iScience, Volume *23*

Supplemental Information

Hexosamine Pathway Activation Improves Protein Homeostasis through the Integrated Stress Response

Moritz Horn, Sarah I. Denzel, Balaji Srinivasan, Kira Allmeroth, Isabelle Schiffer, Vignesh Karthikaisamy, Stephan Miethe, Peter Breuer, Adam Antebi, and Martin S. Denzel

Supplemental Figures

Figure S1 (related to Figure 1)

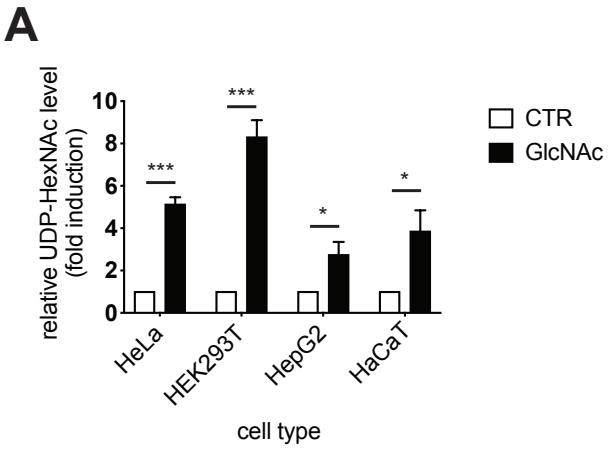

Figure S1- related to Figure 1:

A) UDP-HexNAc levels of indicated cell lines with and without 10 mM GlcNAc treatment for 24h. Mean +SEM ($n \geq 3$), * $p<0.05$ (t-test).

FigureS2 (related to Figure 3)

