MICROBIAL EFFECTS ON C. ELEGANS MODELS OF ALZHEIMER'S

UNCOVERING MECHANISMS BEHIND MICROBIOTA-INDUCED NEUROPROTECTION IN C. ELEGANS MODELS OF ALZHEIMER'S DISEASE

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TITLE: Uncovering mechanisms behind microbiota-induced neuroprotection in *C. elegans* models of Alzheimer's disease

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Lay abstract

Alzheimer's Disease (AD) is the most common neurodegenerative disorder in the world. Causes and preventions of AD are difficult to find because many things affect neurodegeneration. For example, resident gut bacteria can change brain health. To study this, we use *Caenorhabditis elegans* worms as animal models that display traits of AD. *C. elegans* eat bacteria, have a nervous system, and share many genes with humans. We identified bacteria from the human microbiota that improved neuron health in *C. elegans* AD models. We examined *C. elegans* gene expression, and found a group of genes, tau tubulin kinases (TTBKs), were shut down by these bacteria. Removal of the TTBK gene from *C. elegans* improved nervous system health. Since we observed that TTBK is shut down in response to the microbiota, and loss of TTBK improves neuron health, we have found new evidence for how the microbiota can promote a healthier nervous system.

Abstract

Alzheimer's Disease (AD) is the most common neurodegenerative disorder worldwide. The risk of developing AD is influenced not only by genetic factors, but also the environment. The multi-factored pathogenesis that leads to AD development poses a challenge for identifying causal factors that promote or protect against neurodegeneration. We use *Caenorhabditis elegans* as a model of AD to measure the impact of human microbiota species on AD-related phenotypes. The two hallmarks of AD are amyloid-β (Aβ) plaques and neurofibrillary tangles composed of the protein tau. Using a *C. elegans* model overexpressing Aβ, we identified a group of *Enterobacteriaceae* species that significantly reduce paralysis. To validate these findings, we used another *C. elegans* model that pan-neuronally expressed aggregate-prone tau (AP-tau). We observed decreased neurodegeneration in response to most of the same bacteria protective against Aβ-induced paralysis, providing additional evidence of microbiota-promoted neuroprotection. To explore the underlying host mechanisms, we examined gene expression changes in animals exposed to neuroprotective bacteria. Numerous biological processes were differentially regulated in response to the neuroprotective microbiota species, including innate immunity, stress responses, and protein phosphorylation. Several *C. elegans* orthologs of human tau tubulin kinase genes, TTBK1 and TTBK2, were downregulated in response to neuroprotective microbiota species. RNAi-mediated knockdown of *C. elegans ttbk*s sufficiently induced neuroprotection in AP-tau animals. Further, *Enterobacter* caused decreased abundance of a tau species phosphorylated at S422, a TTBK1 direct phosphorylation site. These

findings suggest that species from the human microbiota can reduce tau phosphorylation, and mediate neuroprotection through downregulation of *ttbk.* Overall, by studying the impact of the human microbiota on models overexpressing Aβ or AP-tau, we have uncovered a potential mechanism by which microbiota-mediated neuroprotection can occur. In doing so, we also gain a greater understanding of conserved pathways involved in gene-environment interactions promoting development of AD.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER TWO - Human microbiota bacteria can protect against neurodegeneration by reducing phosphorylation of tau

CHAPTER THREE - Identification of novel microbiota-influenced genes that influence neurodegeneration in *C. elegans*

LIST OF TABLES

CHAPTER TWO - Human microbiota bacteria can protect against neurodegeneration by reducing phosphorylation of tau

CHAPTER THREE - Identification of novel microbiota-influenced genes that influence neurodegeneration in *C. elegans*

LIST OF ABBREVIATIONS

Abbreviation	Definition
AD	Alzheimer's disease
AP	Aggregate-prone
APOE4	Apolipoprotein E4
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Aβ	Amyloid-beta
BDNF	Brain-derived neurotrophic factor
BHI	Brain heart infusion
Cdk-5	Cyclin-dependent kinase 5
CFS	Cell free supernatant
CGC	Caenorhabditis Genetics Center
COLQ	Collagen like tail subunit of asymmetric acetylcholinesterase
DAF-16	abnormal DAuer Formation 16
DMSO	dimethyl sulfoxide

DNA	Deoxyribonucleic acid
ERK2	Extracellular signal-regulated kinase 2
FAD	familial AD
FOXO	Forkhead box
FPRL1	formyl-peptide-receptor-like-1
FPS	Frames per second
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase-3
GWAS	Genome-wide association study
HADHA	Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha
HEK	Human embryonic kidney
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun N-terminal kinases
LB	Luria-Bertani
LRRK2	Leucine Rich Repeat Kinase 2

MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with collagenous structure
NCBI	National Center for Biotechnology Information
NGM	Nematode growth medium
NMD	Nonsense mediated decay
PBS	Phosphate buffered saline
PLM	Posterior lateral microtubule
PMK	P38 Map Kinase family
PMSF	Phenylmethylsulfonyl fluoride
PolyQ	Polyglutamate
PP1	Protein phosphatase 1
PPP1CB	PPP1CB protein phosphatase 1catalytic subunit beta
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PTM	Post-translational modification
PTPN9	Tyrosine-protein phosphatase non-receptor type 9

RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic acid
RNAi	RNA-interference
SCFA	Short-chain fatty acid
SCF	Skp, Cullin, F-box
SDS	Sodium dodecyl-sulfate
SFTPD	Surfactant protein D
SMG	Suppressor with Morphological effect on Genitalia
SMO-1	SUMO (ubiquitin-related) homolog
SNAP25	Synaptosome Associated Protein 25
SUMO	Small ubiquitin-like modifier
SV2A	Synaptic vesicle glycoprotein 2A
T2D	Type-2 diabetes
TBS	Tris-buffered saline
TDP	Transactive response DNA binding protein

TRPC3	Transient receptor potential cation channel subfamily C member 3
TRPC6	Transient receptor potential cation channel subfamily C member 6
TRPC7	Transient receptor potential cation channel subfamily C member 7
TSSK6	Testis specific serine kinase 6
TTBK1	Tau tubulin kinase 1
TTBK2	Tau tubulin kinase 2
UTR	Untranslated region

CHAPTER ONE - Introduction

Alzheimer's Disease

Alzheimer's Disease (AD) is the most common neurodegenerative disorder, affecting more than 46 million people worldwide, and the incidence rate continues to increase with aging populations (Wortmann, 2015). AD is characterized by the oligomerization of toxic amyloid-beta (Aβ) peptides (Karran & De Strooper, 2022) and accumulation of neurofibrillary tangles consisting of hyperphosphorylated and insoluble tau (Arnsten et al., 2021). Aβ and tau pathogenesis results in hippocampal and cortical neuron loss (Bobinski et al., 1998; D'Amelio & Rossini, 2012; Roy et al., 2016). There are two types of AD: Early onset AD, where disease onset is before the age of 65, and late onset AD, which occurs in individuals aged 65 and over (Bekris et al., 2010). Approximately 60% of early onset AD patients have a family history of AD (Bekris et al., 2010; Campion et al., 1999; van Duijn et al., 1994). Familial AD is attributed to the inheritance of causal mutations in genes including *APP*, which encodes the amyloid precursor protein, and *PSEN1*, or *PSEN2*, which encode catalytic subunits of the γ-secretase complex (Bekris et al., 2010; Bertram & Tanzi, 2004; Bettens et al., 2013; Dunn et al., 2019).

Until recently, AD development was largely attributed to the amyloid cascade hypothesis, which suggests that deregulated sequential cleavage of APP by β-secretase and γ-secretase promotes aggregation of Aβ peptides into extracellular plaques, initiating tau hyperphosphorylation to cause neuron death (Small & Duff, 2008). The belief of the Aβ cascade driving tau pathology and neurodegeneration led to the development of drugs

aiming to clear Aβ plaques or prevent Aβ production, which all ultimately failed (Karran & Hardy, 2014; Yiannopoulou et al., 2019). The evidence that targeting Aβ alone will not cure AD revealed that there are still gaps in knowledge on how to treat AD. This triggered some movement back towards prioritizing fundamental research focused on understanding the mechanisms that cause AD. There are also multiple ongoing clinical trials for pharmaceuticals, including novel acetylcholinesterase inhibitors, GABA agonists, and serotonin-mimetic compounds (Cummings et al. 2022).

In healthy individuals, tau is a soluble microtubule-associated protein that maintains and stabilizes microtubules to modulate neuronal signal transmission and axonal transport (Dehmelt & Halpain, 2005). The central nervous system contains six isoforms of tau, which are products from alternative splicing of a single gene, MAPT (Goedert et al., 1989; Himmler et al., 1989). Tau contains an N-terminal projection domain, a proline-rich domain, a repeat domain, and a C-terminal domain. The N-terminal domain, which depending on the isoform can have either one or two inserts, dimerizes with the N-terminus and the proline-rich domains of adjacent tau proteins to organize microtubule spacing (Rosenberg et al., 2008). The repeat domain, which has either three or four repeat sequences depending on the isoform, is the microtubule-binding region (Goedert et al., 1989; Himmler et al., 1989). The basic residues in both the proline-rich domain and repeat domain enable tau to efficiently bind to microtubules (Goode et al., 1997; Kadavath et al., 2015). The function of tau is to protect against the dissociation of tubulin along the lattice and at either ends of the microtubules, thus stabilizing microtubules (D. B. Murphy et al., 1977; Trinczek et al., 1995). Tau can get phosphorylated at more than 40 different epitopes

along the length of the protein (Drepper et al., 2020; Hanger et al., 2007; Morishima-Kawashima et al., 1995). In the normal brain, the intricate regulation of tau phosphorylation and dephosphorylation modulates the stability of the axonal cytoskeleton to regulate neurite growth (Matsuo et al., 1994). Abnormal hyperphosphorylation of tau results in destabilised microtubule networks, which is implicated in tauopathies such as frontotemporal dementia or AD. In AD, tau becomes hyperphosphorylated, causing it to become insoluble and dissociate from microtubules, forming neurotoxic tau aggregates, or neurofibrillary tangles (Arnsten et al., 2021).

The role of tau tubulin kinases in neurodegeneration

Neurotoxicity and neurodegeneration in AD are regulated by cellular responses to tau accumulation (Guthrie et al., 2009; Kraemer et al., 2006; Kraemer & Schellenberg, 2007; Sano et al., 2007; Walenta et al., 2001). Several signaling pathways can contribute to hyperphosphorylation of tau and resistance to degradation of neurotoxic tau aggregates (Obulesu et al., 2011). The abundance of hyperphosphorylated tau in neurofibrillary tangles suggests an imbalance of activity from kinases and phosphatases that act on tau. Tau is phosphorylated by multiple kinases, including glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinase 5 (Cdk5) (Arioka et al., 1993; Ishiguro et al., 1992; Takashima et al., 1995). Tau tubulin kinase-1 (TTBK1) also directly phosphorylates tau at AD-associated epitopes including AT8 (S202, T205) and S198, as well as the disease-specific epitope S422 (Dillon et al., 2020; Lund et al., 2013; Xu et al., 2010). Mutations in TTBK1 are linked to decreased risk of developing AD (Vázquez-Higuera et al., 2011; Yu et al., 2011). TTBK1 is regulated by autophosphorylation (Bao et al., 2021). Aside from

tau, TAR DNA-binding protein 43 (TDP-43) and synaptic vesicle glycoprotein 2A (SV2A) are the only other identified proteins known to be directly phosphorylated by TTBK1 (Taylor et al., 2018; N. Zhang et al., 2015). TTBK1 knockdown in mouse cortical neurons resulted in significant changes in phosphopeptide enrichment (Bao et al., 2021). This analysis revealed additional putative TTBK1 phosphorylation targets, which include proteins involved in the organisation of the neuronal cytoplasm, organisation of the cytoskeleton, and microtubule dynamics. More efforts are required to characterize these targets and determine whether they are direct or indirect targets of TTBK1 (Bao et al., 2021). Currently, pathways regulating TTBK1 expression and activity have not been described. The TTBK1 kinase domain is conserved between species, including vertebrates, *Drosophila melanogaster*, and *Caenorhabditis elegans*, suggesting normal TTBK function is biologically indispensable (Ikezu & Ikezu, 2014). Its conservation also makes it possible to study the influence of TTBK on neurodegeneration in model organisms.

Alzheimer's Disease is multifactorial

Sporadic AD makes up over 90% of all AD cases (Bertram & Tanzi, 2004; Bettens et al., 2013), and is widely considered a multifactorial disease, where along with genetic predispositions, development of the disease is influenced by the patient's environment and lifestyle (De Strooper & Annaert, 2010). In the continual pursuit of identifying potential causes and cures for AD, researchers have found many pathological events associated with disease development in addition to Aβ and tau pathology, such as oxidative stress, endoplasmic reticulum (ER) stress, insulin resistance, and changes to microbiota composition (Samanta et al., 2022). The multi-factored pathogenesis behind AD

development poses a challenge for determining cellular and genetic associations between functional pathways that cause features of disease. Further investigations are needed to understand the cause-effect relationship between the gut microbiota and neurodegeneration in AD.

The human gut microbiota

The human gastrointestinal tract is home to many microorganisms, collectively known as the gut microbiota, which can influence host health and physiology. The human gut microbiota is a complex community. It remains challenging to completely identify and characterize all the archaea, eukarya, and bacterial species present in the gastrointestinal tract (Thursby & Juge, 2017). It consists of over 1000 bacterial species, and over approximately 4 trillion bacteria, with their collectively expressed genes outnumbering our own (Sender et al., 2016). Many factors can influence microbiota composition, including infection (Thabane et al., 2010), antibiotic use (O'Mahony et al., 2014; Verdu et al., 2008), diet (Gentile & Weir, 2018), and host genetics (Qin et al., n.d.).

The gut microbiota can provide a range of benefits to hosts through numerous mechanisms, such as maintaining intestinal barrier integrity (Natividad & Verdu, 2013), assisting with metabolism (den Besten et al., 2013), and modulating resistance to infection (Bäumler & Sperandio, 2016). Thus, alterations in the composition of the gut microbiota have the potential to influence health. Efforts have been made to identify specific bacteria that benefit the host to help characterize a "healthy" microbiota. However, the fluctuation in species associated with a multitude of factors causes microbiota populations to be heterogeneous (Rothschild et al., 2018). This poses many challenges for identifying causal

mechanisms underlying diseases that are influenced by the microbiota. Overall, the complex factors that influence microbiota composition and the nature of how those changes impact host health remain understudied.

The gut-brain axis

An important component that is impacted by the environment and influences the nervous system, is the digestive tract; specifically, the resident microbes of the gut. Through the gut-brain axis, shifts in the microbiota can impact the physiology of the nervous system. The bidirectional communication between the gut and the brain occurs through many routes, including the vagus nerve (Bonaz et al., 2018; Breit et al., 2018), the enteric nervous system (Bessac et al., 2018; Carabotti et al., 2015), and the immune system (Cryan & Dinan, 2012). At the molecular level, pathways assembling the gut-brain axis are regulated through the production of metabolites by the microbes in the gut flora, which can be absorbed into the blood and affect activity of cells at the blood-brain barrier.

In recent years, it has become increasingly apparent that the human microbiota and collection of signaling pathways constructing the gut-brain axis play a pivotal role in human health and disease. Changes in microbiota composition and exacerbated gut pathology have been associated with neurodegenerative diseases including AD (Bernstein, 2017; Breit et al., 2018; C.-H. Chen et al., 2016; Naseer et al., 2014; Sampson et al., 2016; Scheperjans et al., 2015; Unger et al., 2016). Similar trends in gut pathology changes in AD patients suggest that the digestive system can influence the nervous system. A population-based study using the Taiwan National Health Research Database found that

patients with Irritable Bowel Syndrome have a 1.76-fold increased risk of developingAD (C.-H. Chen et al., 2016). Also, changes in microbiome composition have been observed in patients with Type-2 Diabetes (T2D) and obesity, and in addition, vascular effects of obesity and T2D have been argued to have a role in the development of AD (Naseer et al., 2014). However, biochemical networks connecting specific bacterial species to neurodegenerative disease have remained largely unexplored.

Studying the human microbiota to treat neurodegenerative disease

Different methods have been employed to identify members of gut microbiota communities in AD patients. 16S rRNA sequencing approaches revealed that AD is associated with a less diverse microbiota, and bacterial composition changes occur at both the taxa and genus level (Vogt et al., 2017; Zhuang et al., 2018). Reduced microbiota diversity by an age-associated increase in the abundance of *Proteobacteria* and *Erysipelotrichaceae* was also observed in a double transgenic AD mouse model that expresses human APP and PSEN1(dE9) (Bäuerl et al., 2018; Shen et al., 2017; L. Zhang et al., 2017). These findings suggest that shifts in microbial populations or increased overall microbiota diversity could reduce AD pathogenesis, and can serve as an avenue for treatment or preventative therapeutics for AD.

Existing treatment strategies for AD include suggesting patients make changes in their diets. This may include prescribing pre- or probiotics with the aim of promoting shifts in the gut microbiota towards a microbial composition that is associated with better health. Strong adherence to the “Mediterranean diet”, which has the foundation of plant-based

foods like whole grains, nuts, fruits, and vegetables, was associated with a slowed progression of Aβ accumulation in adults predisposed to AD (Berti et al., 2018). Plant-based diets such as the Mediterranean diet are correlated with increased abundance of species belonging to *Firmicutes* and *Bacteroidetes* families, which degrade carbohydrates indigestible to humans (De Filippis et al., 2016). The Mediterranean diet was also associated with increased faecal short-chain fatty acids (SCFAs) (De Filippis et al., 2016). SCFAs like butyrate, propionate, and acetate are associated with protection against a range of inflammatory diseases of the gut (Louis et al., 2014; Smith et al., 2013; Thorburn et al., 2014; Vipperla & O'Keefe, 2012). SCFAs can also cross the blood brain barrier (Oldendorf, 1973), and decreased SCFAs are associated with AD. For example, propionic, butyric, and isobutyric acids are reduced in faecal samples of double transgenic APP/PS1 AD mouse models compared to wild type (L. Zhang et al., 2017; Zheng et al., 2019). Decreased abundance of butyrate-producing gut microbiota is also associated with carrying the apolipoprotein ε4 (APOE4) AD risk allele (Tran et al., 2019). Altogether, these data suggest that increasing the concentration of accessible SCFAs to the host can play a protective role against the development of AD. This can be achieved by adopting diets that increase SCFA-producing bacteria.

Diet supplementation with different formulations of *Lactobacillus* and *Bifidobacterium* probiotic species can successfully decrease inflammation by modulating stress responses in mouse models of AD (Naomi et al., 2021). Similarly, in humans, ingestion of *Lactobacillus* and *Bifidobacterium* is associated with slowed early AD progression and improved cognition in patients (Naomi et al., 2021). This neuroprotection

and changes in neurosignaling can occur via many host mechanisms that are influenced by *Lactobacillus* and *Bifidobacterium* species products, which include neurotransmitters such as acetylcholine (Stanaszek et al., 1977), GABA (Barrett et al., 2012; Cho et al., 2007; Komatsuzaki et al., 2005; Pokusaeva et al., 2017; Siragusa et al., 2007), norepinephrine (Tsavkelova et al., 2000), serotonin (Özoğul et al., 2012), and dopamine (Tsavkelova et al., 2000). Overall, this suggests that treatment with probiotic supplementation could prevent AD progression, making it a promising therapeutic approach. However, no probiotic formulations have been approved as a treatment by major medical regulation agencies. More research is needed to better characterize how probiotic species promote neuroprotection, and to identify ways to mitigate potential negative side effects, which include reduced microbiota diversity (Grazul et al., 2016; Kabbani et al., 2017; Oliveira & Widmer, 2018; Spinler et al., 2016; Suez et al., 2018), and serotonin syndrome (Bermúdez-Humarán et al., 2019; Boyer & Shannon, 2005; Ng et al., 2018) in predisposed individuals. This is especially a concern in AD patients, who typically have additional comorbidities (Miklossy & McGeer, 2016; Suez et al., 2018).

With the debated efficacy and safety of shifting microbiota populations as a treatment for AD, studying specific members of the microbiota may be beneficial. There are few studies that have provided evidence of specific microbial effects on neurodegeneration in AD. *Porphyromonas gingivalis*, a Gram-negative pathogen in chronic periodontitis, was found present in the brain tissue of AD patients by identification of its specific toxic proteases lysine gingipain (Kgp), arginine-gingipain A (Rgp A) and arginine-gingipain B (Rgp B) (Dominy et al., 2019). Increased levels of these gingipains

in the brain tissue were correlated with increased levels of abnormally fragmented tau. Interestingly, the oral administration of a gingipain inhibitor rescued neurodegeneration of hippocampal cells in mice infected with *P. gingivalis*, demonstrating that neurodegenerative bacterial products can be a drug target to reduce neurodegeneration of the host *in vivo*. These findings support the idea that a singular resident microbe in the human body can influence neurodegeneration. However, the human microbiota is vast. Elucidating the specific pathways and interactions impacted by the gut's numerous resident bacteria remains to be challenging, and further investigations are needed.

Using *Caenorhabditis elegans* as a neurodegenerative model

Caenorhabditis elegans are free-living, soil-dwelling nematodes that have a short, hermaphroditic reproductive generation cycle of ~3 days, a short life cycle of ~3 weeks, and grow to ~1-2 mm in length in adulthood (Brenner, 1974). After being originally isolated from compost soil, geneticist Dr. Sydney Brenner laid the foundation for easily cultivating this nematode in a lab environment. The self-fertilizing nature of *C. elegans* hermaphrodites, its high fecundity (~300 progeny per animal), alongside the ability to perform genetic crosses with males, lended to the convenience and affordability of using *C. elegans* as a genetic model. *C. elegans* is also transparent, making it easy to use basic microscopy to study their physiology and notice slight phenotypic changes. In the lab, *C. elegans* populations can be maintained at ambient temperatures on agar plates seeded with a mutant B strain of *Escherichia coli* called OP50. *E. coli* OP50 is auxotrophic for uracil, causing it to grow bacterial lawns thin enough to easily visualise transparent *C. elegans* under a microscope.

Sydney Brenner used functional genomics to map the first version of the *C. elegans* genome, which included over 100 loci spanning six chromosomes (Brenner, 1974).This laid the groundwork for later genome sequencing and discovery of genes and pathways homologous to other model systems and humans. Breakthrough advances in techniques like the creation of fluorescent transcriptional reporters and RNA-interference (RNAi) drove the success of using *C. elegans* as an *in vivo* model. From there, *C. elegans*work contributed to developing the genetic and molecular framework of pathways involvedin development, pathogenesis, and ageing.

The hermaphroditic *C. elegans* nervous system is composed of 300 neurons that are grouped into two independent systems: 20 pharyngeal neurons located in the head region, and 280 somatic neurons consisting of several ganglia in the tail and a stretch of neurons along the body in a spinal cord-like ventral cord (Altun & Hall, 2005; Stefanakis et al., 2015). Sydney Brenner and Nichol Thomson first constructed the *C. elegans* wild type hermaphrodite nervous system using electron microscopy in the 1960s. Further analysis of the neural circuitry in behavioural and locomotor mutants laid the foundation for completely characterizing the synaptic connections and functions of each neuron in the simple *C. elegans* nervous system. Gene regulatory factors and transcription factor-neuronal target gene interactions are conserved between *C. elegans*, *Drosophila*, and vertebrate organisms. This makes *C. elegans* a powerful model for studying pathways involved in neurogenesis and neurodegeneration (Flames & Hobert, 2009; Hobert, 2010; Laurençon et al., 2007). In fact, at least 53% of human genes have a *C. elegans* ortholog, enabling the use of *C. elegans* as a model for experimental approaches that are not feasible

in mammalian models (Alvarez et al., 2022; Kim et al., 2018). This led to the development of numerous *C. elegans* models of neurodegenerative diseases, such as transgenic strains that overexpress human polyglutamates (polyQ) to study Huntington's disease (Faber et al., 1999, 2002), α-synuclein to study Parkinson's disease (PD) (Cao et al., 2005; Lakso et al., 2003), and Aβ or tau to study AD (Alexander et al., 2014).

Despite the consistent presence of Aβ plaques across all AD patients, the specific molecular role of Aβ peptides and oligomers in AD remains unclear. In *C. elegans*, overexpression of transgenically introduced genes encoding features of AD, human Aβand tau protein aggregates, also lead to AD-related phenotypes and neurodegeneration (Ayyadevara et al., 2015; Fatouros et al., 2012). Cellular stress pathways are commonly induced in response to Aβ-induced cytotoxicity. Changes in stress-related gene expression have been observed in *C. elegans* models of AD, including mediators of the p38/PMK-1/MAPK innate immune response (Brehme et al., 2014; Dimitriadi & Hart, 2010; Stringham et al., 1992) and insulin-like signaling pathway (IlS) (Cohen et al., 2006; Kimet al., 2007; C. T. Murphy et al., 2003; Parker et al., 2005; Singh & Aballay, 2006; Tullet, 2015). Altogether, this allows us to study elements of human disease in a simpler and relevant model.

Using *C. elegans* as a model for studying the gut-brain axis

The gut-brain axis is composed of complex signaling pathways that influence the systemic production and secretion of hormones and neurotransmitters that can act on the nervous system. In humans, signaling molecules such as dopamine or serotonin can be derived from the gastrointestinal tract, and members of the gut flora can both directly

produce and modulate endogenous levels of these through stimulating enteroendocrine cells (Bertaccini, 1960; Clarke et al., 2014; Dinan & Cryan, 2017; Eisenhofer et al., 1997; Yano et al., 2015). However, the specific bacteria-host interactions and resulting neurosignaling mechanisms impacting neurodegeneration are still largely undescribed.

To investigate the impact of the human microbiota, high-throughput methods must be employed to allow efficient screening and analysis of the large number of bacterial mechanisms involved. Compared to the use of gnotobiotic mice, studying host-microbe interactions using *C. elegans* has proven to be advantageous. Neuroendocrine signaling also occurs in *C. elegans*, and receptors for microbiota-regulated neurotransmitters like dopamine and serotonin are conserved in the worm (Chase, 2007; Suo et al., 2004). The comprehensive mapping of the *C. elegans* connectome allowed well-logged characterisation of phenotypes associated with neurosignals and genes. Further, the relatively simple biology of *C. elegans* makes it possible to study host-gut mechanisms to an extent that is not yet possible in other model organisms. Large populations of age-synchronised germ-free animals can be easily generated by hypochlorite treatment to disintegrate animals, leaving behind embryos, which are bleach resistant. We can then selectively control the bacterial exposure of hatched larvae to study the effects of specific bacteria on the host. The transparent *C. elegans* body allows easy visualisation of fluorescently labelled bacteria to study real-time intestinal passage and colonisation (Hsiao et al., 2013; Rezzoagli et al., 2019).

Its bacterivorous nature makes *C. elegans* an appropriate model organism for identifying and characterizing the neuroactive potential of the gut microbiota and metabolome. The few studies that use *C. elegans* to study the effects of gut bacteria on the nervous system have almost exclusively been performed in models of PD (S. G. Chen et al., 2016; Goya et al., 2020; Ray et al., 2014). Signals from the gastrointestinal tract originating from the microbiota can influence PD progression. Preclinical studies revealed that probiotic supplementation is associated with improved symptoms in patients (Braak et al., 2003; Castelli et al., 2020; Klann et al., 2022; Scheperjans et al., 2015). In a *C. elegans* model of PD, which overexpresses aggregated human α-synuclein in the body wall muscle, the probiotic strain *Bacillus subtilis* PXN21 strain inhibited α-synuclein aggregation and cleared toxic aggregates in a DAF-16/FOXO-dependent manner (Goya et al., 2020). This was mediated by bacterial biofilm formation and metabolite production in the worm gut (Goya et al., 2020). *B. subtilis* biofilm formation also confers stress resistance and enhanced longevity in *C. elegans*, which is evidence that bacterial diets can have systemic effects (Donato et al., 2017; Smolentseva et al., 2017). A metabolite produced by *Streptomyces venezuelae* induced age- and dose-dependent dopaminergic neuron degeneration in the *C. elegans* Leucine Rich Repeat Kinase 2 (LRRK2) G2019S mutant PD model, providing evidence that bacterial products can influence neurodegeneration in *C. elegans* (Ray et al., 2014). Importantly, the translatability of findings in *C. elegans* to mammals was demonstrated when bacterial curli proteins induced α-synuclein aggregation in *C. elegans* and caused the same effect in a mammalian model (S. G. Chen et al., 2016).

The impact of the gut microbiota on development or progression of neurodegenerative diseases involves numerous complex host processes, including an

immune response (S. G. Chen et al., 2016; Sampson et al., 2016, 2020). *C. elegans* lacks an adaptive immune system but has an innate immune system to defend itself against pathogens (Oikonomou & Shaham, 2011). Although this means we cannot use *C. elegans* to study all aspects of disease pathology, AD-associated protein interaction networks are still conserved (Wang et al., 2021). Therefore, *C. elegans* is still a valuable starting model for the identification of bacteria, genes, and metabolites that influence neurodegeneration. The foundation laid by *C. elegans* work can help inform novel strategies for medical intervention of multifactorial neurodegenerative diseases.

Central hypothesis and summary of study aims

These complex mechanisms of communication between the digestive and nervous systems are still being explored, and gene expression and impacts of involved signaling pathways mediating this relationship requires further elucidation. This project's main goal was to identify microbiota species that may impact AD and understand how they have an effect using *C. elegans* as a model. We hypothesise that bacteria isolated from the human microbiota can influence neurodegeneration through regulation of signaling pathways that mediate communication between the gut-residing bacteria and the nervous system in *C. elegans*. This study focused on characterizing the changes in host gene expression and signaling pathways, allowing us to identify what processes were regulated by the microbiota, and changes in gene expression that promoted neurodegeneration. Further, given the abundance of bioactive molecules in bacteria, the discovery of neuroprotective compounds produced by species from the human microbiota may lead to novel therapeutic approaches and compounds. Overall, we proposed to identify host mechanisms thatregulate

neuroprotection, and microbial factors that contribute to neuroprotection.

Summary of studies

1) Identified changes in *C. elegans* gene expression associated with protection against neurodegeneration through RNA-Seq analysis of wild-type and tau aggregate-prone strains to compare changes based on both exposure to bacteria and presence of tau aggregates (Chapter 2).
2) Investigated if microbiota species impact neurodegeneration by mechanisms involving tau phosphorylation and pathways involved in ageing and lifespan (Chapter 2).
3) Tested neuroprotective capacity of bacterial extracts in *C. elegans* to identify potentially neuroprotective metabolites (Chapter 2).
4) Identified novel mediators of neurodegeneration by performing an RNAi screen of genes that were suppressed by neuroprotective bacteria (Chapter 3).

CHAPTER TWO - Human microbiota bacteria can protect against neurodegeneration by reducing phosphorylation of tau

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Declaration: Research presented as part of this chapter has been prepared for publication.

Contributions: AT performed NGM paralysis assay and preliminary work leading to development of this project. KBP, TL, and AM performed sequencing analyses. KBP and SC made the bacterial extracts. KBP performed all other experiments. MGS provided bacterial isolates. KBP and LTM designed the experiments and wrote the manuscript. KBP made the figures.

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Abstract

Alzheimer's Disease (AD) is the most common neurodegenerative disorder. The multi-factored pathogenesis that leads to AD development poses a challenge for identifying causal factors that promote or protect against neurodegeneration. Using an Aβ-overexpressing *C. elegans* model, we identified *Enterobacteriaceae* species that significantly reduce paralysis. In another *C. elegans* model that pan-neuronally expressed human aggregate-prone tau, we observed most of the same bacteria also decreased neurodegeneration. Examination of host gene expression changes revealed numerous biological processes that were differentially regulated in response to the neuroprotective microbiota species, including innate immunity, stress responses, and protein phosphorylation. Notably, several *C. elegans* orthologs of human tau tubulin kinase isoforms, TTBK1 and TTBK2, were downregulated in response to neuroprotective microbiota species. RNAi-mediated knockdown of *C. elegans ttbk* genes significantly induced neuroprotection and rescued locomotor speed in tau aggregate-prone animals. These findings suggest that bacterial species from the human microbiota can mediate neuroprotection through down-regulation of *ttbk*. *Enterobacter* diets also decreased levels of an htau species phosphorylated at S422, an AD-specific epitope that is phosphorylated by TTBK1. Overall, by studying the impact of the human microbiota on models overexpressing Aβ or aggregate-prone tau, we have uncovered a potential mechanism by which microbiota-mediated neuroprotection can occur. In doing so, we also gain a greater understanding of conserved pathways involved in gene-environment interactions promoting development of AD.

Introduction

Alzheimer's Disease (AD) is the most common neurodegenerative disorder, affecting more than 46 million people worldwide, and the incidence rate continues to increase with aging populations (Wortmann, 2015). The most common form, called sporadic AD, is a multifactorial disorder with genetic and environmental factors both contributing to the disease (De Strooper, 2010). Several studies have reported correlations between microbiome composition or gut pathology and disease state in neurodegenerative disorders (Bäuerl et al., 2018; Chen et al., 2016; Naseer et al., 2014; Sampson et al., 2016; Scheperjans et al., 2015; Shen et al., 2017; Unger et al., 2016; Vogt et al., 2017; L. Zhang et al., 2017; Zhuang et al., 2018). However, it is still unclear whether these changes contribute to neurodegeneration or whether they are a product of the disease.

The human microbiota is estimated to consist of over 1000 species and trillions of bacteria, with their collectively expressed genes outnumbering our own (Sender et al., 2016). The complexity of the microbiota makes elucidating specific pathways impacted by the gut's resident bacteria challenging. Therefore, biochemical mechanisms connecting specific bacterial species to neurodegenerative disease have remained largely unexplored.

Human-associated bacteria can exist in biofilms, which contribute to theirgrowth and survival (Deo & Deshmukh, 2019). *Enterobacteriaceae* species are able to produce biofilms (Da Re & Ghigo, 2006; Zogaj et al., 2003). Curli proteins within bacterialbiofilms are found in gastric infections and disease but are also found in biofilms of commensal bacteria (Tytgat et al., 2019). In biofilms produced by *E. coli*, the amyloid curli

protein plays an essential role in constructing the biofilm, therefore comprising a large proportion of proteins in the extracellular matrix (Hung et al., 2013; McCrate et al., 2013; Olsén et al., 1989). Bacterial amyloid curli proteins are structurally similar to human amyloids, and the human immune system can also recognize bacterial amyloids through the same receptors that recognize human amyloid (Cheng et al., 2008; Fowler et al., 2007; Liu et al., 2012; Maury, 2009). Increasing amounts of evidence suggest that interactions between microbial and eukaryotic amyloids may drive the initiation of neurodegeneration. For example, colonisation of a murine Parkinson's Disease (PD) model with curli-expressing *E. coli* increases toxic alpha-synuclein aggregation (Sampson et al., 2016, 2020). Although it remains unclear if bacterial curli directly increase alpha-synuclein seeding or neurodegeneration, it is possible that altering levels of microbiotal factors like amyloid may be an avenue for treatment of neurodegenerative disease.

While the effects of probiotic (Goya et al., 2020; Naomi et al., 2021) and laboratory bacterial strains have been examined in different models of neurodegeneration, the effects of commensal residents of the human body have not. We use *Caenorhabditis elegans* as a model of AD and other tauopathies to measure the impact of human microbiota species on AD-related phenotypes. Despite the consistent presence of amyloid-β (Aβ) plaques across all AD patients, the specific molecular role of Aβ peptides and oligomers in AD remains unclear. Cellular stress pathways are commonly induced in response to Aβ-induced cytotoxicity, and changes in stress-related gene expression have been observed in *C. elegans* models of AD, including regulation of genes encoding heat-shock proteins (Brehme et al., 2014; Dimitriadi & Hart, 2010; Stringham et al., 1992) as well as genes

involved in and downstream of the insulin-like signaling pathway (E. Cohen et al., 2006; Kim et al., 2007; Murphy et al., 2003; J. A. Parker et al., 2005; V. Singh & Aballay, 2006; Tullet, 2015).

