O-GlcNAc cycling mutants modulate proteotoxicity in Caenorhabditis elegans models of human neurodegenerative diseases

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O-GlcNAcylated proteins are abundant posttranslationally modified by the enzymes O-GlcNAcase (OGA) and O-GlcNAcase (OGT) in the brain, where they play important roles in the regulation of protein homeostasis (7, 8). O-GlcNAc cycling dramatically modulated the severity of the phenotype in transgenic models of tauopathy, amyloid-β-peptide, and polyglutamine expansion (9, 10). Inhibition of O-GlcNAcase alleviated, whereas loss of OGT function exacerbated, the phenotype of multiple neurodegenerative disease models. The O-GlcNAc cycling mutants act in part by altering DAF-16-dependent transcription and modulating the protein degradation machinery. These findings suggest that O-GlcNAc levels may directly influence neurodegenerative disease progression, thus making the enzymes of O-GlcNAc cycling attractive targets for neurodegenerative disease therapies.

Results

The O-GlcNAc Cycling Mutants Affect Toxicity of Two HD Models. We first examined the influence of O-GlcNAc cycling on proteotoxicity with Htr-Q150, an HD model with a fusion protein of the 171 N-terminal amino acids of the human huntingtin protein and a tract of 150 glutamine residues (Htr-Q150) expressed in a small subset of sensory neurons (ASH) and pharyngeal muscles (11–13). In contrast, two ogt-1(ok1474) and oga-1(ok2071) mutants, and demonstrated that the loss of OGT function alleviated, whereas loss of OGA function exacerbated, the proteotoxicity associated with aggregate-prone proteins. The mechanism of this protective effect of decreased O-GlcNAc on proteotoxicity may be linked to deregulation of the protein homeostasis machinery, signaling pathways and DAF-16, a key regulator of the stress response and proteotoxicity in C. elegans.

Proteotoxicity is a common pathological feature of human neurodegenerative diseases. The disease-associated protein aggregates include senile plaques from amyloid-β-peptide, neurofibrillary tangles from hyperphosphorylated tau in Alzheimer’s disease (AD) (1, 2), and huntingtin aggregates from mutant huntingtin protein containing polyglutamine repeats in Huntington disease (HD) (3). The precise mechanism involved in triggering protein aggregation in neurodegeneration is less clear. Recent work has suggested that changes in insulin-like signaling, cell cycle progression, or protein clearance mechanisms may all be involved in the progression of neurodegenerative diseases (4–6).

To facilitate genetic analysis of this group of diseases, many well-characterized Caenorhabditis elegans transgenic models have been developed, and have been used to investigate the roles of insulin and protein degradation pathways in the regulation of proteotoxicity (21–23). C. elegans also provides unique genetic advantages to study the effects of altered O-GlcNAc cycling: loss of function of Ogt in vertebrate systems results in embryonic lethality (24, 25). However, C. elegans animals bearing null mutations of ogt-1 or oga-1 are alive and fertile and appear phenotypically wild type (WT) with respect to development, growth, and movement (26, 27). In the present study, we examined the phenotype of neurodegenerative proteotoxicity models in O-GlcNAcylated null mutants, and demonstrated that the loss of OGT function alleviated, whereas loss of OGA function exacerbated, the proteotoxicity associated with aggregate-prone proteins. The mechanism of this protective effect of decreased O-GlcNAc on proteotoxicity may be linked to deregulation of the protein homeostasis machinery, signaling pathways and DAF-16, a key regulator of the stress response and proteotoxicity in C. elegans.


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addition to enhancing the severity of the Htn-Q150 toxicity, 

*oga-1(ok1207)* also enhanced the onset of the dye-filling defect. In animals at day 3 of adulthood, more degenerative ASH neurons (40.6%) were observed in the *oga-1(ok1207)* mutant compared with the control strain (17.7%). In contrast, an alleviated dye-filling defect was observed in the *oga-1* mutants (Fig. S1).

