An Investigation into Mitigating Proteotoxicity In Vitro and In Vivo

By
Gautam Shrivastava

A thesis submitted to Johns Hopkins University in conformity with the requirement for the degree Master of Science.

Baltimore, Maryland
June 2015

Abstract

Neurodegenerative diseases are often associated with issues in protein degradation brought on by protein aggregates and misfolded proteins that yield proteotoxicity. Amyotrophic Lateral Sclerosis (ALS) serves as the main focus for our lab, as we perform experiments in vitro and in vivo to try and reduce aggregation of mutant super oxide dismutase 1 (SOD1). Two approaches used for undertaking study of protein degradation: a small molecule SIRT2 inhibitor drug trial in G93A mice to test motor skills and survival, and a series of experiments inducing DNA damage and proteotoxicity to discern fluctuations in UBE4B and LSD1. Through 15-17 weeks of drug injections in both male and female cohorts, there was a small improvement in overall lifespan with the drug that was not statistically significant, and no significant differences between the drug and control groups in motor ability. Following inducing stress through various means, there were consistent patterns of reductions in key ubiquitin ligases and associated proteins across four cell lines: H293T, HCT P53 +/+, HCT P53 -/-, and NSC34. These findings offer affirmation in the potential that these proteins work synergistically in protein quality control pathways.

Acknowledgements

I would like to thank the Wang Lab, especially Dr. Periz and Dr. Wang for their guidance, support, and encouragement in undertaking these projects. I also appreciated the support of Dr. Ugolino for her help with the mouse work and optimizing genotyping protocols.

I am also thankful for the collaboration with the Bert Vogelstein lab for supplying the P53 knockout Human Colon Cancer cell line, which was important to these experiments that were focused on understanding the effects of P53 on other proteins. I also appreciate the help from Tocris Laboratories in supplying us with the drug Tenovin-1, as well as Sigma Aldrich for their help with protocols on how to mix drugs.

Table of Contents

List of Figures

Introduction

Environmental stressors and inevitable gene replication errors present challenges to the structure and integrity of macromolecules, such as DNA and proteins, which are remedied by different internal mechanisms. Errors in the integrity of proteins yielding misfolded products or aggregates – termed proteotoxicity – are a common feature among neurodegenerative disease; including Alzheimers disease, Parkinsons disease, Huntingtons disease, and Amyotrophic Lateral Sclerosis (ALS) (Prusiner, 2012). Amyotrophic Lateral Sclerosis (ALS), which is characterized by a loss of upper and lower motor neuron function in the brain and spinal cord, has been an active area of research in the past 25 years. One major mutation that is believed to cause ALS is in the TDP-43 gene, with reports of 19 missense and 1 truncating mutation occurring in TDP-43 that have been correlated with ALS patients (Strong et al., 2007). Mutation of the TDP-43 gene is believed to be an impetus in the formation of a mutant form of Superoxide Dismutatse 1 (SOD1) that is prone to aggregation – a characteristic that induces a high level of proteotoxicity that is believed to lead to the decline of motor neuron function (Arai et al., 2006). The internal mechanisms in place to combat proteotoxicity, specifically the aggregation associated with ALS, represent a complex set of effectors regulated by protein-protein interactions, components of which serve as the objective for this study.

Protein Quality Control and Mutant SOD1 in ALS

With estimates suggesting that 30% of newly synthesized proteins are incorrectly folded and promptly degraded, understanding the mechanisms behind these controls serves as an important stepping stone in discerning patterns behind pathologies involving proteotoxicity (Shubert et al., 2000). As with other biological systems aimed at maintaining

homeostasis, the protein quality control systems have two major lines of defense against misfolded or aggregating proteins – molecular chaperones for disposal, the Ubiquitin-Proteasome Pathway (UPS) (Cookson, 2005).

Molecular chaperones, which represent the first line of defense against proteotoxicity, facilitate the efficient folding and reverse unfolding of proteins (Hershko & Ciechanover, 1998). They work effectively by binding to the aggregation-prone sections of proteins – often hydrophobic stretches – and change local conformation by non-covalent interactions to stabilize them against irreversible aggregation (Kopito, 2000). An important class of chaperones is the Heat Shock Proteins (HSPs), which are ubiquitous elements of cellular machinery that offset a variety of stresses (Uversky, 2009). For example, the characteristic poly-glutamine induced toxicity of Huntington's disease is mitigated by the protective effects of HSP70 (Klucken et al., 2004).

The Ubiquitin-Proteasome Pathway (UPS), widely considered the second line of defense against proteotoxicity, selectively conjugates and targets abnormally structured proteins for degradation via covalent modification (polyubiquitination) (Morimoto et al., 1989). In order to function this way the system requires certain ligases that receive ubiquitin from E2 ligases and transfers it to targeted proteins by creating multi-ubiquitin chains (Wu & Leung, 2011). Special features of the UPS include differing signals for protein degradation: K-48 linked polyubiquitination serve as the classical sign for proteasomal degradation as it leads the ubiquitinated protein to be recognized by the 26S Proteasome (Navon, 2009). K-63 linked polyubiqutination serves as signal for autophagy into the Autophagy – Lysosome Pathway (ALP), which leads to degradation by engulfing the protein within a double membrane that forms an autophagosome (Bence et al., 2001).

Cu/Zn Superoxide dismutase 1 (SOD1) normally serves a protective role as it destroys free radicals and superoxides. However, mutant Cu/Zn SOD1 has been linked to roughly 20% of familial ALS cases, and is characterized by a tendency to misfold and aggregate in motor neurons (Wang et al., 2002). The wild type (WT) SOD1 is characterized by a stable β-barrel structure with a two-state folding process, however mutant SOD1 has a far higher propensity to improperly fold and aggregate in vitro and in vivo (Lindberg et al., 2005). Here we used the G85R mutant human SOD1 ($SOD1^{G85R}$), which clinically leads to aggregation into misfolded soluble oligomers, and larger insoluble aggregates – highly toxic proteins that lead to age-dependent synaptic dysfunction, neurodegeneration, and impaired motor abilities in *C. elegans* and mice (Wang et al., 2009).

Effects of UBE4B, LSD1, and USP7 on P53-mediated Protein Quality Control Measures

Within the protein quality control mechanisms in place to deal with proteotoxicity, there are several notable contributors to chaperone proteins, the UPS system, and the ALP pathway. The UPS system is made up of several classes of enzymes that facilitate its functionality in tagging targets: E1 refers to ubiquitin-activating enzymes, E2 refers to ubiquitin conjugating enzymes, and E3 refers to ubiquitin protein ligases (Johnson et al., 1997). A newer addition to the family is the E4 class, which is necessary for the degradation of certain proteins via the ubiquitin fusion degradation pathways. E4 enzymes specifically catalyze the elongation of a polyubiquitin chain, thereby allowing targeting of polyubiqutinated substrate proteins for degradation by the proteasome (Kuhlbrodt et al., 2005).

One E4 enzyme of particular focus is ubiquitin factor E4B (UBE4B), which is located in the 1p36 region and has been tied across several studies to p53 (Hatakeyama et al.,

2001). Recently, studies have shown that UBE4B promotes polyubiqtuitination and degradation of p53, while also inhibiting p53-dependent transactivation and apoptosis (Wu et al., 2011) (Shi et al., 2009). In a molecular context, UBE4B is amplified to abnormal levels in a variety of cancers: including colon cancer, breast cancer, and promyelocytic leukemia (Heuze et al., 2008; Hosada et al., 2005). Further results from studies showed that deletion of UBE4B from cancer cell lines resulted in the marked apoptosis of tumor cells, suggesting that reducing UBE4B may lead more efficient greater protein quality control machinery. The most recent studies have suggested that one of the central targets for UBE4B is actually p53, which seems to support the notion that UBE4B's inhibition leading to a more active protein quality control mechanism rests heavily on p53's activity (Zeinab et al., 2012).

Another route for impacting p53-mediated protein quality control machinery is through genetic/epigenetic modifications. While methylation of lysines on histone tails produces a silencing effect, these residues can be demethylated by variety of lysine-specific demethylases (LSDs) (Anand & Marmorstein, 2007). LSD1 was the first histone demethylase discovered, and it was primarily participated in gene repression as a component of CoREST (Co-repressor for Element-1 Silencing Transcription Factor) and NuRD (Nucleosome Remodeling and Histone Deacetylation) co-repressor complexes (Shi et al., 2007). LSD1 works by removing methyl groups from mono- and di-methylated lysine-4 residues of histone H3, one of the five main euchromatin histone proteins, and can catalyze de-methylation of histone H3 lysine-9 in cooperation with the androgen receptor (Wang et al., 2009; Chen et al., 2006). Lys-4 and lys-9 demethylations make LSD1 a significant component of transcriptional co-repressor complexes, and the demethylase been found to play an important role in silencing neuron-specific genes in non-neuronal cells (Schulte et al., 2009; Forneris et al., 2006). LSD1's interactions with H3 lysine-9 include gene activation via

demethylation of several non-histone substrates, including p53 (Metzger et al., 2005). More recently within this lab results have shown that a knockdown of LSD1 and UBE4B in wild type SOD1 and mutant $SOD1^{G85R}$ transfected HEK293T cells result in a reduction of mutant misfolded proteins. Additionally, in agreement with previous studies in *C. elegans* and *Drosophila,* the knockdown of UBE4B/LSD1 enhanced protein clearance beyond mutant $SOD1^{G85R}$ to include reductions in other aggregation-prone proteins, including $TDP\text{-}43^{Q331K}$, suggesting a more centralized effect on protein quality control (Periz et al., 2015).

Stabilization of P53 represents another option for enhancing protein quality control measures that are driven by the "guardian of the genome". P53 is kept in an autoregulatory feedback loop by Mouse double minute-2 homolog (MDM2) – an E3 ubiquitin ligase protein – by polyubiquitinating p53 in the absence of detected DNA damage (Chen et al., 1996). The process of stabilizing p53, however, can be achieved by deubituitinating the protein – a task a special class of enzymes are capable of: Deubiquititylating enzyme ubiquitin specific peptidases (USPs) (Li et al., 2004). One such ubiquitin specific peptidase is USP7 – a herpes virus-associated ubiquitin-specific protease (also called HAUSP) that acts as a cysteine protease, and was originally identified as a binding partner for the Herpes Simplex Virus (Everett et al., 1997). Crystallography analysis demonstrated that MDM2 and p53 both bind the N-terminal tumor necrosis factor-receptor associated factor (TRAF)-like domain of USP7 in a mutually exclusive manner, but USP7 retains a higher affinity for MDM2 than p53 (Hu et al., 2006). Further cell culture studies have illustrated that a genetic blockade of USP7 results in a destabilization of the autoregulatory feedback loop of MDM2/p53, and led to an up-regulation of p53 with concomitant cell cycle arrest (Cummins et al., 2004). These findings together suggest that USP7's ability to inactivate MDM2 and elevate P53 activity

may factor into greater protein quality control mechanisms in place to deal with the types of protein aggregates that are characteristic of ALS.

