Gut barrier defects, increased intestinal innate immune response, and enhanced lipid catabolism drive lethality in N-glycanase 1 deficient *Drosophila*

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Abstract

Intestinal barrier dysfunction leads to inflammation and associated metabolic changes. However, the relative impact of infectious versus non-infectious mechanisms on animal health in the context of barrier dysfunction is not well understood. Here, we establish that loss of Drosophila N-glycanase 1 (Pngl) leads to gut barrier defects, which cause starvation and increased JNK activity. These defects result in Foxo overactivation, which induces a hyperactive innate immune response and lipid catabolism, thereby contributing to lethality associated with loss of Pngl. Notably, germ-free rearing of Pngl mutants did not rescue lethality. In contrast, raising Pngl mutants on isocaloric, fat-rich diets improved animal survival in a dosage-dependent manner. Our data indicate that Pngl functions in Drosophila larvae to establish the gut barrier, and that the immune and metabolic consequences of loss of Pngl are primarily mediated through non-infectious mechanisms.

Keywords

Innate immune response, lipid metabolism, N-glycanase 1, Gut mucus barrier, NGLY1, deglycosylation, N-glycosylation
Introduction

Intestinal barrier dysfunction allows various pathogens and non-infectious stimuli to induce the innate immune response\(^1\)\(^{-5}\). Although the goal of innate immune response induction is to restore intestinal homeostasis, its hyperactivation can have local and systemic adverse effects and is implicated in the pathogenesis of human diseases including autoimmune and neurodegenerative disorders\(^6\)\(^{-8}\). In addition, hyperactive immune response can be accompanied by profound changes in metabolism including high energy demand and subsequent depletion of the nutrient depot\(^8\),\(^10\). For example, induction of chronic inflammation results in the aggravation of metabolic disorders in mice models of obesity\(^11\). However, the relative contribution of infectious versus non-infectious mechanisms to detrimental consequences caused by gut barrier dysfunction is not well understood.

The gut mucus layer is one of the key components regulating intestinal barrier functions\(^12\). Equivalent to the mammalian gut mucus layer, a peritrophic matrix (PM) is present in the \textit{Drosophila} intestine\(^13\). The PM is composed of highly glycosylated proteins and chitin, and is continuously secreted from a group of specialized cells called peritrophic matrix-forming ring (PR) cells in the proventriculus region at the junction of foregut and midgut\(^13\),\(^14\). There is strong evidence that in addition to chitins and mucin-type O-glycans, PM also contains N-glycoproteins\(^15\),\(^16\). Moreover, lectin-based studies in another insect suggest that N-glycoproteins might control the functional properties of PM like its permeability\(^17\). However, genetic evidence for the contribution of N-glycoproteins to gut barrier function in \textit{Drosophila} is lacking.
The deglycosylating enzyme N-glycanase 1 (NGLY1) removes N-glycan from misfolded proteins and is thought to function in the endoplasmic reticulum-associated degradation (ERAD) pathway\(^{18-20}\). Recessive mutations in human NGLY1 cause a congenital disorder of deglycosylation named NGLY1 deficiency\(^{21-23}\). It is an ultra-rare disorder that leads to global developmental delay and affects multiple organ systems including the nervous system and the gastrointestinal system. Loss of the Drosophila homolog of human NGLY1 (PNGase-like or Pngl) results in semi-lethality, as less than 1% homozygous mutant animals finish the larval and pupal development and eclose as an adult organism\(^{24,25}\). We have previously reported that loss of Pngl in the visceral mesoderm impairs signaling pathways mediated by decapentaplegic (Dpp; homolog of human bone morphogenetic protein 4, BMP4) and adenosine monophosphate-activated protein kinase (AMPK) in the larval intestine, which lead to structural and functional intestinal phenotypes and contribute to the lethality of Pngl\(^{-/-}\) animals\(^{25,26}\). However, impaired BMP and AMPK signaling due to mesodermal loss of Pngl did not fully explain the lethality of Pngl mutants, suggesting critical roles for Pngl in other biological processes and potentially in other cell types.

Here, we report that loss of Pngl leads to increased expression of the innate immune genes in the Drosophila larval intestine and a systemic increase in lipid catabolism, compromising developmental progression and leading to lethality. We find that loss of Pngl causes intracellular accumulation of N-glycoproteins in the PR cells, accompanied by abnormalities in peritrophic matrix and impairment in the gut barrier function. Our data suggest that the gut barrier defects result in increased activation of Foxo in the gut epithelial cells, both via enhanced stress-induced JNK signaling and...
through a systemic starvation response. In addition, we observe that Pngl is required cell-
automonomously in enterocytes and in the fat body to prevent aberrant Foxo activation and
to repress lipid catabolism. Importantly, while germ-free rearing does not rescue the
lethality of Pngl mutants, increasing the lipid content in isocaloric diets improves their
survival to adulthood, suggesting that the mutant animals lack sufficient energy stores to
reach the adult stage. Altogether, our data suggest that Pngl is required to establish the
gut barrier in Drosophila larvae and that the lethality associated with gut barrier defects
in Pngl mutants is primarily caused by non-infectious mechanisms.

Results

Loss of Pngl is associated with upregulation of immune response-related genes in
the midgut

To determine the biological processes that contribute to lethality in Pngl mutants, we
performed transcriptomic analysis using RNA sequencing (RNA-seq) on third instar larval
midguts of Pngl+/– animals and three control strains: y w (Pngl+/-), Pngl+/-, and Pngl+/-;
Pngl Dp/+, which lacks endogenous Pngl function, but harbors one copy of a Pngl
genomic duplication shown to fully rescue the lethality of Pngl+/- animals26. We first
identified the genes differentially expressed between Pngl+/- and each control, as those
showing an absolute fold-change of at least 1.5 and a P-value less than 0.01. To increase
the stringency of our analysis, we focused on those differentially expressed genes that
were overlapping among these three pairwise comparisons: (1) Pngl+/- vs y w, (2) Pngl+/-
vs Pngl+/-, and (3) Pngl+/- vs Pngl+/-; Pngl Dp/+. Using this strategy, we found 453
upregulated and 455 downregulated genes in Pngl+/- larval midguts, when compared to
all controls (Fig. 1a and Supplementary Table 1). Using the Database for Annotation, Visualization and Integrated Discovery (DAVID\textsuperscript{27,28}, we performed functional gene ontology (GO) analysis on the differentially expressed genes and identified various biological processes significantly altered in \textit{Pngl}\textsuperscript{−/−} midguts (Fig. 1b). We found proteasome-mediated processes as the topmost significantly downregulated gene category (Fig. 1b; top panel), in agreement with previous reports on the regulation of proteasomal gene expression by \textit{Pngl} and its homologs\textsuperscript{29-33}.

Interestingly, a number of significantly upregulated gene categories were related to immune response (Fig. 1b; bottom panel). In \textit{Drosophila}, humoral innate immune response relies on the production of different antimicrobial peptides (AMPs), which can be released systemically or can be synthesized locally in tissues, including the intestinal epithelium, in response to allergens and infection\textsuperscript{34,35}. Many of the upregulated genes in the immune response gene categories encode for AMPs and some of them encode for pattern recognition receptor proteins such as peptidoglycan recognition proteins (PGRPs). To confirm the RNA-seq data, we performed qRT-PCR analysis on 13 innate immune response genes from the list and found all of them to be significantly upregulated in \textit{Pngl} mutant midguts (Fig. 1c). Addition of a genomic copy of \textit{Pngl} fully rescued the immune gene expression (Fig. 1c). We conclude that loss of \textit{Pngl} leads to a significant increase in the expression of multiple immune response-related genes in the larval midgut.

