The role of the nascent polypeptide-associated complex in Caenorhabditis elegans

Paul T. Arsenovic
James Madison University

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The Role of the Nascent Polypeptide-Associated Complex in *Caenorhabditis elegans*

Paul Arsenovic

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Abstract

The nascent polypeptide-associated complex (NAC) is a highly conserved protein complex known to play an important role in the development of metazoan organisms, but its molecular function is not well understood. Recent evidence from experiments using *Saccharomyces cerevisiae* as model supported the hypothesis that the NAC is either a chaperone or a component of the cytosolic chaperone network that interacts with nascent peptides emerging from the ribosome. We tested this model in *C. elegans* and found that the homologues of the NAC, *icd-1* and *icd-2*, behave like chaperones in the worm. Lack of *icd-1* or *icd-2* altered the worms stress response to heat and led to a dramatic up-regulation of *hsp-4*; the homologue of the human ER chaperone BiP. Worms lacking the ER stress signalling protein *xbp-1* generated a higher proportion of defective embryos and had a lower survival rate compared to wildtype populations during *icd-1*(RNAi). Furthermore, *icd-1*(RNAi) increased the size of lysosomes in wildtype worms and embryo gut cells, indicating an up-regulation of ER-mediated autophagy. These results suggest that disruption of the NAC by RNAi causes ER stress in the worm and is likely the cause of embryonic apoptosis that was previously observed in the worm.
1 Introduction

1.1 Background on Protein Folding and Molecular Chaperones

All living organisms must produce functional proteins in order to survive, grow, and reproduce. Proteins are the most abundant and structurally diverse macromolecules, amounting to 15% of the net weight of organisms. One of the fundamental tenets of molecular biology maintains the structure of proteins determine their function. Thus, the survival of all living organisms requires strict control over the structural integrity and quantity of proteins produced. [Hartl et al., 2011] [Vabulas et al., 2010]

To a first approximation, protein structure is determined from the composition and ordering of amino acids, the building block of proteins. The linear sequence of amino acids in a protein from beginning to end is termed its primary structure. In all organisms, protein production occurs on the ribosome, the protein factory of cells. RNA sequences are threaded through the ribosome which translates RNA code into amino acids linked together by a peptide bond. As amino acids emerge from the ribosome, they immediately begin to fold into a three-dimensional shape or a tertiary structure. Ultimately, the tertiary structure of a protein determines its function.

Since structure is vital to protein function, understanding how proteins fold is an important biological problem. Proteins that misfold tend to be biologically dysfunctional and can be toxic if they reach high concentrations in a cell. Since protein quality is a necessary condition for nearly every cellular process, all organisms contain sets of genes dedicated to monitoring and assisting proper protein formation.

Protein structure is believed to be mainly determined by the primary sequence of amino acids encoded in processed mRNA. [Fandrich et al., 2001] Excluding environmental factors, simple proteins structurally evolve to a low energy state; a state that minimizes the bonding energy of intermolecular contacts between amino acids.
Generally, small proteins will fold into a specific conformation at the lowest energy state which is a function of amino-acid composition and order. [Zhang, 2008] Folding dynamics become more complicated with larger proteins and the addition of environmental variables.

Large proteins tend to have multiple low energy states or structural conformations, some of which are non-native or biologically dysfunctional. [Lazaridis and Karplus, 1997] Studies estimate that partially folded intermediates form in 90% of proteins larger than 100 amino acids as they emerge from the ribosome. [Hartl et al., 2011] Partially folded proteins can pose a problem for cells if they bind to other partially folded proteins, forming insoluble aggregates that can become toxic. Organisms prevent protein aggregation by expressing structurally stable, stress resistant proteins called chaperones.

Chaperones are thought to guide proteins to their native state by non-covalently binding to hydrophobic surfaces on partially folded protein intermediates that have the potential to aggregate. In the absence of chaperones, partially folded intermediates have a greater tendency to form amorphous protein complexes. These complexes have a minimal energy state, meaning it is difficult to reverse and refold proteins inside these aggregates (see Figure 1). Furthermore, aggregation complexes can form amyloid fibrils, a hallmark of neurodegenerative pathology, that are thermodynamically very stable and neuro-toxic.

As shown in figure 1 aggregation increases as a function of molecular crowding or as the concentration of proteins surrounding folding peptides increases. Experimentally, Dobson et al. demonstrated the macromolecular crowding effect on protein folding using lysozyme as a reporter. They found that reduced lysozyme refolds in a concentration dependent manner and the rate of refolding dramatically increases in the presence of ER chaperone protein disulfide isomerase. [van den Berg et al., 1999]

Thus, the demand for chaperones is dependent on the cellular environment and the
complexity of translating proteins.

Cellular stresses such as heat, UV radiation, or oxidative stress can also induce protein misfolding and increase the demand for molecular chaperones. In response to translational or stress-induced misfolding, cells up-regulate a set of highly conserved chaperones called heat shock proteins (HSPs). HSPs respond to general protein stress that may compromise the structural integrity of native proteins.

HSPs were discovered in the context of heat shock experiments in *Drosophila Melanogaster* cell culture. Researchers named HSPs according to their molecular weight. [Spradling et al., 1975] They are among the most abundant cellular proteins whose sequence and structure is highly conserved from prokaryotes to mammals. [Robert, 2003] While mainly known for their up-regulation in response to heat stress, HSPs also exhibit functional roles in a diverse set of cellular processes. Four major processes include translational protein folding, stress-induced protein refolding, protein trafficking, and proteolytic degradation. [Hartl et al., 2011]

A subset of HSPs, including HSPs 70, 90, and 60, are termed translational chaperones that guide aggregation-prone proteins to their native state as they emerge from the ribosome. Specifically, these HSPs bind on and off to hydrophobic patches on folding proteins in an ATP-dependent manner. HSP 70 and 90 have ATPase enzyme activity, when ATP is hydrolyzed to ADP they bind to proteins in the cytosol. If HSP 70 or 90 fail to guide nascent peptides to their native state, HSP 60 (sometimes referred as a chaperonin) can enclose partially folded proteins in a protective nanocage. Two highly characterized bacterial chaperonins Gro-EL and Gro-ES work in this manner. [Chakraborthy et al., 2010]

Together, Gro-EL and Gro-ES (homologous to HSP 60 and 10) bind in an ATP-dependent mechanism that encapsulates folding proteins. The restricted environment inside the chaperonin complex encourages compact protein structures, forcing hydrophobic residues into the interior of the folding peptide. Once the protein folds
Figure 1: **Protein folding energetics in the cellular environment.** Large proteins emerging from the ribosome have a tendency to form intermediate folded states, shown by local minima on the energy diagram. The folded state of a protein is largely dependent on the total bonding energy between intramolecular contacts in the peptide. However, as the concentration of the cytosol increases, partially folded proteins are prone to aggregating. Aggregated proteins reach low energy states, making it energetically expensive to reverse. Chaperones prevent aggregation by binding to hydrophobic patches on proteins, forcing hydrophobic residues into the interior of the protein. Figure taken from [Hartl et al., 2011].
properly, hydrolysis of ATP enables dissociation of Gro-EL from Gro-ES and the folded peptide leaves the chaperonin complex. [Horwich et al., 2007]

Generally, HSP 70, 90, and 60 respond to folding demands at the ribosome where nascent peptides emerge. Another overlapping set of HSPs are up-regulated as a result of protein denaturation caused by severe environmental stress. For example, dramatic changes in heat or pH can denature proteins that are already folded in the cytosol. To prevent aggregation of denatured proteins, cell up-regulate stress inducible heat shock proteins such as HSP 104, HSP 70 and HSP 40. [Glover and Lindquist, 1998]

Researchers using *Saccharomyces cerevisiae* as a model have shown all three HSPs work synergetically to renature aggregated proteins. Studies also suggest not all stress induced chaperones can efficiently prevent aggregation as individual units. For example, *in-vitro* experiments show HSP 104 has limited chaperone capabilities in isolation. HSP 70 and 40 also have limited disaggregation activity, however the addition of HSP 104 with HSP 70/40 complex increases disaggregation efficiency and their capacity for larger proteins. [Bosl et al., 2006] A proposed model suggests that the HSP 70/40 complex presents aggregated peptides to HSP 104, a large heximeric protein with a centrally located translocation pore. ATP hydrolysis enables HSP 104 to unfold the aggregated substrate and thread the substrate peptide through its pore to refold at the tunnel exit. [Doyle and Wickner, 2009] Adding a layer of complexity, HSP 40 regulates the ATPase activity on HSP 70, increasing its binding affinity for hydrophobic patches. [Fan et al., 2003, Landry, 2003] These results imply that chaperone function cannot necessarily be inferred by studying single proteins, instead chaperones should be viewed as a functional network.

In yeast, HSP 104, 70, and 40 form a chaperone network which prevents aggregation and encourages renaturing of stressed proteins in the cytosol.\(^1\). However, a large proportion of proteins fold outside of the cytosol in organelles such as the ER and

\(^1\)Cytosol here refers to “The fluid component of cytoplasm, excluding organelles and the insoluble, usually suspended, cytoplasmic components.” Merriam Webster
mitochondria. The chemical environment of these organelles is dramatically different from the cytosol, requiring organelle specific HSPs. The ER has a strong demand for chaperones due to its crowded and oxidative environment that is similar to the extracellular environment. [Nishikawa et al., 2005] Protein quality in the ER is paramount for cellular homeostasis because all secretory and membrane proteins fold in the ER, amounting to roughly 1/3 of the proteome. [Ghaemmaghami et al., 2003] The ER essentially mimicks the stresses proteins face in the extracellular environment while simultaneously guiding proteins to their native state by expressing resident heat shock chaperones BiP and Grp94 (HSP 70 and HSP 90 family members), chaperone lectins, and folding enzymes known as foldases. [Nishikawa et al., 2005, Feige and Hendershot, 2011]

BiP and Grp94, much like cytosolic HSP 70 and HSP 90, have ATP dependent binding affinity for hydrophobic patches on folding proteins in the ER. Chaperone lectins, on the other hand, are calcium dependent chaperones that bind to oligosaccharide ‘chaperone tags’ on folding proteins. [Rutkevich and Williams, 2011] 80% of proteins that enter the ER are tagged with oligosaccharides (also referred to as N-linked glycolisation) as nascent peptides enter the ER lumen to fold. The sugar tag enables recognition by chaperone lectins and co-chaperone ER p57, which together prevent aggregation and mis-paired di-sulfide bonds. Before proteins exit the ER to the golgi, paired cysteine amino acids that span large distances in folding peptides must covalently bond to provide internal stability. Without assistance from foldase chaperones, unpaired cysteine residues on folding peptides can lead to terminally mis-folded proteins. [Feige and Hendershot, 2011]

Foldases are composed of Peptidyl-prolyl cis or trans isomerases (PPIs), and protein-disulfide isomerases (PDIs). PPIs and PDIs, in cooperation with chaperone lectins, enzymatically increase di-sulfide bond formation. [Schroder, 2006] Mechanistically, oxidized (active) PDIs act as an electron donor for reduced, unpaired sulfides.
The oxidation of thiol groups forces the formation of di-sulfide bonds in folding peptides. [Gruber et al., 2006]

Under normal cellular conditions all three ER chaperone classes are expressed as a buffer against potential protein mis-folding. As mis-folded proteins start to accumulate in the ER, the concentration of free (unbound) chaperones decreases as they bind to aggregation prone peptides. ER membrane proteins act like stress sensors and detect the lowered amount of unbound chaperones. The ER responds by shuttling terminally mis-folded proteins out of the ER lumen into the cytosol for degradation. [Nishikawa et al., 2005] Degrading terminally mis-folded ER proteins involves a complex, multi-step process termed ER-associated degradation (ERAD).