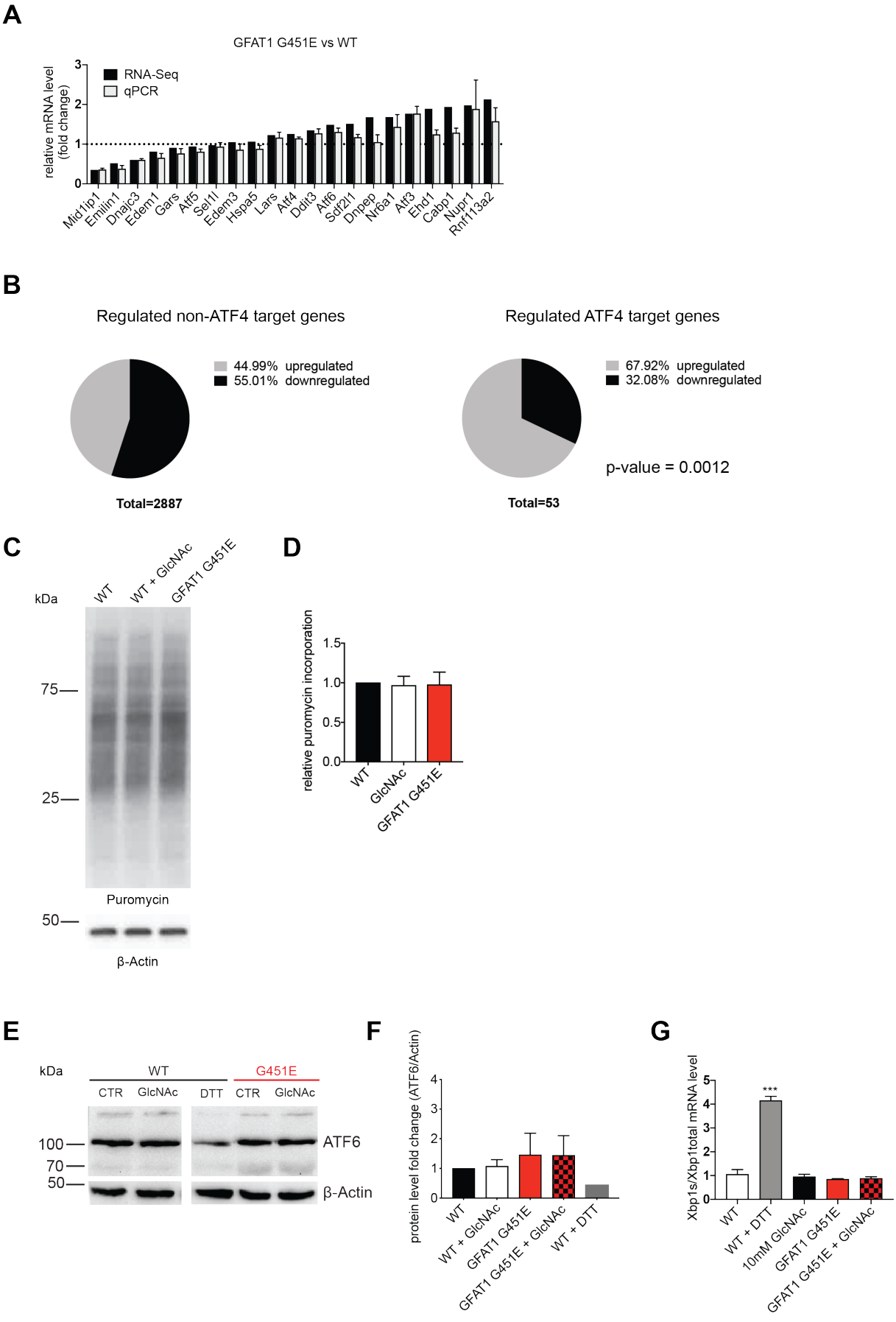

Figure S2 – related to Figure 3:

A) Analysis of differential expression of selected mRNAs between WT and GFAT1 G451E engineered N2a cells from RNA-Seq dataset and analysis by qRT-PCR.

B) Analysis of differentially regulated genes (p-value <0.05) from RNA-Seq experiment with WT and GFAT1 G451E N2a cells. Regulation of ATF4 target genes is compared to non-ATF4 targets (n=3). p-value is indicated (Fisher's exact test).

C) Western blot analysis of puromycin incorporation in WT, GFAT1 G451E, and WT N2a cells treated with 10 mM GlcNAc for 24h.

D) Quantitation of Western blot analysis from Figure S2C. Mean +SD ($n \geq 5$).

E) WT and GFAT1 G451E cells were treated with 10 mM GlcNAc for 16h as indicated and processed for Western blotting to detect full length ATF6. A representative set of n=4 experiments is shown. Image sections are cropped from the same film.

F) Quantification of Western blot analysis from Figure S2C. Mean +SD (n=4).

G) WT and GFAT1 G451E cells were treated with 10 mM GlcNAc for 24h as indicated and processed for qRT-PCR analysis. Treatment with 5 mM DTT for 6h functions as positive control.

Figure S3 (related to Figure 4)

