Adaptor protein WDFY3 is neuroprotective by counteracting age-dependent decline in proteostasis

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**ADAPTOR PROTEIN WDFY3 IS NEUROPROTECTIVE BY
COUNTERACTING AGE-DEPENDENT DECLINE IN PROTEOSTASIS**

XIU HUI FUN

SCHOOL OF BIOLOGICAL SCIENCES

2020

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XIU HUI FUN

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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- I prepared the manuscript drafts. The manuscript was revised together with Asst Prof Guillaume Thibault.

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Summary

Age-dependent decline in proteostasis increases vulnerability of neuronal cells to oxidative stress, altered energy homeostasis and protein aggregation. Recent studies have suggested an adaptor role for BEACH-domain containing proteins (BDCPs) in autophagy-mediated clearance of protein aggregates in neurodegenerative models. Alterations in BDCPs result in protein aggregation and shortened lifespan in *Drosophila*. However, the exact mechanism by which they regulate autophagy and alleviate neurodegenerative toxicity remains unclear. Here, we report the characterisation of WDFY-3, a BDCP in *Caenorhabditis elegans*. Like its *Drosophila* homologue blue cheese, WDFY-3 is widely expressed in the nerve ring and along the entire length of axonal projections, supporting the possibility of having neuron-specific functions. Our results show that *wdfy-3* is not required to induce autophagy genetically and at the protein levels in aged HD animals. Additionally, loss of *wdfy-3* shortens the lifespan and reduces motility of *C. elegans*. Surprisingly during aging, there was a reduction in neuronal aggregates in the absence of *wdfy-3*, suggesting the protective role of WDFY-3 as a stabiliser that sequesters polyglutamine aggregates into inclusion bodies, further reducing toxicity. Together, our data point to the potential role of WDFY-3 in sequestering toxic aggregates upon age-dependent decline in proteostasis.

Abbreviations

Abbreviation	Description
AD	Alzheimer's disease
ALFY	Autophagy-linked FYVE protein
ALS	Amyotrophic lateral sclerosis
APP	β -amyloid precursor protein
ATF-6	Activating transcription factor 6
ATF-6(N)	N-terminal transcription factor
A β	Amyloid-beta
A β_{40}	A β peptide that is 40 residues in length
A β_{42}	A β peptide that is 42 residues in length
<i>bchs</i>	Blue cheese gene
Bchs	Blue cheese protein
BDCPs	BEACH-domain containing proteins
BEACH	Beige and Chediak-Higashi
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CCT	Chaperonin containing TCP-1
CMA	Chaperone mediated autophagy
CNS	Central nervous system
D0	Day 0
D1	Day 1
D4	Day 4
D7	Day 7
eIF2 α	Eukaryotic translation initiation factor 2a
ER	Endoplasmic reticulum
fALS	Familial ALS
FUDR	5'-fluorodeoxyuridine
GFP::LGG-1-PE	PE-conjugated GFP::LGG-1
HD	Huntington's disease

HDAC6	Histone deacetylase 6
Hsc70	Heat-shock cognate protein of 70 kDa
HSE	Heat-shock element
HSF1	Heat shock factor 1
HSPs	Heat shock proteins
HSR	Heat shock response
<i>HTT</i>	Huntingtin gene
HTT	Huntingtin protein
IPTG	Isopropyl- β -D-thiogalactoside
IRE-1	Inositol-requiring enzyme 1
L1	Larva stage 1
L4	Larva stage 4
LB	Luria-Bertani
LBS	Lipid bilayer stress
LGG-1-PE	PE-conjugated LGG-1
LIR	LC3 interacting region
<i>lof</i>	loss-of-function
mHTT	Mutant huntingtin
NAC	Non-amyloid- β component of plaques
NFTs	Neurofibrillary tangles
NGM	Nematode growth media
PAS	Phagophore assembly site
PC	Phosphatidylcholine
PD	Parkinson's disease
PDME	Phosphatidylmethylethanolamine
PE	Phosphatidylethanolamine
PEK-1	Protein kinase (PKR)-like ER kinase
PH	Pleckstrin homology
PI3P	Phosphatidylinositol-3-phosphate

PMME	Phosphomonomethylethanolamine
polyQ	Polyglutamine
qPCR	Quantitative PCR
RIDD	Ire1-dependent decay
RNAi	RNA interference
S1P	Site-1 protease
S2P	Site-2 protease
SNpc	<i>substantia nigra pars compacta</i>
SOD	Superoxide dismutase
SOD1	Superoxide dismutase 1
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
WDFY-3	WD repeat and FYVE domain containing 3 protein
<i>wdfy-3</i>	WD repeat and FYVE domain containing 3 gene
WT	Wild type
XBP-1	x-box binding protein 1

Chapter 1. Introduction

1.1 Neurodegenerative diseases

One of the common characteristics among neurodegenerative diseases is the accumulation of intracellular toxic aggregates in the central nervous system (CNS) [1]. These aggregates include α -synuclein in Parkinson's disease (PD), amyloid-beta ($A\beta$) and tau in Alzheimer's disease (AD), superoxide dismutase (SOD) in Amyotrophic lateral sclerosis (ALS), and mutant huntingtin (mHTT) in Huntington's disease (HD) [2, 3]. Despite the common theme of protein misfolding and aggregation among different neurodegenerative diseases, the pathology of each age-related misfolding disorders can be affected or activated by disease-protein-specific subset of molecular processes [1].

1.1.1 Huntington's disease

HD is an autosomal dominant neurodegenerative disease characterised by the expansion of a CAG trinucleotide repeat within exon one of the huntingtin gene (*HTT*). The length of polyglutamine (polyQ) tract determines the severity of disease with a pathogenic threshold of 35 repeats and above [4-7]. Large CAG repeats results in the formation of an elongated polyQ tract at the amino terminus of the huntingtin protein (HTT) that plays a role in protein aggregation and a gain-of-function toxicity [8]. The polyQ expansions form tightly linked β -sheets through hydrogen bonding between the main chain amides and polar side chains which can function as "polar zippers", leading to the precipitation and aggregation of proteins [9, 10]. Being highly aggregation prone, mHTT forms inclusions throughout the brain [11, 12]. Slow accumulation of aggregates can further trigger neuronal cell death, leading to the development of HD [13].

1.1.2 Parkinson's disease

Presence of Lewy bodies and Lewy neurites in affected neurons and progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) are neuropathological hallmarks of PD. α -synuclein protein encoded by the SNCA gene is the main component of Lewy bodies and Lewy neurite found in PD patients [14]. Mutations in the SNCA gene leads to autosomal dominant forms of PD and are fundamentals to the development of sporadic PD [15]. α -synuclein consists of three domains, namely the N-terminal, non-amyloid- β component of plaques (NAC), and C-terminal domains [16]. Point mutations within the N-terminal region of α -synuclein result in the development of autosomal dominant

familial PD and PD-associated syndromes, a consequence of the misfolding and/or aggregation of mutant α -synuclein protein [14, 17-23]. In addition, the NAC domain supports α -synuclein aggregation by preventing its removal and stimulating formation of fibrils [18]. Lastly, the C-terminal is required to preserve the solubility of α -synuclein [21, 24]. As C-terminally truncated α -synuclein aggregates rapidly as compared to the full-length protein, it further indicates its regulatory role in amyloid aggregation [25-27]. Despite its ability to modulate aggregation, the C-terminus is not integrated into the core structure of α -synuclein fibrils but located on their surface [28, 29]. All three domains are involved in protein aggregation and have the potential to affect one another, either by supporting or impeding formation of fibrils, further resulting in PD [30].

1.1.3 Alzheimer's disease

AD is characterised by the accumulation of extracellular plaques deposits of A β and neurofibrillary tangles (NFTs) of the microtubule binding protein tau [31-33]. A β is generated via endoproteolysis of β -amyloid precursor protein (APP) via sequential cleavage by groups of enzymes or enzyme complexes namely α -, β -, and γ -secretases. Cleavage and processing of APP can be divided into a non-amyloidogenic and an amyloidogenic pathway. In the non-amyloidogenic pathway, cleavage of APP by α -secretase occurs within the A β domain, in turn preventing the production and secretion of A β [34, 35]. Contrarily, sequential cleavage by β - and γ - secretases generates insoluble A β fibrils [36]. Of the A β peptide produced, most of them are 40 residues in length (A β_{40}) and a smaller proportion of 42 residue variant (A β_{42}). Being more hydrophobic, A β_{42} is more prone to fibril formation as compared to A β_{40} and is also the predominant isoform found in cerebral plaques [37, 38]. Being produced as monomer, A β can readily aggregates to form oligomers and fibrils. A β_{40} and A β_{42} aggregation leads to blockage of ion channels, disrupted calcium homeostasis, elevated mitochondrial oxidative stress, reduced energy metabolism and glucose regulation, which leads to the deterioration of neuronal health and eventually neuronal cell death [39].

1.1.4 Amyotrophic lateral sclerosis

ALS is a fatal adult-onset neurodegenerative disease characterised by progressive muscle paralysis as a result of motor neurons degeneration [40]. It can occur either as a familial or a sporadic form, depending on family history of the disease [41]. Presence of proteinaceous aggregates containing

superoxide dismutase 1 (SOD1) in the motor neurons of post-mortem familial ALS (fALS) patients and transgenic mice suggested that SOD1 is related to the pathology of SOD1-linked fALS. However, the pathology does not seem to be caused by alteration of SOD1 enzyme activity [42]. Transgenic mice harbouring fALS-associated SOD1 mutations develop ALS-like symptoms despite having higher expression of SOD1 activity [43]. Additionally, ALS-like symptoms were not observed in *SOD1* knockout mice. Hence, development of ALS is not a consequence of a non-functional enzyme, but a gain of toxic function, consisting of mutations that subsequently result in SOD1 aggregation [44-47]. Although SOD1 protein is widely expressed, it causes motor neuron degeneration despite the presence of SOD1 aggregates in other cell types present in ALS patients [48]. Similar with the prion hypothesis, SOD1 has been shown to drive progression of ALS by inducing the misfolding of natively folded protein through a template-dependent manner [49-51]. Injection of mutant SOD1 homogenates into genetically vulnerable SOD1 transgenic mice induces widespread spinal inclusion pathology [52]. Similarly, wild type (WT) and mutant SOD1 proteins can be transmitted between motor neurons that might occur through cell types such as oligodendrocytes that can further enhance aggregate toxicity of SOD1 [53]. Together, these evidences showed that symptoms of ALS can be spread through neuroanatomical pathway to affect the motor nervous system and the rate at which the symptoms spread appears to be one of the primary drivers of disease progression in ALS.

1.2 Cellular stress response pathways

Protein quality control systems consist of a complex and comprehensive network of cellular signalling pathways that are responsible in protecting against proteotoxic insults. Combination of these proteostasis network maintains proteome homeostasis by ensuring the balance between the production and disposal of proteins [54]. The network consists of pathways which regulates biogenesis, folding, trafficking and degradation of proteins [54]. Upregulation of molecular chaperone helps in maintaining proteostasis. In addition, other protective mechanisms include the isolation of misfolded proteins in compartments within the cell, degradation of proteins by the ubiquitin-proteasome system (UPS), or via autophagy [54]. Proper regulation of these processes can act at the cellular or organelle-specific level to reduce toxicity caused by the accumulation of mutant, misfolded or damaged proteins [54].

1.2.1 Heat shock response (HSR)

Presence of chronic or acute proteotoxic insults within the cytosol leads to the activation of the heat shock response (HSR) in cells [55-57]. HSR can be induced upon the activation of transcription factor heat shock factor 1 (HSF1), which switches its conformation from an inactive monomer to an active trimer that binds DNA with high affinity (Fig. 1). Binding of trimeric HSF-1 on the heat-shock element (HSE) in the promoter region of downstream target genes leads to an increase in the expression of stress response genes, such as heat shock proteins (HSPs) molecular chaperones (Fig. 1). In its monomeric inactive state, HSF1 localises to the cytosol by association with chaperones including Hsp90 [58] and Hsp70 [59]. Increased expressions of HSP90 and HSP70 negatively regulate HSF1 and prevent trimer formation upon heat shock, further indicate the regulatory role of multi-chaperone complex on the activation of HSR [60]. Some studies have reported the neuroprotective role of HSPs based on their ability to either alleviate atypical protein interactions that promote aggregation, either by (i) refolding of unfolded proteins or (ii) directing proteins for degradation [54, 61, 62]. Recent study has also shown that chaperones can interact with protein aggregates and mediate their packaging into inclusions, thereby preventing decline in proteostasis upon aging and promote lifespan extension in *C. elegans* [63-68]. These chaperones may play two critical roles in (i) preventing aggregate fragmentation which can results in the formation of cytotoxic oligomers associated with protein aggregation [69] and (ii) assisting the degradation of protein aggregates through UPS and chaperone mediated autophagy (CMA) [54]. Despite being essential to overcome acute proteotoxic stress, the role of HSR has been studied extensively in unicellular organisms and tissue culture cells, but to a lesser extent in higher organisms [70]. Comprehensive validation and genetic screening reinforce the evolutionary conservation of HSR in *C. elegans*, making it an ideal model organism for further analysis of HSR regulation in age-related neurodegenerative diseases [70].

Figure 1. Activation of the HSR in *C. elegans*.

Under stress condition, intact proteins within the cytosol begin to aggregate and results in the formation of damaged proteins. Damaged proteins further bind to the HSPs to release the monomeric HSF-1 which subsequently changed its conformation into its hyperphosphorylated trimer. The active form of HSF-1 binds with high affinity to the HSE in the promoter of downstream gene targets such as *hsp-70* and *hsp-16.2* in *C. elegans*.

1.2.2 Unfolded protein response (UPR)

The endoplasmic reticulum (ER) is an organelle specialising in folding and post-translational modification of newly synthesized proteins destined for secretion, integration into the plasma membrane or distribution to different organelles [71]. Accumulation of unfolded or misfolded proteins in the ER lumen leads to the activation of a homeostatic intracellular signalling network known as the unfolded protein response (UPR) which coordinates the recuperation of ER function, a process well conserved from yeast to mammals [72].

In *C. elegans*, as in higher organisms, the UPR is regulated by three ER membrane sensors; double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PEK-1), activating transcription factor 6 (ATF-6) and inositol-requiring enzyme 1 (IRE-1) that are activated upon ER stress (Fig. 2) [73]. Activation of these three transmembrane sensors induces specialised transcriptional programs mediated by distinct transducer such as ATF-4, cleaved ATF-6 and spliced x-box binding protein 1 (XBP-1) (Fig. 2) [74, 75].

IRE-1 is a type I ER transmembrane sensor and is the most evolutionary conserved UPR branch. Upon ER stress, oligomerisation of the luminal domain of IRE-1 brings the cytosolic kinase domains into proximity to allow their trans-autophosphorylation, thus allosterically activating itself and revealing the endoribonucleolytic site [76-78]. Activation of cytoplasmic kinase and endoribonuclease domains initiates the cleaving of the mRNA encoding the transcription factor XBP-1 [79, 80]. This in turn drives the transcription of ER resident molecular chaperones and protein modifying enzymes (Fig. 2A) [81]. In addition, IRE-1 activation independently induces the degradation of mRNAs to alleviate load on the ER through an alternative pathway known as regulated IRE-1-dependent decay (RIDD) (Fig. 2A) [82].

Similar to IRE-1, type 1 transmembrane protein PERK-1 oligomerises and undergoes autophosphorylation during ER stress. Subsequently, phosphorylation of the α -subunit of the translation initiation factor (eIF2 α) results in translation inhibition and selectively increases translation of *atf-4*, a transcription factor that regulates stress responses (Fig. 2B) [83, 84].

ATF-6 is a type II transmembrane protein and is constitutively expressed in the ER [85]. Upon ER stress, ATF-6 is transported to the Golgi apparatus for the cleavage of the N-terminal cytosolic domain by Site-1 protease (S1P) and Site-2 protease (S2P), yielding active transcription factor (Fig. 2C) [84, 86, 87]. Cleaved ATF-6 further induces the expression of genes that increases the folding capacity of the ER (Fig. 2C) [84].

Activation of the UPR serves as a protective and adaptive response, but unresolved, can result in the neuronal dysfunction and eventually neurodegeneration [88]. In the post-mortem analysis of the brains of AD, PD, HD, and ALS patients, UPR markers were shown to spatiotemporally correlate with aberrant protein aggregation and the onset of neurodegeneration [89]. Many studies have shown the association between the occurrence of neuropathological features and upregulation of ER stress markers in animal models of AD, PD, ALS and HD, further suggesting the emerging role of UPR in neurodegeneration [90-93]. However, UPR activation can either augment or alleviate neurodegeneration, depending on the type of disease and specific UPR signalling being activated. Hence, the activation of UPR in neurodegenerative diseases can either be favourable or detrimental, most likely dependent on its crosstalk with other cellular stress response pathways [89].

Figure 2. The UPR signalling pathways in *C. elegans*.

Accumulation of misfolded proteins and lipid bilayer stress (LBS) trigger a cellular stress response pathway known as the UPR. (A) Upon ER stress, IRE-1 forms dimers or higher oligomers and subsequently undergoes trans-autophosphorylation. Following that, splicing of *XBP-1* mRNA results in the translation of transcription factor XBP-1 that regulates a subset of UPR genes. Persistent ER stress activates RIDD that further cleave a subset of mRNA to reduce mRNA load. Activation of JNK and NF-κB by IRE-1 initiates transcription of proinflammatory genes. (B) Oligomerisation of PEK-1 phosphorylates itself and eIF2α. Phosphorylated eIF2α attenuates general protein translation while activating transcription factor ATF-4 which subsequently induces a subset of genes including pro-apoptotic factor CHOP and GADD34. (C) Activation of ATF-6 occurs when it undergoes cleavage in the Golgi apparatus where the N-terminal transcription factor [ATF-6(N)] is released by intramembrane proteolysis. ATF-6(N) further regulates a subset of UPR genes. (Adapted and modified from reference [94]).

