Defects in the Neuroendocrine Axis Contribute to Global Development Delay in a Drosophila Model of NGLY1 Deficiency

Tamy Portillo Rodriguez, Joshua D. Mast,1 Tom Hartl, Tom Lee, Peter Sand, and Ethan O. Perlstein
Perlara PBC, 6000 Shoreline Court, Suite 204, South San Francisco, California 94080
ORCID IDs: 0000-0002-2246-0580 (J.D.M.); 0000-0002-4734-4391 (E.O.P.)

ABSTRACT N-glycanase 1 (NGLY1) Deficiency is a rare monogenic multi-system disorder first described in 2014. NGLY1 is evolutionarily conserved in model organisms. Here we conducted a natural history study and chemical-modifier screen on the Drosophila melanogaster NGLY1 homolog, Pngl. We generated a new fly model of NGLY1 Deficiency, engineered with a nonsense mutation in Pngl at codon 420 that results in a truncation of the C-terminal carbohydrate-binding PAW domain. Homozygous mutant animals exhibit global development delay, pupal lethality and small body size as adults. We developed a 96-well-plate, image-based, quantitative assay of Drosophila larval size for use in a screen of the 2,560-member Microsource Spectrum compound library of FDA approved drugs, bioactive tool compounds, and natural products. We found that the cholesterol-derived ecdysteroid molting hormone 20-hydroxyecdysone (20E) partially rescued the global developmental delay in mutant homozygotes. Targeted expression of a human NGLY1 transgene to tissues involved in ecdysteroidogenesis, e.g., prothoracic gland, also partially rescues global developmental delay in mutant homozygotes. Finally, the proteasome inhibitor bortezomib is a potent enhancer of global developmental delay in our fly model, evidence of a defective proteasome “bounce-back” response that is also observed in nematode and cellular models of NGLY1 Deficiency. Together, these results demonstrate the therapeutic relevance of a new fly model of NGLY1 Deficiency for drug discovery and gene modifier screens.

KEYWORDS N-glycanase 1 NGLY1 Pngl Drosophila disease model

Rearranged loss-of-function mutations in the evolutionarily conserved gene NGLY1 result in an ultra-rare genetic disease called NGLY1 Deficiency, which is characterized by global developmental delay, seizures, small head and extremities, chronic constipation, lack of tears, and floppy body (Enns et al. 2014). NGLY1, short for N-glycanase 1, encodes a deglycosylating enzyme that hydrolyzes N-linked glycans from asparagine residues of glycoproteins, liberating oligosaccharides for degradation and recycling (Suzuki et al. 2016). A comprehensive clinical snapshot by National Institutes of Health (NIH) established potential measurable clinical endpoints and a baseline of disease progression in a cohort of 12 patients (Lam et al. 2017).

The pathophysiology of NGLY1 Deficiency has not yet been fully resolved. Before 2014, little was known about NGLY1 function in animal development. Its elucidation is the focus of numerous research groups employing a diversity of disease models, including model organisms and human cells. Two models of the pathogenesis of NGLY1 Deficiency have been proposed. The first model is rooted in biochemistry and defects in cellular glycoprotein homeostasis (Huang et al. 2015). NGLY1 is an essential component of the conserved protein quality control system known as endoplasmic-reticulum-associated degradation (ERAD), bridging p97/VCP-mediated retrotranslocation of proteins from the ER to the cytoplasm for bulk deglycosylation and subsequent degradation by the ubiquitin-proteasome system (UPS) (Suzuki 2015). The absence of cytoplasmic N-glycanase activity has been proposed to result in inappropriate cleavage of N-glycans from glycoproteins by the downstream cytoplasmic enzyme endo-β-N-acetylglucosaminidase (ENGase), whose
normal substrate is soluble oligosaccharide liberated by NGLY1. Such glycoproteins misprocessed by ENGase would retain a single GlcNAc residue that may destabilize proteins and promote their aggregation. Suzuki and colleagues observed evidence of N-GlcNAc misprocessing and accumulation in vitro in NGLY1−/− mouse embryonic fibroblasts (Huang et al. 2015; Fujihira et al. 2017). Based on the collective work of Suzuki and colleagues, inhibition of ENGase has been proposed as a therapeutic thesis for NGLY1 Deficiency (Bi et al. 2017; Fujihira et al. 2017). Indeed, a NGLY1−/− ENGase−/− double mutant mouse is viable while a NGLY1−/− single mutant displays varying degrees of lethality depending on genetic background (Fujihira et al. 2017). However, a NGLY1−/− ENGase−/− double mutant mouse is not healthy. Additional pathogenic mechanisms are required to explain NGLY1 Deficiency.

