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A New Role for Laminins as Modulators of Protein Toxicity in *Caenorhabditis elegans*

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Summary

Protein misfolding is a common theme in aging and several age-related diseases such as Alzheimer's and Parkinson's disease. The processes involved in the development of these diseases are many and complex. Here, we show that components of the basement membrane, particularly laminin, affect protein integrity of the muscle cells they support. We knocked down gene expression of *epi-1*, a laminin α-chain, and found that this resulted in increased proteotoxicity in different *Caenorhabditis elegans* transgenic models expressing aggregating proteins in the body wall muscle. The effect could partially be rescued by decreased insulin-like signaling, known to slow the aging process and the onset of various age-related diseases. Our data points to an underlying molecular mechanism involving proteasomal degradation and HSP-16 chaperone activity. Furthermore, *epi-1* depleted animals had altered synaptic function and displayed hypersensitivity to both levamisole and aldicarb, an acetylcholine receptor agonist and an acetylcholinesterase inhibitor, respectively. Our results implicate the basement membrane as an extracellular modulator of protein homeostasis in the adjacent muscle cells. This is in agreement with previous research showing that imbalance in neuromuscular signaling disturbs protein homeostasis in the postsynaptic cell. In our study, proteotoxicity may indeed be mediated by the neuromuscular junction which is part of the basement membrane, where laminins are present in high concentration, ensuring the proper microenvironment for neuromuscular signaling. Laminins are evolutionarily conserved and thus the basement membrane may play a much more causal role in protein misfolding diseases than currently recognized.

Keywords

amyloid-beta; α-synuclein; neuromuscular junction; protein aggregation; neurodegenerative disease; extracellular matrix

Introduction

Protein aggregation is a hallmark of a range of neurodegenerative diseases, where deposits of beta-sheet structured proteins form in the brain, e.g. amyloid-beta (Aβ) in Alzheimer's disease (AD) and α-synuclein in Parkinson's disease (PD) (Stefani & Dobson 2003; Lashuel & Lansbury 2006). The nematode *Caenorhabditis elegans* (*C. elegans*) has proven an

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excellent model system in which aging and age-related diseases can be studied and many genes, signaling cascades and biological processes that can delay the aging process have been identified (Antebi 2007; van Ham *et al.* 2009; Harrington *et al.* 2010; Kenyon 2010). Various disease models in *C. elegans* have been made to mimic age-related human neurodegenerative diseases, including AD (Link 1995; Kraemer *et al.* 2003), PD (Cao *et al.* 2005; Kuwahara *et al.* 2006; Gitler *et al.* 2008; Kuwahara *et al.* 2008; van Ham *et al.* 2008) and Huntington's disease (Parker *et al.* 2001; Morley *et al.* 2002; Nollen *et al.* 2004). Interestingly, many of the interventions that extend lifespan also delay the onset of disease in these models (Morley *et al.* 2002; Hsu *et al.* 2003; Cohen *et al.* 2006). One widely used *C. elegans* model for AD is a transgenic worm where human Aβ is expressed in body wall muscles, leading to a progressive paralysis with age (Link 1995). Although Aβ is expressed intracellularly in muscle rather than nervous system, this model has identified several molecular mechanisms and molecules involved in Aβ processing and toxicity such as insulin signaling (Cohen *et al.* 2006; Cohen *et al.* 2009), heat shock proteins (HSPs) (Fonte *et al.* 2008), AIP-1 (Hassan *et al.* 2009), tetracycline (Diomede *et al.* 2010), and Thioflavin T (Alavez *et al.* 2011). PD models where α-synuclein is expressed in body wall muscles are also available and like the AD model, these have successfully contributed to our understanding of the genetics behind α-synuclein toxicity (Hamamichi *et al.* 2008; van Ham *et al.* 2008). These models have the advantage of GFP or YFP fused expression of α-synuclein enabling the forming aggregates to be monitored easily with age. However, for some reason these strains overexpressing α-synuclein do not exhibit age-dependent paralysis as observed for the AD model.

In multicellular organisms all tissues are covered with a structure of extracellular matrix (ECM) called the basement membrane (BM). Besides providing structural support to the tissues, the BM is involved in cell polarization, survival and tissue organization (Li *et al.* 2003). The composition and function of the BM is well conserved across species (Hutter *et al.* 2000) and in *C. elegans* and other animals, it consists of collagen IV, laminins and associated proteins (Table 1,2). Laminins are evolutionarily conserved cross-like heterotrimeric molecules composed by α-, β- and γ-chains (Table S1). In *C. elegans* laminin comes in two different isoforms, made from three of the four present laminin genes, *lam-1* (β), *lam-2* (γ), *lam-3* (αA) and *epi-1* (αB), while in vertebrates, there are eleven genes, giving rise to several additional isoforms (Kramer 2005).

Laminin is essential for assembly of the BM and cell polarization (Huang *et al.* 2003; Li *et al.* 2003). Furthermore, the BM is critical for proper formation of the neuromuscular junction (NMJ) between muscle cells and innervating neurons. In the NMJ, laminins are present in high concentrations and coordinate post-with pre-synaptic maturation (Noakes *et al.* 1995; Patton *et al.* 2001; Dixon *et al.* 2006; Nishimune *et al.* 2008). In *C. elegans* it has previously been shown that alterations in the signaling from neurons to the postsynaptic muscle cells affect protein homeostasis in the muscle cell, leading to aggregation of polyglutamine as well as metastable proteins not related to disease (Garcia *et al.* 2007). We therefore speculated whether disturbance of laminins could mediate such protein folding effects in muscle cells through their role in the development and support of the NMJ. We here report that inactivation of laminins indeed confers proteotoxicity in *C. elegans* when assayed using different transgenic models where toxic aggregating proteins are expressed in body wall muscles.

Results

Laminins modulate Aβ toxicity

We found that inactivation of *epi-1* using RNAi caused sterility and higher frequency of vulva explosions which is in agreement with previous studies of *epi-1* mutants (Huang *et al.*

2003). Using the transgenic reporter strain for basement membrane integrity IM253 expressing GFP fused to LAM-1 (β-chain) (Kao *et al.* 2006; Hagedorn *et al.* 2009) we confirmed that RNAi against *epi-1* also caused disruption of the basement membrane (Fig. 1A). We then evaluated the effect of *epi-1* RNAi on Aβ toxicity by studying paralysis in the *C. elegans* strain CL2006 (hereafter Aβ-worms) expressing Aβ in body wall muscle (Link 1995). Knock down of *epi-1* expression significantly accelerated the onset of paralysis in Aβ-worms ($P < 0.0001$, $PT_{50} = 8$ days (time at which 50% were paralyzed), Fig. 1B) compared to control Aβ-worms fed empty vector (EV) RNAi ($PT_{50} > 14$ days, Fig. 1B). The Aβ-strain has a morphological marker caused by a mutation in the body wall muscles, making the worms roll and move in circular paths, termed roller mutants. This may result in a differential uptake of food (RNAi bacteria) compared to wild type worms and thus may influence the penetrance of the paralysis phenotype. To control for this effect, we used a non-Aβ expressing strain with a roller mutation as control in the paralysis assays rather than the traditional wild type N2 strain. Inactivation of *epi-1* in these control worms resulted in a slightly age-related uncoordinated (Unc) phenotype and eventually paralysis (Fig. 1B), also previously reported by others (Huang *et al.* 2003; Dixon *et al.* 2006). However, the onset of paralysis was significantly delayed in these control worms with no transgenic expression of aggregation prone proteins compared to Aβ-worms (Fig. 1B) as it took 11 days before 50% of the tested control worms were paralyzed ($P < 0.0001$). Paralysis and lack of movement is also observed with age in wild-type worms and even in long lived mutants (Collins *et al.* 2008). This suggests that *epi-1* RNAi is enhancing a naturally occurring process. In *C. elegans* early embryonic lethality is often seen when ECM proteins are mutated. For example *pat-3* mutants die as early embryos due to defects in muscle cell attachment to the ECM and muscle filament assembly (Hresko *et al.* 1994). We speculated that defective muscle attachment could perhaps explain paralysis following RNAi of *epi-1*. However, phalloidin staining did not reveal any differences in muscle structure and attachment between EV control worms and *epi-1(RNAi)* worms neither in young worms (4 days old) nor in paralyzed worms (7 and 8 days old) (Fig. S1). This is consistent with our finding that paralysis due to RNAi of *epi-1* occurs with age and not immediately during early development.

