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ORIGINAL ARTICLE

Transcriptome and functional analysis in a *Drosophila* model of *NGLY1* deficiency provides insight into therapeutic approaches

Katie G. Owings[1], Joshua B. Lowry[1], Yiling Bi[2], Matthew Might[3,4,†] and Clement Y. Chow[1,*]

[1]Department of Human Genetics, University of Utah School of Medicine, Salt Lake City, UT 84112, USA, [2]Department of Medicinal Chemistry and [3]Department of Pharmaceutics & Pharmaceutical Chemistry, University of Utah College of Pharmacy, Salt Lake City, UT 84112, USA and [4]School of Computing, University of Utah, Salt Lake City, UT 84112, USA

*To whom correspondence should be addressed at: Department of Human Genetics, University of Utah School of Medicine, EIHG 5150, 15 North 2030 East, Salt Lake City, UT 84112, USA. Tel: 801-585-3314; Fax: 801-581-7796; Email: cchow@genetics.utah.edu

Abstract

Autosomal recessive loss-of-function mutations in *N-glycanase 1* (*NGLY1*) cause *NGLY1* deficiency, the only known human disease of deglycosylation. Patients present with developmental delay, movement disorder, seizures, liver dysfunction and alacrima. NGLY1 is a conserved cytoplasmic component of the Endoplasmic Reticulum Associated Degradation (ERAD) pathway. ERAD clears misfolded proteins from the ER lumen. However, it is unclear how loss of *NGLY1* function impacts ERAD and other cellular processes and results in the constellation of problems associated with *NGLY1* deficiency. To understand how loss of *NGLY1* contributes to disease, we developed a *Drosophila* model of *NGLY1* deficiency. Loss of *NGLY1* function resulted in developmental delay and lethality. We used RNAseq to determine which processes are misregulated in the absence of *NGLY1*. Transcriptome analysis showed no evidence of ER stress upon *NGLY1* knockdown. However, loss of *NGLY1* resulted in a strong signature of NRF1 dysfunction among downregulated genes, as evidenced by an enrichment of genes encoding proteasome components and proteins involved in oxidation–reduction. A number of transcriptome changes also suggested potential therapeutic interventions, including dysregulation of GlcNAc synthesis and upregulation of the heat shock response. We show that increasing the function of both pathways rescues lethality. Together, transcriptome analysis in a *Drosophila* model of *NGLY1* deficiency provides insight into potential therapeutic approaches.

Introduction

N-glycanase 1 (*NGLY1*) deficiency is a recently identified, rare metabolic disorder, caused by loss of function mutations in the *NGLY1* gene (1,2). Patients with *NGLY1* deficiency display a range of developmental and metabolic symptoms, including alacrima, choreoathetosis, liver disease, developmental delay, hypotonia, peripheral neuropathy, EEG abnormalities and microcephaly (2,3). Currently no treatment exists. NGLY1 is a cytosolic enzyme with deglycosylation activity and is thought to participate in the endoplasmic reticulum (ER) associated degradation (ERAD) process (2,4–7).

[†]Present address: Division of Internal Medicine and the Hugh Kaul Personalized Medicine Institute, Birmingham School of Medicine, University of Alabama, Birmingham, AL 35233, USA.

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ER stress occurs when misfolded proteins accumulate in the ER lumen (8). ERAD removes misfolded proteins by translocating misfolded substrates from the ER lumen to the cytosol for proteasomal degradation (9). NGLY1 is thought to be responsible for deglycosylating misfolded proteins prior to degradation in the cytosol (5,10). NGLY1 catalyzes the cleavage of the amide bond between the proximal N-acetylglucosamine (GlcNAc) and the asparagine of the glycoprotein, resulting in a deglycosylated peptide and a free intact N-glycan (5,10). The deglycosylated, misfolded protein is then degraded by the proteasome and the free glycan is recycled in the cell (5,9). It is unknown if NGLY1 is required to deglycosylate all misfolded proteins or just a subset. Further, it is unknown if loss of NGLY1 would result in abnormal ERAD or excessive ER stress.

In addition to its putative ERAD role in clearing misfolded proteins, NGLY1 appears to be important for certain ERAD processes that regulate normal cellular signaling. For example, NGLY1 deglycosylation activity is required for regulating NRF1 function (11,12). NRF1 is a transcription factor that responds to a number of cellular stresses, including proteasomal stress and upregulates transcription of proteasomal subunits and general stress response genes (13,14). NRF1 is translated into the ER membrane and glycosylated. Under non-stressed conditions, NRF1 is constitutively degraded by ERAD. Under stress conditions, however, NRF1 undergoes NGLY1-mediated deglycosylation, followed by proteolytic cleavage and translocation into the nucleus as an active transcription factor (11,12). If NGLY1 function is absent, NRF1 remains glycosylated and cannot properly regulate its target stress response genes (11,12).

It is currently unknown how misregulation of ERAD, NRF1 signaling or other as yet unidentified NGLY1 functions may contribute to the pathogenesis of *NGLY1* deficiency. To address this gap in knowledge, we developed a *Drosophila* model of *NGLY1* deficiency. We used ubiquitous RNAi knockdown of *Pngl* (*Drosophila* ortholog of *NGLY1*; herein referred to as *dNGLY1*) to mimic loss of *NGLY1* function. Knockdown of *dNGLY1* resulted in significant developmental delay and both larval and adult lethality. To understand what might be misregulated in the absence of *dNGLY1* function, we undertook RNAseq in *dNGLY1* knockdown flies and compared them with wild-type control flies. Transcriptome analysis showed no evidence of ER stress in the *dNGLY1* knockdown flies. Knockdown flies, however, did show a strong signature of *Cap 'n' collar* (*cnc*; *Drosophila* ortholog of *NRF1*) dysfunction among downregulated genes, as evidenced by an enrichment of *cnc/NRF1*-regulated genes, including those encoding proteasomal components and proteins involved in oxidation–reduction. Strikingly, a number of transcriptome changes in the *dNGLY1* knockdown flies suggested potential therapeutic interventions. We found evidence for dysregulation of GlcNAc synthesis in *dNGLY1* knockdown flies. We also observed upregulation of a number of components of the heat shock response. We demonstrate that increasing the function of both of these pathways in *dNGLY1* knockdown flies rescues the developmental delay and lethality. Both pathways are already currently under investigation as therapeutic targets for other conditions. Together, transcriptome analysis in a *Drosophila* model provides insight into potential therapeutic approaches for patients with *NGLY1* deficiency.

Results

dNGLY1 knockdown results in developmental delay and lethality

We used the ubiquitous *tubulin-Gal4* to drive *UAS-RNAi* against *dNGLY1* to model the loss of *NGLY1* function in *Drosophila melanogaster*. This resulted in nearly complete knockdown of *dNGLY1* transcript (>95% reduction or >6-fold reduced expression; Fig. 2 and Table 1). Ubiquitous knockdown of *dNGLY1* resulted in significant lethality during larval development, with only ∼30% of expected adult flies eclosing (Fig. 1A). *dNGLY1* knockdown flies also demonstrated developmental delay (Fig. 1B). *dNGLY1* knockdown flies began eclosion nearly 7 days after control flies in the same cross. Lethality was also observed throughout development, confirming previous reports (15,16). Similar rates of lethality and developmental delay were

