Auxin-dependent modulation of the Unfolded Protein Response in *Caenorhabditis elegans*.

A thesis

submitted to Indian Institute and Science Education and Research, Pune in partial fulfilment of the requirements for BSMS Dual Degree Program by

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Epigenetic regulation of cell identity and environmental stress response.

LBMC, ENS de Lyon.

Certificate

This is to certify that this dissertation entitled "**Auxin dependent modulation of the Unfolded Protein Response in *Caenorhabditis elegans*.**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by **Anupam Bhoi** at the "**École normale supérieure (ENS) de Lyon**" under the supervision of "**Dr Francesca Palladino, Principal Investigator at the Laboratoire de Biologie et Modélisation de la Cellule (LBMC)"** during the academic year 2019-2020.
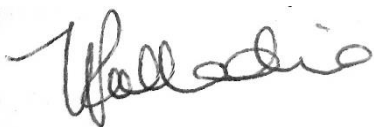
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Declaration

I hereby declare that the matter embodied in the report entitled "**Auxin dependent modulation of the Unfolded Protein Response in *Caenorhabditis elegans***" are the results of the work carried out by me at **Laboratoire de Biologie et Modélisation de la Cellule (LBMC), École normale supérieure (ENS) de Lyon,** under the supervision of **Dr Francesca Palladino** and the same has not been submitted elsewhere for any other degree.

Signature of the supervisor

Signature of the student

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Contents

Abstract

The Proteostasis network, through a co-ordinated action of an intricate intracellular signaling network, serves to maintain a state of balanced proteome referred to as protein homeostasis or proteostasis. Endoplasmic Reticulum (ER) is responsible for proper folding and assembly of almost one-third of all secreted and membrane proteins thus contributing to maintaining cellular and organismal proteostasis. However, perturbations due to genetic or environmental factors can disrupt ER homeostasis and cause the accumulation of misfolded proteins in the ER lumen (ER stress). To cope up with the burden of misfolded proteins in its lumen, the ER upregulates a set of conserved and highly specific intracellular signal transduction pathways collectively known as the Unfolded Protein Response (UPR). The UPR is characterized by the induction of protein folding chaperones, degradation of irreversibly misfolded proteins, protein translation attenuation and activation of apoptosis under prolonged ER stress. A decline in proteostasis is one of the hallmarks of aging and is a major cause for many age related diseases such as neurodegenerative, cardiovascular, and others. My findings indicate that the plant hormone, indole acetic acid commonly known as auxin, can modulate the UPR in the nematode *Caenorhabditis elegans,* providing resistance to ER stress. In my thesis work, I have shown that auxin provides tolerance against ER stress by upregulating the transcription of some of the well-established UPR target genes via the IRE-1/XBP-1 branch of the UPR. Since auxin is an indole derivative and indoles have recently been shown to promote organismal healthspan, my results point to the auxin-dependent activation of the UPR as a mechanism to improve healthspan.

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List of abbreviations

AHR	Aryl Hydrocarbon Receptor
ATF6	Activating Transcription Factor 6
BiP	Binding immunoglobulin Protein
CDC42	Cell Division Control protein 42
CHOP	C/EBP homologous protein
CKB-2	Choline Kinase B2
Ct	Threshold cycle
eIFα	eukaryotic Initiation Factor α
ER	Endoplasmic Reticulum
ERAD	Endoplasmic reticulum Associated Degradation
ERSE	Endoplasmic Reticulum Stress Response Elements
GADD34	Growth Arrest and DNA damge-inducible 34
GRP94	Glucose Regulated Protein 94
HSP-4/3	Heat shock Protein-4/3
IRE-1	Inositol Requiring Enzyme-1
PERK	Protein Kinase like ER Kinase
PN	Proteostasis network
qRT-PCT	quantitative Real Time PCR
RIDD	IRE-1 Dependent Decay
SRP	Serpin
Tg	Thapsigargin
Tm	Tunicamycin
UPR	Unfolded Protein Response
XBP-1	X-box binding protein-1

Introduction

The Proteostasis network.

Proteins must fold and remain folded in their well-defined 3D structures throughout their lifetime to perform their biological functions. Moreover, their abundance and functionality in each cell must be carefully and tightly controlled. This state of a balanced proteome is called protein homeostasis also known as proteostasis which is regulated by an extensive network coordination between protein biogenesis, molecular chaperones, proteolytic systems, and their regulators. This proteostasis network coordinates the functions of its three interlinked arms: protein synthesis and folding, conformational maintenance and degradation, to maintain a balanced proteome in a cell (Figure-1, Hipp, 2019). However robust the network may be, cellular proteostasis is disturbed by various external and internal factors like for example; during environmental stress, DNA damage, protein misfolding, aging etc.
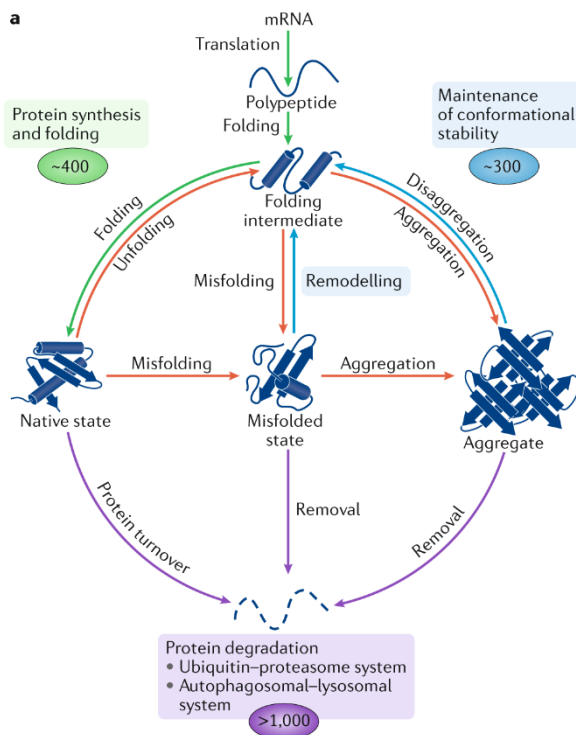

Figure 1: The components of the proteostasis network.

The quality control pathways work at every step to prevent the formation of protein aggregates.

ER stress

The fact that under normal physiological conditions proteins do not misfold, aggregate or accumulate is due to the presence of protein "quality control" mechanisms. The cellular organelle endoplasmic reticulum (ER) has its own mechanism to maintain protein homeostasis (Kaushik and Cuervo, 2015). The ER is a multifunctional organelle that serves as an essential compartment for synthesis, folding and maturation of the secretory and transmembrane proteins (Stress, 2015). The ER provides an oxidizing environment suitable for proper protein folding and it is the site of synthesis and folding of about one-third of the total proteome of the cell. ER located proteins regulate accurate translation, and proper quality control through the activity of its multiple chaperons, foldases, and co-factors that assists the folding of nascent or misfolded proteins and eventually transports improperly folded proteins through a degradation pathway, thus preventing abnormal protein aggregation and resultant proteotoxicity(Ron, 2002). Perturbations of the protein folding by environmental or genetic factors lead to misfolding of proteins and accumulation of misfolded protein in the lumen of the ER a condition known as ER stress. Different types of cellular stress such as perturbations in calcium homeostasis, elevated protein synthesis, glucose deprivation, altered glycosylation, redox changes, ischemia, viral infection, cholesterol overload lead to the accumulation of unfolded proteins that disrupt the ER buffering capacity and poses a fundamental threat to the cells (Zhang and Kaufman, 2004). As a consequence, the cells engage a highly conserved signaling pathway collectively called the Unfolded Protein Response (UPR) to limit the accumulation of unfolded proteins and restore the normal functioning of the ER(Hetz et al., 2015).

The Unfolded Protein Response

The UPR is a transcriptional and translational intracellular stress response activated by the accumulation of unfolded proteins in the lumen of the ER. (shen et al 2001). It is a way of a cell to respond to environmental or endogenous stress which disrupts the process of protein folding taking place in the ER. The UPR operates as a homeostatic control circuit that regulates the protein folding and secretion capacity of the cell according to needs. (Walter et al., 2006). It monitors the status of the ER and when senses an inefficiency in ER's protein folding capacity and the threats of misfolding,

communicates this information to gene expression machinery which triggers three kinds of cellular responses(Walter and Ron, 2012).

i.   Upregulation of expression of ER chaperones and folding proteins to assist in protein folding.

ii. Attenuation of de novo protein translation to reduce the load on the ER.

iii. Degradation of misfolded proteins by proteasome by a process called ER associated degradation (ERAD) (Brown and Naidoo, 2014).

iv. Direct the cell to programmed cell death - apoptosis if the cell cannot be salvaged from proteotoxicty.

The UPR activation upregulates genes whose products expand the ER membrane, increases ER abundance and equips the expanded organelle with newly synthesized protein folding machinery with the primary aim to re-establish protein homeostasis (Schuck et al., 2009). The control of gene expression by the UPR depends upon cellular context and stimuli since the UPR regulated transcription factors can interact with other proteins to drive specific responses. In a nutshell- the UPR, under ER stress, reprograms the cell towards adaptation, sustaining cellular functions by refolding and degrading unfolded proteins or to resort to cell death programs under irreversible conditions (Mart et al., 2017). The UPR activates autophagy in order to remove the aggregates of misfolded proteins that cannot be degraded by the ERAD pathway (Ogata et al., 2006).

The UPR is initiated by three ER membrane sensors that transduce ER stress signals across the ER membrane. The three transmembrane signaling components- Inositol Requiring Enzyme 1 (IRE1), Protein kinase like ER kinase (PERK) and Activating transcription factor-6 (ATF6) are the three principal transducers of the UPR and operate in parallel using their unique mechanisms of signal transduction (Walter and Ron, 2012). Under non stress conditions, all these three sensors are kept in an inactive state at the ER membrane bound to an ER chaperone known as Binding Immunoglobulin protein (BiP) which serves as the master regulator of the UPR. Upon accumulation of unfolded proteins in the lumen of the ER, BiP dissociates from the sensors to attend to the misfolded proteins, allowing the activation of one or more of these signal transducers(Shen et al., 2004).

