Phenotypic Analysis of *pash-1ts* Mutant to Understand the Roles of miRNAs in UPR^{ER}

by

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ABSTRACT

MiRNAs in ~22 nucleotide long, noncoding RNAs that are post transcriptional gene regulators. Recent studies using conditional temperature-sensitive (ts) mutant of the miRNA pathway, *pash-1(ts),* have established that post-developmental expression of miRNA is essential for normal lifespan and physiology. Due to the tight link between reduced lifespan and protein quality control, these observations suggest a potential involvement of miRNAs in the regulation of protein homeostasis (proteostasis).

During their lifetime, cells are faced with the variety of stresses which are the reason of protein misfolding and aggregation. Throughout evolution, organisms improve stress response mechanisms to deal with the disruption of protein homeostasis. One of the stress response mechanisms is endoplasmic reticulum unfolded protein response (UPR^{ER}). UPR^{ER} is crucial not just as a stress response mechanism but also as a regulator of amino acid metabolism, the energy homeostasis and also it is indispensable for the embryonic development.

The overall aim of my thesis is to investigate and establish the mechanisms by which miRNAs are involved in the regulation of UPR^{ER} upon activation of the stress response mechanisms. This has been examined by testing the effects of *pash-1(ts)* mutant on expression and regulation of stress genes involved in the cellular protein quality control and testing the ability of *pash-1(ts)* mutants to respond to ER stress challenges that induce the UPR^{ER}.

Our results demonstrated that compromised miRNA production leads to the activation of HSP-4, *C. elegans* homolog of BiP, in various tissues. Surprisingly, *pash-1ts* mutants were resistant to chronic applications of ER stressors in detrimental concentrations, which normally lead to the activation of pro-apoptotic branch of UPR^{ER}. So that our data suggest a model in which the absence of miRNA production may confer

resistance to the upregulation of apoptotic pathways upon ER stress although it needs to be further studied. We also showed that XBP-1s, a cell-non-autonomous regulator of UPR^{ER} leading to the extension of lifespan, exhibited no change in lifespan of the worms lacking miRNA production. These results suggest that *pash-1ts* animals can be used as a new tool to study miRNA functions in the non-autonomous cell signaling in the future.

ÖZET

Mikro RNA'lar 22 nükleotid uzunluğunda, kodlamayan transkripsiyon sonrası gen düzenleyicileridir. Güncel araştırmalar *pash-1ts* suşunun yüksek sıcaklıklarda idame ettirilmesiyle, miRNA biyosentezinin ilk aşamasının engellenmesinden dolayı, olgun miRNA üretemediğini göstermiştir. Yine aynı çalışmada miRNA'ların kurtçukların normal fizyolojik yapıları ve normal yaşama süreçleri için gerekli olduğu gösterilmiştir. miRNAların sentezlenmemesi durumundaki azalan yaşama ömrü ve fizyolojik malformasyonlar gözlemlendiğinde, miRNA'ların proteostatik mekanizmalardaki fonksiyonları gündeme gelmektedir.

Hücreler hayatları boyunca çeşitli streslerle karşı karşıya kalırlar ve bu streslerle baş etmek için bazı mekanizmalar geliştirmişlerdir. Bunlardan bir tanesi endoplazmik retikulumdaki katlanmamış protein tepkisidir (KPT^{ER}). KPT^{ER} sadece protein katlanmasında değil bağzı farklı fizyolojik durumlarda da önemlidir; örneğin amino asit metabolizması, enerji homeostasisi ve embryonik gelişim.

Tezimin amacı ER stresi altındaki miRNA'ların stres yanıtı mekanizmalarının aktivasyonu ve KPT^{ER}ın düzenlenmesindeki rollerini aydınlatmaktır. Bu mekanizma pash-1ts mutantındaki stres genlerinin farklı ER streslerine gösterdiği tepkiyle araştırılmıştır.

Sonuçlarımız göstermiştir ki, miRNA üretiminin durdurulması HSP-4, memelilerdeki BiP homoloğu, aktivasyonuna neden olmaktadır. Fakat ilginç bir şekilde pash-1ts mutantları kronik ER stresine karşı resistant fenotip göstermiştir. Normalde ölümcül ER stres konsantrasyonları apoptotik yolakların aktive olmasına neden olurken gözlemlediğimiz bu sonuç miRNA'ların olmayışının apoptotik yolak aktivasyonunda gecikmelere yol açabileceğini göstermiştir. Sonuçlarımızın doğruluğunun ispatı daha çok araştırma ile mümkündür. Ayrıca normalde yaşam ömrünü uzatan aktif XBP-1 proteininin *pash-1ts* suşunun yaşam ömrüne bir etkisi olmadığı gözlemlenmiştir.

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INTRODUCTION

1.1. ER stress and Unfolded Protein Response

The endoplasmic reticulum (ER) is involved in the myriad of metabolic processes because of its spatial arrangement in the cell. The ER is composed of extensive tubular structure which has a connection with nuclear envelope and this channeled structure extends toward to the cytoplasmic membrane. The place that ER covers in the cell is likely to depend on type of the cell. Especially for the secretory cells, the efficiency of ER is highly crucial. Besides secretory pathways, ER participates in lipid, steroid and glycogen biosynthesis, detoxification reactions and calcium homeostasis. Additionally, many post-translational modifications such as C-terminal glycosyl phospatidyl inositol (GPI) anchor, S-prenylation, disulfide bond formation and N-glycosylation take place in ER [1].

Because of the high protein production, protein folding mechanisms in the ER are strictly regulated. A nascent chain of protein entering into ER is subjected to various different covalent modifications until it reaches its final 3-D shape. Newly synthesized proteins can enter into ER either with the processes called co-translational translocation or post-translational translocation. In co-translational translocation, ribosomes synthesizing proteins are bound to the central core of translocon associated with a signal recognition particle (SRP) receptor which recognizes the signal sequence at the tip of the nascent chain. This recognition process is triggered by the binding of GTP molecules to the SRP and the SRP receptor. SRP molecules are dissociated from both the translocon and mRNA-ribosome complex upon hydrolysis of GTP molecules to GDP. Signal sequence is

associated with the translocon complex and entrance of the nascent protein into ER lumen is initiated. After growing protein chain enters into ER, signal sequence is exposed to proteolytic cleavage in order to prevent its interference with protein folding because of its hydrophobic nature [2]. On the other hand, some proteins are transported to the ER after they are completely synthesized in cytosol with a process called post-translational translocation.

Unlike cytoplasm (100g/L), the ER lumen has a concentrated amount of protein which is nearly 300-400g/L[3]. Because of this situation, growing nascent chains tend to be misfolded or aggregated. In order to cope with the high amount of protein load in the ER, there are ER resident molecular chaperones. Molecular chaperones are described as proteins that help nascent proteins in developing their native shape but not included in the final protein structure[4]. Additional to classical molecular chaperones, ER also has carbohydrate-binding chaperone system. Classical chaperone system can be found in almost all cellular compartments including mitochondria and cytoplasm and they are mainly the heat shock proteins interacting with the newly synthesized proteins to make them acquire their final shape. On the other hand, carbohydrate-binding chaperone system is only found in the ER and its mechanism depends on the hydrophilic nature of glycans which results in more soluble proteins to prevent aggregation [2].

1.1.1 Molecular Chaperones

In mammalian cells, there are different kinds of molecular chaperones either specific for an organelle or found in the cytoplasm. The working principle of chaperones based on the recognition of hydrophobic sides, which are normally embedded in the native proteins, however they are revealed in the aggregation-prone or misfolded proteins [2]. Molecular chaperones are composed of two domains: an amino-terminal nucleotide-binding domain (NBD), and a carboxyl-terminal substrate-binding domain (SBD). Substrates of a chaperone interact with the substrate binding domain. Binding of molecular

chaperones to misfolded proteins is assisted with the ATP hydrolysis. The affinity of chaperones for their substrates is determined according to state of ATP molecules. When nucleotide exchange factor (NEF) catalyzed the binding of ATP to the chaperones, substrate is released.

One of the organelles which has its protein quality control system is the ER. The ER has various different chaperones including Hsp70 and Hsp90 families of molecular chaperones. Hsp70 family of chaperones, most abundant ER chaperone, is named as Binding Immunoglobulin Proteins (BiP) in metazoans. In normal physiological conditions, BiP is associated with the luminal parts of the ER stress sensors. When there is ER stress, BiP is separated from the receptors and subsequently binds to the accumulating misfolded proteins which activates the downstream pathways of three arms of the UPR which will be mentioned in detail in the following sections[5]. There are two homolog proteins of BiP in *C. elegans,* which are HSP-3 and HSP-4. Due to highly complex mechanism of mammalian UPR, discovery of these conserved homolog proteins in *C. elegans* opened a new window to apply genetic analysis in a simpler way while still dissecting the physiological function of UPR in an animal as opposed to cell culture analysis. In the ER, there is also a specific type of protein quality control mechanism which is carbohydrate-binding chaperone system[2]. N-linked glycosylation is the main post-translational modification, involved in the proper protein folding. This modification begins with the en bloc transfer of an oligosaccharide from dolichol phosphate. Transferred sugar moiety is then attached to a consensus Asn-X-Ser/Thr residue in the nascent polypeptide chain [6]. The binding of an N-glycan to a protein can provide ER resident lectins (calnexin and calreticulin) with information about the folding state of the protein [7].

There are additional organelle specific chaperones, such as mitochondrial chaperones (HSP-6 and HSP-60). During oxidative respiration reactive oxygen species are formed due to the utilization of oxygen in the electron transport chain. ROS is harmful for

the cells so that mitochondria developed its own protein quality control system during evolution[8]. Upon perturbations in the protein folding in the mitochondria, HSP-6 and HSP-60 act in the matrix of the mitochondria to promote protein folding.

Although cell or tissue culture studies give an insight into UPR^{ER} mechanism, it is important to dissect the components of UPR^{ER} in multicellular organisms and determine how UPR^{ER} affect the survival the whole organism.

The main mechanism lying under the control of the UPR^{ER} by these mechanisms is to sense the protein status of ER. In mammals, BiP, which is homologous to HSP-70 in *C.elegans*, locates on the cytoplasmic part of UPR^{ER} sensor transmembrane and inhibits their activation. Misfolded protein accumulation in ER lumen leads BiP to separate from the UPR^{ER} sensor transmembranes, which in turn activates the sensors [9]. UPR^{ER} is also tightly associated with Endoplasmic Reticulum Associated Degradation (ERAD) which forms a connection between UPR^{ER} and proteasome in the cytosol. ERAD is activated when UPR^{ER} is overwhelmed. If ER stress cannot be alleviated then the cell undergoes apoptosis.

1.2. The Unfolded Protein Response Sensors

There are several feedback loops that sense the environmental conditions and adjust the protein production. When the protein folding capacity is exceeded in the ER, misfolded proteins start to aggregate and adaptive stress response mechanisms are triggered against the ER stress. The first study examining the stress response mechanisms in ER has revealed that there is a transcriptional upregulation of ER chaperones[10]. Recent studies have investigated that an increase in the number of ER chaperones is an outcome of unfolded protein response (UPR). UPR in the endoplasmic reticulum (UPR^{ER}) is an adaptive mechanism to cope with the excessive protein load in the ER lumen. UPR^{ER} ensures an information flow to the nucleus and to cytosol about the protein folding capacity of ER in

order to prevent further protein synthesis [1]. According to the level of misfolded protein load in the ER lumen, the cell can further activate either pro-apoptotic or pro-survival signaling pathways.

In mammals, UPR^{ER} is mediated by three major ER-resident transmembrane proteins; PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6, α and β isoforms), and inositol requiring enzyme 1 (IRE1, α and β isoforms).

1.2.1 PKR-like endoplasmic reticulum kinase (PERK)

PERK is a type I transmembrane protein, whose ER-luminal domain is regulated by the ER stress resulting in oligomerization of PERK through its luminal domain. ın normal physiological conditions, PERK's luminal domain interacts with Binding Immunoglobulin Protein (BiP). BiP is also called as HSP70, which is a molecular chaperone found in the ER lumen, whose function is the folding of newly synthesized proteins. Upon protein aggregation in ER lumen, BiP binds to misfolded proteins and dissociates from the luminal domain. BiP removal causes to PERK activation, initiated upon its dimerization and subsequent trans-autophosphorylation. There are two downstream targets of PERK: eukaryotic translation-initiation factor 2α (eIF2 α) and bZIP Cap'n' Collar transcription factor Nrf2 [1]. eIF2α phosphorylation leads to very quick reduction in the rate of protein translation that provides a pro-survival feedback loop [11]. Paradoxically, there is an mRNA increasing in amount upon eIF2α phosphorylation, namely activating transcription factor 4 (ATF4). ATF4 is a transcription factor has functions in different cellular processes, involved in amino acid metabolism, control of cellular redox and apoptosis [1]. Nrf2, on the other hand, is located in the cytoplasm in its inactive state, forming a complex with a cytoskeletal anchor Keap1. When cells are under the ER stress, Nrf2 gets activated by PERK phosphorylation and leading to its separation from Keap1. Subsequently, Nrf2 is localized into nucleus and upregulates the genes of the antioxidant response element (ARE) [1].

1.2.2 Activating Transcription Factor 6 (ATF6)

ATF6 is a transcription factor located in the ER transmembrane. It has two isoforms which are ATF6α and ATF6β. Different than canonical transcription factors, ATF6 is produced as an ER transmembrane precursor. In normal physiological conditions, luminal domain of ATF6 is bound to BiP and it is inactive. However, when misfolded proteins are accumulated in the ER lumen, BiP dissociates from ATF6 leading to be transported to the Golgi apparatus. After translocation to Golgi, its membrane anchor is cleaved by S1P/S2P (site-1 protease/site-2 protease) proteases[12]. Following the cleavage, ATF6 is located to nucleus where it activates the transcription of ER chaperones, ERAD components and XBP1 [13] ATF6 may also be involved in other UPR^{ER} mechanisms through the reduction of disulfide bonds in its luminal domain. [14]

1.2.3. Inositol Requiring Enzyme 1 (IRE1)

Like ATF6, IRE1 also has two isoforms, IRE1α and IRE1β. IRE1α is expressed in almost all tissues, while expression of IRE1β is limited to intestine. IRE1 is the most evolutionary conserved protein among aforementioned ER stress receptors. Different than other ER stress receptors, there are two functions assigned to IRE1's cytoplasmic part, a kinase and a C-terminal RNase domain. When there is an ER stress, IRE1 is oligomerized leading to its trans-autophosphorylated to amplify its activation (**see Figure 1**). The RNase domain in turn cleaves the mRNA encoding the transcription factor X-box binding protein-1 (XBP1) [15]. The resulting molecule of this unconventional splicing phenomenon is X-box binding proteim-1 spliced (XBP-1s), which is a more stable and active transcription factor involved in the regulation of protein folding and ERAD mechanisms [16].

