Aggregation-Promoting Factors in Neurodegenerative Diseases
Insights from a *C. elegans* Model

Olga Sin
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Aggregation-Promoting Factors in Neurodegenerative Diseases

Insights from a *C. elegans* Model

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1. Aim

Neurodegenerative diseases are complex human diseases that are still poorly understood in terms of their etiology and pathogenesis. The objective of this work is to zoom in on the molecular and cellular events that lead to a distinctive characteristic shared by several neurodegenerative diseases: protein aggregation. We take advantage of the nematode Caenorhabditis elegans (C. elegans) to discover genes that modify protein aggregation and toxicity (proteotoxicity) and to further examine how these genes contribute to the aggregation process. In addition, we aim to validate our observations in the mammalian system, by testing whether the mammalian orthologs of the new genes have similar effects on protein aggregation. Ultimately, we hope that the discovery of these modifiers will add to insights in the field, thereby providing a better understanding of how the aggregation process works, which cellular functions are involved and, importantly, how these are affected in the context of disease.
2. Outline of the thesis

In Chapter II we start this thesis by highlighting the importance of a balanced proteome and describing the cellular processes involved in its proper maintenance. We then explain how this balance is disrupted when protein misfolding and aggregation occurs, exemplified by neurodegenerative diseases. Next, we demonstrate the value of using small model organisms including yeast, nematode and fly when identifying genetic modifiers of proteotoxicity and highlight the validation of such modifiers in higher organisms. From a different perspective, we also present an emerging concept, namely the contribution of non-coding RNAs to neurodegeneration, and exemplify how impaired RNA metabolism can lead to neurological disorders.

In Chapter III, we focus on C. elegans models of neurodegenerative diseases and explain how these can be used to discover modifiers of proteotoxicity in high-throughput genetic screens. We describe the two most frequently applied methods of genetic screening – EMS mutagenesis and RNA interference – and compare these methods by explaining the advantages and disadvantages relative to each other. We conclude by providing examples of genetic screens that enabled the identification of modifiers of proteotoxicity in C. elegans models of Parkinson's, Alzheimer's and polyglutamine disease.

In Chapter IV, we describe the characterization of MOAG-2, a modifier of aggregation that was previously identified in a forward genetic screen performed in a C. elegans model of polyglutamine diseases. We discovered that MOAG-2 corresponded with LIR-3, a protein of unknown function previously identified in C. elegans. We observed that a mutation or a partial deletion in this gene suppressed aggregation in our model. MOAG-2/LIR-3 has two non-canonical C2H2 domains, which are commonly found in transcription factors. This suggested that MOAG-2/LIR-3 might regulate protein aggregation by functioning as a transcription factor. We discovered that MOAG-2/LIR-3 is indeed an RNA Polymerase III-associated transcription factor that regulates the transcription of small non-coding RNAs, including small nucleolar RNAs and transfer RNAs. In this study, we discovered that polyglutamine expansion proteins suppress MOAG-2/LIR-3-mediated transcriptional regulation and convert the MOAG-2/LIR-3 protein into an aggregation-promoting factor.

In Chapter V, we focus on how cells respond to proteotoxic stress provoked by the expression of aggregation-prone proteins. To investigate the cellular pathways involved in this stress response, we performed whole transcriptome profiling in
wild type worms and worms expressing polyglutamine expansion proteins. By combining differential gene expression analysis with gene ontology enrichment analysis, we found that the genes that responded to the presence of aggregation-prone proteins in the cell were genes involved in the unfolded protein response, the immune response and oxidative stress. Parallel to this, we also found that aggregation-prone proteins affect *C. elegans* development.

Another modifier of aggregation identified during previous genetic screens was MOAG-4. MOAG-4 has two human orthologs: SERF1A has 50% amino acid similarity to MOAG-4 and SERF2 has 54%. In Chapter VI we follow up on previous studies of SERF2 by Van Ham et al. We aimed to study the effect of SERF2 on amyloid-beta aggregation in the brain of mice and discovered that a full-body *Serf2* knockout resulted in embryonic lethality with incomplete penetrance. Indeed, we observed that the *Serf2* knockout allele was not segregated according to the expected Mendelian ratios, suggesting a crucial function for *Serf2* in mouse development. This observation led us to generate a brain-specific *Serf2* knockout mouse, which is viable and fertile.

In Chapter VII, we critically analyze this work and discuss how it has helped to address our initial aims. We further address any questions that remain open and discuss future perspectives that may follow from this work.
CHAPTER II

Regulation of protein homeostasis in neurodegenerative diseases: the role of coding and non-coding genes

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Abstract
Protein homeostasis is fundamental for cell function and survival, because proteins are involved in all aspects of cellular function, ranging from cell metabolism and cell division to the cell’s response to environmental challenges. Protein homeostasis is tightly regulated by the synthesis, folding, trafficking and clearance of proteins, all of which act in an orchestrated manner to ensure proteome stability. The protein quality control system is enhanced by stress response pathways, which take action whenever the proteome is challenged by environmental or physiological stress. Aging, however, damages the proteome, and such proteome damage is thought to be associated with aging-related diseases. In this review, we discuss the different cellular processes that define the protein quality control system and focus on their role in protein conformational diseases. We highlight the power of using small organisms to model neurodegenerative diseases and how these models can be exploited to discover genetic modulators of protein aggregation and toxicity. We also link findings from small model organisms to the situation in higher organisms and describe how some of the genetic modifiers discovered in organisms such as worms are functionally conserved throughout evolution. Finally, we demonstrate that the non-coding genome also plays a role in maintaining protein homeostasis. In all, this review highlights the importance of protein and RNA homeostasis in neurodegenerative diseases.
Protein homeostasis

Protein folding
Maintaining a healthy proteome is important to ensure cell survival and function. The cell maintains a healthy proteome through a series of complex and tightly regulated surveillance systems (Fig. 1). These systems ensure that each protein is properly folded or assembled in a state that is required for it to perform its function in the cell.

After the synthesis of a nascent polypeptide chain, the protein's amino acid sequence determines whether or not the protein becomes folded, and whether or not chaperone proteins are required for its folding (Fig. 1a, b). Some proteins are thought to exist in a predominantly “unfolded”, “disordered” or “intrinsically unstructured” state ([1], also reviewed in [2, 3]). Such proteins are typically involved in transcription, in signaling pathways and in protein networks ([4], also reviewed in [5, 6]). In mammals, about half of all possible proteins are predicted to have long disorganized regions and about 25% are estimated as being intrinsically unstructured [2]. Other proteins have domains within their amino acid sequence that can fold spontaneously, whereas other large, multi-subunit proteins require molecular chaperones to assist in folding to their native state, as shown in in vitro studies [7-11].

The molecular chaperones that cooperate in the de novo folding or refolding process are subdivided into different classes, which include the Hsp70 system, the small chaperones, the chaperonins and the Hsp90 system [11-14]. In the case of de novo synthesis, chaperones protect the nascent polypeptide chain from aberrant contacts with other domains of the same proteins and from aggregation with other proteins (Fig. 1b) ([13, 14] , also reviewed in [12, 15]). As a protein is synthesized, it is transiently unfolded and its hydrophobic regions are exposed. Hsp70 is able to recognize these regions and it binds to the protein substrate via its peptide-binding site in an ATP-dependent manner (reviewed in [12, 15, 16]). Hsp70 holds the substrate in an extended conformation, stabilizing it and preventing premature misfolding and aggregation. Next, the substrate can be transferred to another chaperone system, such as the
chaperonins, where folding takes place and a three-dimensional structure is acquired (reviewed in [12, 16, 17]).

When misfolded proteins accumulate, unfolded protein responses can increase the levels of chaperones, which are then able to restore the proteins to their properly folded form (Fig. 1c, d, reviewed in [16], [18-21]). Such an accumulation of misfolded protein is just one of the types of stress that can trigger unfolded protein responses. Unfolded protein responses are mechanisms that are highly conserved from yeast to humans and that are induced upon environmental and physiological stress, such as thermal or oxidative stress (reviewed in [22-24]). In one of these pathways thought to respond to the accumulation misfolded proteins in the cytosol, heat shock factor 1 (HSF-1) acts as a master transcriptional regulator. HSF-1 is activated upon phosphorylation, after which it translocates from the cytosol to the nucleus to bind to so-called heat shock elements, thereby upregulating the transcription of heat shock genes. These genes are then translated into proteins that assist in the refolding of misfolded proteins into functionally active proteins, in preventing unspecific interactions, or in mediating their degradation (Fig. 1d) (reviewed in [19, 22]).

Another strategy used by the cell to restore protein homeostasis is the unfolded protein response that is associated with the endoplasmic reticulum (ER) (Fig. 1e, also reviewed in [18, 25, 26]). The ER is the organelle where proteins enter the secretory pathway to acquire post-translational modifications, after which they are delivered to their corresponding organelle, fixed in the plasma membrane or shuttled outside of the cell to perform their function [27]. If misfolded proteins accumulate, the ER-associated degradation (ERAD) pathway is activated through signal transduction pathways that are mediated by three upstream effectors: inositol-requiring protein 1 (IRE1), activating transcription factor (ATF)-6 and PKR-like endoplasmic reticulum kinase (PERK).

IRE1, ATF-6 and PERK mediate three distinct pathways. Firstly, IRE1 is a transmembrane protein kinase that activates itself by auto-phosphorylation and mediates splicing of Hac1 in yeast and XBP-1 in eukaryotes [28-32]. IRE1 is known to promote the transcription of three groups of genes: stress-responsive genes including molecular chaperones and folding enzymes, genes
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Figure 1. Quality control of cellular proteins. When a protein is synthesized, it can acquire its native state in a chaperone-independent (a) or dependent (b) manner. Upon environmental stress or mutations, the protein may either not acquire its native state or lose it, both leading to misfolding (c). Here, the misfolded protein can be refolded back to its functional conformation with the aid of chaperones (d); or sent to degradation via the ERAD (e), the ubiquitin-proteasome system (f) or autophagy (g). Alternatively, it can be redirected to the JUNQ for posterior refolding or degradation by the proteasome (h) or it can be permanently sequestrated in the IPOD (i) or aggresome (j).

involved in ERAD and genes involved in ER trafficking [33-35]. Secondly, ATF-6 is a transmembrane protein with a transcription factor domain (leucine zipper) that translocates from the ER lumen to the Golgi apparatus to be cleaved by proteases [36, 37]. This proteolysis releases the ATF-6 cytosolic fragment, which then enters the nucleus to induce the transcription of ER-resident chaperones and the transcription factor XBP-1, thereby increasing ER protein quality control capacity [29, 37-39]. Thirdly, PERK is a transmembrane kinase protein that phosphorylates the alpha-subunit of the eukaryotic translation initiation factor 2a (eIF2a), thus preventing the binding of the initiator tRNA(Met) to the ribosomal complex, necessary for translation initiation [40-42]. This results in an overall reduction in protein synthesis, thereby attenuating the accumulation of misfolded proteins at the ER.
Protein degradation

If an aberrant protein cannot be folded back into its native state by the molecular chaperones, then it can be eliminated by two proteolytic systems, the proteasome and autophagy (Fig. 1f, g). In the degradation via the ERAD pathway, the ER cooperates tightly with the ubiquitin-proteasome system (UPS) to recognize, mark and traffic the misfolded proteins to the cytosol for degradation (Fig. 1e, reviewed in [18, 43-45]). The exact mechanisms that allow the cell to discriminate misfolded proteins from correctly folded proteins are not fully understood (reviewed in [44, 46, 47]). However, the current notion is that misfolded proteins can be recognized by molecular chaperones (the HSP70 family of proteins) and co-chaperones (the DnaJ/HSP40 family of proteins) [48-51].

An example that illustrates this recognition is the immunoglobulin binding protein (BiP), an HSP70 chaperone that recognizes and binds to the hydrophobic regions of misfolded proteins, thereby preventing their aggregation [49-53]. The binding of the ERAD substrate to BiP and its subsequent release depends on the conversion of ADP to ATP, a process regulated by ERdj proteins, which are part of the DnaJ/Hsp40 family of co-chaperones, and the nucleotide exchange factors GRP170 and BAP/Sil1 [48, 52]. These factors stimulate the ATPase activity of BiP and stabilize its binding to the misfolded protein [54-58].

The ERdj co-chaperones have also been shown to bind directly to unfolded proteins, thus maintaining them in a soluble state to be later recruited by BiP [48, 59]. After the misfolded protein has been identified, it is poly-ubiquitinated to be subsequently targeted for degradation [60-62].

Ubiquitination is a sequential three-step process that marks proteins destined for the proteasome (Fig. 1f). It starts with the activation of ubiquitin (a small 76 amino acid protein) by the activating enzyme E1, followed by binding of ubiquitin to the active site of the ubiquitin-carrier protein E2 and, finally, transfer of the ubiquitin molecule to the substrate in a reaction catalyzed by the ubiquitin protein ligase E3. At least four ubiquitin molecules must be bound to the ERAD substrate for it to be later recognized by the proteasomal machinery [63, 64]. Following this step, the misfolded proteins are delivered to the proteasome (a process called retrotranslocation) and the ubiquitin molecules are removed.
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from the substrate prior to degradation by the deubiquitinating enzymes and recycled [65-67]. The proteasome is a barrel-shaped, multicatalytic proteinase where proteolysis occurs and proteins are cleaved into peptides 2 to 30 amino acid long [68].

The second proteolytic system, autophagy ("self-eating"), is a cellular degradation mechanism that eliminates cytosolic components, organelles and pathogens via lysosomes (Fig. 1g, [69-72]). It is the part of the cell that ensures protein and organelle turnover, where old cellular components are degraded and recycled molecules become available for cell metabolism [70, 71, 73]. For the purpose of this review, we discuss only the role of autophagy as a protein quality control system.

Autophagy can be classified into three categories: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). In macroautophagy, a newly formed double membrane vesicle engulfs the cytosolic material, forming the autophagosome. The autophagosome then fuses with an endosome or lysosome, giving rise to the autolysosome where degradation takes place through the action of hydrolytic enzymes (Fig. 1g) [71]. The double membrane that surrounds the autophagosome is derived from the ER, the mitochondria or the plasma membrane [74-78]. In yeast, autophagy is a multi-step process that requires at least 37 autophagy-related (ATG) genes [79-89]. The majority of the ATG genes have shown to be functionally conserved in mammals [90, 91]. In microautophagy, small molecules from the cytoplasm are internalized by the lysosome through invagination of its own membrane [70, 73]. In contrast to autophagy and CMA, much less is known about microautophagy [92].

CMA differs from the former two forms of autophagy in that it does not involve membrane reorganization. Instead, substrates with a KFERQ amino acid motif are recognized by an HSP70 cytosolic chaperone, Hsc70, that binds and delivers them to the CMA receptor at the lysosome [93-96]. Here, the substrate is unfolded before it is translocated into the lumen of the lysosome for degradation, which is assisted by Hsc73, an intralysosomal HSP70 chaperone [97, 98].
Cross talk exists between the UPS and autophagy. Chronic low-level proteasomal inhibition is known to be sufficient to activate autophagy and it has been suggested that ubiquitinated proteins may also be eliminated through this pathway [99-101]. It has also been proposed that macroautophagy may occur as a compensatory mechanism when either the UPS or CMA is impaired [102, 103].

**Protein compartmentalization**

An alternative pathway for misfolded proteins is the sequestration into specialized protein quality control compartments where they can be either recovered or permanently sequestered (Fig. 1h, i, j) ([104-109], also reviewed in [110, 111]). Distinct quality control compartments harbor different species of misfolded proteins and are evolutionary conserved from yeast to mammals [105, 107-109, 112, 113]. Ubiquitinated misfolded cytosolic proteins are assigned to the juxtanuclear quality control compartment (JUNQ, Fig. 1h). These soluble, mobile misfolded proteins can subsequently be recovered by the molecular chaperone Hsp104 and either refolded back into functionally active proteins or degraded by the proteasomes localized nearby (Fig. 1h) [108, 112]. Non-ubiquitinated misfolded proteins – comprising amyloidogenic proteins – are redistributed to the insoluble protein deposit (IPOD, Fig. 1i). This compartment is localized at the cell periphery and is known to contain insoluble and immobile species, which are not recoverable and seem to remain terminally sequestered there (Fig. 1i) [108]. More recently, it has been proposed that there are no pre-existing compartments in the cell, and that soluble ubiquitinated misfolded proteins (but not the non-ubiquitinated amyloidogenic type) may coalesce and form transient structures termed ‘Q bodies’ that eventually mature into the JUNQ compartments [104].

Much research has focused on finding out whether the redistribution of misfolded proteins to these spatial cytosolic compartments is a random event or whether it depends on the concerted action of sorting factors. Evidence suggests that the latter is the case, and that sorting factors interact with chaperones to deliver misfolded proteins to each compartment [105]. For example, upon physiological stress, Btn2 (a Hook family protein involved in
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linking organelles to microtubules) was shown to associate either with the yeast small heat shock protein Hsp42 to assign misfolded proteins to the IPOD or with the chaperone Sis1 to guide misfolded proteins to the JUNQ [105, 107].

Another type of cytosolic compartment – the aggresome – is localized at the microtubule organizing center (MTOC) and is formed when the proteasome is unable to clear misfolded proteins properly (Fig. 1j) [114]. Aggresome formation is accompanied by redistribution of vimentin, an intermediary filament that acquires a cage-like structure in the aggresome. Ubiquitinated misfolded proteins depend on microtubules to be transported to the aggresome, this being done by the dynein/dynactin complex (Fig. 1j) [115]. Interestingly, the JUNQ shares several similarities with the aggresome, including its perinuclear localization, the presence of chaperones and ubiquitinated misfolded proteins [108, 114]. It was also recently shown to functionally associate with the MTOC and vimentin [112]. Indeed, the overwhelming accumulation of misfolded proteins to the JUNQ is thought to render it an immobile, insoluble aggresome over time [112].

Similar structures to aggresomes are the so-called aggresome-like induced structures (ALIS), which were originally discovered in dendritic cells but were later also found in other type of cells [109, 116]. The ALIS is a transient structure with peripheral and juxtanuclear localization. It is induced under a wide variety of stress conditions (e.g. heat shock, starvation, oxidative stress, inflammation) and clusters newly synthesized, ubiquitinated misfolded proteins [106, 109]. ALIS substrates can also be cleared by the proteasome and lysosome [106].

Cell division could be considered as yet another protein quality control system that sequesters misfolded, aggregated proteins (reviewed in [117, 118]). Studies in bacteria and yeast have shown that accumulation of protein aggregates reduces the fitness of these cells, a problem partially resolved by asymmetric division: these protein deposits are retained in the aging mother cell while the daughter cells are freed from damaged proteins, a process also known as replicative rejuvenation [119-123]. In budding yeast, it has been shown that misfolded proteins sorted either to the JUNQ or IPOD remain in the mother
cell after asymmetric cell division, thus avoiding passage of these species onto the daughter cells [124]. Follow-up work from the same group extended this observation to mammalian cells, where the JUNQ (but not the IPOD) continues to be inherited asymmetrically, thereby always freeing one of the two daughter cells from proteotoxicity [112].

While much is now known about the sophisticated quality control mechanisms that the cell has evolved to ensure proper protein homeostasis, several questions remain to be answered. We know that the cell counts on the concerted action of chaperones to avoid an unfolded or misfolded protein interacting aberrantly with other proteins until it can be refolded back into its native state. In case this is not possible, the aberrant protein is sent to be degraded via the ubiquitin-proteasome system or by autophagy. However, it is still not known how the cell chooses one mechanism of degradation over the other or whether the two mechanisms occur simultaneously. Another unknown relates to protein compartmentalization – yet another strategy for putting away proteins that need to be degraded or permanently sequestered. It has not yet been established how the cell can differentiate between degradable and non-degradable proteins and shuttle them to different subcellular compartments. Finally, another important question is how protein quality control changes during aging. Aging itself may be the contributing factor for progressive deterioration of protein homeostasis, impairing the ability of the protein quality control system to handle the equilibrium between protein folding and degradation.

**Protein misfolding and aggregation in neurodegenerative diseases**

The effects of progressive deterioration of protein homeostasis are thought to play a role in age-related neurodegenerative diseases. The presence of protein aggregates in the brain is namely a hallmark shared by several neurodegenerative diseases, including Parkinson’s (PD), Alzheimer’s (AD) and Huntington’s disease (HD) (reviewed in [125, 126]). In these diseases it is not yet clear why proteins accumulate into aggregates and how this relates to pathogenesis.

Protein aggregation and its relationship to aging and neurodegeneration have
also been widely studied in animal models. Evidence from several animal models suggests that, as the animal ages, the cell’s stress response systems become less efficient and less capable of maintaining a balanced proteome [127-133]. This could lead to the progressive accumulation of cytotoxic aggregation-prone disease proteins that cannot be cleared, ultimately resulting in toxicity and cell death [100, 134-137]. In the roundworm Caenorhabditis elegans, a model organism much used to study aging, protein aggregation has been shown to occur during aging and to affect the lifespan of the organism [138-140]. As previously discussed, when a protein misfolds it exposes its aggregation-prone domains to the cellular environment – domains that would otherwise be structurally concealed – thereby facilitating the likelihood of aberrant interactions with other proteins, potentially leading to proteotoxicity. Such proteotoxicity is proposed to play a role in protein conformational diseases in humans, including PD, AD and HD.

The type of aggregates that are formed varies for different neurodegenerative diseases. Frontotemporal lobar degeneration with fused in sarcoma is an example of a neurodegenerative disease that is characterized by the presence of amorphous, non-amyloidogenic aggregates ([141, 142], also reviewed in [143]). On the other hand, the common neuropathological feature of PD, AD and HD is the presence of an aggregation-prone disease protein that acquires amyloidogenic properties, causing it to form intracellular amyloid aggregates or extracellular amyloid plaques in the brains of patients (reviewed in [125, 126, 144]). The amyloids present in these neurodegenerative diseases can be distinguished from other amorphous, unstructured aggregates because they are organized, insoluble fibrils with a cross-beta structure and because they can be detected by specific amyloid-binding dyes, namely Congo red and thioflavin T (reviewed in [145, 146]). It is interesting to note that—despite their differences in amino acid sequence and function—several unrelated aggregation-prone disease proteins have one thing in common: in disease they are present as amyloid. This suggests that their ability to form amyloid is related to disease and that they may cause proteotoxicity in a similar manner.

*In vitro* studies have made clear that virtually any protein can form amyloid fibrils under certain conditions. Such conditions include low pH, high temperature
and high pressure [147-154]. Native proteins are known to exist in equilibrium with their partially unfolded state, and when they are destabilized by certain conditions or mutations, the equilibrium shifts towards amyloid formation. Predicting aggregation-prone regions in proteins is now possible using bioinformatic tools. Examples of such tools are TANGO, which can specifically identify regions prone to form beta sheets, and Waltz, which can distinguish between amyloid sequences and amorphous beta-sheet aggregates [155, 156].

A proposed mechanism for amyloid formation is depicted in Fig. 2. Most of our understanding of this pathway has come from in vitro studies of aggregation-prone proteins, including amyloid-beta (seen in AD) and alpha-synuclein (seen in PD) but also from studies of globular proteins, including human lysozyme, superoxide dismutase 1, transthyretin and the acylphosphatase from the archaea Sulfolobus solfataricus (reviewed in [125, 146]). One common step of amyloid formation appears to be the conversion of the monomeric, native state protein into an oligomeric intermediate state (Fig. 2). An oligomer is a small and transient cluster of protein molecules that has no fibrillar structure and is of low molecular weight [157-159]. These oligomers can then form protofibrils, which are fibrils 6 to 8 nm in diameter, about 200 nm in length and known to contain beta sheets detectable by Congo red and thioflavin T staining (Fig. 2) [160, 161]. Protofibrils can then convert into amyloid fibrils (Fig. 2) [160]. Of all these aggregation intermediates, it is currently thought that the early ones are cytotoxic and that aggregation may be a neuroprotective response to permanently sequester these intermediates, thereby preventing potentially toxic interactions with other proteins in the cellular milieu [162-165]. In support of this hypothesis, it has been shown that proteins rich in beta-sheet structures aggregate with newly synthesized proteins that have not yet become folded or with intrinsically unfolded proteins, thereby reducing the availability of these proteins to perform their normal function [135]. Further evidence demonstrating that oligomeric or protofibrillar forms of aggregation-prone disease proteins contribute to cell toxicity and death is reviewed elsewhere [144, 146, 166-168].

In a nutshell, the amyloid pathway has only just started to be described and it is not fully understood how protein aggregation correlates with disease. At
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Figure 2. Proposed mechanism for amyloid formation. A protein loses its monomeric native state by conversion into an oligomer which can grow further into amyloidogenic fibrils and ultimately into insoluble amyloid aggregates.

At the clinicopathological level, it is striking that there are individuals with high AD pathology (i.e. abundant amyloid deposits and neurofibrillary tangles) and yet do not display any cognitive impairment (reviewed in [169]). This fact makes it difficult to discern what are the boundaries between normal aging and disease pathogenesis. At the cellular and molecular level, what structural properties do aggregation-prone proteins acquire that make them toxic? This question is further complicated by the fact that aggregation-prone proteins such as amyloid-beta, huntingtin or alpha-synuclein do not share sequence, structure or function. A second question is that of how long neuronal cells can deal with these aggregation-prone proteins. And is their slow accumulation in the brain a reflection of an impaired protein quality control system? Finally, the majority of our knowledge about aggregation intermediates has come from in vitro studies. It remains to be shown whether oligomeric and fibrillar species exist in vivo and what their relevance to pathogenesis is.

