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Determining Protein-Protein Interactions of ALS-Associated SOD1

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DETERMINING PROTEIN-PROTEIN INTERACTIONS OF ALS-ASSOCIATED SOD1

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requirements for the degree of
Master of Science

By

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B.S., Wright State University, 2013

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Leah A. Shurte ENTITLED Determining Protein-Protein Interactions of ALS-Associated Protein SOD1. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Determining Protein-Protein Interactions of ALS-Associated Protein SOD1.

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder that occurs due to the death of motor neurons and leads to paralysis and death within three to five years after symptoms present (Byrne et al., 2013). Superoxide Dismutase 1 (SOD1) was first identified to be associated with ALS in 1993. The objective of this study is to determine which proteins interact with wild type and mutant SOD1 and find any similarities or differences between them. ALS is attributed to a gain of toxicity, therefore abnormal protein interactions in mutant SOD1 are important. The results of this study will provide insight on the protein-protein interactions of SOD1, as well as how important these interactions are in association with ALS. Initially, the plan was to use yeast two-hybrid screening (Y2H) to identify the protein-protein interactions, then confirm the interactions with a pull down assay (immunoprecipitation). However, the Y2H was unable to obtain results. Instead, a combination of a pull-down assay and mass spectrometry were used to identify protein-protein interactions. Fifty one proteins were identified to interact exclusively with wild type SOD1 and thirteen proteins interacted with both wild type and A4V SOD1.

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INTRODUCTION

Neurodegenerative Disease

As age expectancy for the average person increases, so do the consequences of age (Byrne et al. 2013). Neurodegenerative diseases are neurological disorders that have very different clinical features and pathology as a result of the progressive loss and death of neurons (Przedborski et al., 2003). The most common neurodegenerative diseases include Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis, and Huntington's disease, which all appear to be related to protein aggregation.

Increasing age is the most consistent risk factor in neurodegenerative diseases, though the causes of these diseases are essentially unknown. There is controversy about whether the initiation of these diseases are mainly genetic or environmental. Due to the high percentage of sporadic diseases in comparison to the diseases with a genetic component, toxic environmental factors are thought to play a role (Przedborski et al., 2003). For example, some studies suggest gut microbiota play a role in neurodegeneration (Ghaises et al., 2016). Many of these diseases, though different, share similarities such as protein misfolding, excitotoxicity, mitochondrial malfunction, and altered RNA levels (Rezaei-Tavirani et al., 2016; Cudkowics et al., 1997), suggesting related mechanisms.

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is neurodegenerative disease in which the motor neurons in the brainstem, cerebral cortex, and cervical and lumbar spinal cord degenerate and die (Ringel et al., 1993; Tandan and Bradley, 1985). Figure 1 compares a healthy motor neuron to a motor neuron effected by ALS. The dying motor neuron cannot get signal from the brain to the muscle, so the muscle becomes paralyzed and atrophies. Peak onset for this disease is between 55 and 75 years of age with a life expectancy of three to five years and interestingly, a decrease in likelihood of getting ALS after age 80 (Byrne et al., 2013). The physical manifestations of this disease are weakness and paralysis due to progressive muscle atrophy. Seventy-five percent of ALS cases first present in the limbs and result in patients having trouble walking or issues with fine motor skills. They also may drag one foot due to the asymmetrical nature of this disease. Twenty-five percent of ALS patients experience bulbar onset ALS and have difficulty speaking clearly and swallowing. Symptoms include tight and stiff muscles, exaggerated reflexes and involuntary muscle twitches (alsa.org). Patients with bulbar-onset ALS are also more likely to have Frontotemporal Lobar Degeneration (FTLD) along with ALS. This results in some cognitive impairment, such as deficits in verbal flexibility, memory for visual and verbal materials, and abstract reasoning (Strong et al. 1999). Death typically occurs due to respiratory failure (Tandan and Bradley, 1985) or the inability to eat and drink due to the paralysis of the tongue and other oropharyngeal muscles, leading to nutritional insufficiency (Robbins, 1987; Silani et al., 1998).

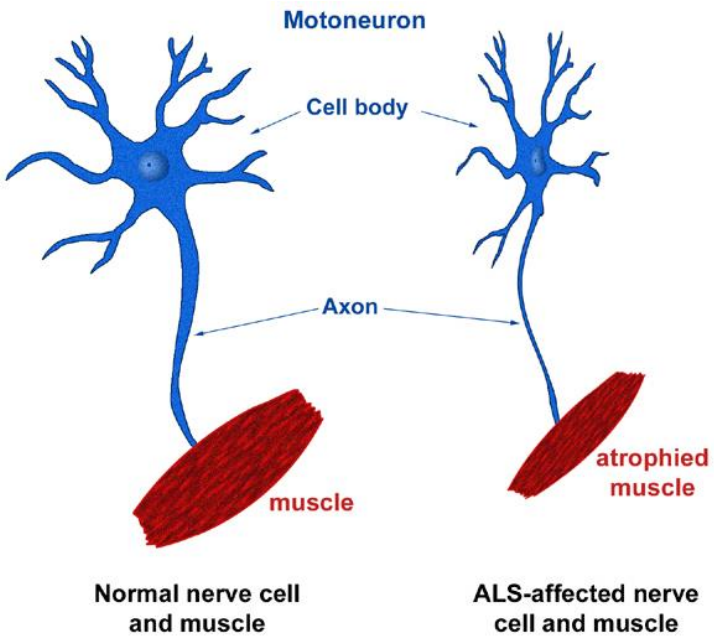


Figure 1: Motor Neuron Death. The motor neuron on the left is healthy and has a normal, working muscle. The motor neuron on the right cannot get a signal from the brain to move the muscle, therefore that muscle becomes paralyzed and atrophies. (Adapted from http://www.visembryo.com/story898.html)

Advances have been made in our understanding of the genetics of ALS, however the mechanism remains unknown (Al-Chalabi and Hardiman, 2013). There are two types of ALS: familial and sporadic (Figure 2). Ninety percent of ALS cases are sporadic ALS (sALS), meaning there was no identified family history of ALS (Kaur et al., 2016). Familial ALS (fALS) makes up the remaining ten percent of ALS patients and is often autosomal dominant in inheritance, however, it is possible that that some fALS cases are misrepresented as sALS cases as ALS is a difficult disease to diagnose. It is also possible that some sALS cases are actually the origin of a line of fALS cases.

There are currently over eighty-two genes associated with ALS (Abel et al., 2012), several found in both fALS and sALS cases including SOD1, FUS, TDP43, and C9ORF72. SOD1, identified in 1993, was the first gene to be associated with ALS and covers 20% of fALS and 3% of sALS cases (Figure 2) (Hayashi et al., 2016). FUS and TDP43, two genes encoding DNA/RNA protein, were independently found and each appeared to be mutated in 5% of fALS and 1% of sALS cases (Robberecht and Phillips, 2013). C9orf72 is the most recent gene associated with a significant number of cases; up to 44% of fALS and 10% of sALS.

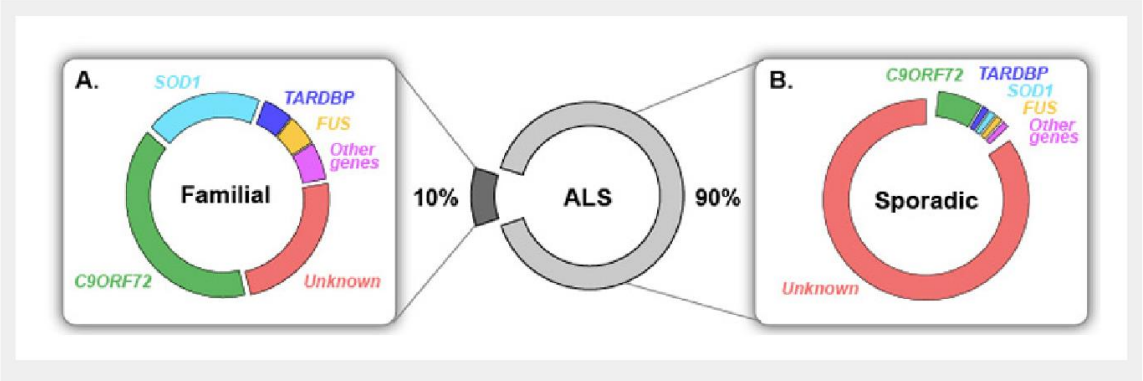


Figure 2: Genetics of ALS. Ten percent of ALS cases are familial, while ninety percent are sporadic. This figure shows the genes that affect each type of disease. Mutations in SOD1 are associated with 20% of fALS cases and 3% of sALS cases. (Laferriere and Polymenidou, 2015)

Once the mechanism of ALS is determined, an effective treatment can be developed. As of now, Riluzole is the only FDA-approved treatment available to patients, and at best provides patients with an additional three months. Riluzole works by inhibiting N-methyl-D-aspartate (NMDA) receptors, kainite receptors (KARs), and tetrodotoxin (TTX) sensitive sodium channels which prevents excessive stimulation and a toxic influx of calcium ions associated with apoptosis. (Miller et al., 2012; Mitsumoto et al., 2014). Riluzole has changed our view of what the mechanism behind ALS may be and suggests that simply preventing excitotoxicity is not enough to treat the disease.

Mitochondria are neuronal energy producers and are linked to several neurodegenerative diseases, therefore it is thought that they could be good target for new treatments. Dexpramipexole is a drug that enhances mitochondrial function. Dexpramipexole made it to a phase 3 clinical trial, but experimental results were no different than placebo (Corcia and Gordon, 2012). Some studies suggest ALS is a not purely a motor neuron disease, but rather a multisystem degeneration and should be treated as such (Huebers et al., 2016). Others suggest stem cell therapy as a new treatment of study that could easily transferred into clinical settings (Mazzini et al., 2003). Though opinions differ on the details of the treatments, scientists know the importance of finding a cure for ALS. Determining the true mechanism of ALS will point scientists in the right direction to develop a treatment.

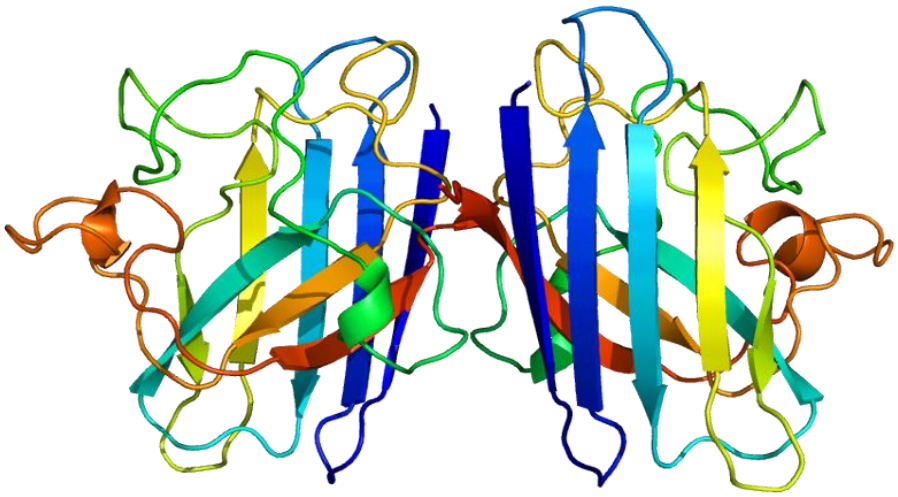


Figure 3: Superoxide Dismutase 1. SOD1 is a homodimer that binds copper and zinc and converts superoxide radicals to oxygen and hydrogen peroxide. (Adapted from Wikimedia Commons)

Superoxide Dismutase 1

In 1993, Rosen and colleagues identified the first gene to be associated with ALS, Superoxide dismutase 1 [Cu-Zn] (SOD1) (Rosen et al., 1993). The SOD1 gene is located on chromosome 21 and is one of the three human superoxide dismutases that convert toxic superoxide radicals to oxygen and hydrogen peroxide. SOD2 and SOD3 are currently not linked to any human disease. They exist as tetramers whereas SOD1 forms a homodimer (Figure 3). The SOD family also differ in localization, with SOD1 found predominantly in the cytosol, SOD2 in the mitochondria, and SOD3 being extracellular, though SOD1 can also be found in the mitochondria (Kawamata and Manfredi, 2008; Son and Elliot, 2014).

