Establishing a Human Transgene Yeast Proteotoxicity Model of Lafora Disease

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Abstract

Lafora Disease (LD) is an autosomal recessive, neurodegenerative, myoclonus epilepsy that is characterized by the formation of polyglucosan inclusion bodies, called Lafora bodies (LB). Previous studies of LD using gene knockout mouse models have found a direct link between laforin, a tyrosine dual specificity phosphatase, and malin, an E3 ubiquitin ligase, with LB formation, though the underlying biochemical mechanism remains unknown. Yeast models have been successfully used to study other neurodegenerative disease as a human transgene proteotoxicity model and may be useful in understanding the mechanisms of LD. Here, we report the elementary steps in the establishment of a human transgene yeast proteotoxicity model specific for LD. Wildtype human laforin and malin have been introduced into yeast strains using centromeric and non-centromeric plasmids. Additionally, genes containing three disease-causing mutations have been individually incorporated into plasmids (K87A and C226S for laforin and D146N for malin). Foci formation has been observed in the laforin C226S strain under fluorescence microscopy in nitrogen starvation conditions. The observation of aggregates validates the strong potential of this model. The characterization of disease relevant mutants in an established model will aid in the investigations of the underlying molecular mechanisms involving glycogen metabolism and the pathogenesis of LD.
Introduction

Neurodegenerative diseases are a family of both inherited and sporadic disorders that are prevalent throughout the population. Reports from the World Health Organization indicate that, as the aging population continues to grow, these diseases will become more common, stressing the need for better treatments and cures to offset the increasing health care costs. Research into these diseases has progressed in part due to the usefulness of the budding yeasts, *Saccharomyces cerevisiae*. Due to its conservation of basic cellular processes and a range of biochemical and genetic tools available, the yeast model system has been effective in the study of several neurodegenerative diseases including Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, and ALS (Franssens et al. 2010; Ju et al. 2011; Meriin et al. 2002; Treush et al. 2011). These models have successfully assisted the discovery of the molecular mechanisms underlying pathology as well as the identification of candidate genes and potential small molecules with therapeutic value.

Lafora disease (LD) is the primary form of adolescence-onset progressive myoclonus epilepsy (Turnball et al. 2012). Despite being described over one hundred years ago, the molecular basis involved in the pathology remains unknown (Lafora and Glueck 1911). Symptoms of LD arise during adolescence beginning with headaches, spontaneous myoclonus, and convulsive seizures which are followed by dysarthria, ataxia, and visual hallucination (Ganesh et al. 2002). These seizures occur concurrently with neurodegeneration in the form of dementia and loss of cerebellar signs leading to death within ten to fifteen years (Striano et al. 2008). There is no cure for LD and treatment is solely symptomatic, aimed at reducing the intensity and frequency of seizures (Minassian et al. 2001). LD is inherited in an autosomal recessive manner caused by over two hundred mutations in the *EPM2A* gene, encoding laforin, a
dual specificity phosphatase, or the \textit{EPM2B} gene which encodes for malin, an E3 ubiquitin ligase (Minassian \textit{et al.} 2002).

The hallmark feature of LD is the accumulation of Lafora Bodies (LB), which are large aggregates of insoluble glycogen called polyglucosans. The aggregates are composed of glycogen consisting of glucose units joined by $\alpha$-1,4-glycosidic linkages with branching by $\alpha$-1,6-glycosidic linkages (Gentry \textit{et al.} 2006). However, one hypothesis is that branches in LBs occur less frequently than normal glycogen or contain an increased amount of phosphates that cumulates to the molecular destabilization of glycogen. Due to these molecular properties, LBs are water-insoluble as compared to normal glycogen. LB formation increases over time along with the number of seizures and neuronal cell death; thus it is hypothesized that LBs are responsible for LD symptoms (Yokoi \textit{et al.} 1968).

LBs accumulate in multiple tissue types yet cell death has only been observed in neurons. Neurons, although having the ability to synthesize glycogen, do not have the machinery to degrade it (Brown 2004). Normally, neurons do not produce glycogen and mutations in either \textit{EPM2A} or \textit{EPM2B} could possibly lead to increase accumulation of glycogen, leading to the formation of LBs in neurons that ultimately lead to cell death. In the brain, glycogen storage is mostly handled by astrocytes, which are able to degrade glycogen and they do not develop LBs (Gruetter 2003).

\textbf{EPM2A and Laforin}

\textit{EPM2A} encodes laforin, a 331-residue protein consisting of a carbohydrate binding motif (CBM; residues 1-124) and a C-terminal dual specificity phosphatase domain (DSP; residues 157-326). Laforin is expressed in all tissues, although brain, muscle, skeletal muscle, heart, and
liver have higher expression levels (Serratosa et al. 1999). In the brain, laforin is expressed predominately in the hippocampus, frontal cortex, cerebellum, and olfactory bulb. Laforin expression increases throughout life reaching a maximum level during adulthood and this may contribute to the disease pathology of LD (Ganesh et al. 2001).

Alternative splicing in EPM2A results in two laforin isoforms that are identical from residues 1-309 with different C-terminal domains. The most abundant isoform, laforin-331 has active phosphatase activity and is localized to the cytoplasm and endoplasmic reticulum when overexpressed. The minor isoform, laforin-317 does not have an active phosphatase domain and localizes to cytoplasm and nucleus. These isoforms have been shown to form a heterodimer that lacks phosphatase activity and may suggest that the minor isoform regulates the major one (Dubey and Ganesh 2008). A recent study by Dubey, Parihar, and Ganesh (2009) has also found three other isoforms of varying lengths, although their biological and physiological relevance is unknown.

