Université de Montréal

Rôle du métabolisme énergétique dans un contexte de vieillissement chez *C. elegans*

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Thèse présentée à la Faculté De Médecine
en vue de l'obtention du grade de Philosophiae Doctor (Ph.D.)
en Pathologie et Biologie cellulaire
option Système nerveux

Décembre 2014

Université de Montréal

Cette thèse intitulée:

Rôle du métabolisme énergétique dans un contexte de vieillissement chez *C. elegans*

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Résumé

L'incidence constante des maladies liées à l'âge reflète un réel enjeu dans nos sociétés actuelles, principalement lorsqu'il est question des cas de cancers, d'accidents cérébraux et de maladies neurodégénératives. Ces désordres sont liés à l'augmentation de l'espérance de vie et à un vieillissement de la population. Les coûts, estimés en milliards de dollars, représentent des sommes de plus en plus importantes. Bien que les efforts déployés soient importants, aucun traitement n'a encore été trouvé. Les maladies neurodégénératives, telles que la maladie d'Alzheimer, de Parkinson, d'Huntington ou la sclérose latérale amyotrophique (SLA), caractérisées par la dégénérescence d'un type neuronal spécifique à chaque pathologie, représentent un défi important. Les mécanismes de déclenchement de la pathologie sont encore nébuleux, de plus il est maintenant clair que certains de ces désordres impliquent de nombreux gènes impliqués dans diverses voies de signalisation induisant le dysfonctionnement de processus biologiques importants, tel que le métabolisme. Dans nos sociétés occidentales, une problématique, directement lié à notre style de vie s'ajoute. L'augmentation des quantités de sucre et de gras dans nos diètes a amené à un accroissement des cas de diabètes de type II, d'obésité et de maladies coronariennes. Néanmoins, le métabolisme du glucose, principale source énergétique du cerveau, est primordial à la survie de n'importe quel organisme.

Lors de ces travaux, deux études effectuées à l'aide de l'organisme *Caenorhabditis elegans* ont porté sur un rôle protecteur du glucose dans un contexte de vieillissement pathologique et dans des conditions de stress cellulaire. Le vieillissement semble accéléré dans un environnement enrichi en glucose. Cependant, les sujets traités ont démontré une résistance importante à différents stress et aussi à la présence de protéines toxiques impliquées dans la SLA et la maladie de Huntington. Dans un deuxième temps, nous avons démontré que ces effets peuvent aussi être transmis à la génération suivante. Un environnement enrichi en glucose a pour bénéfice de permettre une meilleure résistance de la progéniture, sans pour autant transmettre les effets néfastes dû au vieillissement accéléré.

Mots clés : Glucose, Métabolisme, *C. elegans*, Neurodégénérescence, Épigénétique

Abstract

The constant increase of the cases of age-related diseases, including cancers, cerebral accidents and neurodegenerative diseases raises a real problem in our current societies. These disorders are very strongly linked to the increase of life expectancy and to the ageing population. The costs, estimated in billion dollars, requiring vast medical resources and very few treatments exist today. Neuronal diseases, such as the Alzheimer's, Parkinson's, Huntington's disease and amyotrophic lateral sclerosis (ALS) are characterized by the degeneration of various types of neurons. This represents an important challenge because besides the lack of understanding the underlying mechanisms related to their pathology, it is now clear that some of these disorders involve several genes and lead to the dysfunction of fundmental biological processes such as metabolism. In western societies lifestyle and dietary practices may contribute to disease. The increased quantities of sugar and fat in western diets are thought to contribute to the rise of metabolic disorders, including Type II diabetes, obesity and coronary diseases. Nevertheless, it is important to understand that the metabolism of glucose, the brain's main energy source, is essential for survival.

In this thesis, two studies using the model organism *Caenorhabditis elegans* investigated a potential protective role of the glucose in a context of pathological ageing and in conditions of cellular stress. Although ageing seems accelerated in a glucose enriched environment, the test subjects demonstrated an improved resistance to numerous stresses including against toxic proteins involved in the ALS and Huntington's disease. Secondly, it appeared that these effects can be heritably transmitted to successive generations of animals. Thus, a glucose enriched environment allows for increased stress resistance in the offspring, without transmitting the negative effects of accelerated ageing.

Key Words: Glucose, Metabolism, *C. elegans*, Neurodegeneration, Epigenetic

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Sigles et Abréviations

ADN : Acide Désoxyribonucléique

ALS/SLA : Amytrophic Lateral Sclerosis / Sclérose Latérale Amyotrophique.

ARN : Acide Ribonucléique

ARNlnc : ARN long non codant

ARNnc : ARN non codant

ATP: Adénosine tri-phosphate

BDNF: Brain Derived Neutrophic Factor

CH : Huntington Disease / Chorée de Huntington

FTD/ DFT : Frontotemporal Dementia / Démence Fronto-Temporale

GE : Glucose enrichment / Enrichissement en glucose

IIS : IGF/Insulin signalling / Voie IGF/insuline

LDH : Lactate Déshydrogénase

MA : Alzheimer's Disease / Maladie d'Alzheimer

MCT : Monocarboxylate Transporter / Transporteurs moncarboxylés

miARN : micro ARN

MP : Parkinson's Disease / Maladie de Parkinson

NES : Nuclear Export Signal / Signal d'Exportation Nucléaire

NLS : Nuclear Localization Signal / Signal de Localisation Nucléaire

NMDA : *N-methyl-D-asparte*

PRC2 : Polycomb Repressive Complex 2 / Complexe de Répression Polycomb 2

RC : Restriction calorique

ROS : Reactive Oxygen species / Espèces Réactives à l'Oxygène

RRM : RNA Recognition Motif / Motif de Reconnaissance de l'ARN

SNC : Système Nerveux Central

TARDBP : Transactive Response DNA Binding Protein

TOR : Target of Rapamycin

Dédicace

À celle qui aura changé ma vie durant 7 années
et qui m'a éclairé dans les moments les plus
sombres.

Remerciements

WOW, on y est presque! 14 ans au Québec…et presque la moitié au CHUM! Il est loin l'automne 2007, lors duquel je visitais les locaux du CHUM Notre Dame à la recherche d'un stage de fin d'études. Difficile alors d'imaginer que je perdrais la raison et me lancerais dans un projet de doctorat…mais bon ce qui est fait...est fait!

Il est impossible de traverser les différentes étapes du doctorat sans l'appui et la confiance de son superviseur. Je commencerais donc par remercier le Dr. Alex Parker pour m'avoir accueilli dans son laboratoire il y a maintenant 6 ans, pour ses conseils avisés, sa disponibilité et sa rigueur scientifique. Il m'a ouvert la porte à un monde enrichissant et stimulant. Je dois aussi le remercier de m'avoir permis de réaliser mon rêve d'athlète de haut niveau, sachant le temps que je passerais loin de mes obligations scientifiques.

On ne fait jamais de recherche seul, l'équipe qui nous entoure est importante. Au cours de mon cheminement, j'ai eu l'occasion de rencontrer et de collaborer avec des personnes d'exception. J'aimerais remercier mes collègues du laboratoire Parker rencontré au fil des ans avec un merci tout particulier à Alexandra Vaccaro, ma première et seule collègue durant 3 ans. Bien plus que l'équipe de choc que nous formions, je me souviens de nos pauses capuccinos avec Anè (les 3A), nos déjeuners au Petit Extra et les moments d'anthologie de nos stagiaires respectifs.

La recherche ne consiste pas seulement à apprendre, mais aussi à transmettre. Durant les 5 dernières années j'ai eu la chance de côtoyer et de participer activement à la formation de stagiaires provenant du CEGEP de St-Jean sur Richelieu et de divers programmes de l'université de Montréal. Un grand merci à chacun pour votre aide, tout particulièrement à Sarah Gosselin, une stagiaire en or.

Lors de mes premiers pas au CHUM, notre espace était partagé avec le laboratoire du Dr. Christine Vande Velde, que je remercie pour ses conseils. Merci à mes collègues du

laboratoire d'à côté, particulièrement au Dr. Anaïs « Anè » Aulas pour les nombreux conseils, le support au quotidien et pour cette belle complicité développée au fil des ans.

Parce que j'ai eu l'occasion de faire de belles rencontres au fil des ans, un grand merci à mes collègues de l'axe de neurosciences, Guillaume, Vi, Alexandra (la blonde), Loïc, Maxime, Catherine-Alexandra et Marc-André pour les nos activités extrascolaires.

On dit souvent de moi que je suis un vrai drogué de sport…c'est vrai! Merci à mes coéquipiers escrimeurs, Jean, Marc-Antoine, Milosz, Yann, Louise, Ariane, Carl, Fred et Guillaume. Merci à Jacques, Iulian et Jean-Marie, mes entraineurs. J'ai passé de belles années à partager souffrances d'entrainement, voyages au travers du pays et de l'Europe pour certains. Merci aux sabreurs, on ne vous comprend pas toujours, mais on vous apprécie quand même un peu. Tous m'avez aidé à me développer comme personne et athlète.

À ma famille, il est très difficile de trouver les mots pour vous dire à quel point vous avez, et êtes encore, les fondations sur lesquelles j'ai bâti ce que je suis. À mes Montréalais favoris, Papa, Maman et Nico, je sais que je ne suis pas toujours facile à vivre, mais vous êtes toujours présent quand j'en ai besoin et ce même quand que je brûle la chandelle par les deux bouts. Il y a 14 ans nous avons mis un océan entre nous et pourtant les liens sont restés forts, malgré les épreuves. Tous et chacun, Mamie, Grand-père Morillon, Manou, Grand-père Montauban (j'imagine que c'est ça), Didier, Martine, Olivier, Xavier, Catherine, Élisa, Pierre-André, Goeffrey et Maxime, vous m'avez témoigné votre soutien et votre affection.

Je pense avoir fait le tour, j'ai probablement oublié certaines personnes, je m'en excuse et leur dis merci. Une nouvelle étape commence et je l'espère aussi riche que celle que je termine.

MERCI À TOUS!

Chapitre I : Introduction

L'étude présentée dans cette thèse s'articule autour de 4 axes. Le premier abordé est le vieillissement, élément clé dans l'étude des maladies neurodégénératives, 2^{e} axe de ce manuscrit, et du métabolisme. Ce dernier est le principal sujet décripté dans ce travail, étant étroitement lié au vieillissement, mais aussi, à certains égards, aux maladies neuronales. Le dernier axe couvert dans cette prémice est le modèle utilisé dans nos différentes études, le nématode *Caernorhabditis Elegans.*

I Vieillissement

Tout organisme vieillit. Bien que l'espérance de vie varie de manière importante entre espèces, il n'en demeure pas moins que tout être qui nait doit inévitablement disparaitre. De plus le vieillissement apparait comme le principal facteur de risque dans le déclenchement de certains problèmes de santé tels que le cancer, les maladies cardiovasculaires et les désordres neurodégénératifs. Durant les 20 dernières années, le travail effectué sur différents organismes modèles a permis d'identifier plusieurs voies de signalisation ayant la capacité de moduler les phénotypes liés au vieillissement (1,2). Il est globalement accepté que les principales voies de signalisation sont : la voie insuline/IGF (IIS), le statut nutritionnel et l'intégrité mitochondriale.

I.1 Voie Insuline/IGF

I.1.1 Identification :

L'étude du phénotype dauer, alternative au développement du nématode dans un environnement hostile, a mené à l'identification de plusieurs gènes impliqués dans la voie de signalisation Insuline/IGF (IIS), régulant la longévité (3-5). Cette voie influence différentes étapes du développement ainsi que la prolifération cellulaire lors des stades larvaire et adulte. La fonction de la voie IIS inclue également le développement des cellules germinales (6,7). Son rôle sur la longévité est tout aussi important, puisqu'une mutation du récepteur insuline/IGF *daf-2* double la durée de vie du nématode (3). Cette dernière se fait via l'activation d'une voie de signalisation impliquant différents gènes tels que *age-1*/PI3K (Phospho-inositol-3 kinase) (8), *akt-1*/AKT et le facteur de transcription *daf-16*/FOXO3A (Forkhead box O) (3,8-10). Cette voie centrale est très bien conservée au cours de l'évolution puisqu'elle est retrouvée chez la Drosophile (11,12), la souris (13,14) et potentiellement chez l'humain où des populations présentant des polymorphismes pour le facteur de transcription FOXO3A (15,16) ont une espérance de vie supérieure à la moyenne.

I.1.2 Voie insuline, longévité et résistance au stress :

La voie IIS joue aussi un rôle important dans la résistance au stress. En effet, une inactivation de cette dernière par mutation du récepteur IGF augmente de manière significative la résistance au stress thermique, oxydatif et osmotique, ainsi qu'une meilleure résistance aux pathogènes et aux UV. Cette protection est, par ailleurs, dépendante des facteurs de transcription *daf-16*, et *hsf-1*/HSF1 (Heat Shock Factor 1) (17-20).

En parallèle, la voie IIS est aussi un modulateur important de la protéotoxicité, caractéristique des maladies neurodégénératives. Cette toxicité est liée à l'accumulation cellulaire de protéines mal conformées, entrainant la dysfonction et la mort de l'organisme. Ainsi, des modèles de la maladie d'Alzheimer (21,22), de la sclérose latérale amyotrophique (SLA) (23,24), de maladies à répétitions polyglutamine telles que la chorée de Huntington (19,25) ou différentes ataxies spinocérébelleuses (26), ont montré une sensibilité au niveau de l'activation de la voie IIS et du facteur de transcription *hsf-1*.

I.1.3 Voie insuline et métabolisme :

La voie IIS est aussi une composante importante de la réponse aux changements métaboliques. Si son rôle dans le métabolisme est encore mal compris, elle semble être impliquée lors de modifications du statut nutritionnel. En effet, les effets liés à un enrichissement en glucose semblent dépendant de la voie IIS (27,28), alors que dans le cas de la restriction calorique, les effets positifs sont indépendants de la voie du récepteur IGF, mais dépendants de HSF-1 (29,30). Enfin, certaines études suggèrent que seulement certains types de restriction calorique voient leurs effets contrôlés par la voie IIS (31).

I.2 Métabolisme et vieillissement :

I.2.1 Restriction calorique

Parmi les modulateurs de la durée de vie et du vieillissement, le seul qui est environnemental demeure la restriction calorique (RC); une réduction de 30 % à 40 % de

l'apport nutritif. Les effets de la RC ont été mis en évidence sur de nombreux modèles, allant de la levure au primate (32-39). Les organismes soumis à la RC montrent une longévité accrue mais subissent, en contrepartie, une diminution de leur fécondité (40,41). Bien que la RC soit étudiée depuis près de 80 ans, les voies génétiques qui sous-tendent le(s) mécanisme(s) sont encore floues, peut-être en partie parce que les méthodes d'application de la RC varient de manière importante entre les différentes espèces.

D'un point de vue génétique, les voies les plus communément impliquées dans le vieillissement sont indépendantes de la RC. Les données obtenues sur *C. elegans* ou chez la Drosophile suggèrent que les effets de la RC ne dépendent que partiellement de la voie IIS (30,42,43). Néanmoins, les souris mutantes pour le récepteur de l'hormone de croissance, qui produit un phénotype similaire à la RC, voient leur longévité augmenter en conditions restrictives (44).

I.2.2 Sirtuines :

La famille d'histones deacétylase de classe III est plus connue sous le nom de sirtuines (SIRT1-7). Elle a été rapidement impliquée dans la RC via Sir2p, l'orthologue de la levure de SIRT1 chez le mammifère. Une mutation de SIR2 chez la levure bloque l'augmentation du niveau de réplication (Replicative lifespan) observé lors de restrictions en glucose (45). Cet effet a été confirmé chez d'autres modèles, où l'extension de durée de vie observée était abolie par une mutation dans *sir-2.1* (*C. elegans*) ou d*Sir2* (Drosophile) (46-48). Il existe toutefois des données qui vont à l'inverse de ces observations. De plus amples investigations seront nécessaires afin de conclure de manière définitive quel rôle est joué par les sirtuines (46,49,50).

I.2.3 AMPK et mTOR :

Une autre voie génétique est communément associée à la RC. Une diminution de la synthèse protéique ou de la prise d'acides aminés peut augmenter la durée de vie dans divers modèles et ce via la voie de la kinase TOR (Target of Rapamycin) dont l'inhibition augmente aussi la longévité (51-58). Cette modulation peut aussi intervenir via un autre censeur, l'AMPK (Adenosine Monophosphate-activated Protein Kinase) (59). Dans des conditions métaboliques déficientes, AMPK active des voies de signalisation permettant la production d'énergie et inhibant des voies anaboliques telles que celle de TOR (60,61). De plus AMPK agit sur plusieurs voies impliquées dans le métabolisme et qui, dans un contexte indépendant de la RC, peuvent aussi moduler la durée de vie (62).

I.2.4 *Pha-4*/FOXA et *skn-1*/NRF2 :

Finalement les effets de la RC sont aussi contrôlés par deux autres facteurs de transcription identifiés chez *C. elegans*, *pha-4* et *skn-1*. Le premier est impliqué dans le développement du pharynx et des cellules intestinales. Il est le seul orthologue du facteur de transcription FOXA (Forkheard box A) (63) chez le mammifère. Une perte de fonction de *pha-4* a pour effet de bloquer l'extension de durée de vie provoquée par un mutant *eat-2*, connu pour mimer la restriction calorique (64). Cette perte de fonction n'a, pour autant, aucun effet sur la longévité du mutant *daf-2*, ce qui implique un rôle spécifique dans la restriction calorique. De plus *pha-4* possède plusieurs cibles transcriptionnelles en commun avec *daf-16*, l'orthologue de FOXO3A. Cet état en fait un candidat idéal pour la transcription de facteurs impliqués dans la survie cellulaire, dans la croissance et le métabolisme (65,66). En ce qui a trait à *skn-1*, il est l'orthologue du vers pour Nrf2 (Nuclear factor erythroid 2–related factor 2),

impliqué dans la transcription d'enzyme de détoxification de phase II, en réponse à un stress oxydatif ou xénobiotique (67,68). *skn-1* est exprimé au niveau des neurones ASI, qui ont un rôle de censeur nutritif dans l'environnement. Lorsque ces derniers sont non-fonctionnels ou que *skn-1* est muté, l'effet de la RC est aboli (41).

En somme, il existe plusieurs voies impliquées dans la modulation du vieillissement par la RC. Chacune de ces voies, prises de manière indépendante, ne peut pas expliquer la totalité des effets observés. Cela suggère une interaction importante des différents facteurs impliqués dans ces dernières **(Figure 1)**.

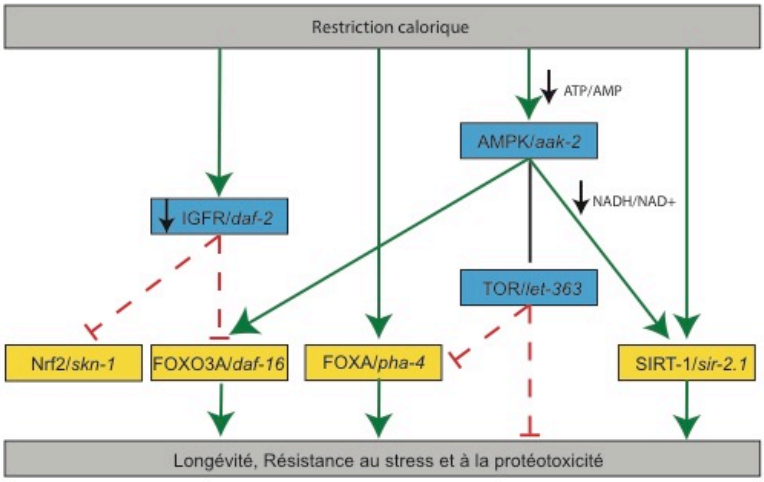

Figure 1: Voies de signalisation liées à la restriction calorique.
Les voies de signalisation (en bleu) et les facteurs de transcriptions qui y sont reliés (en jaune) influencent la réponse à la restriction calorique tout en pouvant intéragir entre elles.

I.3 Mitochondries et vieillissement

La mitochondrie joue un rôle important dans le vieillissement de par sa capacité à fournir l'énergie à la cellule. En fait, la fonction mitochondriale fournit de l'adénosine triphosphate (ATP) à la cellule mais en contrepartie produit aussi des espèces réactives à l'oxygène (ROS) dans des conditions de métabolisme oxydatif. Ces sous-produits ont rapidement été identifiés comme une source de dommages importante au niveau protéique, lipidique et des acides nucléiques (69,70). Ces observations ont mené à la mise en place de la

théorie des Radicaux Libre dans le vieillissement (TRLV) dans les années 50 (71). Cette théorie soutient que l'accumulation de radicaux libres est responsable du vieillissement cellulaire et par ricochet de celui de l'organisme (72,73). Cependant, depuis maintenant plusieurs décennies, cette théorie a été mise à l'épreuve par d'autres observations qui supportent que les ROS ne sont pas la cause, mais plutôt des messagers d'un stress. Ainsi, plusieurs mutants de la chaîne de respiration mitochondriale chez *C. elegans* montrent une hausse de ROS (74-76), mais aussi une longévité accrue par rapport à un animal sauvage (74,77). D'autre part, Les ROS participent à la réponse protective en tant que messagers de différentes voies de signalisation cellulaires (78,79). Il est toutefois admis qu'une accumulation trop importante de ROS apparait dans des organismes vieillissant et que les ROS soient potentiellement nocifs à long terme (80).

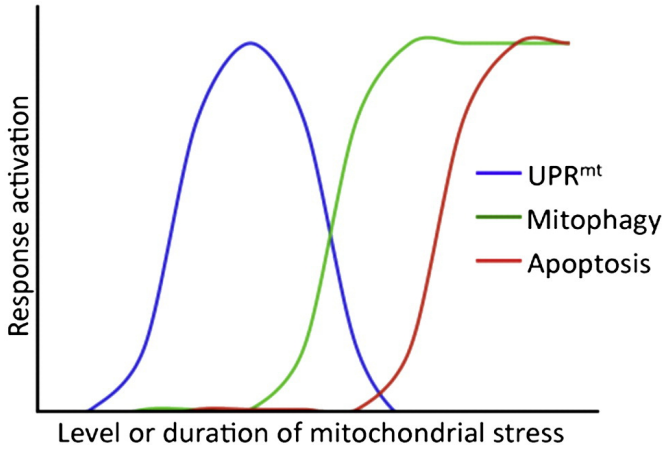

Figure 2: Réponse au stress mitochondrial
Tirée de ***Pellegrino et al., Biochim Biophys Acta, 2013***.
La mitochondrie réagit de manière différente à la durée et au niveau de stress, induisant un réponse progressive qui peut aller de la réponse UPRmt dans des cas légers, à la mitophagie lorsque la cellule peut être sauvée mais pas la mitochondrie et à la mise à mort par apoptose de la cellule.

Il est important de mentionner que plusieurs facteurs, intra ou extracellulaires, peuvent influencer de manière importante la présence de ROS. Ainsi, l'accumulation de mutations d'ADN au niveau somatique et de protéines agrégées peut nuire au bon fonctionnement mitochondrial et ainsi influencer la capacité cellulaire ou celle de l'organisme à répondre de manière adéquate à une exposition au stress.

Dans un contexte de stress, la mitochondrie a plusieurs façons de réagir. Premièrement elle induit une augmentation de l'expression de chaperonnes via la « Unfolded Protein Response » (1,81). Cette réponse est activée lors d'une augmentation de ROS et a pour objectif de réduire la charge nocive. Dans un deuxième temps, les fonctions de fusion et fission mitochondriale sont activées afin de permettre l'élimination d'organelles via un processus connu sous le nom de mitophagie, consistant à l'élimination de mitochondries défectueuses par un processus autophagique (82-84). Enfin, si la cellule ne peut contrer la charge croissante de stress, la voie apoptotique est activée afin de réduire le risque de dommages irréversibles à un tissu ou à l'organisme **(Figure 2)**.

II Troubles neuronaux

Une population vieillissante est sujette à une augmentation des problèmes de santé. Depuis maintenant plusieurs années, le nombre d'individus atteints de troubles neurologiques est en constante augmentation. Parmi ces derniers, les accidents vasculaires cérébraux, mais aussi les maladies neurodégénératives; des pathologies encore mal comprises et pour lesquelles il n'existe pas encore de traitements. Les plus connues sont les maladies d'Alzheimer (MA), de Parkinson (MP), la sclérose latérale amyotrophique (SLA) et la chorée de Huntington (CH). Ces désordres ont en commun la présence d'agrégats insolubles, causés par différentes mutations d'ADN, affectant différentes régions du système nerveux central. Ainsi, les neurones corticaux du lobe temporal et de l'hippocampe dans la MA, les neurones dopaminergiques de la substance noire dans la MP, les neurones moteurs dans la SLA et les neurones du striatum (ganglion de la base) dans la CH vont présenter des agrégats dans leur

cytoplasme. D'un point de vue génétique certains désordres sont liés à la mutation d'un gène (CH), alors que d'autres sont expliquées par plusieurs causes génétiques (MA, SLA). Dans l'étude présentée dans cette thèse, la SLA et la chorée de Huntington ont été modélisées chez *C. elegans*.

II.1 Sclérose Latérale Amyotrophique

II.1.1 Découverte et observations

Les premières observations relatives à la maladie connue sous le nom de sclérose latérale amyotrophique (SLA) ont été effectuées par le Dr Charcot au XIXe siècle. La SLA se caractérise par une dégénérescence des neurones moteurs supérieurs et inférieurs dans le cortex et la moelle épinière, aboutissant la plupart du temps au décès par perte d'innervation des muscles respiratoires (85). Les premiers signes de la pathologie sont une faiblesse musculaire dans les cas de déclenchement épineux (spinal), représentant 70% des cas, et des difficultés à déglutir et à parler dans les cas de déclenchement « bulbaire » (25% des cas). Les dommages causés aux neurones induisent plusieurs dysfonctions parmi lesquelles la dysphagie, la dysarthrie, une atrophie et une faiblesse musculaire.

Le déclenchement de la pathologie se situe entre 47 et 60 ans (86) et affecte 3 individus /100 000 par an. La SLA se décline sous deux formes, soit une forme familiale et une forme sporadique, qui correspondent respectivement à 10 % et 90 % des cas. Toutefois les deux types de SLA sont en tout point similaires au niveau symptomatique (87). Une fois le diagnostic effectué, la progression de la pathologie est souvent très rapide et l'espérance de vie des patients se situe généralement entre 3 et 5 ans (88).

II.1.2 Génétique des cas familiaux

Il est très difficile d'étudier les cas sporadiques principalement à cause de la diversité des causes génétiques qui sous-tendent la pathologie. Ces derniers composent malheureusement la forte majorité des cas référencés. A l'inverse, les 10% de cas familiaux ont permis de mieux comprendre les mécanismes de toxicité qui sous-tendent la SLA. Le premier gène identifié, en 1993, est SOD1 (Superoxide dismutase 1) et les mutations l'affectant regroupent 20% des cas familiaux (89). Près de 170 mutations ont été identifiées sur ce gène, soit plus que la totalité des 154 acides aminés qui composent la protéine.

Dans les dernières années, d'autres gènes liés à la SLA ont été identifiés **(Tableau I)**. Le gène qui sera plus couvert dans cette étude est TARDBP, une protéine liant l'ARN et encodant pour la protéine TDP-43.

Gène	**Proportion des cas familiaux**	**Orthologue *C. elegans***	**Référence**
TARDBP	4%	*tdp-1*	(90,91)
FUS/TLS	5%	*fust-1*	(92)
SOD1	20%	*sod-1*	(89)
C9ORF72	35-50%	*alfa-1*	(93)

Tableau I : Gènes dont la prévalence est la plus importante dans les cas familiaux de SLA, ainsi que leur orthologue chez *C. elegans*.

II.1.3 TARDBP

II.1.3.1 Découvert et mis en évidence dans la SLA

Le gène TARDBP (Transactive response DNA Binding Protein) a été identifié en 1995 lors d'une étude visant à identifier des répresseurs transcriptionnels du VIH (90). Ce n'est qu'en 2006 que TARDBP a été identifié dans les agrégats ubiquitinés de patients SLA et présentant une démence frontro-temporale (DFT) (91) et seulement en 2008 que les premières mutations de TDP-43 ont été identifiées chez des patients (94). Suite à cette découverte plusieurs travaux ont mis en évidence le rôle de la protéine TDP-43 dans le développement (95) et le maintien de la fonction du système nerveux (96,97).

II.1.3.2 Structure de TDP-43

TDP-43 est une protéine majoritairement nucléaire, pouvant se déplacer dans le cytosol au besoin et dont la structure récapitule bien son rôle connu de liaison à l'ARN. Elle possède 2 motifs de liaison à l'ARN, RRM (RNA recognition motif) 1 et 2, contient des signaux de localisation (NLS) et d'exportation (NES) nucléaire et une portion C-terminale riche en glycine, qui permet l'interaction inter-protéines (98,99). Cette région présente la majorité des mutations affectant TDP-43. Elle joue un rôle important, tant pour la tendance de TDP-43 à s'agréger que pour sa capacité d'autorégulation (100). La structure de TDP-43 est très conservée au cours de l'évolution, facilitant le développement de modèles animaux, visant la compréhension de sa fonction, de sa toxicité et des moyens potentiels d'interférer avec la toxicité résultante des mutations référencées **(Figure 3)**.

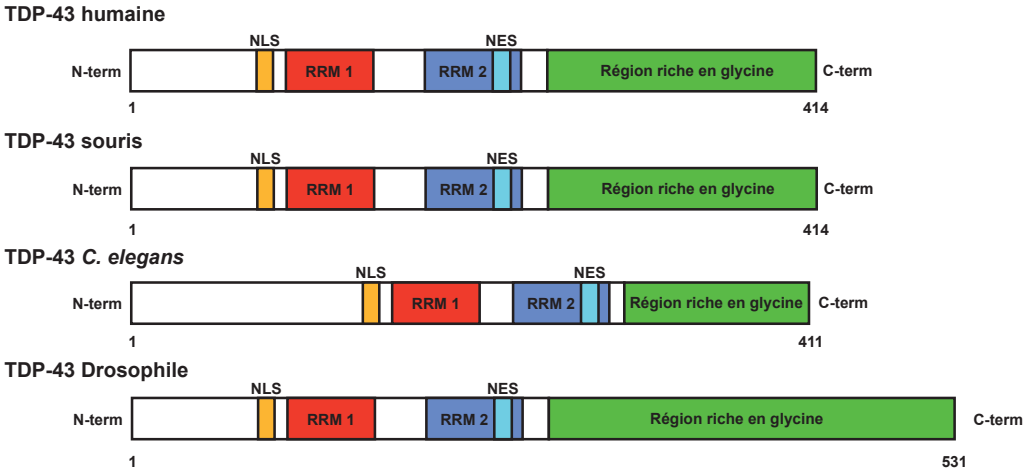

Figure 3: Schéma représentant les différents homologues de TDP-43.
La sequence de *TARDBP*/TDP-43 est très conservée entre l'humain (hTDP-43), la souris (mTDP-43), la Drosophile (dTDP-43) et *C. elegans* (ceTDP-43). On retrouve les motifs de reconnaissance à l'ARN (RRM 1 et 2), les signaux de localisation et d'export nucléaires (NLS et NES) ainsi que la région riche en glycine, ressenssant la plupart des mutations liées à la SLA.

II.1.3.3 Métabolisme de l'ARN

TDP-43 a été identifiée comme un répresseur de la transcription du VIH (90), mais son rôle dans la régulation de la transcription ne se limite pas à ce facteur. Il a en effet été démontré que TDP-43 peut se lier à l'ADN, avec une affinité particulière pour les répétitions TG, qu'il soit simple ou double brin et influencer l'expression, l'épissage et la stabilité des transcrits de différents gènes (101).

Plusieurs travaux ont mis en évidence que TDP-43 interagit avec près de 6 000 ARNm (102-104), avec une affinité particulière lorsque ces derniers possèdent de longues séquences introniques ou lorsque la région 3'-UTR de certains ARN est riche en répétitions UG. Le domaine RRM1 possède une affinité importante pour ces répétitions UG et cette dernière est proportionnelle au nombre de répétitions (105).

II.1.3.3.1 Épissage par TDP-43

L'épissage de l'ARN est nécessaire au bon fonctionnement cellulaire et permet une diversité de transcrit importante, permettant une adaptation importante à l'environnement (revu en 106). La capacité de TDP-43 à lier de longs introns et à participer à l'épissage est important compte tenu du fait que la plupart des gènes possédant de longues séquences introniques sont en nombre important dans le système nerveux (102,107). Parmi les ARN cibles se trouvent CFTR (cystic fibrosis transmembrane regulator) dont l'exon 9 est excisé, menant à une protéine non fonctionnelle, aboutissant à la dysfonction de plusieurs organes, incluant les poumons, les intestins et le pancréas (108). Inversement, TDP-43 peut aussi permettre l'inclusion d'un exon, comme dans le cas de l'ARNm de SMN (Survival motor neuron). Ce dernier possède deux isoformes (SMN1 et SMN2) et il a été montré que TDP-43 favorise l'inclusion de l'exon 7 dans SMN2 (109). SMN est la protéine responsable de la SMA (Spinal muscular atrophy). Elle est aussi importante dans la formation de complexes GEM (Gemini of cajal bodies) permettant l'épissage de l'ARNm (110).

II.1.3.3.2 Stabilité, Transport par TDP-43

TDP-43 possède aussi la capacité de se lier à la région 3'-UTR de certains ARNm (111,112). Par ailleurs, il est maintenant établi que TDP-43 peut réguler sa propre expression, puisqu'une augmentation de son expression induit une réduction de sa transcription, via une interaction avec une région en 3'-UTR de son propre ARNm (100,113). TDP-43 influence aussi la stabilité de plusieurs ARN, incluant celui du neurofilament hNFL (100,114,115) ainsi que leur transport et traduction (116,117). De plus, des études ont démontré que TDP-43 est localisé au niveau des synapses et que les mutations M337V, A315T et Q343R, liées à la SLA,

induisent une perte de mobilité des granules d'ARN, induisant une accumulation au niveau cytoplasmique. En plus des éléments mentionnés précédemment, TDP-43 colocalise avec la protéine Stauffen, liant l'ARN et impliquée dans le transport axonal (118,119). Ceci est en accord avec d'autres travaux montrant qu'une diminution de l'expression de TDP-43 mène à une malformation de plusieurs structures, incluant les synapses (120,121).

II.1.3.3.3 TDP-43 et ARN non codants

Des travaux ont montré que TDP-43 ne se lie pas uniquement à l'ARNm, mais aussi à différents types d'ARN non codants (ARNnc). Ces derniers sont impliqués dans différents processus cellulaires, incluant le remodelage de la chromatine et la régulation de la transcription (122-125). TDP-43 peut lier des ARNnc longs (> 200 nt), tels que NEAT1 (Nuclear-enriched autosomal transcript 1) et MALAT1 (Metastasis-associated lung adenocarcinoma transcript 1), dont l'expression est augmentée dans des cas de FTD-TDP-43 (103). TDP-43 peut aussi interagir avec d'autres ARNnc lors d'un stress afin d'en limiter l'expression tels que Cdk6 (126). Une autre catégorie d'ARNnc est influencée par TDP-43; les micros ARN (miARN). Leur rôle dans le développement du système nerveux et des maladies neurodégénératives est maintenant bien établi (127-129). TDP-43 influence le métabolisme des miARNs en interagissant avec Drosha et Dicer, impliqués dans la maturation des miARN, en favorisant la production des ARNnc (130,131).

II.1.3.4 Mutations de TDP-43 et Dysfonction cellulaire

La mise en évidence de TDP-43 dans la SLA et FTD a eu lieu en 2006, lorsque deux équipes ont montré la présence d'inclusions ubiquitinées de TDP-43 (91,132). TDP-43 est présente dans les inclusions chez 97 % des patients atteints de SLA (133). Néanmoins, le(s) mécanisme(s) de toxicité de TDP-43 reste(nt) encore flou(s). Actuellement il reste encore à déterminer si la toxicité de TDP-43 est due à une perte ou un gain de fonction de la protéine, ou encore à un complexe mélange des deux.

II.1.3.4.1 Perte de fonction

Cette hypothèse se base sur le fait que la protéine TDP-43, normalement nucléaire, a une localisation cytoplasmique dans un contexte pathologique de SLA (91). Ce concept implique que la perte de localisation nucléaire influence la fonction de TDP-43. Ceci est soutenu par des études dans lesquelles une perte de TDP-43 nucléaire influence le métabolisme de l'ARN de certains gènes dont l'expression est enrichie dans le système nerveux et qui sont liés à la SLA ou FTD. Cette liste comprend FUS/TLS, EAAT2, VABP, ALSIN, VCP, FIG4, CHMP2B (102,103). Une perte du complexe d'épissage (spliceosome) a aussi été observée dans des neurones moteurs et dans des tissus de cas sporadiques de SLA (134). Aussi l'expression de la protéine humaine TDP-43 mutante (mTDP-43), pour les mutations Q331K et M337V, induit une dérégulation de l'épissage dans un modèle de souris ayant des dysfonctions motrices, sans agrégation de mTDP-43 (135).

Un modèle de nématode a montré que la mutation du NLS de TDP-43, induisant une localisation cytoplasmique ne cause pas de toxicité (136), alors que la perte de *tdp-1* (l'orthologue de TDP-43) augmente la sensibilité du ver au stress (23). Chez la souris, la perte

totale de TDP-43, que ce soit au niveau du développement embryonnaire (95,137) ou post-natal (138), induit des dysfonctions majeures menant à la mort, alors que des graves déficits moteurs et morphologiques sont induits par une perte de TDP-43 chez la Drosophile (120,139,140).

II.1.3.4.2 Gain de fonction

TDP-43 a une tendance « naturelle » à s'agréger *in vitro* (141) et cela est d'un intérêt certain puisque plusieurs modèles animaux et cellulaires montrent la présence d'agrégats positifs pour TDP-43 (142-144). Cette capacité à s'agréger est aussi retrouvé dans plusieurs pathologies, où la présence de TDP-43 est confirmée dans les agrégats spécifiques à ces pathologies (revue en 145). Parce que la présence d'agrégats a été mis en évidence dans des cerveaux de patients et dans différents modèles et que dans beaucoup de cas une forme tronquée de la protéine est présente (91,132), cela a mené à l'hypothèse que ces inclusions ont une toxicité intrinsèque indépendante de la fonction de TDP-43.

II.1.3.4.3 Un rôle pour la forme tronquée de TDP-43?

La forme tronquée de TDP-43 se décline sous différentes tailles de 35 kDa et de 25 kDa, dont l'agrégation est toxique dans des lignées cellulaires HEK293 et M17, exprimant différentes constructions de TDP-43 (146). Ces agrégats sont cytoplasmiques, potentiellement obtenus après activation de la caspase-3 (147,148) et sujets à plusieurs modifications post-traductionnelles. En effet, TDP-43, pleine longueur ou tronquée, est retrouvée ubiquitinée et phosphorylée dans les agrégats. L'ubiquitination est impliquée dans la dégradation protéique et

une inhibition du protéasome induit une hausse de l'agrégation cytoplasmique de TDP-43. De plus, la présence de certains gènes impliqués dans la voie de dégradation et mutés dans la SLA, confirme cet aspect (102). La phosphorylation de TDP-43 a été démontré dans différentes lignées cellulaires neuroblastome M17 exprimant TDP-43 et semble contribuer à l'agrégation de la protéine (149). Chez *C. elegans*, une mutation des sérines 409/410 en alanine (phospho inactives) réduit la toxicité de TDP-43 exprimée dans un modèle pan-neuronal (promoteur snb-1) (150).

II.1.3.4.4 Une question de localisation cellulaire?

La présence d'agrégats cytoplasmiques, bien que confirmée dans plusieurs modèles et dans des tissus humains, n'est peut-être pas la cause de toxicité de TDP-43. En effet, la présence de fragments 35 kDa solubles chez la souris (142) et le fait que toutes les souris ne présentent pas de fragments malgré la présence de phénotypes similaires (143) suggèrent que la forme tronquée de TDP-43 n'est peut-être pas la cause primaire de toxicité. La localisation cytoplasmique serait responsable de la toxicité de TDP-43. Ce concept est soutenu par certains modèles de Drosophile (140,151).

Des travaux ont mis en évidence que la perte du NLS, induit une localisation cytoplasmique et une agrégation de TDP-43 (143,152). Ce phénomène est aussi présent lorsque la cellule subit un stress (153,154) ou un dommage cellulaire (139), cohérent avec le rôle connu de TDP-43 dans la formation des granules de stress (155). De plus, la toxicité de TDP-43 semble dépendante du niveau cytoplasmique dans des neurones primaires de rats (97) et son expression toxique chez la Drosophile lorsque son NLS est muté (156).

II.1.3.4.5 L'autorégulation en cause?

Le niveau d'expression de TDP-43 est finement régulé dans la cellule. En effet une augmentation ou une baisse de son expression sont toutes deux toxiques. Une des capacités de TDP-43 est de lier et réguler certains ARN, incluant le sien (100,114,115). Ainsi, lorsque TDP-43 est surexprimée, elle réduit la traduction de son propre ARNm (136,142).

Par contre dans une situation pathologique, TDP-43 est relocalisée au niveau cytoplasmique, laissant le noyau avec peu ou pas de moyen de contrôler son expression. De ce fait, il est possible que cette perte d'autorégulation induise une expression accrue de TDP-43 (157), qui va finir par sortir à son tour du noyau et induire un cercle vicieux menant à la mort de la cellule.

II.2 Chorée de Huntington

II.2.1 Découverte et observations

Les premières observations liées à la chorée de Huntington remontent au XIVe siècle, où des cas de « manie dansante » ont été répertoriés. Le terme « chorée », correspondant à un phénotype de mouvements involontaires similaires à une danse, ne fera son apparition qu'un siècle plus tard. C'est en 1872 que George Huntington décrit la pathologie qui porte maintenant son nom.

La chorée de Huntington se traduit de manière symptomatique par un changement de personnalité, une perte de fonctions cognitives, incluant des pertes de mémoire et une difficulté à entreprendre une action, dans des stades précoces. Plus tard apparaissent des dysfonctions motrices et de la démence (158). Le diagnostic est souvent établi entre 35 et 50

ans et les patients décèdent généralement de problèmes secondaires telles qu'une infection des voies respiratoires ou une pneumonie. La classification des stades de développement de la pathologie, établie par Jean-Paul Vonsattel en 1985, se fait au travers de 5 stades (0-4) qui indiquent des stades croissants de dysfonction et de mort cellulaire au niveau du striatum, mais aussi dans d'autres régions telles que le cortex, le thalamus et la substance noire.

Anatomiquement, la chorée de Huntington se manifeste par une dégénérescence massive au niveau des ganglions de la base, principalement au niveau du striatum, et dans le cortex (159). Plus précisément, la principale population neuronale affectée est celle des neurones épineux moyens striataux, cellules composant 95 % du striatum et dont la fonction est surtout inhibitrice via relâchement de GABA.

II.2.2 IT15 et Huntingtine

Le lien génétique de cette pathologie est resté inconnu jusqu'à ce qu'un consortium de scientifiques identifie, il y 20 ans, une augmentation de répétitions Cytosine-Adénosine-Guanine (CAG) encodant pour l'acide aminé glutamine (Q) dans l'exon 1 du gène IT15, qui sera renommé Huntingtine (HTT) par la suite. Lorsque cette répétition CAG est inférieure à 35 acides aminés, les porteurs sont sains alors qu'une longueur de 36-40 répétitions engendrera une pénétrance partielle des symptômes. Enfin, un nombre de répétitions supérieur à 40 résidus entraine une pénétrance complète du phénotype.

La transmission génétique autosomique dominante engendre aussi un phénomène d'anticipation, qui implique une augmentation potentielle de la longueur de répétitions lorsque celle-ci est transmise par le père (160). Cela implique que des individus masculins sains avec

un allèle intermédiaire peuvent avoir une progéniture qui possèdera une longueur pathologique d'huntingtine.

La chorée de Huntington fait partie d'une famille de 9 désordres liés à des extensions de répétitions polyglutamine (polyQ), dont font partie les ataxies spinocérébelleuse de type (1, 2, 3, 6, 7 et 17), ainsi que la SMBA (Spinal and bulbar muscular atrophy) et la DRPLA (Dentatorubral-pallidoluysian atrophy) (161).

II.2.3 Fonctions de Huntingtine

Bien que la protéine soit connue depuis bientôt deux décennies, sa fonction précise reste encore floue. Néanmoins, puisque les différents désordres polyQ affectent des populations neuronales distinctes, il est fort probable que la fonction normale de la protéine en cause ait un rôle important dans la progression de la maladie.

II.2.3.1 HTT et Survie cellulaire

La mort cellulaire rapportée dans un contexte de CH est induite par une hausse des niveaux d'apoptose puisque certains modèles cellulaires, dont l'expression de HTT est réduite, ont une activité caspase-3 plus importante (162) et que la surexpression de HTT protège contre des stimuli toxiques en inhibant l'activation de la pro-caspase-9 notamment (163,164). Ces résultats ont aussi été rapportés dans des modèles de souris hétérozygotes pour *hdh* (homologue de htt chez la souris) (165) et dans un modèle de HD-YAC (Yeast Artificial Chromosome) (166).

Le rôle de HTT dans la survie cellulaire ne se limite pas à un rôle anti-apoptotique,

puisque cette dernière, en association avec le complexe REST/NRSF (167,168), module l'expression du facteur de croissance BDNF (Brain Derived Neutrophic Factor). Le BDNF est synthétisé dans le cortex, mais sa fonction dans la survie cellulaire est importante au niveau du striatum (revue en 169). Lorsque HTT est manquante ou mutée, REST/NRSF pénètre dans le noyau et se lie à RE1/NRSE, entrainant la répression de plusieurs gènes, dont le BDNF (168).

II.2.3.2 HTT et transport vésiculaire

La protéine huntingtine est impliquée dans le transport vésiculaire axonal, en interagissant avec ses partenaires HAP1, HAP40 (Huntington associated protein 1 - 40) et la dynéine (170). Une baisse de l'expression de HTT induit une dysfonction du transport axonal chez la Drosophile (171). Huntingtine est aussi associée au transport mitochondrial dans les neurones striataux (172), possiblement en régulant le complexe moteur sur les microtubules.

II.2.3.3 HTT et fonction synaptique

Le rôle de HTT dans la fonction synaptique a été mis en évidence dans plusieurs modèles (173-175), et chez les mammifères la liaison de HTT à la protéine PSD95 (Post-synaptic density 95) semble jouer un rôle dans la pathogénèse. Cette liaison est déficiente lors de l'expansion polyglutamine entrainant une hausse d'activité de PSD95. Cette augmentation engendre une hyperactivation du récepteur glutamate NMDA *(N-methyl-D-aspartate)* et participe ainsi au phénomène d'excitotoxicité sur lequel nous reviendrons dans une autre section (173).

II.2.4 Modifications post-traductionnelles

Diverses modifications post-traductionnelles peuvent affecter la protéine HTT. Certains sites sont importants pour le clivage de HTT et semblent protecteurs lorsque l'expansion polyQ est présente. Les principaux sites de phosphorylation incluent les sérines 13 & 16, 421 et 434. L'activité de Cdk5 (cyclin-dependent kinase 5), est diminuée chez la souris mutante de mHTT (176), alors que la phosphorylation induite par AKT réduit la toxicité de mHTT dans un modèle cellulaire. Enfin la phosphorylation peut aussi affecter la dégradation de HTT par le protéasome suite à une expansion polyglutamine (177).

Une des principales caractéristiques cellulaires de la CH est la présence d'agrégats ubiquitinés. Le processus d'ubiquitination est impliqué dans plusieurs mécanismes cellulaires, l'un d'entre eux étant la dégradation de protéines âgées ou mal conformées (178). La présence de l'ubiquitine dans les agrégats suggère une dysfonction de la voie du protéasome, ce dernier influençant la toxicité de mHTT (179-181). De plus, mHTT interagit avec différentes composantes de la voie du protéasome tel que HRD1 et Cullin3, influençant la dégradation et l'agrégation de la protéine mutante (182,183), mais aussi en inhibant le protéasome directement (184,185).

Le dernier type de modification majeure est la SUMOylation, qui à l'inverse de l'ubiquitination, a pour rôle de limiter la dégradation d'une protéine via le protéasome en liant les résidus lysines. Dans le cas de mHTT, la SUMOylation permet de réguler la localisation, l'activité, et de réduire le niveau d'agrégation de cette dernière (186). Il en résulte une augmentation de la toxicité de mHTT, ce phénomène étant aussi rapporté dans un autre modèle de maladie polyQ (187).

II.2.5 Huntingtine mutante et dysfonctionnement cellulaire

II.2.5.1 Altération transcriptionnelle

Plusieurs travaux ont démontré que mHTT peut se lier directement à l'ADN et réprimer l'expression de certains facteurs de transcription (188) alors que d'autres ont montré que la fraction soluble de mHTT peut interférer avec d'autres facteurs de transcription clés, tels que CBP (CREB Binding Protein) et p53 (189).

La protéine mHTT entraine un dysfonctionnement transcriptionnel, qui se traduit par une diminution d'expression de certains neuropeptides et neurotransmetteurs, dès les stades précoces de la maladie (190-192). En 2000, une étude regroupant un nombre important de scientifiques, a identifié une quantité importante de gènes dont les niveaux d'ARNm sont modifiés lorsque HTT est mutée. Ces ARNm sont impliqués dans des processus tels que la transmission synaptique, l'intégrité du cytosquelette, les protéines structurales et l'homéostasie calcique (193-196). Des études subséquentes ont aussi montré des anomalies transcriptionnelles dans différents modèles murins exprimant la protéine mHTT (197), qui par ailleurs ont aussi été identifiées dans le tissu musculaire (194).

II.2.5.2 Clivage, Agrégation et conformation

Une des caractéristiques les plus étudiées dans la chorée de Huntington est le clivage de la partie N-terminale de la protéine HTT par différentes protéases résultant en un fragment toxique (198). Ce clivage est provoqué par certaines caspases, principalement la caspase 6 et la caspase 3, mais aussi par les calpaines, protéases dépendantes du calcium (199-201). Cette modification est présente à la fois dans des situations *in vitro*, mais aussi dans des modèles de

souris et dans des tissus post-mortem de patient(s). La caspase 6 est celle qui produit le fragment le plus toxique dans le modèle murin YAC128 (202).

La formation de fragments de plus petite taille mène à la genèse d'agrégats insolubles nucléaires (203). Cette présence d'agrégats constitue un marqueur commun à la plupart des pathologies neurodégénératives, bien que leur composition puisse varier. Dans le cas de la chorée de Huntington, ces agrégats sont retrouvés dans les neurones épineux moyens (NEM) mais aussi dans des populations neuronales du cervelet. Une des caractéristiques majeures de ces agrégats est la présence d'ubiquitine liée à mHTT, cette présence exprimant la volonté de la cellule de se débarrasser de ces agrégats via la voie du protéasome mais dans l'incapacité de le faire en raison de la charge toxique accumulée trop importante pour ce système (204).

L'expansion de résidus polyglutamine dans mHTT conduit à la formation d'agrégats nucléaires. Ces derniers sont impliqués dans la pathogénèse, en autre en séquestrant plusieurs protéines impliquées dans le transport axonal, incluant la kinésine et la dynéine. Cette séquestration nuit au transport de vésicules permettant le bon fonctionnement des synapses (171,174,205). Par contre, une quantité significative de données suggère que les agrégats ne seraient pas toxiques, mais au contraire, protecteurs (206). Cette hypothèse a été avancée après observation que l'agrégation et la mort cellulaire ne sont pas liées (207,208).

II.2.5.3 BDNF

Les neurones les plus affectés dans la chorée de Huntington, les NEM, sont dépendants de l'apport de BDNF par le cortex. Chez les patients, le niveau de BDNF cortical ou striatal, est fortement diminué. Les mêmes réductions d'expression ont été observées dans un modèle murin YAC72 (209) mais aussi dans du tissu post-mortem de patients (210). La perte

d'expression de BDNF induit une réduction de taille des NEMs chez la souris (211) et aggrave le phénotype du modèle R6/2 (212). Cette diminution des niveaux de BDNF peut être le résultat d'une perte de fonction de la protéine mHTT qui réprime le complexe REST/NRSF *(Voir section Htt et survie cellulaire II.2.3.1)*, ou d'une dérégulation de la transcription de BDNF en altérant la fonction de CREB (cAMP Responsive Element Binding), de CBP et d'autres facteurs de transcription liés à BDNF (revue en 213).

L'Huntingtine est impliquée dans le transport axonal et des données suggèrent que mHTT affecte le transport vésiculaire, incluant celui de BDNF et empêchant la transmission de facteur de croissance nécessaire à la survie neuronale (214). Il est cependant important de mentionner que ces données sur le transport de BDNF ne font pas l'unanimité puisqu'une autre étude sur un modèle *knock-in* hdh-CAG150 n'a montré aucune altération du transport au niveau cortical, principale région de production de BDNF (215).

II.2.5.4 Dysfonctionnement mitochondrial

La protéine mHTT peut se lier de manière directe aux mitochondries et affecter leur fonctionnement (216). Le dysfonctionnement mitochondrial observé se manifeste par une altération du métabolisme cellulaire *(Voir section métabolisme chorée de Huntington III.10.3.3)*. L'interaction entre mHTT et la mitochondrie entraine aussi une perte de potentiel membranaire et une sensibilité accrue au calcium (216), qui à forte dose peut entrainer l'apoptose.

La protéine mutée est aussi impliquée dans la dérégulation de la transcription de façon générale dans la cellule. La mitochondrie n'échappe pas à ce phénomène. En effet, mHTT augmente les niveaux de p53 et, par le fait même, de protéines pro-apoptotiques BAX (Bcl-2

associated X protein) (217), tout en réduisant l'expression de PGC-1α (proliferator-activated receptor gamma coactivator-1) impliquée dans la biogénèse de facteurs mitochondriaux et de protéines impliquées dans le métabolisme, telles que FOXO1A et GLUT4 (218-222).

Les défauts répertoriés dans les sections relatives au rôle de BDNF et à la dysfonction mitochondriale dans la CH suggèrent que le métabolisme neuronal est un acteur important dans la pathogénèse. L'étude du métabolisme énergétique peut donc constituer un sujet concret dans la compréhension, et possiblement, dans la réduction de pathologies telles que la CH, mais aussi la SLA et la MA.

II.6 Les maladies neurodégénératives et l'épigénétique

Les modifications épigénétiques ont un rôle important dans le développement de l'organisme et bien sûr dans celui du système nerveux (223,224). Le bon fonctionnement des DNMTs (DNA Methyl Transferase) (225) est requis pour le maintien de la plasticité synaptique et dans la consolidation de la mémoire, avec l'aide de l'acétylation de l'histone 3. Ce type de modification, sur un gène comme BDNF, influence certains comportements, comme la réaction aux chocs électriques (temps d'immobilisation après le choc) (226-228).

Il existe maintenant plusieurs liens entre le vieillissement et la méthylation de l'ADN (229-232). Il est aussi maintenant bien établi que différents troubles dégénératifs comme la SLA et la CH sont liés à des changements de méthylation d'ADN pour différents gènes impliqués, directement ou non, dans la pathologie.

La pathologie la plus décrite quant à ce phénomène est la maladie d'Alzheimer. Les niveaux de méthylation d'ADN sont globalement réduits dans la maladie (233,234) en accord avec la perte de méthylation liée au vieillissement (235). Cependant, il existe des données

suggérant le contraire (234,236). Cette variation est peut-être explicable par le stade de progression de la maladie ou l'âge des sujets. Au niveau cellulaire, ces variations épigénétiques se traduisent, par contre, par une hyperméthylation d'enzymes telle que neprilysin, impliquée dans la dégradation du peptide Aβ (233,237). De plus, une diminution des facteurs requis pour le processus de méthylation, induit une hausse des enzymes BACE et PS1 (Présénilin 1) (238). Cette perte de cofacteurs, comme la vitamine B, est aussi rapportée comme responsable de la hausse de phosphorylation de Tau via GSK3β dans une lignée cellulaire de neuroblastome SK-N-BE (239).

II.6.1 Épigénétique et Sclérose Latérale Amyotrophique

L'étude des gènes SOD1 et VEGF indique une hypométhylation de leur promoteur respectif (240). Cependant dans un modèle murin BAC-GLT1 couplé à GFP (241), une hyperméthylation du promoteur de GLT1, principal transporteur de glutamate des astrocytes, induit une chute d'expression de ce dernier. Néanmoins ce phénomène n'est peut-être pas conservé chez des patients (242). Les DNMT1 et DNMT3a sont liées à une mort neuronale accélérée dans un modèle neuronal NSC34 et sont surexprimées dans des tissus de patients atteints de formes sporadiques de la SLA, suggérant un rôle possible de la méthylation d'ADN dans la SLA (243). Des études plus récentes effectuées sur des tissus ou des échantillons sanguins de patients porteurs de mutations génétiques dans le gène C9*orf*72 montrent une réduction de son expression. Cette réduction résulte d'une augmentation des niveaux de méthylation de l'histone 3 sur différents résidus lysine : H3K9me3, H3K27me3 et H3K79me3, toutes connues pour leur rôle de répression de transcription (244,245).

II.6.2 Épigénétique et Chorée de Huntington

Les premières observations d'un changement de méthylation dans un modèle de la chorée de Huntington datent de 1988 (246). Les informations relatives aux changements épigénétiques sont moins bien établies que dans la maladie d'Alzheimer. Des changements de méthylation d'ADN ont été mis en évidence chez des patients et dans des modèles murins de la maladie de Huntington (247-249), affectant surtout des gènes impliqués dans la neurogenèse. De plus il a été suggéré que des modifications de méthylation du gène du facteur de croissance BDNF (Brain derived Neutrophic Factor) soient impliquées dans la pathologie (250).

En parallèle, il existe plusieurs rapports qui font état de modifications post-traductionnelles au niveau de l'histone 3. La protéine mHTT a la capacité de séquestrer CBP qui joue un rôle HAT (Histone Acetyl Transferase) (251). Cette séquestration entraine l'hypoacétylation de l'histone 3, ainsi que sa triméthylation sur la lysine 9 (H3K9me3) via l'augmentation d'expression de la méthyltransférase SETDB1 (252). Ces deux phénomènes conduisent à l'altération transcriptionnelle dans la CH (252-255). La perte de méthylation H3K4me3 est liée à une diminution de l'expression de BDNF dans un modèle R6/2 (256).

Finalement, plusieurs ARNnc voient leur expression chuter dans la pathologie de la CH (129,257). Il est aussi plausible de croire qu'une modification d'expression de certains micro ARN (miARN) entraine des changements d'expression importants, comme par exemple les miARN-125b et miARN-15, qui sont responsables de réprimer p53, impliqué dans la pathologie de la CH (217).

III Métabolisme général et neuronal

La survie d'un organisme, peu importe la complexité de ce dernier, est dépendante de nombreux facteurs et processus qui régissent le bon fonctionnement des différents organes et organelles qui le composent. Parmi ces processus, le métabolisme joue un rôle clé dans le maintien de différentes fonctions, en traitant différentes composantes de l'alimentation, permettant un apport énergétique ou structurel essentiel à la cellule.

III.1 Métabolisme général du glucose

Dans le métabolisme énergétique, la molécule principale demeure le glucose. Ce composé à six carbones est la source principale d'énergie de l'organisme. Il est même la seule source d'énergie dans le cas du système nerveux central (SNC). Néanmoins, le glucose en lui-même ne donne pas accès à toutes les voies métaboliques connues. Ce rôle est joué par le glucose-6-phosphate. En effet, l'hexokinase qui régit la première étape de la glycolyse, phosphoryle le glucose sur le carbone 6, afin d'obtenir le glucose-6-phosphate. C'est à partir de ce dernier que la plupart des voies métaboliques telles que la glycogenèse, la voie des pentoses et le métabolisme oxydatif (qui suit la glycolyse) démarrent (258) **(Figure 4)**.

III.2 Métabolisme oxydatif:

Les différentes voies liées au glucose ont toutes un rôle important, tant au niveau de la production et du stockage d'énergie que de la synthèse de nouveaux acides nucléiques. La voie la plus connue est celle du métabolisme oxydatif. Ce dernier fait suite à la glycolyse et démarre avec l'utilisation du pyruvate, via sa déshydrogénation, qui entre dans le cycle de l'acide citrique (auparavant appelé cycle de Krebs) et se condense avec l'oxaloacétate pour

permettre la formation du citrate. Une fois dans le cycle, une série de réactions enzymatiques modifie le composé qui y entre, en favorisant la production de facteurs tels que le NADH et le $FADH_2$ qui, une fois produits, sont dirigés vers la mitochondrie où ils sont oxydés sous forme de NAD^+ et $FADH^+$ par la chaine de transport des électrons. Le gradient membranaire mitochondrial permet la production d'adénosine triphosphate (ATP), composé énergétique par excellence. En tout et pour tout, le métabolisme oxydatif a pour résultat de fournir 32 ATP par molécule de glucose (258).

III.3 Glycogénèse:

En parallèle, le glucose peut aussi être stocké, dans l'optique d'une utilisation subséquente en cas de manque important dans la circulation sanguine (hypoglycémie). Pour ce faire, le glucose-6-phosphate est transformé en glucose-1-phosphate via la phosphoglucomutase, et puis transformé en UDP-glucose. L'UDP-glucose est la sous-unité de base qui sert à construire un polymère appelé glycogène, une molécule énergétique qui est stockée principalement dans le foie et les muscles pour le système périphérique, et dans les astrocytes pour le système nerveux. Le niveau de glycogène est très faible dans le système nerveux (3-10 μmol/g) comparativement au foie (200-400 μmol/g) (259) et aux muscles (80 μmol/g) (260). Cependant le niveau de glycogène dans le cerveau est important lors de périodes d'hypoglycémie majeures; durant lesquelles a été observé un phénomène de surcompensation qui entraine une surdose de formation de glycogène lorsque le niveau de glucose sanguin revient à la normale (261). L'équilibre entre la formation et la dégradation du glycogène dépend du niveau de glucose sanguin et dépend de certains neurotransmetteurs,

principalement le glutamate et du niveau sanguin d'hormones telles que l'insuline et le glucagon (262-265).

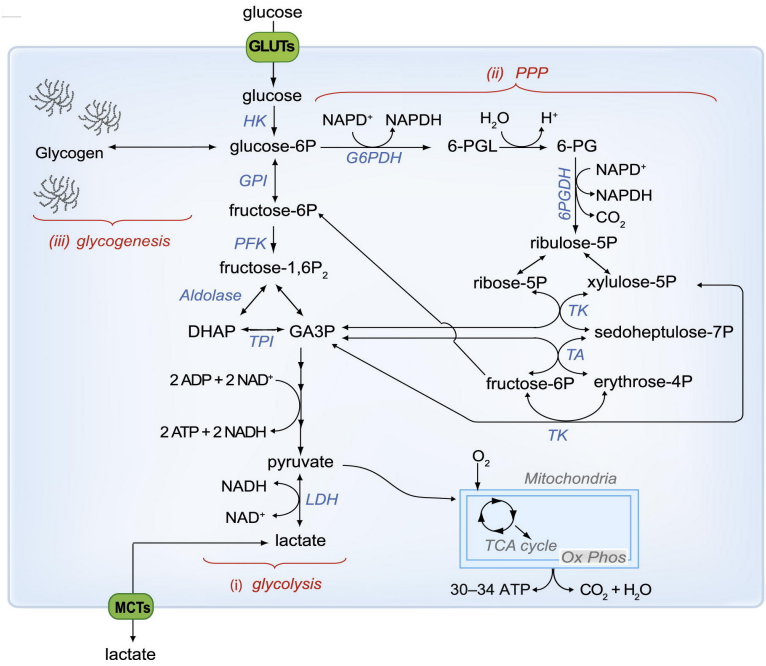

Figure 4: Voies classiques du métabolisme Adaptée de ***Bélanger et al., Cell Metab., 2011***
Représentation des voies majeures du métabolisme énergétique:

i) La glycolyse
ii) La glycogénèse
iii) La voie des pentoses

III.4 Voie des pentoses phosphate

Enfin, le glucose peut aussi servir à la synthèse des acides nucléiques via la voie des pentoses. Ce processus mène aussi à la formation de trois pentoses : ribose, ribulose et xylulose-5-phosphate, toujours à partir de glucose-6-phosphate. La voie des pentoses se fait en deux phases : la première est oxydative et mène à la formation de NADPH via trois étapes : glucose-6-phosphate, 6-phosphogluconolactone, 6-phosphogluconate et ribulose-5-phosphate. La deuxième étape est non oxydative et mène à la formation de ribose-5-phosphate, l'unité de base des acides nucléiques (258).

