1 Glycosylation of gigaxonin regulates intermediate filaments: Novel molecular
2 insights into giant axonal neuropathy

3 Po-Han Chen^{1,2,3}, Timothy J. Smith¹, Jimin Hu¹, Samuel Pan^{2,3}, Alexander Smith^{2,3},
 4 Annie Lu^{2,3}, Jen-Tsan Chi^{2,3,*} and Michael Boyce^{1,*}

5

6 ¹Department of Biochemistry, ²Department of Molecular Genetics and Microbiology
 7 and ³Center for Genomic and Computational Biology, Duke University School of
 8 Medicine, Durham, NC 27710

9 *Co-Correspondence: michael.boyce@duke.edu and jentsan.chi@duke.edu

10

11 Conflict of interest: The authors have declared that no conflict of interest exists.

12

13

14

15

16

17

18

19

20

21

22

23

24

25

Abstract

Gigaxonin (also known as KLHL16) is an E3 ligase adaptor protein that promotes the ubiquitination and degradation of intermediate filament (IF) proteins. Mutations in human gigaxonin cause the fatal neurodegenerative disease giant axonal neuropathy (GAN), in which IF proteins accumulate and aggregate in axons throughout the nervous system, impairing neuronal function and viability. Despite this pathophysiological significance, the upstream regulation and downstream effects of normal and aberrant gigaxonin function remain incompletely understood. Here, we report that gigaxonin is modified by O-linked β -N-acetylglucosamine (O-GlcNAc), a prevalent form of intracellular glycosylation, in a nutrient- and growth factor-dependent manner. Mass spectrometry analyses of human gigaxonin revealed nine candidate sites of O-GlcNAcylation, two of which – serine 272 and threonine 277 – are required for its ability to mediate IF turnover in novel gigaxonin-deficient human cell models that we created. Taken together, these results suggest that nutrient-responsive gigaxonin O-GlcNAcylation forms a regulatory link between metabolism and IF proteostasis. Our work may have significant implications for understanding the non-genetic modifiers of GAN phenotypes and for the optimization of gene therapy for this disease.

Introduction

Giant axonal neuropathy (GAN)

Giant axonal neuropathy (GAN) is a rare neurodegenerative disease

characterized by abnormally large and dysfunctional axons (1). GAN first manifests as severe peripheral motor and sensory neuropathy during infancy and later evolves into central nervous system pathology, including seizures and cognitive impairment (2-4). Most individuals afflicted with GAN become non-ambulatory early in the first decade of life and eventually develop widespread polyneuropathy and dementia (1, 5). Death usually occurs from respiratory failure by age 40 (1, 5).

GAN is caused by loss-of-function mutations in the *GAN* gene, which encodes the gigaxonin protein (2). Gigaxonin (also known as KLHL16) belongs to the Kelch-like (KLHL) protein family, which typically contains BTB, BACK, and Kelch domains (6). Structural and functional studies in representative KLHL family members have demonstrated that the BTB domain interacts with CUL3, a component of E3 ubiquitin ligase complexes, while the KLHL Kelch domains recruit substrates for polyubiquitination (7). Therefore, KLHL proteins are generally considered to be adaptors for CUL3-containing E3 ligase complexes, regulating the ubiquitination and proteolysis of specific substrates.

Gigaxonin promotes the ubiquitination and turnover of IFs, and defective gigaxonin in GAN patients causes neurofilament-L (NF-L) and other IF proteins to accumulate in the axons of neurons (2, 8, 9). Interestingly, this function of gigaxonin is not restricted to the nervous system. For example, gigaxonin is required for the normal proteolysis of vimentin and keratins in fibroblasts, and GAN patients often exhibit curly hair due to high keratin levels (10, 11). Disease-causing *GAN* mutations disable gigaxonin by several mechanisms, including reducing the abundance of its mRNA, destabilizing the protein and/or impairing its biochemical activity (9). Reciprocally, experimental re-expression of gigaxonin in wild type or *GAN*-deficient cells results in the clearance of accumulated IFs (12).

While the regulation and function of gigaxonin remain incompletely understood, several lines of evidence suggest possible connections to cellular metabolism. For example, *GAN*-deficient cells exhibit dysregulated mitochondrial distribution and behavior (10, 13). In *GAN* loss-of-function cells, mitochondria often co-localize with ovoid IF aggregates, frequently found near the nucleus, and mitochondrial motility is reduced (10, 13). Although mitochondrial inner membrane potential is not affected by loss of gigaxonin function (13), the oxygen consumption rate is increased in *GAN* knockout, *GAN* knockdown and *CUL3* knockdown cells (10), consistent with functional links among gigaxonin/CUL3 complexes, IFs and mitochondrial physiology. Furthermore, while ovoid IF aggregates are observed in 3 to 15% of *GAN*-deficient cells grown under standard culture conditions, this phenotype is significantly exacerbated (to 48 to 88%) by serum starvation (8), indicating that nutrient or growth factor signaling impacts on gigaxonin function. Therefore, the severity of *GAN* phenotypes may be modulated by such non-genetic factors as metabolic status. Together, these observations suggest that nutrient cues affect the activity of gigaxonin and its regulation of the IF cytoskeleton, but the underlying mechanisms remain unknown.

O-GlcNAcylation and the KLHL family

In previous work, we discovered a connection between the KLHL protein family and O-GlcNAcylation (14). O-GlcNAc is an abundant form of intracellular glycosylation that reversibly decorates serines and threonines of thousands of nuclear, cytoplasmic and mitochondrial proteins (15, 16). In mammals, O-GlcNAc is added by O-GlcNAc transferase (OGT) and removed by O-GlcNAcase (OGA) in response to a wide range of physiological signals (15-18). UDP-GlcNAc, the nucleotide-sugar

cofactor used by OGT, is created by the hexosamine biosynthetic pathway (HBP) from multiple essential metabolites, including glucose, glutamine, acetyl-coenzyme A, uridine and ATP (19-23). Therefore, fluctuations in these nutrients or growth factors affect the levels of both UDP-GlcNAc and O-GlcNAc, making the modification a sentinel of cell metabolism (16, 23-31). O-GlcNAc cycling controls myriad processes, including cell metabolism, cell cycle progression and cell death (15, 16, 18) and is essential, as genetic ablation of OGT or OGA in mice is lethal (32-34). Aberrant O-GlcNAcylation is also implicated in numerous human diseases, including various forms of neurodegeneration, further underscoring its functional significance (23, 35-43).

Recently, we reported that the KLHL family member KEAP1 (also known as KLHL19) is O-GlcNAcylated. Specific O-GlcNAc sites are necessary for the ability of KEAP1 to mediate the ubiquitination and destruction of NRF2, its best-known target and a master transcriptional regulator of redox stress responses (14, 44, 45). Due to the structural and functional similarities among the KLHL proteins, we speculated that other family members may also be O-GlcNAcylated. Indeed, a pilot experiment suggested that gigaxonin is a potential O-GlcNAc substrate (14). Here, we report that gigaxonin is O-GlcNAcylated on up to nine specific residues in a nutrient-responsive manner. Using novel human gigaxonin loss-of-function neuroblastoma and fibroblast model systems that we created, we identified two specific gigaxonin O-GlcNAc sites that are required for its ability to interact with IF proteins and to promote their turnover. Our results provide new molecular insight into the potential metabolic regulation of the IF cytoskeleton through gigaxonin O-GlcNAcylation and may inform our understanding of GAN disease modifiers and the optimization of GAN gene therapy.