Inactivation of NFE2L1 (NFE2 like bZIP transcription factor 1) in NGLY1-deficient \textit{Drosophila} and mouse embryonic fibroblasts (MEFs) leads to impaired proteasomal and mitophagy gene expression\textsuperscript{26,30,32}. Moreover, we and others have shown that treatment
with an NFE2L2 activator called sulforaphane can rescue these transcriptional defects\textsuperscript{26,30,36}. To examine whether NFE2L1 inactivation can explain the innate immune hyperactivation in \textit{Pngl}–/– larval midguts, we grew \textit{Pngl}–/– larvae on food containing sulforaphane. Despite rescue of proteasomal gene expression by sulforaphane, it did not significantly reduce the expression of innate immunity genes in these animals (Supplementary Fig. 1a and 1b). Moreover, we have reported that increasing the gene dosage of \textit{AMPKα} can significantly rescue the lethality of \textit{Pngl}–/– larvae\textsuperscript{26}. However, adding an extra copy of \textit{AMPKα} did not rescue the upregulation of most immune response genes in \textit{Pngl}–/– midguts and only mildly reduced the expression of some (Supplementary Fig. 2a). Together, these observations indicate that NFE2L1 inactivation and \textit{AMPKα} reduction cannot explain the severe increase in the expression of innate immunity genes in \textit{Pngl}–/– larval midguts.

Enhanced innate immune response in midgut contributes to the lethality of \textit{Pngl} mutants

Excessive immune response in the gut can lead to tissue damage and contribute to lethality in adult flies\textsuperscript{37,38}. Accordingly, we hypothesized that hyperactive immune response might contribute to the lethality in \textit{Pngl} mutant larvae. Expression of AMPs and other innate immune genes in \textit{Drosophila} is regulated by two signaling pathways, the Toll pathway and the immune deficiency (IMD) pathway\textsuperscript{37}. Moreover, in the intestinal epithelium, the forkhead transcription factor Foxo can induce the expression of AMPs in response to starvation, energy deprivation and infection\textsuperscript{39,40}. To determine if the increased immune gene expression contributes to the lethality of \textit{Pngl} mutants, we sought to reduce
immune activation in these animals by decreasing the gene dosage of foxo, Rel (encodes Relish, which is the NF-κB transcription factor in IMD pathway) and Tl (encodes the Toll receptor). Loss of one copy of foxo in Pngl–/– larvae resulted in a statistically significant reduction in the midgut expression of all 13 immune response genes examined in our experiments, although in most cases the expression levels did not fully return to control levels (Fig. 2a). Loss of one copy of Rel and Tl also significantly decreased the expression of immune response genes in Pngl mutants (Fig. 2a). Notably, we observed a more robust improvement in immune gene expression upon reducing the gene dosage of foxo compared to Rel and Tl, both in terms of the number of genes whose expression was affected and in terms the level of reduction in gene expression (Fig. 2a). Pngl–/– animals rarely emerge from the pupal case\(^\text{24,25}\). However, reducing the gene dosage of foxo rescued the lethality of Pngl mutants by 40%, while decreasing the gene dosage of Rel and Tl rescued the lethality of Pngl–/– animals by 19% and 21%, respectively (Fig. 2b). Moreover, combined heterozygosity for foxo and Rel or Tl in Pngl–/– animals did not further increase the degree of lethality rescue achieved by reducing foxo gene dosage alone (Fig. 2b). These observations suggest that these genes contribute to Pngl–/– lethality through a common mechanism, likely the induction of innate immune genes. Of note, adding one genomic copy of AMPKα further increased the survival of Pngl–/–; foxo+/– animals (Supplementary Fig. 2b). Together, these observations suggest that foxo-mediated hyperactivation of innate immune genes is a major contributor to the lethality in Pngl mutants and further suggest that the detrimental effects of innate immune hyperactivation is distinct from the adverse effects of reducing AMPKα signaling in these animals.
So far, our gene expression analysis has been performed on whole midguts after removing neuronal and tracheal connections, and our genetic manipulations were performed by using germline mutations. *Drosophila* midgut consists of endoderm-derived epithelium and mesoderm-derived visceral musculature. Therefore, we next performed tissue-specific knockdown experiments to determine the midgut cell type(s) in which hyperactivation of the innate immune response results in *Pngl*–/– lethality. We also included fat bodies in our analysis, given their prominent role in systemic release of AMPs41. To knockdown foxo, *Rel* and *Tl* in the endoderm, mesoderm and fat bodies of *Pngl* mutants, we used *Myo1A-GAL4*, *Mef2-GAL4* and *r4-GAL4* drivers, respectively. Endoderm-specific knockdown of foxo (*Myo1A>foxoRNAi*) resulted in a lethality rescue of ~28% in *Pngl* mutants, while fat body specific knockdown (*r4>foxoRNAi*) and mesoderm specific knockdown (*Mef2>foxoRNAi*) resulted in ~11% and ~5% lethality rescue, respectively (Fig. 2c). Knockdown of *Rel* in endoderm (*Myo1A>RelRNAi*), fat body (*r4>RelRNAi*), and mesoderm (*Mef2>RelRNAi*) showed a lethality rescue of ~8%, 4% and 2%, respectively, in *Pngl* mutants, while knockdown of *Tl* in endoderm (*Myo1A>TlRNAi*) and fat body (*r4>TlRNAi*) showed ~3% and ~4% lethality rescue, respectively (Fig. 2c). Taken together, these data suggest that the detrimental effects of immune hyperactivation in *Pngl* mutants primarily results from Foxo-mediated induction of immune response genes in enterocytes and to some extent in the fat body.

Using knockdown and rescue experiments with *Mef2-GAL4* and *how24B-GAL4* drivers, we previously showed a major requirement for Pngl in the mesoderm during *Drosophila* development25,26. However, knockdown and rescue experiments had suggested that Pngl might not play an important role in the endoderm25,26. Given the
above observations, we revisited the issue of Pngl requirement in endoderm by using the enterocyte-specific driver employed in this study and also examined its requirement in the fat body. Overexpression of Pngl using Myo1A-GAL4 and r4-GAL4 drivers led to ~19% and ~11% rescue of Pngl–/– lethality, respectively (Fig. 2d). These observations indicate that in addition to its role in mesoderm, Pngl is also required in the endoderm and fat body during Drosophila development. Notably, enterocyte-specific overexpression of Pngl in Pngl mutants led to a significant reduction of innate immune gene expression but did not fully rescue this phenotype (Fig. 2e). These data suggest that although the hyperactive immune response in Pngl mutant midguts is primarily due to the loss of Pngl in the enterocytes, other cell types and tissues contribute to the induction of immune gene expression in the midgut as well.

Pngl mutant larvae exhibit overactivation of Foxo in their intestine and fat body

Based on the strong rescue of lethality and immune response gene expression achieved by foxo heterozygosity in Pngl mutants, we examined whether loss of Pngl affects Foxo activation. Foxo is negatively regulated by insulin receptor (InR) signaling42. Activation of insulin signaling results in phosphorylation of the Akt kinase, which then phosphorylates Foxo to promote its nuclear export43. Under conditions of starvation or energy deprivation, decrease in insulin signaling allows the nuclear localization of Foxo and subsequent induction of its target genes44. Therefore, we examined the level, subcellular localization and phosphorylation status of Foxo in Pngl–/– midguts. Immunostaining in the midguts of control and Pngl mutant larvae revealed increased nuclear localization of Foxo in Pngl mutant midguts (Fig. 3a). In agreement with this observation, western blot analysis
showed significantly decreased phospho-Foxo level in Pngl mutant midguts (Fig. 3b). These observations suggest increased Foxo activation in midgut upon loss of Pngl.

