Research Paper

GCN2 Mediates Access to Stored Amino Acids for Somatic Maintenance during *Drosophila* Aging

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Many mechanistic theories of aging argue that a progressive failure of somatic maintenance, the use of energy and resources to prevent and repair damage to the cell, underpins aging. To sustain somatic maintenance, an organism must acquire dozens of essential nutrients from the diet; this includes essential amino acids (EAAs), which are physiologically limiting for many animals. In *Drosophila*, adulthood deprivation of each individual EAA yields vastly different lifespan trajectories, and adulthood deprivation of one EAA, phenylalanine (Phe), has no associated lifespan cost; this is despite each EAA being strictly required for growth and reproduction. Moreover, survival under any EAA deprivation depends entirely on the conserved amino acid sensor general control nonderepressible 2 (GCN2), a component of the integrated stress response (ISR), suggesting that a novel ISR-mediated mechanism sustains lifelong somatic maintenance during EAA deprivation. Here we investigated this mechanism, finding that flies chronically deprived of dietary Phe continue to incorporate Phe into new proteins and that challenging flies to increase the somatic requirement for Phe shortens lifespan under Phe deprivation. Furthermore, we show that autophagy is required for full lifespan under Phe deprivation and that activation of the ISR can partially rescue the shortened lifespan of *GCN2*-nulls under Phe deprivation. We therefore propose a mechanism by which GCN2, via the ISR, activates autophagy during EAA deprivation, breaking down a larvally acquired store of EAAs to support somatic maintenance. These data refine our understanding of the strategies by which flies sustain lifelong somatic maintenance, which determines length of life in response to changes in the nutritional environment.

Introduction

Aging is defined as the progressive loss of physiological function over time that leads to death[1]; almost all mechanistic theories of aging attribute this loss of physiological function to more damage being inflicted upon the cell than is repaired[2–6]. This is thought to be an issue of metabolic resource management, where nutrients are either consumed at insufficient levels to support somatic maintenance or are in adequate supply but are prioritized for consumption by other processes (e.g., reproduction), which makes them unavailable to support long life[7].

There is a middle ground at which point nutritional resources are prioritized for somatic maintenance; reducing levels of food intake to ~20%–50% of the amount eaten by choice is beneficial for lifespan[8]. This moderate dietary restriction (DR) extends lifespan across taxa, from yeast to primates[9–13]. Since DR levels of food intake also compromise reproduction[14–16], it has been assumed that moderate DR extends lifespan by re-prioritizing resources away from reproduction and toward life-preserving

somatic maintenance[7,17]. While a great deal of research has been devoted to the molecular switches that control resource reallocation[18,19], little is known about which processes comprise somatic maintenance. One strategy to understand how DR extends lifespan is to define the nutritional requirements for sustained life and then characterize the set of somatic processes which are fueled by these nutrients (i.e., somatic maintenance).

Lifespan extension by DR in *Drosophila melanogaster* and mice can be achieved by reducing the abundance of protein, relative to other macronutrients and micronutrients, in the diet[20–23]. Within an organism, protein causes physiological changes in part via intracellular signaling that is triggered by the abundance of the constituent amino acids (AAs)[24], and as such, considerable effort has been directed to understanding how individual AAs interact with AA sensors to alter lifespan[25,26]. In contrast to the lifespan extension observed under partial AA restriction, it was recently shown that complete removal of each essential AA (EAA) from the diet of adult *Drosophila* elicits vastly different reductions in lifespan[27].

Specifically, removal of the EAA methionine (Met) reduces the lifespan to approximately 25% of fully fed flies, while removing the EAA threonine (Thr) yields a lifespan approximately 50% that of fully fed flies. Most strikingly, removal of the EAA phenylalanine (Phe) has no significant impact on lifespan compared to a diet with a full complement of AAs. This variation is surprising because each of these EAAs is strictly required in the diet of *Drosophila* for growth and reproduction[28,29], suggesting that somatic maintenance processes have different requirements for each individual EAA compared to processes involved in growth and reproduction.

This variation in survival response to each EAA deprivation is entirely dependent on general control nonderepressible 2 (GCN2)[27], an intracellular AA sensor which is conserved from yeast to mammals[30]. GCN2 acts during periods of AA deprivation to repress global translation, slowing growth and preserving AAs, while also activating a specific subset of genes involved in the integrated stress response (ISR)[31–36]. *GCN2*-null flies show a normal lifespan on a complete diet but have equally short lifespans when any one EAA is removed from the diet[27]. Thus, GCN2 serves to protect flies against individual EAA deprivation, but the extent of this protection varies in an AA-specific manner.

Here, we utilize this EAA/GCN2 interaction to interrogate the somatic maintenance processes that sustain adult lifespan in *Drosophila melanogaster*. In particular, we use proteomics, dietary AA manipulations, and transgenic interventions to investigate how flies survive without dietary Phe. These data reveal a likely process by which flies store protein during development for later use in somatic maintenance. Future work to characterize this storage and its uses is likely to be important for uncovering the causes of aging.

Methods

Fly husbandry

An outbred, white-eyed Dahomey strain of *Drosophila melanogaster* (wDah) was used for all experiments unless otherwise specified. The *GCN2*-null flies used were obtained as a gift from Dr. Sebastian Grönke (Max Planck Institute for Biology of Ageing). The S1106-GeneSwitch driver line[60] was obtained as a gift from Prof. Linda Partridge (University College London). The UAS-Atg1-RNAi line[61] was obtained as a gift from Dr. Donna Denton (University of South Australia). Knockdown of *Atg1* by this line has been verified as highly effective by Denton et al.[61] (see their fig. S7), where a near ten-fold reduction in *Atg1* expression was measured in the midgut by quantitative polymerase chain reaction. All flies were reared at a controlled population density using eggs laid by age-matched mothers, as described by Linford et al.[37], and developed to adulthood on a standard sugar–yeast (SY) medium, as described by Bass et al.[38]. Newly emerged adult flies were allowed to mate for two days on the SY medium to standardize the mating status of all flies, then 48-hour-old adult female flies were sorted under light CO_2 anesthesia (<30 min) and placed onto their respective experimental diet. During both rearing and experimental stages, flies were kept in a controlled environment of 25 °C, 70% humidity, and a 12-hour light/dark cycle. wDah stocks were maintained at 25 °C, 70% humidity, and a 12-hour light/dark cycle, while *GCN2*-null, S1106-GeneSwitch, and UAS-Atg1-RNAi stocks were maintained at 18 °C, 60% humidity, and a 12-hour light/dark cycle.

Diets

Flies were developed to adulthood on a standard SY medium as described by Bass et al.[38]. Chemically defined synthetic (holidic)

diets were prepared as described by Piper et al.[39,40], following the exome-matched FLYAA formula. Where antibiotics were used, an antibiotic mix was prepared as described by Consuegra et al.[29], consisting of kanamycin (50 µg/mL), ampicillin (50 µg/mL), tetracycline (10 µg/mL), and erythromycin (5 µg/mL). Stock solutions of each antibiotic were made in either MilliQ water (ampicillin and kanamycin) or ethanol (erythromycin and tetracycline) based on their solubility, such that adding 1 mL stock to 1 L holidic media resulted in the above final concentrations. Immediately before dispensing the media, each antibiotic solution was added with a micropipette under constant stirring to promote even mixing. A separate length of tubing was used to dispense the food containing antibiotics, ensuring no antibiotics were inadvertently introduced to the antibiotic-free media. Where rapamycin was used, 30 µL absolute EtOH (Sigma-Aldrich) containing 1000 µM rapamycin (Sigma-Aldrich) was pipetted on top of 3 mL of holidic food for a final rapamycin concentration of 10 µM. We added 30 µL of EtOH to the control vials. The food was then left at room temperature for 24 hours to allow the drug to disperse into the food before use. Where the S1106-GeneSwitch driver was used, 30 uL of absolute EtOH (Sigma-Aldrich) containing 1.67 mM RU486 (Sigma-Aldrich) was pipetted on top of 2 mL of holidic food for a final RU486 concentration of 25 µM. Control vials received 30 µL of EtOH. The food was then left at room temperature for 24 hours to allow the drug to disperse into the food before use.

