Frameshift mutations of YPEL3 alter sensory circuit function in Drosophila

Jung Hwan Kim1,*, Monika Singh1, Geng Pan2, Adrian Lopez1, Nicholas Zito1, Benjamin Bosse1, Bing Ye2,*

1 Department of Biology, University of Nevada, Reno, Reno, Nevada 89557
2 Life Sciences Institute and Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, MI 48109

* Corresponding authors

Correspondence should be addressed to Jung Hwan Kim, University of Nevada, Reno, 1664 Virginia Street, Mailstop-0314, Reno, NV 89557-0314. Email: jungkim@unr.edu, and to Bing Ye, University of Michigan, 210 Washtenaw Avenue, Room 5183A, Ann Arbor, MI 48109. Email: bingye@umich.edu.

Abbreviated title: A Drosophila model of human YPEL3 mutation
ABSTRACT

A frameshift mutation in Yippee-like (YPEL) 3 was recently found from a rare human disorder with peripheral neurological conditions including hypotonia and areflexia. The YPEL gene family is highly conserved from yeast to human, but their functions are poorly defined. Moreover, the pathogenicity of the human YPEL3 variant is completely unknown. To tackle these issues, we generated a Drosophila model of human YPEL3 variant by CRISPR-mediated In-del mutagenesis. Gene-trap analysis suggests that Drosophila YPEL3 (dYPEL3) is predominantly expressed in subsets of neurons, including nociceptors. Analysis on chemical nociception induced by allyl-isothiocyanate (AITC), a natural chemical stimulant, revealed a reduced nociceptive response in dYPEL3 mutants. Subsequent circuit analysis showed a reduction in the activation of second-order neurons (SONs) in the pathway without affecting nociceptor activation upon AITC treatment. Although the gross axonal and dendritic development of nociceptors was not affected, the synaptic contact between nociceptors and SONs were decreased by dYPEL3 mutations. Together, these suggest that the frameshift mutation in human YPEL3 causes neurological conditions by weakening synaptic connection through presynaptic mechanisms.
INTRODUCTION

YPEL3 belongs to the Yippee gene family that is composed of a number of genes present in various eukaryotic species ranging from yeast to human (Hosono et al., 2004), which suggests that they are involved in fundamental biological processes. However, only a handful of studies have hinted at the biological roles of YPEL3. YPEL3 was initially identified as a small unstable apoptotic protein because of its low protein stability and the ability to induce apoptosis when overexpressed in a myeloid cell line (Baker, 2003). Subsequent studies implicate YPEL3 as a tumor suppressor. YPEL3 expression correlates with p53 activity (Kelley et al., 2010). Overexpression and knockdown analyses suggest that YPEL3 suppresses the epithelial-to-mesenchymal transition in cancer cell lines by increasing GSK3β expression (Zhang et al., 2016). Other studies have shown the role of YPEL genes in development. The loss-of-function mutations of YPEL orthologs in ascomycete fungus altered fungal conidiation and appressoria development (Han et al., 2018). In zebrafish, a morpholino-mediated targeting of YPEL3 altered brain structures (Blaker-Lee et al., 2012).

Recently, a mutation in human YPEL3 was found in a patient with a rare disorder that manifests a number of neurological symptoms (the NIH-Undiagnosed Diseases Program). The mutation was caused by a duplication of a nucleotide in a coding exon of human YPEL3, resulting in a frameshift and consequently a premature stop codon. The clinical observation showed that the patient had normal cognition but manifested peripheral symptoms, including areflexia and hypotonia. While these findings indicate significant functions of YPEL3 in the peripheral nervous system, little is known about YPEL3’s functions in the nervous system. Furthermore, the pathogenicity of the identified YPEL3 mutation in the nervous system is completely unknown.

In the present study, we generated a Drosophila model of the human condition caused by the disease-relevant YPEL3 variant using CRISPR/CAS9-mediated in-del mutations. Our gene-trap analysis suggests that subsets of neurons, including nociceptors, express the Drosophila homolog of YPEL3 (dYPEL3). Subsequent analysis revealed reduced nociceptive behavior in dYPEL3 mutants. Consistently, we found that dYPEL3 mutations impaired the activation of second-order neurons (SONs) in the nociceptive pathway and reduced the synaptic contact between nociceptors and these SONs. These findings suggest that the identified human YPEL3 mutation presents its pathogenicity at neuronal synapses.
RESULTS

