**Measurement of Protein Aggregation Levels in Mutant VAPB gene in Transformed Yeast to Determine whether Ubiquitination Proteasome System of the Unfolded Protein Response can be Inhibited**

**I. Introduction**

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that affects both upper and lower motor neurons. The gradual degeneration/death of motor neurons is the main cause of ALS. Motor neurons are a specific type of nerve cell which is associated with voluntary movement. This includes anything from walking to talking and chewing. They are located in the brain and they go from the spinal cord to the muscles in the body. The primary function of the motor neurons are to provide pathways for communication between the brain and the voluntary muscles of the body.[1][2]

ALS patients typically develop muscle weakness as well as paralysis. The muscle weakness or paralysis can be in either the limbs (limb onset) or in the bulbar muscles (bulbar onset). The limb onset of ALS is more prevalent with 80% of cases having upper or lower limb symptoms. Patients with the bulbar onset of ALS have symptoms more along the lines of dysarthria and dysphagia (slurred or slow speech and difficulty swallowing), though limb symptoms may also occur during the course of the disease. The onset of the disease is typically around 55 years of age and progresses at a quick rate. The average life span of a patient diagnosed with ALS is around 3-5 years after the onset, though there are cases where the disease progresses at a somewhat slower rate.[1] According to the NIH, the disease occurs in 2 to 5 per 100,000 individuals worldwide (Fig 1).[2]

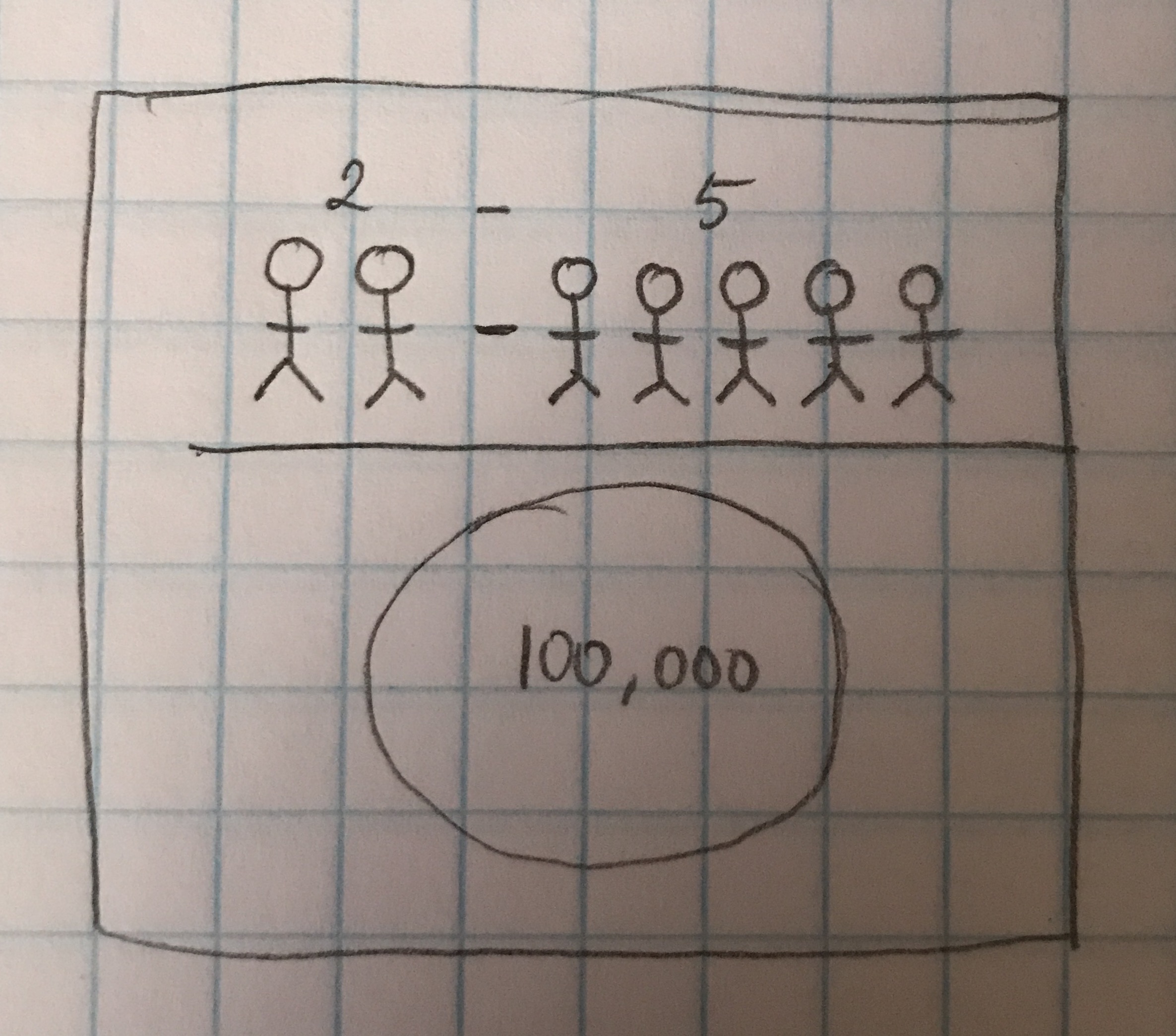


Figure 1. ALS occurs in 2 to 5 people per 100,00 people worldwide [21]

