

Increased longevity mediated by yeast NDI1 expression in *Drosophila* intestinal stem and progenitor cells

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Abstract: A functional decline in tissue stem cells and mitochondrial dysfunction have each been linked to aging and multiple aging-associated pathologies. However, the interplay between energy homeostasis, stem cells, and organismal aging remains poorly understood. Here, we report that expression of the single-subunit yeast alternative NADH dehydrogenase, *ndi1*, in *Drosophila* intestinal stem and progenitor cells delays the onset of multiple markers of intestinal aging and extends lifespan. In addition, expression of *ndi1* in the intestine increases feeding behavior and results in organismal weight gain. Consistent with increased nutrient uptake, flies expressing *ndi1* in the digestive tract display a systemic reduction in the activity of AMP-activated protein kinase (AMPK), a key cellular energy sensor. Together, these results demonstrate that *ndi1* expression in the intestinal epithelium is an effective strategy to delay tissue and organismal aging.

INTRODUCTION

Identifying the molecular and cellular mechanisms that underlie organismal aging represents an urgent biomedical challenge. Towards this goal, considerable attention has been focused on the progressive decline in stem cell functions [1] and, separately, mitochondrial activity [2] that occurs during aging. Fundamental questions remain, however, regarding the relationships among mitochondrial activity within stem cell populations, tissue homeostasis, and organismal aging. Nutrient intake is closely related to energy homeostasis, stem cell maintenance and lifespan determination [3]. Indeed, moderate dietary restriction (DR) can delay the onset of pathology and extend lifespan in diverse species, from yeast to primates [4]. Similarly, many of the genetic mutations that have been reported to extend

organismal lifespan are thought to decrease the activity of nutrient signaling pathways, such as the insulin/insulin-like growth factor signaling (IIS), and the target of rapamycin (TOR) signaling pathways [5]. Critically, the specifics of how alterations in tissue or organ homeostasis affects nutrient signaling pathways and aging of the whole organism remain poorly understood.

The integrity of the intestinal epithelium is essential for maintaining barrier function, nutrient uptake, metabolic homeostasis, and hence, organismal health and survival. In *Drosophila*, the midgut epithelium is maintained by multipotent intestinal stem cells (ISCs), which are distributed along the basement membrane [6, 7]. Division of an ISC gives rise to one daughter cell that retains stem cell fate and another daughter cell that

becomes an enteroblast (EB). During aging, there is a dramatic increase in ISC proliferation which is accompanied by the accumulation of cells that express markers of both ISCs and terminally differentiated daughter cells [8, 9]. In addition, loss of intestinal barrier function has been shown to accompany aging across a range of *Drosophila* genotypes and environmental conditions [10]. Moreover, the age-dependent loss of intestinal integrity is linked to multiple markers of organismal aging, including systemic metabolic dysfunction, increased expression of immunity-related genes, reduced spontaneous physical activity and, critically, is a harbinger of death [10]. Recently, we have characterized the role of the *Drosophila* PGC-1 homolog (*dPGC-1/spargel*), a key regulator of mitochondrial energy metabolism, in the maintenance of ISC quiescence, intestinal integrity, and lifespan determination [11]. More specifically, up-regulation of *dPGC-1* in ISC/EBs delays the onset of markers of intestinal aging and confers increased longevity. However, given the diverse roles that PGC-1 plays in metabolism [12], the question of whether an increase in mitochondrial activity alone, in ISC lineages, is sufficient to confer these phenotypic outcomes remains to be determined.

The single subunit alternative internal NADH dehydrogenase (*ndi1*) from *Saccharomyces cerevisiae*, which lacks a conventional electron transport chain (ETC) complex I, can function in *Drosophila* mitochondria and is able to complement and supplement endogenous ETC complex I [13-15]. Here, we expressed *ndi1* in *Drosophila* somatic stem cell lineages and examined its impact on tissue and organismal aging. *ndi1* expression in ISCs/EBs improves tissue homeostasis in the aging intestine and confers increased longevity at the organismal level, demonstrating that increased NADH dehydrogenase activity alone is sufficient to produce these beneficial effects. Among other phenotypes associated with increased longevity, we find that flies with ISC/EB-specific *ndi1* expression display increased feeding behavior and whole body alterations in metabolic signaling pathways. Consistent with an increase in nutrient intake, long-lived *ndi1* flies show a systemic reduction in the activity of AMP-activated protein kinase (AMPK), a key cellular energy sensor [16]. Our results reveal novel roles for a NADH dehydrogenase in modulating stem cell behavior and intestinal homeostasis during aging. Moreover, we show that enhanced mitochondrial complex I activity in ISC lineages can simultaneously alter feeding behavior in adult flies and prolong lifespan.

RESULTS

Expression of *ndi1* in intestinal stem and progenitor cells extends lifespan

The intestine is a critical target organ with respect to genetic manipulations that can extend longevity [17], as has been shown previously with *dPGC-1* upregulation [11]. To better understand the relationships among mitochondrial respiratory chain activity, intestinal homeostasis, and lifespan determination, we expressed a previously described *UAS-ndi1* construct [14, 15] in the *Drosophila* intestine using the intestine-specific RU486-inducible Gene-Switch driver line *TIGS-2* [18]. Unlike the endogenous *Drosophila* ETC complex I which is sensitive to rotenone inhibition but insensitive to flavone, ND11 is insensitive to rotenone but inhibited by flavone [19]. Induced expression of *ndi1* in the adult intestine produced a robust rotenone-insensitive, flavone-sensitive NADH dehydrogenase activity in mitochondria isolated from intestines (Figure 1A). Control flies from the same background strain that were not provided RU486 did not show detectable levels of rotenone-insensitive, flavone-sensitive NADH dehydrogenase activity, supporting the fidelity of the Gene-Switch system [20, 21] and functionality of the *ndi1* transgene and ND11 protein in the adult fly intestine.

We used this system to examine the impact of intestine-specific expression of *ndi1* on *Drosophila* lifespan. Induced expression of *ndi1* using the *TIGS-2* driver throughout the life of the fly resulted in a significant increase in lifespan in female flies (Figures 1B and S1A) and no major effect in male flies. RU486 produced no major effects on longevity in control flies (Figure S1B). To examine the impact of targeted expression of *ndi1* in intestinal stem cell lineages (ISCs and EBs), we first used the constitutive *esgGal4* driver line and observed a significant extension of lifespan in both female (Figures 1C and S1C-D) and male flies (Figures S1E-F) compared to controls. *esgGal4* expression is restricted to ISCs and EBs in the intestine, however, it is also expressed in stem cells within malpighian tubules, germline and somatic stem cells in the testis, and in salivary glands [22]. Therefore, to validate and extend this finding we took advantage of the RU486-inducible *5961GS* driver which recapitulates the *esgGal4* expression pattern in the digestive tract (ISCs/EBs and malpighian tubule stem cells) [22, 23] but is not expressed in salivary glands [22] or testis (C.L.K. and D.L.J., unpublished data). Induced expression of *ndi1* during adulthood, via *5961GS*, resulted in a significant lifespan increase in females (Figures 1D and S1G-I) but not in males (Figures S1J-

S1K). Expression of *ndi1* during adulthood using a Gene-Switch driver that is expressed in EBs and post mitotic enterocytes (ECs) (*5966GS*, [23]) failed to increase lifespan (Figures S1L-M), implicating expression in ISC as the major contributor to longevity. The largest and most consistent lifespan extension phenotypes using *ndi1* expression were observed with female flies. Therefore, unless noted otherwise, we focused our studies to female flies for the remainder of this study.

***ndi1* expression in ISCs/EBs improves markers of intestinal homeostasis during aging**

Homeostasis of the digestive tract has been shown to play a central role in lifespan determination in *Drosophila* [10, 11, 17, 22]. Therefore, we examined markers of intestinal homeostasis in flies that express *ndi1* in ISCs/EBs. First, we set out to determine whether

ndi1 could delay the onset of markers of ISC proliferation and the accumulation of misdifferentiated ISC daughter cells reported to occur in the aged midgut [8, 9]. Consistent with improved intestinal tissue homeostasis, examination of aged flies that express *ndi1* in ISCs/EBs along with an *esg* reporter (*UAS-gfp*) revealed a significant decrease in the number of *esg* positive cells in the midgut relative to controls (Figure 2A-B). In addition, we also observed a delay in the precocious activation of ISC proliferation, as measured by phosphorylation of histone H3 (pHH3), a marker of cell cycle progression through mitosis. Female flies, 50 days post eclosion, expressing *ndi1* under the control of *esgGAL4* driver contained significantly fewer pHH3⁺ cells, when compared to controls (Figure 2C). No difference in the number of pHH3⁺ cells was observed in 10 day old flies, indicating that *ndi1* expression specifically delays the age-related increase in ISC proliferation.

