SPECIFIC AIMS

Neurodegenerative diseases (NDs), characterized by a loss of neuron function, reduce the quality and length of life for millions of people globally, resulting in an enormous burden on society and on today's health care system as their incidence is increasing worldwide. Neurons require significant energy to fuel cellular pathways necessary for neural transmission and evidence is mounting in NDs that metabolic homeostasis is disrupted in both neuronal and non-neuronal cell populations. Amyotrophic Lateral Sclerosis (ALS) presents clinically as a rapid loss in motor function associated with degeneration and death of both upper and/or lower motor neurons. Frontotemporal Dementia (FTD) is characterized by changes in cognition, personality, as well as motor function, resulting from a progressive degeneration and loss of neurons in the frontal and temporal lobes of the brain. These two ND disorders share a genetic basis providing the opportunity to address the cellular dysfunctions shared between FTD and ALS. With the identification of genetic modifiers of these disorders, and the molecules and pathways that mitigate these dysfunctions, directed and more effective treatments will be possible for the complex ALS/FTD spectrum.

Studies have shown that both ALS and FTD are marked by an increase in glycolytic flux and a decrease in mitochondrial respiration but the field lacks an understanding of what is responsible for these imbalances and how they can be recalibrated. Using genetic models defined by patient alleles in *Drosophila*, we have identified a number of genetic modifiers of neurodegeneration across multiple models of ALS/FTD that encode regulators of cellular metabolism and mitochondrial function. A central player in coordinating energy demands with glucose utilization and nucleic acid biosynthesis is transketolase (TKT), the key enzyme in the non-oxidative branch of the pentose phosphate pathway (PPP). **In our studies, the *Drosophila* ortholog, *tkt (CG8036)*, has emerged as a robust genetic suppressor of neurodegeneration associated with four different models of ALS/FTD based on patient-defined alleles in TDP-43, FUS, SOD1, and the expanded G_4C_2-repeats in *C9orf72*.** We have further determined using models of the two most common forms of ALS/FTD, SOD1 and G_4C_2-repeat expansion associated with *C9orf72*, that increasing *tkt* restores motor function, extends lifespan, while also ameliorating mitochondrial dysfunction in both motor and sensory neurons. In ALS/FTD, it appears that Tkt, a normally effective metabolic rheostat is not able to maintain the balance between glucose metabolism, nucleotide biosynthesis, and the production of energy and antioxidants, yet an increase in *tkt* expression is sufficient to improve neuronal function. Our strong preliminary evidence indicates that altering these metabolic pathways underlies the mechanistic basis of how neural function can be maintained in the face of ALS/FTD mutations known to cause neurodegeneration.

Our central hypothesis is that differential flux through PPP can restore neural circuit function in the context of ALS/FTD. We propose that energy homeostasis, and thus, neuronal health and motor function, can be restored by the central regulator, Tkt. We take advantage of genetic tools in *Drosophila* to accomplish two important goals: first, to reveal the mechanistic basis by which the PPP prevents or delays cellular dysfunctions associated with ALS/FTD; and second, identify genes critical for PPP-mediated suppression that will accelerate progress towards the development of interventions and therapeutics. Our specific aims:

Aim 1: *Determine the requirement for oxidative PPP in tkt-mediated amelioration of ALS/FTD neurodegeneration*. We will test the hypothesis that increased levels of *tkt* redirects abnormally elevated glycolytic flux through the oxidative PPP branch to re-establish redox homeostasis. Does the loss of G6PD and 6PGD negate rescue by increased *tkt*? Is the effect on oxidative stress and motor function cell-type specific?

Aim 2: ***Investigate the functional intersection of non-oxidative PPP and glucose metabolism in in suppression of ALS/FTD-associated defects.*** The critical links between nucleotide synthesis, the AMP/ATP pool, and metabolic demands in *tkt*-mediated ALS/FTD rescue will be determined.

Aim 3: *Define the role of nucleotide biosynthesis in tkt-mediated suppression*. The functional requirements for glycolytic enzymes that act before and after the PPP shunt, i.e. hexokinase and glucose transporters vs. pyruvate kinase, in PPP-mediated suppression will be established, as will cell-type specific glucose utilization and energy production.

In each aim, we will identify the cellular and molecular consequences of *tkt*-induced suppression of neuronal defects using genetic epistasis, cell-specific measurements of *in vivo* biosensors, and comparative metabolic and transcriptomic profiling of mutant vs suppressed genotypes. Together, these data will advance our knowledge of the neuroprotective capabilities of regulated cellular metabolism and will identify the key targets for development of therapeutics that are most likely to prolong neuronal health and maintain motor function.

RESEARCH STRATEGY

Significance Neurodegenerative diseases (NDs) reduce the quality and duration of life for millions of individuals. NDs are characterized by the functional loss of discrete neuronal populations due to complex sources of cellular dysfunction. Frontotemporal dementia and amyotrophic lateral sclerosis (ALS) were originally thought to be clinically distinct because FTD is characterized by loss of neurons in the frontal and temporal lobes, and ALS is defined by upper and lower motor neuron death[1]. It is now clear that these NDs share a genetic basis as well as many pathological features[2]. ALS, designated the most common motor neuron (MN) disease in the world (2/100,000 cases/year)[2-4], is marked by rapid loss of motor control, resulting in paralysis and respiratory failure 3-5 years from diagnosis. FTD is marked by changes in behavior, a decline in language, but also a loss in motor function[5]. Given the overlap in gene mutations associated with ALS and FTD, the disease spectrum is now referred to as ALS/FTD. Genes associated with ALS/FTD represent a diversity of cellular functions[6-11], but when mutated, common defects result, including compromised neuromuscular junction (NMJ) function, mitochondrial dysfunction, protein and RNA aggregates, and the ultimate death of neurons[12-18]. Few effective treatments for ALS/FTD are available and none alleviate symptoms or prolong life more than 3 months[19,20].

The biggest impediment to developing therapeutics for ALS/FTD is our limited understanding of the genetic and molecular mechanisms underlying disease progression, and what types of interventions prevent the cellular defects responsible for loss of motor function and cognition. We have identified a set of genetic modifiers that reduce neurodegenerative phenotypes across four different models of ALS: TDP43, FUS, SOD1, and G_4C_2-repeat expansions in *C9orf72*. The modifier genes act in three metabolic pathways: the pentose phosphate pathway (PPP), nucleotide biosynthesis, and glycolysis, with Transketolase (Tkt) the key enzyme in the non-oxidative PPP that coordinates flux through oxidative PPP, coupled with glycolysis, and nucleotide biosynthesis. In essence, Tkt acts as a rheostat controlling metabolic flux through these pathways, by catalyzing reversible reactions that provide the building blocks for sugars, nucleotides, lipids and amino acids, and ensuring that ample levels of NADPH are produced to maintain the antioxidant capacity of the cell. Given the high metabolic demands of neurons, our discovery that suppressors of ALS/FTD models converge on central energy production pathways is very encouraging and the fact that *tkt* acts as a genetic suppressor across multiple models of ALS/FTD provides us with a hook to determine if the molecular and cellular pathways responsible for its neuroprotective activity are shared across different causes of ALS/FTD. The *long term goal of our research program* is to determine the molecular basis of neurodegeneration and neuroprotective mechanisms. The *specific goals of this proposal* are to use genetic modifiers of ALS/FTD in well-characterized *Drosophila* models to clarify the interdependence of metabolic pathways in restoring motor function so that future translation to mammalian models is more effective and efficient. Our research plan will take advantage of *Drosophila* models of ALS/FTD to determine the functional requirement of each metabolic pathway. Using a comparative approach that integrates genetic epistasis, assays of motor function, live cell biosensor imaging, and comparative metabolite and transcript profiling, we will determine the mechanistic basis for improved motor function and the restoration of cellular processes that prevent or delay neurodegeneration.

Imbalances in energy metabolism have been reported in ALS/FTD patients, most often as an increase in glycolytic flux and impaired oxidative phosphorylation (OXPHOS)[21,22,23,24]. Metabolic profiling of spinal cords from the $hSOD1^{G93A}$ mouse model highlights changes in metabolites associated with energy metabolism[25], with evidence that PPP dysregulation is apparent at symptom onset[26]. We hypothesize that manipulations of non-oxidative PPP, as the central regulator of energy production, will recalibrate imbalances in cellular metabolism seen in ALS/FTD disease, to prolong neuron life and motor circuit function. Results from our studies will clarify the role of different metabolic pathways and identify key targets for effective therapeutic interventions aimed at reducing cellular dysfunctions that lead to neuron loss.

Innovation Our work is innovative because we use genetic suppressors of four different ALS models that are thought to have distinct causes of neuronal dysfunction. The consistency with which Transketolase achieves suppression of ALS/FTD-associated neurodegeneration promises to identify shared aspects of underlying the different causes of ALS/FTD. Further, the relative ease of manipulation and the plethora of genetic tools in *Drosophila* allows us to conduct these studies *in vivo,* in whole animals, in target tissues, across multiple genotypes that will contribute substantially to the complex genetics of ALS/FTD. This model organism approach allows integration across pathways that likely underlie the dysregulation of metabolism in ALS/FTD neurons, and will do so in ways that can accelerate translational research towards treatments. We leverage these modifiers to perform comparative studies of the two most frequent causes of ALS, SOD1 and G_4C_2-repeat expansions in *C9orf72*. Our *Drosophila* knock-in *Sod1* (*superoxide dismutase*) models best mimic dosage of familial ALS (fALS) mutations seen in patients, with the $dSod1^{A4V}$ model exhibiting progressive loss of motor function in the adult, an

important new tool for the field. At face value, the etiology of these two ALS mutations appear distinct, yet they share cellular dysfunctions of many NDs that can be corrected by the action of the same modifier gene. This indicates the broad applicability that our results will have in treating diseases that compromise neuronal health. Our research program is also innovative because we exploit the advantages of multiple experimental approaches with the integration of knowledge about metabolites, the molecules that execute cellular processes, and the presence of enzymes and proteins that control them.

