Long-lived *Indy* induces reduced mitochondrial reactive oxygen species production and oxidative damage

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Communicated by Leon N. Cooper, Brown University, Providence, RI, December 12, 2008 (received for review October 14, 2008)

Decreased *Indy* activity extends lifespan in *D. melanogaster* without significant reduction in fecundity, metabolic rate, or locomotion. To understand the underlying mechanisms leading to lifespan extension in this mutant strain, we compared the genome-wide gene expression changes in the head and thorax of adult *Indy* mutant with control flies over the course of their lifespan. A signature enrichment analysis of metabolic and signaling pathways revealed that expression levels of genes in the oxidative phosphorylation pathway are significantly lower in *Indy* starting at day 20. We confirmed experimentally that complexes I and III of the electron transport chain have lower enzyme activity in *Indy* long-lived flies by Day 20 and predicted that reactive oxygen species (ROS) production in mitochondria could be reduced. Consistently, we found that both ROS production and protein damage are reduced in *Indy* with respect to control. However, we did not detect significant differences in total ATP, a phenotype that could be explained by our finding of a higher mitochondrial density in *Indy* mutants. Thus, one potential mechanism by which *Indy* mutants extend life span could be through an alteration in mitochondrial physiology leading to an increased efficiency in the ATP/ROS ratio.

electron transport chain | mitochondria | oxidative phosphorylation | *Drosophila* | aging

Understanding the biological and physiological underpinnings of aging and the development of interventions to ameliorate its deleterious effects has been the subject of much interest. Identification and examination of specific genetic alterations that extend life span is one common approach for uncovering mechanisms underlying normal aging. A number of single gene alterations that extend healthy life span in model organisms have been isolated, but delineating the specific physiological changes responsible for their life span extending effects has been challenging (1).

High throughput genomic analyses have emerged as an unbiased method for providing information of the physiological changes involved in mediating complex biological phenomena such as longevity. In particular, whole genome transcriptional studies (microarrays) to identify specific genes or physiological systems important in longevity determination have been successfully used in both nematodes and flies. Microarray studies of *daf-2* long-lived nematodes identified specific genes that were then functionally verified as causally related to the *daf-2* life span extension (2).

Restricting the examination of microarrays to only those genes identically shared between different interventions may limit the ability to detect some of the physiologically relevant changes important in complex biological phenomena such as life span extension. As more data becomes available from high throughput gene expression studies, analyses have shifted from a gene centric model to a pathway centric approach. It has been realized that reproducibility of experiments, and comparison across interventions that should have resulted in similar outcomes but did not, has been greatly improved by the grouping of genes into categories defined by their functional relatedness (3). A major factor restricting the use of these approaches by the general scientific community has been a limitation of well characterized gene set databases. Recently, this has improved as increased numbers of genes and pathway databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) (4) and Gene Ontology (5) have become available.

Mutations in the *Indy* gene in *Drosophila* and RNAi studies of *Indy*-like homologs in *C. elegans* have demonstrated that a reduction in normal INDY activity is associated with extension of life span (1, 6, 7). An important feature of the *Indy* long-lived phenotype is that it extends life span with very few tradeoffs in other principal physiological systems. For example, *Indy* long-lived flies show no reduction in resting metabolic rate or early or late life fecundity under normal laboratory rearing conditions and no decrease in maximal flight velocity, negative geotaxis, or 24-hour activity levels has been detected (8–10). INDY is a transmembrane transporter of Krebs cycle intermediates, primarily found at the plasma membrane in the midgut, fat body, and oenocytes of flies (1, 11). The predicted protein sequence and cellular localization at the principal sites for uptake, utilization, and storage of nutrients suggest that INDY may be involved in intermediary metabolism in the fly. Although it has been proposed that a decrease in INDY might extend life span by affecting intermediary metabolism, perhaps by creating a metabolic state that mimics calorie restriction (CR) (1), it is not yet understood how an alteration in the level of expression of INDY could result in life span extension.

We made use of high-throughput gene expression profiling to explore potential molecular and physiological mechanisms underlying life span extension in *Indy* mutant long-lived flies. Examination of differences in gene expression between long-lived *Indy* mutants and control flies over the course of their life span lead to our identifying aspects of mitochondria physiology as a potentially important difference between *Indy* and control flies. We found that the activity of the electron transport chain (ETC) in *Indy* mutants is significantly lower by midlife than in control flies, yet ATP homeostasis is maintained, possibly through an increase in mito-

Author contributions: N.N., P.-Y.W., A.S.B., K.P.W., B.R., and S.L.H. designed research; N.N., P.-Y.W., H.H.N., K.P.W., and B.R. performed research; K.P.W. contributed new reagents/analytic tools; N.N., P.-Y.W., A.S.B., B.R., and S.L.H. analyzed data; and N.N., P.-Y.W., B.R., and S.L.H. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0812484106/DCSupplemental.