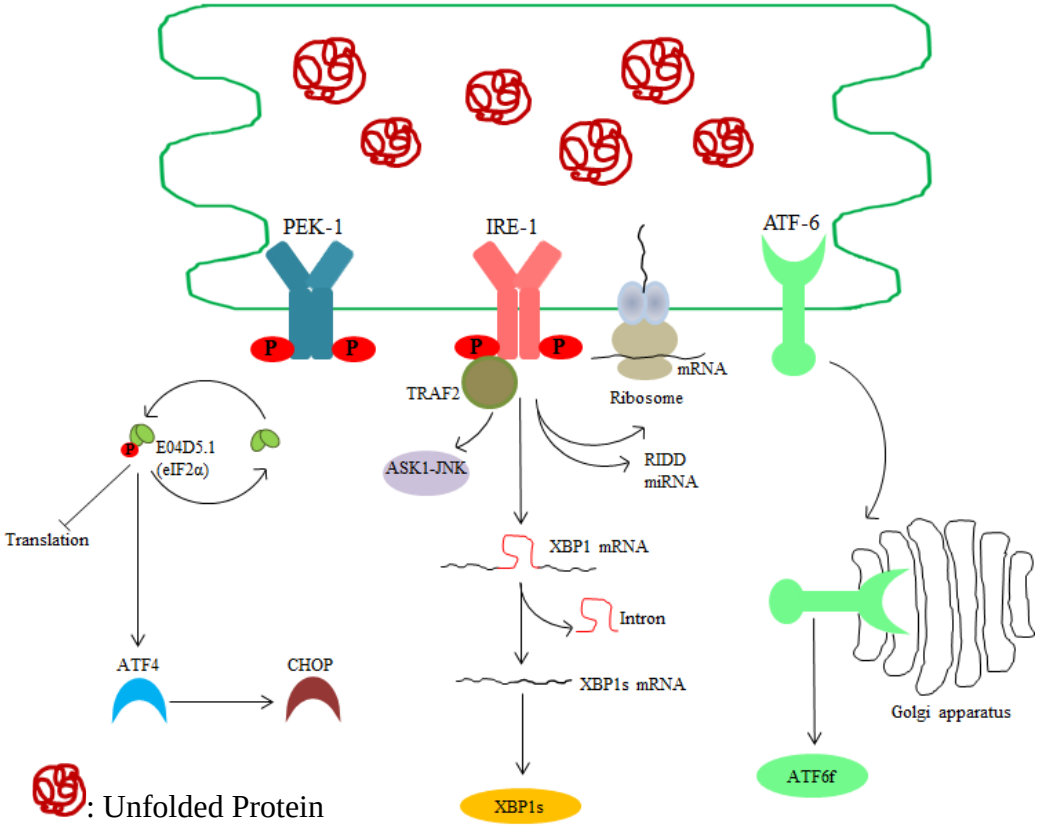


Figure 1: ER stress and unfolded protein response

1.2.4. ER-associated degradation pathway

These three aforementioned unfolded protein response sensors so far, act to cope with the unfolded proteins. However, the proteins does not gain their native shapes by ER-resident chaperones and they have to be eliminated for a cell to survive. Such irrecoverable proteins are processed through ER-associated degradation pathway (ERAD) or eventually engulfed by lysosomes to be degraded by autophagy. The proteins that are targeted for ERAD are exported from ER lumen to the cytosol via an ER-bound protein complex and they act as targets of the UPS. UPS ubiquitinates its targets with an ATP-dependent manner. Ubiquitnylation involves the catalytic activation of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin protein ligase) [17]. The-

ER bound protein complex is involved in the ERAD pathways and it is composed of two proteins called SEL-1 and SEL-11 in *C. elegans* (**see Figure 4**)[18]. SEL-1 is a glycoprotein acting as a cofactor of the SEL-11 that regulates the replacement of proteins from ER lumen to the cytosol via its ubiquitin ligase activity. Recently, SEL-1 protein levels are shown to be alleviated 2-fold upon reinforcement in the hexosamine biosynthetic pathway which results in an increase in the lifespan of *C. elegans.*

1.6. N-linked protein glycosylation in the ER

There is a highly complex mechanism in a cell regulated by thousands of proteins which is far beyond the gene number in the cell[19]. These complex mechanisms are provided particularly by carbohydrates. Carbohydrates are involved in the complex processes including development and physiology of living organisms by modifying proteins post-translationally[20].

Carbohydrates are especially important for the multicellular organisms which are composed of cells needed to be interacted both with each other and with the extracellular matrix for a correct assembly of the cells. Almost all cell surfaces contain an attached sugar (monosaccharide) or a sugar chain (oligosaccharide) both of which are called as glycans. Recent bioinformatics analysis showed that more than 50% of all proteins in a eukaryotic cell are glycoproteins, 90% of which bear an N-linked glycan[21]. Nearly all glycoproteins, the carbohydrate structures are adhered to the protein either by N- or O-glycosylation reactions[21]. N-glycosidic bond always occurs at the amide of an asparagine, which is found in the consensus sequence Asn-X-Ser/Thr where X can be any amino acid except proline. On the other hand, O-glycans are attached to the oxgen residue of serine or threonine amino acids without any determined consensus. This significant amount of glycan proportion of the cell shows how important they are in the cellular signaling. Specifically in the signaling pathways, N-glycan tags on the proteins are recognized by a range of different carbohydrate-binding proteins (lectins).

The first step of the N-glycosylation modification of the newly synthesized proteins by a multimeric enzyme oligosaccharyltransferase (OST) [22]. OST is an integral membrane protein that provides the transmission of pre-assembled glycans made up of a glucose$_3$-mannose$_9$-*N*-acetyl-glucosamine$_2$ ($Glc_3Man_9GlcNAc_2$) structure to the amine group of asparagine (**see Figure 2**).

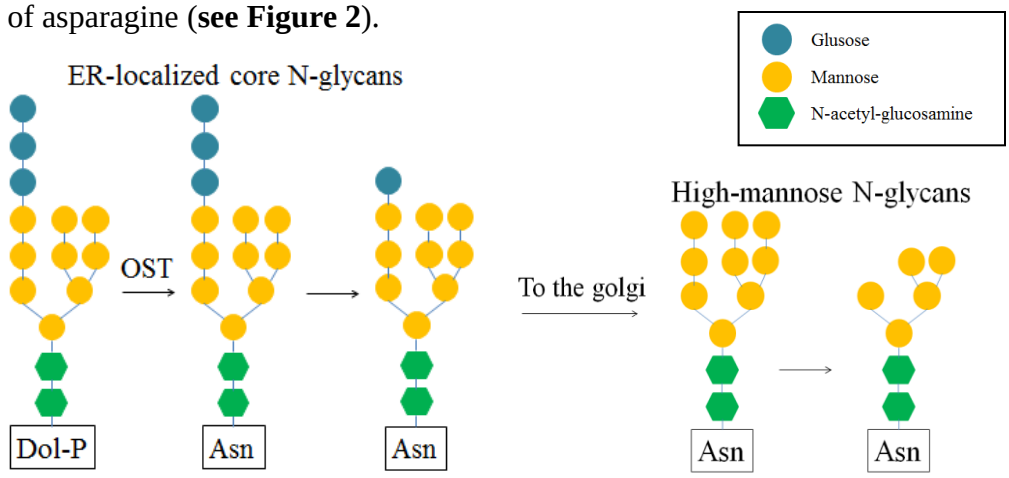


Figure 2: N-glycosylation synthesis adapted from[23]

1.3. Biological functions of miRNAs

MicroRNAs are 21-24 nucleotide long small noncoding RNAs base pairing with their target messenger RNAs to downregulate the gene expression. MiRNAs can act as endogenous regulator of their target mRNAs either via direct degradation or repression. According to recent bioinformatics analysis, 30-75% of human protein-encoding genes have an interaction with at least one miRNA[24]. Because of their high abundance in molecular mechanisms, miRNAs are involved in various fundamental processes both at cellular and organismal level. These processes include developmental timing, cell proliferation [25], differentiation and apoptosis [26]. Since miRNAs have widespread functions in different cellular processes, aberrant miRNA biogenesis leads to variety of diseases, such as cancer [27], obesity, diabetes and neurodegenerative disorders.

1.3.1 Biogenesis of miRNAs

MiRNAs genes can reside in the introns of a protein coding genes or in the intergenic regions. Canonical miRNA biogenesis pathway starts with the transcription of primary miRNA (pri-miRNA) by RNA polymerase II. In order to produce the second precursor, precursor miRNA (pre-miRNA), pri-miRNA is cleaved by the microprocessor complex containing Drosha and DGCR8. The resulting pre-miRNA is transported into cytosol through Exportin 5-Ran GTP(XPO5). Another endoribonuclease Dicer cuts the hairpin loop of pre-miRNA generating a double-stranded RNA. The guide miRNA is loaded into Argonaute proteins (AGO), forming RNA induced silencing complex (RISC) complex. Then this mature miRNA is delivered to 3'UTR of its target mRNA either to carry out translational repression or degradation (**see Figure 3**).

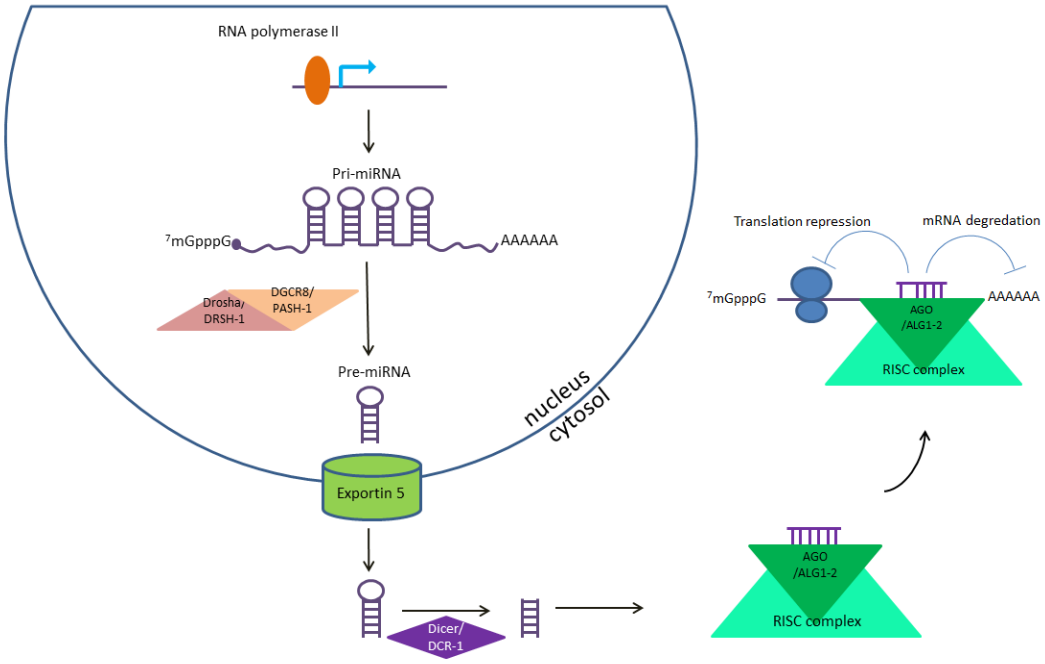


Figure 3: Canonical miRNA biogenesis pathway

1.5. Contribution of miRNAs in UPR^{ER} regulatory pathways

Recent findings in the miRNA field have highlighted the contribution of miRNAs in ER^{UPR}. One of the evidence is that AGO was observed in stress granules when the cells are exposed to ER stress. Almost all AGO proteins are equally distributed through the cytoplasm unless otherwise the cells are subjected to ER stress and they are localized to the stress granules[28],[29]. Stress granules are active macrostructures gathering the translationally inactive mRNA/protein complexes and they are particularly assembled when the cells are experiencing stressful conditions[30]. Upon ER stress, PERK is auto-phosphorylated then subsequently results phosphorylation of translation initiation factor eIF2α and translation is ceased. MRNAs cannot be released from polysomes and stress granules are started to nucleate[31]. The other evidence is revealed by using different cell lines, in which ER stress was shown to change miRNA expression profiles in comparison to non-stressed cells. Behrman et al. used mouse embryonic fibroblasts to treat with two different ER stress inducers thapsigargin and tunicamycin. When wild type cells are compared with the ER-stressed cells, 11 miRNAs are found differentially expressed[32]. In another study, human airway epithelial cells were used to perform miRNA microarray profiling again upon tunicamycin treatment. In this study expression of 47 miRNAs are enhanced upon ER stress[33]. Additionally, Belmont et al. conducted miRNA array of RNA extract from the hearts of ATF6 transgenic mice and found 13 ATF6-regulated miRNAs[34]. All in all, evidences demonstrate that one of the mechanisms of the miRNA expression is dependent on the ER stress.

Although UPR^{ER} is simply taken as a stress response mechanism; it has many important functions in the metabolism of lipid and cholesterol and energy homeostasis [35]. As it can be implied from its wide-ranging functions, the molecular mechanism regulating UPR^{ER} is highly complex. Such that there are puzzling feedback loops between stress response and metabolic pathways. Under the light of this information, highlighting the

functions of miRNAs in the UPR^{ER} mechanisms is very important. There are many individual miRNAs contributing to the cell fate decision under ER stress (**see Figure 4**); however, the effect of global absence of miRNAs to a whole organism under the ER stress has not been studied yet. In multicellular organisms, stress response mechanisms are regulated cell-nonautonomously which indicate a possible crosstalk between different tissues [36]. However, the regulatory molecules of this crosstalk are still unknown. It is in consideration that miRNAs may contribute to ER homeostasis either carried somehow between tissues or by affecting the molecules that make connections between different tissues.

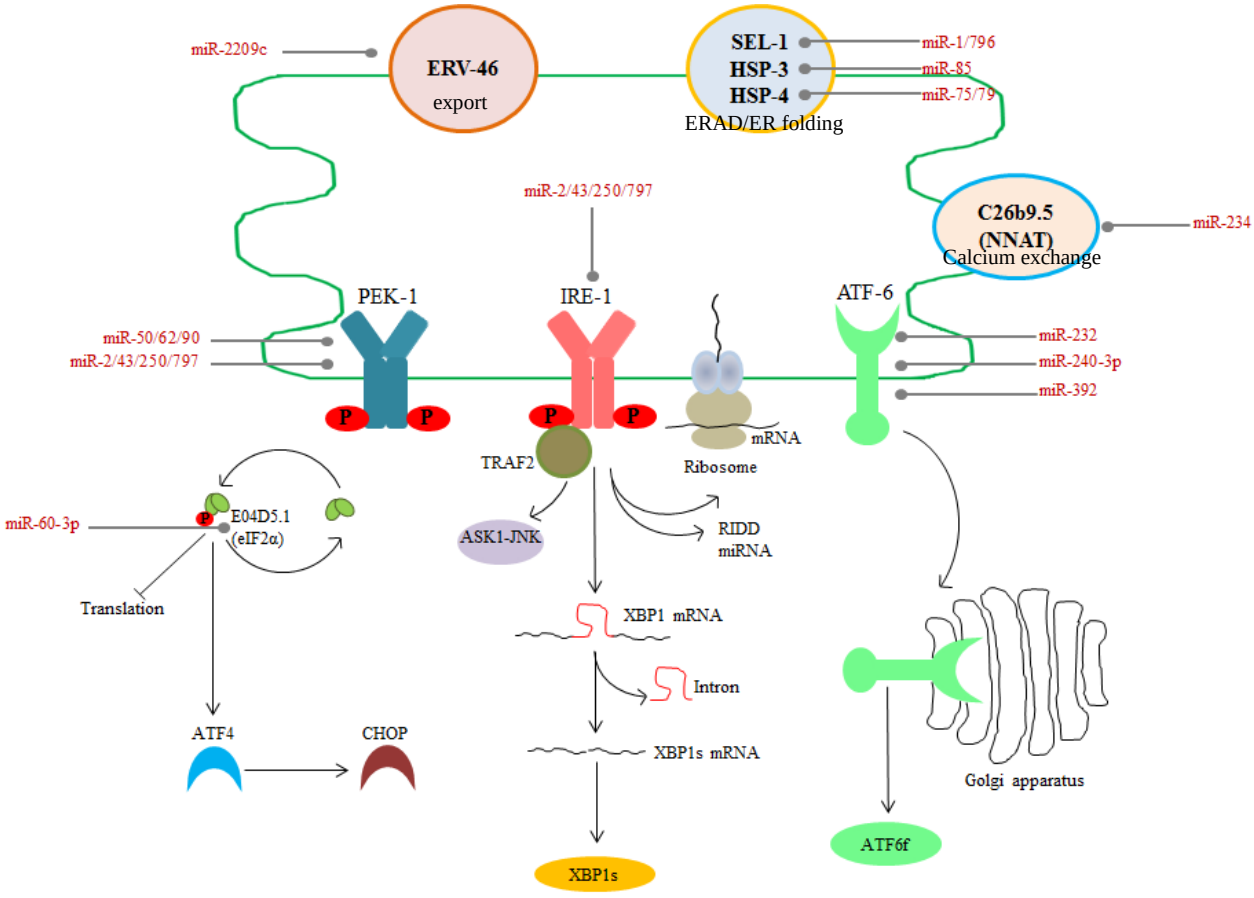


Figure 4: Conserved miRNAs involved in the regulation of UPR^{ER} in *C. elegans*

1.4. *Caenorhabditis elegans* (*C. elegans*) is a powerful organism to study miRNA functions

1.4.1 C.elegans as a model organism

Caenorhabditis elegans (C. elegans) is a model organism that belongs to phylum of Nematoda. They are the roundworms having the smooth-skinned cylindrical body shape. *C. elegans* can reach 1 mm in length at most and it feeds on bacteria. It can be easily isolated from rotten fruit and rotten root [37]. *C. elegans* was first used by Sydney Brenner in 1965 and then scientists started to use it in almost all fields of modern biology. Now it is a well-established organism used in different aspects of cellular biology and ageing. Owing to its convenient and costless cultivation, short life span and considerably high fertilization rate, *C. elegans* is a popular model organism especially in the ageing field. It has approximately 15-20 days post reproduction so that life span experiments take relatively short amount of time comparing other model organisms. *C. elegans* is maintained on the agar plates in which hermaphrodites (XX) compromised the greater part of the population while only 0.1% is males (X0) being a result of rare non-dysjunction in the hermaphrodites germ line. Males and hermaphrodites are anatomically different (**see Figure 5**) [28].