SOD1 is not an essential gene, as shown in the study done by Reaume *et al.* (1996). In this study, an SOD1 knockout mouse model showed no sign of ALS by six months of age, indicating ALS is not caused by a loss of SOD1 function. The mice in this study, however, appeared to age quickly, which is likely due to their inability to detoxify superoxide radicals. The overexpression of SOD1 in mice, however, gave the mice ALS-like symptoms and they became terminally ill after 370 days (Graffmo et al., 2013). This suggests ALS is related to gain of toxicity rather than a loss of function.

SOD1 Mutations

The SOD1 gene is 154 amino acids long and it has over 180 known mutations covering the whole length of the protein (Figure 4), some of which are more strongly

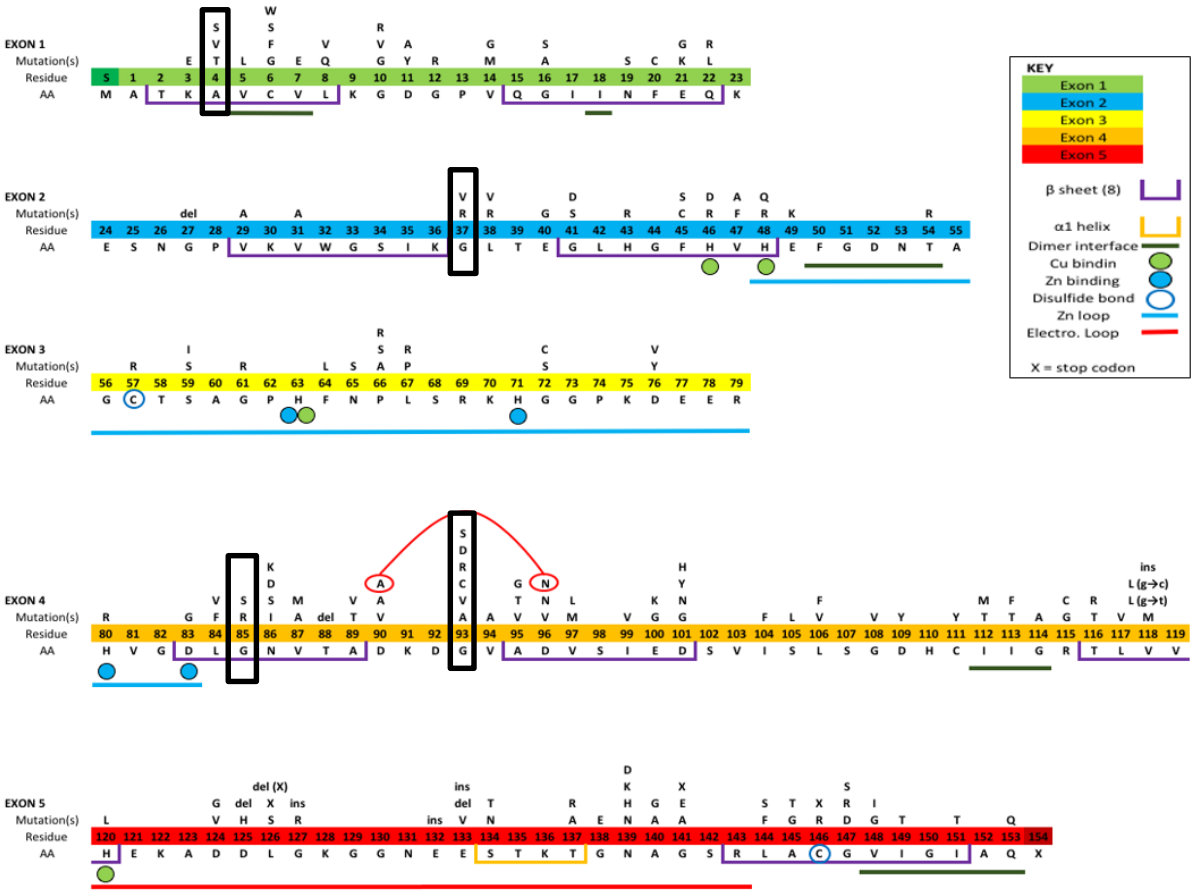


Figure 4: SOD1 Mutations. The above figure shows the many places where mutations can occur on SOD1. The black rectangles indicate the locations of the mutations chosen for this study. (Andrew Koesters)

associated to ALS than others (Kaur et al., 2016). Studying mutant SOD1 will be informative because so many different SOD1 mutants are associated with ALS. Finding similarities between those mutants could possibly point in the direction of a mechanism. Studies have shown that mutations in SOD1 are found in 20% of fALS and 3% of sALS cases. The SOD1 mutants studied in my thesis were: alanine mutated to valine at codon 4 (A4V), glycine to arginine at codon 37 (G37R), glycine to arginine at codon 85 (G85R), and glycine to alanine at codon 93 (G93A).

These mutations do have slight differences from each other. Patients with the A4V mutation have a variable age of onset, but they all have a rapid progression of the disease with average survival after onset of 1.4 years. The A4V mutation is the most common SOD1 mutation found in the United States (Prudencio et al., 2009). The G37R mutation exhibits full enzymatic ability and a lower propensity to aggregate than other mutants (Prudencio et al., 2009; Bruijn et al., 1997). The G85R mutation is enzymatically inactive, but has a late onset and a rapidly aggressive disease progression (Bruijn et al., 1997). The G93A mutation is rare in the population, but it has been well studied as it was the first ALS mutation to be modeled in mice (Prudencio et al., 2009). G93A is another mutation that keeps enzymatic activity completely intact.

Protein-Protein Interactions

Some proteins known to interact with wild type SOD1 are FUS (Fused in Sarcoma), CCS (copper chaperone), Bcl-2 (B-cell lymphoma 2), and CSTB (cystatin B)

(Casareno et al., 1998; Pasinelli et al., 2004; Ulbrich et al., 2014). CCS shuttles copper to the SOD1 protein (Williams et al., 2016). Mutant SOD1 induces expression of Bcl-2 in motor neurons through a redox sensitive transcription factor (Iaccarino et al., 2011). CSTB is a protease inhibitor and is associated with progressive myoclonus epilepsy, a rare syndrome that includes progressive neurological decline. CSTB aggregation is dependent on the redox environment, which is related in part to SOD1 (Ulbrich et al., 2014; Cipollini et al., 2008). Mutations in FUS are also associated with ALS. Although these protein-protein interactions are the only ones as of yet to have been found for wild type SOD1, it is likely that there are many more interactions that have yet been identified. This study is being done in order to find more proteins that interact with wild type SOD1, therefore providing a more complete picture of the protein function. Also, a study on the protein-protein interactions of mutant SOD1 has not previously been done. Consequently, this will allow mutant SOD1 interactions to be compared to wild type SOD1 interactions.

Yeast Two Hybrid Screening

The yeast two-hybrid screen (Y2H) is used to discover protein-protein interactions by testing for physical interactions between a protein of interest and often a genetic library (Figure 5). The transcriptional activator GAL4 is split into a DNA-binding domain (DB) and an activating domain (AD). The bait protein is attached to DB and the prey protein is connected to AD. When the DB and AD are separate, transcription cannot

occur. However, when the bait and prey proteins interact, the DB and the AD are brought together, reconstituting a fully functional transcriptional activator (Young, 1998).

The DB vector contains a *LEU2* marker, which means the yeast containing this vector can grow without leucine in the medium. The AD vector contains a *TRP1* marker allowing growth on medium lacking tryptophan. Two different types of haploid yeast must be used for mating to occur. After the mating process, the now diploid yeast contain both AD and DB vectors and can be grown on leucine and tryptophan dropout plates (LT-). If the proteins interact in the yeast cell, the AD and DB are close enough to activate the reporter gene, *HIS3*. When *HIS3* is activated, it allows for the biosynthesis of histidine, which can be used as a third selection marker. Using all three markers allows for detection of protein-protein interactions.

The number of colonies on an LT- plate corresponds to the number of proteins in an AD tagged cDNA library that were screened against the DB tagged SOD1 (DB-SOD1). The cDNA libraries contain most or all of the human proteins in that system and were made from the mRNA of brain tissues. Adult brain and Fetal Brain cDNA libraries were used. Differences in protein-protein interactions may be found due to the differences between proteins expressed in a fetal brain and in an adult brain.

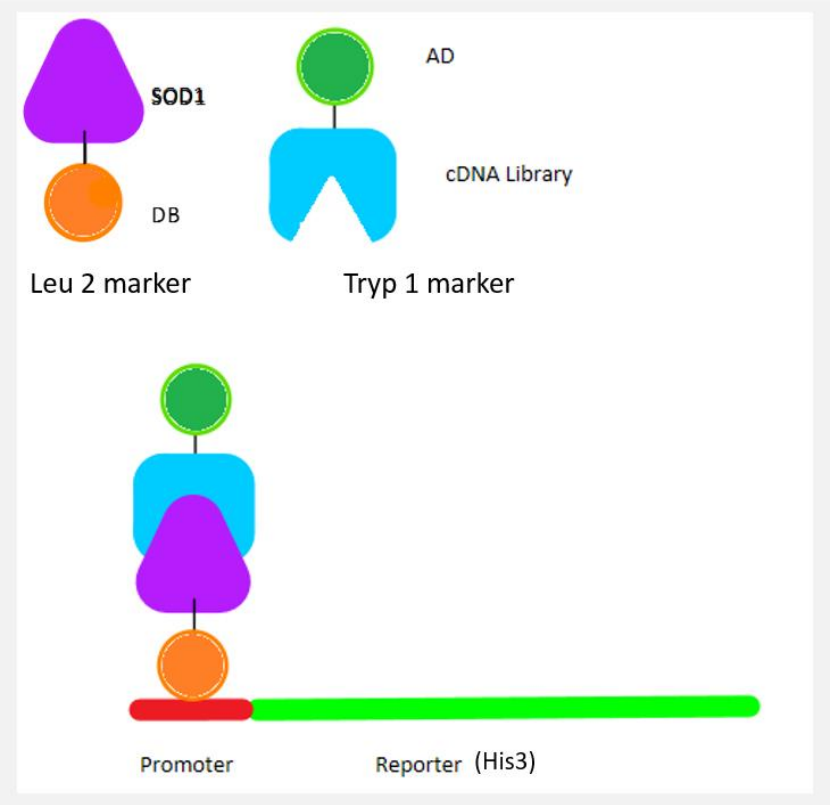


Figure 5: Yeast Two-Hybrid Screening. This figure shows how Y2H works. In the figure above, DB-SOD1 has a leucine prototroph selective marker and the AD- cDNA Library has a tryptophan prototroph selective marker. When SOD1 interacts with a library protein, AD and DB are brought together, allowing for transcription of the reporter gene, *HIS3*.

Pull Down Assay

The pull down assay, or immunoprecipitation, uses an antibody that binds to a specific protein complex to precipitate a desired antigen out of solution (Figure 6). The antibody binds to a known protein in a complex and pulls the entire protein complex out of solution. The unknown proteins in the complex can then subsequently be identified using mass spectrometry. SOD1 was tagged with GST, which can easily bind to and be pulled down by GST resin. The pull down assay is performed using a human cell line (HEK293), therefore, the human SOD1 is where it is naturally found, as opposed to in the Y2H where hSOD1 is transformed to a yeast cell that contains its own SOD1. After extensive washing, material bound to GST resin was subjected to mass spectrometry to identify proteins present in each sample.