1.2.3 Ubiquitin-proteasome system (UPS)

Coordinated action of chaperones and proteolytic systems helps in maintaining protein quality control. However, different physiological and pathological conditions may overwhelm the homeostatic function of the chaperone network and lead to protein aggregation [95-97]. Under such conditions, activation of proteolytic systems such as UPS and autophagy help in eliminating those unstable proteins and further recycle their amino acids [98]. Despite being thought to be largely distinct catabolic pathways, both systems share a series of essential steps, as well as joint accessory proteins and even substrates [99]. Growing evidence supports that proteolytic degradation systems not only share substrates, but also effectors and molecular regulators, thereby allowing one system to modulate functioning of the other [100]. A study done in a *Drosophila* model of neurodegeneration showed that activation of autophagy could compensate the impairment of UPS in a histone deacetylase 6 (HDAC6)-dependent manner [101].

In addition, overexpression of HDAC6 rescued UPS impairment induced neurodegeneration in an autophagy dependent fashion [101]. This further indicates the ability of proteolytic systems to play compensatory role in response to the failure of another. Hence, the maintenance of a functional proteome is dependent on the highly dynamic proteolytic systems which can rapidly adapt to cellular requirements at certain times [100].

The UPS functions in the clearance of ubiquitinated, soluble and short-lived proteins. Ubiquitination of substrates for degradation requires the presence of three enzymes, namely E1, E2 and E3 to covalently add ubiquitin to a target protein [102]. Substrates undergo several rounds of ubiquitylation that lead to the formation of polyubiquitin chain. Ubiquitinated substrates serve as a signal for degradation by the proteasome, a multi-protein complex including a 20S core particle and 19S regulatory particles [103]. For degradation, the proteasome unfolds the substrates and allows the polypeptide chains to pass through the inner channel where they are cleaved into short peptides [103]. Subsequently, peptides are released from the barrel and rapidly processed into amino acids by cellular aminopeptidases and recycled [104]. Larger protein aggregates or inclusions fail to penetrate through the narrow proteasomal barrels and instead further increase protein aggregation by halting proteasomal activity [105]. However, UPS has been demonstrated to be involved in the degradation of various disease-related proteins, namely tau, SOD1, polyQ-huntingtin and α -synuclein [106].

1.2.4 Autophagy lysosome degradation system

Large protein aggregates are unable to pass through the proteolytic cavity of the narrow proteasomal barrels and can further lead to clogging of the protease which affects degradation [107]. Hence, autophagy is necessary in the clearance of these aggregates. Autophagy can be subdivided into microautophagy, macroautophagy and CMA. They deliver substrates to the lysosomes for degradation by different means. However, microautophagy mainly assist in the sequestration of damaged cell organelles through direct invaginations of the lysosomal membrane (Fig. 3) [108]. Hence, protein degradation is mostly mediated by CMA and macroautophagy. CMA is a selective form of autophagy bypassing autophagosome. Instead, selective clearance by CMA is conferred by the presence of a KFERQ-like targeting motif within the substrates. Hydrophobic surfaces of misfolded substrates are recognised by specific cytosolic chaperone protein (heat-shock cognate protein of 70 kDa, Hsc70)

(HSP-1 in *C. elegans*) and transfer them into the lysosome via the lysosomal membrane protein type-2A, LAMP-2A (LMP-2 in *C. elegans*). Once taken into the lysosomes, the substrates are rapidly degraded by proteases (Fig. 3).

Figure 3. Overview of autophagy pathways in *C. elegans*.

There are three different modes of autophagy in *C. elegans* which include macroautophagy, microautophagy and CMA. Different pathways deliver substrates into the lysosome lumen in different ways. In all three processes, the autophagic cargo is degraded by lysosomal hydrolases once they enter the lysosome (Adapted and modified from reference [109]).

On the other hand, macroautophagy is the most common type of autophagy and well-studied. There are three main steps involved in the formation of autophagosome which include initiation, nucleation and expansion of isolated membranes [109]. The initiation step begins at the phagophore assembly site (PAS) where proteins such as UNC-51 kinase complex initiate the formation of autophagosome (Fig. 4) [109, 110]. Subsequent nucleation step requires the activated UNC-51 kinase complex to target a class III PI3K complex (which is composed of BEC-1, LET-512, ZK930.1) to promote the production of a pool of phosphatidylinositol-3-phosphate (PI3P) specific to autophagosome (Fig. 4) [109, 110]. Lastly, in the expansion step, ATG-5/ATG-3/ATG-16 complex is recruited to the autophagosome membrane to facilitate lipidation of LGG-1 with phosphatidylethanolamine (PE) (Fig. 4) [109, 110]. PE-conjugated LGG-1 (LGG-1-PE) is required for the expansion of autophagic membrane, recognition of cargoes and the fusion of autophagosome with lysosomes [109]. Autophagosome can further fuse to endocytic and lysosomal compartments that result in the formation of autophagolysosome.

Figure 4. Formation of autophagosome in *C. elegans*.

The formation of autophagosome involves a series of steps such as initiation, nucleation and expansion of the autophagosome. These steps are controlled by four different functional groups of proteins: (1) initiation complex, (2) nucleation complex, (3) ATG-5/ATG-3/ATG-16 conjugation system and (4) LGG-1 lipidation system. ATG-7 is an E1-like activating enzyme required for the activation of LGG-1. Both ATG-10 and LGG-3 are E2-like ubiquitin conjugating enzymes. Lastly, ATG-4 is a cysteine protease that cleaves LGG-1 at the peptide bond on the glycine residue at the C-terminus, thereby allowing the conjugation of LGG-1 to PE. GFP::LGG-1-PE allows the autophagosome elongation that eventually result in the formation of autophagosome. Mammalian homologues are in brackets.

LGG-1 is the *C. elegans* ortholog of mammalian LC3 and yeast Atg8 which are commonly used autophagy markers in yeast and mammalian systems [110]. PE-conjugated GFP::LGG-1 (GFP::LGG-1-PE) localises to inner and outer membrane of the autophagosome, serving as an indicator of autophagy in *C. elegans* (Fig. 5) [111, 112]. In addition, the use of GFP fusion proteins allowed analysis of autophagosome formation [113]. Upon fusion with lysosomes, GFP::LGG-1-PE is degraded by lysosomal enzymes, releasing GFP fragment which is less sensitive to lysosomal proteolysis (Fig. 5) [113]. Hence, autophagy flux can be determined by changes in the amount of GFP::LGG-1-PE, cytosolic GFP::LGG-1 and cleaved GFP (Fig. 5). In addition, ATG-9 plays an essential role in the retrieval step by supplying growing phagophore with donor membranes via cycling between the site of expansion and the donor [114]. Most of the proteins involved in the retrieval step play a role in phagophore and autophagosome formation, but instead of being included in the completed vesicle, they are released into the cytosol for recycling [114]. Upon the sealing of the autophagosome, it subsequently fuses with lysosomes to form autophagolysosome where content is degraded by lysosomal hydrolases [109]. Recent study has demonstrated that mutations in autophagy related genes

and altered autophagic flux can lead to the development of neurodegenerative disorders [115]. Hence, there is a need to maintain proper autophagy flux by ensuring a balance between autophagosome formation and degradation [115].

Figure 5. Lipidation of GFP::LGG-1 for detection of autophagy flux in *C. elegans*.

Upon PE conjugation, GFP::LGG-1-PE is recruited onto the inner and outer membrane of the autophagosome to assist in the expansion of the vesicle. Fusion with lysosome to form autophagolysosome will enable the degradation of GFP::LGG-1-PE, liberating free GFP. Different forms of the autophagosome marker such as the cytosolic GFP::LGG-1, membrane bound GFP::LGG-1-PE and the cleaved GFP can be used to determine changes in autophagy flux in *C. elegans*.

1.3 BEACH-Domain Containing protein WDFY-3 in aggrephagy

Autophagy provides neuroprotection by promoting degradation of disease-associated proteins and their aggregates. Neurodegenerative diseases often involve the accumulation of protein aggregates or inclusions and require selective autophagy to help in clearance.

Aggrephagy, a term used for selective degradation of protein inclusions by the autophagic pathway has been shown to be an important mechanism for clearance of aggregates [116-124]. The selectivity of specific cargos is dependent on autophagy receptors which recognise cargo marked with degradation signals. Autophagy receptor such as p62 (SQST-1 in *C. elegans*) contains ubiquitin-interacting domains, as well as the LC3 interacting region (LIR) to facilitate the autophagic degradation of ubiquitinated cargo [125, 126]. The LIR region allows the interaction between the autophagy cargo receptor to Atg8 [109]. However, certain cargo can still undergo selective autophagy despite the absence of ubiquitin and receptor can directly recognise cargo independently of ubiquitin [125]. Autophagy cargo receptor serves to target ubiquitinated substrates for degradation via the autophagy lysosomal pathway, further

complemented by the UPS [109]. Once the cargo is being recognised by the receptor, adaptor proteins need to be present for the proper formation of the autophagosome around the cargo targeted for degradation (Fig. 6).

Figure 6. Interaction between core autophagy machinery, ALFY and autophagy receptor p62 to bring about expansion and sealing of autophagosome.

ALFY associates with the autophagy cargo receptor p62 through its PH-BEACH domains. Through ALFY, core autophagy machinery begins the formation of autophagosome that surrounds the cargo to be degraded. Interactions between the adaptor protein ALFY, autophagy cargo receptor p62 and LC-II mark the cargo for degradation through the autophagy lysosomal system. Homologues of *C. elegans* are in brackets.

Adaptor proteins such as Autophagy-linked FYVE protein (ALFY) (WDFY-3 in *C. elegans*) interact with both the core autophagic machinery and autophagy receptor to facilitate autophagosome formation and subsequent degradation within the autophagolysosome. The role of ALFY is well characterised in the mammalian system, as well as its homolog blue cheese (Bchs) in *Drosophila*. Sharing 37% homology to ALFY, WDFY-3 contains similar domains that are also present in ALFY and Bchs (Fig. 7). Despite the lack of predicted functional domains in the N-terminus of ALFY, the C-terminus consists of domains involved in autophagy; a FYVE domain, five WD40 repeats, a Pleckstrin homology (PH) domain, as well as the Beige and Chediak-Higashi (BEACH) domain [117]. Among these domains, PH-BEACH associates with autophagy receptor p62, FYVE binds PI3P which is an essential lipid required for different forms of autophagy and WD40 repeats are necessary for its interaction with Atg5 (ATG-5 in *C. elegans*) [117]. The Atg12-5 complex (ATG-5/ATG-3 complex in *C. elegans*) is needed to initiate the formation of autophagosome, and together with membrane-bound Atg16 (ATG-16 in *C. elegans*) functions as an E3 ligase to facilitate the lipidation of LC3, allowing the formation of autophagosome

[127]. In addition, the PH-BEACH domain interacts with the autophagy receptor p62 [117]. Loss of ALFY prevented Atg5 and LC3 to be recruited to mHTT aggregates, further suggesting the role of ALFY in ensuring proper interaction between core autophagy components and protein inclusions [117]. As the C-terminus of ALFY consists of domains that all have functions in connecting the cargo to the autophagic machinery, it is not surprising that the loss of ALFY can lead to the accumulation of ubiquitinated inclusions in the brain of mutated *bchs* flies [127]. In addition, presence of ALFY is needed for the macroautophagic degradation of cytoplasmic protein aggregates related to HD and PD but not required for starvation-induced bulk macroautophagic degradation [117]. Lastly, the overexpression of ALFY leads to a decrease in protein inclusions and exhibit protective effect against polyQ toxicity. With similar domains present as its homologues, the modulation of WDFY-3 could be a way to eliminate accumulating proteins in neurodegenerative models.

Figure 7. Homologues of WDFY-3 with similar domains involved in autophagy.

The C-terminal end contains several domains, namely PH, BEACH, WD40 and FYVE that are similar across different homologues of WDFY-3.

1.4 Disruption and collapse of proteostasis network in neurodegenerative diseases

One of the common hallmarks among neurodegenerative is the presence of detergent-insoluble aggregates in neurons. Despite the lack of similarity across HD, PD, AD and ALS, these structures consist of amyloids and fibrils that are enriched in cross- β -sheet proteins. In *C. elegans*, the expression of polyQ fused to yellow fluorescent protein in muscle, intestine and neurons results in aggregate formation that is dependent on both polyQ length and age [128]. In addition, chronic expression of polyQ disrupts the proteostasis network which results in misfolding of endogenous metastable proteins [129]. These observations support that the dysregulation of proteostasis network can be affected upon the presence of aggregation-prone proteins.

In vivo and *in vitro* studies involving polyQ disease demonstrated that levels of chaperones and co-chaperones decline with protein aggregation [130, 131]. Presence of mHTT or polyQ aggregates cause

transcriptional dysregulation and sequestration of chaperones which leads to an overall reduction in chaperone levels [130-132]. Similar reduction in soluble chaperones and small HSPs were observed in both *in vivo* and *in vitro* models of AD and PD [133-137]. Because of that, the overall chaperone pool available for maintenance of cellular function will be greatly reduced, leading to global proteostasis collapse and eventually development of neurodegenerative diseases.

Initial biochemical studies indicated that polyQ expansion directly impedes function of the proteasome [138] which results in the accumulation of lysine 48- and 63- linked ubiquitin chains in both HD patients and mice [139]. Nonetheless, it has been demonstrated that effect of mHTT on the proteasome is not a result of direct negative interactions, but rather the progressive titration of chaperones from ubiquitylated client proteins. Titration of chaperones from misfolded proteins prevents them from being degraded through the proteasome, thereby exacerbating proteostasis collapse [140, 141]. Like molecular chaperones, dysregulation of the UPS is due to the sequestration of chaperones that leads to an accumulation of ubiquitinated conjugates in models of HD [140]. Since chaperone sequestration occurs in AD, PD and ALS models, it is not surprising that this mechanism can contribute to decline of proteostasis in different neurodegenerative disease.

Besides their role in the impairment of proteasome, modulation in autophagy is also closely associated with health and disease progression. In mice, lack of autophagy genes Atg5 and Atg7 display severe neurodegeneration as a result of differential inhibitory effect on autophagic pathways induced by disease-associated proteins [142, 143]. Furthermore, impairment of macroautophagy initiation and accumulation of autophagic vacuoles were observed in the tissues of AD patients as a consequence of the failure to target vacuolar ATPase to the lysosome [144, 145]. Besides macroautophagy, mutant α -synuclein may cause the dysfunction of CMA, suggesting that effect of aggregate-induced impairment affects multiple autophagic arms that could eventually result in PD [146, 147]. Contrarily, autophagosome formation and lysosomal fusion were unaffected upon the expression of mHTT, but the inefficient engulfment of cytosolic cargo by autophagosomes resulted in accumulation of aggregates in model of HD [148]. Together, these findings indicate that proper protein degradation pathways need to be in place to prevent cellular disruption observed in various neurodegenerative diseases.

1.5 Age-dependent decline in proteostasis

Aging is an inevitable process that affects cellular processes which can lead to the onset of several neurodegenerative diseases [149]. As neuronal cells are more vulnerable to aging compared to other cell types in the brain, they tend to experience increased amount of oxidative stress, altered energy homeostasis and accumulation of protein aggregates [150]. The age-related decline in protein homeostasis can enable disease related proteins to adopt an abnormal tertiary structure that can further associate to form insoluble aggregates, resulting in cellular dysfunction and neuronal cell death [151]. Systemic proteomic analyses through the lifespan of *C. elegans* provide a greater insight into the changes in proteome composition upon aging [67, 152-155]. Large-scale proteome profiling further revealed an age-dependent proteome remodelling and development of severe proteome imbalance that is accompanied with changes in the abundance of proteins [67]. The protein imbalances are mainly due to increase in the accumulation of potentially harmful and aggregation-prone species [67]. In addition, the abundance of protein inversely correlates to the aggregation propensities, whereby protein of low abundance tends to aggregate more as compared to abundant proteins [67, 156]. With age, many cells and organs gradually lose their ability to maintain proteostasis under basal and stress conditions [157]. The activity of the main players involved in proteostasis maintenance such as chaperones, UPS and autophagy lysosome systems are affected upon aging, further contributing to the increasing proteotoxic loads [62, 158-161]. However, the overexpression of proteasome subunits or essential autophagy genes help to promote overall proteostasis with an extension of lifespan and resistance to stress in *Saccharomyces cerevisiae*, *C. elegans* and *Drosophila* [162, 163]. Similarly, the overexpression of essential autophagy gene Atg5 in mice displayed lifespan extension and leanness as a result of increased autophagic activity [164]. With increasing evidences showing the need to maintain cellular proteostasis to ensure longevity, a better understanding of the involvement of WDFY-3 in macroautophagy can be help in delaying signs of aging and progression of age-related diseases.

1.6 Aims of this study

Given the intimate relationship between regulation of cellular stress responses and neurodegenerative diseases, how the macroautophagy adaptor protein WDFY-3 interacts with the proteostasis network in neurodegenerative disorders remains unknown. While the function of the homologues of WDFY-3 in relation to clearance of proteins is well characterised, its relevance to cellular stress responses

pathways is largely unknown. In this study, we make use of *C. elegans* neurodegenerative models with *wdfy-3* loss-of-function (*lof*) mutants to determine the function of *wdfy-3* in two closely related cellular stress responses, namely the HSR and autophagy. Having potential domains that are involved in the clearance of aggregates, we attempt to first determine the function of uncharacterised *wdfy-3* and further understand its role upon age-dependent decline in proteostasis and clearance of aggregates in various neurodegenerative diseases.

Chapter 2. Materials and methods

2.1 *C. elegans* strains and RNAi constructs

Strains used and created in this study are listed in Table 1. Strains were grown under standard condition at 20°C on the nematode growth media (NGM) with the addition of *Escherichia coli* OP50 as the food source [165]. For RNA interference (RNAi) experiments, animals were grown on HT115 bacteria as previously described [166], and RNAi clones were obtained from the Vidal RNAi library [167]. All RNAi clones were sequenced to confirm their identity.