To establish if increased Aβ toxicity was specific for *epi-1* we inactivated the three other laminin genes in *C. elegans*, *lam-1*, *lam-2* and *lam-3*, using RNAi. When either *lam-1* or *lam-2* expression was knocked down the onset of paralysis was significantly ($P < 0.0001$) earlier compared to worms fed EV control RNAi as seen for *epi-1* RNAi (Fig. 1C). Since *lam-3* is not expressed in the basement membrane of muscle cells (Huang *et al.* 2003) we did not expect to find any effect on paralysis for this gene. Indeed, *lam-3* RNAi was not significantly different from EV ($P = 0.6965$, Fig. 1C). Inactivation of both *lam-1* and *lam-2* simultaneously by feeding the worms with both bacteria at the same time (double feeding) did not result in a stronger phenotype (data not shown). Inactivation of multiple genes using RNAi is often ineffective (Gonczy *et al.* 2000) which is likely also to be the case for double feeding of *lam-1* and *lam-2* RNAi. Since the penetrance of the paralysis phenotype of *lam-1* and *lam-2* was quite variable between experiments we primarily focused our studies on *epi-1* RNAi whose resulting phenotypes (paralysis, sterility) were always reproducible. We expect that results from *lam-1* and *lam-2* RNAi would yield similar results as *epi-1* RNAi since the functional laminin unit in muscle BM is a heterotrimer of these subunits (Kao *et al.* 2006).

Reduced insulin signaling can rescue paralysis caused by *epi-1* RNAi

The progressive and age-related nature of the paralysis observed following *epi-1* knock down made us hypothesize that lifespan extending mutations should be able to ameliorate paralysis. The insulin/IGF-1 signaling pathway is one of the best characterized signaling cascades known to influence lifespan both in *C. elegans* and higher organisms (Kenyon *et*

al. 1993; Bluher *et al.* 2003; van Heemst *et al.* 2005). The insulin/IGF-1 receptor homolog DAF-2 negatively regulates the transcription factor DAF-16 (homolog of the human Forkhead FOXO transcription factor) which in turn regulates multiple stress responsive genes (McElwee *et al.* 2003; Murphy *et al.* 2003; Oh *et al.* 2006). Inhibition or mutation of DAF-2 gives stress resistant and long lived worms (Kenyon *et al.* 1993; Lithgow *et al.* 1995), and in Aβ-worms, *daf-2* RNAi delays the onset of disease (Cohen *et al.* 2006). We confirmed that *daf-2(e1368)* mutants expressing Aβ display a significantly delayed onset of paralysis ($P < 0.0001$, $PT_{50} >> 17$ days, Fig. 2A) compared to control Aβ-worms ($PT_{50}= 13$ days). Importantly, we also found that the *daf-2(e1368)* mutation partially rescued the accelerated paralysis resulting from *epi-1* knockdown, where PT_{50} was delayed from 9 days for Aβ-worms on *epi-1* RNAi, to 11 days for Aβ;*daf-2(e1368)* double mutants ($P < 0.0001$, Fig. 2A).

The *daf-2(e1368)* mutation is a class 1 allele located in the L2 domain of the extracellular part of the insulin/IGF-1 receptor DAF-2. To verify that the protection was indeed mediated via reduced insulin signaling we also examined the class 2 allele *daf-2(e1370)* found in the kinase domain of the intracellular part of the DAF-2 receptor. We found the *daf-2*(*e1370)* allele also protects against accelerated paralysis resulting from *epi-1* knockdown with $PT_{50} = 10$ for Aβ-worms on *epi-1* RNAi compared to 13 days for Aβ; *daf-2(e1370)* double mutants ($P < 0.0001$, Fig.2B). Longevity and stress resistance following reduced insulin signaling is dependent of the FOXO transcription factor DAF-16 (Lin *et al.* 1997; Ogg *et al.* 1997) and we therefore predicted that DAF-16 would be required for protection against paralysis. Indeed we found, that a null mutation (*mu86*) of *daf-16* completely abolishes the protective effects of reduced insulin signaling on paralysis ($P < 0.0001$, Fig. 2B). In fact, we found that lack of DAF-16 causes an earlier onset of paralysis in both control Aβ-worms, in agreement with previously reported studies (Cohen *et al.* 2006), and *epi-1* RNAi treated worms (Fig. 2B). These results indicate that genes in the insulin signaling pathway are able to buffer the deleterious effects caused by *epi-1* depletion. This further supports the notion that paralysis is not simply due to developmental defects or a general frailty of the worms but rather that *epi-1* RNAi is enhancing a naturally occurring process related to aging.

epi-1 knockdown results in higher concentration of small molecular Aβ bands

To test if the strong effect of *epi-1* RNAi on Aβ toxicity (estimated as paralysis) correlated with Aβ levels, we performed Western blotting using anti-Aβ antibody 6E10 of *epi-1* or EV RNAi worms 7 days old. Several Aβ-species in *epi-1* RNAi worms were upregulated compared to EV, especially low molecular weight Aβ-bands possibly corresponding to Aβ monomers (~5 kD) (Fig. 3 shows a blot representative of three independent experiments). Anti-Aβ immunostaining of whole animals of the same age and treatment did not reveal any qualitative differences in the distribution or expression of aggregates (Fig. S2). This indicates that the observed paralysis is not due to presence of more total Aβ, but rather a disturbance of the processing of Aβ and the resulting differences in Aβ species. It is currently unclear if soluble oligomeric Aβ species or Aβ aggregates are causing paralysis in this model since both have been implicated (Fay *et al.* 1998; Link 2001; Cohen *et al.* 2006; Diomede *et al.* 2010).

epi-1 RNAi affects α-synuclein distribution and accelerates paralysis in a GFP::degron model

In order to investigate if protein aggregation was affected more generally by knock down of *epi-1* we turned to a transgenic model expressing α-synuclein fused to YFP in body wall muscle. In this model an increasing number of immobile α-synuclein inclusions is observed with age (van Ham *et al.* 2008). Several genes and biochemical pathways have been identified that can either enhance or prevent formation of α-synuclein inclusions in this

model, for example *sir-2.1* (van Ham *et al.* 2008). We found that *epi-1* depletion lead to a lower number of small inclusions per worm than in empty vector controls, while large inclusions were more prevalent (Fig. 4 A,C) (Mann-Whitney U-test, $P = 0.005$ for same # of inclusions below 15 μm^2 and $P < 0.0009$ for same # of inclusions above 15 μm^2), with the total level of α-synuclein unaltered as verified by Western blotting (Fig. 4B). In fact, nearly all examined *epi-1* (RNAi) treated worms had inclusions larger than 50 μm^2, while such large inclusions were not found in any of the control worms. As for the results in the Aβ-worms, this suggests that RNAi of *epi-1* interferes with the distribution of proteins rather than total amount. To further study the effect of *epi-1* on the toxicity of aggregating proteins, we applied a model of toxic protein aggregation, where GFP with a C-terminal 16 residue addition, "degron peptide" (Bence *et al.* 2001) is expressed in body wall muscle of *C. elegans* (Link *et al.* 2006). This leads to formation of stable aggregates and rapid paralysis and thus may serve as an aggregating control protein. Consistent with our Aβ and α-synuclein results we find that the GFP::degron induced paralysis is also significantly ($P < 0.0001$) exacerbated due to RNAi of *epi-1* RNAi compared to EV controls (Fig. 4D).