In *C. elegans* models of AD, neurotoxicity and neurodegeneration can be regulated through alteration of downstream cellular responses to tau accumulation (Guthrie et al., 2009; Kraemer et al., 2006; Kraemer & Schellenberg, 2007; Krämer & Phistry, 1999; Sano et al., 2007; Szebenyi, Hall, et al., 2007; Szebenyi, Wigley, et al., 2007; Walenta et al., 2001). Signalling between multiple pathways, such as the Wnt signaling pathway and oxidative stress response can also alter levels of tau-induced aggregates and neurodegeneration (An et al., 2005). A recent study has suggested that bacteria can affect neurodegeneration in AD. *Porphyromonas gingivalis*, a Gram-negative pathogen in chronic periodontitis, was present in the brain tissue of AD patients (Dominy et al., 2019). Increased levels of gingipains in the brain tissue were correlated with increased levels of abnormally fragmented tau, supporting the idea that resident microbes in the human body can alter mechanisms that cause neurodegeneration.

Tau can be processed and modified in numerous ways to become neurotoxic. Under normal circumstances, human tau undergoes different post-translational modifications (PTMs) to aid in its role of binding to and stabilizing neuronal microtubules (Wegmann et al., 2021).Tau can become phosphorylated at more than 40 different epitopes along the entire length of the protein (Drepper et al., 2020; Hanger et al., 2007a; Morishima-Kawashima et al., 1995a). The abundance of hyperphosphorylated tau in neurofibrillary tangles suggests an

imbalance of activity from kinases and phosphatases that act on tau. Tau is phosphorylated by multiple kinases, including glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinase 5 (Cdk5) (Arioka et al., 1993; Ishiguro et al., 1992; Takashima et al., 1995). Tau tubulin kinase-1 (TTBK1) also directly phosphorylates tau at AD-associated epitopes AT8 (S202, T205) and S198, as well as the disease-specific epitope S422 (Dillon et al., 2020a; Lund et al., 2013; Xu et al., 2010). However, TTBK1 regulators and substrates have not been comprehensively studied. Targeting TTBK1 may have therapeutic potential because mutations in TTBK1 are linked to decreased risk of developing AD (Vázquez-Higuera et al., 2011; Yu et al., 2011). Its kinase domain is conserved between species, including vertebrates, *Drosophila melanogaster*, and *C. elegans*, enabling the future characterization of TTBK1 in fundamental AD research (Ikezu & Ikezu, 2014).

Tau also undergoes other PTMs, including ubiquitination,acetylation, methylation, and SUMOylation, and the abnormal abundance of these modifications can prevent normal tau function (Alquezar et al., 2020; Wegmann et al., 2021). In fact, acetylation, methylation, and SUMOylation can promote tau aggregation and the formation of neurofibrillary tangles (Balmik & Chinnathambi, 2021; T. J. Cohen et al., 2011; Luo et al., 2014). These observations emphasise the importance of characterizing the host mechanisms that act on tau to investigate biological processes that influence neurodegeneration.

Here, we show that diets of clinical bacterial isolates from the human gut reduce neurodegeneration in *C. elegans* AD models that overexpress human Aβ or aggregate-

prone forms of tau. Extracts of these bacteria also induce neuroprotection, suggesting the effect is caused by production of a metabolite. Despite being neuroprotective, these bacteria shortened lifespan in *C. elegans*, and we show that this occurs independently of p38/PMK-1 signaling. Transcriptomic analysis revealed that the neuroprotective microbiota species alter several host biological processes, including stress response and phosphorylation. Orthologs of human TTBK1 and TTBK2 were downregulated, and we demonstrated that suppression of individual TTBK genes sufficiently induced neuroprotection in *C. elegans* expressing aggregate-prone tau (AP-tau). Further, microbe-induced neuroprotection may occur through reducing abundance of a tau species phosphorylated at S422, an AD-specific epitope and direct TTBK1 phosphorylation site. Phosphorylation of S422 occurs early in disease, before the appearance of NFTs, and is strongly correlated with disease pathology (Vana et al., 2011). Altogether, our findings suggest that microbiota species from the human gut can modulate host responses to influence tau pathology and neurodegeneration in *C. elegans*.

Results

To examine the effects of bacteria from the human microbiome on neurodegeneration, we exposed *C. elegans* to species of bacteria found in the human microbiota. To account for potentially fastidious bacteria, we use a diluted version of brain heart infusion (BHI) media. We assessed the impact of members of the family *Enterobacteriaceae* on phenotypes in *C. elegans* models of AD. We first measured paralysis in a model of Aβ toxicity where a strong muscle-specific promoter and

temperature-inducible system are used to express the human 42 amino acid amyloid beta peptide (C. Link, 2003). We observed decreased paralysis in *C. elegans* populations grown on *Enterobacter cancerogenus*, *Enterobacter hormaechei*, *Klebsiella pneumoniae*, *Citrobacter freundii* and two human isolates of *E. coli* compared to populations grown on *E. coli* OP50 after 72 hours of Aβ overexpression (Figure 1A). To verify that protection was not the result of decreased transgene expression, we exposed animals carrying a transgene with identical construction,but expressing a GFP reporter in lieu of Aβ (C. D. Link et al., 2006), to each of the bacterialstrains. GFP expression was not decreased in response to the protective strains (Supplementary Figure 1), suggesting that decreased paralysis was not a result of altered transgene expression.

To explore the nature of the protection observed, we asked whether exposure to protective bacteria was required throughout development or whether exposure later in life was sufficient to reduce paralysis. Animals were grown on *E. coli* OP50 until they reached L3 and were then transferred to test bacteria before shifting plates to 25˚C to induce expression of Aβ. Paralysis was examined at 48 and 72 hours post-upshift. Although the magnitude of the protection was reduced, the ability to promote protection in this experiment suggests that the protection is not due to a developmental effect on the worms (Figure 1B).

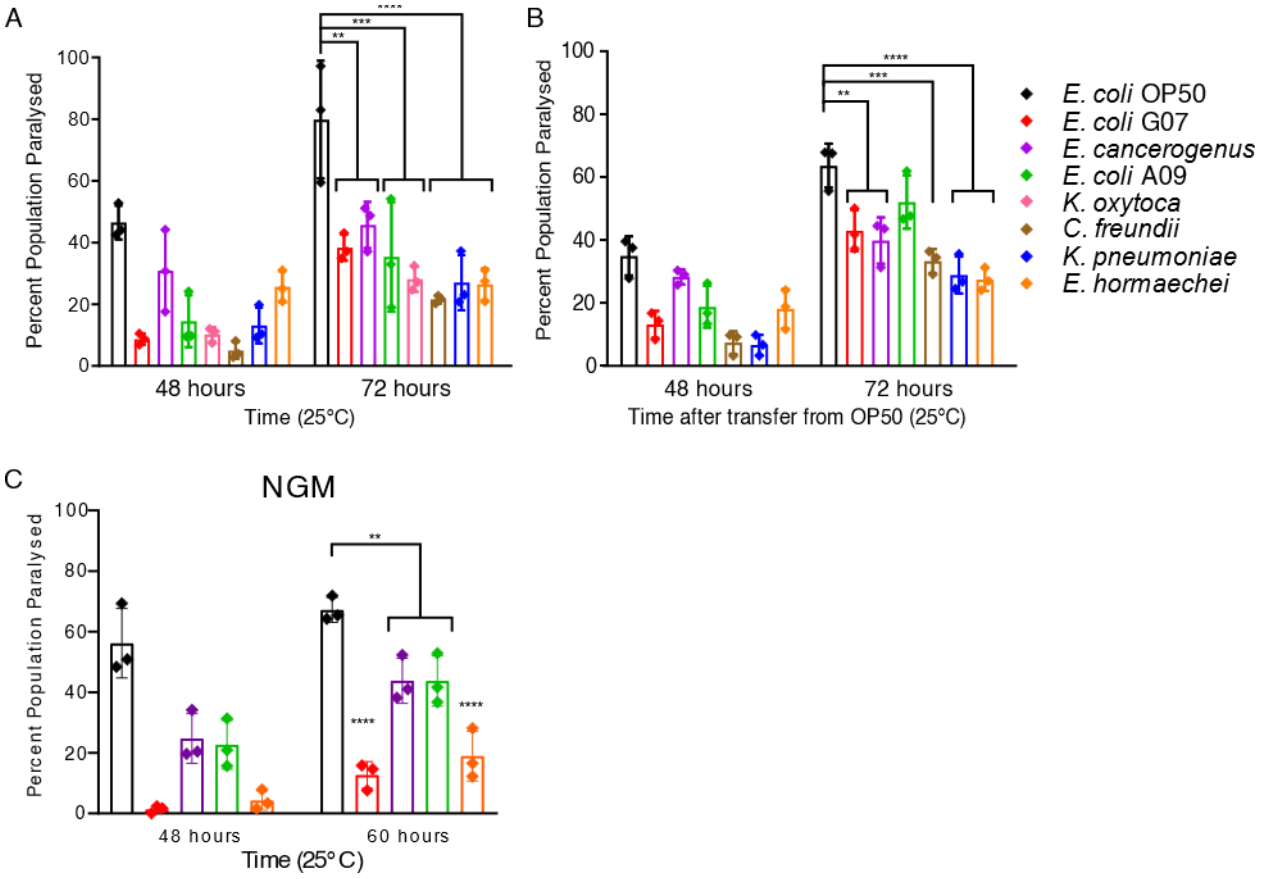


Figure 1. Microbiota species reduce paralysis in Aβ42-overexpressing *C. elegans*. A. Severity of defects in a model of AD is impacted by bacterial exposure. Percent population paralyzed at 48 and 72 hours post induction of Aβ42 expression in response to indicated bacteria. **B.** Percent population paralyzed at 48 and 72 hours post induction of Aβ42 in animals exposed to indicated bacteria only post-L3. **C.** Severity of defects in a model of AD is impacted by bacterial exposure on NGM. Percent population paralyzed at 48 and 60 hours post induction of amyloid beta expression in response to indicated bacteria. One-way ANOVA performed with post-hoc Dunnett's multiple comparisons test, OP50 as control. ****=p<0.0001, ***=p<0.001, **=p<0.01. Bars represent standard deviation in three separate trials. Each data point represents a population of >60.

Additionally, we found that *Enterobacter* and human-isolated *E. coli* strains were still protective when animals were grown on the standard nematode growth media (NGM) (Figure 1C), as well as the richer BHI medium (Figure 1A and 1B). This demonstrates that protection against paralysis is sufficiently induced even when the isolates are grown on a

more limiting medium, and the bacteria can produce adequate amounts of paralysis-reducing factors in different growth conditions.

Neuroprotection is observed in an independent model of tauopathy

To validate our findings, we used a second, independent tau-based model of neurodegeneration, BR5706 (AP-tau animals) (Fatouros et al., 2012). Although tau mutations are not observed in AD, tau aggregation is a defining feature of AD. BR5706/AP-tau animals pan-neuronally express two mutant, aggregate-prone forms of human tau (F3 0N4R ΔK280 and 1N4R V337M) (Figure 2A). The V337M and ΔK280 mutations are associated with frontotemporal dementia with parkinsonism (FTDP-17), a tauopathy that shares pathology with AD (Alonso et al., 2004; Hutton, 2000; Y. P. Wang et al., 2007). The tau F3 fragment is prone to forming β-sheet structures while also inducing nucleation of full-length mutant tau, and this aggregation is exacerbated by the ΔK280 mutation (Y. Wang et al., 2009; Y. P. Wang et al., 2007). This *C. elegans* model recapitulates several features of AD, including tau aggregation, synaptic loss and neurodegeneration (Fatouros et al., 2012).

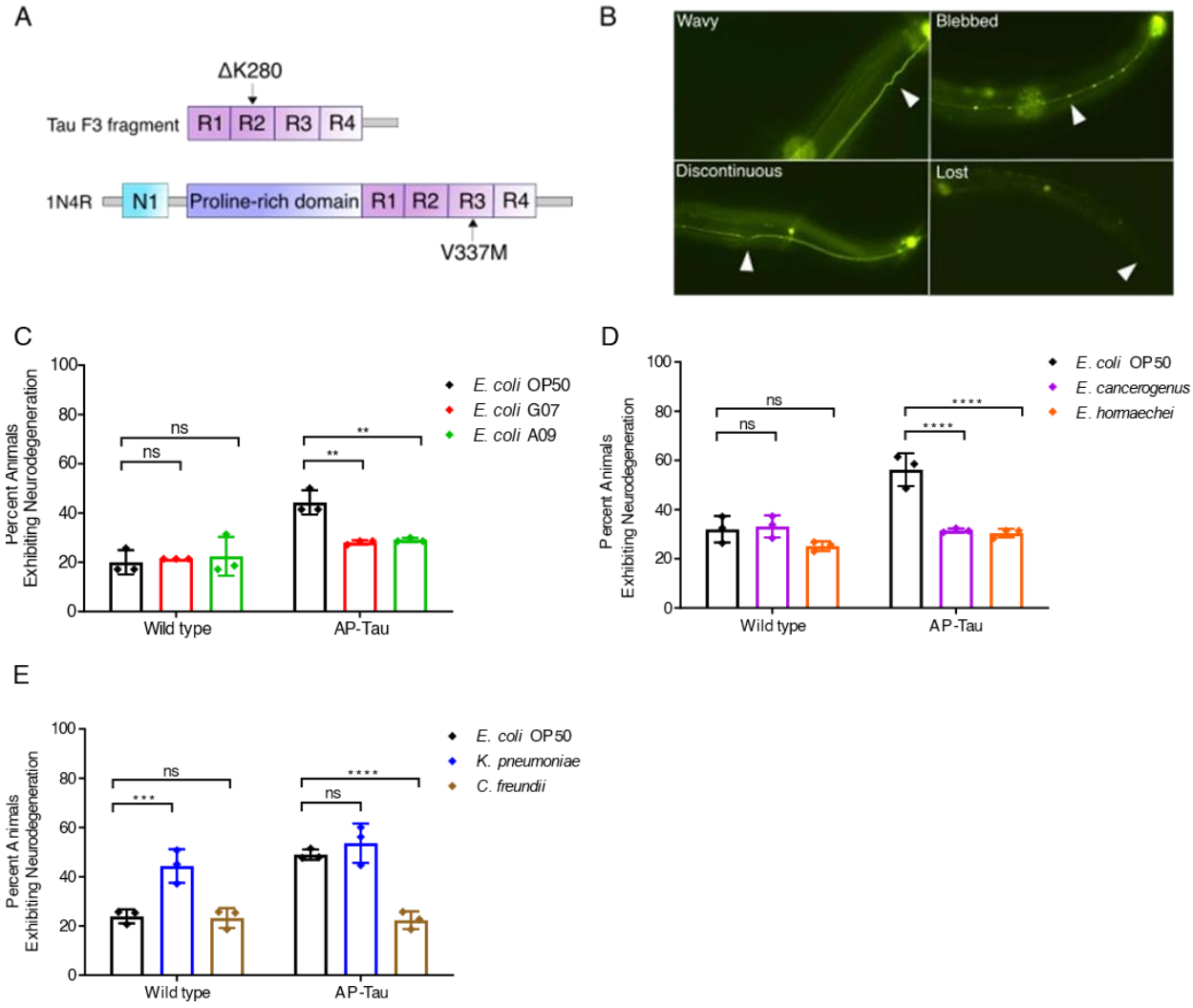


Figure 2. Microbiota species induce neuroprotection in *C. elegans*. A. Tauopathy model used to examine effects of neurodegeneration. Both forms of human tau are expressed from pan-neuronal promoters. **B.** Neurodegeneration of PLM neurons in AP-tau animals. *mec-7p::GFP* is used to visualize the PLM neurons. Arrows indicate signs of neurodegeneration. Axons are scored as wavy when multiple ripples are observed, blebbed when multiple focal swells are observed, discontinuous when a break is found along the axon and cell death is recorded when the cell body is absent. Anterior is left in all photos. **C-E.** Neurodegeneration in wild-type animals was not affected by exposure to human microbiota species. Neurodegeneration was decreased in AP-tau animals grown on microbiota-isolated *E. coli*, *E. cancerogenus*, *E. hormaechei*, or *C. freundii* relative to those grown on *E. coli* OP50. *K. pneumoniae* did not induce neuroprotection in AP-tau animals. For Figures 2C-E, one-way ANOVA performed with post-hoc Dunnett's multiple comparisons test, with respective *E. coli* OP50 as control for each strain. **= p<0.01, ***=p<0.001, ****=p<0.0001. Bars represent standard deviation of the mean. Each graph point represents a population of >60 animals from separate trials scored on different days.

We monitored neurodegeneration of the posterior lateral microtubule (PLM) neurons in day one adults. These neurons undergo age-dependent neurodegeneration even in wild-type animals and have long, easy-to-visualise axons (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). Cell loss and abnormal axonal morphology, including discontinuous, wavy, or blebbed axons, were considered features of degeneration (Wu et al., 2015) (Figure 2B). Approximately 50% of the AP-tau animals fed *E. coli* OP50 showed signs of neurodegeneration at day 1 of adulthood (Figure 2C, 2D, 2E). By contrast, AP-tau animals exposed to *E. cancerogenus*, *E. hormaechei*, or *C. freundii* displayed low levels of neurodegeneration that were similar to those observed in wild type (Figure 2D and 2E). Despite inducing protection against paralysis in the Aβ model, exposure to *K. pneumoniae* increasedneurodegeneration in wild-type animals and did not influence degeneration levels in AP- tau animals (Figure 2E). With the exception of *K. pneumoniae*, bacteria that were protective in the Aβ model were also protective in the AP-tau model.

Neuroprotective bacteria reduce lifespan independently of p38/PMK-1 activation

Since neurodegeneration is a disease of aging, we asked whether conditions that promote neuroprotection also increase lifespan in *C. elegans*. Using *glp-1(g60)* temperature-sensitive sterile animals, we measured lifespan in response to protective bacteria (Figure 3A and Supplementary Table 1). Interestingly, all bacterial strains that were protective in either model of neurodegeneration significantly reduced lifespan in *glp-1(g60)* animals relative to animals grown on *E. coli* OP50. We observed a similar effect in

the AP-tau animals, which have a shortened lifespan that was further decreased in response to the test bacteria when compared to animals fed *E. coli* OP50. (Figure 3A).

While neuroprotective effects induced by probiotic bacteria are associated with an increased lifespan (Donato et al., 2017; Goya et al., 2020; Smolentseva et al., 2017), the opposing effects on survival and neurodegeneration we observed could be consistent with reduced innate immune activation through p38/MAPK. Increased activation of the p38 MAPK, PMK-1, is associated with increased neurodegeneration, and loss of *pmk-1* can reduce neurodegeneration in certain contexts (E. Cohen et al., 2006; E, Lezi, et al., 2018; Vérièpe et al., 2015). However, loss of *pmk-1* shortens lifespan (Park et al., 2018; Portal-Celhay et al., 2012). In our model, if bacteria decreased p38 activation, this could reduce neurodegeneration while decreasing lifespan. We first examined the role of PMK-1 in lifespan determination by investigating whether bacterial exposure would further shorten lifespan in the absence of PMK-1. Lifespan of *pmk-1* mutants grown on either *Enterobacter* strain, *C. freundii*, or *K. pneumoniae*, was shorter than that of *pmk-1* worms grown on *E. coli* OP50 suggesting that the decreased lifespan induced by *Enterobacter* is not the result of *pmk-1* modulation (Figure 3B).

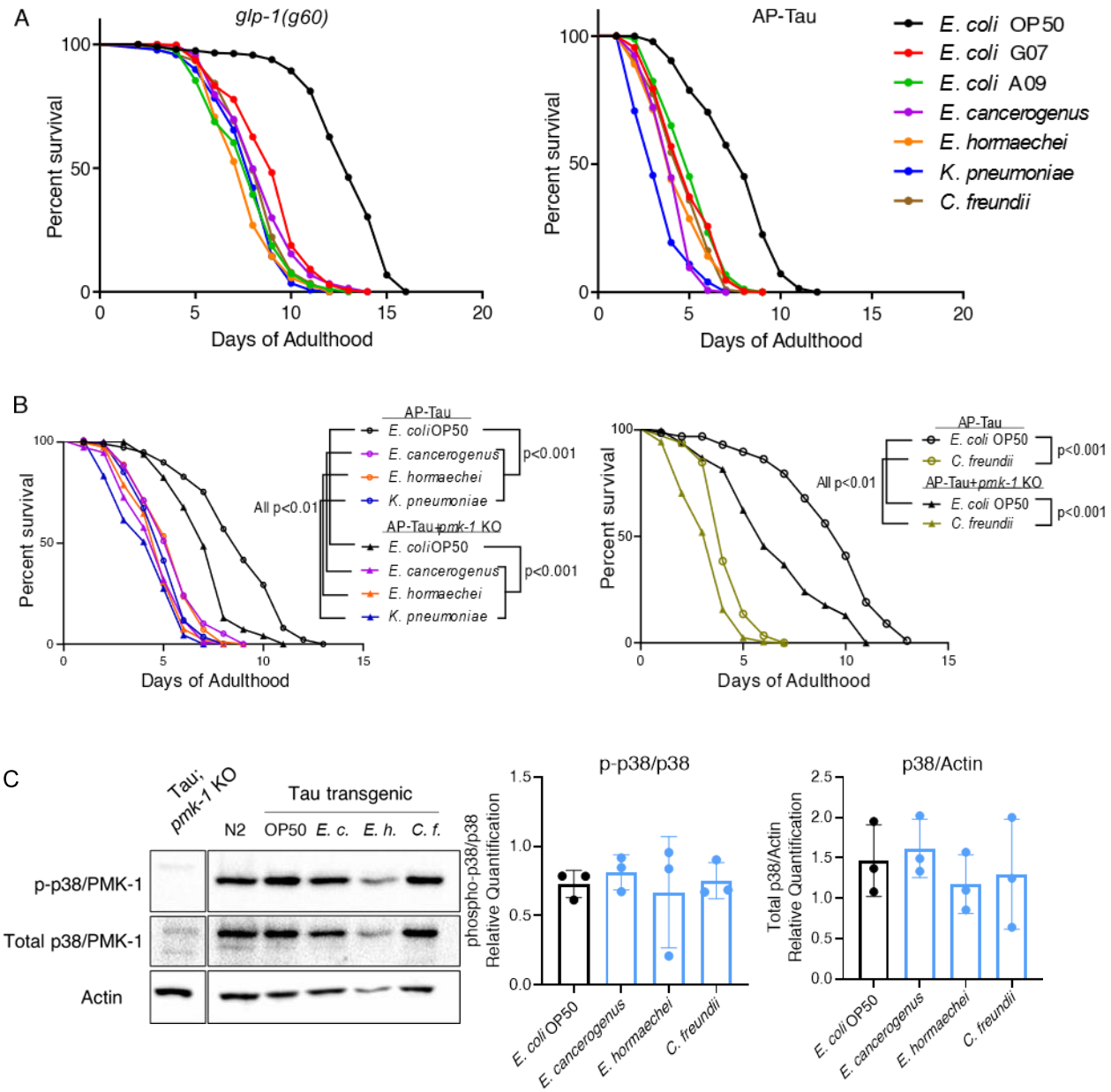


Figure 3. Neuroprotective microbiota species reduce lifespan independent of p38/PMK-1 in *C. elegans*. **A.** Survival of temperature-induced sterile (GG60 strain) *C. elegans* populations of >100 animals exposed to human-isolated bacteria, with *E. coli* OP50 as control (Left). All microbiota species protective against Aβ-induced paralysis also significantly reduced lifespan ($p<0.001$, Log-rank test performed with Bonferroni correction). (Right) Survival of AP-tau *C. elegans* populations of >100 animals exposed to human-isolated bacteria, with *E. coli* OP50 as control. All microbiota species tested significantly reduced population lifespan ($p<0.001$, Log-rank test performed with Bonferroni correction). Time 0 = L4 larval stage, live and dead animals were counted every day until the end of experiment. **B.** Survival of AP-tau *C. elegans* and AP-tau *pmk-1* null populations of >100 animals exposed to human-isolated bacteria, with *E. coli* OP50 as control. All microbiota species tested significantly reduced population lifespan ($p<0.001$, Log-rank test performed with Bonferroni correction). Time 0 = L4 larval stage, live and dead animals were counted every day until

the end of the experiment. **C.** Representative Western blot of phospho-p38/PMK-1 in AP-tau *C. elegans* fed *E. coli* OP50, or neuroprotective diets. Levels of phosphorylated p38/PMK-1 were unchanged. Lysate from N2 wild-type animals were used as positive control, and lysate from *pmk-1* null AP-tau *C. elegans* was used as negative control. One-way ANOVA performed with post-hoc Dunnett's multiple comparisons test using *E. coli* OP50 as control. E. c. = *E. cancerogenus*, E. h. = *E. hormaechei*, and C. f. = *C. freundii*.

Further, we measured PMK-1 expression and activation in response to all bacterial diets. Exposure to the neuroprotective diets did not alter levels of activated PMK-1 in AP-tau animals compared to a diet of *E. coli* OP50 (Figure 3C). This indicates that the reduced levels of neurodegeneration and shortened lifespan in response to the *Enterobacter*s and *C. freundii* likely occurs independently of PMK-1.

Bacteria-induced changes in gene expression in AP-tau *C. elegans*

To investigate how exposure to different bacteria might influence neurodegeneration, we performed global gene expression analysis by RNA-Seq in both wild-type and AP-tau animals exposed to *E. coli* OP50, *E. cancerogenus, E. hormaechei, C. freundii* and *K. pneumoniae*. To understand the effects that neuroprotective and neurodegenerative bacteria have on gene expression in a model of neurodegenerative disease, we compared gene expression in AP-tau animals provided a test bacterial diet or the control diet *E. coli* OP50.

Relative to AP-tau animals grown on *E. coli* OP50, the expression of 986 genes was increased and 713 decreased by exposure to any neuroprotective diet (Supplementary Figure 2). Of the upregulated genes, 290 genes were changed in response to at least two diets, with 84 genes upregulated in response to all three diets. 395 genes were uniquely

upregulated in response to *C. freundii*. Of the 713 downregulated genes, 94 were changed in response to at least two neuroprotective bacteria, with the largest overlap being in the genes that were downregulated by both *Enterobacter*s. We also found that most of the downregulated genes were uniquely downregulated in response to *E. cancerogenus*.

Using WormCat (Holdorf et al., 2020) to identify enriched gene categories, we found there are several common WormCat categories that were enriched among the genes that were upregulated in response to not only neuroprotective diets, but also *K. pneumoniae* when compared to *E. coli* OP50 (Supplementary Table 2). We saw enrichment for the categories "Proteolysis Proteosome: E3: F box" and "Stress response: heat" in response to all three neuroprotective bacteria and *K. pneumoniae*, suggesting that tau aggregation may promote a universal proteolysis and heat stress response on all diets when compared to *E. coli* OP50. The category "Stress response" was also enriched in the downregulated gene sets in response to all neuroprotective bacteria but not *K. pneumoniae*, although the types of stress responses differed between neuroprotective diets. Downregulated genes in response to *E. cancerogenus* and *E. hormaechei* were enriched for "Stress response: detoxification". From the genes involved in detoxification, a diet of *E. cancerogenus* specifically caused enrichment of "ugt", or UDP-glucuronosyltransferases, while *E. hormaechei* caused enrichment of "cyp", or Cytochrome P450s. *C. elegans* UGTs and CYPs are classes of enzymes that have the conserved function of neutralising consumed toxic xenobiotic compounds (Lindblom & Dodd, 2006). The downregulation of UGTs and CYPs suggests that the *Enterobacter* diets suppress the ability of AP-tau *C. elegans* to respond to the intake or accumulation of xenobiotics, although it is unclear if this could be

related or causal to the neuroprotection that we've observed. It is possible that the neuroprotective diets downregulating genes involved in these stress responses triggers a compensating upregulation of genes encoding heat shock proteins involved in the unfolded protein response, and F-box proteins involved in the ubiquitination of proteins that undergo proteasomal degradation (Kipreos & Pagano, 2000). F-box proteins are substrate-recruiting components of the Skp, Cullin, F-box (SCF)-complex that mediate ubiquitination and subsequent degradation of proteins. Tau can be modified by ubiquitination and this modification promotes its degradation. The increase in heat shock proteins and ubiquitin-mediated protein degradation could therefore cause clearance of tau aggregates and lead to neuroprotection.

Many more categories were enriched in the downregulated genes in AP-tau animals provided a non-neuroprotective diet of *K. pneumoniae*, and the impacted biological processes appear relatively diverse compared to the responses to the other bacteria. Notably, *K. pneumoniae* causes enrichment of stress response in the upregulated gene set, but different from the response to neuroprotective diets, we do not see enrichment for stress response from the downregulated genes. We also found upregulation of F-box proteins and heat shock proteins in response to a non-neuroprotective diet of *K. pneumoniae*. It is possible the suppression of other stress response genes is required for heat shock proteins and ubiquitin-mediated protein degradation to effectively reduce the load of neuronal tau aggregates to decrease neurodegeneration. These findings emphasise the difference in the effects *K. pneumoniae* has on neurodegeneration compared to the other bacteria in AP-tau animals.

Bacteria-induced changes in gene expression in wild-type *C. elegans*

One caveat of examining gene expression in populations of animals with different degrees of neurodegeneration is the potential for changes in gene expression to be a result of the neurodegeneration itself rather than a cause of these differences. To address this issue, we compared gene expression in response to the same bacteria in wild-type N2 *C. elegans*. In addition to our initial RNA-seq experiment, we performed a second, independent experiment to obtain a high-confidence set of genes whose expression changed in response to each bacterial diet (Figure 4, Supplementary Table 3). Altogether, 1040 genes were significantly changed in response to at least one protective bacterial diet (Figure 4A), with the most changes occurring in response to *C. freundii*. Few (29) genes were changed in response to all three protective bacterial diets (Figure 4A) and no significantly enriched functional categories were found for these genes.

Although exposure to *Enterobacter* species and *C. freundii* decreased neurodegeneration, it is not clear whether they accomplish this through the same process. In our WormCat analysis of the gene expression datasets, we observed some of the same enriched biological processes, although they were not always representative of the same gene set. Category changes in response to *Enterobacter* species were more similar to each other than to those observed with *C. freundii* (Figure 4B, Supplementary Table 4). This limited overlap may suggest that the mechanism of protection differs between bacterial species or alternatively, that the protection is mediated through the same process, but by inducing changes in different genes. Consistent with the latter scenario, we observed

enrichment of similar and overlapping categories between datasets (Figure 4B). Genes that were upregulated in response to either *E. cancerogenus* or *C. freundii* were enriched for stress response, and genes upregulated in response to *E. hormaechei* and *C. freundii* were enriched for terms related to proteolysis. Downregulated genes in response to *E. cancerogenus* and *E. hormaechei* were enriched for transmembrane transporters, and terms related to signaling and phosphorylation. Stress response and proteolysis were enriched from genes downregulated in response to *C. freundii*. Notably, we found significant enrichment of stress response from genes upregulated by *E. cancerogenus* and *C. freundii*.

From our analyses, although animals from both genetic backgrounds shared the same diets, bacteria-induced gene expression changes in wild-type or AP-tau *C. elegans* mostly differed, with the exception of some similarities, such as the regulation of multiple stress responses. Since the upregulation of genes encoding heatshock proteins and genes involved in proteolysis was common between all diets in the AP-tau model, it is possible that the presence of pan-neuronal tau aggregates significantly alters the way host mechanisms are regulated to favour the management of toxic proteins, while biological processes directly affected by specific diets are more subtly changed. It is also possible that the upregulation of proteolysis and heat stress response genes are a result of neuroprotective mechanisms triggered by the bacteria.

Figure 4. RNA-Seq of wild-type *C. elegans* provided diets of neuroprotective microbiota species *E. cancerogenus*, *E. hormaechei* or *C. freundii*. A. Numbers of reproduced, significant, and differentially regulated genes from two RNA sequencing experiments of wildtype *C. elegans* exposed to neuroprotective bacteria. Venn diagrams display quantification of all genes that were significantly changed in the same direction under the same comparisons from both the 2018 and 2019 RNA-Seq experiments. E. c. = *E. cancerogenus*, E. h. = *E. hormaechei*, C. f. = *C. freundii*. **B.** WormCat categories enriched from genes that were reproducibly, significantly downregulated (left) or upregulated (right) in wild-type animals provided neuroprotective bacterial diets. Only categories enriched with Bonferroni-corrected p-value <0.05 shown. P-values calculated by WormCat using Fisher's exact test, and Bonferroni-corrected p-values with FDR <0.01 also displayed.

Moving forward, we could use the reproduced and significantly changed genes from the analysis of wild-type animals to identify biologically relevant genes that cause

neuroprotection and neurodegeneration in response to bacterial diets. We can complement the wild type data and validate our findings by investigating the overlapping significantly changed genes between the wild type and AP-tau gene sets. Although the gene expression changes in response to diets can be compared between genetic backgrounds, the WormCat analysis highlights the differences in responses from animals that are healthy compared to animals that are sensitized to neurodegeneration. Gene expression changes in wild-type animals are indicative of how the bacteria can prevent neurodegeneration in already-healthy *C. elegans*, while the gene expression changes in AP-tau animals display the tau-bacteria interactions that potentially reduce neurodegeneration within a neurodegenerative genetic background. Therefore, the two analyses can also be evaluated separately and provide insight into how animals of different genetic backgrounds respond to the bacterial diets.

DAF-16 (Urrutia et al., 2020) and PMK-1 have been implicated in bacterial influence on neurodegeneration. To determine whether the effects we observed with microbiome bacteria were mediated through modulation of these pathways, we first compared gene expression with known targets of these genes. PMK-1 activation is associated with neuroprotection in dopaminergic neurons (Chikka et al., 2016), and neurodegeneration in motor and mechanosensory neurons (E, Lezi, et al., 2018; Vérièpe et al., 2015). We asked whether PMK-1 target gene expression was altered in response to bacterial diet, and if there are correlations between their expression and the changed levels of PLM neuron degeneration we observed.

Using a list of previously reported PMK-1 target genes (Troemel et al., 2006), we examined expression of these genes in our datasets (Supplementary Figure 3). Of 121 genes reported to show differential expression in *daf-2; pmk-1* mutants relative to *daf-2(e1368)* alone, 24 were recovered as changed in at least one of our wild type datasets (Supplementary Figure 3, Supplementary Table 4). However, the direction of the change in gene expression would suggest that *C. freundii*, although protective against neurodegeneration, promotes expression of some *pmk-1* target genes. Increased PMK-1 activity would be expected to promote neurodegeneration rather than protecting against it. However, since we observed that both the reduced levels of neurodegeneration and shortened lifespan in response to *C. freundii* are not caused by altered activation of PMK-1 (Figure 3B and 3C), it is likely that the PMK-1 targets are not responsible for the neuroprotection caused by *C. freundii*, and their expression is induced by other pathways. This further suggests that the differential expression of PMK-1 targets found from transcriptomic profiling is likely not due to a change in PMK-1 activation.

Tau tubulin kinases promote neurodegeneration

Protein kinases phosphorylate substrates and phosphatases dephosphorylate substrates, forming a coordinated system by operating antagonistically to regulate many neuronal activities such as survival, differentiation, axon guidance, and synaptic plasticity (Finegan et al., 2009; Li et al., 2000; Pagani & Merlo, 2019; Sun et al., 2001; Wagner et al., 1991; Zang et al., 2021). The ubiquitous nature of phosphorylation events in neuron

function and physiology can unfortunately favour opportunities for deregulated phosphorylation to play a key role in disease development.