Table 1. *C. elegans* strains used in this study.

Strain	Genotype	Strain origin
N2	Wild-type (WT)	CGC
AM49	<i>rmls172 [f25b3.3p ::q19 ::cfp]</i>	CGC
AM44	<i>rmls190 [f25b3.3p ::q67 ::cfp]</i>	CGC
RB991	<i>c26h9a.2(ok912) IV</i>	CGC
DA2123	<i>adls2122 [lgg-1p::gfp::lgg-1 + rol-6(su1006)]</i>	CGC
OH9625	<i>otls292 [eat-4::mCherry + rol-6(su1006)]</i>	CGC
XE1347	<i>wpls39 [unc-47p:mCherry] X</i>	CGC
OH15568	<i>unc-17(ot907[unc-17::mKate2::3xflag]) IV</i>	CGC
NL5901	<i>pkls2386 [unc-54p::alphasynuclein::gfp + unc-119(+)]</i>	CGC
AM263	<i>rmls175 [unc-54p::Hsa-sod-1 (WT)::yfp]</i>	CGC
AM725	<i>rmls290 [unc-54p::Hsa-sod-1 (127X)::yfp]</i>	CGC
GRU101	<i>gnals1 [myo-2p::yfp]</i>	CGC
GRU102	<i>gnals2 [myo-2p::yfp + unc-119p::Abeta1-42]</i>	CGC
CGT241	<i>wdfy-3p::gfp, rol-6(su1006)</i>	This study
CGT262	<i>mCherry::wdfy-3</i>	This study
CGT154	<i>c26h9a.2(ok912) IV</i> (backcrossed 4 times)	This study
CGT169	<i>c26h9a.2(ok912) IV; rmls190 [f25b3.3p ::q67 ::cfp]</i>	This study
CGT184	<i>c26h9a.2(ok912) IV; rmls172 [f25b3.3p ::q19 ::cfp]</i>	This study
CGT216	<i>adls2122 [lgg-1p::GFP::lgg-1 + rol-6(su1006)]; rmls190 [f25b3.3p ::q67 ::cfp]</i>	This study
CGT217	<i>adls2122 [lgg-1p::GFP::lgg-1 + rol-6(su1006)]; rmls172 [f25b3.3p ::q19 ::cfp]</i>	This study
CGT259	<i>unc-17(ot907[unc-17::mKate2::3xflag]) IV; wdfy-3p::gfp, rol-6(su1006)</i>	This study
CGT263	<i>otls292 [eat-4::mCherry + rol-6(su1006)]; wdfy-3p::gfp, rol-6(su1006)</i>	This study
CGT272	<i>wpls39 [unc-47p:mCherry] X; wdfy-3p::gfp, rol-6(su1006)</i>	This study
CGT298	<i>rmls172 [f25b3.3p ::q19 ::cfp]; mCherry::wdfy-3</i>	This study
CGT299	<i>rmls190 [[f25b3.3p ::q67 ::cfp]; mCherry::wdfy-3</i>	This study
CGT300	<i>jsls682 [rab-3p::gfp::rab-3 + lin-15(+)]; mCherry::wdfy-3</i>	This study

2.2 RNAi by feeding

RNAi was carried out as previously described [168, 169]. Briefly, HT115 bacteria were grown in Luria-Bertani (LB) medium containing 100 µg/µl ampicillin at 37°C until log phase before seeding them on NGM plates containing 50 µg/µl carbenicillin, 50 µg/µl tetracycline and 1 mM isopropyl-β-D-thiogalactoside (IPTG). Seeded plates were incubated overnight at 37°C for the induction of dsRNA expression. Gravid worms were subjected to hypochlorite treatment and eggs were allowed to hatch overnight in M9 medium (28 mM Na₂HPO₄, 22 mM KH₂PO₄, 86 mM NaCl, 1 mM MgSO₄) at 20°C to obtain a synchronised population of L1 worms. Hatched worms were transferred onto NGM plates seeded with dsRNA expressing HT115 bacteria and grown until day 0 of adulthood before harvesting. To obtain aged worms, the worms were transferred on alternate days to plates containing 5'-fluorodeoxyuridine (FUDR) (Active Biochem) until day 7 of adulthood before harvesting.

2.3 qPCR

Quantitative PCR (qPCR) was performed as previously described [168]. In brief, worms were harvested and subsequently lysed by bead beating. Total RNA was isolated using TRIzol reagent (ThermoFisher, Waltham, MA) and purified using NucleoSpin RNA columns before an additional DNase treatment step (Macherey-Nagel) following the manufacturer's protocol. cDNA synthesis (1-2 µg RNA per sample) was performed using RevertAid reverse transcriptase (ThermoFischer, Waltham, MA) following manufacturer's protocol. qPCR reactions were performed in triplicates using QuantiNova SYBR Green PCR kit (Qiagen) in a CFX96 Touch™ Real-time PCR Detection system (Bio-Rad). Each reaction contained 60 ng of cDNA and 50 nM of paired-primer mix. Relative mRNA levels were determined with the comparative Ct method ($\Delta\Delta C_t$), using the housekeeping gene *act-1* as a normalisation control. Oligonucleotide primers used in this study are listed in Table 2.

Table 2. Oligonucleotide primers used in this study.

Gene	Sequence (5' to 3')
<i>act-1</i>	GAGCACGGTATCGTCACCAA (Forward) AGGACTGGGTGCTCTTCTGG (Reverse)
<i>lgg-1</i>	TCGTGATGGTCCTGGTAGAGT (Forward) ACGCATCCAACTTCGTCCA (Reverse)
<i>wdfy-3</i>	CCCGAGTTCTTCACATTACCCGA (Forward) CGCTGGCAGAAGCACATCAT (Reverse)
<i>atg-9</i>	GCTCATCCCGAAACTACGGC (Forward) AGGGATGATTCTACGGCGG (Reverse)
<i>sqst-1</i>	AGGAAAGAGAACCTGTTGTCGAGG (Forward) TGATGTCACCGAGGCAAGCA (Reverse)

2.4 Immunoblotting

Autophagy analysis was performed as previously described [168]. Worms were collected and washed in M9 medium before lysing by bead beating three times at 6500 rpm for 30 s in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS) containing freshly added 1x protease inhibitor cocktail (Roche, Basel, Switzerland). Samples were then spun down at 12,000 rpm for 5 min at 4°C to pellet worm debris. The protein concentration of the cleared lysate was measured by using a BCA assay kit (ThermoFisher, Waltham, MA). Either 30 µg or 60 µg of total proteins were loaded into a 10% SDS-PAGE gels to detect both GFP::LGG-1 and GFP::LGG-1-PE. Proteins were transferred to nitrocellulose membranes and subsequently stained with REVERT total protein stain for normalisation (Li-COR Biosciences, Lincoln, NE, catalogue number 925-32210). Subsequently, membranes were blocked with Odyssey blocking TBS buffer (Li-COR Biosciences, Lincoln, NE) for 1 h at room temperature, before incubating membranes with monoclonal anti-GFP antibody (1:1,000) overnight at 4°C (Roche, catalogue number 11814460001). Membranes were washed three times for 5 min each in PBST before the incubation with IRDye 800CW anti-mouse IgG antibody (1:10,000) (Li-COR Biosciences, Lincoln, NE, catalogue number 925-32210). Subsequently, membranes were washed three times for 5 min each in PBST before a final wash with PBS for 5 min. Lastly, membranes were left to dry before scanning with the Odyssey CLx imaging system (Li-COR Biosciences, Lincoln, NE).

2.5 Analysis of aggregates in different neurodegenerative models

Animals were raised at 20°C until day 0, day 5 or day 7 before harvesting for microscopy. Mounting of animals were performed with the use of anaesthesia with 0.5 M of NaN₃ in M9 medium. The number of

aggregates was counted between the two pharyngeal bulbs of individual animals with ImageJ software version 1.52p. Further statistical analysis was done using Student's t-test (Graphpad Prism 8.0.1).

2.6 DAPI and LysoTracker treatment

DAPI staining was performed as previously described [170]. Animals were raised at 20°C until day 0 and day 7 before harvesting for microscopy. Worms were picked onto a drop of M9 buffer on a glass slide before draining off any excess liquid with a soft tissue. Next, 10 µl of 95% ethanol were added onto the worms and subsequently air dry. Immediately after the ethanol evaporated, the same fixation step was repeated. Lastly, 10 µl of 10 µg/mL DAPI was added to the worms and coverslip was put on before imaging.

For LysoTracker staining, harvested worms were picked onto a 12-well plate containing 1 ml of NGM that contains 25 µM of LysoTracker Green (ThermoFisher, catalogue number L7526) and a drop of OP50. Thereafter, the plate was incubated at 20°C overnight for 24 hours before imaging the stained worms. Imaging was performed with FITC/eGFP channel the following day after mounting the worms with 0.5 M of NaN₃ in M9 medium.

2.7 Image acquisition

All the images acquired in this study were taken using the Zeiss LSM710 Laser Confocal Microscope at 10 x, 63 x, or 100 x magnification. For quantification of aggregates, Z-stacks were acquired at 2 µm per slice intervals. For other images that were used to determine colocalisation, single slice of each image at which the pharyngeal bulbs were in focus were obtained at 100 x magnification.

2.8 Thrashing assay

Synchronised worms were grown until day 0, day 5 or day 7 before harvesting after three times of washing with M9 medium. Number of worms were adjusted in M9 medium with 10 worms in every 10 µl-drop. With the use of SMZ1500 Nikon Stereomicroscope, the thrashing of the worms was tracked at 0.8 x magnification for 30 seconds per video. Further analysis was done with the wrMTck plugin in the ImageJ software version 1.52p. Statistical analysis was done using Student's t-test (Graphpad Prism 8.0.1).

2.9 Chemotaxis assay

Chemotaxis assay was performed as previously described with some modifications [171, 172]. Synchronised worms were grown until day 0, day 5 or day 7 before harvesting after three times of washing with M9 medium. Approximately 30 worms were dispensed at the middle of the plate, with equal distance to the solvent and chemoattractant spots. The chemoattractant (1:100 benzaldehyde) and ethanol (solvent) were diluted with equal volume of 0.5 M NaN₃ in M9 medium. Subsequently, 2 µl of ethanol (solvent) was spotted on one end and 2 µl of chemoattractant (1:100 benzaldehyde) was spotted on the opposite side (Fig. 8). Once worms, solvent and chemoattractant spots were absorbed into the agar, the plates were inverted and kept in the dark for 60 min at room temperature. Next, the plates were kept at 4°C before counting to reduce mobility of the worms. Chemotaxis indices were then calculated as $\frac{((B1+B2)-(E1+E2))}{\text{Total number of worms on plate}}$. Statistical analysis was done using Student's t-test (Graphpad Prism 8.0.1).

Figure 8. Illustration of template used for chemotaxis assay.

A 5 cm plate is divided into four quadrants, each marked with either E (solvent) or B (chemoattractant). The inner circle consists of the origin point at which worms are being dispensed. Quadrants are arranged in alternate pattern to prevent any biasness caused by the initial placement of worms. Schematic examples of solvent and chemoattractant are shown on the right. Worms that move out of the inner circle into either one of the quadrants are being scored in order to prevent immobile worms from skewing the data, while worms within the inner circle are being excluded in the analysis.

2.10 Lifespan assay

Lifespan assays were performed at 20°C as previously described [173]. Animals were grown in S medium containing 50 µg/mL carbenicillin, 0.1 µg/mL amphotericin B and 3 mg/mL OP50 suspension. Number of worms were adjusted in M9 medium with 10 worms in every 10 µl. In a 96-well plate, each well contains 110 µl of S medium mixed with 10 µl of worms. Plate was sealed with a tape sealer to avoid contamination and evaporation. Subsequently, the plate was incubated at 20°C until 120 mM FUDR was added once the animals reached larva stage 4 (L4) to prevent development of progenies. Adults were scored manually as dead or alive every 2-3 days. Composition of reagents used in this assay are listed in Table 3.

Table 3. Composition of reagents used for lifespan assay.

Reagent	Composition
Trace metal solution, 1 L	1.86 g Na ₂ EDTA 0.69 g FeSO ₄ .7H ₂ O 0.20 g MnCl ₂ .4H ₂ O 0.29 g ZnSO ₄ .7H ₂ O 0.016 g CuSO ₄
S basal, 1 L	5.9 g NaCl 50 ml of 1 M Potassium phosphate, pH 6.0 1 ml Cholesterol (5mg/mL in ethanol) (to be added after autoclaving)
S medium, 1 L	977 ml S Basal 10 ml 1M Potassium citrate, pH 6.0 10 ml Trace metal solution 3 ml 1M CaCl ₂ 3 ml 1M MgSO ₄

2.11 RNAi screening

Synchronised worms were grown until L4 on NGM supplemented with OP50 before harvesting after three times of washing with M9 medium. Library of RNAi clones were inoculated into 96-well plate with LB medium containing 100 µg/µl ampicillin at 37°C overnight. Subsequently, 80 mM IPTG were added into the 96-well plate to induce the RNAi clones at 37°C for 2 h. The 96-well plate was then spun down at 2500 g for 5 min to concentrate the bacteria before the addition of worms. Number of worms were adjusted to 10 worms in every 40 µl of S medium containing 50 µg/mL ampicillin, 4 mM IPTG. Each well was resuspended with 40 µl of the suspension before incubating at 20°C for the next 5 days before the ranking of the worms based on phenotypic changes, sterility and behaviour.

Chapter 3. Results

3.1 WDFY-3 expression levels increase with age

Despite the ability to maintain a functional proteome in young and healthy individuals, the proteostasis network declines over time and loses its ability to combat the accumulation of proteotoxic insults. Ultimately, age-mediated decline in proteostasis leads to the loss of proteome balance and widespread protein aggregation that can result in degenerative pathologies [157, 159, 174-176]. Studies have shown that both Bchs in *Drosophila* and ALFY in human are mostly expressed in neurons [177, 178]. *Drosophila bchs* mRNA was found to be enriched, but not restricted in the embryonic brain and ventral nerve cord [177]. To validate the neuronal expression of *wdfy-3*, we first determine its expression pattern by generating a GFP reporter driven under a *wdfy-3* promoter (*wdfy-3p::gfp*). GFP expressed mostly in the nerve ring and along the entire axonal projection. To further determine its expression upon aging, expression of *wdfy-3* was observed in day 0 (D0), day 1 (D1), day 4 (D4) and day 7 (D7) animals, in which GFP intensity increased upon aging (Fig. 9). Similarly, the mRNA levels of *wdfy-3* increased with age (Fig. 10). Age-dependent increase in the neuronal expression of *wdfy-3* suggests that it has potential role in the regulation of aggregates that are commonly found in neurodegenerative diseases.

The loss of ALFY homologue Bchs protein in *Drosophila* resulted in neurodegenerative phenotype characterised by the build-up of insoluble aggregated proteins and a shortening of lifespan [179], further indicate the importance of ALFY in the degradation of protein inclusions *in vivo*. Additionally, *in vitro* study also revealed that presence of ALFY was needed for the clearance of aggregates via autophagy [117]. Surprisingly, while the loss of ALFY prevents clearance of inclusions, overexpression of a C-terminal fragment of ALFY resulted in a compelling decrease in the number of polyQ aggregates in both *in vivo* and *in vitro* studies [117]. Similarly, both full-length and C-terminal of Bchs reversed neurodegenerative phenotype in a *Drosophila* eye model of polyQ toxicity [117]. These data suggest that the elevated expression of ALFY can drive the clearance of aggregate proteins, further helping to re-establish cell homeostasis. As the number of glutamine repeats determine the severity of HD, we made use of strains expressing pan-neuronal polyQ aggregates (*rgef-1p::cfp-q67*) in comparison to its soluble counterpart (*rgef-1p::cfp-q19*) to better understand the role of *wdfy-3* in aggregate clearance [129, 180]. To determine if the expression of *wdfy-3* can be induced by the accumulation of aggregates, we measured *wdfy-3* mRNA levels in young and old worms expressing CFP-Q19 or CFP-Q67 (Fig. 11).

Worms expressing the insoluble protein Q67 exhibited a higher expression of *wdfy-3* as compared to Q19. In addition, the expression of *wdfy-3* was further increased in D7 worms as compared to D0. With similar domains involved in clearance of aggregates in its homologues, the increase in *wdfy-3* mRNA expression in the presence of aggregates suggest that there could be *de novo* synthesis of *wdfy-3* to deal with proteotoxic polyQ aggregates in the HD model. Further increase in *wdfy-3* expression in aged animals supports its potential role upon age-mediated decline in proteostasis.

Figure 9. *wdfy-3* is widely expressed in the nerve ring and along axonal projections.

Expression of *wdfy-3* is observed across WT *C. elegans* of 0-, 1-, 4-, and 7-days-old with GFP expression driven under *wdfy-3* promoter (*wdfy-3p::gfp*). Day 0, 1, 4, 7 are denoted as D0, D1, D4 and D7 respectively. Strains were harvested on the same day and imaged with eGFP filter. Scalebar, 100 μ m. Images shown are representation of three independent experiments.

Figure 10. *wdfy-3* mRNA expression increases with age in D7 worms.

Worms were harvested at D0 and D7 of adulthood and subjected to quantitative RT-PCR for analysis. The fold change for *wdfy-3* was normalised to internal control *act-1* before normalising to the WT control. Error bars are based on three independent experiments and represents the mean $\pm$ SEM. P values were calculated by two-tailed Student's t-test.

Figure 11. Expression of *wdfy-3* increases upon the presence of aggregates and aging.

Worms were harvested at D0 and D7 of adulthood and subjected to quantitative RT-PCR for analysis. The fold change for *wdfy-3* was normalised to internal control *act-1* before normalising to the *q19::cfp* (D0) control. Data shown is the mean $\pm$ SEM from two independent experiments.