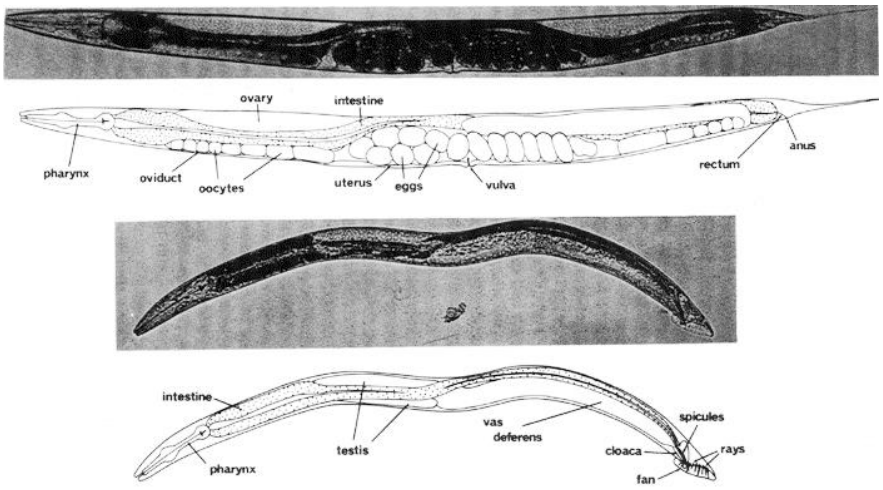


Figure 5: Anatomical differences between male and hermaphrodite

In the first five days of adulthood, each hermaphrodite lays 200-300 embryos. After embryos hatch, they follow through the 4 larval stages L1-L4 before becoming adults in 72 hours at 20°C[38] (**see Figure 6**). The lifecycle of *C. elegans* is sensitive to environmental conditions such as temperature, food accessibility and crowdedness. For example, when they are growing at 25°C, worms have shorter life-span and fecundity is reduced [38]. If worms experience unfavorable conditions, they may enter into two of the arrested stages; one of them is L1 arrest and the other one occurs during the third larval stage which is called as the dauer stage.

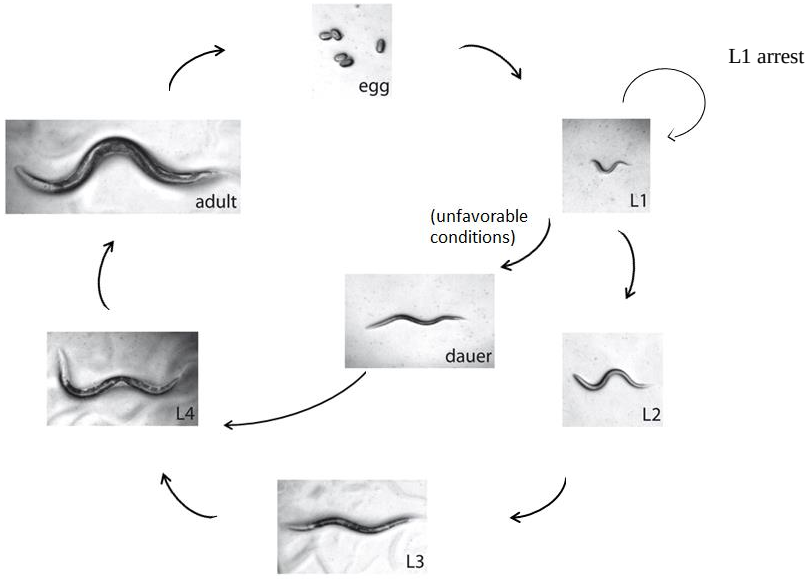


Figure 6: Life cycle of *C. elegans* adapted from [39]

1.4.2 C. elegans miRNAs

MicroRNAs are involved in the regulation of almost all molecular mechanisms in the cell including development, metabolism, cell fate, and cell death [40]. They are the second largest gene family after transcription factors [41]. Discovery of miRNAs has led to different approaches in the gene regulation. The first miRNA, *lin-4*, was discovered by Victor Ambros in *C. elegans.* [42]. Unexpectedly, there was no protein expressed from the *lin-4* gene but instead there was a small RNA regulating its target mRNA by imperfect base-pairing resulting in the inhibition of its targeted mRNA's gene expression. *lin-4* is a heterochronic gene upregulated at the end of L1 stage resulting in the translation inhibition of its target mRNA *lin-14*. When *lin-4* is knocked out or the gain of function mutation of *lin-14* is used, worms cannot pass through the second larval stage L2 [42]. Besides their functions during the larval development, *lin-4* and *lin-14* have also important regulatory functions in the adult. A loss of function (lf) mutation of *lin-4* causes a severe phenotype

shortening the lifespan while over expression of *lin-4* increases the lifespan through the insulin/insulin-like growth factor-1 (IGF-1) pathway [43].

6.A.2 pash-1ts as a genetic tool to study the post- developmental miRNA functions in C. elegans

After the discovery of miRNAs in *C. elegans,* the studies focused on the functions of miRNAs in the development of organisms, as the first miRNA *lin-4,* discovered as a heterochronic gene regulating the timing of the development of *C. elegans.* Additionally, scientists have found that there are also miRNAs as regulators of lifespan. For example, *mir-71* deletionshortens lifespan [44], whereas *mir-239* mutants live longer than the wild type worms. Although, *mir-71* and *mir-239* have been implicated in lifespan studies, their activity in a developing or in an adult animal is not known. There are 434 mature miRNAs in *C. elegans* listed in miRBase and knockouts of 92 of them were obtained, phenotypically characterized. ~15 of the knockouts lead to abnormal phenotypes, many of them looked similar to wild type animals. many miRNAs regulate genes redundantly, knock-out studies with such miRNAs would not may not lead to visible defects. Therefore, to circumvent this, a global downregulation of miRNAs can be achieved by knocking out important miRNA biogenesis factors, such as DICER or DGCR8, Such knockouts have been obtained in various organisms, which resulted in embryonic lethality or severe developmental defects, hampering further studies.

Recently, in a genetic screen a conditional allele of DGCR9/pash-1 was discovered which provides reversible and quick deactivation of miRNA biogenesis (**see Figure 7**). In this project, we used this mutant to examine the post-developmental functions of miRNAs in the ER^{UPR}.

Permissive temperature Restrictive temperature

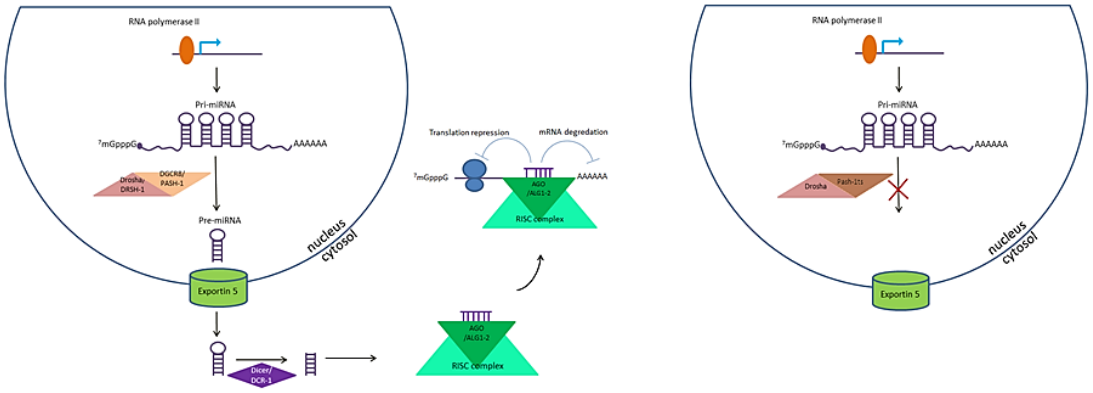


Figure 7: miRNA biogenesis is ceased in *pash-1ts* at the restrictive temperature

Chapter 2

MATERIALS AND METHODS

2.1 *C. elegans* strains and culture

Worms were maintained on NGM agar plates spotted with *E. coli HB101* bacterial lawn at 15°C unless otherwise noted. Standard Nematode Growth Media (NGM) agar containing agar, NaCl and bacto peptone was prepared and autoclaved. After its cool down, $MgSO_4$, $CaCl2_2$ and cholesterol were added due to their heat labile feature (**see Table 1**). Two different sizes of glass plates were used according to the aim of usage. Maintenance is done in large plates (90 mm) and experiments were carried out in small plates (60 mm). Poured plates were dried at least 4 days at room temperature, before potted with bacteria. For long term storage, plates were kept at 4°C. Nematodes were transferred from one plate to another with a thin platinum wire.

Table 1: Ingredients of standard NGM agar

Ingredients	**Quantity in 500 ml**
NaCl (MERCK 1.06400)	50 mM
Agar (CONDA 1802)	1.7%
Bactopepton (CONDA 1616)	0.25%
Double distilled H_2O	486 ml
After autoclave	
5mg/ml Cholesterol (SIGMA C-8503)	0.5 ml
1M KH_2PO_4 (MERCK 1.04873)	12.5 ml
1M $CaCl_2.2H_2O$ (SIGMA 12022)	0.5 ml
1M $MgSO_4.7H_2O$ (194833)	0.5 ml

2.2 *C. elegans* feeding

Worms were fed with HB101 strain of *E. coli.* Bacteria from its glycerol stock were aseptically streaked on LB agar (**see Table 2**) containing streptomycin as a selective antibiotic for HB101. One day after, a single colony was inoculated in B-broth (**see Table 3**) to grow overnight at 37°C. Prepared bacterial liquid culture was applied to plates using a serological pipette near to flame. Plates with bacterial lawn were allowed to dry and grown for 2 days. For long term storage plates were placed at 4°C.

Table 2: Luria Bertani (LB) agar, pH 7.5

Ingredients	**Concentration**
Trypton (marka)	1%
Yeast extraxt	0.5%
NaCl	1%
Agar	2%
Autoclave	
Streptomycin	5 mg/ml

Table 3: B-broth content

Ingredients	**Quantity in 2000 ml**
Bacto tryptone	20 g
NaCl	10 g
Double distilled H_2O	up to 2000ml

2.3 Cleaning of contaminated *C. elegans* and synchronization

Worms occasionally encounter bacterial or fungal contamination. These kinds of contaminations should be eliminated before starting an experiment. Hypochlorite solution

was used to decontaminate *C. elegans* stocks. 3 or 4 plates containing large numbers of gravid adults were washed with M9 and worms were placed in a 15 ml falcon tube. Nematodes were washed 3 times for 2 minutes 2000 rpm at 15°C. After washing, 6 ml of hypochlorite solution was added and solution was vortexed ~6 minutes at maximum speed until the entire adult worms were dissolved and embryos remained because their cuticle is resistant to harsh conditions. 6 ml of hypochlorite solution was completed to 15 ml with M9 and embryos were washed 3 times to remove bleach. At the last step, 300µl bleach was added to remaining pellet of embryos and the resulting solution with embryos were placed onto the edge of HB101bacterial lawn on the NGM plates. After embryos hatch, L1 larvae crawl to bacterial lawn and agar spot containing the bleach can be removed by a sterilized spatula aseptically. This procedure is called as double bleach, as it involves2 steps that bleach is used.

For synchronization, all the steps in double bleach procedure were followed except the last step. In the last step 1 ml of M9 was added to embryos and falcon tubes were put on a rotator. The next day embryos became L1 larvae, which can be placed onto NGM plates with food source. Because *pash-1ts* mutants and other strains with *pash-1ts* background were growing slower than other strains they were spotted 8 hours earlier than the others.

Another cleaning method was spot, bleach which was used when there was no need to high amount of worms. 20µl hypochlorite solution was added on the border of a plate and 25-30 gravid adults were placed onto it. Adult worms were dissolved in bleach and hatched L1 larvae reached to bacterial source the next day. Again bleached agar piece was cut by the spatula near Bunsen burner.

Table 4: Hypochlorite solution content

Ingredients	Quantity in 50 ml
NaOCl	4 ml
10M NaOH	4 ml
Double distilled H_2O	42 ml

2.4 *C. elegans* freezing protocol for long term storage

C. elegans can be kept at -80°C in the frozen state for the long term storage [45]. Freshly starved clean worms in 90 mm plates were collected with 3 ml of M9 buffer (**see Table 2**) and 2.5 ml out of this 3 ml was put into 15 ml falcon tube containing 2.5 ml of freezing buffer (**see Table 3**). 5 cryo tubes were labelled with the strain name and the genotype of the freezing strain. Worms in the 5 ml mixture of M9 and freezing buffer was placed as 1 ml aliquots in 5 separate labelled tubes. Tubes were put in a Styrofoam container before they were located at -80°C for a gradual cooling. To check the success of freezing, one of the tubes was thawed next day and checked whether worms were recovered successfully. For the thawing procedure a vial from -80°C was removed and was allowed to thaw at RT till all the ice was liquidized. Worms then were pipetted onto a large plate with bacterial lawn. A week after nematodes were recovered, they were transferred to a fresh plate to eliminate any contamination.

Table 5: M9 buffer content

Ingredients	Quantity in 5000 ml
Na_2HPO_4	30 g
KH_2PO_4	15 g
NaCl	25 g
Double distilled H_2O	up to 5000ml
Autoclave	
1M $MgSO_4$	100 µl per 100ml bottle

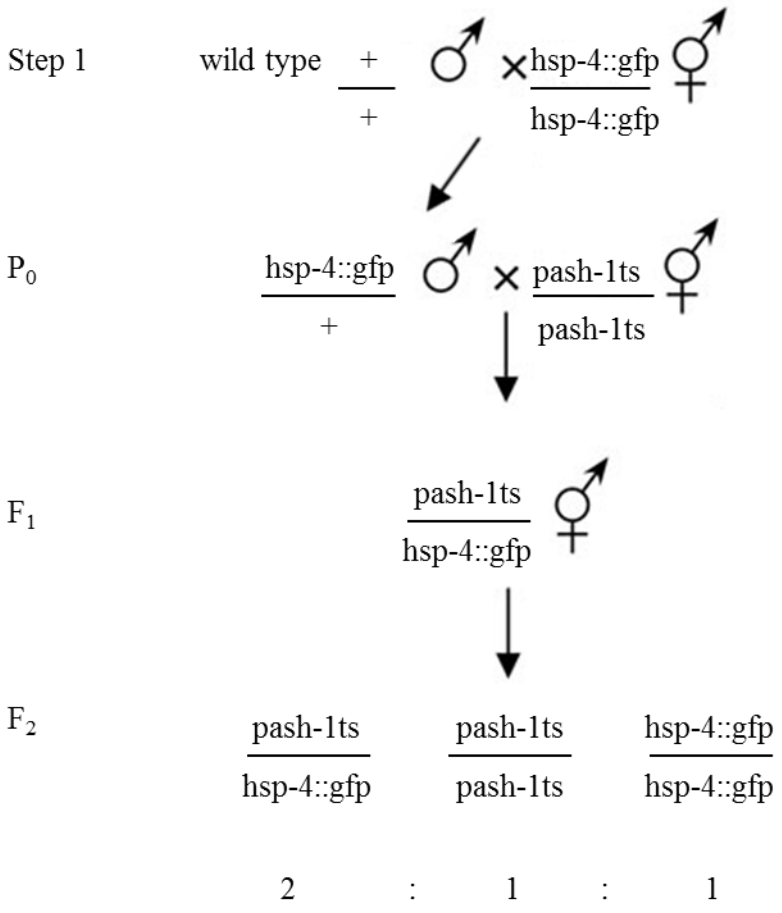
Table 6: Freezer buffer content

Ingredients	Quantity in 2000 ml
1M NaCl	200 ml
1 M KH_2PO_4 pH 6	100 ml
Glycerol (marka)	600 ml
Double distilled H_2O	up to 2L
Autoclave	
0.1 $MgSO_4$	300 µl per 100ml bottle

2.5 Genetic manipulation by setting up crosses with *pash-1ts* mutant

In *C. elegans,* double or triple mutants are easily generated by mating male and hermaphrodites. Unfortunately, males are found much less frequently in wild-type population. In order to generate male abundant populations, we used mild heat shock to late L4 hermaphrodites for 1.5 hours at 34°C water bath. The males of *pash-1ts* mutants were unsterile so that we first crossed wild type male worms with mid-L4 hermaphrodites of the strain desired to be crossed with *pash-1ts*. The success of the cross was understood from the number of males in the progeny which should be around 50%. The males in the

population were heterozygotes of the strain that we want to cross with *pash-1ts.* Mid-L4 hermaphrodites of *pash-1ts* mutants were then crossed with these males. Again the male abundancy in the F_1 generation shows that our cross worked. From F1 generation, 4-6 hermaphrodites were picked into separate plates for self-fertilization to generate homozygotes of both *pash-1ts* and the desired mutant or transgenic line. At least 16 hermaphrodites from F_2 generation were examined at restrictive temperature, 25°C, to see whether they are 100% embryonically lethal (*pash-1ts* homozygotes) or not. After *pash-1ts* homozygotes were handled, they were tested for the second mutation or in some cases for transgenic line. For the transgenic lines, genetic markers are used to distinguish transgenic lines phenotypically. For example, there is a GFP expressed under the promoter of HSP-4 in one of the transgenic lines that I used. By checking the *pash-1ts* worms for GFP markers, *pash-1ts;hsp-4::gfp* transgenic lines were produced.