Proteomics

Adult female flies were starved of Phe for 40 days, then the lysine in the food was completely replaced by a heavy isotope of lysine in which all six carbons are 13C (Silantes). After 4, 9, and 19 days of exposure to the label, 10 flies per condition were sampled and snap-frozen in liquid nitrogen. Protein was extracted from the samples using sodium deoxycholate solubilization, essentially as described previously[41]. Using a Dionex UltiMate 3000 RSLCnano system equipped with a Dionex UltiMate 3000 RS autosampler, an Acclaim PepMap RSLC analytical column (75 µm × 50 cm, nanoViper, C18, 2 µm, 100Å; Thermo Scientific), and an Acclaim PepMap 100 trap column (100 µm × 2 cm, nanoViper, C18, 5 µm, 100Å; Thermo Scientific), the tryptic peptides were separated by increasing concentrations of 80% acetonitrile/0.1% formic acid at a flow of 250 nl/min for 158 min and analyzed with an Orbitrap Fusion mass spectrometer (ThermoFisher Scientific). The instrument was operated in data-dependent acquisition mode to automatically switch between full-scan MS and MS/MS acquisition. Each survey full scan (m/z 375–1575) was acquired with a resolution of 120,000 (at m/z 200) in the Orbitrap after accumulating ions with a normalized automatic gain control (AGC) target of 1e6 and a maximum injection time of 54 ms. The 12 most intense multiply charged ions (z ≥ 2) were sequentially isolated and fragmented in the collision cell by higher-energy collisional dissociation with a resolution of 30,000, an AGC target of 2e5, and a maximum injection time of 54 ms. Dynamic exclusion was set to 15 sec.

The raw data files were analyzed with the MaxQuant software suite v1.6.2.10[42] and its implemented Andromeda search engine[43] to obtain protein identifications using a Uniprot *Drosophila* database downloaded in September 2018. Carbamidomethylation of cysteine residues was selected as a fixed modification, while oxidation of Met and acetylation of protein N-termini were set as variable modifications. Up to three missed cleavages were permitted;

the multiplicity was set to two considering Lys6 as a heavy labels; and a false discovery rate of 1% was allowed for both protein and peptide identification. The proteomics data were further analyzed with Perseus v1.6.2.3[44].

Functional profile analysis

The list of these labeled (newly synthesized) proteins was imported to R Studio[45], and the "enrichKEGG" and "dotplot" functions of the "clusterProfiler" package[46] were used for functional profile analysis and visualization, respectively. To create the distribution of the mean Phe content of the proteins, 10,000 groups of 100 random proteins were sampled from both the list of 1,749 heavy lysine-labeled proteins and from the fly exome (obtained from FlyBase[47]), and the mean Phe content of each group of 100 proteins was plotted.

Lifespan experiments

After being raised and allowed to mate on SY food, 48-hour-old adult female flies were sorted under light CO_2 anesthesia (<30 min) and placed onto the various diets at a density of 10 females per vial. From this day, the protocol of Linford et al.[37] was followed, with flies transferred to fresh food every 2 days and any deaths or censors recorded using the dLife software[37]. Constantly providing fresh food decreased the likelihood of larvae hatching and digging in the food, which can cause adult flies to drown. Flies that drowned or escaped during food changes were censored at that time point.

Statistical analyses

Functional profile analysis

Overrepresentation of proteins from specific gene ontology categories was determined using the "enrichKEGG" function of the "clusterProfiler" package[46], using a strict adjusted p-value cutoff of 0.05. Significant values identified groups of proteins that were overrepresented in the protein set as compared to their representation in the *Drosophila* genome sourced from the Kyoto Encyclopedia of Genes and Genomes[48] (KEGG, organism code = "dme," accessed 2 September 2021).

Lifespan data

Raw data generated by the dLife software[37] was analyzed in R Studio[45] using the Cox Proportional-Hazards Model ("coxph") function of the "Survival" package[49] to find significant differences between genotypes and diets. The analysis of variance ("Anova") function of the "car" package[50] was used to assess genotype by diet interactions, and the "contrast" and "emmeans" functions from the "emmeans" package[51] were used for post hoc pairwise comparisons (Lenth, 2021).

Results

Phe is unlikely to be provided by commensal bacteria

Recent work has found that Phe can be removed from the diet of adult *Drosophila melanogaster* without imposing any cost on their lifespan[27]. This is surprising because Phe is classified as an EAA and is strictly required for development and reproduction[27,29].

One explanation for this is that adult flies obtain Phe from a source other than their diet, such as their gut microbiota. Indeed, dietary depletion of single EAAs can be partially rescued by commensal bacteria supplementation during preadulthood—effects that are abolished by treatment with an antibiotic mixture comprising kanamycin, ampicillin, tetracycline, and erythromycin[29]. We therefore treated our adult flies with the same antibiotic mixture to investigate whether their ability to survive without Phe is similarly compromised (Fig. 1A). Antibiotic addition to the diets of adult flies had no effect on lifespan (p = 0.19, ANOVA) whether or not the food contained Phe (p = 0.19) (Fig. 1B), indicating that commensal or food-borne microbes are unlikely to be supplying Phe to the flies for sustaining lifespan.

Phe-deprived adult *Drosophila* readily incorporates Phe into new proteins

An alternative explanation for why adult flies can survive in the absence of a Phe supply is that they don't require it; the proteins they express to sustain adult lifespan may require little to no Phe. The fly exome codes for 201 proteins without any Phe, and the remaining proteins contain a Phe content between 0.07% and 20.7%, with an average of 3.7% (FlyBase, 2023).

To investigate the nature of the expressed proteome, we maintained adult *Drosophila* on Phe-free food for 40 days (late middle age), at which point we replaced dietary lysine with a heavy isotope of lysine while maintaining a Phe-free diet. After 4, 9, and 19 days on the lysine-labeled, Phe-free food, we assessed the proteome via mass spectrometry (Fig. 2A) to identify newly synthesized proteins as judged by the incorporation of heavy lysine. We identified a total of 1,749 proteins containing the heavy lysine
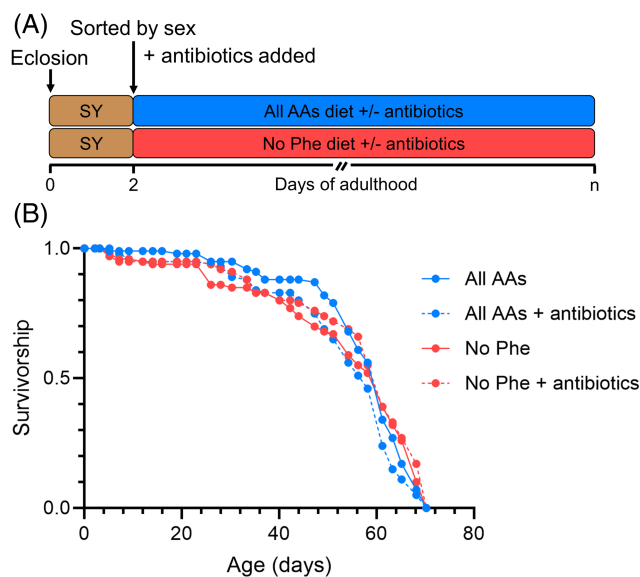