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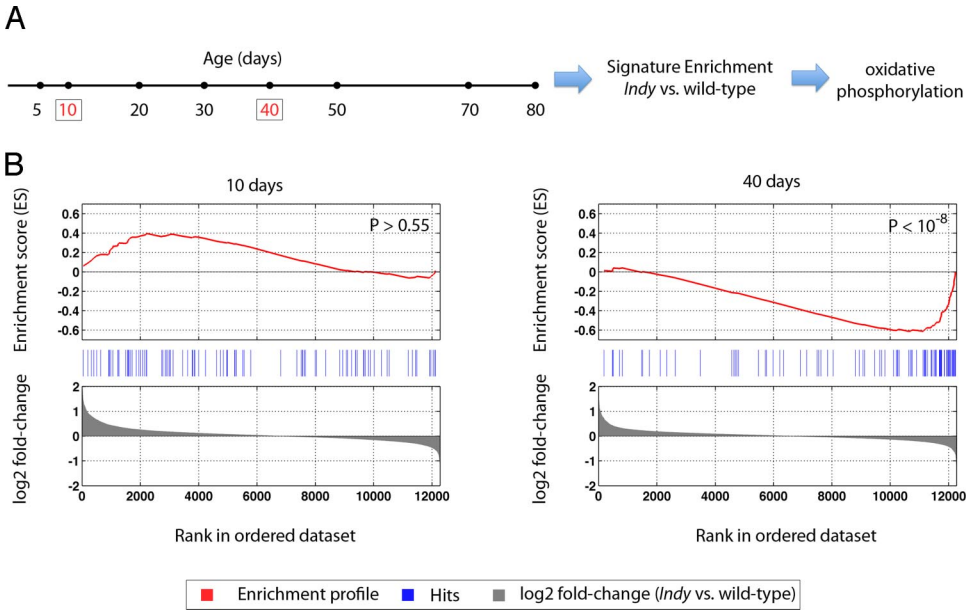

Fig. 1. Gene expression profiling design, analysis strategy, and OXPHOS. (*A*) mRNA expression levels were measured at 5, 10, 20, 30, 40, 50, 70, and 80 days of age in *Indy* mutants and controls; signature enrichment of gene sets from the KEGG pathway database was computed at each time point via the GSEA algorithm. The OXPHOS pathway emerged as significantly enriched for genes down-regulated in *Indy* versus controls. (*B*) Comparison of enrichment profiles of the OXPHOS genes at day 10 (*Left*) and day 40 (*Right*). Enrichment score (ES) from the GSEA algorithm (*Top*, red curve). Location of the OXPHOS genes in the rank list (*Middle*, blue lines). Log_2 fold-change in *Indy* vs. controls of all of the genes on the microarray (*Bottom*, gray); genes were ranked according to their log_2 fold-change. A positive ES indicates a bias toward up-regulation in *Indy* whereas a negative value reflects a bias toward *Indy* down-regulation. Although day 10 showed a slight bias toward up-regulated genes in the OXPHOS set (not significant, $P > 0.55$), at day 40, the OXPHOS pathway was significantly enriched ($P < 10^{-8}$) for genes down-regulated in *Indy* versus control.

chondrial biogenesis. Furthermore, generation of reactive oxygen species (ROS) from mitochondria and accumulation of mitochondrial protein damage is significantly lower in the long-lived *Indy* mutant, two findings consistent with predictions of the oxidative stress hypothesis (12). Based on these findings, we speculate that a coordinated change in mitochondrial activity and density could alter the ATP/ROS ratio, contribute to a delay or reduction in age-related damage, and extend healthy life span.