ERAD can be thought of as the ER’s constitutively expressed protein quality control program. Translation errors or unfavorable folding conditions occur under normal cellular conditions, forming terminally mis-folded proteins at a controlled rate. Normally, ERAD can process mis-folded proteins and maintain ER homeostasis. ERAD disposes of damaged proteins by either proteosomal degradation or the autophagy/lysosomal pathway. [Fujita et al., 2007] Proteosomal degradation utilizes the ubiquitin proteosome machinery, while lysosomal-mediated ERAD involves the export of misfolded proteins into double-membrane vesicles (autophagosomes) that fuse to lysosomes. The canonical ERAD pathway involves proteosomal degradation, which has been researched in great depth.

The process of proteosomal ERAD can be broken down into four basic steps: 1) substrate recognition 2) membrane targeting 3) retro-translocation and 4) proteosomal degradation. First, mis-folded proteins are recognized by ER chaperone Bip via hydrophobic affinity. If substrates fail to fold after multiple binding cycles with BiP, HSP 40 and BiP together target the substrate to the ER membrane for retrotranslocation. During retrotranslocation, the substrate is threaded out of the ER lumen through specific membrane pores into the cytosol for degradation.
The formation of membrane pores can occur through multiple mechanisms. One model proposes ER protein Sec61, a known translocation membrane protein, also forms a retrograde membrane pore for exiting ERAD substrates. Studies have shown that retrotranslocation for specific proteins can be disrupted by blocking Sec61 pores. Additionally, genetic deletions of Sec61 in yeast slow the rate of ERAD. Since deletion of Sec61 slows but doesn’t eliminate ERAD, redundant pathways must exist for retrotranslocation. It has been proposed that Hrd1, a ubiquitination enzyme in yeast, forms membrane pores and tags proteins with ubiquitin for proteosomal degradation.

The last step of ERAD involves ubiquitination of substrate proteins and protein disposal through proteosomal degradation. The proteosome is a highly conserved protein complex which breaks down ubiquitin tagged substrates by enzymatically cleaving their peptide bonds. The process of tagging substrates for the proteosome is termed polyubiquitination, another highly conserved process that requires a specific class of enzymes called ubiquitin ligases.

During polyubiquitination, E1, E2 and E3 ubiquitin ligases in a series of sequential steps covalently link ubiquitin to substrate proteins. Carrier proteins with Ubl or Uba structural domains bring the targeted protein to the proteosome, where it is enzymatically degraded and the resulting amino acids are recycled (Figure 2).

If ERAD fails to lower protein stress through proteosomal degradation, the ER responds with the Unfolded Protein Response (UPR). The UPR is a conserved genetic program which induces the transcription of a host of genes that have the net effect of decreasing the influx of proteins to the ER and increasing the ER’s capacity to process existing proteins. [Chakrabarti et al., 2011] Unlike ERAD and the ubiquitin proteosome system which operate under normal cellular conditions, the UPR is triggered only during high levels of ER stress. The function of the UPR is to prevent proteotoxicity by 1) lowering protein production 2) increasing chaperone levels 3) increasing protein degradation and 4) triggering apoptosis or autophagy if the previous
steps fail to resolve proteomic stress.

In mammals, 3 ER membrane receptors can independently initiate the UPR (Figure 3). The receptors are activating transcription factor 6 (ATK6), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and inosital requiring kinase 1 (Ire1). The latter receptor Ire1 is the most conserved, with homologues spanning from yeast to humans (Figure 3).

The principal regulator of Ire1 is Bip. With low levels of unfolded protein in the ER, excess Bip chaperones bind to the luminal domain Ire1, preventing its activation. Increases in unfolded protein tritrate away Bip, activating the Ire1 receptor. Ire1 activation causes the receptor to dimerize and autophosphorylate its c-terminal domain. [Kawaguchi and Ng, 2011] Phosphorylation activates its RNase domain which splices an intron from constitutively expressed XBP-1 mRNA. The splicing of XBP-1mRNA causes a frame shift that leads to the translation of XBP-1 protein, a potent ER stress transcription factor. [Iwawaki and Akai, 2006] XBP-1 up-regulates a large network of genes which increase chaperone expression, improve the efficacy of ERAD, regulate golgi and ER synthesis, and up-regulate pro-apoptotic and pro-autophagic elements (Figure 3).

Both apoptosis and autophagy are conserved processes that can determine cellular fate in response to ER stress. Apoptosis is a well characterized process that leads to the orderly destruction of a stressed cell that is dangerous or potentially dangerous for an organism. Autophagy is a less characterized cellular phenomenon that involves the degradation and recycling of cellular material at the lysosome.

The core genetic program of apoptosis was elucidated in the model organism C. elegans by the Horvitz lab. These researchers determined that through a signalling cascade, activation of cysteine aspartic proteases (caspases) ultimately led to apoptotic cell death in nematodes. Horvitz et. al showed that developmental (normally occurring) apoptosis could be completely abrogated upon deletion of the major cas-
Figure 2: **Diagram of the ubiquitin proteosome system.** The process of ubiquitination requires multiple ubiquitin conjugating proteins that transfer ubiquitin molecules to the target protein. First, E1 conjugating enzyme transfers activated ubiquitin to an E2 enzyme. E2 and E3 enzymes form a complex and catalyze isopeptide bond formation between ubiquitin and the target protein. E4 enzymes facilitate poly-ubiquitin chain formation. Typically, target proteins are phosphorylated before they are tagged with ubiquitin. Poly-ubiquitylated target proteins interact with UBA-UBL domains on shuttling proteins as they are brought to the proteasome. DUB proteins (deubiquitination enzymes) facilitate recycling of ubiquitin protein. Figure taken from [Lehman, 2009].
pase in *C. elegans* CED-3. Since Horvitz *et al.*’s initial characterization, multiple apoptotic pathways initiated by various stimuli have been observed in several model organisms and humans.

Unlike apoptosis, autophagy is not as well understood and our understanding of its cellular role is currently evolving. Although autophagy is classified as type II programmed cell death, many researchers argue it has pro-survival roles. [Moretti *et al.*, 2007, Ogata *et al.*, 2006, Kourtis and Tavernarakis, 2009] Autophagy involves the formation of small double membrane organelles called autophagosomes. Dysfunctional organelles and proteins are collected by autophagosomes and fused to lysosomes where their materials are broken down by proteases. Thus, the lysosome is analogous to a ‘cellular recycling bin’ and autophagy is the process by which proteins or organelles are brought to the cellular recycling bin. [Cherra *et al.*, 2010]

Autophagy and apoptosis both share functional similarities and common signalling pathways (Figure 4). Cell stresses like acute radiation or oxygen deprivation can trigger autophagic or apoptotic responses. As shown by Figure 4, several apoptotic signalling proteins are also autophagic. Thus, cell fate under acute ER stress likely depends on the stoichiometric balance between regulators of apoptosis and autophagy. The mechanism controlling the switch from autophagy to apoptosis is poorly understood, however some researchers propose EIF2A may be a primary molecule that biases the cell toward autophagy or apoptosis in a time dependent manner (Figure 4). [Moretti *et al.*, 2007]

EIF2A is an initiation factor that is a necessary component for translation. Under ER stress, PERK kinase phosphorylates EIF2a, which inhibits its binding to ribosomal complexes. In mammalian models the net effect of EIF2a phosphorylation is to: 1) halt translation, 2) up-regulate LC3 conversion (pro-autophagic regulator), and 3) activate CHOP (ER pro-apoptotic transcription factor). LC3 conversion is a necessary condition for the formation of autophagosomes. Similar to apoptotic signaling,
Figure 3: **Metazoan ER stress pathways.** Three highly conserved ER membrane receptors trigger the unfolded protein in metazoan animals. Ire1 receptor is the most conserved; homologues span from yeast to humans. Protein stress changes the concentration of unbound resident chaperones such as BiP, activating ER stress receptors PERK, ATF6, and IRE1. IRE1 activation causes receptor dimerization and autophosphorylation leading to the processing of Xbp-1 mRNA. Translated XBP-1 is a transcription factor that up-regulates chaperones, ER and golgi biogenesis, ERAD, and Apoptosis and Autophagy. Activated PERK leads to the phosphorylation of translation initiation factor EIF2a (P-EIF2a). P-EIF2a stops translation by disassociating from the ribosome, and leads to the translation of constitutively expressed ATF4 mRNA. ATF4 is a leucine zipper transcription factor which up-regulates chaperones, metabolic and redox factors, and apoptosis and autophagy. Finally, activated ATF6 translocates to the golgi after ER stress, where it is cleaved by proteases. Cleaved ATF6 leads to transcriptional changes effecting chaperones, ER and golgi biogenesis, and ERAD. Figure taken from [Hetz and Glimcher, 2009].
LC3 conversion requires the cleavage of pro-LC3 (similar to pro-caspases) to LC3-I in the cytosol. Next, LC3-I is modified with the addition of phospho-lipids to LC3-II, which forms the membrane structure of autophagosomes. [Tanida et al., 2004]

Unlike autophagy, ER mediated apoptosis is triggered by negative transcriptional regulation of Bcl-2 by CHOP. If the suppression of Bcl-2 reaches a certain threshold, cytochrome c is released from the mitochondria like a binary switch, irreversibly leading to apoptotic cell death. Because ER-mediated apoptosis requires transcriptional modulation, cells likely initiate autophagy before switching to apoptosis. Evidence from mammalian cell culture supports the view that short-lived ER stress triggers autophagy, not apoptosis. For example, one study showed that neuroblastoma cells exposed to tunicamycin for 1hr (ER stressor) up-regulate autophagosomes, with low levels of cell death. On the other hand, 24hr exposure to tunicamycin leads to dramatic increases in cell death. [Ogata et al., 2006] Thus, cellular fate after ER stress likely depends on the strength and duration of the activation of EIF2a or other major ER stress regulators.

The complicated signalling pathway involving EIF2a illustrates how autophagy and apoptosis should not be viewed independently in the context of ER stress. Ultimately, cells with compromised protein folding machinery experience ER stress and initiate the UPR, which in turn regulates many cellular processes that are often viewed as independent systems. Therefore, proteins with known effects on cellular systems regulated by the UPR, such as translation, apoptosis, autophagy, and chaperone regulation, could be candidate initiators of the UPR. The nascent polypeptide associated complex (NAC) for example is a highly conserved protein complex that has been correlated with many processes regulated by the UPR. The following section reviews literature on the NAC and how its molecular function may be connected to the UPR.
Figure 4: Cross talk between Autophagy and Apoptosis during ER Stress in cancerous cell lines. Exposure to γ-radiation induces ER stress in cancer cell lines that have over-active PI3k. PI3K relays survival signals to cancerous cells by inhibiting pro autophagic and apoptotic factors through mTOR and Akt. Inhibition of mTOR, phosphorylation of eIF2a or activation of JNK can all increase LC3 conversion and autophagy. Similarly, JNK activation, eIF2a phosphorylation and calcium release increase apoptosis signalling. Thus, both apoptotic and autophagic signalling are not mutually exclusive under ER stress. Figure taken from [Moretti et al., 2007].
1.2 Previous Studies on the NAC

The NAC is a heterodimeric complex (subunits named α-NAC and β-NAC) located near the amino-acid exit tunnel in ribosomes. Similar to co-translational chaperones, the NAC is thought to associate with nascent peptides as they emerge from the ribosome. [Reimann et al., 1999] Although the NAC is known to bind to ribosomes, its molecular function has not been precisely defined. Observations of the NAC in many different cellular systems have made it difficult to specifically characterize. To date, the NAC has been implicated as 1) transcription regulator 2) translation regulator 4) ribosomal chaperone 5) ER translocater 6) mitochondrial translocator 7) negative regulator of apoptosis and 8) regulator of autophagy. [Rospert et al., 2002] [Kanki et al., 2010] These seemingly unrelated observations and the NAC’s structural conservation across the eukaryotic domain suggests its molecular function is fundamental to cellular homeostasis.