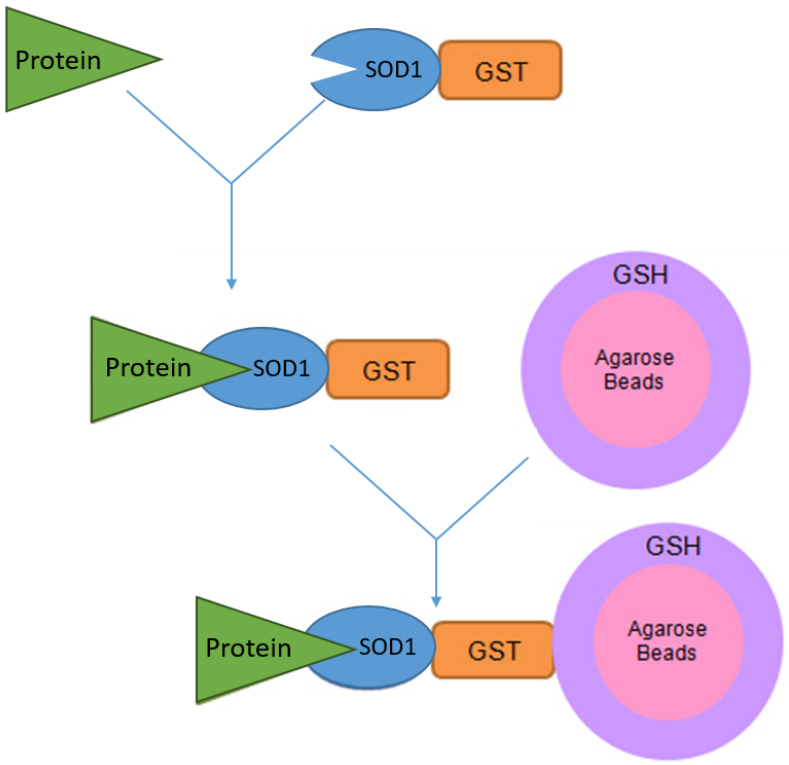


Figure 6: Pull Down Assay. This figure shows how the GST pull down works. GST tagged SOD1 interacts with proteins in the cell lysate. Reduced Glutathione (GSH) beads are mixed with the proteins and bind with GST. Because the GSH beads are heavy, they can be pulled down out of solution, bringing GST tagged SOD1 and its associated proteins with them.

Hypothesis

SOD1 associated ALS is thought to be attributed to a gain of toxicity. For this reason, I expect the SOD1 mutants may have abnormal protein-protein interactions compared to wild type SOD1, either a gain of additional interactions or a loss of interactions that result in a gain of toxicity. In addition, I hypothesize the SOD1 mutants may share common interactions. This is important because determining specific protein-protein interactions that are associated with ALS can broaden treatment concepts. For example, if an abnormal protein-protein interaction triggers the pathology that leads to ALS, a viable treatment mechanism would be breaking that protein-protein interaction. Conversely, if a loss of an interaction causes SOD1 to function differently and gain toxicity, that interaction could possibly be brought back to alleviate that toxicity.

Specific Aims

Aim 1: To determine what other proteins wild type SOD1 interacts with.

Aim 2: To determine if SOD1 mutant proteins have different protein-protein interactions than the wild type.

Materials and Methods

Yeast

Yeast used was haploid Y8800 (a) and Y8930 (α). Two different types of haploid yeast (a and α) were needed in order for the yeast to mate and become diploid (a/α). Yeast was grown from glycerol stock and streaked on new YPD plates every week.

Site-Directed Mutagenesis

Mutagenesis was performed on wild type SOD1 to create point mutations in the gene that corresponded to common ALS associated mutants A4V, G37R, G85R and G93A. PCR primers described in Table 1 were designed using Agilent's Quick-Change Primer Design. Reaction conditions included denaturing for thirty seconds at 90C, annealing for one minute at 55C, and elongation for one minute per kilobasepair of plasmid length at 68C for eighteen cycles. After PCR, a DpnI digestion was done to remove the methylated parental DNA. The newly synthesized DNA was transformed to DH5α. The mutations were confirmed by sequencing.

Gateway Cloning

Gateway is a cloning method that uses specific sequences known as att sites and two enzyme mixes called LR clonase and BP clonase. Gateway cloning ensures the correct reading frame by transferring DNA into different cloning vectors with corresponding att sites (Figure 7). A BP reaction was performed to

Table 1: Primers for Site-Directed Mutagenesis. This table details the forward and reverse primers used to create the A4V, G37R, G85R, and G93A SOD1 mutants.

Mutant	Primer Sequence
A4V Forward	cagcacgcacacgaccttcgtcgccat
A4V Reverse	atggcgacgaaggtcgtgtgcgtgctg
G37R Forward	ccttcagtcagtcttttaatgcttccccacacct
G37R Reverse	aggtgtggggaagcattaaaagactgactgaagg
G85R Forward	tcagcagtcacattgcgcaagtctccaacatgc
G85R Reverse	gcatgttggagacttgcgcaatgtgactgctga
G93A Forward	gacacatcggccacagcatctttgtcagcagtc
G93A Reverse	gactgctgacaaagatgctgtggccgatgtgtc

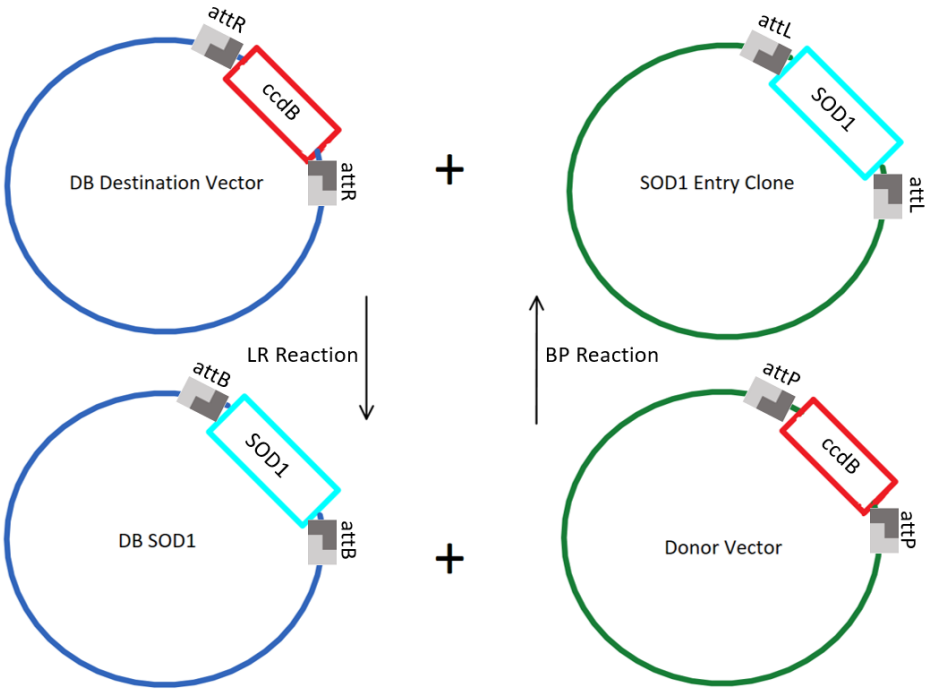


Figure 7: Gateway Cloning Mechanism. The Gateway reaction cuts at the att sites in the plasmids. The BP reaction inserts the SOD1 into an entry clone. That entry clone can then be used in an LR reaction to transfer SOD1 into any Gateway destination vectors. Any segment of DNA with the correct att sites can be cloned using Gateway.

put the SOD1 sequence into an entry clone. An LR reaction was then performed to transfer the SOD1 sequence from the entry clone to the destination vectors.

For yeast two hybrid screening, the gateway AD and DB destination clones were made from wild type and mutant SOD1 on pDONR223 entry clones. The same entry clones were used for the high expression homodimer pairwise test with 212 (DB) and 213 (AD) destination clones. Expression clones (pDEST27) for pull down were made from the same entry clones.

Polymerase Chain Reaction

The amplification procedure consisted of an initial denaturing step at 95 ºC for 2 minutes, followed by 30 cycles of denaturing at 95 ºC for 15 seconds, annealing at 54 ºC for 15 seconds and elongation at 72 ºC for 30 seconds. For PCR of SOD1-GFP, annealing temperature was dropped to 50 ºC and elongation time was increased to 1 minute. Pfu DNA polymerase (Invitrogen) was used because proofreading was important to ensure new mutations were not introduced.

Transformation of Bacteria

All plasmids were transformed into DH5α *Escherichia coli* for amplification. DH5α (50µl) was mixed with DNA (2µl) and incubated on ice for 20 minutes, heat shocked at 42 ºC for 30 seconds, and allowed to recover on ice for five minutes. SOB (500µl) was added and the mixture was incubated at 37 ºC shaking for one hour and then grown on selective LB agar plates. A Qiagen miniprep kit was used to extract the plasmids from the bacteria.

Transformation of yeast

All yeast was transformed using the One-Step method. Freshly grown yeast and the DNA plasmid of interest (150ng/µl) were added to the One-Step buffer (40% PEG, 10% LiAc, 10% DTT), incubated at 45 °C for one hour, and plated on selective plates. Transformants typically would appear within three days after transformation.

Yeast Two Hybrid Screening

Yeast (Y8930) with DB-SOD1 was grown in selective medium to an optical density (O.D. 600) of three. This yeast was mated with yeast containing the brain or fetal brain AD-cDNA libraries (Y8800) at the same optical density on YPD for four to six hours. The cells were then washed off the plate using dH_2O. A 1:10,000 dilution was plated on glucose-containing medium lacking leucine and tryptophan to determine the amount of yeast cells that were able to mate and the number of proteins that were successfully screened against SOD1. The remainder of the cells were plated on plates lacking leucine, tryptophan, and histidine to determine the proteins that interact with SOD1. The eight cDNA libraries used were made from the mRNA of tissues from of adult brain, fetal brain, lung, muscle, spleen, liver, heart, and Hela.

Homodimer Pairwise Test

Corning Costar 96 well plates were used to perform a homodimer test with wild type and mutant AD and DB-SOD1. SOD1 is known to form a homodimer, so this pairwise test serves as a control that the Y2H system is working as intended. We

	AD Empty Vector	AD Wild Type SOD1	AD A4V SOD1	AD G37R SOD1	AD G85R SOD1	AD G93A SOD1
DB Empty Vector	AD EV + DB EV	AD WT + DB EV	AD A4V + DB EV	AD G37R + DB EV	AD G85R + DB EV	AD G93A + DB EV
DB Wild Type SOD1	AD EV + DB WT	AD WT + DB WT	AD A4V + DB WT	AD G37R + DB WT	AD G85R + DB WT	AD G93A + DB WT
DB A4V SOD1	AD EV + DB A4V	AD WT + DB A4V	AD A4V + DB A4V	AD G37R + DB A4V	AD G85R + DB A4V	AD G93A + DB A4V
DB G37R SOD1	AD EV + DB G37R	AD WT + DB G37R	AD A4V + DB G37R	AD G37R + DB G37R	AD G85R + DB G37R	AD G93A + DB G37R
DB G85R SOD1	AD EV + DB G85R	AD WT + DB G85R	AD A4V + DB G85R	AD G37R + DB G85R	AD G85R + DB G85R	AD G93A + DB G85R
DB G93A SOD1	AD EV + DB G93A	AD WT + DB G93A	AD A4V + DB G93A	AD G37R + DB G93A	AD G85R + DB G93A	AD G93A + DB G93A

Figure 8: Homodimer Pairwise Test Outline. All possible combinations of AD-SOD1 and DB-SOD1 were compared. The cross of AD and DB wild type SOD1 should indicate an interaction because SOD1 is a homodimer. The mutants may exhibit differences in their ability to interact.

compared all possible combinations of DB wild type, A4V, G37R, G85R, and G93A SOD1 with their AD counterparts (Figure 8). Colonies of DB-SOD1 in yeast Y8930 and AD-SOD1 in yeast Y8800 were picked and grown overnight in leucine dropout and tryptophan dropout selective media respectively. The AD and DB were then allowed to mate overnight in YPD. The following day, the cells were transferred to leucine and tryptophan dropout media and grown overnight. The cells were then grown on leucine and tryptophan dropout plates as a control and on leucine, tryptophan, and histidine dropout plates to determine if DB-SOD1 and AD-SOD1 interact. This was also repeated using the multicopy high expression AD (212) and DB (213) vectors.