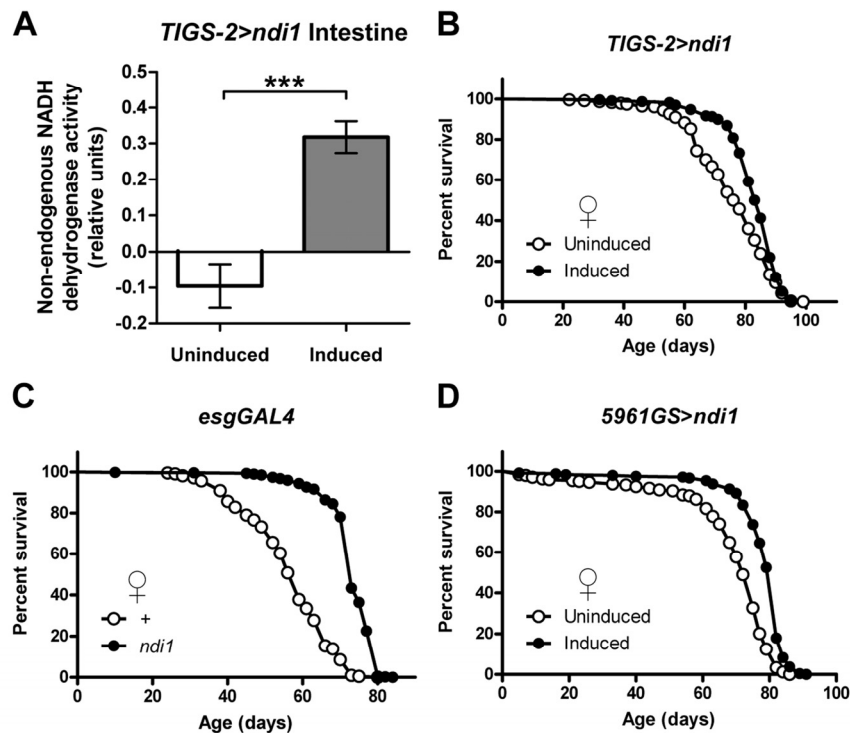


Figure 1. Intestine-specific expression of *ndi1* increases lifespan. (A) Analysis of NDI1 enzymatic activity in mitochondria isolated from intestines. NDI1 is expressed by transgenic expression of an *ndi1* cDNA under control of the intestine-specific *TIGS-2* driver (*TIGS-2>ndi1*). Transgenic expression is induced by exposure of flies to the drug RU486 (100mg/l). Expression of *ndi1* is sufficient to confer flavone sensitive, rotenone insensitive NADH dehydrogenase activity to mitochondria isolated from intestines. (***) $p < 0.001$, t test, 5 replicates per condition, mitochondria from 10 dissected intestines from female flies per replicate). (B) Survival curves of female *TIGS-2>ndi1* flies with or without RU486-mediated transgene induction. Constitutive expression of *ndi1* by RU486 exposure (10mg/l during development, 50mg/l during adulthood) increases lifespan ($p < 0.0001$, log-rank test, at least 200 flies per condition). (C) Survival curves of female *esgGAL4>ndi1* flies compared to isogenic controls. *UAS-ndi1* and the isogenic control strain (*w¹¹¹⁸*) were crossed to *esgGAL4*. A 50% increase in mean survival was observed in response to *ndi1* expression ($p < 0.0001$, log-rank test, at least 200 flies per condition). (D) Survival curves of female *5961GS>ndi1* flies with or without RU486-mediated transgene induction. Adult-onset expression of *ndi1* by RU486 exposure (0.5mg/l) increases fly lifespan ($p < 0.0001$, log-rank test, at least 200 flies per condition).

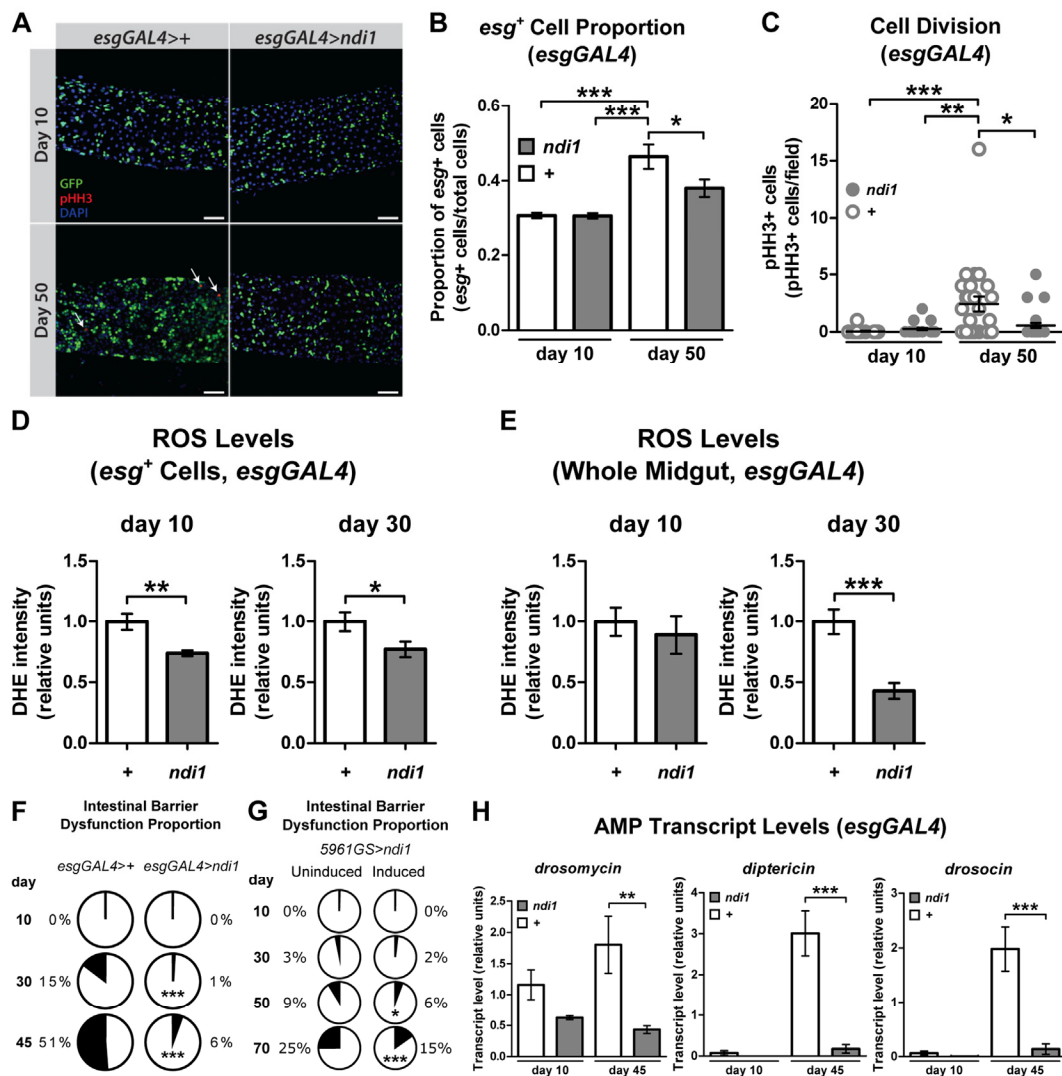


Figure 2. *ndi1* expression maintains intestinal homeostasis during aging. (A) Immunofluorescence images evaluating intestinal homeostasis during aging. Control flies (*esgGAL4>+*, upper panel) and *ndi1* expressing flies (*esgGAL4>ndi1*, lower panel) were assayed for *esg*⁺ cells (GFP⁺ cells) and mitotic cells (pHH3⁺ cells, arrows) 10 days and 50 days post eclosion. Scale bars=50μm. (B) Quantification of proportion of *esg*⁺ cells. The proportion of *esg*⁺ cells (GFP⁺ cells) in all cells (DAPI stain) was increased in aged control flies (“+”, *esgGAL4>+*), but not in *ndi1* expressing flies (“*ndi1*”, *esgGAL4>ndi1*). (*p<0.05, ***p<0.001, One-way ANOVA with Tukey’s post hoc test, at least 22 flies per condition). (C) Quantification of mitotic cells per field of view. The median number of mitotic events (pHH3⁺ cells per field of view) is elevated in aged control flies (“+”, *esgGAL4>+*), but not in *ndi1* expressing flies (“*ndi1*”, *esgGAL4>ndi1*). (*p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test followed by Dunn’s multiple comparisons, at least 22 flies per condition). (D) Quantification of ROS levels per area in ISCs/EBs. *ndi1* expression (“*ndi1*”, *esgGAL4>ndi1*) decreases DHE fluorescence within *esg*⁺ cells (GFP⁺ cells, arrows in figure S2A) in both 10 and 30 day old intestines compared to isogenic controls (“+”, *esgGAL4>+*). (*p<0.05, **p<0.01, t test, at least 3 images per gut, 10 guts per condition). (E) Quantification of ROS levels per area in midguts. *ndi1* expression in ISCs/EBs (“*ndi1*”, *esgGAL4>ndi1*) results in decreased DHE fluorescence in gut tissues relative to isogenic controls (“+”, *esgGAL4>+*) at 30 days post eclosion. (***p<0.001, t test, at least 3 images per gut, 10 guts per condition). (F) Proportion of flies showing loss of intestinal integrity as a function of age, assayed using blue dye no. 1. Aged flies that express *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) show reduced levels of intestinal barrier dysfunction relative to controls (*esgGAL4>+*). (***p<0.001, binomial test, at least 190 flies per condition). (G) Proportion of flies showing loss of intestinal integrity as a function of age in *5961GS>ndi1* flies with or without RU486-mediated transgene induction. Adult-onset expression of *ndi1* by RU486 exposure (0.5mg/l) improves maintenance of intestinal integrity during aging. (*p<0.05, ***p<0.001, binomial test, at least 140 flies per condition). (H) Systemic expression of *Drosomycin*, *Drosocin* and *Diptericin* in 10 and 45 day old flies. Aged flies that express *ndi1* in ISCs/EBs (“*ndi1*”, *esgGAL4>ndi1*) show reduced expression of antimicrobial peptides (AMPs) relative to controls (“+”, *esgGAL4>+*). (**p<0.01, ***p<0.001, t test, 5 replicates per condition, 5 flies per replicate).

An increase in reactive oxygen species (ROS) has been implicated in the loss of tissue homeostasis in the aged fly intestine [24, 25]. Previously, we reported that pan-neuronal expression of *ndil* can reduce ROS levels in the aged brain [15]. It is unclear, however, whether *ndil* expression only in progenitor cells of a tissue is sufficient to cause such changes throughout the tissue. To test this idea, we examined the endogenous levels of ROS in the intestines of control and *esgGAL4>ndil* flies using dihydroethidium (DHE), a redox-sensitive dye that exhibits increased fluorescence intensity when oxidized [26]. Targeted expression of *ndil* in ISCs/EBs led to a reduction of DHE fluorescence in these cells and throughout the aged intestine (Figures 2D-E, S2A).

Loss of intestinal integrity can be assayed in living flies by monitoring the presence of non-absorbed dyes (e.g., FD&C blue No. 1) outside of the digestive tract after feeding [10, 11]. To determine whether *ndil* can delay the onset of intestinal barrier dysfunction, we examined flies of different ages fed FD&C blue No. 1 for evidence of this dye outside of the digestive tract. The proportion of aged flies with dye outside of the intestine was significantly lower in flies with ISC/EB *ndil* expression (Figure 2F). This was not a result of altered development, as adult onset induction of *ndil* in ISCs/EBs, using the *596IGS* driver, was sufficient to decrease the proportion of flies with dye outside of the digestive tract with age (Figure 2G and S2B). Loss of intestinal integrity has been linked with a systemic increase in expression of immunity-related genes [10]. Hence, we assayed systemic expression levels of several anti-microbial peptides (AMPs) genes in *esgGAL4>ndil* and control flies during aging. In line with decreased intestinal barrier dysfunction, flies that express *ndil* in ISCs/EBs show significantly lower expression of multiple AMPs in whole bodies later in life (Figure 2H). Taken together, our findings show that ISC/EB-specific expression of *ndil* leads to improved intestinal homeostasis during aging.