We found that the expression of kinases and phosphatases were differentially regulated in response to the neuroprotective microbiota species in wild type, particularly *E. cancerogenus*, which reproducibly induced the downregulation of 30 kinases and 29 phosphatases in wild-type animals (Figure 5A). Two kinases, Y38H8A.3 and C25A8.5, and three phosphatases, W03F11.4, M70.3, and Y57G7A.6, were downregulated in response to both *Enterobacter*s and *C. freundii*, however, the gene expression signature of kinases and phosphatases are relatively unique to each bacterial diet. Fewer kinases and phosphatases were affected in AP-tau animals in response to neuroprotective diets overall (Figure 5B). *E. cancerogenus* influences expression of the most genes, downregulating 13 kinases and 15 phosphatases, while *C. freundii* downregulates the expression of only *rol-3*, the only kinase that was significantly changed in response to all three diets (Figure 5B). *E. hormaechei* also had relatively little impact on the expression of kinases and phosphatases in AP-tau animals, downregulating the expression of three kinases and one phosphatase (Figure 5B).

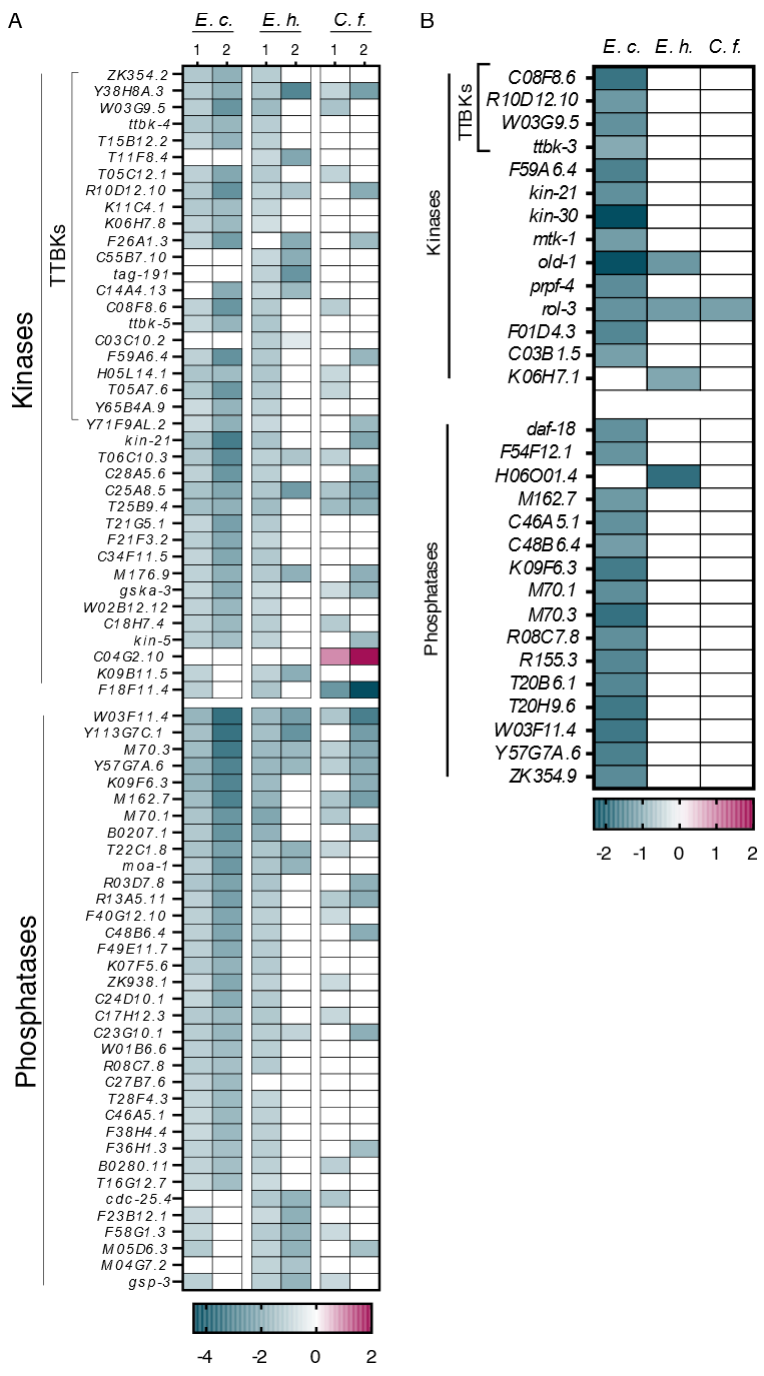


Figure 5. Neuroprotective microbiota species cause changes in the expression of kinases and phosphatases in *C. elegans*. A. Expression of kinases and phosphatases in wildtype *C. elegans* exposed to different bacterial diets from two independent RNA-Seq datasets. Log_2(fold change) is shown. **B.** Expression of kinases and phosphatases in AP-tau *C. elegans* from RNA-Seq. Log_2(fold change) is shown. E. c. = *E. cancerogenus*, E. h. = *E. hormaechei*, C. f. = *C. freundii*.

High but non-pathological levels of tau phosphorylation occurs during hibernation in squirrels (Arendt et al., 2003), sleep in mice (Guisle et al., 2020), and brain development in rats (Brion et al., 1994). The normal, reversible phosphorylation of tau is dependent on the regulation of phosphorylation and dephosphorylation processes. Human tau can get phosphorylated at more than 40 different epitopes along the entire length of the protein (Drepper et al., 2020; Hanger et al., 2007b; Morishima-Kawashima et al., 1995b). In AD, tau becomes hyperphosphorylated, causing it to become insoluble and dissociate from microtubules, forming neurotoxic tau aggregates, or neurofibrillary tangles (Arnsten et al., 2021). Tau tubulin kinases (TTBKs) can influence neurodegeneration, and their ability to phosphorylate tau is consistent with higher levels of expression promoting neurodegeneration in this model. Expression of human TTBK in *C. elegans* increased phosphorylation of human tau and promoted neurodegeneration (Taylor et al., 2018). The TTBK family is expanded in *C. elegans*, with the genome containing 32 TTBKs (Manning, 2005) - and an additional 42 are categorized as TTBKs by WormCat (Holdorf et al., 2020). Of these genes, 22 were downregulated in response to one or both *Enterobacter* species (Figure 5A); RNAi clones were available for 18 of these. To investigate if suppressing *ttbk* is causal for bacteria-mediated neuroprotection, we knocked down these genes by RNAi and examined the impact on paralysis and neurodegeneration in the AD models. In the Aβ-overexpressing model, only knockdown of ZK354.2 protected against paralysis (Figure 6A). Similar to the Aβ transgene in *C. elegans* CL4176, ZK354.2 is expressed in the body wall muscle (Blazie et al., 2017). It is possible that ZK354.2 suppression decreases the abnormal protein oxidation and cellular oxidative stress that triggers paralysis in response

to Aβ overexpression (Drake, 2003). In contrast to the effects on Aβ-induced paralysis, knockdown of all 18 individual *ttbk*s was protective against tau-induced degeneration of the PLM neurons (Figure 6B).

In the AP-tau transgenics, AP-tau is pan-neuronally expressed, so animals also experience decreased locomotor speed compared to wild-type animals, a symptom of motor neuron degeneration (Fatouros et al., 2012). To test if *ttbk* suppression also induces neuroprotection in the motor neurons, we performed RNAi-mediated knockdown of *ttbk*s in the AP-tau animals and measured locomotor speed. Suppressing 14 of the tested 18 *ttbk*s significantly increased locomotor speed in two independent trials, suggesting that decreasing the expression of most tested *ttbk*s also induces neuroprotection in the motor neurons (Figure 6C). This finding provides evidence that *ttbk* knockdown improves neuron function, in addition to improving neuron morphology in the PLM mechanosensory neurons.

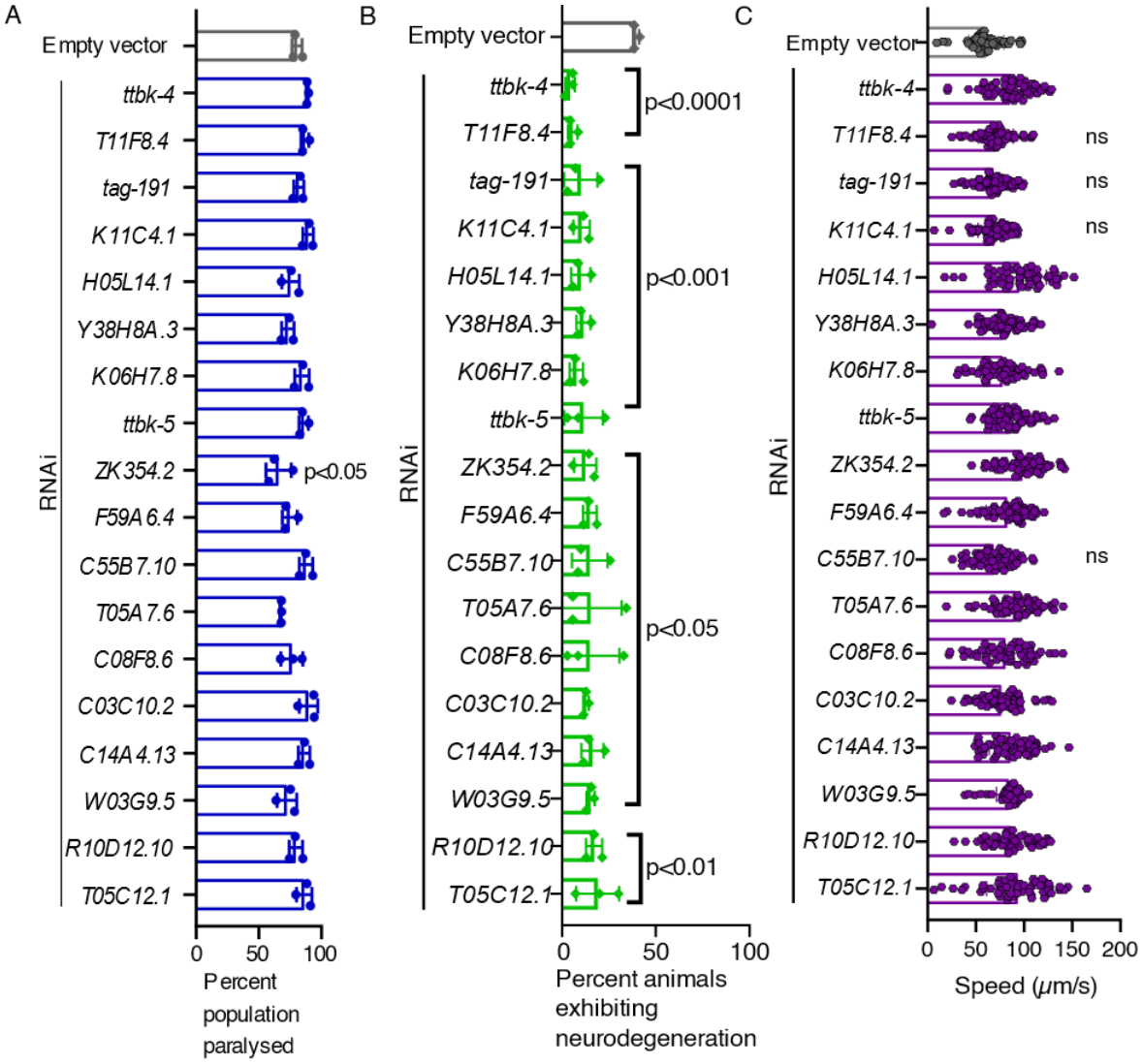


Figure 6. Downregulation of *C. elegans* TTBK1/2 orthologs induces neuroprotection. A. Paralysis observed in populations of *C. elegans* CL4176 after 72 hours of temperature-induced expression of Aβ42, following knockdown of different *ttbk* genes. Levels of paralysis did not significantly differ after knockdown of any tested *ttbk* gene when compared to empty vector (L4440), except ZK354.2. Each graph point represents a population of >60 animals. **B.** Neurodegeneration of AP-tau *C. elegans* at day 1 of adulthood was reduced in response to RNAi-mediated knockdown of 18 *ttbk* genes compared to empty vector. Animals were exposed to *E. coli* HT115 producing dsRNA targeting specified genes for the entirety of assays from embryo to experiment completion. Each data point represents a population of >60 animals. **C.** Speed (μm/s) was quantified in neuronal RNAi-sensitized [(*myo-2p::mCherry*) + *unc-119p::sid-1*)] AP-tau *C. elegans* at day 1 of adulthood in response to RNAi-mediated knockdown of 18 *ttbk* genes compared to empty vector control (L4440). Knockdown of each *ttbk* was performed on a *C. elegans* population that was on a single plate per gene, with 50 animals per condition. Only track durations of 120-180 seconds were included in analysis. Each graph point represents an individual worm. One-way ANOVA and Dunnett'smultiple comparisons test performed with empty vector as control.

Neuroprotection induced by knockdown of TTBK1/2 orthologs is not caused by inherent variables of RNAi

C. elegans neurons are intrinsically less sensitive to RNAi because they express fewer dsRNA transporters, specifically the SID-1 transmembrane protein, compared to cells in other tissues (Calixto et al., 2010). Neuronal overexpression of SID-1 enhances dsRNA uptake and RNAi in *C. elegans* (Calixto et al., 2010). Therefore, we used a *C. elegans* strain that overexpresses SID-1 through the neuron-specific promoter *unc-119*, and crossed it with AP-tau animals, creating a neurodegenerative model that is neuronally sensitized to RNAi. When we knocked down individual *ttbk*s in this model, not only was neurodegeneration significantly decreased, but more consistently overall (Supplementary Figure 5, Figure 6B). Although neuroprotection was not enhanced, we observed narrower confidence intervals in this experiment. This suggests that the increased statistical significance is likely due to the RNAi-sensitized strain experiencing decreased variability between trials, compared to the non-RNAi-sensitized AP-tau animals (Supplementary Table 8). The resulting smaller standard deviation is potentially caused by a more potent RNAi response in the neurons, which allows a more consistent induction of neuroprotection in *C. elegans* populations. Overall, observing a more consistent neuroprotective response to RNAi-mediated knockdown of individual *ttbk*s in neuronally RNAi-sensitized *C. elegans* supports the neuroprotective role of *ttbk* suppression.

A potential explanation for this broad neuroprotective effect from *ttbk* RNAi is that RNAi-mediated knockdown can suppress additional genes. Each Ahringer library RNAi

clone contains a 1-1.5 kb fragment of the target gene, and 80% similarity across 200 bp is predictive of gene silencing (Kamath & Ahringer, 2003). Therefore, the expression of genes that share high sequence similarity with the target gene may be inadvertently suppressed. To investigate if a single *ttbk* RNAi clone is possibly suppressing the expression of multiple *ttbk*s to induce neuroprotection, we sequenced the plasmids of each tested clone and performed NCBI Blast against the *C. elegans* genome. 12 of the 18 tested *ttbk* RNAi clones had >95% sequence similarity to ≥200 bp only to the target gene (Supplementary Table 9). Four clones designed to target *ttbk-4*, ZK354.2, T05A7.6, or Y38H8A.3 have additional high sequence similarities (>80%) with other *ttbk*s, and W03G9.5 and K11C4.1 have an additional high sequence similarity with an uncharacterized gene and a predicted kinase respectively (Supplementary Table 9). Therefore, the neuroprotection from knock-down of W03G9.5, *ttbk-4*, ZK354.2, T05A7.6, Y38H8A.3, and K11C4.1 could be attributed to non-specific suppression of additional genes and will need to be validated using a newly designed gene-specific RNAi clone or a knockout mutant. In contrast, the neuroprotection caused by suppressing expression of the other 12 tested *ttbk*s is likely gene-specific and not due to off-target RNAi-mediated gene silencing.

A caveat of performing RNAi-mediated gene knockdown in *C. elegans* by feeding is having to rely on the use of a bacterium that could acquire mutations. Although we try to prevent this by inoculating from frozen glycerol bacterial stocks for every experiment, it is possible the stress imposed on the bacterial population by incremental thawing and re-freezing canallow mutations to arise over time (Calcott & Gargett, 1981; Kwon et al.,

2018). Genetic changes in the bacterium could affect *C. elegans* phenotypes, which can be inaccurately attributed to RNAi. We sought to confirm that potential changes in the *E. coli* HT115 bacterial clone that carried the empty vector was not the unintended cause of the differencein *C. elegans* neurodegeneration levels. To do this, we transformed the RNAi L4440 emptyvector into *E. coli* HT115 obtained from the original frozen lab stock, as opposed to the 'working stock', then measured the resulting levels of neurodegeneration in AP-tauanimals. When compared to the control and standard diet of *E. coli* OP50, neither the new nor the originally cloned *E. coli* HT115 empty vector controls affected neurodegeneration (Supplementary Figure 6A). Overall, this is evidence that the neuroprotection observed after *ttbk* knockdown is not due to an inadvertent difference in the *E. coli* HT115 bacteria used in prior neurodegeneration assays, but in fact the effect of suppressing *ttbk* gene expression.

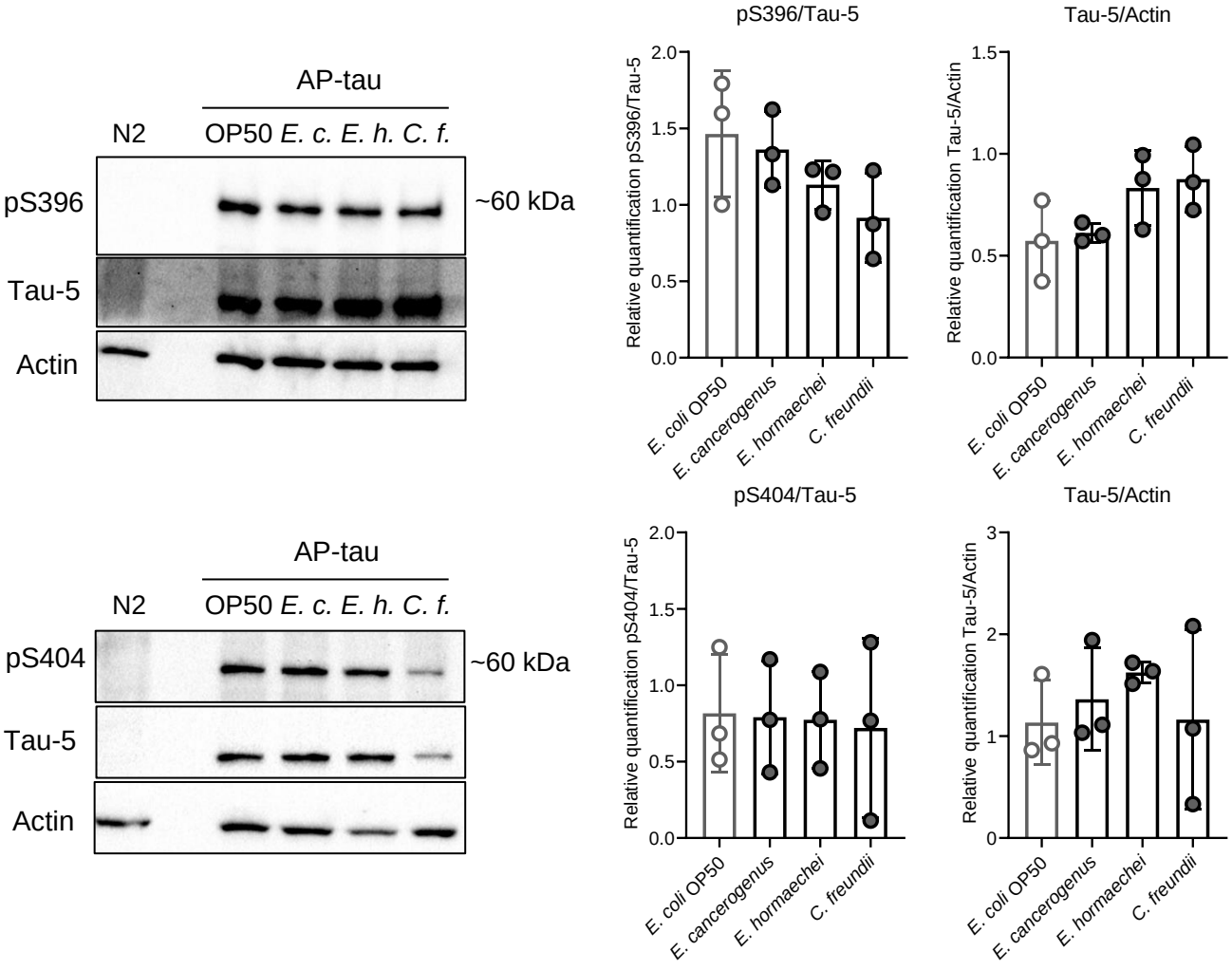
Some *C. elegans* homologs of RDE-1, the primary Argonaute protein that binds to exogenous dsRNA to trigger RNAi, are also involved in embryogenesis and larval development (Grishok et al., 2001). Therefore, the RNAi machinery is involved in endogenous pathways that affect different *C. elegans* phenotypes. This implies that RNAi machinery may potentially influence neurodegeneration. Therefore, we wanted to ensure that neuroprotection following RNAi-mediated knockdown of *ttbk* is not inadvertently caused by triggering the RNAi pathway. To do this, we tested the effects of RNAi-mediated knockdown of two *C. elegans* pseudogenes, T05G5.6 and F09C6.5, which do not encode a functional protein. This way, we were activating RNAi machinery without suppressing the expression of a gene that could cause a phenotypic change in *C. elegans*. Suppressing

the expression of the pseudogenes, as expected, did not cause a change in neurodegeneration when compared to the empty vector control, unlike when we knocked down expression of *ttbk-4*, which caused neuroprotection (Supplementary Figure 6B). This confirms that triggering RNAi does not intrinsically reduce neurodegeneration in AP-tau *C. elegans*. Taken together with our RNAi clone sequence confirmation (Supplementary Table 9), and the confirmation that neuroprotection is not a result of isolate-specific *E. coli* HT115 (Supplementary Figure 6), these findings support our hypothesis that neuroprotection can be induced by suppressing the individual expression of at least 12 specific *ttbk* genes, and that the resulting neuroprotection is not caused by other variables from RNAi.

Neuroprotection is associated with decreased phosphorylation of specific tau species

Increased tau aggregation contributes to the formation of neurofibrillary tangles in AD and other tauopathies (Obulesu et al., 2011). Hyperphosphorylation of tau promotes aggregation (Arnsten et al., 2021; Obulesu et al., 2011). To identify the mechanism of neuroprotection, we examined the effects of these diets on tau phosphorylation and aggregation. Several phosphorylation sites of tau have been identified, including those that are specific to, or enriched in, AD brains. Tau epitopes S404 and S396 are phosphorylated in both mature neurofibrillary tangles and early neuronal tau aggregates before detectable neurodegeneration, therefore being effective markers for early AD pathology (Mondragón-Rodríguez et al., 2014). We measured phosphorylation at these sites to test if neuroprotective diets prevent phosphorylation events that precede tau oligomerization. Phosphorylation of S404 or S396 was not decreased in AP-tau *C. elegans* fed

neuroprotective diets relative to animals fed a diet of *E. coli* OP50 (Figure 7C and 7D). This suggests that the neuroprotection caused by the *Enterobacter*s and *C. freundii* does not require reduced phosphorylation at S404 or S396 to prevent neurofibrillary filament formation. It is possible that the neuroprotective bacteria may not prevent total tau phosphorylation at AD-associated epitopes in every neuron, but they may reduce phosphorylation in specific neurons, such as the PLM neurons that we observed neuroprotection in. Neuron-specific changes in phosphorylation would be challenging to capture from lysis of whole *C. elegans*.



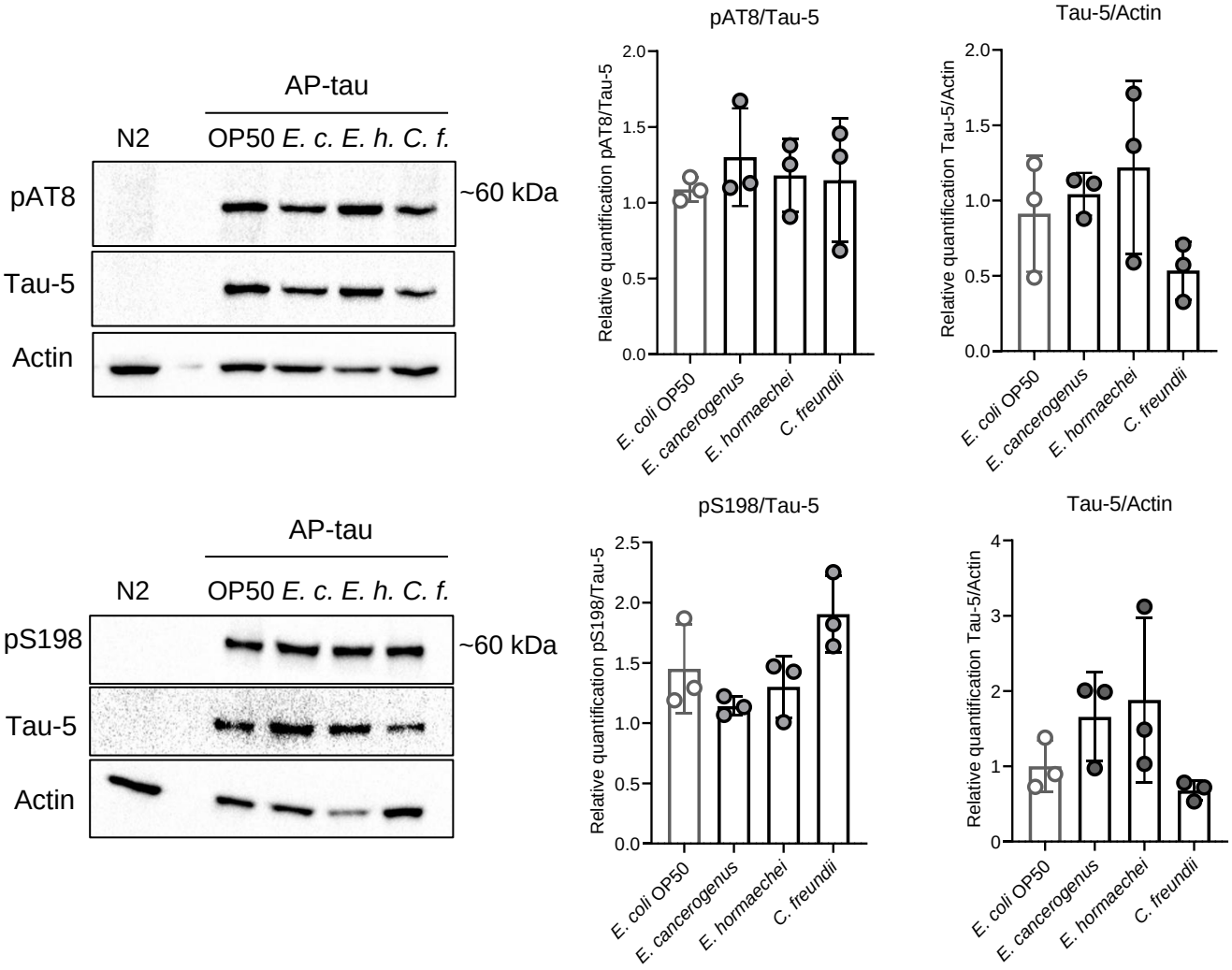


Figure 7. Tau phosphorylation at S396, S404, AT8 (S202/T205), or S198, are unchanged in response to neuroprotective bacteria. Representative blots of pS396 (**A**), pS404 (**B**), pAT8 (S202/T205) (**C**), or pS198 (**D**) tau in AP-tau *C. elegans* fed *E. coli* OP50, or neuroprotective diets. Levels of phosphorylated tau and total levels of tau were unchanged in response to neuroprotective diets. Each band was quantified separately using the mean-grey method in ImageJ. Actin (clone C4) used as loading control. ptau/tau-5 densities were used to measure levels of phosphorylated tau, and tau-5/Actin densities used to measure levels of total tau. One-way ANOVA performed with post-hoc Dunnett's multiple comparisons test using *E. coli* OP50 as control. E. c. = *E. cancerogenus*, E. h. = *E. hormaechei*, and C. f. = *C. freundii*.

We also used phospho-specific antibodies to pAT8 (Ser202, Thr205), pS198, and pS422, to measure phosphorylation at tau epitopes associated with disease (Barthelemy et al., 2019; Campbell et al., 2015; Cao et al., 2018; Dillon et al., 2020a). Phosphorylation at

AT8 or S198 was not reduced in response to the neuroprotective diets compared to a diet of *E. coli* OP50, suggesting bacteria-induced neuroprotection is not mediated through differential phosphorylation of these epitopes (Figure 7C and 7D). On the other hand, S422 phosphorylation of a high molecular weight form of tau was reduced in response to *Enterobacter* diets (Figure 8A and 8B). Tau is post-translationally modified by acetylation, methylation, glycosylation, and SUMOylation; phosphorylation can promote some of these modifications. The higher molecular weight band recognized by the pS422 antibody may be SUMOylated tau protein. SUMO is a 12 kDa protein, and when using SDS-PAGE, SUMOylated substrates typically increase in their apparent size by approximately 20 kDa (Daniel et al. 2017). Phosphorylation promotes SUMOylation that in turn inhibits tau degradation (Luo et al., 2014). A decrease in SUMOylated tau could therefore explain a neuroprotective effect.

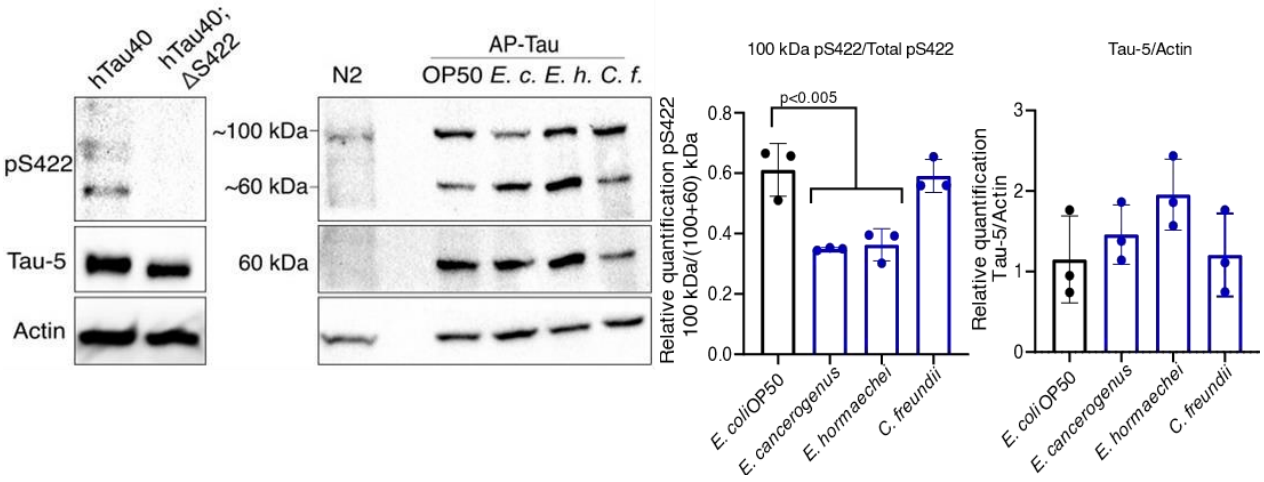


Figure 8. AP-tau *C. elegans* experience decreased abundance of tau species phosphorylated at S422. Representative blot of pS422 in htau40 overexpressing cell line and S422 mutant (left blot), and in N2 or AP-tau *C. elegans* (right blot). Two bands were observed when blotting for pS422, at ~100 kDa and ~60 kDa. Levels of 100 kDa S422-phosphorylated tau relative to total pS422 tau were reduced in response to E. c. and E. h. Levels of total tau were unchanged in response to neuroprotective diets. Each band was quantified separately using the mean-grey method in ImageJ. Actin (clone C4) used as loading control. Tau-5/Actin densities were used to measure levels of total tau. One-way ANOVA performed with post-hoc Dunnett's multiple comparisons test using *E. coli* OP50 as control. E. c. = *E. cancerogenus*, E. h. = *E. hormaechei*, and C. f. = *C. freundii*.

Phosphorylation of both serine and threonine in the AT8 epitope has been reported by ERK2 and p38/MAPK, while phosphorylation of the serine can also be mediated by JNK and TTBK1 (Hanger et al., 2007c; Sato et al., 2006). The only kinase reported to phosphorylate S422 is TTBK1 (Sato et al., 2006). The decreased expression of *ttbk*s in response to *Enterobacter* species makes them good candidates to explain, at least in part, the neuroprotective effects of these bacteria and the decrease in tau phosphorylation that occurs in response to them. *E. cancerogenus* and *E. hormaechei* may therefore induce neuroprotection by decreasing tau phosphorylation at disease-associated epitopes.

Crude extracts of microbiota species sufficiently induce neuroprotection

It is important to find and characterize what bacterial factors either lead to protection against, or cause neurodegeneration. This will allow us to determine what bacterial genes, compounds or activities directly induce neuroprotection. We asked if exposure to only the cell free supernatants (CFS) of the protective microbiota species were sufficient to induce protection against paralysis in *C. elegans* CL4176, or if the bacteria needed to be present for paralysis to be suppressed. CFS from bacterial cultures contain soluble and secreted secondary metabolites from the bacteria, but not the bacterial cells themselves. Animals were provided control diets of *E. coli* OP50 without any supernatant, and OP50 with OP50 supernatant, while the rest of the samples are worms fed OP50 on plates supplemented with protective supernatants. We found that exposure to the supernatants was not sufficient to induce protection against paralysis,

which suggests that the protective factors the bacteria produce are not secreted, and that they may need to be live and ingested to affect paralysis (Figure 9).

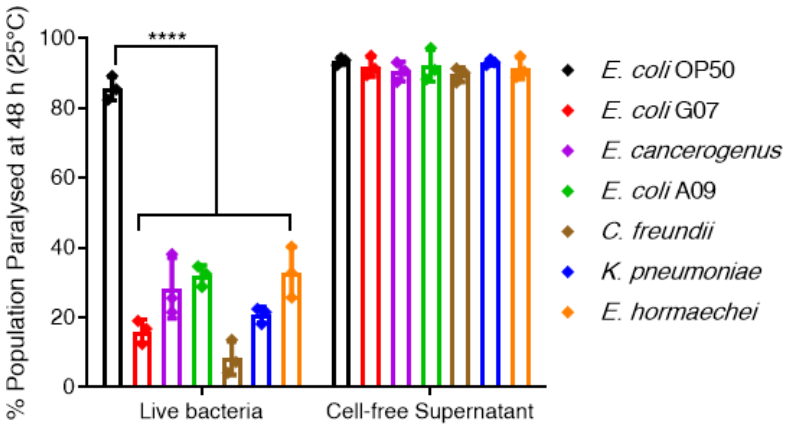


Figure 9. Bacterial supernatants were unable to recapitulate the protective effects of microbiota bacteria. Levels of paralysis observed in populations of CL4176 animals after 72 hours of temperature-induced overexpression of Aβ42 in response to indicated bacterial diets and supplementation with cell-free supernatants. Populations of CL4176 fed control diet of *E. coli* OP50 supplemented with supernatants of protective diets did not experience reduced paralysis like when fed live bacteria. One-way ANOVA run with post-hoc Dunnett's multiple comparisons test, OP50 as control. ****=$p<0.0001$. Bars represent standard deviation of the mean.

