Functional analysis of the role of GCN2 kinase in longevity and amino acid homeostasis in *Drosophila melanogaster*

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ABBREVIATIONS

AA	Amino acid
APC	Anterior piriform cortex
ASNS	Asparagine synthetase
ATF	Activating transcription factor
BCAAs	Branched chain amino acids
CBS	Cysthathionine-beta-synthase
CGL	Cysthathionine-gamma-synthase
CHOP	CCAAT/enhancer-binding protein homologous protein
DA	Dopaminergic neurons
DR	Dietary Restriction
EAA	Essential amino acids
ER	Endoplasmic reticulum
eif2α	Eukaryotic initiation factor-2 alpha
F	Phenyalanine
FGF21	Fibroblast growth factor 21
GCN2	General control non-derepressible-2
GSH	Glutathione
H	Histidine
H_2S	Hydrogen sulfide
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
M/C-R	Methionine and/or cysteine restriction
ORF	Open reading frame
PEK	Pancreatic eif2α kinase
PLP	Pyridoxal-phosphate
R	Arginine
SAA	Sulfur containing amino acids
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine

SYA	Sugar Yeast agar
T	Threonine
TAG	Triacylglycerides
TOR	Target of rapamycin
TSP	Transsulfuration pathway
V	Valine
W	Trytophan
w^{Dah}	white Dahomey

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ZUSAMMENFASSUNG

Die eIF2α-Kinase General Control Non-derepressible 2 (GCN2) detektiert die Abwesenheit von Aminosäuren und koordiniert die Translation mit der Verfügbarkeit der Aminosäuren, wodurch die Zellen den Stress durch Nahrungsmangel bewältigen. Es gibt zunehmende Anzeichen dafür, dass Aminosäuren eine wichtige Rolle in der Modulierung von Langlebigkeit und Gesundheit in diversen Spezies spielen, die zugrundeliegenden Mechanismen bleiben jedoch unklar. GCN2 ist ein evolutionär konservierter Sensor für den Mangel an Aminosäuren und ein potentieller Kandidat für die Regulierung von Aminosäure-abhängigen physiologischen Reaktionen.

Im ersten Teil dieser Studie habe ich die *in vivo*-Funktionen der GCN2-Kinase unter Entzug jeder der 10 essentiellen Aminosäuren (EAS) untersucht, wobei ich eine *Drosophila Gcn2* Null-Mutante und eine vollständig definierte chemische Nahrung verwendet habe. Die vorliegende Studie zeigt, dass die Funktion von GCN2 für die Fliegenentwicklung und das Überleben unter Entzug individueller EAS essenziell ist. Des Weiteren könnte GCN2 bei längerem Entzug einer EAS eine kompensatorische Nahrungsaufnahme induzieren, die zu erhöhten Fettleveln führt. Diese Fettreserven sind wahrscheinlich ursächlich für die erhöhte Resistenz der Fliegen unter vollständigem Nahrungsentzug. Interessanterweise zeigen die *Gcn2* Null-Mutanten und Wildtyp-Fliegen ähnliche physiologische Reaktionen unter Methionin-Entzug, was impliziert, dass einzig der Entzug von Methionin durch einen GCN2-unabhängigen Mechanismus detektiert wird. Außerdem habe ich ermittelt, ob erhöhte Expression von Activating Transcription Factor 4 (ATF4), welcher der GCN2-Kinase nachgeordnet ist, ausreichend ist, um die Phänotypen der GCN2 Null-Mutante unter Mangel verschiedener EAS zum Wildtyp-Phänotypen wiederherzustellen. Die Überexpression von ATF4 kann die GCN2-abhängigen Funktionen unter Mangel spezifischer EAS teilweise wiederherstellen, und die Effizienz der Wiederherstellung hängt davon ab, welche EAS in der Nahrung fehlt. Ein weiterer wichtiger Fund dieser Studie ist, dass ein vollständiger Entzug von Aminosäuren sich von dem Entzug einzelner EAS unterscheidet und wahrscheinlich unabhängig von GCN2 detektiert wird. Ich habe außerdem gezeigt, dass die Funktion von GCN2 in Fliegen, im Gegensatz zu Nematoden, für eine Verlängerung der Lebensspanne durch Rapamycin-induzierte Inhibition von Target Of Rapamycin (TOR) nicht essenziell ist.