IRE-1

IRE-1 is the most conserved of the ER sensors being found in all eukaryotes (Cox et al.,1993). IRE-1 is a bifunctional transmembrane protein that has a cytoplasmic domain with kinase and nuclease activity. Upon activation, IRE-1 oligomerizes that in turn leads to its trans-autophosphorylation which activates its endoribonuclease domain. (Sidrauski and Walter, 1997). IRE-1 endoribonuclease activity unconventionally excises an intron from the mRNA encoding X-box binding protein 1 (XBP-1), generating the functional spliced variant XBP-1s (Calfon et al., 2002). Spliced XBP-1 functions as a potent transcription factor that activates genes involved in protein maturation, protein folding, ER expansion as well as export and degradation of misfolded proteins via ERAD (Yamamoto et al., 2007); Lee et al., 2002). IRE-1 also degrades ER bound mRNA encoding secretory proteins, via a process called regulated IRE-1 dependent decay (RIDD) to reduce protein influx into the ER lumen and alleviate the organelle work load (Hollien and Weissman., 2006). The UPR activates autophagy in order to remove aggregates of misfolded proteins that cannot be degraded by the ERAD pathway (Brown and Naidoo, 2014).

PERK

As IRE-1, the second sensor PERK is a transmembrane serine threonine kinase held in an inactive monomeric state by binding to BiP. Upon activation by ER stress, PERK homodimerizes and phosphorylates itself as well as the ubiquitous translation initiation factor eIF2α on Ser-51 indirectly inactivating the activity of its own guanine nucleotide exchange factor eIF2β, which results in the formation of a stalled 43s ternary complex inhibiting mRNA translation (Harding et al., 2000). As a consequence global protein synthesis is reduced and also the flux of protein entering the ER. Notably, phosphorylation of eIF2α also promotes the translation of some selected mRNA that contains short open reading frames in their 5' untranslated regions, such as those coding for Activating transcription factor 4 (ATF4) and BiP (Ordonez et al, 2015); (Harding et al., 2000). ATF4 activates genes encoding amino acid transporters and antioxidant factors that contribute to the cell's adaptation to stress. In mammals ATF4 also induces expression of transcription factor CHOP and GADD34 (Novoa et al., 2001). CHOP controls genes encoding components involved in apoptosis. Thus the PERK branch is protective at a moderate level of stress, it may also signal for cell

death under prolonged stress. GADD34 is involved in eIF2α dephosphorylation which counteracts PERK and complets a negative feedback loop promoting the recovery of protein translation (Novoa et al., 2001; Novoa et al., 2003)

ATF6

ATF6 is an ER resident transmembrane 90kDa basic leucine zipper transcription factor with N terminal domain in the cytosol and a large C-terminal ER luminal domain that senses ER stress. When activated during stress, ATF-6 is translocated to the Golgi apparatus where it is cleaved by site1 and site2 proteases (S1P and S2P) (Haze et al., 1999). The liberated 50 KDa N-terminal cytosolic fragment translocates to the cell nucleus where it binds to the ER stress response elements (ERSE) and activates UPR target genes (Yoshida et al., 1998). ATF6 upregulates predominantly the expression of ER resident proteins like BiP, Glucose Regulated Protein 94 (GRP94) to improve the protein folding capacity of the ER(Okada et al., 2002). However, it also activates other gene targets such as XBP1, CHOP and protein disulfife isomerase (PDI)(Marciniak et al., 2004). Hence ATF6 branch of the UPR mostly works by improving the protein folding capacity of the ER. The 3 UPR branches extensively crosstalk and synergistically work together to provide protection against ER stress.
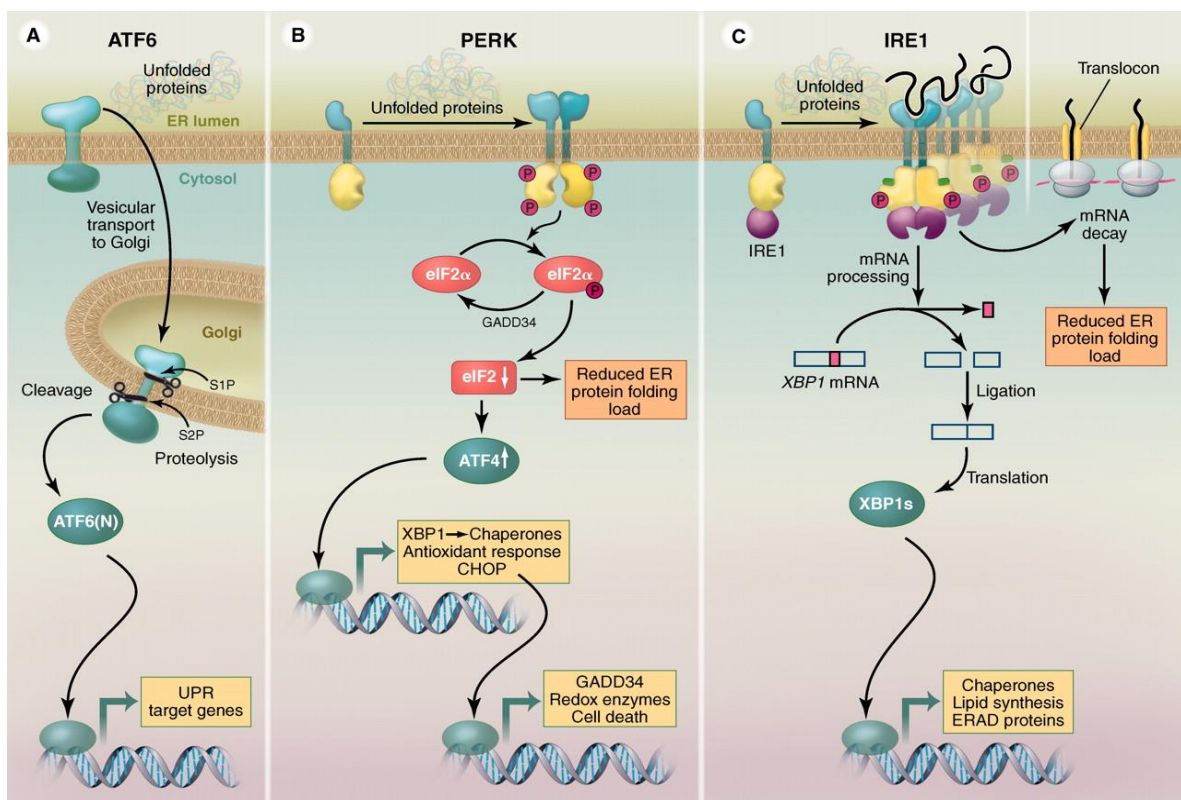

Figure 2: The three UPR branches and their action pathways(Walter and Ron, 2012)

ER stress in human diseases.

A major and common pathological marker for many neurodegenerative disorders such as Alzheimer's, Parkinson's, ALS and Huntington's diseases, is the accumulation of unfolded proteins in the neurons (Skovronsky et al., 2006; Hetz and Saxena, 2017). A decline in UPR activation and proteostasis maintenance by ER is also one of the major causes of ageing and many age related disorders such as atherosclerosis, cardiovascular diseases, and metabolic disorders like diabetes and obesity.

Alzheimer's is a neurodegenerative disease that exhibits the accumulation of β-amyloid and tau proteins (Katayama et al., 2004). Studies have shown that accumulation of β-amyloid in ER lumen disturbs calcium homeostasis in the ER that leads to activation of UPR which in turn induces apoptosis of neurons (Hetz and Saxena, 2017). Similarly, Parkinson's is caused by an accumulation of α-synuclein in ER impacting ER homeostasis including depletion of ER chaperones and inhibiting ER to Golgi trafficking (Bellucci et al., 2011; Cooper et al., 2006). Recent studies have also linked ER stress to Huntington's disease- Polyglutamine increases the accumulation of unfolded proteins by causing defects in lysosome mediated protein degradation (Vidal et al., 2011) in the neurons.

Obesity is also known to be associated with increased ER stress and activated UPR in many tissues such as the hypothalamus, liver, muscle and adipose tissues (Cnop et al., 2012). Activated UPR plays an important role in the development of obesity related disorders particularly type-2 diabetes. During high insulin demand, pancreatic β cells exceed their protein folding capacity and generate ER stress. Prolonged ER stress leads to UPR mediated apoptosis in β cells, eventually causing insulin deficiency (Song et al., 2008; Wang and Kaufman, 2012).

Recent studies in cancer fields have demonstrated that ER stress and UPR are upregulated in various tumor types and are associated with cancer cell survival (Wang et al., 2010). The adaptive arm of UPR provides survival signals that are conducive to tumor growth, whereas apoptotic arm that contributes to cell death and normal growth, is suppressed. Cancer cells evade the apoptotic pathways to differentially activating UPR branches.

Hence, studying the stress sensors for developing therapeutics drugs that reduce ER stress by promoting correct folding of proteins, improving the efficiency of ERAD and/or enhancing detection of misfolded proteins may prove useful in delaying or preventing some of those disorders. Targeting UPR can offer some interesting therapeutic opportunities for cancer treatment.

Caenorhabditis elegans

To study the effect of ER stress and its modulation by external factors I used one of the most versatile model organisms- the nematode Caenorhabditis elegans. *C. elegans* is a widely used model organism that has been extensively applied to research focusing on development, apoptosis, aging, neurodegenerative diseases to name a few for decades. Its ease of handling and genetic manipulation, fully sequenced genome, and short lifespan, make the organism highly advantageous over traditional in vivo animal models. *C. elegans* provides a great platform for studying disease pathologies and conditions related to ER stress and the UPR. Moreover, *C. elegans* mirrors the basic biology of vertebrates more than 80% of its proteome is homologous to that of humans and about two-thirds of its genes are related to human disease (Dexter et al., 2012). More than 3000 available genetic mutants, effortless RNA interference (RNAi), microinjection, as well as genetic crosses, make this animal a powerful toolbox in many life sciences research.

C. elegans, exist as both males and hermaphrodites, has a short lifecycle of about 3 days at 20°C and a relatively mean lifespan of about 2 to 3 weeks, accompanied by a large brood size of around 300 progeny. In addition to that, the worm has a transparent body due to which all its internal systems and tissues are easily visible by a simple light microscope which allows easy examination of cellular as well organ development and changes due to mutation or altered environment. It is easy to maintain on solid or liquid media where it feds non-pathogenic Escherichia coli OP50.

