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Fission Yeast as a Model Organism for FUS-Dependent YCytotoxicity in Amyotrophic Lateral Sclerosis

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FISSION YEAST AS A MODEL ORGANISM FOR FUS-DEPENDENT CYTOTOXICITY IN AMYOTROPHIC LATERAL SCLEROSIS

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Alan J. Cone ENTITLED Fission Yeast as a Model Organism for FUS-Dependent Cytotoxicity in Amyotrophic Lateral Sclerosis BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Cone, Alan J. M.S., Department of Biological Sciences, Wright State University, 2016. Fission Yeast as a Model Organism for FUS-Dependent Cytotoxicity in Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative motor neuron disease that causes progressive paralysis and death by asphyxiation. There is no cure or effective treatment; however, previous research has identified several genes that appear related to the pathology of ALS. When mutated, these genes result in proteins that gain toxic functions and disrupt normal cellular processes. Fused in Sarcoma (hFUS) is a human transcription factor in the nucleus that binds to DNA and RNA. Mutations in hFUS are associated with both familial and sporadic cases of ALS, frontotemporal lobar degeneration (FTLD), and cancer. In ALS and FTLD, hFUS is mislocalized to the cytosol where it interacts with stress granules and forms aggregates. This aggregation and cytotoxicity has been previously studied in budding yeast; however, study in fission yeast may provide unique information. Fission yeast has several genetic advantages over budding yeast for modeling mammalian cell biology, such as 43% of genes contain introns and they posess a similar alternative splicing mechanism. Mammalian and fission yeast cells also both contain microRNA as well as similar cell growth cycles. In this project, I established a fission yeast model of hFUS and showed that hFUS is toxic when overexpressed in fission yeast. Both localization to the nucleus and mislocalization to the cytosol occurred during overexpression of hFUS. In addition, fission yeast homologues to previously identified budding yeast toxicity suppression proteins were able to suppress hFUS toxicity, suggesting the suppression mechanism is conserved.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ALS	Amyotrophic Lateral Sclerosis
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate with Nitro Blue Tetrazolium Chloride, for Western Blot Development
bECM32	*S. cerevisiae* DNA dependent ATPase/DNA helicase
bGAL1	*S. cerevisiae* galactokinase in galactose catabolism pathway
bNAM8	*S. cerevisiae* RNA binding protein
bSKO1	*S. cerevisiae* ATF/CREB family transcription factor
bVHR1	*S. cerevisiae* transcriptional activator
cDNA	Complementary DNA
CFP	Cyan Fluorescent Protein
DAPI	4',6-diamidino-2-phenylindole, stains A-T rich regions of DNA
DIC	Differential Interference Contrast Microscopy
EMM	Edinburgh Minimal Medium
fALS	Familial Amyotrophic Lateral Sclerosis
fATF1	*S. pombe* ATF/CREB family transcription factor
fCSX1	*S. pombe* RNA binding protein
FTLD	Frontotemporal Lobar Degeneration
fUPF1	*S. pombe* ATP-dependent RNA helicase
GFP	Green Fluorescent Protein
hATF2	Human ATF/CREB family transcription factor
hFUS	Human RNA binding protein
hTDP-43	Human DNA binding protein
hTRNAU1AP	Human tRNA Selenocysteine Associated Protein 1
hUPF1	Human ATP-dependent RNA helicase
hUPF2	Human nonsense-mediated decay transcript regulator
LB	Lysogeny Broth
LiOAc	Lithium Acetate
NLS	Nuclear Localization Sequence
NMT	No message in thiamne promoter
OD	Optical Density at 600 nanometers
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol 3350
pREP	*S. pombe* expression vector with a thiamine-repressible promoter

PVDF	Polyvinylidene fluoride
PY NLS	Proline/Tyrosine Nuclear Localization Sequence
RFP	Red Fluorescent Protein
sALS	Sporadic Amyotrophic Lateral Sclerosis
SDS	Sodium Dodecyl Sulfate
TBST	Tris-Buffered Saline with 0.1% Tween20 detergent
tk7	*S. pombe* strain with nonfunctional *ade6* and *leu2* genes
YES	Yeast Extract + Supplements
YFP	Yellow Fluorescent Protein

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I. INTRODUCTION

NEURODEGENERATIVE DISEASE

Neurodegenerative diseases are characterized by dysfunction and death of neurons. These diseases have a major impact on the lives and wellness of patients, and frequently involve muscular dysfunction, paralysis, drastic mood changes, or cognitive impairment. Well-known examples of neurodegenerative diseases include Alzheimer's, Parkinson's, Amyotrophic Lateral Sclerosis, and Huntington's. In all of these diseases protein aggregates are seen in degenerating neurons (Table 1). Some of the diseases may have limited treatment, but no neurodegenerative disease has a cure. Another hallmark of neurodegenerative disease is that typically only one type of neuron or few areas are affected while the rest of the nervous system is spared. This is of particular interest considering that mutated protein aggregates in several diseases can be detected throughout the brain, even though there is only an obvious degeneration in one region. While incidence rates across the entire population for neurodegenerative disease is 2%, the risk skyrockets to 20% at age 65[1-4]. Since it appears that neurodegenerative diseases have a similar pathology, current literature suggests that if one disease mechanism can be identified for one disease then that mechanism may be applicable to the other diseases, leading to a burst of effective treatments, or even cures for these debilitating maladies.

Table 1. **Neurodegenerative diseases and their incidence rates**.

Disease	**Associated Genes**	**Aggregates**	**Global Pop.**	**>65 Pop.**
Alzheimer's	*APP, Presenilin, APOEε4*	Yes	2%	11%
Parkinson's	*SNCA, PRKN, LRRK2*	Yes	0.2%	5%
ALS	*C9orf72, SOD1, FUS, TDP-43*	Yes	0.005%	4%
Huntington's	*HTT*	Yes	0.002%	3%

Neurodegenerative diseases are fairly rare across the global population, however as people age, especially above 65 years old the rates drastically increase such that almost one-fifth of people will face disease. Global population rates are based on incidence rates for all ages combined, while the older than 65 years old population is calculated based on the number of people with the disease living in the United States[1-4]. Protein aggregation has been observed in every disease, suggesting its potential role in disease.

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic Lateral Sclerosis (ALS) is the unifying name given to a set of related motor neuron diseases which appear to have a similar pathology, but differ in inheritance, age of onset, and progression. Motor neurons in the cerebral cortex and spinal cord progressively degenerate, resulting in muscle atrophy and paralysis. Eventually, those with ALS die from asphyxiation as the respiratory muscles become incapable of movement,[5] while neurons outside of the motor cortex and spinal cord appear to be left functioning. However, a few recent studies also suggest sensory neurons[6] and areas involved with grammar may be mildly affected[7], and certain mutations give rise to altered gene expression in a number of areas[8-9]. After diagnosis, progression of the disease can range from a few months to fifty years[10], although the majority of patients die within 39 months[5-6]. Ninety-five percent of cases are sporadic, meaning they are the first in their family to have the disease, while five percent are familial[11], and there is a global incidence rate of 5.4 per 100,000 people[3]. Since inherited types of ALS are clinically indistinguishable from sporadic cases, researchers have focused on inherited cases in an attempt to find a genetic cause within families that are prone to developing ALS.

GENETIC FACTORS OF ALS

While the exact mechanisms of disease are not clear, likely candidates include protein aggregation, oxidative stress, and extracellular signaling. The majority of the genetic mutations associated with ALS are autosomal dominant and directly related to protein aggregates (Table 2). The most frequently mutated genes related to both familial and sporadic ALS from most to least common are: *C9orf72*, *SOD1*, *FUS*, and *TDP-43*[12]. *C9orf72* mutants contain a hexanucleotide repeat expansion $(GGGGCC)_n$ in the intron

before its translation start codon, which when spliced out forms RNAs that can undergo translation themselves, even without their own start codon. This process likely results in interference with protein function, potentially by sequestering and forming protein aggregates[13-14]. *C9orf72* mutants are found in the majority of familial and sporadic cases with a known genetic component; however, due to the nature of the mutation and its recent discovery in 2011[13], studies are limited. *SOD1* was the first gene associated with ALS, linked to 14% of familial cases and up to 1% of sporadic cases[12]. SOD1 is normally able to convert superoxide radicals to molecular oxygen and hydrogen peroxide, serving as a critical antioxidant in cells[15]. Hypotheses quickly formed suggesting that ALS stemmed from excessive oxidative stress since the critical antioxidant was mutated. However, further studies show otherwise, as mice without functional *SOD1* are viable, although they age faster[15], and many *SOD1* mutants do not lose their enzymatic activity, suggesting ALS pathology is more complicated [16]. *TDP-43* and *FUS* were the next genes found to be associated with ALS in 2006 and 2007 respectedly, and each are linked to 4% of familial and 1% of sporadic ALS cases[17-18]. FUS and TDP-43 are both RNA-binding proteins, further supporting the hypothesis that ALS results from more than oxidative stress. FUS is thought to play a role in ALS pathology when mutants mislocalize from the nucleus to the cytosol and aggregate with prion-like properties[17]. Wild-type TDP-43 aggregates are found in many cases of ALS, although they are not thought to be causative as these aggregates co-occur with aggregates formed by other mutated genes related to ALS [18]. However, mutant TDP-43 is associated with ALS pathology as it forms aggregates in absence of mutations in other ALS-linked genes[18]. Loss-of-function TBK1 mutants, also appear at an incidence rate of 4% in familial ALS cases, but in less than 1% of sporadic cases[19]. *TBK1* is unique, as the majority of mutated genes associated with ALS show a gain of

toxic function rather than a loss of function[19]. Normally, TBK1 plays a role in autophagy mediation and physically interacts with optineurin, another protein associated with neurodegenerative disease. When TBK1 is mutated, it loses its interaction with optineurin required for mediating autophagy, leading to a disruption of homeostasis[19]. *TUBA4A,* associated with ALS in 2014, codes for major components of microtubules and is associated with 1.1% of familial ALS and less than 1% of sporadic cases[20]. When *TUBA4A* is mutated, Tubulin Alpha 4A Protein limits the ability of microtubules to repolymerize, resulting in a dysfunctional network of microtubules and cytosolic aggregates[20]. *TUBA4A* continues the trend of other frequently-seen mutations of forming aggregates, however it does not appear to influence RNA synthesis or processing as other mutants related to ALS may (Table 2).

Table 2. Genes related to at least 1% of familial Amyotrophic Lateral Sclerosis cases.

Gene[12][21-22]	**Function**[12][21-22]	**Aggregates**[22]	**RNA**	**fALS**[3][12]	**sALS**[3][12]
C9orf72	Guanine nucleotide exchange factor[23]	Yes[14]	Yes[14]	40%	7%
SOD1	Critical antioxidant	Yes[16]	Yes[9]	14%	1%
TDP-43	RNA Metabolism	Yes[17]	Yes[17]	4%	1%
FUS	RNA Metabolism	Yes[18]	Yes[18]	4%	1%
TBK1	Autophagy Mediator	Yes[19]	Yes[19]	4%	<1%
TUBA4A	Major component of microtubules	Yes[20]	No[20]	1.1%	<1%

Genes are listed from most to least common, with the majority related to abnormalities in RNA processing or production, protein aggregation, and are autosomal dominant in inheritance. Familial ALS (fALS) has more cases with identified mutations than sporadic cases do, however sporadic ALS (sALS) is harder to pinpoint mutations in. Many of these proteins also form aggregates themselves or are associated with wild-type TDP-43 and p62 inclusions.