We next examined the phosphorylation status of Akt in Pngl mutants. Western blot analysis showed a significant decrease in phospho-Akt levels in Pngl mutants (Fig. 3c), suggesting reduced Akt phosphorylation contributes to overactivation of Foxo in these animals. In support of this notion, we observed a lethality rescue of ~11% and ~17% by enterocyte-specific overexpression of wild-type Akt (AktWT) and constitutively active Akt (AktCA), respectively, in Pngl mutants (Fig. 3d). Furthermore, overexpression of AktCA in enterocytes also led to a significant reduction in innate immune gene expression in Pngl mutants (Fig. 3e). Together, these data suggest that reduced Akt activation in Pngl mutant midguts contributes to Foxo overactivation and subsequently increased immune gene expression and lethality.

As mentioned before, Akt is regulated by insulin signaling, and Foxo is a key transcriptional effector of the insulin signaling pathway43. Moreover, in agreement with a previous report45, we observed a small body size phenotype (based on body weight) in Pngl mutant third instar larvae (Fig. 3f, g). This phenotype, which is a signature of decreased insulin signaling in flies46, was also rescued by decreasing the gene dosage of foxo (Fig. 3f, g). Accordingly, our data suggest that reduced insulin signaling in the midgut might contribute to the lethality of Pngl mutants. To test this notion, we examined the effects of overexpressing insulin ligands or receptor on Pngl−/− phenotypes. The Drosophila insulin-like peptide 6 (Dilp6; official name Ilp6) is primarily expressed in third instar larval fat body and is essential for animal growth47. However, overexpression of dilp6 in the fat body did not rescue the lethality of Pngl mutants (Fig. 3h). Analysis of the
Drosophila Dilps by NetNGlyc - 1.0 server (https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0) indicates that none of the Drosophila Dilps harbor predicted N-glycosylation sites except for Dilp6, which has a predicted N-glycosylation sequon at $^\text{VT87}$. We have recently reported that the enzymatic activity of Pngl and its mammalian homolog is required for deglycosylation of misfolded Dpp/BMP4 ligands and secretion of mature Dpp/BMP4 is some cell types. Therefore, to account for a potential effect of loss of Pngl on the secretion of Dilp6, we also overexpressed Dilp2 in the fat bodies of Pngl mutants. Dilp2 overexpression by r4-GAL4 did not rescue the Pngl–/– lethality (Fig. 3h). Overexpression of dipl2 and dilp6 did not improve the small body size phenotype either (Fig. 3i). Next, we overexpressed wild-type InR (InRWT) and constitutively activate InR (InRCA) in Pngl mutants using Myo1A-GAL4 and r4-GAL4 drivers. We observed ~8% and ~10% rescue of the lethality upon overexpression of InRWT and InRCA in the enterocytes of Pngl mutants, respectively (Fig. 3j). A lower degree of rescue was observed upon InRWT and InRCA overexpression in the fat body (Fig. 3j). Overexpression of InRWT and InRCA in enterocytes and fat body also exhibited a partial rescue of body size in Pngl mutants (Fig. 3k). Moreover, we observed a significant decrease in the expression of InR in the midgut of Pngl mutants (Fig. 3l). These observations suggest that reduced InR signaling in the midgut and potentially fat body contributes to the lethality of Pngl mutants.

Reduced InR signaling can result from starvation. Therefore, we examined whether Pngl–/– larvae show any evidence for starvation. One of the hallmarks of starvation in Drosophila larvae is an increase in lipid catabolism. Importantly, one of the upregulated gene categories in the RNA-seq analysis was related to lipid catabolic...
process (Fig. 1b; bottom panel and Fig. 3m), which was confirmed by qRT-PCR experiments showing a significant increase in the expression of several lipases from this list (Lip3, CG6271, CG8093 and CG6277) (Fig. 3n). These observations suggest that starvation contributes to reduced InR signaling and Foxo activation in Pngl mutant larvae.

**Loss of Pngl is associated with gut barrier defects, which contribute to the lethality in Pngl mutants**

Gut barrier defects can lead to activation of the intestinal innate immune response\(^2,3\). Therefore, we examined whether Pngl\(^{-/-}\) larvae exhibited any gut barrier dysfunction. We fed the control and Pngl-mutant larvae on food containing FITC-labeled high molecular weight (500-kDa) dextran and imaged their midguts to visualize the dextran. In the midgut of control larvae, the FITC signal was restricted to the central parts of the lumen area, but the Pngl\(^{-/-}\) midguts failed to retain the FITC signal in the lumen area and showed strong FITC signal filling the spaces among neighboring gut epithelial cells (Fig. 4a and 4b). Around 55% of the examined Pngl-mutant third instar larvae (but none of the WT) showed gut barrier defect, and providing one genomic copy of Pngl fully rescued the phenotype (Fig. 4c). As shown in Supplementary Fig. 2a, increasing the gene dosage of AMPKa reduced the expression of some innate immunity genes in Pngl\(^{-/-}\) larval midguts. However, adding one copy of AMPKa did not rescue the gut barrier defects in Pngl\(^{-/-}\) larvae (Supplementary Fig. 2c). These observations indicate that Pngl is required for normal gut barrier formation in Drosophila larvae independently of the AMPKa defects previously reported in these animals.
Given the abnormality observed in the gut barrier function of \textit{Pngl}–/– larvae, we sought to examine the integrity of peritrophic matrix (PM) in these larvae. To this end, we marked the PM in \textit{Pngl}–/– and control larvae with Helix pomatia agglutinin (HPA) lectin, which selectively binds to \(\alpha-N\)-acytelygalactosamine residues and is specific for O-glycans\textsuperscript{51,52}, and performed confocal imaging. In control larvae, PM separated a central luminal area from a peripheral “peritrophic” space adjacent to the apical surface of the midgut epithelium (Fig. 4d), an arrangement which is thought to ensure that abrasive food particles and microorganisms pass through the gut without contacting the epithelial cells\textsuperscript{13}. However, although PM can clearly be observed in the midgut of \textit{Pngl}–/– larvae, it is highly disorganized and appears to be collapsed on itself (Fig. 4d). This is accompanied by epithelial irregularities, as evidenced by defects in apical phalloidin staining and detachment of some epithelial cells (Fig. 4d). In addition, analysis of midgut sections by light microscopy showed the lumen area lined with intact PM and a peritrophic space between enterocytes and PM in control animals. In contrast, \textit{Pngl} mutant midguts displayed a dense lumen content (potentially due to the gut clearance defect previously reported in these animals\textsuperscript{26}), disorganized PM, and loss of peritrophic space (Fig. 4e). These observations indicate impaired gut barrier function in \textit{Pngl}–/– larvae and abnormalities in PM.

To determine if loss of \textit{Pngl} in PR cells contributes to the gut barrier defects observed in \textit{Pngl}–/– larvae, we overexpressed \textit{Pngl} in PR cells of \textit{Pngl} mutants and fed the larvae with 500-kDa FITC-dextran. We found that the penetrance of gut barrier phenotype in \textit{Pngl} mutant larvae was reduced from 55\% to \(~20\%\) upon PR-specific overexpression of \textit{Pngl} (Fig. 4f). This was accompanied by a lethality rescue of \(~11\%)
(Fig. 4g). Importantly, foxo knockdown in PR cells did not lead to any rescue of lethality
(Fig. 4g). Together, these data suggest that Pngl is required in PR cells to ensure the
integrity of the PM and gut barrier and to promote the survival of Drosophila larvae.