Table 1. Top up- and downregulated genes in *dNGLY1* knockdown flies

	Symbol	FlyBase ID	Log 2 fold change	P-value	P_{adj}
Upregulated	*Jon66Ci*	FBgn0035886	3.41	5.39E−71	1.83E−67
	Hsp70Bb	FBgn0013278	2.89	6.12E−52	1.66E−48
	y	FBgn0004034	2.15	1.03E−47	2.00E−44
	CG31041	FBgn0051041	2.11	2.73E−25	1.24E−22
	Hsp23	FBgn0001224	1.81	9.32E−38	1.15E−34
	CG11912	FBgn0031248	1.57	1.79E−34	1.62E−31
	PGRP-SC1b	FBgn0033327	1.37	4.08E−11	4.40E−09
	CG1648	FBgn0033446	1.37	4.24E−43	6.39E−40
	CG13618	FBgn0039203	1.35	4.92E−17	1.15E−14
	CR44922	FBgn0266227	1.35	3.50E−15	6.50E−13
	CG14499	FBgn0034317	1.32	5.10E−11	5.45E−09
Downregulated	*Sfp24Ba*	FBgn0259951	−1.68	4.56E−50	1.03E−46
	Sfp24Bc	FBgn0261054	−1.77	4.70E−36	5.32E−33
	CR44039	FBgn0264831	−1.78	4.51E−17	1.07E−14
	CG2663	FBgn0037323	−1.81	6.16E−35	5.97E−32
	CG43218	FBgn0262854	−1.94	1.42E−27	8.02E−25
	CG30008	FBgn0050008	−2.00	7.12E−85	4.83E−81
	CG7341	FBgn0036777	−2.09	1.24E−29	8.89E−27
	mthl8	FBgn0052475	−2.20	2.36E−25	1.10E−22
	Pngl	FBgn0033050	−2.60	4.84E−74	2.19E−70
	CG6788	FBgn0030880	−4.21	2.11E−108	2.86E−104

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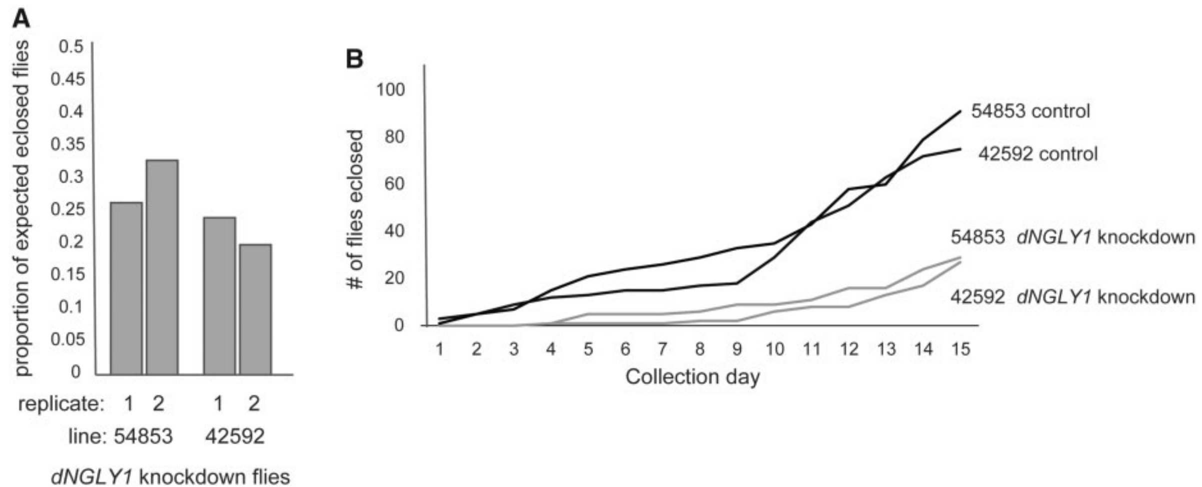

Figure 1. Ubiquitous knockdown of *dNGLY1* results in lethality. (**A**) Proportion of expected *dNGLY1* knockdown flies collected at adult eclosion. Replicates for two different RNAi lines are shown. (**B**) Developmental delay is observed in *dNGLY1* knockdown flies compared with control flies. Representative results of more than four experiments are shown for both RNAi strains and their respective controls. RNAi expression is driven by the ubiquitous *tubulin-GAL4* driver.

observed with two different RNAi lines (Fig. 1A and B). The rest of the analysis was only performed in one of the RNAi lines (54853).

dNGLY1 knockdown results in numerous transcriptional changes

A number of proteostasis-related functions are hypothesized to contribute to the pathogenesis underlying *NGLY1* deficiency in humans (2,4,10,17). To understand if any of these pathways are perturbed in flies lacking *dNGLY1* function, we performed RNAseq on adult *dNGLY1* knockdown and healthy control flies. When compared with controls, *dNGLY1* knockdown flies showed upregulation of 96 genes and downregulation of 290 genes (≥1.5-fold change at FDR 5%; Fig. 2; Table 1; Supplementary Material, Table S1). Confirming the efficacy of the RNAi, *dNGLY1* was the second most downregulated gene (Table 1; Supplementary Material, Table S1; Fig. 2). These RNAseq results provide several insights into the pathogenesis of *NGLY1* deficiency and will be discussed below. We provide some general observations here.

Gene ontology (GO) analysis of the 96 upregulated genes demonstrated enrichment for genes involved in the heat shock response (GO: 0009408) and innate immunity (GO: 0045087), among other categories related to general stress responses (Table 2; Supplementary Material, Table S2). Twenty-six of these genes showed 2-fold or more upregulation. GO analysis of the 290 downregulated genes demonstrated enrichment for genes involved in oxidation–reduction (GO: 0055114), the proteasome (GO: 0043161) and lipid metabolism (GO: 0030497) (Table 2; Supplementary Material, Table S2). Sixty-one genes were downregulated 2-fold or more.

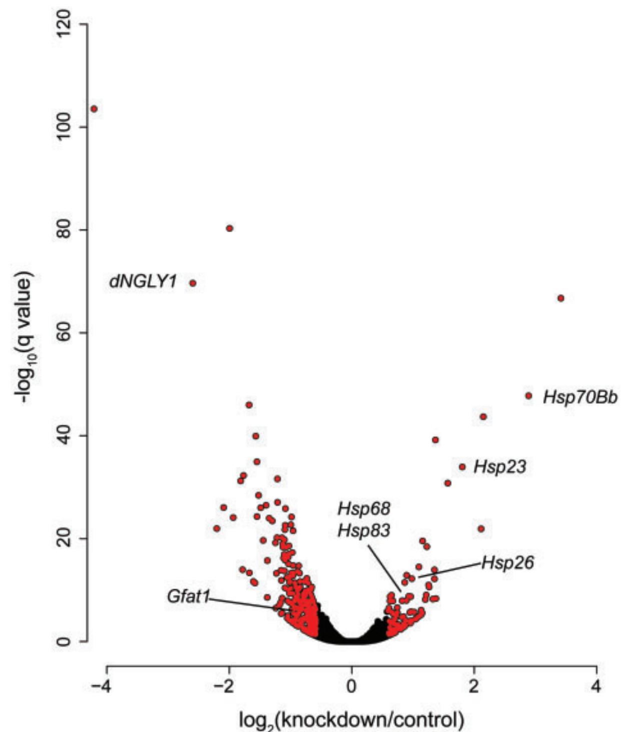

Figure 2. Numerous genes are misregulated in *dNGLY1* knockdown flies. Standard volcano plot showing the genes that are misregulated in *dNGLY1* knockdown flies as compared with control flies, as measured by RNAseq. Relevant genes are labeled. Red=genes with 1.5-fold change and at an FDR of 5%. *q* value=FDR.