Approach *Drosophila* is an incomparable system for elucidating the function of individual genes *in vivo*, defining the pathways in which they act, and revealing if and how they intersect[27]. More than 60% of human disease genes are highly conserved in *Drosophila*, making *Drosophila* models of human disease particularly powerful for the elucidation of molecular and cellular dysfunctions associated with disorders from cancer to obesity[28-35]. The functional organization of the different cellular components comprising the nervous system in *Drosophila* and mammals is remarkably similar[36] enabling impactful studies of neurological disease[37]. Coupled with its utility for pathway analysis and the near complete conservation of metabolic processes, *Drosophila* has recently proven to be especially powerful for addressing questions centered in metabolism[38,39]. The relative abundance of different metabolites is an indicator of the physiological status of a cell. Metabolic enzymes are the work horses driving biochemical reactions, yet precisely how their regulation impacts metabolic pathways is still poorly understood[40]. We use genetic manipulations in *Drosophila* to test hypotheses based on observations made from ALS patients, their cells or mammalian models to provide a mechanistic understanding. We couple subcellular to behavioral phenotypic analyses, using *in vivo* reporters of cellular physiology, profiling of changes in metabolites and gene expression as a means to measure their response to specific genetic alterations. Such profiling will reveal the molecular underpinnings of the neuroprotective versus neurodegenerative state, providing a set of biomarkers that will be indicative of restored neural function.

BACKGROUND & PRELIMINARY STUDIES

Our research plan takes advantage of *Drosophila* models for the two most common forms of familial ALS (fALS) mutations in *SOD1* and GGGGCC-repeat expansions in *C9orf72*[41]. Missense mutations in *SOD1* constitute 20% of ALS cases, with G_4C_2 repeat expansions in a *C9orf72* intron making up 50-72% of familial ALS cases[42-46]. While *SOD1* mutations and G_4C_2 expansions also contribute differentially to FTD[47-49], ALS and FTD exhibit overlapping symptoms suggesting their etiology may be related[50,51]. Hence, ALS/FTD, now considered the same disease, manifests clinically as a spectrum from motor to cognitive dysfunction. At face value, mutations in *SOD1* and the expanded G_4C_2 repeats produce very different molecular disruptions. The immediate effect of disruptions in SOD1 activity is an increase in reactive oxygen species (ROS), while the expansion of G_4C_2-repeats produces high levels of hairpin-forming RNAs and stretches of repeated dipeptides (DPRs) as a result of RAN translation[41,52,53]. In both cases, abnormal proteins show a propensity to form cytoplasmic aggregates, and both result in compromised neuronal function, impaired cellular physiology, and the ultimate denervation and death of motor neurons[54].

Many human diseases are modeled by the over- or mis-expression of human genes harboring patient alleles. In an effort to avoid artefacts inherent in heterologous overexpression, we generated knock-in mutations of the two most common patient alleles in North America, $SOD1^{G85R}$ and $SOD1^{A4V}$[55], at the highly conserved orthologous site in the endogenous *Drosophila Sod1* gene[56,57]. These CRISPR-Cas9-induced mutant lines, $dSod1^{G85R}$ (hereafter *G85R)* $dSod1^{A4V}$ (hereafter *A4V*), and the wild type control, $dSod1^{WT(CR)}$, were generated on the same genetic background to eliminate line to line heterogeneity. Of multiple G_4C_2-expansion models in *Drosophila* [15,53,58-60], we have made use of targeted expression of $(G_4C_2)_{30}$[15] and $(G_4C_2)_{49}$[53] and both cause strong degenerative phenotype. For experiments proposed here, we focus on 49 G_4C_2-repeats following a UAS sequence for GAL4 induced expression, albeit without an ATG, such that DPR production is dependent on RAN translation[61]. We have performed an extensive characterization of these models, demonstrating that they recapitulate progressive loss of motor control, degeneration of MNs, mitochondrial dysfunction, and shortened lifespan, characteristic of human ALS[53,56,57](data from a graduate student K. Russo and postdoc M. Bartoletti; manuscripts in preparation). Our *A4V* knock-in and the controlled expression of $(G_4C_2)_{49}$ provide two powerful adult models for comparative studies of cells, tissues and whole animals at pre- and post-symptomatic stages. Our use of *G85R* and the expression of uninterrupted (G_4C_2)-repeats allows analysis of well characterized larval neurons that provide powerful means to quantify NMJs and live imaging of mitochondrial dynamics.

Drosophila ALS/FTD models: *A4V* homozygotes eclose as adult flies (Fig 1A) that show a progressive decline in motor function (negative geotaxis/climbing) (Fig 1B), accompanied by a reduction in leg MN axonal tracts (Fig 1C), and a shortened lifespan (Fig 1D). *G85R* homozygous adults fail to eclose (Fig 1A) likely due to the severe degeneration of neuromuscular junctions (NMJs) innervating abdominal muscles whose forceful contractions

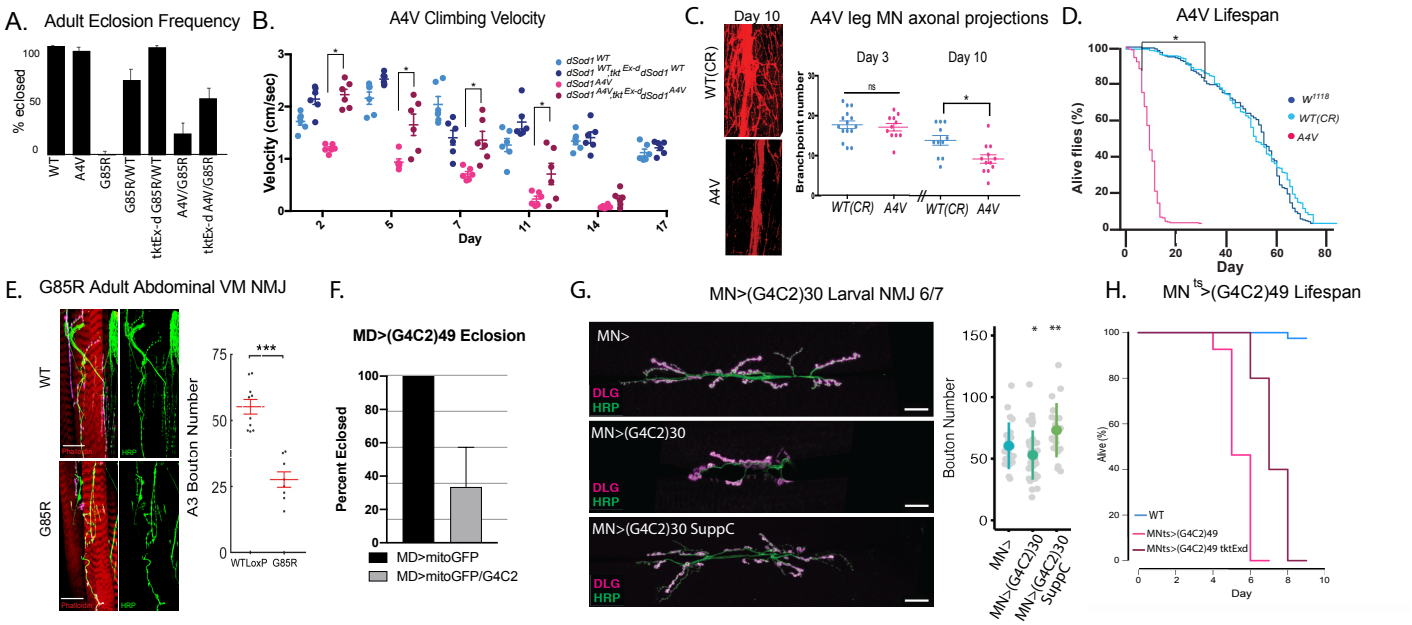

Fig 1 Characteristics of *dSod1* knock-in and G_4C_2 ALS/FTD models. A. Adult eclosion frequency of different allelic combinations. G85R effect on eclosion is dominant. A4V/G85R provides a sensitized background for assessing genetic modifiers (n>85, p<0.0001, Fisher's exact test) B. Climbing velocity of adults of indicated genotypes measured over 17 days (n>60, p<0.01, ANOVA with Hochberg correction for multiple comparisons) C. OK371>mcd8RFP, MN projections in femur of 10 day old adults, number of primary branches off main nerve bundle quantified for 3 day and 10 day females WT(CR) and A4V D. A4V adult lifespan, number dead counted daily, n=100/genotype/cage, 3 replicates. Survival curves by Kaplan-Meier method, Stats: Chi-square log rank test. E. Confocal z-stack adult abdominal VM muscle stained with phalloidin(red), presynaptic membrane anti-HRP(green), scale bar= X. NMJ6/7 boutons counted, blinded for genotype (n>12, one-way ANOVA,*p<0.05,**p<0.01) F. Adult eclosion frequency, (n= stats?) G. Confocal Z-stack 3rd instar larval NMJ6/7 A3, presynaptic membrane stained