***ndil* expression in ISCs/EBs does not affect fertility or physical activity but changes sensitivity to some stresses**

To gain further insight into intestinal *ndil*-mediated longevity, we examined a number of physiological and behavioral parameters in long-lived *esgGAL4>ndil* flies and controls. Neither male nor female flies that express *ndil* in ISCs/EBs showed consistent alterations to fertility (Figures S2C-G). Resistance to oxidative stress, assayed by survival under hyperoxia (80% O₂), was similarly unaffected (Figure S2H), suggesting that ROS levels in the intestinal epithelium are not limiting

for survival under severely hyperoxic conditions. Survival in elevated environmental temperatures (37°C) and water-only starvation showed considerable differences, with *ndil* expressing flies showing significantly greater sensitivity to elevated temperatures (Figure S2I), and greater resistance to starvation (Figure S2J). These changes were not correlated with significant differences in either spontaneous locomotor activity per time of day (Figure S2K) or cumulative activity over 24-hour periods (Figure S2L). Together, these data indicate that intestinal *ndil*-mediated longevity is not associated with a general increase in stress resistance or a decline in reproductive output.

***ndil* expression in ISCs/EBs stimulates feeding behavior**

A moderate reduction in food intake, dietary restriction (DR), can extend lifespan in diverse organisms, possibly by reducing the intake of specific nutrients [4]. To determine if a gross difference in food intake could play a role in *ndil*-mediated longevity, we assayed feeding behavior in *esgGAL4>ndil* flies and controls. Surprisingly, total food consumption, measured using a capillary feeding (CAFE) assay [27], revealed an overall increase in feeding in flies that express *ndil* in ISCs/EBs at both young and aged time points (Figure 3A). An independent assay of feeding using a modified dye-tracking assay [28] was used to parse the feeding behavior into the proportion of flies that feed within the assay period and the meal size of flies that feed. Expression of *ndil* in ISCs/EBs resulted in significant increases in both the proportion of flies that feed (Figure 3B) and their meal sizes (Figure 3C) in both young and aged flies. 24 hour activity profiles of *ndil* expressing flies are similar to controls, suggesting that an altered activity at different times of day is not responsible for the increased feeding during the assay period (Figure S2K). Moreover, adult-onset expression of *ndil* for 10 days in ISCs/EBs, using the *596IGS* driver, was sufficient to confer an increase in total food consumption (Figure 3D) and meal size (Figure 3E). The presence of the inducing drug itself had no significant effect on total feeding (Figure S3B) or meal size (Figure S3C).

To determine whether increased feeding was associated with alterations in defecation, we examined the material excreted by *esgGAL4>ndil* flies and controls. Although *ndil* expressing flies ate significantly more than controls, excreta number were not significantly different than controls, at both young and aged time points (Figure S3D). Recent work has shown that qualitative analysis of excreta can provide insight into intestinal transit and fluid homeostasis [29]. Specifically, flies

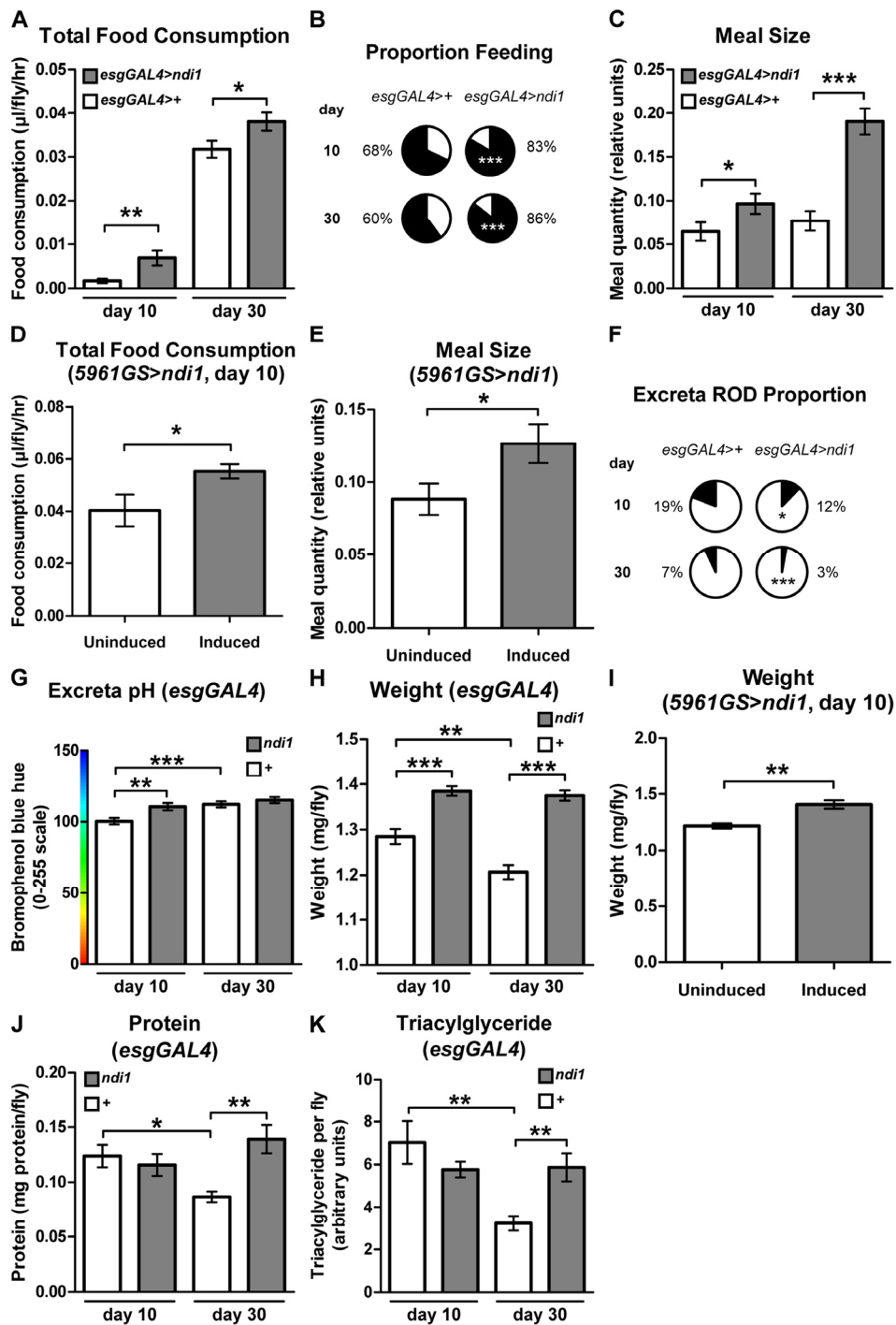


Figure 3. *ndi1* expression in the intestine stimulates feeding behavior. (A) Analysis of total food consumption using a capillary feeding (CAFE) assay. Flies expressing *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) consume significantly more food relative to controls (*esgGAL4>+*). (* $p < 0.05$, ** $p < 0.01$, t test, 10 replicates per condition, 10 flies per replicate). (B) Analysis of feeding proportion using a colorimetric dye-tracking assay. Flies that express *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) had a significantly greater proportion of flies that fed during the assay period relative to controls (*esgGAL4>+*). (***) $p < 0.001$, binomial test, approximately 90 flies per condition). (C) Analysis of meal size using a colorimetric dye-tracking assay (constitutive *ndi1* expression). Of those flies that ate during the assay period in (B), meal size was significantly greater in flies that express *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) relative to controls (*esgGAL4>+*). (* $p < 0.05$, ***) $p < 0.001$, t test, 50-95 flies that ate from B per condition). (D) Analysis of total food consumption using a CAFE assay in *5961GS>ndi1* flies with or without RU486-mediated transgene induction. Ten days of induced *ndi1* expression in ISCs/EBs of adults by exposure to RU486 (0.5mg/l) increases total food consumption relative to uninduced controls. (* $p < 0.05$, t test, 6 replicates per condition, 10 flies per replicate).

Continue. (E) Analysis of meal size using a colorimetric dye-tracking assay in *5961GS>ndi1* flies with or without RU486-mediated transgene induction. Ten days of induced *ndi1* expression in ISCs/EBs of adults by exposure to RU486 (0.5mg/l) increases meal size relative to uninduced controls. (* $p < 0.05$, t test, approximately 85 flies that ate during the assay period in Figure S3A). (F) Analysis of intestinal function by assaying excreta shape. The proportion of oblong deposits (RODs, Figure S3E, arrow) in excreta is significantly lower in deposits from flies expressing *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) relative to controls (*esgGAL4>+*). (* $p < 0.05$, *** $p < 0.001$, binomial test, at least 180 deposits per condition). (G) Analysis of intestinal function by excreta pH. Flies expressing *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) had more alkaline excreta relative to controls ("*+*", *esgGAL4>+*) at day 10 of adulthood in colorimetric analyses of excreta pH after feeding on BPB medium. (** $p < 0.01$, *** $p < 0.001$, t test, at least 180 deposits per condition). (H) Weights of flies with constitutive *ndi1* expression in ISCs/EBs as a function of age. Flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) are heavier than isogenic controls ("*+*", *esgGAL4>+*) and maintain their weight through day 30 of adulthood. (** $p < 0.01$, *** $p < 0.001$, t test, 12 replicates per condition, 5 flies per replicate). (I) Weights of *5961GS>ndi1* flies with or without RU486-mediated transgene induction. Ten days of adulthood only induction of *ndi1* in ISCs/EBs by exposure to RU486 (0.5mg/l) is sufficient to significantly increase weight relative to uninduced controls. (** $p < 0.01$, t test, 6 replicates per condition, 10 flies per replicate). (J) Protein content as a function of age. Protein content is significantly decreased in control flies ("*+*", *esgGAL4>+*) at 30 days of adulthood, but is maintained in flies expressing *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*). (* $p < 0.05$, ** $p < 0.01$, t test, 4 replicates per condition, 5 flies per replicate). (K) Triacylglyceride content as a function of age. Thin-layer chromatography (Figure S3G) and densitometry for triacylglyceride content show a significant decrease in control flies ("*+*", *esgGAL4>+*) at 30 days of age whereas flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) maintain triacylglyceride stores with age. (** $p < 0.01$, t test, 5 replicates per condition, 5 flies per replicate).

that are starved for nutrients and fluids, as during times of high fecundity in females, were shown to have increased frequency of "reproductive oblong deposits" (RODs) and lower fecal pH. Closer examination of excreta shape of young and aged *esgGAL4>ndi1* flies showed a significant reduction in the frequency of RODs (Figures 3F and S3E) indicating improved fluid availability, less concentrated intestinal contents, and quicker intestinal transit [29]. Similarly, fecal pH analysis of flies maintained on bromophenol blue (BPB) containing diets showed less acidic fecal deposits in *ndi1* expressing flies at the young time point (Figure 3G), consistent with a quicker transit through the intestinal tract. Together, these findings indicate that expression of *ndi1* in ISCs/EBs in addition to improving tissue homeostasis, improves intestinal function.

