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Protein Quality Control at the Endoplasmic Reticulum and Nucleus in Caenorhabditis elegans

Daniel Hassell
Western Washington University, danielhassell97@gmail.com

Protein Quality Control at the Endoplasmic Reticulum and Nucleus in *Caenorhabditis elegans*

Daniel Stephen Hassell

Research conducted in the laboratory of Dr. Lina Dahlberg

Western Washington University Department of Biology

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Abstract:

Cells need proteins to carry out molecular functions, but proteins can become damaged or misfolded, which inhibits their function and can cause aggregation. Misfolded protein aggregation is associated with degenerative diseases in mammals. In Eukaryotic cells, chronically misfolded proteins can be destroyed by the Ubiquitin Proteasome System (UPS) as a way to prevent aggregation and disease. For my thesis work, I have investigated two pathways that participate in the UPS.

First, I investigate Endoplasmic Reticulum Associated Degradation (ERAD), a UPS pathway is active in the Endoplasmic Reticulum (ER) of eukaryotic cells. ERAD is well characterized in *Saccharomyces cerevisiae* (yeast), but has only recently been studied in multicellular systems. The Dahlberg Lab has begun to characterize the ERAD pathway regulating GLR-1, a glutamate receptor in the neurons of *Caenorhabditis elegans*. GLR-1 is a substrate for the E3 Ubiquitin ligase HRDL-1 in ERAD, and it accumulates in the cell when HRDL-1 is non-functional. We characterize location of accumulating GLR-1 in the absence of HRDL-1 with organelle level resolution. By determining the glycosylated state of GLR-1, we find that additional GLR-1 remains in the ER when HRDL-1 is nonfunctional. Our finding suggests that GLR-1 is likely accumulating in a misfolded state when ERAD is impaired.

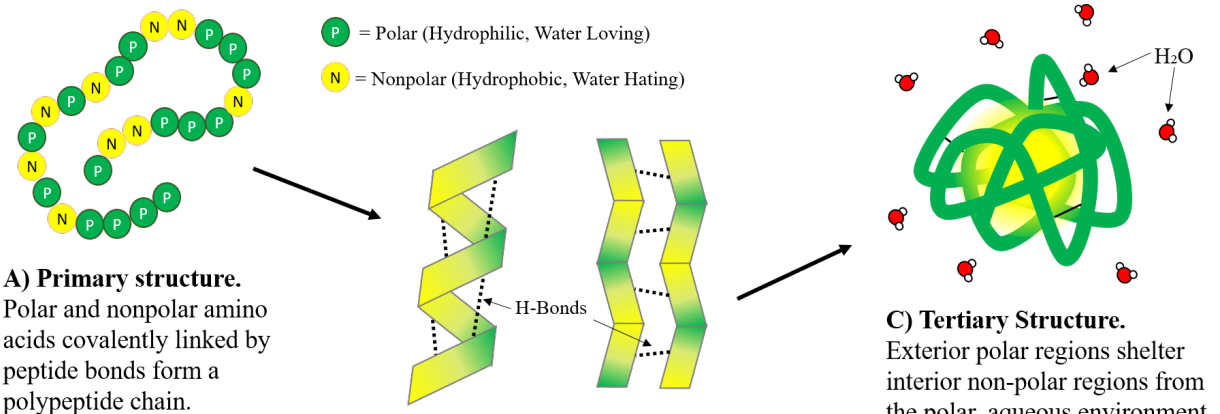
Second, we begin to characterize another branch of the UPS that acts in the nucleus of Eukaryotic cells. A nuclear UPS pathway has also been investigated in *S. cerevisiae*, but a homologous pathway in a multicellular system has not been characterized. Using *C. elegans*, we demonstrate that aggregation prone proteins accumulate in the nuclei of body wall muscle cells, and that nuclear accumulation increases between the second and third day of the worm's life.

Studying protein quality control in *C. elegans* can help us understand how it might function more generally in metazoan systems. Human diseases involving protein aggregation, such as Parkinson's, Alzheimer's, Huntington's, and other neurodegenerative diseases, highlight the importance of protein quality control mechanisms in metazoan systems. Learning about protein quality control in a *C. elegans* may help us understand protein aggregation diseases in humans.

Introduction

Protein Synthesis and Folding

Proteins carry out necessary biological functions in cells. Proteins are made of amino acids covalently linked together via peptide bonds to form long chains called polypeptides. There are 20 commonly occurring amino acids, which can be categorized as polar or non-polar. The sequence of amino acids in the chain is known as the primary structure of the protein (Figure 1A). The pattern of polar and non-polar amino acids in the primary structure leads to secondary structure elements such as α-helices and β-sheets that have polar and non-polar regions (Figure 1B). Nonpolar regions of a nascent protein are driven together by the hydrophobic effect (Figure 2). The protein reaches a single low energy conformation known as the native structure, where hydrophobic residues are hidden from the aqueous environment of the cell (Figure 1C). Other proteins, called molecular chaperones, can also be recruited to direct the polypeptide into its fully-folded state (Figure 3). Unique native conformations allow proteins to have specific functions, allowing cells to accomplish a wide variety of tasks.



A) Primary structure. Polar and nonpolar amino acids covalently linked by peptide bonds form a polypeptide chain.

B) Secondary structures. Hydrogen bonding, dipole-dipole, and other non-covalent interactions between amino acids form secondary structures, including α-helices (left) and β-sheets (right). Regions of polarity (green) and non-polarity (yellow) begin to form.

C) Tertiary Structure. Exterior polar regions shelter interior non-polar regions from the polar, aqueous environment, creating a stable native structure. Disulfide (covalent) bonds help stabilize the tertiary structure of the protein.

Figure 1. Protein structure and folding patterns in an aqueous environment.

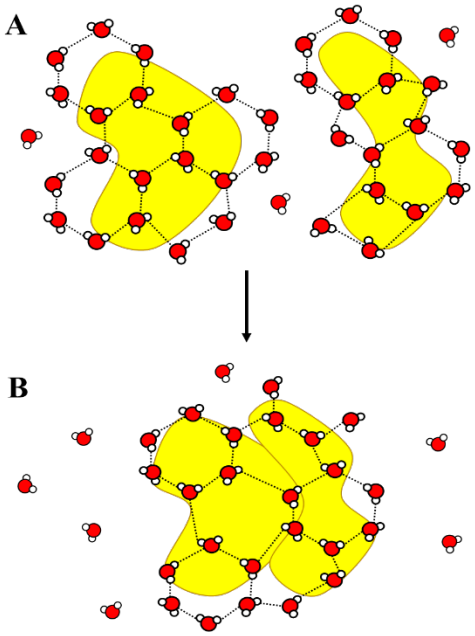


Figure 2. The hydrophobic effect. Clathrate structures made of hydrogen bonds between water molecules surround nonpolar, hydrophobic molecules. (A) Two separate hydrophobic molecules require more water to form the clathrate structure. (B) When two hydrophobic molecules move together, water molecules between them are released. The resulting increase in entropy (disorder) of the aqueous environment is energetically favorable.

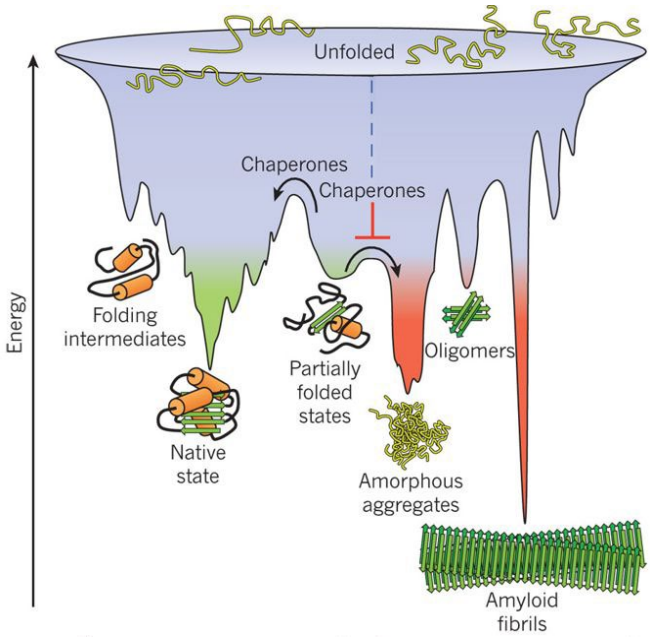


Figure 3. The protein folding funnel. Unfolded, unstable proteins at the top of the funnel fold into lower energy states through a series of intermediate steps. Molecular chaperones help fold proteins into their native state (green tip of the funnel) and prevent proteins from irreversibly forming amorphous aggregates, oligomers, or amyloid fibrils (red tips of the funnel). (Figure adapted from Hartl, Bracher, and Hayer-Hartl 2011).

Proteins Can Become Misfolded

Some synthesized proteins are not folded correctly as a consequence of environmental conditions in the cell, erroneous modification, or mutations in the gene that codes for the protein, among other factors (Pedersen and Heegaard 2013). Up to 30% of synthesized proteins never achieve their naturally folded state and cannot function normally (Schubert et al. 2000). In a misfolded or unfolded state, hydrophobic residues that normally make up the interior of a correctly folded protein are exposed to the aqueous environment of the cell. This is thermodynamically unfavorable and can result in hydrophobic regions of multiple different proteins clumping together in a process called aggregation. The formation of aggregates reduces

contact between hydrophobic residues and the aqueous environment, which is energetically favorable and can make protein aggregates extremely stable structures that are highly resistant to refolding with molecular chaperones (Figure 3).

Aggregation can be extremely toxic to cellular health. Two common human neurodegenerative diseases, Alzheimer's disease (AD) and Huntington's disease (HD), are associated with protein aggregation and demonstrate the serious consequences of protein misfolding. AD is characterized by the loss of cortical brain tissue and presence of amyloid plaques and neurofibrillary tangles, forms of protein aggregation (Ferri et al. 2005). Patients with AD suffer from severe memory loss and lose the ability to carry out everyday tasks. A defining characteristic of HD is the formation of inclusion bodies (protein aggregates) in the nucleus of cells in the central nervous system (Arrasate and Finkbeiner 2012). As a result, HD patients suffer from a loss in motor control and balance. Neurodegenerative diseases are late onset; for example, about 1% of 60 year olds are afflicted with AD, with incidence doubling every five years, so that 30-50% of people over 85 years have AD (Pedersen and Heegaard 2013). AD and HD both exemplify the toxicity of protein aggregation and highlight the importance of protein quality control mechanisms in the cell that remove damaged and misfolded proteins.