enable the adult to escape from their pupal case[56] (Fig 1E). The continuous expression of $(G_4C_2)_{49}$ from embryogenesis also produces an eclosion defect (Fig 1F) and when expressed in MNs, larvae exhibit disruptions in their NMJs, with a significant reduction in synaptic boutons (Fig 1G). When motor neuron or pan-neuronal expression of $(G_4C_2)_{49}$, is limited to the adult, we find a shortened lifespan (Fig 1H) with a similar profile to *A4V*. Such controlled expression of $(G_4C_2)_{49}$ is accomplished using the *Gal4/Gal80^{ts}* system[62]: a neuron-specific Gal4 is combined with a temperature-sensitive repressor *Gal80^{ts}* and a temperature shift to 29˚C on day1 of adult life inactivates the GAL80, allowing neuron expressed GAL4 to activate G_4C_2-repeat expression. The same abbreviated lifespan is observed using pan-neuronal expression: *elav-Gal4 Tub-Gal80^{ts}>$(G_4C_2)_{49}$* (abbreviated as *elav^{ts}>$(G_4C_2)_{49}$*) or motor neuron expression: *OK371-Gal4 Tub-Gal80^{ts}>(G4C2)$_{49}$* (abbreviated as *OK371^{ts}>$(G_4C_2)_{49}$*) accompanied by atrophied leg muscles (Bartoletti, in prep), consistent with the climbing defect (negative geotaxis) observed following pan-neuronal adult expression[53].

Abnormal mitochondrial function is a common feature of NDs and evident in MNs of ALS patients[63,64]. Defects in axonal transport of mitochondria have also been observed in mammalian models of TDP43, FUS, and G_4C_2, including in CCAP motor axons in *Drosophila* G_4C_2 models[65]. Using live imaging of GFP tagged mitochondria in larval filets (Fig 2B), we see a reduction in mitochondrial movement in both sensory and motor axons in *G85R* mutants, with significant reduction in total mitochondria in motor neurons (Fig 2A). Interestingly, we find fewer mitochondria at the axon terminals, but not in the motor neuron cell body (Fig 2B) suggesting differential turnover or production of mitochondria in different regions of the neuron. Importantly, these mitochondrial defects are evident prior to abnormalities in motor function, ie. pre-symptomatic for locomotor loss. We did not find defects in axonal transport *per se*, as our in-depth analysis failed to detect abnormalities in the function of either motor proteins, adapter proteins that link the mitochondria to microtubules, or the transport of other types of vesicles (data from graduate student Y. Nemtsova, in prep). Instead, the defect in movement of mitochondria most likely reflects a dysfunctional organelle, whose role in OXPHOS and as the main source for ATP production is disrupted, failing to maintain energy homeostasis in ALS[66]. Indeed, we found that mitochondria isolated from *G85R* larvae have lower levels of NADH, and both *G85R* and *A4V* show reduced levels of ATP (Fig 2C). Increased levels of mitophagy at G85R axon terminals may account for the reduced mitochondrial content at synapses (Fig 2E).

<u>*Genetic suppression of Drosophila ALS/FTD models:*</u> We reasoned that despite the different causes of ALS/FTD, the fact that genetic and environmental disruptions result in similar clinical manifestations, some commonality in the mechanistic basis must exist, and it is very likely to extend to other NDs. We previously had shown that expression of the *Drosophila* BMP5/6/7, *gbb*, suppresses neurodegeneration of *G85R*, *A4V* and $(G_4C_2)_{49}$ to restore motor circuit function[56], and in an effort to better understand the basis of neuroprotection, we tested a

subset of Exelixis insertions identified as modifiers of *GMR>hTDP-43^{M337V}*, *GMR>hFUS^{R521C}* and *GMR>$(G_4C_2)_{30}$* overexpression models[14,15,67,68], for a modification of the G85R failure to eclose phenotype, in collaboration with colleagues at Biogen. Here we focus on three modifiers that suppressed all ALS/FTD models. The affected genes act in metabolic pathways required for energy homeostasis: d05884, a UAS-containing P-element insertion in *CG8036* (henceforth *tkt*), the ortholog of Transketolase (TKT), a key enzyme in the pentose phosphate pathway (PPP)[69]; f03242, an insertion in *Glut8/CG10960* which encodes a sugar transporter upstream of PPP[70]; and e04545 an insertion in *Paics* which encodes an enzyme in purine biosynthesis downstream of PPP. Of the three, *tkt* is unique in that it is the central regulator of glycolysis, PPP and nucleotide biosynthesis based on its ability to catalyze a number of reversible reactions linking the three pathways. The Exelixis mutation *tkt^{d05884}* (abbreviated *tkt^{Ex-d}*) is an insertion of a UAS-containing P-element which, when driven by *tubulin-Gal4* (*tub>tkt^{Exd}*), qPCR showed a 12.38-fold increase (p=1.9e-10) in *CG8036/tkt* mRNA. In the absence of *Gal4*, *tkt^{Exd}* is leaky and allows low level *tkt* expression. *tkt^{Ex-d}* is remarkable in its ability to not only suppress three overexpression models of ALS/FTD but to also suppress numerous phenotypes associated with the both *G85R* and *A4V* knock-in models (Fig 1,2). We tested an independent *UAS-tkt* line (*UAS-tkt^{EY04835}*) and found that it too, suppresses *G85R* and *A4V* phenotypes when driven by various *Gal4* lines (Bartoletti, in prep.). *tkt^{Ex-d}* not only enables *G85R* and *A4V/G85R* adult eclosion (Fig1A), but it restores reduced climbing and lifespan of *A4V* and *$(G_4C_2)_{49}$*, as well as reduced mitochondrial movement in *G85R* and ATP levels in *G85R* and *A4V* (Fig1,2). The ability of *tkt^{Ex-d}* to restore the number and movement of mitochondria in *G85R* multidendritic (MD) sensory neurons and CCAP-MN neurons (CCAP neurons that innervate body wall muscles) (Fig 2A), coupled with its ability to restore ATP levels supports our conclusion that the defect in mitochondrial movement is a reflection of compromised function in energy production, and when the balance of metabolic flux is reestablished, they recover. In summary, we find that the upregulation of *tkt* in MN or SN/IN ameliorates multiple defects in *G85R and A4V* knock-in animals analogous to its ability to reduce degeneration associated with *hTDP43^{M337V}*, *hFUS^{R521C}* and *$(G_4C_2)_{30}$* overexpression in the eye[68]. Coupled with our finding that genetic modifications of *Glut8*/*CG10960* and *Paics* also suppress across multiple models of ALS/FTD, we capitalize on the central role that Tkt plays in maintaining metabolic flux. Specifically, our research program leverages the neuroprotective role of Tkt to interrogate how realignment of flux through PPP, nucleotide biosynthesis, and glycolysis is beneficial for restoring motor function in ALS/FTD.

While not the immediate focus of the proposed research, we have shown that Gbb, the BMP5/7 ortholog is a

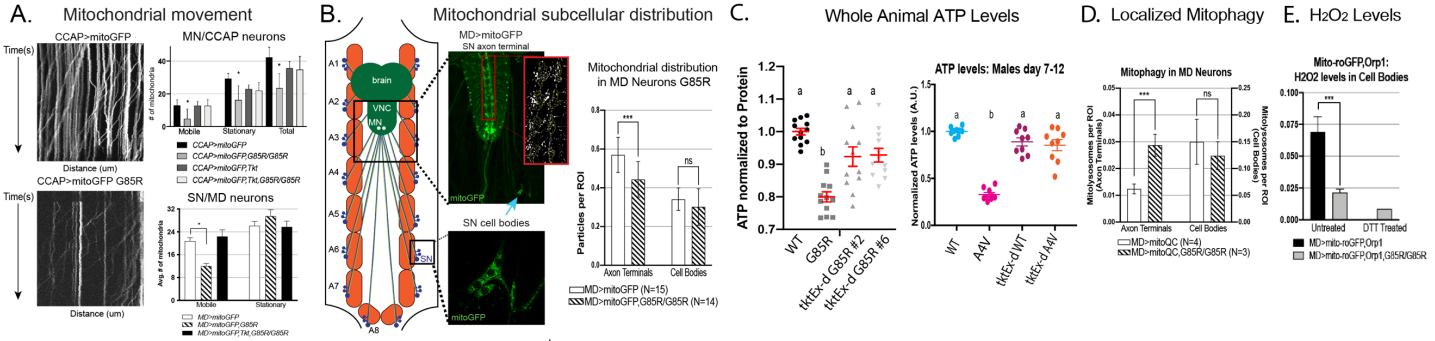

Fig2 Mitochondrial dysfunction A. Live imaging of mitoGFP in late 3rd larval MD axons visualized in kymograph; quantification of mobile, stationary mitochondria in CCAP MNs & MD sensory neurons, *tktExd* rescues decrease in mobile pool in *G85R*. B. 3rd larval filet maintains intact motor circuit innervation of muscles (orange), mitoGFP expression in MD sensory neurons allows visualization/quantification in cell bodies and axonal terminals in VNC (spinal cord equivalent), inset=mitoGFP particles in terminals (***p<0.0001, ns=not significant, Student's t test). C. ATP quantification whole body, larvae (*G85R*), adults (*A4V*) in presence *tktEx-d* Oneway ANOVA Tukey test for multiple comparisons, b significantly different from a. D. Mitolysosome quantification in MD neurons shows elevation in axon terminals, not cell bodies, in *G85R* compared to WT (Student's ttest, ***p<0.001) E. H_2O_2 levels decreased in cell bodies MD neurons in *G85R*, efficacy of mito-roGFP-Orp1 shown by treatment with reducing agent DTT (Student's ttest, ***p<0.001).