Results

Gene Set Enrichment Analysis Reveals an Alteration in Oxidative Phosphorylation (OXPHOS) in *Indy*. As a means of gaining insight into how a decrease in the level of a plasma membrane transporter of Krebs cycle intermediates, INDY, could lead to life span extension, we made use of the unbiased approach of measuring the genome-wide transcriptional profile of long-lived *Indy* mutant flies and their genetically matched controls. mRNA was collected from heads and thoraces of male *Indy206* and a genetically matched normal-lived control from the same mutagenesis (1). We used male heads and thoraces to minimize the effect of age-related reproductive changes and reduce tissue heterogeneity. By restricting the mRNA isolation to heads and thoraces, we can enrich for a few important tissue types including the entire central nervous system (brain including optic and olfactory systems and thoracicoabdominal ganglion), head capsule fat body, and flight muscles. mRNA samples were collected from the head and thorax of *Indy206* and genetically matched control male adult flies at day 5, and every 10 days from day 10 to day 80 (day 60 excluded) and hybridized to two-color microarrays (Fig. 1*A*). *Indy206* heterozygote males have an ≈90% increase in mean and an ≈50% increase in maximum life span (1). Three biological replicates were measured at each time point, and a universal reference sample was obtained by pooling replicates of whole control flies from all days (13).

Because of the potential role of INDY in metabolism (1), we wanted to investigate how the metabolic state of *Indy206* mutant flies may differ from normal flies over the course of their adult life. Hence, we defined a collection of gene sets by grouping genes belonging to a common metabolic pathway, as defined by the KEGG database (4), and other pathways related to genetic, cellular, and environmental information processing (208 gene sets). Of the total 12,268 genes on the arrays, 1,617 were annotated as belonging to at least one pathway. At each time point, each gene set was tested for differences in gene expression between *Indy* mutant and control flies via the gene set enrichment analysis algorithm (GSEA) (14). This algorithm ranks genes from a microarray by the fold-change between treatment and controls, looking for sets in which genes are preferentially at the top or at the bottom of the ranked gene list. Those genes in the top rank of the category are, on average, up-regulated, and the ones in the lower part of the ranked list are, on average, down-regulated. The GSEA system also makes use of a Monte Carlo strategy to test that the observed up- or down-regulation is statistically significant and not just a consequence of random noise in the data.

We included in the analysis all of the gene sets with at least 15 but no more than 500 *D. melanogaster* genes (93 pathways total). Of these, 61 pathways were significantly different in *Indy* with respect to control flies in at least one time point ($P < 0.05$, NES > 1.5; P values were corrected for multiple testing) (15). In particular, OXPHOS (ko00190) was among the top-ranking pathways that was consistently altered across time (day 20 through day 70) and was enriched for genes down-regulated in *Indy* with respect to control. Fig. 1*B* shows the results from the GSEA in the case of OXPHOS genes at day 10 (*Left*) and day 40 (*Right*). At day 10, the OXPHOS genes (*Middle*, blue vertical bars) are distributed roughly uniformly ($P > 0.55$) over the list of all genes on the array ranked by fold-change (*Bottom*, vertical gray bars). At day 40, the genes show a clear bias toward down-regulation in *Indy* with respect to control ($P < 10^{-8}$). A similar decrease in OXPHOS gene expression is seen at day 20, 30, 50, and 70, whereas day 5 and 80 do not show any significant bias (*SI Appendix*).

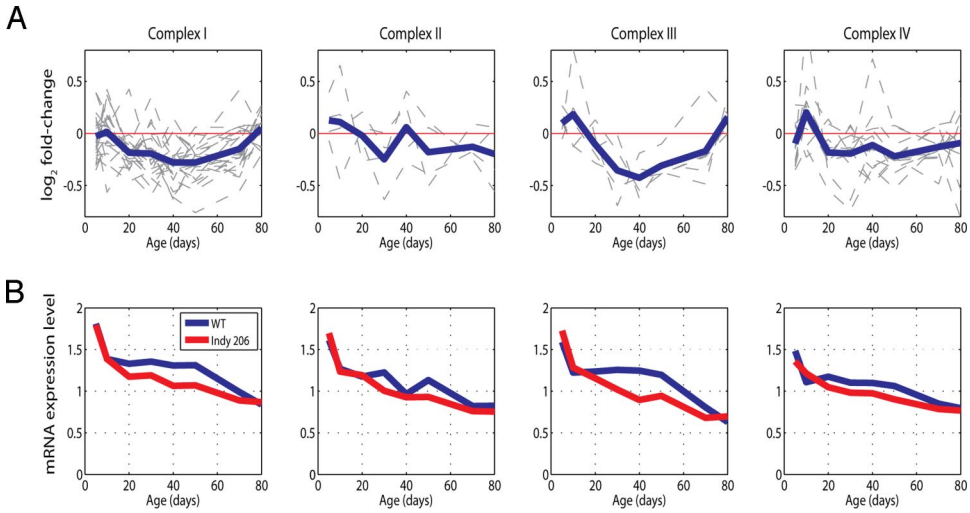

Fig. 2. Expression of ETC complexes I and III is decreased in *Indy*. (*A*) Log_2 fold-change in mRNA expression (microarray data) between *Indy* and wild-type flies for each subunit of OXPHOS complexes I–IV over the course of time (gray dashed lines). The blue solid line represents the complex average log_2 fold-change. Most complexes showed an average down-regulation in Indy versus controls starting at day 20. (*B*) Average mRNA expression level (log_2 fold-change with respect to universal control) of all of the subunits in each complex in control (blue) and *Indy* flies (red). Expression of all complexes decreases with age in both fly strains, with a larger decrease in *Indy* flies at mid age.