Figure 1. Antibiotic addition has no effect on lifespan under phenylalanine (Phe) deprivation. To investigate how flies survive when deprived of dietary Phe, we explored whether Phe could be provided by bacteria living in the food media; commensal bacteria have been shown to support *Drosophila* development when essential amino acids (EAAs) are absent. (A) We added an antibiotic mix to the adult food media, which is known to remove these bacteria. (B) Wild-type lifespan was not affected by antibiotic addition (p = 0.19, ANOVA), nor was there any interaction between antibiotic addition and diet (p = 0.19). These data indicate that it is unlikely that Phe-deprived flies receive Phe from commensal bacteria. Blue lines represent flies fed a full complement of amino acids (AAs), and red lines represent flies fed a diet missing Phe. Solid lines represent a diet with no antibiotics, and dotted lines represent a diet with antibiotics added.
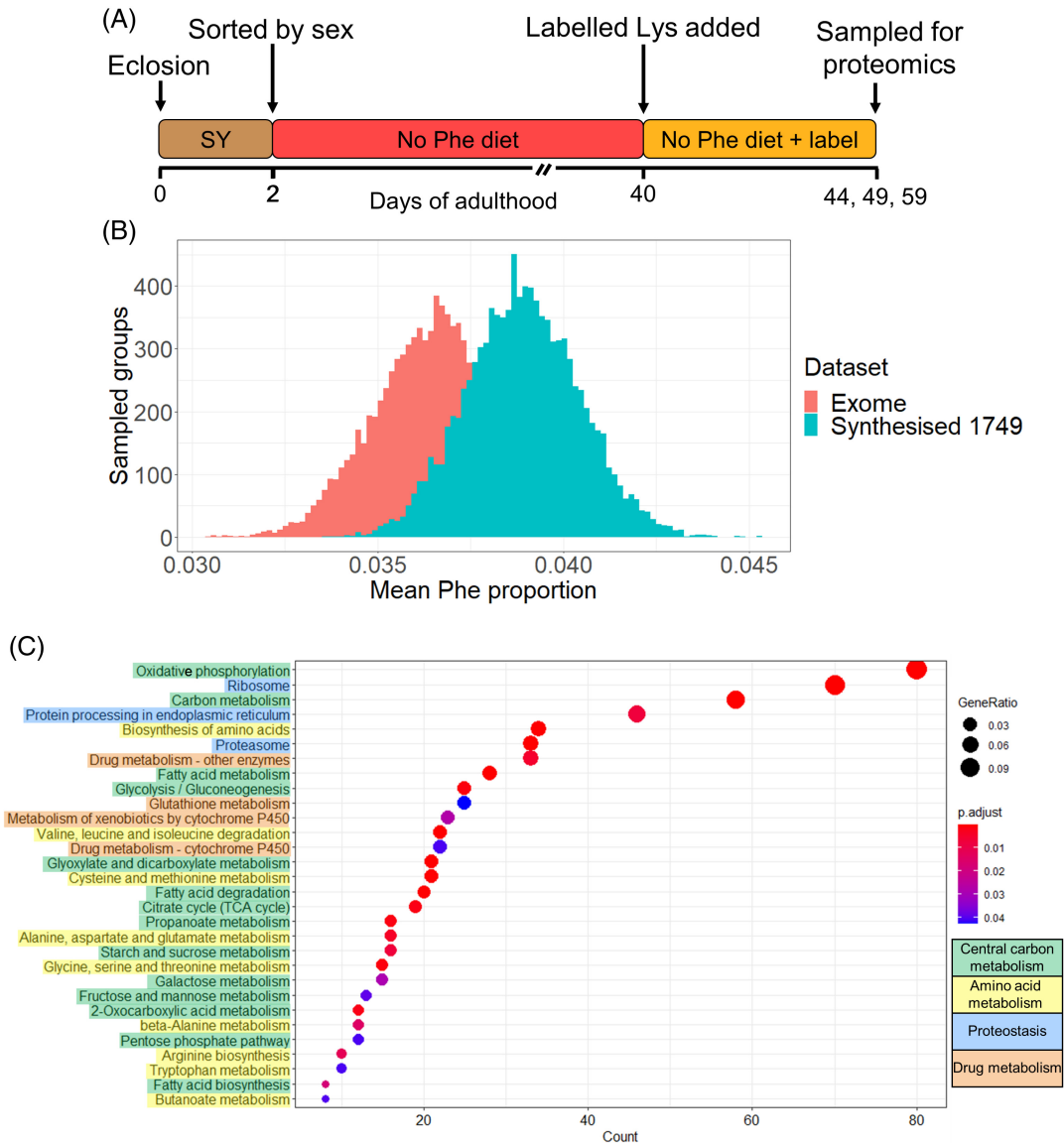
Figure 2. Phe-starved flies continue to synthesize Phe-rich proteins. To determine whether Phe-starved flies continue to use Phe for protein synthesis. **(A)** We starved female adult *Drosophila* of Phe for 40 days before labeling and detecting newly synthesized proteins by mass spectrometry. **(B)** The 1749 proteins found to be newly synthesized after 40 days of Phe deprivation were richer in Phe than randomly sampled proteins from the exome (p < 0.001, t-test). **(C)** We performed functional enrichment analysis on the list of 1749 proteins. The size of each dot represents the proportion of the total protein set which belongs to each respective gene ontology category. The color of the dot represents the probability of achieving the observed proportions by randomly sampling the proteome for the same-sized sets of proteins. Proteins related to central carbon metabolism, AA metabolism, and translation were found to be overrepresented, as were detoxification proteins, which made up >14% of all proteins identified in Phe-deprived flies.

label at all time points (**Table S1**). Surprisingly, rather than being low in Phe, these proteins had a significantly higher Phe content than randomly selected, similarly sized groups of proteins encoded by the fly exome (p < 0.001, t-test) (**Fig. 2B**). Thus, despite being deprived of Phe for more than half of their adult lifespan, the flies continue to express a Phe-enriched proteome, suggesting that flies possess a reservoir of Phe that remains accessible for later-life protein synthesis.

As this set of proteins continues to be expressed even after extended EAA deprivation, we reasoned that this set of proteins may be particularly important for sustaining life. Functional enrichment analysis of the heavy lysine-containing proteins revealed functional overrepresentation of central carbon metabolism (in particular, metabolism of fatty acids as well as sugars

through glycolysis, the pentose phosphate pathway, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation), AA metabolism, and proteostasis (including various AA metabolic pathways, protein breakdown via the proteasome, protein synthesis due to ribosome expression, and protein processing via the ER) (p < 0.05) (**Fig. 2C**). In addition to these core cellular functions, proteins related to the removal of xenobiotic (foreign) and endogenous metabolic toxins were also overrepresented, comprising >14% of all proteins detected. It is therefore possible that during Phe deprivation, flies selectively sustain the expression of proteins with functions related to core metabolic pathways as well as functions related to detoxification capacity, and these represent the core set of proteins required to sustain life and protect against molecular damage accumulation.

Phe deprivation does not protect against Thr deprivation

If chronic Phe deprivation acts as a specific trigger to activate lifespan-preserving processes, perhaps Phe deprivation can protect fly lifespan against simultaneous deprivation of another EAA that would normally shorten lifespan. To test this, we combined dietary Phe deprivation with dietary Thr deprivation, where Thr deprivation alone shortens lifespan of about 50% of fully fed controls[27]. However, rather than ameliorating the cost to lifespan imposed by Thr deprivation, concurrent deprivation of Phe with Thr slightly worsened this cost and reduced lifespan (p = 0.002, Tukey's HSD) (Fig. 3A).