III.6 Récepteurs et transporteurs

Au niveau cellulaire, le glucose est transporté via différents transporteurs. On retrouve principalement les transporteurs GLUT (Glucose transporter), qui permettent un passage passif de la molécule de glucose d'un compartiment à un autre selon le gradient de concentration, puisque le niveau de glucose dans la circulation sanguine est plus important que dans les cellules. Il existe plusieurs isoformes exprimés à différents niveaux selon les tissus examinés. Par exemple, GLUT1 est le plus ubiquitaire, responsable de l'absorption et du maintien des niveaux de glucose basal (266). L'isoforme GLUT2 possède une affinité faible pour le glucose mais une capacité de transport importante (267). Ceci n'en fait pas un transporteur de choix pour l'absorption du glucose alimentaire mais plutôt un contrôleur de la balance osmotique. Il est retrouvé au niveau du foie, du pancréas, des reins et de l'intestin où il permet la réabsorption du glucose (268,269). Au niveau neuronal, le transporteur le plus fréquent est le GLUT3 (266,270), alors que GLUT4 est le transporteur qui est le plus sensible à l'insuline et qui est retrouvé dans les tissus adipeux et les muscles striés (266,271). Il est intéressant de noter que les transporteurs GLUT3 et GLUT4 sont exprimés de manière moins importante chez des patients diabétiques (272,273). La deuxième famille de transporteurs est composée des SGLT (Sodium-Glucose Linked Transporter), qui sont dépendants du sodium et sont trouvés plus fréquemment au niveau de l'intestin et des reins, pour l'absorption du glucose fourni par la diète et du glucose urinaire (274).

Depuis maintenant 70 ans, les travaux effectués dans le but de comprendre le métabolisme du glucose ont utilisé des isotopes, surtout le carbone 14 (^{14}C) et l'hydrogène 3 (^{3}H) (235). Ces derniers ont permis de comprendre comment les hexoses, principalement

composés de carbone, sont utilisés via les différentes enzymes du métabolisme. De plus, des techniques de TEP (tomographie à émission de positons) ont permis de mesurer les niveaux de métabolisation du glucose, principalement de sa phosphorylation via l'hexokinase. Pour ce faire, un dérivé du D-glucose, le 2-déoxy-D-glucose, lié ou non à un fluorophore est utilisé. Ce dernier ne peut pas être métabolisé par les étapes subséquentes de la glycolyse. Plus récemment, l'utilisation de résonance magnétique nucléaire (RMN) a permis de mesurer le niveau du métabolisme oxydatif via l'utilisation d'isotopes carbone du glucose, permettant de comprendre et de quantifier de manière plus adéquate plusieurs concepts comme le cycle glutamine-glutamate des astrocytes (275,276).

III.7 Le glucose dans le système nerveux

Le métabolisme du système nerveux est une part importante de l'énergie totale utilisée par l'organisme. En effet, 25% du métabolisme total est destiné au cerveau, qui ne représente pourtant que 2% de la masse totale de l'organisme (277). Les cellules neuronales ont des capacités de métabolisme oxydatif importantes et peuvent utiliser le glucose comme source énergétique (278) alors que les astrocytes ont une activité glycolytique importante. La plupart des cellules du SNC expriment des transporteurs GLUT. Cependant, les neurones sont incapables de stocker le glucose sous forme de glycogène et dépendent donc d'un apport en énergie continuel à partir des cellules les entourant, principalement des astrocytes. Il est donc important de comprendre le rôle des astrocytes dans le maintien métabolique.

III.8 Métabolisme des acides gras et des acides aminés dans le SNC

Le métabolisme des hydrates de carbone constitue la source principale d'énergie dans le SNC, cependant les acides gras jouent un rôle important dans le métabolisme général. Ce rôle n'est cependant pas conservé dans le SNC (279), le métabolisme des acides gras étant plutôt présent dans le développement neuronal (280). Pour expliquer cette différence entre SNC et organisme global, plusieurs hypothèses ont été avancées. La première veut que les acides gras non estérifiés ne passent pas au travers de la barrière hémato-encéphalique. De ce fait il serait difficile d'imaginer que cette source de nutriment soit utile aux cellules neuronales. Cette hypothèse a toutefois été mise de côté quand il a été prouvé qu'au contraire, les acides gras traversent la barrière hemo-encéphalique (281-283). La deuxième hypothèse est liée à une différence d'expression de plusieurs enzymes impliquées dans la β-oxydation. Il apparait que les mitochondries au niveau cérébral ont des capacités de β-oxydation inférieures à celles retrouvées dans des tissus avec un haut niveau d'énergie comme les muscles et le coeur (284). De plus, la carnitine palmitoyl transferase (CPT1), enzyme clé dans le passage des acides gras dans la mitochondrie, est faiblement exprimée dans le système nerveux en comparaison du foie (285). Dans un deuxième temps, l'utilisation d'acide gras a pour effet de réduire l'activité de la chaine de transport des électrons, impliquant une hausse des ROS ainsi qu'une dépolarisation de la membrane mitochondriale (286-288).

Finalement, les neurones étant des cellules sensibles au niveau d'oxygène, la dégradation d'un acide gras demande 15% plus d'oxygène que pour une molécule de glucose, induisant un risque important d'hypoxie neuronale entrainant des dommages tissulaires. Ce concept est observé dans un tissu tel que le cœur, qui reçoit un apport d'oxygène important, puisque ça consommation d'ATP est très importante (289,290).

III.9 Neurones, astrocytes et métabolisme

Les neurones et les astrocytes peuvent absorber le glucose et le métaboliser via la glycolyse, la voie des pentoses et la phosphorylation oxydative. D'ailleurs, les cellules astrocytaires comptent pour 30% de la consommation d'oxygène liée au métabolisme oxydatif (275,276,291), ce qui correspond au volume occupé par ce type cellulaire.

Les différences majeures entre les deux types cellulaires se situent à deux niveaux :

1) Le type de métabolisme. Les neurones ont un métabolisme oxydatif important et une activité glycolytique déficiente, principalement dû à la dégradation de la 6-phosphofructose-2-kinase/fructose-2,6-biphosphate-3 (Pfkfb3), qui agit indirectement sur l'expression de la fructokinase, impliquée dans la glycolyse (292,293). De leur côté, les astrocytes ont un métabolisme principalement anaérobique basé sur la glycolyse (294,295), à cause de l'expression d'enzymes liées à la glycolyse et à la faible expression de facteurs favorisant le métabolisme oxydatif (294,296). De plus, une activation forcée de la glycolyse au niveau neuronal entraine un phénomène d'apoptose (297).

2) Les neurones sont dépourvus de capacité de stockage, il leur est donc impossible d'utiliser le glucose à des fins de stockage de glycogène. Ce rôle est assumé par les astrocytes, pouvant stocker de grandes quantités de glycogène, particulièrement dans les formations plus distales où les mitochondries ne peuvent pas se localiser (297) et qui composent près de 80% de la surface couverte par les astrocytes (298,299). Ce glycogène demeure de manière stable (300,301) jusqu'à l'activation cérébrale (302). Par ailleurs, l'importance du glycogène dans le métabolisme des astrocytes est démontrée par une chute du ratio d'utilisation de l'oxygène sur l'utilisation de glucose (CMR_{O2}/CMR_{glc}) qui se situe théoriquement à 6:1 (6 molécules de CO_2

libérées pour 1 molécule de glucose utilisée), indiquant une utilisation de glucose via des voies non oxydatives (303).

III.9.1 La relation neurone/astrocyte

Au-delà de l'aspect purement métabolique, les astrocytes ont un rôle de soutien primordial dans l'activité synaptique. En effet, leurs fonctions dans le recyclage des neurotransmetteurs, la conservation des gradients ioniques et la formation de tissu cicatriciel ,après mort neuronale, sont très importantes pour le fonctionnement du système nerveux (304,305). Lorsqu'un neurotransmetteur excitateur, principalement le glutamate, est relâché dans la fente synaptique, il est important de limiter son accumulation pour éviter un

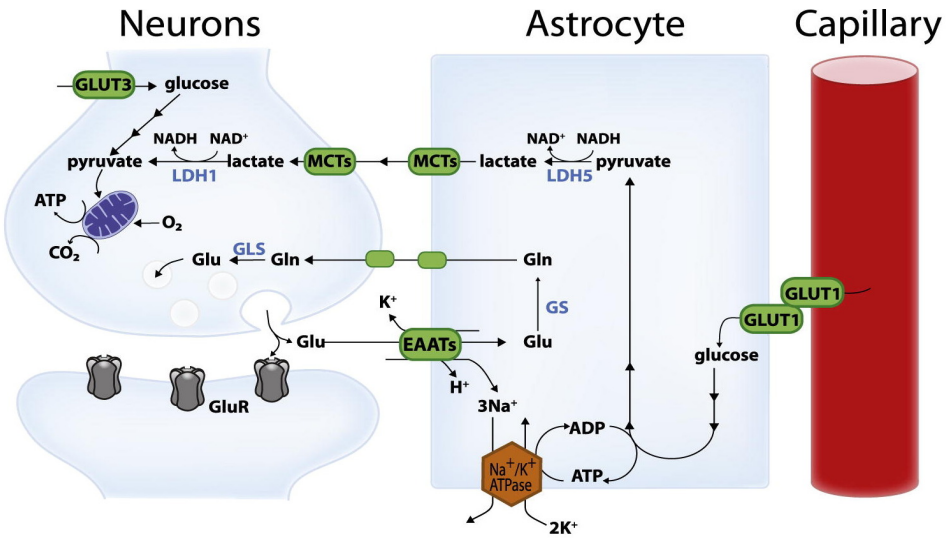

Figure 5: Interaction métabolique Neurone et Astrocytes
Adaptée de ***Bélanger et al, Cell metab. 2011.***
Schéma récapitulatif du concept de navette lactate entre neurones et astrocytes (ANLS). L'activation synaptique induit une relâche de lactate par les astrocytes qui permet un support métabolique important pour les neurones.

phénomène d'excitotoxicité. Les astrocytes, via leurs transporteurs EAAT2 (Excitatory amino acid transporter 2)/GLT1 (Glial glutamate transporter) et EAAT1/GLAST permettent cette récupération et le recyclage, en le transformant en α-cétoglutarate (α-CG) pour une utilisation dans le cycle de l'acide citrique, et en glutamine destinée à revenir aux neurones (276) **(Figure 5)**. Aussi, la déshydrogénation du pyruvate ne permet pas la production de nouveaux composants du cycle de l'acide citrique. Ce rôle étant joué par la pyruvate carboxylase, dont

l'absence dans les neurones est généralement acceptée (306,307), malgré certaines données y suggérant sa présence (308). Le problème découlant de ces processus est donc que les neurones ne récupèrent pas la totalité du glutamate expulsé et s'exposent à une diminution importante d'α-CG. Dans les astrocytes, la pyruvate carboxylase permet la formation d'α-CG, qui a pour but de permettre la formation de glutamine supplémentaire qui est transmise aux neurones pour éviter une chute trop importante du niveau d'α-CG (309).

Finalement, les capacités de stockage du glycogène par les astrocytes jouent un rôle plus important qu'il n'y parait. En effet, la glycogénolyse permet la transmission de lactate aux neurones, permettant la formation d'une mémoire à long terme (LTP), ce phénomène étant perdu après injection d'un inhibiteur de la dégradation du glycogène et promu par une injection de lactate (310).

III.9.2 Dysfonction astrocytaires et maladies neurodégénératives

La principale manifestation d'une dysfonction astrocytaire est l'excitotoxicité. Ce phénomène est produit par une accumulation de L-glutamate dans la fente synaptique lorsque les astrocytes ne sont plus en mesure de recycler le neurotransmetteur. La finalité de ce dysfonctionnement est une sur-activation du neurone post-synaptique conduisant à sa mort. Dans le cadre du vieillissement, même sans la contribution d'une protéine toxique, une perte de fonction progressive des astrocytes est observée chez la souris (311).

Le concept d'excitotoxicité n'est pas spécifique à une pathologie, ce mécanisme de toxicité est référencé dans la SLA et la CH. Il n'en demeure pas moins un mécanisme de toxicité important de ces pathologies. Dans le cas de la SLA et de la CH, il s'explique par une réduction d'expression du transporteur EAAT2/GLT-1, responsable de la récupération du

glutamate. Ce constat est autant valide pour des modèles murins que dans des analyses de tissus post-mortem de patients (312-316). Spécifiquement dans la SLA, l'expression de la protéine SOD1 mutante dans les astrocytes influence la progression de la pathologie et ce, même si les neurones n'expriment pas la forme mutée de la protéine (317,318).
Les travaux sur la CH indiquent une hausse de l'activité glutamatergique alors que les stades tardifs montrent le contraire (319).

Les neurones post-synaptiques exprimant les récepteurs glutamatergiques sont susceptibles d'être affectés par une stimulation liée à un trop haut niveau de glutamate dans la fente synaptique. Dans le cas de la chorée de Huntington il est accepté qu'une sur-activation des récepteurs NMDA conduise à la mort neuronale. Cette observation est soutenue par le résultat de plusieurs travaux montrant que l'injection d'un agoniste du récepteur NMDA induit un phénotype similaire à Huntington chez la souris. De plus, une stimulation glutamatergique dans des modèles YAC72 et YAC128 induit une réponse synaptique de plus grande amplitude que des lignées sauvages (320,321). Au niveau structurel, le rôle de la sous-unité GluN2B, une des quatre sous-unités du récepteur NMDA, dans la spécificité de la dégénérescence des neurones du striatum, est corroboré par plusieurs travaux effectués dans des modèles murins. En effet, cette sous-unité, de par sa capacité à lier la protéine PSD-95 de manière plus important à la sous-unité GluN2A, fragilise le neurone face au phénomène d'excitotoxicité rapporté dans la CH (322,323).

III.9.3 Glucose ou Lactate comme source énergétique

Quand il s'agit d'expliquer quelle molécule est la plus importante dans le métabolisme cérébral, plusieurs écoles de pensées s'opposent. D'un côté, l'école traditionnelle, qui estime

que le glucose est la source principale d'énergie pour les neurones (303,324-326), et de l'autre côté il y a l'hypothèse de l'utilisation du lactate comme source principale d'énergie neuronale (327-329). Cette dernière est très liée à l'hypothèse de la navette lactate, décrite dans les années 90 (330), qui soutient que le glucose est utilisé par les neurones en état de repos, mais qu'un autre processus intervient lors d'une activation synaptique. En effet, le glutamate relâché par les neurones, et recyclé par les astrocytes, induirait la production de lactate dans ces derniers; le lactate serait ensuite acheminé vers les neurones via les transporteurs monocarboxylés (MCT) (331), qui à l'instar des transporteurs de glucose, permettent un passage facilité au travers de la membrane. Le lactate, mais aussi le pyruvate, seraient ensuite utilisés comme source d'énergie. Cependant, bien que le concept soit intéressant, il est encore très controversé, surtout quand vient le temps de l'appliquer à des cellules neuronales dont le neurotransmetteur n'est pas le glutamate. Aussi, cela n'explique pas la présence très importante au niveau des astrocytes de transporteurs du glucose GLUT3, qui permettent un passage très rapide de l'hexose – sept fois supérieur à celui de GLUT1 (332,333). De plus, la lactate déshydrogénase (LDH), qui permet la formation de lactate, ne présente pas une hausse d'activité importante lors d'une stimulation neuronale, ce qui semble indiquer que ce n'est pas un joueur clé dans l'apport énergétique pour le neurone.

III.10 Le glucose dans les troubles neurologiques

III 10.1 Généralités

Le désordre le plus commun en terme de métabolisme est le diabète. Il en existe deux types, le premier étant dû à un désordre auto-immun qui provoque une perte des cellules β du

pancréas (productrices d'insuline) et le deuxième est provoqué par une incapacité du pancréas à produire assez d'insuline pour traiter le glucose absorbé dans la diète. Les facteurs prédisposant à son développement sont principalement : l'âge, une diète déséquilibrée et un manque d'exercice. Chez un patient diabétique, le pancréas réagit moins à l'augmentation de glucose sanguin, ce qui entraine une hyperglycémie. Cette hausse importante en glucose circulant peut provoquer différentes complications, qui incluent des dysfonctions rénales et visuelles, des problèmes cardiaques et des neuropathies périphériques (334). Bien que ce phénomène soit particulièrement présent dans les organes métabolisant les sucres, tels que les muscles, le foie et le pancréas, le cerveau était, croyait-on, un organe insensible à l'insuline. Depuis, des travaux ont mis en évidence la présence de récepteurs au niveau de la barrière hémato-encéphalique (335-338). De plus il a été identifié que le bulbe olfactif, le cortex, l'hypothalamus et l'hippocampe sont parmi les régions du cerveau murin les plus réceptives à l'insuline (339).

III.10.2 Rôle de l'insuline dans le SNC

L'action insulinémique est importante pour plusieurs raisons. La première est qu'elle régit la prise alimentaire en agissant sur les neurones de l'hypothalamus en réduisant le relâchement des peptides anti-anorexigènes AgRP (Agouti related protein) et NPY (Neuropeptide Y) et en augmentant le relâchement de facteur anorexigène POMC (Proopiomelanocortin). Dans certains cas, lorsque les niveaux d'insuline sont insuffisants, la prise alimentaire n'est pas limitée et entraine un certain niveau d'obésité, tel qu'observé dans des animaux ayant une déficience du récepteur à l'insuline (IR) (340).

L'insuline n'est cependant pas limitée à un rôle anorexigène, mais est aussi impliquée

dans la plasticité neuronale à un certain degré. Plusieurs modèles murins de diabète ont des capacités mémorielles déficientes (341,342). Ce phénomène peut en partie être expliqué par le fait que l'insuline agit au niveau de l'expression de récepteur NMDA du glutamate et permet la formation de nouvelle mémoire (LTP) au niveau de l'hippocampe (343,344). Ceci est confirmé par le fait que la déficience en insuline est liée à une perte de volume cérébral, une diminution des fonctions cognitives, des changements électrophysiologiques, morphologiques et une sensibilité à certains désordres neurologiques (345-350). Bien qu'une hyperinsulinémie périphérique chronique ait des effets délétères sur l'organisme, une injection aigüe d'insuline a pour effet d'augmenter les capacités mémorielles et de concentration (351,352). De plus, bien que le métabolisme du glucose ne soit pas modifié par une augmentation d'insuline de manière globale (353), il semble que certaines régions, parmi lesquelles l'hypothalamus, le cortex et l'hippocampe, y sont sensibles (354-356).

L'enzyme agit aussi au niveau des astrocytes, principaux supports métabolique des neurones, en stimulant l'expression de transporteur du glutamate et la synthèse de cholestérol (357-359). Cette synthèse est importante puisqu'une réduction des niveaux de SREBP (Sterol Response Element Binding Protein), une enzyme clef dans la synthèse du cholestérol, induit une perte de transmission synaptique et une réduction des capacités mémorielles (360).

III.10.3 Troubles neurodégénératifs et métabolisme

Les études portant sur la maladie d'Alzheimer et le métabolisme sont nombreuses, principalement dues au fait que la littérature est riche en travaux montrant un lien entre une insulinorésistance et des symptômes cognitifs (361,362). En parallèle, le rôle de l'insuline dans le support métabolique des neurones serait essentiel. L'hormone permet une réduction de

l'oxydation des transporteurs GLUT3, ainsi que l'augmentation de l'entrée de glucose et l'expression de l'hexokinase (363,364), favorisant l'apport en glucose et son métabolisme dans les neurones

Les patients souffrants de MA ont souvent une baisse d'expression du récepteur à l'insuline (365), ainsi qu'une chute des niveaux d'insuline dans le liquide céphalo-rachidien, alors qu'en contrepartie les niveaux d'insuline augmentent dans le plasma. En accord avec le principe que les niveaux d'insuline diminuent dans la maladie d'Alzheimer, une administration d'insuline intra-nasale semble entrainer une diminution des symptômes cognitifs (366). Dans un deuxième temps, une modification de la signalisation cellulaire de l'insuline, possiblement via une voie MAPK, semble augmenter l'élimination du peptide Aβ et ainsi limiter les niveaux de réactivité des astrocytes et les déficits cognitifs dans des modèles murins (347,367,368). Il existe un autre facteur pouvant influencer la susceptibilité à la maladie d'Alzheimer, puisqu'un des facteurs de risque les plus communs dans les cas de la MA demeure le statut génétique de la protéine APOE4 (369). Son rôle dans la maladie est peu connu (369,370) mais, parce que ce type de protéine semble être impliqué dans la synaptogénèse, il est possible qu'une déficience entraine une dysfonction à ce niveau.

Ce phénomène insulinodépendant semble aussi impliqué dans la maladie de Parkinson où les neurones dopaminergiques sont fragilisés face aux toxines s'ils sont traités avec une diète riche en gras (371,372). Par ailleurs, les neurones dopaminergiques de la substance noire traités avec une diète enrichie en gras développent une résistance à l'insuline conduisant à une diminution de l'activité synaptique (373). Ce principe est aussi soutenu par une étude montrant que les patients atteints de MP ont plus de risques de développer une résistance à l'insuline (374).

III.10.3.2 Le cas de la sclérose latérale amyotrophique

Le lien entre la résistance à l'insuline, le diabète de type II et la neurodégénérescence n'est pas aussi clair chez les patients atteints de la sclérose latérale amyotrophique (SLA). Cependant il est maintenant établi que le métabolisme de ces derniers est affecté. En effet, les patients SLA sont souvent maigres, avec un indice de masse corporelle relativement faible (375), indiquant un déséquilibre entre la prise et l'utilisation d'énergie, favorisant la perte musculaire des patients. Ce déséquilibre peut être expliqué par le fait que les patients souffrent de dysphagie, mais surtout que le métabolisme consomme plus d'énergie qu'il n'en accumule, ce phénomène étant connu sous le nom d'hypermétabolisme (376). Certains patients ont un niveau de lipides sanguins plus important, qui peut s'expliquer par la dysfonction des mitochondries dans des tissus comme le muscle. Ceci pourrait cependant être un mécanisme protecteur étant donné qu'une perte de poids est un facteur de risque pour la SLA et que l'hyperlipidémie est associée à une augmentation du taux de survie. (377-379).

Il est difficile de vérifier les dysfonctions métaboliques à des stades précoces de la pathologie chez l'humain et pour y pallier, les modèles de souris exprimant des protéines telle que SOD1 mutante sont utilisés. Chez ces dernières, il apparait que le métabolisme est augmenté et que leur poids est réduit, comparativement aux souris sauvages, et ce avant l'apparition des symptômes (380). Bien que décrite comme une façon de réduire la progression du vieillissement et des maladies à déclenchement tardif, la restriction calorique accélère la dégénérescence neuronale et la mort dans le modèle de souris SOD1 (381,382). Dans un autre modèle, la perte d'expression de TDP-43 entraine une chute importante des niveaux de tissu adipeux, alors qu'une surexpression de TDP-43 cause des dysfonctions mitochondriales (383).

Le concept de dysfonction mitochondriale implique une chute de la production d'ATP (384,385), présent dans des modèles de la SLA, mais aussi chez des patients (386,387).

III.10.3.3 Le cas de la chorée de Huntington

Le rôle du métabolisme dans la Chorée de Huntington est établi et ce à plusieurs niveaux. Il est accepté que la forme mutée d'huntingtine (mHTT) influence l'intégrité des mitochondries. En effet, mHTT peut se lier à la membrane mitochondriale, induisant une dysfonction qui mène à une chute de la production d'ATP (388). Plusieurs études ont aussi montré que mHTT a un rôle néfaste dans la relation astrocytes-neurones, en réduisant les niveaux des transporteurs de l'acide ascorbique, ce dernier permettant l'entrée de lactate dans la cellule neuronale aboutissant à un déficit énergétique fragilisant la cellule (389). Plusieurs études sur des tissus post-mortem et sur des modèles murins indiquent une chute d'absorption du glucose dans plusieurs régions du cerveau (390,391). Dans un autre ordre d'idées, lors d'une demande plus importante en énergie, les capacités d'utilisation du glucose sont limitées dans des cellules striatales primaires exprimant la protéine mHTT (392), alors que leur niveau d'activité glycolytique est moins important (393). Finalement, à l'instar de la SLA, la présence d'hypermétabolisme est répertoriée dans des patients Huntington (394).

III.11 Transmission Épigénétique et métabolisme

Le concept d'épigénétique implique une modification de l'épigénome, induisant des changements transcriptionnels et traductionnels importants chez un individu. Cependant la question relative à la transmission de ces changements aux générations subséquentes reste un

débat ouvert.

III.11.1 Une transmission intergénérationnelle

Le principal argument s'opposant à ce concept de transmission transgénérationnelle est que lors de la fécondation du zygote, l'épigénome est effacé (395). Les premières observations d'une transmission non mendélienne sont le fruit de travail sur les plantes (396). Par la suite, la découverte que certaines marques épigénétiques ne sont pas effacées lors de la fertilisation ou le fait que certains gènes, principalement impliqués dans le métabolisme développemental sont sujets à une empreinte génomique. Dans ce cas, les allèles parentaux ne possèdent pas le même statut transcriptionnel (397,398). De plus, il apparait que la transmission de caractères phénotypiques tels que la couleur du pelage ou de la queue peuvent avoir un mode de transmission non mendélien (399-401), consolidant l'hypothèse d'une transmission de caractères épigénétiques chez la souris. La méthylation de l'histone 3 sur certains promoteurs dans les spermatozoïdes semble aussi jouer un rôle dans l'hérédité épigénétique, puisqu'un faible pourcentage de la marque H3K27me3 est conservé et demeure dans une structure de nucléosome (402,403).

Des données récentes suggèrent que des facteurs non nucléaires, particulièrement des ARNnc, pourraient jouer un rôle important dans ce phénomène (404). Ces ARNnc incluent des miARN, qui ont déjà été mis en évidence comme des facteurs importants dans la transmission de caractères épigénétiques (127,405), et des piARN (PIWI-interacting RNA) trouvés en haute concentration dans le sperme et qui influencent l'empreinte paternelle (paternal imprinting) (406-408).

Chez la drosophile et *C. elegans*, la transmission épigénétique est bien établie et n'agit pas uniquement sur des phénotypes de couleur (409). Elle a aussi un impact sur la longévité et sur

la fécondité impliquant la méthylation de l'histone 3 lysine 4 (229,410). Chez la mouche et le ver, la transmission épigénétique est dépendante de la lignée germinale. D'ailleurs une mutation des gènes *fem-3* et *pgl-1*, essentiels dans le fonctionnement de la lignée germinale, bloque la transmission de caractères génétiques chez *C. elegans* (229).

III.11.2 Transmission de caractères métaboliques

La transmission d'un effet sur une ou deux générations relève d'une transmission intergénérationnelle plutôt que transgénérationnelle, dont l'effet marque plus profondément les générations subséquentes. Il est bien établi aujourd'hui que la diète parentale a des effets majeurs sur la physiologie de la progéniture. Plusieurs travaux suggèrent que l'apport nutrionnel d'un parent influence globalement la physiologie métabolique de la progéniture, souvent de manière négative. Une diète enrichie en gras a pour effet de diminuer la sensibilité à l'insuline et provoque une augmentation du glucose sanguin (411-413). Les études sur le phénomène opposé (restriction calorique) montrent une augmentation de la synthèse du cholestérol, avec une hausse de SREBP (414) et un retard de croissance chez le rat (415). Une diète réduite en protéines induit des problèmes cardio-métaboliques, incluant de l'hypertension, une hausse lipidique, des dysfonctions vasculaires, un déficit du système immunitaire et une susceptibilité accrue au stress oxydatif (416-418)

Les études sur des cohortes Hollandaises, victimes d'un manque nutritionnel durant la Deuxième Guerre mondiale, et Suédoises sujettes à de graves carences, montrent que les générations subséquentes ont une incidence plus importante de désordres métaboliques (419-421). D'autres études ont mis en avant que le sexe du parent subissant une variation diététique influence différentiellement une progéniture femelle ou mâle, ce phénomène étant conservé chez l'humain (411,420,422).

L'hérédité épigénétique est contrôlée par différentes composantes mentionnées précédemment. La méthylation de l'ADN (411,413,423), avec par exemple l'hypomethylation de l'IGF2 (424), a été constatée dans le cas de la population Hollandaise soumise à des carences importantes durant la guerre. La transmission de caractères épigénétiques par une diète déséquilibrée se fait aussi par la modification de l'histone 3, incluant la méthylation de la lysine 4 (229,410), ou par l'inhibition de gènes impliqués dans le complexe polycomb qui est responsable de la triméthylation de la lysine 27, une modification répressive de la transcription (425). Enfin, les ARNnc, incluant miARN et piARN influencent aussi cette transmission de caractères génétiques au travers des générations, et ce, chez l'humain comme chez le nématode *C. elegans* (127,405,426-431). Ces miARNs, tel miR-124 influencent, entre autres, la croissance embryonnaire et adulte chez la souris (127).

IV Modèle expérimental : *Caenorhabditis Elegans*

La recherche biomédicale implique l'utilisation d'organismes modèles afin de comprendre les mécanismes qui sous-tendent certains processus biologiques. Ces modèles permettent aussi dans certains cas de mettre à jour des moyens de combattre des "dysfonctions" biologiques.

L'utilisation de *C. elegans* remonte aux années 70 lorsque le biologiste Sydney Brenner a commencé à travailler sur ce ver rond dans le but d'obtenir un modèle génétique simple pour l'étude du système nerveux, mais aussi de différents systèmes biologiques, incluant un système digestif et reproducteur. *C. elegans* est un modèle avantageux par son cycle de reproduction court (3,5 jours), une durée de vie de 20 à 30 jours, son développement génétique invariable et

sa transparence. De plus, sa petite taille (1 mm à l'âge adulte) en fait un modèle très facile d'utilisation. Les différents stades de développement sont bien définis et se divisent en quatre stades larvaires (L1 au L4) et un stade adulte. Enfin près de 80 % de gènes du ver trouvent un homologue chez l'homo sapiens (humain) (432). Le ver se présente sous deux formes sexuées, une forme hermaphrodite et une forme mâle. Cette dernière est peu fréquente dans une population standard (0,1%) et liée à une non-disjonction au niveau des gonades, donnant un génotype XO qui diffère du XX lié à l'hermaphrodite. Cependant, le mâle joue un rôle important dans les études génétiques menées en laboratoire.

IV.1 Anatomie de *C. Elegans*

Le nématode possède une structure simple consistant en deux tubes concentriques séparés par une cavité remplie de liquide, le pseudocoelom, le tout maintenu par une pression hydrostatique interne (433). Parmi les structures importantes du tube externe se trouve la cuticule, une couche de collagène qui vise à protéger le nématode contre diverses agressions, l'hypoderme ainsi que les systèmes musculaires, neuronaux et excrétoires. Le système musculaire du ver, constitué de quatre bandes attachées à la cuticule via l'hypoderme, permet les mouvements sinusoïdaux de l'animal, en se contractant et se relaxant de manière coordonnée. En ce qui concerne le tube interne, il est composé du pharynx, de l'intestin et des gonades **(Figure 6)**.

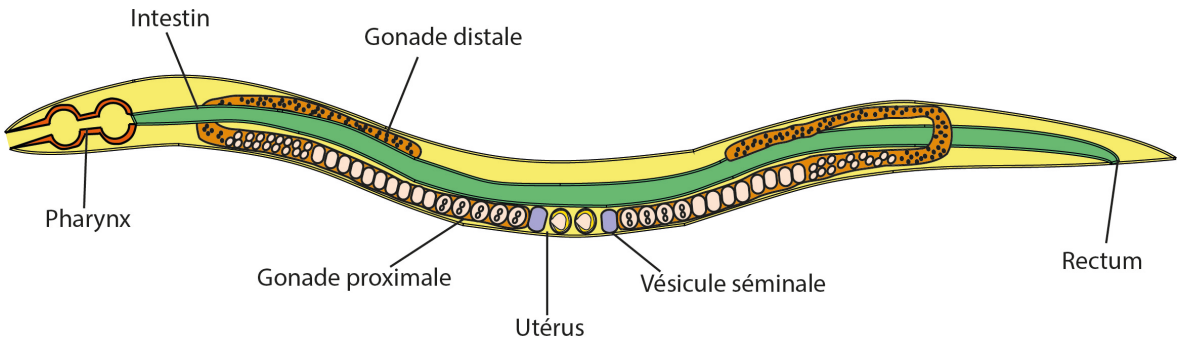

Figure 6: Schéma anatomique de *C. elegans*
Représentation des principales structures anatomiques de C. elegans sous sa forme hermaphrodite. On retrouve un système digestif et un système reproducteur, en plus du système nerveux.

IV.2 Système nerveux du ver

L'ensemble du système nerveux de *C. elegans* se résume en 302 cellules neuronales, de 118 types différents et 52 cellules gliales. Ces différentes cellules s'arrangent de manière systématique et invariable (434). La plupart des gènes impliqués dans la neurogenèse, la migration ou la différenciation cellulaire ont été mis en évidence via des criblages génétiques ayant pour objectif d'identifier des mutants présentant des déficiences dans ces mécanismes (435,436).

Le concept de lignées cellulaires stéréotypées a pour définition un modèle de division et développement cellulaire qui se produit de manière invariable. Chez *C. elegans*, ce modèle se produit 13 fois le long de l'axe antérieur-postérieur lors du développement moteur, bien que certaines différences soient observées, tels que le type de connexion, le type de croissance des neurites ou encore les neurotransmetteurs produits.

Le système locomoteur est principalement contrôlé par la corde neurale ventrale (CNV) où les neurones cholinergiques permettent une contraction des muscles de manière unilatérale et l'inhibition de ceux controlatéraux via l'activation de neurones inhibiteurs de type

GABAergique (437,438). Le système moteur se compose de 113 cellules nerveuses, le mouvement du ver étant contrôlé par 4 types de neurones moteurs interconnectés sous forme d'un motif, soit 2 GABAergiques VD et DD et 2 cholinergiques VB et DB qui permettent l'inhibition controlatérale des muscles et ainsi une propagation du signal électrique **(Tableau II)**. Ce système permet un mouvement ondulatoire qui varie selon le type de stimuli auxquels le ver est confronté. Ainsi, toute stimulation sur les neurones antérieurs (au niveau de la tête) entraine une propagation du signal contractile vers l'extrémité postérieure, induisant un mouvement arrière. Une stimulation à l'extrémité postérieure a l'effet inverse (437).

Neurotransmetteurs libérés	Type de neurones
Acétylcholine	**A** et **B** (corde ventrale (V) ou dorsale (D)) AS (corde dorsale uniquement
GABA	**D** (corde ventrale (V) ou dorsale (D)) **RME** (tête uniquement) **AVL** et **DVB** (entérique)

Tableau II : Nomenclature des neurones impliqués dans la locomotion chez *C. elegans*.
Les neurones cholinergiques sont principalement de type A et B alors que les neurones GABAergiques sont de type D. Ces neurones sont présents dans la corde dorsale (DA, DB et DD) et ventrale (VA, VB et VD).

IV.2.1 GABA, Acétylcholine et locomotion

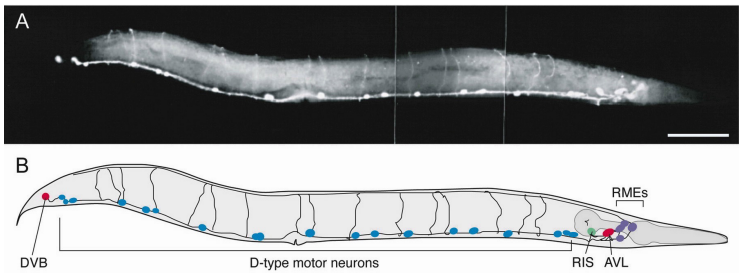

Figure 7 : Représentation de la circuiterie GABAergique chez *C.elegans*. ***Adapté de McIntire et al, Nature. 1993***
Le nematode possède 26 neurones GABAergiques qui contrôlent la motricité de l'animal en réduisant l'activité des neurones cholinergiques.

Chez *C. elegans,* comme dans la plupart des organismes, la transmission synaptique se fait de manière rapide via relâchement de neurotransmetteurs d'un élément pré-synaptique vers un autre post-synaptique. Cependant, chez le nématode, il existe peu de spécialisation au niveau de la portion post-synaptique, c'est donc la proximité des éléments qui permet la communication entre les cellules neuronales. Ainsi un neurone pré-synaptique peut activer un nombre important de cellules à proximité (439). Les neurotransmetteurs impliqués de manière importante dans la locomotion sont le GABA et l'Acétylcholine. Les neurones GABAergiques sont au nombre de 26 et répartis comme suit : 6 DD (dans la corde dorsale), 13 VD (dans la corde ventrale), 4 RME (innervant les muscles de la tête), AVL, DVB (neurones entériques) et RIS (un interneurone) **(Figure 7)**. La fonction du GABA est de manière générale inhibitrice, mais dans le cas des neurones AVL et DVB, la relâche de ce neurotransmetteur permet la contraction des muscles entériques et la défécation (440).

Dans le cas des neurones cholinergiques, ils sont principalement retrouvés dans la CNV, où ils composent 6 des 8 classes de neurones (VA, VB, VC, DA, DB, AS), mais aussi dans le pharynx (neurones M1, M2 et M5) et sont de classe A ou B. Ces derniers produisent des influx de type excitateur, soit au niveau de la jonction neuromusculaire ou sur les neurones GABAergiques dans le but d'inhiber toute contraction au niveau controlatéral. L'acétylcholine

est le seul neurotransmetteur qui est essentiel, puisqu'une mutation dans le gène *cha-1*, encodant la choline acétyle-transférase, entraine une létalité embryonnaire.

IV.3 *C. elegans* comme modèle d'étude

La facilité d'utilisation du nématode en a fait un modèle de choix dans l'étude de différentes pathologies, qu'elles soient neuronales ou non. Il est estimé que 42% des gènes impliqués dans des maladies propres à l'humain ont un orthologue chez *C. elegans* (441). Le développement d'outils tels que l'ARN interférant (ARNi), une grande quantité de lignées mutantes (parfois même de plusieurs mutants pour un même gène), et le développement de lignées transgéniques, permettent des approches diversifiées, auxquelles s'ajoute une autre caractéristique unique, la transparence du ver. Cette dernière permet l'utilisation de protéines fluorescentes *in vivo*, sans intermédiaire de marquages successifs. Dans cette étude, il sera question de lignées neuronales et de leur étude dans un contexte de vieillissement. De plus, plusieurs orthologues de gènes impliqués dans des pathologies humaines sont répertoriés dans le génome du ver. Dès lors qu'il est possible de le faire, l'utilisation de mutations orthologues à celles de l'humain permet l'étude rapide des fonctions potentielles de ces protéines.

Au cours des 20 dernières années, de nombreux modèles ont fait leur apparition, modélisant différentes pathologies incluant la maladie d'Alzheimer, la maladie de Parkinson, mais aussi les deux pathologies couvertes dans notre étude, la sclérose latérale amyotrophique et la chorée de Huntington **(Tableau III)**. Les différentes lignées développées ont des caractéristiques bien définies qui permettent une approche différente quant aux questions posées sur les mécanismes pathologiques sous-jacents. En effet, l'utilisation de promoteurs différents pour un même gène permet de déterminer la spécificité et la susceptibilité cellulaire

propre à une pathologie.

IV.3.1 Sclérose latérale amyotrophique

Les modèles représentant la SLA sont nombreux, dûs au nombre de gènes impliqués dans les cas familiaux. Ainsi, des modèles de SOD1, de FUS/TLS et de TDP-43 ont vu le jour dans la dernière décennie. Ces modèles ont la capacité d'exprimer les protéines transgéniques d'intérêt dans différentes lignées neuronales, incluant les neurones moteurs (442-447), mais aussi l'ensemble des neurones de l'organisme (24,136,150).

En parallèle, les gènes humains introduits dans les différentes lignées transgéniques de SLA ont un orthologue chez *C. elegans*. Ainsi, TDP-43, FUS et SOD1 sont respectivement *tdp-1*, *fust-1* et *sod-1* chez le nématode. S'il n'existe que peu de données sur *fust-1*, *tdp-1* et *sod-1,* ils ont tous été étudiés avec intérêt. Dans le cas de *tdp-1,* le gène est très conservé, homologue à 74% et similaire à 39% à l'orthologue humain TARDBP **(Figure 2).** Une perte de fonction telle que la mutation *tdp-1(ok803)* induit une variation transcriptionnelle, touchant principalement des gènes responsables de la durée de vie, de la locomotion et de la réponse au stress endoplasmique (24). Au niveau physiologique, une perte de fonction de *tdp-1* augmente l'espérance de vie de l'animal mais accroit sa sensibilité au stress cellulaire (23).

IV.3.2 Modèles de ver de la Chorée de Huntington et maladies à répétitions polyglutamine

Les modèles transgéniques de *C. elegans* de la chorée de Huntington sont moins nombreux que ceux relatifs à la SLA. Le modèle utilisé dans les prochaines sections exprime

la partie N-terminale de la protéine hutingtine (HTT) dans des lignées neuronales mécanosensorielles (promoteur *mec-3*) (174), causant une perte de sensation tactile liée à l'âge. D'autres modèles impliquant des maladies polyglutamine ont été développés. Le plus décrit et utilisé, exprimant une répétition glutamine dans des lignées musculaires sous contrôle d'un promoteur *unc-54* (orthologue de la chaine lourde de la myosine) (448), a permis de mieux comprendre les tenants et aboutissants de ces pathologies. Dans le cadre des autres pathologies polyQ, la maladie de Machado-Joseph, connue sous le terme d'ataxie spinocérebelleuse 3 (SCA3) a été modélisée à plusieurs reprises (26,449).

IV.3.3 Quel rôle pour les modèles *C. elegans*?

Le rôle des modèles transgéniques développés s'est manifesté par l'identification ou la confirmation de l'implication de voies de signalisation et de facteurs impliqués dans la pathogénèse. Un des exemples majeurs est sans aucun doute le rôle de la voie Insuline/IGF (IIS). En effet, une réduction de la voie IIS, par mutation du récepteur *daf-2* (IGFR chez l'humain), ralentit la progression de différentes pathologies via le facteur de transcription *daf-16* (FOXO3A chez l'humain) (22,444,450,451). De nombreux travaux ont aussi établi un rôle protecteur du mutant *daf-2* dans la restriction calorique (revue en 452) ainsi que dans l'effet de différents composés biologiques ou synthétiques (453-455).

Pathologie	Gène impliqué	Transgène	Phénotype	Référence
Alzheimer	Aβ	*unc-54* ::A β $_{1-42}$	Paralysie	(456)
	Tau	*aex-3* ::tau	Problèmes moteur	(457)
		rab-3 ::tau	Dysfonction neuronale	(458)
SLA	SOD1	*hsp-16.2* ::SOD1	Sensibilité accrue au stress	(446)
		snb-1 ::SOD1	Déficit locomoteur	(443)
		unc-54 ::SOD1 ::YFP	Paralysie	(445)
		unc-25 ::SOD1	Paralysie	(444)
	TDP-43	*snb-1* ::TDP-43	Déficit moteur	(136)
		snb-1 ::TDP-43 ::YFP	Déficit locomoteur	(459)
		unc-47 ::TDP-43	Paralysie	(442)
	FUS	*unc-47* ::FUS	Paralysie	(442)
		rgef-1 ::GFP ::FUS	Nage affectée	(447)
Huntington	Huntingtine-polyQ	*unc-54* :polyQ	Nage affectée	(448)
		mec-3 ::Htt-polyQ	Réponse tactile défectueuse	(174)
		unc-54 :htt-polyQ	Paralysie	(460)
Parkinson	α-synucleine	*dat-1 ::α-syn*	Dysfonction des neurones dopaminergiques	(461)
		aex-3 :: α -syn et unc-30 :: α -syn	Nage affectée	(461)
		unc-54 :: α -syn ::YFP	Agrégation	(462)

Tableau III : Liste des principaux modèles *C. elegans* développés pour des maladies neuronales liées à l'âge. Inspiré de Li, J. et Le, W. *Exp. Neurol*. (2013)

V. Introduction de l'étude et contribution aux travaux

La voie Insuline/IGF et la restriction calorique influencent le vieillissement dans les modèles les plus simples, comme dans les plus complexes (11,13,30,463). Cette modulation s'étend à la protection contre des protéines toxiques, impliquées dans des troubles neuronaux comme la SLA, la CH et MA (21,22,26,29).

Les travaux effectués sur *C. elegans* dans le cadre de la restriction calorique, dans un contexte pathologique, ont utilisé des modèles exprimant les protéines toxiques dans un système musculaire (29). Ce type cellulaire est bien différent des cellules neuronales impliquées dans les pathologies humaines, et le développement de modèles exprimant les protéines d'intérêts dans un système neuronal demeure une approche plus représentative des pathologies comme la SLA, la CH et la MA.

Nos travaux ont porté sur l'étude de la restriction calorique dans des modèles transgéniques exprimant la protéine mHTT dans des neurones mécanosensoriels et mTDP-43 dans des neurones moteurs. Notre hypothèse de départ était que la restriction calorique serait protectrice dans le cadre d'un vieillissement physiologique, mais que dans une situation pathologique, ce mécanisme ne serait peut-être pas un moyen efficace ou réaliste de moduler la protéotoxicité. Par ailleurs plusieurs données soutiennent cette hypothèse (38,381).

Nos travaux ont donc porté sur le rôle du métabolisme dans un contexte d'enrichissement en glucose, en lien avec le vieillissement physiologique (27,464), puis pathologique. Dans un deuxième temps nous avons exploré le rôle physiologique d'une diète enrichie en glucose dans la transmission de caractères physiologiques au travers de diverses

générations et obtenu des résultats surprenant quant à la transmission de résistance à différents stress.

Participation aux travaux effectués

À titre personnel, j'ai été impliqué de manière importante dans les travaux présentés ici. La première étude intitulée *Glucose delays age-dependent proteotoxicity* est la base de ma thèse de doctorat et à ce titre j'ai participé aux différentes étapes inhérentes à la publication de l'article dans la revue *Aging Cell*. Mon implication inclut principalement la production des données pour les figures 2 à 5 ainsi que les figures supplémentaires 1 à 3 et 5 à 9. Ma collègue Alexandra Vaccaro a contribué à la production des données pour les figures 1, 2, 3 et 6 ainsi que les figures supplémentaires 4 et 10. Anaïs Aulas a participé au développement du protocole pour les immuno-buvardages présentés dans les figures 2, 5 et 6, et la figure supplémentaire 3. Le Dr. Vande Velde a contribué aux discussions et commenté les résultats. Le Dr. Parker a écrit le manuscrit, décidé des expériences à effectuer et discuté des résultats, en collaboration avec Alexandra et moi-même.

En ce qui a trait à la deuxième étude intitulée *Heritable transmission of stress resistance by high dietary glucose in Caenorhabditis elegans*, mon implication est similaire à celle du premier article. Cependant, j'ai été le seul à effectuer les différentes expériences présentées et j'ai participé à la conception des expériences et aux discussions relatives aux résultats obtenus. De plus j'ai participé de manière active à la rédaction du manuscrit avec l'aide de mon superviseur, le Dr. Parker.

Chapitre II

Glucose delays age-dependent Proteotoxicity

Tauffenberger, A., Vaccaro, A., Aulas, A., Vande Velde, C., & Parker, J. A. (2012). Glucose delays age-dependent proteotoxicity. *Aging Cell*, *11*(5), 856–866. doi:10.1111/j.1474-9726.2012.00855.x

Glucose delays age-dependent proteotoxicity

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Key words:

C. elegans, neurodegeneration, aging, metabolism, protein folding, proteotoxicity

SUMMARY

Nutrient availability influences an organism's life history with profound effects on metabolism and lifespan. The association between a healthy lifespan and metabolism is incompletely understood, but a central factor is glucose metabolism. Although glucose is an important cellular energy source, glucose restriction is associated with extended lifespan in simple animals and a reduced incidence of age-dependent pathologies in humans. We report here that glucose enrichment delays mutant polyglutamine, TDP-43, FUS and amyloid-b toxicity in *C. elegans* models of neurodegeneration by reducing protein misfolding. Dysregulated metabolism is common to neurodegeneration and we show that glucose-enrichment is broadly protective against proteotoxicity.

The accumulation of misfolded proteins in the nervous system is a hallmark of many late-onset neurodegenerative diseases. Cellular mechanisms to combat proteotoxicity decline with age suggesting that aging may directly impact neurodegenerative disease onset and progression. Proteotoxicity is associated with a number of phenotypes including oxidative stress, transcriptional and metabolic disturbances. Consistently, a number of mechanisms linked to aging and neuroprotection include genes and pathways that regulate metabolism and energy production including dietary restriction (DR) (Kenyon, 2005).

Glucose is a major energy molecule whose levels are actively regulated. Disrupted glucose homeostasis can lead to obesity, type 2 diabetes and cardiovascular diseases in humans (Venn and Green, 2007). Consistently, in simple models like worms, both DR and glucose restriction slow aging while exposure to high glucose shortens lifespan (Kenyon, 2005). Furthermore, in many systems DR improves glucose homeostasis and ameliorates insulin insensitivity with concomitant health improvements. However, glucose is the main energy source in human neurons and the brain is estimated to consume over 50% of total glucose in the body (Fehm et al., 2006). Neurons have limited energy storage capacity despite their high-energy demands and are susceptible to energy fluctuations and glucose homeostasis abnormalities are observed in amyotrophic lateral sclerosis (ALS) and Huntington's Disease (HD) (Lalic et al., 2008; Podolsky and Leopold, 1977; Pradat et al., 2010). We set out to examine the contribution of either DR or elevated glucose levels in *C. elegans* proteotoxicity models.

RESULTS

Caloric restriction is ineffective against neuronal proteotoxicity

We investigated DR in worms with two methods: by removing their bacterial food source (BD: bacterial deprivation), and with the genetic surrogate *eat-2* mutation that reduces the worms' ability to ingest food (Lakowski and Hekimi, 1998; Smith et al., 2008). As previously reported BD extends the lifespan of wild type worms (**Fig. 1A** and **Table S1**) and suppressed paralysis phenotypes of worms expressing an amyloid-β fragment ($A\beta_{1\text{-}42}$) in body wall muscle cells (**Fig. 1B**) (Kaeberlein et al., 2006; Steinkraus et al., 2008). We examined neuronal proteotoxicity with a well-characterized expanded polyglutamine (polyQ, with 128Q) model in *C. elegans* touch receptor neurons (Parker et al., 2005; Parker et al., 2001) and with worm strain that expresses full length human mutant TDP-43[A315T] (mTDP-43), a mutation linked to ALS (Kabashi et al., 2008) in *C. elegans* GABAergic motor neurons (Vaccaro et al., 2012). mTDP-43 transgenics show adult-onset motility defects leading to progressive paralysis, aggregation of mTDP-43 in motor neurons, and neurodegeneration with no effects on lifespan (Vaccaro et al., 2012). Neither BD nor *eat-2* reduced polyQ or mTDP-43 toxicity in neurons (**Fig. 1C-F**) leading us to conclude that DR conditions cannot ameliorate neuronal proteotoxicity in our models. Of note, caloric restriction was recently shown to shorten the lifespan of an ALS mouse model based on the expression of mutant superoxide dismutase (Patel et al., 2010) and was ineffective in reducing neuronal dysfunction in a Drosophila model of amyloid-β toxicity (Kerr et al., 2011).

Glucose delays proteotoxicity in age-dependant disease models

We next examined conditions opposite to DR, namely high glucose concentrations on proteotoxicity. Using culture conditions shown to have physiological effects (Lee et al., 2009), worms grown from hatching on plates containing 2% D-glucose (GE: glucose enrichment) showed an approximate 4-fold increase of internal glucose levels (**Supplementary Fig. 1A**). We measured glycolytic flux of GE by examining the levels of pyruvate, which is a downstream intermediate metabolite of glycolysis and we observed that worms grown on GE conditions had increased internal pyruvate levels (**Supplementary Fig. 1B**).

We then tested whether GE modulated proteotoxicity in our three different worm models: 128Q in mechanosensory neurons, mTDP-43 in motor neurons, and a second ALS model based on the expression of an ALS-associated FUS mutation (FUS[S57Δ] or mFUS) (Belzil et al., 2011) in motor neurons that also shows age-dependent paralysis and motor neuron degeneration phenotypes (Vaccaro et al., 2012). We tested transgenic worms grown on glucose from hatching (early GE) as well as animals transferred to 2% glucose plates at the late L4 larval stage (late GE). Treatment with early or late GE improved touch insensitivity in animals expressing 128Q in touch receptor neurons (**Fig. 2A**). The 128Q strains show age-dependent degeneration of axonal processes along and the formation of insoluble polyQ proteins in protein extracts from whole worms (Parker et al., 2001). GE reduced neuronal degeneration in the 128Q animals and immunoblot analysis showed a decrease in the amount of insoluble protein from 128Q worm extracts (**Fig. 2B,C**). Looking at our motor neuron models, we observed that GE reduced the paralysis and neuronal degeneration phenotypes caused by the expression of mutant mTDP-43 or mFUS and reduced the fraction of insoluble mutant proteins (**Fig. 1D-I**) suggesting that GE may reduce the cellular load of toxic misfolded

proteins. GE had no effect on transgenic strains expressing wild type polyQ, TDP-43 or FUS proteins (**Supplementary Fig. 2**). Additionally, GE reduced paralysis phenotypes and the amount of toxic oligomers in transgenic worms expressing $A\beta_{1-42}$ in body wall muscle cells (**Supplementary Fig. 3**)

Neuroprotection by glucose requires glycolysis

To determine if protection against proteotoxicity required glycolysis, we tested L-glucose, which is an enantiomer of D-glucose that cannot be phosphorylated by hexokinase and thus cannot enter the glycolysis pathway. We observed that 128Q, mTDP-43, and mFUS transgenics grown on 2% L-glucose were indistinguishable from untreated transgenics demonstrating that D-glucose specifically rescues proteotoxicity (**Supplementary Fig. 4**). These data are consistent with a previous study showing L-glucose had no discernable effects in *C. elegans* lifespan studies (Lee et al., 2009). To directly test if glycolysis was essential for neuroprotection we focused on three glycolytic enzymes represented by a single member in the *C. elegans* genome including *enol-1* (enolase 1), *gpi-1* (glucose 6-phosphate isomerase), and *tpi-1* (triosephosphate isomerase). In each case RNAi against these genes completely blocked the rescuing effect of GE against mutant TDP-43 paralysis (**Fig. 3**). Thus glucose metabolism is essential for the protective effects of GE against proteotoxicity.

Glucose reduces lifespan and progeny numbers

The toxic effects of high dietary glucose are well documented and are known to decrease lifespan in worms (Lee et al., 2009; Mondoux et al., 2011). In *C. elegans* long-lived *daf-2* insulin/IGF receptor mutants and wild type worms grown from adulthood under GE

conditions are reported to have decreased lifespan, but GE did not further reduce the lifespan of the short-lived FOXO transcription factor *daf-16* mutants (Lee et al., 2009). We grew worms under early and late GE conditions and confirmed that both early and late GE reduced the lifespan of *daf-2* mutants (**Supplementary Fig. 5A**, and **Supplementary Table 1**) but did not reduce the lifespan of wild type N2 worms or *daf-16* mutants (**Supplementary Fig. 5B,C** and **Supplementary Table 1**). Furthermore we observed no reduction from GE on the lifespan of 128Q, mTDP-43, mFUS, or $A\beta_{1-42}$ transgenic animals (**Supplementary Fig. 5D-G**, and **Supplementary Table 1**).

We further explored glucose's negative effects on lifespan and protective effects against proteotoxicity by testing different concentrations of glucose in lifespan and proteotoxicity assays. We tested wild type N2 worms and long-lived *daf-2* mutants grown from hatching on plates with different concentrations of glucose including: no glucose, 0.1%, 1%, 2%, 4% and 10% GE. In N2 worms we observed no negative effects of GE on lifespan at concentrations of 0.1%, 1%, or 2% while significant decreases in lifespan were observed for 4% and 10% GE (**Fig. 4A**). For *daf-2* worms we observed that all concentrations of GE reduced lifespan with the most severe effects noted for 4% and 10% GE (**Fig. 4B**). Thus, GE has a dose dependent effect on lifespan but there may also be a contribution from the duration of exposure; the apparent negative effects of 0.1%, 1% and 2% GE in *daf-2* worms may not have time to manifest in the shorter lived N2 strain. Looking at the GE concentrations and proteotoxicity we observed that 0.1% and 1% GE had no effect on the rate of paralysis for mTDP-43 worms while 2%, 4% and 10% GE all significantly reduced mTDP-43 toxicity (**Fig. 4C**). Finally, glucose is reported to reduce progeny numbers of wild type worms (Lee et al., 2009; Mondoux et al., 2011) and we observed all concentrations of glucose reduced brood size

with the greatest effects for 4% and 10% GE (**Fig. 4D**). These data suggest glucose has dose dependent effects and that the protective effects of GE against proteotoxicity can be uncoupled from the negative effects on lifespan and reproduction.

Glycerol does not reduce neuronal dysfunction

Besides its role as an energy source in glycolysis, glucose can be metabolized to glycerol, and increased glycerol levels are believed to prevent the aggregation of proteins damaged during hypertonic stress (Choe and Strange, 2008; Lamitina et al., 2004). We observed that N2 worms exposed to GE had increased concentrations of internal glycerol, as did worms exposed to glycerol on plates (**Supplementary Fig. 6A,B**). To directly test if glycerol was responsible for neuroprotection we grew mTDP-43 worms on plates supplemented with 2% glycerol but saw no reduction in paralysis compared to untreated controls (**Supplementary Fig. 6C**). Thus we conclude the neuroprotection observed from GE is not due to a concomitant increase of internal glycerol levels.

Additional sugars reduce proteotoxicty

To learn if neuroprotection was limited to glucose we examined several other sugars including sucrose, galactose and fructose. We observed that all three sugars reduced paralysis phenotypes of mTDP-43 worms although galactose was the least effective (**Supplementary Fig. 7A**). Next we looked at lifespan and consistent with what we observed for glucose all three sugars reduced the lifespan of wild type N2 worms (**Supplementary Fig. 7B**). Thus the neuroprotective and lifespan limiting phenotypes we observe may be a feature common to many sugars.

Glucose restores protein homeostasis and protects against environmental stress

We further explored the notion that glucose reduces protein misfolding with a number of misfolding sensors by using worms with temperature sensitive (*ts*) destabilizing mutations including: *gas-1*, a mitochondrial complex I subunit, *let-60*/RAS, *unc-15*/paramyosin and *unc-54*/myosin. These animals are viable at the permissive temperature (15°C) but their gene products are impaired at restrictive temperatures (20°C or higher) and produce a range of phenotypes including sterility, lethality, or impaired movement (Gidalevitz et al., 2006). When these *ts* mutants were grown on GE plates from hatching and shifted to non-permissive temperatures as adults and scored, we observed that GE rescued many *ts* phenotypes. Sterility for *gas-1* and *let-60* mutants at 25°C was rescued by GE (**Fig. 5A,B**). Furthermore, GE rescued the slow movement phenotype of *unc-15* (**Fig. 5C**) and *unc-54* (**Supplementary Fig. 8**) mutants at all temperatures tested. These data suggest that glucose has a previously unrecognized ability to counteract protein misfolding.

Looking beyond genetically encoded proteotoxicity and given the links between metabolism, longevity and the cellular stress response, we wondered if GE protected against environmentally induced protein damage. Elevated temperatures can cause widespread protein damage within the cell (Morimoto, 2008) and we observed that worms subjected to GE from hatching were more resistant to heat-induced mortality than worms maintained on normal plates (**Fig. 5D**). Juglone is a natural compound from the black walnut tree that increases intracellular concentrations of superoxide resulting in oxidative stress and covalent damage to proteins (Ferguson et al., 2010; Van Raamsdonk and Hekimi, 2009). Juglone causes near complete mortality of wild type worms by 14 hours in our assay and wild type N2 worms

subjected to GE from hatching and transferred to juglone plates as young adults were highly resistant to juglone-induced mortality (**Fig. 5E**). GE also protected wild type worms against hypertonic stress (**Fig. 5F**). Hypertonic stress from high NaCl concentrations is known to cause extensive protein damage that can be assayed by staining for ubiquitin conjugation (Choe and Strange, 2008). GE greatly reduced the amount of high molecular weight ubiquitin conjugates compared to worms grown on NaCl alone (**Fig. 5G**). These data suggest that glucose protects proteins against misfolding and damage from multiple environmental stresses.

We next directly tested if GE could reduce the amount of misfolded proteins. Worm strains that express GFP in the cytoplasm (*sod-3p::GFP*) or in the mitochondria (*myo-3p::GFP*^{mt}) were subjected to stress resulting in compartmental specific protein misfolding (Yoneda et al., 2004). Tunicamycin is a compound that interferes with the ER-specific N-linked glycosylation leading to ER stress and exacerbates protein misfolding (Yoneda et al., 2004). Immunoblot analysis of *sod-3p::GFP* worms grown on tunicamycin plates showed a large amount of the GFP signal resided in the pellet or insoluble fraction indicating protein misfolding had occurred and that treatment with GE greatly reduced the GFP signal in the insoluble fractions (**Fig. 5H**). Similarly, ethidium bromide disrupts protein processing in the mitochondria leading to increased protein misfolding (Yoneda et al., 2004). Immunoblotting from *myo-3p::GFP*^{mt} worms grown on ethidium bromide plates showed a large GFP signal in the insoluble fraction and that treatment with GE reduced the amount of insoluble proteins (**Fig. 5I**). These data show that GE has a cellular-wide ability to counteract protein misfolding and reduce the amount of insoluble proteins.

Neuroprotection by glucose requires DAF-16 and HSF-1

The accumulation of misfolded proteins is a hallmark of neurodegeneration and a natural consequence of aging (David et al., 2010). Among other mechanisms, cells utilize chaperone networks to maintain protein homeostasis and central to this are the Insulin/IGF and Heat Shock Factor 1 (HSF1) pathways (Morimoto, 2008). In worms, downstream from insulin/IGF signalling via *daf-2* are *daf-16* and *hsf-1*, both of which have been shown to modulate the aggregation and toxicity of misfolded proteins (Cohen et al., 2006; Hsu et al., 2003; Morley et al., 2002; Parker et al., 2005; Zhang et al., 2011). To directly test their role in glucose-mediated neuroprotection we crossed our mTDP-43 transgenics with the loss of function mutants *daf-16(mu86)* and *hsf-1(sy441)*. Mutation in either gene abolished the protective effects of glucose compared to controls (**Fig. 6A,B**). Mutation in *daf-16* or *hsf-1* also blocked the protective activity of glucose against motor neuron degeneration (**Fig. 6C,D**) and suppressed glucose's capacity to reduce the levels of insoluble TDP-43 mutant proteins (**Fig. 6E,F**). These data suggest that GE requires the protein homeostasis activities of *daf-16* and *hsf-1* to protect neurons against protein misfolding. Multiple glucose concentrations were tested but none of them further reduced the lifespan of *daf-16* and *hsf-1* mutants suggesting these genes are downstream effectors of glucose activity (**Supplementary Fig. 9A,B**). Using N2 worms, we tested if GE affected transcription of *daf-16* and *hsf-1*, plus a number of *daf-16* and *hsf-1* transcriptional targets, but observed no difference between GE and non-treated controls (**Supplementary Fig. 9C-E**). These data suggest that GE does not induce the transcription of protein quality control genes but may generally require a functional protein homeostasis network for its neuroprotective properties. To confirm this idea we examined if deletion of genes required for additional protein homeostasis mechanisms were required for

protection against mTDP-43 paralysis. *ire-1* encodes the *C. elegans* orthologue of the Endoplasmic Reticulum (ER) transmembrane protein inositol-requiring kinase 1 (IRE1) and is essential for a branch of the ER unfolded protein response (Calfon et al., 2002). Mutation of *ire-1* blocks the rescuing effects of glucose against mTDP-43 (**Supplementary Fig. 10A**). WWP-1 is an E3 ubiquitin ligase important for ubiquination and proteasomal degradation of proteins (Carrano et al., 2009) and mutation in *wwp-1* also blocks the protective effects of glucose against mutant TDP-43 (**Supplementary Fig. 10B**). Thus, GE likely employs multiple protein homeostasis mechanisms to reduce the toxicity of misfolded proteins.

DISCUSSION

Model organisms are providing insights into the links between metabolism, longevity and age-dependent pathologies. DR is a well-studied lifespan-enhancing phenomenon under examination for its potential to delay age-dependent afflictions. However DR failed to ameliorate neuronal phenotypes in our polyQ and ALS models leading us to question the role of DR mimetics as therapeutics for neurodegeneration. A major mitigating factor may be the relative effects of DR in different tissues; we observed no benefit from DR in neuronal proteotoxicity models, but we and others have observed the protective effects of DR against proteotoxicity in muscle based expression systems (Steinkraus et al., 2008). Indeed there is evidence that *C. elegans* muscles and neurons are not equivalent in their chaperone activity in handling protein misfolding during aging (Kern et al., 2010).

