Ptth regulates lifespan through innate immunity pathway in *Drosophila*

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Abstract

The prothoracicotropic hormone (Ptth) is well-known for its important role in controlling insect developmental timing and body size by promoting the biosynthesis and release of ecdysone. However, the role of Ptth in adult physiology is largely unexplored. Here we show that *Ptth* null mutants (both males and females) show extended lifespan and healthspan, and exhibit increased resistance to oxidative stress. Transcriptomic analysis reveals that age-dependent upregulation of innate immunity pathway is attenuated by *Ptth* mutants. Intriguingly, we find that Ptth regulates the innate immunity pathway, specifically in fly oenocytes, the homology of mammalian hepatocytes. We further show that oenocyte-specific overexpression of Relish shortens the lifespan, while oenocyte-specific downregulation of ecdysone signaling extends lifespan. Consistently, knocking down *torso*, the receptor of Ptth in the prothoracic gland also promotes longevity of the flies. Thus, our data reveal a novel function of the insect hormone Ptth in longevity regulation and innate immunity in adult *Drosophila*.

Significance

The function of Ptth is heavily studied in the insect development stage, especially molting and metamorphosis. However, we know very little about the role of Ptth in adult physiology. In this study, we find that Ptth regulates lifespan through the regulation of the innate immunity pathway in a tissue-specific manner in *Drosophila*. Similar to mammalian growth hormone, Ptth might be the key growth factor that controls longevity by targeting liver inflammation.

Introduction

Insects experience extraordinary morphological changes when they pass from larva through pupa to adult. Insect hormones are the factors that prompts and regulates those changes including physiology, behavior and development aspects. Most of insect hormones are peptide hormones that regulates different functions in various tissues. Prothoracicotropic hormone (Ptth) is a brain neuropeptide hormone that stimulates the secretion of the molting hormone, ecdysone, from the prothoracic glands (PG) in insect larvae [1]. Once ecdysone is released into the hemolymph, it is converted to an active form, 20-hydroxyecdysone (20E), by a P450 monooxygenase at downstream target tissues [2]. 20E is the primary molting hormone that binds to a nuclear receptor (EcR) and initiates various gene expression cascades, which ultimately lead to the physiological, morphological, and behavioral changes associated with molting and metamorphosis [3]. In *Drosophila* Torso is reported as a functional receptor of Ptth in PG to control the biosynthesis and release of ecdysone [4]. The steroid hormone ecdysone is the master regulator of insect developmental transitions, including molting and metamorphosis. Ecdysone receptor (EcR) was identified as a nuclear receptor that is activated by ecdysone [5, 6]. Besides regulating molting and metamorphosis, recent studies show that EcR is also involved in 20E-induced immune potentiation [7]. Furthermore, EcR signaling is the first pathway known to regulate lifespan in *Drosophila* [8]. Inactivating EcR only in adulthood can also extend lifespan of both female and male flies [9]. In contrast, the role of Ptth in the adult physiology remains unexplored.

Fruit flies, *Drosophila melanogaster*, have been used as a powerful organism model in biological and genetic research due to the following facts. First, it has a short and simple reproduction cycle. It takes about ten days at 25 °C to produce one generation. There are four stages in its life cycle, embryo, larva, pupa and adult. Second, it requires a small space and little

expense to culture fruit flies in the laboratory. The fly culture media consist of simple source of carbohydrates (cornmeal) and proteins (yeast). Third, the whole genome sequence of the *Drosophila* was completed in 2000. Its genome is about 170 million base pairs long. There are about 14 thousand protein-coding genes. With Gal4/UAS system, it is easy to knock down or overexpress certain genes in a tissue specific manner.

In *Drosophila*, innate immunity is the first-line of defense upon stress or infection. It is regulated by immune deficiency pathway (Imd) as well as Toll pathway. In *Drosophila*, it is the Imd pathway that controls the expression of most of the antimicrobial peptides (AMPs), a group of small peptides with unique inhibitory effects against bacteria, fungi, and other pathogens. Imd pathway regulates the AMP expressions through the activity of Relish (*Drosophila* homolog of NF-KB). Upon pathogen infection, PGN (peptidoglycan) is recognized and bound by PGRPs (PGN-recognition proteins), which results in recruitment of a signaling complex consisting of Imd, dFadd and Dredd. Once Dredd is ubiquitinated by Iap2 (E3-ligase inhibitory of apoptosis 2), it cleaves Imd, which results in the recruitment and activation of Tab2/Tak1 complex. The Tab2/Tak1 complex phosphorylates and activation of *Drosophila* IKK complex. Relish is activated by the phosphorylation of multiple sites at N-terminus by the Ikk protein Kenny. The phosphorylation of Relish is required for the translocation into nucleus, where Relish interacts with RNA polymerase II to control the promotors of Relish target genes (such as antimicrobial peptide genes, DptA (diptericin) and Cec (Cecropin)) [10].

In this study, we examine the role of Ptth in lifespan regulation and innate immunity pathway. We show that Ptth null mutants (both males and females) show extended lifespan and exhibit increased resistance to oxidative stress. Consistently, Ptth mutants show well-preserved muscle and cardiac function at old ages. RNA-seq and qPCR analyses show that Ptth mutants

attenuate age-dependent activation of Imd pathway, especially in adult oenocytes. We further show that overexpression of Relish in oenocyte shortened lifespan. Taken together, our results reveal a novel role of Ptth in regulating longevity through oenocyte-specific innate immune pathway in *Drosophila*.

Results

Prothoracicotropic hormone (Ptth), the key insect growth factor, regulates both developmental timing and longevity in *Drosophila*.

In *Drosophila*, Ptth was first identified as a key hormonal factor regulating insect ecdysteroid biogenesis, which determines the timing of molting and metamorphosis [1]. Through TALEN genome editing technique, O'Connor lab generated two *Ptth* deletion lines and found that Ptth specifically controls the duration of last instar larval growth and maturation [11]. By examining Ptth gene expression across the development stage (L1, L2, L3, wandering L3, pupa) and adult stage (both female and male), we found that although the peak of *Ptth* expression is at pupa stage, there are some expression in young adult flies including both female and male (Figure 1A).

To examine the role of Ptth in longevity control, we first backcrossed the two *Ptth* loss-of-function mutant lines. During the backcross, we isolated another allele with 7 base pairs deletion at a different position of the final exon. Together, a total of three *Ptth* alleles were backcrossed into a wild type ($w^{[OB]}$) for 5 generations. We renamed these lines as $Ptth^{[8BC1]}$ (2 amino acids deletion), $Ptth^{[120BC2]}$ and $Ptth^{[120BC3]}$ (truncated protein due to premature stop) (Figure 1B). We also included another Ptth allele from thte Bloomington Stock Center in the lifespan. Five generations of backcrosses to its wild type control flies yw^{R} was performed as well. It is also a loss function allele. Similar to the previous study [11], all four backcrossed *Ptth* mutants showed delayed

pupariation, or extended larval-pupal growth duration (**Fig. S1A and Fig. S1B**). Interestingly, these slow-growing *Ptth* mutants were all long-lived compared to their match controls (both females and males, **Fig. 1C-1F**). Consistent with extended lifespan, *Ptth* mutants exhibited increased resistance to paraquat-induced oxidative stress, both female (**Fig. S1C**) and male (**Fig. S1D)** flies, and slower decreasing in the age-dependent climbing ability in both female flies (**Fig. S1E**) and male flies (**Fig. S1F**) as well as preserved cardiac function (e.g., lower arrhythmia at old ages) (**Fig. S1G**). Thus, *Drosophila* Ptth regulates lifespan and healthspan, in addition to its role in developmental timing.

