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Tracing the NGLY1 footprints: insights from *Drosophila*

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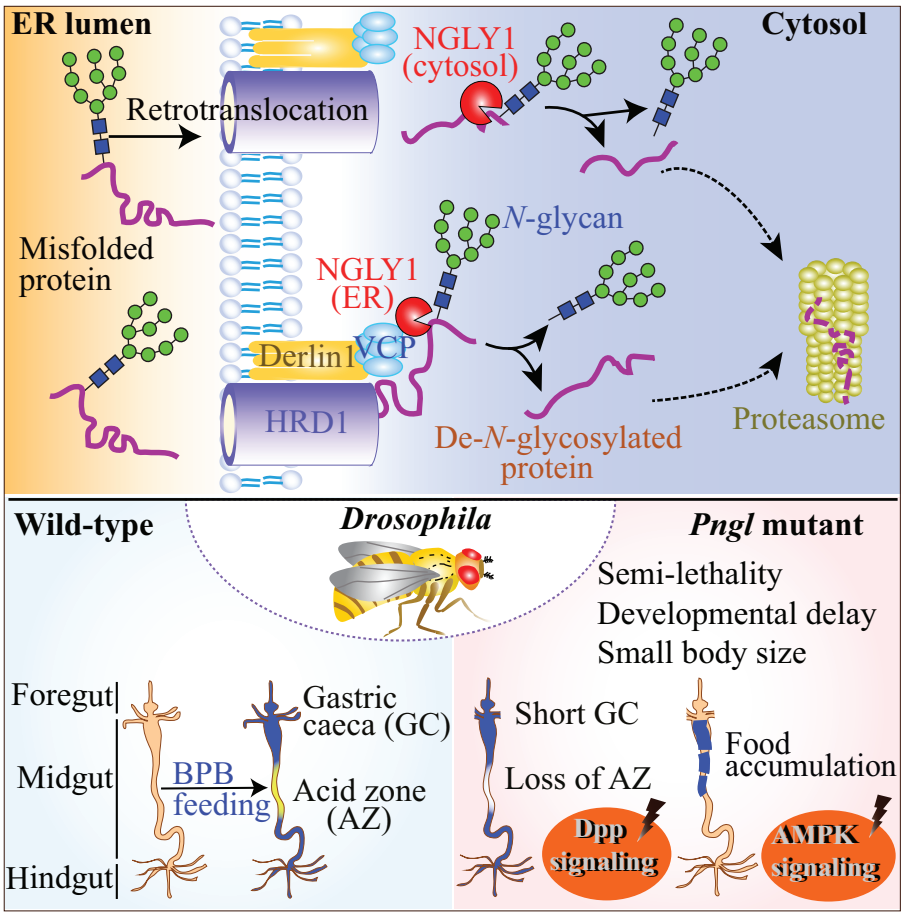
Ashutosh Pandey [1] **and Hamed Jafar-Nejad** [1,2,3,*]

[1]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA; [2]Development, Disease Models and Therapeutics Graduate Program, Baylor College of Medicine, Houston, TX 77030, USA; and [3]Genetics and Genomics Graduate Program, Baylor College of Medicine, Houston, TX 77030, USA

*Hamed Jafar-Nejad, Department of Molecular and Human Genetics, One Baylor Plaza, Room 919E, BCM225 Houston, TX 7703, USA. Tel.: +1-713-798-6159, fax: +1-832-825-1270, email: hamedj@bcm.edu

Recessive mutations in human *N*-glycanase 1 (*NGLY1*) cause a multisystem disorder with various phenotypes including global developmental delay. One of the models utilized to understand the biology of NGLY1 and the pathophysiology of NGLY1 deficiency is *Drosophila melanogaster*, a well-established, genetically tractable organism broadly used to study various biological processes and human diseases. Loss of the *Drosophila NGLY1* homolog (*Pngl*) causes a host of phenotypes including developmental delay and lethality. Phenotypic, transcriptomic and genome-wide association analyses on *Drosophila* have revealed links between NGLY1 and several critical developmental and cellular pathways/processes. Further, repurposing screens of Food and Drug Administration (FDA)-approved drugs have identified potential candidates to ameliorate some of the *Pngl*-mutant phenotypes. Here, we will summarize the insights gained into the functions of NGLY1 from *Drosophila* studies. We hope that the current review article will encourage additional studies in *Drosophila* and other model systems towards establishing a therapeutic strategy for NGLY1 deficiency patients.

