Research Paper

Indy reduction decreases aging-related dysbiosis in *Drosophila*

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ABSTRACT

Reduction in the *Indy (I'm not dead yet)* gene, a plasma membrane citrate transporter, in *Drosophila* and its homolog in worms extends lifespan by promoting metabolic homeostasis. *Indy* reduction delays the onset of aging-associated pathology in the fly midgut, including preservation of intestinal barrier integrity and intestinal stem cell homeostasis. Gut microbiota has broad impacts on host metabolism, health, and aging. Age-related dysbiosis impairs intestinal barrier function and drives mortality. However, the underlying mechanisms that link increased microbial load to frailty and negative effects on health remain mostly unclear. Here we show that *Indy* heterozygote flies have significantly lower bacterial load and increased diversity during aging compared to controls. However, the presence of the microbiota was not required for *Indy* lifespan extension, though removal of microbes did enhance the effects of *Indy* reduction on longevity, suggesting potential interactions between the microbiota and *Indy*. *Indy* down-regulation was linked to reduced expression of Upd3 and Upd2 in the midgut of young flies and Stat92E in old *Indy* flies, while no change in other members of the JAK/STAT signaling pathway observed. Furthermore, flies double heterozygous for $Indy^{206}$/+ and $upd3^{Delta}$/+ alleles lived longer than single heterozygous flies, suggesting synergistic effects on longevity of *Indy* and upd3 pathways. Altogether, our results suggest that *Indy* reduction impacts microbiota load and composition, which together with effects of *Indy* on midgut metabolism contributes to preserved gut homeostasis and extended lifespan.

INTRODUCTION

Indy and microbiota have vital roles in health and longevity

The aging process in organisms is characterized by loss of homeostasis, accumulation of cell damage, and decline in physiologic function [1–3]. Key factors that contribute to aging and its related pathologies have been identified using model organisms including mice, worms, and *Drosophila melanogaster* [3–5]. Microbiota imbalance has been linked to a growing number of human disorders including obesity, cardio-vascular disease, type-2-diabetes and inflammatory bowel disease. One of the major risk factors for dysbiosis is aging. Age-associated dysbiosis of the intestinal microbiota has been implicated in intestinal barrier impairment, activation of intestinal immune response and increased frailty. Increased microbial load in the fly midgut has been associated with age-related intestinal dysfunction and increased mortality [6–10].

The *Indy (I'm not dead yet)* gene encodes the fly homolog of a mammalian SLC13A5 plasma membrane citrate transporter [11–14]. Reduction of *Indy* gene activity in flies or its homologs in worms, rats, and mice has beneficial effects on energy balance by affecting the levels of cytoplasmic citrate, which plays a central role in metabolism [15–21]. Cytoplasmic citrate levels affect glucose and lipid metabolism, and energy production by mitochondria. In flies, INDY protein is highly expressed in the midgut, fat body, and oenocytes, all of which are important sites of fly metabolic regulation [11–13, 22–24]. *Indy* reduction alters the metabolism of flies, worms, mice and rats, in a manner akin to changes associated with caloric restriction (CR) [11, 15–17, 19, 25–28]. Reduction in *Indy* gene expression extends longevity of $Indy^{206}$/+ heterozygous flies and in other *Indy* heterozygous alleles, compared to control flies, delays the onset of intestinal barrier disfunction, and preserves intestinal stem cell homeostasis, and midgut integrity and function [11, 22, 23, 25, 26, 29]. Maximal life span extension was observed in *Indy* heterozygous flies, and their lifespan is not further extended by CR, providing further evidence that *Indy* and longevity pathways overlap. While *Indy* homozygous flies live longer than control flies when aged on a conventional laboratory diet or a high caloric diet, their lifespan is shorter than *Indy* heterozygous flies most likely because they are malnourished [20]. CR without malnutrition delayed the onset of aging associated pathologies, and extended lifespan in a variety of species [30–33]. Beneficial effects of CR on the microbiota and global host metabolism have been reported [34, 35]. Transplantation of the microbiota from female mice aged for 8 weeks on a very-low calorie diet to obese female mice increased recipient microbiota alpha diversity, decreased their body fat accumulation, and improved glucose tolerance compared to mice that received microbiota from ad libitum fed mice [34]. In addition, antitumor effects of CR have been linked to changes in the gut microbiota [36]. However, the level of CR is important for the effects on microbiota, as severe CR improved metabolic health in a randomized human interventional study, but led to a decrease in bacterial abundance and altered microbiota composition [37]. Transplantation of the post-CR diet microbiota decreased adiposity and body weight of recipient mice by impairing nutrient absorption and enriching for pathobionts including, *Clostridioides difficile*. In another randomized controlled study, CR was shown to affect microbiota composition and shift plasma metabolites to a longevity-related metabolic pathway signature [38]. These studies highlight the importance of interactions between nutrients and microbiota and their effects on metabolism.

The JAK/STAT signaling pathway has a key role in epithelium renewal [6, 39–41]. *Drosophila* cytokines Upd3, Upd2 and Upd are released from different cells type in the intestine to promote stem cell proliferation through activation of the JAK/STAT pathway. While JAK/STAT is important for initiating damage repair pathways in response to stress, sustained activation of JAK/STAT, as occurs in old age, disrupts homeostatic processes of the gut, contributing to organismal death [42]. These age-related increases in the JAK/STAT signaling are attributed in large part to increased microbiota density, a commonly reported phenotype in aging *D. melanogaster* [7, 8, 10, 41, 43]. Over-expression of the JAK/STAT pathway leads to increased enterocyte damage and aberrant ISC proliferation resulting in midgut hyperplasia and reduced lifespan. Age-related increases in gut damage and microbiota density are associated with higher ROS production further contributing to activation of the JAK/STAT pathway. Previously, we reported that the midgut of *Indy* flies has reduced ROS accumulation and increased expression of genes encoding ROS-detoxification enzymes, which reduces age-associated hyperproliferation of ISCs [23, 24]. Given the impact of the microbiota on gut damage in aging and JAK/STAT activity, it is possible that the beneficial effects of *Indy* reduction are mediated through impacts on the fly microbiota and reduced JAK/STAT signaling pathway activation. Therefore, we hypothesized that reduction in *Indy* gene activity could also alter the gut microbiota, which in turns reduces JAK/STAT signaling. As such, reduced *Indy* gene expression would lead to lower bacterial load during aging, translating to reduced JAK/STAT signaling, which would be consistent with the previously reported preservation of intestinal homeostasis into old age, and lifespan extension in heterozygous flies [22, 23].

To address our hypothesis, we characterized the microbiota of young and old control and $Indy^{206}$/+ flies and found a significant decrease in bacterial load and increased bacterial diversity in aging $Indy^{206}$/+ flies compared to age-matched control flies. Additionally, we compared lifespan of control and $Indy^{206}$/+ flies with and without a microbiota to determine if lifespan extension in *Indy* heterozygotes was dependent on the presence of the microbiota. We found that the presence of the microbiota was not required for $Indy^{206}$/+ lifespan extension, but removal of microbes did enhance the effects of *Indy* reduction on longevity. In addition, transcriptomic analysis on the midgut of control *yw* and *Indy* on conventional and AX conditions revealed reduction in expression of the JAK/STAT signaling ligand Upd3, and Upd2 in young $Indy^{206}$/+ flies, which leads to slower activation of epithelial cell turnover in the gut and likely contributes to preserved intestinal stem cell homeostasis of *Indy* flies. Taken together, our study shows that microbiota is not required for longevity extension in *Indy* flies, however, reduced *Indy*

expression impacts microbiota load and diversity, which likely add to beneficial effects of *Indy* reduced gene activity on gut homeostasis and longevity.

RESULTS

Presence of the microbiota augments but is not required for lifespan extension in *Indy* heterozygotes

To determine if the previously reported differences in lifespan between control and $Indy^{206}$/+ flies depend on the presence of the microbiota, we assessed lifespan of both conventional (containing an un-altered microbiota) and axenic (containing no microbes) flies. Flies were passaged twice per week. Median lifespan of $Indy^{206}$/+ exceeded that of controls by 12 days in males (21%) and 8 days in females (21%) in conventional conditions (Figure 1A, 1B and Supplementary Table 1). In axenic conditions, median lifespan of $Indy^{206}$/+ flies was 7 days (10%) longer than controls in males and 16 days (50%) longer in females (Figure 1C, 1D and Supplementary Table 2 and Supplementary Figure 1A–1D), suggesting that removal of bacteria impacted control and *Indy*/+ flies to different extents. Thus, while the microbiota is not necessary for $Indy^{206}$/+ lifespan extension, microbes may impact control and *Indy* flies in different ways, potentially indicating direct interactions between the microbiota and *Indy* and/or citrate metabolism. While both male and female $Indy^{206}$/+ and male *yw* live longer in axenic condition compared to conventional, control female lifespan was not significantly different in axenic conditions (Supplementary Figure 1C and Supplementary Table 3). Lifespan studies were repeated by passing flies daily. Median lifespans of $Indy^{206}$/+ male flies were significantly increased compared to *yw* control male flies in conventional and in AX condition, while female $Indy^{206}$/+ flies showed small changes in median lifespan in conventional condition, and a significant increase in median lifespan in AX conditions compared to *yw* control females (Figure 1E, 1F and Supplementary Figure 1E–1H and Supplementary Table 3). Consistent with lifespans of flies that were passaged twice per week, males and female $Indy^{206}$/+, and *yw* male lived longer in AX conditions, while *yw* female lived shorter in AX condition compared to aging on a conventional diet (Figure 1E, 1F and Supplementary Table 4).

Bacterial load is lower in aging $Indy^{206}$/+ compared to control flies

It has been reported that aging-associated gut pathology is exacerbated by increased bacterial load in aging flies [7, 40, 43]. To determine the extent to which delayed aging phenotypes observed in $Indy^{206}$/+ flies may be attributed to microbiota load or diversity, we performed culture-dependent microbiota analysis of young and old *yw* controls and $Indy^{206}$/+ flies via plating of whole surface-sterilized fly homogenates on MRS agar (Figure 2A) and culture-independent analysis (Figure 2B). We found that while bacterial load was similar in the two genotypes at 7 days old, 40-day old $Indy^{206}$/+ flies had a roughly 10-fold lower microbiota load compared to their *yw* counterparts (Figure 2C). Repeated analysis showed that the total bacterial load was lower in $Indy^{206}$/+ female flies at both 7 and 40 days (Supplementary Figure 2A). In addition, we observed some differences in microbiota composition between the two genotypes, especially at 40 days old, as $Indy^{206}$/+ flies had a higher abundance of a specific isolate (isolate 2) that we determined by 16S rRNA sequencing (V3/V4 region) was most closely related to *Acetobacter pomorum* or *pasteurianus* (Figure 2D). Both genotypes contained similar levels of another isolate, closely related to either *A. malorum* or *A. orleanensis* (Figure 2D).

