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Analyzing the effect of apoptotic mutations on the state of the nascent-polypeptide associated complex in Caenorhabditis elegans

Monica Gerber

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Analyzing the Effect of Apoptotic Mutations on the State of the Nascent-Polypeptide Associated Complex in *Caenorhabditis elegans*

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by Monica Marie Gerber
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PUBLIC PRESENTATION

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Abstract

Cells experiencing misfolded protein stress can become debilitated and die, contributing to the onset of disease. The nascent polypeptide-associated complex (NAC) is a heterodimeric translational chaperone that protects against misfolded protein stress by mediating proper protein folding and localization during translation. Depletion of this complex results in misfolded protein accumulation in the endoplasmic reticulum (ER). To determine the importance of the NAC to proteostasis, we have previously depleted the complex in C. elegans via RNA interference and observed numerous dose-dependent effects, including apoptosis of neuronal cells and changes in gene expression of hypodermal cells. While we have observed these cell-specific responses to misfolded protein stress by depleting the NAC artificially, we are also investigating the state of the NAC in cells undergoing misfolded protein stress faced in biologically relevant conditions. Preliminary studies indicate that the beta subunit of the NAC is cleaved in worms undergoing chemically and heat induced misfolded protein stress putatively at a caspase cleavage site functionally conserved in other organisms. We are determining if this cleavage is a step in the apoptotic cell death triggered by misfolded protein stress and therefore dependent on the core elements of the C. elegans apoptotic pathway. By understanding the cell-specific responses to misfolded protein stress induced by NAC depletion in combination with the fate of the NAC in the face of chemically induced stress, we hope to elucidate the role of this highly conserved complex in proteostasis and biologically relevant cell survival.
Introduction

When proteins become misfolded, the results can be detrimental to almost all cell types, especially if the cell is unable to mitigate the response. High levels of misfolded proteins form intermolecular interactions that lead to the formation of aggregates that, if unresolved, can lead to the impairment and ultimate death of the cell. To this end, cells engage numerous pathways in response to misfolded protein stress (MPS) in the endoplasmic reticulum (ER) collectively known as the unfolded protein response (UPR). In some cases, the cell-saving mechanisms of the UPR are not always able to resolve the stress, resulting in the cell killing mechanisms of the UPR triggering apoptosis. The tipping point between this life or death outcome in the face of MPS may in fact be cell-specific, i.e. some cell types may survive MPS more readily than others specific to the UPR mechanisms engaged in those cells. The nature of these cell-specific responses, in addition to the cell death mechanisms engaged once apoptosis is initiated may provide important insights into why certain cell types survive MPS more effectively than others.

The round worm and model organism Caenorhabditis elegans (C.elegans) is ideal for studying cell-specific responses to MPS because the fate of every cell is known throughout the life of the animal, providing a baseline for MPS-induced effects that may change the nature and/or viability of the different cell types expressed in the worm. An essential component for managing protein folding in the worm is the Nascent Polypeptide-Associated Complex (NAC), a heterodimeric translational chaperone hypothesized to play an important role in preventing MPS in the ER and therefore induction of apoptosis as a result of this stress. My studies hope to further elucidate the role of the NAC in prevention of apoptosis during MPS and its fate in those cases where apoptosis is engaged as a result of MPS.
Importance of Proteins

Proteins are arguably the most important macromolecule in any organism simply because they serve a variety of functions in all cell types. Some of these functions include providing cell structure, facilitating resource transport, and driving enzymatic action. The unique qualities and functions of proteins are due to the four levels of structure that arise during translation. The primary structure of proteins is the linear manifestation of polypeptide chains formed through the generation of peptide bonds between amino acids. Secondary structure is achieved through single chain intramolecular interactions to form specific layouts such as a beta-sheet or an alpha-helix. Tertiary structure, the terminal structure of most proteins, is formed through interactions between the secondary structures on a single polypeptide chain. Finally, quaternary structure is formed in some cases, e.g. hemoglobin, when a cluster of chains interacts with each other to form a complex with separate polypeptide-chain subunits. High fidelity of protein folding is essential for cell viability as the structure of a protein defines its function in the cell. The diversity of protein structures observed in any given cell allows for the vast range of protein functions (Berg et al. 2002).

In the process of protein folding during translation, alternate non-functional structures can form and become stable because of their low energy state; to prevent this occurrence, proteins called chaperons bind to nascent polypeptide chains and prevent inappropriate interactions within the chain and/or neighboring proteins (Bobori et al. 2017). Chaperones also refold misfolded proteins during times of stress to retain their functionality and prevent aggregations. Occasionally, if the accumulation of misfolded proteins is acute or chronic, chaperones assist with misfolded protein degradation by escorting them to proteasomes for breakdown (Bobori et al. 2017).
The UPR (Unfolded Protein Response)