The Ubiquitin Proteasome System Degrades Misfolded Proteins

The ubiquitin proteasome system (UPS) is a protein quality control mechanism that is responsible for degrading chronically misfolded proteins in eukaryotic cells. For a misfolded protein to be degraded by the UPS, a small protein called ubiquitin must be covalently added to the misfolded protein via Lysine, or less frequently Serine or Threonine, residues (Figure 4). Adding ubiquitin requires a cascade of reactions involving E1, E2, and E3 ubiquitin ligase proteins. An E1 ligase covalently binds one ubiquitin molecule and activates its C-terminal

Glycine amino acid residue in an ATP dependent manner. This activated form of ubiquitin is then transferred to an E2 ligase, and E3 ligase then catalyzes the transfer of the ubiquitin molecule from the E2 to the target protein (Figure 4). In most organisms, there is one known E1 ligase, dozens of E2 ligases, and hundreds of E3 ligases. Because there is a multitude of E3 ligases that associate with specific target proteins, ubiquitin can be transferred with high specificity. Additional ubiquitin can be added to the misfolded protein in a chain, with each new ubiquitin protein linked to a Lysine amino acid residue on the subsequent ubiquitin (Hershko and Ciechanover 1998). There are 7 different Lysine residues on ubiquitin that can be utilized as links in polyubiquitylation, and specific linkage patterns signal for specific protein fates. The UPS predominantly uses the Lysine-48 linked polyubiquitin chains, which is a signal that the tagged protein must be degraded in the 26S Proteasome (Coux, Tanaka, and Goldberg 1996).

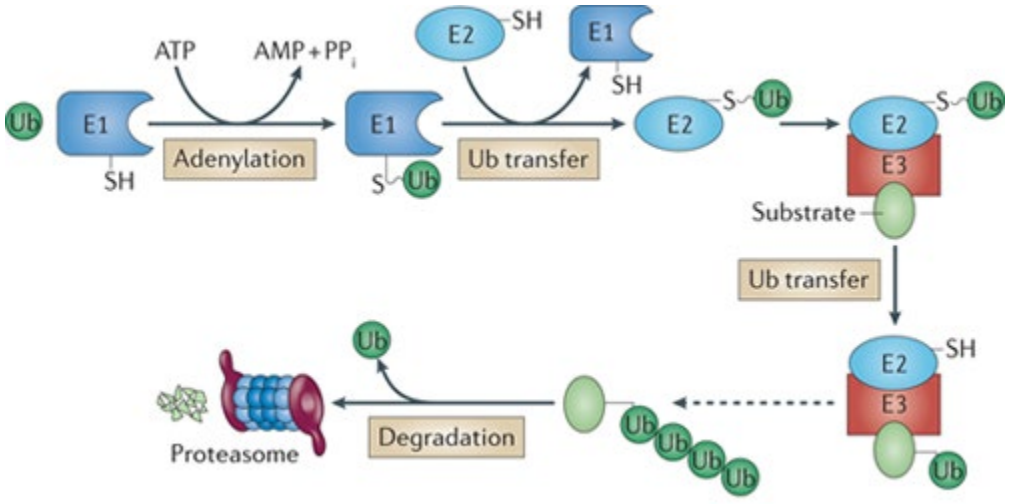


Figure 4. The Ubiquitin Proteasome System. A Cysteine residue on the E1 ligase binds ubiquitin in an ATP-dependent manner. The ubiquitin is then transferred to a Cysteine residue on an E2 ligase. An E3 ligase with its target substrate binds the E2 with ubiquitin, and the ubiquitin is transferred to the misfolded substrate. A polyubiquitin chain with Lysine-48 linkages is a signal for proteasomal degradation. (Maupin-Furlow 2012)

The polyubiquitylated misfolded target protein must be sent to the 26S proteasome for degradation. Although the mechanisms are largely uncharacterized in metazoan systems, the

degradation pathway of ubiquitylated proteins in the Endoplasmic Reticulum (ER) has been researched in yeast. A multi-protein complex that includes cell division control protein 48 (CDC48), ubiquitin fusion degradation protein 1 (UFD1), and nuclear localization protein 4 (NPL4) is involved in extracting polyubiquitylated substrates from the ER membrane and transferring it to the 26S proteasome (Baek, Kim, and Rao 2011; Jarosch et al. 2002; Baek et al. 2013; Wolf and Stolz 2012).

The 26S proteasome itself is comprised of three major particles – one core particle and two regulatory particles. The regulatory particles, which cap the two ends of the cylindrical core particle, are comprised of proteasome regulatory particles (RPNs), some of which de-ubiquitinate the substrate so that ubiquitin molecules can be reused. The inside of the core particle contains 28 protein subunits with catalytic sites that hydrolyze (break) the peptide bonds of the substrate protein into short peptides to be recycled as amino acids, which can be used for protein synthesis or generating metabolic intermediates (Bedford et al. 2010).

Part One: Protein Degradation at the Endoplasmic Reticulum

Background

Transmembrane Protein Maturation - Synthesis and Folding

Transmembrane proteins comprise a diverse class of proteins imbedded in the membranes of a cell. They are important for cell to cell communication by serving as signal receptors, among many other functions. In Eukaryotic cells, membrane proteins are synthesized in the Endoplasmic Reticulum (ER), a membranous organelle that is contiguous with the nuclear membrane. As membrane proteins mature in the ER, they receive modifications to help them to fold and mature correctly. An important modification is the addition of sugar moieties to Asparagine (N) amino acid residues, a process called glycosylation. These tags form a "glyco-code" which displays the folding status, allowing unfolded and folded proteins to be sorted accordingly (Alberts et al. 2002; Amm, Sommer, and Wolf 2014). Specific N-linked glycan tags are unique to proteins found in the ER, although additional glycosylic modifications are made throughout the lifespan of the protein at various locations in the cell. In addition to glycosylation, ER processing includes binding by molecular chaperone proteins to help nascent proteins fold correctly, and the formation of covalent disulfide bonds between Cysteine amino acid residues help stabilize the protein in its folded conformation. Folded, functional membrane proteins leave the ER to further mature in the Golgi Apparatus, and undergo further trafficking to other regions of the cell, including the plasma membrane.

Unfolded Protein Response (UPR)

A variety of stressors can cause protein misfolding or unfolding. One well characterized stress is heat-shock, where an increase in temperature destabilizes the non-covalent bonds in a folded protein and causes it to lose its native conformation. When protein folding and re-folding

in the ER becomes too inefficient, unfolded proteins build up and the unfolded protein response (UPR) is activated. In the ER, three different membrane bound signaling proteins are responsible for detecting high quantities of unfolded protein: the inositol requiring enzyme-1 (IRE1), the PRKR-like ER kinase (PERK), and activating transcription factor-6 (ATF-6) (Bravo et al. 2013)(Figure 5). In unstressed conditions, each sensor is bound to the molecular chaperone BiP (Binding Protein). When misfolded protein concentrations increase in the ER, BiP is released from the luminal (interior) domain of each sensor. This release causes conformational changes in the cytosolic domain of the sensors, activating UPR pathways. Through a variety of molecular interactions, these pathways attenuate overall protein translation rates, cause a genetic response that results in ER expansion, and increase production of chaperone and protein degradation machinery, which helps the cell survive by reducing the concentration of misfolded proteins through refolding or by degrading terminally misfolded proteins (Bravo et al. 2013).

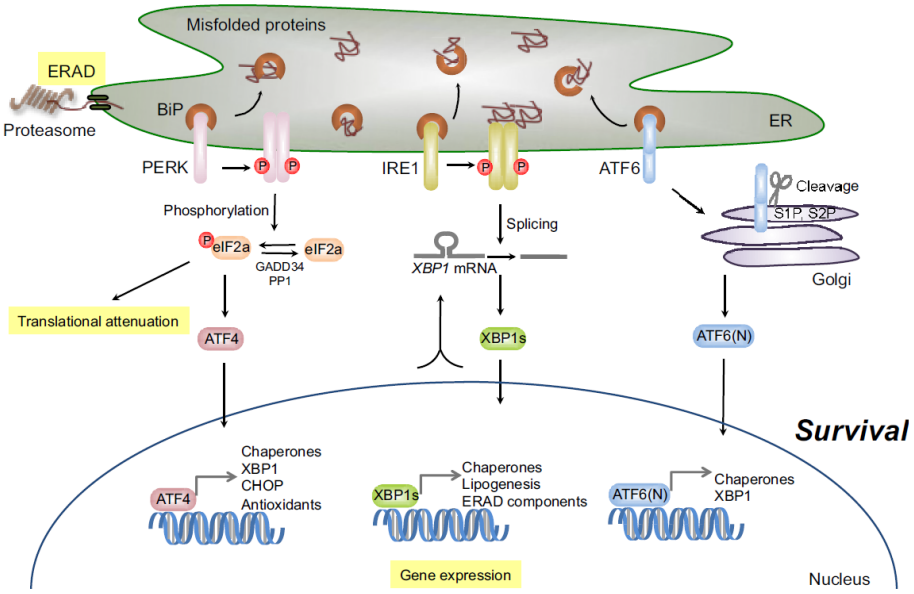


Figure 5. The Unfolded Protein Response. The initiation of PERK, IRE1, and ATF6 ER stress signaling pathways depends on Binding Protein (BiP) dissociation from the luminal domain of each signaling protein. Downstream, potent transcription factors upregulate protein folding and degradation machinery, including ERAD components. (Image from Bravo et. al. 2013)

Endoplasmic Reticulum Associated Degradation (ERAD)

ER associated degradation (ERAD) is an ER-specific branch of the UPS that is activated by the UPR. When BiP chaperones dissociate from the luminal domain of IRE-1 sensors during ER stress, IRE-1 subunits dimerize, and autophosphorylation causes conformational changes that activate an RNA cleaving active site in the cytosolic domain. This site modifies mRNA transcripts that code for potent transcription factors, including XBP-1 that activate the synthesis of ERAD machinery, including E1, E2, and E3 proteins (Kadowaki and Nishitoh 2013) (Figure 5).