Lifespan extension of $Indy^{206}$/+ flies is not dependent on presence of *Acetobacter spp*

To determine if the presence of either cultured *Acetobacter* isolate was responsible for the lifespan differences between conventional control and $Indy^{206}$/+ flies, we generated gnotobiotic flies by inoculating axenic embryos with isolate 1 (*A. pomorum/pasteurianus*), isolate 2 (*A. malorum/orleanensis*), or a mix of both. We recorded lifespan of each gnotobiotic condition and found that, regardless of bacterial treatment, $Indy^{206}$/+ lifespan exceeded that of controls as seen in both conventional and axenic conditions (Figure 2E–2J). These results suggest either that: 1) neither *Acetobacter* isolate impacts lifespan extension of $Indy^{206}$/+ *flies*, or 2) that both isolates impact longevity in a similar fashion.

Microbiota composition differs between control and *Indy* flies

To further investigate microbial community composition in each genotype, we performed culture-independent 16S rRNA microbiota sequencing on *yw* controls, $Indy^{206}/Indy^{206}$ homozygous, and $Indy^{206}$/+ heterozygous flies (Figure 2B). While this analysis could not distinguish between species of *Acetobacter* as in Figure 2, it did reveal the presence of a member or members of the *Lactobacillus* genus, which are common members of the *D. melanogaster* microbiota. In both male and female flies, *Lactobacillus* was most prevalent in $Indy^{206}/Indy^{206}$, and $Indy^{206}$/+ flies, and was only present in a few control flies, particularly at 7 days old (Figure 3A). By 40 days, most $Indy^{206}/Indy^{206}$, and $Indy^{206}$/+ flies were still associated with *Lactobacillus spp*., whereas it was mostly absent

from *yw* aged-matched controls (Figure 3A). This difference in *Lactobacillus* presence between genotypes was reflected in the beta and alpha diversity metrics, which show greater differences between $Indy^{206}$/+ and *yw* controls than between *Indy* heterozygous and homozygous flies (Figure 3B-3E and Supplementary Figure 2B). Furthermore, $Indy^{206}/Indy^{206}$ and $Indy^{206}$/+ male and female flies have increased Shannon diversity compared to control yw flies (Figure 3F, 3G and Supplementary Figure 2C). A similar increase in Shannon diversity was confirmed in young and old $Indy^{206}$/+ female flies

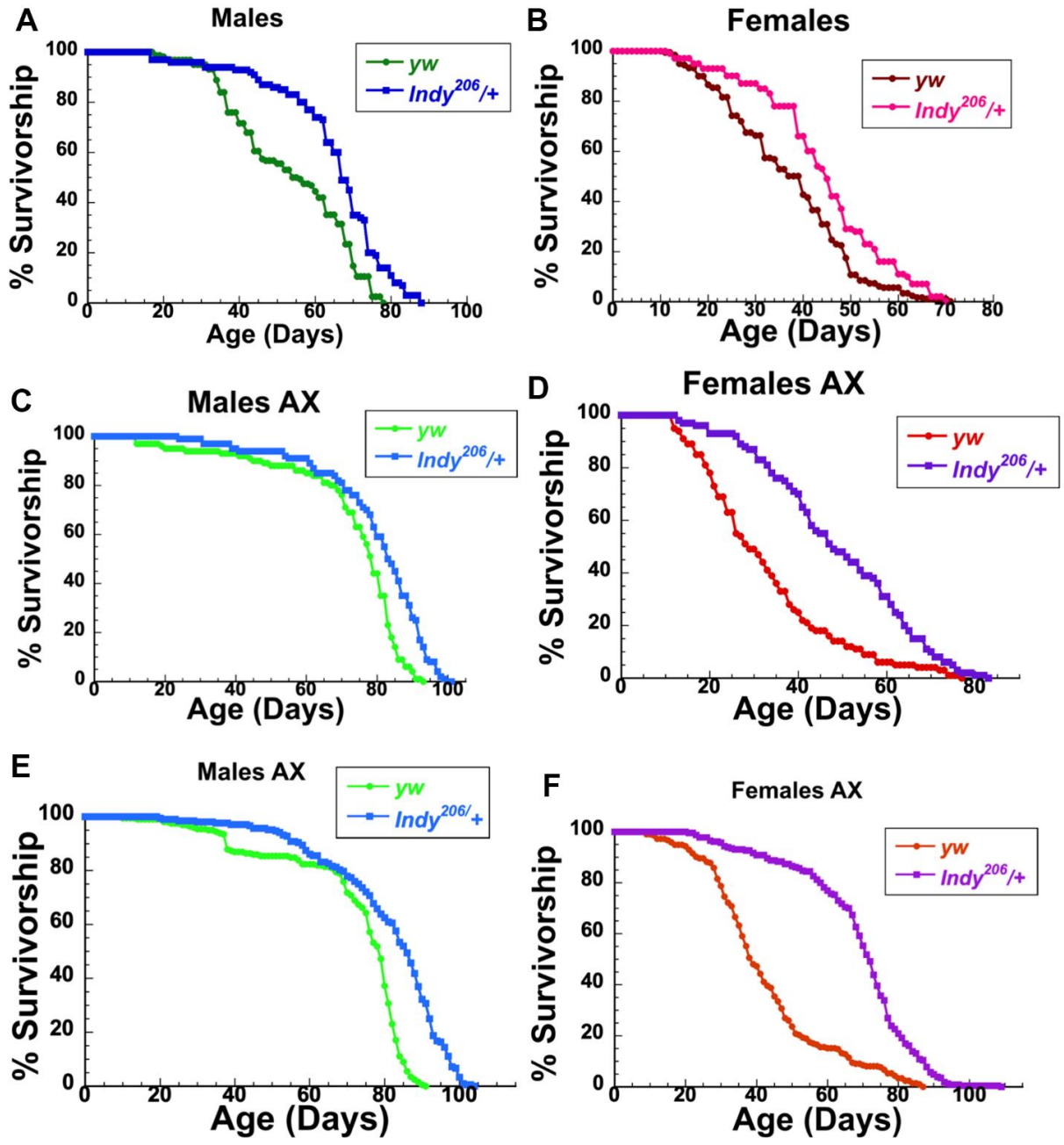

Figure 1. (**A**–**D**) Lifespan of conventional and axenic *yw* control and $Indy^{206}$/+ heterozygous flies passed twice per week (Supplementary Dataset 1). Longevity of conventional (**A**, **B**) and axenic (**C**, **D**) *yw* control (green (**A**), brown (**B**) light green (**C**), orange (**D**)) and $Indy^{206}$/+ (blue (**A**, **C**), magenta (**B**), purple (**D**)) male (**A**, **C**) and female (**B**, **D**) flies passed twice per week expressed as Kaplan-Meier survival curves. N= 131-187 individual flies per condition spread across 10 vials that were passed twice weekly. (**E**–**F**) Effects of axenic culture on lifespan of *yw* control and $Indy^{206}$/+ flies passed daily. Longevity of axenic *yw* control ((light green (**E**), orange (**F**)) and $Indy^{206}$/+ ((blue (**E**), purple (**F**)) male (**E**) and female (**F**) flies passaged daily expressed as Kaplan-Meier survival curves. AX: axenic.