To determine if WDFY-3 protein expression correlates to the genetic expression during aging, endogenous WDFY-3 was tagged at the N-terminal with mCherry (*mCherry::wdfy-3*) using CRISPR/Cas9. As expected, WDFY-3 protein levels increase with age (Fig. 12). Unlike the genetic expression of *wdfy-3*, the overall protein expression pattern of WDFY-3 seems to be less concentrated within the head region and highly expressed within the intestinal region upon aging. The difference between the genetic and protein expression of *wdfy-3* could be due to the stability of fluorophores or the protein itself. The increase in protein expression of *wdfy-3* upon aging suggests that it possess age-dependent role in proteostasis to aid in the clearance of aggregates.

Figure 12. Age-dependent changes in the protein expression of WDFY-3.

Protein expression of WDFY-3 in both D0 and D7 worms. The head region is further zoomed in to observe changes in the expression of WDFY-3 upon aging with 100 x magnification (bottom). Scalebar, 100 μ m (10 x magnification) and 10 μ m (100 x magnification). Images shown are representative of three independent experiments.

The localisation of WDFY-3 was further validated by mating *mCherry::wdfy-3* with a pan-neuronal reporter strain (*rab-3p::gfp::rab-3*) (Fig. 13). As the expression of WDFY-3 is more concentrated at the head region, we observed the colocalisation of RAB-3 and WDFY-3 between the two pharyngeal bulbs of *C. elegans*. Similar to the genetic expression of *wdfy-3p::gfp*, WDFY-3 also colocalises with neurons, further supporting its possible regulatory role upon aging as observed by an age-dependent increase in protein expression levels.

Figure 13. Pan-neuronal expression of WDFY-3 between two pharyngeal bulbs of *C. elegans*.

Protein expression of WDFY-3 colocalises with pan-neuronal reporter strain *rab-3p::gfp::rab-3* in both D0 and D7 worms. Scalebar, 10 μ m. Images shown are representative of three independent experiments.

3.2 Tissue specific expression and subcellular localisation of WDFY-3

To validate WDFY-3 expression in specific tissues, we incorporated *wdfy-3p::gfp* in animals expressing endogenously mKate2 tagged cholinergic locus in both motor- and inter- neurons (*unc-17::mKate2::3xflag*), together with mCherry in the sensory and head ganglia neurons (*eat-4::mCherry*) [181, 182]. In both D0 and D7 animals, the expression of a subpopulation of *wdfy-3* reporter overlaps with cholinergic (Fig. 14, S1) and sensory neurons (Fig. 15, S2) but not with GABA neurons (Fig. 16, S3). Tissue-specific expression of WDFY-3 suggests that it has potential neuron-specific functions that are involved in the clearance of aggregates that tends to accumulate within the cholinergic and sensory neurons.

Figure 14. Subpopulation of *wdfy-3* reporter is expressed in cholinergic neurons.

Strains co-expressing *wdfy-3p::gfp* and *unc-17::mKate2::3xflag* were harvested at both D0 and D7 and used for imaging. Scalebar, 100 μ m. Images shown are representative of three independent experiments. Insets are zoom-in images of the indicated square areas.

Figure 15. Subpopulation of *wdfy-3* reporter is expressed in sensory neurons.

Strains co-expressing *wdfy-3p::gfp* and *eat-4::mCherry* were harvested at both D0 and D7 and used for imaging. Scalebar, 100 μ m. Images shown are representative of three independent experiments. Insets are zoom-in images of the indicated square areas.

Figure 16. Absence of *wdfy-3* in GABA neurons.

Strains co-expressing *wdfy-3p::gfp* and *unc-47p::mCherry* were harvested at both D0 and D7 and used for imaging. Scalebar, 100 μ m. Images shown are representative of three independent experiments. Insets are zoom-in images of the indicated square areas.

As ALFY was shown to localise predominantly in the nuclear envelope [117, 183], we asked if WDFY-3 localises to the nucleus. Based on our experimental approach, there was no colocalisation between that of DAPI and *mCherry::wdfy-3* for D0 and D7 animals (Fig. 17). Despite having similar domains as its homologues, WDFY-3 does not localise to the nuclear envelope under basal condition and could probably be found within the cytosol where most aggregates tend to accumulate to exert its function.

Both *Drosophila* Bchs and mammalian ALFY proteins play a role in aggrephagy by physically interacting with the autophagy machinery to promote protein aggregate clearance [117, 179, 183-185]. To determine if the role of WDFY-3 in autophagy is conserved, we assessed *mCherry::wdfy-3* localisation into lysosomes using the LysoTracker Green (Fig. 18). A subpopulation of *mCherry::wdfy-3* was found to colocalise with the lysosome. With the close association with WDFY-3 and the lysosome, it suggests that WDFY-3 could potentially be involved in lysosome-mediated processes.

Figure 17. WDFY-3 does not localise to the nucleus under basal condition.

Both D0 and D7 worms were harvested and subjected to ethanol fixation and staining with DAPI before imaging. Scalebar, 10 μ m. Images shown are representative of three independent experiments.

Figure 18. WDFY-3 partially localises to the lysosomes under basal condition.

Both D0 and D7 worms were harvested and subjected to staining with LysoTracker Green for 24 hours before the region between the anterior and posterior pharyngeal bulbs was imaged at 100 x magnification. Scalebar, 10 μ m. Images shown are representative of three independent experiments.

3.3 WDFY-3 is dispensable to maintain basal autophagy flux

To maintain homeostasis, there is a need to have a balance between protein degradation and synthesis from single cells to whole organisms [186]. Aging proteome can significantly impair protein folding capacity, in which the proteostasis network becomes overwhelmed by the combination of metastable proteins and damage accumulated during aging [187]. Degradation of cytosolic proteins is performed by the ubiquitin-proteasome and lysosome systems [117]. Particularly, the lysosome machinery permits the elimination of cytosolic protein aggregates across various cell types, including neurons [188-191]. ALFY (homologue of WDFY-3) plays a role in degradation of protein aggregates mediated via macroautophagy by facilitating the interaction of the autophagy cargo receptor p62 with the membrane bound LC3 [117]. The WD40 repeats of ALFY are crucial for its interaction with the autophagy effector Atg5 (ATG-5 in *C. elegans*) and the subsequent formation of the Atg12-5 complex is required to facilitate the conjugation of LC3 proteins to PE for autophagosome formation [127, 192]. *Drosophila* Bchs protein has been well characterised in terms of its role in autophagic degradation of aggregate-prone proteins while the role of WDFY-3 in autophagic clearance of protein aggregates in *C. elegans* still remains to be explored. To further examine the role of *wdfy-3* in autophagy, we performed quantitative analysis of

autophagy related genes in D0 and D7 of WT and *wdfy-3(lf)* animals. To prevent contamination with progenies, worms were transferred to plates containing FUdR from L4 onwards and were harvested post-reproduction after 7 days from young adult stage. Expression levels of genes involved in early step of autophagosome formation *atg-9*, autophagosome marker *lgg-1* and cargo receptor *sqst-1*, were not affected upon the loss of *wdfy-3* in young worms (Fig. 19A). To further determine the role of *wdfy-3* in the regulation of autophagy related genes, we made use of aged worms which has been shown to have an imbalance of proteostasis system [187]. Similarly, there were no change in the expression levels of three autophagy related genes upon the loss of function of *wdfy-3* (Fig. 19B). This result showed that *wdfy-3* is not required for the regulation of autophagy genes at the transcriptional levels.

Figure 19. *wdfy-3* is dispensable to modulate autophagy genes in both D0 and D7 worms. Quantitative RT-PCR of D0 (A) and D7 (B) WT and *wdfy-3(lf)* worms. Fold change of *wdfy-3(lf)* samples was normalised to internal control *act-1* before normalising to the WT control. Data shown is the mean $\pm$ SEM from three independent experiments. P values were calculated by two-tailed Student's t-test. n.s, not significant.

Neurodegenerative diseases are generally characterised by an aberrant low autophagic flux that can contribute to the accumulation of autophagy substrates and eventually proteotoxicity [193] as observed in HD cell model [194]. To determine if WDFY-3 plays a role in the clearance of aggregates, we visualised the colocalisation of WDFY-3 with HD polyQ aggregates pan-neuronal model CFP-Q67 in comparison to its soluble counterpart CFP-Q19 [129, 180]. There was partial colocalisation between soluble polyQ and WDFY-3 in both D0 and D7 animals (Fig. 20). However, the extent of colocalisation was more prominent for Q67 aggregates and WDFY-3, regardless of the age difference (Fig. 21). Despite the colocalisation between WDFY-3 and soluble Q19, the expression pattern of WDFY-3 differs

depending on the presence of soluble or insoluble polyQ. With the presence of insoluble polyQ, localisation of WDFY-3 changes from a diffuse pattern to the formation of puncta as observed in the presence of aggregates (Fig. 21). To further verify the degree of colocalisation between *wdfy-3* with either soluble or insoluble polyQ proteins, Manders' colocalisation coefficient was used for the analysis. The Manders' colocalisation coefficient for the mCherry channel (M1) (Fig. S4A) and CFP channel (M2) (Fig. S4B) were consistent across soluble and insoluble polyQ proteins regardless of age, indicating that their association with *wdfy-3* remains the same. Despite that, with the inclusion of more animals can further help to determine mild difference between the association of *wdfy-3* with soluble and insoluble polyQ proteins. A previous study has shown that mammalian ALFY coimmunoprecipitated with the insoluble polyQ aggregates and are not required for the elimination of soluble polyQ proteins [117]. The change in localisation pattern of WDFY-3 suggests that it might have specific roles in association with aggregates to assist with the clearance and/or stabilisation rather than the soluble form.

Figure 20. Colocalisation of WDFY-3 and soluble polyQ upon aging.

Worms co-expressing *rgef-1p::q19::cfp* and *mCherry::wdfy-3* were harvested at D0 and D7 of adulthood and the region between the anterior and posterior pharyngeal bulbs was imaged at 100 x magnification. Scale bar, 10 μ m. Images shown are representative of three independent experiments.

Figure 21. Colocalisation of WDFY-3 and insoluble polyQ upon aging.

Worms co-expressing *rgef-1p::q67::cfp* and *mCherry::wdfy-3* were harvested at D0 and D7 of adulthood and the region between the anterior and posterior pharyngeal bulbs was imaged at 100 x magnification. Scale bar, 10 μ m. Images shown are representative of three independent experiments.

As WDFY-3 homologues play a role in aggregophagy, we asked if WDFY-3 modulates the expression of key autophagy genes in HD animal models by comparing *wdfy-3* mutants to WT. Despite the presence of aggregates, the absence of *wdfy-3* did not affect those autophagy related genes (Fig. 22A). To further push the system, we observed the same genes in aged HD animals. Similarly, there were no significant changes in autophagic genes *atg-9*, *lgg-1* and *sqst-1* upon the absence of WDFY-3 or presence of insoluble CFP-Q67 (Fig. 22B). Since the loss of *wdfy-3* does not affect autophagic genes despite the presence of aggregates or aged animals, it suggests that it does not modulate autophagy at the transcriptional levels.

Figure 22. *wdfy-3* is dispensable to modulate autophagy genes in both D0 and D7 HD models. Worms were harvested at D0 (A) and D7 (B) of adulthood and subjected to quantitative RT-PCR for analysis. The fold change for *wdfy-3* was normalised to internal control *act-1* before normalising to the Q19 control. Data shown is the mean $\pm$ SEM from at least three independent experiments. P values were calculated by two-tailed Student's t-test. n.s., not significant.

As autophagy flux can be modulated independently of the autophagy genetic transcriptome, we asked if WDFY-3 plays a role in modulating autophagy flux by attenuating its genetic expression using RNAi, in which GFP::LGG-1 is separated from the autophagosome marker, GFP::LGG-1-PE [195-200]. GFP::LGG-1 forms puncta in cells with large population of autophagic vesicles which can serve as an indication of autophagy in *C. elegans* [201-203]. GFP::LGG-1-PE levels were unchanged in *wdfy-3(RNAi)* D0 and D7 animals (Fig. 23). Similarly, GFP::LGG-1-PE levels were unchanged in *wdfy-3(RNAi)* D0 and D7 animals compared to control with the expression of CFP-Q19 or CFP-Q67 (Fig. 24). As WDFY-3 is only expressed in a subset of neurons, it should be noted that the detection of WDFY-3-modulated GFP::LGG-1-PE might be below the detection limits in whole animals. Hence, a different approach of isolating specific cell types, such as neurons, can be performed to detect WDFY-3-modulated GFP::LGG-1-PE, thereby allowing us to better understand the role of *wdfy-3* in modulating autophagy at the transcriptional and protein levels.

Figure 23. *wdfy-3* is dispensable for the modification of GFP::LGG-1-PE in both D0 and D7 animals.

(A) Quantification of GFP::LGG-1-PE expression with GFP antibody in D0 and D7 worms subjected to *wdfy-3(RNAi)*. (B) The fold change upon the knockdown of *wdfy-3* was normalised to REVERT total protein stain before normalising to the WT vector control. Data shown is the mean $\pm$ SEM from three independent experiments.

Figure 24. *wdfy-3* is dispensable for the modification of GFP::LGG-1-PE in both D0 and D7 HD models.

(A) Quantification of GFP::LGG-1-PE expression with GFP antibody in D0 and D7 worms subjected to *wdfy-3(RNAi)*. (B) The fold change upon the knockdown of *wdfy-3* was normalised to REVERT total protein stain before normalising to the Q19 vector control. Data shown is the mean $\pm$ SEM from three independent experiments.

3.4 WDFY-3 is dispensable for the clearance of polyQ aggregates upon aging

Misfolded proteins can eventually mature into small protein aggregate or continue to multimerise into larger inclusions, a common characteristic of neurodegenerative disorders [204, 205]. Being the first line of defence against conformational disorders, chaperones maintain the solubility of misfolded disease-related proteins by dismantling amyloid-like aggregates and resolubilise them. However, under certain conditions when the chaperone network fails to solubilise those protein aggregates, they will be directed for degradation either by the UPS or lysosomal-mediated autophagy. Although the UPS is a well characterised protein degradation pathway, the narrow barrel-shaped proteasome is not able to degrade large protein aggregates [105]. Hence, insoluble protein aggregates, in different neurodegenerative models, must undergo selective autophagy for degradation. Protein aggregates must be recognised by specific receptor complex that interacts with the adaptor protein, ALFY, to ensure proper cargo loading and further degradation through autophagy. To further characterise the role of WDFY-3 as an adaptor protein in the clearance of protein aggregates, the number of aggregates were quantified in WT and *wdfy-3(lox)* mutants expressing CFP-Q67. Aggregates located between the anterior and posterior pharyngeal bulbs of the animal were counted in both D0 and D7 animals (Fig. 25) [206]. In young worms, the number of polyQ aggregates remained constant regardless of the absence of *wdfy-3*. On the other hand, a significantly reduction in the number of aggregates was found in D7 *wdfy-3(lox)* worms compared to WT. This observation contradicts a previous study in which the lack of *Drosophila* Bchs results in the accumulation of ubiquitinated inclusions and subsequent development of neurodegenerative phenotype [179]. However, the role of WDFY-3 and its homologues during age-mediated decline in proteostasis still remains unexplored. The WDFY-3-dependent accumulation of aggregates indicates that it might have a different role upon aging.

Figure 25. *wdfy-3* reduces the clearance of amyloids in aged HD model.

(A) Representative images of animals expressing *rgef-1p::q67::cfp* in the absence of *wdfy-3* in D0 and D7 animals. Yellow arrowheads are pointing to puncta representing insoluble aggregates. Scalebar, 10 μ m. Images shown are representative of three independent experiments. (B) Quantification of the number of aggregates in between the anterior and posterior pharyngeal bulbs of both D0 and D7 *q67* or *q67;wdfy-3(lof)* mutants. $n = 44$ animals. Data shown is the mean $\pm$ SEM from three independent experiments. P values were calculated by two-tailed Student's t-test. n.s., not significant.

3.5 WDFY-3 is required to maintain healthspan and lifespan in HD model upon aging

HD is an inherited degenerative disorder of the CNS that leads to progressive deterioration of mobility and cognition [207]. Previous studies have shown the correlation between protein aggregates and mobility in *Drosophila* and *C. elegans* HD models [208, 209]. To determine if WDFY-3 protects against polyQ insoluble proteins, we performed lifespan assays. Unlike its homolog *Drosophila* Bchs, the lack of *wdfy-3* extended longevity of the animals within a range of 1 to 5 days (Fig. 26 and Table 4). This suggests that *wdfy-3* might have a different role as compared to its homologues as its absence does not affect the lifespan of *C. elegans*. To further assess function of *wdfy-3* in the maintenance of longevity in the presence of aggregates, we performed lifespan assays in Q19 and Q67 animals. Similarly, the absence of *wdfy-3* extended the lifespan of Q19 animals which express the soluble polyQ protein by 3 to 4 days (Fig. 27, Table 4) [179]. Surprisingly, there was an extension of longevity observed in the presence of insoluble polyQ protein in Q67 animals, despite having polyQ aggregates that has been shown to promote toxicity [210]. The absence of *wdfy-3* shortened the lifespan of Q67 animals. This

further indicates that *wdfy-3* is critical to maintain the longevity of *C. elegans* in the presence of aggregates.

Figure 26. *wdfy-3* is dispensable to maintain lifespan in *C. elegans*.

Lifespan analysis of WT and *wdfy-3(lof)* mutants. In a 96-well plate, synchronised L1 worms were added into a mixture of S medium with bacteria OP50, amphotericin B and ampicillin. Number of worms were counted every 2-3 days to monitor lifespan of the animals until the last animal died.

Figure 27. *wdfy-3* is required to maintain lifespan in HD model.