C. elegans is a free living nematode 1 to 1.5 mm in length in its adult stage. Its 3 days life cycle includes an embryonic stage, 4 larval stages - L1 to L4 and the adult stage. The embryos or the eggs have an impermeable shell that isolates and protects them from the outside environment. Eggs hatch into L1 stage worms which with adequate

food grow into L2 to L4 stages and eventually adults during the developmental process. Worms molt and the cuticle of the worms reformed at the end of each larval stage. Under unfavorable conditions such as the absence of food, high temperature or overcrowding the worms arrest their development and enter into a dormant state called the "dauer larva" stage (Corsi et al., 2015)They can survive in the "dauer larva" stage for approximately 3 months and once the favorable environmental condition is reestablished, they can resume growth to adulthood.

The presence of well-defined tissues and organs makes this organism ideal for tissue specific studies. The reproductive tissue of the adult hermaphrodites is comprised of the somatic gonad and houses the germline and egg laying apparatuses. Almost 99% of the worms are self-fertilizing hermaphrodites, which helps to preserve homozygous clones, hence genetic mutant strains are easy to maintain without regular crossings.
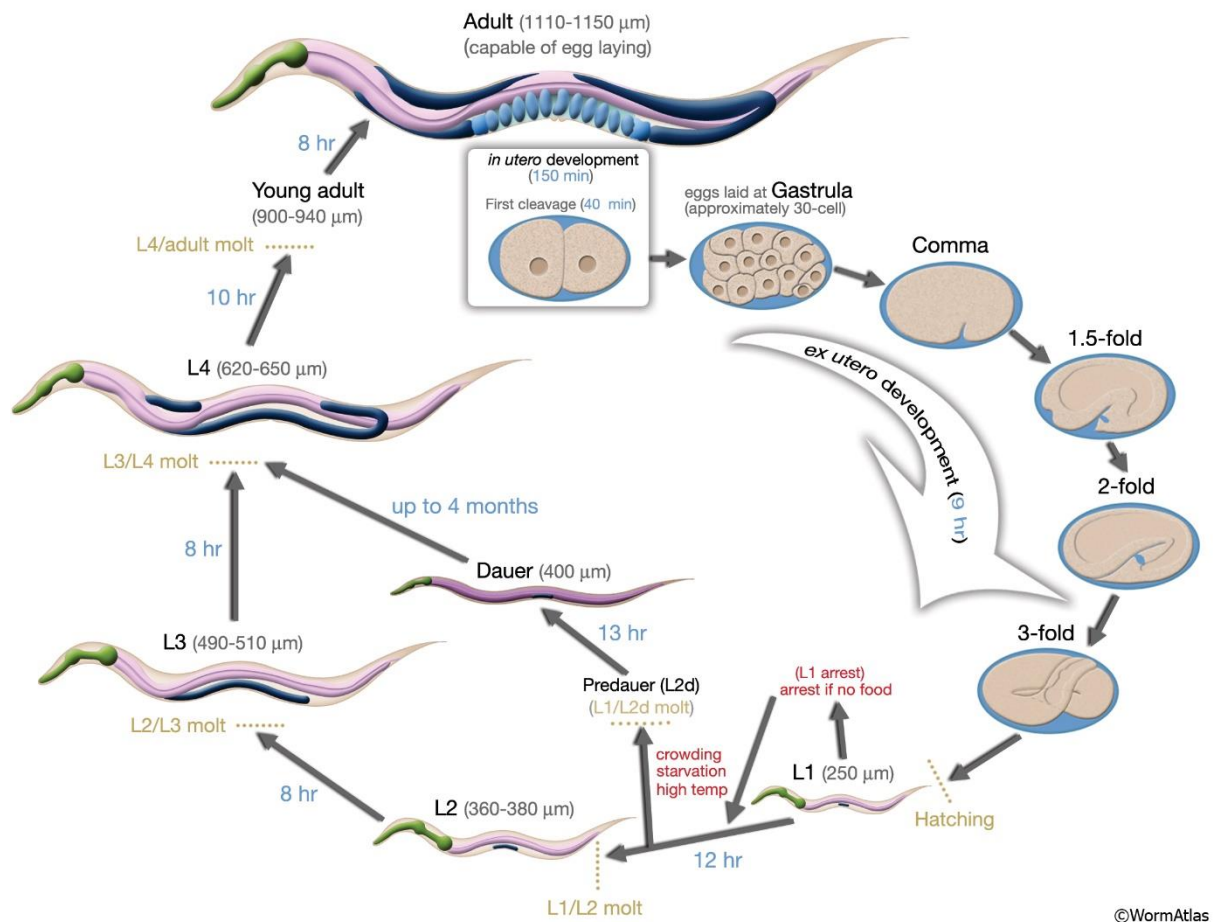

Figure 3: *C.elegans* life cycle

It shows all embryonic phase, all the larval stages, and the adult. Dauer larva is an alternative L3 developmental pathway that the worm enters under unsuitable environmental conditions. The adult worm is reproductive for 2-3 days but lives for 2-3 weeks.

UPR in *C. elegans*

C. elegans has two homologs of mammalian BiP, HSP-3, and HSP-4. The most prominent and studied pathway in *C elegans* is the XBP-1/IRE-1 pathway. Similar to mammalian system XBP-1 is spliced by the endoribonuclease activity of IRE-1. Spliced XBP-1s can upregulate the HSPs and other target proteins in worms as well as ERAD components and other chaperones. C. elegans responds to agents that specifically disrupt protein folding in the ER by transcriptional induction of *hsp-3* and *hsp-4*

The HSPs or Heat shock proteins are a ubiquitous family of gene products present in cells under unstressed conditions for which they function as molecular chaperones. They are expressed in much higher concentration owing to the presence of stress. The HSPs play a critical role in normal homeostasis to assist protein folding, direction of newly formed proteins to target organelles, the assembly or disassembly of protein complexes, inhibition of improper protein aggregation, such as may occur owing to crowding or thermal denaturation and activation of the initial immunological system in response for selected disease. In response to stress, HSPs assist in refolding and repair of denatured proteins as well as facilitating synthesis of new proteins to replace damaged ones.

The PEK-1 branch in worms works in a similarly to that of mammalian system, activating eIF2α and in turn ATF-4, however unlike mammalian system, the apoptotic pathway is absent in worms. The specific downstream targets of ATF-6 and PEK-1 branches are still not known, however, in *C. elegans, atf-6* acts synergistically with *pek-1* to complement the developmental requirement for *ire-1* and *xbp-1*(Shen et al., 2005).

Nematodes are ideal for elucidating the UPR because these pathways are similar but simpler than those in mammals and worms with mutations in these pathways are viable and convenient for analysis. Study of downstream targets in worms should provide insight into how these signaling pathways act in disease and health and they provide an ideal model to identify and evaluate molecules that influence the UPR signaling for drug development.

Indoles Promote healthspan in *C. elegans* and other species

Indoles are aromatic heterocyclic ring compounds, having a bicyclic structure consisting of a benzene ring and a pyrrole ring. Indoles are widely distributed in the natural environment and are produced by a wide variety of bacteria. Indoles and its derivative are important molecules in bacterial physiology regulating various processes such as spore formation, biofilm formation, drug resistance, virulence. Indoles have applications in a wide range of pathophysiological conditions such as cancer, microbial and viral infections, depression, inflammation, hypertension, and emesis and so on (Chadha and Silakari, 2018). Indole is a prominent phytoconstituent across almost all plant species. Essential amino acid tryptophan is a derivative of indole, which is the precursor of the neurotransmitter serotonin. Indole is biosynthesized by the shikimate pathway in plants and bacteria.

Sonowal et al., 2017 have shown that indoles derived from commensal bacteria increase healthspan in *C. elegans*, D. Melanogaster, and mice. They demonstrate that indole acting via the aryl hydrocarbon receptor (AHR), extend reproductive lifespan and contribute to maintaining a youthful gene expression profile in aged worms. They have concluded that indoles produced from microbiota function to promote healthy aging in different species.

The Palladino lab has previously discovered that the plant hormone Indole acetic acid, an indole derivative commonly known as auxin, protects worms against ER stress induced by the N-glycosylation inhibitor Tunicamycin. Lifespan and healthspan extension is often associated with resistance to stress in a range of model organisms and in some cases a causative role for stress resistance in the regulation of longevity has been demonstrated (Haigis and Yankner, 2010)

In my thesis work, I have explored the role of auxin in the regulation of the ER stress response. My results indicate that auxin provides tolerance against ER stress by upregulating some of the well-established UPR target genes via the IRE-1/XBP-1 branch of the UPR and provide novel clues to understand the mechanisms responsible for the effect of indoles on organismal healthspan.

Materials and Methods:

Caenorhabditis elegans strains used

a) N2 (Bristol)

b) *xbp-1 (zc12)*

c) *xbp-1 (tm2457)*

d) *ire-1 (ok799)*

e) *atf-6 (ok551)*

f) *pek-1 (ok275)*

g) *ckb2::gfp (BC14636)*

h) *phsp-4::gfp (zcIs-4)V (SJ4005)*

i) *unc-54::SRP2^{H302R}::gfp (PP657)*

Culture medium and M9 buffer

a. All nematode strains were maintained on solid nematode growth medium (NGM) prepared as follows:

Distilled water containing 2.0% agar, 0.3% NaCl, and 0.25% Bactopeptone is autoclaved before adding 1mM $CaCl_2$, 1mM $MgSO_4$, 25mM pH-6 KPO_4, 5mg/ml cholesterol. The medium is poured into sterile petri dishes of 35mm, 60 mm or 90 mm as needed. Appropriate drugs are added to the solution before pouring onto plates and then the media is allowed to solidify. A Liquid LB culture of E. coli strain OP50 is seeded on the surface of the agar plates which were then allowed to dry for 24 hours at room temperature. Plates containing auxin were protected from light since auxin is light sensitive.

b. M9 buffer is used for washing or synchronizing worms contains 3% KH_2PO_4, 7.5% Na_2HPO_4, 5% NaCl (all w/v). The solution is sterilized by autoclaving before adding 1%$MgSO_4$.

c. Drugs used in experimental cultures- Tunicamycin (Tm) and Thapsigargin (Tg) are ordered from Enzo Life-Sciences in units of 10mg and 5mg solid pellets. 10mg/ml Tm and 10mM Tg stocks were prepared by dissolving the solid pellets in 1ml and

768µl of Dimethyl Sulfoxide (DMSO) respectively. Aliquots of smaller volumes are made and stored at -20°C as working stocks. The stocks are prepared as fresh as possible before any experiment to ensure maximum effect. A 400 mM of Auxin stock is prepared by dissolving indole acetic acid (IAA powder), from Sigma Aldrich, in absolute ethanol. The solution is stored at 4°C and used for no longer than 2 to 3 weeks.