OGT and OGA are multidomain proteins capable of mediating other potential functions in addition to catalyzing addition and removal of O-GlcNAc (26, 27). To confirm that alterations in the proteotoxicity observed in the *oga-1* and *oga-1* null mutant backgrounds depended upon deregulation of the O-GlcNAc modification per se, we compared the proteotoxic phenotype of Htn-Q150 in the *oga-1* single mutants to those in the *oga-1* single mutants. In O-GlcNAc cycling, the need for OGA activity is wholly dependent on the presence of functional OGT to generate the O-GlcNAcylated protein substrate. Thus, similar phenotypes should be observed in the *oga-1* single mutant and the *oga-1* OGT double mutants. Indeed, the *oga-1* OGT double mutants completely rescued the ASH cell death phenotype associated with Htn-Q150 proteotoxicity in the *oga-1* (*ok1207*) single mutant (Fig. 1D and Fig. S1).

To test whether the effect of O-GlcNAcylation on PolyQ proteotoxicity is specific for Htn-Q150, we used a second HD model. In this model, a fusion protein containing 40 glutamine residues fused to YFP (Q40-YFP) is expressed in somatic muscles (i.e., Aβ1–42 peptide was more severe with earlier onset in *oga-1* (*ok1207*). Most *Aβ1–42:oga-1* (*ok1207*) (31). Aβ1–42 peptide expression in the muscles (i.e., Aβ1–42) (31). Aβ1–42 animals show a variety of phenotypes, including age-dependent paralysis in adults (31) (Movie S1), constipation caused by disruption of the defecation muscle functions, and egg-laying defects (Egl) in hermaphrodites caused by defects in sex muscle functions (Fig. S2). Severe Egl phenotypes can lead to early adult death as a result of progeny hatching inside the hermaphrodite uterus (i.e., bagging).

Approximately 50 *Aβ1–42* or *Aβ1–42:oga-1* (*ok1207*) animals were scored daily for the phenotypes from days 1 to 10 of adulthood. The paralysis phenotype caused by the *Aβ1–42* peptide was more severe with earlier onset in *oga-1* (*ok1207*). Most *Aβ1–42:oga-1* (*ok1207*) adults (>80%) were paralyzed at day 1 of adulthood, compared with less than 20% at day 5 of adulthood in the *Aβ1–42* animals (Fig. 2A and B). In addition, many animals showed a strong Egl phenotype, and quickly died from bagging, with all animals dead by day 6 of adulthood (Fig. 2B). WT N2 or *oga-1* (*ok1207*) single mutant animals lacking *Aβ1–42* did not show a paralyzing phenotype, and most animals were still alive at day 5 of adulthood under the same conditions (Fig. S3), suggesting that the toxic *Aβ1–42* peptide was the cause of the paralysis. Several efforts to cross the *Aβ1–42* strain into the *oga-1* mutant background were unsuccessful. As an alternative, we carried out *oga-1* feeding RNAi experiments on the *Aβ1–42* strain, but no obvious effects were seen, possibly as a result of incomplete knockdown of OGT activity.

### Null Alleles of oga-1 Are Neuroprotective in C. elegans Frontotemporal Dementia with Parkinsonism Chromosome 17 Tauopathy Model

As a final test of the influence of O-GlcNAc cycling on proteotoxicity,
we selected a neuronal tauopathy model. A mutation (V337M) in the microtubule-associated protein tau has been identified as the defect in human frontotemporal dementia with parkinsonism chromosome 17 (FTDP-17) (32). FTDP-17 is one of many human tauopathies in which characteristic neurofibrillary tangles are formed from hyperphosphorylated tau. The *C. elegans* transgenic model expressing the tau V337M throughout the nervous system shows phenotypes of uncoordinated locomotion, axon degeneration, and neuronal death (33). A simple, yet effective, method to assay neuronal function in *C. elegans* is by assessing “thrashing” behavior. When placed in liquid, WT animals thrash actively by repeatedly moving the head and tail to the same side of body (Movie S2), the rate of which can be determined by counting the number of body bends per minute. WT and *ogt-1* or *oga-1* single mutant animals exhibit a similar thrashing rate of 100 to 120 bends per minute (Fig. 3A). In contrast, tau V337M transgenic animals showed a significant decrease in the number of thrashing movements (Fig. 3A), with a kinked tail unable to move coordinately with the head (Movie S3) as a result of the severe neurotoxicity of this mutant protein, as previously described (33).

