Spring 2018

The state of the translational chaperone ICD-1 during apoptosis in caenorhabditis elegans

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The State of the Translational Chaperone ICD-1 During Apoptosis in Caenorhabditis elegans

An Honors College Project Presented to
the Faculty of the Undergraduate
College of Science & Mathematics
James Madison University

by Kyle Vincent Cicalese
April 2018

Accepted by the faculty of the Biology and Chemistry & Biochemistry Departments, James Madison University, in partial fulfillment of the requirements for the Honors College.

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PUBLIC PRESENTATION

This work is accepted for presentation, in part or in full, at BioSymposium and Honors Symposium on April 13, 2018 and April 18, 2018, respectively.
# Table of Contents

List of Figures and Tables  
4

Acknowledgements  
5

Abstract  
6

Introduction  
7

Methods & Materials  
17

Results  
21

Discussion  
27

Suggestions for Further Research  
31

References  
33
List of Tables and Figures

Diagram of ER nascent polypeptide folding functions 8
A simplified feedback diagram of the three branches of the UPR 10
Conserved apoptotic pathways for three distantly related organisms 11
Overview of the initiation of apoptosis in C. elegans 12
Amino acid sequence alignments of βNAC homologs 15
Western blot with 4°C overnight incubation of anti-βNAC antibody 21
Western blot of 24-hour ICD-1 RNAi time course with anti-βNAC antibody 22
Western blot measuring the effect of the misfolded-protein stressors 23
Western blot analysis of ICD-2 in wild type C. elegans 24
Western blot analysis of ICD-1 in a native protein gel with anti-βNAC antibody 25
Western blot with of the D. melanogaster ICD-1 homolog, bicaudal 26
Acknowledgements

I would like to thank the James Madison University Biology and Chemistry and Biochemistry Departments and the James Madison University Honors College for giving me the opportunity to conduct research through a guided thesis project. I would specifically like to thank Jared Diener and Dr. Phil Frana from the Honors College for building personal connections with me through HON 300, checking in on the progress of my research this past year, and for granting me an extension on the thesis.

I would also like to thank my research advisor, mentor, and professor, Dr. Bloss, for allowing me to join his research team in the Spring of 2016. Dr. Bloss not only patiently and effectively explained difficult concepts associated with my project, but he also continuously challenged me to think critically and independently develop new paths to take with the research. In addition, I appreciate Dr. Bloss for being very flexible with my lab time because of my busy class schedule with two majors. I am grateful to have been mentored by a person like him, and I feel very prepared for professional school and further research because of my time in his lab.

I would also like to thank my committee members, Drs. Kimberly Slekar and Nathan Wright, for reviewing my proposal and providing input on my final paper (especially in the very short amount of time I had allotted for them). I would also like to acknowledge Dr. Wright for being a role model, believing in my work ethic, and inspiring my love of biochemistry. I would also like to thank him dearly for doing any favor I asked of him literally the same day I asked. In addition, I would like to thank Dr. Slekar for allowing me to use her lab bench while I ran western blots, despite their being plenty of space on Dr. Bloss’.

I would also like to thank Dr. Marquis Walker for significantly improving the efficiency of my western blot analyses through the use of his semi-dry membrane transfer cell. While I unfortunately never took a class with him, Dr. Walker has also served as a phenomenal teacher by succinctly explaining the specific details of various lab techniques as well as areas of his own research to me. In addition, Dr. Walker’s tremendous work ethic has inspired me to become an even more dedicated student.

I would also like to thank my lab partners Claire Gormley, Lars Farber, Kyle Perez, Kristen Hoffman, Rana Ihsan, Kyle Perez, Marina Barmanova, Taylor Hutchinson, and Monica Gerber for helping me transition to lab, passing on detailed information on previous experiments, and working alongside me these past two years.

I would also like to thank my family for supporting my college experience. Whether that be through funds, moral support, proofreading (thank you, Giggy), etc., I appreciate your support nonetheless.

Finally, I would like to thank my friend, Adrian E. Jimenez, for believing in me and inspiring a greater love for academics and research in me since the start of our friendship years ago. I look forward to continuing on our hardworking path of becoming medical doctors.
Abstract

The unfolded protein response (UPR) is a signal transduction cascade that mitigates low levels of misfolded protein stress in the endoplasmic reticulum (ER) in an effort to save the affected cell, while prolonged and/or acute ER stress leads to UPR-initiated apoptosis (programmed cell death). One putative step driving apoptosis is the cleavage of chaperones, proteins tasked to help misfolded proteins refold, by caspases, proteases essential to the execution of apoptosis. We are studying the nascent polypeptide-associated complex (NAC), a heterodimeric chaperone complex essential for viability, to determine if its beta subunit is cleaved by caspases during apoptosis to prevent the NAC from refolding proteins in a cell fated to die. To this end I am developing a western analysis protocol to assess the state of the βNAC homolog inhibitor of cell death-1 (ICD-1) in *C. elegans* experiencing misfolded protein stress. Initial results have identified an antibody with high affinity for a protein of similar molecular weight as ICD-1 that is depleted in animals treated with ICD-1 RNA interference (RNAi). Protein lysates isolated from animals experiencing chemically induced ER-specific misfolded protein stress have displayed altered western profiles consistent with cleavage of ICD-1. Further studies, including immunoprecipitations and protein sequencing, will provide independent confirmation of the specificity of our ICD-1 western analyses, while experiments in stressed *C. elegans* mutants defective for apoptosis will determine the causal relationship of apoptosis and cleavage of ICD-1. If ICD-1 is cleaved by caspases during UPR-induced apoptosis, it will be the first evidence of the targeting of chaperones during the initiation and/or propagation of apoptosis, identifying a novel strategy that helps ensure cell death by eliminating proteins tasked with saving the cell.
Introduction