C. elegans maintenance

Worms are maintained on NGM plates between 15°C and 25°C. Most of my experiments were performed at 20°C. AT this temperature worms develop approximately 1.3 fold faster than at 15°C. If the worms are starved i.e., the plates do not have any OP50 left as food or contaminated, a few healthy/young worms are transferred to a fresh plate by using a sterile titanium pick or by transferring a chunk of agar from the old plate to a fresh one. Healthy, unstarved worms growing on uncontaminated plates are used for experiments.

Synchronization by bleaching

NGM plates containing large numbers of gravid adult worms and unhatched embryos are washed with M9 media. The plate is stirred and gently scratched by a pipette tip to resuspend the worms and the embryos. The media is then collected in a falcon tube and centrifuged at 800 RCF for 1 min. The supernatant is removed by a means vacuum pump and discarded. 10 ml of Bleach solution freshly prepared by mixing 1 mM NaOH, H_2O and 5% NaOCl (bleach) in the ratio 5:3:2, is added to the pellet and vigorously vortexed for 5-7 min or until all the worms have disintegrated and only unhatched embryos are left in the mix. The tube is spinned and the supernatant is discarded leaving a pellet of only unhatched embryos which are then washed atleast 3 times with M9. After the last wash, the pellet is resuspended with M9, transferred to a 50ml conical flask and incubated overnight at 20°C with gentle shaking. After overnight incubation, most of the embryos are hatched but due to lack of nutrients, they are arrested at the L1 stage and hence synchronized at the L1 stage. The content of the flask is recovered by centrifugation resuspended in approximately 30 µl. Synchronized worms are then transferred onto a fresh plate for them to grow.

Developmental stress assays

4 to 6 day-1 adult worms are allowed to lay eggs on 3cm NGM plates containing either Tunicamycin or Thapsigargin as needed. Tunicamycin is a potent ER stress inducer that causes protein misfolding by inhibiting N-linked glycosylation. Thapsigargin inhibits ER Ca^{2+}-ATPase (SERCA pumps), thus blocking the ability of the cell to transport calcium from cytosol to ER. This causes depletion of Calcium in the ER that leads to ER stress. Drug concentration varied according to the experiment and is mention in the results section. After 3 to 4 hours, the adult worms were removed and the number of embryos was counted and noted down. They were allowed to develop at an incubation temperature of 20°C. On day 4 after egg laying, the number of L4 – The adults were counted. The results are expressed as the ratio between L4-adults and laid embryos per strain per condition and are represented with bar graphs. Each experiment was done in replicates of 6 plates per strain per condition and a minimum of atleast 3 such independent experiments were conducted for statistical analysis.

Adult stress assays

Worms are synchronized by bleaching and allowed to grow until reaching adulthood. The adult worms are then transferred to plates containing the stress inducers. I used 30-40ug/ml of Tunicamycin as stress inducing drug. 150 worms in total were transferred on 6 plates, 25 worms/plate. The number of dead, alive and censored worms were counted for each plate and noted down every day until they are all dead. Censored worms are those A Kaplan Mayer curve showing the percent alive over time was plotted using the Oasis POSTECH application for survival analysis. The software provides Log Rank analysis and significance based on each graphical point.

Hormesis assays-

Hormesis or hermetic stress resistance is a phenomenon by which organisms develop a better adaptive response to high intensity stress when exposed to mild stress beforehand (Salminen and Kaarniranta, 2010). Low, mild stress may increase resistance and longevity whereas prolonged overwhelming exposure has detrimental effects (Gems and Partridge, 2008). For my hormesis tests, L4-stage worms were placed on plates with low doses of drugs (in this case auxin) for 24 hours as

pretreatment. Pretreated and control animals were transferred to 3 cm Tm containing NGM plates for developmental stress resistance assays.

Protein aggregation assays

For these assays, I used the PP657 strain of worms as mentioned above. This strain expresses SRP2^{H302R}:YFP transgene exclusively in the muscle cells. SRP-2 is one of the 9 serpins like proteins expressed in worms which is the closest homolog to the mammalian neuroserpin. The H302R point mutation (histidine to arginine exchange) at codon 302 of SRP2 protein causes it to misfold, polymerize and aggregate. SRP-2 aggregates are visible as bright fluorescent particles under a microscope (figure-4) due to the YFP tag added at the C-terminal of the protein. In a standard experiment, worms are allowed to lay eggs on NGM plates containing TM, auxin or both. After 3-4 hours the adults are removed and the embryos are allowed to develop at 20°C. 5 to 10 worms are taken from the plate, paralyzed on an agar pad using levamisole or sodium azide and observed under the microscope. For my observations, I used a fluorescence microscope (Axioplan from zeiss) equipped with a differential interference contrast (DIC) system and various fluorescence filters. The number of aggregate particles is counted in the head region of 20 worms at each developmental stage per experimental condition and a time course graph representing the average number of aggregates corresponding to each life-stage of worms is plotted. The amount of aggregates provides a quantitative representation of the protein aggregation in response to different stimuli.
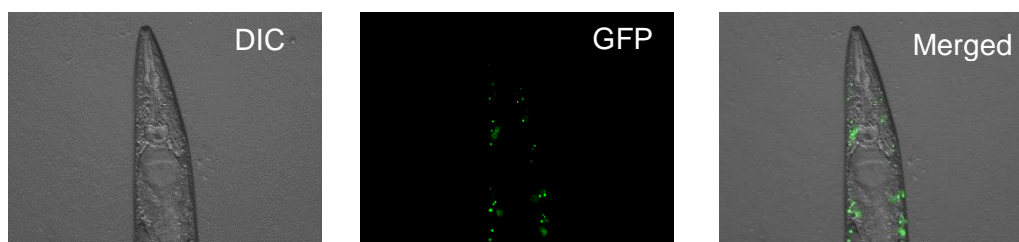

Figure 4: Serpin aggregation

DIC, GFP and merged image of head region of *Unc 119::unc54::srp2H3092R::yfp* strain. The microscope image shows the SRP2^{H302R} aggregated particles as green fluorescent dots.

Protein extraction and western blot-

50 ml stock of Solublizing buffer TNET composed of 50mM pH8 Tris HCl, 300mM Nacl, 1mM EDTA and 0.5%(w/v) Triton X-100 was prepared. Worms are collected in M9 buffer and washed multiple times. After the final wash worms are transferred to a 1.5ml eppendorf tube and centrifuged to get a pellet of 40 to 50 µl and the supernatant is discarded. Lysis buffer is prepared by adding 1µl PMSF and 20µl protease inhibitors to 1ml of TNET buffer. 150ul of lysis buffer (TNET+PI+PMSF) buffer is added to each of the pellets and later added half of the total volume of Lysis matrix Y beads (MP biomedical). The worms are ruptured using a Precellys tissues homogenizer apparatus by subjecting to 2 rounds of vigorous vortexing at 6000 RPM for 20 sec with a 10 sec interval. The tubes are centrifuged at 1400 RPM for 2 mins to settle the beads. The supernatant is collected with a 25G needle into a 1ml syringe and transferred to a 500µl of Eppendorf tubes. They are then subjected to sonication at high power for 4 cycles of 20 sec on and 20 sec off. The tubes are then centrifuged at 14000 RPM for 7 mins. The supernatant is collected by using a 1 ml syringe carefully avoiding the lipid layer at the surface and transferred the protein extract in a clean eppendorf tube. An aliquot of the protein sample is taken and diluted 5 times for quantification by the Bradford method.

For western blot, I used different amounts of proteins of each sample to be run on precasted Invitrogen bis-tris gradient gels. The gel was transferred into Optitran BA-S nitrocellulose membrane from Schleicher and Schuell, using the Bio-rad trans-blot turbo apparatus. The blot was then incubated for 10 min with Miser antibody extender solution that improves the use of primary antibody at a higher dilution. It was washed 5 times with double distilled H_2O. The membrane was blocked in 5% w/v low fat milk in PBSt for 60 mins. Primary antibody probing was done by incubating the membranes with antibody diluted in PBSt at 4°C overnight. After incubation, they were washed 4 times for 7 mins each with a blocking buffer before incubating with secondary antibodies for 1 hour at room temperature. For image development, the blots were incubated for 5 mins in a 1:1 mix of Super signal west Pico chemiluminescent substrate and stable peroxide solution. The membranes were separately wrapped in a transparent plastic sheet and imaged using the ChemiDoc imaging apparatus.

RNA extraction-

Synchronized worms, exposed to various stress depending upon the experiment, were collected in M9 media in 15 ml falcon tubes. 3 separate biological replicates for each condition are used, each coming from 3 separate 9cm plates. The tubes were centrifuged at 800 RCF and washed 3 times with M9 before before finally washing with distilled water. The falcon tubes were centrifuged and the supernatant was discarded leaving the pellet. The pellet was resuspended with a very little amount of water and transferred to 1.5 ml Eppendorf tubes, which are agaoin centrifuged at around 5000 RCF for 5 minutes and the remaing liquid supernatant is discarded. The pellet was resuspended in twice volume of Trizol reagent and the worms were lysed by vortexing. Total RNA was extracted using the Qiagen RNA extraction Kit. RNA quantification and purity was measured by drop-sense quantification software.

Gene expression analysis by RT-qPCR.