Functionality Complementation Assay

Serial dilutions (1:10 dilutions) were performed and spotted on selective (leucine dropout) agar plates. One leucine dropout plate was grown at 30 ºC (control), one was grown at 37 ºC (heat toxicity), and one was grown in the presence of tunicamycin (endoplasmic reticulum stress inducer).

SOD1-GFP Plasmid Construction

The SOD1-GFP construct was made by PCR using SOD1 forward primer 5'-GGGGACAACTTTGTACAAAAAAGTTGGC ATGGCGACGAAGGCC - 3' with Gateway cloning sites (attb1) and SOD1 reverse primer 5'-CTTCTCCTTTGCTGGCCAT TTGGGCGATCCCAATTAC -3' with 19 nucleotides of GFP. The GFP forward primer 5'- GTAATTGGGATCGCCCAA

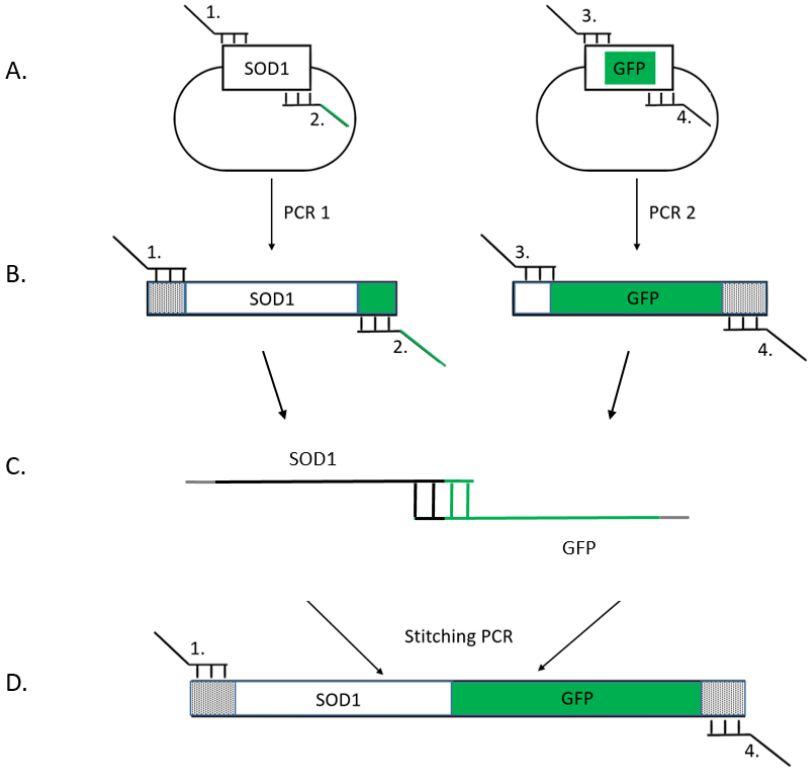


Figure 9: Construction of SOD1-GFP. GFP was tagged to the C terminus of SOD1 using PCR. Primer 1 is a forward primer that includes a Gateway att site with the beginning of the SOD1 sequence. Primer 2 is a reverse primer that includes the beginning of the GFP sequence and end of the SOD1 sequence. Primer 3 is a forward primer that includes the end of the SOD1 sequence and the beginning of the GFP sequence. Primer 4 is a reverse primer that includes a Gateway att site and the end of the GFP sequence. A: Primers were added to plasmids containing SOD1 and GFP. B: The first PCR annealed the primers to the genes and amplified them. C: The second PCR annealed the SOD1 and GFP sequences together. D: This created a SOD1-GFP construct with Gateway att sites.

ATGGCCAGCAAAGGAGAAG -3' contained 18 nucleotides at the end of SOD1 and the GFP reverse primer 5'- GGGGACAACTTTGTACAAGAAAGTTGA TTA GTAGAGCTCATCCATGCCATG -3' included a Gateway cloning site (attb2). The primers designed for this experiment were constructed using the OligoPerfect™ Designer (Invitrogen Corp. Carlsbad, CA). PCR was run first on SOD1 and GFP separately with their respective primers, then together with the SOD1 forward primer and the GFP reverse primer to join GFP to the C terminus of SOD1 (Figure 9).

Cell Culture

HEK-293 mammalian cells were cultured. Transfection of wild type and mutant SOD1 was done using the calcium-phosphate mediated method. Cells were transfected when they were 60% confluent. Forty eight hours after transfection, the cells were lysed with lysis buffer (0.5% Triton X-100, 20 mM Tris, 150 mM NaCl, 1 mM EDTA) and the protein lysate stored at -80 °C.

Pull Down Assay

Wild type and mutant SOD1 were cloned into pDEST27 Gateway vectors containing an N-terminus Glutathione *S*-transferase (GST) tag. GST has an extremely high affinity for GST-Bind™ resin reduced glutathione (GSH), therefore when GSH coated beads were added to the protein mixture, the GST tagged SOD1 adhered to the beads and was isolated from the rest of the protein in the lysate by centrifugation (5 min, 4 °C, 2000 rpm). Any proteins that were physically interacting with SOD1 are expected

to be pulled down by GSH. The GSH beads were boiled in Laemmli buffer (1% SDS, 100mM Tris) for five minutes to detach SOD1 and its interactors from the resin and then centrifuged. Supernatant is expected to contain proteins that interact with SOD1. Gel loading tips were used during wash steps to avoid aspiration of the pellet.

Western Blot

Triplicate protein gels were run to confirm the presence of protein in each step. One gel was stained with coomassie blue to determine if samples contained proteins. Another gel was silver stained using the Pierce® Silver Stain Kit from Thermo Scientific in order to detect low-abundance proteins. Once the presence of protein was confirmed in the samples, western blotting with a rabbit polyclonal antibody to SOD1 (ab16831, Abcam) was used to confirm the presence of GST tagged SOD1 in samples.

Mass Spectrometry

Once the GST control, WT SOD1, and A4V SOD1 protein samples were prepared, they were sent to the mass spectrometry and proteomic core facility at Ohio State University for analysis. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1). Mascot was set up to search the SwissProt_ID_2016_03 database (selected for Homo sapiens). Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 10.0 PPM. Scaffold (version Scaffold_4.5.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide

Prophet algorithm (Keller, et al., 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides.

Results

I. Yeast Two Hybrid Screening (Y2H)

A. Y2H

Screening the brain and fetal brain libraries by Y2H resulted in colony growth on the plates lacking leucine and tryptophan (LT-), but not on the plates lacking leucine, tryptophan, and histidine (LTH-) (Table 2). This experiment was repeated with all available cDNA libraries to determine if there was a degradation problem with the brain and fetal brain libraries due to multiple freezing and thawing. The results from the experiment with the lung, muscle, spleen, liver, heart and Hela cDNA libraries did not differ from the Y2H with the brain and fetal brain libraries (data not shown). To rule out human error in the experiment, a positive control was added. The FUS protein has several known protein-protein interactions and has been successfully used in yeast two hybrid screenings previously in our lab. A Y2H was performed with wild type and mutant DB-SOD1 using DB-FUS as the positive control, as shown in Table 2. In this experiment, there was a similar amount of colony growth on all of the LT- plates, growth on the LTH- plate for the FUS control, but still no growth on the LTH- plates for DB-SOD1, suggesting a problem of the Y2H screening for SOD1.

B. Homodimer Pairwise Test

To further rule out human error, a homodimer pairwise test was performed. Since SOD1 is an obligated homodimer, we know it interacts with itself. If we cross AD-SOD1 and DB-SOD1 directly with each other, we expect they should interact. If the Y2H does

Table 2: Yeast Two Hybrid with Fetal Brain Library. The number of library proteins screened were determined by the number of colonies on the LT- plates. The number of protein-protein interactions were determined by the number of colonies on the LTH- plates. The number of clones screened by wild type and mutant SOD1 were comparable to the positive control (DB-FUS), but no interactions with SOD1 were found.

Construct	# Screened	# Interactions
DB-WT SOD1	1,940,000	0
DB-A4V SOD1	1,120,000	0
DB-G37R SOD1	1,250,000	0
DB-G85R SOD1	1,360,000	0
DB-G93A SOD1	1,320,000	0
DB-FUS (+ control)	1,460,000	87

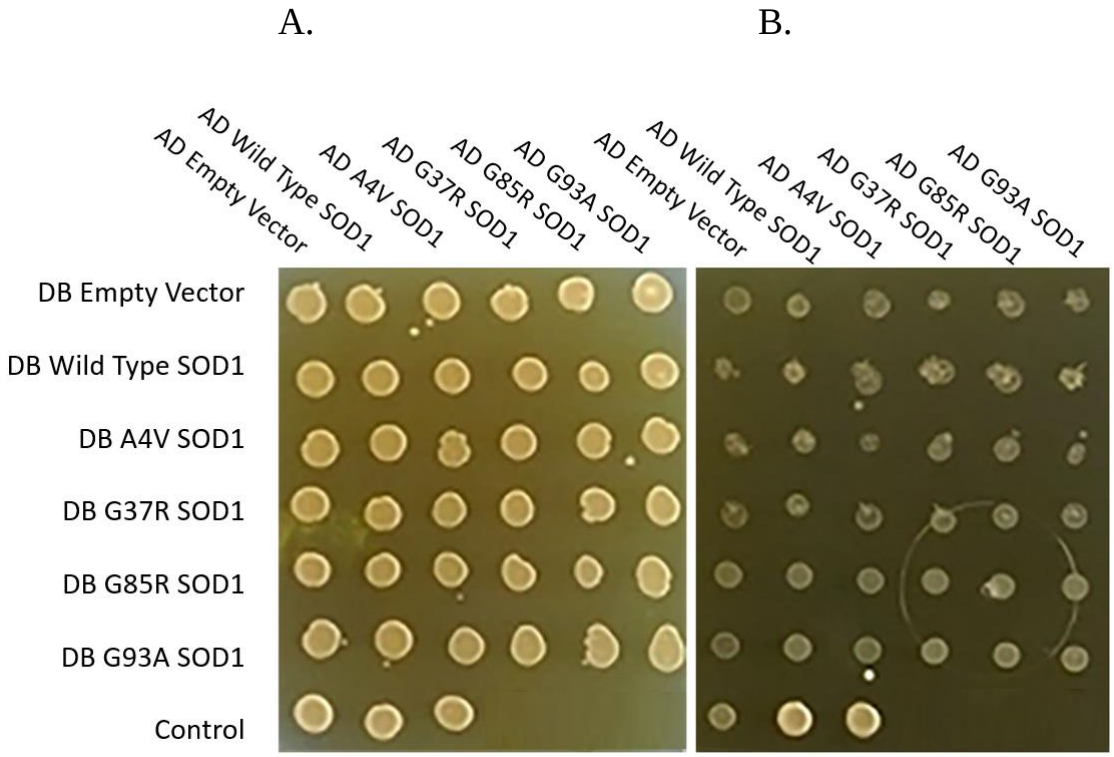


Figure 10: Homodimer Pairwise Test. A: Growth on LT- plates indicate yeast mating was successful. B: The absence of growth on LTH- plates indicate SOD1 does not homodimerize in the AD and DB vectors, or there is a problem in the Y2H screen. The control lane indicates no interaction, weak interaction, and strong interaction respectively on the LTH- plate.

not indicate an interaction, it suggests that the problem is with SOD1 and the Y2H screen itself.

To determine if all SOD1 mutants were still able to dimerize, wild type and mutant SOD1 were cloned into both AD and DB vectors and tested against themselves (Figure 10). Growth on the LT- plate indicated the yeast cells were able to mate successfully, and growth in the controls indicate the experiment worked properly. However, there was no growth on the LTH- plate, suggesting either DB-SOD1 did not dimerize with AD SOD1 or, more likely, the pairwise test was not able to detect the interaction.

