ROLE OF THE GABARAP TUMOR SUPRESSOR IN THE CONTROL OF E.R. STRESS AND CELL APOPTOSIS

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By SAMANTHA ASSEE, B.Sc

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AUTHOR: Samantha Assee, B.Sc. (McMaster University)

CO-SUPERVISORS: Dr. André Bédard and Dr. Ana Campos

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ABSTRACT

In response to starvation, mis-folded proteins accumulate in the endoplasmic reticulum (E.R.) causing E.R. stress. This triggers a series of signaling pathways known as the unfolded protein response (UPR). The response helps to both enhance protein folding capacity and initiate mis-folded protein degradation, reducing E.R. stress. Alternatively, misfolded proteins are degraded and nutrients are recycled through autophagy. Thus, E.R. homeostasis depends on both UPR and autophagy. However, if E.R. stress is not resolved, UPR and autophagy can also cause apoptosis by mechanisms that are not fully understood.

In chicken embryo fibroblasts, gamma-aminobutyric acid receptor-associated protein or GABARAP (a protein involved in autophagy) can promote apoptosis in conditions of prolonged starvation (Maynard et al. 2015). In these conditions, the down-regulation of GABARAP by shRNA/RNA interference reduces the expression of the pro-apoptotic CHOP (CAAT-enhancer-binding protein homologous protein) transcription factor (a marker of E.R. stress) and enhances cell survival. This suggests that elevated levels of autophagy compromises E.R. homeostasis and promotes the expression of CHOP in UPR lethal pathways. While GABARAP induction and processing/activation has been linked to the expression of CHOP upon prolonged starvation (Maynard et al. 2015), nothing is known about the pathway mediating CHOP expression and the relationship with other pathways of the UPR in cells with GABARAP mis-expression. Understanding these pathways will allow us to determine if GABARAP is a general

determinant of E.R. stress or acts specifically on the expression of CHOP to control cell survival.

Elucidating mechanisms which are involved in E.R. stress and the cellular transition between pro-survival to pro-apoptotic roles can allow understanding of processes associated with several pathological conditions like cancer and neuro-degenerative diseases. Additionally, establishing a role for GABARAP tumor suppressor in the control of the UPR and cell fate is also important.

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ABBREVIATIONS

ASK1	Apoptosis signal regulating kinase 1
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATG	Autophagy-related genes
Bcl2	b-cell lymphoma 2
BIM	bcl 2 interacting mediator of cell death
BiP	Binding immunoglobin protein
BNIP3L	BCL2 and adenovirus E1B 19 kDa-interacting protein 3-Like
Caspase	Cysteine-aspartic proteases
CEF	Chicken embryonic fibroblasts
CHOP	CAAT-enhancer-binding protein homologous protein
DDX47	DEAD (Asp-Glu-Ala-Asp/His) box polypeptide 47
DFCP1	Double FYVE Containing Protein 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
eIF2α	Eukaryotic translation initiation factor

ERAD	E.R-associated degradation
E.R.	Endoplasmic reticulum
ERSE	E.R stress response element
FADD	Fas-associated protein with death domain
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GADD34	Growth arrest and DNA damage-inducible 34
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IRE1	Inositol requiring enzyme 1
IRES	Internal Ribosome Entry Site
JNK	cJUN NH2-terminal kinase
LC3	Microtubule-associated protein 1A/1B-light chain 3
p62	Nuceloporin 62
P38MAPK	P38 mitogen-activated protein kinase
PAS	Phagophore Assembly Site
PE	Phosphatidylethanolamine
P-EIF2α	Phospho-eukaryotic translation n initiation factor
PERK	Protein kinase R (PKR)-like endoplasmic reticulum kinase

PI3P	Phosphatidylinositol 3-phosphate
PolyQ	polyglutamine 72 repeat
PP1C	Protein phosphatase 1 catalytic subunit gamma
SDS-page	SDS polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
shRNAi	Short hairpin RNA interference
TBP	TATA-binding protein
TRAF2	Tumor necrosis factor receptor associated- factor 2
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
ULK1 complex	Unc-51 like autophagy activating kinase 1 complex
UPR	Unfolded protein response
Vps34	Vacuolar protein sorting 34
XBP1	Xbox binding protein

CHAPTER 1: INTRODUCTION

Topics in this chapter will consist of E.R. stress, the mechanisms and factors behind the unfolded protein response, the process of autophagy (specifically GABARAPs role) and a primary focus on the recent studies involving the proteins GABARAP and CHOP and their involvement in cell apoptosis and E.R. stress.

1.1 Unfolded Protein Response

The cell can initiate a variety of adaptive stress responses promoting cell survival in conditions where nutrients are depleted. Newly made polypeptides must be folded and modified properly before they can be transported to their final destination (Gardner et al. 2013). To prevent protein aggregation in the E.R. lumen, many E.R. chaperones and folding enzymes ensure basic E.R. quality control that will assist in the maturation of proteins (glycosylation, disulfide bond formation and folding enzymes) (Araki and Nagata 2012). If unfolded proteins are left for a significant amount of time a machinery targeting for E.R.-associated degradation (ERAD) is activated (Smith, Ploegh, and Weissman 2011). Retro-translocation of unfolded proteins moves them back into the cytosol where they are targeted for degradation via a ubiquitin-proteasome system. Although basic quality control mechanisms do help with relieving the accumulation of mis-folded proteins (E.R. stress) and protein maturation, conditions of prolonged starvation can result in cell death.

When starvation ensues, a large accumulation of mis-folded proteins builds within the E.R. creating a significant amount of E.R. stress. This occurs because the cell is lacking

the appropriate amino acids, sugars and energy to complete the processes of protein maturation and associated post-translational modifications. To maintain E.R. homeostasis, eukaryotic cells have created an emergency stress system called the unfolded protein response (UPR). This triggers a series of pathways that can involve transcriptional regulation, increasing E.R. protein folding capacity and the upregulation of pro-survival proteins. The UPR can also regulate protein translation via translational repression which helps to decrease the protein folding load (Walter and Ron 2011). Therefore, the UPR is considered a pro-survival mechanism as it allows for cells to adapt to changes in nutrient availability. On the other hand, depending on the nature and duration of the stress, starvation can ultimately lead to cellular apoptosis as conditions can become too severe or prolonged. A failure to restore E.R. homeostasis can lead to UPR lethal pathways inducing apoptosis and the expression of pro-apoptotic factors such as CHOP (Tabas and Ron 2011). The dual function of the UPR in cell survival and cell death explains why it is involved in a variety of diseases (Walter and Ron 2011). If the UPR starts to induce apoptotic mechanisms due to its inability to maintain E.R. homeostasis, it can lead to killing cells and several pathologies. On the contrary, due to UPR pro-survival function, rogue cells that may be detrimental to the organism can survive (Walter and Ron 2011).

Within each UPR pathway there is an E.R. integral membrane protein (IRE1, PERK and ATF6) that senses irregular E.R. conditions in the E.R. lumen and transmits this information across the E.R. membrane to the cytosol. Once the signal reaches the cytosol a specific group of transcription factors working alone or competitively (b-ZIP

transcription factors) move to the nucleus to activate UPR genes (Gardner et al. 2013; Walter and Ron 2011).

Studies involved with the activation of each pathway of the UPR are incomplete and several mechanistic details remain elusive. However, all pathways begin with a process of protein activation. This involves the dissociation of BiP from three key transmembrane proteins (IRE, PERK, and ATF6) resulting in a change in their oligomerization (Gardner et al. 2013). BiP or "binding immunoglobulin protein", is a protein folding chaperone that contains an ATPase domain which regulates binding to exposed hydrophobic regions of unfolded proteins (Gething 1999). Normally, BiP is bound to the UPR transmembrane proteins blocking activation. As a substantial amount of unfolded proteins start to accumulate within the E.R., BiP disassociates from IRE, PERK and ATF6 (leading to their conformational change and activation) and binds onto the unfolded proteins aiding them in their proper folding (Gething 1999).

In the following sections each UPR branch will be touched upon. More specifically, a description of how each branch can lead to survival or alternatively, apoptosis. It is important to note that all 3 branches of the UPR leads to upregulation of the pro-apoptotic factor CHOP if E.R. stress is prolonged and homeostasis can no longer be maintained by the UPR (Tabas and Ron 2011; Ron and Walter 2007). The UPR pathways are depicted in Figure 1.

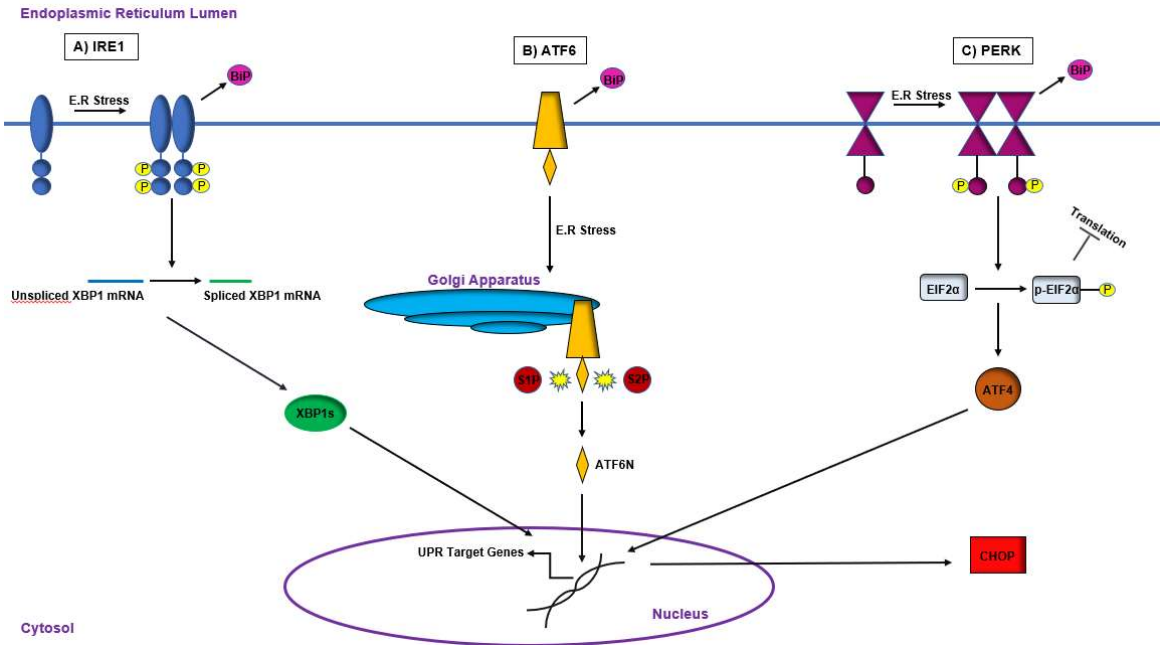


Figure 1: The Unfolded Protein Response. Upon aggregation of unfolded proteins, BiP disassociates from E.R. receptors (PERK, ATF6, and IRE1). A) Activation Initiates IRE1 dimerization and activates its cytoplasmic endonuclease domain that splices XBP1 precursor mRNA. The spliced XBP1 mRNA is translated into a transcription factor that is translocated to the nucleus for UPR gene expression. B) When ATF6 receptor is activated, it is transported to the Golgi apparatus and is proteolyzed by both S1P and S2P proteases. Cleaved ATF6 (ATF6N) can then be translocated to the nucleus for gene expression. C) Finally, PERK is activated by auto transphosphorylation and phosphorylates eIF2α. Translational block by p-eIF2α will enable only particular translation to occur with specific mRNAs, one being ATF4. ATF4 transcription factor will then upregulate target UPR stress proteins and in later prolonged stress activate proapoptotic factors like CHOP. Note that all three branches are involved in the upregulation of CHOP if E.R. stress is prolonged, and homeostasis can no longer be maintained.

