IN PURSUIT OF A MOLECULAR FOUNTAIN OF YOUTH: THE IDENTIFICATION AND CHARACTERIZATION OF LIFESPAN REGULATORS IN DROSOPHILA

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To my family,

all of them.

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Over the past century, average human lifespan has experienced steady increase despite lack of substantial intervention or understanding of the aging process. In fact, many organisms have the latent potential to live much longer than they normally do. This indicates lifespan determination is an active process subject to regulation. Components of this impending longevity are beginning to unravel through dietary and genetic studies in model systems. To date, several pathways indicate human lifespan extension through direct molecular intervention may be feasible, however, important limitations persist. A common thread among these conserved lifespan regulators is metabolism. Therefore, further insight into lifespan extending mechanisms may lie within tissues governing important metabolic processes.

Here we describe a multi-tiered, strategy to identify *Drosophila melanogaster* mutants with extended lifespan based upon enrichment for insertions in genes that are expressed in

metabolic tissues. Our results indicate metabolically relevant tissues are a rich source of genetic longevity regulation.

We identified a regulator of G protein signaling (RGS) domain containing sorting nexin, termed *snazarus* (*sorting nexin lazarus*, *snz*). Flies with insertions into the 5' untranslated region of *snz* live up to twice as long as controls. Transgenic expression of UAS-*Snz* from the *snz* Gal4 enhancer trap insertion, active in metabolic tissues, rescued lifespan extension. Notably, old *snz* mutant flies remain active and fertile indicating that *snz* mutants have prolonged youthfulness, a goal of aging research. Since mammals have *snz*-related genes, it is possible that the functions of the *snz* family may be conserved to humans.

Next, we identified the two key adenosine monophosphate (AMP) biosynthetic pathways as regulators of *Drosophila* longevity. We found that heterozygous mutation of anabolic components of the *de novo* as well as the salvage AMP biosynthesis pathways extend lifespan. These pathway mutations, and caloric restriction, increased adenosine mono- and diphosphate to adenosine triphosphate (ATP) ratios. Consistent with the altered ratios, lifespan extension was dependent on functional adenosine monophosphate-activated protein kinase (AMPK). Supplementing the diets of adult mutants with adenine restored adenosine nucleotide ratios and rescued lifespan extension. These data establish *de novo* and salvage AMP biosynthesis as determinants of adult lifespan. The dosage sensitivity and enzymatic nature of *de novo* and salvage AMP biosynthesis, and the conserved aspects of adenosine nucleotide derivatives and lifespan extension, indicate that these pathways are potentially amendable drug targets worth continued exploration.

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INTRODUCTION

What is Aging?

Aging is complex. In fact, a metaphor of the field's complexity can be found in the disagreement over the proper spelling of the term "aging" (or ageing)[1]. Although additional controversy exists over the precise definition, aging can be broadly defined as the collection of changes that decrease functional capacity and cause progressively increasing likelihood of death[2]. These changes occur at the cellular, tissue, organ, and whole organism level; however, a paucity of direct measurements of the rate and type of physiological decline remain, leaving an inadequate chronologic description of age. Consequently, the age of individual cells, tissues, and organs do not necessarily correlate with the age of an organism. While this may be due to inconsistent criteria, it may also indicate aging occurs at different rates within an organism [3]. Among a population, however, the lifespan of an individual species is both consistent and characteristic; concurrently, lifespan between species varies dramatically. For example, the common house mouse (*Mus musculus*) rarely exceeds a lifespan of 3 years under protected laboratory conditions, while certain species of bat, also a mammal of similar body size and metabolic rate, can live over forty years in captivity[4-7]. Outside of class, lifespan estimates of wild species can range from 72 hours in the mayfly to the bowhead whale which can live over two centuries[8,9]. This represents a fold variation in lifespan among the animal kingdom greater than 25,000. Adding to the variation, evidence suggests the simple multi-cellular organism, hydra, do not show increased mortality with age, indicating the possibility of an immortal organism[10]. Beyond "what is aging?" these observations lead to questions regarding why and how we age. In fact, we may not need to fully understand what aging is in order to identify ways to delay it.

Theory of Aging

The earliest descriptions of aging are likened to the gradual and inevitable deterioration over time, like that of a mechanical object, leading ultimately to breakdown, failure, and death. Certain aspects of human biology do deteriorate (e.g., tooth enamel and nonexudative age-related macular degeneration). Unlike a machine, however, an organism self-generates, maintains homeostatic flux, and is capable of self-repair. Therefore, it is likely that this breakdown occurs due to the organism's failure to repair itself and is not a product of aging. Furthermore, in direct opposition to the prospect of wearing out, continual challenging use, like exercise, can increase efficiency and strengthen cardiac and skeletal muscle and not age them.

August Weismann, although a proponent of aging as mechanical decline, was the first to advance the theory of aging by proposing an explanation for why organisms age. He concluded that aging was an evolutionarily selected mechanism designed to eliminate older individuals from competing against their progeny[11]. This was the first description of aging as genetically regulated process. Weismann's theory, however, lacked the details of how natural selection could produce aging as a mechanism for death.

From an evolutionary stand point the presence of aging is somewhat paradoxical. First, for the majority of species in natural habitats, aging doesn't occur. The threat of predation, infectious disease, and fluctuations in climate and nutrient availability produce a high natural mortality rate. Populations in the wild simply die before they grow old[12]. Thus, natural selection's direct influence over the aging process is limited. In these species, strong selective pressure exists to develop rapidly and reproduce early with high fecundity, however, alleles that either enhance or diminish an individual's rate of aging past maturity would not significantly contribute to the subsequent gene pool. Second, for the few long-lived species (e.g., large

mammals) aging is deleterious to individual fitness. Any gene contributing to the process of aging would not be maintained. In fact, individuals with inactivating mutations in such a gene would have a selective advantage providing pressure to eliminate the gene within the population and result in a continuous increase in lifespan. These points maintain that if natural selection were directly functional aging would not exist.

Resolution of the presence of aging paradox began with the work of John Burdon Sanderson Haldane, in 1941. While studying Huntington's disease, Haldane questioned why around age forty, a single genetic allele leading to increasing loss of mental and physical capacity would survive obvious negative selection. He proposed ancestral populations did not survive into their forties and therefore natural selection would have no impact on the presence of a genetic disease which presented later in life[13]. Peter Medawar further developed this notion into the mutation accumulation theory of aging. The theory states that the force of natural selection peaks prior to reproductive maturity at which point a progressive weakening occurs with increasing age[14]. This waning effect allows mutation and genetic drift to act increasingly unchecked and lead to the higher frequency of various alleles with deleterious actions later in life that ultimately cause aging[2]. Mutation accumulation theory implies that the process of aging developed randomly; however, the characteristic lifespan of individual species and the vast range of lifespan among different species argue against randomness. In addition, the American biologist, George Williams, asserted that although the force of natural selection declines with age, it should still function at a capacity strong enough to select against the significant human aging that occurs between the ages of thirty and forty regardless of the presence or absence of genetic alleles that lead to diseases like Huntington's.

In 1957, George Williams published the idea now known as the antagonistic pleiotropy theory of aging. Williams emphasized that the adverse effects of aging on fitness require another force to counter act negative selection and promote the presence of aging. He postulated the root of this force is an indirect result of genes with multiple functions and opposite effects on fitness at different ages[15]. When coupled with the notion that the force of natural selection declines with age[16], this allows genes with even modest beneficial effects early in life to be favored by natural selection despite the same genes causing aging later in life. This theory conceptualized the notion of a life-history trade-off in which later survival is sacrificed for a more rapid fitness benefit. Consequently, antagonistic pleiotropy reconciles the trend of short lived species having rapid sexual maturity with heightened fecundity and longer-lived species with extended maturation periods and relatively small brood sizes.

Recent attempts to further explain mechanisms of aging center around dietary and genetic studies which prove lifespan extension is possible. The first, known as the disposable soma theory, suggests allocation of limited metabolic resources available to an organism is divided between maintenance of somatic cells and reproduction via germ cells[17]. Genes which provide or require additional resources to accelerate reproductive development or efficiency can be selected at the expense of resources reserved for somatic repair[18]. This selection leads to increased rates of somatic deterioration which manifests as aging. A corollary of this theory suggests mechanisms exist to redistribute resources away from reproduction, perhaps during unfavorable reproductive periods, into somatic maintenance thereby extending life. This is supported by several studies in model systems which demonstrate methods of lifespan extension are related to reductions in fecundity[19,20]. Mounting evidence, however, suggests aging and fecundity may be controlled independently[21]. A second explanation, termed the hormesis

hypothesis, claims mild stressors activate a survival response within the organism causing lifespan extension by increased defense against the causes of aging. However, it is unclear why natural selection would refrain from chronic activation of such pathways. Without some type of negative trade-off the hormesis hypothesis is inconsistent with the antagonistic pleiotropy theory of aging.

Over the past century, we have seen continual evolution of the theories behind aging. Current explanations lack cohesion, and perhaps a unified theory of aging is unattainable due to the complex and potentially multifaceted process of aging. However, a theoretical base allows hypotheses to develop into testable predictions which provide a usable model for experimentation, and will ultimately lead to further elaboration and validation.

Models of Human Aging

The first study of human aging is credited to Benjamin Gompertz who was interested in what he called "an increased inability to withstand destruction."[22] Gompertz showed that after a period of maturation the probability of death increased exponentially as a function of age across four British cities in the mid 1820s[22]. This work defined the first model of aging in mathematical terms. Furthermore, it established the importance of mortality rate and the utility of mortality curves as the basis of lifespan analysis. A clear mathematical characterization helped to define aging in terms that can be quantified and analyzed. In addition, it provided means of testing whether lifespan can be extended.

Experimental analysis of human longevity is difficult. Studying human life history is impractical, cost prohibitive, and has yet to be attempted. The examination of genetic variations

overrepresented in human centenarians is often confounded by lack of adequate controls, irreproducibility, and environmental variation[23,24]; however, when coupled with candidate gene approaches these studies have achieved reasonable insight[25]. Fortunately, the conserved nature of methods found to increase lifespan suggest the advantages of model organisms can be used to study the aging process as it applies to human biology. In fact, the value and relevance of model organisms in the study of aging is indisputable, and allows for greater experimental control, genetic manipulation, and screening techniques that have rapidly advanced longevity research.

One important model in aging research is the single celled budding yeast, *Saccharomyces cerevisiae*. Yeast lifespan is categorized in two ways: replicative and chronological. Replicative lifespan (RLS) is determined by the number of daughter cells produced before senescence [26]. Chronological lifespan (CLS) is measured by the amount of time a yeast cell can remain viable in non-dividing conditions [27]. The yeast system allows for rapid experimental progress due to the speed at which its lifespan can be quantified. Yeast perform the most basic biologic functions allowing for the unique opportunity to study aging at the cellular level, without anatomical complexities. However, recent data indicate that culture conditions ultimately cause chronologic death in yeast, indicating studies examining yeast CLS may not be representative of aging in other systems [28]. RLS ends when a cell is deemed post-mitotic and not necessarily dead, which may more accurately represent a measure of fecundity not lifespan. Furthermore, a fundamental cause of replicative aging is thought to be related to extrachromosomal ribosomal DNA circles, which do not exist in cells of higher organisms [29 30]. This calls into question the relevance of yeast lifespan in human aging. Despite the evolutionary conserved aspects of some pro-longevity mechanisms from yeast to mammals and the potential value of studying aging at the cellular level, the influence of differentiated tissues and coordinated intercellular

communication absent from unicellular organisms coupled with clear yeast-specific mechanisms of aging limits this otherwise powerful system.

The round worm, *Caenorhabditis elegans*, has provided aging researchers a successful intermediate between the speed of a quick lifespan and the relevance of a patterned multi-cellular organism. In addition to mortality curves, worms are subject to increasing senescence of mobility and fertility throughout life and can be used to further quantify the aging process [31] [3]. However, important biological distinctions exist. Worms are hermaphrodites, which allows tremendous genetic control, but precludes potential sexually dimorphic aspects of aging. Simplified cellular patterning in worms limits identification of tissue specific lifespan regulation, particularly in tissues with high metabolic activity. In addition, developing *C. elegans* can enter into an immobile quiescent state known as dauer formation not seen in many higher eukaryotes. It remains unclear whether some aspects of lifespan extension in worms involve entrance into a unique partial duaer-like state that does not exist in humans. Despite these criticisms, several genetic and dietary manipulations that extend longevity identified in worms have provided key insight into mechanisms that modulate aging in mammals.

The unique advantages of the *Drosophila melanogaster* model system have been extensively used throughout numerous aspects of aging research. The long history of *Drosophila* genetics allowed George Williams to cite studies involving *Drosophila* in support of his original description of antagonistic pleiotopy[15]. Extension of *Drosophila* lifespan through artificial selection of laboratory populations confirmed the role of natural selection in aging[32]. *Drosophila* provides a short lifespan and more differentiated, functionally conserved tissue structure than other invertebrate models. In addition, a wide variety of genetic tools and mutations available to researchers allow for extensive gain and loss of function studies with which to elucidate

mechanisms of aging. *Drosophila* helped identify important environmental and molecular regulators of lifespan, as well as, provide evidence of evolutionary conservation[33,34 35]. Increased organ complexities, not found in other model systems, helped elucidate tissue specific lifespan regulation in *Drosophila*[36 37]. Physiological, behavioral, and disease-related alterations that occur with aging have also been studied with the *Drosophila* model system[38,39].

While invertebrate models led to the elucidation of numerous conserved mechanisms involved in lifespan extension, mammalian model systems have brought tremendous validation and practical application to the aging field. Longevity research in these systems is costly and time intensive given their long lifespan (i.e., mice and rats can live three or four years; primates live upwards of 30 years); however certain questions can only be asked in these systems. For example, invertebrate cause of death remains largely unknown regardless of lifespan, where as age-associated pathologies in mammals can be more insightful post mortem. Specifically, invertebrate models are composed of mostly post-mitotic cells and do not develop cancer, therefore, pro-longevity affects on age-associated increased cancer risk are only possible in mammals.

Mechanism of Lifespan Extension are Linked to Metabolism

In 1934, McCay *et al.* documented a fifty percent lifespan extension in rats fed a thirty-five percent reduction in calories [40]. This study was the first to indicate the aging process is not too complicated to escape manipulation. In fact, it proved lifespan is under active regulation and can be extended by relatively simple alteration of experimental conditions. Subsequent studies in species ranging from the single celled yeast, *Saccharomyces cerevisiae*, to the Rhesus macaques

(*Macaca mulatta*), a nonhuman primate with approximately ninety-three percent sequence identity to the human genome[41], confirm a reduced diet without malnutrition known as caloric restriction can lower age-related mortality rate approximately three fold[42-44]. The consistency with which caloric restriction extends lifespan across species indicates the presence of an ancient, evolutionarily conserved pathway that regulates longevity. This suggests molecular mechanisms may coordinate downstream of caloric restriction and provide multiple targets to delay aging. It also questions whether additional independent longevity pathways exist. The identification and mechanistic understanding of these pathways remains the priority of aging research.

The first molecular pathway shown to influence aging was the insulin/insulin-like growth factor signaling (IIS) pathway. The IIS pathway is a component of the endocrine system and functions to help coordinate organismal responses influencing growth and energy metabolism at the tissue and cellular level. In *Drosophila*, mutation of the insulin receptor substrate (IRS) homolog, *chico*, extends lifespan nearly fifty percent[21,45]. Inhibition of downstream pathway components functioning to propagate IIS[46] or activation of functional IIS inhibitors also extend lifespan[47,48] (Figure 1). Subsequent studies across various model systems have confirmed the lifespan benefit of decreased IIS pathway activity is evolutionarily conserved[49-51]. In *C. elegans*, the lifespan extension of IIS inhibition is orchestrated through forkhead box O (FoxO), heat-shock factor (HSF), and Nrf-like transcription factors[52-55]. These transcription factors coordinate up or down regulation of a diverse gene array that acts cumulatively to produce longevity. Interestingly, individual genes among this array function in a variety of cellular responses that can be thought of as protective, including defense against infection and prevention, repair, or disposal of macromolecular damage, while the function of others remains unknown[56].