Recent studies suggest the NAC is a translational chaperone or co-chaperone that interacts with cytosolic HSPs. [Koplin et al., 2010a, Karbstein, 2010, del Alamo et al., 2011]. This is based on evidence from structural studies using NAC homologues in yeast, egd1 and egd2. Wegrzyn et al. showed the n-terminus of egd1 bound to the ribosome close to the amino acid exit tunnel. [Wegrzyn et al., 2006] Following this work, Pech et al. showed the n-terminus of β-NAC specifically binds to ribosome protein rpl-31 and this binding property could be conferred to a non-ribosomal chimeric protein fused with β-NACs n-terminus. [Pech et al., 2010] They also show α-NAC can bind to rpl-17, a neighboring protein of rpl-31. Both rpl-31 and rpl-17 are in close proximity of newly folding peptides and surrounded by rRNA. The crystal structure of human NAC revealed a nucleic-acid binding region in α-NAC that had stronger affinity for ssRNA than DNA, suggesting the NAC may also bind to rRNA surrounding the amino-acid exit site. [Beatrix et al., 2000] [Liu et al., 2010a] Furthermore, rpl-31 is a contact point for the co-chaperone complex RAC and the SRP receptor (an ER
translocation protein) [Peisker et al., 2008] [Halic et al., 2006]. Koplin et. al. showed that double deletions of the RAC chaperone partner SSB and the NAC led to synergistic growth defects and increased sensitivity to translation inhibitors. [Koplin et al., 2010a] Most recently, Frydman et. al. showed that yeast NAC associates with nearly all nascent peptides on translating ribosomes and modulates SRP binding to nascent peptides. [del Alamo et al., 2011] All of these observations are consistent with the hypothesis that the NAC is a translational chaperone or co-chaperone that modulates the binding of the SRP receptor to the ribosome, effecting ER translocation.

If the NAC is a translational chaperone that effects SRP receptor binding, one would expect its removal to increase protein stress, possibly mis-routing proteins to the ER and trigger the UPR. Activation of the UPR is known to have multiple downstream effects such as: transcription modulation, translation attenuation, chaperone up-regulation, an increase in ERAD, metabolic changes, and an increase in apoptosis or autophagy.

Many of these effects have already been observed in studies involving the NAC. For example, removal of the NAC by RNAi increases apoptosis in *C. elegans* embryos, human cell culture, and drosophila cell culture. [Tim A. Bloss, 2004, Bloss et al., 2003, Creagh et al., 2009, Hotokezaka et al., 2009] There is extensive evidence the NAC binds to ribosomes and interacts with translation machinery. [Freire, 2005, Sakakibara et al., 2008, Braat et al., 2004] Numerous studies have shown the up-regulation of the NAC and various heat shock proteins. [Hoffrogge et al., 2007, Park et al., 2010, del Alamo et al., 2011, Murphy and Pinto, 2010] Collectively, all of these observations support a chaperone role for the NAC and its removal possibly engages the multifaceted unfolded protein response.

To date, the role of the NAC in *C. elegans* has only been studied in the context of its effect on apoptosis. RNAi removal of the $\beta$-NAC homologue, icd-1 (inhibitor of cell death 1), in C. elegans caused an increase in embryonic apoptosis. [Bloss et al.,
2003] Removal of the α-NAC homologue, icd-2, also caused an increase in apoptosis in C. elegans embryos [Tim A. Bloss, 2004]. The observed cell death was independent of CED-3, the primary death caspase in the worm, indicating the presence of an unidentified caspase. The cause of increased apoptosis in icd-1(RNAi) embryos remains an open question. The purpose of this research is to test the chaperone model of the NAC in C. elegans and determine whether NAC removal triggers the unfolded protein response in the worm leading apoptosis in embryos.

1.3 Experimental Strategy and Predictions

To test the hypothesis that the NAC is a molecular chaperone in C. elegans, we designed a series of experiments where we removed icd-1 and icd-2 by RNAi feeding, examined the worm for chaperone deficient phenotypes, and monitored the expression of known chaperones. The following list explains our experimental strategy and the predictions associated with each experiment.

1. Determine worm viability during protein denaturing heat stress. Worm viability was measured as function of worm movement and survival over time during heat stress.

   (a) We expected icd-1 or icd-2(RNAi) populations to be more sensitive to heat stress compared to wild-types as measured by decreased movement or survival over time.

   (b) If the knockdown strains respond to heat stress similarly to wild-types, then it is less likely the chaperone hypothesis is correct.

2. Determine if icd-1(RNAi) leads to an increase in chaperone expression in the worm. We measured chaperone expression in vivo using GFP reporters.

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2All subsequent experiments, except the ubiquitination assay, examine icd-1 (RNAi). We focused on icd-1 to minimize the use of time and resources during the following experiments.
(a) We expected to see an increase in chaperone expression in icd-1(RNAi) worms because we reasoned the worm compensates for chaperone dysfunction by up-regulating other chaperones.

i. Using HSP-4::GFP and HSP-16::GFP reporter strains, we compared chaperone levels in icd-1(RNAi) worms to untreated reporter strains.

3. Determine if reducing chaperone expression in the worm decreases worm viability during icd-1(RNAi).

   (a) We expected HSF-1 (KO) worms fed icd-1(RNAi) to:

      i. Show increased sensitivity to heat stress if icd-1(RNAi) increases chaperone expression through activation of HSF-1.

      ii. Show decreased sensitivity to heat stress if icd-1(RNAi) increases chaperone expression independently of HSF-1.

      iii. Show no difference in heat stress sensitivity relative to wild-type;icd-1(RNAi) worms if icd-1(RNAi) only up-regulates non-cytosolic chaperones.

4. Search for phenotypic markers of protein misfolding or abnormally high protein degradation in icd-1(RNAi) affected embryos. We expected morphologically defective embryos to show evidence of either protein aggregation or high levels of protein turnover.

   (a) Using a poly-glutamine-YFP reporter strain, we expected icd-1(RNAi) worms to show an increase in protein aggregation or

   (b) We expected to see evidence of protein turnover by monitoring morphologically defective embryos for stress-related granules such as aggresomes, p-bodies, or stress granules.

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3Chaperone compensation was previously observed in E.coli after Trigger Factor deletion, and in C. elegans after ER chaperone deletions. [Kapulkin et al., 2005, Teter et al., 1999]
2 Methods

2.1 Maintenence of Worms

C. elegans strains are maintained in an incubator at 22°C on Carolina Nemotode Growth Agar seeded with OP50 bacteria. OP50 feeder is cultured overnight shaking at 37°C in liquid LB broth. Overnight cultures are derived from frozen stocks stored at -70°C. Worms are transferred to new plates every two to four days to maintain growth.

2.2 Mounting C. Elegans Embryos or Worms for Microscope Viewing

Embryos were mounted onto agar pads made with .05g of agar/mL of dH20. 8-10uL of M9 buffer [3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 1 ml 1 M MgSO4, H2O to 1 litre. Autoclave.] was distributed evenly across the agar to provide a transfer medium for embryos. For live worm viewing, M9 solution was mixed with levamisole at 10% concentration to prevent worm mobility.

2.3 RNAi Plate Recipe

RNA interference experiments utilize genetically modified feeder bacteria which express double-stranded RNA (dsRNA) specific to the gene of interest. To express dsRNA, feeder bacteria contain an engineered plasmid containing the gene of interest under the control of an IPTG promoter. Engineered feeder bacteria were grown from single colonies in liquid LB by shaking overnight at 37°C. During the last 4 hours of shaking IPTG was added to the overnight culture to a final concentration of 20µM. Cultured feeder is seeded onto RNAi plates. RNAi plates were made by combining 4.5g NaCl, 25.5g agar, 3.75g peptone, 1.5mL of 2mg/mL uracil, 0.22g CaCl 0.75g
of 10mg/ml cholesterol in 1463mL of dH2O. This mixture was autoclaved, allowed to cool, and 37.5mL of phosphate buffer (pH 6), 1.5mL of 1M MgSO4, 15uL of .1M IPTG and 1.5mL of 25mg/mL carbenicillin was added. Seeded plates were dried in an incubator for 24 hours at 37C.

2.4 Heat Shock Assays

Three independent heat shock experiments were performed. Measurement parameters and methodolgies were not identical each assay, thus experiments are described seperately.

2.4.1 Heat Shock Trial 1

Gravid Adult wildtype worms were synchronized by bleaching worms in 1:1 NaOH and bleach. Worms were bleached in 10uL of lysis solution on the perimeter of medium sized OP50 plates. 28-30hrs later synchronized F1 worms were moved to their respective plates. Worms were moved to 3 plate types: 1) icd-1(RNAi) 2) icd-2(RNAi) or 3) OP50. 50 worms were moved to each plate type. Plate types were made in duplicate in order to have temperature controls maintained at 20C. All worm populations were grown at 20C for 30hrs. After 30hours of growth, heat shock populations were moved to a 36C incubator. Worms were heat shocked continuously for 6 hours. 6 hours was chosen because Walker et al previously showed heatshocked worms at 36C remain viable until 6 hours of heat stress. [Walker et al., 2003] After 6 hours of heatshock, worms were periodically removed from the incubator and prodded with a platinum pick to examine their mobility. Responses were recorded as movement or lack of movement. Worms were sampled every hour. Movement was measured as displacement or positional change on the plate.
2.4.2 Heat Shock Trial 2

Gravid adult wildtype worms were synchronized by bleaching worms in 1:1 NaOH and bleach. Worms were bleached in 10uL of lysis solution on the perimeter of medium sized OP50 plates. 27-28 hours later synchronized F1 worms were moved to their respective plates (RNAi plates were identical to trial 1, however freshly made OP50 plates were used for the control populations). 50 worms were moved to their respective plates and grown for 30 hours at 20C. Treatment populations were transferred to a 36C incubator and heat shocked for 6 hours. After 6 hours of heat shock, worms were periodically removed from the incubator and prodded with a platinum pick to access their survival by observing the presence or absence of pharyngeal pumping.

2.4.3 Heat Shock Trial 3

Gravid wildtype and hsf-1(KO) (cgc PS3551) worms were bleached by placing worms in freshly made 1:1 NaOH and bleach. Hsf-1(KO) worms express non-functional hsf-1, which is the major cytosolic HSP transcription factor [Morley and Morimoto, 2004]. Worms were bleached in 10uL of lysis solution on the perimeter of medium sized OP50 plates. 28-29 hours later F1 larva were moved to their experimental plates (OP50 or icd-1(RNAi) feeder). The worms were moved by washing the larva off the plate with 1mL of M9 using a 10mL pipetter. The M9 worm solution was transferred to a 15mL tube. The volume of the worm solution was measured with a 1000uL pipette. Once the total volume was determined, half of the solution (wildtype and HSF-1 worms solutions) was transferred to OP50 plates and the other half to icd-1 plates. Worms were washed, rather than picked from plates, in order to maximize the population sample for each plate. Worms were grown on treatment plates for 30 hours and transferred into an incubator set at 35-36C. Worms were heat shocked for seven hours before examining their movement. Percent movement was determined by prodding worms with a platinum pick every hour. Movement was measured as
displacement or positional change on the plate.