1.1.2 Unfolded Protein Response Pathways – PERK

Protein kinase R (PKR)-like endoplasmic reticulum kinase or PERK is a transmembrane kinase which is activated upon E.R. stress (Figure 1). PERK's lumenal stress sensing domain detects an accumulation of mis-folded proteins and the cytosolic kinase domain, free from the disassociated BiP, is trans-phosphorylated (Gardner et al. 2013). Once trans-phosphorylated, PERK phosphorylates the eukaryotic translation initiation factor (EIF2α) becoming P-EIF2α (phospho-eIF2α). This inhibits EIF2α's original function, which is the initiation of mRNA translation (Gardner et al. 2013). This helps to reduce general protein synthesis as there is an overall decrease of protein load entering the E.R. (Gardner et al. 2013). Translation attenuation is then followed by degradation of proteins that have accumulated by ERAD (E.R.-associated degradation) and the expression of pro-survival genes (Chakrabarti, Chen, and Varner 2011). It is also important to note that although mRNA translation for a lot of proteins are inhibited, certain stress-response proteins are also upregulated when EIF2α is limiting (Walter and Ron 2011). Specifically, these are mRNAs contain inhibitory short upstream reading frames in their 5'-untranslated regions (Jackson, Hellen, and Pestova 2010). They also have IRES (internal ribosome entry sites), that allow for CAP-independent translation (Thakor and Holcik 2012). An example of this class of mRNA is that of activation transcription factor 4 (ATF4). ATF4 regulates the expression of both CHOP and DNA damage-inducible 34 (GADD34) proteins (Ron and Walter 2007). GADD34 encodes a regulatory subunit of the phosphatase PP1C (protein phosphatase 1 catalytic subunit gamma) complex that dephosphorylates P-EIF2α creating a negative feedback loop and reversing the

translational attenuation regulated by PERK (Novoa et al. 2001). CHOP is a member of the C/EβP family of transcription factors and is well known for its role in transcriptional activation of apoptotic genes. Therefore, it's important to note that although the PERK pathway is initially pro-survival, it can also lead to a proapoptotic response in a CHOP-dependent manner due to prolonged starvation. The dualism of the PERK pathway is likely regulated by the level of phosphorylated EIF2α as shown through several experiments that inhibit the GADD34-PPIC complex. Specifically, when GADD34-PPIC is selectively inhibited by a small molecule or deletion of GADD34, cells are protected from E.R. stress by prolonging low levels of p-eIF2α (Marciniak et al. 2004; Tsaytler et al. 2011).

1.1.3 Unfolded protein Response Pathways – ATF6

ATF6 (activating transcription factor 6) is another key pathway of the UPR involved in the upregulation of chaperones, foldases and components of the ERAD pathway (Adachi et al. 2008; Bommiasamy et al. 2009) (Figure 1). ATF6 is a transmembrane protein with a carboxy-terminal E.R. stress sensing domain within the E.R. lumen and an amino-terminal bZIP transcription factor domain within the cytosol (Gardner et al. 2013). Once activated by E.R. stress, ATF6 is transported from the E.R. to the Golgi apparatus within transport vesicles that pinch off the E.R. and is proteolyzed by both site-1 and site-2 proteases (Schindler and Schekman 2009; Haze et al. 1999). This releases ATF6s amino-terminal transcription factor domain. Processed ATF6 or ATF6(N) translocates to the nucleus and binds to E.R. stress response elements (ERSE) on UPR gene promoters which finally initiates transcription of UPR target genes (Yoshida et al. 1998; Haze et al.

1999). ATF6 is also heavily involved with another branch of the UPR named IRE1, specifically with the induction of XBP1 expression (an important component to the IRE1 pathway) (Yoshida et al. 2001). Although little is known about the deactivation of the ATF6 pathway, authors have speculated that the unspliced RNA transcript of XBP1 translates into a protein that acts as a proteasomal tag and negative regulator (Yoshida, Uemura, and Mori 2009). The unspliced pre-cursor XBP1 RNA translates into a protein (pXBP1u) that binds onto the matured XBP1 transcription factor (pXBP1s) (Tirosh et al. 2006; Yoshida et al. 2006) and activated ATF6 (Yoshida, Uemura, and Mori 2009). This makes them both prone to proteasomal degradation. Together, these two steps have been shown to be involved in the recovery phase of E.R. stress.

1.1.4 Unfolded Protein Response Pathways – IRE1

IRE1 (inositol-requiring kinase 1) is present in all eukaryotes and is therefore known to be the most well conserved branch of the UPR (Hetz and Glimcher 2009). IRE1 transmembrane protein has both an amino-terminal E.R. lumenal domain and carboxy-terminal cytoplasmic endoribonuclease and kinase domains (Gardner et al. 2013). Due to the accumulation of misfolded proteins, E.R. stress starts to build and IRE1 is activated (BiP disassociation from the IRE1 transmembrane protein occurs initially) through dimerization of the molecule, allowing for higher-order oligomerization to occur in the E.R. lumenal domains (Lee et al. 2008; Korennykh et al. 2009) (Figure 1). After these conformational changes, activation occurs within the cytosolic domains which leads to the activation of the endoribonuclease domains as well (Walter and Ron 2011). When the endoribonuclease domains become activated it cleaves the RNA precursor encoding the

UPR transcription factor XBP1. Processing by IRE1 removes the intron in the XBP1 RNA precursor promoting translation of the XBP1 transcription factor and the expression of genes that encode for E.R. quality control proteins (Yoshida et al. 2001; Sidrauski, Cox, and Walter 1996; Travers et al. 2000).

IRE1 has also been seen to be associated with cellular apoptosis. For instance, IRE1 dimers will interact with adaptors like TRAF2 (tumor necrosis factor receptor associated- factor 2) to allow for the activation of ASK1 (apoptosis signal regulating kinase 1) which subsequently leads to JNK (cJUN NH2-terminal kinase) and p38MAPK (P38 mitogen-activated protein kinase) activation (Urano 2000). JNK is involved in the activation of the pro-apoptotic protein BIM (BCL2 interacting mediator of cell death) (Lei and Davis 2003; Putcha et al. 2003) and the inhibition of BCL2 (b-cell lymphoma 2), a pro-survival protein (Yamamoto, Ichijo, and Korsmeyer 1999).

1.2 Autophagy

Autophagy is an alternative process that helps to promote cell survival and allows for the elimination of mis-folded proteins that escape the E.R. Recognized as a key catabolic process, it occurs as a response to extracellular stress (hypoxia, starvation, temperature) and intracellular stress (starvation and accumulation of both damaged organelles and components of the cytoplasm) (Levine and Klionsky 2004). In autophagy, dysfunctional organelles and proteins are trapped into double membrane vesicles named autophagosomes. Autophagosomes fuse with lysosomes promoting both degradation and recycling of the contents within the vesicle (Levine and Klionsky 2004).

1.2.1 Core Mechanism in Autophagy

A predominant number of molecular components are involved in the initiation and development of autophagosomes. They have been discovered in multiple model organisms leading to the identification of several AuTophaGy-related (ATG) genes (Klionsky et al. 2003) as well as other pre-discovered genes which are involved in the process. Although there are several steps involved in the development of autophagosomes, key steps govern the process of their production.

The first step involves a group of proteins called the ULK complex (Unc-51 Like Autophagy Activating Kinase 1 complex). This is initiated by specific signals of stress that induce the process of autophagy (Fleming et al. 2011). This then will activate another complex named the Vps34 complex (Vacuolar Protein Sorting 34) that is responsible for the initiation of a double-layered membrane within the cytosol by phosphorylating an essential lipid component called PI3P (Phosphatidylinositol 3-phosphate). PI3P phosphorylation allows for docking of essential proteins involved in membrane growth. Next, a well-established complex consisting of three Atg proteins (the Atg5-Atg12-Atg16L complex) is activated and colocalized to the forming membrane where its function is to help catalyze a covalent binding reaction between the LC3-I (Microtubule-associated protein 1A/1B-light chain 3) protein and membranous phosphatidylethanolamine (Fujita et al. 2008). This process is called lipidation as LC3-I is bound onto the membrane creating its conjugated form LC3-II. Lipidated LC3-II has been shown to play an effective part in enclosing the membrane (Yoshimori 2010; Sou et al. 2008). As this occurs, the membrane will grow and enwrap a portion of the cytosol

creating a fully developed autophagosome. In the final steps of autophagy, the autophagosomes will fuse with lysosomes creating autolysosomes. Once fused, the lysosomes will release its lysosomal hydrolases into the vesicle degrading the contents within (Fleming et al. 2011). The contents are then recycled and used again to build new components. The Mechanism of Autophagy is depicted in Figure 2.

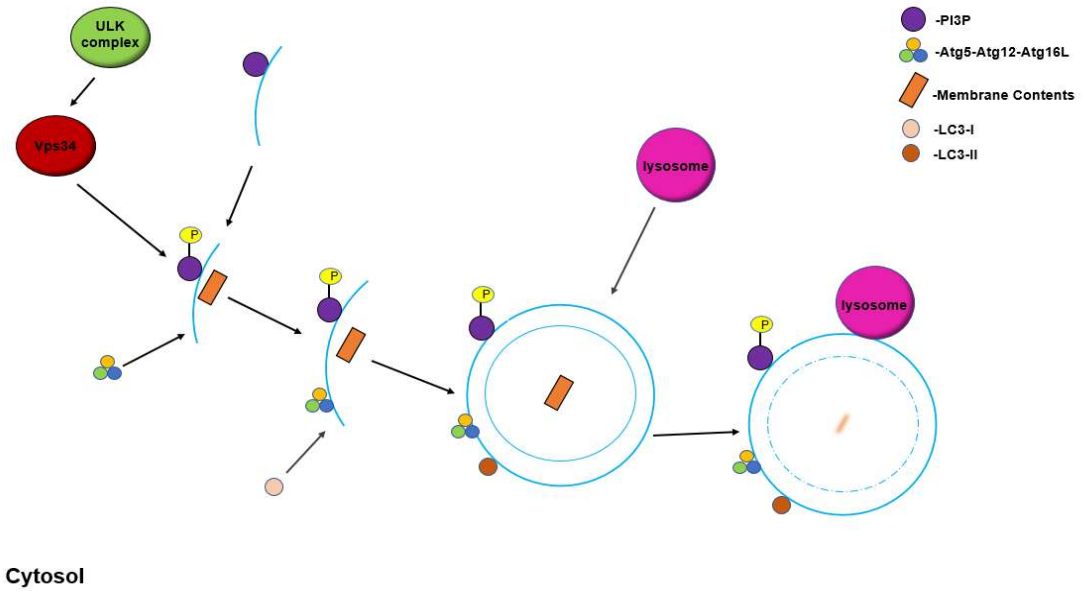


Figure 2: Core Mechanism of Autophagy. The first step involves a group of proteins called the ULK complex which is initiated by specific signals of stress. This activates another complex named the Vps34 that initiates double-layered membrane growth by phosphorylating the essential lipid component PI3P. Next, specific Atg proteins (the Atg5-Atg12-Atg16L complex) are activated and colocalized to the forming membrane. Its function is to help catalyze a covalent binding reaction between the LC3-I protein and membranous phosphatidylethanolamine, creating lipidated LC3-II. Lipidated LC3-II then plays an effective part in enclosing the membrane. As this occurs, the membrane enwraps a portion of the cytosol creating a fully developed autophagsome. In the final steps, autophagosomes fuse with lysosomes creating autolysosomes. Once fused, the lysosomes will release lysosomal hydrolases degrading the contents within.

It is also important to note that autophagosome origin and initial processing are still highly debated. In regards to where membrane material originates from, several morphological studies showed association with the E.R. (Hayashi-Nishino et al. 2009; Ylä-Anttila et al. 2009). For example, it was shown that the E.R. forms a structural cradle for the formation of the autophagosome (Hayashi-Nishino et al. 2009). The study used overexpression of the inactive mutant Atg4B that disallowed LC3-I to be lipidated into LC3-II, creating deformed and unclosed premature autophagosomes. Using electron microscopy and tomography, an abundance of so-called E.R.-IM (E.R. innermembrane) structures (the E.R. created its own subdomain encircling these premature structures) was observed, establishing a connection between the E.R. and the inner membranes (Hayashi-Nishino et al. 2009). In another recent paper, scientists looked at a autophagy protein named Atg14L as it's known to localize to the E.R. in the initial steps of autophagosome production (Matsunaga et al. 2010). In this study, Atg14L was mutated at a single residue, which disallowed it to localize to the E.R. This mutant background led to defective autophagy induction implying a key role for Atg14L in the recruitment of an important membrane kinase (P13 kinase) which is involved in autophagosome biogenesis. This showed that two important proteins involved in early autophagosome production must be targeted to the E.R. for autophagy production (Matsunaga et al. 2010). Additionally, DFCP1 (Double FYVE Containing Protein 1), a marker for autophagosome assembly sites, has been shown to have a E.R. targeting domain and is heavily associated with the E.R. (Axe et al. 2008). These are just a few examples that establish a connection between components that are involved in early autophagosome biogenesis and the E.R.