epi-1 depletion impairs cellular responses to aggregating proteins

Cellular survival and integrity depend on the proper folding of proteins. Several defense mechanisms have evolved to protect against protein damage, these include refolding and repair by chaperones, the unfolded protein response (UPR) as well as removal by processes such as autophagy and proteasomal degradation (Gregersen & Bross 2010; Haigis & Yankner 2010). The expression of the small heat shock protein HSP-16 is controlled by the FOXO transcription factor DAF-16 (McElwee *et al.* 2003; Murphy *et al.* 2003) as well as the transcription factor HSF-1 (GuhaThakurta *et al.* 2002). Both DAF-16 and HSF-1 are implicated in the aging process (Link *et al.* 1999; Walker *et al.* 2001), maintenance of protein homeostasis (Ben-Zvi *et al.* 2009; David *et al.* 2010) as well as Aβ toxicity (Cohen *et al.* 2006). Since HSP-16 is upregulated and colocalize with Aβ in the Aβ-worms (Fonte *et al.* 2002) we speculated whether the proteotoxic effects of *epi-1* RNAi are caused by a failure to fully mount the HSP-16 response and/or other defense mechanisms. To address this issue, we used reporter strains expressing DAF-16 fused to GFP and GFP expressed under the promotor of *hsp-16.2*. Under normal conditions, DAF-16::GFP is mostly localized in the cytoplasm, but upon stress, DAF-16::GFP will relocate to the nucleus. Reducing the expression of *epi-1* by RNAi did not cause any detectable nuclear accumulation of DAF-16::GFP nor induction of *hsp-16.2* (not shown). When challenged with heat stress, *epi-1* RNAi did not affect the ability to relocate DAF-16 to the nucleus, but *hsp-16.2*::GFP expression in *epi-1(RNAi)* worms was significantly lower than EV (t-test, $P < 0.0001$, day 4, Fig. 5A,B). This shows that *epi-1* depletion interferes with heat stress induction of *hsp-16.2*. If *hsp-16.2* induction is compromised we would expect *epi-1(RNAi)* worms to be heat sensitive. Indeed, we found that *epi-1* RNAi resulted in significantly heat sensitive worms when compared to EV controls (Fig. 5C, $P < 0.0001$).

The UPR of the ER responds to the presence of misfolded proteins also when present outside the ER (Rao & Bredesen 2004). We therefore also assayed ER stress using a *hsp-4*::GFP reporter. HSP-4 is part of the unfolded protein response (UPR) and as such a marker for ER stress (Urano *et al.* 2002). The GFP-intensity in *epi-1* knocked down worms was not significantly different from EV (Day 4: t-test, $P = 0.989$; Day 6: $P = 0.0643$, not shown). The proteasome is a large protein complex responsible for the degradation of misfolded proteins by proteolysis. Due to the effects on proteotoxicity and *hsp-16.2* response, we wished to study if this correlates with proteasome activity. We therefore estimated the proteasome activity as chymotrypsin-like activity (Lima & Rattan 2010) in *epi-1* and EV RNAi treated worms of the RNAi sensitive strain *rrf-3(pk1426)*. The activity was measured in 3 days old worms, still unaffected by the *epi-1* RNAi treatment and in

worms 6 days old, when movement defects were starting to appear. We found that *epi-1* RNAi significantly reduced the activity of the proteasome over time (t-test, $P = 0.008$) while the activity in EV worms did not change during this time period (t-test, $P = 0.525$) (Fig. 5D). Thus, in addition to impaired expression of molecular chaperones, *epi-1* RNAi may also lead to impaired removal of damaged proteins by lowering proteasome activity.

Other basement membrane components do not induce Aβ specific paralysis except *fbl-1*

epi-1 knockdown has a massive effect on the BM, which is almost completely destroyed, resulting in a punctuated pattern, consistent with earlier reports (Kao *et al.* 2006)(Fig. 1A). Intriguingly, already at day 3 where there is no gross effect on movement, the BM as visualized by LAM-1::GFP is massively disturbed. These observations led us to speculate whether inactivation of other BM components or BM receptors have the same effect as the laminins. We therefore tested the effect of RNAi knockdown of the other major BM components and their receptors on the development of paralysis in Aβ-worms (Table 1,2). Paralysis caused by RNAi of fibulin (*fbl-1*, $P < 0.0001$, $PT_{50} = 14$, Fig. S3A), dystroglycan (*dgn-1*, $P < 0.05$, $PT_{50} = 13$, not shown), perlecan (*unc-52*), and the two integrins *pat-2* and *pat-3* were significantly (all $P < 0.0001$, $PT_{50} < 4$, Fig. S4) different from EV. Fibulin binds to laminins and nidogens of the BM (Kramer 2005) and the assembly of *fbl-1* at mechanosensory neuron attachments is dependent on *epi-1* (Muriel *et al.* 2006) , which may explain the observed effect on paralysis. Similarly, dystroglycan is a receptor for *epi-1* (Johnson *et al.* 2006). However, *dgn-1(RNAi)* induced paralysis in Aβ-worms was not significantly different from paralysis in control worms not expressing Aβ (not shown).

Interestingly, PAT-2 and PAT-3 have previously been suggested to interact with UNC-52 (Kramer 2005) as well as laminin to regulate muscle arm extension and thereby NMJ development in *C. elegans* (Dixon *et al.* 2006). However, although the effect on paralysis of *unc-52, pat-2* and *pat-3* RNAi was highly significant, it had a dramatic onset already at the time of early adulthood, in contrast to that of *epi-1* RNAi occurring later in life. This made us hypothesize that this early paralysis was due to severe developmental disorders caused by RNAi of *unc-52, pat-2* and *pat-3* during the larval stages. We therefore tested a control strain without Aβ expression as well as the Aβ;*daf-2(e1368)* double mutant under the prediction that the paralysis would not be Aβ specific and that a *daf-2* mutation should not be able to rescue the effect if caused by developmental defects. Indeed, we found this to be the case for all three genes ($PT_{50} < 4$ days. Fig. S4). We therefore believe that the *unc-52*, *pat-2* and *pat-3* RNAi induced paralysis phenotypes are different (i.e. caused by other factors and not age-related) than the progressive paralysis seen following RNAi of *epi-1*. The onset of paralysis of following RNAi of *fbl-1* and *dgn-1* is observed late in life and given the known interaction of these genes with *epi-1* we expected that the underlying mechanisms of paralysis would be similar. However, we found that reduced insulin signaling could only rescue inactivation of *dgn-1* (not shown) and not *fbl-1* (Fig S3B).In fact, Aβ;*daf-2(e1370)* double mutants treated with *fbl1-1* RNAi were paralyzed much earlier ($PT_{50}= 8$) than control Aβ;*daf-2(e1370)* worms ($PT_{50} >> 13$, $P < 0.0001$) and had pale and thin appearance suggesting a detrimental developmental effect.

epi-1 knockdown sensitizes worms to neuroactive drugs

Neuronal input to muscles occurs at the NMJ where the presynaptic motor neuron is separated from the postsynaptic muscle cell by the basement membrane with laminin as a major component (White *et al.* 1986; Noakes *et al.* 1995). Therefore, we next asked whether *epi-1* depletion affects responsiveness to the neuroactive drugs, levamisole and aldicarb. Aldicarb is an acetylcholinesterase inhibitor that causes acetylcholine accumulation in the NMJ. This leads to overstimulation of acetylcholine receptors and paralysis of wild type worms (Rand 2007). Levamisole is an acetylcholine receptor agonist that also leads to

paralysis of wild type worms. The drugs are commonly used to assay and distinguish between defects in release of acetylcholine by the motor neuron and the responsiveness of the muscles to acetylcholine (Lackner *et al.* 1999; Rand 2007). We assayed the acute sensitivity of *epi-1* depleted worms to levamisole and aldicarb in *rrf-3(pk1426)* worms by scoring paralysis over time after drug exposure. *epi-1* depletion resulted in significant hypersensitivity to both levamisole (t-test, $P < 0.001$, Fig. 6) and aldicarb (t-test, $P < 0.05$, Fig. 6). This observation indicates that *epi-1* negatively regulates aspects of neurotransmission either by negatively regulating acetylcholine release at the neuromuscular junction or by promoting GABA secretion in the neuromuscular junction (Vashlishan *et al.* 2008; Locke *et al.* 2009).