ERAD has been well characterized in yeast. The E3 ubiquitin ligase DOA10 functions with E2 ligases UBC6 and UBC7 to polyubiquitylate misfolded substrates using Lysine-48 linkages. CDC48 is involved in the extraction and of DOA10 ubiquitylated substrates from the ER membrane and subsequent translocation to the 26S proteasome for degradation (Weber et al. 2016; Carvalho, Goder, and Rapoport 2006). Another ERAD E3 ligase called HRD1 has also been characterized in yeast. Although both DOA10 and HRD1 function at the ER, they are not functionally redundant. HRD1 targets substrates that are misfolded in their luminal or transmembrane domains, while DOA10 generally targets proteins that are misfolded in their cytosolic domains (Zattas et al. 2016). ERAD has also been studied in human cell culture, though to a lesser extent. MARCH6 is the human ortholog to yeast DOA10, and both MARCH6 and DOA10 both contain a C-terminal element (CTE) that is required for DOA10/MARCH6 to function (Zattas et al. 2016).

***Caenorhabditis elegans* as a model organism**

Although ERAD has been characterized in yeast, a single celled eukaryotic organism, the Dahlberg Lab is interested in knowing how it functions in complex multicellular organisms. To

do this, we have opted to use *C. elegans*, a microscopic nematode worm. The three-day reproductive lifecycle of *C. elegans* is ideal for performing many experiments in rapid succession. In addition, they are non-intensively maintained on a bed of *E. coli* bacteria and agar, and they are predominantly hermaphroditic and capable of self-propagating genetic clones, which is useful for genetic studies.

A useful aspect of *C. elegans* as a model organism is the relative ease of studying cell-type specific biology in living organisms. They have exactly 959 somatic cells, all of which are mapped for tissue type and location, and many of which are well studied (Corsi 2015). *C. elegans* was the first multicellular organism to have its genome completely sequenced, and 60-80% of human genes have an ortholog in *C. elegans*, so research using *C. elegans* has relevance to human biology (Corsi 2015). Specifically, the proteins relevant to this study have orthologues in humans, allowing us to infer potential protein-protein interactions in humans based on our findings in *C. elegans* (Table 1). Many cutting-edge molecular biology tools that are used in yeast research can also be used on *C. elegans*. Their transparent cuticle allows for effective microscopy using fluorescently-labelled fusion proteins and reporter genes, and current genomic editing tools like CRISPR can be used for genetic experiments. In addition, RNA channels in the gut of *C. elegans* allows researchers to conduct RNA interference (RNAi) experiments (Corsi 2015).

	Orthologous Protein		
Function	**Yeast (*S. Cerevisiae*)**	***C. elegans***	**Humans (*H. sapiens*)**
E3 Ligase (ERAD)	HRD1 DOA10	HRDL-1 HRD-1 MARC-6	Gp78 HRD1 MARCH6
E2 Ligase (ERAD)	UBC6 UBC7	UBC-6 UBC-7	UBE2J1 UBE2G1
Glutamate Receptor	--	GLR-1	GLUR1
Membrane Protein Extraction	CDC48	CDC48.1 CDC48.2	p97

Table 1. Protein homology in yeast, *C. elegans*, and humans. Data organized from www.wormbase.org.

Our lab has begun to characterize ERAD in a multicellular organism, using *C. elegans*. We have chosen to study the well characterized membrane protein GLR-1 as an ERAD substrate. GLR-1 is a transmembrane glutamate receptor that is essential for food seeking behavior and is expressed in a subset of interneurons in *C. elegans*. Prior to my work, the Dahlberg Lab had already begun to characterize how GLR-1 is regulated by ERAD. We have studied the quantity and localization of GLR-1 by using genetic tools to fuse it Green Fluorescent Protein (GFP), creating a single polypeptide chain (GLR-1::GFP). This allows us to use fluorescent microscopy to visualize GLR-1 in living *C. elegans* and to conduct biochemical assays which help us quantify GLR-1 abundance.

The E3 ligase HRDL-1 appears to be involved in the polyubiquitylation of GLR-1. Microscopic and biochemical analysis suggest that GLR-1 abundance increases when functional HRDL-1 is not present (Witus 2016, unpublished). This suggests that GLR-1 may be a substrate

for HRDL-1 in ERAD. However, it remains unclear if the additional GLR-1 in *hrdl-1* mutant animals is misfolded or if it resides at the ER.

Research Question:

The purpose of this study is to identify the location of GLR-1 aggregation in the absence of functional HRDL-1 with organelle level resolution. We hope to distinguish if GLR-1 is accumulating in the ER, or if it is allowed to leave the ER and accumulate elsewhere.

Hypothesis:

ERAD functions to degrade misfolded proteins in the ER, so we hypothesize that disrupting the GLR-1 ERAD pathway would result in GLR-1 accumulation at the ER, likely in a misfolded state. In order for a protein to be exported from the ER, it needs to display an ER exit signal that can be recognized by proteins associated with COP vesicles (Alberts et al. 2002). These signals include specific glycosylations that indicate the protein is correctly folded. A correctly folded protein will receive glycosylation modifications and be exported to continue maturation at the Golgi Apparatus, where it receives different glycosylations. We think that we will find ER-type glycosylations on a larger proportion of GLR-1 in *C. elegans* with no functional HRDL-1, which would suggest that accumulated GLR-1 is in the ER membrane and likely misfolded.

Methods

Worm Strains

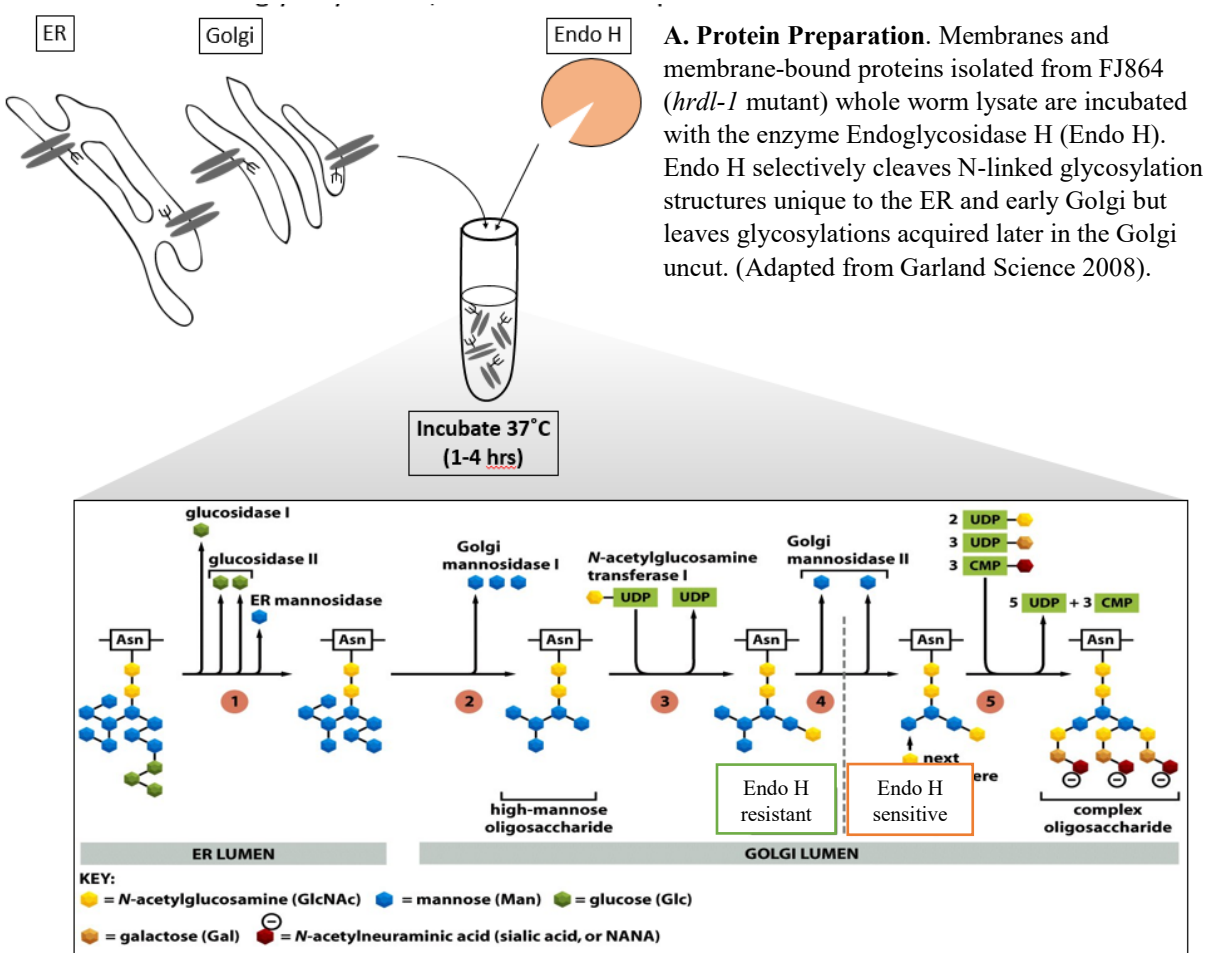
FJ354: *glr-1::gfp*(nuIs24)(IV)

This strain is a control for GLR-1 abundance with normal (wild-type, WT) ERAD function. It also contains an integrated transgene of *glr-1* with a near C-terminal fusion to green fluorescent protein (GFP), which we use as an antigen in a western blot assay (described below).