Lifespan analysis of animals expressing *rgef-1p::q19::cfp* and *rgef-1p::q67::cfp*, with and without the loss of function of *wdfy-3*. In a 96-well plate, synchronised L1 worms were added into a mixture of S medium with bacteria OP50, amphotericin B and ampicillin. Number of worms were counted every 2-3 days to monitor lifespan of the animals until the last animal died.

Previous studies have shown the correlation between protein aggregates and mobility in *Drosophila* and *C. elegans* HD models [208, 209]. Similar to humans, *C. elegans* display decline in physical ability with age. This is manifested by body movement which is assessed via the frequency of thrashes in liquid. To determine neuronal specific function of *wdfy-3*, thrashing assay was performed in WT and *wdfy-3(lf)* animals. Regardless of age, the absence of *wdfy-3* did not affect motility of WT animals (Fig. 28). Similarly, in young worms, the loss of function of *wdfy-3* did not affect the motility of Q19 animals (Fig. 29A). However, there was a slight improvement in the motility of Q67 with *wdfy-3(lf)* despite having similar number of aggregates as Q67 in young animals (Fig. 29A). In contrast, *wdfy-3* is dispensable to maintain motility of Q19, but significantly reduced the movement of aged worms expressing insoluble polyQ (Fig. 29B). Despite having lesser aggregates in Q67 aged animals with *wdfy-3(lf)*, loss of ability to maintain motility suggests that *wdfy-3* is indispensable to maintain healthspan in HD model upon aging.

Figure 28. *wdfy-3* is dispensable to maintain motility in WT animals.

Synchronised WT and *wdfy-3(lf)* worms were harvested at D0 and D7 of adulthood. Quantification was performed by normalising the number of body bends per second. $n = 32$ animals. Data shown is the mean $\pm$ SEM from three independent experiments. P values were calculated by two-tailed Student's t-test. n.s, not significant.

Figure 29. *wdfy-3* is required to maintain motility in aged HD model.

Synchronised *q19*, *q19;wdfy-3(lof)*, *q67* and *q67;wdfy-3(lof)* worms were harvested at D0 (A) and D7 (B) of adulthood. Quantification was performed by normalising the number of body bends before second. $n \geq 30$ animals. Data shown is the mean $\pm$ SEM from three independent experiments. P values were calculated by two-tailed Student's t-test. n.s, not significant.

To determine if the loss of function of *wdfy-3* affects the phenotypes associated with proteotoxicity, the function of neurons can be measured by chemotaxis that assess the ability of worms towards an attractive volatile odorant [171]. The loss of *wdfy-3* did not affect chemotaxis of worms regardless of age (Fig. 30). Similarly, CFP-Q19 and CFP-Q67 young worms expressing neuronal polyQ proteins showed no changes in chemotaxis in the absence of *wdfy-3* (Fig. 31). However, there was a significant reduction in chemotaxis in aged worms expressing CFP-Q67, whether it is in the presence or absence of *wdfy-3* (Fig. 31). Together, these findings argue that *wdfy-3* is necessary to suppress the toxicity of aggregates in aged animals.

Figure 30. *wdfy-3* is dispensable to maintain chemotaxis in aged model.

Synchronised WT and *wdfy-3(lof)* worms were harvested at D0 and D7 of adulthood. $n \geq 100$ animals for each strain and condition. Data shown is the mean $\pm$ SEM from three independent experiments. P values were calculated by two-tailed Student's t-test. n.s, not significant.

Figure 31. Loss of *wdfy-3* did not further affect the chemotaxis in aged HD model.

Synchronised *q19*, *q19;wdfy-3(lof)*, *q67* and *q67;wdfy-3(lof)* worms were harvested at D0 and D7 of adulthood. $n \geq 100$ animals for each strain and condition. Data shown is the mean $\pm$ SEM from three independent experiments. P values were calculated by two-tailed Student's t-test. n.s, not significant.

3.6 Potential crosstalk between WDFY-3 with cellular stress pathways

Apart from autophagy, there are other pathways that are known to play a role in the clearance of cytosolic aggregates, namely the HSR and UPR [211, 212]. Activation of the HSR helps in the re-folding and assembling of proteins during stress as well as to facilitate the degradation of irreversibly stress-induced denatured proteins by macroautophagy [213]. Hence, both HSR and macroautophagy serve at the two ends of the spectrum of proteostasis network and are likely to complement one another under stress conditions [213]. To further look into specific autophagy and/or HSR factors that promote WDFY-3-dependent stabilisation of polyQ aggregates, we performed a RNAi screening with selective autophagy and HSP related genes. The screening was performed in WT and *wdfy-3(lf)* mutants, as well as animals expressing both neuronal soluble and insoluble polyQ proteins in the presence or absence of *wdfy-3(lf)* mutant (*q19::cfp*, *q19::cfp;wdfy-3(lf)*, *q67::cfp*, *q67::cfp;wdfy-3(lf)*) (Fig. 32, Table 5 and 6). Accumulation of polyQ expansion in *C. elegans* has been shown to directly corresponds to motility defect that can serve as a read out for the screen [128]. Hence, RNAi knockdown of autophagy and HSP related genes which can lead to the accumulation of polyQ aggregates that result in observable phenotypic changes. With that, selective genes with obvious phenotypic changes can be targeted on solid NGM plates to allow further quantitative analysis. As certain genes are required for development, we performed the RNAi screening for 5 days at the post-developmental stage when worms reached young adult before assessing their phenotypic changes, sterility and behaviour (Fig. 32). Within the first 3 days, worms were grown on normal NGM plates with bacteria OP50 before being transferred on bacteria expressing RNAi clones. For positive control, we included *pmt-2* RNAi that shows phenotypic developmental defect [168, 214]. Following RNAi treatments, phenotypes were assigned with 0 (slight changes in brood size and growth), 1 (sick and/or smaller brood size), or 2 (very sick and/or sterile) in comparison to either WT or CFP-Q19 animals with empty vector [168]. From the 75 RNAi clones, two were found to reduce fecundity in the presence of amyloids as compared to CFP-Q19 (Fig. 33). Loss of *wdfy-3* significantly reduces the number of progenies production in *q67;wdfy-3(lf)* mutants as compared to other strains when either *cyn-11* or *cct-5* is being knockdown (Fig. 33). In addition, there were no detrimental defects observed and the progenies appeared to be healthy. Being a co-chaperone, *cyn-11* has been shown to negatively regulate the HSR [70, 215]. Knockdown of *cyn-11* reduces polyQ aggregates and upregulates downstream targets of the HSR such as *hsp-70* and *hsp-16.2* [70, 215]. In contrast, autosomal recessive mutations of *CCT5* and *CCT4* subunits

resulted in sensory neuropathy [216, 217]. Recent study revealed the close relationship between CCT complex and autophagy whereby the integrity of CCT is essential for autophagosomes degradation in cells and *Drosophila* [218]. Although the knockdown of *cct-5* or *cyn-11* can activate HSR constitutively in the muscles, it might not be enough to deal with polyQ aggregates that are being expressed within the neurons, in addition to the loss of function of *wdfy-3* [70]. As age-related collapse of proteostasis are associated with reduced brood size or sterility, the maintenance of proteostasis upon *C. elegans* adulthood comes at the cost of a reduction in reproductive capacity [219, 220]. An increase in the transcriptional levels of *cct-5* and *cyn-11* in *q67;wdfy-3(lof)* mutants could indicate the presence of compensatory roles that helps in maintaining proteostasis. Hence, further transcriptional and aggregates quantification can be done to determine if *cct-5* and/or *cyn-11* are involved in the regulation of aggregates upon the absence of *wdfy-3*.

Figure 32. Schematic model for the RNAi screening for potential compensatory pathway upon the loss of *wdfy-3*.

Synchronised L1 worms grown on NGM plate supplemented with OP50 before subjecting them to 96-well plate comprising of RNAi targeting HSP and autophagy related genes from young adult for a total of 5 days. Ranking of the worms were done based on phenotypic changes, sterility and behaviour compared to control strain subjected to empty vector.

Figure 33. *cct-5* and *cyn-11* are required for production of progenies in the presence of amyloids.

Synchronised young adults were subjected to vector, *cct-5* or *cyn-11* RNAi for 5 days before the ranking of the worms were done based on phenotypic changes compared to control strain (*q19::cfp*) subjected to empty vector. Red boxes showed the groups of progenies produced for all strains and conditions, except for *q67;wdfy-3(lof)* animals subjected to RNAi knockdown of *cct-5* and *cyn-11* which lack progenies. Red arrowheads are pointing to adult animals within each well, except for two wells which lack adult animals.

3.7 WDFY-3 is dispensable to maintain fitness in other neurodegenerative models upon aging

Despite the common theme of protein misfolding and aggregation among different neurodegenerative diseases, the pathology of each of the age-related misfolding disorders can be affected or activated by disease-protein-specific subset of molecular processes [1]. Based on the HD model, we have shown that WDFY-3 is required to maintain proper healthspan and lifespan in the presence of polyQ aggregates. In addition, WDFY-3 functions as a stabiliser to prevent toxicity of polyQ aggregates upon aging. To determine if WDFY-3 can generally stabilise other form of aggregates, we performed a knockdown of *wdfy-3(RNAi)* and subsequently monitored changes in protein aggregates as well as the healthspan of models expressing various forms of aggregates.

Like other neurodegenerative diseases, presence of inclusions in the brains containing disease-specific protein α -synuclein can be found in PD. Co-immunoprecipitation experiments from previous study revealed that α -synuclein interacts with Atg5 and ALFY [117]. This further suggests that the role of WDFY-3 can be extended to other diseases that are closely related to aggregate-prone proteins. To visually trace the expression of α -synuclein, we used a strain that expressed α -synuclein fused to YFP in *C. elegans* under the control of the *unc-54* promoter (*unc-54p::alphasynuclein::yfp*) that drives expression to the body wall muscle cells [221]. The expression of α -synuclein in the muscle allows better visualization and efficient knockdown with RNAi as compared to neuronal expression [221]. As the number of α -synuclein proteins relocate to discrete inclusions and increase with aging, worms were subjected to whole life RNAi and imaged at adulthood day 5 to examine the role of *wdfy-3* in the clearance of α -synuclein [221]. In the absence of *wdfy-3*, level of α -synuclein protein remained similar as compared to the empty vector control (Fig. 34). This further indicate that the presence of *wdfy-3* is dispensable for the proper clearance of these aggregates upon aging. To determine if the absence of *wdfy-3* affects neuronal function in PD model, chemotaxis and thrashing assays were performed. Although the knockdown of *wdfy-3* did not affect the formation of α -synuclein aggregates, there was a slight decrease in motility (Fig. 35A). However, chemotaxis (Fig. 35B) ability was not affected in aged PD animals upon the loss of function of *wdfy-3*. Unlike HD model with neuronal expression of polyQ aggregates, absence of *wdfy-3* did not affect healthspan of the PD model, thereby suggesting that it might have different roles upon aging and/or possess tissue-specific function. As *wdfy-3* is widely

expressed within neurons, it further suggests the possibility of having regulatory role that is associated with neurons, therefore restricting observations upon its absence in other tissues.

Figure 34. *wdfy-3* is dispensable for accumulation of α -synuclein in the muscles of day 5 PD model.

Synchronised *unc-54p::alphasynuclein::yfp* worms were subjected to vector and *wdfy-3(RNAi)* from L1 to day 5 of adulthood and harvested for imaging at 10 x magnification. Scale bar, 100 μ m. Images shown are representative of three independent experiments. Insets are zoom-in images of the indicated square areas.

Figure 35. *wdfy-3* is dispensable to maintain healthspan in aged PD model.

Synchronised *unc-54p::alphasynuclein::yfp* worms were subjected to vector and *wdfy-3(RNAi)* from L1 to day 5 of adulthood and harvested for thrashing (A) and chemotaxis (B) assays. $n=129$ animals (A), $n \geq 100$ animals for each strain and condition (B). Data shown is the mean $\pm$ SEM from three independent experiments for each assay. P values were calculated by two-tailed Student's t-test. n.s, not significant.

Apart from PD, ALFY has been shown to also assist in the clearance of intracellular mutant SOD1, TDP43, and its C-terminal fragment TDP25 aggregates that are found in ALS [222]. Like previously demonstrated, ALFY predominantly localises within the nuclear membrane of the motor neurons and colocalises with cytoplasmic aggregated mutant SOD1 proteins in mice [222]. Similarly, the overexpression of the C-terminal of ALFY significantly reduced aggregated mutant SOD1 proteins, while ALFY knockdown prevented the elimination of these proteins [222]. To determine if the function of *wdfy-3* as a stabiliser is conserved in the ALS model expressing mutated form of human SOD-1 in the body wall muscle, we performed *wdfy-3(RNAi)* from L1 stage to adulthood day 7 before harvesting them for changes in the expression of aggregates and assays to assess their neuronal function in the absence of *wdfy-3* [223]. Unlike HD, the disease progression of ALS is dependent on the accumulation of mutated SOD1, rather than the number of aggregates [224]. To determine changes in the expression of aggregates, the overall distribution of the aggregates was compared against empty vector control based on intensity [224]. Upon the loss of *wdfy-3*, there was no change in SOD1 expression within the worm's body wall muscle cells (Fig. 36A, 36B). This observation showed that *wdfy-3* is dispensable for the clearance of SOD1 aggregates expressed within the muscles. To further correlate this, both thrashing and chemotaxis assays were used to assay neuronal function upon loss of *wdfy-3*. Similarly, knockdown of *wdfy-3* did not affect the motility (Fig. 37A) and chemotaxis index (Fig. 37B) of the aged ALS model. Similar to the PD model, *wdfy-3* might have specific role in regulating aggregates within certain cell types, for example neurons, therefore its loss of function effect was not observed in muscle specific SOD1 aggregates.

Figure 36. *wdfy-3* is dispensable for the aggregation of muscle-specific SOD-1 in ALS model.

(A) Synchronised *unc-54p::Hsa-sod-1(WT)::yfp* and *unc-54p::Hsa-sod-1(127x)::yfp* worms were subjected to vector and *wdfy-3(RNAi)* from L1 to day 7 of adulthood and harvested for imaging. Scale bar, 100 μ m. Images shown are representative of two independent experiments. Insets are zoom-in images of the indicated square areas. (B) Quantification of SOD-1 YFP expression in (A). $n = 12$ animals (B). Data shown is the mean $\pm$ SEM from two independent experiments. P values were calculated by two-tailed Student's t-test. n.s, not significant.

Figure 37. *wdfy-3* is dispensable to maintain healthspan in the ALS model.

Synchronised *unc-54p::Hsa-sod-1(WT)::yfp* and *unc-54p::Hsa-sod-1(127x)::yfp* worms were subjected to vector and *wdfy-3(RNAi)* from L1 to day 7 of adulthood and harvested for thrashing (A) and chemotaxis (B) assays. $n \geq 70$ animals (A), $n \geq 100$ animals for each strain and condition (B). Data shown is the mean $\pm$ SEM from at least three independent experiments for each assay. P values were calculated by two-tailed Student's t-test. n.s, not significant.

Unlike PD and ALS, neither the role of ALFY nor Bchs in AD have not been explored. Therefore, we also conducted similar behavioural assays to determine the role of *wdfy-3* in aged AD model expressing pan-neuronal A β 1-42. Despite being a widely used model organism for several neurodegenerative diseases, *C. elegans* AD model with fluorophore tagged is not available and therefore *in vivo* changes in aggregates cannot be monitored. Instead, behavioural and/or lifespan assays can still be performed. In line with previous study, strain expressing pan-neuronal A β 1-42 displayed lower motility as compared to control strain expressing *myo-2p::yfp* (Fig. 38A) [225]. Surprisingly, *wdfy-3(RNAi)* did not affect either motility (Fig. 38A) or chemotaxis (Fig. 38B) index of aged AD model as compared to the empty vector control. Despite having pan-neuronal expression of A β , the loss of function of *wdfy-3* did not further aggravate the behaviour of aged AD model, unlike its stabiliser role in aged HD model. This indicates that the role of *wdfy-3* could be different and is specific for the regulation of certain types of aggregates that differ across various types of neurodegenerative diseases upon aging.

Figure 38. *wdfy-3* is dispensable to maintain healthspan in the AD model.

Synchronised *gnals1*[myo-2p::yfp] and *gnals2*[myo-2p::yfp+unc-119p::Abeta1-42] worms were subjected to vector and *wdfy-3*(RNAi) from L1 to day 7 of adulthood and harvested for thrashing (A) and chemotaxis (B) assays. $n \geq 40$ animals (A), $n \geq 100$ animals for each strain and condition (B). Data shown is the mean $\pm$ SEM from three independent experiments for each assay. P values were calculated by two-tailed Student's t-test. n.s., not significant.

Chapter 4. Discussion

Accumulation and turnover of disease-specific proteins are common hallmarks among neurodegenerative disease. However, changes upon age-dependent decline in proteostasis can also lead to proteome remodeling and severe proteome imbalance that contribute to increasing pool of aggregate-prone proteins. Degradation of misfolded and aggregated proteins found within the nucleus or cytoplasm are directed to the two main intracellular system, namely UPS and autophagy [226-229]. Autophagy serves as a compensatory pathway in the event when the UPS is impaired and overwhelmed [101, 189]. However, decrease in proteolytic activity is responsible for the accumulation of damaged cellular components in almost all the tissue of aged organisms. Indeed, failure of the protein quality control mechanism results in detrimental effect in post-mitotic cells that no longer undergo cell division, such as neurons, in turn resulting in sporadic late-onset neurodegeneration [230]. Despite extensive studies on the process of autophagy initiation, autophagosome formation and subsequent fusion with the lysosome, the function of receptors and adaptor proteins that are involved in selective autophagy remains relatively unknown until recently [231]. Among the adaptor proteins in mammals, ALFY has been shown to bridge the autophagic machinery with protein aggregates to facilitate formation of autophagosomes around the cargo to be degraded, by binding to autophagy receptor p62 via its PH-BEACH domain, Atg5 through its WD40 repeats and PI3P via its FYVE domains [117, 184]. Similarly, biochemical studies have demonstrated that physical interaction between *Drosophila* Bchs with other components of the autophagic machinery, further supporting its role as aggrephagy adaptor and p62-binding BEACH domain protein. Despite the well characterised role of its homologues, the function of WDFY-3 remains unknown and its involvement upon aging has yet to be explored *in vivo*. Using a *C. elegans* HD model, we find that the role of *wdfy-3* switches from an adaptor protein to a stabiliser of non-degradable accumulating aggregates upon aging, in which is important to maintain both fitness and lifespan in the presence of polyQ amyloids.