No evidence of ER stress or ERAD dysfunction

We found no enrichment among upregulated genes for GO categories related to the ER stress response in the *dNGLY1* knockdown flies (Table 2; Supplementary Material, Table S2). There was no change in expression of any of the canonical markers of the ER stress response (Supplementary Material, Table S1), suggesting that loss of *dNGLY1* is not sufficient to induce ER stress. To further test this, we crossed an UAS-*Xbp1-GFP* fusion fluorescent marker of ER stress (18,19) onto *dNGLY1* knockdown flies. Under ER stress, *Xbp1* is spliced by IRE1 to generate a transcription factor that regulates the expression of ER stress response genes (20). Under ER stress, *Xbp1-GFP* splicing results in a strong increased GFP signal (18,19). We found no increase in GFP signal in *dNGLY1* knockdown flies, when compared with control flies (Supplementary Material, Fig. S1), confirming that knockdown of *dNGLY1* is not sufficient to induce strong ER

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Table 2. GO analysis of up- and downregulated genes in *dNGLY1* knockdown flies

	GO term	Fold enrichment	*q* value
Upregulated	GO: 0009408: response to heat	18.6	2.65E−04
	GO: 0009617: response to bacterium	23.5	3.63E−04
	GO: 0019835: cytolysis	72.1	9.26E−04
	GO: 0016998: cell wall macromolecule catabolic process	55.5	0.002
	GO: 0050830: defense response to Gram-positive bacterium	16.1	0.007
	GO: 0045087: innate immune response	10.4	0.006
	GO: 0042594: response to starvation	14.3	0.008
	GO: 0034644: cellular response to UV	77.3	0.012
	GO: 0009631: cold acclimation	67.6	0.014
	GO: 0034605: cellular response to heat	36.1	0.044
	GO: 0009411: response to UV	36.1	0.044
Downregulated	GO: 0055114: oxidation–reduction process	4.8	1.58E−11
	GO: 0030497: fatty acid elongation	18.6	1.60E−04
	GO: 0040003: chitin-based cuticle development	5.2	2.44E−04
	GO: 0043161: proteasome-mediated ubiquitin-dependent protein catabolic process	6.6	0.001
	GO: 0006030: chitin metabolic process	5.9	0.002
	GO: 0009072: aromatic amino acid family metabolic process	29.2	0.010
	GO: 0006013: mannose metabolic process	26.0	0.013
	GO: 0005975: carbohydrate metabolic process	5.0	0.013
	GO: 0032504: multicellular organism reproduction	2.9	0.040
	GO: 0042744: hydrogen peroxide catabolic process	16.7	0.036
	GO: 0006517: protein deglycosylation	16.7	0.036

stress under physiological conditions. It is still possible that there is a small subset of cells or a specific developmental window where ER stress is induced. Our analysis presented here would miss these specific instances.

NGLY1 is thought to be a component of the ERAD pathway (2,7,10). Loss of function of a number of ERAD components results in decreased clearance of misfolded proteins and subsequent ER stress (7,9). To test whether ERAD is functioning properly, we knocked down *dNGLY1* in the presence of a misfolded protein. We expressed a misfolded protein (*UAS-Rh1^{G69D}*) in the larval eye disc using the *GMR-GAL4* driver, which leads to ER stress, cell death and a degenerated adult eye, a proxy for ER stress-induced apoptosis (19). Previous studies demonstrated that enhancement of ERAD improves this ER stress eye phenotype and inhibition of ERAD drastically worsens the eye phenotype (21). We hypothesized that if dNGLY1 has a major role in ERAD, we would observe a change in the eye degeneration phenotype in this model. Knockdown of *dNGLY1* in the presence of this misfolded protein resulted in no change in the degenerated eye phenotype when compared with control flies (Supplementary Material, Fig. S2). dNGLY1 appears to not be involved in ERAD, at least in this simple model. This does not rule out a role for dNGLY1 in ERAD function in other cell- or tissue-specific or developmental contexts.

Evidence of cnc/NRF1 dysfunction

Two broad patterns emerged from genes downregulated in the *dNGLY1* knockdown flies. There was a significant enrichment for genes encoding oxidoreductases (GO: 0055114; $q = 1.35\times 10^{-10}$) and genes encoding proteasomal subunits (GO: 0043161 and GO: 0010498; $q = 0.009$ and 0.01; Tables 2 and 3; Supplementary Material, Table S2). This pattern of enrichment is consistent with dysregulation of the Cap 'n' collar (cnc) transcription factor (22–24). We compared the genes downregulated in *dNGLY1* knockdown flies with a previous study that identified genes upregulated by *cnc* activity (22). Nearly 15% of downregulated genes in *dNGLY1* knockdown flies are also upregulated by *cnc* activity (40 genes; Table 3; Supplementary Material, Table S3). This overlap between *dNGLY1* knockdown and cnc overexpression represents a significant enrichment of common genes, compared with two identically sized random lists of genes from the genome (Fisher's exact test, $P = 2.05\times 10^{-13}$). *cnc* is the fly ortholog of mammalian *NRF1* and *NRF2* (24). NRF1 regulates proteasomal levels in response to proteasomal stress (13). NRF2 regulates oxidoreductases and antioxidant genes (25). Our data is consistent with previous findings demonstrating that NGLY1 is required for proper cnc/NRF1 pathway signaling (11,12).

GlcNAc supplementation partially rescues lethality associated with *dNGLY1* knockdown

RNAseq analysis demonstrated that *Gfat1* transcript levels were ~1.7-fold downregulated in *dNGLY1* knockdown flies compared with control flies (Figs 2 and 3A; Supplementary Material, Table S1). Gfat1 is an enzyme that controls the rate limiting step in the UDP-GlcNAc biosynthetic pathway (26), suggesting that UDP-GlcNAc production might also be downregulated. Notably, there were no changes in transcript levels of the other components of the UDP-GlcNAc biosynthesis pathway, including *Gfat2* (Fig. 3A). *Gfat2* is a paralog of *Gfat1* in *Drosophila* (26). Gfat1 and Gfat2 likely play similar roles in the production of UDP-GlcNAc, but the two genes have partially non-overlapping expression patterns (http://flyatlas.gla.ac.uk/flyatlas/index.html).

Downregulation of *Gfat1* should result in reduction of UDP-GlcNAc levels. The fact that the expression level of *Gfat2* is unchanged, suggests that there is no compensation in the pathway. UPD-GlcNAc is a sugar involved in a number of posttranslational modifications and signaling and proteostasis pathways. Because of the number of processes that UDP-GlcNAc is involved in (27), including, but not limited to glycosylation of most secreted proteins, altered levels of UDP-GlcNAc might contribute to the lethality observed in *dNGLY1* knockdown flies.

Table 3. Examples of *cnc*-regulated genes downregulated in *dNGLY1* knockdown flies

	Symbol	FlyBase ID	Log 2 fold change	P-value	P_{adj}
Downregulated proteasomal genes	*Prosalpha6*	FBgn0250843	−1.12	1.12E−21	3.62E−19
	Prosbeta4	FBgn0032596	−0.78	5.72E−12	7.19E−10
	Prosbeta5	FBgn0029134	−0.72	7.13E−13	9.97E−11
	Prosbeta2	FBgn0023174	−0.63	3.36E−09	2.82E−07
	Prosalpha4	FBgn0004066	−0.61	1.96E−08	1.42E−06
	Prosalpha3	FBgn0261394	−0.59	7.25E−09	5.72E−07
	Prosalpha7	FBgn0023175	−0.56	8.66E−09	6.76E−07
Top downregulated *cnc*-regulated genes	*CG7341*	FBgn0036777	−2.09	1.24E−29	8.89E−27
	Ugt86Dd	FBgn0040256	−1.45	5.77E−23	2.18E−20
	Cyp313a3	FBgn0038007	−1.24	3.87E−09	3.19E−07
	CG18179	FBgn0036023	−1.15	5.72E−08	3.79E−06
	Pxd	FBgn0004577	−1.15	7.02E−15	1.29E−12
	CG16884	FBgn0028544	−1.14	1.69E−23	6.76E−21
	CG5326	FBgn0038983	−1.10	4.24E−23	1.64E−20
	CG9514	FBgn0030592	−1.10	8.55E−17	1.93E−14
	CG15414	FBgn0031542	−1.04	1.69E−07	1.01E−05
	CG17562	FBgn0038449	−1.03	3.52E−20	1.04E−17

We hypothesized that GlcNAc supplementation might rescue lethality in *dNGLY1* knockdown flies. To test this, we used two different GlcNAc supplementation approaches. First, we tested whether GlcNAc supplementation during larval development rescued developmental lethality. We allowed females to lay eggs on standard non-supplemented media or media supplemented with GlcNAc (100 μg/ml). Larvae were allowed to develop on their respective media. Larvae raised on media supplemented with GlcNAc showed a significant partial rescue in developmental lethality, as measured by the proportion of adult flies that eclosed (Fig. 3B). By the end of 11 days of collecting newly eclosed adult flies, we recovered nearly three times the number of flies when compared with no GlcNAc supplementation (Fig. 3C). This partial rescue effect was specific to GlcNAc and not a general sugar effect, as supplementation with dextrose did not rescue lethality (Supplementary Material, Fig. S3).