Next, we set out to determine whether *esgGAL4>ndi1* flies show systemic physiological changes that are consistent with increased nutrient uptake. Whole body weight measurements indicated that *esgGAL4>ndi1* flies are heavier than controls, and maintain their weight during aging (Figure 3H). As with feeding behavior, adult-onset expression of *ndi1* for 10 days in ISCs/EBs, using the *5961GS* driver was sufficient to confer an increase in body weight (Figure 3I), and this was not a result of the inducing drug itself (Figure S3F). Moreover, aged *esgGAL4>ndi1* flies display increased protein levels and triglyceride stores relative to controls at aged timepoints (Figures 3J-K, S3G). Unlike triglycerides, levels of glycogen declined similarly in both *ndi1* expressing flies and controls with age (Figure S3H).

***ndi1* expression in ISCs/EBs alters systemic metabolic signaling pathways**

We set out to further characterize the physiology of long-lived *ndi1* flies by examining steady state effects of *ndi1* expression on systemic nutrient sensitive pathways. AMP-activated protein kinase (AMPK) is a crucial metabolic gauge that is activated by low cellular energy status [16]. Since expression of *ndi1* in ISCs/EBs stimulates feeding, we reasoned that these flies may show reduced systemic AMPK activity. Indeed, Western blots specific for phosphorylated AMPK revealed significantly decreased phosphorylation at Thr184 in whole bodies of *esgGAL4>ndi1* flies relative to controls (Figures 4A-B and S4A). AMPK activation has been shown to stimulate sirtuin1 (SIRT1) activity, which deacetylates FOXO and increases its transcriptional activity [30]. Consistent with this, systemic dFOXO transcriptional activity, assayed by measuring transcript levels of multiple direct downstream targets of dFOXO in whole bodies, was significantly decreased in *esgGAL4>ndi1* flies (Figure 4C).

FOXO activity is independently regulated by a number of different signaling pathways, including the insulin/insulin-like growth factor signaling (IIS) pathway [31]. To determine whether the observed decrease in dFOXO activity in *esgGAL4>ndi1* flies was also associated with an increase in systemic IIS, we assayed the activation state of the direct mediator of FOXO activity in the IIS pathway, phosphoinositide-3-OH-kinase-dependent serine/threonine protein kinase (AKT). In whole bodies of *esgGAL4>ndi1* flies the

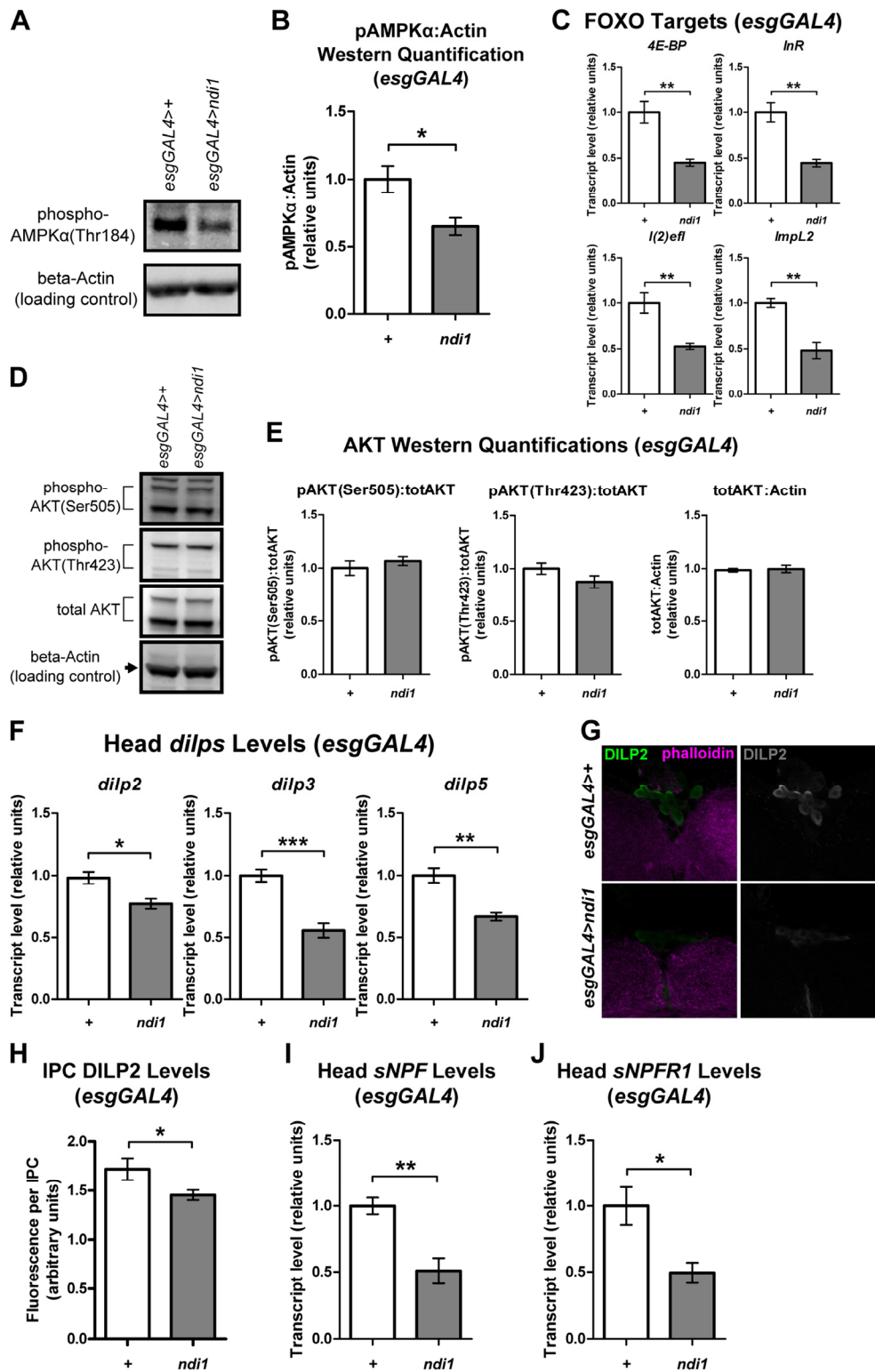


Figure 4. *ndi1* expression in the intestine produces alterations in systemic metabolic signaling pathways. (A-B) Western blot (A, Figure S4A) and densitometric analysis (B) of AMPK α phosphorylation at Thr184. AMPK α phosphorylation (normalized to a loading control, beta-Actin) is significantly decreased in flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4*>*ndi1*), relative to isogenic controls ("+", *esgGAL4*>+) at day 10 of adulthood. (* $p < 0.05$, t test, 5 replicates per condition, 15 flies per replicate).

Continue. **(C)** Transcript levels of downstream targets of dFOXO. *4E-BP*, *InR*, *I(2)efl*, and *ImpL2* transcript levels are significantly lower in flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) relative to isogenic controls ("*+*", *esgGAL4>+*) at day 10 of adulthood. (***p*<0.01, t test, 5 replicates per condition, 5 flies per replicate). **(D-E)** Western blot (D, Figure S4B) and densitometric analysis (E) of AKT phosphorylation at Ser505 or Thr423, and total AKT levels. AKT phosphorylation (normalized to total AKT) is not altered in flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) relative to isogenic controls ("*+*", *esgGAL4>+*) at day 10 of adulthood. Total AKT levels (normalized to beta-Actin) are also unchanged. (n.s., t test, 5 replicates per condition, 5 flies per replicate). **(F)** Transcript levels of head *dilp* genes. *dilp2*, *dilp3*, and *dilp5* transcript levels from heads of flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) are significantly lower than those of controls ("*+*", *esgGAL4>+*) at day 10 of adulthood. (**p*<0.05, ***p*<0.01, ****p*<0.001, t test, 5 replicates per condition, 30 heads per replicate). **(G-H)** Immunofluorescence staining (G) and quantification (H) of DILP2 levels in insulin producing cells (IPCs). DILP2 fluorescence in IPCs of flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) are significantly lower compared to that of controls ("*+*", *esgGAL4>+*) at day 10 of adulthood. (**p*<0.05, t test, at least 170 IPCs from 12 different brains). **(I-J)** Transcript levels of *sNPF* (I) and its cognate receptor, *sNPF1* (J) in heads. Flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) have significantly lower *sNPF* and *sNPF1* transcript levels in heads than controls ("*+*", *esgGAL4>+*) at day 10 of adulthood. (**p*<0.05, ***p*<0.01, t test, 4-5 replicates per condition, 30 heads per replicate).

phosphorylation of AKT was not significantly affected at either the IIS phosphorylation site (Thr423) or the TORC2 phosphorylation site (Ser505), nor was there a difference in total AKT protein levels (Figures 4D-E and S4B). Similarly, Western blot analysis of S6K, a component of the TOR pathway revealed no significant changes in phosphorylation at the TORC1 target residue (Thr398) or in total S6K levels in whole bodies of *ndi1* expressing flies (Figures S4C-D). Thus, the observed decrease in FOXO transcriptional activity in *esgGAL4>ndi1* flies is not associated with alterations in systemic IIS/TOR pathway activities.

ndi1 expression affects both feeding behavior and longevity, both of which have been shown to be regulatable by *Drosophila* insulin-like peptides (*dilps*) signaling [32-34]. Therefore, we checked the expression levels of several *dilps* to determine if *dilp* levels in heads of *esgGAL4>ndi1* flies were altered. Transcript levels in heads of *dilp2*, *dilp3* and *dilp5*, that are expressed in the insulin producing cells (IPCs) upon feeding, were significantly decreased in *esgGAL4>ndi1* flies (Figure 4F) while the transcript level of *dilp1*, which is not expressed in adult heads [32], was not altered significantly (Figure S4E). Direct quantification of DILP2 protein levels in IPCs by immunofluorescence showed a similar decrease in DILP2 fluorescence in IPCs of *esgGAL4>ndi1* flies (Figure 4G and 4H). The *Drosophila* ortholog of mammalian neuropeptide Y, short neuropeptide F (*sNPF*), is expressed in the nervous system and regulates food intake [35] and along with its cognate receptor, *sNPF1*, regulates the expression of *dilps* in the fly brain [36]. To determine if decreased *dilp* transcription in heads of *esgGAL4>ndi1* flies is linked to altered *sNPF*/*sNPF1* expression, we measured transcript levels of *sNPF* and *sNPF1* in heads. In line with *dilp* transcript levels in heads, both *sNPF* and *sNPF1* transcript levels were decreased in heads of *esgGAL4>ndi1* flies relative to controls (Figure 4I and 4J).