FUSED IN SARCOMA (FUS)

In 2006 and 2007, focus shifted to novel mutations in genes associated with RNA processing, including *hTDP-43*[18] and *hFUS*[17]. hFUS contains an RNA-binding domain with no known ALS-associated mutations, a zinc-finger motif, a transcriptional activation domain with prion-like properties, and a proline/tyrosine nuclear localization sequence[17] (Figure 1). The majority of mutations related to ALS are missense mutations that do not cause a frame shift or truncate the protein (Table 3). The high mutation rate in the nuclear localization sequence (NLS) suggests that cytotoxicity may come from mislocalization and probably a gain of toxic function. If overexpressed, both wild-type and mutated hFUS will mislocalize to the cytosol and can form aggregates[17][24-25]; however, mutated hFUS will interact with stress granules to delay their assembly and accelerate their disassembly[26]. Mutations occur across the gene except in the RNA Recognition Motif and Zinc Finger, again suggesting core function still persists, but a mutation elsewhere results in new and toxic functionality. hFUS can be sequestered to the cytosol by excessive glutamate signaling, often a precursor to cell death in itself[27-28].

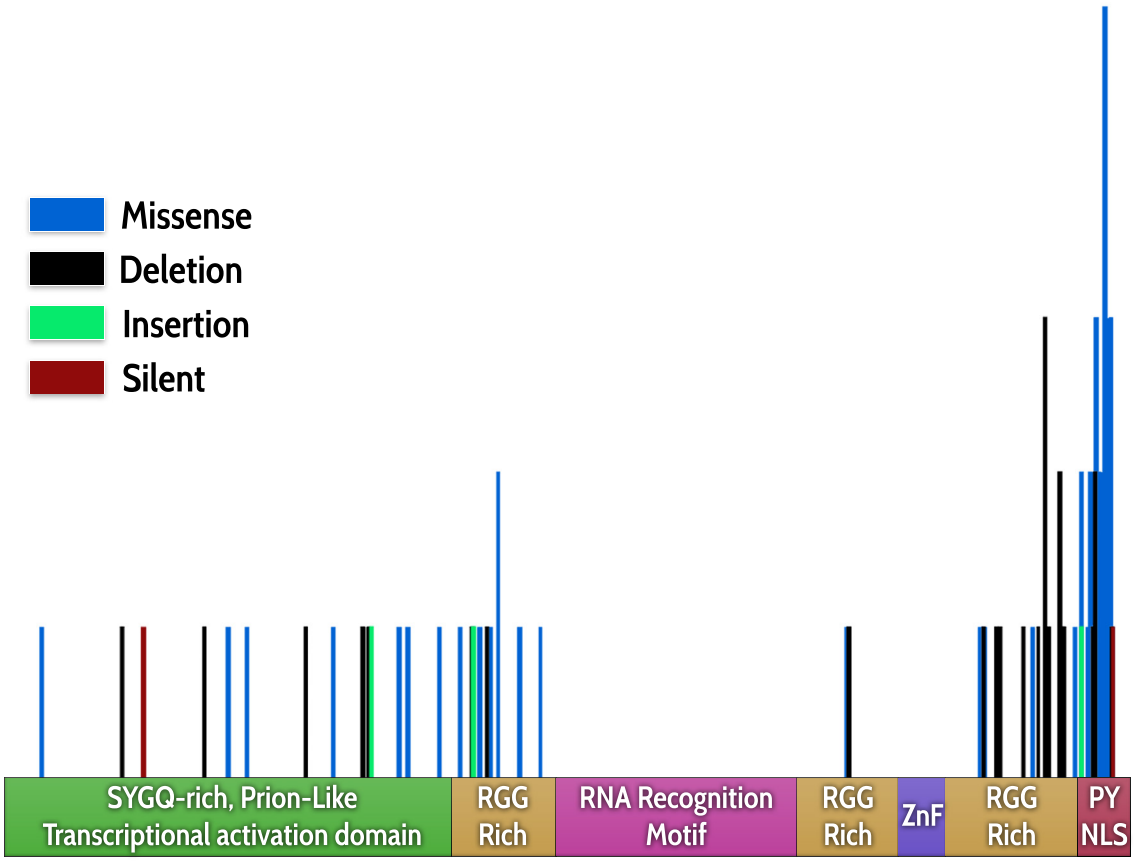

Figure 1. hFUS schematic and mutation histogram. ALS related mutation histogram paired with functional domains of the hFUS protein[17]. The number of different mutations at a given amino acid residue ranges from zero to five, and the most common type of mutation is missense followed by deletion with frameshift[29-37].

Table 3. **hFUS mutations and phenotypes**.

Mutation	Type	Functional Domain	Phenotype
P18S[29]	Missense	Prion-Like / SYGQ Rich	FALS, SALS
S57X[30]	Deletion	Prion-Like / SYGQ Rich	FALS, SALS
Y66Y[31]	Silent	Prion-Like / SYGQ Rich	SALS
S96X[32]	Deletion	Prion-Like / SYGQ Rich	FALS, Cognitive Impairment
P106L[29]	Missense	Prion-Like / SYGQ Rich	ALS / FTLD
S115N[29]	Missense	Prion-Like / SYGQ Rich	SALS
G144-Y149X[29]	Deletion	Prion-Like / SYGQ Rich	FALS
G156E[30]	Missense	Prion-Like / SYGQ Rich	FALS with Dementia
G171-G175X[33]	Deletion	Prion-Like / SYGQ Rich	ALS, Not Specified
G174-G175[34]	Insertion	Prion-Like / SYGQ Rich	FALS
G174-G175X[32]	Deletion	Prion-Like / SYGQ Rich	FALS, ALS / FTLD
G187S[29]	Missense	Prion-Like / SYGQ Rich	FALS
G191S[30]	Missense	Prion-Like / SYGQ Rich	FALS
G206S[32]	Missense	Prion-Like / SYGQ Rich	ALS / FTLD
R216C[30]	Missense	RGG Rich Region 1	FALS
G222insG[29]	Insertion	RGG Rich Region 1	FALS
G223-G226X[32]	Deletion	RGG Rich Region 1	FALS
G225V[30]	Missense	RGG Rich Region 1	FALS
G230C[30]	Missense	RGG Rich Region 1	FALS
G230X[29]	Deletion	RGG Rich Region 1	SALS
R234L[30]	Missense	RGG Rich Region 1	FALS
R234C[30]	Missense	RGG Rich Region 1	FALS
R244C[30]	Missense	RGG Rich Region 1	FALS
M254V[20]	Missense	RGG Rich Region 1	ALS / FTLD
G399V[29]	Missense	RGG Rich Region 2	SALS
S402-P411Xins-GGGG[29]	Deletion and Insertion	RGG Rich Region 2	FALS
S462F[29]	Missense	RGG Rich Region 3	FALS
M464I[29]	Missense	RGG Rich Region 3	SALS
G466VfsX479[29]	Deletion, Frameshift	RGG Rich Region 3	SALS

G472VfsX527[29]	Deletion/ frameshift	RGG Rich Region 3	FALS
G474VfsX528[29]	Deletion/ frameshift	RGG Rich Region 3	FALS
Y485AfsX514[32]	Deletion/ frameshift	RGG Rich Region 3	FALS
R487C[29]	Missense	RGG Rich Region 3	SALS
G492EfsX527[29]	Deletion/ frameshift	RGG Rich Region 3	SALS
R495QfsX527[29]	Deletion/ frameshift	RGG Rich Region 3	SALS
R495X[32]	Deletion	RGG Rich Region 3	FALS, SALS
R495EfsX527[32]	Deletion/ frameshift	RGG Rich Region 3	FALS
G497AfsX527[32]	Deletion/ frameshift	RGG Rich Region 3	FALS
R502fsX15[29]	Deletion/ frameshift	RGG Rich Region 3	SALS
D502EfsX516[29]	Deletion/ frameshift	RGG Rich Region 3	FALS
G503WfsX12[29]	Deletion/ frameshift	RGG Rich Region 3	SALS
G504WfsX12[29]	Deletion/ frameshift	RGG Rich Region 3	SALS
G507D[29]	Missense	RGG Rich Region 3	FALS, SALS
K510E[29]	Missense	NLS	FALS
K510R[29]	Missense	NLS	FALS
K510WfsX517[32]	Deletion/ frameshift	NLS	FALS
S513P[29]	Missense	NLS	FALS
R514S[30]	Missense	NLS	FALS, SALS

R514G[35]	Missense	NLS	FALS
G515C[30]	Missense	NLS	FALS
E516V[29]	Missense	NLS	FALS, SALS
H517D[33]	Missense	NLS	FALS
H517Q[30]	Missense	NLS	FALS
H517P[29]	Missense	NLS	FALS
R518Xfs519[36]	Deletion/ frameshift	NLS	Juvenile ALS (SALS)
R518K[30]	Missense	NLS	FALS
R518G[29]	Missense	NLS	FALS, SALS
Q519IfsX527[29]	Deletion/ frameshift	NLS	FALS
Q519X[29]	Deletion	NLS	FALS, SALS
R521S[37]	Missense	NLS	FALS
R521C[32]	Missense	NLS	FALS, SALS
R521G[32]	Missense	NLS	FALS, SALS
R521H[32]	Missense	NLS	FALS, SALS
R521L[32]	Missense	NLS	FALS, SALS
R522G[30]	Missense	NLS	FALS
R524T[30]	Missense	NLS	FALS
R524W[29]	Missense	NLS	FALS
R524S[32]	Missense	NLS	FALS
P525P[31]	Silent	NLS	SALS
P525L[34]	Missense	NLS	FALS, SALS
X527YextX[29]	Extension	NLS	FALS
r.spl[32]	Splicing	Splicing	FALS

Previously reported hFUS mutations, type of mutation, related domain, and type of ALS resulting from the mutation.

YEAST MODEL OF FUS CYTOTOXICITY

Cytotoxic behavior of hFUS has been demonstrated in budding yeast (*Saccharomyces cerevisiae*)[24-25]. Shulin Ju integrated a sequence coding for hFUS fused with Green Fluorescent Protein (GFP) on the N-terminus into the budding yeast's genome. Inducible expression of hFUS could be controlled extracellularly by growing the yeast on medium containing 2% galactose. Glucose represses expression while galactose activates it in a tightly-regulated fashion by interacting with the *bGAL1* promoter. This simple method of induction makes the *bGAL1* promoter a common choice for protein expression studies in budding yeast. When *hFUS* is expressed in budding yeast, the protein localizes to the cytoplasm and forms aggregates that are visualized as punctate structures (Figure 2). hFUS aggregates are then thought to play a role in cytotoxicity as spotting assays show that in budding yeast even one integrated copy of *hFUS* is enough to be extremely toxic (Figure 3). Aaron Gitler and colleagues performed similar experiments, notably showing that *hFUS* will co-localize with p-bodies and stress granules, suggesting this interaction is part of the pathology (Figure 4)[25]. In general, *S. cerevisiae* is a well established model organism for science and recreation, with a long history of use; however, fission yeast (*Schizosaccharomyces pombe*) offers several unique advantages (Tables 4-8), including undergoing a cell cycle similar to mammalian cells[38-40], mammalian-like alternative splicing mechanisms[41], and the presence of microRNA[39][41].