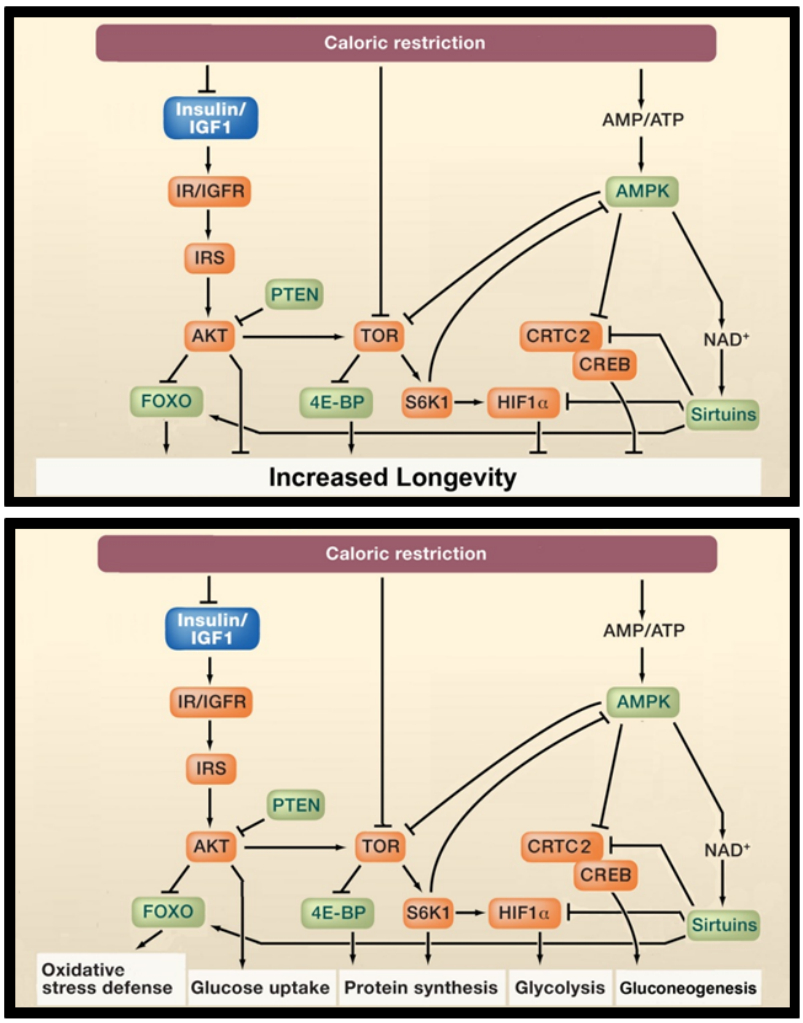


Figure 1. Regulation of lifespan is linked with metabolism. Top panel: Simplified model of known lifespan regulatory pathways elucidated in various model systems and effected by caloric restriction. For simplicity mammalian homologs are depicted and representative components shown. Orange boxes represent lifespan determinants that extend lifespan when functionally reduced. Green boxes depict regulators that promote longevity when over-expressed or activated. Blue box symbolizes other protien. Insulin Receptor or Insulin-like Growth Factor Receptor (IR/IGFR), Insulin Receptor Substrate (IRS), Phosphatase and Tensin Homolog (PTEN), Akt, Forkhead Box transcription factor family (FOXO), Target of Rapamycin (TOR), Eukaryotic translation initiation factor 4E-binding protein (4E-BP), Ribosomal S6 Kinase1 (S6K-1), Hypoxia-inducible factor 1 alpha (HIF-1α), Adenosine Monophosphate-activated Protein Kinase (AMPK), Cyclic-AMP Response Element-Binding (CREB), CREB regulated transcription coactivator 2 (CRTC2), SIRT1 (Sirtuins). Bottom Panel: Lifespan regulators also function in a variety of critical metabolic process establishing a link between metabolism and lifespan determination.
*Role of Sirtuins in lifespan extension is inconsistent, and therefore remains in green but lacks a direct arrow to increased longevity. *Modified from Houtkooper,et. al. (2010).*

An additional pathway found to influence longevity is focused on the target of rapamycin (TOR) kinase. TOR signaling functions as a major amino-acid and nutrient sensing pathway that stimulates cellular growth via proliferation, transcription, protein biosynthesis, and blockade of catabolism when activated[57]. Like IIS, inhibition the TOR pathway increases lifespan in many species, from yeast to mice[37,58,59]. In fact, TOR kinase can be activated cell-autonomously by the IIS pathway[60]; however, studies in worms suggest the longevity of decreased TOR activity is independent of FoxO, indicating distinct downstream paths to longevity[61]. Lifespan extension via reduced TOR signaling depends in part on relieved inhibition of eukaryotic initiation factor 4E binding protein (4E-BP) and decreased levels of hypoxia inducible factor-1 (HIF-1) caused by lower ribosomal subunit S6 kinase activity[37,62 63] (Figure 1). These alterations function in a variety of physiological responses which promote survival and efficient resource utilization, including stress-response, mitochondrial function, protein translation, and protein recycling via autophagy.

A third longevity pathway involves the intracellular energy sensor *adenosine monophosphate-activated protein kinase* (*AMPK*). Intracellular energy is stored in the chemical bonds of adenosine triphosphate (ATP). As energy is used, ATP is converted into its lower energy precursors, adenosine diphosphate (ADP) or adenosine monophosphate (AMP). Increased AMP:ATP and ADP:ATP ratios activate AMPK [64,65]. In this manner, AMPK functions in response to alterations in cellular energy to coordinate enzymes and transcription factors shifting metabolic processes between energy utilization and energy conservation/generation [66-68]. Activation of AMPK extends lifespan [69 70]. Activated AMPK leads to the inhibition of TOR signaling, which may account for some of the lifespan benefit [71]. In addition, AMPK activation in C. elegans increases longevity via disassociation of the cyclic

AMP response element binding (CREB) transcription factor and its coactivator (CRTC) [72] (Figure 1). In mammals, an analogous complex can regulate important metabolic and stress related functions. However, it is unknown which are important in the aging process [73 74 75].

Activation of nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylases known as sirtuins, primarily Sir2, have been reported to increase lifespan in yeast, worms, and flies[76-78]. NAD^+ is an important co-factor for the electron transport chain and its concentration is linked to the cellular metabolic state [79]. Thus, surtuin activity responds to metabolic alterations by modification of protein targets and transcriptional changes through chromatin remodeling. Sirtuins can link several longevity pathways (Figure 1). For example, activated AMPK can indirectly increase sirtuin activity, which can, in turn, also inhibit CRTC2[73,80,81]. Sir2, or the mammalian homolog SIRT1, can activate FoxO and HSF1 transcription factors[76,82,83]. SIRT1 can also deacetylate and inhibit HIF1α[84]. Although interaction with lifespan determinants and a role in metabolism is clear, recent data seriously question the pro-longevity effects of sirtuins [85]. Until definitive studies in mammalian systems are complete the role of sirtuins in lifespan determination remains inconsistent[86].

Aging research has begun to unravel cascades that control longevity[34,87-89]. Lifespan regulators, including insulin and IGF-1 hormones, TOR kinase, AMPK, and perhaps sirtuins, are also key sensors assigned to adjust and maintain metabolic homeostasis in response to nutrient flux. While one could not dispute that caloric restriction acts on each of these pathways to promote longevity, it remains unclear to what extent. This is confounded by the functional relation and considerable interaction among lifespan regulators. What has become clear is that metabolic processes and their relevant tissues are central components of lifespan regulation [90-92]. Furthermore, these pathways, as well as their affects on lifespan determination, are conserved

over great evolutionary distances. Questions do remain regarding whether or not these findings are feasible and can be successfully applied to reduce initial mortality rate in humans.

Therapeutic Potential of Lifespan Determinants

With aging comes increased susceptibility to many diseases, and certain age-related diseases (e.g., cancer, heart disease, sarcopenia, and neurodegeneration) are especially prevalent among elderly populations [93 94 95]. In fact, you are ten times more likely to develop a tumor after the age of 65 than before[96] . Animals such as mice and dogs are also significantly more cancer-prone after approximately two and ten years of age, respectively [97 98]. Therefore, a similar increased risk of cancer occurs at approximately seventy-five percent of life expectancy in mice and dogs, as in humans. This indicates increases in cancer risk, and perhaps other diseases, are directly related to rates of aging. Therefore, a potential therapeutic application of aging research is the manipulation of longevity pathways to delay age-associated disease onset or progression.

While conclusive evidence of pro-longevity effects in humans is lacking, caloric restriction is the best documented and robust lifespan extending intervention throughout the animal kingdom. In addition to lifespan extension of wild-type animals, caloric restriction also delays the onset of numerous age-associated diseases models, including cancer, heart disease, sarcopenia, and neurodegenerative diseases[3,42,99-101 102 19]. However, even if additional studies substantiated a similar effect in human patients, several difficulties still impede its widespread usage. Markedly reduced caloric intake is unpleasant and difficult to maintain [103 104]. In addition, data suggests the decreased mortality rate benefits are immediately abolished upon deviation from a calorically restricted diet. Therefore, the prospect of successful treatment of human

disease through a regiment as demanding as caloric restriction is low. A potentially more practical approach may be the pharmacological manipulation of pro-longevity molecular pathways (e.g., IIS, AMPK, and TOR) downstream of caloric restriction.

Reduced IIS extends lifespan. Treatment with chemical inhibitors of IIS in *Drosophila* results in slight, inconsistent increases in lifespan [105]. Insufficient dosage may account for this tepid longevity response; however, higher dose could cause severe insulin insensitivity and risk development of serious metabolic disorders, like diabetes. Fortunately, other IIS inhibitors remain to best tested, and the numerous transcriptional targets of IIS may hold additional therapeutic potential.

Metformin is a highly prescribed antidiabetic known to activate AMPK [106]. Metformin treatments in worms and mice indicate a potential lifespan benefit, however, the effect is inconsistent and further experimentation is needed [70 107 108 109]. In addition, it is not yet certain that AMPK is the sole target of metformin. Other AMPK activators, like 5-aminoimidazole-4-carboxamide riboside (AICAR), exist however their ability to extend lifespan is undocumented.

Similar to sirtuins themselves, a chemical activator of sir2 called resveratrol was originally reported to extend the lifespan of yeast, worms, and flies[110,111]. However, additional studies report no lifespan benefit with resveratrol treatment in flies and only a slight lifespan increase in worms[112]. In addition, evidence suggests that resveratrol does not directly affect Sir2, but may have an indirect effect via AMPK activation which also promotes longevity [113,114]. Where resveratrol and other sirtuin activators have succeeded is in treatment of metabolic disease [115]. Although the role of sirtuins in lifespan determination has been deemphasized, if not discarded, their initial ties to longevity lead to links in metabolic pathways and practical therapeutic application. This supports the notion that because of the close link between

longevity pathways and metabolism the potentially broad benefits of pro-longevity drugs may have a more focused capacity to treat metabolic disease.

The most promising proof of drug inducible lifespan extension involves the TOR inhibitor rapamyicin. Rapamycin treatment in yeast, flies, and mice significantly increases lifespan [59,116,117]. Furthermore, studies conducted in mice indicate rapamycin administration at middle age promotes lifespan extension[59]. This indicates rapamycin is functional in adults, an important criterion for approval. Rapamycin is already approved for use in humans, and is widely prescribed as an immune suppressant. It is difficult to reconcile the immunosuppressive activity with potential life extending actions of rapamycin, and this will likely preclude rapamycin from clinical trials involving age-related disease. In spite of this, rapamycin stands as proof of principle toward the identification of longevity drugs effective in mammals.

Currently, any method to extend longevity cannot be approved by the Federal Drug Administration (FDA). Aging is not recognized by the FDA as a disease. Thereby, aging cannot be an indication for any drug. Pro-longevity drugs will only be approved if they affect disease. Pharmacological manipulation of longevity pathways to produce lifespan extension is possible. However, approval of individual therapeutic strategies remains problematic due to potentially harmful side effects or questionable efficacy.

Rational For Studies

Aging is markedly complex. Although inroads have identified a few dietary and genetic mechanisms as conserved routes to lifespan extension, much remains unclear. However, a well established theme common among pro-longevity mechanisms is a functional role in metabolic

processes. Therefore, tissues of metabolic relevance may be a unique source of lifespan regulators.

Mechanisms shown to extend lifespan also delay several negative aspects accompanying old age in model systems, including onset of cancer, diabetes, and heart disease[118]. Today, therapeutic application of these findings involves unfavorable potential side effects and barriers to clinical trials inhibiting widespread use. Again, the metabolic link of lifespan determination offers potential road to clinical application through treatment of metabolic disease. In fact, the unique overlap between lifespan regulation and metabolism suggests any findings regarding aging may be relevant to metabolic function and disease. Therefore, identification of additional lifespan regulators is beneficial to the further elucidation of mechanisms involved in lifespan determination as well as potential therapeutic insight into treatment of age-associated and metabolic disease.

Drosophila has a proven record of successful contributions vital to the field of aging. The short generation time, small genome size, comprehensive collection of allelic variants, and functionally conserved physiology make *Drosophila* highly amenable to the study of aging. In addition, the GAL4 activation system creates the ability to target or identify gene expression restricted to specific cells or tissues within *Drosophila* [119]. This provides a unique opportunity to take advantage of the established link between aging and metabolism in order to direct the search for novel components of lifespan regulation to specific, metabolically relevant tissues.

MATERIALS AND METHODS

Fly Stocks and Culture

Flies were reared in uncrowded conditions in standard cornmeal-dextrose-agar-yeast media with sprinkled yeast granules unless otherwise noted. The original GAL4 enhancer trap insertion line, *pGawB/ FM7*, the transposase line *CyOHop2*, and the *w⁻; UAS-eGFP; UAS-eGFP* reporter line were previously described (contribution from Dr. Claude Desplan) [120]. *w⁻; noc^{Sco}/CyO; TM6B, Tb^{1}/MKRS* was from the Bloomington Stock Center (stock #3703). *w^{1118}* and *mth^{1}* (generous contributions from Dr. Seymour Benzer) were used as controls[121]. Dcg-GAL4 is a fat body GAL4 driver (contribution from Dr. Charles Dearolf). Lines that were identified to have extended lifespan, including *C32* and *F71* (see below) were backcrossed at least ten generations into the *w^{1118}* (control) background prior to further experimentation by mating twenty mutant virgin flies, homozygous for the first cross and heterozygous for subsequent crosses, to twenty *w^{1118}* males for ten generations and then heterozygous females and hemizygous males were intercrossed to produce homozygous stocks. The P-element mutation was selected by following the eye-color marker, mini-white. In the case of *F71* the *TM6B, Tb^{1}/MKRS* balancer stock was backcrossed to *w^{1118}* ten generations in parallel as described before re-introduction of either *TM6B, Tb^{1}* or *MKRS* alleles to maintain *F71* stock heterozygosity. Balancer alleles were removed prior to experimentation by single cross of isogenic *F71/MKRS or F71/ TM6B, Tb^{1}* stocks to *w^{1118}*. *G1409* (*P(Mae-UAS.6.11)CG1514,* gift from Dr. Ulrich Schafer) and *SZ4089* (*P(RS5)5-SZ-4089,* Szeged stock center) contain P-insertions into the *CG1514* locus. The precise location of P-insertion sites for all lines with lifespan extension was determined by inverse PCR[122] and/or plasmid rescue. The *SZ4089* line was backcrossed into the *w^{1118}* (control) background. The *G1409* line does not have an eye color

marker and thus was used in experiments without further backcrossing. The additional *AdSS* insertional line, *HA2022* (*P{RS5}cCG17273*$^{5\text{-}HA\text{-}2022}$), was obtained from the Drosophila Genetic Resource Center. *AdSL* (*PBac{w*$^{+mC}$*=PB}CG3590*^{c02781}*/TM6B, Tb*^{1}), *AdenoK* (*P{ w*$^{+mC}$ *y*$^{+mDint2}$*=EPgy2}CG11255*^{EY07694}), *Aprt* (*ru*^{1} *Aprt*^{5} *h*^{1}), and *AMP deam* (*P{w w*$^{+mC}$*=EPg}CG32626*^{HP10734}) lines were obtained from the Bloomington Stock Center. *Adk2*, (*P{EP}Adk2*^{EP2149}) was obtained from the Exelixis Collection at Harvard Medical School. All lines were backcrossed at least ten generations into the *w*^{1118}, which was used as the control unless otherwise noted.

Enhancer Trap Screen

The X-linked enhancer trap P-element, *PGawB*, was mobilized to generate new insertions as described previously[120]. Briefly, *pGawB/FM7* females were mated to *CyoHop2* males to generate female jumpstarter flies, *pGawB/w-;CyoHop2/+*, which were then mated to the *w-; UAS-eGFP; UAS-eGFP* reporter line. F1 larvae resulting from the jumpstarter-UAS-eGFP reporter cross were flushed from media with water, cleared of debris by floatation on NaCl solution, rinsed in water, and screened for fat body GFP expression under a fluorescence dissecting microscope using a GFP filter. Individual F1 larvae with fat body expression were grown to adults in individual food vials and then mated to the *w-; noc*^{Sco}*/CyO; TM6B, Tb*^{1}*/MKRS* balancer stock and the resulting GFP fluorescing, *CyO*, and *MKRS* F2 progeny were intercrossed to generate lines. Chromosomal assignment for each insertion was achieved by examining segregation of the GFP fluorescence in males and females for X insertions, and by crossing *CyO* and *MKRS* balanced F2 males to *w-; UAS-eGFP; UAS-eGFP* and examining segregation of GFP fluorescence against either the *CyO* or *MKRS* balancer. In the case of X insertions, lines were generated by crossing to *FM7* balancer stocks

Lifespan Assays

Adults emerging from uncrowded cultures were mated and the resulting larvae were again grown in uncrowded conditions to produce offspring used in lifespan assays. Flies that emerged within a two day period were pooled and aged for an additional three days under standard culture conditions before subjecting to lifespan assays. Approximately eighty males and eighty females were placed in demography cages in duplicate or triplicate cohorts per trial. During initial screening of the enhancer trap collection, pools of ten lines were collected and mortality curves determined at either 25°C or 30°C. Specifically, ten flies per line per sex were combined together in standard food vials and quadruplicate samples were assessed for mortality every other day and then placed into fresh vials. For later lifespan analysis demography cages were kept at room temperature (22–23°C) as described previously[123] and mortality scored daily when changing to fresh food vials.