Experimental Pursuits with p53 Activation and Proposed Protein Quality Control Pathways

The experiments associated with this study examined the issue of proteotoxicity in ALS from two different standpoints – in vivo and in vitro. The in vivo aspect of this study dealt with the use of Tenovin-1, a SIRT1/2 inhibitor that increases acetylation and stability of p53, in cohorts of litter-matched male and female cohorts of G93A mice (Lain et al., 2008). Through a 15-week drug or control injection period, the mice were observed for changes in mass, motor skills, grip strength, and overall survival. Building from experiments in cell culture where Tenovin-1 was shown to reduce $SOD1^{G85R}$ protein aggregates, it was hypothesized that the elevated p53 levels would reduce mutant SOD1 aggregates and extend motor abilities, improve weight retention, and extend overall survival beyond the control.

The in vitro aspect of this study sought to examine the actors involved in protein quality control mechanisms mediated by p53. Aforementioned studies indicate that inhibiting UBE4B, USP7, and LSD1 result in a more robust reduction of mutant SOD1 aggregates, which has been replicated by experiments in this laboratory using tumor cells in cell culture, *C. elegans,* and *Drosophila* as model organisms. The latest findings in this laboratory also implicate a synergistic relationship between UBE4B and LSD1 in a hypothesized protein quality control pathway (the SUNS pathway) mediated by p53 that suppresses proteotoxicity-associated neurodegeneration (Periz et al., 2015). In order to understand potential mechanisms involved with the pathway, experiments imparting different types of proteotoxic stresses on cells were devised to test levels of these proteins tested against controls. Through the use of doxorubicin damage (DNA intercalation),

transfection with WT SOD1 and SOD1^{G85R} (protein aggregates), and heat shock as methods of stressors, the effects were observed in cell lines via western blot.

Methods and Materials

Cell Culture

For the cell culture experiments performed, four lines of cells were used. Human Embryonic Kidney 293T cells (HEK293T) were used as controls due to availability of high yield transfections protocols and robust growth potential. Human Colon Cancer P53 wild type cells (HCT+/+) and Human Colon Cancer P53 Knockout cells (HCT-/-) were used to contrast the impact of P53 against its absence on protein quality control mechanisms as stressors were applied, including protein aggregation. Mouse Motor Neuron-like hybrid cells (NSC34) were used to analyze whether the results found from the other cell lines could be replicated in motor neuron-like cells, which are most affected by protein aggregation in the pathology of Amyotrophic Lateral Sclerosis. The cell lines were kept in 10% Fetal Bovine Serum (FBS) Dulbecco's Modified Eagle Medium (DMEM), with added non-essential amino acids, β-mercaptoethanol, and glutamax.

Heat Shock

The four cell lines used (HEK293T, HCT +/+, HCT -/-, NSC34) were plated in 60 mm dishes pre-treated with Polyethylenimine (PEI) for one hour. The cell concentrations used were 2 ml of 3 x 10^5 cells/ml in DMEM. The dishes were made in duplicates; one was for extracting protein while the other was to extract RNA. The dishes were divided into three groups: control plates were kept at 37˚C, 1 hour heat shock plates kept at 43˚C, and 2 hour heat shock plates were kept at 43˚C for two hours. Immediately following the end of heat shock treatment, the cells were placed on ice and lysed with RIPA lysis buffer for proteins, and RNA was extracted.

Transfection

The four cell lines used (HEK293T, HCT +/+, HCT -/-, NSC34) were plated in 6 well dishes pre-treated with PEI for an hour. The cell concentrations used were 2 ml of 7.5x 10^4 cells/ml. The dishes, made in duplicates for RNA and protein extraction separately, were left to incubate overnight before transfection via lipofectamine 3000. They were transfected with either wild type (WT) Superoxide Dismutase 1 (SOD1) or G85R. After confirming high transfection efficiency via GFP, the cells were lysed to extract protein and RNA for further analysis.

Doxorubicin DNA Damage

The three cell lines used (HEK293T, HCT -/-, NSC34) were plated in 6 well dishes pre-treated with PEI for one hour. The cell concentrations used were 2 ml of 1.5 x 10^5 cells/ml. The dishes were divided into control and experimental, and made in duplicates to allow for both protein and RNA extraction. The experimental cells were treated with 16 ml of DMEM mixed with 4µl of Doxorubicin – an agent that intercalates DNA and is often used in chemotherapy – in order to induce a stress on the cells.

Rotarod

We obtained the Rotarod Machine (LE8200) from Harvard Apparatus (Holliston, MA). Mice were trained on the rotarod at increasing speeds from 6 rpm up to 16 rpm for 5 minutes at three separate times, in one-hour intervals in a day, the day before testing and injections. Mice that fell off during training were put back onto the rotating rod for the continued duration of five minutes. In testing, mice were placed on the rod in an accelerating

speed, over the course of 5 minutes, as it rose from 4 rpm to 40 rpm. Once the mice fell from the rod, their times were recorded and they were placed back into their cages. Before and after each run, the rod and rip plates were wiped down with 70% ethanol. During the course of the drug trial, both control and experimental groups of mice underwent training once a week and testing twice a week, with injections only occurring after testing. The training and testing was concluded for mice that were no longer able to stay on the rod for more than two seconds.

Intraperitoneal Injections

In the drug trial, intraperitoneal (IP) injections of SOD G93A mice with either the Tenovin-1 drug or a control solution began once the mice reached 60-64 days old (average 63 days). IP injections were administered twice a week for 15 weeks. Tenovin-1, a P53 activator (Tocris), was dissolved in a 70% 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, St. Louis, MO) water solution to make for a total drug concentration injected of 40.9 mg/kg, which came out to 1.818 mg/ml of solution we injected. The 250 μl of the 1.818 mg/ml solution of Tenovin-1 and 70% Cyclodextrin was combined with 400 μl of Phosphate Buffer Saline (PBS) and mixed, then 400 μl was injected into each mouse in the drug group. For the control group, the 400 μl injection was composed of a mixture of 250 μl of 70% Cyclodextrin and 400 μl of PBS.

Grip Test

We utilized the Bioseb Grip Test (Vitrolles, France) in order to obtain additional data on the grip strength of mice using the slanted mesh attachment. Following previous

literature on strategies to use the test that detailed forelimb and hind-limb strength, we opted for measuring total limb strength by holding the mouse by its tail and lowering it onto the mesh until all limbs had grasped the slanted mesh. Then we would pull the mouse by the tail perpendicular to the slanted mesh, three times, and average the trials for data points. Data from the Grip test was not presented because of issues in reproducibility, consistency, and a lack of differences between groups.

Mouse Survival

For the purposes of the drug trial, the disease endpoint was defined by the mouse not being able to right itself after ten seconds of being placed on its back (Dardiotis et al., 2013). This was discerned by testing the mice three times when they had visible trouble moving or performing on the rotarod, and if two of the three tests showed they could not right themselves within ten seconds they were deemed to be at the endpoint.

Tissue Harvesting

Mice in the trial, from both drug and control groups that had reached the disease endpoint were removed from their cages and euthanized with carbon dioxide. Immediately following euthanization, the brain, spinal cord (dissected into left and right), and muscle taken from the legs were all removed and immediately dry-frozen in liquid nitrogen. These tissues were kept in -80˚C storage for potential future use and analysis.

Tenovin-1 Drug Trial in G93A Mice

Following successful knockdown of mutant G85R SOD aggregates in vitro, Tenovin-1 was used to launch a drug trial in the transgenic G93A line of mice. The dosing of the drug was based on a previous chemotherapeutic use in literature, where a dosage of 92 mg/kg was used, followed by dilutions to sustain mouse survival for longer periods of time. Following an optimization process in which various concentrations were formulated, the ultimate planned dosage cut the chemotherapeutic use's concentration in half to 46 mg/kg – which resulted in use of a 40.9 mg/kg dosage once mixed.

In the design of the study, the G93A positive males and females were divided, then the gender groups were further divided into a drug and control cohort where the groups were matched by litter. Thus, each pair of G93A positive mice was split so one would go enter the control group and the other the drug group. The mice in the drug and control groups were assigned randomly. They were then kept in cages with their littermates until they reached 60-64 days of age, when they began training and testing on the Rotarod machine and injections. The mice were first trained on the machine for five minutes, and then tested the following day. Data points consisted of three trials of testing on the Rotarod machine, each one hour apart, and a mass measurement following the third trial. The mice were then injected and released back into their cage. Mice were trained and tested once a week, and injected twice a week for up to fifteen weeks if they survived. The total number of mice in the female cohort for which data is presented is 32 (16 drug and 16 control) while the total number of mice in the male cohort for which data is presented is 10 (5 in the drug group and 5 in the control group).

Figure 1:

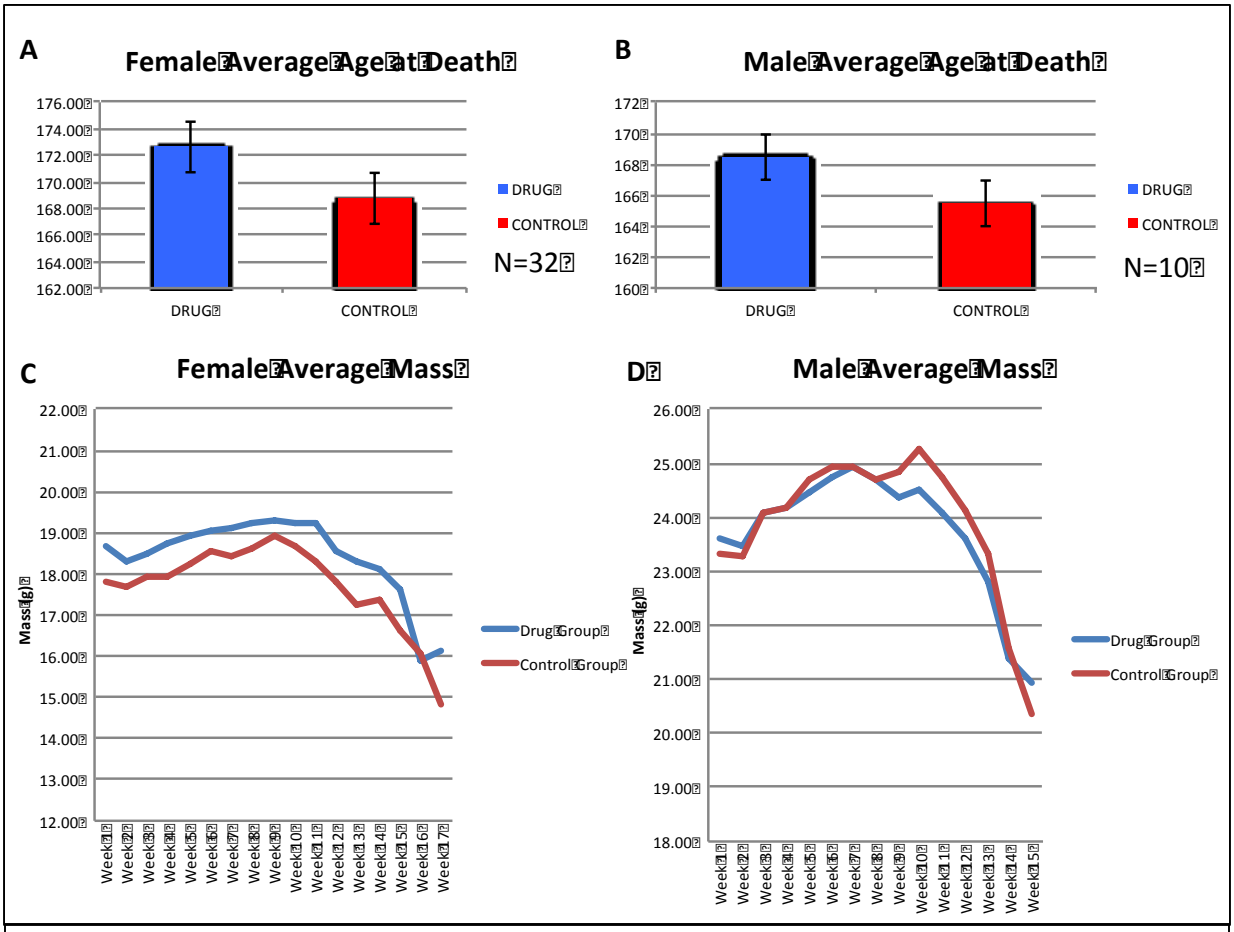

Figure 1: A+B: Drug group in both female and male cohorts demonstrated longer survival over control groups, but difference was not significant at $p<.05$. Death was measured by discovery of dead body (checked daily) or failure of survival test (inability to correct self in 10 seconds when turned over).

C+D: Differences in mass for both drug and control groups in both cohorts were not significant at $p<.05$. Mass retention was used as a proxy for overall health, and was used as a prognostic indicator for when symptoms began. Mass was measured following Rotarod testing each week.

Drug Treatment Offered Small but Non-Significant Improvement in Lifespan and Mass Retention

The results of long term survivorship and mass retention can be seen in Figure 1, with slight differences between the female and male cohorts. In the female cohort, consisting of 32 mice, it can be seen that the average age at death for the drug group was 172.67 days while the control group's value was 168.78 – a difference that was not found to be significant by T-Test at $p<.05$. Furthermore, the female drug group shows a consistently higher average mass than the control group through the duration of the 17 weeks of data collection. In the male cohort, consisting of 10 mice, the average age at death for the drug group was 168.5 days while it was 165.5 days for the control group – a slight difference that was also found to be not significant by T-Test at $p<.05$. Unlike the female cohort, the male cohort's control group held a higher average mass than the drug group for the duration of the 15 weeks of data collection. It is important to note that the age at death refers to either the actual death of the mouse or the failure of the survival test. On several occasions, mice that fail the survival test perished within a day, affirming confidence with these values for average age at death. It is also important to note that the differences in average mass between the drug and control groups may have been an effect of the random selection of heavier mice in one group or the other, and not necessarily the effects of the drugs.

Figure 2

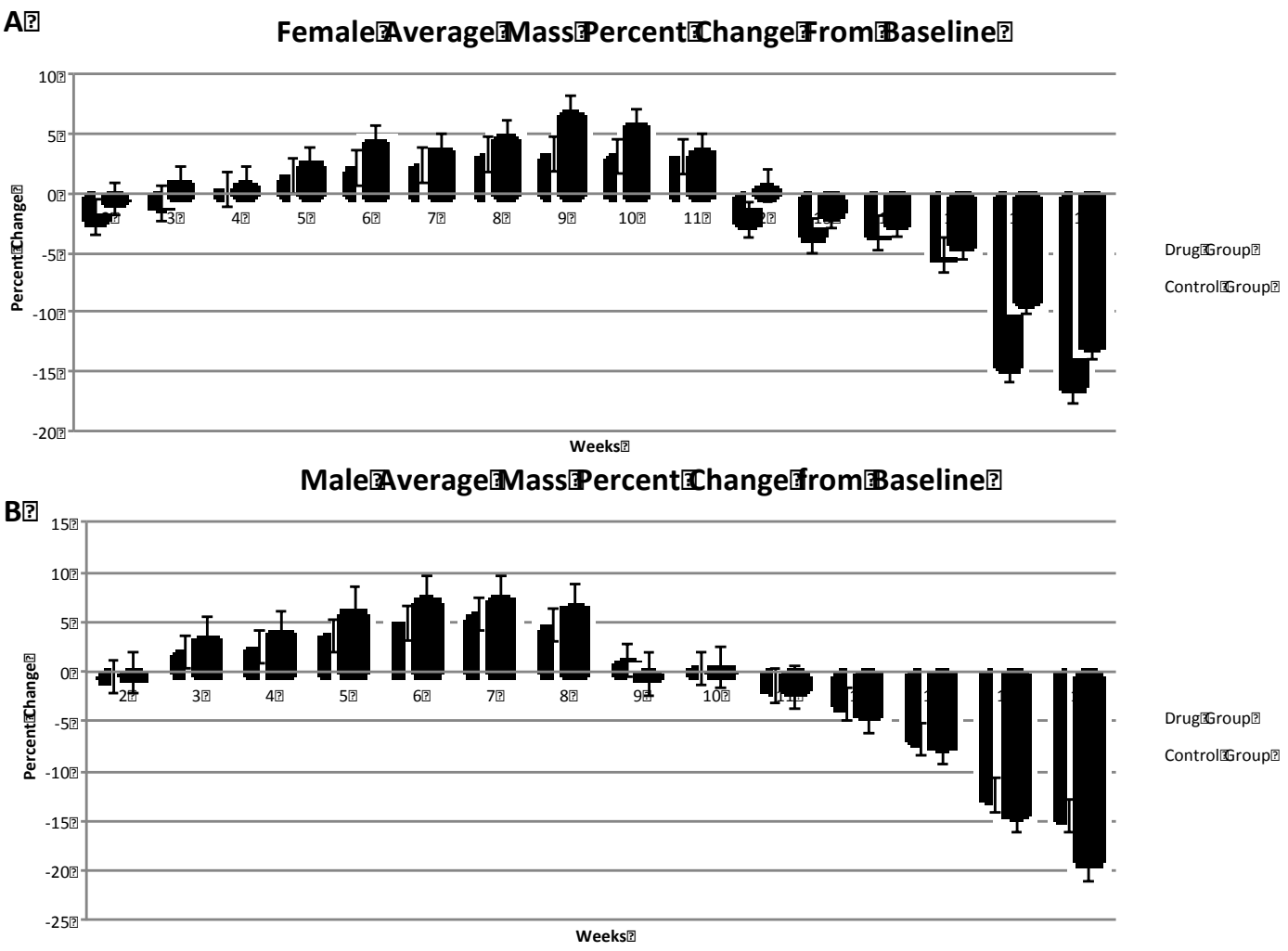

Figure 2: A: The drug group in the female cohort (N=32) demonstrated a negative effect on weight gain and retention, and accelerated weight loss across the 17 weeks of observation. Despite maintaining a lower average mass (Figure 1), the control group more easily put on weight, retained weight, and wasted away slower than their drug treated counterparts. Differences between the drug and control groups were not significant at $p<.05$.

B: The male cohort (N=10) showed smaller differences between drug and control groups, but followed the same pattern. The drug group mice were unable to put on and maintain weight as well as the control group for the first 8 weeks, however the wasting period (8-15 weeks) showed little difference between drug and control groups. Differences between the groups were not significant at $p<.05$.

Drug Treatment Hindered Mass Building and Had Mixed Effects on Wasting Later in Life

Despite the mass differences mentioned earlier from Figure 1, Figure 2 A and B show that the drug group had effects in both the female and male cohorts in terms of mass retention and shedding. In Figure 2A, the female cohort is seen building mass for several weeks following the start of the drug trial at about 63 days of age – but the drug group lags behind the control group in building mass through week 12 of the trial. Following week 12, approximately when the mice become symptomatic, the drug group also had an increased mass shedding compared to the control group through the end of data collection at week 17. These differences were analyzed by two sample T-Test but the resultant p-value was 0.310 – indicating that these differences are not significant.

In the male cohort shown in Figure 2B, a similar trend can be seen to the female cohort – initially the control males are more able to put on mass through week 8. However the difference here is that the drug group males lose mass less quickly than control – it appears that the drug may help prevent the wasting associated with symptomatic ALS in the later stages for the male cohort. The differences were analyzed by two sample T-Test, and the resultant p-value was found to be 0.924 – indicating that these differences are also not significant.

Drug Treatment Showed No Significant Differences in Motor Ability

Assessment of motor skills by Rotarod showed similar results for the male and female cohorts across the duration of data collection. Figure 3A shows the female cohort's progress, through average trial time, across the 17 weeks of data collection. The drug and control group appear to have the same motor skills throughout the 17 weeks, with a slightly higher average trial time for an 8-week span in the drug group. Once analyzed by two-sample

T-Test, the differences in the female cohort had a p-value of .524, indicating they were not significant.

The male cohort shown in Figure 3B shows a similar trend with slightly more volatility as indicated by the sharper curves going up and down for both groups. Unlike in the female cohort, the drug group in the male cohort appears to have a higher average trial time by up to 20 seconds at the peak, and the drug group appears to sustain the higher average for almost the entire duration of the trial. Upon further analysis by two sample T-Test, the p-value was found to be 0.324, indicating that the differences between groups are not significant. However, this may be due to the small sample size (N=10) and it stands to reason that these differences may be more striking and closer to significance in a future trial with more males samples included.