FJ864: *glr-1::gfp*(nuIs24)(IV);*hrdl-1*(gk28)(I)

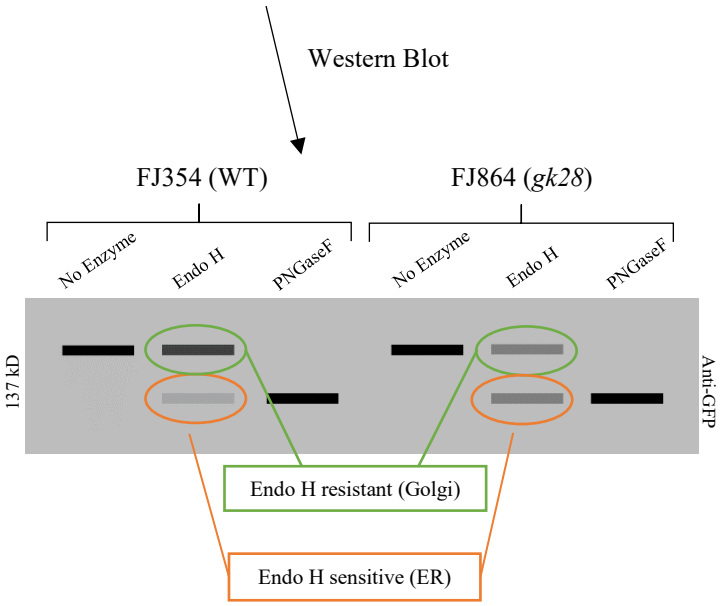
This strain contains a 1981bp deletion in the *hrdl-1* gene. This significant deletion effectively knocks out HRDL-1, disrupting the ERAD pathway for GLR-1. This strain also contains the integrated transgene *glr-1::gfp*, allowing it to be detected with the same antibodies as the control strain in a western blot assay.

Figure 6. Experimental Overview



A. Protein Preparation. Membranes and membrane-bound proteins isolated from FJ864 (*hrdl-1* mutant) whole worm lysate are incubated with the enzyme Endoglycosidase H (Endo H). Endo H selectively cleaves N-linked glycosylation structures unique to the ER and early Golgi but leaves glycosylations acquired later in the Golgi uncut. (Adapted from Garland Science 2008).

B. Protein Detection. The Endo H treated membrane proteins are then denatured, separated by molecular weight with SDS-PAGE, and transferred to a nitrocellulose membrane (western blot). Using a sequence of antibodies that are (1) anti-GFP and (2) chemiluminescent, GLR-1::GFP protein can be visualized and quantified. Performing the procedure in parallel with FJ354 (WT) whole worm lysate provides a basis for relative quantification. Negative (no enzyme) and positive (PNGaseF) glycosylation-cleaving control reactions flank Endo H treated membrane proteins. The upper (un-cleaved) to lower (cleaved) band density ratio in the Endo H treated GLR-1::GFP can be compared to analyze the relative amount of GLR-1 at the ER membrane.



To determine if GLR-1 is accumulating at the ER when a *hrdl-1* mutation compromises the GLR-1 ERAD pathway, we analyzed the glycosylated state of GLR-1 proteins in worms with and without a *hrdl-1* mutation. The glycosylation patterns of proteins at the ER are unique compared to those on proteins that have left the ER and continued to mature. Given this, we adapted a three-part protocol: (1) Isolate the membrane proteins from a population of worms, (2) digest the membrane proteins with a selective glycosidase enzyme, and (3) use a Western blot to visualize and quantify GLR-1::GFP (Figure 6) (original protocol adapted from Chun et al. 2008).

Membrane Isolation

Worms grown in a liquid culture are lysed with freeze-thaw cycles. The lysis solution is treated with sonication to break open the membranes of cells. A low velocity spin eliminates worm cuticle (protective outer layer) and any un-lysed material, followed by a high velocity spin that separates membrane with membrane-bound proteins from cytosol and cytosolic components.

Glycosidase Digest

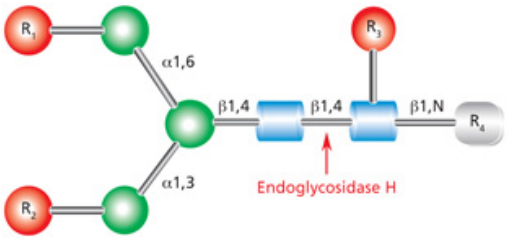


Figure 7. Endoglycosidase H cleaves β(1,4) linkages between two GlcNac subunits near the base of ER-type the glycosylations. (Figure from Sigma-Aldrich)

Because glycosylation modifications given to proteins at the ER membrane are modified as proteins move to other organelles, detection of ER specific modification can describe where a protein was at the time of lysis. Endoglycosidase H (Endo H) is an enzyme that recognizes and shortens (cleaves) only N-linked glycosylations on proteins present in the ER, changing their overall molecular weight (Figure 7). Thus, GLR-1 proteins with ER modifications will have a smaller molecular weight than other GLR-1 proteins (at the Golgi, cell membrane, etc.) after the Endo H digest (Figure 6B).

Western Blot

A Western blot is used to visualize and quantify specific proteins from the lysate. Endo H treated membrane proteins are solubilized with Sodium Dodecyl Sulfate (SDS), which associates with hydrophobic regions on proteins and negatively charges them, and beta-mercaptoethanol (βME) which reduces (breaks) disulfide bonds. This denatures the tertiary and secondary structure of the membrane proteins, breaking them down to their primary structure. The denatured, negatively charged membrane proteins are loaded into a polyacrylamide gel, and an electric current is passed through the gel, causing them to migrate with the current. Smaller proteins migrate through the gel matrix faster than large proteins, separating all membrane proteins by size. The membrane proteins are then transferred from the gel and onto a nitrocellulose membrane. To detect the GLR-1::GFP fusion proteins on the membrane, anti-GFP antibodies are washed over the membrane and attach to GFP. A secondary antibody with chemiluminescent tags attach to the first antibody, and the protein can be visualized and quantified (Figure 6B).

Results

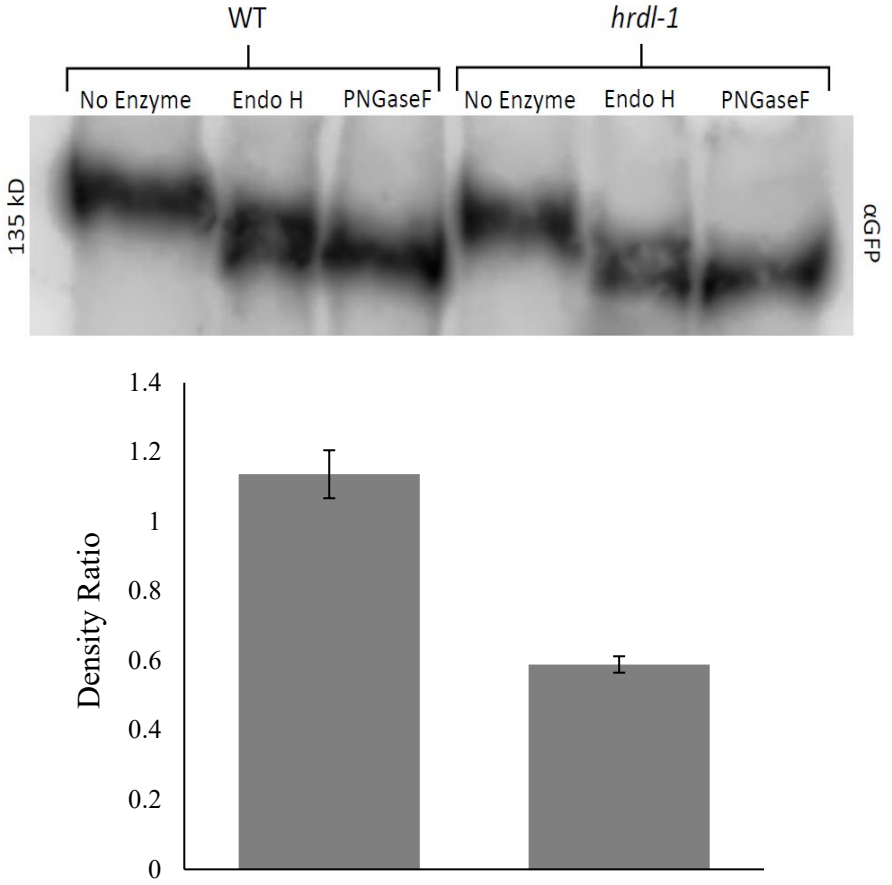


Figure 8. Western blot probed with α-GFP antibody to detect GLR-1::GFP fusion protein in WT (FJ354) and *hrdl-1* mutant (FJ864) worm strains. Quantification of the Endo H resistant to Endo H sensitive protein ratio reveals that a greater proportion of GLR-1::GFP is found at the ER membrane in *hrdl-1* mutants compared to WT (n=2).

To quantify my western blot data, I measured the pixel intensity of the upper (Endo H resistant) and lower (Endo H sensitive) bands in the Endo H treated lanes and found the upper to lower band density ratio. I found that a higher proportion of GLR-1::GFP protein is sensitive to Endo H cleavage in *C. elegans* without functional E3 ubiquitin ligase HRDL-1 (*WT* SD=0.069, *hrdl-1* SD=0.024, *n*=2). GLR-1::GFP is only sensitive to Endo H when it has ER-type glycosylations; thus, I find that a higher proportion of GLR-1::GFP is present in the ER compared to the rest of the cell when the *hrdl-1* gene is mutated.

Discussion

The current model predicts that ERAD substrates will remain at the ER when ERAD is compromised. Thus, I predicted that GLR-1, an ERAD substrate, would remain at the ER when the E3 responsible for targeting misfolded GLR-1 for degradation, HRDL-1, is non-functional. The data I collected indicates that a higher proportion of GLR-1 has ER glycosylations in *C. elegans* without functional HRDL-1, suggesting that more GLR-1 resides at the ER when ERAD is compromised. This data supports the current model.

The additional GLR-1 protein in *hrdl-1* mutants is likely misfolded. The Dahlberg lab conducts behavioral assays using worms with E3 mutations. One phenotype associated with a *glr-1* mutation in *C. elegans* is a decrease food seeking behavior. In the absence of diacetyl, an odorant released by their food source, worms will reverse and turn another direction to continue food seeking. This is called a "reversal," and the frequency of reversals depends on glutamatergic signaling in the interneurons using GLR-1. *Glr-1* mutants reverse less often compared to wild-type worms. Worms without functional HRDL-1 have slightly lower reversal rates rather than an increased reversal rate, even though we find that GLR-1 is more abundant in these animals (Chapman, unpublished; Witus, unpublished). Combined with my finding that additional GLR-1 resides at the ER and not at the synapse in worms without functional HRDL-1, we hypothesize that additional GLR-1 is likely misfolded.