One explanation for this failure of Phe deprivation to rescue Thr deprivation is that sustained life under dietary EAA deprivation requires access to internally stored EAAs, and lifespan will be limited if these EAAs are not stored or cannot be accessed in sufficient amounts. In this case, the flies' pool of stored Thr is relatively small compared to the amount required to sustain life, whereas the pool of stored Phe is sufficiently large to sustain protein synthesis for a full lifespan (as supported by our proteomics data). Thus, combinations of EAA restrictions may limit lifespan to
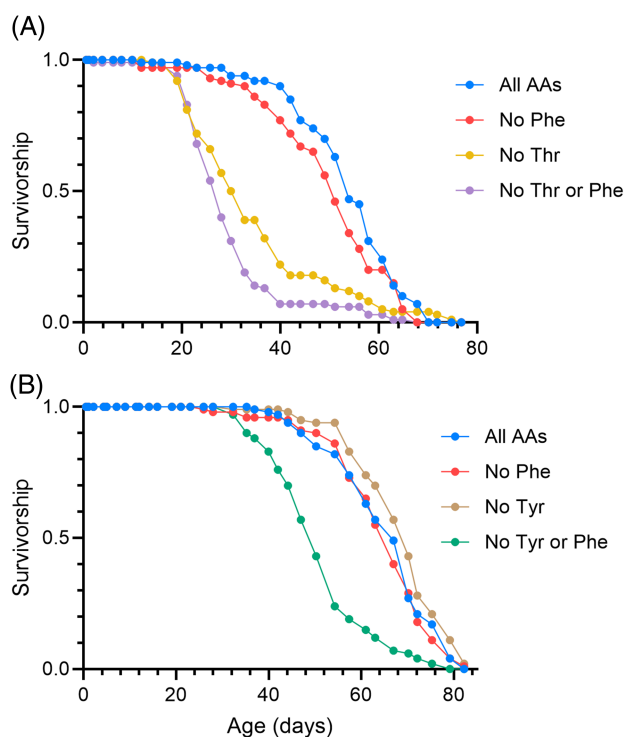
a level that can only be sustained to the extent supported by the most limiting EAA.

Increasing the requirement for Phe reduces lifespan under Phe deprivation

If each individual EAA deprivation limits lifespan according to an intrinsic relationship between its level accessible from stores and the specific requirement for that EAA to support life, it should be possible to shorten the lifespan of Phe-deprived flies by accelerating the depletion of their Phe reservoir. In *Drosophila*, the non-EAA tyrosine (Tyr) is synthesized from Phe via an irreversible reaction catalyzed by Phe hydroxylase[52]. Thus, maintaining flies on food lacking both Phe and Tyr may shorten lifespan because Phe will be depleted more rapidly to meet the need for both Phe and its catabolite Tyr. By contrast, depriving flies of either Phe or Tyr alone should not impose a cost to lifespan because sufficient stores of Phe exist to meet the need for each one individually.

Consistent with our storage hypothesis, removing Phe or Tyr alone resulted in lifespans that were no different from fully fed controls (p > 0.170, Tukey's HSD), whereas depriving flies of both Phe and Tyr resulted in a 25% reduction in lifespan when compared to flies fed all AAs (p < 0.001) (Fig. 3B). Together, these data indicate that while Phe is usually stored in sufficient amounts to sustain lifelong somatic maintenance, this storage can become limiting when somatic maintenance requirements for Phe are increased. This further supports our hypothesis that lifespan under each EAA deprivation is determined by a balance between the amount of EAA stored and the amount required for somatic maintenance.

Alternative activation of nutritional stress signaling partially restores the lifespan of *GCN2*-null flies under Phe deprivation

Recent data has shown that the ability of flies to maintain a full lifespan without dietary Phe requires the presence of the AA sensor and signaling kinase GCN2[27]. When *GCN2*-null flies are fully fed, they are equally as long-lived as fully fed controls, but when deprived of dietary Phe, their lifespan drops to about 30% of wild-type flies maintained with or without Phe (p < 0.001 for all comparisons of Phe-deprived *GCN2*-nulls versus controls, Tukey's HSD) (Fig. 4A). When activated by AA deprivation, GCN2 initiates the ISR, which involves repression of general translation as well as coordinating enhanced expression of a subset of genes involved in protecting the flies against nutrient stress[31,33–36]. *GCN2*-null flies may therefore be protected against Phe deprivation by alternative activation of the ISR.

Recent work has shown that Tyr deprivation can activate the ISR independently of GCN2[53], meaning that Tyr deprivation should protect *GCN2*-null flies against Phe deprivation by restoring activation of the ISR. In line with this prediction, *GCN2*-null flies deprived of both Phe and Tyr lived 33% longer than *GCN2*-nulls deprived of Phe alone (p = 0.017) (Fig. 4B). While this is a significant enhancement of lifespan, it is far from a complete restoration. This may occur if Tyr deprivation only partially activates the ISR or because Tyr deprivation can only provide a limited benefit for lifespan; our previous data with wild-type flies show that its absence becomes deleterious later in life when Phe is also missing from the diet (Fig. 3B).

The mild protective effect of Tyr depletion for *GCN2*-nulls may be caused by activation of the ISR to break down AA stores
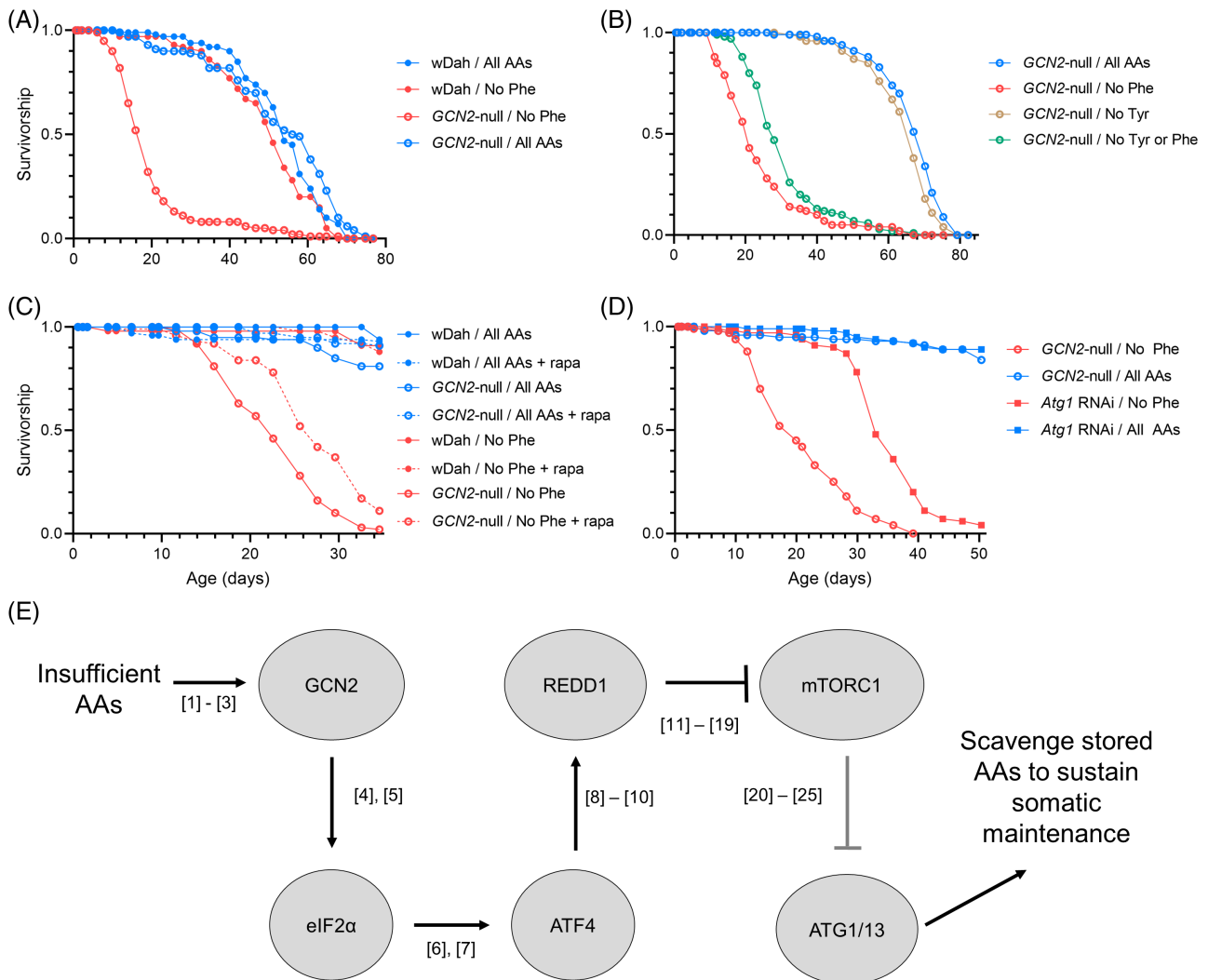