Figure 3:

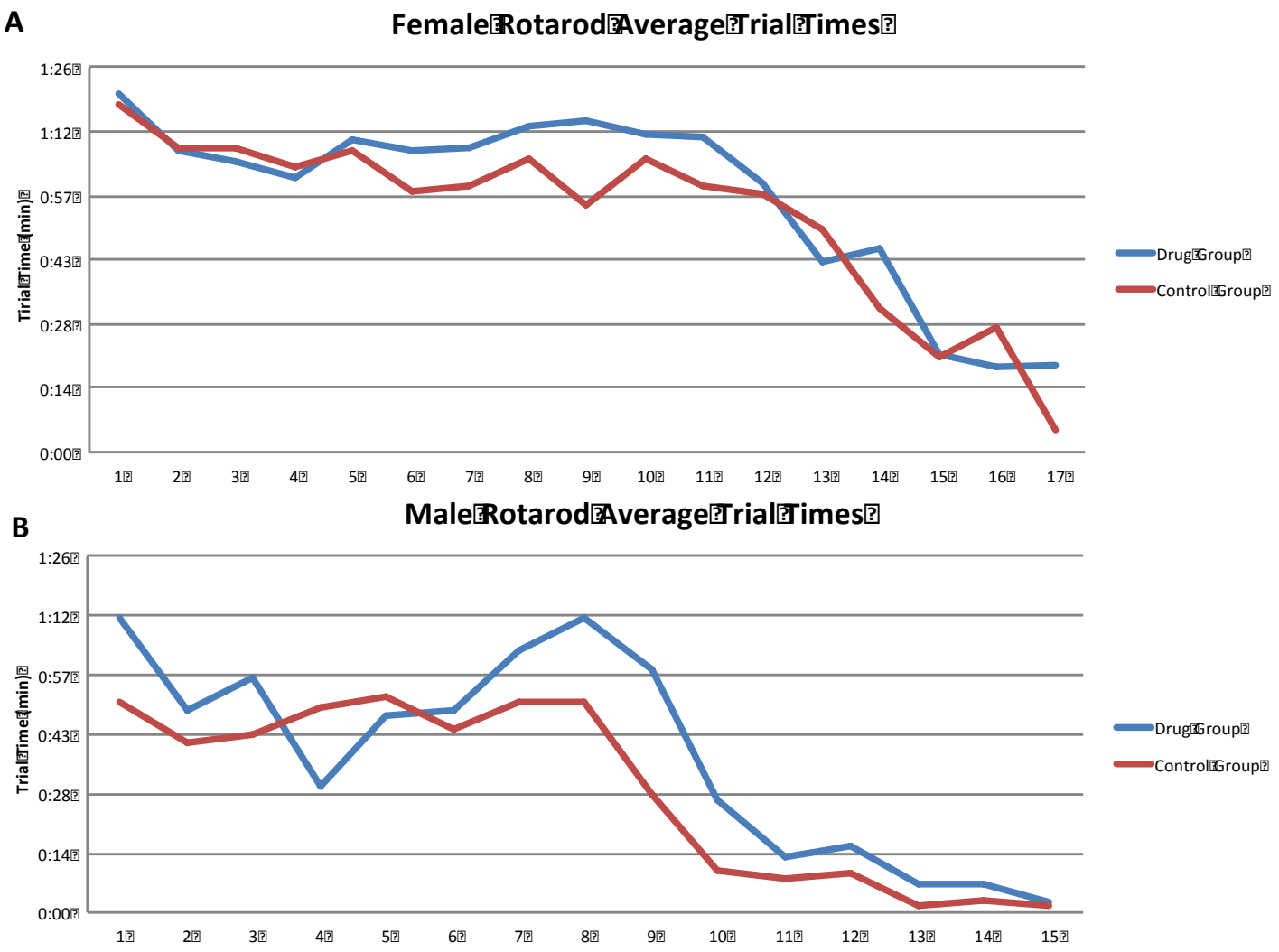

Figure 3: A: Average Rotarod trials for female cohort (N=32) show little difference between drug and control groups over 17 week data collection period. The decline in average Rotarod trial time begins when the mice appear to become symptomatic – at 12 weeks (~148 days old) for the female cohort.

B: Average Rotarod trials for the male cohort (N=10) shows the drug group with slightly higher average trial times over the 15 weeks of data collection, however differences were not significant at $p<.05$. The decline in average Rotarod trial time begins when the mice appear to become symptomatic – at 9.5 weeks (~128 days old) for the male cohort.

Figure 4:

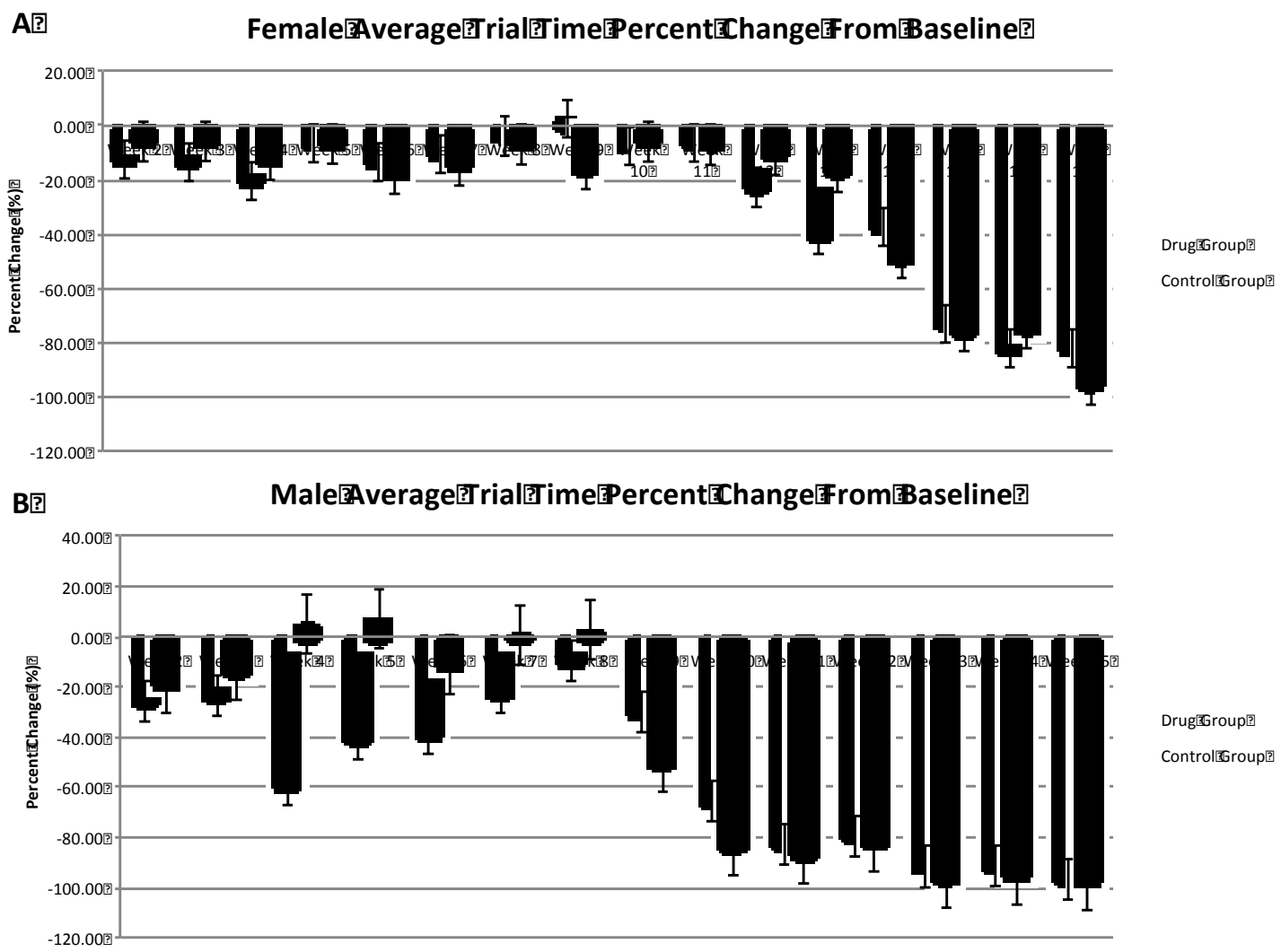

Figure 4: A: Week by week analysis of motor ability trends show the drug group had earlier decay in motor abilities through week 8, before both groups began evenly dropping from week 9 onwards. However, there were no significant differences ($p<.05$) in percentage change for female cohort ($N=32$).

B: Week by week analysis of motor ability trends show that the drug cohort initially decreased strongly until week 8, after which both the drug and control cohorts decayed at similar rates. However, there were no significant difference ($p<.05$) in percent change for the male cohort ($N=10$).

Figure 4 provides a deeper analysis into how the drug and control groups differed across the span of the drug trial with respect to how much the average trial time differed from the baseline – the first recorded test. In Figure 4A, there appears to be very little difference between the drug group and the control group in terms of average trial time percent change from the baseline, a pattern that stays consistent from the beginning to the end of the data collection period. Analysis by two-tailed unequal variances T-Test showed a p-value of 0.97, indicating that the little differences are in fact not significant.

In Figure 4B, the male cohort shows a slightly different pattern in average trial time percent change from the baseline. The drug group had a greater negative percent change than the control group for the first 8 weeks of the drug trial, before the control group began having similar negative percent changes for the seven weeks that followed. The differences, when analyzed using a two-tailed unequal variances T-Test, had a p-value of 0.49, indicating that they were not significant.

It is also important to note that another means of collecting data that we had planned, using the Bioseb Grip Test device, offered no useful data to discriminate between drug and control groups. There was a high degree of variability in data produced, much of it drawn from factors beyond our control that dealt with the mice – how hard they grasped, whether they randomly pulled strongly or weakly, and whether they tried to escape rather than hang on to the mesh. The measurements lacked consistency, and the multitude of confounding factors made this medium of obtaining data unreliable and unfit for presentation.

DNA Damage and Proteotoxicity Experiments

To assess the expression of certain genes that have been implicated in the proteotoxicity repair pathways – LSD1 and UBE4B – and the ubiquitin specific protease USP7, a series of experiments were devised to damage DNA and impose stress and potential proteotoxicity to see how cells deal with the effects. The three-pronged approach for these experiments included: inducing DNA damage via a DNA intercalating agent (Doxorubicin), transfecting cells with wild type and mutant SOD1, and exposing cells to one and two hours of heat shock. The cell types used were: Human Embryonic Kidney 293T Cells (HEK293T), Wild Type Human Colon Cancer Cells (HCT116 P53 +/+), P53 Knockout Human Colon Cancer Cells (HCT116 P53 -/-), and a Mouse Motor Neuron-like hybrid cell line (NSC34).