Genetic modifiers of proteotoxicity

Genetic screens in small model organisms for protein aggregation in disease

The current understanding of how protein misfolding and aggregation contributes to neurodegeneration is far from complete. Molecular and cellular mechanisms that may regulate neurodegenerative disorders have been discovered in small organisms, the major ones being yeast (Saccharomyces cerevisiae), fly (Drosophila melanogaster) and nematode (C. elegans) (Table 1). In general, these small organisms are easy to grow and manipulate; their genomes are fully sequenced and accessible in public databases; and they provide information relatively quickly due to their short life cycle. Moreover,
the availability of resources such as genome-wide mutant libraries (deletion, overexpression or RNAi-based) further adds to the attraction of using these organisms as powerful genetic tools. Indeed, well-established models of several neurodegenerative diseases, including AD, PD and polyglutamine diseases, have now been generated in each of these small organisms [170]. Of note is that expression of an exogenous aggregation-prone protein typically exclusive to mammals can faithfully mimic some neuropathological features, namely the protein aggregation and toxicity phenotype seen in the diseased brain [170, 171]. And it is this that makes models in small organisms so attractive in the search for evolutionary conserved modifiers of proteotoxicity. These modifiers will provide insight in disease pathology and can be further explored as targets for therapy.

Finding modifiers of proteotoxicity in such models can be relatively quick: researchers can take advantage of high throughput screening techniques using genome-wide overexpression, deletion, or RNAi libraries or using chemical mutagenesis. These resources are unbiased methods that can be used to screen for genes that – when mutated, overexpressed or suppressed – contribute to an increase or decrease of protein aggregation and toxicity. Some of the hits that result from these screens may very well be genes that have already been associated with disease in humans. On the other hand, it is also a way of identifying previously unknown regulators of proteotoxicity – such findings may provide mechanistic insights into that particular disease. It should be noted, however, that genes shown to strongly suppress or enhance aggregation in one model do not always have a similar effect in other models, possibly due to the inherent differences between species or between the methods employed. Nevertheless, functionally conserved genetic modifiers of aggregation and toxicity have been identified across species.

In the end, to establish the value of genes discovered to be involved in aggregation and toxicity in small organisms, the results will have to be reproduced in human neurons and in mammalian animal models. If the function of modifiers of proteotoxicity identified in small organisms is evolutionarily conserved, their mammalian counterparts may become therapeutic targets worthy of future pharmacological investigation.
At the same time, small model organisms provide a simple platform that can be used not only to understand the basic mechanisms underlying the causal gene of disease but also as a pharmacological screening tool. Below we describe examples of genetic modifiers that have been studied in different model organisms for PD, AD and polyglutamine diseases.

### Parkinson’s disease models

Alpha-synuclein is the major constituent of the protein aggregates found in the brains of PD patients, which are also known as Lewy bodies [172]. It is a 140 amino-acid protein that is mostly expressed in the brain and is thought to have a function at the synapse (reviewed in [126, 173]).

The aggregation phenotype is successfully recapitulated in the budding yeast *S. cerevisiae*, where heterologous expression of alpha-synuclein induces toxicity in a concentration-dependent manner and is associated with the formation of cytoplasmic protein aggregates similarly to those observed in the human brain [174]. The characteristics that make yeast a powerful genetic tool for studying neurodegenerative disorders are reviewed elsewhere [171, 175].

In yeast, Cooper et al demonstrated that overexpression and subsequent accumulation of alpha-synuclein impairs vesicle transport from the ER to the Golgi (Table 1) [176]. In the same study, a genome-wide overexpression screen identified the small GTPase Ypt1 as a modifier of alpha-synuclein toxicity. Overexpression of Ypt1p was sufficient to prevent alpha-synuclein toxicity, by enabling forward trafficking from the ER to the Golgi. This observation was further extended to *Drosophila* and *C. elegans* models of PD as well as in rat midbrain primary neurons, where Rab1 – the functionally conserved orthologue of Ypt1p – suppressed dopaminergic neuron loss (Table 1) [176].

Another modifier of proteotoxicity identified from the same original yeast screen was YPK9, an orthologue of the human lysosomal P-type ATPase ATP13A2 (also known as PARK9), an enzyme known to be associated with early onset parkinsonism (Table 1). YPK9 overexpression prevented alpha-synuclein-induced toxicity by reducing intracellular aggregation and restoring alpha-synuclein localization to the plasma membrane [177]. The same study showed...
that the *C. elegans* orthologue CATP-6 partially prevented dopaminergic neuron loss, and that knockdown of CATP-6 increased alpha-synuclein misfolding in an age-dependent manner. Finally, in rat primary neuron cultures transduced with a lentivirus carrying the familial alpha-synuclein A53T mutation, heterologous expression of human ATP13A2 prevented neuronal loss (specifically dopaminergic neurons). Notably, this study was the first to show a link between environmental and genetic causes of PD, since YPK9 protected against manganese toxicity in yeast, a heavy metal thought to be risk factor for PD. Indeed, YPK9 was later shown to regulate manganese tolerance via diverse cellular processes, such as vesicle transport, vacuolar organization and chromatin remodeling in yeast (Table 1) [178].

The important role of vesicle-mediated transport in alpha-synuclein toxicity has also been demonstrated by other studies [179, 180]. In a screen performed by Kuwahara et al, the authors discovered ten neuroprotective genes, four of which were involved in endocytosis. Knockdown of two of these genes (*apa-2* and *aps-2*, encoding two different subunits of the AP-2 adaptor protein which mediates clathrin-dependent endocytosis) revealed that deficiencies at synaptic vesicles led to alpha-synuclein neurotoxicity [179].

Several modifiers of proteotoxicity have also been identified using RNAi screens in *C. elegans* models of PD [179-181]. Follow up on this work has revealed *tdo-2* as a general regulator of proteotoxicity and lifespan [182].

Genetic screens not only help us to identify novel modifiers of proteotoxicity, they can also be useful for rediscovering genes that were previously known to be associated with disease. Such an example comes from work by Hamamichi et al, where an RNAi screen identified the autophagy-related gene *Atgr7* as protecting against alpha-synuclein-induced toxicity in *C. elegans* dopaminergic neurons [181]. The mammalian orthologue of *Atgr7* has previously been implicated in neurodegeneration in mice, where it was found to cause axonal degeneration and dystrophy when ablated, thereby highlighting the importance of neuronal autophagy in preventing degeneration (Table 1) [183].
Table 1. Overview of a selection of genetic modifiers of protein aggregation and toxicity that are conserved between small model organisms, mammalian cells and animal models.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Organism</th>
<th>Transgene</th>
<th>Tissue</th>
<th>Modifier</th>
<th>Mammalian orthologue</th>
<th>Cellular process</th>
<th>Transposed to</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD</td>
<td>S. cerevisiae</td>
<td>alpha-synuclein</td>
<td>n.a.</td>
<td>YPT1P</td>
<td>RAB1</td>
<td>ER-to-Golgi vesicular trafficking</td>
<td>D. melanogaster, C. elegans and rat midbrain primary neurons</td>
<td>[176]</td>
</tr>
<tr>
<td></td>
<td>S. cerevisiae</td>
<td>alpha-synuclein</td>
<td>n.a.</td>
<td>YPK9</td>
<td>ATP13A2</td>
<td>Manganese homeostasis</td>
<td>C. elegans and rat midbrain primary neurons</td>
<td>[177, 178]</td>
</tr>
<tr>
<td></td>
<td>C. elegans</td>
<td>alpha-synuclein</td>
<td>Body wall muscle and dopaminergic neurons</td>
<td>ATGR7</td>
<td>ATG7</td>
<td>Autophagy</td>
<td>Mouse</td>
<td>[181, 183]</td>
</tr>
<tr>
<td></td>
<td>C. elegans</td>
<td>amyloid-beta</td>
<td>Body wall muscle</td>
<td>DAF-2</td>
<td>IGFR1</td>
<td>Insulin/IGF–1 signaling pathway</td>
<td>Mouse</td>
<td>[195, 197]</td>
</tr>
<tr>
<td>AD</td>
<td>S. cerevisiae</td>
<td>amyloid-beta</td>
<td>n.a.</td>
<td>YAP1802</td>
<td>PICALM</td>
<td>Endocytosis</td>
<td>C. elegans, rat cortical neurons, human</td>
<td>[198]</td>
</tr>
<tr>
<td></td>
<td>D. melanogaster</td>
<td>tau</td>
<td>Eye</td>
<td>GLUT1</td>
<td>SLC2A14</td>
<td>Glucose metabolism</td>
<td>Human</td>
<td>[199]</td>
</tr>
<tr>
<td></td>
<td>C. elegans</td>
<td>polyglutamine</td>
<td>Body wall muscle</td>
<td>MOAG-4</td>
<td>SERF1A and SERF2</td>
<td>Unknown</td>
<td>Human HEK293 and SK-N-SH cells</td>
<td>[203]</td>
</tr>
<tr>
<td></td>
<td>C. elegans</td>
<td>polyglutamine</td>
<td>Touch receptor neurons</td>
<td>662 modifiers</td>
<td></td>
<td>Diverse</td>
<td>CHL2 and R6/2 mouse models</td>
<td>[205]</td>
</tr>
<tr>
<td>PolyQ</td>
<td>C. elegans</td>
<td>polyglutamine</td>
<td>Body wall muscle</td>
<td>CCT</td>
<td>TRIC</td>
<td>De novo protein folding</td>
<td>In vitro, S. cerevisiae, mouse N2A neurons, human HeLa cells</td>
<td>[202, 206, 207]</td>
</tr>
<tr>
<td></td>
<td>S. cerevisiae</td>
<td>huntingtin fragment with expanded polyglutamine</td>
<td>n. a.</td>
<td>BNA4</td>
<td>KMO</td>
<td>Kynurenine pathway of tryptophan degradation</td>
<td>D. melanogaster, mouse</td>
<td>[208-210]</td>
</tr>
</tbody>
</table>

Abbreviations: PD - Parkinson's disease; PolyQ - Polyglutamine diseases; AD - Alzheimer's disease; n.a. - not applicable.
Alzheimer’s disease models

The brains of patients with AD are characterized by the presence of amyloid plaques and neurofibrillary tangles, which develop as a result of an accumulation of extracellular deposition of two different proteins: amyloid-beta in the plaques and intracellular hyperphosphorylated tau in the tangles (reviewed in [184, 185]). The disease can be caused by a mutation in the gene for amyloid precursor protein (APP), or in presenilin 1 or presenilin 2, all of which alter amyloid production (reviewed in [184, 186]).

*Caenorhabditis elegans* has been a fundamental tool for dissecting the pathways that link lifespan to AD (Table 1). Specifically, one of the major pathways that regulates lifespan is the insulin/IGF-1 signaling (IIS) pathway – a pathway that has been validated in nematodes, flies and mice and strongly implicated in humans [187-193]. In one of the models that recapitulates AD, *C. elegans* expresses a human amyloid-beta protein fragment (peptide 3-42) in the body wall muscle and progressive paralysis is used as readout for amyloid-beta toxicity [194]. In this model, knockdown of the insulin/IGF-1 receptor DAF-2 not only significantly extended lifespan but also prevented amyloid-beta toxicity by delaying the onset of paralysis, identifying a link between the mechanisms of aging and proteotoxicity [195]. Modulation of lifespan by DAF-2 was also found to be highly dependent on HSF-1 and DAF-16, two transcription factors known to drive the expression of longevity genes [196]. Curiously, while both blocked proteotoxicity, they did so through opposing effects: while HFS-1 promoted disaggregation, DAF-16 pushed aggregation forward, possibly as a means of sequestering the amyloidogenic protein from the cellular milieu [195].

The observation that inhibition of the IIS pathway protects against proteotoxicity was further confirmed in an AD mouse model with haploinsufficiency of IGFR-1, the mammalian orthologue of DAF-2 (Table 1) [197]. Here, reducing only half the expression of IGFR-1 (and thereby the IIS pathway) was sufficient to prevent amyloid-beta toxicity, namely by reducing inflammation and neuron loss. The AD mice with reduced IGFR-1 also performed better in memory and learning tasks than their age-matched AD controls did and this was found to be correlated with the formation of densely packed aggregates in the brain. This
supports the idea that aggregation is a protective mechanism to permanently sequester smaller, soluble oligomeric amyloid-beta species that are proteotoxic.

The importance of modeling neurodegenerative diseases in small organisms has been further reinforced by Treusch et al, who have identified modifiers of amyloid-beta toxicity that are conserved from yeast to humans (Table 1) [198]. Taking advantage of a yeast model of AD, they performed an unbiased genetic screen for modifiers of amyloid-beta toxicity. Of the identified modifiers, six were found to be risk factors for AD in humans – either validated or potential – that had been previously identified from family-based genome-wide association studies (GWAS). These modifiers were specific to amyloid-beta, in that in yeast they did not prevent toxicity induced by another aggregation-prone protein, alpha-synuclein. Another modifier of amyloid-beta toxicity identified by Treusch et al is YAP1802, a suppressor of amyloid-beta proteotoxicity that is involved in clathrin-mediated endocytosis. Its human homolog PICALM is also involved in endocytosis and has been validated as a high risk factor for AD (Table 1). YAP1802 prevents amyloid-beta toxicity in yeast and the human homolog PICALM prevents amyloid-beta toxicity in rat cortical neurons. Notably, this study identifies a causal gene for susceptibility to AD and proposes defective endocytosis as a contributing factor in AD pathology, with a possible role for PICALM.

In another independent study, GWAS data for AD was combined with a functional screen in *Drosophila* (Table 1) [199]. From a set of GWAS variants obtained from patients with AD, Shulman et al found 19 evolutionarily conserved orthologues in the fly that either enhanced or suppressed neurotoxicity associated with tau. Six of these interacted with tau in vivo, including the glucose transporter GLUT1, found to be functionally conserved in the human orthologue SLC2A14, further supporting a role for this risk factor as a disease modifying factor (Table 1) [199].

**Polyglutamine disease models**

In addition to models for PD and AD, there are several other models for aggregation-prone proteins, which include those for human polyglutamine diseases such as Huntington’s disease. In polyglutamine diseases trinucleotide
repeats cause expanded tracts of the amino acid glutamine in the encoded protein. In one *C. elegans* model, the animals express expanded glutamine repeats fused to a fluorescent protein in the body wall muscle. Expression of 35 to 40 glutamines is sufficient to cause aggregation, which increases with aging and is correlated with toxicity [200]. This model has been used in at least two genome-wide RNAi screens performed to search for suppressors and enhancers of proteotoxicity [201, 202]. These screens identified genes involved in RNA metabolism, as well as in protein synthesis, folding, trafficking and degradation as polyglutamine modifiers. In a subsequent screen to look for more modifiers, it was found that polyglutamine aggregation is not always coupled with proteotoxicity [201].

In an EMS screen to find genes that drive aggregation, Van Ham et al identified MOAG-4 (modifier of aggregation) as an aggregation-promoting factor in disease models expressing polyglutamine, alpha-synuclein and amyloid-beta, establishing MOAG-4 as a general regulator of proteotoxicity (Table 1) [203]. MOAG-4 is thought to be active during the early steps of the aggregation process, where it drives the formation of compact aggregation intermediates [203]. MOAG-4 is functionally conserved in two human orthologues, SERF1A and SERF2, which have the same aggregation-promoting function in human cell-based models of polyglutamine diseases (Table 1) [203]. Recent insights into the function of one of these proteins, SERF1A, suggest that it acts as an amyloid-promoting factor [204]. In this study, SERF1A recognized a broad range of aggregation-prone proteins (alpha-synuclein, huntingtin, amyloid-beta, prion protein) and mediated their conversion into amyloid *in vitro* [204]. It was further demonstrated that, to do this, SERF1A interacted directly with the monomeric form of the protein to seed amyloid growth, therefore supporting the hypothesis that MOAG-4/SERF1A acts on the early intermediates of the amyloid pathway [204]. SERF1A did not promote aggregation of non-amyloidogenic proteins.

An RNAi screen performed by Lejeune et al identified 662 modifiers that regulate polyglutamine-induced proteotoxicity in *C. elegans* touch receptor neurons, 49 of which were found to be differentially expressed in two mouse
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models of HD (Table 1) [205].

Another protein originally identified as a suppressor of polyglutamine aggregation in a C. elegans model is the chaperonin CCT [202]. It is composed of eight subunits and, together with HSP70, is involved in de novo folding of newly synthesized proteins [12]. Its orthologue, TRiC (also known as TCP), was shown to cooperate with HSP70 to prevent proteotoxicity by promoting the formation of non-toxic, soluble polyglutamine oligomers in a yeast model [206]. TRiC also modulated proteotoxicity in mouse and human cell models (Table 1) [207]. The subunit CCT1 was also shown to physically interact with polyglutamine to suppress aggregation in vitro, supporting the hypothesis that TRiC binds to polyglutamine to prevent it from acquiring a potentially toxic conformation [207].

Finally, a modifier identified in yeast is the kynurenine 3-monooxygenase BNA4, whose deletion prevented proteotoxicity induced by mutant huntingtin [208]. Follow-up work showed that genetic ablation or pharmacological inhibition of the orthologue KMO prevented toxicity in a fly and mouse model for HD (Table 1) [209, 210].

In summary, small model organisms including yeast, flies and nematodes are powerful tools for identifying genes involved in protein aggregation and toxicity. Several examples where small animal organisms complement findings from human cell models or mouse models further validate the importance of using these small animal models.

Non-coding RNA in neurodegeneration

When the Human Genome Project started in 1990, it was estimated that 30,000 to 40,000 protein coding genes would be found in the human genome [211]. When the project was completed in 2001, researchers were surprised to find far fewer protein coding genes than expected, namely 21,000, representing only about 2% of the total genome – with the remaining 98% being considered as “junk DNA” [212, 213]. However, it soon became clear that this “junk DNA” actually contained regulatory elements such as non-coding RNA (ncRNA), transcription factor binding sites or certain chromatin structures that govern
gene expression. These conserved functional elements in the human genome were subsequently comprehensively identified and characterized [214]. Within these conserved functional elements, many classes of ncRNA were identified and the list has been growing ever since ([215, 216], also reviewed in [217]). Indeed, the number of ncRNA transcripts are far greater than those coding for proteins and the list of all existing ncRNAs is not yet complete [218]. What we do know is that there are different classes of ncRNA with essential functions in gene transcription, RNA processing and translation, a selection of which is presented in Table 2 (a more complete list can be found in [217]). Indeed, impaired RNA metabolism has been correlated with several neurodegenerative diseases. For example, abnormal expansion repeats in the non-coding regions of disease-related genes induce toxic RNA gain-of-function in myotonic dystrophy, amyotrophic lateral sclerosis and frontotemporal dementia [219, 220]. For the purpose of this review, we focus on a few examples of ncRNAs that have been directly implicated in neurological or neurodegenerative diseases (Table 2).

**microRNAs**

Over the past few years, it has become evident that ncRNAs are key players in the development and maintenance of the nervous system. Of all classes of ncRNAs identified so far, microRNAs (miRNAs) are those that have been most extensively studied and documented. The function of miRNAs is to bind to the 3’-untranslated region (3’ UTR) of messenger RNA and inhibit its translation or target it for degradation (Table 2) [221]. *In situ* hybridization studies in mouse and zebrafish have revealed miRNA to be expressed throughout the brain; these studies have also demonstrated that miRNA expression is spatiotemporally controlled, supporting a biological function for miRNAs in the central nervous system [222-224]. Indeed, several hundreds of miRNAs are involved in brain development [225–229]. miRNAs play a role in virtually every aspect of brain function including neurogenesis, neural differentiation and maintenance, and synaptic plasticity, all of which are described extensively elsewhere [230–232].
Table 2. Classes of RNAs and their localization, size and function.

<table>
<thead>
<tr>
<th>Class</th>
<th>Localization</th>
<th>Transcribed by</th>
<th>Function</th>
<th>Size</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Messenger RNA (mRNA)</td>
<td>Nucleus and cytoplasm</td>
<td>Pol II</td>
<td>Gene expression into protein</td>
<td>2-5 kb</td>
<td>n.a.</td>
</tr>
<tr>
<td>Ribosomal RNA (rRNA)</td>
<td>Cytoplasm</td>
<td>Pol I</td>
<td>Translation, is part of the small or large subunit of the ribosome</td>
<td>70S (prokaryotic) or 80S (eukaryotic)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Micro RNA (miRNA)</td>
<td>Nucleus and cytoplasm</td>
<td>Pol II or Pol III</td>
<td>Inhibition of translation or degradation of mRNA</td>
<td>21 – 23 bp</td>
<td>AD, PD, HD, PHC-2, PHC-4, neurodevelopmental and neurological disease</td>
</tr>
<tr>
<td>Transfer RNA (tRNA)</td>
<td>Cytoplasm</td>
<td>Pol II or Pol III</td>
<td>Translation, brings cognate amino acid of mRNA to nascent polypeptide chain</td>
<td>70 - 90 bp</td>
<td>PHC-2, PHC-4, neurodevelopmental and neurological disease</td>
</tr>
<tr>
<td>Long non-coding RNA (lncRNA)</td>
<td>Nucleus and cytoplasm</td>
<td>Pol II or Pol III</td>
<td>Diverse regulatory roles of other RNAs and proteins, chromatin remodeling</td>
<td>&gt; 200 bp</td>
<td>AD, SCA7</td>
</tr>
<tr>
<td>Small nuclear RNA (snRNA)</td>
<td>Nucleus</td>
<td>Pol II or Pol III</td>
<td>Splicing of pre-mRNA</td>
<td>100 - 300 bp</td>
<td>SMA</td>
</tr>
</tbody>
</table>

Abbreviations: AD - Alzheimer's disease; PD - Parkinson's disease; HD - Huntington's disease; PHC - pontocerebellar hypoplasia; SCA - spinocerebellar ataxia; SMA - spinal muscular atrophy; n.a. - not applicable

miRNAs have also been associated with various aspects of aging and neurodegenerative diseases (Table 2) [233–237]. For example, Northern blotting experiments in the hippocampi of fetuses, adults, and AD patients have shown that miR-128 were upregulated in the AD development and during aging [237]. In these experiments, miR-9 and miR-107 had increased expression in the AD disease and neurodevelopmental and neurological disease. miR-29a and miR-29b-1 are involved in the amyloid-beta 1-42 generation in the brain, which is lost in the diseased brain due to the reduced expression of these miRNAs (Fig. 4). In these studies, the expression of miR-107, miR-29a, and miR-29b-1 was decreased in the AD brain while expression of miR-34a. is increased. In a mouse model for AD, miR-34a has been shown to play a role in the amyloid-beta 1-42 generation.
inhibit bcl-2, an anti-apoptotic gene that prevents cell death provoked by amyloidogenic species (Fig. 4b) [234]. Additionally, miR-124 has been found to regulate APP alternative splicing in neurons [233].

In PD, downregulation of the miR-34b/c cluster is correlated with downregulation of DJ-1 and Parkin, two genes implicated in the pathogenesis of PD, although a causal link has yet to be determined [238]. It has recently been shown in a cell model that this same cluster directly represses alpha-synuclein mRNA levels and consequently aggregate formation, establishing that miRNAs can have a direct effect on the expression of an aggregation-prone protein [239].

Several miRNAs have also been found to be dysregulated in polyglutamine diseases (Table 2) [240–244]. In HD, REST is a transcription factor that negatively regulates neuronal gene expression and has been found to repress brain-specific miRNAs in mouse and human brains [241, 242]. Two of these miRNAs, miR-9 and miR-9*, have been identified as targeting the REST complex in a negative feedback loop [244]. In a cell model of spinocerebellar ataxia type 1, miR-19, miR-101 and miR-130 cooperatively regulate ataxin-1 expression levels by binding to its 30 UTR [243]. Inhibition of these miRNAs leads to ataxin-1 accumulation in cells and subsequent cell death [243].

**tRNAs**

Transfer RNAs are essential for mRNA translation into a protein, as they are responsible for transporting the cognate amino acid to the nascent polypeptide chain (Table 2) [245, 246]. Due to the degeneracy of the genetic code, there can be up to five tRNAs per amino acid—termed isoacceptors—that have distinct anticodons for recognizing the same amino acid [245, 246]. On the other hand, tRNAs that share the same anticodon but have distinct body sequences are termed isodecoders, and their number vary greatly [246].

Growing evidence suggests that mutations in individual tRNAs—or in the enzymes involved in their biosynthesis—are a contributing factor in neurodegeneration (Fig. 3) [247–252]. For example, a point mutation (4274T>C) in the mitochondrial tRNA for isoleucine was identified in a patient suffering
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from motor neuron disease, although the mechanism by which this mutation might lead to disease is unknown (Fig. 3a) [252]. In a recent study, loss of function of one of the brain-specific tRNA isodecoders for arginine was found to be correlated with neurodegeneration in mice (Fig. 3a) [247]. Specifically, a point mutation (50C>T) in the T loop of the arginine tRNA provoked ribosome stalling, which is normally offset by GTPBP2. However, simultaneous impairment of GTPBP2 in these mice disabled its function as a so-called rescue factor, subsequently resulting in neurodegeneration [247].

Other impairments in the tRNA biosynthesis pathway are seen in pontocerebellar hypoplasia (PHC). PHC is an autosomal recessive neurodegenerative disorder that has six subtypes (PHC1-6) and is generally characterized by hypoplasia and atrophy of the cerebellum and pons [253]. PHC2 and PHC4 arise from impaired tRNA splicing endonuclease (TSEN) activity. TSEN is composed of two catalytic subunits (TSEN 2 and TSEN34) and two non-catalytic subunits (TSEN54 and TSEN15) (Fig. 3c) [250, 254]. It is thought that mutations in both

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**Figure 3.** Mutations in the tRNA biosynthesis pathway that lead to neurodegeneration. The point mutation (50C>T) in the T loop of one tRNA isoacceptor for arginine (Arg) provokes neurodegeneration. Another described point mutation (4274T>C) in the mitochondrial tRNA for isoleucine (Ile) has also been associated with motor neuron disease (a). Following transcription, the 50 leader sequence of the pre-tRNA is removed by RNaseP, the 30 end is processed by RNase Z and the trinucleotide CCA is added to the 30 end by a nucleotidyl transferase (b). Different bases of the RNA transcript can undergo chemical modifications (c). The introns of the pre-tRNA are spliced out by a tRNA splicing endonuclease (TSEN). Mutations in these enzymes have been associated with pontocerebellar hypoplasia (PHC) and mutations in their co-factor CLP-1 with motor neuron loss (d). Finally, the mature tRNA is loaded with an amino acid (aa) via tRNA synthetases.
catalytic subunits and in TSEN54 may prevent proper complex formation, leading to misplicing of premature tRNAs (pre-tRNAs) into their mature form, thereby unbalancing the tRNA repertoire for protein synthesis [248, 250]. PHC6 results from a mutation in the intronic region of the mitochondrial pre-tRNA synthetase gene for arginine [251].