Graphical Abstract



Keywords: AMPKα; BMP signalling; deglycosylation; rare disease.

Abbreviations: BMP4, bone morphogenetic protein 4; BTZ, bortezomib; Cnc, cap-n-collar; DMSO, dimethyl sulfoxide; ERAD, endoplasmic reticulum-associated degradation; HSPs, heat shock proteins; NFE2L1, nuclear factor erythroid 2 like-1; RNAseq, RNA sequencing; ROS, reactive oxygen species; TGFβ, transforming growth factor beta; VCP, valosin containing protein.

Recessive mutations in a human de-*N*-glycosylating enzyme, *N*-glycanase 1 (NGLY1), cause a congenital disorder of deglycosylation called NGLY1 deficiency (OMIM # 615273) (*1*). NGLY1 is a cytosolic enzyme which de-*N*-glycosylates (removes *N*-glycans or simply deglycosylates) *N*-glycoproteins (Fig. 1A). NGLY1 has been functionally established as a part of endoplasmic reticulum-associated degradation (ERAD) pathway, and a small number of yeast proteins have been shown to be NGLY1-dependent substrates for ERAD (*2*). However, deglycosylation by NGLY1 does not necessarily promote the degradation of its target proteins (*3–5*). Moreover, to date only two established NGLY1 target proteins, *i.e.* bone morphogenetic protein 4 (BMP4) and nuclear factor erythroid 2 like-1 (NFE2L1; also called NRF1), have been shown to be functionally dependent on NGLY1's enzymatic activity (*6–9*). NFE2L1 is the first established evolutionarily conserved target of NGLY1 (*8–10*). It is essential for proteasomal gene expression during the proteasome bounce back response (*11*). Studies on mammalian cells and worms showed that cytosolic NGLY1 deglycosylates NFE2L1 to promote its transcriptional activity (*8–10*).

NGLY1 deficiency was first identified in 2012 by whole-exome sequencing (*12*). Since then, over 100 patients have been identified (personal communication from Matt Wilsey and Selina Dwight, Grace Science Foundation). It is a multisystemic disorder with various clinical phenotypes including global developmental delay, movement disorders, seizures, orthopaedic manifestations, microcephaly, feeding difficulty, chronic constipation, elevated liver enzymes and intellectual disability (*1*, *13–18*). In addition to cell-based assays using NGLY1 deficiency patient fibroblasts and *Ngly1* mutant mouse embryonic fibroblasts (MEFs), a number of model organisms including mouse, rat, *Caenorhabditis elegans* and *Drosophila* have been used to understand the functions of NGLY1 and to identify the molecular mechanisms underlying NGLY1 deficiency phenotypes (*6*, *7*, *10*, *19–23*). Findings from these studies have also