very effective suppressor of ALS/FTD associated neurodegeneration when upregulated[56]. Gbb is required for maintaining the expression of a number of genes encoding PPP and glycolytic enzymes, including five PPP genes, *tkt* (*CG8036*), *Taldo, Zw* (*G6PD*), *Pgd*, and *Rpi*, and three glycolysis genes, *Pgi*, *GAPDH* (*CG9010*), *Pgk* based on RNAseq analysis comparing *gbb* null (*gbb^{1}/gbb^{2}*) vs wild type (*w^{1118}*) animals. While the rescue of *A4V* climbing and lifespan by neuronally-expressed *gbb* is quite extraordinary, we know from our own research that Gbb/BMP signaling is regulating a number of other cellular functions beyond PPP and glycolysis, and therefore, here, *we focus our mechanistic analysis on the actions of downstream effectors of Gbb signaling, that are direct regulators of cellular metabolism.*

EXPERIMENTAL PLAN

Our overarching hypothesis is that differential flux through PPP can restore neuron function, and thus, motor circuit activity in the context of ALS/FTD. We will test this hypothesis by comparing genetic manipulations of genes in the oxidative and non-oxidative branches of the PPP, nucleotide biosynthesis and glycolysis in each

ALS/FTD mutant vs wild type backgrounds to assess how phenotypes and cellular dysfunctions associated with ALS/FTD are impacted. We propose that disruptions in cellular metabolism documented in the literature as an underlying factor of the etiology of ALS/FTD and thought to trigger neurodegeneration[23,26,71,72], can be rebalanced by changing the level of specific enzymes and metabolites. The challenge comes as to when and where that change must occur. As a critical first step we use spatial and temporal genetic manipulations possible in the *Drosophila* system to identify how pathways can be manipulated in the context of ALS/FTD to reestablish the proper balance of metabolites required to sustain the energy demands of neurons over time. Our results will be used throughout the project period to refine our hypotheses in the most efficient and effective means to provide a thorough understanding of the mechanistic actions of PPP in the context of whole organism and intact motor circuit. Acquiring this knowledge is critical for future investments in mouse and human models of ALS/FTD in their *in vivo* context, which is often more difficult and, in most cases, impossible

We will compare models of the two most common forms of ALS/FTD that exhibit very similar phenotypes, albeit whose underlying causes may be quite different. Suppression of motor dysfunction and reduced lifespan could be accomplished at a point where the two converge or by a manipulation that broadly impacts cell function. This research plan will clarify these possibilities. Comparing the effects of genetic manipulations on adult motor activity and lifespan in *A4V* and *neural^{ts}>$(G_4C_2)_{49}$* models is particularly powerful given their similar decline in motor function and survival. Comparing how such manipulations influence compromised NMJs, dysfunctional mitochondria, and failure to eclose evident in *G85R* and MN>$(G_4C_2)_{49}$ animals will provide insight into specific cellular functions necessary for motor activity. In each case, we have extensive experience conducting these quantifiable assays and have demonstrated feasibility of each. Genetic backgrounds are controlled with comparisons between genotypes varying by one genetic element, with multiple genetic recombinants tested, as well as multiple RNAi lines. More than 50 individuals of each genotype are scored for eclosion with 3 biological replicates (stats: Fischer's exact, with Tukey HSD corrections), 3 replicates of 100 flies of each genotype are monitored for viability every other day to determine survival curves (stats: Kaplan-Meier, post-hoc pair wise comparison), and 6 vials of 10 flies of each sex and genotype are imaged using an automated system for tracking rate of climbing (negative geotaxis) using local linear regression with at least biological replicates (Spierer 2021) (stats: ANOVA with Tukey correction for multiple comparisons). Larval or adult filets are done followed by immunohistochemistry to analyze the morphology of different NMJs[56] bouton counts, synapse area, branch length and number of active zones of 8-10 individuals are quantified from different genotypes blinded to the researcher. Live imaging of mito-GFP expressed in specific sets of neurons provides a powerful readout for mitochondrial function in an in vivo setting. Kymograph visualizations are quantified from more than 10 individuals.To assist the reader and to avoid long lists of nearly redundant genotypes, we will use *dALS^{mdl}* to designate that the same genotype will be examined with each of the models: *G85R*, *A4V*, MN>$(G_4C_2)_{49}$, and conditionally expressed *MN^{ts}>UAS-$(G_4C_2)_{49}$*. We will follow standard nomenclature for Gal4/UAS directed expression. *ChAT>tkt* indicates a *tkt* gene construct (*UAS-tkt*) is transcriptionally activated in cholinergic neurons (*ChAT-Gal4*). **Gal4* indicates that multiple Gal4 drivers will be tested separately with the same genetic elements in that genotype, with specific Gal4 lines to be tested noted in the text.

tkt influences both glucose metabolism and nucleotide biosynthesis as the key pentose phosphate pathway (PPP) enzyme where it catalyzes reversible reactions between ribose 5-phosphate (R5-P), the precursor of nucleotide biosynthesis, glycolytic intermediates: glyceraldehyde 3-phosphate (Ga3-P) and fructose 6-phosphate (F6-P), and other sugar phosphates (Fig 3). The improvement in motor function seen when *tkt* expression is increased in ALS/FTD models could result from: 1) increased flux through oxidative PPP, 2) a boost in

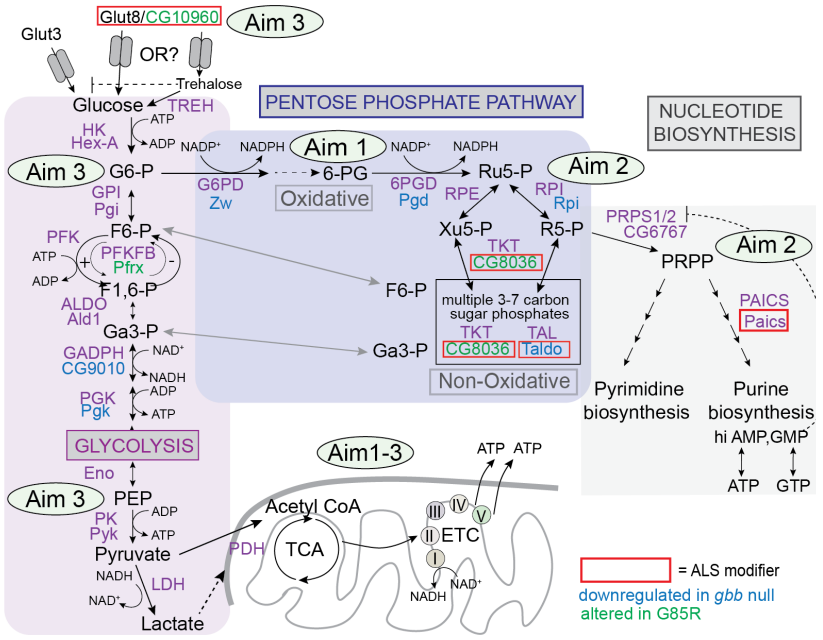

Fig3 Pentose Phosphate Pathway, Glycolysis, Nucleotide Biosynthesis
Metabolites (black), genes encoding enzymes (human (top), Drosophila (bottom) , purple), genetic modifier of four ALS models (red box), gene down regulated in *gbb* null (blue), genes misregulated in G85R (green).

nucleotide biosynthesis, and/or 3) increased glycolytic flux. The requirement for each pathway in *tkt*-induced suppression will be tested in ameliorating ALS/FTD-associated deficits.

Aim 1: *Determine the requirement for oxidative PPP in tkt-mediated amelioration of ALS/FTD neurodegeneration*

Following the conversion of glucose into glucose 6-phosphate (G6P), the first step of glycolysis, G6P can be funneled into the oxidative PPP to produce NADPH, a key reducing equivalent, that has two important functions, 1) driving *de novo* synthesis of macromolecules (nucleotide, fatty acids, amino acids) required by the cell, and 2) as the key regulator of antioxidant defense pathways. Neurons are highly sensitive to oxidative stress and mechanisms that increase the shunt of G6P through oxidative PPP to increase NADPH will be beneficial for neuronal health. Similarly, the production by the PPP of ribose 5-phosphate (R5P), the backbone of nucleic acids, serves are the entry point into nucleotide biosynthesis. In this aim we will clarify the requirement of these two pathways in *tkt*-mediated suppression of degenerative phenotypes.

Aim 1A: Requirement for oxidative PPP in *tkt*-mediated suppression Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the conversion of G6P to 6-phospho-gluconate, the rate-limiting step in the oxidative phase of the PPP. A variety of disease phenotypes are associated with deficiencies in G6PD activity that result in lower levels of NADPH and thus, increased oxidative stress[73]. Long-lived flies have increased levels of G6PD which correlates with improved neuronal proteostasis[74] Overexpression of G6PD in mice reduces oxidative damage from ROS, reduce mitochondrial damage, prevent age-related hearing loss, and improve healthspan[75,76]. Increases in Tkt are known to stimulate the oxidative branch of PPP producing NADPH, critical for maintaining redox homeostasis under stressful conditions, while also generating ribulose 5-phosphate (Ru5-P) [69,77].