The second model of pathogenesis is rooted in genetics and defects in the deglycosylation of specific glycoprotein clients, including but not limited to the master regulator of the conserved 26S proteasome “bounce-back” response, NFE2L1 (the transcription factor nuclear factor erythroid 2 like 1 also known as Nrf1) (Radhakrishnan et al. 2010). NRF1 belongs to the ancient basic leucine zipper family of transcription factors that regulate many developmental and stress response pathways in animals (Kim et al. 2016). The fly NRF1 homolog, cap-n-collar (cnc) increases the expression of proteasome subunit genes, as well as oxidative and redox stress response pathways (Grinberg et al. 2011). In an unbiased screen for genetic modifiers of the proteasome bounce-back response in nematodes, Lehrbach and Ruvkun discovered that the nematode homolog of NGLY1, PNG-1, deglycosylates the ER-membrane bound isoform of the nematode homolog of NRF1, SKN-1A. They further demonstrated that deglycosylation of SKN-1A by PNG-1 is required for SKN-1A translocation to the nucleus, and transcriptional activity (Lehrbach and Ruvkun 2016).

In a complementary study, Bertozzi and colleagues revealed that NGLY1 activity is required for NRF1 signaling in mouse embryonic fibroblasts mice in the same manner that PNG-1 activity is required for SKN-1A function in nematodes (Tomlin et al. 2017). In fact, they showed that chemical inhibition of NGLY1 function potentiates cytotoxicity caused by proteasome inhibition in human cancer cell lines (Tomlin et al. 2017), which mirrors the observation in nematodes that pNG-1 loss-of-function mutants are extremely hypersensitive to proteasome inhibition by bortezomib (Lehrbach and Ruvkun 2016). Jafar-Nejad and colleagues showed that the fly NGLY1/Pngl is required during embryonic and larval development in Drosophila for post-translational processing of Dpp, the fly homolog of the conserved bone morphogen protein (BMP) family (Galeone et al. 2017), opening up the possibility that NGLY1 is required for the function of multiple glycoprotein clients.

Here we carried out phenotyping, high-throughput assay development and a chemical-modifier screen on a new fly model of NGLY1 Deficiency, herein referred to as Pngl01. Of ~2,560 bioactive compounds, the ecysderoid insect molting hormone 20-hydroxyecdysone (20E) partially suppressed global developmental delay in mutant homozygous. Expression of a human NGLY1 transgene in the prothoracic gland (PG) and sites of ecysderoidogenesis partially rescued global developmental delay in mutant homozygous. These data indicate that defects in ecdysone-producing tissues contribute to the global developmental delay of the Pngl01 flies. Pngl01 flies are also hypersensitive to the proteasome inhibitor bortezomib and the organic solvent dimethyl sulfoxide (DMSO). Together, these observations combined with other results in the literature (Owings et al. 2018) can be accommodated by a model wherein NGLY1/Pngl is required for NRG1/cnc function in the Drosophila neuroendocrine axis.

**METHODS**

**Pgnl allele generation**

A cassette containing a stop codon and mw+ was cloned into a modified version of pUC57. Homology arms to the ngl1 locus were cloned upstream and downstream of the cassette. The guide RNA (GCTGAG-GAATAACCTTGAG CCG) was cloned into pCDF3 (Port et al. 2014). pCDF3 and pUC57 with Pngl homology arms, stop codon, and mw+ were injected into vas-Cas9 (Bloomington stock #51323). Two independent mw+ F1 strains were established and balanced stocks were created. Sequencing confirmed the integration of the stop codon and mw+ (Figure 1A) immediately 3′ to bp 1906699 (Release 5.57) in chromosome 2R (NT_033778.3).

**Rate of eclosion and rescue by human ngl1 transgene studies**

w, Pngl01/CyO, actin-switch-GAL4 males were crossed to Pngl01/CyO, Act-GFP, UAS-human-NGLY1 virgin females. The phenotypes of eclosing flies were recorded on days 9-14 after parents were mated. We found that the actin-switch-gal4 transgenic strain expressed GAL4, despite the absence of RU486.

**Fly strains**

Actin5C-switch-GAL4 (stock #9431) was obtained from the Bloomington stock center. Pngl excision alleles and UAS-human-NGLY1 were obtained from Dr. Hamed Jafar-Nejad. Two independent mw+ PnglPL/CyO, actin-switch-GAL4 flies were obtained from Dr. Kaye Suyama. phm-GAL4 and spok-GAL4 drivers were obtained from Dr. Michael O’Connor.

**Larval size measurements**

0-4 hr old larvae were placed into petri dishes with standard fly food media for 3 days at 25°C. Then, larvae were removed from the food, rinsed in PBS, and placed in PBS to be imaged. The areas of nineteen heterozygous and twenty homozygous larvae were measured in ImageJ.

**Plate preparation for chemical modifier screening**

100nL compound or DMSO (vehicle) was dispensed from mother plates into wells of 96 well daughter plates using the Echo acoustic dispenser (LabCyte). Then, 100nL compound or DMSO (vehicle) was dispensed from mother plates into wells of 96 well daughter plates using the Echo acoustic dispenser (LabCyte). Then, 100nL compound or DMSO (vehicle) was dispensed from mother plates into wells of 96 well daughter plates using the Echo acoustic dispenser (LabCyte). Then, 100nL compound or DMSO (vehicle) was dispensed from mother plates into wells of 96 well daughter plates using the Echo acoustic dispenser (LabCyte).