Generation of a disease-relevant variant of YPEL3 in Drosophila

Although the discovery of a YPEL3 variant in a patient underscores the importance of YPEL3 in human health, whether this variant causes any defects in the nervous system is unknown. There are five YPEL genes in human. YPEL1, 2, 3, and 4 are highly homologous to each other (up to 96% identity at amino acid sequences), while YPEL5 has only ~40% homology to the other members (Hosono et al., 2004). We found two YPEL homologs in Drosophila, Yippee and CG15309, using an ortholog search (Hu et al., 2011). The predicted amino acid sequences of CG15309 showed a 88% similarity (81% identity) to human YPEL3 (Figure 1A), while that of Yippee showed a 65% similarity (53% identity) (Data not shown). Yippee appears to be an ortholog of YPEL5 because it is more closely related to YPEL5 than YPEL3 with 87% similarity and 73% identity to YPEL5 (Data not shown). Therefore, we named CG15309 as dYPEL3.

The variant identified in the human patient introduces an extra nucleotide in the middle of the coding exon, which produces a frameshift and consequently results in the incorporation of the 37 ectopic amino acids followed by a premature stop codon (Figure 1B). To generate a Drosophila model of the human variant, we took advantage of the CRISPR/CAS9 technology to induce In-del mutations (Port et al., 2014). The entire coding sequence of dYPEL3 is in a single exon. We designed a guide RNA that targets the middle of the coding exon (Figure 1C, top) and successfully isolated two dYPEL3 frameshift mutants named dYPEL3T1-6 and dYPEL3T1-8 (Figure 1C, middle). dYPEL3T1-6 has a 2-nucleotides deletion at 121 nucleotides downstream of a start codon, which generated a premature stop codon at 153 downstream of start codon, while dYPEL3T1-8 carries a 4-nucleotides deletion at 118 and generated a premature stop codon at 145 downstream of a start codon. Similar to the human variant, the mutations introduced additional amino acids followed by a premature stop codon (Figure 1C, middle). The ectopic amino acids in dYPEL3T1-6 closely resemble those of the human variant (Figure 1C, bottom panel).

**dYPEL3 is expressed in subsets of neurons**

We did not find any gross developmental defects in dYPEL3T1-6 or dYPEL3T1-8 flies. Homozygotes were viable and fertile, and showed normal growth under standard culture condition (data not shown). This raises the possibility that dYPEL3 is expressed in a subset of cells in the body. Our efforts of generating antibodies against dYPEL3 failed in two independent trials, precluding the use of immunostaining for identifying the cell types that express dYPEL3. We thus took advantage of a GAL4 enhancer-trap line, CG15309-GAL4 (dYPEL3-GAL4) (Gohl et al., 2011), to study the expression pattern of dYPEL3 in flies. This line contains a GAL4 insertion in the first intron of dYPEL3, which places the GAL4 under the control of the endogenous dYPEL3 promoter and enhancers (Figure 2A, top). As a result, the expression pattern of GAL4 represents that of dYPEL3. We expressed a membrane GFP reporter (mouse CD8::GFP or mCD8::GFP) to visualize dYPEL3 expression pattern in Drosophila larvae. A small number of cells in the larval central nervous system (CNS), including the ventral nerve cord (VNC) and brain, were labeled by mCD8::GFP (Figure 2A). These cells extended fine processes that cover most of the neuropil area in the larval CNS, suggesting that they are neurons. To identify the cell types that express dYPEL3, dYPEL3-GAL4 > mCD8::GFP samples were co-immunostained with the neuron marker anti-Elav and the glial marker anti-Repo (Figure 2B). About 85% of cells that were labeled with dYPEL3-GAL4 were positive for Elav, but none was positive for Repo (Figure 2C). This result
suggests that dYPEL3 is predominantly expressed in neurons, but not in glia. Interestingly, dYPEL3-GAL4 also labeled a subset of sensory neurons, including the class IV da neurons (nociceptors), class III da neurons and chordotonal neurons (both mechanosensors), but not the class I da neurons (proprioceptors) (Figure 2B-ii and -iii). dYPEL3 was not expressed in muscles nor epidermal cells (supplement of Figure 2).

The disease-relevant mutations of dYPEL3 cause defective nociceptive behavior

The human patient shows symptoms mainly in the peripheral nervous system (PNS), including areflexia and hypotonia (the NIH-Undiagnosed Diseases Program). We thus focused our analysis on dYPEL3-positive neurons in the PNS (Figure 2B-ii and -iii). The enhancer-trap analysis suggests that both the nociceptors and mechanosensors express dYPEL3. We performed an immunostaining experiment with the nociceptor marker anti-Knot antibody (Hattori et al., 2007; Jinushi-Nakao et al., 2007) and confirmed the presence of dYPEL3 in nociceptors (Figure 3A).