Materials and methods

Cell culture

SH-SY5Y, HEK 293T, HeLa, and MDA-MB-231 were obtained from the Duke Cell Culture Facility (CCF) and BJ5ta was obtained from American Type Culture Collection (ATCC). Media used were: SH-SY5Y, DMEM/F-12 (Gibco #11320), with HEPES (Gibco #15630); HeLa, 293T and MDA-MB-231, DMEM (Gibco #11995), with HEPES; BJ5ta, 4:1 ratio DMEM (Gibco #11965) and Medium 199 (Gibco #11150). All cell lines were maintained at 37 °C supplied with 5% CO₂ following standard guidelines from the Duke CCF or ATCC and were cultured in medium supplied with 10% fetal bovine serum (FBS) and penicillin/streptomycin. For serum starvation, SH-SY5Y cells were first washed twice with phosphate-buffered saline (PBS) and then supplied with medium containing 10% or 0.1% FBS for 72 h (8). For glutamine deprivation, HA-gigaxonin-transfected (24 h) 293T cells were first washed twice with PBS and then supplied medium (Gibco #210130-24; 10% dialyzed FBS, Sigma #F0392; L-methionine, Sigma #M5308, final 30 mg/L; and L-cystine, Sigma #C6727, final 63 mg/L) with or without L-glutamine (Gibco #25030081, 100X) for an additional 22 h before collecting lysates.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of gigaxonin O-GlcNAcylation

Purified gigaxonin-myc-6xHis was separated by SDS-PAGE and Coomassie-stained. Stained bands of the correct molecular weight were subjected to standard in-gel trypsin digestion

(https://genome.duke.edu/sites/genome.duke.edu/files/In-gelDigestionProtocolrevised_0.pdf). Extracted peptides were lyophilized to dryness and resuspended in 12 µl of 0.2% formic acid/2% acetonitrile. Each sample was subjected to chromatographic separation on a Waters NanoAquity UPLC equipped with a 1.7 µm BEH130 C₁₈ 75 µm I.D. X 250 mm reversed-phase column. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Following a 4 µl injection, peptides were trapped for 3 minutes on a 5 µm Symmetry C₁₈ 180 µm I.D. X 20 mm column at 5 µl/minute in 99.9% A. The analytical column was then switched in-line and a linear elution gradient of 5% B to 40% B was performed over 60 minutes at 400 nl/minute. The analytical column was connected to a fused silica PicoTip emitter (New Objective, Cambridge, MA) with a 10 µm tip orifice and coupled to a QExactive Plus mass spectrometer (Thermo) through an electrospray interface operating in data-dependent acquisition mode. The instrument was set to acquire a precursor MS scan from m/z 350-1800 every three seconds. In data-dependent mode, MS/MS scans of the most abundant precursors were collected following higher-energy collisional dissociation (HCD) fragmentation at an HCD collision energy of 27%. Within the MS/MS spectra, if any diagnostic O-GlcNAc fragment ions (m/z 204.0867, 138.0545, or 366.1396) were observed, a second MS/MS spectrum of the precursor was acquired with electron transfer dissociation (ETD)/HCD fragmentation using charge-dependent ETD reaction times and either 30% or 15% supplemental collision energy for ≥ 2+ precursor charge states. For all experiments, a 60-second dynamic exclusion was employed for previously fragmented precursor ions.

Raw LC-MS/MS data files were processed in Proteome Discoverer (Thermo Scientific) and then submitted to independent Mascot searches (Matrix

Science) against a SwissProt database (human taxonomy) containing both forward and reverse entries of each protein (20,322 forward entries). Search tolerances were 5 ppm for precursor ions and 0.02 Da for product ions using semi-trypsin specificity with up to two missed cleavages. Both y/b-type HCD and c/z-type ETD fragment ions were allowed for interpreting all spectra. Carbamidomethylation (+57.0214 Da on C) was set as a fixed modification, whereas oxidation (+15.9949 Da on M) and O-GlcNAc (+203.0794 Da on S/T) were considered dynamic mass modifications. All searched spectra were imported into Scaffold (v4.3, Proteome Software) and scoring thresholds were set to achieve a peptide FDR of 1% using the PeptideProphet algorithm. When satisfactory ETD fragmentation was not obtained, HCD fragmentation was used to determine O-GlcNAc residue modification, using the number of HexNAcs identified in combination with the number of serines and threonines in the peptide.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (46) via the PRIDE (47) partner repository with the dataset identifier PXD012488. Reviewer account details:

Username: reviewer85399@ebi.ac.uk

Password: 6yY8YKqf

CRISPR and shRNA

To generate *GAN* knockout models, the lentiviral CRISPR/Cas9 system developed by the Zhang lab was used (Addgene #52961) (48). Three different guide RNAs (gRNAs) targeting the human *GAN* gene were selected using the CHOPCHOP website (<http://chopchop.cbu.uib.no>) (gRNA1-F:

200 CACCGGCAGAAGAACATCCTGGCGG, gRNA1-R:
 201 AAACCCGCCAGGATGTTCTTCTGCC; gRNA2-F:
 202 CACCGGGTGCAGAAGAACATCCTGG, gRNA2-R:
 203 AAACCCAGGATGTTCTTCTGCACCC; gRNA3-F:
 204 CACCGCGGCCAGCCCGTACATCAGG, gRNA3-R:
 205 AAACCCTGATGTACGGGCTGGCCGC). SH-SY5Y and BJ5ta cells were infected
 206 with gRNA/Cas9-expressing lentivirus and selected with puromycin to generate
 207 *GAN*-deficient cell lines. The expression of gigaxonin and Cas9 was validated by
 208 Western blotting after puromycin selection. Cell lines were maintained in
 209 puromycin-containing media.

210 To knock down *GAN* expression, a lentiviral shRNA system was purchased
 211 from Sigma (TRCN0000083858), targeting the *GAN* 3' UTR, with shRNA
 212 CCGGCCACATAATATGGGATGCAATCTCGAGATTGCATCCCATATTATGT
 213 GGTTTTTTG.

214

215 *Immunofluorescence assay (IFA)*

216 To visualize IFs, control or *GAN*^{-/-} cells were seeded onto chamber slides
 217 (Thermo) and transfected using Lipofectamine 3000 (Invitrogen) for 72 h. Cells were
 218 fixed in 4% formaldehyde for 10 minutes, followed by three washes with 1X PBS,
 219 blocking at room temperature for 1 h (5% bovine serum albumin in 1X PBS, 0.3%
 220 Triton X-100), and incubation with anti-vimentin (Cell Signaling #5741, 1:200 in
 221 blocking solution) or anti-HA (Santa Cruz #sc-7392, 1:100 in blocking solution)
 222 antibody at 4 °C overnight. Nuclei were marked by DAPI (ThermoFisher #S36938)
 223 and F-actin was visualized by phalloidin staining (Alexa Fluor 594 phalloidin
 224 #A12381), if applied. The above staining protocol was also applied for NF-L (Cell

Signaling #2837, 1:100), GM130 (Cell Signaling #12480, 1:100) and the V5 epitope tag (Cell Signaling #13202, 1:100). All images were acquired on a Zeiss 780 inverted confocal microscope at the Duke Light Microscopy Core Facility.