I. Doxorubicin DNA Damage

In the first leg of the DNA damage and proteotoxicity experiments, only the HCT+/+, HCT -/-, and NSC cells were used to assess how pathways worked in the absence of p53 and in neural-like cells. Following incubation of 3.0 x 10^6 cells overnight, cells were treated with 4 μl of Doxorubicin for ten minutes before they were lysed to extract protein and RNA. The samples were then run on a gel, with the consequent membrane incubated with antibodies for USP7, UBE4B, and LSD1, and the loading control of GAPDH. Figure 5A shows the results from the Doxorubicin DNA damage portion of the experiment. For quantification, the samples were normalized to the loading controls (GAPDH) found at the bottom of the figure. For USP7, there is a decrease across each of the cell lines: a 60% decrease in the HCT+/+ cells, an 83% decrease in the HCT -/- cells, and a 23% decrease in the NSC34 cells. For UBE4B, there was a similar decrease across all the cell lines: a 15%

decrease in HCT +/+ cells, a 28% decrease in HCT -/- cells, and a 13% decrease in NSC cells. In LSD1 there was a change in the pattern: a 95% decrease in HCT +/+ cells, a 20% decrease in HCT -/- cells, and a 32% decrease in NSC34 cells. For USP7 and UBE4B the P53 knockout HCT -/- line had the greatest decrease in protein, but the line had the smallest decrease for LSD1.

These findings suggest that the decrease in UBE4B and LSD1, which previous literature had found to result in a robust increase in clearance of DNA and proteotoxic damage, were replicated by this experiment. The reduction in UBE4B, LSD1, and USP7 – apparent across all cell lines – suggests that their repression (either at the transcription level, or the protein level) is necessary for reducing the toxicity of the damage wrought by the DNA intercalating agent. Future directions with this experiment will involve discerning whether there is repression at the transcription level through analysis of the RNA present when the cells were lysed.

Figure 5:

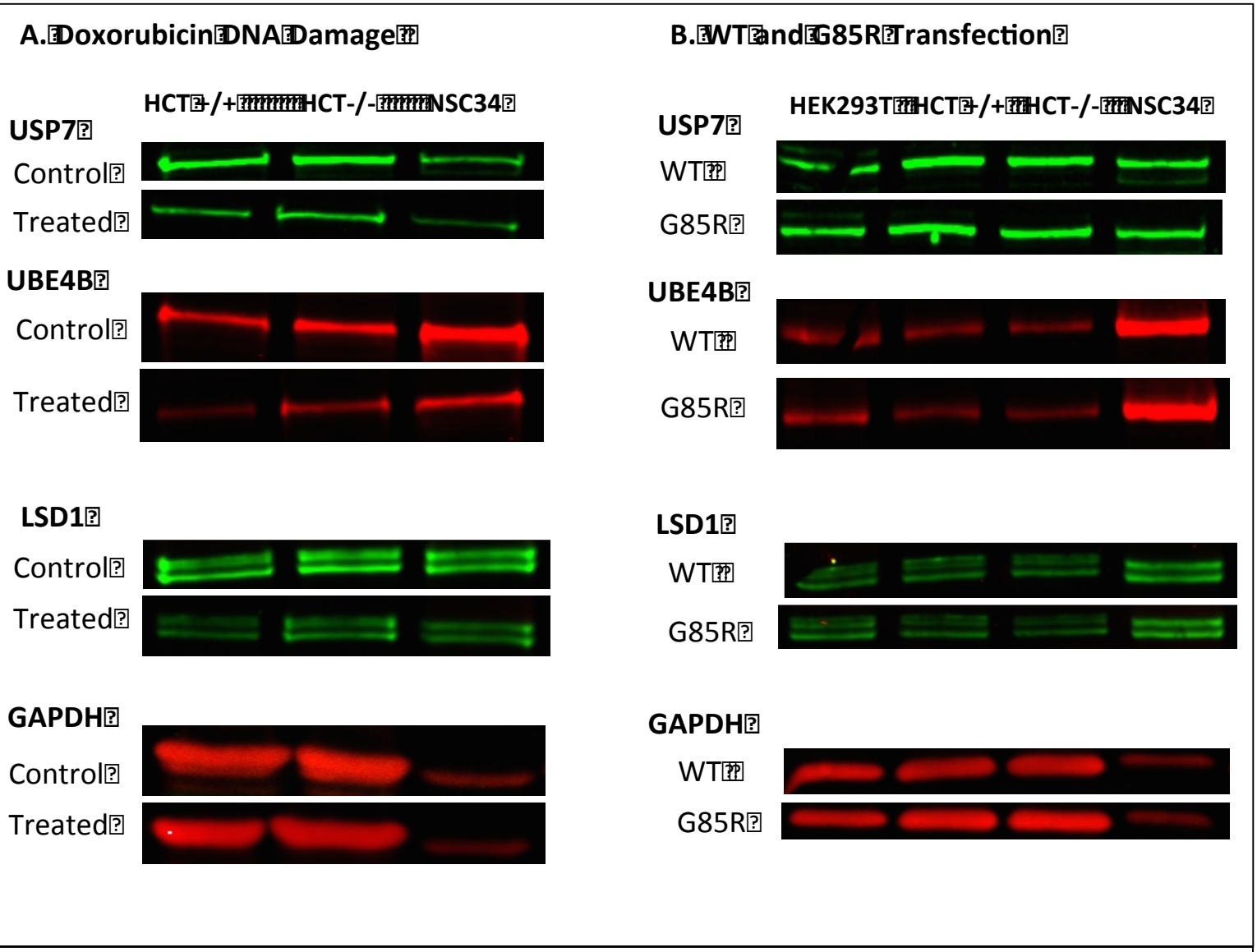

Figure 5: A: Doxorubicin Treatment results – all images taken from same membrane with same exposure.
USP7: 60% decrease in HCT +/+, 83% decrease in HCT -/-, 23% decrease in NSC34.
UBE4B: 15% decrease in HCT +/+, 28% decrease in HCT -/-, 13% decrease in NSC34.
LSD1: 95% decrease in HCT +/+, 20% decrease in HCT -/-, 32% decrease in NSC34.

B) Transfection with WT and G85R SOD1 results – all images taken from same membrane with same exposure.
All percentages reflect G85R increase/decrease over WT transfected cells (control).
USP7: 40% increase in HEK293T, 45% decrease in HCT +/+, 50% decrease in HCT -/-, 10% decrease in NSC34.
UBE4B: 42% decrease in HEK293T, 35% decrease in HCT +/+, 21% decrease in HCT -/-, 44% increase in NSC34.
LSD1: 20% increase in HEK293T, 45% decrease in HCT +/+, 45% decrease in HCT -/-, 4% decrease in NSC34.

II. Wild Type and G85R Mutant SOD1 Transfection

For this set of the DNA damage and proteotoxicity experiments, four cell lines (HEK294T, HCT +/+, HCT -/-, and NSC34) were used for which 1.5 x 10^5 cells were incubated overnight before transfection with the WT SOD1 and G85R mutant SOD1 the following day. The samples, transfected via the Lipofectamine 3000 kit (Life Technologies), were then scanned for GFP to identify relative transfection efficiencies – all of which were at least 50%. The cells were then lysed to extract protein and RNA. Following the run of protein via western blot, the band strength was quantified through normalizing to the loading control GAPDH.

Figure 5B shows the results from the western blot run using the extracted proteins for each of the cell lines. For USP7, there was a slight mix of effects : compared to the WT transfected cells, the $SOD1^{G85R}$ transfected HEK293T cells had an increase in USP7 of 40%, while the mutant transfected HCT +/+ had a decrease of 45%, the mutant transfected HCT -/- had a decrease of 50%, and the mutant transfected NSC34 cells had a decrease in USP7 of 10%. For UBE4B, there was a similar pattern to USP7: a 42% decrease in the $SOD1^{G85R}$ transfected HEK293T cells compared to the WT transfection, a 35% decrease in UBE4B for the HCT+/+ line, a 21% decrease in the HCT -/- line, and a 44% increase in UBE4B in the NSC34 cell line. The pattern for LSD1 in the G85R transfected line resembled USP7: there was a 20% increase in LSD1 in the G85R transfected HEK203T cell line, but a 45% decrease in LSD1 in the mutant transfected HCT+/+ and HCT -/- cell lines, and a 4% decrease in LSD1 in the mutant transfected NSC34 cell line.

The findings in bulk suggest that there is an overall decrease in USP7, UBE4B, and LSD1 in the mutant G85R SOD1 transfected cells – affirming results from previous studies (including Periz et al. from the same laboratory) signaling that down-regulation of these

proteins are necessary measures to improve clearance of protein aggregates. Questions arise from the reduction in levels of UBE4B, LSD1, and USP7 that are of similar magnitude for the HCT p53 WT and knockout lines, suggesting that the presence of p53 may not play as active of a role as hypothesized. Another question comes from the least robust decline of these protein levels in the NSC34 cell line, the most clinically relevant for ALS study. Future research directions will analyze the RNA samples associated with this experiment, looking into whether the proteins were down regulated at the transcriptional level or the protein levels within the cell. Another set of experiments will also be necessary to discern the roles played by p53.

III. Heat Shock Treatment

The final leg of the series of experiments was using the four cell lines for heat shock treatments so that we could induce a temperature stress on the cells to see how they would counteract it. We incubated 6 x 10^5 cells overnight, and divided them into three groups – control (remains at 37°C), one-hour heat shock (at 43°C) and two-hour heat shock (at 43°C). Immediately following the end of the sample's assigned time in the 43°C heat, the cells were lysed to retrieve protein and RNA. They were then run on a gel, and the quantification of their signals was normalized against the loading control GAPDH. This experiment was replicated, and the findings discussed below reference both replicates.

The results for each of the three target proteins (USP7, UBE4B, and LSD1) were consistent with the findings from the other two legs of the experiment in showing decreases to a large extent for each of the cell lines.

Heat Shock Brings Steep USP7 Decline Across All Cell Lines

For USP7, the first replicate of this experiment in HEK293T cells, shown in Figure 6A, had an overall decrease of 70% in the protein following heat shock – the average between two replicate had a decrease of 75% for the one hour heat shock and a decrease of 65% for the two hour heat shock. The HCT +/+ line, shown in Figure 6B, had a 94% decrease overall in USP7 averaged between two replicates, with a 94.5% decrease coming with the one hour treatment and a 93% decrease coming with the two hour treatment. The HCT -/- line, shown in Figure 7A, had a similar decrease in USP7, at approximately 50% for both one and two hour treatments in the averaged across both replicates. The NSC34 cell line, shown in Figure 7B, had a robust decrease in USP7 of almost 98% for both the one and two hour treatments averaged across both replicates.

These findings on the decline of USP7 corroborate previous studies mentioned in which a decline USP7, a p53 deubiquitinating enzyme, correlates with improved protein quality control efficiencies. The protein level declines from control levels in HCT +/+ and NSC34 lines were also shown to be significant at the $p<.05$ level, suggesting that the heat shock stress in p53 wild type cells and motor-neuron like cells down regulates USP7 further in order to improve protein quality control machinery. Further analysis into the nature of the USP7 decline, whether transcriptional or at the protein level, will involve RT and QPCR techniques applied to the RNA extracted from the lysed cells.