Loss of NGLY1 and its homologs affects the degradation of some misfolded N-
glycoproteins and lead to their cytosolic accumulation\(^{19,53-55}\). Moreover, we have
previously reported that upon loss of NGLY1, misfolded BMP4 molecules fail to be
deglycosylated and retrotranslocated from the ER to the cytosol, leading to accumulation
of misfolded BMP4 in the ER and a failure in the secretion of properly folded BMP4\(^{49}\). To
assess whether there is an accumulation of N-glycoproteins in the PR cells of Pngl mutant
larvae, we stained the proventricular regions of these animals and control third instar
larvae with two lectins: wheat germ agglutinin (WGA), which recognizes N-
acetylglucosamine in O-glycans, chitins, and N-glycans, and concanavalin A (Con A),
which primarily binds high-mannose N-glycans\(^{15,56}\). Notably, Pngl mutants show an
accumulation of WGA\(^+\) and ConA\(^+\) intracellular puncta in the PR cells, suggesting that
loss of Pngl might affect the trafficking and/or secretion of some N-glycoproteins (Fig. 4h
and 4i). These observations provide a potential mechanism for the PM defects observed
in Pngl\(^{-/-}\) larvae.

**Germ-free rearing does not rescue the gut barrier defect and lethality of Pngl
mutants**

The gut barrier defects and upregulation of multiple microbial recognition peptides in
Pngl\(^{-/-}\) larvae (Fig. 4 and Fig. 1c) suggest that the gut microbiota might contribute to some
of the Pngl\(^{-/-}\) phenotypes. Moreover, altered gut microbiota can precede the intestinal
barrier defects in aging adult *Drosophila*\textsuperscript{57}. To directly examine whether gut microbiota play any roles in *Pngl*-mutant phenotypes, we generated germ-free *Pngl*\textsuperscript{−/−} and control larvae. Germ-free rearing of *Pngl* mutants did not lead to any improvement in their gut barrier phenotype (Fig. 5a), suggesting that the PM defects caused by loss of *Pngl* are not due to altered gut microbiota.

The germ-free *Pngl*\textsuperscript{−/−} larvae showed a significant increase in the percentage of larvae which reach the pupal stage compared to conventionally reared *Pngl*\textsuperscript{−/−} larvae (Fig. 5b), suggesting a role for gut dysbiosis in the developmental delay of *Pngl* mutants. However, germ-free rearing did not lead to any rescue in the lethality of *Pngl* mutants (Fig. 5c). Of note, germ-free rearing of *Pngl*\textsuperscript{−/−}; *foxo*\textsuperscript{+/-} larvae resulted in further improvement in their survival compared to conventionally reared *Pngl*\textsuperscript{−/−}; *foxo*\textsuperscript{+/-} animals (Fig. 5c, also compare to Fig. 2b). Quantitative RT-PCR showed that expression of the genes involved in the recognition of microorganisms is not statistically different between germ-free *Pngl*\textsuperscript{−/−} and germ-free *yw* larval midguts (Fig. 5d). However, the germ-free *Pngl* mutants still exhibit a significant increase in the expression of most of AMPs compared to germ-free control larvae (Fig. 5d). Together, these data suggest that altered gut microbiota is not the major inducer of AMP gene expression in *Pngl* mutants, and that the contribution of gut microbiome to the lethality in *Pngl* mutants is redundant to that of Foxo hyperactivation.

**Gut barrier defects lead to starvation and contribute to Foxo-dependent innate immune gene induction and lethality in *Pngl* mutants**
So far, our data suggest that gut dysbiosis cannot explain the detrimental effects of gut barrier defects on the survival of Pngl\(^{-/-}\) larvae. To gain mechanistic insight into the role of gut barrier defects in the lethality of Pngl\(^{-/-}\) larvae and to separate the effects of gut barrier defect from the cell-autonomous effects of loss of Pngl in the midgut and fat body, we induced gut barrier defects in control (Pngl\(^{+/+}\)) animals by feeding them with polyoxin D (Poly D), a chitin synthase inhibitor that disrupts PM formation in insects\(^{58,59}\). Poly D feeding for 48 hours resulted in a 43%-penetrant gut barrier phenotype, ~19% lethality, and increased expression of immune genes in y w animals (Fig. 6a-c), confirming that this strategy can impair PM formation in Drosophila larvae.

Poly D fed foxo\(^{+/}\) larvae showed a 38%-penetrant gut barrier phenotype, indicating that as expected, loss of one copy of foxo does not affect the ability of Poly D to impair gut barrier integrity (Fig. 6a). However, removing one copy of foxo rescued the lethality caused Poly D feeding and significantly reduced the expression of most immune genes induced by the chemical (Fig. 6b and 6c). Further, western blot analysis revealed a significant reduction in the relative levels of phospho-Foxo in midguts of Poly D-fed larvae, suggesting Foxo activation (Fig. 6d). These data strongly suggest that the detrimental effects of impaired gut barrier on Drosophila larval development primarily result from Foxo-mediated induction of innate immunity in the midgut.

In dextran feeding assays (Fig. 4) and bromophenol blue feeding assays\(^{25}\), loss of Pngl did not appear to affect the amount of food intake in Drosophila larvae. Moreover, quantification of the number of mandibular movements per second suggests a comparable feeding behavior in control and Pngl-mutant larvae (Supplementary Fig. 3). Therefore, the starvation signature observed in these animals is not likely to be due to
reduced feeding. Importantly, the Poly D-fed y w larvae showed a statistically significant increase in the expression of lipases in the midgut, suggesting a starvation condition (Fig. 6e). Together, these observations indicate that gut barrier defects can lead to a starvation-like condition in Drosophila larvae and suggest that Foxo likely operates downstream of this starvation-like phenotype to mediate the lethality associated with gut barrier defects.

Overexpression of InR or Akt in the midgut was less effective in rescuing the Pngl−/− lethality compared to foxo knockdown by using the same driver (compare Fig. 3d and 3j to Fig. 2c). Therefore, in addition to reduced InR signaling, other pathways are likely to contribute to Foxo activation and lethality in Pngl−/− midguts. The Jun-N-terminal Kinase (JNK) pathway is a potential candidate for these effects, as it is activated in epithelial tissues by a variety of extrinsic and intrinsic stressors and can induce the nuclear localization and activation of Foxo. Indeed, feeding Poly D to wild-type larvae led to the activation of JNK signaling in larval midgut, as evidenced by the accumulation of phospho-JNK (pJNK) in their midgut epithelial cells (Fig. 6f). Similarly, Pngl−/− larvae accumulated pJNK in their midgut epithelial cells (Fig. 6g), supporting the notion that gut barrier defects induce JNK signaling in Pngl−/− midguts.

To evaluate the functional significance of JNK activation in Pngl−/− larvae, we overexpressed double-stranded RNA for the Drosophila JNK homolog basket (bsk) in the enterocytes of Pngl−/− larvae. We observed a lethality rescue of 22% upon bsk knockdown in midgut epithelial cells of Pngl−/− larvae (Fig. 6h). This was accompanied by a significant decrease in immune gene expression and a significant decrease in Foxo nuclear localization in midguts (Fig. 6i and 6j). Together, these observations suggest that gut
barrier defects lead to increased JNK signaling in $Pngl^{-/-}$ midgut epithelial cells, which itself contributes to Foxo activation and lethality in these animals.