The Unfolded Protein Response (UPR) is a collection of intracellular signal transduction cascades triggered by the accumulation of misfolded proteins within the ER lumen to mitigate MPS. If MPS is not effectively resolved through the cell-saving mechanisms of the UPR, this stress response will subsequently trigger the death of the affected cell via apoptosis. One function of the ER (specifically the rough ER or RER) is to fold and modify proteins destined for secretion or to become integral membrane proteins; as such, about 30% of all proteins synthesized in a typical cell are located to the ER for these purposes. When proteins are not folded properly in the ER, they are bound by chaperones for refolding or exportation to the cytoplasm to be degraded by the proteasomes (Walter and Ron, 2011) (Figure 1). The balance between these two mechanisms changes during times of misfolded protein stress induced by a variety of stressors such as chemical exposure and environmental changes, e.g. heat. (Naresh and Haynes, 2019).
Figure 1: The Two Different Outcomes of Protein Synthesis within the ER begins with the translation of messenger RNA on a ribosome associated with the endoplasmic reticulum. The nascent polypeptide is released into the ER lumen where it will either properly fold or become misfolded. If it is improperly folded the protein will be transported out of the ER to be degraded by the Ubiquitin Proteasome System. If the protein is properly folded and modified, it will bud off via a vesicle to be processed in the Golgi apparatus. Figure adapted from Dobson, 2003.

In the face of high levels of ER-specific MPS, the UPR activates up to three signaling pathways that mitigate the misfolded protein stress or induce apoptosis if the misfolded protein stress outpaces attempts to resolve it (Walter and Ron, 2011). Cell saving activities induced by the UPR include, but are not limited to, attenuation of translation, the upregulation of chaperone expression and the increase of misfolded protein degradation. The inability of these mechanisms to resolve the MPS in a timely fashion results in the UPR-mediated activation of apoptosis (Acosta-Alvear et al. 2018).

One pathway engaged during the UPR is initiated by the dimerization of the inositol requiring enzyme 1 (IRE1) signal transducer. IRE1 is a transmembrane kinase that, when activated, engages downstream targets that initiate the expression of chaperones as well as
proteins associated with ER biogenesis and ER-associate protein degradation (ERAD) (Figure 2C). These responses mitigate MPS in the ER by reducing the concentration of misfolded protein in this organelle, preventing the formation of aggregations. This branch of the UPR is the most highly conserved throughout evolution and is commonly employed as a response to misfolded protein stress. Another signal transducer engaged by the UPR is activating transcription factor-6 (ATF6) (Figure 2A). As with IRE1, ATF6 is an ER transmembrane activated in the presence of unfolded proteins within the ER lumen; specifically, ATF6 is transported to the Golgi apparatus where it is cleaved and released as a transcription factor to affect the expression of UPR target genes primarily tasked with many of the same cell-saving outcomes initiated by IRE1. The third arm of the UPR, protein kinase RNA (PKR)-like ER kinase (PERK), is initiated through homo-dimerization that ultimately leads to affecting the expression of both cell-saving and cell-killing proteins (Figure 2B).
Figure 2: Mechanistic Pathways of the Unfolded Protein Response depicted in 3 panels. Panel A demonstrates the action of activating transcription fact-6 (ATF6) as it is transported to the Golgi where it is cleaved. The cleavage product then acts as a transcription factor for genes that regulate protein folding machinery in the ER. Panel B demonstrates the transcriptional regulation of protein kinase RNA-like ER kinase (PERK). Here, phosphorylation of PERK initiates a cascade of signals that ends up increase the transcription of the target genes. Panel C depicts the decrease of ER protein folding load and increase of transcriptional activators that are initiated through the inositol-requiring enzyme 1 (IRE1) mechanistic pathway of the UPR. Figure adapted from Walter and Ron, 2011.

All three arms of the UPR can work in parallel to manage the homeostatic conditions of the ER for the maintenance of the nascent proteins being folded and modified there, mitigating mild and/or short-lived MPS to ensure the survival and functionality of the affected cell (Walter and Ron, 2011). However, in cases of acute and/or chronic ER stress that cannot be mitigated through cell-saving mechanisms, the UPR can lead to cell death via apoptosis. While the exact mechanism for UPR-induced apoptosis is not well-defined, it is thought that all three arms of the UPR have the potential to initiate apoptosis. This has been shown through studies involving the silencing of one out of the three UPR pathways and the continuation of apoptosis in its absence.
(Tabas and Ron, 2011). The model organism C. elegans contains homologues of all three arms of the UPR; in addition, experimental induction of MPS in the ER of C. elegans is well-established, making this organism optimal for studying ER-specific MPS and subsequent outcomes in different cell types (Mori, 2009).

**Apoptosis and its role in C. elegans**

Apoptosis is a well-defined and tightly controlled cell death mechanism important for the elimination of cells that are damaged beyond repair, including those experiencing prolonged ER-associated misfolded protein stress. Apoptosis is also essential for proper development in organisms as evolutionarily distant as humans and worms. During development, C. elegans generates just over 1,000 somatic cells per worm; approximately 130 of those cells will undergo cell death via apoptosis engaging a core apoptotic pathway that consists of proteins well conserved throughout a wide variety of organisms (Figure 3). As such, studying apoptosis in C. elegans gives insight into apoptosis in general, including mechanisms that inhibit or stimulate programmed cell death during times of stress (Arvanitis et al. 2013).
Figure 3: Conserved Apoptotic Pathways for *C. elegans*, *Drosophila*, and mammals. In all three pathways, homologues of similar proteins are depicted as the same color. Red boxes represent initiator caspases, yellow represents caspase activator proteins, blue represents anti-apoptotic BCL-2 family member proteins, and purple represents conserved pro-apoptotic BCL-2 family members. The other colors (orange and light blue) are proteins unique to mammals and drosophila. Figure adapted from Shi, 2011.