The inability of DR to delay neuronal proteotoxicity in our systems led us to investigate glucose metabolism. Our work identifies a new role for the ubiquitous energy molecule glucose in protein folding and neuroprotection. Glucose metabolism is central to biology and

the maintenance of glucose homeostasis has profound effects on development, health, fertility and lifespan of many organisms. Work with simple models like *C. elegans* is beginning to shed light on conserved aspects of excess glucose intake, or the glucose stress response (Lee et al., 2009; Mondoux et al., 2011; Schulz et al., 2007). In worms elevated glucose intake has pleiotropic effects with negative impacts on lifespan, fertility and dauer formation (Lee et al., 2009; Mondoux et al., 2011). In humans excessive intake of glucose and other sugars is associated with a number of health problems including obesity, type II diabetes and neuronal toxicity (Giacco and Brownlee, 2010). Importantly, our data demonstrate that the negative effects on lifespan from excessive glucose can be separated from neuroprotection. We clearly show dose-dependent effects where neurons expressing toxic proteins respond favourably to glucose even though high glucose concentrations lead to decreased lifespan among other outcomes.

Our data suggest that glucose achieves neuroprotection by reducing the levels of misfolded mutant proteins within the cell. This activity requires the network of chaperone proteins under control of the stress and cellular survival genes *daf-16* and *hsf-1* since deletion of either of these genes blocked glucose's neuroprotective effects. These findings are consistent with the known role of these genes in protecting against neuronal proteotoxicity (Parker et al., 2005; Zhang et al., 2011). Even though *daf-16* and *hsf-1* are required for neuroprotection, GE does not increase the expression of these genes. Furthermore, decreased lifespan from GE is dependent on *daf-16* and *hsf-1* since lifespan is not further reduced in these mutants consistent with previous findings (Lee et al., 2009).

In simple systems like worms mutations that extend lifespan are often associated with increased cellular stress resistance (Kenyon, 2010). A consequence of these life-extending

mutations is often extensive metabolic reprogramming (Artal-Sanz and Tavernarakis, 2008) and our work suggests that certain metabolic outcomes are more favourable to neurons expressing toxic proteins than others. Future work carefully examining the metabolic profiles of diseased and aging neurons may help delineate pathogenic mechanisms and potential therapeutic approaches. In conclusion we describe an unexpected role for glucose in cellular protection and strategies to supplement neuronal glucose levels may reduce neuronal proteotoxicity.

Materials and Methods

Worm strains and genetics

Standard methods of culturing and handling worms were used. Worms were maintained on standard NGM plates streaked with OP50 *E. coli.* In some experiments D-glucose or L-glucose was added to NGM plates (All products from Sigma). All strains were scored at 20°C unless indicated. Mutations and transgenes used in this study were: *daf-2(e1370), daf-16(mu86), dvIs2[unc-54::Aβ_{1-42};rol-6(su1006*)], *eat-2(ad465), gas-1(fc21), igIs1[mec-3::htt57Q128::CFP;mec-7::YFP;lin-15(+)], igIs245[mec-3::htt57Q19::CFP;mec-7::YFP;lin-15(+)], let-60(ga89), muIs84[sod-3::GFP], unc-15(e1402), unc-54(e1301), zcIs14[myo-3::GFP(mit)], xqIs133[unc-47::TDP-43[A315T];unc-119(+)]* and *xqIs98[unc-47::FUS[S57Δ]*. Most of the strains were obtained from the *C. elegans* Genetics Center (University of Minnesota, Minneapolis). Mutants or transgenic worms were verified by visible phenotypes, PCR analysis for deletion mutants, sequencing for point mutations or a combination thereof. Deletion mutants were out-crossed a minimum of three times to wild type worms prior to use.

Worm behavioural tests

Touch tests were conducted and scored as previously described (Parker et al., 2007). Briefly, for late GE experiments 128Q animals were transferred to glucose plates at the L4 stage while early GE animals were exposed to glucose from hatching. The animals were assayed for touch responsiveness at the L4 stage and subsequently tested at adult days 1-11. For worms expressing Aβ_{1-42}, mutant TDP-43 or FUS animals were counted as paralyzed if they failed to

move upon prodding with a worm pick. Worms were scored as dead if they failed to move their head after being prodded in the nose and showed no pharyngeal pumping. For the paralysis tests worms grown on glucose from hatching were transferred to the appropriate experimental plate for scoring.

Fluorescence microscopy

For scoring of neuronal processes from 128Q, mTDP-43 and mFUS transgenics, animals were selected at days 1, 5 and 9 of adulthood for visualization of mechanosensory (128Q) or motor neurons (mTDP-43 and mFUS) processes *in vivo*. Animals were immobilized in M9 with 5 *mM* levamisole and mounted on slides with 2% agarose pads. Neurons were visualized with a Leica 6000 and a Leica DFC 480 camera. A minimum of 100 animals was scored per treatment over 4-6 trials. The mean and SEM were calculated for each trial and two-tailed t-tests were used for statistical analysis.

Measurement of glucose, glycerol and pyruvate

Extracts from synchronized worms were done as described previously (Lamitina et al., 2004; Lee et al., 2009). Glucose levels were quantified with the Amplex Red Glucose/Glucose Oxidase kit (Molecular Probes-Invitrogen, CA, USA). Glycerol levels were quantified with Glycerol Free Reagent (Sigma, MO, USA). Pyruvate levels were quantified with a Pyruvate assay kit (Biovision, CA, USA). Protein levels were quantified with a BCA assay kit and used for normalization of glucose or pyruvate levels. The mean and SEM were calculated for each trial and two-tailed t-tests were used for statistical analysis.

Stress assays

For oxidative stress tests, worms were grown on NGM or NGM + 2% glucose and transferred to NGM plates + 240 μM juglone at adult day 1. For thermal resistance worms were grown on NGM or NGM 2% glucose and put at 37°C as adult day 1. For osmotic resistance worms were grown on NGM or NGM 2% glucose and put on 400 mM NaCl plates at adult day 1. For all assays, worms were evaluated for survival every 30 min for the first 2 hours and every 2 hours after up to 14 hours. Nematodes were scored as dead if they were unable to move in response to heat or tactile stimuli. For all tests worms, 20 animals/plate by triplicates were scored.

Lifespan assays

Worms were grown on NGM or NGM + glucose and transferred on NGM-FUDR or NGM-FUDR + glucose. 20 animals/plate by triplicates were tested at 20°C from adult day 1 until death. Worms were declared dead if they didn't respond to tactile or heat stimulus. For bacterial deprivation experiments hypochlorite-extracted N2 worms were grown on UV killed OP50 bacteria. LB cultures were streaked with UV-killed bacteria and grown overnight to confirm their inability to grow. At day 1 of adulthood N2 worms were transferred to NGM plates without bacteria. Worms were transferred to new NGM plates without bacteria in a flow hood to avoid bacterial contamination that could act as a food source.

RNAi Paralysis tests

RNAi-treated strains were fed with *E. coli* (HT115) containing an Empty Vector (EV), *enol-1(*T21B10.2*), tpi-1(*Y17G7B.7) *and gpi-1(* Y87G2A.8) RNAi clones from the ORFeome RNAi library. RNAi experiments were performed at 20°C. Worms were grown on either NGM

or NGM + 2% glucose both enriched with 1 mM Isopropyl-b-D-thiogalactopyranoside (IPTG). Worms were scored for paralysis as described before from adult days 1 to 12.

Progeny tests (glucose dosage)

For scoring progeny under different glucose conditions, 10 L4 worms were grown on NGM (0% glucose) plates enriched with either 0.1%, 1%, 2%, 4% or 10% glucose and placed at 20°C. Over the next two days individual worms were transferred on new plates and L1 larvae were scored for each plate.

Bacterial deprivation experiments

Following a previously described protocol (Kaeberlein et al., 2006) hypochlorite-extracted worm population was grown on a UV killed bacteria to avoid any contamination. Worms were transferred to NGM plates with no bacteria and tested from adult day 1 to adult day 12. Nematodes were moved to a new empty plate every day to avoid any bacterial contamination that could act as a food source. Worms were scored as paralyzed if they were not able to move in response to tactile stimulus and as dead if they were not able to move their head after tactile stimulus. All the experiment were run at 20°C, 20 worms/plate by triplicates. Touch tests were done as described above.

Temperature sensitive strains experiments

For scoring *gas-1(fc21)* and *let-60(ga89)* progeny, five synchronized L4 worms were grown on NGM plates with or without 2% glucose (1 animal/plate) and were placed at 15, 20, or 25°C overnight to lay eggs. The next two days the worms were individually transferred to new

plates: L1 worms were scored for each plate. For the *unc-15(e1402)* and *unc-54(e1301)* slow movement phenotypes sixty synchronized L4 animals were grown from hatching on NGM plates with or without 2% glucose (20 animals/plate) and were placed at 20°C. Over twelve days, worms were transferred to a new plate everyday, placed into a 1 cm diameter circle and after 5 minutes animals that did not move from the circle were scored. The mean and SEM were calculated for each trial and two-tailed t-tests were used for statistical analysis.

Protein ubiquitination after NaCl treatment

N2 worms were grown on NGM or NGM + 2% glucose plates, washed with M9 and grown for 24h on 400mM NaCl plates. The next day worms were collected with M9 and pellets were made for western blotting.

GFP solubility experiments

For *sod-3::GFP*, worms were grown on NGM or NGM + 2% glucose plates, washed with M9 and grown for 24h on 5mg/ml Tunicamycin plates. The next day worms were collected with M9 and pellets were made for western blotting. For *myo-3::GFP*^{mt}, worms were grown on NGM or NGM + 2% glucose plates, washed with M9 and grown for 24h on 125mg/ml Ethidium Bromide plates. The next day worms were collected with M9 and pellets were made for western blotting.

Worm lysates

Worms were collected in M9 buffer, washed 3 times with M9 and pellets were placed at -80°C overnight. Pellets were lysed in RIPA buffer (150mM NaCl, 50mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) + 0.1% protease inhibitors (10 mg/ml leupeptin, 10

mg/ml pepstatin A, 10 mg/ml chymostatin LPC;1/1000). Pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged at 16000*g*. Supernatants were collected.

For 128Q, TDP-43 and FUS transgenics soluble/insoluble fractions, worms were lysed in Extraction Buffer (1M Tris-HCl pH 8, 0.5M EDTA, 1M NaCl, 10% NP40 + protease inhibitors (LPC;1/1000)). Pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged at 100,000*g* for 5 min. The supernatant was saved as "S" (Soluble) fraction and the remaining pellet was resuspended in extraction buffer, sonicated and centrifuged at 100,000g for 5 min. The remaining pellet was resuspended into 100 µl of RIPA buffer, sonicated until the pellet was resuspended in solution and saved as sample "P" (pellet). For *sod-3::GFP* and *myo-3::GFP*^{mt} soluble/insoluble fractions, worms were lysed in Extraction Buffer (1M Tris-HCl pH 8, 0.5M EDTA, 1M NaCl, 10% NP40 + protease inhibitors (LPC;1/1000)). Pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged 16,000g. The supernatant was saved as "total protein extract" and the remaining pellet was resuspended in extraction buffer, sonicated and centrifuged at 100,000g for 5 min. The supernatant was saved as "S" (Soluble) fraction and the remaining pellet was resuspended in extraction buffer, sonicated and centrifuged at 100,000g for 5 min. The remaining pellet was resuspended into 100 µl of RIPA buffer, sonicated until the pellet was resuspended in solution and saved as sample "P" (pellet).

Protein quantification

All supernatants were quantified with the BCA Protein Assay Kit (Thermo Scientific. MA, USA) following the manufacturer instructions.

Immunoblot

Worm RIPA samples (175 μg/well) were resuspended directly in 1x Laemmli sample buffer, migrated in 12.5% or 10% polyacrylamide gels, transferred to nitrocellulose membranes (BioRad, CA, USA) and immunoblotted. Antibodies used: rabbit anti-GFP(1:1000, ab6556, AbCam, MA, USA), rabbit anti-TDP-43 (1:200, Proteintech, IL, USA), rabbit anti-FUS/TLS (1:200, AbCam, MA, USA), mouse anti-ubiquitin (1:500; BD, NJ, USA), mouse anti Amyloid Beta 6E10 (1:1000, Calbiochem, Merck, GE) and mouse anti-actin (1:10000 MP Biomedicals, OH, USA). Blots were visualized with peroxidase-conjugated secondary antibodies and ECL Western Blotting Substrate (Thermo Scientific, MA, USA). Densitometry was performed with Photoshop (Adobe, CA, USA).

Statistics

Statistics of nematode touch-test data were performed using one-way ANOVA, with correction for multiple testing by Tukey-Kramer's Multiple Comparison Test. Data were expressed as mean ± SD for >100 nematodes in each group. All experiments were repeated at least three times. $P < 0.05$ was considered significant. For paralysis and stress-resistance tests, survival curves were generated and compared using the Log-rank (Mantel-Cox) test, and a 60-100 animals were tested per genotype and repeated at least three times.

ACKNOWLEDGMENTS

We would like to thank C. Néri and INSERM for the polyQ worms and the CGC for providing additional strains used in these experiments. We thank S. Peyrard and E. Bourgeois for technical support and H. Catoire for critical reading of the manuscript. J.A.P is a CIHR New Investigator. A.T. is supported by a CIHR and Huntington's Society of Canada fellowship. The CHUM Foundation, a CIHR Catalyst Grant, the Frick Foundation for ALS Research, and the Bernice Ramsay Discovery Grant from the ALS Society of Canada supported this work.

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SUPPORTING INFORMATION:

Supplementary Fig. 1. Worms grown on glucose plates have elevated levels of internal glucose and pyruvate.

Supplementary Fig. 2. Glucose has no effect on wild type polyglutamine, TDP-43 or FUS strains.

Supplementary Fig. 3. Glucose reduces amyloid-b toxicity.

Supplementary Fig. 4. L-glucose does not reduce neuronal proteotoxicity in *C. elegans* transgenics.

Supplementary Fig. 5. GE reduces lifespan of long-lived *daf-2* mutants but not strains with shorter lifespans.

Supplementary Fig. 6. Glycerol does not reduce mutant TDP-43 toxicity.

Supplementary Fig. 7. Other sugars reduce proteotoxicity and lifespan.

Supplementary Fig. 8. Glucose rescues *unc-54* mutant phenotypes.

Supplementary Fig. 9. Glucose does not affect *daf-16*, *hsf-1* or target gene expression.

Supplementary Fig. 10. Glucose neuroprotection requires protein homeostasis genes.

Supplementary Table 1 Lifespan analysis for all experiments.

FIGURE LEGENDS

Figure 1 Dietary restriction does not reduce neuronal proteotoxicity

(**A**) Bacterial deprivation (BD) of adult animals increased the lifespan compared to normally fed (*ad libitum*) wild type N2 animals.

(**B**) BD delayed the paralysis phenotype caused by the expression of $A\beta_{1-42}$ in worm body wall muscles compared to *ad libitum* transgenics ($P < 0.001$).

(**C**) BD had no effect on the loss of touch sensitivity induced by the expression of mutant polyglutamine (128Q) in worm mechanosensory neurons compared to *ad libitum* transgenics.

(**D**) The progressive paralysis phenotype caused by the expression of mutant TDP-43 in worm motor neurons was unaffected by BD conditions compared to *ad libitum* transgenics.

(**E**) The *eat-2(ad465)* mutation reduces feeding and induces dietary restriction conditions in worms but had no effect on 128Q toxicity in worm mechanosensory neurons.

(**F**) Mutant TDP-43 toxicity in worm motor neurons was unaffected by the *eat-2(ad465)* mutation.

Figure 2 Glucose enrichment delays proteotoxicity in *C. elegans.*

(**A**) Touch sensitivity was improved by early glucose enrichment (GE) for L4 stage 128Q transgenics and for 128Q animals subjected to either early or late GE at adult days 1-3 relative to untreated 128Q worms (*P<0.05).

(**B**) GE reduced axonal degeneration phenotypes in adult day 9 128Q animals (*P<0.05 compared to untreated 128Q).

(**C**) Soluble supernatant (S) and insoluble pellet (P) fractions from 128Q worms grown with or without GE were compared by immunoblotting against GFP. GE reduced the amount of insoluble 128Q::CFP fusion protein levels compared to 128Q worms grown without GE.

(**D**) GE reduced mTDP-43 associated age-dependent paralysis (P<0.001 for GE transgenics versus untreated transgenics).

(**E**) Early GE reduced mTDP-43 associated age-dependent motor neuron degeneration. (*P<0.001 at days 5 and 9 of adulthood compared to untreated transgenics).

(**F**) GE reduced the amount of insoluble mTDP-43 protein in pellet (P) fractions compared to TDP-43 worms not grown on GE.

(**G**) GE reduced mFUS associated paralysis (P<0.001 for early or late GE versus untreated transgenics).

(**H**) Early GE reduced the progressive neuronal degeneration observed in mFUS transgenics (*P<0.001 at days 5 and 9 compared to untreated transgenics).

(**I**) GE reduced the amount of insoluble mFUS protein in pellet fractions compared to mFUS worms not grown on GE.

Figure 3 Glycolysis genes are required for glucose neuroprotection

mTDP-43 worms grown in plates with 2% glucose enrichment (GE) and empty vector (EV) RNAi had reduced levels of paralysis compared to untreated EV mTDP-43 worms ($P<0.001$). RNAi against genes involved in glycolysis including (**A**) *enol-1*, (**B**) *gpi-1* or (**C**) *tpi-1* all blocked the protective effects of GE compared to EV + GE controls ($P<0.001$).

Figure 4 Dose-dependent effects of glucose on lifespan and proteotoxicity.

Strains were grown on plates from hatching with either 0% (NGM), 0.1%, 1%, 2%, 4% or 10% glucose enrichment (GE).

(**A**) Wild type N2 worms grown on 0.1%, 1%, or 2% GE had lifespans indistinguishable from worms on regular NGM media. N2 worms grown on 4% or 10% had significantly shorter lifespans than worms grown on regular media.

(**B**) *daf-2(e1370)* worms grown on all GE concentrations had significantly decreased lifespans compared to *daf-2* worms grown on normal media. The largest decrease in lifespan was for worms grown on 4% and 10% GE plates.

(**C**) mTDP-43 worms grown on 0.1% or 1% GE had similar rates of paralysis compared to mTDP-43 transgenics grown on normal media. TDP-43 worms grown on 2%, 4%, or 10% had significantly reduced rates of paralysis compared to mTDP-43 worms grown on normal media.

(**D**) Wild type N2 worms grown on 0.1%, 1%, 2%, 4% or 10% had reduced brood sizes compared to worms grown on normal NGM media. N2 animals grown on 4% and 10% had the fewest progeny (*$P<0.001$ compared to N2 worms grown on NGM).

Figure 5 Glucose protects against protein misfolding and environmental stress.

(**A**) GE rescued the low-progeny phenotype of the temperature-sensitive *gas-1* mutant at the restrictive temperature of 25°C (*P<0.001 versus untreated *gas-1* mutants).

(**B**) GE rescued the low-progeny phenotype of the temperature-sensitive *let-60* mutant at the restrictive temperature of 25°C (*P<0.001 versus untreated *let-60* mutants).

(**C**) GE reduced the progressive, age-dependent slow-movement phenotype of *unc-15* mutants at 20°C (P<0.05 versus untreated *unc-15* mutants) and 25°C (P<0.001 versus untreated *unc-15* mutants).

(**D**) N2 worms grown on GE plates were resistant to thermal stress compared to untreated controls (P<0.001).

(**E**) Wild type N2 worms grown on GE plates were resistant to juglone-induced mortality compared to untreated controls (P<0.001).

(**F**) Glucose protected wild type N2 worms against high NaCl toxicity (P<0.001) compared to untreated controls.

(**G**) Representative western blot and quantification of high molecular weight ubiquitin conjugates of worms exposed to NaCl or grown on GE plates before exposure to NaCl stress. Treatment with GE reduced the amount of ubiquitinylation levels caused by hypertonic stress.

(**H**) Representative western blot of protein extracts (total, supernatant or pellet) from SOD-3::GFP transgenic worms exposed to tunicamycin, or tunicamycin with 2% GE. The amount of insoluble GFP in the pellet fraction was reduced in animals grown under GE conditions.

(**I**) Representative western blot of protein extracts (total, supernatant or pellet) from GFP^{mt} transgenic worms exposed to ethidium bromide ± 2% GE. The amount of insoluble GFP in the pellet fraction was greatly reduced in animals grown under GE conditions.

Figure 6 Glucose neuroprotection is *daf-16* and *hsf-1* dependant

(**A**) GE rescued mTDP-43 paralysis (P<0.001 versus non-treated mTDP-43 worms). *daf-16(mu86)* enhanced paralysis (P<0.001 versus mTDP-43 alone) and abolished the protective effect of GE against mTDP-43 associated paralysis in transgenic worms (P<0.001 mTDP-43;*daf-16(mu86)* + GE vs. mTDP-43 + GE).

(**B**) GE reduced mTDP-43 paralysis (P<0.001 versus non-treated mTDP-43 worms) and this was dependent on *hsf-1* (P<0.001 mTDP-43;*hsf-1(sy441)* + GE vs. mTDP-43 + GE)

(**C**) GE suppressed motor neuron degeneration at adult days 5 and 9 in worms expressing mTDP-43, but this protective effect was lost in animals mutant for *daf-16* or (**D**) *hsf-1* (*P<0.001 versus mTDP-43 alone.

mTDP-43 proteins remained highly insoluble after glucose treatment in (**E**) *daf-16* and (**F**) *hsf-1* mutants.

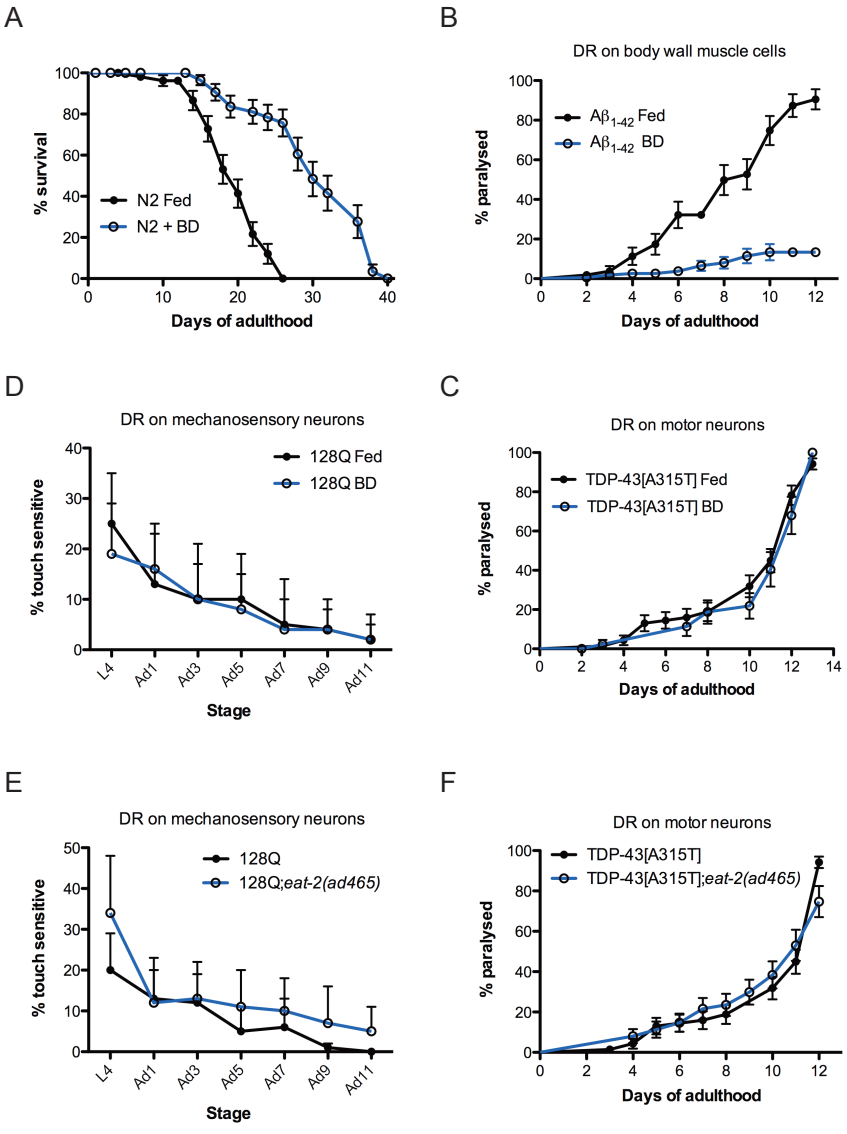

A
% survival
N2 Fed
N2 + BD
Days of adulthood
B
DR on body wall muscle cells
% paralysed
Aβ1-42 Fed
Aβ1-42 BD
Days of adulthood
D
DR on mechanosensory neurons
% touch sensitive
128Q Fed
128Q BD
L4
Ad1
Ad3
Ad5
Ad7
Ad9
Ad11
Stage
C
DR on motor neurons
% paralysed
TDP-43[A315T] Fed
TDP-43[A315T] BD
Days of adulthood
E
DR on mechanosensory neurons
% touch sensitive
128Q
128Q;eat-2(ad465)
Stage
F
DR on motor neurons
% paralysed
TDP-43[A315T]
TDP-43[A315T];eat-2(ad465)
Days of adulthood

Figure 1

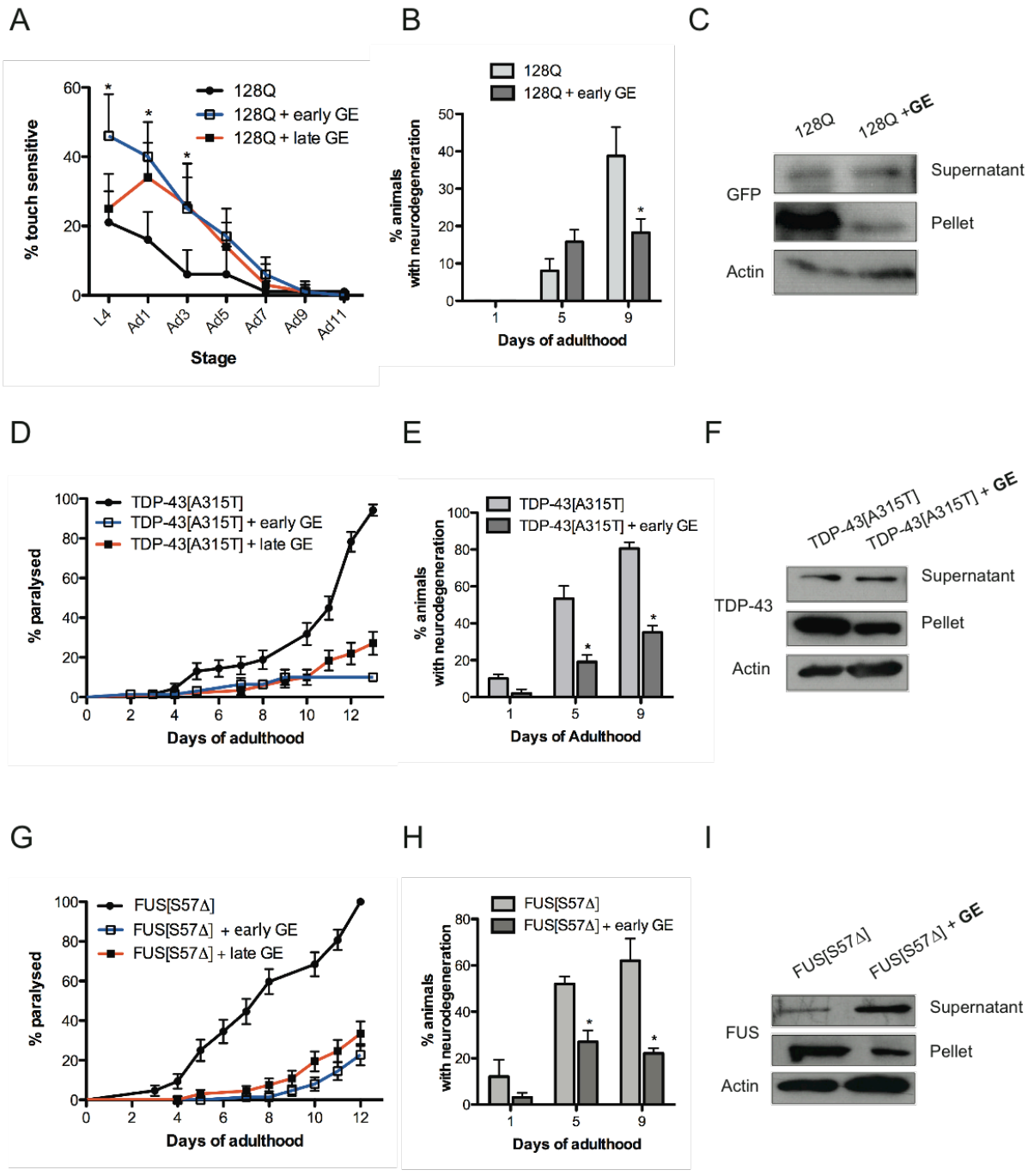
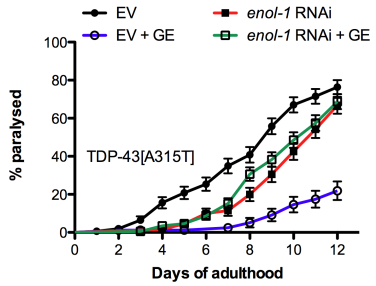
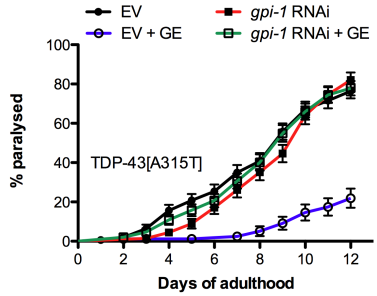

A
B
C
% touch sensitive
128Q
128Q + early GE
128Q + late GE
L4
Ad1
Ad3
Ad5
Ad7
Ad9
Ad11
Stage
% animals with neurodegeneration
Days of adulthood
128Q
128Q +GE
GFP
Supernatant
Pellet
Actin
D
E
F
% paralysed
TDP-43[A315T]
TDP-43[A315T] + early GE
TDP-43[A315T] + late GE
Days of adulthood
Days of Adulthood
TDP-43
G
H
I
FUS[S57Δ]
FUS[S57Δ] + early GE
FUS[S57Δ] + late GE
FUS

Figure 2

A


EV
EV + GE
enol-1 RNAi
enol-1 RNAi + GE
% paralysed
TDP-43[A315T]
Days of adulthood

B


EV
EV + GE
gpi-1 RNAi
gpi-1 RNAi + GE
% paralysed
TDP-43[A315T]
Days of adulthood

C

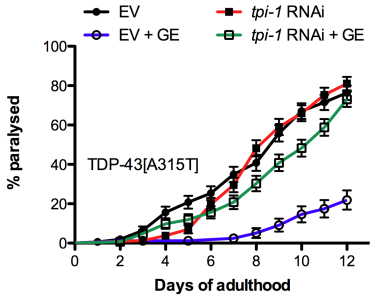

EV
EV + GE
tpi-1 RNAi
tpi-1 RNAi + GE
% paralysed
TDP-43[A315T]
Days of adulthood

Figure 3

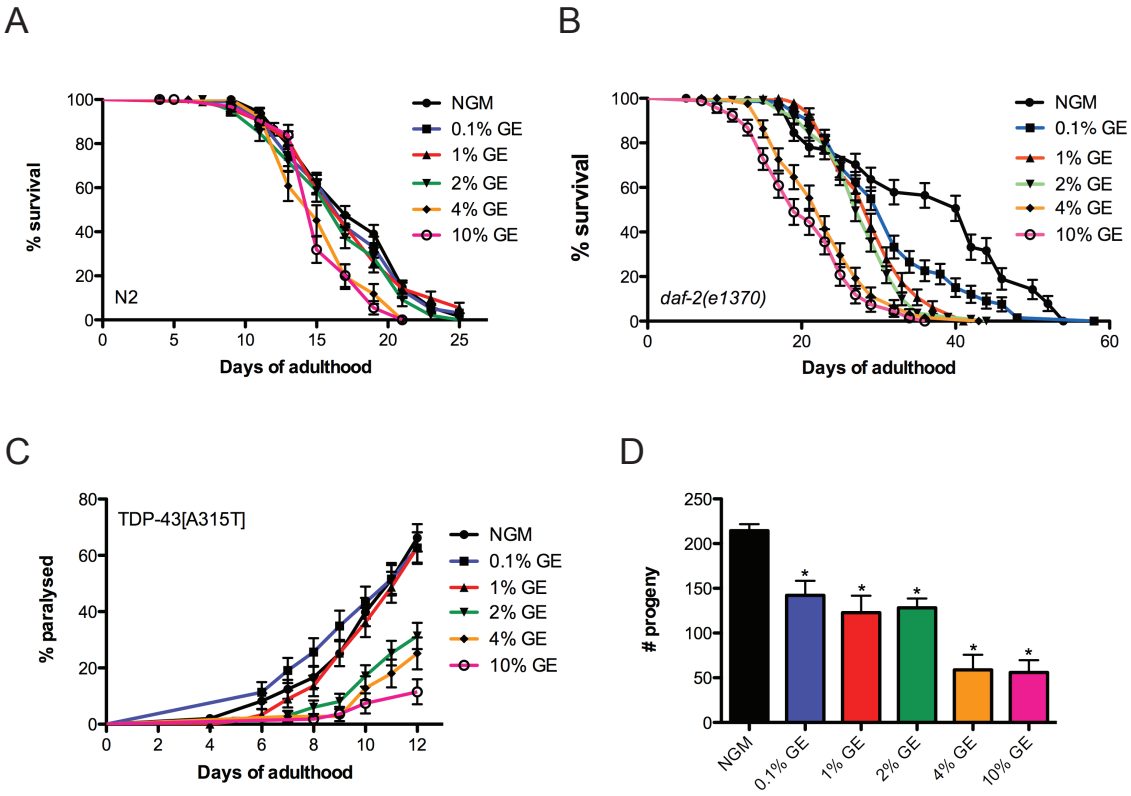

A
B
C
D
% survival
N2
Days of adulthood
NGM
0.1% GE
1% GE
2% GE
4% GE
10% GE
daf-2(e1370)
% paralysed
TDP-43[A315T]
progeny

Figure 4

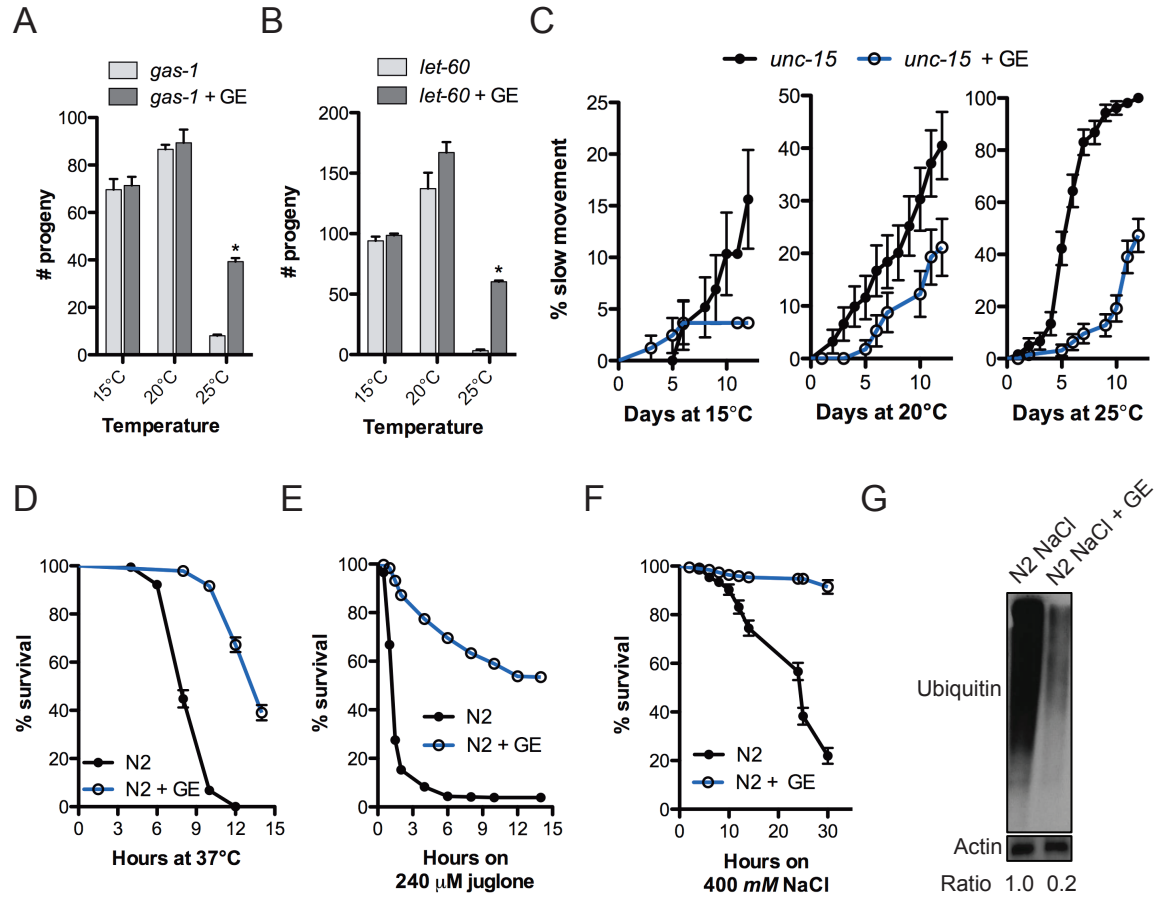

A
gas-1
gas-1 + GE
progeny
Temperature
15°C
20°C
25°C
B
let-60
let-60 + GE
progeny
Temperature
C
unc-15
unc-15 + GE
% slow movement
Days at 15°C
Days at 20°C
Days at 25°C
D
% survival
N2
N2 + GE
Hours at 37°C
E
% survival
N2
N2 + GE
Hours on
240 μM juglone
F
% survival
N2
N2 + GE
Hours on
400 mM NaCl
G
N2 NaCl
N2 NaCl + GE
Ubiquitin
Actin
Ratio 1.0 0.2
H
T sod-3::GFP
T sod-3::GFP + GE
S sod-3::GFP
S sod-3::GFP + GE
P sod-3::GFP
P sod-3::GFP + GE
GFP
I
T GFPmt
T GFPmt + GE
S GFPmt
S GFPmt + GE
P GFPmt
P GFPmt + GE
GFP

Figure 5

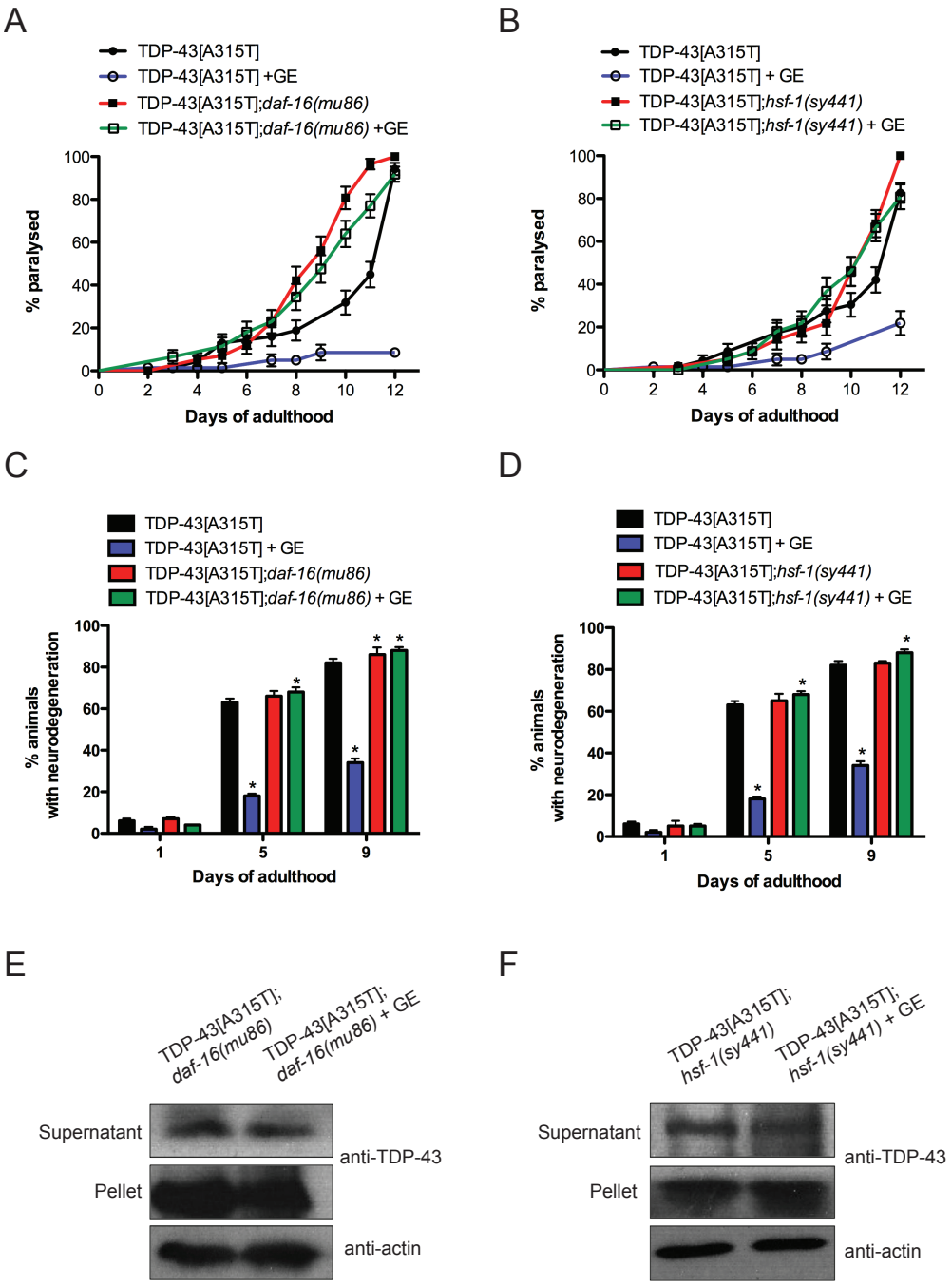
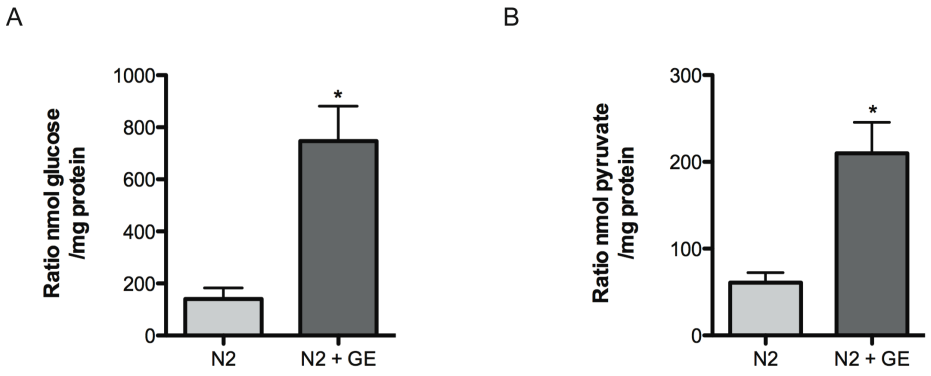

A
TDP-43[A315T]
TDP-43[A315T] +GE
TDP-43[A315T];daf-16(mu86)
TDP-43[A315T];daf-16(mu86) +GE
% paralysed
Days of adulthood
B
TDP-43[A315T]
TDP-43[A315T] + GE
TDP-43[A315T];hsf-1(sy441)
TDP-43[A315T];hsf-1(sy441) + GE
% paralysed
Days of adulthood
C
TDP-43[A315T]
TDP-43[A315T] + GE
TDP-43[A315T];daf-16(mu86)
TDP-43[A315T];daf-16(mu86) + GE
% animals with neurodegeneration
Days of adulthood
D
TDP-43[A315T]
TDP-43[A315T] + GE
TDP-43[A315T];hsf-1(sy441)
TDP-43[A315T];hsf-1(sy441) + GE
% animals with neurodegeneration
Days of adulthood
E
TDP-43[A315T]; daf-16(mu86)
TDP-43[A315T]; daf-16(mu86) + GE
Supernatant
Pellet
anti-TDP-43
anti-actin
F
TDP-43[A315T]; hsf-1(sy441)
TDP-43[A315T]; hsf-1(sy441) + GE
Supernatant
Pellet
anti-TDP-43
anti-actin

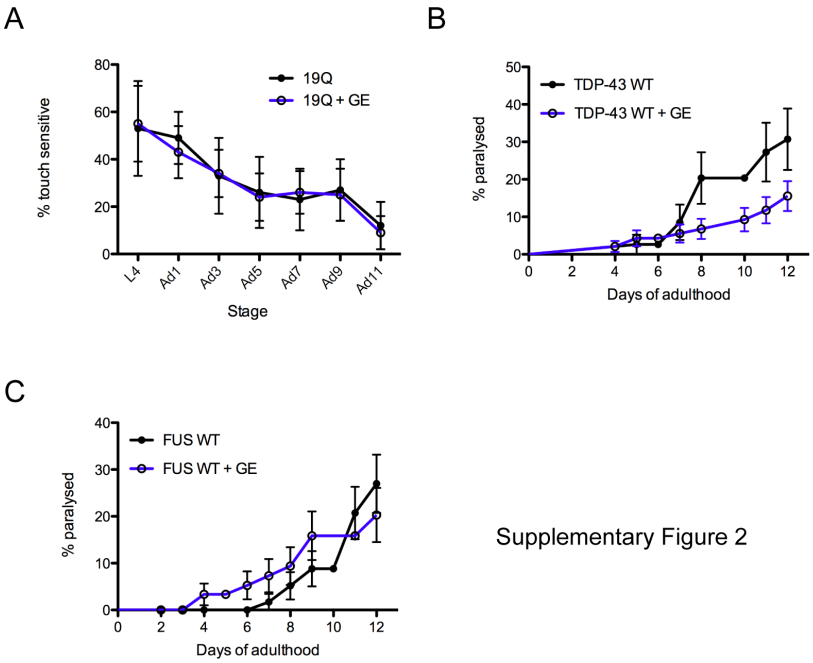
Figure 6



Supplementary Figure 1

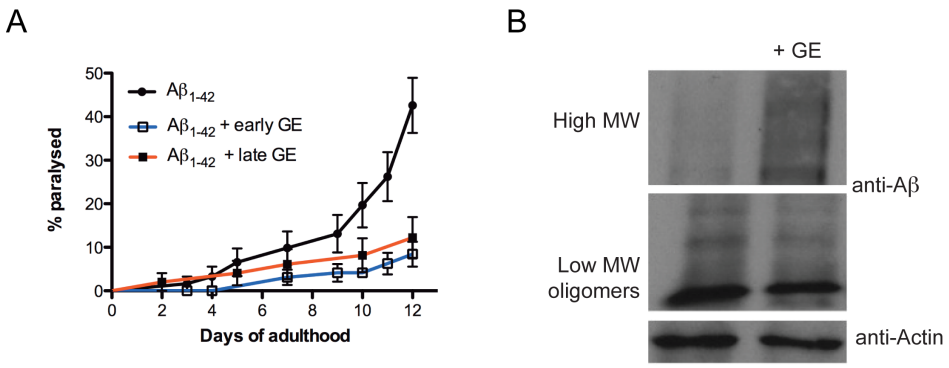
Supplementary Figure 1. Worms grown on glucose plates have elevated levels of internal glucose and pyruvate.

Worms were grown on 2% glucose plates from hatching, harvested as young adults and assayed for glucose and pyruvate levels. (A) GE increased internal glucose levels (*P< 0.001 versus untreated). (B) GE increased internal pyruvate levels (*P<0.001 versus untreated).



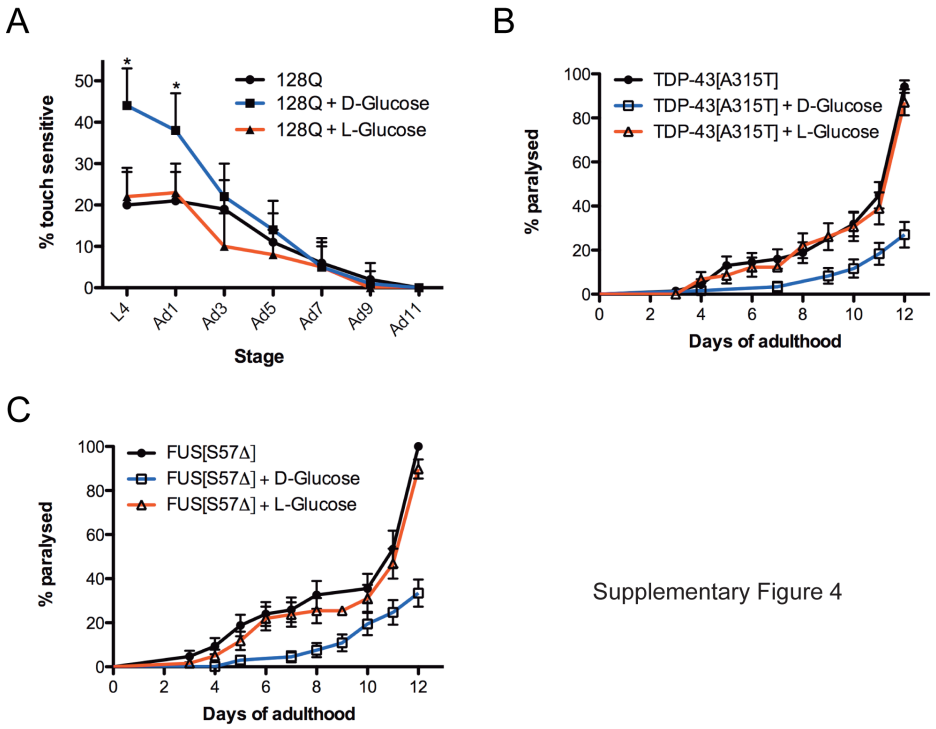
Supplementary Figure 2. Glucose has no effect on wild type polyglutamine, TDP-43 or FUS strains.

Treatment with glucose from hatching (early GE) had no effect on (A) the touch responsiveness of animals expressing 19Q in mechanosensory neurons or the rates of paralysis for strains expressing (B) wild type TDP-43 or (C) wild type FUS in motor neurons.



Supplmentary Figure 3

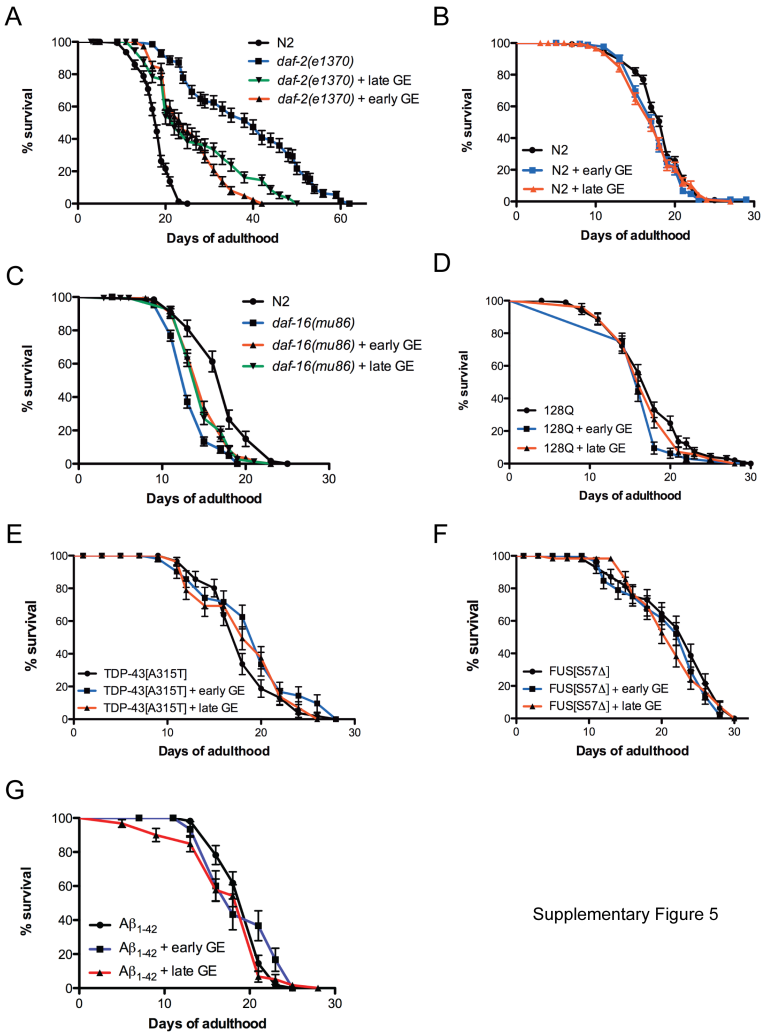
Supplementary Figure 3. Glucose reduces amyloid-β toxicity.
(A) $A\beta_{1-42}$ worms grown on either early or late GE plates had significantly reduced rates of paralysis compared to untreated control transgenics ($P<0.001$). (B) $A\beta_{1-42}$ worms exposed to GE showed a reduction in the amount of toxic low molecular weight (MW) oligomers and an increase in the amount of protective high MW species.



Supplementary Figure 4

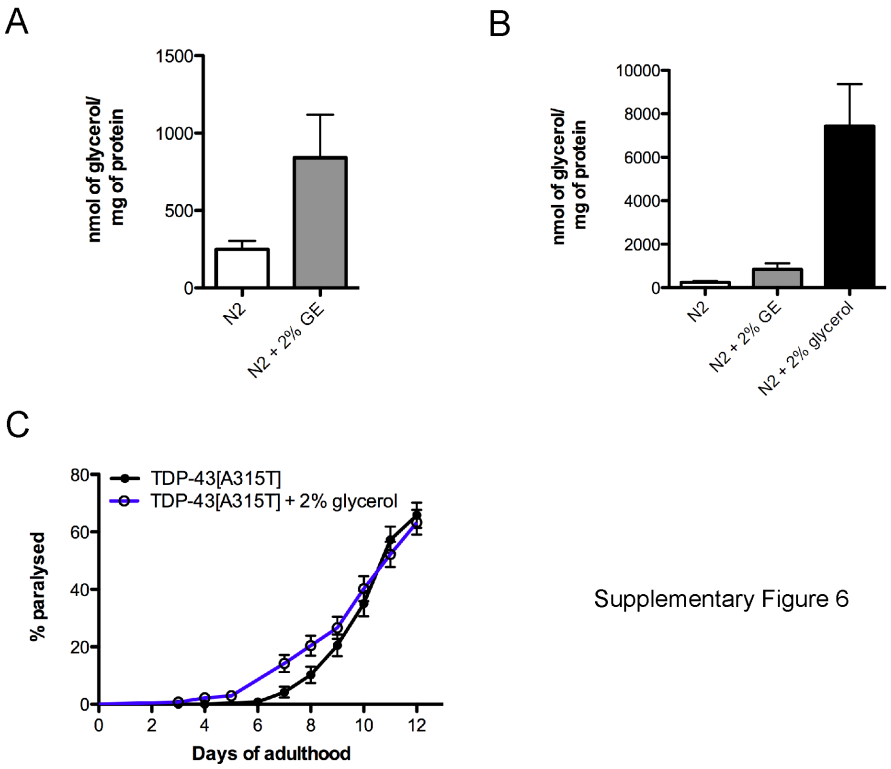
Supplementary Figure 4. L-glucose does not reduce neuronal proteotoxicity in *C. elegans* transgenics.

Animals were grown on 2% D-glucose, 2% L-glucose, or normal NGM from hatching and assayed for proteotoxicity through adulthood. (A) 2% D-glucose rescues touch insensitivity of 128Q animals at the larval L4 stage and day 1 of adulthood (*P < 0.01 versus untreated worms or animals treated with 2% L-glucose). (B) D-glucose rescues mTDP-43 induced paralysis compared to untreated worms or animals treated with 2% L-glucose (P<0.001). (C) D-glucose rescues mFUS induced paralysis compared to untreated worms or animals treated with 2% L-glucose (P<0.001)



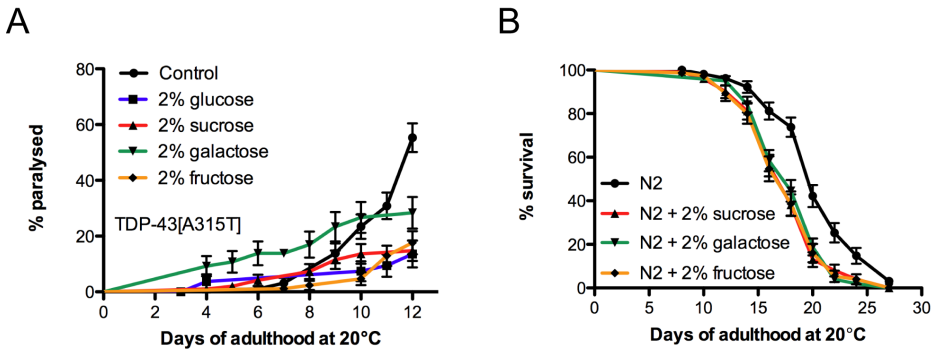
Supplementary Figure 5

Supplementary Figure 5. GE reduces lifespan of long-lived *daf-2* mutants but not strains with shorter lifespans. Lifespan was tested for multiple strains using either early or late GE (at 2% glucose). (A) Both early and late GE reduced the lifespan of long-lived *daf-2(e1370)* animals (P< 0.001 early or late GE versus untreated *daf-2* mutants). (B) The lifespan of wild type N2 animals was unaffected by early or late GE. (C) Neither early or late GE reduced the lifespan of *daf-16(mu86)* mutants. (D) There was no effect on lifespan by early or late GE for (D) 128Q, (E) mTDP-43, (F) mFUS or (G) $A\beta_{1-42}$ transgenics compared to untreated control strains.



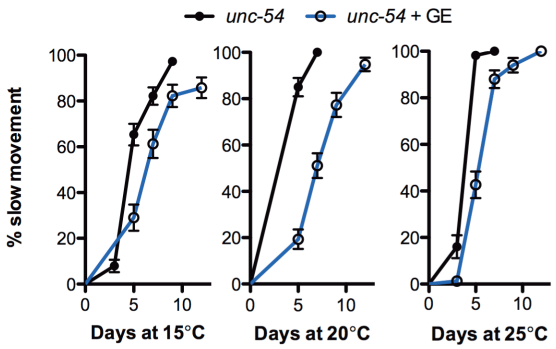
Supplementary Figure 6

Supplementary Figure 6. Glycerol does not reduce mutant TDP-43 toxicity.
(A) N2 worms exposed to 2% glucose enrichment (GE) have increased internal glycerol levels as do (B) worms exposed to 2% glycerol. (C) 2% glycerol does not reduce mutant TDP-43 paralysis



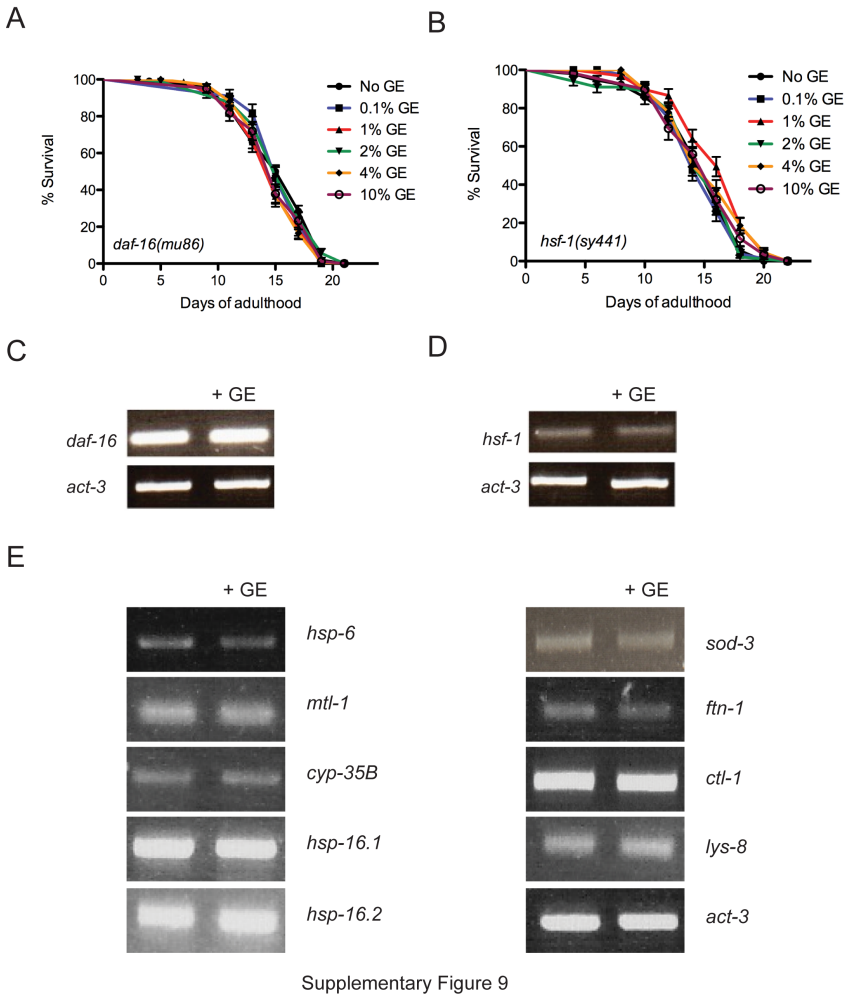
Supplementary Figure 7

Supplementary Figure 7. Other sugars reduce proteotoxicity and lifespan.
(A) Mutant TDP-43 exposed to glucose, sucrose, galactose or fructose had significantly reduced rates of paralysis compared to untreated TDP-43 worms ($P<0.001$ for glucose, sucrose and fructose vs. untreated controls, $P<0.01$ for galactose vs. untreated controls). (B) All sugars tested reduced the lifespan of N2 worms compared to untreated controls.



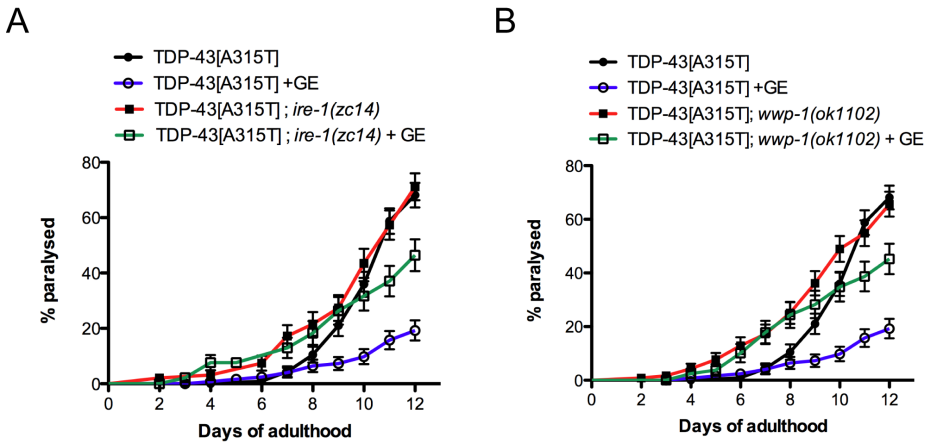
Supplementary Figure 8

Supplementary Figure 8. Glucose rescues *unc-54* mutant phenotypes.
Glucose enrichment (GE) rescued the progressive slow movement phenotype of *unc-54* mutants at all temperatures tested (P<0.001 versus untreated *unc-54* mutants).