Im zweiten Teil dieser Studie habe ich die Rolle der GCN2-Kinase während reduzierter Nährstoffaufnahme (RN) und im Transsulfurierungs-Stoffwechselweg (TSW) analysiert.

Aminosäuren, insbesondere Methionin und Cystein, spielen eine wichtige Rolle für Lebensspannen- und Gesundheits-Vorteile durch RN. Der TSW ist für den Stoffwechsel von Methionin und Cystein verantwortlich und kontrolliert möglicherweise die RN-induzierte Langlebigkeit durch H_2S, welches ein Metabolit des TSW ist. Ein Anstieg in der Produktion von H_2S während RN wurde in Hefe, Nematoden, Fruchtfliegen und Nagetier-Modellen beobachtet. Interessanterweise zeigt die vorliegende Studie, dass die durch RN und Methionin- und/oder Cystein-Restriktion induzierte Langlebigkeit in Fliegen unabhängig von GCN2 ist, aber dass die Produktion von H_2S zumindest teilweise von GCN2 abhängig ist. Daher habe ich den Zusammenhang zwischen RN-induzierter Langlebigkeit und der Produktion von H_2S erneut untersucht und fand überraschenderweise, dass eine erhöhte Lebensspanne nicht mit erhöhter Kapazität für die Produktion von H_2S korreliert.

SUMMARY

The eIF2α kinase, General Control Non-derepressible-2 (GCN2), senses amino acid starvation and coordinates cellular translation with amino acid availability allowing cells to cope with the nutritional stress. Accumulating evidence shows an important role of amino acids in modulation of longevity and healthspan in diverse species, however the underlying mechanisms remain elusive. With its function as an evolutionarily conserved amino acid starvation sensor, GCN2 kinase is a potential candidate to be involved in regulation of amino-acid-dependent physiological responses.

In the first part of this study, I have analyzed the *in vivo* functions of GCN2 kinase under deprivation of each of the 10 essential amino acids (EAAs) using a *Drosophila Gcn2* null mutant and a fully defined chemical diet. The present study shows that GCN2 function is essential for ensuring fly development and survival under deprivation of individual EAAs. Furthermore, this study suggests that GCN2 induces a compensatory feeding response in flies under long-term nutritional deprivation of an EAA, leading to increased lipid level, which is probably causal for the increased resistance of flies under full starvation. Interestingly, *Gcn2* null mutant and wild-type control flies exhibit similar physiological responses upon methionine deprivation, suggesting that methionine is the only EAA whose deprivation is sensed by a GCN2-independent mechanism. Furthermore, I tested whether enhanced expression of the downstream effector of GCN2 kinase, activating transcription factor-4 (ATF4), in the absence of GCN2 is sufficient to rescue the phenotypes of *Gcn2* null mutant under starvation of different EAAs. The present study shows that ATF4 activation partially rescues GCN2 mediated functions under deprivation of specific EAAs and that the rescue efficiency upon ATF4 activation depends on which EAA is being deprived from the diet. Another important finding of this study is that an EAA starvation is different from full amino acid starvation and probably sensed by GCN2-independent mechanisms. In addition, this study shows that in contrast to worms, GCN2 function in flies is not essential for lifespan extension conferred by rapamycin-mediated *Target of Rapamycin* (TOR) inhibition.

In the second part of this study, I have analyzed the role of GCN2 kinase in dietary restriction (DR) and the transsulfuration pathway (TSP). Amino acids, particularly methionine and cysteine, play an important role in mediating longevity and health benefits of DR. Recently, it has been suggested that the TSP, responsible for methionine and cysteine metabolism, controls DR-mediated longevity through one of its metabolite, H_2S.

An increase in the TSP-mediated H_2S production was seen in yeast, worm, fruit fly, and rodent models of DR. Interestingly, the present study shows that DR- and methionine and/or cysteine restriction (M/C-R)- induced longevity is GCN2-independent in flies but TSP-mediated H_2S production, at least in parts, is dependent on GCN2. I, therefore re-examined the connection between DR-induced longevity and TSP-mediated H_2S production and surprisingly found that increased lifespan do not correlate with increased H_2S production capacity in flies.