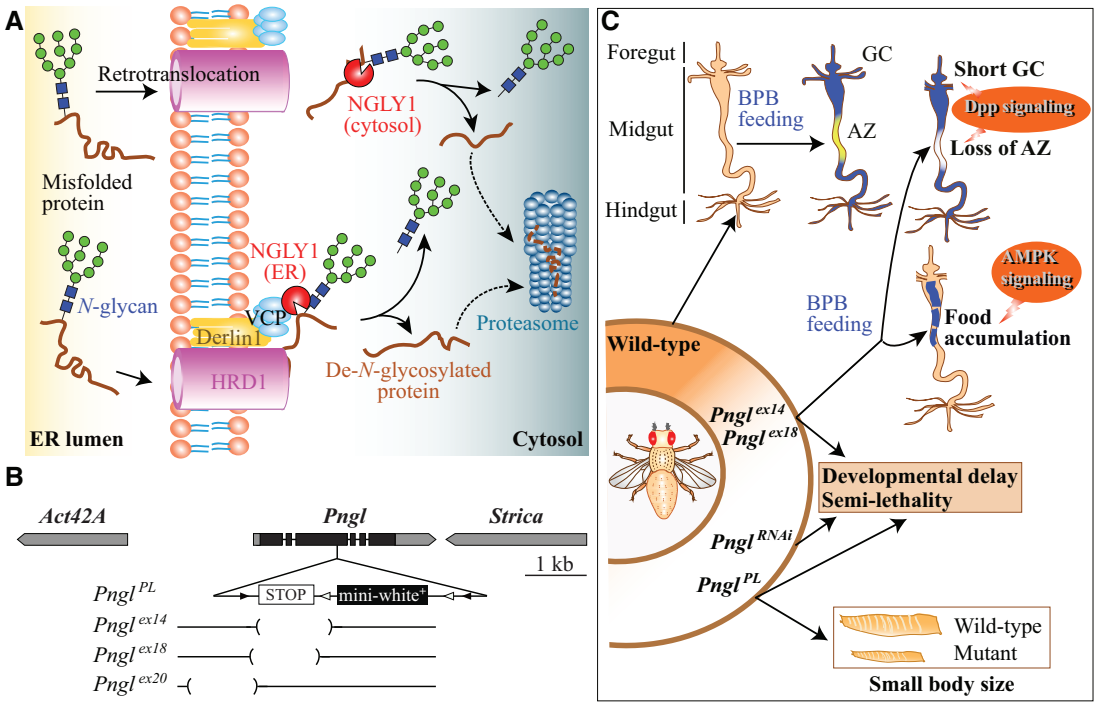


Fig. 1. (**A**) Cartoon representation of retrotranslocation of misfolded *N*-glycosylated proteins from the ER lumen to the cytosol through a membrane-associated complex including HRD1, Derlin1 and VCP. These *N*-glycosylated proteins are deglycosylated either by cytosolic NGLY1 (top) or ER membrane-recruited NGLY1 (bottom) and thereafter undergo proteosomal degradation. (**B**) Schematic representation of the *Pngl* genomic region and different *Pngl* alleles generated so far. The dark area in *Pngl* represents the coding region. $Pngl^{PL}$ is generated through CRISPR-*Cas9* gene editing and harbours a nonsense mutation at codon 420 followed by the mini-white^{+} insertion. $Pngl^{ex14}$, $Pngl^{ex18}$ and $Pngl^{ex20}$ are microdeletions generated through imprecise excision of a *P*-element insertion in the *Pngl* locus. (**C**) Cartoon representation of some of the *Pngl* loss-of-function phenotypes and the impaired signalling pathways associated with these phenotypes. Not all alleles have been examined for all phenotypes. AZ, acid zone; GC, gastric caeca; BPB, bromophenol blue.

provided some basis towards developing therapeutic approaches for this disease.

As a well-established genetically tractable animal, *Drosophila* has been used to model various human diseases for many years (*24*) including NGLY1 deficiency. This review is intended to highlight the contributions of *Drosophila* studies towards understanding the biological functions of NGLY1 and pathophysiology of NGLY1 deficiency.

Drosophila as a Model for NGLY1 Deficiency

The *Drosophila* homolog of human NGLY1 is called PNGase-like (Pngl) and was first characterized by the Tadashi Suzuki group in 2010 (*25*). Structurally conserved with human NGLY1, fly Pngl possesses a core transglutaminase-like (TG) domain harbouring its catalytic site, a PUB (peptide: *N*-glycanase/UBA or UBX-containing proteins) domain and a C-terminal carbohydrate binding PAW domain (*25*). Alignment of Pngl with human NGLY1 using the Clustal W method (DNASTAR Lasergene 10) shows 33.2% amino acid sequence identity between full-length proteins and 60.3% identify between their TG domains. Initial *in vitro* assays using a radioactively labelled glycopeptide substrate did not detect deglycosylation activity for Pngl (*25*). However, a more sensitive *in vivo* assay later confirmed that Pngl possesses a deglycosylation activity comparable to that of human NGLY1 (*7*).