We first determined whether the function of nociceptors were altered by the dYPEL3 mutations. The nociceptors detect various stimuli including noxious heat, touch, and chemical and initiate the neural pathway that cumulates in the larval rolling and curling behavior (Hwang et al., 2007; Ohyama et al., 2015). Allyl-isothiocyanate (AITC), a natural chemical stimulant, is known to cause larval nociceptive behavior though nociceptors (Kaneko et al., 2017; Xiang et al., 2010; Zhong et al., 2012). We applied AITC to the wild-type control, dYPEL3T1-6, and dYPEL3T1-8, and found a significant reduction in nociceptive rolling behavior in the dYPEL3 mutants (Figure 3B). The extent of decrease in nociceptive rolling was not different between the two mutant alleles of dYPEL3, which are almost identical except for the sequences in the ectopic stretch of amino acids (Figure 1C). This suggests that the truncation of dYPEL3, but not the presence of the ectopic amino acid sequences, is responsible for the observed phenotype. dYPEL3T7-8 represents a simpler version since it only has a few ectopic amino acid incorporation (Figure 1C). Therefore, we focused our analysis on dYPEL3T7-8 for further analysis.

How does dYPEL3 mutation affect the sensory function? We first looked into whether the dYPEL3 mutation affects the development of nociceptors. We expressed mCD8::GFP specifically in nociceptors in wild-type and dYPEL3T1-8 larvae using the nociceptor-specific driver pppk-GAL4 (Grueber et al., 2007). The gross morphology and total length of dendrites were not affected in dYPEL3T1-8 (Figure 4A). Next, we tested whether the presynaptic terminals of nociceptors are defective in dYPEL3 mutants. To this end, a flip-out mosaic experiment was performed to label single nociceptive presynaptic arbors (Yang et al., 2014). The quantification of the total presynaptic arbors revealed that dYPEL3T7-8 did not affect the development of presynaptic arbors of nociceptors (Figure 4B).

The disease-relevant mutations of dYPEL3 reduce the synaptic transmission from nociceptors to their postsynaptic neurons

Next, we assessed the synaptic transmission from nociceptors to their postsynaptic neuron Basin-4, a key second-order neuron in the nociceptive pathway (Ohyama et al., 2015). The activation of Basin-4 elicits nociceptive behavior even in the absence of nociceptor activation, while silencing these neurons suppresses nociceptive behavior (Ohyama et al., 2015). The genetically encoded calcium indicator GCaMP6f was selectively expressed in Basin-4 for recording intracellular calcium, a proxy of neuronal activity (Chen et al., 2013) (Figure 5A). Larvae were dissected in insect saline as a fillet preparation with intact PNS and CNS (Kaneko et al., 2017). The nociceptors were stimulated using AITC. There are two
Basin-4 neurons in each segment of the VNC, one on the left side and the other on the right side (Kaneko et al., 2017; Ohyama et al., 2015). We found that the cumulative GCaMP signals from Basin-4 neurons were significantly decreased in dYPEL3T1-8 mutants, as compared to wild-type control (Figure 5A, ~55% decrease). By contrast, GCaMP measurement in nociceptor axon terminals showed that dYPEL3 mutations did not change nociceptor activation by AITC (Figure 5B).

The disease-relevant mutations of dYPEL3 reduce the synaptic contact between nociceptors and their postsynaptic neurons

How do the dYPEL3 mutations reduce the nociceptor-to-Basin4 synaptic transmission? To address this, we employed a synaptic-contact-specific GFP reconstitution across synaptic partners (GRASP) technique, termed sybGRASP (Macpherson et al., 2015), to assess the synaptic contact between the presynaptic terminals of nociceptors and the dendrites of Basin-4 neurons. The GRASP technique utilizes two separate fragments of GFP molecule – split-GFP1-10 (spGFP1-10) and split-GFP11 (spGFP11), which can be detected by a specific anti-GFP antibody only when the two fragments are in close proximity to reconstitute a complete GFP. In sybGRASP, spGFP1-10 is fused to the synaptic vesicle protein synaptobrevin and expressed in the presynaptic neurons, while spGFP11 is fused to a general membrane tag and expressed in postsynaptic neurons. Two independent binary gene expression systems, GAL4-UAS and LexA-LexAop, were used to drive the expression of spGFP1-10 and spGFP11 in different cell types (del Valle Rodríguez et al., 2012).