We crossed the transgenic tau V337M mutant strain into the *ogt-1* or *oga-1* null mutant background. The uncoordinated locomotion phenotype was completely rescued and the thrashing rate was significantly improved when the tau V337M was crossed into the *ogt-1* (ok430) mutant background (Fig. 3A and Movie S4). The *oga-1* (ok1207) did not worsen the thrashing defect of the tau V337M transgenic animals, as observed in other neurodegenerative models tested (Fig. 3A and Movie S5). The tau protein is modified by O-GlcNAc and phosphorylation (14, 34), and hyperphosphorylated tau forms the characteristic neurofibrillary tangles (1). To examine whether the phenotypes seen in the O-GlcNAc cycling mutants result from changes in the phosphorylation state of tau V337M protein, we examined the accumulation of the total and phosphorylated tau by using specific antisera by Western blotting. In general, the levels of the phosphorylation-independent tau were decreased in *ogt-1* and *oga-1* null mutants. Interestingly, there was less pS396 and pS202/pT205 in *ogt-1* (ok430) and increased pS202/pT205 in *oga-1* (ok1207) (Fig. 3B). We concluded that the *ogt-1* mutation decreased the overall level of the tau protein and multiple phosphorylated forms thus alleviated tau V337M proteotoxicity. The *oga-1* mutation did not further worsen the existing severe phenotype even though it increased tau phosphorylation as detected by certain tau phospho-specific antibodies.

For multiple *C. elegans* neurodegenerative disease models, loss of O-GlcNAc modulation increased the proteotoxicity of the exogenous, aggregate-prone proteins whereas an excess of O-GlcNAc modulation enhanced it (except for the tau V337M model; Fig. 4). Interestingly, O-GlcNAc modulation modulated the proteotoxicity when the toxic protein was expressed in neurons (Htn-Q150 and tau V337M) or muscles (Q40-YFP and Ap1-42). Additionally, the effect of O-GlcNAc modulation was not universal to all types of proteotoxicity. O-GlcNAc had no effects on a generic proteotoxicity model (GFP-degron; Fig. S4) (35). Quantitative measurements (i.e., with quantitative RT-PCR)
of the expression of different transgenes suggested that differential expression of the toxic transgene was not responsible for the phenotypes observed in the O-GlcNAc cycling mutants (Fig. S5). We next tested whether the effects of disrupted O-GlcNAc cycling on these proteotoxicity models were linked to protein stability and turnover.

**O-GlcNAc Cycling Influences Pathways of Protein Degradation: Proteasome and Autophagy.** Many cellular protein aggregates are ubiquitinated and proteins involved in several neurodegenerative diseases are known substrates of the ubiquitin proteasome system (36, 37). To explore the possibility that O-GlcNAc cycling modifies proteotoxicity by regulating proteasome activity, we examined the accumulation of polyubiquitinated proteins in WT and the O-GlcNAc cycling mutants. As shown in Fig. 5 A and B, there was a slight (15%) decrease in the level of polyubiquitinated proteins observed in the *ogt-1* mutants compared with WT animals, consistent with a modest activation of proteasome activity and the reduction in proteotoxicity observed in these mutants. However, we did not see significant changes in the level of polyubiquitinated proteins in the *oga-1;ogt-1* double mutants (Fig. 5 A and B), suggesting regulation of proteasome activity is not the only mechanism responsible for the effects of O-GlcNAcylation on proteotoxicity.