Through studying ERAD in the nervous system of *C. elegans*, a Eukaryotic multicellular organism, we can begin to understand how protein degradation systems function in higher eukaryotes, such as humans. Up to 30% of synthesized proteins never achieve their naturally folded state and cannot serve their designated purpose (Schubert et al. 2000). If not degraded, misfolded proteins can form toxic protein aggregates in the cell which may lead to cell death.

This phenomenon is observed in neurodegenerative diseases like Alzheimer's and Huntington's. Ongoing and future studies will further characterize the GLR-1 ERAD pathway by investigating the effects of E2 ligase *ubc-6* and *ubc-7* mutations on the localization and quantity of GLR-1 in interneurons. Additionally, the Dahlberg lab has begun studies of GLR-1 ERAD regulation under ER stress with high rates of protein misfolding. By increasing the rate of protein misfolding at the ER, we can determine which proteins appear to have the most important role in ER stress response.

Part Two: Protein Degradation at the Nucleus

Background

The UPS acts at the Nucleus

While endoplasmic reticulum (ER)-associated degradation pathway has been well characterized, the ubiquitin-proteasome system (UPS) of the nucleus has only recently been characterized in yeast. Dr. Rich Gardner and his lab at the University of Washington discovered a degradation pathway that relies on the E3 ubiquitin ligase San1 to identify and ubiquitinate misfolded proteins at the nucleus (Gardner, Nelson, and Gottschling 2005). Like E3 ubiquitin ligases that act at the ER, San1 contains a RING (Really Interesting New Gene) domain that catalyzes the transfer of ubiquitin from E2 ubiquitin conjugating enzymes to the misfolded protein. Unlike ER-associated E3s, the San1 RING domain contains a nuclear localization sequence (NLS) that is necessary and sufficient for San1 to be localized to the nucleus (Gardner, Nelson, and Gottschling 2005) (Figure 9).

Figure 9. The San1 protein RING domain. The larger black letters represent Cysteine and Histidine amino acid residues that bind Zinc ions for conformational stability. The large gray letters represent the bipartite nuclear localization sequence, while the entire sequence is underlined (Figure adapted from Gardner, Nelson, and Gottschling 2005)

In ERAD, Cdc48 is required to extract membrane proteins from the ER membrane due to the insolubility of the transmembrane domains of the substrate. In the nucleus, Cdc48 is necessary to relocate some, but not all, San1 targeted misfolded proteins to the nuclear proteasome; the more insoluble the misfolded protein is, the more dependent it is on Cdc48 for

translocation to the proteasome (Gallagher, Clowes Candadai, and Gardner 2014). San1 recognizes misfolded proteins based on the length of exposed hydrophobic sequences. A sequence of five hydrophobic amino acid residues or longer is sufficient for San1 recognition, and shorter sequences are less efficiently recognized and degraded (Fredrickson, Gallagher, et al. 2013).

Identifying a San1 Analogous Metazoan PQC Mechanism

The primary structure of San1 is poorly conserved, so using virtual bioinformatics search tools to identify orthologues in other organisms in is largely inefficient. However, potential candidates for San1 orthologues in humans were identified using two unique characteristics: (1) San1 has comparatively few Lysine amino acid residues outside of its RING domain, and (2) San1 is highly disordered outside of its RING domain (Fredrickson, Clowes Candadai, et al. 2013; Boomsma et al. 2016)(Figure 10). Ten possible candidates for San1 orthologues have also been suggested in *C. elegans* using these two parameters (Rich Gardner, personal communication). The identified San1 orthologues have not yet been tested for involvement in PQC activity in the nucleus.

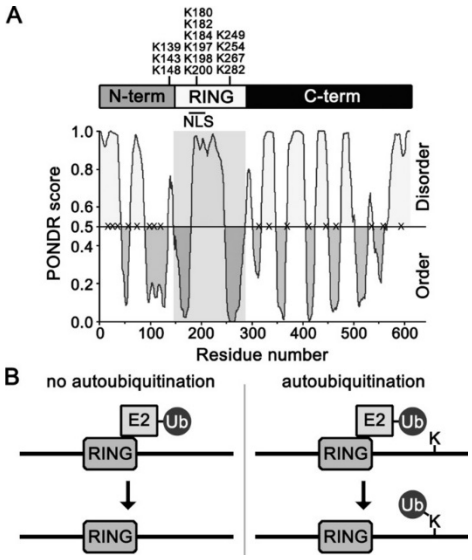


Figure 10. (A) San1 is highly disordered in the N- and C- terminal regions, making sequence homology very low between potential orthologues. However, the high degree of disorder itself can be used to identify potential orthologues. San1 contains very few Lysine (K) residues outside of the RING domain, which is another defining characteristic that is used to find orthologues. **(B)** Adding Lysine (K) residues into the C- or N- terminal regions of San1 can cause autoubiquitination, where San1 transfers ubiquitin to itself rather than the target protein, resulting in San-1 degradation rather than substrate degradation. (Figure from Fredrickson et al. 2013)

Based on biochemical evidence from the yeast San1 pathway, San1 substrate polypeptides were engineered in order to test potential San1 orthologues in human cells. These polypeptides include a chain of Isoleucine amino acid residues, a nuclear localization sequence (NLS), and green fluorescent protein (GFP). Isoleucine is non-polar and highly insoluble, so attaching a chain of it to GFP^{NLS} makes the polypeptide aggregation prone which signals for degradation of the protein. The NLS causes the protein to be translocated to the nucleus, and GFP allows the construct to be visualized. This substrate is degraded in the nucleus of human cells, but the proteins involved in the pathway have not been identified (Morimoto and Shibata, unpublished).

In human patients, symptoms of neurodegenerative disease becomes more common with age (Pedersen and Heegaard 2013). This suggests that PQC becomes less effective with age, either because protein misfolding increases or because PQC mechanisms function more poorly

over the lifespan of an individual. This age-dependent phenotype should also occur in a model system if the molecular mechanisms are conserved.

Research Question / Rationale

The primary goal of this study was to engineer a model system in which we can study nuclear PQC mechanisms. Using *C. elegans*, a multicellular eukaryotic organism, may help us understand how it functions in higher Eukaryotes (such as humans). Through this model, we investigated if (1) a San1 substrate behaves similarly in *C. elegans* as it does in a yeast model, and (2) if nuclear PQC shows significant loss of efficacy over the lifespan of *C. elegans*. Human neurodegenerative diseases typically have late onset; for example, Alzheimer's disease incidence is about 1% in 60-year-olds and doubles every 5 years, demonstrating that PQC mechanisms may become less effective over time (Pedersen and Heegaard 2013). If nuclear PQC mechanisms are conserved from *C. elegans* to humans, we predict that protein homeostasis in the nucleus will become worse over time. The Dahlberg Lab's research on ER-associated degradation provides evidence that PQC mechanisms at the ER are conserved from yeast to *C. elegans*. In addition, PQC mechanisms in the mitochondria and ER are conserved across many eukaryotic organisms, including yeast and humans (Bohovych, Chan, and Khalimonchuk 2015; Ruggiano, Foresti, and Carvalho 2014). Thus, it is reasonable to predict that a nuclear protein quality control mechanism is also conserved from yeast to *C. elegans*.

Methods

Making a Model for Nuclear PQC

We used the same blueprint for a San1 substrate engineered polypeptide sequence used in yeast to generate a *C. elegans* analog. Using a circular piece of DNA (plasmid), we placed the sequence for the San1 substrate under the control of a myosin promoter that is active in the body wall muscle of *C. elegans* (Figure 11).

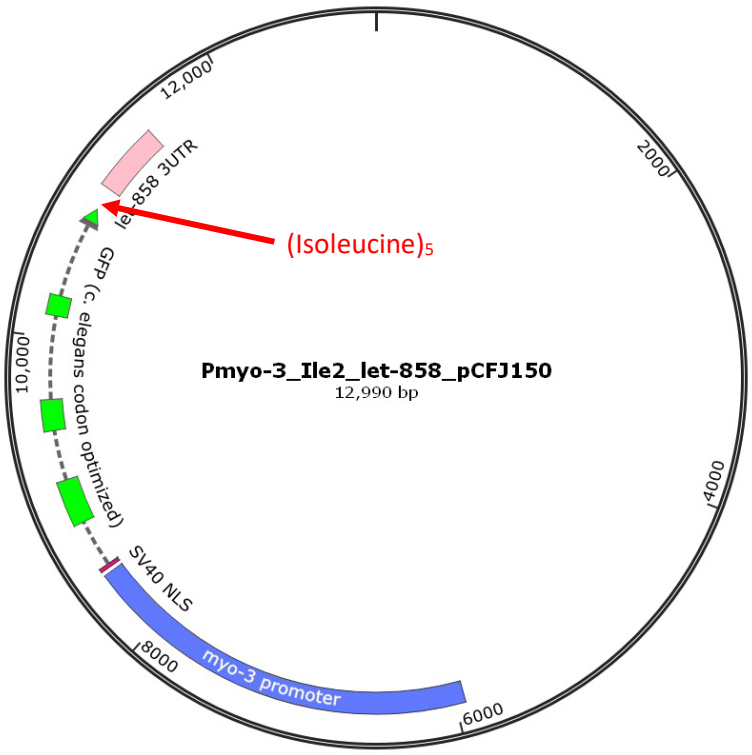


Figure 11. Plasmid map. The *myo-3* promoter is shown in blue, just upstream of the SV40 NLS, a viral nuclear localization sequence. GFP exons are shown in green, and the location of 5 Isoleucine codons at the terminus of the GFP sequence are indicated with the red arrow.