Synaptic vesicle exocytosis from presynaptic terminals exposes spGFP1-10 onto presynaptic cleft where it reconstitutes the functional GFP molecule by associating with postsynaptic spGFP11 molecules. This technique has been used widely to visualize synaptic contact between two identified neuron types.

The spGFP1-10 and spGFP11 were specifically expressed in nociceptors and Basin-4 neurons, respectively (Figure 6A). The resulting GRASP signal was measured in each segmental neuropil, and normalized by the spGFP1-10 intensity in wild-type and in dYPEL3T1-8 (Figure 6A, top). We detected a mild, but significant, decrease (23%) in the GRASP signals in dYPEL3T1-8, as compared to those in wild-type control (Figure 6A, bottom right). This suggests that the synaptogenesis between nociceptors and its synaptic target Basin-4 is compromised by the dYPEL3 mutations.

While the nociceptors express dYPEL3 (Figure 3A), dYPEL3 does not seem to be expressed in Basin-4 neurons, because dYPEL3-GAL4 was not expressed in Basin-4 neurons labeled by a Basin-4-selective LexA (Figure 6B). Thus, dYPEL3 acts presynaptically in nociceptors to regulate the synaptogenesis between nociceptors and their postsynaptic neurons.
DISCUSSION

The biological functions of YPEL3 or YPEL gene family are largely unknown. Moreover, the pathogenicity of the identified YPEL3 frameshift mutation is completely unknown. Drosophila provides a powerful tool to analyze disease-relevant human gene mutations (Bellen et al., 2019). In this study, we generated a Drosophila model of human YPEL3 mutation and demonstrated that the disease-relevant YPEL3 frameshift mutations cause pathogenesis in the nervous system.

YPEL gene family is highly conserved across the eukaryotic species ranging from yeast to human. Likewise, our homology analysis indicated a strikingly high sequence homology between human and Drosophila YPEL3 (80% identity, Figure 1B). Interestingly, it appears that the sequence homology extends even to the nucleotide level since the analogous frameshift mutation gave rise to the generation of similar amino acid sequences in the ectopic sequences in dYPEL3T1-6 (Figure 1C). Given such high sequence homology, we envision that the functions of human YPEL3 and Drosophila YPEL3 are also conserved. YPEL family can be subdivided into two categories. Human YPEL1, 2, 3, and 4 belong to one with high homology with each other, while YPLE5 constitute a distinct family (Hosono et al., 2004). In Drosophila, there is only a single homolog of human YPEL1 to 4, CG15309 (Figure 1B). Because the tissue expression patterns of YPEL genes are complex in human and mice (Hosono et al., 2004), the single YPEL gene makes Drosophila advantageous as a model for studying YPEL3-induced pathogenesis.

In human and mice, YPEL3 is ubiquitously expressed as based on RT-PCR method (Hosono et al., 2004). Northern blot analysis on murine tissues indicated relative enrichment of YPEL3 in brain and liver tissue (Baker, 2003). Our results based on a gene-trap Drosophila line indicates that dYPEL3 is expressed in subsets of neurons, but not in glia (Figure 2B and C). The human patient exhibited multiple neurological symptoms in the PNS, but had normal cognition (the NIH-Undiagnosed Diseases Program). Interestingly, dYPEL3-GAL4 was selectively expressed in nociceptors and mechanosensors in the PNS. Furthermore, YPEL3 frameshift mutations reduced nociceptive behavior (Figure 3B). This suggests that the neurological symptoms in the human patient originates from the neural tissues that normally express YPEL3.