Figure 6:

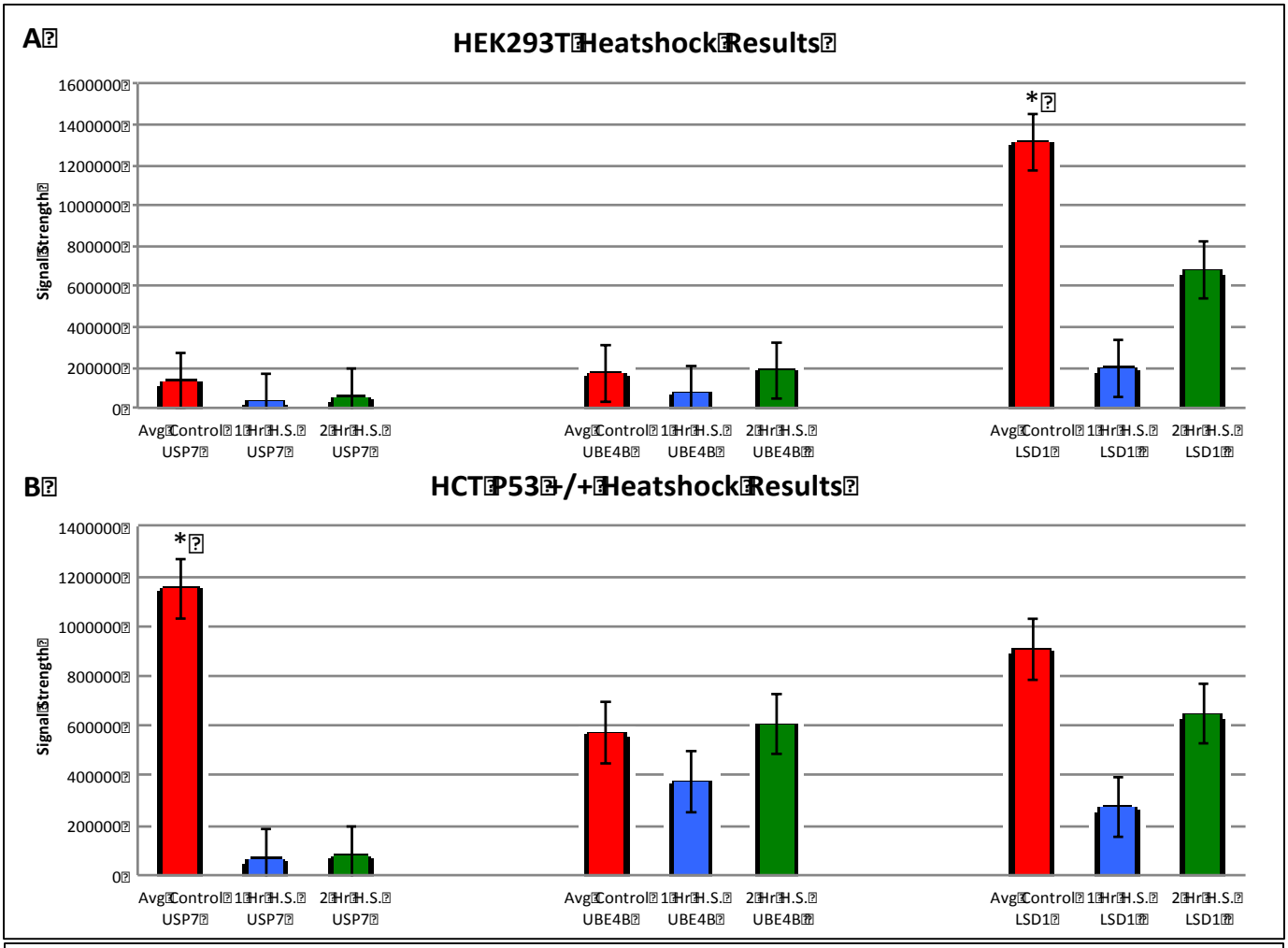

Figure 6: All results are avg. protein signals from two replicates. (*Indicates significance at the p<.05 level)
A: HEK293T results from heat shock experiment:
USP7: Compared to control - 75% decrease in 1-hour heat shock, 70% decrease in 2-hour heat shock.
UBE4B: Compared to control – 50% decrease in 1-hour heat shock, 10% increase in 2-hour heat shock.
LSD1: Compared to control – 80% decrease in 1-hour heat shock, 50% decrease in 2-hour heat shock.

B: HCT P53 +/+ (WT) results from heat shock experiment:
USP7: Compared to control – 94.5% decrease in 1-hour heat shock, 93% decrease in 2-hour heat shock.
UBE4B: Compared to control – 35% decrease in 1-hour heat shock, 6% increase in 2-hour heat shock.
LSD1: Compared to control – 70% decrease in 1-hour heat shock, 30% decrease in 2-hour heat shock.

Figure 7:

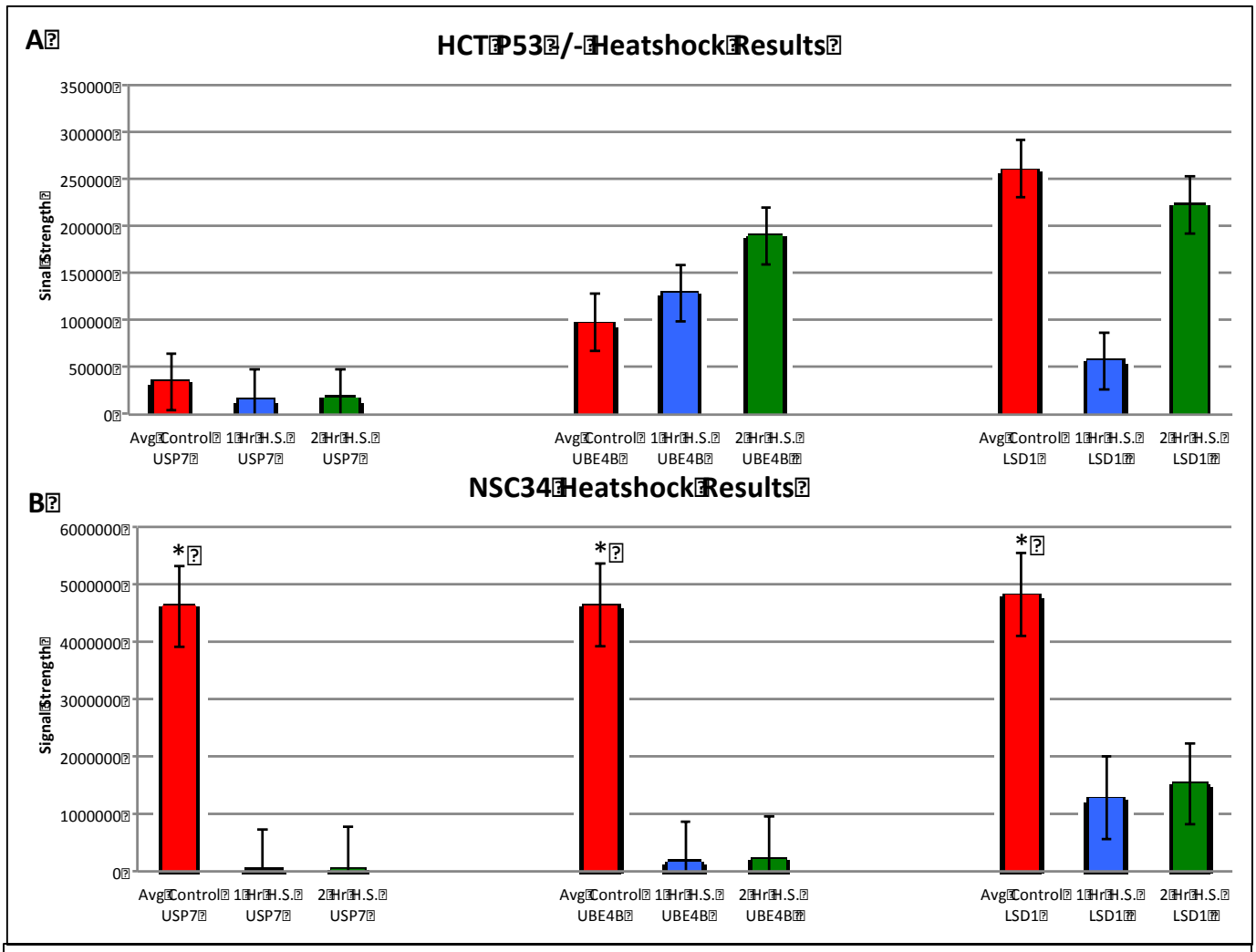

Figure 7: All results are avg. protein signals from two replicates. (*Indicates significance at the p<.05 level)
A: HCT P53 -/- (P53 Knockout) results from heat shock experiment:
USP7: Compared to control - 54% decrease in 1-hour heat shock, 50% decrease in 2-hour heat shock.
UBE4B: Compared to control – 33% increase in 1-hour heat shock, 96% increase in 2-hour heat shock.
LSD1: Compared to control – 80% decrease in 1-hour heat shock, 15% decrease in 2-hour heat shock.

B: NSC34 results from heat shock experiment:
USP7: Compared to control – 99.3% decrease in 1-hour heat shock, 99% decrease in 2-hour heat shock.
UBE4B: Compared to control – 96% decrease in 1-hour heat shock, 95% decrease in 2-hour heat shock.
LSD1: Compared to control – 75% decrease in 1-hour heat shock, 70% decrease in 2-hour heat shock.

UBE4B Has a Mixed Decline in Some Heat Shock Affected Cell Types

In the HEK293T cell line (shown in Figure 6A), the average between two replicate had a 50% decrease following the one-hour heat shock treatment, but a 10% increase over the control following the two-hour treatment. The average between replicates for the HCT +/+ line (Figure 6B) also demonstrated that pattern – a 35% decrease following one hour of heat shock, but a 6% increase over the control in the two-hour heat shock treatment. In the HCT -/- line (Figure 7A) average between replicates showed an increase of UBE4B up to 35% following the 1-hour heat shock, and a 96% increase over the control following the 2-hour heat shock treatment. The NSC34 cell line average between replicates, as shown in Figure 7B, had a 96% decrease in UBE4B following the one hour treatment and a 95% decrease in UBE4B following the two hour treatment.

The decline in UBE4B across most cell lines indicates that the findings from previous papers, in which a decline in UBE4B was strongly correlated with improved cell damage repair, are supported by these series of experiments. The most robust decline in UBE4B compared to the control was in the NSC34 cells, which was found to be significant at $p<.05$. The hypothesis that a decline in UBE4B works in conjunction with other proteins in a p53-medaiated protein quality control pathway is supported by this experiment's demonstrated decline in UBE4B because p53 serves as one of the major targets for UBE4B's polyubiquitinating activity. This notion is also supported by the measured increase in UBE4B in the HCT p53 knockout cells – suggesting that the absence of p53 as a target led to an increase in UBE4B presence within cells. Further studies will also require RNA analysis to discern whether the reductions occur transcriptionally or in protein levels, as well as more replicates with the p53 WT and Knockout lines to discern the role played by p53 in the pathways that involve UBE4B.