Misfolded protein stress can trigger cell damage and death if the stress is not mitigated by cellular responses. Misfolded protein stress in the endoplasmic reticulum (ER) is often the source of this stress, and the cell has robust responses that can help resolve this stress and save the cell. Sometimes, though, the cell accumulates so much damage that killing the cell is preferable to saving it. In such cases, apoptosis is triggered to eliminate the damaged cell, and this process activates proteases called caspases that cleave the target proteins to ensure the death of the cell. I am testing the hypothesis that one target of activated caspases is a chaperone complex called the NAC and that this cleavage dismantles a cell-saving stress responder to ensure efficient cell death.

The Unfolded Protein Response

The ER is the organelle responsible for synthesis and modification of proteins and lipids. Before being transported to the cell surface, secretory or membrane-bound proteins first enter the ER lumen as unfolded polypeptide chains. Resident protein folding components called chaperones in the lumen help fold these nascent polypeptides into their native states, and only when they have reached this conformation are they transported via ER exit vesicles\(^1\) to the cell membrane to perform their specific functions (Fig. 1)\(^2\). Protein folding, however, is a slow and inefficient process with several polypeptide chains never reaching their functional tertiary structure\(^3\). The cell must therefore monitor the nascent proteins that enter the ER and retro-translocate any that are likely to be terminally misfolded so they can be degraded by the 26S proteasome in the cytosol\(^1,2\). This process of targeting misfolded proteins for ubiquitination and subsequent proteasome degradation is known as ER-associated degradation (ERAD)\(^3\).
Figure 1 Diagram of ER nascent polypeptide folding functions. Unfolded polypeptides enter the ER lumen through transmembrane channels called translocons. Resident chaperone proteins such as BiP, calnexin, and protein disulfide isomerase (PDI) assist in proper folding of nascent polypeptides by preventing protein aggregations, monitoring the processing of the highly branched glycans, and forming intramolecular disulfide bonds between methionine residues. Various intracellular and extracellular conditions can lead to an imbalance of nascent polypeptide management in the ER, leading to the initiation of the unfolded protein response. Figure adapted from Faitova et al. 2006.

Cell differentiation, extracellular conditions, and the physiological state of the cell all affect the flux of nascent polypeptides into the ER lumen. These factors, especially cytotoxic condition, can lead to an imbalance between nascent polypeptide load and protein folding machinery, resulting in an accumulation of misfolded proteins in the ER. This cellular condition is referred to as “ER stress.”

The unfolded protein response (UPR) is an intracellular signal transduction cascade that regulates gene expression pathways in the ER to mitigate misfolded protein stress. It first acts to reestablish homeostasis by mediating the expansion of the ER lumen and increasing the expression of chaperone proteins to occupy that expanded space to thereby expand the organelles capacity for protein folding. The UPR also attenuates translation by decreasing the flux of nascent polypeptide into the lumen and increasing ERAD. If ER stress is prolonged and the UPR’s cell saving mechanisms are unable to manage the increased unfolded protein load, the
UPR can initiate apoptosis (programmed cell death) to eliminate the damaged cell and prevent further harm to the organism\(^1\).

The UPR is composed of three branches that act in parallel to increase gene expression pathways of chaperones to enhance protein folding. Each branch is defined by a class of ER-resident transmembrane proteins that mediate its intracellular signaling pathways: inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK)\(^4\). All three sensors act to monitor the conditions of the ER and initiate signal transduction cascades that increase the expression of genes that increase the protein folding machinery in the ER. In addition, IRE1 and PERK act to decrease the load of nascent polypeptide chains entering the ER lumen through regulation of translation. Ultimately, these functions act as feedback loops to mitigate misfolded protein stress (Fig. 2)\(^1\). Though it is not clear which mechanism(s) of the UPR lead to apoptosis upon prolonged ER stress, a study suggests that one possibility is that the signaling of these three branches eventually becomes unsynchronized over time and the balance between rectifying the cell and terminating it shift towards the latter as misfolded protein stress prolongs\(^1\).
Figure 2 A simplified feedback diagram of the three branches of the UPR. IRE1, ATF6, and PERK sense ER stress levels and initiate signal transduction pathways to increase the protein folding capacity of the ER lumen. IRE1 and PERK also signal pathways to decrease the amount of protein loaded into the lumen. If these rectifying functions cannot reestablish homeostasis and ER stress persists, the UPR will eventually initiate apoptosis to terminate the cell and prevent damage to the overall organism. Figure adapted from Walter & Ron (2011).