Western blot (WB) and immunoprecipitation (IP)

To measure vimentin levels after genetic or chemical manipulation, cells were first lysed in radioimmunoprecipitation assay (RIPA) buffer (ThermoFisher; 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). Whole-cell lysates were then sonicated (>20 pulses) on ice until all insoluble material was dissolved (i.e., no aggregates visible after centrifugation). Protein concentrations were determined by bicinchoninic acid assay and used to normalize all samples. After boiling in sample buffer, the lysates were analyzed by Western blot (WB). For most co-IP experiments, cells were lysed in Pierce IP buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and 25 mM Tris-HCl pH 7.5) supplemented with protease and phosphatase inhibitors. 250 µg to 500 µg (final volume: 250 µl; 1 to 2 µg/µl) protein lysates were used for IPs. For co-IP of EGFP-vimentin and HA-gigaxonin, cells were transfected with the indicated constructs for 24 h and lysed in Pierce IP buffer. Lysates were sonicated gently (15 pulse) to disrupt aggregates and spun down at 4 °C to collect the soluble fraction (discarding visible, insoluble EGFP-positive aggregates). 500 µg of soluble protein extract was incubated with HA or GFP antibody for 6 h before addition of Dynabeads (ThermoFisher) for an overnight incubation at 4 °C. Beads were washed four to five times with Pierce IP buffer. After washing, samples were eluted in 2X sample buffer at 95 °C for 5 minutes. Antibodies used in this study include CUL3 (Bethyl #A301-109A), O-GlcNAc (Santa Cruz RL2, #sc-59624s; ThermoFisher 18B10.C7, #MA1-038), β-tubulin (Cell Signaling #2128), Flag (Sigma

#F1840), MYC (Santa Cruz #sc-40), HA (Santa Cruz #sc-7392), ubiquitin (Santa Cruz #sc-8017), gigaxonin (Sigma #SAB4200104), NF-L (Cell Signaling #2837), vimentin (Cell Signaling #5741), vinculin (Santa Cruz #sc-73614), GAPDH (Santa Cruz #sc-25778), and GFP (Santa Cruz #sc-8334).

Results

Mass spectrometry identifies candidate O-GlcNAc sites on gigaxonin

In a previous study focused on KEAP1 glycosylation (14), we discovered initial evidence of gigaxonin modification by O-GlcNAc. We speculated that gigaxonin O-GlcNAcylation might regulate its function in IF protein turnover, analogous to the role of KEAP1 O-GlcNAcylation in governing the ubiquitination and stabilization of its target NRF2 (14). To test this hypothesis, we first confirmed the O-GlcNAcylation of endogenous gigaxonin in a variety of cell types. In SH-SY5Y neuroblastoma cells, gigaxonin is dynamically O-GlcNAc-modified, as the degree of glycosylation was reduced by treatment with a small molecule inhibitor of OGT (5SGlcNAc) and enhanced by a small molecule inhibitor of OGA (Thiamet-G) (Figure 1A). Because gigaxonin regulates IF proteins in non-neuronal tissues as well (49-51), we asked whether these observations extended to other cell types. Indeed, we observed dynamic gigaxonin O-GlcNAcylation in HeLa (Figure 1B) and MDA-MB-231 cells (Figure 1C) as well, supporting gigaxonin O-GlcNAcylation as a broad phenomenon across disparate cell types.

To identify O-GlcNAcylated residues on gigaxonin, we expressed myc-6xHis-tagged gigaxonin (MH-gigaxonin) in 293T cells, a facile human expression system, and obtained highly purified protein via tandem IP using anti-myc

antibody and immobilized metal affinity chromatography (Figure 1D). Purified gigaxonin from two biological replicates was digested in-gel and analyzed by mass spectrometry (MS) to identify O-GlcNAc sites.

In our first MS experiment, we identified nine candidate O-GlcNAc sites on gigaxonin. Five sites (T207, S219, S224, S228 and S229) reside in the BACK domain, three (S249, S254, S272) lie in the junction between the BACK and the first Kelch domain and the last site (T277) is within the first Kelch domain (Figure 1E). In a second MS analysis of an independently expressed and purified gigaxonin sample, we again observed the glycosylation of S228, S229, S272 and T277, underlining the reproducibility of the methodology and providing strong evidence for the O-GlcNAcylation of these residues.

To identify the major *in vivo* O-GlcNAcylation site(s) on gigaxonin, we expressed wild type or unglycosylatable (Ser/Thr→Ala) point-mutant constructs in 293T cells and analyzed them by IP/WB. No single point mutation dramatically reduced total detectable gigaxonin O-GlcNAcylation under standard culture conditions, possibly due to the simultaneous modification of multiple sites or to technical limitations of the available anti-O-GlcNAc antibodies (data not shown). On the other hand, upon inhibition of OGA by Thiamet-G, the S272A and T277A mutants, when compared to wild type, showed modestly impaired increases in O-GlcNAc signal (Figure S1). This result suggests that inducible gigaxonin O-GlcNAcylation may occur primarily at S272 and/or T277.

Gigaxonin loss-of-function neuroblastoma and fibroblast cells provide novel GAN model systems

Primary fibroblasts from GAN patients have been powerful tools for

dissecting the functional impact of gigaxonin mutants, but they are genetically heterogeneous and often exhibit variable IF phenotypes (e.g., 3 to 15% of cells displaying ovoid aggregates), which may confound the interpretation of some experiments (8, 52). Mouse models of GAN have also been valuable but exhibit less severe neurodegeneration than do human patients, suggesting potentially important species-dependent differences (53-55). Therefore, we sought to use CRISPR genome engineering to ablate endogenous gigaxonin expression in human cells, establishing *GAN*^{-/-} systems that reliably recapitulate cellular GAN disease phenotypes and permit functional tests of gigaxonin mutants. We envisioned that such tractable human cell lines would provide a useful complement to existing GAN primary cell and mouse systems. We chose SH-SY5Y and BJ5ta (immortalized human fibroblasts) cells as appropriate models based on previous reports of gigaxonin function and IF protein accumulation in these cell types (12). Multiple guide RNAs (gRNA) targeting *GAN* exon 1 were designed and sub-cloned into a lentiCRISPR vector (56). After selection, *GAN*^{-/-} SH-SY5Y cells exhibited reduced proliferation and often grew as individual cells instead of the clusters seen in the parental cell line (Figure S2C).

We evaluated gigaxonin and vimentin expression in *GAN*^{-/-} cells by WB. Most gigaxonin protein was depleted in the *GAN*-targeted cells (Figure 2A), with a corresponding dramatic increase in endogenous vimentin (Figure 2A and S2A). By IFA, most *GAN*^{-/-} SH-SY5Y (Figure 2B) and BJ5ta (Figure S2B) cells showed single, perinuclear and ovoid-shaped vimentin aggregates, similar to previously reported phenotypes in primary cells from GAN patients (Figure 2B) (8, 12). Aggregates co-localized with the microtubule organizing center but not with Golgi markers (Figure 2D-E). Consistent with a previous report (8), F-actin distribution was not affected by gigaxonin loss (Figure 2C), demonstrating the specificity of these effects

on the IF compartment.