Finally, CLP-1 is a mammalian kinase that cooperates with the TSEN complex to remove the intronic loop of pre-tRNAs (Fig. 3c) [255]. Loss of CLP-1 results in severe impairment of spinal motor neurons in mice, ultimately leading to respiratory failure [255]. CLP-1 mutations in affected patients have been correlated with neurodevelopment and neurological symptoms in both the central and peripheral nervous system [256, 257].

In summary, these studies demonstrate a crucial role for tRNAs in neuronal function, as either mutations in their transcript or defective post-transcriptional modifications can affect their proper processing and function, ultimately leading to neurodegeneration.

Other ncRNAs
The other non-coding RNAs shown in Table 2 have been less well studied but are nevertheless worthy of mention. Long non-coding RNAs (lncRNAs) are more than 200 nucleotides long and are mostly expressed in the nervous system (Table 2) ([223], also reviewed in [258]). Three lncRNAs have been suggested to be involved in neurodegenerative diseases. Firstly, BACE-1 anti-sense transcript is an lncRNA that competes with miR-485-5p for binding to the BACE-1 mRNA to stabilize it (Fig. 4c) [259]. In AD, the levels of BACE-1 anti-sense transcript are elevated, thereby stabilizing BACE-1 mRNA and enhancing its expression, which further promotes the generation of toxic amyloid-beta 1–42 (Fig. 4c) [259]. Secondly, in spinocerebellar ataxia type 7 (SCA7), IncSCA-7 crosstalks with miR-124 to regulate transcript levels of atxn7 [260]. Thirdly, Abhd11os is an lncRNA that has been shown to be neuroprotective against mutant huntingtin in two mouse models for HD, although the exact mechanism of how this occurs remains to be determined [261].
Small nuclear RNAs (snRNAs) exist as small nuclear ribonucleoproteins (snRNPs) and are major components of the pre-mRNA splicing machinery (Table 2) [262, 263]. The survival motor neuron protein (SMN) is directly involved in the generation of snRNPs [264]. In a mouse model of spinal muscular atrophy, SMN deficiency affects the snRNA pool in a tissue-specific manner, ultimately leading to pre-mRNA splicing defects in a diverse range of genes [264]. Further evidence for the involvement of snRNAs in neurodegeneration comes from work by Jia et al., who revealed that a mutation in a U2 snRNA gene impairs alternative splicing of pre-mRNA which is directly responsible for neuron loss in the cerebellum and hippocampus of mice [265].

**Figure 4.** Impaired BACE-1 regulation contributes to AD. miR-107, miR-29a and miR29-b-1 were shown to be decreased in the brain of AD patients while BACE-1 mRNA and protein levels were elevated (a). In an AD mouse model, elevated levels of miR-34a negatively correlate with BCL-2 protein levels, which normally prevent apoptosis induced by amyloid-beta (b). BACE-1 anti-sense transcript was reported to be upregulated in the brain of AD patients. BACE-1 anti-sense transcript stabilizes BACE-1 mRNA thereby facilitating its expression, which ultimately results in the generation of more amyloid-beta (c).
Neurodegeneration is clearly not exclusively caused by imbalances in protein coding genes—it can also arise from dysregulation of ncRNAs. Over the past two decades, we have begun to understand that ncRNAs are not just “transcriptional noise” and have started to define their role in the CNS and in neurodegeneration. Several reports have shown that different classes of ncRNAs influence the expression levels of the disease protein and that each class of ncRNA does so either by affecting the protein post-transcriptionally or through crosstalk with other classes of ncRNAs (miRNAs, lncRNAs). Maintaining a proper environment for protein synthesis is crucial to ensure that each mRNA molecule is effectively spliced and translated into a protein (through tRNAs and snRNAs). To establish the causal relationships between changes in ncRNAs and disease phenotypes, the targets of these ncRNAs need to be uncovered. Understanding the role of ncRNAs will provide insight into the mechanisms of neurodegenerative diseases, which enables the identification of targets for therapeutic interventions.

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Regulation of protein homeostasis in neurodegenerative diseases: 
the role of coding and non-coding genes
CHAPTER III

Genetic screens in *Caenorhabditis elegans* models for neurodegenerative diseases

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Abstract

Caenorhabditis elegans comprises unique features that make it an attractive model organism in diverse fields of biology. Genetic screens are powerful to identify genes and C. elegans can be customized to forward or reverse genetic screens and to establish gene function. These genetic screens can be applied to “humanized” models of C. elegans for neurodegenerative diseases, enabling for example the identification of genes involved in protein aggregation, one of the hallmarks of these diseases. In this review, we will describe the genetic screens employed in C. elegans and how these can be used to understand molecular processes involved in neurodegenerative and other human diseases.
1. Introduction

1.1 Caenorhabditis elegans as a model organism

Sydney Brenner first introduced the nematode *C. elegans* as a genetic model organism in 1965 and since then the model has been extensively used in very diverse fields of research, from developmental biology to ecotoxicology, aging and neuroscience [1]. This has resulted in several breakthroughs in biomedical science, which include the discovery of genetic regulators of programmed cell death, the use of the green fluorescent protein as a protein marker, and the discovery of RNA interference. Indeed, this nematode combines a number of characteristics that make it an advantageous model, anatomically and genetically, which are summarized in Box 1. Moreover, the characteristics of this invertebrate make it an easy experimental model to study biological processes in a relatively cheap, quick, and easy way.

*C. elegans* is a small, free-living nematode of about 1–1.5 mm in length that can be found in temperate soil environments feeding on different bacteria, including *Escherichia coli*. It exists in two sexual forms, as a hermaphrodite or as a male. The former is self-fertile, able to produce its own sperm and eggs and is the predominant adult form. Although males are rare (about 0.02%), their abundance in the offspring can be increased to 50% by mating with

Box 1

**Advantages of using Caenorhabditis elegans as a model organism**

1. small size (1-1.5 mm long)
2. short reproductive cycle
3. short lifespan
4. translucent body
5. precise, predetermined anatomy
6. ease of culture
7. small genome
8. whole genome sequenced
9. RNAi library available
10. deletion mutant database
hermaphrodites [2]. The length of the life cycle of wild type N2 C. elegans strains and its lifespan depends on the growth temperature. Grown at 20°C, hermaphrodites usually lay 300–350 eggs and once the eggs hatch, it takes about three days to develop from a larva to an adult. The average lifespan of this organism can vary between 18 and 20 days [3,4]. At higher temperatures, the life cycle is shortened and the lifespan decreased. One major advantage of C. elegans is that it has a well-dissected and predetermined anatomy. The adult hermaphrodite has exactly 959 somatic cells and 302 neurons [1,5,6]. Its transparent body enables one to easily follow cell fate or expression of fluoroscently tagged proteins of interest in the living animal. Moreover, C. elegans was the first multicellular organism to have the complete genome sequenced and this gave rise to several databases and resources that are currently available online for the scientific community ([7], see “Online links” at the end of this article).

Genetic screens are widely used in C. elegans to discover gene function. It can be easily applied to discover which gene mutations are responsible for a specific phenotype of interest (forward genetics) or, conversely, the gene function can be purposely altered to assess what is the consequence in terms of development, behavior or alterations in specific biological processes (reverse genetics). The two major genetic screens employed are ethyl methane sulfate (EMS) screens and (genome-wide) RNAi screens. They have been fundamental not only to dissect nematode genetics but also to identify genes involved in aging, development, DNA damage response, and signal transduction, amongst other biological processes [8–13].

1.2 C. elegans homology to humans
For the scope of this review, we shall explore the rationale and the basic procedures for both methods not only highlighting their advantages but also pinpointing the drawbacks. Next, we will explore how genetic screens can help us gain insight into the molecular and cellular mechanisms of human diseases. Specifically, we will focus on the application of genetic screens to discover potential disease-modifier genes by exemplifying studies on C. elegans models for neurodegenerative diseases.
There are a significant number of proteins that are evolutionary conserved between *C. elegans* and humans. At the time that the *C. elegans* genome sequencing was complete, 36% of *C. elegans* proteins (from a set composed of 18,891 protein sequences) were found to have homologs in humans (set composed of 4979 protein sequences), by pairwise comparison (The *C. elegans* Sequencing Consortium, 1998 [7]). Thereafter, this percentage was increased to 83% due to the much larger human gene dataset available to perform the comparison [14]. A more recent study estimated that 38% of the 20,250 *C. elegans* protein-coding genes had unique corresponding functional orthologs in humans (7663 unique hits) [15]. In a nutshell, biological processes unraveled in the invertebrate *C. elegans* can provide insight into human biology.

2. Genetic screens

Genetic screens in *C. elegans* are well-established and commonly used to assess gene function in any biological process of interest. High-throughput (semi-) automatized setups and screening methods enable hundreds of parallel experiments in microtiter plates. In a screen, wild type animals are mutagenized or treated with RNAi and then scored for phenotypical changes. Below we describe two types of genetic screens that are most frequently used: EMS mutagenesis and RNA interference (RNAi). The characteristics of both type of screens are summarized in Table 1.

<table>
<thead>
<tr>
<th>EMS mutagenesis</th>
<th>RNA interference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivation or alteration of gene function</td>
<td>Reduction or depletion of gene function</td>
</tr>
<tr>
<td>Requires identification of gene mutation</td>
<td>Candidate gene is known</td>
</tr>
<tr>
<td>Permanent mutation</td>
<td>Possible to select developmental stage for depletion</td>
</tr>
<tr>
<td></td>
<td>No effects on embryos in the first generation</td>
</tr>
<tr>
<td>Can select for non-essential genes</td>
<td>Can identify roles of essential genes in a post-developmental process</td>
</tr>
<tr>
<td></td>
<td>Limited penetrance to neurons</td>
</tr>
<tr>
<td></td>
<td>Limited efficiency if the protein that is encoded by the targeted gene is very stable</td>
</tr>
</tbody>
</table>
2.1 EMS mutagenesis

The most commonly used method to mutate the genome of *C. elegans* is the treatment with EMS. The mutagen induces mutations in the sperm and oocytes of hermaphrodites. Sydney Brenner tested systematically different mutagens, but researchers are mostly using EMS because of its relatively low toxicity and relatively good efficiency (summarized in [16]). The hermaphroditism of *C. elegans* allows easy maintenance of a mutation, as a homozygous worm will pass it to all the progeny through self-fertilization.

Mutations can be identified using a simple F2 screen firstly described by Brenner in 1974 [1]. Thousands of copies of any particular gene can be analyzed in a typical EMS screen. The frequency of a null mutation at any particular locus of the genome is one for every 2000 copies by using standard concentrations (50 mM) of the mutagen. That means that one can expect to identify 6 mutations per particular gene in a typical experiment of 12,000 haploid genomes (reviewed in [16]). The mutagenized worms are placed on Petri dishes and grown for two generations to produce homozygous mutants (Fig. 1). Worms from the F2 generation showing a specific phenotype of interest are further singled to new plates to determine whether the phenotype is transmitted to the next generation.

Once a worm with a specific phenotype is isolated, the responsible mutation needs to be identified. By using single nucleotide polymorphisms (SNPs) of the Hawaiian wild type strain in comparison to the Bristol strain (natural variation wild type) it is possible to map a mutation first to a certain chromosome [17] and then in several steps to a specific region on that chromosome. When a mutation is mapped to a gene region, sequencing or the specific knockdown of every single gene in that area by RNAi can be used to identify the mutated gene. The development of new sequencing methods like deep sequencing in the last decade facilitates the identification of mutations and can save laborious fine mapping [18–20]. It is important to keep in mind that an isolated, mutated animal can have several mutations at different loci. For further interpretation, it is therefore necessary to backcross the animals several times with wild type strains. The importance for controlling the genetic background was shown by
Burnett and colleagues. They demonstrated that a described lifespan extension [21] by overexpression of SIR-2 disappeared after several backcrossings [22]. Tissenbaum and Guarente further showed that the overexpression of SIR-2.1 slightly increases lifespan but to a much lesser extent than the transgenic animals used in their first publication [23]. Deep sequencing methods can be
used to monitor mutations in the background. Preferentially, one should use independent mutant or deletion alleles of a gene to confirm results.

In the first EMS screens, 619 mutants were identified with visible phenotypes especially from the uncoordinated class [1]. This group of genes impairs wild type movements when mutated. Under laboratory conditions proper moving is not essential as food is plentiful and sex is dispensable so therefore maintenance and characterization of mutants that may not survive in non-laboratory conditions are possible. Many of these mutants have revealed important information about molecules and mechanisms involved in human disease. For example, one gene of the uncoordinated class is $unc-2$ (uncoordinated 2) and encodes for a homolog of the voltage-sensitive calcium-channel alpha-1 subunit (human P/Q calcium channel CACNA1A) [24]. Missense mutations in the CACNA1A calcium channel in humans are associated with a rare form of migraine [25], which is often associated with low levels of serotonin [26]. $unc-2$ mutants show neuronal migration defects similar to serotonin-deficient mutants [27] and UNC-2 is required for the desensitization to the two neurotransmitters dopamine and serotonin [28]. Studies using $C. elegans$ showed that UNC-2 interacts with the transforming growth factor (TGF)-β, a pathway that is required for movements through regulation of serotonin levels probably through the modulation of the expression of $tph-1$ (tryptophan hydroxylase), the enzyme that converts tryptophan into serotonin [29]. There are elevated levels of TGF-β1 in migraine patients compared to those of pain-free individuals [30].

In addition to mutations in the uncoordinated class, Brenner also identified mutants with aberrant appearance like animals with small bodies, blistered cuticles, twitching muscles, rolling locomotion, long bodies, dumpy bodies, forked heads or bent heads [1].

EMS screens are often used to identify different mutations with the same phenotype to further investigate if those genes function in the same processes. Using this approach, John Sulston and H. Robert Horvitz searched e.g. for mutants that show defects in the differentiation of a vulva from epidermal cells [31]. Molecular follow-up studies revealed that animals that lack a vulva had
mutations in two signaling pathways: the epidermal growth factor (EGF)/RAS pathway and the Notch signaling pathway (reviewed in [32–34]), both having major roles in cell fate determination. These studies in C. elegans have increased the understanding of these molecular pathways involved in oncogenesis in humans (reviewed in [35,36]).

Another possibility to find genes of the same genetic pathways are enhancer or suppressor screens. In this case the mutagenesis occurs on a non-wild type strain whose genetic composition is known and causes a defined phenotype. Like this, one can screen for mutations in this genetic background that enhance or suppress (reverse) that phenotype. With this approach one is able to show that two genes not only act in the same pathway but also their hierarchy which means that one is acting upstream of the other (summarized in [16]). However, one should still keep in mind that it might also be possible that some proteins result in the same phenotype when mutated even though they do not necessarily function in the same pathway.

Although EMS mutagenesis is a powerful tool to generate a high number of mutations and high-throughput screens to identify mutants with a specific phenotype it also has some limitations. It has been estimated that about 30% of the genes in C. elegans can be mutated to a visible phenotype [37] (some mutations might result e.g. in a lethal phenotype as it is the case for a number of developmental genes) and it needs to be mentioned that the identification of the same mutations which indicates a saturation of the screen is no guarantee that some other genes might be missed in this screen. High-throughput screens are only a starting point for further detailed experiments at molecular levels.

2.2. RNA interference

RNA interference was first discovered and investigated in C. elegans and published in 1998 by Andrew Fire et al. [38] (Nobel Prize in Physiology and Medicine in 2006). The discovery of dsRNA-mediated gene silencing has revolutionized genetic studies in C. elegans, as well as in other model organisms. Similar to EMS screens, RNAi screens can be used to identify genes that, when depleted, result in a certain phenotype or enhance or suppress a mutant phenotype.
RNAi in *C. elegans* is systemic, which, to date, is not the case for any other animal models. Therefore it is sufficient to introduce dsRNA into one specific tissue to get RNA silencing also in distant cells because of an amplification process called transitive RNAi [39]. This systemic effect is advantageous for large-scale genome-wide RNAi screens in *C. elegans*.

There are different methods that describe how to silence gene expression in *C. elegans*. The dsRNA can be delivered into the worm by (1) injection into any region [38], (2) feeding with dsRNA-producing bacteria [40], (3) soaking in dsRNA [41] or (4) in vivo production of dsRNA from transgenes under the control of specific promotors [42]. Cell-specific factors seem to regulate thereby the entry and export of dsRNA [39,43,44]. Some cell types (e.g. neurons) seem not to respond well to systemically delivered RNAi [42]. The use of RNAi enhanced mutants (e.g. *eri-1* mutant or mutants of the retinoblastoma pathway that are described to enhance RNAi especially in nervous tissue) might circumvent this problem [45,46]. In addition, Calixto and colleagues generated transgenic animals overexpressing the transmembrane protein SID-1 which is an essential component for systemic RNAi in the neurons. This modification increased the response to dsRNA delivered by feeding. It seemed that the expression of SID-1 in the neurons, on the other hand, decreased the RNAi effect on non-neuronal cells which might be useful for studying the function of essential genes in the neurons. This effect could be even increased when using a *sid-1* mutant background [47]. Durieux and colleagues further used this mutant that is insensitive for systemic RNAi to investigate the knockdown of one of the cytochrome c oxidase-1 subunits in specific tissues by controlling the expression of dsRNA via tissue-specific promotors [48]. The tissue-specific expression of SID-1 (not only in neurons) in a *sid-1* knockout background probably also enables to study tissue-specific effects especially of essential genes.

Especially the possibility to feed animals with dsRNA-producing bacteria enables to perform high-throughput RNAi screens in *C. elegans* (Fig. 2) [49,50]. For efficient induction of RNA interference the choice of the dsRNA-coding region is essential. In *C. elegans*, long dsRNA fragments (more than 100 bp) trigger gene silencing via RNAi. For most genes dsRNA is about 200–1000 nucleotides or even longer and covers exon-regions of the targeted gene. The
fragment should only target one gene. Once the coding region is chosen it can be cloned into a specific vector encoding the production of the specific dsRNA (summarized in [51]). The L4440 vector contains two bacteriophage T7 RNA polymerase promotors flanking the multiple cloning site in which the cDNA of a specific gene has been inserted. The construct can be transformed into *E. coli* strain HT115 (ED3). This strain is deficient for the bacterial RNA polymerase III and its production of bacteriophage T7 polymerase from the construct can be induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). The bacteria are then synthesizing two complementary RNA strands that form a duplex RNA which can mediate RNAi [38].

RNAi libraries are commercially available which includes one library of bacteria clones containing cDNAs of 17,575 genes which represents about 87% of the *C. elegans* genome [49] and one library including clones of 11,800 *C. elegans* genes [52]. Positive scored clones subsequently can be sequenced to confirm that they target the predicted gene. To prevent any further off-target effects of the dsRNA and therefore false-positive results, one should consider generating a second RNAi construct targeting the mRNA of the same gene [52]. Besides the coding regions, the 3′UTR of mRNA might as well be a suitable target as RNA localization elements for the transport of the mRNA or regulation elements of

![Figure 2. High-throughput RNAi screen in *C. elegans*: Age-synchronized animals are transferred to microtiter plates containing different clones of HT115 *E. coli* bacteria. Every clone produces a specific dsRNA which is taken up by the nematodes and induces a knockdown of the corresponding gene. Positive hits in the phenotypic screen are finally confirmed by sequencing the bacterial clone and repeating the specific knockdown in single experiments. Starting point of the RNAi feeding (possible at any developmental stage) and time point of the phenotypic scoring depend on the experiment setup](image)
eukaryotic gene expression are typically located in this region (summarized in [53,54]).

Dissolving adult hemaphrodites with hypochloride (bleach) will yield only fertilized eggs and can be used to age-synchronize the animals for a screen. Gene knockdown by RNAi can be induced at different developmental stages in contrast to EMS mutagenesis, which generates stable mutations that are present at all stages. Thus, the examination of the function of a gene that is transcribed at different developmental stages is possible. This is especially interesting when an active gene is essential at early developmental stages [55]. To investigate the effect of gene depletion by RNAi during embryonic development it is necessary to feed the parental worm with the specific bacterial strain.

Another possibility to study the effect of gene depletion at a certain timepoint was previously described by Calixto and colleagues, by performing a temperature-sensitive conditional knockdown. They could induce the knockdown of a gene by controlling via temperature the expression of RDE-1, a C. elegans argonaut protein which is required for RNA interference. This resulted in active RNAi at 15°C but not at 25°C. Furthermore, they observed that the switching ON and OFF is much faster than transferring animals from RNAi-mediating bacteria to non-RNAi inducing bacteria and vice-versa [56].

The dilution of bacteria containing a specific RNAi construct with non-RNAi mediating bacteria may decrease the efficiency of knocking down a certain gene. In that case of mild RNA interference, lethality effects and other very strong phenotypes are reduced and it might still be possible to study the function of these special genes.

The target of RNAi is known. This is one major difference to EMS mutagenesis that cannot be directed to specific genes. Therefore, besides genome-wide RNAi screens, one can also screen in a smaller subset of candidate genes for example based on previous microarray data, GWAS data, interactome studies, etc. Colaiácovo and colleagues performed for example a RNAi screen to check for germline phenotypes in a subset of genes that were generated by
Genetic screens in Caenorhabditis elegans models for neurodegenerative diseases

a previous microarray analysis of Reinke et al. that was focusing on germline-enriched gene expression [57,58]. In another screen we were looking for genes that, when knocked down, increased the number of alpha-synuclein inclusions in a Parkinson’s disease C. elegans model [59]. This group of 80 genes was then further used for a second RNAi screen in order to find candidates that, when knocked down, induced motility changes in the disease background [60].

RNAi efficiency of bacterial clones in the library can differ. Whereas some dsRNAs induce gene silencing closely to a knockout of a gene, others only generate a mild knockdown. One should always be aware that RNAi is only silencing gene activity and that it is not a full knockout of a gene. It is estimated that about 10–30% of candidates are scored as false negatives as the RNAi is not efficient enough to result in an obvious phenotype. On the other hand the percentage of false positives is relatively low (0.4%) [61]. It is also important to keep in mind that RNA interference is acting at the mRNA level and therefore only influencing the expression of a protein. That means that the stability of a protein is highly influencing the RNAi effect as already generated proteins and their activity are not affected anymore.

Results can also differ from one experiment to the other using the same bacteria clone to silence a specific gene. For example the freshness of the material can be crucial (IPTG, Ampicillin, RNAi construct containing bacteria) [51]. In contrast, a knockout mutant e.g. by EMS mutagenesis has the advantage that it results in a stable genotype. Results of the RNAi screen should therefore be confirmed by single experiments with the candidate genes, preferentially with a gene mutant strain.

A clear phenotype for scoring is mandatory for any successful screen. An obvious easy-to-recognize phenotype as well as automation of scoring facilitates the screening process.

The small size of the animals, the variety of simple phenotypes that are often results of one single gene disruption or silencing, the hermaphroditic reproduction, the homology to higher organisms (see above) and the knowledge of the C. elegans genome, cell-distribution and nematode anatomy
make this animal an optimal model organism to identify the function of genes via any kind of high-throughput screen.

3. From genome to function: what have genetic screens taught us?
One of the advantages of *C. elegans* is that it is amenable to generate “humanized” models of human diseases. For the purpose of this review, we will describe as an example *C. elegans* models of neurodegenerative diseases. Neuropathological hallmarks found in the human brain can be successfully recapitulated in the nematode, such as protein aggregation [62]. Indeed, one of the common features in neurodegenerative diseases is the presence of protein aggregates in the brains of affected patients. These structures originate from protein misfolding and aggregation of so-called “aggregation-prone proteins”. To name a few, these can be the amyloid-beta in Alzheimer’s disease (AD), mutant huntingtin in Huntington’s disease (HD) and alpha-synuclein in Parkinson’s disease (PD) [63]. By mechanisms that are still to be unraveled, these aggregation-prone proteins adopt a distinct conformation, which is thought be a toxic gain-of-function [64,65]. The general understanding is that aggregation (or inclusion formation) renders cellular protection by sequestering misfolded proteins, therefore preventing potentially toxic protein–protein interactions [65,66].

Several nematode models have been generated to recapitulate molecular aspects of diseases, including HD, PD, AD and muscular dystrophy [59,67–72]. Although they do not feature clinical aspects of the disease, they provide the means to understand the molecular mechanisms in these diseases. Genetic screens performed in some of these models represent quick, unbiased methods
Table 2. Summary of genetic screens performed in *C. elegans* models of neurodegenerative diseases.

