Insights from *Dictyostelium*: Examining the role of cellular stress in Batten disease

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Abstract

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The neuronal ceroid lipofuscinoses (NCLs), commonly known as Batten disease, are a family of fatal neurodegenerative disorders that primarily affect children. Several subtypes of NCLs have been reported, each being caused by a mutation in a distinct ceroid lipofuscinosis neuronal (*CLN*) gene; this results in aberrant lysosome function and the accumulation of lipoprotein aggregates (known as ceroid lipofuscin) within cells. Several innate cellular pathways exist to alleviate the stress caused by the buildup of aggregates. The endoplasmic reticulum (ER) is an essential organelle in this process because it is responsible for maintaining cellular homeostasis through protein production, quality control, and regulating several signalling pathways. The unfolded protein response (UPR) consists of several conserved pathways devoted to attenuating ER stress caused by an accumulation of misfolded proteins or aggregates; at the center of this stress response is GRP78, a molecular chaperone that binds to misfolded proteins to facilitate proper folding. The social amoeba *Dictyostelium discoideum* is an excellent model system for studying NCLs as it encodes more CLN-like proteins when compared to other classical model organisms (e.g., yeast, worm, fruit fly). In this study, *D. discoideum* was used to elucidate the effects of ER stress and build an understanding of how cells cope with increased stress. Beyond this, ER stress in *D. discoideum* models for CLN3 disease and CLN5 disease were evaluated. First and foremost, during the induction of ER stress by tunicamycin, there was an increase in intracellular and extracellular amounts of Grp78 accompanied by an increase in stress-related changes to the ER. Furthermore, models of CLN3 disease and CLN5 disease displayed increased amounts of Grp78 as well as a disrupted ER morphology. Interestingly, wildtype *D. discoideum,* AX3 cells, treated with tunicamycin displayed a similarly disrupted ER when compared to CLN models. Finally, when subjected to tunicamycin-induced ER stress, these NCL models displayed a trend towards increased Grp78 amounts, however, these cells appear to have a reduced sensitivity to tunicamycin-induced stress compared to wild-type cells. In summary, this study highlights *D. discoideum* as a model for studying ER stress through the conserved role of Grp78 in the stress response and concludes that an aberrant ER stress underlies the pathology of the NCLs.

Keywords: Batten disease, BiP, CLN3, CLN5, *Dictyostelium discoideum*, endoplasmic reticulum, ER stress response, GRP78, neuronal ceroid lipofuscinoses (NCLs), unfolded protein response

Acknowledgments

The study of the natural world began as we evolved complex thought, igniting a pursuit to understand fundamental principles governing our universe, giving birth to science. Science, in its essence, is an art; it is through relentless practice, continuous refinement, adaptation, and empirical validation that scientists have meticulously shaped our comprehension of the world. Inspired by diverse muses, we channel our energy towards the betterment of our world. I am sincerely grateful for the opportunity to be part of this collaborative endeavor.

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1. Introduction

1.1 Endoplasmic Reticulum (ER)

The endoplasmic reticulum (ER) is a continuous membrane-bound organelle that synthesizes most of the proteins produced by the cell (Dallner et al, 1966; Caro and Palade; 1964). Emerging from the nuclear envelope, the ER extends throughout the cell forming contact points with the plasma membrane (PM) and mitochondria (West et al, 2011). Traditionally, the ER has been described as having two structural domains: membrane cisternae (commonly referred to as "sheets") and tubules (Veratti,1961; Palade and Porter, 1954). The arrangements of sheets and tubules not only vary throughout the cell but across different cell types within the same organism (Veratti, 1961; Palade and Porter, 1954). The perinuclear region of the ER, adjacent or juxtaposed to the nucleus, is concentrated with ER sheets decorated with ribosomes to form the rough ER, creating a hub for protein synthesis (Krebich and Sabatini, 1992). As the ER extends distally through the cell, the concentration of ribosome-less tubules increases and intertwines with a portion of sheets, forming the peripheral or smooth ER (Veratti, 1961; Palade and Porter, 1954). It has been proposed that sheet regions may be responsible for protein synthesis based on their high ribosome density (Krebich and Sabatini, 1992), while tubules are responsible for lipid synthesis and calcium ion (hereafter referred to as calcium) homeostasis (English and Voeltz, 2013; Friedman and Voeltz, 2011). Finally, the cortical ER is a region of the peripheral ER that forms connections with the PM to aid in secretion as well as lipid and signal exchange (West et al, 2011).

The ER performs an array of functions by populating the membrane and lumen with versatile proteins and cofactors (ions such as calcium and magnesium) to create a specialized, yet

adjustable microenvironment to facilitate protein folding. However, for many reasons, the cell may not be able to adequately adjust, resulting in prolonged stress. The dynamic nature of the ER is maintained by various proteins and signals that regulate how the cell responds to stress to maintain homeostasis. One such protein, glucose regulatory protein 78 (GRP78, also known as binding immunoglobin protein, BiP) mediates the stress response through sensing, communicating, and regulating multiple stressors including the accumulation of misfolded proteins, reactive oxygen species, and environmental stressors (changes in heat, salinity, and environmental toxins) (Haas and Wabl 1983; Munro and Pelham, 1986; Rose et al, 1989; Morai et al, 1992; Lee, 1987; Lentz and Sadler, 1991; Wu et al, 1993; Gewandter et al, 2009). GRP78 is classified as a molecular chaperone protein, which aids in protein folding (Kassenbrock and Kelly, 1989). Additionally, GRP78 is a member of the heat shock protein (HSP) family and is further subcategorized into the HSP70 group due to its molecular weight, N-terminal ATPase domain, and C-terminal peptide-binding domain (Lee, 1987; Munro and Pelham, 1986; Määttänen et al., 2010).

The ER is ubiquitous throughout the eukaryotic domain of life; conserved proteins have been identified that regulate its structure and function including: reticulons (RTN), a class of proteins that promote ER membrane curvature and are required for the degradation of ER tubules; Alastin (ATL), a family of GTPases responsible for mediating ER fusion; homologs of ATL include Sey1 in *D. discoideum* and *S. cerevisiae*, as well as Root hair defective 3 found in plants (*A. thaliana*) (Hu et al, 2009; Chen et al, 2011; Liu et al, 2007; Liu et al 2012; Liu et al, 2012; Zurek et al, 2011; Steiner et al, 2017; van Anken et al, 2014; Mishiba et al, 2019; Yu et al, 2022; Dominguez-Martin et al, 2018). Beyond this, the ER is comprised of various homologs of molecular chaperone proteins.

1.1.1 Molecular chaperones: GRP78, calnexin, and calreticulin

Molecular chaperones are highly conserved proteins, existing to mediate the folding of polypeptides or to target polypeptides for degradation through the ubiquitin-proteasome system (UPS), ER associated degradation (ERAD), or autophagy (Bukau et al., 2006; Kampinga and Craig, 2010; Ellis, 1990). However, proteins can escape these cycles leading to aggregation and further collapse of protein homeostasis (Hartl et al., 2011). In tandem with chaperone-mediated protein folding, many molecular chaperones are calcium-binding proteins, acting to buffer calcium ions within the cell (Prins and Michalak, 2011).

GRP78 can interact with misfolded proteins by directly interacting with hydrophobic regions on misfolded proteins through the C-terminal substrate binding domain of GRP78 which can be regulated by ATP binding to the N-terminal nucleotide-binding domain (Kassenbrock and Kelly, 1989; Lee, 2001; Szabo et al, 1994; Wang et al, 2017). In the ATP-bound state, there is a low affinity for substrate binding (Szabo et al, 1994). Once hydrolyzed to ADP, the affinity for misfolded protein substrates increases (Szabo et al, 1994). Additionally, the nucleotide-binding domain can also increase or decrease ATPase activity through binding magnesium or calcium, respectfully (Sriram et al, 1997; Wei and Hendershot, 1995; Wang et al, 2017). Together, this allows GRP78 to facilitate the proper folding of proteins, prevent misfolded proteins from aggregating, or target misfolded proteins for degradation (Sazabo et al, 1994; Simons et al, 1995).

GRP78 is an integral cog in the ER stress pathways as it is shuttled from site to site to signal stress, aid in protein folding, and regulate calcium flow (Lee, 1987; Pobre et al, 2019). GRP78 can carry out these tasks through various levels of regulations or can be assisted by

cochaperone proteins such as members of the DnaJ family, who aid GRP78 by activating binding sites or by acting as a liaison between GRP78, misfolded proteins, and equipment specialized for degradation (Misselwitz et al,1998; Jin et al, 2008; Ushioda et al, 2008 Fritz et al, 2014).

Despite having an ER signal peptide and localizing predominantly to the ER, GRP78 can be directed to the cytosol, nucleus, mitochondria, or PM, and can be secreted out of the cell (Suzuki et al, 1991; Sun et al, 2006). The localization and function of GRP78 are influenced by post-translational modifications (PTMs) such as arginylation (Cha-Molsttad et al, 2015). GRP78 performs many functions including aiding in protein folding, UPR activation, targeting misfolded proteins for ERAD, maintaining calcium homeostasis, and regulating mitochondrial stress all of which can be regulated by various PTMs that influence interactions with different proteins (Ma and Hendershot 2004; Otero et al. 2010; Cesaratto et al, 2020). Further evidence suggests a role in autophagy, a cellular process that degrades protein and cellular components in eukaryotic cells. For example, overexpression of GRP78 has been shown to promote autophagosome formation (Khaminets et al, 2015; Itakura and Mizushima, 2010 (Cook and Clarke, 2012).

A separate class of chaperones, namely lectin chaperones, including calnexin and calreticulin, bind to glycoproteins to aid in folding (Hammond et al, 1994). Calnexin is an ER membrane protein and is found at several ER-organelle contact sites including sites with mitochondria (Wada et al, 1991; Lynes et al, 2013; Paskevicius et al, 2023). Calreticulin resides within the ER lumen and participates in lectin chaperone activity (Smith and Koch, 1989; reviewed in Michalak et al, 1992). Both proteins have binding sites for calcium and also function to maintain calcium homeostasis (Ostwald and MacLennan, 1974; Wada et al, 1991; Schrag et al. 2001; Kozlov et al. 2010).

Conserved chaperone proteins in eukaryotes include orthologs of the HSP family- HSP70 and HSP90 (GRP78 and GRP94), calnexin, calreticulin, and ER oxidoreductases PDI, which catalyze the formation of disulfide bonds (Ulrich and Hartl et al, 2011). Together, molecular chaperones have a profound effect on cellular stress and disease and function to aid in proper protein folding and prevent protein aggregation across all domains of life.

1.2 Endoplasmic reticulum stress

Cellular homeostasis is fundamental to survival. Various stimuli can cause the cell to become stressed while negatively impacting other vital cellular mechanisms. However, the ER is able to sense and coordinate several innate pathways to reestablish homeostasis with the help of GRP78.

1.2.1 Sensing stress: the unfolded protein response (UPR)

Within the ER lumen, GRP78 is bound to ER membrane proteins: activating transcription factor 6 (ATF6), inositol-requiring 1α (IRE1α), and protein kinase-like ER kinase (PERK) in metazoans (Figure 1A), Ire1p in yeast, IreA in *D. discoideum*, and IRE1A, IRE1B, IRE1C in plants (*Arabidopsis thaliana* homologs of IRE1, while other species of plants such as rice have one homolog of IRE1), as well as basic leucine zipper transcription factor (bZIP)17 and bZIP28 (plant homologs of ATF6) (Sidrauski and Walter, 1997; Mishiba et al, 2019; Haze et al., 1999; Liu et al., 2007). In response to various stressors, GRP78 disassociates from these complexes to initiate various signaling cascades).

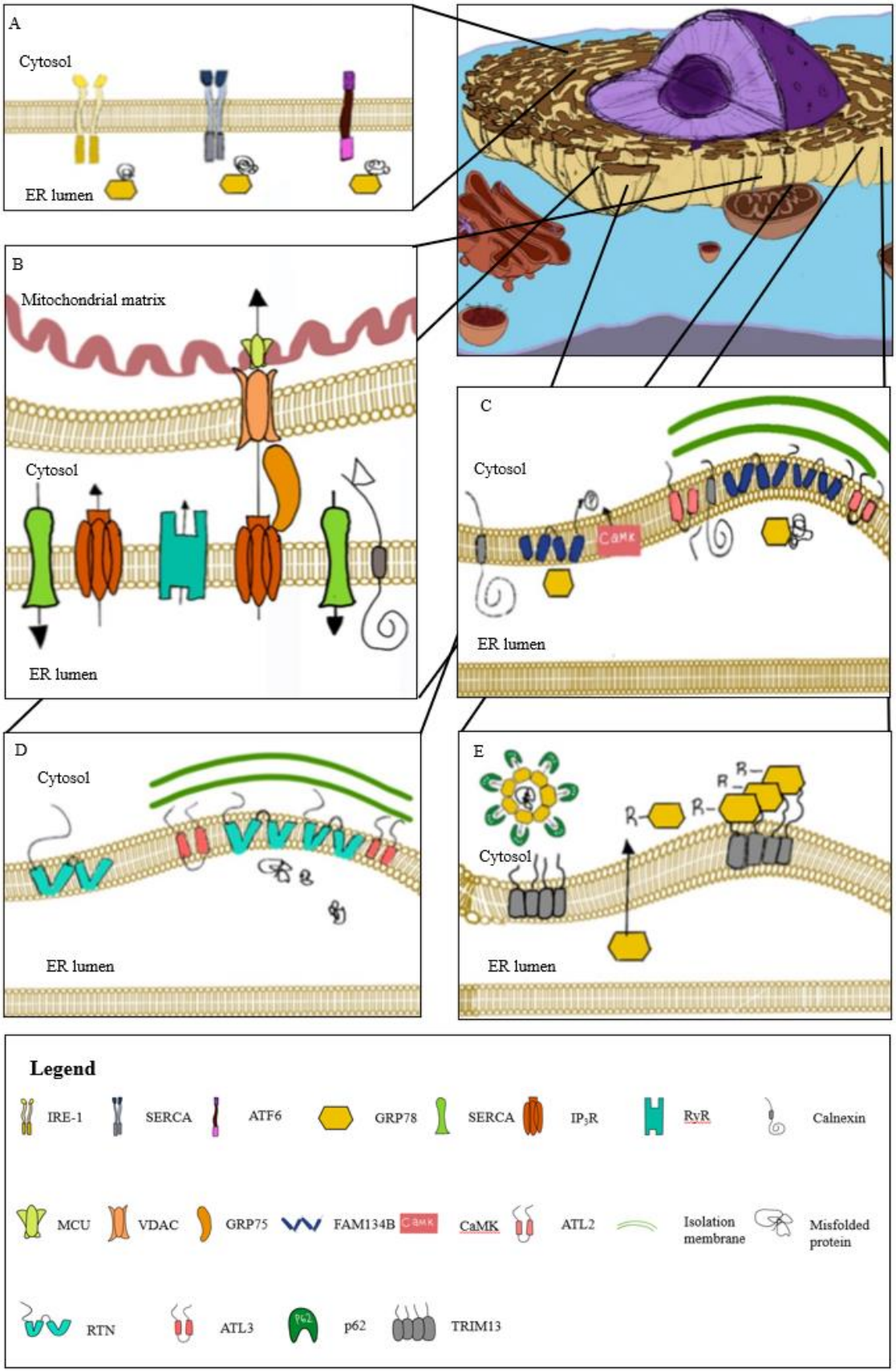
A
Cytosol
ER lumen
B
Mitochondrial matrix
Cytosol
ER lumen
C
Cytosol
CaMK
ER lumen
D
Cytosol
ER lumen
E
R
Cytosol
ER lumen
Legend
IRE-1
SERCA
ATF6
GRP78
SERCA
IP3R
RyR
Calnexin
MCU
VDAC
GRP75
FAM134B
CaMK
ATL2
Isolation membrane
Misfolded protein
RTN
ATL3
p62
TRIM13

Figure 1: Cellular pathways involved in regulating endoplasmic reticulum stress.
(A) UPR: interaction with misfolded proteins causes GRP78 to dissociate from IRE1, PERK, and ATF4, this then triggers UPR signaling based on the activated signal transducer.
(B) Calcium transport at MAMs: SERCA actively transports calcium into the ER. IP_3R and RyR pump calcium into the cytosol. IP_3R forms a complex with GRP78, VADC, and MCU to transport calcium from the ER to mitochondria.
(C) ER-phagy via RTN: RTN family of proteins influences ER turnover by influencing ER biogenesis and ER-phagy through oligomerization. For example, through homo-oligomerization, RTN3L clusters and induces ER fragmentation leading to ER-phagy through interactions with LC3 and recruitment of ATL3 to the region. In contrast, RTN3L heterooligomerization with RTN3S promotes ER tubular biogenesis.
(D) ER-phagy via FAM134B: ER stress promotes the oligomerization of FAM134B through the activation of calcium/calmodilin-dependent protein kinases and subsequent phosphorylation of FAM134B. Additionally, FAM134B colocalized with ATL2 and has been suggested that the two proteins work in series to promote ER-phagy. Although ER-phagy has been accepted as helpful means to prevent over ER expansion excessive ER degradation through FAM134B mediated ER – phagy also results in a loss of ER homeostasis by indiscriminately degrading the ER to the point where function is hindered.
(E) The degradative role of Arg/N-degerdon: argnylation of GRP78 allows for binding to p62 (green, missing from trim) leading to oligomerization. Oligomerized p62 targets protein aggregates for degradation and interacts with TRIM13 to induce ER-phagy.

ATF6 is a transmembrane protein embedded in the ER. The C-terminal region, located within the ER lumen, senses misfolded proteins through interactions with GRP78, while the N-terminal region faces the cytosol and functions as a bZIP transcription factor once activated (Haze et al., 1999; Yoshida et al., 1998). When the UPR is activated, GRP78 disassociates from the C-terminus of ATF6 leading to proteolysis of ATF6 and transport to the Golgi. This activates the bZIP portion of ATF6, which then translocates to the nucleus, leading to further *GRP78* transcription (Haze et al., 1999; Shen et al., 2002). ATF6 also increases the expression of other major ER chaperone proteins by forming a heterodimer with X-box-binding protein (XBP1), as found in mice embryonic fibroblasts (MEFs), allowing for the induction of ERAD (Yamamoto, et al, 2007).

IRE1 is anER transmembrane protein where the N-terminal region interacts with GRP78 on the luminal side of the ER. Stimulation by misfolded proteins causes GRP78 to dissociate, resulting in IRE1 oligomerization and/or dimerization to activate endoribonuclease (RNase) and serine-threonine kinase activity (Shamu and Walter, 1996; Tirasophon et al., 2000; Bertolotti et al, 2000). This RNase activity allows for the excision and activation of the mRNA transcript for XBP1(Yamamoto et al., 2007). Prolonged ER stress induces a decrease in RNase specificity causing a decrease in overall mRNA translation through regulated IRE1-dependent decay (RIDD) (Hollien et al., 2009; Hollien and Weissman, 2006). If ER stress continues to persist, IRE1 may trigger apoptosis through the activation of caspases and other proapoptotic factors and protein kinases (Urano et al., 2000; Tabas and Ron, 201; Han et al, 2009).

Like IRE1, PERK is a transmembrane protein and monitors ER stress through N-terminal interactions with GRP78 within the ER lumen (Harding et al., 1999). Stimulation by misfolded proteins and subsequent PERK dimerization activates the cytosolic kinase activity of PERK

leading to the phosphorylation of serine 51 on the α-subunit of eukaryotic initiation factor 2α (eIF2α) (Harding et al., 1999; Starck et al., 2016). This prevents the translation of mRNA transcripts, increases *GRP78* transcription, and compromises the formation of the GTP·eIF2α·Met-tRNA$_i$ ternary complex resulting in a delay in translation re-initiation in mammalian cell lines (Starck et al., 2016). As a result, ribosomes will have a greater chance of "misreading" upstream inhibitory reading frames and will promote ATF4 translation, with stalled or misreading ribosomes being targeted for ERAD (Vattem and Wek, 2004; Starck et al., 2016). ATF4 is a bZIP transcription factor involved in the upregulation of UPR target genes for efficient protein folding, antioxidant response, amino acid biosynthesis, and pro-apoptotic factor CCAAT-enhancer-binding protein homologous protein (CHOP) (Harding et al, 2000; Huggins et al, 2016; reviewed in Han et al, 2012). A 2004 study by Marciniak et al, linked CHOP to calcium-mediated apoptosis by hyperoxidizing the ER lumen and sending ERO1-alpha (ER oxidase 1 α) to the ER lumen. Oxidation can induce the activation of the ER calcium release through inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R), a channel for transporting calcium ions, which is regulated by IP_3 (Higo et al., 2005). IP_3-induced calcium releases from the ER and the stress responses are inhibited when ERO1-alpha is knocked down (Li et al, 2009).