**Apoptosis and C. Elegans**

Apoptosis is a ubiquitous metazoan cell death mechanism that is not only crucial in maintaining organismal homeostasis in response to prolonged ER stress, but it is also important in development and removal of harmful cells. *Caenorhabditis elegans* (*C. elegans*) are microscopic, hermaphroditic nematodes that can reproduce rapidly, generating 250-300 genetically identical progeny. They are also transparent and easily visible under a microscope. The main effectors of both the UPR and apoptosis present in *C. elegans* are highly conserved in mammalian organisms, including humans.°
are nematodes whose UPR and apoptotic effectors are highly conserved in humans, making them excellent model organisms for studies in either process (Fig. 3).

**Figure 3** Conserved apoptotic pathways for three distantly related organisms. The *C. elegans* apoptotic effectors have been highly conserved despite evolution. The various colors of boxes encasing the name of the effectors represent the homologues among these three organisms, describe what the colors represent generally (e.g. purple boxes represent conserved pro-apoptotic BCL-2 family members, blue represent anti-apoptotic BCL-2 family members, yellow represent caspase activated proteins, red represent caspases, green represent pro-apoptotic elements released from mitochondria during apoptosis, light blue represents anti-apoptotic elements that exist in the cytoplasm, and orange is Cytochrome C, which is released by the mitochondria to initiate apoptosis.). Figure adapted from Shi (2001).

Nematodes are eutelc organisms, producing the exact number of cells during development that they will have throughout adulthood. In *C. elegans*, 1090 cells are produced to form an adult worm, and exactly 131 undergo apoptosis during development\(^8\)-\(^10\). These 131 apoptotic cells use the same mechanisms to undergo apoptosis (Fig. 4). In a majority of cells where apoptosis is not occurring, egg-laying defective EGL-1 is not expressed, allowing cell death abnormal CED-9 to bind to CED-4 dimers, inhibiting apoptosis\(^10\). EGL-1 and CED-9 are members of the Bcl-2 family, where CED-9 is an anti-apoptotic protein with four Bcl-2
homology (BH) domains (BH1, 2, 3, and 4), and EGL-1 is a pro-apoptotic BH3-only-domain protein\textsuperscript{9,10}. To initiate apoptosis, the targeted cell will begin to express EGL-1, which physically interacts with CED-9 to dissociate the interaction intermolecular interaction between CED-9 and CED-4. CED-4 then forms tetramers that recruit, bind, and subsequently facilitate the activation of the caspase CED-3, the protease that executes apoptosis in \textit{C. elegans}. The oligomerized CED-3/CED-4 complex is called the apotosome, which is necessary for cell death\textsuperscript{9-12}. One of the anti-apoptotic proteins putatively cleaved by CED-3 is inhibitor of cell death 1 (ICD-1), the homolog of human BTF3/\(\beta\)NAC, which is the beta subunit of the nascent polypeptide-associated complex (NAC)\textsuperscript{13-17}. The possible cleavage of ICD-1 by CED-3 is one of the main investigations of this study.

\textbf{Figure 4} Overview of the initiation of apoptosis in \textit{C. elegans}. When the cell receives the appropriate developmental cues that require cell termination, EGL-1 will bind to the CED-9/CED-4 complex, which will release and activate dimeric CED-4, forming a tetramer that will bind to Pro-CED-3 to facilitate the initiation of apoptosis. Figure adapted from Lettre & Hengartner (2006).
The Nascent-Polypeptide Complex

The Nascent-Polypeptide Complex (NAC) is a ribosomal associated protein involved in nascent polypeptide folding. It is also suggested that the NAC acts as a translational chaperone in both normal protein folding and the stress response. The NAC is a member of the HSP 70/40 family of chaperones. As a translational chaperone, the NAC promotes protein localization away from the ER and to other parts of the cell during translation. This function of the NAC suggests that it is important in cell stress management by preventing the aggregation of misfolded proteins in the ER.

The NAC is a heterodimer consisting of α- and β-subunits, both of which are thought to have distinct functions when they are not associated with the NAC heterodimer, e.g. the α-NAC homodimer has a special structure that allows it to interact with nucleic acids, serving as a transcriptional factor. The heterodimeric form of the NAC does not interact with nucleic acids, and it is suggested that an excess in β-NAC can suppress the concentration of the α-NAC homodimer, thus indicating that β-NAC may serve as a transcriptional regulator. Also while β-NAC/ICD-1 can serve addition to serving as a transcriptional regulator, its role as a translational chaperone subunit is the main topic of this proposed research.