**Domains of EPM2A**

The carbohydrate binding motif (CBM) is a noncatalytic domain that binds to carbohydrates, and enzymatically alters it with a secondary domain (Boraston et al. 2004). The CBM in laforin is part of the CBM20 family, which are 90-130 residues long and are one of the most described CBM families (Wang et al. 2002). CBM20 domains are highly heterogeneous at the primary sequence level but contain fairly well-conserved aromatic residues (Machovic et al. 2005). The CBM20 domain in laforin has been shown to bind glycogen and LBs. The secondary domain that modifies carbohydrate is the dual specificity phosphatase (DSP) domain.
The DSP domain is part of the protein tyrosine phosphatase (PTP) family that consists of cysteine-dependent phosphatases (Alonso et al. 2004). The PTP families have a conserved CX$_3$R motif that they use to cleave phosphoester bonds (Tonks 2006). It has been shown that laforin like other cysteine-dependent enzymes, is inactive under oxidative conditions and requires a reduced environment to be biologically functional (Dukhanade et al. 2011). Loss-of-function point mutations in either the CBM or the DSP domain ultimately result in LD (Singh et al. 2009).

Currently, the three-dimensional structure for laforin has yet to be experimentally determined. However, Roma-Mateo et al. (2011) created a homology model using *Geobacillus stearothermophilus* cyclodextrin glycosyltransferase (PDB: 1CYG) for the CBM domain and human DUSP22 phosphatase (PDB: 1WRM) for the DSP. The model for DSP domain model suggests that DSP folds into the αβα fold that is characteristic of PTP proteins. This fold consists of four to five β-sheets surrounded by α-helices (Anderson et al. 2001). In the CBM20 domain, it was observed that there are conserved aromatic residues for carbohydrate binding: Trp32, Trp 85, and Trp 99. These residues form a suitable hydrophobic binding area for glycogen binding. In the model they have created, Roma-Matero have found that the PTP loop, containing the active cysteine (Cys266), and the conserved aspartic acid (Asp235) point towards the catalytic domain. Although these models provide some information, a crystal structure of laforin itself would be required to determine how carbohydrates are positioned and altered through the CBM and DSP domain.

Laforin has also been shown to create dimers in cell culture, tissue, and recombinant bacteria (Castanheria et al. 2010; Liu et al. 2006; Fernandez-Sanchez et al. 2003). A study done by Liu et al. (2009), has demonstrated that SDS-resistant laforin forms in vitro and in vivo.
Additionally phosphatase activity is only seen in the homodimerized form of laforin while the monomeric laforin was biologically inactive. Furthermore Liu et al. found laforin oligimerization that resulted in the abolishment of phosphatase activity. These finding was challenged, however, when laforin was studied in normal reduced conditions where it was found that monomeric laforin was the most abundant form of phosphatase. The study also observed that the difference between phosphatase activities was negligible between monomeric and dimeric laforin (Dukhade et al. 2011). The discrepancy between the two studies can probably be attributed to the Liu et al. using little to no reducing agent in their experiments resulting in laforin dimerization.

**Laforin and Malin**

Malin is a 395 residue E3 ubiquitin ligase that contains an N-terminal RING (Really Interesting New Gene) finger domain and 6 NHL (NCL-1, HT2A, Lin-41) repeat protein-protein interaction motifs (Lohi et al. 2005). Several past studies have shown that laforin and malin form a complex. Laforin will recruit substrates to be ubiquinated by malin, and some of these substrates are enzymes that regulate glycogen synthesis. Malin will also ubiquitinate laforin after removing phosphates from glycogen. Previous studies have shown for example that the laforin-malin complex binds and ubiquitinates the muscle isoform glycogen synthase and protein targeting to glycogen (PTG) (Vilchez et al. 2007; Vernia et al. 2009) (Fig. 1A). This targeted degradation resulted in decreased protein and glycogen levels. The role of laforin as an adaptor protein is not necessary for its role as a glucan phosphatase, as a catalytically inactive point mutant (C266S) still recruits malin and targets enzymes involved in glycogen synthesis (Solaz-Fuster et al. 2008; Worby et al. 2008). Additionally, one group has found that malin also ubiquitinates glycogen debranching enzyme (Cheng et al 2007).
Figure 1: Glycogenesis mechanism (A) Biochemical regulation pathway of glycogen synthase. (B) Glucose molecules assemble on the glycogen primer or glycogenin protein by glycogen synthase via α-1:4 glycosidic units. Branching enzyme forms α-1:6 glycosidic bond forming branches. (C) Imbalances in glycogen synthase and glycogen branching enzyme leads to overextension of branch arm lengths but too few branches causing decrease glycogen solubility. Past studies have shown that an overexpression of malin and laforin down-regulate PTG, which reduces glycogen synthase levels.
Previous experiments utilized mostly cell culture experiments in which laforin, malin, and other glycogen synthesis enzymes were overexpressed. However, other groups using mouse models have found varied results. Tagliabracci et al. (2008) demonstrated that in three month old laforin knockout mice did not have an increase in levels of glycogen synthase or PTG. Two other groups demonstrated that there was no increase in glycogen synthase, PTG, or glycogen branching enzyme in malin-deficient mice at three to six months of age (DePaoli-Roach et al. 2010; Turnbull et al. 2010). Another group, however, using malin-deficient mice found higher levels of glycogen synthase in brain extract at 11 months (Valles-Ortega et al. 2011). The discrepancy between these results suggests that age may be the underlying reason for the differences.

**Laforin and Malin, ER Stress and Protein Protection**

Previous studies have shown that the laforin-malin complex plays a role in protecting cells from ER stressors. One study by Wang et al. (2013) used laforin-depleted cell cultures and found that these cells were more susceptible to apoptosis via ER stressors such as tunicamycin. Tunicamycin is an inhibitor of the N-glycosylation of proteins, a common post-translational modification, causing decreases to protein stability in ER. The group has also shown that in mice, the laforin-malin complex protects against ER-stress induced apoptosis and that in the absence of laforin, ER stress drastically accelerates LB formation and accumulation. Additionally, after supplementation of the drug there was an observed decrease in proteasome activity and an increase in apoptosis. Another supporting piece of evidence is that mice lacking laforin and human LD tissue were also similarly susceptible to ER stress (Vernia et al. 2009). Similar results have also been found using malin-deficient cell culture (Rao et al. 2010).
Laforin itself has been shown to induce ER stress. Overexpression of laforin has been observed to aggregate and localize with ubiquitin, ER chaperones, and proteasome inhibitors (Mittal et al. 2007). This aggregation is increased when there is co-expression of mutant laforin leading to increased apoptosis and ER stress response (Liu et al. 2009). Previous studies have shown that laforin has also been shown to protect against buildup of misfolded protein. Garyali et al. (2009) demonstrated that when they overexpress aggregate-prone proteins with laforin, malin, and heat shock 70 protein that the aggregate proteins were degraded, protecting the cell against cytotoxicity. They found that the laforin-malin complex ubiquitinated the misfolded proteins and targeted for degradation in the proteasome.