1.2.2 Autophagy and Apoptosis

There have been a variety of studies performed on cells and organisms depicting that autophagy is involved in mechanisms of survival (as expected) as the loss of function in autophagy genes showed decreased viability when starvation arose in several cell types and organisms. (Lum, DeBerardinis, and Thompson 2005; Komatsu et al. 2005; Tsukada and Ohsumi 1993). What is fascinating is although autophagy is a pro-survival mechanism and its processes have opposite outcomes to apoptosis, several studies have concluded that autophagy and apoptosis are connected in many ways (Rubinstein and Kimchi 2012). One example is the regulation of apoptosis by autophagy. In some cases, proteins involved in autophagy regulate the apoptotic pathway by direct interaction with components involved in apoptotic machinery. This includes proteins that are well known to be involved in autophagosome formation (Rubinstein and Kimchi 2012). Examples of such proteins include Atg5, which is known to mediate the release of cytochrome c, a hemeprotein involved in the initiation of apoptosis once released from the mitochondria (Yousefi et al. 2006). Another is Atg12, which has been shown to be involved in caspase activation (a group of protease enzymes that play an essential role in cell death) in a variety of apoptotic stresses (Rubinstein et al. 2011). Both Atg5 and Atg12 regulate apoptotic processes by interacting with the anti-apoptotic protein family BCL2, inhibiting its function and allowing for apoptosis to begin. Another interesting case involves autophagosomes used as a platform for caspase 8 activation (Rubinstein and Kimchi 2012). There are two different routes involving the recruitment of caspase 8 to the autophagosome membrane. One includes ubiquitylated caspase 8 binding to p62

(nucleoporin 62, a autophagic cargo receptor) using the ubiquitin binding domain of p62 (Jin et al. 2009; Young et al. 2012). The other route involves the interaction of caspase 8 with adapter protein FADD (Fas-associated protein with death domain) and Atg5 (Young et al. 2012; Pyo et al. 2005).

One interesting possibility as to why autophagic proteins are involved with apoptotic regulation is specific autophagy proteins may act like a rheostat, sensing the metabolic state of the cell (Rubinstein and Kimchi 2012). When the stress conditions of the cell become too severe, autophagy proteins may send signals to activate apoptotic proteins creating a switch between early autophagic response and late apoptotic response. Another possibility is activation of apoptotic proteins by autophagy proteins involve deactivation of their autophagic function allowing apoptosis to be activated and pro-survival autophagy functions to be suppressed (Rubinstein and Kimchi 2012). Much like the UPR, autophagy has been shown to switch between roles of survival and death depending on the cells environmental conditions.

1.3 UPR and Autophagy Crosstalk

When the UPR fails to maintain homeostasis, cells also initiate autophagy (Bernales, McDonald, and Walter 2006). It has been shown that some E.R. stress sensors in the UPR act as effectors to help initiate autophagosome formation. One example includes the UPR factor P-EIF2α (Y. Kouroku et al. 2007). As previously mentioned, the Atg5-**Atg12**-Atg16 complex helps to convert LC3-I into the LC3-II lipidated form on nascent autophagosome membranes (Fujita et al. 2008). In the experiment, the LC3 conversion

process was induced by polyQ (polyglutamine 72 repeat) aggregation (a malfolded protein) within the E.R. as the aggregation was shown to induce E.R. stress and vesicle formation (autophagosomes) (B. Ravikumar 2002; Brinda Ravikumar et al. 2004; Yoriko Kouroku et al. 2002; Nishitoh et al. 2002). In control cells, they saw that when polyQ aggregation was induced mRNA and protein expression of Atg12 was upregulated. On the other hand, when a substitution mutation was induced on EIF2α eliminating phosphorylation in the same conditions, Atg12 mRNA and protein expression was inhibited ultimately preventing LC3 conversion. This was good indication that the PERK system is involved in the process of autophagosome formation in conditions of E.R. stress caused by polyQ aggregation.

Another example involves the IRE1 pathway in the UPR – specifically with XBP1 transcript splicing. Studies showed that XBP1 mRNA splicing induced autophagy in endothelial cells (Margariti et al. 2013). Endothelial cells were infected with an adenovirus expressing a XBP1 spliced transcript and characterized by electron microscopy. Several structures representing autophagic vesicles were observed in these conditions. When the cells were infected an adenovirus encoding the unspliced XBP1 transcript, little to none of these structures were observed (Margariti et al. 2013). Additionally, when there was an overexpression of spliced XBP1transcript there was also an increase in mRNA and protein levels with two autophagy genes (BECLIN-1 and LC3β) (Margariti et al. 2013). Lastly, the authors also showed that mature XBP1 binds directly to the gene promoter of Beclin-1, suggesting that autophagic induction may be due to XBP1 transcriptional regulation of the BECLIN-1 gene (Margariti et al. 2013).

1.4 GABARAP

Gamma-aminobutyric acid receptor-associated protein or GABARAP is involved with several functions within the cell. One function deals with neurotransmission, cell synapsis and ion channel regulation. GABARAP is directly associated with microtubules and microfilaments indicating that it is involved with the coordination and interaction of $GABA_A$ receptors within the cells cytoskeleton. This suggests that GABARAP helps its associated $GABA_A$ receptors in receptor trafficking, anchoring and synaptic clustering (Chen et al. 2000; Wang and Olsen 2000).

Recently, there has been much focus on GABARAPs involvement with autophagy and its role in autophagosome development. GABARAP has been identified as part of the ATG8 family along with LC3. In Yeast, ATG8 levels correlate with the size of the autophagosome as the reduction of ATG8 leads to smaller autophagosomes and the attenuation of autophagy processes (Xie, Nair, and Klionsky 2008). This indicates that the protein is involved in the maturation of later stages of the phagophore as it develops into the autophagosome. For ATG8 to be activated, there needs to be a covalent conjugation to phosphatidylethanolamine (PE) allowing for ATG8 to be anchored onto the autophagosomal membrane to perform its function (Ichimura et al. 2000). In mammals, GABARAP function is very similar to ATG8 function. It has been shown that cells that are deficient in GABARAP do display impaired autophagosome formation (Weidberg, Shvets, et al. 2010) and that GABARAP is involved in phagosome elongation (Schaaf et al. 2016). Much like ATG8, GABARAP must first be cleaved and conjugated to the lipid phosphatidylethanolamine and anchored onto the autophagosomal membrane

(GABARAP II) where it will facilitate membrane expansion (provided by the E.R.) (Weidberg, Shpilka, et al. 2010). It is also important to mention that GABARAP and its other ATG8 family members (LC3) associate with autophagy adaptor proteins that allows for cargo selection, targeting and degradation (Schaaf et al. 2016).

1.4.1 GABARAP and Cell Death

A pathway that leads to the induction of autophagy mediated death in chicken embryo fibroblasts has been described in our laboratory focusing on both ATG8 family members GABARAP and LC3 (Maynard et al. 2015). In CEF, LC3 lipidation is rapid in response to starvation while GABARAP is only expressed and lipidated in conditions of *severe* nutrient depletion and unlike LC3, GABARAP I&II levels have been shown to increase upon starvation. The downregulation of LC3 by RNAi leads to apoptosis in all conditions demonstrating that it is required for cell survival. On the other hand, cells with complete downregulation of GABARAP induced by RNAi interference are viable and survive for extended periods of starvation with reduced levels of apoptosis (Maynard et al. 2015). It is also important to note that the expression of the CHOP transcription factor (a pro-apoptotic factor and indicator of prolonged E.R. stress) is reduced in the absence of GABARAP (Maynard et al. 2015). This suggests that increasingly high levels of autophagy can compromise E.R. homeostasis and can lead to cell death.

GABARAP has been shown to be involved with other pro-apoptotic factors such as DEAD (Asp-Glu-Ala-Asp/His) box polypeptide 47 (DDX47) (Jeong, Seung, and Chun 2005) and BNIP3L (BCL2 and adenovirus EIB 19 kDa-interacting protein 3-Like)

(Schwarten et al. 2009). It is important to mention that direct interaction between GABARAP and DDX47 was investigated in our laboratory. Unfortunately, the experiments were unsuccessful at confirming the previous studies found in CEF. GABARAP and its role in cancer has also been investigated. For example, GABARAP has been classified as a tumor suppressor as there is a reduction of GABARAP expression in numerous breast cancer cell lines (Klebig et al. 2005). As suggested by Maynard and colleagues, due to GABARAPs involvement with the modulation of CHOP expression in conditions of prolonged starvation and E.R. stress, it plays a role in the regulation of cell fate and therefore, this information may help us to understand the mechanism behind its tumor suppressor function (Maynard et al. 2015).

1.5 RATIONALE

Based on recent data, we hypothesize that GABARAP controls the level of autophagy ("autophagic flux") of the cell and E.R. homeostasis (Maynard et al. 2015). This implies that increased levels of lipidated GABARAP ultimately impairs E.R. function, activating UPR lethal pathways. As cells are starved, the autophagic flux progressively increases inducing GABARAP expression. When the autophagic flux is lower, new nutrients are provided to the cell by autophagy which allows the cell to survive. However, as the flux starts to increase, the accumulation of GABARAP occurs and the demands on the E.R. (which is the source of membrane formation of autophagosomes) become too severe impairing E.R. function (Maynard et al. 2015). This results in high E.R. stress and eventually cellular apoptosis. It is important to acknowledge that autophagy does help to

decrease the level of misfolded proteins, but becomes deleterious when it accumulates in response to prolonged starvation.

1.6 OBJECTIVES

Maynard and colleagues suggest that GABARAP downregulation reduces E.R. stress and CHOP expression upon prolonged starvation (Maynard et al. 2015). However, it is not known if GABARAP levels affect multiple pathways in the UPR. Our primary objective is to look at key factors involved with the UPR in conditions of GABARAP down-regulation and prolonged starvation. This will be done by Western blotting analysis using antibodies for P-EIF2α, ATF6, ATF4 and CHOP. Since XBP1 expression is controlled at the level of splicing, we can quantitate the levels of spliced and unspliced XBP1 transcripts by qRT-PCR (quantitative reverse-transcriptase polymerase chain reaction). These experiments will help us to determine if a reduction in GABARAP expression attenuates all pathways of the UPR, thus attenuating total E.R. stress on a global level, or GABARAP down-regulation *only acts* to block CHOP expression in conditions of prolonged starvation.

CHAPTER 2: DETAILED MATERIAL AND METHODS

2.1 Tissue Culture

Chicken embryonic fibroblasts (CEFs) were cultured in DMEM high glucose media (Sigma #D6429) enhanced with 5% Tryptose Phosphate Broth (Sigma #T-9157), 5% Heat Inactivated Cosmic Calf Serum (Hyclone #SH30087.03), 1% Penicillin/Streptomycin (Sigma #P4333) and 1% L-Glutamine (Sigma #G7513) making complete DMEM media. Cells were kept within an incubator at 41.5°C and in 5% medical grade CO_2. CEFs were split every 2 days into 100 mm plates (Falcon #353003) using 0.05% Trypsin-EDTA (Sigma #T3924). Split into 1:3 dilution, cells were usually kept until the 9^{th} passage. Once at that point, cells were discarded and new primaries were thawed for use.

2.2 E.R. Stress Treatment

CEF that were treated for E.R. stress were first split into 1:3 and incubated in their respective conditions (41.5°C and in 5% medical grade CO_2) until confluence was reached. The cells were then treated with 1 µg/mL of Tunicamycin (Sigma #T7765), an E.R. stress inducing drug (glycosylation inhibitor), for a duration of 18-22 hours. The control sample CEF were treated with DMSO diluent.