1.2.2 Stress-related degradation mediated by GRP78

In response to an increase in misfolded proteins, the cell triggers the UPR to increase the protein folding capacity of the cell by inducing the transcription of additional ER chaperones, while decreasing the transcription of other proteins and increasing protein degradation (Ma and Hendershot 2001; Mori et al 1998; Mori et al, 2000; Patil and Walter 2001). While the UPR gauges the stress caused by misfolded proteins, proteins can be extracted from the ER, tagged,

and degraded with the aid of ERAD, the UPS, and autophagy (Hwang and Qi, 2018; Itakura and Mizushima, 2010; Klionsky et al., 2000). ERAD is responsible for recognizing misfolded proteins in the ER and directing them for degradation by the UPS (Tsai and Ye, 2002; Hwang and Qi, 2018). One function of ERAD is to recognize misfolded proteins and polypeptides stalled at ribosomes and transport them out of the ER to be targeted and delivered for proteasome degradation (Christianson et al, 2011; Hwand et al, 2017). The proteasome is a complex of enzymes that carry out protein degradation (reviewed in Adams, 2003). GRP78 is also involved in mediating the movement of ribosome-stalled proteins from the ER to the cytosol for degradation, with the loss of GRP78 in HEK293T cells leading to the ER retention of stalled ribosomes and proteins (Cesaratto et al, 2020). Proteins can be targeted to the proteasome through ubiquitination, a PTM where ubiquitin is covalently attached to a protein; here ubiquitin governs protein fate by influencing protein interactions (reviewed in Yau and Rape, 2016; Pergolizzi et al, 2019). Together, ubiquitin and the proteasome form the UPS, which is composed of ubiquitin, E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, E3 ubiquitin–ligase enzymes, and the proteasome to degrade an array of proteins (reviewed in Schwartz and Ciechanover, 2009). Additionally, the clearance of large protein aggregates and cellular compartments can be done through autophagy (Rusten et al, 2008). Autophagy involves the receptor-driven detection of cargo for degradation through the formation of an isolation membrane, which grows into an autophagosome (Suzuki, 2001). Autophagosomes and lysosomes then fuse, combining cargo with lysosomal enzymes for degradation (reviewed in Zaffagnini and Martens, 2016). A study by B'Chir et al (2013), showed that the PERK-eIF2α branch of the UPR was essential for stress-induced autophagy in MEFs. Consequently, the aberrant function and localization of GRP78 can lead to the dysfunction of these pathways

resulting in substrate accumulation as well as cellular and clinical abnormalities. The ER, and the connections it holds with several pathways and organelles, make it a hub for signalling; as a result, altercations to ER stress can affect homeostasis and several additional signal pathways.

1.2.3 Endoplasmic reticulum stress and calcium signalling

Beyond its function as the manufacturing center of the cell, the ER functions as a major store of calcium (Pietrobon et al, 1990). Additionally, mitochondria are also known to modulate various cellular communications through calcium signalling (reviewed in Wallace, 2012). Specialized proteins govern the release of calcium at the mitochondria-associated membrane (MAM) (Figure 1B), a specialized region of the ER that forms connections with the mitochondria through proteinaceous tethers and specialized calcium transport protein to govern mitochondrial bioenergetics, lipid synthesis, calcium signaling, and autophagosome assembly (reviewed in Wilson and Metzakopian, 2021). In Chinese hamster ovary (CHO) cells, GRP78 forms a complex with IP_3R3 and Sig-R (a ligand-operated calcium-sensitive chaperone) to transport calcium at the MAMs (Hayashi and Su, 2007). Additionally, studies in MA-10 cells (cell line derived from mouse Leydig cell tumor) show that under ER stress, GRP78 can also localize to the MAMs to facilitate the flow of calcium and regulate mitochondrial metabolism (Prasad et al, 2017). Taken together, the calcium binding and shuttling activity of GRP78 in concert with its known interactions with calcium transport proteins underscores a role in regulating calcium homeostasis and calcium transport. As a result, calcium transporter activity has a complex effect of cellular stress and GRP78.

1.2.3.1 Sarcoplasmic/Endoplasmic reticulum calcium ATPase (SERCA)

Sarcoplasmic/Endoplasmic reticulum calcium ATPase (SERCA) is an active calcium transporter that moves calcium from a low concentration area, such as from the cytosol, to high concentration regions, like the ER (Wu et al, 1995; reviewed in de Meis and Vianna; 1979). A 2013 study by White et al evaluated the effect of SERCA inhibition on ER stress in glioma cells, which demonstrated an increase in GRP78 amounts and ER caspase 3 and 4 activation accompanied by the release of calcium (White et al, 2013). The calcium transport ability of SERCA is regulated through interactions with post-translationally modified calnexin (Roderick et al, 2000; Lyne et al, 2013). For example, phosphorylated calnexin was shown to inhibit SERCA activity when calcium stores were full (Chevet et al, 1999). Under these conditions, phosphorylated calnexin also binds to ribosomes to aid protein translation in canine pancreatic microsomes and Rat-2 cells (Chevet et al, 1999). Whereas the dephosphorylation of calnexin reinstates calnexin-SERCA activity in Xenopus oocytes, reducing calcium flow (Roderick et al, 2000). Furthermore, calnexin can be modified by palmitoylation, a post-translational modification that attaches a saturated 16-carbon fatty acid chain to specific cysteines via a thioester bond (O'Dowd et al, 1988; Stoffel et al, 1994). Lynes and colleagues observed that palmitoylated calnexin is concentrated at MAMs in HeLa cells; however, upon ER stress induction by TN, a reduction in MAM-associated calnexin can be seen in HeLa cells (Lynes et al, 2012). Although previous reports support that acute ER stress can increase ER-mitochondrial contact points (Bravo et al, 2011), Lynes et al hypothesize that the movement of calnexin away from MAM promotes a specific function of calnexin (Lynes et al, 2012). Acute ER stress induced by either DTT or TN resulted in the depalmitoylation of calnexin but not dephosphorylation; thereby reducing calnexin-SERCA interactions while maintaining calcium flow in HeLa cells and MEFs at a reduced rate (Lynes et al, 2013). By comparing the behaviours

of wildtype calnexin with a mutated form of calnexin in MEFs that cannot be palmitoylated, it was concluded that palmitoylation is required for calnexin-augmented calcium flow through SERCA (Lynes et al, 2013). Additionally, under non-palmitoylatable conditions, increased interactions between calnexin and the oxidoreductase, ERp57, mediate an increase in protein folding and ER quality control (Zapun et al, 1998; Elliott et al, 1997; Lynes et al, 2012). Finally, Deniaud et al (2008) demonstrated that chemical stressors such SERCA inhibitor, thapsigargin (TG), and tunicamycin (TN) sustain a high flow of calcium from the ER to the mitochondria which was mediated by IP_3R, and voltage dependent anion channel (VDAC) in HeLa and HCT116 cells, resulting in the activation of proapoptotic pathways and increases in mitochondrial membrane permeability regulated by B-cell lymphoma 2 (Bcl-2) family members (Deniaud et al, 2008).

1.2.3.2 Inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R)

IP_3R is a family of intracellular calcium channels that transport calcium out of the ER (Supattapone et al, 1988; Berridge and Irvine, 1984). Although IP_3R can be found alone in the ER membrane, it can also be tethered to MAMs to transport calcium into the mitochondria (Bartok et al, 2019). Calcium flow from the ER to the mitochondria via IP_3R is required for the suppression of autophagy, while decreased calcium flow is accompanied by the activation of AMPK to induce autophagy (Cardenas et al, 2010). IP_3R can be coupled to VDAC through proteaceous tethers provided by another protein of the GRP family, GRP75, which in turn connects to mitochondrial calcium uniporter (MCU) to mediate calcium flow from the ER to the mitochondria at the MAM (Figure 1B) (Hajnoczky et al, 1995). Beyond physically connecting

IP_3R to VADC, GRP75 (*Hspa9)*, this complex also affects the stress response such that downregulation of VDAC protects against ER stress induced by TN and improves stress-induced atrial remolding in mouse atrial cardiomyocytes (HK-1 cells) and in cardiac-specific *Hspa9* knockout mice (Yuan et al, 2022). The inhibition of IP_3R in ventilator-induced lung injury mice models through 2-aminoethoxydiphenyl borate, reduced ER stress-induced inflammation and improved mitochondrial dysfunction by stimulating calcium release from the ER resulting in inflammation, ER stress, and mitochondrial dysfunction (Ye et al, 2021). Furthermore, overexpression of GRP78 in NSC34 cells support the neuroprotective effect of GRP78, which the authors attributed to the restoration of mitochondrial respiration, ROS levels, mitophagy through IP_3R activity due to GRP78 overexpression (Leiva-Rodriguez et al, 2021). Finally, in a study by Ferreiro et al (2006), the treatment of cortical neuron cultures with synthetic prions and beta-amyloid peptides (prone to aggregation) was accompanied by increased amounts of GRP78, caspase-12 activation as well as the early release of calcium from IP_3R and RyR (Rayanodine receptor) leading to the loss of calcium homeostasis.

1.2.3.3 Ryanodine receptor (RyR)

The RyR family of calcium channels are located at the ER membrane (Lai et al, 1988). Inhibition of cytochrome oxidase (COX) activity induces an increase in ER calcium release through RyR that can be augmented by TG (Costa et al., 2013). Short-term TN-induced stress increases RyR activity, however, chronic treatment leads to a reduction in RyR activity. By inhibiting RyR and IP_3R activity, stress-mediated ER calcium loss can be reduced and delay the activation of the UPR in INS-1 β cells, mouse islets, and Akita islets (Yamamoto et al., 2019).

Remarkably, RyR dysfunction was induced by the accumulation of misfolded proteins demonstrated by beta-amyloid protein accumulation in cortical neurons, INS-1 β cells, mouse islets and Akita islets; subsequently triggering RyR-medicated calcium release in cortical neurons (Ferreiro et al, 2006; Yamamoto et al, 2019). This hints that misfolded proteins may interact with RyR and increase calcium flow (Ferreiro et al, 2006; Yamamoto et al, 2019). Another means of regulating calcium flow is through interactions with calcium-binding proteins such as calmodulin (CaM) or calcium/calmodulin-dependent protein kinase II (CaMKII).

1.2.3.4 Calmodulin (CaM)

Calcium dynamics can be used to regulate protein function through calcium-binding proteins; one such protein is calmodulin (CaM) (Cheung, 1970; Teo and Wang, 1973). Calcium binding to CaM triggers a conformational change allowing it to bind to CaM-binding proteins and govern cellular pathways (Cheung, 1980). Early on, CaM was shown to regulate phosphodiesterase and brain adenylate cyclase activity, as well as the synthesis and degradation of cyclic AMP in the brain (Cheung et al, 1978; Cheung et al, 1975). Additionally, CaM is involved in regulating calcium flow from the ER (reviewed in Balshaw et al 2002). For example, CaM was shown to interact with IP_3R1 (a member of the IP3R family) to inhibit channel activity in a calcium-dependent manner (Hirota et al, 1999; Patel et al, 1997). However, CaM is not essential for IP_3R regulation (Sun and Taylor, 2008). Studies on calcium-activated CaM binding to RyR detected a decreased rate of calcium release when calcium is bound to CaM, while calcium apo-CaM (calcium-free CaM) weakly activated RyR1 while inhibiting RyR2 (Moore et al, 1999; Balshaw et al, 2001; Yamaguchi et al, 2001; Huang et al, 2012). Furthermore, Uchida et al (2022) propose a model where ER stress induced by TN causes the translocation of CaM

away from RyR and into the nucleus based on mouse aortic vascular smooth muscle cells. It is noteworthy that dantrolene, a compound that enhances CaM-RyR interactions and stabilizes the closed state of the channel to prevent calcium leak, has been shown to lower TN-induced *GRP78* expression (Balshaw et al, 2001; Uchida et al, 2022). Interestingly, dantrolene treatment restored the binding of CaM to RyR, prevented nuclear CaM accumulation, and restored ER calcium dynamics, thereby adding resilience to ER stress (Uchida et al, 2022). Finally, the disassociation of CaM from RyR2 was associated with Alzheimer's disease phenotypes including increased ER stress, decreased ER calcium content, and Aβ accumulation in murine models (Nakamura et al, 2021). However, by re-establishing CaM-RyR binding with dantrolene treatment, ER stress can be reduced; for example, dantrolene treatment can prevent TN-induced decreases in ER calcium content (Nakamura et al, 2021).

1.2.3.4 Calcium/calmodulin-dependent protein kinase II (CaMKII)

Another calcium-binding protein is calcium/calmodulin-dependent protein kinase II (CaMKII). Sustained calcium flow out of the ER due to the induction of ER stress, activates CaMKII activity leading to programmed cell death pathways (Feng and Anderson, 2016; Timmins et al 2009; Roe and Ren, 2013), while inhibiting CaMKII activity prevents ER stress in TN-induced cardiomyopathy models (Feng and Anderson, 2016). In addition, CaMKII can phosphorylate the ER based, autophagy receptor protein, FAM134B, leading to its oligomerization, ER-fragmentation, and delivery for autophagic degradation (Khaminets et al; 2015; Jiang et al, 2020).

In a study by Liu et al (2020), CaMKII over-expression in C57BL/6 mouse and human neuroblastoma (SH-SY5Y) cells correlated to an increase in oxidative stress by phosphorylating and activating CERB (cAMP-responsive element-binding protein). This led to mitochondrial calcium overload and increases in ROS (Liu et al, 2020). However, inhibition of CaMKII downregulates MCU expression and reduces oxidative damage (Liu et al, 2020). CaMKII activation also participates in cardiotoxicity caused by the chemotherapeutic doxorubicin; here doxorubicin encourages calcium leaks from the ER leading to altered calcium dynamics (Sag et al, 2011; Tscheschner et al, 2019). GRP78 overexpression followed by treatment with doxorubicin in neonatal rat ventricular cardiomyocytes led to dose-dependent effects on CaMKII activity, with low levels of GRP78 overexpression and the accompanied reduction in CaMKII activation and p53 accumulation being beneficial (Tscheschner et al, 2019). However, a 3-fold increase in GRP78 expression resulted in caspase 3 activation and calcium-dependent increase in CaMKII activity which contribute to cytotoxicity (Tscheschner et al, 2019). These findings underscore the delicate balance required for GRP78 modulation, suggesting a potential therapeutic strategy involving precise regulation of GRP78 to optimize its protective effects while mitigating potential adverse consequences caused by excessive GRP78 over-expression.

1.2.3.6 Calcium and caspase activation

Enhanced calcium flow from the ER to mitochondria induces ER stress, mitochondrial dysfunction, and apoptosis (Kruman et al, 1998; Biagioli et al, 2008). Apoptosis is a form of cell death regulated by the caspase family of aspirate-specific cysteine proteases (Cohen, 1997). This is further governed by mitochondria through the Bcl-2 family of apoptosis suppressors (Fujita

and Tsuruo, 1998). To activate the proapoptotic activity, most proteins in the caspase family require the cleavage of a prodomain (Nakagawa et al., 2000). For example, in HEK 293T cells, caspase-12 remains inactive by forming a complex with caspase-7 and GRP78 to remain in the ER, however, under prolonged periods of cellular stress, GRP78 disassociates and causes caspase-7 to cleave caspase-12, thereby activating cell death (Rao et al, 2001). Additionally, caspase-12 is released from Tumor Necrosis Factor Receptor-associated Factor 2 (TRAF2) under ER stress through c-Jun N-terminal kinase (JNK) modulation of IRE1-TRAF2 resulting in apoptosis (Yoneda et al, 2001). Caspase 12 can further induce apoptosis through cytochrome c in parallel to the JNK activation through caspase 8 (Jimbo et al, 2003). Caspase 8 mediates crosstalk between the ER and mitochondria through BAP31, a Bcl-2 binding transmembrane protein. When cleaved, the p20 form of BAP31 causes an early release of calcium from the ER, mitochondrial recruitment of dynamin-related protein 1, as well as fragmentation and fission of the mitochondrial network inducing the release of cytochrome-c resulting in apoptosis (Breckenridge et al., 2003).

In summary, several ER stress signalling cascades rely heavily on calcium signalling and communications with mitochondria to mitigate stress and enhance degradation. Moreover, GRP78 has demonstrated a profound effect to these pathways, resulting in protein aggregation, ER stress, and mitochondrial dysfunction; thereby disrupting the ability of the ER to perform its duties.

1.3 Endoplasmic reticulum dynamics

The plasticity of the ER allows it to be remodeled and arranged to meet the current demands of the cell. One such mechanism in plants, yeast, and mammalian cells relies on the

interactions between the ER and the cytoskeleton to rearrange structures to cope with stress and the changing environment (Ueda et al, 2010; Fehrenbacher et al, 2002). Moreover, evidence from mammalian cells also suggests that interactions between the ER and microtubules are required to maintain ER structures and contribute to ER turnover and biogenesis (Shibata et al, 2008; Waterman-Storer and Salmon, 1998; Lee and Chen, 1988). Failure of the ER to properly remodel results in the inability to cope with stress, mutations, and diseases leading to neurodegeneration, cancers, cardiovascular disease, respiratory diseases, and diabetes (Bergmann et al., 2017; Cabral-Miranda and Hetz, 2018; reviewed in Choi and Song, 2019).

1.3.1 Endoplasmic reticulum-phagy

To mitigate stress, the UPR signals for an increase in the size of the ER and alters the concentrations of its resident proteins (Hosoi and Ozawa, 2010; Basseri and Austin, 2012). However, to prevent excessive expansion due to stress and return the ER to a resting state, the cell digests portions of the ER through a subset of autophagy devoted to digesting the ER, known as ER-phagy (Figure 1C-E) (reviewed in Fregno and Molinari, 2018). ER-phagy has been observed in euglena, yeast, plants, and mammalian cells (de Duve and Wattiaux, 1966). Early on, several studies established that ER degradation and clearance were mediated by lysosomes; this was enhanced by several stimuli including chemical stress, nutrient starvation, and development/differentiation (Hruban et al., 1963; Noovikoff et al, 1964; Locke and Collins, 1965; Bolender and Weibel 1973). Alterations to ER-phagy, and more generally autophagy, have been implicated in neurodegenerative disease, cardiovascular disease, and cancers (Boellaard et al, 1989; Davidson et al, 2012; Viswanath et al, 2018; Linxweiler et al, 2012).

To date, studies across models have pinned down the involvement of autophagy (*ATG*) gene products in the recruitment and degradation of the ER as part of ER-phagy. This involves the partition and fragmentation of ER regions overpopulated with misfolded proteins from the main ER in preparation for ER-phagy (Hamasaki et al, 2005). Specifically, ER degradation was blocked in yeast Δatg16 cells noted by the accumulation of ER fragments within autophagosomes (Hamasaki et al, 2005) and mammalian ATG5 and ATG7 were connected to degrading misfolded proteins from the ER as well as the maintenance of protein and calcium homeostasis (Fujita et al., 2007; Ishida et al., 2009; Kamimoto et al., 2006; Antonucci et al., 2015). It is noteworthy that these degradative processes are not always due to stressful or toxic stimuli, ER-phagy (as well as autophagy, ERAD, and the UPR) exist to maintain homeostasis within a healthy cell and is a key component in development and differentiation. RTN, ATL, FAM134B, calnexin, and GRP78 are a few proteins known to be involved in the maintenance of ER structures and have been shown to be involved throughout the process of ER fragmentation and ER-phagy (Fumagalli et al, 2016; Grumati et al, 2017; Chen et al, 2019; Forrester et al, 2019). Although the full mechanism of ER-phagy, fragmentation, and degradation is unclear, reticulophagy regulator 1 (RETREG1)/FAM134B, FAM134A/RETREG2, and FAM134C/RETREG3 have been identified as receptors of ER-phagy and may be involved in the autophagosome recruitment process (Khaminets et al, 2015; Reggio et al, 2021). Together, the oligomerization of FAM134 or RTN proteins, as well as their interactions with ATL proteins are required for ER-phagy in mammalian cells (Figure 1 C-E) (Liang et al, 2018; Grumati et al, 2017; (Khaminets et al, 2015; Reggio et al, 2021). While in yeast, Atg 40, a reticulon-like protein, acts as an ER-phagy receptor similar to FAM134B and interacts with Atg8 to mediate remodeling (Mochida et al, 2020).