I designed primers specific for some of the UPR target genes namely- hsp-4, hsp-3, ckb-2, xbp-1, and xbp-1s. 500ng of extracted RNA from each sample was used for reverse transcription to get the cDNA using a Thermo Fisher Revert-aid first strand cDNA synthesis kit. The reverse transcription step is done in duplicates of 20µl for each biological sample and later pooled together and diluted 5 times to prepare the working stock. The cDNA is then used as the template for PCR.

a. Primers used for RT-qPCR

UPR Target genes	Forward primer	Reverse primer
hsp-4	cttctctgtgtttccacttctcc	tcaaaatcaacgagaaaactttcata
hsp-3	gaggataagggaaccggaaa	gggctgagtctgttctggtc
xbp-1 unspliced	ccgatccacctccatcaac	accgtctgctccttcctcaatg
xbp-1 spliced	tgcctttgaatcagcagtgg	accgtctgctccttcctcaatg
ckb-2	cctggaatcaagatgaatgga	atggtgaacggtttttgagc
Reference genes		
actin	gctggacgtgatcttactgattacc	gtagcagagcttctccttgatgtc
cdc-42	ctgctggacaggaagattacg	ctcggacattctcgaatgaag

b. Standard curve analysis of primer pairs-

The target genes are amplified by PCR amplification using the cDNA reverse transcribed from RNA extracted from every experimental sample as a template and specific primer pairs for each target. The PCR amplification product is first checked by agarose gel electrophoresis. 10-fold serial dilutions from 10^{-2} to 10^{-9} are made of each amplified product. These diluted cDNAs are then used as the template for qRT-PCR using the Takyon No-Rox SYBR qPCR master mix (Eurogentec), to get the efficiency curve of the primers. I used a Bio-Rad CFX96 T1000 real time system for all qRT-PCR runs and the Bio-Rad CFX manager application to analyze the results. The efficiency of primers is calculated by the equation $e = 10^{\wedge}(-1/m)$ where "e" is the efficiency and "m" is the slope of the standard curve automatically calculated by the software. Primers designed to be used in qRT-PCR must have an efficiency between 1.85 and 2.10 to get trustful results. Once the efficiency of primers is established, they are then used for qRT-PCR to determine the mRNA expression levels of the genes. The cDNA from each replicate sample of each experimental condition are used as templates in technical duplicates. The Takyon mix mentioned above is used for all qPCR experiments.

c. Fold change calculation-

The Threshold cycle (C_t) is the cycle number at which the fluorescent signal of the reaction crosses the fluorescence threshold, a fluorescent signal significantly above the background fluorescence. The C_t value is inversely proportional to the starting amount of the target or the original relative expression level of the gene. After the run, the C_t values of each of the samples are exported to the MS excel spreadsheet and used to calculate the relative mRNA amount that is the fold change (increase or decrease) in the expression of targets with respect to control (untreated worms). The C_t of all biological and technical replicates are averaged and the relative fold change of each gene target is calculated by the equation- $F_{tar} = e^{\wedge}(C_{t(control)} - C_{t(treated)})$, where "e" is the efficiency of the target primer calculated from the standard curve. The fold change is normalized by using two reference genes- $F_{norm} = F_{tar} / F_{hk}$. The normalized fold change (F_{norm}) is represented in bar graphs for each target

gene. F_{tar}, F_{hk}, F_{norm} denote fold change of target genes, housekeeping genes and normalized fold change respectively.

For each target gene, its fold change under treatment of auxin, Tm and both, is relative to its expression in untreated (control) worms where the expression is standardized to value 1.

Results

The plant hormone Auxin (indole-3-acetic acid) provides resistance to ER stress inducer Tm during development.

I performed stress resistance assays on wild type N2 worms using Tunicamycin alone or in combination with auxin. I found that 1mM auxin significantly improved survival in the presence of Tunicamycin (Tm). I used 2µg/ml and 3µg/ml of Tm and 1mM auxin. On average 20% of the embryos reached the L4-adult stage on Tm after 4 days while approximately 40% of the embryos developed into L4-adults when auxin was present in combination with 3µg/ml Tm. The survival was approximately 40% (without auxin) and 80% (with auxin) when 2µg/ml of Tm was used. As expected 100% of the embryos developed into adults on control plates containing DMSO. Also, 100% of embryos developed into adults on plates containing auxin alone (Figure-5a), suggesting auxin do not affect normal development.

Resistance due to auxin is concentration dependent.

I performed developmental stress resistance assays on wildtype N2 worms keeping the concentration of Tm constant at 3µg/ml but using increasing concentrations of auxin- 0.1mM, 0.2mM, 0.5mM and 1mM. Noticeably, the effect of protection on stress increases with the dose of auxin. (Figure-5b)

The protective effect of auxin extends to Thapsigargin.

To confirm that auxin protects against ER stress, I performed stress resistance assays using a different stress inducer- Thapsigargin. When exposed to 70 µM of Tg approximately 50% of the embryos reached adulthood. However in presence of 1mM of Auxin the embryos developing into L4 adults reached 80% (figure-5c) Together my experiments show that auxin increases resistance to two well-characterized ER stress inducers, proving that auxin does not specifically inhibit the effect of one drug by, for example, preventing its uptake by the worms.

Auxin provides ER stress resistance via hormesis like effect.

I pretreated the adult worms with 0.1mM, 0.2mM, 0.5mM and 1mM of auxin for 24 hrs prior to stress resistance assay with 3µg/ml of Tm. I observed that the progeny of pretreated worms were more resistant to Tm than that of worms that had not been pretreated. Similar to the results of the auxin gradient experiment, the progeny of worms pretreated with a higher dose of auxin perform better under Tm stress (figure-5 D). This data indicates that auxin does not need to be provided together with Tm in order to impart its protective effect and that auxin alone per se might activate the UPR.
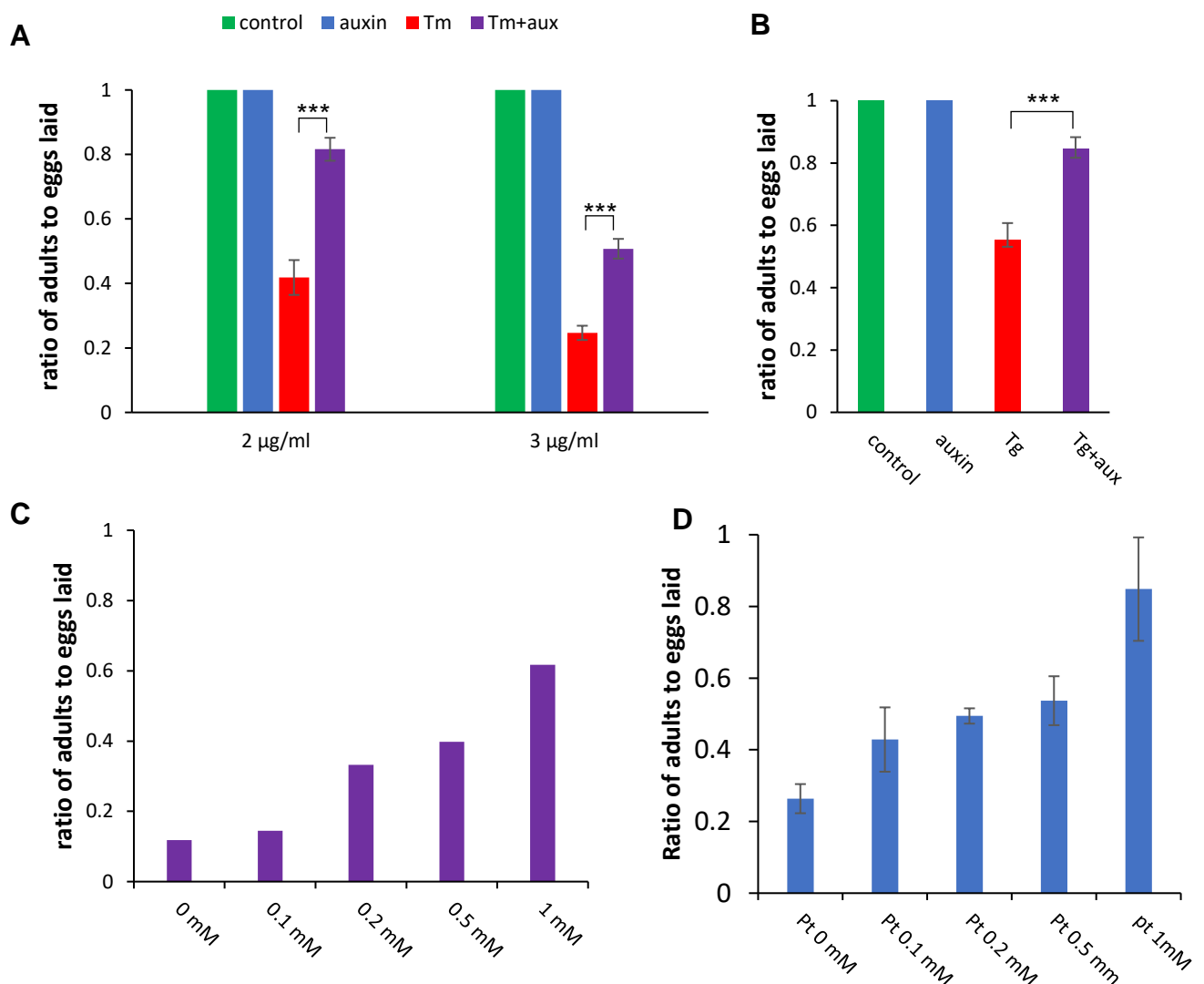

Figure 5: Auxin imparts resistance against ER

A- Stress resistance assays of wildtype worms in 2 and 3 ug/ml Tm with and without 1 mM auxin. **B**- The same assay with Tg as stress inducer. Auxin imparts resistance to ER stress caused by 2 different drugs. **C**-The protective effect of auxin to Tm stress increases with increasing dosage of auxin supplement. X axis represent concentration of auxin. **D**- Auxin can confer resistance to ER stress by hormesis. Pt- pretreated. Worms are pretreated with varied concentration of auxin and their progeny was resistant to ER stress compared to untreated worms. Statistical significance was calculated from 3 independent experiments. (***$p<0.001$). Error bars represent standard error of the mean.

Auxin requires the xbp-1/ire-1 branch of the UPR to promote ER stress resistance.