2.3 Retroviral shRNAi Vector Construct Generation

It is important to note that the retroviral vector system used in these experiments are RCAS (Replication Competent ALV LTR with a Splice acceptor) vectors. These vectors are different from most other vectors because they encode replication-competent viruses

(most vectors being replication-defective) and are derived from a parental virus of avian origin (Hughes 2004). Replication-competent vectors spread rapidly in a short time and will infect essentially all cells (Hughes 2004).

The target sequence of the GABARAP gene was selected using the shRNAi design tool at www.genescript.com/ssl-bin/app/rnai. The Hairpins for the first miRNA cloning site *(Nhe1/MluI)* of the pRFPRNAiC(U6-) miRNA cassette were created by polymerase chain reaction (PCR) using 10ng of the corresponding gene-specific oligonucleotide along with 100ng of two flanking oligonucleotides A and B (Table 1) using Q5 High-Fidelity 2X Master Mix (NEB M0492S). Oligonucleotides C and D (Table 1) were used to generate the shorthair pins for GABARAP. The PCR protocol for amplification was as follows: 5 minutes at 95°C, 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 4 minutes for a total of 25 cycles. PCR protocol was completed using an Applied Biosystems GeneAmp PCR system 2700. The PCR product were cleaned with the GeneElute™ PCR Clean-Up Kit (Sigma NA1020) following manufacturer instructions. Next, the Purified PCR product was digested at the *NheI* and *MluI* restriction sites and subcloned into the pRFPRNAiC(U6-) miRNA cassette. The miRNA expression cassette harboring the GABARAP targeting sequence was subsequently subcloned into a RCASBP(A)-RNAi vector which utilized *NotI-ClaI* digestion. See Table 1 for the oligonucleotides used.

Table 1: Oligonucleotides Sequences Used for shRNA Vector Construction

A	5'-GGCGGGGCTAGCTGGAGAAGATGCCTTCCGGAGAGGTGCTGCTGAG CG-3'
B	5'-GGGTGGACGCGTAAGAGGGGAAGAAAGCTTCTAACCCCGCTATTCA CCACCACTAGGCA-3'
C	5'-GAGAGGTGCTGCTGAGCGACGCTCTTCTTCTTCGTCAACATAGTGAA GCCACAGATGTA-3'
D	5'-ATTCACCACCACTAGGCAGCGCTCTTCTTCTTCGTCAACATACATCTG TGGCTTCACT-3'

2.4 Transfection

Calcium phosphate transfection of the shorthairpin RNA interference retroviral vectors were performed prior to starvation and analysis. DNA precipitation prior to transfection included 10µg of the DNA vector with 20µg of salmon sperm per 100 mm plate after ethanol precipitation overnight at -20°C. Recovering the DNA involved centrifugation at 13,000 rpm for 10 minutes at 4°C, discarding the supernatant, rinsing in 70% ethanol and centrifuging again at 13,000 rpm for 5 minutes. Supernatant was discarded, pellet was dried in a speed vac for 3 minutes and each DNA vector sample was resuspended in 200ul of ddH_20 and 62ul of $CaCl_2$. Next, 238ul of ddH_20 was added to bring the total volume to 500ul. CEF were split 1:3 one day prior to transfection and complete DMEM medium was replaced with fresh complete DMEM medium 4 hours prior to transfection. Cells are usually 40-60% confluent for transfection. 500µl of 2X HBSP pH 7.12 buffer (1.5mM Na_2HPO_4, 10mM KCl, 280mM NaCl, 12mM glucose, and 50mM HEPES) was added drop-wise while vertexing to initiate DNA precipitation. DNA was left to precipitate for 30

minutes and 1 mL of total DNA precipitate mix was added to each 100 mm plate. Plates were then incubated for 5 hours. After incubation, cells were glycerol shocked (4mL of 15% of glycerol in HBSP buffer) for 1 minute. Next, each plate was washed twice in complete DMEM medium and a final 9mL of complete DMEM media was added to each plate. The cells were passaged a total of three times to ensure full infection of cells by the replication-competent shRNA retrovirus.

2.5 Western Blotting

2.5.1 Cell Culture Conditions

GABARAP shRNAi (short hairpin RNA interference), CHOP shRNAi and control RCASBP(A)RNAi vectors were transfected into CEFs and protein samples were prepared for Western blotting analysis. Cells in each plate were split into a 1:3 dilution, amplified and left to starve in the incubator for a period of time without replacement of new medium (usually until cells started to look stressed and elongated). Conditions in the incubator were normal at 41.5°C and in 5% medical grade CO2. Samples were then harvested at several time points, starting with cycling conditions and ending with a number of days post confluence throughout the starvation period.

2.5.2 Cell Lysate Preparation

After incubation cells were collected for lysate preparation. Cells were washed with 1XPBS pH 7.4 (137 mM NaCl, 4.3 mM Na_2HPO_4, 2.7 mM KCL and 1.47 mM KH_2PO_4) three times. Next, they were scraped and collected into a microcentrifuge tube in a total of 1mL of 1XPBS using a cell scraper and then pelleted in a microcentrifuge at 6500 RPM for

5 min at 4°C. Remaining 1XPBS supernatant was pipetted out and cells were lysed with 100-200ul of 1X SDS sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 60 mM Tris pH 6.8) and 1% added Halt protease and phosphatase inhibitor cocktail (Halt #1861281). Cells were mixed thoroughly with pipette and then vortexed for 20 secs. Once lysed, samples were boiled for 3 mins and placed back into the centrifuge at 13,000 RPM for 5 mins at 4°C, pelleting cellular debris. The supernatant was collected and placed into a fresh new microcentrifuge tube where it was stored in the -80°C freezer.

2.5.3 Bradford Assay

First, a standard curve was prepared by adding an increment volume (0-12ul) of bovine albium serum (1ug/ul) to a total mixture of 200ul ddH_20 and 2ul SDS sample buffer in each microcentrifuge tube. 800ul of Bradford reagent was added to each tube and optical densities were measured at 595nm generating a standard curve. Next, the sample readings were prepared by adding 2ul of protein lysate to 200ul of ddH_20 and 800ul of Bradford reagent. Optical densities were measured at 595nm and the protein concentrations were measured utilizing the standard curve.

2.5.4 SDS – page and Western Blotting

Samples for Western blotting were prepared with 30ug of total protein in a total volume of 40ul of 1X SDS sample buffer. Samples were run on 14% SDS – polyacrylamide gel and then transferred to a nitrocellulose membrane (BIO-RAD #162-0115). Once transferred, the membranes were blocked in milk or BSA solution (5% skim milk/BSA powder dissolved in 1X TBS solution pH 7.6 (150mM NaCL and 50mM Tris-HCL)) for

45 minutes at room temperature. Next, the membranes were incubated with the appropriate primary antibody overnight at 4°C. The following day, the nitrocellulose membrane went through a washing cycle that consisted of 1 wash of 1X TBS, 2 washes of 1X TBST (1X TBS with 0.1% tween) and a final two washes of 1X TBS, each for 5 minutes. A second incubation with the desired secondary antibody was done for a duration of 2 hours at room temperature. After, the membranes were washed for a second time following the same washing cycle as mentioned above and was visualized via Luminata Forte Western HRP Substrate (Millipore #WBLUF0500) and hyperfilm (GE Healthcare #28906839) following the protocol of the manufacturer.

2.5.5 Antibodies

The following primary antibodies and their appropriate dilutions were used in each experiment; anti-GABARAP (MBL: M135-3) at a dilution of 1:200, anti-CHOP ('Tulip Bleed 2' provided by Dr. André Bédard, plasma used as the source of antibody) at a dilution of 1:750, anti-ATF4 also known as CREB-2 (Santa Cruz: sc-200) at a dilution of 1:50, anti-p-eIF2α (Invitrogen: 710292) at a dilution of 1:750 and anti-ERK2 (Millipore: 05-157) with a dilution of 1:1000.

The following secondary antibodies and their appropriate dilutions were used in each experiment; HPR conjugated anti-Mouse IgG at a dilution of 1:25000 (Cell Signaling: 7076), HPR conjugated anti-Rabbit IgG at a dilution of 1:25000 (Cell Signaling: 7074S) and a HPR conjugated anti-bovine/goat IgG with a dilution of 1:25000 (Santa Cruz: 2378).

2.6 qRT-PCR

2.6.1 Cell Culture Conditions

GABARAP shRNAi vector and RCASBP(A)RNAi control vector were all transfected into CEFs for RNA sample preparation. Cells were split at 1:3 dilution, amplified and left to starve in the incubator for a period of time without replacement of new media (usually until cells started to physically look stressed and elongated). Conditions in the incubator were normal at 41.5°C and in 5% medical grade CO_2. Samples were harvested at several time points, starting with cycling conditions and ending with a number of days post confluence throughout the prolonged starvation period.

2.6.2 RNA collection

RNA sample collection of downregulated GABARAP shRNAi cells and RCASBP(A)RNAi(-) control vector cells were done using the Qiagen RNeasy Mini Kit protocol (Qiagen #74104). Samples (one 100mm plate per sample collection) were prepared via a direct lysis method by washing the cells with 1XPBS followed by 600ul of lysis buffer provided by the Qiagen RNeasy Mini Kit. Cells were then homogenized by passing them through a 20-gauge blunt ended needle at least 5 times. Finally, the samples were loaded onto the provided RNA columns and washed and eluted through the columns following the protocol of the manufacturer.

2.6.3 cDNA Synthesis

After RNA samples were collected, their concentrations were measured by utilizing their optic density at a wavelength of 260 nm. After, the RNA was treated with DNAse I. The process consisted of the appropriate volume of RNA in ul for 2ug (dependent on the

concentration of sample), 1ul of DNAse 1, 2ul of 10X reaction buffer ($MgCl_2$) and a variable amount of DEPC H_2O to bring the final volume to 20ul for each sample. Samples were then placed in a warm bath at 37°C for 30 minutes. After, 1ul of 25mM of EDTA was added to each sample and they were placed on the heat block at 70°C for 5 minutes. The second step of the process consisted of cDNA synthesis with the DNAse treated RNA. Reagents used for this process were provided in the ProtoScript® First Strand cDNA Synthesis Kit (New England Bio Labs #E6300S). This consisted of 6ul of the treated RNA, 2ul of the d(T)23Vn oligos, 10ul of 10X buffer mix and 2ul of 2X enzyme mix, all provided by the cDNA synthesis kit. The samples were incubated in a water bath at 42°C for 1 hour and soon after placed in a heat block at 80°C for 5 minutes. Finally, 30ul of DEPC H_20 was added to each sample and samples were stored in the -20°C freezer.

2.6.4 PCR Protocol

The PCR reaction for each sample was as follows: 3.5ul of ddH_20, 12.5ul of Q5 High-Fidelity (2X) master mix (New England Bio Labs #M0492S), 4ul of template DNA (cDNA synthesis) and 2.5ul of both forward and reverse oligos. The following XBP1 primers were used in PCR amplification:

XBP1 Forward: 5'-GTGCGAGTCTACGGATGTGA-3'

Reverse: 5'-AAGCCGAACAGGAGATCAGA-3'

The Following TBP (TATA-binding protein) primers were used in PCR amplification:

TBP Forward1: 5'-AGCGACACAGGGAACATCTG-3'

Reverse1: 5’-GTACAGAGGTGTGGTTCCCG-3'

TBP Forward2: 5'-CTGTACCCGTCCCCAATGAC-3’

Reverse2: 5'-GGCACGAAGTGCAATGGTTT-3'

The Following HPRT (Hypoxanthine-guanine phosphoribosyltransferase) primers were used in PCR amplification:

HPRT Forward1: 5'-GCATCGTGATTG GCGATGAT-3'

Reverse1: 5'-GTCCTGTCCATGATGAGCCC-3'

HPRT Forward2: 5'-CATTGTGCTGGAAAGCAGCAG-3

Reverse2: 5'-GCAGAACAAGTCCAGGTCGT-3'

PCR samples were then placed in the thermocycler (Applied Biosystems GeneAmp PCR system 2700) at the following specific conditions: 95°C for 2 minutes, 95°C for 20 seconds, 56°C for 20 seconds, 72°C for 30 seconds and finally 72°C for 2 minutes (in that order). The entire cycle was repeated 35 times. After PCR amplification, the samples were run in an 2% agarose gel. The gel was then visualized via ethidium bromide staining and an UV transilluminator.