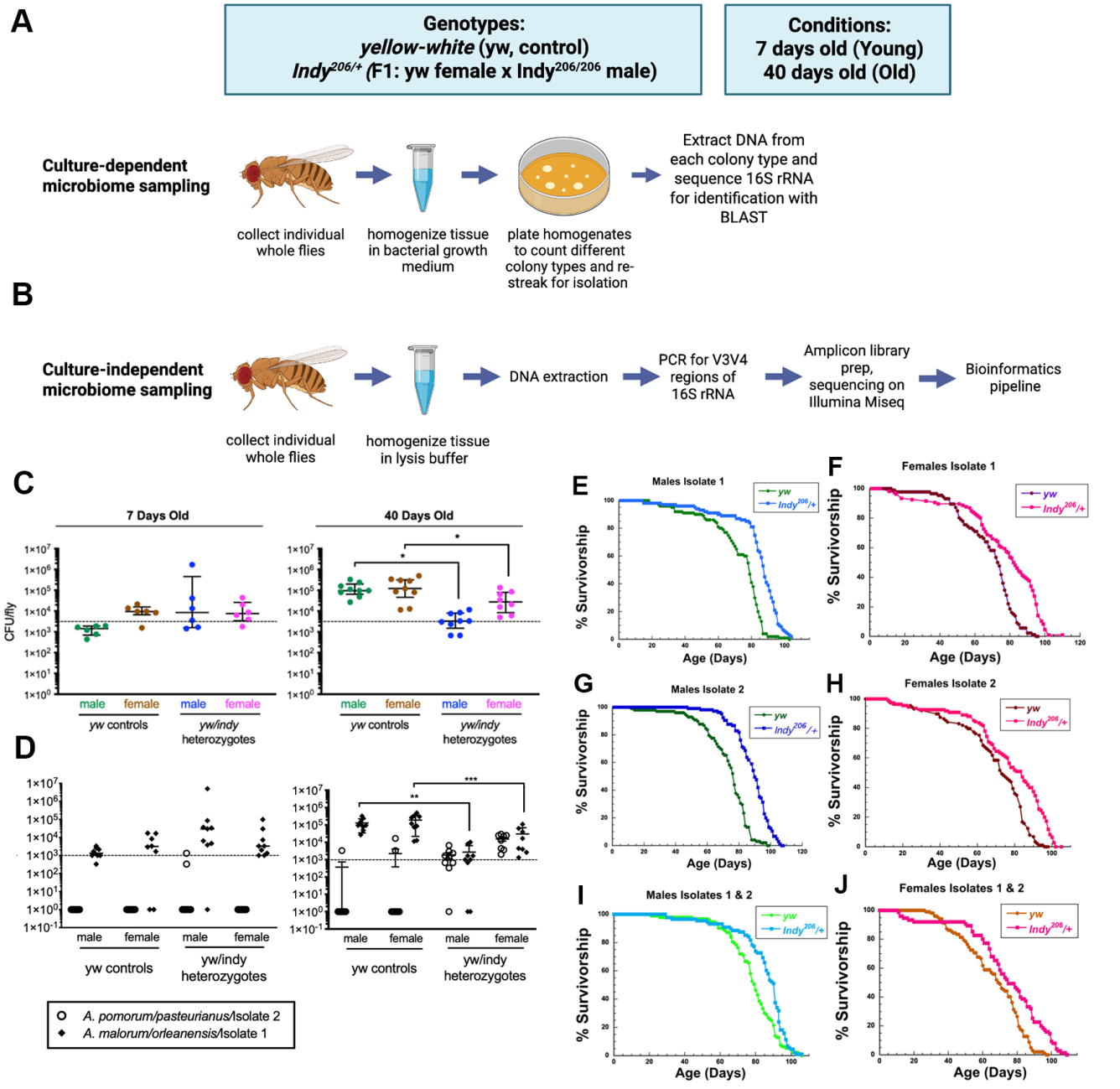

Figure 2. Experimental diagram: Workflow of culture-dependent (**A**) and culture-independent (**B**) microbial sequencing from individual whole flies, which were homogenized in bacterial growth media, plated and extracted DNA was sequenced (**A**), or DNA was extracted from homogenate, PCR for V4 region of 16S rRNA and sequenced (**B**). (**C**) Total bacterial load in 7 day and 40-day old *yw* controls and *Indy*^{206}*/+* flies. Bacterial counts are expressed as colony forming units (CFU) per fly, as determined by growth of colonies on MRS agar (Supplementary Dataset 2). Points represent individual flies; lines and error bars represent median and interquartile range of data points. (**D**) Load of bacterial isolates 1 and 2 detected in 7 day and 40 day old *yw* controls and *Indy*^{206}*/+* flies (Supplementary Dataset 3). Bacterial counts are expressed as colony forming units (CFU) per fly, as determined by growth of colonies on MRS agar. (**C**, **D**) Points represent individual flies; lines and error bars represent median and interquartile range of data points. Statistical differences between *yw* controls and *Indy*^{206}*/+* CFU counts were determined via two-way ANOVA with Bonferroni multiple comparisons tests. N= 3 replicate experiments collecting 3 individual flies per treatment group (n=9 flies per genotype/age). Statistical results not shown were non-significant. Significance is expressed as follows: ns, not significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. (**E**–**J**) Lifespan of gnotobiotic *yw* control and *Indy*^{206}*/+* heterozygous flies. Longevity of gnotobiotic control *yw* and *Indy*^{206}*/+* male (**E**, **G**, **I**) and female (**F**, **H**, **J**) flies containing Isolate 1 (**E**, **F**), Isolate 2 (**G**, **H**), or a mix of both isolates (**I**, **J**) expressed as Kaplan-Meier survival curves (Supplementary Dataset 4). Statistical differences between control and heterozygote lifespan were determined via Log-rank analysis. N= 84-125 individual flies per condition spread across 7-13 vials passed twice weekly. All survivorships of *Indy*^{206}*/+* were significantly longer compared to *yw* control flies P≤0.0001=****. het: heterozygote *Indy*^{206}*/+*.

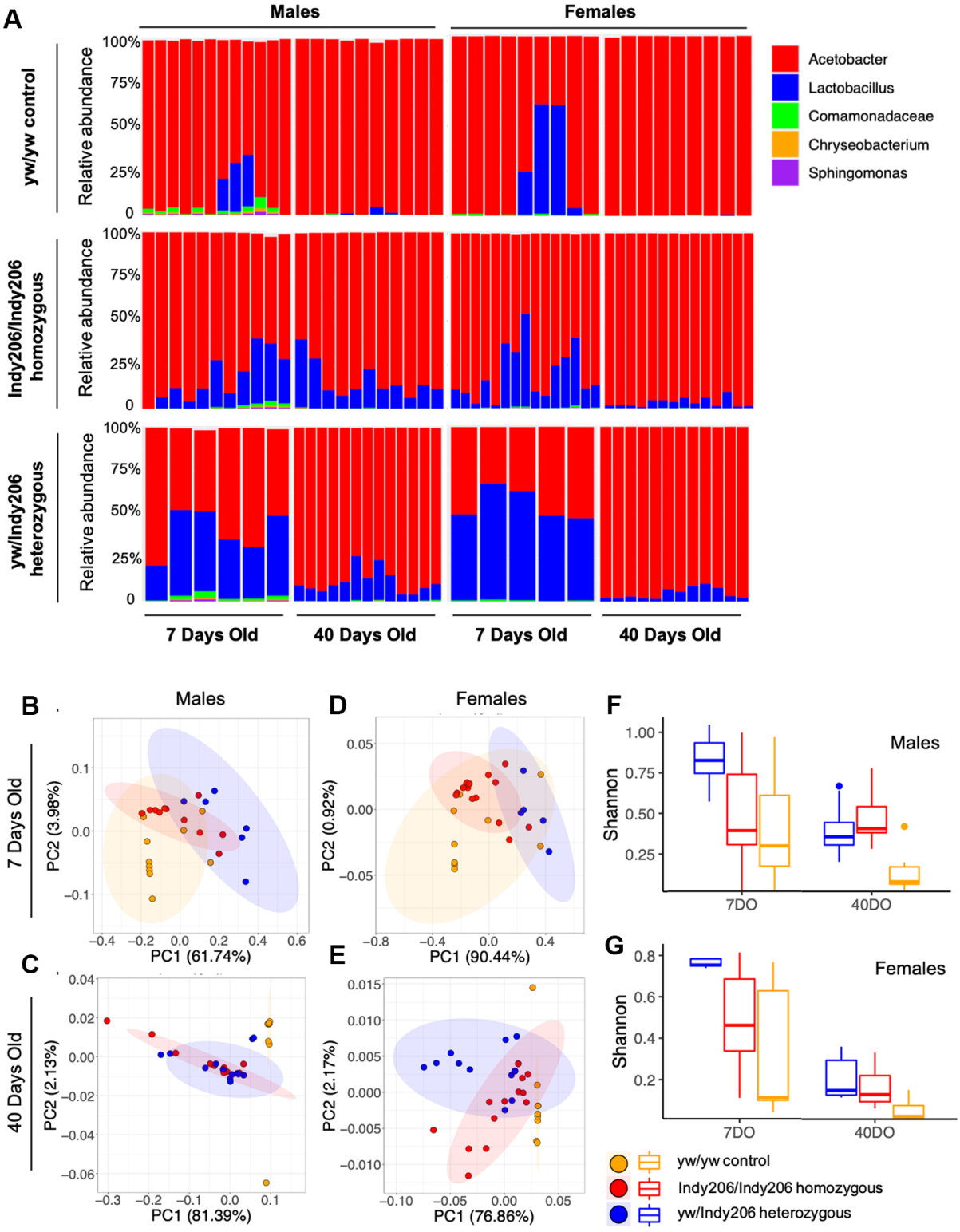

Figure 3. (**A**) Microbial community composition of young and old *yw* control, *Indy*^{206}/*Indy*^{206}, and *Indy*^{206}/+ flies. Plots represent relative abundance of top five genera of bacteria present in individual whole flies. (**B**–**G**) Beta and alpha diversity of young and old *yw* control, *Indy*^{206}/*Indy*^{206}, and *Indy*^{206}/+ flies (Supplementary Dataset 5). (**A**–**D**) Principal component analysis (PCoA) of Bray-Curtis distances between microbiotas of individual fly samples. Genotypes are indicated by different colors. Shaded ellipses represent 95% confidence intervals for clusters of each group. (**F**, **G**) Shannon diversity of each condition separated by males (**F**) and females (**G**) (Supplementary Dataset 6). Boxes range from the 25th to 75th percentile values for individual samples within each group; middle line shows the median; whiskers represent minimum and maximum values; individual points outside of minimum and maximum show outliers.

compared to controls in a second set of experiments (Supplementary Figure 2B). Along with our culture-dependent analysis, these differences in microbial diversity between aging *yw* controls and *Indy*^{206}/+ suggest that microbial diversity, in addition to total microbial load, may be impacting aging.

Indy reduction has a significant impact on host transcriptome

To determine molecular mechanisms and pathways underlying longevity extension and the effects of the reduced microbiota load in *Indy*^{206}/+ flies, we performed RNA-Seq analysis. We determined RNA-Seq profiles using three biological replicates at each time point and of each genotype. Bulk RNA was extracted from dissected midguts of female *Indy*^{206}/+ and *yw* control flies at 7 and 40 days of age, reared under conventional or axenic conditions. A sample-to-sample distance heatmap shows genotype and age to be the driving force for distance, indicating distinct transcriptional profiles across age, genotypes and rearing condition (Supplementary Figure 3A). This is corroborated by transcriptome-wide principal component (PC) analysis, which indicates that biological samples from the same age and genotype cluster together and are clearly distinct (Figure 4A). PC analyses of samples from the AX and conventional reared flies are more similar at age 7 and partially overlap, while samples of AX and conventional reared flies at 40 days are more distant (Figure 4A).

Difference in age-associated DE between control *yw* and *Indy* in conventional and AX conditions

The generation of axenic fly lines eliminates both gut and substrate specific microbes, as well as their compounds, therefore, our study can distinguish between effects of microbiota and *Indy* reduction on transcriptome of the midgut. To search for common upregulated and downregulated age-associated DE genes, we compared *yw* control and *Indy*^{206}/+ age-specific DE genes in flies reared in AX and conventional conditions (CR): Up:*yw* CR 7-v-40, Up:*yw* AX 7-v-40, Up: *Indy*^{206}/+ CR 7-v-40 and Up: *Indy*^{206}/+ AX 7-v-40 (Figure 4B). Similar comparison was performed for downregulated common and unique DE (Figure 4C). A Venn diagram shows only 66 commonly upregulated and 52 commonly downregulated DE genes across control and *Indy*^{206}/+ flies in AX and conventional conditions (FDR 0.02 (Figure 4B, 4C). Common upregulated genes belong to four KEGG pathways including metabolic pathways, carbon metabolism, cytoskeleton and motor proteins. Highest number of unique upregulated and downregulated DE genes are observed in *yw* CR 7-v-40 females N=370, and N=293, respectively (Figure 4B, 4C). This illustrates that *yw* flies reared with microbiota experienced the largest age-associated changes in midgut gene expression. As a consequence of increased bacterial load, there is an upregulation of the members of Cytochrome P450 family, including Cytochrome P4504g1, Cyp6a22, Cyp12d1-p, and Cyp12d1-d. The number of age-associated DE genes is much lower in both AX and conventional *Indy*^{206}/+ flies, consistent with lower bacterial load observed in aging *Indy*^{206}/+ flies (Figure 4B, 4C). There are 31 common upregulated DE genes in *Indy*^{206}/+ in AX and conventional conditions, that include Glutathione S transferase E10, a member of the glutathione pathway, and the Phosphoenolpyruvate carboxykinase 2 (Pepck2), a mitochondrial enzyme that is the key regulator in gluconeogenesis. Common downregulated DE genes in *Indy*^{206}/+ AX and conventional aging flies include tobi (target of brain insulin).

