP, particularly HSPB1, is reported form the literature that it will be phosphorylated before dissemble from big oligomers into small pieces (Rogalla *et al.*, 1999). With the measurement of the phosphorylated sHSPs, we might able to know more about whether the sHSP are activated or not during ER stress. UPR proteins like IRE1α should also measure its phosphorylated protein level since it will phosphorylated under ER stress and activate its downstream pathway to alleviate the accumulation of unfolded protein in ER (Preissler and Ron, 2019; Bashir *et al.*, 2021).

4.6 Conclusion

Based on the findings in the present study and existing literature, our current understanding of sHSP and ER stress in skeletal muscle remains in its infancy. Here, we have outlined the literature and our own preliminary data that shape our understanding of sHSP and ER stress, highlightened the recent findings made in the field and finally, suggest future directions for the studies can take for further exploration. Importantly, the previous data provided a limited information, still they have helped to lay down the foundations with respect to the types of sHSP that have a greater potential to perform a role under ER stress. For now, it seems that only some typical types of sHSP may involve in regulating the protein aggregates under ER stress. Despite little is known about sHSP and ER stress relationship in skeletal muscle, it

seems as though they can act and response independently but not with the same oligomerization complexes. Our data show that two sHSP, namely HSPB1 and HSPB6 are decreased under ER stress via two different types of ER-stress inducer, Tunicamycin and Thapsigargin, with two different amount of dosage respectively. Furthermore, this thesis also demonstrate the response of three individual UPR stress pathway ,and the well-known ER stress markers under four different types of ER stress condition(two ER stress inducer with two amount of dosage respectively) in C2C12 myotubes. Finally, the discussion within the thesis pointed out the current limitation and propose a few possible future direction to continue the project. Overall, these data demonstrate this thesis explore the knowledge of the response of small heat shock protein under endoplasmic reticulum stress in C2C12 skeletal myotubes.

CHAPTER 5

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