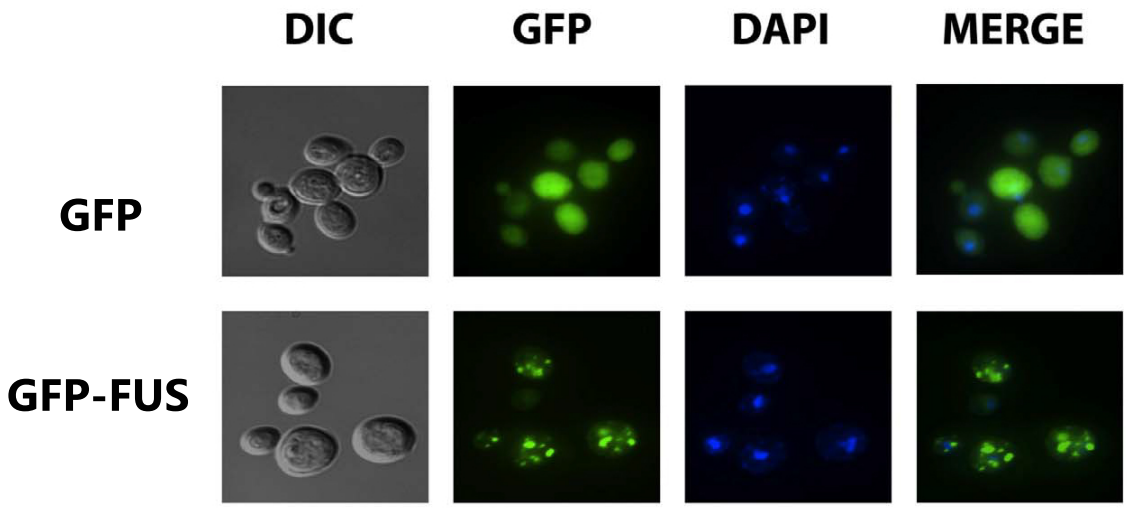

Figure 2. **FUS aggregates and mislocalizes in budding yeast.** Localization and appearance of GFP alone (top) and GFP-FUS (bottom) proteins are viewed via fluorescence microscopy. Differential interference contrast (DIC) microscopy was used to visualize the yeast cells. 4',6-diamidino-2-phenylindole (DAPI) is a stain that binds to A-T rich regions of DNA to make the nucleus fluoresce. *GFP* codes for a protein that fluoresces green and can be seen in fluorescence microscopy. GFP is expressed ubiquitously throughout the cell; however, when it is attached to FUS it is unable to be localized to the nucleus, instead forming aggregates in the cytosol, as indicated by several bright green spots (Adapted from Ju et al., 2011).

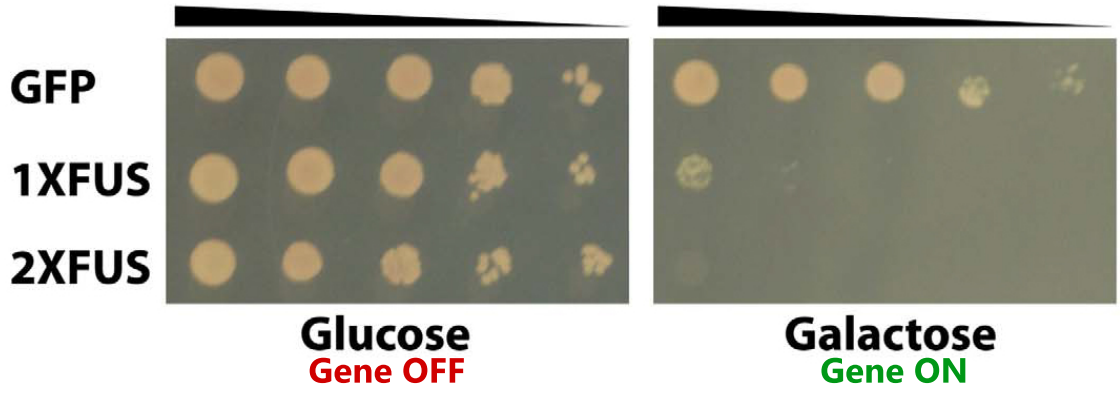

Figure 3. **FUS is toxic when overexpressed in budding yeast**. Ten-fold serial dilutions (most to least concentrated yeast drops) with yeast containing one or two copies of FUS were grown on glucose-containing and galactose-containing media. Galactose induces expression of FUS, which results in the lethal phenotype. Pictures were taken after two days of growth at 30°C (Adapted from Ju et al., 2011).

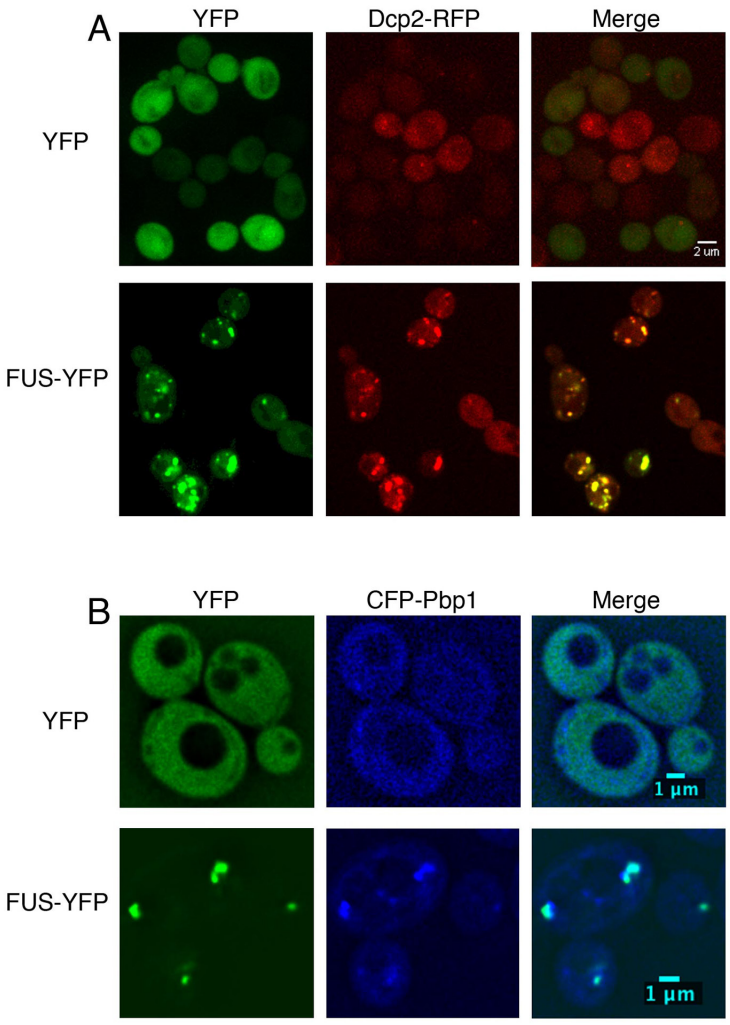

Figure 4. **FUS co-localizes with p-bodies and stress granules**. Fluorescence microscopy images showing the localization of FUS along with a p-body (A) or stress granule (B) markers strongly suggest that FUS contributes to both p-body and stress granule composition. (A) Fluorescence microscopy images showing FUS tagged with Yellow Fluorescent Protein (YFP), shown in green, co-localizing with Dcp2, a p-body marker, tagged with Red Fluorescent Protein (RFP). Any areas on the merged panel showing yellow suggests both proteins are in the same place. (B) Fluorescence microscopy image showing FUS tagged with YFP, shown in green, and stress granule marker Pabp1 tagged with Cyan Fluorescent Protein (CFP), shown in blue. Light blue regain on the merge suggest that both proteins are in the same location (Adapted from Sun et al., 2011).

Table 4. **Genetic makeup of *S. cerevisiae* and *S. pombe***.

Attribute	***S. cerevisiae***	***S. pombe***
Sequenced genome	Yes[42]	Yes[43]
Chromosomes	16[42]	3[43]
Number of Genes	~5,600[42]	~4,900[43]
Genes with Introns	5%[43]	43%[43]
Centromeres	Small[42][44]	Elongated with Repeats[43-44]

Both yeasts have a sequenced genome, however *S. pombe* contains many genes with introns while *S. cerevisiae* only has relatively few.

Table 5. **Cell cycle attributes of *S. cerevisiae* and *S. pombe***.

Attribute	***S. cerevisiae***	***S. pombe***
Mitotic Spindle Present	G1/S to Division[39]	M Phase[38]
Chromosome Condensation	Little[39]	Significant[39]
Cell Division Regulation	G1 to S[39]	G1 to S and G2 to M[39]
Generation Time	1.25 - 2 hours[42]	2 - 4 hours[43]
Synchronize Cells Based on Cell Cycle Phase	Bud Emergence[39]	Centrifugation as size indicates age[39]

S. pombe has a cell cycle similar to the one found in mammalian cells, so labs with interests in cell cycle regulation usually use *S. pombe* rather than *S. cerevisiae* as a model organism.

Table 6. Transcription and translation attributes of *S. cerevisiae* and *S. pombe*.

Attribute	***S. cerevisiae***	***S. pombe***
Promoter Region Location	10 base pairs upstream[39]	25-35 base pairs upstream[39]
Mammalian Promoter Active	No[39]	Yes including HIV1, SV40, and CaMV35S[39]
Yeast Artificial Chromosome	Yes[39]	Yes[45-47]
Human gene expression	Yes	Yes
Human gene substitution	Yes	Yes[48]
Structure of Small Nuclear U2 and U6 RNA	Similar to Fungi[39]	Similar to higher eukaryotes[49] [50]
RNA 5' Splicing Site	Similar to Fungi[39]	Like Mammals[39]
RNA 3' End Formation	AT-Rich; lack AAUAAA motif[39]	AT-Rich; lack AAUAAA motif[39]
Alternative Splicing	Similar to Fungi[50]	Similar to higher eukaryoes[50-52]
Codon usage	Similar to Fungi[53]	Similar to Fungi[53]
MicroRNAs	No / Not Detected	Yes[41][51]

S. pombe is similar to mammalian cells in its processes from initiation of gene transcription to post-translational processing.

Table 7. **Signaling in *S. cerevisiae* and *S. pombe***.

Attribute	***S. cerevisiae***	***S. pombe***
Drug-Metabolizing Enzyme Expression (cytochrome P450)	Yes, 1% of Cell[39]	Yes, >10% of Cell[39]
Heat Shock Response	Heat Shock Factor constitutively bound to Heat Shock Element[54]	Heat Shock Factor responsive to heat shock[54]
Recognition of ER retention KDEL signal	No[39]	Yes[39]
Classic NLS Recognition	Yes[39]	Yes[39]
PY NLS Recognition	No[24]	Yes (this study)

Cellular stress studies and protein folding likely would differ based on the yeast used due to a difference in the heat shock response.

Table 8. **Other properties of *S. cerevisiae* and *S. pombe***.

Attribute	***S. cerevisiae***	***S. pombe***
Commonly Used Inducible Promoter	Tight Control, in presence of galactose using *GAL1* promoter[24-25]	Tight control, delayed repression (16 hours) with thiamine using *nmt* promoter[55]
Age Determination	Bud Emergence[39]	By Size[39]
G-protein coupled receptor	Limited[39]	Yes, can perpetuate signals[39]
Sexes	a and α[56]	h+, h-, and h90[39]
Switching Sex	Both[56]	h90 only[57]
Mating Induction	Rich Medium[39]	Nitrogen Starvation[39]
Mammalian-like apoptosis	No[58]	Yes[58-59]
Cell Fusion with Mouse Cells	No[39]	Yes[39]

Current methods of working with yeast favor *S. cerevisiae* as it is more commonly used; however, small changes to protocols can adapt them for *S. pombe*. *S. pombe*'s ability to utilize G-protein coupled receptors, undergo a mammalian-like apoptosis, and fuse with mouse cells suggests that many characteristics of *S. pombe* are conserved in mammalian systems.

FISSION YEAST

Budding yeast are a commonly used model with a wide range of protocols and genetic tools while fission yeast models typically have methods derived from budding yeast versions. Despite a lack of established protocols, the genetic and cellular advantages of fission yeast provide reason to increase their use as a model for studying neurodegenerative disease-related proteins. Presence of introns, alternative splicing machinery, and microRNA allow fission yeast to regulate and alter gene expression at more levels than just transcription and translation, along with higher precision. Similar to mammals, fission yeast produce alternatively spliced mRNAs. Fission yeast also contain a well-formed Golgi apparatus[39] permitting post-translational modifications such as prenylation and addition of a terminal galactose, resulting in profound differences in the localization of proteins[60]. As an example, assume there is a gene that codes for the word and punctuation "Awesome." Budding yeast would be able to translate this to always make the word "Awesome." If that "Awesome" gene however is set up so the DNA is "Awe-[intron]-some", then fission yeast would be able to transcribe that same gene into "Awe," "Some," and "Awesome." In addition to the alternative forms, a greater number of possible post-translational modifications in fission yeast could change "Awesome" to "Awesome!" or "Awesome?" resulting in profoundly different versions of the same gene.

