Hexosamine Pathway Activation Improves Protein Homeostasis through the Integrated Stress Response

HIGHLIGHTS
Hexosamine pathway (HP) activation induces the integrated stress response (ISR)
HP activation ameliorates poly-glutamine aggregation via the ISR and autophagy
In C. elegans, the HP/ISR axis improves cell autonomous protein homeostasis
The proteoprotective role of longevity-associated HP is evolutionarily conserved

DATA AND CODE AVAILABILITY
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Hexosamine Pathway Activation Improves Protein Homeostasis through the Integrated Stress Response

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SUMMARY
Activation of the hexosamine pathway (HP) through gain-of-function mutations in its rate-limiting enzyme glutamine fructose-6-phosphate amidotransferase (GFAT-1) ameliorates proteotoxicity and increases lifespan in Caenorhabditis elegans. Here, we investigate the role of the HP in mammalian protein quality control. In mouse neuronal cells, elevation of HP activity led to phosphorylation of both PERK and eIF2α as well as downstream ATF4 activation, identifying the HP as a modulator of the integrated stress response (ISR). Increasing uridine 5′-diphospho-N-acetyl-D-glucosamine (UDP-GlcNAc) levels through GFAT1 gain-of-function mutations or supplementation with the precursor GlcNAc reduces aggregation of the polyglutamine (polyQ) protein Ataxin-3. Blocking PERK signaling or autophagy suppresses this effect. In C. elegans, overexpression of gfat-1 likewise activates the ISR. Consistently, co-overexpression of gfat-1 and proteotoxic polyQ peptides in muscles reveals a strong protective cell-autonomous role of the HP. Thus, the HP has a conserved role in improving protein quality control through modulation of the ISR.

INTRODUCTION
During aging, organisms undergo a progressive decline in physiological function that results in an elevated risk for numerous diseases (Niccoli and Partridge, 2012). A limited number of conserved molecular pathways control life- and healthspan across species and represent potential entry points for treatments of age-related diseases (Kenyon, 2010). However, only few potential drug targets have so far been identified and their role in human aging remains poorly understood. Considering the burden of age-related diseases, it is paramount to identify and understand conserved mechanisms that extend healthspan and promote longevity.

Among the pathways controlling the rate of aging, the upstream signaling nodes including insulin/IGF-1 signaling and the mTOR pathway are well understood, and downstream effectors are partially known. These include the protein homeostasis network, controlling protein synthesis and degradation as key processes. With age, the fidelity of the protein homeostasis network declines (Labbadia and Morimoto, 2015) and by enhancing protein homeostasis, e.g., by boosting autophagy, lifespan can be extended in mice (Pyo et al., 2013). In turn, autophagy is required for lifespan extension in numerous paradigms ranging from caloric restriction to insulin signaling and rapamycin treatment (Madeo et al., 2015). However, there is a gap in knowledge regarding the links between upstream signaling pathways and outputs that orchestrate the coordinated and diverse changes required for extended health of an organism.

The protein homeostasis network comprises all processes that define the dynamics of the proteome: protein synthesis, protein folding, and recycling by the proteasome or through autophagy (Labbadia and Morimoto, 2015). Protein homeostasis is required under normal conditions but is particularly important to be maintained under proteotoxic stress. To detect challenges to proteome stability, cellular compartments possess specialized protein quality surveillance mechanisms that trigger protective responses upon proteotoxic stress. The endoplasmic reticulum (ER) has an unfolded protein response (UPR) with three parallel pathways that are activated by luminal misfolded proteins (UPRER). First, the IRE1 sensor is an endonuclease that specifically triggers mRNA splicing of transcription factor XBP1, permitting expression of downstream stress response genes such as the HSP4/BiP chaperone (Calfon et al., 2002). Second, activated ATF6 is cleaved upon transport to the Golgi, which enables its translocation into the nucleus to act as a...
transcription factor (Haze et al., 1999). Both pathways coordinate expression of genes that reinstate ER homeostasis. The third UPR<sup>ER</sup> pathway is controlled by PRKR-like endoplasmic reticulum kinase (PERK), which is an ER membrane-localized stress sensor as well. Upon activation, PERK phosphorylates its sole known client, the α subunit of eukaryotic initiation factor-2 (eIF2α), which is a key regulator of protein synthesis (Harding et al., 1999). While PERK activation thus suppresses overall mRNA translation to reduce the burden on the protein folding machinery, specific transcripts that are regulated by upstream open reading frames (uORFs) become induced (Harding et al., 2000; Hinnebusch, 1993; Hinnebusch et al., 1988). These include the stress response gene activating transcription factor (ATF4), which is a key regulator of autophagy (Rzymski et al., 2009). Together, PERK phosphorylation, as an output of cellular stress, thus triggers a coordinated program termed the integrated stress response (ISR).