Imprecise excision of a *P*-element insertion in the *Pngl* locus resulted in the generation of three microdeletion alleles $Pngl^{ex14}$, $Pngl^{ex18}$ and $Pngl^{ex20}$ (Fig. 1B). These alleles exhibited developmental delay and were semi-lethal, as <1% of homozygotes were able to finish the pupal stage and eclose from the pupal case as adult flies. Moreover, the adult escapers are short-lived and sterile. These observations provided the first set of evidence suggesting a critical role for an NGLY1 homolog in animal development and survival (*25*). Ubiquitous expression of wild-type mouse and human NGLY1 rescued the lethality of *Pngl* mutant animals, suggesting functional conservation between Pngl and its mammalian homolog NGLY1 (*7*, *25*). In addition to the developmental delay and lethality, a study from our lab reported shortening of gastric caeca in the midgut of $Pngl^{ex14/ex14}$ and $Pngl^{ex18/ex18}$ third instar larvae. Further, feeding these larvae with food mixed with bromophenol blue revealed the loss of a specialized region in the *Drosophila* gut called the acid zone and a failure to empty the gut or the food accumulation phenotype [(*7*); Fig. 1C].

To model NGLY1 deficiency, the Perlstein group (*26*) used the CRISPR/Cas9 technology to generate another *Pngl* allele called $Pngl^{PL}$, which harbours a nonsense mutation at codon 420 and is predicted to remove the C-terminal carbohydrate binding domain (Fig. 1B). $Pngl^{PL}$ homozygotes also exhibited developmental delay and semi-lethality, as only 32% of the animals reached to adult stage when reared at 25°C. Both the $Pngl^{PL}$ homozygous larvae and the eclosed adult flies had significantly smaller body size compared to heterozygous animals. The developmental delay and small body size phenotypes of $Pngl^{PL/PL}$ animals were rescued upon ubiquitous expression of human *NGLY1* (*26*), further indicating the functional similarity between *Drosophila* Pngl and human NGLY1. In another report by the Chow group (*27*), ubiquitous knockdown (~95% reduction in gene expression) of *Pngl* was carried out to model *NGLY1* loss-of-function in *Drosophila*. *Pngl* knockdown resulted in lethality (only ~30% of adult flies survived) and significant developmental delay (*27*).

The consistent phenotypes observed upon analysis of independently generated *Pngl* loss-of-function alleles and RNAi-mediated *Pngl* knockdown, combined with evidence for functional similarity between *Drosophila* and human NGLY1 rationalized further usage of the fly model to study NGLY1.

Regulation of animal development and physiology by NGLY1: insights from phenotypic analysis in Drosophila

As mentioned above, $Pngl^{ex14/ex14}$ and $Pngl^{ex18/ex18}$ third instar larvae had short gastric caeca in their midgut and exhibited loss of acid zone and food accumulation. These phenotypes were rescued by restoring *Pngl* expression (*7*). To determine the molecular mechanisms by which *Drosophila* NGLY1 regulates animal development, these phenotypes were further analysed as described below.

Regulation of BMP/Dpp retrotranslocation and signalling by NGLY1 BMPs are member of the transforming growth factor beta (TGFβ) superfamily of signalling proteins and play critical roles during animal embryonic development and adult maintenance (*28*). BMP ligands can bind their receptors as homodimers and also as heterodimers, with heterodimers generally showing stronger signalling activity compared to homodimers (*29–32*). *Drosophila* genome encodes three BMP ligands, one of which called decapentaplegic (Dpp) plays key roles during midgut development (*33*). During BMP maturation the newly synthesized inactive precursor proteins form dimers in the ER and then undergo one or more cleavages along the secretory pathway to form active BMP dimers, which bind to BMP receptors on neighbouring cells to activate signalling (*30*). Most if not all BMP and TGFβ ligands are predicted (and in some cases shown) to be *N*-glycosylated, and *N*-glycans affect various aspects of BMP/TGFβ function, including receptor binding strength, processing and the balance between heterodimer versus homodimer formation (*34–36*). However, whether BMP proteins are subjected to ERAD and whether deglycosylation regulates BMP signalling was not known.