2.7 Survival and Proliferation Assay

Confluent CEF were seeded into 60 mm plates at a dilution of 1:3. 60 mm plates were incubated in their respective conditions (41.5°C and in 5% medical grade CO_2). Cells were left in the incubator to starve (media was not replaced) and collected for a cell count at each

time point. Each sample was treated with 1 mL of trypsin and diluted in 9 mL of ISOTON® II Dilutent (Beckman Coulter #8546719). For statistical significance, each cell sample was counted in triplicates using a Beckman Coulter model Z2 Coulter Counter (Coutler Corporation, Miami, FL).

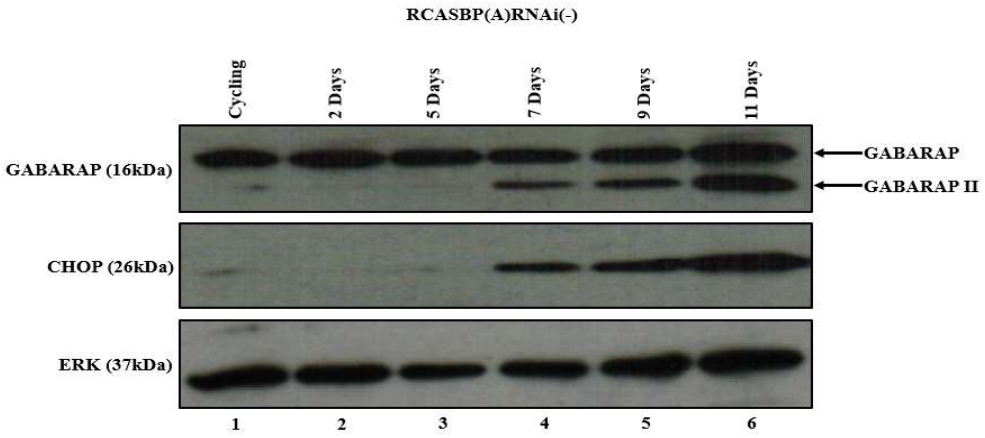
CHAPTER 3: RESULTS

3.1 GABARAP Inhibition by shRNA/RNA interference

Our first experiment was to validate the efficacy of our replication – competent retroviral vectors (see Detailed Material and Methods) for GABARAP down-regulation by short hairpin RNA interference (shRNAi). RCASBP-GABARAP-RNAi, a short hairpin retroviral vector, was used for GABARAP shRNAi downregulation. The parental vector, RCASBP(A)RNAi (-) was used as a control. Following transfection and retrovirus infection of the monolayer, both the control and GABARAP shRNAi downregulated cells were left to starve (left in the incubator, media not replaced) and protein lysates were prepared at specific time points until the cells started to show significant signs of cell death. The time points were as followed: cycling cells, 2 day post confluence cells, 5 day post confluence cells, 7 day post confluence cells, 9 day post confluence cells and 11 day post confluence cells. Through Western blot analysis and incubation of the appropriate GABARAP specific antibody, we analyzed GABARAP expression between both treatments over their starvation period (Figure 3). The RCASBP(A)RNAi(-) control showed a growing accumulation of GABARAP and the GABARAP-II isoform. This accumulation over time (particularly in the later days of starvation) indicates that

autophagy was initiated in response to stress. It should be noted that there is a modification that occurs in autophagy where GABARAP is cleaved and processed into its lipidated form GABARAP II in the later stages of autophagosomal development. GABARAP II has been shown to be associated with the membrane of the autophagosome (Kabeya 2004). A striking difference was observed with the GABARAP shRNAi samples. As expected, a decrease in both GABARAP and GABARAP II expression was seen throughout all the allotted time periods regardless of the prolonged starved conditions. ERK specific antibody was utilized as the loading control for each blot and Image J software was used to quantify the blots.

3A .



3B.

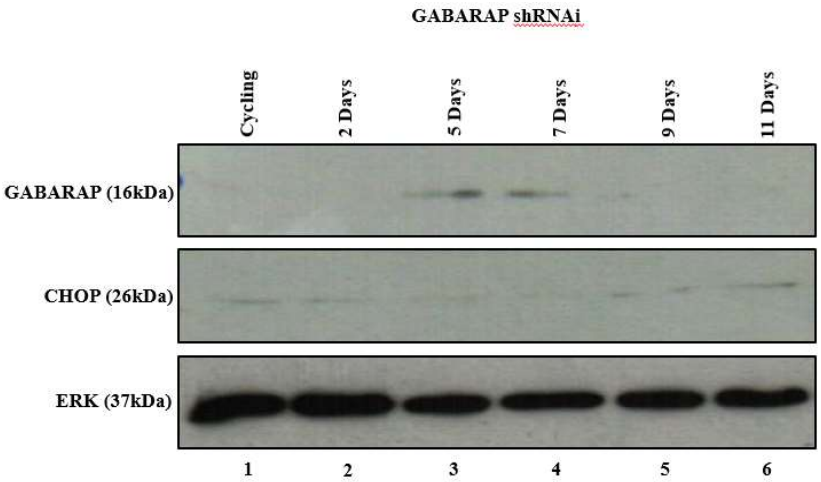


Figure 3: GABARAP Downregulation Results in Decreased CHOP Expression. Western Blot Analysis using GABARAP, CHOP and ERK antibodies. After transfection, RCASBP(A)RNAi control (3A) and GABARAP shorthairpin RNAi (3B) samples were prepared during the cycling growth phase and after a number of days post confluence as starvation ensued. GABARAP was cleaved and modified as GABARAP II in later days of starvation (days 7,9 and 11) during autophagosomal development indicating autophagy within the RCASBP(A)RNAi control. CHOP expression was also expressed in RCASBP(A)RNAi control (also days 7,9 and 11) indicating E.R. stress. Decreased expression of both GABARAP and CHOP was observed in the GABARAP shorthairpin RNAi samples after several days of starvation unlike its control. Quantification analysis of blots is shown in panels 3C and 3D.

3C.

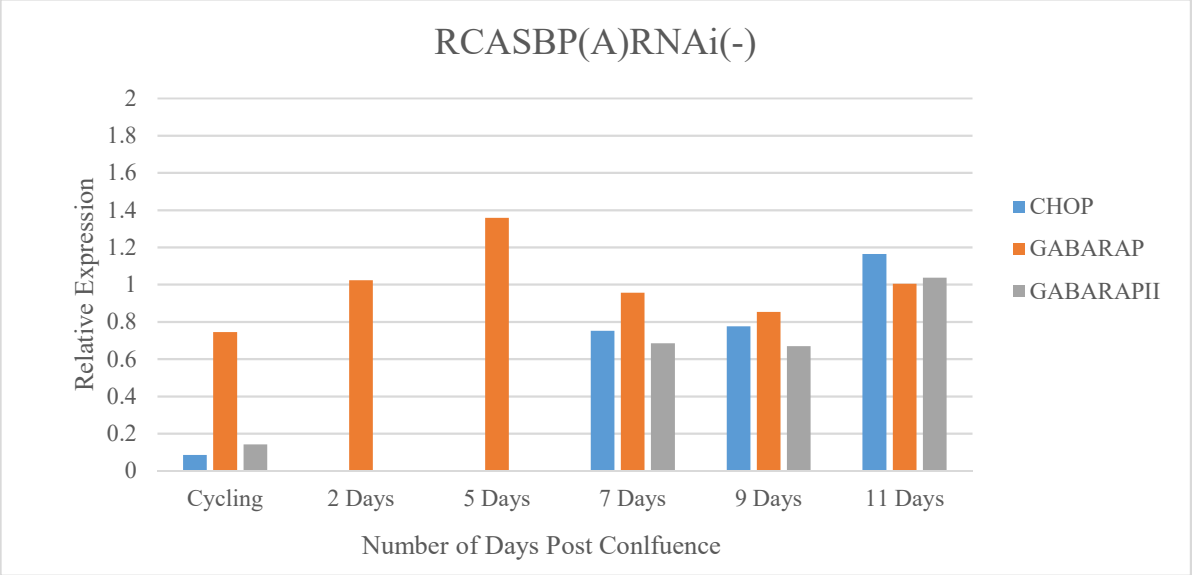


Figure 3C: Quantification of Western Blot Analysis by Image J of Results shown in Panel 3A.

3D.

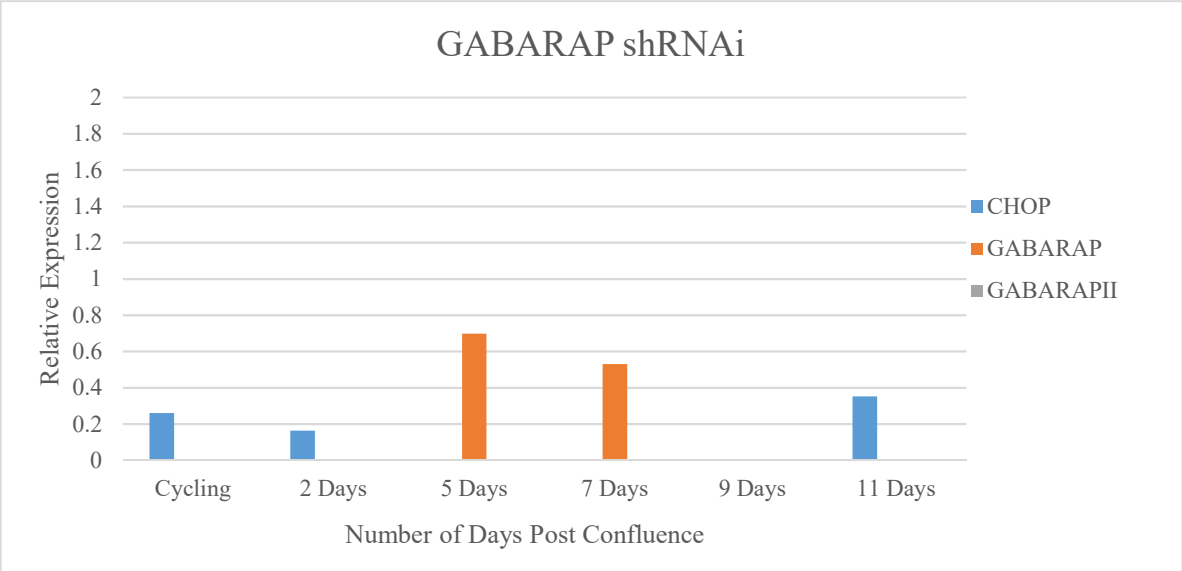


Figure 3D: Quantification of Western Blot Analysis by Image J of results shown in Panel 3B.