To confirm our CRISPR results by an independent method, we also established stable shRNA-mediated *GAN* knockdown cell lines (shGAN). As expected, we observed vimentin increases and aggregation in shGAN SH-SY5Y cells using both WB (Figure S3A) and IFA (Figure S3B). Importantly, shGAN cells showed similar cellular morphology as *GAN*^{-/-} cells, and re-expression of wild type shRNA-resistant gigaxonin (V5-GAN^R; R: shRNA-resistant) abolished both vimentin accumulation (Figure S3A and B) and the cellular morphology phenotype (Figure S3C). Rescue of these GAN-like phenotypes by gigaxonin re-expression confirms the specificity and validity of our cell systems.

Taken together, these results show that engineered *GAN*-deficient neuroblastoma and fibroblast cell lines phenocopy pathophysiologically relevant characteristics of GAN patient cells (8), indicating that they are tractable and appropriate models for evaluating the function of gigaxonin O-GlcNAcylation.

S272 and T277 O-GlcNAc sites are required for gigaxonin function

To evaluate the functional significance of the gigaxonin O-GlcNAc sites we identified, we harnessed the disease-relevant vimentin aggregation phenotype of *GAN*^{-/-} cells (Figure 2) to assess the function of unglycosylatable gigaxonin point-mutants. First, we expressed HA-tagged wild type or S52G mutant gigaxonin (a known loss-of-function GAN disease allele, as a control (12)) in *GAN*^{-/-} SH-SY5Y cells and performed IFA. As before, *GAN*^{-/-} SH-SY5Y cells exhibited dramatically increased vimentin levels, as compared to controls (Figure 3A, upper two rows). Re-expression of wild type gigaxonin (HA-WT-gigaxonin) markedly repressed vimentin levels (Figure 3A, 3rd row), whereas the disease-causing S52G mutant did

not (Figure 3A, 4th row) (12). These results confirm that the vimentin aggregation phenotype of *GAN*^{-/-} cells is an appropriate assay for assessing the function of wild type and mutant gigaxonin.

Next, we used *GAN*^{-/-} cells to test the function of the nine unglycosylatable gigaxonin point-mutants. Interestingly, among all mutants, only S272A and T277A consistently failed to clear vimentin aggregates across three independent experiments, comparable to the S52G mutant (Figure 3B). These results indicate that the S272 and T277 O-GlcNAcylation sites are critical for the ability of gigaxonin to regulate IF proteins. Given the evidence that S272 and T277 may also be the primary sites of inducible gigaxonin O-GlcNAcylation (Figure S1), the dynamic, site-specific glycosylation of S272 and T277 may be required for normal gigaxonin activity in cells.

Because gigaxonin and CUL3 interact genetically and biochemically, we hypothesized that loss of gigaxonin O-GlcNAcylation at specific sites might affect these functional interactions, analogous to our prior results with KEAP1 (14). To test this possibility, we co-expressed wild type or unglycosylatable gigaxonin mutants with CUL3 in 293T cells and examined their effects endogenous IF proteins. Overexpression of gigaxonin significantly reduces vimentin levels even in cells that express endogenous gigaxonin (12). However, CUL3 overexpression alone had no noticeable effect on vimentin (Figure S4A). Co-expression of CUL3 and gigaxonin further reduced vimentin levels, as predicted by their established functional interaction (Figure S4A). In contrast, the co-expression of the S52G GAN mutant failed to reduce vimentin and NF-L levels, compared to wild type gigaxonin (Figure 4 and S4A), in agreement with a previous report (12). Consistent with our results in *GAN*^{-/-} cells (Figure 3), co-expression of the S272A or T277A mutant with CUL3

failed to reduce vimentin and NF-L levels in three biological replicates (Figure 4).

Collectively, these data highlight the importance of the S272 and T277 gigaxonin

O-GlcNAcylation sites in governing the degradation of IF proteins.

Gigaxonin O-GlcNAcylation sites are required for interaction with IF proteins

Gigaxonin is thought to be an adaptor for CUL3-containing E3 ligase complexes, mediating the ubiquitination and degradation of IF proteins. Gigaxonin interacts with both CUL3 (7) and the E3 ligase RBX1 through its BTB domain (57). Truncation mutants and biochemical experiments have demonstrated that the gigaxonin Kelch domains interact with ubiquitination targets, such as tubulin cofactor B (58, 59) and vimentin (60). However, the specific residues on gigaxonin critical for interacting with these targets remain unidentified.

Based on our prior studies of KEAP1 glycosylation (14), we hypothesized that O-GlcNAcylation of gigaxonin might influence its biochemical interactions with CUL3 or with IF proteins themselves. In support of this idea, STRING analysis, based on direct and indirect interactome databases (61), suggested that gigaxonin interacts with several E3 ligase components, including CUL3 and RBX1 (Figure S4B). We experimentally confirmed the biochemical interaction between endogenous CUL3 and gigaxonin via co-IP and WB (Figure S4C). Interestingly, the CUL3/gigaxonin interaction was not affected by the S272A or T277A mutations (Figure 5A), likely because these residues lie outside the BTB domain thought to interact with CUL3 (7).

Next, we tested whether the interactions between gigaxonin and its substrates vimentin or NF-L were affected by O-GlcNAc site mutations. The molecular weight of endogenous vimentin (54 kDa) is close to IgG heavy chain, which would complicate the interpretation of IP/WB results. Therefore, we used a previously

validated EGFP-vimentin construct (62) for these experiments. EGFP-vimentin and HA-gigaxonin constructs (wild type, S272A or T277A mutants) were co-transfected into 293T cells. When overexpressed, most EGFP-vimentin occurs in assembled IFs, which are insoluble in standard IP lysis buffer (62, 63). After gentle sonication and centrifugation, the soluble fraction of lysates was used for co-IP assays, which confirmed a robust interaction between wild type gigaxonin and vimentin (Figure 5B). However, the interaction between vimentin and S272A or T277A gigaxonin, as compared to wild type, was consistently reduced when immunoprecipitated by either HA (gigaxonin) or GFP (vimentin) (Figure 5B). A similarly reduced interaction was also observed between soluble endogenous NF-L and expressed S272A or T277A gigaxonin (Figure 5B). Moreover, the S272A and T277A gigaxonin mutants triggered less polyubiquitination of vimentin, as compared to wild type (Figure 5C), indicating a functional impact of this reduced biochemical interaction. Together, these data demonstrate that the S272 and T277 gigaxonin glycosylation sites are critical for its optimal interaction with IF proteins, leading to polyubiquitination and protein turnover.

Nutrient-responsive O-GlcNAcylation of gigaxonin

Because O-GlcNAc is a nutrient-sensitive and stress-responsive modification (18, 22, 64), we hypothesized that metabolic conditions or stresses might affect gigaxonin O-GlcNAcylation. In a previous study, Bomont and Koenig showed that the vimentin aggregation observed in *GAN* loss-of-function cells was aggravated by serum starvation or microtubule depolymerization (8), treatments also known to induce O-GlcNAc changes (59, 65-67). Therefore, we tested whether gigaxonin O-GlcNAcylation is impacted by serum starvation. Interestingly, IP/WB experiments

revealed that serum starvation reduced gigaxonin glycosylation, as judged by two different anti-O-GlcNAc monoclonal antibodies (Figure 6A). In the same samples, we also observed the accumulation of vimentin under serum starvation (Figure S5A), reminiscent of the enhanced vimentin aggregation when GAN patient fibroblasts were similarly treated (8). In another, independent test of our hypothesis, gigaxonin O-GlcNAcylation was considerably reduced by deprivation of glutamine, an essential nutrient feeding the HBP (Figure 6B). Taken together, these results demonstrate that gigaxonin O-GlcNAcylation is responsive to serum or glutamine levels.