Lastly, laforin has been shown to be a positive regulator of autophagy. In cell and mouse models of knock out laforin, it has been observed that there is a decrease in autophagy resulting from the impaired formations of autophagosomes. The underlying biochemical and molecular basis for this is unknown. However, in these knockout cells the mammalian target of the rapamycin pathway is overactive (Aguado et al. 2010). Similar results have been found using malin knockouts (Criado et al. 2012). Due to this decrease in autophagy, substrates that usually are degraded may remain in the cell, which could ultimately lead to cell death and the observation of LD pathogenesis.

**Biological Mechanisms of Laforin and Malin in Lafora Disease**

Previous studies have demonstrated laforin and malin are involved in glycogen metabolism (Garyali et al. 2009). Glycogen synthase is the enzyme responsible for the synthesis of glucose into glycogen. During glycogen synthesis, phosphate ions are incorporated into glycogen through a sporadic error, roughly 1 in 10,000. Laforin may serve as a quality control
enzyme, binding to glycogen and removing the phosphate (Tagliabracci et al. 2011). Malin subsequently ubiquinates laforin targeting laforin for degradation (Gentry et al. 2005). Under this hypothesis, LD would be caused by loss of function mutations in laforin and malin. This hypothesis is supported by evidence where mutations in laforin and malin lead to the same histology and disease seen in mice and human LD patients. As laforin and malin have a link to glycogen, it seems logical that the loss of function of these proteins would result in the indirect or direct aggregation of glycogen. The roles that laforin and malin play highlight that the key to LD progression involves either interaction or regulation of glycogen metabolism. Current evidence supports two different hypotheses for the mechanism of LD pathogenesis. Laforin and malin have been shown to form a complex that also interacts with PTG, which targets the pleiotropic phosphatase protein phosphatase 1 (PP1) to dephosphorylate and activate glycogen synthase, one of two enzymes responsible for glycogen synthesis (Fernandez-Sanchez et al. 2003). Some studies have shown that an absence of laforin and malin leads to increased PTG which ultimately causes increased glycogen synthase activity (Vilchez et al. 2007). An increase in glycogen synthase activity without an increase branching enzyme activity would lead to an imbalance in glycogen branching and elongation, leading to the formation of LB by decreasing glycogen solubility (Fig 1C).

An alternative hypothesis is that a loss of function mutation in laforin would prevent the removal of phosphate ions. Additionally, a loss of function mutation in malin would prevent the ubiquitination and subsequent protein degradation of laforin (Fig. 2). Phosphate ions are incorporated into glycogen through a sporadic error are thought to destabilize the tight packing of the glycogen branch arms by exposing branch hydrophobic surfaces, thus leading to decreased glycogen solubility and possible aggregation (Turnbull 2012). Laforin, untargeted by malin for
degradation, would remain bound to glycogen and cause decreased packing of glycogen leading to decreased solubility and aggregate formation. Mouse models show support for both of these hypotheses but the specifics of the mechanisms are unclear. It is known, however, that LBs play a critical role in LD pathogenesis, as LB formation in mice leads to myoclonus and neurodegeneration (Turnbull et al. 2011).
**Figure 2: Proposed lafora body formation.** Laforin removes bound phosphates on glucose subunits. Malin subsequently ubiquitinates laforin, targeting it for proteosomal degradation. Loss of function malin would increase laforin bound to glycogen and loss of function laforin would leave phosphates improperly bound. Either mechanisms of glycogen structural destabilization could lead to LB formation through promotion of aggregation.
Possible Therapeutic Approaches for Lafora Disease

When considering possible therapies for LD, there are many factors to consider. There are multiple possible pathways that drive the pathogenesis of LD and the data currently does not give one clear and definite solution. So far, current results suggest that interactions with glycogen metabolism enzymes, cellular stress, and glycogen phosphorylation are all relevant in LD progression. However, much is still unknown about these pathways and how they interact. This uncertainty, compounded by the fact that there is still much debate on what is the physiological and biological role of laforin, leads to a vast amount of work still needed to be done.

One biological role that is highly supported by the literature is that laforin functions as a glucan phosphatase. Without its phosphatase activity phosphates remain on glycogen and disrupt the stable network of carbohydrate leading to LB formation. Laforin has been clearly demonstrated in vitro to remove phosphate ions but its other functions are still unclear. However, with laforin’s ability to form a laforin-malin complex, it is reasonable to suggest that this complex may have a part in other pathways such as autophagy and/or ER stress. For example, LB formation resulting from misfolded laforin could lead the cell to ubiquinate the aggregate. These aggregates could then alter the normal mechanisms surrounding cell transport and autophagy. Since the central hallmark of LD is glycogen metabolism deficiencies, it is critical to observe changes throughout the glycogen synthesis pathway resulting from misfolded laforin to better understand the mechanism by which these changes result in pathogenesis. Despite these gaps in knowledge, researchers have made strides in therapeutic approaches.
One therapeutic approach is to regulate the glycogen synthesis pathway. The Turnbull group (2008) for example, has demonstrated that depletion of PTG in knockout laforin mice resulted in down regulation of glycogen synthesis, decreasing the amount of LBs, neuronal cell death, and myoclonic epilepsy. By down regulating PTG, an activator of glycogen synthesis and inhibitor of glycogen phosphorylase, a decrease in the levels of glycogen synthesis was observed. This ultimately results in a lower level of glycogen that may have slowed the progression of the disease.