<table>
<thead>
<tr>
<th>Model</th>
<th>Transgene</th>
<th>Tissue</th>
<th>Genetic screen</th>
<th>Modifier hits</th>
<th>Mammalian ortholog</th>
<th>Cellular process(es)</th>
<th>Transposed to</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PolyQ</td>
<td>Q35-YFP</td>
<td>Body wall muscle</td>
<td>Genome-wide RNAi screen</td>
<td>186 genes</td>
<td>n.a.</td>
<td>RNA synthesis and processing, protein synthesis, folding, transport, degradation, components of the proteasome</td>
<td>n.a.</td>
<td>[83]</td>
</tr>
<tr>
<td>PolyQ</td>
<td>Q35-YFP</td>
<td>Body wall muscle</td>
<td>Genome-wide RNAi screen</td>
<td>88 genes</td>
<td>n.a.</td>
<td>Cell cycle, cell structure, protein transport and energy and metabolism</td>
<td>n.a.</td>
<td>[84]</td>
</tr>
<tr>
<td>PolyQ</td>
<td>Q40-YFP</td>
<td>Body wall muscle</td>
<td>EMS screen</td>
<td>MOAG-4</td>
<td>SERF1A and SERF2</td>
<td>Unknown</td>
<td>Human cell models</td>
<td>[76]</td>
</tr>
<tr>
<td>PD</td>
<td>alpha-</td>
<td>Body wall muscle</td>
<td>Genome-wide RNAi screen</td>
<td>80 genes</td>
<td>n.a.</td>
<td>Vesicle trafficking, lipid metabolism, lifespan</td>
<td>n.a.</td>
<td>[59]</td>
</tr>
<tr>
<td>PD</td>
<td>alpha-</td>
<td>Body wall muscle</td>
<td>RNAi</td>
<td>TDO-2</td>
<td>TDO</td>
<td>Tryptophan metabolism</td>
<td>n.a.</td>
<td>[60]</td>
</tr>
<tr>
<td>PD</td>
<td>alpha-</td>
<td>Body wall muscle and DA neurons</td>
<td>Hypothesis-based RNAi screen</td>
<td>ATGR7</td>
<td>ATG7</td>
<td>Autophagy</td>
<td>Mice</td>
<td>[69, 78]</td>
</tr>
<tr>
<td>PD</td>
<td>alpha-</td>
<td>Whole nervous system</td>
<td>RNAi screen</td>
<td>APA-2; APS-2</td>
<td>n.a.</td>
<td>Vesicular trafficking</td>
<td>n.a.</td>
<td>[70]</td>
</tr>
<tr>
<td>AD</td>
<td>tau</td>
<td>Whole nervous system</td>
<td>Genome-wide RNAi screen</td>
<td>60 genes</td>
<td>38 have human homologs</td>
<td>Kinases and phosphatases; protein folding, stress response and degradation; transcription; proteolysis; neurotransmission and signaling; neuronal regeneration</td>
<td>n.a.</td>
<td>[75]</td>
</tr>
</tbody>
</table>
that have enabled insights into the underlying mechanisms of neurodegeneration. Indeed, many of the disease modifiers discovered in *C. elegans* were found to be reproducible in human cell-based models and other animal models such as mice, strengthening the validity of using this small organism to study complex human diseases, as summarized in Table 2 [69,73–78].

### 3.1. *C. elegans* models for polyglutamine diseases

Polyglutamine diseases comprise a subset of neurodegenerative disorders that include HD, spinocerebellar ataxias (− 1, − 2, − 6, − 7, −17), Machado–Joseph disease (also known as spinocerebellar ataxia 3) and spinobulbar muscular atrophy [79]. The common characteristic of polyglutamine diseases is an abnormal expansion of CAG triplets (which encode glutamine) in the coding region of the disease gene. Although the length of the CAG repeat may vary from individual to individual, the threshold to develop disease is around 40 CAG repeats (except for SCA6), which cause a polyglutamine expansion in the protein that is prone to aggregate. The larger the CAG repeat the earlier onset will occur and the more severe the disease phenotype will be. A more detailed and complete information on polyglutamine diseases is reviewed elsewhere [79]. In *C. elegans*, several different models have successfully recapitulated protein aggregation. Similarly to what occurs in humans, the length of the CAG repeats also determines the aggregation phenotype in *C. elegans*. At least three models have been generated to induce polyglutamine-associated toxicity in neurons by expressing expanded polyglutamine stretches in ASH sensory neurons, touch receptor neurons or the entire nervous system of *C. elegans* [68,80–82]. Polyglutamine aggregation has been modeled in the body wall muscle cells of *C. elegans* [68]. In this model, expanded polyglutamine stretches are fused to a yellow fluorescent protein (YFP) under the *unc-54* promotor, which is specific to the body wall muscle. The aggregation and toxicity phenotype is polyglutamine length-dependent. As the animal ages, the accumulation of protein aggregates increases, which is associated with toxicity [68]. This model has been widely used for genetic screens to discover enhancers and/or suppressors of polyglutamine proteotoxicity. Two genome-wide RNAi screens revealed modifier genes and classified them according to their biological function [83,84]. In the first screen, Q35 animals were fed with
dsRNA-producing bacteria and scored for genes that, when downregulated, provoked premature polyglutamine aggregation [83]. The major functional classes included RNA synthesis and processing, protein synthesis, folding, transport and degradation and components of the proteasome. In the second screen, the authors sought for genes that drive aggregation in Q35 animal and therefore the selection was made for genes that suppressed polyglutamine-induced aggregation when downregulated [84]. With this study, a new subset of modifier genes was recently found to belong to broader biological functions, namely cell cycle, cell structure, protein transport and energy and metabolism [83,84]. Therefore, proteotoxicity is not derived only from protein-related processes but rather a more diverse spectrum of biological functions that also have an effect on protein misfolding and aggregation. Interestingly, nine of these recently identified modifier genes were able to fold misfolded proteins back into the native state when constitutively expressed in misfolding mutants [84].

Forward genetics have also been used to identify modifiers of proteotoxicity. One such screen consisted in treating Q40-expressing worms with EMS. The aim was to find positive regulators of aggregation by selecting genes that suppressed protein aggregation when chemically mutated by EMS. The screen revealed MOAG-4 (modifier of aggregation) as a general aggregation-promoting factor in polyglutamine, Parkinson's and Alzheimer’s disease models [76]. Inactivating MOAG-4 alleviated from polyglutamine-induced aggregation and toxicity; moreover, this effect was functionally conserved in the human orthologs SERF1A and SERF2. A recent follow-up on one of these orthologs showed that SERF1A is a specific aggregation-promoting factor, since it was able to bind specifically to amyloidogenic proteins, including alpha-synuclein, prion protein, amyloid-beta and huntingtin, but not to non-amyloidogenic proteins [85].

Genetic screens have also been used to find regulators of proteotoxicity using the C. elegans neuronal system. An RNAi screen performed in a C. elegans model expressing 128 polyQ stretches in the touch receptor neurons resulted in 662 genes that either enhanced or suppressed neuron toxicity, as measured by loss of touch response [77]. Comparison of these disease modifier genes to gene
expression data in two mouse models of HD showed that there was an overlap of 49 genes that were dysregulated in the striatum of either model, emphasizing the power of using *C. elegans* to find novel regulators of proteotoxicity relevant in human diseases.

### 3.2. *C. elegans* models for Parkinson’s disease

Parkinson’s disease (PD) is the second most common neurodegenerative disease (after Alzheimer’s disease) that affects 1% of the population over the age of 50. Clinically, it is characterized by resting tremors, rigidity, bradykinesia and postural instability [86,87]. The defects in the motor system result from the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc), which project and innervate the neurons in the caudate and putamen. Consequently, there is a reduction of dopamine levels, which is the neurotransmitter that plays a role in the coordination of body movements. Besides motor disabilities, PD patients can experience non-motor symptoms such as autonomic dysfunction, sleep disturbances and neuropsychiatric symptoms [88]. Most cases of PD are sporadic (about 95%) with unknown etiology. It has been suggested that disease can result from the accumulation of toxins (pesticides and heavy metals) over the years. Only 5% of PD has a familial origin and is associated with genetic mutations [88]. However, there are neuropathological hallmarks common to both sporadic and familial forms of PD. These are the loss of dopaminergic neurons in the SNpc, that result from the degeneration of the nigrostriatal pathway which leads to the motor symptoms described earlier as well as the formation of intraneuronal protein aggregates known as Lewy bodies and Lewy neurites in the surviving neurons, which contain alpha-synuclein.

Alpha-synuclein is a small (140 amino acids) natively soluble, monomeric protein that is predominantly expressed in the brain and is enriched in presynaptic terminals [89]. Although the precise function of this protein remains unclear, it is thought to be involved in the regulation of dopamine neurotransmission, vesicular trafficking and modulation of synaptic function and plasticity [90–92]. Three different mutations in the alpha-synuclein gene (A53T, A30P and E46K) cause autosomal-dominant PD [93–95] and genomic duplications and triplications of the gene have also been identified; suggesting
that overproduction of wild type alpha-synuclein is sufficient to cause disease [96,97].

Genetic screens performed with this model have been supporting an important relationship between alpha-synuclein and vesicle transport. The “humanized” model of C. elegans for PD expresses the human alpha-synuclein fused to YFP in the body wall muscle. Phenotypically, immobile YFP-positive foci can be seen in the muscle cells and these foci increase in number and correlate with age-dependent toxicity. An unbiased genome-wide RNAi screen with this model showed 80 modifier genes that, when suppressed, provoked premature alpha-synuclein inclusion formation [59]. A follow-up on those modifier genes revealed tdo-2, a gene involved in tryptophan degradation, as a general regulator of protein homeostasis during aging [60]. Moreover, 49 of the original 80 modifier genes had human homologs, which were enriched for genes related to vesicular trafficking functions. In another screen using a similar model, nematode genes orthologous to human familial PD genes were preselected to perform a hypothesis-based RNAi screen [69]. A subset of candidate genes from the initial screen was then further analyzed in another C. elegans model, expressing alpha-synuclein in the dopamine neurons, in order to assess their relevance at the neuronal level. This study revealed five candidate genes that were able to protect from alpha-synuclein-induced dopaminergic neurodegeneration. Again, the most representative class of genes here was associated with vesicular trafficking, with the exception of the autophagy-related gene Atgr7, of which the mammalian ortholog (Atg7) was previously implicated in neurodegeneration in mice [78]. Also, a serine/threonine kinase involved in axonal elongation, UNC-51, was found to be homologous to the previously associated risk factor ULK-2, as revealed by a genome-wide association study performed in PD patients [98]. Parallel to these findings, Kuwahara et al. were able to pinpoint two genes, apa-2 and aps-2, that when knockdown by RNAi increase alpha-synuclein-induced neurotoxicity in a C. elegans model expressing the transgene in the whole nervous system [70]. These two genes encode for subunits of the AP-2 adaptor complex, which mediates the internalization of cargo into the cell from the extracellular space via clathrin-mediated endocytosis [99].
3.3. *C. elegans* models for Alzheimer’s disease

Alzheimer’s disease (AD) is the most prevalent neurodegenerative disease, which is predicted to affect 66 million people worldwide by 2030 [100]. It represents the most common form of dementia, leading to clinical symptoms such as memory loss and mood swings. Aging and lifestyle are risk factors for development of AD, but 70% of the cases are attributable to genetics [101]. The main neuropathological features are the presence of extracellular amyloid-beta plaques, which consist of an accumulation of aggregated amyloid-beta, and intraneuronal tangles of hyperphosphorylated tau. Mutations in several genes can lead to the development of AD, including mutations in genes encoding for the amyloid-precursor protein (APP), presenelin 1 (PSEN1) and presenelin 2 (PSEN2). These genes are part of the APP cleavage pathway and mutations in these genes promote the processing of APP towards the amyloidogenic pathway, promoting the formation of amyloid-beta. Amyloid-beta peptides can have different lengths, including 40 or 42 amino acids. Amyloid-beta 42 is the most common species found in the amyloid plaques, indicating its propensity to rapidly aggregate in comparison to amyloid-beta 40.

Tau is encoded by the microtubule-associated tau protein (MAPT) gene and predominantly expressed in the nervous system. As to its function, it is known to associate and stabilize microtubules. It has been already classified as one of the risk genes for developing AD by at least two independent studies [102,103].

There are several models in *C. elegans* that express either human amyloid-beta or tau. In the first case, the worms express amyloid-beta 3–42 in the body wall muscles which causes the progressive accumulation of amyloid-beta 3–42 in the muscle cells and paralysis, which worsens with aging [71,104]. There have been several variations to this model, either combined with inducible systems, driving expression in neurons or, more recently, expressing full-length amyloid-beta 1–42 [105–107]. On the other hand, tau-expressing models have been specific to neuronal cells and the phenotype is either worsening of uncoordinated movement or insensitivity to the touch response due to transgene expression [75,108,109].
Genetic screens in Caenorhabditis elegans models for neurodegenerative diseases

Genetic screens in C. elegans models for AD have been scarce. So far, there has been no genetic screen performed in any of the models expressing amyloid-beta. There is only one report on genome-wide RNAi done in a tau-expressing model [75]. Sixty modifier genes were discovered to belong to several functional classes including, kinases, chaperones, proteases and phosphatases. Of these, 38 had homologs in humans but, more importantly, 6 had already been associated with disease, either in humans or other animal models. One of these modifiers was the nicotinic acetylcholine receptor alpha-7 (nAchR), a ligand-gated ion channel expressed in the human brain and known to contribute to tau phosphorylation [110].

4. Final considerations

Genetic screens are powerful means to find genes involved in a certain biological process of interest and their function. The fact that C. elegans is a tractable system to model human diseases further allows one to perform genome-wide screenings in a relatively quick and unbiased manner. Genetic screens can have two outcomes, both being equally informative. On the one hand, new genes are discovered and therefore novel pathways are implicated, giving fresh perspectives on the biological process being studied. On the other hand, genetic screens that reveal genes already known to be associated with disease strengthen the importance of those genes in pathogenesis. Many screens that start with a genome-wide approach end up with an extensive list of candidate genes that are classified according to their functional class. From here, a selection of these genes should be refined and prioritized in order to study further their individual contribution to pathogenesis. One of the critical points is considering those that might have significance at the mammalian level. Additionally, if the human gene can replace the function of the endogenous one, it demonstrates evolutionary conservation of function and enables one to extrapolate findings from small organisms to complex human diseases. It is, therefore, essential to validate the genes from the screen in higher organisms. However, it is also often that a screen might reveal genes that do not have a direct sequence homolog in mammals. Nevertheless, they may be indicators of other genes that may be functional orthologs or otherwise regulators of genes with a role in human disease (e.g. transcription factors).
Another contribution of genetic screens may be to provide novel targets for drug development [111]. Although not all features of a complex human disease are fully recapitulated in the nematode, one can argue that its simplicity can be advantageous. Especially, because analysis of the expression of the causative gene and its interactors or modifiers can be done without other confounding factors inherent to the complexity of the human biology.

Genome-wide association studies (GWAS) have gained importance in the last ten years, becoming one of the forefront strategies to find common genetic factors associated with susceptibility to develop disease. One challenge now is to establish the functional consequences of these genetic variations. Coupling genetic screens or candidate gene approach in *C. elegans* to find causative genes may represent a quick and inexpensive way to assess functional relevance of associated variations and consequently obtain concrete targets to act upon. For instance, a genome-wide toxicity screen in yeast revealed 6 modifiers of amyloid-beta toxicity that were previously identified as risk factors in GWAS [112]. Importantly, those modifiers were functionally conserved from yeast, to *C. elegans* and to rat. Another study by Shulman et al. showed, for the first time, a link between an AD risk factor and a causative gene by functional screening in the fly [113].

Although this review focused in *C. elegans* models for neurodegenerative diseases, it should be noted that *C. elegans* is a model organism for other human diseases as well. *C. elegans* has been used to model certain aspects of cancer, diabetes, obesity, polycystic kidney disease, muscular dystrophy and innate immunity, to name a few. A more complete view of these different disease models is summarized elsewhere [111].

All in all, genetic screens in small organisms such as *C. elegans* can not only aid to dissect fundamental biological questions but also have the versatility of being adapted to model complex human diseases, such as neurodegenerative diseases. Moreover, its attributes make it a tractable system to drug target discovery and compound screening, emphasizing the potential of this organism to extrapolate findings from small organisms to higher vertebrates.
Online links
- Textpresso, a full text literature searches of *C. elegans*  
  (http://www.textpresso.org/)
- Worm Interactome Database  
  (http://interactome.dfci.harvard.edu/C_elegans/index.php)
- The Caenorhabditis Genetic Center, with an extensive list of strains  
  (http://www.cbs.umn.edu/CGC/)
- Wormbase, a complete database of genetics, genomics and biology of  
  *C. elegans* (www.wormbase.org)
- Wormbook, a comprehensive, open-access collection of original, peer-reviewed chapters covering topics related to the biology of *C. elegans* and other nematodes (http://wormbook.org)
- *C. elegans* Gene Knockout Consortium, which creates knockout strains  
  (http://celeganskoconsortium.omrf.org/)
- National Bioresource Project, which generates, collects, stores and distributes deletion mutants of *C. elegans*  
  (http://www.shigen.nig.ac.jp/c.elegans/index.jsp)
- Wormatlas, a database of behavioral and structural anatomy of *C. elegans*  
  (http://www.wormatlas.org)

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Chapter III


Genetic screens in *Caenorhabditis elegans* models for neurodegenerative diseases
CHAPTER IV

Functional sequestration of MOAG-2/LIR-3 by polyglutamine expansion proteins

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Abstract

Aging-related protein aggregation is one of the hallmarks of neurodegenerative disorders such as Alzheimer’s, Parkinson’s and polyglutamine diseases. The cellular processes that drive protein aggregation in these diseases have remained largely unknown. Using a forward genetic screen in a C. elegans model for polyglutamine aggregation, we here identified lin-26-related gene 3 (lir-3) as modifier of protein aggregation (moag-2) that promotes protein aggregation. A mutation or a deletion in moag-2/lir-3 reduced protein aggregation. moag-2/lir-3 encodes a protein of unknown function with a putative nuclear localization domain and two domains that are homologous to nucleotide-binding C2H2 zinc finger domains. By combining chromatin immunoprecipitation sequencing and RNA sequencing, we found that MOAG-2/LIR-3 is preferentially associated with promoter regions of RNA Polymerase III-regulated non-coding RNAs and regulates their expression in wild type N2 nematodes. This regulation is lost in worms that express proteins with expanded polyglutamine tracts. These results suggest that, by driving polyglutamine aggregation, MOAG-2/LIR-3 can no longer execute its normal function thus resulting in a transcriptional change of small non-coding RNAs. Together, our results show that aggregation-prone disease proteins can turn benign cellular proteins into aggregation-promoting factors, at the expense of their biological function. The functional switch can be explored as a therapeutic target to interfere with aggregation events in neurodegenerative diseases.
Introduction
Neurodegenerative diseases like Alzheimer’s, Parkinson’s or polyglutamine diseases are a major health concern and demand a better understanding of the disease pathogenesis in order to make disease-modifying treatments available. One core pathological hallmark of neurodegenerative diseases is the presence of protein aggregates in different brain areas of affected patients [1]. These insoluble, macromolecular structures are particularly enriched in aggregation-prone proteins which, by exposing regions of their amino acid sequence, begin to self-associate or aggregate with other proteins of the cell, thereby hampering normal cellular function [2-4]. Depending on whether these proteins remain soluble and misfolded or acquire amyloidogenic properties they will be localized to different cellular compartments where they can either be degraded and recycled by the ubiquitin-proteasome machinery or form insoluble aggregates [5, 6]. It is not resolved whether these protein aggregates are causing disease. The current view is that soluble oligomeric or fibrillar precursors to these aggregates are cytotoxic and that aggregation is a protective measure to sequester these harmful species [7-10].

The cellular factors that drive protein aggregation are poorly understood. Direct aggregation-promoting factors have been identified [11-14]. SH3GL3 (or endophilin-3) is a SH3-domain GRB2-like 3 protein that was suggested to interact directly with the polyglutamine stretch within exon 1 of the huntingtin gene to promote aggregation [14]. This interaction was proposed to be dependent on the polyglutamine length of mutant huntingtin or its aggregated form [14, 15]. MOAG-4/SERF was identified as a direct modifier of polyglutamine, alpha-synuclein and amyloid-beta-induced aggregation [11, 12]. MOAG-4/SERF is a predominantly disordered protein of unknown function that was shown to drive aggregation by transient binding to aggregation-prone proteins at the early stages of the aggregation pathway [11, 12]. Cell non-autonomous regulation of protein aggregation has been described with the example of unc-30, a homeodomain transcription factor involved in the synthesis of the inhibitory neurotransmitter GABA [16]. Mutation of unc-30 impairs neuronal signaling at the neuromuscular junction and enhances protein aggregation in a C. elegans model for polyglutamine diseases [16].
A wide variety of genetic screens have been performed to find genes that regulate aggregation and aggregation-associated toxicity in animal models for neurodegenerative diseases [12, 17-22]. Here, we describe a forward genetic screen where we sought for genes that normally drive protein aggregation in a C. elegans model for polyglutamine diseases. We identified moag-2/lir-3 as a modifier of aggregation that, when mutated, reduced aggregation up to 51% in a polyglutamine model. We demonstrate that MOAG-2/LIR-3 is a transcriptional regulator that promotes the transcription of small non-coding RNAs (ncRNA), including small nucleolar RNAs (snoRNAs) and transfer RNAs (tRNAs), in wild type nematodes. We propose that MOAG-2/LIR-3 shifts its role in the presence of polyglutamine from a transcriptional regulator of ncRNAs to an aggregation-promoting factor.

Results
Inactivation of MOAG-2/LIR-3 reduces polyglutamine aggregation
To identify genes that drive protein aggregation, we performed a forward genetic screen in a C. elegans model that expresses an aggregation-prone polyglutamine stretch of 40 residues fused to YFP (Q40-YFP) in the body-wall muscle cells [12]. We screened for mutants that reduced polyglutamine aggregation, which we named modifiers of aggregation (moag) ([12] and unpublished data). moag-2(pk2183) (hereafter designated simply as “lir-3(pk2183)”) presented a 51% reduction in the number of aggregates relative to the wild type Q40 animals in their fourth larval stage (Figure 1A, B). SNP-mapping and genome sequencing allowed us to fine-map the causal mutation and revealed six genes as putative candidates for moag-2 (Figure S1B). For one of these, which mapped to lir-3 (lin-26 related; sequence: F37H8.1; accession number: NC_003280), the causative mutation was in the start codon, replacing the first methionine with an isoleucine (Met1Ile) (Figure 1C, S1B). lir-3 encodes a LIN-26-like zinc finger protein of unknown function (http://wormbase.org, March 2014). It is predicted to have two zinc finger domains of the C2H2 type (residues 191-214 and 224-247) at the carboxyl terminus (Figure S1C) and a nuclear localization signal spanning amino acid residues 132 to 141 (http://nls-mapper.iab.keio.ac.jp/, March 2014). LIR-3 shares two non-canonical C2H2 zinc finger motifs with LIN-26 (31 to 35% identical), LIR-1 (20 to 31% identical) and LIR-2 (25% identical) [23].
To assess whether \textit{moag-2} was \textit{lir-3}, we generated a \textit{lir-3}(tm813) deletion mutant (hereafter designated simply as “\textit{lir-3}(tm813)”) and crossed it with the polyglutamine animals. This strain has a 795 bp deletion that spans residues 276 to 1070 of the F37H8.1 sequence that causes a premature stop codon (Figure 1C, S1C). The partial deletion of \textit{lir-3} caused a 35% reduction of aggregates relative to the wild type animals (Figure 1D). Animals heterozygous for the \textit{lir-3} deletion allele exhibited a similar number of aggregates as wild type, supporting that the reduction of aggregation was recessive and due to the loss of function of \textit{lir-3} (Figure 1D). We next asked whether overexpression
of \textit{lir-3} could restore the aggregation phenotype. We injected a rescue construct with full-length \textit{lir-3} including a 1.5 kb sequence upstream of the start codon to include its endogenous promoter as well as 330 bp downstream to include the 3’UTR (Figure 1C). The expression of the \textit{lir-3} rescue fragment in Q40;\textit{lir-3}(tm813) animals was able to restore the aggregation phenotype by 2-fold (p<0.05), therefore confirming \textit{lir-3} as the gene responsible for driving aggregation in our polyglutamine model (Figure 1E). In summary, these results demonstrate that \textit{lir-3} is the \textit{moag-2} gene.

One property of aggregates in the brains of neurodegenerative disease patients, which is also captured by the \textit{C. elegans} polyglutamine model, is that they are typically resistant to strong detergents, such as sodium dodecyl sulfate (SDS) [24-26]. To establish whether MOAG-2/LIR-3 promoted aggregation of SDS-insoluble polyglutamine aggregates, we performed a filter retardation assay on lysates of wild type and \textit{moag-2/lir-3} mutant polyglutamine animals [27, 28]. This assay enables the detection of SDS-insoluble protein aggregates while smaller, soluble species are not captured. Both Q40;\textit{moag-2/lir-3}(pk2183) and Q40;\textit{moag-2/lir-3}(tm813) mutants presented less SDS-insoluble aggregates than their corresponding Q40 controls (Figure 1F). The reduction of SDS-insoluble aggregates was more pronounced in the point mutant (43%; p=0.491) than in the deletion mutant (26%; p=0.1828) (Figure S1D). Mutation or partial deletion of \textit{moag-2/lir-3} did not detectably reduce the transcription or the protein expression level of Q40-YFP, indicating that \textit{moag-2/lir-3} does not reduce aggregation by reducing expression level of the Q40-YFP protein (Figure 1F, G). Together, these results indicate that \textit{moag-2/lir-3} drives the formation of SDS-insoluble aggregates.