We also tested whether adult longevity of *dNGLY1* knockdown flies was enhanced with GlcNAc supplementation. We tested four different combinations of conditions (Fig. 4A): with or without GlcNAc supplementation during larval development and with or without GlcNAc supplementation during adult maintenance (100 μg/ml). We tested the adult longevity of *dNGLY1* knockdown flies under all four combinations of GlcNAc supplementation. Under normal culture conditions, with no supplementation, *dNGLY1* knockdown flies showed maximal survival of 65 days, representing a significant difference in longevity when compared with control flies ($P = 1.1 \times 10^{-16}$). At 65 days, control flies with no supplementation showed 85% survival.

GlcNAc supplementation during adult maintenance always resulted in enhancement of longevity in *dNGLY1* knockdown flies (Fig. 4). This effect was independent of GlcNAc supplementation status during larval development. *dNGLY1* knockdown flies that were raised during larval development with GlcNAc supplementation and maintained with GlcNAc supplementation as adults had enhanced longevity over those that had no supplementation during larval development or adulthood (Fig. 4B; $P = 0.021$). *dNGLY1* knockdown flies raised during larval development with no supplementation, but switched to GlcNAc supplementation during adult maintenance showed a significant enhancement in longevity, when compared with *dNGLY1* knockdown flies with no supplementation during larval development or adulthood (Fig. 4C; $P = 0.013$). Flies raised with GlcNAc supplementation during larval development and with GlcNAc supplementation during adult maintenance had a longer longevity than flies that were raised during larval development on GlcNAc, but switched to no supplementation during adulthood (Fig. 4D; $P = 0.049$). Additionally, we observed that flies supplemented with GlcNAc during larval development showed a large immediate die off (up to 20%) during the first day after eclosion (Fig. 4B and D). We did not observe this die off if the flies were raised with no supplementation during larval development (Fig. 4B and C). This suggests that GlcNAc supplementation during larval development is likely allowing sicker flies to survive to eclosion, but they die shortly after eclosion. Control flies showed no significant difference in longevity under any of these conditions. Together, these experiments indicate that supplementing GlcNAc during development and during adult maintenance can partially rescue lethality associated with loss of *dNGLY1* function.

GlcNAc supplementation does not rescue *dNGLY1* knockdown transcriptional changes

To understand how GlcNAc supplementation results in a partial rescue of lethality in *dNGLY1* knockdown flies, we performed RNAseq in adult flies that were supplemented with GlcNAc during larval development. Strikingly, GlcNAc supplementation did not result in major transcriptional changes in either control or *dNGLY1* knockdown flies. For either genotype, a within genotype comparison revealed that GlcNAc supplementation resulted in fewer than 10 genes that showed significant changes at an FDR of 5%, but none of these transcriptional changes were larger than 1.5-fold (Supplementary Material, Tables S4 and S5). Importantly, in *dNGLY1* knockdown flies, GlcNAc supplementation did not alter *dNGLY1* knockdown efficiency. When we compared knockdown with control flies with or without GlcNAc supplementation, we found that the correlation between *Gfat1*, proteasome genes and heat shock genes was very strong, indicating that GlcNAc supplementation does not affect any of these differences ($r^2 = 0.997$; $P = 2.0 \times 10^{-16}$; Supplementary Material, Fig. S4). GlcNAc rescue of *dNGLY1* knockdown lethality likely occurs through post-translational mechanisms.

A number of post-translational mechanisms involve glycosylation. However, in *Drosophila*, reagents to measure global changes in these glycosylation-related post-translational mechanisms are

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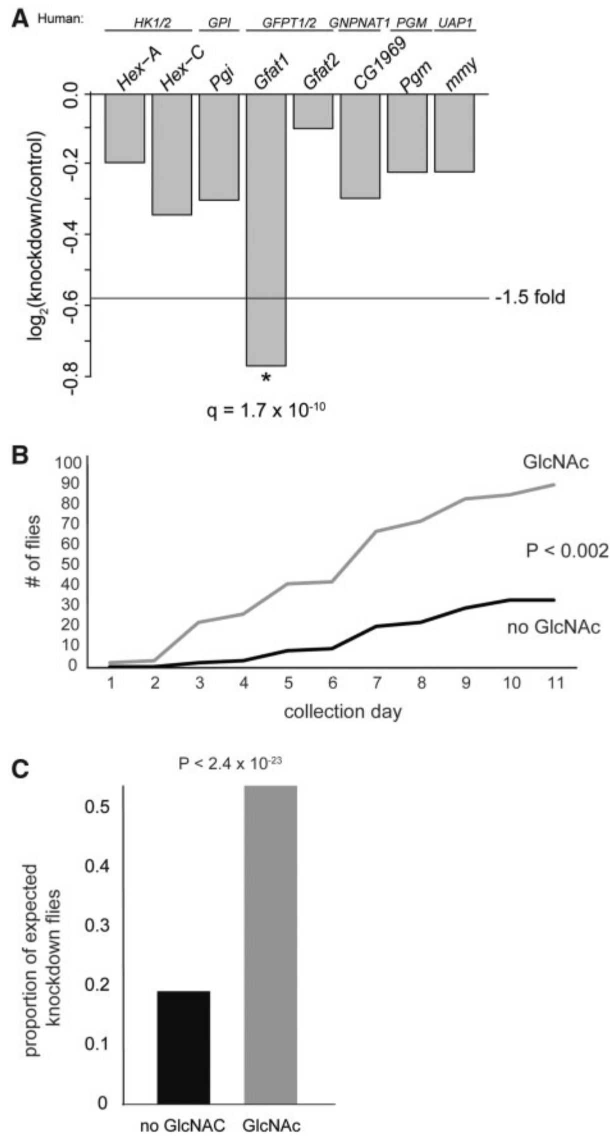

Figure 3. GlcNAc supplementation partially rescues *dNGLY1* knockdown lethality. (**A**) Expression changes in components of the UDP-GlcNAc biosynthetic pathway between *dNGLY1* knockdown and control flies. *Gfat1* is the only component that shows a significant change. *Gfat1* is nearly 2-fold downregulated in *dNGLY1* knockdown flies ($q = 1.7 \times 10^{-10}$). Human orthologs of *Drosophila* components shown above. Black horizontal line represents 1.5-fold downregulation. (**B**) Dietary GlcNAc supplementation during development rescues developmental delay ($P < 0.002$). Cumulative number of flies counted on each collection day is plotted. (**C**) At the end of 11 days of collecting, there was a significant increase in the proportion of expected *dNGLY1* knockdown flies raised on GlcNAc, when compared with no GlcNAc supplementation ($P < 2.4 \times 10^{-23}$). All data are representative of at least three independent experiments.

lacking. We can only reliably probe global O-GlcNAcylation changes in *Drosophila*. Thus, we tested whether GlcNAc supplementation alters global O-GlcNAcylation levels in *dNGLY1* knockdown flies. Western blot analysis, using an antibody to the O-GlcNAcylation tag, identified no consistent global differences between control flies and *dNGLY1* knockdown flies, with or without GlcNAc supplementation (Supplementary Material, Fig. S5). This does not rule out a tissue-specific, development-specific or protein-specific change in O-GlcNAcylation that we are unable to assay in adult flies.