Supplementary Figure 9

Supplementary Figure 9. Glucose does not affect *daf-16*, *hsf-1* or target gene expression. Glucose enrichment (GE) at all concentrations did not reduce the lifespan of (A) *daf-16(mu86)* or (B) *hsf-1(sy441)* mutants. 2% GE did not alter the expression of (C) *daf-16* or (D) *hsf-1*. (E) 2% GE did not alter expression levels of a number of *daf-16* and *hsf-1* target genes. Hsp-6



Supplementary Figure 10

Supplementary Figure 10. Glucose neuroprotection requires protein homeostasis genes. Glucose enrichment (GE) reduces mutant TDP-43 paralysis compared to untreated control strains but this was blocked by mutation in (A) *ire-1* or (B) *wwp-1.*

	Strains	Mean LS	p Value	75th Percentile (Days)	Max. LS	Total Number of Animals Died/Total
Figure 1	N2	19		21	26	49/65
	N2 + BD	29	<0.0001	36	40	34/80
Figure 4	N2	17		20	25	138/142
	N2 + 0.1%	17	n.s. 0.9388	20	25	94/97
	N2 + 1%	17	n.s. 0.9333	20	25	87/96
	N2 + 2%	17	n.s 0.0741	20	25	99/101
	N2 + 4%	15	<0.0001	16	21	51/57
	N2 + 10%	15	<0.0001	16	21	59/65
	daf-2(e1370)	40		45	54	73/112
	daf-2(e1370) + 0.1%	30,5	0.0006	35	58	84/118
	daf-2(e1370) + 1%	28	<0.0001	32	41	79/108
	daf-2(e1370) + 2%	27	<0.0001	29.5	44	114/117
	daf-2(e1370) + 4%	22	<0.0001	24	43	72/85
	daf-2(e1370) + 10%	18	<0.0001	24	36	73/91
Figure S5	*daf-2(e1370)*	39		49.5	62	126/151
	daf-2(e1370) + EGE	23.5	<0.0001	30	41	138/155
	daf-2(e1370) + LGE	21.5	<0.0001	34	50	128/150
	daf-16(mu86)	15		16	21	289/295
	daf-16(mu86) + EGE	14	n.s. 0,5832	16	23	147/149
	daf-16(mu86) + LGE	14	n.s. 0,2059	16	23	151/160
	N2	18		20	27	240/254
	N2 + EGE	17	n.s. 0.2639	18	29	165/176
	N2 + LGE	17	n.s 0.1415	17.5	27	166/189
	128Q	15		19	30	173/178
	128Q + EGE	16	n.s. 0.0509	15	29	152/156
	128Q + LGE	16	n.s. 0.2326	17	28	156/165
	TDP-43 [A315T]	17		17	28	54/66
	TDP-43[A315T] + EGE	19	n.s. 0.0620	21	28	39/62
	TDP-43[A315T] +LGE	18	n.s. 0.3616	21	26	50/62
	FUS[S57Δ]	13		26	30	48/63
	FUS[S57Δ] + EGE	12	n.s. 0.2750	24	28	51/63
	FUS[S57Δ] + LGE	15	n.s. 0.3745	24	30	54/62
Figure S7	N2	20		22	27	93/111
	N2 + 2% Sucrose	18	<0.0001	19	24	100/101
	N2 + 2% Galactose	18	<0.0001	19	24	101/101
	N2 + 2% Fructose	18	<0.0001	19	24	75/75

Figure S9	*daf-16(mu86)*	16		18	21	208/208
	daf-16(mu86) + 0.1%	15	n.s. 0,5812	16	19	65/66
	daf-16(mu86) + 1%	15	n.s. 0,4632	16	19	71/72
	daf-16(mu86) + 2%	15	n.s. 0,4587	16	21	89/90
	daf-16(mu86) + 4%	15	n.s. 0,3698	16	21	134/136
	daf-16(mu86)+ 10%	15	n.s. 0,3333	16	21	82/82
	hsf-1(sy441)	16		17	20	90/98
	hsf-1(sy441) + 0.1%	14	n.s. 0,0745	16	22	78/89
	hsf-1(sy441) + 1%	16	n.s. 0,0901	17	22	97/101
	hsf-1(sy441) + 2%	14	n.s. 0,4955	15	22	91/102
	hsf-1(sy441) + 4%	15	n.s. 0,4021	17	22	86/98
	hsf-1(sy441) + 10%	16	n.s. 0,3952	17	22	59/59

Supplementary Table 1. Lifespan analysis for all experiments. Related to Figure 4, and Supplementary Figures, 7 and 8 and 9. Animals that died prematurely (ruptured, internal hatching) or were lost (crawled off the plate) were censored at the time of scoring. LS (lifespan), n.s. not significant

Chapitre III :

Heritable Transmission of Stress Resistance by High Dietary Glucose in *Caenorhabditis elegans*

Tauffenberger, A., & Parker, J. A. (2014).

Heritable Transmission of Stress Resistance by High Dietary Glucose in Caenorhabditis elegans. *PLoS Genetics*, *10*(5), e1004346.

doi:10.1371/journal.pgen.1004346.s004

Heritable transmission of stress resistance by high dietary glucose in *Caenorhabditis elegans*

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ABSTRACT

Glucose is a major energy source and is a key regulator of metabolism but excessive dietary glucose is linked to several disorders including type 2 diabetes, obesity and cardiac dysfunction. Dietary intake greatly influences organismal survival but whether the effects of nutritional status are transmitted to the offspring is an unresolved question. Here we show that exposing *Caenorhabditis elegans* to high glucose concentrations in the parental generation leads to opposing negative effects on fecundity, while having protective effects against cellular stress in the descendent progeny. The transgenerational inheritance of glucose-mediated phenotypes is dependent on the insulin/IGF-like signalling pathway and components of the histone H3 lysine 4 trimethylase complex are essential for transmission of inherited phenotypes. Thus dietary over-consumption phenotypes are heritable with profound effects on the health and survival of descendants.

AUTHOR SUMMARY

Nutritional state has major effects on health and longevity, and investigations into the mechanisms of dietary restriction have taken the lion's share of recent genetic discoveries. We used *Caenorhabditis elegans* to investigate the role of diet on nematode physiology and report the surprising finding that exposure to high glucose at one generational time point has heritable effects in descendent progeny. Glucose promotes resistance against cellular stress and neurodegeneration in parental and descendent progeny, while reducing lifespan only in the parental generation. Furthermore, we found that glucose mediated protection is dependent on well known metabolic and stress response genes. Numerous strategies have evolved to ensure reproductive success in the face of changing and challenging environments. It is believed that extended lifespan phenotypes observed under dietary restriction conditions maximize an organism's survival until environmental conditions improve allowing for reproduction. We discovered a novel diet-influenced reproductive advantage; animals subjected to high dietary glucose are resistant to protein damaging stress, and this resistance is transmitted to their progeny. The trade-off for stress-resistant progeny is decreased lifespan and fecundity in the parental strain suggesting that this strategy may be adaptive under nutrient rich conditions.

INTRODUCTION

Aging is an inevitable process that affects all organisms and a better understanding of the underlying biological mechanisms is relevant to human health [1]. In nature, organisms struggle against environmental conditions to survive and hopefully reproduce. This is an energetically costly and persistent process, thus nutrient availability greatly influences an organism's life history with profound affects on survival, reproduction and lifespan. The life history of most organisms naturally consists of periods of low nutrient availability and core mechanisms have evolved to deal with nutrient stress, namely starvation or near-starvation. Research into the genetic underpinnings of nutritional state on health and longevity is an active area of research, with the mechanisms of dietary restriction taking the lion's share of recent genetic discoveries [2]. Modern industrialized societies no longer live in fear of famine, but instead live in conditions of a near perpetual feast. Unfortunately, diets high in sugar are linked to numerous health problems in humans [3]. However, we wondered why animal species will over-consume resources if given the opportunity and hypothesized there may be an adaptive benefit to such behavior.

Using *Caenorhabditis elegans* to investigate over-consumption phenotypes we discovered that exposure to high glucose concentrations at one generational time point, the parental generation, had persistent and heritable effects in descendent progeny. Glucose promotes resistance against cellular stress and neurodegeneration in parental and descendent progeny, while reducing lifespan in the parental generation only. Furthermore, we found that glucose mediated phenotypes are dependent on known metabolic genes including components of the Insulin/IGF-like pathway, the sirtuin *sir-2.1*, and AMPK, while the transgenerational inheritance of glucose-directed phenotypes are dependent on histone methylation enzymes.

Thus, dietary glucose can induce the transmission of heritable, cellular phenotypes with profound consequences on health and survival.

RESULTS

Heritable diminution of progeny from glucose exposure in the parental generation

We first focused on reproduction since in *C. elegans* the average number of progeny is a strong physiological phenotype showing transgenerational inheritance [4], and wild type N2 worms cultured under glucose enrichment (GE) conditions have reduced progeny numbers [5-7]. Consistently we observed that parental generation (P0) N2 worms exposed to GE had reduced total progeny numbers compared to untreated controls [5] (Figure 1A), and that this effect extended to the descendent F1 and F2 generations (Figure 1B-D). Thus, glucose can induce a heritable, transgenerational phenotype on progeny from a single exposure of the P0 generation.

The insulin/IGF-signalling pathway is an evolutionarily conserved network of genes regulating an organism's response to nutritional states and is a major conserved regulator of aging [2], thus we investigated its contribution to the transgenerational effects of GE on progeny numbers. DAF-16 is a forkhead transcription factor and the major downstream regulator of the insulin/IGF-like signalling pathway [8,9] and we observed that GE did not further reduce progeny in *daf-16* mutants at any generational time point (Figure 1). We then examined genes known to respond to nutritional status and interact with both *daf-16* and the insulin/IGF-like signalling pathway including *aak-2,* which encodes the alpha subunit of the AMP-activated protein kinase (AMPK) [10], and *sir-2.1* that encodes an orthologue of the histone deacetylase SIRT1 [11]. Similar to *daf-16* mutants, we observed that mutation in either *aak-2* or *sir-2.1* likewise blocked the progeny reducing effects of GE. Finally, we examined

hif-1, the *C. elegans* orthologue of mammalian hypoxia induced factor 1 (HIF1) [12], a protein that regulates glucose metabolism and the cellular response to low oxygen conditions and observed that GE continued to reduce progeny numbers in the P0 and F1 generation of *hif-1* mutants.

Exposure to high dietary glucose reduces the lifespan of wild type N2 worms, as well as the long-lived phenotype of worms with hypomorphic mutations in the gene encoding the worm's sole Insulin/IGF-like receptor DAF-2 [5-7]. We confirmed that GE reduced the lifespan of N2 and *daf-2* worms in the P0 generation but found no evidence that this was a transgenerational phenotype since the F1 and F2 descendent progeny had lifespans similar to untreated control worms (Figure S1, Table S1). In total, these data suggest that specific components of the insulin/IGF-like signalling pathway regulate the negative effects of GE on reproduction in *C. elegans* and that the heritable effects on reproduction can be separated from lifespan.

Transgenerational inheritance of resistance to oxidative stress

Despite the negative effects on fecundity and lifespan, we previously reported that GE strongly protected worms against environmental stress [5]. We tested for resistance to oxidative stress using juglone, a natural product from the black walnut tree that produces intracellular oxidative stress and decreases the survival of N2 worms [13]. Treating P0 N2 worms with glucose provided potent protection against oxidative stress induced lethality, and this protection persisted into the F1 generation of N2 worms even though these F1 animals were never exposed to glucose (Figure 2A). Glucose-mediated resistance against oxidative stress was not transmitted further since the F2 generation of N2 worms was sensitive to

juglone (Figure 2A). Having observed that a carbohydrate supplemented diet augmented stress resistance phenotypes, we wondered if this was due to a contribution from the worms' bacterial food source and/or and if other dietary supplements like protein or fat would also promote stress resistance. We discounted possible bacterial effects since GE continued to promote resistance to oxidative stress for worms grown on heat-killed bacteria (Figure S2A). Additionally, we observed no augmented resistance to oxidative stress for worms grown on plates supplemented with either methionine or oleic acid [14] (Figure S2B) suggesting the stress resistance phenotypes may be limited to dietary sugars.

We then tested if insulin/IGF-like pathway genes were required for protection against juglone by glucose and observed that similar to the effects on progeny numbers, *daf-16*, *aak-2* and *sir-2.1* were all required for the heritable protective effects of GE against oxidative stress (Figure 2B-D). Consistent with the effects of GE on progeny and lifespan, *hif-1* was not required for the glucose-mediated protection against glucose (Figure 2E). To further confirm that these genes were required for transmission, we treated F1 animals with RNAi against *daf-16, aak-2 and sir-2.1* and RNAi knockdown of each of these genes blocked the heritable transmission of stress resistance (Figure 2F). These data suggest that key components of the insulin/IGF-like pathway are required for the heritable, protective effects of glucose against oxidative stress. Recent work showed a possible hormetic protection in response to oxidative stress, as a mild induction of the cellular stress response could increase long term resistance and longevity [15]. To investigate the possible role of glucose as a stress inducer, we stained the worms with dihydrofluorescein, a marker of oxidative stress [16]. We observed comparable levels of fluorescence for N2 worms treated with glucose compared to untreated

control worms (Figure S2C) suggesting that glucose does not induce a generalized oxidative stress phenotype.

Parental exposure to glucose provides transgenerational protection against neurodegeneration

Aging is a risk factor for many diseases including late-onset neurodegenerative disorders [17]. To determine whether or not glucose could provide generational protection against age-dependent proteotoxicity we turned to a well-characterized *C. elegans* model of TAR DNA-binding protein 43 (TDP-43) motor neuron toxicity [18-21]. TDP-43 is a conserved RNA/DNA binding protein with mutant variants being causative for amyotrophic lateral sclerosis [22]. *C. elegans* expressing mutant TDP-43 in motor neurons show adult onset, age-dependent paralysis and neurodegeneration phenotypes that are diminished by treatment with glucose [5]. We observed that P0 generation TDP-43 worms treated with glucose had reduced rates of paralysis (Figure 3A) and axonal degeneration (Figure 3B) compared to untreated controls, and this protective effect persisted into the F1 generation. Thus, GE can reduce genetically encoded proteotoxicity and this effect is heritable. We next confirmed that GE did not reduce the paralysis rate through a hormetic stress response as dihydrofluorescein levels were indistinguishable from mutant TDP-43 animals treated with glucose (Figure S2C), but also because glucose-mediated neuroprotection was not lost after treatment with the antioxidant N-acetyl cysteine (Figure S2D).

Transmission of glucose phenotypes requires H3K4me3 components

Work from Brunet and colleagues demonstrated that components of the histone H3 lysine 4 trimethylation (H3K4me3) complex are essential to the transmission of transgenerational effects on longevity [23]. Thus, we hypothesized that genes encoding the H3K methyltransferase *set-2*, and the H3K4me3 complex component *wdr-5.1* would be required for the heritable transmission of glucose phenotypes on neurodegeneration, stress resistance and fecundity. First, we observed that H3K4me3 methylation was increased in P0 animals compared to untreated controls (Figure 4A). We observed this methylation mark in both young worms at the L3 larval stage and adults, thus suggesting that tissue heterogeneity or the presence of eggs in older animals do not contribute to this phenomenon. However, the increased H3K4me3 methylation observed in P0 animals was not transmitted to the F1 or F2 generations (Figure 4A). These observations are in agreement with work from Brunet and colleagues [23], where the H3K4me3 complex is associated with the transgenerational inheritance of longevity, but the H3K4me3 mark is not heritable. One interpretation is that H3K4me3 may be an indirect effect arising from other functions of the H3K4me3 complex in regulating global physiological changes.

We further investigated the genetic requirements of *set-2* and *wdr-5.1* for the transmission of glucose-mediated phenotypes. First we observed that GE suppressed the rate of paralysis in mutant TDP-43; *set-2* and mutant TDP-43; *wdr-5.1* mutants in the P0 generation, but suppression of mutant TDP-43-induced paralysis was not transmitted to the descendent F1 generation (Figure 4B and 4C). Accordingly, GE reduced axonal degeneration in P0 animals but failed to rescue the degeneration in F1 animals carrying *set-2(ok952)* or *wdr-5.1(ok1417)* mutations (Figure S3A). These data suggest that *set-2* and *wdr-5.1* are required for the heritable transmission of stress resistance phenotypes, but are not necessary for the

stress resistance itself. To confirm this we tested *set-2* and *wdr-5.1* mutants, subjected to GE, against oxidative stress and observed that GE continued to provide protection against juglone in the P0 generation, but this protection was lost in the F1 generation (Figure 4D and 4E) and this phenomenon was confirmed by RNAi treatment against *set-2* and *wdr-5.1* (Figure S3B and S3C). Likewise with the negative effects on reproduction, *set-2* and *wdr-5.1* mutants treated with GE had reduced numbers of progeny in the P0 generation, but progeny numbers returned to normal levels in the F1 descendants and subsequent F2 and F3 generations (Figure 4F and 4G).

However, the requirement of *set-2* and *wdr-5.1* for the transmission of glucose phenotypes may be independent of H3K4me3 methylation status. We confirmed that *set-2* and *wdr-5.1* are indeed required for the increased H3K4me3 methylation induced by glucose of the P0 generation of animals, since this methylation mark was abolished by *set-2* or *wdr-5.1* mutations (Figure S3D). Despite this, we observed that H3K4me3 methylation was not transmitted from P0 to F1 animals (Figure 4A) suggesting that this mark is not associated with the heritable transmission of glucose-induced phenotypes. Thus *set-2* and *wdr-5.1* may have activities independent of H3K4me3 methylation for the heritable transmission of glucose-associated phenotypes.

The germline is required for transmission of glucose protection

The transgenerational inheritance of longevity requires a functional germline [23]. Thus, we investigated the role of the germline in transmission of stress resistance after GE by using feminized mutants *fem-3(e2006)* that are not able to produce mature eggs at restrictive temperatures [24] and *pgl-1(bn102)* that is not able to form a functional germline at restrictive

temperatures [25]. Both strains showed increased resistance to juglone under GE conditions in the P0 and F1 generation at 15°C, but the transmission was lost at 25°C in the F1 generation, indicating that the glucose-induced transmission of stress resistance is dependent on a functional germline (Figure 5).

DISCUSSION

Numerous strategies have evolved to ensure reproductive success for organisms in the face of changing and challenging environments [1]. It is believed that extended lifespan phenotypes observed under dietary restriction conditions maximize an organism's survival until environmental conditions improve allowing for reproduction. We discovered a novel diet-influenced reproductive advantage; animals subjected to high dietary glucose are resistant to protein damaging stress, and this resistance is transmitted to their progeny. The trade-off for stress-resistant progeny is decreased lifespan and fecundity in the parental strain suggesting that this strategy may be adaptive under nutrient rich conditions.

In the P0 generation, high dietary glucose leads to negative effects on lifespan and reproduction along with increased protection against protein-damaging stress. The resulting F1 progeny are resistant to stress via an epigenetic mechanism along with a small reduction in fecundity, but these animals do not suffer negative lifespan effects. This dietary induced adaptation may be favorable from an evolutionary standpoint where in times of plenty an epigenetic program is initiated to maximize the survival of progeny at the expense of less total progeny and a decrease in parental health. This mechanism is reminiscent of antagonistic pleiotropy, where fitness in early life is increased at the expense of negative effects on health and fitness in late life [2]. Here, excess energy from high dietary glucose may be used to maintain proteostasis under stress conditions [5], but only the parental P0 hermaphrodites pay

the price of shortened lifespan. The recently proposed theory of hyperfunction may help explain these contradictions [26], where the continued activity of developmental and growth programs later in life leads to decreased fitness and longevity [2]. Indeed, the majority of genes regulating lifespan have metabolic functions essential for development or growth, and perhaps the continued activity of these networks driven by excess dietary glucose leads to negative phenotypes. If true, we would expect that only those animals directly exposed to glucose would suffer negative effects and this is seen by the large reduction of lifespan in P0, but not F1 animals.

While the reduced progeny phenotype persists for two generations, the stress resistance phenotypes last only until the F1 generation, suggesting maternal inheritance rather than a transgenerational inheritance mechanism. Furthermore, we observe increased H3K4me3 methylation only in the parental P0 animals exposed to glucose, but not in the descendent F1 and F2 progeny. Thus, while the stress resistance phenotypes are passed to the F1 generation, the glucose induced methylation mark is not, so perhaps maternally deposited RNAs contribute to the glucose-induced phenotypes of the F1 progeny. One interpretation might be that H3K4me3 occurs on specific loci, and thus is impossible to detect by western blot. Alternatively, the complex may initiate large-scale physiological changes, and H3K4me3 may be an indirect, non-heritable effect simply indicating open chromatin and increased transcription. Finally, it is possible that an additional methylation mark, like H3K36me3 is involved [27] in the transmission of glucose-associated phenotypes. In any event, both histone modifying enzymes and the insulin/IGF-like pathway are necessary for the transmission of glucose-induced phenotypes. This is likely an adaptive phenotype since while the dietary

status of the P0 animals may be predictive for the immediate environment of F1 animals, this may not be the case for F2 generations and beyond.

Of interest is the comparison of phenotypes of high dietary glucose conditions versus nutrient stress from reduced feeding. Worms experiencing dietary restriction are long-lived [28], have reduced fertility [29], show resistance to thermal stress [30] but are not resistant to genetically encoded neuronal proteotoxicity [5]. These observations suggest that the bulk of dietary-restricted animals' resources are used to maintain survival, minimizing reproductive potential until environmental conditions improve. In contrast, high dietary glucose may represent a nutrient rich environment, and these animals have the luxury to enlist genetic programs promoting survival of their progeny.

Given that an organism's life history is greatly affected by nutritional status, an open question is how far do cellular changes go in response to nutritional state. The emerging field of transgenerational epigenetic inheritance is providing evidence that certain epigenetic modifications persist over several generations [31]. Furthermore, the investigation of heritable phenotypic effects from sustained dietary changes is a developing field that may have implications on human health [32-34]. The investigation of the biological consequences of early environmental influences has a long history [35], and there are numerous studies into the effects of prenatal diet on the health of offspring, including mammals [36], but the molecular mechanisms are not well known. Future studies in genetically tractable models like *C. elegans* will be a powerful approach to unravel the epigenetic mechanisms of nutrient stress on healthy aging.

Materials and Methods

Worm strains and genetics

Standard methods of culturing and handling worms were used. Worms were maintained on standard NGM plates streaked with OP50 *E. coli*. In some experiments D-glucose was added to NGM plates (all products from Sigma-Aldrich). All strains were scored at 20°C unless indicated. Mutations and transgenes used in this study were: *daf-16(mu86), sir-2.1(ok434), aak-2(ok524), hif-1(ia4), set-2(ok952), wdr-5.1(ok1417), fem-3(e2006), pgl-1(bn102) and xqIs133[unc-47::TDP-43[A315T];unc-119(+)]*. Some strains were provided by the *C. elegans* Genetics Center (University of Minnesota, Minneapolis), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Mutants or transgenic worms were verified by visible phenotypes, PCR analysis for deletion mutants, sequencing for point mutations or a combination thereof. Deletion mutants were out-crossed a minimum of three times to wild type worms prior to use.

Worm behavioral tests

Mutant TDP-43 animals were scored for paralysis and counted as positive if they failed to move upon prodding with a worm pick. Worms were scored as dead if they failed to move their head after being prodded on the nose and showed no pharyngeal pumping. For the paralysis tests worms were grown on NGM or NGM +2% glucose and transferred to NGM-FUDR or NGM-FUDR +2% glucose. For the F1 generation, L4 animals were transferred from NGM +2% glucose to NGM and their progeny used as the F1 generation. The same method was used for the F2 generation.

Fluorescence microscopy

For scoring of neuronal processes from mTDP-43 transgenics, animals were selected at days 1, 5 and 9 of adulthood for visualization of motor neurons processes *in vivo*. Animals were immobilized in M9 with 5 *mM* levamisole and mounted on slides with 2% agarose pads. Neurons were visualized with a Leica 6000 and a Leica DFC 480 camera. A minimum of 100 animals was scored per treatment over 4-6 trials. The mean and SEM were calculated for each trial and two-tailed t-tests were used for statistical analysis.

Stress assays

For oxidative stress tests, worms were grown on NGM or NGM with a dietary supplement (glucose, oleic acid or methionine) and transferred to NGM plates + 240 μM juglone at adult day 1. For the F1, L4 worms from NGM + 2% glucose plates were transferred on NGM and their progeny used as F1 generation. The same process was used for the F2 generation. Worms were evaluated for survival every 30 min for the first 2 hours and every 2 hours after up to 14 hours. Nematodes were scored as dead if they were unable to move in response to heat or tactile stimuli. For all tests worms, 20 animals/plate by triplicates were scored. Temperature sensitive mutant *fem-3(e2006)* or worms were maintained at 15°C and switched to 25°C at hatching and kept at this temperature until tested on juglone. Temperature sensitive *pgl-1(bn102)* were maintained at 15°C and switched to 25°C at the L4 larvae stage and kept at this temperature until tested on juglone.

Lifespan assays

Worms were grown on NGM or NGM + 4% glucose and transferred on NGM-FUDR or NGM-FUDR + glucose. For the F1 generation, L4 animals from the NGM + 4% glucose plates were transferred to NGM plates and progeny used as the F1 generation on NGM-FUDR. The same process was used to prepare the F2 generation. 20 animals/plate by triplicates were tested at 20°C from adult day 1 until death. Worms were scored as dead if they didn't respond to tactile or heat stimulus.

Progeny tests

For scoring progeny, 10 L4 worms were grown on NGM or NGM +2% glucose and placed at 20°C. Over the next three days individual worms were transferred to new plates and the L1 larvae were scored for each plate. For the F1 generation, 10 L4 larvae from the P0 were transferred to new NGM plates without glucose and this process was repeated for the F2 and F3 generations.

Dihydrofluorescein Diacetate Assay

For visualization of oxidative damage in the transgenic strains the worms were incubated on a slide for 30 minutes with 5 μM dihydrofluorescein diacetate dye (Sigma-Aldrich) and then washed with 1X PBS three times. After the slide was fixed fluorescence was observed with the Leica system described above.

Worm lysates

Worms were collected in M9 buffer, washed 3 times with M9 and pellets were placed at -80°C overnight. Pellets were lysed in RIPA buffer (150mM NaCl, 50mM Tris pH 7.4, 1% Triton X-

100, 0.1% SDS, 1% sodium deoxycholate) + 0.1% protease inhibitors (10 mg/ml leupeptin, 10 mg/ml pepstatin A, 10 mg/ml chymostatin LPC;1/1000). Pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged at 16000*g*. Supernatants were collected.

Protein quantification

All supernatants were quantified with the BCA Protein Assay Kit (Thermo Scientific) following the manufacturer's instructions.

Immunoblot

Worm RIPA samples (50 µg/well) were resuspended directly in 1X Laemmli sample buffer, migrated in 14% polyacrylamide gels, transferred to nitrocellulose membranes (BioRad) and immunoblotted. Antibodies used: rabbit anti-Histone H3 Total (1:1000, ab1791 Abcam), rabbit anti-Histone tri-methylated (1:1000, ab8580 Abcam), and mouse anti-actin (1:5000 , MP Biomedicals). Blots were visualized with peroxidase-conjugated secondary antibodies and ECL Western Blotting Substrate (Thermo Scientific). Densitometry was performed with Photoshop (Adobe).

RNAi experiments

RNAi-treated strains were fed with *E. coli* (HT115) containing an Empty Vector (EV), *set-2* (C26E6.9)*, wdr-5.1* (C14B1.4)*, daf-16* (R13H8.1) or *aak-2* (T01C8.1) RNAi clones from the ORFeome RNAi library and *sir-2.1* (R11A8.4) clone from the Ahringer RNAi library. RNAi experiments were performed at 20°C. Worms were grown on either NGM or NGM + 2% glucose both enriched with 1 mM Isopropyl-b-D-thiogalactopyranoside (IPTG).

Statistics

For paralysis and stress-resistance tests, survival curves were generated and compared using the Log-rank (Mantel-Cox) test, and 60-100 animals were tested per genotype and repeated at least three times.

ACKNOWLEDGMENTS

We thank Sarah Peyrard for technical support, and Dr. Jean-Claude Labbé for RNAi clones.

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Figure Legends

Figure 1. Heritable diminution of progeny from glucose exposure in the parental generation.

(A) In parental P0 generation animals, glucose enrichment (GE) decreased the average number of progeny of wild type N2 and *hif-1* mutant worms compared to untreated controls. GE had no effect on *daf-16*, *aak-2* or *sir-2.1* mutants. **P<0.01 versus untreated *hif-1* controls, ****P<0.0001 versus untreated N2 controls

(B) F1 generation N2 and *hif-1* descendants had reduced progeny numbers compared to F1 descendants from untreated P0 controls. *P<0.05, ***P<0.001

(C) N2 worms in the F2 generation from P0 parents exposed to GE also had reduced progeny numbers. *P<0.05

(D) F3 generation descendants from GE treated P0 parents had comparable progeny numbers compared to animals descendent from untreated P0 parents.

Figure 2. Transgenerational inheritance of resistance to oxidative stress.

(A) N2 animals exposed to GE are highly resistant to juglone-induced lethality and this resistance was transmitted to descendent progeny in the F1 and generation, $P<0.0001$ versus untreated animals.

(B-E) Resistance to oxidative stress by GE was lost in the P0 and F1 generations in animals mutant for (B) *daf-16*, (C) *aak-2*, or (D) *sir-2.1*. (E) GE continued to provide resistance to P0 and F1 animals mutant for *hif-1*, $P<0.0001$ versus untreated animals.

(F) GE increased oxidative stress resistance in P0 N2 animals but this effect was lost in F1 animals treated with *daf-16, aak-2* or *sir-2.1* RNAi clones.

Figure 3. Parental exposure to glucose provides transgenerational protection against neurodegeneration.

(A-B) GE reduces TDP-43 mediated age-dependent (A) paralysis, $P<0.0001$ versus untreated animals and (B) neurodegeneration in P0 animals and their F1 descendants, $^{*}P<0.0001$ versus untreated animals.

Figure 4. Transgenerational inheritance of glucose phenotypes requires H3K4me3 components.

(A) N2 animals treated with 2% GE had an increased H3K4me3 mark and this effect was lost in subsequent generations.

(B-C) GE delayed late onset paralysis in P0 but not in F1 generation of mTDP-43; *set-2(ok952)* (B) and mTDP-43; *wdr-5.1(ok1417)* (C) animals compared to untreated control and this protection was lost in F1 generation.

(D-E) Stress resistance was increased in P0 but not in F1 generation of COMPASS (Complex Proteins Associated with Set1) mutants (D) *set-2(ok952)* and (E) *wdr-5.1(ok1417)*.

(F-G) Total progeny numbers were reduced in P0 but not in F1 generations of (F) *set-2* and (G) *wdr-5.1* mutants.

Figure 5. The germline is required for transmission of glucose protection.

(A-B) Glucose increased stress resistance in P0 and F1 generation of *fem-3(e2006)* mutants at 15°C, but failed in the F1 generation at 25°C.

(C-D) Glucose increased resistance to juglone in P0 and F1 generation of *pgl-1(bn102)* mutants at 15°C but failed in the F1 generation at 25°C.

Table S1. Lifespan analysis for all experiments.

Related to Figure S1.

Animals that died prematurely (ruptured, internal hatching) or were lost (crawled off the plate) were censored at the time of scoring. All control and experimental animals were scored and transferred to new plates at the same time. n.s. not significant

Figure S1. Lifespan reduction by glucose is not transmitted over generation.

(A-B) GE reduced lifespan in the P0 generation of (A) N2 and (B) *daf-2(e1370)* animals, $P<0.0001$, but failed to reduce lifespan in the F1 and F2 generations. (Related to Figure 1).

Figure S2. Glucose protection is independent of a hormetic increase of oxidative stress.

(A) N2 animals exposed to GE on dead OP50 bacteria (DB) were highly resistant to juglone-induced lethality and this resistance was transmitted to descendent progeny in the F1 and generation, $P<0.0001$ versus untreated animals.

(B) Methionine (0.1%) and oleic acid (0.45 mM) failed to increase resistance to juglone.

(C) Images of adult worms stained with 5 μM dihydrofluorescein diacetate. N2 worms do not show increased fluorescence after treatment with glucose. mTDP-43 animals experience high levels of oxidative stress and strongly fluoresce when stained with dihydrofluorescein diacetate.

(D) GE reduced the paralysis rate of mTDP-43 animals and treatment with N-acetyl cysteine did not block the suppression of paralysis.

Figure S3. COMPASS genes are required for methylation increase by glucose

(A) Glucose failed to increase the methylation H3K4me3 mark in *set-2(ok952)* and *wdr-5.1(ok1417)* mutated animals (Related to Figure 4).

(B) Glucose enrichment reduced axonal degeneration in mTDP-43; *set-2(ok952)* ($P<0.001$) and mTDP-43; *wdr-5.1(ok1417)* ($P<0.0001$) P0 animals but failed to rescue the phenotype in the F1 generation.

(C-D) RNAi against (C) *set-2* and (D) *wdr-5* failed to block GE protection against juglone in P0 animals, but blocked the transmission in the F1 generation. (Related to Figure 4D and 4E). The N2 control was used for both experiments.

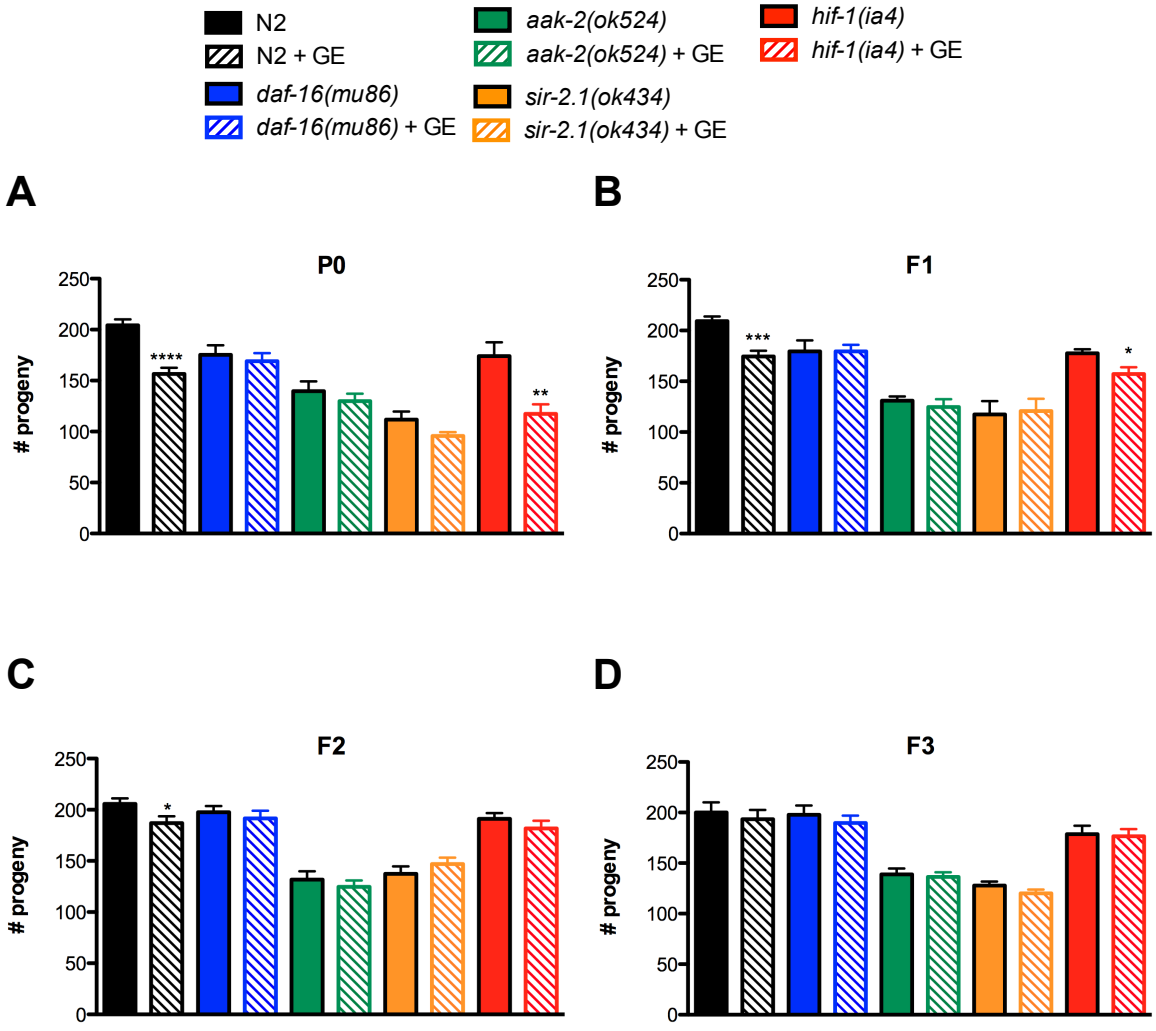

N2
N2 + GE
daf-16(mu86)
daf-16(mu86) + GE
aak-2(ok524)
aak-2(ok524) + GE
sir-2.1(ok434)
sir-2.1(ok434) + GE
hif-1(ia4)
hif-1(ia4) + GE
A
P0
B
F1
C
F2
D
F3
progeny
0
50
100
150
200
250
****
**
***
*
*

Figure 1

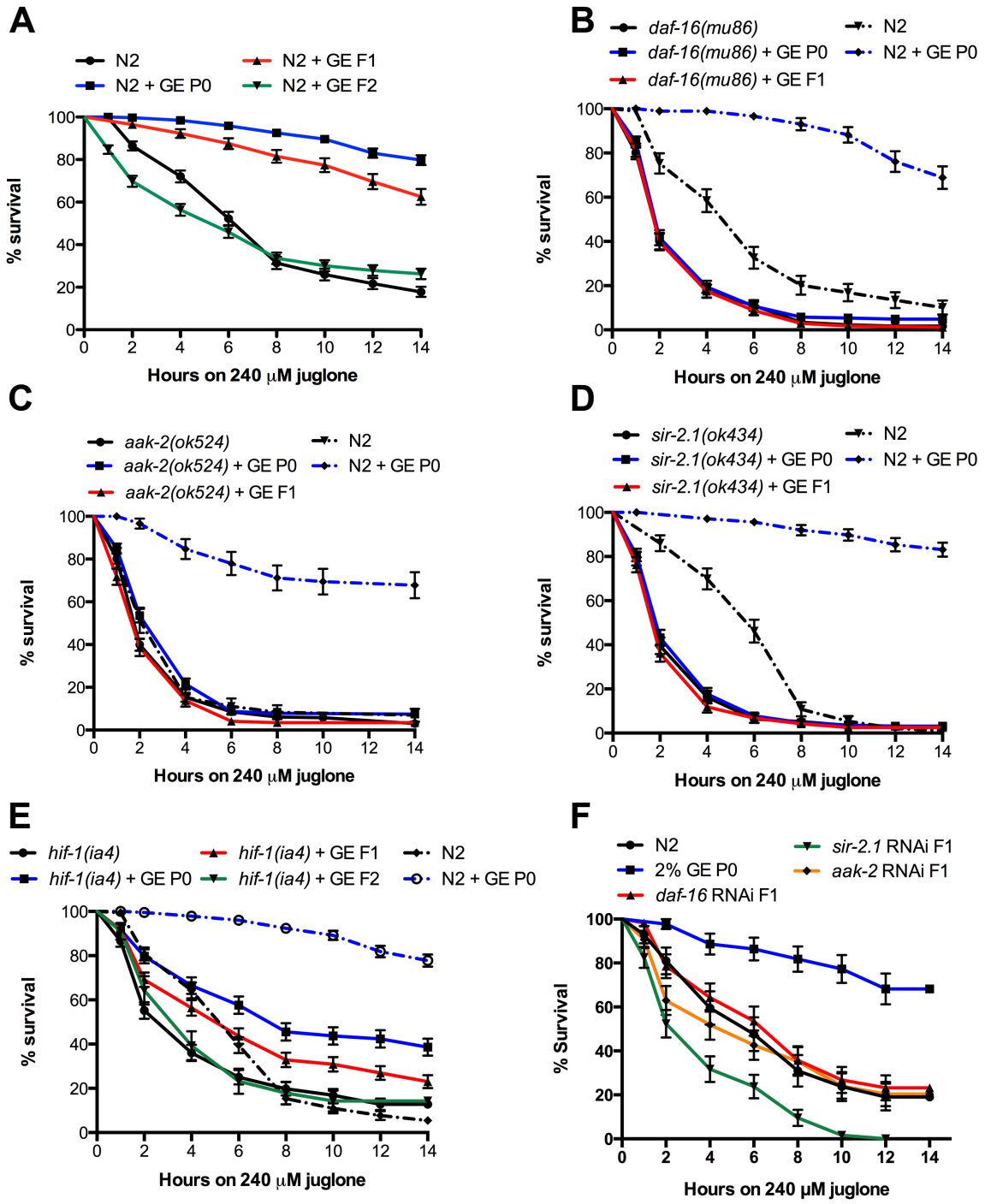
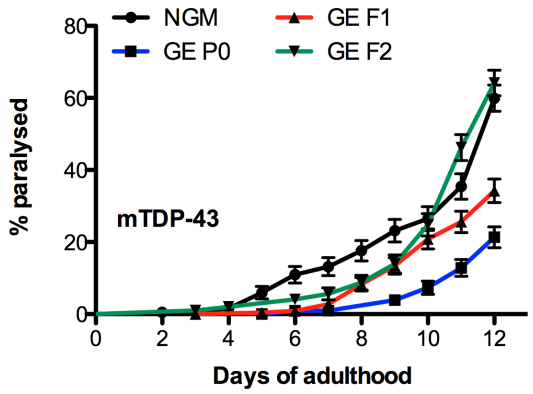

A
N2
N2 + GE P0
N2 + GE F1
N2 + GE F2
% survival
Hours on 240 μM juglone
B
daf-16(mu86)
daf-16(mu86) + GE P0
daf-16(mu86) + GE F1
N2
N2 + GE P0
% survival
Hours on 240 μM juglone
C
aak-2(ok524)
aak-2(ok524) + GE P0
aak-2(ok524) + GE F1
N2
N2 + GE P0
% survival
Hours on 240 μM juglone
D
sir-2.1(ok434)
sir-2.1(ok434) + GE P0
sir-2.1(ok434) + GE F1
N2
N2 + GE P0
% survival
Hours on 240 μM juglone
E
hif-1(ia4)
hif-1(ia4) + GE P0
hif-1(ia4) + GE F1
hif-1(ia4) + GE F2
N2
N2 + GE P0
% survival
Hours on 240 μM juglone
F
N2
2% GE P0
daf-16 RNAi F1
sir-2.1 RNAi F1
aak-2 RNAi F1
% Survival
Hours on 240 μM juglone

Figure 2

A


NGM
GE F1
GE P0
GE F2
% paralysed
mTDP-43
Days of adulthood

B

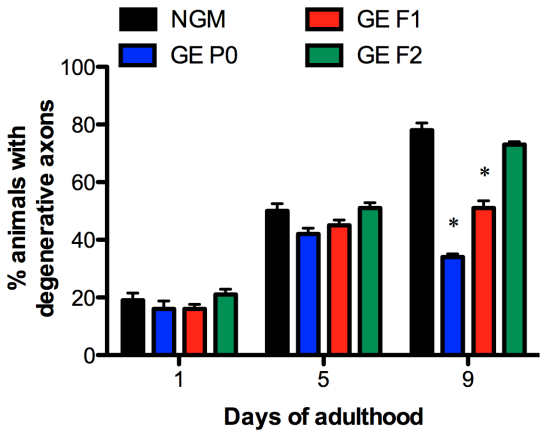

NGM
GE F1
GE P0
GE F2
% animals with degenerative axons
Days of adulthood

Figure 3

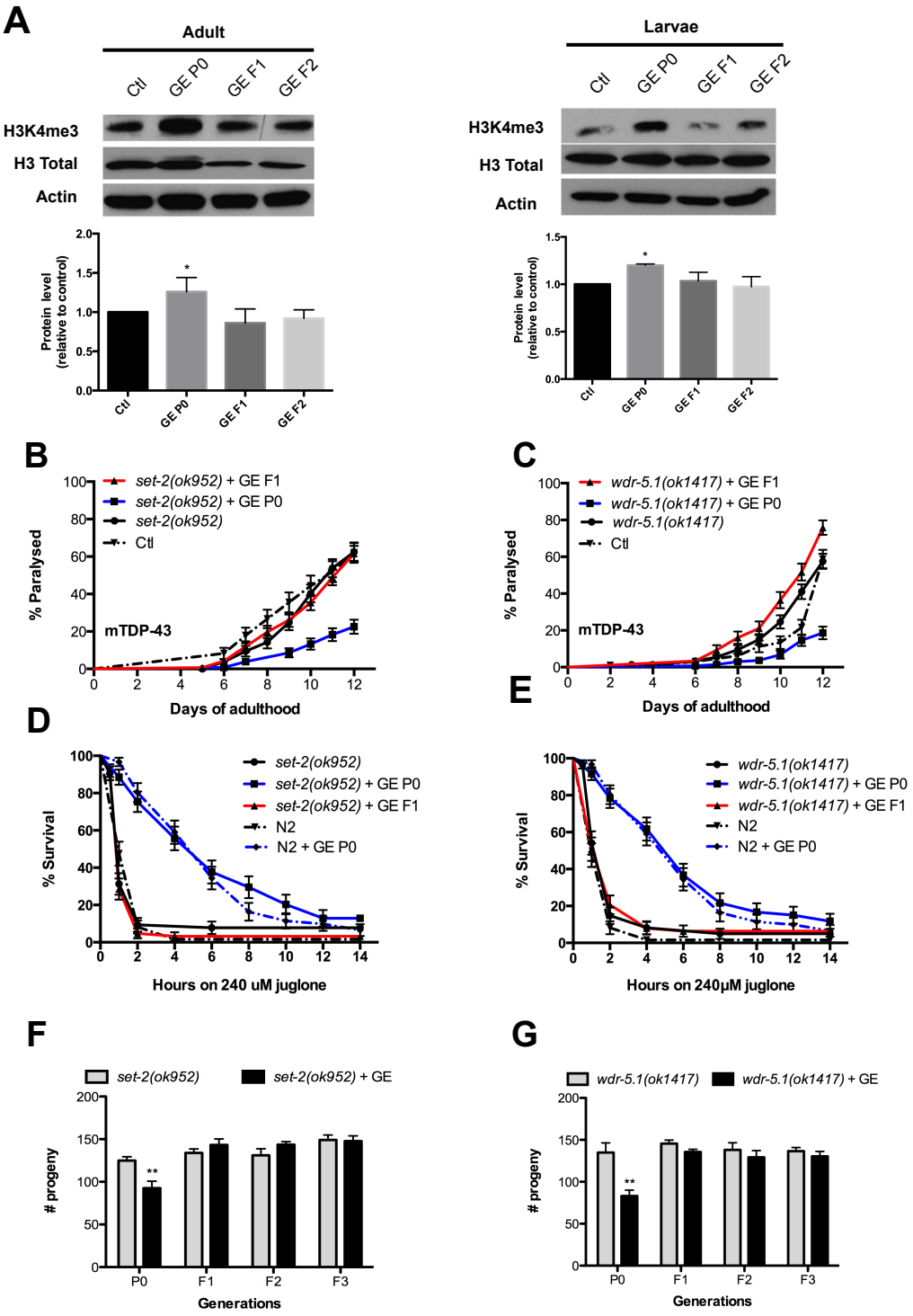

A
Adult
Ctl
GE P0
GE F1
GE F2
H3K4me3
H3 Total
Actin
Protein level (relative to control)
Larvae
B
set-2(ok952) + GE F1
set-2(ok952) + GE P0
set-2(ok952)
Ctl
% Paralysed
mTDP-43
Days of adulthood
C
wdr-5.1(ok1417) + GE F1
wdr-5.1(ok1417) + GE P0
wdr-5.1(ok1417)
D
% Survival
N2
N2 + GE P0
Hours on 240 uM juglone
E
Hours on 240µM juglone
F
set-2(ok952) + GE
progeny
P0
F1
F2
F3
Generations
G
wdr-5.1(ok1417) + GE

Figure 4

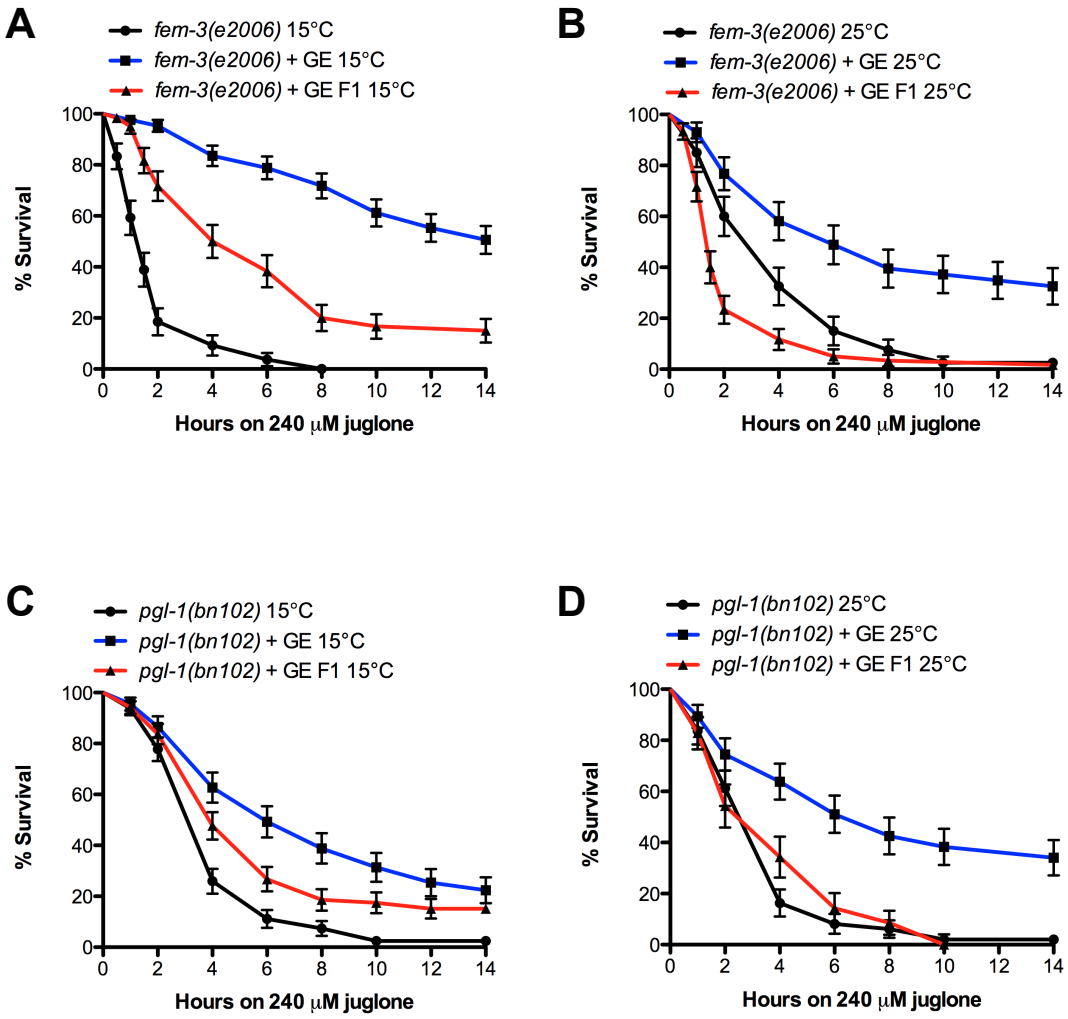
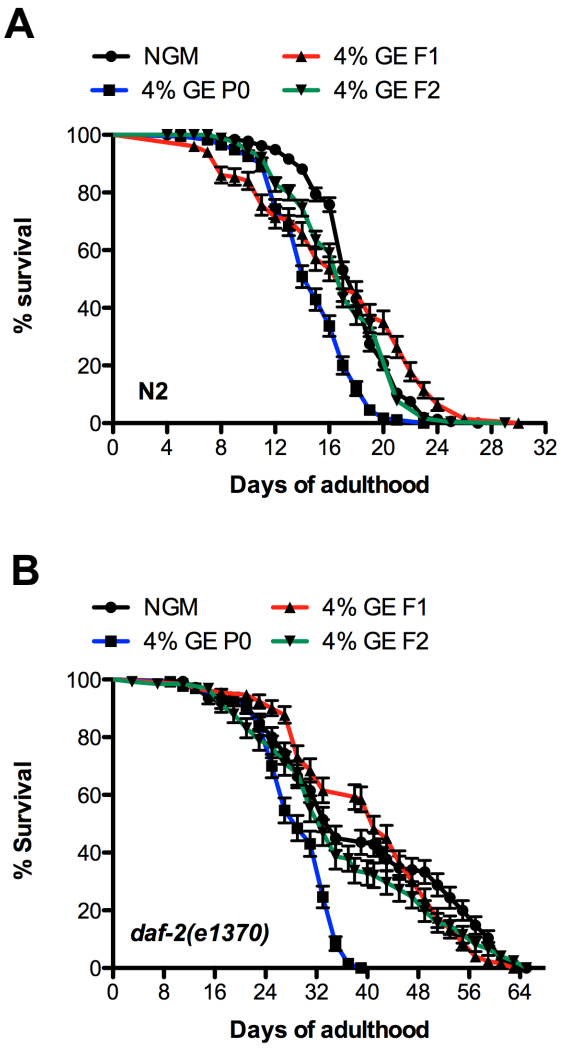

A
fem-3(e2006) 15°C
fem-3(e2006) + GE 15°C
fem-3(e2006) + GE F1 15°C
% Survival
Hours on 240 μM juglone
B
fem-3(e2006) 25°C
fem-3(e2006) + GE 25°C
fem-3(e2006) + GE F1 25°C
% Survival
Hours on 240 μM juglone
C
pgl-1(bn102) 15°C
pgl-1(bn102) + GE 15°C
pgl-1(bn102) + GE F1 15°C
% Survival
Hours on 240 μM juglone
D
pgl-1(bn102) 25°C
pgl-1(bn102) + GE 25°C
pgl-1(bn102) + GE F1 25°C
% Survival
Hours on 240 μM juglone

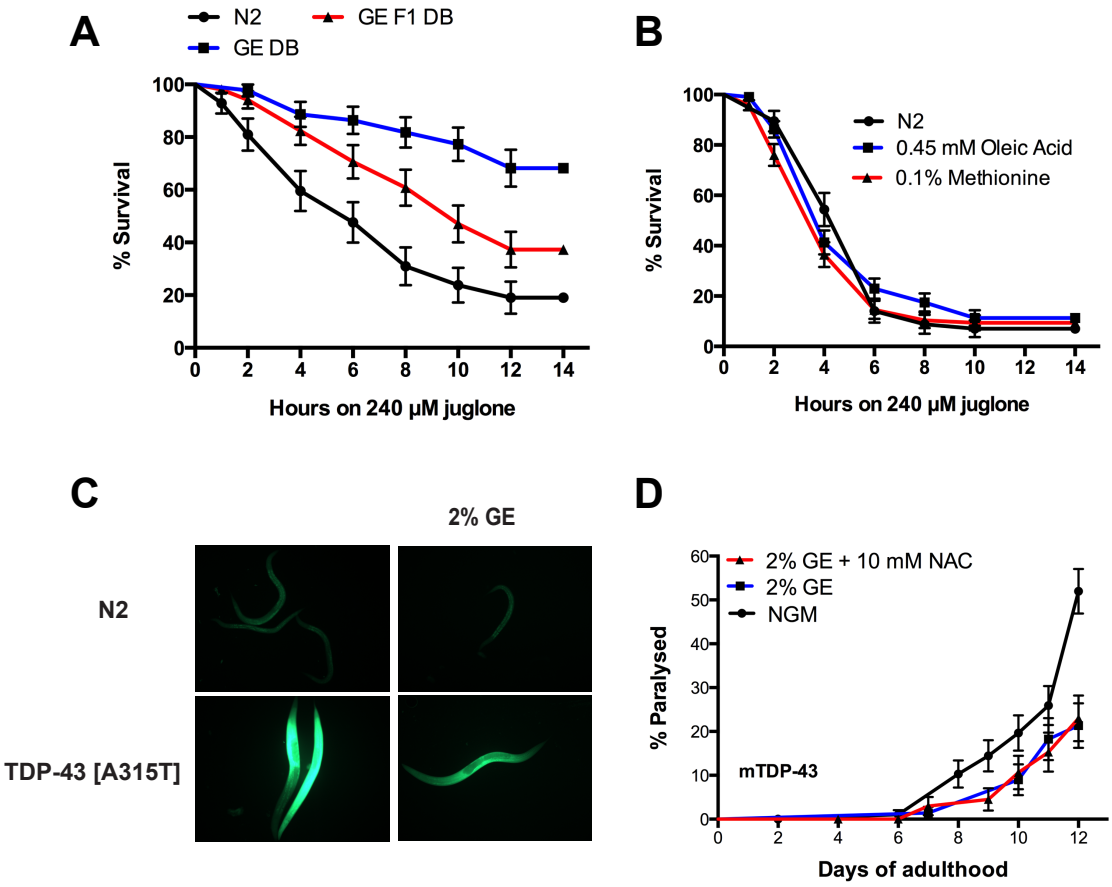
Figure 5

Strains	Mean lifespan	pValue	75th percentile (days)	maximum lifespan	Total number of death/total
N2	18		18	27	309/317
N2 + 4% GE P0	15	**<0,0001**	16	21	175/175
N2 + 4% GE F1	17	n.s. 0,1083	19	29	142/152
N2 + 4% GE F2	17	n.s. 0,2327	18	30	244/257
daf-2(e1370)	35		53	65	138/142
daf-2(e1370) + GE P0	29	**<0,0001**	33	39	130/132
daf-2(e1370) + GE F1	41	n.s. 0,2965	49	63	128/132
daf-2(e1370) + GE F2	33	n.s. 0,1353	47	65	122/129

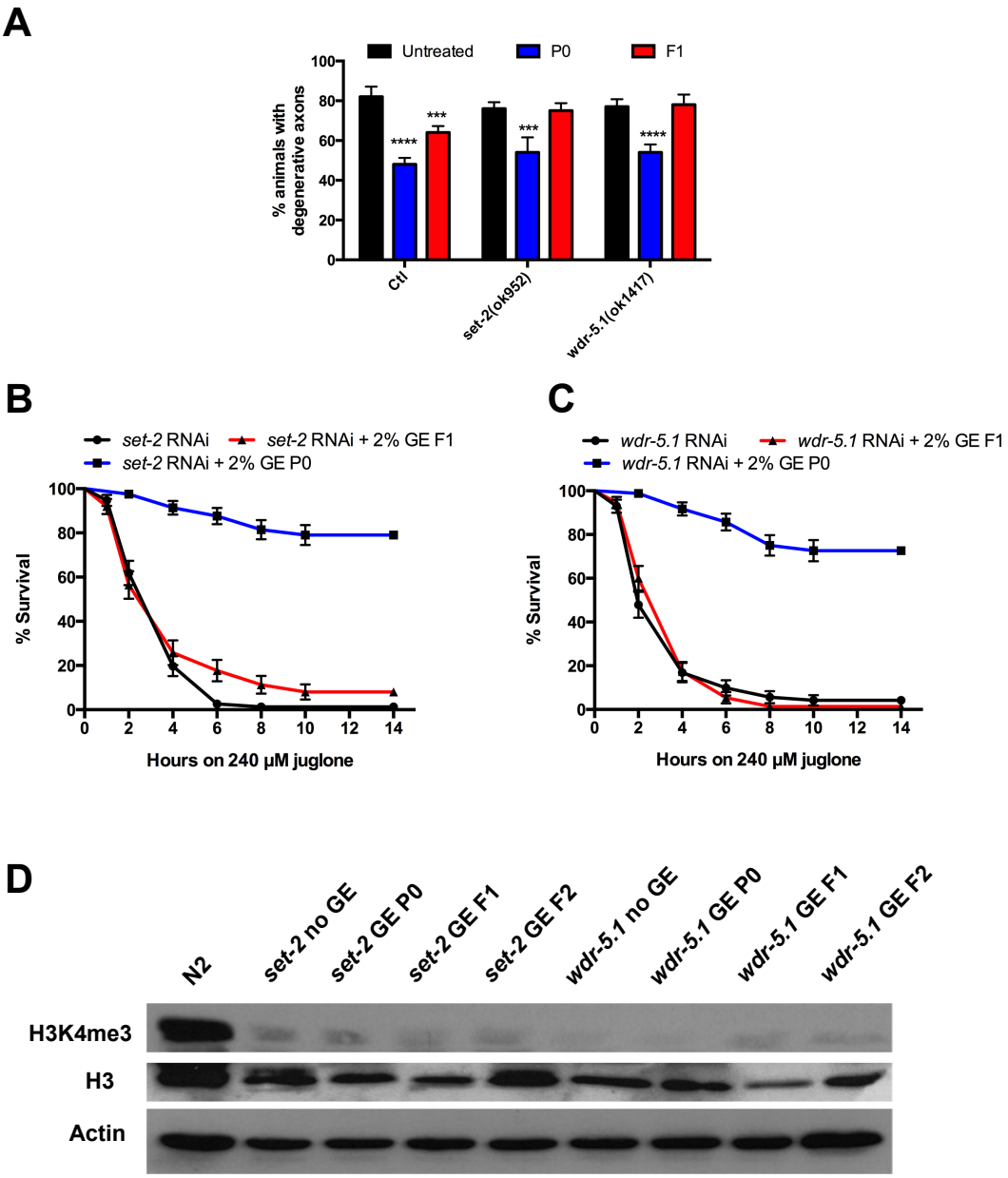
Supplementary Table I



Supp. Figure 1



Supp. Figure 2



Supp. Figure 3

Discussion et Conclusion

I. Glucose, métabolisme et vieillissement

Les études présentées dans ce manuscrit ont mis en lumière une nouvelle fonction de la molécule de glucose. Cette dernière est bien connue pour son rôle dans le métabolisme, particulièrement celui du système nerveux. Malheureusement, elle est aussi connue pour son rôle dans différentes pathologies reliées à un excès calorique. Le rôle du sucre dans les problèmes d'obésité, de maladies coronariennes et le diabète de type II est bien établi, et certains considèrent le glucose comme un poison. La nouveauté ici vient du rôle neuroprotecteur et de la capacité à réduire la protéotoxicité de manière plus globale. Ce rôle n'avait pas été mis en évidence auparavant et soulève plusieurs questions, incluant le mécanisme permettant cet effet.

II. Restriction et Enrichissement calorique

Depuis maintenant 30 ans les études sur la restriction calorique ont prouvé que la prise alimentaire module l'espérance de vie (29,463). La restriction de prise alimentaire améliore la qualité de vie, mesurée par l'accumulation de marqueurs de vieillissement, comme la lipofuscine et la durée de vie moyenne des organismes modèles étudiés (30,465). Dans notre première étude, nous avons testé si la restriction calorique pouvait réduire la toxicité des modèles transgéniques pour la SLA et la CH. Nous avons utilisé deux souches transgéniques exprimant la protéine TDP-43[A315T] dans une lignée de neurones moteurs (442) et la protéine mutante HTT_{1-17}-128Q dont l'expression est contrôlée par le promoteur *mec-3*,

spécifique aux neurones mécanosensoriels (174). Dans les prémices de notre étude, un modèle $A\beta_{1-42}$ dont l'expression est contrôlée par un promoteur musculaire a aussi été utilisé. Le choix des modèles neuronaux, particulièrement celui de TDP-43 dans les neurones moteurs, est lié à notre volonté de représenter le plus fidèlement possible la spécificité des cellules impliquées dans la SLA chez l'humain. En effet, cette dernière est caractérisée par la perte de neurones moteurs.

La restriction calorique, consistant en un retrait de la principale source nutritionnelle, a bel et bien augmenté la durée de vie de la lignée sauvage N2 et réduit la progression du phénotype de paralysie de $A\beta_{1-42}$. Cependant, elle n'a eu aucun effet sur le phénotype des deux lignées neuronales TDP-43[A315T] et 128Q. Cette différence pourrait s'expliquer par le type cellulaire impliqué. En effet, alors que les modèles TDP-43[A315T] et 128Q expriment leur protéine mutante respectivement dans les neurones moteurs et mécanosensoriels, le modèle $A\beta_{1-42}$ l'exprime dans les cellules musculaires. Les neurones et les muscles ont des capacités métaboliques différentes. Les premiers nommés sont incapables de stocker l'énergie, alors que les cellules musculaires sont parmi les plus efficaces dans la formation de réserves énergétiques sous forme de glycogène. Dans une situation de stress cellulaire, où la cellule a besoin d'énergie, il est plausible qu'une réserve d'énergie permette un meilleur maintient des fonctions cellulaires.

L'élément inédit dans notre étude provient des effets mesurés lors d'un enrichissement au glucose. Des concentrations croissantes (0,1%, 1%, 2%, 4% et 10%) de glucose diététique ont produit des effets physiologiques importants chez les nématodes traités. Comme prévu, la durée de vie et les niveaux de progénitures sont réduits après traitement au glucose (27,464,466). Cependant, cette hausse diététique augmente la résistance au stress cellulaire

(oxydatif, osmotique et thermique) et réduit la protéotoxicité liée à l'expression de protéines impliquées dans la SLA et la CH.

III. Le rôle du métabolisme dans la protection induite par le glucose

L'efficacité du métabolisme diminue lors du vieillissement (revu en 467). En réduisant l'expression de trois enzymes impliquées dans la glycolyse, soit *gpi-1* (Glucose-6-Phosphate Isomerase), *tpi-1* (Triose Phosphate Isomerase) et *enol-1* (ENOLase) et en utilisant différents composés alternatifs tels que le glycérol, le tréhalose et le L-glucose (l'énantiomère du D-glucose), nous avons montré que ce processus métabolique est requis pour maintenir l'effet du glucose sur la toxicité neuronale de TDP-43[A315T] et 128Q. Récemment, le laboratoire du Dr Caldwell a confirmé que la présence d'une glycolyse fonctionnelle était requise pour la protection neuronale dans différents modèles de la maladie de Parkinson (468). À l'inverse, l'équipe du Dr Ristow a démontré qu'une diminution de l'expression de *gpi-1* augmente la durée de vie chez le ver (464). Ces données contradictoires suggèrent un rôle différent du métabolisme dans des conditions physiologiques et pathologiques. Une vérification des enzymes impliquées dans le cycle de l'acide citrique plus approfondie pourrait aussi permettre de mieux comprendre les voies métaboliques dont dépend une diète riche en glucose.