***Ptth* mutants repress age-dependent upregulation of AMP expression**

To understand the molecular mechanisms underlying Ptth-regulated longevity, we performed an RNA-Seq analysis to characterize the transcriptomic changes in young and aged female wild-type (w[OB]) and Ptth mutants ($Ptth^{120BC2}$). There are 731 highly differentiated genes between young Ptth mutants and wild-type (Fold change>1.5 or <-1.5, FDR<0.05) (Fig. 2A). Among those genes, 129 of them are highly upregulated (Fold change>1.5, FDR<0.05) while 602 of them are highly downregulated (Fold change <-1.5, FDR<0.05). Those genes are enriched with development pathway (Fig. 2C). There are 610 highly differentiated genes between old Ptth mutants and wild-type (Fold change>1.5 or <-1.5, FDR<0.05) (Fig. 2B). Those genes are enriched with immune response pathway (Fig.2D). Scatter-plots show that some genes highly induced in wild type flies by age are suppressed in Ptth mutants (Fig. 2E). Venn Diagram shows that among those 1220 genes differentially expressed between young and aged wild-type (Fold change>2, FDR<0.05), 754 of them were not significantly changed in Ptth mutants. (Fig. 2F) GO analysis of those age upregulated genes among 754 differentiated genes shows that

"immune response" and "defense response" are highly enriched (Fig.G). Interestingly, heatmap of AMP gene express in the young and aged wild type and Ptth mutant flies shows that the age-related upregulation of AMP genes involved in innate immunity (Toll/Imd) pathway significantly suppressed by Ptth mutants (Fig. 2H). In addition, Ptth mutants did not show age-dependent induction of inflammatory cytokines (data not shown). Consistently, Gene Ontology analysis showed that 'immune response' and 'Innate Immune response' were two of the top enriched pathways that were differentially expressed in aged flies (Fig. 2D), but not in young flies (Fig. 2C).

To further confirm above results, we performed qRT-PCR to validate the expression of several Toll/Imd genes, such as Imd transmembrane receptor Peptidoglycan recognition protein LC (PGRP-LC) and antimicrobial peptide Diptericin A (DptA). As shown in **Fig. 2I-2J**, the expression of both *PGRP-LC* and *DptA* was significantly upregulated upon normal aging, while *Ptth* mutants alleviated these age-related inductions. The hyperactivation of innate immune pathways is a hallmark of chronic inflammation (inflammaging). Our preliminary study suggests that Ptth might regulate animal aging through innate immunity pathway, and reduced Ptth signaling suppresses inflammaging.

***Ptth* regulates Imd pathway specifically in fly hepatocytes (oenocytes).**

Ptth is a neuropeptide hormone secreted by a cluster of neuronal cells in the brain. To identify the target tissue through which Ptth regulates longevity and Imd pathway, we screened various fly tissues using the gene expression pattern of PGRP-LC and DptA as screening markers. First, we separated heads, thorax and abdomen and checked the expression of PGRP-LC and DptA (**Fig. 3SA-B**). We found that age-induced PGRP-LC and DptA expression in wild type was

suppressed in *Ptth* mutants. Second, we dissected the tissues in the abdomen including fat body, heart, oenocyte and gut, we found that age-related induction of PGRP-LC and DptA expression was only blocked in oenocytes dissected from *Ptth* mutants (**Fig. 3A-3B**).

Relish, the fly homolog of NF-kB, is the key transcription factor of Imd pathway that regulates the expression of AMP genes (e.g., PGRP-LC and DptA). Upon activation of innate immune response (such as aging and bacterial infection), Relish is cleaved into two parts by rapid proteolytic cleavage, a 68 kDa N-terminal fragment (Rel68) and 49 kDa C-terminal fragment (Rel49). Rel49 is degraded in cytoplasm, while Rel68 is translocated to nucleus to activate the transcription of AMP genes, such as DptA gene expression. This process requires the kinase Kenny and caspase Dredd, respectively [12]. To monitor Relish activation, we performed immunostaining by using the antibody against Rel68 to monitor its nuclear translocation in young and old oenocytes. Consistently with PGRP-LC and DptA expression, age-dependent increases in nuclear localization of Relish, was attenuated in oenocytes of *Ptth* mutants (**Fig. 3C**), but not in other tissues such as fat body (**Fig. 3D**), muscle **(Fig. 3E)** and gut **(Fig. 3F)**. Taken together, these findings sugessts that Ptth regulates imd pathway specifically in fly oenocytes, the homolog of mammalian hepatocytes.

Ptth binding to Torso in PG regulates longevity and imd pathway

As the receptor of Ptth, torso expresses in different tissues including oenocytes. However, when we constitutively knocked down *torso* in oenocytes, the lifespan of flies was shortened (**Figure 4A**). This suggests that Ptth does not target oenocytes directly. Torso is reported as the receptor of ligand Ptth in PG, which initiate Ecdysone biosynthesis and release in PG [5]. To confirm the role of torso in lifespan control through its expression in PG, we constitutively

knocked down *torso* in PG and examined the lifespan **(Figure 4B)**. Interestingly we found constitutively knocking down *torso* in PG extended lifespan in female flies. To examine if ecdysone also plays a role in this process, we performed a longevity assay with constitutively knocking down EcR in the oenocytes (**Figure 4C**). EcR activity was reported increased in oenocytes comparing to the other tissues [13]. As we expected, those flies lived longer than the wild type female flies. We also performed immunostaining against Relish 68 antibody after constitutively knocking down Torso in PG (**Figure 4D**) and EcR in oenocytes (**Figure 4E**). In both case constitutively knocking down Torso in PG and EcR in oenocytes blocked the age-induced translocation of Rel68 to nuclei that happened in the control female flies accordingly. All of those evidences suggest that Ptth regulates longevity and Imd pathway through binding to the receptor torso in PG.

Relish is required for the extended lifespan in *Ptth* mutants

To further confirm the role of Imd pathway in the Ptth regulated longevity, we combined Ptth mutant with oenocytes tissue driver. Then overexpressed Relish in the oenocytes with Ptth mutant background, surprisingly we found that the extended longevity caused by Ptth mutation was rescued (**Figure 5**). Thus, our data suggest that Ptth may target its receptor torso in PG to control the release of ecdysone, which will in turn regulate innate immunity pathway specifically in oenocytes to control lifespan and healthspan in *Drosophila*.

Discussion

In *Drosophila*, Ptth (prothoracicotropic hormone) is secreted by a few of neuroendocrine cells located in each brain hemisphere. These neuronal cells are also called PG neurons, which is

in accordance with their innervation pattern. The PG neuronal cells project their axons to terminate on the PG (prothoracic gland). Ptth ligand binds to torso receptor in PG cells and initiates the biosynthesis and release of ecdysone. Ecdysone is the steroid hormone that regulates molting and metamorphosis. Loss of function mutation Ptth flies delay L3 larval molting to pupa, around 24 hours (**Figure 1SA~B**). Interestingly we found that this delayed development from L3 to pupa also slows down aging (**Figure 1C~F**) through repressing age-induced Imd pathway, especially in oenocyte tissue.