CHAPTER 1

General Introduction

"I shall live forever or die trying."

1.1 Ageing: the process of growing old

Biologically, ageing is a gradual decline of physiological functions due to accumulation of molecular and cellular damages over time. By 2050, 22% of the world's population will be over 60 years (WHO). This puts an immense pressure on our society as ageing is the major risk factor for age-associated pathologies including neurodegeneration, sarcopenia, cardiovascular disorders and diabetes, which account for roughly two-third of all the deaths occurring worldwide (Campisi, 2013). Therefore, it is crucial to understand the underlying mechanisms of ageing and its associated pathologies and to identify interventions for long and healthy life.

Understanding ageing is however challenging. The inherent complexity of ageing has lead to more than 300 different theories explaining why and how ageing occurs (Kirkwood, 2005). Ageing results from intricate interactions between diverse factors including genetic background, environmental changes and epidemiological conditions (Montesanto et al., 2012). It exhibits great phylogenetic diversity and broad inter-individual variability (Christensen et al., 2006). Despite the inherent complexity of ageing, there have been substantial advances in ageing research over recent years, providing crucial insight into metabolic pathways and biochemical processes involved in ageing. Recently, a seminal study identified and categorized nine cellular and molecular hallmarks of ageing that together determine the ageing process (López-Otín et al., 2013). The existence of several species in nature including rockfish, sturgeon, turtles, bivalves and lobsters that show negligible or no signs of ageing (Kirkwood and Austad, 2000), itself demonstrates that ageing is not inevitable. Indeed, recent work has shown that ageing can be ameliorated by nutritional, genetic and pharmacological interventions (Fontana et al., 2010; Partridge, 2009).

1.2 Nutrition and ageing

Nutritional intervention is probably the most robust approach to combat ageing in laboratory animals. Dietary, genetic or pharmacological manipulations that decrease nutrient signaling extend lifespan (Fontana et al., 2010; López-Otín et al., 2013). An evolutionarily conserved nutritional intervention that delays ageing and improves lifespan is dietary restriction (DR). DR, classically defined as reduced food intake without malnutrition, extends lifespan in diverse species including yeast, worms, flies, fish, rodents and rhesus monkeys (Fontana et al., 2010). DR also improves metabolic fitness and protects against age-related decline in function and diseases including obesity, diabetes, cardiovascular disease, cancers and neurodegeneration in rodents and primates (Fontana and Partridge, 2015). In humans, DR significantly decreases body weight and abdominal fat and causes a robust improvement in glycemic control and insulin sensitivity (Soultoukis and Partridge, 2016; Weissa et al., 2006). DR encompasses different interventions varying widely in both dietary composition and timing of food intake (Mair and Dillin, 2008). The beneficial effects of DR can be obtained by reduced intake of specific macronutrients including carbohydrates, fats or protein in the diet rather than simple reduction of total calories (Lee et al., 2014b; Mair et al., 2005; Piper et al., 2005). Among the macronutrients, proteins have been shown to play a causal role in determining DR-mediated longevity.

1.2.1 Dietary protein and amino acids in ageing

Proteins are made up of 20 different amino acids. In multicellular organisms, half of them cannot be synthesized de novo, and are hence called essential amino acids (EAAs), and must be acquired through dietary means (Wu, 2016) (essential and non-essential amino acids are listed in Table 1.1). Dietary guidelines recommend high intake of proteins, especially derived from animal products, to combat obesity and promote health (Arentson-Lantz et al., 2015). However, over the last decade, studies in different model organisms have demonstrated that restriction of dietary protein and specific amino acid plays a key role in modulating health and longevity benefits of DR. In *Drosophila*, reduction of dietary yeast, the sole source of protein, extends lifespan to much higher extent than isocaloric reduction of sucrose (Mair et al., 2005). In rodents, reduction in the protein content of the diet (decreased protein to carbohydrate ratio) also maximizes lifespan (Solon-Biet et al., 2014).