This method of genetic engineering in model organisms is extremely common. A promoter is a sequence of DNA to which transcription factors bind and regulate the transcription of the downstream gene. As a result, select genes are actively transcribed in specific cell types. The myosin-3 protein is an isoform of myosin, a protein necessary for muscle contraction that is expressed in the body wall muscle of *C. elegans* (Okkema et al. 1993). By putting our construct downstream of the myosin-3 promotor, we can express our protein in the body wall muscle of *C. elegans*. The plasmid DNA is injected into the gonad of an adult worm, which allows it to be passed on to future generations (Evans 2006) (Figure 12). Dr. Irini Topalidou at the University of Washington completed the microinjection of our plasmid.

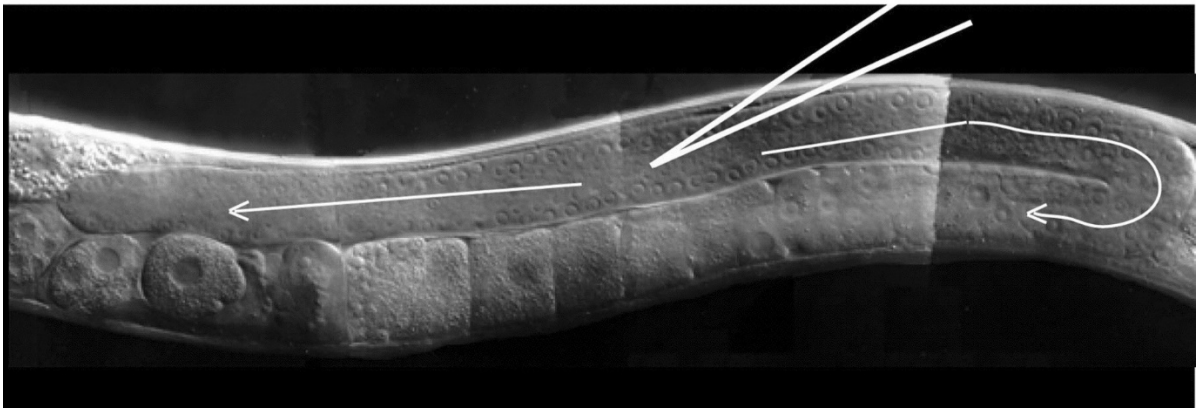


Figure 12. Microinjection of Plasmid DNA into the distal arm of the gonad in *C. elegans*. The white point represents the tip of the needle, and the arrows show the flow of injected DNA. At the injection site, there is not yet a plasma membrane surrounding developing egg cells, so the plasmid can diffuse into nuclei with a higher success rate (Image from Evans 2006)

Plasmid DNA is extrachromosomal, meaning it is not included in the genomic, chromosomal DNA of the organism. Because the plasmid is not part of the genome, it has variable inheritance. Thus, some offspring inherit many copies of the plasmid, while a fraction of offspring inherit no copies. To mitigate this, we integrated our plasmid DNA into the genome of *C. elegans*. We used UV light to induce double-stranded DNA breaks in the genomic and plasmid DNA. Naturally occurring DNA repair pathways then integrate the plasmid DNA into

the genome (Evans 2006) (Figure 13). Once the plasmid is integrated into the genome, it is reliably passed to offspring through chromosomal DNA; offspring receive the same number of copies of the plasmid in every cell.

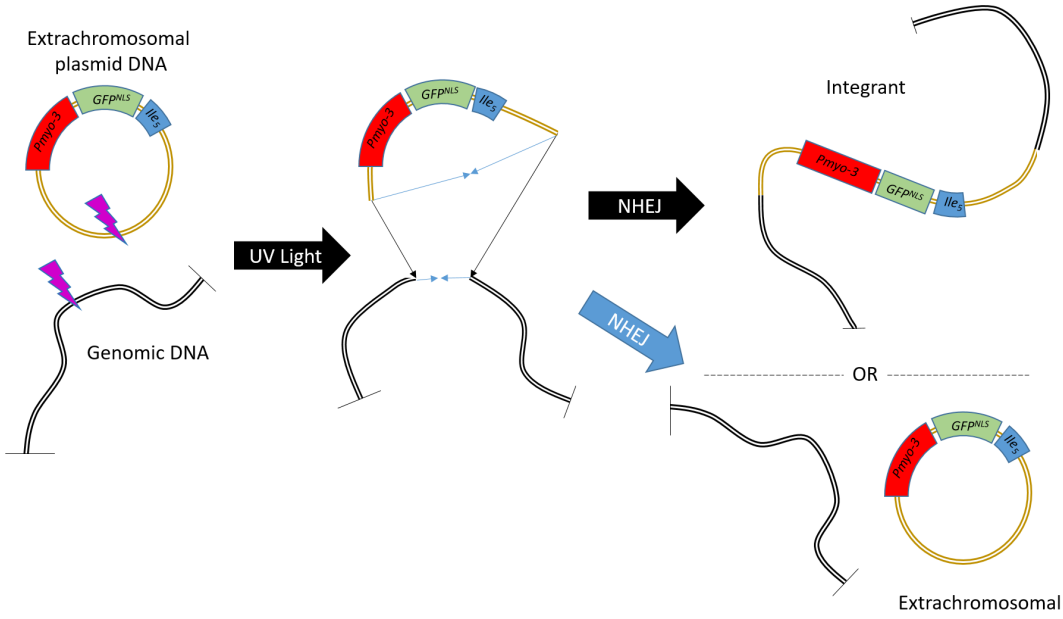


Figure 13. Plasmid integration. UV light is directed at a population of worms containing the plasmid, causing double-strand DNA breaks in both the plasmid DNA and genomic DNA in the nuclei of the worm. The non-homologous end joining (NHEJ) DNA repair pathway can either (1) rejoin genome-genome double strand breaks (non-integrant), or (2) join the plasmid and genome double stranded breaks, integrating the plasmid DNA into the genome of the worm.

The probability that DNA plasmid will successfully integrate into the genomic DNA of *C. elegans* is low, so a large scale screen must be conducted to find "integrant" worms. UV exposed worms are picked individually onto their own plate and allowed to self-fertilize, creating genetic clones of themselves. All offspring of an integrant parent will express the genetic construct from the plasmid DNA, while a non-integrant parent will pass the un-integrated plasmid to some (but not all) of the offspring. After screening dozens of potential integrants, I identified one worm that contained a successfully integrated plasmid.

We followed the same procedure to integrate a second plasmid with GFP^{NLS} linked to a Phenylalanine repeat instead of an Isoleucine repeat. Phenylalanine is another non-polar, hydrophobic amino acid, and the repeat sequence was also used in yeast and human cell studies to test for San1 orthologues (Fredrickson, Gallagher, et al. 2013). However, I failed to identify an integrant strain of worms, yet found a strain with high penetrance – a high rate of inheritance from parent to offspring – that I also continued to test for an aging phenotype, as described below. Because the strain wasn't a true integrant, the data is less reliable and is not included in this manuscript.

Testing for Age-Dependent PQC Efficiency

To test if nuclear PQC becomes less effective over time, we use fluorescent microscopy and western blotting. To ensure all worms we use are the same age in each age group, I synchronized the lifecycle of a population of worms. Age synchronization is done by moving 10 egg-laying adult worms to a new plate, allowing them to lay eggs for 4 hours, then removing the adults from the plate. The result is a population of offspring that are within 4 hours of the same age. I imaged the heads and tails of 2, 3, and 6 day old worms and quantified the appearance of green fluorescent nuclei in the body wall muscle. A worm with a functioning nuclear PQC mechanism should have fewer green nuclei, as its cells are able to degrade the green glowing aggregation prone polypeptide (GFP^{NLS}::Ile_5) efficiently. A worm with poorly functioning nuclear PQC will have more green nuclei because its cells are unable to degrade the aggregation prone polypeptide.