The protein homeostasis network is challenged not only by external stimuli such as heat shock but also by endogenous metastable proteins. Although these undergo proper protein folding or degradation under normal conditions, they tend to aggregate when the protein homeostasis network becomes insufficient. This not only leads to loss of function of the affected protein but can also result in a gain of toxic function that is often associated with aggregation (Dragatis et al., 2000; Duyao et al., 1995; Oikawa et al., 2016). Metastable proteins include Aβ amyloid protofibrils, superoxide dismutase, and polyglutamine (polyQ) tracts that form in some proteins such as Huntingtin due to unstable CAG repeats (Harper et al., 1997; Khare et al., 2004; MacDonald et al., 1993). These aggregation-prone proteins are causally linked to Alzheimer’s disease, amyotrophic lateral sclerosis, and Huntington’s disease, respectively (Glenner and Wong, 1984; Hazeki et al., 1999; Rosen et al., 1993; Wang et al., 2008). Furthermore, the expansion of CAG repeats in the coding region of Ataxin-3 (ATX3) leads to its aggregation, which is causative of Machado-Joseph disease (MJD) (also known as spinocerebellar ataxia-3) (Bevivino and Loll, 2001; Kawaguchi et al., 1994). MJD is a progressive autosomal dominant neurodegenerative disease that causes cerebellar ataxia, and currently there is no effective treatment available (Coutinho and Andrade, 1978; Duarte-Silva and Maciel, 2018).

In a genetic screen for proteotoxic stress resistance in C. elegans we have previously identified the metabolic hexosamine pathway (HP) as a regulator of aging (Denzel et al., 2014). Specifically, we showed that single amino acid substitutions in the pathway’s key enzyme glutamine fructose-6-phosphate amidotransferase (GFAT-1) result in gain of function and elevated cellular levels of the HP’s product UDP-GlcNAc. This leads to increased activity of protein degradation processes such as ER-associated degradation, proteasome activity, and autophagy. Although these processes are induced and required for the longevity of GFAT-1 gain-of-function mutants, how UDP-GlcNAc triggers the coordinated response of the protein homeostasis network remained unknown (Denzel et al., 2014). Moreover, it was unclear if the HP has a conserved role in mammalian protein homeostasis. Here, we show that HP activation triggers an ER stress response in mammalian cells that results in a significant reduction of aggregated polyQ expanded ATX3 through PERK signaling and the ISR. Using the nematode C. elegans we demonstrate that HP activation modulates the ISR and ameliorates polyQ toxicity in a conserved cell-autonomous manner.

RESULTS
HP activation through specific gain-of-function mutations in GFAT-1 (such as the G451E substitution) as well as GlcNAc supplementation was previously shown to increase lifespan and counter proteotoxicity in the nematode C. elegans (Denzel et al., 2014). To test the impact of HP activation on toxic protein aggregation in mammalian cells, we first established strategies to increase HP flux in mammalian systems (Figure 1A). GFAT1 is highly conserved, and we engineered the G451E point mutation in N2a cells using Crispr/Cas9 (Figure 1B). This gain-of-function substitution increases levels of the HP product UDP-GlcNAc by 4- to 5-fold in mouse N2a cells (Ruegenberg et al., 2020). Supplementation with 10 mM GlcNAc likewise increased the cellular UDP-GlcNAc concentration (Figure 1C). Notably, the two interventions were additive. Consistent with our previous work in the nematode, increased HP flux conferred resistance to the drug tunicamycin in N2a cells (Figures 1D and 1E). Tunicamycin is an inhibitor of UDP-GlcNAc: Dolichylphosphate GlcNAc-1-Phosphotransferase, which catalyzes the first step of N-glycan synthesis utilizing UDP-GlcNAc as substrate (Heifetz et al., 1979). Presumably, elevated UDP-GlcNAc levels “outcompete” tunicamycin and counter the inhibitory effect. Importantly, GlcNAc supplementation also increased UDP-GlcNAc levels in other mammalian systems including mouse primary keratinocytes and multiple human cell lines (Figures 1F and S1A). Moreover, we generated GFAT1 overexpression mice and tested HP
activation in primary keratinocytes. Like GlcNAc supplementation, GFAT1 overexpression led to elevated UDP-GlcNAc levels (Figure 1F).