Our results suggest that dYPEL3 is expressed in nociceptors, but not in their postsynaptic target Basin-4 neurons (Figure 3A and 6B). Then, how does the YPEL3 frameshift mutation lead to neuronal pathogenesis? Our GCaMP experiments showed that the activation of nociceptors by AITC was not altered in dYPEL3T1-8 mutants (Figure 5B). Rather, dYPEL3T1-8 mutations reduced Basin-4 responses to nociceptor stimulation (Figure 5A). The gross neuronal development of nociceptors was not altered by the dYPEL3 mutations (Figure 4). This suggests that the neurotransmission from nociceptors to their projection neurons is altered by the dYPEL3 frameshift mutation. Interestingly, we found that the synaptic contact between nociceptors and Basin-4 was reduced (Figure 6A). Because Basin-4 activation is central to nociceptive behavior (Ohyama et al., 2015), the reduced synaptic transmission from nociceptors to Basin-4 is likely responsible for the reduction in nociceptive behavior in dYPEL3 mutants. It is intriguing that human patient has peripheral symptoms of hypotonia and areflexia, both may arise from reduced synaptic transmission. Our results suggest that nociceptors, but not Basin-4, express dYPEL3 (Figure 3A and 6B), implying that dYPEL3 mutations affected presynaptic function. It will be important to identify neuron types that express YPEL3 in human in future studies.
How does YPEL3 frameshift mutation generate pathogenicity? The mutations in human patient and in our Drosophila model introduce premature stop codons, which may induce the non-sense mediated decay resulting in YPEL3 loss-of-function. However, the analysis of gene structure indicates that the frameshift mutation may escape from the non-sense mediated decay, because the premature stop codons are present in the last coding exons both in human and Drosophila YPEL3. This may lead to the generation of a truncated version of YPEL3 proteins. If this is the case, the truncation, rather than the introduction of ectopic amino acid sequences, may play a role in pathogenesis. This notion is supported by our finding that both dYPEL3T1-8 and dYPEL3T1-6 altered the nociceptive behavior to the same extent (Figure 3B).

The molecular function of YPEL3 is not clear. It has predicted zinc-finger motifs (Hosono et al., 2004). A recent study demonstrated that the zinc-finger motifs in a YPEL domain of Mis18 is important for the overall folding of YPEL domain that mediates the centromeric localization of Mis18 proteins (Subramanian et al., 2016). The YPEL domain in Mis18 has about 20% sequence similarity to YPEL proteins. YPEL3 suppresses the epithelial-mesenchymal transition when overexpressed by altering GSK3β protein expression (Zhang et al., 2016). Since many regulators of gene expression contain zinc-finger motifs, we envision that the mutation may cause changes in gene expression. It will be important to investigate how YPEL3 mutation affects the gene expression that is involved in synapse formation and maintenance in future studies.

Taken together, we generated a Drosophila model of human YPEL3 mutation and found that the YPEL3 variant can generate nervous system dysfunction. To our knowledge, this is the first report on the pathogenicity that is caused by the frameshift mutation of YPEL3. Our model will be instrumental for future investigations that may lead to effective treatments for the disorders caused by YPEL3 mutations.
MATERIALS AND METHODS

Drosophila melanogaster genetics

Drosophila strains were kept under standard condition at 25 °C in a humidified chamber. The following strains were used in the study: w1118 (3605), ppk-GAL4 (Grueter et al., 2007), ppp- LexA (Gou et al., 2014), UAS-syb::spGFP1-10 (Macpherson et al., 2015), LexAop-CD4::spGFP11 (Macpherson et al., 2015), UAS-FRT-rCD2-stop-FRT-CD8::GFP (Wong et al., 2002), hs-FLP (Nern et al., 2011) (55814), UAS-CD4-GFP (35836), UAS-GCaMP6f (Mutlu et al., 2012) (42747), LexAop-GCaMP6f (Mutlu et al., 2012) (44277), CG15309-GAL4 (Gohl et al., 2011) (62791), nos-Cas9 (Port et al., 2014) (54591), GMR57F07-GAL4 (Jenett et al., 2012) (46389), GMR57F07-lexA (Pfeiffer et al., 2010) (54899). The number in the parentheses indicates the stock number from the Bloomington Drosophila Stock center.

The generation of dYPEL3 frameshift mutants

The CRISPR/CAS9-mediated In-del mutation was used to generate dYPEL3 frameshift mutant flies. A guide RNA construct was generated in pCFD:U6:3 (Port et al., 2014) with a guide RNA sequences that target the middle of dYPEL3 coding exon. The standard transformation procedure was done to generate a transgenic line. The transformants were crossed with nos-Cas9 (Port et al., 2014) flies to induce in-del mutations in germ cells. The resulting progeny were screened for the desired mutations by the genomic PCR of CG15309 following the Sanger sequencing.

AITC-induced nociceptive behavior

Allyl-isothiocyanate (AITC, Sigma-Aldrich) was prepared in DMSO, dissolved in water as final 25 mM concentration, incubated on a rocker for 3 days before use. Fly embryos were grown for five days in 12 hour light/dark cycle at 25 °C, humidified incubator. The third instar larvae were moved to room temperature for an hour, gently scooped out of food, washed in tap water, and placed on a grape-agar 24 well plate that is covered with 300 µl AITC solution (25 mM). Both male and female larvae were used for the test. The behavior was recorded with a digical camera for 2 min and the number of larvae showing a complete rolling behavior (minimum 360° rolling) and curling (curling plus any rolling that is under 360°) was manually analyzed (Honjo et al., 2012). The experiments were paired for the wild-type control (w1118) and dYPEL3 homozygous mutant larvae. The experiments were repeated three times in different days with different AITC preparation. All three trials were combined for statistical analysis.