In *C. elegans*, all cells express the proteins necessary to initiate apoptosis, while only a small subset of these cells will die by apoptosis during embryonic development. In cells not fated to die, CED-9, a homologue of BCL-2, binds to dimers of CED-4, a homologue of apoptotic protease activating factor-1 (Apaf-1) at the mitochondrial membrane, preventing CED-4 from activating the caspase proCED-3, a protease that, when activated, executes apoptosis. In cells fated to die, the pro-apoptotic protein EGL-1 is expressed and binds to CED-9, releasing it from CED-4. Dimers of CED-4 oligomerize with proCED-3, placing these zymogens in close proximity to each other, allowing them to auto-activate (Figure 4). Once activated, CED-3 will target proteins for cleavage that will lead to the death and dismemberment of the cell, generating membrane-encased “apoptotic bodies” that are then engulfed and recycled by surrounding cells (Conradt et al. 2016).
Figure 4: Core Apoptotic Pathway of *C. elegans* initiated through developmental cues and stress signals. When the cell receives an overwhelming large amount of apoptotic stimuli, EGL-1 is transcriptionally activated. The expression of EGL-1 causes the release of a CED-4 dimer from mitochondrion bound CED-9. The CED-4 dimer is then able to dimerize with itself to form the apoptosome that activates CED-3 to initiate cell death. Figure adapted from Nehme and Conrad, 2009.

This well-established cell-death pathway drives developmental apoptosis in *C. elegans*, but its role in stress-induced apoptosis is less well-defined, particularly in response to misfolded protein stress. My studies are designed to determine the putative role(s) of the core apoptotic pathway in the cell death associated with MPS. Specifically, I will induce misfolded protein stress in CED-3 and CED-4 knockout strains of *C. elegans* and determine stress-related outcomes, specifically effects on the functionality of the NAC. These knockout strains are morphologically normal, but do not exhibit any apoptosis. The benefit to using these strains is the ability to induce MPS in worms missing elements of the core apoptotic pathway and determining those members necessary and sufficient for any stress-induced apoptosis observed in the presence of MPS (Ellis and Horvitz, 1986).

Role of the Nascent Polypeptide-Associated Complex

I hypothesize that a target of activated CED-3 during apoptosis is inhibitor of cell death-1 (ICD-1), the beta subunit of the *C. elegans* NAC. As mentioned earlier, the NAC is a highly conserved heterodimeric translational chaperone involved in the folding of nascent polypeptides and localization of those proteins away from the ER during translation (Lauring et al. 1995). The
NAC consists of an α and β subunit that work together to bind to the ribosomal complex and ensure proper protein folding and localization as the polypeptide emerges from the complex (Figure 5). More recent evidence indicates that in addition to the complex’s role as a translational chaperone, the NAC subunits will dissociate from both the ribosome and each other in response to the formation of protein aggregates formed during misfolded protein stress (Kirstein-Miles et al. 2013). These subunits, once dissociated, are thought to each play a rescuing role in response to MPS, but the nature of these roles remain largely undefined.

Figure 5: Crucial Protein Chaperones associated with the ribosome including the nascent polypeptide associated complex (NAC) and the mammalian ribosomal-associated complex (mRAC). The black line represents the mRNA being transcribed by the small and large subunits of the ribosome (gray ovals). The red and the yellow pieces form the α and β subunits of the NAC, while the two blue subunits form the mRAC. The green piece represents heat shock protein-70 (Hsp70) which acts as an unbound chaperone that assists with the nascent polypeptide (orange line) exiting the ribosome. Figure adapted from Kirstein-Miles et al. 2013.

In experiments where the NAC subunits become dissociated, each subunit appears to show activity (Beatrix and Wiedmann, 2000). When mouse βNAC is depleted, αNAC has the ability to act as a co-activator for the regulation of genes associated with bone cell differentiation (Akhouayri et al. 2005). When human or Drosophila αNAC is dissociated from βNAC, βNAC serves as a caspase 3 substrate in vitro (Kogan and Gvozdev, 2013). It is the ability of βNAC to be cleaved by caspases that is consistent with our hypothesis that C. elegans βNAC is targeted...
for cleavage by CED-3 during the progression of apoptosis in the worm, and testing this hypothesis is the primary focus of my studies.

In *C. elegans*, βNAC is known as inhibitor of cell death-1 (ICD-1) while αNAC is known as inhibitor of cell death-2 (ICD-2). In previous studies, the loss of ICD-1 through RNA-mediated interference (RNAi) leads to large increases in apoptosis throughout embryonic development (Bloss et al. 2003). This apoptosis was found to be dependent on the caspase activator CED-4, but not the caspase CED-3. In addition, overexpression of ICD-1 will suppress some developmental apoptosis, consistent with ICD-1’s role as an inhibitor of apoptosis. Where ICD-1 fits into the core apoptotic pathway as an inhibitor of apoptosis is not yet determined (Bloss et al. 2003).