Results

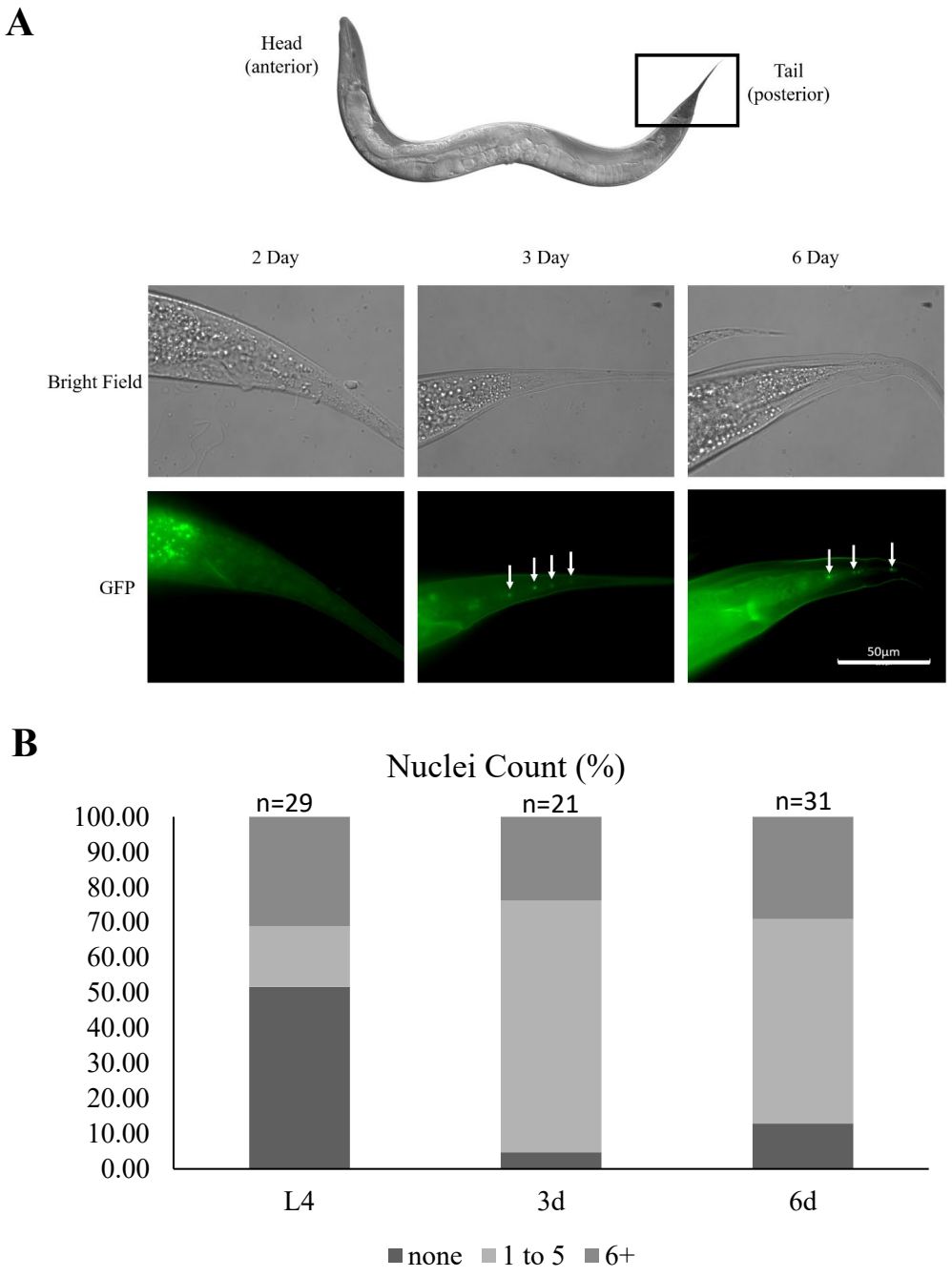


Figure 14. Age-dependent nuclear accumulation of SAN1 substrate Ile_5::GFP^{NLS}. (**A**) Representative images depicting tails of *C. elegans* with integrated Ile_5::GFP^{NLS} from each age group. Arrows indicate GFP fluorescent nuclei. (**B**) Images were analyzed for number of GFP-fluorescent nuclei (n= images analyzed), then grouped into categories based on number of nuclei observed (no nuclei, between 1 and 5 nuclei, and 6+ nuclei). Graph indicates percentage of total images in each category.

We found that the number of nuclei with 1-5 GFP^{NLS}::Ile_5 inclusions increased between images of 2 and 3 day old worms, but not between images of 3 and 6 day old worms (Figure 14B). Additionally, the number of images showing no nuclear GFP^{NLS}::Ile_5 inclusions decreased sharply between 2 and 3 day old worms. Combined, this data suggests that the majority of worms develop nuclear inclusions of GFP^{NLS}::Ile_5 between 2 and 3 day old worms.

Discussion

Imaging data suggests that the extent of nuclear accumulation of GFP^{NLS}::Ile_5 is age dependent, suggesting that we have created a model to study nuclear PQC within an aging paradigm. Although there were worms with many (6+) and no nuclear inclusions of GFP^{NLS}::Ile_5 in each age group, we find that more worms developed nuclear inclusions between 2 and 3 days of age (Figure 14). It is not clear if this increase is caused by overloading properly functioning nuclear PQC systems, or if nuclear PQC mechanism are beginning to malfunction. The lifespan of *C. elegans* is about two weeks; by imaging worms that are closer to the end of their lifespan (e.g. 10-14 days of age), we may see another increase in GFP^{NLS}::Ile_5 nuclear accumulation that is a result of the nuclear PQC mechanism malfunction. This might contribute more useful understanding of human age-related aggregation diseases.

Although the nuclear localization sequence in GFP^{NLS}::Ile_5 should cause the polypeptide to localize in nucleus, we seek to develop a nuclear reporter to visualize the body wall muscle cell nuclei. This will (1) confirm that the accumulations we see are in nuclei and (2) characterize the accumulation in nuclei. While imaging, it appeared that some nuclei were diffuse with GFP^{NLS}::Ile_5, while others had smaller, brighter puncta. This may be because some nuclei were just out of the plane of focus, causing the GFP fluorescence to appear diffuse, or that some nuclei

have more defined GFP^{NLS}::Ile_5 inclusions than others. To test this, we could express a fluorescently tagged histone in the nuclei of the body wall muscle cells. Histones are DNA binding proteins that help organize and regulate expression of chromosomal DNA in the nucleus. If we fluorescently label histones, we will be able to visualize the nuclei of the worm, which will help further characterize GFP^{NLS}::Ile_5 nuclear accumulation. If GFP^{NLS}::Ile_5 protein and fluorescently labelled histones overlap in the same image, it provides evidence that GFP^{NLS}::Ile_5 is in the nucleus. The histone marker will also help determine which nuclei are in focus, which will allow us to determine if some nuclei have more diffuse GFP^{NLS}::Ile_5 than others.

A western blot analysis can also be done to quantify the total amount of GFP^{NLS}::Ile_5 in each age group. If biochemical analysis suggests that GFP^{NLS}::Ile_5 abundance increases from 2-day to 3-day-old worms, it would agree with my microscopic imaging data, which suggests that GFP^{NLS}::Ile_5 nuclear accumulation increases after the third day.

GFP^{NLS}::Ile_5 is a model substrate for San1 mediated degradation, and it has been used in human cells to characterize yeast and human San-1 orthologues (Fredrickson, Gallagher, et al. 2013; Morimoto and Shibata, unpublished). Future studies will determine if San1 orthologues are important for GFP^{NLS}::Ile_5 degradation in *C. elegans* using RNA interference (RNAi). The *C. elegans* genome contains 152 proteins with RING domains, ten of which have some homology to San1 (Kipreos 2005). We will knock out the expression of each protein using RNAi and determine which knockouts cause an increase of nuclear accumulation of GFP^{NLS}::Ile_5. Previous studies indicate that Cdc48 plays a role in nuclear degradation of highly insoluble misfolded proteins in the nucleus of yeast (Gallagher, Clowes Candadai, and Gardner 2014). We will knock out both copies of Cdc48 orthologues in *C. elegans* (*cdc48.1* and *cdc48.2*) using RNAi and determine if either play a role in nuclear protein quality control for GFP^{NLS}::Ile_5.

Summary

Cells need proteins to carry out molecular functions, but proteins can become damaged or misfolded. Misfolded proteins can become toxic to cells if they accumulate and aggregate, which is evident in human neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's, among others. The Ubiquitin Proteasome System (UPS) is a conserved pathway that degrades terminally misfolded proteins, preventing aggregation and disease.

In the first part of my thesis work, I further characterized Endoplasmic Reticulum Associated Degradation (ERAD), a UPS pathway that acts in the Endoplasmic Reticulum (ER) of eukaryotic cells. The Dahlberg Lab had already begun to characterize the ERAD pathway regulating GLR-1, a glutamate receptor in the neurons of *Caenorhabditis elegans* that is necessary for reversal behavior. GLR-1 is a substrate for the E3 Ubiquitin ligase HRDL-1 in ERAD, and it accumulates in the cell when HRDL-1 is non-functional (Witus, unpublished). One would expect that an increase in GLR-1 abundance might cause worms to have increased reversal behavior. However, behavioral analysis suggests that worms without functional HRDL-1 reverse less often than wild-type, suggesting that accumulating GLR-1 is non-functional (Chapman, unpublished). My work connects these two observations by suggesting that the additional GLR-1 in worms without functional HRDL-1 is not localized to the synapse where it functions, but is held at the ER, suggesting it may be misfolded. Altogether, our work demonstrates that impairing ERAD causes a misregulation of important membrane proteins and impairs cell function.

In the second part of my thesis work, I began to characterize a metazoan protein quality control (PQC) mechanism that acts in the nucleus. A nuclear UPS pathway has been investigated in *S. cerevisiae* (yeast) that requires the E3 San1, but a homologous pathway in a multicellular

(metazoan) system has not been characterized. Using an aggregation prone target protein that is a known target of a nuclear UPS in yeast (GFP^{NLS}::Ile_5), I helped generate a *C. elegans* model to study metazoan nuclear PQC. With this model, I found evidence that GFP^{NLS}::Ile_5 accumulates in the nuclei of body wall muscle cells, and that nuclear accumulation increases between the second and third day of the worm's life. These finding suggest that there is a nuclear PQC mechanism in the nucleus of *C. elegans* that functions similarly to nuclear PQC in yeast, and future work will use this model to continue research on proteins involved in the mechanism.

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Supplementary Materials: Protocols

Membrane Preparation Protocol:

1. Grow up 10-12 plates of worms, until plates are full but not starved. If starting with worm powder from liquid culture, skip to step 5.
2. Wash 3 plates of worms serially with 1mL M9. Centrifuge at 4k RPM and pull off M9, being careful not to pipette worms. Repeat until all plates are washed. Do a final rinse of the pellet by adding 1.5mL M9 directly to the tube. After final spin, put the microfuge tube on ice to consolidate worm pellet, and pull off all M9.
3. Add 100µL of freezing buffer (1:200 PMSF, 1:100 PI). Freeze in liquid nitrogen.
4. Using a mortar and pestle, grind the frozen pellet into a fine powder, making sure to keep the worm powder frozen throughout the process. Put powder back into the microfuge tube, re-freeze in liquid nitrogen. This can be stored at -80˚C, or continue on to step 5.
5. Thaw worm powder on ice, then add Buffer A (1:200 PMSF, 1:100 PI) for a total volume of 225µL.
6. Sonicate for 10 seconds at setting 3-4, then rest on ice for 1 minute. Repeat 5 times.
7. If needed, add Buffer A (1:200 PMSF, 1:100 PI) to 250µL.
8. Centrifuge at 7k RPM for 12 minutes at 4˚C.
9. Carefully take the supernatant and pipette into an ultracentrifuge tube. Balance tubes to be within 500µg of each other.
10. Centrifuge using Rotor 3 (AT120) at 82k RPM (303k rcf max) for 40 minutes at 4˚C.
11. Completely remove the liquid supernatant, and carefully obtain the waxy pellet. Dounce the pellet into 100µL Buffer A (1:200 PMSF, 1:100 PI, 1:100 DTT) and pipette into a microfuge tube.