Furthermore, certain amino acids have distinct effects on longevity. In yeast,

chronological lifespan can be extended by selective restriction of asparagine, glutamate or methionine in the medium (Wu et al., 2013a; 2013b). In flies, adding back EAAs, in particular methionine (or to a lesser degree tryptophan) to the diet of DR flies abrogates lifespan extension (Grandison et al., 2009). Furthermore, reduced methionine content under low protein status leads to lifespan extension in flies (Lee et al., 2014a). In rodents, diets restricted for methionine and tryptophan have also been shown to increase lifespan (McIsaac et al., 2016; Miller et al., 2005; Orentreich et al., 1993). Interestingly, DR in flies leads to increase in the activity of the transsulfuration pathway (TSP) (Kabil et al., 2011) The TSP controls the conversion of methionine into cysteine and is the primary source of endogenous hydrogen sulfide (H_2S) (Stipanuk and Ueki, 2010). Recently, increased TSP-mediated H_2S production has been suggested to be causative for DR benefits including extended lifespan, in yeast, worms, flies and rodents (Hine et al., 2015). Surprisingly, besides methionine and tryptophan, effects of other EAA-restricted diets on lifespan are not known, although some amino acids have been shown to induce other benefits of DR. A recent study indicated that restriction of branched-chain amino acids (leucine, isoleucine and valine) is sufficient to improve glucose tolerance and body composition equivalently to a protein restricted diet (Fontana et al., 2016). Recent years have seen significant progress with regard to the influence of amino acids on lifespan, however more work is needed to understand the underlying mechanisms and pathways regulating this effect.

Table 1.1: List of essential and non-essential amino acids
(*Arginine is a conditionally essential amino acid in humans)

Essential amino acids (EAAs)	Non-essential amino acids (NEAAs)
Methionine (M)	Alanine (A)
Trytophan (W)	Cysteine (C)
Leucine (L)	Aspartic acid (D)
Isoleucine (I)	Glutamic acid (E)
Valine (V)	Glycine (G)
Histidine (H)	Asparagine (N)
Phenyalanine (F)	Proline (P)
Threonine (T)	Glutamine (Q)
Lysine (K)	Serine (S)
Arginine* (R)	Tyrosine (Y)

1.3 Amino acid starvation sensing pathway

Cells have evolved different signal transduction mechanisms to sense intracellular amino acid levels. The presence of amino acids is sensed by *Target of rapamycin* (TOR) kinase whereas lack of amino acids is sensed by GCN2 kinase (Gallinetti et al., 2013). The focus of this thesis is particularly the role of GCN2 kinase under amino acid starvation.

1.3.1 GCN2 – an eIF-2α kinase

The ability of an organism to survive depends on its ability to trigger responses against diverse stresses. In eukaryotes, an important strategy to deal with different stresses is to down-regulate protein synthesis, allowing cells to conserve resources and maintain cellular proteostasis (Spriggs et al., 2010). Stress-dependent inhibition of protein synthesis is attained by a family of kinases that phosphorylates eukaryotic initiation factor-2 alpha (eIF2α). In mammals, four different eIF-2α kinases have been identified and each of them is activated in response to a different set of stress signals (Wek et al., 2006). Heme-regulated inhibitor (HRI) is induced by heme deprivation, heat and oxidative stress, Protein kinase double stranded RNA dependent (PKR) is activated by double stranded RNA binding and participates in an anti-viral defense mechanism, Pancreatic eIF2α kinase (PEK/PERK) is triggered by endoplasmic reticulum (ER) stress and General control non-derepressible-2 kinase (GCN2) is activated primarily by amino acid starvation but can also be triggered by other stresses including UV irradiation and proteasome inhibition (Wek et al., 2006).

GCN2 is the only eIF-2α kinase that is evolutionary conserved at the structural and functional levels across diverse species ranging from yeast to mammals (Dever et al., 1993; Wek et al., 2006). It primarily senses amino acid starvation and coordinates cellular translation with amino acid availability, thus allowing cells to cope with the amino acid starvation stress. However, GCN2 is not only relevant for controlling protein synthesis and maintaining amino acid homeostasis but also has been implicated in several other biological processes. In higher eukaryotes, GCN2 mediates feeding behavior (Maurin et al., 2005), energy storage (Guo and Cavener, 2007), long-term memory formation (Costa-Mattioli et al., 2005), tumor cell survival (Ye et al., 2010), immune response (Murguía and Serrano, 2012) and intestinal inflammation (Ravindran et al., 2016). Moreover, in humans GCN2 mutations have been linked to pulmonary veno-occlusive disease, a form of pulmonary hypertension (Eyries et al., 2013).