Another potential therapeutic avenue is to target the protein folding and autophagy pathways. Previous studies suggest autophagy is down regulated in the absence of laforin, therefore the use of therapeutics to enhance and protect autophagy pathways may be an interesting approach. Likewise, a method that could enhance protein folding by increasing the fidelity and upregulation of chaperone protein and proteosomal activity may have similar effects. Lastly, one additional approach is to use gene therapy to express EPM2A or treatment with a Trojan horse liposomes (called PEGylated immunoliposomes) containing the genes of interest (Pardrige et al. 2010).

Yeast Models for Neurodegenerative Diseases

Previous studies of LD that made use of gene knockout mouse models and cell culture have ascertained a direct link between laforin and malin with LB formation though the exact biochemical mechanisms remain elusive. Furthermore, examination of laforin and malin disease-causing mutations, especially in vivo studies, has been lacking.

Yeast models have been successfully used to study various neurodegenerative diseases and may provide a useful tool set for the study of LD (Franssens et al. 2010; Ju et al. 2011;
Meriin et al. 2002; Treush et al. 2011; ). Yeast do not contain homologous genes of human laforin and malin but do have a conserved glycogen synthesis pathway. Nonetheless, despite the lack of pathogenic genes, use of the yeast model is attractive for numerous reasons: the entire genome of *S. cervisae* has been sequenced, genetic manipulation is relatively easy, yeast can be subjected to a variety of biochemical tools, and additionally yeast cell lines are well-established and inexpensive. Human transgene yeast models of neurodegenerative diseases have led to the discovery of biological mechanisms behind cell toxicity and have been used to screen for suppressors and enhancer genes and small molecule therapeutics (Ju et al. 2011). Building upon the previous success of yeast models in studying neurodegenerative diseases we aim to develop a human transgene yeast proteotoxicity model in order to explore the underlying biochemical mechanisms involved in LB formation and cell toxicity which leads to LD.

Here, we report the elementary steps in the establishment of a yeast proteotoxicity model to investigate the effects of human laforin and malin and their disease relevant mutations on glycogen metabolism. We have made three point mutations to explore the role of each functional protein domain of the pathogenic proteins. These include mutations in the carbohydrate binding motif and the dual phosphate domain in laforin, and a mutation in malin which disrupts the laforin-malin complex formation. Additionally, we have introduced wildtype human laforin and malin into a yeast system and have verified expression of these genes. We have identified conditions where LB formation initiates in the yeast model using one of the disease relevant mutations.
Materials and Methods

Plasmids and strain creation

Human laforin was amplified by PCR using forward primer (caccatgcgcgtcgcgttggg), and reverse primer with or without stop codon (ctacaggctacacagaagaacga, caggtcagcacagaagaagc). Human malin was amplified by PCR using primers (caccatgcgcgtcgcgaagcctc) and (ctaaccagttacactttagaagtt, acactttagaagtt). Gateway technology was utilized to introduce PCR products into the pDONR221 gateway system entry vector through the BP clonase (Invitrogen) reaction. Site-directed mutagenesis was used to incorporate two mutations into laforin K87A (ctccgcgttccaggaacgcgttacagctgtcc, ggacagttcggccgttctgaagccggg) and C266S (accccagcgttgctgtgcacgtacagcgtgtcagctgtgcacg). Site-directed mutagenesis was performed using standard methods.

A gateway LR clonase reaction (Invitrogen) was used to incorporate laforin and malin genes into yeast destination vectors (pAG413Gal-ccdb-eGFP, pAG414Gal-ccdb-eGFP, pAG423Gal-ccdb-eGFP, and pAG423Gal-ccdb-HA) for laforin and (pAG415Gal-ccdb, pAG415Gal-ccdb-eGFP, pAG424Gal-ccdb, pAG424Gal-ccdb-eGFP) for malin. Mach1 cells were used in bacterial transformations, performed using standard protocols.

Media and Growth Conditions

Synthetic media and agar lacking histidine (-His), tryptophan (-Trp), leucine (-Leu), histidine and tryptophan (-His- -Trp) and histidine, tryptophan, and leucine (-His- -Trp- -Leu) and containing 2% glucose, galactose, or raffinose were used for the respective yeast strains. As
listed, strains were supplemented with drugs following an overnight growth in media lacking ammonium sulfate and containing 0.1% proline. Strains were grown media containing 0.003% SDS for three hours and then drugs for 4 hours before harvesting. Concentrations of 75uM MG132 (sigma) or 1mM phenylmethanesulfonylfluoride (PMSF) (sigma) were used. Media was supplemented using 2ug/ml Tunicamycin (sigma) or 2mM 2-deoxyglucose (sigma) where listed. Nitrogen starvation conditions were grown with yeast nitrogen base lacking ammonium sulfate (Fisher).

Yeast cells were grown in 30°C incubators (plates) or 30°C shakers (media). Wild-type plasmids and mutants were transformed into W303α strain (MATα can1-100, his3-11,15, leu2-3,112,trp1-1, ura3-1, ade2-1). Yeast transformations were done using standard Polyethylene glycol (PEG)/Lithium acetate (LiAc) transformation methods. Briefly, cells from 50-milliliter overnight culture plus DNA construct was mixed with transformation buffer (240µl 50% PEG3350, 36µl 1M LiAc, 56µl water and 18µl dimethyl sulfoxide (DMSO)), followed by incubation at 30°C water bath for 30 minutes and then a 42° water bath for 30 minutes. Cells were recovered in YPD for one hour at 30°C. Afterwards, cells were spread onto respective dropout mix and grown at 30°C for 2-3 days.