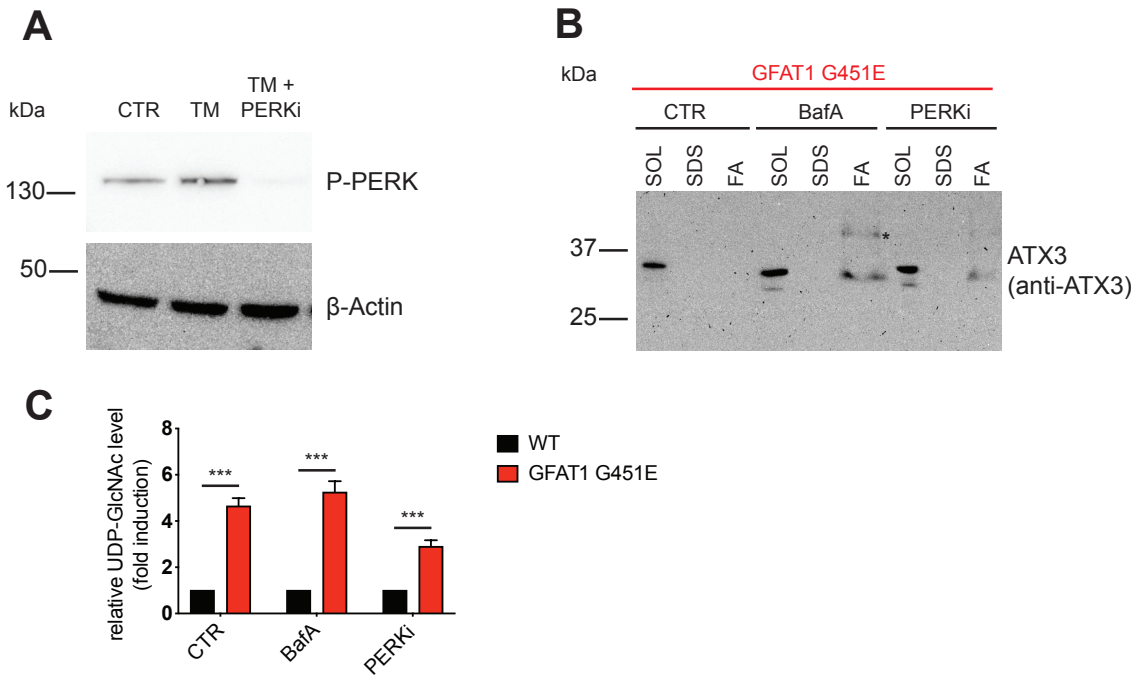

Figure S3 - related to Figure 4:

A) Western blot analysis of N2a cells treated with 10 µg/µL TM or 10 nM PERK inhibitor for 3h.

B) GFAT1 G451E N2a cells transiently expressing the C-terminal fragment described in Figure 2A were treated with the indicated compounds (160 nM BafA, 10 nM PERK inhibitor) and processed for fractionation and Western blotting. * partially formic acid soluble material.

C) Relative UDP-GlcNAc level from WT and GFAT1 G451E N2a cells treated with the indicated compounds (160 nM BafA, 10 nM PERK inhibitor) for 24h.

Figure S4 - related to Figure 6

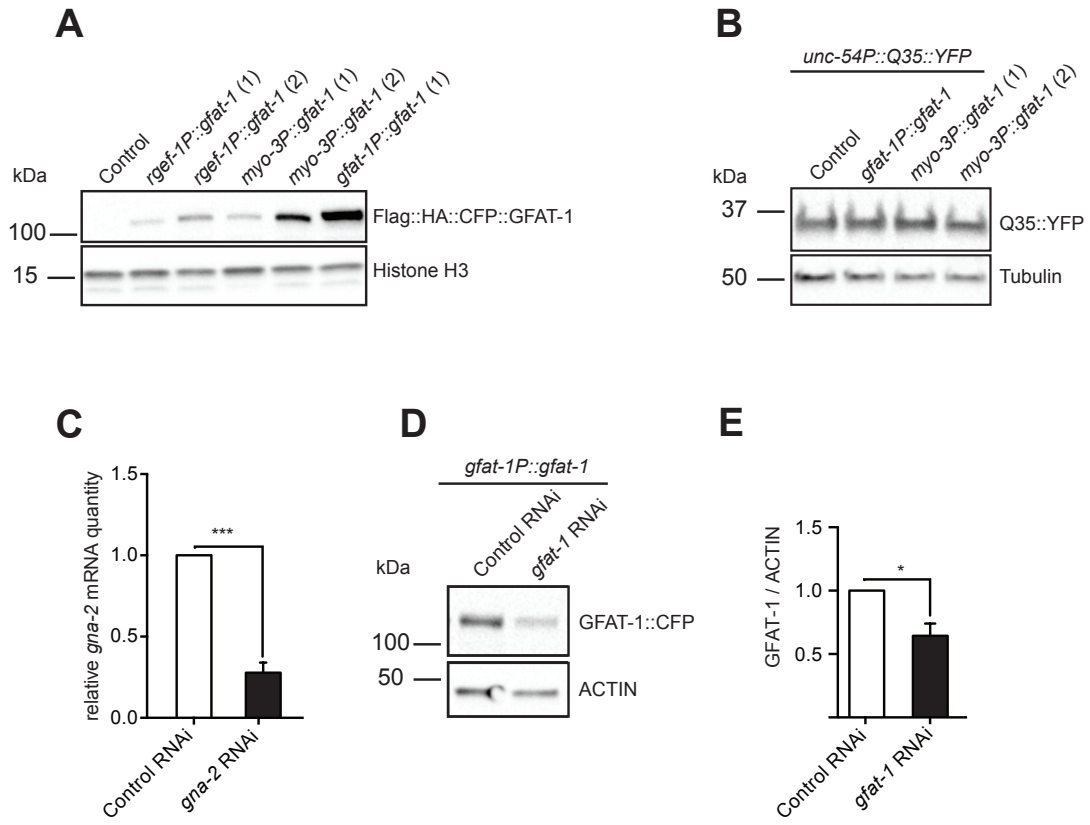

Figure S4 - related to Figure 6:

A) Representative Western blot from L4 larvae showing level of GFAT-1 transgene expression in distinct *gfat-1* OE lines.