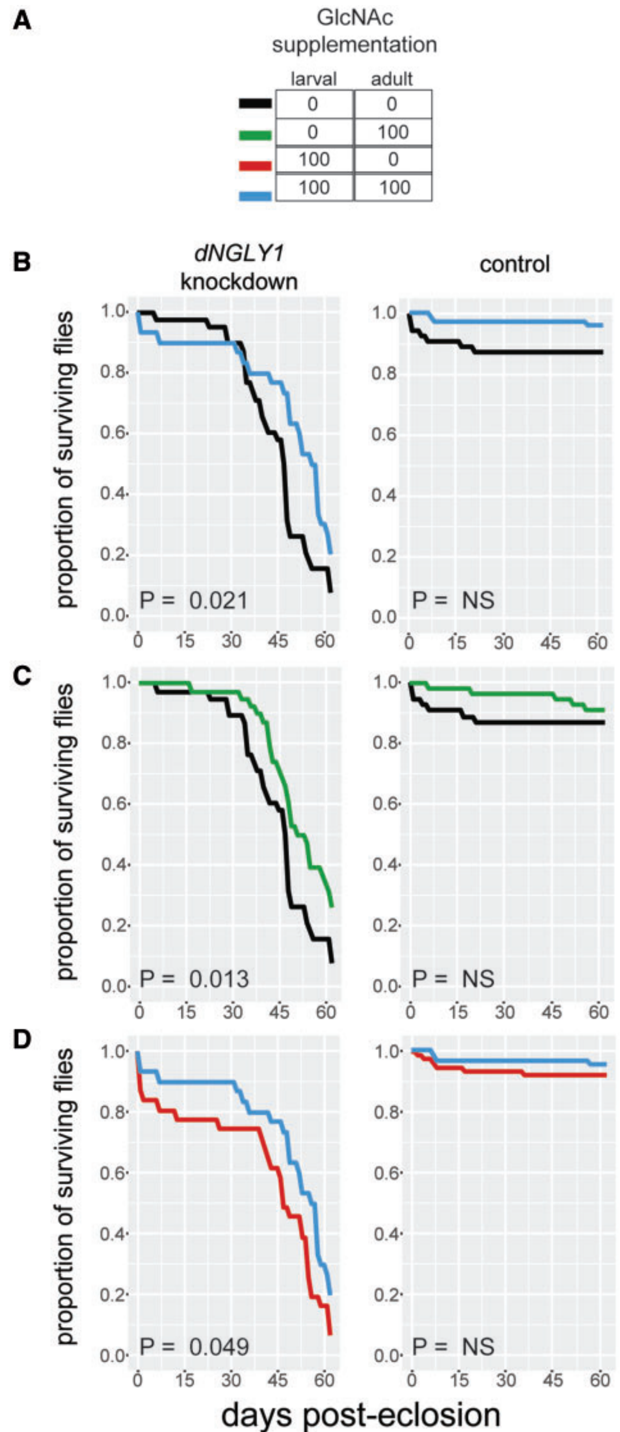

Figure 4. GlcNAc supplementation improves longevity in *dNGLY1* knockdown adult flies. (**A**) GlcNAc supplementation scheme used during larval development or adult maintenance (0 or 100 μg/ml). Colors represent the different combinations. Adult GlcNAc supplementation improves longevity, irrespective of larval supplementation status. Comparisons of *dNGLY1* knockdown adult longevity (days post-eclosion) for (**B**) no GlcNAc supplementation versus complete GlcNAc supplementation ($P = 0.021$); (**C**) no GlcNAc supplementation versus adult only supplementation ($P = 0.013$); (**D**) larval only GlcNAc supplementation versus complete supplementation ($P = 0.049$). GlcNAc supplementation had no significant effect on control fly longevity (B–D; right).

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UDP-GlcNAc levels in *dNGLY1* knockdown flies

We hypothesized that there might be abnormal levels of UDP-GlcNAc in *dNGLY1* knockdown flies. We also hypothesized that GlcNAc supplementation might normalize abnormal UDP-GlcNAc levels in *dNGLY1* knockdown flies. To test this, we used HPLC analysis to compare UDP-GlcNAc levels in control flies and *dNGLY1* knockdown flies under no supplementation and GlcNAc supplementation conditions. We used 1-day-old adult flies, identical to the RNAseq analysis. UDP-GlcNAc levels were calculated as a percent of total by normalizing the UDP-GlcNAc peak area to the total ethanol extraction peak level. Under normal rearing conditions with no GlcNAc supplementation, we found that levels of UDP-GlcNAc were consistently increased in *dNGLY1* knockdown flies when compared with control flies (UDP-GlcNAc/total $\times$100; control = 0.489–0.539; *dNGLY1* = 0.868–0.873; Supplementary Material, Fig. S6). Under GlcNAc supplementation conditions, control flies and *dNGLY1* knockdown flies showed similar UDP-GlcNAc levels (UDP-GlcNAc/total$\times$100; control: 0.550–0.636; *dNGLY1* knockdown: 0.482–0.491; Supplementary Material, Fig. S6). Because UDP-GalNAc and UDP-GlcNAc can be interconverted, we also measured UDP-GalNAc levels and across all conditions. We found no changes in UDP-GalNAc levels (Supplementary Material, Fig. S6). It appears that supplementation had little effect on UDP-GlcNAc levels in control flies, but supplementation seems to have normalized levels in *dNGLY1* knockdown flies. The normalization of UDP-GlcNAc levels might explain the partial rescue we see with GlcNAc supplementation. It is still unclear why we see increased levels under no supplementation. More analysis with higher sensitivity assays will be needed to identify mechanism by which UDP-GlcNAc levels are changing in the *dNGLY1* knockdown flies.

Heat shock response is important for survival in *dNGLY1* knockdown flies

RNAseq analysis in the *dNGLY1* knockdown flies also revealed upregulation of a number of heat shock genes (Fig. 2; Fig. 5A; Supplementary Material, Table S1), representing several classes of heat shock genes, including *HSP70*, *HSP20* and *HSP90*. We hypothesized that upregulation of heat shock genes might be an adaptive stress response in *dNGLY1* knockdown flies and that a stronger heat shock response might rescue lethality. Because most lethality occurs during larval development, we subjected knockdown and control larvae to a standard heat shock treatment at the beginning of the first, second or third instar stages. Following heat shock, larvae were allowed to develop normally and adults were counted after eclosion. Heat shock treatment during all three instar stages resulted in rescue of lethality (Fig. 5B). The most effective rescue occurred with heat shock at the first instar stage.

We next hypothesized that the upregulated heat shock genes were required for survival of *dNGLY1* knockdown flies. To test this, we used RNAi to systematically knockdown each upregulated heat shock gene. Individual knockdown of *Hsp70Bb*, *Hsp23* or *Hsp26* on the *dNGLY1* knockdown background resulted in increased lethality compared with *dNGLY1* knockdown alone (Fig. 5C). Strikingly, knockdown of *Hsp70Bb*, which was one of the highest upregulated genes (>7-fold) (Figs 2 and 5A), showed complete lethality (Fig. 5C). This suggests that *Hsp70Bb* might be especially critical to survival in the absence of dNGLY1 activity. Knockdown of *Hsp68* on the *dNGLY1* knockdown background did not alter survival (Fig. 5C). Knockdown of *Hsp70Bb*, *Hsp23*, *Hsp26* or *Hsp68* alone on a control background did not result in lethality, indicating that the increased lethality observed, is an interaction between heat shock genes and *dNGLY1* (Fig. 5D). Knockdown of *Hsp83* alone on a control background resulted in near complete lethality (Fig. 5D), making it impossible to evaluate its role on the *dNGLY1* knockdown background. There appears to be an important genetic interaction between the heat shock response and *dNGLY1*.