Figure 3. Lifespan is limited by the insufficient supply of individual EAAs relative to demand. Given that Phe deprivation is the only EAA deprivation with no lifespan cost, we asked whether Phe deprivation could protect against other EAA deprivations. **(A)** We deprived adult female *Drosophila* of both Phe and another EAA threonine (Thr). Wild-type flies deprived of both Phe and Thr were shorter lived than those deprived of only Thr (p = 0.002, Tukey's HSD), indicating that Phe deprivation cannot protect against Thr limitation. To test whether increasing the physiological demand for Phe can limit lifespan, **(B)** We deprived adult female *Drosophila* of both Phe and Tyr, which are synthesized from Phe. Wild-type flies simultaneously deprived of Phe and Tyr were indeed shorter-lived than flies fed all AAs (p < 0.001) or deprived of Phe (p < 0.001) or Tyr (p < 0.001) alone. These data suggest that lifespan is limited by the insufficient supply of individual EAAs relative to their somatic maintenance demand.

Figure 4. General control nonderepressible 2 (GCN2)-mediated autophagy may release stored EAAs under EAA deprivation. (A) Starving *GCN2*-null flies of Phe shortens lifespan in comparison to *GCN2*-null flies fed all AAs and wild-type flies fed all AAs or no Phe (p < 0.001 in all comparisons). **(B)** To test whether lifespan of *GCN2*-null flies under Phe deprivation can be rescued by integrated stress response (ISR) activation, we deprived flies of both Phe and Tyr and measured lifespan. Deprivation of Tyr partially rescued *GCN2*-null lifespan from the cost of Phe deprivation (p = 0.017). **(C)** The ISR can also be activated via pharmacological inhibition of mTOR. The addition of the mTOR inhibitor rapamycin to the food partially rescues *GCN2*-null lifespan under Phe deprivation (p < 0.001). **(D)** ISR activation ultimately results in an upregulation of autophagy. To test whether autophagy is required for survival under Phe deprivation, we expressed Atg1 RNAi in the fat body and gut and then deprived the flies of Phe. Atg1 RNAi flies deprived of Phe had a reduced lifespan compared to those fed all AAs (p < 0.001). **(E)** These lifespan data suggest that under EAA deprivation, GCN2 may activate autophagy to release stored EAAs and thus sustain lifelong somatic maintenance. Our proposed model for this mechanism combines many individually well-characterized signaling events from studies in an array of model systems to form a pathway that connects AA deprivation to autophagy and encompasses the two major AA sensors (GCN2 and mTOR). Gray ovals represent proteins involved in the signaling pathway. Each protein interaction is accompanied by a reference in square brackets for a study in which the interaction has been observed, and these interactions are expanded upon in **Table S2**.

for the synthesis of new proteins and/or by sparing the flies from investing in the proteome by generally suppressing translation. Both of these effects can also be achieved by inhibiting the AA sensor and growth activator mTORC1[54–59]. We therefore added the mTOR inhibitor rapamycin to the food of the flies to see if mTORC1 suppression would rescue the lifespan of *GCN2*-nulls on food without Phe. Rapamycin addition extended the lifespan of *GCN2*-nulls on Phe-free food by 21% (p < 0.001, Tukey's HSD) and, to the extent that we measured it, had no effect on the lifespan of wild-type flies when fed diets with all AAs or lacking Phe, nor did it affect the lifespan of *GCN2*-nulls fed all AAs (p > 0.12 in all comparisons) (**Fig. 4C**).

Autophagy knockdown reduces lifespan under Phe deprivation

If retrieval of AAs from protein stores is a key element of these lifespan effects, then activation of autophagy may be required for flies to survive EAA deprivation. To test this, we knocked down the autophagy protein ATG1 specifically in the fly fat body and gut[60,61], which are major metabolic tissues and are known to be important reservoirs for nutrients[62,63]. *Atg1* knockdown significantly shortened lifespan of flies on food without Phe (p < 0.001), but not to the same extent as caused by the loss of *GCN2* (p < 0.001) (**Fig. 4D**). By contrast, *Atg1* RNAi flies were substantially longer-lived when maintained on a complete diet and, to the

extent of our experiment, were no different from fully fed *GCN2*-null flies (p = 0.39).

Together, our data point to flies possessing a developmentally acquired store of EAAs, which they access during dietary EAA deprivation by activation of the ISR to trigger autophagy. The EAAs liberated by this process are then utilized for the synthesis of proteins required for lifelong somatic maintenance.

Discussion

To sustain life, an organism must employ a specific set of processes required for physiological function and ongoing somatic maintenance. Sustaining these processes requires resources that are originally sourced from the diet. Because these resources are generally thought to be limiting, the way in which they are differentially allocated between fitness traits lies at the heart of explaining the constraints under which aging has evolved[17,64–66]. In seeking mechanistic explanations for aging, many studies have focused on defining the nutritional elements that limit organismal fitness; nitrogen limitation, which for flies takes the form of limitation for an EAA, has recently become a central focus of these studies[20–22]. Here, our data, alongside those of Srivastava et al.[27], indicate how limiting amounts of dietary AAs determine adult *Drosophila* lifespan. Overall, our data are consistent with a model in which flies store AAs during development, which they can later access to protect themselves while they find a better food source to support reproduction. We propose that the ability to sustain life under each EAA deprivation could be a read out of the extent to which an EAA is stored relative to the degree to which it is required for somatic maintenance.

These data shed light on the mechanism used by flies to protect themselves during the period of time between experiencing an EAA/total protein deficit and finding a proteinaceous food source. Deprivation of any AA is thought to result in a buildup of uncharged tRNA molecules, which directly bind to and activate GCN2[31,32,67,68]. Given that *GCN2*-null flies quickly succumb to any EAA deprivation, GCN2 may be responsible for providing access to stored EAAs for sustained somatic maintenance during EAA deprivation. Our data indicate that this breakdown occurs via autophagy, the process of degrading and recycling cellular components, which is the most prominent route for bulk AA recycling[69].