Discussion

The BM in multi cellular organisms is involved in tissue organization, cell polarization and -signaling and is primarily composed of type IV collagens, laminins, nidogens and perlecan (Kramer 2005). In this study we have identified laminins as modulators of protein homeostasis in *C. elegans* disease models transgenically overexpressing aggregation prone peptides. To our knowledge, our study is the first to report effects on protein integrity as result of laminin depletion. We have found that depletion of laminin, particularly the gene *epi-1*, accelerates onset of paralysis in Aβ-worms and worms expressing toxic GFP aggregates. Furthermore, *epi-1* depletion results in an altered distribution of Aβ and α-synuclein, while the total load seems unaffected. *epi-1* knockdown is also associated with a failure to mount an appropriate increase in *hsp-16.2* expression in response to misfolded proteins, lowered proteasome activity and hypersentitivity to the neuroactive drugs aldicarb and levamisole.

Reduced insulin signaling results in increased lifespan and stress resistance (Kenyon *et al.* 1993; Lithgow *et al.* 1995) and has previously also been shown to confer protection against protein aggregation stress in worm models for Huntington's disease (Morley *et al.* 2002; Hsu *et al.* 2003), AD (Cohen *et al.* 2006) as well as naturally aggregating proteins (David *et al.* 2010). In agreement with these reports we find that mutation of *daf-2* protects against Aβ toxicity and that this fully depends on the FOXO transcription factor DAF-16. Interestingly, we find that the age-related paralysis induced by *epi-1* RNAi in Aβ-worms can also be partially rescued by reduced insulin signaling. Amelioration of paralysis requires DAF-16. In fact, if *daf-16* and *epi-1* are inactivated at the same time paralysis is greatly accelerated. Most *daf-2* alleles are pleiotropic and influence several phenotypes such as fertility, development, dauer formation, stress resistance and longevity (Gems *et al.* 1998). Since some *daf-2* alleles confer slower development compared to wild-type worms this could perhaps account for the delayed onset in *epi-1* RNAi induced paralysis. However, we have seen protection with both the class 2 allele *daf-2(e1370)* and class 1 *daf-2(e1368)* allele and since only the *e1370* allele slows development we can rule out a difference in development time as causal factor.

Due to the greatly increased lifespan of *daf-2* mutants compared to wild-type worms it is difficult to directly compare their chronological ages. Keeping the difference in biological age versus chronological ages in mind is important not only for studies of neuroprotection but for all aging studies. Is the delayed paralysis simply due to the longer lifespan of the *daf-2* mutants? Since the underlying mechanisms involved in protein aggregation stress and the aging process are likely to overlap it is difficult, if not impossible, to separate the two phenomena. The same is true for example for compounds extending lifespan and protecting against Aβ toxicity (Gutierrez-Zepeda *et al.* 2005; Alavez *et al.* 2011). We suggest that the observed differences in our study and previous studies are not simply due to differences in lifespan but rather an altered biological response to protein toxicity. The clearest example

supporting this view is the onset of paralysis observed when DAF-16 is removed which occurs much earlier than one would predict based on the well documented only slightly reduced lifespan of *daf-16* mutants compared to wild-type worms (Lin *et al.* 1997; Ogg *et al.* 1997).

Mutations in BM molecules cause a variety of tissue defects and neuromuscular junction (NMJ) abnormalities in worms and higher organisms (Noakes *et al.* 1995; Ackley *et al.* 2003; Yurchenco *et al.* 2004; Dixon *et al.* 2006; Kao *et al.* 2006). Consequently, numerous diseases are caused by mutations in BM and extracellular matrix (ECM) proteins including cardiovascular diseases, connective tissue diseases (Bateman *et al.* 2009) and neurodegenerative diseases (Bonneh-Barkay & Wiley 2009; Gardiner 2011). The BM offers both structural support as well as receptor – ligand mediated signaling across the BM. For most diseases of the BM and ECM it is currently unclear if structural changes or altered signaling or perhaps both are causally involved in their etiology. Similarly, structural changes of the BM, altered signaling or both may account for the enhanced proteotoxicity observed in our study following *epi-1* knock down. We propose a model where lack of laminin in the BM negatively effects the intracellular environment, making aggregation prone proteins more unstable resulting in early paralysis in Aβ-worms. Young Aβ-worms are able to buffer these deleterious effects by various quality control systems. However, when these systems become exhausted as the worm ages, paralysis eventually occurs. Proteins also aggregate with age in wild-type worms but it occurs much later in life (David *et al.* 2010). Consistent with this model we observe a decrease in proteasome activity and a failure to induce the chaperone *hsp-16.2* when laminin is scarce. If worms are further challenged by additional protein stress through expression of transgenic Aβ, α-synuclein or GFP::degron, the effect is exacerbated and the onset of paralysis occurs earlier. The negative effects from laminin depletion are likely related to the aging process since paralysis can be rescued by lowering insulin/IGF signaling known to slow the normal aging process. Interestingly, disturbance of the BM is not sufficient to cause paralysis in young worms rather age-related paralysis is accelerated. Loss of protein homeostasis is likely mediated via the NMJ where laminins are critical for proper differentiation (Nishimune *et al.* 2008). Disturbed NMJ signaling has elegantly been shown to affect protein homeostasis in the postsynaptic muscle cell (Garcia *et al.* 2007). In this study, overexcitation of postsynaptic cells led to increased polyglutamine aggregation as well as aggregation of metastable proteins not related to disease. This model could explain the occurrence of locomotion defects (paralysis due to effects on muscle function) as well as the protein handling problems we observe. The hypersensitivity of *epi-1* depleted worms to aldicarb and levamisole supports the idea that neurosignaling in worms with an impaired BM is disturbed.

Many BM proteins are essential for normal development and muscle attachment (Dixon & Roy 2005). One would therefore expect that inactivation of these should also disturb neuromuscular signaling and alter proteotoxicity. Interestingly, of all the BM proteins investigated we found that only knock down of laminins resulted in a dramatically accelerated age related paralysis in the Aβ worms. There could be several reasons for this observation. The lack of effect following inactivation of some BM proteins could be due to redundant functions or inefficient RNAi treatment. Inactivation of other BM proteins did cause paralysis already during development and early adulthood which is probably due to developmental defects in muscle attachment rather than protein aggregation. It is possible that the level of RNAi knock down of BM gene expression needs to be at a certain critical low level in order to avoid developmental defects and uncover the protein aggregation phenotype. Alternatively, the paralysis is caused by disturbance of a laminin dependent signaling event.

Several studies have implicated laminin in neurodegeneration (Bonneh-Barkay & Wiley 2009). Laminin promotes neurite outgrowth and differentiation (Sanes 1989) and laminin has also been shown to be involved with neuroprotection in a mouse model for human temporal lobe epilepsy induced by Kainic acid (KA) excitotoxicity (Chen & Strickland 1997). In this model laminin degradation precedes neuronal loss after KA treatment and regions of the brain with high laminin expression are more resistant to excitotoxin-induced neuronal cell death. Laminin has also been reported to bind the amyloid precursor protein APP (Kibbey *et al.* 1993; Swistowski *et al.* 2009). Laminin has also been associated with Aβ plaques and is induced in the brain of AD patients, although its role in pathogenesis has not been elucidated (Murtomaki *et al.* 1992; Palu & Liesi 2002). Furthermore, laminin inhibits Aβ fibril formation *in vitro* and in cell culture (Castillo *et al.* 2000; Morgan *et al.* 2002). An increasing number of studies are showing that synaptic activity can modulate Aβ homeostasis, where an increase in activity leads to an increase in Aβ secretion (reviewed by (Tampellini & Gouras 2010). That synaptic activity can contribute to the development of AD supports the idea that structural defects in the synapse can play an important role in the pathogenesis. Although these reports link AD with laminin as a potential modulator of the disease through a direct association, the effects we observe in our study are not specific for Aβ but rather seems as a general phenomenon affecting age-related proteotoxicity. Our study therefore offers a novel mechanism of action via proteotoxicity by which mutations of laminin could be causally linked to neurodegeneration. Supporting this idea laminin was recently found as a biomarker for Parkinson's disease (Grunblatt *et al.* 2010) but whether this relates to our findings on proteotoxicity needs to be further studied. *C. elegans* models of modified BM and NMJ may be useful for further studying the relationship between BM proteins, proteotoxicity and neurodegeneration and provide strong screening platforms for identifying novel biomarkers and targets for intervention.