Innate immunity pathway is evolutionally conserved between *Drosophila* and mammal. The transcription factor, Relish, in *Drosophila* innate immunity pathway is homolog to the transcription factor, NF-kB in mammal innate immunity pathway. Our data suggests that loss of Ptth reduces the Relish translocation from cytoplasm to nucleus that is usually induced by aging (**Figure 4D**). Thus reducing growth factor, such as Ptth, could reduce age induced immunity response, further lead to reduced inflammation during aging, also known as inflammaging.

Ptth is one of the key hormones in the insect endocrine system. Our findings suggest that endocrine growth factor like Ptth plays a role in regulating tissue aging, especially oenocyte aging, by modulating innate immunity pathway. Repressing growth factors can attenuate age-dependent induction of innate immunity response and inflammation. Like mammalian liver, insect oenocyte is the center for lipid metabolism. Recent studies show that oenocytes function similarly to mammal liver [14]. In mammal, liver is the target tissue of growth hormone. Together with IGF1 (Insulin-like Growth Factor 1), growth hormone promotes muscle mass and lipid metabolism. Lack of growth hormone in mice results in smaller body size (Ames Dwarf Mouse) and prolonged lifespan [15]. Consistent with our data, growth hormone also regulate innate

immunity pathway. Studies in macrophages also show that growth hormone receptor (GH-R) plays a role in maintaining immune system homeostasis in aging [16].

As early as in 1995, it is reported that in *Bombyx* Ptth shares a common molecule with the vertebrate growth factors Beta-nerve growth factor (beta-NGF), transforming growth factor-beta2 (TGF-beta22) and platelet-derived growth factor- BB (PDGF-BB) and belongs to the growth factor superfamily [17]. Our findings suggest that insect Ptth may share many common mechanisms with mammalian growth hormone in longevity regulation.

Materials and Methods

Fly Husbandry and Stocks

Flies were maintained at 25°C, 60% relative humidity and 12-hour light/dark. Adults were reared on agar-based diet with 0.8% cornmeal, 10% sugar, and 2.5% yeast (unless otherwise noted). Fly stocks used in the present study are: *Ptth8K1J*, *Ptth120F2A*, *Ptth RNAi* (V102043), *torso RNAi* (V36280), *phm-GAL4*, *EcR RNAi* (BDRC#29374), *Attp40 RNAi* (BDRC #36304), and $Ptth^{T1}$ (BDRC #84568). yw^{R} control line is a gift from the Tatar Lab (Brown university, Providence, RI, USA).

Developmental Timing Analysis

To synchronize development for timed experiments, parental flies were allowed to lay eggs for 6 hours on an apple juice agar plate coated with a thin layer of yeast paste. Hours after egg laying (AEL) was measured from the end of this time. Fertilized eggs were collected on apple juice agar plates and put into small 35mm x 10mm at 30~50 eggs per dish, petri dishes (Falcon) filled with about 2.5 ml standard fly food. Larvae were raised in groups of 30 to prevent

crowding. Larvae for all experiments were raised inside an insulated and moist chamber at 25°C, 60% relative humidity and 12-hour light/dark cycle. For developmental progression, pupa were scored in 4 hour intervals every day till all larvae molt into pupae.

RNA extraction and QRT-PCR

Adult tissues (oenocyte, heart, gut, fat body) were all dissected in 1 × PBS before RNA extraction. For oenocyte dissection, we first removed fat body through liposuction and then detached oenocytes from the cuticle using a small glass needle. Tissue lysis, RNA extraction, and cDNA synthesis were performed using Cells-to-CT Kit (Thermo Scientific). Whole body flies were collected on CO_2 and transferred to 1.7 ml centrifuge tube with a stainless steel ball and 500ml Trizol(Thermo Fisher Scientific, Waltham, MA, USA), tissuelyzer was used to smash the flies. About 15 flies were used in a biological replicate. DNase-treated total RNA was quantified by Nanodrop and about 500 ng of total RNA was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA).

QRT-PCR was performed with a Quantstudio 3 Real-Time PCR System and PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Two to three independent biological replicates were performed with two technical replicates. The mRNA abundance of each candidate gene was normalized to the expression of *RpL32* for fly samples, by the comparative C_T methods. Primer sequences are listed in the following: PGRP-LC forward TTTAACCTTCCTGCTGGGTATC and reverse TTGTCTGTAATCGTCGTCATCTC, DptA forward TTGCCGTCGCCTTACTTT and reverse CCTGAAGATTGAGTGGGTACTG.

Immunostaining and imaging

To examine the co-localization of Relish and nucleus, adult oenocytes were dissected from young (5~7 days old) and old (38~40 days old) female flies in 1X PBS and then fixed in 4% paraformaldehyde for 15 min at room temperature. Tissues were washed with 1x PBS with 0.3% Triton X-100 (PBST) for three times (~5 min each time), and blocked in PBST with 5% normal goat serum for 30 min. Tissues were then incubated overnight at 4 °C with primary antibodies anti-Relish (RayBiotech RB-14-0004,1:500) diluted in PBST, followed by the incubation with secondary antibodies obtained from Jackson Immuno Research for 1 h at room temperature next day. After three times washes again, tissues were mounted using ProLong Gold antifade reagent (Thermo Fisher Scientific) and imaged with an FV3000 Confocal Laser Scanning Microscope (Olympus). DAPI or Hoechst 33342 was used for nuclear staining.

Lifespan Assay

Flies were collected under brief CO_2 anesthesia and placed in food vials at a density of 25~30 females/males flies per vial, with a total of 150~300 flies for most conditions. Flies were passed to fresh food every other day and dead flies were scored and counted. Lifespan and log-rank p value were determined using JMP for survival analysis. For the use of GeneSwitch flies, flies were fed with 200 μM RU486 in food to activate the gene knock down at adult stage.

RNA-seq and Bioinformatics

For all groups, total RNA was collected from ~15 female wholebody flies, three biological replicates each condition, using Trizol method(described as above) and following by DNase treatment(Ambion). RNA concentration was quantified by Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA Catalog number: Q10210). RNA-Seq libraries were constructed using 300 ng of total RNA and NEBNext Ultra Directional RNA Library Prep

Kit for Illumina (New England Biolabs (NEB), Ipswich, MA, USA. Catalog number: E7420). RNA concentrations were measured using Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA Catalog number: Q10210). Poly(A) mRNA was isolated using NEBNext Oligo d$(T)_{25}$ beads and fragmented into 200 nt in size. After first strand and second strand cDNA synthesis, each cDNA library was ligated with a NEBNext adaptor and barcoded with an adaptor-specific index. Twelve libraries were pooled in equal concentrations, and sequenced using Illumina HiSeq 3000 platform (single-end, 50 bp reads format).

The RNA-Seq data processing was performed on Ubuntu system. FastQC was first performed to check the sequencing read quality and Fastx is used to filter the bad quality read from fastq. Then the raw reads were mapped to *D. melanogaster* genome (*Drosophila*_melanogaster.BDGP6.22.98.chr.gtf) using Star(https://github.com/alexdobin/STAR.git). Htseq-count was used to count the number of mapped reads on each gene and DE-seq2 (R package) was used to generate normalized data. After normalization, differentially expressed protein-coding transcripts were obtained using following cut-off values, false discovery rate (FDR) ≤ 0.05 and fold-change ≥ 2. Non-coding gene and low expressed genes (FPKM< 0.01) were excluded from the analysis. RNA-Seq read files have been deposited to NCBI ‘s Gene Expression Omnibus (GEO) (Accession # GSE112146). To review GEO files: Go to https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112146.