Having established independent routes of HP activation we asked whether this activation could alleviate the aggregation of metastable proteins. To this end, we established two independent ATX3-PolyQ expression systems that carry a C-terminal fragment (amino acids 257–360) of ATX3 with a polyQ stretch. First, we assessed the amount of insoluble ATX3-polyQ71 in an inducible Tet-Off expression system in mouse N2a cells (Figure 2A). Upon activation of ATX3-polyQ71 expression by removal of doxycycline, the aggregation-prone fragment was detected in the SDS-insoluble formic acid (FA) fraction (Figure 2B). Treatment with 10 mM GlcNAc, however, fully prevented aggregation of ATX3-polyQ71, whereas supplementation of the negative control D-Arg had no effect (Figure 2B). Second, we transiently expressed tagged ATX3-PolyQ80 in N2a WT and GFAT1 G451E gain-of-function mutant cells (Figure 2C). In line with the results from the inducible system, HP activation by GlcNAc supplementation or genetic GFAT1 gain of function significantly reduced the amount of SDS-insoluble ATX3-PolyQ80 (Figures 2D and 2E). These data demonstrate a conserved role of the HP in improving protein homeostasis and counteracting protein aggregation in mammalian cells.

To understand the cellular and molecular changes that signal activation of the HP to improve protein homeostasis, we performed RNA sequencing in N2a WT and N2a GFAT1 G451E mutant cells. We focused our analysis primarily on regulatory circuits within the protein homeostasis network. Analysis of previously
described ATF6, XBP1, and ATF4 target genes (https://chip-atlas.org/target_genes) revealed differential expression of UPRER target genes in GFAT1 G451E mutant cells versus WT controls, which we selectively validated by qPCR (Figure S2A). About 30% of the 177 detected ATF4 target genes were changed in the GFAT1 gain-of-function cells, significantly more than the regulated fraction of all detected genes (23% of 12,626 genes, Figure 3A). About two-thirds of the regulated ATF4 targets were significantly upregulated indicating an ISR induction (Figure S2B). Furthermore, a subset of XBP1 and ATF6 target genes were differentially regulated as well. However, the transcriptional outputs of these other two UPRER branches were slightly underrepresented compared with all regulated genes (20% of XBP1 target genes and 19% of all ATF6 target genes were regulated, Figure 3A).

Consistent with an activation of the ISR, HP activation through GFAT1 gain of function and GlcNAc supplementation elevated phosphorylation of the ER-stress sensor PERK and the mRNA translation regulator eIF2α (Figures 3B and 3C). Accordingly, ATF4 protein levels were strongly increased, whereas mRNA levels remained largely unaffected, indicating activation of uORF-regulated translation (Figures 3B, 3C, and S2A). Notably, overall translation rates as assessed by puromycin incorporation were not altered upon any of the treatments (Figures S2C and S2D). We also investigated the status of ATF6 and XBP1 to address the other UPR branches. Upon GlcNAc supplementation or GFAT1 activation, we detected only minor or no effects regarding ATF6 activation and XBP1 splicing (Figures S2E–S2G). Differential regulation in a subset of the respective target genes might be explained by overlapping regulation of UPRER target genes. 

Figure 2. HP Activation Ameliorates Protein Aggregation in Mammalian PolyQ Models

(A) Schematic representation of a vector expressing an aggregation-prone ATX3 fragment. A C-terminal fragment of ATX3 cDNA corresponding to amino acid residues 257–360 in the full-length protein was cloned under the control of a tetracycline responsive element (TRE) and tagged with a C-terminal c-myc epitope as described elsewhere (Haacke et al., 2006).

(B) N2a cells stably expressing a Tet-Off operator (tTA) were transiently transfected with the construct described above and treated as indicated (10 mM D-Arg, 10 mM GlcNAc, 1 μg/mL doxycycline). Cells were lysed and processed for fractionation 24 h after treatment and transfection. * Partially formic acid-insoluble material detected by the ATX3 antibody; 1, endogenous ATX3; 2, exogenous ATX3 fragment.