Experimental Procedures

Nematode strains

Strains N2 (*C. elegans* wild-type Bristol strain), CL2006 (*dvIs2*[pCL12(*unc-54*/human Abeta peptide 1-42 minigene) + pRF4]), NL5901 (*pkIs2386*[P*unc-54*::alphasynuclein*::yfp* + *unc-119*(+)], CL2070 (*dvIs70 Is*[*hsp-16.2::gfp;rol-6(su1006)*] , TJ356 (*zIs356 Is*[*daf-16*::*daf-16-gfp;rol-6*]), SJ4005 (*zcIs4* [*hsp-4*::*gfp*] V), and NL2099 (*rrf-3(pk1426)* II) were obtained from *Caenorhabditis Genetics Center*, University of Minnesota, (Minneapolis, MN, USA). Note that CL2006 is actually expressing Abeta 3-42 rather than 1-42 (McColl *et al.* 2009). OLS30: (*dvIs2; daf-2(e1368)*), OLS44 (*dvIs2*;*daf-2(e1370)*), OLS45 (*dvIs2;daf-2(e1370);daf-16(mu86)*) were constructed as described in the supporting material. IM253 (*urEx131*[IM#px41.1 (*lam-1*::*gfp*), pRF4 (*rol-6*)]) was a kind gift from Dr William Wadsworth (Robert Wood Johnson Medical School, Piscataway, NJ, USA) and CL2337 (*smg-1*ts*(cc546)*;*dvls38*[pCL60(*myo-3*/*gfp*::degron/long3' UTR)+pRF4]) was kindly provided by Dr Christopher Link (University of Colorado, Boulder, CO, USA) and maintained at 16 °C. All other strains were maintained at 20 °C on standard Nematode Growth Medium (NGM) spotted with *Escherichia coli* strain OP50.

Paralysis assays

RNAi was performed by feeding the worms *E. coli* strain HT115 making gene-specific dsRNAs (Timmons & Fire 1998). RNAi plates were NGM plates containing 1 mM isopropyl thiogalactoside (IPTG) and ampicillin (50 μg/ml). Worms feeding on HT115 bacteria containing the empty vector pL4440 (referred as EV) were used as controls. Paralysis assays using CL2006, OLS30, OLS44 and OLS45 were performed at 20 °C by placing freshly laid eggs on RNAi plates spotted with the appropriate bacteria clone. Adults were scored daily for onset of paralysis. Worms were scored as paralyzed if they responded

to touch (nose movement) but failed to move their entire body when gently touched by a platinum wire. As an additional control CL2070 worms were also tested for paralysis. CL2070 are not expressing Aβ but are roller mutants as CL2006. The tested RNAi clones can be seen in Table 1 and Table 2 and were obtained from the Ahringer (Source BioScience, Nottingham, UK), OpenBiosystems (Thermo Fisher Scientific, Huntsville, AL, USA) RNAi Libraries or generated by standard cloning techniques and inserted into pL4440 and expressed by HT115 cells (see Table S2 for the used primers to generate inserts). All RNAi clones were verified by sequencing the insert using a L4440 specific primer (5'-GAGTGAGCTGATACCGCTC-3'). All clones were tested in the second generation of RNAi treatment to minimize any maternal rescue, except when otherwise stated. For paralysis scoring of the temperature sensitive strain CL2337, freshly laid eggs developed at 16 °C on *epi-1* or EV RNAi plates. After 36 h (at the third larval stage), the plates were shifted to 25 °C and scored for paralysis beginning 19 h after the upshift as in (Link *et al.* 2006). In general, all herein used strains were affected by *epi-1* RNAi giving rise to sterility, vulva explosions and a progressive paralysis, setting in when worms were approximately 7 days old (except for *daf-2* worms). For this reason most of the assays were performed around this time point.

Heat stress tolerance

Thermotolerance was determined as previous described (Olsen *et al.* 2006). Briefly, freshly laid eggs of strain NL2099 were placed on RNAi plates spotted with bacteria containing *epi-1* or EV. After 4 days at 20 °C, adult worms were heat stressed at 35 °C. The number of surviving animals was scored every hour, beginning 4 h after the upshift.

Immunoblotting

EV or *epi-1* RNAi of Aβ-worms was carried out as described above. At the age of 7 days, 25 adult worms per replicate were transferred to tubes containing 15 μl S-basal [0.1 M NaCl, 0.05 M H_2PO_4 (pH 6)], snap frozen in liquid nitrogen, and stored at -80 °C until further analysis. Bio-Rad (Copenhagen, Denmark) sample buffer and reducing agent were added to the tubes, boiled for 5 min before fractionated on Bio-Rad CriterionXT precast gels (12%), and electroblotted onto a nitrocellulose membrane using a Bio-Rad Trans-blot cell and incubated with 2% skim milk in 1×PBS. Membranes were probed with mouse anti-Aβ antibody clone 6E10 (Eurogentec, Medinova Scientific, Glostrup, Denmark) as primary antibody and HRP-conjugated, rabbit anti-mouse antibody (DakoCytomation, Glostrup, Denmark) as secondary antibody. HRP was visualized using Amersham ECL plus detection systems (GE Healthcare, Brøndby, Denmark). Anti-actin antibody (clone JLA20, Calbiochem, Merck Chemicals, Hellerup, Denmark) was used to control for equal loading. A similar protocol was used for GFP-immunoblotting of strain NL5901 (α-synuclein::YFP). Primary antibody was anti-GFP antibody (Roche Applied Science, Hvidovre, Denmark) and secondary antibody as above. NL5901 worms (30 worms per replicate) were collected after being kept on *epi-1* or EV RNAi for 6 days.

Fluorescence microscopy

NL5901 (α-synuclein worms) were grown on *epi-1* or EV RNAi for two generations. At day 6, worms were mounted on agarose pads and α-synuclein::YFP inclusions were visualized on a Zeiss Axiovert 200M inverted microscope with LSM 510 Meta confocal system. The number and size of aggregates from the tip of the nose till the end of the first pharyngeal bulb was estimated using the Ziess LSM Image Browser software (Zeiss 1997-2006) for 8 individuals of each treatment.

The structure of the basement membrane was visualized on the Meta confocal system as above, using a LAM-1::GFP reporter strain (IM253) kept on *epi-1* or EV RNAi for 3 and 6

days from eggs, respectively. DAF-16::GFP localization was determined using strain TJ356 kept for 6 days on *epi-1* or EV RNAi from eggs. Expression level of *hsp-16.2::*GFP (strain CL2070) of *epi-1* or EV RNAi treated adult worms (4 days old) with or without a prior heat shock at 33 °C for 2 h followed by 2-3 h recovery was also investigated. Images of the heat induced worms were analyzed for GFP-intensity using the software ImageJ 1.43. The number of analyzed individuals was 14 for each treatment.

Investigation of ER stress was performed using strain SJ4005 (Urano *et al.* 2002) kept on EV or *epi-1* RNAi for 4 or 6 days from egg. The images were analyzed for GFP-intensity using ImageJ as above. The number of analyzed individuals on day 4 was 18 and 17 for *epi-1* and EV RNAi respectively, and for day 6 it was 16 and 18, respectively. All images were acquired on a Leica DMI3000B inverted microscope equipped with an Olympus DP72 camera and Cell imaging software (Olympus) unless otherwise stated.