B) Representative Western blot of total Q35::YFP expression in distinct *gfat-1* OE lines relative to control at day 1 adult worms, n=2.

C) Relative mRNA abundance in day 1 adult worms grown on RNAi from egg onward as measured by qRT-PCR, shown is mean +SEM, values normalized to *ama-1*, n=3, *** $p<0.001$ (t-test).

D) Representative Western blot of GFAT-1::CFP and actin loading control in day 1 adult *gfat-1P::CFP::gfat-1* strains after RNAi treatment from egg onward.

E) Quantification of GFAT-1::CFP expression relative to actin in day 1 adult worms from Western blot, shown is mean +SEM, n=3, * $p<0.05$ (t-test).

Supplemental Table 1- related to Figure 3.

qPCR primer list	
Gene/Primer	Sequence (5'→3')
qATF4_m_FWD1	GGGTTCTGTCTTCCACTCCA
qATF4_m_REV1	AAGCAGCAGAGTCAGGCTTTC
qHSPA5_m_FWD1	TTCAGCCCAATTATCAGCAAACTC
qHSPA5_m_REV1	TTTTCTGATGTATCCTCTTCACC
qATF3_m_FWD1	ATAAACACCTCTGCCATCGG
qATF3_m_REV1	GCCTCCTTTTCCTCTCATCTTC
qATF5_m_FWD1	AATTGAGGTGTATAAGGCCCG
qATF5_m_REV1	GGATAGGAAAGTGGAATGGAGG
qATF6_m_FWD1	GAGGCTGGGTTCATAGACATG
qATF6_m_REV1	GCTAGTGGTTTCTGTGTACTGG
qCabp1_m_FWD1	ATGGACGAGTGGACTTTGAAG
qCabp1_m_REV1	GTACATCCTGGGCTTTCGG
qDdit3_m_FWD1	TGTTGAAGATGAGCGGGTG
qDdit3_m_REV1	AGGTTCTGCTTTCAGGTGTG
qDnajc3_m_FWD1	TGGAGTAAATGCGGATGTGG
qDnajc3_m_REV1	ACGGTCGCTCTCCTATAGTATG
qDnpep_m_FWD1	CCTATCTTGGCTTCTCGACTG
qDnpep_m_REV1	ACAGAAGGGAACAGCTCAAAG
qEdem1_m_FWD1	CAATGAAGGAGAAGGAGACCC
qEdem1_m_REV1	GCATCTTCCACATCCCCTATC
qEdem3_m_FWD1	TGGGAGAAAAGACAGCGAAG
qEdem3_m_REV1	GCAAGACATAGGCTTTCAACAG
qEhd1_m_FWD1	ATCTCATTCCACCCTCCAAAC
qEhd1_m_REV1	CCCCAATTTCTGCCCCTC
qEmilin1_m_FWD1	CAAGCCAGACTATCAACCCTG
qEmilin1_m_REV1	CATAACCCTGACAGCACCTC
qGARS_m_FWD1	GAGGGAGATGAACAGAGAACG
qGARS_m_REV1	CAGTGGAAGGACAGAACATTTG
qLARS_m_FWD1	CGAGCTGGAACACATAGAAGTC
qLARS_m_REV1	CGATGGCTGAGGATTTACTAGG
qMid1ip1_m_FWD1	AGATCGGCTTCAGTAATTGGG
qMid1ip1_m_REV1	ACTTTACAGTGTGCCCTTCG
qNr6a1_m_FWD1	TCAGGATGAATTGGCAGAGC
qNr6a1_m_REV1	ACAGATGAGACAGGTTCGTTG
qNupr1_m_FWD1	TGCTGACCAAGTTCCAGAAC
qNupr1_m_REV1	CTGGGTGTGATGTCCTGTATC
qRnf113a2_m_FWD1	AGTATCGAAAAGCAGAGGGTG
qRnf113a2_m_REV1	ACTGTGATTGCTTTGGTTTGAG
qSdf2l1_m_FWD1	TCGCCGCTATCCAACAAC
qSdf2l1_m_REV1	TCCACAGGTCCAGGTCATC
qSel1l_m_FWD1	GGCGTGGAGTAGAACAGAATC
qSel1l_m_REV1	AGTGAAGTGCCGTCTCATTAC
qXBP1_spliced_m_FWD1	CTGAGTCCGAATCAGGTGCAG
qXBP1_spliced_m_REV1	GTCCATGGGAAGATGTTCTGG
qXBP1_total_m_FWD1	TGGCCGGGTCTGCTGAGTCCG
qXBP1_total_m_REV1	GTCCATGGGAAGATGTTCTGG