Discussion

Here we report that the KLHL protein gigaxonin is O-GlcNAcylated in a nutrient- and serum-responsive manner. Of nine candidate O-GlcNAc sites we identified, serine 272 and threonine 277 are reproducibly glycosylated and required for gigaxonin's full interaction with, and ubiquitination of, IF proteins. Our new loss-of-function *GAN* models displayed classical ovoid vimentin aggregates in a consistent and robust manner, enabling genetic complementation experiments to measure the impact of individual point-mutations on IF protein turnover. These experiments further confirmed the functional significance of the S272 and T277 O-GlcNAc sites. Together, our results suggest that stimulus-induced O-GlcNAcylation of gigaxonin may be required for its optimal ability to regulate IF protein turnover, perhaps by inducing a conformational change in gigaxonin that promotes IF protein binding (Figure 7).

New culture models of GAN

Current models for GAN rely primarily on patient-derived fibroblasts (8, 12, 52), induced pluripotent stem cells (68) or *GAN*^{-/-} mice (13, 53-55). Though valuable, patient-derived cells have heterogeneous genetic backgrounds and display incompletely penetrant IF phenotypes (e.g., 3 to 15% of cells displaying ovoid aggregates), which may complicate the interpretation of mechanistic and functional studies. *GAN*^{-/-} mice have also been powerful systems but do not fully phenocopy human GAN symptoms, suggesting important species-dependent differences (13, 53-55). Using CRISPR-mediated gene deletion, we developed *GAN*^{-/-} models in both GAN-relevant human fibroblast and neuroblastoma cell lines (12), in which more than 80% of *GAN*^{-/-} cells display ovoid-shaped vimentin aggregates (Figure 2). These human *GAN*^{-/-} cell lines are easier to propagate and manipulate experimentally than are patient-derived cells or mice and will therefore complement existing systems and facilitate the characterization of the biochemical regulation of gigaxonin and IF aggregation. Importantly, our cell systems may also enable future high-throughput chemical or genetic screens to identify candidate drugs and drug targets to ameliorate IF protein aggregation in GAN.

Gigaxonin O-GlcNAc sites in human disease

Our results suggest novel hypotheses to test in GAN disease contexts. For example, gigaxonin O-GlcNAcylation may be dysregulated in GAN patients, leading to reduced gigaxonin stability or reduced interaction with, and ubiquitination of, soluble IF proteins. While none of the nine identified candidate O-GlcNAc sites is reported to be mutated in the GAN patients sequenced to date, it is possible that mutations at other sites may affect O-GlcNAcylation by inducing conformational changes that affect the access of OGT or OGA. More work will be required to gain

mechanistic insight into how O-GlcNAcylation regulates gigaxonin function in GAN-relevant contexts. For instance, it will be important to analyze the O-GlcNAcylation status of gigaxonin in human patients, which may be helpful in predicting protein function *in vivo*.

Beyond GAN, 276 different gigaxonin mutations have been identified in human tumor samples in the cBioportal database (69, 70). Among these somatic mutations in cancer, an S272F mutation was identified in a cutaneous melanoma and an S254C mutation in prostate adenocarcinoma. Our results predict that the S272F mutation, at least, would impair gigaxonin function. IFs – especially vimentin – participate in multiple cancer-relevant processes, including cell adhesion, migration, epithelial-mesenchymal transition and metastasis (71, 72). Therefore, the biological significance of gigaxonin in cancer or other diseases may be underappreciated, and the function of gigaxonin in tumor biology could be an interesting subject of future work (73).

Nutrient sensing and O-GlcNAcylation as potential modifiers of gigaxonin function and GAN phenotypes

Nutrient sensing is thought to be a prominent function of O-GlcNAcylation in intracellular signaling (16, 19-31). In a previous study, we reported that O-GlcNAcylation of the KLHL protein KEAP1 varies dynamically with glucose levels (14). Given these results, it is intriguing that gigaxonin O-GlcNAcylation also fluctuates with metabolic cues (Figure 6). Together, these observations suggest that nutrient status and IF proteostasis might be linked through the glycosylation of gigaxonin in particular, and that O-GlcNAcylation may be a widespread regulatory modification of KLHL proteins in general.

Here we show that deprivation of serum or glutamine (which feeds the HBP) reduces gigaxonin O-GlcNAcylation (Figure 6). Interestingly, serum starvation also potentiates IF aggregation in *GAN* loss-of-function cells, but the underlying mechanism remained unclear (8). We propose that nutrient or growth factor starvation may trigger gigaxonin deglycosylation, reducing its interaction with its substrates, such as vimentin or NF-L, and eventually leading to IF accumulation and aggregation (Figure 7). Serum starvation induces a variety of signaling events that may directly or indirectly regulate OGT, OGA or UDP-GlcNAc availability. The integration of these factors in the O-GlcNAcylation, regulation and function of gigaxonin will be a key focus of future studies. In the longer term, it will be important to determine whether changes in metabolic cues and gigaxonin glycosylation affect its function *in vivo*, and whether this signaling is dysregulated in GAN patients, potentially contributing to the variability of disease severity.

Therapeutic implications

Finally, our findings may have important implications for GAN therapy. Currently, there are no treatments to mitigate symptoms or disease progression, due partly to our imperfect understanding of the molecular etiology of GAN. However, gene therapy approaches have shown significant potential. For example, Gray and colleagues used engineered adeno-associated virus (AAV) vectors to restore wild type gigaxonin expression in *GAN*^{-/-} mice and achieved persistent transgene expression in the central and peripheral nervous systems for more than one year, accompanied by reduced neuronal IF protein aggregation (74). More recently, the Gray lab conducted clinical trials of intrathecal delivery of a scAAV9/JeT-GAN vector to re-express wild type gigaxonin in GAN patients (NCT02362438). Despite this clear promise, potential

obstacles remain. For example, endogenous gigaxonin is normally expressed at low levels and its ectopic overexpression obliterates the entire IF network, leading to cell dysfunction (11, 12). Therefore, improved knowledge of gigaxonin regulation – through O-GlcNAcylation and other mechanisms – is needed to understand its activity in healthy neurons and, perhaps, to tune its activity during GAN gene therapy (51, 75).

Author contributions

PHC, TJS, SP, AS, AL, and JH performed the experiments. PHC, JTC, and MB designed the experiments and interpreted the results. PHC, JH, JTC, and MB wrote the manuscript. All authors reviewed and approved the manuscript. JTC and MB supervised all aspects of the work.

Acknowledgements

We thank the members of the Boyce and Chi labs for critical discussion and technical support and Dr. Erik Soderblom and the Duke Proteomics and Metabolomics Shared Resource for O-GlcNAc site-mapping analyses. This study was supported by NIH grants R01GM118847 (to MB), GM124062 (to JTC), DOD grants (W81XWH-17-1-0143, W81XWH-15-1-0486 to JTC), Duke Cancer Institute (DCI) pilot fund (to MB and JTC) and by a gift from the Hannah's Hope Fund (to MB and JTC).