To test whether the effect of auxin is dependent on the UPR, I used different strains carrying mutations that inactivate specific branches of the UPR. In my initial experiments, I used 3µg/ml of Tm. However, since this dose killed all the mutant embryos, the experiment was repeated with Tm- 2µg/ml. Using this dose I observed that in the case of pek-1 mutant worms around 10% of embryos reached adulthood on Tm but 40% of worms reached adulthood when auxin was present in combination with Tm. Surprisingly, atf-6 mutant, on the other hand, was found to be more resistant (60%) to Tm as compared to wildtype worms (25%). In the presence of both auxin and Tm, a slight improvement in resistance was observed in this mutant as compared to Tm only condition. As shown by others worms lacking either ire-1 or xbp-1 were highly sensitive to Tm (Taylor and Dillin, 2013), with the percentage of worms reaching adulthood being less than 10% and the ratio being unaltered by the presence of auxin. I used two different xbp-1 mutants, one carrying the null allele (*zc12*) which is more sensitive to Tm than the one carrying the partial loss of function allele (*tm2457*). (Figure-6). Together these results indicate that only the IRE-1/XBP-1 branch of the UPR is necessary for auxin mediated resistance to ER stress.
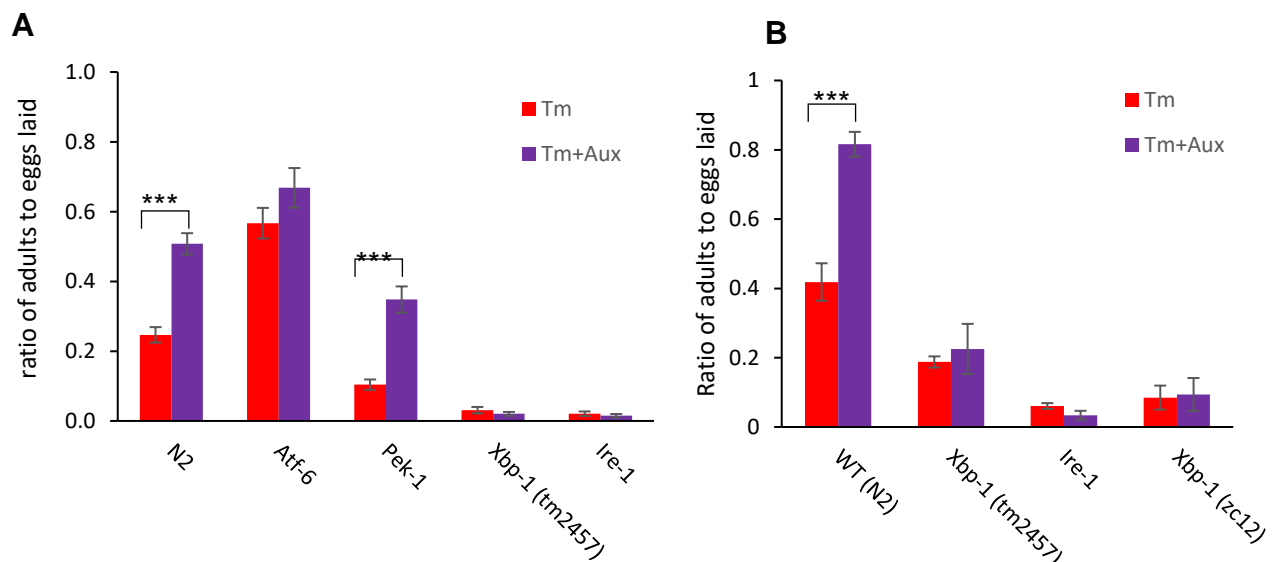

Figure 6: Stress resistance assay of UPR mutants
Stress resistance assays performed on **A-** 3µg/ml Tm and **B-** 2µg/ml Tm with and without 1mM Auxin. The XBP-1 and IRE-1 mutants were highly sensitive to Tm and their sensitivities were unaltered by auxin, suggesting auxin requires the IRE-1/XBP-1 branch of the UPR. Error bars represent standard error of the mean. Statistical analysis were done from 3 independent experiments having 3 biological replicate in each experiment.

Auxin protects adult worms from ER stress.

I performed adult survival assays on worms treated with Tm- 40ug/ml, with and without auxin. This high dose was used because the adult worms are more resistant than developing larvae. As expected, worms on Tm plates survived shorter than control worms (data not shown) with a mean survival of approximately 9 days. However, worms on plates containing both auxin and Tm were healthier and survived significantly longer than those on Tm only with a mean survival of 10 days (Figure-7) By contrast xbp-1 mutants which were more sensitive to Tm and short lived as compared to wildtype worms (with a mean survival of around 7 days) were not affected by auxin suggesting that the IRE-1/XBP-1 signaling cascade is required for auxin to promote survival in presence of Tm.

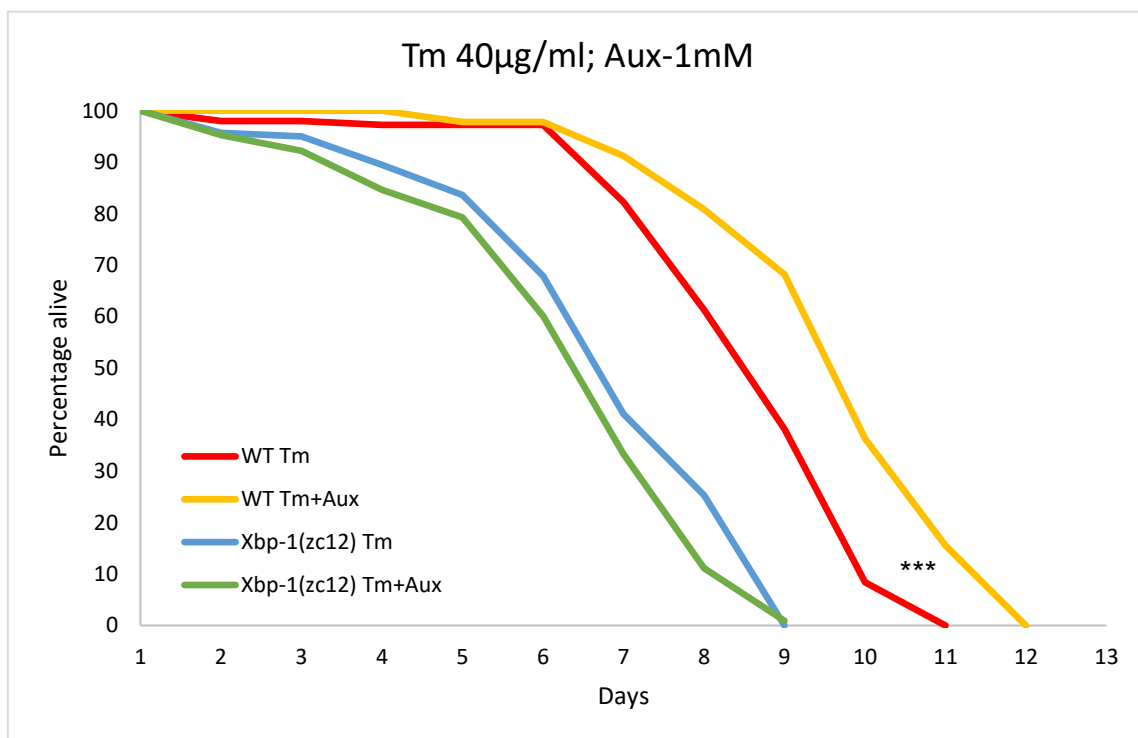**Figure 7: Adult survival assay of N2 and xbp-1 mutant (zc12).**

Kaplan-Meier plot of wildtype N2 worms and xbp-1 mutant exposed to 40µg/ml Tm with and without auxin supplement. N2 worms were significantly long survived when auxin is supplied in combination with Tm (***$p < 0.001$) whereas xbp-1 mutant was short survived (mean life span- 7 days) and auxin has no effect on its survival ($p = 0.1193$)

HSP-4 upregulation by auxin was undetectable by western blot.

For quantifying the expression of HSP-4 I used the strain SJ4005 *hsp-4::gfp(zcls4)V* that expresses a hsp-4::gfp transcriptional reporter (Calfon et al., 2002). This reporter gene tracks the expression of endogenous hsp-4 in worms. It has a relatively low basal green fluorescent protein (GFP) expression but the expression is strongly induced in the gut and hypodermis after treatment with Tm. A reporter gene is a gene that is tagged or attached to a regulatory sequence of a gene of interest (target gene). In this case, the reporter gene (gfp) is placed under the control of the target gene (HSP-4) promoter and the reporter gene's activity can be observed and in turn measure the target gene expression.

In order to test the theory that auxin enhances the expression UPR genes in a manner similar to Tm but without causing stress, I exposed the SJ4005 worms to 1mM auxin for 48 hrs and checked the expression of GFP in both auxin treated and control worms by western blot, using anti-GFP antibodies.

When normalized with the housekeeping gene tubulin, no difference was observed in the blot between auxin treated and control animals (figure 8A). The image intensity was quantified using imageJ and the ratio of *hsp-4::gfp* expression intensity in auxin treated worms and control worms is depicted in table below (figure 8B). Quantification ratio is near to 1 suggesting that the *hsp-4::gfp* expression level is similar in both conditions. This result suggests that auxin might be causing a very mild upregulation of HSP-4*,* which is undetectable by western blot. Hence I turned to qRT-PCR, a far sensitive technique, to quantify the expression of UPR target genes including with *hsp-4*, under the influence of auxin.

A
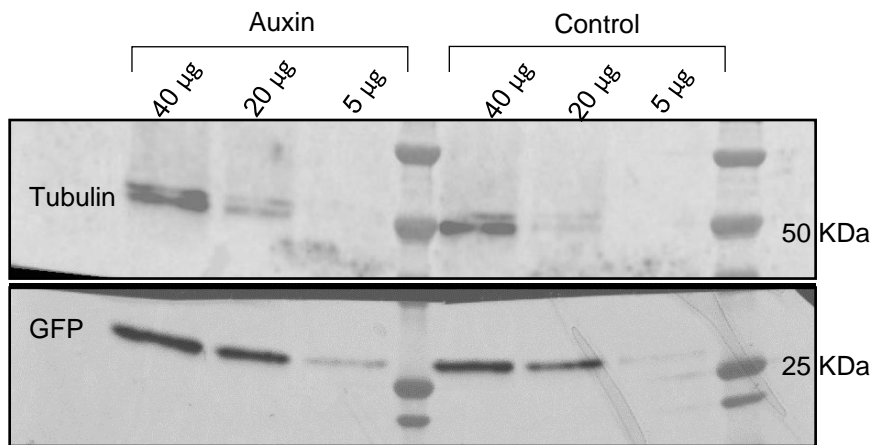
B

load µg	hsp-4:gfp ratio of auxin to control
40	1.21820
20	1.27069
5	1.11122

Figure 8: Western blot quantification of GFP expression in auxin treated and control worms.