Transparent Methods

Cell maintenance, cell viability, and puromycin incorporation assays

All cell lines were cultured in DMEM containing 4.5 g/L glucose (Gibco or Biochrom AG) supplemented with 10% fetal bovine serum (Life technologies or Biochrom AG) and Penicillin Streptomycin (50 U/mL).

Relative cell viability was assessed using the XTT cell proliferation Kit II (Roche) according to the manufacturer's instructions. Tunicamycin treatment with the indicated concentrations was performed for 48h, starting 24h after cell seeding. XTT turnover was normalized to untreated control cells.

Puromycin incorporation was performed for exactly 10 min at a final puromycin concentration of 10 µg/mL. Cells were subsequently lysed in RIPA buffer, pH 7.4 (50 mM TrisCl, 150 mM NaCl, 0,2% TX 100, 10 mM $MgCl_2$ and 1x protease inhibitor (Thermo Scientific or Roche)) and analyzed by Western blotting.

Generation and husbandry of transgenic mice

Generation of transgenic GFAT1 mice was performed by Taconic Biosciences (Cologne, Germany). A gene trap cassette was inserted in the ROSA26 locus using recombination-mediated cassette exchange in embryonic stem cells. The gene trap cassette encodes a loxP-flanked transcription termination cassette upstream of the human GFAT1 (hGFAT1) open reading frame. Upon cre-mediated deletion of the transcription termination cassette, hGFAT1 (also GFPT1) is expressed under the control of the chicken beta-actin promoter. hGFAT1 is N-terminally tagged with FLAG-HA.

Animals were housed on a 12:12h light:dark cycle with *ad libitum* access to food under pathogen-free conditions in individually ventilated cages. All animals were kept in C57BL/6J background. Animal care and experimental procedures were in accordance with the institutional and governmental guidelines and were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen. For breeding, $hGFAT1^{flox/wt}$ males were crossed with transgenic females expressing the cre recombinase under the control of the CMV promoter (CMV-$cre^{+/-}$). The offspring was genotyped using a primer pair specific for FLAG-HA-hGFAT1 (hGFAT1_fwd: CGGTGGAGGTTACCCATACG; hGFAT1_rev: CGAGCTTGGCAATTGTCTCTG) and a primer pair detecting the cre recombinase gene (Cre_fwd:

GCCAGCTAAACATGCTTCATC; Cre_rev: ATTGCCCCTGTTTCACTATCC). Double positive animals were considered to overexpress hGFAT1 in all tissues. WT, hGFAT1$^{flox/wt}$, and CMV-cre$^{+/-}$ mice served as controls.

Isolation and culture of primary keratinocytes

Primary murine keratinocytes were cultured in MEM/HAM's F12 (FAD) medium with low Ca^{2+} (50 µM) (Biochrom AG), supplemented with 10% fetal bovine serum (chelated, ThermoFisher Scientific), penicillin/streptavidin (ThermoFisher Scientific), L-glutamine (ThermoFisher Scientific), ascorbic acid (50 µg/mL, Sigma), adenine (0.18 mM, Sigma), insulin (5 µg/mL, Sigma), hydrocortisone (0.5 µg/mL, Sigma), EGF (10 ng/mL, Sigma), and cholera enterotoxin (10 ng/mL, Sigma) on collagen G-coated (30 µg/mL in PBS, Biochrom AG) tissue culture plates. The cells were grown at 32°C in 5% CO_2.