ALS has two forms of genetic onset. The first type is called sporadic ALS (sALS) and has about 90% of all cases, making it the most common type. Sporadic ALS means that the onset is caused by a random mutation in the proteins associated with the motor neurons and there is no hereditary background of ALS in the affected person. The second type is called familial (fALS) and has about 10% of all cases. Familial ALS means that the onset is due to it being hereditary with one of the parents being a carrier of the mutant gene causing the disease.[1]

Each fALS case is caused by a mutation in a different gene. Many fALS cases are due to mutations in the SOD1 (20%), C90RF72 (30%), and TARDB and FUS (4-5%) genes (Fig. 2). There are, however, a number of cases associated with mutations in other genes such as the vesicle associated membrane protein associated protein B (VAPB).[1][3] The VAPB protein is an integral membrane protein located in the endoplasmic reticulum (ER). It has many functions including protein folding, lipid transport, vesicle movement between cells, as well as the unfolded protein response (UPR) which detects the unfolded/misfolded proteins, degrades the misfolded proteins[3] via the ubiquitination proteasome system (UPS)[11], and attempts to correct the proteins by increasing the production of molecular chaperones which are involved in protein folding.[3]

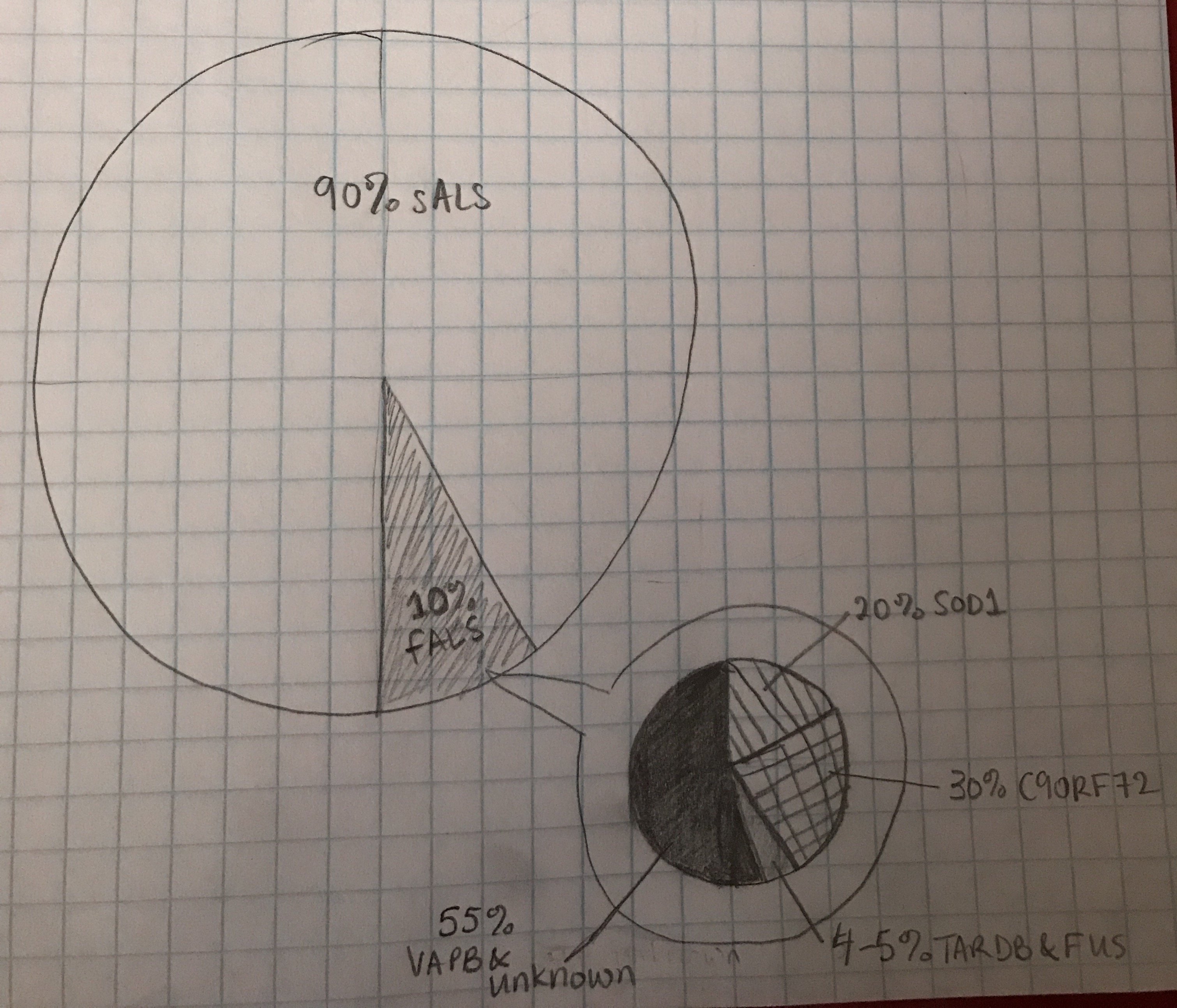


Figure 2. Percentage of sALS vs fALS and percentage of fALS caused by most prominent mutations [22]

The fALS caused by mutations in the VAPB protein is called ALS8 and was first found in a large Brazilian family with many members, both male and female, affected across four generations. [1][4] The mutation found in the VAPB gene showed that an amino acid called proline was being replaced by another amino acid called serine at the codon number 56.[1][4] This mutation caused an increase in the number of unfolded or misfolded proteins in the ER and caused an aggregation of protein to form. The aggregation of proteins is a common sign in neurodegenerative diseases. The aggregation, under normal circumstances, would be taken care of by the UPR and UPS systems. This would use the UPS to degrade misfolded proteins and increase molecular chaperone proteins that would fold the unfolded proteins via the UPR system. In the mutated form, the aggregates are not removed or fixed and continue to accumulate. Theoretically, this accumulation of the aggregates would interfere with the UPS of the UPR system and ultimately cause the motor neurons degeneration (Fig. 3 [20]) [1]. The Qiu et al (2012) experiment showed that an increase the amount of protein aggregation did not interfere with the overall function of the UPR/UPS system. This was specifically in the P56S mutation of VAPB [1].

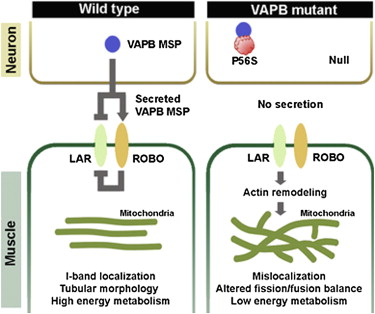


Figure 3. **a)** Non-ALS8 interactions in the ER **b)** ALS8-affected interactions in the ER [20]