In previous experiments, the induction of high levels of apoptosis in *C. elegans* depleted of ICD-1 was produced via RNAi, an artificial loss of protein expression that may not be recapitulated in the worm in a biologically relevant context. Cleavage of ICD-1 during the initiation of stress-induced apoptosis, however, would theoretically produce the same outcome, an increase in apoptosis, and therefore may be an initial step in the process of killing cells experiencing overwhelming MPS. Support for this model is found in the behavior of βNAC in other organisms: *bicaudal*, the βNAC/ICD-1 homologue in *Drosophila melanogaster*, is a conserved substrate for mammalian caspases in vitro (Creagh et al. 2009). In addition, human βNAC isolated from cell lines is cleaved by caspase 3 in vitro. In both cases, the cleavage site for these caspases is found towards the carboxyl terminus of the protein, and as such is putatively and functionally conserved in ICD-1 (Figure 6).
To determine the state of ICD-1 in cells undergoing strong MPS that leads to apoptosis, I have developed a western blot analysis that detects the length of ICD-1 in control and environmentally stressed cells. Previous work by undergraduate students Lars Farber and Kyle Cical _e been established by the ability to detect the depletion of ICD-1 via RNAi using western blot analysis (Figure 7), and I have used their protocol as a starting point for my own studies. Ultimately I want to determine if ICD-1, which likely has the potential to be cleaved by a caspase, is in fact cleaved by a caspase to initiate and/or propagate during stress-induced apoptosis in _C. elegans_. Such a finding would be the first evidence of an apoptotic strategy that involves the elimination of an anti-apoptotic protein through direct caspase-cleavage.

**Experimental Design**

The purpose of this study is to test the hypothesis that ICD-1/βNAC is targeted for cleavage by CED-3 during stress-induced apoptosis. Specifically, I will induce ER-specific MPS in _C. elegans_ using heat or chemicals and determine the nature of ICD-1 in these affected populations via western blot analysis. Ultimately I hope to determine if ICD-1 is being cleaved during MPS and whether this cleavage correlates with an increase in apoptosis.
Figure 7: Polyclonal antibody raised against mouse βNAC recognizes C. elegans βNAC. Mixed population worm lysates were generated over a βNAC RNAi time-course and exposed to polyclonal antibody generated against the βNAC homologue in mouse. Lane 2 represents lysates isolated at time zero while lanes 3, 4 and 5 represent lysates isolated after 4, 9 and 48 hours of βNAC (RNAi) exposure respectively. C.elegans βNAC is predicted to be ~22 kD (arrows) and is undetectable 4 hours into the icd-1 RNAi experiment. Arrowheads denote a 75 kD protein that also becomes undetectable 4 hours into the icd-1 RNAi experiment. Figure adapted from Cicalese Undergraduate Senior Thesis, 2018.

If ICD-1 cleavage is detected in populations experiencing MPS, I will then perform these experiments in apoptotic mutant backgrounds to determine the core apoptotic elements that may be required for this cleavage. In previous studies, RNAi depletion of ICD-1 initiated a pathway of apoptosis that is not dependent on the caspase CED-3, but is dependent on CED-4, indicating the presence of another apoptotic pathway reliant on a caspase other than CED-3 (Bloss et al. 2003). We hypothesize that just as increased apoptosis was seen in the absence of CED-3 during ICD-1 RNAi, we will observe cleavage of ICD-1 in worms under stress that is dependent on CED-4, but not CED-3. These results would confirm that not only is ICD-1 targeted for cleavage to facilitate apoptosis during stress, but this stress-induced apoptotic pathway is different from the canonical developmental apoptosis pathway in that it relies on a caspase other than CED-3.
This investigation has the potential to be beneficial in understanding the importance of cellular proteostasis in humans. Misfolded protein stress has been found to play a role in the onset of many diseases such as neurodegenerative diseases, stroke, cardiovascular diseases, obesity, diabetes, cancer, immune disorders, atherosclerosis and liver diseases (Kim et al. 2008). Studies have even gone further into detail on the regulation of this stress through analysis of BTF3, the human homologue of βNAC. In particular, BTF3 has been found to be a proto-oncogenic protein, indicating that when overexpressed it can cause many types of cancer, such as pancreatic cancer, lung cancer and breast cancer (Kusumawidjaja et al. 2007). Additionally, the alpha subunit of the NAC is decreased in patients with various neurodegenerative disorders, including Alzheimer’s and Down syndrome (Kim et al. 2008). Furthermore, the NAC has already been found to be a promising target for treatment of these diseases (Jeon et al. 2016). With this information, it is clear that studying the inhibitor of cell death complex of C. elegans has the potential to be very beneficial in understanding many diseases and providing further insight into the NAC, its human homologue.
Materials and Methods

Care and Maintenance of C. elegans

Four strains of *C. elegans* (N2 wild-type, MT3002 (ced-3 mutant), MT1522 (ced-3 mutant), MT2547 (ced-4 mutant) were grown on Nematode Growth Medium (NGM) (Carolina Biological). In 60mm petri dishes, 200 μL of *Escherichia coli* strain OP50-1 was used as the food source. Plates were sealed and stored inverted at 22 °C for up to seven days. Stock populations were maintained by transferring 10-20 worms of varying age onto a new NGM plate with fresh food every 5-7 days.

Bacterial Maintenance

*E. coli* strain OP50-1 was streaked onto Luria Broth Agar Plates with streptomycin at 1μL:100 mL Agar and grown at 37 °C for a minimum of 10 hours. The plate was sealed and stored at 4 °C. To prepare the liquid culture for use on the NGM plates, 10 mL of Luria Broth (Thermo Fischer Scientific) with 10 μL of streptomycin was used to incubate 1 OP50-1 colony for 12 hours in the shaker at 37 °C. After the minimum of 12 hours, the culture is transferred to a sterile 15 mL conical tube and stored at 4 °C until needed.