\textbf{MOAG-2/LIR-3 is C2H2-domain protein associated with RNA Polymerase III promoters}

Having established that mutation of \textit{moag-2/lir-3} reduces polyglutamine aggregation, we next investigated the endogenous function of the protein. C2H2 zinc finger domains are predominantly associated with DNA-binding transcription factors but may also have other functions such as mediating protein-protein interactions or binding to RNA [29-31]. Bioinformatic analysis combined with manual curation predicted MOAG-2/LIR-3 to be a
Figure 2. **MOAG-2/LIR-3 preferentially binds to promoters of small ncRNAs.** (A) Comparison between the composition of the gene biotypes bound by MOAG-2/LIR-3 (1kb around TSS) and those distributed genome-wide. See also Figure S2A. (B) MOAG-2/LIR-3 binding sites within -1000 and +1000 bp from TSS for protein-coding, ncRNA, tRNA and snoRNA genes. (C) Enriched consensus DNA motifs for MOAG-2/LIR-3 with p value. (D) Number of MOAG-2/LIR-3 binding sites containing Box A and Box B. (E) Heat map showing the binding of different transcription factors to promoters of *C. elegans* protein-coding, snoRNA and tRNA genes. The hierarchical clustering was generated using the average linkage cluster method with a binary metric distance.
transcription factor [32, 33]. To explore this possibility, we took advantage of chromatin immunoprecipitation followed by deep sequencing (ChIP seq) as previously described, using L4-staged animals that express an integrated construct of *lir-3* fused to a GFP tag [34, 35]. This analysis yielded a total of 678 unique MOAG-2/LIR-3 binding sites, of which 404 overlapped with 813 *C. elegans* genes. Further analyses of these genes revealed that MOAG-2/LIR-3 binding was enriched in the transcription start sites (TSS) of tRNA genes (35.7%, p<0.001), snoRNA genes (6.3%, p<0.001), rRNA genes (2.5%, p<0.001) and snRNA genes (2.2%, p<0.002) (Figure 2A, B, S2A). While MOAG-2/LIR-3 was also found in the vicinity of protein-coding and other ncRNA genes, this binding was not significantly enriched (Figure 2A, B).

We then asked whether the binding sites were enriched in any consensus sequence motif that could be recognized by MOAG-2/LIR-3. From the 678 binding sites initially identified in our ChIP seq, more than half of the sites contained Box A and Box B sequence motifs, 301 of which contained both motifs (Figure 2C, D). Box A and Box B constitute the canonical type 2 promoter site recognized by the RNA Polymerase (Pol) III complex [36, 37]. Pol III is responsible for the transcription of structural or catalytic small nuclear RNAs (snRNAs), of tRNAs and of snoRNAs, which mediate chemical modifications of other RNA molecules [37-40].

These findings led us to hypothesize that MOAG-2/LIR-3 could bind to the same target promoters as Pol III. Several *C. elegans* transcription factors have been shown to bind in the proximity of non-coding genes, including PHA-4, PQM-1 and GEI-11 [41]. This prompted us to ask whether the association of MOAG-2/LIR-3 with the RNA Pol III complex resembled the binding of these transcription factors to the promoters of non-coding genes. To answer this question, we collected publicly available ChIP seq data for known transcription factors of *C. elegans* (http://www.modencode.org; September 2014) and analyzed their binding to the promoters of protein-coding genes, snoRNA and tRNA genes (Figure 2E). While there is little association of MOAG-2/LIR-3 with the promoters of protein-coding genes, there is a strong similarity of MOAG-2/LIR-3 binding profile with a group of factors clustered together with representative components of the RNA Pol III complex, including
Pol III, the TATA binding protein (TBP-1), two subunits of the transcription factor for Pol III C (TFC-1 and TFC-4) and the nuclear pore proteins NPP-3 and NPP-13, which were recently shown to associate with the RNA Pol III complex to regulate tRNA and snoRNA splicing [36]. The binding of these factors is very abundant in the promoters of snoRNAs as well as tRNAs (Figure 2E, arrowhead), in contrast to the majority of the other transcription factors. Together, we demonstrated that MOAG-2/LIR-3 binds to the promoters of small ncRNA genes suggesting that MOAG-2/LIR-3 is associated with Pol III transcription.

MOAG-2/LIR-3 is a positive regulator of the RNA Pol III-mediated transcription of small ncRNAs
Because MOAG-2/LIR-3 bound to the promoters of small ncRNA genes, we next asked what consequence this binding would have for the transcription of the Pol III downstream targets. We therefore compared the RNA expression in wild type animals to \textit{moag-2/lir-3} mutant animals by transcriptome profiling. In line with the absence of MOAG-2/LIR-3 at Pol II promoter sites, we did not find any differentially expressed protein-coding genes in the mutants compared to the wild-type N2 animals, thereby excluding MOAG-2/LIR-3 as a transcriptional regulator of protein-coding genes (Figure 3C, S3A). Mutations in \textit{moag-2/lir-3}, however, did result in the downregulation of snRNAs (p<0.001), snoRNAs (p<0.001) and tRNAs (p<0.001) in both mutants, demonstrating that MOAG-2/LIR-3 is required for Pol III-mediated transcription of these small ncRNAs (Figure 3C, S3A). We next asked where MOAG-2/LIR-3 was positioned relative to RNA Pol III complex, by comparing the positions of the ChIP seq signals of MOAG-2/LIR-3 relative to the different components of the RNA Pol III complex. For both the tRNA and snoRNA genes, all factors localized to the Box A and Box B containing promoter region, consistent with previous reports (Figure 3A, B) [36]. MOAG-2/LIR-3 was positioned to the same sites (Figure 3A, B). Together, these results indicate that MOAG-2/LIR-3 functions as a positive regulator of the Pol III-mediated transcription of small ncRNAs in \textit{C. elegans}.
Figure 3. MOAG-2/LIR-3 regulates transcription of small ncRNAs. (A) Boxplot showing the relative expression of different gene biotypes in *lir-3(pk2183)* relative to the wild type N2 background. Coding: protein coding genes; ncRNA: non-coding RNA; Pseudo: pseudogenes; snRNA: small nuclear RNA; snlRNA: snRNA-like RNA, snoRNA: small nucleolar RNA; tRNA: transfer RNA. See also Figure S3A. ***p<0.001 (B) Positions of ChIP seq signal maxima relative to TSS (right y axis) with maximum normalized read count (left y axis) for the 51 snoRNA genes and the 290 tRNA genes picked in this study. Bottom box represents the motif position of Box A and Box B relative to snoRNA and tRNA genes. See also Figure S2B. (C) Diagram showing the positions of the RNA Pol III factors and MOAG-2/LIR-3 estimated from data presented in panel (B).
Regulation of protein aggregation by MOAG-2/LIR-3 is independent of its role as a transcriptional regulator

Next, we asked whether MOAG-2/LIR-3 regulated protein aggregation via the RNA Pol III–mediated transcription of the small ncRNAs. We therefore knocked down by RNAi, one-by-one, the individual components of the RNA Pol III complex in both the wild type and the \textit{moag-2/lir-3} (\textit{pk2183}) mutants. To confirm RNAi knockdown, we also looked for RNAi-associated phenotypes other than aggregation. If Pol III-mediated transcription would play a role, this would result in a reduction in the amount of aggregates in the wild type Q40 animals but not in the \textit{moag-2/lir-3} mutants. As a control, knockdown of Pol II decreased the amount of aggregates in both the wild type and the mutant animals, indicating that a reduction in the expression of coding genes has an effect independent of \textit{moag-2/lir-3} (Figure 4A). Knockdown of Pol III, TBP-1, TFC-1 and TFC4 did not alter aggregation in the Q40 nor in the Q40;\textit{moag-2/lir-3} mutant strains, indicating that reduction of Pol III-mediated transcription in the absence of \textit{moag-2/lir-3} is not responsible for the reduction of aggregation (Figure 4A). Reduction of \textit{npp-13} or of tRNA processing enzymes also did not alter the aggregation phenotype, indicating that neither small RNA processing nor the availability of mature tRNAs plays a role in reduction of aggregation (Figure 4A, B). These results indicate that the regulation of protein aggregation by MOAG-2/LIR-3 is separate from its role in RNA transcription with the RNA Pol III complex.

Polyglutamine expansion proteins suppress transcription of small ncRNAs

We next aimed to address why the role of MOAG-2/LIR-3 in driving protein aggregation was independent of its role as a transcriptional regulator. We therefore first compared the RNA expression profiles of wild type Q40 animals to \textit{moag-2/lir-3} mutant Q40 animals. In contrast to wild type N2 animals, in the presence of polyglutamine there was no longer a change in the relative expression levels of the small ncRNAs (Figure 4C). When we then measured the absolute levels of all RNAs, we found that, in contrast to the protein-coding RNAs, pseudogenes and other ncRNAs, the expression of snRNAs, snoRNAs and tRNAs was already strongly reduced in wild type Q40 animals, indicating that polyglutamine expansion proteins downregulated ncRNA expression...
Figure 4. MOAG-2/LIR-3 drives aggregation by converting into an aggregation-promoting factor. (A) Number of aggregates measured upon RNAi knockdown of individual components of the RNA Pol III complex in Q40 and Q40;lr-3(pk2183) animals. Aggregate counting was performed at young adult stage. As an internal quality control for RNAi, squares indicate penetrance (100% [closed] and 0% [open]) of all associated visible RNAi phenotypes other than aggregation. See also Figure S4A. (B) Number of aggregates measured upon RNAi knockdown of tRNA processing enzymes in Q40 and Q40;lr-3(pk2183) animals. Aggregate counting was performed at L4 stage. In panels A and B, data are represented as mean ± SEM and significance was calculated using two-tailed unpaired t-test. (C-E) Boxplot showing the relative expression of different gene biotypes in (C) Q40 and Q40;lr-3(pk2183); in (D) N2 and Q40 wild type animals (Q40 wild type outcrossed from pk2183); and in (E) lr-3(pk2183) in the N2 and Q40 genetic backgrounds. See also Figure S4C-D. Coding: protein coding genes; ncRNA: non-coding RNA; Pseudo: pseudogenes; snRNA: small nuclear RNA; snRNA-like RNA, snoRNA: small nucleolar RNA; tRNA: transfer RNA.*p<0.05; **p<0.01; ***p<0.001, ns is not significant. In all panels, the average of three biological replicates is represented. (F) Working hypothesis for the role of MOAG-2/LIR-3 as an aggregation-promoting factor. In wild type animals, MOAG-2/LIR-3 normally regulates the transcription of small ncRNAs, namely snRNAs, snoRNAs and tRNAs. However, when polyglutamine expansion proteins are expressed, MOAG-2/LIR-3 switches its function from transcriptional regulator to an aggregation-promoting factor, thereby driving polyglutamine aggregation.
Functional sequestration of MOAG-2/LIR-3 by polyglutamine expansion proteins

(p<0.001; Figure 4D). We observed this reduction in expression to a lesser extent in the *moag-2/lir-3* mutant Q40 animals compared to the mutant N2 animals, suggesting that part of the downregulation in the wild type animals was caused by polyglutamine inhibition of MOAG-2/LIR-3 (p<0.001; Figure 4E). Indeed, this finding explained why mutations in *moag-2/lir-3* could no longer reduce the expression in these Q40 animals (Figure 4C, S4C).

Altogether, these data suggest a switch of function of MOAG-2/LIR-3 in presence of polyglutamine expansion proteins from its biological role as a transcriptional regulator to a pathological role as an aggregation promoter at the expense of its own function (Figure 4F).

**Discussion**

Aggregation-prone disease proteins have been found to cause age-related neurodegenerative diseases but the cellular processes that drive their aggregation and toxicity are still not fully understood. Using a genetic screen in a *C. elegans* model for disease, we here identified MOAG-2/LIR-3 as regulator of RNA Pol III transcription that — in presence of polyglutamine expansion proteins — turns into a positive regulator of polyglutamine aggregation.

The role of MOAG-2/LIR-3 as a transcription factor has been previously suggested by others [23, 32, 33]. Its initial discovery as a putative transcription factor was due to its structural similarity to the C2H2 zinc fingers of LIN-26, a fate regulator responsible for the differentiation of non-neuronal ectodermal cells and somatic gonad epithelium [23, 42, 43]. By combining ChIP seq with RNA sequencing, we provide the first experimental evidence of MOAG-2/LIR-3 being a regulator of transcription.

In *C. elegans*, several transcription factors have been shown to bind to more than 10% to the promoters of small ncRNAs, but only one (GEI-11) is predicted to regulate ncRNAs [41]. We showed that MOAG-2/LIR-3 is required for the transcription of snRNA, snoRNA and tRNA genes and — unlike the majority of the other transcription factors — MOAG-2/LIR-3 bound to the same target genes as the RNA Pol III complex.
MOAG-2/LIR-3 expression in *C. elegans* has been described to localize to the nucleus of body wall muscle cells, the vulval muscles, the spermatheca, the head and tail ganglia and the ventral nerve cord, from embryogenesis throughout adulthood (www.transgeneome.mpi-cbg.de, March 2014 and [32, 33]). A microarray performed in two distinct mechanosensory neurons — the touch receptor neurons and the FLP neurons — revealed that *moag-2/lir-3* was upregulated in the FLP sensory neurons, suggesting that *moag-2/lir-3* is required for FLP differentiation [44]. Because the expression of MOAG-2/LIR-3 is limited to a subset of cell types, this suggests that MOAG-2/LIR-3 could be a tissue-specific regulator of transcription rather than a core component of the RNA Pol III machinery. Such cell type specific regulators of RNA Pol III have been described for human cells and proposed to accommodate tissue-specific needs for small non-coding RNAs ([45, 46], also reviewed in [39, 47]). Whether MOAG-3/LIR-3 has a similar role remains to be established.

In *C. elegans*, the nuclear pore protein NPP-13 has been described to associate with the RNA Pol III complex, which regulates the efficient processing of snoRNA and tRNA transcripts [36]. Knockdown of NPP-13 results in abnormally long snoRNA and tRNA transcripts that cannot be processed into their mature form [36]. We did not find unprocessed transcripts neither for snoRNA nor tRNAs in *moag-2/lir-3* mutant animals (data not shown), which excludes the possibility that MOAG-2/LIR-3 is required for RNA Pol III transcript processing. Moreover, knockdown of NPP-13 did not alter aggregation, which suggests that mutations in *moag-2/lir-3* do not alter aggregation by interfering with the nuclear pore complex.

In this study, expression of an aggregation-prone protein downregulated the levels of snRNAs, snoRNAs and tRNAs, demonstrating that aggregation-prone proteins also affect the non-coding genome. One explanation for this downregulation is that the aggregation-prone proteins sequestered or changed the localization of MOAG-2/LIR-3, and perhaps also components of the RNA Pol III complex. Several intrinsically disordered proteins, which include proteins involved in transcriptional regulation, are known to be sequestered by aggregation-prone proteins into aggregates [3, 48-51]. One example
is Sp1, which can no longer bind to its DNA targets due to sequestration by mutant huntingtin [52, 53]. Because MOAG-2/LIR-3 is a small (284 aa), predominantly disordered protein (75.5%) (http://bip.weizmann.ac.il/fldbin/findex, June 2015), its sequestration could explain the lower levels of small ncRNAs observed in presence of aggregation-prone proteins. Nevertheless, the fact that aggregation-prone proteins affect small ncRNA expression poses the interesting question whether this is correlated their toxicity. It will also be interesting to find whether the cellular downregulation of small ncRNAs is a consequence specific for polyglutamine expansion proteins or shared by other aggregation-prone proteins such as alpha-synuclein and amyloid-beta.

Individual knockdown of the components of the RNA Pol III complex did not affect aggregation in Q40 animals, indicating that RNA Pol III transcription is not related to the effect of moag-2/lir-3 mutations on protein aggregation. Impaired processing of precursor tRNA into its mature form or mutations in the tRNA body sequence have been implicated in neurodegeneration [54-59]. In C. elegans, mutations in the tRNA body sequence can result in protein misfolding, protein aggregation and neurological defects [60, 61]. We found, however, that knockdown of tRNA processing enzymes as well as factors known to cooperate with those enzymes (rtcb-1, clpf-1 and gtbp2 homolog) did not alter the aggregation phenotype in Q40 animals, also indicating that impaired tRNA processing is not the cause for protein aggregation in our model. The glutaminyl tRNA that specifically recognizes the CAG codon is required in high demand during translation of the expanded CAG tract in huntingtin, eventually resulting in its depletion [62]. According to our data, there is no evidence for a preferential enrichment or depletion of glutaminyl pre-tRNAs in Q40 animals (data not shown). In addition, while we cannot account for mature tRNAs present in the body wall muscle cells, which would require detection in a tissue-specific manner, the polyglutamine expression levels are not decreased in the moag-2/lir-3 mutant animals, reducing the possibility that the availability of glutaminyl tRNAs plays a role.

Several transcription factors have been implicated in protein aggregation, but none with known function related to RNA Pol III transcription [53, 63]. Our results indicate that MOAG-2/LIR-3 regulated protein aggregation independently of its
role in small ncRNA transcription and processing, as shown by RNAi knockdown experiments. One possibility is that MOAG-2/LIR-3 drives aggregation directly. Precedents for aggregation-promoting factors include SERF1A, which can drive aggregation of a variety of disease proteins via a transient direct interaction with early aggregation intermediates and SH3GL3, which was shown to bind physically to the huntingtin protein or its mutated form through the SH3 domain [11, 12, 14, 15].

Components of the RNA Pol II complex have been reported to be part of protein aggregates. Such an example comes from TBP, which was localized to nuclear inclusions of SCA-1,-2,-3 and neurofibrillar tangles of Alzheimer’s disease [64-66]. A regulatory role for TBP in protein aggregation, as we observe here for MOAG-2/LIR-3, however, has not been reported.

In summary, this work reveals that protein aggregation can affect the non-coding genome by altering the expression of ncRNA genes available in the cell. Secondly, this work opens yet another perspective on how aggregation-prone disease proteins can impair cellular homeostasis by inhibiting transcription of non-coding RNAs and converting normal cellular proteins into aggregation-promoting factors at the expense of their function.

**Methods**

**Strains and genetics**

Standard methods were used for culturing *C. elegans* at 20°C [67]. The LIR-3::GFP strain (OP312) was generated by biolistic transformation to produce an integrated, low-copy transgene of the WRM0637aB05 fosmid, recombineered with GFP:3XFLAG in frame at the carboxy terminus of the *lir-3* locus [34]. To synchronize animals, eggs were collected from gravid hermaphrodites by hypochlorite bleaching and hatched overnight in M9 buffer. The desired amount of L1 animals were subsequently cultured on nematode growth medium agar plates (#633185, Greiner Bio-One) seeded with OP50 bacteria. The following strains were used or generated: wild type N2 (Bristol), AM141 *rls133[P(unc-54)Q40::YFP]X* [68], OP312 *wgls312[P(lir-3)::TY1::EGFP::3xFLAG + unc-119(+)]* [35, 69], OW1002 *lir-3(tm0813)I*, OW1003 *rls133[P(unc-54)Q40::YFP]X;lir-3(tm813)II*, OW1004 *rls133[P(unc-54)Q40::YFP]X*, OW1019...
moag-2 (pk2183), OW1020 rmls133[P(unc-54)Q40::YFP]X; moag-2(pk2183)II, OW1021 rmls133[P(unc-54)Q40::YFP]X, OW1086 rmls133[P(unc-54)::Q40::YFP]X, OW1087 rmls133[P(unc-54)::Q40::YFP]X; zgEx221[P(myo-3)::CFP + P(lir-3)::lir-3]; lir-3(tm813)II, OW1090 rmls133[P(unc-54)::Q40::YFP]X, OW1091 rmls133[P(unc-54)::Q40::YFP]X; zgEx226[P(myo-3)::CFP + P(lir-3)::lir-3]; lir-3(tm813)II, OW1100 rmls133[P(unc-54)::Q40::YFP]X; wgls312[P(lir-3)::TY1::EGFP::3xFLAG + unc-119(+)].

EMS Mutagenesis and Mapping
Mutagenesis was performed using standard *C. elegans* ethyl methanesulfonate (EMS) methodology [70]. Eight thousand mutagenized genomes were screened for suppressors of aggregation. *moag-2*(pk2183) was identified by single-nucleotide polymorphism mapping to a region between base 9,400,743 and 11,827,697 on linkage group II [71]. Next generation sequencing was performed in that region to identify candidate genes for *moag-2*. CLC Bio (Qiagen, http://www.clcbio.com) and MAQGene softwares were utilized for mapping the mutation in F37H8.1 [72].

Creation of transgenic strains
For the rescue experiment, a genomic construct of *lir-3* spanning 1500bp upstream to 330bp downstream of F37H8.1 was amplified from N2 genomic DNA by nested PCR using primers F1: CGCTCACAGTCAACGTCG; R1: CCATGCGATTTGACACATTTCG; F2: CGGCATTGCTCTTGTCGTGC and R2: GCATCTCATGAAACCAGACGC. The resulting PCR fragment was cloned into the pGEM-T Easy Vector (Promega) and sequenced. Transgenic lines were made by injecting ~20 ng/ul of construct along with ~10 ng/ul of pPD136.61 [P(unc-54::CFP)] in N2 animals.

RNAi experiments
RNAi experiments were performed on NGM agar plates containing 1 mM IPTG and 50 mg/ml ampicillin that were seeded with RNAi bacteria induced with IPTG to produce dsRNA. Animals were synchronized by hypochlorite bleaching and L1 animals were grown on RNAi plates (#628103, Greiner Bio-One) and used for the experiments at L4 stage, unless stated otherwise.
Quantification of aggregates
The number of aggregates present in whole animals was counted using a fluorescence dissection microscope (Leica Microsystems, Wetzlar, Germany). A minimum of 20 animals was counted in three biological replicates, unless stated otherwise.

Filter retardation assay
The protocol was adapted from Wanker et al [27, 28]. Briefly, crude worm lysates from synchronized L4 animals were resuspended in FTA Sample Buffer (10 mM Tris-Cl pH 8.0; 150 mM NaCl; 2% SDS) and disrupted using a bead-beater (FastPrep 24, MP Biomedicals) for 7 cycles of 20 seconds bead beating/5 minutes rest per cycle. Supernatants were transferred to new 1.5 ml tubes and protein concentration was determined using the BCA Protein Assay Kit (#23227; Thermo Scientific Pierce). To detect SDS-insoluble aggregates, 100 ug of total protein was mixed with 1 M DTT and FTA Sample buffer (final concentration 40 ug/100 ul) and heated for 98ºC for 5 minutes. Samples were filtered through a 0.22 micron cellulose acetate membrane using a Bio-Dot microfiltration apparatus (Biorad) and 100 ug of total protein was used followed by five-fold serial dilutions. Proteins were blocked for 30 minutes with 5% milk in TBS-T. Membranes were incubated with GFP (#632381, Clontech) or α-tubulin (#T6074-200UL, Sigma) primary antibodies at a 1:5000 dilution overnight at 4ºC. Incubation with secondary antibody anti-mouse was applied at a 1:10.000 dilution for 1 hour at room temperature. Antibody binding was visualized with an ECL kit (#RPN2232, Amersham).

Chromatin immunoprecipitation sequencing
ChIP assays were conducted as previously described [35, 41]. Worm staging was achieved by bleaching and L1 starvation. Arrested L1 animals were plated on peptone-enriched NGM plates seeded with OP50 bacteria and grown for 48 hours for L4 collection at 20ºC. Samples were crosslinked with 2% formaldehyde for 30 minutes at room temperature and then quenched with 1 M Tris pH 7.5. The pelleted worms were subsequently flash frozen in liquid nitrogen and stored at -80ºC. Samples were sonicated using a microtip to obtain mostly 200 to 800 bp DNA fragments. For each sample, 2.2 or 4.4 mg of cell
extract was immunoprecipitated using a goat anti-GFP, GoatV (gift from Kevin White). The enriched DNA fragments and input control (genomic DNA from the same sample) for two biological replicates were used for library preparation and sequencing as previously described [73]. Briefly, samples were libraried and multiplexed using the Ovation Ultralow DR Multiplex Systems 1-8 and 9-16 (NuGEN Technologies Inc., San Carlos, CA) following the manufacturer’s protocol except Qiagen MinElute PCR purification kits were used to isolate the DNA. Library size selection in the 200-800 bp range was achieved using the SPRselect reagent kit (Beckman Coulter, Inc., Brea, CA) and sequencing was performed on the Illumina HiSeq 2000 platform. To search for MOAG-2/LIR-3 specific binding sites, only binding sites consistent across both replicates and within a range of -400 bp to +100 bp distance from TSS were considered. In addition, we excluded highly occupied target regions to avoid false positives in our analysis [74]. The ChIP seq data used was obtained from modENCODE DCC (http://www.modencode.org; September 2014). All ChIP-seq data have been deposited in Gene Expression Omnibus (GEO), under accession number (in preparation).

**RNA sequencing**

Worms were grown to L4 stage and total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer’s description. For polyA RNA sequencing, the TruSeq Sample Preparation V2 Kit was used (Illumina). For non-coding RNA sequencing (mRNA, snoRNA, tRNA and large ncRNA), a protocol has been described previously [36]. Briefly, total RNA was treated with DNAsel and depleted from rRNA with the Ribominus Eukaryote Kit (#A10837-08, Invitrogen). Fragmentation of RNA was performed using a Fragmentation Buffer (#AM8740, Ambion) and cDNA was generated using the Superscript II Kit (Invitrogen). cDNA libraries were subjected to high-throughput single-end sequencing (50 bp) in an Illumina HiSeq 2500 instrument. The RNA sequencing dataset has been submitted to GEO, under accession number (in preparation). RNA-sequencing data was mapped to WS220 genome reference using TopHat 2.0.9 program [75] and gene annotation from Ensembl release 66. Per gene expression data was normalized as fragments per million mapped (FPM). Data visualization and statistical tests were conducted using R scripts.
Chapter IV

**Bioinformatic Analysis**
Conserved domains were identified using SMART (Simple Modular Architecture Research Tool) [76, 77]. Prediction of nuclear signal localization was done with NLS Mapper [78]. Prediction of protein folding was done with FoldIndex [79]. The algorithm used for motif discovery was The MEME Suite [80]. Orthologs were identified using protein BLAST search and aligned with T-Coffee multiple sequence alignment tool [81]. Amino acid predictions were performed using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi).