Fat Body Visualization and Nile Red Staining

Larvae or adult flies were submerged in methanol prior to microscopic analysis or dissection of the fat body. GFP expression in whole larvae or adults was documented under a fluorescence dissecting scope with a GFP filter. Fat bodies were carefully dissected, fixed in formalin, permeabilized in 0.2% Triton X-100 and stained with Nile Red and documented under a fluorescence dissecting scope using a rhodamine filter.

Mammalian Adipogenic Cell Culture

3T3-L1 murine preadipocytes were purchased from the American Type Culture Collection and maintained in growth media (DMEM with 10% calf serum, 10 units/ml penicillin, 10 µg/ml streptomycin) at 37°C in 5% CO_2. Cells were passed before confluence and discarded

after 10 passages. Media was changed every other day during cell maintenance and adipogenesis. 3T3-L1 cells were induced to undergo adipogenesis as described [124 125].

RNA Extraction, cDNA Synthesis and Real-time PCR

Total RNA from cultured cells, mouse adipose depots, or ten-day old adult males was extracted with Trizol (Invitrogen), RNase-free DNAse I-treated, and reverse-transcribed using random hexamers and MMLV-reverse transcriptase (Invitrogen) to obtain cDNA. Gene expression was measured by quantitative real-time PCR analysis using SYBR Green Master Mix reagent (Applied Biosystems, 7500 Real-Time PCR System). Real-time PCR values of target gene expression were normalized over endogenous *ß-actin* or *ribosomal protein 49* expression for cell culture and mouse or *Drosophila* experiments respectively. All real-time primer sequences were validated for specificity and efficiency prior to use. Real-time primer sequences are available upon request.

Mouse Studies

Mice were housed in a 12:12 light:dark cycle. For genetic obesity modeling, fat depots were removed from six month-old *ob/ob* mice and control littermates fed 4% fat chow (Teklad) [124]. Veterinary care was provided by the Division of Comparative Medicine. All animals were maintained under the guidelines of the U.T. Southwestern Medical Center Animal Care and Use Committee according to National Institute of Health guidelines.

Transgenic Flies

The cDNA clone AT01932 contains the full-length *Snz* (CG1514) cDNA within the pOTB7 vector. Full-length *Snz* cDNA was PCR amplified and cloned into pUAST[119] to generate pUAST-*Snz*. Transgenic lines harboring pUAST-*Snz* were generated using P-element-mediated

germ line transformation as described previously[126,127]. Primer sequences used for PCR amplification and sequence verification are available upon request. Full-length AdSS cDNA was PCR amplified and cloned into pUAST from the cDNA clone RE23826 to generate pUAST-AdSS. Transgenic lines harboring pUAST-AdSS were generated using P-element-mediated germ line transformation as described previously [126,127]. Primer sequences used for PCR amplification and sequence verification are available upon request. The dominant negative AMPK allele, UAS-AMPK^{DN}, (*P{w^{+mC}=UAS-SNF1A.K57A}2*) was previously described[128] and acquired from the Bloomington Stock Center. Controls for all experiments involving transgenic flies were UAS allele alone.

P-element Revertant Screen

Females from the C32 enhancer trap line that contained an insertion in the 5′-UTR of *snz* (CG1514), *snz^{C32}*, were crossed to FM7/y;*CyOHop2/+* (transposase source) males to mobilize the P-element. The resulting *snz^{C32}*/FM7; *CyoHop2/+* female jumpstarter flies were crossed to *FM7/Y* males. Male white-eyed progeny, lacking the *FM7* balancer, were selected and mated to *pGawB/FM7;+;+* females to generate lines. After mating, genomic DNA was harvested from individual males and PCR amplified with primers flanking the original insertion site to examine the potential P-excision site. *w^{1118}* males were used as controls and candidate excision lines were sequenced to assess the excision event. Through this P-excision screen, we obtained multiple lines in which excision at the CG1514 insertion site had occurred, but in all cases a small fragment (25–45 bp) of residual P-element was still present at the excised locus. Revertant lines were maintained without further backcrossing due to the lack of an eye-color marker.

Oxidative and Heat Stress Assays

Adult flies emerging on the same day from non-crowded cultures were collected and further cultured in identical conditions on standard fly food for ten days before stress analyses. For oxidative stress tests, groups of twenty flies per sex per genotype were subject to control, 5% H_2O_2, or 20 mM paraquat conditions and death was scored every twelve hours[129]. Heat stress assays were performed by placing groups of twenty flies per sex per genotype in a 37°C incubator and scoring for mortality every two hours. Stress tests were performed at least three times and each test was performed in triplicate.

Negative Geotaxis Assays

Ten flies of the indicated ages were put into empty extended vials. Vials were gently shaken and flies tapped down to the bottom of the vial. The time required for each individual fly to climb 9.5 cm was measured, averaged, and used to calculate speed. Flies that at any point flew were excluded from the trail. Five consecutive trails were preformed on three independent groups. The experiment was performed twice.

Fertility and Fecundity Assays

Flies were grown under standard culture conditions and aged as described in lifespan assays. At various time points five females per genotype were isolated from cultures, introduced to five one week old w^{1118} males in separate bottles, and provided apple juice agar supplemented with yeast paste. After acclimating for twenty-four hours, flies were given a fresh apple juice plate with yeast paste and allowed to lay eggs for twenty-four hours. Egg collections were repeated for four twenty-four hour periods. After each period the total number of eggs and subsequent larvae were counted. Viability was the number of larvae produced divided by the

number of eggs laid. Male fertility was assessed in a similar manner using five aged males paired with five one-week old virgin w^{1118} females. All experiments were performed at least in triplicate.

Nucleotide Measurements

Ten-day old adult flies were isolated in sex-specific groups of forty, washed in ddH_2O, and placed on ice. 250 microliters of ice-cold 8% (v/v) $HClO_4$ was added and samples immediately subjected to three intervals of thirty sec sonication and thirty sec on ice. The solution was then neutralized with 1 N K_2CO_3 and centrifuged to pellet debris. The supernatant was passed through a 0.2-μm filter, and subjected to reversed phase chromatography using a Targa C18 250 × 4.6 mm 5-μm column as described [130]. Nucleotides were detected at 254 nm, and peak areas were measured using 32 Karat software. Nucleotide identities were confirmed by co-migration with known standards. Calorie restricted flies (Cal Res) were given water only for forty-eight hours prior to measurement. Adenine supplemented (plus Ade) flies were given either 0.05% w/v adenine supplemented or control diet (see below) during the ten day maturation period before analysis.

Adenine Supplementation

Standard diet contained approximately 64.2 g cornmeal (MP Biomedicals), 16.0 g dry active yeast (Fleischmann's), 12.4 g agar (MoorAgar), 85.5 mL molasses (Grandma's), 8.5 ml Tegosept (240 g l^{-1} in ethanol; Genesee), and 2.4 ml Propionic Acid (Fisher) per liter. Required 10X adenine hemisulfate (Sigma #A9126) stock solutions were prepared fresh in ddH_2O with 30% (w/v) autolysed yeast powder (Genesee) added in order to avoid additional yeast supplementation. Stock solution was then added to 55 °C molten standard diet to generate proper

1X concentration. An equal amount of autolysed yeast solution alone added to standard diet was used as control. For larval lethality experiments, w^{1118};;*TM6B,Tb*^{1}/+, *F71/TM6B,Tb*^{1}, or *AdSL/TM6B,Tb*^{1} adults were cultured in bottles containing adenine supplemented diet for three separate three day intervals. The percentage of homozygous pupae was calculated based on the absence of the *TM6B,Tb*^{1} balancer chromosome and the tubby phenotype. For simplicity, the calculated percent was normalized to the expected homozygous Mendelian ratio of one third to give the expected survival percentage. These experiments included between three and eight hundred total pupae per replicate and were repeated twice. For lifespan assays 4 mL aliquots of 0.05% adenine supplemented food was dispensed into vials, prepared fresh twice throughout the experiment, and stored at 4 °C.

RESULTS I: METABOLIC ENHANCER TRAP LONGEVITY SCREEN

To focus the search of novel regulators of lifespan, a collection of lines with mutational insertions in genetic loci expressed in metabolically relevant tissues were first isolated (Figure 2A). To achieve this, enhancer trap lines generated by transposition of the pGawB construct containing an enhancerless *GAL4* gene were crossed with a reporter line harboring an upstream activating sequence (UAS) coupled with an enhanced green fluorescence protein (eGFP) transgene [119]. Using a fluorescence dissecting microscope and isolated lines with mutations in tissues of metabolic importance the eGFP expression in F1 progeny was documented. The primary focus was *Drosophila* fat body expression, however, co-expression with other tissues provided fat body expression that was relatively strong, and therefore, is also included. This technique allowed simultaneous production of tools for tissue specific transgenesis while generating mutations[119,120]

Approximately 700,000 F1 larvae were screened and 591 lines generated with metabolic eGFP expression. 102 of these lines displayed fat body-selective GFP expression, while others had some co-expression in other tissues including those that are critical in metabolism (i.e. fat body and oenocytes; fat body and anterior midgut; fat body, oenocyte, and anterior midgut)[131,132]. This comprised our collection of "metabolically–enriched" mutants. Approximately eighty additional lines were selected that did not have any GFP expression in the fat body, but did express GFP in non-fat tissues such as brain or muscles. This was done, in part, to compare the frequency of possible long-lived flies in the two collections as a potential assessment of whether the regulation of lifespan might be biased towards one tissue or another.

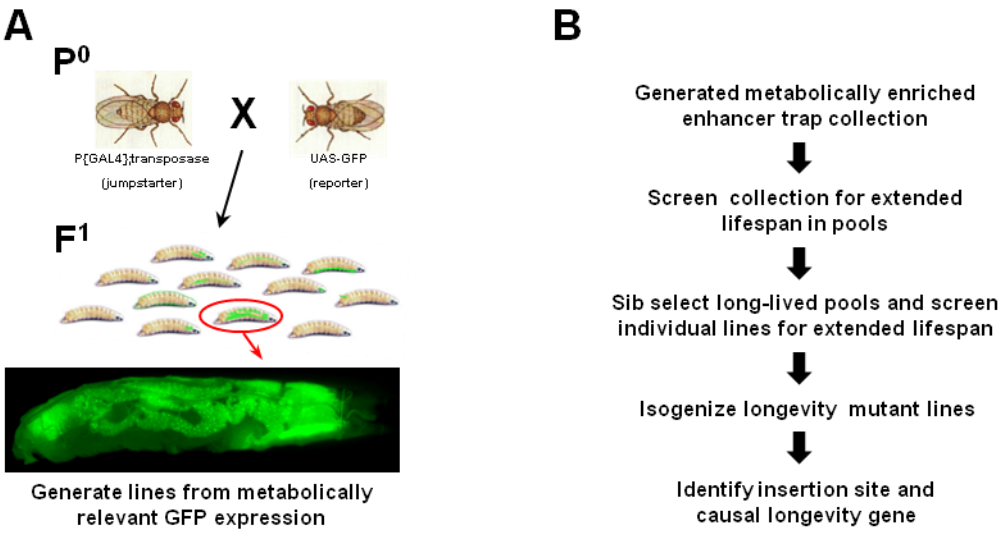


Figure 2. Metabolically Enriched Enhancer Trap Longevity Screen. (**A**) Cartoon of the two-component enhancer trap screen designed to isolate mutational insertions in loci expressed in metabolically relevant tissues. (**B**) Schema of the longevity screen.

The entire enhancer trap collection was then subjected to repeated longevity screening (Figure 2B). For this screen, it was required that extended maximum or median lifespan could be reproduced at a threshold of >30% increase compared to controls, be present in both sexes, and at both 25°C and 30°C. Assuming that lifespan extension at this stringency would be infrequent, and because in such a collection only about 15 percent of the lines (i.e. ~100) are projected to have any mutant phenotype, the lines were initially screened in pools. The positive pools were sib selected and individual lines screened several times. From this, ten lines that displayed a consistent and significant increase in lifespan were identified (Figure 3). All of the lines that displayed increased longevity came from the "metabolically–enriched" enhancer trap lines and none derived from lines that we selected for lack of fat body expression, although this sample size was significantly smaller.

To decrease the likelihood that second site mutations were the cause of the extended longevity and to avoid potential background or modifier effects the ten long-lived lines were backcrossed >10 generations into a control w^{1118} strain. To identify the genomic location of the P-element insertion in each of the longevity lines, DNA isolated from flies was sequenced using plasmid rescue and inverse PCR at both the 5′ and 3′ ends. These approaches identified only a single chromosome insertion in each line, and database searches identified the predicted gene affected by the individual P-element insertions (Table 1).

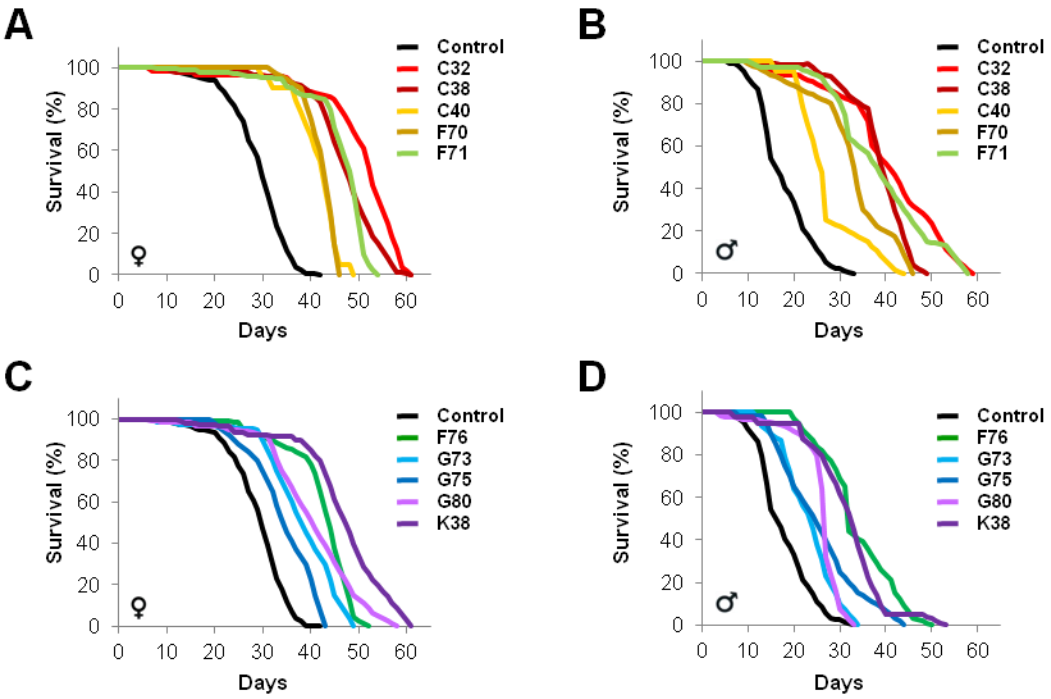


Figure 3. Ten metabolic enhancer trap lines were long lived. (**A** and **B**) Representative mortality curve of five isolated female and male longevity lines. (**C** and **D**) Representative mortality curve of additional five isolated female and male longevity lines. Note lines are separated for aesthetics. Assays performed at 30° C. (log rank, $P<0.00001$ in any line compared to control)