The translation chaperone role of β-NAC has been studied in its nematode homolog, inhibitor of cell death-1 (ICD-1). Bloss et al. (2003) used RNA-mediated interference (RNAi) to determine the effect of ICD-1 depletion in *C. elegans*. Depletion of ICD-1 by RNAi resulted in an increase in apoptosis in the embryo and larva stage of the worm. The cell type that decreased the most in these stages were neurons, however, apoptosis also occurred in other cell lineages. Therefore, Bloss et al. (2003) also examined the effect of IDC-1 depletion on lineages where apoptosis does not naturally occur (i.e., gut cells). They found that ICD-1 depleted gut
cells could die upon the depletion of ICD-1. To further delineate the association of ICD-1 with apoptosis, they found that overexpressing ICD-1 suppresses developmentally programmed cell death. These findings strongly suggest that ICD-1 plays an anti-apoptotic role in the life of cells in *C. elegans*; how ICD-1 interacts with other core apoptotic elements in *C. elegans*, including CED-4 and CED-3 was not known. To determine potential interactions, knockout strains of CED-3 or CED-4 were depleted of ICD-1 to determine if an increase in apoptosis was still observed in the absence of these important pro-apoptotic proteins. It was found that CED-4 loss-of-function mutations eliminated all apoptosis in *icd-1* (RNAi) mutants, indicating that CED-4 is necessary for increased apoptosis upon depletion of ICD-1; unexpectedly, CED-3 was not required for the increase in apoptosis observed. This result was unexpected because CED-3 was originally considered to be required for all apoptosis in *C. elegans*, indicating the existence of a putative CED-3-independent apoptotic pathway that is activated by CED-4, and suppressed by ICD-1 and or the NAC\(^9,10,13\).

The results from the study above are significant, however, depletion of ICD-1 in these experiments was implemented in an artificial manner, i.e. via RNA interference. The purpose of this study in particular is to determine if a depletion of ICD-1 in the cell occurs in a more biologically relevant context. More specifically, the purpose of this investigation was to determine if ICD-1 is actively depleted during misfolded protein stress by the caspases activated during apoptosis, thereby promoting further misfolded protein stress and reinforcing the apoptotic signal. In support of this model, human βNAC is known to be cleaved by caspases both *in vitro* and in cell lines undergoing apoptosis; in addition, the Drosophila homologue of βNAC/ICD-1, Bicaudal, is also cleaved in vitro by caspases\(^{13,18,19}\). ICD-1 shares with these homologues a putative caspase cleavage in the carboxyl region of the protein (Fig. 5)\(^{13}\).
Figure 5 Amino acid sequence alignments of βNAC homologs: *bicaudal* (CG3644) and βNACtes coding genes (CG18313 and CG32601) of *D. melanogaster*, CAB56249 of *Arabidopsis thaliana*, ICD 1 (Q18885) of nematode *Caenorhabditis elegans*, CAA52200 of *Homo sapiens*, and Egd1 (CAA55371) and Btt1 (P40314) of *Saccharomyces cerevisiae*. The NAC domain is outlined. The six fragments that form the α helices of the caspase 3 recruitment domain (CARD) in nematode βNAC are indicated with spiral lines below the bottom line of the alignment. The amino acid sequences cleaved by caspase 3 (human QSVD and *D. melanogaster* GDDD) are framed with a solid line; putative caspase cleavage sites [71] are framed with a dashed line. Figure and legend adapted from Kogan and Gvozdev et al. (2014).

Experimental Strategy

In order to determine the state of ICD-1 during ER stress-induced apoptosis, I developed a western blotting protocols to visualize the relative expression patterns of intact ICD-1 at 17.5 kD and its putatively cleaved state consisting of two bands of approximately 5 kD and 12.5 kD. Before these western blots could be performed, an antibody specific to ICD-1 needed to be identified and a western protocol needed to be optimized for it. This investigation used a polyclonal anti-βNAC antibody (Invitrogen) to determine the specificity for ICD-1 in a western blot of wild type lysates. ICD-1 RNA interference (RNAi) trials were further confirm the
specificity of the antibody to ICD-1 predicting that upon ICD-1 depletion, the band visualized by our antibody should also be depleted if it is ICD-1. The optimized western protocol was used to determine the state of ICD-1 during misfolded protein stress by exposing *C. elegans* populations to misfolded-protein-stress-inducers, such as tunicamycin (TN), which causes an increase in misfolded glycoprotein aggregation in the ER and thapsigargin (TG), which is a SERCA-inhibitor that decreases Ca$^{2+}$ levels in ER, debilitating calcium-dependent ER chaperones.

This investigation is of interest because understanding how ICD-1 is depleted during misfolded protein stress in *C. elegans* may allow for a greater understanding of the role of the human homologue, βNAC, in management of cellular proteostasis. These studies are also important in that they may identify for the first time the targeting of a cell-saving stress response by a caspase, revealing a previously unrealized strategy engaged during apoptosis. In addition, these studies could ultimately provide further insight into the consequences of the βNAC depletion observed in neurodegenerative disorders like Alzheimer’s and βNAC overexpression in certain types of cancer$^{16}$. 
Materials & Methods

Care and Maintenance of *C. elegans*

*C. elegans* strains were maintained on Nematode Growth Medium (NGM) (Carolina Biological). 200 µL *Escherichia coli* strain OP50-1 was used as a food source on each wild type plate. Each strain was sealed and stored at room temperature 22 °C. *C. elegans* colonies were replenished every seven days by transferring worms to new NGM plates.