2.5 ER stress survival assays

All stress survival assays were examined with young adult animals as t=0. Since the restrictive temperature is 25°C for *pash-1ts*, which is the required temperature to cease miRNA production, experiments were performed at 25°C. Worms were placed on plates with the stress initiator chemicals as staged young adults. A platinum wire was utilized to prod an animal from both head and tail. The worm was scored as dead when it did not react to a mechanical input. When the worm was alive but unable to complete a sinusoidal movement, it was accounted as paralyzed.

2.5.1 DTT stress survival assay

For the first DTT survival assay, large NGM agar plates were dried for 4 days before they were seeded with 500 µl of 300 mM DTT (AppliChem) stock solution to achieve 10 mM. in final concentration . DTT was dissolved in ddH_2O and stocks older than a week were not used in the experiments as DTT is prone to be oxidized by air. Because of its intensive odor and aseptic concerns, DTT plates were prepared in laminar flow hood and kept 2 days before spotted with bacteria. For each set of experiments, at least 50 young adult worms for each strain were transferred to DTT plates and their survival were checked after 16 hours, 21 hours and 41 hours..

DTT sensitivity assay was also carried out the same way in the presence of 40µM FUDR to prevent the detrimental effect of DTT on egg laying of the worms. As egg laying defective animals could not be analyzed due to internal hatching and subsequent death.

2.5.2 Tunicamycin survival assay

12-well plates were used to test the effects of tunicamycin (TM) (AppliChem). A vial of 5 mg of TM was dissolved in 5 ml DMSO making the stock concentration 1mg/ml. For the experiments carried out with 20µg/ml TM, 1mg/ml stock solution was directly added to 150 ml of standard NGM agar after autoclaving. To avoid the adverse effects of heat on TM, bottles containing the agar were kept at 55°C water bath before TM was added. For the TM stress survival assays carried out with 30µg/ml, 6mg/ml stock solution was prepared to decrease final DMSO concentration in agar. Each well was filled with 2 ml agar and plates were spotted after 3 days. In order to circumvent any cross contamination between strains, every 12-well plates contained the worms from the same strain, as it is shown in the **Figure 8.**

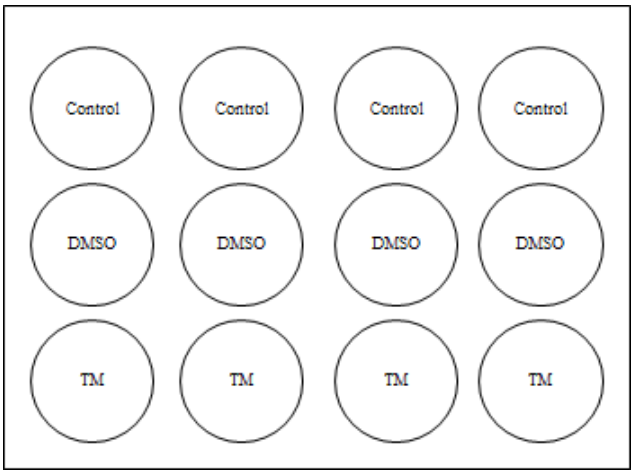


Figure 8: Representation of the 12-well plate

2.5.3 Thapsigargin survival assay

Similar to tunicamycin experiments, 12-well plates were used for thapsigargin survival assay. This time drug has been added later on NGM agar plates because of its heat labile structure. A 10 µl droplet of thapsigargin stock solution, in which thapsigargin was dissolved in DMSO one day before the experiment, was added on the center of agar plates assuming it completely fused in the plate. After 12 hours the plates were spotted with 10µl of HB101 by targeting the thapsigargin drop. ~20 worms were examined in each well and they were transferred every second day.

2.6 N-acetylglucosamine lifespan assay

Lifespan analyses with N-acetylglucosamine were performed at 25°C on UV-killed *E. coli* HB101 with young adults as t=0. 70-100 L4 animals for each strain were picked and placed to 15°C till they become young adults (16 hours) then they were replaced to 25°C to stop miRNA production (16 hours) before they were put in N-acetylglucosamine plates (**see Table 6)**.

Table 7: Preparation of 5mM N-acetylglucosamine plates

Ingredients	Quantity in 500 ml
200 mM N-acetylglucosamine (Carbiosynth,)	12,5 ml
55°C standard NGM agar	487,5 ml

2.7 Embryo count experiments

Staged L4 staged worms were picked and grown till young adult stage at 15°C. Temperature was shifted to 25°C, which is a restrictive temperature for the activity of PASH-1TS protein. Worms then transferred in each well individually and their embryos were counted every 12 hours.

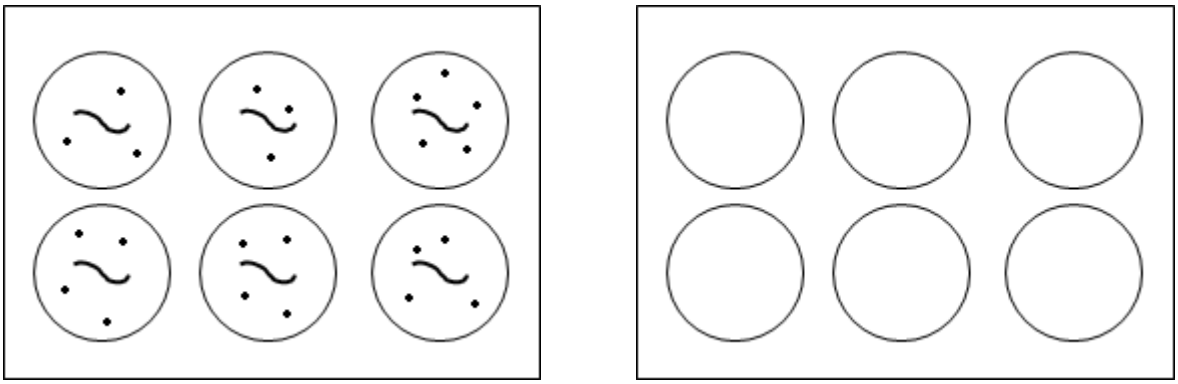


Figure 9: Representation of 6-well plates in embryo count experiments

2.8 Epifluorescence microscopy

2% agarose was prepared and melted agarose was pipetted in glass tubes in 1 ml aliquots. In order to keep the agarose in liquid state, heat block was set at 65°C. Two glass slides were covered with tape to be thicker than the actual slide to help its removal under the other slide which serves as a top cover to give agar a circular shape. After 2-3 minutes the agar padded slide was removed under the cover slide gently. For adult worms, 10µl of 20mM NaN_3 was used as an anesthetized. Pictures were taken at 20X with 100ms exposure

unless otherwise noted. For embryo images, embryos were collected with a small amount of food at the tip of a platinum wire and put into M9 buffer. Embryo images were taken at 100X with 100ms unless otherwise noted.

2.9 Time required for some of the strains used in lifespan analyses to become L4

Since *pash-1ts* mutant slowly grows even at 15°C, it was hard to synchronize the worms. After bleaching, I applied L1 larvae according to the time seen in the **Table 8**. For example, I spotted pash-1ts and pash-1ts;rab-3p::xbp-1s L1s to food containing plates nearly 12 hours before wild type, *gfat-1(dh784)* and *rab-3p::xbp-1s*. This may be applied to the *pash-1ts* double mutants which likely to behave similarly to *pash-1ts* demonstrated in **Table 8.**

Table 8: Required time for depicted strains until they become L4

Development at 15°C					
	wild type	*pash-1ts*	*rab-3p::xbp-1s*	*pash-1ts;rab-3p::xbp-1s*	*gfat-1(dh784)*
Egg laid	0 h	0 h	0 h	0 h	0 h
Larval stage 4	62 h	76 h	64 h	76 h	65 h

2.10 Strain list

Strain	Genotype
N2	Wild type
SX1137	*pash-1(mj100)I*
SX1359	*pash-1(mj100)I; mjEx331*
SX1483	*pash-1 (mj100)I; mjEx365*
SX1501	*pash-1 (mj100)I; mjEx383*
SJ4005	*zcIs4 [hsp-4::GFP] V*
SJ4058	*zcIs9 [hsp-60::GFP]*
AA2560	*gfat-1(dh784)II*
AGD927	*uthIs270[rab-3p::xbp-1s+ myo-2p::tdTomato]*
MT14119	*nDf67 mir-52(n4114) IV/nT1 [qIs51] (IV;V;nDf58X.*
SAR38	*pash-1(mj100) I; zcIs13[hsp-6::GFP] V*
SAR39	*pash-1(mj100) I; zcIs4[hsp-4::GFP] V*
SAR40	*Pash-1(mj100)I; gfat-1(dh784)II*
SAR51	*Pash-1(mj100)I; uthIs270[rab-3p::xbp-1s+ myo-2p::tdTomato]*

Chapter 3

RESULTS

After *pash-1ts* mutant is isolated in a genetic screen, many phenotypical and genetic characterizations of the mutant animals were carried out. One of the prominent characteristic of this mutant is its short lifespan, which lasts about 8 days. While the reason of this short lifespan is in question, their necessity in normal physiology of the worms is highlighted. In the absence of miRNAs, degeneration in the muscle tissue have been observed, which was rescued by reintroducing the wild type copy of the gene[46]. We hypothesized that the worms lacking miRNAs have elevated proteotoxic stress due to the absence of miRNA regulation. Our results show that one of the amyloid-binding compounds increases the health-span of *pash-1ts* mutants (Gulseren N., unpublished data). Amyloids are insoluble fibrous protein aggregates causing to degenerative diseases such as Alzheimer. This amyloid binding compound was proposed to bind aggregation prone proteins to stabilize them and in addition enhances their proteosomal degradation [47]. These results made us to take into consideration the involvement of other unfolded protein response mechanisms. Other than proteasome system, endoplasmic reticulum and mitochondria have their own unfolded protein response mechanisms; UPR^{ER}, UPR^{MT}. Since the lifespan extension of mutants having reduction of mitochondrial functions was not dependent on miRNA synthesis, we decided to examine UPR^{ER} response in *pash-1ts* animals.

3.1. *pash-1ts* mutants are not sensitive to ER stress inducers

ER stress resistance or sensitivity can be examined by growing worms on plates containing chemicals that are known to induce ER stress. Three different chemicals were

used to test the importance of the presence of miRNAs under ER stress. These three ER stressors were selected to see the outcomes of different kinds of ER stresses. DTT and tunicamycin create ER stress by directly affecting negatively the protein folding. These stressors create protein aggregation in the ER lumen. On the other hand, thapsigargin has an indirect effect on the protein folding. It causes the excessive Ca^{2+} release from the ER lumen by inhibiting the Ca^{2+} ATPase which causes protein folding defects.

3.1.1 Dithiothreitol (DTT) has a modest effect on the miRNA deficient worms

Dithiothreitol (DTT) is a very potent reducing agent and it prevents the disulfide-bond generation, which causes the accumulation of proteins in the ER. However, this chemical also interrupt the disulfide bond formation of nascent proteins in the cytosol so that, it is not a specific ER stressor. I have used *gfat-1(dh784)* and *rab-3p::xbp-1s* as positive controls, as both mutant animals are known to be resistant to ER stress.

gfat-1(dh784) is one of the gain of function mutant of *glutamine-fructose-6-phosphate amino-transferase 1* , which is the rate-limiting enzyme of hexosamine pathway. *gfat-1(dh784)* was identified with other additional three alleles of it in a forward genetic screen using ethyl methanesulfonate (EMS) to induce random mutations. Then tunicamycin selection was applied to distinguish the *C. elegans* mutants that can survive in lethal concentrations of tunicamycin. Further characterization of *gfat-1*alleles showed HSP-4 expression was decreased upon tunicamycin exposure in comparison to wild type animals, which is the indication of enhanced protein quality control in the ER. Additionally, ERAD and autophagy mechanisms were improved to cope with protein overload in the ER lumen. I picked *gfat-1(dh784)* allele to perform my experiments since it was the most resistant allele to tunicamycin[48].

My second positive control was *rab-3p::xbp-1s,*which is the constitutively active form of XBP-1s expressed under the neuronal promoter *rab-3.* Solely, neuronal XBP-1s

was sufficient to recover ER stress, lengthen lifespan, and trigger the UPR^{ER} mechanisms in intestinal cells in a cell non-autonomous manner[49].

In our initial analysis, compared to wild type and positive controls, *pash-1ts* mutant animals were found to be the most resistant strain to 10 mM DTT exposure at all-time points that I investigated (**see Figure 1**). For 16 hours exposure, there is not much difference between strains. We started to see the differential effect of DTT after 21 hours exposure. The percentage of alive *pash-1ts* mutants were nearly 98% while 43% of *gfat-1*mutants were alive. The reason of this sharp decrease in *gfat-1* comparing to wild type and other positive control *rab-3p::xbp-1s* may be the high fecundity rate of this mutant since DTT exposure caused egg laying defect in wild type, *gfat-1* and *rab-3p::xbp-1s* mutant animals. Due to the high reproductive rate of *gfat-1* mutants, internal hatching phenotype may be more severe than the others. DTT may cause to vulval muscles to be not properly contracting since it blocks the disulfide bond formation in proteins. Egg laying defect leads to death due to internal hatching of embryos. Therefore, this could skew the results and cause *pash-1ts* to be more resistant than the positive controls, as it has its embryonic lethal phenotype and do not die due to internal hatching.

To prevent internal hatching, I have used 40µM 5-Fluoro-2'-deoxyuridine (FUDR) and I increased the DTT concentration to 15 mM (**see Figure 2**). FUDR is a commonly used drug in the ageing field of *C. elegans* because it inhibits cell division so that animals do not produce embryos. This also eliminates the need for daily transfer of worms to separate the progeny from the mothers [50]. I also increased the DTT concentration to observe the differences in ER stress sensitivity between the strains. However, I observed still a significant amount of “bag of worms” which are the worms containing a lot of early larvae inside the mothers. I did not further increase the concentration of FUDR since it has been shown as an enhancer of the proteostasis in *C. elegans* and this would hamper the further analysis [50].

In these experiments, I observed highly variable results between two different data sets for *pash-1ts; gfat-1(dh784)* double mutants. In order to have the double mutant, I set up a cross between *gfat-1(dh784)* and *pash-1ts*. After the *pash-1ts* was confirmed from its embryonic lethal phenotype (see materials and methods), *gfat-1(dh784)* was determined by TM selection at 15°C which is the permissive temperature of *pash-1ts* mutants allowed us to track only the *gfat-1(dh784)* gain of function mutant. TM resistant candidate double-mutant homozygotes were selected and assumed as they were the *pash-1ts; gfat-1(dh784).* I also performed PCR to see the single nucleotide change in GFAT-1 gof mutation; however, it did not work (data not shown). Finally, I decided to exclude this strain from my further experiments.