Electron Transport Complex Expression Is Decreased in *Indy*. To further dissect the differences between the two genotypes in the OXPHOS pathway, we grouped genes in the ETC according to the specific complex they contribute to (complexes I, II, III, and IV). Fig. 2*A* shows the log_2 fold-change between *Indy* and control gene expression from the microarray data in complexes I–IV as a function of age. The decrease in expression of several of the genes encoding subunits of the ETC was confirmed by QPCR (Table S1). The majority of the genes in most complexes have a lower expression level in *Indy* starting at day 20 (gray dashed lines). This behavior is especially pronounced in complexes I and III, with maximum absolute fold-change at day 40. Despite the significant decrease in fold-change for complexes I and III by day 20 and throughout most of adult life, by Day 80 there is virtually no difference between *Indy* and control.

The apparent equilibration of the ratio of fold-change in the ETC mRNA levels between *Indy* and control at later ages was examined in greater detail. One possibility is that at older ages, the mRNA levels of the ETC complexes in *Indy* could be increasing. However, because the fold-change seen represents the ratio of absolute level of expression between *Indy* and control, an alternative possibility is that mRNA expression of ETC complex in the control is also decreasing with age but at a slower rate then *Indy*, and by later ages they both reach a similar minimal level of expression. A decrease in ETC complex mRNA has been reported in *D. melanogaster* and other organisms and is considered a part of the aging signature (16, 17). As seen in Fig. 2*B*, examination of the average mRNA expression level (log_2 fold-change with respect to universal control) of all of the nuclear encoded subunits in each complex in control and *Indy* flies (Fig. 2*B*) shows that there is an age-dependent decline in both fly types, but the decrease is earlier in the *Indy* flies.

Electron Transport Complex Activity Is Decreased in *Indy*. One of the consequences of a decrease in expression of ETC in the mitochondria could be a decrease in the enzymatic activity associated with these complexes in each mitochondrion. To examine whether a decrease in expression levels of ETC genes corresponds to an actual decrease of the ETC activity in *Indy* flies, we extracted the mitochondrial protein from heads and thoraces of male *Indy* and control flies and measured the enzymatic activity of complex I, II, III, and IV as a function of total mitochondrial protein at day 20 (the earliest time at which we detect a difference by microarray). As suggested by the microarray data, Fig. 3 shows that the enzymatic activity of complexes I–IV appear lower in *Indy* than in controls, although only complexes I and III show a highly significant ($P < 0.01$) difference. Thus, in *Indy*, the decrease in mRNA expression, at least for complex I and III, corresponds to a decrease in the ETC enzymatic activity per mitochondrion.

ROS Production Is Reduced in the Mitochondria of *Indy* Long-Lived Flies. We sought to assess what the consequence of a decrease in expression of ETC genes and ETC activity in *Indy* might effect. A decrease in ETC activity decreases mitochondrial membrane po-

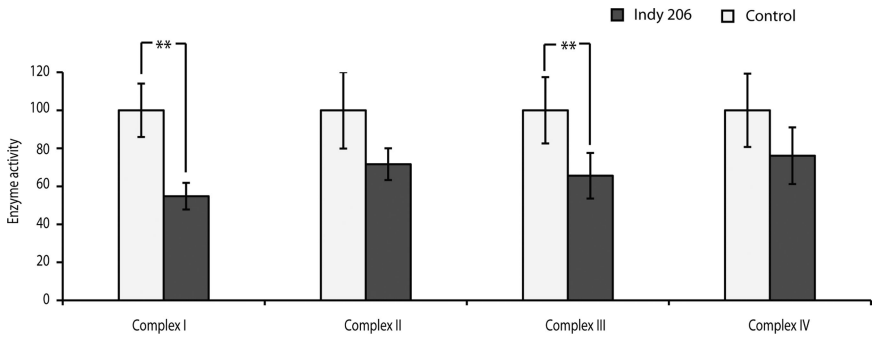

Fig. 3. Activity of ETC complexes I and III is decreased in *Indy*. In respiratory complexes I and III, 20-day old *Indy* mutants have significantly lower activity. Respiratory complex activities were measured in isolated mitochondria from 20-day old flies. Data shown are mean ± SD (3 biological replicates). Each biological replicate sample contained head and thoraxes from more than 75 male flies. **, $P < 0.01$, paired Student's *t* test.