IV. Le glucose dans un contexte de vieillissement

Il est possible d'imaginer que le vieillissement physiologique, durant lequel l'organisme perd progressivement ses fonctions, ne soit pas tout à fait similaire à celui que nous décrivons comme pathologique dans cet ouvrage (453). En effet, le vieillissement pathologique semble être accéléré dû aux dommages inhérents à la dysfonction des protéines mutantes exprimées

dans nos modèles. A partir de cette hypothèse, il est envisageable que dans ces conditions, l'organisme requière davantage d'énergie pour activer les voies de signalisation et de protection requises. De ce fait il est donc possible que la restriction calorique puisse être efficace dans certains types cellulaires et dans des conditions physiologiques, mais que dans les cas de maladies neurodégénératives, un apport en énergie puisse être une meilleure solution pour aider les neurones à survivre (381).

Afin de décrire l'effet du glucose, dans un contexte de vieillissement, nous avons utilisé différentes lignées mutantes, incluant celle du récepteur *daf-2* (IGFR) et des facteurs de transcription *daf-16* (FOXO3A) et *hsf-1* (HSF1). Ces trois gènes sont impliqués dans différents processus métaboliques et de longévité, incluant la restriction calorique dans le cas de *daf-16*. Il apparait, d'après nos résultats, que les deux facteurs de transcriptions *daf-16* et *hsf-1* sont liés à l'effet produit par le glucose; leur perte de fonction abolit les effets mentionnés précédemment. Par ailleurs, il apparait que les mécanismes impliqués dans une situation d'enrichissement en glucose sont les mêmes que ceux requis pour la restriction calorique. Donc, le statut énergétique engage des mécanismes cellulaires qui, selon que l'on soit en condition de restriction ou de surplus calorique, produisent des effets différents.

V. Le glucose, durée de vie et résistance au stress

Une diète enrichie en glucose avec une concentration de 2% (100 mM) est responsable d'une diminution de l'espérance de vie chez *C. elegans* (27,464). Différents groupes, incluant le nôtre, ont mis en évidence que cette réduction était dépendante de facteurs de transcription *daf-16* et *hsf-1* (27). La survie des vers traités n'est pas affectée par un ajout de glucose. En ce qui concerne le récepteur insuline/IGF *daf-2*, une mutation entraine une sensibilité accrue au glucose. Des doses même très réduites (0,1% et 1%), amplifient l'effet délétère observé sur

l'espérance de l'animal. Ces données indiquent que l'activation de la voie insuline/IGF (IIS) module le rôle du glucose dans la durée de vie. En contrepartie, *daf-2* est aussi impliqué dans les effets positifs mentionnés dans ce manuscrit, incluant la réponse au stress et à la protéotoxicité. Il est aussi envisageable que les effets du glucose sur les souches mutantes de *daf-16* et *hsf-1* ne soient pas visibles, étant donné que la durée de vie est déjà réduite. Dans le même ordre d'idées, les effets du glucose sur la durée de vie chez le mutant *daf-2* pourraient être potentialisés parce que ces derniers ont une durée de vie augmentée.

Une étude publiée par l'équipe du Dr Solari a mis en lumière le rôle d'un orthologue des transporteurs monocarboxylés (MCT) dans la durée de vie (469). Cette étude sur le rôle mimétique de *slcf-1 (*SoLute Carrier Family) quant à la restriction calorique, montre que l'inactivation du gène entraine une augmentation de l'espérance de vie. Cependant, cette mutation induit une sensibilité accrue au stress oxydatif chez le jeune adulte et ce, malgré une hausse des niveaux d'espèces réactives à l'oxygène. Cette tendance s'inverse durant le vieillissement, jusqu'à ce que l'inactivation de *slcf-1* entraine une résistance supérieure au stress oxydatif. Ces résultats révèlent que le métabolisme, dans le développement, influence la résistance de l'organisme et que ce rôle lors du vieillissement n'a plus la même importance. Il est envisageable que le maintien des voies de signalisation actives chez l'adulte, parce que non requises, influence le vieillissement et la réponse au stress. Ce maintien induirait une toxicité plus importante lors d'un contexte de stress cellulaire ou de vieillissement pathologique. Ces données, parce que les effets *slcf-1* semblent liés à *daf-16* et *hsf-1*, suggèrent que leur expression, à différents stades de développement, influence la résistance au stress. Ces résultats sont en accord avec le rôle de *daf-16* et *hsf-1* à des étapes de vieillissement différentes chez *C. elegans* (470,471). Des données récemment obtenues dans le laboratoire suggèrent

qu'une diète enrichie en glucose agirait de la même manière. La durée de vie du nématode est réduite s'il est sujet à un enrichissement pour toute la durée de sa vie. Cependant, un traitement uniquement dispensé au stade larvaire induit une augmentation de l'espérance de vie alors que le glucose la réduit lorsque administré durant le stade adulte **(Annexe 1)**.

VI. Le glucose induit une neuroprotection

Le lien entre la résistance au stress et la protéotoxicité est robuste. Les organismes modèles ont établi que certaines voies de signalisation comme la voie IIS ou certains gènes, incluant SIRT1/*sir-2.1* et AMPK/*aak-2*, ont un rôle important dans la longévité. Notamment dans la progression de plusieurs pathologies neurodégénératives (26,450). Dans notre étude, nous avons utilisé le modèle TDP-43 développé dans le laboratoire (442) et testé différents mutants, impliqués dans le vieillissement et sensibles au statut nutritionnel de l'organisme. Parmi ces gènes nous avons mesuré les effets de *daf-2*/IGFR, *daf-16*/FOXO3A, *sir-2.1*/SIRT1, *aak-2*/AMPK et *hsf-1*/HSF1, dans un contexte d'enrichissement en glucose. Les effets observés lors d'un traitement à une concentration de 2% de glucose, sur la durée de vie, la résistance au stress, la protéotoxicité et, à moindre effet, les niveaux de progénitures, sont dépendants de ces différents facteurs de transcription. Le lien entre les maladies neurodégénératives et le métabolisme est, jusqu'à présent, peu ou pas décrit chez *C. elegans*. Toutefois, ce lien a déjà été établi chez la souris et les patients atteints de SLA et de la CH. En effet, il a été démontré que l'utilisation du glucose est réduite chez les patients atteints de la CH (390,391,472) et de la SLA (376,377,379,380). Ces données suggèrent que les cellules neuronales sont en manque d'énergie et que la restriction calorique semble même être délétère dans un modèle de souris exprimant la protéine SOD1 (381). Il est donc envisageable de

penser que “l’apport d’énergie” soit une clé pour limiter la progression des symptômes de la SLA et de la CH.

Si nos travaux sur le ver sont encourageants, il est peu probable que l’ajout de glucose diététique, à des doses telles que mentionnées dans ce manuscrit (100 mM), soit envisageable dans des modèles complexes. Une barrière importante s’élève car l’ajout de glucose peut induire une résistance à l’insuline lorsque administré à fortes doses. Cette résistance est, par ailleurs, un facteur important dans la maladie d’Alzheimer. Il serait donc plus pertinent de vérifier par quels moyens il serait possible d’acheminer un apport accru d’énergie vers les cellules neuronales. L’exemple des transporteurs du glucose, dont l’expression est modulée dans la maladie d’Alzheimer (473,474), mais pour lesquels il existe peu de données pour la SLA et la maladie de Huntington, est un sujet qui mérite qu’on s’y attarde.

VII. Le glucose réduit le niveaux de progéniture

Plusieurs groupes ont mis en évidence les effets délétères d’une diète enrichie en glucose sur les niveaux de progéniture (27,464,466). Plus globalement, il est établi que le diabète entraine des problèmes de fertilité ainsi que des complications lors de la grossesse (475). Dans le but de comprendre le mécanisme lié à la diminution de progéniture par un traitement au glucose, nous avons, comme pour la neurodégénérescence liée à TDP-43 mutante, testé différents mutants impliqués dans différentes voies de signalisation liées au métabolisme et à la croissance. Les résultats indiquent que cette diminution des niveaux de progénitures est dépendente de *daf-16*, *aak-2*, *sir-2.1* et *daf-2*. L’équipe du Dr Krause a mis en évidence que les effets liés à la réduction de progéniture étaient augmentés lorsque le gène *ogt-1*, impliqué dans la O-N-acétylglucosaminylation (O-GlcNac) était muté (466). Cette voie de

signalisation est connue pour son rôle de censeur du statut métabolique, étant notamment impliquée dans la résistance à l'insuline (476). Plus particulièrement, les niveaux d'O-GlcNac augmentent lors d'un stress cellulaire (477) et il est maintenant connu que cette modification post-traductionnelle est aussi liée à la chromatine et à la réduction de transcription de certains gènes impliqués dans le métabolisme comme PGC-1α ou FOXO (revue en 478). Toutes ces informations suggèrent qu'une diète enrichie en glucose provoque des changements transcriptionnels au niveau métabolique et il serait intéressant de vérifier les niveaux d'O-GlcNac lors d'un traitement au glucose et d'étudier si les effets présentés dans cette étude sont dépendants de cette modification.

VIII. Le glucose comme un stress?

Des résultats obtenus en parallèle des études présentées ici suggèrent aussi que l'ajout de glucose requiert un environnement riche en oxygène pour augmenter la résistance au stress. Les vers traités à une dose de 2% de glucose sont plus sensibles au stress hypoxique **(Annexe 2)**. Un environnement pauvre en oxygène est nocif pour les neurones parce que leur métabolisme oxydatif requiert une quantité d'oxygène importante pour fonctionner. La possibilité qu'une diète enrichie en glucose puisse induire une hausse du métabolisme oxydatif, pourrait impliquer une augmentation de la production de ROS. Cette hausse peut induire un léger stress (74,479), conduisant à l'expression de facteurs protecteurs permettant de protéger les neurones.

Dans l'optique d'un rôle d'agent stressant, nous avons vérifié la possibilité que le glucose induise une activation de certaines voies de signalisation impliquées dans la réponse au stress. Nos données montrent une activation d'expression de *hsp-6* (HSP60), une chaperonne

impliquée dans la réponse UPR mitochondriale (mitoUPR). Cette activation semble spécifique puisque qu'aucune autre chaperonne, qu'elle soit impliquée dans la réponse UPR du réticulum endoplasmique (*hsp-4*/BiP) (erUPR) ou dans la réponse au stress thermique (*hsp-16.2*), ne montre une activation semblable suite à un traitement au glucose **(Annexe 3)**.

La réponse mitoUPR active plusieurs chaperonnes, influence la résistance au stress et la durée de vie (480-482). Dans une situation d'enrichissement en glucose, où les facteurs régissant la signalisation de la voie UPR - DVE1 (Defective proVEntriculus in Drosophila homolog), UBL5 (UBiquitin-Like family) et ATFS1 (Activating Transcription Factor associated with Stress) - sont absents, le glucose ne peut activer HSP6 et perd aussi la capacité de réduire la protéotoxicité liée à l'expression de TDP-43[A315T] **(Annexe 4)**. Ces données suggèrent que le glucose, en plus de son activité métabolique, agirait comme un stress induisant une réponse mitochondriale. Cette réponse au stress suggère une réponse hormétique soutenue par l'accumulation de données ayant conduit à l'établissement de la théorie de mito-hormèse (483). Comme dans le cas de la réponse hypoxique, cette hypothèse pointe vers un rôle d'agent stressant et il est possible qu'une activation modérée de la voie mitoUPR puisse avoir des effets transitoires, alors qu'une sous-activation, de même qu'une sur-activation puisse être néfaste.

IX. Transmission des effets du glucose

Dans notre deuxième étude, nous nous sommes intéressés à la possibilité que les effets mentionnés dans la 1^{e} étude puissent avoir un effet plus profond dans la résistance au stress. Nos données montrent effectivement que les effets reliés au glucose peuvent être transmis à la génération suivante. Cet effet intergénérationnel ne se produit que sur une seule génération,

impliquant davantage un effet maternel qu'une transmission transgénérationnelle. Néanmoins, cette transmission des effets, produits par le glucose ne s'applique qu'à certains de ces aspects, la diminution de la durée de vie n'étant pas transmise à la génération suivante. Cela suggére un mécanisme permettant une adaptation d'une population qui est confrontée à un environnement hostile, de sorte que la progéniture, qui dans nos conditions expérimentales n'est pas sujette à cet environnement, se voit transmettre une résistance accrue au stress.

Parmi les mécanismes potentiels examinés, la tri-méthylation de la lysine 4 de l'histone 3 (H3K4me3) semble être requise pour la transmission des effets. Cette modification épigénétique est connue pour être une marque reliée à une hausse de transcription, dont le rôle dans le vieillissement a été mis en évidence par l'équipe du Dr Brunet (229). Cependant, dans notre étude les effets ne se transmettent qu'à la génération suivante et l'augmentation de H3K4me3 n'apparait que pour la génération traitée au glucose. L'ablation du complexe de gènes impliqués dans la H3K4me3, la méthyltransférase *set-2* et le membre du complexe *wdr-5.1* sont requis pour la transmission des effets. Cela implique un rôle fonctionnel qui pourrait impliquer un autre type de modification épigénétique requérant la présence du complexe composé de *set-2* et *wdr-5.1* (484). Le rôle de la déméthylase *rbr-2*, connu pour faire contrepoids à *set-2,* reste à étudier et il serait intéressant de vérifier si une mutation de cette dernière entraine une potentialisation de la transmission sur plus d'une génération.

D'un point de vue populationnel, il est possible que cette transmission des effets induits par le glucose soit un mécanisme de protection visant à favoriser la survie de la génération suivante lors d'un stress. Ainsi la progéniture se voit transmettre des caractères visant à augmenter sa résistance au stress, mais sans pour autant subir les effets sur leur espérance de vie.

Limites, Perspectives et Conclusion

I. Le modèle

La force du modèle *C. elegans*, sa simplicité, est aussi sa faiblesse. Il est estimé que 42% des pathologies humaines peuvent être modélisées chez le nématode (441). Cependant, dans le cas du métabolisme, certains aspects ne peuvent être transposés, incluant la résistance à l'insuline et l'obésité. Une hausse de glucose diététique entraine bel et bien une accumulation de tissu adipeux (485) mais les nématodes ne sont pas de taille supérieure à leurs congénères non traités et ne semblent pas développer de résistance à l'insuline, facteur important dans l'étude du métabolisme énergétique et principale dysfonction du diabète de type II.

Dans le cas du métabolisme et de l'activité neuronale, les synapses du nématode sont dites « en passant » parce qu'à la différence des mammifères, les synapses ne sont pas isolées à l'aide de cellules gliales. Ces dernières sont aussi un élément important de la synapse et leur rôle est encore peu décrit chez le ver, malgré plusieurs données quant à leur localisation et leur rôle dans l'excitotoxicité (486,487). Cette différence organisationnelle est un élément limitant dans l'étude plus spécifique des troubles neurodégénératifs.

II. Étude du métabolisme au sens large

Nous avons montré un rôle novateur pour le glucose mais il reste une quantité importante d'informations à mettre à jour. Les facteurs de transcriptions *daf-16* et *hsf-1* sont impliqués, bloquant la majorité des effets liés à l'ajout de glucose, mais nous n'avons pas

établi quelle activité transcriptionnelle est primordiale dans ce contexte. Un criblage pour des variations d'expression entre des individus traités au glucose ou non pourrait permettre de mieux comprendre les mécanismes sous-jacents à ces effets. Une analyse des gènes contrôlés par les facteurs de transcriptions mentionnés précédemment (*daf-16* et *hsf-1*), aurait aussi un intérêt, puisque nous n'avons pas vérifié de manière précise leur niveau d'expression.

Dans un deuxième temps, il serait pertinent de vérifier les métabolismes protéiques et lipidiques. L'étude du rôle des acides gras dans la longévité et dans une potentielle neuroprotection est une idée à ne pas écarter (377-379). Leur rôle dans le métabolisme et sur la longévité ayant déjà été établi par d'autres (488-490). Le métabolisme du glucose influence de manière importante celui des acides gras et une hausse de glucose dans la diète entraine une hausse de la lipogenèse, phénomène impliqué dans la résistance à l'insuline via une diminution du transporteur du glucose GLUT4, sensible à cette dernière (491-493). Un deuxième aspect pourrait aussi être lié à l'utilisation d'acides gras non estérifiés omega-3 (n-3-AGNE) puisqu'une diminution du ratio n-6/n-3 des acides gras réduit la résistance à l'insuline chez la souris. En effet, l'expression du gène *C. elegans fat-1*, dont la fonction est de transformer les acides-gras n-6 en n-3, chez la souris améliore les symptômes d'obésité et d'inflammation liés à la résistance à l'insuline (494,495).

III. Transport du glucose

L'ajout de glucose tel que présenté dans cette étude est difficilement conciliable et transposable à des modèles plus complexes comme les mammifères, les complications secondaires à une diète trop riche en hydrate de carbone étant importantes. Cependant, plusieurs composés diététiques ont montré des effets importants sur l'activation du

transporteur GLUT4, suggérant ainsi qu'il est possible de modifier la physiologie de ces transporteurs via plusieurs mécanismes (revue en 496). Ces données sont particulièrement intéressantes parce qu'elles impliquent une possibilité de voie thérapeutique. En effet, le principal transporteur du glucose dans le système nerveux est GLUT3, l'isoforme avec le plus d'affinité et le plus de capacité catalytique (revue en 270) parmi les transporteurs de classe I (GLUT1-4). Son expression est modulée lors de désordres métaboliques ainsi que dans la maladie d'Alzheimer (473,474), suggérant un rôle prépondérant dans le métabolisme neuronal. Dans le cas de *C. elegans*, le seul transporteur identifié à ce jour est nommé *fgt-1* (facilited glucose transporter 1) et semble avoir une affinité similaire à celle de GLUT2. Il serait intéressant de vérifier les effets induits par le glucose dans un contexte de mutation de *fgt-1* et de vérifier si son expression change dans des conditions pathologiques avec une expression de TDP-43[A315T] ou de 128Q, et si tel est le cas, de vérifier si certains composés ont la capacité de moduler cette expression (454,496).

IV. Modulation épigénétique

La publication de travaux sur la transmission épigénétique (229,232) de la longévité chez *C. elegans* nous a amenés à envisager ce concept dans nos conditions d'enrichissement en glucose. Notre travail portant sur la transmission de certaines résistances cellulaires liées à un environnement enrichi en glucose a mis en évidence une dépendance de cette transmission à la modification épigénétique H3K4me3. Cette modification est liée à une hausse de l'activité transcriptionnelle, au stress et au vieillissement. Dans un avenir proche, l'étude plus approfondie des différentes modifications épigénétiques pourrait donner une image plus claire des schémas de transmissions liés au métabolisme. Récemment, plusieurs groupes ont montré

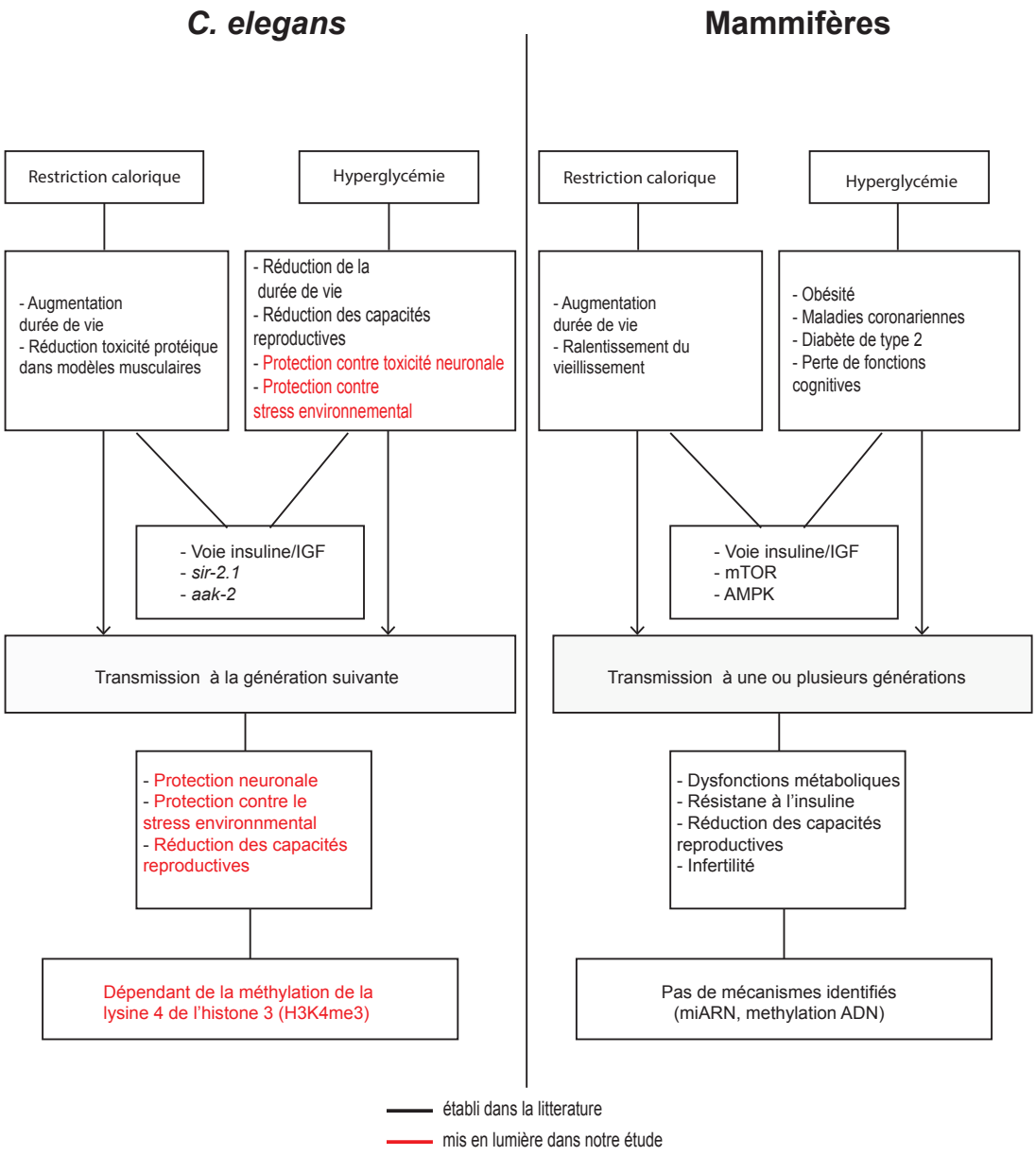
un lien entre différents facteurs métaboliques, tels que FOXO3A (497) et la modification post-traductionnelle O-GlcNac (498,499) et différents composants de la transmission dite épigénétique comme le complexe de remodelage SWI/SNF. Le métabolisme du glucose est intimement lié à ces deux composants et il serait pertinent de vérifier si l'ajout de glucose dans la diète favorise l'augmentation ou la répression de la transcription de certains gènes.

V. Conclusions

Les études présentées ici ont mis en lumière un rôle particulier du glucose. En effet, il est évident que cette molécule, bien qu'essentielle à la survie d'un organisme, nécessite un contrôle important. Les effets négatifs liés à une diète trop riche ne sont plus à démontrer et il serait fort peu avisé de conseiller à des patients d'augmenter leur consommation de glucose. Néanmoins, dans des conditions pathologiques il est possible qu'une hausse des niveaux énergétiques puisse aider le système à combattre l'accumulation de protéines ou de dommages cellulaires. L'utilisation d'un organisme-modèle comme le nôtre a permis cette modélisation, comme par exemple l'expression d'une protéine mutante TDP-43 ou encore HTT_{1-17}-128Q. Dans un deuxième temps, l'ajout de glucose dans la diète semble activer des voies de signalisation qui favorisent l'expression de certaines chaperonnes impliquées dans la réponse au stress. Lorsque ces voies sont activées à des stades précoces du développement (stade larvaire dans le cas du ver) et pour une courte durée (jusqu'au début du stade adulte), il est envisageable que cela puisse favoriser la survie d'un organisme. Ces données soutiennent l'hypothèse d'hyperfonction liée au vieillissement (500), qui veut que des mécanismes cellulaires activés durant le développement et nécessaires au bon fonctionnement de l'organisme, continuent d'être activés chez l'adulte et soient la cause du vieillissement. Ce

stress pourrait, par ailleurs, favoriser la transmission de facteurs génétiques à une ou plusieurs générations, permettant une augmentation de la résistance à un environnement hostile et ce via des mécanismes épigénétiques qui sont encore à démontrer.

Ces études révèlent que, malgré ses effets nocifs, le glucose a la capacité d'influencer la survie d'un organisme **(Figure 8)**. Cette hausse de résistance cellulaire ne se fait malheureusement pas sans effets secondaires importants. À l'avenir, il sera primordial de s'attarder à ce métabolisme et aux possibilités qu'il peut offrir dans le traitement de maladies neurodégénératives.



<u>Figure 8: Schéma recapitulatif des effets liés à un diète riche en glucose</u>.
Les effets liés à un restriction calorique ou à un hyperglycémie sont variés et ont des effest souvent néfastes. Néanmoins, dans certains cas pathologiques, l'ajout de glucose à la diète peut avoir des effets positifs sur l'organisme *C. elegans*. En noir les effets connus dans la litterature et en rouge les effets mis en lumière dans notre étude.

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Annexe 1 : L'influence sur longévité d'un environnement enrichi en glucose dépend du stade de développement du nématode.

Plusieurs données suggèrent que la réponse au stress n'est pas constante au fil du développement d'un organisme et que des stades plus avancés ont une réponse de moins grand amplitude lorsque confrontés à un stress (501). Le glucose ayant la capacité d'induire la réponse mitoUPR, nous avons vérifié si la toxicité de ce dernier était dépendante du stade de développement des animaux traités dans un environnement enrichi en glucose.

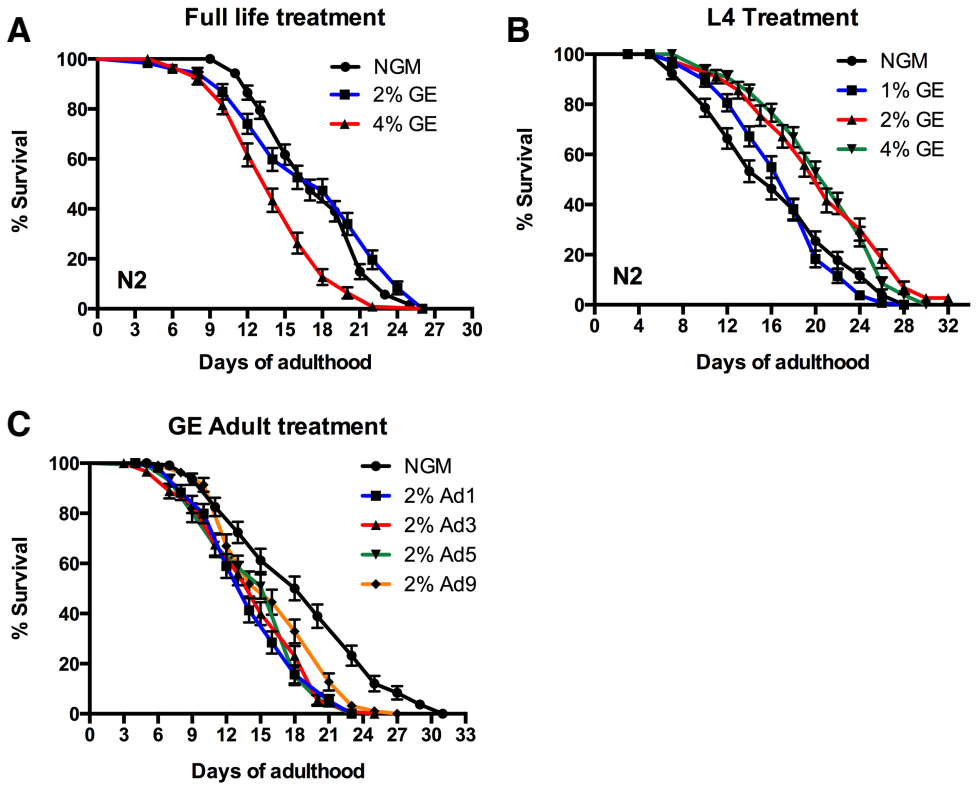

Figure 1 : L'influence sur longévité d'un environnement enrichi en glucose dépend du stade de développement du nématode
Une diète enrichie en glucose induit différents effets dépendant du stade de développemement durant lequel le traitement est appliqué. **A)** Un traitement tout au long de la durée de vie aura des effets délétères sur la durée de vie, **B)** alors qu'un traitement au stade larvaire l'augmente et **C)** qu'un traitement à l'âge adulte uniquement induite une réduction significative, même à 2% de concentration.

Annexe 2 : Une diète enrichie en glucose induit une sensibilité accrue au stress hypoxique.

L'ajout de glucose dans la diète semble avoir des effets positifs sur la réponse au stress cellulaire, incluant les stress oxydatifs, osmotiques et thermiques. Cependant, des tests préliminaires effectués relativement au stress hypoxique semble indiquer qu'un environnement enrichi en glucose augmente la sensibilité des sujets traités. Le ver sauvage montre une hausse importante de mortalité après 24h de traitements et cette toxicité est plus importante pour des sujets mutants pour le gènes *hif-1*, alors que le mutant *daf-2*, connu pour être résistant à différents stress, voit sa sensibilité augmenter mais de manière très limité.

Cette condition de stress est la seule répertoriée dans nos travaux conduisant à l'augmentation de la mort des vers traités. Ces données suggèrent un besoin important d'oxygène lorsque la diète est enrichie en glucose qui, lorsque combiné à un environnement extérieur hypoxique, cause une diminution de la survie.

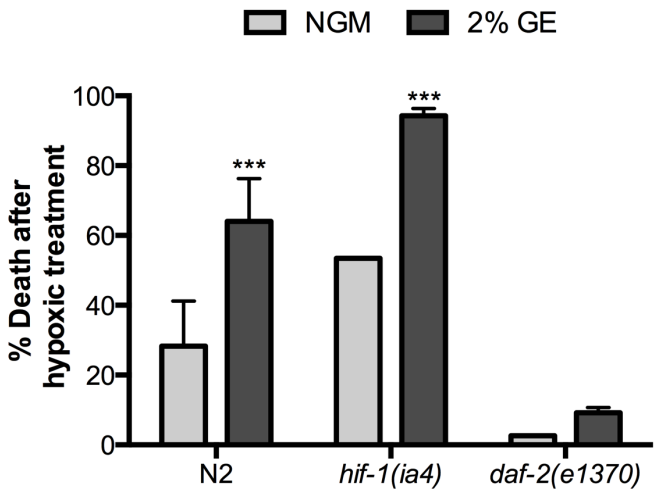

Figure 2 : Le glucose augmente la sensibilité au stress hypoxique.
Les vers traités en conditions d'enrichissement en glucose (2%) montrent une augmentation de leur sensibilité après après 24h stress hypoxique. ***P<0.001

Annexe 3 : Une diète enrichie en glucose induit l'expression d'une chaperonne mitochondriale.

Lors de nos différentes expériences nous avons établit qu'un environnement enrichie en glucose induit une augmentation de la résistance à différents stress cellulaires. La question suivant a été de vérifier si cette protection est médié par l'augmentation de certaines chaperonnes. Nous avons utilisé des rapporteurs transcriptionnels de chaperonnes (502) répondant à différents stress : *hsp-6* et *hsp-60* (stress mitochondrial), *hsp-4* (stress du réticulum endoplasmique) et *hsp-16.2* (stress thermique).

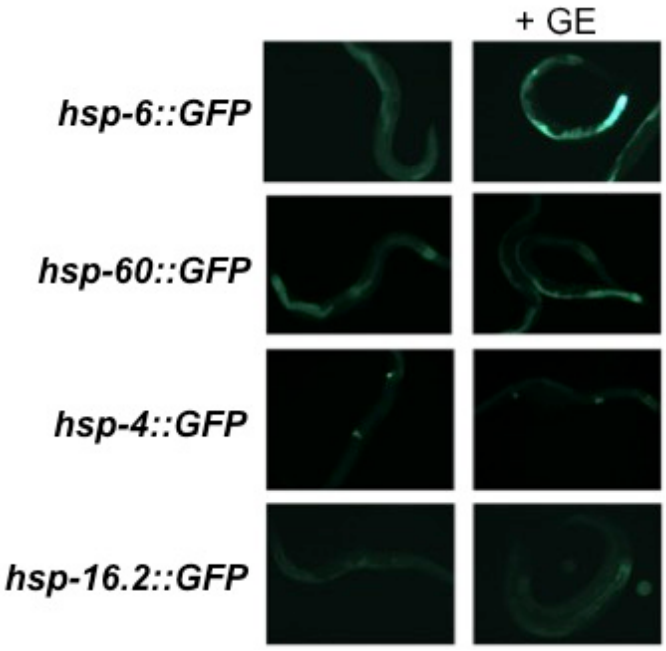

Figure 3 : Une diète enrichie en glucose induit l'expression de la chaperonne mitochondriale HSP6.
Une diète enrichie en glucose induit l'expression de *hsp-6*p ::GFP, mais n'a aucun effet sur les rapporteur des chaperonnes *hsp-60* (mitochondrie), *hsp-4* (réticulum endoplasmique) et *hsp-16.2* (thermique).

Annexe 4 : La glucoprotection contre TDP-43 est dépendante de la réponse mitoUPR.

L'induction de la réponse mitoUPR par une diète enrichie en glucose pose la question de savoir si cette dernière est responsable de l'induction pour moduler la toxicité inhérente au glucose, ou alors, si cette induction est causé par un mécanisme métabolique lié au métabolisme du glucose. Dans le cas de la protéotoxicité lié à l'expression de la protéine TDP-43[A315T], la perte de fonction des gènes impliqués dans la réponse mitoUPR (482) induit une perte des effets lié au glucose, indiquant que cette réponse est bel et bien nécessaire à la protection par l'hydrate de carbone.

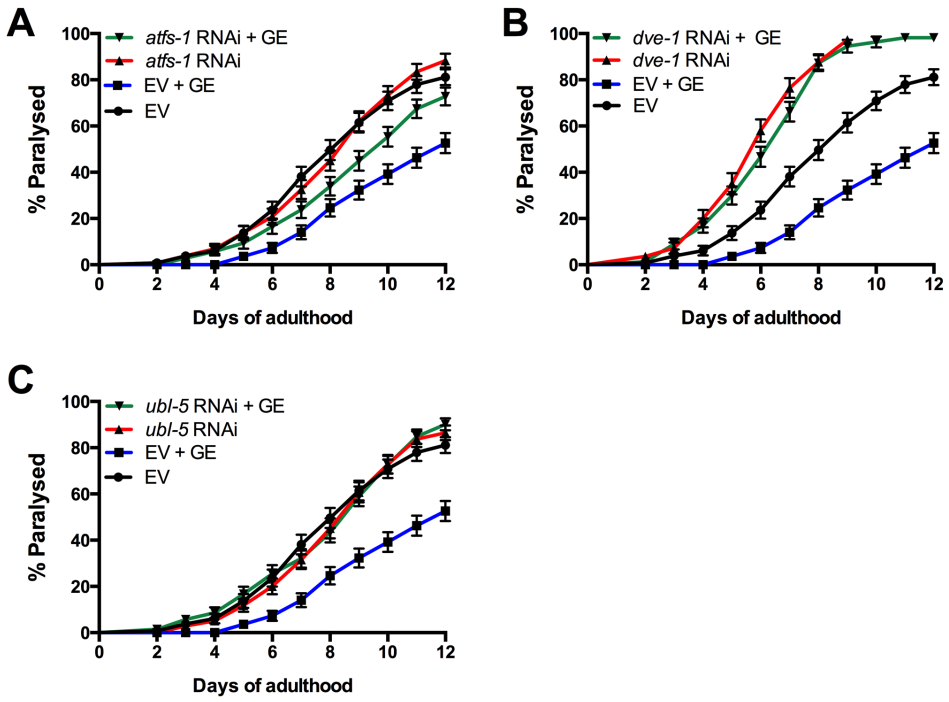

Figure 4 : La glucoprotection contre la toxicité de TDP-43 est dépendante de la réponse mitoUPR.
Le glucose réduit la toxicité liée à mTDP-43 lorsque traité avec l'ARNi contrôle, mais cette protection est perdue lors d'un traitement avec de l'ARNi pour **A)** *atfs-1*, **B)** *dve-1* et **C)** *ubl-5*.

Annexe 5:

Mutant TDP-43 and FUS Cause Age-Dependent Paralysis and Neurodegeneration in C. elegans.

Vaccaro, A., **Tauffenberger, A**., Aggad, D., Rouleau, G., Drapeau, P., & Parker, J. A. (2012).
PLoS ONE, *7*(2), e31321. doi:10.1371/journal.pone.0031321.s013

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PLoS one

Mutant TDP-43 and FUS Cause Age-Dependent Paralysis and Neurodegeneration in *C. elegans*

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Abstract

Mutations in the DNA/RNA binding proteins TDP-43 and FUS are associated with Amyotrophic Lateral Sclerosis and Frontotemporal Lobar Degeneration. Intracellular accumulations of wild type TDP-43 and FUS are observed in a growing number of late-onset diseases suggesting that TDP-43 and FUS proteinopathies may contribute to multiple neurodegenerative diseases. To better understand the mechanisms of TDP-43 and FUS toxicity we have created transgenic *Caenorhabditis elegans* strains that express full-length, untagged human TDP-43 and FUS in the worm's GABAergic motor neurons. Transgenic worms expressing mutant TDP-43 and FUS display adult-onset, age-dependent loss of motility, progressive paralysis and neuronal degeneration that is distinct from wild type alleles. Additionally, mutant TDP-43 and FUS proteins are highly insoluble while wild type proteins remain soluble suggesting that protein misfolding may contribute to toxicity. Populations of mutant TDP-43 and FUS transgenics grown on solid media become paralyzed over 7 to 12 days. We have developed a liquid culture assay where the paralysis phenotype evolves over several hours. We introduce *C. elegans* transgenics for mutant TDP-43 and FUS motor neuron toxicity that may be used for rapid genetic and pharmacological suppressor screening.

Citation: Vaccaro A, Tauffenberger A, Aggad D, Rouleau G, Drapeau P, et al. (2012) Mutant TDP-43 and FUS Cause Age-Dependent Paralysis and Neurodegeneration in *C. elegans*. PLoS ONE 7(2): e31321. doi:10.1371/journal.pone.0031321

Editor: Leonard Petrucelli, Mayo Clinic, United States of America

Received October 11, 2011; **Accepted** January 5, 2012; **Published** February 21, 2012

Funding: This research was supported by: Montreal University Hospital Foundation (http://fondationduchum.com/fr) to JAP, Natural Sciences and Engineering Research Council Discovery Grant (http://www.nserc-crsng.gc.ca) to JAP, Canadian Institutes of Health Research New Investigator (http://www.cihr-irsc.gc.ca) to JAP, Frick Foundation for ALS Research (http://frick-fondation.ch) to PD and JAP and a Bernice Ramsay Discovery Grant from the ALS Society of Canada (http://www.als.ca/) to JAP. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Amyotrophic Lateral Sclerosis (ALS) is a late-onset progressive disease affecting motor neurons ultimately causing fatal paralysis [1,2]. Most cases are sporadic, but ~10% of patients have an inherited familial form of the disease. Dominant mutations in SOD1 (copper/zinc superoxide dismutase 1) account for ~20% of familial ALS cases and ~1% of sporadic cases [1]. The recent discovery of mutations in TAR DNA-binding protein-43 (TDP-43) and Fused in sarcoma (FUS, also named TLS) in both familial ALS and frontotemporal dementia (FTD) has shifted research into disease mechanisms and potential therapeutics [3–9].

TDP-43 and FUS are evolutionarily conserved DNA/RNA binding proteins that shuttle between the nucleus and cytoplasm having multiple roles including DNA transcription and RNA processing [3,9–12]. Mutant TDP-43 and FUS (mTDP-43 and mFUS) are found in cytoplasmic inclusions in the disease state while the accumulation of wild type TDP-43 and FUS (wtTDP-43 and wtFUS) are observed in an increasing number of disorders including Alzheimer's Disease, Parkinson's Disease and the polyglutamine diseases (reviewed in [10]). The pathogenic mechanisms for mutant TDP-43 and FUS age-dependent neuronal toxicity remain unclear. As of now there is no consensus whether mutant TDP-43 and FUS employ a loss-of-function, a gain-of-function, or both in motor neuron cell death.

Since TDP-43 and FUS are evolutionarily conserved we used the nematode *Caenorhabditis elegans* to investigate mutant TDP-43 and FUS age-dependent neurodegeneration. We created transgenic nematodes that express full-length wild type or mutant TDP-43 and FUS in the worm's GABAergic motor neurons. Transgenic TDP-43 and FUS worms recapitulate a salient feature of ALS; they display adult-onset, age-dependent, progressive paralysis and degeneration of motor neurons. Importantly, mTDP-43 and mFUS, but not wtTDP-43 and wtFUS, strains show the presence of insoluble proteins in extracts from whole animals suggesting that protein misfolding may be a primary cause of toxicity. We introduce a genetically tractable platform to investigate motor neuron toxicity caused by mutant TDP-43 and FUS that can be used for suppressor screening.

Results

Transgenic worms expressing full-length human TDP-43 or FUS in motor neurons display age-dependent paralysis

Since ALS is a motor neuron disease we expressed wild type and mutant human TDP-43 and FUS proteins in the worm's 26 GABAergic motor neurons with the vesicular GABA transporter (*unc-47*) promoter (Figures 1A, B) [13]. Multiple transgenic strains carrying extrachromosomal arrays were obtained by microinjection and stable lines with chromosomally-integrated transgenes

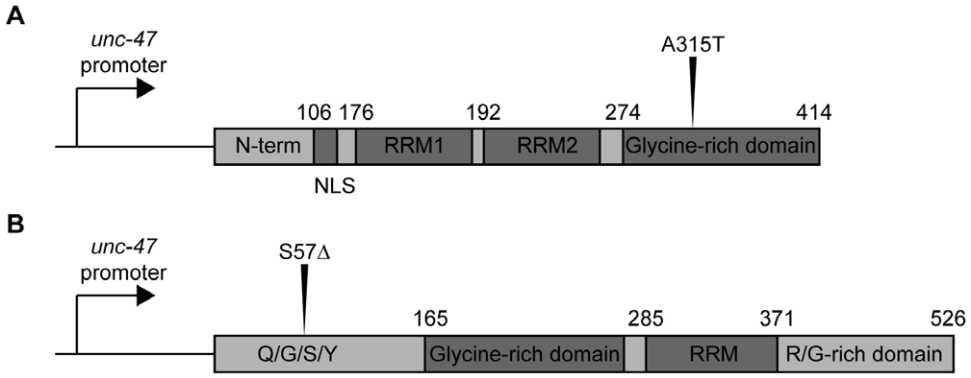

Figure 1. TDP-43 and FUS transgene constructs. (A) Full-length wild type human TDP-43 and the clinical mutation A315T were cloned into a vector for expression in motor neurons by the *unc-47* promoter and injected into *C. elegans*. (B) Full-length wild type human FUS and the clinical mutation S57Δ were cloned into the *unc-47* expression vector and injected into *C. elegans*. RRM (RNA Recognition Motif), Q/G/S/Y (Glutamine-Glycine-Serine-Tyrosine-rich region), R/G (Arginine-Glycine-rich region), NLS (Nuclear localization signal).
doi:10.1371/journal.pone.0031321.g001

were isolated after UV-irradiation [14]. Both wild type TDP-43 and the ALS-associated A315T mutant proteins were expressed in transgenic worms as detected by immunoblotting of worm protein extracts with a human specific TDP-43 antibody (Figure 2A) [4]. Similarly, using a FUS antibody we confirmed the expression of wild type and the ALS-linked S57Δ mutant proteins by western blotting (Figure 2B) [15].

All strains were morphologically normal and showed no adverse phenotypes during development. However, during adulthood the transgenic strains begin to display uncoordinated motility phenotypes that progressed to paralysation. Paralysis was age-dependent and occurred at higher rate for mTDP-43 and mFUS worms compared to wtTDP-43 and wtFUS transgenics (Figures 3 A, B). Typically, after 12–13 days on plates 100% of the mTDP-43 and mFUS worms were paralysed while only 20% of the wtTDP-43 and wtFUS worms were affected. The low rate of paralysis for wtTDP-43 and wtFUS strains is comparable to what is observed in transgenics expressing GFP from the same *unc-47* promoter (Figure 3C). Additionally, the paralysis assay is widely used to study age-dependent degenerative phenotypes and is not observed in wild type non-transgenic worms until they reach advanced age (approximately 20 days) [16–18]. Finally, motility defects and adult onset paralysis have been previously observed in worms with degenerating GABAergic motor neurons suggesting that mTDP-43 and mFUS may negatively affect GABAergic neuronal function and survival [19].

TDP-43 and FUS transgenics have normal lifespans

One of the signs of aging in worms is decreased motility [18,20]. Thus the progressive paralysis phenotypes observed in the TDP-43 and FUS transgenics may be due to overall decreased health from the expression of toxic non-native proteins leading to accelerated mortality, a part of which is a decline in motility. We conducted lifespan analyses and observed that all of the transgenics had lifespans indistinguishable from non-transgenic wild type N2 worms (Figures 4A, B and Table S1). These observations suggest that the paralysis observed in our models is specific to the expression of TDP-43 and FUS in motor neurons and not due to secondary effects from general sickness and reduced lifespan.

TDP-43 and FUS cause neuronal dysfunction

The progressive paralysis phenotypes caused by mTDP-43 and mFUS suggest there may be motor neuron dysfunction and/or

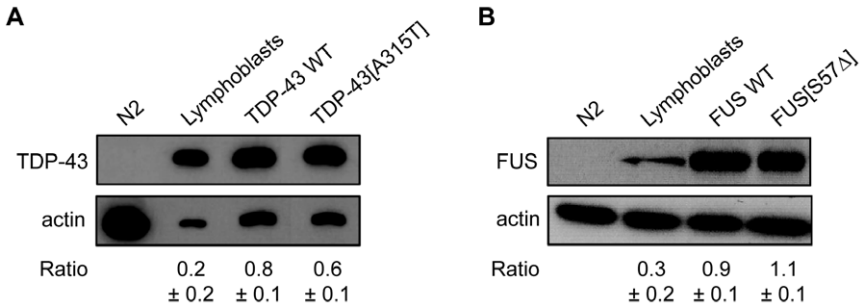

Figure 2. Expression of human TDP-43 and FUS proteins in *C. elegans* transgenics. (A) Total protein levels from non-transgenic worms, human lymphoblast cells and transgenic worms expressing wtTDP-43 or mTDP-43. Staining with a human TDP-43 antibody showed no signal for non-transgenic worms but a signal corresponding to full-length human TDP-43 at ~45 kDa in size was observed in extracts from human cells and the two transgenic TDP-43 worm strains. wtTDP-43 and mTDP-43 strains showed comparable protein expression levels. (B) Total protein levels from non-transgenic worms, human lymphoblast cells and transgenic worms expressing wtFUS or mFUS. Using a human FUS antibody, no signal was detected in non-transgenic worms, but a signal corresponding to full-length human FUS at ~75 kDa in size was observed in extracts from lymphoblast cells and the transgenic FUS worm strains. wtFUS and mFUS worms showed identical levels of protein expression. For all experiments actin staining was used as a loading control and expression ratios ± SEM of TDP-43 or FUS to actin was determined from 3 independent experiments. Representative western blots are shown.
doi:10.1371/journal.pone.0031321.g002

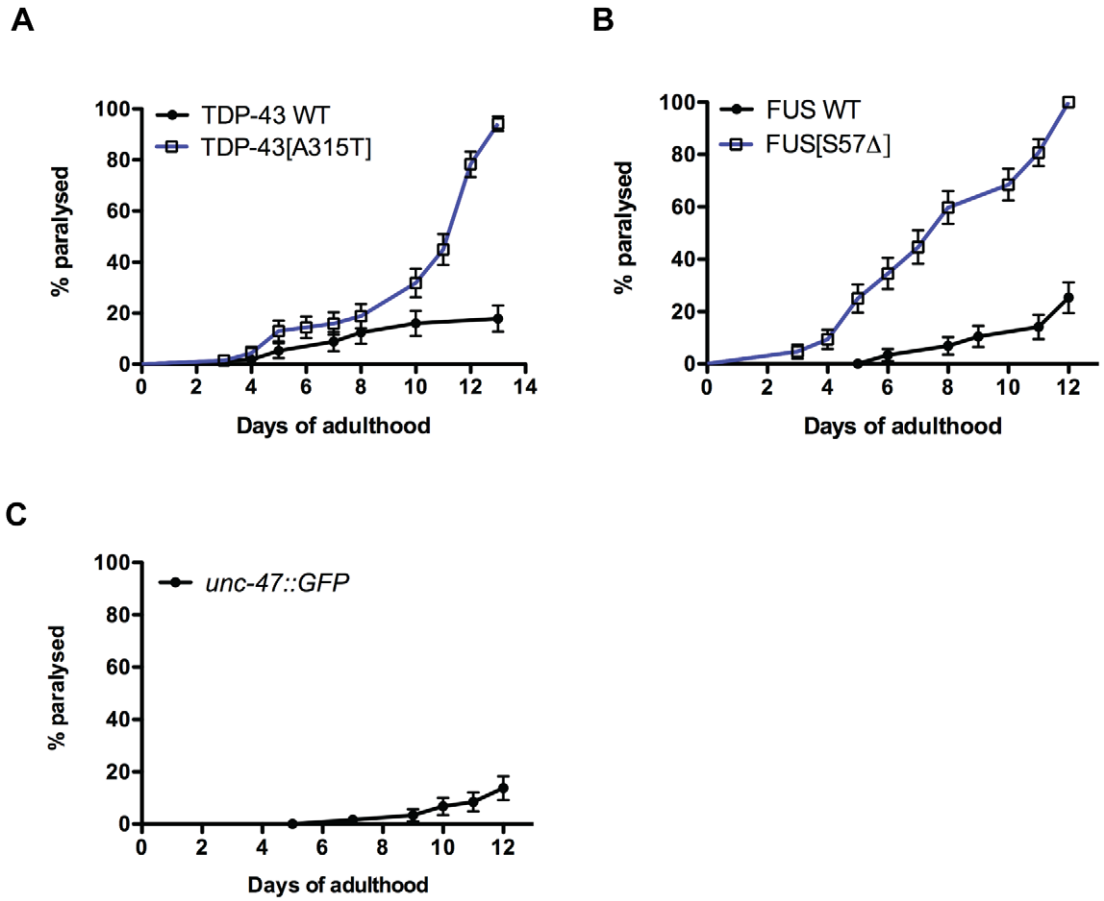

Figure 3. Mutant TDP-43 and FUS cause adult-onset, age-dependent paralysis in *C. elegans*. Transgenics were monitored from the adult stage and scored daily for paralysis. (A) mTDP-43 worms show a rate of progressive paralysis that is greater than transgenics expressing wtTDP-43 ($P<0.001$). (B) Transgenics expressing mFUS become paralysed significantly sooner than wtFUS control transgenics ($P<0.001$). (C) Transgenic worms expressing GFP in motor neurons show low levels of paralysis.
doi:10.1371/journal.pone.0031321.g003

degeneration in these animals. *C. elegans* body wall muscle cells receive excitatory (acetylcholine) and inhibitory (GABA) inputs to coordinate muscle contraction/relaxation and facilitate movement [21,22]. Body wall muscle activity can be measured indirectly with the acetylcholinesterase inhibitor aldicarb [23]. Exposure to aldicarb causes accumulation of acetylcholine at neuromuscular junctions resulting in hyperactive cholinergic synapses, muscle hypercontraction, and acute paralysis [23]. Hypersensitivity to

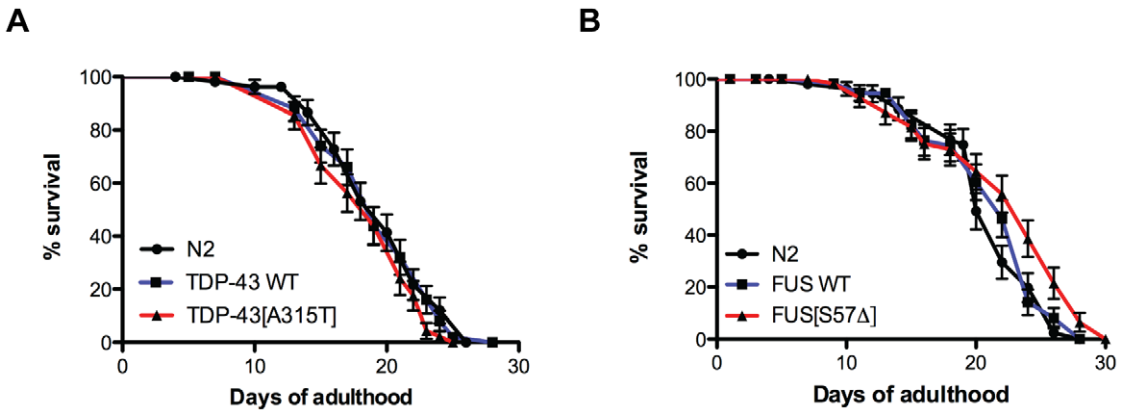

Figure 4. TDP-43 and FUS transgenes do not affect lifespan. Beginning at Day 1 of adulthood we tested the lifespans of wild type non-transgenic N2 worms and transgenics expressing (A) wtTDP-43 and mTDP-43 as well as (B) animals expressing wtFUS and mFUS. Animals expressing TDP-43 or FUS transgenes had lifespans indistinguishable from N2 worms.
doi:10.1371/journal.pone.0031321.g004

aldicarb-induced paralysis has been used to identify genes that increase acetylcholine secretion or decrease inhibitory GABA signalling [24]. For example mutants lacking genes required for GABA transmission like the vesicular GABA transporter *unc-47* are hypersensitive to aldicarb-induced paralysis [25]. To investigate if our TDP-43 and FUS transgenics had abnormal activity at the neuromuscular junction we exposed the animals to aldicarb. We observed that, like *unc-47* mutants, mTDP-43 and mFUS animals were hypersensitive to aldicarb-induced paralysis, while wtTDP-43 and wtFUS transgenics showed a rate paralysis identical to non-transgenic N2 worms (Figures 5A, B). These data suggest that the inhibitory GABA signalling is impaired in mTDP-43 and mFUS transgenics. *unc-47* mutants are classically described as having a "shrinker" phenotype, where in response to touch the worm does not move away but instead the whole body undergoes longitudinal shortening [21], and we observed that the shrinker phenotype was weakly penetrant in adult mTDP-43 and mFUS worms. To determine if impaired GABAergic neurotransmission contributed to the paralysis phenotype we examined two *unc-47* loss-of-function mutants and they both showed age-dependent paralysis, a phenotype not previously reported for *unc-47* (Figure 5C) [21]. Thus, mTDP-43 and mFUS cause neuronal dysfunction in GABA neurons leading to progressive motility defects culminating in paralysis, a phenotype similar to animals deficient in GABAergic signalling.

TDP-43 and FUS cause progressive degeneration of motor neurons

Many neurodegenerative diseases are characterized by neuronal dysfunction prior to degeneration [26]. To investigate if the progressive paralysis phenotypes in our TDP-43 and FUS transgenics were accompanied by neurodegeneration we crossed all of the transgenics with an integrated reporter (*unc-47p::GFP*) that expresses GFP in the same GABAergic motor neurons [13] (Figures 6A, B). Similar to reports from another *C. elegans* TDP-43 toxicity model [27], we observed gaps/breaks in motor neuron processes in TDP-43 and FUS animals compared to animals expressing *unc-47p::GFP* alone (Figures 6 C–F). We extended our analysis by scoring degeneration in living GFP, wtTDP-43, mTDP-43, wtFUS and mFUS transgenics at days 1, 5 and 9 of adulthood. We observed that degeneration was age-dependent and occurred at higher rate for the mTDP-43 and mFUS animals compared to the wtTDP-43 and wtFUS transgenics (Figure 6G). Thus our TDP-43 and FUS transgenics mimic the adult-onset, gradual decline of neuronal function ultimately resulting in age-dependent motor neuron degeneration seen in diseases like ALS.

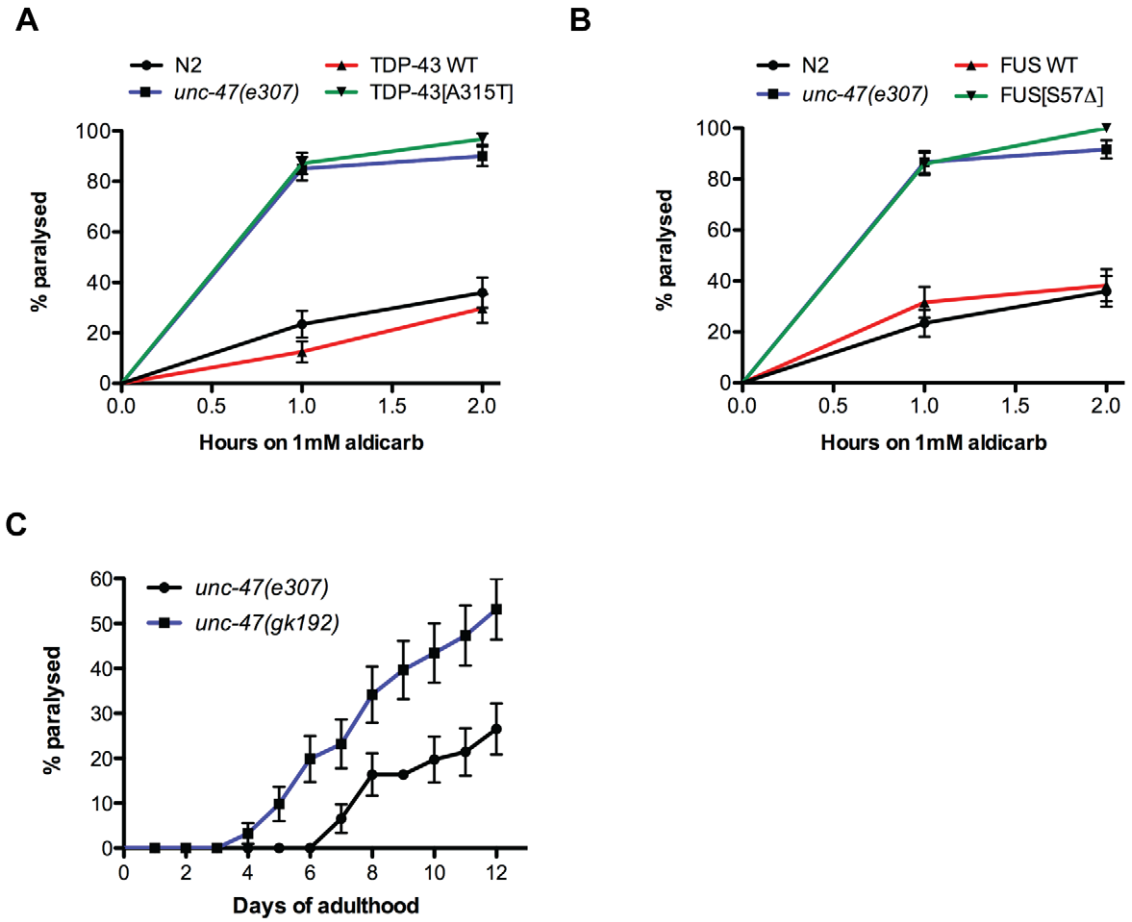

Figure 5. Mutant TDP-43 and FUS impair synaptic transmission. (A) Cholinergic neuronal transmission was measured by determining the onset of paralysis induced by the cholinesterase inhibitor aldicarb. *unc-47(e307)* mutants and mTDP-43 transgenics were hypersensitive to aldicarb-induced paralysis compared to either wtTDP-43 transgenics or N2 worms ($P<0.001$ for *unc-47* or mTDP-43 compared to N2 or wtTDP-43 worms). (B) mFUS transgenics and *unc-47(e307)* mutants were more sensitive to aldicarb induced paralysis compared to either wtFUS transgenics or N2 controls ($P<0.001$). (C) *unc-47* mutants grown on regular worm plates showed age-dependent progressive paralysis.
doi:10.1371/journal.pone.0031321.g005

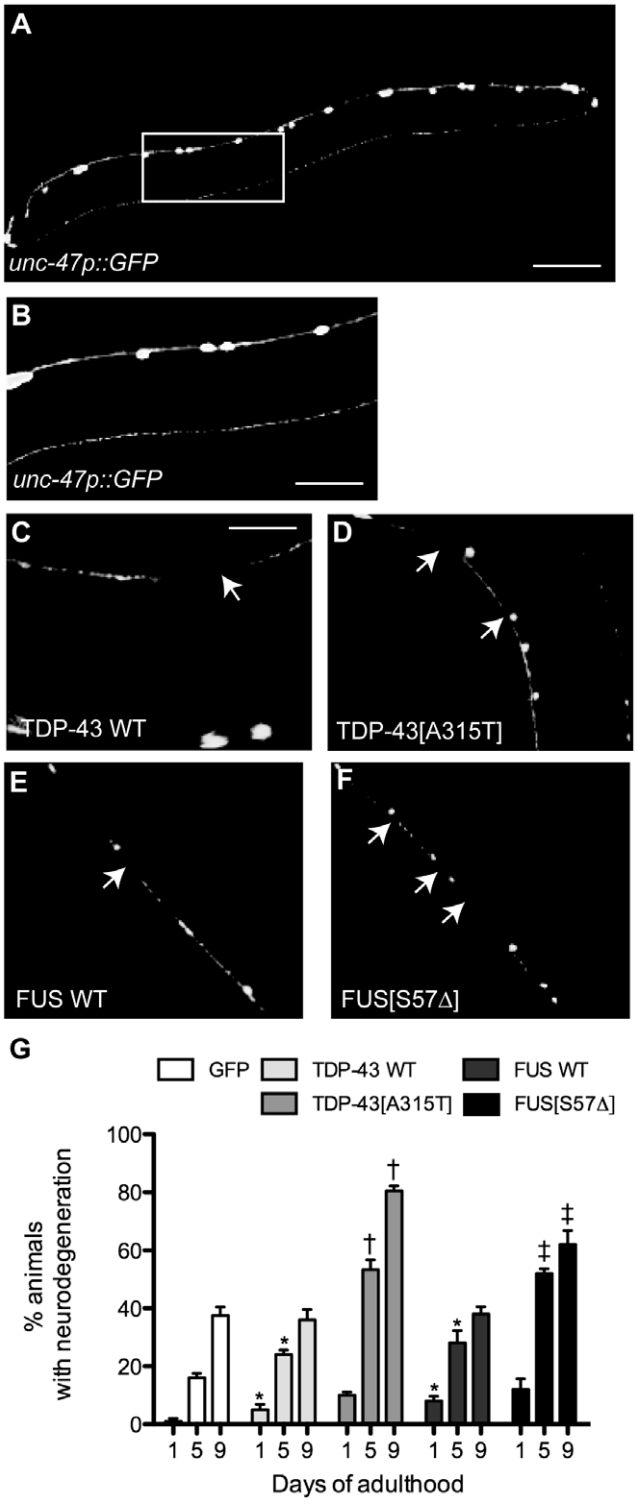

Figure 6. Mutant TDP-43 causes motor neuron degeneration. Shown are representative photos of living, adult *unc-47p::GFP*, *unc-47p::GFP;TDP-43*, and *unc-47p::GFP;FUS* transgenics. (A) Image of an entire *unc-47p::GFP* worm showing the GABAergic motor neurons. Scale bar represents 50 μm. (B) High-magnification of the framed area from (A) showing wild type morphology of motor neurons. Scale bar represents 20 μm. High magnification of motor neurons labelled with *unc-47p::GFP* in (C) wtTDP-43, (D) mTDP-43, (E) wtFUS and (F) mFUS transgenics showing gaps along neuronal processes (arrows). Scale bar represents 10 μm for photos (C) to (F). (G) Quantification of neurodegeneration in transgenic worms at days 1, 5 and 9 of adulthood. * wtTDP-43 and wtFUS have a higher rate of neurodegeneration compared to *unc-47p::GFP* controls at days 1 and 5 of adulthood ($P<0.05$). †mTDP-43 transgenics have a higher rate of neurodegeneration at days 5 and 9 compared to wtTDP-43 transgenics ($P<0.001$). ‡mFUS transgenics show an enhanced rate of neurodegeneration at days 5 and 9 of adulthood in compared to wtFUS transgenics ($P<0.001$).
doi:10.1371/journal.pone.0031321.g006

Mutant TDP-43 and FUS are highly insoluble

Since TDP-43 and FUS are prone to aggregation in several model systems including *C. elegans*, we tested if the same was true for our transgenics [27–33]. To examine if protein misfolding is more pronounced for strains expressing mTDP-43 and mFUS, we used a biochemical assay to detect protein aggregation. Homogenized protein extracts from transgenic worms were separated into supernatant (detergent-soluble) and pellet (detergent-insoluble) fractions [30]. Immunoblotting the TDP-43 transgenics with a human TDP-43 antibody revealed the accumulation of mTDP-43 in the pelleted, insoluble fraction, while wtTDP-43 proteins were predominantly detected in the supernatant, or soluble fractions (Figure 7A). Similar results were obtained for the FUS transgenics where immunoblotting with a human FUS antibody showed that mFUS accumulated in the insoluble pellet fraction while wtFUS proteins remained soluble (Figure 7B). These data suggest that mTDP-43 and mFUS proteins are susceptible to misfolding leading to insolubility and aggregation that may contribute to motor neuron dysfunction and degeneration.