Preparation of Bacterial Culture

Cultures of *E. coli* OP50-1 and bacterial strains expressing dsRNA targeting ICD-1 mRNA were prepared using Luria Broth culture (Fischer Scientific). 15 mL of Luria Broth was incubated with a colony of the desired *E. coli* strain and 15 µl of antibiotics (streptomycin for OP50-1 cultures and ampicillin for RNAi cultures) at 50 µM to prevent growth of other microorganisms at 37 °C for 12 hours in the shaker. After 12 hours the cultures were transferred to sterile 15 mL tubes and refrigerated at 4 °C until needed.

Lysate Production

Two *C. elegans* plates were each rinsed repeatedly into a 1.5 mL Eppendorf tube with 1 mL of M9 solution [3 g KH$_2$PO$_4$, 11.3g Na$_2$HPO$_4$·7H$_2$O, 5g NaCl, 1 mL 1M MgSO$_4$, 1 L dH$_2$O] and then centrifuged, with the supernatant being discarded. 100 µL of protein extraction buffer were added to the pellet per plate with 1% protease inhibitor (Thermo Scientific). Liquid nitrogen was used to freeze drops of solution, and each solid droplet was crushed with mortar and pellet and added back to the Eppendorf tube. Once thawed, the solution was centrifuged and 40 µL aliquots were prepared for western blots.
ICD-1 RNAi Plate Production and Time Course Design

RNAi plates were prepared as follows: 4.5 g NaCl, 25.5 g agar, 3.75 g peptone, 1.5 mL of 2mg/mL uracil, 0.22 g CaCl, 0.75 g of 10mg/mL cholesterol in 1462 mL of diH2O. The solution was autoclaved, allowed to cool, and 37.5 mL of phosphate buffer (pH 6), 1.5 mL of 1M MgSO4, 15 µL of 0.1M IPTG (required for induction of dsRNA expression) and 1.5 mL of 25 mg/mL ampicillin (for selection of dsRNA-expressing plasmid) were added.

To perform the ICD-1 RNAi time course, approximately 100 N2 adult worms were transferred from wild type plates to RNAi plates. After 24 hours of exposure to ICD-1 RNAi, plates of stressed *C. elegans* populations were used to make lysates that were then used for western blot analyses. These lysates were created for three days, resulting in lysate populations with 24, 48, and 72 hours of exposure to ICD-1 RNAi.

SDS PAGE Gel Electrophoresis

4-20% grade Mini-POTEAN® TGX Stain-Free™ Gels (Bio-Rad) were used to separate proteins and ladder. Western run buffer consisting of 14.1 g glycine, 6.7 g tris, and 0.1% SDS was used to run the gel. 5 mL of Precision Plus Protein Standard Kaleidoscope (Bio-Rad) was used as a standard. Each well consisted of a solution of 20 µL of lysate, 5 µL of 4x Laemmli Sample Buffer, and 2 µL dithiothreitol. Gels were run at 120 V for 1 hr, then transferred to Polyvinylidene difluoride (PVDF) membrane (Thermo Scientific) using a semi-dry transfer.

Native Gel Electrophoresis

4-20% grade Mini-POTEAN® TGX Stain-Free™ Gels were used to segregate proteins and ladder. Western run buffer consisting of 14.1 g glycine and 6.7 g tris were used to run the gel. 5 mL of Precision Plus Protein Standard Kaleidoscope was used as a standard. Each well
consistent of a solution of 20 µL of lysate and 5 µL of Native Sample Buffer. Gels were run at 120 V for 1 hr, then transferred using a semi-dry transfer.

**Western Blot Analyses**

To blot and incubate the antibody, the PVDF membrane with transferred protein was allowed to dry and then wet in 100% ethanol until saturated. The membrane was then washed in 1x PBST (100 mL of 10x PBS, 900 mL of dH₂O, and 5 mL of tween 20) twice for 5 min each. The membrane was then washed in 3% blocking buffer (0.6 g dry milk and 20 mL 1x PBST) for 60 min. The membrane was then washed in 1x PBST twice for 5 min each. The membrane was incubated with diluted anti-βNAC (10 µL primary antibody and 4 mL 3% blocking buffer) overnight (minimum of 14 hrs) at 4ºC. The membrane was then washed in 1x PBST twice for 5 min each. A diluted horse radish peroxidase (HRP) secondary antibody (Invitrogen) (10 µL secondary antibody and 4 mL 3% blocking buffer) at room temperature for 1 hr. The membrane was then washed in 1x PBST twice for 5 min each.