$Pngl$ mutants exhibit an increase in lipid catabolism and isocaloric dietary lipid supplementation partially rescues the lethality in these animals

Starvation can lead to lipid mobilization$^{50,62}$. In line with the significant increase in the expression of multiple lipase genes in $Pngl^{-/-}$ midguts (Fig. 3l and 3m), we observed a significant reduction in the lipid storage in fat body and midgut of $Pngl$ mutants (Fig. 7a). Triacylglycerol (TAG) is the main energy reservoir in *Drosophila*$^{63}$. In accordance with the dramatic increase in the expression of multiple lipase genes and reduction of fat storage in $Pngl^{-/-}$ tissues, we found a significant decrease in TAG level in hemolymph and midgut of $Pngl$ mutant throughout the third instar larval period (Fig. 7b). This was accompanied by a significant increase in free fatty acid levels in hemolymph of early third instar $Pngl$ mutants (Fig. 7c, 72 hr). By mid-third instar larval stage, free fatty acid levels return to normal (Fig. 7c, 108 hr), potentially suggesting the depletion of energy reserve as the development of $Pngl^{-/-}$ larvae proceeds. Loss of one copy of $foxo$ improved all of these phenotypes (Fig. 7a-c). Together, these data indicate that $Pngl^{-/-}$ larvae experience a significant degree of starvation associated with a Foxo-dependent increase in lipid catabolism.

In the standard fly food used in our experiments, only 2% of the total energy contents is provided by lipids. The depletion of lipid storage in $Pngl^{-/-}$ midgut and fat body prompted us to examine whether increasing the fat content of the food can promote the survival of $Pngl^{-/-}$ larvae. To this end, we used two additional isocaloric food compositions
with different fat contents in our experiments: a “high-protein intermediate-fat diet” (HPIFD) and a “high-fat diet” HFD (Fig. 7d and Supplementary Table 2). On our standard diet (SD), 14.8% of Pngl–/– larvae reached the pupal stage (Fig. 7e). However, when grown on isocaloric HPIFD and HFD, 38.3% and 74.1% of Pngl–/– larvae reached the pupal stage, respectively (Fig. 7e). Further analysis indicated that the developmental delay of Pngl–/– larvae was partially rescued by HPIFD and fully rescued by HFD (Supplementary Fig. 4A). Remarkably, HPIFD and HFD also led to Pngl–/– lethality rescues of 14.5% and ~36%, respectively (Fig. 7f). Immunostaining indicated that HFD significantly reduced the Foxo nuclear localization in midgut and fat body of Pngl mutants (Supplementary Fig. 4B), and partially rescued innate immune gene overexpression in Pngl mutants (Supplementary Fig. 4c). These data underscore the contribution of metabolic abnormalities and energy depletion to the developmental delay and lethality in Pngl mutants.

Discussion

We previously reported that Pngl is required in the midgut visceral mesoderm for the regulation of Dpp and AMP kinase signaling in Drosophila. However, impairment of these two pathways only partially explained the lethality of Pngl–/– animals. Here, we report that Pngl plays critical roles in several other cell types (PR cells, enterocytes, and fat body cells). Loss of Pngl leads to gut barrier defects, causing suppression of InR signaling and activation of JNK signaling in enterocytes. These alterations result in overactivation of Foxo in enterocytes and fat body, which in turn leads to hyperactivation of intestinal innate immune genes and increased lipid catabolism, ultimately causing
lethality (Fig. 7g). Pngl mutants show significant developmental delay, with less than 1% of the animals reaching the end of the pupal stage and eclosing as an adult\textsuperscript{24,25}.

Intriguingly, growing Pngl mutants on an isocaloric high-fat diet fully rescued their developmental delay and allowed 33% of the animals to reach adulthood, strongly suggesting that depletion of energy depot during the larval and pupal development plays a critical role in the lethality of Pngl mutants. We note that a high-fat diet also suppressed Foxo overactivation in the fat body and midgut of Pngl\textsuperscript{-/-} larvae. This observation suggests that by worsening the starvation status, enhanced lipid catabolism engages in a vicious cycle with Foxo overactivation and negatively impacts the survival of Pngl\textsuperscript{-/-} animals.

Our data provide strong evidence that hyperactivation of innate immune genes contributes to lethality in Pngl mutants. First, decreasing the gene dosage of foxo, Rel and Tl, which are the known regulators of innate immune gene expression, partially rescued the lethality in Pngl mutants. In addition, in all of our other genetic and diet-induced rescue experiments, rescue of lethality was accompanied by reduced immune gene expression. It has recently been reported that overexpression of AMPs results in cytotoxicity and cell death in aging Drosophila\textsuperscript{7}. Our data suggest immune hyperactivation can also induce lethality during development and that Pngl prevents immune hyperactivation by helping establish a normal gut barrier.

Although foxo heterozygosity had the biggest rescue effect in Pngl mutants, loss of one copy of Rel and Tl also showed some degree of lethality rescue. Since pathogens are thought to be the main inducers of the Imd and Toll pathways in Drosophila\textsuperscript{64}, we anticipated that gut microbiota contribute to the lethality of Pngl\textsuperscript{-/-} animals. Rather
surprisingly, germ-free rearing of *Pngl* mutants did not rescue lethality at all, even though it dramatically rescued the developmental delay in these animals. How can one reconcile these observations? Since germ-free rearing also removes commensal bacteria, one possibility is that commensal bacteria are important for the gut homeostasis in *Pngl* mutants. Regardless of the reason for this discrepancy, our data indicate that upon gut barrier defect in *Pngl* mutants, infectious insults are not the primary mediators of hyperactive immune response.

Mammalian NGLY1 is shown to depend on its deglycosylation activity to mediate its biological functions\textsuperscript{29,49}. Initially, it was thought that the *Drosophila* Pngl might lack deglycosylation activity\textsuperscript{24}. However, it was later demonstrated that the fly Pngl has a deglycosylation activity comparable to human NGLY1 and that a point mutation in the catalytic domain of the fly Pngl fully abolished its ability to rescue the lethality of *Pngl*-- animals\textsuperscript{25}. About one third of the proteome in animals is thought to be decorated with *N*-glycans\textsuperscript{65}. Therefore, theoretically, thousands of proteins can be potential targets for NGLY1 and its homologs. To date, two animal proteins have been shown to depend on the deglycosylation activity of NGLY1 for their function, SKN-1/NFE2L1 and Dpp/BMP4\textsuperscript{29,31,49}. We have previously shown that Pngl regulates Dpp in the visceral mesoderm, not endoderm\textsuperscript{25}. Moreover, our current data suggest that impaired NFE2L1 activity cannot explain the hyperactivation of innate immune response genes in *Pngl* mutant midguts (Supplementary Fig. 1). Therefore, the direct targets of Pngl mediating the phenotypes studied in this work remain to be identified. Accumulation of ConA\textsuperscript{+} and WGA\textsuperscript{+} puncta in PM-secreting cells in *Pngl*-- larvae suggests that loss of Pngl affects the secretion of some *N*-glycoproteins. Components of the *Drosophila* larval PM have not
been systematically identified. However, studies in other insects indicate that some PM components are $N$-glycoproteins$^{16,17}$. Therefore, the gut barrier defects observed in $Pngl^{-/-}$ larvae might result from a failure in the deglycosylation of key $N$-glycoprotein components of the PM. Human insulin receptors harbors 19 predicted $N$-glycosylation sites, 14 of which have been experimentally verified$^{66}$. Importantly, $Drosophila$ InR is predicted to have 12 $N$-glycosylation sites ($NetNGlyc 1.0$ server prediction). Therefore, while the reduction in InR signaling in $Pngl^{-/-}$ larvae can be explained by starvation and reduced expression of $InR$, it is also plausible that the InR protein itself is a direct target of Pngl and shows abnormal trafficking and/or function upon loss of Pngl-mediated deglycosylation. Further studies are required to identify and characterize the biologically relevant targets of NGLY1/Pngl in the $Drosophila$ larval intestine and fat body.