Sheet ER-phagy occurs through FAM134B oligomerization by the activation of calcium/calmodulin-dependent protein kinases and subsequent phosphorylation of FAM134B and interactions with ATL2 to promote ER membrane fragmentation (Figure 1C) (Khaminets et al. 2015; Jiyoung et al, 2020). Alternatively, tubular ER-phagy is governed by interactions between RTN proteins and ATL3 through oligomerization of RTN (Figure 1D) (Shibata et al, 2008; Voeltz et al, 2006; Grumati et al, 2017). For example, through homo-oligomerization, RTN3L clusters and induces ER fragmentation leading to ER-phagy by recruiting ATL3 to the region, inturn allowing for interactions with LC3(Grumati et al, 2017; Chen et al, 2019). In contrast, RTN3L hetero-oligomerization with RTN3S promotes ER tubular biogenesis (Grumati et al, 2017).

Although ER-phagy has been accepted as helpful means to prevent over ER expansion (Schuck et al, 2014), excessive ER degradation through ER-phagy also results in a loss of ER homeostasis by indiscriminately degrading the ER to the point where function is hindered (Khaminets et al, 2015; Liao et al, 2019). Interestingly, cancer cells may benefit from increased ER-phagy by maintaining an unstressed environment (Chipurupalli et al, 2022). FAM134B is suggested to form a complex with GRP78 to mediate ER-phagy, with the depletion of GRP78 leading to a decrease in FAM134B-dependent ER-phagy and a decrease in cancer cell viability as cells are no longer able to cope with increased stress (Chipurupalli et al, 2022). This further supports the hypothesis that ER-phagy plays a role in reducing ER stress and hints that the dysregulation of ER-phagy through aberrant GRP78 function leads to the onset of disease.

1.4 Endoplasmic reticulum stress and disease

Under conditions of abnormal stress, degradation is hindered and cytotoxic aggregates accumulate in the cell, leading to disease (Mattson et al, 1999; Hamilton and Terentyev, 2018; Wiersma et al, 2017; Arif et al, 2014). Although acute stressors can enhance protection, prolonged stress can disrupt the attenuative side of ER stress leading to the dysregulation of life-saving cellular processes. Depending on the cause, ER stress can manifest as various pathologies in a multicellular system. For example, the pathogenesis underlying neurodegenerative disorders, cardiovascular disease, respiratory illness, diabetes, and cancers can be attributed to an abnormal ER stress response and can be traced back to aberrant GRP78 function (Scheuner and Kaufman, 2008; Fernandez et al, 2000; Smith et al, 2005; Cunnea et al, 2011; Okada et al, 2004; Xu et al, 2012; Wiersma et al, 2017). Prolonged stress can stem from many causes such as the accumulation of misfolded proteins, mutations, errors in the UPR, or altercations to protein degradation; a single or combination of these causes can be sufficient in causing disease. Several lines of evidence suggest that by manipulating the stress response, these stressors can be mitigated to treat disease. For example, activation of the UPR through Salubrinal (a synthetic inhibitor of eIF2a de-phosphorylation - phosphorylated eIF2a is involved in upregulating the UPR) can reduce aggregation in models of α-synucleinopathies, a class of neurodegenerative disease characterized by aberrant α-synuclein accumulation (Colla et al, 2012).

1.4.1 α-synucleinopathies

α-synucleinopathies are a subset of neurodegenerative disorders classified by the build up of α-synuclein in neurons and glial cells, these include Parkinson's disease (PD), dementia with Lewy bodies (DLB), and multiple systems atrophy (MSA) (Siwecka et al, 2023). Synucleins are

a family of small aggregation prone proteins and have been implicated in neurodegenerative diseases and cancers (Reviewed in Surguchov and Surguchev, 2022). α-synuclein (encoded by: *SNCA)* localizes to the nucleus, cytosol, and presynaptic nerve terminals (Maroteaux et al, 1988). α-synuclein has been reported to function as a molecular chaperone,interact with Hsp70 and Hsp40 (DnaJ family) to coordinate neurotransmitter releases, prevent neurodegeneration, and is involved in reducing protein kinase C activity (serine-threonine kinases) to suppress apoptosis (Park et al, 2002; Jin et al, 2011; Chandra et al, 2005). Furthermore, reports have indicated that CaM can regulate the formation of α-synuclein fibrils while α-synuclein can also regulate the activation and inhibition of CaM activity (Martinez et al, 2003). Although these diseases are all characterized by α-synuclein accumulation, the origin of accumulation varies. α-synuclein can accumulate, aggregate, or increase in amounts due to mutations in the *SNCA* gene that can increase the aggregability of the gene product or the amount of the product (common in hereditary variants) (Book et al, 2018; Edwards et al, 2010; Jowaed et al, 2010). In addition, district variants or strains of α-synuclein can form proteinase K resistance, with variants from MSA being more detrimental (Shahnawaz et al, 2020). Alternatively, mutations in genes that affect cellular degradation pathways or transport can also lead to accumulation (common in sporadic forms of α-synucleinopathies). For example, mutations in *GBA* lead to a decreased β-glucosidase function leading to the accumulation of glucocerebroside and glucosylsphingosine. Although mutations in *GBA* commonly cause Gaucher disease (a lysosomal storage disorder with neurodegenerative effects), mutations in *GBA* can also interfere with autophagy and lysosomal degradation. Additionally, PD, DLB, and MSA and have been associated with increased levels of UPR markers: GRP78, p-PERK, and p-eIF2a (Gegg et al, 2012; Wernick et al, 2020, reviewed in Velayati et al, 2010; Segarane et al, 2009). Alternatively, α-synuclein may also interfere with

the trafficking of the *GBA* gene product, from the ER to lysosomes (Granek et al, 2023). Additionally, *GBA* mutations can impact α-synuclein accumulation by blocking autophagy, resulting in further protein accumulation and ER stress (Stojkovska et al, 2018; Kuo et al, 2022).

An alternative route for accumulation can be seen in MSA. While the cellular hallmark of MSA is the accumulation of glial cytoplasmic inclusions (CGIs) in oligodendrocytes containing α-synuclein, an additional component of CGI is p25α (tubulin polymerization promoting protein, TPPP), and histone deacetylase 6 (HDAC6) (Tu et al, 1998; Wakabayashi et al 1998; Kovacs et al, 2004; Miki et al, 2011). In MSA, p25α localizes towards the cell body rather than the myelin sheath leading to α-synuclein accumulation and can stimulate α-synuclein polymerization; therefore, authors Ejlersokv et al (2013) proposed that the p25α may influence autophagosome processing (Lindersson et al, 2005; Song et al, 2007; Ejersokv et al, 2013). Furthermore, p25α, it can also inhibit HDAC6 leading to tubulin acetylation and change in microtubule arrangements (Lindersson et al, 2005). HDAC6 has also been proposed to be involved in the loading and transport of misfolded proteins to aggresomes (a pre-autophagic structure where misfolded proteins and aggregates can be sequestered) with its loss impairing the clearance of misfolded proteins (Kawraguchi et al, 2003). Interestingly, HDAC inhibition can lead to the transcriptional induction of GRP78 (Baumeister et al. 2009). To date, several studies have evaluated HDAC6 modulation on the progress of α-synucleinopathies (reviewed in Lemos and Stefanova, 2020). Together it would appear that aberrant interactions with microtubules and degradative components may contribute to MSA as well as other neurodegenerative diseases; however, more direct evidence is needed. Additionally, mutations in the *LRRK2* gene can lead to α-synucleinopathies. *LRRK2* encodes for the protein leucine-rich repeat kinase 2 (LRRK2). Although the function is unclear, this gene has been shown to offer a protective role by

upregulating GRP78 while its loss has been linked to impaired protein degradation (Tong et al, 2010; Yuan et al, 2011). Furthermore, gene delivery of GRP78 was able to reduce α-synuclein neurotoxicity in rat models of PD (Gorbatyuk et al, 2012). Taken together, the relationship between ER stress and α-synucleinopathies is a complex and supports that several independent pathways can contribute to the cellular stress, pathogenesis, and disease.

1.4.2 Lysosomal storage disorders (LSD)

Lysosomal storage disorders (LSD), such as Gaucher disease, Tay-Sachs disease, and the neuronal ceroid lipofuscinoses (NCLs), are a class of metabolic diseases caused by mutations in genes encoding for lysosomal enzymes, membrane proteins, and proteins involved in the trafficking and regulation of lysosomes; as a result, there is an accumulation of substrates leading to neurodegeneration, and in most cases, cell death (reviewed in Wraith, 2002; Platt, 2018). Moreover, recent studies show lysosomes as more complex organelles, involved in several processes beyond metabolism such as calcium and stress signalling, leading to a greater effect on disease (reviewed in Morgan et al, 2011). Lysosomes represent a place in the stress response that is vital for mitigating stress where any altercation in various pathways can lead to LSD and neuronal degeneration by preventing degradation. Direct studies on ER stress in LSD are still scarce, however, lysosomal enzyme deficiency, lysosome autophagosome fusion, and with have been shown to elicit a stress response and have been implicated in disease.

In summary, ER stress can underlie neuronal cell death or degeneration despite originating from different stressors such as the inherent propensity of a protein or its mutated variants to aggregate as well as the inability of the cell to properly degrade cytotoxic aggregates. Research geared toward mitigating ER stress through these lenses of protein accumulation,

aggregation, and degradation will help combat not only neurodegenerative diseases but also allow scientists to manipulate the stress response and control the response of stressful or toxic stimuli.

1.5 Neuronal ceroid lipofuscinoses (Batten Disease)

The NCLs are a family of neurodegenerative LSDs that affect all ethnicities and ages; however, they are predominantly seen in children (Mole & Cotman, 2015). Commonly known as Batten Disease, several different subtypes of this disease have been reported with each subtype caused by mutations in distinct ceroid lipofuscinosis neuronal (*CLN*) genes (*PPT1/CLN1, TPP1/CLN2, CLN3, DNAJC5/CLN4, CLN5, CLN6, MFSD8/CLN7, CLN8, CTSD/CLN10, PGRN/CLN11, ATP13A2/CLN12, CTSF/CLN13, KCTD7/CLN14)* (Mole and Cotman, 2015; Cárcel-Trullols et al, 2015). These mutations lead to the accumulation of autofluorescent material consisting of lipid-protein aggregates and ceroid lipofuscin due to aberrant lysosomal function (Palmer et al., 1992; Radke et al., 2015). As a result, patients present with seizures, progressive vision loss, a decline in movement and cognitive ability, as well as premature death (Schulz et al., 2013). Further analysis of ceroid lipofuscin deposits revealed that a major component was mitochondrial ATP synthase subunit c (Palmer et al., 1989, Palmer et al., 1992). This finding sparked the idea that mitochondria are implicated in the pathology of the disease. In support of this, morphological studies have shown enlarged mitochondria in CLN3 disease that may be attributed to metabolic stress, altered trafficking, or defects in ATP synthase subunit c (Palmer 1989; Palmer et al, 1992). In healthy human fibroblasts, calcium regulates ATP synthase, however, *CLN* gene mutations can display altered calcium homeostasis (Das et al,

1994; Das et al, 1999) suggesting that alterations to calcium signalling may affect cellular functions leading to pathogenesis.

Several mechanisms are affected leading up to the development of neurodegenerative pathologies; they include those directly involved with the function of the mutated proteins as well as those involved in downstream cellular responses that attempt to alleviate the stress caused by said mutated protein(s). These two pathways act as a positive feedback loop, supporting disease progression. Although researchers are still in the process of characterizing the exact links between each mutated CLN protein, their cellular effects, and the pathology of the NCLs, there is ample evidence that ER stress and aberrant signaling are caused by these mutations.

Research suggests that mutations in *CLN* genes invoke cellular and ER stress. Of the *CLN* genes, mutations in *CLN1*, *CLN3*, *CLN6*, and *CLN8* strongly yield ER stress; others such as *CLN4*, *CLN5*, *CLN11*, and *CLN13* have been hypothesized to affect ER stress based on existing research suggesting that they affect processes caused by ER stress or attenuate ER stress through pathways like autophagy (reviewed in Marotta et al, 2017).

1.5.1 CLN3 disease

The most common form of NCL is a juvenile-onset form caused by mutations in the *CLN3* gene (Schulz et al., 2013; Mole and Cotman, 2015). The precise function of CLN3 is unknown but it has been linked to a variety of cellular processes including endosomal, lysosomal, and Golgi trafficking, as well as calcium homeostasis, and mitochondrial function (Luiro et al., 2004, Chandrachud et al, 2015; Fossale et al., 2004; Cotman and Stropoli, 2012; Metcalf et al., 2008; Calcagni et al, 2023; Uusi-Rauva et al., 2012). Within mammalian cells,

CLN3 localizes to mitochondria, lysosomes, endosomes, and Golgi apparatus (Jarvela et al, 1998; Calcagni et al, 2023; Katz et al, 1997; and Kyttala et al, 2004). Furthermore, the yeast homolog of CLN3, Btn2p, interacts with transport proteins, Yif1p, to form a complex with Yip1p and Rab1/Ypt1p (Chattopadhyay et al 2003; Barrowman et al, 2003). Building on this study, Luiro et al (2004) provide evidence for this interaction in mammalian cells based on interactions between CLN3 and microtubule-binding proteins, Hook1-3, and in turn Rab GTPases. Together this suggests that abnormal trafficking may be due to interactions between CLN3 and the cytoskeleton (Luiro et al 2004).

Puranam et al (1999) determined a possible link between CLN3, ceramide, and apoptosis such that over-expression of CLN3 leads to increases in cell survival in NT2 cells (Puranam et al, 1999). Ceramide activity has been linked to apoptosis through both caspase-dependent and independent pathways (Smyth et al, 1996; Park et al, 2011). Wu and colleagues (2014) further investigated the role of CLN3 in the ER stress signalling pathway by transfecting SH-SY5Y cells with vectors containing full-length *CLN3* cDNA, mutant cDNA $CLN3^{\Delta ex7/8}$, siCLN3, and siCLN3-scramble. Overexpression of CLN3 was shown to increase cell proliferation when compared to cells expressing the mutant or empty vectors. In turn, knockdown cells generated with siCLN3 and siCLN3-scramble demonstrated decreased viability (Wu et al, 2014). To assess how these lines respond to ER stress, the cells were exposed to TN resulting in an increase in unfolded proteins as well as activating the mTOR signalling pathway. As expected, TN exposure reduced cell viability in control cells. Interestingly, cells overexpressing CLN3 were resistant to TN-induced death at 2.5 µg/mL and 5 µg/mL concentrations and displayed decreased amounts of GRP78, while cells expressing mutant CLN3 displayed sensitivity and decreased amounts of GRP78 (Wu et al, 2014). Next, TN-induced apoptosis in these cells was evaluated with Hoechst

33254 staining and showed a reduction in apoptosis in cells overexpressing CLN3, while mutant CLN3 and the downregulated lines showed an increase in apoptosis when compared to controls (Wu et al, 2014). However, in the absence of TN treatment, there were no measurable changes in apoptosis suggesting that the protective effect was only invoked during TN-induced stress (Wu et al, 2014).

The progression of ER stress in murine models can also be monitored by levels or ER marker genes *Grp78* and *Chop*. mRNA levels of *Grp78* and *Chop* were evaluated in lines overexpressing CLN3, mutant CLN3, as well as in knockdown cells and revealed that levels of *Grp78* were greater when CLN3 was overexpressed, while it was similarly reduced in both mutant and knockdown lines (Wu et al, 2014). In contrast, *Chop* levels were decreased in cells overexpressing CLN3 and increased in mutant CLN3 and knockdown cells such that *Chop* levels in knockdown cells displayed the greatest reduction compared to mutant CLN3 and controls (Wu et al, 2014). These findings suggest that CLN3 does not play a direct role in the ER response, however, the presence of the mutation impairs the cell's ability to respond to stress as seen by the reduction in *Grp78* and the push towards TN-induced apoptosis (Wu et al, 2014).

1.5.2 CLN5 disease

The onset of CLN5 disease varies based on the specific mutations in the *CLN5* gene, with symptoms presenting at late infantile, juvenile, and adult-onset stages of life (Savukoski et al, 1998; Cannelli et al., 2007; Xin et al., 2010; Mancini et al, 2015; Mole and Cotman, 2015). CLN5 localizes to the ER as a type II transmembrane protein, and is then subsequently cleaved by members of the signal peptide peptidase family of proteases, yielding a soluble glycoprotein protein (Savukoski et al, 1998; Isosomppi et al, 2002; Vesa et al, 2002; Holmberg et al,

2004; Jules et al., 2017). In this state, CLN5 localizes to lysosomes (Isosomppi et al, 2002). The variations seen in symptom intensity and onset may be due to the differences in the distinct *CLN5* mutation or variants by altering the protein structure, function, and/or trafficking; however, it appears that the localization of CLN5 does not necessarily correlate to disease onset (Schmiedt et a, 2010). For example, mutations affecting the N-linked glycosylation of CLN5 have demonstrated aberrant trafficking that varies based on the mutation site, while others lead to increases in ER localized or Golgi localized CLN5 (Schmiedt et al, 2010; Moharir et al, 2012). To date, research describes CLN5 as a versatile protein, as such mutations in *CLN5* may favour a specific function over another, thereby leading to the varying manifestations of CLN5 disease.

Recently, CLN5 has been reported to be a bis(monoacylglycerol)phosphate (BMP) synthase (Medoh et al, 2023). Reduced amounts of BMP have been linked to dysfunctional lipid metabolism, lysosomal dysfunction, and neurodegeneration, and can stimulate autophagy (Alcalay et al, 2020; Ilnytska et al., 2021; Medoh et al 2023). Interestingly, LRRK2 mutants also display elevated levels of BMP (Alcalay et al, 2020). Human CLN5 has also been reported to function as a S-depalmitoyase through thioesterase activity (Luebben et al, 2022). Palmitoylation is involved in regulating protein localization and function with mutations in palmitoylation genes leading to neurological disorders and other human diseases (reviewed in Hornemann, 2014). Models of CLN5 disease display downstream cellular effects that may indicate elevated levels of ER stress or the inability to attenuate ER stress based on autophagic dysfunction as previous studies support autophagy activation downstream of ER stress (Wiersma et al, 2017). Impaired autophagy can, in-turn, trigger neuronal degeneration (reviewed in Yin et al, 2017). Since the specific *CLN5* mutation affects the function of the protein in differing ways, it is expected that will translate into various levels of stress in the variants of CLN5 disease.

1.6 *Dictyostelium discoideum*: a model for biomedical research

From the dawn of science, model organisms have been used to help deepen our understanding of biology. *Dictyostelium discoideum* is an established model for understanding conserved cellular and developmental processes, offering both cellular and multicellular insights due to its ability to form a multicellular organism under nutrient deprivation (Mathavarajah et al, 2017; Huber et al, 2022; Dickinson et al, 2012; Raper, 1984). When nutrients are readily available, *D. discoideum* cells exist in a single cell state; however, when prompted by starvation, they enter a multicellular development cycle by chemotactically migrating toward pulses of cyclic adenosine monophosphate (AMP) to form a spore-containing, multicellular, fruiting body (Raper, 1984; Gerisch and Hess, 1974). *D. discoideum* is considered a facultative multicellular organism as it only triggers multicellularity in response to a specific environmental cue. Yet, it can continue to proliferate in a unicellular state if nutrients are available (Raper, 1984; Dickinson et al, 2012). Because if its ability to form multicellular aggregates through cellular communications with single cells, *D. discoideum* is also known as the social amoeba.

An updated view of the eukaryotic tree of life connects the classes *Dictyostelia* and animals together by proposing the new taxon, Amorphea (Burki et al, 2019), formerly referred to as unikonta (He et al, 2014). Amorphea encompasses subtaxons including: Obazoa, a novel subtaxon including opisthikonts (an established clade containing animals and fungi), heterotrophic flagellates (breviate and apusomonds), and Amoebazoa (amoeba and slime molds including Dictyostelia) (Katz and Grant 2015; Brown et al, 2013; Katz et al, 2012; Adl et al, 2012). Since facultative multicellularity is not unique to the class *Dictyostelia* and has been observed in other members of amorphea, Dickinson et al propose that an ancestral facultative

multicellular amorphea/unikont diverged into obligate unicellular, obligate multicellular, and facultative lineages; thereby justifying the cellular and molecular parallels that can be seen in other eukaryotic models (Medina et al, 2003; Sánchez-Martínez and Pérez-Martín, 2001; Paps and Trillo, 2010). This is further supported by remarkable similarities seen in *D. discoideum* α-catenin behaviour and the characterization of epithelial tissue similarities between *D. discoideum* and animals (Dickinson et al, 2012). The deep phylogenetic connection metazoans share with *D. discoideum* supports its use as a model organism for biomedical research. To date, *D. discoideum* has allowed scientists to study gene mutations and interpret how these results translate to mammalian models with a strong degree of translatability; contributing to our understanding of neurodegenerative diseases, metabolism, mitochondrial dysfunction, and cancer (Mathavarajah et al, 2017; Carilla-Latorre et al, 2010; Chernivec et al., 2018; Gross and Pears, 2021; Rosenbush et al, 2021; Huber et al, 2022; Pearce et al, 2019; Dickinson et al, 2012).