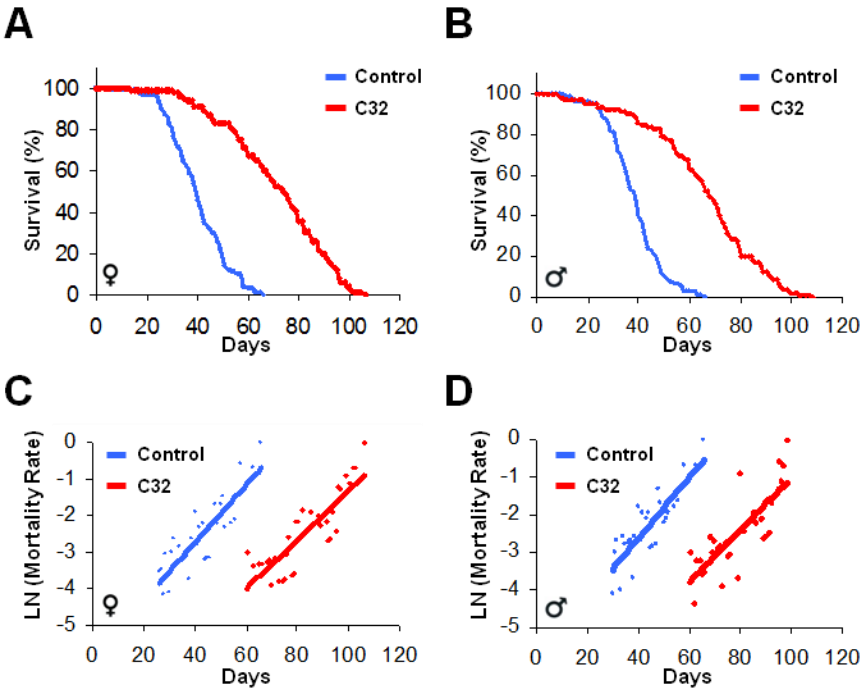
Line	Position	Insertion Site	Effected Gene	Expression Pattern	Gene Function	Homolog
C32	7C2-3	5' UTR of *snz* (*CG1514*)	*snazarus*	FB; Anterior MG	RGS contaiining sorting nexin family member; endocytosis[151]; signal transduction[153]; phosphatidylinositol binding[129]	SNX13, SNX14, SNX25
C38	10E3-4	30 bp 5' of Hsc70-3 (*CG4147*)..	*Heat shock protein cognate 3*	FB; MG	stress response[168]; ER chaperone[169]; sleep wake cycle[170]	BiP
C40	18E2-3	5' UTR of *meso18E*	*meso18E*	FB	regulated by Dmef2[171]; mesoderm development[172]	None identified
F70	12A9	1st intron of *Dmel NFAT*	*drosNuclear Factor of Activated T cells*	FB; MG	transcription factor; negative regulation of synaptic vesicle exocytosis[173]; response to salt stress[174]	NFAT5
F71	93A1	5' UTR of *AdSS* (*CG17273*)	*Adenylosuccinate Synthetase*	FB; Ubq.	*de novo* AMP biosynthesis	AdSS1 and 2
F76	6E4	1st intron of *ogre* (*CG3039*)	optic ganglion reduced	FB	Innexin family member; imaginal CNS morphogenesis[175]:. Phototransduction[176]	*Pannexin* and *Connexin* families
G73	61C7	3' UTR of *CG42719*	*CG42719*	FB MG discs	Unknown	none identified
G75	46E1	3rd exon of *CG1371*	*CG1371*	FB MG	carbohydrate binding; carboxypeptidase activity	nodal modulator 1, 2, and 3
G80	12C3	5'UTR of *Ahcy13* (*CG11654*).	*Adenosylhomo-cysteinase at 13*	FB	component of the one-carbon metabolic process important for methylation[177]	Adenosylhomo-cystein hydrolase
K38	18F5	Largest intron of *CG32529*	*CG32529*	FB MG	Unknown	none identified

Table 1. Ten metabolic enhancer trap lines were long lived. Information regarding each line is presented including, cytogenetic band position (Position) and insertion site (Insertion Site) relative to predicted gene effected (Effected Gene). Third instar larval expression pattern of effected gene is estimated by individual line insertional Gal4 crossed to UAS-eGFP (Expression Pattern) and listed according to relative strenght; fat body (FB), midgut (MG), ubiquitous (Ubq), imaginal discs (discs). Gene function is based on protein structure or existing literature (Gene Function). Protein sequence analysis generated closest mammalian homolog (Homolog). Polypeptide binding protein (BiP), Nuclear factor of activated T-cells (NFAT). Note: F76 is 700 bp 5' of CG14430 and therfore may also affect it. K38 may also effect *amnesiac* (*amn*)[178].

RESULTS II: AN RGS-CONTAINING SORTING NEXIN CONTROLS LIFESPAN

The C32 Enhancer Trap Line is Long-lived

We further characterized the enhancer trap line *C32* as it had the longest lifespan of the ten lines identified in the screen and because old *C32* flies appeared vigorous and fertile (see below). After creating a more isogenic background through a >10 generation backcross into a control w^{1118} strain, we analyzed the lifespan of multiple cohorts of female homozygous and male hemizygous *C32* mutants and controls. *C32* flies in the w^{1118} background of either sex lived up to 100% longer than control w^{1118} flies (Figure 4, A to E). Mortality analyses indicated that the primary effect of the *C32* mutation was a reduction of initial mortality rate (Figure 4C and D). These results show that the *C32* mutant allele leads to increased lifespan in both a mixed genetic background, i.e., the original enhancer trap mutant strain, and in the w^{1118} inbred background.



E

Genotype	Sex	N(*n*)	Median Lifespan[a]	Maximum Lifespan[a]
C32/C32	F	4(365)	72.50 ± 3.23**	109.00 ± 2.04**
w^{1118}/w^{1118}	F	4(355)	44.75 ± 1.84	72.50 ± 3.38
C32/Y	M	4(506)	78.25 ± 4.09*	113.00 ± 3.70**
w^{1118}/Y	M	4(465)	42.25 ± 1.89	69.50 ± 2.36

Figure 4. *C32* flies are long-lived. (**A**, **B**) Adult female (**A**) or male (**B**) *C32* and control w^{1118} flies were identically reared and survival was assessed daily. (n>80 per group, p<0.0001 by log-rank test) (**C**, **D**) Log mortality plots for adult female (**C**) or male (**D**) *C32* and control w^{1118} flies identically reared. (n>80 per group, p<0.0001 by log-rank test) (**E**) Table summarizing four independent lifespan analyses of *C32* and control flies. [a]Values are mean of the median and maximum lifespan of C32 and w^{1118} control female (F) and male (M) flies ± standard error of the mean. N, number of replicates. *n*, total number of flies examined. * p <0.001, ** p <0.0003 by student's t-test.

C32 Inserted into the 5′ UTR of *snazarus*, an RGS-containing Sorting Nexin

DNA sequence isolated from *C32* flies using plasmid rescue and inverse PCR at both the 5′ and 3′ ends identified a single P-element insertion in cytogenetic band position 7C2-3 on the X chromosome. Database searches indicated the *C32* P-element inserted into the 5′UTR of *CG1514* (Figure 5A), a hypothetical gene that encodes a member of the sorting nexin (Snx) family, which we termed *snazarus-sorting nexin lazarus* (*snz*, pronounced snaz). Snx proteins are defined by the presence of a phospholipid binding (PX) domain (Figure 5B) [133 134]. CG1514 also has two predicted transmembrane domains, a PX associated (PXA) domain, and a regulator of G-protein signaling (RGS) domain (Figure 5B). Database searches also identified three mammalian homologs of CG1514, termed SNX13, SNX14, and SNX25 (Figure 5B) [134].

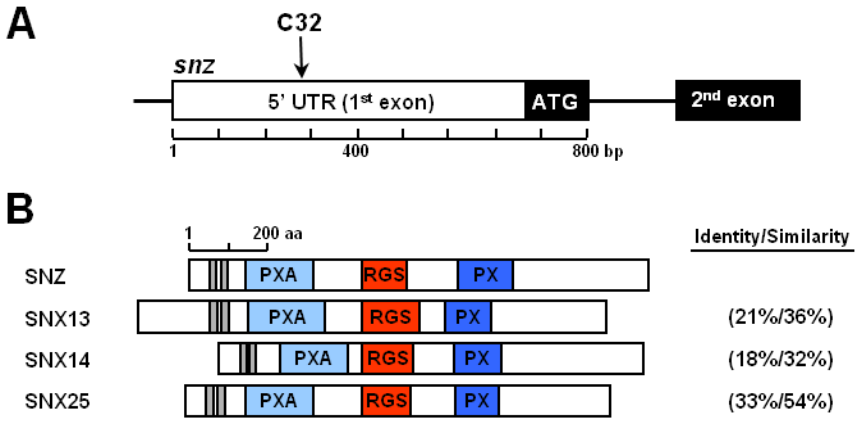


Figure 5. *C32* enhancer trap P-element inserted into the 5′UTR of *snazarus*. (**A**) Location of the *C32* P-element insertion into the first exon of the *snazarus (CG1514)* gene. (**B**) Domain structure and alignment of *D. melanogaster* Snz and the three mammalian homologs. Grey rectangles represent hydrophobic patches (potential transmembrane domains).

RGS-containing Sorting Nexins have Conserved and Dynamic Adipocyte Expression

We examined the pattern of expression of *snazarus* by using the C32-Gal4 insertion to drive expression of UAS-eGFP. We detected strong expression of GFP in the fat body, the organ structure homologous to mammalian adipose tissue, from larval stages and throughout life (Figures 6, A to C). There was also some heterogeneous and lower level expression in the anterior midgut where fatty acid synthase and several other genes central to fat biology are expressed (Figure 6D) [135]. To address the potential metabolic relevance inferred by our screening parameters, we examined the expression of the three mammalian homologs of *snazarus* in 3T3-L1 preadipocytes, which can be induced to differentiate into adipocytes [136], and in fat depots from control and *ob*/*ob* mice, a genetic model of obesity [137]. We found dynamic expression of *snz* homologs in 3T3-L1 cells and murine fat pads with higher levels in differentiated adipocytes (Figure 6E) and in *ob*/*ob* fat depots (Figure 6F).

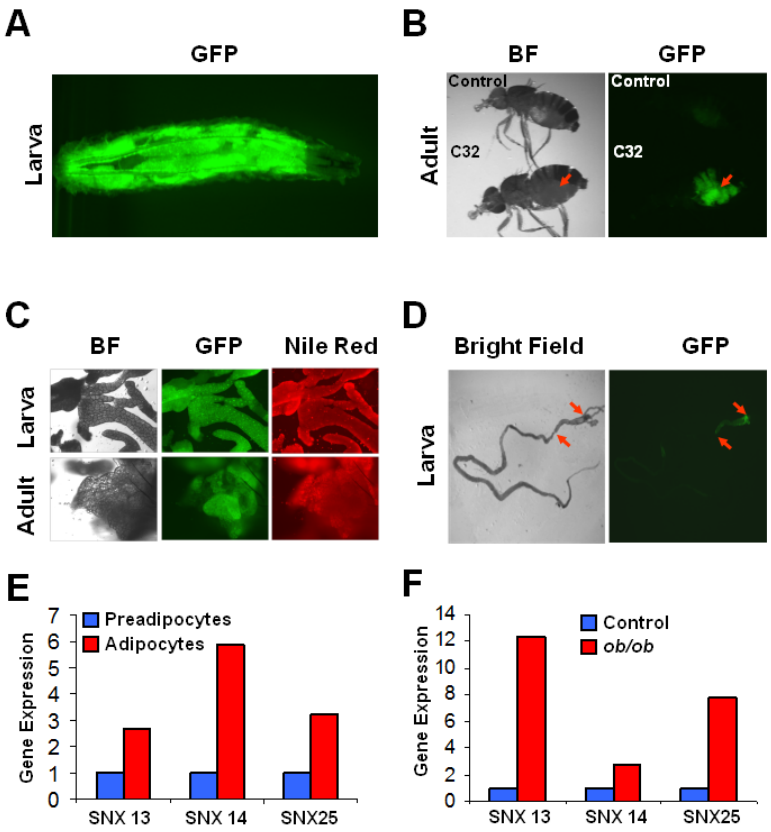


Figure 6. RGS-containing Sorting Nexins have conserved and dynamic adipocyte expression. (**A**) *C32* (minimal promoter Gal4); *UAS-eGFP* larvae were examined and photographed under GFP fluorescent microscopy, which showed strong expression in the fat body. (**B**) Adult control or *C32; UAS-eGFP* flies were photographed under bright field (BF) or GFP fluorescent microscopy. Arrow indicates the abdomen, which contains much of the adult fat body. Slight background fluorescence in control fly is due to the presence of the yeast, which is used as a food source, in the gut. (**C**) The fat body of *C32; UAS-eGFP* larvae or adults was removed, incubated with the fat specific fluorescent dye Nile Red and photographed under bright field (BF) as well as GFP and Nile Red fluorescent microscopy. (**D**) The intestinal tract of *C32; UAS-eGFP* larvae were removed and then examined and photographed with GFP fluorescent microscopy, which showed that portions of the anterior midgut (segment between arrows) express GFP. (**E**) SNX13, SNX14 and SNX25 mRNA expression levels were quantified with real-time PCR in uninduced 3T3-L1 preadipocytes and in induced 3T3-L1 adipocytes (n = 1). (**F**) Fat pads were removed from control and genetically obese *ob/ob* mice and the levels of SNX13, SNX14 and SNX25 expression were assessed with real-time PCR (n = 1).

Snz Transgensis Rescues C32 Lifespan Extension

To determine if *Snz* can rescue *C32* lifespan extension, we generated transgenic flies that contain full-length *Snz* cDNA under the control of an upstream activating sequence (UAS) that can be activated with Gal4, which was present in the P-element mobilized in our fat body enhancer trap screen. The *C32-Gal4* enhancer trap displayed strong expression in the larval and adult fat body and had some co-expression in the larval anterior midgut, a region that regulates fat storage (Figure 6, A to D) [132]. As *C32* hemizygous males were long-lived, the presence of the Gal4 in the *C32* P-element allowed us to determine the lifespan effects of *Snz* transgenesis simply by crossing *UAS-Snz* males with *C32* mutant virgin females and then comparing lifespans of *C32*, *UAS-Snz*, and *C32;UAS-Snz* male siblings. The mortality curves indicated that *Snz* transgenesis, from the promoter active in metabolic tissues, rescued the *C32* lifespan extension (Figure 7A). However, UAS-Snz expressed from the Dcg-Gal4 fat body driver did not alter longevity (Figure 7B), and together with the relatively modest reduction in *C32;UAS-Snz* lifespan compared to control suggests that the reduction in longevity was neither non-specific nor due to Snz toxicity. The accumulated data support the notion that *Snz* regulates longevity and indicate that it does so in tissues central to fat biology.

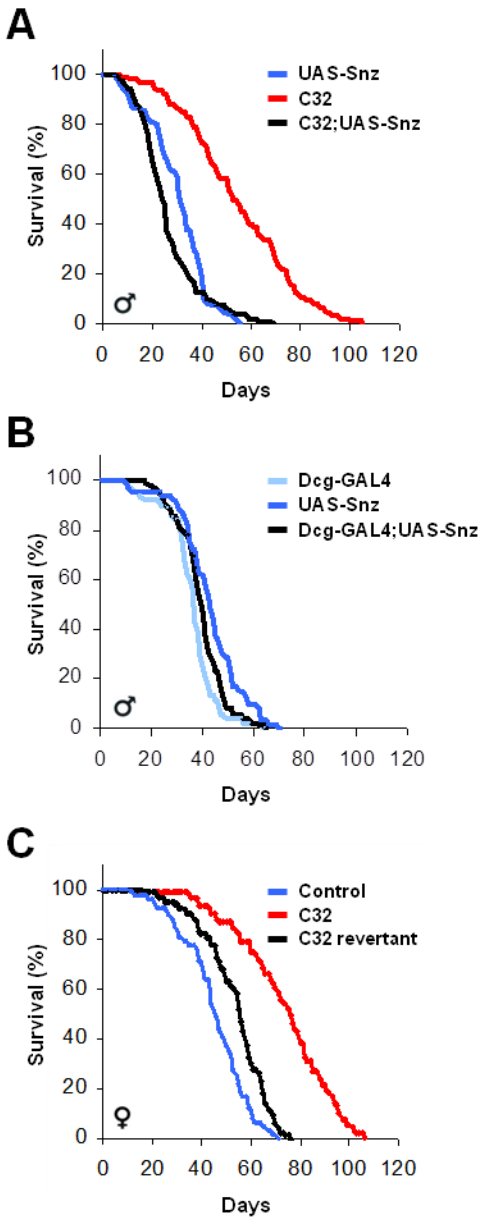


Figure 7. *Snz* transgenesis and *C32* excision reduces lifespan extension. (**A**) The lifespan of male control, *C32*, and *C32; UAS-Snz* transgenic flies was plotted. (n>80, p<0.0001 by log-rank test between *C32* and *C32; UAS-Snz*) (**B**) The lifespan of male *Dcg-GAL4*, UAS-Snz, Dcg-GAL4;UAS-Snz transgenic flies were plotted. (n>80) (**C**) Female control, *C32*, and *C32* revertant flies were cultured and survival was plotted. (n>80, p<0.0001 by log-rank test between *C32* and *C32* revertant) The lifespan of the male excision lines also reverted towards normal. Representative data from multiple experiments is shown.