In humans and other animals, the VAPB protein and its function is conserved. Another organism that carries a protein that functions in a similar way to VAPB is yeast. Yeast carries a homologue of VAPB called scs2. Scs2 functions in a similar way as VAPB does in mammals and mutation in this gene produces similar results as mutation in the VAPB gene [7]. Since the genome of yeast is much smaller, transforming human or mammalian VAPB into yeast would be ideal to create a new library of possible mutations within that gene. The purpose of the experiment described in this proposal would be to test whether one of the mutations from the library of forced mutations would produce protein aggregates that would interfere with the UPS of the UPR system and would ultimately cause aggregate accumulation leading to ALS8.

**II. Experiment**

The experiment’s purpose is to determine the level of protein aggregation that would cause the onset of ALS8 via interference of the UPS of the UPR system in the mutant VAPB gene and to compare this to the amount of protein aggregation that would normally occur in the original gene of VAPB. If the level of protein aggregation was higher than the normal level present in the original VAPB gene, then I would expect there to be an interference in the UPS of the UPR system, signifying the onset of ALS8.

II.A Creating VAPB Mutants in Yeast

In order to determine the levels of protein aggregation in the mutant VAPB, mutant VAPB needs to be inserted into plasmids that would be transformed into yeast. This is done because the yeast genome is much smaller than that of other organisms and can easily be sequenced. Inserting VAPB into the plasmids allows the proper characteristics of the gene to be present when the plasmid is transformed into yeast.

Polymerase chain reaction (PCR) is the traditional method to create mutants. This experiment would use error-prone PCR (EP-PCR). EP-PCR allows random mutations to be introduced into a long segment of DNA (Fig 4 [18]). Wilson et al (2001) described a technique for mutagenizing a library of sequences using random mutagenesis via PCR. In this protocol, they started with a large DNA template concentration and performed four cycles of EP-PC, transferring around 10% of the results into a new EP-PCR reaction. This step should be repeated until a good number of molecules are produced. Wilson et al (2001) described this technique to give around 50 EP-PCR doublings (doubled DNA molecules) with around 3.5% of the nucleotide positions in the DNA template having mutations. For the purposes of this experiment, I will be using this process on the VAPB gene. The products derived from this technique will then be sequenced and primers will be determined from this. The primers determined from the EP-PCR products must be homologous to the plasmid in which I would insert the VAPB gene as well as its mutations into. This would be necessary because inserting non-homologous primers into a plasmid would cause it to not bind with those primers and thus not taking in the VAPB gene which would be necessary when transforming it into yeast.