Whole-cell extracts, which contain bacterial metabolites that can induce neuroprotection, were produced from lysing the test bacteria. DMSO is a commonly used solvent for producing crude extracts because it solubilizes both polar and non-polar compounds. In *C. elegans* grown on solid media, exposure to 0.5-2% DMSO alone extends lifespan by regulating expression of DAF-16/FOXO targets, while concentrations of 0.05-0.2% DMSO do not alter *C. elegans* lifespan (X. Wang et al., 2010).

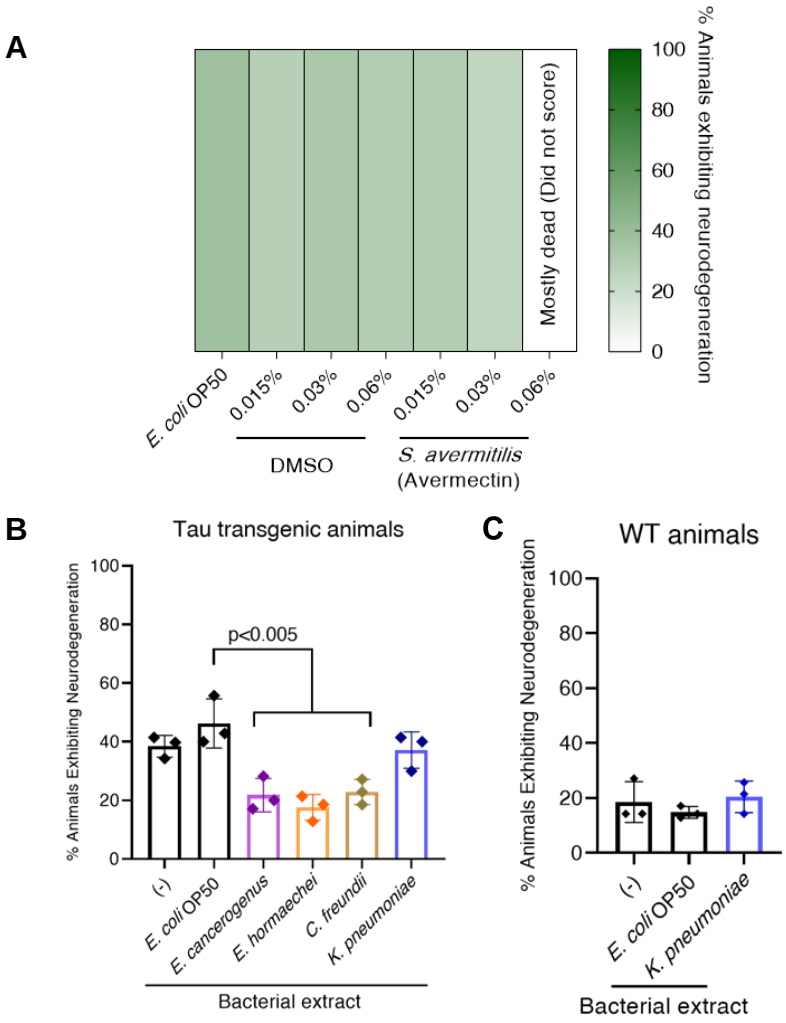


Figure 10. Crude extracts of microbiota species sufficiently induce neuroprotection in AP-tau *C. elegans*. A. Preliminary trial measuring levels of neurodegeneration in AP-tau animals, in response to increasing concentrations (v/v) of DMSO or whole cell extract from *S. avermitilis* containing avermectin. B. Neurodegeneration is reduced when the E. coli OP50 diet is supplemented with crude extracts from neuroprotective bacteria at a final concentration of 0.06%. *K. pneumoniae* extract did not influence neurodegeneration. C. *K. pneumoniae* extract did not alter levels of neurodegeneration in wild type.

To determine an appropriate crude extract concentration, we first determined if low concentrations of DMSO affect neurodegeneration (Figure 10A). As a positive control, we tested the effects of an extract from *Streptomyces avermitilis*, which produces the anthelmintic avermectin (Burg et al., 1979). Neurodegeneration in AP-tau animals was consistent in response to *E. coli* OP50 alone, and OP50 supplemented with DMSO

concentrations of 0.015-0.06% (Figure 10A). The *S. avermitilis* extract caused death in most of the *C. elegans* population at a concentration of 0.06% or higher, demonstrating that supplementation with a crude extract at this concentration can sufficiently induce a phenotype in *C. elegans*. Taken together, these findings suggest that the amount of DMSO in 0.06% of the extract sample does not influence neurodegeneration, while the concentration of causal bacterial metabolite present in 0.06% of crude extract can be enough to induce a phenotype in *C. elegans*.

To test if the crude extracts influence neurodegeneration, we supplemented an *E. coli* OP50 control diet with a final concentration of 0.06% crude extract, then measured neurodegeneration in the AP-tau animals. Extracts from the neuroprotective *Enterobacter*s and *C. freundii* sufficiently induced neuroprotection in the AP-tau animals (Figure 10B), while extract from the neurodegenerative *K. pneumoniae*, did not induce neuroprotection (Figure 10C). This suggests that the neuroprotection induced by the microbiota species is likely caused by the production of neuroprotective metabolites, which could be species-specific. Future work to fractionate and purify the crude extracts is needed to identify the causal metabolite and its required dose to induce neuroprotection. *K. pneumoniae* extract caused no changes in wild type neurodegeneration, suggesting that live *K. pneumoniae* is required to induce neurodegeneration in wild-type animals, or if *K. pneumoniae* does induce neurodegeneration by production of a metabolite, it could be that a higher dose is required than what was provided in this experiment.

Discussion

The human microbiota is a complex community that remains largely undescribed due to the challenges in complete identification and characterization of all the bacteria species and strains present in the gastrointestinal tract. We sought to identify specific human microbiota species that protect against features of AD and elucidate the genetic and cellular mechanisms connecting bacterial exposure to neurodegenerative disease. The comprehensively mapped *C. elegans* connectome and well-logged characterisation of phenotypes associated with neurosignals and genes, as well as its bacterivorous nature, makes *C. elegans* instrumental in identifying and characterizing the neuroactive potential of the gut microbiota and metabolome.

For complex disorders, characterizing changes in host gene expression signatures helps us identify genes that are sensitive to the environment and provides insight on biological responses that may contribute to disease development. In our study, by investigating bacteria-induced changes in gene expression, we identified environmentally responsive genes that contribute to neurodegeneration. Specifically, exposure to two different *Enterobacter* species, *C. freundii*, or extracts of these bacteria, promoted neuroprotection in a model of AD and tauopathies. Exposure to these bacteria promoted extensive changes in gene expression, notably, of genes involved in phosphorylation. Exposure to *Enterobacter* produced changes in the expression of a prominent proportion of the uncharacterized *C. elegans* TTBK gene class family, and reduced abundance of a putatively neurotoxic tau species phosphorylated at S422, an AD-specific site reported to

be directly phosphorylated by TTBK1 in humans (Dillon et al., 2020b). These data are consistent with a model whereby protective bacteria decrease the expression of TTBKs and in turn reduce tau phosphorylation, processing, and subsequent neurodegeneration. Although we did not see changes of tau phosphorylation levels at non-disease specific AT8 (S202/T205), S198, S396, or S404 epitopes, there may be a complex interplay between phosphorylation sites. For example, phosphorylation at some sites may be required to allow phosphorylation at secondary sites. This has been shown for GSK3β sites, where the phosphorylation of neighbouring sites "primes" tau and promotes phosphorylation by GSK3β (Cho & Johnson, 2004; Gong et al., 2005; T. J. Singh et al., 1995; Steinhilb et al., 2007). TTBKs are reported to phosphorylate multiple serine residues, however we only measured phosphorylation at five sites, and observed differences at S422. It is possible that TTBKs phosphorylate additional causal tau epitopes to promote neurodegeneration. It is also possible that the additional phosphorylation of priming residues by TTBKs may promote S422 phosphorylation by other TTBKs, or the other phospho-sites may be regulated by several kinases (Cavallini et al., 2013).

Mutations in TTBK1 are linked to decreased risk of developing AD (Vázquez-Higuera et al., 2011; Yu et al., 2011), while TTBK2 mutations are associated with spinocerebellar ataxia type 11 (Houlden et al., 2007). TTBK1 is regulated by autophosphorylation (Bao et al., 2021), and aside from tau, TDP-43 and SV2A are the only other identified proteins known to be directly phosphorylated by TTBK1 (Taylor et al., 2018; N. Zhang et al., 2015). TTBK1 knockdown in mouse cortical neurons resulted in significant changes in phosphopeptide enrichment (Bao et al., 2021). This analysis revealed

additional putative TTBK1 phosphorylation targets belonging to enriched biological processes including organisation of the neuronal cytoplasm, organisation of the cytoskeleton, and microtubule dynamics. More efforts are required to characterize these targets to determine whether they are direct or indirect targets of TTBK1, and how changes in their phosphorylation affect neuron health (Bao et al., 2021). Currently, pathways regulating TTBK1 expression and activity have not been described, but the TTBK1 kinase domain is conserved in vertebrates, *Drosophila melanogaster*, and *C. elegans*, enabling the investigation of TTBK1 in fundamental AD research (Ikezu & Ikezu, 2014). WormCat defines 74 *C. elegans* genes as TTBK1/2 orthologs (Holdorf et al., 2020), and they could be a result of gene duplication. A third of the *C. elegans* genome consists of duplicated genes that lead to expansion of gene families (Cavalcanti et al., 2003; Cutter et al., 2009; Friedman & Hughes, 2001; Gu et al., 2002). Evolutionarily, gene duplication events occur when a replicate copy of an ancestral gene acquires mutations, then undergoes relaxed selection to eventually cause genetic drift (Ohno, 2013). Thus far, the *C. elegans* TTBK gene family is largely undescribed, with the only work to date describing some members as putative cytoskeletal regulators involved in *C. elegans* sperm motility (Holdorf et al., 2020; Ortiz et al., 2014; Reinke et al., 2000), or being involved in pheromone-induced dauer formation (Neal et al., 2016). Transcriptional reporters of *ttbk-3*, *ttbk-4*, *ttbk-5*, *ttbk-6*, and *ttbk-7* revealed that these TTBKs are expressed in neurons (Neal et al., 2016). Expression of *ttbk-3*, *ttbk-4*, and *ttbk-5* was in either or both of the ASI and ASK sensory neurons and was weak and variable in additional neurons (Neal et al., 2016). These findings suggest that *ttbk* genes can differ in their individual expression patterns, which isa potential explanation for why there are numerous *ttbk* genes in *C. elegans*. However, how*ttbk*s may function to influence neuronal health have not been studied. Our work is the first

to study *C. elegans ttbk*s in the context of neurodegeneration, and further, the first to demonstrate microbe-induced changes in TTBK gene expression of any animal model. This work provides insight on which specific tau kinases can be targeted by microbiota species to modulate neurodegeneration.

Cellular stress pathways are commonly induced in response to Aβ-induced cytotoxicity, and changes in stress-related gene expression have been observed in *C. elegans* models of AD, including mediators of the p38/MAPK innate immune response (Brehme et al., 2014; Dimitriadi & Hart, 2010; Stringham et al., 1992) and insulin-like signaling pathway (IlS) (E. Cohen et al., 2006; Kim et al., 2007; Murphy et al., 2003; A. J. Parker et al., 2005; V. Singh & Aballay, 2006; Tullet, 2015). *E. coli* strain-specific effects on neurodegeneration in an IlS-dependent manner have also been observed in *C. elegans* (Urrutia et al., 2020). Other studies found bacterially-induced neuroprotection associated with increased lifespan in a Parkinson's disease model fed *Bacillus subtilis* (Cogliati et al., 2020; Goya et al., 2020), but we did not observe this in the AP-tau animals with the human microbiota isolates tested. We also did not observe clear correlations between bacteria-induced changes in neurodegeneration and the expression levels of affected PMK-1 or DAF-16 targets. It is likely that if PMK-1 or IlS is responsible for the differences in the neurodegeneration we've observed, it could be dependent on the activation or suppression of specific downstream targets, and future work would need to investigate the genes individually to confirm this. Our findings are not the first to demonstrate that neurodegeneration and lifespan do not always correlate (Apfeld & Fontana, 2017; E, Zhou, et al., 2018; Vérièpe et al., 2015), suggesting that although they can be related, processes

that control aging and neurodegeneration can work independently of each other, and these processes differ based on environmental stimulus and the disease model.

Our work identified bacteria that influence expression of host genes that may contribute to AD development. This may be instrumental in the identification of bacterial factors and characterization of cellular processes related to pathogenesis in the AD patient. Taking cognizance of the complex interplay between Aβ- and tau-induced pathologies in AD, we prioritised studying bacteria that are protective against pathologies caused by both histopathological features of AD. Given the abundance of bioactive molecules produced by bacteria, the discovery of neuroprotective compounds produced by species from the human microbiota may lead to novel therapeutic approaches. Overall, this research helps us understand how environment can impact neurodegenerative disease and provides potential for employing the human microbiota as a source of therapy for AD.

Methods

C. elegans strains

C. elegans were maintained on nematode growth media (NGM) seeded with *E. coli* OP50. Several *C. elegans* strains were obtained from the CGC, including CL4176 *smg-1(cc546ts)*I; *dvIs27*[pAF29(*myo-3*::A-Beta 1-42::let-851 3'UTR) + *rol-6*(*su1006*))] (C. Link, 2003). AP-tau strain used was BR5706 byIs193 [*rab-3p*::F3DK280 + *myo2p::mcherry*]; *bkIs10* [*aex-3p*::h4R1NtauV337M +*myo2p::GFP*] (Fatouros et al., 2012). A *mec-7::GFP* transcriptional reporter was introduced into BR5706 to generate

LMN008 (byIs193[*rab-3p*::F3DK280 + *myo2p::mcherry*]; *bkIs10* [Paex-3::h4R1NtauV337M+*myo2p::GFP*]; muIs32[*mec-7::GFP + lin-15(+) + him-5*].

AP-tau *pmk-1* mutant strain was created by crossing *C. elegans* LMN008 (byIs193[*rab-3p*::F3DK280+*myo2p::mcherry*]; bkIs10[*aex-3p*::h4R1NtauV337M+*myo2p::GFP*]; [*mec-7::GFP + lin-15(+) + him-5*]) with *C. elegans* KU25 *pmk-1(km25)*.

AP-tau RNAi-sensitized strain was created by crossing *C. elegans* expressing [(*myo-2p::mCherry*) + *unc-119p::sid-1*] with *C. elegans* LMN008, then selecting for progeny with GFP-positive and mCherry-positive pharynx and *mec-7::GFP*. This strain expresses only the bkIs10[*aex-3p*::h4R1NtauV337M+*myo2p::GFP*] tau transgene.

N2, obtained from the Caenorhabditis Genetics Centre (CGC), was used as wild type. CL4176 worms were maintained at 15℃, and all other strains at 20℃ unless otherwise specified.

Bacterial strains

Enterobacter and *Citrobacter* isolates were obtained from faecal samples collected from healthy individuals by Dr. Michael Surette's lab at McMaster University. *E. coli* OP50 was obtained from the CGC. All experimental bacteria were inoculated from frozen and grown overnight in liquid Brain Heart Infusion (BHI) media (Criterion) 37℃, 5% CO_2 before seeding onto plates. For all *C. elegans* experiments, bacteria were seeded onto a modified and diluted version of Brain Heart infusion media (¼ BHI). Media contained

(0.00176 g/mL NaCl , 0.00925 g/mL, BHI dehydrated media (Criterion), 17 g/L agar (Fisher Bioreagents), 0.001 mol/L $CaCl_2$, 0.001 mol/L MgSO4, 0.005 g/mL cholesterol (Amresco) in 100% ethanol, and 0.018 mol/L potassium phosphate buffer (pH 6.0). 16S genes from all isolates were sequenced to verify identity (Supplementary Table 6).

Paralysis and Neurodegeneration assays

60 mm ¼ BHI plates were prepared and seeded with 50 μL of bacteria in triplicate, then incubated overnight at 37℃, 5% CO_2 for paralysis and neurodegeneration assays. *C. elegans* CL4176 eggs were collected by hypochlorite treatment (Stiernagle, 2006). Approximately 150-200 eggs were added to each experimental plate and incubated at the permissive temperature of 15℃ for 48 hours. Plates were then shifted to 25℃ and paralysis was scored at 48 and 72 hours. Animals with either absolutely no movement or no movement except for the head were scored as paralysed.

Eggs were collected from LMN008 animals and wild-type animals expressing *mec7p::GFP*, and LMN008 worms were bleach synchronised, and approximately 400 LMN008 eggs, and 150 N2 eggs were pipetted on corresponding experimental plates. Plates were incubated at 20℃ until worms were developed to adulthood (day 1 adult). Worms were anaesthetised with 0.1% sodium azide, and GFP fluorescence was observed and captured using a Nikon Eclipse Ni-U. All experiments were performed as three separate trials. Animals were scored as exhibiting neurodegeneration when displaying blebbed, wavy, or discontinuous axons, as reported in (Wu et al., 2015).

RNA extraction, purification, and sequencing

10 cm petri plates were prepared with ¼ BHI media. Assay plates were seeded with 500 µL of bacteria and incubated overnight at 37°C in 5% CO2. Wild-type N2 eggs were collected by hypochlorite treatment, and eggs were plated to allow development of approximately 5000 worms per plate. Worms were incubated at 25°C for approximately 45 hours, at which time the worms were late L4 or early adult. Test plates were then washed off, and RNA extraction was performed with TRIzol Reagent (Thermo Fisher Scientific) as per manufacturer's instructions. RNA samples were DNAse treated and further purified using the PureLink RNA purification Kit (Thermo Fisher Scientific).

Raw FASTQ files from multiple lanes corresponding to the same samples were concatenated and quality controlled using FASTQC, and samples with overrepresented sequences were identified. Trimmomatic's tools Illuminaclip and Sliding Window were used to remove residual TruSeq3 adapter sequences and Poly-T sequences. Samples were run through FASTQC once again to ensure quality and successful trimming. Mate-paired sequences were mapped to *C. elegans* reference genome (WBcel 235) using HISAT2 with default settings. Splice sites .gtf file was uploaded onto GalaxyLab. Maximum intron length in spliced alignment parameters were set to 110,000 and first strand (R/RF) orientation was used. Reads mapped to annotated genes from BAM files were counted using ht-seq with parameters set to dUTP-based method, "Reverse" used for stranded mode and force sorting of BAM by NAME = True. Rate of concordant pairs mapped to annotated genes checked to reach cut off of <80%. At this stage, two files did not meet the required

cut off; one N2-*C. freundii* replicate had an annotation rate of 71.52%, and one N2-*E. cancerogenus* replicate had an annotation rate of 2.18% after poly-T trimming. These samples were excluded from all subsequent steps. htseq files were input into DESeq2 ANOVA performed to normalize and compare reads obtained from triplicate test conditions to control *E. coli* OP50. Experimental condition was set to first factor and control *E. coli* OP50 was second factor. All DESeq2 files were downloaded, and samples were filtered for genes with read counts ≥10 and adjusted p-values ≤0.05 after multiple hypothesis testing with Benjamini-Hochberg procedure controlling false discovery rate. Differential gene expression analysis of the second RNA-Seq experiment was performed in Multiple experiment viewer (MeV). Rank Products statistical testing was used, performing a two-class unpaired experiment. Fold changes were Log_2-transformed. Rates of used and aligned reads were recorded to assure quality of sequencing (Supplementary Table 7 and Supplementary Table 8).

Gene lists were compared to identify overlapping and condition-unique gene expression in response to all three test bacteria. Wormcat (Holdorf et al., 2020) was used for functional annotation of genes and identification of human orthologs.

RNAi-mediated gene knockdown paralysis and neurodegeneration assays

All RNAi clones were obtained from the Ahringer library (Kamath & Ahringer, 2003). Bacteria expressing dsRNA were inoculated from frozen, grown at 37°C overnight in LB supplemented with ampicillin (50 μg/mL). Overnight cultures were subcultured 1/100 in fresh LB/Amp for 6 hours. 200 μL was seeded on prepared media to grow onto

NGM media supplemented with carbenicillin (25 μg/mL) and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) plates at 37°C overnight. *C. elegans* CL4176 eggs were added to plates and incubated at 15°C for 48 hours before shifting to 25°C. Paralysis was measured 48 hours after shift to the restrictive temperature. Animals with either absolutely no movement or no movement except for the head were scored as paralysed.

Eggs were collected from LMN008 animals and wild-type animals expressing *mec7p::GFP*, LMN008 worms were bleach synchronised, and approximately 400 LMN008 eggs and 150 N2 eggs were pipetted onto corresponding experimental plates. Plates were incubated at 20°C until worms were developed to adulthood (day 1 adult). Worms were anaesthetised with 12mM levamisole, and GFP fluorescence was observed and captured using a Nikon Eclipse Ni-U. All experiments were performed as three separate trials. Animals were scored as exhibiting neurodegeneration when displaying blebbed, wavy, or discontinuous axons, as reported in (Wu et al., 2015). RNAi clones that caused neuroprotection were all sequenced (Supplementary Table 9).

Lifespan assays

Lifespan assays were performed at 25°C using *C. elegans* strain, G660, *glp-1(g60)* temperature-sensitive sterile mutant. Test bacteria was inoculated in liquid BHI from frozen stock overnight at 37°C, 5% CO_2. 50 μL of overnight culture was seeded on ¼ BHI plates in triplicate and incubated overnight at 37°C, 5% CO_2. *C. elegans* eggs were added to plates following isolation by hypochlorite treatment, allowing populations of 100-200 animals to develop per plate. Animals scored at Day 0 were at stage L4.

C. elegans AP-tau animals were grown on *E. coli* OP50 until they reached L4. FUdR-treated (50 mM) ¼ BHI plates were seeded with 200 μL of experimental bacteria in triplicate and incubated overnight at 37°C, 5% CO_2. BR5706 animals developed to L3 were transferred to FUdR-treated plates. Animals scored at Day 0 were at stage L4. Animals were incubated at 20°C for the experiment.

Protein extraction and immunoblotting

Populations of 150000-200000 worms were developed from embryo to young adulthood on control or test bacterial lawns grown on ¼ BHI media, per replicate. Worms were washed off with M9 buffer and washed 3-5 times. Worms were pelleted, M9 supernatant was removed, and RIPA buffer (150 mM NaCl, 1% NP40 (Igepal), 0.5% deoxycholate, 0.1% SDS, 50 mM Tris) was added to a final volume of 0.5 mL, with the addition of protease inhibitor cocktail (Sigma (P8340) 100 μL/mL), PMSF (0.5mM), and phosphatase inhibitors NaF (10mM) and $Na_3VO_4 \cdot 2H_2O$ (1mM). Samples were lysed at 4°C using a bead homogenizer (Fisherbrand Bead Mill 4 Homogenizer) in 2mL tubes containing 5-10 abrasive silicon carbide beads at the highest frequency for 30 seconds, at least 12 times, with 15 second pauses in between. Samples were observed under the microscopeto ensure lysis of worms. Samples were stored at -80°C. Crude protein extracts were spun down at 16,000g for 20 minutes at 4°C, and supernatants were transferred for use. Protein concentrations were measured using ThermoScientific Pierce Detergent Compatible Bradford Assay Reagent and a Nanodrop spectrophotometer.

All samples were normalised to 30 μg of protein. Samples were boiled at 95°C for 10 minutes before being spun down and loaded onto a gel. The gel was run at 80V for 20

minutes to stack samples, then 120V for up to one hour (7.5% resolving gel). Samples were transferred onto a PVDF membrane using semi-dry apparatus (Bio-Rad Trans-Blot SD cell, Cat. # 170-3940), then transferred at 10V for 30 minutes. Membranes were blocked in 7% milk powder and PBS-T or TBS-T for 30-60 minutes at room temperature. Primary antibodies used: Tau-5 (1:1000) (ab80579), AT8 (1:1000) (Invitrogen, MN1020), pS422 (1:10000) (Abcam, ab79415), pS198 (1:50000) (ab79540), pS404 (1:1000) (D2Z4G), pS396 (1:50000) (ab109390), actin (1:5000) (Sigma, MAB1501). Secondary antibodies used: goat-anti-mouse (1:10000) (Invitrogen, G-21040), goat-anti-rabbit (Invitrogen, G-21234). Blots were imaged using Clarity Max Western ECL Substrate (BioRad) and BioRad ChemiDoc XRS+ system. Blots were quantified using ImageJ using the grey averaging technique.

Using WormLab Solid Tracker for C. elegans movement assay in response to RNAi

NGM was supplemented with carbenicillin (25 μg/mL) and 1 mM isopropyl β-D- 1-thiogalactopyranoside (IPTG). RNAi bacteria from the Ahringer library (Kamath and Ahringer, 2003) were streaked out onto LB/Amp (50 μg/mL) plates for further use. Bacteria were inoculated and grown at 37°C overnight in LB supplemented with ampicillin (50 μg/mL). Overnight cultures were subcultured 1/100 in fresh LB/Amp for 6 hours. 200μL were seeded on prepared media to grow on plates at 37°C overnight. One 60mm plate was used per gene knockdown. Eggs from RNAi-sensitized AP-tau animals were collected and plated. A MBF Bioscience WormLab Imaging System was used to record three-minute videos for each condition at 7.5 FPS. Videos underwent tracking, and the tracking summary was exported to an Excel file. Subjects were filtered to only include objects that were tracked for 120-180 seconds. The speed (um/s) [track length/time] values of individual subjects

were used per condition for one-way ANOVA statistical testing with Dunnett's test, using empty vector as control. Two separate trials were performed as biological replicates, the first replicate using 30 subjects, and the second replicate using 50subjects per condition.

Bacterial supernatant paralysis assay

60 mm Petri plates were prepared with 1/4BHI, then seeded with 50 µL of bacteria in triplicate, and incubated overnight at 37°C, 5% CO_2. *C. elegans* CL4176 worms and eggs were washed off maintenance plates with M9 buffer (3 g/L KH_2PO_4 (Anachemia), 6 g/L Na_2HPO_4 (Anachemia), 5 g/L NaCl (Sigma-Aldrich), 1 ml/L 1M $MgSO_4$ (Anachemia)), then bleach synchronized with bleach mix (20% sodium hypochlorite solution (Fisher Chemical) and 10% 5M NaOH diluted in ddH_2O). Bacterial supernatants from separate overnight cultures were obtained using a syringe and 0.2 µm filter. 300 µL of each supernatant was added to plates with lawns of *E. coli* OP50. Approximately 150-200 isolated eggs were then plated on each experimental plate and incubated at the permissive temperature of 15°C for 48 hours. Plates are then upshifted to 25°C for another 48 hours. At 48 hours at 25°C, each experimental plate is scored under compound microscope, where worms exhibiting paralysis were counted then divided by number of total worms for calculation of percentage of paralysis. Plates are replaced back at 25°C for another 24 hours, then were scored again at a total of 72 hours at 25°C. Animals with either absolutely no movement or no movement except for the head were scored as paralysed.

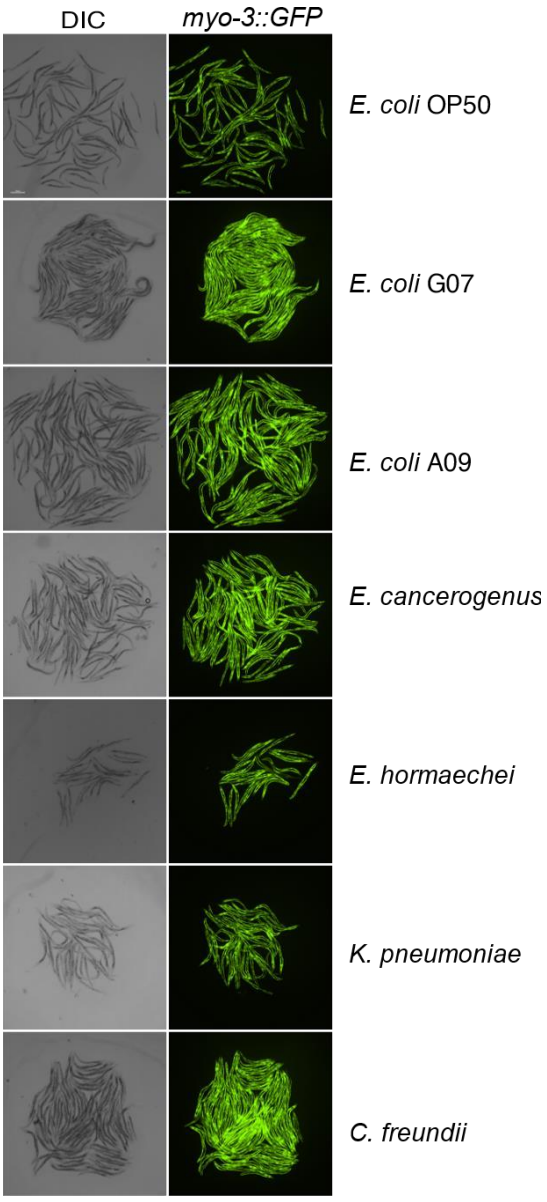
Making bacterial extracts and testing impact on neurodegeneration

The solid media methanol extraction protocol was adapted from "Standard Protocol – Reproduction of Natural Product Library Extracts" (Perry et al., 2015). Bacteria were inoculated from a frozen stock into BHI liquid media. 300 µL of overnight culture was seeded onto 10 cm ¼ BHI plates, one plate per bacterium. Plates were incubated at 37°C

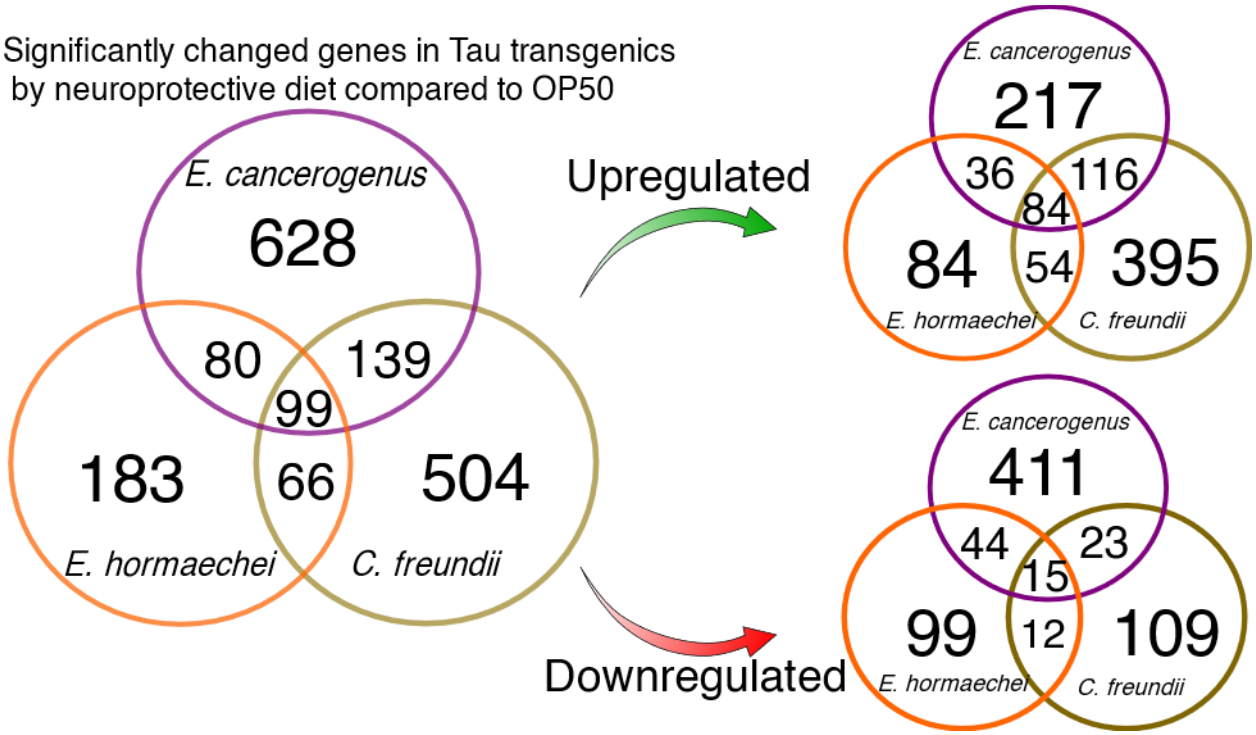
overnight. Bacteria and media were broken up using a sterile scoop and transferred into a sterile 50 mL syringe. Bacteria and media were pushed through the syringe into a 50 mL Falcon tube to create a slurry. 100% methanol was added to the slurry and incubated at 20°C with shaking overnight. Using a milk filter disk, slurries were filtered into glass test tubes, and samples were vacuum-dried using Genevac (Genevac Ltd). Dried extracts were dissolved in 100% DMSO (VWRV0231) and left at room temperature for 48-72 hours. Extracts were transferred to Eppendorf tubes and spun down at 13,200 rpm for 5 minutes and stored at -80°C. Supernatants of crude extracts were used for experiments.

60 mm ¼ BHI plates were poured with exact volumes of 10 mL each. For each test condition, 44 µL of *E. coli* OP50 overnight culture was supplemented with 6 µL extract, then the mixture was seeded onto plates for experiments. Populations of AP-tau LMN008 were bleach-synchronised, and approximately 400 LMN008 eggs were pipetted onto corresponding experimental plates. Plates were incubated at 20℃ until worms were developed to adulthood (day 1 adult). Worms were then paralyzed on the slide with 0.1% sodium azide, then GFP fluorescence was observed and captured using a Nikon Eclipse Ni-U. All experiments were performed as three separate trials. Animals were scored as exhibiting neurodegeneration when displaying blebbed, wavy, or discontinuous axons, as reported in (Wu et al., 2015).