**Culturing fly larvae in 96-well plates**

Pngl01/CyO, Act-GFP flies were cultured in a large population cage (Genesee) for up to two weeks, where they laid eggs on grape juice agar trays (Genesee) coated with a thin strip of active yeast paste. Egg collections were restricted to ~6 hr during morning hours and were placed into 25°C for ~24 hr. ~0-6hr old 1st instar larvae were rinsed off of the trays with room temperature water and collected in funnel-attached 40 micrometer sieves typically used for cell straining. To remove embryo contamination, the larv...
suspension. Embryos from the mixture's solution can therefore be removed with a 10ml pipette. Embryos were then added to sorting solution (Polyethylene glycol, COPAS 200x GP sheath reagent, Tween20) and drawn into a BioSorter (Union Biometrica) for sorting and dispensing into 96 well compound/media bearing plates. Three GFP negative homozygotes were gated from heterozygotes and dispensed into the 11 left most columns of the plate, and wells G12 and H12. Three GFP positive heterozygotes were dispensed into wells A12, B12, C12, D12, E12, and F12. Plates were then covered with permeable adhesive seals and incubated at 25°C for three days. Approximately 18 plates were dispensed into per day, and larval viability was not affected by their time submerged in dispensing solution (<4 hr).

Preparation of plates for imaging
On the terminal day of the assay (day 3), gas permeable seals were removed and plates were dispensed with 20% sucrose solution made acidic with hydrochloric acid (pH 2) and carrying defoamer (Five Star Defoamer 105-2 oz). Plates were then vortexed and more solution was added to suspend larvae to a fixed focal plane. Plates were then imaged.

Image and data analysis
The Fly Imager uses a Sony a7r ii camera, controlled over USB by the gphoto software to generate full plate images that are then run through a larval detection algorithm. The algorithm first builds an image of what the well would look like when it is empty. To do this it excludes areas near edges (since those are probably larvae) and interpolates across the gaps. It then looks at the difference between the image and estimated empty background, selecting areas with a high difference to be larvae.

20E feeding and effect on developmental timing
20E (Enzo) was dissolved in 100% ethanol and added to molten standard fly food media at 200 μM and added to vials. An equivalent set of vials with an equal amount of ethanol was added for negative controls. 0-6 hr larvae were dispensed with the BioSorter into the vials and incubated at

Figure 1 Genotyping and phenotyping PnglPL allele. A) We used CRISPR to create an allele of Pngl with a stop codon and white + transgene inserted upstream of the PAW domain. Sequencing confirmed the integration of the stop codon and mw+ immediately 3' to bp 1906699 (Release 5.57) in chromosome 2R (NT_033778.3). B) The fraction of PnglPL/+ heterozygote, PnglPL/PL homozygote, or PnglPL/PL;Actin > NGLY1 flies that eclose over time normalized to the total surviving flies for each genotype. Flies were grown on standard untreated media. The ratio of flies emerging in this experiment for PnglPL/+; PnglPL/PL, Act > hNGLY1, and PnglPL/CyO is 15:40:137 individuals. Ubiquitous expression of a human NGLY1 transgene rescued the 2-day development delay to eclosion observed in PnglPL homozygotes. C) PnglPL homozygous adult flies are smaller than homozygous siblings expressing the human NGLY1 transgene. D) PnglPL homozygotes are 25% smaller than their heterozygous siblings (P < 0.01; Student’s t-test).
25°C for 12 days. The number of larvae that pupariated were recorded on key days at 8 AM, 12 PM, and 4 PM, and the number of adults that eclosed were recorded on days 9–12 at a single time.

Rescue of developmental delay with ring and prothoracic gland expression of human NGLY1

Ring gland expression of human NGLY1 was driven using the GAL4/UAS system (Brand and Perrimon 1993) and ring gland drivers 2_286-GAL4, spok-GAL4, and phm-GAL4 in PnglPL/Ex20 compound heterozygotes. Adults were allowed to lay eggs for 8-16 hr, and the genotypes for all eclosing flies were recorded. The number of ring gland rescued flies eclosed was normalized to the number of eclosed sibling homozygotes not carrying the driver.

Bortezomib feeding and effect on developmental timing

Bortezomib (Selleck Chem) was dissolved in DMSO at 100mM stock concentration and added to molten standard fly food media to a set of 35mm petri dishes at testing concentrations while maintaining a final 0.025% DMSO concentration. An equivalent set of 35mm petri dishes with an equal amount of DMSO was added for negative controls. 0-6 hr larvae were dispensed with the biosorter into the 35mm petri dishes and incubated at 25°C for 3 days. The number of larvae that were still alive were recorded, and imaged to quantify the sizes with FIJI software.

Data Availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. All strains are available upon request. Supplemental material available at Figshare: https://doi.org/10.25387/g3.6227189.