4.1 *wdfy-3* is required to maintain healthspan and lifespan in aged *C. elegans* HD model independently of basal autophagy.

Both Bchs and ALFY are large and highly conserved proteins that aid in the removal of cytoplasmic ubiquitinated protein aggregates by promoting clearance through macroautophagy. *Drosophila* Bchs and mammalian ALFY were shown to be highly expressed in the neurons and brain respectively [179, 183]. Similarly, *wdfy-3* is widely expressed along the entire axonal projections, as well as the cholinergic and sensory neurons (Fig. 9, 13-16). Neurodegenerative diseases are characterised by the deposition of specific protein aggregates in neurons and glia which can lead to an impairment of neuronal proteostasis. In a *C. elegans* model of tauopathy, paralysis, axonal degeneration of cholinergic motor neurons, presynaptic defects, synapse loss and mitochondrial transport defects occur early in adulthood [232]. In addition, accumulation of human wild type SOD1 protein in motor neurons of *C. elegans* led to oxidative stress-induced loss of cholinergic motor neurons [233]. Similarly, expression of a huntingtin fragment containing 150 polyQ repeats in the amphid sensilla of head sensory neurons resulted in nerve degeneration [234]. As various forms of protein aggregates are closely associated with neuronal degeneration and presynaptic defects, the presence of *wdfy-3* within the sensory and cholinergic neurons could suggest its potential role in mediating the aggregate-induced proteotoxicity within neurons that are post-mitotic and prone to degeneration [232-234]. Additionally, *wdfy-3* expression increases upon aging, further supporting its role in the maintenance of age-dependent decline in proteostasis which eventually contributes to the development of various neurodegenerative diseases (Fig. 9, 10, 12). Upon cellular stress such as proteasome inhibition, ALFY which is predominantly located in the nucleus gets recruited to cytoplasmic ubiquitinated polyQ proteins [183]. Unlike ALFY, nuclear staining showed no colocalisation between the nucleus and WDFY-3 in both young and aged animals expressing *mCherry::wdfy-3* at the basal condition (Fig. 17). However, lysotracker staining revealed partial colocalisation between lysosomes and WDFY-3 (Fig. 18). These observations indicate that WDFY-3 does not localise to nucleus predominantly and most likely resides within the cytosol in which it is closer to lysosomes which assists with clearance via macroautophagy under basal condition.

Overexpression of full length ALFY or its C terminus that consists of p62 and Atg5 binding sites reduced protein aggregation in primary neurons expressing polyQ Htt and *Drosophila* eye model of polyQ disease, implies that elevated ALFY levels can induce aggrephagy. In line with that, flies deficient in

Bchs resulted in an accelerated accumulation of ubiquitin-positive protein aggregates and neurodegeneration [179]. The association between WDFY-3 with soluble and insoluble polyQ proteins were shown to differ in terms of localisation (Fig. 20, 21). Upon the accumulation of insoluble aggregates, WDFY-3 switches from a diffuse pattern to form puncta that is reflected by its colocalisation with aggregates in both young and aged worms, further indicating that it functions in the regulation of aggregates rather than the soluble forms of polyQ regardless of age. The colocalisation of amyloids with WDFY-3 and the upregulation of *wdfy-3* mRNA levels in aged HD model suggest that WDFY-3 could potentially play an important role in regulating polyQ aggregates (Fig. 11, 20-21, S4).

Sharing 37% homology with ALFY, the C-terminal end of WDFY-3 contains domains required for autophagic clearance, thereby suggesting potential role in clearance of aggregates in *C. elegans* [235]. Accumulating evidences support the role of mammalian ALFY and *Drosophila* Bchs as autophagic adaptors which physically interact with autophagy machinery that assist in the clearance of aggregates [117, 179, 183]. Consistent with previous findings, *wdfy-3* is dispensable to maintain basal autophagy at both the transcriptional and protein level, as depicted by the similar expression levels of autophagic genes involved along the initiation and elongation steps of autophagy as WT (Fig. 19A) [117, 236]. Neuronal-specific knockout of Atg5 or Atg7 in mice leads to the accumulation of ubiquitinated cytoplasmic inclusion bodies [142, 143]. Furthermore, absence of the PI3K regulator or Atg8/LC3 has been shown to result in the accumulation of ubiquitinated and p62-positive aggregates in flies [237-239]. Therefore, the presence of core autophagy proteins is required to ensure proper clearance of protein aggregates. WDFY-3 contains domains at the C-terminal end that can potentially connect protein aggregates to the autophagic machinery via SQST-1 (homologue of p62) and ATG-5 (homologue of Atg5). Hence, presence of aggregates could induce the increase in transcripts of autophagic genes in WT animals but not in *wdfy-3(lop)* mutants. However, similar expression of autophagic genes *atg-9*, *lgg-1* and *sqst-1* were observed upon the presence of amyloids in young HD model, regardless of the presence of *wdfy-3* (Fig. 22A). Although the expression of disease-associated proteins was shown to interfere with cellular proteostasis, the ability to maintain similar genetic expression of autophagic genes suggests that an intact proteostasis network is still in place to support the clearance of aggregates in young HD animals [67, 240]. Aging in *C. elegans* alters several components of the proteostasis network, most distinctly protein biosynthesis and protein degradation

[67]. This is supported by the age-dependent decrease in levels of cytosolic and mitochondria ribosomal proteins together with an overall reduction in protein synthesis in *C. elegans* [67]. Despite the increase in proteasomal subunits and corresponding increase in proteasome activity, the age-dependent accumulation of surplus and aberrant protein species overwhelms the proteostasis network [67]. In addition, degradation pathways such as macroautophagy and CMA decrease with age [230, 241]. This further emphasise on the presence of compromised autophagy flux with age that contributes to the development of age-related diseases such as neurodegeneration and metabolic defects [143, 242]. Similar to young animals, age-dependent decline in proteostasis did not affect the induction of autophagic genes in aged *C. elegans* upon the absence of *wdfy-3* (Fig. 19B). In fact, a combination of aging and amyloids did not modulate autophagy genes in aged HD models, further indicating that *wdfy-3* does not modulate basal autophagy at the transcriptional level (Fig. 22B). In line with these observations, expression of the autophagosomal marker GFP::LGG-1-PE remains the same, regardless of age or presence of aggregates upon the knockdown of *wdfy-3* (Fig. 23, 24). As WDFY-3 is expressed in certain subsets of neurons, neuronal specific GFP::LGG-1 reporter such as *rgef-1p::gfp::lgg-1* can be used for the detection of WDFY-3-modulated GFP::LGG-1-PE expression that might be below the detection limits in isolates of whole animals. The use of neuronal specific autophagic reporter can help in limiting detection of only neuronal GFP::LGG-1-PE and subsequent quantification through immunoblotting with both macroautophagy and proteasome inhibitors can better help in determining the potential role of WDFY-3 in modulating autophagy.

As autophagosomes are required for the packaging and eventually degradation of the aggregates, quantification of the polyQ aggregates were performed in aged HD model. Despite the colocalisation between insoluble polyQ with WDFY-3, the number of polyQ aggregates were unaffected upon the absence of *wdfy-3* in young HD worms (Fig. 25). Surprisingly, aged HD worms showed a significant decrease in the number of polyQ aggregates (Fig. 25). The increase in *wdfy-3* expression upon the presence of aggregates, especially with the additional factor of aging, could indicates its potential in associating with these aggregates to prevent them from further aggregation. Formation of fibrils involved a nucleation-dependent polymerisation pathway which includes the various intermediate soluble, oligomeric and protofibrillar states that are structurally less ordered than the fibrils. Surprisingly, amyloid fibrils are not necessarily harmful to cells, but toxicity arises upon the presence of various

aggregation intermediates states primarily soluble oligomers during the process of fibril assembly [243-245]. Being structurally dynamic, having exposed hydrophobic amino acid residues and unpaired β strands to the solvent, these oligomers can have high degree of interactivity with cellular proteins and membranes [246-249]. Chaperone systems have been shown to prevent aberrant interactions by shielding pre-existing aggregates or converting toxic soluble oligomeric species into large inclusion bodies by reducing their reactive surfaces for toxic interactions with other cellular components [250-259]. Although the absence of *wdfy-3* does not affect young worms expressing insoluble aggregates, the shortening of lifespan and reduced fitness of the animals reflect the importance of *wdfy-3* upon aging (Fig. 27, 31, and 33). Despite having lesser number of aggregates in *q67;wdfy-3(lf)* aged animals, the shortening of lifespan and reduction in motility could indicate the presence of toxic aggregates. The toxicity effect observed seems to be dependent on WDFY-3 as observed in *q67* aged animals, thereby suggesting that WDFY-3 could play similar role as chaperones in associating with these aggregates to prevent aberrant interactions with other cellular components or aggregates which can otherwise result in cellular proteotoxicity. Unlike its homologues, the role of *wdfy-3* is dispensable to maintain proteostasis in young worms, while it switches to being a stabiliser that facilitates the sequestration of aggregates, thereby stabilising non-degradable accumulating polyQ aggregates and subsequently resulting in better healthspan upon aging (Fig. 39).

4.2 *cyn-11* and *cct-5* play potential role in maintenance of fecundity upon the loss of function of *wdfy-3* in HD model

Despite being functionally distinct, yet complementary, both HSR and autophagy are two systems that have similar goals of maintaining protein homeostasis under stress conditions. A recent study demonstrated the need of autophagy induction to extend the lifespan of *C. elegans* by subjecting them to either early exposure of heat shock or through the overexpression of HSF-1 [206]. Similarly, *in vitro* and *in vivo* studies showed the upregulation of autophagy upon heat stress, further highlighting the importance of crosstalk between HSR and autophagy in response to stress and lifespan regulation [260-263]. Molecular chaperones have been shown to promote protein folding, disaggregation and targeting terminally misfolded proteins for proteolytic degradation. However, knockdown of co-chaperones that negatively regulate those chaperones, can further enhance the activity of chaperones and subsequently suppress polyQ aggregation or protein misfolding [264-267]. Consistently, co-

chaperones and co-factors were shown to negatively regulate the folding activity of Hsp70 [268, 269]. Hence, enhancement of folding can be attained either by both positive and negative regulation of chaperones, or compensatory response that upregulates other chaperones. Previously shown to negatively regulate the HSR, knockdown of co-chaperone *cyn-11* should enhance the activity of chaperones and subsequently improves proteostasis. In the presence of insoluble polyQ proteins, combination of *wdfy-3(lox)* with knockdown of *cyn-11* resulted in a reduction of progenies as compared to soluble proteins (Fig. 33). Although almost all the negative regulators are ubiquitously expressed, they displayed tissue-specific expression of the HSR, of which knockdown of *cyn-11* resulted in the activation of the HSR only within the muscles [70]. However, muscle-specific activation of HSR was insufficient to improve the sterility of HD model with the combination of *cyn-11(RNAi)* and *wdfy-3(lox)*. This observation could be a combination of insufficient activation of the HSR that plays compensatory role and maintenance of proteostasis that occurred at the cost of reproduction upon aging [219, 220].

Besides co-chaperones, one other key player that aids in protein folding is the chaperonin containing TCP-1 (CCT/TRIC), which is a cytosolic ATP-dependent eukaryotic chaperonin consisting of two stacked octameric rings. Several studies have demonstrated the neuroprotective function of CCT in neurodegenerative diseases such as HD [250, 254, 258, 270]. Surprisingly, knockdown of TRIC components has been shown to have protective effect in model of A β toxicity [271]. Therefore, it is hypothesised that maintaining low levels of misfolded proteins may induce or retain activity of regenerative pathways that mediates toxicity of aggregates. Similar to *cyn-11*, knockdown of *cct-5* in combination with *wdfy-3(lox)* also reduced number of progenies in HD animals (Fig. 33). In this case, the loss of *cct-5* seems to be less protective and that it might be important to maintain proteostasis when *wdfy-3* is absent.

Maintaining the amount of co-chaperones and chaperonins are important in counteracting aggregate toxicity. Expression of co-chaperones has been shown to alleviate phenotypes associated with protein aggregation in various cellular and organismal models, including yeast, *C. elegans*, *Drosophila*, mammalian cells and mice [133, 250, 266, 272-280]. Interaction between TRIC and Hsp70/Hsp40 system have also been shown to prevent aggregation early within the protein aggregation pathway by promoting assembly of larger soluble polyQ oligomers (400-500 kDa), in contrast to the

conformationally distinct HTT oligomers (~200 kDa) [250, 254, 258]. Apart from that, studies have also shown that J-proteins that interact with the Hsp70s or Hsp70 and Hsp110 machineries, are required to promote activity of disaggregase to help in preventing aggregation. Disruption of the interactions between J-protein co-chaperones and the Hsp70s machineries can affect fundamental disaggregase activities and thereby resulting in a reduction in fecundity of *C. elegans* [281-285]. This further suggests that the maintenance of protein quality control in metazoan plays a central role in aggregates clearance, stress recovery and maintaining organismal fitness upon aging. The knockdown of *cct-5* and *cyn-11* significantly reduce the number of progenies production in *q67;wdfy-3(lox)* animals which could be due to the aggravation of inefficient protein quality control mechanisms present in *wdfy-3(lox)* mutants expressing insoluble polyQ proteins. Further quantification of the transcriptional or protein expression of *cyn-11* and *cct-5* in *wdfy-3(lox)* mutants can help us to better understand how these HSP components function in relation to *wdfy-3*.

4.3 Differential role of WDFY-3 in other neurodegenerative models upon aging

Several studies performed in *Drosophila* Bchs and mammalian ALFY were mainly with pharmacological inhibitors and overexpression of Bchs domains, with less emphasis on other forms of neurodegenerative diseases other than diseases involving polyQ proteins [179, 183, 184, 222, 286]. Interestingly, endogenous Htt was found in complex with Atg5, LC3 and p62 regardless of samples from healthy and HD patients [117]. This suggests that ALFY forms a complex with the autophagic machinery and aggregates independently of the type of aggregates present. In addition, knockdown of ALFY inhibited the clearance of aggregated forms of SDS-insoluble α -synuclein proteins. Furthermore, ALFY was shown to be forming a complex with Atg5 and α -synuclein proteins [117]. These findings suggest that ALFY is required for the elimination of different types of aggregated proteins. As the role of its homologues can be extended to different types of aggregated proteins, we wondered if the stabiliser role of WDFY-3 can be extended to other models of neurodegenerative diseases upon aging. Hence, aged animals of different neurodegenerative disease models were used for behavioural assays and microscopy to assess whether *wdfy-3* is required to maintain fitness and neuronal function.

Unlike the role of *wdfy-3* in the HD model, *wdfy-3* is dispensable for the accumulation of inclusions in aged PD, AD and ALS models (Fig. 34-38). These observations were supported by either slight or no

significant changes in the number of aggregates, chemotaxis and motility assays upon the knockdown of *wdfy-3*. Unlike that of the HD model, different neurodegenerative models were generated through the expression of either human α -synuclein, mutant SOD1 or A β ₁₋₄₂ [210, 221, 223, 225]. Although ectopic expression of polyQ was used for the HD model, its expression alone was sufficient to aggregate in body wall muscle cells or neurons in a polyQ-length dependent manner, subsequently result in cellular dysfunctions in those tissues [128, 210]. The morphological characteristics of amyloidogenic proteins such as A β , α -synuclein and mHTT are similar, despite variation in amino acid composition and molecular size [287]. As such, these proteins are likely to adopt the same aggregation mechanism as they undergo conversion from monomers that associate to form oligomers which can further generate protofibrils and fibers [287]. However, the use of ectopic expression of polyQ repeat serves as a generic model of polyQ toxicity and might not behave similarly to that of mutant huntingtin. Hence, its stabiliser role might not be well reflected in those neurodegenerative models expressing human mutant proteins. In addition, *wdfy-3* is widely expressed within neurons and its function in models expressing muscle-specific aggregates might not be as apparent if it lacks non-autonomous signalling. However, neuronal expression of human A β ₁₋₄₂ still shows the dispensable role of *wdfy-3* as a stabiliser in the maintenance of fitness. This further supports the hypothesis that the stabiliser role of *wdfy-3* is specific towards aggregate-prone polyQ proteins and not other types of insoluble aggregates upon aging.

Figure 39. Model for the putative role of *wdfy-3* as a stabiliser that maintain healthspan and lifespan upon aging.

The neuroprotective role of WDFY-3 serves as a secondary cellular pathway that help in counteracting proteotoxic-induced cell death as primary proteostasis pathways decline with age. By understanding the governing mechanisms between the proteostasis network and WDFY-3 upon aging can further provide therapeutic approaches for neurodegenerative diseases.

Chapter 5. Future Work

5.1 Characterise the role of WDFY-3 in aggregophagy of HD model

The role of *Drosophila* Bchs and mammalian ALFY as adaptor proteins that promote clearance of aggregates have been well characterised [117, 178, 179, 183, 235, 286, 288, 289]. However, our findings suggested that WDFY-3 has a surprising neuroprotective role in the stabilisation of age-dependent accumulation of aggregates in *C. elegans* HD model. Despite its dispensable role to modulate basal autophagy genetically and at the protein levels, WDFY-3 still partially colocalises to lysosomes in both young and aged animals. Moreover, WDFY-3 changes its localisation as it switches from being diffuse to puncta formation in the presence of polyQ aggregates despite age, indicating its potential role in macroautophagy. Besides that, expression of WDFY-3 increases with age and was found to be highly expressed in neurons. As homologues of WDFY-3 play an important role in the development of neurons, we can further investigate its role in autophagy flux through *C. elegans* developmental and aging processes with the use of different polyQ aggregates HD models [236, 290].