Serial Dilution and Spotting Assay

Yeast strains were grown overnight at 30°C to mid-log phase. Cultures were then normalized to OD$_{600} = 0.10$ and 5X serially diluted and spotted onto respective dropout plates containing 2% glucose or galactose.

Yeast Total Cell Protein Extract
After culturing for 24hr, cells were resuspended in 1:1 TCA Buffer (10% Tris pH 8, 12.5% 4M NH₄OAc, 0.4% 0.5 M ethylenediaminetetraacetic acid (EDTA), 1x proteinase inhibitor cocktail (Roche), 10% 100mM PMSF) 20% TCA. Yeast cells were lysed using 300mg of glass beads and cells were vortexed and recovered on ice after each vortex for 30 seconds, six times. Cells were then washed with TCA Buffer with 10% TCA twice, and supernatants were combined. Supernatant was centrifuged 5’ at 15,000g and the resulting pellet was solubilized using 6% SDS, 250mM Tris base. Protein concentrations were normalized with a Bradford bovine serum albumin assay (Biorad, 2012) and then ran on a 9% SDS-PAGE gel.

Glycogen Assay

50 OD₆₀₀ cell samples were subjected to a 30 minute lysis in 30% KOH followed by three washes of 66%, -20°C ethanol washes. Glycogen quantifications were done using the EnzyChrom™ Glycogen Assay Kit (BioAssay Systems, 2008).

Immunoblotting

Yeast protein extracts were subjected SDS-PAGE, and protein was transferred onto PVDF membrane (Millipore), followed by 60 minute incubation with 1% Tween containing TBS. The anti-GFP (molecular probes) antiserum was used at 1:10,000 concentration and anti-HA antiserum (Invitrogen) was used at 1:5000 concentrations.

Fluorescence Microscopy

Cells were grown in SC media containing 2% raffinose to early log phase, and induced with 2% galactose for protein expression for 24 hours. Cells were harvested and formaldehyde
fixed for 1 hour. Cells were then washed twice with PBS before viewing by fluorescence microscopy.

**Solubilization Assay**

Yeast strains were grown overnight in selective media and 10 OD$_{600}$ of yeast cells were harvested. Cells were washed with 10 mM Na$_3$ and resuspended into 1.4M Sorbitol, 0.1M Hepes pH7.5, 10mM Na$_3$. Cells were treated with 1 mg of zymolase for 90 minutes at 30°C to create spheroblasts. Spheroblasts were collected by centrifugation at 1500g for 10’ and osmotically lysed in 0.8M Sorbitol, 1mM EDTA pH8.0, 0.1M Hepes pH7.5, 1x proteinase inhibitor cocktail (Roche), 10% 100mM PMSF by mechanical pipetting. Unbroken cells were removed by centrifugation at 1500g for 10’. Insoluble fractions were isolated from total cell lysate by centrifugation at 14,000g for 25’. Fractions were resuspended in lysis buffer.

**Results**

**Centromeric Expression of Human Laforin in Yeast Results in no Toxicity in Localization and Cell-Growth Assays**

To create a S. cerevisiae human transgene proteotoxicity model to be used in the investigation of Lafora Disease, yeast strains expressing laforin and malin were generated. The human genes *EPM2A* and *EPM2B*, which encode for laforin and malin respectively, were analyzed to determine which mutants would be most useful in the creation of this model. The *EPM2A* gene encodes for a protein that is 37 kDa and contains a carbohydrate binding motif and a dual specificity phosphatase domain. The *EPM2B* gene encodes for a protein that is 42 kDa that consists of an N-terminal RING domain and 6 NHL repeat protein-protein interactions.
motifs. The disease relevant mutant-types (K87A and C266S in \textit{EPM2A} and D146N in \textit{EMP2B} [Fig. 3A]) were selected as representatives for each of the domains in the two genes of interest. The two genes were initially cloned from vectors present in the lab into the pDONR221 yeast gateway entry vector. The disease relevant mutations were individually made and the mutants were subsequently cloned into the PRS41X centromeric destination vectors. The stop codon was removed from a second set of the mutant genes by site directed PCR and subsequently cloned into centromeric destination vectors containing a c-terminal green fluorescence protein (GFP) tag. The destination plasmids placed the genes of interest under the control of a \textit{GAL1} promoter, whereby expression of the proteins of interest can be tightly regulated by changing sugar sources. Thus, in these strains by switching the carbon source to galactose, laforin and malin expression is induced.

After creation of the plasmids containing wild type or mutant laforin and malin, the plasmids were transformed into wild type haploid W303\(\alpha\) and \(\Delta\text{reg1}\) yeast strains. \textit{REG1} is a gene that acts on the negative regulation of glucose repressible genes including the yeast AMP-activated protein kinase, SNF1, which induces glycogen synthesis. The \(\Delta\text{reg1}\) strain has higher levels of basal glycogen synthesis, a growth defect phenotype dependent upon the synthesis of glycogen and increase sensitivity to chemical stresses such as benomyl and tunicamycin. By comparing \(\Delta\text{reg1}\) and W303\(\alpha\) strains, we can assess the specific effects of glycogen synthesis. In the first set of experiments, the yeast strains tested contained a single copy laforin, double copy of laforin, single copy laforin with malin, and a double copy of laforin with malin.