Next focusing on the mTDP-43 and mFUS transgenics we fixed whole *unc-47p::GFP;mTDP-43* and *unc-47p::GFP;mFUS* worms and respectively stained them with human TDP-43 and human FUS antibodies. We detected mTDP-43 and mFUS in both the nuclei and cytoplasm of motor neurons (Figure 8). The cytoplasmic accumulation of mTDP-43 and mFUS in our transgenics is consistent with findings in patients suggesting that these proteins misfold leading to intracellular build-up and aggregation [10].

Finally, we noticed that the fixed mTDP-43 and mFUS showed gaps or breaks along the GFP labelled neuronal processes similar to what was observed in living animals (Figures 6D, F). To confirm that neurodegeneration was not simply due to loss of GFP signals, we stained whole *unc-47p::GFP;mTDP-43* and *unc-47p::GFP;mFUS* worms for GABA [22]. We observed that the gaps along the processes as visualized by a loss of GFP signal likewise corresponded to a loss of GABA staining (Figure 9). Altogether these data suggest that the expression of TDP-43 and FUS lead to degeneration of motor neurons as has been observed for TDP-43 in other worm models [27].

Paralysis phenotypes are enhanced in liquid culture

One goal in developing these transgenics is for use in genetic and pharmacological suppressor screens. TDP-43 and FUS transgenics may have decreased inhibitory GABA signalling ultimately causing muscle hypercontraction leading to paralysis. When grown on solid media the mTDP-43 and mFUS paralysis phenotypes manifest over a period of 5 to 13 days (Figure 3). Worms grown in liquid culture exhibit a stereotypical swimming motion that is considerably more vigorous than worms crawling on solid media [34]. We hypothesized that placing worms in liquid culture would increase activity at the neuromuscular junction and precipitate paralysis phenotypes much earlier than worms grown on solid media.

Using age-synchronized worms we transferred young adult TDP-43 and FUS transgenics to 96-well plates with liquid media and scored their motility every 2 hours. We observed a rapid

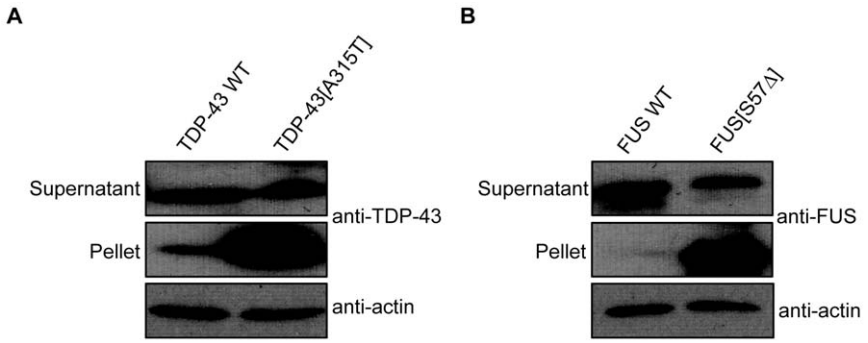

Figure 7. Mutant TDP-43 and FUS are highly insoluble. Shown are representative images from western blotting of the soluble supernatant and insoluble pellet fractions of protein extracts from transgenic TDP-43 and FUS strains. (A) Blotting against TDP-43 shows that a large proportion of the TDP-43 signal resides in the insoluble fraction for mTDP-43 worms, while the signal is largely soluble for the wtTDP-43 samples. (B) Immunoblotting with a human FUS antibody revealed that mFUS proteins primarily resided in the insoluble fractions while wtFUS proteins were exclusively soluble. Immunoblotting for actin was used as the loading control.
doi:10.1371/journal.pone.0031321.g007

onset of paralysis for the mTDP-43 and mFUS lines with approximately 80% of the population becoming immobile after 6 hours progressing to 100% paralysis after 12 hours (Figure 10A, B Videos S1, S2, S3, S4). wtTDP-43 and wtFUS animals also showed increased paralysis but at a much lower rate, with approximately 20% of the animals immobile after 6 hours moving to 80% paralysis after 12 hours (Figure 10, Videos S5, S6, S7, S8). Non-transgenic N2 animals showed a very low rate of paralysis of approximately 15% after 12 hours (Figure 10C, Videos S9, S10). In comparison, approximately 50% of transgenic *unc-47p::GFP* control animals were paralysed after 12 hours, a rate intermediate between non-transgenic N2 worms and transgenic wtTDP-43 and wtFUS animals (Figure 10C, Videos S11, S12). The difference between wild type and mutant transgenic lines is easy to distinguish, particularly at 6 hours, and suggests that this phenotype may be used for rapid genetic and chemical screening.

Discussion

Here we introduce a novel *C. elegans* platform for investigating mechanisms of motor neuron toxicity caused by mTDP-43 and mFUS. To more closely model human disease we chose to express full-length human TDP-43 and FUS without additional tags since the inclusion of tags like GFP can mask or enhance the phenotypes of wild type and mutant proteins [35,36]. Additionally, we reasoned that restricting expression to a smaller set of neurons might produce phenotypes less severe, or later, than observed in other *C. elegans* models [27,29,30,33]. Since ALS is characterized by degeneration of the motor neurons we engineered strains expressing human TDP-43 and FUS in the animal's 26 GABAergic neurons [13,22]. Additionally, ALS patients show cortical hyperexcitability that may be due to reduced inhibitory signalling from the GABAergic system [37,38]. We believe our transgenic mTDP-43 and mFUS worms recapitulate this patho-

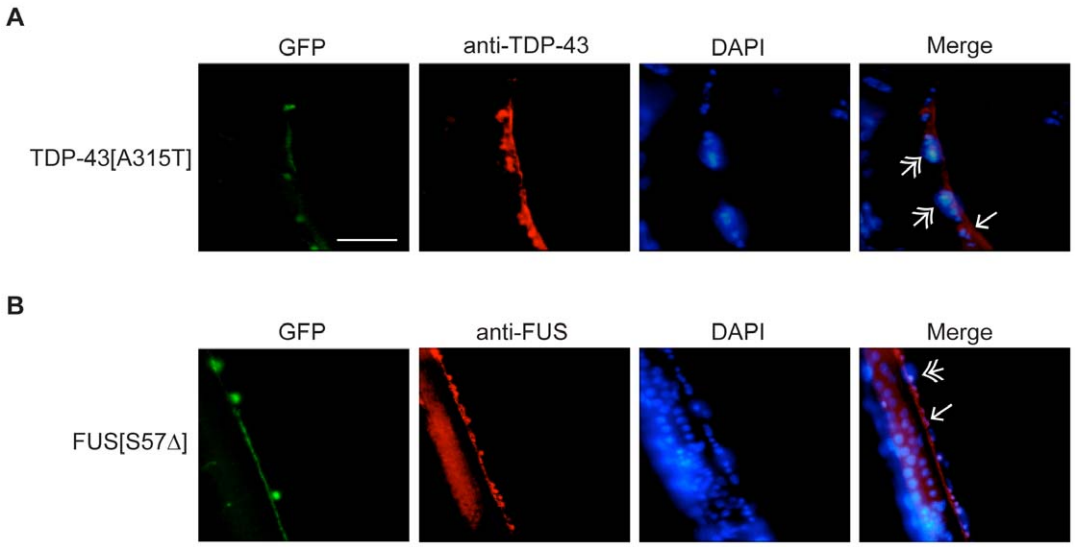

Figure 8. Mutant TDP-43 and FUS aggregate in vivo. (A) Representative image of a fixed *unc-47p::GFP;mTDP-43* worm stained with a human TDP-43 antibody. The green channel shows GFP labelled motor neurons. Antibody staining (red signal) revealed aggregation of TDP-43 signals in motor neurons. Staining of motor neuron nuclei with DAPI (blue signal) revealed that TDP-43 is both cytoplasmic (single arrowhead) and nuclear (double arrowhead). Scale bar represents 10 μm for all photos. (B) Staining of *unc-47p::GFP;mFUS* worms with a human FUS antibody (red signal) and DAPI (blue signal) revealed cytoplasmic (single arrowhead) and nuclear (double arrowhead) accumulations in motor neurons.
doi:10.1371/journal.pone.0031321.g008

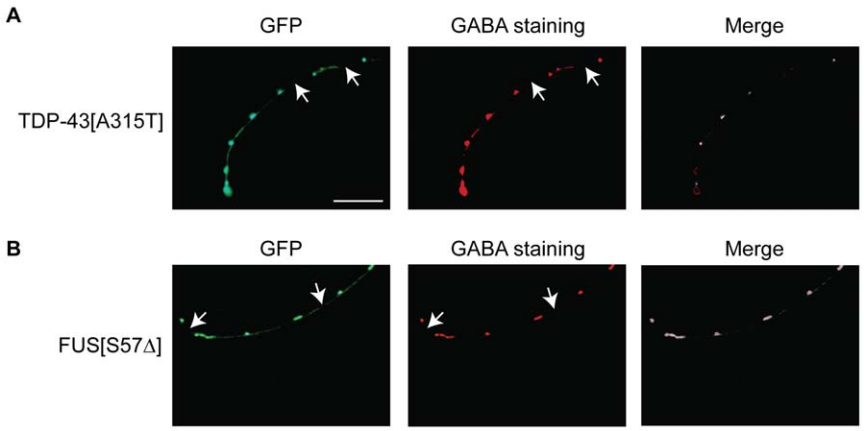

Figure 9. Decreased GABA staining in mutant TDP-43 and FUS worms. (A) Fluorescent micrograph of a fixed *unc-47p::GFP;mTDP-43* worm stained with a GABA antibody revealed neurodegeneration in motor neurons that mirrors the loss of GFP signals. Scale bar represents 10 μm for all photos. (B) Staining of *unc-47p::GFP;mFUS* worms also showed loss of GABA signals similar to the loss of GFP in the motor neurons.
doi:10.1371/journal.pone.0031321.g009

physiological mechanism; they show decreased GABA staining and are hypersensitive to the acetylcholinesterase inhibitor aldicarb, suggesting a reduction of inhibitory GABA input at neuromuscular junctions [24,25]. In our models sensitivity to aldicarb can be detected in day 1 adult worms, while paralysis and motor neuron degeneration can first be detected starting at day 5 of adulthood demonstrating that similar to ALS, neuronal dysfunction occurs prior to neurodegeneration [39].

Importantly, our transgenic TDP-43 and FUS animals only begin to show motility defects once they have reached adulthood a feature absent from other models [27,29,30,33]. Thus our models mirror a prominent clinical feature of ALS, they display adult-

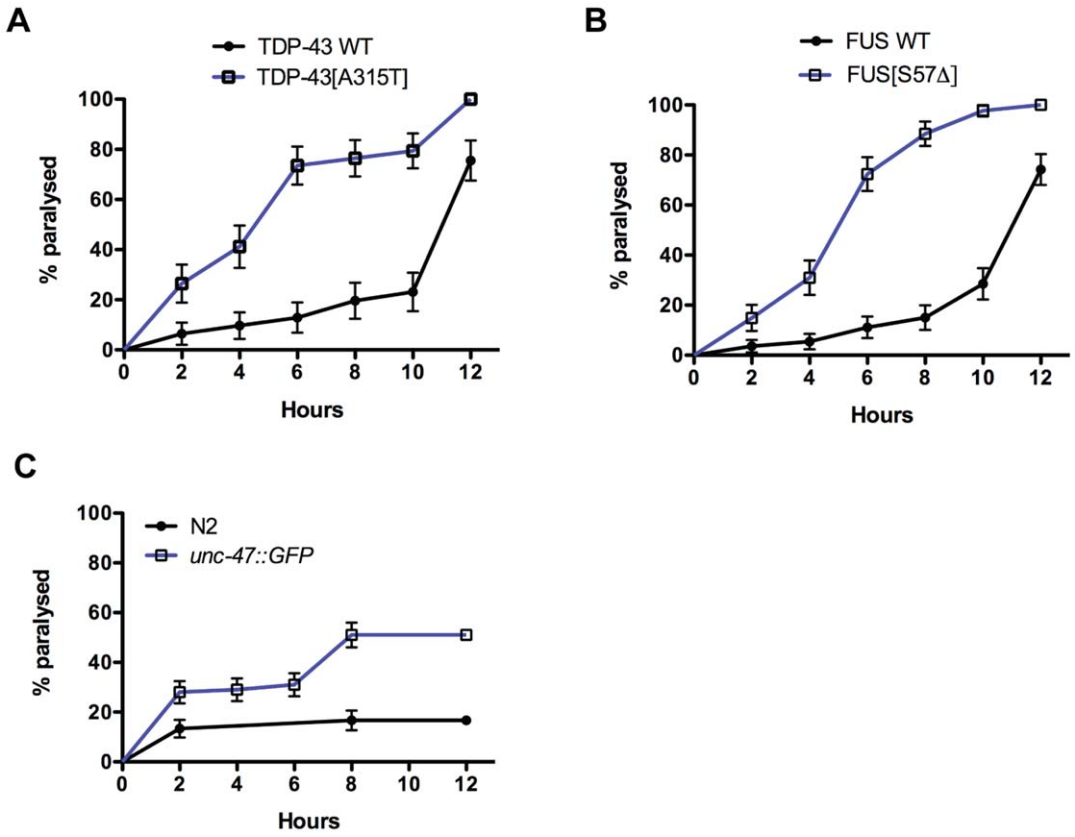

Figure 10. Accelerated paralysis phenotypes for TDP-43 and FUS transgenics in liquid culture. (A) Paralysis phenotypes resolve over a number of hours for wtTDP-43 and mTDP-43 worms grown in liquid culture. mTDP-43 worms have a faster rate of paralysis compared to wtTDP-43 transgenics ($P<0.001$). (B) Transgenic mFUS worms show motility defects and become paralysed at a rate faster than wtFUS controls ($P<0.001$). (C) *unc-47p::GFP* transgenics have an increased rate of paralysis compared to non-transgenic N2 worms ($P<0.001$).
doi:10.1371/journal.pone.0031321.g010

onset, age-dependent, progressive paralysis [40,41]. Additionally, unlike previously described TDP-43 and FUS models based on pan-neuronal expression [27,30,33] our transgenics do not show reduced lifespan suggesting the behavioural phenotypes observed in our transgenics are not influenced by general sickness. Our transgenics do share many features with other neuronal-based models, notably the aggregation and insolubility of mutant TDP-43 and FUS as well as degeneration of motor neurons suggesting there may be common mechanisms of toxicity amongst the models [27,29,30,32,33,42–45]. However, cytoplasmic aggregation of TDP-43 and FUS is a prominent feature of the human pathologies and this is seen in a recently described worm FUS model [33], but is absent from previously reported TDP-43 models [27,29,30]. We detect TDP-43 and FUS in both the nucleus and the cytoplasm of motor neurons from young adult (Day 1) transgenics. The preferential toxicity of mutant TDP-43 and FUS alleles along with their cytoplasmic accumulation suggests our models may recapitulate aspects of neurotoxicity relevant to the disease state.

With no clear mechanism for TDP-43 and FUS neuronal toxicity it is currently not possible to design *in vitro* assays for high-throughput drug screening. Thus the further development and characterization of *in vivo* models for neurodegeneration will guide studies in mammalian systems. We believe our models strike an optimal balance between strong, age-dependent phenotypes and the expression of mutant proteins in relatively few neurons and may be useful for modifier screening. In terms of sensitivity, genetic mechanisms and/or small molecules need only to work on 26 neurons to achieve suppression. In terms of speed, our transgenics offer the possibility of medium-throughput suppressor screening based on the accelerated paralysis phenotype of mTDP-43 and mFUS worms grown in liquid culture. mTDP-43 and mFUS cause neuronal dysfunction in advance of motor neuron degeneration. The path from protein misfolding to neuronal dysfunction and cell death takes many decades in humans and it may be more efficient to target therapies to early pathogenic stages. Thus using simple systems to screen for suppression of neuronal dysfunction may be useful to prevent subsequent neurodegeneration.

A number of models for TDP-43 and FUS toxicity in various systems have been described, but there is still no clear answer whether TDP-43 and FUS neuronal toxicity are due to a loss/gain of function of these proteins individually or together in some common genetic pathway [44–46]. Furthermore it is still unclear if all TDP-43 and FUS mutations share similar pathogenic mechanisms but having similarly constructed models for each may address this question. Now that we have validated the *unc-47* motor neuron approach for modelling toxicity, future work will focus on the development of new transgenics with additional TDP-43 and FUS mutations.

We present here novel transgenics for investigating age-dependent motor neuron toxicity caused by mutant TDP-43 and FUS. We expect these strains will be useful for identifying genetic and chemical suppressors to give insights into disease mechanisms and support the development of new therapies for age-dependent neurodegeneration.

Materials and Methods

Nematode strains

Standard methods of culturing and handling worms were used [47]. Worms were maintained on standard NGM plates streaked with OP50 *E. coli*. Strains used in this study were obtained from the *C. elegans* Genetics Center (University of Minnesota, Minneapolis) and include: N2, *oxIs12[unc-47p::GFP+lin-15]*, *unc-47(e307)*, *unc-47(gk192)* and *unc-119(ed3)*.

Transgenic TDP-43 and FUS worms

Human cDNAs for wild type and mutant TDP-43[A315T], and wild type and mutant FUS-TLS[S57Δ] were obtained from Dr. Guy Rouleau (CRCHUM, Université de Montréal). The cDNAs were amplified by PCR and cloned into the Gateway vector pDONR221 following the manufacturer's protocol (Invitrogen). Multisite Gateway recombination was performed with the pDONR TDP-43 and FUS clones along with clones containing the *unc-47* promoter (kind gift from Dr. Erik Jorgensen, University of Utah), the *unc-54* 3′UTR plasmid pCM5.37 (Dr. Geraldine Seydoux, Johns Hopkins, Addgene plasmid 17253) and the destination vector pCFJ150 to create *unc-47*::TDP-43 and *unc-47*::FUS expression vectors. Transgenic lines were created by microinjection of *unc-119(ed3)* worms, multiple lines were generated and strains behaving similarly were kept for further analysis. Transgenes were integrated by UV irradiation and lines were outcrossed to wild type N2 worms 5 times before use. The main strains used in this study include: *xqIs132[unc-47::TDP-43-WT;unc-119(+)]*, *xqIs133[unc-47::TDP-43[A315T];unc-119(+)]*, *xqIs173[unc-47::FUS-WT;unc-119(+)]*, and *xqIs98[unc-47::FUS[S57-Δ];unc-119(+)]*.

Paralysis assays on plates

For worms expressing TDP-43 or FUS, 20–30 adult day 1 animals were picked to NGM plates and scored daily for movement. Animals were counted as paralyzed if they failed to move upon prodding with a worm pick. Worms were scored as dead if they failed to move their head after being prodded in the nose and showed no pharyngeal pumping. All experiments were conducted at 20°C.

Lifespan assays

Worms were grown on NGM-FUDR plates to prevent progeny from hatching. 20 animals/plate by triplicates were tested at 20°C from adult day 1 until death. Worms were declared dead if they did not respond to tactile or heat stimulus. Survival curves were produced and compared using the Log-rank (Mantel-Cox) test.

Aldicarb test

To evaluate synaptic transmission, worms were grown on NGM and transferred to NGM plates +1 mM aldicarb at adult day 1. Paralysis was scored after 1 and 2 hours on aldicarb plates. Animals were counted as paralyzed if they failed to move upon prodding with a worm pick. All tests were performed at 20°C.

Liquid culture protocol

Synchronized populations of worms were obtained by hypochlorite extraction. Young adult worms were distributed in 96-wells plate (20 µl per well; 20–30 worms per well), containing DMSO or test compounds and incubated for up to 6 h at 20°C on a shaker. The motility test was assessed by stereomicroscopy. Videos of worms were taken with on an Olympus S7x7 stereomicroscope equipped with a Grasshopper GRAS-03K2M camera using Flycap software (Point Grey Research) at a rate of 300 frames per second.

Immunostaining of whole worms

Age synchronized, adult day 1, whole worms were fixed and stained as described in WormBook [48]. Antibodies used include:

rabbit anti-TDP-43 (1:50, Proteintech), rabbit anti-FUS/TLS (1:50, AbCam), and rabbit anti-GABA (1:50, Proteintech).

Fluorescence microscopy

For scoring gaps/breaks from TDP-43 and FUS transgenics, synchronized animals were selected at days 1, 5 and 9 of adulthood for visualization of motor neurons *in vivo*. Animals were immobilized in M9 with 5 *mM* levamisole and mounted on slides with 2% agarose pads. Motor neurons were visualized with a Leica 6000 microscope and a Leica DFC 480 camera. A minimum of 100 animals was scored per treatment over 4–6 trials. The mean and SEM were calculated for each trial and two-tailed t-tests were used for statistical analysis.

Worm lysates

Worms were collected in M9 buffer, washed 3 times with M9 and pellets were placed at -80°C overnight. Pellets were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate)+0.1% protease inhibitors (10 mg/ml leupeptin, 10 mg/ml pepstatin A, 10 mg/ml chymostatin LPC; 1/1000). Pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged at 16000*g*. Supernatants were collected.

Protein quantification

All supernatants were quantified with the BCA Protein Assay Kit (Thermo Scientific) following the manufacturer instructions.

Protein solubility

For TDP-43 and FUS transgenics soluble/insoluble fractions, worms were lysed in Extraction Buffer (1 M Tris-HCl pH 8, 0.5 M EDTA, 1 M NaCl, 10% NP40+protease inhibitors (LPC; 1/1000)). Pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged at 100000*g* for 5 min. The soluble supernatant was saved and the remaining pellet was resuspended in extraction buffer, sonicated and centrifuged at 100000g for 5 min. The remaining pellet was resuspended into 100 µl of RIPA buffer, sonicated until the pellet was resuspended in solution and saved.

Immunoblots

Worm RIPA samples (175 µg/well) were resuspended directly in 1× Laemmli sample buffer, migrated in 12.5% or 10% polyacrylamide gels, transferred to nitrocellulose membranes (BioRad) and immunoblotted. Antibodies used: rabbit anti-human-TDP-43 (1:200, Proteintech), rabbit anti-human-FUS/TLS (1:200, AbCam), and mouse anti-actin (1:10000, MP Biomedical). Blots were visualized with peroxidase-conjugated secondary antibodies and ECL Western Blotting Substrate (Thermo Scientific). Densitometry was performed with Photoshop (Adobe).

Statistics

For paralysis and stress-resistance tests, survival curves were generated and compared using the Log-rank (Mantel-Cox) test, and 60–100 animals were tested per genotype and repeated at least three times. For image analysis statistical significance was determined by Student's t-test and the results shown as mean ± standard error. Prism 5 (GraphPad Software) was used for all statistical analyses.

Supporting Information

Table S1 Lifespan analysis for all experiments.
(PDF)

Video S1 mTDP-43 worms in liquid culture at time 0.
(MOV)

Video S2 mTDP-43 worms after 6 hours in liquid culture.
(MOV)

Video S3 mFUS worms in liquid culture at time 0.
(MOV)

Video S4 mFUS worms after 6 hours in liquid culture.
(MOV)

Video S5 wtTDP-43 worms in liquid culture at time 0.
(MOV)

Video S6 wtTDP-43 worms after 6 hours in liquid culture.
(MOV)

Video S7 wtFUS worms in liquid culture at time 0.
(MOV)

Video S8 wtFUS worms after 6 hours in liquid culture.
(MOV)

Video S9 N2 worms in liquid culture at time 0.
(MOV)

Video S10 N2 worms after 6 hours in liquid culture.
(MOV)

Video S11 *unc-47p::GFP* worms in liquid culture at time 0.
(MOV)

Video S12 *unc-47p::GFP* worms after 6 hours in liquid culture.
(MOV)

Acknowledgments

We thank S. Peyrard, E. Bourgeois and S. Al Ameri for technical support, H. Catoire for critical reading of the manuscript, and Dr. E. Jorgensen for the *unc-47* plasmid.

Author Contributions

Conceived and designed the experiments: JAP. Performed the experiments: AV AT DA. Analyzed the data: AV JAP. Contributed reagents/materials/analysis tools: GAR PD. Wrote the paper: AV JAP.

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Annexe 6 :

Reduction of polyglutamine toxicity by TDP-43, FUS and progranulin in Huntington's disease models.

Tauffenberger, A., Chitramuthu, B. P., Bateman, A., Bennett, H. P., & Parker, J. A. (2013). *Human Molecular Genetics*, *22*(4), 782–794. doi:10.1093/hmg/dds485

HMG Advance Access published November 29, 2012

Human Molecular Genetics, 2012 **1–13**
doi:10.1093/hmg/dds485

Reduction of polyglutamine toxicity by TDP-43, FUS and progranulin in Huntington's disease models

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Received October 18, 2012; Revised October 18, 2012; Accepted November 13, 2012

The DNA/RNA binding proteins TAR DNA-binding protein 43 (TDP-43) and fused-in-sarcoma (FUS) are genetically linked to amyotrophic lateral sclerosis and frontotemporal lobar dementia, while the inappropriate cytoplasmic accumulations of TDP-43 and FUS are observed in a growing number of late-onset pathologies including spinocerebellar ataxia 3, Alzheimer's and Huntington's diseases (HD). To investigate if TDP-43 and FUS contribute to neurodegenerative phenotypes, we turned to a genetically accessible *Caenorhabditis elegans* model of polyglutamine toxicity. In *C. elegans*, we observe that genetic loss-of-function mutations for nematode orthologs of TDP-43 or FUS reduced behavioral defects and neurodegeneration caused by huntingtin exon-1 with expanded polyglutamines. Furthermore, using striatal cells from huntingtin knock-in mice we observed that small interfering ribonucleic acid (siRNA) against TDP-43 or FUS reduced cell death caused by mutant huntingtin. Moreover, we found that TDP-43 and the survival factor progranulin (PGRN) genetically interact to regulate polyglutamine toxicity in *C. elegans* and mammalian cells. Altogether our data point towards a conserved function for TDP-43 and FUS in promoting polyglutamine toxicity and that delivery of PGRN may have therapeutic benefits.

INTRODUCTION

Neurons are a cell subtype sensitive to protein homeostasis and protein misfolding can lead to proteotoxic stress and eventually cell death (1). The phenomenon has been associated with many adult-onset neurodegenerative diseases including Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD). Two proteins that have attracted widespread interest in the field of neurodegeneration are the DNA/RNA binding proteins TAR DNA-binding protein 43 (TDP-43) and fused-in-sarcoma (FUS). Mutations in TDP-43 and FUS account for up to 6% of familial ALS, but the mislocalization of TDP-43 and FUS is observed in a growing number of age-dependent disorders including frontotemporal lobar degeneration (FTLD), Alzheimer's disease and the polyglutamine (polyQ) diseases (2). TDP-43 and FUS are predominantly nuclear DNA/RNA-binding proteins found in cytoplasmic inclusions in the disease state (2). TDP-43 and FUS have numerous roles in RNA homeostasis including: transcription, splicing, miRNA processing, stress granule dynamics, nucleocytoplasmic shuttling and RNA transport with local translation (2–10). However, it is difficult to link the normal biological function of TDP-43 and FUS to pathogenesis, since it is still unclear whether the pathogenic mechanism follows a loss of function, a toxic gain of function or both (11,12).

To learn more about the potential roles of TDP-43 and FUS as modifiers of age-dependent neurodegeneration, we asked whether these proteins participated in polyQ toxicity. The polyQ disorders comprise nine different orders including several ataxias as well as HD (13). In each case, neuronal dysfunction is caused by an expansion of a polymorphic CAG tract coding for glutamine that over a specific threshold causes disease (14). Expanded polyQs are associated with a number of phenotypes including neuronal dysfunction, oxidative stress, intracellular protein aggregation and ultimately cell

death (15). A prominent feature of HD is the aggregation of mutant huntingtin protein and the recent identification of both TDP-43 (16) and FUS (17) as components of these aggregates suggests that these proteins may contribute to pathogenesis.

Since TDP-43 and FUS are evolutionarily conserved, we studied the potential role of the *Caenorhabditis elegans* orthologs *tdp-1*/TDP-43 and *fust-1*/FUS in regulating polyQ toxicity in a well-defined transgenic model based on the expression of human huntingtin exon-1 like proteins in worm mechanosensory neurons (18–20). In parallel, we examined huntingtin toxicity in mammalian cells after small interfering ribonucleic acid (siRNA) knockdown of TDP-43 or FUS. We report that a reduction of TDP-43 or FUS reduced polyQ toxicity in both worm and mammalian cell models. Furthermore, the suppression of polyQ toxicity by reduction of TDP-43 was dependent on the neurotrophic factor progranulin (PGRN) in both worms and mammalian cells. Our findings suggest that TDP-43 and FUS may actively contribute to polyQ toxicity and may be new targets for therapeutic development.

RESULTS

tdp-1 and *fust-1* mutations diminish neuronal dysfunction in *C. elegans*

To investigate the possible interaction of *tdp-1, fust-1* and polyQ proteins, we used the well-characterized deletion mutants *tdp-1(ok803)* (21,22) and *fust-1(tm4439)* (23), both of which are predicted to be null alleles (Fig. 1A). We examined the contribution of *tdp-1(ok803)* and *fust-1(tm4439)* to polyQ toxicity using a transgenic worm model expressing mutant huntingtin with 128 polyQ repeats (128Q) in mechanosensory neurons (19). Transgenic 128Q animals show progressive, age-dependent loss-of-touch sensitivity compared with the wild-type polyQ control (19Q) animals or non-transgenic wild-type N2 (Fig. 1B). Mutated *tdp-1(ok803)* resulted in a significant delay of age-dependent touch insensitivity (Fig. 1C), a phenotype we also observed with a second deletion mutant *tdp-1(ok781)*. Similarly, the *fust-1* mutation had a small but still significant protective effect on the 128Q neuronal touch response (Fig. 1D). We tested whether the suppression of 128Q toxicity was due to any potential effects on lifespan by the *tdp-1* and *fust-1* mutations. First, we observed that the expression of polyQ transgenes had no effects on lifespan compared with non-transgenic wild-type N2 worms (Supplementary Material, Fig. S1A and Table S1). As reported previously, we observed that *tdp-1(ok803)* mutants had extended lifespan compared with wild-type N2 worms (22,23), while *fust-1(tm4439)* mutants had lifespan indistinguishable to N2 worms and the lifespan of either mutant was unchanged by the expression of 128Q transgenes (Supplementary Material, Fig. S1B and Table S1). These data suggest that the suppression of 128Q neuronal dysfunction is not due to non-specific lifespan effects. Additionally, we tested the effects of the *tdp-1(ok803)* or *fust-1(tm4439)* mutations on the general touch response in the absence of 128Q transgenes. Since we observe the greatest suppression of 128Q toxicity up to day 3 of adulthood in 128Q worms, we performed touch response assays on N2, *tdp-1(ok803)* and *fust-1(tm4439)* animals over this time period and observed comparable levels of touch sensitivity (Supplementary Material, Fig. S1C). Finally, the suppression of 128Q touch insensitivity was not due to transgene effects since neither *tdp-1(ok803)* or *fust-1(tm4439)* affected 128Q protein or transgene expression levels (Fig. 1E and F, Supplementary Material, Fig. S1D).

tdp-1 and *fust-1* mutations reduce axonal degeneration

HD patients and animal models display protein aggregation and neuronal degeneration (15). The 128Q transgenic worms show several progressive phenotypes including axonal degeneration and protein aggregation (19). These animals express soluble yellow fluorescent proteins (YFP) in mechanosensory neurons that appear as a continuous fluorescent signal along the axonal processes in young animals (Fig. 2A). As the 128Q animals age, the YFP signal becomes discontinuous and punctate indicating degeneration of the axonal processes (Fig. 2B). Additionally, in this model mutant 128Q proteins are tagged with cyan fluorescent protein (CFP), making it possible to observe aggregation phenotypes *in vivo* (Fig. 2C). Crossing the 128Q transgene into the *tdp-1(ok803)* background, we observed that age-dependent axonal degeneration phenotypes were significantly delayed in *tdp-1(ok803); 128Q* versus 128Q animals (Fig. 2D). Furthermore, *tdp-1(ok803);*128Q animals had a lower incidence of progressive axonal aggregation compared with 128Q controls (Fig. 2E). To test whether the *C. elegans* FUS ortholog *fust-1* behaved similarly to *tdp-1*, we constructed *fust-1(tm4439);*128Q strains and observed that the deletion of *fust-1* reduced axonal degeneration (Fig. 2F) and the aggregation of 128Q fusion proteins (Fig. 2G). These data suggest that wild-type *tdp-1* and *fust-1* promote polyQ-mediated neurodegeneration and aggregation phenotypes in *C. elegans* neurons.

TDP-1 protection is independent of HDAC6

TDP-43 is involved in many aspects of RNA metabolism. Recent work has shown a role for TDP-43 in the regulation of histone deacetylase-6 (HDAC6) mRNA when TDP-43 levels are modified (24,25). As HDAC inhibitors have been proposed as a therapeutic target for HD, and that HDAC6 more specifically has been reported to play a role in autophagic clearance of misfolded huntingtin proteins (26,27), we wondered whether HDAC6 was involved in *tdp-1*-mediated protection against polyQ toxicity. To address whether the *C. elegans* HDAC6 ortholog *hdac-6* played a role in the delay of proteotoxicity when *tdp-1* was absent, we constructed a *tdp-1;hdac-6;*128Q strain. Interestingly, the *hdac-6* mutation had no effect on the *tdp-1*-mediated rescue of 128Q phenotypes including touch sensitivity and axonal degeneration (Supplementary Material, Fig S2A and B). Furthermore, *hdac-6* transcription was unchanged by the deletion mutants *tdp-1(ok803)* and *tdp-1(ok781)* (Supplementary Material, Fig. S2C) suggesting that the suppression of 128Q toxicity by *tdp-1* is independent of *hdac-6*/HDAC6 in our model.

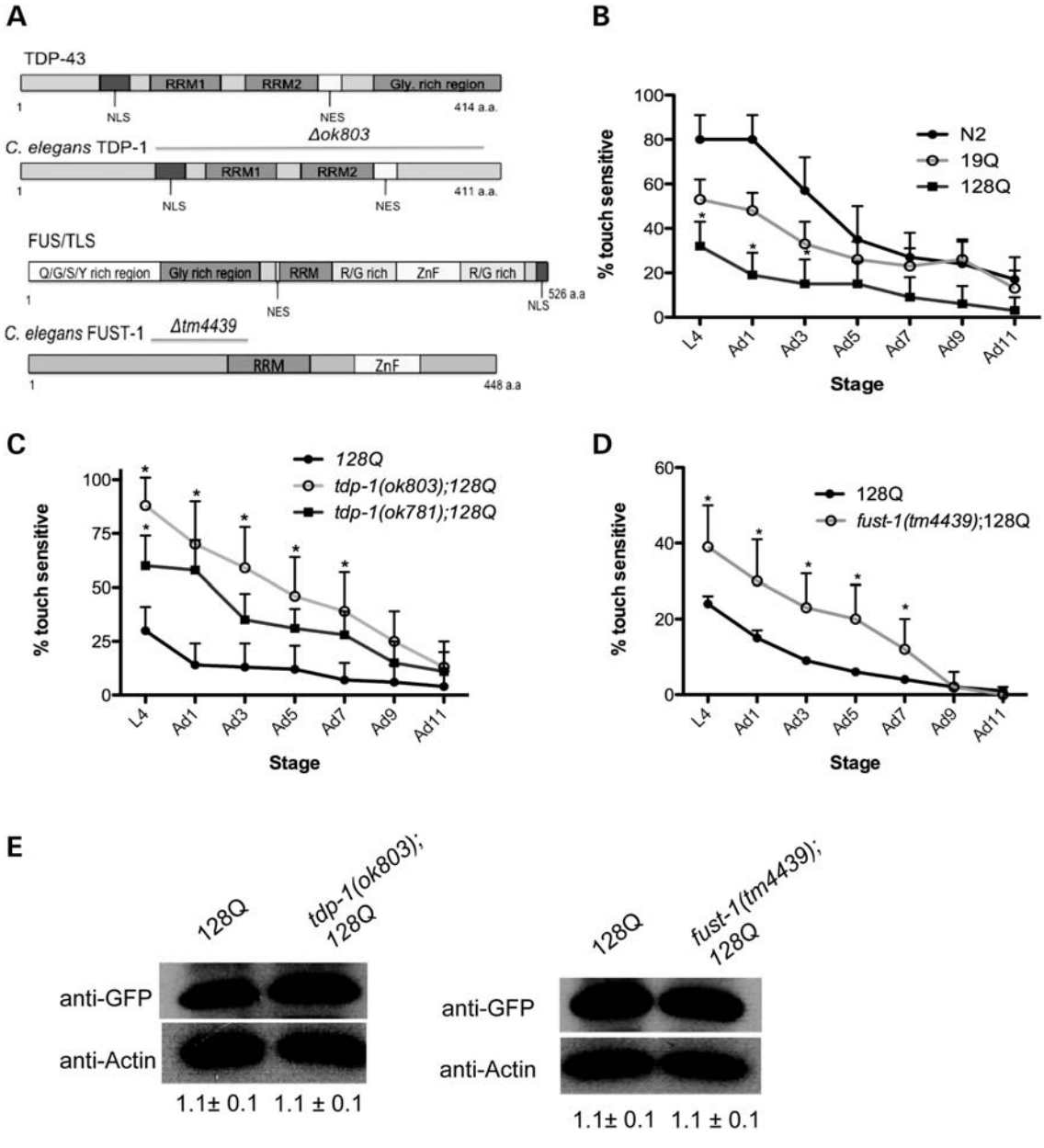

Figure 1. *tdp-1* and *fust-1* mutations reduce neuronal dysfunction in worms expressing mutant polyQ. (**A**) Schematic representation of human TDP-43 with worm ortholog TDP-1 and human FUS with nematode FUST-1 along with the extent of *C. elegans* deletion mutations. (**B**) 128Q animals display an age-dependent loss-of-touch sensitivity compared with wild-type animals ($^*P < 0.05$). (**C**) Mutation of *tdp-1* rescues 128Q toxicity up to Ad 9 compared with 128Q controls ($^*P < 0.05$). (**D**) Mutation of *fust-1* rescues polyQ toxicity compared with 128Q controls ($^*P < 0.05$). (**E**) Mutation in either *tdp-1* or *fust-1* has no effect on 128Q protein expression.

Suppression of polyQ toxicity by *tdp-1* is dependent on progranulin

Looking at another known TDP-43 target (28) we turned to PGRN, a well-known growth factor expressed in many tissues including the central nervous system (29), with roles in tissue repair, inflammatory response and potentially in neuronal growth (30). Moreover, PGRN loss of function has been identified as responsible for FTLD with ubiquitinated inclusions (FTLD-U) (31,32) and PGRN was identified in FTLD cases with TDP-43 proteinopathy (33). The *C. elegans* ortholog of PGRN is PGRN-1 (30) and to address the possible role of PGRN-1 in *tdp-1*-mediated protection, we used the *pgrn-1(tm985)* null mutant in our 128Q animals (30). Constructing *tdp-1(ok803);pgrn-1(tm985);*128Q and *fust-1(tm4439);pgrn-1(tm985);*128Q strains we observed that mutation in *pgrn-1* completely abolished *tdp-1*-mediated neuronal protection including improved touch sensitivity (Fig. 3A), but *pgrn-1* had no effect on the suppression of touch insensitivity by mutation of *fust-1* (Fig. 3B). Likewise, we observed that a loss of *pgrn-1* blocked the suppression of axonal degeneration by *tdp-1(ok803)* (Fig. 3C), while *pgrn-1(tm985)* had no effect on the suppression of axonal degeneration by

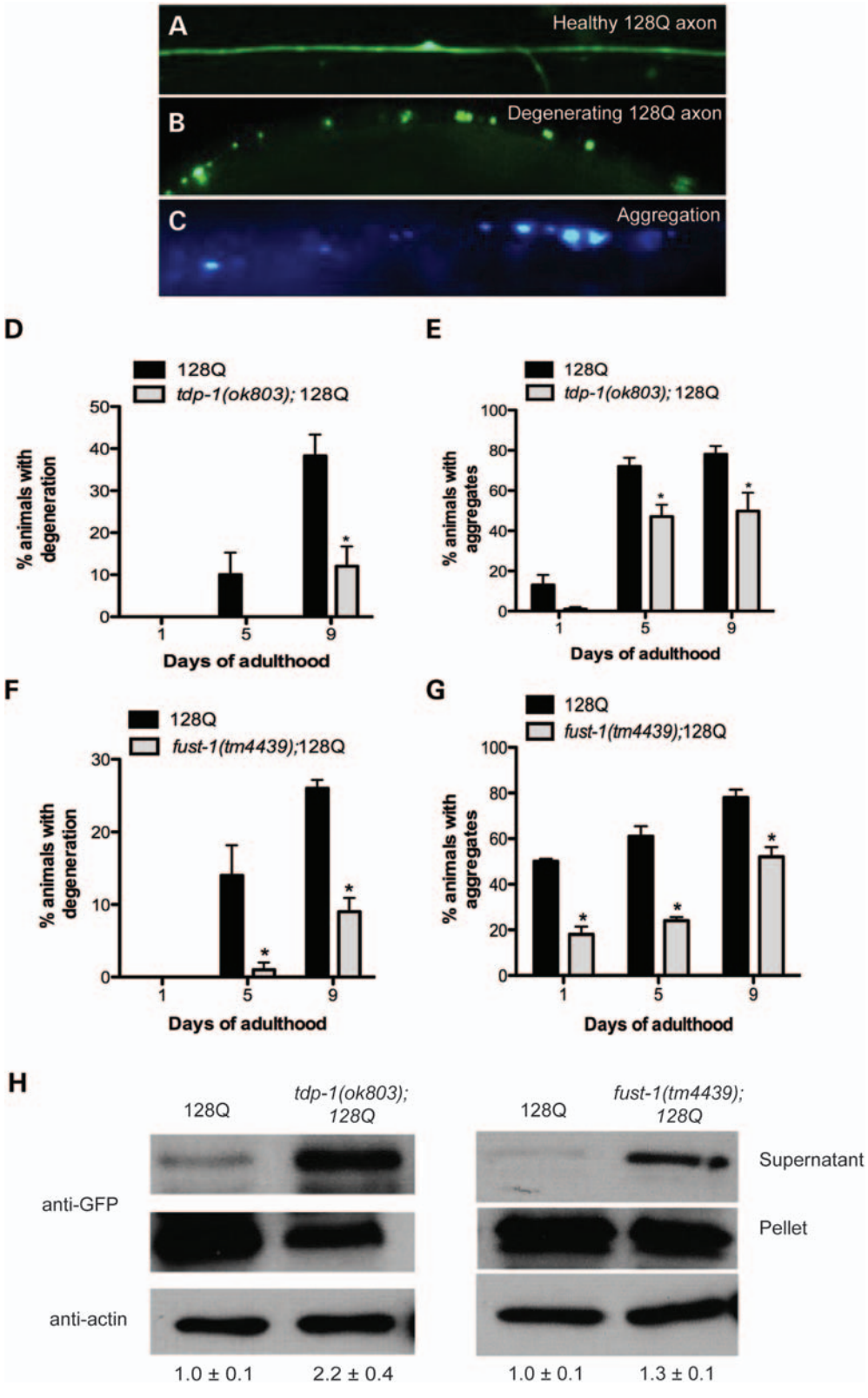

Figure 2. *tdp-1* and *fust-1* mutations reduce mutant polyQ neurodegeneration and aggregation phenotypes in *C. elegans*. (**A**) Young adult 128Q animals expressing the *mec-7p::YFP* transgene display continuous and uniform fluorescence along the axonal processes. (**B**) Old *mec-7p::YFP*;128Q animals display discontinuous and punctate fluorescent signals. (**C**) Transgenic 128Q::CFP animals display axonal aggregation in PLM cells. (**D**) Mutation of *tdp-1* reduces axonal degeneration in 128Q worms models compared with 128Q alone ($^*P < 0.05$). (**E**) Mutation of *tdp-1* reduces axonal aggregates in 128Q transgenics compared with 128Q alone ($^*P < 0.05$). (**F**) Mutation of *fust-1* reduces axonal degeneration in 128Q worms compared with 128Q alone ($^*P < 0.05$). (**G**) Mutation of *fust-1* reduces axonal aggregates in 128Q transgenics compared with 128Q alone ($^*P < 0.05$). (**H**) Mutation of *tdp-1* and *fust-1* decreases the amount of insoluble 128Q protein, and increases the amount of soluble 128Q protein compared with 128Q alone.

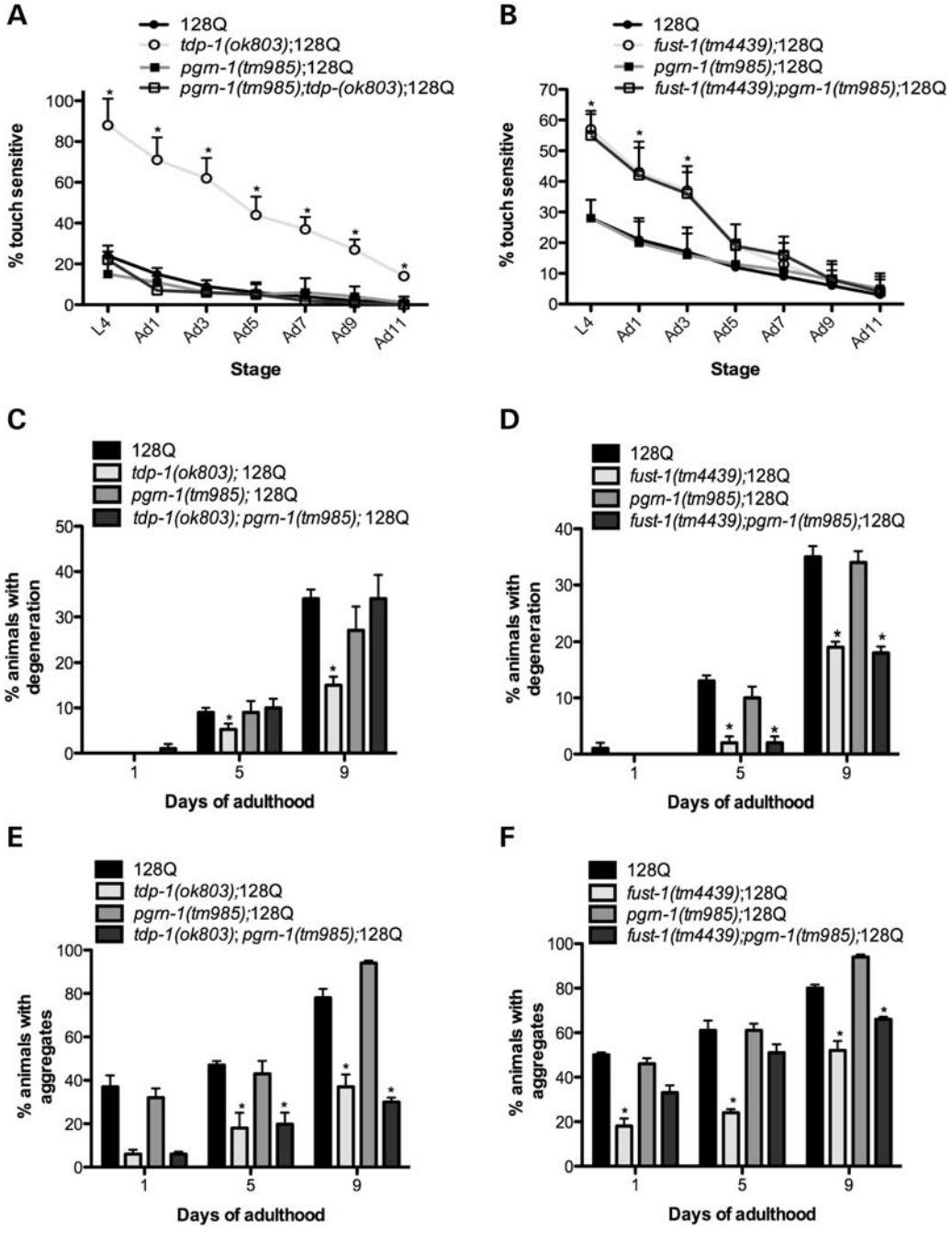

Figure 3. Suppression of mutant polyQ toxicity by *tdp-1*, but not *fust-1*, is dependent on PGRN. (**A**) *tdp-1*;128Q animals have increased touch sensitivity compared with 128Q animals ($^*P < 0.05$), but this protection was lost by mutation of PGRN in *pgrn-1;tdp-1*;128Q mutants. (**B**) *tdp-1*;128Q animals have decreased axonal degeneration compared with 128Q animals ($^*P < 0.001$), but this protection was lost by mutation of PGRN in *pgrn-1;tdp-1*;128Q mutants. (**C**) Mutation of *fust-1* continues to suppress 128Q toxicity by a deletion of *pgrn-1* ($^*P < 0.05$). (**D**) Mutation of PGRN does not block the suppression of axonal degeneration caused by *fust-1* in *fust-1(tm4439)*;128Q animals ($^*P < 0.0001$ compared with 128Q, or *pgrn-1*;128Q). (**E**) Mutation of PGRN fails to block the suppression of aggregation caused by *tdp-1* in *tdp-1(ok803)*;128Q animals ($^*P < 0.0001$ compared with 128Q, or *pgrn-1*;128Q). (**F**) Mutation of PGRN does not block the suppression of aggregation caused by *fust-1* in *fust-1(tm4439)*;128Q animals ($^*P < 0.05$ compared with 128Q, and $P < 0.0001$ with *pgrn-1*;128Q).

fust-1(tm4439) (Fig. 3D). Finally, we looked at aggregation and observed that a loss of *pgrn-1* did not block the suppression of 128Q aggregation by *tdp-1(ok803)* (Fig. 3E). We observed similar results for the *fust-1(tm4439);pgrn-1(tm985);* 128Q strain, where *pgrn-1(tm985)* partially diminished the suppression of aggregation by *fust-1(tm4439)* in animals at days 1 and 5 of adulthood, but *fust-1(tm4439);128Q* and *fust-1(tm4439);pgrn-1(tm985);*128Q showed a similar suppression of aggregation by day 9 of adulthood (Fig. 3F).

These data suggest that the suppression of 128Q toxicity by *tdp-1*/TDP-43 is dependent on *pgrn-1*/PGRN, while the suppression of toxicity by *fust-1*/FUS does not require

pgrn-1/PGRN, thus placing *tdp-1*/TDP-43 and *fust-1*/FUS in separate pathways. Furthermore, these data suggest that *pgrn-1*'s role in modulating neuronal dysfunction and neurodegeneration caused by 128Q can be uncoupled from aggregation. A loss of *pgrn-1* had negligible effects on aggregation in *tdp-1(ok803);pgrn-1(tm985);*128Q and *fust-1(tm4439); pgrn-1(tm985);*128Q worms, despite the fact that *pgrn-1* is required for the suppression of 128Q-mediated neuronal dysfunction and degeneration by *tdp-1(ok803)*.

Reducing TDP-43 and FUS protect against mutant huntingtin toxicity in mammalian cells

Since HD primarily affects striatal and cortical neurons, we turned to cell lines generated by the conditional immortalization of striatal precursors from embryos of the Hdh^{Q111} knock-in mouse. These cells are known as ST$Hdh^{Q111/Q111}$ and express full-length huntingtin with an expanded mutant polyQ allele (111 glutamines). These cells, along with the wild-type ST$Hdh^{Q7/Q7}$ controls containing a non-pathogenic polyQ repeat (7 glutamines), express full-length huntingtin from its normal chromosomal location at endogenous, physiological levels (34).

To investigate whether TDP-43 or FUS promotes mutant huntingtin toxicity, we opted to reduce the expression of these genes using a siRNA approach. These ST*Hdh* cells undergo cell death when stressed (35), providing a convenient system to investigate whether TDP-43 or FUS contributes to polyQ toxicity. We observed that two out of the three test siRNAs significantly reduced the level of TDP-43 protein compared with control siRNA, in both the ST$Hdh^{Q7/Q7}$ and ST$Hdh^{Q111/Q111}$ cells (Fig. 4A). Interestingly, we observed a significant reduction in cell death for ST$Hdh^{Q111/Q111}$ cells treated with the validated TDP-43 siRNAs, with no effect on the ST$Hdh^{Q7/Q7}$ control cell line (Fig. 4B). We observed similar results for FUS, where two out of the three test siRNAs effectively reduced the level of FUS protein that likewise corresponded to decreased cell death for ST$Hdh^{Q111/Q111}$ cells, with no effect on the ST$Hdh^{Q7/Q7}$ controls (Fig. 4C and D). We confirmed these findings with an alternate method to score cell death using flow cytometry, where we again observed that siRNAs against TDP-43 or FUS reduced cell death in ST$Hdh^{Q111/Q111}$ cells with no effect on ST$Hdh^{Q7/Q7}$ control cells (Supplementary Material, Fig. S3). As in worms, these data demonstrate that wild-type TDP-43 and FUS promote mutant huntingtin toxicity suggesting that this may be a conserved pathogenic mechanism.

Progranulin protects against mutant huntingtin toxicity

Data from our *C. elegans* 128Q model demonstrated that *pgrn-1* modulates polyQ toxicity. To test whether this was conserved in our mammalian St*Hdh* model, we first examined the consequences of siRNA-mediated inhibition of PGRN. We observed that one of three PGRN siRNAs tested enhanced cell death in ST$Hdh^{Q111/Q111}$ cells with no significant effect on ST$Hdh^{Q7/Q7}$ controls (Fig. 5A and B). Consistently, we observed that overexpression of human PGRN suppressed cell death in ST$Hdh^{Q111/Q111}$ cells with no significant effect on ST$Hdh^{Q7/Q7}$ control cells (Fig. 5C and D). Collectively, these data suggest that PGRN is a conserved modifier of mutant huntingtin toxicity.

TDP-43, but not FUS, requires PGRN for neuroprotection against mutant huntingtin

Our genetic analysis of polyQ toxicity in worms showed that PGRN is required for neuroprotection by deletion of TDP-43, but that PGRN is dispensable for suppression of toxicity via FUS loss of function. First, we confirmed the efficacy of downregulation of protein levels by single or double siRNA treatments in ST$Hdh^{Q7/Q7}$ and ST$Hdh^{Q111/Q111}$ cells (Fig. 6A). We confirmed our previous results showing that TDP-43 or FUS siRNA reduced cell death, while PGRN siRNA enhanced cell death in ST$Hdh^{Q111/Q111}$ cells, and no significant effects were observed for any siRNA treatment in ST$Hdh^{Q7/Q7}$ cells (Fig. 6B). Looking at the dual siRNA experiments for the ST$Hdh^{Q111/Q111}$ cells, we observed that PGRN siRNAs abolished the protective effects of TDP-43 siRNAs (Fig. 6B). Meanwhile, siRNAs against PGRN failed to block the protective effects of FUS siRNAs against cell death in the ST$Hdh^{Q111/Q111}$ cells. In sum, these data are consistent with our *C. elegans* data and demonstrate that TDP-43 and PGRN work in the same pathway to regulate mutant huntingtin toxicity, while FUS may operate in a separate or parallel pathway to PGRN.

DISCUSSION

Late-onset diseases are responsible for a growing number of fatal and untreatable disorders within our aging population. Thus, finding effective treatments is becoming a major societal challenge. Despite the fact that TDP-43 and FUS are becoming increasingly linked to late-onset diseases (2), very little is known about their role as modifiers of disease.

In this work, we demonstrated that *tdp-1 and fust-1*, the orthologs of human TDP-43 and FUS, respectively, have the capacity to reduce protein toxicity in a nematode model of polyQ toxicity by reducing age-dependent neuronal dysfunction, neurodegeneration and aggregation phenotypes. The reduction of neuronal proteotoxicity by *tdp-1* is not restricted to mutant polyQ as *tdp-1* mutants have been reported to suppress toxicity caused by mutant SOD1 (23) as well as TDP-43 and FUS (22). However, *fust-1* was not observed to modify SOD1 toxicity in worms (23), while we see the suppression of mutant polyQ phenotypes. Nonetheless, TDP-43 and FUS may be general modifiers of late-onset proteotoxicity.

We validated the neuroprotective effects of reducing TDP-43 or FUS against mutant polyQ toxicity in mammalian neurons, suggesting that this may be a highly conserved mechanism worth examining in mouse models or HD patients. There is great interest in determining the genetic and molecular interactions of TDP-43 and FUS in relation to their normal biological functions as well as their roles in several late-onset neurodegenerative diseases (12). Genetically tractable model organisms including worms (22,23), flies (36,37) and fish (38) have begun to explore the interactions of the wild-type and mutant forms of these proteins. In particular, studies

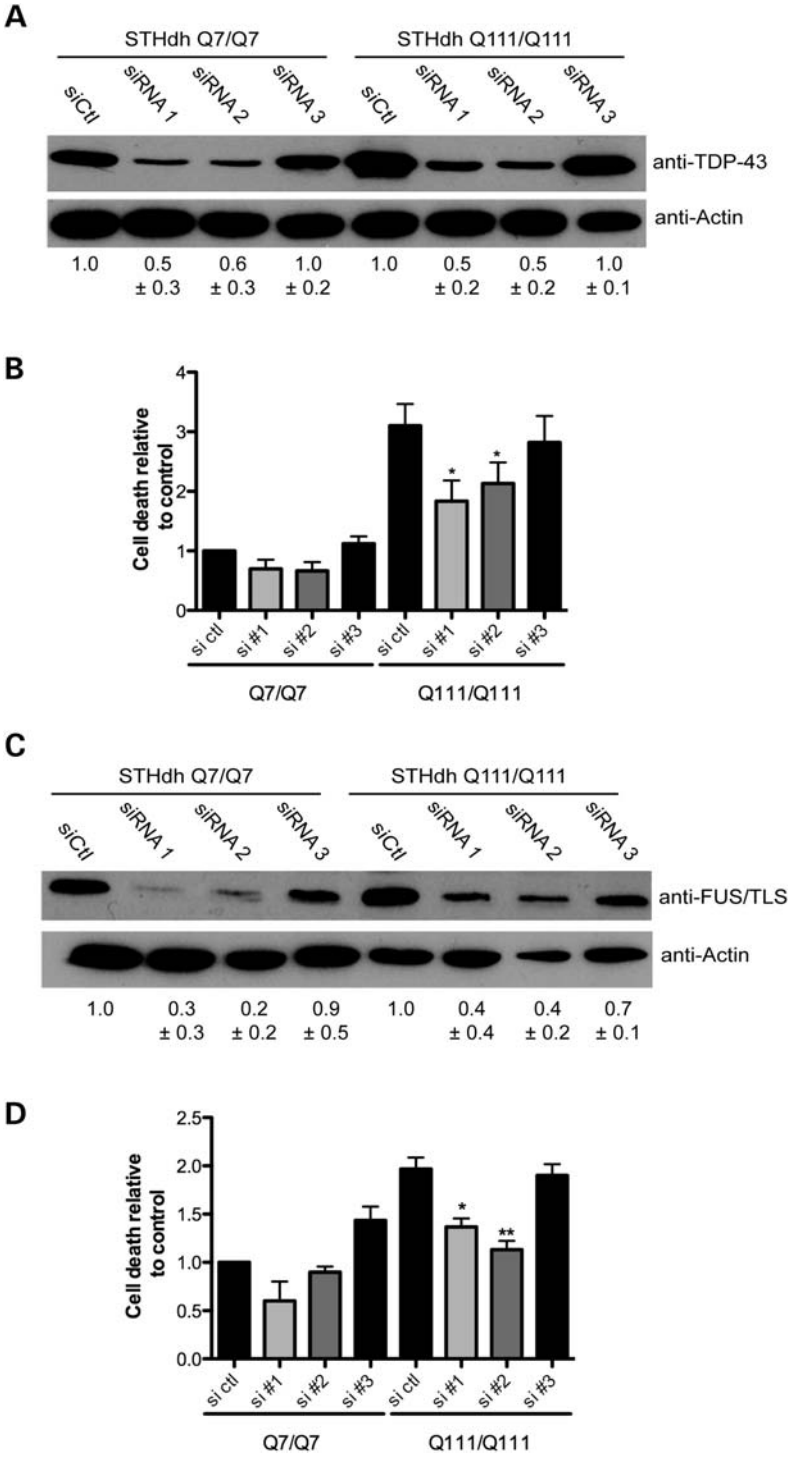

Figure 4. Reducing the expression of TDP-43 and FUS protects against mutant huntingtin toxicity in striatal cells. (**A**) Validation of TDP-43 siRNAs (20 μM) showing efficient reduction of TDP-43 protein levels for two of three siRNAs compared with control siRNA. (**B**) Reduction of TDP-43 by siRNA rescues cell death in ST*Hdh*$^{Q111/Q111}$ cell lines compared with control siRNA ($^{*}P < 0.05$). TDP-43 siRNA had no significant effect on ST*Hdh*$^{Q7/Q7}$ cells. (**C**) Validation of FUS siRNAs (20 μM) showing efficient reduction of FUS protein levels for two of three siRNAs compared with control siRNA. (**D**) Reduction of FUS by siRNA rescues cell death in ST*Hdh*$^{Q111/Q111}$ cell lines compared with control siRNA ($^{*}P < 0.05$; $^{**}P < 0.01$). FUS siRNA had no significant effect on ST*Hdh*$^{Q7/Q7}$ cells.

from flies (36) and fish (38) place FUS genetically downstream from TDP-43 in terms of their normal biological function. Our previous study examining the neurotoxic effects of mutant TDP-43 and FUS proteins on *C. elegans*' motor neurons places wild-type *tdp-1*/TDP-43 downstream from the mutant proteins (22), suggesting that the biologically

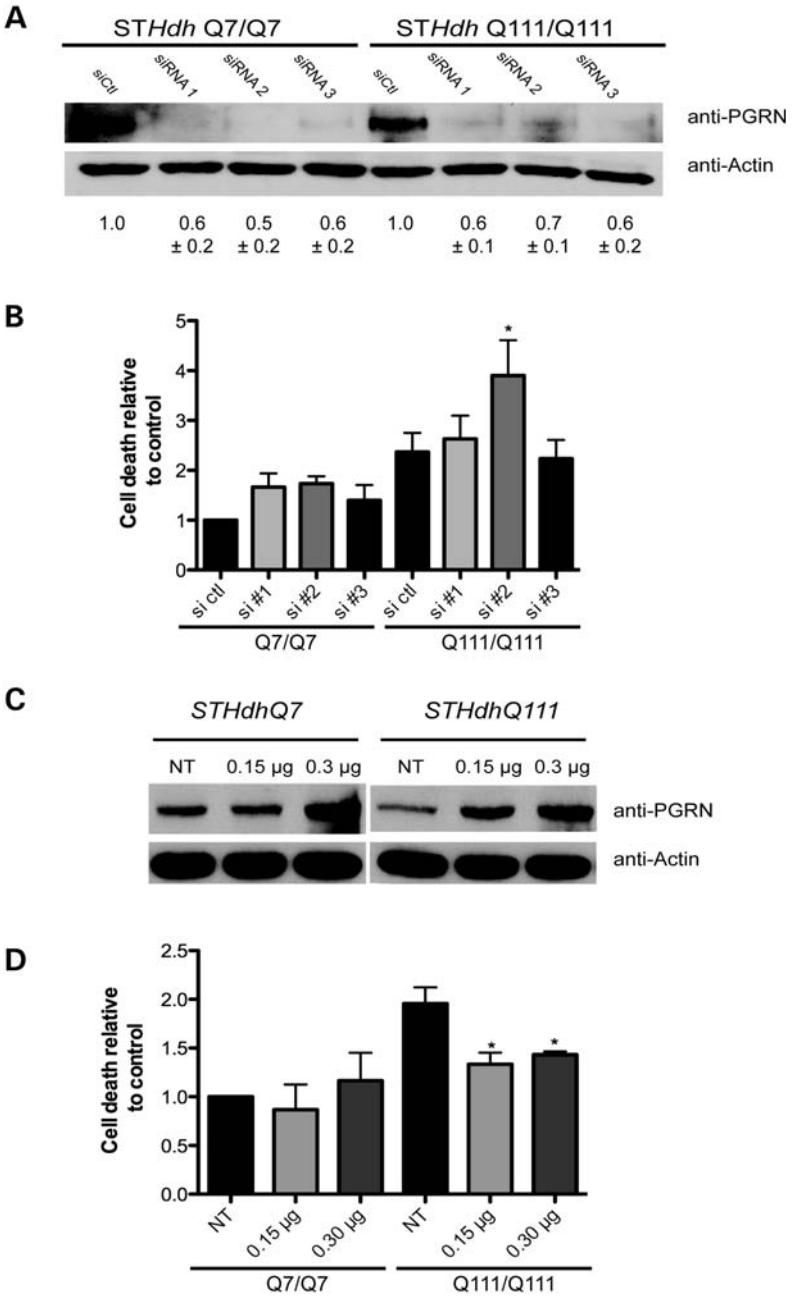

Figure 5. Human PGRN influences huntingtin toxicity in striatal cells. (**A**) Validation of PGRN siRNAs (20 μM) showing efficient reduction of PGRN protein levels compared with control siRNA. (**B**) Reduction of PGRN by siRNA increases cell death in ST*Hdh*$^{Q111/Q111}$ cell lines ($^{*}P < 0.05$). PGRN siRNA had no significant effect on ST*Hdh*$^{Q7/Q7}$ cells. (**C**) Validation of human PGRN transfection showing efficient increase in protein levels compared with non-transfected cells. (**D**) Overexpression of PGRN had no effect on ST*Hdh*$^{Q7/Q7}$ but reduced cell death in ST*Hdh*$^{Q111/Q111}$ cell lines ($^{*}P < 0.05$).

normal and pathological states may not be genetically equivalent. In this current study, we place wild-type *tdp-1*/TDP-43 and *fust-1*/FUS genetically downstream from mutant polyQ toxicity.