RESULTS

Generation of the PnglPL flies and characterization of its phenotypes

We used CRISPR/Cas9 to create a new allele of Pngl with a premature stop codon and a selectable marker inserted upstream of the PAW domain, herein referred to simply as “PnglPL” (Figure 1A). We rationalized that this truncated allele better reflects patient alleles, as no NGLY1 null alleles have been observed in individuals affected by NGLY1 deficiency. We benchmarked PnglPL against the previously described Pngl genetic null mutant generated by P-element excision (Pngllox20), which causes developmental defects and semi-lethality with few adult escapers (Funakoshi et al. 2010). PnglPL homozygotes are delayed in the larval-to-pupal transition by one day, and delayed to eclosion by 2 days when grown on standard media (Figure 1B). As three-day-old larvae, PnglPL homozygotes are ~75% the size of their heterozygote siblings (P < 1X10^-10, Student’s t-test) (Figure 1D).

Consistent with semi-lethality observed in other Pngl mutants, PnglPL homozygote mutants survive to pupation, but only 32% of flies emerge as adults when reared at 25°C (Funakoshi et al. 2010, Galeone

Figure 2 PnglPL homozygotes are hypersensitive to dimethyl sulfoxide (DMSO). A) An image of PnglPL homozygous larvae grown in 96-well plate format on food dosed with different concentrations (0–0.4%) of DMSO. PnglPL homozygous larvae are hypersensitive to DMSO, a decrease in larval size is observed starting at 0.025% DMSO and larval size is decrease as the dose of DMSO is increased. The time to pupation of B) Pngllox20 (n = 3 replicates, 20-30 individuals/replicate) or C) PnglPL (n = 5 replicates, 20-30 individuals/replicate) homozygous larvae reared on food treated with DMSO. Homozygous mutant larvae exhibit hypersensitivity to DMSO showing a delayed time to pupariate and increased lethality.
et al. 2017). We also found the degree of this lethality in Pngl<sup>PL</sup> homozygote mutants was temperature dependent. Specifically, the pupal lethal phenotype was cold-sensitive, and at temperatures at or below 21° no adults emerged. Pngl<sup>PL</sup> homozygotes that survive to adulthood are sterile, which is also consistent with the effects of previously characterized alleles, Pngl<sup>b30</sup> and Pngl<sup>b14</sup> (Funakoshi et al. 2010, Galeone et al. 2017).

Time to eclosion in Pngl<sup>PL</sup> homozygotes is completely rescued by ubiquitous expression of a human NGLY1 (hNGLY1) transgene (Figure 1B). The small body size phenotype of Pngl<sup>PL</sup> homozygote adults is also rescued by ubiquitous expression of hNGLY1 (Figure 1C). These results when flies are reared in standard vials suggested phenotypes suitable for high-throughput, whole-organism phenotypic screening at each stage of fly development from 1<sup>st</sup> instar larvae onwards.

We decided to optimize a high-throughput larval size assay in 96-well plates for several reasons. Assay miniaturization from 30mL vials to 96-well plates involves reducing the number of animals tested by a factor of 10, e.g., 30 animals per vial vs. 3 animals per well. The larval size difference between Pngl<sup>PL</sup> heterozygote larvae vs. Pngl<sup>PL</sup> homozygote larvae was more significant in 96-well plates than the timing to pupation difference or the timing to eclosion difference. A 3-day assay vs. a 7-11 day assay allowed for faster optimization cycle times. Time to pupation and time to eclosion would be secondary assays in vial format to ensure primary screening hits rescue global developmental delay, not just developmental delay in larvae.

As a prelude to drug screening, we established the tolerability of Pngl<sup>PL</sup> homozygote larvae to dimethyl sulfoxide (DMSO), the organic solvent for almost every compound library, including the Microsource Spectrum collection. The maximum tolerated dose of DMSO, therefore, sets a ceiling on the final assay screening concentration. Unexpectedly, we observed that Pngl<sup>PL</sup> homozygotes are extremely hypersensitive to DMSO compared to the heterozygote control (Figure 2). We estimated a maximum tolerated dose in the Pngl<sup>PL</sup> homozygote of 0.1% v/v DMSO (14mM) (Figure 2A, C). Surprisingly, larvae homozygous for Pngl<sup>PL</sup> are several fold more sensitive than those homozygous for the null allele, Pngl<sup>b30</sup> (Figure 2B). The Pngl<sup>PL</sup> truncates the protein before the PAW domain, which binds mannose (Suzuki et al. 2016), but leaves the catalytic domain intact. This suggests to us that at least some of the DMSO sensitivity we observe is not simply due loss of Pngl activity.

In comparison, wild-type Drosophila exhibit adult lethality starting at 1% v/v DMSO, larval lethality ranging from 2% v/v to 3% v/v DMSO, and a “no observed adverse effect level” (NOAEL) dose of 0.3% v/v DMSO (Figure S1; Nazir 2003). DMSO could be acting as a general stressor, or possibly inducing oxidoreductive stress, specifically. For the purposes of drug screening, we exploited DMSO as a sensitizer in the larval size assay. Exposure of mutant larvae to 0.1% v/v DMSO, which would be at 0.3% v/v DMSO in standard vials, would allow us to take advantage of existing lab automation for managing multi-well plates in high-throughput screening (HTS) applications, including a whole-organism sorter and dispenser. Our method includes steps to dissolve and dilute pre-existing fly food so that larvae can be floated to a fixed focal plane and then imaged with custom plate imager. Software was written to measure the overall area of floated larvae to enable the identification of small molecules that significantly increase the size of Pngl<sup>PL</sup> homozygote larvae vs. vehicle-fed larvae.