For keratinocyte isolation newborn mice (P0-P3) were sacrificed by decapitation. The corpus was incubated in 50% betaisodona/PBS (Mundipharma GmbH) for 30 min at 4°C before being washed in different solutions for 2 min each: PBS (ThermoFisher Scientific), 0.1% octenidin in ddH_2O (Serva Electrophoresis), PBS, 70% ethanol, PBS, antibiotic-antimycotic-solution in PBS (ThermoFisher Scientific). Tail and legs were removed and the tail tip was used for genotyping. Complete skin was separated from the body and incubated in 2 mL dispase II solution (5 mg/mL in 50 mM HEPES/KOH pH 7.4, 150 mM NaCl) over night at 4°C. The skin was placed in 500 µL FAD medium and the epidermis was separated from the dermis as a sheet. The epidermis was transferred dermal side down onto 500 µL TrypLE (ThermoFisher Scientific) and incubated for 20 min at RT. The keratinocytes were washed off the epidermis using 3 mL FAD medium. After centrifugation, the keratinocytes were resuspended in FAD medium and seeded on collagen G-coated tissue culture plates.

Small molecule LC/MS/MS Analysis

UDP-HexNAc concentrations were measured as described previously (Denzel et al., 2014). In brief, cells were trypsinized, lysed in water by freeze/thaw cycles, and subjected to chloroform/methanol extraction. Absolute UDP-HexNAc levels were determined using an Acquity UPLC connected to a Xevo TQ Mass Spectrometer (both Waters) and normalized to total protein content.

Expression constructs

Cloning of the ATX3 (257c)-Q71-MYC construct was described elsewhere (Haacke et al., 2006). The C-terminal fragment of ATX3 (257c) – Q80 with a FLAG-HA tag was cloned into BamHI and XhoI sites within the MCSI of the pcDNA3.1(+)-IRES-GFP backbone (Life technologies) using In-Fusion cloning (TaKaRa Bio).

Plasmids for tissue-specific overexpression of *C. elegans gfat-1* in the nematode were constructed using a GeneArt® Seamless PLUS Cloning and Assembly Kit (Invitrogen) according to manufacturer´s instructions. All promoter fragments for tissue-specific *gfat-1* expression were amplified from genomic DNA of wildtype *C. elegans*. The *gfat-1* ORF and 3UTR of 3,354 bp as well as the pDC6 vector were amplified from the *gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1-3´UTR* plasmid (Denzel et al., 2014).

Target		Sequence
myo-3P	FW	CAGGTCGACTCTAGATATGGTGGCCGATTTTGAGT
	RV	TACCGGATCCTCTAGATTAGATGGATCTAGTGGTCG
rgef-1P	FW	TCTAGAATCCCGTTTGGGACAAGAA
	RV	TACCGGATCCTCTAGACGTCGTCGTCGTCGATGCCG
gfat-1 ORF	FW	ATGTGCGGAATTTTCGCCTA
	RV	TTACTCGACGGTAACTGACT
vector 1 (tags)	FW	CTAGAGGATCCGGTACCGGTA
	RV	GAAAATTCCGCACATACCGATCCCACCTCCGCCTTTG
vector 2 (backbone)	FW	GTTACCGTCGAGTAAAGCGTCCCGTCTTCTGCCCA
	RV	TCTAGAGTCGACCTGCAGGC

Fractionation experiments

For fractionation experiments cell lysates containing 1 µg/µL total protein were centrifuged at 22,000xg for 30 min at 4 °C. Pellet fractions were separated from supernatants (Triton X-100-soluble fraction) and homogenized in 150 µl RIPA buffer containing 2% SDS followed by a second centrifugation step at room temperature. The supernatants (SDS-soluble fraction) were removed, and the remaining pellets were incubated for 16 h in 100% formic acid at 37 °C (Hazeki et al., 2000). Homogenates were vacuum dried and dissolved in 50 µL Laemmli buffer (SDS-insoluble fraction, FA) followed by pH adjustment with 2 M Tris-base or 1 M NaOH for SDS-PAGE analysis.

Western Blot analysis

Cell lysates (RIPA buffer) were subjected to SDS PAGE following a sonication step. *C. elegans* synchronized gravid day 1 adult worms were collected in M9 or in case of total Q35 quantification in urea/SDS buffer (8 M urea, 50 mM Tris pH 8, 2% SDS,

50 mM DTT), snap frozen in liquid nitrogen and lysed by addition of 4x LDS sample buffer (Thermo Fisher) containing 50 mM DTT. After a boiling and a sonication step, equal volumes were subjected to SDS-PAGE. A ChemiDoc MP Imaging System (BioRad) and films were used for detection.