Discussion

Therapeutic development for rare diseases is often hampered by a lack of research. Hypotheses about a particular rare disease or rare disease gene are formed based on a small body of research that is likely incomplete. This can be counterproductive for understanding pathogenesis and for therapeutic development. The identification of *NGLY1* as the causative gene for a previously undiagnosed disease brought *NGLY1* biology into the spotlight (1,2,28). Since its initial identification in a single family (1), we have learned more about possible pathogenesis underlying this devastating disease. Previous to this study, it was hypothesized that NGLY1 functions to deglycosylate all misfolded proteins as they are extracted by ERAD (2,4,5,10,17). It was hypothesized that if NGLY1 function was absent, ER stress would occur and glycans may be inappropriately removed by other enzymes (10). The findings we report here suggest that at least some components of this hypothesis may not be entirely correct.

We utilized RNAseq in a *Drosophila* model to get a fuller picture of the pathogenesis underlying *NGLY1* deficiency. Strikingly, we found no evidence of ER stress or abnormal ERAD in the absence of *NGLY1*. This suggests that, at least under normal physiological conditions, *NGLY1* may not be required for ERAD to clear misfolded proteins. A previous study demonstrated that loss of *NGLY1* in cultured mouse embryonic fibroblasts resulted in abnormal ERAD processing of an artificial model substrate, but no increased ER stress was reported (5). This difference might be attributable to subtle species differences, but more likely, this might be explained by different experimental approaches. While we used an *in vivo* model, the previous study relied on cultured fibroblasts expressing a non-endogenous ERAD substrate (5). We cannot rule out a cell-type-specific or developmental-specific role for NGLY1 in ER stress or ERAD, as our approaches may not have been sensitive enough to detect these types of effects.

While ERAD is responsible for clearing misfolded proteins in general, ERAD processing is also required for the normal function of a small select group of glycoproteins (9). For example, in Caenorhabditis *elegans*, loss of *NGLY1* results in a neuronal branching defect that is attributed to the absence of an unknown processed glycoprotein (29). More recently, it has been demonstrated that *NGLY1* in *C. elegans* is required for proper processing of the stress-responsive transcription factor, NRF1 (11,12). Under non-stressed conditions, NRF1 is translated into the ER membrane and is quickly degraded by ERAD. However, under conditions like proteasome stress, NRF1 is deglycosylated by NGLY1, which allows NRF1 to be further processed and enter the nucleus to regulate stress response genes (11,12). In the absence of *NGLY1*, NRF1 remains glycosylated and cannot function properly (11,12). Indeed, we found evidence that *cnc*, the *Drosophila* ortholog of *NRF1*, is also misregulated in the *dNGLY1* knockdown flies. We found entire classes of cnc/NRF1 target genes downregulated, including genes encoding proteasome subunits and proteins with oxidation–reduction functions. Our data further support the idea that NGLY1 may function in very specific ERAD contexts.

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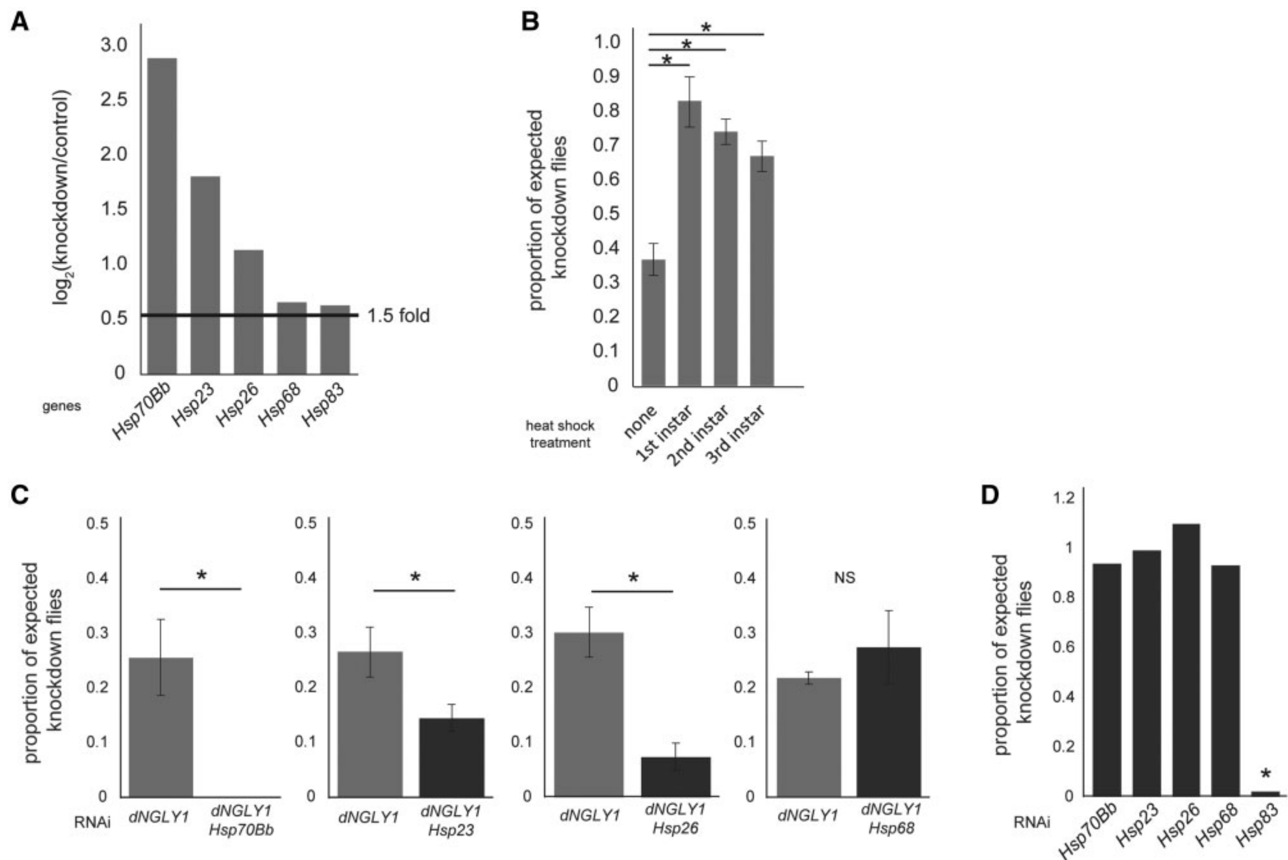

Figure 5. Heat shock rescues lethality in *dNGLY1* knockdown flies. (**A**) Upregulation of heat shock genes in *dNGLY1* knockdown flies when compared with control flies. All genes shown are significantly upregulated ($q<0.05$). Black horizontal line=1.5-fold upregulated. (**B**) Heat shock treatment during larval development recues lethality. Heat shock during each of the three larval instar stages significantly improves survival (first instar: $P=6.9\times10^{-6}$; second instar: $P=1.7\times10^{-9}$; third instar: $P=2.4\times10^{-5}$). None=no heat shock treatment. (**C**) Heat shock genes are required for survival in *dNGLY1* knockdown flies. Knockdown of *Hsp70Bb* ($P<3.3\times10^{-12}$), *Hsp23* ($P<8.5\times10^{-5}$) and *Hsp26* ($P<4.8\times10^{-5}$) on the *dNGLY1* knockdown background significantly reduced survival. Knockdown of *Hsp68* on the *dNGLY1* knockdown background did not impact survival. These experiments were performed under normal developmental conditions, without heat shock treatment. (**D**) Ubiquitous knockdown of *Hsp70, Hsp23, Hsp26* or *Hsp68* alone had no effect on survival. Knockdown of *Hsp83* alone resulted in near complete lethality ($P=1.7\times10^{-18}$). These experiments were performed under normal developmental conditions, without heat shock treatment. Mean±standard deviation. All data represent at least three independent experiments.