1.6.1 The *D. discoideum* endoplasmic reticulum stress

D. discoideum provides researchers with unique insights into the molecular and cellular mechanisms of the cell and how these mechanisms are affected by mutations and external stimuli. Nonetheless, only a select few studies address the direct impact of stress at the cellular or multicellular level. Without fully understanding this, scientists are unable to determine whether mutated proteins are directly involved in pathogenesis or if the pathology is a secondary result due to a change in stress levels. For example, a mutation may directly hinder viability or increase cellular stress levels which in turn hinder viability.

Currently, IreA is the only identified UPR signal transducer in *D. discoideum* (Dominguez-Martin et al, 2018). Dominguez-Martin et al (2018) used an IreA-GFP fusion to

visualize the ER and observed the ER emanating from the nuclear envelope, forming concentrated sheet domains in perinuclear region. As the ER extends, sheet regions transition into a tubular labyrinth that connects to cortical ER sheets near the PM (Dominguez-Martin et al, 2018). Upon induction of ER stress by TN, IreA was observed to form clusters, the authors interpreted theses as high-order oligomers. A similar oligomerization was seen in yeast, where Ire1p formed clustered structures to aid the UPR by recruiting *HAC1*(*XBP1* in mammalian cells) and enhancing RNase efficiency. Conversely, in animal cells IRE1 activity leads to XBP splicing and decays mRNA through RIDD to reduce protein load (Hollien et al, 2009). ER stress in *D. discoideum* is linked to IreA-dependent changes in mRNA transcripts through an unknown pathway; however, this does not account for the total change in mRNA transcripts, alluding to the existence of a more complex stress response pathways in *D. discoideum*, where ER stress regulation goes beyond IreA. Further studies on Grp78 in *D. discoideum* support a conserved role for the protein in the cellular stress response, such as stress induced by mercury (a heavy metal and inducer of reactive oxygen species) or tunicamycin (prevents N-linked glycosylation by inhibiting the first step of N-linked glycosylation) which causes the over expression of Grp78 (Boatti et al, 2016; Domínguez-Martín et al, 2018).

1.6.1.2 *D. discoideum* proteasome

The *D. discoideum* 20S proteasome was characterized in 1993 and shares similarities with other eukaryotic proteasomes (Schauer et al, 1993). Proteins and aggregates can be transported to the nucleus to be degraded by the proteasome (Schauer et al, 1993). In eukaryotic cells, the proteasome depends on the cell cycle, which allows the proteasome to relocate from

various protein quality control compartments such as from the nucleus to the cytosol (e.g., dissolution of the nuclear envelope during mitotic division) (Amsterdam et al, 1993). Stress also has an effect on proteasome localization and the movement of aggregates (Malinovaka et al, 2015; reviewed in Enenkel et al, 2020). A 2015 study by Malinovaka et al, examined the proteome of *D. discoideum* with bioinformatics to quantify prion-like protein domains to evaluate how prone the organism was to form protein aggregates. *D. discoideum* has a higher number of aggregation-prone proteins when compared to other organisms; however, these aggregation-prone proteins accumulate and are degraded in the nucleus and not in the cytosol as seen in other models (Malinovaka et al, 2015). It was found that endogenous and heterologous prion-like proteins do not aggregate, remaining soluble under normal cellular conditions through interactions with molecular chaperones (Malinovaka et al, 2015). However, the induction of stress through heat or the inhibition of molecular chaperones cause cytotoxic aggregates to form in the cytosol (Malinovaka et al, 2015). Taken together this suggests that *D. discoideum* possess a means to prevent the aggregation of the prone proteins that can disrupted by stress.

1.6.1.3 Autophagy D. discoideum

Autophagy is required for *D. discoideum* multicellular development, with the loss of autophagy-related genes leading to disrupted development (Otto et al, 2003). Research by King et al (2011) suggests an evolutionary connection between autophagy in *D. discoideum* and human cells based on their observations of autophagosome formation emanating from the ER membrane under the induction of mechanical stress. Additionally, the autophagosome marker, LC3/ATG8, colocalizes with vacuole membrane protein 1 (VMP1) in mammalian cells and is required for autophagosome development (Ropolo et al, 2007). Mammalian VMP1 and *D.*

discoideum Vmp1 both localize to the ER (Dusetti et al, 2002; Calvo-Garrido et al, 2008). VMP1 and Vmp1 share remarkable functional conservation, allowing mammalian VMP1 to complement the phenotype and localization of Vmp1 when Vmp1 was mutated (Calvo-Garrido et al, 2008). In a follow up study, Calvo-Garrido et (2010), observed the colocalization of Vmp1 with autophagic marker, Atg8, and provided evidence that protrusions from the ER transition into omegasomes suggesting that this may serve as a platform for autophagosome formation (Axe et al, 2008; Simonsen and Stenmark, 2008). Although not indicated in this study, this observation may depict the recruitment of Atg8 positive autophagosome membrane to the ER membrane through interactions with ER membrane proteins. For example, studies in mammals and yeast suggest that LC3(ATG8)/Atg8 may bind to another receptor protein on the ER surface, FAM134B/Atg40 (Mochida et al, 2015; Khaminets et al, 2015). As a result, protein aggregates positive in both autophagic markers, including p62 and Atg8, as well as ubiquitin-positive substrates manifest in cells lacking Vmp1 (Calvo-Garrido et al, 2008). Human and *D. discoideum* p62 share several functional motifs including domains required for oligomerization, interactions with ubiquitin-associated domains, and ZZ-type finger domain (Calvo-Garrido et al, 2010; Moscat and Diaz-Meco, 2009). Interestingly, the ZZ-type finger domain of p62 binds with N-terminally arginylated proteins, such as R-GRP78 (Cha-Molstad et al, 2015). The binding of R-GRP78 to the ZZ- domain of p62 initiated oligomerization of p62 and targets substrates for autophagosomes (Figure 1E) (Cha-Molsttad et al, 2015).

1.6.2 D. discoideum as a model for the NCLs

D. discoideum, has proven to be a vital model for studying human neurological disorders (Eichinger et al, 2005). Not only has *D. discoideum* aided scientists in developing insights into proteins associated with Alzheimer's disease, Parkinson's disease, and Huntington's disease, but it is an esteemed model organism for the NCLs, as *D. discoideum* encodes homologs of 11 of the 13 CLN proteins including CLN3, CLN5, and MFSD8 (Huber, 2020). Mutations in human CLN3 and CLN5 lead to CLN3 disease and CLN5 disease, respectively, while mutations in MFSD8 cause CLN7 disease (Huber, 2020).

Although many aspects of the *D. discoideum* degradation pathway are yet to be clearly enlightened, *D. discoideum* offers unique insights into ER stress and protein homeostasis, allowing researchers to better understand how these pathways behave in eukaryotes. Through the lenses of NCLs, *D. discoideum* has contributed to our understanding of the nature of CLN3 and CLN5 disease at the molecular and cellular level.

In *D. discoideum,* Cln3 localizes to the contractile vacuole system, and *cln3*-deficiency is linked to defects in osmoregulation, characterized by hypersensitivity to both hypotonic and hypertonic stress (Huber et al, 2014; Mathavarajah et al, 2018). Additionally, loss of *cln3* alters the *D. discoideum* secretome, demonstrating that several secreted proteins are involved in ubiquitin-dependent protein catabolic process, protein translation, and proteolysis (Huber, 2017).

D. discoideum cells deficient in *cln5* display increased autophagic flux based on an increased number of autophagosomes and ubiquitin-positive substrates (McLaren et al, 2021). Similarly, Adams et al (2019) detected increased autophagic flux in CLN5 disease patient fibroblasts and cln5-deficient HeLa cells. Additionally, Cln5 interacts with chaperones, as well as proteins involved in RNA and protein processing, proteolysis, catabolism, and ubiquitination (Huber and Mathavarajah, 2018).

1.7 Thesis aims, hypotheses, and rationale

The cellular stress response is complex and dynamic in nature, affecting several cellular and multicellular processes through various signalling cascades; at the confluence of these pathways is the GRP78. Together, GRP78 and the ER coordinate stress signals to attenuate various stressors such as unfolded proteins to maintain cellular homeostasis by regulating protein production, calcium ion homeostasis, reactive oxygen species, and various additional roles depending on the cell type. The UPR provides the cell with options to alleviate stress, however, prolonged stress and/or mutations will off set cellular function, leading to pathogenesis and disease. Given the importance of *D. discoideum* to biomedical research, this study aims to further characterize Grp78 and ER stress to better understand the cellular and organismal effects of chemical, biological, and genetic stressors in *D. discoideum*. Findings from this study will provide the basis for understanding the relationship between the ER, Grp78, stress, and disease. This will provide insights into how Grp78 behaviours may be a key factor contributing to the pathogenesis of several diseases. Going beyond this, an aberrant stress response is associated with neurodegenerative disease; this study aims to explore the effects of mutations in conserved *CLN* genes in *D. discoideum* (*cln3* and *cln5*) on Grp78 and ER stress. Previous studies have characterized the ER stress response in *D. discoideum* based on cellular Grp78 levels; however, a deeper understanding is required to design methods to attenuate the downstream effects of stress. The present study, hypothesizes that Grp78 plays an intricate role in communicating cellular stress, predicting that the chemical induction of ER stress, the genetic loss of *cln3* and *cln5*, as well as the combination of the two will lead to alterations in the Grp78, cellular stress, and ER dynamics.

2. Materials and Methods

2.1 Cell culturing, chemicals, media, and antibodies

AX3, the parental line of *cln3*$^-$ and *cln5*$^-$, and AX4, the parental line of *hsp70*$^-$, *hspE*$^-$, and *mhsp78*$^-$ cells, were obtained from the Dicty Stock Center. *cln3*$^-$ and *cln5*$^-$ were created by homologous recombination (Huber et al, 2014; Huber and Mathavarajah, 2018; Faix et al., 2016), while the *hsp70*$^-$, *hspE*$^-$, and *mhsp78*$^-$ cell lines were purchased from the Genome Wide *D. discoideum Insertion* (GWDI) Bank through the Dicty Stock Center (Fey et al, 2019). All cell lines were grown on Sussman Maurice (SM) agar with *Klebsiella aerogenes* and maintained at 21ºC (Fey et al., 2007). Additionally, cells were grown axenically in HL5 and supplemented with ampicillin (100 μg/mL) and streptomycin sulfate (300 μg/mL) by placing spores from fruiting bodies into HL5 media (Formedium, Hunstanton, Norfolk, UK) at 21°C, allowing them to germinate and grow to confluency. Once confluent, cells were transferred to Erlenmeyer flasks containing ampicillin (100 μg/mL) and streptomycin sulfate (300 μg/mL). These flasks were maintained at 21°C on a rotary shaker at 150 rpm (Bioshop, Burlington, ON, CA) (Fey et al., 2007). Knockout cell lines, *cln3*$^-$ and *cln5*$^-$, carried a blasticidin S resistance (*bsr*) cassette and were selected using blasticidin S hydrochloride (10μg/mL) (Bioshop, Burlington, ON, CA). Cells used for experimentation were harvested at a density of 1-5 x 10^6 cells/mL (mid-log phase) (Fey et al., 2007). HL5 and low-fluorescence HL5 were purchased from Formedium (Hunstanton, Norfolk, UK). KK2 buffer (0.7 g/L of anhydrous K2HPO4, 2.2 g/L of anhydrous KH2PO4, pH 6.5) was used to starve cells. Mouse monoclonal anti-calnexin (270-390-2), mouse monoclonal anti-calreticulin (252-234-2), mouse monoclonal anti-VatC (224–256-2), and mouse monoclonal anti-p80 (H161) were purchased from the Developmental Studies Hybridoma Bank

(University of Iowa, Iowa City, IA, USA). Mouse monoclonal anti-β-actin was purchased from Santa Cruz Biotechnology Incorporated (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated and Alexa Fluor-conjugated secondary antibodies were purchased from New England Biolabs (Whitby, ON, CA) and Fisher Scientific Company (Ottawa, ON, CA), respectively.

To create the stock TN solution used in these experiments, 5 mg of TN (Cell Signaling Technologies Inc, Whitby, ON, CA) was dissolved in DMSO to make a stock of 21,025 µg/mL (25 mM). This stock was further diluted to 2628.125 µg/mL with KK2 (1:8). After 2 hours of being starved in 5 mL of KK2 buffer 0.95 µL of 1:8 TN solution was added to the existing KK2 creating a 0.5 µg/mL working solution of TN-KK2.

2.2 Grp78 antibody

An antibody against Grp78 was commissioned from GenScript and produced using in two New Zealand Rabbits using an antigen corresponding to amino acids 41-440 from *D. discoideum* Grp78. Two affinity purified antibodies were provided by GenScript, one from each of the immunized rabbits.

2.3 Collection of intracellular and extracellular samples

Cells were harvested during the mid-log phase of their growth and allowed to grow until confluency on 60 mm x 15 mm polystyrene Petri dishes with antibiotic supplemented HL5. Once confluent (8.0×10^6 cells), the HL5 was removed, and cells were washed twice with KK2 before incubating with the desired treatment (KK2 to induce starvation or KK2 supplemented with TN or DMSO) for 4 hours. After this incubation period, the conditioned buffer was decanted and concentrated using Amicon Ultra-4 10 KDa molecular cutoff centrifugation units (Fisher

Scientific Company, Ottawa, Ontario, Canada) at 4200 ×g for 25 min at 4°C. Adhered cells were lysed using a buffer containing 150 mM NaCl, 50 mM Tris, and 0.5% NP-40, set at a pH of 8.3, and supplemented with a Pierce™ protease inhibitor tablet (Fisher Scientific Company, Ottawa, ON, CA). The protein concentration of the samples was quantified using Qubit Protein Assay Kit and Qubit 2.0 Fluorometer (Fisher Scientific, Whitby, ON, CA).

2.4 SDS-PAGE and western blotting

Whole-cell lysates and samples of conditioned buffer were diluted in a 1:1 ratio with 2X Laemmlli sample buffer (120 mM Tris-HCl, 4% sodium dodecyl sulfate, 20% glycerol, 0.004% bromophenol blue, 10% 2-mercaptoethanol, pH 6.8) and heated at 95ºC for 5 minutes. Once heated, samples were separated by SDS-PAGE and transferred onto Immun-Blot® PVDF membrane for 90 minutes at 90V (Bio-Rad Laboratories Limited, Mississauga, ON, CA) through western blotting using the wet electroblotting technique. Membranes were blocked for 45 minutes using a solution consisting of 5% milk, tris-buffered saline (TBS), and 1% Tween20 (TBST). Membranes were then washed three times with TBST solution before incubating for 2 hours with the following primary antibodies: anti-Grp78 (1:1000), anti-β-actin (1:1000), or anti-countin (1:1000) diluted in a 5% milk TBST blocking buffer. Prior to moving on to the secondary antibody incubation, the membrane was washed three times using TBST. After which membranes were incubated with either a secondary antibody solution containing either HRP-conjugated rabbit antibodies (1:2000) or HRP-conjugated mouse antibodies (1:2000) diluted in a 5% milk TBST blocking buffer. The membranes were than washed three times using TBST and imaged by applying Clarity or Clarity Max enhanced chemiluminescence (ECL) to the membrane and imaging the membrane with ChemiDoc Imaging System (Bio-Rad Laboratories

Limited, Mississauga, ON, CA). Gels not subjected to protein transfer were stained using the Pierce™ Silver Stain Kit were imaged using the Invitrogen iBright Imaging Systems to evaluate protein loading (Thermo Fisher Scientific, Whitby, ON, CA). Both anti-Grp78 antibodies detected the same protein on western blots and marked the same intracellular structures in immunofluorescence experiments. Therefore, one antibody was randomly selected and used in all subsequent experiments. The intensity of the protein bands was quantified using Fiji/Image J and standardized against the levels of β-actin (Schindelin et al, 2012).

2.5 Immunolocalization

Growth-phase cells (0.35×10^6 cells total) were deposited onto flame-sterilised coverslips and placed inside separate wells of a 12-well dish. After allowing cells to adhere for 1 hour, coverslips were submerged overnight in low-fluorescence HL5. The following day, cells were fixed by carefully pipetting 1 mL of -81°C methanol onto cover slips, allowing them to be fully submerged for 45 minutes. Immunolocalization of starved cells was done by replacing the low-fluorescence HL5 with KK2 ± chemicals for 2 hours prior to fixation. Once fixed, the coverslips were removed from the wells and washed in KK2 buffer by submerging the coverslips at a 45° angle 20 times and placed cell side down on 40 μL of blocking buffer (KK2 buffer, 0.2% gelatin, 0.1% Triton 29 X-100) for 1 hour. Following the blocking stage, cells were washed using the same method before being incubated for 1 hour with the respective primary and secondary antibodies. The following primary antibodies were used for immunolocalization: anti-Grp78 (1:100), anti-calnexin (1:50), anti-calreticulin (1:50), anti-VatC (1:50), anti-p80 (1:50). Anti-Grp78 was coupled with anti-rabbit Alexa Fluor 488 secondary antibody (1:200) while the anti-calnexin, anti-calreticulin, anti-VatC, and anti-p80 were coupled with anti-mouse Alexa Fluor

555 (1:100). Coverslips were then washed for a final time and mounted onto slides using Prolong Gold Anti-Fade Reagent with DAPI (Fisher Scientific Company, Ottawa, ON, CA) and sealed with clear nail polish. Fixed cells were imaged using a Nikon Ts2R-FL inverted microscope equipped with a Nikon Digital Sight Qi2 monochrome camera (Nikon Canada Incorporated Instruments Division, Mississauga, On, Canada) Images were viewed with NIS Elements BR 5.02, and then merged with ImageJ/Fiji.

3. Results

3.1 Detection of Grp78 in *D. discoideum*

To further elucidate the role of Grp78 in *D. discoideum*, an antibody was commissioned from GenScript and raised against amino acids 41-440 of the endogenous Grp78. This antibody was then used to examine the expression and localization of Grp78 in *D. discoideum*. The amino acid sequence of human GRP78 was inputted into BLASTp for comparison with Grp78 through the online bioinformatic resources available through dictyBase (http://www.dictybase.org); this showed 65% exact identities and 80% positive identities (Figure 2A). To determine how Grp78 levels vary across the *D. discoideum* life cycle, the gene expression profile was obtained from dictyExpress, which showed that *grp78* expression peaks after 4 hours of starvation (https://dictyexpress.research.bcm.edu/landing/) (Figure 3A). To confirm this at the protein level, *D. discoideum* cells were starved in KK2 buffer and lysed after two-hour increments over a period of 12 hours (Figure 3B). In support of dictyExpress, data from these western blots showed that Grp78 levels peak between 4-6 hours of starvation (Figure 3B). When starved, *D. discoideum* cells secrete lysosomal enzymes and proteases leading to increased proteolytic activity outside the cell (Rossomando et al, 1978). Rossomando and colleagues suggested that this extracellular proteolytic activity may help in the transition of cells from a single to multicellularity by altering the PM protein composition (Rossomando et al, 1978; Ashworth and Quance, 1972). Moreover, a similar rate of secretion can be seen when comparing proteases and glycosidases of lysosomal origin suggesting that this process may be lysosome dependent (Rossomando et al, 1978). However, further examination revealed that this is not consistent for all lysosomal enzymes, and that proteolytic activity may be attributed to different functional

classes of lysosomes which sequester different groups of enzymes at different rates, leading to variations in different enzyme activities in the conditioned buffer (Dimond et al, 1981). This is supported by the observation that different inhibitors affect the extracellular activity of specific enzymes differently (Dimond et al, 1981). The relationship between *D. discoideum*, secretion, and lysosomes makes this window of time between the onset of starvation and migration vital in understanding LSDs. Additionally, ER stress and Grp78 have been shown to be differentially affected by changes in proteolytic activity, further warranting research into how this window of time affects the stress response. Therefore, for the purposes of this study, samples were collected during growth as well as after 2 and 4 hours of starvation.