Excision of the C32 P-element Shortens Lifespan Extension

To further examine the possibility that *snz* is the gene that regulates lifespan in the *C32* line, we mobilized the *C32* P-element with an activated transposase and scored for excision of the P-element based upon an eye color marker [119]. We then evaluated progeny lines with altered eye color using a PCR-screening strategy designed to identify precise excision alleles. However, in even the most precise excision events, small alterations containing between twenty-five and forty-five bases of the original P-element still remained. This is not uncommon with P-element excision techniques[138]. We compared the lifespan of the three lines that most closely resemble the wild-type genomic sequence with w^{1118} controls and the parent *C32* line and found that their longevity was more similar to control (Figures 7C, not shown).

Independent Insertions into *snz* have Increased Longevity and Display Transheterozygous Lifespan Extension

Several consortia have undertaken large-scale P-element insertional mutagenic screens[139-141]. To reduce the possibility that a linked second-site mutation was responsible for the *C32* lifespan extension, and to examine the generality of the effect, we obtained two independently derived P-element *snz* insertions (*G1409* and *SZ4089*). Both *G1409* and *SZ4089* insertions are present in the 5′UTR (Figure 8A). Comparison of control, *C32*, *G1409*, and *SZ4089* lifespan indicated that females and males of all three lines with an insertion into the *snz* locus lived substantially longer than controls (Figures 8B and 8C). Next, we performed complementation tests with females, and observed lifespan extension in transheterozygotes of *C32* with either *G1409* or *SZ4089* (Figure 8D and 8E). The X-chromosome location of *snz* precluded this same analysis in males. Of note, very old *C32/G1409* transheterozygous females remained fertile and produced viable offspring (Figure 8E, arrowheads).

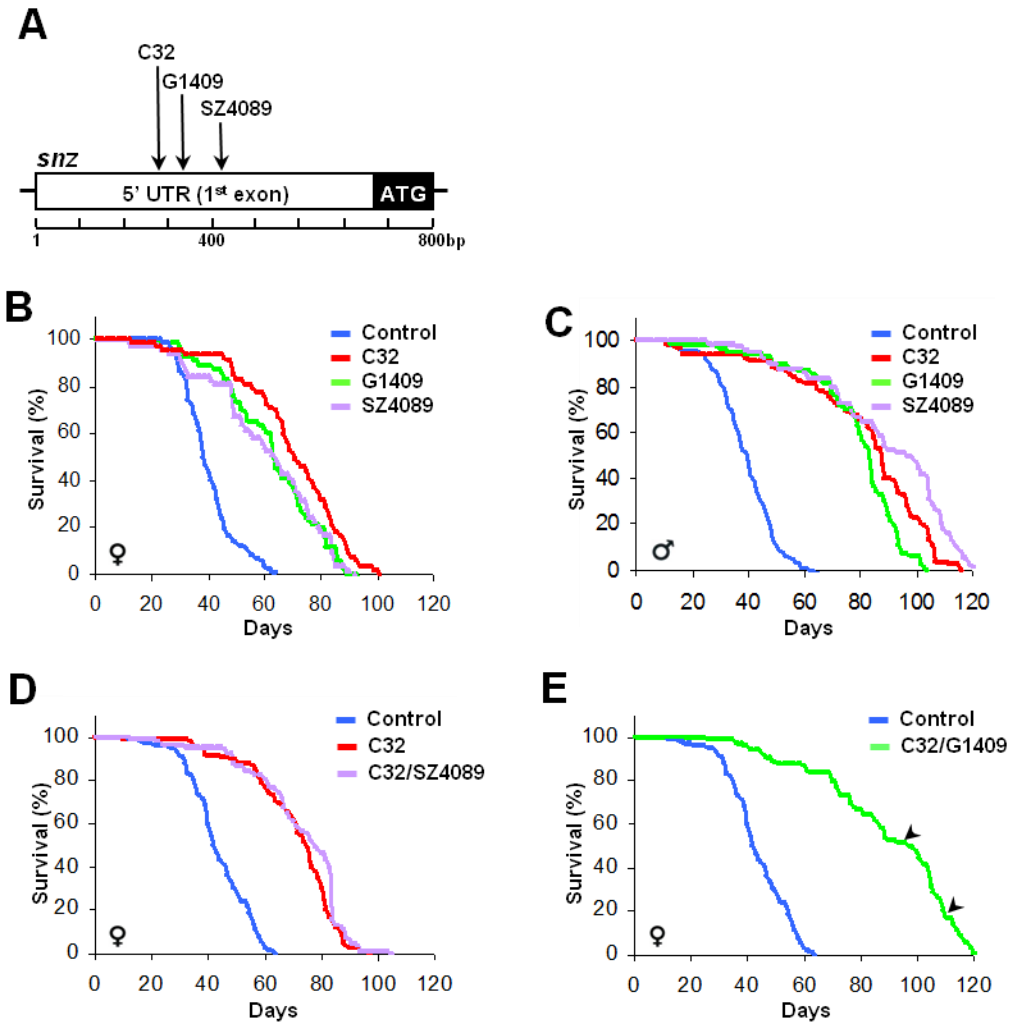


Figure 8. Independent *snz* alleles are long-lived. (**A**) Location of the *C32*, *G1409*, and *SZ4089* P-element insertions in the *snazarus* locus. (**B**, **C**) Female (**B**) and male (**C**) control, *C32*, *G1409*, and *SZ4089* flies were cultured and survival was plotted. (n>80) (**D**, **E**) The lifespan of control, *C32* as well as *C32/SZ4089* (**D**) and *C32/G1409* (**E**) female transheterozygotes was assessed and plotted. (n>80) $p<0.0001$ by log-rank test between control and all *snz* mutant alleles. Arrowhead indicates fertility at observed time-points. Representative data from multiple experiments is shown.

Snz Mutant Flies are Active and Fertile

The observation that very old *C32* flies, at ages when no control flies were alive, produced offspring was noteworthy as invertebrates and vertebrates have substantial reductions in fertility with aging and also because calorically restricted animals and many long-lived genetic mutants appear to shift from reproduction to somatic maintenance until conditions are again favorable for procreation[142,143]. The extended fertility of the *C32* flies might reflect a delay in the onset of egg laying. So, we quantified the number of eggs produced by one week-old control and 1week-old *C32* flies but found no difference (Figure 9A). Next, we analyzed the viability of eggs produced by one week-old control and *C32* flies as well as cohorts at roughly fifty percent survival (6.5-weeks old for controls versus 9-weeks old for *C32* mutants), a time point for *C32* flies at which virtually all control flies are dead (Figure 3). We found that at young ages the viability appeared indistinguishable (Figure 9B). However, the eggs produced by 6.5-week controls had substantially reduced viability while *C32* eggs were resistant to this effect (Figure 9B). Further, 9-week old *C32* flies produced approximately four times more progeny than 6.5-week controls (not shown). We also compared the ability of control and *C32* males at fifty percent survival to fertilize one-week old virgin control females and found that the percentage of viable eggs was equivalent (Figure 9C). Of note, development of *C32* flies proceeds normally based upon morphology and the timing of egg laying to eclosion.

In flies, reduced activity can extend lifespan and a goal was to assess whether *C32* may be such a "refrigerator" mutation [144]. Casual observations indicated that young and old *C32* flies were at least as, if not more, active than young controls in a variety of behaviors including feeding, flying, courting, and crawling. To quantify activity, we performed negative geotaxis assays [145], in which we analyzed the rate at which control and *C32* flies crawl from the bottom to

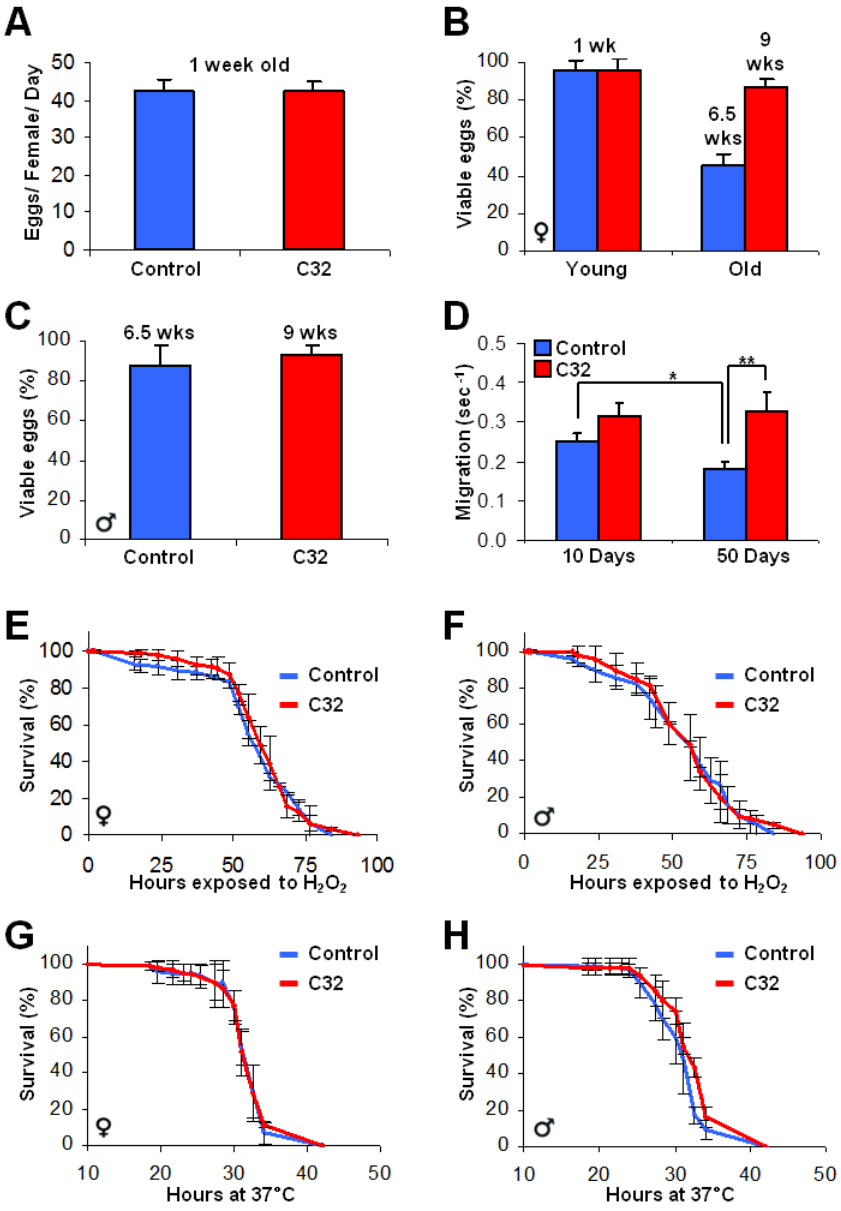


Figure 9. Old *C32* flies are active and fertile. (**A**) Egg production was measured for 1-week old control and *C32* females. (n = 15 per group) (**B**) Egg viability was assessed for control and *C32* females at the indicated ages. (n = 15 per group) (**C**) Male 6.5 week old controls and 9-week old *C32* flies were cultured with young control virgins and the percentage of viable eggs was evaluated. (n = 15 per group) (**D**) Activity, scored as crawling rate in a negative geotaxis assay, was analyzed in control and C32 flies at the indicated ages. (n = 30 per group) (**E**, **F**) 5 day-old adult *C32* and control flies, cultured in identical conditions, were incubated with H_2O_2 and female (**E**) and male (**F**) survival was plotted. (n = 60 per group) (**G**, **H**) Female (**G**) and male (**H**) control and *C32* flies were incubated at 37°C and survival was scored and plotted. (n = 60 per group) * $p<0.02$, ** $p<0.03$ by student's t-test. Error bars represent SEM.

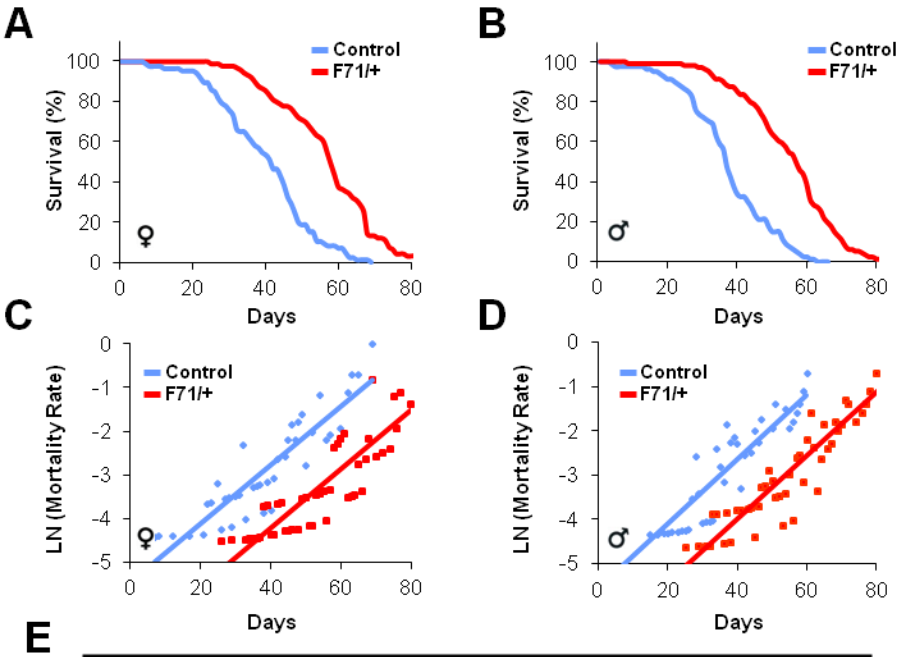
the top of a vial. We found that *C32* flies were equally, if not slightly more, vigorous as controls (Figure 9D), supporting the idea that the lifespan extension observed in *C32* flies is not secondary to decreased physical activity. Further, it appeared that the *C32* mutation might ameliorate the decline in activity observed with aging (Figure 9D). Taken together, these data indicate that *C32* flies have an extension of not only lifespan, but also healthspan as assessed by fertility and activity.

The ability to handle environmental stress often decreases with age and many long-lived strains have improved stress responses, so we subjected *C32* and control flies to a variety of stressors[34,36,121,146]. However in response to 5% H_2O_2, an oxidative stress, or elevated temperature, male and female *C32* flies had equivalent responses as sibling controls (Figures 9, E to H). These data suggest that *C32* flies have normal stress resistance.

RESULTS III: AMP Biosynthesis Pathways Regulate Lifespan

F71 Heterozygous Flies are Long-lived.

Line *F71* emerged as a primary focus because it had one of the longest lifespans of the ten lines identified in the screen and the longevity benefit was present in the heterozygous state. After backcrossing *F71* greater than ten generations into a control w^{1118} strain to remove potential second site mutations and avoid background or modifier effects, we determined whether the increased lifespan was present in more than one background. The lifespan of isogenic male and female heterozygous *F71* mutants was approximately twenty percent longer than sibling controls (Figure 10, A to E). Homozygous *F71* mutants were larval lethal. The heterozygous lifespan increase was due to reduced initial mortality rate, indicating a delay in aging (Figure 10, C and D).



E

Genotype	Sex	N (*n*)	Median Lifespan	Maximum Lifespan
w^{1118}	F	8 (656)	42.02 ± 1.91	69.89 ± 0.51
F71/+	F	8 (649)	52.59 ± 2.01**	82.63 ± 1.89**
w^{1118}	M	8 (661)	40.44 ± 1.16	65.56 ± 1.45
F71/+	M	8 (654)	50.03 ± 1.44**	77.00 ± 4.45*

Figure 10. *F71* heterozygous flies are long-lived. (**A** and **B**) Heterozygous *F71* P-element insertion extends lifespan of adult female and male flies (log rank, P<0.00001 in either case). (**C** and **D**) Log mortality plots denote consistent and constant lifespan benefit in *F71* heterozygous females and males, respectively. (**E**) Eight (N) replicates involving roughly 655 (*n*) isogenic flies show that the heterozygous *F71* insertion significantly increases both median and maximum lifespan. Data presented ± s.e.m. (* P<0.05, ** P<0.01, student's tTest).

F71 Heterozygous Mutants are Healthy

To assess overall health, and because aging and longevity can effect activity, fertility, and stress resistance we examined several physiological parameters of *F71* heterozygotes. Activity and fertility were not reduced by *F71* heterozygosity. In fact *F71* heterozygotes were significantly more active that controls. This indicates that the mutation led to increased "health-span" of the flies (Figure 11, A and B). Unlike some long-lived animals[36,121,146], *F71* heterozygotes do not have altered resistance to oxidative stress (Figure 11, C and D). Together, these results indicate that in addition to being long-lived, flies carrying a heterozygous *F71* mutation are healthy.