Human patients with NGLY1 deficiency display an array of symptoms including global developmental delay, lack of tears, and chronic constipation$^{67}$. Some of the NGLY1 deficiency patients were reported to have recurrent, severe respiratory infections, while others were reported to have higher than expected antibody titers against rubella and rubeola after Measles, Mumps and Rubella (MMR) vaccination$^{22}$. Moreover, global gene profiling in $Ngly1$-deficient melanoma cells showed upregulation of cytokines such as interferon $\beta1$ and interleukin 29 (ref. $^{68}$), and $Ngly1^{-/-}$ mouse embryonic fibroblasts exhibit increased expression of the interferon genes$^{30}$. These observations suggest an association between loss of NGLY1 and altered immune response in various contexts. Moreover, altered glycosylation in intestinal epithelial cells is implicated in chronic inflammatory diseases including inflammatory bowel disease$^{69}$. The critical roles uncovered here for $Drosophila$ Pngl in regulating the gut mucus barrier, innate immune
response, and metabolic homeostasis warrant further studies to explore whether loss of
NGLY1 in other systems or alterations in N-glycosylation machinery exert similar immune
and metabolic effects.

Methods

Drosophila strains and culture

Animals were reared at room temperature on standard food containing cornmeal,
molasses, and yeast in all experiments except for those involving high-protein,
intermediate-fat diet (HPIFD) and high-fat diet (HFD). Detailed composition of these diets
is listed in Supplementary Table 2. The following Drosophila strains were used in the
study: (1) y w, (2) foxo^{Δ94}/TM6B, Tb^{1}, (3) Tl/TM3, Sb^{1}, (4) Rel^{E38}, (5) UAS-foxo^{RNAi}, (6)
UAS-Tl^{RNAi}, (7) y^{1} sc^{v} v^{1} sev^{21};UAS-Rel^{RNAi}, (8) Myo1A-GAL4, (9) r4-GAL4, (10) c135-
GAL4, (11) UAS-dipl2, (12) UAS-InR^{WT}, (13) UAS-InR^{A1325D} (constitutively activated UAS-
InR^{CA}), (14) UAS-Akt^{WT}, (15) UAS-Akt^{ΔPH} (constitutively activated UAS-Akt^{CA}), and (16)
UAS-bsk^{RNAi} (Bloomington Drosophila Stock Center); (17) Mef2-GAL4 (ref. 70); (18)
Pngl^{ex14} and (19) UAS-Pngl^{WT} (ref. 24); (20) PBac[Pngl^{M1}]VK31 (Pngl duplication; ref. 26);
and (21) UAS-dipl6 (ref. 71).

Transcriptomic analysis/RNA sequencing

Midgut tissue from third instar larvae was dissected and homogenized in group of 25 in
cold solution of Tri-reagent (Sigma-Aldrich, T9424); the amount of RNA in each sample
was determined by Nanodrop, and RNA quality was analyzed using agarose gel
electrophoresis (1.2%). The samples were prepared on a Beckman FXP using the
Illumina TruSeq stranded mRNA chemistry and sequenced on a NextSeq 500 (Mid Output flowcell) in paired-end mode. Raw paired-end sequencing reads were trimmed using cutadapt v1.12 to remove Illumina adapters and low-quality bases. The processed reads were then aligned to the Drosophila dm6 genome using STAR v2.5.3a. Gene-level counts, based on GTF annotations from Flybase Release 6.20, were tabulated by totaling all reads overlapping the collapsed set of exons for each gene following previously published methods. Genes with a counts per million reads mapped (CPM) of less than 5 in less than two samples were excluded. Differentially expressed genes were identified by using the edgeR glmFit model. The false discovery rate (FDR) was controlled by applying the Benjamini–Hochberg procedure to the p-values. Significantly expressed genes were defined as those exhibiting an absolute fold-change of at least 1.5 and an FDR of less than or equal to 0.01. The raw data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE206229.

**Lethality rescue assay**

Lethality rescue was examined according to previously published report. We scored the number of eclosed progeny (observed). The estimated total number (expected) was calculated based on Mendelian inheritance for each genotype. The observed/expected ratio is presented as lethality rescue percentage.

**Real time quantitative RT-PCR analysis**

Total RNA was extracted from 5 larval midguts with Trizol (Invitrogen) and dissolved in 25 μL of RNase-free water. cDNA was then synthesized from 1 μg total RNA using...
amfiRivert II cDNA Synthesis Master Mix (R5500, GenDEPOT), and qPCR was carried out using amfiSure qGreen Q-PCR Master Mix, Low ROX (Q5601, GenDEPOT). Expression levels were normalized to Actin (endogenous control). Relative gene expression was calculated as fold change using the $2^{-\Delta\Delta Ct}$ method. The oligonucleotides used to assess target genes expression are listed in Supplementary Table 3.

**Dissections, staining, Image acquisition and processing**

Larval midgut and fat body tissues were dissected and fixed in 4% paraformaldehyde. Antibodies were rabbit anti-dFoxo 1:250 (ab195977, abcam) and rabbit anti-SAPK/JNK 1:500 (Cat No. 559309, Sigma-Aldrich). Lectin staining in the proventriculus region was performed using helix pomatia agglutinin (HPA), Alexa Fluor™ 488 conjugate 1:1000 (cat No. L11271, Invitrogen), wheat germ agglutinin (WGA) CF®488A 1:1000 (Cat No. 29022, Biotium), and concanavalin A (ConA) CF®488A 1:1000 (Cat No. 29016, Biotium). Confocal images were acquired using a Leica TCS-SP8 microscope and processed with Amira5.2.2. Image quantifications were performed using ImageJ. All images were processed in Adobe Photoshop CC. Figures were assembled in Adobe Illustrator CC.

**Western blotting**

Protein samples were prepared from larval midguts in lysis buffer containing Halt™ Phosphatase Inhibitor Single-Use Cocktail (Thermo Fisher Cat. No. 78428) and Protease Inhibitor Cocktail (Promega Cat. No. G6521). The following antibodies were used: rabbit anti-pFoxo1 1:1000 (Cat No. 9461, Cell Signaling Technology), rabbit anti-Foxo1 1:1000 (Cat No. 2880, Cell Signaling Technology), rabbit anti-Akt 1:1000 (Cat No. 4691, Cell
Signaling Technology), rabbit anti-pAkt 1:1000 (cat No. 4060, Cell Signaling technology) and mouse anti-actin 1:1000 (DSHB Cat. No. 224236-1). Western blots were developed using Clarify ECL Western Blotting Substrates (BioRad). The bands were detected using an Azure Biosystems c280 digital imager using chemiluminescent detection of HRP. Three independent immunoblots were performed for each experiment.

**Feeding behavior assay**

Larval feeding behavior method was adapted from ref. 73. Third instar larvae were placed in sucrose-agar plates (5% sucrose mixed in 3% agar medium) and allowed to settle for 15 min. Number of mouth-hook contractions per minute were counted. A total number of 30 larvae were scored for each group.

**Generation of germ-free animals**

Germ-free animals were generated by following the previously published method74. Flies were kept for egg laying in grape juice agar plate for 3-4 hr. Eggs were collected and dechorionated with 2.7% sodium hypochlorite for 2-3 minutes. Dechorionated eggs were washed twice in 70% ethanol and thrice in water, and then transferred to sterile fly food containing tetracycline (50 µg/mL). Flies were reared on sterile food vials for three generations. Absence of bacteria in germ-free flies was confirmed by 16S rRNA amplification using the 27F (5'-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’ GGTTACCTTGGTACGT 3’) primers.