Statistical analysis

GraphPad Prism 6 (GraphPad Software, La Jolla, CA) was used for statistical analysis. To compare the mean value of treatment groups versus that of control, one-way ANOVA was performed using Dunnett’s test for multiple comparison. The effects of mutants on starvation responses was analyzed by two-way ANOVA, including Tukey multiple comparisons test.

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Author Contributions

Conceived and designed the experiments: PK HB. Performed the experiments: PK PL JK MB AK TM HB. Analyzed the data: PK PL MB HB. Wrote the paper: PK HB. All authors reviewed and approved the final version of this manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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Figures:

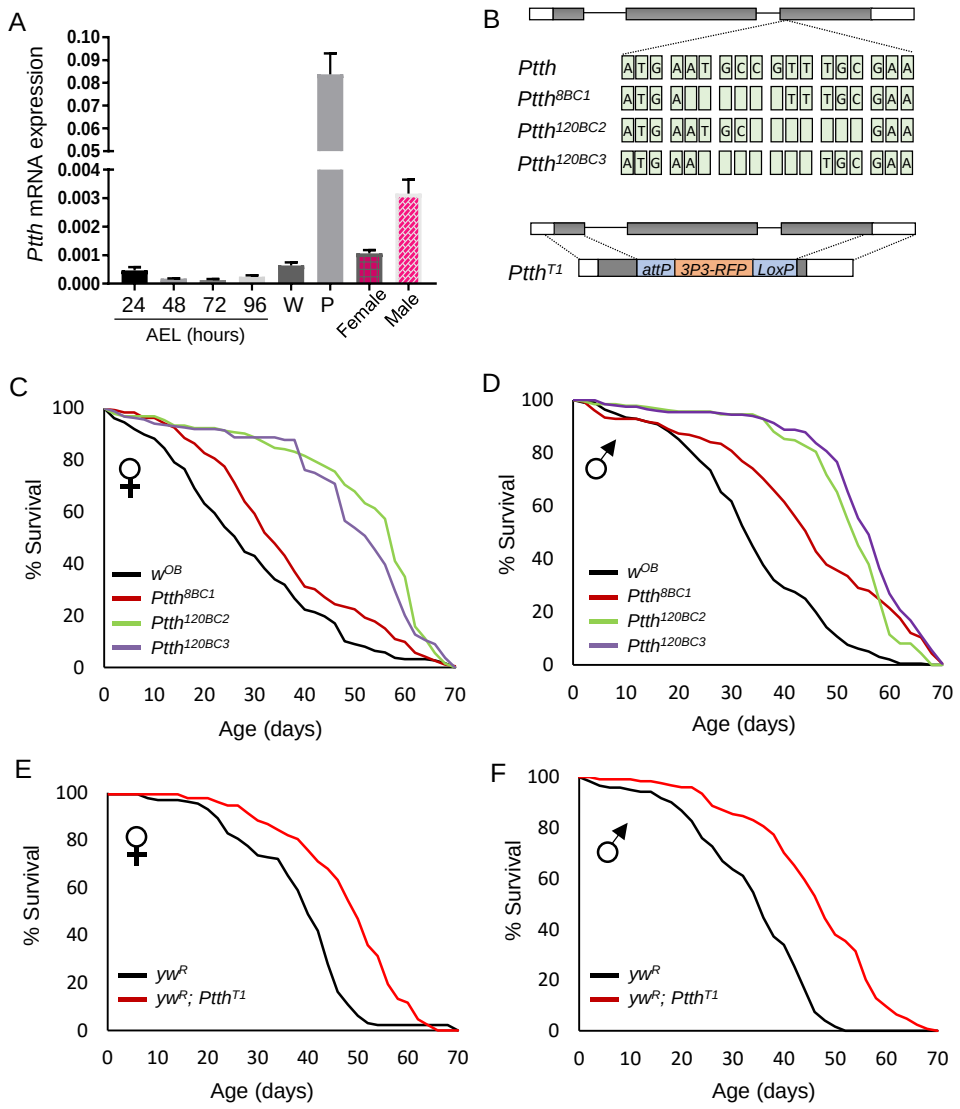
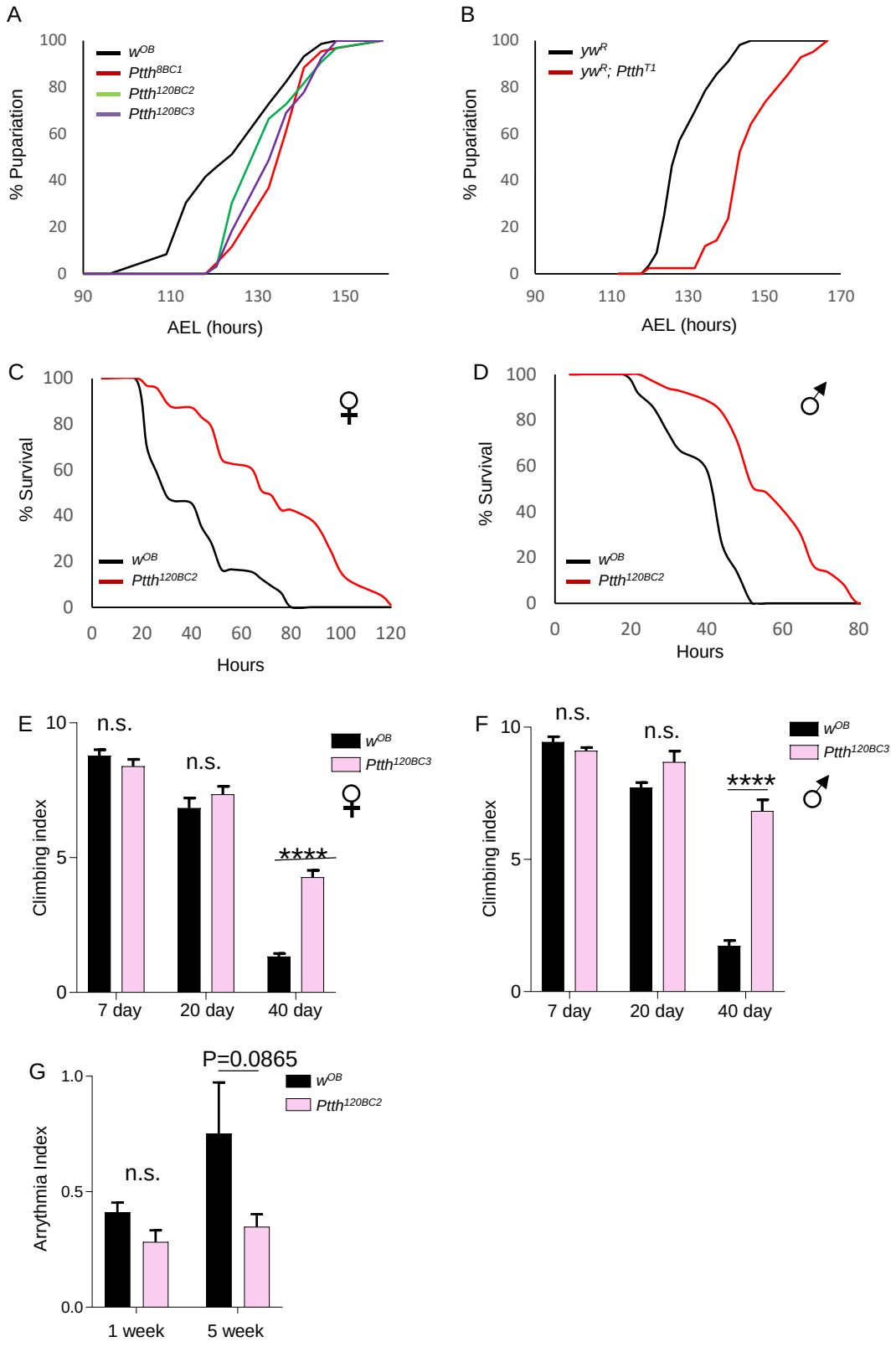