(C) Schematic representation of a vector expressing an aggregation-prone ATX3 fragment including an internal ribosomal entry site (RES) followed by GFP.

(D) Fractionation of cell lysates from WT, GFAT1 G451E engineered N2a cells, and both treated with 10 mM GlcNAc 48 h after transfection with the ATX3-PolyQ construct (Figure 2C). SOL, SDS-soluble fraction; SDS, SDS-insoluble fraction; FA, SDS-insoluble formic acid fraction.

(E) Quantification of fractionation experiments from Figure 2D. Insoluble fraction was normalized to GFP expression. Mean ± SD (n ≥ 4), *p < 0.05, **p < 0.01 (ANOVA).
genes. Taken together, HP activation induces the PERK/\(eIF2\alpha\) arm of the UPR\(ER\) resulting in a transcriptional response.

As HP activation triggered the ISR, we investigated whether this stress response was required for the amelioration of PolyQ aggregation. Again, we first used the ATX3-polyQ71 expression system in mouse N2a cells and found that the GlcNAc-mediated reduction of aggregation was reversed by inhibition of PERK with the specific inhibitor GSK2606414 or by blocking autophagic flux with bafilomycin A (Figures 4A and S3A). Next, we tested polyQ aggregation using both the Flag-HA tagged ATX3-polyQ80 construct containing a GFP internal control and the ATX3-polyQ71 expression system in GFAT1 G451E N2a cells during PERK and autophagy inhibition. In all these approaches, PERK inhibition and reducing autophagy restored polyQ aggregation demonstrating that the ISR-autophagy axis mediates HP-induced benefits (Figures 4A, 4B, and S3B). To rule out possible effects of GSK2606414 or bafilomycin A on the HP, we tested whether the PERK and autophagy responses were downstream of HP flux. Liquid chromatography-mass spectrometry analysis showed that UDP-GlcNAc levels in GFAT1 G451E cells remain significantly elevated after GSK2606414 or bafilomycin A treatment compared with WT N2a cells (Figure S3C).

Having uncovered the link between HP flux and protein quality control in a mammalian system we wondered whether the beneficial effects are cell-autonomous or transmitted in a cell non-autonomous
manner. Given that protein quality control mechanisms including the ISR are evolutionarily conserved across species from *C. elegans* to humans, we established a system to test the tissue-specific role of the HP in protein homeostasis in the nematode. First, we assessed ISR activation upon HP activation by either GFAT-1 overexpression under its endogenous promoter (Figure 5A) or by GlcNAc supplementation and found increased phosphorylation of eIF2α in both cases (Figures 5B–5E). Consistently, we found elevated ATF-5 levels (homolog of ATF4) in GFAT-1 overexpressing worms using a GFP reporter strain (Figures 5F and 5G).

We then generated *C. elegans* overexpressing GFAT-1 specifically in body wall muscle or in neurons (Figures 6A and S4A) and crossed them to Q35::YFP transgenic worms that drive the expression of metastable polyQ proteins solely in the body wall muscle (Morley et al., 2002). We found that polyQ aggregation was significantly reduced by local HP activation (Figures 6B and 6C). This was not the case when GFAT-1 was overexpressed in neurons under the *rgef-1* promoter or under its endogenous promoter, which shows only minor activity in the body wall muscle (Figures 5A, 6A, and 6C). Importantly, aggregate reduction occurred without a decrease in total polyQ protein in *gfat-1* transgenic animals (Figure S4B). Q35::YFP transgenic animals are well known to show a progressive paralysis phenotype that can be assessed by motility assays (Morley et al., 2002). We found that muscle overexpression of GFAT-1 preserved motility, whereas neuronal GFAT-1 overexpression did not (Figure 6D). Overexpression of GFAT-1 under its endogenous promoter slightly improved motility over control conditions, consistent with a mild induction of HP
activity in muscle tissue. To test whether this beneficial effect was linked to GFAT-1 and HP activity, we used RNAi to downregulate *gfat-1* and *gna-2* expression. GNA-2 is the enzyme downstream of GFAT-1 in the HP.