We hypothesize that *tkt*-induced suppression of ALS phenotypes will be compromised by a reduction in oxidative PPP flux. A loss of G6PD activity is predicted to reduce Ru5-P levels, compromising production of Tkt substrates, Xu5-P and R5-P, that support the influx of sugars into glycolysis and nucleotide biosynthesis. To block oxidative PPP and eliminate its contribution to NADPH production, we have obtained a strain null for both *Zw*, synonymous for *G6PD*, and *Pgd*, (6PGD (6-phosphogluconate dehydrogenase)) from BDSC (Bloomington Drosophila Stock Center). *Zw-G6PD and Pgd-6PGD* null alleles each demonstrate a dosage dependent effect on enzyme activity. Double homozygotes (hereafter ZwPgd) are viable indicating that survival is possible without oxidative PPP [78], as also true in humans who inherit an X-linked G6PD deficiency but suffer from forms of anemia due to the fact that red blood cells lack mitochondria, the alternate source of NADPH. We will test for the ability of increased *tkt* expression to suppress reduced eclosion frequency, climbing ability and survival exhibited by our ALS/FTD models in the context of *ZwPgd* mutants. Both single copy *ZwPgd/++* and copy *ZwPgd/ZwPgd* knockdown will be tested for an impact on *tkt*-induced rescue. Specifically, we will test for an oxidative PPP requirement in when *tkt* is expressed in MNs (*OK371-Gal4*) and in SN/INs (*ChAT-Gal4*), as well as with more restricted drivers *MD-Gal4* and *CCAP-Gal4* (a subset of MNs), since *MD>tkt* is sufficient to rescue eclosion, as well as mitochondrial transport (Fig 2A). **Gal4>UAS-tkt dALS^{mdl}* will be compared to *ZwPgd;*Gal4>UAS-tkt dALS^{mdl}*, with the phenotypes of *ZwPgd* and *ZwPgd *Gal4>UAS-tkt* as controls.

If knocking down oxidative PPP has no effect on *tkt*-induced suppression, it is possible that elevated levels of *tkt* can in some way compensate for the loss of oxidative PPP's contribution to macromolecular biosynthesis and/or NADPH production by the mitochondria may be sufficient. If oxidative PPP is required, we will see a failure of suppression, suggesting that *tkt*-induced rescue is sensitive to a disruption in carbon flux through oxidative PPP. Blocking oxidative PPP, could alone enhance degeneration, and this would be detectable in *ZwPgd dALS^{mdl}* animals. Should such an enhancement be so severe to cause lethality, we will knockdown ZwPgd in specific cells using UAS-RNAi lines for each gene driven by the *Gal4. In this case, to control for the number of UAS-containing transgenes, UAS-lucRNAi or UAS-GFPRNAi will be included in the genetic constructions of the experimental lines used.

An increase in *Zw/G6PD* levels could alone improve motor function. To test for a suppression of ALS phenotypes, G6PD (*UAS-G6PD*)[79] will be driven by *OK371-Gal4*, *ChAT-Gal4* and *MD-Gal4* in **Gal4>UAS-G6PD dALS^{mdl}*. Eclosion, climbing, and survival frequencies (as described above) will be compared to **Gal4>UAS-G6PD* and the **Gal4* as controls in each ALS model, as will mitochondrial movement and NMJ morphology. We will also explore the possibility that co-expression of *Zw/G6PD* and *tkt* could provide a more robust suppression of motor defects. If not, assays for molecular readouts will inform as to the source of imbalance between these branches of PPP. These experiments will test our central hypothesis of the role for oxidative PPP in ALS/FTD suppression and clarify the individual contribution of *tkt* and *Zw-G6PD* to this pathway's effect.

Aim 1B: Measure NADPH and redox couples as outputs of oxidative PPP manipulations using *in vivo* redox biosensors We expect manipulations of *Zw-G6PD* and *Pgd-6PGD* to alter the redox state of cells in

response to changes in NADPH production, given that pyridine nucleotides are regulators of energy metabolism, mitochondrial function, calcium turnover, apoptosis, and many other biological processes, including the defense against ROS[80]. We expect increased *Zw-G6PD* expression to produce more NADPH, which facilitates the detoxification of reactive oxygen species (ROS). *Zw-G6PD* loss will result in a decrease in NADPH enabling increases in levels of oxidant species such as H_2O_2 or oxidized glutathione, GSSG. For some time, increases in oxidative stress and alterations in redox couples have been linked to disease progression in ALS [63,81]. Measures of reduced and oxidized forms of NADP+/NADPH have been performed on extracts of whole tissues [82], however, to detect changes in redox state in a cell type-specific manner, as well as in specific sub-cellular locations, we have optimized the *in vivo redox-GFP* (*ro-GFP*) biosensors for glutathione and H_2O_2 [83]. We have tested four transgenic constructs to *in vivo* monitor redox species, the GSH/GSSG redox couple and H_2O_2, in the cytosol or mitochondria[83]. *roGFP-Grx1* reports on glutathione redox couple the major antioxidant protection system, and *ro-GFP-Orp1* reflects the major oxidant species, H_2O_2. We have verified the efficacy of each construct when expressed in MD neurons by measuring the 405/488nm ratio in the absence and presence of the reducing agent DTT. In each case, we observe a clear decrease in the ratio when the sensor is reduced by DTT (Fig 2E).

Oxidative stress has been implicated in many neurodegenerative diseases like ALS, but little is known about quantitative changes *in vivo*, at the cellular level. Our preliminary data show a marked decrease in mitochondrial H_2O_2 levels in MD cell bodies in *G85R*, with no significant difference observed at axon terminals, indicating subcellular differences in mitochondrial function in diseased neurons (Fig 2F). How these measures of oxidative stress change in response to *tkt*-expression +/- *Zw-G6PD* function and oxidative PPP flux will determine their ability to eliminate imbalances in redox potential in different neuron types and in different subcellular locations, at the synapse vs the cell body. The difference we detect in H_2O_2 is interesting, as we find a clear increase in mitophagy in the axon termini but no change in cell bodies (Fig 2E), indicating a higher rate of mitochondrial turnover at the axon terminal not seen in the cell body. We do not yet understand the relationship between these subcellular measures but our ability to make measurements in these different compartments over time and in different genetic backgrounds will allow us to identify their molecular basis in an in vivo context of the diseased and suppressed state.

Aim 1C. Define molecular intersection of transcriptional regulation and metabolism associated with neuroprotective nature of ALS suppressors We have shown that by manipulating metabolic regulators, neurodegeneration associated with ALS/FTD can be suppressed. The key to developing effective therapeutics is to identify the critical metabolites and pathways where interventions can be made. Results from experiments in Aims 1A&B will clarify the functional importance of oxidative PPP. The next critical step is to obtain a comprehensive understanding of the changes in the metabolic intermediates, that are at the crux of a cell's physiology, as a consequence of our genetic manipulations in ALS/FTD backgrounds. Metabolic enzymes and other proteins are one step away from the actionable molecules, the metabolites, yet their levels drive the reactions and pathways. Thus, it is important to measure both, through comparative metabolomic and transcriptomic analyses over time, providing the complete landscape of metabolic regulators and their products. We will first compare metabolite and gene expression profiles from *tkt-suppressed* nervous systems at pre- and post-symptomatic stages to *A4V* and $(G_4C_2)_{49}$ mutants of the same age, with a comparison to wild type animals. As our starting material, *tkt* will be expressed pan-neuronally and adult heads will be harvested on specific days to capture the pre-symptomatic (normal climbing velocity/intact motor neuron projections) and post-symptomatic (partially compromised climbing/reduced axonal projections) states to infer changes in metabolite pools with symptoms. Males and females will be separated, as *A4V* males show a more rapid decline than females. The set of adult flies used in this comparative analysis are delineated in Table 1 by genotype, sex and age. We fully acknowledge that the proteome lies between the transcriptome and the metabolome, and will pursue protein (e.g., PPP enzyme) analyses in the future. The joint analyses of metabolites and gene expression provides the most efficient test of our hypotheses and will enable candidate enzyme approaches in subsequent analyses.

Aim 1Ca. Elucidate metabolomic profile associated with *tkt*-induced suppression of ALS models

Thirty fly heads of each genotype (Table 1) in 5 replicates will be flash frozen, and sent to our collaborator Dr. Nav Chandel, an expert in metabolism and mitochondrial function, at Northwestern Feinberg School of Medicine, for extraction and analysis using their optimized protocols for *Drosophila* metabolomics[84]. Samples will be normalized by mass and the inclusion of internal standards. To focus on the immediate response to *tkt*-suppression, a targeted analysis will capture carbohydrates, glycolytic and PPP intermediates, purine and pyrimidine bases, and TCA cycle metabolites. We will examine multivariate patterns of metabolite variation by performing a principal component analysis on the different genotype/sex/age metabolomes. This will reveal general patterns. For each metabolite, we will fit the following model: metabolite level = μ + *ALS* + *tkt* + *age* +

*ALS*tkt + ALS *age + tkt*age + ALS*tkt*age + ε,* where ALS represents the 3 genotypes (WT, A4V, or $G_4C_2)_{49}$), tkt is the *tkt* suppressor state (control vs. overexpressed), and age the two time points. The remaining terms are the pairwise and three-way interactions, plus model mean (μ) and error (ε). The interaction terms are informative about the impact of the suppressor on that metabolite in each control or ALS models. The three-way term captures the genotype-specific neurodegenerative effects over time. Data processing and statistical analysis will be carried out in close collaboration with Nav Chandel's group with Mathieu Bartoletti in my lab group. David Rand's lab group at Brown Univ. has experience working with both metabolomic and transcriptomic data sets, and both the Rand lab and the Computational Biology Core at Brown University will serve as an additional resource for statistical analysis and data visualization.