TDP-43 (16,39) and FUS (17) are known to colocalize to polyQ aggregates in patient tissue and cell culture models, but the molecular basis for these interactions is not fully known. However, more is known about TDP-43 where it has been shown that TDP-43 contains a glutamine/asparagine (Q/N)-rich domain in its C-terminal that is responsible for binding to polyQ aggregates (39). It has been shown that mutant polyQ sequesters TDP-43 from the nucleus into cytoplasmic inclusions. This leads to toxicity because TDP-43 is depleted from the nucleus, and it can no longer perform its normal RNA processing functions. In this model, toxicity can be partially alleviated by increasing TDP-43 expression to offset its cytoplasmic entrapment (39). These findings are not completely compatible with our findings, but this likely reflects the many complex cellular roles of TDP-43. For instance, many groups have shown that increased expression

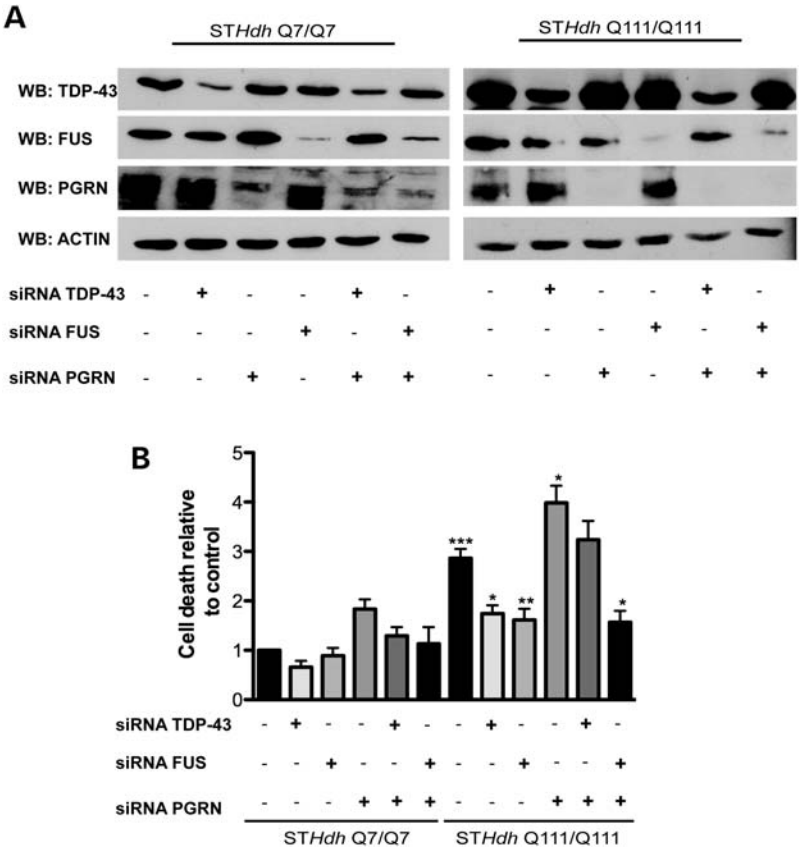

Figure 6. Suppression of polyQ toxicity by TDP-43, but not FUS, is dependent on PGRN. (**A**) Validation of TDP-43, FUS and PGRN siRNAs (20 μM) showing efficient reduction of TDP-43, FUS and PGRN protein levels compared with control siRNA. (**B**) ST*Hdh*$^{Q111/Q111}$ cells had a higher rate of cell death compared to ST*Hdh*$^{Q7/Q7}$ controls (***$P < 0.001$). Transfection of TDP-43 or FUS siRNA reduced ST*Hdh*$^{Q111/Q111}$ cell death compared to control siRNA (*$P < 0.05$ and **$P < 0.01$ respectively), while PGRN siRNA increased ST*Hdh*$^{Q111/Q111}$ cell death compared to ST*Hdh*$^{Q111/Q111}$ cells treated with control siRNA (*$P < 0.05$). ST*Hdh*$^{Q111/Q111}$ cells treated with TDP-43 siRNA had less cell death than cells co-transfected with TDP-43 and PGRN siRNAs (**$P < 0.01$). ST*Hdh*$^{Q111/Q111}$ cells transfected with FUS sRNAi or co-transfected with FUS and PGRN siRNAs had comparable levels of cell death, both of which were less than control siRNA treated ST*Hdh*$^{Q111/Q111}$ cells (*$P < 0.05$).

of wild-type TDP-43 is cytotoxic and is sometimes as toxic as mutant TDP-43 (40–49). Furthermore, we have shown that a loss of *tdp-1*/TDP-43 function renders worms sensitive to cellular stress, perhaps by nuclear depletion, but alleviates proteotoxic stress caused by mutant TDP-43 or FUS (22). Thus, we propose that wild-type *tdp-1*/TDP-43 contributes to cellular toxicity propagated by mutant proteins including polyQ, TDP-43 and FUS. In this context, we consistently observe that inhibition of *tdp-1*/TDP-43 suppresses proteotoxicity.

TDP-43 and FUS have many thousands of targets (50) but nothing is known about which, if any, contribute to polyQ toxicity. As an initial foray into this area, we looked at two targets, HDAC6 and PGRN, in our 128Q worm model. HDAC6 is already known to modify polyQ toxicity (26,27) and was an obvious first choice. However, *hdac-6* had no effect on the *tdp-1* mediated suppression of 128Q toxicity. The second gene we examined was PGRN, a known partner of TDP-43 in FTLD (33,51). In parallel, PGRN has also been identified as a TDP-43 mRNA target (28), which indicated a potential interaction between the two genes. PGRN is a well-studied factor involved in many biological processes including neuroprotection (52). Work from *C. elegans* and cultured cells suggests a critical role of PGRN in cell survival, as mutation of worm *pgrn-1* results in increased apoptotic clearance in *C. elegans* (30) and increased sensitivity to oxidative stress and excitotoxicity in cell culture (53). The genetic accessibility of our models allowed us to identify *pgrn-1*/PGRN as a downstream effector of *tdp-1*/TDP-43 in regulating polyQ toxicity. Of interest is the observation that *pgrn-1*/PGRN does not appear to play a role in the modulation of polyQ toxicity by *fust-1*/FUS. We validated these genetic interactions in mammalian neurons again demonstrating a link between TDP-43 and PGRN (but not FUS and PGRN) in regulating huntingtin toxicity, suggesting that this may be a highly conserved mechanism worth examining in more advanced mammalian models and HD patients. Summarizing our findings we propose two pathways regulating polyQ toxicity, where PGRN is downstream from TDP-43, while FUS operates independently of PGRN (Fig. 7).

Recent reports from zebrafish models show that PGRN promotes motor neuron development (54) and that PGRN overexpression can rescue TDP-43-dependent neuropathy (55). However, the molecular relationship between TDP-43 and PGRN is unclear, since it has been reported that a mutation in PGRN causes caspase mediated TDP-43 cleavage (56), but this has not been observed in all instances (57,58).

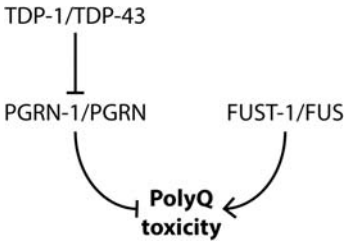

Figure 7. Simple model of TDP-43, FUS and PGRN in regulating polyQ toxicity. Wild-type TDP-43 or FUS promotes the toxicity of mutant polyQ proteins. Reduction of TDP-43 or FUS reduces polyQ toxicity, but for TDP-43 this is dependent on PGRN. Thus, TDP-43 and PGRN function in a common pathway to regulate polyQ toxicity, while FUS acts in a parallel pathway.

Furthermore, while PGRN has the capacity to modulate toxicity of TDP-43, PGRN overexpression had no effect on SOD1, another ALS related protein, suggesting a more diverse role of TDP-43 in neurodegeneration.

We do not believe that the regulation of polyQ toxicity by *pgrn-1*/PGRN is linked to aggregation, as *pgrn-1* had negligible effects on 128Q aggregation in worms whether neuronal dysfunction and neurodegeneration phenotypes were rescued or not. Furthermore, despite expressing full-length huntingtin, the ST*Hdh* cells do not display aggregation phenotypes (34); thus, the modulation of cell death by TDP-43, FUS or PGRN is likely independent of aggregation dynamics.

In summary, *tdp-1*/TDP-43 and *fust-1*/FUS are conserved modifiers of mutant polyQ toxicity. More work is required to fully understand the molecular mechanisms but targeted manipulation of TDP-43, FUS and/or their specific targets may have therapeutic relevance. Furthermore, delivery of PGRN to diseased polyQ neurons may be a promising strategy to boost neuronal survival against misfolded proteins.

MATERIALS AND METHODS

C. elegans experiments

Worm strains and maintenance

Worms were cultured and handled by standard methods. All experiments were done at 20°C. The mutant and transgenic strains used in the experiments were: *igIs1*[*mec-3::htt57Q128::CFP;mec-7::YFP;lin-15(+)*], *igIs245*[*mec-3::htt57Q19::CFP;mec-7::YFP;lin-15(+)*] (characterized in (16)), *tdp-1(ok803), fust-1(tm4439), hdac-6(ok3203) and pgrn-1(tm985).* Mutant strains were obtained from the *C. elegans* Genetics Center (CGC, University of Minnesota, Minneapolis, USA) or from National Bioresource Project (Japan). The deletion mutants crossed to polyQ transgenic strains were verified by PCR and each had been outcrossed a minimum of three times prior to use. All strains were maintained at 20°C on *Escherichia coli* OP50 bacteria.

Behavioral tests

Touch tests were conducted as described previously (59). Sensitivity to mechanical sensations was scored at adult days (Ad) 1 to 11 by touching the nematode's tail 10 times and analyzed as the percentage of response. A minimum of 100 animals was tested per genotype. The mean and standard deviation are reported for each group of worms per age tested.

Fluorescence microscopy test

Axonal counts were scored in worms at days 1, 5 and 9 of adulthood for axonal dystrophy, axonal degeneration and axonal aggregation. Worms were immobilized in 5 mM levamisole and placed on a 2% agarose pad to carry out *in vivo* axonal examination. A minimum of 100 worms was scored over 4–6 trials for each condition. Nematodes were examined on a Leica DM6000 microscope using a Leica DFC480 camera.

Lifespan assays

Worms were grown on nematode growth media (NGM) and transferred to NGM-FUDR plates. Twenty animals per plate by triplicates were tested at 20°C from Ad 1 until death. Worms were declared dead if they did not respond to tactile or heat stimulus.

RNA extraction and reverse transcription-PCR

Worms were grown on standard NGM media, washed with M9 buffer and the pellet was stored at −80°C overnight. The frozen pellet was thawed on ice and 400–500 µl of Trizol (Invitrogen) reagent was added and the solution was incubated for 5 min at RT. Then, 150 µl of chloroform was added and the worm solution was incubated for 5 min at RT. After incubation, the solution was centrifuged at 12 000*g* for 15 min at 4°C. RNA (aqueous phase) was transferred into a new tube and precipitated with 500 µl of isopropyl alcohol with incubation for 15 min at RT. RNA/alcohol solution was then centrifuged at 12 000*g* for 15 min at 4°C. The supernatant was eliminated and the pellet washed with 500 µl of 75% ethanol, vortexed and centrifuged at 7500*g* for 15 min at 4°C. The supernatant was eliminated and the pellet air-dried. RNA was resuspended in 50 µl of RNase free water, incubated at 55°C for 10 min and stored at −80°C. For reverse transcription (RT) PCR, complementary DNA (cDNA) was obtained using a QuantiTect Reverse Transcription kit (QIAgen). To perform the RNA cleaning, 1 µl of genomic deoxyribonucleic acid wipeout, 500 ng of RNA and H_2O for a total volume of 7 µl were incubated for 2 min at 42°C. For the reverse transcription, the 7 µl of the previous mix was added to a PCR mix (0.5 µl of primers, 0.5 µl of reverse transcriptase and 2 µl of RT buffer). The mix was incubated for 15 min at 42°C and 3 min at 95°C.

Worm lysates

Worms were collected in M9 buffer, washed three times with M9 and the pellets were placed at −80°C overnight. The pellets were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) + 0.1% protease inhibitors (10 mg/ml leupeptin, 10 mg/ml pepstatin A, 10 mg/ml chymostatin LPC;1/1000). The pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged at 16 000*g*. The supernatant was collected. For 128Q transgenic protein solubility, the worms were lysed in extraction buffer (1 M Tris–HCl, pH 8,

0.5 M EDTA, 1 M NaCl, 10% NP40 + protease inhibitors (LPC;1/1000)). The pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged at 100 000*g* for 5 min. The supernatant was stored as 'S' (soluble) fraction and the remaining pellet was resuspended in extraction buffer, sonicated and centrifuged at 100 000*g* for 5 min. The remaining pellet was resuspended in 100 ml of RIPA buffer, sonicated until the pellet was resuspended in solution and stored as sample 'P' (pellet). The pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged at 16 000 g. The supernatant was stored as 'total protein extract' and the remaining pellet was resuspended in extraction buffer, sonicated and centrifuged at 100 000*g* for 5 min. The supernatant was stored as 'S' (soluble) fraction and the remaining pellet was resuspended in extraction buffer, sonicated and centrifuged at 100 000*g* for 5 min. The remaining pellet was resuspended in 100 ml of RIPA buffer, sonicated until the pellet was resuspended in solution and stored as sample 'P' (pellet).

Striatal cell experiments

TDP-43, FUS and PGRN experiments

Mouse striatal cells ST*Hdh*$^{Q7/Q7}$ and ST*Hdh*$^{Q111/Q111}$ were grown in Dulbecco's modified Eagle's medium (4.5 g/l glucose, 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM glutamine) at 33°C, 5% CO_2. The cell were plated (1×10^5) on six-well dishes and grown for 24 h at 33°C, 5% CO_2. RNAi MAX reagent (Invitrogen) and 125 pmol of TDP-43, FUS or PGRN *stealth RNAi* siRNA were used according to the manufacturer's instructions. Transfection media were replaced after 5 h by standard growth media and the cells were analyzed after 72 h. TDP-43 1 (MSS214148): 5′-cgaaaggguuuggcuuuguucgauu-3′, TDP-43 2 (MSS214149): 5′-gcaaucugguauauguugucaacua-3′, TDP-43 3 (MSS214150): 5′-gaaauaccaucagaagacgauggga-3′, FUS 1 (MSS214763): 5′-gggauaugguuccacuggaggcuau-3′, FUS 2 (MSS214764): 5′-acaauaccaucuucgugcaaggccu-3′, FUS 3 (MSS214765): 5′-cccagaguggagguuaugucaaca-3′, PGRN 1 (MSS204932): 5′-ccaugauaaccagaccuguuguaaa-3′, PGRN 2 (MSS204933): 5′-ggaaccaaguguuugcgaaagaaga-3′, PGRN 3 (MSS204934): 5′-ggaccugugagaaggaugucgauuu-3′. For transfection of human PGRN, the cells were maintained and plated as described previously. Lipofectamine LTX reagent (Invitrogen) and 0.15 μg or 0.3 μg of human PGRN construct were used according to manufacturer's instructions. Transfection media were replaced after 3 h by standard growth media and the cell were analyzed 24 h later.

Cloning of human PGRN

A cDNA sequence encoding the human PGRN (NP_002078.1) with a C-terminal polyhistidine tag was amplified by PCR, cloned into a pcDNA3.1/TOPO vector (Invitrogen), and ampicillin-resistant colonies were identified, cultured, verified by restriction digest and DNA sequencing. Bacterial colonies with plasmid containing human PGRN were cultured and purified using a MiniPrep kit (Roche) and were expressed.

Cell death assay

After 72 h of siRNA treatment, the cell media were replaced by normal media with 200 μM sodium arsenite. After 6 h treatment, the media, which contains dead cells, were recuperated and the cells were harvested with 0.05% trypsin (Multicell). The cells were then centrifuged for 3 min, 5000 rpm at 4°C and the pellet was resuspended in cold phosphate buffered saline. A fraction of the cell suspension was mixed with an equal volume of Trypan blue (Multicell) and placed on a hemacytometer. Cell death proportions were calculated by counting the number of dead cells, appearing blue because of the dye and the number of living cells.

Fluorescence-activated cell sorting

The fluorescence-activated cell sorting experiments were performed using the PE AnnexinV Apoptosis detection kit I (BD Bioscience) following the manufacturer's instructions. Annexin + cells are shown in Supplementary Material, Fig. S3. Data acquisition was performed with a BD LSR II flow cytometry (BD Biosciences) and the data were analyzed with FlowJo (Treestar, Ashland, OR, USA).

Cell lysates

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) + 0.1% protease inhibitors (10 mg/ml leupeptin, 10 mg/ml pepstatin A, 10 mg/ml chymostatin LPC;1/1000). The cells were lysed, incubated on ice for 10 min, then moved at RT for 10 min and finally centrifuged at 16 000*g* for 10 min. Protein quantification was performed using a BCA protein assay kit (Thermo Scientific).

Immunoblot

For cell experiment, the following antibodies were used: rabbit anti-TDP-43 (1/1000; Proteintech), rabbit anti-FUS (1/2000; ab23439 Abcam), sheep anti-PGRN (1/100; Abcam), rabbit anti-PGRN (1/300; Invitrogen) and mouse anti-actin (1/400 000; MP Biomedicals). For worms, rabbit anti-GFP (1/5000; ab6556, Abcam) and mouse anti-actin (1/2000; MP Biomedicals) were used.

Statistics

Statistical analysis of nematode touch-test data and cell death assays were performed using one-way ANOVA, with correction for multiple testing by Tukey–Kramer's multiple comparison test. For the touch-test experiments, data were expressed as mean ± SD for >100 nematodes in each group. All experiments were repeated at least three times. $P < 0.05$ was considered significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We would like to thank C. Néri (INSERM, France) for the polyQ worms, as well as the National Bioresource Project (Japan), and the CGC for providing additional strains used

in these experiments. We thank S. Peyrard and A. Aulas for technical support and H. Catoire for critical reading of the manuscript.

Conflict of Interest statement. None declared.

FUNDING

A.T. is supported by a CIHR-Huntington's Society of Canada fellowship. J.A.P. is a CIHR New Investigator. A CIHR Catalyst Grant, the CHUM Foundation and the Bernice Ramsay Discovery Grant from the ALS Society of Canada supported this work.

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Annexe 7 :

Evaluation of longevity enhancing compounds against transactive response DNA-binding protein-43 neuronal toxicity.

Tauffenberger, A., Julien, C., & Parker, J. A. (2013). *Neurobiology of Aging*, *34*(9), 2175–2182. doi:10.1016/j.neurobiolaging.2013.03.014

Neurobiology of Aging xxx (2013) 1–8

Contents lists available at SciVerse ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Evaluation of longevity enhancing compounds against transactive response DNA-binding protein-43 neuronal toxicity

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ARTICLE INFO

Article history:
Received 21 September 2012
Received in revised form 2 March 2013
Accepted 11 March 2013

Keywords:
TDP-43
Amyotrophic lateral sclerosis
C. elegans
Neurodegeneration
Longevity
Proteotoxicity

ABSTRACT

In simple systems, lifespan can be extended by various methods including dietary restriction, mutations in the insulin/insulin-like growth factor (IGF) pathway or mitochondria among other processes. It is widely held that the mechanisms that extend lifespan may be adapted for diminishing age-associated pathologies. We tested whether a number of compounds reported to extend lifespan in *C. elegans* could reduce age-dependent toxicity caused by mutant TAR DNA-binding protein-43 in *C. elegans* motor neurons. Only half of the compounds tested show protective properties against neurodegeneration, suggesting that extended lifespan is not a strong predictor for neuroprotective properties. We report here that resveratrol, rolipram, reserpine, trolox, propyl gallate, and ethosuximide protect against mutant TAR DNA-binding protein-43 neuronal toxicity. Finally, of all the compounds tested, only resveratrol required *daf-16* and *sir-2.1* for protection, and ethosuximide showed dependence on *daf-16* for its activity.

1. Introduction

For more than 75 years, people have been fascinated by the discovery that rats living on a restricted diet (dietary restriction) showed increased lifespan (McCay et al., 1989), a phenomenon that is under investigation in primates (Colman et al., 2009; Mattison et al., 2012). Of great interest is the fact that not only do many organisms show increased lifespan under dietary restriction conditions but they also show decreased incidences of age-related pathologies (Anderson and Weindruch, 2012). Additional mechanisms that regulate longevity have been discovered including mitochondrial function and the insulin/insulin-like growth factor (IGF) signaling pathway. Molecular and genetic approaches have begun to decipher the cellular mechanisms of lifespan extension and this has led to the development of an industry hoping to find and develop longevity mimetics as potential therapeutic agents against age-related disease (Mercken et al., 2012). Work from model organisms like *C. elegans* has identified numerous compounds that extend lifespan by influencing conserved longevity mechanisms and we wondered if these compounds would be effective against age-dependent proteotoxicity. To evaluate these compounds we turned to a *C. elegans* model of age-dependent motor neuron toxicity (Vaccaro et al., 2012a) and tested 11 compounds reported to extend lifespan. We identified 6 compounds that reduced mutant transactive response (TAR) DNA-binding protein-43 (TDP-43) neuronal toxicity and might be useful as candidates for testing and drug development in mammalian models of neurodegeneration.

2. Methods

2.1. C. elegans *strains and genetics*

Standard methods of culturing and handling worms were used. Worms were maintained on standard nematode growth media plates streaked with OP50 *E. coli*. All strains were scored at 20 °C. Mutations and transgenes used in this study were: *daf-16(mu86)*, *hsf-1(sy441)*, *rrf-3(pk1426)*, *sir-2.1(ok434)*, and *xqIs133[unc-47::TDP-43[A315T];unc-119(+)]*. Most of the strains were obtained from the *C. elegans* Genetics Center (University of Minnesota, Minneapolis, MN, USA). Mutants or transgenic worms were verified by visible phenotypes, polymerase chain reaction analysis for deletion mutants, sequencing for point mutations, or a combination thereof. Deletion mutants were outcrossed a minimum of 3 times to wild type N2 worms before use.

2.2. Paralysis assays

Worms were counted as paralyzed if they failed to move when prodded with a worm pick. Worms were scored as dead if they

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failed to move their head after being prodded in the nose and showed no pharyngeal pumping. For the paralysis tests worms grown on the specific compound from hatching were transferred to the appropriate experimental plate for scoring.

2.3. Neurodegeneration assays

For scoring of neuronal processes, TDP-43 transgenic animals were selected at day 9 of adulthood for visualization of motor neurons processes in vivo. Animals were immobilized in M9 with 5 mM levamisole and mounted on slides with 2% agarose pads. Neurons were visualized using a Leica DM6000 microscope and a Leica DFC 480 camera. A minimum of 100 animals were scored per treatment over 4–6 trials. The mean and standard error of the mean were calculated for each trial and 2-tailed *t* tests were used for statistical analysis.

2.4. RNAi experiments

RNA interference (RNAi)-treated strains were fed *E. coli* (HT115) containing an empty vector or *skn-1* (T19E7.2) RNAi clones from the ORFeome RNAi library (Open Biosystems). RNAi experiments were performed at 20 °C. Worms were grown on Nematode Growth Media enriched with 1 mM isopropyl-ß-D-thiogalactopyranoside. All RNAi paralysis tests were performed using a TDP-43 [A315T];*rrf-3(pk1426)* strain. To minimize developmental effects, L4 worms were grown on plates with either *skn-1*(RNAi) or empty vector and assayed for paralysis as adults. *skn-1*(RNAi) activity was confirmed by the observation of lethal and sterile phenotypes in the progeny of treated animals.

2.5. Protein extraction

Worms were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate) plus 0.1% protease inhibitors (10 mg/mL leupeptin, 10 mg/mL pepstatin A, 10 mg/mL chymostatin. Nematodes were lysed with a $27_{1/2}$ syringe 10–15 times, incubated on ice for 10 minutes then moved at room temperature for 10 minutes and finally centrifuged at 16,000*g* for 10 minutes. Protein quantification was performed using a BCA protein assay kit (Thermo Scientific). For TDP-43 transgenic worms, soluble and insoluble fractions were obtained using methods previously described (Liachko et al., 2010; Neumann et al., 2006), with modifications. Briefly, worms pellets were homogenized with a pellet mixer (Disposable Pellet Mixer and Cordless Motor, VWR) in 1 volume (wt/vol) of low-salt buffer (Benedetti et al., 2008) (10 mM Tris, 5 mM Ethylene Diamine Triacetic Acid (EDTA), 10% sucrose, pH 7.5) and centrifuged at 25,000*g* for 30 minutes at 4 °C. The supernatant represents the low salt (LS) fraction, containing the soluble proteins.

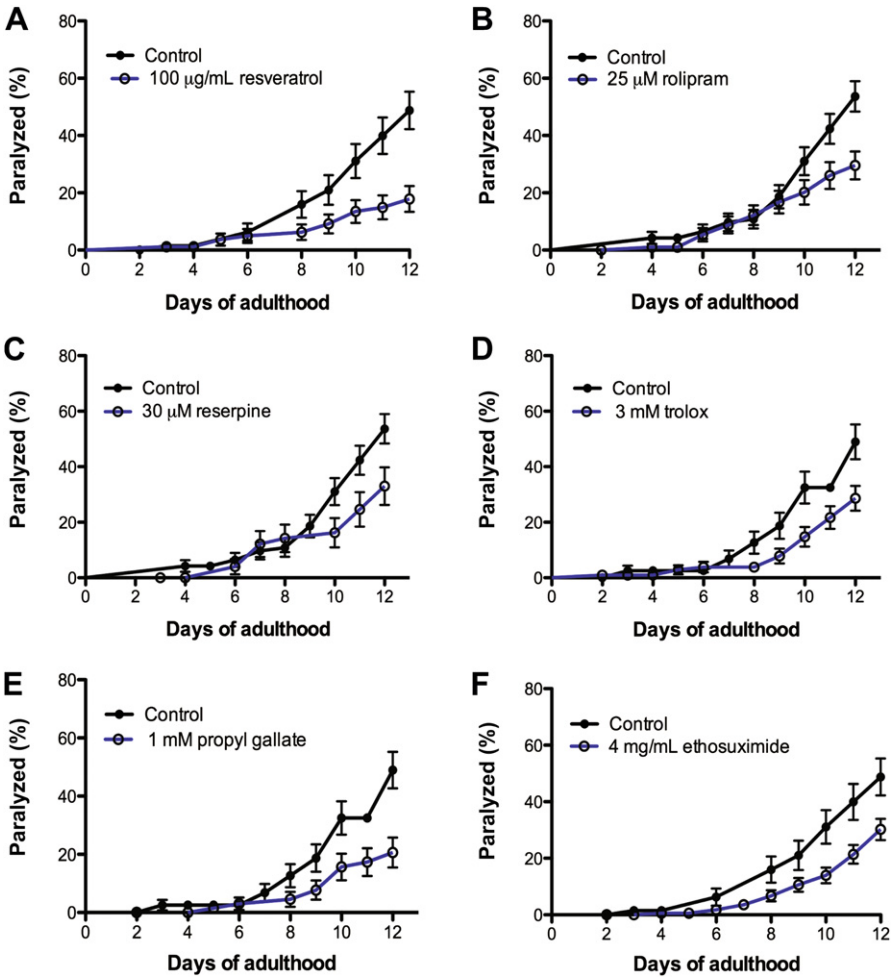

Fig. 1. Lifespan-extending compounds reduce mutant TAR DNA-binding protein–43-induced paralysis. Compounds that reduced mutant TAR DNA-binding protein–43-induced motility defects and paralysis compared with untreated control animals included: (A) 100 µg/mL resveratrol ($p < 0.001$); (B) 25 µM rolipram ($p < 0.001$); (C) 30 µM reserpine ($p < 0.05$); (D) 3 mM trolox ($p < 0.01$); (E) 1 mM propyl gallate ($p < 0.01$); (F) 4 mg/mL ethosuximide ($p < 0.01$). See also Supplementary Table 1.

The pellet was washed with the same volume of LS, centrifuged again, and the supernatant was discarded. The remaining pellet was re-extracted with the same volume of Triton buffer (LS with 1% Triton X-100 and 0.5 M NaCl), centrifuged at 180,000 *g* for 30 minutes at 4 °C. The resulting pellet was re-extracted with the same volume of myelin floatation buffer (Triton buffer containing 30% sucrose) and centrifuged at 180,000 *g* for 30 minutes at 4 °C. The remaining pellet was re-extracted in the same volume of Sarkosyl buffer (LS with 1% N-Lauroyl-sarcosine and 0.5 M NaCl), incubated with agitation for 1 hour at 22 °C and centrifuged at 180,000 *g* for 30 minutes at 22 °C. The detergent-insoluble pellet was weighted and solubilized in 5 volumes (wt/vol) of urea buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1- propanesulfonate, pH 8.5) and sonicated for 5 minutes. All buffers contained 1 mM Dithiothreitol DTT and protease inhibitors (LPC; 1/1000). The soluble LS and the insoluble urea fractions were quantified with the Bradford Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions.

3. Results

3.1. Neuroprotection from select longevity-enhancing compounds

We investigated neurodegeneration with a well-characterized transgenic *C. elegans* strain that expresses the full-length human TDP-43 with the A315T mutation associated with amyotrophic lateral sclerosis in the worm's GABAergic motor neurons (Vaccaro et al., 2012a). These animals display adult-onset motility problems leading to progressive paralysis and neuronal degeneration that can be assessed over a period of 9–12 days (Vaccaro et al., 2012a). With this model, we then tested 11 compounds reported to increase lifespan in *C. elegans* for whether they could suppress the progressive paralysis caused by mutant TDP-43 (mTDP-43). The compounds tested included: the antioxidants propyl gallate (PG), trolox (TRO), and α-lipoic acid (Benedetti et al., 2008), the polyphenols resveratrol (RSV) (Morselli et al., 2010) and quercetin (Kampkotter et al., 2008), the anticonvulsant ethosuximide (ETX)

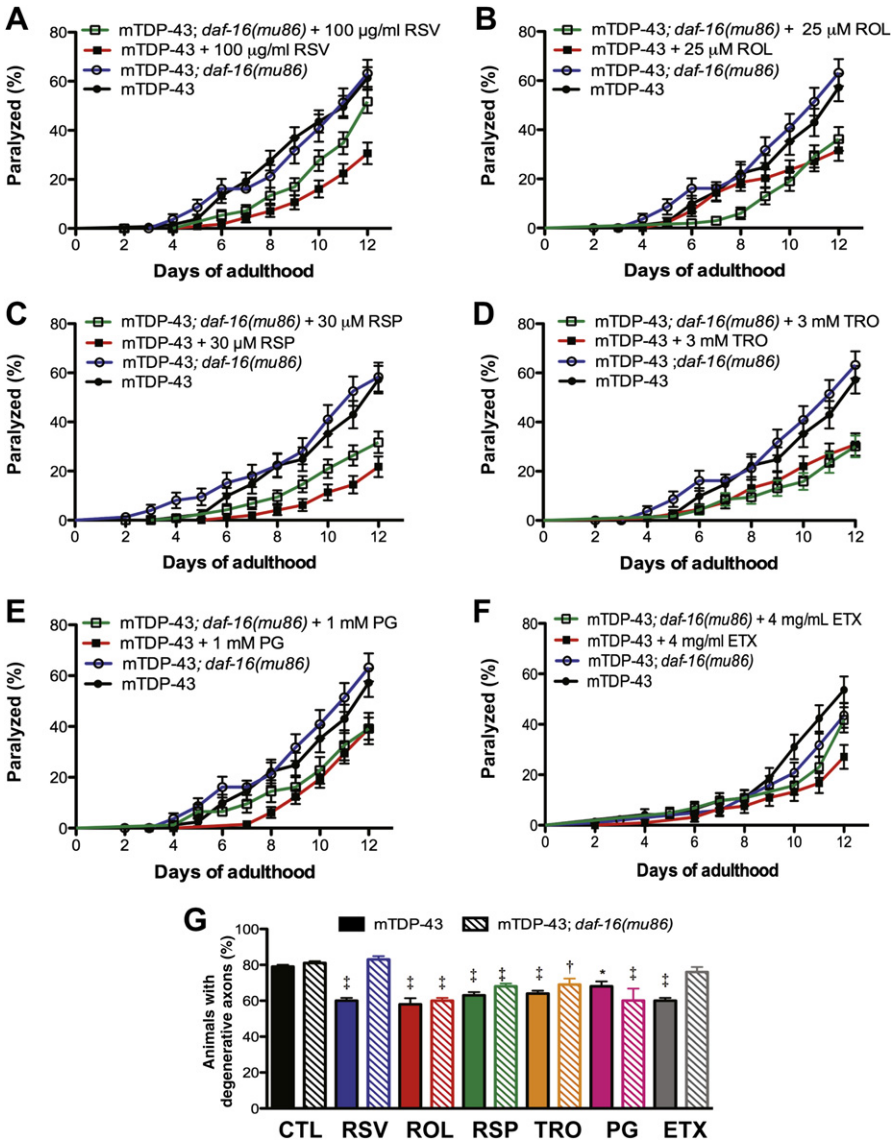

Fig. 2. Transcription factor *daf-16*/Forkhead box O (FOXO mediates RSV and ETX neuroprotection. (A) RSV delayed mutant TAR DNA-binding protein-43 (mTDP-43) paralysis and this effect was dependent on *daf-16*. (B–E) ROL, RSP, TRO, and PG all reduced mTDP-43 paralysis compared with untreated control animals independent of *daf-16*. See Supplementary Table 1 for statistical information. (F) ETX delayed mTDP-43 paralysis and this was dependent on *daf-16*. (G) RSV and ETX failed to rescue neuronal degeneration in *daf-16* mutant animals but all other compounds reduced neuronal degeneration at adult day 9 in *daf-16* mutant animals compared with untreated transgenic animals. * $p < 0.05$; † $p < 0.001$; ‡ $p < 0.0001$. Abbreviations: ETX, ethosuximide; PG, propyl gallate; ROL, rolipram; RSP, reserpine; RSV, resveratrol; TRO, trolox.

(Collins et al., 2008), reserpine (RSP) (Srivastava et al., 2008), spermidine (Eisenberg et al., 2009), valproic acid (Evason et al., 2008), and thioflavin (Alavez et al., 2011), and rolipram (ROL), a Phosphodiesterase 4 inhibitor that mimics the effects of RSV on mitochondrial function and glucose tolerance (Park et al., 2012) (please see Supplementary Table 1 for official names and suppliers). Interestingly, only 6 compounds rescued TDP-43 toxicity: RSV, ROL, RSP, TRO, PG, and ETX (Fig. 1, Supplementary Table 2) rescued mTDP-43 proteotoxicity at dosages previously used to increase lifespan. The 5 remaining compounds did not delay paralysis in the transgenic mTDP-43 worms (Supplementary Fig. 1, Supplementary Table 2). We also tested whether these 6 neuroprotective compounds extended lifespan in our worms and found that all compounds except for RSV increased lifespan (Supplementary Fig. 2, Supplementary Table 3). We also tested whether the reported effects were dependent on changes on protein expression. We found no differences in global protein expression after treatment with the 6 compounds in our transgenic worms (Supplementary Fig. 3). Interestingly, we also observed a reduction in mTDP-43 insolubility in animals treated with TRO, PG, and ETX suggesting these compounds might aid the cellular clearance of toxic protein species (Supplementary Fig. 3). Our data reveal an imperfect correlation between the ability of a compound to extend lifespan and reduce neuronal proteotoxicity.

3.2. *Involvement of* daf-16, hsf-1, sir-2.1, *and* skn-1 *pathways in compound-mediated neuroprotection*

Some of the key regulators of aging and stress signaling in *C. elegans* include the forkhead transcription factor *daf-16*, the heat shock factor transcription factor *hsf-1*, the sirtuin deacetylase *sir-2.1* (Kenyon, 2010), and the Nuclear respiratory factor transcription factor *skn-1* (An and Blackwell, 2003; Bishop and Guarente, 2007). To test if the 6 active compounds functioned within these pathways, we crossed our mTDP-43 transgenic animals with loss-

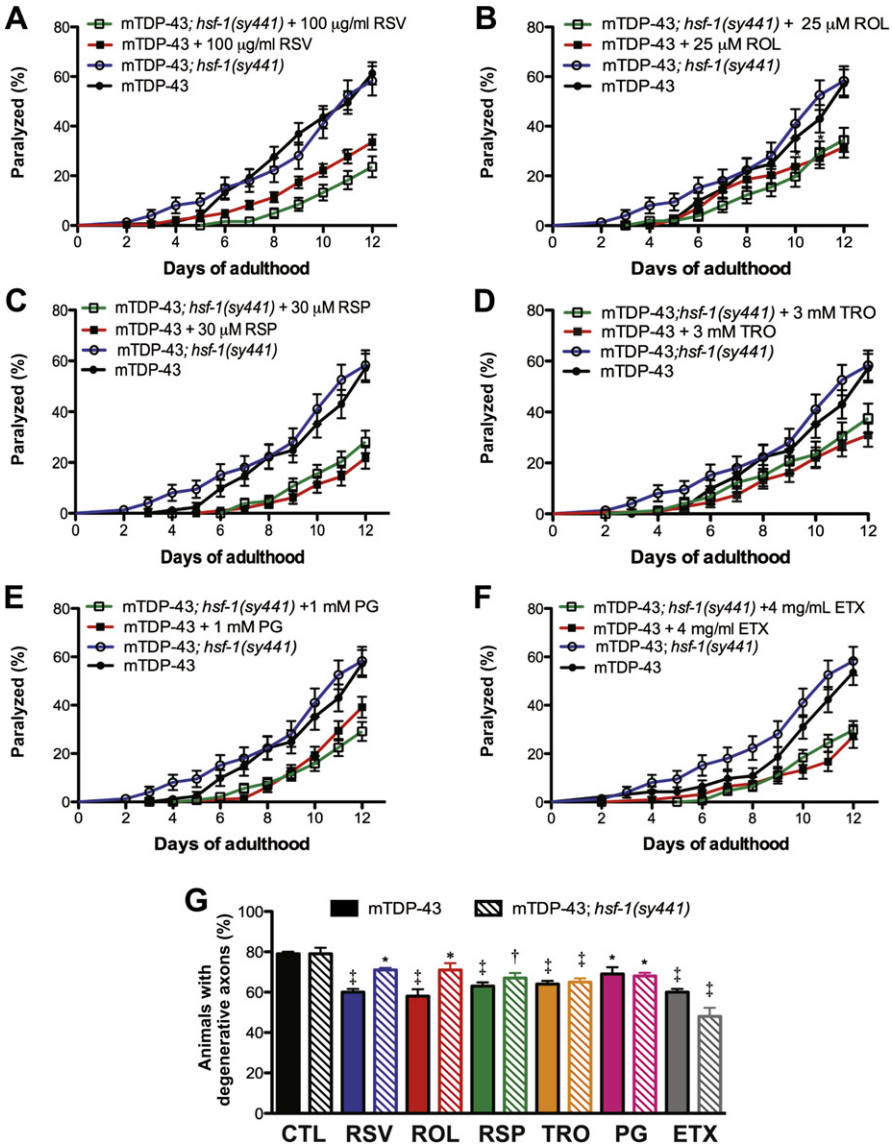

Fig. 3. Neuroprotective effects by the compounds are independent of *hsf-1*. (A–F) All compounds reduced mutant TAR DNA-binding protein-43 (mTDP-43) paralysis compared with untreated control animals independently of *hsf-1*. See Supplementary Table 1 for statistical information. (G) All compounds reduced neuronal degeneration in mTDP-43 animals compared with untreated control animals. * $p < 0.05$; † $p < 0.001$; ‡ $p < 0.0001$. Abbreviations: ETX, ethosuximide; PG, propyl gallate; ROL, rolipram; RSP, reserpine; RSV, resveratrol; TRO, trolox.

of-function mutations or RNAi for each gene and tested if the compounds maintained neuroprotective activity.

3.2.1. *Transcription factor* daf-16 *mediates resveratrol and ETX neuroprotection*

Reduced insulin/IGF pathway signaling has been implicated in aging and stress resistance (Kenyon, 2005; Kenyon et al., 1993). The downstream effector *daf-16*/Forkhead box O has been identified as a key target of RSV neuroprotective effects in polyglutamine toxicity (Parker et al., 2005), and consistently, RSV was less effective at reducing mTDP-43-induced paralysis and axonal degeneration (Fig. 2A and G). Of the remaining compounds we observed that ETX was less effective at suppressing paralysis and neurodegeneration in mTDP-43;*daf-16* mutants suggesting that part of this compound's neuroprotective activity might rest within the insulin/IGF signaling pathway (Fig. 2F and G).

3.2.2. *Neuroprotective effects by compounds are independent of* hsf-1

Chaperones are key regulators of the cellular stress response and the heat shock factor *hsf-1*/Heat shock factor 1 (HSF1) has been implicated in dietary restriction and proteotoxicity (Cohen et al., 2006; Teixeira-Castro et al., 2011). We tested the different compounds in mTDP-43;*hsf-1(sy441)* mutants and observed no differences in the rates of paralysis or neurodegeneration compared with untreated mTDP-43 control animals (Fig. 3 and Supplementary Table 2). Thus, although *hsf-1* is important for proteotoxicity modification, it seems that neuroprotection by the 6 compounds tested here is independent of *hsf-1*.

3.2.3. *Resveratrol reduces neurotoxicity in a* sir-2.1*-dependent manner*

The polyphenol RSV is naturally produced by certain plant species in response to environmental stress (Signorelli and Ghidoni,

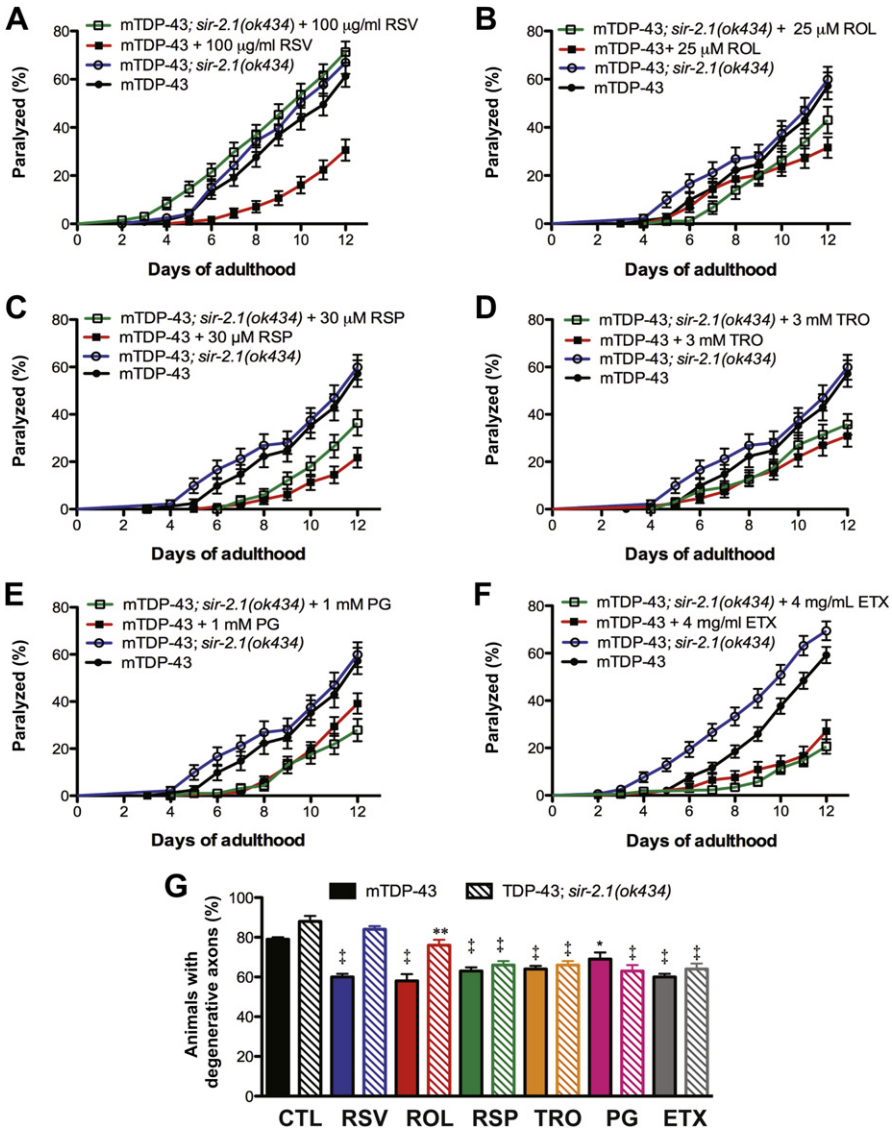

Fig. 4. Resveratrol reduces neurotoxicity in a *sir-2.1*-dependent manner. (A) RSV delayed mutant TAR DNA-binding protein-43 (mTDP-43) paralysis and this was dependent on *sir-2.1*. (B–F) ROL, RSP, TRO, PG, and ETX all reduced mTDP-43 paralysis compared with untreated animals independently of *sir-2.1*. See Supplementary Table 1 for statistical information. (G) RSV failed to rescue axonal degeneration in *sir-2.1* mutant animals but all other compounds reduced neuronal degeneration in mTDP-43 animals compared with untreated control animals. * $p < 0.05$; ** $p < 0.01$; ‡ $p < 0.0001$. Abbreviations: ETX, ethosuximide; PG, propyl gallate; ROL, rolipram; RSP, reserpine; RSV, resveratrol; TRO, trolox.

2005). Subsequent studies have shown that RSV requires sirtuins, a class of nicotinamide adenine dinucleotide-dependent deacetylases, for lifespan extension using dietary restriction (Lin et al., 2000), and was able to rescue neurodegeneration in different late age of onset disease in a Sirtuin 1-dependent manner (Kim et al., 2007; Parker et al., 2005). Thus, consistent with previous studies, RSV failed to rescue paralysis and neurodegeneration in mTDP-43;*sir-2.1(ok434)* mutants (Figs. 4A, 2G and Supplementary Table 2). However, the remaining 5 compounds continued to suppress paralysis and neurodegeneration phenotypes in the absence of *sir-2.1* (Figs. 4B–F and 2G), suggesting they function through alternative pathways.

3.2.4. *Neuroprotective effects by compounds are independent of* skn-1

Many genes involved in aging modulation and stress resistance overlap. We investigated whether the transcription factor *skn-1*, an Nuclear respiratory factor-like response factor that regulates stress resistance and longevity (Tullet et al., 2008; Wang et al., 2010), was required for rescue of neuronal phenotypes in mTDP-43 transgenic worms after treatment with the 6 compounds. However, all the compounds continued to rescue paralysis and axonal degeneration in the absence of *skn-1* (Fig. 5 and Supplementary Table 2).

4. Discussion

Understanding the cellular mechanisms of lifespan extension is an active area of research, as are efforts to apply these findings to age-related pathologies. Because extending lifespan by genetic or dietary methods delays age-associated negative phenotypes from worms to primates, the quest to identify chemicals replicating these effects is a promising area of therapeutic investigation. Supporting this notion are studies from *C. elegans* demonstrating that dietary restriction (Steinkraus et al., 2008), insulin/IGF signaling (Cohen et al., 2006; Morley et al., 2002), *hsf-1* (Cohen et al., 2006; Hsu et al., 2003), *sir-2.1* (Parker et al., 2005), or *skn-1* (Wang et al.,

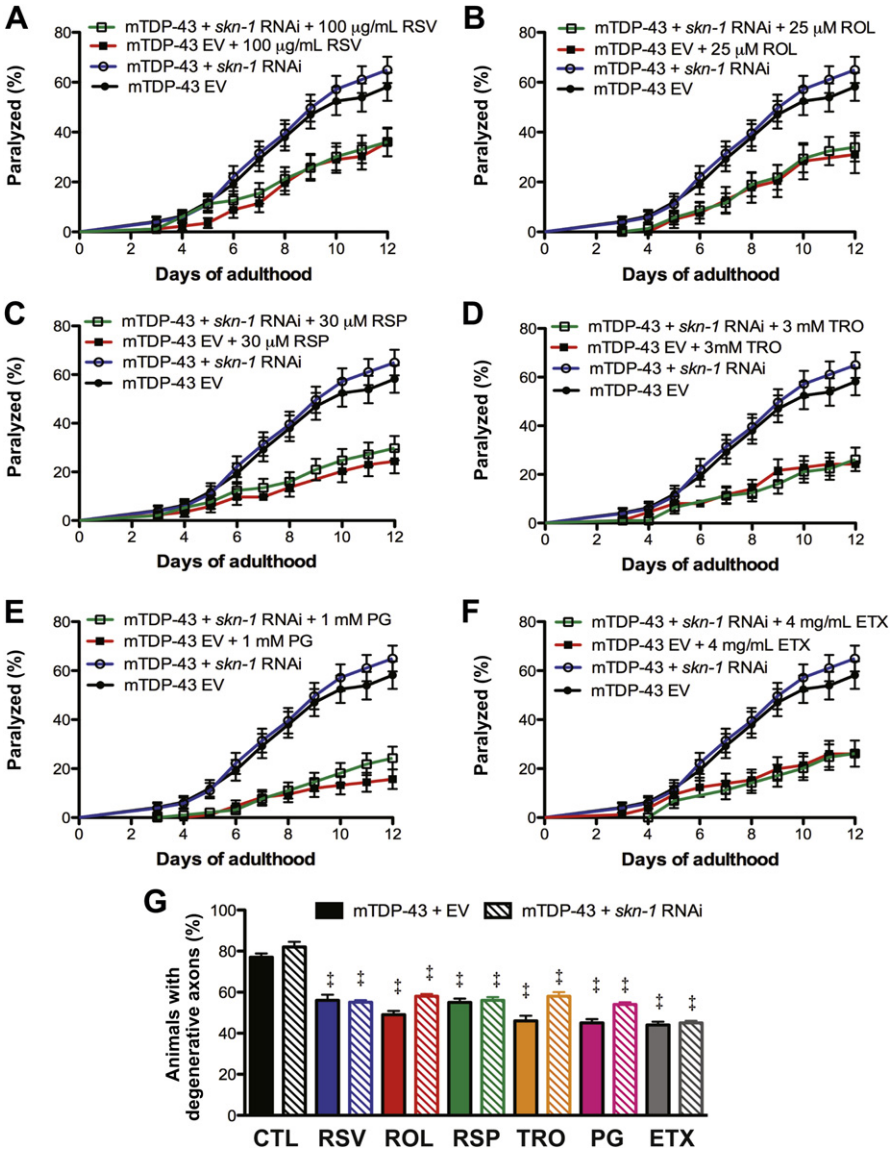

Fig. 5. Neuroprotective effects by compounds are independent of *skn-1*. (A–F) All compounds reduced mutant TAR DNA-binding protein-43 (mTDP-43) paralysis compared with untreated control animals independently of *skn-1*. See Supplementary Table 1 for statistical information. (G) All compounds reduced neuronal degeneration in mTDP-43 animals compared with untreated control animals. ‡ $p < 0.0001$. Abbreviations: ETX, ethosuximide; EV, Empty Vector; PG, propyl gallate; RNAi, RNA interference; ROL, rolipram; RSP, reserpine; RSV, resveratrol; TRO, trolox.

2010) modify proteotoxicity. With this goal in mind, we examined a number of compounds shown to extend lifespan, or in some cases, to reduce polyglutamine or amyloid-ß proteotoxicity in *C. elegans*, and investigated if they could suppress neuronal mTDP-43 toxicity. We identified 6 compounds that suppressed neuronal toxicity, but of these only RSV and ETX showed dependence on the aging genes *daf-16* and *sir-2.1*.

Our data suggest that lifespan extension might not be a strong predictor for neuroprotection. Indeed, recent work has shown that dietary restriction is ineffective against delaying neurodegeneration caused by mTDP-43 or polyglutamine in worms (Tauffenberger et al., 2012), amyloid-ß or tau toxicity in flies (Kerr et al., 2011), or mutant superoxide dismutase 1 in mice (Patel et al., 2010). Regarding the insulin/IGF pathway, reduced signaling has been shown to rescue (Cohen et al., 2006) or enhance proteotoxicity (Vaccaro et al., 2012b), suggesting this pathway might be difficult to manipulate for therapeutic benefit. Part of the discrepancy might be that earlier studies investigating longevity and proteotoxicity relied on models expressing mutant proteins in *C. elegans* body wall muscle cells. Similar results have been observed for *sir-2.1*, where deletion of *sir-2.1* exacerbates polyglutamine toxicity in neurons (Bates et al., 2006; Parker et al., 2005), but rescues polyalanine and α-synuclein toxicity in muscle cells (Catoire et al., 2008; van Ham et al., 2008). The disparity between muscle and neuronal models might apply to drug screening as well, where compounds like thioflavin have been shown to reduce amyloid-ß and polyglutamine toxicity in muscle cells (Alavez et al., 2011), but we observed no activity in our neuronal TDP-43 model. Neurons and muscle cells have different functions and metabolic requirements so it might not be surprising that they respond differently to manipulations promoting overall lifespan extensions in the context of proteotoxicity. Indeed, neurons and muscle cells appear to have different capabilities in responding to protein misfolding during aging (Kern et al., 2010). Thus, the predictive value of muscle-based models for neurodegenerative disorders needs to be interpreted with caution.

Although we describe an imperfect correlation between longevity and neuroprotection, we have identified 6 compounds that protect against mTDP-43 toxicity in motor neurons. Further investigation and validation in vertebrate systems will be required to gauge the effectiveness of these compounds as early leads for drug discovery and development in amyotrophic lateral sclerosis and other late-onset proteinopathies.

Disclosure statement

All authors have no conflicts of interest to disclose.
No approvals were required for the work described in this report.

Acknowledgments

The authors thank S. Peyrard for technical support. A.T. is supported by a CIHR and Huntington's Society of Canada fellowship. J.A.P. is a CIHR New Investigator. The CHUM Foundation, a CIHR Catalyst Grant, the Bernice Ramsay Discovery Grant from the ALS Society of Canada, and the Congressionally Directed Medical Research Program (CDMRP) Amyotrophic Lateral Sclerosis Research Program (USA) supported this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2013.03.014.

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Annexe 8 :

TDP-43 Toxicity Proceeds via Calcium Dysregulation and Necrosis in Aging Caenorhabditis elegans Motor Neurons.

Aggad, D., Vérièpe, J., **Tauffenberger, A**., & Parker, J. A. (2014). *Journal of Neuroscience*, *34*(36), 12093–12103. doi:10.1523/JNEUROSCI.2495-13.2014

Neurobiology of Disease

TDP-43 Toxicity Proceeds via Calcium Dysregulation and Necrosis in Aging *Caenorhabditis elegans* Motor Neurons

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Amyotrophic lateral sclerosis (ALS) is a heterogeneous disease with either sporadic or genetic origins characterized by the progressive degeneration of motor neurons. At the cellular level, ALS neurons show protein misfolding and aggregation phenotypes. Transactive response DNA-binding protein 43 (TDP-43) has recently been shown to be associated with ALS, but the early pathophysiological deficits causing impairment in motor function are unknown. Here we used *Caenorhabditis elegans* expressing mutant TDP-43^{A315T} in motor neurons and explored the potential influences of calcium (Ca^{2+}). Using chemical and genetic approaches to manipulate the release of endoplasmic reticulum (ER) Ca^{2+} stores, we observed that the reduction of intracellular Ca^{2+} ($[Ca^{2+}]_i$) rescued age-dependent paralysis and prevented the neurodegeneration of GABAergic motor neurons. Our data implicate elevated $[Ca^{2+}]_i$ as a driver of TDP-43-mediated neuronal toxicity. Furthermore, we discovered that neuronal degeneration is independent of the executioner caspase CED-3, but instead requires the activity of the Ca^{2+}-regulated calpain protease TRA-3, and the aspartyl protease ASP-4. Finally, chemically blocking protease activity protected against mutant TDP-43^{A315T}-associated neuronal toxicity. This work both underscores the potential of the *C. elegans* system to identify key targets for therapeutic intervention and suggests that a focused effort to regulate ER Ca^{2+} release and necrosis-like degeneration consequent to neuronal injury may be of clinical importance.

Key words: ALS; *C. elegans*; calcium; ER; necrosis; TDP-43

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the selective loss of both upper and lower motor neurons in the cortex and spinal cord (Wijesekera and Leigh, 2009). ALS is an age-dependent, rapidly progressive disease with life expectancy typically being between 2 and 5 years after onset. Thanks to recent genetic advances, causative mutations for ALS have been discovered in over a dozen genes (Renton et al., 2014), including one encoding transactive response DNA-binding protein 43 (TDP-43; Kabashi et al., 2008) among others.

Evidence is mounting that pathways regulating protein degradation may influence ALS pathogenesis (Blokhuis et al., 2013). We previously reported that mutant TDP-43^{A315T} proteins expressed in *Caenorhabditis elegans* motor neurons are susceptible to misfolding, leading to insolubility, aggregation (Vaccaro et al., 2012a), and activation of the endoplasmic reticulum (ER) unfolded protein response (UPR^{ER}; Vaccaro et al., 2012b, 2013). Induction of the UPR^{ER} by mutant TDP-43 suggests that the capacity of the ER to properly fold proteins may be exceeded, leading to cellular dysfunction and death (Walker and Atkin, 2011).

The ER constitutes a Ca^{2+} store whose uptake and release are extensively regulated to maintain cellular Ca^{2+} homeostasis, and disrupted ER function can induce Ca^{2+} depletion (Burdakov and Verkhratsky, 2006). Altered Ca^{2+} homeostasis has been investigated as a mechanism to distinguish motor neurons that are vulnerable or resistant to degeneration in ALS (Palecek et al., 1999; Vanselow and Keller, 2000). Indeed, ALS-vulnerable motor neurons in mice display Ca^{2+} buffering capacities that are five to six times lower compared with those found in ALS-resistant oculomotor neurons (Vanselow and Keller, 2000), while a more recent study has shown that altered Ca^{2+} buffering may be a risk factor for SOD-1 toxicity (von Lewinski et al., 2008).

We investigated the role of cellular Ca^{2+} balance in our TDP-43 models to learn more about the mechanisms of Ca^{2+}-mediated cellular demise. We report that a null mutation in calreticulin (CRT-1), a central regulator of ER Ca^{2+} homeostasis, suppresses both paralysis and the neurodegeneration caused by mutant TDP-43^{A315T} in motor neurons. Furthermore, deletion of the Ca^{2+} binding ER protein calnexin (CNX-1), the ER Ca^{2+} release channels UNC-68 (ryanodine receptor), or ITR-1 (inositol 1,4,5 triphosphate receptor) suppressed TDP-43 toxicity. Consistently, pharmacological manipulations modulating ER Ca^{2+} release and/or uptake suppressed TDP-43 toxicity. Downstream from perturbed Ca^{2+} homeostasis, we discovered that mutations in the Ca^{2+}-regulated calpain protease TRA-3 and aspartyl protease ASP-4 also suppressed TDP-43 toxicity.

Received June 12, 2013; revised July 24, 2014; accepted July 28, 2014.

Author contributions: D.A., J.V., A.T., and J.A.P. designed research; D.A. and J.V. performed research; A.T. contributed unpublished reagents/analytic tools; D.A., J.V., and J.A.P. analyzed data; D.A. and J.A.P. wrote the paper.

Some strains were provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). This research was supported by the CHUM Foundation, the ALS Society of Canada, the Canadian Institutes of Health Research, the Fonds de Recherche Santé Québec, the Muscular Dystrophy Association (U.S.), and the Congressionally Directed Medical Research Program Amyotrophic Lateral Sclerosis Research Program (U.S.), and a Canadian Institutes of Health Research New Investigator Award to J.A.P. We thank S. Peyrard for technical help.

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DOI:10.1523/JNEUROSCI.2495-13.2014

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Our findings suggest that the regulation, and possibly release, of ER Ca^{2+} stores are required for neurotoxicity of TDP-43 in *C. elegans*. It is generally believed that caspase-driven neuronal apoptosis is an underlying pathogenic mechanism in many late-onset neurodegenerative diseases including ALS (Martin, 1999), but the executioner caspase CED-3 is dispensable for neurodegeneration in our TDP-43 model. Instead, the involvement of calpain and aspartyl proteases is more similar to necrosis-mediated cell death (Syntichaki et al., 2002). We propose that misfolded mutant TDP-43 increases $[Ca^{2+}]_i$ by disrupting ER function and activates calpain proteases, which in turn activates killer aspartyl proteases, leading to cell destruction.

Materials and Methods

C. elegans *strains and methods.* Standard culturing and genetic methods were used (Stiernagle, 2006). Animals were maintained at 20°C unless otherwise indicated. Unless otherwise stated, the strains used in this study were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN) and include the following: *asp-4(ok2693)*, *ced-3(ok2734)*, *cnx-1(nr2009)*, *cnx-1(nr2010)*, *crt-1(bz30)*, *crt-1(jh101)*, *itr-1(sa73)*, *kbIs7 [nhx-2p::rde-1 + rol-6(su1006)]*, *kzIs20[pDM#715(hlh-1p::rde-1) + pTG95(sur-5p::nls::GFP)]*, *oxIs12 [unc-47p::GFP + lin-15(+)]*, *rde-1(ne219)*, *sid-1(pk3321)*, *tra-3(ok2207)*, *uIs69 [pCFJ90(myo-2p::mCherry) + unc-119p::sid-1]*, and *unc-68(e540)*. Genetic crosses generated transgenic/mutant combinations, and the presence of transgenes and mutations was confirmed by PCR, visible markers, sequencing, or a combination thereof.

Transgenic lines expressing mutant TDP-43^{A315T}, wild-type TDP-43 (TDP-43^{WT}), *unc-47p*::GFP; TDP-43^{A315T}, and *unc-47p*::GFP; TDP-43^{WT} were previously described (Vaccaro et al., 2012a) and created as follows: human cDNAs for TDP-43^{WT} and TDP-43^{A315T} (a gift from Dr. Guy Rouleau, McGill University, Montreal, QC, Canada) were amplified by PCR and cloned into the Gateway vector pDONR221 following the manufacturer's protocol (Invitrogen). Multisite Gateway recombination was performed with the pDONR TDP-43 clones along with clones containing the *unc-47* promoter (a gift from Dr. Erik Jorgensen, University of Utah, Salt Lake City, UT; and Dr. Marc Hammarlund, Yale University, New Haven, CT), the *unc-54* 3′ UTR plasmid pCM5.37 (Addgene plasmid 17253; a gift from Dr. Geraldine Seydoux, Johns Hopkins University, Baltimore, MD), and the destination vector pCFJ150 (Addgene plasmid 19329; a gift from Dr. Erik Jorgensen, University of Utah) to create *unc-47p::TDP-43* expression vectors. Transgenic lines were created by microinjection of *unc-119(ed3)* worms, multiple lines were generated, and strains behaving similarly were kept for further analysis. Transgenes were integrated by UV irradiation, and lines were outcrossed to wild-type N2 worms five times before use. Several strains showing comparable phenotypes and transgene expression levels were kept and the strains used in this study include the following: *xqIs132*[*unc-47p::TDP-43^{WT}*; *unc-119*(+)] and *xqIs133*[*unc-47p::TDP-43^{A315T};unc-119*(+)].