FISSION YEAST HOMOLOGUES OF ECM32, NAM8, & SKO1

After assessing cytotoxicity of hFUS, Ju and colleagues also performed a library overexpression screen to identify budding yeast genes that when co-expressed with hFUS would suppress hFUS cytotoxicity[24]. Ninety-five percent of the budding yeast genome was screened, yet only a few genes appeared to suppress hFUS cytotoxicity, including

bECM32, bNAM8, and bSKO1 (Tables 9 & 10). bECM32, a homologue to the human protein hUPF1, is associated with nonsense mediated decay. bNAM8, which is potentially regulating the *bGAL1* promoter rather than suppressing hFUS toxicity, has the human homologue hTRNAU1AP which can bind to RNA but its exact function is not well understood. bSKO1 is a transcription factor without a human homologue, however there is a fission yeast homologue fATF1. Fission yeast also contain homologues to these genes with equal or higher identity to human homologues as confirmed by Protein BLAST (Figure 5). Functionality among the homologues in each organism is comparable, and they appear to play similar roles, which is beneficial in determining how they manage to suppress FUS cytotoxicity. Shulin Ju also performed a Western Blot to see if suppression was caused by altered hFUS expression, but with every suppression gene, hFUS protein level was not significantly changed, suggesting another mechanism is responsible for toxicity suppression[24].

Table 9. Budding yeast genes that suppress cytotoxicity of hFUS.

Gene	Function	Human Homologue	Function
bECM32	Translation termination	UPF1	RNA Decay
bNAM8	RNA binding protein	TRNAU1AP	Unknown; RNA Binding
bSBP1	RNA Binding Protein	RBM14	Nuclear receptor coactivator
bSKO1	Transcription factor	None	N/A
bVHR1	Transcription activator	None	N/A

These genes are all able to suppress hFUS cytotoxicity when over expressed without altering the levels of hFUS. Full-length hUPF1 and hUPF2 also rescue hFUS cytotoxicity in a budding yeast model[24].

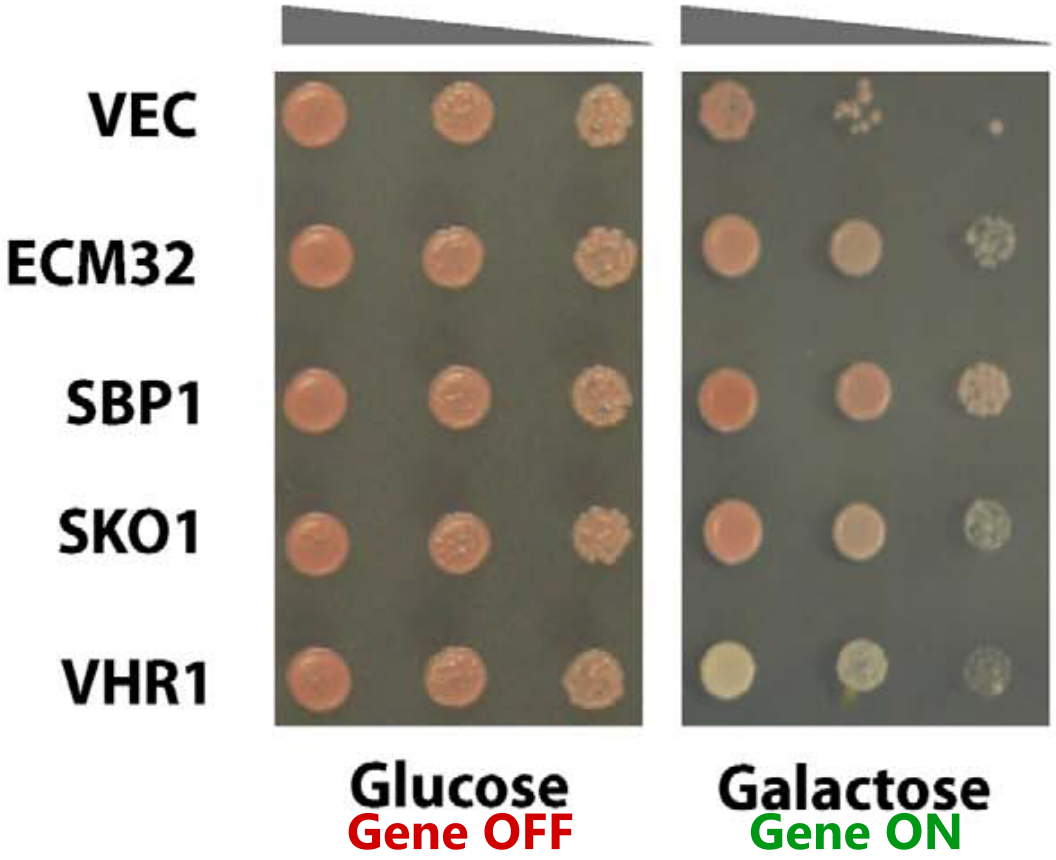

Figure 5. Expression of bECM32, bSBP1, bSKO1, and bVHR1 suppresses hFUS toxicity. When both hFUS and rescue genes are expressed, hFUS toxicity is reduced. The mechanism of how these genes suppress toxicity is unknown, but hFUS levels are not decreased and aggregates still form (Adapted from Ju et al., 2011).

Table 10. *S. pombe* and human homologues to *S. cerevisiae* toxicity suppression proteins.

Suppression Genes			**Identity**		
S. cerevisiae	***S. pombe homologue***	***H. sapiens homologue***	***S. cerevisiae to S. pombe***	***S. pombe to H. sapiens***	***S. cerevisiae to H. sapiens***
bNAM8	fCSX1	hTRNAU1AP	35% (5e-51)	44% (3e-40)	48% (2e-26)
bSKO1	fATF1	hATF2	44% (2e-07)	44% (2e-19)	*none*[24]
bECM32	fUPF1	hUPF1	34% (2e-61)	59% (0.0)	35% (7e-66)

S. pombe homologues to the genes identified in the *S. cerevisiae* screen add new possibilities to finding and understanding the mechanism of how they are able to suppress FUS cytotoxicity, along with uncovering relevant human homologues.

II. SPECIFIC AIMS

AIM 1: To establish fission yeast (*S. pombe*) as a model organism for FUS-dependent cytotoxicity.

AIM 2: To assess the ability of *S. pombe* homologues of genes previously identified in *S. cerevisiae* to suppress FUS cytotoxicity.

III. MATERIALS AND METHODS

FISSION YEAST STRAINS AND MAINTENANCE

Fission yeast strain tk7 / wild-type, provided as a generous gift from Dr. Yong-jie Xu's lab (Wright State University), was streaked out on Yeast Extract + Supplements (YES) medium. Glycerol stocks were prepared by inoculating a toothpick full of fission yeast in 5 mL liquid YES, growing overnight at 30°C and shaking at 11 x *g*, then the next morning mixing 600 µL of liquid culture with 400 µL of 50% glycerol and storing the mixture at -80°C. When selecting for fission yeast with a specific vector or maintaining the vector, Edinburgh Minimal Medium (EMM) (Sunrise Science Products, San Diego, CA) without leucine or adenine was used to grow the yeast.

VECTORS

S. pombe vectors pREP1, pREP41, and pREP81 (Figure 6) were provided as a generous gift from Dr. Yong-jie Xu's lab as either a bacterial culture containing the vector or pure vector. A version of pREP1 with an adenine selective marker was provided by Dr. Taro Nakamura (Osaka City University) as a pure vector. Pure vectors were transformed into competent *E. coli* cells as previously described[61-62] and selected for by growing at 37°C overnight on Lysogeny broth (LB) with added ampicillin to a final concentration of 100 µg / mL. The next day individual colonies were selected from the plate and inoculated in 5 mL of liquid LB with ampicillin overnight at 37°C, shaking at 11 x *g*. The next morning, 600 µL of liquid culture was mixed with 400 µL 50% Glycerol and stored at -80°C, and vector extraction was performed using QIAGEN (Germantown, MD)[63] or Zymo Research (Irvine, CA)[64] miniprep kits.

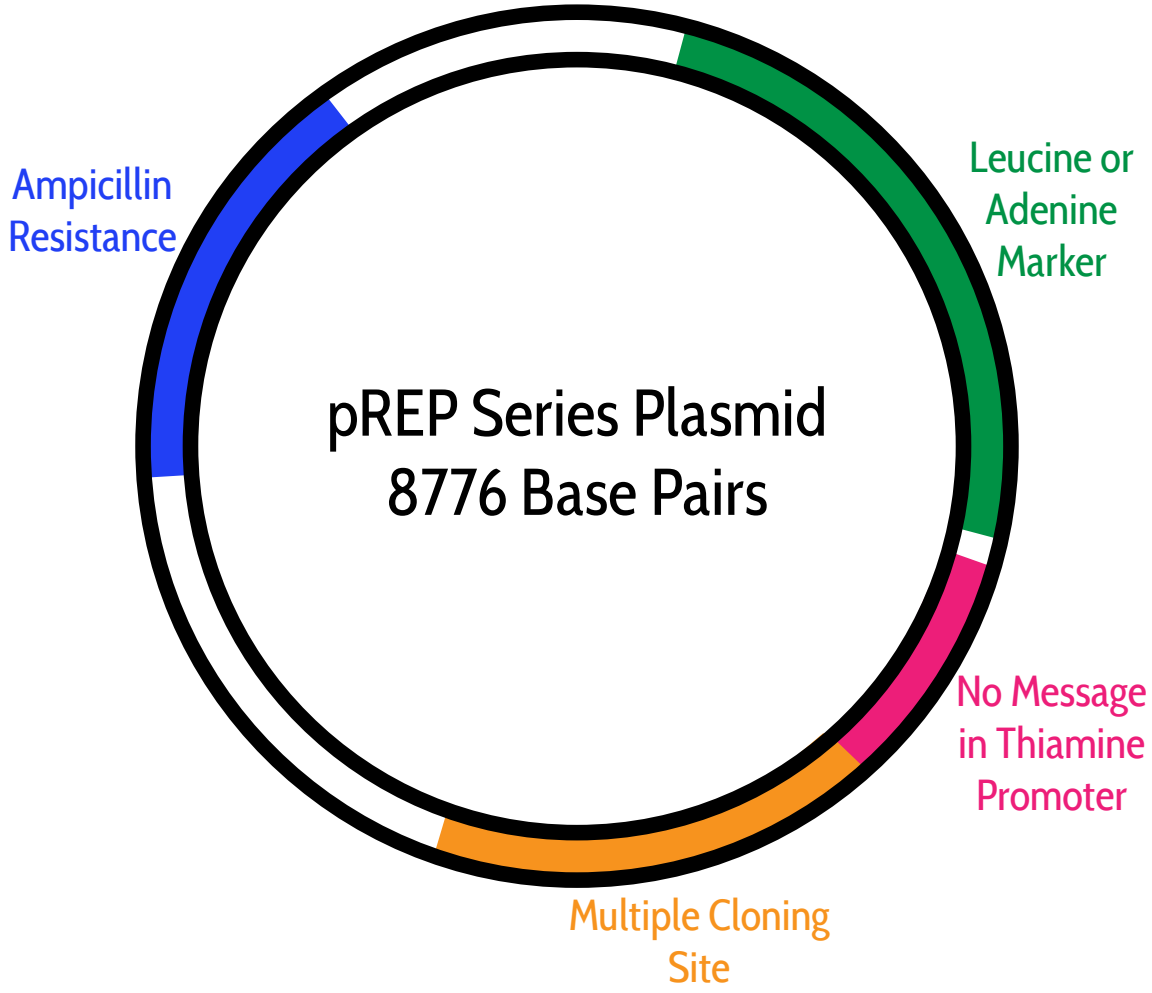

Figure 6. **pREP Series Vector Map.** The pREP series plasmids are 8.7 kb in length and contain a leucine (*leu2*) or adenine (*ade6*) selective marker, ampicillin resistance gene, repressible promoter, and multiple cloning site. The ampicillin resistance enables bacteria containing the vector in medium containing ampicillin. The *leu2* and *ade6* genes enable selection of yeast with the vector. Since the genes code for proteins that can synthesize a missing required amino acid, only the yeast that grow are those which have the vector. The no message in thiamine (nmt) promoter is tightly controlled and repressed by adding 10 μM thiamine to growth media.