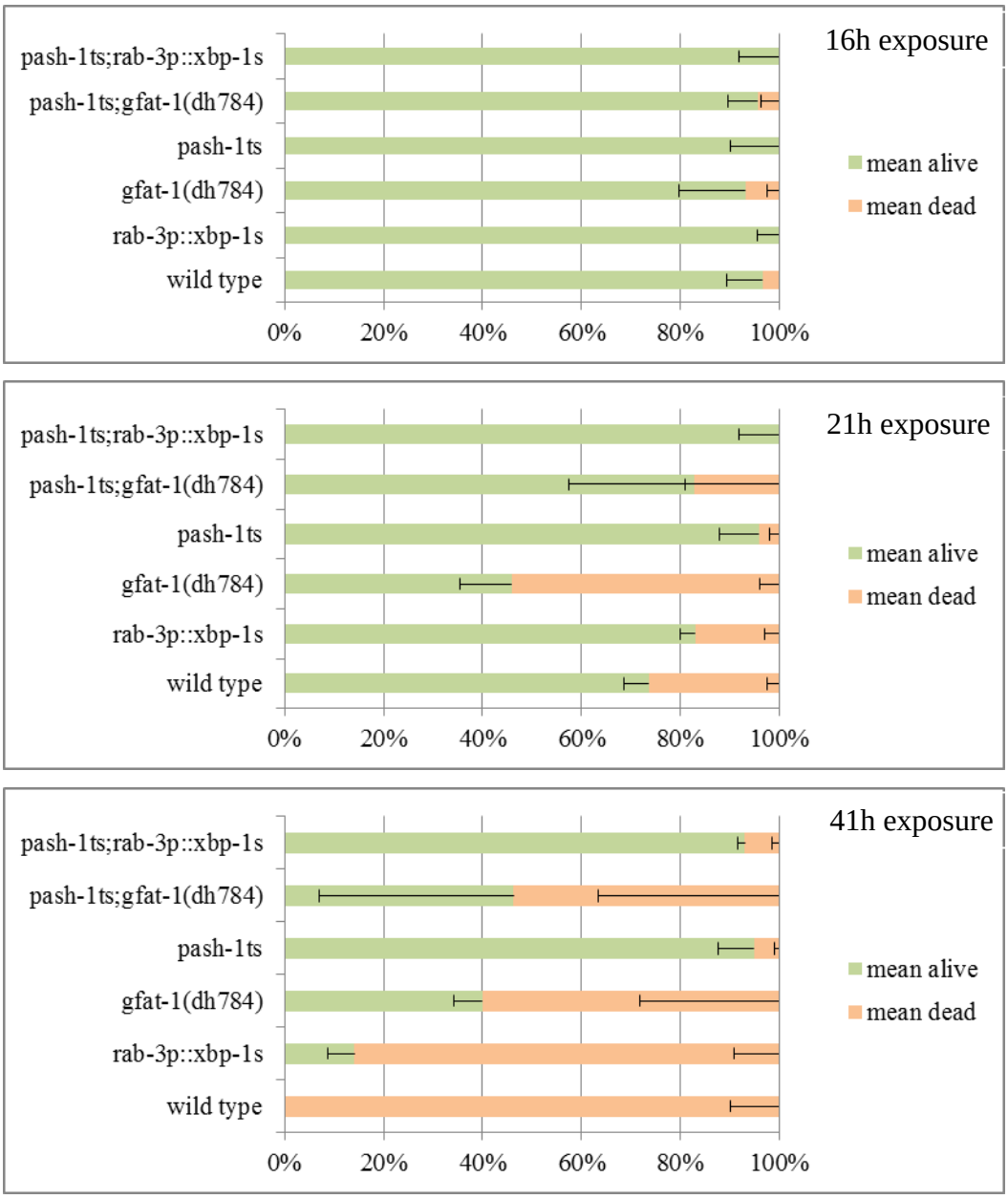


Figure 10: 10 mM DTT stress exposure for the indicated time points: For each strain L4-staged worms were collected and grew till young adult at 15°C. Young adult animals then transferred to 10 mM DTT plates and shifted to 25°C. Dead or alive worms counted after 16, 21 and 41 hours, respectively. For each of the six populations per two

experiments, n=50. Error bars demonstrate one standard deviation from the average of indicated situation (alive or dead) over of two independent experiments.

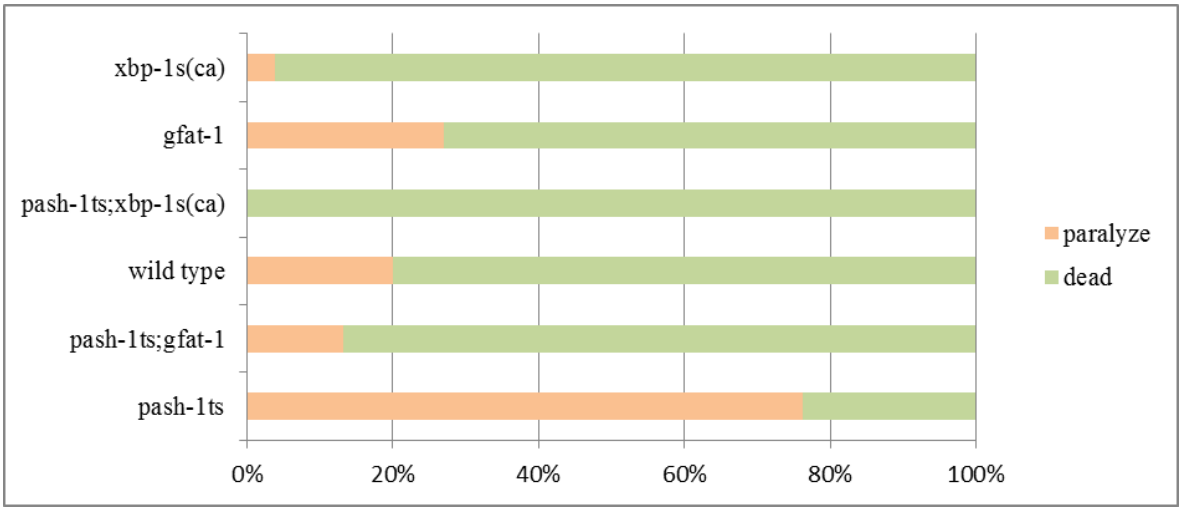


Figure 11: 40 µM FUDR 15 mM DTT stress after 16 hours: FUDR was added in standard NGM agar after autoclaving. The experiment was performed as indicated in the first figure.

3.1.2 Tunicamycin does not have an effect on the lifespan of miRNA deficient worms

The second ER stressor that I used was tunicamycin (TM). Comparing to DTT, TM is a specific stressor of the ER by inhibiting the activity of rate limiting enzyme of hexosamine biosynthetic pathway leading to the inhibition of glycoprotein formation. This rate limiting enzyme is called as GFAT-1, which utilizes fructose-6-phosphate from glycolytic pathways to produce UDP-N-Acetylglucosamine for glycoprotein synthesis.

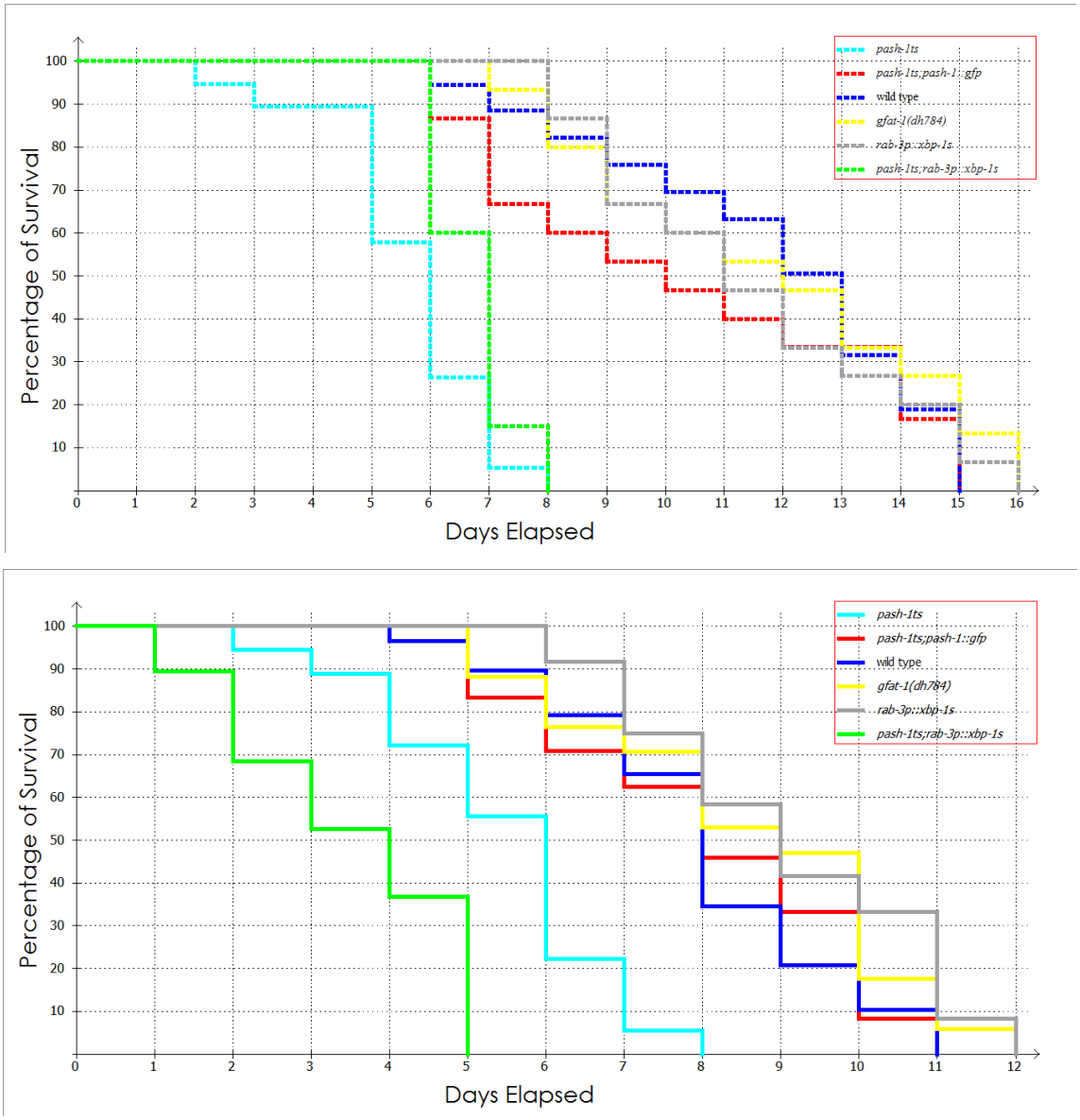


Figure 12: Survival curve of indicated strains upon 30µg/ml TM treatment: TM treatment was initiated when worms were young adults (L4+16 hours at 15°C). After young adult worms were replaced into 12-well TM plates, the temperature was shifted to 25°C to stall the miRNA production in *pash-1ts* mutants. The above graph in top panel

shows the survival analysis in control plates (0.5% DMSO), while the graph in the lower panel contain the survival analysis in the presence of 30µg/ml TM.

Table 9: Percentage analysis of half-life and maximum life upon 30µg/ml TM

	Half life	Maximum life
wild type	-33%	-27%
pash-1ts	0%	0%
rab-3p::xbp-1s	-18%	-25%
pash-1ts;rab-3p::xbp-1s	-43%	-38%
gfat-1(dh784)	-25%	-25%

Upon TM treatment, the half-life of wild type animals decreased by 33% while the half-lives of our positive controls *rab-3p::xbp-1s* and *gfat-1(dh784)* were reduced by 18% and 25%, respectively. When we compare our positive controls with wild type in our control group, the half-life of *rab-3p::xbp-1s* is less than both *gfat-1(dh784)* and wild type. Although, this data seems contradictory to the reference paper of *rab-3p::xbp-1s* [49], they performed their lifespan analyses at 20°C. Because of *pash-1ts* mutant lifespan analyses were carried out at 25°C, which is a mild-heat shock for *C. elegans.* At 25°C, maintenance of proteostasis in *C. elegans* decreases sharply compared to 15°C or 20°C so that *rab-3p::xbp-1s* may not be as functional as it is at 20°C.

Dillin et al. showed that constitutively active form of *xbp-1s* under the neuronal *rab-3* promoter is sufficient to extend the lifespan of worms. Neuronal *xbp-1s* expression leads to activation of UPR in the distal tissues, such as intestine. They propose a model that the neuron specific detection of ER stress and the activation of IRE-1 arm of UPR^{ER} give rise to the secretion of still undefined molecules from small clear vesicles (SCVs) [49]. As protein quality control system is regulated cell-nonautonomously in multicellular

organisms, we further hypothesized that these SCVs may include miRNAs. Our lifespan analysis with *pash-1ts;rab-3p::xbp-1s* animals showed that there is no positive effect of the active form of neuronal *xbp-1s* on the lifespan of miRNA depleted animals. This data suggest neuronal xbp-1s extends lifespan in a miRNA dependent pathway and it may preliminarily propose a model whereby the absence of miRNAs ceases crosstalk between different tissues. Our hypothesis is strengthened by the results in which expression of PASH-1::GFP specifically in neurons prominently increases the lifespan of worms[46].

Interestingly, half-life of *pash-1ts;rab-3p::xbp-1s* was reduced by 43%. Considering both *pash-1* and *rab-3p::xbp-1s* animals are resistant to TM, it was unexpected to observe a decrease in the half-life of the animals. It is hard to interpret this data; however, when we consider the fact that mammalian IRE1 degrades the miRNAs that normally inhibits the translation of Casp2 mRNA which activates apoptotic pathways. Absence of miRNAs together with active Ire-1 pathway may lead to aberrant apoptosis in these animals.

3.1.3 HSP-4 chaperones is differentially expressed in pash-1ts mutants

HSP-4 is the ER stress specific chaperone which resides into ER lumen bound to the luminal domains of ER stress response proteins. When there is aberrant protein folding in the ER, HSP-4 disassociates from the luminal domains of ER membrane bound proteins and assist folding of the proteins to reduce the protein overload in the ER. The level of HSP-4 is mainly regulated by transcriptional response. Therefore, the level of HSP-4 can be visualized by its promoter activity. In our studies,we took the advantage of a transgenic strain in which the *gfp* is expressed under the control of a transcriptional *hsp-4* promoter so that we could analyze the expression patterns of HSP-4 in the *pash-1ts* background. Our results showed that HSP-4 is differentially expressed in various tissues of *pash-1ts* mutant compared to wild type animals. One of the interesting observations was the *hsp-4*

expression at the hypodermal cells of the *pash-1ts* mutants. We observe GFP signal at the positions of *hyp-7, hyp-8, hyp-9, hyp-10* and *hyp-11*.

It is interesting to see that even though we see a relatively high expression of HSP-4 in *pash-1ts* mutants, indicating the presence of ER stress; we do not see a hyper-synthesized phenotype against ER stressors (**see Table 1,2**). Normally, prolonged stress in the ER causes activation of apoptosis, but there might be a delay in this pathway in *pash-1ts* mutants.