Although expression of WDFY-3 increased upon aging, information on the dynamic role of WDFY-3 through development and aging was still lacking. To address this issue, expression of *wdfy-3* at the transcriptional level with reporter strain *wdfy-3p::gfp* can be observed at additional timepoints which include different larva stages and beyond day 7 of adulthood. With the expression pattern of *wdfy-3* established, two different timepoints can be defined at which its expression is at the lowest (T_{low}) and highest (T_{high}). To determine its role in mediating autophagic flux, overexpression of *wdfy-3* neuronally can be performed at T_{low} and ablation of expression with *wdfy-3(lf)* mutant at T_{high} , and subsequently monitored with a tandem-tagged autophagic reporter *rgef-1p::mCherry::gfp::lgg-1* [195]. Besides that, inhibitors and activators of autophagy can be used in strains co-expressing *rgef-1p::mCherry::gfp::lgg-1* reporter with either the overexpression or loss of function of *wdfy-3* to determine how its expression can affect autophagy flux. In addition, autophagy flux can also be monitored *in vivo* with the use of similar tandem-tagged autophagy reporter in strains expressing polyQ proteins with the knockdown of *wdfy-3*. Lastly, interconnection of WDFY-3 with the autophagic machinery can be identified by attenuating different autophagy genes with RNAi in which could play a role leading to WDFY-3-dependent accumulation of aggregate upon aging.

5.2 Dissect cascades leading to WDFY-3-dependent stabilisation of aggregates in HD model

Based on our results, WDFY-3 switches its role as an adaptor protein to a stabiliser in the HD model upon aging as demonstrated with the ability to maintain healthspan and lifespan in the presence of *wdfy-3*. These data further suggest a potential neuroprotective role of *wdfy-3* in stabilising polyQ aggregates and subsequently to prevent accumulation of toxic aggregates. Overexpression of *wdfy-3* or *wdfy-3(lox)* mutant can be used at T_{low} and T_{high} respectively, in combination with different lengths of polyQ strains and changes in the number of aggregates can serve to verify the stabiliser role of WDFY-3. In addition, both lifespan and motility assays can be performed to observe the effect on polyQ aggregates upon the overexpression or ablation of *wdfy-3* at T_{low} and T_{high} . Besides the overexpression of WDFY-3, different domains at the C-terminal end that are involved in clearance of aggregates can be overexpressed in aged polyQ strains with different lengths to determine the specific domains involvement in the WDFY-3-dependent stabilisation of aggregates. Besides autophagy, both HSR and UPR have been shown to be involved in the clearance of cytosolic aggregates [211, 212]. In addition to the autophagy pathway, potential crosstalk with both cellular stress responses can be explored in which attenuation of autophagy by RNAi can be combined with factors involved in age attenuated UPR and HSR. With that, changes in the number of aggregates or behaviour in the HD model can help to identify age-modulated factors that shift WDFY-3 activity from aggregate clearance to a stabiliser of aggregates upon aging.

5.3 Conserved neuroprotective role of WDFY-3 in other neurodegenerative disease models

Although knockdown of *wdfy-3* did not affect the behaviour and aggregates present in the various aged neurodegenerative models, the role of *wdfy-3* across different ages has not been explored. As the type of aggregates vary across different neurodegenerative diseases, the threshold at which WDFY-3 might still function as a stabiliser that possesses neuroprotective role may also differ. Hence, knockdown of *wdfy-3* can be performed at different ages across the different neurodegenerative models. In addition, strain expressing the overexpression of *wdfy-3* can be mated to different neurodegenerative models to determine how the expression of WDFY-3 affects the changes in those aggregates. Further quantification of the protein aggregates with confocal microscopy and immunoblotting can be performed

to verify changes in number of soluble and insoluble proteins upon the overexpression or loss of *wdfy-*

3.

Chapter 6. Conclusion

One of the most common pathological hallmarks of many neurodegenerative diseases is the consistence presence of cellular aggregations of misfolded proteins [291]. With a common theme of misfolded or aggregated proteins in different neurodegenerative diseases, implementation of protein quality control helps to ensure proteome homeostasis. Cellular stress responses such as HSR, UPR and autophagy function to maintain integrity of the proteome in the presence of misfolded or aggregated proteins by regulating biogenesis, folding, trafficking and degradation of proteins [54]. In recent years, BDCPs such as ALFY and Bchs have drawn attention for their adaptor role in assisting with the clearance of protein aggregates via macroautophagy. Being an adaptor protein, it functions as a bridge between aggregates and the autophagic machinery to ensure proper enclosure of the autophagosomes around the cargo which is subsequently directed for degradation. Both ALFY and Bchs were found to be highly expressed either in the brain or neurons, further indicate that these adaptor proteins might be important in non-dividing cells to better help with the clearance of aggregate-prone proteins and preventing accumulation of aggregates [179, 183]. While studies have been focusing on the importance of ALFY and Bchs to prevent accumulation of aggregates, the function of WDFY-3 remains uncharacterised despite having similar domains as its homologues. Although the accumulation and turnover of disease-specific proteins are the focus of neurodegenerative diseases, aging itself can also result in homeostatic changes that affect the balance between protein synthesis and degradation. Several studies have shown that the role of ALFY is not restricted to aggrephagy of disease protein deposits, but also extended to other forms of commonly occurring stress complexes and ubiquitinated structures [117, 235, 292, 293]. Hence, there is a need to also examine the role of WDFY-3 upon aging in which is usually accompanied with an overall decline in proteostasis. In this study, we demonstrated the importance of WDFY-3, a potential adaptor protein which has been previously characterised in mammalian and *Drosophila*, that switches its role to stabilise aggregates upon aging to prevent accumulation of toxic aggregation, subsequently improving lifespan and healthspan in *C. elegans* model of HD. Potential role of WDFY-3 to modify disease-relevant aggregates involved in neurodegenerative diseases has emphasised its function as a stabiliser involved in counteracting age-related neurodegeneration by providing essential neuroprotection. By understanding the governing mechanisms between cellular stress responses and WDFY-3 can further provide invaluable insight to therapeutic approaches for neurodegenerative diseases.

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Appendix

Supplementary Figure 1. Subpopulation of *wdfy-3* reporter is expressed in cholinergic neurons (Insets).

Strains co-expressing *wdfy-3p::gfp* and *unc-17::mKate2::3xflag* were harvested at both D0 and D7 and used for imaging. Images shown are insets of the indicated square areas in Figure 14.

Supplementary Figure 2. Subpopulation of *wdfy-3* reporter is expressed in sensory neurons. (Insets).

Strains co-expressing *wdfy-3p::gfp* and *eat-4::mCherry* were harvested at both D0 and D7 and used for imaging. Images shown are insets of the indicated square areas in Figure 15.

Supplementary Figure 3. Absence of *wdfy-3* in GABA neurons (Insets).

Strains co-expressing *wdfy-3p::gfp* and *unc-47::mCherry* were harvested at both D0 and D7 and used for imaging. Images shown are insets of the indicated square areas in Figure 16.

Supplementary Figure 4. Colocalisation analysis of *mCherry::wdfy-3* with both soluble and insoluble polyQ upon aging.

Colocalisation analysis was performed using ImageJ to determine Manders' colocalisation coefficients M1 (A) and M2 (B) for channels 1 (Ch1) and 2 (Ch2) respectively. Data shown is the mean $\pm$ SEM for n =number of single-slice images. n = 7, 9, 8, 16 for *rgef1p::q19::cfp* (D0), *rgef1p::q67::cfp* (D0), *rgef1p::q19::cfp* (D7) and *rgef1p::q67::cfp* (D7) respectively. P values were calculated by two-tailed Student's t-test.

Table 4. Lifespan analysis

Strains	Mean lifespan $\pm$ SEM (days)	75 %	% change to control	Number of animals	P values versus WT or <i>q19</i>	Figure
WT	14.6 $\pm$ 0.6	9	-	108/108	-	Fig. 26
<i>wdfy-3(lox)</i>	19.6 $\pm$ 0.6	17	34.6	114/115	<0.0001	
WT	18.3 $\pm$ 0.4	12.5	-	223/223	-	biological replicate for Fig. 26
<i>wdfy-3(lox)</i>	19.1 $\pm$ 0.6	14	4.1	145/145	0.0245	
WT	13.0 $\pm$ 0.4	9	-	177/177	-	biological replicates for Fig. 26
<i>wdfy-3(lox)</i>	15.7 $\pm$ 0.5	9	21.3	137/137	<0.0001	
<i>q19</i>	16.1 $\pm$ 0.5	11	-	118/124	-	Fig. 27
<i>q19;wdfy-3(lox)</i>	19.8 $\pm$ 0.3	18	22.8	117/118	0.0022	
<i>q67</i>	19.9 $\pm$ 0.4	18	23.1	109/109	0.0018	
<i>q67;wdfy-3(lox)</i>	11.5 $\pm$ 0.4	9	-28.8	172/172	<0.0001	
<i>q19</i>	11.7 $\pm$ 0.3	9	-	151/151	-	
<i>q19;wdfy-3(lox)</i>	15.0 $\pm$ 0.5	12	27.7	103/108	<0.0001	biological replicate for Fig. 27
<i>q67</i>	14.4 $\pm$ 0.5	10	22.8	74/74	0.0002	
<i>q67;wdfy-3(lox)</i>	9.9 $\pm$ 0.4	7	-15.3	101/101	0.0001	

Table 5. List of genes and hits from RNAi screening in adulthood day 1 of WT and *wdfy-3(lof)* mutants.

RNAi candidates		Phenotypic ranking		Positive clone?	Gene description
		WT	<i>wdfy-3(lof)</i>		
	vector	0	0	NO	RNAi vector control (pL4440).
F54D11.1	<i>pmt-2</i>	0	0	NO	Phosphatidylethanolamine N-methyltransferase. Catalyses phosphomonomethylethanolamine (PMME) to phosphatidylmethylethanolamine (PDME), and PDME to phosphatidylcholine (PC).
D2085.2	<i>atg-10</i>	0	0	NO	Ortholog of human ATG10; E2-like ubiquitin conjugating enzyme involved in autophagy.
Y87G2A.3	<i>atg-4.1</i>	0	0	NO	Ortholog of human ATG4A and ATG4B; exhibits cysteine-type endopeptidase activity; is involved in autophagy and proteolysis.
W03C9.3	<i>rab-7</i>	0	0	NO	Ortholog of human RAB7A; exhibits GTPase activity; is involved in several processes, including body morphogenesis, endosomal transport, and oocyte growth; localizes to the late endosome membrane, lysosome, and phagocytic vesicle membrane.
VF39H2L.1	<i>syx-17</i>	0	0	NO	Ortholog of human STX17; predicted to have SNAP receptor activity and SNARE binding activity.
K02D10.5	<i>snap-29</i>	0	0	NO	Ortholog of human SNAP29; predicted to have SNAP receptor activity and syntaxin binding activity; is involved in intracellular protein transmembrane transport; ovulation; and secretion. Localises to the apical plasma membrane and recycling endosome.
W02C12.3	<i>hlh-30</i>	0	1	NO	Transcription factor TFEB orthologue that regulates expression of multiple autophagy-related, lysosomal genes and modulates autophagy process.
Y39A1A.1	<i>epg-6</i>	0	0	NO	Ortholog of human WDR45. Possess phosphatidylinositol phosphate binding activity and involved in autophagosome assembly.
C03B1.12	<i>Imp-1</i>	1	0	NO	Orthologue of LAMP/CD69 lysosomal membrane proteins. Lysosomal associated membrane glycoprotein. Transmembrane protein localises within the lysosomal membrane that is required for the fusion of autophagosomes with lysosomes during autophagy.

M7.5	<i>atg-7</i>	0	0	NO	Ortholog of human ATG7. E1-like activating enzyme involved in the activation of LGG-1.
ZK593.6	<i>lgg-2</i>	0	0	NO	Ortholog of human MAP1LC3A. Ubiquitin-like modifier involved in autophagosome formation. Through HOPS complex subunit vps-39, it tethers lysosomes with autophagosomes to form autophagolysosomes.
K09A9.2	<i>rab-14</i>	0	0	NO	Ortholog of human RAB14. Predicted to have GTP binding activity and GTPase activity; Involved in phagolysosome assembly involved in apoptotic cell clearance and phagosome acidification involved in apoptotic cell clearance.
T19E7.3	<i>bec-1</i>	0	0	NO	Ortholog of human BECN1. Together with phosphatidyl-3-phosphate kinase vps-34, functions as core subunit of the PI3K complex that mediates formation of phosphatidylinositol 3-phosphate and in turn regulate membrane trafficking.
B0336.8	<i>lgg-3</i>	0	0	NO	Ortholog of human ATG12. Ubiquitin-like protein involved in autophagosome formation. Modifier protein required for a protein conjugation system essential for autophagy.
F37C12.2	<i>epg-4</i>	0	0	NO	Ortholog of human EI24. Involved in macroautophagy and negatively regulate the assembly of autophagosome.
Y60A3A.1	<i>unc-51</i>	0	0	NO	Ortholog of human ULK1 and ULK2. Exhibits protein kinase activity and involved in autophagy.
ZK930.1	<i>vps-15</i>	0	0	NO	Ortholog of human PIK3R4. Predicted to have serine/threonine-protein kinase activity.
D2007.5	<i>atg-13</i>	0	0	NO	Ortholog of human ATG13. Predicted to have protein kinase binding and regulator activity.
Y47D3A.16	<i>rsks-1</i>	0	0	NO	Ortholog of human RPS6KB2. Serine/threonine-protein kinase which regulates mRNA translation. Negatively regulates lifespan and resistance to starvation, oxidative stress, protein aggregation.
T23G11.7		0	0	NO	Ortholog of human VTA1. Predicted to have SNARE binding activity.
K06A1.5	<i>atg-16.2</i>	0	0	NO	Ortholog of human ATG16L1. Function in a complex with ATG12-ATG5 to mediate the conjugation of phosphatidylethanolamine (PE) to LGG-1 to promote autophagosome formation.
T01H3.2		0	0	NO	Ortholog of TECPR1. Predicted to have phosphatidylinositol-3-phosphate binding activity.

M01E5.6	<i>sepa-1</i>	0	0	NO	Adapter protein that connects P-granules in somatic cells with the autophagic machinery. Involved in autophagy.
Y40C5A.1	<i>sqst-2</i>	0	0	NO	Predicted to have K63-linked polyubiquitin modification dependent protein binding activity.
CD4.4	<i>vps-37</i>	0	0	NO	Ortholog of human VPS37B and VPS37C. Component of the ESCRT-I complex, a regulator of vesicular trafficking process. Required for the sorting of endocytic ubiquitinated cargos into multivesicular bodies.
C52B11.5		0	0	NO	Ortholog of human RAB20. Plays a role in apical endocytosis/recycling, maturation and acidification of phagosomes.
C05D9.2	<i>Imp-2</i>	0	0	NO	Ortholog of human LAMP1. Predicted to encode lysosomal associated membrane glycoprotein. Required for the fusion of autophagosomes with lysosomes during autophagy.
M03A8.2	<i>atg-2</i>	0	0	NO	Ortholog of human ATG2A and ATG2B. Involved in autophagosome formation, regulation of lipid droplet morphology and dispersion.
F20D1.6	<i>rbg-1</i>	0	0	NO	Ortholog of human RAB3GAP1. Involved in formation of amyloid fibril and regulation of autophagy.
B0513.9	<i>vamp-8</i>	1	1	NO	Ortholog of human VAMP8. Predicted to have SNAP receptor activity and SNARE binding activity involved in autophagy.
B0025.1	<i>vps-34</i>	0	0	NO	Ortholog of human PIK3C3. Mediates formation of phosphatidylinositol 3-phosphate on intracellular vesicles and regulates membrane trafficking.
T28D9.10	<i>snr-3</i>	1	1	NO	Ortholog of human SNRPD1. Required for pre-mRNA splicing and involved in the formation of stable, biologically active snRNP structures.
C47B2.4	<i>pbs-2</i>	0	0	NO	Ortholog of human PSMB7. Proteasome 20S subunit.
K05C4.1	<i>pbs-5</i>	1	1	NO	Ortholog of human PSMB5 and PSMB8. Proteasome 20S subunit
R186.3		0	0	NO	Ortholog of human SRPRB. SRP receptor β subunit.
C52E4.4	<i>rpt-1</i>	0	0	NO	Ortholog of human PSMC2. Proteasome 19S subunit.
F23F12.6	<i>rpt-3</i>	0	0	NO	Ortholog of human PSMC4. Proteasome 19S subunit.
F23F1.8	<i>rpt-4</i>	1	1	NO	Ortholog of human PSMC6. Proteasome 19S subunit. Involved in proteasome-mediated ubiquitin dependent protein catabolic process and regulation of protein stability.
T09B4.9	<i>tin-44</i>	0	0	NO	Ortholog of human TIMM44 (Translocase of inner mitochondrial membrane 44); Predicted to have chaperone binding activity.