Stress Induction Experiments

To induce a natural stress response, each population was exposed to heat and thapsigargin in separate experiments.

For the heat shock experiments, 10-20 fertile adult worms were placed on two NGM plates each. The plates were stored upside-down at 22 °C for 4-5 days. When the worms were a mixed population and their food source was near depleted, one of the 2 plates were sealed, inverted and incubated at 37 °C for 6 hours. After heat-shocking the experimental plate, mixed population protein lysates were made from each plate.
For the thapsigargin (TG) stress experiments, 10-20 fertile adult worms of each strain were placed on separate TG plates (RECIPE) seeded with OP50-1 culture. The plates were sealed and stored upside-down at 22 °C for 4 days or until there were large populations of worms and the food sources were nearly depleted. At this point, mixed population protein lysates were made for comparison to the control lysates from the heat shock experiments.

**Protein Lysate Production**

To make protein lysates, the prepared plates from the stress induction experiments were rinsed repeatedly with 1.5 mL of M9 solution (3g KH₂PO₄, 11.3 g Na₂HPO₄·7H₂O, 5g NaCl, 1 mL of 1M MgSO₄ in 1 L dH₂O) and placed into a 1.5 mL micro centrifuge tubes. The full tubes were spun for 2 mins and immediately placed on ice for another 2 minutes to slow the movement of the suspended worms. The supernatants were removed as much as possible by pipetting without disturbing the worm pellets. 2 μL of protease inhibitor (Thermo Fisher Scientific) was added to each tube before re-suspending the worms in 200 μL of worm lysis buffer (50 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM MgCl₂, 100 mM KCL, 10% glycerol, 0.05% NP-40). The tubes were incubated on ice for 5 minutes before proceeding.

The worm lysis suspensions were dropped into liquid nitrogen and allowed to freeze into solid droplets. These solid droplets were crushed into a fine powder using a mortar. After the excess liquid nitrogen evaporated, the powder was transferred to a new 1.5 mL Eppendorf tube and allowed to thaw. The thawed samples were spun down in the micro centrifuge for 5 minutes on top speed and the supernatants were stored at -80 °C in 50 μL aliquots to be used in western blot analysis.
**Gel Sample Preparation and SDS-PAGE**

To prepare samples for SDS-PAGE, 12 μL of 4X Laemmli Sample Buffer (Bio-Rad) was added to each 50 μL aliquot. The samples were then placed in a boiling water bath for 5 minutes. 5 μL of Precision Plus Protein Standard Kaleidoscope (Bio-Rad) was used as a standard in 4-20% Mini-PROTEAN® TGX Stain-Free™ Gels (Bio-Rad) for separating the prepared protein samples. 30 μL of each sample was added to its own well and the gels were run at 120V for 1 hour or until there is large separation of the ladder at the bottom of the gel.

This procedure was followed for the first and second blot performed (heat-treatment trials 1 and 2). The last 2 western blot analyses (heat-treatment trial 3 and thapsigargin trial 1) were completed with 35 μL of each sample after being boiled in the presence of 50 mM DTT for 10-15 mins instead of 5 mins.

**Gel Transfer**

To transfer the gel, whatman paper and PVDF membrane (Thermo Fisher Scientific) were cut to size and soaked in transfer buffer (25mM Tris base, 190 mM glycine, 20% ethanol) and 100% ethanol followed by transfer buffer, respectively, until saturated. The transfer sandwich was assembled and locked into its cassette. An ice pack was placed into the tank to keep the transfer area cold and the tank was filled to cover the sandwich in transfer buffer. The transfer was run at 300 mA at 4 °C for 1 hour. The sandwich was disassembled and the membrane was allowed to dry before continuing.

**Blotting and Antibody Incubation**

The PVDF membrane was wet in 100% ethanol until saturated and then washed in 1x PBST (100mL 10x PBS (73.1 g NaCl, 44.5 g Na₂HPO₄·7H₂O, 11.6 g Na₂HPO₄·H₂O), 900 mL dH₂O, 5 mL Tween 20) twice for 2-5 minutes each. The membrane was then soaked in 3%
blocking buffer (heat treatment trial 1 used pre-made blocking buffer (Thermo Fisher Scientific) and the rest of the trials used non-fat dry milk (0.6 g non-fat dry milk and 20 mL 1x PBST)) for 1 hour while shaking. The buffer was dumped off and the membrane washed in 1x PBST twice for 2-5 minutes each wash. The membrane was then washed in diluted goat IgG anti-βNAC primary antibody (20 μL antibody (Thermo Fisher Scientific) with 8 mL blocking buffer (the antibody volume was increased to 25 μL for the final heat treatment trial and the thapsigargin trial)) overnight (minimum of 14 hours) at 4 °C with gentle agitation. The membrane was then washed in 1x PBST twice for 2-5 minutes each wash. The membrane was incubated in diluted rabbit anti-Goat IgG horse radish peroxidase (HRP) secondary antibody (Thermo Fisher Scientific) (20 μL antibody with 8 mL blocking buffer) at 22 °C for 1 hour. The membrane was then washed in 1x PBST twice for 2-5 minutes each wash.