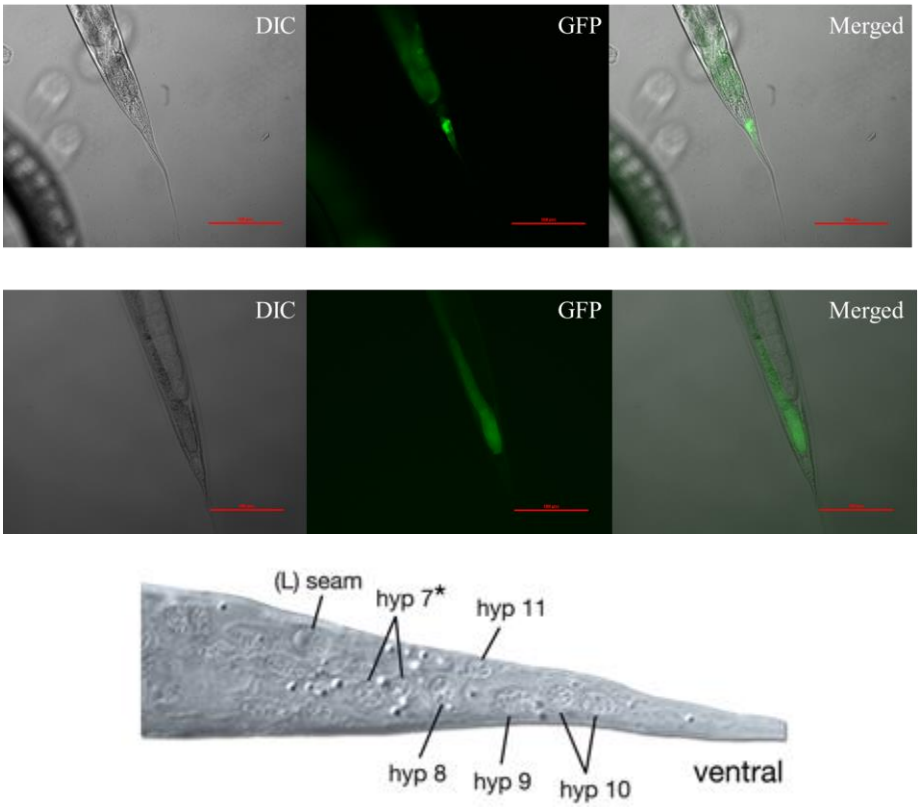


Figure 13: Differential expression of HSP-4 in the hypodermal cells in the posterior of the worms: *hsp-4::gfp* and *pash-1ts;hsp-4::gfp* transgenic worms were picked as L4s and grown at 15°C till they grow young adults (16 hours). Then temperature is shifted to 25°C.

After 14 hours, worms were transferred to 8µg/ml TM containing plates for 6 hours. Images were taken by Nikon80i epifluorescence microscope. Scale bars: 100µm (representative image is taken from wormatlas.org)

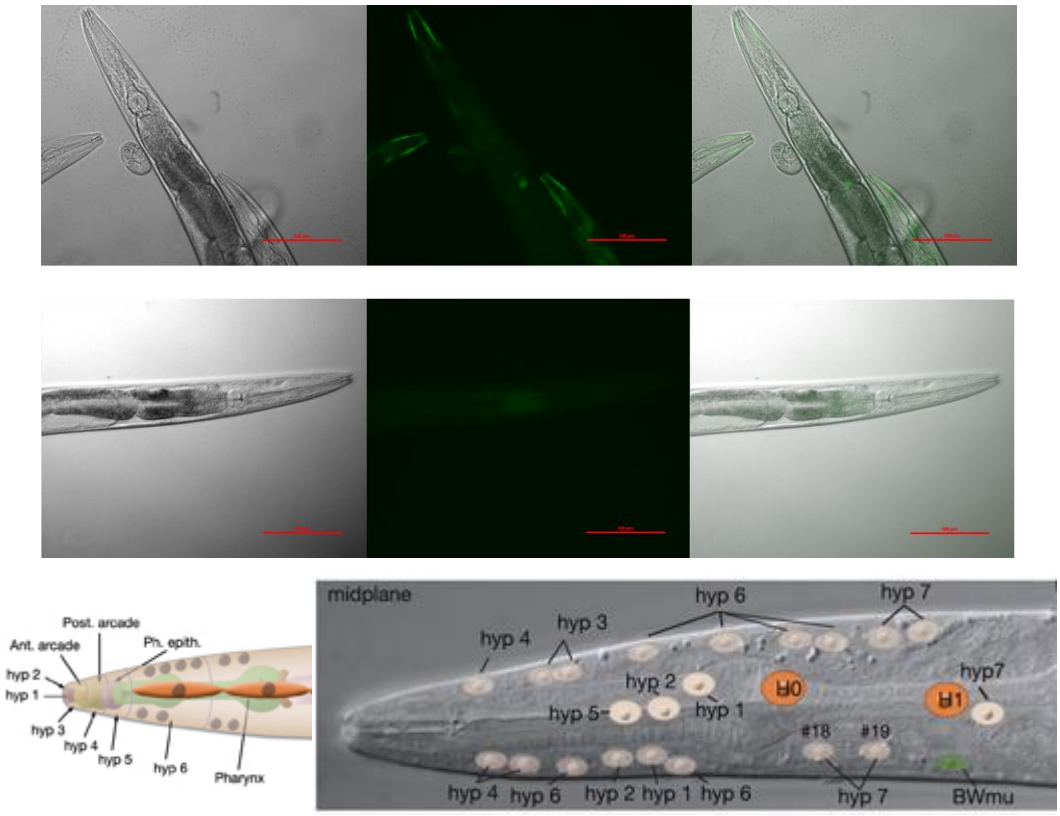


Figure 14: Differential expression of HSP-4 in the hypodermal cells in the anterior part of the worms: The same procedure in the Figure 4 was followed. Scale bars: 100µm (representative image is taken from wormatlas.org)

3.1.4 Thapsigargin has negative effects on pash-1ts animals unlike other ER stressors

Another pharmacological reagent to induce ER stress is thapsigargin (TG). It is a potent inhibitor of the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA). Cytosolic calcium is

transported by the SERCA into the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR). These two organelles have crucial functions for the control of Ca^{2+} concentration in the cytosol. They either absorb Ca^{2+} from the cytoplasm or act as a storage site to release Ca^{2+} ions upon a stimulus. Muscle contraction is highly dependent on Ca^{2+} levels in the cytosol. When Ca^{2+} is released to the cytosol from SR, muscle is contracted. Recently, Zwaal et al. demonstrated that *C. elegans* has a highly conserved SERCA homologue CeSERCA encoded by the gene *sca-1*[51]. Phenotypic analysis of the deletion mutants of CeSERCA revealed defects in the muscle contraction and larval arrest. Usage of thapsigargin results in similar phenotypes due to decrease in the levels of calcium[51]. Low calcium levels negatively affect the calcium-dependent ER resident chaperones, such as calnexin, and proteins start to accumulate. Additionally, chronic application of thapsigargin was shown to activate pro-apoptotic signal of ER^{UPR} with an enhanced transcriptional activation of CHOP[52]. Although there is no identified *C. elegans* homolog of CHOP, we hypothesized that thapsigargin may activate apoptotic pathways independent from CHOP protein so that *pash-1ts* may be hyper synthesized against it supporting our first hypothesis that the disruption in miRNA biogenesis pathway disables the activation of apoptotic signals. If miRNAs are involved in the upstream of apoptotic signal activation upon ER stress, thapsigargin would initiate the programmed cell death directly targeting the pro-apoptotic proteins. When we look at **Table 1** and **Figure 1**, we do not observe any significant motility defect after 24 hours of exposure to thapsigargin. Thapsigargin causes protrusions in the rectum and vulva. *pash-1ts* animals were affected approximately 10% higher than wild type when we compare rectal protrusion defect [53]. On the other hand, the number of the animals that have vulval protrusion was comparable for both *pash-1ts* mutant and wild type animals.

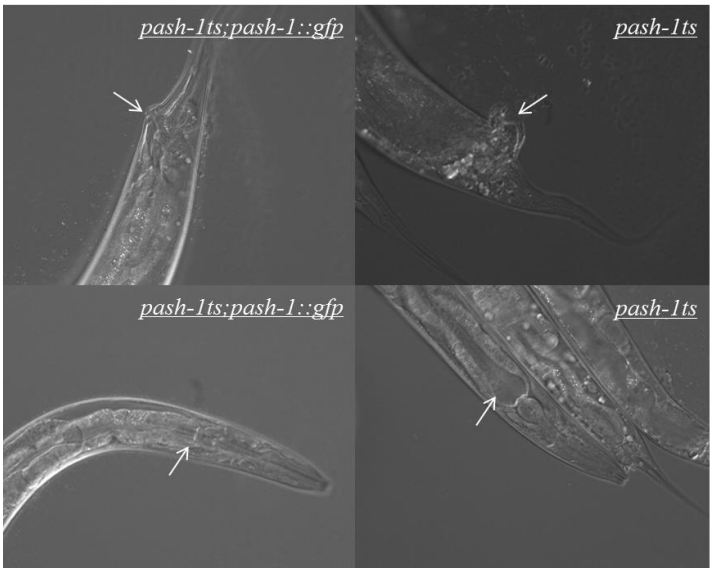


Figure 15: Microscope images of depicted strains to represent the phenotypes in the Table 3

Table 10: **Defective phenotypes upon thapsigargin treatment:** Strains used in this experiment were picked as L4 larvae on standard NGM plates and temperature shifted to 25°C when they reach to young adults. Young adult animals were transferred to thapsigargin or control plates. Phenotypic characterization was carried out after 24h. Data represents two biological replicates with 50 animals.

	% affected animals		
	0 µM	1 µM	1.5 µM
Movement			
wild type	0	3	3
pash-1ts	0	8	10
pash-1ts; pash-1::gfp	0	0	0
gfat-1 (dh784)	0	0	0
rectal protrusion			
wild type	0	72	77
pash-1ts	0	81	85
pash-1ts; pash-1::gfp	0	78	81
gfat-1 (dh784)	0	38	54
vulval protrusion			
wild type	0	61	70
pash-1ts	0	42	57
pash-1ts; pash-1::gfp	0	60	72
gfat-1 (dh784)	0	0	25

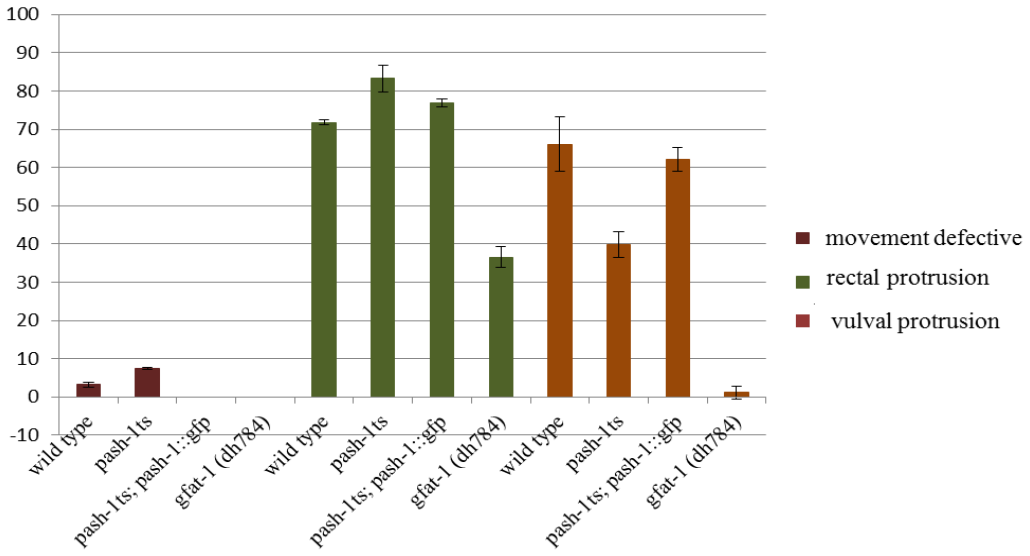


Figure 16: Percentages of the phenotypic characterization in Table 3: The same procedure was followed as in the Table 3.

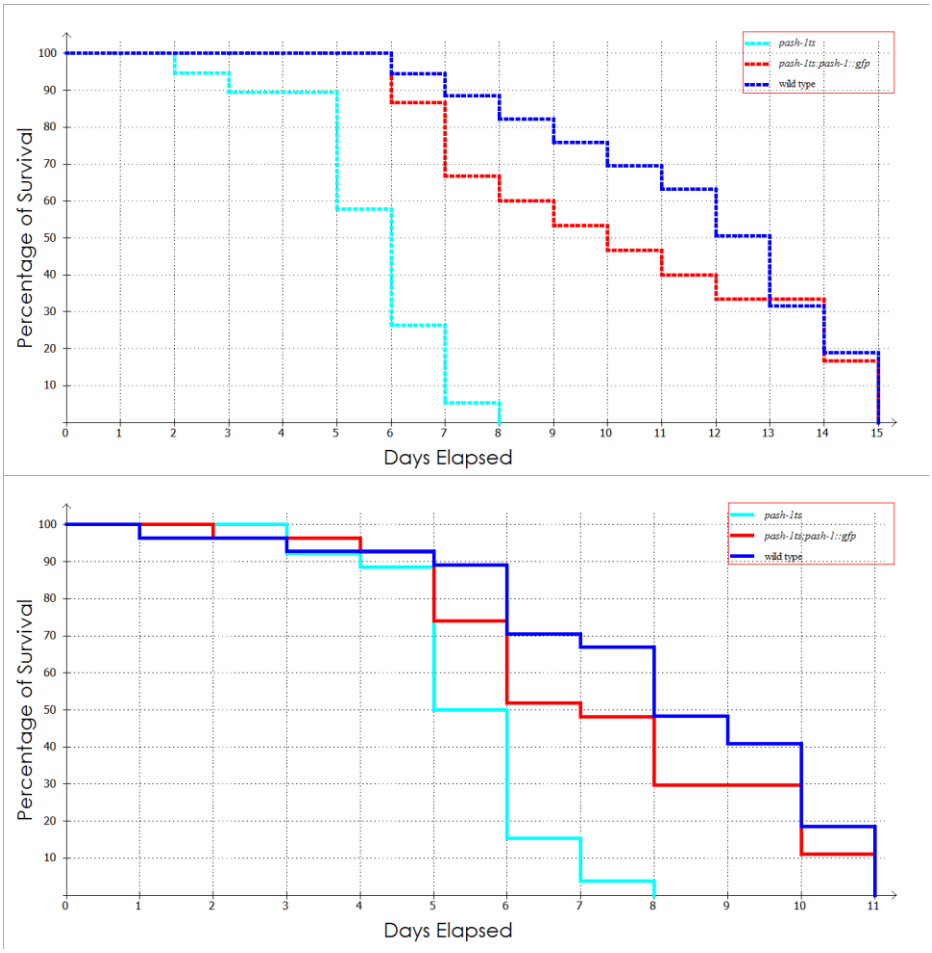


Figure 17: Survival curve of depicted strains upon chronic application of 1,5 µM thapsigargin: L4 stage animals were picked in standard NGM plates and they reached to young adults at 15°C. Young adults were transferred to 12-well plates seeded with final 1,5 µM thapsigargin concentration. Thapsigargin was dissolved in DMSO and control plates Contained 0.5% DMSO do not affect the lifespan of animals[54].

Table 11: Thapsigargin decreases the half-life of *pash-1ts* unlike other ER stressors

	Half life	Maximum life
wild type	-33%	-27%
pash-1ts	-17%	0%
pash-1ts;pash-1::gfp	-30%	-27%

In lifespan analysis on thapsigargin plates, the half-life of *pash-1ts* mutants were decreased by 17% while maximum life did not change. Still, this phenotype was promising due to the fact that other ER stressors did not have any effect on neither half-life nor maximum life of the animals.

3.2. Enhancement in the UPR^{ER} by supplementation with N-acetylglucosamine did not improve the lifespan of miRNA lacking adult worms while embryonic lethality were rescued modestly

N-acetlyglucosamine (GlcNac) is a monosaccharide derivative of glucose which has an amide group instead of hydroxyl group[6]. It is one of the dietary supplements commonly used in the US. Recently, GlcNac was shown as an enhancer of the different cellular degradation pathways by reducing the toxic protein levels so that increases the lifespan in *C. elegans* [48]. *gfat-1* mutation leads to an increase in the GlcNac levels and therefore, serves as another form of GlcNac supplementation.

As a starting point of our hypothesis that *pash-1ts* mutants have prominently shorter life-span partly because of the aberrant proteostasis in the ER, we considered to use a chemical improving the UPR^{ER} functions. GlcNac was shown to enhance the protein folding capacity of ER by boosting the ERAD and autophagy pathways. In order to see the effects of an enhancement in UPR^{ER} would ameliorate the detrimental effects of the absence of miRNA, we carried out lifespan analysis with GlcNac.

3.2.1 N-acetylglucosamine does not have an effect on pash-1ts lifespan

In **Figure 9** and **Table 4**, it is shown that GlcNac does not have an effect on neither maximum life nor half-life of the *pash-1ts* animals. Wild type increases its half-life by 8% while *rab-3p::xbp-1s* decreased its half-life by -7% which might be an experimental error. The most interesting result was the reduction in the both half-life and maximum life of the *pash-1ts;rab-3p::xbp-1s* by -14% and -13% respectively. I observed *pash-1ts* in GlcNac were usually larger than *pash-1ts; rab-3p::xbp-1s* animals, however I did not quantify this observation. During the lifespan analysis, I realized that there are worms hatched in the *pash-1ts; rab-3p::xbp-1s* (**see Figure 10)**. In order to quantify this observation, I determined the percentage of the embryos that hatched in *pash-1ts; rab-3p::xbp-1s* animals in parallel to control animals. ~20% of *pash-1ts;rab-1p::xbp-1s* embryos hatched at 25ºC. It should be noted that *pash-1ts;rab-1p::xbp-1s* animals have egg laying defects, therefore they accumulate embryos in the uterus. This may hamper with our analysis and may cause underestimation of the percentage of hatched embryos.