Heat Shock Treatment Brought Consistent Declines in LSD1 Levels

For the HEK293T cells protein levels averaged across both replicates (Figure 6A), there was a decrease in LSD1 of 80% in the one-hour heat shock treatment and a decrease of 50% in the two-hour treatment. In the HCT +/+ line, as shown in Figure 6B, there was a 70% reduction in LSD1 following the one hour treatment, and a 30% reduction following the two hour treatment. The HCT -/- line, shown in Figure 7A, had an 80% reduction in the one hour treatment, followed by a lesser 15% reduction in the two hour heat shock treatment. For the NSC34 cell line, shown in Figure 7B, there was a decrease in LSD1 of 75% following the one hour treatment and a 70% reduction following the two hour treatment.

The consistent decline in LSD1 with the one and two hour heat shock treatments offers support to previous literature detailing how stressors affecting macromolecules (such as DNA and protein) lead to a decline in LSD1 as the protein quality control mechanisms come into full swing. The LSD1 decline in NSC34 cells, much like the other target proteins in the study, was significant at the $p<.05$ level, suggesting that in neural-like cells the proposed protein quality control pathways that suppress neurotoxic protein aggregation (Periz et al., 2015) are active. Taken together with the decline in UBE4B, the heat shock experiment adds to the body of understanding of the proposed SUNS pathway by demonstrating its overall effect in additional contexts of induced proteotoxicity.

Discussion

With the positive results of the Tenovin-1 drug in cell culture knocking down SOD1 aggregation, the natural next step was to test the drug in vivo. With the drug trial in G93A mice, we looked the effects of potentially knocking down mutant SOD1 aggregation through the lens of motor ability and mass retention – both of which decrease drastically with progression of disease. The data for motor skills, gleaned from performance over weeks on the Rotarod device, indicated that there was no significant difference between the drug and control groups in either the male or female cohort. Additionally, the mass building of mice seemed to fall behind in the drug group, while the wasting common with more advanced degree progression seemed to accelerate with the drug treatment – though all differences were also shown to be not statistically significant. While statistical significance may fall victim to the small sample sizes (N=32 for females, N=10 for males), there may have also been an issue with beginning mice on the trial too late in age for the drug to have a substantial effect on survival and motor skills.

With the potential for optimizing this experiment for the future with younger mice at the start, leaving more time for qualitative data collection after 15 weeks of drug injections, there is promise for positive results. Additionally, this approach of using P53 activating drugs for delaying or potentially subverting neurodegenerative disease caused by misfolded protein aggregation represents an active area of research in a direction that has only recently been explored in basic science, medicine, and public health.

With the experiments that induced DNA damaging stress and proteotoxicity, there is an addition to the body of knowledge on which target proteins found in elevated or repressed quantities – especially as consistent patterns across cell lines. With the knowledge that USP7, UBE4B, and LSD1 are all found in reduced quantities in cells that have suffered

DNA damage, Heat shock damage, or are dealing with mutant SOD1 aggregation, there is greater support for the notion that they may work synergistically as hypothesized in a p53-mediated protein quality control pathway. The nature of the results in the varied cell types – from those used as controls (HEK293T), to those used to discern the impact of p53's presence (HCT +/+, HCT -/-), to those framing the issue in the context of relevant cells to ALS study (NSC34) – suggest that the results are replicable and demonstrate the need for a series of experiments to detail the impacts of the target proteins in a potential mechanism dealing with stressors.

Through elucidating some of the pieces in place that form part of greater mechanisms that deal with proteotoxicity, we can uncover more therapeutic targets that would stymie and/or prevent progression of a multitude of neurodegenerative diseases. Through better understanding of how proteins like UBE4B, LSD1, and USP7 work in conjunction with the "guardian of the genome" P53, it stands to reason that more developments down these lanes hold great promise for discerning the causes of malfunctioning protein quality control that cause a variety of diseases from amyotrophic lateral sclerosis to multiple sclerosis to a plethora of cancers.

Bibliography

Amente S, Lania L, Majello B. The histone LSD1 demethylase in stemness and cancer transcription programs. Biochim Biophys Acta 2013; 1829: 981–6.

Anand, R. and Marmorstein, R. (2007) Structure and mechanism of lysine-specific demethylase enzymes. J. Biol. Chem. 282, 35425□-35429

Arai, T., Hasegawa, M., Akiyama, H., et al. (2006). TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Biochemical and Biophysical Research Communications, 351, 602–611.

Bence NF, Sampat RM, Kopito RR. Impairment of the ubiquitin-proteasome system by protein aggregation. Science 2001; 292: 1552-1555.

Bruijn LI, Houseweart MK, Kato S, Anderson KL, Anderson SD, Ohama E, Reaume AG, Scott RW, Cleveland DW. Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1. Science 1998; 281: 1851-1854.

Chatterjee A, Upadhyay S, Chang X, et al. U-box-type ubiquitin E4 ligase, UFD2a attenuates cisplatin mediated degradation of DeltaNp63alpha. Cell Cycle 2008;7:1231–1237.

Chen, Y., Yang, Y., Wang, F., Wan, K., Yamane, K., Zhang, Y., and Lei, M. (2006) Crystal structure of human histone lysine-specific demethylase 1 (LSD1). Proc. Natl. Acad. Sci. USA 103, 13956□ 13961

Chen, J., Wu, X., Lin, J., and Levine, A. J. (1996) mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. Mol. Cell. Biol., 16: 2445 – 2452

Cookson, M. R. (2005). The biochemistry of Parkinson's disease. Annual Review of Biochemistry, 74, 29–52.

Cuervo, A. M., Wong, E. S. P., & Martinez-Vicente, M. (2010). Protein degradation, aggregation and misfolding. Movement Disorders, 25, S49–S54.

Cummins, J. M., Rago, C., Kohli, M., Kinzler, K. W., Lengauer, C., & Vogelstein, B. (2004). Tumour suppression: Disruption of HAUSP gene stabilizes p53. Nature, 428, 1 pg following 486.

Dardiotis, E., Panayiotou, E., Feldman, M.L., Hadjisavvas, A., Malas, S., Vonta, I., Hadjigeorgiou, G., Kyriakou, K., and Kyriakides, T. (2013). Intraperitoneal melatonin is not neuroprotective in the G93ASOD1 transgenic mouse model of familial ALS and may exacerbate neurodegeneration. Neuroscience Letters 548, 170–175.

De Jong, W. W., Leunissen, J. A., Leenen, P. J., Zweers, A., & Versteeg, M. (1988). Comparison with small heat shock proteins and Schistosoma egg antigen. Journal of Biological Chemistry, 263, 5141–5149.

Everett, R. D., Meredith, M., Orr, A., Cross, A., Kathoria, M., & Parkinson, J. (1997). A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein. EMBO Journal, 16, 1519–1530.

Forneris, F., Binda, C., Battaglioli, E., and Mattevi, A. (2008) LSD1: oxidative chemistry for multifaceted functions in chromatin regulation. Trends Biochem. Sci. 33, 181-189

Hatakeyama S, Yada M, Matsumoto M, et al. U box proteins as a new family of ubiquitin-protein ligases. J Biol Chem 2001;276:33111–33120.

Hershko A and Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998; 67: 425-479.

Heuze ML, Lamsoul I, Moog-Lutz C, Lutz PG. Ubiquitin- mediated proteasomal degradation in normal and malignant hematopoiesis. Blood Cells Mol Dis 2008;40:200–210.

Hosoda M, Ozaki T, Miyazaki K, et al. UFD2a mediates the proteasomal turnover of p73 without promoting p73 ubiquitination. Oncogene 2005;24:7156–7169.

Hu, M., Gu, L., Li, M., Jeffrey, P. D., Gu, W., & Shi, Y. (2006). Structural basis of competitive recognition of p53 and MDM2 by HAUSP/USP7: implications for the regulation of the p53-MDM2 pathway. PLoS Biology, 4, e27.

Huang J, Sengupta R, Espejo AB, et al. p53 is regulated by the lysine demethylase LSD1. Nature 2007; 449: 105–8.

Johnson ES, Ma PC, Ota IM, Varshavsky A. A proteolytic pathway that recognizes ubiquitin as a degradation signal. J Biol Chem 1995;270:17442–17456.

Klucken, J., Shin, Y., Masliah, E., Hyman, B. T., & McLean, P. J. (2004). Hsp70 reduces alpha-synuclein aggregation and tox- icity. Journal of Biological Chemistry, 279, 25497–25502.

Koegl M, Hoppe T, Schlenker S, et al. A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. Cell 1999;96:635–644.

Kopito RR. Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol 2000; 10: 524-530.

Kuhlbrodt K, Mouysset J, Hoppe T. Orchestra for assembly and fate of polyubiquitin chains. Essays Biochem 2005;41:1–14.

Lain, S., Hollick, J.J., Campbell, J., Staples, O.D., Higgins, M., Aoubala, M., McCarthy, A., Appleyard, V., Murray, K.E., Baker, L., et al. (2008). Discovery, in vivo activity, and mechanism of action of a small-molecule p53 activator. Cancer Cell 13, 454–463.

Lee MG, Wynder C, Cooch N, et al. An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature 2005; 437: 432–5.

Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J, et al. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature. 2002;416:648–53.

Li, M., Brooks, C. L., Kon, N., & Gu, W. (2004). A dynamic role of HAUSP in the p53-Mdm2 pathway. Molecular Cell, 13, 879–886.

Lin T, Ponn A, Hu X, et al. Requirement of the histone demethylase LSD1 in Snai1-mediated transcriptional repression during epithelial-mesenchymal transition. Oncogene 2010; 29: 4896–904.

Lindberg MJ, Byström R, Boknäs N, Andersen PM, Oliveberg M (2005) Systematically perturbed fold- ing patterns of amyotrophic lateral sclerosis (ALS)-associated SOD1 mutants. Proc Natl Acad Sci USA 102: 9754–9759. PMID: 15987780

Metzger E, Wissmann M, Yin N, et al. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 2005; 437: 436–9.

Morimoto, R. I., & Santoro, M. G. (1998). Stress-inducible responses and heat shock proteins: New pharmacologic targets for cytoprotection. Nature Biotechnology, 16, 833–838.

Naeem, A., Ahmad, T., Muzaffar, M., Ahmad, S., & Saleemuddin, M. (2011). A partially folded state of ovalbumin at low pH tends to aggregate. Cell Biochemistry and Biophysics, 59, 29–38.