MOLECULAR CLONING

hFUS, *GFP-hFUS*, *fCSX1*, *fATF1*, and *fUPF1* were placed into pREP series vectors for expression in *S. pombe*. *hFUS* was amplified out of the budding yeast vector pRS303GAL1-FUS via a 25 μL polymerase chain reaction (PCR) using Pfu DNA polymerase with the forward primer CACAGTGTCGACATGGCCTCAAACGATTATACCC and reverse primer CACATGGATCCTTAATACGGCCTCTCCCTGC to add SalI and BamHI restriction sites respectively. In order to clone *hFUS* into pREP1 vector with the *ade6* gene, the alternative reverse primer CACATATTTAAATTAATACGGCCTCTC-CCTGC was used to add a SwaI restriction site. After PCR, gel electrophoresis[65] was done to verify that the DNA was properly amplified. In order to purify the new PCR product, Zymoclean Gel DNA Recovery Kit was used[66]. Once a pure product was obtained, the product along with pREP1, pREP41, and pREP81 were digested with SalI (Promega) for two hours in NEBuffer 3.1 (New England Biolabs), and then everything was cleaned and concentrated with the Zymo Research DNA Clean & Concentrator kit[67] immediately after the restriction digest. The cleaned and concentrated DNA for each vector and PCR product was then digested for two hours in BamHI-HF (New England Biolabs) in NEBuffer 4.1 (New England Biolabs), or alternatively SwaI in NEBuffer 3.1 at 25°C (New England Biolabs) for cloning into the adenine vector, and then cleaned and concentrated once more. At this point, typical concentration of the vectors was 75 ng/μL and the gene was around 11 ng/μL. T4 DNA Ligation (New England Biolabs) was performed at room temperature for two hours, or overnight for the adenine selection vector, using a 3:1 molar ratio of vector to insert. After ligation, the new pREP1-FUS, pREP41-FUS, and pREP81-FUS constructs were transformed into chemically competent *E. coli* cells[61], plated on LB plates with ampicillin and grown overnight at 37°C. The next day, a single

colony from each plate of without satellite colonies was inoculated in liquid LB with ampicillin, grown at 37°C with shaking at 11 x *g* overnight. 600 µL of the liquid culture was removed and used to make a 1 mL glycerol stock. The vector DNA was extracted from the remaining 4.4 mL of culture using a QIAGEN Miniprep Kit[63]. Once the new vectors had been purified, microplate DNA quantification readings were taken to assess the concentration of each, typically between 200-800 ng/µL. Confirmation of successful ligation of *hFUS* into the pREP vectors was done by a three hour restriction digest with EcoRI (New England Biolabs) in NEBuffer 4.1 along with a PCR using the previously listed primers.

In order to clone *GFP-hFUS* into the pREP series, two additional restriction enzyme sites had to be added as all of the sites already on the pREP vector also existed in either *GFP* or *hFUS*. The restriction enzyme sites NheI and BglII were selected as both of those enzymes were available and recently purchased from New England Biolabs. A 25 µL PCR with pREP1 was done to amplify the multiple cloning site and add NheI and BglII sites using forward and reverse primers CACATGGATCCGCTAGCCCGGGTA-AAAGG and CACATGGATCTCAGATCTGCATTACTAATAGAAAGG respectively. The 5' end of the forward primer also included a BamHI site while the 5' end of the reverse primer included a SacI site so the amplified product could be digested then ligated into the vectors. After PCR, the amplified DNA was excised from a gel and purified using the Zymo Research Gel DNA Recovery kit[66], then the purified multiple cloning site along with pREP1 was digested with BamHI-HF in NEbuffer 4.1 (New England Biolabs) for two hours at 37°C. Following digestion, both the multiple cloning site and vector were cleaned and concentrated[67], then digested with SacI in CutSmart Buffer (New England Biolabs) for two hours then cleaned and concentrated as previously described.

Using a 3:1 molar ratio of vector to insert, the new multiple cloning site was ligated into pREP1 using T4 DNA Ligase at room temperature for two hours, followed by inactivation of the ligase at 65°C for 10 minutes prior to doing a transformation into chemically competent *E. coli.* Transformants were grown, selected for, then glycerol stocks were made as previously described in this document. Successful addition of restriction enzyme sites was verified by performing a two hour digest with NheI in CutSmart buffer and another two hour digest with BglII in NEBuffer 3.1, then compared on a gel to pREP without these sites. Both enzymes were able to linearize the new vector while leaving the control untouched, indicating successful ligation.

GFP-hFUS was amplified out of the vector pDEST53-GFP-FUS via a 25 μL PCR using the forward primer CACATGGCTAGCATGGCCAGCAAAGGAGAAG with a NheI site and the reverse primer CACGTGAGATCTTTAATACGGCCTCTCCCTGC with a BglII site. Successful amplification was confirmed by running a gel, and then as before the DNA was excised from the gel and purified. Purified *GFP-hFUS* and pREP1 with added restriction enzyme sites were digested with NheI in CutSmart buffer (New England Biolabs) for two hours, cleaned and concentrated, then digested with BglII in NEBuffer 3.1. Another round of cleaning and concentrating was done prior to setting up an identical T4 DNA Ligation and chemically competent *E. coli* transformation, selection, and vector purification as previously performed. *GFP-hFUS* was confirmed to be in pREP1 by amplifying it out of the newly purified vector via PCR with the *GFP-hFUS* primers and then running on it a gel.

Since *fATF1, fCSX1,* and *fUPF1* were not available in the lab, they needed to be amplified from genomic DNA. Genomic DNA extraction was performed with a single-tube Lithium Acetate (LiOAc)-SDS lysis as previously described[68]. *fATF1*, *fCSX1,*

and *fUPF1* were amplified using the primers and conditions described in Table 10. After amplification, *fATF1, fCSX1,* and *fUPF1* along with the pREP1 vector were digested in the same reaction conditions as previously used for *hFUS* and *GFP-hFUS*, but using the restriction enzymes mentioned in Table 10 for each gene, with XmaI digestion performed in CutSmart buffer (New England Biolabs). After digesting with each restriction enzyme, and cleaning and concentrating the DNA between and after digestions, *fATF1, fCSX1,* and *fUPF1* were ligated into pREP1 with T4 DNA Ligase, then transformed and amplified in *E. coli* as done previously for *hFUS* and *GFP-hFUS*. After transformation, vectors were extracted and purified from *E. coli* as before, and then confirmed with both a restriction digest and PCR. Twenty-percent glycerol stocks were made upon confirmation of the gene's ligation into the vector.

Table 11. Primers for fission yeast genes *fATF1*, *fCSX1*, and *fUPF1*.

Gene	Forward Primer	Reverse Primer	Added Sites
fATF1	CACATTGTCGACATGTC-CCCGTCTCCCGTC	GCAAGACCCGGGCTAGTAC-CCTAAATTGATTCTTTGAGC	SalI, XmaI
fCSX1	GCAGTCGTCGACATGTC-TATTGACTGCCTTTATCGC	GCAAGACCCGGGTTATGAATC-GCGTGACAAGC	SalI, XmaI
fUPF1	GGAGTCGTCGACAT-GTCTTTAGGGCTA-CAACCTAATAAT	AATCGAGGATC-CCTAGAACCTAGTAGGTTC-GTCGAACT	SalI, BamHI

These genes are the fission yeast homologues to budding yeast genes identified in a screening to suppress *hFUS* cytotoxicity. *fATF1* and *fCSX1* were amplified with thirty cycles at an annealing temperature of 58°C for thirty seconds and elongation step at 72°C for two minutes and thirty seconds with a final extension time of three minutes. Due to its larger size, *fUPF1* had the same reaction parameters as *fATF1* and *fCSX1*, except the extension time was three minutes and the final extension time was three minutes and thirty seconds.

YEAST TRANSFORMATION

Transforming *S. pombe* with pREP series vectors was done by adapting the budding yeast One-Step Transformation Lithium Acetate method[69] to fission yeast. *S. pombe* was grown overnight on a plate containing EMM. The next day, 90 μL of 50% polyethylene glycol 3350 (PEG) and 10 μL of 1 M LiOAc was added to a 1.5 mL microcentrifuge tube. Fission yeast were then picked up on a toothpick from the EMM plate and resuspended in the PEG and LiOAc solution. One microgram of DNA was added to the 1.5 mL microcentrifuge tube, then the tube was briefly vortexed. The 1.5 mL microcentrifuge tube was then incubated at 43°C for 40 minutes with brief vortexing every 10 minutes. After incubation, the yeast were plated onto EMM dropout plates of the desired selection amino acid (leucine or adenine) and thiamine if needed to repress gene expression, then placed in an incubator to grow at 30°C for four to six days. Once colonies grew, they were picked up off of the plate and resuspended in 5 mL of the same type of liquid growth medium and grown overnight to confirm the presence of vector.

YEAST CRUDE PROTEIN EXTRACTION

S. pombe was grown to the mid-exponential phase in liquid medium then normalized to an optical density (OD_{600}) of 2 in 1 mL of H_2O. The cells were then centrifuged at 5,440 x *g* for two minutes, resuspended in 100 μL of 0.2 M NaOH, and incubated on ice for 15 minutes. Once again the yeast was centrifuged at 5,440 x *g* for two minutes, but then the pellet was resuspended in 100 μL of Yeast Protein Extraction Sample Buffer (2.4 mL 1 M Tris at a pH of 6.8, 20 mL 50% Glycerol, 8 mL 10% SDS, 4 mL of 1% Bromophenol Blue, and 65.6 mL H_2O for 100 mL of buffer). 0.4 μL of 2-mercaptoethanol was added, then the mixture was boiled for five minutes at 95°C followed by a brief

vortexing and three minute centrifugation at 6,720 x *g*. The cells were then either stored at -80°C or loaded directly into an acrylamide gel to minimize protease activity.

GEL STAINING AND WESTERN BLOT

Two gels each composed of a 4% acrylamide / bis-acrylamide (29:1) stacking gel and 10% acrylamide / bis-acrylamide (29:1) separating gel were used to determine that protein was extracted from yeast and confirm the expression of a specific protein. Protein samples were loaded into the wells and the gel was run for five minutes at 50 volts, then the voltage was increased to 150 volts until the PageRuler Plus ladder[70] reached the bottom of the gel.

One gel was then placed in a box with a flat bottom and Coomassie Blue gel staining buffer was poured over it. The gel submerged in buffer was then shaken at room temperature for two hours, at which point the staining buffer was replaced with new staining buffer after briefly washing the gel in water, and shaken again at room temperature for two hours. The buffer was replaced one more time and the staining continued overnight. In the morning, the staining buffer was removed and the gel was washed in water, then destaining buffer (50 mL Acetic Acid, 100 mL Methanol, and H_2O up to a final volume of 500 mL) was poured onto the gel and it was shaken at room temperature until the destaining buffer became blue, or for about one hour. The destaining buffer was then poured off and fresh destaining buffer was added and the gel was shaken at room temperature for another hour. This continued until the bands could clearly be seen and were distinct from the background.

The other gel was placed in Towbin Buffer[71] for 15 minutes. Polyvinylidene fluoride (PVDF) was soaked with 100% methanol briefly, then washed in water and placed

in Towbin Buffer for 15 minutes in order to equalize the osmolarity of both the gel and membrane. Towbin buffer was poured inside the transfer box and both sponges were wet before assembling the transfer sandwich so the gel was on the anode and membrane was on the cathode side[72]. After placing the transfer sandwich in the transfer box, about 800 mL of Towbin Buffer was poured in to cover the transfer sandwich, which was run without interruption at 50 volts for two hours at room temperature.