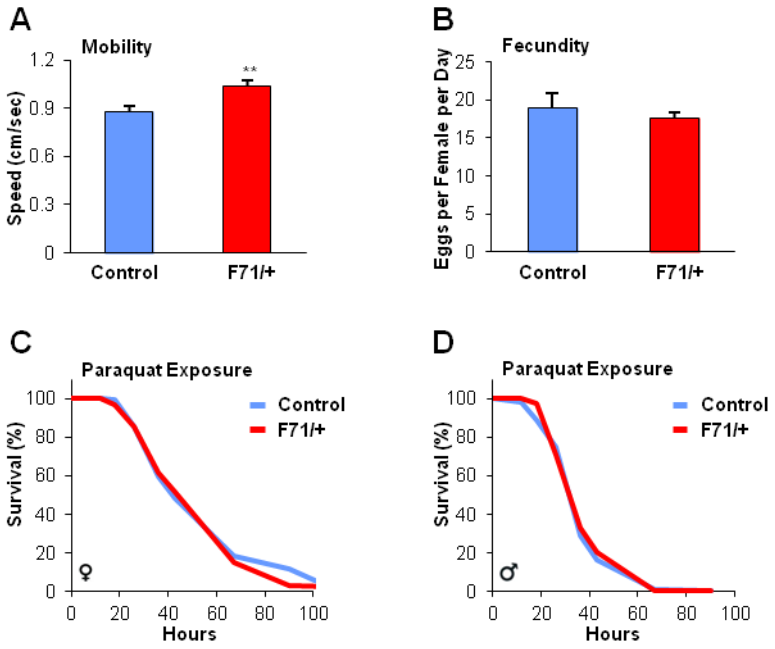


Figure 11. *AdSS* heterozygous mutants are healthy. (**A**) Adult *F71* heterozygotes are more active than controls. (**B**) *F71* heterozygous females have normal fecundity. (**C** and **D**) Survival upon exposure to oxidative stress (e.g., paraquat) is not altered in female or male *F71* heterozygotes compared to controls. Data presented ± s.e.m. (* P<0.05, ** P<0.01, student's tTest).

AdSS Regulates Longevity

To identify the genomic location of the *F71* insertion we used both 5' and 3' inverse PCR and plasmid rescue with DNA sequencing. These techniques identified a single third chromosome insertion at cytogenetic band position 93A1. Database searches demonstrated that the *F71* P-element inserted into the 5' UTR of *CG17273*, twenty-five base pairs upstream of the transcriptional start (Figure 12A). Sequence homology identified *CG17273* as *Drosophila Adenylosuccinate Synthetase* (*AdSS*). To assess the generality of this gene's effect on lifespan we acquired an additional, independently derived, P-element *AdSS* insertion, HA2022 (Figure 12A), and backcrossed over ten generations into a w^{1118} strain. We evaluated AdSS expression levels with qPCR of adult extracts and found *F71* heterozygous insertion decreased *AdSS* levels approximately fifty percent while *HA2022* heterozygotes displayed a twenty-seven percent decreased expression (Figure 12B). We then compared the lifespan of control, *F71* and *HA2022* flies and confirmed that females and males heterozygous for *AdSS* insertions lived significantly longer than controls (Figure 12, C and D). To determine if transgenic expression of *AdSS* rescued the *F71* heterozygous mutant lifespan extension, we generated transgenic flies that contain full-length *AdSS* cDNA under the control of an upstream activating sequence (UAS). Taking advantage of the *GAL4* contained within the *F71* P-element insertion, we attempted to determine whether *AdSS* transgensis rescued the *F71* heterozygous longevity phenotype simply by crossing the UAS-*AdSS* allele with *F71* heterozygotes. The mortality curves of UAS-*AdSS*, *F71*/+, and *UAS-AdSS;;F71*/+ siblings showed that *AdSS* transgensis reversed the *F71* heterozygous lifespan extension (Figure 12, E and F). The accumulated data support the notion that *AdSS* regulates longevity.

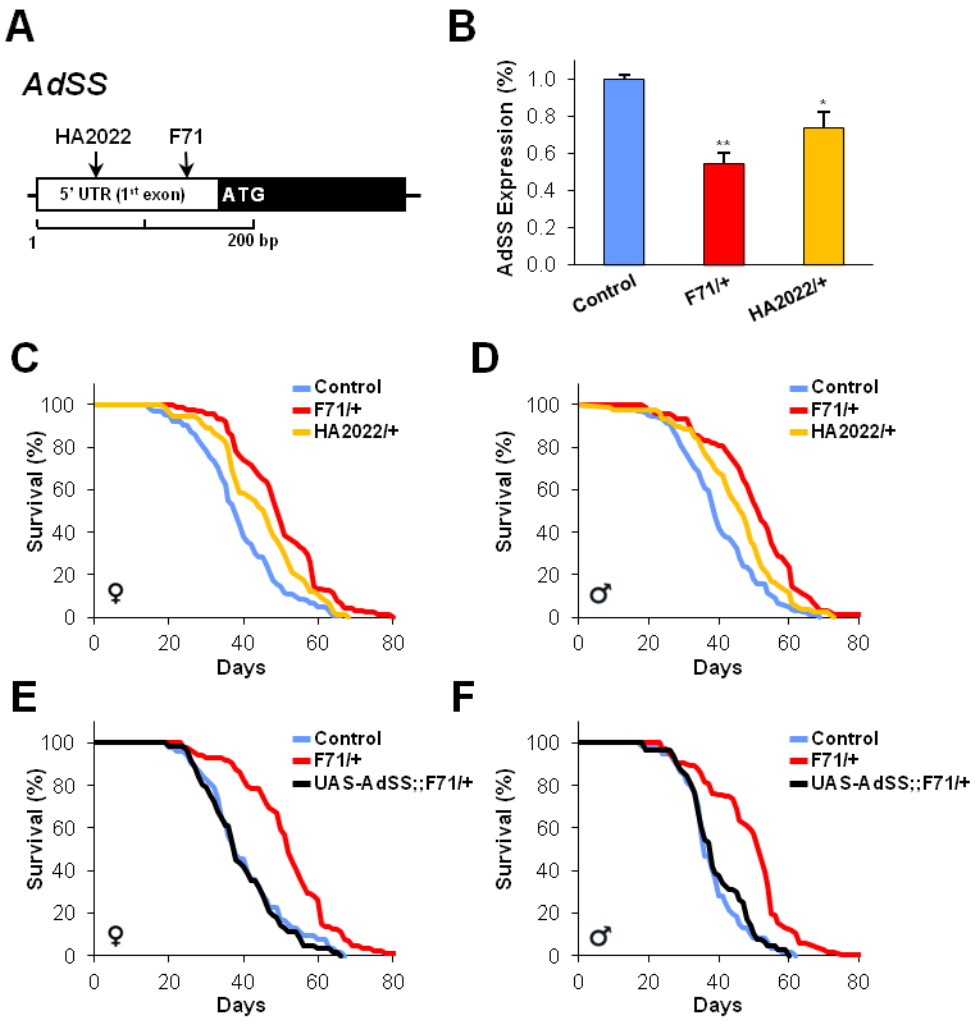


Figure 12. *AdSS* regulates longevity. (**A**) Location of *F71* and *HA2022* insertions in the 5' untranslated region of *AdSS*. (**B**) *AdSS* expression is reduced in *F71* and *HA2022* heterozygotes. (**C** and **D**) Independently derived *AdSS* heterozygous insertions extend the lifespan of females and males (log rank of any heterozygote compared to controls, P<0.008). (**E** and **F**) Transgenic overexpression of wildtype *AdSS* in the *F71* heterozygous background restores lifespan to that of controls.

AdSS is Expressed in Numerous Larval and Adult Tissues

Due to the potentially broad necessity of *AdSS* function we examined the expression pattern of *AdSS* by using the F71-Gal4 insertion to drive expression of UAS-eGFP. As our screen would dictate, we detected strong GFP expression in the larval fat body (Figure 13A). Expression of GFP was also seen in larval gut, muscle, and brain (Figure 13A). This expression pattern was mirrored in the adult (Figure 13B).

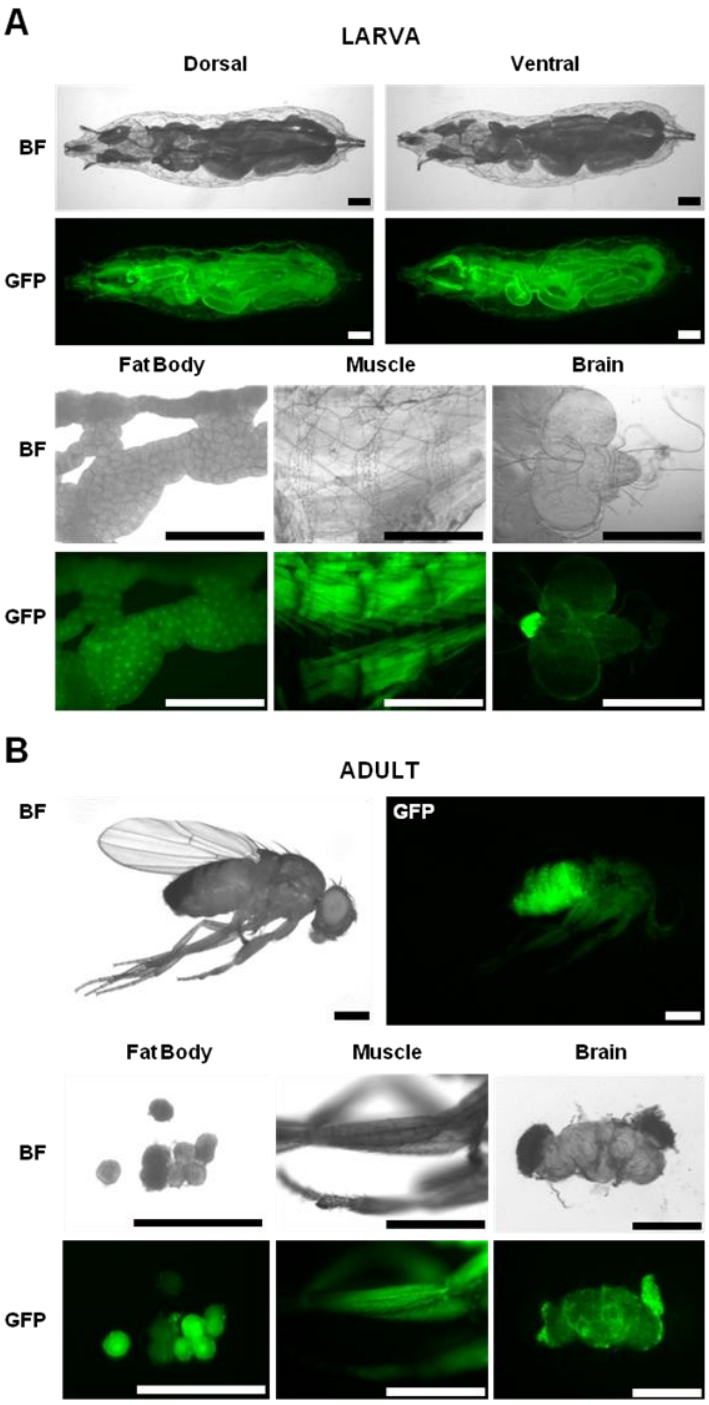


Figure 13. *AdSS* is expressed in multiple lraval and adult tissues. (**A**) *F71* (minimal promoter Gal4); *UAS-eGFP* larvae and (**B**) adult flies were examined and photographed under bright field (BF) or GFP fluorescent microscopy, which showed strong expression in the fat body, gut, muscle, and brain.

***de novo* and Salvage AMP Biosynthetic Enzymes Regulate Lifespan.**

AdSS catalyses the first committed step of *de novo* AMP biosynthesis [147] (Figure 14). We next evaluated the possibility that other components of *de novo* AMP biosynthesis might also extend lifespan. To test this we obtained a P-element insertion located within the *Adenylosuccinate Lyase* (*AdSL*) locus and again backcrossed into w^{1118}. As observed with the *AdSS* alleles, male and female flies carrying heterozygous insertions in *AdSL* displayed extended longevity, and homozygous mutants died as larvae (Figure 15, A and B). In addition to the *de novo* pathway, AMP can be synthesized via a salvage pathway (Figure 14). Therefore we acquired insertional mutations in two AMP salvage pathway components, *Adenosine Kinase* (*AdenoK*) and *Adenine Phosphoribosyltransferase* (*Aprt*), and backcrossed into w^{1118}. Just as with the *de novo* pathway, males and females heterozygous for insertions in *Adenosine Kinase* or *Aprt* were long-lived (Figure 15, C to F).

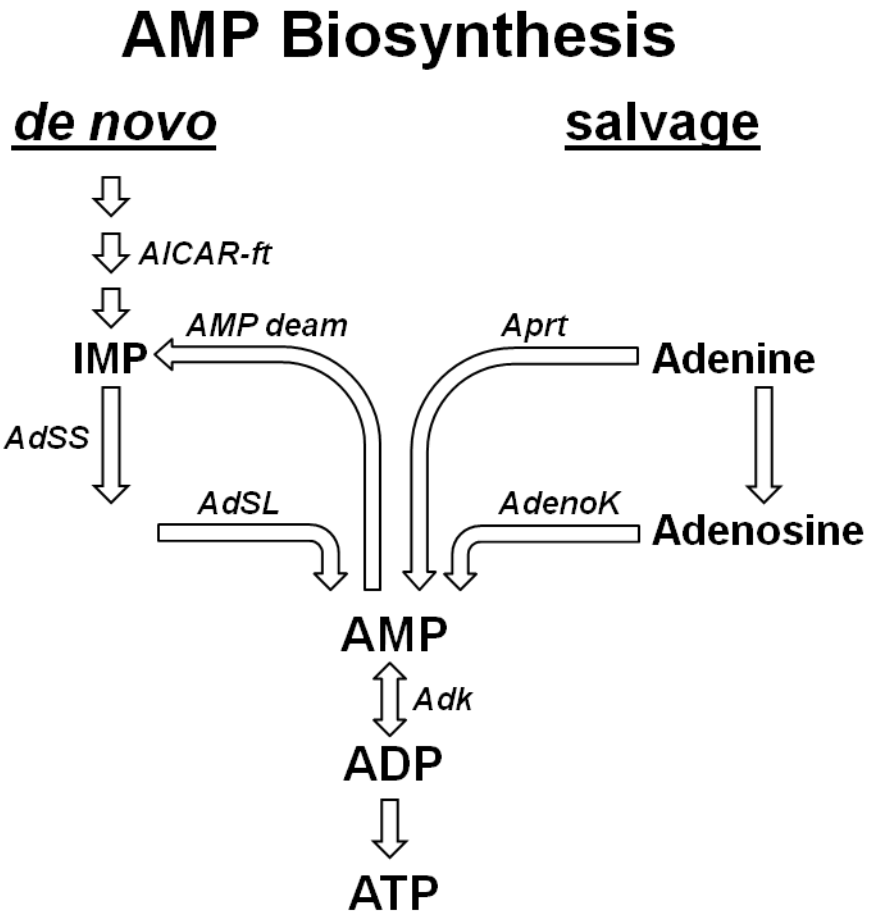


Figure 14. AMP biosynthetic pathways. AMP biosynthesis occurs though two distinct pathways, *de novo* and salvage. *Adenylosuccinate Synthetase* (*AdSS*) catalyzes the first committed step in *de novo* AMP biosynthesis followed by the action of *Adenylosuccinate Lyase* (*AdSL*). *AMP deaminase (AMP deam)* is the reverse of these two reactions, converting adenosine monophosphate (AMP) into inosine monophosphate (IMP). The salvage pathway converts adenine, via *Adenine Phosphoribosyltransferase* (*Aprt*), or adenosine, via *Adenosine Kinase* (*AdenoK*), to AMP. *Adenylate Kinase* (*Adk*) also produces AMP from ADP substrate molecules. Prior to committed AMP biosynthesis Aminoimidazolecarboxamide formyltransferase (*AICAR-ft*) is involved in purine synthesis.

In addition to the two AMP biosynthesis pathways, *Adenylate Kinase* (*Adk*) also generates AMP by catalyzing the conversion of two molecules of ADP into AMP and ATP. Three homologues of *Adenylate Kinase* are present in the fly genome and an insertion into *Adenylate Kinase 2* (*Adk2*) was available, which we backcrossed into w^{1118}. Heterozygous *Adk2* mutant males and females had increased lifespan (Figure 15, G and H). We also tested an insertional mutation of *AMP deaminase,* which catalyzes the hydrolytic deamination of adenosine monophosphate into inosine monophosphate, the opposite direction of the longevity genes (Figure 14). Interestingly, heterozygous insertional mutation of *AMP deaminase* had no effect on lifespan (Figure 15I). Of note, *AMP deaminase* is present on the X chromosome so only females are able to carry a heterozygous insertion. Finally, because AdSL is a bifunctional enzyme and participates in inosine monophosphate (IMP) synthesis prior to AMP specific production[148] we addressed the specificity of lifespan extension and AMP biosynthesis pathway mutation. We examined an insertional mutation of *Aminoimidazolecarboxamide Formyltransferase* (*AICAR-ft*) which catalyses the final two reactions in IMP biosynthesis (Figure 14). AICAR-ft acts downstream of the secondary *AdSL* function, but prior to committed AMP biosynthesis. Heterozygous insertional mutation of *AICAR-ft* had no effect on lifespan (Figure 15, J and K). These data indicate that heterozygous mutations in genes encoding enzymes that function specifically in the formation of adenosine derivatives extend lifespan.