During *Drosophila* development, gastric caeca and acid zone formation is regulated by BMP/Dpp signalling from visceral mesoderm to midgut endoderm through phosphorylated Mothers against dpp (pMad) (*37*). The phenotypic similarity between loss of *Pngl* and the midgut-specific loss of *dpp* (*33*) prompted our group to examine the effects of loss of Pngl on BMP/Dpp signalling. We found that Pngl is primarily required in the mesoderm to ensure proper midgut development and that its enzymatic activity is required

to promote BMP signalling in the visceral mesoderm (*7*). Loss of *Pngl* results in a severe decrease in the level of Dpp homodimers in *Drosophila* larvae, leading to the conclusion that deglycosylation is required for the formation and/or stability of Dpp homodimer (*7*). More recently, we showed a role for NGLY1 in BMP4/Dpp retrotranslocation and signalling and identified BMP4/Dpp as a biologically relevant, direct target of NGLY1 in both flies and mammals (*21*). Upon accumulation of misfolded BMP4 in the ER, some NGLY1 molecules are recruited to the cytosolic side of the ER via NGLY1's interaction with valosin containing protein (VCP) (Fig. 1a, bottom). Our data suggest that removing misfolded BMP4/Dpp molecules from the ER via NGLY1-mediated retrotranslocation allows properly folded BMP4/Dpp molecules in the ER to form productive dimers required for signalling (*6*).

Interestingly, NGLY1 appeared to have different regulatory roles for BMP4/Dpp versus the previously established direct target NFE2L1. First, NFE2L1 can be deglycosylated by free cytoplasmic NGLY1 (Fig. 1A, top), while our data suggest that BMP4/Dpp can only be deglycosylated by the ER-recruited NGLY1 (Fig. 1a, bottom). Second, NFE2L1's retrotranslocation does not seem to depend on NGLY1. However, NGLY1-dependent deglycosylation is required for retrotranslocation of BMP4/Dpp. Lastly, deglycosylation of NFE2L1 by NGLY1 leads to its activation, while deglycosylation of misfolded BMP4/Dpp seems to promote their proteasomal degradation. Upon removal of an *N*-glycan by NGLY1, the asparagine (N) harbouring the glycan is changed into aspartic acid (D) (*5*). Importantly, the Ruvkun lab showed that the N-to-D sequence editing of NFE2L1 *N*-glycosylation sites by NGLY1 is essential for its activation in worms (*10*). In contrast, our data indicate that deglycosylation of misfolded BMP4/Dpp proteins by NGLY1 is sufficient for proper BMP4/Dpp signalling and N-to-D sequence editing is not required for this process (*21*). Misregulated BMP signalling is associated with human pathologies in different organs including gastrointestinal, musculoskeletal and nervous systems (*28*). Since NGLY1 deficiency is a multisystem human disorder (*1*, *12*, *16*), tissue-specific impairment of BMP signalling may account for some of the NGLY1 deficiency phenotypes. This is further supported by the observed BMP signalling dysregulation in the fourth ventricle choroid plexus and the heart in *Ngly1* mutant mouse embryos (*21*) and warrants further studies on potential contribution of impaired BMP signalling to NGLY1 patient phenotypes.

Regulation of AMPKα *expression and AMPK signalling by NGLY1* Loss of Dpp signalling in the midgut does not explain the food accumulation phenotype in *Pngl* mutants and only partially accounts for their lethality (20–30%) (*7*). Following up on the food accumulation phenotype in *Pngl*-mutant larvae, a recent study from our lab reported that this phenotype is associated with impaired gut peristalsis caused by reduced mesodermal expression of *AMP-activated protein kinase α subunit* (*AMPKα*) (*21*). *Pngl* mutants showed altered mitochondrial morphology (abnormal cristae structure) along with decreased ATP and increased reactive oxygen species (ROS) levels in their midgut. These phenotypes are in agreement with the previous reports showing altered mitochondrial function in NGLY1-deficient *C.elegans* and MEFs, and NGLY1 deficiency patient fibroblasts and muscle (*18*, *38*, *39*). Restoration of mesodermal *AMPKα* levels was shown to improve the mitochondrial morphology in the visceral muscle, restore ATP levels and reduce ROS levels in the *Pngl* mutant midgut. Similarly, *Ngly1* mutant MEFs and fibroblasts from NGLY1 deficiency patients showed reduced *AMPKα1* and *AMPKα2* expression. Moreover, pharmacological enhancement of AMPK signalling improved the impaired energy homeostasis in both *Ngly1* mutant MEFs and NGLY1 patient fibroblasts (*21*). Together, these observations suggest that regulation of *AMPKα* level is an evolutionarily conserved function of NGLY1 and that boosting AMPK signalling can ameliorate the mitochondrial energy metabolism defects in several NGLY1 deficient contexts.