C. High Expression Homodimer Pairwise Test

SOD1 was cloned into high expression AD (213) and DB (212) vectors and a homodimer pairwise test was performed. Due to the high copy number of the vectors, homodimerazation was expected to be seen. There was growth on the LT- plates, indicating successful mating, but no growth on the LTH- plates (Figure 11). This either suggests the high expression AD-SOD1 and DB-SOD1 did not dimerize, indicating a problem with the protein, or the test was unable to detect the interaction.

D. DB-SOD1 is Functional

A functional assay was performed to determine if the SOD1 protein was expressed properly in the yeast and functioning. If DB- hSOD1 was not able to rescue the *sod1Δ* yeast from the toxicity, it would suggest DB-hSOD1 was not functioning, which

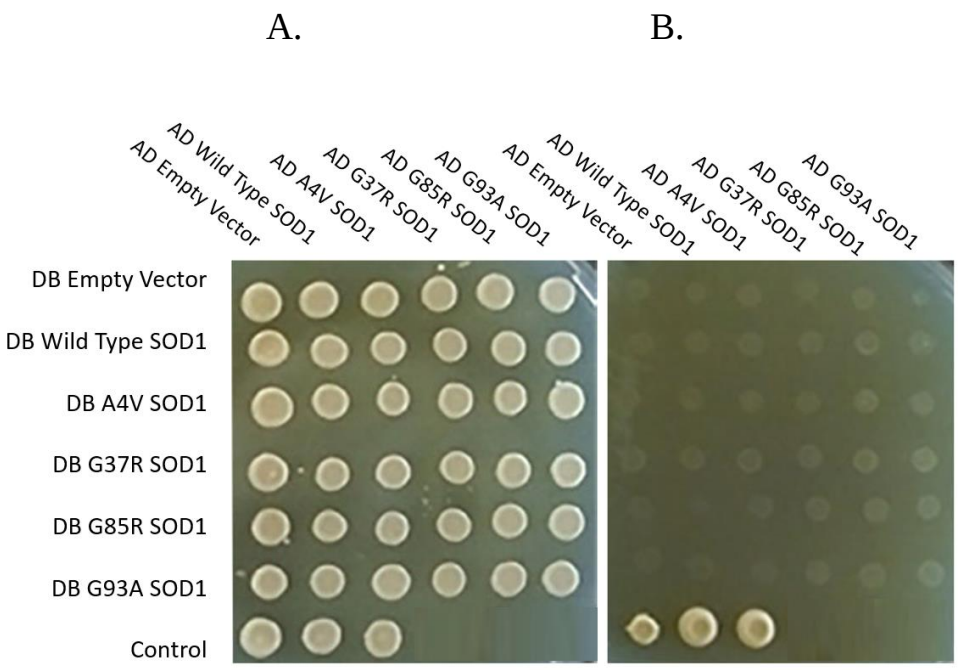


Figure 11: High Expression Homodimer Pairwise Test. A: Growth on LT- plates indicate yeast mating was successful. B: The absence of growth on LTH- plates indicate a problem in the Y2H screen. The control lane indicates no interaction, weak interaction, and strong interaction respectively on the LTH- plate.

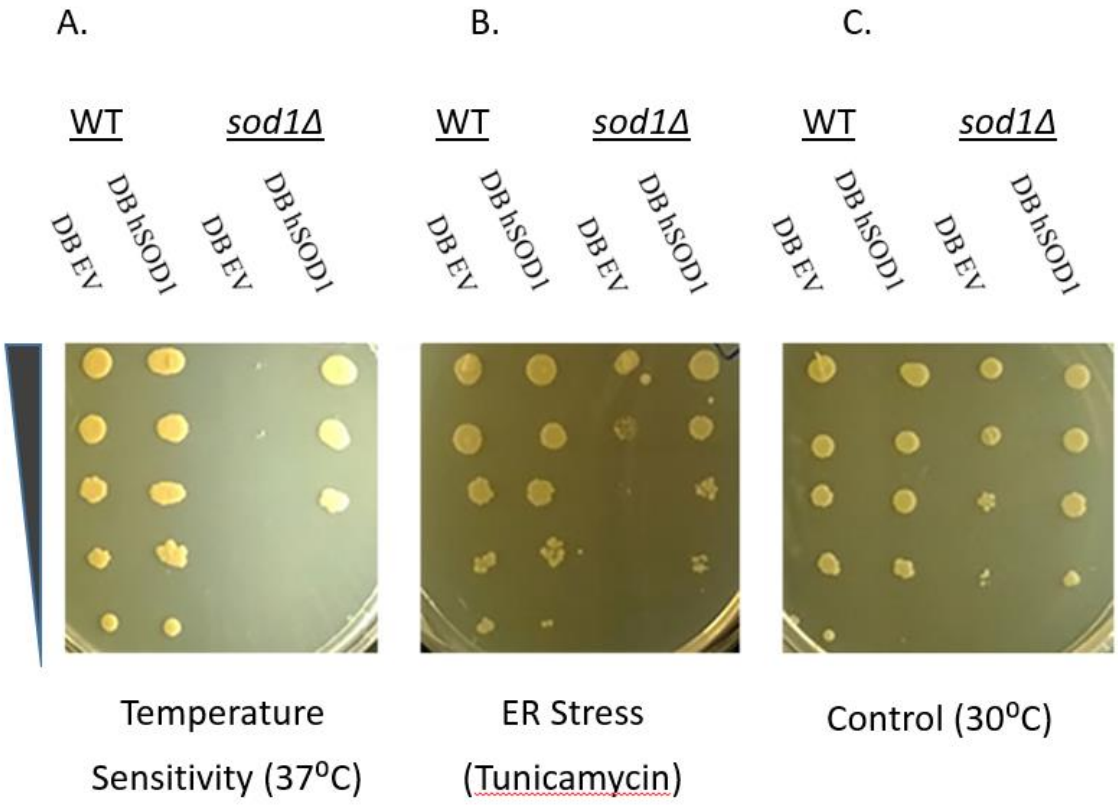


Figure 12: Functional Assay. Serial dilutions were performed and grown in toxic conditions. If DB-SOD1 is able to take over the role of endogenous yeast SOD1 it suggests DB-SOD1 is functional. A: DB- SOD1 was able to rescue growth of *SOD1Δ* at 37°C. B: DB-SOD1 was able to rescue growth of *SOD1Δ* in the presence of 0.75µg/ml tunicamycin. C: Control grown at 30 °C.

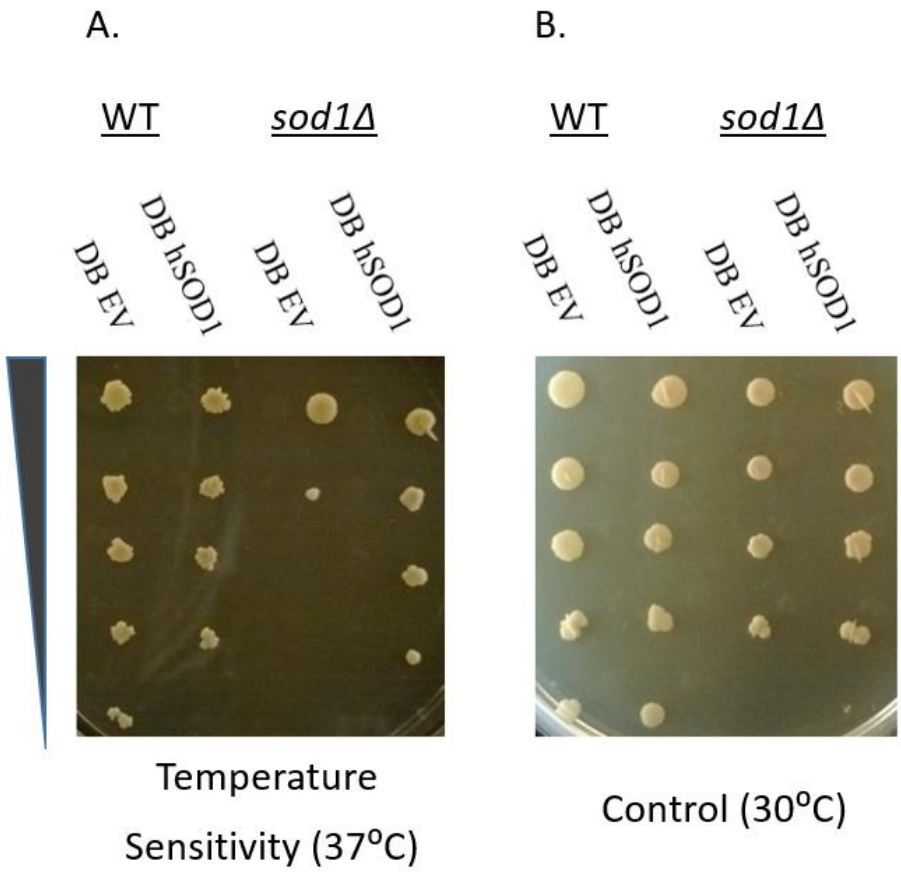


Figure 13: DB-SOD1-GFP Functional Assay. Serial dilutions were performed and grown in toxic conditions. If DB-SOD1 is able to take over the role of endogenous yeast SOD1, it suggests DB-SOD1-GFP is functional. A: DB- SOD1 were able to rescue growth of *SOD1Δ* at 37°C. B: Control grown at 30 °C.

A B. C.

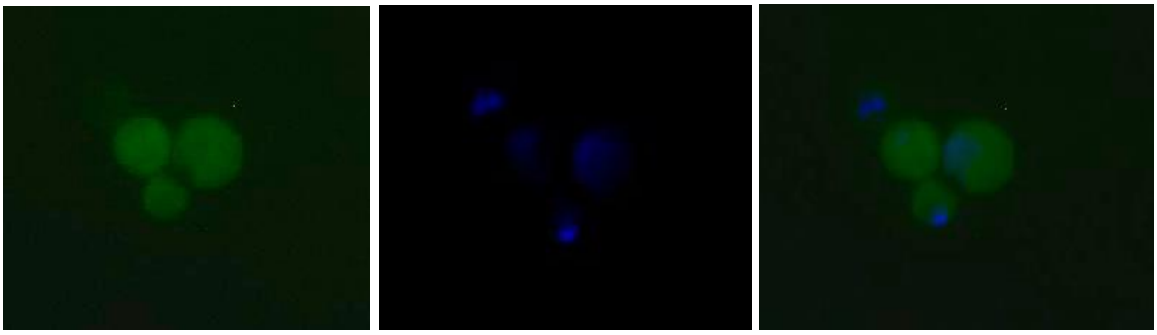


Figure 14: DB-SOD1 Not Abundant in Nucleus. A: The green color shows where DB-SOD1 is in the yeast cell. B: The yeast cell was stained with DAPI to identify the nucleus. C: Images A and B were combined to show DB-SOD1 does not abundantly localize to the nucleus.

could be the reason the Y2H and homodimer pairwise tests yielded no positive results. However, DB hSOD1 was able to rescue the *sod1Δ* yeast, indicating that the protein was functional and the DB vector did not disrupt enzymatic activity (Figure 12). A functional assay was also performed to confirm the functionality of DB-SOD1-GFP (Figure 13).

E. DB-SOD1 Not Localized to Nucleus

In order for the Y2H to work, DB-SOD1 must be localized to the nucleus. For this reason, the DB vector has a nuclear localization signal. If the nuclear localization signal is not strong enough, DB-SOD1 would remain in the cytosol, where SOD1 is primarily found. SOD1 was tagged with GFP and cloned into the DB vector to determine where DB-SOD1 was in the cell. DAPI staining was done to identify the nucleus. As Figure 14 shows, the nuclear localization signal on the DB vector was not strong enough to localize DB-SOD1 in the nucleus, suggesting this is the reason the Y2H did not work.