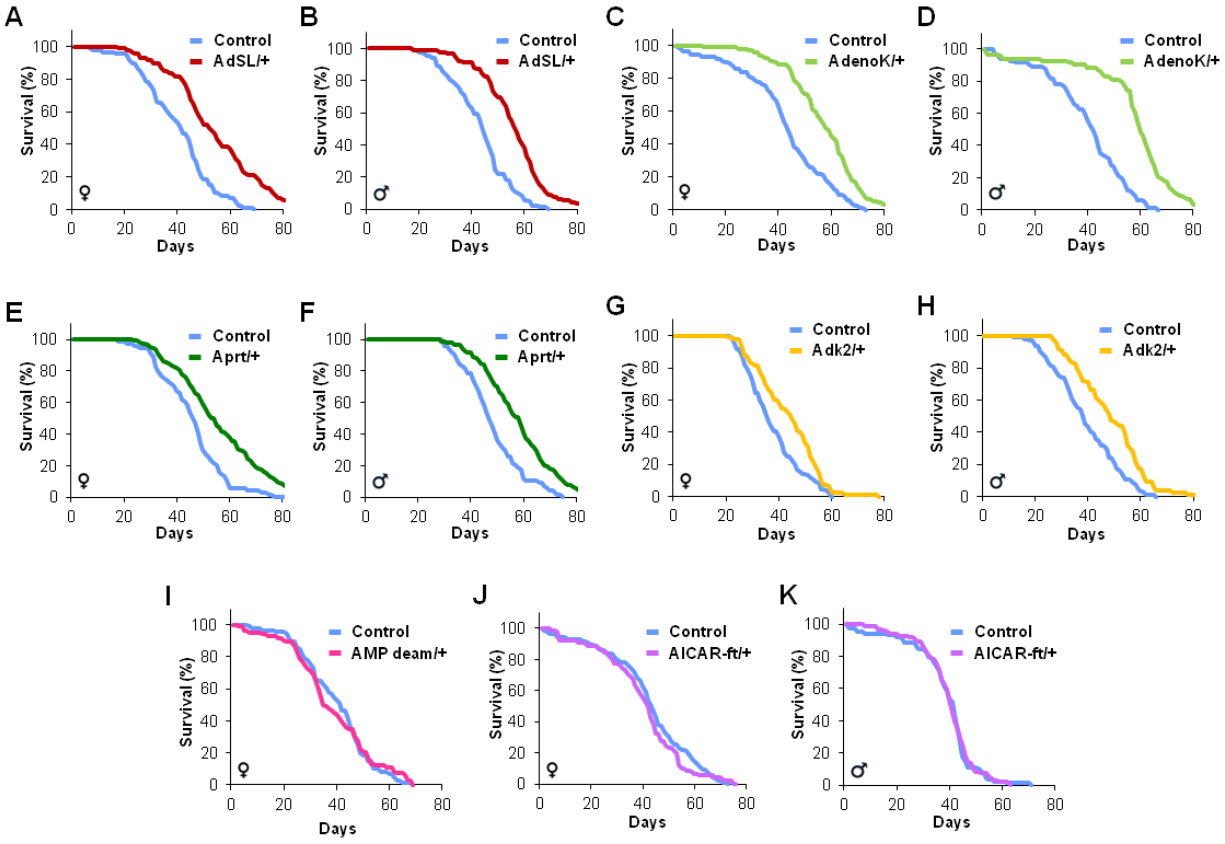


Figure 15. *de novo* and salvage AMP biosynthetic enzymes regulate lifespan. (**A** and **B**) Heterozygous insertional mutation of *AdSL* extends lifespan of adult female and male flies (log rank, P<0.00001 in either case). (**C** to **F**) Heterozygous insertions into AMP salvage pathway biosynthesis components (*AdenoK* and *Aprt*) extend the lifespan of females and males (log rank of any heterozygote compared to controls, P≤0.0002). (**G** and **H**) Female and male flies with heterozygous insertions in *Adk2* have increased longevity (log rank, P<0.004 in either case). (**I**) Heterozygous *AMP deaminase* insertion has no effect on lifespan (P>0.8). (**J** and **K**) Female and male flies with heterozygous insertions in *AICAR-ft* have no alterations in lifespan (log rank, P>0.8 in either case).

de novo and Salvage AMP Biosynthetic Enzymes Regulate Cellular Energy Balance

A plausible consequence of mutating components of adenosine nucleotide synthesis is the altered concentration of the products *in vivo*. In worms, ratios of these products (e.g., AMP:ATP) are increased by caloric restriction and appear predictive of lifespan [69,149]. To test whether AMP:ATP ratios where regulated by the biosynthetic pathways, we performed high-performance liquid chromatography (HPLC) on perchloric acid extracts of adult flies. We found *F71* heterozygous flies had approximately three to four fold higher AMP:ATP ratios than controls (Figure 16A). Interestingly, this increase is similar to that seen in calorically restricted adult flies (Figure 16A). Transgenic expression of *AdSS* in the *F71* heterozygous mutant background, which rescued lifespan extension (Figure 12, E and F), also restored AMP:ATP ratios to that of controls (Figure 16A). Expansion of these studies showed that heterozygous mutation of those genetic components of AMP synthesis that increased lifespan also increased AMP:ATP ratios, but those with normal lifespan (e.g., *AMP deaminase* and *AICAR-ft*) had control ratios (Figure 16A). Since ADP is both a substrate and product during the generation and utilization of ATP, respectively, we also examined ADP:ATP ratios in our normal and long-lived flies. As with AMP:ATP ratios, increased ADP:ATP exactly correlated with increased lifespan (Figure 16B).

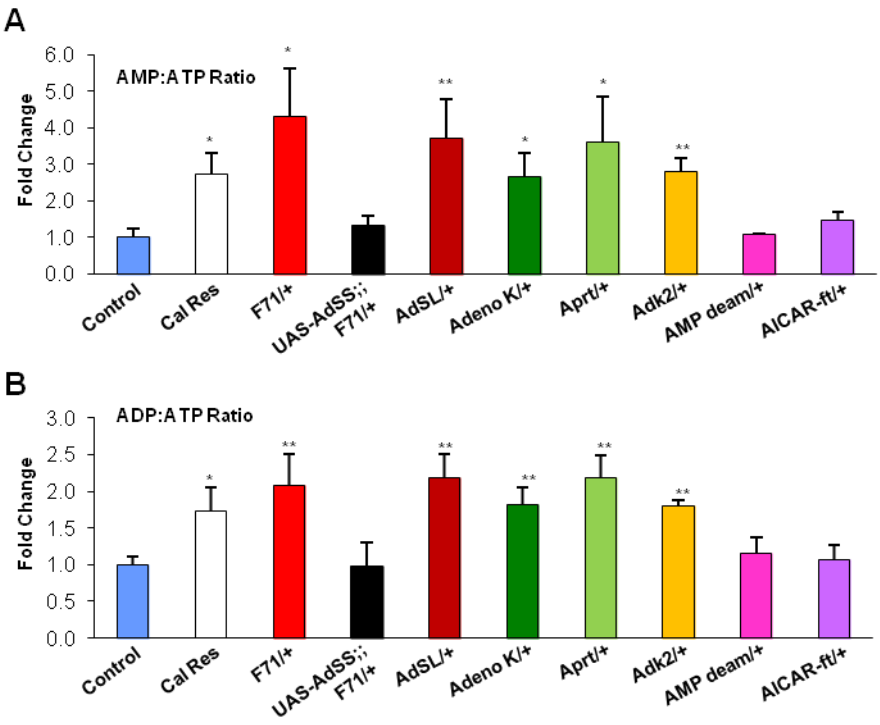


Figure 16. Anabolic *de novo* and salvage AMP biosynthetic enzymes regulate cellular energy balance. (**A** and **B**) Caloric restriction (Cal Res) and those heterozygous mutations of AMP biosynthesis components that increase lifespan also increase AMP:ATP and ADP:ATP ratios compared to controls. The AMP deaminase or AICAR-ft heterozygous mutations do not affect lifespan, AMP:ATP, or ADP:ATP ratios. Transgenic overexpression of wild type AdSS (black bar) rescues the AMP:ATP and ADP:ATP ratio increase observed in F71 heterozygotes. Of note, the increase in AMP:ATP or ADP:ATP ratios observed in the long-lived mutants are equal to or greater than that of calorically restricted (Cal Res) animals. Data presented ± s.e.m. (* $P<0.05$, ** $P<0.01$, student's tTest).

To examine which adenosine nucleotide derivative may play the largest role in these ratio alterations, we also quantified individual adenosine nucleotide levels. ATP levels were only reduced approximately sixteen percent in long-lived and calorically restricted flies compared to controls (Figure 17A). This does not account for the much larger differences in AMP:ATP and ADP:ATP ratios observed. Surprisingly, these same flies displayed ADP and AMP quantities roughly 60% and 160% increased, respectively (Figure 17, B and C). These data indicate that all three adenosine nucleotides contribute to increases in AMP:ATP and ADP:ATP ratios. However, a more dramatic effect may be due to increases in lower energy adenosine derivatives rather than decreased ATP. Of note, no significant alterations in total adenosine derivative pools were reached in any heterozygous mutant, however, we cannot rule out a possible contribution of reduced total adenosine nucleotide derivatives that may escape detection limits.

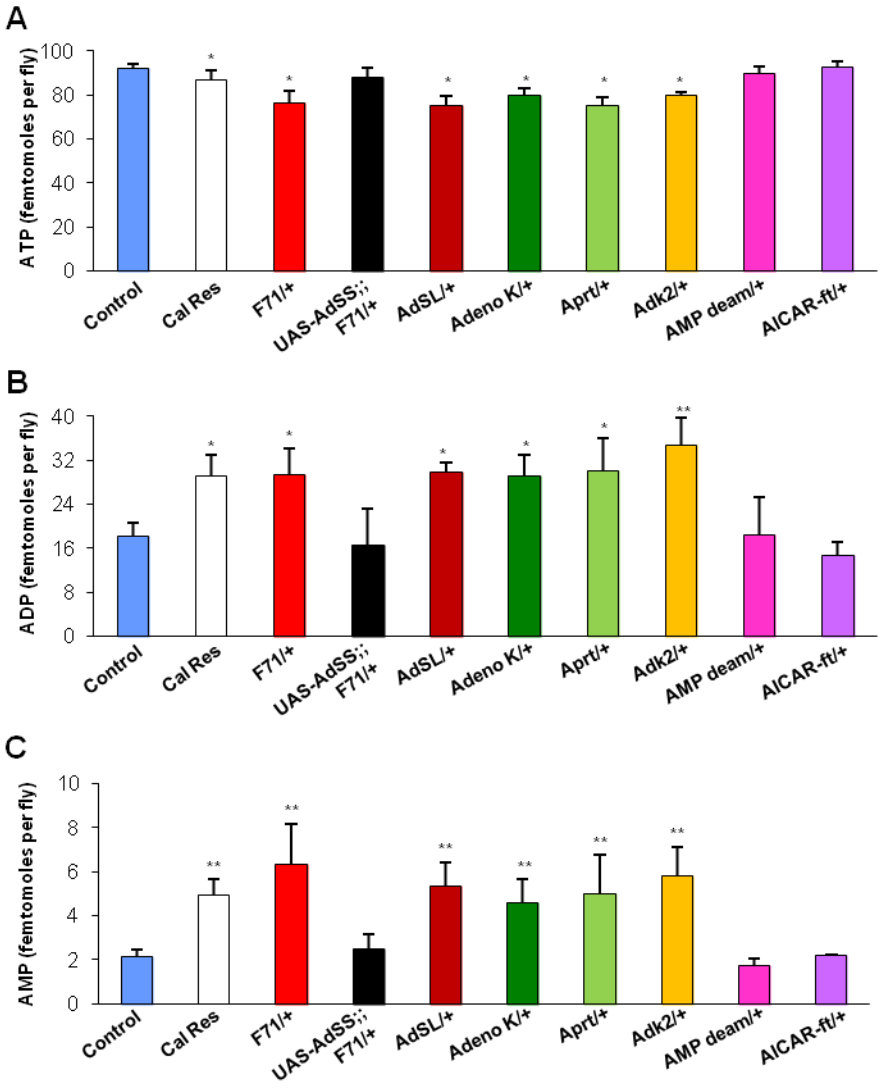


Figure 17. Heterozygous mutation of anabolic *de novo* and salvage AMP biosynthetic enzymes alter adenosine derivative quantities. (**A**) ATP quantities are slightly reduced with Caloric restriction (Cal Res) and those heterozygous mutations of AMP biosynthesis components that increase lifespan. These same alterations dramatically increase (**B**) ADP and (**C**) AMP levels. Transgenic overexpression of wild type AdSS (black bar) rescues these alterations. AMP deaminase or AICAR-ft heterozygous mutations have adenosine nucleotide quantities similar to controls. Data presented ± s.e.m. (* P<0.05, ** P<0.01, student's tTest).

AMP Biosynthesis Pathway Mutation Longevity is Dependent on Functional AMPK

Increased AMP:ATP and ADP:ATP ratios, as observed in the AMP biosynthesis mutants, activate *Adenosine monophosphate-activated protein kinase* (*AMPK*) [64,65]. In this manner, *AMPK* senses cellular energy to coordinate enzymes and transcription factors shifting metabolic processes between energy utilization and energy conservation/generation [66-68]. In worms, transgenic expression of *AMPK* is sufficient to extend lifespan and is necessary for some methods of caloric restriction [69,72,149,150]. To test whether *AMPK* activity is necessary for the lifespan benefit of heterozygous AMP biosynthesis mutants, we examined the lifespan of *F71* heterozygotes driving expression of a UAS-AMPK dominant negative allele (UAS-AMPK^{DN}) in *F71* mutant cells. We found that expression of dominant negative AMPK eliminated the lifespan benefit seen in male and female *F71* heterozygotes (Figure 18, A and B). To examine the potential confounds of additive effects on lifespan or potential deleterious effects of dominant negative AMPK expression, we also compared the lifespan of *F71* heterozygotes driving expression of both UAS-AdSS and UAS-AMPK^{DN} to *F71* heterozygotes driving expression of either UAS-AdSS alone or UAS-AMPK^{DN} alone, but found no significant difference (Figure 18, A and B). These data indicate the lifespan extension of heterozygous AMP biosynthesis mutation is dependent on functional *AMPK* activation.

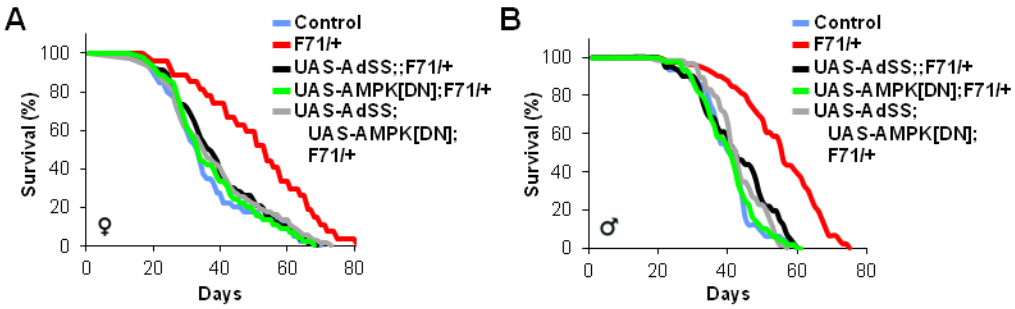


Figure 18. AMP biosynthesis pathway mutation longevity is dependent on functional AMPK. (**A** and **B**) Dominant negative AMPK (AMPK[DN]) rescues F71 heterozygous lifespan extension (log rank, P>0.5 compared to control flies). The lifespan of F71 heterozygotes co-expressing AMPK[DN] and wild type AdSS is the same as heterozygotes expressing either one of the transgenes alone or controls.