DISCUSSION

A decline in mitochondrial activity has been implicated in multiple degenerative diseases of aging [2]. These findings raise the intriguing possibility that strategies to stimulate mitochondrial activity during aging may delay the onset of pathology and extend healthspan. In support of this idea, we recently reported that overexpression of the fly PGC-1 homolog, *dPGC-1*, in ISC lineages is sufficient to preserve intestinal homeostasis during aging and extend fly lifespan [11]. However, due to the extensive interactions that PGC-1 has with multiple aspects of metabolism [12], the possibility persists that endogenous *dPGC-1* interactions, other than its role as a regulator of mitochondrial activity, play a role in the cellular and/or organismal phenotypes that we observed. Unlike *dPGC-1*, *ndi1* is exogenous, from a different kingdom, with no known homologs in animals, so any changes that result from *ndi1* expression can reasonably be expected to be from the function of *ndi1* as an NADH dehydrogenase. A previous study reported that ubiquitous expression of *ndi1* using a constitutive driver line can increase fly lifespan [13]. However, studies of the genetics of aging and lifespan determination are prone to confounding effects due to uncontrolled differences in genetic background between test and control lines [37]. Using an inducible gene expression system, which eliminates this issue, we failed to observe lifespan extension upon ubiquitous expression, but instead observed that neuron-specific expression of *ndi1* can extend lifespan [15]. In the present study, we have extended this approach and show that expression of *ndi1* in adult intestinal stem and progenitor cells can reduce whole tissue ROS levels, improve tissue homeostasis, delay the onset of intestinal barrier dysfunction, and extend the lifespan of flies. Therefore, a major conclusion of this study is that an increase in mitochondrial NADH dehydrogenase activity alone in ISCs/EBs can delay both tissue and organismal

aging, possibly by limiting pro-proliferative ROS levels in the intestinal epithelium.

Long-lived flies expressing *ndil* in ISCs/EBs have behavioral, physiological, and biochemical correlates of increased nutrition, showing increased feeding, weight, metabolic stores, and decreased systemic activation of AMPK. Importantly, *ndil*-mediated weight gain can be observed upon adult-onset expression in ISCs/EBs. Moreover, both increased sensitivity to elevated temperatures, and resistance to starvation of the long-lived flies are wholly consistent with larger flies (with lower surface-to-mass ratios) and improved nutrient absorption and storage. Further studies using radioactive tracers of specific nutrients may provide clues as to whether increased total caloric uptake or differential absorption of specific nutrients play a role in the increased longevity of *ndil* expressing flies. Regardless of whether total caloric intake or absorbed nutrient composition plays a bigger role, one indication that improved nutrition plays a role in increasing lifespan is the ability of flies expressing *ndil* in ISCs/EBs to retain body weight and metabolic stores with age.

Forkhead Box-O (FOXO) transcription factors, inhibited by IIS, have been implicated in metabolic homeostasis and lifespan determination [38]. Indeed, adult-onset and tissue-restricted overexpression of the single *Drosophila* FOXO ortholog (*dFOXO*) can increase longevity [39, 40]. Yet, the relationships between IIS, FOXO activity and organismal health are not straightforward. Reduced IIS in mammals results in diabetes, whose associated pathologies shorten lifespan, and aged flies display markers of impaired IIS, including *dFOXO* activation, which are tightly linked to impending death [10, 41]. In the current study, we show that long-lived flies, expressing *ndil* in ISCs/EBs, show reduced expression of multiple *dFOXO* target genes in whole bodies. However, reduced *dFOXO* activity was not associated with alterations in AKT activation indicating that systemic IIS activity is not altered. Examination of *dilp* levels in *ndil* expressing flies revealed low transcript levels of head *dilps*. Therefore, *ndil* expression in ISCs/EBs may result in uncoupling of DILP signaling from nutritional status. Although the spatially and temporally dynamic nature of feeding and nutrition signaling make definitive interpretations difficult, one possibility that is consistent with our findings is that feeding suppresses AMPK activity, leading to decreased FOXO activity and *sNPF/sNPF1* transcript levels. Without a corresponding increase in DILP levels to inhibit feeding, however, the flies remain in a permissive state for feeding, and even with reduced *sNPF/sNPF1* signaling, eat more.

How do we reconcile our findings with previous work reporting that reduced IIS and/or FOXO activation prolongs lifespan in *Drosophila* [5]? Our observation that long-lived flies expressing *ndil* in ISCs/EBs show reduced expression of *dilps* in heads and DILP2 levels in IPCs may provide some insight. Reduced expression of *dilp2* has been consistently associated with increased lifespan in multiple genotypes in studies from different laboratories [32, 33, 36, 39, 42, 43]. Moreover, deletion of the neurosecretory cells that produce *dilp2*, 3, and 5 produces phenotypes that overlap with ISC/EB expression of *ndil*, including resistance to starvation stress, sensitivity to heat stress, and increased lifespan [32]. Uncovering the mechanism by which *ndil* expression in ISCs/EBs results in altered expression of *dilps* could provide important insights into the role of somatic stem cells in the regulation organismal lifespan, metabolism and behavior. Regardless of the underlying mechanisms, our findings demonstrate that providing exogenous NADH dehydrogenase activity in ISCs/EBs is an attractive strategy to delay markers of intestinal aging and prolong healthy lifespan in fruit flies. Given that *ndil* can be functionally expressed in mammalian cells [44-46], and does not cause an immune response [47], expression of *ndil* in mammalian stem cells may provide a strategy to similarly improve tissue homeostasis and delay the onset of aging.

EXPERIMENTAL PROCEDURES

Unless otherwise specified, mated female flies were used for experimental analyses. For full descriptions of methods used in this study, please see Supporting Information Materials and Methods.

Fly lines, culture, and genotypes. *UAS-ndil* lines [15] were backcrossed 10 times into a *w¹¹¹⁸* background. *TIGS-2* line was provided by L. Seroude, *5961GS* was provided by H. Jasper, and *esgGAL4* was provided by A. Christiansen. Culturing of flies and measurements of lifespan were performed as previously described [11]. See Supporting Information Materials and Methods for details.

Genotypes: *TIGS*>+;+;+;*TIGS-2*/+;*TIGS*>*ndil*:+;*UAS-ndil*/+;*TIGS-2*/+;*esgGAL4*>+;+;*esgGAL4*,*UAS-gfp*/+;+;*esgGAL4*>*ndil*:+;*esgGAL4*,*UAS-gfp*/*UAS-ndil*:+;*5961GS*>+;+;*5961GS*,*UAS-gfp*/+;+;*5961GS*>*ndil*:+;*5961GS*,*UAS-gfp*/*UAS-ndil*:+;*5966GS*>+;+;*5966GS*/+;+;*5966GS*>*ndil*:+;*5966GS*/*UAS-ndil*:+

Feeding Assays. Capillary feeding (CAFE) assays and dye-tracking assays were adapted from previously described protocols with slight modifications [27, 28].

See Supporting Information Materials and Methods for details.

Molecular Biology and Physiology. Protocols and reagents used for complex I and NDI1 activity assays, immunofluorescence staining, ROS staining, quantitative real-time polymerase chain reaction (qRT-PCR), intestinal barrier and transit assays, and measurements of fertility, stress resistance, spontaneous activity, weights, and metabolites are provided in Supporting Information Materials and Methods.

Statistical Analyses. Unless otherwise indicated, significance was determined using a two-tailed, unpaired *t* test from at least three independent experiments and expressed as *p* values. All error bars reflect standard error of the mean.

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Author Contributions

All authors contributed to conception of experiments and interpretation of the data. JHH, JG, CLK, MU, and MR performed the experiments and collected and analyzed the presented data. JHH and DWW wrote the paper.

Conflicts of Interest Statement

The authors declare no conflicts of interest.

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SUPPLEMENTAL MATERIALS AND METHODS

Fly Media. Unless otherwise noted solid food media provided to flies contained 1% agar, 3% yeast, 1.9% sucrose, 3.8% dextrose, 9.1% cornmeal, 1.5% methylparaben, and 1% acid mix wt/vol. For RU486 and control media, a stock solution of RU486 (Cayman Chemicals, Ann Arbor, MI, USA) or ethanol was mixed into the media to specified concentrations after cooling to approximately 65°C.

Lifespan Analysis. Flies that eclosed over a 24-36 hour period were collected and allowed to mate for approximately 60 hours. Female or male flies were collected under light nitrogen-induced anesthesia and maintained at a density of 30 flies per vial in a humidified, temperature-controlled (25°C) incubator with a 12 hour light-dark cycle. Flies were transferred to new vials every 2-3 days and scored for death.

Mitochondrial Isolation. Fly guts were dissected on ice on ice-cold 1XPBS, and mitochondria were purified by differential centrifugation. Dissected guts were homogenized in chilled mitochondrial isolation medium (MIM, 250 mM sucrose, 10 mM Tris (pH 7.4), 0.15 mM MgCl₂) and debris was pelleted by centrifugation (500×g, 5 min at 4°C). Mitochondria were pelleted by centrifugation (5,000×g, 5 min at 4°C) and stored at -80°C.

NADH:Ubiquinone Oxidoreductase Activity Assay. Prepared mitochondria were resuspended in MIM and added to 150 µl of a colorimetric complex I activity assay buffer (1× PBS, 3.5 g/l BSA, 0.2 mM NADH, 0.24 mM KCN, 60 µM DCIP, 70 µM decylubiquinone, 25 mM antimycin A). NADH:ubiquinone oxidoreductase activity was monitored as a drop in DCIP absorbance at 600 nm using an Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA). Flavone or rotenone insensitive activity was measured as the difference in DCIP reduction in the presence of flavone (0.4 mM) or rotenone (2 µM) in the assay buffer, and baseline activity in an assay buffer that contained both inhibitors. All reported activities are normalized to citrate synthase activity.

Citrate Synthase Activity Assay. Prepared mitochondria were diluted into MIM and added to 150 µl of a colorimetric citrate synthase activity assay buffer (50 mM Tris (pH 8.0), 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.3 mM acetyl-CoA, 1 mM oxaloacetic acid). Citrate synthase activity was measured as an increase in DTNB absorbance at 412

nm using an Epoch microplate spectrophotometer (BioTek).

Feeding Assays. Capillary feeding (CAFE) assays were performed as previously described [1] with modifications. Briefly, 10 flies were placed in vials with wet tissue paper as a water source and a capillary food source (5% sucrose, 5% yeast extract, 2.5% FD&C Blue No. 1 (SPS Alfachem, Lexington, MA, USA)). Feeding was monitored from approximately 3 hours after lights on until lights off, with capillaries being replaced and feeding amounts recorded every 2-3 hours.