The following antibodies were used in this study: GFP (ms, Clontech, Living Colors and rb, Cell Signaling (CS), D5.1), HA (rat, Roche, 3F10), Puromycin (ms, Millipore, 12D10), ATF4 (rb, CS, D4B8), PERK (rb, CS, C33E10), P-PERK (Thr980, rb, CS, 16F8), eIF2α (rb, CS, D7D3), P-eIF2α (Ser51, rb, CS, D9G8 and 119A11 (*C. elegans*)) ß-ACTIN (ms, Sigma, AC-74), H3 (rb, Abcam, ab1791), α-TUBULIN (ms, Sigma, DM1A), α-ATX3 (#986, (Haacke et al., 2006)).

qRT-PCR, RNA-sequencing and data analysis

Cells were collected in QIAzol (QIAGEN) and frozen in liquid nitrogen. Total RNA was isolated using the RNeasy Mini Kit (QIAGEN) and cDNA was subsequently generated by iScript cDNA Synthesis Kit (BioRad). RNA quality was assessed on a Bioanalyzer (Agilent).

qRT-PCR was performed with Power SYBR Green master mix (Applied Biosystems) on a ViiA 7 Real-Time PCR System (Applied Biosystems). GAPDH expression functioned as internal control. Primer sequences are listed in Supplementary Table 1.

RNA-Sequencing experiments were performed from three biological replicates of N2a WT and N2a GFAT1 G451E cells.

Libraries were sequenced on an Illumina HiSeq2000. Raw reads were trimmed using FLEXBAR version 2.4 (Dodt et al., 2012) and mapped to the mouse genome GRCm38_81 using HISAT version 0.1.6-beta (Kim et al., 2015). Transcripts were assembled using StringTie version 1.0.4 (Pertea et al., 2015) and quantified using Cufflinks version 2.2.1 (Trapnell et al., 2010). Differential gene expression was calculated using cuffdiff within the Cufflinks suite version 2.2.1 (Trapnell et al., 2013).

The list of ATF4, XBP1 and ATF6 target genes was downloaded from the ChIP-Atlas (https://chip-atlas.org/target_genes), selecting mouse as reference organism and 5k distance from the transcription start site. Furthermore, potential target genes were filtered to have an average MACS2 score of at least 240. These target gene lists were then intersected with the differential expression data to calculate the significance of a shift in the amount of significantly changed genes in the respective target list and the

background (all expressed genes). The p-value and odds ratios were calculated using Fisher's exact test in R (https://www.jstor.org/stable/2342435?seq=1#metadata_info_tab_contents).

Data and Software Availability

Raw sequencing data were deposited to the NCBI Gene Expression Omnibus (GEO) under the accession number GSE140357.

Maintenance and culture of *C. elegans* strains

All *C. elegans* strains were grown at 20°C on nematode growth medium (NGM: 2.5% bacto-agar, 0.225% bacto-peptone, 0.3% NaCl (all w/v), 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KPO_4, 5 µg/mL cholesterol) seeded with the OP50 strain of *E. coli* bacteria unless mentioned otherwise (Brenner, 1974). The strains used in experiments were outcrossed at least 4 times to the N2 Bristol control strain, which served as wild type reference strain.

Synchronization of worm population and gene knockdown

For all experiments, worms were synchronized by short egg-lays. For that purpose, gravid adults were transferred to culture plates and completely removed again after a period of 4h.

For RNAi-mediated knockdown of a specific gene worms were cultured from egg onward on *E. coli* HT115 (DE3) bacteria expressing dsRNA of the target gene under the control of an IPTG-inducible promoter (Kamath et al., 2001; Timmons and Fire, 1998). NGM plates for RNAi experiments contained a final concentration of 100 µg/µL ampicillin and 1 mM IPTG were used. RNAi clones were obtained from either the Ahringer or Vidal library (Kamath and Ahringer, 2003; Rual et al., 2004).