Autophagy has also been suggested to play a role in the clearance of protein aggregates in neurodegenerative diseases (38). The formation of autophagosomes is triggered under conditions of starvation and stress. The O-GlcNAc cycling machinery acts as a nutrient sensor and has been suggested to regulate autophagy (39, 40). To test this hypothesis, we used a marker of autophagy in *C. elegans*, LGG-1-GFP, to investigate the effects of O-GlcNAcylation on autophagy (41, 42). In fed animals, we observed increased expression of LGG-1-GFP in *ogt-1* and *oga-1* mutants, but with little phosphatidylethanolamine (PE)-conjugated isoform (PE-LGG-1-GFP; Fig. 5 C), which is the marker of autophagy induction (41). Under starvation, *ogt-1* and *oga-1*...
neurodegenerative models. Our examine the effects of loss of O-GlcNAc cycling in well-deregulated proteotoxicity models, and a complete list of genes tested. These proteotoxicity in HSF-1 has been demonstrated to have a joint action in regulating CViable mutants in providing a mechanistic link between the hexosamine signaling path- way. Moreover, there was an increase in the active form (PE- marks at their promoters (45). To ask whether the O-GlcNAc cycling mutants regulate proteotoxicity through a DAF-16-de- pending pathway, the expression level ofdaf-16 isoforms and DAF-16 targets was measured with quantitative RT-PCR. The O- GlcNAc cycling mutants significantly altered the transcript levels of isoforms daf-16β and total daf-16. Whereas daf-16β and total daf-16 transcripts were significantly up-regulated in the ogt-1 mutant, daf- 16a was uniquely down-regulated in the oga-1 mutant (Fig. 5E). This deregulation of daf-16 was further confirmed by looking at known DAF-16 targets in the two neuronal proteotoxicity models (Fig. 5E). In the ogt-1 mutant background, multiple DAF-16 targets, including two transthyretin-like genes, were significantly deregulated in the proteotoxic models, whereas hif-1 and hsf-1 were not significantly changed (Fig. 5E and Datasets S1 and S2). HSF-1 has been demonstrated to have a joint action in regulating proteotoxicity in C. elegans with DAF-16 (21, 43). Datasets S1 and S2 provide expression data in the oga-1 mutant, muscular proteotoxici ty models, and a complete list of genes tested. These findings, and our previously published work (45), suggest that O-GlcNAc cycling may alter DAF-16-dependent gene expression, thus providing a mechanistic link between the hexosamine signaling pathway and proteotoxicity.

Discussion

Viable mutants in C. elegans provide a unique opportunity to examine the effects of loss of O-GlcNAc cycling in well-defined neurodegenerative models. Our findings clearly demonstrate that O-GlcNAc cycling can influence the severity of proteotoxic phenotypes in C. elegans. Direct O-GlcNAc modification on amyloid precursor protein regulates its proteolytic processing (15, 46), which was not the focus in the present study because AB1–42 is directly expressed in muscles intracellularly. Tau can be modified by O-GlcNAc and O-phosphorylation (14, 34). Hyperphosphorylated tau forms the characteristic neurofibrillary tangles found in AD and other tauopathies, including FTDP-17 as modeled in our study with tau V337M (1, 33, 47). Based on the extensive crosstalk between these two modifications on tau (11, 34), one working model is that increased O-GlcNAcylation (when OGA is mutated or inhibited) will decrease tau phosphorylation and inhibit aggregate formation. A recent study with Thiamet-G (an O-GlcNAcase inhibitor) in transgenic mice over-expressing P301L tau, another mutation identified in FTDP-17, decreases tau aggregate formation and neuronal loss without altering tau phosphorylation (12). In contrast, we observe that increased O-GlcNAcylation (in oga-1 mutant) has no effect on the already severe tauopathy phenotype, whereas loss of the OGT (in ogt-1 mutant) rescued the tauopathy (Fig. 3A). We also observe unchanged tau phosphorylation at S409, S396, and S214, and increased phosphorylation at S202/T205 in the O-GlcNAcase null mutant (Fig. 3B). Thus, the relationship between tau O- GlcNAcylation and phosphorylation is likely to be more complex than a simple competition model might suggest. The discrepancy observed in FTDP-17 models in mouse and C. elegans could be a result of differences between the model organisms used in these studies. In addition, one must also be cautious in comparing the effects of treating animals with a pharmacological agent blocking O-GlcNAc cycling and the consequences of mutant alleles that have a chronic loss of activity. Adaptive changes in protein homeostasis and gene expression may occur (and even be selected for) in mutant animals, and these may mask physiological effects seen upon relatively acute pharmacological treatments.

O-GlcNAcylation can also modulate protein homeostasis indirectly through the stress response, insulin signaling, and proteon degradation pathways (45, 48). Excessive O-GlcNAcylation has been shown to block proteasome activity and proteosome-dependent degradation of Sp1, p53, and delta-lactoferin (49–52). Moreover, knocking down OGT activity leads to proteasome activation (51). Consistent with this finding, we observe reduced proteotoxicity and overall lower levels of polyubiquitinated proteins in ogt-1 mutants (Figs. 4 and 5A). O-GlcNAcylation has also been proposed to regulate autophagy (39, 40). RNAi knockdown of autophagy components in C. elegans enhances the proteotoxicity of Htt-Q150 and Q40-YFP (23). We show that both O-GlcNAc cycling mutants substantially up-regulate the expression of the autophagy marker LGG-1-GFP and enhance autophagy induction during starvation, whereas ogt-1 and oga-1 mutants regulated proteotoxicity in opposite directions. Thus, modulation of the autophagy and proteotoxicity activity may not provide a full explanation for the altered proteotoxic phenotype in the O-GlcNAc cycling mutants.