We selected the Microsource Spectrum collection for a pilot screen. The library contains 2,532 unique compounds including ~600 FDA approved drugs, ~800 compounds that have reached clinical trial stages in the USA, ~400 drugs that have been marketed in Europe or Japan but not the USA, ~600 bioactive tool compounds, and ~800 natural products. Three larvae per well were cultured in 96-well plates with control wells comprising the two outermost columns. We used Pngl<sup>PL</sup> homozygous larvae fed vehicle (0.1% v/v DMSO) as a negative control. We include two positive control groups: the first group consist of Pngl<sup>PL</sup> heterozygous larvae fed vehicle, and the second group consists of Pngl<sup>PL</sup> homozygous larvae cultured without DMSO. The remaining 80 wells contained Pngl<sup>PL</sup> homozygous larvae fed a unique library compound at 10µM plus 0.1% v/v DMSO. An image of a representative drug screening plate with zoomed-in reference wells is shown in Figure 3.

We performed the screen in triplicate, meaning three independent biological replicates. There was statistically significant separation between positive and negative controls (Figure 4A). On average, Pngl<sup>PL</sup> homozygotes were half the size of Pngl<sup>b30</sup> heterozygotes, although some variation in size was observed in each genotype. 75% of all screening plates (73 of 96) had a Z factor > 0; most of the screening
plates with high variability belonged to the second replicate (Figure 4B). A weak positive correlation existed in pairwise comparisons of each biological replicate when all data points are included (Figure 5A). When we only included wells with Z scores less than -2 or greater than 2, the positive correlation increased significantly on average (Figure 5B). For example, in the pairwise comparison of replicate 1 vs. replicate 2, the correlation improves from $R^2 = 0.05977$ in the full dataset to $R^2 = 0.48171$ in the reduced dataset (with positive and negative controls removed as well).

We initially considered 162 pre-hits with a Z score of $>2$ in two of three biological replicates as primary screening positives (Table 1). Over two-thirds of those pre-hits proved to be false positives for one of two of the following reasons. First, uneaten fly food in the well occasionally increased the image background artificially inflating the calculated area of the larvae. Second, because Pngl mutants are hypersensitive to DMSO, any failure in compound dispensing or variability in DMSO levels due to hydration resulted in larger larvae. Forty-five compounds with a Z-score of $>2$ in two of three replicates were considered further because their wells did not have obvious high background or low/no DMSO. The raw images of the wells containing those 45 compounds were manually inspected, and 18 appeared to have larvae larger than the negative controls in the same plate. Ultimately, these 18 unique compounds were found to have a Z of $>2$ in at least two of three replicates. All 18 of the pre-hits from the screen were ordered, dissolved, and retested in attempt to reproduce rescue in vial format with larger numbers of animals (Table 2). One compound, 20-hydroxyecdysone (20E), partially rescued the developmental delay of PnglPL homozygote larvae development to pupae (Figure 6B), but had no effect on developmental timing of PnglPL heterozygote larvae (Figure 6A). Moreover, the suppressive effect of 20E persisted to adulthood, resulting in a statistically significant fourfold increase in eclosion percentage (Figure 6E; $P < 0.01$ Student’s t-test).

As a control to rule out a simple ecdysone biosynthesis defect, we showed that the 20E precursor 7-hydroxycholesterol (7D) failed to rescue PnglPL homozygote larvae development to pupae (Figure 6D). If synthesis of 20E is faulty, it is likely at a point downstream of 7D. We could not reproducibly validate any of the other 17 pre-hits, so we focused our efforts on understanding the mechanism-of-action (MoA) of 20E. Therefore, this pilot screen had an extremely low hit rate of 0.04% (1/2532).

**20E implicates the fly neuroendocrine axis as particularly sensitive to loss of NGLY1/Pngl function**

20E drives metamorphosis in *Drosophila* and arthropods generally (Faunes and Larraín 2016). Dietary cholesterol forms the basis of 20E, and all 20E precursors are synthesized in the prothoracic gland, an organ that comprises part of the larger ring gland. The immediate 20E precursor, ecdysone or “E”, is packaged into secretory vesicles, secreted, and distributed by the hemolymph throughout the animals. E is converted to 20E in these peripheral tissues, and initiates signaling cascades and gene expression inducing physiological, morphological, and behavioral changes with each molt, or developmental transition.