References

1. Gordon N. Giant axonal neuropathy. *Dev Med Child Neurol*. 2004;46(10):717-9.
2. Bomont P, Cavalier L, Blondeau F, Ben Hamida C, Belal S, Tazir M, et al. The gene encoding gigaxonin, a new member of the cytoskeletal BTB/kelch repeat family, is mutated in giant axonal neuropathy. *Nat Genet*. 2000;26(3):370-4.
3. Berg BO, Rosenberg SH, and Asbury AK. Giant axonal neuropathy. *Pediatrics*. 1972;49(6):894-9.
4. Asbury AK, Gale MK, Cox SC, Baringer JR, and Berg BO. Giant axonal neuropathy--a unique case with segmental neurofilamentous masses. *Acta Neuropathol*. 1972;20(3):237-47.
5. Yang Y, Allen E, Ding J, and Wang W. Giant axonal neuropathy. *Cell Mol Life Sci*. 2007;64(5):601-9.
6. Dhanoa BS, Cogliati T, Satish AG, Bruford EA, and Friedman JS. Update on the Kelch-like (KLHL) gene family. *Hum Genomics*. 2013;7:13.
7. Furukawa M, He YJ, Borchers C, and Xiong Y. Targeting of protein ubiquitination by BTB-Cullin 3-Roc1 ubiquitin ligases. *Nat Cell Biol*. 2003;5(11):1001-7.
8. Bomont P, and Koenig M. Intermediate filament aggregation in fibroblasts of giant axonal neuropathy patients is aggravated in non dividing cells and by microtubule destabilization. *Hum Mol Genet*. 2003;12(8):813-22.
9. Boizot A, Talmat-Amar Y, Morrogh D, Kuntz NL, Halbert C, Chabrol B, et al. The instability of the BTB-KELCH protein Gigaxonin causes Giant Axonal Neuropathy and constitutes a new penetrant and specific diagnostic test. *Acta Neuropathol Commun*. 2014;2:47.
10. Lowery J, Jain N, Kuczmarski ER, Mahammad S, Goldman A, Gelfand VI, et al. Abnormal intermediate filament organization alters mitochondrial motility in giant axonal neuropathy fibroblasts. *Mol Biol Cell*. 2016;27(4):608-16.
11. Cleveland DW, Yamanaka K, and Bomont P. Gigaxonin controls vimentin organization through a tubulin chaperone-independent pathway. *Human Molecular Genetics*. 2009;18(8):1384-94.

12. Mahammad S, Murthy SN, Didonna A, Grin B, Israeli E, Perrot R, et al. Giant axonal neuropathy-associated gigaxonin mutations impair intermediate filament protein degradation. *J Clin Invest.* 2013;123(5):1964-75.
13. Israeli E, Dryanovski DI, Schumacker PT, Chandel NS, Singer JD, Julien JP, et al. Intermediate filament aggregates cause mitochondrial dysmotility and increase energy demands in giant axonal neuropathy. *Hum Mol Genet.* 2016;25(11):2143-57.
14. Chen PH, Smith TJ, Wu J, Siesser PF, Bisnett BJ, Khan F, et al. Glycosylation of KEAP1 links nutrient sensing to redox stress signaling. *EMBO J.* 2017;36(15):2233-50.
15. Hart GW, Slawson C, Ramirez-Correa G, and Lagerlof O. Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. *Annual review of biochemistry.* 2011;80:825-58.
16. Hanover JA, Krause MW, and Love DC. The hexosamine signaling pathway: O-GlcNAc cycling in feast or famine. *Biochim Biophys Acta.* 2010;1800(2):80-95.
17. Hart GW. Three Decades of Research on O-GlcNAcylation - A Major Nutrient Sensor That Regulates Signaling, Transcription and Cellular Metabolism. *Front Endocrinol (Lausanne).* 2014;5:183.
18. Bond MR, and Hanover JA. A little sugar goes a long way: the cell biology of O-GlcNAc. *J Cell Biol.* 2015;208(7):869-80.
19. Hart GW, Housley MP, and Slawson C. Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. *Nature.* 2007;446(7139):1017-22.
20. Wells L, Vosseller K, and Hart GW. Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc. *Science.* 2001;291(5512):2376-8.
21. Love DC, and Hanover JA. The hexosamine signaling pathway: deciphering the "O-GlcNAc code". *Sci STKE.* 2005;2005(312):re13.
22. Bond MR, and Hanover JA. O-GlcNAc Cycling: A Link Between Metabolism and Chronic Disease. *Annual Review of Nutrition.* 2013;33(1):205-29.
23. Bond MR, and Hanover JA. O-GlcNAc cycling: a link between metabolism and chronic disease. *Annu Rev Nutr.* 2013;33:205-29.
24. Mondoux MA, Love DC, Ghosh SK, Fukushima T, Bond M, Weerasinghe GR, et al. O-linked-N-acetylglucosamine cycling and insulin signaling are required

- for the glucose stress response in *Caenorhabditis elegans*. *Genetics*. 2011;188(2):369-82.
25. Kreppel LK, and Hart GW. Regulation of a cytosolic and nuclear O-GlcNAc transferase. Role of the tetratricopeptide repeats. *J Biol Chem*. 1999;274(45):32015-22.
26. Ruan H-B, Han X, Li M-D, Singh Jay P, Qian K, Azarhoush S, et al. O-GlcNAc Transferase/Host Cell Factor C1 Complex Regulates Gluconeogenesis by Modulating PGC-1 α Stability. *Cell metabolism*. 2012;16:226-37.
27. Ruan HB, Dietrich MO, Liu ZW, Zimmer MR, Li MD, Singh JP, et al. O-GlcNAc transferase enables AgRP neurons to suppress browning of white fat. *Cell*. 2014;159(2):306-17.
28. Taylor RP, Geisler TS, Chambers JH, and McClain DA. Up-regulation of O-GlcNAc transferase with glucose deprivation in HepG2 cells is mediated by decreased hexosamine pathway flux. *J Biol Chem*. 2009;284(6):3425-32.
29. Taylor RP, Parker GJ, Hazel MW, Soesanto Y, Fuller W, Yazzie MJ, et al. Glucose deprivation stimulates O-GlcNAc modification of proteins through up-regulation of O-linked N-acetylglucosaminyltransferase. *J Biol Chem*. 2008;283(10):6050-7.
30. Liu K, Paterson AJ, Chin E, and Kudlow JE. Glucose stimulates protein modification by O-linked GlcNAc in pancreatic beta cells: linkage of O-linked GlcNAc to beta cell death. *Proc Natl Acad Sci U S A*. 2000;97(6):2820-5.
31. Cheung WD, and Hart GW. AMP-activated protein kinase and p38 MAPK activate O-GlcNAcylation of neuronal proteins during glucose deprivation. *J Biol Chem*. 2008;283(19):13009-20.
32. Shafi R, Iyer SP, Ellies LG, O'Donnell N, Marek KW, Chui D, et al. The O-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. *Proceedings of the National Academy of Sciences of the United States of America*. 2000;97(11):5735-9.
33. Keembiyehetty C, Love DC, Harwood KR, Gavrilova O, Comly ME, and Hanover JA. Conditional knockout reveals a requirement for O-GlcNAcase in metabolic homeostasis. *The Journal of biological chemistry*. 2015.