This experimental design allows us to identify the changes in metabolite profiles between ALS model, suppressed ALS model and control over time, which will allow cause-effect relationships to be established. While metabolomics has been performed on both patient and ALS/FTD model tissues, the comparison of metabolite profiles between the diseased state and the suppressed state in a highly controlled genetic background has not been done. We expect to see parallels to analyses done on patient cells given the extraordinarily high conservation of metabolic pathways within the animal kingdom. We hypothesize that pan-neuronal expression of *tkt* will increase the abundance of metabolites in oxidative PPP and nucleotide biosynthesis in *A4V* and $elav^{ts}>(G_4C_2)_{49}$ to correct metabolic imbalances caused by each ALS/FTD condition. It is possible that: 1) the immediate effects of an *A4V-SOD1* mutation and the RNA and DPR toxicity produced by G_4C_2 expansions, have similar effects on cellular metabolism, and *tkt* corrects this imbalance; 2) pre-symptomatically *A4V* and $elav^{ts}>(G_4C_2)_{49}$ profiles are different but after degeneration has begun their profiles converge and *tkt* corrects imbalances; or 3) the metabolic profiles of *A4V* and $elav^{ts}>(G_4C_2)_{49}$ are distinct at all points of progressive decline, but as neurons struggle to regain homeostasis in *A4V* and $elav^{ts}>(G_4C_2)_{49}$, *tkt* expression stimulates different mechanisms to regain balance. For example, *tkt* expression could stimulate a response to upregulate oxidative PPP, or it could increase glycolytic flux to produce more pyruvate, essentially acting as a rheostat to realign pathway flux for needed energy production. A comparison of metabolite profiles from *tkt*-suppressed versus *A4V* and $elav^{ts}>(G_4C_2)_{49}$ animals, pre- and post-symptomatic, will identify which of these scenarios is true and the mechanism used. Depending on the results of our genetic studies in all Aims, we will repeat our analysis to test for example, how the knockdown of ZwPgd affects the metabolite profile of *tkt*-suppressed *A4V*, and if that is different from *tkt*-suppressed $(G_4C_2)_{49}$. In this way we gain tremendous strength in correlating functional outputs on motor activity.

	Gal4	tkt	♂	♀	days
WT			X		2, 8
				X	2, 23
WT	elav	X	X		2, 8
				X	2, 23
A4V			X		2, 8
				X	2, 23
A4V	elav	X	X		2, 8
				X	2, 23
$(G_4C_2)_{49}$	$elav^{ts}$		X		2, 5
				X	2, 5
$(G_4C_2)_{49}$	$elav^{ts}$	X	X		2, 5
				X	2, 5

Table 1. Genotypes for metabolomic & transcriptomics

Aim 1Cb. Define and compare cell-type specific gene expression profile underlying neuroprotection

Transcription is the first step in executing changes in the genome, and the transcriptome provides a global view of this regulatory response. To best correlate gene expression profiles with metabolite profiles, total RNA will be extracted from the heads of the same animals listed in Table 1, from 3 biological replicates. RNA will be sent to BGI for library prep and sequencing. 100bp paired end reads will allow gene identification as well as alternatively sliced RNAs that give rise to different enzyme isoforms. Our bioinformatic RNAseq pipeline will involve FastQC[85], read mapping with a two pass STAR protocol[86], DESeq2[85], and p-values adjusted to FDR via Benjamini-Hochberg. Pathway analysis will be done using STRING and gene enrichment analysis (GSEA)[87,88] with GO[89] and REACTOME[90]. DE genes will be compared between pre- vs post-symptomatic animals, *A4V* vs $(G_4C_2)_{49}$, +/- *tkt* expression, for 'reversal genes', whose expression increase in the ALS mutant then decrease in the suppressed tissue, or vice versa. The analyses will be performed in a manner similar to the mode stated above, where each transcript is tested for the effects of the ALS model, *tkt* expression and time point. Analyses done using DESeq2 will be compared to linear models of log transformed quantile-normalized read counts, using the lm function in the R programming language [91].This genome-wide analysis allows us to address the following questions: Do reversal genes highlight particular pathways? What is the direction of change for each DE gene in oxidative PPP, glycolysis, nucleotide biosynthesis, and mitochondrial pathways when the level of a single component/metabolic enzyme is altered in the disease versus wild type state? These metabolomic and transcriptomic analyses will allow us to test very specific hypotheses about homeostasis: namely, are the metabolites that change in the suppression context covarying with altered expression of the enzymes that are adjacent to them in the metabolic pathways. This can be tested with matrix correlation analyses[92,93]. This comparative analysis will provide novel insights into the mechanism responsible for regulating cellular metabolism in the context of improved neuronal function and extension of life.

To date we have found that either MN or SN/IN *tkt*-expression can suppress many phenotypes in ALS/FTD models. However, the first functional defects in *G85R* animals were detected electrophysiologically in SN/INs and not MNs (Held). Therefore, if we find, for example, that downregulating nucleotide biosynthesis in SN/IN mitigates *tkt*-induced rescue in the *$(G_4C_2)_{49}$* model but downregulation of PRPS in MNs has no effect, we will be very interested in the transcriptomic differences between these cell types. Cell type specific RNAseq by INTACT/TAPIN[94-96] will provide transcriptomic data from specific subsets of cells as our data from Aim 1-3 become available. We will use TAPIN to isolate the SN/IN and MN pools of neurons to perform RNAseq to compare: ChAT-Gal80^{ts}>*tkt unc84-2XGFP PRPS-RNAi* $(G_4C_2)_{49}$ to OK371-Gal80ts>*tkt unc84-2XGFP PRPS-RNAi* $(G_4C_2)_{49}$ with appropriate controls. The *unc84-2XGFP* construct localizes GFP to the inner nuclear membrane allowing for isolation of nuclei in targeted cells. Our colleague Karla Kaun has TAPIN working well in her lab and has offered advice. These analyses are preferred to single cell RNAseq, as we are interested in molecular features of different neuronal populations, not individual cells. Moving forward these types of analyses will be important as energy homeostasis relies on metabolic interactions between different cell types, each with its own pattern of metabolic gene expression[24]. Flyscape[97]a software package that allows for the connection of metabolites with enzymes will be used to build network graphs coupling metabolomic and transcriptomic profiles.

Outcome, potential problems & alternative approaches: These studies will provide a comprehensive, integrative analysis of relevant organismal, cellular, and molecular changes associated with patient-defined models of ALS/FTD in the context of biochemical pathways strongly implicated in ALS. This information can be informative about approaches for therapies for ALS that may be metabolite- or expression-based. Notably, observed declines in any metabolite motivate dietary supplement experiments to test for comparable suppression effects of such compounds. We acknowledge that the metabolomic and transcriptomic data will be complex and of high dimension, but each experiment is designed in a factorial manner with explicit hypotheses about interaction of ALS model, suppressor, and age, that has not been done in any model. The challenges of this complexity greatly outweigh the limitations of pursuing 'candidate' metabolites or transcripts one-by-one. We recognize that there are post-transcriptional and -translational modifications that alter function and that various proteomic analyses would address alternative possibilities. But the data at hand confirm that changes to dynamic pathways are a key to the ALS and suppressed phenotypes we have documented, and thus conclude that these approaches are the most efficient given the current state of knowledge.

Aim 2: Define the role of nucleotide biosynthesis in tkt-mediated neurodegeneration suppression

Increases in Tkt and ribose 5-phosphate isomerase (Rpi) activity result in increased carbon flux through oxidative PPP producing higher levels of ribose 5-phosphate (R5-P), the entry point to nucleotide biosynthesis (Fig 3). R5-P is the substrate for the rate-limiting enzyme phosphoribosyl pyrophosphate synthetase (PRPS1/2) to produce phosphoribosyl pyrophosphate (PRPP), the common precursor of the 5-carbon sugar subunit for purine, pyrimidine, and pyridine biosynthesis. Mutations in PRPS1/2 are associated with Art syndrome, Charcot-Marie-Tooth disease and other neurological disorders with links to disruptions between nucleotide synthesis and metabolic demands[98-100]. A recent report indicates that R5-P is reduced in spinal cords of the *hSod1^{G93A}* ALS mouse model[26] suggesting a disruption in PPP. Consistent with this prediction, we find that *tkt* levels are reduced by 2.45 fold in *A4V* animals by qPCR (ttest p=0.038). We predict that R5-P levels would be reduced in *A4V* animals and increased in *tkt*-suppressed *A4V* animals, this will be clarified by our metabolomic profiling in Aim 1Ca. However, it is curious that the *Paics-e04545* Exelixis insertion, identified as a suppressor of TDP43, FUS, $(G_4C_2)_{30}$[68] and *G85R* (Wharton lab), is a loss of function mutation. It is possible that reduced flux through purine synthesis results in a feedback to influence the AMP/ATP pool and influences AMPK-induced autophagy[101] to aid in reducing compromised motor neurons. This possibility will be examined in *ALS^{mdl}* +/- *Paics-e04545* and *Paics-RNAi* but our main focus in this aim will be on modulating nucleotide biosynthesis as a whole.