To test whether the 20E insufficiency in Pngl-deficient mutants is autonomous to the ring gland, we expressed a UAS-driven hNGLY1 transgene that can rescue global developmental delay when expressed ubiquitously (Figure 1B, C), in the ring gland with the 2-286-GAL4 driver. To control for off-target mutations due to strain background confounding our results, we tested the trans-heterozygous combination of Pngl alleles, PnglPL/PnglPL, and PnglPL/PnglPLx20. Most PnglPL/PnglPLx20 larvae not expressing the human NGLY1 transgene eclosed on Day 10. In contrast, most
PnglPL/Pnglex20 larvae expressing human NGLY1 in the ring gland pupated on Day 9 (Figure 7A). Aside from the ring gland, 2-286-GAL4 also drives expression in the salivary gland, fat body, and cuticle in larvae (Timmons et al. 1997). We observed a similar rescue effect when human NGLY1 transgene expression is driven by two ring-gland-specific drivers, phantom-GAL4 (phm) (Figure 7B) and spookier-GAL4 (spok) (Figure 7C). phm and spok encode cytochrome P450 monoxygenases in the ecdysone biosynthetic pathway (Rewitz et al. 2007). Driving hNGLY expression in PnglPL homozygotes with either phm-GAL4 or spok-GAL4 rescued the

![Figure 5](image)

**Figure 5** Positive correlation between 3X replicates. A) The three pairwise comparisons of Z-scores show positive correlations indicating that a set of small molecules could modify the small larval size phenotype. B) The positive correlations between replicates is more evident when only plotting Z-scores of < -2 or > 2. C) Larval size is partially rescued when 20E is fed to PnglPL homozygous larvae.

Table 1 Whittling Screen pre-hits to a set of promising compounds to test in validation studies

| Compounds with Z > 2 in 2 of 3 replicates | High background | Low/No DMSO | Without high background or Low/No DMSO | Clear difference from controls found by manual inspection |
|------------------------------------------|----------------|------------|----------------------------------------|-------------------------------------------------------|
| Replicates 1 and 3                       | 44             | 30         | 3                                      | 11                                                   | 5                                                    |
| Replicates 1 and 2                       | 42             | 12         | 5                                      | 25                                                   | 12                                                   |
| Replicates 2 and 3                       | 76             | 64         | 3                                      | 9                                                    | 5                                                    |
| Total                                    | 162            | 106        | 11                                     | 45                                                   | 22                                                   |

Ultimately 22 compounds were found to have a Z of >2 in two of three replicates and upon manual inspection, the wells with those cpds appeared to have larvae larger than the negative controls. Two of those 22 compounds had a Z of >2 in three of three replicates, were compounded twice in this comparison, and thus the final set of consider further were 18 unique compounds.
Table 2 The 18 compounds that showed promise in the screen and were considered pre-hits. These had a Z-score of >2 in 2 of 3 replicates in the primary screen.

| Common Name | Replicate 1 | Replicate 2 | Replicate 3 | Retest, Phase 1* | Retest, Phase 2 *** | Retest, Phase 3 *** | Retest, Phase 4 **** |
|-------------|------------|------------|------------|----------------|-----------------|-----------------|-----------------|
| CRUSTECDYSONE | 3.36      | 2.24      | 3.03      | Small study indicated that 100μM rescued developmental delay to pupation | Trend supported that small size was partially rescued | Trend supported that small size was partially rescued | Positive – conclusive evidence that larval developmental delay and pupal lethality was rescued. |
| TETRACYCLINE HUROCHLORIDE | 2.84      | 1.01      | 2.39      | Z > 2, 3X @50μM | Trend supported that small size was partially rescued | Trend supported that small size was partially rescued | Negative | Negative |
| HARMINE     | 3.37      | 1.92      | 2.41      | Z > 2 in >1 replicate | Trend supported that small size was partially rescued | Trend supported that small size was partially rescued | Negative | Negative |
| SULFLURAMID | 4.45      | 6.22      | -0.01     | Z > 2, 3X @ 12.5μM | Larvae appeared larger than controls, but the measured areas were not. | Negative | Not tested |
| URSOCHOLANIC ACID | 8.61 | 7.51 | 1.85 | 25 and 50μM could not be tested b/c of insolubility | Larvae appeared larger than controls, but the measured areas were not. | Negative | Not tested |
| 5α-ANDROSTANE | 6.46 | -1.10 | 2.44 | Z > 2 in >1 replicate | Negative | Not tested | Not tested |
| IRIGENIN TRIMETHYLETHER | 0.73 | 5.62 | 4.90 | Z > 2 in >1 replicate | Negative | Not tested | Not tested |
| PATULIN     | -0.43    | 2.22      | 2.63      | Z > 2 in >1 replicate | Negative | Not tested | Not tested |
| ANTIMONY POTASSIUM TARTRATE TRIHYDRATE | 2.30 | 0.57 | 2.66 | Z > 2 in >1 replicate | Negative | Not tested | Not tested |
| ALOGLIPTIN BENZOATE | 2.23 | 8.26 | -2.26 | Z > 2 in >1 replicate | Negative | Not tested | Not tested |
| CEFEPIME HYDROCHLORIDE | 3.25 | -0.12 | 3.85 | Z > 2, 2X, @50μM | Negative | Not tested | Not tested |
| PALMIDROL (5mM) | 3.95 | 1.01 | 5.46 | Larvae appeared larger than controls, but the measured areas were not. | Negative | Not tested | Not tested |
| THIOGUANINE, TIOGUANINE** | 3.51 | -0.91 | 0.80 | Z not >2 in >1 replicate | Not tested | Not tested | Not tested |
| THIOGUANINE, TIOGUANINE | 0.91 | -0.02 | 4.97 | Z not >2 in >1 replicate | Not tested | Not tested | Not tested |
| CAPTAN      | 4.11      | -3.41     | 3.36      | Z not >2 in >1 replicate | Not tested | Not tested | Not tested |
| AGARIC ACID | 3.16      | 1.90      | 4.05      | Z not >2 in >1 replicate | Not tested | Not tested | Not tested |
| ZONISAMIDE  | 0.42      | 3.19      | 2.55      | Z not >2 in >1 replicate | Not tested | Not tested | Not tested |
| BROXALDINE  | 2.05      | -1.05     | 6.62      | Z not >2 in >1 replicate | Not tested | Not tested | Not tested |
| PICEID      | 2.66      | 3.04      | 2.06      | Not tested | Not tested | Not tested | Not tested |