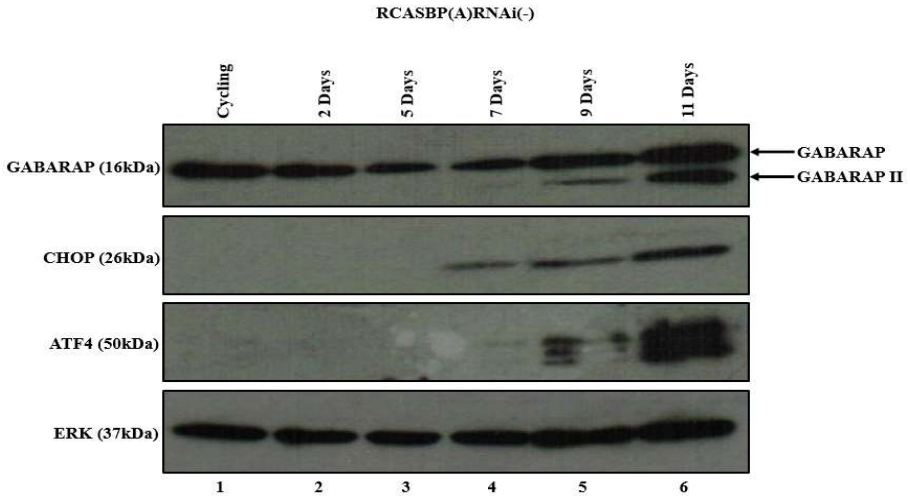
3.2 Decreased Chop Expression in Conditions of GABARAP Downregulation

As cells are starved, E.R. stress increases and UPR lethal pathways are activated. Consequently, there is a striking upregulation of the pro-apoptotic transcription factor CHOP in later days of prolonged starvation. Thus, CHOP can be used as a control to observe if there is E.R. stress occurring within the CEF population as starvation ensues. As mentioned before, when there is GABARAP downregulation there is also a decreased expression of CHOP (Maynard et al. 2015). This was confirmed with the GABARAP shRNAi samples and its corresponding RCASBP(A)RNAi (-) control samples. (Figure 3). Following retrovirus infection, both the RCASBP(A)RNAi (-) control and GABARAP shRNAi downregulated cells were left to starve and protein lysates were prepared at specific time points until the cells started to show signs of cell death. The time points were as followed: cycling cells, 2 day post confluence cells, 5 day post confluence cells, 7 day post confluence cells, 9 day post confluent cells and 11 day post confluence cells. Through Western blot analysis and incubation with the appropriate CHOP specific antibody, we confirmed the lack of CHOP expression in response to GABARAP downregulation in starved CEF. Upregulation of CHOP is seen at 7 day post confluence, 9 day post confluence and 11 day post confluence samples with the RCASBP(A)RNAi (-) vector control. However, there is a near complete lack of CHOP expression in all the time points in conditions of GABARAP shRNAi downregulation, consistent with a reduction of E.R. stress. ERK specific antibody was utilized as the loading control and Image J software was used to quantify the blots.

3.3 ATF4 Expression in Conditions of GABARAP Downregulation

As mentioned previously in the introduction, it has been shown that ATF4 is involved with regulating CHOP expression within the PERK pathway in later stages of prolonged starvation (Ron and Walter 2007). Thus, we examined if the downregulation of GABARAP also attenuates ATF4 expression providing us with a mechanism linking E.R. homeostasis, GABARAP, CHOP and ATF4. Following retrovirus infection, both the RCASBP(A)RNAi (-) control and GABARAP shRNAi downregulated cells were left to starve and protein lysates were prepared at different time points until the cells started to show physical signs of stress and cell death. Using ATF4 antibodies and Western blotting we observed increased expression of GABARAP II, CHOP and ATF4 proteins in the later days of starvation (7, 9 and 11 day post confluent cells) in RCASBP(A)RNAi (-) control samples (Figure 4). In contrast, there was decreased expression of GABARAP, GABARAP II, CHOP and ATF4 observed upon downregulation of GABARAP in the GABARAP shRNAi cells. ERK specific antibody was utilized as the loading control for each blot and Image J software was used to quantify the blots.

4A.



4B.

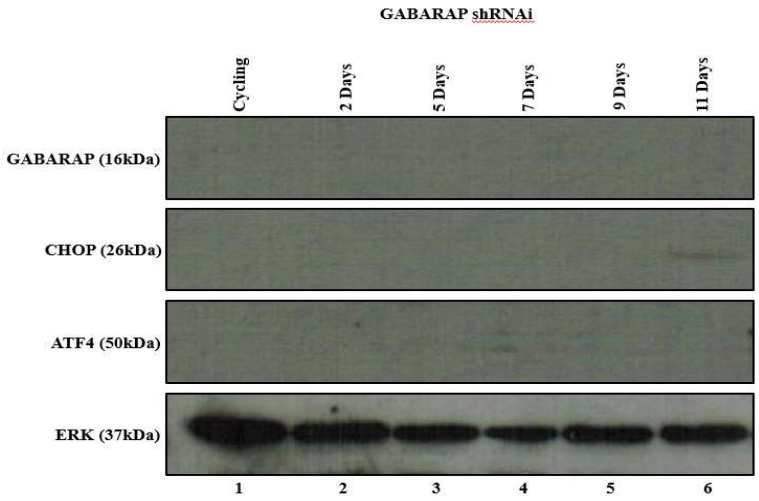


Figure 4: Downregulated GABARAP Causes a Decrease in ATF4 Expression. Western Blot Analysis using ATF4, GABARAP, CHOP and ERK antibodies. After transfection, RCASBP(A)RNAi control (4A) and GABARAP shorthairpin RNAi (4B) samples were prepared during the cycling growth phase and after a number of days post confluence as starvation ensued. In the RCASBP(A)RNAi control GABARAP II, CHOP and ATF4 are all similarly increasing in expression as starvation continues through days 7-9 due to amplified cell stress. When compared to the GABARAP shRNAi cells, there is little to no expression of all three proteins in conditions of prolonged starvation. Quantification analysis of blots is shown in panels 4C and 4D.

4C.

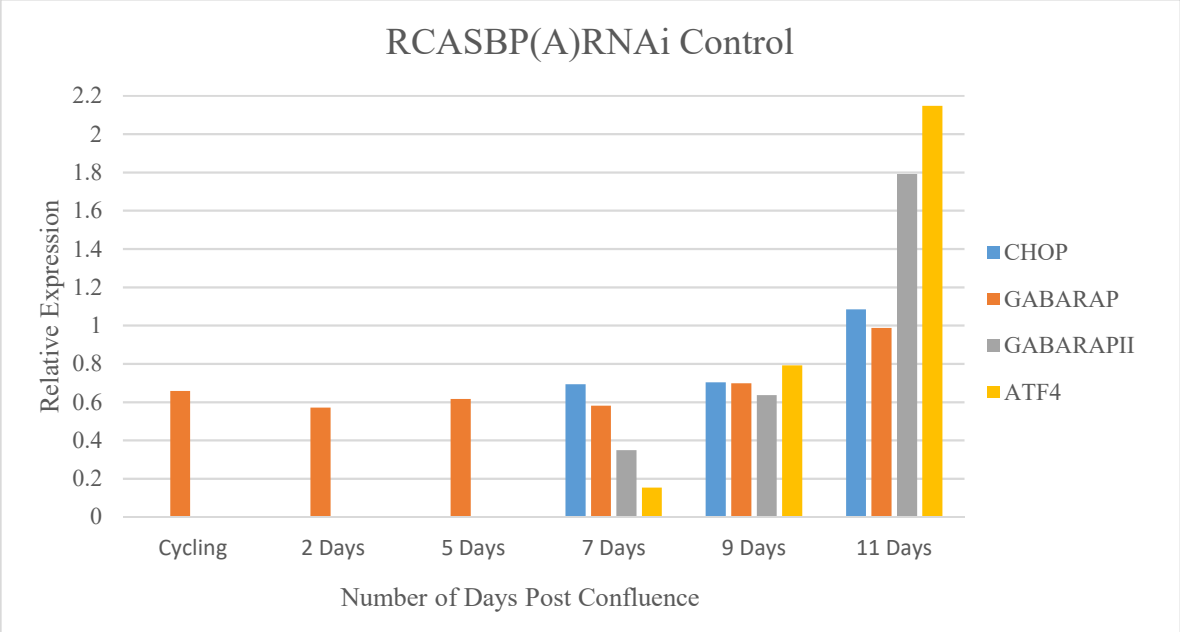


Figure 4C: Quantification of Western Blot Analysis by Image J of Results Shown in Panel 4A.

4D.

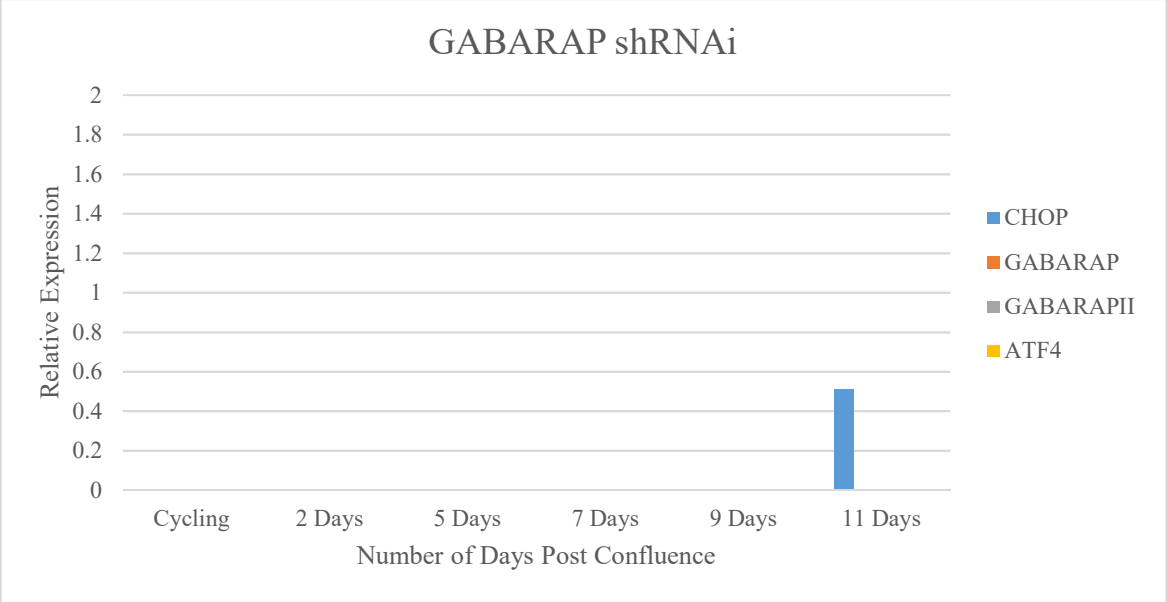


Figure 4D: Quantification of Western Blot Analysis by Image J of Results shown in Panel 4B.

3.4 CHOP Downregulation

To confirm the role of CHOP in the induction of apoptosis upon prolonged starvation, shRNA vectors for CHOP were transfected and analyzed in cycling and starved CEF. Our experiment was to validate the efficacy of our retroviral vectors for CHOP down-regulation by short hairpin RNA interference (shRNAi). RCASBP-CHOP-RNAi, a short hairpin retroviral vector, was used for CHOP downregulation. The parental vector, RCASBP(A)RNAi (-) was used as the control. Two different RCASBP-CHOP-RNAi Short hairpin vectors named CHOP shRNAi #1 and CHOP shRNAi #2 were transfected into CEF. To validate how each retrovirus worked, drug treatment of both sets of transfected cells was implemented once they were passed 3 times and reached confluence. E.R. stress was induced by treating cells with the drug tunicamycin (1ug/ml), a potent inducer of E.R. stress. Cells treated with the diluent DMSO was used as a control. Lysates were prepared 18 hours after treatment. Using the CHOP specific antibody, a Western blot Analysis was completed. When blots were compared and analyzed it was evident that there was decreased CHOP expression in the CHOP shRNAi downregulated tunicamycin treated cells when compared to its tunicamycin RCASBP(A)RNAi (-) control (with both CHOP shRNAi vectors) (Figure 5). The DMSO diluent control had a less amount of CHOP expression in both the CHOP shRNAi downregulated cells and its RCASBP(A)RNAi (-) control, indicating that the tunicamycin drug had worked. ERK specific antibody was utilized as the loading control and quantitation of blots was done via image J software.