Considering the potential impact of R5-P and nucleotide biosynthesis on ALS motor function, the most effective means of testing whether a reduction in this pathway alters *tkt*-induced suppression is to knock down the rate-limiting enzyme, PRPS, using loss-of-function *PRPS* alleles, or UAS-*PRPS-RNAi*[102]. We hypothesize that **Gal4>UAS-tkt UAS-PRPS-RNAi dALS^{mdl}* will negate the rescuing ability of *tkt* (**Gal4>UAS-tkt dALS^{mdl}*). *PRPS* homozygous nulls are lethal, therefore, we will first determine if *tkt*-induced suppression is sensitive to a single gene copy reduction of *PRPS* in our motor assays (climbing and eclosion) which are particularly sensitive to dosage. If we do not see a change, *PRPS-RNAi* will give us more control over levels of knock-down and the ability to test cell type specific responses by driving it at different temperatures. Given the demonstrated roles for both *PRPS* and *Paics* in regulating nucleotide biosynthesis, cellular autophagy and response to ROS, we will perform *in vivo* measurements of ATP using the UAS-AT1.0NL1 biosensor[103,104] we have used to quantify relative

levels of ATP in neurons, anti-LC3 to assess autophagy, and H2DCFDA (Molecular Probes C6827), a fluorescent probe to measure ROS levels. For more in-depth assessments of *in vivo* redox coupling, the *cyto-ro-GFP* and *mito-roGFP* lines will be used. As an example, these cellular studies will be performed initially in *G85R* and *$(G_4C_2)_{49}$* to assess potential changes to *tkt*-suppression efficacy (*Gal4>*UAS-tkt dALS*^{mdl}) when each gene is knocked down, e.g., *Gal4>*UAS-tkt UAS-PRPS-RNAi dALS*^{mdl}, or overexpressed. Measurements will be compared to those from downregulated PRPS alone, in each model **Gal4>UAS-PRPS-RNAi dALS*^{mdl}, and **Gal4 dALS*^{mdl}, to assess the effect of modulating of nucleotide biosynthesis.

Given that *R5-P* is the key metabolite linking nucleotide biosynthesis and oxidative PPP, one may expect that a reduction in R5-P will attenuate the ability of *tkt* expression to suppress eclosion, climbing and survival deficits when knocking down Rpi, the enzyme responsible for the conversion of Ru5-P to R5-P, in **Gal4>tkt Rpi-RNAi dALS*^{mdl} compared to **Gal4>tkt dALS*^{mdl}. However, the knockdown of *Rpi* has been shown to produce more NADPH by what appears to be the *tkt*-mediated conversion of excess Ru5-P to F6P, which is shunted back to oxidative PPP[105]. Whether downregulation of *rpi* has the same effect in the context of ALS/FTD, or in the *tkt*-suppressed state, is not known. We will test this possibility using *rpi-RNAi* and *rpi* loss of function alleles. If it does, we would predict that co-knock down of Rpe would eliminate the observed feedback to oxidative PPP, and severely compromise *tkt*-induced suppression. Regardless, our genetic analysis in an ALS/FTD-associated degenerative state will delineate the specific enzymes that can or cannot compensate for *tkt*'s regulatory role, and their impact on autophagy, ATP production and ROS levels in *tkt*-suppressed ALS/FTD.

Outcomes, potential problems & alternative approaches: This analysis will relate the organismal response in terms of motor function and survival with cellular response. If *PRPS* is required for *tkt*-induced suppression, we will test if an increase in nucleotide biosynthesis promoted by increased *PRPS* expression can suppress *dALS*^{mdl} phenotypes, especially in in the absence of *tkt*. Resolution of alternative molecular outcomes can be assessed with additional metabolite and transcriptional profiling to related these findings to those from Aim 1.

Aim 3: **Investigate the functional intersection of *non-oxidative PPP and* glucose metabolism in suppression of ALS/FTD-associated defects**

As one of its roles in non-oxidative PPP, *tkt* catalyzes sugar phosphates to feed glycolysis (Fig 3). Glucose is the primary source of energy for neurons and ALS is characterized by increased glycolytic flux. One hypothesis to explain heightened glycolytic flux in ALS/FTD is that the cell is compensating to meet energy demands that cannot be met due to dysfunctional mitochondria. Glycolysis breaks down monosaccharides to produce pyruvate and in so doing achieves a net gain of 2 ATP molecules. In contrast, OXPHOS in the mitochondria generates 30 ATP molecules. While glycolysis can generate energy quickly and in the absence of oxygen, it requires high inputs of glucose due to its inefficiency. Given this small gain in energy production (2 ATP), glycolysis alone may not be the most efficient means by which cells overcome energy demands, and in ALS/FTD it is possible that other pathways with higher yields and added antioxidant capabilities would be preferrable. However, the apparent inability of neurons in ALS/FTD to make use of such pathways, suggests a fundamental pathway block, or a disruption in the central controller, thus, preventing homeostasis. In this aim, we again take advantage of *tkt*-induced suppression, but we also dissect the role of *CG10960*, which encodes a hexose transporter and like *tkt*, emerged as a genetic modifier of all four ALS/FTD models: TDP43, FUS, G_4C_2 and SOD1 (Fig. 3)

Aim 3A. **Determine effect of glycolytic flux on neuroprotective role of *tkt*** When increased, *tkt* is predicted to increase fructose 6-phosphate (F6-P) and glyceraldehyde 3-phosphate (Ga3-P) input to glycolysis. Here we ask: does *tkt*-suppression require flux through glycolysis to produce more energy, or are F6-P and Ga3-P shunts back into oxidative PPP? We will test genes that encode enzymes acting at the first step in glycolysis, above the split between glycolysis and oxidative PPP (GCK, Glucokinase), and below the point where Ga3-P and F6-P enter glycolysis (PK, pyruvate kinase) to address their requirement for *tkt*-induced suppression. *Drosophila* HexA, a GCK ortholog, results in hyperglycemia, increased ROS, and increased DSBs when silenced[106]. Pyk, *Drosophila* PK, and HexA catalyze unidirectional reactions, making them key regulatory enzymes, with dose-sensitize effects in the cell. *Pyk* and *HexA* will be knocked down by available deficiencies, loss of function mutations and/or RNAi lines[107,108]. We will focus our analysis at the cellular level to avoid more general systemic effects, manipulating *Pyk* and *HexA* function in specific neurons using *OK371*, *ChAT* or *MD* analogous to Aim1, while activating *tkt* expression. Gal80^{ts} will be employed to provide temperature-dependent temporal control over cell-specific Gal4 expression to bypass lethality, as in[107]. HexA converts glucose to G6P, the metabolite linking glycolysis and oxidative PPP, therefore we predict that a reduction in *HexA* will significantly compromise *tkt*-induced rescue. *Pyk* acts at the end of glycolysis to generate pyruvate which is converted to acetyl-Co-A in the mitochondria, and the requirement for its function in *tkt*-mediated suppression will be determined. To ensure proper pan-neuronal knockdown of *Pyk*, pyruvate levels will be measured in brains/VNCs. To date, we do not

detect abnormalities in pyruvate levels in either *G85R* or *tkt*-rescued *G85R* animals (Bartoletti, data not shown), suggesting that glycolysis is not severely disrupted in *G85R* mutants or that *tkt*-mediated suppression results in a significant upregulation. Alternative pathways can generate acetyl-Co-A in which case *Pyk* knockdown may not be required for *tkt*-induced rescue, indicating glycolysis can be bypassed.

Aim 3B. **Determine importance of glucose uptake in role of Glut8 in modulation of degeneration**. Multiple studies have shown glycolytic flux is increased in ALS/FTD patients, as well as in *Drosophila hTDP43* or *hTDP43*^{G298S} overexpression models where levels of PEP and pyruvate and *Pfk* transcription were increased[72]. Despite this increase they observed overexpression of glucose transporters *Glut1* and *Glut3* provide some amelioration TDP43 phenotypes. Given these results it may seem surprising that we identified a loss of function mutation in *Glut8*, a hexose transporter, as a suppressor of neurodegeneration, especially since a knock down in GLUT8 in mammalian systems has been shown to compromise glucose uptake[109,110]. It is important to remember that the *Glut8/CG10960* mutation is effective in mitigating degenerative phenotypes in all four ALS models (TDP43, FUS, G_4C_2, SOD1). The UAS-containing P-element insertion is oriented such that the inserted UAS initiates transcription to produce an antisense *Glut8/CG10960* RNA causing a 6.8fold reduction in *Glut8/CG10960* RNA levels (p=0.0462, ttest). *Glut8*^{f03242} enables a high percentage of *G85R* adults to eclose and restores the reduced numbers of mitochondria at the synapse, as well as mitochondrial mobility in axons (Fig 4). Given *Glut8*'s role in glucose transport these data suggest that a reduction in glucose can alleviate neuronal dysfunction in multiple models of ALS/FTD, consistent with the demonstration that a deletion of GLUT8 increases locomotion in mice [111]. These conflicting results could be explained by differential flux through glycolysis versus PPP achieving the same end goal, indicating a more in-depth analysis is warranted. Our multipronged analysis in the context of the intact nervous system with models that reflect molecular stoichiometry will clarify this apparent discrepancy.