KEGG pathway analysis revealed upregulated fatty acid (FA) metabolism in samples from *Indy*^{206}/+ flies when compared with controls in AX and conventional conditions at 7 or 40 days of age (Figure 4D). Upregulated biological processes include FA biosynthetic processes, FA elongation, peroxisome, pentose phosphate pathway and ribosome (Figure 4D). FA metabolism is upregulated in *Indy* flies due to the shift to using FA oxidation as the preference for substrate oxidation. Similar changes in fat metabolism were reported in calorie restricted flies and rodents [30, 44]. Comparison of the transcriptome of 7-day old *Indy*^{206}/+ flies to 40-day old *Indy*^{206}/+ kept in both AX and conventional conditions, revealed similar upregulation of carbon metabolic processes and FA metabolic processes as *Indy* flies age (Figure 4E). Hippo signaling pathway is downregulated in 40 days old *Indy*^{206}/+ flies compared to *yw* control flies in CR conditions, which includes downregulation of cell proliferation, consistent with our previous report that *Indy* flies have reduced age-associated increase in intestinal stem cell (ISC) proliferation [23]. There is also downregulation of Hippo signaling pathway in *Indy* flies when 7- and 40-days old flies aged in AX condition were compared. In addition, *Indy* flies have downregulated bacterial invasion of epithelial cells, as well as Toll and Imd signaling pathways, in agreement with reduced bacterial load observed in 40 days old *Indy* flies (Figure 4E).

The role of the JAK/STAT signaling pathway in *Indy* longevity

We next examined if there is a link between reduced *Indy* expression and the JAK/STAT signaling pathway. We determined the levels of expression of Upd3, and

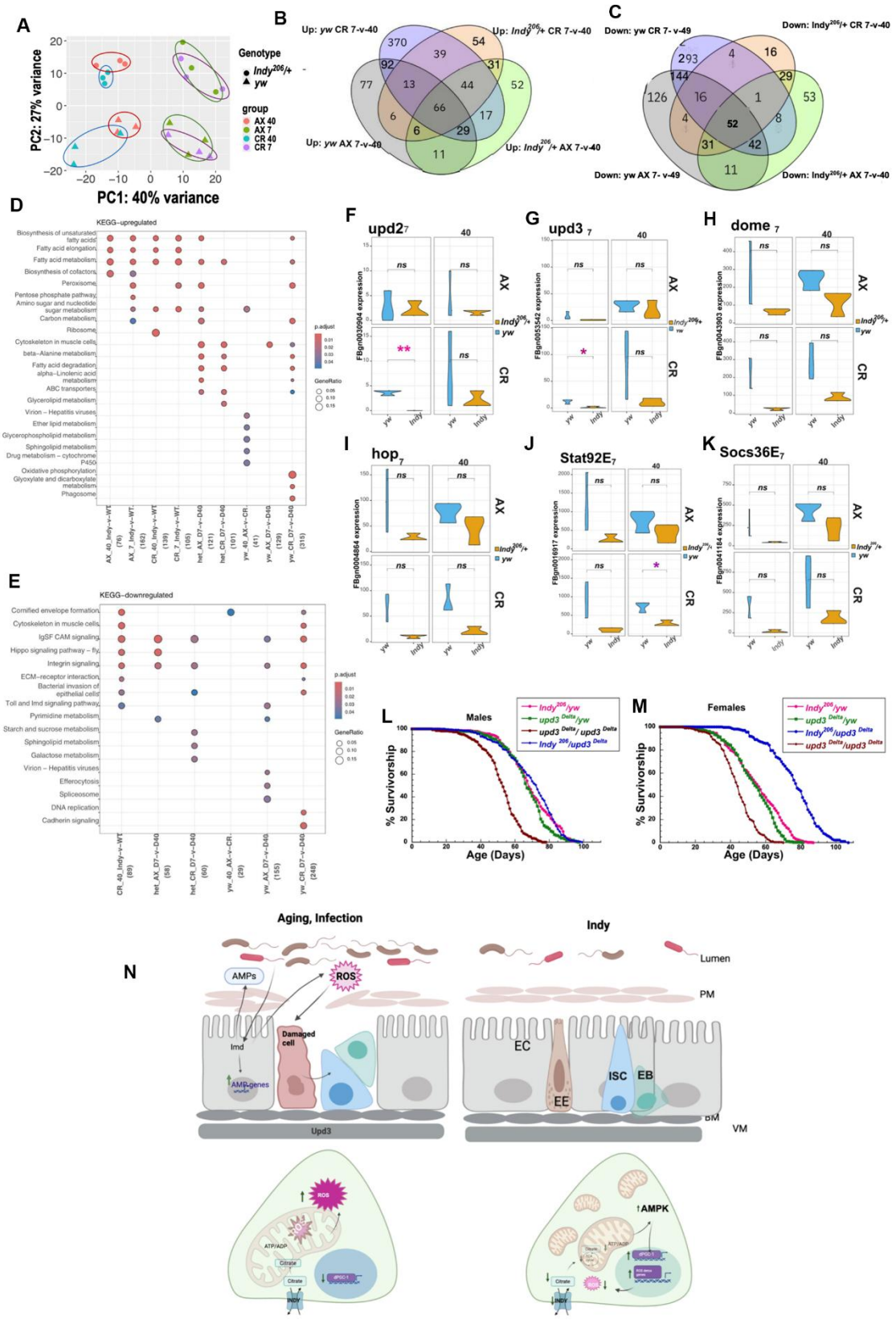

Figure 4. Effects of *Indy* reduction, age and AX condition on female midgut transcriptome. (**A**) PCA plots of differentially expressed genes in midgut samples from *Indy*^{206}/+ and *yw* control female flies in conventional (CR) and axenic (AX) condition at 7 and 40 days of age. (**B**, **C**) Venn diagrams show common or unique upregulated (Up) (**B**) or downregulated (Down) (**C**) DE genes between *yw* control and

Indy^{206}/+ in flies reared in AX or conventional conditions: Up: yw CR 7-v-40, Up: yw AX 7-v-40, UP: *Indy*^{206}/+ CR 7-v-D-40 and Up: *Indy*^{206}/+ AX 7-v-40. (**C**) The same comparison shown for downregulated DE genes. FDR threshold: 0.02, logFC threshold: 0 (**D**, **E**) Functional enrichment analysis shows KEGG pathways of upregulated (**D**) or downregulated (**E**) DE genes associated with *Indy* reduction, aging, conventional vs. AX conditions compared to *yw* controls. logFC threshold: 0.2. (**F**–**K**) *Indy* reduction affects JAK/STAT signaling pathway. *Indy*^{206}/+ and *yw* (control) differentially expressed gene analysis of count data of Upd2 (**F**), Upd3 (**G**), dome (**H**), hop (**I**) (Supplementary Dataset 7), Stat92E (**J**) and Socs36E (**K**) at 7 and 40 days of age in conventional (CR) and AX conditions. (**L**, **M**) The epistatic relationship between the *Indy* and JAK/STAT longevity pathways. Survivorships of male (**L**) and female (**M**) *upd3*^{Delta}/*upd3*^{Delta}(brown), *upd3*^{Delta}/+ (green), *Indy*^{206}/+ (magenta), *and upd3*^{Delta}/+;*Indy*^{206}/+ (black) flies expressed as Kaplan-Meier survival curves. Survivorship was determined by log-rank analysis using JMP16 program. N= 253-293 individual flies per genotype across 12 vials that were passed daily. (**N**) Proposed model of beneficial effects of *Indy* reduction on intestinal stem cell homeostasis. Intestinal integrity is preserved in aging *Indy* flies, compared to aging control flies. *Indy* reduction decreases bacterial load, and increases microbial diversity. Lower ATP/ADT ratio in Indy flies increases AMPK gene expression that leads to increased gene expression of the PGC1α, ROS detoxification genes and lower ROS production in mitochondria in the midgut, which all lead to preserved ISC homeostasis and longer lifespan.

Upd2, which are released from different cells type and promote proliferation by activation of the JAK/STAT pathway and epithelial cell turnover in the gut. We found significant decrease in the expression levels of Upd2 and Upd3 in *Indy*^{206}/+ flies aged in a conventional condition, but not in flies kept in AX condition at 7 days of age (Figure 4F, 4G). We also determined expression levels of other members of JAK/STAT signaling (Dome, Hop, Stat92E, and Socs36E) in the midguts of *Indy* and control flies (Figure 4H–4K). Hop is *Drosophila* homologue of mammalian JAK, Stat92E is *Drosophila* homologue of STAT transcriptional factor and Socs36E is suppressor of cytokine signaling. There is a significant decrease in the Stat92E levels of expression levels at age 40 in CR condition (Figure 4J). No differences were found in levels of Upd2, Upd3, Hop, and Socs36E at age 40 days in females that were kept in either conventional or axenic conditions (Figure 4H–4K). The levels of Upd2 and Upd3 were very low in *Indy*^{206}/+ flies compared to control flies at 7 days of age kept in conventional condition consistent with lower bacterial load in *Indy* flies at 7 days (bacterial load Figure 2C). While the levels of expression of *Dome* and *hop* in *Indy* flies were lower compared to control flies, the levels were not significantly different in females reared on either conventional condition or AX conditions (Figure 4H, 4I). Heatmap illustrate trend of lower expression levels of Upd2, Upd3, Hop, Dome, Socs36E and Stat92E in all *Indy*^{206}/+ samples compared to *yw* control with exception of *yw* samples isolated from 7 days old female in AX condition. These results are consistent with the lack of a microbiota in *yw* AX females and lower microbial count in *Indy*^{206}/+ flies (Supplementary Figure 3B).