The membrane was then rinsed in water and blocked in TBST with milk (5% Powdered Milk, 1x TBS, and 0.1% Tween20)[72] for one hour of shaking at room temperature. After blocking, the primary antibody was added to fresh TBST and incubated with rocking at 4°C overnight. The next morning, the membrane was rinsed with TBST and shaken at room temperature for five minutes, then rinsed. This was repeated four more times. After the final rinse, the secondary antibody was added to fresh TBST with 5% milk just covering the membrane and shaken for one hour at room temperature. As before, five rinses for five minutes were done in TBST, then BCIP/NBT (WorldWide) was poured onto the membrane and shaken at room temperature until bands developed.

SERIAL DILUTION AND SPOTTING ASSAY

Fission yeast transformed with a vector were grown overnight in 5 mL of liquid selective EMM containing 10 μM Thiamine to repress vector gene expression. The next day, the OD was read by measuring absorbance at 600 nm to estimate the concentration of yeast cells. The higher the OD, the more yeast are present. Cells were washed with water and ODs were normalized to 3 by taking the multiplicative product of the desired final volume and desired OD, then dividing by the measured OD. That number corresponds to the volume of culture which needs to be centrifuged for three minutes at 12,100 x *g* to

pellet cells which were then resuspended in the desired volume of water, thereby resulting in the desired cell concentration. The ODs for all samples must be consistent, otherwise phenotypes may simply be due to differing yeast concentrations among samples. The serial dilutions were set up by filling four tubes with 180 μL of H_2O, then transferring 20 μL by pipette from the tube with the immediately higher concentration of yeast to that tube, mixing by pipetting up and down, and then doing the same for the next tube on down (Figure 7). After the dilutions, starting with the least concentrated and going to the most concentrated, 1.5 μL of each dilution was pipetted onto the proper agar plate and allowed to dry before inverting the plate and incubating at 30°C. Plates were observed and photographed daily.

FLUORESCENCE MICROSCOPY AND IMAGING

Fluorescence microscopy and imaging were performed with a Nikon Eclipse TE2000-S microscope and images were collected then analyzed using Meta Imaging Series 6.1. Slide preparation was performed by adding 5 μL of live culture of mid-log phase yeast to a VWR VistaVision Microscope Slide, then placing a Gold Seal Cover Glass over top, laying down one side and then carefully bringing the rest of the glass down as to prevent air bubble formation. The slide was then immediately taken to the microscope where yeast cells were identified and viewed in the eyepiece. If the nucleus needed to be visualized, then 333 μL of overnight culture was added to a 1.5 mL microcentrifuge tube and mixed with 666 μL of 100% Ethanol. The mixture was incubated at room temperature for 30 minutes and yeast were collected by spinning them down for one minute at 420 x *g* to avoid displacing the nucleus. The pellet was then washed in 1x Phosphate-buffered saline (PBS), then resuspended in 1 mL of 1x PBS with 0.5 μL of 4',6-diamidino-2-phenylin-

dole (DAPI) stock at a concentration of 2.5 mg/mL. One drop of this solution was placed onto a slide as previously described. Images were obtained using Meta Imaging Series 6.1 after exposing the samples to the laser in order to excite GFP or DAPI.

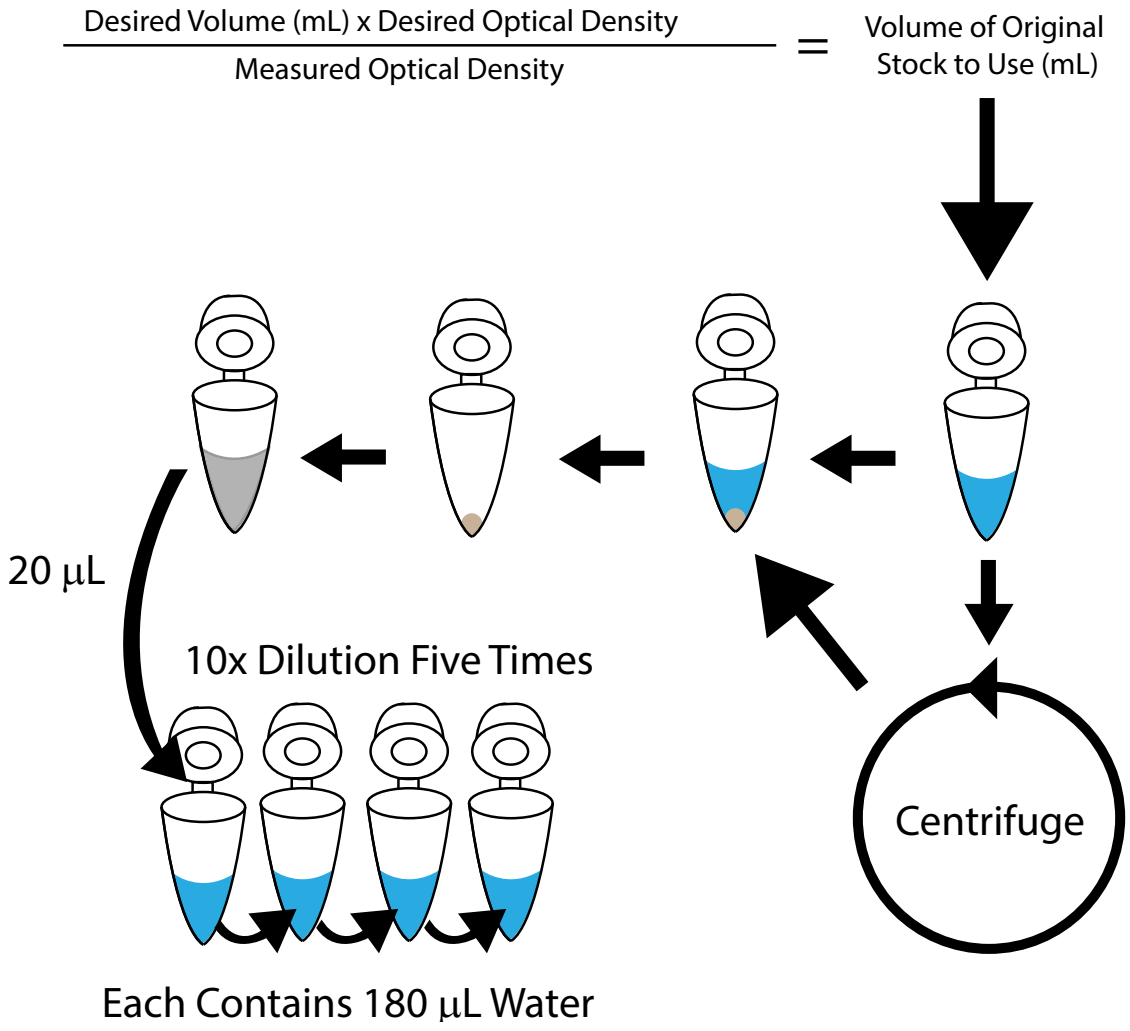

Figure 7. Serial dilution of fission yeast. Figure depicting the centrifugation and dilutions. This is a standard method for assessing yeast phenotypes, and allows for observation of phenotype across several logarithmic concentrations of yeast.

IV. RESULTS

OVEREXPRESSION OF hFUS IS TOXIC IN FISSION YEAST

Fission yeast were transformed with the high (pREP1), medium (pREP41), or low expression vectors (pREP81) containing *hFUS* and were selected on minimal medium with 10 μM thiamine and no leucine. Serial dilutions were done with strains containing one of the three expression levels of *hFUS*, or an empty pREP1 vector as a control, then were spotted onto leucine dropout plates. The control plate also had 10 μM of thiamine to repress expression of *hFUS*. At the highest expression level, similar to the budding yeast model, hFUS is toxic to fission yeast, as demonstrated by weak to no growth in each spot (Figure 8). The medium and low expression levels were less toxic than the high expression, but still showed limited growth compared to the control plate (Figure 8). The high expression level is expected to be 300 times the medium expression level[73]. In order to confirm expression of hFUS, a Western Blot was run with the crude extract from fission yeast transformed with the pREP1 vector containing *hFUS* in the presence of 0 μM, 0.2 μM, and 10 μM thiamine, along with crude extract from *S. cerevisiae* with an integrated copy of *hFUS*. The proteins were separated by electrophoresis on two gels, one of which was stained with Coomassie Blue to determine the total amount of protein loaded, while the other was transferred to a PVDF membrane for Western Blot analysis using an antibody specific to hFUS (Abcam, Cambridge, MA). As shown in Figure 9, hFUS is expressed at a level compareable to budding yeast. Thiamine repression worked as intended, since only conditions with low concentrations or lacking thiamine showed hFUS protein expression. These experiments have been repeated five times, confirming hFUS toxicity in fission yeast.

FUS LOCALIZES TO THE NUCLEUS AND CYTOSOL

Fission yeast transformed with pREP1 containing *GFP-hFUS* were grown overnight in synthetic medium with 10 μM thiamine and lacking leucine, then the next day cells were centrifuged for three minutes at 420 x *g* and washed twice in H_2O. After washing, the pellet was resuspended in synthetic medium lacking leucine and thiamine and grown overnight so GFP-hFUS could be expressed. The next morning the cells were either fixed, or, for live imaging, 5 μL were placed on a microscope slide, and observed under the Nikon Eclipse TE2000-S microscope at 40x and 60x. GFP-hFUS could be seen both inside and outside of the nucleus (Figure 10). Aggregation was also observed. The Western Blot and fluorescence microscopy data together suggest that hFUS is likely the agent of cell death, as the protein is expressed and forms aggregates. To check whether GFP-FUS is toxic, a spotting assay was performed. The addition of GFP to the N-terminus of hFUS does not appear to alter toxicity of hFUS (Figure 8).

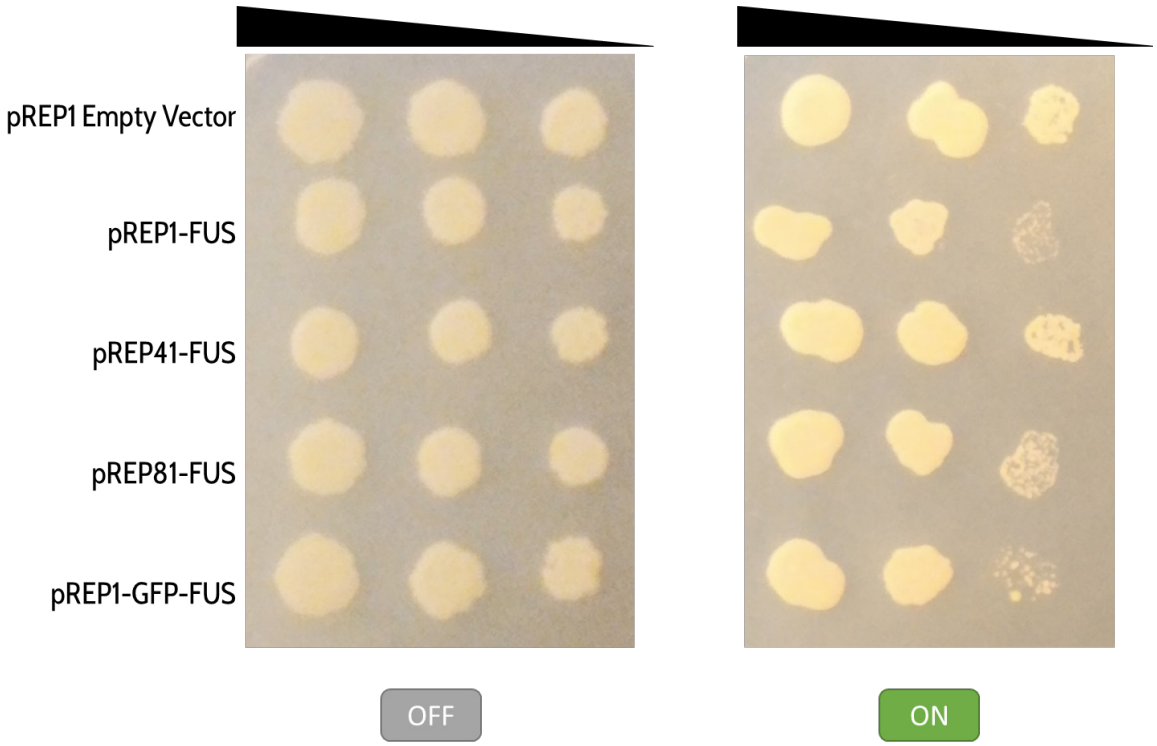

Figure 8. Expression of hFUS is toxic in fission yeast. The image on the left is of a minimal medium plate lacking leucine and including 10 μM of thiamine so gene expression is repressed. The image on the right also is of a minimal medium plate lacking leucine, but gene expression is not repressed so hFUS should be expressed. The highest expression level vector shows clear toxicity as growth is limited compared to the other conditions, while the medium and low expression levels showed clear, but less intense toxicity. Toxicity of GFP-hFUS on the high expression level vector is comparable to the untagged high expression level of hFUS. Pictures were taken after three days of growth at 30°C.