2.5 Monitoring hsp-4 expression during icd-1 RNAi

To monitor hsp-4 expression in live embryos during an icd-1 knockdown, worms expressing an hsp-4-GFP fusion protein were used (cgc SJ4005). The zaIs4 reporter transgene is inserted into chromosome V, and is unlinked to the endogenous hsp-4 gene on chromosome II. Previous experiments confirmed the strain reliability emits a strong GFP signal in the gut and hypodermis upon heat stress or treatment with tunicamycin [Calfon et al., 2002]. Tunicamycin inhibits n-linked glycolisation, causing ER stress [Noda et al., 1999]. L4s from strain SJ4005 were moved to an icd-1 RNAi plate and an OP50 control. Worms were grown at 20C for 48 hours. Adults were transferred to fresh plates after 24hrs to differentiate old and young embryos. After 48hrs, randomly picked embryos were mounted on glass slides (see material and methods 2.2) and viewed under Nikon C1 confocal microscope for fluorescence.

2.6 Quantification of hsp-4 Expression

Flourescent images of embryos expressing hsp-4 were captured with a Nikon C1 confocal microscope. Images were captured using brightfield optics and analyzed with NIS Elements software. Flourescent intensity was measured with the “line-scan” measurement function. The line-scan measurement tool was centered and oriented with the long-axis of embryos. This measurement outputs flourescent intensity for each pixel in the line-scan. Data was exported into excel and the average intensity was calculated for OP50 and icd-1 treatments. After random selection, 16 wildtype and 13 xbp-1 (KO) embryos were scanned and quantified.
2.7 Monitoring hsp-16 expression during icd-1(RNAi)

To monitor hsp-16 expression in live embryos during an icd-1 knockdown, worms expressing an hsp-16::GFP reporter were used. HSP-16 is a small heat shock protein expressed in the cytosol. L4s containing the hsp-16::GFP reporter were moved to an icd-1(RNAi) plate and an OP50 control. Worms were grown at 20C for 48 hours. Adults were transferred to fresh plates after 24hrs to differentiate old and young embryos. After 48hrs, randomly picked embryos and worms were mounted on glass slides (see material and methods 2.2) and viewed under Nikon C1 confocal microscope for fluorescence. Severally affected embryos were grown on icd-1(RNAi) plates for up to 96hours.

2.8 xbp-1(KO) Embryo Profiling

To test if icd-1 deficient embryos increase hsp-4 expression as a result of an activated unfolded protein response, an engineered worm strain with mutant XBP-1 coupled with an hsp-4 reporter was used (cgc xbp-1 (KO)). Xbp-1(KO) or (zc12) animals contain a nonsense mutation at residue 11 in the predicted xbp-1 protein. Animals are unable to induce hsp-4::GFP in response to treatment by tunicamycin or heat shock. [Calfon et al., 2002]L4 XBP-1 mutants were transferred to OP50 and icd-1(RNAi) plates. Embryos were monitored under the microscope over a 48 hour period. Embryo profiles were generated by classifying embryos morphologically as 1) young 2) comma or two-fold 3) pretzel or 4) morphologically defective. Profiles were used to determine the extent of the RNAi effect by comparing embryo stages at different time points during the RNAi. The experiment was duplicated using the same methodology.
2.9 Comparing xbp-1(KO) and wildtype embryos throughout *icd-1* (RNAi)

2.9.1 20C embryo profiles

After establishing XBP-1 mutants were severely effected by *icd-1* (RNAi), the wildtype embryonic response was compared with the XBP-1 embryonic response. 50 pretzel-staged wildtype and xbp-1 (KO) worms were moved to icd-1 RNAi plates, representing time 0 of the experiment. Worms were incubated at 20C. Wildtype and xbp-1 (KO) adult worms began laying embryos day 3 and day 4 respectively. Embryos were sampled randomly from worm plates, mounted on agar pads in M9 solution (see 2.2), and imaged with Nomarski optics. The population was profiled by categorizing embryos as 1) pretzel 2) comma or two-fold 3) young or 4) defective. The purpose of profiling embryos was to 1) ensure the icd-1 RNAi was affecting adult worms and 2) to make comparisons between wildtype and xbp-1 (KO) populations over time.

2.9.2 18C embryo profiles

A second identical experiment was carried out at 18C with the purpose of maximizing the embryo output of xbp-1 (KO) worms since their fertility rate is significantly lower than wildtypes. We hypothesized xbp-1 (KO) worms, which have limited capacity for misfolded proteins, would output more embryos at a lower temperature. The opposite occurred and embryos were not observed until 5 days after pretzel-staged parents were moved to their respective plates.

2.9.3 18C embryo profile replication

Experiment 2.9.2 was replicated at 18C. xbp-1 (KO) worms failed to lay embryos and wildtype worms showed a similar embryo profile as 2.9.2.
2.10 Lifespan comparison of wildtype an XBP-1 worms throughout icd-1 RNAi

Pretzel-staged wildtype and xbp-1 (KO) embryos were moved to icd-1 RNAi plates and the percent survival of worms was determined daily. Survival was measured by prodding worms manually with a platinum pick and viewing for pharyngeal pumping. To determine normal viability, control xbp-1 (KO) and wildtype strains were grown on OP50.

2.10.1 20C Lifespan Assay

The lifespan assay and embryo profile experiments were run in parallel. Thus, worms were incubated at 20C in the first lifespan assay. Initial populations sizes for wildtype and xbp-1 (KO) strains was 51 and 50 respectively. Worm viability was measured for 11 days.

2.10.2 18C Lifespan Assay 1.0

For the second lifespan assay, strains were moved to an incubator set at 18C with the purpose of increasing the embryo output of heat sensitive xbp-1 (KO) worms. Initial populations sizes for wildtype and xbp-1 (KO) strains was 31 and 28 respectively. Worm viability was measured for 9 days.

2.10.3 18C Lifespan Assay 2.0

Experiment 2.10.3 replicated the conditions of 2.10.2. Worm viability was measured on day 1, 7, and 10. The initial population sizes for wildtype and xbp-1 (KO) strains was 35 and 30 respectively.
2.11 Identifying Lysosomes

Pretzel-staged wildtype worms were grown on icd-1 RNAi plates at 20C. After 5 days of growth, embryos were mounted on glass slides and viewed with Nomarski Optics. Morphologically defective embryos were examined at 100x using a Zeiss Axioskop up-right microscope. Lysosomal granules are bi-refringent under DIC optics and can be filtered from other objects in the embryo by setting the polarizer and analyzer to maximum extinction (a condition where the polarizer and analyzer transmission axes are oriented 90 degrees to each other).

2.11.1 Staining lysosomal granules for DNA

To test if the granules contained DNA, mounted defective embryos were stained with DAPI (4',6-diamidino-2-phenylindole), a fluorescent dye that binds to A-T rich DNA. Around 10% of morphologically defective embryos have weak membranes and the osmotic pressure under the cover slip causes these embryos to burst open. Bi-refringent granules remain intact and can be stained with fluorescent dye. DAPI stain was diluted to 1ug/ml and pipetted to the edge of the glass cover slip, with embryos mounted underneath. The opposite side of the cover-slip was blotted with a kimwipe, drawing the staining solution across the slide through capillary action. The slide was stained for 5 minutes, without light exposure. Fluorescent images were captured with a Nikon C1 confocal microscope under brightfield settings.

2.11.2 Using Glo-1 lysosomal reporter strain

Previously, researchers identified bi-refringent granules in the guts of C. elegans that are believed to contain lysosomes. This theory is based on observations in which bi-refringent material is misrouted to the gut lumen after the deletion of known lysosomal genes. Furthermore, genetically modified worms expressing GFP controlled by the Glo-1 promoter visually correlate with bi-refringent gut granules. Glo-1 is a known
rab GTPase expressed in lysosomes. [Hermann et al., 2005, Zhang et al., 2010] To determine the identity of the bi-refrangent objects in icd-1 induced defective embryos, Glo-1 reporter worms (cgc VS17) were grown on icd-1 and OP50 feeder as previously described (see 2.11). Morphologically defective embryos were mounted on glass slides and viewed with Nomarski and widefield flourescent optics using a Nikon C1 Confocal microscope. DIC and GFP flourescent channels were overlayed with NIS elements software.

### 2.12 Monitoring Protein Aggregation with Poly-Q mutants

To test if icd-1(RNAi) induces an aggregation phenotype in worms, a genetically modified strain (Q35) expressing poly-glutamate protein in muscles (cgc AM140) was used. Poly-glutamate proteins are known to aggregate and cause neuro-toxicity. Specifically, we used a strain that expresses an aggregation prone protein composed of 35 glutamate (Q35) amino acids attached to yellow flourescent protein (YFP). Previous experiments show synchronized L1 worms fed HSF-1 RNAi for 72hrs exhibit aggregation that can be visualized as punctate YFP signals. [Nollen et al., 2004a] In our experiment, worms were synchronized to pretzel stage and fed icd-1 RNAi or OP50 bacteria for 96hrs. Next, adult worms were mounted on glass slides (see 2.2). Using a Nikon C1 confocal microscope, widefield flourescent images of Q35 strains fed OP50 or icd-1 were captured using identical exposure settings.

### 2.13 GST-ICD-2 Protein Purification

The GST Purification kit (Amersham Biosciences) utilizes an engineered plasmid which is cloned with a gene of interest. The gene of interest is fused with the tag protein Glutathione S-transferase (GST). First, the gene of interest is inserted into the pGEX plasmid in frame with the GST gene, under the control of an IPTG promoter. IPTG induced E. coli express the gene of interest which is fused to GST. Protein is
extracted and the GST-fusion protein is purified using Glutathione Sepharose beads packed into a spin column. Non-fusion proteins are washed off the column and the GST protein is eluted with a wash buffer. Purification is verified with a protein gel.

Starting with an engineered *e. coli* strain with a pGEX plasmid containing the ICD-2 gene, the ICD-2 vector was grown in LB media overnight to a density of $A_{600} = 0.6$. After reaching target density, the culture was induced with 1mM IPTG for 4hrs. After the induction, the culture was alloquated into 25mL conical tubes and frozen to -20C. Two 25mL bacteria pellets, thawed from the 4hr induction, were re-suspended in 1mL of BPER lysis solution/pellet, provided by the BPER GST-Purification Kit (Thermo Scientific). Pellets were mixed with the lysis solution by vortexing. 30ul of Halt protease inhibitor was added to 3ml BPER lysis solution. Soluble proteins were isolated by centrifuging lysed bacteria in 2.0mL micro-centrifuge tubes at 16,000xg for 15minutes. The supernatant was poured off the pellet carefully. The pellet was not completely solid nor condensed toward the bottom, so only the top part of supernatant was extracted in 1.5ml ependorf tubes. Immobilized Glutathione was shaken and 200uL was pipetted into the protein extract. Extracts were shaken for 10 minutes in the shaker. After shaking, extracts were centrifuged in 1.5mL ependorf tubes @ 4000rpm (1200xg) for 5 minutes. The supernatant was discarded (unbound protein) and the remaining gel (gluthione beads) was resuspended with 150uL of wash buffer. Approximate volume was 300uL. The gel slurry was transferred to a Spin Cup Column inserted in a Collection tube. From here the thermo scientific protocol was followed exactly (from step 10) until the end. 8. Samples were stored at 4C overnight. The following day 280nm absorption was recorded with a Nanodrop for all elution volumes. There were two samples (from 2 pellets) and four elution volumes, 500ul each. Therefore a total of eight tubes (500uL of eluted protein, each subsequent elution had less protein content).