Insulin/insulin-like growth factor–like signaling (IIS) has been linked to neurodegenerative diseases and associated proteotoxicity (21, 53, 54). O-GlcNAc cycling is a key regulator of the insulin pathway in C. elegans, impacting dae’s formation and longevity (26, 27, 45). Decreased IIS signaling, and the resulting release of inhibition of the downstream transcription factor, DAF-16, ameliorate the phenotype of C. elegans proteotoxicity models; conversely, RNAi knockdown of DAF-16 enhances it (21, 22, 43). Here, we have shown that daf-16 isoforms are differentially regulated in the O-GlcNAc cycling mutants, resulting in deregulation of multiple downstream targets of DAF-16 in the proteotoxicity models. The deregulated targets include the transthyretin-like genes, whose products are generally believed to be neuroprotective proteins in AD (55). Thus, modulation of the IIS pathway is likely to be one of the key mechanisms by which changes in O-GlcNAcylation influences proteotoxicity. Our findings extend the growing body of evidence linking insulin signaling to neurodegeneration (4, 56). The striking effects of O-GlcNAcylation on multiple neurodegenerative disease models open the possibility of exploiting the O-GlcNAc cycling pathway therapeutically for these types of disorders.

Materials and Methods

Strains. O-GlcNAc cycling mutant strains used in this paper include ogt-1 (ok430) (26), ogt-1(ok14747) (45), and oga-1(ok1207) (27). Details of C. elegans proteotoxic models are provided in SI Materials and Methods.

Scoring Degeneration and Death of ASH Neurons. The proteotoxicity of Htt- Q150 in ASH neurons was assessed as described previously with minor modification (28). Day-3 or day-8 adult animals were incubated in the fluorescent dye DiI (20 μg/mL) for 1 h at room temperature. Then, after a 1-h recovery on NGM plates to remove extra dye attached to the body surface, the animals were mounted on 2% (w/vol) agarose pads. The DiI dye staining and OSM-10: GFP expression in ASH neurons were scored with a confocal spin disk microscope.

Aggregate Quantification of Q40-YFP Animals. Synchronized first larval stage animals were kept at 20 °C for 2 d, and the resulting L4 animals were observed under a stereomicroscope with an epifluorescence illuminator. Aggregates of Q40-YFP protein were counted as described previously (29).

Paralysis Assay of Animals Expressing Amyloid β-Peptide or GFP-Degron in Body Wall Muscles. Fifty synchronized adult animals were scored daily for their movement phenotype from days 1 to 10 of adulthood (35). The
phenotypes were scored in three categories: normal, paralyzed, and dead. Animals that moved freely on the plate or responded well to light touch (i.e., moving in the opposite direction of the touch) were scored as normal; those that did not respond promptly or had a "halo" formed around their head (as a result of limited body movement and feeding on the bacterial lawn) were scored as paralyzed; and those that could not move at all and did not have pharyngeal pumping were scored as dead.

**Worm Thrashing Assay.** L4 larvae were picked and placed in 15 µL of M9 buffer on a glass slide. The animals were allowed to acclimate for 1 to 2 min, after which their movements in the liquid were recorded with a camera attached to a stereomicroscope. The number of thrashes per minute was counted in a slow-motion analysis of the recording for each animal.

**Western Blot Analysis.** Ten 1-d-old adult animals were lysed at 95 °C in the presence of LDS sample buffer and β-mercaptoethanol. After brief centrifugation, the proteins in the lysates were separated on a 4% to 12% NuPAGE Bis-Tris gel. The procedures of blocking and blotting followed the standard protocol for the Odyssey infrared imaging system (LI-COR Biosciences). Primary antibodies used in the study are listed in **SI Materials and Methods**.

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