Endoglycosidase H Protocol:

1. Add 1.5 volumes of 2X Denature Buffer to P2 membranes.
2. Denature at 90˚C for 10 minutes.
3. Add 5 volumes of enzyme buffer to appropriate tubes:
    a. Endo H: 1X Glycobuffer 3
1% NP-40
1:1000 β-ME
1:100 PMSF
1:1000 PI

    b. PNGaseF: 1X Glycobuffer 2
1% NP-40
1:1000 β-ME
1:100 PMSF
1:1000 PI
4. Let rest on ice for 5 minutes, then add enzymes.
5. Incubate at 37˚C for 1-3 hours.
6. Add 1 volume 20% TCA to each tube. Let rest on ice for 10 minutes.
7. Centrifuge at 14k for 10 minutes. Remove as much supernatant as possible.

8. Wash the pellet with 1.5mL acetone without disturbing it, then remove all acetone.
9. Add SDS sample buffer. If the buffer turns yellow, add Tris-HCl pH 8.8 until blue again. Continue on to western blot protocol or freeze in -20˚C.

Western Blotting Protocol:

1. Load samples and MW markers (10 uL) on poly acrylamide gel. In each well: 15 ul of 15 ul sample + 3 ul 6X SDS buffer mix (some will be left over but this allows for pipette error)
2. Boil samples at 95 degrees C for 5 mins
3. Do a gentle spindown to send any droplets remaining to the bottom.
4. Run samples on gel for desired time (standard 4h @80V)
5. Open the plates that hold the gel and gently remove the stacking gel and any unused lanes using a razor blade.
   -Make sure you can see the MW marker.
6. Make transfer buffer
   100 mL10 X transfer buffer

   100 mL MeOH (this is dangerous stuff!)

   800 mL H20

   1 L

7. Make the sandwich:
   a. Cut 2 pieces of Whatman (filter) paper and one piece of nitrocellulose -Use gloves, fingerprints will ruin your data.
      -Snip the corner above the MW marker on both the gel and nitrocellulose so you can keep track of the orientation throughout the protocol.

   b. Pour some transfer buffer into a shallow dish and put down 1 layer of filter paper.
      -Avoid trapping any air bubbles at any step after this.

   c. VERY gently ease the gel from the glass plate onto the filter paper.
   d. Nudge the gel into position, keeping everything under the buffer the whole time.
   e. Gently lay the nitrocellulose on top.
      -The gel and nitrocellulose cling to each other so don't plan to do any adjustments after you lay the nitrocellulose down. You still can it's just a pain and you might damage the gel.

   f. Cover with the final piece of filter paper.
      -Do a final check for air bubbles

   g. Place the sandwich in the Western cassette, between the two sponges.
      a. Remember: The nitrocellulose MUST be on the side that faces the cathode.
      b. The final order should be:
         Cathode; cassette; sponge; paper; nitrocellulose; gel; paper; sponge; cassette; anode.

      -If you aren't 100% confident you did this step right it's ok to take it out of the cassette and check.

8. Run the western overnight at 10 V for 10-12 hours with a stir bar and ice pack in the container.

9. Remove the membrane and check that the pre-stained markers transferred.
10. Stain the nitrocellulose membrane with Ponceau S for ~5 minutes.
11. Rinse briefly with water and mark the sites of MW markers with a pencil.
12. Cut the nitrocellulose down as much as possible without cutting your sample.
13. Make up 5% *nonfat* milk solution in TBS-T.
    -Make sure milk is homogenous or it will ruin the protein.
14. Block the membrane in the milk solution for ~10 minutes. (**optional**)
15. Wash the membrane 3 times with TBS-T to remove extra milk.
16. Probe the western overnight using PRIMARY antibodies (mouse anti-GFP) at a 1:1000 dilution. This should be done in the cold room with moderate shaking.
    -When inserting the western into the 15 mL falcon tube, avoid crinkling it by loosely rolling it up, placing a corner in the tube, then as simultaneously insert and roll the western into the falcon tube.
17. Wash the membrane 3X10 minutes with TBS-T, at room temperature.
18. Probe the membrane with SECONDARY antibody (anti-mouse HRP) at 1:10,000 in TBS-T + 5% (w/v) milk. Do in the cold room with moderate shaking for 1.5-4 hours.
    -Ideal if you can use a flat container for this
19. Wash the membrane 3X10 minutes with TBS-T.
20. Mix Pierce ECL (Femto) solutions 1:1 in a microfuge tube. (0.5 mL each).
21. Apply the ECL reagent to the membrane while the imager is warming up (2-5 min incubation at RT).
22. Take pictures for 30 sec to 5 min, as necessary. BE SURE TO GET AN IMAGE OF THE MW MARKERS.
23. Print images and save the TIFF files to the server for further processing.

Plasmid Integration Protocol:

C. elegans Transgene Integration, adapted from Darrell Killian, https://personalwebs.coloradocollege.edu/~dkillian/Home/Transgene_Integration.html

"When transgenes are injected into the C. elegans germ line, the resultant transgenic animals carry the transgenes as extrachromosomal arrays. Extrachromosomal arrays not always transmitted to the next generation and often the transgenic animals are mosaic; they have some cells with the array and some without. In some cases the transgenes must be stably integrated into the genome before they can be reliably evaluated. This protocol is for using UV irradiation to cause double-stranded breaks in the genome and the endogenous DNA repair mechanisms will sometimes insert the extrachromosomal array into the genome."

1. Pick about 150 L4 transgenic animals to one (or several) NGM plates without bacteria. David Fay (University of Wyoming) reports that OP50 bacteria acts like sunscreen and lowers the dosage of UV irradiation experienced by the worms.

2. Remove the lids (they block UV) and place into the UV Stratalinker 1800 with the open side facing up.

3. Program the FB UVXC-1000 to deliver 30,000 micro joules (300 x 100 uj) of energy: Press "ENERGY", press 3-0-0, and then press "START". It should take about 20-30 seconds and beep when it is done. The FB UVXC-1000 has 5 bulbs calibrated to 265 nm.

4. Place 5 irradiated L4 animals on each of up to 30 OP50-seeded NGM plates. Use all of the irradiated worms.

5. Let the worms starve out those plates. Note that some of these plates will not starve out due to the irradiated animals either dying or becoming sterile. It may take several days longer than expected to starve out the plates since the worms will be unhealthy.

6. Chunk each starved plate onto a seed plate and label it so it can be traced back to the original plate. For example: Label a starved plate as "1" and chunk onto a new plate labeled "1a".

7. 1-2 days after chunking, single 5 ***transgenic*** L4 animals to 5 seeded NGM plates (one per plate). Also label these plates so they can be traced back to the original chunked plate. For example: From plate "4a", label the 5 plates made from this plate as "4a-1", "4a-2", "4a-3" etc.....

8. About 4 days later examine each plate and select plates with 100% transgenics and throw out any plates with non-transgenic offspring. If you get more than one plate with 100% transgenics from the same parent plate (that was chunked) they are probably not separate integration events unless they map to different chromosomes. Generally only keep one independent integration event. The rate of integration is 0-5%.

9. After an integrated line is established, it must be given a new allele designation and frozen as a new strain. Also, since UV irradiation causes DNA damage, the strain must be outcrossed several times to remove any background mutations.

Microscopic Imaging Protocol:

SLIDE PREPARATION (In Dahlberg lab using 2,3-butanedione monoxine, BDM)

1) Turn on heat block to 50-60 ˚C
2) Melt 2% (w/v) agarose in ddH_2O in the microwave. Transfer some of the agarose into a glass tube and place on the heat block. Cover the vial with a piece of aluminum foil. Melted agarose is good for one day on the heat block.
3) Quickly, pipet ~2 drops of the agarose onto a glass slide and cover the droplet with another glass slide. This will produce a thin agarose pad. If the agarose solidified too quickly, wipe it off with a Kim-wipe and try again. Try to minimize bubbles. Worms like to aggregate around bubbles and are no good for imaging. Allow the pad to dry for ~2 minutes.
4) In an Eppendorph tube, Make up a solution of 30 mg/mL BDM in M9 (i.e. 0.006 grams BDM in 200 µL M9). This solution is good for one day on ice.
5) On the dissecting microscope stage, place 4-5 µL of the BDM solution onto a glass cover slip, and cover with a 60 mm petri-dish to prevent the liquid from evaporating.
6) Pick ~20 worms and swish the tip of pick in the BDM drop to dislodge worms. Return the petri dish cover, and allow worms to paralyze for 5-6 minutes.
7) Once worms are paralyzed, place the glass slide with the agarose pad on top of the BDM drop. The slide and the cover slip should adhere and you can remove the slide with the cover slip from the stage. Remove excess liquid with a Kim-wipe.

IMAGING (Leica DMi6000 inverted microscope)

1) BE CAREFUL. This is a $50k piece of equipment, and the 63x objective alone cost $7k
2) Remove microscope cover, make sure that the objective that pointed up is at 40X or lower (it should always be returned to 10x at the end of imaging)
    a. If the objective was left at a higher magnification (63x or 100x), remove the detachable slide stage before turning on the microscope
3) Turn on the microscope computer, fluorescence box (if needed), and the microscope control box. Allow the stage to calibrate.
4) Turn on the computer and open the Leica LASX software for the configuration:
    a. Choose DefaultDynamicWidefieldTree if taking images
    b. Choose SimulatorAF6000 if you do not want to connect to the microscope and just want to use the software
5) At 10x magnification in DIC optics, place the slide in the mount with the glass cover slip facing down
    a. Place the glass slide all the way to one side of the mount. This way, if you have to take it off and back on, the location of your worms in mark and find will be the same
6) At 40x magnification, focus the edges of the pharynx and mark the location. Move to the anterior of the worm, and focus so that the edges of the worm are in focus. Repeat for all worms on slide.
7) Once the position of all worms are marked, move the stage, move the objective all the way down, and switch to the 63x objective. Carefully put one drop of immersion oil onto the lens of the objective.
8) Move the slide back over the objective lens, and move to the first marked location.
9) Imaging:
    a. Set one channel to blue fluorescent light to capture GFP, using a 0.5 second exposure.
    b. Set a second channel to green fluorescent light to capture RFP, setting an appropriate exposure length with the over/under setting.
    c. Set a third channel to DIC, setting an appropriate exposure length with the over/under setting.
10) Take images of all marked locations, being mindful of photo-bleaching the GFP.
11) When you are done, clean and dry objective lenses and return the turret to 10x.
12) Put on the cover