The concentration of purified protein was estimated by plotting the 280nm absorp-
tion against each sequential elution (Figure 5). The first elution extracted the most protein, subsequent elutions have lower protein concentrations. Third and fourth de-
lutions have nearly identical absorption values, thus their abs values were averaged and assumed to represent background levels. Averaging elutions 1 and substracting the approximated background (averaged value of elutions 3 and 4) yielded a concentration of 0.42mg/ml. If this approximation is accurate, then the protein production efficiency with this protocol is 6.7mg GST protein/mL of induced culture. To ensure isolated ICD-2-GST was pure, a coomassie protein gel was run.

### 2.14 ICD-2 Western Blot

15uL of purified ICD2-GST (see 2.13) was mixed with 5 uL of 4x LDS sample buffer. The sample was boiled at 100C for 1 minutes. The Bio Rad SDS gel was loaded with 10uL of 250kD marker and 20uL of the protein sample. The SDS gel was run at 130V for 20 minutes. PVDF membrane was wetted in methanol for 15 seconds and equilibrated in transfer buffer. Transfer apparatus was run for 1hr at 100V with a stir bar and ice pack filled with western transfer buffer [39mM glycine, 48mM Tris Base, 0.037% SDS, 20% methanol]. PVDF membrane was stored at room temperature sandwiched between blotting paper. Dry PVDF membrane was wetted in Methanol for 15 seconds. 1uL of .5mg/μL pAb was spotted on the upper right corner of the membrane to control for the staining procedure. The membrane was washed with blocking solution [5mL of 10x PBS, 2.5g of NFDM (dry milk), brought to 50mL with dH20] for 30 minutes on a shaker. Blocking solution was washed twice in 1xPBST, 5 minutes each on the shaker. Stock GST-pAB (HRP conjugated) was diluted to a final concentration of .5ug/mL. The antibody solution was incubated with the PVDF membrane for 1 hour on a shaker at 37C and 1 hr at room temperature. The membrane was washed 4x, 5 minutes each in 1xPBST. The membrane was incubated in 6mLs of the luminol/enhancer solution (BioRad HRP Chemoluminesence kit) for
4:30 seconds. Excess luminol substrate was drained off and additional liquid was removed with a paper towel by touching the bottom of the membrane. The membrane was sandwiched in plastic wrap and the picture was taken using Bio Rad Chem Doc XRS. ICD-2 GST bands were visible after 800s of exposure.

2.15 *In-vitro ubiquitination assay*

Using Boston Biochem’s K-960 conjugation kit, the conjugation reaction mixture was assembled with the following concentrations: 10 μL of 10X Energy Solution, 10 μl Conjugation Fraction A, 40 μl Conjugation Fraction B, 40 μl Ubiquitin: 150 μM, and 2.5μL of ddH20. The reaction was initiated by adding 1 μl ICD2-GST suspended protein: (400ng of protein). The reactions were stopped in a time-course experiment to determine the optimal incubation required. 20μL of the reaction mixture was removed at each time point. Quenched samples were stored at room temperature after addition of 10mM EDTA final concentration. The samples were run on an SDS-gel and ubiquitination was measured by western blotting (see 2.14).
Figure 5: Concentration of Purified ICD2-GST as determined by Nanodrop. Each data point represents the average absorption of 3 samples of purified ICD-2 GST elutions. Two independent isolations were performed, termed elution 1 and elution 2 in the figure legend. Elutions refer to the resuspended protein extracted from the GST column trap. The first and second elutions extracted nearly all the immobilized protein. The average value of elutions 3 and 4 defined the background signal absorption of the resuspension buffer.
3 Results

3.1 Heat shock sensitivity is reduced in \textit{icd-1} (RNAi) worms

Heat shock is known to cause an up-regulation of chaperones due to the denaturing effect of thermal stress. [Prahlad and Morimoto, 2009] To determine whether the NAC has a chaperoning function in \textit{C. elegans}, worms were exposed to extended heat stress after an \textit{icd-1} or \textit{icd-2} gene knockdown. We hypothesized the removal of the NAC by RNAi would increase worm sensitivity to heat stress. Correspondingly, if \textit{icd-1} or \textit{icd-2} RNAi had no differential effect on the response to thermal stress relative to wildtypes, the chaperone model is likely invalid. We measured worm sensitivity to heat stress by monitoring their movement in response to a physical stimulus.

3.1.1 Heat Shock 1.0

Unexpectedly, removal of \textit{icd-1} decreased worm sensitivity to heat. After 750 minutes of heat stress, 90% of worms lacking \textit{icd-1} show movement. In comparison, only 38% of control wildtypes move after 750 minutes of heat stress. \textit{Icd-2} was even more heat resistant than \textit{icd-1}, with 90% of worms retaining movement over the entire length of the heat shock experiment (Figure 6).

3.1.2 Heat Shock 2.0

The heat shock experiment was replicated, however we measured worm survival as a function of pharyngeal pumping instead of percent movement. Measuring percent survival was a more stringent way to determine worm viability. Thus, many worms that showed lack of movement still exhibited pharyngeal pumping. In the second heat shock assay, \textit{icd-2} (RNAi) and to a lesser degree \textit{icd-1} (RNAi) populations showed a decrease in sensitivity to heat stress, but the magnitude of heat resistance was smaller than the first experiment (Figure 7).
Figure 6: **Movement of heat stressed worms after icd-1 or icd-2(RNAi).** Bleach synchronized worms were heat shocked for 900 minutes in an incubator set to 36°C. Control worms were maintained at 20°C to ensure all worms strains were mobile. Movement was measured as lateral movement in response to prodding with a platinum pick. Treatment worms were grown on RNAi plates for 30 hours before they were heat shocked. To measure movement, worms were removed from heat shock and counted at room temperature.

Figure 7: **Survival of heat stressed worms after icd-1 or icd-2(RNAi).** Bleach synchronized worms were heat shocked for 900 minutes in an incubator set to 36°C. Control worms were maintained at 20°C to ensure all worms strains remained viable. Survival was measured as pharyngeal pumping in response to prodding with a platinum pick. Treatment worms were grown on RNAi plates for 28 hours before they were heat shocked. To measure movement, worms were removed from heat shock and assessed at room temperature.
3.2 \textit{hsp-4}, the homologue of BiP in \textit{C. elegans}, is up-regulated after \textit{icd-1} RNAi

To determine if the decreased sensitivity of \textit{icd-1} or \textit{icd-2} deficient worms to heat stress was a result of chaperone up-regulation, we used an hsp-4::GFP reporter strain (see 2.5). \textit{Hsp-4} is the \textit{C. elegans} homologue of human ER chaperone BiP. For 48hrs, adult worms were fed \textit{icd-1} RNAi and embryos were monitored for \textit{hsp-4} expression. \textit{Icd-1} removal led to a large increase in hsp-4::GFP reporter expression in embryos (Figure 8). To compare the signal strength of \textit{icd-1} deficient embryos against OP50 controls, the fluorescent intensity of random samples of embryos was quantified. The peak fluorescent intensity in \textit{icd-1} rnai embryos was 4.2 times greater than controls (Figure 9). On average, the fluorescent intensity of entire embryos from \textit{icd-1} rnai is 3.4 times greater than controls (Figure 10).

To determine the range of HSPs up-regulated after \textit{icd-1} removal, HSP-16::GFP was tested. HSP-16 is homologous to human alphaB-crystallin, a member of the small heat shock protein family. In \textit{C. elegans}, HSP-16 is up-regulated in response to various stressors, including protein stress and is tightly correlated with worm longevity. [Hartwig et al., 2009] In almost all embryos examined, HSP-16 was not expressed above background fluorescence levels during RNAi experiments (Figure 11). In a small proportion of severely affected embryos past comma stage, fluorescent signals were detected in the pharynx region of the worm in the late stages of an \textit{icd-1} knockdown (Figure 12).
Figure 8: **hsp-4::GFP embryo expression in DIC and widefield fluorescent channels.** Images were acquired under 10x magnification and identical exposure settings using a Nikon C1 confocal. Embryos were mounted on agar pads after parental worms were fed either OP50 (Image A) or icd-1(RNAi) (Image B) feeder for 48hrs. Embryos in images A and B were genetically identical, containing an hsp-4::GFP reporter construct (cgc SJ4005).

Figure 9: **Quantified hsp-4::GFP fluorescent intensity of icd-1 RNAi and control embryos.** The fluorescent intensities of hsp-4 and icd-1(RNAi); hsp-4 embryos were quantified using the line scan intensity function in NIS elements software. Each line represents the average fluorescent intensity as a function of scanned pixels of all embryos from a population. Images were acquired with identical optical settings.
Figure 10: Quantified whole embryo average HSP-4::GFP fluorescent intensity of icd-1 and control embryos. The fluorescent intensity of HSP-4::GFP; icd-1 (RNAi) and HSP4-GFP embryos were quantified using the line scan intensity function in NIS elements software. Each bar represents the average pixel intensity for all embryos in each population. The error bars represent one standard deviation.

Figure 11: Example images of HSP-16 expression in larva and worms. Parental worms were fed icd-1(RNAi) or OP50 feeder for 48hrs. Resulting larva and embryos were mounted on agar pads and viewed on a Nikon C1 confocal microscope. Images are overlays of DIC and fluorescent channels. Image A and B are representative images of hsp-16::GFP embryos and larva respectively.
Figure 12: **3D projection of severally effected embryo expressing HSP-16 in the pharynx and gut.** Parental worms were fed *icd-1(RNAi)* feeder for 60hrs and morphologically defective embryos were mounted on agar pads. Embryos were viewed with a Nikon C1 confocal microscope. A dual channel (DIC and green fluorescent) z-stack was captured and the image was transformed into a 3D topological projection using NIS elements software. A) shows DIC, B) shows GFP, and C) shows an overlay of GFP and DIC channels.
3.3 *icd-1* RNAi sensitivity as a function of chaperone levels

After confirming hsp-4::GFP expression was significantly higher in *icd-1* (RNAi) embryos, we tested the effects of HSF-1 dependent chaperone up-regulation on worm and embryo viability. First, to determine the effect of cytosolic HSP expression on worm viability during *icd-1* (RNAi), an HSF knockout (KO) strain was heat-shocked during *icd-1* (RNAi) and worm viability was measured as a function of movement. HSF-1 is the major cytosolic HSP transcription factor in *C. elegans* which up-regulates HSPs in response to stress. [Shamovsky and Nudler, 2008] As expected, HSF(KO) worms were highly sensitive to heat stress; approximately 90% of HSF(KO) worms lacked movement after 750 minutes of heat stress (Figure 13). As previously shown (see Figure 6), wildtype worms lacking *icd-1* were the least sensitive to heat after 750 minutes of stress. Interestingly, HSF(KO) worms lacking *icd-1* were considerably less sensitive to heat relative to HSF(KO) controls. After 750 minutes of heat stress, 45% of HSF (KO) worms treated with *icd-1*rna showed movement, compared to 7% in the HSF(KO) control group (Figure 13).