Figure 1. Ptth regulates lifespan in Drosophila. (A) Relative PTTH expression across *Drosophila* development stage and early adult stage. (B) Skematic diagram of ptth mutants : *Ptth*^{8BC1} *Ptth*^{120BC2} , *Ptth*^{120BC3} , *Ptth*^{T1} (C) Lifespan of : *Ptth*^{8BC1}(Log-Rank test p=0.0139, n=200), *Ptth*^{120BC2} (Log-Rank test p<0.001, n=200), *Ptth*^{120BC3} (Log-Rank test p<0.001, n=200) , female. (D) Lifespan of : *Ptth*^{8BC1}(Log-Rank test p<0.001, n=200), *Ptth*^{120BC2} (Log-Rank test p<0.001, n=200), *Ptth*^{120BC3} (Log-Rank test p<0.001, n=200), male (E) Lifespan of *Ptth*^{T1} (Log-

Rank test $p<0.001$, $n=125$), female (F) Lifespan of *Ptth*^{T1}(Log-Rank test $p<0.001$, $n=125$), male. Log-Rank test was performed by JMP.


A
% Pupariation
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C
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Ptth120BC2
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wOB
Ptth120BC3
F
Climbing index
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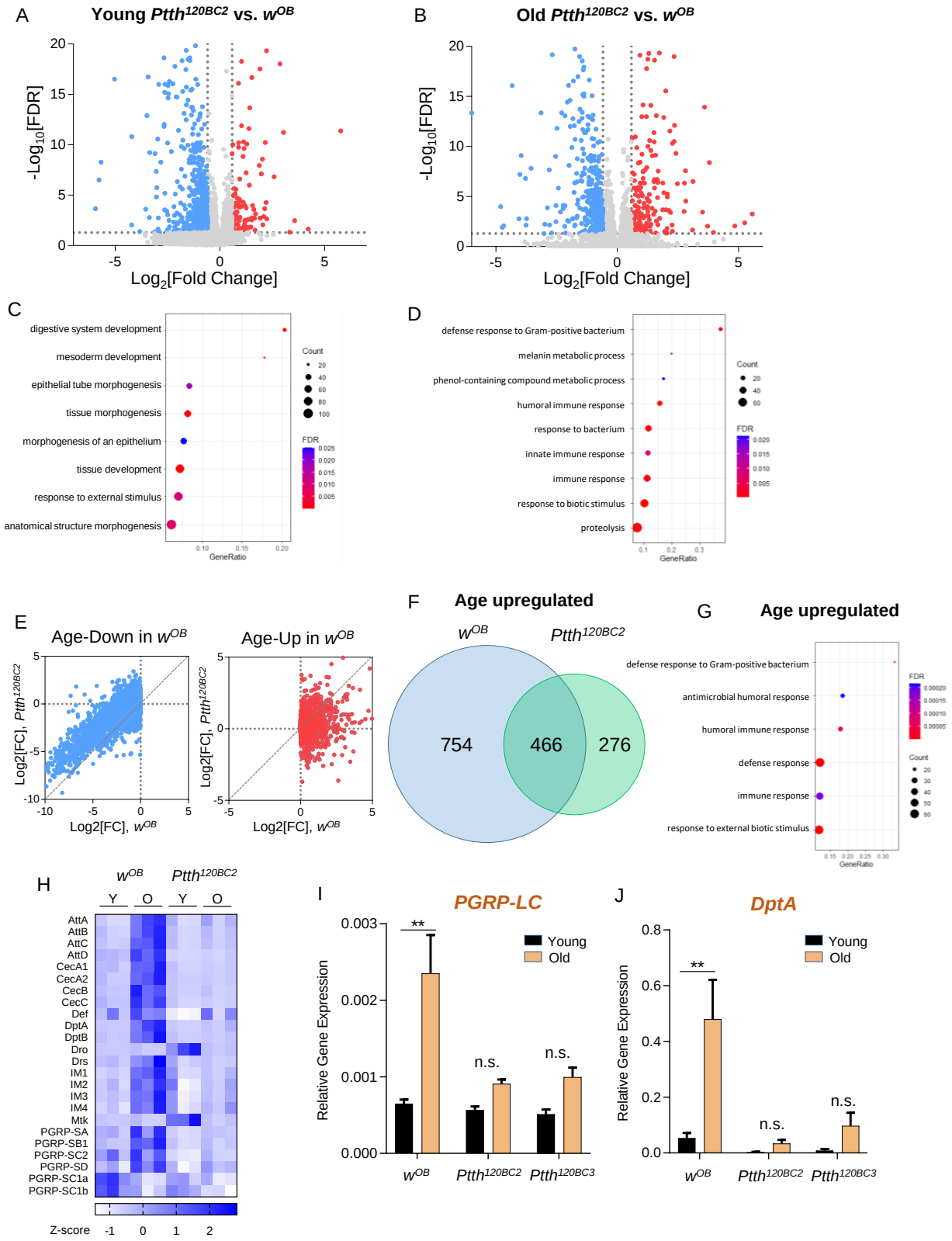
n.s.
****
7 day
20 day
40 day
wOB
Ptth120BC3
G
Arrythmia Index
P=0.0865
n.s.
1 week
5 week
wOB
Ptth120BC2

Figure S1. Ptth regulates healthspan in Drosophila. (A) Developmental timing of *Ptth*^{8BC1}, *Ptth*^{120BC2}, *Ptth*^{120BC3} and wild type w^{OB} (three replicates for each genotype, about 30~40 eggs in each plate). (B) Developmental timing of *Ptth*^{T1} and wild type yw^{R}(three replicates for each genotype, about 30~40 eggs in each plate).(C) The survival plot of *Ptth*^{120BC2} female flies after treated 20mM paraquat. (Log-Rank test, $p<0.001$, n=100).(C) The survival plot of *Ptth*^{120BC2} male flies after treated 20mM paraquat. (Log-Rank test, $p<0.001$, n=100). (E) climbing assay *Ptth*^{120BC3}, female, two-way Anova followed by Turkey with multiple comparison, $p<0.0001$, n=30 (F) climbing assay *Ptth*^{120BC3}, male, two-way Anova followed by Turkey with multiple comparison, $p<0.0001$, n=30 (G) Arrhythmia Index of young(7 days old) and old(35 days old) *Ptth*^{120BC2} female flies. One-way Anova followed by Turkey with multiple comparison, $p=0.0865$, n=15~20.