Dye tracking assays were performed as described previously [2] with slight modifications. Approximately 30 flies were placed in vials with solid media supplemented with 2.5% dye (FD&C Blue No. 1) from 3 hours after lights on until 5.5 hours after lights on at 25°C. Possibly due to the low yeast concentration in our standard medium (0.3% yeast), flies required approximately 2.5 hours to show appreciable feeding. Even after 2.5 hours, significant proportions of flies still had not eaten (determined as described below), suggesting that our assay conditions were not saturating. Flies were frozen, decapitated, and homogenized separately in 200µl H₂O. Cell debris was pelleted via centrifugation, and absorbance (629nm) of a 1:2.5 dilution of the supernatant was used to determine whether the fly ate (flies were categorized as having eaten if they had A₆₂₉nm greater than 110% of the absorbance outside of the dye absorption range, at 800nm), and if so, the relative meal size (A₆₂₉-A₈₀₀).

qRT-PCR. RNA isolation, cDNA synthesis, and quantitative PCR were performed on whole flies or dissected body parts as previously described [3]. Briefly, total RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA) following manufacturer protocols. Samples were treated with DNase I and used for cDNA synthesis using a Fermentas RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative RT-PCR was performed using a Power SYBR Green PCR Master Mix (Life Technologies) in a 45 cycle reaction in a Applied Biosystems 7300 Thermal Cycler (Life Technologies). Amplicons of actin5C were used as a reference for normalization. Primers:

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l(2)efl:CAGACGCGTTTATCCAAGTG,  
ATCCCACCAGTCACGGAAC,  
dilp1:GCTTTAATACGCTGCCAAGG,  
CGGATCCGTACAGATTGGTT,  
dilp2:ATCCCGTGATTCCACACAAG,  
GCGTTCCGATATCGAGTTA,
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dilp3:AGAGAACTTTGGACCCCGTGA,
TGAACCGAACTATCACTCAAC,
dilp5:GCCTTGATGGACATGCTGA,
TCATAATCGAATAGGGCCCAAG,
sNPF:CCCGAAAACCTTTTAGACTCA,
TTTTCAAACATTTCCATCGT,
sNPF1:CTGGCCATATCGGACCTACT,
GGCCAGTACTTGGACAGGAT,
actin5c, drosomycin, dipterin, drosocin, InR, 4E-BP,
ImpL2 primers were previously described [3].

Immunofluorescence and Cell Quantification. Fixation and staining of *Drosophila* midguts were carried out as previously described [4]. Midguts were co-labeled with chicken anti-GFP (1:5000, #GFP-1010, Aves Labs, Tigard, Oregon, USA) and rabbit anti-phospho-histone H3 (1:200, #06-570, EMD Millipore, Darmstadt, Germany) before mounting in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) containing 4',6-diamidino-2-phenylindole (DAPI). Images of the posterior midgut (approximately 250 μ m anterior to the pyloric ring) were acquired with a 20x objective on a Zeiss LSM 780 confocal microscope. Cells that stained for either GFP, phospho-histone H3 (pHH3+), or DAPI were counted manually from at least 22 samples per treatment group. For GFP+ counts, One-way ANOVA was used to determine statistical significance; means of each treatment were compared using Tukey's post hoc test. To determine significant differences in the median number of pHH3+ cells per posterior midgut, a Kruskal-Wallis test was used followed by a Dunn's post hoc test.

Dihydroethidium (DHE) Staining. ROS levels were detected in live tissues as previously described [4]. Briefly, guts were dissected in ice-cold Schneider's medium (Caisson Labs, North Logan, UT, USA) and briefly fixed in 4% formaldehyde in Schneider's medium for 3 minutes at room temperature. Guts were washed twice in Schneider's medium for 30 seconds and stained in 50 μ M DHE (Life Technologies) in Schneider's medium for 7 minutes at room temperature in a light protected chamber. Samples were washed three times for 5 minutes in Schneider's medium at room temperature, mounted in Prolong Gold with DAPI (Life Technologies), and imaged immediately at 100X magnification, 200–500 μ m anterior to the pylorus.

Intestinal Barrier Dysfunction. Flies were tested in groups of approximately 30 per vial, as previously described [3]. Briefly, flies were transferred to solid media supplemented with 2.5% dye (FD&C Blue No. 1) for 9 hours and then checked visually under a dissecting microscope for evidence of dye outside of the gut.

Weight. Flies were weighed in groups of 5 or 10 in pre-weighed microcentrifuge tubes, using an analytical scale (Torbal, Clifton, NJ, USA).

Triacylglyceride. Lipids were extracted from whole flies in a chloroform:methanol:water solution (2:5:1 by volumes) and nonpolar lipids were separated on thin layer chromatography plates (Analtech, Newark, DE, USA) with a n-hexane:diethylether:glacial acetic acid solution (70:30:1 by volumes). Plates were air-dried, stained (0.2% Amido Black 10B in 1M NaCl), and lipid bands were quantified by photo densitometry using ImageJ as described [4].

Glycogen and Protein. Decapitated flies were homogenized in ice-cold water, cleared and used for glycogen quantification using a Glycogen Colorimetric/Fluorometric Assay Kit (BioVision, Milpitas, CA, USA) following manufacturer protocols. Protein content was quantified using a μ BCA kit (Thermo Fisher Scientific) following manufacturer protocols using the same clarified homogenates.

Spontaneous Activity. Spontaneous activity was measured as previously described [5]. Briefly, flies were placed in a *Drosophila* activity meter (TriKinetics, Waltham, MA, USA) in vials at a density of 30 flies per vial. Movements were recorded continuously under normal culturing conditions for at least 24 hours on a 12 hour light-dark cycle.

Fertility. Virgin female flies were collected over a 24-hour period and matured for an additional 60 hours before mating to w¹¹¹⁸ flies, either for a single day or continuously. Flies were transferred to new vials every 1-2 days and fertility was measured over the entire lifespan by counting total adult progeny that had eclosed from the old vials after 2 weeks at 25°C. Continuously mated flies were maintained at a density of 10 females and 10 males per vial and single day mated flies were maintained at a density of 10 females per vial.

Stress Resistance. All stress assays were performed with mated female flies 10 days post eclosion at a density of 25-30 flies per vial. For hyperoxia resistance, flies were maintained in a humidified chamber maintained at 85% O₂ and survival was assayed at least once per day. For heat stress resistance, flies were maintained in a humidified 37°C chamber and survival was scored every 2 hours. For wet starvation, flies were maintained on water only medium (1% agar in ddH₂O wt/wt) and maintained in a 25°C incubator with 12 hour light-dark cycles. Survival was scored multiple times per day.

Fecal Deposit Number, Shape, and pH. Excreta assays were performed as previously described [6] with modifications. Flies were maintained on solid media supplemented with 0.5% bromophenol blue, sodium salt (BPB, Sigma-Aldrich, St. Louis, MO, USA) for 24 hours before the assay. Groups of 10 flies were maintained in 60X15mm petri dishes with 2ml BPB medium for 72 hours, and the center of the lid was photographed and analyzed using ImageJ [7] for fecal number, shape, and hue.

Western Blot. Western blot analyses were performed using standard procedures for Polyvinylidene difluoride (PVDF) membranes using the following antibodies and dilutions: phospho-AMPK α (Thr184) (1:1000, #2535, Cell Signaling Technology, Danvers, MA, USA), beta-Actin-peroxidase (1:5000, #a3854, Sigma-Aldrich), phospho-AKT (Ser505) (1:1000, #4060, Cell Signaling Technology), phospho-AKT (Thr423) (1:1000, #2965, Cell Signaling Technology), total AKT (1:1000, #4691, Cell Signaling Technology), phospho-S6K (Thr398) (1:500, #9209, Cell Signaling Technology), total S6K (1:750, #sc-230, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit IgG-peroxidase (1:2000, #a6154, Sigma-Aldrich). Densitometry and quantification were performed using ImageJ [7].

DILP2 Immunofluorescence. Dissected brains were fixed, stained, and mounted following published protocols [8] with DILP2 antibodies provided by P. Leopold (1:800), rat IgG (#A11077, 1:250, Life Technologies), and phalloidin (#A12379, 1:100, Life Technologies). Approximately 10-20 images per brain were taken with a Zeiss CLS microscope and analyzed using ImageJ [7] to measure total fluorescence of individual IPCs in slices with maximum nuclear area.

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SUPPLEMENTAL FIGURES

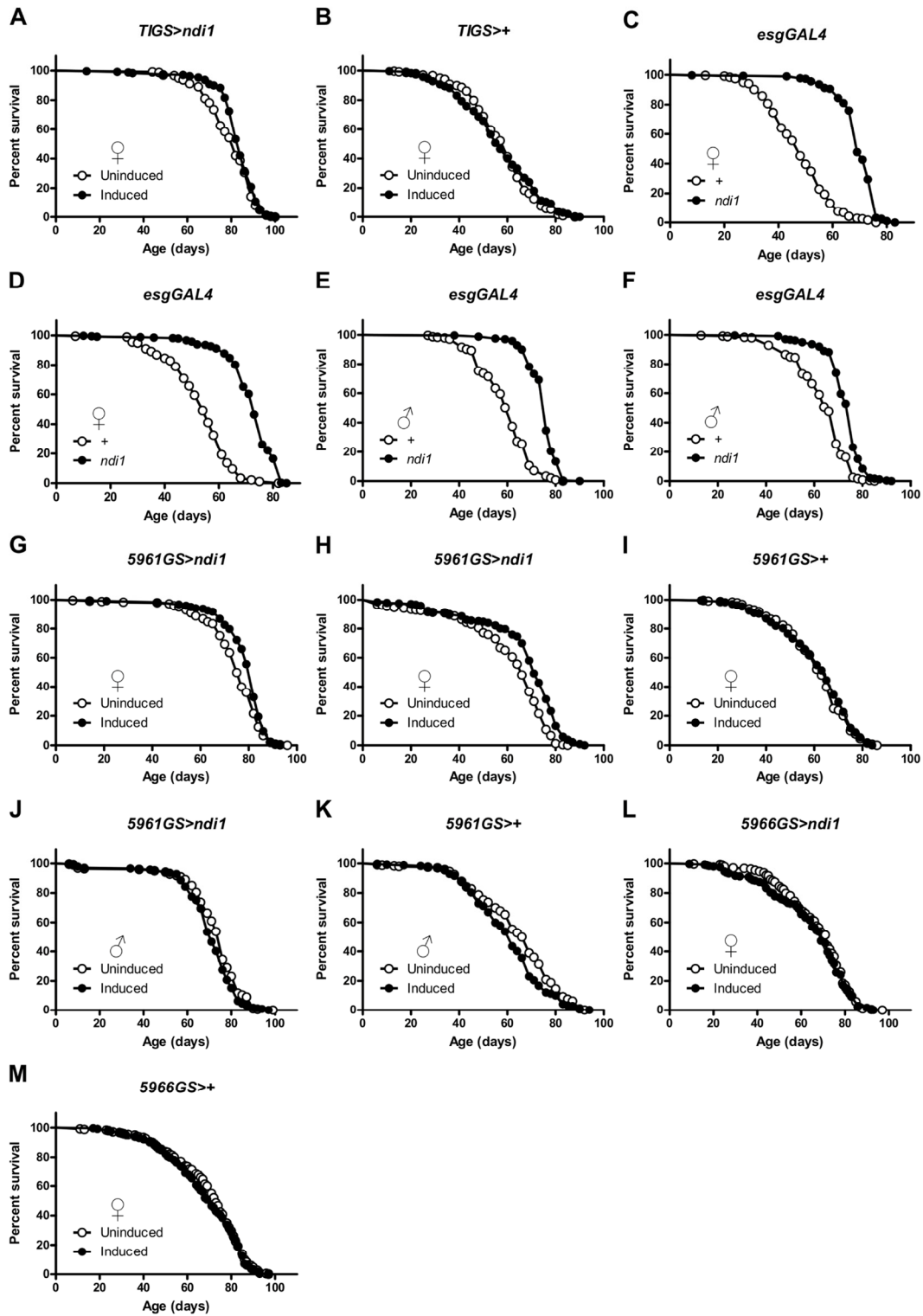


Figure S1. Survival curves of tissue specific *ndi1* expression. (A) Replicate survival curve of female *TIGS>ndi1* flies with or without induced transgene expression. Constitutive induction of *ndi1* by RU486 exposure (“Induced”, 10mg/l during development, 50mg/l during adulthood) increases lifespan relative to uninduced controls. ($p < 0.001$, logrank test, 4.2% increase in mean, at least 200 flies per condition).