We first assayed whether expression of human laforin and malin were toxic to the yeast cells. Single and two copies of laforin, with and without, malin were assayed in a colony dilution assay. The strains were diluted and spotted onto plates with glucose (expression off) and
galactose (expression on) (Fig. 3B). After 48 hours of growth, it was observed that all the strains had colonies of similar sizes, indicating that no observable toxicity phenotype was exhibited from expression of human laforin and or malin in yeast. Furthermore, the growth of the strains at 37°C to increase cell stress through the induction of the heat shock pathway did not result in a growth defect phenotype.
Figure 3: Human laforin is not toxic in yeast. (A) Schematic map of disease relevant laforin and malin mutations. Mutations were created by gateway cloning with the pDONR221 destination vector into pAG4x vectors of interest then subsequently transformed into yeast. Transformants were verified through genetic sequencing. (B) Colony dilution assay performed with different laforin and malin mutants. Strains were spotted in 5X serial dilutions. W303α control strain shows similar growth to mutant strains.
As no growth defect phenotype was observed in the colony dilution assay, we next wanted to investigate whether the induction conditions in previous assays result in robust and stable expression of human laforin and malin. We assayed expression of laforin using fluorescence microscopy and western blot. For microscopy, yeast strains were created as described above but with the addition of an N-terminally fused green fluorescent protein (GFP). Fluorescence microscopy of yeast with two copies of laforin and reg1Δ strain showed that the presence of laforin was minimal (Fig. 4A). No difference between wild type and laforin mutants was observed. In order to assess the expression of laforin, protein expression analysis was done by western blot after induction by 2% galactose for 24 hours (Fig. 4B). Western blot revealed that the GFP fusion tag does result in a detectable level of signal indicating that laforin protein expression is being achieved.
Figure 4: Laforin is detectable using a green fluorescence protein tag. (A) Cells were induced with 2% galactose for 24 hrs and were then fixed and viewed with fluorescence microscopy. (B) Western blot performed with different strains of laforin and malin. Protein expression was induced with 2% galactose for 24 hours. eGFP tagging cells was found to identify the presence of laforin.
Nitrogen Starvation Conditions Increase Laforin Levels In Yeast

We next wanted to determine if the laforin expression profile changes in conditions where glycogen is abundant in the cell. Nitrogen starvation conditions lead to an increase in basal levels of glycogen in yeast. Thus, we grew wildtype yeast with mutants in nitrogen starvation conditions for 24 and 74 hours and then quantified laforin expression by western blot (Fig. 5A and B). The strongest signal was observed in the Δreg1 strain containing two copies of laforin-eGFP and malin. However, the phenotype of the Δreg1 strain leads to inconsistencies due to repeat culturing through transformations and assays (data not shown). We wanted to determine the growth conditions for W303α strains containing laforin that would lead to the strong signal that was found in the reg1Δ background strain. Nitrogen starvation for 72 hours did increase laforin expression as compared to normal media control conditions and a 24-hour nitrogen starvation. However a 72 hour starvation condition, when laforin was induced in the last 30 hours, resulted in poor expression of laforin. In conditions of 72 hours of nitrogen starvation followed by a 24 hour normal nitrogen media recovery, we saw an increased amount of laforin expression as compared to the induction in the last 30 hours (Fig. 5C and 5D, Lanes 2, 3, 5, 7). None of these conditions provided the ideal level of laforin expression for our purposes.
Figure 5. Nitrogen starvation does increase levels of laforin. Western blot were done with different strains of laforin and malin in nitrogen starvation conditions with and without recovery. Protein expression was induced with 2% galactose for 24 hours.
ER Stressors And Proteasome Inhibitors Do Not Increase Laforin Expression Levels

We next used ER stressors and proteasome inhibitors to determine if these conditions change laforin expression profile. ER stressors such as dimethyl sulfoxide, tunicamycin, and 2-deoxyglucose and proteasome inhibitors have been shown to drastically increase the rate of Lafora body formation. Glycogen assays were done to determine the amount of soluble glycogen in the cells and Western Blots were done to determine expression levels as a result of these treatments. Results indicate ER stress did not induce changes in laforin levels in our yeast strains (Fig. 6) and did not change glycogen solubility in cells. However, an increase in glycogen solubility was observed using proteasome inhibitor MG132. The results of the drug stress and the nitrogen starvation assays suggested that centromeric plasmids did not provide enough protein for us to systematically reproduce a phenotype representative of LD.
Figure 6: Proteasome inhibition and ER stress do not increase levels of laforin in W303α laforin two copy with malin strain. Protein expression was induced with 2% galactose for 24 hrs. Drugs were applied for 4 hours. (A) Protein expression in proteasome inhibitors. (B) Protein expression in ER stressors. (C and D) Glycogen assay.
High Expressing Strains Using 2 Micron Plasmids Generate Mild Toxicity Phenotype

Centromeric plasmids contain sequence that yeasts recognize as a chromosome-like structure which causes yeast to retain 1-2 copies. 2-micron plasmids lack this structure causing the yeast to retain 10-100 copies of plasmid that causes a resultant increase in the expression of protein. By using the expression system described previously, 2-micron plasmids were utilized for yeast with wildtype human laforin, malin and with mutations of interest. In the generation of these high copy plasmids, a new protein tag was utilized, human influenza hemaglutinin (HA) tag mutants were created along with green fluorescence protein tag (GFP) (Fig. 7A, B, C) HA tagged mutants were created for immunoblotting assays due to the clarity of the westerns as compared to using GFP antiserum. High expressing strains were tested for growth defect phenotypes in order to determine the baseline toxicity of laforin in mutant strains. Yeast cultures were grown for 24 hours and were spotted on glucose and galactose containing plates. A minor growth defect was observed in all strains containing both wild type and mutant laforin coexpressed with malin.

Laforin C266S Mutant Forms LB Precursors

Fluorescence microscopy of high expressing strains at 24 and 72 hours of nitrogen starvation conditions revealed abnormal growth in the C266S mutant with morphological phenotypes exhibited by irregular cell wall shape. Strong localization of laforin was found in the C266S suggesting aggregation (Fig. 8). However, at 24 hour recovery in nitrogen rich conditions, the foci dissipate suggesting that they are unstable. Regardless of their stability, identification of a condition of initial LB formation for the laforin C266S mutant validates the usefulness of our model.
Figure 7: Human laforin in 2 micron plasmids generate a mild toxicity phenotype in mutant yeasts. (A) Protein expression was induced with 2% galactose for 24 hrs. HA tagged yeast were noticeably cleaner than eGFP tagging. (B) Western blot of HA tagged 2 μm mutant strains. (C) Colony dilution assay of 2 μm mutant strains at in glucose (left) and galactose (right). Strains were grown for 48 hours.
Figure 8: Microscopy of nitrogen starved 2 micron mutants reveal foci in laforin C226S strain. Cells were induced with 2% galactose for 24 hours and then were fixed and viewed with fluorescence microscopy. (A) W303 ccdb. (B) W303 Laforin Gal eGFP Malin 424 (C) W303 Laforin K87A Gal eGFP Malin 424 (D) W303 Laforin C266S 423 Gal eGFP Malin 424.
Levels Of Soluble And Insoluble Laforin Are Similar In All Strains