For the amplification portion of the western blot, the membrane was incubated in 4 mL of Bio-Rad Amplification Reagent [2mL amplification diluent (Bio-Rad), 1 mL Amplification reagent (Bio-Rad), and 1 mL ddH₂O] for 10 min. Next, the membrane was washed in 20% Dimethyl sulfoxide (DMSO)/PBST (3 mL DMSO, 1.5 mL 10x PBST, and 10.5 mL ddH₂O) times for 5 min each wash. The membrane was then washed in 1x PBST twice for 5 min each. The membrane was then incubated in diluted streptavidin-HRP [4 µL streptavidin-HRP (Bio-Rad) and 4 mL 3% blocking butter] for 30 min.

For colorimetric detection, the membrane was incubated in 5 mL Opti4CN substrate dilution [0.5 mL diluent (Bio-Rad), 4.5 mL ddH₂O, and 0.100 mL substrate (Bio-Rad)] for 15 min or until desired signal appeared (usually approximately 7-10 min). The membrane was then
washed in ddH₂O for 15 min and then allowed to dry away from sunlight (note: PVDF is sensitive to UV light) for analysis.
Results

The purpose of this study was to investigate the state of ICD-1 during misfolded protein stress in the ER of *C. elegans*. Before performing experiments related to the state of ICD-1 during apoptosis, it was essential to find an antibody with high affinity for our target protein. A goat polyclonal anti-βNAC antibody was expected to display a high affinity for ICD-1 through a western blot analysis, thus a protocol to optimize the signaling for ICD-1 was necessary to proceed with the investigation. Many different protocols were attempted, a 4°C overnight incubation of primary anti-βNAC antibody displayed the highest specificity for a band in the molecular weight range of approximately 17.5 kD (Fig. 6). Two other proteins with molecular weights of approximately 15 and 21 kD appear to have a specificity for the anti-βNAC antibody as well as two other bands with molecular weights of approximately 45 kD and 54 kD.
Figure 6 Western blot with 4°C overnight incubation of anti-βNAC antibody. Lane 1 contained 5 µL of Bio-Rad Precision Plus Protein Kaleidoscope ladder. 20 µL of wild type lysate with laemmli buffer and dithiothreitol (DTT) were added to lanes 2-3.

To further determine the specificity of the anti-βNAC antibody for ICD-1, RNA interference (RNAi) was performed with ds RNA species specific for ICD-1 mRNA. Lanes 3-5 and 8-10 display an absence of a prominent band of 21 kD that is present in wild type lanes 2 and 7, with the retention of the 15 and 17 kD bands in both wild type and ICD-1 RNAi lysate populations (Fig. 7). In addition, relatively prominent bands at 34 kD and 75 kD lanes (2 and 7) were not present in the ICD-1 RNAi populations. Finally, a 50 kD band was only apparent in the 72 hour ICD-1RNAi exposed lysate populations (lanes 5 and 10).

Figure 7 Western blot of 24-hour ICD-1 RNAi time course with anti-βNAC antibody. Lanes 1 and 6 contained 5 µL of Bio-Rad Precision Plus Protein Kaleidoscope ladder. Lanes 2 and 7 contained N2 control lysate samples. Lanes 3 and 8 contained samples of *C. elegans* with 24-hour exposure to ICD-1 RNAi, 4 and 9 had 48 hours of exposure, and 5 and 10 had 72 hours of exposure. 30 and 20 µL of samples containing the respective lysates as well as laemmli buffer and DTT were loaded into lanes 2-5 and 7-10, respectively. Black arrows point to the gradual absence of a 21 kD band through ICD-1 RNAi exposure. Green arrows point to the gradual
depletion of a 37 kD band through ICD-1 RNAi exposure. Blue arrows point to the appearance of a 50 kD after 72 hours of ICD-1 RNAi exposure.

To determine the state of ICD-1 in worms experiencing misfolded protein stress, western analysis was performed in *C. elegans* populations exposed to tunicamycin (TN) and thapsigargin (TG). Results indicate the presence of a band of similar molecular weight to ICD-1 in all three conditions. Trials with TN stressor indicate the presence of a significant band at 75 kD that is not present in other stressors (Fig. 8).

![Figure 8](image-url)

**Figure 8** Western blot measuring the effect of the misfolded-protein stressors, tunicamycin (TN) and thapsigargin (TG), on ICD-1. Lane 1 contained Bio-Rad Precision Plus Protein Kaleidoscope ladder. Lane 2 contained N2 control lysate samples. Lane 3 contained TN-stressed sample. Lane 4 contained TG-stressed sample. 40 µL samples containing the respective lysate as well as laemmli buffer and DTT were loaded into lanes 2-4.
To determine the state of ICD-1’s binding partner, ICD-2, during my experiments, a western blot protocol for the optimization of anti-ICD-2 antibody was attempted. Amongst two trials with varying durations of primary antibody incubation, the second trial with a 4°C overnight incubation appeared to produce the most significant signal, but no band at the expected site of ICD-1 was observed (Fig. 9). Lanes 2-4 contained similar concentrations of lysate, and the western blot indicates cross reactivity with little specificity.

![Western blot analysis of ICD-2 in wild type C. elegans.](image)

**Figure 9** Western blot analysis of ICD-2 in wild type *C. elegans*. The expected molecular weight for ICD-2 is approximately 22.1 kD. Lane 1 contained 5 µL of Bio-Rad Precision Plus Protein Kaleidoscope ladder. Lanes 2-4 contained 20 µL of a solution containing N2 lysate as well as laemmlis buffer and DTT.