Evidence from a variety of sources and study systems indicates that GCN2 activation can lead to autophagy induction via mTORC1 inhibition and, therefore, the breakdown of stored protein ([Fig. 4E] and **Table S2**). Under this mechanism, GCN2 activation enhances the translation of selected transcripts, including that of the transcription factor ATF4[31,33–36]. While there is currently no evidence that GCN2 or ATF4 can activate autophagy directly, evidence from murine cell culture indicates that ATF4 upregulates *REDD1*, which encodes a kinase known to suppress the AA sensor complex mTORC1[70,71], in turn activating autophagy[54–59]. This is consistent with our data in which Phe deprivation activates GCN2, which indirectly induces autophagy to liberate stored EAAs for ongoing survival and somatic maintenance.

Given the proposed role of GCN2 in freeing stored EAAs to support lifespan under EAA deprivation, the variability in lifespan response to each EAA deprivation can be explained by the difference between the amount of that EAA that is stored and the amount that is required for somatic maintenance. In our study, we focused on Phe deprivation since, unlike adulthood deprivation of any one of the other EAAs, omitting Phe from the adult diet has no effect on wild-type fly lifespan[27]. This indicates that Phe is atypical among the EAAs in that it is stored in excess of its requirement for lifelong somatic maintenance. By contrast, dietary Thr deprivation shortens lifespan considerably, and dietary Met deprivation shortens it to an even greater extent[27]. By our model, the stored amounts of these EAAs fall far short of the levels required for flies to sustain lifelong somatic maintenance.

One of the proteins found in our proteomics data to be continuously synthesized in flies despite suffering from chronic Phe deprivation is larval serum protein 2 (LSP2), which is strongly induced during late larval development and is particularly rich in Phe[72]. It therefore represents a candidate protein to store larvally acquired Phe that can be used to fuel ongoing somatic maintenance. Knockdown of *Lsp2* in developing flies should reduce this store and therefore reduce lifespan under adult Phe deprivation.

Our proteomics data can also reveal the nature of the processes that underpin somatic maintenance. We found an overrepresentation of proteins involved in central carbon metabolism, proteostasis, and cellular detoxification. These proteins may represent the baseline requirement for keeping flies alive. If it is indeed true that fueling cellular detoxification is required to keep cellular damage in check to sustain life[6], knockdown of cap-n-collar (*cnc*) (the *Drosophila* homolog of the xenobiotic response transcription factor Nrf2) should reduce activity of the cellular detoxification processes and shorten lifespan under Phe deprivation. Such a response has been observed in *C. elegans*, where loss of the *Nrf2* ortholog *skn-1* shortens lifespan and *skn-1* overexpression increases lifespan[73,74].

Conclusions

In this study, by investigating the relationship between EAA deprivation, GCN2, and lifespan, we identify EAA storage and retrieval as a likely key regulator of adult *Drosophila* lifespan. We point to a new approach for identifying the resources and molecular processes that are required to sustain life. Future studies that identify and characterize these processes are likely to enhance our understanding of the mechanisms that modulate aging.

Acknowledgments

Author Contributions

J.N.J., M.D.W.P., C.K.M., and T.K.J. conceived the project. R.B.S. performed the mass spectrometry runs and analyzed the

raw data. J.N.J. performed the other experiments and analyzed the data. J.N.J. wrote the original article, and M.D.W.P., C.K.M., and T.K.J. assisted in reviewing and editing. M.D.W.P., C.K.M., and T.K.J. supervised the project. All authors approved the final article.

Supplementary Materials

References

1. López-Otín C., Blasco M.A., Partridge L., Serrano M., & Kroemer G. (2013). The hallmarks of aging. *Cell* **153**(6), 1194–1217. PMID: 23746838; doi: 10.1016/j.cell.2013.05.039.

2. Weismann A. (1882). Ueber die Dauer des Lebens. *Am. J. Psychology* **3**(1), 105. doi: 10.2307/1411530.

3. Harman D. (1956). Aging: A theory based on free radical and radiation chemistry. *J. Gerontology* **11**(3), 298–300. PMID: 13332224; doi: 10.1093/geronj/11.3.298.

4. Gensler H.L., & Bernstein H. (1981). DNA damage as the primary cause of aging. *Q. Rev. Biology* **56**(3), 279–303. PMID: 7031747; doi: 10.1086/412317.

5. Bjorksten J. (1968). The crosslinkage theory of ageing. *J. Am. Geriatr. Soc.* **16**(4), 408–427. PMID: 4868749; doi: 10.1111/j.1532-5415.1968.tb02821.x.

6. Gems D., & McElwee J.J. (2005). Broad spectrum detoxification: The major longevity assurance process regulated by insulin/IGF-1 signaling? *Mech. Ageing Dev.* **126**(3), 381–387. PMID: 15664624; doi: 10.1016/j.mad.2004.09.001.

7. Kirkwood T.B.L., & Holliday R. (1979). The evolution of ageing and longevity. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* **205**, 531–546. PMID: 42059; doi: 10.1098/rspb.1979.0083.

8. Weindruch R., Walford R.L., Fligiel S., & Guthrie D. (1986). The retardation of aging in mice by dietary restriction: Longevity, cancer, immunity and lifetime energy intake. *J. Nutrition.* **116**(4), 641–654. PMID: 3958810; doi: 10.1093/jn/116.4.641.

9. McCay C.M., Crowell M.F., & Maynard L.A. (1935). The effect of retarded growth upon the length of life span and upon the ultimate body size. *J. Nutrition.* **10**(1), 63–79. doi: 10.1093/jn/10.1.63.

10. Klass M.R. (1977). Aging in the nematode Caenorhabditis elegans: Major biological and environmental factors influencing life span. *Mech. Ageing Dev.* **6**, 413–429. PMID: 926867; doi: 10.1016/0047-6374(77)90043-4.

11. Chapman T., & Partridge L. (1996). Female fitness in Drosophila melanogaster: An interaction between the effect of nutrition and of encounter rate with males. *Proc. Royal Soc. Lond. Ser. B Biological Sci.* **263**(1371), 755–759. PMID: 8763795; doi: 10.1098/rspb.1996.0113.

12. Jiang J.C., Jaruga E., Repnevskaya M.V., & Jazwinski S.M. (2000). An intervention resembling caloric restriction prolongs life span and retards aging in yeast. *FASEB J.* **14**(14), 2135–2137. PMID: 11024000; doi: 10.1096/fj.00-0242fje.

13. Colman R.J., Anderson R.M., Johnson S.C., Kastman E.K., Kosmatka K.J., Beasley T.M., … Weindruch R. (2009). Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science* **325**(5937), 201–204. PMID: 19590001; doi: 10.1126/science.1173635.

14. Partridge L., Gems D., & Withers D.J. (2005). Sex and death: What is the connection? *Cell* **120**(4), 461–472. PMID: 15734679; doi: 10.1016/j.cell.2005.01.026.

15. Piper M.D.W., Partridge L., Raubenheimer D., & Simpson S.J. (2011). Dietary restriction and aging: A unifying perspective. *Cell Metab.* **14**(2), 154–160. PMID: 21803286; doi: 10.1016/j.cmet.2011.06.013.

16. Simpson S.J., Couteur D.G.L., Raubenheimer D., Solon-Biet S.M., Cooney G.J., … Fontana L. (2017). Dietary protein, aging and nutritional geometry. *Ageing Res. Rev.* **39**, 78–86. PMID: 28274839; doi: 10.1016/j.arr.2017.03.001.

17. Holliday R. (1989). Food, reproduction and L'ongevity: Is the extended lifespan of calorie-restricted animals an evolutionary adaptation? *Bioessays* **10**(4), 125–127. PMID: 2730632; doi: 10.1002/bies.950100408.