Calcium imaging

Live calcium imaging was done using GCaMP6f (Mutlu et al., 2012). Briefly, the wandering third instar larvae – the wild-type control male or dYPEL3 frameshift hemizygous were dissected in a modified hemolymph-like 3 (HL3) saline (Stewart et al., 1994) (70 mM NaCl, 5 mM KCl, 0.5 mM CaCl2, 20 mM MgCl2, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES, pH 7.2). Glutamate (10 mM) were added to the HL3 solution to prevent muscle contractions and sensory feedback. The GCaMP signal was recorded in the entire volume of nociceptor axon terminals or Basin-4 cell bodies. The live imaging was done with a Leica SP5 confocal system equipped with an extra-long-working distance 25X water objective a 1 or 2 µm step-sizes. The membrane tdTomato proteins were expressed along with GCaMP6f and used as an internal normalization control for both lateral and focus drifting. The basal GCaMP signal was recorded for a duration of 30 sec to generate baseline fluorescence (F0), then the
samples were treated with AITC (25 mM) in the HL-3 while continuous recording. The 3D
time-lapse images were collapsed to 2D time-lapse by using the maximum Z-projection in
the imageJ software. The region of interest was selected either in the axonal projection of
nociceptors or in the cell bodies of Basin-4. The ImageJ Time Series Analyzer plugin (NIH)
was used to quantify the fluorescence intensity of GCaMP6f.

Immunostaining
The immunostaining was done essentially as previously reported (Kim et al., 2013). The
primary antibodies used are: chicken anti-GFP (1:5000) (Aves Laboratories), Rabbit anti-
RFP (1:5000) (Rockland), rat anti-Elav (1:100) (Developmental hybridoma bank, clone
9F8A9), mouse anti-Repo (1:100) (Developmental hybridoma bank, clone 8D12). The
secondary antibodies used as 1:500 and are from the Jackson ImmunoResearch: Cy2 or
Cy5 conjugated goat anti-chicken, Cy2 or Cy5 conjugated goat anti-mouse, Cy5 conjugated
goat anti-Rabbit, Cy3 conjugated goat anti-rat. The confocal imaging was done with a Leica
SP8 confocal system equipped with a 63X oil-immersion objective with 0.3 µm step-size.
The resulting 3D images were projected into 2D images using a maximum projection
method.

In order to report the relative synaptic contact between the nociceptors and their
post-synaptic partners, the sybGRASP was performed in the male larvae from a wild-type
control (w^{1118}) or dYPEL3^{T1-8} hemizygous. The Syb::split-GFP1-10 (Macpherson et al., 2015)
was expressed in nociceptors. The CD4::split-GFP11 (Macpherson et al., 2015) was
expressed in Basin-4 neurons. The polyclonal chicken anti-GFP antibody (Aves
Laboratories) recognizes the split-GFP1-10 and the reconstituted GFP protein while the
mouse anti-GFP antibody (1:100) (Sigma-Aldrich, clone GFP-20) recognizes only the
reconstituted GFP. Therefore, the mouse anti-GFP antibody was used to measure the
GRASP signal (anti-GRASP) and the polyclonal chicken anti-GFP antibody was used as an
internal control for normalizing the GRASP signal. The fluorescence images were acquired
to minimum signal saturation for quantitation. The mean fluorescence intensities of anti-
GRASP and anti-split-GFP1-10 from each hemi-neuropil segment (segments 4, 5, and 6)
were measured from the confocal images.

Assessment of dendrite development in nociceptors
The membrane GFP, mCD8::GFP, was specifically expressed in nociceptors using ppk-
GAL4 in a wild-type control (wt) and dYPEL3 frameshift mutants (dYPEL3^{T1-8}). The wild-type
control male or dYPEL3^{T1-8} hemizygous larvae were used for the analysis. Total length of
dendrites was measured from the male larvae of wt and dYPEL3^{T1-8} using Simple neurite
tracer plugin (Longair et al., 2011) in the ImageJ software.