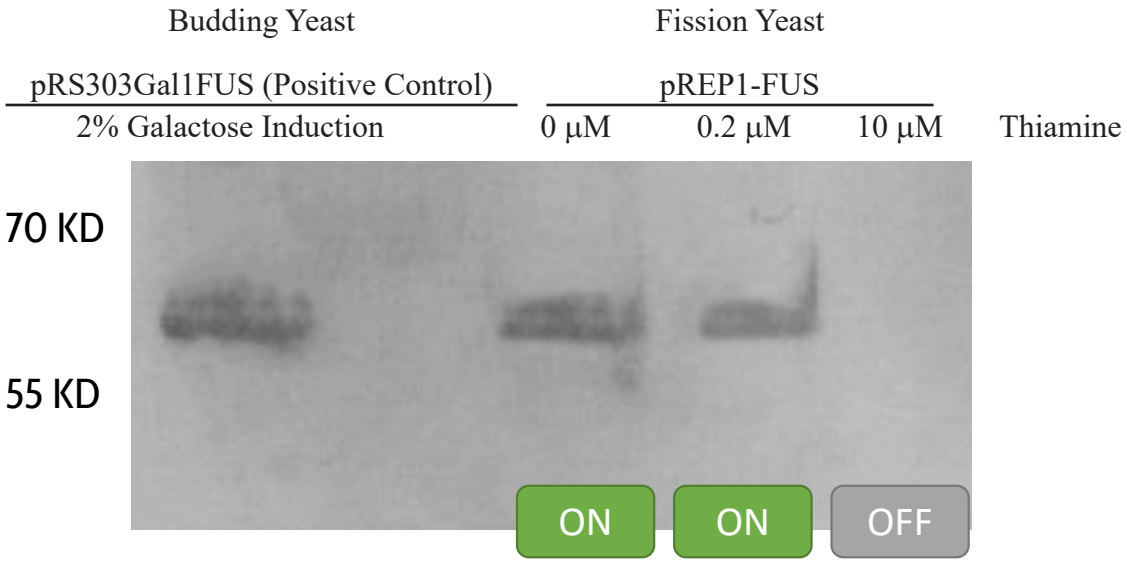

Figure 9. Westerm blot detecting hFUS expression in fission yeast. hFUS was detected in every sample except pREP1-FUS in 10 µM thiamine, where it was expected and confirmed to be repressed. The hFUS protein appears 62 KD, the expected size of hFUS. This confirms that hFUS is expressed in fission yeast after induction.

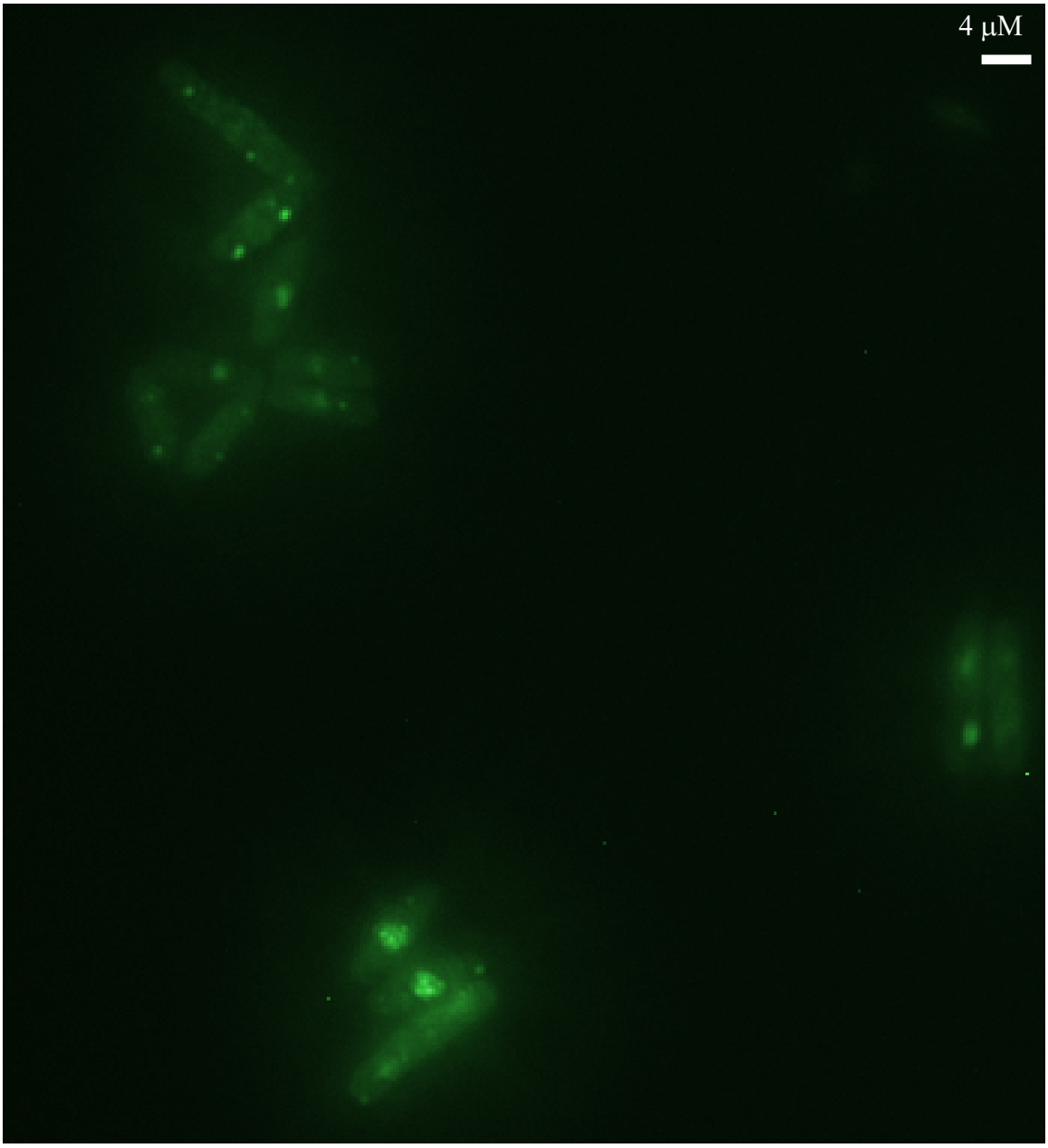

Figure 10. hFUS localizes to the nucleus and cytosol, and forms aggregates. Fluorescence microscopy image (40x) of fission yeast expressing GFP-hFUS (green). GFP-hFUS localizes to the nucleus, appears to aggregate, and is also detected in the cytosol.

FISSION YEAST HOMOLOGUES SUPPRESS TOXICITY

Fission yeast homologues *fATF1*, *fCSX1*, and *fUPF1* to *bNAM8*, *bSKO1*, and *bECM32* respectively, were amplified by PCR from *S. pombe* genomic DNA and cloned onto pREP1 vectors containing a leucine selection marker. To test for the ability of these genes to suppress hFUS toxicity, first transformations were done of just the homologue itself, empty vectors with leucine or adenine as a selection marker, and then each homologue on a pREP1 leucine selection vector paired with *hFUS* on a pREP1 adenine selection vector. fATF1 and fCSX1 when overexpressed by themselves are cytotoxic (Figure 11). fATF1 has a similar toxicity to hFUS while fCSX1 appears to be more toxic than hFUS or fATF1. Overexpression of fUPF1 was not toxic to fission yeast. fATF1, fCSX1, and fUPF1 when co-expressed with hFUS are able to suppress hFUS cytotoxicity (Figure 12). If fATF1,fCSX1, or hFUS are overexpressed alone they are toxic, but co-expression suppresses toxicity of each protein. Presence of both vectors was confirmed by extracting the vectors from fission yeast and successfully amplifying hFUS and each suppression gene via PCR.

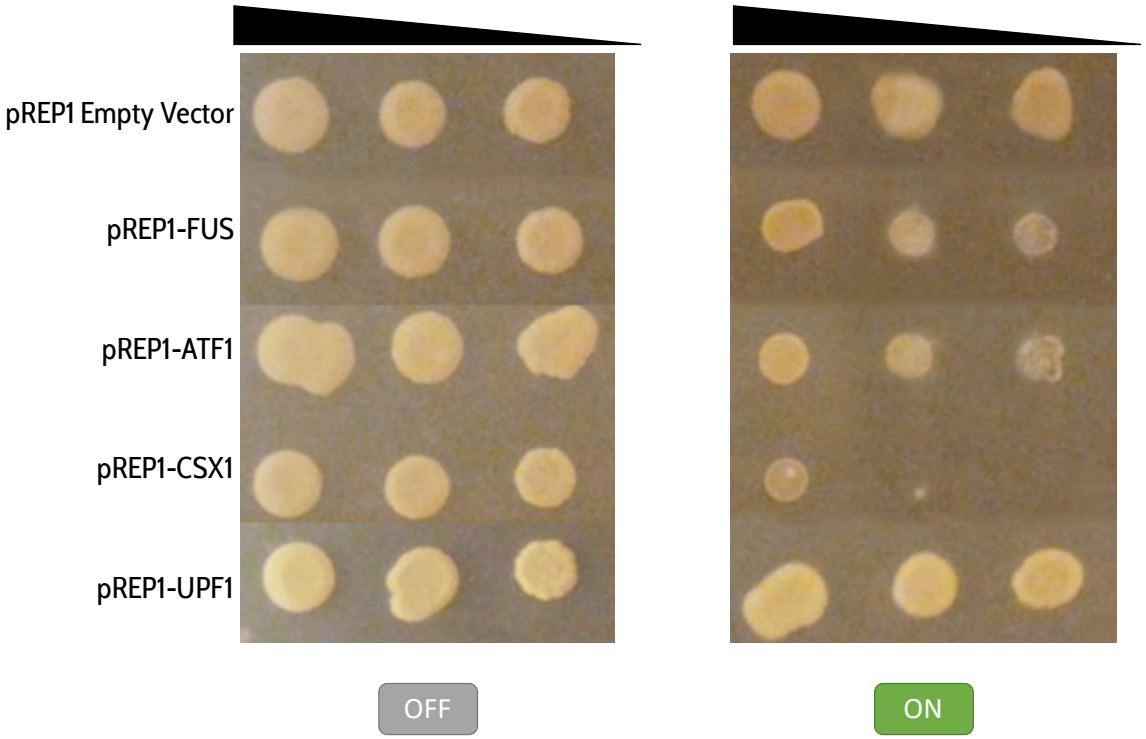

Figure 11. fATF1 and fCSX1 are toxic in fission yeast. The picture of the plate on the left lacks leucine and contains 10 μM thiamine to repress gene expression while the plate pictured on the right has no leucine or thiamine to permit gene expression. From top to bottom, pREP1 empty vector, hFUS, fATF1, fCSX1, and fUPF1 were spotted out to determine their toxicity when over-expressed. fATF1 and fCSX1 both appear to be toxic, as does hFUS. fATF1 has a toxicity similar to hFUS, while fCSX1 is more toxic than either fATF1 or hFUS. fUPF1 is not toxic when overexpressed in fission yeast. Pictures were taken after four days of growth at 30°C.