Proteasome activity

The activity of the proteasome was evaluated as the *in vitro* chymotrypsin-like enzymatic activity as in (Lima & Rattan 2010). Worms of the strain NL2099 (*rrf-3(pk1426)*) were grown on *epi-1* or EV RNAi at 25 °C from eggs and triplicates of 150 worms were collected at day 3 and 6. Samples were washed three times in S-basal and once in assay buffer [50 mM NaCl, 10 mM HEPES (pH 8), 250 mM sucrose, 1 mM EDTA, and 0.2% Triton X-100]. Each sample (~30 μl) was then snap-frozen in liquid nitrogen and thawed on ice 5 times. Cold assay buffer (220 μl) was added, vortexed and centrifuged 1 min at 4000 g at 4 °C. The protein concentration of the supernatant was measured by the Bradford protein assay. Five μg of total protein were added to a black-shaded 96-well plate with 50 μM succinyl-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC) as substrate (Sigma-Aldrich, Brøndby, Denmark) and assay buffer to a total volume of 200 μl. As a negative control, 50 μM of the proteasome inhibitor MG132 (Sigma-Aldrich) was incubated with a sample and substrate as above. The fluorescence intensity was measured at excitation 355 nm and emission 460 nm for 60 min at room temperature, using a fluorometer (POLARstar optima, BMG LABTECH, Hoffenberg, Germany). After omitting the first few non-linear cycles, the rate of increase in fluorescence intensity was used as a measure of activity. Duplicate measurements were made of all samples.

Sensitivity to levamisole and aldicarb

Sensitivity to levamisole and aldicarb was determined by assaying the number of paralyzed worms over time following acute exposure to the drug as in (Lackner *et al.* 1999; Mahoney *et al.* 2006). Eggs from NL2099 worms were placed on *epi-1* or EV RNAi. After 3 days at 25 °C adult worms were then transferred to *epi-1* or EV seeded RNAi plates containing 100 μM levamisole or 0.5 mM aldicarb (Sigma-Aldrich, Brøndby, Denmark) and paralysis scored after 2 h.

Statistical analysis

For statistical analysis of paralysis and heat stress tolerance the Graphpad Prism 5.01 software was used (GraphPad Software, San Diego, Ca, USA). For comparison of survival curves the Log rank test (Mantel Cox) was used with *P* values below 0.05 being significant. For pairwise comparison (Student's t-test or Mann-Whitney U-test) of aggregate data, proteasome activity, GFP-intensity and drug sensitivity the statistical package PAST (Hammer *et al.* 2001) was used, with *P* values below 0.05 considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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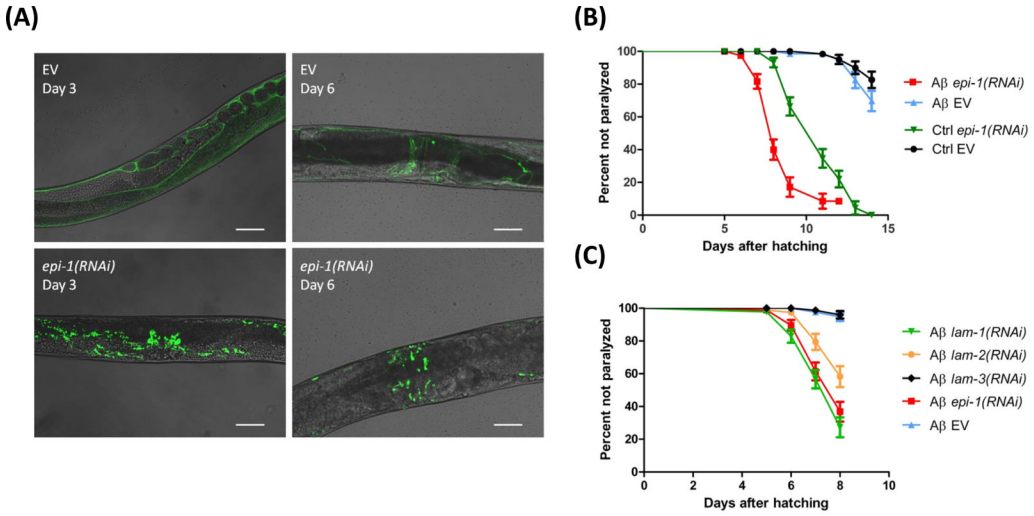

Fig. 1. *epi-1* RNAi targets LAM-1 distribution and causes enhanced paralysis of Aβ-worms

A) Images of the midsection of LAM-1::GFP tagged adult worms grown on empty vector (EV) or *epi-1* RNAi for 3 or 6 days from eggs. *epi-1* RNAi leads to a punctuated pattern of LAM-1. Scale bar: 50 μM.**B)** RNAi of *epi-1* results in early onset of paralysis in transgenic Aβ-worms, compared with EV RNAi. *epi-1* RNAi of a control worm not expressing Aβ also results in paralysis. **C)** Gene knockdown of *lam-1* and *lam-2* also changes the onset of paralysis in Aβ-worms. Error bars are SEM. Note that X-axes differ in scale in B) and C).

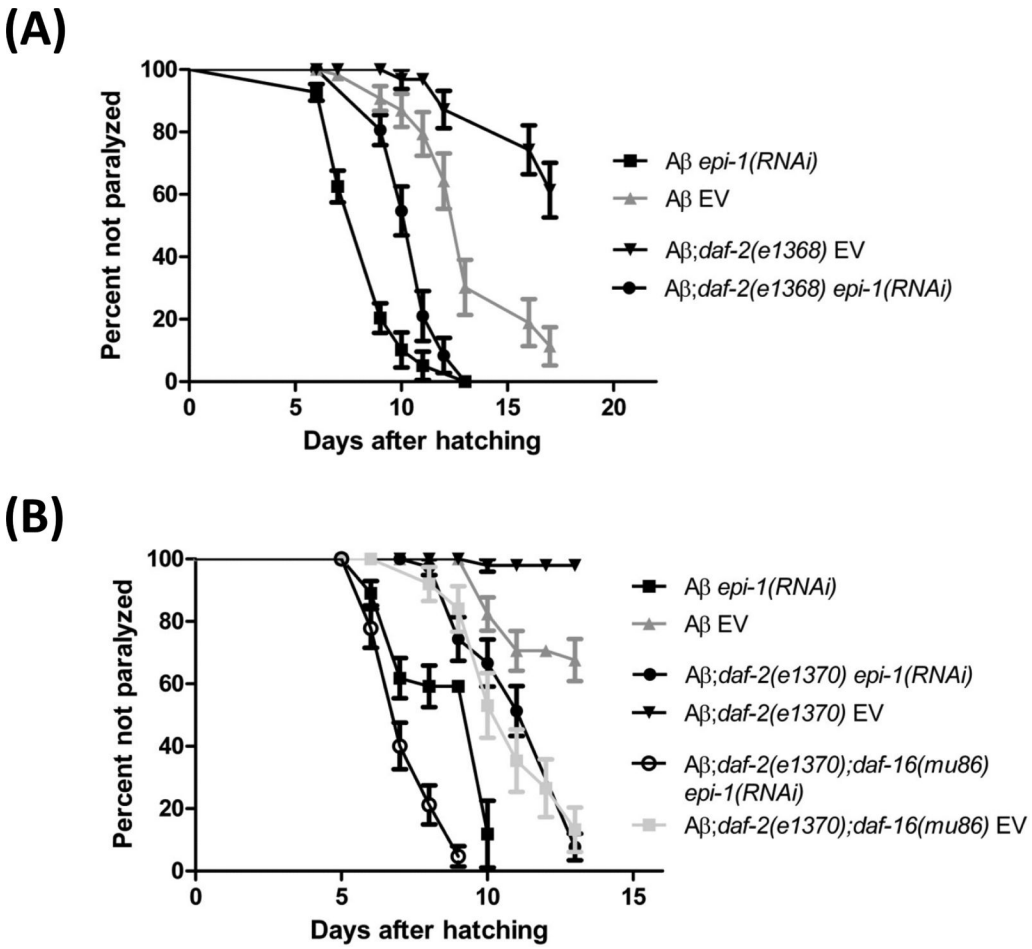

Fig. 2. The paralysis effect caused by *epi-1* RNAi can be partially rescued by mutation in *daf-2*

Paralysis assay of Aβ-worms and Aβ;*daf-2(e1368)* **(A)** or Aβ;*daf-2(e1370)* and Aβ;*daf-2(e1370);daf-16(mu86)* **(B)** mutants grown on *epi-1* or empty vector (EV) RNAi from eggs. Error bars are SEM.