*Phase 1 was 3 or 6 reps of a dose response (3.125, 6.25, 12.5, 25, and 50μM) in the primary screen/96 well format and/or tests for rescue of developmental delay (CRUSTECDYSONE and PALMIDROL).

**The drug library had thioguanine in 2 different wells. ***Phase 2 and 3 tested whether promising pre-hits could rescue larval size at 10μM or 50μM in petri-dish experiments. ****Phase 4 tested whether promising pre-hits could rescue larval developmental delay and pupal lethality at 22.6°C.
pupal lethality phenotype (11.4% and 9.8% eclosed, respectively) compared to sibling controls (6.5% and 6.4% eclipsed, respectively). Together, these data suggest that NGLY1/Pngl is necessary for normal function of the ring gland to enable proper levels of 20E to circulate throughout the developing animal and drive developmental transitions.

**Figure 6** 20-hydroxyecdysone (20E), but not an earlier ecdysone pathway precursor (7-dehydrocholesterol), partially rescues developmental delay and lethality in PnglF<sup>−/−</sup> mutants. The time to pupation of A) Pngl<sup>FL/+</sup> heterozygous or B) Pngl<sup>FL</sup> homozygous larvae reared on food treated with different concentration of 20E (n = 10 replicates, 20-30 individuals/replicate). 20E did not impact developmental rate of heterozygous Pngl<sup>FL</sup> larvae to pupation, but partially rescued development delay of homozygous Pngl<sup>FL</sup> larvae to pupation at 200 μM. The time to pupation of C) Pngl<sup>FL/+</sup> heterozygous or D) Pngl<sup>FL</sup> homozygous larvae reared on food treated with different concentration of 7-d (n = 3 replicates, 25-30 individuals/replicate). 7-d did not impact developmental rate of heterozygous or homozygous Pngl<sup>FL</sup> larvae to pupation. E) The fraction of animals surviving to eclosion. 20E, but not 7-d, rescued larval lethality of Pngl<sup>FL</sup> homozygous at 200 μM. (n = 10 replicates, 20-30 individuals/replicate; n = 3 replicates, 25-30 individuals/replicate, respectively).
NGLY1/Pngl deficient flies are hypersensitive to proteasome inhibition

As mentioned above, loss of NGLY1 sensitizes nematodes and human cancer cell lines to proteasome inhibition. We predicted that Pngl\textsuperscript{PL} homozygote mutants would exhibit hypersensitivity to bortezomib. Indeed, bortezomib caused 100% lethality of Pngl\textsuperscript{PL} homozygous larvae at 5μM, while lethality was not observed in Pngl\textsuperscript{PL/+} heterozygous larvae until a dose of 25μM (Figure 8). In addition, the size of Pngl\textsuperscript{PL} homozygous mutants was reduced by <50% when treated with 1μM bortezomib, and to ~50% Pngl\textsuperscript{PL/+} heterozygous larvae at ~10μM bortezomib. These data indicate that the half-maximal inhibitory concentration (IC\textsubscript{50}) of bortezomib to reduce larval growth is ~10X less in Pngl\textsuperscript{PL} homozygote.

Treatment of NGLY1/Pngl deficient flies with 20E partially rescues the effect of bortezomib

Given that both 20E and bortezomib can modify the phenotypes associated with mutations in Pngl, we decided to test if treatment with 20E could rescue some of the effect of bortezomib. Pngl\textsuperscript{PL} homozygote mutant larvae grown on food treated with both bortezomib and 20E were significantly larger than those grown on food treated with bortezomib alone (Figure 8C).

DISCUSSION

We successfully generated a new Drosophila model of NGLY1 Deficiency, optimized a high-throughput whole-animal phenotypic assay of larval size, and then performed a proof-of-concept drug repurposing screen. While 20E itself should not be considered a drug candidate for NGLY1 Deficiency in humans, the fact that it is a chemical suppressor implicates the neuroendocrine axis in the pathophysiology of NGLY1 deficiency in flies. In other words, even though 20E is an insect-specific developmental hormone, the neuroendocrine axis and steroid-derived developmental hormones are conserved in mammals and may play a role in NGLY1 Deficiency in humans. Collectively, our findings – most strikingly, hypersensitivity of the Pngl\textsuperscript{PL} mutant both to bortezomib and to DMSO – align with results observed in nematodes (Lehrbach and Ruvkun 2016) and human cells (Tomlin et al. 2017) that point to the essential and conserved role of NGLY1 in regulating the function of glycoprotein clients, specifically NRF1 and the proteasome backresponse.