A I loaded three different amounts of protein per type on the gel for PAGE. The blot was incubated with anti-GFP and anti-tubulin antibodies. In auxin treated worms expression of both the GFP and tubulin were equally higher compared to control worms.
B Table showing the ratio of *hsp-4:gfp* expression intensity in auxin treated worms and control worms after normalization with tubulin as quantified using imageJ. The ratio is near to 1.

Auxin causes upregulation of UPR target genes

In order to test if auxin upregulates any of the UPR target genes I performed chronic stress assays by growing a synchronized batch of wildtype worms on 1mM auxin, 0.5 µg/ml Tm and both 0.5 µg/ml and 1mM Auxin, from L1 to L4 larval stages. I performed chronic stress to mimic the long-term exposure of the developmental stress resistance assay, although I lowered the concentration of Tm to prevent the high levels of toxicity observed with 3µg/ml Tm. After 48hrs treatment, qRT-PCR was performed on extracted RNA to determine the fold change in expression of UPR target genes- *hsp-3, hsp-4, ckb-2* along with the expression of *xbp-1* and its spliced product- *xbp-1s* in auxin and Tm treated worms and control worms. I observed that, whereas the expression of *hsp-3* and *xbp-1spliced* were not significantly altered by auxin-only treatment after 48 hrs, *hsp-4* and *ckb-2* were upregulated 1.5 fold and 1.35 fold respectively. Importantly, both *hsp-4* and *ckb-2* were upregulated almost 2-fold (compared to control) when auxin is administered in combination with Tm, which is more pronounced when compared to that observed in Tm-only treated worms (figure-9-A). These data suggest an additive effect of auxin and Tm in the upregulation of stress response genes.

Since I found that *hsp-3* and *xbp-1s* expression were unchanged after 48hrs I hypothesized that the expression levels may initially increase but then rapidly alleviates to basal expression. In order to test the hypothesis, I performed acute stress assays by treating synchronized L4 staged worms to 1mM Auxin, 5 µg/ml Tm and both for 3 and 6 hours exposure period. I observe the stress response genes tested were all upregulated under Tm treatment except *total xbp-1* expression. Furthermore, the expression of *hsp-3* and *ckb-2* was upregulated in auxin-only conditions and an additive effect was observed when auxin was provided in combination with Tm (figure 9-A and 9-B). The expression of both *xbp-1 total* and *xbp-1spliced* were not increased after 3 hrs exposure but was significantly higher after 6 hrs when auxin is provided in combination with Tm, further supporting the theory of the additive effect of auxin and Tm.
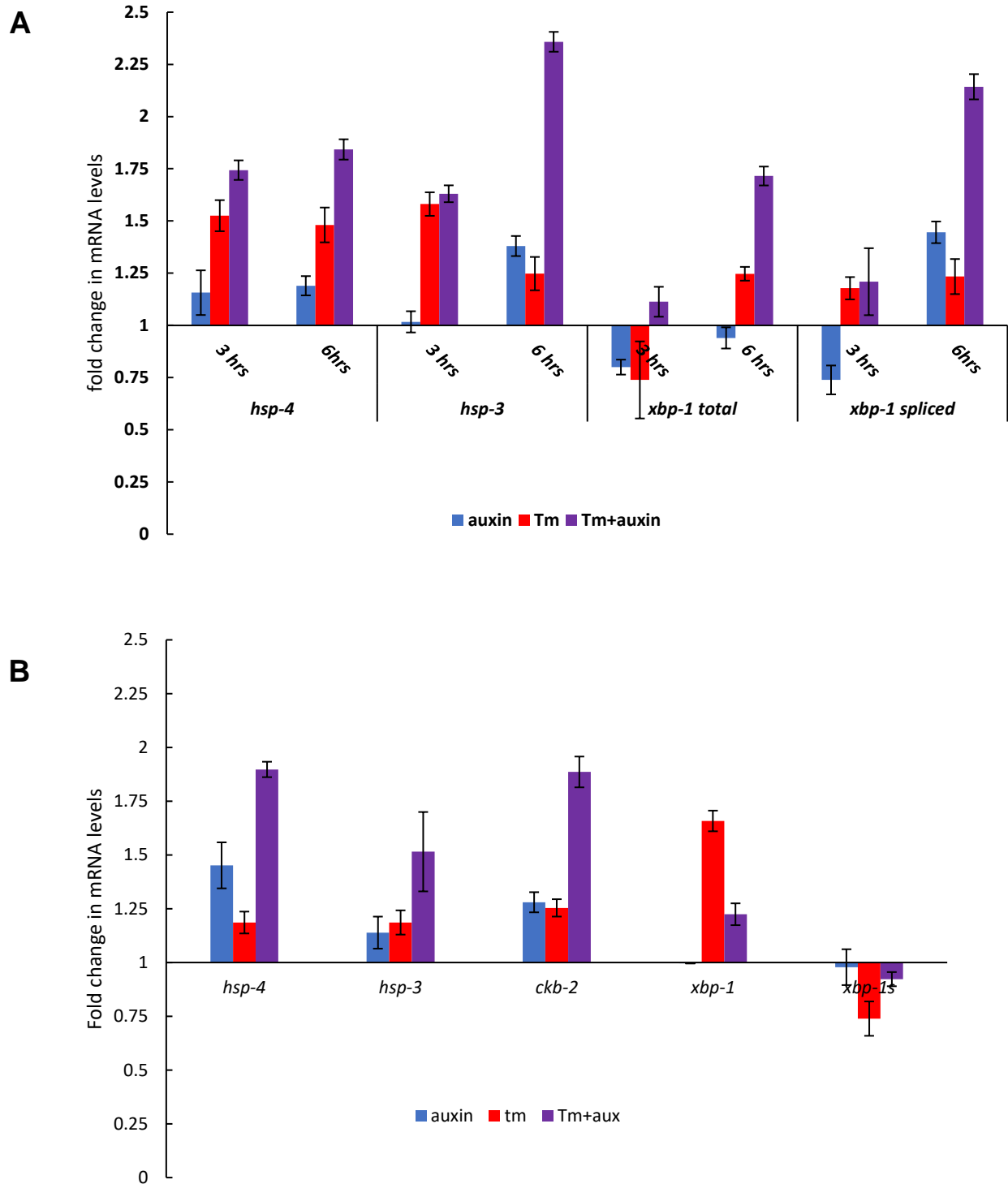
Figure 9: mRNA expression profiles of UPR target genes

(A) Acute stress: L4 worms were treated with 5ug/ml Tm, 1mM auxin and both for and the expression of genes were quantified after 3 hours and 6 hours of treatment.

(B) Chronic stress expression profiles. The worms were exposed to 0.5ug/ml Tm, 1mM Aux and both for a period of 48 hrs during development from L1 to L4. Acute stress expression profiles. Error bars show standard error of the mean.

Protein aggregation is unaffected by auxin.

To check whether auxin has a more general role in maintaining protein homeostasis I performed a protein aggregation assays on worms treated with- 1mM auxin, Tm-2μg/ml, and both auxin and Tm. I used the PP657 strain that expresses SRP2^{H302R}:YFP transgene exclusively in the muscle cells. The aggregated particles are visible as bright dots under fluorescent microscope I monitored the number of protein aggregates in worms over a time course from the L2 larval stage to day-2 adults.

All together there were no significant changes in the number of aggregates counted in worms incubated with auxin, Tm or both. However I observe a significant reduction in number of aggregates under auxin treatment in only L4 larval stage but the number of aggregates under all conditions reaches a plateau after a sharp increase in the larval stages and no difference was observed thereafter.

Figure 10: protein aggregation assay
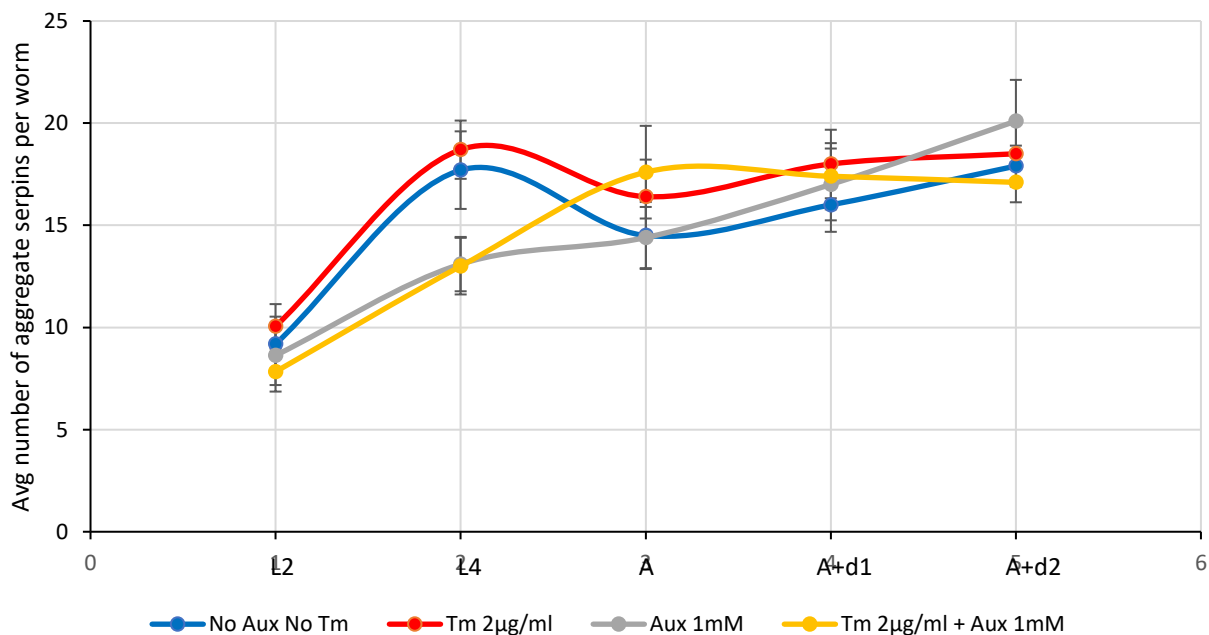
Comparison of serpin aggregation with and without tm and auxin at different stages of the worm. It shows how the number of aggregates varies along with age. The aggregations are counted in individual worms and an average number among 20 worms is plotted. Error bars, showing standard error over mean, depict that the differences between the conditions are not significant p>0.1.