Both interventions largely prevented the motility improvements (Figures 6E and S4C–S4E). Finally, we tested if enhanced motility during GFAT-1 overexpression was dependent on *atf-5*. Knockdown of *atf-5* indeed suppressed the motility phenotype in GFAT-1 transgenic animals (Figure 6F). Together, these findings suggest that cell autonomous HP activation provokes the ISR, reduces polyQ aggregation, and improves motility.

**DISCUSSION**

Here, we examined the role of the HP in protein homeostasis in nematodes and mammalian cells. Previously, we showed that activating point mutations in GFAT-1 elevate UDP-GlcNAc levels, suppress...
proteotoxicity, and enhance lifespan in the nematode *C. elegans*. However, it remained unclear how elevated levels of the metabolite UDP-GlcNAc modulate protein homeostasis and if this function is conserved across species. Therefore, we investigated the consequences of HP activation in mammalian cells. We show that gain-of-function mutations causing specific substitutions of conserved amino acids elevate the HP product UDP-GlcNAc in mouse cells. In cultured neuronal cells, we found that SDS-insoluble polyQ-fused ATX3 was drastically reduced upon elevation of HP activity. Transcriptomics pointed toward...
an activation of the UPRER, and our biochemical analysis showed strong PERK phosphorylation, whereas XBPT and ATF6 remained largely unaffected. Downstream of PERK, we noted an induction of the ISR in GFAT1 gain-of-function cells, which was required for the suppression of ATX3-polyQ aggregation. In a C. elegans model of polyQ proteotoxicity in the body wall muscle, we showed that specifically muscle-targeted overexpression of GFAT-1 suppressed toxicity indicating a cell-autonomous mode of action. These results reveal that the HP modulates the ISR to locally improve protein quality control.

Our data establish a link between the metabolic status of the HP and the ISR. It remains unclear, however, how elevated HP metabolites might modulate the key upstream ISR regulators PERK and elf2\(\alpha\). UDP-GlcNAc is a precursor for O-GlcNAc modifications, but a knockdown of O-GlcNAc transferase OGT, which is essential for this posttranslational modification, did not reduce the capacity of HP activation to clear polyQ aggregates (data not shown). Previous work has shown that glucosamine treatment can lead to ER stress (Lombardi et al., 2012). Moreover, glucosamine supplementation leads to PERK activation and elf2\(\alpha\) phosphorylation (Kline et al., 2006). Together with our work, this suggests a mild HP-induced ER stress that results in PERK activation and elf2\(\alpha\) phosphorylation. Using N2a cells, we indeed observed significant downstream ATF4 activation; however, we did not observe a reduction in bulk protein synthesis or cell growth. This is consistent with a mild effect of HP activation on ER stress that does not achieve a level of full UPR activation.

ATF4 is a key uORF-regulated downstream target of the ISR. ATF4 regulated processes include amino acid metabolism, response to oxidative stress, and apoptosis. GCN4, the yeast ortholog of ATF4, was previously shown to mediate lifespan extension under ribosome subunit depletion (Steffen et al., 2008). In addition, ATF4 has been implicated in mammalian longevity as it is upregulated in distinct types of slow-aging mutant mice (Li et al., 2014; Li and Miller, 2015) and it stimulates production of the pro-survival hormone FGF21 (Salminen et al., 2013; Notte et al., 2015). Consistent with an activation of autophagy downstream of the ISR, the elimination of polyQ aggregates upon HP activation was suppressed by treatment with the autophagy inhibitor bafilomycin A. Supporting this model, a previous study has demonstrated that ER stress modulates autophagy through elf2\(\alpha\) phosphorylation to degrade polyQ aggregates (Kouroku et al., 2007). Furthermore, enhancing elf2\(\alpha\) phosphorylation pharmacologically reduces SDS-insoluble Huntingtin aggregates in a mouse model of Huntington’s disease (Krzyzosiak et al., 2018).

Why might enhanced metabolite flux in the HP lead to improved protein quality control? The HP is optimally positioned to reflect the availability of numerous metabolites of key energy pathways. HP flux is only maximized under conditions of sufficient energy and macronutrient availability and is then limited through UDP-GlcNAc-mediated feedback regulation of GFAT1. Protein synthesis and degradation are very energy-consuming pathways, and it is plausible that the HP might signal energy availability. ISR induction typically reduces protein synthesis, whereas this was not the case when the HP was activated. Rather, our data point to enhanced protein degradation and thus, potentially, turnover. HP activation might thus signal conditions supporting proteome maintenance under which toxic protein aggregates are degraded or fail to form toxic conformations in the first place.