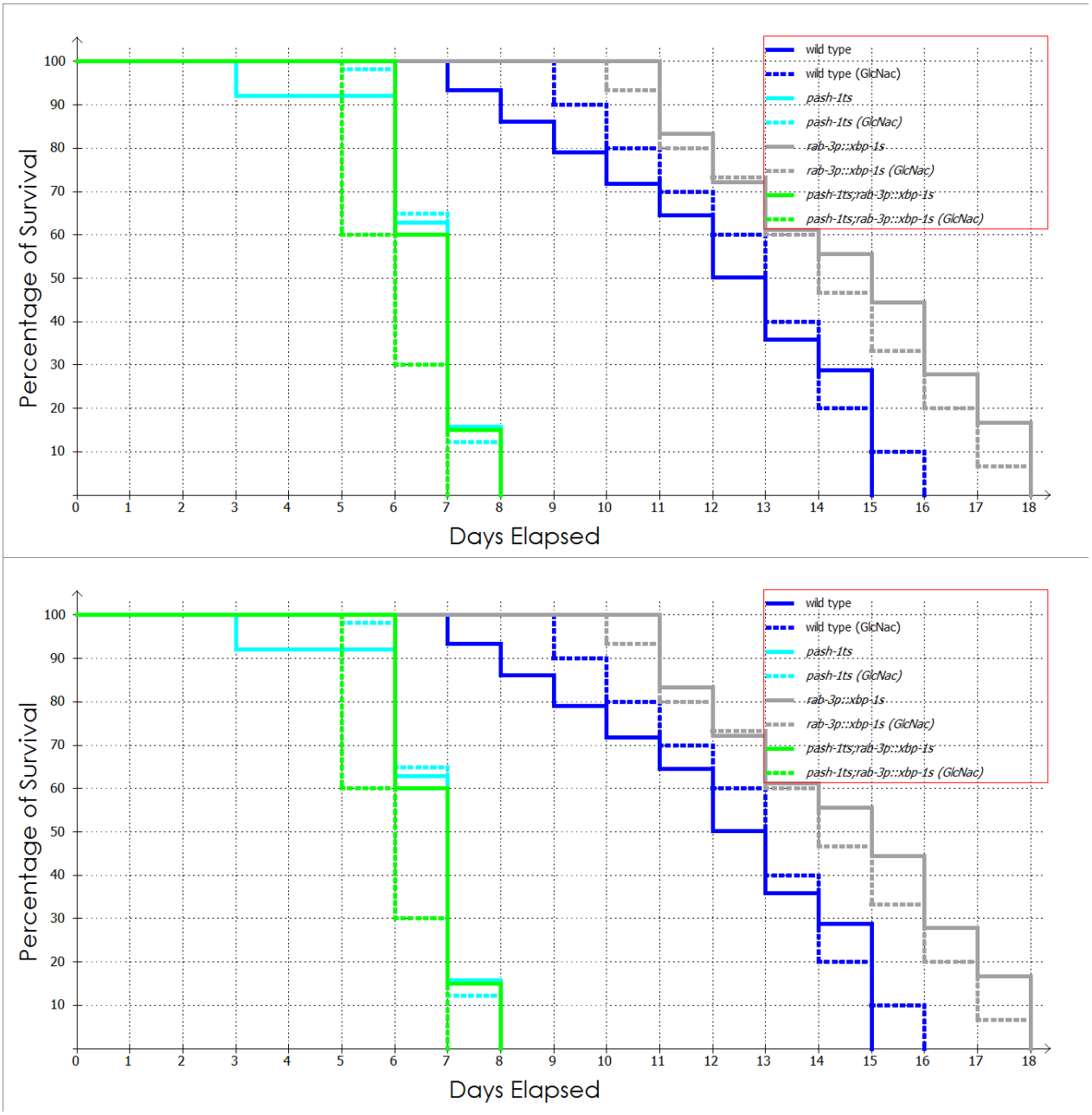


Figure 18: Survival analysis of indicated strains with the supplementation of N-acetylglucosamine: Experiments were started with the L4-staged worms at 15°C till they become young adults. Until then, the temperature is shifted to 25°C for 14 hours. Young

adult animals were transferred to 5mM GlcNac plates. Worms were counted every or every second day as whether they are dead or paralyzed. For *pash-1ts* and *pash-1ts;rab-3p::xbp-1s* animals, average life spans of 3 independent repeats on each they were represented while for other strains the repeat number is 2. (N=70-80 in each experiments for pash-1ts while N=40-50 for other strains.)

Table 12: GlcNac does not affect the life-span of *pash-1ts*

	Half life	Maximum life
wild type	8%	7%
pash-1ts	0%	0%
rab-3p::xbp-1s	-7%	0%
pash-1ts;rab-3p::xbp-1s	-14%	-13%

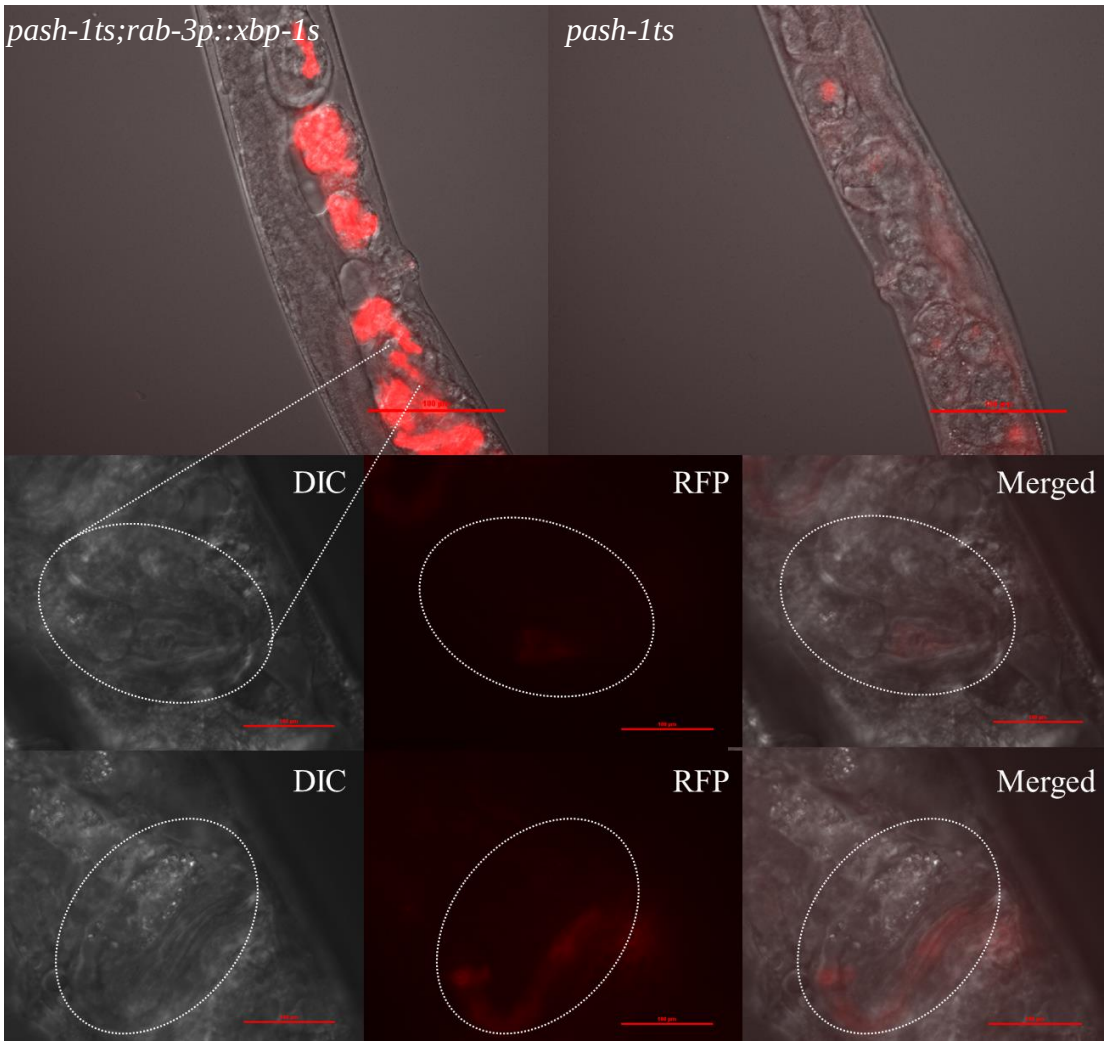


Figure 19: *pash-1ts:rab-3p::xbp-1s* worms live shorter than *pash-1ts* due to the internal hatching of embryos in *pash-1ts:rab-3p::xbp-1s* animals: Animals synthesized with 20 mM NaN_3 on 2%agar pad. 2 of the embryos shown in the figures are belonging to *pash-1ts;rab-3p::xbp-1s.*

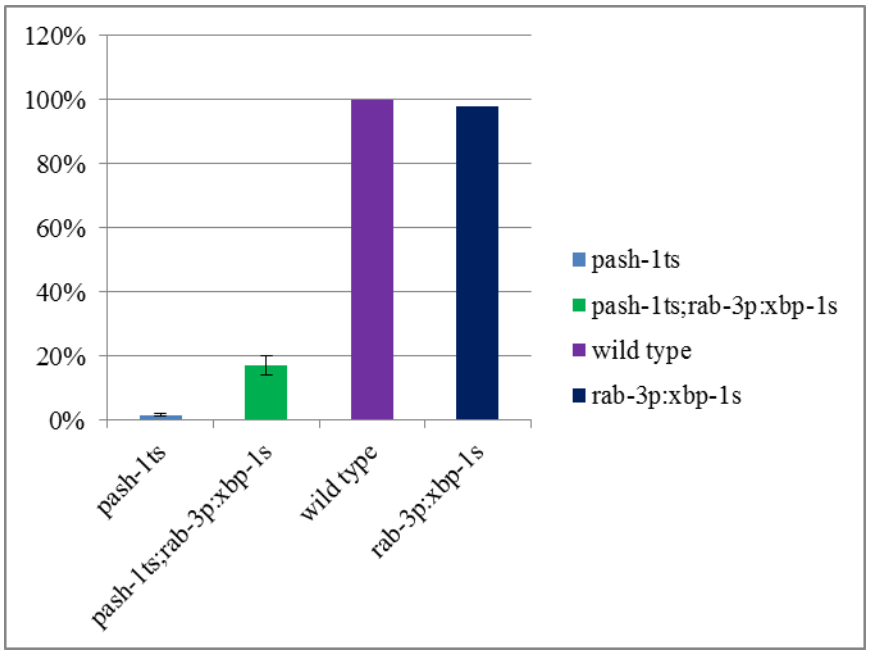


Figure 20: Neuronal active *xbp-1s* has moderate effect on the hatching of embryos in the *pash-1ts* background: 6 L4 worms from each strain were grown at 15°C until they become young adults. Young adult worms were kept at 25°C before 6 worms from each strain put in 6-well plates individually. Embryos were counted every 12 hours.

3.2.2 N-acetylglucosamine has a modest effect on embryonic lethality phenotype of pash-1ts

Neuronally active *xbp-1s* increased the percentage of hatched embryos in *pash-1ts* animals, we wanted to ask whether GlcNac would have similar effect. In **Figure 12**, we see nearly 12% rescue of the embryonic lethal phenotype of *pash-1ts* mutants. In order to see the specificity of this phenotype, we used another allele of pash-1ts, *pash-1(pk2083),* which is independently isolated from *pash-1(mj100).* With this allele the effect of GlcNac was prominent, which is 45%. This may be the result of maternally contributed miRNAs in the *pash-1(pk2083),* which positively regulate the UPR^{ER} pathway. Then we used a deletion of *mir-35* miRNA family (*nDf50*) which is also embryonically lethal to see whether the effect

of GlcNac is miRNA specific and we saw 12% increase in the embryonic hatching, which is similar to the *pash-1ts* embryos.

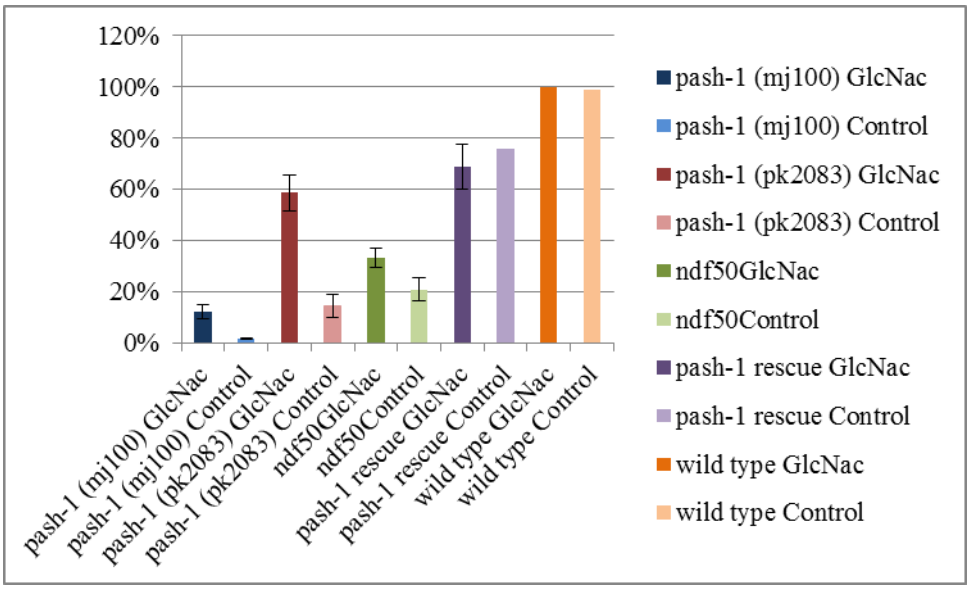
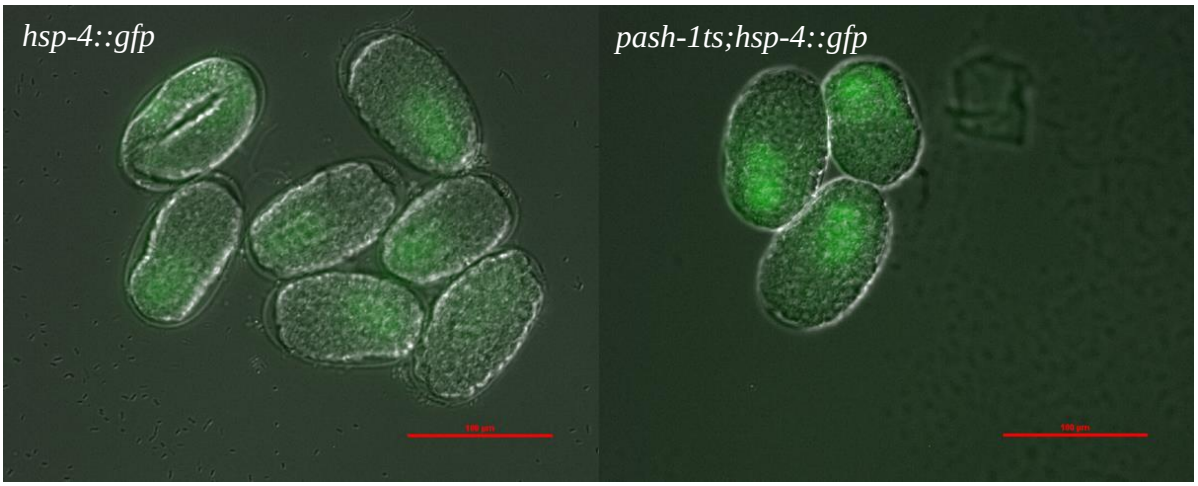


Figure 21: The effects of GlcNac on depicted strains: The same procedure was followed as in Figure 11.