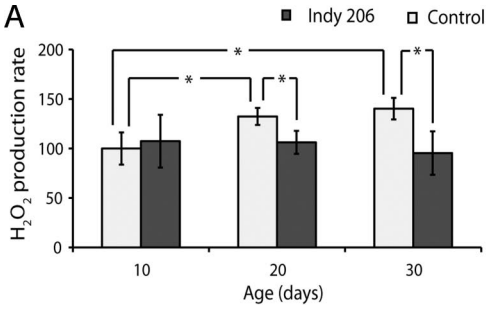

Fig. 4. ROS and oxidative damage is lower in *Indy*. Long-lived *Indy* mutant flies have a lower ROS production rate and less oxidative damage. Indy mutants show lower H_2O_2 production rate (*A*) and protein oxidation (*B*) as compared with controls. Data shown are mean ± SD (3 biological replicates). Each sample contained head and thoraxes from more than 75 male flies. *, $P < 0.05$; **, $P < 0.01$, paired Student's *t* test.

tential (protonmotive force), which has been linked to a reduction in the mitochondrial generation of ROS (18). Therefore, we measured the rate of production of hydrogen peroxide (H_2O_2) in live mitochondria from the heads and thoraces of male *Indy* and control flies at 10, 20, and 30 days of age (Fig. 4*A*). At day 10,the amount of H_2O_2 produced by *Indy* and control mitochondria is similar. However, by day 20, the rate of H_2O_2 production is significantly lower in the *Indy* mitochondria as compared with control mitochondria. The significantly lower production of H_2O_2 continues in *Indy* until at least day 30. We conclude that one consequence of a decrease in expression of ETC mRNA and mitochondrial ETC activity in *Indy* flies is a decrease in the production of H_2O_2 from the mitochondria, as compared with controls.

Accumulation of Oxidative Damage Is Decreased in *Indy* Flies. The oxidative stress hypothesis suggests that a decrease in the generation of mitochondrial ROS is associated with a reduction in accumulation of oxidative damage (12). An association between a decrease in mitochondrial production of H_2O_2 and a decrease in the rate of accumulation of oxidative damage has been shown in *Drosophila* (19). To assess whether there is a difference in the accumulation of oxidative damage in the *Indy* long-lived flies, we examined the amount of protein carbonyls in the mitochondrial protein extract from heads and thoraces of *Indy* and control flies at different ages. Using the OxiSelect Protein Carbonyl ELISA kit (Cell Biolabs), we found that by Day 40, there is a significant decrease in the amount of mitochondrial protein carbonyls in *Indy* as compared with control flies (Fig. 4*B*).

ATP Levels Are Normal in *Indy* Flies. A potential consequence of a reduction in ETC activity could be a reduction in the synthesis of ATP. A decrease in ATP synthesis would likely have a negative effect on the normal physiology of the adult fly and offset some of the advantages of a reduction in ROS generation or oxidative damage. Surprisingly, we found that despite a significant decrease in ETC activity, the amount of ATP in the heads and thoraces of *Indy* and control flies is similar (Fig. 5*A*). In *Indy* flies, some alteration has occurred to decrease the amount of ETC activity per mitochondrion without negatively impacting total cellular ATP synthesis.

Mitochondrial Density Is Increased in *Indy* Flies. It has been suggested that the life span extending effect of CR in mammals may involve a reduction in mitochondrial OXPHOS and ROS generation with maintenance of normal ATP levels (20). A mechanism by which a balance between lower ETC activity and normal ATP levels is achieved appears to include an increase in the density of mitochondria. We determined the density of mitochondria in *Indy* and control flies by measuring the ratio of mitochondrial DNA to nuclear DNA (21–23). As shown in Fig. 5*B*, the number of mitochondrial DNA copies, and perhaps mitochondria density, is increased by at least 25% in the heads and thoraces of male *Indy* flies as compared with controls by Day 20. A higher density of mitochondria would allow *Indy* mutant flies to make up the difference in ATP production resulting from a decrease in ETC activity per mitochondrion.