We identified specific changes in the levels of immune and stress response genes in the midgut of control and *Indy* flies reared under conventional and axenic conditions. In aged flies, intestinal barrier dysfunction is associated with increased expression of immunity-related genes such as antimicrobial peptides (AMPs). To examine the role of the immune response in preserved intestinal integrity of *Indy* flies we determined expression levels of AMPs responsive to the microbiota and with activity predominately against Gram-negative bacteria (Diptericin A (DptA), Diptericin B (Dpt B)), as well as Drosocin (Dro), which is mostly active towards Gram-positive bacteria, and Drosomycin (Drs)), an antifungal peptide, using mRNA isolated from *yw* and *Indy*^{206}/+ females. There was no significant difference in levels of mRNA between *Indy* and control samples, however heatmap illustrates a trend of lower levels of AMPs genes in *Indy*^{206}/+ flies (Supplementary Figure 3C–3G).

Synergistic effects of Upd3 and *Indy* in longevity extension

To determine if *Indy* and JAK/STAT longevity pathways overlap we examined the epistatic relationship between these two pathways. Reducing *Indy* or JAK/STAT signaling extends fly lifespan, while overexpressing JAK/STAT activity reduces lifespan. If *Indy* and JAK/STAT pathways overlap, longevity of flies with simultaneous *Indy* and JAK/STAT reduction will be similar to longevity of flies with reduction in only *Indy* or Upd3. In contrast, if the two pathways are independent, longevity of flies with simultaneous reduction in both genes will be longer compared to a single mutant. To examine the role of Upd3 in longevity extension of *Indy* flies, we determined lifespan of *Indy*^{206}/+, *upd3*^{D}/+ heterozygous and *upd3*^{Delta}/*upd3*^{Delta} homozygous flies, and flies double heterozygous for *Indy*^{206}/+ and *upd3*^{Delta}/+. *upd3*^{Delta}/+ allele has a 13,464 bl deletion that removes the first three exons of Upd3 (including the start ATG) made by imprecise excision of P{XP}upd3(d00871) and is shown to lack function [45]. Both male and female *upd3*^{Delta}/*upd3*^{Delta} homozygous flies have the shortest median and maximal lifespan. *upd3*^{Delta}/+ male and female flies have 26% and 23% longer median lifespan compared to homozygous flies respectively (Figure 4L, 4M and Supplementary Table 5). Median longevity of *Indy*^{206}/+ flies is similar to *upd3*^{Delta}/+ flies when compared to homozygous *upd3*^{Delta} flies, (Median lifespan: males live 38%, females 27% longer.)

However, *upd3*^{Delta}/+;*Indy*^{206}/+ male flies have a median lifespan 38% longer, while female flies live 77% longer compared to homozygous *upd3*^{Delta} flies (Supplementary Table 5). These finding suggest that mechanisms underlying longevity extension observed in *Indy*^{206}/+ and *upd3*^{Delta}/+ partially overlap, but also have independent effects on longevity, more notably in females.

DISCUSSION

Indy and microbiota have vital roles in health and longevity

Here, we show that *Indy* reduction is associated with lower bacterial load and higher bacterial diversity in aging *Indy* flies compared to controls. We hypothesize that INDY as a plasma membrane citrate transporter affects microbiota via its impacts on cellular citrate levels. Reduction of SLC13A5 (the mammalian homolog of Indy) results in reduced intracellular citrate levels in mice [15]. INDY has affinity for several other intermediate metabolites including succinate, malate, and fumarate, however, its highest affinity is for transporting citrate [12, 13]. Cytoplasmic citrate is the substrate of ATP-citrate lyase (ACLy), generating cytosolic Acetyl-CoA and oxaloacetate. Acetyl-CoA is a building block for the synthesis of fatty acids and cholesterol, but it also affects gene expression and epigenetics [17]. Nonhuman primates on high fat, high sucrose diet for 2 years showed increase in hepatic *mIndy* levels, and their liver microarray analysis showed increase in hepatic lipid metabolism and inflammation [46]. Mice fed citrate exhibited increased inflammation in liver and white adipose tissue, as well as elevated insulin resistance [47]. Another factor contributing to levels of Krebs cycle intermediates in the gut is the presence of microbes. Based on their genomes, *Acetobacter* is predicted to produce succinate while *Lactobacillus* likely consumes both succinate and citrate [48, 49], meaning that cross-feeding between gut microbes likely contributes to metabolite flux, resulting in downstream effects on nutrient cycling [50, 51]. While citrate can promote growth of microbes such as *Lactobacillus*, it has also been shown to have bactericidal effects, possibly due to its ability to lower pH [52, 53]. Thus, changes to citrate levels may result in alterations to diversity depending on how different bacteria are able to tolerate these shifts. A recent study has shown that increased citrate consumption by young mice increases SLC13A5 levels, increases bacterial load, decreases microbiota diversity, and shifts composition characterized by decreased Bacteroidetes and increased Firmicutes [54]. Citrate consumption could alter gut pH promoting growth of acid tolerant microbiota [54]. Citrate consumption also supports short-chain FA synthesis, which when internalized in enterocytes activates HIF-1a, in a non-hypoxic dependent pathway leading to increased colon permeability [54].

The mechanism by which age-associated increases in microbiota load decreases ISC homeostasis and contributes to shorter lifespan is thought to be tied to constitutive activation of the immune response, including release of reactive oxygen species and antimicrobial peptides, which damage enterocytes [7]. This damage activates the JAK/STAT signaling pathway to repair and renew the gut epithelium, however, age-associated increases in bacterial load and dysbiosis are thought to lead to continuous JAK/STAT activation, resulting in hyperproliferation and loss of ISC homeostasis which causes further gut deterioration and dysplasia, and subsequently host death [42]. We hypothesized that *Indy* reduction preserves midgut integrity and prevents intestinal barrier dysfunction by affecting host-microbiota interactions, thus preventing the negative effects of age-associated increase of JAK/STAT signaling pathway (Figure 4N). In response to increased microbiota load with age, *Drosophila* cytokines Upd, Upd2 and Upd3 are released from different cell types and activate the JAK/STAT pathway. We found that *Indy* reduction prevented age-associated increases in microbiota load in male and female *Indy*^{206}/+ flies compared to *yw* control at 7 days of age. We also found that *Indy* reduction increases microbiota diversity across the fly lifespan. This is consistent with our findings that *Indy* reduction decreases transcriptional levels of Upd2, and Upd3, but not other members of the JAK/STAT (Dome, 9STAT92E, Soc36E) pathway in the midgut of young conventionally reared *Indy* flies. To examine the epistatic relationship between *Indy* and the JAK/STAT signaling pathway we determined lifespans of the *upd3*^{D} mutant flies as homozygous, heterozygous and double heterozygous *upd3*^{Delta}/+; *Indy*^{206}/+. Homozygous *upd3*^{Delta} flies have the shortest lifespans compared to heterozygous *upd3*^{Delta}/+ and *Indy*^{206}/+, which have similar and long lifespans. There is an additive effect on longevity when female flies are double heterozygous suggesting that the effect of *Indy* and *upd3* on female longevity only partially overlaps and are synergistic. Our data suggest that *Indy* reduction has downstream effects on the microbiota of the fly, preventing bacterial overgrowth and altering microbiota diversity, which impacts longevity at least partially in a JAK/STAT-mediated fashion.

Age-related increases in ROS production contribute to activation of JAK/STAT pathway, and ISC proliferation. We previously showed that *Indy* flies have reduced ROS levels and increased transcription of genes

encoding ROS-detoxification enzymes in the midgut of *Indy* heterozygous and homozygous flies, which could reduce JAK/STAT activity [23]. Another phenotype of *Indy* reduction in *D. melanogaster* is preserved mitochondrial function, which leads to reduced levels of reactive oxygen species in the fly midgut [23]. *Indy* heterozygous flies have increased spargel/dPGC1 mRNA expression levels, which lead to increased mitochondrial biogenesis as measured by increased mtDNA copies, increased number and decreased size of mitochondrial examined by electron microscopy (EM), increased mRNA levels of *l(2)neo, Pdsw* encoding components of complex I and *Cytochrome C oxidase* encoding a component of complex IV, and enhanced mitochondrial activity [23]. Genetic interventions that preserve mitochondrial capacity and lower ROS production promote regenerative homeostasis in the *Drosophila* midgut [55–57]. Recent studies have shown that transient exposure to low doses of oxidants in developing *D. melanogaster* results in the reduction of *Acetobacter* spp. in the gut, which culminates in both extended gut healthspan and extended lifespan [58]. In mice, mitochondrial function was shown to regulate reactive oxygen species production and, as a result, microbiota diversity [59], providing evidence that the effects of *Indy* reduction on the microbiota may have ties to its impacts on mitochondria and reactive oxygen species levels.

Despite the differences in the microbial communities of control flies and $Indy^{206}$/+, our lifespan analysis comparing conventional and axenic flies make it clear that the microbiota is not required for *Indy*'s effects on longevity. However, given that total removal of the microbiota resulted in an even greater lifespan extension in $Indy^{206}$/+ compared to control flies over that observed in conventional flies, we expect that the microbiota does factor into *Indy*'s effects on the host. It is possible that the microbiota prevents $Indy^{206}$/+ flies aged in conventional conditions from reaching their maximum lifespan by, along with increasing ROS and JAK/STAT signaling, affecting upstream levels of citrate or other Krebs cycle intermediates, which translates to downstream effects of *Indy* on energy homeostasis [23]. With the microbiota removed in axenic flies, these processes would be allowed to proceed unhindered, leading to a lifespan extension attributable to both the complete lack of microbiota and microbiota-independent factors. It is worth noting that effects of microbiota on flies' longevity depend on fly age, diet, environment and microbiota composition. In some studies, flies in axenic conditions live longer compared to siblings kept in conventional conditions [10]. Previously axenic flies live longer if they are exposed to microbiota early in life, but not when exposed late in life [60]. In a previous study, when germ-freen flies were fed different combinations of a the five core *Drosophila* microbiota, their resulting bacterial loads, development, reproduction and aging differed [51]. As such, in our study, we did not observe a significant lifespan extension of axenic control flies (yw Ax), as has been reported for other control genotypes. It is possible that microbiota isolates 1 and 2, or interactions between the different isolates improved lifespan in our gnotobiotic assays and may explain the conventional yw lifespan. Considering that flies with reduced *Indy* gene activity live longer when kept in both conventional and in axenic conditions, it would be useful in the future to compare activity levels, energy expenditure, or reproduction in axenic versus conventional flies. Previously, we reported that reducing *Indy* gene activity increases spontaneous physical activity [20], but does not affect fight velocity or resting metabolic rate [28]. *Indy* heterozygous flies have higher fecundity compared to genetic control in standard laboratory condition, but they have reduced fecundity on a reduced-calorie diet, which is consistent with hypothesis that reduced Indy gene activity phenocopies the CR condition [28]. Similarly, mice living in axenic conditions can exhibit positive or negative effects on longevity. Reduced lifespan of axenic mice compared to conventionally raised mice has been attributed to reduced immune development and increased susceptibility to infection. Other studies have reported altered neurodevelopment, stress responses, and anxiety-like behaviors in axenic mice that have been linked to microbiota-gut-brain axis [61]. Whether *Indy* reduction has a similar interaction with microbiota in flies would be an interesting follow up to our work.