A
Young Ptth120BC2 vs. wOB
-Log10[FDR]
Log2[Fold Change]
B
Old Ptth120BC2 vs. wOB
-Log10[FDR]
Log2[Fold Change]
C
digestive system development
mesoderm development
epithelial tube morphogenesis
tissue morphogenesis
morphogenesis of an epithelium
tissue development
response to external stimulus
anatomical structure morphogenesis
GeneRatio
Count
FDR
D
defense response to Gram-positive bacterium
melanin metabolic process
phenol-containing compound metabolic process
humoral immune response
response to bacterium
innate immune response
immune response
response to biotic stimulus
proteolysis
GeneRatio
Count
FDR
E
Age-Down in wOB
Age-Up in wOB
Log2[FC], Ptth120BC2
Log2[FC], wOB
F
Age upregulated
wOB
Ptth120BC2
754
466
276
G
Age upregulated
defense response to Gram-positive bacterium
antimicrobial humoral response
humoral immune response
defense response
immune response
response to external biotic stimulus
GeneRatio
FDR
Count
H
wOB
Ptth120BC2
Y
O
Y
O
AttA
AttB
AttC
AttD
CecA1
CecA2
CecB
CecC
Def
DptA
DptB
Dro
Drs
IM1
IM2
IM3
IM4
Mtk
PGRP-SA
PGRP-SB1
PGRP-SC2
PGRP-SD
PGRP-SC1a
PGRP-SC1b
Z-score
-1
0
1
2
I
PGRP-LC
Relative Gene Expression
Young
Old
**
n.s.
n.s.
wOB
Ptth120BC2
Ptth120BC3
J
DptA
Relative Gene Expression
Young
Old
**
n.s.
n.s.
wOB
Ptth120BC2
Ptth120BC3

418

419

Figure 2. Ptth mutants repress age-dependent upregulation of antimicrobial peptide (AMP) expression. (A) Volcano plot showing genes significantly upregulated and downregulated by PTTH mutant in young whole body, 5 days old, as determined by RNA-seq. (B) Volcano plot showing genes significantly upregulated and downregulated by PTTH mutant in old whole body, 38 days old, as determined by RNA-seq. (C) Dot Plot Analysis showing pathway differentially expressed between young PTTH mutants and wild type. (D) Dot Plot Analysis showing pathway differentially expressed between old PTTH mutants and wild type. (E) Scatter Plot comparing the age-upregulated gene between wild type and PTTH mutant. (F)Venn diagram showing genes induced by age in wild type and genes induced by age in PTTH mutant. (G) Dot Plot Analysis with genes upregulated by age, 754 genes, showing pathways differentially expressed with those genes. (H) Heat map showing gene expression with genes identified in innate immunity pathway, comparing young and old wild type and PTTH mutant flies. (I) qPCR results showing relative PGRP-LC(receptor of imd pathway) expression in female whole body flies, wildtype and PTTH mutant, young (5 days old)and old flies(38 days old), , two-way Anova followed by Turkey with multiple comparison, p=0.0014 , three biological replicates.(J) qPCR results showing relative DptA (down stream gene of imd pathway) expression in female whole body flies, wildtype and PTTH mutant, young(5 days old) and old (38 days old)flies, two-way Anova followed by Turkey with multiple comparison, p=0.0038, three biological replicates.

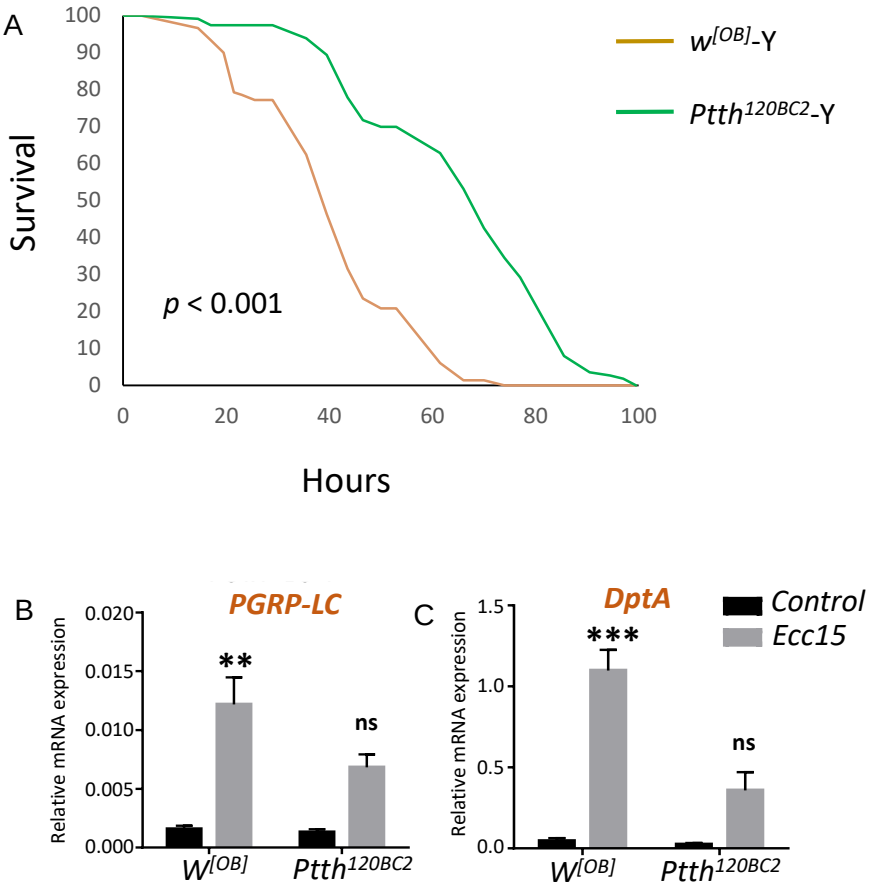


Figure 2S. Ptth mutants attenuates Ecc15-induced antimicrobial peptide (AMP) expression. (A) The survival plot of young(14 days old) *Ptth*^{120BC2} and wild type female flies after treated by Ecc15 (O.D.600 = 200)(Log-Rank test, p<0.001, n=125). qPCR results showing relative (B) PGRP-LC(receptor of imd pathway) expression and (C)Relative DptA (down stream gene of imd pathway) expression in female whole body, wildtype and PTTH mutant, young flies(5 days old) after fed by Ecc15(O.D.600=200) or 5% sucrose over night(~16 hours). (three biological replicates, two-way ANOVA Tukey's multiple comparison test, alpha=0.05)