PGC-1-alpha Expression Is Increased in *Indy* Flies. In mammals, one of the central proteins regulating mitochondrial biogenesis is PGC-1 alpha. An increase in PGC-1 alpha activity is known to enhance mitochondrial biogenesis (24, 25). Using QPCR, we found that the level of the putative fly homolog of PGC-1 alpha, CG 9809 (26), is increased in the heads and thoraces of *Indy* by >40%, as compared with controls at day 20 (Fig. 5*C*). An increase in PGC-1 alpha levels coupled with the increase in mDNA suggests that by day 20, the *Indy* long-lived mutant has a higher density of mitochondria per cell than controls.

Discussion

High-throughput genomic studies, such as whole genome microarrays, are powerful tools for exploring the biological and physiological mechanisms underlying complex biological phenomena such as aging. Most analyses of whole genome microarrays focus on a gene centric approach that concentrates on identifying specific genes whose level of expression has changed in response to the life span altering intervention. This analysis is followed by an evaluation of the phenotypic impact of changes in expression of the specific

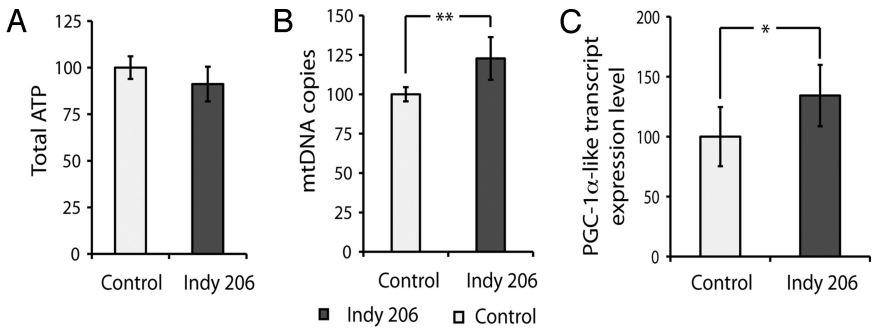

Fig. 5. ATP levels are normal and mitochondrial density and PCG-1 alpha-like mRNA levels are increased in *Indy*. (*A*) Long-lived *Indy* mutant flies produce the same amount of total ATP as control flies at day 20. (*B*) Indy flies have a higher mitochondria density than wild-type flies at 20 days. mtDNA copies were measured by using qPCR as number of COI relative to GAPDH (21–23). (*C*) The PGC-1α-like transcript, which is involved in mitochondrial biogenesis, has a higher expression in Indy flies at 20 days. Data shown are mean ± SD (3 biological replicates for *A*, and 6 biological replicates for *B* and *C*). Each sample contained head and thoraxes from more than 75 male flies. *, $P < 0.05$; **, $P < 0.01$, paired Student's *t* test.

gene(s) in order to determine the physiological systems that may be involved in mediating life span extension.

Another approach to analyzing the information obtained from microarrays is a pathway centric method such as GSEA (14). This approach uses annotated sets of physiological pathways (KEGG and Gene Ontology) to determine what physiological pathways are affected in the conditions being examined. Starting by identifying a physiological pathway, rather than individual genes, may provide some advantages because it immediately suggests the likely importance of specific pathways in the condition being studied. Another advantage is that by looking at the summation of changes throughout an entire pathway, the analysis is not dependent on the preservation of a change in the level of expression of a single gene across different but potentially related interventions or genetic backgrounds. Studies testing the efficacy of the pathway approach indicate that it is both more sensitive and robust than the gene centric approach when used to compare across different experimental protocols and different laboratories (3). Such an approach should be particularly valuable for understanding the underlying changes important in complex biological phenomena such as aging.

We used a pathway centric approach to predict changes in physiological pathways that may be causally related to the life span extending effect of the *Indy* long-lived mutant. One of the major alterations seen in *Indy* long-lived mutants is an early reduction in the expression of the nuclear genes coding for elements of the ETC involved in mitochondrial OXPHOS, in particular complexes I and III. The selective reduction in expression of complexes I and III does not occur until after Day 10. It is accompanied by a corresponding decrease in the enzymatic activity of complexes I and III, a preservation of the low level of mitochondrial ROS production and, by Day 40, a decrease in mitochondrial protein carbonyls. Despite the decrease in mitochondrial OXPHOS, ATP levels in *Indy* flies continue to be maintained at normal levels, most likely because of an increase in mitochondrial density, as reflected by an increase in PGC-1 alpha and mDNA.