In summary, here we show that *Indy* reduction prevents age-related dysbiosis, which typically is associated with an increase in bacterial load and decrease in microbiota diversity. We found reduced expressions of Upd2 and Upd3 in young *Indy* flies and Stat92E in old *Indy* flies reared in conventional condition, while no change in other members of the JAK/STAT signaling pathway were observed. Flies double heterozygous for $Indy^{206}$/+ and $upd3^{Delta}$/+ alleles lived longer than single heterozygous flies, suggesting synergistic effects *Indy* and upd3 pathways on longevity. Together with our prior work, an interesting model is emerging that changes in metabolism associated with *Indy* reduction lead to increased intestinal homeostasis and health that is characterized by preserved mitochondrial function and reduced ROS production, reduced aberrant intestinal stem cell proliferation, preserved gut integrity, and reduced age-associated dysbiosis, leading to healthier and longer life of *Indy* flies (Figure 4N). As the role of INDY and microbiota are conserved across organisms, our study provides a framework to study

underlying mechanisms of the effects of reduced *Indy* and microbiota on health and longevity.

MATERIALS AND METHODS

Fly stocks and rearing

The *yellow white (yw)* control line was obtained from the Bloomington Stock Center (Stock number 6599, *y(1(w(67c23)*). *Indy*^{206} flies were originally obtained from Tim Tully [11, 62], *w(*)upd3)Delta)* was obtained from the Bloomington Stock Center (Stock number 55728). *yw* and *Indy*^{206} flies were reared on food containing 25 mg/mL tetracycline for 3 generations to eliminate *Wolbachia*. This treatment was followed by growing flies for at least 10 generations in tetracycline-free food. Flies were recovered from antibiotics for at least six months prior to experiments to allow for regrowth of the microbiota. To addressed genetic background *Indy*^{206} flies were backcrossed to *yw* flies for 10 generations prior experiments. Heterozygous *Indy*^{206}/+ flies were the F1 progeny of 10 virgin *yw* female crossed with 9 *Indy*^{206}/*Indy*^{206} male flies. Flies were reared on food containing per liter of water: 28 g Brewer's yeast, 49 g yellow cornmeal, 113 g sucrose, 8.1 g Drosophila agar, and 2.4 g Tegosept (Methyl4-hydroxybenzoate) dissolved in 10.7 mL of 100% ethanol as in [63]. Food was autoclaved prior to addition of Tegosept. Stocks were maintained in a 25 °C incubator with a 12-hour light:dark cycle, and newly emerged flies were passaged to fresh vials every 3-4 days.

Generation of axenic flies

Using embryo cages, *yw* and *Indy*^{206}/*Indy*^{206} flies laid eggs overnight on grape juice agar plates smeared with yeast paste. The next morning, plates were rinsed with 70% ethanol, then PBS, and embryos were lifted from the agar with sterile swabs. Embryos were poured into cell strainers and rinsed with 5% bleach until dechorionation was confirmed under a dissecting microscope. Embryos were then rinsed well with sterile water and suspended in 100% ethanol before being transferred via pipette to sterile fly food to develop. Axenic flies were maintained by passaging flies only in a biosafety cabinet or next to a flame. To mate axenic *yw* and *Indy*^{206}/*Indy*^{206} flies or collect axenic flies for experiments, CO_2 fly pad and paintbrush were thoroughly bleached, then treated with 70% ethanol, then put under UV light for 15 minutes in a PCR cabinet, where flies were then collected. Stocks and experimental vials containing axenic flies were regularly screened visually (axenic tubes have a distinct darkening of the media and no visible microbial growth as described in [64]) and via plating to ensure axenic status was maintained (all fly microbiome members in these studies were culturable).

Lifespan studies

Newly emerged *yw* or F1 *Indy*^{206}/+ (cross described in Fly Stock Rearing) flies were collected and passed onto fresh food twice per week (Figure 1A–1D) or daily (Figure 1E, 1F). Each vial contained 20 male and 20 female flies, and 10 replicate vials per treatment were established. Deceased flies were recorded regularly until all flies in each vial were dead. Heterozygous flies used in survivorship analysis described in Figure 2H, 2I were generated by crossing 10 virgin 4-9 days old female to 9 male 4-9 days old flies. Parents were passed to a new vials every 2 days: a) *Indy*^{206}/+ were F1 generation from crosses in which virgin *Indy*^{206}/*Indy*^{206} homozygous female flies were crosses to *yw* males; b) *upd3*^{D}/+ were F1 generation of cross in which virgin *upd3*^{D}/*upd3*^{D} homozygous female flies were crossed to *yw* males; c) *upd3*^{D}/+; *Indy*^{206}/+ were F1 generation from crosses in which virgin *upd3*^{D}/*upd3*^{D} homozygous female were crossed to *Indy*^{206}/*Indy*^{206} homozygous male flies. F1 progeny for lifespan studies were collected within 24 hours following eclosion using CO_2 and transferred to a vial at density of 25 male and 25 female flies per vial containing conventional corn diet. 12 replicate vials were established. Flies were maintained in a humidified temperature-controlled environmental chamber at 25 °C (Percival Scientific) on a 12-hour light:dark cycle. Flies were passed every day, and the number of dead flies were counted. Longevity data were censored for early mortality (1-10 Days) to remove death due to post-eclosion maturation, or other deaths that are not related to aging. The number of censored flies is listed in brackets in Supplementary Tables 1, 2, and 5. Survivorship curves were analyzed by log-rank test JMP16 program.

Culture-dependent microbiota analysis

Flies were surface sterilized in 70% ethanol to remove external microbes, then washed in sterile PBS. Individual whole flies were collected in 1.5 mL screw-top vials containing 1 mm glass beads and 500 µL of sterile PBS. Samples were homogenized via bead-beating for 45 s at 4000 rpm. Homogenate was diluted to 10^{-5} in PBS using 96-well plates, then 3 µL of each dilution was pipetted onto MRS agar plates. Colony growth was recorded after 3-5 days. Colony types were determined visually, and colonies of different morphologies were re-streaked for isolation, then subjected to DNA extraction using the Epicentre MasterPure kit. Purified bacterial DNA was submitted to Eurofins for Sanger sequencing using primers 27F (5'-AGAGTTTGATYMTGGCTCAG-3') and 519R

(5'-GTATTACCGCGGCKGCTG-3'). DNA sequences were run through BLAST to obtain genus-level identification predictions (or species-level if possible).

16S rRNA microbiota sequencing

Flies were surface sterilized in 70% ethanol and washed in sterile PBS. Individual whole flies were collected in 1.5 mL screw-top vials containing a 1:1 mix of 1 mm glass beads and 0.5 mm glass beads and 300 µL of tissue and cell lysis buffer with proteinase K (Epicentre MasterPure DNA kit). Samples were homogenized via bead-beating for 45 seconds at 4000 rpm, then frozen at -80 °C. Upon thawing, samples were again homogenized as performed previously to ensure tissue degradation. DNA extraction was performed following the Epicentre MasterPure protocol and 16S rRNA library preparation and sequencing was performed by the University of Connecticut Microbial Analysis, Resources, and Services (MARS) facility. Briefly, the V4 region of the 16S rRNA gene was PCR amplified using 515F and 806R primers with Illumina adapters and dual indices. Libraries were cleaned following the Omega Bio-Tek Mag-Bind Beads protocol and sequenced on the Illumina MiSeq (2x250 base pair kit).

Bacterial culturing and generation of gnotobiotic flies

Bacterial strains isolated from the culture-based microbiota analysis were kept at -80 °C in 50% glycerol for long-term storage. To generate gnotobiotic flies, bacteria were streaked on MRS agar plates, then single colonies were transferred to flasks containing a 1:1 mix of MRS broth and mannitol broth. Liquid cultures were grown shaking at 29 °C, 200 rpm, for up to 24 hours. Cultures were set to an OD_{600} of 0.5 (about 10^5 cells per mL), and 150 mL of a 1:1 mix of diluted culture and 2.5% sucrose was fed to axenic adults by dispensing it onto the food to soak into the diet before fly transfer. After several days of laying, adults were discarded and F1 progeny were collected for lifespan analysis.

Bioinformatics and statistical analyses

16S rRNA sequences were processed using Mothur v. 1.39.4 following the MiSeq SOP [65]. Sequences were clustered at 97% similarity and rarified to 10,000 reads for alpha and beta diversity metrics. Taxonomy plots, Shannon alpha diversity plots, and Bray-Curtis beta diversity principal component analysis plots were generated in R using ggplot2. Lifespan was analyzed in GraphPad Prism using Kaplan-Meier survival; statistical significance of differences in survival between genotypes was determined via Log-rank test. Statistical differences between *yw* controls and *Indy* heterozygote CFU counts were determined via two-way ANOVA using GraphPad Prism. Significance is expressed as: P>0.05=ns, P≤0.05=*, P≤0.01=**, P≤0.001=***, P≤0.0001=****.