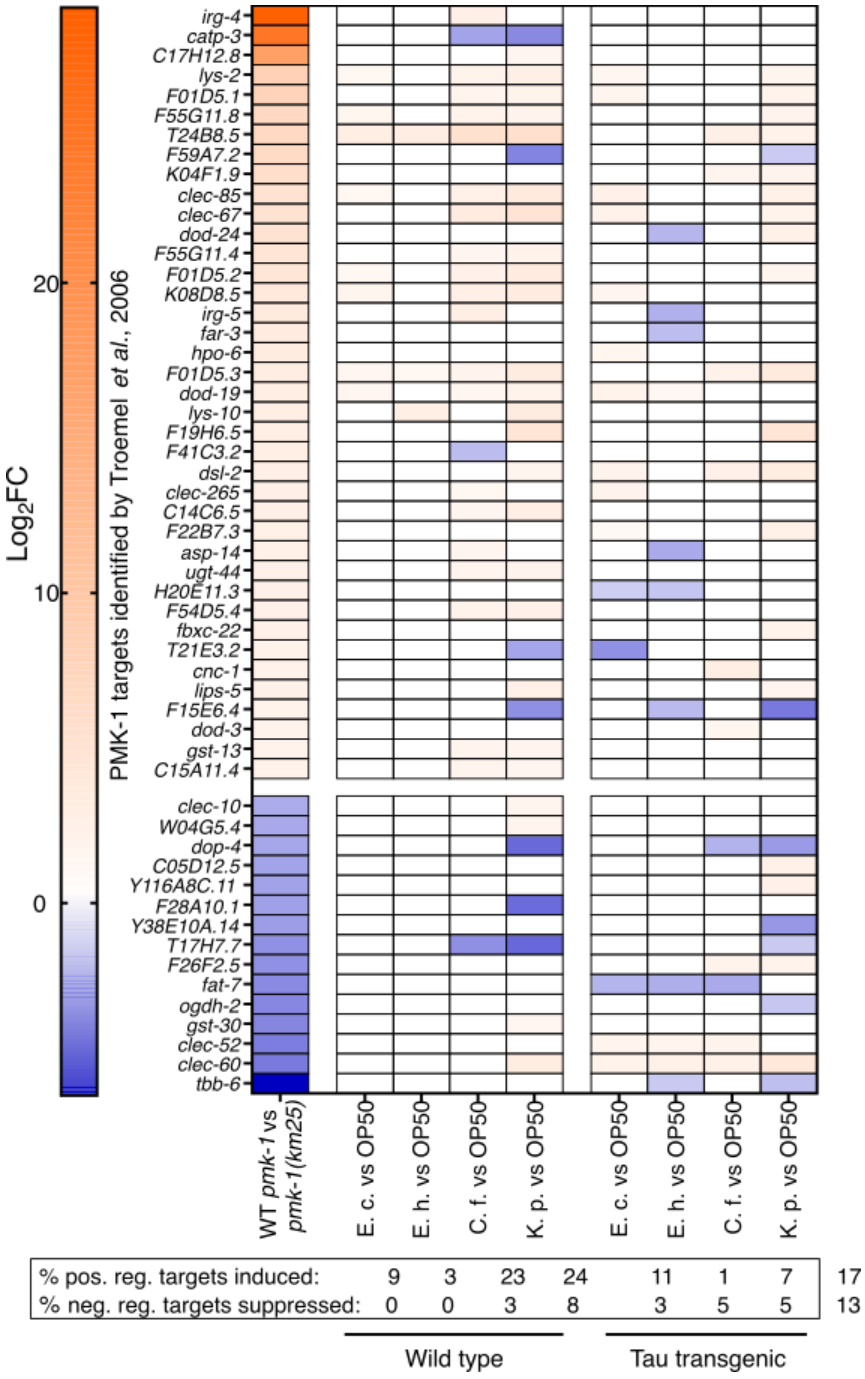
Supplementary



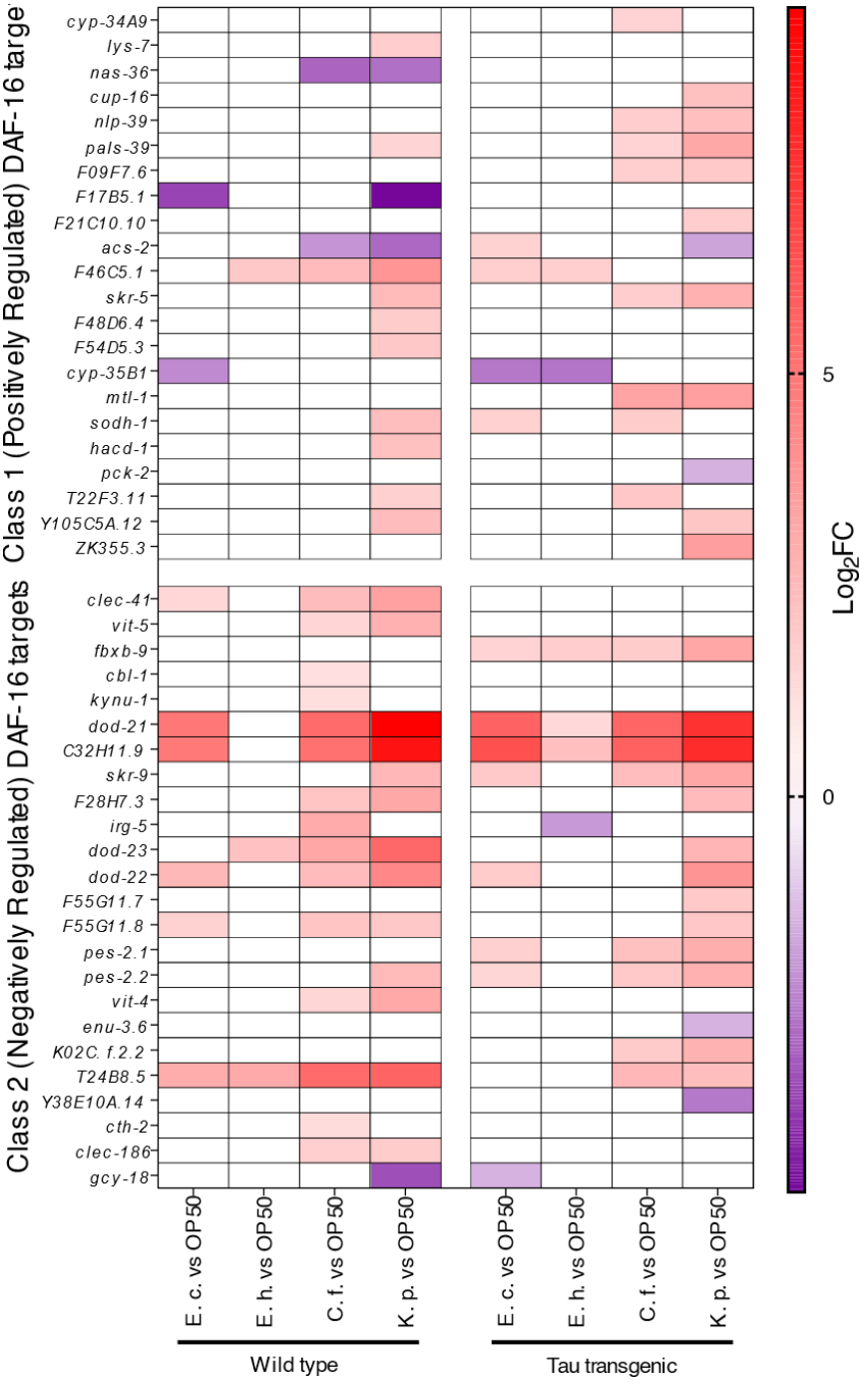
Supplementary Figure 1. *C. elegans* CL2179 exposed to protective microbiota species.



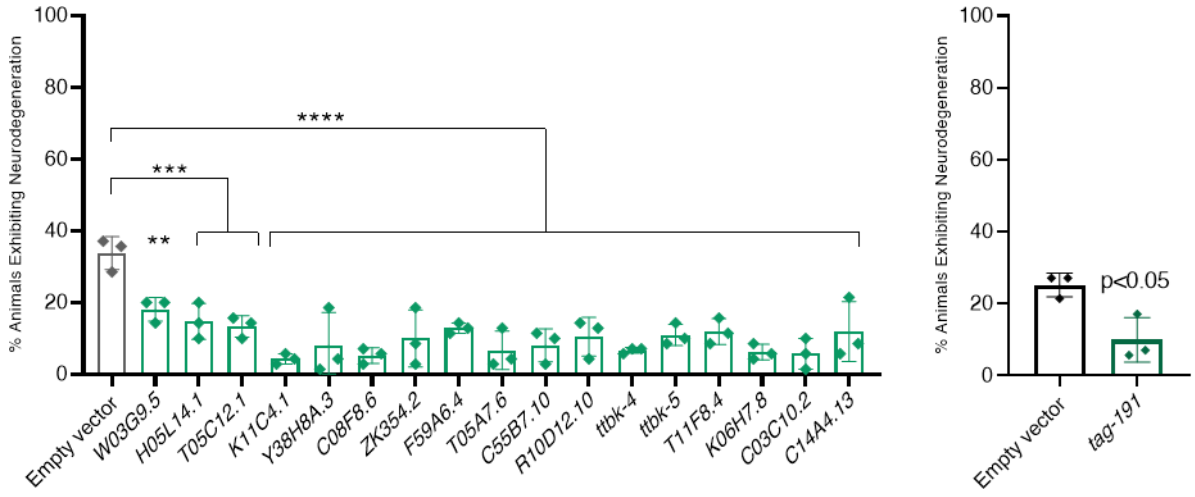
Supplementary Figure 2. Number of regulated genes from RNA-Seq of AP-tau BR5706 *C. elegans* fed neuroprotective microbiota species.



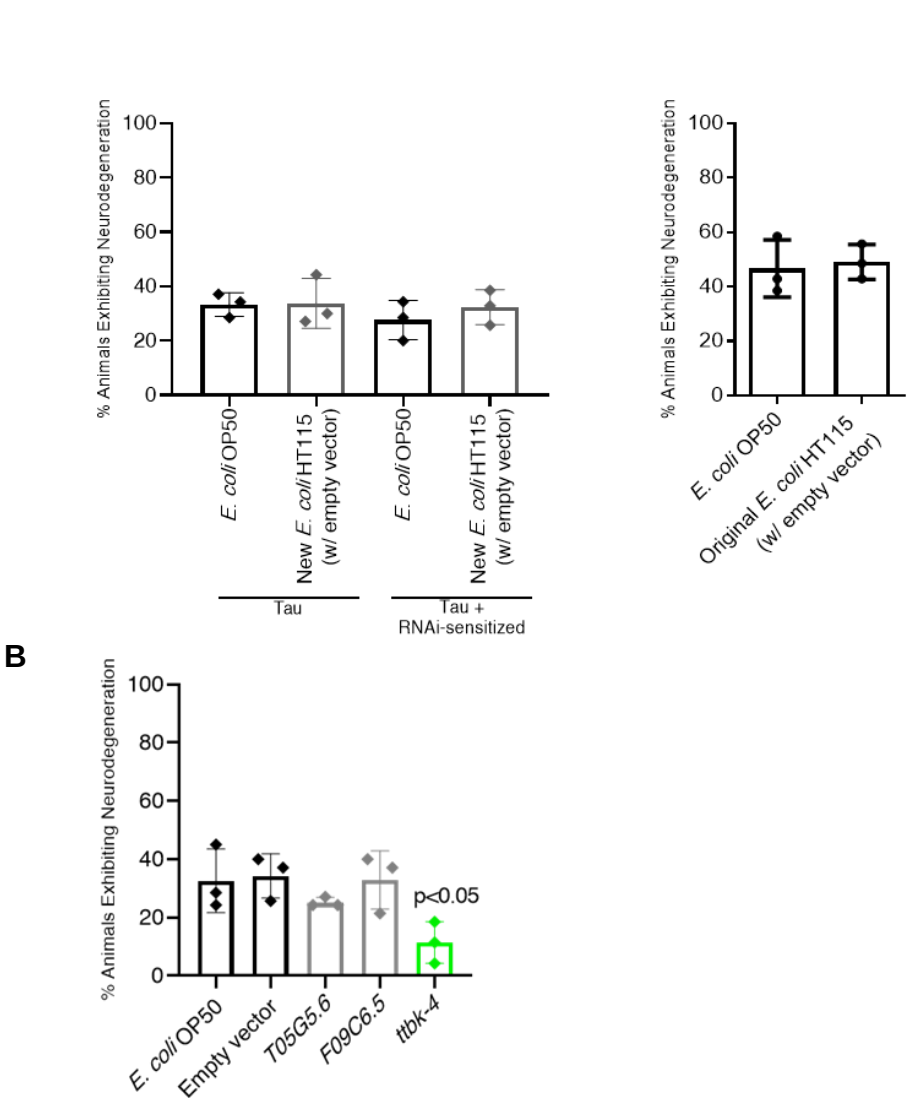
Supplementary Figure 3. Bacteria-induced changes in expression of PMK-1 targets in *C. elegans*.



Supplementary Figure 4. Bacteria-induced changes in expression of DAF-16 targets in *C. elegans*.



Supplementary Figure 5. Neuroprotection induced by TTBK knockdown occurs in neuronal RNAi-sensitized AP-tau *C. elegans*. Each graph point represents a population of >60 animals from separate trials scored on different days. One-way ANOVA performed with Dunnett's multiple comparisons test to compare neurodegeneration levels caused by *ttbk* RNAi to empty vector. **=p<0.01, ***=p<0.001, ****=p<0.0001. Bars represent standard deviation of the mean.



Supplementary Figure 6. Neither the (A) source of *E. coli* HT115, nor the (B) general triggering of RNAi, inadvertently trigger neuroprotection in AP-tau *C. elegans*

Supplementary Table 1. Lifespan assays raw data across trials

		Trial 1				Trial 2				Trial 3			
Genetic Background	Bacterial Diet	Median Survival (days)	Total subjects at T_0	Number of deaths	Number censored	Median Survival (days)	Total subjects at T_0	Number of deaths	Number censored	Median Survival (days)	Total subjects at T_0	Number of deaths	Number censored
Wildtype (*glp-1(g60)*)	*E. coli* OP50	13	356	319	37	15	147	103	44				
	E. coli G07	9	401	357	44	10	249	192	57				
	E. coli A09	8	396	357	39	9	276	229	47				
	E. cancerogenus	8	494	406	88	10	265	208	57				
	E. hormaechei	8	437	380	57	9	275	237	38				
	C. freundii	8	427	407	20	10	282	231	51				
	K. pneumoniae	8	320	298	22	9	275	250	25				
Tau transgenic (Fig. 3A)	*E. coli* OP50	8	919	557	362	8	318	258	60				
	E. coli G07	5	886	649	237	7	281	207	74				
	E. coli A09	5	703	512	191	7	379	287	92				
	E. cancerogenus	4	687	548	139	5	206	193	13				
	E. hormaechei	4	936	742	194	6	248	224	24				
	C. freundii	5	567	464	103	5	347	278	69				
	K. pneumoniae	3	711	617	94	4	353	329	24				
Tau transgenic + *pmk-1* KO (Fig. 3B)	*E. coli* OP50	7	233	134	99	6	253	100	153	6	290	164	126
	E. cancerogenus	5	188	102	86	3	197	153	44				
	E. hormaechei	5	212	108	104	3	186	118	68				
	C. freundii	6	204	130	74	4	267	215	52	6	236	140	96
	K. pneumoniae	5	190	119	71	3	136	91	45	5	285	283	102

Supplementary Table 2. WormCat categories enriched from significant genes that were differentially regulated in AP-tau animals provided neuroprotective bacterial diets.

WormCat Analysis of Significantly Changed Genes in Tau Transgenic C. elegans in response to bacteria

Category Number	Categories upregulated in response to *E. cancerogenus*	# genes from gene set	Total # genes assigned to category	Corrected p-value	PValue
1	Proteolysis proteasome	63	738	2.19E-24	8.77E-26
	Extracellular material	44	485	2.84E-18	1.14E-19
	Stress response	46	810	5.08E-12	2.03E-13
2	Proteolysis proteasome: E3	63	598	1.73E-28	2.54E-30
	Extracellular material: collagen	35	185	2.05E-23	3.02E-25
	Stress response: heat	8	30	4.13E-06	6.07E-08
	Stress response: C-type Lectin	18	257	1.11E-05	1.64E-07
	Stress response: pathogen	13	177	3.28E-04	4.83E-06
3	Proteolysis proteasome: E3: F box	60	445	2.79E-32	3.10E-34
	Extracellular material: collagen	35	185	2.71E-23	3.02E-25
	Stress response: heat	8	30	5.46E-06	6.07E-08
	Stress response: C-type Lectin	18	257	1.47E-05	1.64E-07
	Stress response: pathogen: CUB	5	25	5.75E-03	6.38E-05

Category Number	Categories upregulated in response to *E. hormaechei*	# genes from gene set	Total # genes assigned to category	Corrected p-value	PValue
1	Proteolysis proteasome	38	738	6.90E-16	3.63E-17
	Stress response	31	810	5.23E-10	2.75E-11
2	Proteolysis proteasome: E3	38	598	2.52E-18	5.36E-20
	Stress response: heat	7	30	1.01E-06	2.15E-08
	Stress response: C-type Lectin	10	257	4.38E-03	9.31E-05
3	Proteolysis proteasome: E3: F box	36	445	1.98E-20	3.04E-22
	Stress response: heat	7	30	1.40E-06	2.15E-08
	Proteolysis general: aspartate: cathepsin	4	22	3.81E-03	5.85E-05
	Stress response: C-type Lectin	10	257	6.05E-03	9.31E-05

Category Number	Categories upregulated in response to *C. freundii*	# genes from gene set	Total # genes assigned to category	Corrected p-value	PValue
1	Proteolysis proteasome	85	738	1.11E-30	4.82E-32
	Stress response	57	810	2.80E-12	1.22E-13
	Unknown	270	8160	1.30E-08	5.67E-10
2	Proteolysis proteasome: E3	85	598	4.02E-36	5.59E-38
	Unknown	270	8160	4.08E-08	5.67E-10
	Stress response: pathogen	20	177	5.03E-07	6.98E-09
	Stress response: heat	6	30	6.09E-03	8.46E-05
3	Proteolysis proteasome: E3: F box	79	445	1.73E-39	1.61E-41
	Stress response: pathogen: caenacin	6	11	8.04E-05	7.52E-07
	Unknown	185	5830	9.55E-05	8.92E-07
	Unknown: regulated by multiple stresses	82	2248	6.99E-04	6.54E-06
	Stress response: heat	6	30	9.05E-03	8.46E-05

Category Number	Categories upregulated in response to *K. pneumoniae*	# genes from gene set	Total # genes assigned to category	Corrected p-value	PValue
1	Proteolysis proteasome	183	738	1.57E-62	5.80E-64
	Unknown	587	8160	1.77E-18	6.55E-20
	Stress response	99	810	7.27E-15	2.69E-16
2	Proteolysis proteasome: E3	182	598	4.26E-73	4.21E-75
	Unknown	587	8160	6.61E-18	6.55E-20
	Stress response: pathogen	36	177	3.12E-10	3.09E-12
	Non-coding RNA: linc	24	174	5.68E-04	5.63E-06
	Stress response: C-type Lectin	30	257	8.95E-04	8.86E-06
	Stress response: heat	9	30	3.29E-03	3.26E-05
3	Proteolysis proteasome: E3: F box	169	445	1.36E-79	9.10E-82
	Unknown	403	5830	5.58E-11	3.75E-13
	Unknown: regulated by multiple stresses	181	2248	1.74E-09	1.17E-11
	Stress response: pathogen: other	20	96	1.85E-05	1.24E-07
	Non-coding RNA: linc	24	174	8.38E-04	5.63E-06
	Stress response: C-type Lectin	30	257	1.32E-03	8.86E-06
	Stress response: heat	9	30	4.85E-03	3.26E-05

Category Number	Categories downregulated in response to *E. cancerogenus*	# genes from gene set	Total # genes assigned to category	Corrected p-value	PValue
1	DNA	20	178	2.30E-09	7.66E-11
	Unknown	194	8160	4.51E-05	1.50E-06
	Stress response	30	810	1.27E-03	4.22E-05
2	Stress response: detoxification	17	151	1.69E-07	1.83E-09
	DNA: repair	11	67	3.67E-06	3.99E-08
	Extracellular material: chitinase	9	38	3.99E-06	4.34E-08
	Unknown	194	8160	1.38E-04	1.50E-06
3	Unknown: regulated by multiple stresses	84	2248	4.84E-09	3.69E-11
	DNA: repair	11	67	5.23E-06	3.99E-08
	Extracellular material: chitinase	9	38	5.68E-06	4.34E-08
	Stress response: detoxification: ugt	9	69	4.91E-04	3.74E-06
	Signaling: phosphatase: Y	10	91	5.87E-04	4.48E-06

Category Number	Categories downregulated in response to *E. hormaechei*	# genes from gene set	Total # genes assigned to category	Corrected p-value	PValue
1	Stress response	27	810	6.80E-11	3.40E-12
	Metabolism	23	1577	1.90E-03	9.50E-05
2	Stress response: detoxification	9	151	2.03E-05	4.42E-07
	Stress response: pathogen	7	177	4.55E-03	9.89E-05
3	Stress response: detoxification: CYP	6	82	7.55E-04	1.20E-05

Category Number	Categories downregulated in response to *C. freundii*	# genes from gene set	Total # genes assigned to category	Corrected p-value	PValue
1	Stress response	18	810	1.50E-05	7.87E-07
	Extracellular material	12	485	3.15E-04	1.66E-05
2	Extracellular material: matrix	6	67	1.10E-04	2.39E-06
	Stress response: C-type Lectin	9	257	6.10E-04	1.33E-05
3	Extracellular material: matrix	6	67	1.39E-04	2.39E-06
	Stress response: C-type Lectin	9	257	7.69E-04	1.33E-05

Category Number	Categories downregulated in response to *K. pneumoniae*	# genes from gene set	Total # genes assigned to category	Corrected p-value	PValue
1	Extracellular material	70	485	4.10E-24	1.41E-25
	Transmembrane transport	74	861	6.07E-14	2.09E-15
	Metabolism	88	1577	3.08E-07	1.06E-08
	Signaling	65	1193	3.92E-05	1.35E-06
	Cytoskeleton	29	369	6.12E-05	2.11E-06
2	Extracellular material: collagen	43	185	3.31E-21	2.52E-23
	Signaling: hedgehog-like	19	90	1.65E-08	1.26E-10
	Cytoskeleton: cadherin	6	11	4.75E-04	3.63E-06
	Transmembrane transport: calcium channel	7	20	7.39E-04	5.64E-06
	Metabolism: lipid	35	525	8.58E-04	6.55E-06
	Extracellular material: matrix	11	67	1.05E-03	8.00E-06
	Cytoskeleton: other	11	77	3.37E-03	2.57E-05
	Transmembrane transport: ABC	9	51	4.15E-03	3.17E-05
	Metabolism: 1CC	7	30	6.76E-03	5.16E-05
3	Extracellular material: collagen	43	185	5.10E-21	2.52E-23
	Signaling: hedgehog-like	19	90	2.55E-08	1.26E-10
	Cytoskeleton: cadherin	6	11	7.32E-04	3.63E-06
	Transmembrane transport: calcium channel	7	20	1.14E-03	5.64E-06
	Extracellular material: matrix	11	67	1.62E-03	8.00E-06
	Non-coding RNA: small RNA: argonaute	7	22	1.91E-03	9.45E-06
	Cytoskeleton: other	11	77	5.19E-03	2.57E-05
	Transmembrane transport: ABC	9	51	6.41E-03	3.17E-05

Supplementary Table 3. Log_2fold change values of reproduced and significant genes from RNA-Seq of wild-type *C. elegans* provided neuroprotective bacterial diets. Lists ordered by ranking top 100 up- and down-regulated genes in response to each diet.

Top <u>upregulated</u> reproduced genes in response to *E. cancerogenus*

WBID	Gene name	Log2FC 2018	Log2FC 2019	Avg Log2FC
WBGene00007873	dod-21	4.278656034	5.62549537	4.9520757
WBGene00007872	C32H11.9	5.148542996	4.60557926	4.877061129
WBGene00011979	T24B8.5	3.199434191	2.83539989	3.01741704
WBGene00001477	fmo-2	2.404199633	3.20148278	2.802841209
WBGene00044376	Y49F6B.12	1.542350891	3.75969052	2.651020708
WBGene00010125	dod-22	2.639826405	2.54558178	2.592704094
WBGene00018601	F48C1.9	1.381737926	2.76830148	2.075019702
WBGene00019563	K09C6.9	2.000685338	2.10283195	2.051758646
WBGene00019914	clec-150	1.328701632	2.63125837	1.979980001
WBGene00012583	clec-4	1.563189433	2.16681007	1.864999751
WBGene00017294	F09E10.1	1.871828589	1.83182788	1.851828237
WBGene00000670	col-95	1.640611294	2.05510976	1.847860529
WBGene00018643	drd-50	1.428177641	2.22103243	1.824605036

WBGene00010659	K08D8.5	1.396525244	2.1075899	1.752057574
WBGene00012457	acbp-6	1.020905989	2.46056463	1.740735308
WBGene00023493	nspc-12	1.618289577	1.85160014	1.73494486
WBGene00009488	oac-20	1.531128086	1.81277379	1.671950938
WBGene00001786	gst-38	1.230722302	2.08345856	1.657090433
WBGene00013931	clec-97	1.25687415	2.04071518	1.648794665
WBGene00010128	F55G11.8	1.460641839	1.79343153	1.627036687
WBGene00008360	nspc-19	1.132220936	2.0882837	1.610252318
WBGene00011190	swt-6	1.175702955	2.03516943	1.605436193
WBGene00010745	dod-17	1.37746779	1.81511494	1.596291367
WBGene00017376	F11C7.2	1.10981941	2.03405715	1.571938281
WBGene00020052	R13A5.10	0.811465587	2.28825882	1.549862206
WBGene00021797	plep-1	1.32377286	1.76855838	1.546165618
WBGene00009397	clec-66	1.304498107	1.7196388	1.512068452
WBGene00008494	F01D5.3	1.170248747	1.8109738	1.490611271
WBGene00206386	K10C2.12	1.03334576	1.90041195	1.466878857
WBGene00007153	clec-41	1.027603641	1.88632052	1.45696208

WBGene00022644	dod-19	0.915240991	1.9890888	1.452164894
WBGene00009640	nspc-10	1.260624922	1.61731232	1.438968623
WBGene00021872	clec-85	1.006577191	1.82788612	1.417231654
WBGene00008493	F01D5.2	1.03912666	1.7427707	1.39094868
WBGene00001775	gst-27	0.802641926	1.96194094	1.382291432
WBGene00003091	lys-2	0.804588461	1.91657668	1.360582571
WBGene00008304	C54D10.10	0.723260096	1.89576397	1.309512035
WBGene00016658	C45B2.1	0.805155748	1.75839294	1.281774346
WBGene00021619	Y47D7A.6	0.888951066	1.62375336	1.256352215
WBGene00006533	tba-7	0.795265433	1.71343676	1.254351097
WBGene00014244	ZK1307.1	0.776083182	1.41922856	1.097655872

Top <u>downregulated</u> reproduced genes in response to *E. cancerogenus*

WBID	Gene name	Log2FC 2018	Log2FC 2019	Avg Log2FC
WBGene00009706	argk-1	-6.33692675	-8.81373174	-7.57532924
WBGene00010928	clec-258	-2.21608362	-7.05089521	-4.63348941
WBGene00006075	str-7	-2.45804766	-5.35372181	-3.90588474
WBGene00008905	F17B5.1	-1.97989249	-5.05077395	-3.51533322
WBGene00003995	pgp-1	-4.575799	-1.89139133	-3.23359517
WBGene00009048	cth-1	-2.78558252	-3.37924791	-3.08241522
WBGene00011800	T16G1.6	-3.24658373	-2.89261292	-3.06959832
WBGene00021007	W03F11.4	-1.87517176	-3.58768327	-2.73142751
WBGene00009471	F36D3.5	-2.14552531	-3.20252899	-2.67402715
WBGene00007381	C06C6.7	-1.39853247	-3.80916624	-2.60384936
WBGene00009149	F26D2.10	-1.48691077	-3.70975587	-2.59833332
WBGene00013771	Y113G7C.1	-1.58870794	-3.54123997	-2.56497395
WBGene00011798	T16G1.4	-2.9553304	-2.11899212	-2.53716126
WBGene00019785	M70.3	-1.68582444	-3.36676468	-2.52629456
WBGene00021969	Y57G7A.6	-1.91082492	-3.11168788	-2.5112564

WBGene00019586	K09F6.3	-1.88114623	-3.07908766	-2.48011694
WBGene00018134	F37A4.4	-1.90173464	-3.03632136	-2.469028
WBGene00019216	H20J04.1	-1.23539604	-3.68105768	-2.45822686
WBGene00012115	T28B8.4	-1.78480465	-3.10710035	-2.4459525
WBGene00002204	kin-21	-1.58468015	-3.29386501	-2.43927258
WBGene00010933	M162.7	-1.65734443	-3.14187416	-2.39960929
WBGene00022090	Y69A2AR.19	-1.98980782	-2.72166256	-2.35573519
WBGene00000133	amt-1	-2.84277914	-1.86447907	-2.35362911
WBGene00020353	T08B6.4	-1.7773412	-2.86059306	-2.31896713
WBGene00014179	ZK1010.5	-1.75132785	-2.87710472	-2.31421629
WBGene00007946	ugt-33	-2.26499446	-2.34185183	-2.30342315
WBGene00004909	snf-10	-1.55464998	-3.00831711	-2.28148355
WBGene00019089	clec-206	-2.0208102	-2.52571509	-2.27326264
WBGene00012689	Y39B6A.30	-1.80734595	-2.65078864	-2.2290673
WBGene00015795	C15F1.2	-1.8779842	-2.54593108	-2.21195764
WBGene00019472	cyp-35B1	-1.6011962	-2.79621851	-2.19870735
WBGene00011910	alg-3	-1.59759396	-2.79046679	-2.19403038

WBGene00000019	abt-1	-1.34898847	-3.01608517	-2.18253682
WBGene00011671	cyp-13A4	-1.76719826	-2.5568371	-2.16201768
WBGene00000485	che-3	-1.23910433	-3.06691982	-2.15301207
WBGene00018548	clec-79	-1.64696068	-2.65801715	-2.15248891
WBGene00020414	T10E9.4	-1.50526863	-2.79863497	-2.1519518
WBGene00007248	catp-4	-1.65079237	-2.64367157	-2.14723197
WBGene00020289	T06C10.3	-1.39443356	-2.87900926	-2.13672141
WBGene00014238	ZK1225.4	-1.34663192	-2.8564724	-2.10155216
WBGene00001839	hdl-1	-1.55495351	-2.6169671	-2.0859603
WBGene00014102	best-25	-1.28782728	-2.88256677	-2.08519702
WBGene00019783	M70.1	-1.48383519	-2.66248293	-2.07315906
WBGene00011191	R10D12.10	-1.34387371	-2.7555067	-2.0496902
WBGene00021579	clec-73	-1.56097504	-2.53601336	-2.0484942
WBGene00019337	K02F6.3	-1.55064292	-2.53192865	-2.04128578
WBGene00015026	B0207.1	-1.37535088	-2.6967489	-2.03604989
WBGene00010915	M110.7	-1.94199993	-2.12795791	-2.03497892
WBGene00021678	Y48G1C.5	-1.41120651	-2.65601585	-2.03361118

WBGene00017177	F02E8.4	-0.99544076	-3.06191175	-2.02867625
WBGene00020707	wago-10	-1.36500462	-2.68305358	-2.0240291
WBGene00003026	lin-41	-0.92327884	-3.10308052	-2.01317968
WBGene00011173	acs-18	-1.55762795	-2.46331654	-2.01047224
WBGene00013651	Y105C5B.11	-1.41332573	-2.60410973	-2.00871773
WBGene00020223	T05A7.6	-1.38465599	-2.62996706	-2.00731152
WBGene00017554	nep-9	-1.66752382	-2.30225564	-1.98488973
WBGene00004904	snf-5	-1.54528218	-2.4000846	-1.97268339
WBGene00021016	W03G9.5	-1.24894433	-2.68615783	-1.96755108
WBGene00013700	Y106G6D.3	-1.33372916	-2.59832243	-1.9660258
WBGene00001414	fer-1	-1.29342642	-2.63599484	-1.96471063
WBGene00019086	F59A6.4	-1.16641525	-2.75716784	-1.96179155
WBGene00009708	F44G3.7	-1.33330204	-2.57676049	-1.95503127
WBGene00004969	spe-15	-1.5549177	-2.33861668	-1.94676719
WBGene00007081	AH6.3	-1.26312919	-2.62761766	-1.94537343
WBGene00016753	oac-9	-1.68828626	-2.18988193	-1.9390841
WBGene00011918	T22C1.8	-1.50240703	-2.36902883	-1.93571793

WBGene00014197	ZK1053.2	-1.5288448	-2.33529903	-1.93207191
WBGene00020116	moa-1	-1.2635495	-2.60016958	-1.93185954
WBGene00006449	alg-4	-1.44185839	-2.421648	-1.93175319
WBGene00007448	C08F8.6	-1.14922007	-2.6696481	-1.90943409
WBGene00007791	C28A5.6	-1.1681035	-2.64362999	-1.90586675
WBGene00012595	Y38E10A.17	-1.29630551	-2.51321052	-1.90475802
WBGene00009884	F49C12.15	-1.42126082	-2.3830806	-1.90217071
WBGene00019015	F57F4.1	-1.50208946	-2.2875664	-1.89482793
WBGene00004901	snf-2	-1.64696823	-2.11301804	-1.87999313
WBGene00012684	Y39B6A.25	-1.38707056	-2.37056993	-1.87882024
WBGene00010992	R03D7.8	-1.43804157	-2.31717946	-1.87761051
WBGene00016085	C25A8.5	-1.53243911	-2.20999232	-1.87121572
WBGene00016675	C45G9.4	-1.55165848	-2.18935295	-1.87050571
WBGene00011114	R07E5.6	-1.46168401	-2.27247575	-1.86707988
WBGene00019920	acs-15	-1.18671748	-2.53940141	-1.86305944
WBGene00008230	pfk-1.2	-1.2817877	-2.41938326	-1.85058548
WBGene00004976	spe-41	-1.63109862	-2.02216689	-1.82663276

WBGene00017179	wht-4	-1.48628772	-2.15398629	-1.82013701
WBGene00021288	clec-123	-1.6150866	-2.01360509	-1.81434584
WBGene00017802	F26A1.3	-1.1297565	-2.48675712	-1.80825681
WBGene00012010	T25B9.4	-1.63152757	-1.98218476	-1.80685617
WBGene00010241	F58D2.2	-1.47112519	-2.141119	-1.8061221
WBGene00019997	R10F2.6	-1.01765413	-2.5854083	-1.80153122
WBGene00020053	R13A5.11	-1.18617035	-2.3998109	-1.79299062
WBGene00017955	F31E8.5	-1.5242738	-2.05962793	-1.79195087
WBGene00008063	C41G7.6	-1.2785715	-2.30440001	-1.79148576
WBGene00008508	F01G10.5	-1.24692841	-2.32571082	-1.78631961
WBGene00010920	M117.4	-1.31419767	-2.2527715	-1.78348459
WBGene00019257	dhhc-13	-1.64373078	-1.90525915	-1.77449497
WBGene00006409	hdl-2	-1.5582792	-1.98018854	-1.76923387
WBGene00014239	ZK1225.5	-1.26905909	-2.25276623	-1.76091266
WBGene00018898	F55F10.1	-1.01021034	-2.51100477	-1.76060756
WBGene00020533	T16A1.2	-1.49515338	-2.01130596	-1.75322967
WBGene00011478	T05D4.5	-0.93388021	-2.54901757	-1.74144889

Top <u>upregulated</u> reproduced genes in response to *E. hormaechei*

WBID	Gene name	Log2FC 2018	Log2FC 2019	Avg Log2FC
WBGene00016670	ilys-3	4.20271752	3.11792978	3.66032365
WBGene00007758	C27A7.8	2.4836314	4.53752979	3.51058059
WBGene00044376	Y49F6B.12	2.58772344	4.18910692	3.38841518
WBGene00011979	T24B8.5	2.40506812	3.78774781	3.09640797
WBGene00010256	hrg-3	2.87258596	3.23175879	3.05217238
WBGene00000670	col-95	2.00797159	3.94010506	2.97403833
WBGene00016669	ilys-2	3.29302254	2.63494022	2.96398138
WBGene00003099	lys-10	1.81928942	3.86282876	2.84105909
WBGene00013334	nlp-56	1.34519753	4.17396328	2.75958041
WBGene00007605	hrg-7	2.31115775	3.03352412	2.67234094
WBGene00008360	nspc-19	1.84217889	3.23586279	2.53902084
WBGene00011046	R05H10.7	2.88495455	2.14784659	2.51640057
WBGene00013931	clec-97	1.20171207	3.62818488	2.41494847
WBGene00021263	Y22D7AR.10	1.41732636	3.31993609	2.36863122
WBGene00015355	C02F12.5	1.84228413	2.86896297	2.35562355