34. Yang YR, Song M, Lee H, Jeon Y, Choi EJ, Jang HJ, et al. O-GlcNAcase is essential for embryonic development and maintenance of genomic stability. *Aging cell*. 2012;11(3):439-48.
35. Wang P, and Hanover JA. Nutrient-driven O-GlcNAc cycling influences autophagic flux and neurodegenerative proteotoxicity. *Autophagy*. 2013;9(4):604-6.
36. Shin SH, Love DC, and Hanover JA. Elevated O-GlcNAc-dependent signaling through inducible mOGT expression selectively triggers apoptosis. *Amino Acids*. 2011;40(3):885-93.
37. Lazarus BD, Love DC, and Hanover JA. O-GlcNAc cycling: implications for neurodegenerative disorders. *Int J Biochem Cell Biol*. 2009;41(11):2134-46.
38. Hanover JA, and Wang P. O-GlcNAc cycling shows neuroprotective potential in *C. elegans* models of neurodegenerative disease. *Worm*. 2013;2(4):e27043.
39. Akan I, Olivier-Van Stichelen S, Bond MR, and Hanover JA. Nutrient-driven O-GlcNAc in proteostasis and neurodegeneration. *J Neurochem*. 2018;144(1):7-34.
40. Yuzwa SA, Shan X, Macauley MS, Clark T, Skorobogatko Y, Vosseller K, et al. Increasing O-GlcNAc slows neurodegeneration and stabilizes tau against aggregation. *Nat Chem Biol*. 2012;8(4):393-9.
41. Vaidyanathan K, Durning S, and Wells L. Functional O-GlcNAc modifications: implications in molecular regulation and pathophysiology. *Critical reviews in biochemistry and molecular biology*. 2014;49(2):140-63.
42. Yuzwa SA, and Vocadlo DJ. O-GlcNAc and neurodegeneration: biochemical mechanisms and potential roles in Alzheimer's disease and beyond. *Chemical Society reviews*. 2014;43(19):6839-58.
43. Zhu Y, Shan X, Yuzwa SA, and Vocadlo DJ. The emerging link between O-GlcNAc and Alzheimer disease. *The Journal of biological chemistry*. 2014;289(50):34472-81.
44. Chen PH, Chi JT, and Boyce M. KEAP1 has a sweet spot: A new connection between intracellular glycosylation and redox stress signaling in cancer cells. *Mol Cell Oncol*. 2017;4(6):e1361501.
45. Chen PH, Chi JT, and Boyce M. Functional crosstalk among oxidative stress and O-GlcNAc signaling pathways. *Glycobiology*. 2018;28(8):556-64.

46. Deutsch EW, Csordas A, Sun Z, Jarnuczak A, Perez-Riverol Y, Ternent T, et al. The ProteomeXchange consortium in 2017: supporting the cultural change in proteomics public data deposition. *Nucleic Acids Res.* 2017;45(D1):D1100-D6.
47. Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu DJ, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* 2019;47(D1):D442-D50.
48. Sanjana NE, Shalem O, and Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods.* 2014;11(8):783-4.
49. Soomro A, Alsop RJ, Negishi A, Kreplak L, Fudge D, Kuczmarski ER, et al. Giant axonal neuropathy alters the structure of keratin intermediate filaments in human hair. *J R Soc Interface.* 2017;14(129).
50. Treiber-Held S, Budjarjo-Welim H, Reimann D, Richter J, Kretzschmar HA, and Hanefeld F. Giant axonal neuropathy: a generalized disorder of intermediate filaments with longitudinal grooves in the hair. *Neuropediatrics.* 1994;25(2):89-93.
51. Johnson-Kerner BL, Roth L, Greene JP, Wichterle H, and Sproule DM. Giant axonal neuropathy: An updated perspective on its pathology and pathogenesis. *Muscle Nerve.* 2014;50(4):467-76.
52. Bomont P. Degradation of the Intermediate Filament Family by Gigaxonin. *Methods Enzymol.* 2016;569:215-31.
53. Dequen F, Bomont P, Gowing G, Cleveland DW, and Julien JP. Modest loss of peripheral axons, muscle atrophy and formation of brain inclusions in mice with targeted deletion of gigaxonin exon 1. *J Neurochem.* 2008;107(1):253-64.
54. Ganay T, Boizot A, Burrer R, Chauvin JP, and Bomont P. Sensory-motor deficits and neurofilament disorganization in gigaxonin-null mice. *Mol Neurodegener.* 2011;6:25.
55. Armao D, Bailey RM, Bouldin TW, Kim Y, and Gray SJ. Autonomic nervous system involvement in the giant axonal neuropathy (GAN) KO mouse: implications for human disease. *Clin Auton Res.* 2016;26(4):307-13.
56. Sanjana NE, Shalem O, and Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods.* 2014;11(8):783-4.

57. Allen E, Ding J, Wang W, Pramanik S, Chou J, Yau V, et al. Gigaxonin-controlled degradation of MAP1B light chain is critical to neuronal survival. *Nature*. 2005;438(7065):224-8.
58. Wang W, Ding J, Allen E, Zhu P, Zhang L, Vogel H, et al. Gigaxonin interacts with tubulin folding cofactor B and controls its degradation through the ubiquitin-proteasome pathway. *Curr Biol*. 2005;15(22):2050-5.
59. Slawson C, Zachara NE, Vosseller K, Cheung WD, Lane MD, and Hart GW. Perturbations in O-linked beta-N-acetylglucosamine protein modification cause severe defects in mitotic progression and cytokinesis. *J Biol Chem*. 2005;280(38):32944-56.
60. Johnson-Kerner BL, Garcia Diaz A, Ekins S, and Wichterle H. Kelch Domain of Gigaxonin Interacts with Intermediate Filament Proteins Affected in Giant Axonal Neuropathy. *PLoS One*. 2015;10(10):e0140157.
61. Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, et al. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res*. 2017;45(D1):D362-D8.
62. Tarbet HJ, Dolat L, Smith TJ, Condon BM, O'Brien ET, 3rd, Valdivia RH, et al. Site-specific glycosylation regulates the form and function of the intermediate filament cytoskeleton. *Elife*. 2018;7.
63. Ridge KM, Shumaker D, Robert A, Hookway C, Gelfand VI, Janmey PA, et al. Methods for Determining the Cellular Functions of Vimentin Intermediate Filaments. *Methods Enzymol*. 2016;568:389-426.
64. Zachara NE, and Hart GW. O-GlcNAc a sensor of cellular state: the role of nucleocytoplasmic glycosylation in modulating cellular function in response to nutrition and stress. *Biochim Biophys Acta*. 2004;1673(1-2):13-28.
65. Slawson C, Lakshmanan T, Knapp S, and Hart GW. A mitotic GlcNAcylation/phosphorylation signaling complex alters the posttranslational state of the cytoskeletal protein vimentin. *Mol Biol Cell*. 2008;19(10):4130-40.
66. Chou CF, and Omary MB. Mitotic arrest-associated enhancement of O-linked glycosylation and phosphorylation of human keratins 8 and 18. *J Biol Chem*. 1993;268(6):4465-72.
67. Haltiwanger RS, and Philipsberg GA. Mitotic arrest with nocodazole induces selective changes in the level of O-linked N-acetylglucosamine and