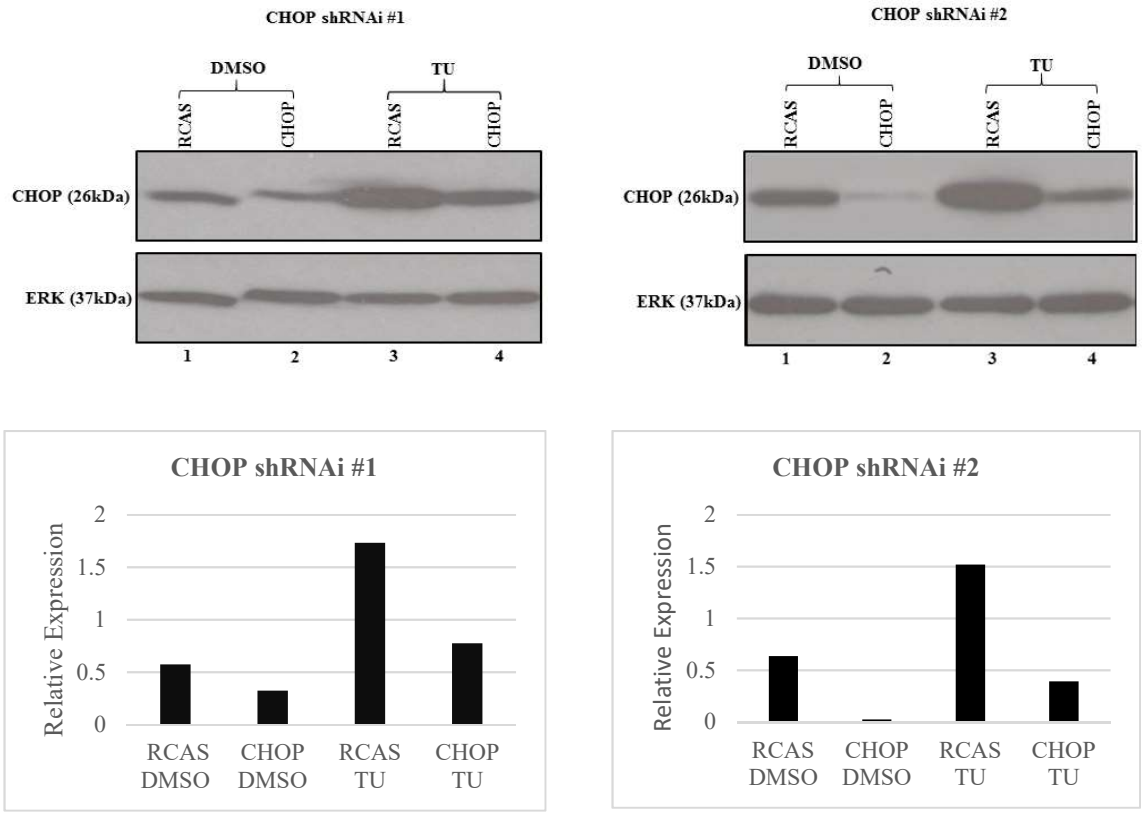


Figure 5: Western Blot Analysis of Proteins Expressed upon CHOP downregulation by shRNAs. After transfection, protein lysates were prepared after 4 passages and CHOP and ERK antibodies was used for Western Blot analysis. Lysates of each CHOP shorthairpin RNAi (CHOP) and their control RCASBP(A)RNAi (RCAS) expressing cells were prepared after treatment in various conditions (tunicamycin (TU) drug treated cells (a known E.R stress inducer) and their DMSO diluent controls). The cells were treated for a total of 18 hours. Shown in each blot, is decreased expression of CHOP protein in the CHOP shorthairpin RNAi samples as compared to their RCAS control samples after drug treatment. This validated the downregulation of both CHOP shorthairpin RNAi vectors. Quantification of each blot was produced using image J software and each graph is shown beneath their corresponding western blot.

3.5 CHOP Downregulation and Survival

Once downregulation was validated we performed survival proliferation assays with both CHOP short hairpin vectors (CHOP shRNAi #1 and #2) and the control cells RCASBP(A)RNAi(-) (Figure 6). Samples were left in an incubator to starve (media was not replaced) over a period of time and cells were counted on a Coulter counter and recorded every other day. More cells survived in response to prolonged starvation in CHOP shRNAi samples specifically from days 5-9. Within that time interval, the RCASBP(A)RNAi (-) control started to decrease in cell number while both the CHOP shRNAi vector cell numbers stabilized.

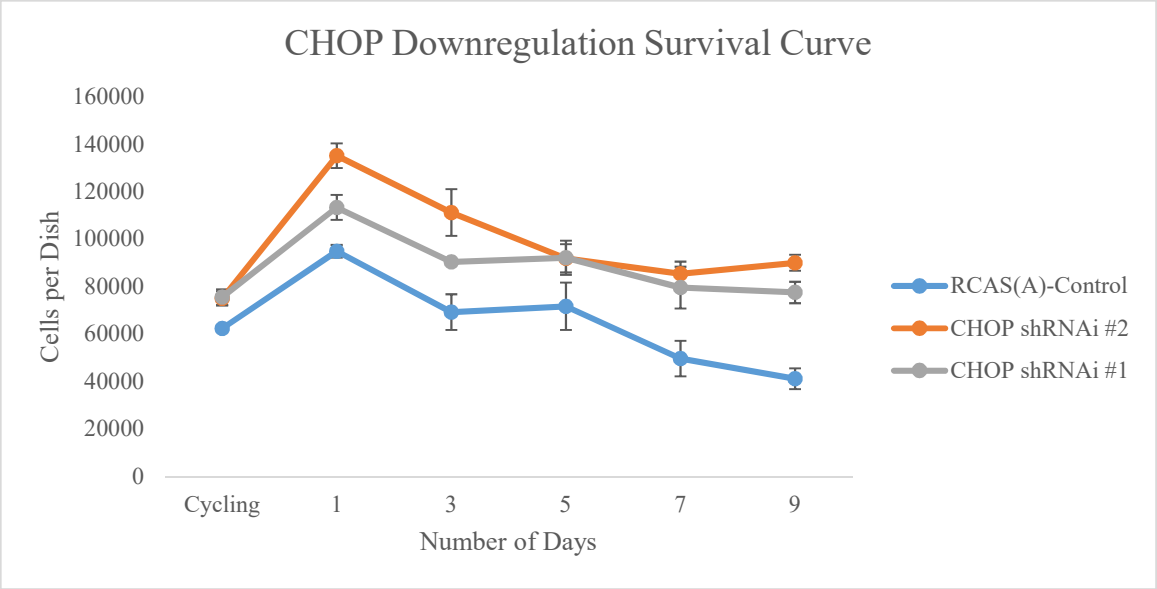


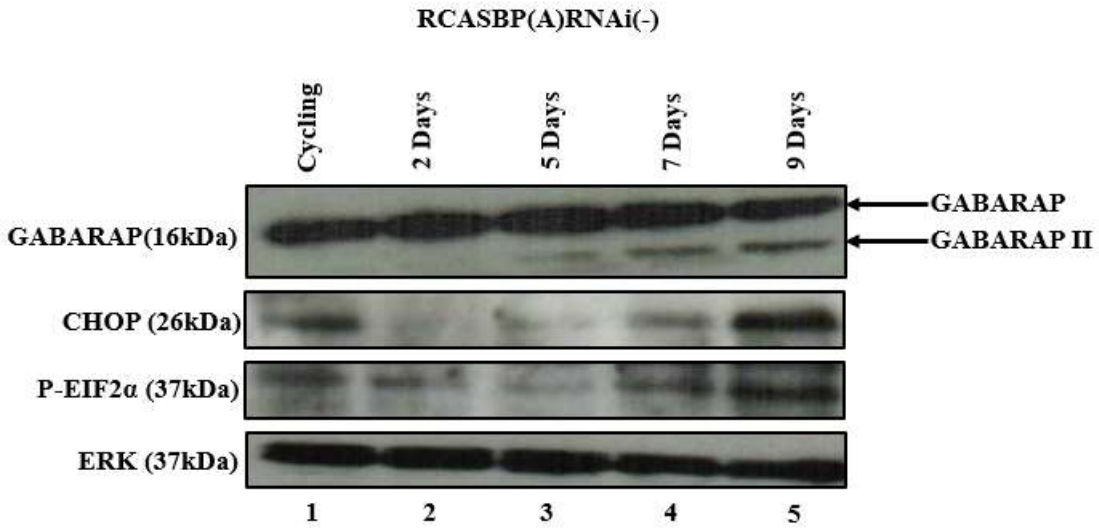
Figure 6: Survival Proliferation Assay of Cells Expressing CHOP shRNAi #1 and #2 and their associated RCAS control. Cells were left in an incubator to starve (media was not replaced) over a period of several days, counted on a Coulter counter and recorded every other day. Samples were collected in triplicates for statistical value. More cells survived in response to prolonged starvation in CHOP shRNAi samples specifically from days 5-9. RCAS(A) control started to decrease in cell number while both the CHOP shRNAi cell numbers stabilized.

3.6 p-EIF2α Levels in Conditions of GABARAP Downregulation

Although it has been demonstrated that GABARAP could be involved in UPR proapoptotic pathways by regulating the expression of ATF4 and CHOP, there is also the possibility that the downregulation of GABARAP could be helping to reduce overall E.R. stress in the UPR. This may be caused by a reduction in autophagy production allowing less stress to be placed on the E.R. to engage in the production of autophagosomes in response to starvation. Thus, it would be pertinent to investigate if GABARAP inhibition also affects other signaling pathways of the UPR including phosphorylation of EIF2α (P-EIF2α).

Once the retrovirus infection was complete, both the RCASBP(A)RNAi (-) control and GABARAP shRNAi downregulated cells were left to starve and protein lysates were prepared at specific time points until the cells started to show physical signs of stress and cell death. There was increased expression over time of both CHOP and GABARAP II proteins in control cells in the later days of starvation (5, 7 and 9 day post confluence cells) (Figure 7). Although detectable in cycling cells, levels of P-EIF2α increased over time as starvation ensued. When compared to the GABARAP shRNAi downregulated cells, there was little to no expression of GABARAP, GABARAP II and CHOP in conditions of prolonged starvation. Although P-EIF2α was observed at all time points, protein expression was reduced as starvation ensued. ERK specific antibody was utilized as the loading control for each blot and image J software was used to quantify the blots.

7A.



7B.

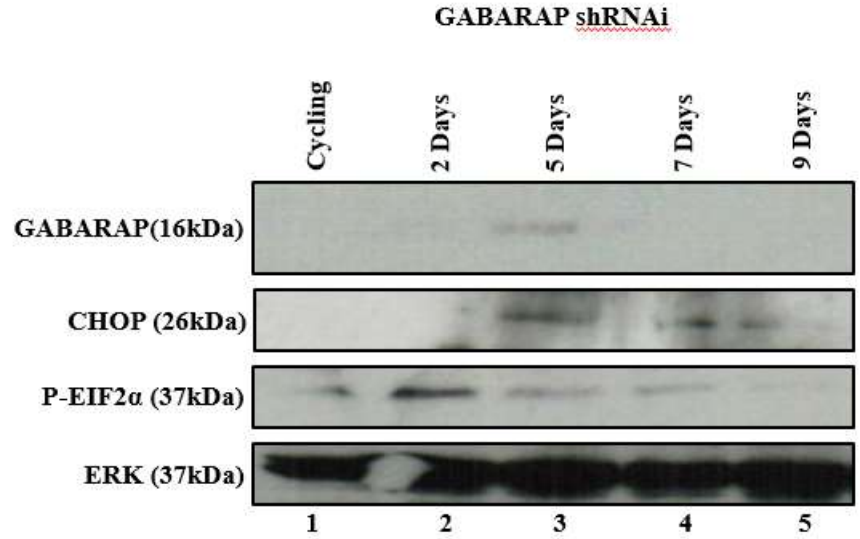


Figure 7: Downregulation of GABARAP Results in Decreased P-EIF2α Expression. Western Blot Analysis using P-EIF2α, GABARAP, CHOP and ERK antibodies. After transfection, RCASBP(A)RNAi control (7A) and GABARAP shorthairpin RNAi (7B) samples were prepared during the cycling growth phase and after a number of days post confluence as starvation ensued. In the RCASBP(A)RNAi control GABARAP II, CHOP and P-EIF2α are all similarly increasing in expression as starvation continues through days 5-9. When compared to the GABARAP shRNAi cells, there is little to no expression of GABARAP and CHOP protein and a decreased expression over time of P-EIF2α. Quantification analysis of blots is shown in panels 7C and 7D.

7C.