The amplification portion of the western blot analysis was completed with the Western Blot Amplification Module from Bio-Rad. The membrane was first incubated in 8 mL of amplification reagent (4 mL amplification diluent (Bio-Rad), 2 mL amplification reagent (Bio-Rad), and 2 mL ddH2O) for 10 minutes. Next, the membrane was washed in 20% dimethyl sulfoxide (DMSO) solution (3 mL DMSO, 1.5 mL 10x PBST, and 10.5 mL ddH2O) twice for 5 minutes each wash. The membrane was then washed in 1x PBST twice for 2-5 minutes each wash. Finally, the membrane was incubated in diluted streptavidin-HRP (8 μL streptavidin-HRP (Bio-Rad) with 8 mL blocking buffer) for 30 minutes. The membrane was then washed in 1x PBST twice for 2-5 minutes wash.

Signal Detection

Colorimetric detection was completed with the Opti-4CN™ Substrate kit from Bio-Rad. The membrane was incubated in 10 mL of the substrate dilution (1 mL diluent (Bio-Rad), 4.5
mL ddH₂O, and 200 μL substrate (Bio-Rad)) for approximately 7-10 minutes or until the desired signal appears. To remove excess substrate, the membrane was quickly rinsed in ddH₂O 3-4 times. The membrane was placed on a piece of whatman paper and allowed to dry for analysis.
Results

In these experiments, I tested the hypothesis that ICD-1/βNAC is targeted for cleavage during apoptosis triggered by high levels of MPS in *C. elegans*, and that this cleavage requires both CED-4 and CED-3. To test this hypothesis, I performed western blot analysis to determine the size of ICD-1 in untreated control populations and experimental populations exposed to stressors known to cause ER-specific MPS. To determine the apoptotic elements putatively necessary for the cleavage of ICD-1, I performed the same experiments in animals defective for canonical elements of the *C. elegans* apoptotic pathway, specifically CED-3 or CED-4.

To determine the state of ICD-1 in both untreated and stressed worms, I first optimized a western blot analysis used to detect the protein in the collected lysates. Previous protocols had resulted in the detection of a band at the size appropriate for ICD-1 (22 kD) that is missing in lysates isolated from worms treated with ICD-1 (RNAi), indicating the specific recognition of ICD-1 by our antibody (Figure 7). In addition to the band at 22 kD, a band at 75 kD present in control lysates disappeared in ICD-1 RNAi lysates. There are also bands at approximately 50 kD and 35 kD that are not consistent with any previously acquired data (Figure 7).

A repeat of this protocol produced similar results in my initial experiment (Figure 8A). The bands at 22 and 75 kD were once again detected, but numerous other bands were also present. To reduce background signal, a second experiment increased the stringency of the blocking solution, resulting in decrease in background signal with a concomitant loss of specific signal in most samples (Figure 8B). Nonetheless, the 22 kD and 75 kD bands thought to be specific to ICD-1 were much clearer, as well as a 40 kD band that does not diminish in ICD-1 RNAi lysates (Figure 7). To optimize the strength of the specific signals in my western blot analysis, a third experiment increased the amount of lysate loaded into the gel to present the
optimal balance between visualization of specific ICD-1 signal while reducing background signal dramatically (Figure 8C and Figure 9).

In *C. elegans* exposed to prolonged periods of heat, the full length version of ICD-1 appears to predominate regardless of the genetic background of the worm. Heat is a generator of misfolded protein, and previous work in our lab has shown *C. elegans* exposed to heat results in higher mortality and less vitality relative to untreated controls (Arsenovic et al. 2012). If ICD-1 is targeted for cleavage in the presence of high levels of MPS, we might expect to see a change in the length of ICD-1 worms exposed to heat relative to untreated controls. Specifically, cleavage of ICD-1 at its putatively conserved caspase cleavage site would produce two fragments of 17 kD and 5 kD, one of which would still be putatively recognized by our ICD-1 antibody. There is a faint band at approximately 17 kD in both an untreated and heat-treated CED-3 knockout protein lysates (Figure 8, Lanes 7 and 8 respectively) but the strength of this band doesn’t appear to increase in the population exposed to heat (lane 8).
Figure 8: Western Blot Assessing the Effects of Heat Shock on the detection of ICD-1 in four different strains of *C. elegans*. In all panels, lanes 1 and 6 contain 5 μL of Bio-Rad Precision Plus Protein Kaleidoscope ladder and all other lanes contained either protein of a control or heat treatment population. Lanes 2 and 3 correspond to the $N_2$ strain of wild-type worms, lanes 4 and 5 correspond to MT2547, a CED-4 knockout mutant strain, lanes 7 and 8 correspond to MT1522 and lanes 9 and 10 correspond to MT3002, both of which are CED-3 knockout mutant strains. The black arrows correspond to the predicted location of the ICD-1 protein at approximately 22 kD and the arrowhead indicates the predicted location of cleaved ICD-1 at approximately 17 kD. Panel A was the first blot performed with a commercially made blocking buffer, Panel B was created with the same samples as Panel A except a 3% non-fat dry milk blocking buffer was used, and finally Panel C was a repetition of Panel B but with increased loading volumes and increased exposure to the primary antibody.
As with *C. elegans* exposed to prolonged heat, those exposed to thapsigargin don’t show a detectable shift from full length ICD-1 to cleaved ICD-1 regardless of genetic background. Thapsigargin is a known inducer of MPS in the ER through the disruption of calcium homeostasis in the ER (Amodio et al. 2011). It is a commonly used reagent in *C. elegans* for the elucidation of stress responses triggered by MPS in the ER. If ICD-1 is targeted for cleavage in the presence of ER-specific MPS, one might expect to see a strong shift from full length ICD-1 to its shorter version in wild type animals exposed to thapsigargin relative to the untreated controls. This does not appear to be the case. Consistent with the heat treatment results, the CED-3 knockout lysates display bands at both the 22 kD and 17 kD sizes, but there is no discernible increase in the amount of the putative cleaved ICD-1 as a result of exposure to thapsigargin (Figure 9, lanes 7 and 8).