Navon A and Ciechanover A. The 26 S proteasome: from basic mechanisms to drug targeting. J Biol Chem 2009; 284: 33713-33718.

Neumann, M., Sampathu, D. M., Kwong, L. K., et al. (2006). Ubiquitinated TDP-43 in fronto temporal lobar degeneration and amyotrophic lateral sclerosis. Science, 314, 130–133.

Nicholson B, Suresh KK. The multifaceted roles of USP7: new therapeutic opportunities. Cell Biochem Biophys. 2011;60:61–8.

Pandey, U. B., Nie, Z., Batlevi, Y., et al. (2007). HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. Nature, 447, 859–863.

Pauli, D., Tonka, C. H., Tissieres, A., & Arrigo, A. P. (1990). Tissue-specific expression of the heat shock protein HSP27 during Drosophila melanogaster development. Journal of Cell Biology, 111, 817–828.

Periz G, Lu J, Zhang T, Kankel MW, Jablonski AM, Kalb R, et al. (2015) Regulation of Protein Quality Control by UBE4B and LSD1 through p53-Mediated Transcription. PLoS Biol 13(4): e1002114.

Prusiner SB (2012) Cell biology. A unifying role for prions in neurodegenerative diseases. Science 336: 1511–1513. doi: 10.1126/science.1222951 PMID: 22723400

Robinson, J. L.; Geser, F.; Stieber, A.; Umoh, M.; Kwong, L. K.; Van Deerlin, V. M.; Lee, V. M.; Trojanowski, J. Q. Acta Neuropathol. 2013, 125, 121−131.

Rocha MC, Pousinha PA, Correia AM, Sebastião AM, Ribeiro JA (2013) Early Changes of Neuromuscular Transmission in the SOD1(G93A) Mice Model of ALS Start Long before Motor Symptoms Onset. PLoS ONE 8(9): e73846. doi:10.1371/journal.pone.0073846

Rubinsztein, D. C. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature, 443, 780–786. 71. Pandey, U. B., Nie, Z., Batlevi, Y., et al. (2007). HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. Nature, 447, 859–863.

Schubert U, Anton LC, Gibbs J, Norbury CC, Yewdell JW, Bennink JR. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. Nature 2000; 404: 770-774.

Schulte, J.H., Lim, S., Schramm, A., Friedrichs, N., Koster, J., Versteeg, R., Ora, I., Pajtler, K., Klein- Hitpass,L.,Kuhfittig-Kulle,S.,Metzger,E.,Schu¨le, R., Eggert, A., Buettner, R., and

Kirfel, J. (2009) Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Cancer Res. 69, 2065-2071

Shi D, Pop MS, Kulikov R, et al. CBP and p300 are cytoplasmic E4 polyubiquitin ligases for p53. Proc Natl Acad Sci USA 2009;106:16275–16280.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., Casero, R.A., and Shi, Y. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119, 941-953

Shi, Y., Sawada, J., Sui, G., Affarel, B., Whetstine, J.R., Lan, F., Ogawa, H., Luke, M.P., Nakatani, Y., and Shi, Y. (2003) Coordinated histone modifications mediated by a CtBP co-repressor complex. Nature 422, 735-738

Shi, Y.J., Matson, C., Lan, F., Iwase, S., Baba, T., and Shi, Y. (2005) Regulation of LSD1 histone demethylase activity by its associated factors. Mol. Cell. 19, 857-864

Strong, M. J., Volkening, K., Hammond, R., et al. (2007). TDP43 is a human low molecular weight neurofilament (hNFL) mRNAbinding protein. Molecular and Cellular Neuroscience, 35, 320–327.

Thomas, P. J., Shenbagamurthi, P., Sondek, J., Hullihen, J. M., & Pedersen, P. L. (1992). The cystic fibrosis transmembrane conductance regulator effects of the most common cystic

fibrosis-causing mutation on the secondary structure and stability of a synthetic peptide. Journal of Biological Chemistry, 267, 5727–5730.

Uversky, V. N. (2009). Intrinsic disorder in proteins associated with neurodegenerative diseases. Frontier Bioscience, 14, 5188–5238.

Wagstaff, M. J., Collaco-Moraes, Y., Smith, J., De Belleroche, J. S., Coffin, R. S., & Latchman, D. S. (1998). Protection of neuronal cells from apoptosis by Hsp27 delivered with a herpes simplex virus-based vector. Journal of Biological Chemistry, 274, 5061–5069.

Wang J, Xu G, Borchelt DR (2002) High molecular weight complexes of mutant superoxide dismutase 1: age-dependent and tissue-specific accumulation. Neurobiol Dis 9: 139–148. PMID: 11895367

Wang, J., Hevi, S., Kurash, J.K., Lei, H., Gay, F., Bajko, J., Su, H., Sun, W., Chang, H., Xu, G., Gaudet, F., Li, E., and Chen, T. (2009) The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. Nat Genet. 41, 125 129

Wang J, Farr GW, Hall DH, Li F, Furtak K, et al. (2009) An ALS-linked mutant SOD1 produces a loco- motor defect associated with aggregation and synaptic dysfunction when expressed in neurons of Caenorhabditis elegans. PLoS Genet 5: e1000350–e1000350. doi: 10.1371/journal.pgen.1000350 PMID: 19165329

Wu H, Leng RP. UBE4B, a ubiquitin chain assembly factor, is required for MDM2-mediated p53 polyubiquitination and degradation. Cell Cycle 2011;10:1912–1915.

Wu H, Pomeroy SL, Ferreira M, et al. UBE4B promotes Hdm2- mediated degradation of the tumor suppressor p53. Nat Med 2011;17:347–355.

Zeinab, R., Wu, H., Sergi, C., and Leng, R. (2012). UBE4B: A Promising Regulatory Molecule in Neuronal Death and Survival. Ijms 13, 16865–16879.

GAUTAM SHRIVASTAVA

15 Woodcrest Drive ● Syosset, NY 11791 ● gautam.shrivastava@nyu.edu ● (516) 835-1528

Education

Johns Hopkins Bloomberg School of Public Health, *Baltimore, MD* 09/13 – 06/15
- *Master of Science* in Biochemistry and Molecular Biology

New York University, College of Arts and Science**,** *New York, NY* 09/09 – 05/13
- *Bachelor of Arts* in Politics with Minor in Chemistry
- NYU in London – Fall 2011

Syosset High School, *Syosset NY* 09/05 – 06/09

Professional Experience and Internships

Wang Laboratory, Johns Hopkins Bloomberg School of Public Health, Baltimore**,** MD, *S.c.M. Student* 04/14 – 07/15
- Thesis projects overseen by Dr. Goran Periz in Dr. Jiou Wang's Laboratory focused on neurodegenerative disease
- Ran SIRT 1/2 inhibitor drug trial in mice aimed at in vivo knockdown of SOD1 aggregation, managed overall mouse colonies
- Tested small molecule inhibitors of E3/E4 Ub-ligases, examined protein quality control responses to various proteotoxic stresses

Generation Tomorrow, Johns Hopkins Center For AIDS Research, Baltimore**,** MD, *Research Assistant* 10/13 – 05/14
- Gained certification in testing and counseling for HIV in Maryland, partnered with Baltimore Behavioral Health
- Planned and lead focus groups on HIV/HCV disease transmission, risk factors, stigma associated with disease, treatment options
- Co-coordinated logistics and outreach for HIV testing days at Baltimore Behavioral Health for patients and community members
- Co-developed health education curriculum for HIV/HCV, presented to leadership of Johns Hopkins CFAR

Malaria No More, Programs Department, New York, NY**,** *Intern* 02/13 – 06/13
- Provided research and technical support to Tanzania and Cameroon diagnostic and treatment country teams
- Researched Malaria diagnostic and treatment policy in Africa, prepared briefings for ongoing projects
- Conducted background research on potential partner organization and individuals for expansion into Nigeria
- Assisted in data organization for annual reports and "Nightwatch" initiative evaluations for Cameroon and Tanzania

Clinton Global Initiative, Department of Finance and Operations**,** New York, NY, *Intern* 09/12 – 12/12
- Reviewed invoices for all ongoing CGI Programs via Financial Edge, oversaw payment processing for vendors
- Analyzed and adjusted budgetary allocations for different departments based on annual expenditures and calculated expansion
- Detailed payments for all events in FE, worked with director and departments on cutting expenses and staying within budget

J.C.E. NICU Summer Scholar Program, New York University School of Medicine**,** New York, NY, *Scholar* Summer 2011
- Awarded Best Undergraduate Scholar in J.C.E Summer Scholar Program
- Project: "The Outcome of Term Infants with Hypoxic-Ischemic Encephalopathy Evaluated for Hypothermia Treatment"
- Reviewed and organized test data from patient chart records, compiled and presented findings, called patients for follow-ups
- Shadowed in Pediatric Neurosurgery, General Surgery, Anesthesiology, and Neonatology at Bellevue and NYU

New York City Free Clinic, New York University School of Medicine**,** New York, NY, *Volunteer* 02/11 – 02/12
- Worked with patients to schedule new appointments, responded to phone calls and messages, organized testing materials
- Shadowed clinical teams of doctors and medical students treating patients

Molecular Cardiology (Gelb Laboratory), Mount Sinai School of Medicine, New York, NY, *Volunteer Researcher* 05/10 – 02/11
- Worked under the direction of a Ph.D. student on a gene correction project via adeno-associated virus vectory.jh.edu
-
- Trained in basic laboratory skills, working with bacteria, virus vectors, PCR, gel electrophoresis, and cell culture

Activities and Community Service

SOURCE HIV Counseling Program, Johns Hopkins Bloomberg SPH**,** Baltimore, MD**,** *Site Leader/Volunteer* 09/14 – 06/15
- Serve as site leader for Sisters Together and Reaching (STAR) – coordinate scheduling, liaise with site staff, troubleshoot
- Conduct HIV testing & counseling on STAR's mobile unit, recruit individuals from high risk communities across Baltimore
- Awarded 2015 Student Outreach Resource Center (SOURCE) Community Service Award

Red Cross Club, New York University, *President, Executive Board* 09/09 – 05/13
- Organized and coordinated food delivery events, soup kitchen days, disaster relief fundraisers, CPR/First Aid trainings
- Treasurer for two years, maintained finances, allocated funding to programs run, liaised with club administrators at NYU
- Vice-President for one year, connected with community organizations, maintained connection to RC chapter of Greater NY

Skills and Interests

- Languages: Fluent and literate in Hindi, conversational in Spanish
- Skills: Proficient in MS Office, SPSS, R, Financial Edge; Certified in First Aid, CPR/AED, HIV Testing & Counseling -Maryland
- Interests: Tennis, world history, politics, reading, debate, travel, film