In fact, we can already propose a mechanism to link NRF1 function to ecdysteroidogenesis and the neuroendocrine axis in flies. The fly homolog of NRF1 is the longest isoform of cnc, Cnc\textsubscript{C}, which contains a conserved N-terminal leucine rich transmembrane region targeting Cnc\textsubscript{C} to the ER (Grimberg et al. 2011). Specific loss of Cnc\textsubscript{C} in the prothoracic gland reduces the expression of edcsyne biosynthetic genes and results in delayed timing to pupation (Deng and Kerppola 2013). RNA interference of the Colorado potato beetle homolog of Cnc\textsubscript{C} also reduced ecdysteroidogenesis pathway gene expression and delayed timing pupation, which could be rescued by 20E supplementation (Sun et al. 2017). We would predict then, that as the functional equivalent of SKN-1A in nematodes and NRF1 in mammals, Cnc\textsubscript{C} might also be a substrate for deglycosylation by Pngl in flies. A second testable prediction is that the fly homolog of nematode DDI1 and human DDI2, rings lost (ringo), acts downstream of Pngl to proteolyze Cnc\textsubscript{C}, generating a mature, nuclear-active species.

There are other potential explanations for how 20E might partially rescue global developmental delay in the Pngl\textsuperscript{PL} mutant that do not directly involve NRF1/Cnc\textsubscript{C}, or that may contribute alongside loss of NRF1/Cnc\textsubscript{C} activity. For example, Decapentaplegic (Dpp) signaling is impaired in the ventral mesoderm of Pngl mutant larvae, leading to developmental defects that contribute to the lethality observed in these mutants (Galeone et al. 2017). Aside from local signaling, Dpp also regulates developmental timing. Dpp acts in the prothoracic gland to suppress edcsyne release by repressing the expression of genes required for ecdysteroidogenesis (Setiawan et al. 2017). It is possible defects in Dpp signaling in the prothoracic gland of Pngl mutants could cause the misregulation of edcsyne production.

Alternatively, Pngl\textsuperscript{PL} mutants may not package edcsyne into secretory vesicles properly, or may be defective in secreting edcsyne.

Figure 7 Pngl is necessary for normal function of the ring gland. The fraction of Pngl\textsuperscript{PL} / Pngl\textsuperscript{E20} compound heterozygotes eclosed with human NGLY1 driven by the ring gland driver (blue) A) 2_286-GAL4 B) spookier-GAL4, or C) phantom-GAL4 compared to sibling controls lacking a driver (red). The reported values are normalized to the total number of eclosed Pngl\textsuperscript{PL} / Pngl\textsuperscript{E20} compound heterozygotes for each experiment. A) Compound heterozygous Pngl\textsuperscript{PL} / Pngl\textsuperscript{E20} larvae expressing 2_286 > NGLY1 eclosed earlier and had lower lethality than control flies (64 Pngl\textsuperscript{PL} / Pngl\textsuperscript{E20}; 2_286-GAL4 individuals compared to 120 Pngl / CyO siblings; 74 Pngl\textsuperscript{PL} / Pngl\textsuperscript{E20}; 2_286 > hNGLY1 individuals compared to 185 Pngl / CyO siblings). Compound heterozygous Pngl\textsuperscript{PL} / Pngl\textsuperscript{E20} larvae expressing A) spookier > NGLY1 or B) phantom > NGLY1 had lower lethality at eclosion than control flies (23 Pngl\textsuperscript{PL} / Pngl\textsuperscript{E20}; spook-GAL4 individuals compared to 337 Pngl / CyO siblings; 43 Pngl\textsuperscript{PL} / Pngl\textsuperscript{E20}; spook > hNGLY1 individuals compared to 398 Pngl / CyO siblings); and 19 Pngl\textsuperscript{PL} / Pngl\textsuperscript{E20}; phm-GAL4 individuals compared to 235 Pngl / CyO siblings; 63 Pngl\textsuperscript{PL} / Pngl\textsuperscript{E20}; phm > hNGLY1 individuals compared to 459 Pngl / CyO siblings, respectively).
Second, signal transduction mediated by the interaction between 20E and the ecdysone receptor (EcR) might not be fully operational, and so extra 20E boosts this flawed signaling. Third, ecdysone secretion by the ring gland is induced by two neurons that synapse onto the prothoracic gland and secrete the neuropeptide prothoracicotropic hormone, or PTTH. PTTH contacts the receptor tyrosine kinase Torso to initiate signaling leading to ecdysone secretion. Perhaps there is a flaw in PTTH secretion or Torso signaling. Fourth, damage to developing larval tissues, or starvation, may impinge on 20E to fine tune organism development so that a properly proportioned and nourished animal can develop fully to adulthood. The PnglPL mutant may have damaged tissues, for example through protein-aggregate toxicity or proteasome stress; or it may have some degree of starvation, for example if the gut cannot attain nutrients properly. 20E feeding may bypass delays induced by this hypothetical tissue damage/starvation.

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