Continue. **(B)** Survival curve of *TIGS-2* control female flies with or without RU486 exposure. Exposure of *TIGS-2* driver control flies (*TIGS>+*) to RU486 (same conditions as Figure 1B and S1A) does not significantly alter lifespan. (n.s., logrank test, at least 200 flies per condition). **(C-D)** Replicate survival curves of female *esgGAL4>ndi1* flies and controls. Expression of *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) increases lifespan relative to controls ("*+*", *esgGAL4>+*). ($p < 0.001$, logrank test, 46% (C) and 35% (D) increase in mean, at least 170 flies per condition). **(E-F)** Survival curves of male *esgGAL4>ndi1* flies and controls. Expression of *ndi1* in ISCs/EBs and testis ("*ndi1*", *esgGAL4>ndi1*), increases lifespan in mated male flies relative to controls ("*+*", *esgGAL4>+*). ($p < 0.001$, logrank test, 27% (E) and 16% (F) increase in mean, at least 200 flies per condition). **(G-H)** Replicate survival curves of female *5961GS>ndi1* flies with or without adult-onset transgene induction. Adult-onset induction of *ndi1* expression in ISCs/EBs by RU486 exposure (0.5mg/l) increases lifespans. ($p < 0.001$, logrank test, 5.6% (G) and 10.5% (H) increase in mean, at least 200 flies per condition). **(I)** Survival curve of *5961GS* control female flies with or without adult-onset transgene induction. RU486 (same conditions as Figure S1G and S1H) exposure in *5961GS* control flies (*5961GS>+*) does not alter lifespan. (n.s., logrank test, at least 200 flies per condition). **(J)** Survival curve of male *5961GS>ndi1* flies with or without adult-onset transgene induction. Adult-onset induced *ndi1* expression by RU486 exposure (0.5mg/l) results in a mild decrease in lifespan relative to uninduced controls. ($p < 0.01$, logrank test, 2% decrease in mean, at least 200 flies per condition). **(K)** Survival curve of *5961GS* control male flies with or without RU486 exposure. Exposure of *5961GS* driver control flies (*5961GS>+*) to RU486 (same conditions as Figure S1J) decreases lifespan. ($p < 0.01$, logrank test, 6% decrease in mean, at least 200 flies per condition). **(L)** Survival curve of female *5966GS>ndi1* flies with or without adult-onset transgene induction. Adult-onset induction of *ndi1* expression by RU486 exposure (0.5mg/l) using a geneswitch driver for EBs and ECs, *5966GS*, did not increase lifespan relative to uninduced controls. (n.s., logrank test, at least 200 flies per condition). **(M)** Survival curve of *5966GS* control female flies with or without RU486 exposure. Exposure of *5966GS* control flies (*5966GS>+*) to RU486 (same conditions as Figure S1L) does not alter lifespan. (n.s., logrank test, at least 200 flies per condition).

Figure S2. *ndi1* expression does not alter fertility or activity, but affects some stress resistances. **(A)** Single confocal section images of DHE stained midguts evaluating ROS levels during aging. *esgGAL4>ndi1* flies and controls (*esgGAL4>+*), 10 and 30 days post eclosion, were assayed for changes in ROS levels within *esg⁺* cells and per area. (arrows=*esg⁺* cells). **(B)** Intestinal integrity in *5961GS* control flies with and without RU486 exposure. Intestinal integrity is not altered by RU486 exposure (same conditions as Figure 2G) in control flies (*5961GS>+*). (n.s., binomial test, at least 120 flies per condition). **(C-D)** Fertility measurements in continuously mated female **(C)** and male **(D)** flies that express *ndi1* in ISCs/EBs. No consistent differences in reproductive output were observed in *esgGAL4>ndi1* flies compared to controls (*esgGAL4>+*). (** $p < 0.01$, * $p < 0.05$, t test, 7-8 replicates per condition, 10 male and 10 female flies per replicate). **(E-F)** Fertility measurements in single day mated female **(E)** and male **(F)** flies that express *ndi1* in ISCs/EBs. No consistent differences in reproductive output were observed in *esgGAL4>ndi1* flies compared to controls (*esgGAL4>+*). (** $p < 0.001$, t test, 7-8 replicates per condition, 10 female flies per replicate). **(G)** Total lifetime reproductive output of flies that express *ndi1* in ISCs/EBs. Expression of *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) did not alter total lifetime fertility relative to controls ("*+*", *esgGAL4>+*). (n.s., t test, 7-8 replicates per condition, as described in Figure S2C-F). **(H)** Survival curve of *esgGAL4>ndi1* flies and controls under hyperoxic conditions (80% O₂). Flies that express *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) and controls (*esgGAL4>+*) showed similar survival under hyperoxia. ($p < 0.05$, logrank test, 1.6% increase in mean, at least 200 flies per condition). **(I)** Survival curve of *esgGAL4>ndi1* flies and controls under heat stress (37°C). Flies that express *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) are more sensitive to heat stress relative to controls (*esgGAL4>+*). ($p < 0.001$, logrank test, 18% decrease in mean, at least 170 flies per condition). **(J)** Survival curve of female *esgGAL4>ndi1* flies and controls under wet starvation. Flies that express *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) are more resistant to water-only starvation conditions than controls (*esgGAL4>+*). ($p < 0.001$, logrank test, 6% increase in mean, at least 200 flies per condition). **(K)** Spontaneous activity profiles of female *esgGAL4>ndi1* flies and controls. At day 10 of adulthood, activity profiles of flies that express *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) and controls (*esgGAL4>+*) are similar. (3 replicates per condition, approximately 30 flies per replicate). **(L)** Average 24-hour spontaneous activities of female *esgGAL4>ndi1* flies and controls. Flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) and controls ("*+*", *esgGAL4>+*) show similar 24-hour spontaneous activities at day 10 and 30 of adulthood. (n.s., t test, 3 replicates per condition, approximately 30 flies per replicate).

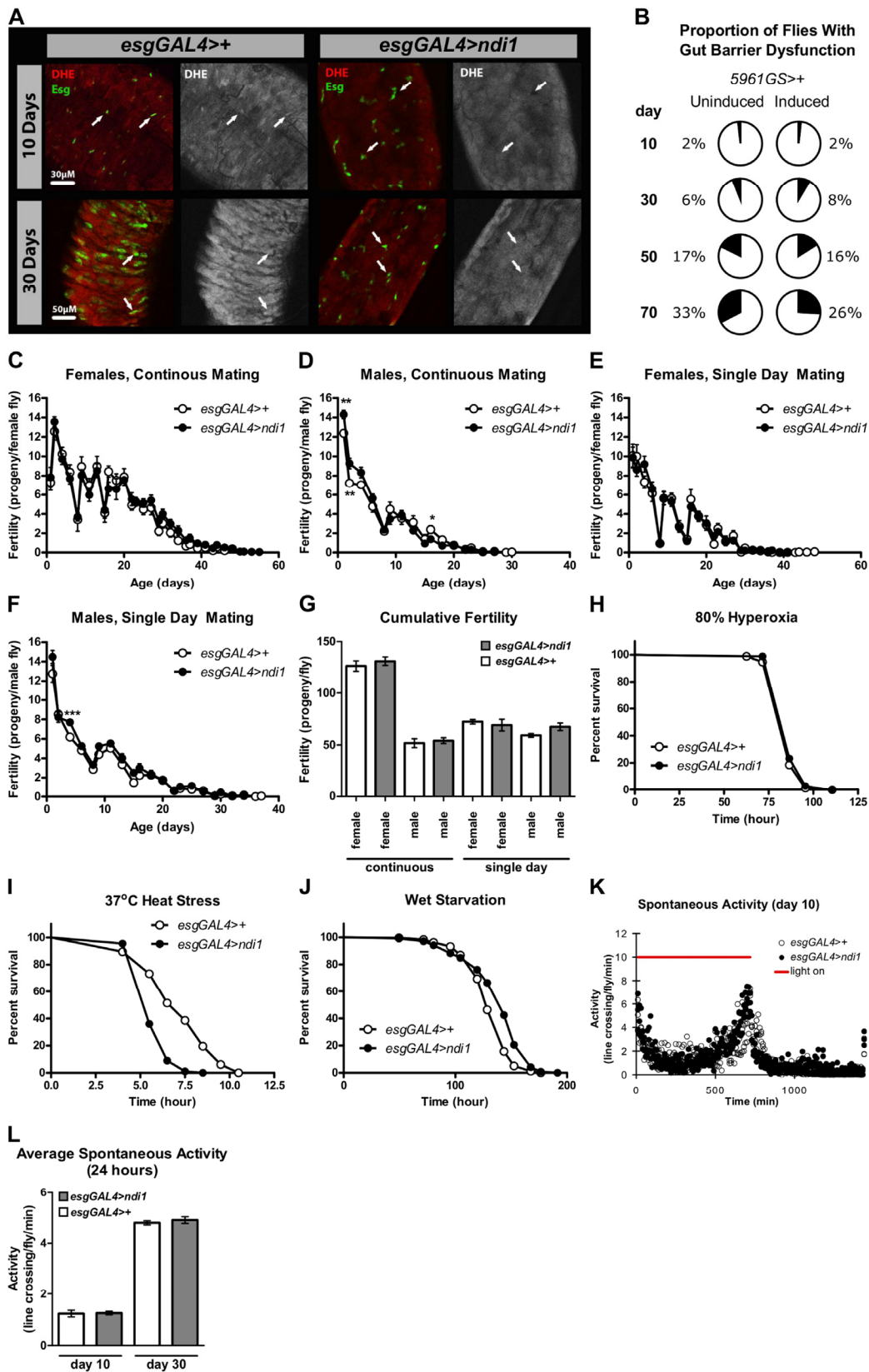


Figure S2. *ndi1* expression does not alter fertility or activity, but affects some stress resistances. See figure legend above.