A)

>DDB0233663|DDB_G0276445 |Protein|gene: DDB_G0276445 on chromosome: 2 position 6984336 to 6986396

Length = 658

Score = 776 bits (2003), Expect = 0.0
Identities = 413/629 (65%), Positives = 508/629 (80%), Gaps = 4/629 (0%)

```
Query: 28  GTVVGIDLGTTYSCVGVFKNGRVEIIANDQGNRITPSYVAFTPEGERLIGDAAKNQLTSN 87
           G+V+GIDLGTTYSCVGVFK G+VEII NDQGNRITPSYVAFT E ERLIG+AAKNQ T N
Sbjct: 32  GSVIGIDLGTTYSCVGVFKKGKVEIIPNDQGNRITPSYVAFT-ETERLIGEAAKNQATLN 90

Query: 88  PENTVFDAKRLIGRTWNDPSVQQDIKFLPFKVVEKKTKPYIQVDIGGGQTKTFAPEEISA 147
           PENT+FD KRLIGR ++D  VQ+D+K LP+K+V K  KPY+ V + G + KT++PEEISA
Sbjct: 91  PENTIFDIKRLIGRRFDDEEVQRDMKLLPYKIVSKNNKPYVVVKVKG-EEKTYSPEEISA 149

Query: 148 MVLTKMKETAEAYLGKKVTHAVVTVPAYFNDAQRQATKDAGTIAGLNVMRIINEPTAAAI 207
           M+L +MKE AEA LGK VTHAVVT PAYFNDAQR ATKDAG IAGL V+R+INEPTAAA+
Sbjct: 150 MILGRMKEIAEASLGKTVTHAVVTCPAYFNDAQRAATKDAGVIAGLEVLRVINEPTAAAL 209

Query: 208 AYGLDKR-EGEKNILVFDLGGGTFDVSLLTIDNGVFEVVATNGDTHLGGEDFDQRVMEHF 266
           AYG D   + EKNILV+DLGGGTFDVS+L+I++GVFEV +TNGDTHLGGEDFDQRVM+HF
Sbjct: 210 AYGFDATGDKEKNILVYDLGGGTFDVSVLSIEDGVFEVRSTNGDTHLGGEDFDQRVMKHF 269

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           + +++KKTGKD  KD +++QKLRR  E AKR LS+  Q ++EIE+F++G+D  ETLTRAK
Sbjct: 270 LTVFQKKTGKDASKDKKSLQKLRRAAENAKRILSTSPQTQLEIENFFDGKDLIETLTRAK 329

Query: 327 FEELNMDLFRSTMKPVQKVLEDSDLKKSDIDEIVLVGGSTRIPKIQQLVKEFFNGKEPSR 386
           FEELNMDLF+ T+ PV+KVLED+  KKS I E+VLVGGSTRIPKIQQL+K+FFNGKEP+R
Sbjct: 330 FEELNMDLFKKTLDPVKKVLEDAKFKKSQIHEVVLVGGSTRIPKIQQLLKDFFNGKEPNR 389

Query: 387 GINPDEXXXXXXXXXXXXLSGDQDTGDLVLLDVCPLTLGIETVGGVMTKLIPRNTVVPTK 446
           G++PDE            + ++ T  LVLLD+ PLTLGIETVGGVMT LIPR T VPTK
Sbjct: 390 GVHPDEAVAYGAAVQGGIFTNEEGTDTLVLLDIAPLTLGIETVGGVMTALIPRGTFVPTK 449

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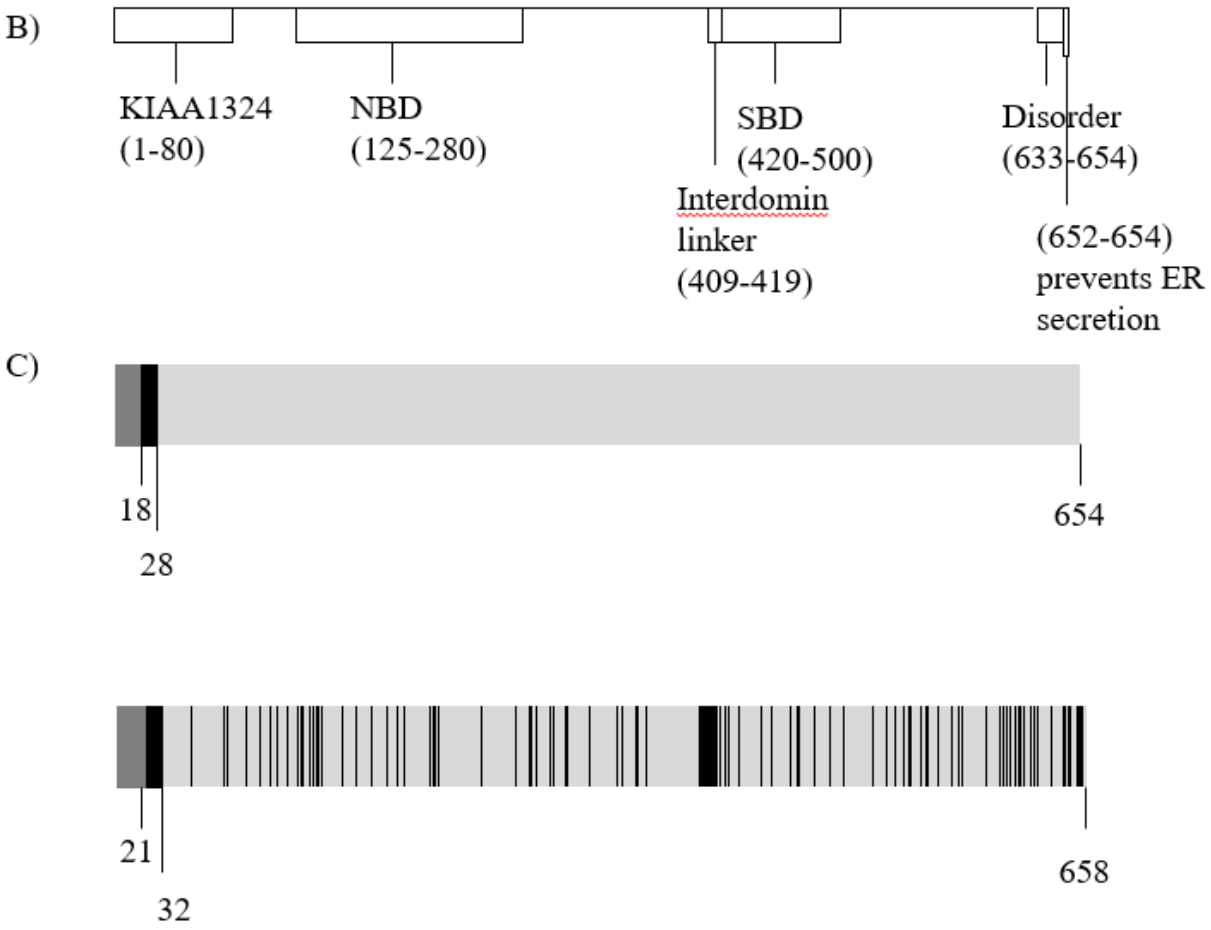

Figure 2: Bioinformatic analysis highlighting conserved regions between Human GRP78 and *D. discoideum* Grp78. (A) alignment of Human GRP78 (Uniport P11021) against *D. discoideum* Grp78 (DictyBase DDB_G0276445) showing 65% identities and 80% similarities. (B) Domian architecture of human GRP78 based on information interpreted from UniProt. (C) Similarities between Human GRP78 and *D. discoideum* Grp78 based on information obtained from UniProt, dictyBase, and DictyBLASTp. Dark grey: signal peptide, black: unconserved, regions, light grey: conserved.

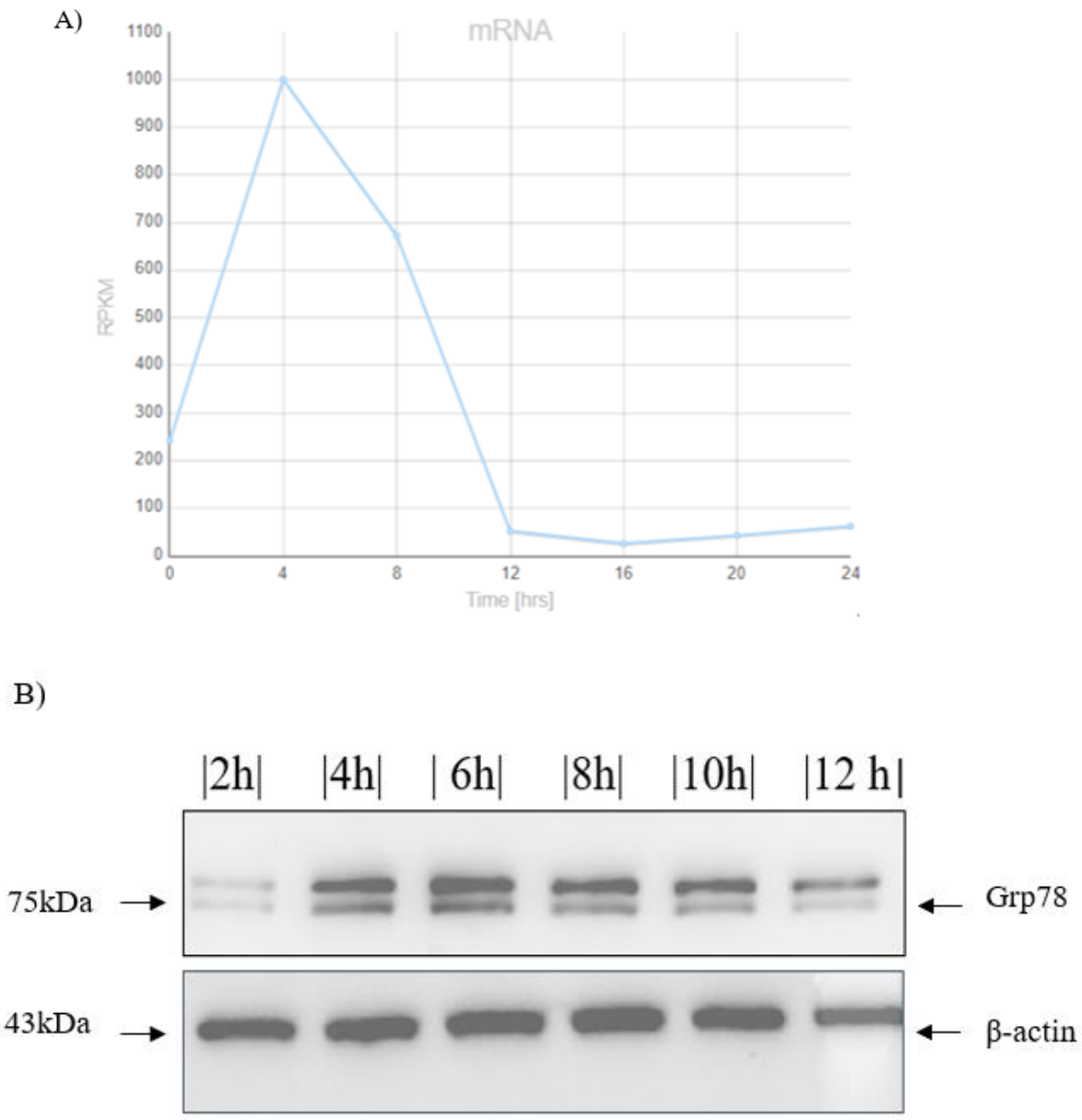

Figure 3: The expression and amount of Grp78 protein peaks after 4 hours of starvation. **(**A) Gene expression profile measured in RPKM (reads per kilobase of transcripts per million reads) of *grp78* during the *D. discoideum* life cycle obtained from dictyExpress (https://dictyexpress.research.bcm.edu/landing/). (B) Whole cell lysates obtained 2 hours apart over 12 hours were run on SDS-PAGE and analysed by western blotting with anti-Grp78 and anti-β-actin (loading control). Intracellular Grp78 levels were normalized against β-actin levels.

Western blotting of AX3 whole cell lysates with the anti-Grp78 antibody revealed a 75 kDa protein (Figure 3B) that aligns with the expected molecular weight of Grp78 (dictyBase). The antibody detected two protein bands on western blots (doublet), which is consistent with previous studies that used GRP78/Grp78 antibodies to detect Grp78 in mice and *D. discoideum* whole cell lysates via western blotting (Figure 3B) (Kasetti et al; 2016; Dominguez-Martin et al, 2018). However, previous studies did not examine if the double banding was due to PTMs. PTMs can affect the folding, activity, and trafficking of proteins including lysosomal enzymes; one such PTM is N-linked glycosylation where asparagine residues are modified by the addition of glycans increasing the molecular weight of the protein by approximately 10 kDa (reviewed in Pohl et al, 2009). The glycosylation of lysosomal enzymes affects lysosomal transport through the addition of a mannose-6-phosphate moiety on the glycan (reviewed in Pohl et al, 2009). Alterations to N-liked glycosylation patterns have been shown to affect the lysosomal trafficking of TTP1 and CLN5 (Wujek et al, 2004; Steinfeld et al, 2004; Moharir et al, 2013). Based on sequence analysis, *D. discoideum* Grp78 has conserved N-linked asparagine residue at aa position 611(Uniprot) sparking the question: is Grp78 affected by N-linked glycosylation leading to the Grp78 doublet seen in western blots. Therefore, samples were treated with PNGase F to remove N-linked oligosaccharides and then analyzed by western blotting to see if this was the cause for the doublet. Treatment with PNGase F did not show any changes to the Grp78 banding pattern (Figure 4A). When these samples were probed for countin, a known N-glycosylated protein, a noticeable shift in banding was observed such that PNGase F-treated countin was significantly smaller in size when compared to the control. In a 2013 study, Mohair et al studied the N-linked glycosylation of CLN5 and observed a difference in the size of CLN5 from cells treated with TN verses cells treated with PNGase F, such that PNGase F treated CLN5 was

larger than TN treated CLN5. The authors proposed that PNGase F treatment may lead to polypeptide property changes or because PNGase F further modifies asparagine to aspartic acid contributing to the size difference (Moharir et al, 2013). This may explain why a shift in Grp78 protein size is not detected after PNGase treatment in our study; however, treatment with TN in the present study did not lead to changes in Grp78 protein size, indicating that the doublet is not due to N-linked glycosylation.

An alternative explanation for the doublet observed in this study may be due to similarities between the antigen sequence used to generate the Grp78 antibody and sequences from other proteins in the HSP family, leading to non-specific binding. BLASTp analysis of the Grp78 antigen sequence against the *D. discoideum* proteome revealed 3 possible non-specific binding partners: Hsp70, HspE, and mHsp78. To determine if our Grp78 antibody was specific for Grp78 and not these proteins, *D. discoideum* knockouts for *hsp70*, *hspE*, and *mhsp78* were obtained from the Genome Wide Dictyostelium Insertion (GWDI) mutant bank (remiseq.org). Whole cell lysates of these cells were analyzed by western blotting using anti-Grp78. This analysis still detected two bands, thus providing further support that anti-Grp78 is specific for Grp78 (Figure 4B).

Next, the localization of Grp78 was determined in AX3 cells by immunofluorescence microscopy. In AX3 cells, Grp78 primarily localized to the perinuclear region, as well as tubular structures that extend out towards the cell periphery (Figure 5); this patterning typically indicates ER localization (Zheng et al, 2018; West et al 2011; Hegde, 2022). To confirm that Grp78 localizes to the ER, immunofluorescence was used to see if Grp78 co-localized with established ER markers, calnexin and calreticulin (Opas et al, 1991; Wada et al, 1991; Nomura et al, 2010; Lakkaraju et al 2012). Grp78 localized to calnexin- and calreticulin-stained regions, indicating

that Grp78 localizes primarily to the ER (Figure 5). Taken together, these observations show that the anti-Grp78 antibody detects a doublet at ~75 kDa on western blots and detects an ER resident protein with a similar localization to calnexin and calreticulin. Based on these findings, we concluded that the anti-Grp78 antibody is specific for *D. discoideum* Grp78.

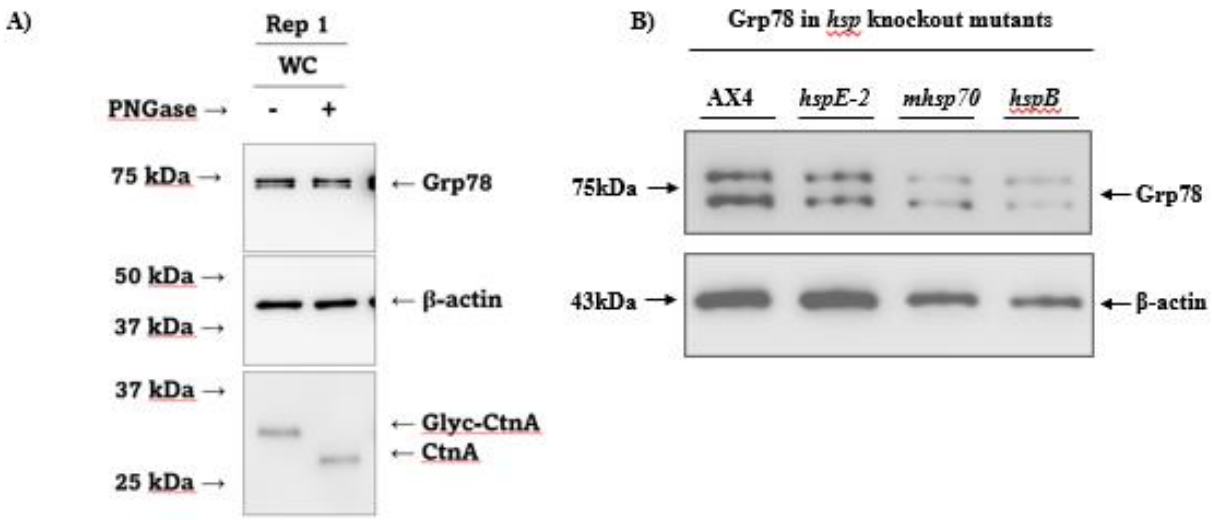

Figure 4: Grp78 is not glycosylated and the Grp78 antibody does not bind other HSP proteins. (A) Whole cell lysates (2 µg) from cells starved for 4 hours were incubated in the presence and absence of PNGase F. All protein samples were separated by SDS-PAGE and analyzed by western blotting with anti-Grp78 and anti-countin (PNGase F positive control) (B) Whole cell lysates (2 µg) from AX4, the parental line for *hsp* mutants: *hsp E-2*, *mhsp70*, *hspB.* Growth-phase cells were harvested, lysed subsequently proteins were separated by SDS-PAGE and analyzed by western blotting with anti-Grp78 as well as an anti-β-actin (loading control). Intracellular Grp78 levels were normalized against β-actin levels.

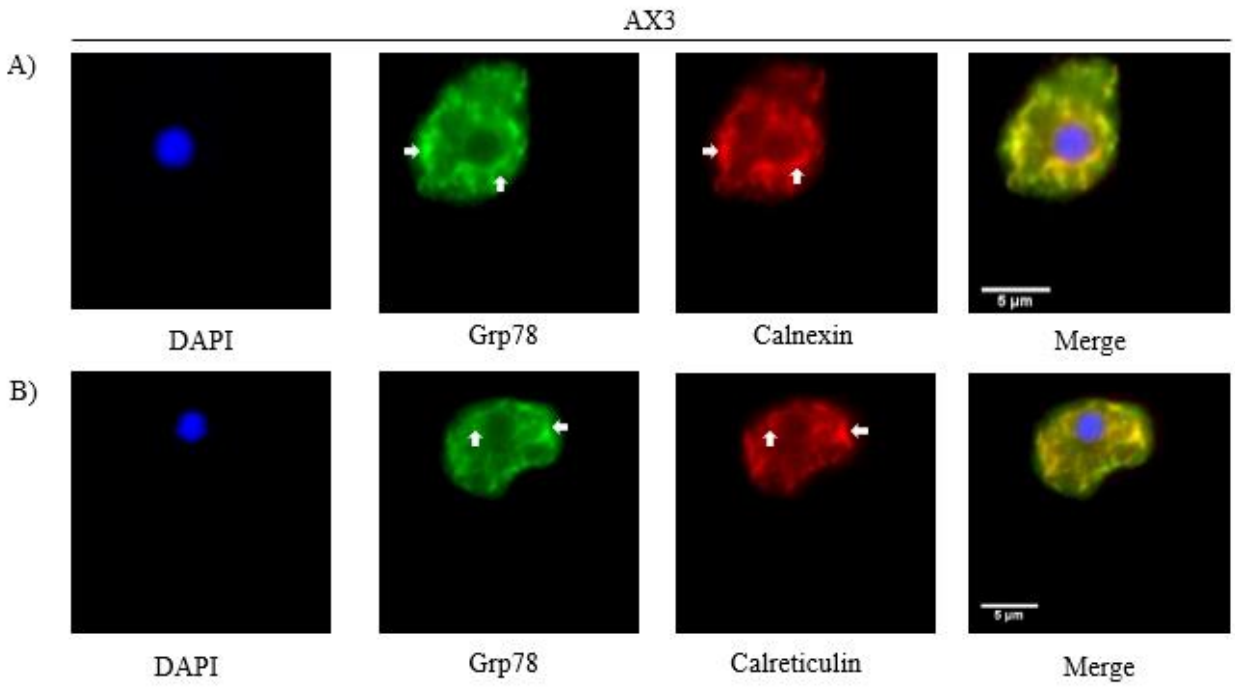

Figure 5: Grp78 localizes to the endoplasmic reticulum. Immunofluorescence of cells starved for 2 hours and probed with anti-Grp78, and (A) anti-calnexin or (B) anti-calreticulin primary antibodies followed by Alexa 488 (green) and Alexa 555 secondary antibodies (red). Cells were then stained with DAPI to reveal nuclei (blue). Images were merged with ImageJ/Fiji. White arrows: Normal ER structure. Scale Bar = 5 µm.

3.2 Localization and amounts of Grp78 during tunicamycin-induced stress

Elevated levels of cellular stress, in particular ER stress, has been detected in several models of neurodegenerative and cardiovascular disease coupled with deviating levels of GRP78, supporting that stress has a profound effect on cellular integrity (reviewed in Cabral-Miranda and Hetz, 2017; reviewed in Casas, 2017). Existing studies on GRP78/Grp78/KAR2 across models have shown that it is upregulated due to various stressors such as TN (Dorner et al, 1992; Domínguez-Martín et al, 2018). To establish a baseline for studying ER stress in *D. discoideum*, ER stress was induced by starving AX3 cells for 4 hours in KK2 and then supplementing the starvation buffer with 0.5 μg/mL of TN after the first 2 hours of starvation. Western blotting revealed an increased amount of Grp78 inside cells (Figure 6A). Immunofluorescence was used to determine if the localization of Grp78 in AX3 cells was altered after starving cells for 2 hours in KK2 buffer supplemented with 0.5 μg/mL of TN. In contrast to untreated cells, where Grp78 formed a complex network of sheets and tubules extending from the perinuclear space to the cell periphery (Figure 6 B and C), TN-stressed cells displayed a fragmented network of ER tubules seen as an abnormal arrangement of calnexin, calreticulin, and Grp78 marked puncta or structure in the cytosol, as well as puncta stained solely with Grp78 (Figure 6D and 6E).

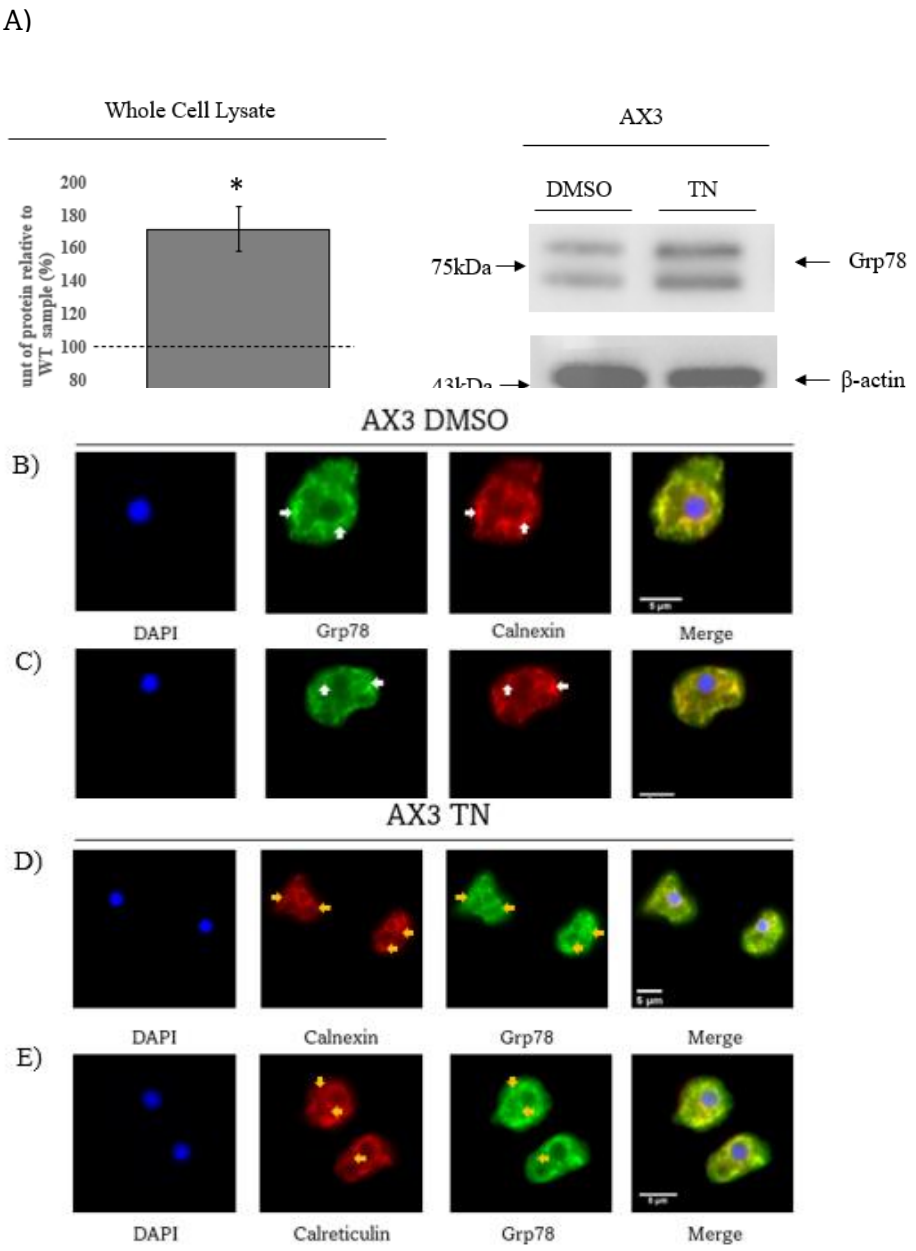

Figure 6: Tunicamycin treatment increases intracellular Grp78 amounts and alters Gr p78 localization in AX3 cells. (A) Western blotting of AX3 cells incubated in the presence and absence of TN. Cells were grown to confluency and starved for 4 hours after the 2 hours KK2 was supplemented with 0.5µg/mL DMSO (control) or TN for the remaining time then harvested and lysed. Whole cell lysates (2 µg of protein) were separated by SDS-PAGE and analyzed by western blotting with anti-Grp78 and anti-β-actin (loading control). Intracellular Grp78 levels were normalized based on levels of β-actin (n=3). Cells were then starved for 2 hours in KK2 buffer supplemented with 0.5µg/mL DMSO (B and C) or 0.5µg/mL of TN (D and E) and then fixed in ultra-cold methanol (-80ºC) and probed with anti-Grp78 primary antibody followed by an anti-rabbit secondary antibody conjugated to Alexa 488 (green). Subsequently, cells were co-stained with mouse anti-calnexin (B and D) or mouse anti-calreticulin (C and E) primary antibody followed by an anti-mouse secondary antibody conjugated to Alexa 555 (red). Lastly, cells were stained with DAPI to reveal nuclei (blue). Images were merged with ImageJ/Fiji. Co-localization in the merged images appears yellow. White arrow: healthy ER structure yellow arrow: aberrant ER structure. *p-value < 0.05.

3.3 Altered Grp78 localization and endoplasmic reticulum morphology in *cln3*$^{-}$ and *cln5*$^{-}$ cells