Dietary Adenine Supplementation Restores Energy Balance and Lifespan

To probe whether the observed lifespan extension of the AMP biosynthesis mutants might be a consequence of increased adenosine nucleotide ratios, we attempted to restore these ratios in the heterozygous mutants. Since AMP biosynthesis can occur through the modification of adenine via the salvage pathway (Figure 14), we hypothesized that dietary adenine supplementation might affect adenosine derivative pools. To identify an appropriate concentration of adenine, we exploited the larval lethality of *AdSS* and *AdSL* homozygosity to determine whether adenine supplementation could rescue the mortality. We found that adding 0.05% and 0.1% (w/v) adenine to the diet produced Mendelian ratios of both *AdSS* and *AdSL* homozygous pupae (Figure 18A). We then supplemented the diet of adult control and *F71* heterozygotes with 0.05% (w/v) adenine and evaluated AMP:ATP ratios. Adenine supplementation reduced the ratio of AMP:ATP in *F71* heterozygotes to that of unsupplemented controls (Figure 19B). To determine the effect of this AMP:ATP ratio restoration on aging we measured the lifespan of *F71* heterozygous and control adults given a diet with or without 0.05% (w/v) adenine supplementation. Adenine feeding had no effect on control lifespan, indicating that the supplementation was not significantly toxic (Figure 19, C and D). Notably, 0.05% (w/v) adenine did rescue the lifespan extension of female and male *F71* heterozygotes (Figure 19, C and D). These data indicate that supplemental dietary adenine can rescue the increased AMP:ATP ratio of long living *de novo* AMP biosynthesis heterozygous mutants and consequently reverses the lifespan extension. Notably, this effect was observed when adenine was added to adult flies.

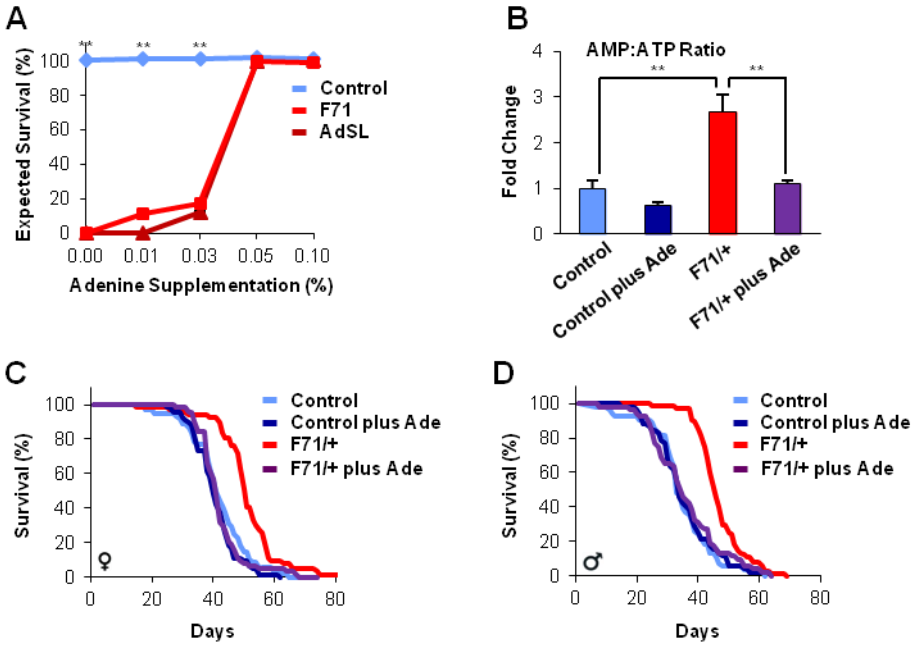


Figure 19. Dietary adenine supplementation restores energy balance and lifespan. (**A**) Dietary adenine supplementation [0.05% or 0.1% (w/v)] restores expected Mendelian ratios of homozygous AdSS and AdSL mutant lethality. (**B**) Adding 0.05% (w/v) adenine to the diet of adult F71 heterozygotes restores AMP:ATP ratios to control levels. (**C** and **D**) Supplementing the diet of adult F71 heterozygotes with 0.05% (w/v) adenine rescues lifespan extension, but does not alter control longevity (log rank, $P>0.3$). Data presented $\pm$ s.e.m. (* $P<0.05$, ** $P<0.01$, student's tTest).

DISCUSSION

Metabolically Targeted Identification of Novel Lifespan Regulators

For centuries humans have searched for keys to long-life and recent experiments in a variety of model systems support the notion that metabolic tissues are important in longevity[37,123,151,152]. Therefore, we hypothesized sites that regulate metabolism may be a source of lifespan regulators. To identify these potential regulators of lifespan, we designed a multi-tiered approach, in which the initial step was a minimal promoter-Gal4; UAS-eGFP metabolic enhancer trap screen [120], followed by extensive lifespan analysis of the mutant collection. We identified ten lines that had significantly increased longevity during multiple assays in both males and females.

Interestingly, none of the genes identified have been directly implicated in lifespan regulation. Perhaps the stringency at which tissue expression patterns dictated those mutants analyzed for lifespan extension excluded components of known longevity pathways. However, if gene mutations predicted to cause lifespan extension based on insertional site location for our screen are confirmed, many of the predicted genes may function in processes previously linked to aging, including stress response and protein turn over. Other genes identified may link previously unrelated processes to aging such as methylation or developmental signaling. Still others have completely unknown gene function.

These data prove that targeted screening of a "metabolically-enriched" mutant collection is a reasonable methodology to identify genes important in lifespan control. Our preliminary analyses indicate metabolically relevant tissues are a potent source of lifespan regulators. However, our enhancer trap screen did not approach saturation, so there are likely to be a diverse

array of other genes yet identified. In fact, related approaches could provide substantial insight into lifespan control. While our screen suggests lifespan regulators may be preferentially expressed in metabolic tissues further expansion is needed for statistical confirmation. Furthermore, in addition to repeat metabolic tissues directed screens, primary screening for mutational insertions in genetic loci specifically expressed in brain or muscle might also be appropriate in the search for genes that regulate longevity. Furthermore, the identification of insertional mutations which are homozygous lethal, but extends life when present in the heterozygous state (i.e., AMP biosynthesis enzymes) emphasizes the importance of gene dosage in complex phenotypes, such as longevity. Screening methods able to titer transcript or protein levels may be successful in further elucidation of lifespan determination.

An RGS-Containing Sorting Nexin Controls Lifespan

Among our long-lived enhancer trap lines, *C32* had the greatest lifespan extension and remained active and fertile at extremely old age. Specifically, insertional mutants of the *C32* locus survived up to twice as long as controls. This remains one of the longest living gene mutations in *Drosophila* to date and effectively extends the theoretical limits of lifespan. If similar results could be directly applied to current human populations the average lifespan would reach approximately 160 years, which is roughly forty years longer than any human as every survived (122 years, Jeanne Louise Calment, 1875-1997). Interestingly, these mutants are more active and fertile than controls of corresponding mortality rate despite significantly higher chronological age. This indicates an extension of "health-span" a major goal of the aging field. Additional comparison of old and young *C32* mutants suggests the senescence of mobility and fecundity typically seen with age is completely abolished by *C32* mutation. It is unclear whether

this effect is at all related to the lack of senescence suggested in the simple fresh-water hydra. However, additional quantification of lifetime fecundity and expansion of studies into other phenotypes with characteristic age-related senescence, like behavioral patterns and heart tube function, are needed to substantiate claims of agelessness.

C32 inserted into the 5′UTR of the hypothetical gene *CG1514*, predicted to encode an RGS domain containing sorting nexin, that we termed *snazarus* (*snz*) for *sorting nexin lazarus*. Several lines of evidence support the notion that *snz* is responsible for the longevity phenotype. For example, multiple techniques identified a single unique mutation site that persisted with lifespan extension after isogenic reduction of potential background effects and second site mutations. Transgenically over-expressed wild-type *snz* in the mutant background reversed the *C32* longevity phenotype. While, similar *snz* over-expression in a wild-type background had no effect on lifespan, indicating transgenic rescue is not due to aberrant or toxic effects. Furthermore, removal of the *C32* P-element caused longevity to revert towards controls. These excision strains had longer life than control w^{1118} flies, which may be secondary to background effects inherent in the methodology, the inability to backcross the revertants into w^{1118} due to the loss of the eye-color marker, the presence of the remaining piece of the P-element, local hopping of the P-element into other regions of the *snz* gene, or other factors. Of note, two other independently derived P-element insertions in the *snz* locus, *G1409* and *SZ4089*, also conferred long life and display transheterozygous lifespan extension. Thus, the accumulated data are consistent with the idea that *snz* regulates lifespan.

In additional to identifying a novel role of *snz* in lifespan determination, we also confirmed that by targeting enhancer trap lines with insertions expressed in metabolically

relevant tissues our screening method was simultaneously able to identify genetic mutations with important metabolic consequences. Our data indicates *snz* and its closest mammalian homologs, SNX13, SNX14, and SNX25, are highly expressed in analogous adipocyte tissue types, i.e. the *Drosophila* fat body and mammalian fat pad. Furthermore, *in vitro* and *in vivo* studies indicate *snz* homologs are dynamically regulated in response to adipocyte differentiation and obesity.

Snz is a member of the sorting nexin (Snx) family, defined by the presence of a PX, phospholipid binding domain. A general theme of the Snx family is that they regulate various aspects of endocytosis, important in internalization and in modulating signal transduction[133,134]. Many mammalian Snxs direct trafficking of surface receptors including tyrosine kinase receptors, in some cases increasing and in others reducing signal transduction [153-156]. Snz and the three related mammalian homologs, SNX13, SNX14, and SNX25, are a subgroup of the Snx family that, in addition to the signature PX domain, all contain an RGS domain indicating potential additional roles in signal transduction. RGS family proteins attenuate heterotrimeric G-protein signaling [157,158]. The RGS domain of the Snz homolog SNX13 is unique among tested RGS domains in the ability to reduce signaling from Gαs proteins that regulate cAMP levels and thereby protein kinase A (PKA) action [155]. A recent study showed that activating the PKA pathway increased lifespan [159]. Therefore, the RGS-containing Snx subgroup could control lifespan or metabolism by regulating protein trafficking and/or by modulating G protein signaling. Structure-function studies with Snz, such as attempting rescues with forms of Snz in which the PX or RGS domain is mutated, may help to clarify these notions.

AMP Biosynthesis Pathways Regulate Lifespan

The goal of this study was to identify novel regulators of lifespan functioning within metabolically relevant tissues. An additional goal of the field is to translate molecular insight gained from longevity pathway dissection into rational therapeutics targeting age-related or metabolic diseases. To that end, a variety of pharmacological approaches have been undertaken[160]. However, these efforts have been hampered by the relative paucity of identified molecular pathways that when altered can extend lifespan[161,162]. In addition, current pro-longevity drugs carry potentially severe side effects or convey unimpressive lifespan benefits. One highly attractive characteristic of a potential drug target is dosage sensitivity, which is often indicated by heterozygous phenotypes. Interestingly, from our collection of metabolically relevant lifespan regulators *F71* was identified as one of the longest living enhancer trap lines and the only mutation found to extend lifespan in the heterozygous state.

Initial analysis of *F71* indicated the lifespan extension was caused by P-element insertion roughly twenty-five bp upstream of the *Drosophila* homolog of *Adenylosuccinate Synthetase*. In addition, *F71* heterozygotes displayed normal fertility, slightly increased activity, and normal response to oxidative stress. Interestingly, an independently derived *AdSS* insertional mutation, *HA2022*, also displayed heterozygous lifespan extension. Gene expression analysis of the two *AdSS* alleles showed that *F71* heterozyotes maintained roughly fifty percent transcript levels while *HA2022* heterozygotes conferred a twenty-seven percent reduction compared to controls. Interestingly, the hypomorphic effect of each *AdSS* allele on transcript levels corresponded to a slightly less dramatic lifespan extension seen in *HA2022* heterozygotes. Lastly, transgenic over-expressed wild-type *AdSS* in the heterozygous mutant background completely rescued the

lifespan extension of *F71* heterozygotes. These data support the notion that *AdSS* regulates lifespans in a dosage-sensitive manner.

The Gal4 insertion within *AdSS* indicates expression in numerous tissues throughout life, including fat body, gut, muscle, and brain. It would be interesting to note if mutation in any of these tissues is either necessary or sufficient to extend lifespan. In addition, changes in AMP biosynthesis pathway component expression may by altered with age and related to predicted AMP:ATP ratios seen in aging *C. elegans*.

AdSS catalyzes the first committed step of *de novo* AMP biosynthesis. Interestingly, maintenance of adenosine derivatives (e.g., AMP, ADP, and ATP) at relatively constant levels is a major function of metabolism[163]. ADP and ATP are formed from AMP [163]. AMP is also generated by an additional parallel enzymatic processes called the salvage AMP biosynthesis pathway[164]. Expansion of studies initiated by *F71* indicated heterozygous mutation of anabolic components of *de novo* and salvage AMP biosynthesis extend lifespan. Thus, *de novo* and salvage AMP biosynthesis pathways regulate longevity. The mechanism of lifespan extension involves increased AMP:ATP ratios and depends on functional AMPK. Studies in worms and yeast also amplify the role of AMP biosynthesis in longevity.

A recent yeast expression profiling screen identified many potential genes important in lifespan determination including purine import and biosynthesis[165]. This suggests the role of AMP biosynthesis in lifespan regulation is conserved. However, important differences exist with the work presented here and that of the yeast screen. Our studies indicate a specific dosage-sensitive role of AMP biosynthesis resulting from alteration of cellular energy status. In yeast, several components of purine synthesis, prior to AMP specific branching, extended lifespan

while mutation of the *de novo* AMP synthesis pathway component *AdSL* was lethal. In addition, the authors note extrinsic media alterations, unrelated to aging in higher organisms, may be the leading cause of lifespan extension. Our studies suggest pro-longevity mutations of purine synthesis may be yeast specific and the CLS extension seen in yeast *de novo* and salvage AMP biosynthesis mutations may function through additional mechanisms related to AMP:ATP ratios. Alternatively, perhaps the *AICAR-ft* allele used in our studies inadequately affected purine synthesis and subsequent adenosine nucleotide pools leaving a role in *Drosophila* lifespan determination unidentified. It would be interesting to quantify adenosine nucleotide concentrations in these yeast mutants and determine whether CLS extension depends on homologous AMPK function[166]. However, interpretation of these experiments may be difficult due to direct functional differences found in the yeast AMPK homolog, SNF1[167].

In worms, AMP:ATP ratios appear predictive of lifespan, and over-expression of AMPK increases lifespan [69,72,149]. Thus the fly data presented here support the role of AMP biosynthesis, AMPK, and AMP:ATP ratios as lifespan regulators that can function across broad evolutionary distances. In addition, recent evidence has confirmed a role of ADP:ATP ratios as important for AMPK activity[65]. Our data indicate, ADP:ATP ratios may also contribute to lifespan determination via AMPK activity. Currently, it is unclear which adenosine nucleotide derivative has the greatest influence on AMPK activity and thereby longevity. It is known, and our data support, that the vast majority of the adenosine nucleotide pool consists of ATP, while the lowest percentage is present as AMP. Our data indicate that AMP biosynthesis pathway mutation simultaneously decreases ATP levels while increasing ADP and AMP levels. This creates a paradox in which genetic reduction of an enzymatic pathway results in the increased concentration of some of its final products. The specific regulation of these dynamics lies at a

complex intersection between biochemistry and genetics and would be a fascinating, albeit difficult, area of future study.

The roles of *de novo* and salvage AMP biosynthesis in flies appear dosage-sensitive and functional in adults based upon genetics and dietary adenine supplementation. These results suggest that interventions reducing adenine conversion to nucleotides might be a plausible method to extend lifespan. It is possible that reducing dietary adenine, which may be less arduous than caloric restriction, and may account for some of the lifespan benefits conferred by caloric restriction, might be a new approach. In addition, the dosage sensitivity and enzymatic nature of *de novo* and salvage AMP biosynthesis, and the conserved aspects of adenosine nucleotide derivatives and lifespan extension, indicate that these pathways are potentially amendable targets. Hadacidin can inhibit *AdSS* activity and AP5A can block *Adk* function. Provided identification of the correct dose, these compounds may be important pro-longevity drugs. In addition, high thorough put screening methods to identify small molecules capable of increasing AMP:ATP or ADP:ATP ratios may also increase lifespan.

Concluding Remarks

Utilizing the unique advantages of *Drosophila melanogaster*, these experiments provided a targeted mutagenic screening approach resulting in the identification of molecular lifespan regulation within metabolically relevant tissues. Characterization and mechanistic insight into two of the mutations found in this screen uncovered new potentially conserved roles for a family of molecules, the sorting nexins, and two classic biochemical pathways, *de novo* and salvage AMP biosynthesis, in lifespan regulation. The remaining genes identified undoubtedly hold additional insight regarding previously unknown connections between other metabolically

important processes and lifespan determination. Characterization of these lines will be of great interest to the aging field.

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Table 1 References [168] [169] [170] [171] [172] [173] [174] [175] [176] [177,178]

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