List of *C. elegans* strains

N2 Bristol (wildtype)

AA4135 *dhIs941[gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1 3'UTR; myo-2::mCherry]*

AM140 *rmIs132[unc-54P::Q35::YFP]*

AA4234 *rmIs132[unc-54P::Q35::YFP]; dhIs941[(gfat-1P::FLAG::HA::cfp::gfat-1::gfat-1 3'UTR); myo-2::mCherry]*

AA4230 *rmIs132[unc-54P::Q35::YFP]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (1)
AA4233 *rmIs132[unc-54P::Q35::YFP]; dhIs1042[(myo-3P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry]* (2)
AA4296 *rmIs132[unc-54P::Q35::YFP]; dhEx1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry*] (1)
AA4297 *rmIs132[unc-54P::Q35::YFP]; dhEx1017[(rgef-1P::HA::FLAG::CFP::gfat-1::gfat-1 3´UTR); myo-2::mCherry*] (2)
AA4423 *ldIs[atf-5P::GFP::unc-54 3'UTR]* (kindly provided by Dr. Keith Blackwell)
AA4474 *ldIs[atf-5P::GFP::unc-54 3'UTR]; dhIs941[(gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1 3'UTR); myo-2::mCherry]*

Worm imaging

Worm stacks were arranged on unseeded NGM plates on ice and images were taken on a Leica M165FC fluorescence microscope using a Leica DFC 3000G camera and Leica Application Suite. High magnification images of single worms were obtained on a Carl Zeiss Axio Imager Z1 connected to a Zeiss Axiocam 506 mono camera using AxioVision software. Pictures were processed using Adobe Photoshop CS5.

PolyQ aggregate quantification

Quantification of polyQ aggregates in *C. elegans* muscle was done from images of synchronized *unc-54P::Q35* worms taken at day 4 adult stage. Images of 10 worms were taken for each biological replicate and aggregate quantification was performed blinded.

Motility assay

C. elegans motility was assessed in synchronized worm populations at day 8 of adulthood. Worms responding to mild touch stimulus on culture plates were transferred to M9 buffer on unseeded NGM plates and were allowed to adjust to new conditions for about 30 sec. Afterwards full body bends within a 30 sec interval were recorded by counting. More than 10 worms per genotype and condition were analysed for each biological replicate. Experiments were performed blinded.

Supplemental References

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71-94.

Denzel, M.S., Storm, N.J., Gutschmidt, A., Baddi, R., Hinze, Y., Jarosch, E., Sommer, T., Hoppe, T., and Antebi, A. (2014). Hexosamine pathway metabolites enhance protein quality control and prolong life. Cell *156*, 1167-1178.

Dodt, M., Roehr, J.T., Ahmed, R., and Dieterich, C. (2012). FLEXBAR-Flexible Barcode and Adapter Processing for Next-Generation Sequencing Platforms. Biology *1*, 895-905.

Haacke, A., Broadley, S.A., Boteva, R., Tzvetkov, N., Hartl, F.U., and Breuer, P. (2006). Proteolytic cleavage of polyglutamine-expanded ataxin-3 is critical for aggregation and sequestration of non-expanded ataxin-3. Hum Mol Genet *15*, 555-568.

Hazeki, N., Tukamoto, T., Goto, J., and Kanazawa, I. (2000). Formic acid dissolves aggregates of an N-terminal huntingtin fragment containing an expanded polyglutamine tract: applying to quantification of protein components of the aggregates. Biochem Biophys Res Commun *277*, 386-393.

Kamath, R.S., and Ahringer, J. (2003). Genome-wide RNAi screening in Caenorhabditis elegans. Methods *30*, 313-321.

Kamath, R.S., Martinez-Campos, M., Zipperlen, P., Fraser, A.G., and Ahringer, J. (2001). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans. Genome biology *2*, Research0002.

Kim, D., Langmead, B., and Salzberg, S.L. (2015). HISAT: a fast spliced aligner with low memory requirements. Nature methods *12*, 357-360.

Pertea, M., Pertea, G.M., Antonescu, C.M., Chang, T.C., Mendell, J.T., and Salzberg, S.L. (2015). StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nature biotechnology *33*, 290-295.

Rual, J.F., Ceron, J., Koreth, J., Hao, T., Nicot, A.S., Hirozane-Kishikawa, T., Vandenhaute, J., Orkin, S.H., Hill, D.E., van den Heuvel, S.*, et al.* (2004). Toward improving Caenorhabditis elegans phenome mapping with an ORFeome-based RNAi library. Genome research *14*, 2162-2168.

Timmons, L., and Fire, A. (1998). Specific interference by ingested dsRNA. Nature *395*, 854.

Trapnell, C., Hendrickson, D.G., Sauvageau, M., Goff, L., Rinn, J.L., and Pachter, L. (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. Nature biotechnology *31*, 46-53.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature biotechnology *28*, 511-515.