During ER stress, GRP78 is known to relocate to different subcellular domains (cytosol, mitochondria, nucleus, etc) (Li et al, 2023; Tsai et al 2002; Sun et al. 2006; Li et al, 2013). Immunofluorescence microscopy was used to visualize Grp78 in *cln3*$^{-}$ and *cln5*$^{-}$ cells after two hours of starvation. In AX3 cells, Grp78 localized to subcellular compartments marked by calnexin and calreticulin. In contrast, *cln3*$^{-}$ and *cln5*$^{-}$ cells display an altered ER structure with regions of the ER appearing fragmented, presenting as a discontinuous network with isolated vesicular structures and puncta stained with both Grp78 and calnexin or calreticulin in *cln3*$^{-}$ and *cln5*$^{-}$ cells. However, fewer regions of fragmented ER are observed in *cln5*$^{-}$ cells coupled with greater regions of discontinuity in ER network (Figure 7A-F). Previous studies focused on ER fragmentation as a step prior to ER degradation or ER-phagy showed that overexpression of FAM134 and yeast homolog Atg40 lead to ER fragments and assembly with autophagic markers LC3B and Atg8, respectfully(Khaminets et al, 2015; Mochida et al, 2020).These markers are associated with the isolation of ER fragments for degradation leading to decreases in ER continuity (Khaminets et al, 2015; Mochida et al, 2020). Imaging of ER fragments from these studies show similar disturbances to ER structure seen in the present immunofluorescence study of ER proteins, Grp78, calnexin, and calreticulin in TN treated *D. discoideum* (Figure 6) and *D. discoideum* models of CLN3 and CLN5 disease (Figure 7A-F).

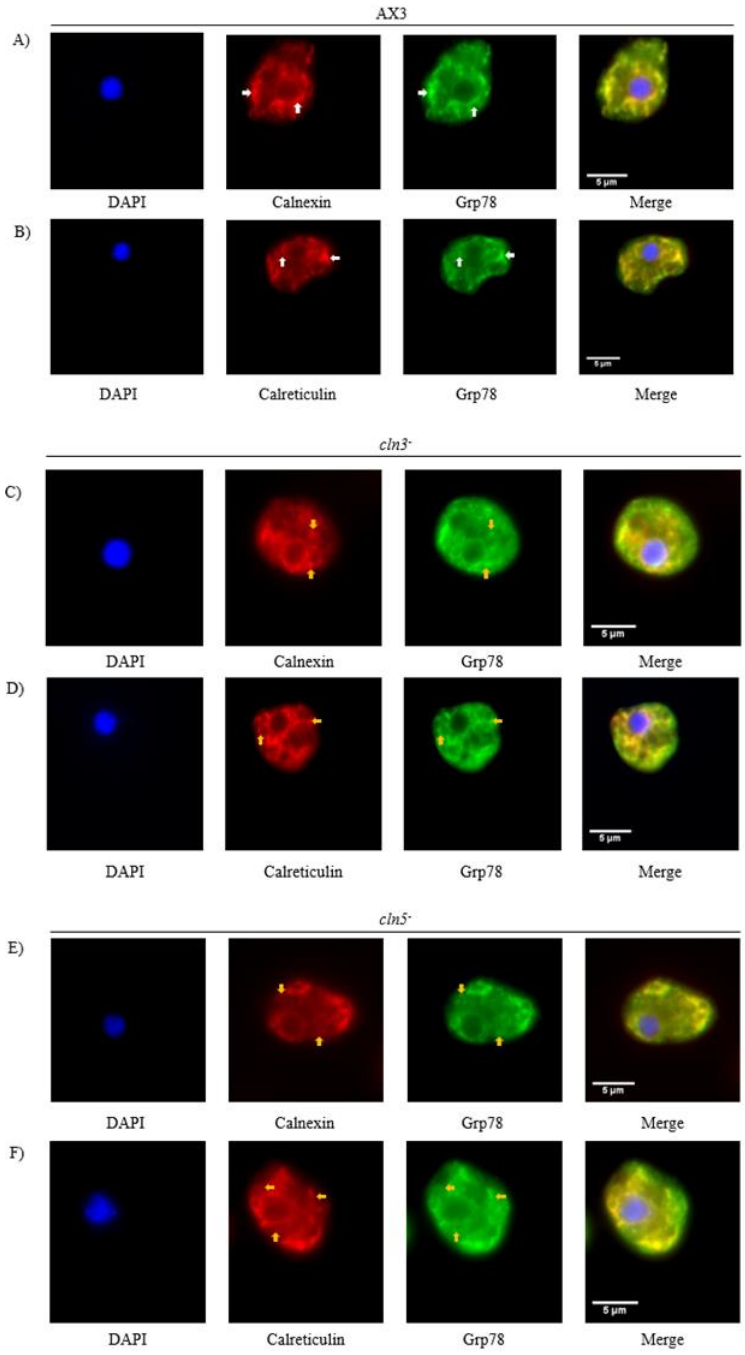

Figure 7: Loss of *cln3* and *cln5* alters the localization of Grp78 by driving the formation of aberrant ER structure. Cells were starved for 2 hours, fixed in ultracold menthol (-80ºC), and probed with anti-Grp78 primary antibody followed by anti-rabbit secondary antibody conjugated to Alexa 488 (green). Subsequently, the cells were probed with either anti-calnexin (A, C, and E) or anti-calreticulin (B, D, and F) primary antibodies followed by an anti-mouse secondary antibody conjugated to Alexa 555 (red). Lastly, cells were stained with DAPI to reveal the nuclei (blue). Images were merged with ImageJ/Fiji. Co-localization in the merged images appears yellow. White arrows: healthy ER structure, ellow: aberrant ER structures. Scale bar =5µm.

3.4 Altered Grp78 localization is not present in acidic vesicles or secretory lysosomes

The endocytic pathway of *D. discoideum* involves the uptake of particles at the PM, transfer through early acidic endocytic compartment/lysosomes, followed by maturation to acidic post-lysosomes, and finally exocytosis though exosomes (reviewed in Maniak, 1999). GRP78 can be secreted from colon cancer cells through exosomes (Li et al, 2016). This secretion can be decreased by inhibiting HDAC6 resulting in acetylation and the activation of the UPR (Li et al, 2016). Taken together, these findings contribute to the hypothesis that GRP78 can be secreted via exosomea during periods of stress. Inhibition of HDAC6 results in GRP78 aggregation in the ER and prevents its sorting into multivesicular bodies (Li et al, 2016). Moreover, these GRP78 ER aggregates did not colocalize with early endosome marker, EEA1 (Li et al, 2016). Additionally, studies on human colon and lung cancer cells have shown that GRP78 can be unconventionally trafficked to the cell surface (PM) upon ER stress through endosomal transport (Van Krieken et al, 2021). To determine if the endocytic pathway was a responsible for the altered localization of Grp78 or Grp78 secretion in *D. discoideum*, the subcellular localization of Grp78 in *cln3*$^{-}$ and *cln5*$^{-}$ cells was observed with immunofluorescence, specifically if the loss of either *cln3*$^{-}$ or *cln5*$^{-}$ led to co-localization of Grp78 with components of the endocytic pathway with endosome marker, protein 80 (p80, a putative copper transporter), or subunit C of the *D. discoideum* homolog of human V-ATPase, VatC, which localizes to acidic intracellular compartments such as contractile vacuoles, endosomes, and lysosomes (Journet et al., 1999; Ravanel et al., 2001). After 2 hours of starvation, Grp78 did not localize to intracellular structures marked by either p80 or VatC. Taken together, these observations confirm that Grp78 does not localize to the endocytic pathway in p80 or VatC-stained vesicles within *cln3*$^{-}$ and *cln5*$^{-}$ cells (Figure 8A-F).

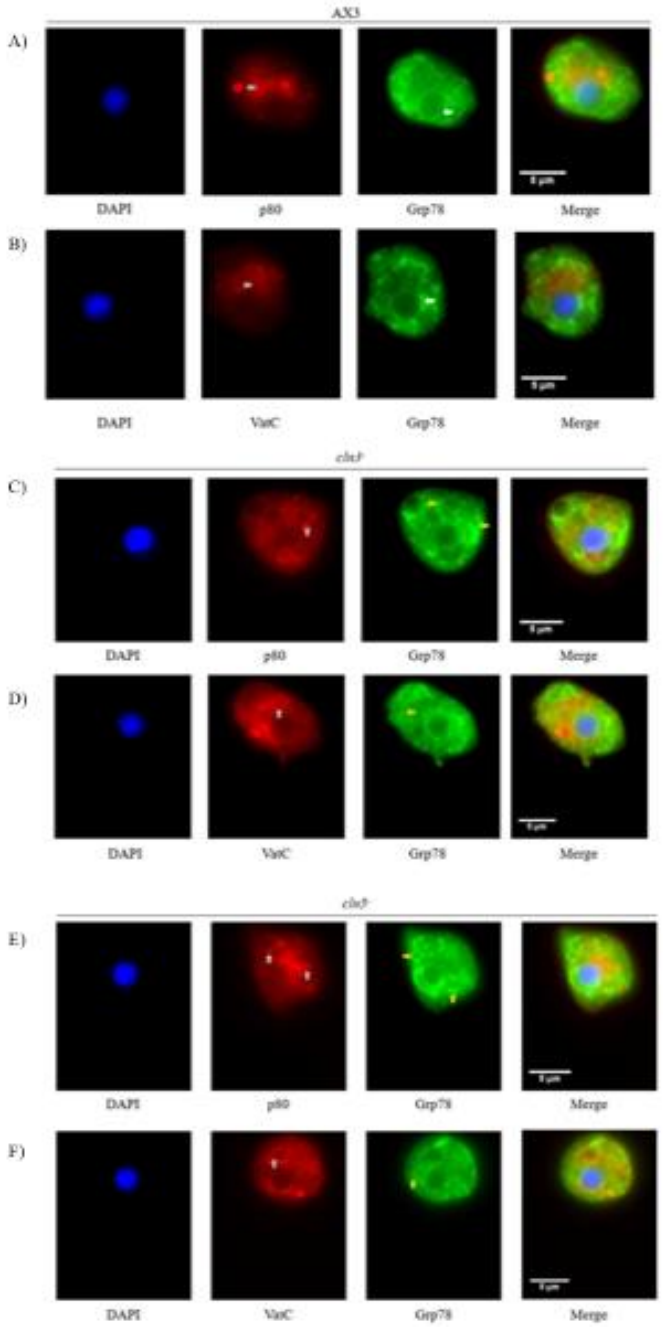

Figure 8: Mis-localized Grp78 localization in *cln3*$^{-}$ and *cln5*$^{-}$ cells is not present in acidic vesicles or secretory lysosomes suggesting that Grp78 secretion involves other compartments or pathways Cells were starved for 2 hours in KK2 buffer and then fixed with ultra-cold methanol (-80ºC) and probed with anti-Grp78 primary antibody followed by anti-rabbit secondary antibody conjugated to Alexa 488 (green). Subsequently, the cells were probed with either anti-p80 (A, C, and E) or anti-VatC (B, D, and F) primary antibodies followed by an anti-mouse secondary antibody conjugated to Alexa 555 (red). Lastly, cells were stained with DAPI to reveal the nuclei (blue). Images were merged with ImageJ/Fiji. Co-localization in the merged images appears yellow. Grey: endocytic vesicle yellow: abbarent Grp78-stained structure.

3.5 *cln3*$^{-}$ and *cln5*$^{-}$ cells display altered levels of intracellular and extracellular Grp78

The pathologies of several neurodegenerative diseases have been attributed to alterations in the cellular stress response; the NCLs are no exception (reviewed in Manotra et al., 2017). Furthermore, models of NCLs have reported altered amounts of Grp78 both intracellularly and extracellularly (Wu et al, 2014; Huber, 2017). To determine if *D. discoideum* models of CLN3 and CLN5 disease display similar hallmarks of ER stress, whole cell lysates and concentrated conditioned buffer samples from 4-hour starved *cln3*$^{-}$ and *cln5*$^{-}$ cells were analyzed by western blotting using anti-Grp78 (Figure 9A). The amount of Grp78 was significantly increased in whole cell lysates and concentrated condition buffer samples from *cln3*$^{-}$ cells, while cells *cln5*$^{-}$ show a trend toward increased Grp78 amounts with greater variation between replicates (Figure 9A). The amount of Grp78 from *cln3*$^{-}$ conditioned buffer displayed a significant increase in Grp78 amounts; in contrast, Grp78 from *cln5*$^{-}$ conditioned buffer displayed increased amounts of Grp78 compared to the wildtype but was less when compared to *cln3*$^{-}$ (Figure 9B).

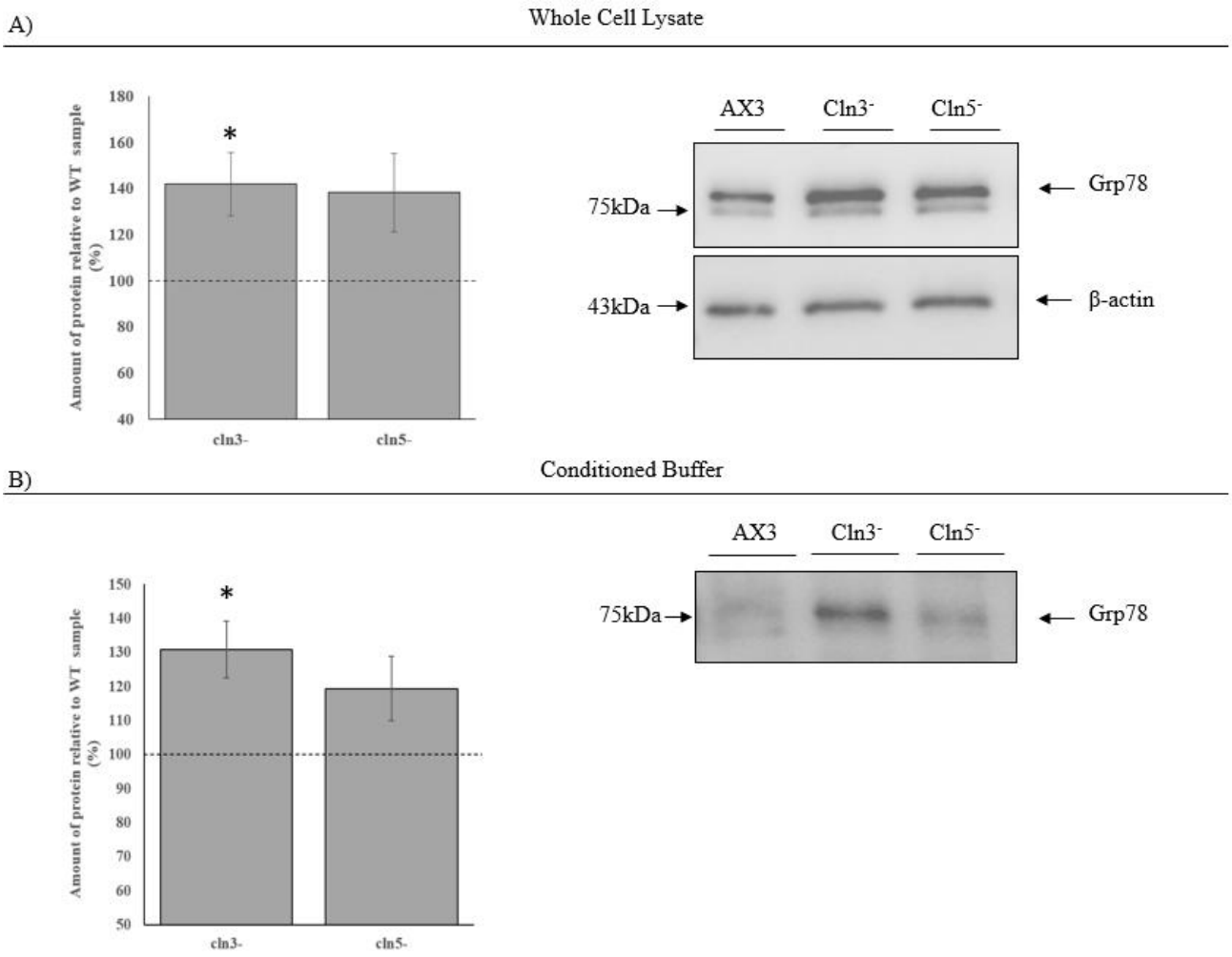

Figure 9. Increased amounts of intracellular and extracellular Grp78 in AX3, *cln3*$^-$ and *cln5*$^-$ indicating an increase in ER stress. AX3, *cln3*$^-$, and *cln5*$^-$ cells were grown to confluency and starved for 4 hours, then harvested and lysed, while the conditioned buffer was decanted and concentrated. (A) Whole cell lysates (2 μg of protein) were separated by SDS-PAGE and analyzed by western blotting with anti-Grp78 and anti-β-actin (loading control). Intracellular Grp78 levels were normalized against β-actin levels. (N_{AX3}= 5) (B) For extracellular amounts of Grp78, conditioned buffer samples (0.5 μg of protein) were then separated by SDS-PAGE and analyzed by western blotting with anti-Grp78 (N_{AX3}= 5). The bands identified in both whole cell lysates and conditioned buffer were quantified with ImageJ/Fiji. Molecular weight markers (in kDa) are shown to the right of each blot. Data in all plots are presented as the mean amount of protein (% AX3) ± SEM. Statistical significance was determined using a one-sample t-test. P-value<0.01 vs. AX3. *p-value < 0.05

3.6 Tunicamycin-induced ER stress in AX3 mimics altered Grp78 localization seen in *cln3*$^{-}$ and *cln5*$^{-}$ cells

TN induced ER stress results in endoplasmic reticulum morphological changes in endothelial cells (Bik et al, 2019). Based on the altered Grp78 localization and ER morphology observed in *cln3*$^{-}$ and *cln5*$^{-}$ cells, these cell lines are expected to cope with increased ER stress in different ways. To gain insight into what this looks like on the subcellular level, immunofluorescence was conducted on cells starved for 2 hours in TN-supplemented KK2. Under these conditions, TN-induced stress in AX3 cells leads to changes in ER structures with increases in aberrant structures stained with calnexin, calreticulin, and Grp78, Grp78-stained puncta, as well as a decrease in ER network (Figure 10A and 10B) when compared to AX3 treated with DMSO (Figure 6B and 6C). Interestingly, these stress-induced structural changes can be seen in the above observations of *cln3*$^{-}$ and *cln5*$^{-}$ cells starved without TN (Figures 7C, 7D, 7E, and 7F). Consistent with the ER stress structure-related changes seen in *cln3*$^{-}$ cells, TN-induced stress in *cln5*$^{-}$ cells also mimics a similar Grp78 distribution (Figure 10E and 10F). However, when comparing these stress-induced structural changes across cell lines, larger puncta were observed in *cln3*$^{-}$ cells, in contrast, the changes in AX3 cells displayed fewer and smaller puncta suggesting that TN treatment affects ER homeostasis differently based on the *cln* mutation. Collectively, this data suggest treatment of *cln3*$^{-}$ and *cln5*$^{-}$ with TN exacerbated ER stress related structural changes and that the loss of *cln3* or *cln5* hinders the cellular stress response to TN-induced stress.

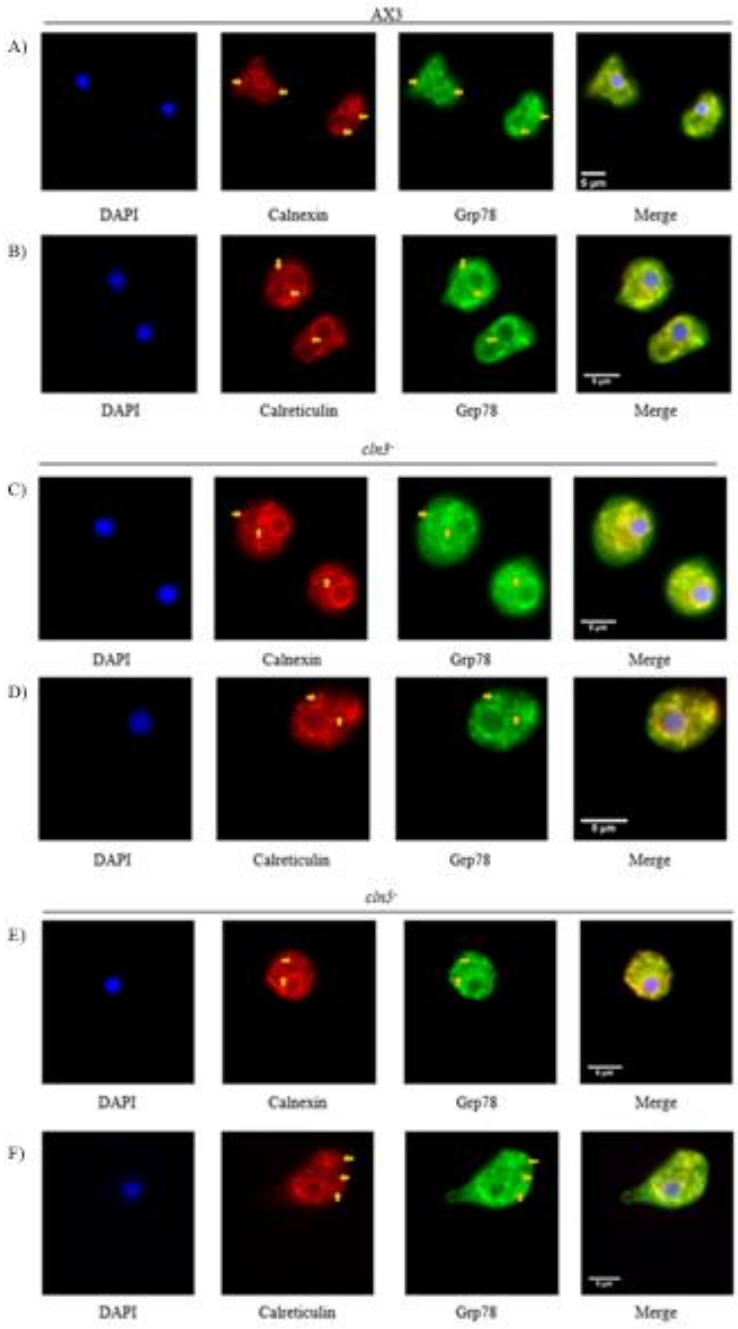

Figure 10: Tunicamycin-induced endoplasmic reticulum stress causes Grp78 localization in AX3 cells to mimic the altered localization seen in untreated *cln3*$^{-}$ and *cln5*$^{-}$ cells while increasing the generation of endoplasmic reticulum stress-related structures in treated cells. Cells were starved for 2 hours in KK2 buffer supplemented with 0.5µg/mL of TN and then fixed with ultra-cold menthol (-80ºC) and probed with anti-Grp78 followed by anti-rabbit secondary antibody conjugated to Alexa 488 (green). Subsequently, cells were co-stained with either anti-calnexin (A, C, and E) or anti-calreticulin (B, D, and F) primary antibody followed by an anti-mouse secondary antibody conjugated to Alexa 555 (red). Lastly, cells were stained with

DAPI to reveal the nuclei (blue). Images were merged with ImageJ/Fiji. Co-localization in the merged images appears yellow. Yellow arrow: aberrant ER structures.

3.7 Tunicamycin-induced ER stress does not cause Grp78 to be present in acidic vesicles or secretory lysosomes in AX3, *cln3*$^{-}$, and *cln5*$^{-}$ cells

Since GRP78 is trafficked through the endosomal pathway during periods of stress (Li et al, 2016; Van Krieken et al, 2021), we sought to confirm that additional stress caused by TN treatment did not cause Grp78 to localize to the endocytic pathway by conducting an additional immunofluorescence experiment on TN-treated cells using antibodies against Grp78, p80 and VatC. This revealed that when AX3, *cln3*$^{-}$, *cln5*$^{-}$ cells are subjected to TN-induced stress there is an increase in Grp78 localization outside of the ER that does not correspond to p80 or VatC stained compartments (Figure 11A-F).

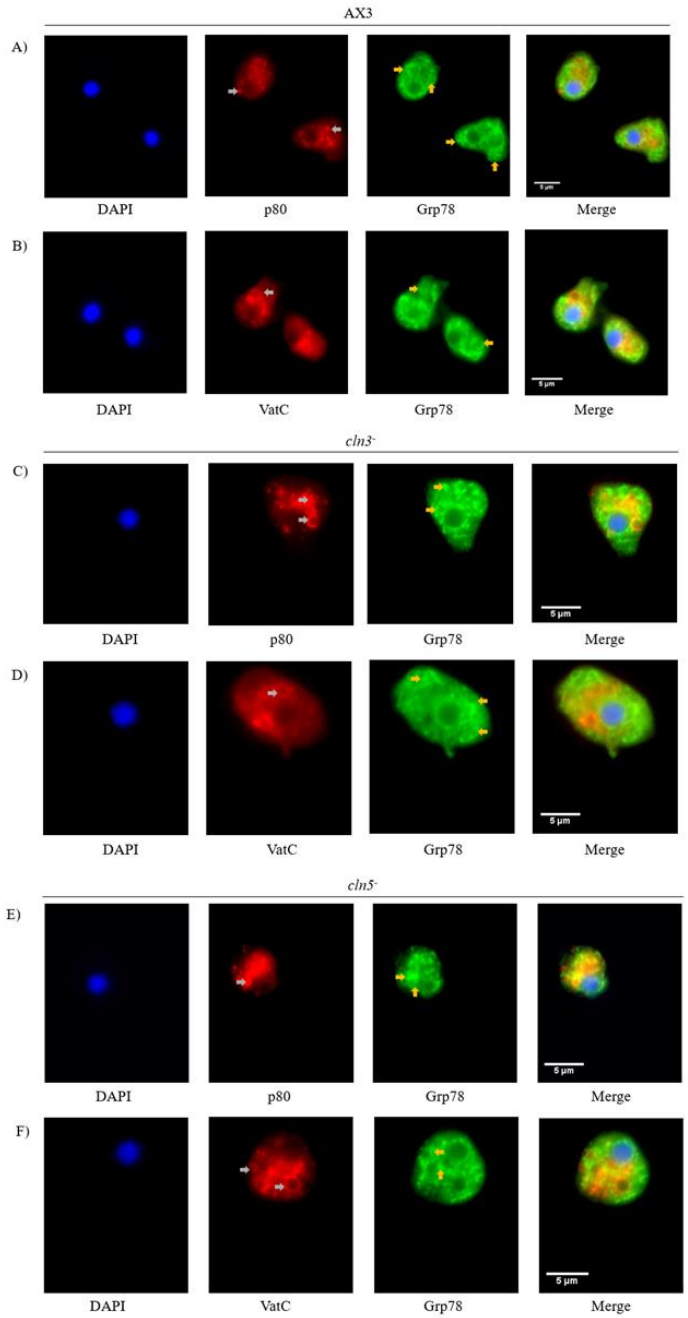

Figure 11: Tunicamycin-induced ER stress does not cause Grp78 to be present in acidic vesicles or secretory lysosomes in AX3, *cln3*$^{-}$, and *cln5*$^{-}$ cells. Cells were starved for 2 hours in KK2 buffer and then fixed with ultra-cold methanol (-80ºC) and probed with anti-Grp78 primary antibody followed by anti-rabbit secondary antibody conjugated to Alexa 488 (green). Subsequently, the cells were probed with either anti-p80 (A, C, and E) or anti-VatC (B, D, and F) primary antibodies followed by an anti-mouse secondary antibody conjugated to Alexa 555 (red). Lastly, cells were stained with DAPI to reveal the nuclei (blue). Images were merged with ImageJ/Fiji. Co-localization in the merged images appears yellow. grey: endocytic vesicle yellow: abbarent Grp78-stained structure.

3.8 Tunicamycin-induced stress in *cln3*$^{-}$ and *cln5*$^{-}$ cells results in a trend towards increased amounts of intracellular and extracellular Grp78

TN- treatment has been shown to elevate levels of GRP78 in multiple models leading and leads to changes in ER structure (Woo et al 2013; Dorner et al, 1992; Domínguez-Martín et al, 2018; Bik et al, 2019). TN-induced stress results in a trend towards increased amounts of intracellular and extracellular Grp78 in *cln3* and *cln5* deficient cell lines when compared to control (Figure 12), however, there was a large amount of variation between replicates of the same cell line. Figures 12A and 12B display the percent change in Grp78 amount relative to the DMSO control cell, supporting that the ER stress response to TN still increases Grp78 levels in *D. discoideum* models for CLN3 and CLN5 disease. Although TN treatment increases Grp78 levels across cell lines, the degree of increase fluctuates.