Analysis of presynaptic arbors of single nociceptors
The flip-out (Wong et al., 2002) experiment was performed to visualize the terminal axon
arbors of single nociceptors. A flip-out cassette (FRT-rCD2-stop-FRT-CD8::GFP) and a
heat-shock inducible Flippase (FLP) was introduced either in a wild-type control (w^{1118}) or in
dYPEL3^{T1-8} mutants along with ppk-GAL4. The three-day-old larvae grown in grape-agar
plate were heat-shocked for 15 min in 37 °C water bath and allowed one more day of growth
at 25 °C before dissected and processed for immunostaining and imaging. The wild-type
control male or dYPEL3^{T1-8} hemizygous larvae were used for the analysis. The total
presynaptic arbor length was manually measured using the ImageJ software. Branches
shorter than 5 µm were excluded from the analysis.
**Experimental design and statistical analysis**

All statistical analysis was performed as two-tailed using GraphPad Prism version 7.04 (GraphPad Software). The Chi-square test was used for nociceptive behavior. The Mann-Whitney test was used for Ca²⁺ imaging and the GRASP experiments. An unpaired Student's test was used for presynaptic arbor size and dendritic development analysis. A p value smaller than 0.05 were considered statistically significant. All p values are indicated as NS; non-significant, *; P < 0.05, **; P < 0.01, and ***; P < 0.001.
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COMPETING INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

J.K. and B.Y. conceptualized and designed research. J.K., M.S., G.P., A.L., N.Z., and B.B. performed research and analyzed data. J.K., and B.Y. wrote the paper. All authors reviewed the manuscript.
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FIGURE LEGEND

Figure 1. The generation of a Drosophila model of YPEL3 frameshift mutation.
(A) CG15309 is the Drosophila homolog of human YPEL3. Sequence alignment between human YPEL3 (YPEL3) and Drosophila CG15309. Shaded in red are the identical amino acid sequences.
(B) Duplication of a cytosine nucleotide in YPEL3 gene from a patient (top). A predicted molecular lesion in human YPEL3 (bottom) introduces an ectopic amino acid sequences (shaded in green). The preserved region is shaded in red.
(C) CRISPR-CAS9 mediated in-del mutation in CG15309/dYPEL3. A guide RNA is designed targeting the middle of coding exon (top). The isolated dYPEL3 in-del mutants (middle). Sequence alignment between a wild-type (wt), dYPEL3T1-6, and dYPEL3T1-8. The introduced ectopic amino acid sequences following a premature stop codon were shaded in green. Bottom, the sequence alignment of the introduced ectopic amino acid sequences from the human YPEL3 frameshift mutants and dYPEL3T1-6. The identical amino acid sequences are shaded in red.

Figure 2. dYPEL3 is neuronal gene.
(A) The expression pattern of dYPEL3 in the CNS. An In-site gene trap line for dYPEL3 was used (CG15309-GAL4/dYPEL3-GAL4). GAL4 transcription factor is inserted in the first intron. The introduction of UAS-mCD8::GFP demonstrates the endogenous expression pattern of dYPEL3. Note that the CG15309-GAL positive cells elaborate fine processes throughout the CNS. Scale bar = 50 µm.
(B) mCD8::GFP was expressed under dYPEL3-GAL4 (magenta) following the immunostaining with anti-Elav (neuronal, green) and anti-Repo (glial, blue) antibodies. (i) the CNS. Cell bodies are magnified in 1 and 2. (ii) and (iii) are chordotonal neurons and a class III da neuron in the PNS (short arrows), respectively. (iii) also shows a class IV da neuron (nociceptor) that is positive for dYPEL3 (long arrow).
(C) Quantitation of the Elav-positive and Repo-positive cells that are labeled with CG15309-GAL4. The majority of dYPEL3-positive cells were Elav-positive, but none were positive for Repo.

Supplement of Figure 2. Muscle and epidermal tissue are negative for dYPEL3 expression.
Red-stinger, a nucleus-targeted red fluorescence protein was expressed under dYPEL3-GAL4 (magenta, anti-RFP). Wandering 3rd instar larvae were dissected to reveal their body wall that contains both larval muscles and epidermis along with the PNS neurons (green, anti-HRP stain). Cell nuclei were labeled with DAPI (left, blue) to identify muscle and epidermal cells. Note that the nuclei from both muscle and epidermal cells are devoid of RFP signal.

Figure 3. dYPEL3 frameshift mutations reduce nociceptive behavior.
(A) Nociceptive/class IV da neurons are positive for dYPEL3. A nuclear GFP (GFP-nls, green) was expressed under dYPEL3-GAL4 following the immunostaining with anti-Knot antibody (blue). Anti-HRP antibody was used to label all PNS neurons (magenta).
(B) The AITC-induced nociceptive behavior was measured in a wild-type control (wt) and dYPEL3 frameshift mutants (dYPEL3T1-8, and dYPEL3T1-6). The number of larvae that exhibited complete rolling behavior, curling, and no response was scored as expressed as a
percentage (n = 252 for each genotype). The Chi-squared test was performed between the groups.