The molecular mechanism through which NGLY1 regulates AMPKα levels remains to be addressed. As a possible mechanism, the NGLY1-NFE2L1 axis was examined. Loss of NGLY1 impairs NFE2L1-mediated proteasome bounce back response. NFE2L2, a paralog of NFE2L1, is known to regulate antioxidant response genes (*40*) and its pharmacological activation by sulforaphane has been shown to compensate for the loss of NFE2L1-mediated gene expression in NGLY1-deficient MEFs (*39*). Similar to *Ngly1* mutant MEFs, *Pngl* mutant flies showed reduced expression of proteasome genes, which was rescued by sulforaphane treatment. However, sulforaphane treatment did not rescue *AMPKα* levels in *Pngl* mutants and *Ngly1*$^{-/-}$ MEFs (*21*). These data, along with other experiments on MEFs, indicated that the reduction in *AMPKα* observed in NGLY1-deficient contexts is independent of loss of NFE2L1 activity or impaired proteasome function. Earlier, *Ngly1* mutant MEFs have been reported to accumulate cytosolic aggregates of an ERAD substrate having *N*-linked *N*-acetylglucosamine monosaccharides (*N*-GlcNAc) (*3*). Accumulation of these *N*-GlcNAc-bearing aggregates can possibly interfere with the function of *O*-GlcNAc, which is the major type of glycan decorating nucleocytoplasmic proteins and regulates a range of cellular processes including transcription (*41*). Therefore, the reduced *AMPKα* level in NGLY1-deficient contexts might be indirectly mediated through impaired *O*-GlcNAc function. Involvement of NGLY1 in the quality control of an *N*-glycosylated cell surface receptor upstream of *AMPKα* transcription also remains a possibility.

Insights into NGLY1 deficiency from transcriptomic and genome-wide association analysis in Drosophila

In addition to studies focussed on characterization of specific *Pngl*-mutant phenotypes, several groups have used systems-level and genome-wide association studies to shed light on *Pngl* biology. In one study, the Chow group performed RNA sequencing (RNAseq)

analysis in *Drosophila* upon *Pngl* knockdown and found upregulation of the genes involved in heat shock response, innate immunity and other types of stress response, along with downregulation of the genes related to oxidation–reduction, proteasome and lipid metabolism (*27*). Downregulation of proteasome subunits and oxidoreductase genes upon *Pngl* loss is consistent with the loss of Cap-n-collar (Cnc), which is the *Drosophila* ortholog of mammalian NFE2L1 and NFE2L2 (*42–44*). Of note, there was no enrichment of the genes associated with ERAD in this RNAseq dataset (27). Further, *Pngl* knockdown did not affect the expression of the Xbp1-GFP ERAD marker and the severity of an ERAD-responsive eye phenotype induced by expression of a misfolded protein (*27*). These data suggest that *Drosophila* Pngl may not be involved in ER stress and ERAD pathway. However, it is possible that the residual *Pngl* remaining in the KD animals masks a role in the ERAD pathway. Moreover, tissue-specific and/or developmental context-specific roles for Pngl in ERAD remain a distinct possibility.

RNAseq analysis in *Pngl* knockdown *Drosophila* also showed upregulation of heat shock proteins (HSPs) including Hsp90, Hsp70 and Hsp20 (*27*). Induction of HSPs through heat shock treatment improved the survival of *Pngl* knockdown animals, while individual knockdown of the HSPs enhanced lethality in *Pngl* knockdown flies. Knockdown of Hsp70Bb led to complete lethality in the *Pngl* knockdown animals (*27*). This suggests a possible role for HSPs (especially Hsp70Bb) in *Pngl* loss-of-function phenotypes. As a molecular chaperone, Hsp70 is involved in cellular housekeeping and stress response including proper folding of nascent proteins, subcellular transport and degradation of misfolded proteins (*45*). Considering the role of HSPs in protein homeostasis, it is possible that in NGLY1 deficient conditions, HSPs can prevent aggregation/accumulation of misfolded glycoproteins and direct them to degradation pathway.