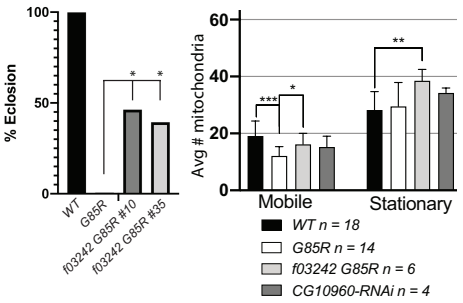

Fig 4 Glut8 mutant suppresses ALS phenotypes. (Left) Two independent recombinants of the Glut8 f03242 mutation allow G85R adults to eclose. (Right) Glut8 f03242 suppress the mitochondrial transport defect in G85R. Stats: Shapiro-Wilk normality test; unpaired t-test for normally distributed, Mann-Whitney U test for non-normally distributed * p ≤ 0.05, **p ≤ 0.01, ***p≤ 0.001

We will first test if glucose uptake is altered in *Glut8* mutant neurons using 2-NBDG, a fluorescently labelled glucose that is taken up and trapped in cells but not metabolized [112]. If glucose uptake is unaffected, it is possible *Glut1,3* are able to compensate. If glucose uptake is reduced in the *Glut8* mutant, we will test if *Glut1,3* knock down can also suppress our ALS/FTD model phenotypes and most importantly if Glut1,3 function is required for *tkt*-mediated suppression. Similarly, we will knock down *Glut8* in a *tkt*-overexpression animal. If glucose uptake is an important element of *tkt*-induced suppression then we would observe reduced/elimination of suppression. Alternatively, if we observe that in the *Glut8*-KD and *tkt*-OE suppression is more robust these genetic variants could be acting additively via the same mechanism. If the result is synergistic, *Glut8* reduction and *tkt* elevation may both be neuroprotective via independent mechanisms. If *tkt*-induced suppression depends on *Glut8* function, we will investigate its molecular basis. It is possible that instead of compromising glucose uptake, the reduction in *Glut8* preferentially affects trehalose uptake. Uptake of trehalose has been shown to depend on GLUT8, and trehalose is now thought act intracellularly to block glucose transport mediated by GLUT1,3 [113]. Thus, if cytosolic levels of trehalose are reduced in the *Glut8*^{f03242}, the block on *Glut1,3* could allow more glucose uptake. If this were indeed the case in neurons, we would expect overexpression of *Glut8* (*UAS-Glut8/CG10960* from BDSC) to increase trehalose and reduce glucose uptake. Following our genetic manipulations of *Glut8/CG10960,* we will test glucose uptake *in situ* with 2-NBDG [112]. If overexpressing *Glut8/*CG10960, and thus increasing trehalose blocks glucose uptake, we would expect that overexpressing *Trehalase* (*Treh*), the enzyme that breaks down trehalose would alleviate the block on glucose uptake.

Invoking a different mechanism, researchers propose that trehalose induces autophagy in MNs, delaying ALS progression[114,115] although, there appears to be some controversy with regard to its neuroprotective mechanism[116,117]. As it is not an immediate goal of this research to resolve the neuroprotective mechanism of trehalose, but with respect to the effective nature of Exelixis insertion *f03242* in *Glut8* to restore function to our *dALS*^{mdl} and the potential effect on autophagy, we will use anti-p62 (SQSTM1) as a measure for its initiation and anti-LC3 for autophagosomes, to assess any effects of *f03242* on autophagy in *dALS*^{mdl}. Brains will be dissected from both pre- and post-symptomatic larvae and adults +/- *f03242*, immunostained, and fluorescence quantified. An independent test for the effect of reduced *Glut8* will be performed with using loss of function alleles and *Glut8* RNAi to titrate down the levels of *Glut8*. Trehalose has also been shown to increase p62 independently of autophagy, and as p62 levels increase, so does the nuclear translocation of *Nrf2* (*ref2p/cnc*), a transcription factor that enhances the expression of antioxidant factors that reduce ROS[118]. *Nrf2* has been shown to directly

regulate the expression of *Tkt* and *Taldo* in A549 lung cancer cells lines [119,120]. These studies will determine if downregulation of *Glut8* impacts *p62* expression and thus, autophagy. We will extend our analysis to examine the relationship of *Glut8* to *tkt* and *taldo* expression, especially with respect to their regulation of autophagy.

Outcomes, potential problems & alternative approaches: These analyses will identify the role of glucose uptake in the disease vs suppressed state. The vast majority of reagents used in Aim 3 have been previously characterized, however, in the event that conditional or localized expression is required, we have vast amounts of experience with such experimental manipulations. Reagents to assay *in situ* for endogenous enzyme expression, ie. Pgi, HexA, Ldh[108] will be employed when appropriate. Should our analysis of glycolysis require clarification of glucose utilization, this will be monitored with the glucose sensor *UAS-FLII12Pglu-700μδ6*[121]. If our metabolomic analysis indicates altered lactate levels as observed in metabolic studies of NSC-34 transfected with SOD1-G93A[63], the lactate sensor UAS-Laconic[108] will be used to measure lactate concentrations in specific subsets of neurons in mutants versus tkt-suppressed animals. As in mammals Drosophila have multiple genes encoding different isoforms of hexokinase/glucokinase. HexA is broadly expressed with known functions in neurons, glial cells and flight muscle, while HexC is restricted to the fat body in larvae with lower expression in adults. Should our results with HexA be inconclusive, we will test down- and up-regulation of HexC as described above as an alternative.

Aim 3C Elucidate epigenetic changes in response to metabolic disruption and restoration A poorly understood consequences of altered metabolism is its ability to affect genome-wide transcription. Metabolite intermediates can directly or indirectly influence the activity of chromatin-modifying enzymes and thus, affect chromatin dynamics and resulting transcription[122,123]. Acetyl-coenzyme A serves as the acetyl donor for histones, mediated by histone acetyltransferases (HAT) and histone deacetylases (HDAC). Alterations in glucose metabolism in muscle stem cells can alter histone acetylation impacting muscle stem cell biology[124]. We predict AcetylCoA availability is limited in ALS neurons, evidenced by reduced ATP and abnormal mitochondrial dynamics apparent in our ALS models. To determine if efficient acetylation of histones is altered, we will map chromatin marks dependent on H3K9ac/H3K14ac and H4K16ac using CUT&RUN, a highly efficient methodology for epigenomic profiling[1125,126,127], in *dALS^{mdl}* and suppressed animals. We will use TAPIN to isolate nuclei from specific neuronal populations (MN vs SN), and sequence to determine how epigenome changes may respond to different metabolic states. Genotypes of high priority are those that modulate flux through oxidative PPP or glycolysis and result in a robust suppression of degeneration. How is genome availability altered between WT, ALS mutant and *tkt*-suppressed ALS mutant? These will be compared to manipulations that change the metabolic profile but do not mitigate ALS phenotypes. CUT&RUN for two acetylated chromatin marks will be a powerful connection between transcriptomic response to metabolic state. We will work with our colleague Erica Larschan on the methodology who has extensive expertise in epigenome profiling using ChIPseq and more recently CUT&RUN. Erica's postdoc will perform the quantitation and statistical analysis of the sequencing reads.

In summary, genetic analyses carried out in this proposal may highlight a single critical pathway essential for restoration of motor function, or they may show that manipulations targeting one pathway leads to compensatory actions by another. The latter is expected as energy homeostasis has evolved as a system to modulate changes in metabolic processes in response to imbalances. In the case of ALS, it is clear that imbalances exist yet the cells are unable to restore homeostasis. The outcome of our experiments will highlight exactly those imbalances, and which pathways are the most or least sensitive to modulations that increase motor function. They will also clarify the extent of overlap between different models, SOD1 and G_4C_2-repeats, as well as different mutations in SOD1, an important contribution to the field. Our studies are innovative because we will address the mechanisms underlying this inability to restore homeostasis in the context of well characterize ALS/FTD models. Our Aims are comprehensive as they integrate effects across different pathways that operate as an adaptive network. The scope of this work is entirely attainable in the Drosophila model, and we anticipate that these studies can provide highly fundamental mechanisms that can accelerate translations approaches to ALS/FTD treatments.

TIMELINE Aim	Year1	Year2	Year3	Year4	Year5
Aim 1A. Determine oxidative PPP requirement for *tkt*-suppression					
Aim 1B. *In vivo* measure of cell state using biosensors					
Aim 1C. Elucidate metabolic & transcriptomic profile of *tkt*-suppression					
Aim 2. Assess the role of nucleotide biosynthesis in *tkt*-suppresson					
Aim 3A Investigate interdependence of PPP & glycolysis					
Aim 3B Determine role of *Glut8* & glucose uptake in neuroprotection					

Use of Human Specimens and/or Data

Does any of the proposed research in the application involve human specimens and/or data *	❍ Yes ● No
Provide an explanation for any use of human specimens and/or data not considered to be human subjects research.	
Are Human Subjects Involved	❍ Yes ● No
Is the Project Exempt from Federal regulations?	❍ Yes ❍ No
Exemption Number	❑ 1 ❑ 2 ❑ 3 ❑ 4 ❑ 5 ❑ 6 ❑ 7 ❑ 8
Other Requested Information	

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