To investigate the effect of *hsp-4* on worm and embryo viability during *icd-1* (RNAi) we used a genetically modified worm strain lacking functional *xbp-1* (*xbp-1* (KO)). Without functional *xbp-1*, worms have a diminished ER stress response and express lower levels of *hsp-4*. This strain also contains an hsp-4::GFP reporter as measure of *hsp-4* expression levels.

*Xbp-1* (KO) worms were fed *icd-1* (RNAi) for 48hrs and embryos were monitored for *hsp-4* expression. On average, hsp-4::GFP expression in *xbp-1* embryos was 67% stronger in *icd-1* deficient embryos compared to controls (Figure 14). In contrast, worm strains with functional *xbp-1* express 340% more *hsp-4* relative to controls during *icd-1* (RNAi) (Figure 9). After confirming *xbp-1* (KO) embryos express decreased levels of *hsp-4* relative to wildtype (N2), we compared the phenotypes of *icd-1* deficient embryos against controls fed OP50.
Figure 13: Movement of heat stressed HSF (KO) and wildtype worms during *icd-1* RNAi. Synchronized worms were heat shocked for 750 minutes at 36°C. All strains developed on *icd-1* (RNAi) or OP50 plates for 30 hrs before heat shock. Movement was measured as positional displacement in response to prodding with a platinum pick. Worm movement was measured every hour after continuous heat shock for 450 minutes. Red and blue lines represent control (OP50) populations and purple and green lines represent *icd-1* (RNAi) populations.

Figure 14: Hsp-4::GFP expression quantified in *xbp-1* (KO) embryos during *icd-1* (RNAi). The fluorescent intensity of *xbp-1* (KO) and *xbp-1* (KO); *icd-1* (RNAi) embryos was quantified using the line-scan intensity function using NIS elements software. Each line represents the average fluorescent intensity as a function of scanned pixels of all embryos from a population. Images of both embryo populations were acquired with identical optical settings.
To compare embryo populations, random embryos were picked from OP50 or *icd-1* feeder plates and viewed with DIC optics. Embryos were categorized by developmental stage and morphologically defective embryos were scored separately (Figure 15). On average, 15% of *xbp-1 (KO)* embryos fed OP50 were morphologically defective. In contrast, 88% of *xbp-1 (KO)* embryos were defective after 48hrs of *icd-1 (RNAi)* (Figure 16).

In order to compare *xbp-1 (KO)* and wildtype embryos after *icd-1 (RNAi)*, parental worms were developmentally synced and resulting embryos were phenotypically classified over a 24 hour sampling period. Since *xbp-1 (KO)* and wildtype worms were treated with *icd-1 (RNAi)*, the differences in their embryo profiles is assumed to be attributed to the presence or absence of functional *xbp-1*. Early F1 embryos were 5% defective in wildtype strains and 15% defective in *xbp-1 (KO)* embryos. On average, control *xbp-1 (KO)* embryos are defective 15% of the time, therefore the RNAi effects were not measurable. Over the subsequent 24hr period, embryo profiles altered dramatically. 95% of *xbp-1 (KO)* embryos were defective and 82% of wildtype embryos were defective (Figure 17). Since *xbp-1 (KO)* embryos lay a higher proportion of defective embryos compared to wildtypes under control conditions (see Figure 16), the rate of change of defective embryos was calculated to compare the wildtype and *xbp-1 (KO)* response to *icd-1 (RNAi)*. The rate of change of defective embryos is nearly the identical between *xbp-1 (KO)* and wildtype populations when grown at 20C (Figure 17).

The experiment was repeated, however the growth temperature was slightly altered. To maximize the embryo output of *xbp-1 (KO)* worms, the growth temperature was lowered to 18C because the *xbp-1 (KO)* is heat sensitive. *Xbp-1 (KO)* worms are heat sensitive because they have a compromised ER stress response and ER stress increases with higher temperatures. At 18C, worm strains developed slower and embryos were not observed until 5 days of growth. Unexpectedly, lowering the
Figure 15: **Comparison of normal and defective embryos at various developmental stages.** A), B), and C) show wildtype early, comma, and pretzel stage embryos respectively. D), E), and F) show defective embryos at early, comma, and pretzel stages respectively.
Figure 16: *xbp-1* (KO) embryo profiles with OP50 or *icd-1(RNAi)* feeder. In two independent experiments, late larval *xbp-1* (KO) worms were moved to OP50 or *icd-1* (RNAi) plates and grown for 48hrs. Embryos were removed after 48hrs and categorized by developmental stage or as morphologically defective shown in Figure 15. Embryos were viewed with a Nikon C1 confocal microscope under 60x magnification.
Figure 17: *xbp-1* (KO) and N2 embryo profiles after *icd-1* RNAi. Late-staged synchronized embryos were transferred to *icd-1* (RNAi) plates and embryos from the resulting adults were monitored. Embryos were removed from plates randomly and the proportion of defective embryos was calculated from the sampled embryos (Figure 15). A) shows *xbp-1* (KO) and N2 populations grown during *icd-1* (RNAi) at 20C; B) shows the same strains grown at 18C.
temperature substantially increased the rate of defective embryos in the \textit{xbp-1} (KO) population. More than half of \textit{xbp-1} (KO) embryos during the first day of fertility were morphologically defective, compared to 15\% of embryos grown at 20C. 90\% of older F1 \textit{xbp-1} (KO) embryos were defective, compared to 45\% in the wildtype population (Figure 17). The rate of change of defective embryos was initially much higher in the \textit{xbp-1} (KO) population, indicating the \textit{icd-1} (RNAi) effect occurs earlier in time and is more severe for worms lacking functional \textit{xbp-1} at a lower growth temperature (Figure 17).

In addition to measuring the rate of morphological defects in embryos, the lifespans of developmentally synchronized wildtype and \textit{xbp-1} (KO) worms were evaluated throughout an \textit{icd-1} (RNAi). Worms were synchronized late in embryogenesis and survival was measured as a function of pharyngeal pumping. After 11 days of growth at 20C, 35\% of \textit{xbp-1} (KO) worms were alive, compared with 66\% of wildtypes (Figure 18). The experiment was repeated, however worms were grown at 18C. Overall, both strains develop slower and survive longer at 18C. However, \textit{xbp-1} (KO) worms consistently had lower survival rates compared to wildtypes. After 9 days of growth at 18C, 80\% of wildtypes survive, compared to 50\% in the \textit{xbp-1} (KO) population (Figure 18).
Figure 18: **Worm survival during an icd-1 (RNAi) at 20 and 18 degrees celsius.** Late-stage synchronized embryos were transferred to *icd-1* (RNAi) plates at time 0. Points on the line represent measurement frequency. Percent survival was measured as a function of pharyngeal pumping. Images a and b show independent survival assays at 20C and 18C respectively.
3.4 Loss of *icd-1* leads to an enlargement of lysosomes

3.4.1 DNA staining of embryo granules

After examining the viability of *xbp-1* deficient worms during *icd-1* (RNAi) we looked for phenotypic markers of unfolded protein in defective embryo populations. We reasoned that the synergistic effect of *xbp-1* (KO) and *icd-1* (RNAi) on worm viability resulted from increased protein stress. Proteotoxicity can lead to protein aggregation and/or increased protein degradation that can be detected in cells microscopically, as is the case with aggresomes, stress granules, or p-bodies [Spector, 2006].

During an *icd-1* knockdown, a significant proportion of embryos have apoptotic phenotypes that occur up to 48hrs into the RNAi treatment. After 48hrs, many embryos become severally degraded as measured by vacuolation and they often contain distinct bi-refringent donut-shaped granules inside cells. Previous research has shown that apoptotic DNA in fibroblasts and neurons have similar morphology to the granules we observed, so we determined the putative DNA content of these structures. [Baskin et al., 2003] Embryos containing bi-refringent objects often lysed from the osmotic pressure underneath the microscope slide; the bi-refringent granules that remained intact were stained with a DNA marker (DAPI) (Figure 19). The bi-refringent objects did not correlate with the DNA stain (Figure 20).

3.4.2 Identifying granules with lysosomal reporter

Frequently, bi-refringent granules observed during *icd-1* (RNAi) were located in the gut region of embryos, a region known to contain lysosome organelles. To determine the putative lysosomal nature of these structures we used a worm strain containing lysosomes marked with GFP. The strain is a GFP construct fused with the promoter for the GLO-1 gene, a known Rab GTPase located in lysosomal membranes. [Hermann et al., 2005]
Figure 19: **Bi-refringent granules from bursted wild-type embryo.** Embryos from wild-type worms were fed *icd-1* (RNAi) for 72hrs were mounted on agar pads and viewed with a Nikon C1 confocal microscope. Approximately 15-20 minutes after mounting defective embryos, osmotic pressure causes a proportion of defective embryo membranes to burst open. Bi-refringent granules were observed with DIC optics, with the polarizer set to extinction. The yellow arrows point to the granules of interest.
Figure 20: **DNA stain of bursted embryo.** Defective embryos were mounted as described in section 2.2. Embryos were stained with DAPI and viewed with a Nikon C1 confocal microscope. A) shows the DIC channel with the area of image C highlighted in yellow, B) shows DIC and UV channels with the area of image D highlighted in yellow. The white arrows point to DNA content, the yellow arrow highlights the bi-refringent granules of interest.
Previous research has shown that ER stress can up-regulate autophagosome formation, leading to an increase in the lysosomal content of stressed cells. [Hetz et al., 2009] It has been proposed that when proteosomal ERAD fails to rid the ER of misfolded protein, autophagy is up-regulated to dispose of protein aggregates at the lysosome. [Ishida et al., 2009] Thus, the appearance of enlarged lysosomes was consistent with the hypothesis that NAC disruption leads ER stress.

To verify the bi-refringent granules were lysosomal structures, Glo-1::GFP worms were fed icd-1 (RNAi) and defective embryos were viewed with DIC and widefield fluorescent optics. In severally effected icd-1 (RNAi) embryos, Glo-1::GFP expression was highly correlated with the bi-refringent granules (Figure 21).

3.5 Monitoring protein aggregation after icd-1 RNAi

After showing that icd-1 removal affects chaperone expression in worms and embryos, we tested whether icd-1 (RNAi) affects protein aggregation in worms. Since the up-regulation of hsp-4 is indicative of protein misfolding at ER, we reasoned that misfolded proteins could potentially aggregate in icd-1 (RNAi) worms. To test this idea, we used a genetically modified worm strain expressing a poly-glutamine repeating peptide (Q35) fused to YFP (yellow fluorescent protein). Huntington’s disease is caused by an expansion of the poly-glutamine (poly Q) repeat region of Huntington protein and expression of poly Q repeating peptides in C. elegans results in age-dependent protein aggregation. [Zhang et al., 2011] Experiments have shown that chaperone expression reduces the aggregation of poly Q protein. For example, hsf-1(RNAi) leads to an early onset of poly Q aggregation and rapid aging in worms. [Nollen et al., 2004a, Hsu et al., 2003] In an age dependent manner, worms expressing poly-glutamine YFP fusion proteins report aggregation as a function of the diffuseness the fluorescent signal emitted (Figure 22). [Brignull et al., 2006] We monitored Q35::YFP expression in worms fed icd-1(RNAi) for 96hrs from pretzel
Figure 21: Overlap of GLO1::GFP reporter and bi-refringent objects in *icd-1* (RNAi); Glo-1::GFP embryo. Embryos from the Glo-1::GFP reporter strain were mounted on agar pads after 72hrs of *icd-1* (RNAi). All images show the same embryo in different channels under 60x magnification captured with a Nikon C1 confocal microscope. A) is an overlay of DIC and GFP channels, b) is an overlay of GFP and extinction DIC. C) is only the GFP channel D) is the DIC channel at extinction. The red outline defines the border of the embryo.
stage. We reasoned that if protein aggregation increases during \textit{icd-1} (RNAi), then Q35::YFP expression would appear as fluorescent foci (Figure 22). No visible differences were discernable between worms fed \textit{icd-1} (RNAi) or control OP50 bacteria (Figure 23).