![Western blot](image)

**Figure 9: Western blot Assessing the Effects of Thapsigargin** on the detection of ICD-1 in four different strains of *C. elegans*. Lanes 1 and 6 contain 5 μL of Bio-Rad Precision Plus Protein Kaleidoscope ladder and all other lanes contained either protein of a control or thapsigargin treatment population. Lanes 2 and 3 correspond to the N₂ strain of wild-type worms, lanes 4 and 5 correspond to a CED-4 knockout mutant strain, lanes 7 and 8 correspond to a CED-3 knockout mutant strain and lanes 9 and 10 correspond to a different CED-3 knockout mutant strain. The lanes are alternating control lysate, TG lysate (i.e. Lane 2 is N₂ control, Lane 3 is N₂ TG treatment etc.). The arrow corresponds to the predicted location of the ICD-1 protein at approximately 22 kD and the arrowhead indicates the predicted location of cleaved ICD-1 at approximately 17 kD. The bracketed region highlights the presence of a 75 kD protein putatively associated with the detection of ICD-1.
Discussion

The purpose of my experiment was to elucidate more information on the state of ICD-1 in *C. elegans* during times of misfolded protein stress and to test the hypothesis that ICD-1 is cleaved in these cells. I also tested the hypothesis that this cleavage is dependent on the initiation of apoptosis involving the caspase activator CED-4 and the caspase CED-3. Throughout these experiments, I used heat exposure and the ER misfolded protein stress inducer thapsigargin to generate misfolded protein in wild-type and apoptotic mutants to determine 1) if ICD-1 is cleaved in *C. elegans* undergoing MPS and 2) if so, is this cleavage dependent on the presence of CED-4 and/or CED-3. To generate interpretable data, I first optimized my western analysis protocol to increase the detection of ICD-1 while decreasing non-specific background signal. Through this optimization, I was able to consistently produce three strong bands at 75, 40 and 22 kD, two of which (75 and 22 kD) are thought to be specific to ICD-1 based on their disappearance in previously performed ICD-1 RNAi experiments (Figure 7). The other bands seen through previous analyses (50 kD and 35 kD) were not detected and therefore deemed to be non-specific to ICD-1 (Figure 7).

The heterodimeric NAC is a translational chaperone that manages protein folding and localization in healthy cells to maintain proteostasis. βNAC’s role in this complex is well-defined in this context, but less is known about the role of βNAC in cells undergoing MPS, and nothing is known about the fate of βNAC in cells undergoing MPS-induced apoptosis. In *C. elegans*, ICD-1, a homologue for βNAC, is important for the regulation of apoptosis as evidenced by the dramatic increase in apoptosis observed in ICD-1 depleted animals (Bloss et al. 2003). If the artificial loss of ICD-1 via RNAi triggers apoptosis in *C. elegans*, it is possible that ICD-1 is purposefully targeted for inactivation via caspase cleavage during biologically relevant
apoptosis i.e. one induced by MPS. Support for this putative outcome is found in the analysis of other βNAC homologues. Human βNAC or BTF3 is known to be depleted in cells undergoing apoptosis, and the protein itself is cleaved by caspase 3 in vitro. The *Drosophila* βNAC homologue, *bicaudal*, is also known to be a caspase substrate in vitro (Creagh et al. 2009). Interestingly, sequence alignment of the three homologues indicates that they all may share the same functionally conserved cleavage sequence (Bloss et al. 2003). At this point, though, βNAC cleavage has not been associated with a cellular event. With the evidence that depletion of ICD-1 induces MPS and rampant apoptosis in *C. elegans* in combination with the possibility of βNAC homologues being susceptible to caspase cleavage, I was lead to the hypothesis that in a naturally induced state of MPS, such as with heat or chemical exposure, ICD-1 is cleaved by a caspase during apoptosis to initiate and/or propagate the death of the affected cells.

To test this hypothesis, we exposed the wildtype, CED-3 knockout mutants, and CED-4 knockout mutants to heat and thapsigargin as MPS inducers and determined the state of ICD-1 in these populations via western analysis. In general the results were inconclusive, but similar patterns of detection were seen in natural stress induction as seen in artificial ICD-1 depletion. Three bands (22, 40 and 75 kD) are observed consistently in my western analyses, with the band at 22 kD being the correct size for ICD-1. The disappearance of the band over the course of an ICD-1 RNAi experiment supports this conclusion. The band at approximately 40 kD was hypothesized to be a homodimeric form of βNAC, but this interpretation is unlikely in light of the fact that this band, unlike the band at 22 kD, is not diminished in ICD-1 RNAi treatments. This is an indication that this protein at 40 kD is not related to ICD-1. The band at approximately 75 kD does appear to be related to ICD-1 based on its diminution in ICD-1 RNAi experiments. Such a size could be indicative of a trimer or tetramer of ICD-1, consistent with
the ability of αNAC to form multimers to act as transcription factors when dissociated from βNAC (Wang et al. 2010).