WBGene00009898	dod-23	1.32203846	3.19542119	2.25872983
WBGene00000716	col-143	1.11168019	3.27651422	2.1940972
WBGene00000620	col-43	1.32896738	3.02459539	2.17678139
WBGene00001477	fmo-2	1.18504276	3.16463953	2.17484115
WBGene00189949	Y48G1C.13	1.50783003	2.80579519	2.15681261
WBGene00019914	clec-150	1.48467203	2.77617208	2.13042205
WBGene00016894	C53B7.3	1.73158473	2.46176933	2.09667703
WBGene00000675	col-101	1.17259364	2.9585783	2.06558597
WBGene00008357	nspc-16	1.97084669	2.15335696	2.06210182
WBGene00000617	col-40	1.40533066	2.68518197	2.04525632
WBGene00017294	F09E10.1	1.78060308	2.29551335	2.03805822
WBGene00009778	F46C5.1	1.1634503	2.80076294	1.98210662
WBGene00008566	acox-1.3	1.39077365	2.5472844	1.96902902
WBGene00021797	plep-1	1.47515789	2.45565994	1.96540891
WBGene00021005	ule-1	1.53705167	2.36944681	1.95324924
WBGene00018601	F48C1.9	1.4297017	2.42415993	1.92693081
WBGene00016434	C35B1.4	0.6866617	3.01055109	1.8486064

WBGene00011103	R07E3.2	1.47454193	2.21973392	1.84713792
WBGene00021070	cpr-8	1.35742855	2.32029909	1.83886382
WBGene00017730	F22H10.6	0.76702381	2.90687138	1.8369476
WBGene00008359	nspc-17	1.2608567	2.36127606	1.81106638
WBGene00011486	T05E12.3	1.01395536	2.59676826	1.80536181
WBGene00009637	nspc-13	1.11481628	2.49168542	1.80325085
WBGene00015495	C05E11.6	1.17188667	2.43253014	1.8022084
WBGene00022191	Y71H2B.1	0.76945096	2.80524704	1.787349
WBGene00023493	nspc-12	1.33027654	2.23954018	1.78490836
WBGene00010174	F56H9.2	1.10185878	2.45145064	1.77665471
WBGene00012688	Y39B6A.29	1.08098347	2.3822495	1.73161649
WBGene00000597	col-8	0.97790172	2.47488569	1.72639371
WBGene00045261	H29C22.1	1.31349771	2.12792576	1.72071173
WBGene00000693	col-119	1.13845554	2.29929154	1.71887354
WBGene00000609	col-20	0.90404648	2.5051284	1.70458744
WBGene00015683	C10G8.4	1.37445269	2.0333701	1.7039114
WBGene00022245	acp-6	0.99735319	2.40397434	1.70066376

WBGene00001444	flp-1	1.392562	1.99130475	1.69193337
WBGene00000754	col-181	0.86978805	2.4900061	1.67989707
WBGene00003380	mnm-2	1.01337338	2.32998024	1.67167681
WBGene00001449	flp-6	1.01121442	2.31231435	1.66176439
WBGene00014103	best-26	1.03206438	2.21486536	1.62346487
WBGene00220076	T21B4.21	1.1512368	2.06050564	1.60587122
WBGene00023477	nspc-1	0.9450079	2.23822655	1.59161723
WBGene00004987	spp-2	1.06408402	1.89087195	1.47747798
WBGene00044696	F52E1.14	0.99991208	1.93481427	1.46736317
WBGene00021135	W10G11.2	1.15455427	1.67560718	1.41508072
WBGene00008494	F01D5.3	0.7383459	2.04132249	1.38983419
WBGene00012457	acbp-6	0.7916784	1.89224146	1.34195993
WBGene00009877	F49C12.7	0.91031072	1.76524831	1.33777952
WBGene00012221	W03C9.5	0.65979895	1.75987647	1.20983771

Top <u>downregulated</u> reproduced genes in response to *E. hormaechei*

WBID	Gene name	Log2FC 2018	Log2FC 2019	Avg Log2FC
WBGene00009706	argk-1	-4.67145254	-6.88452585	-5.7779892
WBGene00010928	clec-258	-3.58826048	-6.5785986	-5.08342954
WBGene00006075	str-7	-2.79465517	-5.77204283	-4.283349
WBGene00206502	T01B7.13	-1.50016419	-6.72831852	-4.11424135
WBGene00018630	F49D11.6	-1.89730673	-6.29575236	-4.09652955
WBGene00010285	F58H1.2	-1.99820862	-6.16656026	-4.08238444
WBGene00003888	osm-8	-2.15121298	-5.77212076	-3.96166687
WBGene00017018	D1014.5	-2.05250815	-5.85538384	-3.953946
WBGene00019154	glf-1	-2.39749354	-5.12542734	-3.76146044
WBGene00012346	W08G11.1	-1.18340762	-6.24138702	-3.71239732
WBGene00012186	mlt-11	-2.69504384	-4.71535826	-3.70520105
WBGene00001691	grd-2	-1.70511783	-5.70320242	-3.70416013
WBGene00004127	abu-12	-1.48899387	-5.80691385	-3.64795386
WBGene00014183	ZK1025.3	-1.81268403	-5.47355001	-3.64311702
WBGene00013171	Y53F4B.25	-1.65356739	-5.49894412	-3.57625575

WBGene00005016	sqt-1	-1.90225555	-5.19658873	-3.54942214
WBGene00010673	K08E7.5	-1.96728913	-5.04367697	-3.50548305
WBGene00050875	bah-1	-2.05164055	-4.95803632	-3.50483844
WBGene00004120	pqn-32	-1.87904548	-5.12472802	-3.50188675
WBGene00003995	pgp-1	-4.43760817	-2.49371128	-3.46565973
WBGene00006952	wrt-6	-2.06413274	-4.85115988	-3.45764631
WBGene00020560	T19C3.2	-1.49398758	-5.38679731	-3.44039245
WBGene00008448	E01G4.6	-1.54437402	-5.32592508	-3.43514955
WBGene00001716	grl-7	-1.64582535	-5.18813394	-3.41697964
WBGene00003057	lon-3	-2.14787624	-4.64714973	-3.39751299
WBGene00007552	C13C12.2	-1.75935085	-4.94113397	-3.35024241
WBGene00022680	ZK180.6	-1.28741944	-5.39229247	-3.33985596
WBGene00012939	subs-4	-1.89524945	-4.777861	-3.33655523
WBGene00017998	F33D4.6	-1.95698056	-4.71506448	-3.33602252
WBGene00000251	bli-1	-2.36298016	-4.29948362	-3.33123189
WBGene00004264	qua-1	-2.31249437	-4.33997579	-3.32623508
WBGene00004133	abu-13	-1.95789763	-4.66623625	-3.31206694

WBGene00007996	C38C6.3	-1.44729496	-5.15115096	-3.29922296
WBGene00000623	col-46	-1.96231285	-4.57727174	-3.2697923
WBGene00000252	bli-2	-2.11346088	-4.4213617	-3.26741129
WBGene00020194	T03G6.1	-1.82340764	-4.68274329	-3.25307546
WBGene00000639	col-63	-1.32013825	-5.17057149	-3.24535487
WBGene00005017	sqt-2	-1.6121605	-4.85825484	-3.23520767
WBGene00008150	mam-2	-1.57186634	-4.85807337	-3.21496985
WBGene00018737	F53B1.4	-1.30750593	-5.08229126	-3.1948986
WBGene00019565	cyp-35A3	-1.96349207	-4.38079252	-3.17214229
WBGene00011530	T06D8.10	-1.48535924	-4.85284831	-3.16910378
WBGene00012257	lpr-4	-2.61850015	-3.71731656	-3.16790835
WBGene00006950	wrt-4	-1.31776033	-4.98851249	-3.15313641
WBGene00018573	oac-30	-0.93688571	-5.32758145	-3.13223358
WBGene00044109	K02E11.10	-1.40532174	-4.85299585	-3.12915879
WBGene00015646	mlt-10	-1.49537052	-4.72985492	-3.11261272
WBGene00004398	rol-8	-1.67791427	-4.47275822	-3.07533625
WBGene00007177	oac-1	-1.18163172	-4.96803674	-3.07483423

WBGene00000606	col-17	-1.32483704	-4.78596414	-3.05540059
WBGene00011800	T16G1.6	-3.61552976	-2.48320006	-3.04936491
WBGene00000399	cdh-7	-3.13522722	-2.95508381	-3.04515551
WBGene00001714	grl-5	-1.65838984	-4.42458768	-3.04148876
WBGene00015300	C01F1.5	-2.20629047	-3.86942644	-3.03785846
WBGene00007479	C09F9.2	-2.48037106	-3.57183669	-3.02610387
WBGene00018572	lin-42	-0.96812212	-5.06394673	-3.01603443
WBGene00008851	F15B9.8	-1.49122427	-4.53791181	-3.01456804
WBGene00001690	grd-1	-1.74562242	-4.27042622	-3.00802432
WBGene00016786	cyp-35A4	-1.37375542	-4.63136445	-3.00255994
WBGene00019272	H42K12.3	-2.10616798	-3.89002971	-2.99809884
WBGene00138711	R07B1.13	-1.74105555	-4.24637511	-2.99371533
WBGene00004397	rol-6	-1.55633794	-4.41757747	-2.9869577
WBGene00000655	col-79	-1.67899914	-4.26698526	-2.9729922
WBGene00009355	F33A8.7	-0.96966277	-4.97539433	-2.97252855
WBGene00004394	rol-1	-0.96355359	-4.97718925	-2.97037142
WBGene00011665	T09F5.1	-1.89364272	-4.00291496	-2.94827884

WBGene00006954	wrt-8	-1.31314324	-4.56798713	-2.94056518
WBGene00020554	T19A5.3	-0.94850757	-4.88354939	-2.91602848
WBGene00017438	F13H8.5	-1.03297011	-4.7896632	-2.91131666
WBGene00000631	col-54	-1.18669606	-4.62379564	-2.90524585
WBGene00009983	cut-2	-1.14120084	-4.66565984	-2.90343034
WBGene00022504	mam-1	-1.51515669	-4.28855428	-2.90185548
WBGene00000626	col-49	-0.8790992	-4.90736742	-2.89323331
WBGene00009133	bed-3	-1.46169172	-4.31219119	-2.88694145
WBGene00000930	dao-4	-1.89429795	-3.84296088	-2.86862942
WBGene00011952	T23F6.5	-1.96917115	-3.76378358	-2.86647737
WBGene00012791	Y43D4A.5	-2.20256131	-3.52054522	-2.86155327
WBGene00019520	K08B12.1	-2.39487426	-3.32621435	-2.8605443
WBGene00001074	dpy-13	-0.94130578	-4.77756692	-2.85943635
WBGene00013173	Y53F4B.27	-1.09918312	-4.58235191	-2.84076752
WBGene00019089	clec-206	-2.51143987	-3.16922618	-2.84033302
WBGene00000133	amt-1	-2.82626856	-2.82537419	-2.82582138
WBGene00000647	col-71	-0.97407367	-4.67391158	-2.82399262

WBGene00077697	F58E6.13	-1.66106789	-3.96996525	-2.81551657
WBGene00003542	nas-23	-1.01247844	-4.61581529	-2.81414687
WBGene00000705	col-131	-1.40332265	-4.20988718	-2.80660492
WBGene00001724	grl-15	-2.02815282	-3.57556407	-2.80185844
WBGene00000402	cdh-10	-2.28613279	-3.30519781	-2.7956653
WBGene00044163	F49C5.11	-1.52364289	-4.06479548	-2.79421918
WBGene00010345	F59F5.7	-1.37153898	-4.21237572	-2.79195735
WBGene00000615	col-38	-0.95911909	-4.62197692	-2.790548
WBGene00000748	col-175	-1.03060091	-4.54633895	-2.78846993
WBGene00000649	col-73	-0.95199696	-4.58494036	-2.76846866
WBGene00001066	dpy-4	-0.79476994	-4.73060176	-2.76268585
WBGene00000711	col-138	-1.03334269	-4.49202812	-2.76268541
WBGene00021095	mlt-8	-1.37996211	-4.12408007	-2.75202109
WBGene00044617	bus-1	-2.23530699	-3.26745836	-2.75138267
WBGene00008605	mlt-9	-2.01486072	-3.46717396	-2.74101734
WBGene00016069	C24H10.3	-1.21211747	-4.26169261	-2.73690504
WBGene00013852	hhat-1	-1.62387061	-3.84270687	-2.73328874

Top <u>upregulated</u> reproduced genes in response to *C. freundii*

WBID	Gene name	Log2FC 2018	Log2FC 2019	Avg Log2FC
WBGene00007873	dod-21	2.90264539	7.97685967	5.43975253
WBGene00011979	T24B8.5	5.0902449	5.67587748	5.38306119
WBGene00007872	C32H11.9	3.73157018	6.68528783	5.208429
WBGene00017501	pud-3	2.54266356	5.49438482	4.01852419
WBGene00017498	pud-4	2.05652516	5.26352322	3.66002419
WBGene00018643	drd-50	2.60259128	4.62554544	3.61406836
WBGene00000213	asm-3	1.96148778	5.16411585	3.56280182
WBGene00018971	clec-67	3.18317789	3.9343192	3.55874854
WBGene00000670	col-95	3.05314582	3.75578158	3.4044637
WBGene00017294	F09E10.1	3.63304482	2.995531	3.31428791
WBGene00009898	dod-23	2.76358855	3.73686921	3.25022888
WBGene00044212	Y68A4A.13	2.77700982	3.6804724	3.22874111
WBGene00007864	irg-6	2.98805404	3.31663583	3.15234493
WBGene00008360	nspc-19	2.71787535	3.53247802	3.12517668
WBGene00001786	gst-38	2.38788579	3.85632435	3.12210507

WBGene00009429	irg-5	3.91629717	2.18615195	3.05122456
WBGene00013931	clec-97	2.51424667	3.53475479	3.02450073
WBGene00017660	F21C10.11	2.5940587	3.43267249	3.0133656
WBGene00007758	C27A7.8	2.92646774	3.10020137	3.01333456
WBGene00021624	Y47D7A.12	1.90846608	3.9998004	2.95413324
WBGene00019368	K03H6.2	2.29943043	3.56905608	2.93424325
WBGene00021263	Y22D7AR.10	2.6734803	3.12224939	2.89786484
WBGene00008068	sdz-6	2.20456641	3.32516534	2.76486587
WBGene00005002	spp-17	2.53881273	2.9765675	2.75769011
WBGene00008584	irg-4	2.93862099	2.55977838	2.74919968
WBGene00010659	K08D8.5	2.35581974	3.14006367	2.7479417
WBGene00013334	nlp-56	2.15857588	3.31798335	2.73827962
WBGene00000731	col-158	2.05393655	3.39318934	2.72356295
WBGene00219849	C36C9.10	1.21464768	4.20631689	2.71048228
WBGene00009069	F23A7.4	1.04188814	4.32358148	2.68273481
WBGene00235133	T26H5.14	2.20136953	3.15663034	2.67899993
WBGene00008357	nspc-16	2.29455915	3.03439198	2.66447556

WBGene00008359	nspc-17	2.36853285	2.95124023	2.65988654
WBGene00010769	asah-1	2.18946242	3.00425308	2.59685775
WBGene00021236	pud-1.2	1.39238226	3.78142235	2.5869023
WBGene00007867	C32H11.4	2.65530146	2.51772043	2.58651095
WBGene00023493	nspc-12	1.89965436	3.26230384	2.5809791
WBGene00021872	clec-85	2.50650181	2.63403535	2.57026858
WBGene00000620	col-43	1.88510044	3.22126652	2.55318348
WBGene00015355	C02F12.5	2.41511354	2.68781761	2.55146557
WBGene00001768	gst-20	2.05765752	3.04443192	2.55104472
WBGene00009637	nspc-13	1.89446612	3.20518749	2.5498268
WBGene00019914	clec-150	2.58146044	2.48060938	2.53103491
WBGene00008566	acox-1.3	2.0761134	2.93070596	2.50340968
WBGene00044357	nspc-6	1.2571301	3.71043198	2.48378104
WBGene00015449	ugt-63	1.44212717	3.52505856	2.48359286
WBGene00010125	dod-22	1.90172799	3.05204312	2.47688556
WBGene00023477	nspc-1	1.58749051	3.359033	2.47326175
WBGene00009640	nspc-10	1.89625424	3.03912516	2.4676897

WBGene00009778	F46C5.1	2.12672507	2.80205857	2.46439182
WBGene00021005	ule-1	2.559713	2.33901428	2.44936364
WBGene00007153	clec-41	1.96667961	2.86804592	2.41736276
WBGene00008358	nspc-18	2.50889218	2.31374056	2.41131637
WBGene00018646	mul-1	1.59451576	3.22260664	2.4085612
WBGene00003090	lys-1	2.30496397	2.48945277	2.39720837
WBGene00008493	F01D5.2	1.65020921	3.11137341	2.38079131
WBGene00009787	F46F2.3	2.24194452	2.50669139	2.37431795
WBGene00021731	Y49G5A.1	2.00445891	2.69979637	2.35212764
WBGene00009638	nspc-14	1.97239767	2.60354076	2.28796922
WBGene00021500	Y40C7B.4	1.6868975	2.877929	2.28241325
WBGene00018647	F49F1.7	1.22482117	3.32491431	2.27486774
WBGene00001731	grl-22	1.48045509	3.04048833	2.26047171
WBGene00189949	Y48G1C.13	1.9719125	2.49437989	2.2331462
WBGene00009397	clec-66	2.02787775	2.38889602	2.20838688
WBGene00000675	col-101	1.89096277	2.50356168	2.19726223
WBGene00009396	clec-65	1.83363277	2.54707789	2.19035533

WBGene00000716	col-143	1.87704148	2.49966879	2.18835513
WBGene00003091	lys-2	1.3465585	3.00646366	2.17651108
WBGene00022125	Y71F9B.1	1.96246252	2.38850224	2.17548238
WBGene00019563	K09C6.9	2.33923568	2.00034079	2.16978823
WBGene00001693	grd-4	1.64575465	2.69068396	2.1682193
WBGene00206386	K10C2.12	1.68979677	2.62671518	2.15825597
WBGene00000617	col-40	1.7281373	2.57922382	2.15368056
WBGene00010050	F54D5.4	2.00928171	2.29494469	2.1521132
WBGene00016636	perm-2	1.86103648	2.4391272	2.15008184
WBGene00012583	clec-4	1.94737056	2.31689684	2.1321337
WBGene00017490	pud-2.1	1.00944989	3.2282578	2.11885384
WBGene00045261	H29C22.1	2.21210093	2.00679573	2.10944833
WBGene00009237	F28H7.3	1.9819049	2.22928935	2.10559712
WBGene00016894	C53B7.3	1.86980878	2.33392749	2.10186813
WBGene00010128	F55G11.8	1.83467346	2.3544886	2.09458103
WBGene00022245	acp-6	1.75459865	2.42491871	2.08975868
WBGene00003756	nlp-18	2.16909996	2.00511077	2.08710537

WBGene00044206	T26H5.9	1.61081463	2.52796145	2.06938804
WBGene00009192	asah-2	1.3271891	2.80677423	2.06698167
WBGene00007180	B0457.6	1.68420724	2.44672826	2.06546775
WBGene00016434	C35B1.4	1.64049661	2.48708461	2.06379061
WBGene00015683	C10G8.4	2.05505902	2.06178828	2.05842365
WBGene00011190	swt-6	1.19203358	2.92165278	2.05684318
WBGene00014253	ZK1320.3	1.78360364	2.3209521	2.05227787
WBGene00000556	cnc-2	1.91091587	2.18193243	2.04642415
WBGene00008494	F01D5.3	1.54804601	2.52267168	2.03535884
WBGene00021797	plep-1	1.89005856	2.14246097	2.01625977
WBGene00022821	ZK813.2	1.80246524	2.21726891	2.00986708
WBGene00005552	sri-40	1.87306405	2.10434688	1.98870546
WBGene00010901	M28.10	1.60180517	2.37433455	1.98806986
WBGene00010982	flp-32	1.66849441	2.26315311	1.96582376
WBGene00001461	flp-18	1.94994947	1.98022431	1.96508689
WBGene00012615	dct-16	1.19671724	2.72075193	1.95873458

Top <u>downregulated</u> reproduced genes in response to *C. freundii*

WBID	Gene name	Log2FC 2018	Log2FC 2019	Avg Log2FC
WBGene00017998	F33D4.6	-5.06578483	-5.35593929	-5.21086206
WBGene00017018	D1014.5	-3.90285097	-5.85687577	-4.87986337
WBGene00004264	qua-1	-4.4235805	-5.10963663	-4.76660857
WBGene00000251	bli-1	-4.92180182	-4.48996101	-4.70588142
WBGene00015646	mlt-10	-4.33978175	-4.98576771	-4.66277473
WBGene00004120	pqn-32	-3.81581402	-5.46735948	-4.64158675
WBGene00008150	mam-2	-4.15464909	-5.011016	-4.58283255
WBGene00008448	E01G4.6	-4.29447953	-4.84211472	-4.56829713
WBGene00011668	clec-47	-4.25317674	-4.81446524	-4.53382099
WBGene00012186	mlt-11	-4.22049109	-4.84689488	-4.53369299
WBGene00012540	Y37A1B.7	-4.44078073	-4.52533149	-4.48305611
WBGene00003888	osm-8	-3.19321027	-5.7308928	-4.46205153
WBGene00000133	amt-1	-3.89713368	-4.96004657	-4.42859012
WBGene00011530	T06D8.10	-3.59186921	-5.21100687	-4.40143804
WBGene00003057	lon-3	-4.38708487	-4.37046267	-4.37877377

WBGene00009133	bed-3	-3.68527815	-5.00714634	-4.34621225
WBGene00019272	H42K12.3	-4.79026663	-3.84031727	-4.31529195
WBGene00014183	ZK1025.3	-3.92427792	-4.62379392	-4.27403592
WBGene00006948	wrt-2	-4.63149805	-3.86712249	-4.24931027
WBGene00000252	bli-2	-4.42423147	-4.07112293	-4.2476772
WBGene00004397	rol-6	-3.89955144	-4.54462353	-4.22208749
WBGene00010285	F58H1.2	-4.08923796	-4.35327809	-4.22125802
WBGene00016196	C28H8.5	-1.87318103	-6.4954404	-4.18431071
WBGene00020571	lpr-7	-3.0772826	-5.28290934	-4.18009597
WBGene00019520	K08B12.1	-4.5139028	-3.83326156	-4.17358218
WBGene00019285	cbn-1	-3.25803473	-5.05457643	-4.15630558
WBGene00000655	col-79	-4.13200478	-4.08705392	-4.10952935
WBGene00022680	ZK180.6	-3.46518717	-4.73210883	-4.098648
WBGene00000402	cdh-10	-4.76473961	-3.43054687	-4.09764324
WBGene00001691	grd-2	-3.03357497	-5.12567025	-4.07962261
WBGene00005016	sqt-1	-3.43011583	-4.70090694	-4.06551139
WBGene00018737	F53B1.4	-3.82578402	-4.27385312	-4.04981857

WBGene00013173	Y53F4B.27	-3.12791297	-4.96123073	-4.04457185
WBGene00020194	T03G6.1	-3.57829586	-4.47547784	-4.02688685
WBGene00012257	lpr-4	-4.25728151	-3.77060785	-4.01394468
WBGene00044109	K02E11.10	-3.10036008	-4.90519701	-4.00277855
WBGene00021931	Y55F3AM.14	-2.73742596	-5.23974746	-3.98858671
WBGene00004133	abu-13	-3.92152887	-4.03721205	-3.97937046
WBGene00001714	grl-5	-3.54627298	-4.37355134	-3.95991216
WBGene00001716	grl-7	-3.55951719	-4.33959192	-3.94955455
WBGene00004398	rol-8	-3.2836201	-4.60680518	-3.94521264
WBGene00005017	sqt-2	-3.5453117	-4.31080946	-3.92806058
WBGene00020560	T19C3.2	-3.49562424	-4.35218446	-3.92390435
WBGene00017991	clec-180	-3.89133059	-3.94967791	-3.92050425
WBGene00017307	F09F9.2	-4.23634028	-3.60424568	-3.92029298
WBGene00010467	K01D12.8	-1.73659182	-6.09373229	-3.91516206
WBGene00000639	col-63	-3.27936467	-4.53931214	-3.9093384
WBGene00008810	cyp-13A12	-2.39761033	-5.41639717	-3.90700375
WBGene00004127	abu-12	-3.41793254	-4.38154016	-3.89973635

WBGene00006950	wrt-4	-3.40659152	-4.37953702	-3.89306427
WBGene00022577	nstp-3	-3.2375904	-4.53860104	-3.88809572
WBGene00009261	F30A10.2	-4.59379599	-3.12740842	-3.8606022
WBGene00000702	col-128	-3.7914361	-3.91421397	-3.85282504
WBGene00019154	glf-1	-3.28196804	-4.41261699	-3.84729251
WBGene00015841	skpo-2	-2.93386404	-4.7566041	-3.84523407
WBGene00044395	R12C12.10	-3.16517248	-4.43187769	-3.79852508
WBGene00007479	C09F9.2	-4.18341555	-3.41117169	-3.79729362
WBGene00006193	str-146	-1.69217614	-5.89216255	-3.79216935
WBGene00022415	Y102A11A.5	-4.02700591	-3.47334526	-3.75017558
WBGene00011548	T06G6.6	-3.66095478	-3.83435687	-3.74765582
WBGene00012346	W08G11.1	-2.63919117	-4.8506305	-3.74491083
WBGene00010267	lips-9	-2.88542283	-4.53755094	-3.71148688
WBGene00013891	ZC434.3	-3.64624093	-3.74325617	-3.69474855
WBGene00001079	dpy-20	-2.82413122	-4.52324096	-3.67368609
WBGene00012255	lpr-6	-3.67071396	-3.66380437	-3.66725917
WBGene00011671	cyp-13A4	-2.88123444	-4.42733057	-3.65428251

WBGene00007552	C13C12.2	-3.22773708	-4.06234287	-3.64503997
WBGene00010714	mam-3	-2.76753045	-4.51603646	-3.64178345
WBGene00001065	dpy-3	-3.64723222	-3.59935492	-3.62329357
WBGene00010673	K08E7.5	-3.02922251	-4.20965517	-3.61943884
WBGene00017438	F13H8.5	-3.12141861	-4.08680742	-3.60411301
WBGene00011235	suro-1	-2.90257444	-4.27090531	-3.58673988
WBGene00020554	T19A5.3	-2.87737846	-4.27686836	-3.57712341
WBGene00012429	Y11D7A.5	-3.3901938	-3.75334044	-3.57176712
WBGene00007530	C11H1.5	-3.86713372	-3.24826393	-3.55769882
WBGene00017577	F18F11.4	-2.6451568	-4.4628836	-3.5540202
WBGene00006956	wrt-10	-4.07920031	-3.02531558	-3.55225795
WBGene00000606	col-17	-2.93248776	-4.17117905	-3.5518334
WBGene00001069	dpy-7	-4.23512474	-2.83907564	-3.53710019
WBGene00014182	ZK1025.2	-2.82752697	-4.23754473	-3.53253585
WBGene00012791	Y43D4A.5	-3.65536555	-3.38917287	-3.52226921
WBGene00000930	dao-4	-3.22186682	-3.80650888	-3.51418785
WBGene00008605	mlt-9	-3.74182908	-3.28031674	-3.51107291

WBGene00005018	sqt-3	-3.0357908	-3.97591183	-3.50585132
WBGene00012648	Y39A1A.9	-3.31200231	-3.66007929	-3.4860408
WBGene00018487	F46C8.8	-2.96071069	-3.97740156	-3.46905612
WBGene00206502	T01B7.13	-3.15852513	-3.7717825	-3.46515381
WBGene00014094	ZK829.3	-2.30754874	-4.62272992	-3.46513933
WBGene00015645	lips-7	-2.6826859	-4.24732034	-3.46500312
WBGene00000631	col-54	-2.89059539	-4.02754712	-3.45907125
WBGene00006954	wrt-8	-3.43413601	-3.45380872	-3.44397236
WBGene00077697	F58E6.13	-3.11711415	-3.75796769	-3.43754092
WBGene00004998	spp-13	-3.73427141	-3.09262775	-3.41344958
WBGene00012635	Y38H8A.1	-3.41277912	-3.4121496	-3.41246436
WBGene00004394	rol-1	-2.74711829	-4.06782458	-3.40747144
WBGene00021389	Y38A10A.2	-2.0398434	-4.76031638	-3.40007989
WBGene00006952	wrt-6	-2.84515726	-3.92848546	-3.38682136
WBGene00000689	col-115	-2.44485983	-4.32871451	-3.38678717
WBGene00019089	clec-206	-2.56713216	-4.19996051	-3.38354634

Supplementary Table 4. WormCat categories enriched from reproduced and significant genes that were differentially regulated in wild-type animals provided neuroprotective bacterial diets.

WormCat Analysis of Reproduced, Significantly Changed Genes in Wild Type *C. elegans* provided neuroprotective die

Category *E. cancerogenus* UP		RGS	AC	PValue	Bonferroni
1	Stress response	17	810	5.34E-14	3.74E-13
2	Stress response: pathogen	8	177	4.23E-10	6.35E-09
	Stress response: C-type Lectin	6	257	2.44E-06	3.67E-05
3	Stress response: pathogen: CUB	5	25	7.29E-10	1.38E-08
	Stress response: C-type Lectin	6	257	2.44E-06	4.64E-05

Category *E. hormaechei* UP		RGS	AC	PValue	Bonferroni
1	Extracellular material	13	485	1.65E-10	1.98E-09
2	Extracellular material: collagen	9	185	6.02E-10	1.26E-08
	Proteolysis general: lysozyme	2	11	0.00032	0.00673904
3	Extracellular material: collagen	9	185	6.02E-10	1.51E-08
	Proteolysis general: lysozyme	2	11	0.00032	0.00802267

Category *C. freundii* UP		RGS	AC	PValue	Bonferroni
1	Stress response	67	810	4.77E-31	1.10E-29
	Ribosome	28	244	1.21E-17	2.79E-16
	Extracellular material	26	485	1.35E-09	3.11E-08
	Neuronal function	19	308	2.39E-08	5.51E-07
	Metabolism	42	1577	5.27E-06	0.00012114
	Proteolysis general	15	395	0.00014	0.00331851
2	Ribosome: subunit	28	92	1.17E-27	7.63E-26
	Stress response: pathogen	30	177	4.60E-23	2.99E-21
	Extracellular material: collagen	19	185	9.08E-12	5.90E-10
	Neuronal function: synaptic function	19	244	6.99E-10	4.54E-08
	Stress response: C-type Lectin	17	257	4.86E-08	3.16E-06
	Stress response: GST	7	52	6.21E-06	0.00040339
	Proteolysis general: inhibitor	7	66	2.56E-05	0.00166707
3	Ribosome: subunit	28	92	1.17E-27	1.06E-25
	Neuronal function: synaptic function: neuropepti	19	139	9.28E-14	8.35E-12
	Extracellular material: collagen	19	185	9.08E-12	8.17E-10
	Unknown: regulated by multiple stresses	69	2248	6.49E-11	5.84E-09
	Stress response: pathogen: CUB	8	25	3.96E-09	3.56E-07
	Stress response: C-type Lectin	17	257	4.86E-08	4.37E-06
	Stress response: pathogen: saposin	5	14	2.26E-06	0.00020296
	Metabolism: mitochondria: complex IV	5	16	3.87E-06	0.00034824
	Stress response: pathogen: other	9	96	4.75E-06	0.0004273
	Stress response: GST	7	52	6.21E-06	0.00055854
	Stress response: pathogen: ShK	3	3	3.12E-05	0.00281159

Category	*E. cancerogenus* DOWN	RGS	AC	PValue	Bonferroni
1	Signaling	53	1193	1.16E-19	2.32E-18
	Cytoskeleton	18	369	1.42E-08	2.85E-07
	Unknown	114	8160	1.16E-05	0.00023129
2	Signaling: phosphatase	32	197	5.39E-28	2.91E-26
	Cytoskeleton: microtubule	17	128	1.81E-14	9.78E-13
	Signaling: Y kinase	10	75	3.72E-09	2.01E-07
	Unknown	114	8160	1.16E-05	0.00062448
	Transmembrane transport: ABC	5	51	0.00012	0.00636058
3	Unknown: regulated by multiple stresses	94	2248	4.14E-30	2.74E-28
	Signaling: phosphatase: Y	22	91	3.64E-23	2.40E-21
	Cytoskeleton: microtubule: tau tubulin kinase	17	74	5.60E-18	3.70E-16
	Signaling: Y kinase	10	75	3.72E-09	2.45E-07
	Signaling: phosphatase: other	10	98	3.84E-08	2.53E-06
	Transmembrane transport: ABC	5	51	0.00012	0.00777404

Category	*E. hormaechei* DOWN	RGS	AC	PValue	Bonferroni
1	Extracellular material	42	485	5.69E-24	1.08E-22
	Signaling	36	1193	2.68E-08	5.10E-07
	Unknown	136	8160	4.01E-07	7.62E-06
2	Extracellular material: collagen	36	185	1.05E-31	5.13E-30
	Signaling: phosphatase	16	197	6.41E-10	3.14E-08
	Signaling: hedgehog-like	11	90	5.82E-09	2.85E-07
	Unknown	136	8160	4.01E-07	1.97E-05
	Transmembrane transport: ABC	6	51	2.04E-05	0.00100085
3	Extracellular material: collagen	36	185	1.05E-31	6.49E-30
	Unknown: regulated by multiple stresses	92	2248	2.43E-25	1.51E-23
	Signaling: hedgehog-like	11	90	5.82E-09	3.61E-07
	Signaling: phosphatase: Y	11	91	6.47E-09	4.01E-07
	Cytoskeleton: microtubule: tau tubulin kinase	7	74	1.57E-05	0.00097217
	Transmembrane transport: ABC	6	51	2.04E-05	0.00126638

Category	*C. freundii* DOWN	RGS	AC	PValue	Bonferroni
1	Extracellular material	63	485	6.00E-43	9.01E-42
	Unknown	136	8160	1.98E-06	2.97E-05
	Stress response	22	810	7.51E-05	0.0011263
	Proteolysis general	14	395	0.0001	0.00157253
2	Extracellular material: collagen	53	185	3.74E-52	1.94E-50
	Signaling: hedgehog-like	15	90	3.44E-13	1.79E-11
	Unknown	136	8160	1.98E-06	0.00010281
	Transmembrane transport: lipid	4	15	3.58E-05	0.00186019
3	Extracellular material: collagen	53	185	3.74E-52	2.54E-50
	Unknown: regulated by multiple stresses	84	2248	2.44E-20	1.66E-18
	Signaling: hedgehog-like	15	90	3.44E-13	2.34E-11
	Transmembrane transport: lipid	4	15	3.58E-05	0.00243256

Supplementary Table 5. 16S sequences of bacteria.