A decrease in the level of transcription of the ETC with age is one of the few whole genome transcriptional changes shared among humans, mice, flies, and nematodes (16, 17). It has been suggested that this decrease could assist in reducing oxidative damage at older ages (16). Our findings of a positive effect on reduction of oxidative damage with an earlier decrease in ETC activity in *Indy* long-lived flies supports the hypothesis that the decrease in ETC activity during normal aging may be a compensatory response rather than a passive deleterious consequence of aging. During aging, an increasing burden of mitochondrial ROS generation may trigger a decrease in ETC activity (27, 28) leading to a decrease in mitochondrial ROS production and delay in accumulation of oxidative damage. In the *Indy* long-lived mutant, the decrease in ETC activity and mitochondrial ROS production is initiated earlier and may contribute to the life span extension seen in *Indy* flies.

The reason ETC activity is not normally lower earlier in adult life may relate to the need for production of ATP. Beneficial effects of reducing ETC on minimizing ROS production might be offset by a simultaneous decrease in ATP production. Early in life, when growth and reproduction are maximal, the requirement for ATP is greatest, and both *Indy* and control flies may have their ETC activity set at a high rate to accommodate this need. In *Indy* flies, by day 20, ETC activity has begun to decrease, and mitochondrial density appears to increase. Although the efficiency of each mitochondrion for generating ATP is reduced, the presence of a greater density of mitochondria may contribute to the production of additional ATP. These modifications, coupled with the likelihood that by day 20 the steady state need for ATP may be lower, could allow *Indy* flies to maintain a normal pool of cellular ATP.

We postulate that a decrease in mitochondrial ETC activity, in combination with a modest increase in mitochondrial density, maintains normal levels of ATP while simultaneously decreasing mitochondrion oxidative damage in *Indy*. This favorable state is a byproduct of the relationship between the ETC activity and mitochondrial ATP and ROS production. Studies of mitochondria physiology suggest that small decreases in the protonmotive force across the inner mitochondrial membrane, as would be expected with a decrease in ETC activity, reduces ATP synthesis in an approximately linear manner while simultaneously decreasing ROS production nearly exponentially (18). For every small decrease in ETC activity there will be a much larger reduction in the amount of ROS produced than ATP. *Indy* long-lived flies may take advantage of this therapeutic window by decreasing ETC activity per mitochondrion so as to greatly reduce ROS production and increasing mitochondrial density to make up for the smaller loss in total cellular ATP production per mitochondrion. The net effect is that a slightly greater density of mitochondria, each working at a lower rate, can generate the same amount of ATP without increasing ROS production (Fig. 6). We currently do not have a clear answer as to why such a state, given its advantages in terms of minimizing ROS production, has not evolved in control flies. This model, however, does not take into account the cost of mitochondrial biogenesis and maintenance, which could significantly impact the net energy balance in a natural environment where flies are subject to many more challenges than in the laboratory setting. That such a fine balance is likely to be the case for *Indy* is evident when the richness of the food normally used in laboratory conditions is reduced. Reducing food quality to conditions more like what might be expected in the wild leads to a more severe decrease in reproduction in the *Indy* long-lived flies than in controls, suggesting there are potential costs to decreasing ETC activity and increasing mitochondrial density, at least early in adult life (9).

Per mitochondrion	OXPHOS ↓	ATP ↓	ROS ↓↓
Per cell	[Mito] ↑	ATP -	ROS ↓

Fig. 6. ATP/ROS tradeoff model. For every small decrease in ETC activity there will be a much larger reduction in the amount of ROS produced than ATP. *Indy* long-lived flies may take advantage of this by decreasing ETC activity per mitochondrion so as to greatly reduce ROS production and increasing mitochondrial density to make up for the smaller loss in total cellular ATP production per mitochondrion. The net effect is that a slightly greater density of mitochondria, each working at a lower rate, can generate the same amount of ATP with less ROS produced.

Maintaining an advantageous ATP/ROS ratio may represent a common mechanism for extending healthy life span. During normal aging in humans, mice, flies, and nematodes, there is a gradual reduction of ETC expression with age, which could provide a favorable balance in the ATP/ROS ratio (16). Mitochondria in mammals undergoing caloric and dietary restrictions have been reported to have an increase in ATP/ROS ratio in which cells generate the same amount of ATP with reduced oxidative stress and damage, and consequently, with potential beneficial effects on life span (20). A similar positive effect on ATP/ROS ratio is seen in adult flies in which mitochondrial uncoupling has been increased in neurons and life span extended (unpublished data) (19). The ATP/ROS ratio may thus serve as a formula for assessing the metabolic efficiency of a cell in the context of aging. Interventions that maintain a beneficial mitochondrial ATP/ROS ratio could provide therapeutic opportunities for extending healthy life span.