As we observed foci for the C266S strain, we wanted to determine if there was a difference between this particular mutant, wildtype laforin, and the other K87A mutant in terms of solubilization. We expect that in the solubilization assay, laforin C266S would be more insoluble than other laforin strains due to the lack of foci in other strains. To further examine the composition of laforin in yeast, the amount of soluble and insoluble laforin was assessed (Fig. 9). Because the foci were only observed at the 72 hour starvation point, we harvested a time point here and then at 24hr recovered, as to compare the difference in solubilization. To determine solubilization, cells were osmotically lysed and an insoluble pellet was collected through 10,000g centrifugation for 20 minutes. The whole cell extract, supernatant, and pellet in each strain were compared. Surprisingly, in almost all the strains the amount of laforin in the pellet (insoluble laforin) was nearly equal to the amount of laforin in the supernatant (soluble laforin) despite the lack of foci signal in fluorescence microscopy for wildtype and K87A laforin. The laforin K87A strain contained relatively equal amounts of insoluble and soluble laforin during starvation and recovery. Arguably, the C266S strain contains more insoluble protein observed for the laforin strains.
Figure 9: The amount of soluble and insoluble laforin is similar for wildtype and mutants. Cells were grown for 72 hours in galactose nitrogen starvation conditions and were recovered in +nitrogen for 24 hours. Cells were converted to spheroeblasts by treatment with zymolase for 1 hour at 30°C and then osmotically lysed. W: Whole cell extract. S: Supernatant. P: Pellet centrifuged at 10,000 RPM for 20 minutes.
ER Stress Does Not Increase Laforin Levels in High Expressing Strains

We wanted to assay if laforin expression results in ER stress in our yeast model. It has been shown that using ER stressors such as tunicamycin and 2-deoxyglucose increase the formation of Lafora bodies in Lafora disease models in mice. Western blot demonstrated that ER stress does not increase laforin expression (Fig. 10). Yeast strains expressed laforin in similar amounts despite ER stresses. Though ER stressors have been shown to induce LB formation, ER stress may not lead to an explicit increase in laforin levels in our model.
Figure 10: ER Stress does not cause increased laforin expression levels. Protein expression was induced with galactose. Mutant yeast strains were grown with 0.1 mM tunicamycin, 2mM 2-deoxyglucose, or no drug for four hours. (1) Molecular Weight Marker. (2) 303 CCDB. (3, 6, 9) 303 423 Laforin eGFP 424 Malin. (4, 7, 10) 303 423 Laforin K87A eGFP 424 Malin. (5, 8, 11) 303 423 Laforin C266S eGFP 424 Malin.
Laforin Mutants Do Not Cause An Increase In ER Stress

Since Laforin did not increase in expression levels after the addition of ER stressors, we wanted to verify that ER stress was being induced in yeast strains. A KAR2 assay was done in order to assess the levels of ER stress (Fig. 11). KAR2 is an ER lumen protein that is a stress marker which binds to unfolded or problematic proteins in ER. An increase in KAR2 levels is suggestive of an increase in ER stress. In immunoblotting of KAR2 we observe no noticeable difference between wildtype and mutant laforin and malin expressing yeast. ER stress is not being induced in these cells and especially in C266S mutant where foci are observed suggesting that foci formation is not enough to cause ER stress, which may prevent the observation of a toxic phenotype.
Figure 11: KAR2 levels in wild type yeast and mutants are consistent. Protein expression of laforin was induced by 2% galactose for 24 hours.
Discussion

Yeast models have been used as a powerful biochemical tool for neurodegenerative diseases such as Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, and ALS. These diseases share a set of similar characteristics with LD such as atypical protein assembly and induced cell death. A human transgene yeast proteotoxicity model for LD can give insight to the mechanisms of LD but also provide information for other neurodegenerative diseases. Lafora bodies (LB) in LD can serve as a potential model for other neurodegenerative diseases such as Lewy bodies in Parkinson’s disease or senile plaques in Alzheimer’s disease. Through this model we hope to elucidate the biochemical pathology in LD while gaining insight into the mechanisms of other neurodegenerative diseases.

In this paper, we report the elementary steps in the establishment of a yeast proteotoxicity model for Lafora disease. We have introduced wild-type laforin and malin into yeast along with three disease-causing mutations (K87A and C266S in laforin and D146N in malin). Through these mutations we hope to systematically determine which steps are being interrupted within the pathology of the disease. Phenotypic differences between mutations may reveal which step in the biochemical pathway of glycogen may be affected and may allow us to determine the pathological mechanism of this specific mutation. If no toxic phenotypic differences are observed between mutations then that could suggest that in our model, any break within the pathway rather than a specific interruption in synthesis or regulation can lead to disease pathology.

Specific mutations in each region have been thought to affect glycogen metabolism in different ways. A mutation in the carbohydrate binding motif (K87A) could cause laforin, which
is a regulator of stable glycogen synthesis, to be unable to bind to glycogen thereby promoting glycogen destabilization and ultimately its aggregation. The dual specificity phosphatase domain in laforin has been proposed to remove phosphates from glycogen and a mutation in this domain (C266S) could result in laforin binding to glycogen but being unable to remove the phosphate which could potentially lead to LB formation due to the destabilizing effects of a highly charged ion within the neutrally charged glycogen. The C266S mutation could cause laforin remain bound to glycogen. Malin has been proposed to ubiquitinate laforin targeting it for degradation. Without the DSP domain, laforin may lack the proper protein signaling to malin leading laforin to become fixed. Lastly, the mutation in the second NHL repeat in malin could lead to the laforin-malin complex being unable to form. NHL repeats have been proposed to function as protein-protein interaction sites and without these; malin may lack the necessary structures to bind to laforin. Similar to the effect of the C266S mutation in laforin, the D146N mutation in malin could lead to laforin being stuck on glycogen leading to aggregation. Through these mutations we hoped to explore the biochemical pathology of Lafora Disease.