RNA-Seq and analysis (RiboZ-Seq)

For RNA-Seq experiments flies were collected and maintained as described above. At ages 7 and 40 d, *Indy*^{206} heterozygous and *yw* flies, aged in conventional and axenic conditions were separated by sex on CO_2 pads and midguts were dissected from female flies. Dissections of female midguts were performed under sterile conditions, which was achieved by cleaning all equipment (Microscope, centrifuge) and forceps with 70% alcohol, RNAse detergent (RNaseZap™, Thermo Fisher Scientific) and using a flame to create a sterile zone around dissecting area. All supplies and solutions in RNA-seq experiment were sterile, RNAse, and DNAse free. Total RNA was isolated from 3 biological replicates from 30-40 female flies in each replicate, except samples from 40 days old *yw* female aged in axenic condition, which had between 17 and 26 guts in each replicate. Total RNA was isolated using Trizol as described [23]. One mg of each RNA sample was used to prepare RNA-seq libraries with the Illumina Tru-Seq Stranded mRNA Library Preparation kit (cat # 20020594) with the IDT for Illumina TruSeq RNA UD Indexes set (cat # 20040871). The libraries were pooled and sequenced on one lane of a NovaSeq 6000 S4 flow cell generating 100bp paired-end reads yielding an average of 80 million reads per sample and a minimum of 20 million reads per replicate.

mRNA-Seq analysis

The quality of the sequencing data was evaluated with FastQC (v0.11.9) and MultiQC (1.9). Sequence reads were mapped to the *Drosophila* genome (GSE97233) with STAR (version 2.71a) and the resulting BAM files were used to generate gene counts with featureCounts using the unique-counting mode. Analyses of sample relationship and differential expression were performed with DESeq2 [66]. To identify specific pathways associated with diet shift, we performed functional enrichment analysis using the DAVID database. Pathway analysis and identification of networks associated with shifting flies were also carried out by Gene Ontology (GO) and the KEGG.

Data sharing plans

All the bulk RNA-Seq data have been deposited at NCBI BioProject and are publicly available as of the date of publication. Submission ID: SUB16031777 BioProject ID: PRJNA1431527.

BioProject and associated SRA metadata are available at https://urldefense.com/v3/__https://dataview.ncbi.nlm.nih.gov/object/PRJNA1431527?reviewer=ikldhgv7huct0c009ub9v0juc5__;!!ADdU39pX!WSXLCZy5lEgmAPDjqFY3I6ZAzYCm1rGIv4n52fiOwtvdZKbYgDOlJ-sGlCSHraBbZkeyzrhogweV1jtG7w$ in red only format.

Original codes used for data analysis are available through GitHub:

https://github.com/graveleylab/Microbiome-Indy-Longevity-in-Drosophila

Raw data for survival and microbiota analysis (CFUs, 16S rRNA pipeline) is available in supplemental data and posted on Figshare (Reviewers access) (DOI to be updated upon publication).

AUTHOR CONTRIBUTIONS

Conceptualization, B.R., N.A.B; Methodology and Investigation, D.N.A.L., S.P, J.M., E.S., K.K., S.O., B.R.G., B.R., N.A.B; Writing – Original Draft and Figure Creation, D.N.A.L., E.S., K.K., S.O., B.R., N.A.B; Writing – Review and Editing, Supervision, D.N.A.L., E.S., S.O., B.R., N.A.B.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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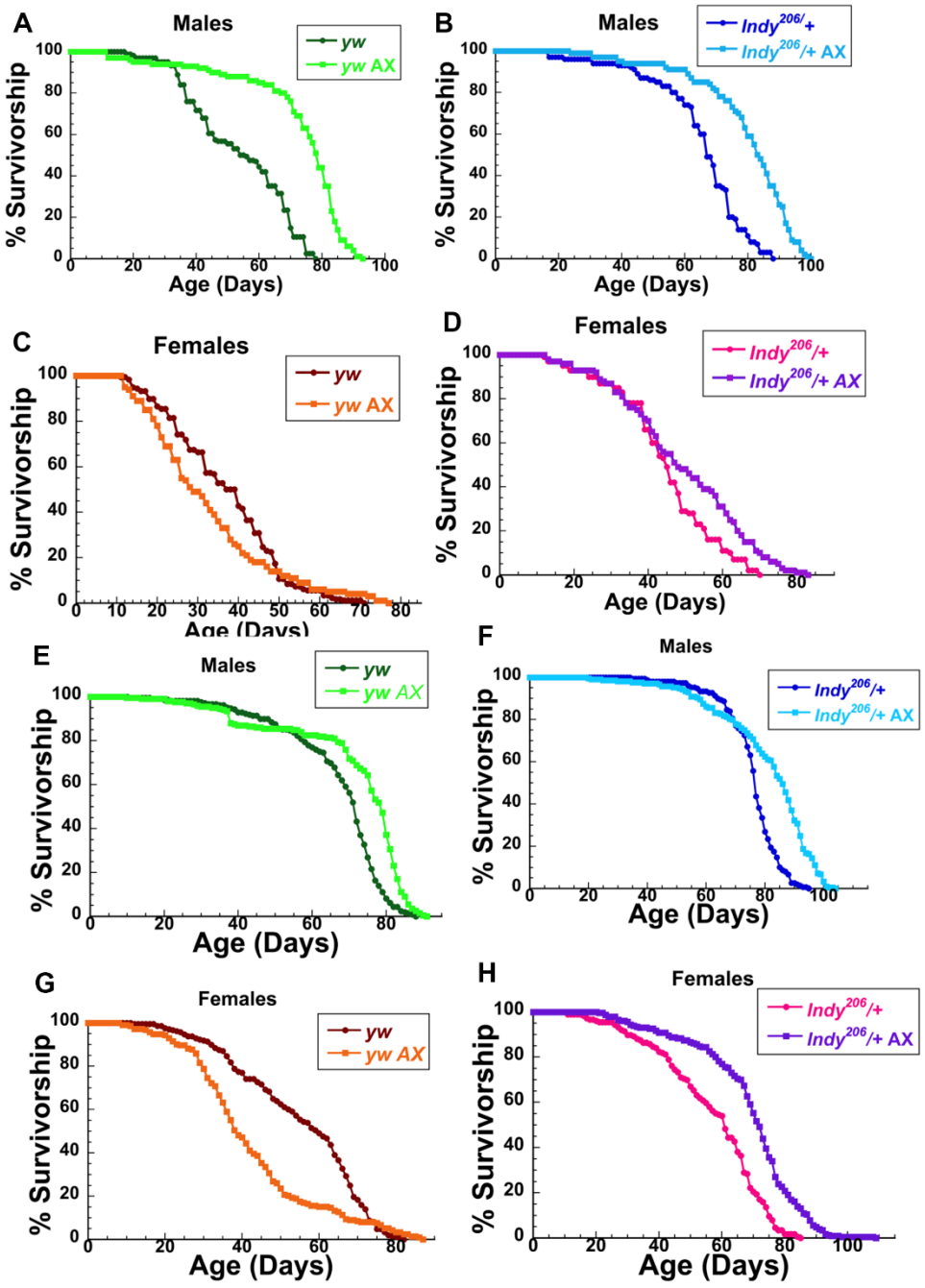
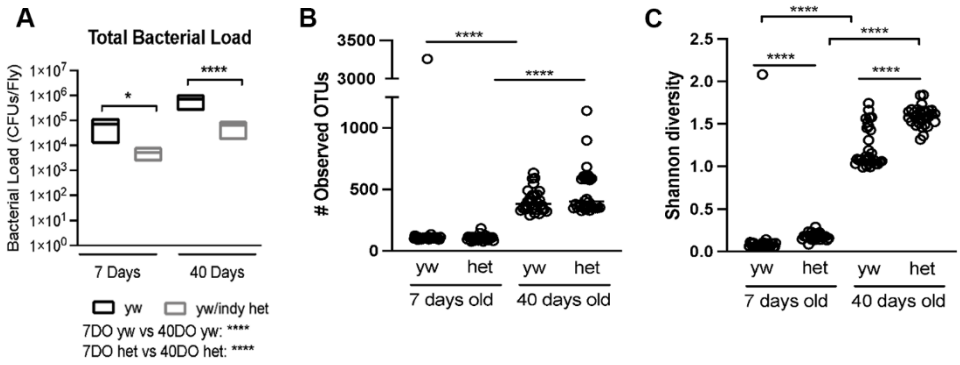
SUPPLEMENTARY MATERIALS

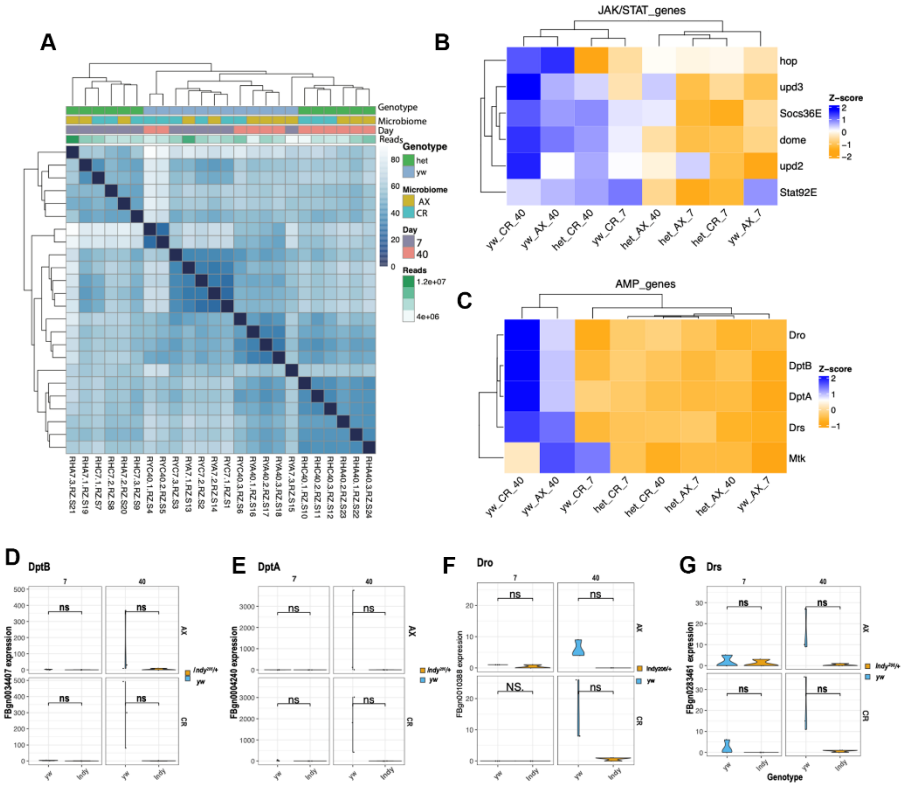
Supplementary Figures



Supplementary Figure 1. Effects of axenic culture on lifespan of control and *Indy*^{206}*/+* flies passed twice per week (**A**–**D**) (Supplementary Dataset 8) or daily (**E**–**H**). Axenic conditions extend lifespan of *yw* (**A**, **E**) and *Indy*^{206}*/+* (**B**, **F**) males, and *Indy*^{206}*/+* females (**D**, **E**), but *yw* females live shorter in axenic condition (**C**, **G**). Longevity of conventional (green) and axenic (light green) *yw* males (**A**, **E**); conventional (brown) and axenic (orange) *yw* females (**C**, **G**); conventional (blue) and axenic (light blue) *Indy*^{206}*/+* males (**B**, **F**); conventional (magenta) and axenic purple *Indy*^{206}*/+* female (**D**, **H**) flies expressed as Kaplan-Meier survival curves. N= 131-187 individual flies per condition spread across 10 vials that were passaged twice weekly. AX: axenic.