Bi-allelic pathogenic variants in *NGLY1* identified so far exhibit phenotypic variability in NGLY1 deficiency patients without an obvious genotype–phenotype correlation (*1*, *16*), suggesting potential contributions by genetic and/or environmental modifiers. To identify the effects of genetic background on phenotypic variability in NGLY1 deficiency, the Chow group screened a panel of genetically heterogeneous fly strains for their ability to modify the lethality phenotype of *Pngl* knockdown flies (*46*). They discovered a broad range of phenotypic modification across the panel, from fully viable to fully lethal. Genome-wide association analysis on this panel helped the investigators identify a *Pngl* modifier gene called *Ncc69*, the fly homolog of mammalian NKCC1 and NKCC2. Ncc69/NKCC1 encodes for a member of the SLC12 family of $Na^+/K^+/Cl^-$ transporters. Digestion with PNGase F suggests that NKCC1 is *N*-glycosylated. Moreover, loss of *Ngly1* in MEFs resulted in slower migration of endogenous NKCC1 in western blots compared to NKCC1 from wild-type MEFs, suggesting increased molecular weight due to the retention of *N*-glycans. Importantly, the function of NKCC1 was reduced upon loss of NGLY1 in MEFs (*46*). Since NKCC1 is widely expressed in secretory epithelia (*47*), its impaired function might potentially explain the lack of sweat and tears observed in NGLY1 deficiency patients. Moreover, the Circadian rhythm, one of the enriched categories in the gene set enrichment analysis in this study, was altered upon tissue-specific *Pngl* knockdown in brain pacemaker neurons (*46*). This suggested a function for Pngl in fly sleeping behaviour and a potential mechanistic link to the sleep abnormalities reported in the NGLY1 deficiency patients (*1*, *16*). Altogether, these two studies demonstrate the power of fly genetic and genomic studies in discovering novel downstream pathways and modifiers of NGLY1.

Potential therapeutic leads for NGLY1 deficiency from drug repurposing/pharmacological screenings in Drosophila

Currently, there is no FDA-approved drugs available for NGLY1 deficiency. To find potential therapies, the Perlstein group performed a drug repurposing screen. They used the small body size phenotype observed in the $Pngl^{PL/PL}$ *Drosophila* [(*26*); Fig. 1C] for a high-throughput, whole-organism phenotypic screening at the larval stage. In this screen, they used a library of 2,532 unique compounds, which included ~600 FDA-approved drugs and ~800 natural products. The screen identified a validated hit, 20-hydroxyecdysone (20E), which partially rescued the developmental delay and small body size phenotype in $Pngl^{PL/PL}$ larvae (*26*). 20E elicits metamorphosis in insects including *Drosophila* (*48*). 20E and its precursors are synthesized from dietary cholesterol in the prothoracic gland, which is part of the ring gland and a component of the fly neuroendocrine system. Ring gland-specific expression of human NGLY1 alleviated developmental delay and pupal lethality in *Pngl*-deficient animals. These data suggest that *Drosophila* Pngl functions in the neuroendocrine axis. How does the loss of Pngl impairs the neuroendocrine axis? Pngl regulates Dpp signalling in some contexts (*7*, *49*), and Dpp limits the expression of genes related to ecdysone synthesis (*7*, *49*). Therefore, impaired neuroendocrine axis in *Pngl* mutants might result from reduced Dpp signalling. Alternatively, given the previous reports on the critical role of Cnc in ecdysone synthesis (*50*), the neuroendocrine phenotype of *Pngl* mutants might result from impaired Cnc activation. Regardless of the molecular mechanisms underlying potential defects in steroid synthesis in *Pngl*-mutant larvae, it is tempting to speculate that there might be a connection between this observation and the adrenal insufficiency or adrenal cortex vacuolation observed in several NGLY1 deficiency patients (*51*, *52*), as the adrenal gland secretes steroid hormones as well.