Bacterium	**16 Sequence**
Enterobacter cancerogenus	>GC60_assembly.fasta-16S_/1-1536 AAGGAGGTGATCCAACCGCAGGTTCCCCTACGGTTACCTTGTTACGACTTCACCCCAGTCATGAATCACAAA GTGGTAAGCGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGT GTGTACAAGGCCCGGGAACGTATTCACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGA GTCGAGTTGCAGACTCCAATCCGGACTACGACATACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCT CTTTGTATATGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCAC CTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAACAAAGGATAAGGGTT GCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGA GTTCCCGAAGGCACCAAAGCATCTCTGCTAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGC ATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCC GTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGA CATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAG TCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGA ATTCTACCCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGAATGCAGTTCCCAGGTTGAGCCCGGGGATTTC ACATCCGACTTGACAGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTA TTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCAATCACCAAGGTTATTAAC CTTAATGCCTTCCTCCTCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCA TCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCC AGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCATTACCCCACCTACTAGCT AATCCCATCTGGGCACATCTGATGGCATGAGGCCCGAAGGTCCCCCACTTTGGTCTTGCGACGTTATGCGGT ATTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCACCCGTCCGCCGC TCGTCACCCGAGAGCAAGCTCTCTGTGTTACCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAAT CTGAGCCATGATCAAACTCTTCAA
Enterobacter hormaechei	>GC61_assembly.fasta-16S_/1-1538 AAGGAGGTGATCCAACCGCAGGTTCCCCTACGGTTACCTTGTTACGACTTCACCCCAGTCATGAATCACAAA GTGGTAAGCGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGT GTGTACAAGGCCCGGGAACGTATTCACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGA GTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCT CTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCAC CTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCTAACCGCTGGCAACAAAGGATAAGGGTT GCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGA GTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGC ATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCC GTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGA CATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAG TCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGA ATTCTACCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGAATGCAGTTCCCAGGTTGAGCCCGGGGATTTC ACATCCGACTTGACAGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTA TTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATCGACAAGGTTATTAAC CTTATCGCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCA TCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCC AGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTACTAGCT AATCCCATCTGGGCACATCCGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGT ATTAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCACCCGTCCGCCAC TCGTCAGCGAAGCAGCAAGCTGCTTCCTGTTACCGTTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCA ATCTGAGCCATGATCAAACTCTTCAA
Citrobacter freundii	>GC62_assembly.fasta-16S_/1-995 TTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAGCAC AGAGGAGCTTGCTCCTTGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCCGATGGAGGGG GATAACTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTT GCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCT GGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA ATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTA CTTTCAGCGAGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATTGACGTTACTCGCAGAAGAAGCACCGGC TAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCA CGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCGAAACTGGCAGGC TAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGT GGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC CCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACG CGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAG CGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATC

Klebsiella pneumoniae 32D9	27f aligned with 1492r (321 bp-1046 bp) GGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTgAAGAAGGCCTTCGGGTtGTAAAGCACTTTCAGCGGGGAGGAAGGCG aTaARGGTTAATAACCTKKCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGG GCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAA TGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGG AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTC CGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTtaAAACTCAAATGAATTGACGGGGGCCCGCAC AAGCGGTGGAGCATGTGGTTTAATTCGATGCAaCGCGAAGAACCTTACCTGGTCTTGACATCCAcAGAACTTTCCAGARW KGRWTKGKTGCCTTCgGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTC CCGCAA
Klebsiella oxytoca 32A3	27f aligned with 1492r (334 bp-1083 bp) GCAGCCATGcCGCGTGTATGAAGAAGGCcTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAGGgTTAATAA CCtgTTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGC GTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGA ACTGCATTCGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCT GGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATT AGATACCCTGGTAGTCCACGCTGTAAACGATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCG TTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGgCCCGCACAAGCGGTGGAGCA TGTGGTTTAATTCGATGCAACGCGAAGAACcTTACCTACTCTTGACATCCAGAGAACTTAgCAGAgATGCTTTGGTGCCT TCGGGAACTCTGAgACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTtGGGTTAAGTCCCGCAACGAGCGC AMCCCTTATCCTTTGTTGCCAGCGgTcCSG
E. coli A09	27f aligned with 1492r (404 bp-1039 bp) AGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGA AATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTA GCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAA AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGG CGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGG GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGT TTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAAT
E. coli G07	27f aligned with 1492r (413 bp-1018 bp) TACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCA AGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTG GGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA TCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGG ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAAC GCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGA GCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTG CCTTCGGGaaCCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCG

Supplementary Table 6. RNA-Seq Experiment 1 alignment rates

Sample	Paired Reads In Raw FASTQ	Paired Reads after Trimmomatic	Reads Passing QC	HISAT2 Overall Alignment Rate	Read Pairs Mapped to a Single Location	Single Location Concordance Rate	htseq-count SAM alignment pairs	no_feature	ambiguous	too_low_aQual	not_aligned	alignment_not_unique	Total RNA-Seq Counts	Mapped Reads Annotation Rate	Genes with RNA-Seq Data	Raw Data Usage Rate
1B06-1	24039678	24039678	100%	98.82%	21826305	90.79%	27134870	98489	468388	366300	94578	4812981	21294134	89.64%	17486	88.58%
1B06-1 (trimmed)	24039678	23895421	99.40%	98.51%	21554162	90.20%	27031403	97064	455418	452082	118536	4871753	21036550	89.37%	17497	87.51%
1B06-2	38632376	38632376	100%	98.40%	33131321	85.76%	47037951	114360	1643079	580362	313284	12962407	31424459	82.66%	16993	81.34%
1B06-2 (trmmed)	38632376	38391944	99.38%	98.13%	32719177	85.22%	46841220	112721	1610615	717137	333828	13021318	31045601	82.41%	17015	80.36%
1B06-3	22474130	22293268	99.20%	98.19%	19543038	87.66%	26168306	129498	605112	429632	173155	5977883	18853026	86.13%	16977	83.89%
B1-1	29734397	29401007	98.88%	97.94%	24023062	81.71%	37759911	52424	1820580	576436	274787	12843771	22191913	77.07%	15609	74.63%
B1-2	23748930	23532907	99.09%	96.32%	18571340	78.92%	30851727	68865	1546781	383212	649314	11218394	16985161	74.93%	12849	71.52%
B1-3	19379159	19379159	100.00%	98.60%	16299641	84.11%	24315481	52237	1082312	284474	120062	7580568	15195828	79.53%	15798	78.41%
B1-3 (trimmed)	19379159	19251097	99.34%	98.25%	16069608	83.47%	24208681	52157	1057984	351475	142539	7613580	14990946	79.26%	15806	77.36%
OP50-1	27671846	27439930	99.16%	96.99%	22372678	81.53%	34932155	111838	1434783	442052	583411	11501157	20858914	78.38%	16281	75.38%
OP50-2	31272467	30961140	99.00%	97.76%	25618412	82.74%	38997276	121360	1641207	634150	336178	12372494	23891887	78.94%	16727	76.40%
OP50-3	23555191	23361221	99.18%	98.20%	19477048	83.37%	29469992	94814	869814	413484	194750	9355612	18541518	80.82%	17032	78.72%
1A05 1	27113652	27078135	99.87%	98.70%	24,490,997	90.45%	30,613,019	23,927,147	19,113	452,315	118,945	5,513,710	581,789	2.18%	11,165	2.15%
1A05 2 (Poly T trimmed)	24,196,758	23942725	98.95%	98.80%	21,756,280	90.87%	26,506,012	104,001	371,189	346,003	107,030	4,257,811	21,319,978	90.13%	17,193	88.11%
1A05 3	24,554,190	24,411,579	99.42%	98.42%	22,173,251	90.83%	27,246,437	96361	377,561	453,291	149,857	4,434,025	21,735,342	90.47%	17,231	88.52%

Supplementary Table 7. RNA-Seq Experiment 2 alignment rates

Sample	Paired Reads in Raw FASTQ	Paired Reads after Trimmomatic	Reads Passing QC	HiSAT2 Overall Alignment Rate	Read Pairs Mapped to a Single Location	Single Location Concordance Rate	htseq-count SAM alignment pairs	no_feature	ambiguous	too_low_aQual	not_aligned	alignment_not_unique	Total RNA-Seq Counts	Mapped Reads Annotation Rate	Genes with RNA-Seq Data	Raw Data Usage Rate
N2 - OP50-1	22544518	22277009	98.81%	88.98%	16095401	72.25%	25563186	128421	82636	4034623	399366	5010038	15908102	80.25%	16549	70.56%
N2 - OP50-2	19366001	19047089	98.35%	88.24%	13791094	72.41%	21328717	109215	77607	3622065	393973	3499184	13626673	81.08%	16719	70.36%
N2 - OP50-3	16507759	16312680	98.82%	88.25%	11938937	73.19%	17965990	84903	67459	3141917	319940	2546094	11805677	82.01%	16911	71.52%
N2 - 1A05-1	25404547	25017413	98.48%	88.39%	18107655	72.38%	28060172	193757	88161	4712251	498336	4710123	17857544	80.76%	16653	70.29%
N2 - 1A05-2	21175987	20865552	98.53%	88.14%	15190307	72.80%	23025898	146446	76945	4038430	418273	3354449	14991350	81.52%	16809	70.79%
N2 - 1A05-3	23035517	22644110	98.30%	87.96%	16496294	72.85%	24868200	151974	85217	4415255	475652	3450286	16289816	81.79%	16865	70.72%
N2 - 1B06-1	24204445	23552620	97.31%	88.41%	16810680	71.37%	26962107	181894	62176	4402440	480166	5237652	16597779	79.71%	16437	68.57%
N2 - 1B06-2	27642245	27180923	98.33%	88.33%	19581616	72.04%	30644525	169359	95422	5185221	533341	5312802	19348380	80.59%	16618	70.00%
N2 - 1B06-3	22439683	22166659	98.78%	87.66%	16178476	72.99%	24032290	174433	98215	4500462	454960	2874275	15929945	81.98%	16905	70.99%
N2 - 32D9-1	26308702	25808199	98.10%	87.55%	18548153	71.87%	28524341	124187	97865	5190675	571894	4183207	18356513	81.24%	16753	69.77%
N2 - 32D9-2	24396930	24025509	98.48%	88.77%	17242094	71.77%	27714115	77384	70025	4381595	455303	5607302	17122506	80.28%	16555	70.18%
N2 - 32D9-3	28013166	27292782	97.43%	88.41%	19204669	70.37%	31986342	91151	76107	5058592	559695	7128546	19072251	79.04%	16299	68.08%
N2 - B1-1	21297182	20919276	98.23%	88.71%	14816966	70.83%	24418727	220005	67697	3801822	417151	5358097	14553955	78.43%	16172	68.34%
N2 - B1-2	19437144	19015628	97.83%	88.28%	13418020	70.56%	22032985	112649	60343	3584991	394588	4611678	13268736	79.04%	16299	68.26%
N2 - B1-3	19733695	19442991	98.53%	88.79%	13761506	70.78%	22753073	75481	61904	3558025	360542	5052406	13644715	79.04%	16298	69.14%
Tau - OP50-1	25159628	24768865	98.45%	87.24%	18080220	73.00%	26539255	215634	145487	5152091	545149	2732282	17748612	82.14%	16938	70.54%
Tau - OP50-2	32440412	31836641	98.14%	87.26%	22997378	72.24%	34678551	309796	181568	6531055	731161	4378496	22546475	81.16%	16736	69.50%
Tau - OP50-3	25175895	24748782	98.30%	86.33%	17849772	72.12%	26158576	230397	149106	5469087	611213	2198415	17500358	81.91%	16890	69.51%
Tau - 1A05-1	28350631	27944118	98.57%	87.16%	20319451	72.71%	29996634	236311	161377	5801997	639886	3203148	19953915	81.93%	16894	70.38%
Tau - 1A05-2	22938211	22546318	98.29%	87.22%	16400698	72.74%	24238191	177940	127403	4646000	518543	2643925	16124380	82.00%	16908	70.29%
Tau - 1A05-3	25813050	25405799	98.42%	87.94%	18389332	72.38%	28170399	200914	129071	4969088	529865	4252586	18088875	80.96%	16696	70.08%
Tau - 1B06-1	23463606	23157487	98.70%	86.81%	16788483	72.50%	24662535	154269	137745	4954247	540712	2353342	16522220	82.19%	16948	70.42%
Tau - 1B06-2	24687865	24388144	98.79%	87.26%	17696918	72.56%	26348635	158437	137619	5051839	540869	3033193	17426678	81.89%	16886	70.59%
Tau - 1B06-3	24427715	24087060	98.61%	86.40%	17388287	72.19%	25449741	176227	144604	5323700	578705	2131651	17094854	82.14%	16939	69.98%
Tau - 32D9-1	23625190	23335573	98.77%	86.33%	16807435	72.02%	24637409	266088	126356	5191990	560558	2051388	16441029	81.61%	16829	69.59%
Tau - 32D9-2	21846726	21500412	98.41%	86.36%	15483336	72.01%	22748044	246567	119109	4734178	532091	1972625	15143474	81.56%	16818	69.32%
Tau - 32D9-3	22647458	22371311	98.78%	86.36%	16163491	72.25%	23597029	226370	124577	4931539	551832	1925255	15837456	81.97%	16904	69.93%
Tau - B1-1	22331677	22042119	98.70%	86.43%	15921212	72.23%	23276541	244906	124677	4852830	530233	1949509	15574386	81.75%	16858	69.74%
Tau - B1-2	30220370	29764764	98.49%	86.63%	21614250	72.62%	31426466	353122	160835	6465093	702815	2613550	21131051	81.95%	16899	69.92%
Tau - B1-3	21001174	20686910	98.50%	87.38%	14948077	72.26%	22589875	198148	114378	4228818	457288	2932987	14658256	81.09%	16722	69.80%

Supplementary Table 8. RNAi-sensitized animals experience more robust neuroprotection with *ttbk* RNAi

	Original Tau transgenic			RNAi-sensitized Tau transgenic		
Dunnett's multiple comparisons test	**Mean Diff.**	**95.00% CI of diff.**	**Adjusted P Value**	**Mean Diff.**	**95.00% CI of diff.**	**Adjusted P Value**
Empty vector vs. *W03G9.5*	24.29	5.355 to 43.22	0.0052	15.71	3.818 to 27.60	0.0041
Empty vector vs. *T05C12.1*	20.48	1.545 to 39.41	0.0271	20.47	8.582 to 32.37	0.0001
Empty vector vs. *C14A4.13*	23.33	4.398 to 42.26	0.008	21.9	10.01 to 33.80	<0.0001
Empty vector vs. *C03C10.2*	26.66	7.732 to 45.59	0.0018	28.09	16.20 to 39.99	<0.0001
Empty vector vs. *K06H7.8*	31.9	12.97 to 50.83	0.0002	27.62	15.72 to 39.51	<0.0001
Empty vector vs. *T11F8.4*	33.81	14.88 to 52.74	<0.0001	21.9	10.01 to 33.80	<0.0001
Empty vector vs. *ttbk-4*	35.24	16.31 to 54.17	<0.0001	27.14	15.25 to 39.04	<0.0001
Empty vector vs. *R10D12.10*	22.38	3.448 to 41.31	0.0122	23.33	11.43 to 35.22	<0.0001
Empty vector vs. *ttbk-5*	28.09	9.162 to 47.02	0.0009	22.85	10.96 to 34.75	<0.0001
Empty vector vs. *C55B7.10*	24.76	5.832 to 43.69	0.0042	25.71	13.82 to 37.60	<0.0001
Empty vector vs. *H05L14.1*	29.53	10.60 to 48.46	0.0005	19.04	7.152 to 30.94	0.0004
Empty vector vs. *T05A7.6*	24.29	5.355 to 43.22	0.0052	20.47	8.582 to 32.37	0.0001
Empty vector vs. *F59A6.4*	24.76	5.828 to 43.69	0.0042	20.95	9.055 to 32.84	<0.0001
Empty vector vs. *ZK354.2*	27.14	8.212 to 46.07	0.0014	23.81	11.91 to 35.70	<0.0001
Empty vector vs. *C08F8.6*	24.76	5.828 to 43.69	0.0042	28.57	16.68 to 40.46	<0.0001
Empty vector vs. *Y38H8A.3*	28.1	9.165 to 47.03	0.0009	25.71	13.82 to 37.60	<0.0001
Empty vector vs. *K11C4.1*	29.05	10.12 to 47.98	0.0006	29.52	17.63 to 41.41	<0.0001

Supplementary Table 9. Sequence results of Ahringer RNAi clones targeting tested *ttbk*s

TTBK gene name	*E. c.* Log2FC	*E. h.* Log2 FC	*C. f.* Log2FC	Clone has >80% Sequence Identity to ONLY target gene?
W03G9.5	-2.686	NC	NC	No, 99% W03G9.5 and 100% W03G9.9 (uncharacterized gene)
C14A4.13	NC	-1.760	NC	Y
C03C10.2	NC	-0.561	NC	Y
T05C12.1	-2.213	NC	NC	Y
ttbk-5	-1.870	NC	NC	Y
ttbk-4	-1.838	NC	NC	No, 100% ttbk-4 and 86% ttbk-6
K06H7.8	-1.759	NC	NC	Y
T11F8.4	NC	-2.221	NC	Y
R10D12.10	-2.756	-1.469	NC	Y
F59A6.4	-2.757	NC	NC	Y
ZK354.2	-1.981	NC	NC	No, 99.6% ZK354.2 and 92.5% Y65B4A.9 (*ttbk*)
C55B7.10	NC	-2.079	NC	Y
H05L14.1	-1.688	NC	NC	Y
T05A7.6	-2.630	NC	NC	No, 99.7% T05A7.6 and 86.4% F59A6.4 (*ttbk*)
C08F8.6	-2.670	NC	NC	Y
Y38H8A.3	-1.990	-3.057	-2.418	No, 100% Y38H8A.3 and 89.5% C39H7.1 (*ttbk*)
K11C4.1	-1.669	NC	NC	No, 99% K11C4.1 and 85% C17C3.11 (predicted kinase)
tag-191	NC	-2.682	NC	Y

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CHAPTER THREE - Identification of novel microbiota-influenced genes that decrease neurodegeneration in *C. elegans*

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Contributions: KBP and LTM designed and performed experiments and wrote the manuscript. KBP made the figures.

Abstract

The heterogeneity of Alzheimer's disease (AD) poses many challenges for studying the causes of disease development. Studies have aimed to elucidate cellular processes that play a role in AD-associated neurodegeneration in the context of the two main molecular features consistently present in AD patients: amyloid-beta (Aβ) plaques, and tau protein-induced neurofibrillary tangles. Previously, we used a *C. elegans* model that overexpressed Aβ in the body wall muscle to identify human microbiotal *Enterobacteriaceae* species that significantly reduced paralysis. Most of these bacteria decreased neurodegeneration in a second *C. elegans* AD model that pan-neuronally expressed human aggregate-prone tau. Examination of host gene expression revealed numerous human orthologs that were differentially regulated. Here, we followed transcriptomic profiling of *C. elegans* exposed to neuroprotective *Enterobacter* with an RNAi screen of downregulated orthologs as a method to identify disease-associated genes in AD. Our findings complement reports that suggest WormCat-defined orthologs PPP1CB, PTPN9, COLQ, MARCO, TRPC3, or TRPC6 impact AD, while also identifying TRPC7, TSSK6, and SFTPD as potential novel mediators of neurodegeneration. Overall, our work uncovered *C. elegans* genes that were differentially regulated by microbiota species and influenced neurodegeneration in models of AD.

Introduction

Mechanisms that promote amyloid-β- (Aβ) and tau-induced neurodegeneration in Alzheimer's disease (AD) need further characterization. Most AD cases occur sporadically, while familial early-onset AD only accounts for <5% of all cases (Bekris et al., 2010; Chouraki & Seshadri, 2014). Familial AD patients have gene variants that are associated with dysregulated Aβ production, including mutants of amyloid precursor protein (APP), or γ secretase subunits presenilin 1 (PSEN1) and presenilin 2 (PSEN2) (Bekris et al., 2010). The APOE ε4 allele (APOE4), present in ~40% of patients, is the strongest known genetic risk factor for sporadic AD (Farrer et al., 1997; C.-C. Liu et al., 2013). APOE4 increases the likelihood of AD development by 3 to 15-fold, with some ethnic groups showing more susceptibility than others (Farrer et al., 1997). Despite these known genetic associations, the heterogeneity of AD demonstrates that there are additional contributing factors to disease development.

Genome-wide association studies (GWAS) provide insight on the genetics of AD, but a large portion of the genetic risk for this disease remains unknown (Harold et al., 2009; Lambert et al., 2013). Some variants enriched in AD populations are associated with processes involved in Aβ deposition, neuron intracellular trafficking, and synaptic development and plasticity (Harold et al., 2009; Lambert et al., 2013). Approaches using machine learning can computationally model AD and predict disease-associated genetic risk factors (Binder et al., 2022; Casanova et al., 2013; Di Deco et al., 2013; Huang et al., 2018; Mirzaei et al., 2016; Pentón-Rol & Cervantes-Llanos, 2018; Signaevsky et al., 2019). These techniques only predict genes that could contribute to AD

development and do not necessarily pinpoint causal variants and genes. Post-GWAS analyses are needed to fully characterize candidate genes and their functional variants to understand their exact roles in the pathophysiology of AD. Therefore, it is necessary to combine computational approaches with biological screening to identify genes with the potential to influence AD. This dual approach has successfully predicted novel AD risk genes (Binder et al., 2022). However, studying the causal mechanisms of AD-associated genes *in vivo* is needed to identify feasible and effective gene targets for the development of therapeutic strategies.

C. elegans models that overexpress human Aβ have been used to validate risk genes identified in human GWAS (Alvarez et al., 2022; Mukherjee et al., 2017). One of the advantages of using *C. elegans* as a screening model for AD is that it has orthologous genes for 53% of human genes, including AD risk genes (Alvarez et al., 2022; Kim et al., 2018). In fact, eight orthologs of AD-associated genes were upregulated in *C. elegans* overexpressing human Aβ and aggregated tau (Twine et al., 2011; C. Wang et al., 2018). The ability to silence *C. elegans* genes by RNAi allows functional characterization of *C. elegans* orthologs of AD-associated genes (Kamath et al., 2003; Kamath & Ahringer, 2003). This approach, using a *C. elegans* model transgenically expressing Aβ in the body wall muscle, identified 13 novel candidates that influence Aβ cytotoxicity (Mukherjee et al., 2017). More investigations are needed to test if these genes may also contribute to tau-induced neurodegeneration in AD.

We identified members of the human gut microbiota that significantly reduce paralysis and neurodegeneration in Aβ-overexpressing *C. elegans* and tau aggregate-

prone *C. elegans*, respectively. Transcriptomic profiling of *C. elegans* exposed to neuroprotective bacteria revealed 1040 genes that were reproducibly changed between two RNA-Seq experiments. From there, 347 genes had available Ahringer RNAi clones. We then prioritized testing genes that were downregulated by *Enterobacter*. Of the 180 genes knocked down, we found that suppression of eight individual genes suppressed paralysis in Aβ-overexpressing animals and verified that seven of these genesalso reduced neurodegeneration in AP-tau animals. Overall, we uncovered novel *C. elegans* genes that are differentially regulated by microbiota species and influence neurodegeneration in models of AD.

Results

Transcriptomic profiling of *C. elegans* reveals neuroprotective *Enterobacter* downregulates numerous human orthologs

To identify genes that influence paralysis and neurodegeneration, we performed two independent global gene expression analyses by RNA-Seq in wild-type N2 worms developed on control *E. coli* OP50, *E. cancerogenus, E. hormaechei,* or *C. freundii*, bacteria previously identified as having a protective effect against Aβ cytotoxicity and aggregated tau-induced neurodegeneration (See Chapter 2). We identified significant gene expression changes and prioritized lists from the 1040 reproducibly changed genes in wild-type animals fed neuroprotective diets. Prioritizing the reproduced, high-confidence dataset allowed us to identify diet-specific host genes that could play a key

role in neuroprotection in an AD-sensitized background. Of these, 635 were downregulated, with 420 genes identified by WormCat as human orthologs. We further prioritized testing the 347 genes that have human orthologs, since these are most likely to have conserved functions in mechanisms that cause neurodegeneration in AD.

RNAi screen of human orthologs identified potential causal genes in Aβ-overexpressing *C. elegans*

Out of the 347 orthologs, we first tested the effects of knocking down 180 genes that decreased in expression in response to both neuroprotective *Enterobacter* diets and had RNAi clones available in the Ahringer library (Figure 1, Supplementary Figure 1). We found that RNAi-mediated knockdown of eight human orthologs that were reproducibly downregulated in response to neuroprotective bacteria also induce protection against paralysis in an Aβ-overexpression model of AD (Table 1). Genes were defined as hits if suppressing their expression caused significantly decreased paralysis in two or more independent trials (Table 1). The RNAi clones corresponding to each hit were sequenced. Two clones intending to target *gsp-3* or *spe-41* have additional high sequence similarities (>80%) with *gsp-4* and *trp-3*, respectively. Therefore, the reduced paralysis from knocking down expression of *gsp-3* and *spe-41* could be attributed to non-specific suppression of additional genes and will need to be validated using a newly designed gene-specific RNAi clone or a knockout mutant. In contrast, the protection caused by suppressing expression of the other six tested genes is likely gene specific.

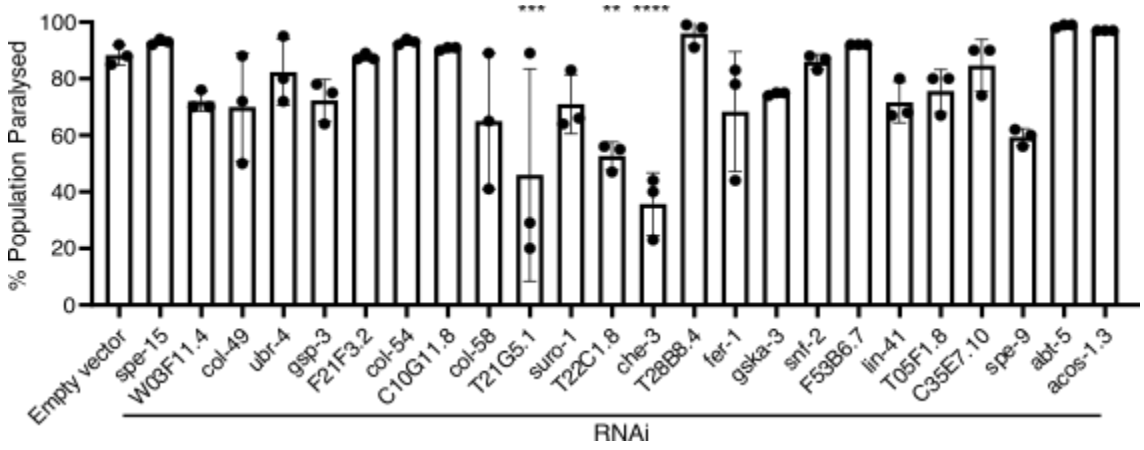


Figure 1. Suppressing *Enterobacter*-downregulated genes using RNAi reduced paralysis in Aβ42-overexpressing *C. elegans*. Representative figure showing levels of paralysis observed in populations of CL4176 animals after 72 hours of temperature-induced overexpression of Aβ42, following knockdown of individual genes. Animals were exposed to *E. coli* HT115 containing an empty vector (control) or producing dsRNA targeting genes of interest. Triplicate populations were scored, each point representing an experimental plate with >60 worms. One-way ANOVA run with post-hoc Dunnett's multiple comparisons test, L4440 as control. **=p<0.001, ***=p<0.001, ****=p<0.0001. Bars represent standard deviation of the mean.

Table 1. RNAi hits. Genes where RNAi-mediated knockdown of their expression caused decreased paralysis in at least two trials after 72 hours of Aβ-overexpression in C. elegans CL4176.

Gene	**WormCat gene description**	**Human ortholog(s) defined by WormCat**
gsp-3	Predicted to have metal ion binding activity and phosphoprotein phosphatase activity; is involved in several processes, including flagellated sperm motility, male meiosis chromosome segregation, and oviposition; localizes to the cytoplasm, nuclear chromatin, and pseudopodium; is expressed in the sperm.	PPP1CB (protein phosphatase 1 catalytic subunit beta)
T22C1.8	Predicted to have protein tyrosine phosphatase activity.	PTPN9 (protein tyrosine phosphatase, non-receptor type 9)

sqt-1	Structural constituent of collagen and cuticulin-based cuticle; is involved in cuticle development involved in collagen and cuticulin-based cuticle molting cycle.	COLQ (collagen like tail subunit of asymmetric acetylcholinesterase)
W02B12.12	Predicted to have ATP binding activity and protein serine/threonine kinase activity.	TSSK6 (testis specific serine kinase 6)
spe-41	Exhibits store-operated calcium channel activity; is involved in calcium ion transport, cellular calcium ion homeostasis, and single fertilization; localizes to the cytoplasmic vesicle, integral component of plasma membrane, and pseudopodium membrane; is expressed in the spermatid.	TRPC3 (transient receptor potential cation channel subfamily C member 3), TRPC6, and TRPC7 (transient receptor potential cation channel subfamily C member 7)
sqt-2	Predicted to be a structural constituent of cuticle\; is involved in cuticle development involved in collagen and cuticulin-based cuticle molting cycle.	MARCO (macrophage receptor with collagenous structure)
col-90	Predicted to be a structural constituent of cuticle.	SFTPD (surfactant protein D)
ech-1.1	Predicted to have 3-hydroxyacyl-CoA dehydrogenase activity and enoyl-CoA hydratase activity.	HADHA (hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha)

Validating role of human orthologs in neurodegenerative model

Of 180 genes knocked down, we found eight reduced paralysis relative to controls. These were chosen to then test the effects of knockdown on neurodegeneration in the aggregate-prone tau (AP-tau) model (see Chapter 2). Neurodegeneration of AP-tau *C. elegans* when compared to control was reduced in response to RNAi of seven of the eight genes that were protective in the screen using the Aβ-overexpressing animals (Figure 2, Table 1).

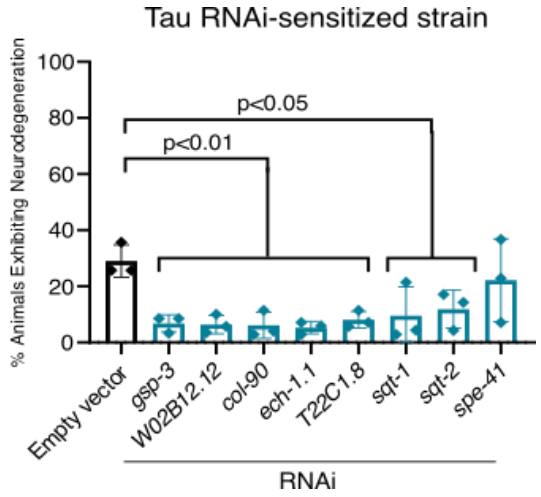


Figure 2. RNAi of human orthologs caused neuroprotection in AP-Tau *C. elegans.* Neurodegeneration was quantified in AP-tau and neuronal *sid-1*-overexpressing *C. elegans* at day 1 of adulthood, in response to RNAi-mediated knockdown of indicated genes compared to empty vector control (L4440). Knockdown of each gene except *spe-41* significantly decreased the percentage of animals exhibiting neurodegeneration. Each graph point represents a population of >60 animals from separate trials scored on different days. One-way ANOVA performed with Dunnett's multiple comparisons test to compare neurodegeneration levels between treatments and control. Bars represent standard deviation of the mean.

The genes that were protective against both paralysis and neurodegeneration range in function. They include kinases or phosphatases (W02B12.12, or *gsp-3* and T22C1.8 respectively) or structural constituents of the cuticle (*sqt-1*, *sqt-2*, and *col-90*). *ech-1.1* is

predicted to have 3-hydroxyacyl-CoA dehydrogenase activity and enoyl-CoA hydratase activity and function in the mitochondria. Overall, these findings demonstrate that initial screening to test the impact of gene knockdown in paralysis assays can lead to identification and validation of genes that are also neuroprotective. This approach thereby allowed identification of genes that can potentially protect against both Aβ-induced and tau-induced pathogenesis of AD.

Discussion

Here, we used transcriptomic profiling of *C. elegans* fed neuroprotective bacteria to identify human orthologs that can be novel genetic mediators of neurodegeneration in AD. Out of 180 genes that were tested, RNAi mediated knockdown of eight genes significantly reduced paralysis in Aβ-overexpressing *C. elegans*, and of these, seven significantly reduced neurodegeneration in *C. elegans* pan-neuronally expressing AP-tau. These genes are therefore candidates for regulators of pathways or biological processes that may impact susceptibility to pathological Aβ and tau in AD.

Downregulation of phosphatase gene PPP1CB, the ortholog of *gsp-3*, is associated with AD (Li et al., 2021; Q. Zhang et al., 2018). PPP1CB encodes the β catalytic subunit of the protein phosphatase 1 (PP1) isoform, PP1β. PP1β is a serine-threonine phosphatase that is involved in synaptic transmission and plasticity by regulating the phosphorylation status of presynaptic proteins such as the vesicle fusion mediator, SNAP25 (Foley et al., 2021; Horváth et al., 2017). Synaptic dysfunction is observed in AD (Marsh & Alifragis, 2018), and this is correlated with increased levels of Aβ oligomers (Lue et al., 1999; McLean et al., 1999). PPP1CB was downregulated in

blood samples of AD patients (Li et al., 2021), and its expression was negatively correlated with AD stage and Aβ plaques (Q. Zhang et al., 2018). It is possible that suppression of PPP1CB prevents PP1β-regulated synapse formation at the synapses of neurons in the presence of Aβ. However, whether Aβ accumulation drives PPP1CB suppression in AD is unknown.

The *C. elegans* ortholog of PPP1CB, *gsp-3*, is also predicted to enable serine/threonine phosphatase activity, but whether *gsp-3* is also expressed at neuronal synapses has not been described in the worm. Interestingly, expression of *gsp-3* was upregulated in response to increased Aβ aggregation over time in the same model (Godini et al., 2019). This suggests that Aβ accumulation promotes *gsp-3* expression. Taken together, despite being orthologs, expression of *gsp-3* or PPP1CB does not correlate with AD. However, these data suggest that deregulation of phosphorylation, whether it is caused by increased or decreased expression of phosphatase genes, can occur in the presence of Aβ. It is possible that the resulting abnormal phosphorylation status of their targets may contribute to AD, and more investigation is required to characterize the impact their substrates can have on neurodegeneration. Based on our findings, it is possible that in this *C. elegans* model, RNAi-mediated knockdown of *gsp-3* reducing Aβ-induced paralysis may be a consequence of correcting the abnormally upregulated processes that induce cytotoxicity and paralysis. If, like PPP1CB, *gsp-3* also regulates synapse formation, it is possible that Aβ overexpression can impair synaptic transmission through dysregulated *gsp-3*.

The phosphatase PTPN9, orthologous to T22C1.8, is also associated with AD (N. Liu et al., 2021). Hippocampal-specific expression of the tyrosine phosphatase PTPN9 is associated with AD, where it was enriched among a group of genes that are involved in dephosphorylation and neurogenesis (N. Liu et al., 2021). PTPN9-deficient mice experience defective neural tube development during brain formation (Y. Wang et al., 2005). It is possible that mutations of PTPN9 cause dysregulation of neurodevelopmental processes, and its hippocampal expression in later life contributes to AD pathogenesis (Alonso et al., 2004; N. Liu et al., 2021). WormCat predicts the function of T22C1.8 to be conserved, although its causal role in neurodegeneration has not been characterized. We hypothesize that similar to PTPN9, expression of T22C1.8 may contribute to Aβ-induced cytotoxicity. This could explain how RNAi-mediated knockdown of T22C1.8 reduces Aβ-induced paralysis. Our finding that knockdown of T22C1.8 or *gsp-3* also promoted neuroprotection in AP-tau animals suggests that these phosphatases play a role in tau pathogenesis as well.

Collagen-like tail subunit (single strand of homotrimer) of asymmetric acetylcholinesterase (COLQ), the human ortholog of *sqt-1*, encodes a subunit of asymmetric acetylcholinesterase (AChE) (Hu et al., 2017). In AD, the increased activity of AChEs are associated with the aggregation of Aβ and formation of neurofibrillary tangles (Carvajal & Inestrosa, 2011; Polydoro et al., 2013; Rees & Brimijoin, 2003; Reyes et al., 2004). Therefore, several AChE inhibitors have been introduced as a treatment of AD with the goal of restoring synaptic levels of acetylcholine and compensating for cholinergic neuron degeneration in patients, (McGleenon et al., 1999;

Mehta et al., 2012). The specific function of COLQ as a subunit of AChE has not been studied in the context of AD. *C. elegans* has four AChE genes (*ace-1*, *ace-2*, *ace-3*, and *ace-4*), and although their functions have not been completely described, *ace-1* and *ace-2* are neuronally expressed (Combes et al., 2001). It is unknown if *sqt-1* may interact with *C. elegans* AChE genes. Our finding that suppressing the expression of *sqt-1*, an ortholog of AChE subunit COLQ, induces protection from Aβ cytotoxicity is in accordance with AChE inhibition as a therapeutic approach for AD. However, given that *sqt-1* encodes a structural constituent of collagen and cuticle, it is possible that the function of COLQ is not conserved in *C. elegans*. More investigations are needed to determine the potential role of *sqt-1* in *C. elegans* AChE activity.