Finally, to test our cellular data in vivo, we asked if HP activation can activate the ISR to counter protein aggregation in C. elegans. Previously, we had shown that activating point mutations in GFAT-1 can counter proteotoxicity by inducing protein quality control. We had not detected major transcriptomic changes in gfat-1 gain-of-function worms (unpublished data). As GFAT-1 is most strongly expressed in the pharynx and seam cells of the worm, we might not have detected a UPRER response from these tissues in whole animal lysates. Genetic gfat-1 activation rescued polyQ-mediated toxicity in neurons and in muscle tissue. It was therefore unclear if the GFAT-1 activation had cell non-autonomous effects on protein homeostasis. In the current study we show that GFAT-1 overexpression or GlcNAc treatment indeed increased elf2\(\alpha\) phosphorylation in the nematode, consistent with our mammalian data. Moreover, co-overexpression of polyQ peptides and GFAT-1 in muscle tissue resulted in clear improvements in motility and aggregate clearance, whereas overexpression in neurons did not protect from muscle-specific polyQ proteotoxicity, consistent with a cell autonomous effect.

In all, our data demonstrate a key conserved role of the HP in protein homeostasis through induction of the ISR. Thus, pharmacological activation of the HP might hold potential in protecting from age-related proteotoxic diseases.
Limitations of the Study
First, although we show that the HP affects the UPRER, we do not mechanistically understand how elevated UDP-GlcNAc triggers PERK phosphorylation. This might be due to mild ER stress caused by changes in N-glycosylation or through a more specific mechanism. Second, although our conclusions are based on experiments in tissue culture and in C. elegans, the question remains whether HP activation might have proteoprotective effects in higher mammals. Studies in mice will address this point. Finally, we have investigated only polyQ toxicity in this study. It will be of interest to study the HP in the context of other toxic protein species.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

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

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.100887.

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AUTHOR CONTRIBUTIONS
M.H., S.I.D., A.A., and M.S.D. designed the research and wrote the manuscript. M.H., S.I.D., I.S., K.A., B.S., V.K., S.M., and P.B. performed the experiments. M.H., S.I.D., B.S., and P.B. performed the data analysis.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

Hexosamine Pathway Activation Improves Protein Homeostasis through the Integrated Stress Response

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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≥3), * p<0.05 (t-test).
Figure S2 (related to Figure 3)

A

GFAT1 G451E vs WT

B

Regulated non-ATF4 target genes

Regulated ATF4 target genes

Total=2887

Total=53

p-value = 0.0012

C

D

kDa

WT

WT + GlnAC

GFAT G451E

rel. uptake

Purysomy

50

β-Actin

E

F

kDa

WT

G451E

CTR

GlnAc

DTT

CTR

GlnAc

100

70

50

ATF6

β-Actin

G

Wnt-5a total mRNA level

WT

WT + DTT

10mM GlnAc

GFAT G451E + GlnAc

GFAT G451E + DTT
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≥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:**

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:

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 | Sequence (5'→3') |
|------------------|------------------|
| qATF4_m_FWD1     | GGGTTCTGTCTCCACTCCA |
| qATF4_m_REV1     | AAGCAGCAGGATCAGGCTTTC |
| qHSPA5_m_FWD1    | TTTCTGATGTATCTCTCTCACC |
| qHSPA5_m_REV1    | TTTTCTGATGTATCTCTCTCACC |
| qATF3_m_FWD1     | ATAAACACTCTGCTCAGAGGC |
| qATF3_m_REV1     | GCCCTCTCCCTCTTCTACCTTC |
| qATF5_m_FWD1     | AATTGAGGTGATGAGCTTCTGG |
| qATF5_m_REV1     | GGTAGCTGGAGGACTTGAAG |
| qATF6_m_FWD1     | GAGGCTGGGTTCATGAATGGAG |
| qATF6_m_REV1     | GCTAGTGTTCTGCTGTACTGG |
| qCabp1_m_FWD1    | ATGGACGAGTGGAGCTTGAAG |
| qCabp1_m_REV1    | GTACATCTGGGCTTTGCAG |
| qDdit3_m_FWD1    | TGTTGAAGTACGCTGAGGAG |
| qDdit3_m_REV1    | AGGTTCTGCTTCCAGGGT |
| qDnajc3_m_FWD1   | TGGAGTAATGCGGAGGGT |
| qDnajc3_m_REV1   | ACAGGTCCTCTCTCATTATG |
| qDnpep_m_FWD1    | CCTATCTGGCTCTCTGGACTG |
| qDnpep_m_REV1    | CACAGAGGAGACAGCTCAAAG |
| qEdem1_m_FWD1    | CAATGAAGGAGAACACAGCAG |
| qEdem1_m_REV1    | GCATCTCCACATCCCCTACT |
| qEdem3_m_FWD1    | TGGAGAAGAAGACACAGGAG |
| qEdem3_m_REV1    | GGAAGCATAGGCTTTCCAGAG |
| qEhd1_m_FWD1     | ATGCTCTCACCCTCCAAC |
| qEhd1_m_REV1     | CCCCAATTCTGCCCTCT |
| qEmilin1_m_FWD1  | CAAGCCAGATACATCAAACCTC |
| qEmilin1_m_REV1  | CATAAACCCTGACAGCACCTC |
| qGARS_m_FWD1     | GAGGAGGAGACAGAGAAGC |
| qGARS_m_REV1     | CAGTGGAAGGACAGACATT TG |
| qLARS_m_FWD1     | CAGACGAGGAGACAGAAGGAG |
| qLARS_m_REV1     | CGATTGAGGGAGATTACTAGG |
| qMid1p1_m_FWD1   | AGATCGGCTTCAATGAGG |
| qMid1p1_m_REV1   | ACTTATCAGTGTCCTCTTG |
| qNr6a1_m_FWD1    | TCAGATGAGATGGAGAGC |
| qNr6a1_m_REV1    | ACAGATGAGAGGGGGTTGTG |
| qNuprl_m_FWD1    | TGCTGACCAAATGTCAGAAAC |
| qNuprl_m_REV1    | CTGGATGTGATGGTGCTGATTC |
| qRnf13a2_m_FWD1  | AGATCGGAAAGGAGAGGAG |
| qRnf13a2_m_REV1  | ACTGTGATGATTGGGAGAGG |
| qSdf2l1_m_FWD1   | TCGCCGCTATCCCAAC |
| qSdf2l1_m_REV1   | TCCACAGGGTCAGGTCATC |
| qSel1l_m_FWD1    | GCGCTGGAGTAGAAACAGATAC |
| qSel1l_m_REV1    | AGTGAAGTGCCCCTCTGATAC |
| qXB1p_spliced_m_FWD1 | CGGTCCAGGGATGAGGTCAG |
| qXB1p_spliced_m_REV1 | GTCCATGGGAGATGTTCTGG |
| qXB1p_total_m_FWD1 | TGCCGGGCTCTGCTGAGTCAG |
| qXB1p_total_m_REV1 | GTCCATGGGAGATGTTCTGG |
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 MgCl₂ 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, hGFAT1floxflox/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: ATTGCCCCCTGTTCACATCC). Double positive animals were considered to overexpress hGFAT1 in all tissues. WT, hGFAT1^floox/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% CO₂.

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 ddH₂O (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 3’UTR 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     | CAGGTCGACTCTAGATATGGTGCCGATTTTGAGT TACCGGATCCTCTAGATTAGATGGATCTAGTGGTCG |
| rgef-1P    | TCTAGAATCCCGTTTGGGACAAGAA TACCGGATCCTCTAGACGTCGTCGTCGTCGATGCCG          |
| gfat-1 ORF | ATGTGCGGAATTTTCGCCTA TTACTCGACGGTAACTGACT                                |
| vector 1   | CTAGAGGATCCCGGTACCCGTA                                               |
| vector 2   | GTTACCCTGAGTAAGACGTCCCGTCTTCTGCCCCA                                 |

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)), β-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 dhls941[gfat-1P::FLAG-HA::cfp::gfat-1::gfat-1 3'UTR; myo-2::mCherry]
- AM140 mls132[unc-54P::Q35::YFP]
- AA4234 mls132[unc-54P::Q35::YFP]; dhls941[(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.
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