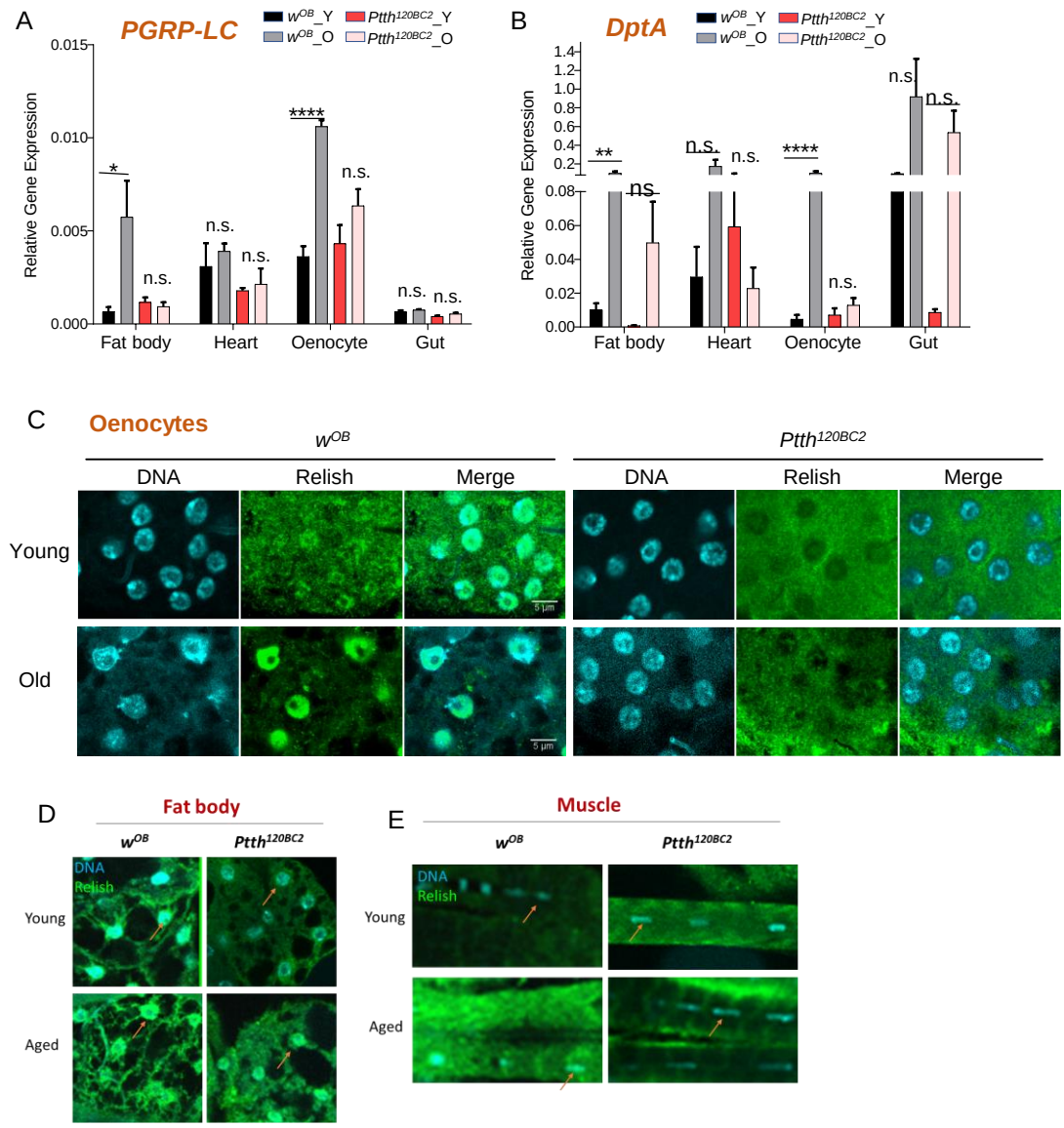


Figure 3. Ptth regulates Imd pathway specifically in fly hepatocytes (oenocytes). (A) Relative PGRP-LC expression in fatbody(p=0.0115), heart, oenocyte(p<0.0001) and gut tissue with young and old wild type and PTTH mutant female flies. , one-way Anova followed by Turkey with multiple comparison, three biological replicates. (B), Relative DptA expression in fatbody(p=0.0054), heart, oenocyte(p<0.0001) and gut tissue with young(5 dyas old) and old(38 days old) wild type and PTTH mutant female flies, one-way Anova followed by Turkey with

multiple comparison, three biological replicates. (C) Relish 68 antibody staining in oenocyte tissue showing age induced translocation of Rel68 is suppressed in PTTH mutants. This phenomenon did not happen in fat body (D), and muscle(E).

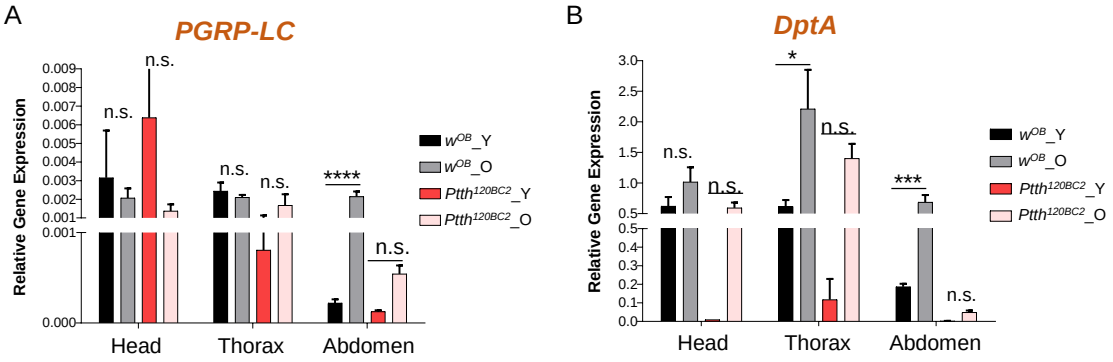


Figure S3. Ptth regulates Imd pathway specifically in fly abdomen. (A) Relative PGRP-LC expression in head, thorax and abdomen($p<0.0001$) tissue with young(5 days old) and old(38 days old) wild type and PTTH mutants, one-way Anova followed by Turkey with multiple comparison, three biological replicates. (B) Relative DptA expression in head, thorax($p=0.0327$) and abdomen($p=0.0003$) tissue with young(5 days old) and old(38 days old) wild type and PTTH mutants, one-way Anova followed by Turkey with multiple comparison, three biological replicates.