18. Saltiel A.R., & Kahn C.R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414**(6865), 799–806. PMID: 11742412; doi: 10.1038/414799a.

19. Virgilio C.D., & Loewith R. (2006). The TOR signalling network from yeast to man. *Int. J. Biochem. Cell Biol.* **38**(9), 1476–1481. PMID: 16647875; doi: 10.1016/j.biocel.2006.02.013.

20. Mair W., Piper M.D.W., & Partridge L. (2005). Calories do not explain extension of life span by dietary restriction in Drosophila. *PLoS Biol.* **3**(7), e223. PMID: 16000018; doi: 10.1371/journal.pbio.0030223.

21. Grandison R.C., Piper M.D.W., & Partridge L. (2009). Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. *Nature* **462**(7276), 1061–1064. PMID: 19956092; doi: 10.1038/nature08619.

22. Solon-Biet S.M., McMahon A.C., Ballard J.W.O., Ruohonen K., Wu L.E., Cogger V.C., … Simpson S.J. (2014). The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. *Cell Metab.* **19**(3), 418–430. PMID: 24606899; doi: 10.1016/j.cmet.2014.02.009.

23. Zanco B., Mirth C.K., Sgrò C.M., & Piper M.D. (2021). A dietary sterol trade-off determines lifespan responses to dietary restriction in Drosophila melanogaster females. *Elife* **10**, e62335. PMID: 33494859; doi: 10.7554/eLife.62335.

24. Tavernarakis N. (2008). Ageing and the regulation of protein synthesis: A balancing act? *Trends Cell Biol.* **18**(5), 228–235. PMID: 18346894; doi: 10.1016/j.tcb.2008.02.004.

25. Babygirija R., & Lamming D.W. (2021). The regulation of healthspan and lifespan by dietary amino acids. *Transl. Med. Aging* **5**, 17–30. PMID: 34263088; doi: 10.1016/j.tma.2021.05.001.

26. Green C.L., Lamming D.W., & Fontana L. (2022). Molecular mechanisms of dietary restriction promoting health and longevity. *Nat. Rev. Mol. Cell Biol.* **23**(1), 56–73. PMID: 34518687; doi: 10.1038/s41580-021-00411-4.

27. Srivastava A., Lu J., Gadalla D.S., Hendrich O., Grönke S., & Partridge L. (2022). The role of GCN2 kinase in mediating the effects of amino acids on longevity and feeding behaviour in Drosophila. *Front. Aging* **3**, 944466. PMID: 35821827; doi: 10.3389/fragi.2022.944466.

28. Sang J.H., & King R.C. (1961). Nutritional requirements of axenically cultured Drosophila melanogaster adults. *J. Exp. Biol.* (4) 793–809. doi: 10.1242/jeb.38.4.793.

29. Consuegra J., Grenier T., Baa-Puyoulet P., Rahioui I., Akherraz H., Gervais H., … Leulier F. (2020). Drosophila-associated bacteria differentially shape the nutritional requirements of their host during juvenile growth. *PLoS Biol.* **18**(3), e3000681. PMID: 32196485; doi: 10.1371/journal.pbio.3000681.

30. Gallinetti J., Harputlugil E., & Mitchell J.R. (2012). Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR. *Biochem. J.* **449**(1), 1–10. PMID: 23216249; doi: 10.1042/BJ20121098.

31. Dever T.E., Feng L., Wek R.C., Cigan A.M., Donahue T.F., & Hinnebusch A.G. (1992). Phosphorylation of initiation factor 2α by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. *Cell* **68**(3), 585–596. PMID: 1739968; doi: 10.1016/0092-8674(92)90193-G.

32. Olsen D.S., Jordan B., Chen D., Wek R.C., & Cavener D.R. (1998). Isolation of the gene encoding the Drosophila melanogaster homolog of the Saccharomyces cerevisiae GCN2 eIF-2alpha kinase. *Genetics* **149**(3), 1495–1509. PMID: 9649537; doi: 10.1093/genetics/149.3.1495.

33. Harding H.P., Novoa I., Zhang Y., Zeng H., Wek R., Schapira M., & Ron D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* **6**(5), 1099–1108. PMID: 11106749; doi: 10.1016/S1097-2765(00)00108-8.

34. Harding H.P., Zhang Y., Zeng H., Novoa I., Lu P.D., Calfon M., … Ron D. (2003). An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell* **11**(3), 619–633. PMID: 12667446; doi: 10.1016/S1097-2765(03)00105-9.

35. Vattem K.M., & Wek R.C. (2004). Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *P. Natl. Acad. Sci. U. S. A.* **101**(31), 11269–11274. PMID: 15277680; doi: 10.1073/pnas.0400541101.

36. Kang M.-J., Vasudevan D., Kang K., Kim K., Park J.-E., Zhang N., … Ryoo H.D. (2017). 4E-BP is a target of the GCN2–ATF4 pathway during Drosophila development and aging. *J. Cell Biol.* **216**(1), 115–129. PMID: 27979906; doi: 10.1083/jcb.201511073.

37. Linford N.J., Bilgir C., Ro J., & Pletcher S.D. (2013). Measurement of lifespan in *Drosophila* melanogaster. *J. Vis. Exp.* PMID: 23328955; doi: 10.3791/50068.

38. Bass T.M., Grandison R.C., Wong R., Martinez P., Partridge L., & Piper M.D.W. (2007). Optimization of dietary restriction protocols in Drosophila. *J. Gerontol. Ser.* **62**(10), 1071–1081. PMID: 17921418; doi: 10.1093/gerona/62.10.1071.

39. Piper M.D.W., Blanc E., Leitão-Gonçalves R., Yang M., He X., Linford N.J., … Partridge L. (2014). A holidic medium for Drosophila melanogaster. *Nat. Methods* **11**(1), 100–105. PMID: 24240321; doi: 10.1038/nmeth.2731.

40. Piper M.D.W., Soultoukis G.A., Blanc E., Mesaros A., Herbert S.L., Juricic P., … Partridge L. (2017). Matching dietary amino acid balance to the in silico-translated exome optimizes growth and reproduction without cost to lifespan. *Cell Metab.* **25**(3), 610–621. PMID: 28273481; doi: 10.1016/j.cmet.2017.02.005.

41. Huang C., Foster S.R., Shah A.D., Kleifeld O., Canals M., Schittenhelm R.B., & Stone M.J. (2020). Phosphoproteomic characterization of the signaling network resulting from activation of the chemokine receptor CCR2. *J. Biol. Chem.* **295**(19), 6518–6531. PMID: 32241914; doi: 10.1074/jbc.RA119.012026.

42. Tyanova S., Temu T., & Cox J. (2016). The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat. Protoc.* **11**(12), 2301–2319. PMID: 27809316; doi: 10.1038/nprot.2016.136.

43. Cox J., Neuhauser N., Michalski A., Scheltema R.A., Olsen J.V., & Mann M. (2011). Andromeda: A peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* **10**(4), 1794–1805. PMID: 21254760; doi: 10.1021/pr101065j.

44. Tyanova S., Temu T., Sinitcyn P., Carlson A., Hein M.Y., Geiger T., … Cox J. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat. Methods* **13**(9), 731–740. PMID: 27348712; doi: 10.1038/nmeth.3901.

45. RStudio Team (2018). *RStudio: Integrated Development Environment for R*. Boston, MA: RStudio, Inc.

46. Yu G., Wang L.-G., Han Y., & He Q.-Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. *Omics J. Integr. Biology* **16**(5), 284–287. PMID: 22455463; doi: 10.1089/omi.2011.0118.