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**Author Contributions**
O.S. and E.A.A.N. designed the research; O.S., M.K., R.S., A.M.C., C.M., A. Z., H.H.W. and W.H. performed the research; O.S., T.v.d.J., R.M.P., V.G. analyzed the data; and O.S. and E.A.A.N. wrote the manuscript.
References


45. Alla RK, Cairns BR (2014) RNA polymerase III transcriptomes in human embryonic stem cells and induced pluripotent stem cells, and relationships with pluripotency transcription
Functional sequestration of MOAG-2/LIR-3 by polyglutamine expansion proteins


Supplemental figures

Figure S1. (A) Representative bright field images of L4-staged Q40 and Q40; lir-3(pk2183) animals. Insets show a magnification of the vulva. Scale bar, 75 μm (B) List of candidate genes for moag-2 obtained by whole genome sequencing. (C) Amino acid sequence of LIR-3 with the predicted nuclear localization signal (NLS, residues 132-141) and C2H2 zinc finger domains (residues 191-214 and 224-247). Grey shadow indicates location of the point mutation and the deleted residues in the lir-3(tm813) mutant. (D) Quantification of filter retardation assays. Immunoblots were quantified by densitometry using ImageJ. Presented is the ratio (fold change) between protein levels of Q40; moag-2/lir-3 mutants relative to their corresponding wild types (corrected to α-tubulin as a loading control). Filter retardation assay was performed at the L4 stage and represented is the average of three biological replicates. Data are represented as mean ± SEM and significance was calculated using one-tailed unpaired t-test. *p<0.05

Figure S2. (A) Number of MOAG-2/LIR-3 binding sites per gene biotype and total number of known C. elegans genes. p value was calculated by permutation test. (B) Number of MOAG-2/LIR-3 binding sites containing Box A and Box B in snoRNA and tRNA genes. (C) Number of snoRNA genes encoded by RNA Pol II or III genome-wide and detected in this study.
Chapter IV

Figure S3. Boxplot showing the relative expression of different gene biotypes in \textit{lir-3(tm813)} relative to the wild type N2 background. Coding: protein coding genes; ncRNA: non-coding RNA; Pseudo: pseudogenes; snRNA: small nuclear RNA; snlRNA: snRNA-like RNA, snoRNA: small nucleolar RNA; tRNA: transfer RNA. ***p<0.001 significance was calculated using two-tailed unpaired t-test.

Figure S4. (A) Number of aggregates measured upon RNAi knockdown of individual components of the RNA Pol III complex in Q40 and Q40; \textit{lir-3(tm813)} animals. Aggregate counting was performed in young adult stage. Represented is the average of four biological replicates. (B) Number of aggregates measured upon knockdown of tRNA synthetases in Q40 and Q40; \textit{lir-3(pk2183)} animals. Asterisk indicates genes previously known to reduce protein aggregation. In panels A and B, an internal quality control for RNAi was performed and squares indicate penetrance (100% [closed] and 0% [open]) of all associated visible RNAi phenotypes other than aggregation. Data are represented as mean ± SEM and significance was calculated using two-tailed unpaired t-test. (C-E) Boxplot showing the relative expression of different gene biotypes in (C) \textit{lir-3(tm813)} relative to its wild type in the Q40 background; in (D) in the N2 and Q40 wild type animals (Q40 control outcrossed from \textit{lir-3(tm813)}); and in (E) \textit{lir-3(tm813)} in the N2 and Q40 genetic backgrounds. Coding: protein coding genes; ncRNA: non-coding RNA; Pseudo: pseudogenes; snRNA: small nuclear RNA; snlRNA: snRNA-like RNA, snoRNA: small nucleolar RNA; tRNA: transfer RNA. *p<0.05; **p<0.01; ***p<0.001; ns is not significant. In panels B to E, the average of three biological replicates is represented.
Table S1. Overview and statistical analysis of aggregate counting experiments.

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ns - not significant
Table S2. List of RNAi foods used in knockdown experiments.

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<th>Gene</th>
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<td>L4440</td>
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<td>Empty vector</td>
</tr>
<tr>
<td>F36A4.7</td>
<td>ama-1</td>
<td>Large subunit of RNA Polymerase II</td>
</tr>
<tr>
<td>C42D4.8</td>
<td>rpc-1</td>
<td>Ortholog of the human RNA Polymerase III</td>
</tr>
<tr>
<td>T20B12.2</td>
<td>tbp-1</td>
<td>Ortholog of the human TATA-box-binding protein (TBP)</td>
</tr>
<tr>
<td>T02C12.3</td>
<td>tfc-5</td>
<td>Ortholog of the human general transcription factor IIIC (TFC-1)</td>
</tr>
<tr>
<td>Y37E3.15</td>
<td>npp-3</td>
<td>Nucleoporin required for normal nuclear pore complex distribution in the nuclear envelope; also described as being part of the RNA Polymerase III complex</td>
</tr>
<tr>
<td>E04A4.4</td>
<td>hoe-1</td>
<td>RNAse Z</td>
</tr>
<tr>
<td>F55B12.4</td>
<td>hpo-31</td>
<td>Predicted to have tRNA nucleotidyl transferase activity</td>
</tr>
<tr>
<td>T06D8.9</td>
<td>-</td>
<td>43% homologous to the human TSEN15 subunit of the tRNA splicing endonuclease</td>
</tr>
<tr>
<td>F52C12.3</td>
<td>-</td>
<td>31% homologous to the human TSEN54 subunit of the tRNA splicing endonuclease</td>
</tr>
<tr>
<td>F16A11.2</td>
<td>rtcb-1</td>
<td>Ortholog of the human tRNA splicing ligase (RTCB)</td>
</tr>
<tr>
<td>C49H3.10</td>
<td>xpo-3</td>
<td>Ortholog of the human importin beta 1</td>
</tr>
<tr>
<td>W06H3.2</td>
<td>-</td>
<td>Putative mitochondrial tRNA synthetase</td>
</tr>
<tr>
<td>F29A2.4</td>
<td>clpf-1</td>
<td>48% homologous to mammalian RNA kinase protein, recently shown to associate with TSEN and drive tRNA splicing</td>
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<td>-</td>
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<td>Y23H5A.7</td>
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<td>Cysteinyl amino-acyl tRNA synthetase</td>
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<td>T08B2.9</td>
<td>frs-1</td>
<td>Phenylalanyl amino-acyl tRNA synthetase</td>
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<td>nrs-1</td>
<td>Asparaginyl amino-acyl tRNA synthetase</td>
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<td>krs-1</td>
<td>Lysyl amino-acyl tRNA synthetase</td>
</tr>
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<td>aars-1</td>
<td>Alanyl amino-acyl tRNA synthetase</td>
</tr>
<tr>
<td>C47D12.6</td>
<td>trs-1</td>
<td>Threonyl amino-acyl tRNA synthetase</td>
</tr>
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<td>C34E10.4</td>
<td>wrs-2</td>
<td>Tryptophanyl amino-acyl tRNA synthetase</td>
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<td>F26F4.10</td>
<td>rrt-1</td>
<td>Arginyl amino-acyl tRNA synthetase</td>
</tr>
<tr>
<td>R74.1</td>
<td>lrs-1</td>
<td>Leucyl amino-acyl tRNA Synthetase</td>
</tr>
<tr>
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<td>dars-1</td>
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<td>Y41E3.4</td>
<td>qars-1</td>
<td>Glutaminyl amino-acyl tRNA synthetase</td>
</tr>
</tbody>
</table>
CHAPTER V

Transcriptional profiling in *C. elegans* reveals developmental and cellular responses to polyglutamine expansion proteins

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*equal contribution

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Work in progress
Abstract
Protein aggregation is a hallmark of neurodegenerative diseases such as Alzheimer’s, Parkinson’s and polyglutamine diseases. Polyglutamine diseases are characterized by the abnormal expansion of CAG repeats in the disease-causing gene. Consequently, when the gene is translated into a protein, the protein is unable to acquire its native conformation, so that it becomes misfolded and aggregation-prone. The aggregation-prone protein interacts aberrantly with other normal, functioning proteins in the cell, ultimately disrupting protein homeostasis. While most research into polyglutamine diseases has focused on the changes that take place after the aggregation-prone protein has been translated, less is known about changes at the transcriptional level. We therefore sought to identify the transcriptional changes that occur when a cell is exposed to aggregation-prone proteins such as polyglutamine expansion proteins. We took advantage of a C. elegans model for polyglutamine diseases – which recapitulates the protein misfolding and aggregation observed in human disease – and performed whole transcriptome analysis at two different stages of larval development in both wild type (N2) and polyglutamine-expressing animals.

Principal component analysis revealed that the major sources of expression variation were related to stage of development (L2/L3 versus L4) followed by the genetic background (wild type N2 versus the polyglutamine model). RNA sequencing-derived data was analyzed by combining standard differential expression analysis with gene ontology enrichment analysis to identify those genes whose expression was affected by aggregation-prone proteins. The genes thus identified were found to be related to the unfolded protein response, oxidative stress and immune response. We also found that aggregation-prone proteins slow down the rate of animal development. We propose that this combination of events may represent a coping strategy that the organism adopts to preserve its fitness as a response to the pathological presence of aggregation-prone proteins.
Introduction

Polyglutamine diseases are a class of neurodegenerative disorders that include Huntington’s disease, spinocerebellar ataxia (types 1, 2, 6, 7, 17), Machado–Joseph disease and spinobulbar muscular atrophy [1, 2]. Due to a loss of neurons, affected patients commonly experience motor dysfunction as well as cognitive and behavioral abnormalities [3]. These diseases are characterized by an abnormal expansion of CAG repeats in the disease-causing genes. The resulting protein has an unusually long stretch of glutamines, which causes it to become misfolded and prone to aggregation, ultimately contributing to protein aggregation [4]. Despite our knowledge of the underlying causes of polyglutamine diseases, we are not yet able to explain why protein aggregation occurs in affected patients. If we can understand how the cell copes with protein aggregation, we can identify and characterize the cellular pathways implicated in pathogenesis and target these for disease-modifying treatments.

Polyglutamine aggregation has been recapitulated in several models in the nematode *Caenorhabditis elegans* [5-8]. In one of those models, an expanded polyglutamine stretch containing 40 CAG repeats is expressed in the body wall muscle cells, whereby the formation of aggregates is proteotoxic and progresses with age [6]. Here we used this model to find answers to the following questions: What transcriptional changes occur in the cell upon expression of polyglutamine expansion proteins? Which pathways are activated? Does this change during the course of worm development? To this end, we performed a global integrative analysis of the transcriptome of a *C. elegans* model of polyglutamine diseases, by employing principal component analysis and differential gene expression analysis. We aimed to identify the pathways that are activated upon pathological overexpression of polyglutamine expansion proteins in order to gain insight into the relevant cellular responses.
Chapter V

Results
Developmental stage and genetic background account for differences in transcriptome profiles

In our analysis of the transcriptome of polyglutamine-expressing (Q40) animals, we selected larval stages two/three (L2/L3) and four (L4), in order to examine transcriptome changes during development. To search for the main sources of variance in our transcriptome profiling data, we employed principal component analysis (PCA). The first principal component yielded a clear distinction between L2/L3 and L4 animals, showing that the developmental stage had a strong effect in our expression data (Figure 1A, C). The following principal component explained the variance between our datasets based on the presence of the transgene (Figure 1B, C). Indeed, while the profile of polyglutamine-expressing animals was very similar to that of wild type N2 animals at the L2/L3 stage, these differences were more pronounced at developmental stage L4 (Figure 1A-C). These results are consistent with the observation that – under our laboratory conditions – expression of polyglutamine expansion proteins slows down worm development (Figure 1D). We performed enrichment analysis based on gene ontology (GO) using the web-based toolset gProfiler (http://biit.cs.ut.ee/gprofiler/, Ensembl 79). This enabled us to learn more about the gene set comprising the first principal component, since this component explained most of the variance of the data (18.7%). GO term analysis of the first principal component revealed that the genes in the first principal component were related to developmental processes (Figure 1E). In all, our transcriptomic profiling data demonstrated that expression variation was determined firstly by developmental stage and secondly by genetic background.

Polyglutamine expansion proteins trigger the ER-associated unfolded protein response

We next used differential gene expression analysis to look for genes that were significantly up- or downregulated due to the presence of aggregation-prone proteins. A false-discovery rate (FDR) cutoff of 0.1% was used to identify such differentially expressed genes. First, we excluded genes differentially expressed between the L2/L3 and L4 stages in the N2 genetic background, thus avoiding developmental effects (and therefore false positives) that might
Transcriptional profiling in *C. elegans* reveals developmental and cellular responses to polyglutamine expansion proteins.

**Figure 1. Effect of aggregation-prone proteins on the *C. elegans* transcriptome.** (A, B) PCA plots of the 23 samples included in this study. Different colors indicate the different time points (L2/L3 or L4) and genetic backgrounds (Q40 or N2). PC, principal component. (C) Heat map showing the distribution of the 23 samples included in this study. The hierarchical clustering was generated using the average linkage cluster method with a Euclidean metric distance. (D) Approximate number of hours from egg hatching until L1 and between successive developmental stages until adulthood in *C. elegans*. (E) Gene ontology (GO) term enrichment analysis for gene set of the first principal component. (F) Results of differential gene expression analysis as measured by the fold change in expression between raw reads of wild type animals in L2/L3 and L4 stage (x axis) and N2 and Q40 animals in L4 stage (y axis). (G) GO term enrichment analysis for genes differentially expressed in the presence of aggregation-prone proteins (blue data points from panel (F)), and the number of genes associated with each GO term. (H) GO term enrichment analysis for genes differentially expressed in the presence of aggregation-prone proteins (green data points from panel (F)), and the number of genes associated with each term.
mask the contribution of the polyglutamine expansion proteins to the cell’s transcriptome (Figure 1F, red). Then, to determine the specific contribution of polyglutamine expansion proteins to the cell’s transcriptome, we looked for genes that were differentially expressed between the Q40 and N2 animals at the L4 stage (Figure 1F, blue). GO term enrichment analysis of this cluster identified genes involved in three types of cellular functions, namely the unfolded protein response (UPR, 14 genes), the pathogen response (35 genes), and the redox process (54 genes) (Figure 1G). Further inspection of the genes involved in the UPR pathway revealed significant downregulation of the *abu* and *pqn* family of genes (Table 1). The *abu* (activated in blocked UPR) family of genes is expressed when the UPR is blocked and it has been suggested that their protein products may protect the cell against aberrantly folded proteins [9]. Both the *pqn* and *abu* genes encode proteins whose amino acid sequences contain glutamine/asparagine-rich (Q/N) domains, a common feature of prion-like domains [10, 11]. This hints at the possibility that polyglutamine expansion proteins trigger the UPR to cope with misfolded proteins in the cell, thereby downregulating the *abu/pqn* genes since they are not necessary.

Parallel to this, in Q40 animals we detected upregulation of the *cdr-4, gad-3* and *C14B9.2* genes (Table 1), all of which are regulated by *xbp-1*, a transcription factor required for the activation of the UPR [9, 12, 13]. While not much is known about *cdr-4* and *gad-3*, *C14B9.2* is known to encode a disulfide isomerase. Protein disulfide isomerases are important for the formation of native disulfide bonds in proteins transiting through the ER, a process known as oxidative protein folding (reviewed in [14]). We observed no activation of other canonical UPR components, such as the worm homologs of BiP/GRP78 (*hsp-3* and *hsp-4*), an ER-associated molecular chaperone [15-17]. Overall, this set of differentially expressed genes is consistent with the cellular response pathways known to be triggered by misfolded, aggregation-prone proteins in the ER [18].

Curiously, GO term enrichment analysis also identified a cluster of 35 genes involved in the pathogen response, including *lys-7, sdd-3* and *cnc-6* (Table 1). Aggregation-prone proteins – and in particular polyglutamine expansion proteins – are not known to trigger a pathogen defense response. Moreover, several genes within this cluster are expressed in the *C. elegans* gut, suggesting
that aggregation-prone proteins can also provoke cell non-autonomous effects. Finally, we identified upregulation of genes within the redox cluster, including genes known to be involved in oxidative stress (cst-2 and sod-3) and belonging to the cytochrome P450 family of monooxygenases, including kmo-1 (Table 1). The yeast homolog of kmo-1 – bna-4 – has been identified as a potent suppressor of polyglutamine-induced proteotoxicity [19]. Taken together, these results point to aggregation-prone proteins inducing cellular stress, resulting in activation of cellular stress response pathways.

Table 1. Genes expressed differentially between wild type and polyglutamine-expressing animals at the L4 stage (blue data points from Figure 1F).

<table>
<thead>
<tr>
<th>Cellular process (no of genes)</th>
<th>Function (no of genes)</th>
<th>Gene examples</th>
<th>Fold change</th>
<th>Short description</th>
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</thead>
<tbody>
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<td>Response to stress (53)</td>
<td>AC3.3</td>
<td>abu-1</td>
<td>-2.66</td>
<td>Transmembrane proteins with a glutamine/asparagine-rich (“prion”) domain</td>
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<td>Unfolded protein response (14)</td>
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<td>abu-6</td>
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</tr>
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<td></td>
<td>C03A7.8</td>
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<td>R09F10.2</td>
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<td>-2.70</td>
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</tr>
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<td>F35A5.3</td>
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</tr>
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</tr>
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<td></td>
<td>C14B9.2</td>
<td>-</td>
<td>1.40</td>
<td>Protein disulfide isomerase</td>
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<td></td>
<td>K01D12.11</td>
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<td>1.79</td>
<td>Predicted transmembrane protein part of the cadmium responsive (CDR) family of proteins</td>
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<td>Pathogen response (35)</td>
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<td>lys-7</td>
<td>2.00</td>
<td>Antimicrobial enzyme</td>
</tr>
<tr>
<td></td>
<td>T08A9.7</td>
<td>spp-3</td>
<td>1.17</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
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<td>cnc-6</td>
<td>7.42</td>
<td>Antimicrobial peptide</td>
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<td>C24A8.4</td>
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<td>Protein kinase</td>
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<td></td>
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<td>sod-3</td>
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<td>Iron/manganese superoxide dismutase</td>
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<td>R07B7.5</td>
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</tbody>
</table>

Polyglutamine expansion proteins affect C. elegans development

While performing differential expression analysis (also using an FDR cutoff of 0.1%), we identified additional clusters of differentially expressed genes (Figure 1F, magenta and green). These clusters of genes were significantly upregulated during development of wild type animals while slightly downregulated in Q40 animals. Further inspection of this cluster identified two sub-groups: one sub-group had more genes that were strongly downregulated (Figure 1F, magenta)
while the other had more genes that were clustered but less downregulated (Figure 1F, green). Since we suspected that this expression pattern may be due to developmental differences provoked by the expression of aggregation-prone proteins, we performed GO term analysis of each cluster individually. For the sub-group with strongly downregulated genes, we could draw no conclusions regarding biological processes (Figure 1F, magenta). However, the clustered genes (Figure 1F, green) were enriched for development and maturation processes, muscle reorganization and lipid storage (Figure 1H). The fact that we observed downregulation of developmental genes as a response to the presence of aggregation-prone proteins is consistent with the aforementioned developmental delay observed in polyglutamine-expressing animals (Figure 1D), suggesting that aggregation-prone proteins can also affect animal development. In all, expression of aggregation-prone proteins alters the expression developmental genes, which slows down *C. elegans* development.

**Discussion**

Here we investigated the cellular responses that are elicited by the presence of aggregation-prone proteins using a *C. elegans* model of polyglutamine diseases. We used differential gene expression analysis to demonstrate that the presence of aggregation-prone proteins – in the form of polyglutamine expansion proteins – triggers the expression of ER-associated UPR genes. This observation is unexpected since polyglutamine expansion proteins do not usually enter the secretory pathway. We speculate that this cellular stress response is activated in an attempt to reduce overall protein synthesis of misfolded polyglutamine expansion proteins and perhaps that of other polyglutamine-rich proteins – thereby explaining the downregulation of *abu/pqn* genes – as has been suggested by others [18].

The presence of polyglutamine expansion proteins is thought to induce the heat shock response (HSR), a cellular stress response that relies on molecular chaperones to prevent protein misfolding and aggregation [20, 21]. We did not, however, detect any differential expression of genes associated with the HSR. One possibility is that the animals in our experiment have undergone adaptive evolution, whereby over successive generations of growth in laboratory conditions the animals have developed the ability to cope with the constitutive
expression of polyglutamine expansion proteins, while maintaining the aggregation-associated phenotypes. Such adaptive evolution has for example occurred in the wild type strain N2, which after continuous maintenance in laboratory conditions has developed “laboratory-derived” alleles as an adaptation process ([22, 23] also reviewed in [24]).

The fact that we saw upregulation of several genes involved in the innate immune response suggests that polyglutamine expansion proteins also affect immune response signaling pathways. The induction of a defense response has not been previously described in polyglutamine diseases. We propose that the presence of polyglutamine expansion proteins is perceived by the worms as an infection, thereby triggering the innate immune system. This was recently shown to be the case for the aggregation-prone proteins TDP-43 and FUS, which induce immune responses in C. elegans models of amyotrophic lateral sclerosis [25]. It is of interest to note that the UPR is also part of the cellular defense in C. elegans and is activated upon pathogen infection, further supporting the notion that polyglutamine expansion proteins induce effects in the organism similar to those induced by pathogens [26-28].

Our findings relating to the induction of cellular stress pathways are not entirely without precedent. Oxidative stress originates from the imbalance between the production and clearance of reactive oxygen species (ROS) and has been linked to Huntington’s disease [29, 30]. Here we identified several genes with redox functions, which points to another cellular stress response activated in the presence of polyglutamine expansion proteins. The fact that we observed the involvement of the UPR, the immune response and oxidative stress pathways suggests that aggregation-prone proteins have a systemic effect in the organism. It will be interesting to further address how these cellular pathways are functionally affected in mammalian neuronal cell or animal models and determine whether these pathways mediate neurodegeneration induced by polyglutamine expansion proteins in these systems.

Finally, we found larval development to be significantly affected in polyglutamine-expressing animals. Others have reported that expression of aggregation-prone proteins slows down the growth rate of yeast [18] and delays
development in another *C. elegans* model expressing polyglutamine expansion proteins in the body wall muscle cells [21]. Collectively, these findings point towards the tentative notion of a delay in organismal development as a result of cell non-autonomous signaling across tissues that is provoked by the presence of aggregation-prone proteins.

In conclusion, we propose that the presence of aggregation-prone proteins activates a cellular stress response that – combined with a delay in development – may impose a fitness cost in this model.

**Methods**

**Strains and genetics**

Standard methods were used for culturing *C. elegans* at 20°C [31]. To synchronize animals, eggs were collected from gravid hermaphrodites by hypochlorite bleaching and hatched overnight in M9 buffer. The desired number of L1 animals were subsequently cultured on agar plates (#633185, Greiner Bio-One) containing nematode growth medium and seeded with OP50 bacteria. The following strains were used or generated: N2 (wild type): AM141 *rmIs133[P(unc-54)Q40::YFP]X* [6]; OW1004 *rmIs133[P(unc-54)Q40::YFP]X*; and OW1021 *rmIs133[P(unc-54)Q40::YFP]X*.

**RNA sequencing**

Worms were grown to larval stages two/three (L2/3) and four (L4) and total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer’s instructions. For polyA RNA sequencing, sequencing libraries were prepared using the TruSeq Sample Preparation V2 Kit (Illumina) and subjected to high-throughput single-end sequencing (50 bp) in an Illumina HiSeq 2500 instrument.

**Data analysis**

Single-end reads obtained by Illumina sequencing were aligned to the *C. elegans* reference genome (Wormbase, version WB235) using Star Aligner [32]. Raw read counts were extracted from the alignments using the HTSeq package. The reads were fragment per million (FPM) normalized, log transformed and null averaged prior to Principal Component Analysis (PCA). Average clustering
Transcriptional profiling in *C. elegans* reveals developmental and cellular responses to polyglutamine expansion proteins was performed using Pearson correlation for the corrected RNA samples and complete clustering was performed with euclidean distances for the read distance, this was plotted with the heatmap2 function in R. Differential expression analysis was performed on the corrected raw reads using EdgeR [33], and gene ontology enrichment analysis was performed using the web-based toolset gProfiler [34, 35].

**Acknowledgements**
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**Author contributions**
O.S. and E.A.A.N. designed the experiments. O.S. and R.S. performed the experiments. O.S., T.v.d.J. and V.G. analyzed the data. O.S., E.A.A.N. and V.G. wrote the manuscript.
References


Transcriptional profiling in *C. elegans* reveals developmental and cellular responses to polyglutamine expansion proteins


## Supplemental Information

### Table S1. Top 100 genes upregulated in polyglutamine-expressing animals at the L4 stage (blue data points from Figure 1F).

<table>
<thead>
<tr>
<th>Sequence name</th>
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<th>Fold change</th>
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Transcriptional profiling in *C. elegans* reveals developmental and cellular responses to polyglutamine expansion proteins.

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Transcriptional profiling in *C. elegans* reveals developmental and cellular responses to polyglutamine expansion proteins

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CHAPTER VI

Loss of Serf2 in mice results in embryonic lethality with incomplete penetrance

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Work in progress
Abstract
Neurodegenerative disorders such as Parkinson’s, Alzheimer’s and polyglutamine diseases are commonly characterized by the presence of protein aggregates in specific neurons. How the formation of these proteinaceous inclusions occurs is still not fully understood, nor is their role in pathogenesis. A positive regulator of aggregate formation has been discovered in *C. elegans* models for aggregation of alpha-synuclein, amyloid-beta and polyglutamine. This protein, known as modifier of aggregation MOAG-4, has two human orthologs, SERF1A and SERF2, which drive polyglutamine aggregation in human cells. This suggests that SERF may drive disease-associated protein aggregation in the mammalian brain. To test this hypothesis, we generated a *Serf2* knockout mouse model in a C57BL/6 background. We found that the homozygous null allele is not inherited in Mendelian proportion, but some individuals are able to survive until adulthood. Our results suggest that full body knockout of *Serf2* is embryonic lethal with incomplete penetrance. Since such mice will be of limited use to study the function of *Serf2* in adult mice, we generated mice harboring a brain-specific knockout for *Serf2*. We report that the ablation of *Serf2* expression from the brain resulted in viable and fertile animals.