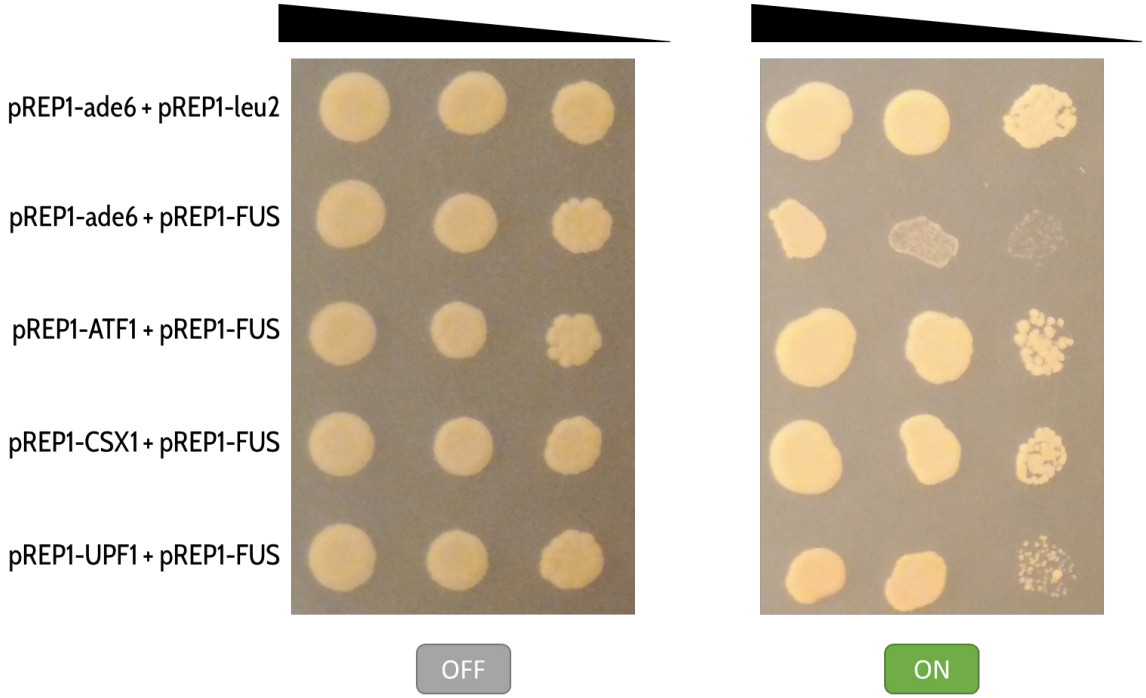

Figure 12. Co-expression of fATF1, fCSX1, or fUPF1 with hFUS suppresses toxicity. The plate on the left lacks leucine and adenine, and contains 10 µM thiamine to repress gene expression while the plate on the right has no leucine, adenine or thiamine, allowing gene expression. fATF1, fCSX1, and fUPF1 were able to partially suppress hFUS cytotoxicity, while hFUS also was inversely able to partially suppress the toxicity of fATF1 and fCSX1. Interestingly, the results indicate that the toxicity of fATF1, fCSX1, and hFUS are not additive, but instead appear to cancel each other out. Pictures were taken after three days of growth at 30°C.

V. DISCUSSION

Neurodegenerative diseases affect 20% of individuals over the age of 65, and share traits including protein aggregation and cell death, yet no cures exist. Treatments are not particularly helpful and manage symptoms rather than treating the underlying disease. Development of a unique model that allows for rapid screening and testing, but contains the complexities of mammalian cells would help with understanding disease.

In my study, *Schizosaccharomyces pombe* (fission yeast) was tested and validated as a novel model organism for studying neurodegenerative diseases at the molecular level, with the efficacy and accuracy provided by traditional systems like *Saccharomyces cerevisiae* (budding yeast). Fission yeast, budding yeast, and humans all diverged one billion years ago, and present day fission yeast and humans share a remarkable set of traits. As a yeast, *S. pombe* is easily, quickly, and cheaply worked with, and shows strong phenotypes. Benefits of fission yeast include similar promoters and cell cycle to mammalian cells, alternative splicing machinery, and microRNA. These traits make fission yeast an advantageous model organism for studying human diseases. Information found from a fission yeast system could then be tested and validated in higher models with confidence that mechanisms will be conserved.

S. pombe AS A MODEL ORGANISM

S. pombe was used to generate a novel model organism for studying the neurodegenerative disease ALS, by assessing how the overexpression of the protein hFUS impacted cell viability. Similarities and differences to the budding yeast model were seen, which validates the generation and use of a new model. As in budding yeast, overexpres-

sion of wild-type hFUS is toxic to fission yeast and will localize to the cytosol where it forms aggregates. In budding yeast, hFUS does not appear to significantly enter the nucleus and remains in the cytosol[25], but in fission yeast hFUS will localize to the nucleus without significant presence of hFUS in the cytosol until aggregates form. This unique difference allows further study to be done in fission yeast utilizing a mutated version of hFUS found in ALS patients to see how the mutation affects localization, toxicity, and the ability for toxicity to be suppressed by other proteins.

The level of toxicity by hFUS on an exogenous vector in fission yeast was similar to the same condition in budding yeast[24]. The level of toxicity shown in the *S. pombe* model allows screening for genes that suppress or enhance toxicity, hopefully aiding in the understanding of the mechanism behind hFUS cytotoxicity. With the genetic advantages of fission yeast, it is also expected that additional proteins may be identified using screens in *S. pombe* that cannot be found in *S. cerevisiae* screens.

fATF1, fCSX1, AND fUPF1

fATF1, fCSX1, and fUPF1 all are able to suppress toxicity induced by hFUS, suggesthing that the suppression mechanism is conserved between *S. pombe* and *S. cerevisiae*. Conversely, hFUS is able to suppress toxicity induced by the overexpression of *fATF1* and *fCSX1*. Interestingly, when identifying and reviewing the fission yeast homologues to the budding yeast suppression genes, they kept appearing together in publications, often related to oxidative stress[74-77].

The human homologue of fCSX1 is not toxic when overexpressed in budding yeast, but is toxic in neuronal cells (communication with the Barmada group, University of Michigan, unpublished data), which supports use of the fission yeast model since

fCSX1 is toxic when overexpressed in *S. pombe*. fUPF1 by itself is not cytotoxic, consistent with the overexpression of budding yeast or human homologues bECM32 and hUPF1 respectively. In budding yeast and human cells, hUPF1 is able to strongly suppress toxicity of hFUS. bUPF1 is unable to suppress toxicity, while bECM32 can. Work should be performed to identify common qualities between bECM32, fUPF1, and hUPF1, along with differences from bUPF1 in an attempt to understand how this suppression occurs. Characterizing and assessing protein-protein interactions is a good place to start as finding binding partners can give considerable information about how a mechanism or pathway may work. Since hFUS levels do not decrease, likely the action of several proteins in a pathway work to limit the toxic effects when both the suppression protein and hFUS are overexpressed.

Now with two models, budding and fission yeast, experiments on UPF1 can be performed and confirmed in two models, thereby validating results quickly. A few places to start include generating fragments of UPF1 or chimeric proteins to determine what regions are required for toxicity suppression. Performing studies in yeasts with different genes knocked out may also yield information on how suppression occurs. When comparing predicted protein interactions via STRING between bUPF1, fUPF1, hUPF1, and bECM32, all four are predicted to interact with UPF2, UPF3, and DCP2, while only the proteins that suppress toxicity, fUPF1, hUPF1, and bECM32, have a predicted interaction with SUP35 homologues. Knocking out *UPF2*, *UPF3*, or both, will help pin down the pathway that suppresses hFUS toxicity, as UPF1, UPF2, and UPF3 are involved in nonsense-mediated decay. If these knockouts result in UPF1 alone being unable to suppress toxicity, then likely it suppresses through the nonsense-mediated decay pathway and not just by an interaction with UPF1 itself. Alternatively, if this is not the case then another

mechanism must be responsible, perhaps involving SUP35. SUP35 is a eukaryotic peptide chain release factor that interacts with bECM32, fUPF1, and hUPF1, but not bUPF1 suggesting the interaction with SUP35 is critical for suppressing toxicity. Unfortunately, SUP35 is an essential gene, so a simple knockout study cannot be performed, and expression must be tightly controlled by another mechanism that enables the cell to grow until the gene can be safely turned off.

These results validate *S. pombe* as a novel model organism. For experiments similar to those performed in *S. cerevisiae*, *S. pombe* appear to be a valid and useful model organism for diseases previously successfully modeled in *S. cerevisiae*. Now with fission yeast confirmed as a useful model, future studies can focus on using the genetic advantages including alternative splicing, microRNA, and greater number of post-translational modifications to determine the impact that those processes have on cytotoxicity of disease associated proteins. Introns, regulation by RNAs, and post-translational modifications have been largely ignored when studying disease because it has not been easy or feasible to do so. *S. pombe* now provide a system which is simple and cheap to grow, but retains the advantages of far more complex and costly systems like human cell culture or mouse models. Integrating full genomic *hFUS*, including introns, into the *S. pombe* genome would shed light onto the role of *hFUS* on the alternative splicing of other genes and how this influence may change with mutated *hFUS*. Currently, only *hFUS* complementary DNA (cDNA) is used which is mRNA that has undergone reverse transcription back into DNA. Problematically, this is a heavily modified version of the gene sequence that lacks introns and the native 5' or 3' untranslated regions. These regions are functionally important for the regulation of proteins downstream, often via RNAs which are generated from introns and may bind to these untranslated regions, typically to repress gene expression.

With these added features in the model, assessing how the gene product of *hFUS* from cDNA differs from *hFUS* from the genome, along with differences in other genes' RNA or protein expression between the two conditions could give great insight into how these previously ignored genetic features may have a large impact on hFUS cytotoxicity, and may lead to new research projects looking beyond protein aggregation and to how genetic expression is altered. Understanding differences between RNA and protein expression in control and disease models should highlight impacted pathways or potentially uncover new ones. These pathways then ideally could be targeted by a compound or other therapy rather than attempting to overexpress a gene as a treatment. Once a mechanism or even potential toxicity suppressor is sufficiently identified in *S. pombe*, then the project could be validated in human cell culture and mouse models, hopefully leading to better options that translate from models to clinical trials.

VI. CONCLUSION

Neurodegenerative diseases increase with age and, while exhibiting distinct symptoms, frequently appear to have similar pathological mechanisms including protein aggregation and cell death. Budding yeast are one model which has been used to study neurodegenerative disease at a cellular and molecular scale because they are simple to grow and handle, results are clear and occur quickly, and it is feasible to quickly screen thousands of proteins for disease-related phenotypes. However, as more research demonstrates the potential role that mRNA, microRNA, and alternative splicing may play in disease, fission yeast offer several advantages to this tried and true model. The current alternative for a faithful representation of protein expression involves using human cell culture, growing neurons, then overexpressing the protein of interest. While all of the genetic advantages are in this system, phenotypes are not clearly observed. Large scale experiments such as genetic screening may be technically possible, but are not feasible due to the amount of money and time required for working with human cells. Fission yeast, however, are cheap and provide comparatively rapid results, enabling large scale experiments to be performed, while retaining genetic attributes similar to human cell models. Data from fission yeast studies could then be validated in human cells, as fission yeast would enable a shift from screening genes to studying a select identified few and associated pathways in the context of ALS.

S. pombe is another kind of yeast shown to have the advantages of working with budding yeast while also possessing genetic advantages of human cell culture. Complementing budding yeast models and possessing advantages of its own, fission yeast allow for large-scale genetic screening and more accurate protein assessment to be done quickly and cheaply. Fission yeast make a great model to study genetic and protein-linked diseas-

es. This study demonstrated fission yeast is a valid model organism for molecular studies of neurodegenerative disease, which allows for future studies to obtain additional information using *S. pombe's* genetic advantages. These future studies in the fission yeast will hopefully increase the understanding of exactly how hFUS contributes to cytotoxicity and enable future studies to uncover mechanisms to suppress toxicity. *S. pombe* is a novel model organism with unique advantages that is compatible with existing models, allowing for cross-validation between models, and insight into new and potentially critical information.

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