3.6 Structural modeling of alpha NAC homologue \textit{icd-2} reveals a UBA domain

Structural studies of NAC homologues from Archea to humans have shown \(\alpha\)-NAC contains a ubiquitin-associated domain (UBA) domain. [Spreter et al., 2005, Liu et al., 2010b] To determine the putative presence of a UBA domain in the \textit{C. elegans} homologue \textit{icd-2}, the protein was modelled using the Phyre server. Phyre is a template-based homology modeling server which predicts protein structure based on existing known structures from SCOP and PDB databases. [Kelley and Sternberg, 2009] The phyre model detected a NAC domain and a c-terminal UBA domain with an internal confidence score or 

The likelihood of ubiquitination increases with the presence of a UBA domain. [Su and Lau, 2009] Numerous proteins with c-terminal UBA domains are also known to interact with poly-ubiquitylated proteins at the proteosome. [N’Diaye et al., 2009] However, a class of proteins with UBA domains show no affinity for poly-ubiquitin chains \textit{in vitro}. \textit{Egd2}, the yeast homologue of \textit{icd-2}, belongs in a class proteins in which the functionality of their UBA domains is not fully understood. [Raasi et al., 2005] To test whether \textit{icd-2} could be ubiquitinated \textit{in vitro}, \textit{icd-2} was purified, incubated with conjugation enzymes and run on an SDS gel and transferred for a western blot. Ubiquitylated \textit{icd-2} would run as a protein smear on the western blot since conjugated ubiquitin increases the weight of its target protein. ICD-2-GST was detected at the predicted location (50 kD), but did not show a protein smear above the predicted band size. The ICD2-GST band signal weakened with respect to the incubation time with
Figure 22: Polyglutamine aggregation in *C. elegans* using various polyglutamine YFP fusion proteins. Panel A shows 4 day old worms expressing polyglutamine::YFP fusions of various lengths in muscle tissue. Panel B is identical to panel A, except it shows polyglutamine expression in neurons around the pharynx. Images Q40, Q82, and Q86 have visible polyglutamine aggregates that appear as fluorescent foci. Figure taken from [Brignull et al., 2006].

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Figure 23: Q35::YFP worms fed OP50 or *icd-1* (RNAi). Synchronized late-stage Q35::YFP embryos were grown on *icd-1* or OP50 feeder for 4 days. Larva were imaged at 20x magnification under identical optical settings using a Nikon C1 confocal microscope. A) and b) show control and *icd-1* (RNAi) larva respectively.
Figure 24: **Predicted structure of ICD-2.** The amino-acid sequence of *icd-2* (accession # CCD71934.1) was sent to Phyre protein modeling server. [Kelley and Sternberg, 2009] The model detected both a NAC and UBA domain. The NAC fold represents the predicted binding site for *icd-1*, forming a beta barrel when *icd-1* and *icd-2* bind as complex. The three alpha helices (chain highlighted in yellow) represent the UBA domain. This arrangement is known to have binding affinity for ubiquitin. [Kozlov et al., 2007]
conjugation enzymes. The disappearance of signal over time was attributed to either 1) protein degradation or 2) a blocked antibody binding site due to ubiquitination. To test these possibilities the experiment was repeated with one reaction containing all the necessary conjugation enzymes without ubiquitin (Figure 25).

The results of the second western blot did not show ubiquitination and indicated instead that ICD-2-GST was degrading over time. Overall, the results of the in vitro ubiquitination were inconclusive and should be repeated.
Figure 25: *In-vitro ubiquitination of icd-2-GST fusion.* Purified *icd-2*-GST protein was incubated with ubiquitin conjugation enzymes and ubiquitin for varying amounts of time. In image A, *icd-2a* and *icd-2b* represent two different purifications of *icd-2* at different concentrations. Numbers at the top of columns red columns represent incubation time. Image b shows the second ubiquitination experiment with one conjugation mixture lacking ubiquitin. Red brackets represent detected protein bands and their cumulative signal intensity.
4 Discussion

4.1 Analysis of Results

The major goal of this project was to test the translational chaperone model of the NAC using *C. elegans* as a model organism. Our model assumes that removal of *icd-1* by RNAi eliminates the functionality of the NAC complex in *C. elegans*, disrupting the folding and routing of proteins. As such, we expected to see an unfolded protein response to compensate for the disruption of the NAC. Previous researchers observed that increased HSP expression masks the effects of NAC deletion in yeast and only double knockouts of HSP-70 and NAC show complementary growth defects [Koplin et al., 2010b]. However, to this date there were no known studies that tested this model in higher order organisms such as *C. elegans*. Our research supports the view that the NAC has chaperone qualities in *C. elegans* and leads to an unfolded protein response in the worm.

The results from heat shock experiments indicated that chaperone levels change in response to the removal of *icd-1*. Initially, we predicted removing *icd-1* would increase worm sensitivity to heat stress. Instead, both worm mobility and survival increased after *icd-1* removal during heat stress. This unexpected result led to the hypothesis that decreasing *icd-1* up-regulates heat shock proteins, conferring heat stress resistance in worms; a finding consistent with studies of the NAC in yeast [del Alamo et al., 2011].

To test whether chaperone levels increase after *icd-1* (RNAi), we monitored *hsp-4* expression *in vivo* with an *hsp-4::GFP* reporter. *hsp-4* is an ER chaperone that is homologous to the human ER chaperone BiP. BiP interacts with folding peptides after they are threaded through the translocation pore of the ER. The mammalian unfolded protein response is initiated when BiP is titrated away from ER stress receptors by unfolded protein [Kawaguchi and Ng, 2011].
Embryos from worms fed *icd-1* dsRNA showed a dramatic increase in *hsp-4* expression. Similar results were seen previously, where Beatrix et. al. observed that human cell lines have elevated BiP levels after knocking down α-NAC, the *icd-2* homologue [Hotokezaka et al., 2009]. Interestingly, expression patterns of *hsp-4::GFP* showed increased expression in regions of the embryo not associated with neuronal development. These results are consistent with previous findings [Bloss et al., 2003] where loss of *icd-1* resulted in preferential death of neurons relative to other cell types. Thus, the lack of expression of *hsp-4* in neuronal regions of embryos may explain why neurons preferentially die by apoptosis during *icd-1* (RNAi).

We propose that removing *icd-1* or *icd-2* disrupts proteostasis by eliminating the putative chaperone functions of the NAC and the worm compensates by increasing the expression of other chaperones to prevent ER mediated apoptosis. Compensatory regulation after single deletions of HSPs have been observed previously in *E. coli* and *C. elegans*. For example, deletion of the bacterial translational chaperone Trigger Factor (TF) leads to an increase in ribosomal related HSPs, preventing the accumulation of unfolded protein in *E. coli* [Teter et al., 1999]. Furthermore, single deletions of ER chaperones in *C. elegans* unexpectedly does not increase their sensitivity to toxic human beta amyloid peptide; instead beta amyloid toxicity is alleviated through a compensatory up-regulation of *hsp-4* [Kapulkin et al., 2005]. Thus, *icd-1* removal may perturb proteostasis causing higher than normal expression of chaperones, which in turn masks its function.

In addition to the up-regulation of ER localized *hsp-4*, our results suggest *icd-1* removal may also increase cytosolic HSPs. An *icd-1* knockdown in worms led to an increase in heat stress resistance in HSF-1(KO) worms. HSF-1 is the major cytosolic HSP transcription factor in *C. elegans* and loss of function mutations cause heat stress sensitivity, protein aggregation, and advanced aging in the worm. [Walker et al., 2003] [Hsu et al., 2003] [Nollen et al., 2004b] The resistance to heat stress in HSF-1
(KO) icd-1 (RNAi) worms could be explained by an up-regulation in cytosolic HSPs, independent of HSF-1. Alternatively, elevated hsp-4 expression and the initiation of the UPR may increase the worms threshold for stress induced unfolded protein even in the absence of increases in cytosolic HSPs.

To determine whether hsp-4 expression protects the worm during icd-1(RNAi), we used a genetically modified worm strain lacking xbp-1, a major ER stress transcription factor responsible for the up-regulation of hsp-4. The xbp-1 strain also contained an HSP-4::GFP reporter to monitor hsp-4 expression. Previous studies showed that xbp-1(KO) worms treated with tunicamycin express significantly less hsp-4 than controls. Tunicamycin prevents n-linked glycosylation, causing ER stress. [Noda et al., 1999] Therefore, we used the xbp-1(KO) strain to determine how the xbp-1-dependent ER stress response and hsp-4 expression affect embryo viability after icd-1(RNAi.)

To determine the effect of icd-1(RNAi) on xbp-1(KO) strain, we compared two embryo populations fed icd-1 RNAi or OP50. xbp-1(KO) embryos were highly sensitive to icd-1(RNAi) and morphologically distinct from wildtypes fed icd-1(RNAi), i.e. most defective embryos from the xbp-1(KO) population were immobile and arrested earlier in development than wildtypes.

To determine if the severity of icd-1(RNAi) in xbp-1(KO) worms resulted from diminished hsp-4 expression, we first had to determine the levels of hsp-4 in the presence or absence of icd-1. hsp-4 was quantified by HSP-4::GFP expression in worms fed control or icd-1 feeder. Xbp-1(KO) embryos showed some hsp-4 expression, but not nearly as much as xbp-1(+) embryos. Thus, the xbp-1(KO) strain expresses much less hsp-4 compared to wildtype, but we found that parallel stress pathways enable limited hsp-4 expression, even in the absence of xbp-1.

After confirming xbp-1 (KO) strains express relatively low levels of hsp-4 after an icd-1 knockdown, we compared the morphology of their embryo populations against wildtypes during icd-1(RNAi). At 20C, the rate at which xbp-1(KO) mutants gen-
generated defective embryos was roughly the same as xbp-1(+) embryos; surprisingly though, at 18C, xbp-1 (KO) embryos were particularly sensitive to icd-1(RNAi) and produce defective embryos at a higher rate than xbp-1(+) strains. It is possible that low temperature embryonic development limits the expression of heat sensitive cytosolic HSPs which may protect embryos lacking icd-1. Thus, elevated HSP expression due to higher growth temperatures may ameliorate stress caused by icd-1(RNAi). Recent experiments have shown that thermosensory neurons control the heat shock response in C. elegans, and this may explain why temperature has such a dramatic effect on xbp-1(KO); icd-1 (RNAi) worms. [Prahlad and Morimoto, 2011] An alternative explanation is that slower growth at a low temperature amplifies the icd-1(RNAi) effect because worms ingest dsRNA for a longer period before they generate embryos.

At 18C or 20C, xbp-1 (KO) adult worms have a lower survival rate compared to wildtypes fed icd-1 rnai as measured by pharyngeal pumping. We expected lifespan reduction in worms lacking icd-1 and a functional UPR because protein stress is known to negatively affect longevity. [Morley and Morimoto, 2004] For example, hsf-1 rnai leads to an early onset of protein aggregation, resulting in a reduced lifespan in worms. [Hsu et al., 2003] It is possible that icd-1 rnai causes rapid ageing in xbp-1 (KO) worms through a similar mechanism. If loss of icd-1 does indeed trigger the accumulation of unfolded proteins, then we should be able to detect evidence of these events icd-1 deficit worms.