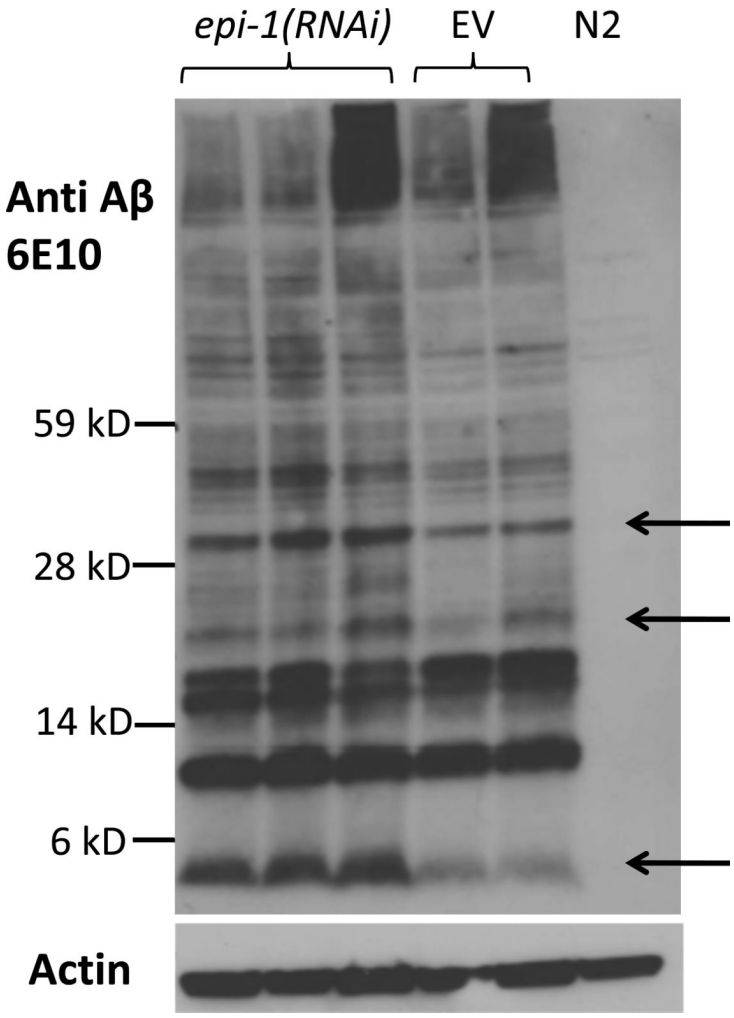

Fig. 3. *epi-1* knockdown results in higher concentration of small molecular Aβ bands

Immunoblot of protein extracts from Aβ-worms kept on *epi-1* and empty RNAi vector (EV) for 7 days, using anti-Aβ antibody (6E10). Loading control is actin. N2 is the wildtype strain, not expressing Aβ. An increase in Aβ levels in several bands (especially low molecular weight bands) is seen in *epi-1* RNAi worms when compared to EV controls (arrows).

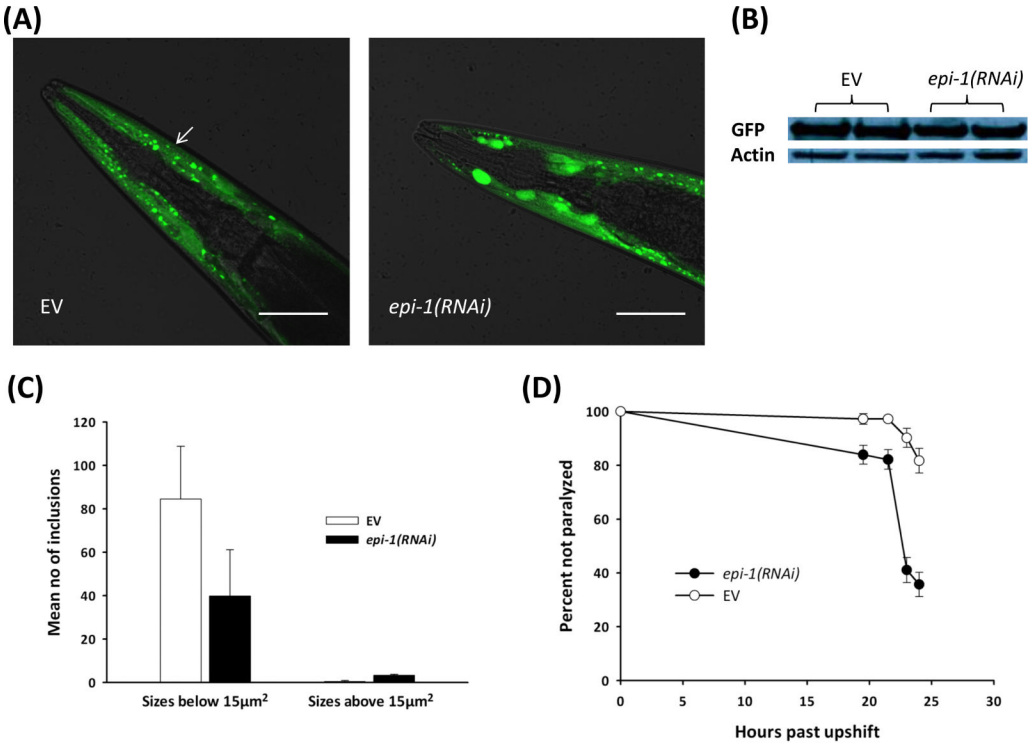

Fig. 4. Depletion of *epi-1* affects number and size of α-synuclein inclusions and enhances sensitivity to aggregating GFP

A) Representative confocal images showing the head region of α-synuclein::YFP transgenic worms fed *epi-1* or empty vector (EV) RNAi for 6 days. Scale bar is 50 μm. **B)** Immunoblot using anti-GFP of worms treated as in A), loading control is actin. **C)** Mean number of inclusions of sizes below and above 15 μm^2 as counted from the tip of the nose to the end of the anterior pharyngeal bulb (marked by an arrow in A)). *epi-1(RNAi)* treated worms have significantly fewer inclusions of sizes below 15μm^2 ($P = 0.005$) but a higher number of large inclusions above 15 μm^2 ($P = 0.0009$) (n=8 per treatment, error bars represent SD), same treatment as in A). **D)** Paralysis assay of a transgenic GFP::degron strain grown on *epi-1* or EV RNAi. The strain expresses an aggregating variant of GFP fused to a "degron" peptide in body wall muscle, resulting in rapid paralysis when upshifted from 16 °C to 25 °C. RNAi of *epi-1* increases paralysis of this model of toxic protein aggregation ($P < 0.0001$).