Discussion

Tm and Tg induce ER stress in *C. elegans* by causing protein misfolding, and disrupting calcium homeostasis respectively, against which the unfolded protein response is activated to cope with the resultant proteotoxicity. However, after prolonged or extreme ER stress, the resistance mechanisms fail to reestablish protein homeostasis and the animals succumb to its effects. My findings from the stress resistance assays performed on different UPR mutant worms show that auxin can modulate specifically the IRE-1/XBP-1 pathway of UPR in nematode *C. elegans* to provide protection against ER stress caused by Tm (figure- 6 A, B) also extending its effect in case of stress caused by Tg (figure-5 B).

To prove my hypothesis that auxin, although not a stress inducing drug, can prompt the same regulatory effect as does Tm or Tg, I performed the hormesis assay. By definition, hormesis is a physiological phenomenon in which exposure to mild stress, enhances the capability of animals to resist severe stress. Pretreating worms with auxin for 24 hours prior to exposing them to Tm, remarkably improves their capability to resist stress (figure 5D). These results suggest that auxin mimics the function of a hormetin and it prompts the upregulation of stress responses that prepare the worms for Tm stress. Speculatively, worms activate their stress regulatory pathways in response to auxin, sensing it as a stress hormone/drug even though auxin itself is not a stress inducer like Tm or Tg. Auxin on its own upregulates the stress response pathways is further supported by mRNA expression profiles showing higher transcription levels of UPR target genes in worms treated with auxin compared to untreated worms. qRT-PCR results show that UPR target genes such as *hsp-4, hsp-3, and ckb-2* are upregulated in worms when treated with auxin (Figure- 9 A,B) . These genes code for their corresponding chaperoning proteins that assist in protein folding and are upregulated during ER stress. Auxin being able to upregulate those genes in worms support the theory that auxin is sensed as a stress hormone, although it is not a stress inducer like Tm.

In order to test whether auxin imparts resistance to ER stress by making use of the *C elegans* UPR system, I performed the stress resistance assays on different UPR mutant worms lacking the specific stress sensors of three UPR branches. My results

show that XBP-1 and IRE-1 loss of function mutants are very sensitive to Tm and auxin does not protect these mutants against Tm induced ER stress (figure- 6 A, B). However, pek-1 mutants were protected by auxin similarly to control worms. These results reveal that specifically the XBP-1/IRE-1 branch of the UPR is required for auxin to have its effect and the PEK-1 branch is not. IRE-1 is a stress sensor that acts as an endoribonuclease responsible for the non-canonical splicing of the *xbp-1* mRNA to the functional spliced product xbp-1s. Based on my results, one possibility is that auxin functions to improve the endoribonuclease activity of IRE-1, producing more XBP-1s, which in turn activate the transcription of UPR target genes. Alternatively, IRE-1 could be specifically sensitive to auxin, compared to other sensors, and it gets activated in the presence of auxin. Both hypotheses are also supported by qRT-PCR results that show relatively higher levels of *spliced xbp-1* in auxin treated worms (figure-9).

It is a known fact that the ability to activate the UPR declines with age from yeast to mammals. In fact, proteostasis impairment is one of the major hallmarks of ageing (Carlos et al., 2013). To study whether auxin imparts its same effect in adult worms as it does in larval stages, I performed the adult stress resistance assays on wild type and *xbp-1* mutant worms at 40µg/ml Tm in combination with 1mM auxin. Previously shown by others (Taylor and Dillin, 2013) the *xbp-1* mutant were short lived in the presence of Tm and I observed that auxin did not improve their survival. Conversely, wildtype worms were healthier and survived 10% longer in presence of auxin (figure-7). These data extend the protective role of auxin via the XBP-1/IRE-1 pathway to adult worms suggesting a possible role of the UPR in improving the healthspan of worms and other species exposed to indoles (Sonowal et al., 2017).

Cellular and organismal proteostasis is maintained by an array of protein quality mechanisms, the ER UPR being one only of them, which declines with aging. To test the hypothesis that auxin may provide its protective effect through a general increase of the proteostasis capacity of the worms, I measured how auxin affects protein aggregation using a *C. elegans* strain that expresses an aggregation-prone version of serpin (SRP-2[H302R]) in muscles cells (Schipanski et al., 2013). As shown in figure 10 auxin does not protect muscle cells against protein aggregation during development. These results seem to rule out a general effect of auxin on proteostasis maintenance. However, it was a preliminary experiment and needs more tests, nevertheless it will

be of interest to test whether auxin contributes to protein homeostasis by affecting autophagy, proteosomal activity or heat shock response.

All those above results are the effects of auxin observed only under stressed conditions i.e. when supplemented with Tm. Western blot image showing the expression bands of GFP tagged HSP-4 in control and auxin treatment show no difference when normalized by tubulin (figure-8). I speculated that the auxin on its own must have a mild effect on the upregulation of target genes which are not observable in a western blot experiment. Hence, to be more accurate and get more sensitive results on target genes upregulation, I then decided to quantify the mRNA expression levels of target genes in the presence of auxin and Tm when exposed for different time periods.

The transcriptional response induced by Tm is well characterized. My data show that auxin can induce upregulation of some UPR target genes and its effect is found to be additive when applied in combination with Tm. Notably, all genes tested show an upregulation under acute treatment with auxin. There was no difference in the expression level of *hsp-4* after 3 hours and 6 hours of treatment with only auxin. However, the upregulation of *hsp-4* by auxin and its additive effect with Tm is evident from the expression profiles. Moreover, *hsp-4* and *ckb-2* upregulation were maintained till 48 hours when auxin was provided with Tm. 48 hours is the maximum time experimentally tested. An Immediate upregulation was not observed in case of *hsp-3* and *xbp-1 spliced* as well as *xbp-1 total* mRNA. Their upregulation was observed post 6 hrs exposure to the combination of auxin and Tm but rapidly attenuates back to near basal expression within 48 hours of treatment.

All the genes tested had their own timing of upregulation and deregulation. The general predicted dynamics of the expression of those genes is shown in a cartoon model (figure-11). All the examined genes were upregulated after 6 hrs of exposure to Tm and auxin. The upregulation is higher when worms were exposed to the combination of Tm and auxin, as compared to treatment to Tm only. Together, these qRT-PCR data indicate that auxin enhances the response to stress by assisting in the upregulation of UPR genes under stressed conditions. However, the effect only lasts for so long before their expression gets normalized to basal level. I observed that within 48 hrs *xbp-1* and *hsp-3* expression gets reduced as compared to their expression at

6hrs. The enhancement of spliced *xbp-1* upregulation when auxin is administered with Tm supports the idea that auxin catalyzes the endoribonuclease activity of IRE-1 and higher spliced xbp-1 in the nucleus may account to the resistance imparted by auxin during Tm stress. Overall the induction of genes is modest in worms treated with auxin, but a more pronounced effect is observed when auxin and Tunicamycin are combined. However more experiments are need to solidify the results

The resistance of worms to stress induced by Tm can be owed to the fact that auxin when supplied with Tm highly upregulates the stress response genes, significantly more than the regular upregulation during stress. This enhancement of stress response genes by auxin, albeit temporary, may be sufficient for the worms to cope to stress as seen in stress resistance assays. This effect of auxin also plays part in delaying the decline in UPR among old aged worms hence increasing the healthspan on the animals. Together these results conclude that auxin provides resistance to ER stress by enhancing the expression of stress response genes, acting via the XBP-1/IRE-1 branch of the UPR in *C. elegans*.
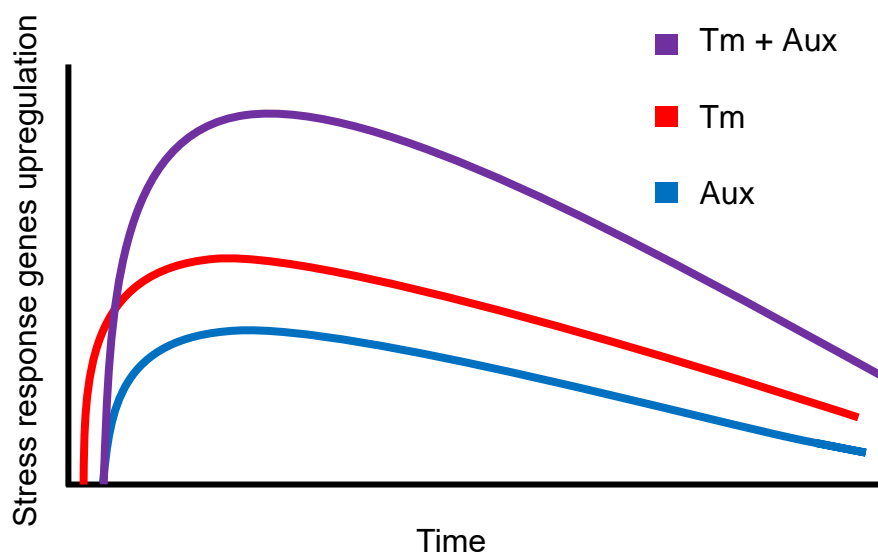

Figure 11: a predicted model of stress response genes regulation

The graph shows a dynamic of gene regulation when treated with Tm, auxin and both. Auxin is able to upregulate some of the UPR target genes at first but the expressions rapidly decrease to basal levels. However, auxin, when provided with Tm, is able to enhance the stress response pathways, showing an additive effect in the upregulation of stress response genes.

Conclusion.

Protein homeostasis is one of the fundamental prerequisites for cell survival. With age, the UPR declines in its efficiency to maintain protein homeostasis. My findings suggest that cellular UPR can be modulated by external agents such as auxin. Auxin can upregulate some of the UPR genes in a manner beneficial to the animal by acting via the XBP-1/IRE-1 branch of the UPR. The UPR sensors can be a potential therapeutic target for the treatment of various diseases including age related diseases such as neurodegenerative diseases. Finding out strategies that reduce ER stress by improving the efficiency of the UPR can prove useful in delaying or preventing many age related diseases as mention in this thesis. My work shed some light on one of the ways the UPR can be made to improve its efficiency and organismal response to stress.

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