Methods

Fly Strains. Long-lived *Indy206* heterozygous males were compared with normal-lived heterozygous control males derived from the original mutagenesis (2216/CS for the microarray study and 1085/CS for the biochemical assays) (1). All flies were made heterozygous by crossing to Canton-S. Flies were maintained in a humidified, temperature-controlled incubator with 12/12 h on/off light cycle at 25° C in vials containing 10% sucrose, 10% yeast, and 2% agar. The flies were passed to new vials every 2 days (29).

Microarrays. Flies were cultured on standard laboratory food as described (29). *Indy206* flies heterozygous over Canton-S (*Indy206*/CS) and genetically matched heterozygous controls (2216/CS) were placed on cold block, and head and thorax were dissected at ages 5, 10, 20, 30, 40, 50, 70, and 80 days. Three replicates of 15 males were homogenized in TRIzol reagent (Invitrogen), and RNA was isolated by standard method of Chomczynski and Sacchi (30). From each sample, 2 μg of total RNA was amplified with T7 RNA polymerase (Promega) by using one-round linear amplification protocol as described (31). Construction of *Drosophila* DNA microarrays as described in ref. 13: Briefly, 13,061 predicted or known genes were represented by PCR amplicons (size range 200–500 bp) from *Drosophila* genomic DNA (strain y1; cn1 bw1 sp1). Transcript levels were quantified by using a universal reference sample of pooled mRNA from control whole male flies representing all ages. Normalization of scanned images was done by using GenePix microarray analysis software (Axon Instruments) (13).

Microarray Statistical Analysis. Microarray GenePix files were processed with the *limma* package (32) to obtain expression scores for 14,422 probes. Probesets corresponding to the same genes were averaged to obtain the expression scores of the 12,268 unique genes. Background correction was performed via the *normexp* method (33), within array normalization was performed via the *print-tip loess* method, and between array normalization by using the *Gquantile* method (34). The GSEA (14) was applied to the microarray data to identify which of the 208 KEGG pathways (4) were significantly up- or down- regulated in *Indy* at each age.

RNA Preparation, cDNA Synthesis, and Real-Time QPCR. Total RNA was prepared for microarray and QPCR studies from the head and thorax of more than 20 adult male flies as described (13). QPCRs were performed by using a 7500 Fast Real-Time PCR System (Applied Biosystems), SYBR Green Master Mix (Applied Biosystems), and gene specific primers (Table S2). A two-step PCR was carried out with denaturation at 95° C for 15 s, and annealing and extension combined at 60° C for one minute in a total of 40 cycles. The uniqueness of amplicons was analyzed by using dissociation.

ATP, Mitochondrial Hydrogen Peroxide, Respiratory Complex Activities, and Protein Carbonyl Measurements. Heads and thoraxes from more than 75 flies at different ages were removed and kept on ice for <30 min before isolation of mitochondria. Heads and thoraxes were homogenized and total ATP measured by using ATP determination kit (Molecular Probes). Isolation of mitochondria was performed as described (19). Hydrogen peroxide production was measured in isolated mitochondria under normal respiration conditions by using the Amplex Red kit (Molecular Probes) as described (19). The methods for measuring mitochondrial complex activity were modified from those used in refs. 27 and 28 and *SI Text*. Mitochondrial protein carbonyls were measured by using OxiSelect Protein Carbonyl ELISA kit (Cell Biolabs) following the protocol provided by the manufacturer. Total ATP was normalized to total protein isolated from the head and thorax. Hydrogen peroxide and protein carbonyls were normalized to mitochondrial protein isolated from the head and thorax. Protein concentrations were measured by using the Bio-Rad Protein assay reagent. All studies were done in triplicate and used more than 75 flies per assay.

Mitochondrial DNA Measurement. Mitochondrial DNA content was determined by the ratio of the gene for cytochrome oxidase subunit I (COI) to a nuclear gene GAPDH (Table S2) (21–23). Total DNA from the heads and thoraxes of more than 20 flies at different ages were isolated by using Maxwell 16 Instrument and tissue DNA purification kit (Promega). DNA copy numbers were measured by using real-time PCR as described above.

ACKNOWLEDGMENTS. We thank Suzanne Kowalski, Debbie Best, and Tine Herreman for technical assistance. This work was supported by National Institute on Aging Grants K25AG028753 (to N.N.); AG16667, AG24353 and AG25277 (to S.L.H.); and AG23088 (to B.R.), and an Ellison Medical Foundation New Scholar Award (to A.S.B.). S.L.H. is an Ellison Medical Research Foundation Senior Investigator and recipient of a Glenn Award for Research in Biological Mechanisms of Aging.

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