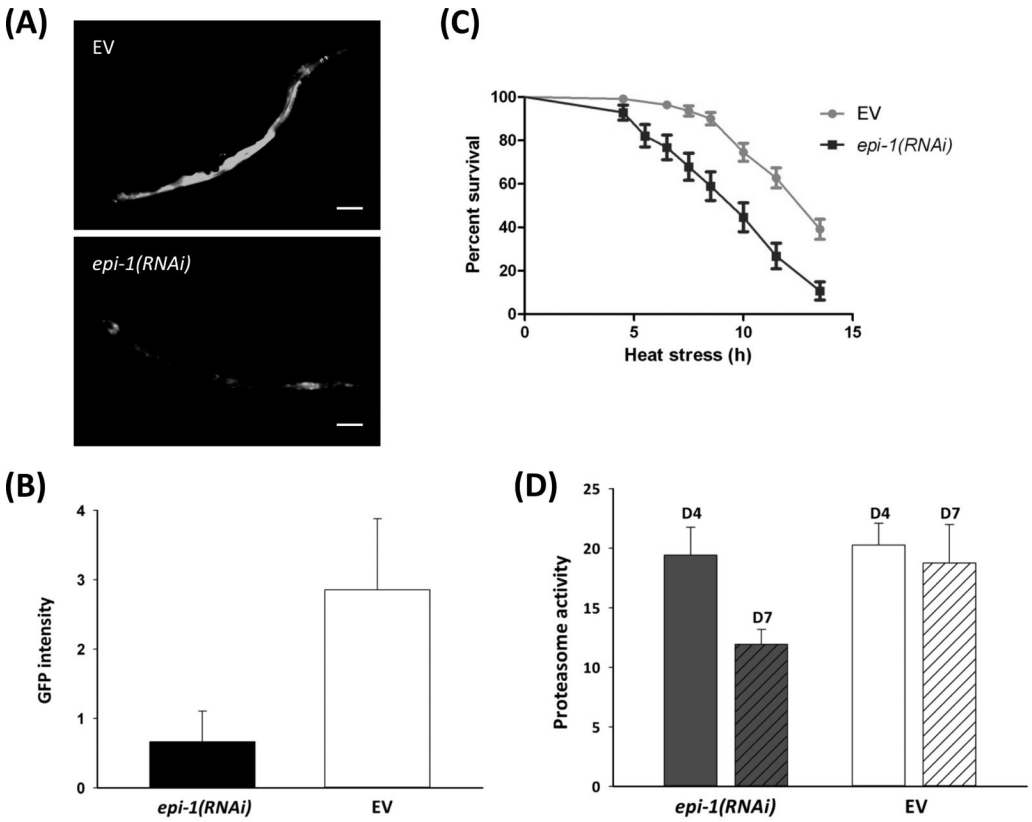

Fig. 5. *epi-1* RNAi results in impaired stress responses as evaluated by *hsp-16.2* heat induction, heat stress resistance and proteasome activity

A) Representative images of *hsp-16.2*::GFP expression after a 33 °C heat shock in worms kept on *epi-1* RNAi or EV for 4 days from eggs. Scale bar is 100 μm **B)** Average GFP intensity in these worms (n=14 for both treatments, error bars are SD). The GFP intensity is significantly lower in worms fed *epi-1* RNAi than in EV ($P < 0.0001$). **C)** Survival to heat stress at 35 °C of adult *rrf-3(pk1426)*. Worms kept on *epi-1* RNAi are significantly more sensitive to heat stress than worms kept on empty vector (EV) ($P < 0.0001$). Error bars are SEM. **D)** Average proteasome activity at day 3 and day 6 of *rrf-3(pk1426)* kept on *epi-1* or EV RNAi (n=3 of 150 worms each, error bars represent SD). The proteasome activity decreases significantly with time when *epi-1* is RNAi knocked down ($P = 0.008$) while EV is not significantly different over time ($P = 0.525$).

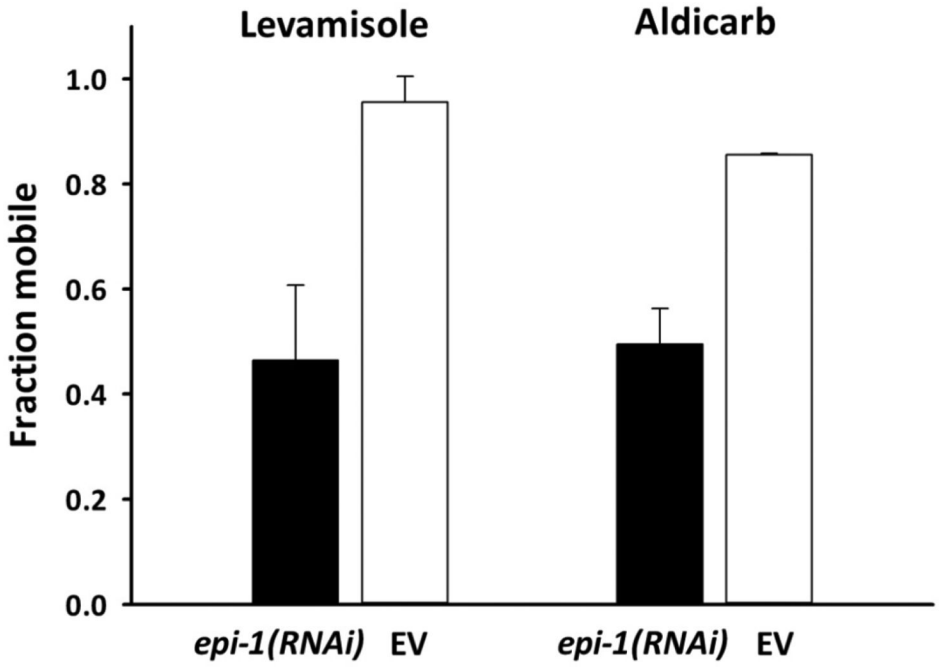

Fig. 6. Knockdown of *epi-1* causes hypersensitivity to levamisole and aldicarb

Acute sensitivities to levamisole and aldicarb measured as the fraction of mobile worms after a 2 h exposure to 100 μM levamisole or 0.5 mM aldicarb. *rrf-3(pk1426)* worms were kept on *epi-1* RNAi or EV for 3 days from eggs at 25 °C before transferred to drug plates. *epi-1* RNAi sensitizes worms to levamisole ($P < 0.001$) and aldicarb ($P < 0.05$). Error bars are SD.

Table 1

Analysis of paralysis after RNAi against BM proteins. Only laminins (except for *lam*-3) and *fbl-1* RNAi knockdown resulted in enhanced paralysis in Aβ-worms.

Common name	Gene	Gene name	RNAi induced paralysis	PT_{50}	Other phenotypes
Collagen IV α1	*emb-9*	K04H4.4	NS	> 14	Lva
Collagen IV α2	*let-2*	F01G12.5	NS	> 14	Egl, Adl
Collagen XVIII	*cle-1*	C36B1.1	NS	> 14	
Fibulin-1	*fbl-1*	F56H11.1	$P < 0.0001$	14	
Hemicentin	*him-4*	F15G9.4	NS	> 14	
Laminin αA	*lam-3*	T22A3.8	NS	13	
Laminin αB	*epi-1*	K08C7.3	$P < 0.0001$	7-9	Ste, Rup
Laminin β	*lam-1*	W08F8.3	$P < 0.0001$	7-8	Ste
Laminin γ	*lam-2*	C54D1.5	$P < 0.0001$	10	Ste
Nidogen	*nid-1*	F54F3.1	NS	> 14	
Papilin	*ppn-1*	C37C3.6	NS	> 14	Egl
Perlecan	*unc-52*	ZC101.2	$P < 0.0001$ *	< 4	

NS: Not significantly different from paralysis of EV treated worms. PT_{50} = the time at which 50% of the worms were paralyzed.

Lva: larval arrest, Egl: egg laying defective, Adl: adult lethal, Ste: sterile, Rup: exploded through vulva.

All genes with a non-significant outcome were tested at least twice, while genes showing statistical significance were verified at least three times. All assays but the four laminins and *unc-52* were performed over two generations to avoid maternal rescue.

*denotes that the observed paralysis phenotype was not specific for Aβ-worms but also prevalent in controls and Aβ;*daf-2* worms.

Table 2

Analysis of paralysis after RNAi against receptors for BM proteins.

Common name	Gene	Gene name	RNAi induced paralysis	PT_{50}	Other phenotypes
Dystroglycan	*dgn-1*	T21B6.1	$P < 0.05$*	13	
Dystroglycan	*dgn-2*	F56C3.6	NS	> 14	
Dystroglycan	*dgn-3*	F07G6.1	NS	> 14	
Glypican	*gpn-1*	F59D12.4	NS	> 14	
Glypican	*lon-2*	C39E6.1	NS	> 14	Lon
Integrin α	*ina-1*	F54G8.3	NS	> 14	
Integrin α	*pat-2*	F54F2.1	$P < 0.0001$*	< 4	Adl
Integrin β	*pat-3*	ZK1058.2	$P < 0.0001$*	< 4	
LAR-RPTP	*ptp-3*	C09D8.1	NS	> 14	
Syndecan	*sdn-1*	F57C7.3	NS	> 14	

NS, Not significantly different from EV. PT_{50} = the time at which 50% of the worms were paralyzed.

Lon: longer and thinner animals, Adl: adult lethal.

All assays but *pat-2* and *pat-3* were performed over two generations to avoid maternal rescue. All genes were tested at least twice.

* denotes that the paralysis phenotype was not specific for Aβ-worms but also prevalent in controls and/or Aβ;*daf-2* worms.