A)



B)

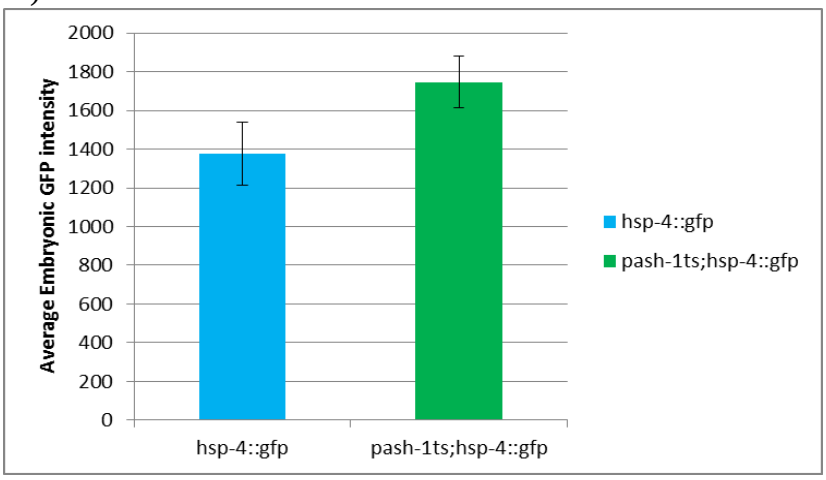


Figure 22: Embryonic expression of *hsp-4* in *pash-1ts* animals: *pash-1ts* and *pash-1ts;hasp-4::gfp* animals were picked as L4 staged worms to grow at 15°C till young adult. Young adult animals were then put at 25°C. Animals were laid eggs for 2 hours. Then embryos were handled with a platinum wire and put at 2% agarose pad. A) Fluoresnce images of embryos. B) Quantification of fluorescence signal in Figure 22.A. Quantification was done by quantification tool of NIS elements software program. Error bars represent the standard deviation of the mean GFP intensity of the embryos. (n=20 embryos for each strain)

In order to see whether the absence of miRNA biogenesis affects ER UPR in the embryos, I examined the expression of chaperones in the embryos of *pash-1ts* animals. *pash-1ts* has a strong embryonically lethal phenotype at the restrictive temperature [46]. If the absence of miRNAs causes in the upregulation of chaperone expression in the adult worms, we might expect to see this outcome during embryogenesis. I crossed pash-1ts animals to two different heat shock protein reporter strains in order to visualize the effects of loss of miRNA production to the levels of ER specific chaperones.

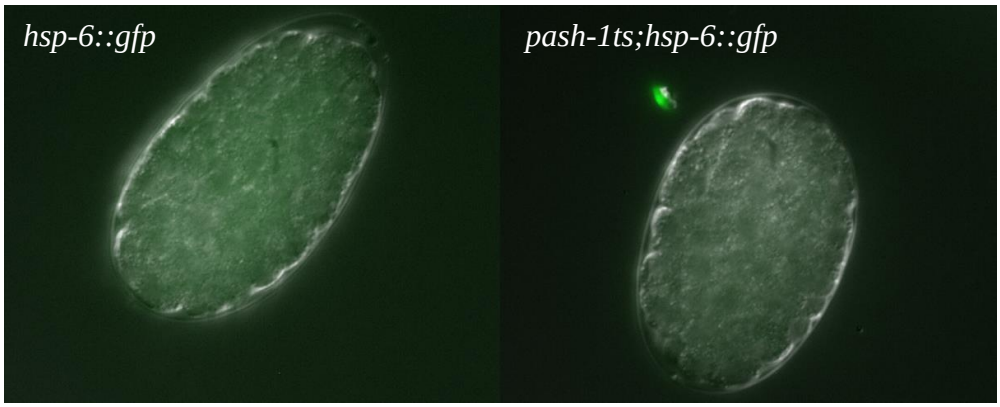


Figure 14: Embryonic expression of *hsp-6* in *pash-1ts* animals: The experiment was carried out in the same way with Figure 13.

hsp-4::GFP showed an up-regulation, at the gastrulation stage of the embryos while *hsp-6*, mitochondrial chaperone, did not show any upregulation in *pash-1ts* animals.

3.2.3 N-acetylglucosamine has a positive effect on the hypodermal+ muscle rescue lines of pash-1ts

Taking into account the cell-nonautonomous regulation of UPR^{ER}, we wanted to test the effect of N-acetylglucosamine on different tissue rescue lines of *pash-1ts. pash-1ts; mjEx365* is a transgenic line which expresses PASH-1 protein under the *col-10* and *myo-3* promoters in the *pash-1ts* background. *col-10* and *myo-3* the hypodermal and muscle specific promoters, respectively. *pash-1ts;mj383* transgenic line that contains *col-10p::pash-1* transgene, that expresses wild type copy of PASH-1 protein only in hypodermal cells. We observe there is no significant effect of GlcNac on *pash-1ts; mj383* (dark blue dashed line), while it increases the half-life of *pash-1ts; mjEx36* (dark green dashed line).

When we think of that GlcNac supplementation did not extend the lifespan of *pash-1ts* animals but it has some positive effects on hypodermal plus muscle rescue lines, we may think of again the contribution of miRNAs on the cell-nonautonomous signaling in ER stress.

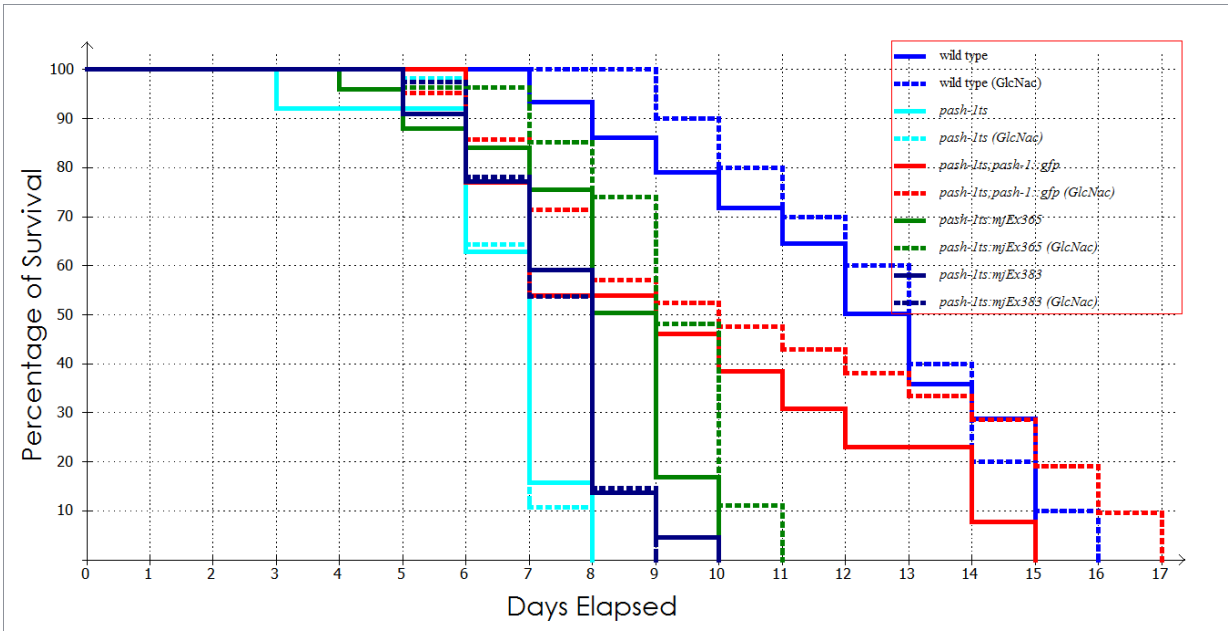


Figure 23: The effect of GlcNac to different rescue line of *pash-1ts*

Table 13: **Table 14: Percentage analysis of half-life and maximum life upon GlcNac**

	Half life	Maximum life
wild type	8%	7%
pash-1ts	0%	0%
pash-1ts;pash-1::gfp	11%	13%
pash-1ts;mjEx365	16%	10%
pash-1ts;mjEx383	0%	11%

Chapter 4

DISCUSSION

In our studies, we saw that the absence of miRNA production did not make worms hyper-synthesized upon ER stress. Indeed, DTT makes *pash-1ts* worms more resistant than the wild type worms. Similarly, TM, an inhibitor of N-glycosylation, did not have an effect on *pash-1ts* mutants' lifespan. This ER stress resistant phenotype of *pahs-1ts* made us to think that there might be a signaling problem for cells to activate the apoptotic cells under prolonged stresses. This hypothesis was likely since cancer cells use miRNA biogenesis down-regulation in order to circumvent the pro-apoptotic signaling pathways[55]. In order to test this hypothesis, I used another ER stressor thapsigargin shown to activate CHOP, a transcription factor activated via PERK branch of the UPR thought to control programmed-cell death of the cell. Although there is no identified CHOP homolog in *C. elegans*, there might be other proteins which are not homologous to CHOP but still activates the pro-apoptotic signals upon thapsigargin treatment. In order to search for CHOP homolog in *C. elegans* western blot analysis may be done with the antibody specific to mammalian CHOP protein.

While I was seeking for an explanation for ER stress resistant phenotype of *pash-1ts* mutants, I saw a paper in which DROSHA knock-down make cells resistant against ER stress[56]. Although these experiments were performed in cell lines, what I observe with *C. elegans* was very similar to their observations.

Apoptosis is a programmed-cell death depending on a family of proteases. There is a cysteine at active site of these proteases and they cleave their target proteins at aspartic acids so that they are called as caspases. The link between ER stress and apoptosis was a mysterious situation until caspase 12 was identified. Caspase-12 has similarities with other

caspases being synthesized as an inactive proenzyme composed of a regulatory domain and two catalytic domains. However, different than other caspases it is highly specific to the damages raised from ER stress and it is not proteolytically triggered by other death inducer [57]. It is suggested that caspase 12 activation is linked to Ire1 pathway. As it was mentioned in the introduction part, Ire1 interact with TRAF2 through its cytosolic domain. When Ire1 is overexpressed, TRAF2 can interact with caspase-12 and initiates its activation[5]. Moreover and most importantly for this research Ire1 overexpression was shown to induce apoptosis when overexpressed[58]. This may be the reason why we observe short lifespan of the *pash-1ts;rab-3p::xbp-1s* animals comparing to *pash-1ts*. The activation of apoptotic pathways due to *xbp-1s* overexpression may act like an apoptotic inducer downstream of the ER dependent apoptotic pathway.

In our experiments with different rescue lines of *pash-1ts* we saw an enhancement in the half-life and the maximum life of the animals when we apply GlcNac. However, we do not see a positive effect of GlcNac in *pash-1ts* animals. *pash-1ts* mutants injected with the wild type copy of PASH-1 protein under the promoter of both hypodermal and muscle tissues, have 16% increase in the half-life and 10%incrase in the maximum life. On the other hand, pash-1ts mutants only with the muscle rescue have no improvement in the half-life but in maximum life. This result may be speculated as the hypodermal rescue line has more prominent effect over muscle rescue when UPR^{ER} is improved. When we consider the fluorescent images of *hsp-4::gfp* animals, ER stress was observed mostly in the hypodermal cells in the absence of miRNAs. Maybe the rescue of this highly stressed tissue has an apparent effect on the whole organism since miRNAs have important functions in the UPR^{ER} of hypodermal cells. Reason why we observe no effect of GlcNac on pash-1ts worms may be because of the some tissues may be affected negatively while some of them are affected positively from GlcNac treatment which cancels out each other. I would like to see the effect of GlcNac on neuronal rescue lines of *pash-1ts* which has the most significant

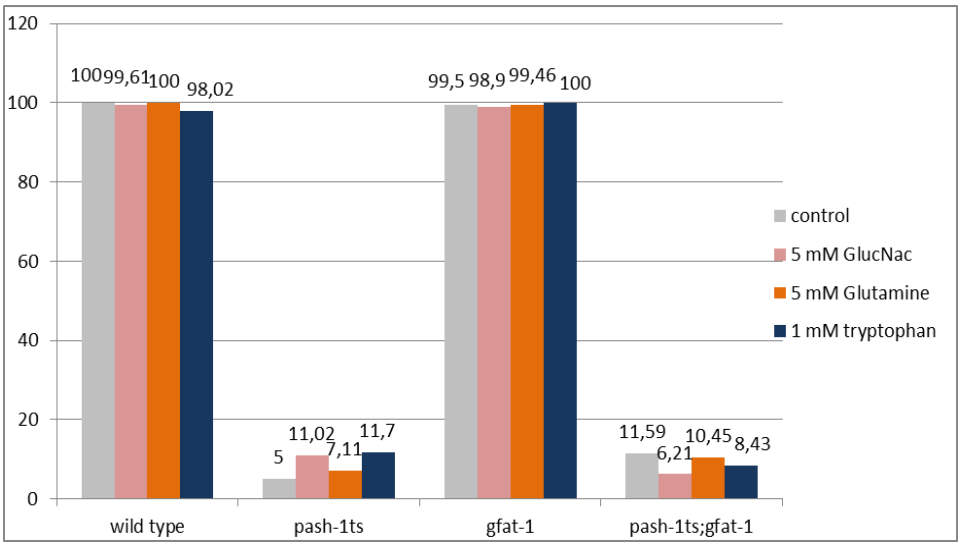
improvement in the lifespan of worms; however, we do not have this strain. I would like to also see the effects of ER stressors on different rescue lines.

Chapter 5

APPENDIX

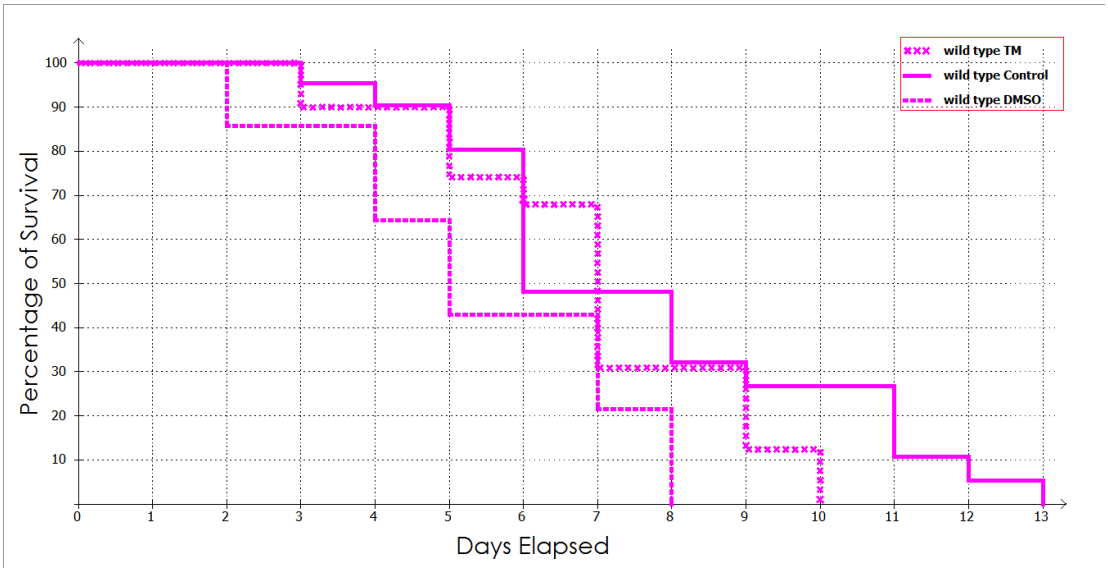
5.1. Glutamine and Tryptophan have no effect on the hatching of embryos

I choose glutamine and tryptophan for a couple reasons to test their effects on embryonic lethality. Glutamine is an amino acid utilized as an amine donor by GFAT-1 enzyme to produce glucosamine 6-phosphate from fructose-6-phosphate. I wanted to test the effect of exogenous supply of glutamine; however, I did not observe any effect. Secondly, I wanted to see the effect of tryptophan on the hatching of embryos since it has been shown a UPR^{ER} upregulator [59]. Again there was a slight difference comparing to control embryos.



5.2. 2% DMSO concentration has an effect on the life span

For the first TM experiments we used 1mg/ml TM stock concentration, which makes final 2% DMSO concentration for the 20µg/ml TM plates. However, we observed an effect to this concentration in wild type worms. So we decided to continue with 6mg/ml stocks to decrease the DMSO concentration under 0.5%[54].



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