While NGLY1 may function in ERAD, there are likely a number of other processes that are misregulated and may contribute to its disease pathogenesis (16,30). This is especially apparent from our RNAseq analysis, as we found nearly 400 genes that were either up- or downregulated in the absence of d*NGLY1*. Indeed, these genes provide clues to downstream misregulated processes and potential therapeutic approaches. We found that the rate limiting step in the UDP-GlcNAc biosynthetic pathway was downregulated nearly 2-fold, suggesting that UDP-GlcNAc levels might be abnormal. We found this to be particularly exciting, as GlcNAc supplementation appeared to be a relatively simple approach to test. Further, if it was successful in *Drosophila*, it might be easily implemented in patients (see below). We hypothesized that abnormal levels of UDP-GlcNAc were contributing to the lethality we observed. Accordingly, we found that GlcNAc supplementation rescued lethality associated with loss of *dNGLY1* function. Strikingly, GlcNAc supplementation can partially rescue lethality without rescuing cnc/NRF1 signaling defects, suggesting that there might be therapeutic approaches to *NGLY1* deficiency that do not necessarily have to address NRF1 dysfunction.

We hypothesized that lower levels of *Gfat1* might lead to lower levels of UDP-GlcNAc. However, we found increased UDP-GlcNAc levels in *dNGLY1* knockdown flies. This can be for multiple reasons, including changes in metabolic flux and other compensatory mechanisms. We also only profiled 1-day-old flies (RNAseq and UDP-GlcNAc measurements). Because we saw such a large rescue of lethality with supplementation, we hypothesized that recently eclosed adult flies might harbor signatures of this rescue. It is entirely possible that we would observe different transcriptional signatures and UDP-GlcNAc levels during larval development or during late adulthood. Different life stages might have different demands for UDP-GlcNAc. Further analysis is needed to explain this observation.

Nevertheless, our results demonstrate that supplementing GlcNAc in the food rescues lethality of *dNGLY1* knockdown flies. It is possible that the rescue effect we observe occurs because this supplementation restores UDP-GlcNAc levels in the flies. Equally possible, GlcNAc supplementation rescue might be through a number of other mechanisms. GlcNAc supplementation can be beneficial under different stress conditions (27,31). Some of these effects include protecting the organism from ER stress, enhancing ERAD and other protein quality control pathways (27,31). It is also possible that all these effects are at play in the *dNGLY1* knockdown flies or there are different developmental- or tissues-specific effects. Further work is needed to understand the mechanism by which GlcNAc supplementation recuses *dNGLY1* knockdown flies.

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RNAseq analysis also revealed that the heat shock response is another pathway that might be targeted for therapy. We demonstrated that heat shock response genes are required for survival in *dNGLY1* knockdown flies. More importantly, we demonstrated that a stronger heat shock response rescues lethality. There are a number of reasons why the heat shock response might be upregulated in *dNGLY1* knockdown flies. Downregulation of the proteasome or abnormal ERAD function might lead to cytoplasmic aggregates. In turn, these cytoplasmic aggregates would lead to induction of the heat shock response which is normally recruited to maintain cytoplasmic protein homeostasis. The heat shock response is also induced under a number of other stress conditions (32).

It is exciting that the GlcNAc and heat shock pathways might be targets for therapeutic development for *NGLY1* deficiency. For GlcNAc, it is particularly exciting that a nutritional supplement might alleviate some symptoms. Various forms of GlcNAc are available in grocery stores and health food stores as 'health supplements', purportedly to treat a variety of health issues. In fact, anecdotal stories from parents of children with *NGLY1* deficiency suggest that GlcNAc is indeed a promising therapeutic. A small number of parents have administered GlcNAc to their children, and they claim that it relieves the alacrima and modulates sleep disturbances (personal communication). We learned about this result after presenting our work at several conferences. It is striking that our studies and patient experiences have independently converged on this possible treatment, underlying the importance of listening to patient experiences. Of course, rigorous human trials will be needed to establish possible efficacy of GlcNAc replacement in *NGLY1* deficiency patients.

Enhancing the heat shock response in *NGLY1* deficiency patients may also hold promise. At least in flies, a simple increase in the response showed a significant increase in survival. It is possible that only certain components of the response are needed to improve survival. For example, we demonstrate that *Hsp70* is the only heat shock gene absolutely required for survival of *dNGLY1* knockdown flies. In other diseases where the heat shock response appears to be beneficial, Hsp70 is also the major component responsible for the positive effects (33–35). More work is needed to determine which minimal components are required for this effect. Nevertheless, a number of drugs are already being used in research and being developed for therapies that alter the heat shock response. Chemical inducers of the heat shock response, including eldanamycin, geldanamycin, geranylgeranylacetone and celastrol, have already been shown to be effective in certain cell and animal models of neurodegenerative diseases (36–39).

Here we present a number of findings that point to new ways we might approach therapeutic development for *NGLY1* deficiency. Approaches like genetic screens and RNAseq, which do not require a strong a priori hypothesis, might be especially important to rare disease research communities because they can bring new insight relatively quickly and can prevent too narrow of a focus. Rare disease patient advocacy groups are especially good at disseminating research to the patients. However, too narrow of a focus may generate hope in findings that may not be the best therapeutic approaches. Our studies demonstrate that these types of approaches, especially in model organisms, like *Drosophila*, can quickly reveal novel findings and potential therapeutic approaches for rare diseases like *NGLY1* deficiency.

Materials and Methods

Drosophila melanogaster fly stocks

All stocks were maintained under standard laboratory conditions on agar–dextrose–yeast medium at 24°C on a 12-h light/dark cycle. The following strains were obtained from the Bloomington *Drosophila* Stock Center (strain ID): *dNGLY1* RNAi (54853 and 42592), *Tubulin-GAL4* driver (5138), *GMR-GAL4* driver (1104), *attp40* control strain (36304), *Hsp70Bb* RNAi (32997 and 33948), *Hsp23* RNAi (30541 and 44029), *Hsp26* RNAi (42610), *Hsp68* RNAi (50637) and *Hsp83* (32996 ad 33947). The *UAS-Rh1*^{G69D} strain has been previously described (19). The *UAS-Xbp1-GFP* marker strain has been previously described (18,19). With the exception of the initial characterization in Figure 1, *dNGLY1* RNAi strain 54 853 was used for all analysis. The corresponding *attP40* control strain was used as our wild-type control.

RNAseq

mRNA sequencing was performed on total RNA from whole male control or knockdown 1-day-old flies raised on control food or GlcNAc supplemented food (4 total genotype ×treatment groups). All 4 groups were sequenced in triplicate (12 total samples). Single-end 50 bp mRNA-seq libraries were made with 1.5 μg of total RNA using the Illumina TruSeq RNA Sample Preparation kit (Illumina, Inc., CA, USA). Samples were prepared by the Huntsman Cancer Institute High-Throughput Genomics Core. The 12 samples were multiplexed and sequenced on a single lane on the Illumina HiSeq 2500 instrument. Image analysis and base calling were performed with the provided Illumina software. RNA-seq reads were aligned to the *Drosophila* genome assembly using TopHat v1.4.1 (40). Total expression level for each transcript, measured in FPKM (Fragments Per Kilobase-pair of exon Model), was calculated based on mapped reads (41).