We conclude that *Serf2* is required for normal mouse development. Mice harboring the *Serf2* conditional knockout allele can now be used to study the biological function of *Serf2* in mouse development. Additionally, by mating these brain-specific knockout mice with mouse models of neurodegenerative diseases, we can also investigate whether *Serf2* is indeed a genetic modifier of protein aggregation.
Introduction
While protein aggregation and toxicity are hallmarks of neurodegenerative diseases such as Parkinson’s (PD), Alzheimer’s (AD) and polyglutamine diseases, their role in these diseases is currently unknown (reviewed in [1, 2]). To better understand the role of protein aggregation in disease, efforts have been made to identify genes and pathways that regulate protein aggregation. The identification of such genes has been aided by genetic screens in Caenorhabditis elegans models expressing aggregation-prone proteins, including alpha-synuclein, amyloid-beta and polyglutamine [3-10]. The function of the genetic modifiers identified in these screens has been shown to be conserved in human cell models and in mice [5, 11, 12].

In C. elegans models for PD, AD and polyglutamine diseases, we previously identified MOAG-4/SERF as a regulator of age-related proteotoxicity [5]. We showed that MOAG-4 promotes polyglutamine aggregation and toxicity and that its function is evolutionarily conserved in the mammalian orthologues SERF1A and SERF2 (small EDRK rich factor 1A and 2) [5]. In human cell models expressing polyglutamine, overexpression of MOAG-4/SERF enhanced aggregation and cell death [5].

The molecular function of the SERF proteins is unknown. These proteins are ubiquitously expressed and are therefore predicted to have a role in general cellular pathways. SERF1A was first identified in a comparative genomics study, where it was found to be a genetic modifier of spinal muscular atrophy in human patients [13]. A transcriptome analysis later revealed that SERF1A is downregulated in the cerebellum of HD patients [14]. Recently, SERF1A was shown to promote amyloid aggregation in vitro [15].

Here we aimed to test the hypothesis that SERF2 drives disease-associated protein aggregation in the mammalian brain. To this end, we generated Serf2 homozygous null mutant mice. By intercrossing Serf2+/- mice, we discovered that the segregation of the knockout allele did not follow Mendelian inheritance, suggesting that the targeted disruption of Serf2 has fundamental implications for mouse development. To overcome this limitation, we generated brain-
specific Serf2 knockout mice that are viable and fertile. We further investigated the distribution of Serf2 expression and found that Serf2 is transcribed in various tissues, namely brain, heart, intestine, kidney and liver.

Results and Discussion

Deletion of Serf2 results in embryonic lethality with incomplete penetrance

To study the function of Serf2, we generated a mouse strain with an integrated promoter-driven selection cassette (Figure 1A) [16]. Serf2 has 3 exons and spans about 4 kb in chromosome 2. Two loxP sites were inserted around exon 2 of the Serf2 gene and its expression was prevented by the presence of an intronic Neo selection cassette that was surrounded by two loxP sites and two FRT sites (Figure 1A). Hprt Cre-induced recombination excised the Neo gene and exon 2 of Serf2, thereby generating a heterozygous reporter knockout for Serf2, with the lacZ reporter transgene under the control of the Serf2 promoter (Serf2+/−, Figure 1A, B).

We next attempted to generate Serf2 null mice (Serf2−/−) by intercrossing Serf2+/− mice. The numbers of Serf2−/− progeny obtained were far lower than those

![Figure 1. Gene targeting strategy for the Serf2 knockout model.](image_url)

(A) Diagram of the Serf2 targeting vector. The Serf2 exon 2 is flanked by two loxP sites. Expression is prevented by an intronic Neo selection cassette surrounded by two loxP sites and two FRT sites. Mating with a Hprt Cre deleter mouse strain removes exon 2, resulting in a reporter knockout whereby the lacZ transgene is expressed under the control of the Serf2 promoter. Diagram adapted from [16]. (B) Genotype of wild type, heterozygous and knockout Serf2 transgenic mice. Primers F3 and R3 were used to distinguish the targeted allele (420 bp) from the untargeted wild type allele (335 bp), identified with primers F4 and R3. (C) Numbers of mice obtained for each genotype from intercrossing Serf2+/− mice or by mating Serf2+/− mice with Serf2−/− mice. Numbers in parentheses represent the expected Mendelian ratio for each genotype.
expected according to Mendelian inheritance and only a small proportion was able to survive throughout adulthood, suggesting embryonic lethality with incomplete penetrance (Figure 1B, C). When we attempted to mate Serf2*+/− mice with Serf2*−/− mice, Serf2*−/− offspring of this cross were also underrepresented (Figure 1C). Together, these results demonstrate that Serf2 is essential for mouse development.

**Serf2 is expressed in disease-related brain areas**

To understand the role of Serf2, we next wanted to identify the tissues in which Serf2 is normally expressed in Serf2*+/+ and Serf2*+/− mice. To do so, primers specific for Serf2 were used to investigate its expression in the brain, heart, intestine, kidney, liver and pancreas by qPCR (Figure 2A). Serf2 was expressed in most the tissues examined in this study. mRNA expression in Serf2*+/− mice

![Figure 2. Expression of Serf2 in different tissues. (A) Expression of Serf2 in tissues of 3-month-old mice. Relative mRNA levels were measured by qPCR in wild type (n=3), heterozygous (n=8) and knockout mice (n=1) for Serf2. (B) Protein expression of Serf2 in liver samples obtained from wild type, heterozygous and knockout mice by Western blot. (C) Protein expression of Serf2 in the cerebellum (Cer) and hippocampus (Hip) of wild type and heterozygous mice by Western blot. In both experiments, actin was used as a loading control. (D) Expression of Serf1 in different brain regions of wild type mice (n=5) and mice heterozygous for the Serf2 knockout allele (n=5). (E) Expression of Serf2 in different brain regions of wild type mice (n=5) and mice heterozygous for the Serf2 knockout allele (n=5). Relative mRNA levels were measured in the olfactory bulb, hippocampus, striatum, cortex, cerebellum and brainstem. **** p<0.0001; *** p<0.001; ns is not significant.](image-url)
was significantly lower (up to 50%) than that in control mice (Figure 2A). This was later confirmed by measuring Serf2 protein levels in the brain and liver (Figure 2B, C).

Knowing that Serf2 was expressed in the brain and aiming to use this model to later generate a Serf2 knockout in a neurodegenerative disease background, we asked in which brain areas Serf2 was expressed. We found that Serf2 was expressed in different brain regions, including the brainstem, the olfactory bulb, the cerebellum, the striatum and, importantly, the hippocampus and the cortex, which are frequently affected in neurodegenerative diseases (Figure 2E). We examined 3 and 6-month old mice and confirmed that Serf2−/− mice had reduced expression of Serf2 while Serf1a expression was unaltered (Figure 2D, E). A reduction in expression of Serf2 was not compensated by the expression of Serf1a, supporting the use of this model to study Serf2 function.

Our findings show that Serf2 is expressed in different tissues, including the brain. Specifically, Serf2 is expressed in brain areas relevant to disease and loss of Serf2 does not affect Serf1a expression.

**Mice with brain-specific knockout for Serf2 are viable and fertile**

Since the full body knockout of Serf2 resulted in embryonic lethality, we could not take advantage of these mice to study the role of Serf2 in protein aggregation in the brain. To overcome this limitation, we specifically eliminated Serf2 expression in the brain. First, mice harboring the Serf2 construct were crossed with FLP deleter mice to generate a conditional Serf2 allele (Serf2floxflox, Figure 3A). Serf2floxflox mice express the transgene as wild type and have normal appearance (data not shown). To specifically eliminate Serf2 expression in the brain, Serf2floxflox mice were mated with mice that express the Cre recombinase under the control of the Sox1 promoter, which restricts expression of Cre to the central nervous system (Figure 3A). Conditional knockout mice (Cre Sox1+ Serf2−/−) derived from this cross were viable and fertile (Esther Stroo, personal communication). To demonstrate that Serf2 expression was eliminated only in the brain, we measured expression levels of Serf2 in the brain and compared them with those in other tissues, including the kidney and the liver. When compared with expression in Serf2floxflox control mice, expression of Serf2 in Cre Sox1+ Serf2−/− mice was almost completely abolished
Loss of Serf2 in mice results in embryonic lethality with incomplete penetrance.

To investigate whether the knockout of Serf2 affected Serf1a expression, we also measured Serf1a in the brain. We detected a statistically significant increase of Serf1a in the brain of Cre Sox1+ Serf2−/− mice, which suggests a compensation mechanism by Serf1a in the absence of Serf2 (Figure 3B).

in the brain but unaltered in the kidney and liver (Figure 3C). Together, these results demonstrate the successful knockout of Serf2 in the brain without its expression in other tissues being affected.

Figure 3. Gene targeting strategy for the brain-specific knockout of Serf2. (A) To generate a conditional allele for Serf2, mice harboring the Serf2 targeting vector were mated with a FLP deleter line to excise the lacZ transgene and the Neo selection cassette. This resulted in a floxed Serf2 allele (Serf2lox/lox), in which Serf2 is expressed as the wild type. Next, Serf2lox/lox mice were mated with Sox1 Cre deleter mice to generate Cre Sox1+ Serf2−/− progeny. Diagram adapted from [16]. (B) Quantitative PCR of Serf1 in the brain (n=4), the liver (n=2) and the kidney (n=2) of Serf2lox/lox and Cre Sox1+ Serf2−/− mice (5 months old). (C) Quantitative PCR of Serf2 in the brain (n=4), the liver (n=2) and the kidney (n=2) of Serf2lox/lox and Cre Sox1+ Serf2−/− mice (5 months old). *** p<0.001; ns is not significant.
Conclusion
In summary, Serf2 is essential for mouse development. We describe the generation of brain-specific Serf2 knockout mice that can be used not only to further study the role of Serf2 in mouse development but also to determine Serf2 involvement in proteotoxicity by mating these knockout mice with mouse models of neurodegenerative diseases.

Methods

Expression construct and generation of transgenic mice
The Serf2 targeting vector was generated by the International Knockout Mouse Consortium (IKMC, Figure 1A) [16, 17]. Briefly, the exon 2 of Serf2 is flanked by two loxP sites its expression is prevented by an intronic Neo selection cassette, which is flanked by two loxP sites and two FRT sites. The construct was injected into C57BL/6N embryonic stem cells [18]. Two founder lines (B11 and G9) were positive for the construct and subsequently expanded for this study. To generate a full body knockout of Serf2, both lines were crossed with a Hprt Cre deleter mouse strain to remove the PGK-Neo cassette and Serf2 floxed exon. Confirmation of correct recombination was performed by PCR genotyping analysis of ear biopsy. All mice were heterozygous with respect to the construct. Animals were backcrossed into the C57Bl/6J background (Charles River) six times.

Genotyping
DNA was prepared from ear biopsy and processed with prepGEM Tissue (#PTI0500, ZyGEM Corporation Ltd). PCR reactions contained three primers, one sense primer specific for the Serf2 transgene F3 (5’-CCGGTGCCTACCATTACCAG-3’); a second sense primer specific for genomic Serf2 F4 (5’-GATGATGGGCTTTCTGCTGC-3’); and one antisense primer present in the transgene and mouse Serf2 R3 (5’-CTTGATATGTAAGCCCTGC-3’). The knockout allele was identified with pair F3 and R3, generating a 420 bp product; and the wild type allele was identified with pair F4 and R3, generating a 335 bp product. Cycling conditions were 2 min at 94°C; 35 cycles of 30 sec at 94°C; 30 sec at 60°C; 1 min at 72°C; and 7 min at 72°C.
Quantitative PCR
Total RNA was extracted using TRizol Reagent (Life Technologies) according to the manufacturer’s description. Total RNA quality and concentration were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific/Isogen Life Science). cDNA was made from 1.5 ug (tissues) or 2 ug (brain regions) total RNA with a RevertAid H Minus First Strand cDNA Synthesis kit (Life Technologies) using random hexamer primers.

Quantitative real-time PCR was performed using a Roche LightCycler 480 Instrument II (Roche Diagnostics) with SYBR green dye (Bio-Rad Laboratories) to detect DNA amplification. Relative transcript levels were quantitated using a standard curve of pooled cDNA solutions. Expression levels were normalized to 18S mRNA levels. The primers for RT-PCR used were Serf1 F2 (5'-TGGCCCGTGAAATCAAAGAGAAA-3'); Serf1 R2 (5'-TGCATGATCTCTGAATCCCTCTGCT-3'); Serf2 F2 (5'-CCGCGGTAACCAGCGAGAGC-3'); Serf2 R2 (5'-TCCGAGTCCCTCTGCTTGCG-3'); 18S F1 (5'-CGGACAGGATTGACAGATTG-3'); 18S R1 (5'-CAAATCGCTCCACCAACTAA-3').

Western Blot
Frozen tissue was homogenized in RIPA buffer (10 mM Tris-Cl pH 8.0; 1 mM EDTA; 0.5 mM EGTA; 1% Triton X-100; 0.1% sodium deoxycholate; 0.1% SDS; 140 mM NaCl). Samples were homogenized with a tissue grinder pestle and incubated on ice for 30 min, followed by centrifugation at high speed for 30 min at 4ºC. The supernatant was transferred to a new tube and protein was quantified using the Pierce BCA Protein Assay kit (#23225, Life Technologies). Approximately 130 ug (cerebellum and hippocampus) or 40 ug (liver) of protein were loaded onto 15% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes and blocked with 5% BSA in PBS-T. Membranes were incubated with primary antibodies for SERF2 at 1:1500 dilution overnight (#11691-1-AP, Proteintech) or actin at 1:10,000 dilution overnight (#3134S, Cell Signaling Technology). Washes were performed with PBS-T. Incubation with secondary anti-rabbit for SERF2 or anti-mouse for actin was done at 1:10,000 dilution for 1 hour at room temperature. Antibody binding was visualized with an ECL kit (Amersham).
Acknowledgements
We thank Rui Costa and Catherine French for the Sox1 Cre transgenic mice. We thank Mathias Jucker and Ehud Cohen for fruitful discussion. We thank Sally Hill for editing the manuscript. Alumni chapter Gooische Groningers facilitated by the Ubbo Emmius Fund. This work was supported by the European Research Council (ERC) starting grant (to E.A.A.N.), the Ubbo Emmius Fonds and the Fundação para a Ciência e Tecnologia (SFRH/BPD/51009/2010) (to O. S.).

Author contributions
O. S., W. H. and E. A. A. N. designed the experiments. O. S. and W. H. performed the experiments. O. S. and E. A. A. N. wrote the manuscript.
Loss of Serf2 in mice results in embryonic lethality with incomplete penetrance

References

CHAPTER VII

Discussion and perspectives
Many neurodegenerative diseases are characterized by clinicopathological features that affect motor capacity and cognition in affected patients. Examples of such diseases are Alzheimer’s, Parkinson’s, polyglutamine diseases and amyotrophic lateral sclerosis (ALS). Unfortunately, no cure is currently available and the risk of developing these diseases increases with aging. Interestingly, a hallmark of post-mortem biopsies from the brains of affected patients is the presence of protein aggregates in neurons. While specific aggregation-prone proteins have been identified for each neurodegenerative disease, their contribution to disease remains unresolved. More specifically, we do not know what triggers protein aggregation and how this is mechanistically linked to pathogenesis. What we do know is that protein homeostasis is affected in these diseases, and understanding how it is altered may help us not only to identify the cellular processes involved but also – in the long term – to target them for potential disease-modifying treatments.

The aim of the work presented in this thesis was to dissect the cellular and molecular aspects of protein aggregation, in order to find mechanisms that help to explain how protein homeostasis correlates with pathogenesis. A main goal was to discover genetic regulators of protein aggregation in an unbiased and hypothesis-free manner, with the expectation of identifying new regulators and therefore new mechanisms in protein aggregation.

We were successful in identifying one such new regulator in the form of MOAG-2/LIR-3. Chapter IV describes the identification and characterization of this modifier of aggregation in a C. elegans model of polyglutamine diseases. We discovered that MOAG-2/LIR-3 is an RNA Polymerase (Pol) III-associated transcriptional regulator that drives polyglutamine aggregation. In this chapter we propose a mechanism whereby aggregation-prone proteins recruit normally functioning cellular proteins to promote their own aggregation. Despite this important finding, this study leaves several questions unanswered – these are discussed below, together with suggestions for directions of future research in the context of MOAG-2/LIR-3 and the link between the non-coding genome and protein aggregation.
1. Aggregation-prone proteins affect the non-coding genome
One surprising finding from this work was that in our model the presence of polyglutamine expansion proteins induced the downregulation of small non-coding RNAs (ncRNAs) – specifically snRNAs, snoRNAs and tRNAs – demonstrating that the non-coding genome is also affected by the presence of aggregation-prone proteins. It is still unknown how this downregulation occurs and we propose two different possibilities that could explain this observation. The first possibility is that misfolded, aggregation-prone proteins directly sequester these small ncRNAs into aggregates, thereby reducing their abundance in the cell. The second possibility is that aggregation-prone proteins sequester MOAG-2/LIR-3, subsequently inhibiting it from driving small ncRNA transcription. To experimentally address these hypotheses, it would be necessary to isolate the polyglutamine aggregates and detect the presence of small ncRNAs (e.g. qPCR) or MOAG-2/LIR-3 (e.g. co-immunoprecipitation).

The next unresolved question is what biological consequences the downregulation of small ncRNAs might have. For example, the reduction of one type of small ncRNAs in the nucleus – the U1 snRNAs – affects the splicing of premature mRNA [1, 2]. In our case, however, downregulation of snRNAs did not affect the splicing of premature mRNA (data not shown). Our observations so far point to the notion that it is possible to retain functional splicing events despite lower levels of splicing factors. The downregulation of another type of small ncRNA – the tRNAs – may also have biological consequences, and it will be interesting to investigate how tRNA function is affected, if at all (e.g. is translation efficiency maintained? does the proteome change?).

Finally, it will also be worth assessing whether the downregulation of small ncRNAs is cell type-specific (e.g. do polyglutamine-expressing cells have a distinct ncRNA repertoire?) and whether altered ncRNA homeostasis is specific to polyglutamine expansion proteins or shared by other aggregation-prone proteins (e.g. alpha-synuclein, amyloid-beta).
2. Is MOAG-2/LIR-3 related to calcium-regulated transcription factors?

Once we discovered that MOAG-2/LIR-3 was a transcription factor we were interested to know whether it shared homology with other mammalian transcription factors. Protein sequence comparisons revealed that MOAG-2/LIR-3 is predominantly conserved among the *Caenorhabditis* genus. It does, however, share 27% homology with the human calcineurin B homologous protein 1 (CHP1) and 37% homology with the human zinc finger protein 64 (ZNF64). The presence of these two functional motifs – a calcium-binding domain and a zinc finger – is typical of human calcium-regulated transcription factors, such as the cAMP-responsive element-binding protein (CREB)-binding protein (CBP), the downstream regulatory element antagonist modulator (DREAM) and FOXO3A [3]. The combination of these two domains – calcium binding and DNA binding – poses the interesting possibility that MOAG-2/LIR-3 may be a calcium-dependent transcription factor that regulates expression of small ncRNAs.

3. What are the proteins that interact with MOAG-2/LIR-3?

We can learn more about MOAG-2/LIR-3 function by identifying the types of proteins with which it interacts or forms complexes. We have shown that MOAG-2/LIR-3 binds to the same promoters as the RNA Pol III complex. We have also shown that inactivation of MOAG-2/LIR-3 reduces the levels of transcribed small ncRNAs, suggesting that MOAG-2/LIR-3 cooperates with the RNA Pol III complex to promote small ncRNA transcription. The question of whether MOAG-2/LIR-3 is physically part of the RNA Pol III complex could be answered by using purified MOAG-2/LIR-3 in co-immunoprecipitation experiments, followed by mass spectrometry. This approach would also enable us to identify other co-immunoprecipitated proteins and establish a protein interaction network for MOAG-2/LIR-3, which would tell us more about MOAG-2/LIR-3 function and whether it cooperates with other co-factors to regulate the expression of small ncRNA genes.
4. Could MOAG-2/LIR-3 be an RNA-binding protein?
The C2H2 zinc fingers of MOAG-2/LIR-3 are structurally homologous to the canonical C2H2 zinc fingers of the transcription factor IIIA (TFIIIA) in *Xenopus laevis* [4]. TFIIIA is a promoter-bound recruitment factor that recruits TFIIIB which, in turn, recruits Pol III to the promoters of genes encoding of 5S rRNA [5]. The binding of TFIIIA to the promoter takes place through physical binding of zinc fingers 4-9 to two specific regions: Box A and the intermediate element [6]. In addition to binding to 5S promoters, TFIIIA also binds to 5S RNA itself through zinc fingers 4-7 [7-9]. The fact that we found MOAG-2/LIR-3 to be frequently bound to 5S promoters suggests that it may also act as a recruitment factor, recruiting other components of the RNA Pol III complex to Box A and Box B. Furthermore, the resemblance of motifs within its structure to the zinc fingers of TFIIIA opens up the possibility that MOAG-2/LIR-3 might bind directly to rRNA or to any other type of RNA, a prospect that will have to be assessed in future studies.

5. Do aggregation-prone proteins alter binding of MOAG-2/LIR-3 to its transcriptional targets?
We have shown that MOAG-2/LIR-3 binds to the promoters of snRNA, snoRNA and tRNA genes to promote their transcription. One open question stemming from this work is how the presence of aggregation-prone proteins modifies the binding of MOAG-2/LIR-3 to its transcriptional targets. We propose that polyglutamine sequesters MOAG-2/LIR-3 or modifies its localization, thereby preventing MOAG-2/LIR-3 binding to its DNA targets such that it can no longer transcribe small ncRNAs with the same efficiency as that in wild type animals. This could explain why we observe lower levels of small ncRNAs in the presence of aggregation-prone proteins. Indeed, it would be interesting to verify whether MOAG-2/LIR-3 can still retain its function as a transcriptional regulator in polyglutamine-expressing animals, a hypothesis which will require testing in additional ChIP seq experiments.

The identification and characterization of MOAG-2/LIR-3 in *C. elegans* offers a major contribution to our understanding of the aggregation pathway in neurodegenerative diseases, and in particular how benign cellular proteins can convert their function to an aggregation-promoting factor. A second major
step focused on the effects of protein aggregation at the transcriptional level. In Chapter V, we showed that the presence of polyglutamine-expansion proteins induced the expression of stress response genes involved in: 1) oxidative stress; 2) the ER-associated unfolded protein response (UPR); and 3) the innate immune response. Oxidative stress results from the imbalance between the production and clearance of reactive oxygen species (ROS), which can cause damage to DNA, proteins and other cellular components (reviewed in [10]). Our results come in line with previous published work implicating oxidative stress in Huntington’s disease [11, 12]. It would be interesting to further explore this correlation and determine what specific cellular events lead to oxidative stress (e.g. mitochondrial dysfunction? overproduction of ROS? insufficient antioxidant production?).

If unfolded proteins accumulate in the ER, the UPR is responsible for restoring protein homeostasis by fine-tuning protein-folding load with protein-folding capacity (reviewed in [13]). Previous published work showed that the UPR contributes to neuroprotection in a C. elegans model expressing alpha-synuclein in the dopaminergic neurons [14]. Although polyglutamine expansion proteins are not expected to enter the secretory pathway, it is still reasonable to consider that their accumulation at the ER (perhaps immediately after protein synthesis) would be sufficient to trigger the UPR as protective response to aggregation-prone proteins.

A surprising finding was the involvement of the innate immune response to polyglutamine expansion proteins. Several genes that encode antimicrobial peptides were upregulated in our study, suggesting that aggregation-prone proteins can also elicit a systemic effect in the organism. It is possible that aggregation-prone proteins are perceived by the organism as a pathogen and thereby elicit the immune system to counteract their toxicity. Indeed, the involvement of the cellular stress responses described here show that the organism takes advantage of distinct cellular defense mechanisms at its disposal to combat the pathogenic presence of aggregation-prone proteins. Following this line of thought, it would be interesting to investigate whether there is a cross talk between these cellular responses to aggregation-prone proteins.
Lastly, we saw that the effects of aggregation-prone proteins go beyond cellular stress responses and can also delay *C. elegans* development. Polyglutamine diseases are not known to be associated with impaired development, therefore suggesting that the developmental delay observed in our polyglutamine model may be specific to nematodes.

A final major step in our understanding of protein aggregation is well under way as a result of our work with MOAG-4 and its mammalian orthologs, SERF1A and SERF2. Below we discuss the contributions made by our study on SERF2 in the brains of mice (Chapter VI) and discuss plans for future experiments in mouse models of Alzheimer’s disease.

The aggregation-prone protein most commonly associated with Alzheimer’s disease (AD) is amyloid-beta (reviewed in [15, 16]). We and others have previously shown that aggregation of amyloid-beta is promoted both *in vitro* and *in vivo* by MOAG-4/SERF [17, 18]. In a *C. elegans* model of amyloid-beta, the transgene is expressed intracellularly in the body wall muscle, where it accumulates and contributes to progressive paralysis in the worm [19]. We have shown that deletion of MOAG-4 ameliorates this paralysis phenotype by reducing the amount of seeding-competent amyloid-beta [17]. In addition, our studies in human cell models have shown that the function of MOAG-4 is conserved in two mammalian orthologs, SERF1A and SERF2 [17]. This led us to hypothesize that SERF proteins could be driving amyloid-beta aggregation in the AD brain. To test this hypothesis, we generated a *Serf2* knockout mouse and crossed it with an AD mouse model. In Chapter VI, we discovered that a full body knockout of *Serf2* results in embryonic lethality with incomplete penetrance. Since this does not allow us to study the role of *Serf2* in adult mice, we generated brain-specific *Serf2* knockout mice instead. As a next step in this project, we plan to mate these animals with AD mouse models.