C. elegans spe-41 is orthologous to three different transient receptor potential cation channel subfamily C members: TRPC3, TRPC6, and TRPC7. These three TRP genes are expressed in the hippocampus and are regulators of Ca2+ homeostasis and cell growth (Duitama et al., 2020; Neuner et al., 2015; Wu et al., 2004). Disturbing neuronal Ca2+ homeostasis can promote development of neurodegenerative diseases such as AD (Small, 2009; Zündorf & Reiser, 2011). In mouse models of aging, high levels of TRPC3 expression are associated with poor spatial memory, and suppressing TRPC3 significantly rescued memory deficits (Neuner et al., 2015). This is consistent with our finding of *spe-41* knockdown being protective in a *C. elegans* model of AD. Literature on TRPC6's influence on neurodegeneration is contradictory, with both overactivation (Chernyuk et al., 2019) and downregulation (Lessard et al., 2005; Popugaeva et al., 2019; H. Zhang et al., 2016) being reported in AD. These conflicting reports suggest the effects of TRPC6

on AD may be heterogeneous. This may be explained by the expression of TRPC6 being sensitive to glucose fluctuation (He et al., 2020), which can vary in AD populations. TRPC6 expression is suppressed in hypoglycemic conditions (He et al., 2020). Therefore, studies investigating TRPC6 activity as a potential risk factor of AD should take care to control for AD subjects based on diet and comorbidities that cause hypoglycemia like diabetes (He et al., 2020). In mice, TRPC7 is expressed in many tissues including the brain, heart, lung, spleen, and testis (Okada et al., 1999). In a model using human embryonic kidney (HEK) cells, mouse TRPC7 was found to be functionally different from TRPC3 despite their structural homology (Okada et al., 1999). The potential of TRPC7 being involved in neurodegeneration has not been explored, and if it plays a role in the pathogenesis of AD, it may be different from TRPC3 and TRPC6. The function of *C. elegans spe-41* in models of neurodegeneration has not been reported, but its function may be conserved since *spe-41* exhibits store-operated calcium channel activity (Singaravelu et al., 2012). Since RNAi-mediated knockdown was protective in the Aβ model but not the AP-tau model, this suggests that *spe-41* may function to stabilize Ca2+ homeostasis perturbed by Aβ specifically.

C. elegans sqt-2 is orthologous to the macrophage scavenger receptor with collagenous structure (MARCO). Microglial cells are the macrophages of the central nervous system. They express numerous receptors that can bind Aβ under both normal and pathological conditions (Alarcón et al., 2005; Coraci et al., 2002; Husemann et al., 2002; Krieger & Krieger, 1994; Marzolo et al., 1999; Wolf & Gonias, 1994; Yan et al., 1996; Yu & Ye, 2015). MARCO is a membrane glycoprotein that functions as a

scavenger receptor on astrocytes and microglia. In rats, MARCO can bind several ligands including pathogens *Neisseria meningitidis* and *Streptococcus pneumoniae* (Braun et al., 2011), or the fucoidan polysaccharide (Alarcón et al., 2005). MARCO-mediated uptake of *N. meningitidis* was demonstrated in mice, providing evidence of its involvement in the elimination of invading pathogens in the nervous system (Mukhopadhyay et al., 2006). On rat glial cells, MARCO can form a complex with the G protein-coupled receptor formyl-peptide-receptor-like-1 (FPRL1) to phagocytose and clear Aβ42 (Brandenburg et al., 2010; Koenigsknecht, 2004; Paresce et al., 1996). However, cellular processes influenced by MARCO-mediated Aβ clearance in AD need further investigation. The immune-regulating functions of glial cells are not conserved in *C. elegans*. Glia in *C. elegans* can phagocytose dying cells during embryogenesis (Sulston et al., 1983), but whether Aβ or bacterial pathogens promote glial phagocytic activity remains to be determined. *C. elegans sqt-2* is a predicted cuticle gene that is expressed in the amphid neurons, which are exposed to the external environment. The amphid socket glia are lined with a cuticle that could be involved in processing signals from the environment (Oikonomou et al., 2011; Ward et al., 1975). Therefore, it is possible that *sqt-2* plays a role in sensing the worm's microbial diet. However, the downstream neuronal responses mediated by *sqt-2*, and how they may promote changes in neurodegeneration, need to be investigated.

Mitochondria supply energy to support neuronal activity, and having healthy mitochondria is necessary to protect against oxidative damage. Disturbances in mitochondrial function are associated with neurodegenerative diseases such as AD (Lin

& Beal, 2006; H. Wang et al., 2020). Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha (HADHA), the ortholog of *C. elegans ech-1.1*, is the alpha subunit of the mitochondrial trifunctional protein, which catalyzes the mitochondrial beta-oxidation of long-chain fatty acids (Orii et al., 1999). HADHA expression was increased in the cortex of 5xFAD AD mice, which overexpress APP and mutated PSEN1 to induce amyloidogenesis (Janelidze et al., 2016; H. Wang et al., 2020). Importantly, cortical upregulation of HADHA coincided with decreased expression in the cerebrospinal fluid of humans and 5xFAD mice, mirroring the expression pattern of pathological Aβ (i.e. increased in cortex and decreased in cerebrospinal fluid) (H. Wang et al., 2020). In fact, 59% of the 37 proteins found to follow this expression signature were mitochondrial proteins, highlighting associations between mitochondria functions, oxidative stress, and AD (W. Wang et al., 2020). *C. elegans ech-1.1* is also predicted to be localized to the mitochondria and have 3-hydroxyacyl-CoA dehydrogenase activity (Van Gilst et al., 2005; Xu et al., 2015). Although *ech-1.1* has not been studied in the context of neurodegeneration, it is possible that its upregulation in response to Aβ is conserved in our model. Our findings are an example of how RNAi-mediated knockdown of a predicted mitochondrial and Aβ-responsive gene can protect against paralysis in a *C. elegans* model of AD.

Overall, our work demonstrates that initial screening using knockdown of single genes in paralysis assays can lead to identification and validation of genes that are also neuroprotective, thereby allowing identification of genes that protect against both Aβ-induced and tau-induced pathogenesis of AD. Subsequent analyses to explore the

biological processes and pathways the *C. elegans* orthologs are involved in may be foundational in characterizing the roles of these genes in AD development and pathogenesis. Our findings from this screen suggest that suppressing orthologs PPP1CB, PTPN9, COLQ, TRPC3, TRPC6, MARCO, or HADHA can be potential therapeutic avenues for AD. To our knowledge, mechanistic studies on TRPC7, testis specific serine kinase 6 (TSSK6) or surfactant protein D (SFTPD) in neurodegeneration have not been performed. Therefore, our findings suggest they can be novel mediators of neurodegeneration. Overall, using transcriptomics and by performing an RNAi screen, we uncovered genes that are linked to Aβ- and tau-induced pathogenesis in *C. elegans* models of AD. Understanding the mechanistic consequences of suppressing these orthologs in the treatment of AD will require future studies.

Materials and Methods

C. elegans strains

C. elegans CL4176 *smg-1(cc546ts)*I; *dvIs27*[*pAF29(myo-3::A-Beta 1-42::let-851* 3'UTR) + *rol-6(su1006)*] was constructed by Dr. Christopher Link's lab, allowing temperature-sensitive expression of the human Aβ1-42 peptide in the body muscle wall (Link, 2003). This expression system is mediated by a temperature-sensitive mutation in *smg-1,* a gene that is required for nonsense mediated decay (NMD) (Grimson et al., 2004). At the permissive temperature (15°C), SMG-1 is functional and NMD recognizes and degrades the Aβ transcript because it carries a long untranslated region. At the restrictive temperature, NMD is inhibited, the transcript is not degraded, and Aβ is produced. (Mango, 2001). *C. elegans* BR5706 *byIs193*[*rab-*

*3p::*F3DK280+*myo2p::mcherry*]; *bkIs10*[*aex-3p::*h4R1NtauV337M+*myo2p::GFP*] was constructed by Dr. Chronis Fatouros. It expresses two mutant tau transgenes. Pan-neuronal expression of the pro-aggregant K280 deletion in 0N4R tau was achieved using a *rab-3* promoter (Fatouros et al., 2012). The K280 deletion is found in patients with Frontotemporal dementia with parkinsonism-17, who have tau pathologies similar to those observed in AD patients (von Bergen et al., 2001). Additionally, a V337M substitution mutation in the 1N4R isoform of human tau is also expressed. Fatouros *et al.* found that the BR5706 worms expressing both mutations recapitulated toxicity and tau pathology observed in mammalian models of disease, such as neuronal defects and uncoordinated movement in adults (Fatouros et al., 2012). The LMN008 worms have the same genotype as BR5706, with an integrated *mec7p::GFP* reporter, allowing visualization of AVM, ALML/R, PVM, and PLML/R mechanosensory neurons.

The AP-tau RNAi-sensitized strain was created by crossing *C. elegans* LMN008 with a strain expressing [pCFJ90(*myo-2p::mCherry*) + *unc-119p::sid-1*]. This strain also expresses the bkIs10[*aex-3p::*h4R1NtauV337M+*myo2p::GFP*] transgene.

The N2 strain was used as wild-type; they were obtained from the Caenorhabditis Genetics Centre (CGC).

All strains were maintained on nematode growth medium (NGM) seeded with *E. coli* OP50. CL4176 worms were grown at 15°C, and all other strains at 20°C unless otherwise specified.

RNAi-mediated gene knockdown paralysis assay

NGM was supplemented with carbenicillin (25 μg/mL) and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). RNAi bacteria from the Ahringer library (Kamath & Ahringer, 2003) were streaked out onto LB/Amp (50 μg/mL) plates for further use. Bacteria were inoculated from frozen, grown at 37°C overnight in LB supplemented with ampicillin (50 μg/mL). Overnight cultures were diluted 1/100 in fresh LB/Amp and grown for 6 hours. RNAi plates were seeded with 200 μL of culture and grown on plates at 37°C overnight. CL4176 eggs were added to plates. Plates were incubated at 15°C for 48 hours until upshift to 25°C. Paralysis was measured after 48 hours at 25°C. Knockdown of GFP was performed as an extra no-target control alongside the empty vector. Animals with either absolutely no movement or no movement except for the head were scored as paralysed.

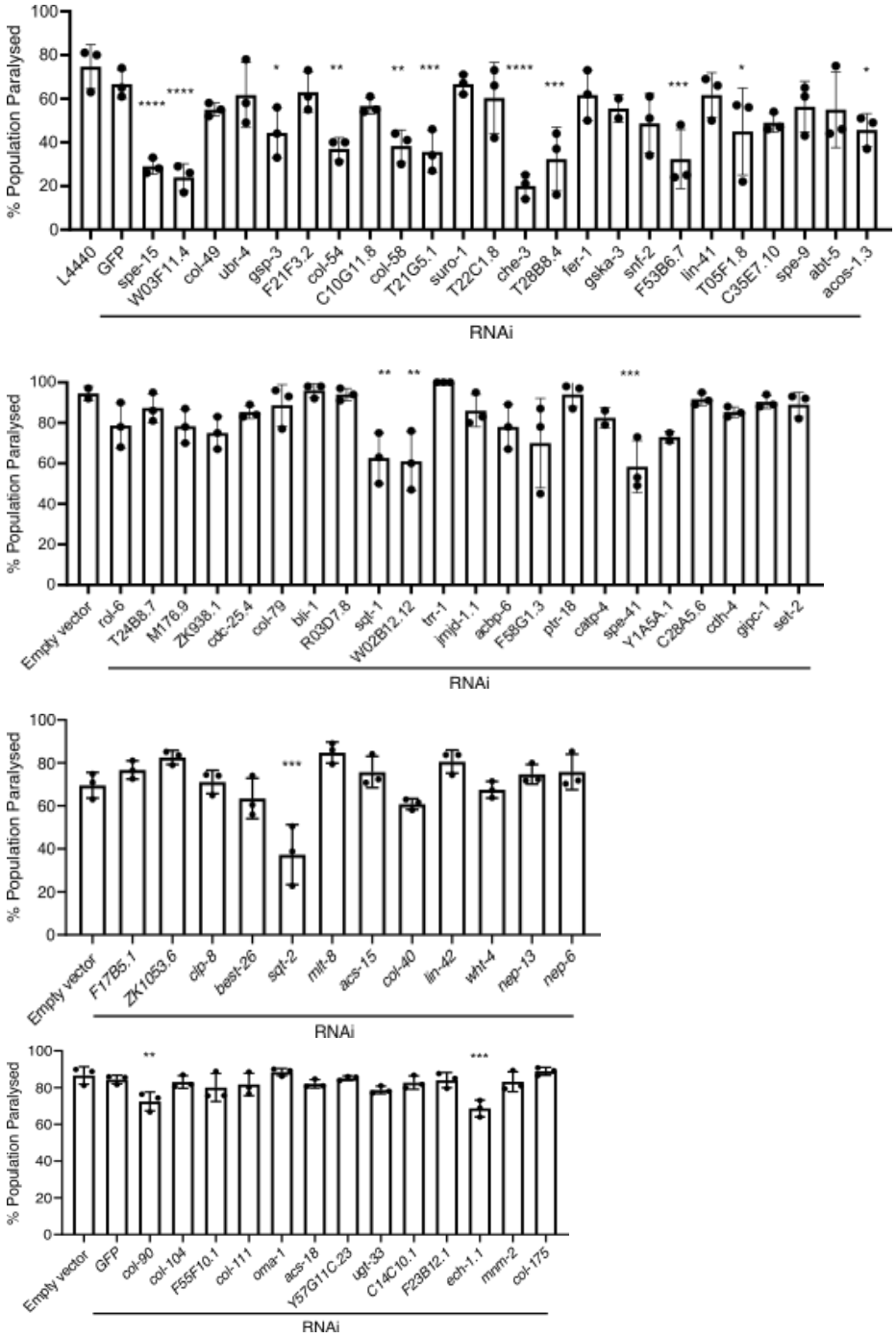
Knockdown of *nhr-23* was performed as a positive control.

RNAi-mediated gene knockdown neurodegeneration assay

Medium was supplemented with carbenicillin (25 μg/mL) and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). RNAi bacteria from the Ahringer library (Kamath & Ahringer, 2003) were streaked out onto LB/Amp (50 μg/mL) plates for further use. Bacteria were inoculated from frozen and grown at 37°C overnight in LB supplemented withampicillin (50 μg/mL) overnight. Overnight cultures were subcultured 1/100 in fresh LB/Amp for 6 hours. 200 μL of subcultures were seeded on prepared media to grow on plates at 37°C overnight. Populations of AP-tau LMN008 expressing a *mec-7p::GFP* marker were bleach-synchronized, and approximately 400 LMN008 eggs were added to

each experimental plate. Plates were incubated at 20℃ until worms were developed to adulthood (day 1 adult). Worms were then paralyzed on a slide with 0.1% sodium azide, then GFP fluorescence was observed and captured using a compound microscope. All experiments were performed as three separate trials. Knockdown of *nhr-23* was performed as a positive control for RNAi effectiveness.

Supplementary



Supplementary Figure 1. RNAi screen of human orthologs identified from RNA-Seq in C. elegans CL4176. Only genes that caused protection when knocked down in ≥2 trials were identified as hits in Table 1.

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CHAPTER 4 – Conclusions

Summary of findings

In the work presented in this thesis, I found that specific members of the human microbiota caused neuroprotection in *Caenorhabditis elegans* models of Alzheimer's disease (AD), and that they may do so by modulating host gene expression. As outlined in Chapter 2, I produced the foundation of this project by identifying human microbiota species that protected against paralysis and neurodegeneration in amyloid-β (Aβ)- and tau-overexpressing *C. elegans*, respectively. From there, we investigated host mechanisms impacted by human-associated bacteria and further focused on characterizing how these mechanisms influenced neurodegeneration. Two independent RNA-Seq experiments identified bacteria-induced changes in gene expression that spanned numerous biological processes. We explored how several of these can induce neuroprotection. The main subset of genes we sought to characterize belonged to the tau tubulin kinase (TTBK) gene family. Their homology with human TTBK1/2 supported our rationale for studying these genes, especially since *C. elegans* TTBKs have not been functionally characterized in a neurodegenerative model. The major challenge in doing socame from the fact that *C. elegans* has 74 TTBKs and, of these, 22 were reproducibly suppressed in response to neuroprotective *E. cancerogenu*s and/or *E. hormaechei* species. Knockdown of individual TTBKs induced neuroprotection and improved locomotor speed in AP-tau *C. elegans*, demonstrating that TTBKs contribute to neurodegeneration and decreased motor function in this model. Our work is the first to study *C. elegans* TTBKs in both Aβ and tau-overexpressing models of AD, and further, the first to

demonstrate microbe-induced changes in TTBK gene expression of any animal model. The suppression of TTBK expression suggested to us that these bacteria may cause decreased tau phosphorylation and subsequently reduce neurodegeneration. Indeed, we found that *Enterobacter* species reduced the abundance of a putatively neurotoxic tau species phosphorylated at S422, an AD-specific site directly phosphorylated by human TTBK1 (Dillon et al., 2020). Tau can be processed and modified in numerous ways to become neurotoxic. In fact, acetylation, methylation, and SUMOylation can promote Tau aggregation and the formation of neurofibrillary tangles (Balmik & Chinnathambi, 2021; Cohen et al., 2011; Luo et al., 2014). Based on our findings that the pS422 Tau species that decreased in response to *Enterobacter* was ~40 kDa larger than monomeric pS422 tau, we hypothesized that pS422 tau had undergone SUMOylation (~11 kDa) and/or ubiquitination (~9 kDa) on at least one site. The processes explaining how not only one type, but a combination of different post-translational modifications (PTMs) of tau affect neurodegeneration remain understudied. My work emphasizes the importance of characterizing the numerous mechanisms that act on tau to investigate biological processes that influence neurodegeneration.

Our findings that neuroprotective bacteria also reduced *C. elegans* lifespan suggested immune signaling was being modulated. We asked if bacteria-induced neuroprotection in *C. elegans* occurred through suppression of p38/PMK-1/MAPK innate immune signaling. Activated PMK-1 upregulates most of its target genes and downregulates a smaller proportion of its targets. All tested microbiota species influenced the expression of some PMK-1 targets in AP-tau animals, but many target genes were not

affected. Out of the targets that were changed, some were expressed in the opposite direction than what would be expected with PMK-1 activation. This was observed in response to neuroprotective bacteria or a neurodegenerative diet of *K. pneumoniae.* Therefore, there were no clear correlations between the expression levels of affected target genes and bacteria-induced changes in neurodegeneration. We also found that decreased lifespan occurred independently of PMK-1, and that bacteria do not affect PMK-1 activation. Altogether, these data suggest that altered PMK-1 signaling is unlikely to be the causal neuroprotective host response to these bacteria. We also asked if bacterial diet differentially regulated insulin-like signaling (IlS) and transcriptional activity of FOXO/*daf-16*. Similar to our investigation of PMK-1 targets, our test bacteria induced changed expression of DAF-16 targets. However, again, there were no clear differences between animals provided a neuroprotective diet vs. *K. pneumoniae.* Overall, although we observed some microbe-specific differential regulation of PMK-1 and DAF-16 target genes, the data suggests neither pathway is solely responsible for the diet-induced changes in neurodegeneration that we observed in *C. elegans*.

To understand the effects that neuroprotective and neurodegenerative bacteria have on gene expression in a model of neurodegenerative disease, we compared gene expression in AP-tau animals provided neuroprotective bacterial diets or a diet of *K. pneumoniae*. Our analyses revealed relatively subtle differences in the enriched biological processes identified from diet-specific gene expression changes. We also found that tau aggregation may promote a universal proteolysis and heat stress response. It is possible that the presence of pan-neuronal tau aggregates significantly altered the

way host mechanisms were regulated to favour the management of toxic proteins. It is also possible that the gene expression changes were a result of neuroprotective mechanisms triggered by the bacteria. Therefore, different from our analyses of wild-type *C. elegans*, these data can provide insight on gene expression changes resulting from tau-bacteria interactions that reduce neurodegeneration within a neurodegenerative genetic background.

This work demonstrates how investigating individual members of the microbiota can reveal factors that influence neurodegeneration. We found that crude extracts of microbiota species sufficiently induced neuroprotection, suggesting that the bacteria reduced neurodegeneration by producing a bioactive metabolite. Our work provides some insight on mechanisms by which microbiota species might influence neurodegeneration, and demonstrates the potential of using the microbiota to identify biological agents that can be used to treat AD.

In Chapter 3, we identified putative causal human orthologs that induce neuroprotection and neurodegeneration in *C. elegans* models of AD. We identified seven genes that, when knocked down, reduced paralysis and neurodegeneration in both Aβ- and tau-overexpressing animals. The *C. elegans* genes that were protective against both paralysis and neurodegeneration ranged in their predicted function: one kinase, two phosphatases, three structural constituents of the cuticle, and a hydroxyacyl-CoA dehydrogenase. The functions of several of their orthologs have been studied in mammals; however future investigations on how they can be targeted in the treatment of neurodegenerative disease are needed. Our work demonstrated that initial screening to

test the impact of knocking down single genes in paralysis assays can allow identification and validation of genes that are also neuroprotective. Subsequent analyses to explore the biological processes and pathways the *C. elegans* orthologs are involved in may be foundational in characterizing the roles of these genes in AD development and pathogenesis.

Overall significance and implications

AD is the most common neurodegenerative disorder in the world, demonstrating the great need for a cure. However, finding a cure for AD has been a decades-long challenge. AD is a multi-billion dollar burden for the world economy (Jia et al., 2018), and is the subject of repeated clinical trial failures (Yiannopoulou et al., 2019). The complexity of AD has imposed conflicting scientific theories (Kametani & Hasegawa, 2018). These issues, caused by the multifactorial nature of AD, make it clear that an interdisciplinary approach is necessary in researching this disease.

Many past studies that modelled AD neglected to study tau pathogenesis, perpetuating the disproportionate assignment of causal disease mechanisms to Aβ. By using models of both Aβ and tau, we can attribute our findings to pathologies of Aβ, tau, or both. Tau-induced neurodegeneration is complex in itself. Phosphorylation is only one of many tau PTMs, and the specific epitopes phosphorylated can affect tau status differently. We found that neuroprotective bacteria can reduce 100 kDa tau phosphorylated at S422, but not 60 kDa, nor other tested AD-associated sites. These findings suggest that pS422 tau is associated with additional PTMs that can drive tau pathogenesis in AD. Our work is part of a growing

number of studies that consider the nuances in neurofibrillary tangle formation (Aragão Gomes et al., 2021; Carroll et al., 2021; Wesseling et al., 2020). We also found that a subset of genes from a class of *C. elegans* kinases can influence tau-induced neurodegeneration and were the first to demonstrate that TTBK expression can be microbially regulated. Altogether, these are novel findings describing host mechanisms that contribute to neurodegeneration.

Studies of patient microbiota can identify bacteria compositional changes at the genera level (Ling et al., 2020; Vogt et al., 2017). These studies are powerful in defining broad microbiota signatures associated with AD. It is necessary to supplement this knowledge by studying the impact of specific species and their products. Existing work that addresses this generally aims to characterize probiotic species and strains of the genera *Lactobacillus*, *Bacillus*, and *Bifidobacterium* (Goya et al., 2020; Naomi et al., 2021). Our work identified novel neuroprotective human-associated bacterial species and determined that they influence neurodegeneration through production of a bioactive metabolite. This in turn provides foundational insight on potentially novel bacterial mediators of neurodegeneration, and this work is an example of how these factors can be derived from specific species of the gut. Altogether, we identified novel host gene expression signatures induced by microbiotal species and their bioactive metabolites, which influence neurodegeneration in *C. elegans* models of AD.

Study limitations and future directions

The work of this thesis provides evidence supporting the neuroprotective role of specific human-associated bacteria in AD, however there are experimental limitations

that can be addressed to improve the strength and impact of our findings. Therefore, our work will benefit from further investigation. We studied microbiota-neurodegeneration effects in both Aβ and tau disease backgrounds. This allowed us to identify bacterial effects specific to either pathology, e.g., *K. pneumoniae* was protective against Aβ- but not tau-induced cytotoxicity. We can use our findings to disentangle neurodegenerative mechanisms involving Aβ vs tau, however our work does not necessarily reflect the complex synergistic processes Aβ and tau influence to promote AD pathogenesis. This may be compounded by our use of a non-mammalian model, which does not have an adaptive immune system. Therefore, it is now crucial to follow our work with translating our findings into a mammalian model that, like AD patients, exhibits both Aβ and tau pathogenesis, and can experience adaptive-innate immunity crosstalk associated with disease progression. An AD mouse model that can be used to study both Aβ and tau lesions is 3xTg-AD ($huAPP_{695}$ (Swe); $MAPT_{4R0N}$ (P301L); $Psen1^{M146V}$ knock-in) (Oddo et al., 2003). These animals exhibit a combination of both Aβ and tau accumulation; therefore, they are considered to be a "complete" transgenic mouse model of AD (Oddo et al., 2003). Translation of our work into a mammalian model is currently underway in collaboration with Dr. Margaret Fahnestock and PhD candidate Mona Abdollahi. We aim to test the effects of neuroprotective bacterial extracts on mammalian models of AD. Mona is currently completing preliminary experiments testing for viable extract doses *in vitro* using wild-type mouse primary hippocampal and cortical cells. Future work for this project includes studying neuron health in response to bacterial extracts using the 3xTg-AD mouse model by measuring potential changes in neuron dendrite integrity, and brain-derived neurotrophic factor (BDNF) expression. Ensuring that our

findings can translate to a mammalian model is necessary. However, an understanding of the fundamental mechanisms involved in AD pathogenesis remains unclear. We propose that using the double transgenic *C. elegans* strain, UM0001 (*snb-1p*::Aβ1–42 + *rab-3p*::F3ΔK280), may be useful in characterizing host mechanisms that influence neurodegeneration caused by the presence of both Aβ and tau (Wang et al., 2018). We can analyze gene expression changes caused by neuroprotective bacteria in this strain. Using a strain that is co-expressing AP-tau and Aβ could capture disease-associated gene expression that we may have missed in our transcriptomic profiling of animals only expressing AP-tau. Further studies evaluating the mechanisms that influence both Aβ and tau pathology will also be necessary. Aβ- and tau- induced deficits in neurodegeneration, lifespan, chemotaxis associated with learning, motility, and accumulation of protein aggregates, can be measured in *C. elegans* UM0001 (Wang et al., 2018). We could follow transcriptomics by using RNAi and making mutants of genes of interest in a double transgenic background, and test for changes in neurodegeneration-associated phenotypes to identify disease-influencing genes. We can compare findings to our existing work to understand how tau-associated mechanisms tie into a model that is also expressing Aβ. This provides potential for investigating processes contributing to other tauopathies in addition to AD, as well as helping pinpoint conserved host mechanisms that cause neuroprotection in AD.

We found that human tau undergoes PTMs in AP-tau *C. elegans*. Specifically, we found that a 100 kDa tau species phosphorylated at S422 is reduced in response to neuroprotective *Enterobacter*. Identifying this tau species will help characterize how the host copes with tau-induced neurotoxicity in response to *Enterobacter*. We hypothesized

that 100 kDa pS422 tau is SUMOylated. To test this, we can perform immunoprecipitation of 1) SMO-1 to blot for pS422, and 2) total Tau to blot for pS422 and SMO-1. The pS422 antibody (ab79415) that detects the 100 kDa band is not suitable for immunoprecipitation. We also created an AP-tau strain expressing a GFP knock-in downstream of *C. elegans* SUMO (*byIs193*[*rab-3p*::F3DK280+*myo2p::mcherry*]; *bkIs10*[*aex-3p*::h4R1NTauV337M+*myo2p::GFP*]; *smo-1*(ot1038[*smo-1::gfp*::3xflag]) to test if the 100 kDa band is SUMOylated. If the GFP fusion results in the shift of the 100 kDa band to approximately 127 kDa (GFP weighs 27 kDa), this would indicate that the heavier pS422 tau species is SUMOylated. Although we could associate tau PTMs identified from this work to the phosphorylation of S422, we are ultimately limited by the number of phosphorylation sites we choose to investigate. Future considerations to address this include using liquid chromatography coupled to high resolution mass spectrometry (LC-MS) to characterize tau species (Wu et al., 2022) that are differentially modified in AP-tau animals fed neuroprotective *Enterobacter*. This technique allows for the identification of phosphosites and other PTMs including acetylation, methylation, and ubiquitination (Wu et al., 2022; Tan et al., 2015; Morris et al., 2015). The enrichment of SUMOylated tau phosphorylated at S422 using the AP-tau *smo-1::gfp*::3xflag knock-in strain (i.e., isolating the 127 kDa band after SDS-PAGE) prior to LC-MS can identify other phosphorylation events associated with tau SUMOylation. Overall, this work would help discriminate post-translationally modified tau species associated with neurodegeneration.

Our work focuses on studying the modifications of human tau in *C. elegans* without considering the possible interactions between human tau and the endogenous tau.

C. elegans tau, PTL-1, is expressed neuronally and is also present in other tissues including the body wall muscle (Davis et al., 2022). PTL-1 contains high sequence similarity with human tau in the microtubule-binding repeat domain, and regulates microtubule assembly *in vitro* (Goedert et al., 1996; McDermott et al., 1996). In the worm, loss of *ptl-1* caused increased neurodegeneration of ALM and GABAergic neurons, and reduced lifespan (Chew et al., 2013). Loss of *C. elegans* PTL-1 exacerbated decreased lifespan caused by transgenically introducing aggregate-prone human tau (Nunez et al., 2022). These data demonstrate that PTL-1 functions to maintain neuronal health, which is consistent with the idea that tauopathies are caused by both 1) the accumulation of neurotoxic tau aggregates, and 2) the loss of normal tau function. Therefore, it is possible that loss of PTL-1 function may synergistically promote neurodegeneration with human tau in AP-tau *C. elegans*. We speculate that this could occur through the seeding of human tau to form aggregates with PTL-1, and/or tau-induced processes promoting hyperphosphorylation and dissociation of PTL-1 from microtubules. Currently, there are no existing antibodies that can detect PTL-1, so we cannot measure potential differences in pathogenic phosphorylation of the endogenous tau. An alternative approach we can take is knocking out *ptl-1* in AP-tau animals to test if bacteria-mediated neuroprotection not only occurs through suppressing TTBK or reducing S422 phosphorylation of tau but is also dependent on normal PTL-1 function. Since *ptl-1* is also expressed in the body wall muscle, we could use the same approach to explore the possible interactions between PTL-1 and Aβ that may influence the bacteria-induced protection against paralysis we observed. Additional work with these *ptl-1* strains by using *ttbk* RNAi or introducing *ttbk* mutations could provide insight on how

TTBKs may act on Aβ, human tau, and/or *C. elegans* tau to modulate neurodegeneration.

We can also investigate the bacterial factors that promote neuroprotection. Our findings that crude bacterial extracts sufficiently promote neuroprotection suggest that this effect is induced by production of a metabolite. To begin identifying the causal metabolite, we can fractionate the crude extracts and test the ability of fractions to induce neuroprotection in AP-tau animals. Neuroprotective fractions will then undergo mass spectrometry to identify the metabolites present, followed by isolation of putative neuroprotective compounds by purification. However, the multifarious nature of crude extracts makes the identification of specific causal metabolites time-consuming and experimentally challenging. A limitation of our study is that we did not test the neuroprotective effects of the bacterial supernatants in AP-tau animals. We can make concentrated methanol extracts of the supernatants from neuroprotective bacteria, test if they are sufficient to induce neuroprotection, and follow with isolating the causal metabolite using the same approach as we would with extracts. Since supernatants contain fewer total metabolites compared to crude whole-cell extracts, using supernatants could allow more efficient identification and isolation of neuroprotective metabolites. This work would identify specific bacteria-produced neuroprotective compounds. Testing the effects of these compounds on our *C. elegans* models, as well as mammalian models, will explore the drug discovery potential of our work.

In response to neuroprotective bacteria, *C. elegans* underwent many different gene expression changes, with my work mostly concentrating on the characterization of *ttbk*s. Although this concentration provided our work with a focus, the exploration of the RNA-Seq experiments we performed remains limited. Our transcriptomic analyses

can be further mined to characterize host responses to the microbiota. This includes studying genes that were upregulated in response to neuroprotective bacteria, genes and gene families involved in the many enriched stress responses, and genes that were uniquely changed by each bacterium. We can also explore the roles of differentially regulated phosphatases to gain a deeper understanding of how the neuroprotective bacteria affect phosphorylation in *C. elegans*, and specifically, how they may regulate the phosphorylation status of tau in AP-tau animals. Our AP-tau datasets can also be compared to wild type datasets to identify changes in host expression caused by an AP-tau genetic background. Overall, the transcriptomic analyses we performed can inform future investigations of bacteria-induced neuroprotection in *C. elegans.*

Our work demonstrated that RNAi-mediated knockdown of *ttbk* sufficiently caused neuroprotection. It was surprising that of 18 *ttbk*s knocked down, all were neuroprotective, and 14 improved locomotor function. After sequencing the RNAi clones, we found that six were predicted to have off-target effects, with four of these being other *ttbk*s, but the 12 remaining clones should selectively target the intended gene. To test this, we can sequence animals experiencing RNAi of one *ttbk*, and use Nanostring to see if expression of other *ttbk*s change. We also need to validate neuroprotection caused by RNAi by knocking out each gene of interest in AP-tau animals. Further, rescuing phenotypes in overexpression mutants would confirm that loss of *ttbk* promotes neuroprotection. These genetic tests can also be performed to validate the neuroprotective capacity of suppressing the seven human orthologs we identified from our RNAi screen.

Concluding statement

Efforts towards developing treatments for symptoms (Cummings et al., 2020; Poudel & Park, 2022), more sophisticated diagnostic tools (Scheltens et al., 2016), and understanding mechanisms that cause AD (Guo et al., 2020) are continuing in the research community. It has become increasingly clear that many factors contribute to AD. In the work of this thesis, we demonstrated that neuroprotective bacteria changed expression of numerous genes involved in a diverse set of biological processes. This highlights how complex microbiota-host interactions can be but offers a foundation for identifying causal mechanisms. We have provided evidence of neuroprotective bacteria suppressing tau kinases and reducing a form of pathogenic tau in *C. elegans*. Further investigations on how suppressing *ttbk* can change tau modifications, and how altered tau status can reduce neurodegeneration, will help inform which mechanisms should be targeted in an AD patient. Although the development of microbially-derived therapeutics for AD are beyond the scope of this thesis, our work demonstrates that bacterial products can significantly cause neuroprotection in an AD model. Identifying metabolites that suppress *ttbk*-driven tau pathogenesis and neurodegeneration would provide an understanding of one mechanism through which host-associated bacteria can cause neuroprotection in AD.

Altogether, the existing research on AD suggests that future patient treatment will require a personalised approach. Treatment and preventative approaches should consider a patient's genetics, comorbidities, and even their microbiota composition. Efforts to characterize AD-associated microbiota communities can help inform preventative strategies in aging populations, such as adopting lifestyle or diet changes. In conjunction

with this, research on how microbes can influence neurodegeneration, and what bacterial products can promote a healthier nervous system, will lead to discoveries that combat AD. The complexity of AD inadvertently made way for widespread discoveries that spanned decades of dedicated research. Continued examination of the many factors contributing to disease may ultimately result in preventative and treatment regimens against AD.

References - Introduction

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References - Conclusions

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