Quantification of total expression and change in expression

Read counts were normalized across all samples using the default normalization method in the DeSeq2 package in R (42). Principal components analysis (PCA) was used to identify outlying samples. PCA is a commonly used method to identify outlying replicates in RNAseq analysis. If a sample is outlying, it suggests that it is transcriptionally different from the other replicates, likely because of a number of technical reasons. If a sample was not clustered with the appropriate genotype (knockdown or control) and GlcNAc supplementation condition (supplementation versus no supplementation), it was removed from analysis. After removal of outlying samples, there remained at least two replicates for each genotype and treatment group. Remaining samples were re-normalized. Gene expression changes were assessed by comparing samples using linear models with the DeSeq2 package in R (42).

ER stress and ERAD tests

To test for increased ER stress, salivary glands from *tubulin* > *dNGLY1-RNAi* and *tubulin* > *Attp40* larvae (wandering L3) carrying the *UAS-Xbp1-GFP* marker were dissected out and imaged on a Nikon A1R laser scanning confocal microscope. As a positive control, *tubulin* > *Attp40* larvae were exposed to 1 μM Thapsigargin in PBS for 30 min. Thapsigargin induces ER stress

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by inhibiting the SERCA Ca^{2+} ATPase. This experiment was performed in triplicate.

To test for ERAD function, a line carrying *GMR-GAL4* and *UAS-Rh1*^{G69D} (43) was crossed to either flies carrying *UAS-dNGLY1 RNAi* or *Attp40* control flies. Eyes from adult knockdown and control flies were imaged and analyzed as previously described (43). Over 10 flies were scored for each genotype.

Developmental lethality experiment

We used *tubulin-GAL4* to drive ubiquitous UAS-*dNGLY1-RNAi*. *tubulin* > *dNGLY1-RNAi* and *tubulin* > *Attp40* control flies were raised on normal control food or food supplemented with 100 μg/ml of GlcNAc (Sigma Aldrich). Flies were collected and scored once a day until eclosion was complete. For each day, the proportion of *dNGLY1* knockdown flies versus control balancer flies was quantified for each group. χ^2 analysis was used to determine deviation from the expected ratio of genotypes (R. Version 2.8.1; R Development Core Team).

Lifespan experiment

Tubulin > *dNGLY1-RNAi* knockdown and control flies were raised on normal control food or food supplemented with 100 μg/ml of GlcNAc. Upon eclosion, adult flies were separated into groups of 15 flies and then placed into new vials. Half the adult flies from each developmental diet were placed onto normal control food and half were placed onto food supplemented with 100 μg/ml GlcNAc. As a control, this procedure was also performed with *tubulin* > *Attp40* control flies. The adult flies were placed onto fresh food every 3–4 days and their lethality was scored every 1–2 days until all flies died. At least 100 flies were included in all genotype and supplementation combinations. Lifespan experiment was performed in triplicate.

Survival analysis was performed in R (Version 2.8.1; R Development Core Team) with the Survival package previously described (44). Kaplan–Meier survival plots were also generated where indicated.

O-GlcNAcylation western blot analysis

Protein was extracted from 10 flies (1 day old; control and *dNGLY1* knockdown, with or without supplementation). Flies were treated identically to the flies used for RNAseq analysis. Supplementation occurred throughout larval development. Western blotting for O-GlcNAcylated proteins was performed with the anti-O-linked *N*-acetylglucosamine antibody (RL2; Abcam) at a 1 : 1000 concentration in 5% BSA for 12 h at 4°. The blot was then incubated for one hour at room temperature using Peroxidase AffiniPure Goat anti-Mouse IgG (H + L) secondary antibody at a concentration of 1:100 000 (Jackson ImmunoResearch Laboratories). The blot was exposed using ECL (Thermo Fisher).

Extraction of UDP-GlcNAc and UDP-GalNAc

The procedure for the extraction of UDP-GlcNAc and UDP-GalNAc from *Drosophila* was optimized using a previously reported method (45). To establish HPLC conditions, we used 10 flies from the w^{1118} stain (a standard *Drosophila* strain). For analysis, we used 25 flies in each sample (1 day old; control and *dNGLY1* knockdown, with or without supplementation). Flies were manually ground down in 120 μl of 75% ethanol and cells were further lysed on ice using a sonic dismembrator (Fisher Scientific). After adding additional 300 μl of 75% ethanol, the samples were incubated with a rotator at 4°C for 10 min. Soluble supernatant was collected by centrifugation (14 500 rpm, 20 min at 4°C). The insoluble fractions were extracted a second time if necessary and the combined supernatant was dried using a SpeedVac (LABCONCO). After solvent evaporation, the samples were resuspended in 50 μl of PBS, then diluted to 150 μl using HPLC grade water. A 100-μl of supernatant was filtered through the 3000 MWCO filter (Millipore) and the filtrate was stored at −80°C until ready for further analysis.

Ion-pair reversed-phase HPLC analysis

In order to control for sample volume between samples, we normalized all UDP-GlcNAc levels or UDP-GalNAc levels to the total ethanol extraction peak area. All samples were analyzed using ion-pair reversed-phase HPLC (Hitachi LaChrom Elite HPLC System). A LaChromUltra C18 column (5 μm, 4.6 × 150 mm, Hitachi) was used for separation. UDP-GlcNAc and UDP-GalNAc were detected by absorbance at 260 nm. The following two buffers were optimized as mobile phase (Buffer A: Buffer B = 99: 1): Buffer A (50 mM ammonium phosphate buffer pH 5.0 with 10 mM tetrabutylammonium hydrogensulfate); Buffer B (99.8% methanol containing 10 mM tetrabutylammonium hydrogensulfate). The flow rate was set at 1 ml/min and the column temperature was maintained at 28°C. UDP-GlcNAc and UDP-GalNAc standards, and all other chemicals were purchased from Sigma-Aldrich. For each sample injection, 1% gradient was used in the entire 60 min run and the column was washed after each run. A 100-μl of w^{1118} sample was used for spiking with standards to identify peaks (Supplementary Material, Fig. S6), and 40 μl of each test sample was subsequently injected and analyzed.

Heat shock experiment

Tubulin-GAL4 virgin females were mated to *UAS-dNGLY1-RNAi* males on standard food overnight. The mated females were then transferred to fresh food and allowed to lay eggs for 5 h. The larvae were collected, counted and heat shocked 24 (first instar), 48 (second instar) or 72 h (third instar) after the end of the egg laying period at 37°C for 1 h. The control group was collected and counted, but not heat shocked. Flies were allowed to develop and eclose. Upon eclosion, the proportion of knockdown flies versus control balancer flies was quantified for each group. χ^2 analysis was used to determine deviation from the expected ratio of genotypes (R. Version 2.8.1; R Development Core Team). At least 100 flies were included in all genotype and heat shock combinations and were performed in triplicate.

To test the effect of knockdown of each individual upregulated heat shock gene, we crossed their respective RNAi strains to the *dNGLY1* knockdown flies. The following experiments were all performed under normal developmental conditions, without heat shock treatment. In this scenario, *tubulin* > *GAL4* drives expression of both *dNGLY* RNAi and heat shock gene RNAi. Adult flies were scored. Survival of *dNGLY1* knockdown flies and double knockdown flies were compared with balancer controls to calculate proportion of surviving flies. To test the effect of heat shock gene RNAi on its own, each RNAi strain was crossed to *tubulin* > *GAL4*. In all cases, when two RNAi lines were available, both were analyzed and results were agreement. At least 100 flies were included in all genotype combinations and

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replicates. χ^2 analysis was used to determine deviation from the expected ratio of genotypes.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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