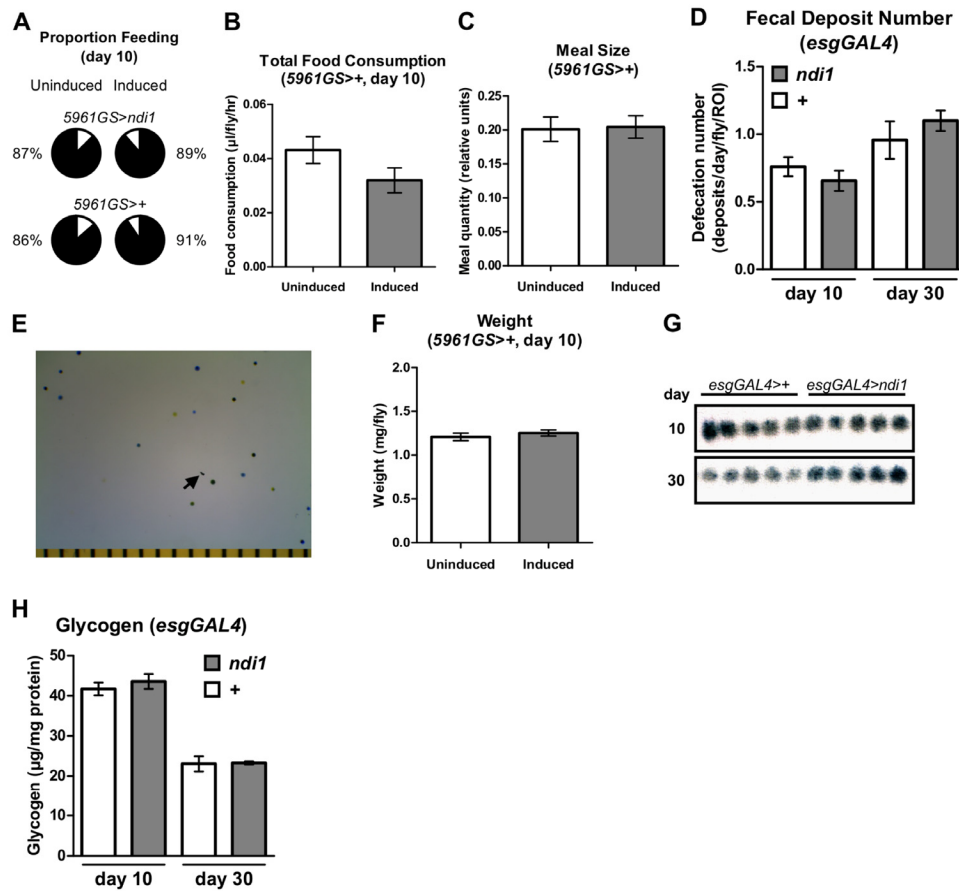


Figure S3. RU486 exposure does not affect feeding or weight in controls, and *ndi1* expression does not alter excreta number or glycogen content. (A) Analysis of feeding proportion using a colorimetric dye-tracking assay (adult only *ndi1* expression). At day 10 of adulthood, the proportions of flies that feed are not altered by adult-onset induction of *ndi1* in ISCs/EBs by exposure to RU486 (*5961GS>ndi1*, 0.5mg/l) or by exposure to RU486 alone (*5961GS>+*, 0.5mg/l). (n.s., binomial test, 96 flies per condition). (B) Analysis of total food consumption in driver controls using a CAFE assay. Exposure to RU486 (same conditions as Figure 3D) does not affect total feeding in control flies (*5961GS>+*) at day 10 of adulthood. (n.s., t test, 6 replicates per condition, 10 flies per replicate). (C) Analysis of meal size in control flies using a colorimetric dye-tracking assay. Meal sizes of control flies (*5961GS>+*) are not altered by exposure to RU486. (n.s., t test, approximately 85 flies that ate during the assay period in (A)). (D) Excreta quantification in *esgGAL4>ndi1* flies and controls. Total number of fecal deposits are similar in flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) and controls ("*+*", *esgGAL4>+*). (n.s., t test, 9-10 replicates per condition, 10 flies per replicate). (E) Representative image of fecal deposits collected from flies cultured on BPB medium. Excreta were used for fecal deposit number (Figure S3D), shape (Figure 3F), and pH analyses (Figure 3G). (Tick marks=1mm, arrow=ROD). (F) Weights of controls with or without RU486 exposure. Adult-onset exposure to RU486 (same conditions as Figure 3I) does not alter weight in controls (*5961GS>+*) at day 10 of adulthood. (n.s., t test, 6 replicates per condition, 10 flies per replicate). (G) Image of thin-layer chromatography for triacylglyceride content. Thin-layer chromatography was used to assay triacylglyceride content in flies that express *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) and controls (*esgGAL4>+*) (densitometry in Figure 3K). (5 flies per lane). (H) Glycogen content as a function of age. Glycogen content is similar in flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) and controls ("*+*", *esgGAL4>+*). (n.s., t test, 5 replicates per condition, 5 decapitated flies per replicate).

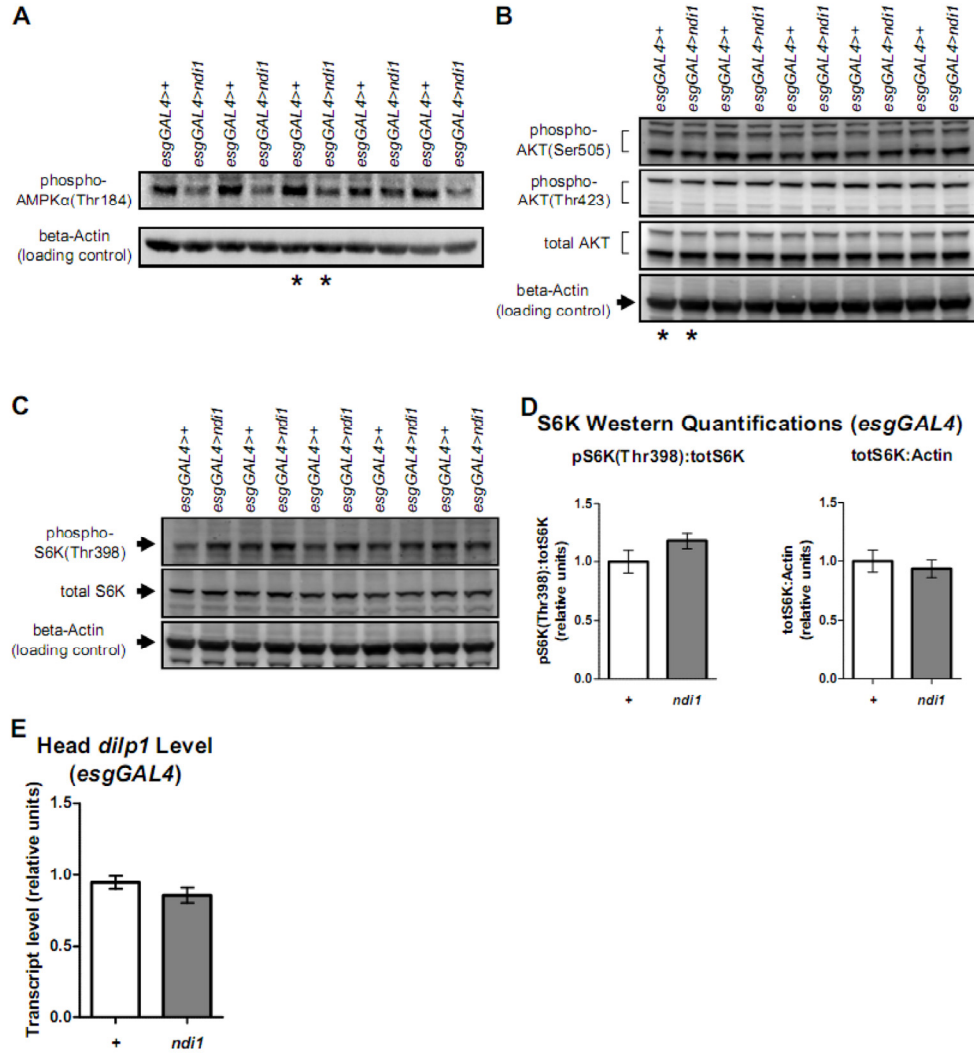


Figure S4. Western blots and head transcript levels of *esgGAL4>ndi1* flies. (A) Western blot detection of phosphorylated AMPK α (Thr184) and beta-Actin in whole flies. Flies that express *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) and controls (*esgGAL4>+*) were assayed at day 10 of adulthood for phosphorylated AMPK α (Thr184) levels by densitometric analysis (Figure 4B). (*=lanes used for representative image in Figure 4A, 15 flies per lane). (B) Western blot detection of phosphorylated AKT(Ser505), phosphorylated AKT(Thr423), total AKT, and beta-Actin in whole flies. Flies that express *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) and controls (*esgGAL4>+*) were assayed at day 10 of adulthood for phosphorylated AKT(Ser505), phosphorylated AKT(Thr423), and total AKT levels by densitometric analysis (Figure 4E). (*=lanes used for representative images in Figure 4D, 5 flies per lane). (C) Western blot detection of phosphorylated S6K(Thr398), total S6K, and beta-Actin in whole flies. Flies that express *ndi1* in ISCs/EBs (*esgGAL4>ndi1*) and controls (*esgGAL4>+*) were assayed at day 10 of adulthood for phosphorylated S6K(Thr398) and total S6K levels by densitometric analysis (Figure S4D). (5 flies per lane). (D) Densitometric analysis of phosphorylated S6K(Thr398). S6K phosphorylation (normalized to total S6K) is not significantly different at day 10 of adulthood in flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) relative to controls ("+", *esgGAL4>+*). Total S6K levels (normalized to beta-Actin) are also similar. (n.s., t test, 5 replicates per condition, 5 flies per replicate). (E) Transcript levels of *dilp1* from heads. Flies that express *ndi1* in ISCs/EBs ("*ndi1*", *esgGAL4>ndi1*) and controls ("+", *esgGAL4>+*) have similar *dilp1* transcript levels in heads at day 10 of adulthood. (n.s., t test, 5 replicates per condition, 30 heads per replicate).