To determine the ability of the anti-βNAC antibody to ICD-1 in native conditions, a western blot using a similar protocol for anti-βNAC staining for SDS-PAGE gels was conducted using a native gel (Fig. 10). Lanes 2-9 all contained 20 µL of sample containing N2 wild type
lysate and native sample buffer with no denaturant or boiling of the sample in an effort to retain the tertiary structure of ICD-1. The proteins in sample lanes do not appear to have separated sufficiently through gel electrophoresis and no discernible bands were observed.

Figure 10 Western blot analysis of ICD-1 in a native protein gel with anti-βNAC antibody. Lanes 1 and 10 contained 5 μL of Bio-Rad Precision Plus Protein Kaleidoscope ladder. Lanes 2-9 contained 20 μL of a solution containing N2 lysate as well as Native buffer. Denaturing agents SDS and DTT were not used in the native gel experiments.

To determine the potential cross reactivity of anti-βNAC antibody to other homologues of ICD-1, a western blot protocol using anti-βNAC antibody was performed on the βNAC homologue, bicaudal, from *Drosophila melanogaster*. The expected molecular weight of bicaudal is approximately 17.7 kD. A significant signal appeared at the 25 kD mark in lane 3 where wild type *D. melanogaster* was loaded (Fig. 11).
Figure 11 Western blot with of the *D. melanogaster* ICD-1 homolog, bicaudal. Lane one contained 5μL of Westernsure ladder. Lane 2 contained 35μl wild type *C. elegans* lysate. Lane 3 contained 35μl wild type flies. Lane 4 contained 35μl trpml mutant flies.
Discussion

The purpose of this investigation was to determine the state of inhibitor of cell death-1 (ICD-1) during misfolded protein stress in C. elegans. To more effectively understand the state of ICD-1, it was necessary to first develop a western blotting protocol for an antibody specific to ICD-1. Previous attempts at optimizing a western blot protocol for an antibody specific for ICD-1 were conducted by former lab members using a monoclonal antibody, however, the antibody they used 1) produced significant noise on the western blot and 2) could not produce a signal specific to ICD-1. The current investigation used a polyclonal anti-βNAC antibody from a goat to optimize the western blotting signal of ICD-1. After several trials of varying durations and temperatures of primary antibody incubation, the 4°C overnight exposure produced the most significant signal of bands at a molecular weight of around 17.5, which is the theoretical molecular weight of ICD-1 (Fig. 6). Despite some cross reactivity of the antibody, the presence of three bands in the range of 15-25 kD indicated that the anti-βNAC antibody was putatively specific for a protein with a molecular weight similar to ICD-1.

To further confirm the specificity of the anti-βNAC antibody for ICD-1, ICD-1 RNAi time course trials were performed. ICD-1 RNAi is expected to deplete the expression of the icd-1 gene by generating ds RNA that complementary-base pairs with ICD-1 mRNA, creating double stranded mRNA that is targeted for degradation, leading to a decrease in and potential absence of ICD-1 expression. If, in the presence of ICD-1 RNAi, ICD-1 expression decreased as expected, then a resulting western blot would not be expected to display the band observed at a molecular weight of approximately 17.5 kD because the protein would not exist and thus could not bind to the anti-βNAC antibody. Figure 7 indicates the absence of a 21 kD band after exposure to ICD-1 RNAi, while the other two bands in the range of 15-25 kD, 15 and 17, respectively, were
retained when ICD-1 RNAi populations. (Fig. 7). While 21 kD is not the exact theoretical molecular weight as ICD-1, proteins do not always run at the same molecular weight in SDS-PAGE gels as their theoretical molecular weights. Specifically, strongly acidic peptides with E and D residue percentages between 11.4-51.1% tend to run at larger molecular weights in SDS-PAGE gels than their expected molecular weights because their acidity can lead to gel mobility shifts. Because ICD-1 is composed of 14.3% acidic residues, it may have run to the 21 kD mark in the SDS-PAGE gel, a larger molecular weight than expected, which would further confirm that the signal at 21 kD is ICD-1. While the ICD-1 RNAi time course provides evidence for the specificity of the anti-βNAC antibody for ICD-1, its specificity can be further confirmed by using the antibody to purify ICD-1 and perform mass spectrometry on this isolate.

In addition to the possible confirmation of the specificity of the anti-βNAC antibody to ICD-1, the RNAi populations produced two additional noteworthy observations (Fig. 7). The first is the absence of an approximately 35 kD band in the RNAi populations (lanes 3-5 and 8-10) that is present in the wild type populations (lanes 2 and 7). While the expected molecular weight of the native NAC complex containing both the alpha and beta subunit, is expected to have a molecular weight of approximately 38 kD, the presence of the denaturing agent, SDS, is intended to disrupt the intermolecular bonds in the complex. Because SDS was used in this western blot, it is not expected that that 35 kD is representing a signal for the beta subunit of the intact NAC.