We looked for protein aggregation in icd-1 fed worms expressing polyglutamine repeats attached to YFP (Q35::YFP). Parental worms fed icd-1 dsRNA did not exhibit aggregation relative to OP50 controls. It is possible that icd-1(RNAi) alone does not lead to an early onset of protein aggregation due to the concomitant upregulation of HSPs; rather icd-1(RNAi) coupled with xbp-1 loss of function may cause aggregation and rapid aging. The generation of a xbp-1(KO) expressing a poly Q reporter would test such a hypothesis. Since icd-1 rnai causes an up-regulation of hsp-4 and possibly
other chaperones, icd-1(KO) alone could potentially suppress aggregation in worms.

Because protein aggregation was not readily apparent in icd-1(RNAi) embryos, we searched for other phenotypic markers of unfolded protein stress. There are at least two potential outcomes during icd-1(RNAi) that could prevent the observation of significant protein aggregation: 1) rapid elimination of affected cells or 2) rapid turnover of misfolded protein. An elevated rate of developmental apoptosis was the only defective phenotype previously described in icd-1(RNAi) embryos in C. elegans. [Bloss et al., 2003] We consistently observed high levels of embryonic apoptosis at specific time frames throughout an icd-1(RNAi). Generally, between 36hrs-52hrs into icd-1(RNAi), embryos fill with corpse-like structures in regions of the embryo populated by neurons. [Bloss et al., 2003] Beyond 52hrs, a significant proportion of embryos contain unusually large bi-refringent cellular objects found in viable intestinal cells. These bi-refringent objects were identified as lysosomal structures using a GLO1::GFP reporter. In the case of lysosomal generation, gut cells provide insights into what appear to be cell specific responses to loss of icd-1; in the case of neurons, loss of icd-1 triggers apoptosis, while gut cells possibly remain viable through the generation of lysomes.

The unfolded protein response is known to trigger pro-autophagic signals, therefore these lysosomal structures may indicate a dramatic increase in autophagy in icd-1(RNAi) embryos. During an unfolded protein response, proteosomal degradation machinery can become overloaded, requiring alternative pathways for protein disposal like autophagy. [Hoyer-Hansen and Jaattela, 2007] Certain cell types in the embryo could be biased toward autophagic or apoptotic fates depending on their physiology and ability to degrade unfolded protein, explaining why both increased apoptosis and enlarged lysosomes are observed during an icd-1 knockdown.

Enlarged lysosomes have been previously correlated with the gut region of the worm when previous researchers observed similar appearing objects in pek-1; xbp-1
(RNAi) knockouts (Pek-1 is the *C. elegans* homologue of Perk kinase). Interestingly, the same researchers found that gut related bi-refringent objects disappear in *ire-1*; *pek-1* double knockouts. [Shen et al., 2001] It is possible these granules are the same type of lysosomal structures observed after *icd-1* (RNAi) and they do not appear in *pek-1*; *xbp-1* (RNAi) mutants because they are regulated by the c-jun kinase (JNK). As shown in Figure 3, JNK can be activated by ER stress, consistent with the *pek-1*; *xbp-1* (RNAi) observations, and up-regulate autophagy independently of *xbp-1*. Recent evidence from a mammalian cell culture model showed that JNK activates autophagy after ER stress through the *IRE-1* pathway and this protects cells, explaining why these structures may disappear in *ire-1* (KO) mutants. The same study showed JNK inhibition biased cells toward apoptosis after ER stress. [Ogata et al., 2006] Interestingly, numerous studies have shown α-NAC interacts with and up-regulates JNK. [Quelo et al., 2002, Moreau et al., 1998, Papachristou et al., 2003, Kim et al., 2002] Future studies should examine whether JNK inhibition in *C. elegans* similarly increases apoptosis after *icd-1* removal.

Inhibition of mTOR (inhibitor of rapamycin) or EIF2 (elongation initiator factor 2) also causes an up-regulation of autofluorescent gut granules that appear similar those in *icd-1* (RNAi) embryos. [Long et al., 2002] In mammals, both mTOR and EIF2 negatively regulate ATG proteins that are essential for autophagic vesicle formation. As shown in Figure 4, ER stress inactivates EIF2a by PERK phosphorylation and pro-autophagic signals are relayed. Similarly, removal of mTOR increases pro-autophagic signaling. Thus, it is possible that *icd-1* (RNAi) causes an up-regulation in autophagy mediated by multiple ER stress responses; i.e. the activation of JNK, phosphorylation of *eif2a*, or by inhibition of mTOR.

Recent studies have shown ubiquitin-associated domains (UBA) could be structurally important for autophagosome association. Interestingly, structural modeling of *C. elegans* NAC revealed a UBA domain in the c-terminus of *icd-2*. Assuming the
structural modeling is accurate, the UBA domain in \textit{icd-2} might be the mechanistic link causing lysosome up-regulation after \textit{icd-1} removal (see Figure 24). Two different autophagy related proteins, Plic-2 and p62, require UBA domains to associate with autophagosomes. Deletion of the UBA domain on \textit{plic-2} protein reduced autophagy rates in human cell lines. [N’Diaye et al., 2009] Similarly, p62’s c-terminal UBA domain is essential for the recognition of substrate proteins destined for lysosomal degradation. Presumably, p62’s UBA domain structurally binds to poly-ubiquitylated substrates, shuttling damaged or old proteins to lysosomes rather than the proteosome. [Bjorkoy et al., 2005] The c-terminal UBA domain in \textit{icd-2} might confer similar properties to the NAC in \textit{C. elegans}. An alternative explanation is that the UBA domain facilitates an interaction between \textit{icd-2} and the proteosome, as suggested by Panasenko et. al. and St. Arnaud et. al. [Panasenko et al., 2009, Quelo et al., 2002] Deletion of the NAC may decrease proteosomal degradation of misfolded protein and subsequently misfolded protein might be routed to autophagosomes.

To understand \textit{icd-2}’s putative role in protein degradation, we set out to determine the ubiquitination state of \textit{icd-2}. Poly-ubiquitination implies protein turnover, while mono ubiquitination is indicative of a signaling function. [Sadowski et al., 2011] Using \textit{in-vitro} ubiquitination assays, we tried to determine if \textit{icd-2} could be poly-ubiquitylated since UBA domains are known to have ubiquitin binding properties. [Kozlov et al., 2007] Our western blot results were inconsistent and we did not see any evidence of poly or mono ubiquitination. It is possible the GST moiety on purified \textit{icd-2} protein interfered with the ubiquitination assay. Alternatively, \textit{icd-2}’s UBA domain might only bind to existing poly-ubiquitin chains, having little or no affinity for ubiquitin polymerization like \textit{p62}’s UBA domain. [Raasi et al., 2005]
4.2 Potential Role of the NAC in Human Pathologies

Using *C. elegans* as a model, we observed increased apoptosis and an increase in phenotypic markers indicative of autophagy in embryos lacking functional NAC. Misregulation of autophagy through chaperone disfunction are hallmarks of neurodegenerative diseases. Parkinson’s, Alzheimers and Huntington’s disease have been shown to alter autophagy kinetics (Figure 26). In Huntington’s and Parkinson’s disease, the degradation of damaged organelles and protein aggregates is impaired and autophagosomes accumulate in degenerating brains. [Wong and Cuervo, 2010] In alzheimer’s disease, it is proposed that the formation of a protein complex composed of Beclin, VPS15, and VPS34 is inhibited, affecting the nucleation of autophagosomes. [Wong and Cuervo, 2010] Since neurodegenative pathologies are tightly linked to autophagic processes, understanding the function of proteins involved in the up-regulation of autophagy may provide pharmeceutical targets for future treatments against neurodegeneration. The up-regulation and enlargement of lysosomes in *C. elegans* lacking icd-1 mimicks neurodegenerative cellular phenotypes and may explain the connection between the down-regulation of α-NAC in brain tissue from alzheimer’s patients . [Kim et al., 2002]

To better understand *icd-1’s* role in autophagy, future studies should examine how NAC deletions mechanistically cause an up-regulation of lysosomes. An *icd-1* knockdown of GFP::LGG-1 reporters could be used to directly quantify the effect of NAC removal on autophagosome regulation. LGG-1 is the *C. elegans* homologue of LC3, the standard marker of autophagy. [Hansen et al., 2008] Furthermore, *icd-1* knockdowns coupled with EIF2, mTOR and JNK knockout strains may elucidate which signaling cascade triggers autophagic changes after the NAC is disrupted. In addition, a more detailed analysis of Q35::YFP expression after *icd-1 RNAi* is warranted since only parental generation *icd-1*(RNAi) strains were examined in this study. Future studies could examine protein aggregation in Q35::GFP reporter strains fed *icd-1*
Figure 26: Proposed mechanisms for autophagy regulation in neurodegenerative diseases. In window 1, the Beclin, Vps15, Atg14, and Vps34 complex is inhibited in Alzheimer’s disease, altering the nucleation of autophagosomes. Window 2 shows how up-regulation of the mTOR protein complex in Huntington’s disease is believed to reduce phagophore formation. Window 3 shows that organelle and amyloid protein disposal by autophagosomes is inhibited in Huntington’s and Parkinson’s disease. Window 4 shows that in both Huntington’s disease and Spinal Muscular Atrophy the fusion of autophagosomes with lysosomes is impaired. Window 5 shows that defects in lysosomal enzymes, such as cathepsin proteases, leads to an accumulation of autophagosomes in lysosomes. Figure taken from [Wong and Cuervo, 2010].
RNAi for multiple generations, since *icd-1* knockdowns tend to amplify in successive generations.

In conclusion, the goal of this research project was to determine the functional role of the NAC in *C. elegans* and understand mechanistically how the disruption of this protein complex alters worm development. Using knowledge gained from structural studies of the NAC homologue in yeast, we tested the hypothesis that *C. elegans* NAC is a translational chaperone. We reasoned that disruption of an essential chaperone could explain the ranging effects associated with the NAC in past studies, such as translation regulation, protein mis-routing, HSP up-regulation, apoptosis induction, and autophagy defects.

First, we determined that *icd-1* or *icd-2* (RNAi) changes the worms ability to cope with heat stress. To understand why *icd-1* (RNAi) worms tolerated heat stress differently than wildtypes, we monitored HSPs *in vivo* using GFP reporter strains. We found that *hsp-4* was dramatically up-regulated in embryos generated from *icd-1* (RNAi) worms. *hsp-4* is the homologue of human BiP, the primary ER chaperone and an important signalling component for ER stress. Knowing that BiP and other ER chaperones are up-regulated by the UPR, we monitored mutant worms that have a genetically impaired UPR during *icd-1* (RNAi). We found that embryo populations lacking *xbp-1* were more acutely effected by *icd-1* (RNAi) compared to wildtypes and this effect was temperature dependent. This result was consistent with the hypothesis that the presence of the NAC prevents protein stress. Next, we analyzed the phenotypes of *icd-1* (RNAi) embryos and found that lysosomes were highly enlarged. It is known that unfolded protein and damaged organelles are processed at the lysosome by autophagy and the UPR can up-regulate this process. Thus, we identified a new phenotypic marker in *icd-1* (RNAi) embryos that is consistent with the chaperone model. Hopefully, the results of this project can be used elucidate the specific signalling pathways that lead to the development defects observed in NAC mutants.
Ultimately, a mechanistic understanding of the NAC and closely related proteins may enable pharmaceutical targets for neurodegenerative disease in the future.
References


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