The ability of ICD-1 to form multimers would shed new light onto the state of ICD-1 in healthy and stressed cells. αNAC is known to dimerize to form a transcription factor involved in bone cell differentiation in mice, but there is no circumstance in which βNAC is known to self-associate. These results may be the first evidence of ICD-1/βNAC forming multimers to perform functions beyond that of a translational chaperone subunit. The role that this might be is worthy of investigation, and may involve, as it does with αNAC, a role in control of gene expression. In fact, if ICD-1 dissociates from both the ribosomal complex and αNAC during MPS, it may form these multimers to control the expression of genes important for cell survival. In this context, cleavage of ICD-1 by a caspase during the initiation of apoptosis may prevent ICD-1 from saving a cell too damaged to function properly, thereby sealing its fate for death.

When comparing apoptotic mutants with the wildtype *C. elegans* in both control and stress inducing environments, there seem to be no differences in ICD-1 western analysis profiles. Considering the heat stress experiments, OCD-1 bands show up consistently in all lanes with no changes in between control and treatment populations. For example, the CED-3 mutants to the right of the gels contain bands at 22 kD and 40 kD in all four lanes (Figure 8 & 9). This qualitative data could indicate that the same thing is happening across all four strains of worms that were tested. If no changes are seen when core apoptotic elements are eliminated from the situation, then perhaps the state of ICD-1 that is seen in these situations are not dependent on the core apoptotic pathway.

Unfortunately, conclusions based on these experiments are preliminary at best because the bands were not consistently detectable from trial to trial though the same protocol was
applied. This inconsistency can be seen in the disappearance and reappearance of bands from panel B to panel C of the heat exposure experiments (Figure 8). This variability could be occurring for a variety of reasons. For instance, the protein concentration could be varying from lysate to lysate in between trials. In some cases, the amount of protein used could be detectable, but in others, there is not enough to visualize the already minute amount of ICD-1 present in mammalian cells. Another reason for the inconsistency between experiments seen here and the experiments previously completed is the variability between batches of ICD-1 antibody. The antibody used within these experiments is polyclonal in nature, meaning they bind to a variety of epitopes of the desired protein. When these antibodies are being generated, there is no guarantee that the host is generating antibodies against the same epitopes in each preparation. Therefore, success in one set of experiments could be skewed by the use of a different batch of polyclonal antibodies in future experiments.

The lack of a change in the state of ICD-1 during MPS could be to the reasons discussed above, but could also be due to suboptimal exposure to stress inducers. For example, the heat treatment parameters used in my experiments were based on previous experiments measuring the viability and movement of worms (Arsenovic et al. 2012). While these parameters may sufficient to detect gross morphological defects in movement and effects on embryonic lethality, they may not be appropriate to induce strong MPS at the cellular level. This theory could also be applied to the thapsigargin experiments. The plates that were utilized could not have had enough chemical in them to cause a MPS response within the three days of exposure time. With these possibilities, experiments should be repeated with increased exposure to the stress inducers where a MPS response is detected.
Conclusions and Further Research

Although these experiments did not elucidate strong conclusions about the state of ICD-1 during biologically relevant stress, the fundamental question of the fate of ICD-1 in healthy and stressed cells remains of interest. Heat exposure and thapsigargin both have the potential to induce MPS in *C. elegans*, but what that response entails for the fate of ICD-1 is still unclear. ICD-1 could be cleaved by CED-3 or another caspase, consistent with the presence of ICD-1’s putatively conserved cleavage sequence; it could also form multimers and perform some secondary function similar to the transcription regulation capability of an αNAC homodimer. These two outcomes are of course not mutually exclusive and may actually be related. Additionally, our hypothesis was that ICD-1 is cleaved during MPS dependent apoptosis through the core apoptotic pathway of *C. elegans*. However, no apparent changes were seen when results were compared between wild-type and mutant strains incapable of apoptosis. This could indicate that ICD-1’s fate in any given cell, healthy or stressed, is not dependent on the developmental apoptotic pathway.

These conclusions are by no means confirmed and repetitions of these experiments should be completed to ensure confirmation of these theories. These experiments should be repeated using higher protein concentrations confirmed by protein quantification assays to ensure that there is enough protein in the lysate to be detected on the blot. The amount of protein does not have to be equal as these experiments are still collecting qualitative data and accurate quantification is not necessary at this point, but verification of the presence of enough protein could save time and ensure clearer results. In addition, as discussed above, I recommend an increase in the exposure to stress inducers to ensure that the response detected is valid and measuring what I hope it is measuring.
Finally, once the protocol is optimized and these results have been confirmed, then further studies on a separate apoptotic pathway and its relationship to the NAC could be completed and even extended to a monoclonal antibody based protocol. In addition, a protocol for the state of ICD-2/αNAC should be optimized to allow for further comparison and characterization of the other subunit of the NAC. These studies will provide substantial insight into apoptosis within *C. elegans* and with time could be used to elucidate an *in vivo* model for the role of the NAC in human diseases, including neurodegeneration and cancer (Kogan and Gvozdev, 2013).
References