$Pngl^{PL}$ homozygotes show significant developmental delay and are not as healthy as their control

siblings. Moreover, they can only tolerate low doses of dimethyl sulfoxide (DMSO), which was used as the vehicle for the screen. These factors likely limited the ability of the above-mentioned screen in identifying additional validated compounds suppressing the *Pngl* phenotype (*26*). To overcome these limitations, the Perlstein group devised another screening strategy to identify suppressors of the *Pngl* heterozygous animals' sensitivity to proteasomal inhibition (*22*). This drug repurposing screen was based on the observation that $Pngl^{+/PL}$ larvae are much healthier than the $Pngl^{PL/PL}$ larvae but are still twofold more sensitive to the proteasome inhibitor bortezomib (BTZ) than wild-type larvae. Also, $Pngl^{+/PL}$ larvae are much less sensitive than the $Pngl^{PL/PL}$ larvae to DMSO, which allowed the usage of fivefold higher doses of test compounds and a reduction in the false negative rate of the screen (*22*). Another strength of this study was that a parallel screen was performed in *png-1*-mutant worms, followed by cross-validation of the hits between the two invertebrate models and with a human cell line (U2OS osteosarcoma cell line). Three categories of chemicals were identified: NFE2L2 inducers, catecholamine boosters and anti-inflammatory drugs. Rescue of BTZ sensitivity by NFE2L2 activators is in agreement with previous reports showing the loss of NFE2L1-associated proteasomal gene expression in NGLY1-deficient backgrounds and its compensation by NFE2L2 (*21*, *39*). It is worth noting that BTZ also induces mitochondrial toxicity and oxidative stress in some cell types (*53*). Therefore, it is possible that the rescue of BTZ sensitivity by NFE2L2 activators is mediated through NFE2L2-mediated enhancement of the antioxidant response rather than through restoration of the proteasomal gene expression.

Identification of a therapeutic potential for both steroidal and nonsteroidal anti-inflammatory drugs in this screen is in line with a recent report on increased expression of interferon-stimulated genes in *Ngly1* mutant MEFs and mice (*39*). In addition, global gene expression profiling showed upregulation of cytokine response genes (such as IFNB1 and IL-29) in melanoma cells upon *NGLY1* knockdown (*54*). Moreover, immune response-related genes were upregulated in *Drosophila* upon *Pngl* knockdown (*27*). To our knowledge, none of the clinical studies have so far examined the status of interferon and cytokine response in these patients. However, some NGLY1 deficiency patients were found to have higher than expected titres of antibodies against rubella and/or rubeola after Measles, Mumps and Rubella vaccination (*1*). Moreover, some affected children seem to be rarely infected by common viral pathogens like common cold (*55*). Therefore, a hyperactive immune response might be a conserved feature of NGLY1 deficiency. Finally, it is worth noting the only compound that ameliorated the NGLY1-deficient phenotypes in both the fly and worm screens and was also validated in U2OS cells was aripiprazole, an atypical antipsychotic medication capable of activating the dopamine receptor (*22*). This observation, along with identification of several other catecholamine-related compounds from the invertebrate screens suggests a possible mechanistic role and/or therapeutic potential for catecholamine boosters in NGLY1 deficiency.

Future Perspectives

Drosophila studies have made important contributions to our knowledge about NGLY1 function and the potential mechanisms underlying the pathophysiology of NGLY1 deficiency. For instance, two important signalling pathways, BMP/Dpp and AMPK signalling, were reported to be impacted upon loss of NGLY1 through studies based on phenotypic analysis in the fly model, followed by work in mammalian models. Moreover, even though the NGLY1-NFE2L1 axis has not been well studied in *Drosophila*, the reduced proteasome gene expression in *Pngl* mutant and knockdown animals and the identification of NFE2L2 induction as a potential therapeutic target to suppress the sensitivity of *Pngl* mutants to a proteasomal inhibitor suggest the conservation of NGLY1-NFE2L1 axis in this model organism. Although restoration of none of these pathways fully rescued the lethality in fly models, their genetic or pharmacological modulation significantly improved the survival of NGLY1 mutant animals, suggesting that multiple downstream events and targets contribute to the overall phenotypes of loss of NGLY1. Lack of complete rescue by these pathways also suggests possible involvement of other pathways yet to be discovered. As studies on *Drosophila* continue to reveal the affected pathways associated with NGLY1 deficiency, it is imperative to expand the efforts to explore their underlying molecular mechanisms and evolutionary conservation in mammals including humans. We hope that these efforts will ultimately facilitate the development of novel therapeutic approaches to NGLY1 deficiency.

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Conflict of Interest

None declared.

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