II. Pull Down and Mass Spectrometry Analysis

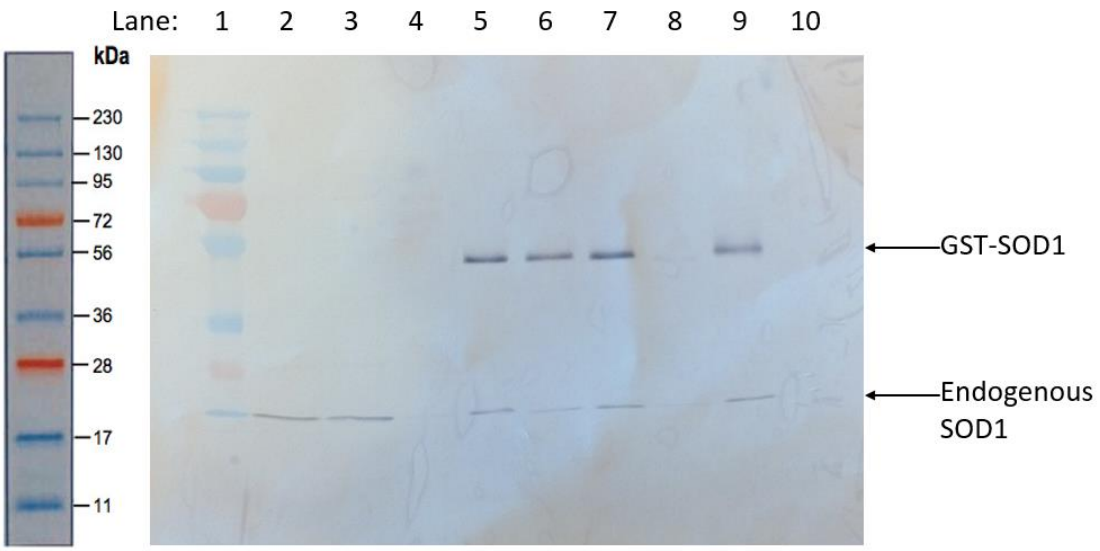
A. Transfection and Cell Lysis

The transfection was performed with a GFP control done alongside to estimate transfection efficiency. Transfection efficiency averaged at 80%. Cells were lysed and proteins were separated by SDS-PAGE and visualized with coomassie blue staining.

B. SDS-PAGE and Western Blot

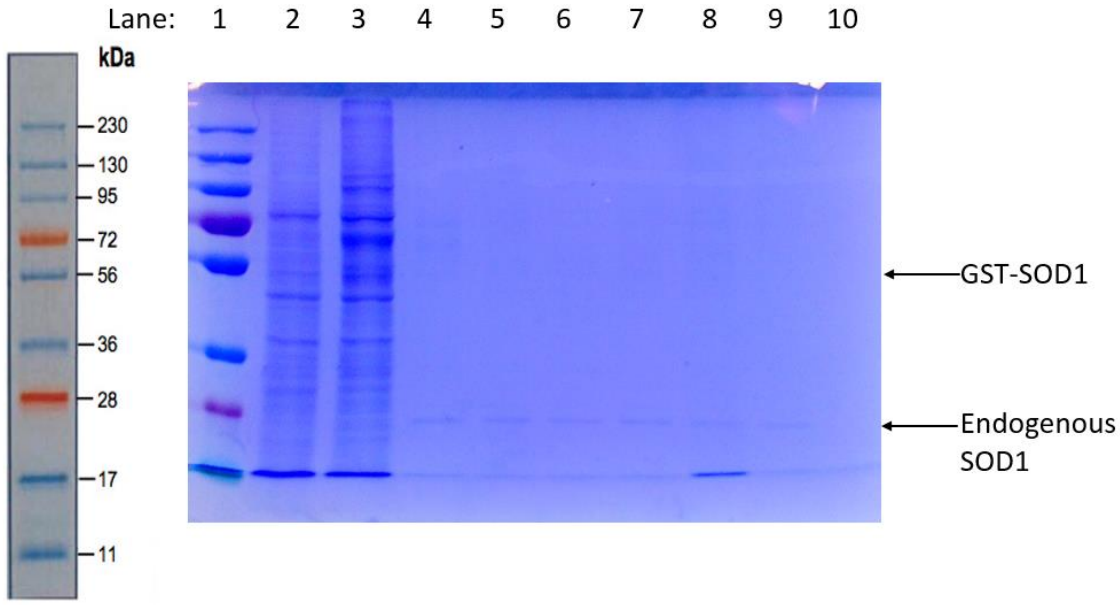
The GST-SOD1 complex of protein-protein interactors was pulled down using GSH beads and then purified from the beads by boiling in Laemmli buffer. GST-SOD1 was then visualized on a western blot (Figure 15). GST-SOD1 was able to pull down endogenous SOD1, suggesting it was able to pull down other proteins as well.

Proteins in the SOD1 complex were applied to SDS-PAGE and gels were stained to detect proteins. The bands on the coomassie stained gel were compared for differences between wild type and mutant SOD1 (Figure 16). The bands on the gel were very light due to a low concentration of proteins and no apparent differences were able to be identified. Therefore, the gel was silver stained to detect proteins of lower concentration (Figure 17). The silver stain did not show any detectable differences in the bands either, so only three samples (GST control, WT SOD1, and A4V SOD1) were sent for further analysis.



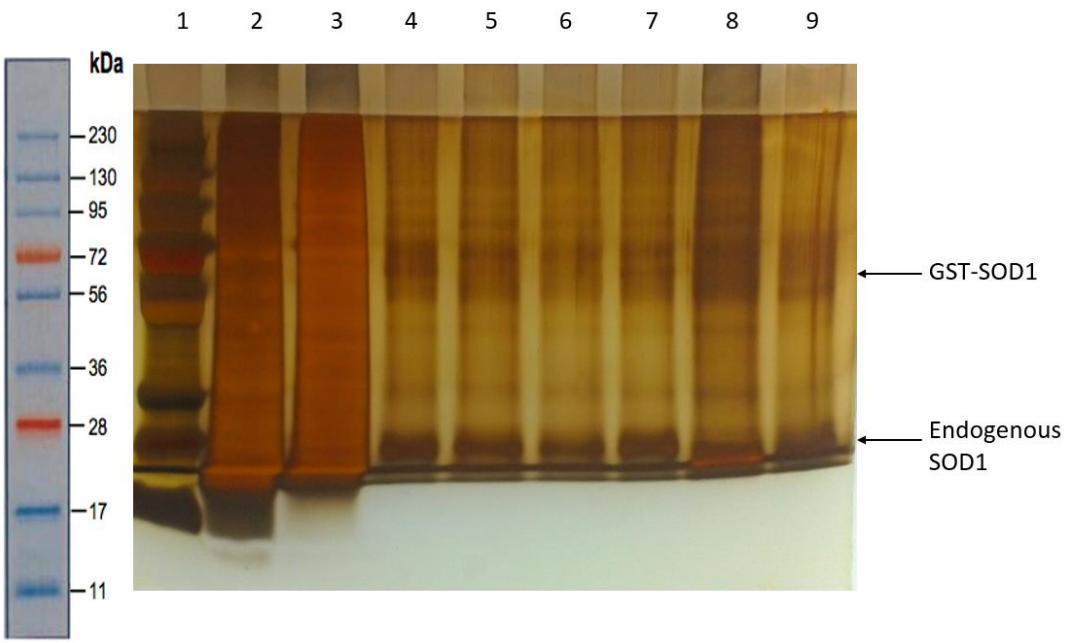
Lane1: Ladder
Lane 2: Not transfected HEK
Lane 3: Unbound Empty Vector (EV)
Lane 4: EV Pull Down
Lane 5: WT Pull Down
Lane 6: A4V Pull Down
Lane 7: G37R Pull Down
Lane 8: G85R Pull Down
Lane 9: G93A Pull Down
Lane 10: Just Buffer Pull Down

Figure 15: Western Blot of Samples from Pull Down Assay. The presence of GST-SOD1 and endogenous SOD1 was confirmed by western blot with the SOD1 antibody.



Lane1: Ladder
Lane 2: Not transfected HEK
Lane 3: Unbound Empty Vector (EV)
Lane 4: EV Pull Down
Lane 5: WT Pull Down
Lane 6: A4V Pull Down
Lane 7: G37R Pull Down
Lane 8: G85R Pull Down
Lane 9: G93A Pull Down
Lane 10: Just Buffer Pull Down

Figure 16: Coomassie Staining. The presence of GST-SOD1 could not be determined when stained with coomassie blue due to its low concentration.



Lane1: Ladder
Lane 2: Not transfected HEK
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Lane 4: EV Pull Down
Lane 5: WT Pull Down
Lane 6: A4V Pull Down
Lane 7: G37R Pull Down
Lane 8: G85R Pull Down
Lane 9: G93A Pull Down

Figure 17: Silver Staining. The presence of GST-SOD1 was difficult to determine through silver staining. GST-SOD1 should be located in the smear of proteins identified by the arrow.

C. Mass Spectrometry Analysis

Mass Spectrometry was used to identify the proteins that were pulled down in the solution. A GST control, GST tagged wild type SOD1, and GST tagged A4V SOD1 (Lanes 4, 5, and 6 respectively in Figures 15, 16, and 17) were sent for mass spectrometry analysis. Any proteins found to interact with both SOD1 and the GST control were assumed to be false positives and not included in the list of protein-protein interactions. Mass spectrometry determined that fifty-one proteins interact exclusively with wild type SOD1 (Table 3). Thirteen proteins were found to interact with both wild type and A4V SOD1 (Table 4). One notable protein interaction was SOD1 (Table 4, #1), suggesting both wild type and A4V SOD1 retain the ability to homodimerize. Zero proteins were found to exclusively interact with A4V SOD1 (Figure 18).

D. GO Term Analysis of Protein-Protein Interactions

Protein-protein interactions were analyzed using PANTHER (**P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships) Gene Ontology (GO). The molecular functions of all the wild type interactions, including the interactions shared by the A4V mutant, is shown in Figure 19. The molecular functions of only the interactions shared between the wild type and A4V SOD1 are shown in Figure 20. Of these interactions, 46.2% are binding proteins, 15.4% are structural proteins, 30.8% relate to catalytic activity and 7.7% relate to antioxidant activity.

The molecular functions of the interactions unique to wild type SOD1 can be seen in Figure 21. These unique wild type interactions also correspond to the protein-protein

interactions lost by the A4V mutant. A4V SOD1 retains all protein-protein interactions relating to antioxidant activity. However, its loss of interactions leads to decreasing functionality in all other areas compared to the wild type.

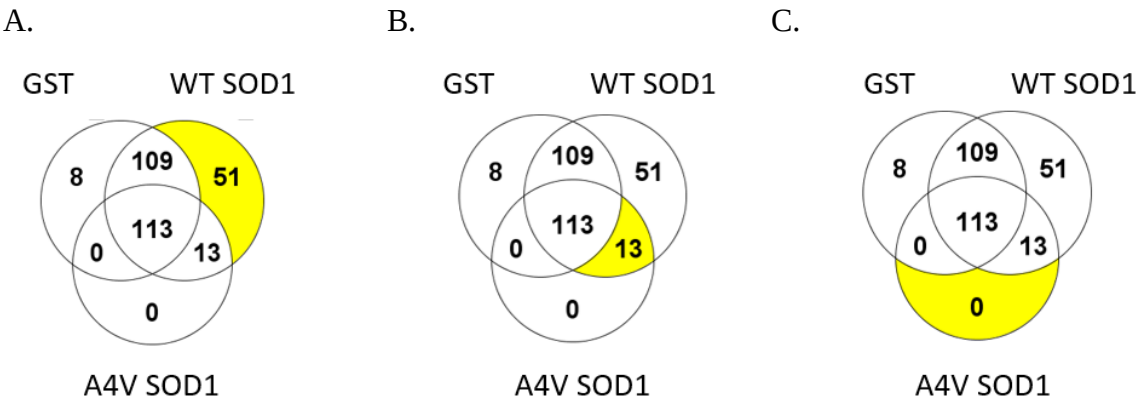

Figure 18: Comparison of Interactors. Protein-protein interactions were determined by mass spectrometry and compared. Any proteins that interacted with both the control (GST) and SOD1 were not tallied as real interactors. A. There were fifty one proteins that exclusively interacted with wild type (WT) SOD1. B. Thirteen proteins were found to interact with both wild type and A4V SOD1. C. Zero proteins were identified that interact exclusively with A4V SOD1.