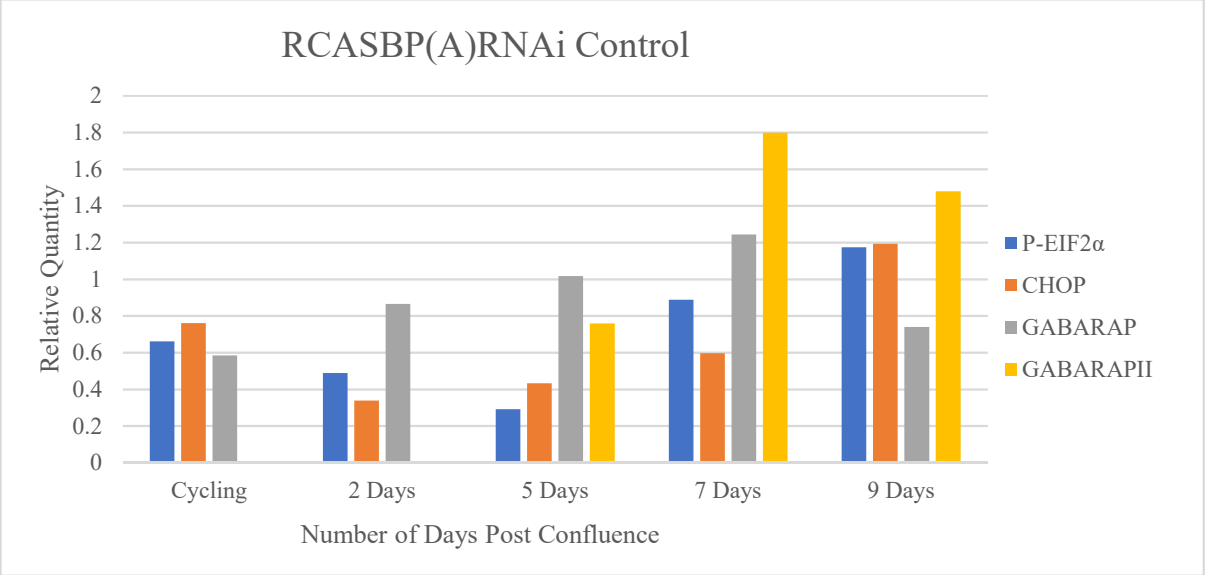
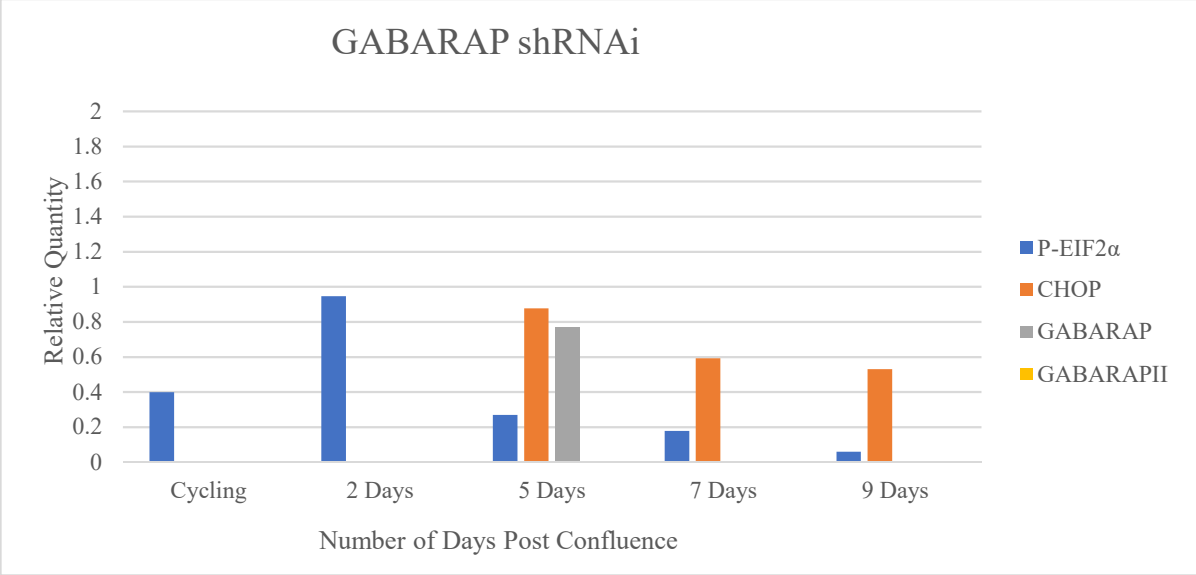


Figure 7C: Quantification of Western Blot Analysis by Image J of Results shown in Panel 7A.

7D.



Figur3 7D: Quantification of Western Blot Analysis by Image J of Results shown in Panel 7B.

CHAPTER 4: DISSCUSSION

The cell can initiate a variety of adaptive stress responses which promote cell survival in conditions of nutrient depletion. As cells starve, an accumulation of mis-folded proteins occurs due to the insufficient amount of amino acids, sugars and energy to complete the processes of protein maturation and their post-translational modifications. Although there are basic quality control mechanisms like E.R. chaperones (Araki and Nagata 2012) and ERAD (Smith, Ploegh, and Weissman 2011) to aid with preventing protein aggregation, sometimes conditions of starvation can become too severe and E.R. stress builds. If E.R. homeostasis cannot be maintained an emergency response is initiated. This is known as the Unfolded Protein Response (UPR).

The UPR has 3 main pathways that involve transcriptional regulation allowing upregulation of pro-survival proteins, some that increase folding capacity or translational repression which helps to decrease the overall protein load (Walter and Ron 2011). The 3 main branches of the UPR are named IRE1, PERK and ATF6. All pathways are activated when there is increased aggregation of mis-folded proteins in the E.R. lumen. IRE1 is a transmembrane that dimerizes and activates its cytoplasmic endonuclease domain splicing XBP1 precursor RNA. Spliced XBP1 is a transcription factor that translocates to the nucleus for UPR gene expression (Walter and Ron 2011). When the ATF6 receptor is activated, it is transported to the Golgi apparatus and is proteolyzed by both S1P and S2P proteases. Processed ATF6 is translocated to the nucleus for UPR gene expression (Haze et al. 1999; Yoshida et al. 1998). Finally, PERK is activated by transphosphorylation which proceeds to phosphorylate EIF2α (Gardner et al. 2013). Translational block by P-

EIF2α reduces protein load to the E.R. while allowing expression of specific mRNA in IRES such as ATF4 (Thakor and Holcik 2012). ATF4 transcription factor upregulates target UPR stress proteins and in prolonged stress activates proapoptotic factors like CHOP (Gardner et al. 2013).

Autophagy is another process that promotes cell survival by helping to eliminate mis-folded proteins that escape the E.R. Dysfunctional organelles and proteins are trapped into a double membrane vesicle called autophagosomes. These vesicles then fuse with lysosomes recycling the contents within by degradation (Levine and Klionsky 2004). There are many proteins that are involved with the process of autophagy when it comes to function and autophagosome development. In this thesis, we focused on one protein that is involved in the developmental stages in autophagosome biogenesis named GABARAP.

GABARAPs main role in autophagy is in the later stages of autophagosome development as there is impairment of autophagosome formation when the cell is GABARAP deficient (Weidberg, Shvets, et al. 2010). Our laboratory has shown that GABARAP is not only involved in autophagy processes but is also heavily involved in cell death through a pathway leading to induction of autophagy mediated death in chicken embryo fibroblasts (Maynard et al. 2015). The study focusses on two ATG8 family proteins, LC3 and GABARAP. In CEF, LC3 lipidation is a rapid response to starvation while GABARAP is expressed and lipidated only in conditions of *severe* nutrient depletion. The downregulation of LC3 by RNAi leads to apoptosis in all conditions as it is needed for cell survival. In contrast, cells with complete downregulation of GABARAP by RNAi interference survive for extended periods of starvation with reduced levels of

apoptosis (Maynard et al. 2015). Additionally, the expression of CHOP transcription factor (a pro-apoptotic factor and indicator of prolonged E.R. stress) is reduced in the absence of GABARAP, suggesting that extremely high levels of autophagy can compromise E.R. homeostasis and can thus lead to cell death (Maynard et al. 2015).

The reoccurring theme that is evident in these pathways (UPR, autophagy and GABARAP) is that although their inherent function at first may be pro-survival in times of stress, when conditions become too severe there can be a switch in their role as they all start to be involved with pro-apoptotic factors. Regarding GABARAP, it is well confirmed that this protein is involved with pro-survival in autophagy. But when there is severe nutrient depletion GABARAP expression is strongly stimulated suggesting that it is highly involved in conditions of severe E.R. stress (Maynard et al. 2015). Thus, when it is downregulated the cell will survive for an extended amount of time even when cells are severely starved and consequently, there is also downregulation of a well-known pro-apoptotic factor and E.R. stress indicator, CHOP (Maynard et al. 2015). With this information confirmed, what remains elusive is how GABARAP is involved with cell death in conditions of starvation, E.R stress and the UPR. Moreover, little is known about the state of activation with UPR pathways in conditions where GABARAP expression is altered. The primary goal of this thesis work was to characterize UPR pathways when GABARAP is downregulated in conditions of prolonged starvation. The results of these experiments would ultimately help to determine if the reduction of GABARAP attenuates *all* pathways of the UPR due to the decrease in overall E.R. stress and/or if GABARAP only acts specifically to block CHOP expression. Based on the experiments provided, we

have concluded that GABARAP may be involved in the decrease of overall E.R stress on a global level.

The rationale is GABARAP aids to regulate the level of autophagy of the cell and therefore E.R homeostasis. Increased levels of lipidated GABARAP will ultimately impair E.R. function and activate lethal UPR pathways (Maynard et al. 2015). When cells starve, there is an increase of autophagy that induces GABARAP expression (an increase in "autophagic flux"). Thus, it is hypothesized that a lower autophagic flux provides nutrients for the cell and the cell can survive. Nevertheless, when the flux starts to increase as a result of the accumulation of GABARAP, the demands on the E.R. (a source for autophagosomes) membrane becomes too severe impairing E.R. function. This results in high E.R. stress and eventually cellular apoptosis. Autophagy does help to decrease the level of misfolded proteins, but becomes deleterious when hyper-activated in response to prolonged starvation.

In these experiments GABARAP was downregulated by shRNA/RNA interference considerably enhancing CEF survival. As expected, the pro-apoptotic factor CHOP had decreased in expression in these conditions (Figure 3). After both confirmations, factors in each UPR pathway were investigated in conditions of GABARAP downregulation and prolonged starvation (ATF6, XBP1 and P-EIF2α). Unfortunately, experiments for both ATF6 and XBP1 were unsuccessful. For the ATF6 protein, the antibody did not appear to detect chicken ATF6 by Western blotting analysis. Multiple trials that involved increasing the concentration of both antibody and protein were performed and ATF6 still remained undetectable on several blots. For XBP1

mRNA, RNA was collected and cDNA synthesis was successful. This was confirmed by performing a PCR on cDNA samples with both HPRT and TBP control oligos. Unfortunately, when PCR was performed on XBP1 oligos we could not get noticeable bands for both unspliced and spliced XBP1 mRNA. Interestingly, ATF4 expression was attenuated in prolonged starvation when there was downregulation of GABARAP (Figure 4). Since ATF4 function has been documented to be involved with regulating CHOP expression in the later stages of prolonged stress, this reinforced the idea that GABARAP may be indirectly involved with pro-apoptotic regulation of CHOP (Ron and Walter 2007). There was also confirmation of CHOPs role of apoptosis in prolonged starvation and E.R. stress via survival proliferation assays. When CHOP was downregulated, cells did survive longer as starvation ensued (Figure 6).

However, there was additional evidence that showed the attenuation of UPR factor P-EIF2α in the same conditions indicating that downregulation of GABARAP by shRNA/RNAi reduces phosphorylation of EIF2α (Figure 7). These results show that a decrease in GABARAP expression may lead to an overall decrease of E.R. stress in the UPR due to the decrease in autophagosome demand on the E.R. Due to the unsuccessful results of both XBP1 and ATF6, the investigation of both these UPR factors in conditions of GABARAP downregulation and prolonged starvation remains unknown. Thus, there is still possibility for further investigation for both these UPR factors and their possible attenuation in these specific conditions.

GABARAPs involvement with cell fate is indeed striking. With discovery of its involvement with cancer as a tumor suppressor (Klebig et al. 2005), perhaps its

involvement in autophagy mediated death and its indirect regulation of E.R. stress could lead to a better understanding of its role in tumorigenesis in the future.

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