- accumulation of incompletely processed N-glycans on proteins from HT29 cells. *J Biol Chem.* 1997;272(13):8752-8.
68. Johnson-Kerner BL, Ahmad FS, Diaz AG, Greene JP, Gray SJ, Samulski RJ, et al. Intermediate filament protein accumulation in motor neurons derived from giant axonal neuropathy iPSCs rescued by restoration of gigaxonin. *Hum Mol Genet.* 2015;24(5):1420-31.
69. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov.* 2012;2(5):401-4.
70. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal.* 2013;6(269):pl1.
71. Richardson AM, Havel LS, Koyen AE, Konen JM, Shupe J, Wiles WG, et al. Vimentin Is Required for Lung Adenocarcinoma Metastasis via Heterotypic Tumor Cell–Cancer-Associated Fibroblast Interactions during Collective Invasion. *Clinical Cancer Research.* 2018;24(2):420-32.
72. Kidd ME, Shumaker DK, and Ridge KM. The role of vimentin intermediate filaments in the progression of lung cancer. *Am J Respir Cell Mol Biol.* 2014;50(1):1-6.
73. Kang JJ, Liu IY, Wang MB, and Srivatsan ES. A review of gigaxonin mutations in giant axonal neuropathy (GAN) and cancer. *Hum Genet.* 2016;135(7):675-84.
74. Bailey RM, Armao D, Nagabhushan Kalburgi S, and Gray SJ. Development of Intrathecal AAV9 Gene Therapy for Giant Axonal Neuropathy. *Mol Ther Methods Clin Dev.* 2018;9:160-71.
75. Mussche S, Devreese B, Nagabhushan Kalburgi S, Bachaboina L, Fox JC, Shih HJ, et al. Restoration of cytoskeleton homeostasis after gigaxonin gene transfer for giant axonal neuropathy. *Hum Gene Ther.* 2013;24(2):209-19.

Figure 1

Figure 1. Site-specific O-GlcNAcylation of human gigaxonin

(A-C) Inhibition of OGT by 50 μ M Ac₄5SGlcNAc (5SG) or of OGA by 25 μ M

Thiamet-G (TMG) decreases or increases endogenous gigaxonin O-GlcNAcylation,

respectively, in SH-SY5Y (24 h), HeLa (24 h), and MDA-MB-231 cells (34 h). Cells were treated as indicated and endogenous gigaxonin was IPed from whole-cell lysates and analyzed by WB. Gigaxonin bands are indicated by arrows. (D) Coomassie blue stain (left) and WB (right) of myc-6xHis-gigaxonin protein tandem-purified from 293T cells for MS site-mapping. (E) MS analysis identified nine candidate O-GlcNAc sites on gigaxonin, indicated in the context of its domain structure. Complete gigaxonin site-mapping are available via the ProteomeXchange database (<http://www.proteomexchange.org>, dataset identifier PXD012488).

Figure 2

Figure 2. Generation of novel GAN model cell systems by CRISPR-Cas9 genome engineering

(A) SH-SY5Y cells were subjected to control or *GAN* gRNA genome editing. Lysates from single cell-derived clones were analyzed by WB, confirming loss of gigaxonin (arrow) and increased vimentin levels (arrows) in *GAN*^{-/-} cells, as compared to controls. ns, non-specific. (B) Vimentin forms ovoid, perinuclear aggregates in *GAN*^{-/-} cells. Endogenous vimentin (green) and nuclei (DAPI, blue) were visualized by IFA in control and *GAN*^{-/-} cells derived from three independent gRNAs. (C) Vimentin aggregates co-localize with the microtubule organizing center in *GAN*^{-/-} cells, as detected by α -tubulin and vimentin IFA. (D) Vimentin aggregates do not co-localize with the Golgi marker GM130, as indicated by IFA in *GAN*^{-/-} cells. (E) F-actin distribution is not affected by gigaxonin loss, as indicated by IFA in *GAN*^{-/-} cells.

Figure 3

A

Figure 3. Unglycosylatable S272A and T277A gigaxonin mutants are impaired in clearing vimentin aggregates in *GAN*^{-/-} cells

(A) Expression of wild type gigaxonin, but not the S52G mutant, clears vimentin aggregates in *GAN*^{-/-} cells. Control (top row) or *GAN*^{-/-} (rows 2-4) SH-SY5Y cells were mock-transfected or transfected with HA-wild type or HA-S52G gigaxonin constructs for 72 h. Expressed gigaxonin (HA, green) and endogenous vimentin (red) were visualized by IFA. (B) *GAN*^{-/-} SH-SY5Y cells were transfected with the indicated HA-tagged unglycosylatable gigaxonin point-mutant constructs for 72 h. Expressed gigaxonin (HA, green) and endogenous vimentin (red) were visualized by IFA.

Figure 4

Figure 4. Unglycosylatable S272A and T277A gigaxonin mutants are impaired in cooperating with CUL3 to reduce IF protein levels in 293T cells

293T cells were mock-transfected or transfected with MYC-CUL3 and wild type or point-mutant HA-gigaxonin constructs, as indicated, for 72 h. Cell lysates were analyzed by WB.

Figure 5

Figure 5. S272A and T277A gigaxonin mutants have reduced interaction with vimentin and NF-L

(A) 293T cells were co-transfected with constructs encoding GFP or wild type, S272A or T277A HA-gigaxonin and MYC-CUL3, as indicated, for 46 h. Lysates were subjected to MYC IP and analyzed by WB. The interaction between S272A or T277A gigaxonin with CUL3 is comparable to wild type. (B) 293T cells were co-transfected with constructs encoding wild type, S272A or T277A HA-gigaxonin and EGFP-vimentin, as indicated, for 30 h. Lysates were subjected to HA or GFP IP and analyzed by WB. The S272A and T277A gigaxonin mutants show reduced interaction with vimentin and NF-L. (C) 293T cells were co-transfected with constructs encoding EGFP-vimentin and wild type, S272A or T277A HA-gigaxonin, as indicated, for 30 h. Lysates were subjected to GFP IP and analyzed by ubiquitin (Ub) WB. Polyubiquitination of vimentin is reduced in S272A or T277A gigaxonin mutant-expressing cells, compared to wild type.

Figure 6

Figure 6. Nutrient-sensitive regulation of gigaxonin O-GlcNAcylation

(A) SH-SY5Y cells were treated with 10% or 0.1% serum for 72 h, as indicated, and lysed. Endogenous gigaxonin was IP-ed and analyzed by WB. Gigaxonin O-GlcNAcylation is reduced after serum starvation, as indicated by two different anti-O-GlcNAc monoclonal antibodies. (B) 293T cells were transfected with wild type HA-gigaxonin for 24 h, treated with control or glutamine-free medium for an additional 22 h, as indicated, and then lysed. HA-gigaxonin was IP-ed and analyzed by WB. Gigaxonin O-GlcNAcylation is reduced after glutamine starvation, as indicated by two different anti-O-GlcNAc monoclonal antibodies.

Figure 7

Figure 7. Proposed model for O-GlcNAc-mediated regulation of gigaxonin

Taken together, our results suggest that O-GlcNAcylation of gigaxonin promotes its optimal interaction with IF proteins (e.g., vimentin or NF-L) to facilitate their polyubiquitination and proteasome-mediated degradation. Nutrient deprivation and/or other stimuli lead to reduced gigaxonin O-GlcNAcylation, especially at S272 and T277, inhibiting its interaction with IFs and leading to their accumulation.