47. Gramates L.S., Agapite J., Attrill H., Calvi B.R., Crosby M.A., Santos Gdos, … Strelets V.B. (2022). FlyBase: A guided tour of highlighted features. *Genetics* **220**(4), iyac035. PMID: 35266522; doi: 10.1093/genetics/iyac035.

48. Kanehisa M., & Goto S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **28**(1), 27–30. PMID: 10592173; doi: 10.1093/nar/28.1.27.

49. Therneau T. (2023). *A Package for Survival Analysis in R*. Version 3.5-7. Available from: https://CRAN.R-project.org/package=survival.

50. Fox J., & Weisberg S. (2019). *An R Companion to Applied Regression*. Third Ed. Thousand Oaks, CA: Sage.

51. Lenth R.V. (2023). *emmeans: Estimated Marginal Means, aka Least-Squares Means*. Version 1.8.7. Available from: https://CRAN.R-project.org/package=emmeans.

52. Geltosky J.E., & Mitchell H.K. (1980). Developmental regulation of phenylalanine hydroxylase activity in Drosophila melanogaster. *Biochem. Genet.* **18**(7–8), 781–791. PMID: 6781472; doi: 10.1007/BF00484593.

53. Kosakamoto H., Okamoto N., Aikawa H., Sugiura Y., Suematsu M., Niwa R., … Obata F. (2022). Sensing of the non-essential amino acid tyrosine governs the response to protein restriction in Drosophila. *Nat. Metab.* **4**(7), 944–959. PMID: 35879463; doi: 10.1038/s42255-022-00608-7.

54. Matsuura A., Tsukada M., Wada Y., & Ohsumi Y. (1997). Apg1p, a novel protein kinase required for the autophagic process in Saccharomyces cerevisiae. *Gene* **192**(2), 245–250. PMID: 9224897; doi: 10.1016/S0378-1119(97)00084-X.

55. Kamada Y., Funakoshi T., Shintani T., Nagano K., Ohsumi M., & Ohsumi Y. (2000). Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J. Cell Biology* **150**(6), 1507–1513. PMID: 10995454; doi: 10.1083/jcb.150.6.1507.

56. Chang Y.-Y., & Neufeld T.P. (2009). An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. *Mol. Biol. Cell* **20**(7), 2004–2014. PMID: 19225150; doi: 10.1091/mbc.e08-12-1250.

57. Ganley I.G., Lam D.H., Wang J., Ding X., Chen S., & Jiang X. (2009). ULK1·ATG13·FIP200 complex mediates mTOR signaling and is essential for autophagy. *J. Biol. Chem.* **284**(18), 12297–12305. PMID: 19258318; doi: 10.1074/jbc.M900573200.

58. Hosokawa N., Hara T., Kaizuka T., Kishi C., Takamura A., Miura Y., … Mizushima N. (2009). Nutrient-dependent mTORC1 association with the ULK1-Atg13–FIP200 complex required for autophagy. *Mol. Biol. Cell* **20**(7), 1981–1991. PMID: 19211835; doi: 10.1091/mbc.e08-12-1248.

59. Jung C.H., Jun C.B., Ro S.-H., Kim Y.-M., Otto N.M., Cao J., … Kim D.H. (2009). ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol. Biol. Cell* **20**(7), 1992–2003. PMID: 19225151; doi: 10.1091/mbc.e08-12-1249.

60. Giannakou M.E., Goss M., Jünger M.A., Hafen E., Leevers S.J., & Partridge L. (2004). Long-lived Drosophila with overexpressed dFOXO in adult fat body. *Science* **305**(5682), 361–361. PMID: 15192154; doi: 10.1126/science.1098219.

61. Denton D., Shravage B., Simin R., Mills K., Berry D.L., Baehrecke E.H., & Kumar S. (2009). Autophagy, not apoptosis, is essential for midgut cell death in Drosophila. *Curr. Biol.* **19**(20), 1741–1746. PMID: 19818615; doi: 10.1016/j.cub.2009.08.042.

62. Zinke I., Schütz C.S., Katzenberger J.D., Bauer M., & Pankratz M.J. (2002). Nutrient control of gene expression in Drosophila: Microarray analysis of starvation and sugar-dependent response. *Embo. J.* **21**(22), 6162–6173. PMID: 12426388; doi: 10.1093/emboj/cdf600.

63. Scott R.C., Schuldiner O., & Neufeld T.P. (2004). Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Dev. Cell* **7**(2), 167–178. PMID: 15296714; doi: 10.1016/j.devcel.2004.07.009.

64. Williams G.C. (1966). Natural selection, the costs of reproduction, and a refinement of Lack's principle. *Am. Nat.* **100**(916), 687–690. doi: 10.1086/282461.

65. Kirkwood T.B.L. (1977). Evolution of ageing. *Nature* **270**(5635), 301–304. PMID: 593350; doi: 10.1038/270301a0.

66. Shanley D.P., & Kirkwood T.B.L. (2000). Calorie restriction and aging: A life-history analysis. *Evolution* **54**, 740–750. PMID: 10937249; doi: 10.1111/j.0014-3820.2000.tb00076.x.

67. Wek R.C., Jackson B.M., & Hinnebusch A.G. (1989). Juxtaposition of domains homologous to protein kinases and histidyl-tRNA synthetases in GCN2 protein suggests a mechanism for coupling GCN4 expression to amino acid availability. *Proc. Natl. Acad. Sci. U. S. A.* **86**(12), 4579–4583. PMID: 2660141; doi: 10.1073/pnas.86.12.4579.

68. Malzer E., Szajewska-Skuta M., Dalton L.E., Thomas S.E., Hu N., Skaer H., … Marciniak S.J. (2013). Coordinate regulation of eIF2α phosphorylation by PPP1R15 and GCN2 is required during *Drosophila* development. *J. Cell Sci.* **126**, 1406–1415. PMID: 23418347; doi: 10.1242/jcs.117614.

69. Mizushima N., & Komatsu M. (2011). Autophagy: Renovation of cells and tissues. *Cell* **147**(4), 728–741. PMID: 22078875; doi: 10.1016/j.cell.2011.10.026.

70. Whitney M.L., Jefferson L.S., & Kimball S.R. (2009). ATF4 is necessary and sufficient for ER stress-induced upregulation of REDD1 expression. *Biochem. Bioph. Res. Co.* **379**(2), 451–455. PMID: 19114033; doi: 10.1016/j.bbrc.2008.12.079.

71. Xu D., Dai W., Kutzler L., Lacko H.A., Jefferson L.S., Dennis M.D., & Kimball S.R. (2019). ATF4-mediated upregulation of REDD1 and Sestrin2 suppresses mTORC1 activity during prolonged leucine deprivation. *J Nutr*. PMID: 31875479; doi: 10.1093/jn/nxz309.

72. Telfer W.H., & Kunkel J.G. (1991). The function and evolution of insect storage hexamers. *Annu. Rev. Entomol.* **36**(1), 205–228. PMID: 2006868; doi: 10.1146/annurev.en.36.010191.001225.

73. An J.H., & Blackwell T.K. (2003). SKN-1 links C. elegans mesendodermal specification to a conserved oxidative stress response. *Genes Dev.* **17**(15), 1882–1893. PMID: 12869585; doi: 10.1101/gad.1107803.

74. Tullet J.M.A., Hertweck M., An J.H., Baker J., Hwang J.Y., Liu S., … Blackwell T.K. (2008). Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans. *Cell* **132**(6), 1025–1038. PMID: 18358814; doi: 10.1016/j.cell.2008.01.030.