Table 3: Unique Wild Type SOD1 Interactors

#	Accession	Protein Name
1	DHX9_HUMAN	ATP-dependent RNA helicase A
2	SSRP1_HUMAN	FACT complex subunit SSRP1
3	TCPD_HUMAN	T-complex protein 1 subunit delta
4	RL24_HUMAN	60S ribosomal protein L24
5	ARF3_HUMAN	ADP-ribosylation factor 3
6	RBBP4_HUMAN	Histone-binding protein RBBP4
7	P53_HUMAN	Cellular tumor antigen p53
8	TKT_HUMAN	Transketolase
9	C1TC_HUMAN	C-1-tetrahydrofolate synthase, cytoplasmic
10	SERA_HUMAN	D-3-phosphoglycerate dehydrogenase
11	XPO2_HUMAN	Exportin-2
12	PPIB_HUMAN	Peptidyl-prolyl cis-trans isomerase B
13	HNRPC_HUMAN	Heterogeneous nuclear ribonucleoproteins C1/C2
14	RAB1A_HUMAN	Ras-related protein Rab-1A
15	ECHA_HUMAN	Trifunctional enzyme subunit alpha, mitochondrial
16	HSP74_HUMAN	Heat shock 70 kDa protein 4
17	IF2B1_HUMAN	Insulin-like growth factor 2 mRNA-binding protein 1
18	ATPG_HUMAN	ATP synthase subunit gamma, mitochondrial
19	SDHA_HUMAN	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial
20	SYDC_HUMAN	Aspartate--tRNA ligase, cytoplasmic
21	MDHC_HUMAN	Malate dehydrogenase, cytoplasmic
22	DDX21_HUMAN	Nucleolar RNA helicase 2
23	TRAP1_HUMAN	Heat shock protein 75 kDa, mitochondrial
24	ANM1_HUMAN	Protein arginine N-methyltransferase 1
25	MATR3_HUMAN	Matrin-3
26	STRAP_HUMAN	Serine-threonine kinase receptor-associated protein
27	ROA3_HUMAN	Heterogeneous nuclear ribonucleoprotein A3
28	CKAP4_HUMAN	Cytoskeleton-associated protein 4
29	AN32E_HUMAN	Acidic leucine-rich nuclear phosphoprotein 32 family E
30	ECHM_HUMAN	Enoyl-CoA hydratase, mitochondrial
31	RS15A_HUMAN	40S ribosomal protein S15a
32	SSBP_HUMAN	Single-stranded DNA-binding protein, mitochondrial
33	RBMX_HUMAN	RNA-binding motif protein, X chromosome
34	RL35A_HUMAN	60S ribosomal protein L35a
35	HMCS1_HUMAN	Hydroxymethylglutaryl-CoA synthase, cytoplasmic
36	RAGP1_HUMAN	Ran GTPase-activating protein 1

37	PSA_HUMAN	Puromycin-sensitive aminopeptidase
38	THIL_HUMAN	Acetyl-CoA acetyltransferase, mitochondrial
39	RUVB1_HUMAN	RuvB-like 1
40	RL34_HUMAN	60S ribosomal protein L34
41	IPYR_HUMAN	Inorganic pyrophosphatase
42	ETFB_HUMAN	Electron transfer flavoprotein subunit beta
43	MIC19_HUMAN	MICOS complex subunit MIC19
44	CBX5_HUMAN	Chromobox protein homolog 5
45	PRKDC_HUMAN	DNA-dependent protein kinase catalytic subunit
46	RS9_HUMAN	40S ribosomal protein S9
47	ACTN4_HUMAN	Alpha-actinin-4
48	RL36_HUMAN	60S ribosomal protein L36
49	HMGA1_HUMAN	High mobility group protein HMG-I/HMG-Y
50	SMC1A_HUMAN	Structural maintenance of chromosomes protein 1A
51	DDX46_HUMAN	Probable ATP-dependent RNA helicase DDX46

Table 3: Unique Wild Type SOD1 Interactors. Mass Spectrometry indicated fifty one protein interactors exclusive to wild type SOD1. Any proteins that interacted with both wild type SOD1 and the GST control are assumed to be interacting with GST and are not included in the table. Interactors shared between wild type and A4V SOD1 are not included in this table.

Table 4: Wild Type and A4V Shared Interactors

	Accession	Protein Name
1	SODC_HUMAN	Superoxide dismutase [Cu-Zn]
2	XRCC6_HUMAN	X-ray repair cross-complementing protein 6
3	PHB_HUMAN	Prohibitin
4	H2A1A_HUMAN	Histone H2A type 1-A
5	H2B1B_HUMAN	Histone H2B type 1-B
6	RL7_HUMAN	60S ribosomal protein L7
7	HBA_HUMAN	Hemoglobin subunit alpha
8	HMGB1_HUMAN	High mobility group protein B1
9	RS25_HUMAN	40S ribosomal protein S25
10	AATM_HUMAN	Aspartate aminotransferase, mitochondrial
11	RL9_HUMAN	60S ribosomal protein L9
12	TIF1B_HUMAN	Transcription intermediary factor 1-beta
13	PDIA6_HUMAN	Protein disulfide-isomerase A6

Table 4: Wild Type and A4V Shared Interactors. Mass spectrometry identified thirteen proteins that interact with both wild type and A4V SOD1. Any proteins that interacted with all wild type SOD1, A4V SOD1 and the GST control are assumed to be interacting with GST and are not included in the table.

A. B.

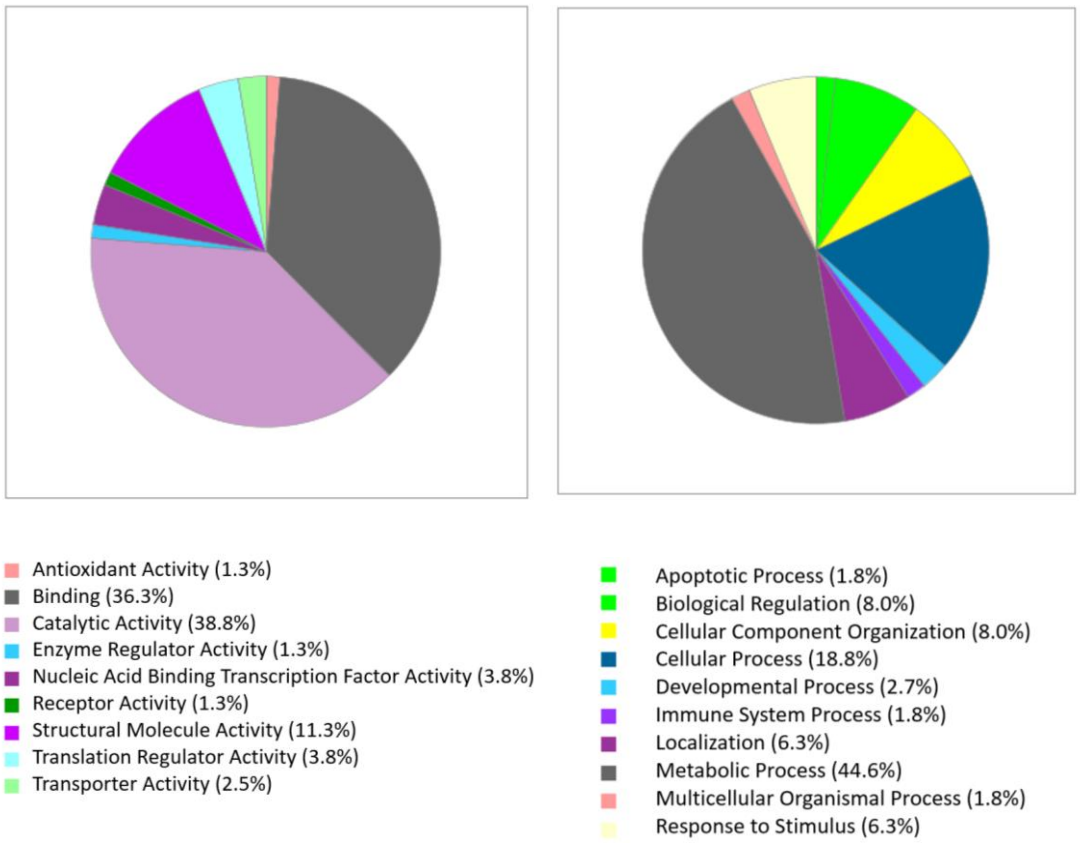

Figure 19: GO All Wild Type Interactions. Processes hypothesized to be impacted by protein-protein interactions of wild type SOD1. A. Distribution and percentages of molecular function of all the proteins that interact with wild type SOD1. B. Distribution of biological processes of all the proteins that interact with wild type SOD1.

A. B.

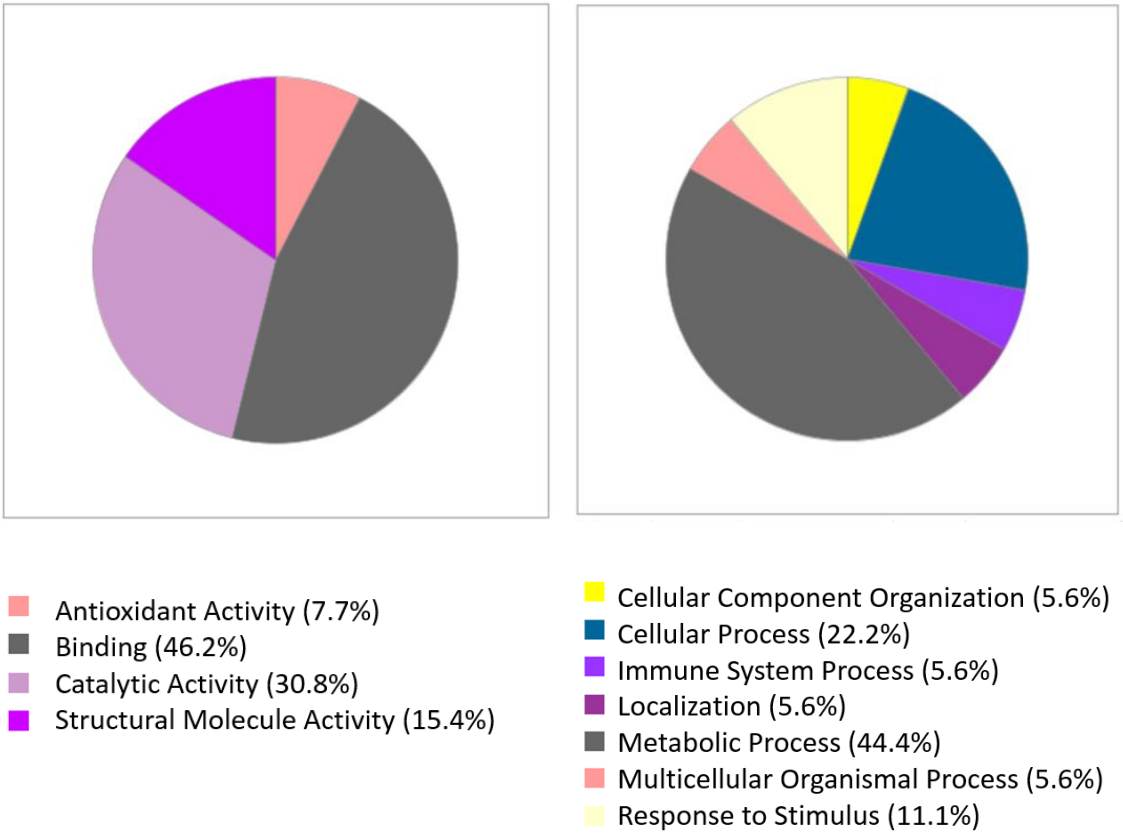

Figure 20: GO Wild Type and A4V Shared Interactions. Processes hypothesized to be impacted by protein-protein interactions of wild type and A4V SOD1. A. Distribution and percentages of molecular function of the proteins that interact with both wild type and A4V SOD1. Enzyme regulator activity, nucleic acid binding, transcription factor activity, receptor activity, structural molecule activity, translation regulator activity, and transporter activity were lost in the A4V mutant. B. Distribution of biological processes of the proteins that interact with both wild type and A4V SOD1.