**Figure 4. The development of nociceptors is not altered by dYPEL3 mutations.**

(A) mCD8::GFP was specifically expressed in nociceptors using ppk-GAL4 in wild-type control (wt) and dYPEL3 frameshift mutants (dYPEL3T1-8). Total length of dendrites was measured (n = 6 for each genotype). Scale bar = 50 µm.

(B) The axon terminals of single nociceptors from wild-type and dYPEL3T1-8 mutants were visualized using the flip-out technique. The total length of axon terminals was measured (n = 12 for wt, n = 14 for dYPEL3T1-8). Scale bar = 10 µm.

Unpaired Student t-test with Welch’s correction were performed. Data is presented as mean ± s.e.m.

**Figure 5. dYPEL3 mutations reduce the synaptic transmission from nociceptors to Basin-4 neurons.**

(A) Basin-4 activation upon AITC treatment was reduced by dYPEL3 mutations. GCaMP6f was expressed in Basin-4 neurons. Nociceptors were activated by 25 mM AITC. The Ca²⁺ increase in Basin-4 was measured by GCaMP fluorescence (n = 12 for wt, n = 18 for dYPEL3T1-8).

(B) Nociceptor activation was not altered by dYPEL3 mutations. GCaMP6f was expressed in nociceptors using ppk-GAL4. Nociceptors were activated by 25 mM AITC. The Ca²⁺ increase in the axon terminals of nociceptors was measured by GCaMP fluorescence (n = 13 for each genotype). Mann-Whitney test. Data was presented as a violin plot.

**Figure 6. dYPEL3 mutations reduces the synaptic contact between nociceptors and Basin-4 neurons.**

(A) The sybGRASP technique was used to report the synaptic contact between nociceptors and Basin-4. The spGFP1-10 and spGFP11 were expressed in nociceptors and Basin-4 respectively (bottom left). The resulting GRASP signal was visualized by anti-GRASP antibody (top left, green, see materials and methods for details). The spGFP1-10 that is expressed in nociceptor axon terminals was used as a normalization control (top right, magenta). n = 36 for wt, n = 34 for dYPEL3T1-8. Data was presented as a violin plot. Mann-Whitney test.

(B) Basin-4 does not express dYPE3. A membrane red fluorescent protein, CD4-tdTomato was expressed under dYPEL3-GAL. GFP was expressed under Basin-4 specific LexA. Note that the cells expressing GFP does not overlap with the cells that are positive for dYPEL3-GAL4. Scale bar = 10 µm.
The frameshift mutation found in human YPEL3

| Transcript | Exon | Type         | Protein                                      |
|------------|------|--------------|----------------------------------------------|
| NM_031477.4(YPEL3):c.273dup | exon2 | insertion frameshift | p.Val92Serfs*38 (STOP codon 37 positions) |

Predicted molecular lesion

The generation of frame-shift mutations in Drosophila YPEL3

The isolated In-del mutations and predicted molecular lesion in dYPEL3

The homology between the extended sequences in human mutant and dYPEL3

62% identity
Figure 2

A

B

C

|       | dYPEL3 positive total | Elav Positive | Repo positive |
|-------|-----------------------|---------------|---------------|
| Repeat-1 | 96                    | 86            | 0             |
| Repeat-2 | 80                    | 65            | 0             |
| Repeat-3 | 85                    | 71            | 0             |
Figure 4

A

wt dYPEL3 \( \text{T}^{\text{T}_{1-8}} \)

---

**NS (P = 0.70)**

Total dendritic length

\( (\mu m) \)

\( \begin{array}{c}
\text{wt} \\
\text{dYPEL3}^{\text{T}_{1-8}}
\end{array} \)

---

B

wt dYPEL3 \( \text{T}^{\text{T}_{1-8}} \)

---

**NS (P = 0.37)**

Presynaptic arbor size

\( (\mu m) \)

\( \begin{array}{c}
\text{wt} \\
\text{dYPEL3}^{\text{T}_{1-8}}
\end{array} \)
Figure 6

A

WT

d'YPEL3^{T1-8}

GRASP

spGFP-10

** (P = 0.0069)

B

Basin-4 > GFP

d'YPEL3 > CD4-tdTomato

Merged

GRASP (fold change)

wt

d'YPEL3^{T1-8}