**Developmental assay**
For developmental assay, the expected ratio was calculated based on Mendelian inheritance for genotypic classes and the observed/expected ratio is reported as a percentage. Crosses made up of 5 virgin females and 5 males were set in each tube after a period for sexual maturation and housing to obtain the maximum fitness level during the three days of egg deposition in a non-overcrowded environment. Crosses were set in triplicate and flies were transferred to fresh vials every 3 days for three times. The total number of pupae for genotypic classes produced over 20 days in each vial was scored.

**Nile red staining**

Midgut and fat body tissues from third instar larvae were dissected and fixed in 4% paraformaldehyde. Fixation and washing of tissues were followed by the incubation in with 1:2500 dilution of 0.5mg/mL Nile red (Cat No. 19123, Sigma Aldrich) for half an hour. Upon incubation, tissues were rinsed in water and mounted in 80% glycerol followed by image acquisition.

**Triacylglycerol and free fatty acid estimation**

Triacylglycerol and free fatty acid levels were estimated from larval midgut and hemolymph. Midgut samples were prepared by homogenizing ~15 midguts in cold 1X PBST (0.05% tween). Hemolymph was collected from 15 larvae and mixed in cold 1X PBST. Triacylglycerol levels were estimated using manufacturer’s protocol (Sigma Aldrich # MAK266). Free fatty acid levels were estimated using manufacturer’s protocol (Sigma Aldrich # MAK044). Triacylglycerol and free fatty acid levels were normalized with the protein content determined by Bradford assay.
Dextran feeding assay and gut barrier defect quantification

Third instar larvae were fed on semi-solid drops of 500 kDa FITC-labelled dextran (Sigma-Aldrich, St. Louis, MO, USA), diluted to 1 mg/mL in sucrose-agar medium (5% sucrose mixed in 3% agar medium) on a petri dish. Larvae were allowed to feed for 15 minutes. Larvae were washed with cold 1X PBS to remove any excess FITC-dextran on the surface. Midguts were dissected out and fixed in 4% paraformaldehyde followed by imaging under fluorescent microscope. For gut barrier defect quantification, larval guts that failed to retain FITC-signal (green) in their lumen were scored. Pharmacological induction of gut barrier defects was achieved by rearing third instar larvae on food containing 100 µM polyoxin D (Sigma Aldrich # 529313) for 48 hr. Gut barrier defects were examined and quantified using the dextran feeding assay described above.

Statistical analysis

Unpaired Student’s t test, one-way ANOVA and two-way ANOVA with the Dunett’ and Bonferroni Post hoc tests, respectively, were used for statistical analyses. Statistical tests, P values and parameters including the sample sizes and replicates are mentioned in Figure Legends. All statistical analyses were performed using GraphPad Prism 9.

Data Availability

Data are available with in the article and supplementary information. RNAseq data set are available through the Gene Expression Omnibus (GEO) accession number GSE206229.
References


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Authors contribution
A.P., A.G., S.Y.H, T.V. and H.J.-N. designed and conceived the project and interpreted the data. W.F.M., B.S. and L.M.S. performed RNAseq and transcriptomic analysis. A.P., A.G., S.Y.H., G.C. performed the experiments. A.P., A.G., T.V. and H.J.-N. wrote the manuscript. All authors read, edited and approved the manuscript.

Competing Interests
The authors declare no competing interest.
**Immune response gene expression**

- **Antimicrobial peptides (AMPs)**
- **Microbial recognition peptides**

**No. of upregulated genes**

- **Pngl[-/–] vs. y w**
- **Pngl[-/–] vs. Pngl[-/+]
- **Pngl[-/–] vs. Pngl[-/–], Pngl Dp/+**

**No. of downregulated genes**

**Downregulated gene categories**
- Proteasome-mediated ubiquitin-dependent protein catabolic process
- Axon guidance
- Proteasomal ubiquitin-independent protein catabolic process
- Proteasomal protein catabolic process
- Peripheral nervous system development
- Mushroom body development
- Homophilic cell adhesion via plasma membrane adhesion molecules
- Positive regulation of transcription from RNA polymerase II promoter
- Imaginal disc-derived wing morphogenesis
- Regulation of establishment of planar polarity
- Cell-cell adhesion mediated by cadherin
- Regulation of tube length, open tracheal system
- Chitin-based embryonic cuticle biosynthetic process
- Motor neuron axon guidance
- Negative regulation of translation

**Upregulated gene categories**
- Glutathione metabolic process
- Innate immune response
- Response to bacterium
- Mannose metabolic process
- Defense response to Gram-positive bacteria
- Humoral immune response
- Hexose transport
- Antibacterial humoral response
- Defense response
- Sphingosine biosynthetic process
- Cytoplasmic translation
- Carbohydrate metabolic process
- Lipid catabolic process
- Defense response to insect
- Proteolysis

**-log10(adjusted P-value)**

**Fig. 1**
**Figure 1. Loss of Pngl is associated with the upregulation of immune response related genes.** 

**a** Venn diagrams showing the overlap of differentially regulated genes in Pngl<sup>+/−</sup> with comparison to control (y w), Pngl<sup>+/−</sup> and Pngl<sup>−/−</sup>, Pngl<sup>Dp/+</sup> in RNA-seq analysis. Number of both upregulated and downregulated genes shown in the Venn diagram were based on >1.5 fold-change. 

**b** Graph presents DAVID functional GO analysis of biological processes (BP) of downregulated (top), and upregulated (bottom) genes based on their −log<sub>10</sub> of adjusted P-value. Numbers next to the bars show the number of genes differentially expressed in each category. 

**c** Graph showing expression of immune response related genes in the indicated genotypes. Values are expressed as fold changes relative to control (y w). Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 and **P<0.01 using one-way ANOVA.
**Fig. 2**

(a) Graph showing fold change in gene expression for various conditions.

(b) Bar graph showing % Expected ratio (survival) for different combinations of Myo1A-GAL4 and Pngl.[+/–] with survival data for foxo, Rel, Tl, and Pngl.[–/–].

(c) Graph showing % Expected ratio for different GAL4 constructs (Mef2-GAL4, Myo1A-GAL4, r4-GAL4) and their effects on Pngl.[–/–] and Pngl.[+/–] conditions.

(d) Graph showing % Expected ratio for Pngl.[–/–] and Pngl.[+/–] conditions treated with FOXO RNAi, REL RNAi, and TL RNAi.