A. B.

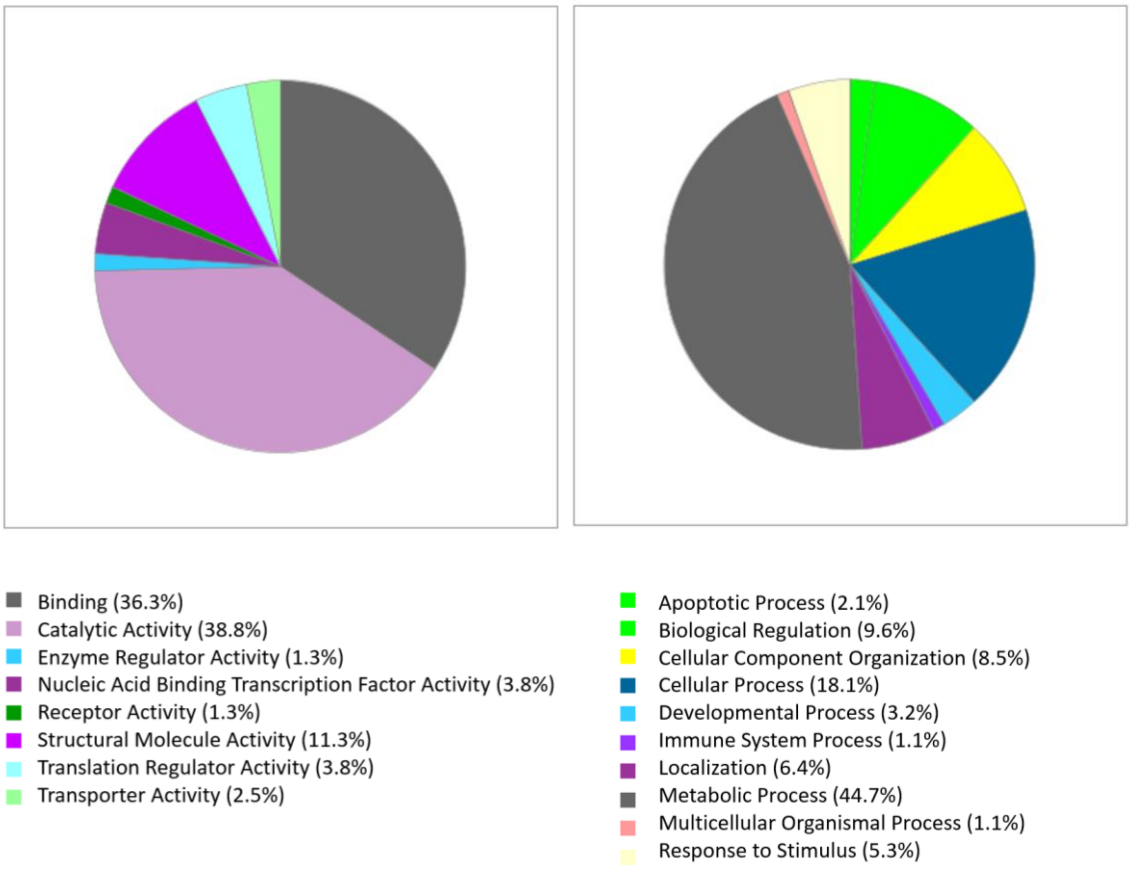

Figure 21: GO Unique Wild Type Interactions. Processes hypothesized to be impacted by protein-protein interactions of unique wild type SOD1. A. Distribution and percentages of molecular function of the proteins that exclusively interact with wild type SOD1. Because this represents proteins only interacting with the wild type, these functions were lost in the A4V mutant. A4V SOD1 retained all antioxidant activity. B. Distribution of biological processes of the proteins that exclusively interact with wild type SOD1. The A4V mutant lost interactions with proteins important in apoptosis, regulation, and development.

Discussion

Based on the results, it seems that the yeast two hybrid screen would not work with the protein SOD1. This could be due to several reasons, many of which I was able to rule out. First, I ruled out the possibility that I was not screening enough of the library proteins against SOD1 by optimizing the experiment, therefore greatly increasing the number of proteins from the library that were able to mate with SOD1. Second, I ruled out the possibility that there was a problem with the libraries or that SOD1 did not interact with any proteins in the brain and fetal brain libraries by performing a Y2H with all the cDNA libraries available to me. Third, I ruled out that it was human error by adding the FUS control and getting an appropriate amount of colonies (Table 1).

Because SOD1 is a homodimer, I performed a homodimer pairwise test to act as a positive control for the Y2H. I tested wild type DB-SOD1 against AD-SOD1 and the SOD1 mutants. I expected wild type SOD1 to interact with itself, if not all of the mutants. However, the homodimer pairwise test (Figure 10) showed that SOD1 did not interact with itself or any of the mutants, differing drastically from the literature and likely indicating an issue with my model. Thinking expression level may be the culprit, I then cloned wild type and mutant SOD1 into the high expression AD and DB vectors in the hopes that these vectors would give a better interaction (Figure 11). However, I obtained the same results as when using the lower expression vectors, suggesting there is a significant problem with the yeast two hybrid screening of SOD1.

These results introduced the possibility that the SOD1 protein was not functional when cloned into the DB vector. It was possible that the DB vector disrupted the folding

of SOD1, therefore blocking its binding sites and disrupting the enzymatic activity of SOD1. If SOD1 was not functioning properly, the Y2H would not work. To determine if this was the case, I performed a functional assay by transforming my DB-SOD1 construct into *sod1Δ* yeast to see if it would rescue growth under toxic conditions. In conditions of both heat (37 °C) and tunicamycin toxicity, DB-SOD1 was able to rescue the *sod1Δ* yeast, indicating DB-SOD1 retained its enzymatic activity and functionality (Figure 12). Therefore, the addition of DB does not affect the functionality of SOD1.

In order for the Y2H to work, SOD1 must be localized to the nucleus. Another possibility that the Y2H was not working was DB-SOD1 was not localized to the nucleus. SOD1 is normally cytosolic, but the DB vector contains a nuclear localization signal. Consequently, when SOD1 is cloned into the DB vector, the DB-SOD1 construct should be localized to the nucleus. Depending on the strength of the nuclear localization signal on the DB vector, however, this may not be the case. To test this, an SOD1-GFP gateway entry clone was created (Figure 9) and cloned into the high expression DB vector. I determined DB-SOD1-GFP was expressing by performing a functional test to see if it would rescue *sod1Δ* yeast (Figure 13). When DB-SOD1-GFP was shown to rescue *sod1Δ*, it was then transformed into Y8930 yeast and stained with DAPI to determine where SOD1 was localized. DB-SOD1-GFP was found to be localized in the cytosol (Figure 14), strongly indicating that this is the reason the Y2H did not work. In order to fix this problem, a stronger nuclear localization signal could be added to DB-SOD1 to force it into the nucleus. Had I gotten this result earlier, I would have been able to test if this truly was the reason the Y2H did not work by adding a stronger nuclear localization signal to DB-SOD1. If DB-SOD1 then localized to the nucleus and the Y2H

worked, I would have continued with the Y2H and not worked with mammalian cells. However, it is possible that SOD1 has a nuclear exclusion signal that prevents it from entering the nucleus. If this is the case, no localization signal could place it in the nucleus. That nuclear exclusion signal would have to be removed from SOD1 by mutagenesis or stitching PCR in order for the Y2H to work.

Because the Y2H system did not seem to be working, I redirected my efforts to find protein-protein interactions using the pull down assay. I transfected wild type and mutant GST-SOD1 into HEK-293 cells and performed a pull down assay to isolate the protein complex of SOD1 interactions followed by mass spectrometry analysis. GST-SOD1 was confirmed to be in the sample by western blot (Figure 15) using SOD1 antibody and proteins visualized on coomassie blue (Figure 16) and silver stained gels (Figure 17).

It was determined that wild type SOD1 interacts exclusively with fifty one proteins (Table 3). Three proteins that were notable were Protein arginine N-methyltransferase 1 (ANM1, Table 3, #24), Matrin -3 (MATR3, Table 3, #25), and RNA-binding motif protein, X chromosome (RBXM, Table 3, #33). These proteins were also identified previously in our lab to interact with FUS (Unpublished Data), another ALS associated protein, and MATR3 is also an ALS associated protein. It is interesting that these proteins interact with wild type SOD1, but not A4V SOD1.

Both wild type and A4V SOD1 share interactions with thirteen proteins (Table 4). One protein to note that interacted with both wild type SOD1 and A4V SOD1 was SOD1 (Table 4, #1). This suggests that the A4V mutant does not lose the ability to homodimerize. A4V SOD1 did not exclusively interact with any proteins (Figure 18).

Therefore, in the case of A4V, no gain of protein-protein interactions were identified with mutant SOD1. However, the A4V mutation resulted in a loss of fifty one protein-protein interactions (Table 3), any of which could result in a gain of toxicity.

GO Term analysis indicated that A4V SOD1 lost many proteins that performed several molecular functions. Those functions lost completely included enzyme regulator activity, nucleic acid binding, transcription factor activity, receptor activity, structural molecule activity, translation regulator activity, and transporter activity. Functionality of binding, catalytic activity and structural molecule activity were only partially lost. Interestingly, the A4V mutant retained all interactions relating to antioxidant activity (SOD1, Table 4, #1), suggesting oxidative stress may not be the cause of SOD1 toxicity. Protein-protein interactions that were lost to A4V SOD1 were shown to be important in biological processes such as apoptosis, regulation, and development.

Any proteins that were shown to interact with both GST and SOD1 were treated as false positives pulled down only by GST. However, it is possible that some proteins interact with SOD1 and GST independently. For example, the FUS protein that has been found previously to interact with SOD1 was identified in this study to interact with both GST and SOD1, and therefore a false positive. Many of the proteins identified by the mass spectrometer as protein-protein interactions may be false positives as well. Depending on the sensitivity of the machine, prior protein samples may still be picked up during sample analysis. Alternatively, the mass spectrometer may not be sensitive enough to identify some low concentration proteins that interact with SOD1. In either case, more experimentation needs to be done to validate protein-protein interactions with SOD1.

As these results were from one pull down experiment followed by one mass spectrometry analysis, this experiment should be repeated. If the same proteins are identified by analysis of the samples from multiple pull down experiments, there is a higher confidence that the interactions are real. To further the confidence of real interactions, an immunoprecipitation with the antibody from each specific protein interaction could be done. Those interactions could then be visualized on a western blot. For full confidence of direct protein-protein interactions, *in vitro* assays with purified proteins should be performed. This will exclude the possibility that any given protein is just binding to another protein that interacts with SOD1.

Conclusion

It was determined that the Y2H screen was not effective at identifying SOD1 binding partners. I believe this is due to DB-SOD1 not localizing to the nucleus, but further study needs to be done to confirm this. Adding a stronger nuclear localization signal and doing another Y2H would be a good way to determine if this is the reason. Every different method to find protein-protein interactions has the opportunity to identify different proteins, therefore performing a Y2H would be a way to confirm positive interactions found using mass spectrometry.

The A4V mutant was found to lose fifty one protein mutations compared to wild type SOD1. It is possible one or more of the proteins lost in the mutant prevents SOD1 from gaining a toxic function. For example, when the protein or complex of proteins bind, SOD1 functions normally. However, if the same proteins are not able to bind due to the mutation, SOD1 develops the toxic function that plays a role in ALS.

It cannot be determined in this study if mutants other than A4V lose normal or gain abnormal protein-protein interactions. Each mutant would need to be analyzed independently to determine if they share the characteristics of A4V SOD1. Due to the different disease characteristics associated with the mutants, it is possible different SOD1 mutants gain or lose interactions differently. The gain or loss of some of these proteins may make SOD1 more or less toxic, thus leading to the differences in onset and progression between the SOD1 mutants. It could be very important to discover if these interacting proteins are also related to FUS or other ALS associated proteins. More study needs to be done to determine if the protein-protein interactions from this study were real and to realize their significance in relation to ALS.

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