Supplementary Figure 2. Bacterial load and diversity in young and old controls and *Indy*^{206}/+ heterozygous flies. (**A**) Total bacterial load in 7-and 40-day old *yw* control and *Indy*^{206}/+ female flies. Bacterial counts are expressed as colony forming units (CFU) per fly, as determined by growth of colonies on MRS agar. Lines and error bars represent median and interquartile range of data points. Statistical differences between *yw* control and *Indy*^{206}/+ female flies CFU counts were determined via two-way ANOVA. N= 3 replicate experiments collecting 3 individual flies per treatment group (n=9 flies per genotype/age). Statistical results not shown were non-significant. Significance is expressed as follows: ns, not significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. (**B**) Alpha diversity of young and old control, and *Indy*^{206}/+ heterozygous female flies determined by sequencing V3V4 region to better reach species level IDs.



Supplementary Figure 3. (**A**) A sample-to-sample distance heatmap shows the driving force for distance in each genotype (*yw*: control, het: *Indy*^{206}/+), age (7, 40 days) and rearing condition (CD: conventional, AX: axenic condition). (**B**) Heatmaps of members of JAK/STAT signaling pathway: Upd3, Upd2, Hop, Dome, Socs36E and Stat92E, in control and *Indy*^{206}/+ flies at 7 and 40 days of age in conventional and AX conditions. (**C**) Heatmaps of members of antimicrobial peptide (AMPs): Diptericin A (DptA), Diptericin B (DptB), Drosocin (Dro), and Drosomycin (Drs), in control and *Indy*^{206}/+ flies at 7 and 40 days of age in conventional and AX conditions. (**D**–**G**) The levels of DptB, DptA, Dro and Drs are not different in the midgut of *Indy* and control female flies.

Supplementary Tables

Supplementary Table 1. Effects of reducing *Indy* gene activity on longevity of flies aged in non-axenic or axenic condition compared to control *yw* flies when passed twice per week.

Genotype	Sex	Diet	N (n censored)	Mean LS (% change)	X^2	p	Maximal LS (% change)
yw	M	non-AX	162 (2)	53.7	38.171	<0.0001*	75.7
Indy^{206}/+	M	non-AX	131 (4)	65.5 (21)			84.7 (12)
yw	F	non-AX	178 (4)	36.9	18.7023	<0.0001*	59.3
Indy^{206}/+	F	non-AX	142 (5)	44.7 (21)			66.6 (12)
yw	M	AX	176 (6)	73.2	44.3441	<0.0001*	88.5
Indy^{206}/+	M	AX	147 (7)	80.4 (10)			96.4 (9)
yw	F	AX	187 (8)	32.9	59.4104	<0.0001*	65.5
Indy^{206}/+	F	AX	160 (5)	49.4 (50)			76.2 916)

M: Male, F: Female, non-AX: non axenic, AX: axenic.
*Statistically significant.

Supplementary Table 2. Effects of axenic culture on lifespan of control and *Indy*^{206}/+ flies passed twice per week.

Genotype	Sex	Diet	N (n censored)	Mean LS (% change)	X^2	p	Maximal LS (% change)
yw	M	non-AX	162 (2)	53.7	-181.8958	<0.0001*	75.7
yw	M	AX	176 (6)	73.2 (36)			88.5 (17)
yw	F	non-AX	178 (4)	36.9	2.5089	0.1132	59.3
yw	F	AX	187 (8)	32.9 (-10)			65.5 (10)
Indy^{206}/+	M	non-AX	131 (4)	65.5	106.5593	<0.0001*	84.7
Indy^{206}/+	M	AX	147 (7)	80.4 (23)			96.4 (14)
Indy^{206}/+	F	non-AX	142 (5)	44.7	14.7471	<0.0001*	66.6
Indy^{206}/+	F	AX	160 (5)	49.4 (11)			76.2 (14)

non-AX: non-axenic, AX: axenic.
*Statistically significant.

Supplementary Table 3. Effects of reducing *Indy* gene activity on lifespan of control and *Indy^{206}/+* flies aged in non-axenic or axenic condition when passed daily.

Genotype	Sex	Diet	N (n censored)	Mean LS (% change)	X^2	p	Maximal LS (% change)
yw	M	non-AX	208 (0)	67.2	49.9147	<0.0001*	82.7
Indy^{206}/+	M	non-AX	149 (1)	75.7 (13)			89.3 (8)
yw	F	non-AX	208 (3)	55.3	1.7025	0.1806	75.5
Indy^{206}/+	F	non-AX	176 (5)	56.7(3)			77.7 (3)
yw	M	AX	198 (3)	72.1	94.1875	<0.0001*	87.0
Indy^{206}/+	M	AX	208 (2)	81.0 (12)			99.8 (6)
yw	F	AX	212 (3)	42.8	169.5708	<0.0001*	76.9
Indy^{206}/+	F	AX	230 (3)	68.8 (61)			91.5 (21)

M: Male, F: Female, non-AX: non-axenic, AX: axenic.
*Statistically significant.

Supplementary Table 4. Effects of axenic culture on lifespan of control and *Indy^{206}/+* flies when passaged daily.

Genotype	Sex	Diet	N	Mean LS (% change)	X^2	p	Maximal LS (% change)
yw	M	non-AX	208 (0)	67.2	56.8646	<0.0001*	82.7
yw	M	AX	198 (3)	72.1 (7)			87.0 (5)
yw	F	non-AX	208 (3)	55.3	27.0522	<0.0001*	75.5
yw	F	AX	212 (3)	42.5 (-23)			76.9 (2)
Indy^{206}/+	M	non-AX	149 (1)	75.7	68.4399	<0.0001*	89.3
Indy^{206}/+	M	AX	208 (2)	81.0 (7)			99.7 (12)
Indy^{206}/+	F	non-AX	176 (5)	56.7	73.5511	<0.0001*	77.7
Indy^{206}/+	F	AX	230 (3)	68.8 (21)			91.5 (18)

M: Male, F: Female, non-AX: non-axenic. AX: axenic.
*Statistically significant.

Supplementary Table 5. Effect of reducing *Indy* gene activity on lifespan of *upd3^{Delta}* flies.

Sex	Genotype	N (n censored)	Median LS (% change)	X^2	p	Maximal LS (% change)
M	*upd3^{Delta}/upd3^{Delta}*	270 (10)	53			68
M	*upd3^{Delta} /+*	280 (1)	67 (26)	221.9	<0.0001*	87 (28)
M	*Indy^{206}/upd3^{Delta}*	253 (4)	73 (38)	235.1	<0.0001*	90 (32)
M	*Indy^{206}/+*	279 (3)	68 (28)	242.2	<0.0001*	90 (32)
F	*upd3^{Delta}/upd3^{Delta}*	263 (19)	44			60
F	*upd3^{Delta}/+*	273 (2)	54 (23)	105.0	<0.0001*	71 (18)
F	*Indy^{206}/upd3^{Delta}*	274 (3)	78 (77)	516.2	<0.0001*	98 (63)
F	*Indy^{206}/+*	293 (1)	56 (27)	130.1	<0.0001*	62 (3)
M	*upd3^{Delta}/+*	280 (1)	67			87
M	*Indy^{206}/upd3^{Delta}*	253 (4)	73 (9)	12.16	0.0005	90 (3)
F	*upd3^{Delta}/+*	273 (2)	54			71
F	*Indy^{206}/upd3^{Delta}*	274 (3)	78 (44)	339.7	<0.0001*	98 (38)

M: Male, F: Female.
**** <0.0001.
P=***.

Supplementary Datasets

Please browse Full Text version to see the data of Supplementary Datasets 1–8.

Supplementary Dataset 1. Survivorships data of conventional and axenic *yw* control and *Indy*^{206}/+ heterozygous flies passed twice per week (**A**–**D**) or passed daily (**E**, **F**). Survivorship data were censored for early mortality (1 – 10 Days) (Figure 1).

Supplementary Dataset 2. Bacterial load in 7 days old young and 40 days old controls and *Indy*^{206}/+ heterozygous flies. Bacterial counts are expressed as colony forming units (CFU) per fly as determined by growth of colonies on MRS agar (Figure 2C).

Supplementary Dataset 3. Bacterial loads of bacterial isolates 1 and 2 detected in 7 days old young and 40 days old *yw* controls and *Indy*^{206}/+ flies. Bacterial counts are expressed as colony forming units (CFU) per fly, as determined by growth of colonies on MRS agar, (Figure 2D).

Supplementary Dataset 4. Survivorships of gnotobiotic *yw* control and *Indy*^{206}/+ heterozygous male and female flies containing Isolate 1, Isolate 2, or a mix of both isolates, Figure 2E–2J.

Supplementary Dataset 5. Microbial community composition of young and old control, *Indy*^{206}/*Indy*^{206} homozygous, and *Indy*^{206}/+ heterozygous flies. Figure 3B–3E (7 days old and 40 days old are uploaded as FASTA file and the codes are available following the link: https://figshare.com/s/b047a29fa0e52c7b52d3; https://figshare.com/s/be61c39aa6ae77ec6bb1.

Supplementary Dataset 6. Shannon diversity of each condition separated by males (**F**) and females (**G**). Original codes used for data analysis are available: https://figshare.com/s/e5a99f43871474c5adf8.

Supplementary Dataset 7. Survivorships of male and female *upd3*^{D}/*upd3*^{D}, *upd3*^{Delta}/+, *Indy*^{206}/+, and *upd3*^{Delta}/+;*Indy*^{206}/+ flies. Survivorship data were censored for early mortality (1 – 10 Days) (Figure 2H, 2I).

Supplementary Dataset 8. Survivorships of *yw* control and *Indy*^{206}/+ flies passed twice per week (**A**–**D**) or daily (E–H) on a conventional or axenic conditions. AX=Axenic (Supplementary Figure 1).