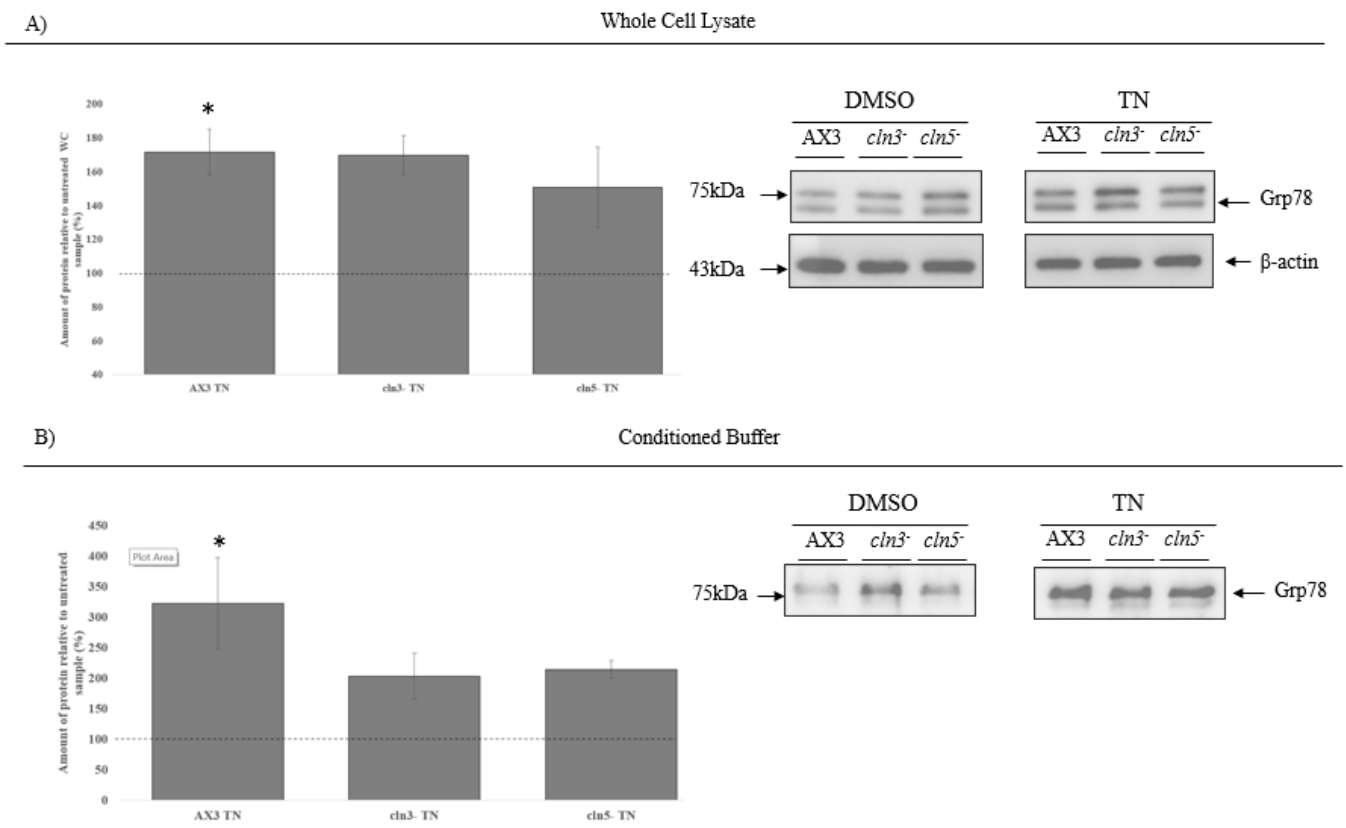

Figure 12: Tunicamycin-induced stress results in a trend towards increased amounts of intracellular and extracellular Grp78 in *cln3*$^-$ and *cln5*$^-$ cells. All cells were grown to confluency and starved for 4 hours. Cells were then harvested and lysed, while the conditioned buffer was decanted and concentrated. (A) Whole cell lysates (2 µg of protein) were separated by SDS-PAGE and analyzed by western blotting with anti-Grp78 and anti-β-actin (loading control). Intracellular Grp78 levels were normalized against β-actin levels, analyzed, and expressed as a percentage of the DMSO control (N=3) (B) Samples of concentrated conditioned buffer (0.5 µg) were separated by SDS-PAGE, analyzed by western blotting with anti-Grp78, and expressed as a percent of the DMSO control. (N_{AX3}=6) The bands identified in both whole cell lysates and conditioned buffer were quantified with ImageJ/Fiji. Molecular weight markers (in kDa) are shown to the right of each blot. Data in all plots are presented as the mean amount of protein relative to wild type ± SEM Statistical significance was determined using a one-sample t-test. *p-value < 0.05.

4. Discussion

In this thesis, the roles of Grp78 in *D. discoideum* were investigated. During both unstressed and stressed conditions, Grp78 was detected in whole cell lysates and in concentrated samples of conditioned buffer. During unstressed conditions, Grp78 localizes to sheet like structures around the perinuclear region and extends as a tubular network outward into the cell periphery. Furthermore, Grp78 was observed to colocalize with calnexin and calreticulin with the exception of a few sole Grp78-stained puncta seen in the cytosol. These findings indicate that, akin to human and mammalian GRP78, *D. discoideum* Grp78 predominantly localizes to the ER. When ER stress was induced by TN, AX3 wildtype cells produced increased amounts of intracellular and extracellular Grp78, as indicated in western blots of AX3 samples. In TN-treated AX3 cells, this resulted in changes to ER structure such as increases in aberrant structures and puncta stained with Grp78, and ER resident proteins, calnexin or calreticulin, as well as an interrupted ER network.

To assess the hypothesis: ER stress contributes to the pathogenesis of neurodegenerative disease, the underlying ER stress levels in AX3 cells as well as *D. discoideum* models for CLN3 and CLN5 disease were evaluated based on the amount and localization of Grp78 protein. Increased amounts of Grp78 were detected in whole cell lysates and conditioned buffer samples in all models; with the degree of increase varying based on the *cln* mutations. Remarkably, a similar ER morphology was seen in *cln3*$^{-}$ and *cln5*$^{-}$ cells when compared to TN-treated AX3 cells. However, in contrast to *cln3*$^{-}$ cells, *cln5*$^{-}$ cells have fewer and smaller aberrant structures; suggesting that these cells are better able to mitigate stress.

4.1 Establishing *D. discoideum* as a model for endoplasmic reticulum stress

4.1.1 Grp78 detection and post translational modifications

To study the role of Grp78 in *D. discoideum,* an antibody was raised against amino acids 41-440 of the endogenous *D. discoideum* Grp78. Western blotting of whole cell lysates and concentrated conditioned buffer with this antibody revealed an approximate 75 kDa protein with two immunoreactive bands; aligning with the molecular weight and banding patterns seen in western blots for both human and *D. discoideum* GRP78/Grp78 (Domínguez-Martín et al, 2018; Fu et al, 2022). Grp78 has a putative N-linked glycosylation site (Uniprot). Modification to N-linked glycosylation sites have been connected to lysosomal enzyme trafficking, with mutations affecting N-linked glycosylation leading to aberrant trafficking (reviewed in Pohl et al, 2009). To determine if N-glycosylated Grp78 was responsible for the double banding observed, whole cell lysates were treated with PNGase F to remove N-linked glycosylation. This did not reveal any changes to the banding pattern. Probing for countin, a known N-linked glycosylated protein, confirmed that PNGase F treatment was successful at removing N-linked glycans, however, it has been suggested that PNGase treatment may further modify proteins leading to smaller changes in protein size, preventing a noticeable change in band positioning. This is not likely the case for *D. discoideum* Grp78 as TN treatment did not result in an observable shift either.

Various PTMs can also be used to tag proteins for degradation. A well studied example of this is the UPS, where the N-terminus is modified by ubiquitin (Chau et al, 1989). Another PTM involved in degradation that also affects protein localization is arginylation (Cha-Molstad et al 2015). Arginylation involves the addition of arginine to an N-terminal amnio acid (Cha-Molstad et al 2015). Arginylation of GRP78 and PDI have been suggested to influence the

localization of these proteins such that arginine-labeled proteins localize away from the ER (Hu et al, 2006). Furthermore, bioinformatic analysis identified molecular chaperons, GRP78, GRP94, and calreticulin as well as oxidoreductases, PDI, and ERdJ5 with the ability to undergo N-terminal argniylation in mammalian cells (Cha-Molstad et al 2015). By raising antibodies specific to arginylated proteins, Cha-Molstad et al (2015) confirmed that GRP78, PDI, and calreticulin are arginylated. Sequence alignment of the N-terminal region of GRP78 and squencelogs (homologs based on sequence) highlighted conserved N-terminal residues at Glu19 of GRP78 (Cha- Molstad et al, 2015). After the removal of the putative signal peptide of Grp78 (1-21) predicted by SignalP-5.0, the N-terminal residue of Grp78 would be Glu22, hinting towards conserved arginylation. Interestingly, a GRP78 doublet can be seen in Cha-Molstad et al (2015) blots of GRP78; however, blotting with a R-specific antibody to the protein in question only showed one immunoreactive band. Arginylated-GRP78 (R-GRP78) localizes to the cytosol where it can interact with p62, sequestome-1, to induce oligermization and mediate autophagic degradation of protein aggregates or ER structures. Interaction between p62 and R-GRP78 as well as the subsequent oligomerization and interactions with ER transmembrane receptor tripartite motif containing 13 (TRIM13) mediate ER -phagy and target the degradation of misfolded cytosolic proteins (Figure 1E) (Cha-Molstad et al, 2015; Ji et al, 2019).

Although the antibody used in this study was created against an antigen specific to Grp78, it is possible that this banding was created by nonspecific binding to similar proteins. BLASTp analysis revealed that this amino acid sequence of the Grp78 antigen shares similarity with three other members of the HSP family and as such, the antibody may have detected other proteins from this family. To test this, *D. discoideum* cell lines deficient in *hspB*, *hspE2*, and *mhsp70* were obtained from the GWDI. These western blots revealed no differences in the

banding patterns, concluding that both bands were due to Grp78, and that this new antibody was specific to Grp78.

4.1.2 Grp78 localization

Immunolocalization revealed that Grp78 is present at the nuclear envelope and extends throughout the cell as sheets in the perinuclear area, with the concentration of sheets diminishing as the ER extends distally across the cell; here Grp78 stained tubular ER structures become more prevalent. Various studies have independently concluded that this localization pattern is linked to the ER; showing that the ER extends from the nuclear envelope as dense sheet regions surrounding the perinuclear region and extends to cell periphery as a network of interconnected tubulars (Veratti, 1961; Palade and Porter, 1954; West et al., 2011). Previous attempts to visualize the *D. discoideum* ER have been done by tagging IreA with GFP (Domínguez-Martín et al, 2018). In said study, IreA-GFP localized to dense sheet-like regions surrounding the nuclear envelope and as a network of thin, less-dense, tubules that extended towards the cell periphery (Domínguez-Martín et al, 2018). Domínguez-Martín et al also established that treating cells with 2 μg/mL of TN resulted in an increase in intracellular Grp78. In the present study, Grp78 was detected in increased amounts in both intracellular and extracellular samples after treatment with 0.5 μg/mL of TN after starvation. Additionally, we observed the colocalization calnexin and calreticulin with Grp78 which is consistent with the ER structure seen by Domínguez-Martín et al, 2018 and augments support for this ER morphology in *D. discoideum*. Additionally, in the present study, the localization of Grp78, calnexin, and calreticulin were examined under TN-induced stress conditions. This led to the detection of altered ER structure

and morphology – seen as a disarray of puncta stained with Grp78 and calnexin, or calreticulin throughout the cell.

4.1.3 Stress-related changes to the endoplasmic reticulum

In mammalian models, GRP78 localizes in increased amounts to the cytosol, cell membrane, mitochondria, and extracellularly, in addition to the ER during stressed conditions (Li et al, 2013; Bruneel et al, 2005; Tsai et al 2002; (Li et al, 2016; Van Krieken et al, 2021). In this study, immunofluorescence of Grp78 under TN-induced stress, showed changes in ER structure based on the localization of calnexin, calreticulin, and Grp78. In concert with this finding, Grp78 staining intensified in these regions of aberrant ER structures and Grp78-stained puncta when compared to calnexin or calreticulin. Moreover, studies detect increased amounts of cytosolic GRP78 in cytosolic fractions of HEK 293T cells when treated with TG, originally, GRP78 only existed in ER fractions supporting that stress causing GRP78 to be relocated away from the ER to the cytosol (Rao et al, 2002). These alterations to ER morphology and increases in fragmented regions, may be due to the cell partitioning off cytotoxic ER regions or regions concentrated by misfolded proteins and upregulating ER-phagy to increase the rate of ER turnover. However, if ER-phagy is not balanced by ER biogenesis there will be a decrease in the overall ER volume and network making the cell prone to stress. Additionally, increased autophagy and excessive ER-phagy can increase ER stress by over-degrading the ER, hindering protein production and cellular signalling. Although this is the first study to display these stress-related ER changes in *D. discoideum,* this phenomenon has still been seen in various other models. For example, a decrease in ER network continuity was coupled with increased ER swelling/dilation as demonstrated in neuronal models that were reversed by suppressing the UPR

(Arruda et al, 2023; Louessard et al, 2017). ER swelling and dilation can be triggered by acute stress to increase the folding capacity of the cell, however, can also cause further issues under prolonged conditions such as fragmentation or changes in ER-phagy/biogenesis (Shreatha et al, 2023; Louessard et al, 2017). Additionally, IreA was observed to cluster together during stressed conditions in *D. discoideum* and yeast; in yeast this was associated with enhanced RNase efficiency (Rubio et al, 2011; Domínguez-Martín et al, 2018). It is also possible the aberrant ER structure observed in the present study serve a similar function by increasing the availability of proteins active sites as suggested in Domínguez-Martín et al, 2018. Based on the conserved Grp78 qualities highlighted here, this study strongly supports *D. discoideum* as a biomedical model for studying cellular stress, however, further research is needed to further elucidate the specific stress related processes of *D. discoideum.*