In order to insert the primer into the plasmid, I would first digest the plasmid with a restriction enzyme. This would help linearize the plasmid and allow it to open for the insertion of the VAPB PCR product. I would then mix both the PCR products and the linear plasmid (Fig. 4 [18]). Mixing both the PCR product and the linear plasmid would allow it to bind and then a transformation process (Fig. 5 [19]) [15, 16] would be used to convert the plasmid into yeast.



Figure 4. Depiction of the process of EP-PCR [18]



Figure 5. Yeast Transformation [19]

II.B Measuring the Protein Aggregation

In the experiment done by Qiu et al (2012), transgenic mice VAPB were used in the process of measuring the protein aggregation levels. They also tagged the mutated VAPB with a 3xFLAG-tag at the N-terminus in order to differentiate between the two VAPB proteins. They then determined the VAPB aggregation levels of each using Western blot. While there are other ways to determine protein aggregation levels, using Western blot allowed them to see the amount of fluorescence formed by both the wild types and the mutants.[1]

In my experiment, I am using the transformed yeast with the VAPB gene in it. In order to measure the protein aggregation within these cells at a better accuracy, I would use a slightly different measuring tool called Fluorescent dye ProteoStat (simply known as ProteoStat). ProteoStat allows for a homogenous assay to monitor the aggregation of proteins within a solution. This technique is related to more traditional techniques such as Thioflavin T (ThT), another fluorescent dye which also displays fluorescence to depict the level aggregation of protein. The lead dye used in the ProteoStat technique is a molecular rotor that attaches itself into the crevices of beta sheets in the aggregated protein. The ProteoStat technique is able to detect a much broader range of protein aggregates, has a brighter signal, and can detect small concentrations of protein aggregates in a concentrated solution. [13] This is because the dye’s red fluorescence emission has a maximum of 600 nm. This amount of emission allows any auto-fluorescence to be reduced. An experiment (Fig. 6) done by Shen et al (2011) shows that ProteoStat was used to detect amyloid plaques which are usually present in many patients with neurodegenerative diseases (this one specifically showing Alzheimer’s disease). The experiment showed a fluorescence emission which enhanced the presence of these plaque, showing that the dye is able to detect a much broader range of protein aggregates [17].

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Figure 6 shows **a)** Detection of amyloid plaques and other background material in patients with Alzheimer’s disease (AD) using Thioflavin T dye (ThT); **b)** Dectection of amyloid plaques in patients with AD using ProteoStat dye—notice that the background material from the **a)** is not seen in **b)** due to the brightness of the ProteoStat dye. [17]

II.C Using the Transformed Yeast with Mutant VAPB and Measuring for Protein Aggregation

Mutant genes for the transformed yeast VAPB will be created via error-prone PCR. These mutant genes will be placed in a solution in the ProteoStat tool and the aggregation level of the protein will be measured through the level of the fluorescent dye present. These levels will then be compared to levels of aggregation of the wild type protein to see whether an adequate amount of aggregation accumulation has developed. This will then help determine whether the onset of ALS8 would potentially occur.

**III. Discussion**

Assuming the experiment goes smoothly, the protein aggregation levels of the mutant VAPB gene within the transformed yeast will be higher than those of the original VAPB gene within the transformed yeast. This would confirm that an interference has occurred and the UPS of the UPR is not properly degrading the proteins that are accumulating. Using these results, I could potentially state that due to the interference of the protein aggregates in the UPS of the UPR system of the mutant VAPB gene, the onset of ALS8 would occur.[1] This conclusion, however, can only be made due to the aggregation levels within the transformed yeast organism. In order to really know whether these levels of aggregations would display symptoms of ALS8, I would have to see human brain cells with both mutated and non-mutated VAPB and test them.

In their experiment, Qiu et al (2012) stated that the levels of protein aggregation in the P56S mutation of the VAPB gene were not enough to show that aggregation alone caused ALS8.[1] The possibility of getting a better level of protein aggregation in these other mutations seems better as the technique yields higher fluorescence which would help detect the level of aggregation.[13] Another part of this experiment for the future would be to test the P56S mutation once more with the ProteoStat technique and see whether the yield is higher to determine whether protein aggregation levels can determine the onset of ALS8.[1][13]

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21. Hand Drawn Image 1. Keerthana Vishwanath (Depiction of Source 2)

22. Hand Drawn Image 2. Keerthana Vishwanath (Depiction of Sources 1 and 3)