The aim of generating a brain-specific *Serf2* knockout mouse in an AD background is to find out whether the function of MOAG-4/SERF in proteotoxicity is conserved from *C. elegans* to mammals. Specifically, we want to know whether removing SERF from the brain in AD mouse models suppresses amyloid aggregation and neurotoxicity. We also want to find out whether
changes in proteotoxicity are accompanied by changes in motor and cognitive functions. The experimental design is depicted in Figure 1. Brain-specific Serf2 knockout mice – Sox1 Cre+ Serf2−/− – will be mated with two distinct AD mouse models. The first strategy involves mating the Sox1 Cre+ Serf2−/− mice with the APPPS1 mouse model [20]. In this model, the human amyloid precursor protein (APP) harboring the K595N/M596L “Swedish” mutation (otherwise known as K670N/M671L) and the human presenelin 1 (PS1) protein harboring the L166P mutation are co-expressed under the Thy1 promoter, which directs transgene expression to the postnatal brain [20]. Both mutations are associated with early onset AD in humans. In this model, amyloid-beta deposition is seen as early as six weeks of age, with cognitive impairment appearing at 7 months of age [20, 21]. From the mating between the Sox1 Cre+ Serf2−/− mice and the APPPS1 mice, we expect to obtain progeny in Mendelian proportions with the following characteristics: 1) wild type for all transgenes; 2) transgenic for APPPS1; 3) wild type lacking SERF2 in the brain; and 4) transgenic for APPPS1 and lacking SERF2 in the brain (Figure 1). Here, we will focus predominantly on brain histology and immunohistochemistry to study the effect of a lack of Serf2 in the brain on amyloid-beta deposition (Figure 1).

In the second strategy, we will mate Sox1 Cre+ Serf2−/− mice with the APPswe/PSEN1dE9 mouse model, from which we also expect to obtain all four desired phenotypes in Mendelian proportions (Figure 1) [22]. In this model of AD, a chimera of mouse and human APP harboring the K595N/M596L “Swedish” mutation (APPswe) is co-expressed with the exon-9-deleted (delta E9) variant of human mutated PS1 protein (PSEN1dE9), the latter also associated with early onset AD [22]. The expression of both transgenes is under the mouse prion promoter, which restricts their expression to the neurons of the central nervous system [22]. In APPswe/PSEN1dE9 mice, amyloid-beta plaques typically appear at 4-6 months of age, and plaque accumulation progresses up to 12 months of age, when cognitive deficits also start to appear [23, 24]. We will subject these mice to a battery of behavioral tests to assess spatial memory reference (e.g. Morris water maze), exploration and anxiety (e.g. open field) and motor capacity (e.g. grip strength) (Figure 1). In parallel, we will perform histological and immunohistochemical assays to assess amyloid deposition and neuronal toxicity. Since AD is a progressive disorder, we want to learn how aggregation
formation, neurotoxicity and cognition are affected during aging. For this reason, we will include both 3 to 4 and 6 to 7-month old animals in our analysis. In all, we hope to uncover the function of Serf2 in the brain and determine whether it acts as a genetic modifier of proteotoxicity and cognition in AD. Results from these studies will provide insight into how the brain copes with protein aggregation during aging and whether this is accompanied by changes in behavior. Ultimately, this study may reveal Serf2 to be a potential therapeutic target for modulation of neurodegeneration.

**Figure 1.** Diagram representing the generation of a brain-specific Serf2 knockout mouse (Sox1 Cre+ Serf2−/−) in two different AD mouse models. In the "APPPS1" strategy, the brain-specific Serf2 knockout mice will be mated with the APPPS1 mice and focus on histological and immunohistochemical analyses at both 1 and 3 months of age. In the "APPswe/PSEN1dE9" strategy, the brain-specific Serf2 knockout mice will be mated with the APPswe/PSEN1dE9 mice. A set of behavioral tests will be performed parallel to histological and immunohistochemical analyses to 3 to 4 and 6 to 7-month old mice.
Outlook
In this thesis, we have demonstrated that aggregation-prone proteins influence ncRNA homeostasis, thereby introducing the concept that aggregation-prone proteins have effects that go beyond protein homeostasis. This demonstrates that it may be worth examining how the non-coding genome is altered in neurodegenerative diseases. We have also identified a new aggregation-promoting factor and hope to have uncovered yet another mechanism exemplifying how protein homeostasis can go awry during pathogenesis. Finally, we have provided a starting point for translational research, in which we intend to explore the possibility of a genetic modifier – discovered in *C. elegans* and conserved throughout evolution – being a potential therapeutic target for neurodegenerative diseases.
References


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Aging-associated neurodegenerative diseases – including Alzheimer’s, Parkinson’s and polyglutamine diseases – are projected to be among the top four causes of burden of disease by 2030 (World Health Organization, 2006). This demands investment in biomedical research to find disease-modifying treatments and to improve quality of life in the long term.

One of the hallmarks of neurodegenerative diseases is the presence of protein aggregates in the brains of affected patients. At the cellular and molecular level, protein aggregation results from the disruption of protein homeostasis, which is essential to maintain the correct synthesis, folding, shuttling and clearance of proteins in the cell. One major hurdle in the field is that we do not know why protein aggregation occurs and how it correlates to pathogenesis. What we do know is that specific aggregation-prone proteins have been identified for the different neurodegenerative diseases, and genetic studies have enabled the identification of key mutations in their corresponding genes. Currently, efforts are being made at the genomic, transcriptomic and proteomic level to understand what drives protein aggregation and how it is mechanistically linked to disease.

In this thesis, my motivation was to understand what drives protein aggregation and toxicity. Specifically, my aim was to discover what cellular mechanisms occur as a reaction to the presence of an aggregation-prone protein in the cell. In Chapter II, we provide a comprehensive overview of different cellular strategies used to cope with aggregation-prone proteins. We show how small model organisms – including yeast, nematodes, and flies – can be valuable tools to identify the genes involved in protein aggregation and toxicity. Importantly, we show that many of these genes are conserved in mice, allowing more complex studies that can be potentially extrapolated to humans. We then introduce an emerging concept – namely the contribution of the non-coding genome to neurodegeneration – and describe the progress made in this field towards a better understanding of the mechanisms of disease. We demonstrate how recently published work has revealed the link between RNA metabolism and neurological and neurodegenerative diseases, and describe how microRNAs, tRNAs and other types of non-coding RNA are dysregulated in these diseases.
One unresolved issue is the lack of a mechanistic explanation for how impaired RNA metabolism determines pathogenesis.

In Chapter III, we highlight the model organism *C. elegans* as a versatile tool for gene function studies. In particular, we focus on “humanized” models of *C. elegans* for neurodegenerative diseases – including Alzheimer’s, Parkinson’s and polyglutamine diseases – and describe how they can be used in forward genetic screens (with ethyl methane sulfate) and in reverse genetics (genome-wide RNAi screens). One aspect of these models worth mentioning is that protein aggregation can be uncoupled from protein toxicity. Next, we present a selection of genetic screens performed in *C. elegans* and show that the genetic modifiers of proteotoxicity identified in these screens are often involved in general cellular processes (e.g. protein quality control system, RNA metabolism, cell cycle), demonstrating that protein aggregation and toxicity can result from different levels of cellular dysregulation.

In line with this, Chapter IV describes our work that stemmed from a genetic screen performed in a *C. elegans* model for polyglutamine aggregation aimed at finding genes that promoted protein aggregation and toxicity. This led to the discovery of the novel genetic modifier of aggregation *moag-2* (modifier of aggregation-2), a gene that when mutated suppresses protein aggregation up to 51%. Whole-genome sequencing revealed the causative gene of *moag-2* to be *lir-3*. We found that *moag-2/lir-3* encodes a protein with a predicted nuclear localization signal and two non-canonical zinc finger domains, which are homologous to those in the transcription factor for RNA Polymerase III A (TFIIIA). Indeed, our data revealed that MOAG-2/LIR-3 binds to the same genomic regions as does the RNA Polymerase III machinery in its regulation of the transcription of small non-coding RNAs, including small nuclear RNAs, small nucleolar RNAs and transfer RNAs. These small non-coding RNAs are largely involved in the regulation of gene expression or in the modification of other RNA molecules. Once we had determined the function of MOAG-2/LIR-3, we were surprised to learn that MOAG-2/LIR-3 promotes polyglutamine aggregation in a manner that is independent of its function as a transcriptional regulator. We thereby propose a scenario where aggregation-prone proteins can trigger a switch of function in cellular proteins and hijack
them to promote aggregation. Another unprecedented finding from this work is that the presence of polyglutamine expansion proteins induces the downregulation of snRNA, snoRNA and tRNA genes in wild type animals. Although we do not yet know the biological consequences of this observation, it suggests that aggregation-prone proteins can also affect the homeostasis of non-coding RNA.

In Chapter V, we explore the transcriptional changes that occur in the cell when it is exposed to aggregation-prone proteins. By performing whole-transcriptome profiling in a *C. elegans* model of polyglutamine diseases, we found that the expression of aggregation-prone proteins delays animal development and, at the same time, triggers genes involved in cellular stress responses.

One important aspect of identifying modifiers of aggregation in small model organisms is their subsequent validation in human cells and mouse models. Previous work from our group identified MOAG-4/SERF as a positive regulator of proteotoxicity in *C. elegans* models of polyglutamine disease, Parkinson's and Alzheimer's disease. In Chapter VI, we describe the generation of a knockout mouse for SERF2, one of the human orthologs of MOAG-4. We found that eliminating Serf2 is not favorable for mouse development, as it frequently results in embryonic lethality. We then explain how this finding led us to generate a brain-specific Serf2 knockout mouse instead, which yielded viable and fertile animals. We further validate the potential use of these knockout mice by demonstrating that SERF2 was successfully eliminated from the brain without affecting its expression in other organs. Finally, we propose that these brain-specific Serf2 knockout mice be used to further study the role of SERF2 as a modifier of aggregation in the brain by mating it with mouse models of neurodegenerative diseases (Chapter VII).

In this thesis, we have identified a new genetic regulator of protein aggregation. The findings described here will improve not only the exploration of new mechanisms but also our understanding of the complex protein aggregation process. This work has also provided a starting point for transposing findings from small model organisms to mammalian systems, paving the way for translational research in the long term.
Samenvatting
Samenvatting

Volgens voorspellingen van de World Health Organization dringen neurodegeneratieve ziekten, zoals de ziekten van Alzheimer en Parkinson, in 2030 door tot de top vier van de aandoeningen die de meeste ziektekosten veroorzaken (World Health Organization, 2006). Biomedisch onderzoek naar aangrijpingspunten voor therapie en naar mogelijkheden om de kwaliteit van leven te verbeteren is daarom van groot belang.

Een van de kenmerken van neurodegeneratieve ziekten is de aanwezigheid van eiwitaggregaten in de hersenen van getroffen patiënten. Op cellulair en moleculair niveau wordt eiwitaggregatie veroorzaakt door een verstoring van de eiwithomeostase in de cel, normaal een samenspel van controle op de synthese, de vouwing, het transport en de afbraak van eiwitten in de cel. Een belangrijk probleem in het veld is dat we niet weten wat eiwitaggregatie aandrijft of hoe het verband houdt met de pathogenese. Wat we wel weten is dat er bij de verschillende neurodegeneratieve ziekten, specifieke aggregatie-gevoelige eiwitten een rol spelen en dat mutaties in hun overeenkomstige genen de oorzaak zijn van erfelijke varianten van deze ziekten. Momenteel wordt veel genetisch, celbiologisch en biochemisch onderzoek gedaan om te achterhalen wat de oorzaak is van de toxiciteit en aggregatie van deze ziekte-eiwitten.

Het doel van mijn proefschrift was om te begrijpen welke biologische processen eiwitaggregatie en toxiciteit aandrijven en welke cellulaire mechanismen optreden als reactie op de aanwezigheid van aggregerende eiwitten in de cel. In Hoofdstuk II, geven we een uitgebreid overzicht van de verschillende cellulaire strategieën die gebruikt worden om aggregatie-gevoelige eiwitten aan te pakken. We laten zien hoe kleine modelorganismen – zoals gist, nematoden, en vliegen – waardevol kunnen zijn om genen te ontdekken die betrokken zijn bij eiwitaggregatie en de toxiciteit. We laten zien dat veel van deze genen evolutionair geconserveerd zijn in zoogdieren, wat betekent dat de vindingen potentiële kunnen worden geëxtrapoleerd naar mensen. Vervolgens introduceren we de bijdrage van het niet-coderende genoom aan neurodegeneratie en beschrijven recente inzichten op dit gebied. Wij beschrijven de relatie tussen RNA metabolisme en neurologische en neurodegeneratieve ziekten, en geven een overzicht van de betrokkenheid
van microRNAs, tRNA’s en andere vormen van niet-coderende RNA bij deze ziekten. Er is nog geen mechanistische verklaring voor hoe een verstoord RNA metabolisme bijdraagt aan de pathogenese.

In **Hoofdstuk III**, hebben we aandacht voor het modelorganisme *C. elegans* als een veelzijdig diermodel om de functie van genen te ontdekken. In het bijzonder richten we ons op “gehumaniseerde” *C. elegans* modellen voor neurodegeneratieve ziekten en hoe ze kunnen worden gebruikt in genetische screens. Interessant is dat in deze modellen eiwitaggregatie en toxiciteit losgekoppeld kunnen zijn. Vervolgens presenteren we een selectie van genetische screens die zijn uitgevoerd in *C. elegans* en die hebben aangetoond dat de genetische regulatoren van eiwitaggregatie en toxiciteit vaak betrokken zijn bij algemene cellulaire processen (bijvoorbeeld eiwithomeostase, RNA stofwisseling, celcyclus), waaruit blijkt dat eiwitaggregatie en toxiciteit kunnen voortvloeien uit verschillende niveaus van cellulaire ontregeling.

**Hoofdstuk IV** beschrijft een vervolg op een genetische screen in een *C. elegans* model voor polyglutamine aggregatie: de identificatie van de genetische mutant *moag-2* (modifier of aggregation-2) die eiwitaggregatie voor meer dan 50% vermindert. Met behulp van genoom sequencing hebben we gevonden dat de mutatie in *moag-2* ligt in het gen *lir-3*, waarvan de functie onbekend is. MOAG-2/LIR-3 lijkt te coderen voor een eiwit met een nucleair lokalisatiesignaal en twee zinkvinger domeinen die verwant zijn aan de zinkvinger domeinen in de transcriptiefactor voor RNA Polymerase IIIA (TFIIIA). Met behulp van een eiwit-DNA interactiestudie, vonden we dat MOAG-2/LIR-3 bindt aan dezelfde DNA sequenties als RNA Polymerase III en ook de transcriptie reguleert van kleine niet-coderende RNA’s, waaronder kleine nucleaire RNA’s (snRNAs), kleine nucleolaire RNA’s (snoRNAs) en transfer RNA’s (tRNAs). Deze kleine RNA’s zijn betrokken bij de regulatie van genexpressie of bij de bouw van andere RNA-moleculen. We waren verrast dat MOAG-2/LIR-3 polyglutamine aggregatie aanstuurt op een manier die onafhankelijk is van zijn functie als regulator van transcriptie. Aggregatie-gevoelige ziekte-eiwitten lijken dus op twee manieren een schadelijk effect te kunnen hebben: ze inactiveren belangrijke cellulaire eiwitten en gebruiken deze om hun eigen aggregatie te versterken. Een andere verrassende ontdekking was dat de aanwezigheid van polyglutamine de
hoeveelheid snRNA, snoRNA en tRNA reduceerde in de worm. Hoewel we nog niet weten wat de biologische gevolgen hiervan zijn en of dit ook te maken heeft met moag-2/lir-3, suggereren de resultaten dat aggregatie-gevoelige eiwitten invloed hebben op de homeostase van niet-coderende RNAs.

In Hoofdstuk V verkennen we de transcriptionele veranderingen die optreden in *C. elegans* wanneer deze aggregerende eiwitten aanmaken. Met behulp van een transcriptoom analyse in een *C. elegans* model van polyglutamine ziekte, vonden we een effect op de expressie van genen die betrokken zijn bij groei en ontwikkeling en op genen die betrokken zijn bij cellulaire reactie op stress.

Om te bepalen of modifiers van aggregatie in kleine modelorganismen ook een rol spelen bij ziekten, is het belangrijk om eerst hun rol in menselijke cellen en zoogdiermodellen te onderzoeken. Eerder identificeerde we MOAG-4/SERF als positieve regulator van eiwitaggregatie en toxiciteit in *C. elegans* modellen voor polyglutamine, Parkinson en Alzheimer. Hoofdstuk VI beschrijft hoe we een knockout muis hebben gemaakt voor SERF2, één van de humane orthologen van MOAG-4. We vonden dat het uitschakelen van SERF2 niet gunstig is voor ontwikkeling van de muis, omdat het meestal leidt tot embryonale letaliteit. We leggen uit hoe we vervolgens een brein-specifieke SERF2 knockout muis hebben gemaakt die wel levensvatbaar en vruchtbaar is. We laten zien dat we SERF2 met succes uit de hersenen hebben gehaald, zonder dat de expressie in andere organen is aangetast. Ten slotte stellen wij voor dat deze hersenspecifieke SERF2 knockout muizen kunnen worden gebruikt voor het verder bestuderen van de rol van SERF2 als modifier van aggregatie in de hersenen door ze genetisch te combineren met muismodellen voor neurodegeneratieve ziekten (Hoofdstuk VII).

Met de identificatie van een nieuwe genetische regulator van eiwitaggregatie opent dit proefschrift mogelijkheden voor onderzoek naar mechanismen die eiwitaggregatie en toxiciteit in cellen kunnen verklaren. Bovendien biedt het met de generatie van een muismodel een uitgangspunt voor het vertalen van bevindingen in *C. elegans* naar zoogdiermodellen voor neurodegeneratieve ziekten, om in de toekomst toe te werken naar translationeel onderzoek.
Resumo
A doença de Alzheimer, a doença de Parkinson e as doenças de expansão de poliglutaminas são doenças neurodegenerativas que se prevê virem a ser a quarta principal causa de custos de saúde em 2030 (World Health Organization, 2006). É, portanto, necessário investir na investigação biomédica destas patologias de forma a encontrar tratamentos que permitam melhorar a qualidade de vida a longo prazo.

Uma característica diagnosticante das doenças neurodegenerativas é a presença de agregados proteicos no cérebro. Estas estruturas resultam de uma incapacidade, a nível celular e molecular, da manutenção da homeostase proteica, que decorre por sua vez de disfunções em vias envolvidas na síntese, folding, tráfego e degradação de todas as proteínas celulares. Ainda não se conhece a razão por que ocorre a agregação proteica observada, e de que modo é que esta última está relacionada com a patologia. São, no entanto, conhecidas, para determinadas doenças neurodegenerativas, as proteínas que têm predisposição para aggregator (as chamadas aggregation-prone proteins) e, através de estudos genéticos, já foram identificadas mutações nos respectivos genes que determinam o desenvolvimento da patologia. Vários estudos realizados a nível genómico, transcriptómico e proteómico estão a ser efetuados no sentido de compreender os fatores envolvidos na agregação proteica e de que modo é que esta está mecanisticamente relacionada com a patologia.

O objectivo principal desta tese foi o estudo dos mecanismos que promovem a agregação e toxicidade proteica em doenças neurodegenerativas. Mais concretamente, procurou-se compreender como é que a célula reage perante a presença de proteínas com predisposição para agregar. O Capítulo II descreve as diferentes estratégias celulares que existem para lidar com este tipo de proteínas. Nesse capítulo também são sublinhadas as vantagens de utilizar modelos animais — nomeadamente leveduras, nemátodes e moscas — como ferramentas versáteis para descobrir genes (ou moduladores genéticos) envolvidos na agregação e toxicidade proteica. Muitos dos genes descobertos nestes organismos estão conservados em ratinhos, o que permite estudos mais complexos e cujas descobertas poderão ser potencialmente aplicáveis a humanos. No Capítulo II, explora-se ainda o conceito emergente da contribuição do genoma não-codificante para a neurodegeneração, e descreve-
se o progresso realizado nesta área de investigação. São apresentados vários exemplos recentes onde se demonstra a relação entre o metabolismo de RNA e doenças neurológicas e neurodegenerativas e, mais concretamente, descreve-se como é que disfunções em microRNAs, tRNAs e outros tipo de RNAs se correlacionam com a patologia. A contribuição das disfunções no metabolismo do RNA para o desenvolvimento da patologia e os mecanismos envolvidos são questões que permanecem em aberto.

No Capítulo III, apresenta-se o modelo animal *C. elegans* como uma ferramenta para descobrir a função de genes. Explora-se como se pode tirar partido de modelos de *C. elegans* para as doenças de Alzheimer, Parkinson ou Huntington, e descreve-se como podem ser utilizados em screens genéticos (utilizando etil metano sulfonato ou RNA de interferência). Um aspeto a mencionar é que, nestes modelos, a agregação e toxicidade proteicas são fenótipos que podem ser analisados independentemente um do outro. Ainda no Capítulo III são apresentados resultados de screens genéticos nos quais se observa que moduladores genéticos de proteotoxicidade estão frequentemente associados a funções metabólicas gerais (sistema de controlo de qualidade proteica, metabolismo de RNA, ciclo celular, etc.), demonstrando que a agregação e toxicidade proteica podem resultar de diferentes níveis de disfunção celular.

O Capítulo IV descreve o trabalho desenvolvido a partir de um screen genético num modelo de *C. elegans* para doenças de expansão de poliglutaminas. Neste screen procuraram-se genes que promovem a agregação e toxicidade proteica, usando a poliglutamina como exemplo de uma proteína com predisposição para agregar. Neste capítulo descreve-se também a descoberta do modulador genético *moag-2* (*modifier of aggregation-*2) que, quando mutado, reduz a agregação proteica até 51%. Após sequenciação, descobriu-se que o modulador genético *moag-2* corresponde ao gene *lir-3*. O gene *moag-2/lir-3* codifica uma proteína de função desconhecida, mas a sua sequência de aminoácida contém um sinal de localização nuclear e dois domínios com “dedos” de zinco (*zinc fingers*) homólogos aos do factor de transcrição para a RNA Polimerase III A (TFIIIA). Neste estudo, descobriu-se ainda que a proteína MOAG-2/LIR-3 se liga aos mesmos locais genómicos
que a maquinaria da RNA Polimerase III para transcrição de pequenos RNAs não-codificantes — entre os quais se incluem os small nuclear RNA (snRNA); small nucleolar RNAs (snoRNAs) e RNAs de transferência (tRNA). A função destes pequenos RNAs não-codificantes é a regulação da expressão genética ou modificação de outras moléculas de RNA. Curiosamente, descobriu-se que a proteína MOAG-2/LIR-3 promove a agregação de poliglutaminas de uma forma que é independente da sua função como factor de transcrição. Assim, proteínas com predisposição para agarrar conseguem sequestrar e converter proteínas celulares normais em factores que promovem agregação proteica. Uma descoberta adicional deste trabalho foi que proteínas com predisposição para agarrar reduzem a abundância de pequenos RNA não-codificantes em animais wild type, nomeadamente snRNAs, snoRNAs e tRNAs. Apesar de serem desconhecidas as consequências biológicas desta observação, os resultados sugerem que proteínas com predisposição para agarrar também podem influenciar a homeostase de RNAs não-codificantes.

O Capítulo V descreve as alterações trascricionais que ocorrem quando células expressam proteínas com predisposição para agregação proteica. Ao comparar o perfil transcriptómico de animais wild type com animais que expressam poliglutamina, descobriu-se que esta atrasa o desenvolvimento animal — consistentemente com observações anteriores de que estes últimos demoram normalmente mais tempo a atingir o mesmo estádio de desenvolvimento do que animais wild type. Resultados obtidos pelo mesmo estudo revelaram ainda quais são as vias de sinalização activadas como consequência da presença de poliglutaminas. Mostra-se também que a expressão de proteínas com predisposição para agarrar induz a activação da unfolded protein response (UPR), uma das principais vias envolvidas na manutenção da homeostase proteica.

Uma vez identificados moduladores em modelos animais como leveduras, nemátodes ou moscas, é necessário validar a sua função em mamíferos ou cultura de células humanas. A proteína MOAG-4/SERF foi identificada, em trabalho previamente publicado pelo nosso grupo, como um modulador genético de agregação proteica em modelos de C. elegans para a doença de Alzheimer, de Parkinson e de expansão de poliglutaminas. O Capítulo VI,
descreve a geração de um modelo de murganho com deleção (*knockout*) do gene SERF2, um dos homólogos de MOAG-4. Descobriu-se que a deleção de SERF2 não é favorável para o desenvolvimento animal, uma vez que resultou frequentemente em letalidade embrionária com penetrância incompleta. Esta observação levou a que se tentassem gerar mutantes condicionais nos quais a expressão de SERF2 é eliminada apenas no cérebro. Esta estratégia foi bem sucedida uma vez que se obtiveram mutantes condicionais viáveis e férteis. Mostra-se ainda que a deleção de SERF2 do cérebro foi conseguida sem afectar a sua expressão noutros órgãos. Finalmente, propomos que estes mutantes condicionais possam ser cruzados com modelos de murganho para a doença de Alzheimer, o que permitirá estudar a função de SERF2 como modulador de agregação proteica na doença de Alzheimer (*Capítulo VII*).

Em suma, esta tese descreve a descoberta e caracterização de um novo modulador genético para a agregação proteica e propõe mecanismos alternativos que podem ajudar a compreender melhor a complexidade do processo de agregação proteica. Paralelamente, o trabalho aqui apresentado deu início ao processo de validação de um modulador genético descoberto em *C. elegans* e a sua transposição para modelos de Alzheimer em murganhos, abrindo caminho a investigação translacional.
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List of publications