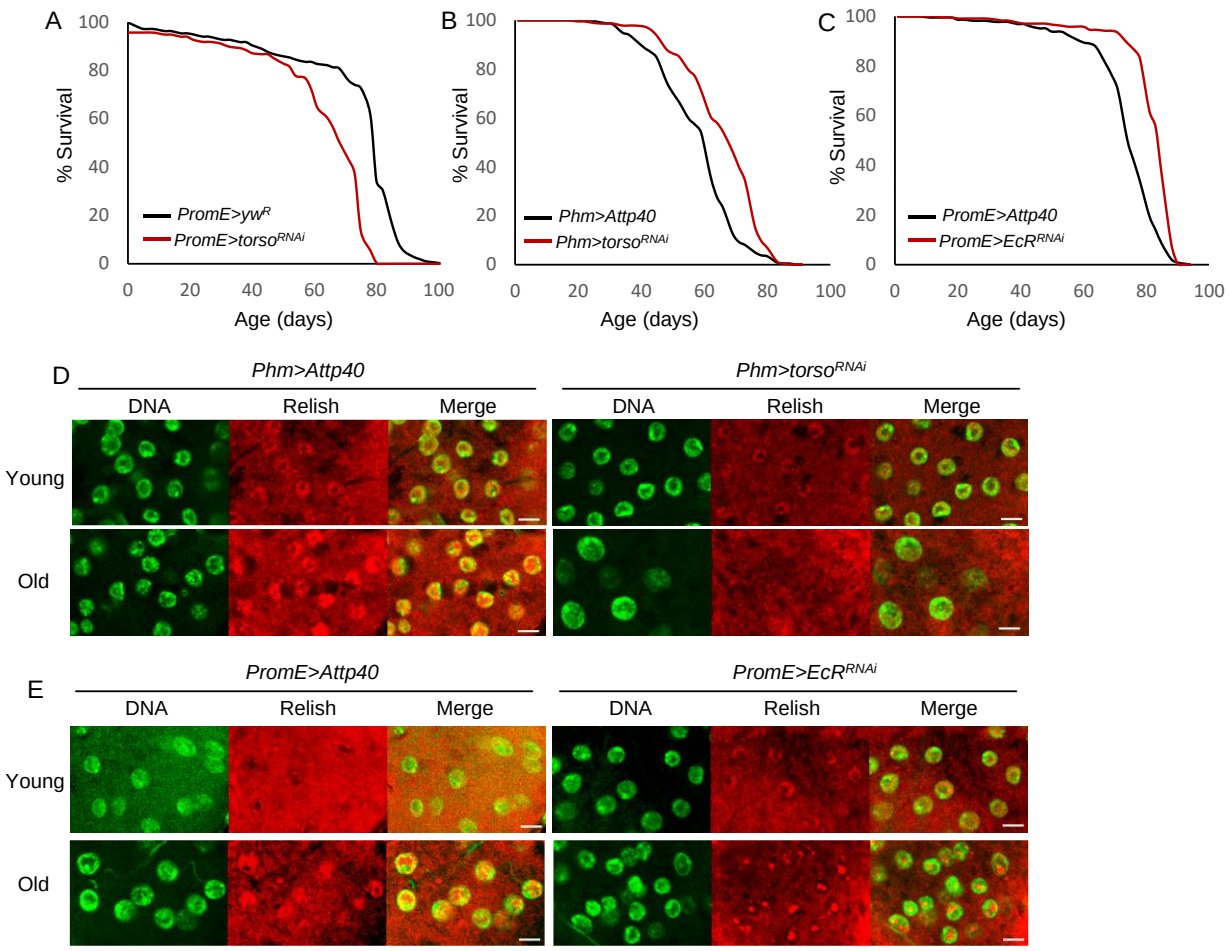


Figure 4. Ptth regulates lifespan through development and its receptor Torso in prothoracic glands (PG). (A) Constitutively knocking down Torso in oenocytes extends female lifespan.)(Log-Rank test, p<0.001, n=200). (B) Constitutively knocking down Torso in PG extends female lifespan.)(Log-Rank test, p<0.001, n=200). (C) Constitutively knocking down EcR in oenocytes extends female lifespan.)(Log-Rank test, p<0.001, n=200). (D) Rel68 antibody staining in female young and old oenocyte tissue after constitutively knocking down Torso in PG showing constitutively knocking down Torso in PG blocks the age induced translocation of Rel68 in wild type. Young is 5 days old and old is 38 days old. n=5. (E) Rel68 antibody staining in female young and old oenocyte tissue after constitutively knocking down

EcR in oenocyte showing constitutively knocking down EcR in oenocyte blocks the age induced translocation of Rel68 in wild type. Young is 5 days old and old is 38 days old. n=5.

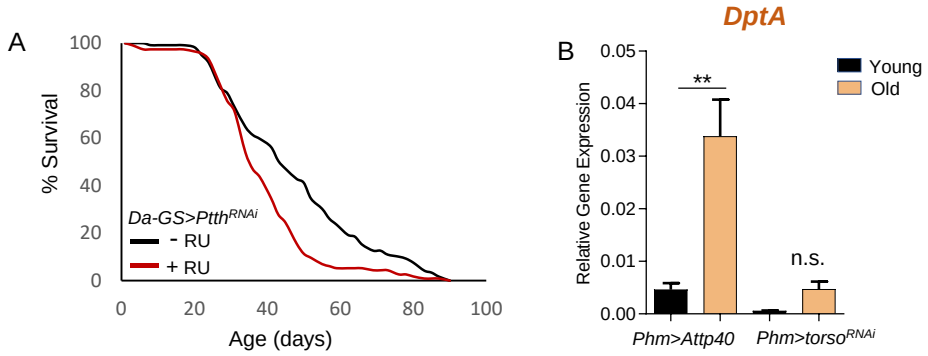


Figure S4. Ptth did not regulate lifespan at the adult stage. PG-specific Torso knockdown blocks age-dependent upregulation of Imd pathways. (A) Lifespan of knocking down PTTH in adult stage showing shorter lifespan than control)(Log-Rank test, $p<0.001$, n=200). (B) Relative DptA expression to RPL32 is induced by age in wild type. Constitutively knocking down Torso in PG blocks this induction. Young is 5 days old and old is 38 days old. (three biological replicates, two-way ANOVA Tukey's multiple comparison test)

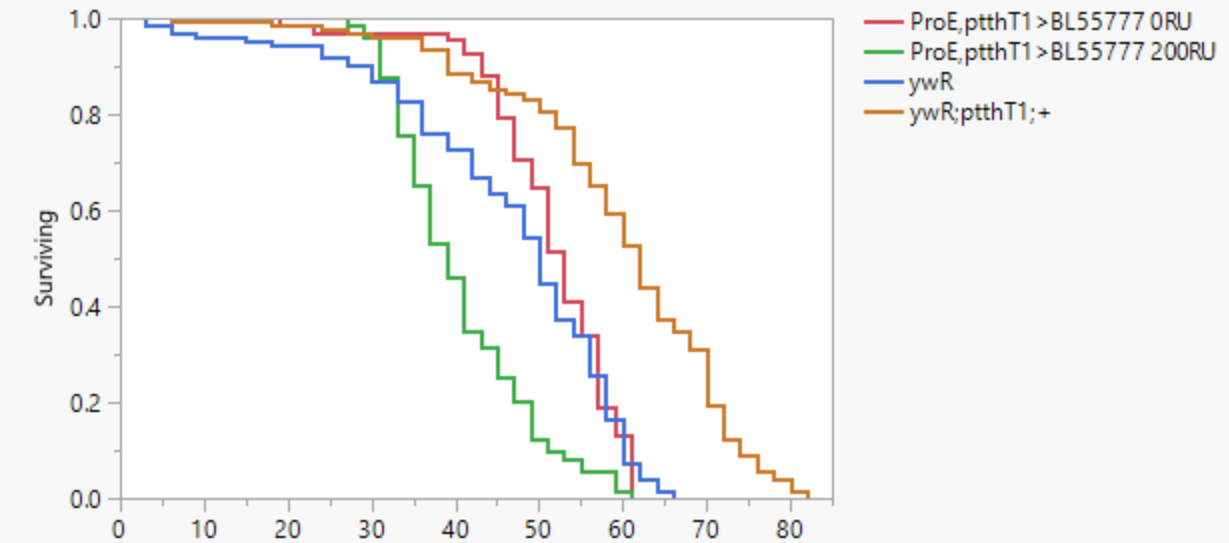


Figure 5. Oenocyte-specific overexpression of Relish rescues the lifespan extension of Ptth mutants. Lifespan of overexpressing Relish in adult stage showing shorter lifespan than control) (Log-Rank test, $p<0.001$, $n=150$).