The second observation is the presence of a prominent 50 kD band in only the 72-hour ICD-1 RNAi exposed populations (Fig. 7). While no literature has investigated this specific band, it does not appear to be noise because it is only present at one time point that was repeated into two samples. Due to the supposed non-randomness of this 50 kD band, one possible
suggestion for its appearance is the expression of apoptotic-related proteins upon persistent exposure to stressed conditions.

The results from the tunicamycin and thapsigargin stressed westerns appear to be inconclusive with regard to the state of ICD-1 during apoptosis (Fig. 8). The western blot from Figure 8 did not display either the 12 kD or 5 kD band that was expected. The 4-20% grade Mini-POTEAN® TGX Stain-Free™ Gels do not allow for significant spacing between low running proteins, which could have affected the display of the putatively cleaved ICD-1 portions. However, if this experiment were performed with a lower graded gel, it may display the cleaved portions of ICD-1, if it is indeed cleaved. However, if this experiment with a lower graded gel were to occur and ICD-1 did not appear at the expected cleaved values, one possible explanation for that result is the possibility that while both chemicals are known to induce misfolded protein stress in the ER, neither is known to increase apoptosis. Therefore, the lack of ICD-1 cleavage would be due to a lack of increase in apoptosis in these experiments. In addition, the epitope for the anti-βNAC antibody may also be cleaved during apoptosis, which may lead to a lack of signal in the expect regions. Because of the latter possibility, either a different antibody specific to an epitope specific to one of the putatively cleaved would be needed to further analyze the state of ICD-1 during apoptosis through the use of western blot analyses. In addition, measuring the rate of apoptosis by means of a transfection incorporating the RNA of one of the putatively cleaved portions of ICD-1 into a healthy population of *C. elegans* may also reveal a greater understanding of the state of ICD-1 during apoptosis.

The results from the anti-ICD-2 antibody appear to be inconclusive. The expected molecular weight of ICD-2 is approximately 20.4 kD. While a band with a similar molecular weight was displayed around the 20 kD mark, more significant bands were displayed at higher
molecular weights and therefore more trials should be conducted to optimize the western blotting protocol for the ICD-2 antibody. Optimizing the western blotting protocol for this antibody could be significant because it could lead to an improved understanding of the relative expressions of ICD-1 and ICD-2 during apoptosis.

A western blot using the optimized anti-βNAC antibody protocol and native gels was conducted to gain a better understanding of the potential intermolecular interactions of ICD-1. Specifically, this experiment was performed to further confirm whether the beta subunit of the NAC is always associated with its alpha subunit or if either (or both) are expressed independent of the NAC. The results from this experiment are inconclusive because the western blot displays antibody signaling for a cluster of proteins at the top of the gel, indicating that the proteins samples did not migrate as expected (Fig. 10). While a native sample buffer and no denaturing agents were incorporated in the samples loaded into each well, a gel specific for NativePAGE most likely should have been used instead because it would have increased protein migration.

The western blot using the optimized anti-βNAC antibody protocol was performed on wild type D. melanogaster populations to determine the specificity of this is antibody for the ICD-1 homologue, bicaudal (Fig. 11). With a theoretical molecular weight of approximately 20 kD, the band with significant signal at 25 kD in lane 3 of this western blot is suspected to be bicaudal. While no definitive conclusions can be drawn regarding the specificity of the anti-βNAC antibody for bicaudal solely off this western blot, bicaudal RNAi time courses similar to the one ran in this investigation are expected to produce similar results as the ICD-1 RNAi time course (Fig. 7).

While the results from this investigation do not provide much additional information about the state of ICD-1 during apoptosis, they do establish a promising protocol for future
western blot investigations of the state of ICD-1 in apoptosis. With a promising western blotting protocol for an antibody specific for ICD-1, several experiments regarding the state of ICD-1 can be conducted. If ICD-1 is, indeed, cleaved during stressed conditions that increase apoptosis, the next step of this experiment is to determine the possible roles of CED-4 and/or CED-3 in this cleavage event. To determine the role of these apoptotic pathway members, CED-4 and CED-3 loss-of-function mutant strains should be exposed to stressors and to determine state of ICD-1 in those conditions. Determining the possible roles of CED-4 and/or CED-3 in cleavage of ICD-1 will provide insights into the apoptotic pathway(s) engaged during misfolded protein stress and subsequent cell death, and may provide further proof for the existence of a novel, CED-3-independent apoptosis in *C. elegans*.

**Suggestions for Further Research**

In addition to determining a potential caspase responsible for the CED-3 independent apoptosis in *C. elegans*, it is also recommended to continue exposing ICD-1 to varying stressed populations to gain a better understanding of potential stress-specific apoptotic responses. It is also recommended to further analyze the specificity for the anti-βNAC antibody for ICD-1 through protein purification and mass spectrometry. In addition, it is recommended to further optimize a protocol for ICD-2 antibody specificity as well as anti-βNAC antibody for native gels because doing so could allow for a better characterization of ICD-2 and
References