(e) Graph showing immune response gene expression for Myo1A-GAL4, Pngl.[+/–], Myo1A-GAL4, Pngl.[–/–], and Myo1A-GAL4 > Pngl.[–/–] conditions.
Figure 2. Enhanced innate immune response in midgut contributes to the lethality of *Pngl* mutants. **a** Graph showing immune response genes in the indicated genotypes. Values are expressed as fold changes relative to control (*y w*). Error bars represent SD of three replicates. Significance is ascribed as *P*<0.05 and **P*<0.01 using one-way ANOVA. ns, not significant. **b** Graph showing % lethality rescue (calculated based on the expected Mendelian ratio) in *Pngl* mutants upon removing one copy (1X) of each immune gene activator. n represents the number of animals scored. **c** Graph showing % lethality rescue in *Pngl* mutants upon tissue-specific knockdown of immune activators using mesoderm- (*Mef2-GAL4*), enterocyte- (*Myo1-GAL4*) and fat body-specific (*r4-GAL4*) drivers. n represents the number of animals scored. **d** Graph showing % lethality rescue in *Pngl* mutants upon enterocyte- and fat body-specific overexpression of *Pngl*. n represents the number of animals scored. **e** Graph showing immune gene expression in the indicated genotypes. Error bars represent SD of three replicates. Significance is ascribed as *P*<0.05 and **P*<0.01 using one-way ANOVA. ns, not significant.
Figure 3. Reduced Akt phosphorylation and enhanced Foxo activation in Pngl mutant midguts. **a** Confocal images showing DAPI (blue) and Foxo staining (red) in wild-type and Pngl mutant midgut. Scale bar is 50µm. **b** Western blot images and quantification showing the increased Foxo level and decreased phospho-Foxo level in Pngl mutant midguts. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using unpaired student’s t-test. **c** Western blot images and quantification showing decreased Akt phosphorylation in Pngl mutants. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using unpaired student’s t-test. ns, not significant. **d** Graph showing % lethality rescue upon tissue specific overexpression of wild-type (AktWT) and constitutively activated (AktCA) form of Akt in Pngl mutants. n represents the number of animals scored. **e** Graph showing expression of immune genes in the indicated genotypes. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 and **P<0.01 using one-way ANOVA. **f** Images of larvae and pupae of the indicated genotypes. **g** Graph showing quantification of larval body weight of the indicated genotypes. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using one-way ANOVA. **h** Graph showing % lethality rescue upon tissue-specific overexpression of Drosophila insulin-like peptides (dilp2 and dilp6) in Pngl mutants. n indicate the number of animals scored. **i** Graph showing quantification of body weight of indicated genotypes. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using one-way ANOVA. **j** Graph showing % lethality rescue upon tissue-specific overexpression of wild-type (InRWT) and constitutively activated (InRCA) forms of InR in Pngl mutants. n indicate the number of animals scored. **k** Graph showing larval body weight upon tissue-specific overexpression of InRWT and InRCA in Pngl
mutants (n>20 for each condition). Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using one-way ANOVA.  

Graph showing the expression of \textit{InR} in the indicated genotypes. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using unpaired student’s t-test.  

List of genes in the “Lipid catabolic process” from the larval midgut RNA-seq, along with their fold increase in expression level in \textit{Pngl}⁻/⁻ compared to \textit{y w} control.  

Graph showing the increased expression of lipases in \textit{Pngl} mutant midgut. Error bars represent SD of three replicates. Significance is ascribed as **P<0.01 using unpaired student’s t-test.
Fig. 4
Figure 4. Loss of Pngl is associated with gut barrier defect which contributes to the lethality in Pngl mutants. a Low magnification fluorescent images of wild-type and Pngl mutant midgut upon FITC-labeled dextran (Green) feeding. b Confocal images showing phalloidin (red) and DAPI (blue) staining in wild-type and Pngl mutant midgut upon FITC-labeled dextran (Green) feeding. Scale bar is 50 µm. c Graph showing the quantification of gut barrier defect phenotype in the indicated genotypes (n=60 for each genotype). d Confocal image showing phalloidin (red), DAPI (blue) and HPA-lectin staining (Green) for peritrophic matrix in wild-type and Pngl mutant midgut. Scale bar is 50 µm. e Light microscopic images of cross-section of wild-type and Pngl mutant midguts. Lumen (L), Peritrophic matrix (PM), Peritrophic space (PS), Enterocytes (EC). f Graph showing quantification of gut barrier defect phenotype in the indicated genotypes (n=60 for each genotype. g Graph showing lethality rescue in Pngl mutants upon foxo knockdown and Pngl overexpression in their PM secretory region. n indicates the number of animals scored. h Confocal images showing Phalloidin (red), DAPI (blue) and WGA lectin staining (Green) in the proventriculus region of wild-type and Pngl−/− third instar larvae (n=4 for each genotype). Insets show close-up of PM-secreting cells. Scale bar is 50 µm. i Confocal images showing Phalloidin (red), DAPI (blue) and ConA lectin staining (Green) in the proventriculus region of wild-type and Pngl−/− third instar larvae (n=5 for each genotype). Brackets mark the PR cells. Scale bar is 50 µm.
**Fig. 5**

**a** % animals with gut barrier defects

**b** % animals reached pupal stage

**c** % Expected ratio

**d** Immune response gene expression
Figure 5. Gut microbial abundance does not explain gut barrier defects and lethality in *Pngl* mutants. 

a) Graph showing quantification of gut barrier defect phenotype in germ-free wild-type and *Pngl* mutants (n=60 for each condition).

b) Graph showing the percentage of germ-free and conventionally reared wild-type and *Pngl*−/− animals that reached the pupal stage (n=90 for each condition). Error bars represent SD of three replicates. Significance is ascribed as **P<0.01 using unpaired student’s t-test.

c) Graph showing % lethality rescue in germ-free and conventionally reared *Pngl*−/− and *Pngl*−/−; foxo+/−. n indicates the number of animals scored.

d) Graph showing immune gene expression in germ-free wild-type and *Pngl* mutants. Error bars represent SD of three replicates. Significance is ascribed as **P<0.01 using unpaired student’s t-test. ns, not significant.
Fig. 6
Figure 6. Gut barrier defects induce starvation and Foxo-dependent induction of innate immune genes and lethality. 

a Graph showing the quantification of gut barrier defect in control and polyoxin-D (Poly D) fed wild-type and foxo+/– mutants (n=60 per condition). b Graph showing % adult eclosion in Poly D fed wild-type and foxo+/– mutants (n=60 per condition). Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using unpaired student’s t-test. ns, not significant. c Graph showing immune response gene expression in control and Poly D fed wild-type and foxo+/– mutant midguts. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 and **P<0.01 using two-way ANOVA. ns, not significant. d Western blot images and quantification graph showing increased activation of Foxo in Poly D fed wild-type midguts. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using unpaired student’s t-test. e Graph showing expression of lipases (Lip3 and CG6277) in Poly D fed wild-type midguts. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using unpaired student’s t-test. f Confocal images showing DAPI (blue) and pJNK staining (red) and quantification in control and Poly D fed wild-type midguts. Scale bar is 50 µm. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using unpaired student’s t-test. g Confocal images showing DAPI (blue) and pJNK staining (red) and quantification in wild-type and Pngl mutant midguts. Scale bar is 50 µm. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using unpaired student’s t-test. h Graph showing % lethality rescue upon enterocyte-specific knockdown of Drosophila JNK (bsk) in Pngl mutants. n indicates number of animals scored. i Graph showing innate immune gene expression in the indicated genotypes. Error bars represent SD of three replicates. Significance is ascribed
as *$P<0.05$ and **$P<0.01$ using one-way ANOVA. j Confocal images showing DAPI (blue) and Foxo staining (red) and quantification of Foxo nuclear localization in the indicated genotypes. Scale bar is 50 µm. Error bars represent SD of three replicates. Significance is ascribed as *$P<0.05$ using one-way ANOVA.
Figure 7. Pngl mutant exhibit increased lipid catabolism and supplementation on dietary lipid partially rescues lethality in Pngl mutants. a Images showing Nile red (red) and DAPI (blue) staining in fat body (top) and midgut (bottom) of the indicated genotypes and their quantification. Scale bar is 50 µm. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using one-way ANOVA. b Triacylglycerol (TAG) level in hemolymph and midgut of indicated genotypes. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 and **P<0.01 using one-way ANOVA. c Free fatty acid (FFA) level in the hemolymph of indicated genotypes. Error bars represent SD of three replicates. Significance is ascribed as *P<0.05 using one-way ANOVA. d Pie charts showing % carbohydrates, proteins and fats in different diet compositions. e Graph showing % animals that reach to pupal stage upon feeding on the indicated diets. n indicates the number of animals scored. f Graph showing lethality rescue in Pngl mutants upon feeding on the indicated diets. n indicates the number of animals scored. g Schematic model showing that loss of Pngl results in Foxo overactivation and subsequent innate immune gene expression and lipid catabolism, leading to lethality.