Pharmacological treatment. All chemicals were obtained from Sigma-Aldrich. Dantrolene and thapsigargin were dissolved in DMSO and added to agar plate to final concentrations of 10 μM and 3 μg/ml, respectively. EGTA was dissolved in 1N NaOH and was added to agar plates to a final concentration of 0.5 mM. MDL-28170 was dissolved in DMSO and added to agar plate to a final concentration of 20 μM.

Paralysis assay. Worms were grown at 20°C on standard Nematode Growth Media (NGM) plates with or without compounds (30 animals/plate, by triplicates) and scored daily for movement. Animals were scored as paralyzed if they failed to move upon prodding with a worm pick. Failure to move their head to touch and the absence of pharyngeal pumping was scored as dead. Statistical analysis was performed using GraphPad Prism software (log-rank Mantel–Cox test).

Neurodegeneration assay. Worms (*unc-47p*::GFP; TDP-43^{A315T} or *unc-47p*::GFP; TDP-43^{WT}, with or without additional mutations listed above) were grown at 20°C on standard NGM plates with or without compounds. Young adult worms were transferred onto seeded NGM plates (with or without compounds), and were selected at days 1, 5, and 9 of adulthood (100 animals/treatment). Live worms were placed on a 2% agarose pad containing 5 mM levamisole in M9 medium to immobilize the worms. Worms were observed under fluorescence microscopy (Leica 6000 microscope) and scored for gaps or breaks in the processes of GABAergic neurons. The mean and SEM were calculated, and ANOVA with Bonferroni correction were used for statistical analyses.

RNA interference experiments. RNA interference (RNAi)-treated strains were fed *Escherichia coli* (HT115) containing an empty vector (EV) or an RNAi clone corresponding to the gene of interest indicated above. All RNAi clones were from the ORFeome RNAi library (Open Biosystems). RNAi experiments were performed at 20°C. Worms were grown on NGM enriched with 1 mM isopropyl-β-D-thiogalactopyranoside. All RNAi paralysis tests were performed using a TDP-43^{A315T}; *unc-47p*::GFP in conjunction with the appropriate mutation and transgenes for tissue-specific silencing in neurons, intestine, or muscle cells based on strains TU3401, VP303, or NR350, respectively. To minimize developmental effects, L4 worms were grown on plates with RNAi bacteria and assayed for paralysis as adults. Worms were transferred every 2 d.

Western blot analysis. Synchronized populations of worms expressing TDP-43 were grown at 20°C on standard NGM plates with or without compounds (15 plates/treatment). Immunoblot analysis of protein levels was performed on whole-animal extracts prepared by washing animals in M9 medium to remove adherent bacteria. The pellets were placed at −80°C overnight and homogenized in 1 ml/g RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) plus 0.1% protease inhibitors (10 mg/ml leupeptin, 10 mg/ml pepstatin A, 10 mg/ml chymostatin; 1:1000). Pellets were sonicated and centrifuged at 16,000 × *g*. Supernatants were collected and were saved as the total fraction. Protein concentrations were determined by the Quick Start Bradford Protein Assay (Bio-Rad). Supernatants, 50 μg/well, were subjected to SDS-PAGE (10%) and transferred to nitrocellulose membranes (Bio-Rad). The immunoblotting analyses were performed using the following antibodies: rabbit anti-TDP-43 (1:1000; Proteintech) and mouse anti-actin (1:20,000 for worms; MP Biomedicals). Proteins were visualized using peroxidase-conjugated secondary antibodies and ECL Western Blotting Substrate (Thermo Scientific). Densitometry was performed with Photoshop (Adobe).

Results

Genetic manipulation of $[Ca^{2+}]$ suppresses TDP-43 toxicity in motor neurons

CRT-1 is a Ca^{2+} binding/storing protein of the ER that serves both as a molecular chaperone and as a central regulator of Ca^{2+} homeostasis (Michalak et al., 1999). Worms expressing mutant TDP-43 in their motor neurons show age-dependent motility defects, leading to paralysis and neurodegeneration (Vaccaro et al., 2012a). To investigate the role of Ca^{2+} balance in TDP-43 neuronal toxicity, we constructed *crt-1(bz30);* TDP-43^{A315T} and *crt-1(jh101);* TDP-43^{A315T} strains, and scored them for paralysis. We observed a significant reduction in the rate of paralysis for *crt-1(bz30);* TDP-43^{A315T} and *crt-1(jh101);* TDP-43^{A315T} animals compared with control TDP-43^{A315T} transgenics (Fig. 1*A*). Focusing on *crt-1(bz30);* TDP-43^{A315T}, we also observed a significant rate of motor neuron degeneration compared with control TDP-43^{A315T} transgenics (Fig. 1*B*).

In the ER, calreticulin works in conjunction with CNX-1 to execute chaperone functions and mediate cellular Ca^{2+} homeostasis (Krause and Michalak, 1997). Given the functional and structural similarities between the two proteins, we tested whether calnexin, encoded by *cnx-1* in *C. elegans*, also influenced TDP-43 toxicity. We observed that introduction of the loss of function mutations *cnx-1(nr2009)* or *cnx-1(nr2010)* into mutant TDP-43^{A315T} transgenics led to a significant decrease in paralysis compared with control TDP-43^{A315T} transgenics (Fig. 1*A*). Focusing on *cnx-1(nr2010);* TDP-43^{A315T}, we also observed a sig-

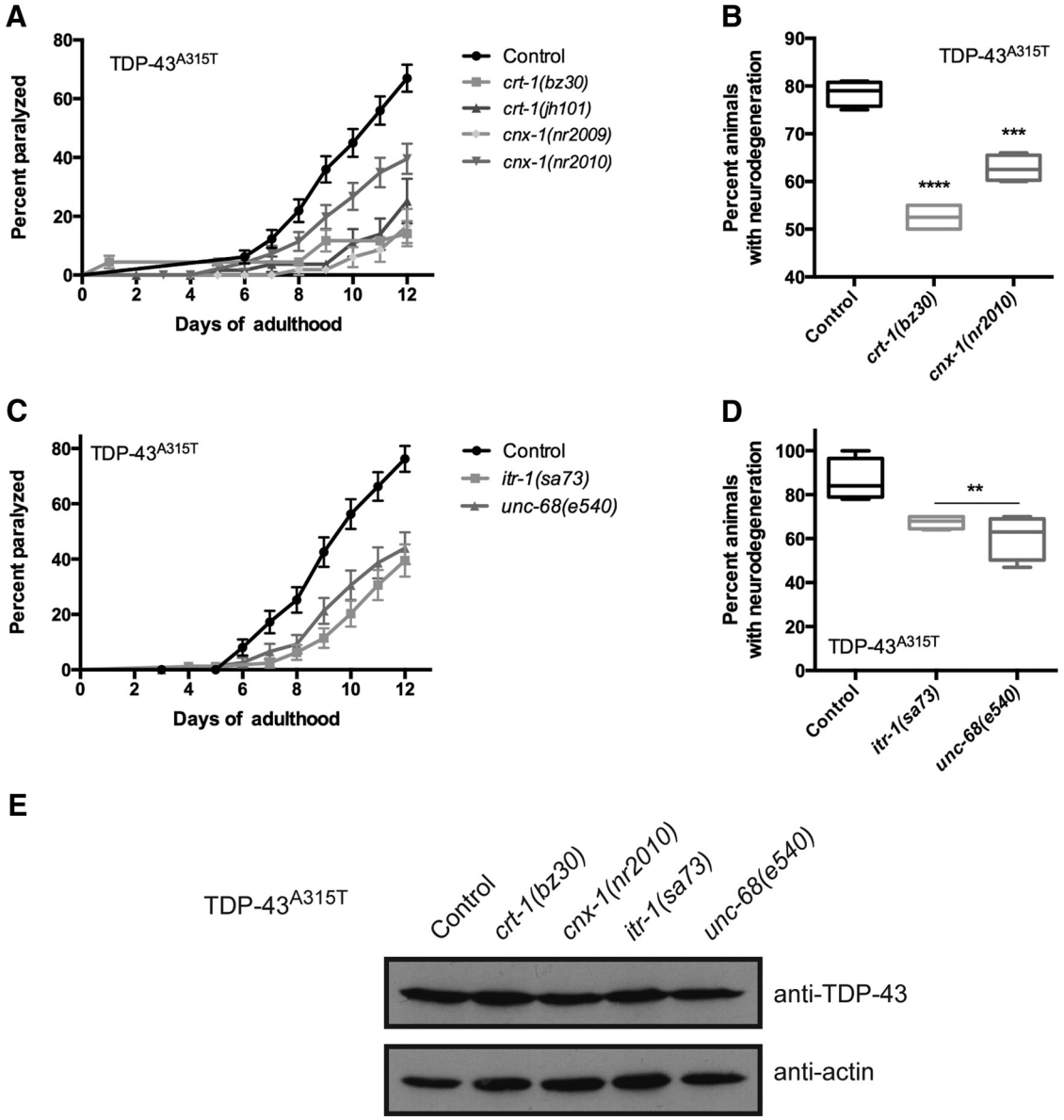

Figure 1. Genes regulating ER calcium release promote TDP-43 neuronal toxicity. ***A***, Null mutations in *cnx-1* or *crt-1* suppress age-dependent paralysis caused by TDP-43^{A315T} compared with transgenic TDP-43^{A315T} controls. $p < 0.0001$ for TDP-43^{A315T}; *cnx-1(nr2009)* versus TDP-43^{A315T}; $p = 0.0002$ for TDP-43^{A315T}; *cnx-1(nr2010)* versus TDP-43^{A315T}; $p < 0.0001$ for TDP-43^{A315T}; *crt-1(bz30)* versus TDP-43^{A315T}; $p < 0.0001$ for TDP-43^{A315T}; *crt-1(jh101)* versus TDP-43^{A315T}. TDP-43^{A315T}, $n = 114$; TDP-43^{A315T}; *cnx-1(nr2009)*, $n = 76$; TDP-43^{A315T}; *cnx-1(nr2010)*, $n = 98$; TDP-43^{A315T}; *crt-1(bz30)*, $n = 90$; and TDP-43^{A315T}; *crt-1(jh101)*, $n = 63$. ***B***, Mutations in *cnx-1* or *crt-1* reduce age-dependent neurodegeneration in TDP-43^{A315T} transgenics compared with TDP-43^{A315T} control animals. ***$p < 0.001$ versus TDP-43^{A315T} at day 9; ****$p < 0.0001$ versus TDP-43^{A315T} at day 9. ***C***, Null mutations in *unc-68* and *itr-1* reduce TDP-43^{A315T}-mediated paralysis compared with control TDP-43^{A315T} transgenics. $p < 0.0001$ for either for TDP-43^{A315T}; *unc-68(e540)* or for TDP-43^{A315T}; *itr-1(sa73)* versus TDP-43^{A315T}. TDP-43^{A315T}, $n = 90$; TDP-43^{A315T}; *itr-1(sa73)*, $n = 88$; and TDP-43^{A315T}; *unc-68(e540)*, $n = 84$. ***D***, Degeneration of motor neurons is reduced in adult day 9 TDP-43^{A315T} transgenics compared with controls. **$p < 0.01$ versus TDP-43^{A315T} at day 9. ***E***, Western blotting with a human anti-TDP-43 antibody revealed comparable levels of protein expression in all strains.

nificant decrease of motor neuron degeneration compared with control TDP-43^{A315T} transgenics (Fig. 1*B*).

To complete the genetic investigation of cellular Ca^{2+} balance, we tested the following two other genes involved in ER regulation of $[Ca^{2+}]_i$: the ER Ca^{2+} release channel inositol triphosphate receptor channel InsP3R, encoded by *itr-1* (Dal Santo et al., 1999), and the ER Ca^{2+} release channel ryanodine receptor channel RyR, encoded by *unc-68* (Maryon et al., 1996). We then investigated the effects of mutations in the InsP3R and RyR genes on TDP-43^{A315T}-mediated paralysis and motor neuron degeneration. Similar to the disruption of calnexin and calreticulin function, *itr-1(sa73);* TDP-43^{A315T} and *unc-68(e540)*; TDP-43^{A315T} strains displayed significantly reduced paralysis and motor neuron degeneration phenotypes compared with TDP-43^{A315T} controls (Fig. 1*C*,*D*). To confirm that the suppression of TDP-43^{A315T} neuronal toxicity was not due to transgene effects, we quantified the level of TDP-43 protein expression by immunoblotting and observed no difference in protein levels for TDP-43 in combination with any of the mutations (Fig. 1*E*).

Pharmacological modulation of $[Ca^{2+}]_i$ regulates TDP-43^{A315T} toxicity in motor neurons

To confirm that altering $[Ca^{2+}]_i$ levels in turn regulates TDP-43 toxicity, we turned to a complementary approach by using chemical reagents to manipulate ER Ca^{2+} release and/or uptake. We first treated TDP-43^{A315T} mutants with EGTA, a Ca^{2+}-specific

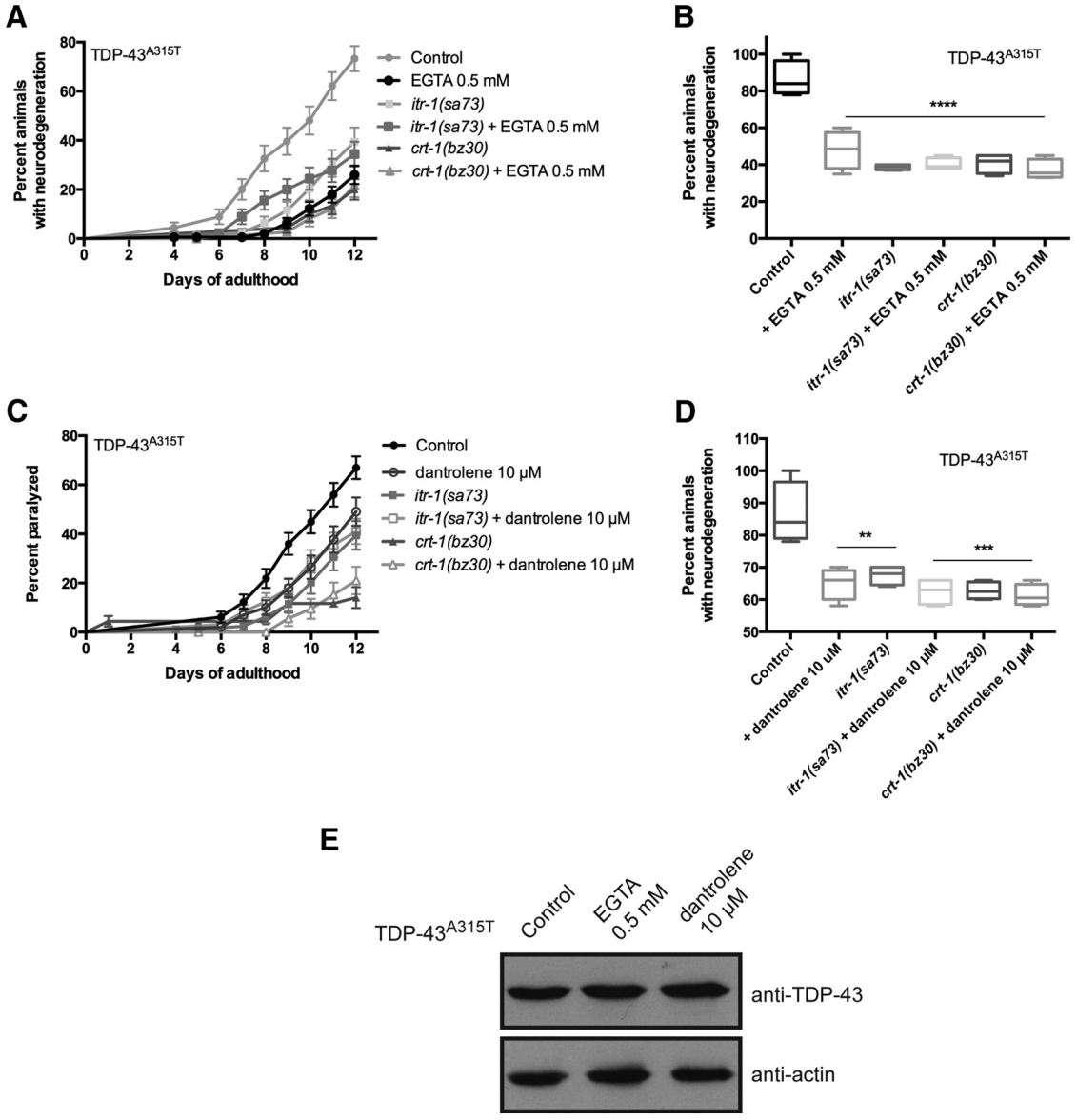

Figure 2. Pharmacological manipulation of $[Ca^{2+}]_i$ reduces TDP-43 neuronal toxicity. ***A***, TDP-43^{A315T} transgenics treated with EGTA showed significantly less paralysis compared with untreated controls ($p < 0.0001$ versus TDP-43^{A315T}). The protective effect of EGTA was not additive to the suppression of TDP-43-mediated paralysis by mutation in either *crt-1* or *itr-1*. $p = 0.8470$ versus TDP-43^{A315T}; *crt-1(bz30)*; and $p = 0.7817$ versus TDP-43^{A315T}; *itr-1(sa73)*. TDP-43^{A315T}, $n = 90$; TDP-43^{A315T} + EGTA, $n = 181$; TDP-43^{A315T}; *itr-1(sa73)*, $n = 90$; TDP-43^{A315T}; *itr-1(sa73)* + EGTA, $n = 90$; TDP-43^{A315T}; *crt-1(bz30)*, $n = 90$; TDP-43^{A315T}; *crt-1(bz30)* + EGTA, $n = 90$. ***B***, Degeneration of motor neurons in TDP-43^{A315T} animals at day 9 of adulthood was reduced to comparable levels in TDP-43^{A315T} transgenics treated with EGTA alone or in combination with mutations in *crt-1* or *itr-1*. ****$p < 0.0001$ versus TDP-43^{A315T} at day 9. ***C***, Treatment with dantrolene suppressed TDP-43^{A315T}-mediated paralysis compared with untreated control animals ($p = 0.0031$ versus TDP-43^{A315T}). Suppression of TDP-43^{A315T}-mediated paralysis by *crt-1* or *itr-1* was not significantly different from these same mutant strains treated with dantrolene. TDP-43^{A315T}, $n = 114$; TDP-43^{A315T} + dantrolene, $n = 100$; TDP-43^{A315T}; *itr-1(sa73)*, $n = 88$; TDP-43^{A315T}; *itr-1(sa73)* + dantrolene, $n = 96$; TDP-43^{A315T}; *crt-1(bz30)*, $n = 90$; TDP-43^{A315T}; *crt-1(bz30)* + dantrolene, $n = 90$. ***D***, Degeneration of motor neurons, in TDP-43^{A315T} animals at day 9 of adulthood was reduced to similar levels in TDP-43^{A315T} transgenics treated with dantrolene alone or in combination with mutations for *crt-1* or *itr-1*. **$p < 0.01$ versus TDP-43^{A315T} at day 9; ***$p < 0.001$ versus TDP-43^{A315T} at day 9. ***E***, TDP-43 protein expression was unchanged by culture conditions with EGTA or dantrolene.

chelator, and observed a clear reduction in the rate of paralysis and neurodegeneration compared with untreated TDP-43^{A315T} transgenics (Fig. 2*A*,*B*). Additionally, EGTA did not further suppress paralysis and neurodegeneration in *crt-1(bz30);* TDP-43^{A315T} or *itr-1(sa73);* TDP-43^{A315T} strains (Fig. 2*A*,*B*), suggesting that *crt-1*, *itr-1*, and EGTA use a common mechanism to reduce TDP-43 toxicity, namely reduced $[Ca^{2+}]_i$.

We next tested whether Ca^{2+} derived from ER stores might contribute to the progressive paralysis caused by mutant TDP-43. We treated TDP-43^{A315T} mutants with dantrolene, a reagent that specifically inhibits Ca^{2+} release from ER stores (Song et al., 1993). In dantrolene-treated animals, paralysis and neurodegeneration were markedly reduced consistent with the hypothesis that ER Ca^{2+} stores contribute to TDP-43 neuronal toxicity (Fig. 2*C*,*D*). Dantrolene treatment did not further suppress paralysis and neurodegeneration phenotypes caused by TDP-43^{A315T} in *crt-1(bz30)* and *itr-1(sa73)* mutants, suggesting a shared mechanism of action (Fig. 2*C*,*D*). The suppression of TDP-43 toxicity by EGTA and dantrolene were not due to reduced transgene expression since similar levels of TDP-43 protein expression were detected by immunoblotting in treated and untreated TDP-43 transgenics (Fig. 2*E*).

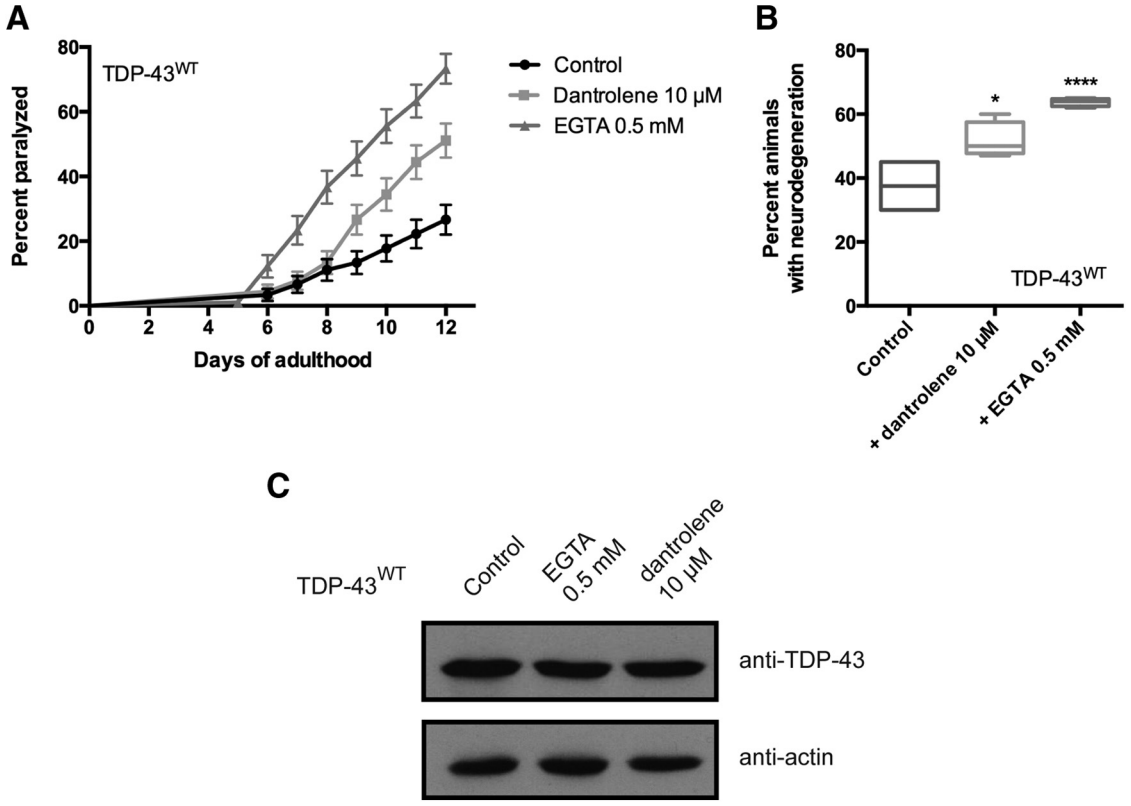

Figure 3. Disrupted Ca^{2+} homeostasis enhances wild-type TDP-43 toxicity. ***A***, Transgenic worms expressing TDP-43 ^{WT} treated with either EGTA or dantrolene had increased rates of paralysis compared with untreated controls ($p < 0.01$ for dantrolene-treated worms versus untreated TDP-43 ^{WT} controls; $p < 0.0001$ for EGTA-treated worms versus untreated TDP-43 ^{WT} controls). TDP-43 ^{WT}, $n = 90$; TDP-43 ^{WT} + dantrolene, $n = 90$; TDP-43 ^{WT} + EGTA, $n = 90$. ***B***, Treatment with dantrolene or EGTA increased neurodegeneration in adults on day 9; TDP-43 ^{WT} transgenics compared with untreated TDP-43 ^{WT} transgenics. *$p < 0.05$ versus TDP-43 ^{WT} at day 9, ****$p < 0.0001$ versus TDP-43 ^{WT} at day 9. ***C***, Similar levels of TDP-43 proteins were detected by Western blotting in untreated TDP-43 ^{WT} transgenics compared with animals treated with EGTA or dantrolene.

We previously reported that worms expressing mutant TDP-43, but not wild-type TDP-43, show elevated ER and oxidative stress (Vaccaro et al., 2013). Ca^{2+} release and uptake is essential for normal cellular function, and we hypothesized that chemically manipulating $[Ca^{2+}]_i$ may only be beneficial to neurons expressing mutant TDP-43 if disrupted Ca^{2+} homeostasis is indeed an underlying mechanism of mutant TDP-43 toxicity. To explore this possibility, we tested transgenics expressing TDP-43 ^{WT} with EGTA or dantrolene, and observed that exposure to either compound greatly enhanced paralysis and neurodegeneration (Fig. 3). Thus, chemically manipulating $[Ca^{2+}]_i$ is beneficial only to neurons expressing mutant TDP-43 proteins.

Thapsigargin-induced ER Ca^{2+} release restores TDP-43 ^{A315T}-dependent cell death in the absence of calreticulin

We next explored whether forced ER Ca^{2+} release might overcome the *crt-1*-induced block on TDP-43-mediated paralysis and neurodegeneration. Here, we sought to reverse *crt-1*-dependent suppression of TDP-43 neuronal toxicity by driving release of the remaining ER Ca^{2+} stores in TDP-43 ^{A315T}; *crt-1(bz30)* animals. We treated TDP-43 ^{A315T}; *crt-1(bz30)* animals, which are fully suppressed for paralysis and neurodegeneration, with thapsigargin, a compound that inhibits the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) ER Ca^{2+} reuptake pump and induces the release of ER Ca^{2+} via the InsP3 receptor channel (Takemura et al., 1989). We found that thapsigargin treatment significantly restored paralysis and neurodegeneration in TDP-43 ^{A315T}; *crt-1(bz30)* mutants compared with chemically untreated controls (Fig. 4*A*,*B*). As a positive control, we exposed transgenics expressing TDP-43 ^{WT} to thapsigargin, and we observed the enhancement of paralysis and neurodegeneration phenotypes (Fig. 4*A*,*B*), suggesting that increased $[Ca^{2+}]_i$ induces cellular damage in *C. elegans*. Finally, using immunoblotting we confirmed that treatment with thapsigargin did not affect the expression of TDP-43 transgenes (Fig. 4*C*). In summary, these data suggest that the elevation of $[Ca^{2+}]_i$ itself may be cytotoxic in nematode motor neurons, supporting a model in which a critical rise in $[Ca^{2+}]_i$ is a causative factor in neurotoxicity, and that CRT-1 is not required for thapsigargin-induced cell death.

Calpain and aspartyl proteases are required for neurodegeneration in a *C. elegans* ALS model

Caspase-dependent apoptosis is a major mechanism promoting cell death in neurodegenerative diseases (Fuchs and Steller, 2011). To determine whether apoptosis was involved in TDP-43 toxicity, we disrupted the main executioner protease, caspase CED-3, which mediates programmed cell death in *C. elegans* (Ellis and Horvitz, 1986). We observed that *ced-3* was not required for paralysis or degenerative phenotypes induced by mutant TDP-43 ^{A315T} in motor neurons (Fig. 5*A*,*B*). Thus, having shown that genetic and pharmacological manipulation of $[Ca^{2+}]_i$ suppresses neurotoxic effects of TDP-43 ^{A315T} in motor neurons, and based on the "calpain–cathepsin" hypothesis described previously (Yamashima et al., 1998), we investigated aspartyl and calpain protease function in paralysis and neurodegeneration. There are 17 genes with similarity to calpain, 7 of which show significant identity to mammalian calpains (Syntichaki et al., 2002), and 7 aspartyl protease genes encoded in the *C. elegans*

genome (Tcherepanova et al., 2000). Based on previous work linking some of these genes to neurodegeneration in *C. elegans* (Syntichaki et al., 2002), as well as the availability of viable loss-of-function mutant strains, we focused on the Ca^{2+}-regulated calpain protease TRA-3 and the aspartyl protease ASP-4. Furthermore, *asp-4* has previously been identified as a modifier of α-synuclein toxicity in *C. elegans* (Qiao et al., 2008). Using null mutants for *tra-3* and *asp-4*, we scored for paralysis and neurodegeneration in strains expressing TDP-43^{A315T}. We observed a significant reduction in the rate of paralysis and the progressive degeneration of motor neurons for TDP-43^{A315T}; *tra-3(ok2207)* or TDP-43^{A315T}; *asp-4(ok2693)* animals compared with control TDP-43^{A315T} strains (Fig. 5*A*,*B*). To confirm that these calpain and aspartyl proteases acted downstream of elevated $[Ca^{2+}]_i$ to regulate mutant TDP-43 toxicity, we treated TDP-43^{A315T}; *tra-3(ok2207)* and TDP-43^{A315T}; *asp-4(ok2693)* strains with thapsigargin. We observed that thapsigargin treatment failed to restore TDP-43^{A315T}-induced paralysis and neurodegeneration when the calpain or aspartyl proteases were absent (Fig. 5*C*,*D*). These data suggest that *tra-3* and *asp-4* are essential for calcium-mediated neurotoxicity associated with mutant TDP-43, and that these proteases may be a terminal effector of neurodegeneration. Furthermore, TDP-43 transgene expression was not affected by mutations in *ced-3*, *tra-3*, or *asp-4* (Fig. 5*E*). We next wanted to determine whether the calpain–aspartyl protease pathway could be a target for small-molecule intervention against TDP-43 toxicity. Z-Val-Phe-CHO (MDL-28170) is a calpain inhibitor previously shown to suppress necrosis in *C. elegans* (Syntichaki et al., 2002). We observed a significant reduction of TDP-43^{A315T}-mediated paralysis in worms treated with MDL-28170 (Fig. 5*F*). These data suggest that calpain and aspartyl proteases may be targeted for preventing neurodegeneration associated with mutant ALS proteins.

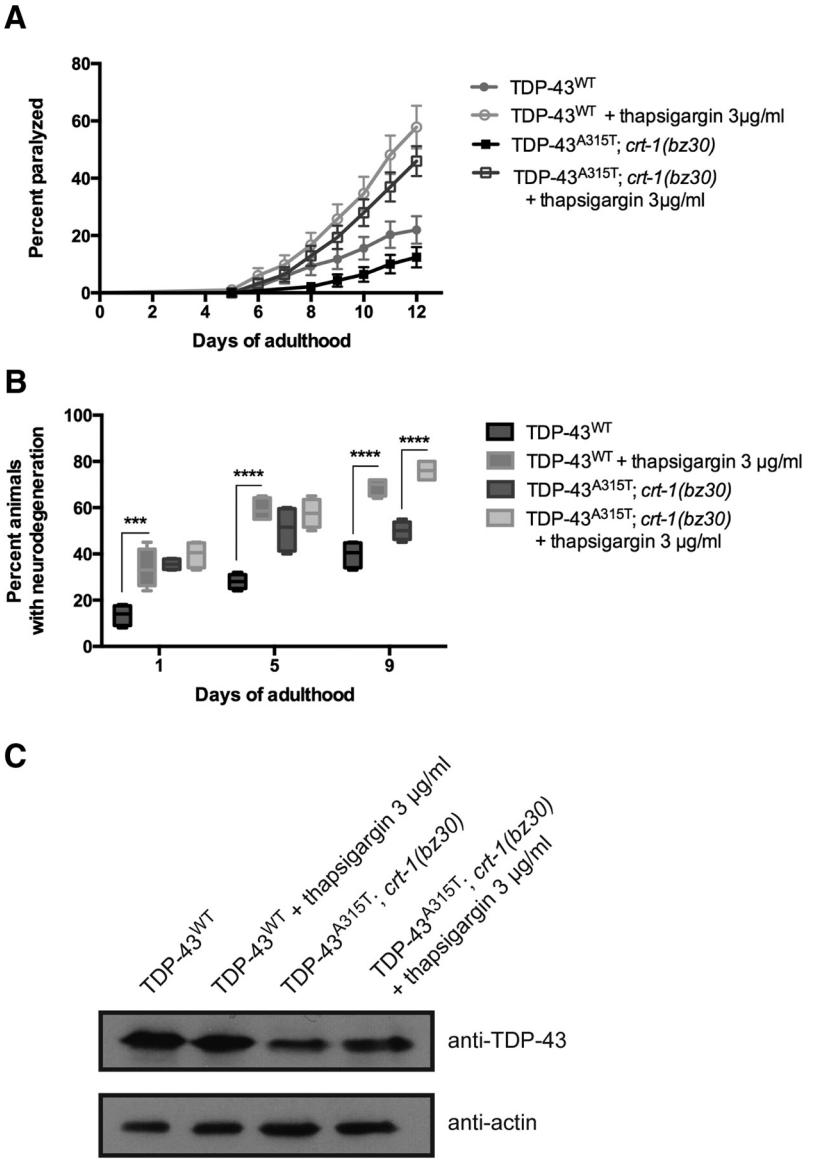

Figure 4. Forced release of ER Ca^{2+} stores enhances TDP-43 neuronal toxicity. ***A***, Paralysis was enhanced in TDP-43^{A315T}; *crt-1(bz30)* and TDP-43^{WT} transgenics treated with thapsigargin compared with untreated transgenic controls. $p < 0.001$ for TDP-43^{A315T}; *crt-1(bz30)* animals versus those treated with thapsigargin; $p < 0.001$ for TDP-43^{WT} versus those treated with thapsigargin. TDP-43^{WT}, $n = 98$; TDP-43^{WT} + thapsigargin, $n = 95$; TDP-43^{A315T}; *crt-1(bz30)*, $n = 96$; TDP-43^{A315T}; *crt-1(bz30)* + thapsigargin, $n = 100$. ***B***, Thapsigargin enhanced neurodegeneration transgenics expressing TDP-43^{WT} at days 1, 5, and 9 of adulthood compared with untreated controls. The suppression of neurodegeneration in TDP-43^{A315T}; *crt-1(bz30)* animals was lost by thapsigargin treatment in adult day 9 transgenics. ***$p < 0.001$ versus TDP-43^{WT} at day 1; ****$p < 0.0001$ versus TDP-43^{WT} or TDP-43^{A315T}; *crt-1(bz30)*. ***C***, Thapsigargin did not affect TDP-43 protein expression in TDP-43^{WT} or TDP-43^{A315T} worms.

Cell-autonomous suppression of TDP-43 toxicity by *tra-3* and *asp-4* in motor neurons

We wondered whether the regulation of TDP-43 toxicity was specific to *tra-3* and *asp-4*, or involved additional calpain and aspartyl proteases. Using RNAi, we conducted a blind screen of five calpain (*clp-1*, *clp-2*, *clp-4*, *clp-7*, and *tra-3*) and seven aspartyl (*asp-1*, *asp-3*, *asp-4*, *asp-6*, *asp-7*, *asp-10*, and *asp-13*) protease genes against TDP-43^{A315T} transgenics engineered for RNAi sensitivity only within the nervous system (Calixto et al., 2010). We observed that RNAi against only *tra-3* or *asp-4* significantly suppressed the paralysis phenotype of TDP-43^{A315T} transgenics (Fig. 6*A*,*B*). In conjunction with our experiments using null mutants, our RNAi experiments suggest that the regulation of TDP-43^{A315T} toxicity is specific to *tra-3* and *asp-4*. We wondered whether the effects of *tra-3* and *asp-4* in mediating TDP-43^{A315T} motor defects were cell autonomous or cell nonautonomous. We conducted *tra-3* or *asp-4* RNAi experiments in TDP-43^{A315T} transgenics sensitized to RNAi only within intestinal cells or body wall muscle cells. We observed no significant reduction of paralysis by *tra-3* or *asp-4* RNAi targeted to intestinal or body wall muscle cells (Fig. 6*C*,*D*). These data suggest that motor defects and degenerative phenotypes caused by TDP-43^{A315T} require the activity of *tra-3* and *asp-4* in the nervous system, and are not likely to be influenced by protease activity in other tissues. Unfortu-

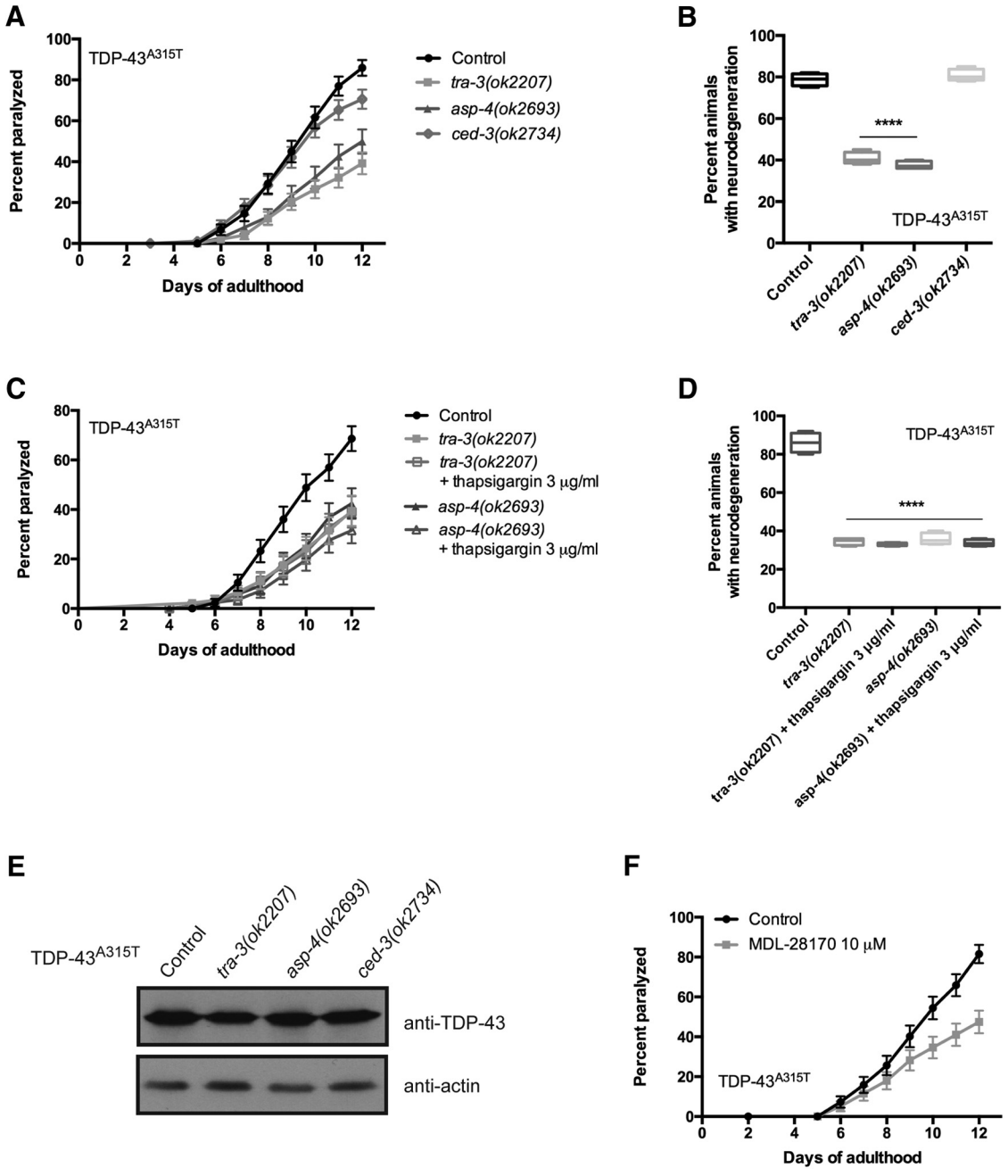

Figure 5. Calpain and aspartyl proteases facilitate TDP-43 neuronal toxicity. ***A***, Null mutations in *tra-3* or *asp-4* suppress age-dependent paralysis in TDP-43^{A315T} transgenics compared with TDP-43^{A315T} controls. Mutation in *ced-3* had no significant effect on paralysis phenotypes compared with TDP-43^{A315T}. $p < 0.0001$ for TDP-43^{A315T}; *tra-3(ok2207)* or TDP-43^{A315T}; *asp-4(ok2693)* versus TDP-43^{A315T} transgenic controls. TDP-43^{A315T}, $n = 90$; TDP-43^{A315T}; *tra-3(ok2207)*, $n = 102$; TDP-43^{A315T}; *asp-4(ok2693)*, $n = 79$; TDP-43^{A315T}; *ced-3(ok2734)*, $n = 96$. ***B***, Neurodegeneration was significantly reduced in adult, day 9, TDP-43^{A315T} transgenics by *tra-3* or *asp-4* null mutations compared with TDP-43^{A315T} alone. A null mutation of *ced-3* failed to suppress TDP-43^{A315T} neurodegeneration. ****$p < 0.0001$ versus TDP-43^{A315T} at day 9. ***C***, The suppression of TDP-43^{A315T}-mediated paralysis by *tra-3* or *asp-4* was unaffected by the addition of thapsigargin. $p < 0.0001$ for TDP-43^{A315T} versus TDP-43^{A315T}; *tra-3(ok2207)* or TDP-43^{A315T}; *asp-4(ok2693)* with or without thapsigargin treatment. TDP-43^{A315T}, $n = 90$; TDP-43^{A315T}; *tra-3(ok2207)*, $n = 90$; TDP-43^{A315T}; *tra-3(ok2207)* + thapsigargin, $n = 90$; TDP-43^{A315T}; *asp-4(ok2693)*, $n = 90$; TDP-43^{A315T}; *asp-4(ok2693)* + thapsigargin, $n = 90$. ***D***, Suppression of age-dependent neurodegeneration in TDP-43^{A315T} transgenics by *tra-3* or *asp-4* mutations was unchanged by thapsigargin treatment. ****$p < 0.0001$ versus TDP-43^{A315T} at day 9. ***E***, Null mutations of *tra-3*, *asp-4*, or *ced-3* did not affect TDP-43 protein expression. ***F***, The calpain inhibitor MDL-28170 reduced paralysis in TDP-43^{A315T} transgenics. $p < 0.001$ for treated versus untreated TDP-43^{A315T} animals. TDP-43^{A315T}, $n = 92$; TDP-43^{A315T} + MDL-28170, $n = 82$.

nately, we could not extend this analysis to Ca^{2+} homeostasis genes since *crt-1* and *itr-1* RNAi were ineffective in our assays.

Ca^{2+} homeostasis and protease genes do not suppress TDP-43^{WT} motor defects

Since our primary assay to identify neuroprotective agents depends on a behavioral assay to detect improved motility of TDP-43^{A315T} transgenics, there is the possibility that we may have identified mutants that nonspecifically augment motility phenotypes. To rule this out, we turned to our TDP-43^{WT} strains that show limited toxicity, with paralysis phenotypes comparable to the expression of GFP alone (Vaccaro et al., 2012a). We crossed *itr-1(sa73)*, *unc-68(e540)*, *crt-1(bz30)*, *cnx-1(nr2010)*, *tra-3(ok2207)*, or *asp-4(ok2693)* mutations into the

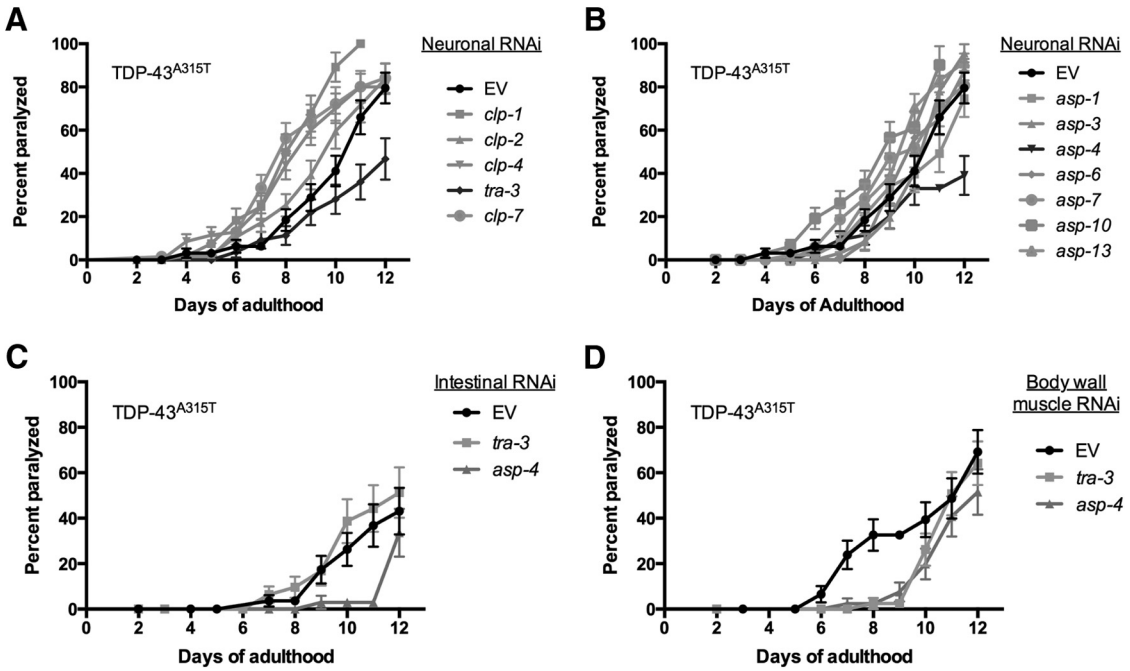

Figure 6. TDP-43-mediated motility defects require *tra-3* and *asp-4* in the nervous system. ***A***, RNAi against *tra-3* suppressed TDP-43^{A315T}-mediated paralysis. $p < 0.05$ for TDP-43^{A315T} treated with *tra-3*(RNAi) versus TDP-43^{A315T} treated with EV control RNAi. TDP-43^{A315T} + EV, $n = 71$; TDP-43^{A315T} + *clp-1*(RNAi), $n = 71$; TDP-43^{A315T} + *clp-2*(RNAi), $n = 84$; TDP-43^{A315T} + *clp-4*(RNAi), $n = 79$; TDP-43^{A315T} + *tra-3*(RNAi), $n = 68$; TDP-43^{A315T} + *clp-7*(RNAi), $n = 69$. ***B***, RNAi against *asp-4* suppressed TDP-43^{A315T}-mediated paralysis. $p < 0.05$ for TDP-43^{A315T} treated with *asp-4*(RNAi) versus TDP-43^{A315T} treated with EV control RNAi. TDP-43^{A315T} + EV, $n = 71$; TDP-43^{A315T} + *asp-1*(RNAi), $n = 54$; TDP-43^{A315T} + *asp-3*(RNAi), $n = 66$; TDP-43^{A315T} + *asp-4*(RNAi), $n = 67$; TDP-43^{A315T} + *asp-6*(RNAi), $n = 59$; TDP-43^{A315T} + *asp-7*(RNAi), $n = 62$; TDP-43^{A315T} + *asp-10*(RNAi), $n = 68$; TDP-43^{A315T} + *asp-13*(RNAi), $n = 58$. ***C***, There were no significant differences in the rates of paralysis for TDP-43^{A315T} sensitized for intestine-specific RNAi by treatment with EV(RNAi), *tra-3*(RNAi), or *asp-4*(RNAi). TDP-43^{A315T} + EV, $n = 77$; TDP-43^{A315T} + *tra-3*(RNAi), $n = 54$; TDP-43^{A315T} + *asp-4*(RNAi), $n = 59$. ***D***, There were no significant differences in the rates of paralysis for TDP-43^{A315T} sensitized for body wall muscle-specific RNAi by treatment with EV(RNAi), *tra-3*(RNAi), or *asp-4*(RNAi). TDP-43^{A315T} + EV, $n = 64$; TDP-43^{A315T} + *tra-3*(RNAi), $n = 51$; TDP-43^{A315T} + *asp-4*(RNAi), $n = 60$.

TDP-43^{WT} strain, but none of the mutations suppressed the paralysis phenotype caused by TDP-43^{WT} (Fig. 7). However, TDP-43^{WT}; *unc-68(e540)* animals had a higher rate of paralysis compared with TDP-43^{WT} controls ($p < 0.05$), suggesting that a general impairment of UNC-68 function may negatively impact the neuronal function and motility observed in TDP-43^{WT} worms. Thus, we conclude that neuroprotective effects of these mutants against TDP-43^{A315T} toxicity are not due to a general improvement of motor function.

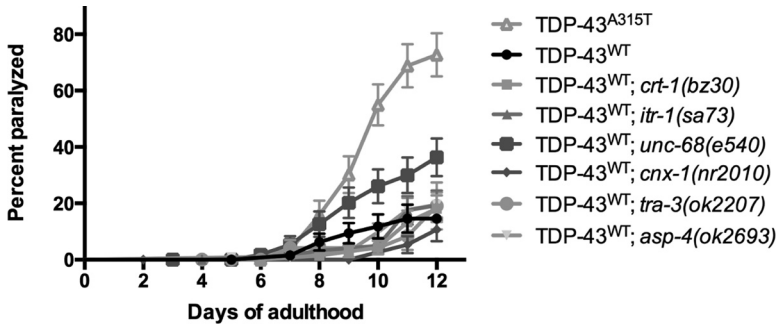

Figure 7. Calcium homeostasis and protease genes do not suppress motility defects in TDP-43^{WT} animals. There was no significant suppression of TDP-43^{WT} motility defects by Ca^{2+} or protease gene mutations. $p < 0.001$ for TDP-43^{A315T} versus TDP-43^{WT}. TDP-43^{A315T}, $n = 81$; TDP-43^{WT}, $n = 65$; TDP-43^{WT}; *crt-1(bz30)*, $n = 71$; TDP-43^{WT}; *itr-1(sa73)*, $n = 71$; TDP-43^{WT}; *unc-68(e540)*, $n = 60$; TDP-43^{WT}; *cnx-1(nr2010)*, $n = 61$; TDP-43^{WT}; *tra-3(ok2207)*, $n = 71$; TDP-43^{WT}; *asp-4(ok2693)*, $n = 115$.

Discussion

Ca^{2+} homeostasis and ALS

Ca^{2+} is an intracellular signaling molecule that regulates many mechanisms in the nervous system (Nikoletopoulou and Tavernarakis, 2012). Cells maintain a tightly controlled resting cytosolic free calcium concentration of ~100 nM by extruding excess Ca^{2+} by pumps and exchangers, and by compartmentalizing Ca^{2+}. The ER is involved in many critical processes, including being a specialized Ca^{2+}-storing organelle (100–800 μM range). The ER is closely involved in the regulation of Ca^{2+} flow within cells and is found in all neurons, occupying cell bodies, and extending toward axons, dendrites, and dendritic spines. In the context of ALS, evidence is mounting that the capacity of the cellular machinery of the ER to properly fold proteins is exceeded (Hetz and Mollereau, 2014), leading cells to react with the UPR^{ER} and that a perturbation of the ER function can induce Ca^{2+} depletion. Studies investigating Ca^{2+} homeostasis in motor neurons have shown that ALS-vulnerable motor neurons in mice display low endogenous Ca^{2+} buffering capacities (Lips and Keller, 1998; Palecek et al., 1999). We hypothesized that dysregulated $[Ca^{2+}]_I$, possibly from the release of ER Ca^{2+} stores, may contribute to mutant TDP-43 neuronal toxicity.

We tested for potential influences of Ca^{2+} in mutant TDP-43^{A315T}-induced degeneration in the following two ways: with genetic mutations that alter release of ER Ca^{2+} stores; and by using chemical reagents to manipulate ER Ca^{2+} release (Fig. 8). We found that null mutations in *crt-1*, a molecular chaperone that plays a critical and complex role in cellular calcium homeostasis as a major site for stored Ca^{2+} (Michalak et al., 1999), suppressed both paralysis and neurodegeneration induced by

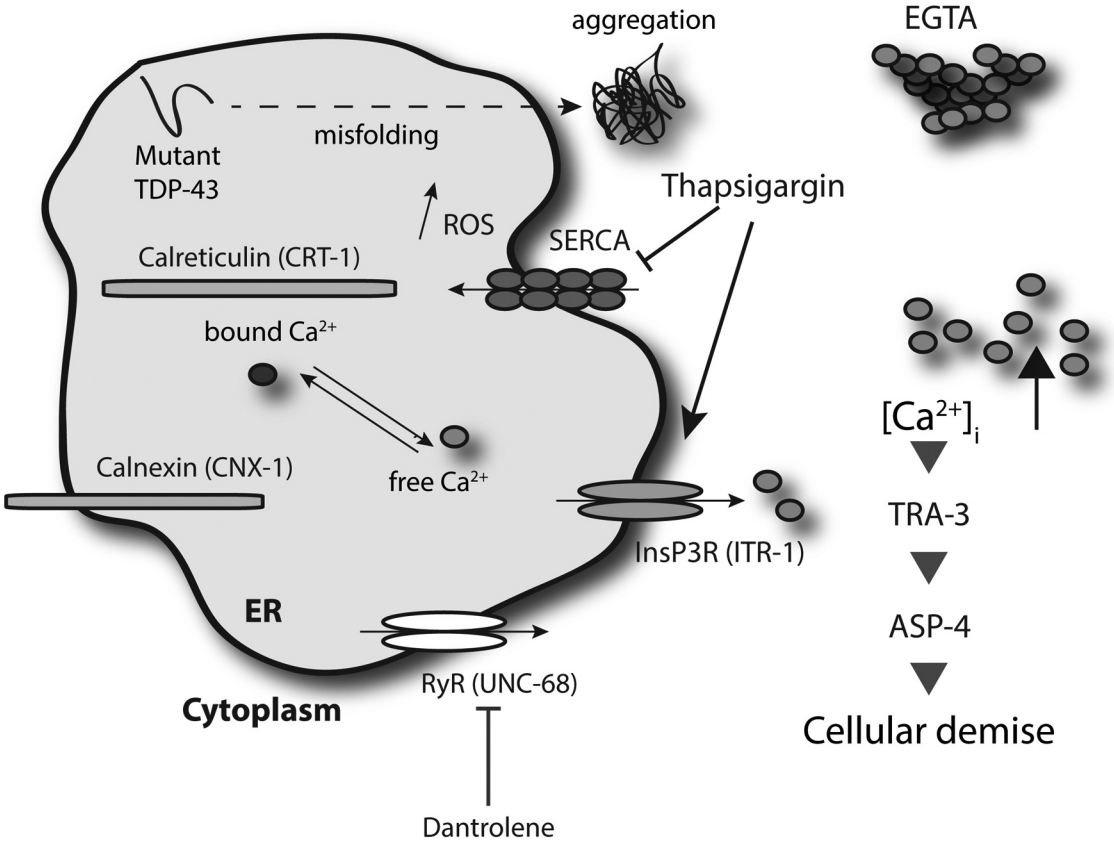

Figure 8. Working model for TDP-43 and Ca^{2+}-dependent necrosis-like neuronal toxicity. The chronic stress induced by protein misfolding and aggregation of mutant TDP-43 proteins may lead to inappropriate release of Ca^{2+} from ER stores into the cytoplasm. The resultant $[Ca^{2+}]_i$ increase is essential for downstream events, including activation of the Ca^{2+}-regulated TRA-3 calpain protease, which in turn mediates leakage of killer aspartyl proteases (ASP-4), leading to neuronal dysfunction and cell death. Mutations affecting ER Ca^{2+} storage (calreticulin and calnexin) or ER calcium release (InsP3R and RYR calcium release channels) disrupt release and are therefore neuroprotective. Pharmacological reduction of $[Ca^{2+}]_i$ by EGTA or dantrolene is also neuroprotective, while a forced increase of $[Ca^{2+}]_i$ by thapsigargin enhances neuronal toxicity. Disabling the activity of calpain or aspartyl proteases also protects against TDP-43-associated neuronal dysfunction and degeneration.

mutant TDP-43. Dysregulation of the ER-resident Ca^{2+} binding protein calreticulin may directly contribute to motor neuron death in ALS models (Bernard-Marissal et al., 2012). Our data showing that calreticulin contributes to neurodegeneration is somewhat at odds with findings for an SOD-1 model showing that reduced calreticulin levels activate the FAS/CD95 pathway to trigger cell death (Bernard-Marissal et al., 2012). However, no clear ortholog of FAS/CD95 exists in the *C. elegans* genome, suggesting that the role of calreticulin in mediating TDP-43 neuronal toxicity in our study may follow different cellular mechanisms. Alternatively, the mechanisms governing the degradation of SOD1 proteins may be distinct from TDP-43 (Mulligan and Chakrabartty, 2013).

However, ER stress has emerged as a mechanism in ALS (Matus et al., 2013; Tadic et al., 2014), and has been linked to the motor neuron vulnerability observed in SOD-1 mouse models (Nishitoh et al., 2008; Saxena et al., 2009), but it remains to be seen whether the ER is a site of action for other ALS mouse models. Furthermore, many aspects of the genetic signaling pathways controlling ER stress response were discovered in *C. elegans* and are conserved in mammalian systems (Mori, 2009). Encouragingly, we previously linked the ER stress response to TDP-43 toxicity (Vaccaro et al., 2013), and identified a number of small molecules that reduce neurodegeneration in *C. elegans* and zebrafish TDP-43 motor neuron models. Further insights into the induction of the ER stress response and neurodegeneration come from a recent report using a *C. elegans* model of SOD1 neuronal toxicity (Thompson et al., 2014). Linking the model organism studies to mammals are the ER stress-protective compounds salubrinal (Saxena et al., 2009) and guanabenz (Jiang et al., 2014), both of which show neuroprotective activity in mouse SOD-1 models. Thus, work from *C. elegans* models may be predictive for mechanisms of motor neuron degeneration in mammalian systems.

Because luminal calreticulin works in conjunction with calnexin to effectuate chaperone functions and mediate cellular Ca^{2+} homeostasis (Krause and Michalak, 1997), we also disrupted calnexin function using loss-of-function mutations and confirmed the suppression of TDP-43 neuronal toxicity. It is known that Ca^{2+} is released from ER stores into the cytoplasm via the InsP3R and the RyR Ca^{2+} channels. We blocked the RyR function by using a null mutation in *unc-68* or by treatment with dantrolene, a reagent that specifically inhibits Ca^{2+} release from ER stores (Song et al., 1993), and InsP3R by using a null mutation in *itr-1*, and we showed the same suppression of TDP-43 toxicity. Thus, our data extend upon and are consistent with recent work showing that inositol triphosphate receptors regulate neurotoxicity in *Drosophila* and cell culture TDP-43 models (Kim et al., 2012). Further highlighting the role of Ca^{2+} homeostasis, treatment of TDP-43^{A315T} mutants with EGTA, a Ca^{2+}-specific chelator, produced a clear reduction of paralysis and neurodegeneration phenotypes. Importantly, the fact that nei-

ther dantrolene nor EGTA enhanced TDP-43 toxicity in TDP-43^{A315T}; *crt-1* or TDP-43^{A315T}; *itr-1* double mutants suggests that the inappropriate release of Ca^{2+} from ER stores may be a common mechanism of TDP-43-mediated neuronal toxicity. Conversely, thapsigargin-induced ER Ca^{2+} release, by activating the InsP3R function and blocking the Ca^{2+} return to the ER from the cytoplasm via the SERCA Ca^{2+} pump, can restore TDP-43^{A315T}-induced cell death in the absence of calreticulin, indicating that Ca^{2+} release and uptake are essential for TDP-43^{A315T} neuronal toxicity, and can in fact also enhance the toxicity of worms expressing TDP-43^{WT} in motor neurons.

The role of endogenous TDP-1/TDP-43 in neurodegeneration

Our group (Vaccaro et al., 2012b), plus another research team (Zhang et al., 2012), previously reported that endogenous TDP-1 (*C. elegans* ortholog of TDP-43) is required for toxicity caused by the transgenic expression of mutant TDP-43 in the *C. elegans* nervous system. Additionally, we also showed that TDP-1/TDP-43 is required for the toxicity of mutant polyglutamine proteins in *C. elegans* and mammalian cell culture models of Huntington's disease (Tauffenberger et al., 2013). These findings suggest that wild-type TDP-1/TDP-43 may actively contribute to neurodegeneration beyond ALS. Our previous work also showed that *tdp-1* is ubiquitously expressed, and is mainly a nuclear protein. Under stress conditions, including ER stress, TDP-1 expression is increased, and this chronic, elevated expression is cytotoxic, leading to decreased lifespan in worms (Vaccaro et al., 2012b). These observations are consistent with the function of TDP-43 as a stress-inducible protein, as is seen in many systems (Janssens and Van Broeckhoven, 2013). Another conserved phenotype of TDP-43 is its cytotoxicity when overexpressed, suggesting that its expression levels are tightly regulated (Buratti and Baralle, 2011). A caveat of our data interpretation in this current study is that a role for TDP-1 in the necrosis-like degeneration of TDP-43^{A315T} motor neurons was not examined. Part of the cascade of molecular events described here leading to neurodegeneration may involve endogenous TDP-1. The cytotoxicity of TDP-1 itself may depend on Ca^{2+} homeostasis and/or protease genes. Additionally, since TDP-1 is a DNA/RNA binding protein, another possibility is that TDP-1 may regulate the expression of Ca^{2+} and protease genes under stress conditions, including for proteotoxicity and ER stress. Future studies will explore the contribution of TDP-1 to these molecular mechanisms.

Necroptosis as a key mechanism of neurodegeneration in ALS

The perturbation of cytosolic Ca^{2+} homeostasis has been implicated in necrotic cell death both in mammals and in *C. elegans* (Sattler and Tymianski, 2000; Xu et al., 2001), but the mechanism by which Ca^{2+} triggers cellular demise remains unclear; so, we investigated relevant signaling pathways based upon the "calpain–cathepsin hypothesis." In 1998, Yamashima et al. formulated the calpain–cathepsin hypothesis, according to which Ca^{2+}-activated cysteine proteases compromise the integrity of the lysosome and cause leakage of acidic hydrolases (Yamashima et al., 1998). We tested the requirement for calpain and aspartyl protease activity in neurodegeneration inflicted by the expression of TDP-43^{A315T} in GABAergic motor neurons, and showed that null mutations in the calcium-regulated *tra-3* calpain protease and aspartyl protease *asp-4* suppress both paralysis and neurodegeneration. Interestingly, TDP-43^{A315T} toxicity was unaffected by a null mutation in the cysteine–aspartate protease CED-3, a protein central to apoptosis in *C. elegans*, and in agreement with previous studies of TDP-43 toxicity in *C. elegans* (Liachko et al., 2010). Thus, in terms of genetic signaling pathways the neuronal toxicity caused by TDP-43^{A315T} in *C. elegans* more closely resembles necrosis than classic apoptosis (Troulinaki and Tavernarakis, 2012).

Recent work has suggested that motor neuron death in models of both sporadic and familial ALS occurs through necroptosis (Re et al., 2014), a form of programmed necrosis that does not require caspases (Ofengeim and Yuan, 2013). Our work is in agreement with this notion as inactivation of a *C. elegans* key executioner caspase, *ced-3*, had no effect on motor neuron dysfunction in our TDP-43 models. Key molecules regulating necroptosis in ALS models include the receptor-interacting serine/threonine-protein kinase 1 and mixed-lineage kinase domain-like, but whether orthologs of these proteins regulate TDP-43 toxicity in our *C. elegans* ALS models requires further investigation. Importantly, work from *C. elegans* detailing programmed necrosis may shed light on mechanisms relevant to neurodegeneration in mammalian settings and perhaps specifically ALS.

Abnormal Ca^{2+} signaling has been linked to multiple neurological disorders, but the challenge remains in identifying disease-specific mechanisms (Bezprozvanny, 2009). We propose that the chronic stress induced by misfolded mutant TDP-43 proteins induces the inappropriate release of Ca^{2+} from ER stores into the cytoplasm is a trigger for subsequent neurodegeneration (Fig. 8). Disrupted Ca^{2+} homeostasis may have multiple downstream, effector mechanisms promoting neuronal dysfunction and cell death, including the inappropriate activation of the Ca^{2+}-dependent proteases identified here, disrupting mitochondrial activity (Tradewell et al., 2011; Parone et al., 2013), or altered Ca^{2+} signaling at the neuromuscular junction (Armstrong and Drapeau, 2013). Thus, restoration of Ca^{2+} homeostasis in ALS motor neurons and/or limiting programmed necrosis may be pursued as potential therapeutic interventions.

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