The redistribution of Grp78 from the ER to the cytosol and other organelles may be attributed to changes in calcium homeostasis. The activation of various stress responses will act to alter intracellular calcium levels to communicate stress (Deniaud et al, 2008). TN-induced ER stress has been shown to elevate intracellular calcium levels by causing calcium release from the ER in mouse aortic smooth muscle cells, increases in intracellular calcium levels in bovine aortic endothelial cells, and contributes to an increase in intracellular calcium flow in hepatocytes (Dong er al, 2021; Buckley and Whorton, 1997; Ziomek et al, 2014). The increase in calcium flow from the ER or other stores into the cytosol in turn can act as a positive feedback loop, further inducing cellular stress and stress-induced pathways (Mohsin et al, 2020). It has also been proposed that calcium influences the activity of Grp78 in the stress signaling pathways by altering the binding affinity between GRP78 and ADP or ATP (Lamb et al, 2006).

4.1.4 Grp78 protein amounts

The role of GRP78/Grp78/KAR2 in the UPR, as a chaperone protein and in stress signalling has been well established, with studies showing that it is increased in response to environmental and chemical stressors in mammalian and human cells, *D. discoideum,* and yeast (Matlack et al 1999; Brown and Naidoo, 2012; Wang er al, 2010; Dong et al, 2008, Domínguez-Martín et al, 2018). To further explore this role in *D. discoideum,* AX3 cells were starved for 4 hours, after 2 hours the starvation media was supplemented with 0.5 μg/mL of TN. Western blotting with anti-Grp78, revealed that TN treatment increased the amount of Grp78 in AX3 whole cell lysates.

4.2 The effects of mutations in *CLN* genes on Grp78 and the endoplasmic reticulum

Neurodegenerative diseases have been shown to have a profound effect on the cellular stress response (reviewed in Peña-Bautista et al, 2020). In general, increased GRP78 amounts and the ability to increase protein folding capacity have been accepted as attenuative; however, this is not always sufficient and can be a sign of increased stress or the inability to mitigate stress. The present study reveals that *cln3*$^{-}$ and *cln5*$^{-}$ cells displayed elevated amounts of Grp78 in intracellular and extracellular samples, demonstrating that the role of Grp78 may extend into the extracellular environment. Although further exploration is needed to determine the role (if existent) of extracellular Grp78 in *D. discoideum,* studies in mammalian cells show that secreted GRP78 interacts with the cell surface and circulatory GRP78 was shown to reduce vascular inflammation in human coronary endothelial cells from patients with coronary artery disease (Repges et al, 2021). Moreover, the loss of *cln3* and *cln5* in *D. discoideum* lead to changes in ER structure. As expected, the degree of Grp78 increase and altered ER structure varied respectively.

4.2.1 CLN3

Prior evidence from *D. discoideum* notes that Cln3 partially localizes to the contractile vacuole, an organelle linked to osmoregulation and is also a major calcium store. Accordingly, the loss of *cln3* results in defects in osmoregulation and in calcium homeostasis (Mathavarajah et al, 2018; Gabriel et al, 1999, Sesaki et al, 1997, Malchow et al, 2006). Interestingly, these calcium abnormalities extend to other organelles in other models such as in mouse cerebellar neuronal progenitor cells displaying alterations in endoplasmic reticulum, mitochondrial, and lysosomal stores leading to aberrant calcium signalling, vesical transport, and the accumulation of autophagosomes (Chandrachud et al, 2015). Moreover, studies on SH-SY5Ycells (neroblastoma cell line) show that CLN3 knockdown cells altered cytosolic free calcium and led to calcium induced cytotoxicity (Chang et al, 2007). *CLN3* deficient astrocytes also display abnormal calcium signalling (Bosch et al, 2019). Additionally, calcium chelation in *D. discoideum* deficient *cln3* cells rescues abnormal phenotypes including abnormal differentiation (slug formation) and migration (Huber, 2014). Mathavarajah et al (2018), observed that after one hour in a hyperosmotic environment, both wildtype and *cln3*$^{-}$ *D. discoideum* cells recovered their ameboid shape, however, after treatment with ammonium chloride, a pH elevating lysosomotrophic compound and inhibitor of autophagy, *cln3*$^{-}$ cells were unable to recover while wildtype cells recovered. As ER-phagy is required to maintain a healthy ER, inhibiting autophagy may disrupt ER structures by preventing ER-phagy, hinting that ammonium chloride treatment exacerbates cellular stress to the point where the cell is unable to recover from stress even after the removal of the stressor, ammonium chloride, in *cln3*$^{-}$ cells resulting in a disrupted ER network and inability to return to ameboid.

In our study, loss of *cln3* in *D. discoideum,* led to elevated amounts of Grp78. Contrary to this finding, CLN3-deficient SH-SY5Y cells as well as SH-SY5Y mutated CLN3 displayed reduced levels of GRP78 (Wu et al, 2014). However, in agreement with Wu and colleagues' results, *cln3* deficient *D. discoideum* displayed a trend towards a diminished stress response to TN when compared to the AX3, supporting that Cln3 may play an indirect role in regulating stress. These findings suggest that GRP78 and CLN3 may interact with one another to regulate stress in SH-SY5Y cells. However, in *D. discoideum*, the loss *cln3* still yields an increase in intrecellular and extracellular Grp78.

Uusi-Rauva et al (2008) observed the immunoprecipitation of CLN3 with GRP78 and PM Na, K-ATPase, a transporter important for calcium homeostasis in *CLN3*-deficient primary mouse neurons; this displayed a reduction in the rate of endocytosis when compared to wild type cells after blocking Na, K-ATPase activity with ouabain treatment (Uusi-Rauva et al, 2008). Ouabain is a steroid derived ligand of Na, K-ATPase that can be used to treat heart disease (Aizman et al, 2001). Ouabain acts as signal transducer by inducing calcium oscillations within cells (Aizman et al, 2001). GRP78 was also shown to be involved in ouabain-induced endocytosis (Kesiry and Liu, 2005). Although, Grp78 was not observed to localize to endocytic compartments marked with p80 or VatC during unstressed or stressed conditions in *D. discoidieum*, it is possible that the secretion of Grp78 observed in *D. discoideum* treated with TN or in *D. discoideum* model of CLN3 and CLN5 disease may be due to Grp78 trafficking to other compartments (exosomes, multivesicular bodies that are not stained with P80 or Vat C) during the *D. discoideum* stress response or that Grp78 secretion and relocalization involves a sperate pathway.

To date, cell surface localization of Grp78 has not been observed in *D. discoideum* suggesting that GRP78 may only localize to the endocytic pathway to traffic GRP78 to the PM in mammalian cells, a property of GRP78 that may not be necessary for *D. discoideum* to communicate stress, however, additional studies are needed to investigate the relationship between CLN3, GRP78, and N, K-ATPase and cellular stress.

4.2.2 CLN5

cln5 deficient cells exhibited a trend towards an increase in Grp78 amounts compared to the untreated wildtype; however, the degree of Grp78 increase in *cln5*$^{-}$ cells is less then the increase seen in *cln3*$^{-}$ cells. This suggest that the loss of *cln5* leads to a milder stress response based on the nature of the protein or that *cln5*$^{-}$ cells are better able to attenuate stress compared to *cln3* deficient cells. It is also possible that real-time altercations and variations in autophagy and proteasome degradation observed in *D. discoideum* models of CLN5 disease may contribute to the varying degrees of Grp78 increase between replicates (McLaren et al, 2021; Kim and Huber, 2022). Under times of adversity, the cell is able to activate alternative routes to compensate for an increase in stress; as such, evidence suggests that *cln5* deficient cells are able to cope with this by increasing ubiquitination (McLaren et al, 2021).

In addition, research suggest that CLN5 functions as a thioesterase, deplaymitolayse, and/or glycoside hydrolyse (Mathavarajah and Huber, 2018; Lubennen et al, 2022). Proteasome inhibition in multiple melanoma cells has been suggested to mediate resistance to stress by upregulating GRP78; in turn cells show an increase in sensitivity to proteosome inhibition when GRP78 is restricted (Ma et al, 2014; Wang et al, 2007). Beyond this, the blockage of GRP78 confers sensitivity in breast cancer cells in murine models treated with the microtubule-

interfering agent, taxol, when originally ineffective (Luo et al, 2010). Taxol was found to increase GRP78 expression; however, GRP78 knock-down or cells treated GRP78 inhibitor, EGCG, sensitized cells to taxol. In studies of multiple myeloma cells, cells that survived treatment with protease inhibitors, displayed elevated amounts of GRP78, suggesting that GRP78 is involved in mediating the cells response to proteasome inhibition (Adomako et al, 2015). Taken together this suggests that insufficient Grp78 increases sensitivity to proteasome inhibition and microtubule alterations (Lou et al, 2010; Adomako et al, 2015).

Recently, Cln5 has been reported to function as a cysteine-based S-depalmitoylase (cysteine palmitoyl thioesterase) (Lubennen et al, 2022). Palmitoylation has been shown to influence the role of calnexin in an ER stress-dependent manner. Calnexin can be palmitoylated to help regulate calcium homeostasis through interactions with SERCA at the MAMs, however, non-palmitoylated calnexin interacts with ERp57 to mediate protein folding (Lynes et al, 2012; Lynes et al, 2013). Although the mechanism of calnexin depalmitoylation remains unknown, evidence suggest that depalmitoylation enzymes from the thioesterase family are regulators of ER stress responses (Lynes et al, 2013; Baekkeskov and Kanaani, 2009). This hints that, *cln5* deficiency might lead to constitutive calcium flow from the ER to the mitochondria and a reduced protein folding capacity. In mammalian cells, increased calcium flow into the mitochondria is essential for the apoptosis through caspase activation (reviewed in Orrenius et al, 2003). Finally, it is possible that the variations seen in CLN5 disease can steam from the cell being able to further regulate vital pathways such as calcium homeostasis and degradation from various other perspectives; depending on the specific mutation certain function of CLN5 maybe affected differently; thereby contributing to the later and variable onset seen in CLN5 disease.

5. Conclusion

Grp78 regulates ER stress induced by chemical stressors such as TN or genetic stressors such as the loss of *cln3* or *cln5* (genes linked to CLN3 disease and CLN5 disease, respectively) in *D. discoideum.* During stressed conditions Grp78 can be detected in increased amounts in both whole cell lysates and samples of concentrated conditioned buffer. Additionally, it was observed that both TN treatment and genetic mutations leading to the loss of *cln3* or *cln5* alter ER structure and morphology due to the increase in cellular stress through ER fragmentation and the formation of aberrant structures marked with Grp78 and calnexin, or calreticulin. This showed that the effect of TN on ER structure in AX3 cells mimics the ER structure caused by the loss of *cln3* and *cln5.* In concert, these findings support the hypothesis that Grp78 plays a critical role in communicating stress based on the increase in Grp78 protein amounts caused by chemical treatment and genetic mutations. Nonetheless, more work is required to elucidate the intricacies of the stress response pathways and their effect on other cellular processes in *D. discoideum* and mammalian models, to understand effect cellular stress has on pathogenesis of disease. Overall, findings from this study have provided novel insights into the role of Grp78 in *D. discoideum,* highlighting conservations in the stress response across biomedical models and strongly support that the stress response is altered in NCL models.GRP78/Grp78 and ER stress is at the confluence of several pathogenetic streams, connecting and adding to several diseases; by investigating pathways to cope, communicate, and alleviate stress future studies may learn how to divert stress related pathologies and possibly prevent or treat multiple diseases, including several neurodegenerative diseases (such as NCLs, LSDs, AD, PD, DLB, MSA, etc.) by manipulating the stress response.

6. References

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