We created a series of wildtype and mutant laforin and malin in centromeric plasmid strains and high expressing strains, and found that laforin dosing is important in generating a toxic phenotype. Colony dilution assay of wildtype human laforin and malin in centromERIC plasmids showed no growth defect between laforin and malin strains and W303α control whereas high-expressing strains showed a minor growth defect (Fig. 3 and Fig. 7B). Overexpression of laforin has been shown to cause aggregate formation in neuroblastoma cell lines and mice which is consistent with our results (Mittal et al. 2007). High-expressing strains in our model were necessary to achieve a mild toxicity effect in yeast most likely because S. cerevisiae have a constitutively active secondary autophagy pathway.
We also expressed a series of laforin and malin constructs to determine levels of laforin and found clean and robust expression throughout all strains (Fig. 4B and 7A). However, none of these strains generated the toxicity phenotype necessary for the LD proteotoxicity model. We next sought to determine conditions in which aggregate formation can be observed in our model. Overexpression of laforin has been shown to induce ER stress (Liu et al. 2009) and ER stress has been shown to accelerate the formation of LBs (Wang et al. 2013). We determined the baseline level of ER stress in our yeast mutants by assessing levels of KAR2. KAR2/BiP is an ER lumen chaperone and functions as an ER stress marker because it binds to misfolded proteins in the ER. If laforin or its mutants stresses the ER we would expect an increase in KAR2 levels. However, we see uniform expression throughout all laforin strains suggesting low levels of ER stress in our strains (Fig. 11). As laforin overexpression did not lead result in ER stress in yeasts, we instead attempted to induce ER stress chemically. We have shown that ER stress and proteasome inhibition does not lead to increase in laforin expression (Fig. 6 and Fig. 10). We then attempted a different approach using nitrogen starvation conditions to induce the formation of aggregates through glycogen level increase.

Nitrogen starvation increases basal levels of glycogen in S. cerevisiae (Küenzi and Flechter 1972). We assayed laforin levels at different points in nitrogen starvation conditions and found that the reg1Δ with two copies of laforin GFP tagged and one copy of malin had the strongest signal. Due to inconsistencies with the reg1Δ background strain, we sought to replicate this signal using conditions in W303α strains containing laforin and malin. However, despite identifying conditions which increased signal, we did not observe any corresponding growth defect phenotype or aggregate formation representative of LB. When we used high expression plasmids with nitrogen starvation conditions, we found a stronger laforin signal in these strains.
than in the centromeric plasmid containing strains which seems promising (Fig. 7A). Due to the increase in laforin levels in nitrogen starvation conditions, we wanted to examine strains in nitrogen starvation conditions with fluorescence microscopy for potential foci formation.

Due to previous studies suggesting a strong link between LB formation and LD pathology, foci formation was the main indication used to validate the usefulness of our model (Yokoi et al. 1968; Turnbull et al. 2012). Conditions under nitrogen starvation seemed the most promising for aggregate formation. The laforin C266S high expression strain, under nitrogen starvation conditions, exhibited irregular cell wall shape and strong laforin signal localization (Fig. 8). Without the nitrogen starvation conditions we were unable to observe foci in the strains (data not shown). With the laforin C266S mutation we have determined a possible condition in that foci are observed. Our results are inconsistent with previous results by Wang et al. (2013) who used ER stress to induce aggregation. We were able achieve conditions of initial LB formation through nitrogen starvation, suggesting that the formation of our aggregates functions through increases in glycogen, rather than ER stress.

Interestingly, we did not observe foci formation in the laforin K87A strain suggesting a difference between these two mutations. K87A is a mutation found on the CBM20 domain of laforin whereas C266S is a mutation found on the DSP domain which may suggests that a loss of function phosphatase activity in laforin may cause aggregate formation in our model but loss of function in carbohydrate binding ability may not. We have not yet characterized the specific mechanism of aggregation in C266S and are in the process if further investigation.

In order to evaluate the differences between C266S and K87A laforin mutations, we used a solubilization assay as a preliminary test to determine the relative levels of soluble and
insoluble laforin in these strains. We expected that because foci are only observed in the laforin C266S strain, we would observe higher levels of insoluble laforin relative to other strains. Surprisingly, laforin was observed to be soluble and insoluble in equal parts in all strains (Fig. 9). We reasoned that all strains may have high levels of insoluble laforin protein due to the cell fractionation procedure that may have resulted in the increased aggregation of laforin.

Here, we have identified a transient condition in that we are able to observe aggregate formation in the human transgene proteotoxicity model. We observed that nitrogen starvation conditions were able to induce foci formation in the laforin C226S strain but not the K87A or wildtype strain. ER stress was not able to accelerate the formation of aggregates as described in previous literature. Further work must be done to determine the mechanisms of aggregate formation in the laforin C226S strain and to optimize aggregate-forming conditions.

One approach to optimize aggregation condition we have considered is to down regulate autophagy pathways in yeast, which can be done genetically or pharmaceutically using rapamycin and dimebon. The use of a Δatg11 or Δatg1 strain would block the yeast CVT (cytoplasm to vacuole) autophagy or general autophagy pathways and should allow for the accumulation of cytosolic glycogen and potentially decrease the degradation of aggregates. Another potentially avenue is to use high levels of amino acids or cyclic adenosine monophosphate, which would down regulate vacuolar degradation inhibiting autophagy and also allowing for glycogen accumulation (Budovskaya et al. 2004). Through these strategies we hope to stabilize our observed aggregates for future characterization.
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