F25H2.9	<i>pas-5</i>	1	1	NO	Ortholog of human PSMA5. Proteasome 20S subunit.
F49C12.8	<i>rpn-7</i>	1	1	NO	Ortholog of human PSMD6. Proteasome 19S subunit.
T21B10.7	<i>cct-2</i>	1	1	NO	Ortholog of human CCT2. Predicted to have ATP binding and unfolded protein binding activities.
F26D10.3	<i>hsp-1</i>	1	1	NO	Ortholog of human HSPA8. HSP70 Chaperone.
C15H9.6	<i>hsp-3</i>	0	0	NO	Ortholog of human HSPA5. HSP70 Chaperone (ER).
F56H1.4	<i>rpt-5</i>	1	1	NO	Ortholog of human PSMC3. Proteasome 19S subunit.
F01F1.8	<i>cct-6</i>	1	1	NO	Ortholog of human CCT6A. Predicted to have ATP binding and unfolded protein binding activities.
F25G6.8		0	0	NO	Ortholog of human SRP14. SRP subunit.
F39H11.5	<i>pbs-7</i>	1	1	NO	Ortholog of human PSMB4. Proteasome 20S subunit.
C02F5.9	<i>pbs-6</i>	1	1	NO	Ortholog of human PSMB1. Proteasome 20S subunit
Y49E10.1	<i>rpt-6</i>	0	0	NO	Ortholog of human PSMC5. Proteasome 19S subunit.
ZK328.2	<i>eftu-2</i>	0	0	NO	Ortholog of human EFTUD2. Predicted to have GTPase and U5 snRNA binding activities.
C47E8.5	<i>daf-21</i>	1	1	NO	Ortholog of human HSP90AA1 and HSP90AB1. HSP90 Chaperone.
C07G2.3	<i>cct-5</i>	0	0	NO	Ortholog of human CCT5. Predicted to have ATP binding and unfolded protein binding activities.
F55C5.8	<i>srpa-68</i>	0	0	NO	Ortholog of human SRP68 (signal recognition. SRP subunit
C23G10.4	<i>rpn-2</i>	1	1	NO	Ortholog of human PSMD1. Proteasome 19S subunit.
R12E2.3	<i>rpn-8</i>	1	1	NO	Ortholog of human PSMD7. Proteasome 19S subunit
K07D4.3	<i>rpn-11</i>	1	1	NO	Ortholog of human PSMD14. Proteasome 19S subunit
C47E12.4	<i>pyp-1</i>	0	0	NO	Ortholog of human PPA1 and PPA2; Predicted to have inorganic diphosphatase activity and magnesium ion binding activity; Involved in innate immune response.
C37H5.8	<i>hsp-6</i>	1	1	NO	Ortholog of human HSPA9. Predicted to have ATP binding and unfolded protein binding activities; Involved in mitochondrial UPR.
F57B9.10	<i>rpn-6</i>	1	1	NO	Ortholog of human PSMD11. Proteasome 19S subunit.
C36B1.4	<i>pas-4</i>	1	1	NO	Ortholog of human PSMA7. Proteasome 20S subunit
F57B10.1	<i>let-607</i>	1	1	NO	Ortholog of human CREB3. Predicted to have DNA-binding transcription factor activity. Involved in protein folding, regulation of growth and signal transduction.
Y38A8.2	<i>pbs-3</i>	1	1	NO	Ortholog of human PSMB3. Proteasome 20S subunit.

R05F9.10	<i>sgt-1</i>	0	0	NO	Ortholog of human SGTA and SGTB. TPR co-chaperone.
F09D1.1	<i>usp-39</i>	0	0	NO	Ortholog of human USP39. Predicted to possess thiol-dependent ubiquitinyl hydrolase and zinc ion binding activities.
T24H7.2		0	0	NO	Ortholog of human HYOU1. Predicted to have ATP binding activity and is involved in IRE-1 mediated UPR.
C26C6.5	<i>dcp-66</i>	0	0	NO	Ortholog of human GATAD2A and GATAD2B. NuRD subunit.
Y55F3AR.3	<i>cct-8</i>	1	1	NO	Ortholog of human CCT8. Predicted to have ATP binding and unfolded protein binding activities.
F38E11.5	<i>copb-2</i>	2	2	NO	Ortholog of human COPB2. COPI β' subunit.
C47E12.5	<i>uba-1</i>	1	1	NO	Ortholog of human UBA1. Predicted to have ATP binding activity and ubiquitin-like modifier activating enzyme activity is involved in several processes, including embryo development, gamete generation, and nematode larva development.
T05C12.7	<i>cct-1</i>	0	0	NO	Ortholog of human TCP1. Predicted to have ATP binding and unfolded protein binding activities.
K01C8.10	<i>cct-4</i>	1	1	NO	Ortholog of human CCT4. Predicted to have ATP binding and unfolded protein binding activities.
T01B7.4	<i>cyn-11</i>	0	0	NO	Ortholog of human PPIH. Cyclophilin co-chaperone.
T10B5.5	<i>cct-7</i>	0	0	NO	Ortholog of human CCT7. Predicted to have ATP binding and unfolded protein binding activities.

Table 6. List of genes and hits from RNAi screening in adulthood day 1 of *q19*, *q19;wdfy-3(lf)*, *q67* and *q67;wdfy-3(lf)* animals.

RNAi candidates		Phenotypic ranking				Positive clone?	Gene description
		<i>q19</i>	<i>q19;wdfy-3(lf)</i>	<i>q67</i>	<i>q67;wdfy-3(lf)</i>		
Transcript	Gene						
	vector	0	0	0	0	NO	RNAi vector control (pL4440).
F54D11.1	<i>pmt-2</i>	0	0	0	0	NO	Phosphatidylethanolamine N-methyltransferase. Catalyses phosphomonomethyl ethanolamine (PMME) to phosphatidyl dimethyl ethanolamine (PDME), and PDME to phosphatidylcholine (PC).
D2085.2	<i>atg-10</i>	0	0	0	0	NO	Ortholog of human ATG10; E2-like ubiquitin conjugating enzyme involved in autophagy.
Y87G2A.3	<i>atg-4.1</i>	0	0	0	0	NO	Ortholog of human ATG4A and ATG4B; exhibits cysteine-type endopeptidase activity; is involved in autophagy and proteolysis.
W03C9.3	<i>rab-7</i>	0	0	0	0	NO	Ortholog of human RAB7A; exhibits GTPase activity; is involved in several processes, including body morphogenesis, endosomal transport, and oocyte growth; localizes to the late endosome membrane, lysosome, and phagocytic vesicle membrane.
VF39H2L.1	<i>syx-17</i>	0	0	0	0	NO	Ortholog of human STX17; predicted to have SNAP receptor activity and SNARE binding activity.
K02D10.5	<i>snap-29</i>	0	0	0	1	NO	Ortholog of human SNAP29; predicted to have SNAP receptor activity and syntaxin binding activity; is

							involved in intracellular protein transmembrane transport; ovulation; and secretion. Localises to the apical plasma membrane and recycling endosome.
W02C12.3	<i>hlh-30</i>	0	0	0	0	NO	Transcription factor TFEB orthologue that regulates expression of multiple autophagy-related, lysosomal genes and modulates autophagy process.
Y39A1A.1	<i>epg-6</i>	0	0	0	0	NO	Ortholog of human WDR45. Possess phosphatidylinositol phosphate binding activity and involved in autophagosome assembly.
C03B1.12	<i>Imp-1</i>	0	0	0	0	NO	Orthologue of LAMP/CD69 lysosomal membrane proteins. Lysosomal associated membrane glycoprotein. Transmembrane protein localises within the lysosomal membrane that is required for the fusion of autophagosomes with lysosomes during autophagy.
M7.5	<i>atg-7</i>	0	0	0	0	NO	Ortholog of human ATG7. E1-like activating enzyme involved in the activation of LGG-1.
ZK593.6	<i>lgg-2</i>	0	0	0	0	NO	Ortholog of human MAP1LC3A. Ubiquitin-like modifier involved in autophagosome formation. Through HOPS complex subunit vps-39, it tethers lysosomes with autophagosomes to form autophagolysosomes.

K09A9.2	<i>rab-14</i>	0	0	0	0	NO	Ortholog of human RAB14. Predicted to have GTP binding activity and GTPase activity; Involved in phagolysosome assembly involved in apoptotic cell clearance and phagosome acidification involved in apoptotic cell clearance.
T19E7.3	<i>bec-1</i>	0	0	0	0	NO	Ortholog of human BECN1. Together with phosphatidyl-3-phosphate kinase vps-34, functions as core subunit of the PI3K complex that mediates formation of phosphatidylinositol 3-phosphate and in turn regulate membrane trafficking.
B0336.8	<i>lgg-3</i>	0	0	0	0	NO	Ortholog of human ATG12. Ubiquitin-like protein involved in autophagosome formation. Modifier protein required for a protein conjugation system essential for autophagy.
F37C12.2	<i>epg-4</i>	0	0	0	0	NO	Ortholog of human EI24. Involved in macroautophagy and negatively regulate the assembly of autophagosome.
Y60A3A.1	<i>unc-51</i>	0	0	0	0	NO	Ortholog of human ULK1 and ULK2. Exhibits protein kinase activity and involved in autophagy.
ZK930.1	<i>vps-15</i>	0	0	0	0	NO	Ortholog of human PIK3R4. Predicted to have serine/threonine-protein kinase activity.
D2007.5	<i>atg-13</i>	0	0	0	0	NO	Ortholog of human ATG13. Predicted to have protein kinase binding and regulator activity.

Y47D3A.16	<i>rsk-1</i>	0	0	0	0	NO	Ortholog of human RPS6KB2. Serine/threonine-protein kinase which regulates mRNA translation. Negatively regulates lifespan and resistance to starvation, oxidative stress, protein aggregation.
T23G11.7		0	0	0	0	NO	Ortholog of human VTA1. Predicted to have SNARE binding activity.
K06A1.5	<i>atg-16.2</i>	0	0	0	0	NO	Ortholog of human ATG16L1. Function in a complex with ATG12-ATG5 to mediate the conjugation of phosphatidylethanolamine (PE) to LGG-1 to promote autophagosome formation.
T01H3.2		0	0	0	0	NO	Ortholog of TECPR1. Predicted to have phosphatidylinositol-3-phosphate binding activity.
M01E5.6	<i>sepa-1</i>	0	0	0	0	NO	Adapter protein that connects P-granules in somatic cells with the autophagic machinery. Involved in autophagy.
Y40C5A.1	<i>sqst-2</i>	0	0	0	0	NO	Predicted to have K63-linked polyubiquitin modification dependent protein binding activity.
CD4.4	<i>vps-37</i>	0	0	0	0	NO	Ortholog of human VPS37B and VPS37C. Component of the ESCRT-I complex, a regulator of vesicular trafficking process. Required for the sorting of endocytic ubiquitinated cargos into multivesicular bodies.
C52B11.5		0	0	0	0	NO	Ortholog of human RAB20. Plays a role in apical

							endocytosis/recycling, maturation and acidification of phagosomes.
C05D9.2	<i>Imp-2</i>	0	0	0	0	NO	Ortholog of human LAMP1. Predicted to encode lysosomal associated membrane glycoprotein. Required for the fusion of autophagosomes with lysosomes during autophagy.
M03A8.2	<i>atg-2</i>	0	0	0	0	NO	Ortholog of human ATG2A and ATG2B. Involved in autophagosome formation, regulation of lipid droplet morphology and dispersion.
F20D1.6	<i>rbg-1</i>	0	0	0	0	NO	Ortholog of human RAB3GAP1. Involved in formation of amyloid fibril and regulation of autophagy.
B0513.9	<i>vamp-8</i>	1	1	1	1	NO	Ortholog of human VAMP8. Predicted to have SNAP receptor activity and SNARE binding activity involved in autophagy.
B0025.1	<i>vps-34</i>	0	0	0	0	NO	Ortholog of human PIK3C3. Mediates formation of phosphatidylinositol 3-phosphate on intracellular vesicles and regulates membrane trafficking.
T28D9.10	<i>snr-3</i>	1	1	1	1	NO	Ortholog of human SNRPD1. Required for pre-mRNA splicing and involved in the formation of stable, biologically active snRNP structures.
C47B2.4	<i>pbs-2</i>	0	0	0	1	NO	Ortholog of human PSMB7. Proteasome 20S subunit.
K05C4.1	<i>pbs-5</i>	1	1	1	1	NO	Ortholog of human PSMB5 and PSMB8. Proteasome 20S subunit
R186.3		0	0	0	0	NO	Ortholog of human

							SRPRB. SRP receptor β subunit.
C52E4.4	<i>rpt-1</i>	0	0	0	0	NO	Ortholog of human PSMC2. Proteasome 19S subunit.
F23F12.6	<i>rpt-3</i>	0	0	0	0	NO	Ortholog of human PSMC4. Proteasome 19S subunit.
F23F1.8	<i>rpt-4</i>	1	1	1	1	NO	Ortholog of human PSMC6. Proteasome 19S subunit. Involved in proteasome-mediated ubiquitin dependent protein catabolic process and regulation of protein stability.
T09B4.9	<i>tin-44</i>	0	0	0	0	NO	Ortholog of human TIMM44 (Translocase of inner mitochondrial membrane 44); Predicted to have chaperone binding activity.
F25H2.9	<i>pas-5</i>	1	1	1	1	NO	Ortholog of human PSMA5. Proteasome 20S subunit.
F49C12.8	<i>rpn-7</i>	1	1	1	1	NO	Ortholog of human PSMD6. Proteasome 19S subunit.
T21B10.7	<i>cct-2</i>	1	1	1	1	NO	Ortholog of human CCT2. Predicted to have ATP binding and unfolded protein binding activities.
F26D10.3	<i>hsp-1</i>	1	1	1	1	NO	Ortholog of human HSPA8. HSP70 Chaperone.
C15H9.6	<i>hsp-3</i>	0	0	0	0	NO	Ortholog of human HSPA5. HSP70 Chaperone (ER).
F56H1.4	<i>rpt-5</i>	1	1	1	1	NO	Ortholog of human PSMC3. Proteasome 19S subunit.
F01F1.8	<i>cct-6</i>	1	1	1	1	NO	Ortholog of human CCT6A. Predicted to have ATP binding and unfolded protein binding activities.
F25G6.8		0	0	0	0	NO	Ortholog of human SRP14. SRP subunit.
F39H11.5	<i>pbs-7</i>	1	1	1	1	NO	Ortholog of human PSMB4. Proteasome 20S subunit.
C02F5.9	<i>pbs-6</i>	1	1	1	1	NO	Ortholog of human PSMB1. Proteasome

							20S subunit
Y49E10.1	<i>rpt-6</i>	0	0	0	0	NO	Ortholog of human PSMC5. Proteasome 19S subunit.
ZK328.2	<i>eftu-2</i>	0	0	0	0	NO	Ortholog of human EFTUD2. Predicted to have GTPase and U5 snRNA binding activities.
C47E8.5	<i>daf-21</i>	1	1	1	1	NO	Ortholog of human HSP90AA1 and HSP90AB1. HSP90 Chaperone.
C07G2.3	<i>cct-5</i>	0	0	0	1	YES	Ortholog of human CCT5. Predicted to have ATP binding and unfolded protein binding activities.
F55C5.8		0	0	0	0	NO	Ortholog of human SRP68 (signal recognition. SRP subunit
C23G10.4	<i>rpn-2</i>	1	1	1	1	NO	Ortholog of human PSMD1. Proteasome 19S subunit.
R12E2.3	<i>rpn-8</i>	1	1	1	1	NO	Ortholog of human PSMD7. Proteasome 19S subunit
K07D4.3	<i>rpn-11</i>	1	1	1	1	NO	Ortholog of human PSMD14. Proteasome 19S subunit
C47E12.4	<i>pyp-1</i>	0	0	0	0	NO	Ortholog of human PPA1 and PPA2; Predicted to have inorganic diphosphatase activity and magnesium ion binding activity; Involved in innate immune response.
C37H5.8	<i>hsp-6</i>	1	1	1	1	NO	Ortholog of human HSPA9. Predicted to have ATP binding and unfolded protein binding activities; Involved in mitochondrial UPR.
F57B9.10	<i>rpn-6</i>	1	1	1	1	NO	Ortholog of human PSMD11. Proteasome 19S subunit.
C36B1.4	<i>pas-4</i>	1	1	1	1	NO	Ortholog of human PSMA7. Proteasome 20S subunit
F57B10.1	<i>let-607</i>	1	1	1	1	NO	Ortholog of human CREB3. Predicted to have DNA-binding

							transcription factor activity. Involved in protein folding, regulation of growth and signal transduction.
Y38A8.2	<i>pbs-3</i>	1	1	1	1	NO	Ortholog of human PSMB3. Proteasome 20S subunit.
R05F9.10	<i>sgt-1</i>	0	0	0	0	NO	Ortholog of human SGTA and SGTB. TPR co-chaperone.
F09D1.1	<i>F09D 1.1</i>	0	0	0	0	NO	Ortholog of human USP39. Predicted to possess thiol-dependent ubiquitinyl hydrolase and zinc ion binding activities.
T24H7.2		0	0	0	0	NO	Ortholog of human HYOU1. Predicted to have ATP binding activity and is involved in IRE-1 mediated UPR.
C26C6.5	<i>dcp-66</i>	0	0	0	0	NO	Ortholog of human GATAD2A and GATAD2B. NuRD subunit.
Y55F3AR.3	<i>cct-8</i>	1	1	1	1	NO	Ortholog of human CCT8. Predicted to have ATP binding and unfolded protein binding activities.
F38E11.5		2	2	2	2	NO	Ortholog of human COPB2. COPI β' subunit.
C47E12.5	<i>uba-1</i>	1	1	1	1	NO	Ortholog of human UBA1. Predicted to have ATP binding activity and ubiquitin-like modifier activating enzyme activity is involved in several processes, including embryo development, gamete generation, and nematode larva development.
T05C12.7	<i>cct-1</i>	1	1	1	1	NO	Ortholog of human TCP1. Predicted to have ATP binding and unfolded protein binding activities.
K01C8.10	<i>cct-4</i>	1	1	1	1	NO	Ortholog of human CCT4. Predicted to have ATP binding and unfolded protein

							binding activities.
T01B7.4	<i>cyn-11</i>	0	0	0	1	YES	Ortholog of human PPIH. Cyclophilin co-chaperone.
T10B5.5	<i>cct-7</i>	0	0	0	0	NO	Ortholog of human CCT7. Predicted to have ATP binding and unfolded protein binding activities.