1.3.2 Domains composition of the GCN2 protein

The ~180 kDa GCN2 protein is composed of five different domains (Figure 1.1) (Dong et al., 2000; Hinnebusch and Natarajan, 2002; Wek et al., 1995). From the N-terminus, the first is the RWD domain (so named from its presence in RING finger proteins, WD-repeat-containing proteins, and yeast DEAD-like helicases). It acts as a binding site for GCN1/GCN20 proteins, which are required to stimulate GCN2 activation. Next to the RWD domain, a pseudokinase domain (ΨK) is present, which is also suggested to help in GCN2 kinase activation. The third is a typical eukaryotic protein kinase (PK) domain, which is inherently inert and its activation depends on the interactions with other domains (Lageix et al., 2015; Qiu et al., 2001). The fourth is a histidyl-tRNA synthetase (HisRS)-related domain, so named because it is highly homologous to the entire sequence of histidyl-tRNA synthetase enzyme. The HisRS-related domain interacts and binds different uncharged tRNAs together with the last domain of GCN2, the C-terminal domain (CTD). The CTD is also required for association with ribosomes and facilitates GCN2 dimerization (Lageix et al., 2015).

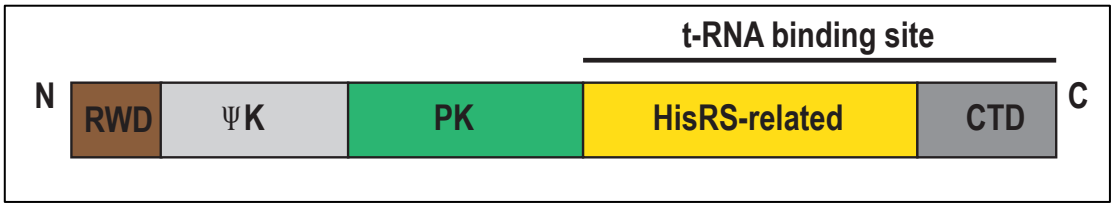


Figure 1.1: Schematic representation of the domain arrangement in yeast GCN2

From the N to C terminus: RWD, pseudokinase (ΨK), protein kinase (PK), HisRS-related, and C-terminal (CTD) domains. Arrow above GCN2 indicates binding site of t-RNA.

1.3.3 Mechanism of amino acid starvation sensing by GCN2 kinase

GCN2 is a serine/threonine kinase that senses and overcomes amino acid deprivation (Castilho et al., 2014; Qiu et al., 2001). The proposed mechanism for amino acid starvation sensing by GCN2 suggests that uncharged t-RNAs serve as an activating signal for this kinase. Amino acid deprivation results in the accumulation of uncharged t-RNAs, which bind to the HisRS-related domain of GCN2 (Figure 1.2). This binding evokes a conformational change, resulting in activation of the adjacent PK domain (Diallinas and Thireos, 1994; Dong et al., 2000; Wek et al., 1995). Subsequently, the activated kinase domain catalyzes auto-phosphorylation of GCN2 and phosphorylation of its only known substrate, eIF2α, at residue serine-51. Phosphorylation of eIF2α reduces the activity of the

guanylate exchange factor, eIF2B, which recycles inactive GDP-bound eif2α to its GTP-bound active form. Reduced availability of GTP-bound eif2α inhibits the formation of ternary complex (eIF2α-GTP-Met-tRNAi), which is essential for delivering the initiator methionyl t-RNA (Met-tRNAiMet) to the small ribosomal subunit in the first step of translation initiation, thereby causing a general inhibition of global translation (Hershey, 1991; Qiu et al., 2001; Wek et al., 1995) (Figure 1.2). However, translation of selective mRNAs with specific regulatory elements in their 5' UTRs (untranslated regions) is increased. These mRNAs code for transcription factors such as GCN4 in yeast (Hinnebusch, 1997), or activating transcription factor 4 (ATF4) in mammals (Harding et al., 2003), which trigger a transcriptional response involving a number of target genes to effectively manage the stress condition and relieve the cell from amino acid starvation (Harding et al., 2003; Sikalidis et al., 2010).

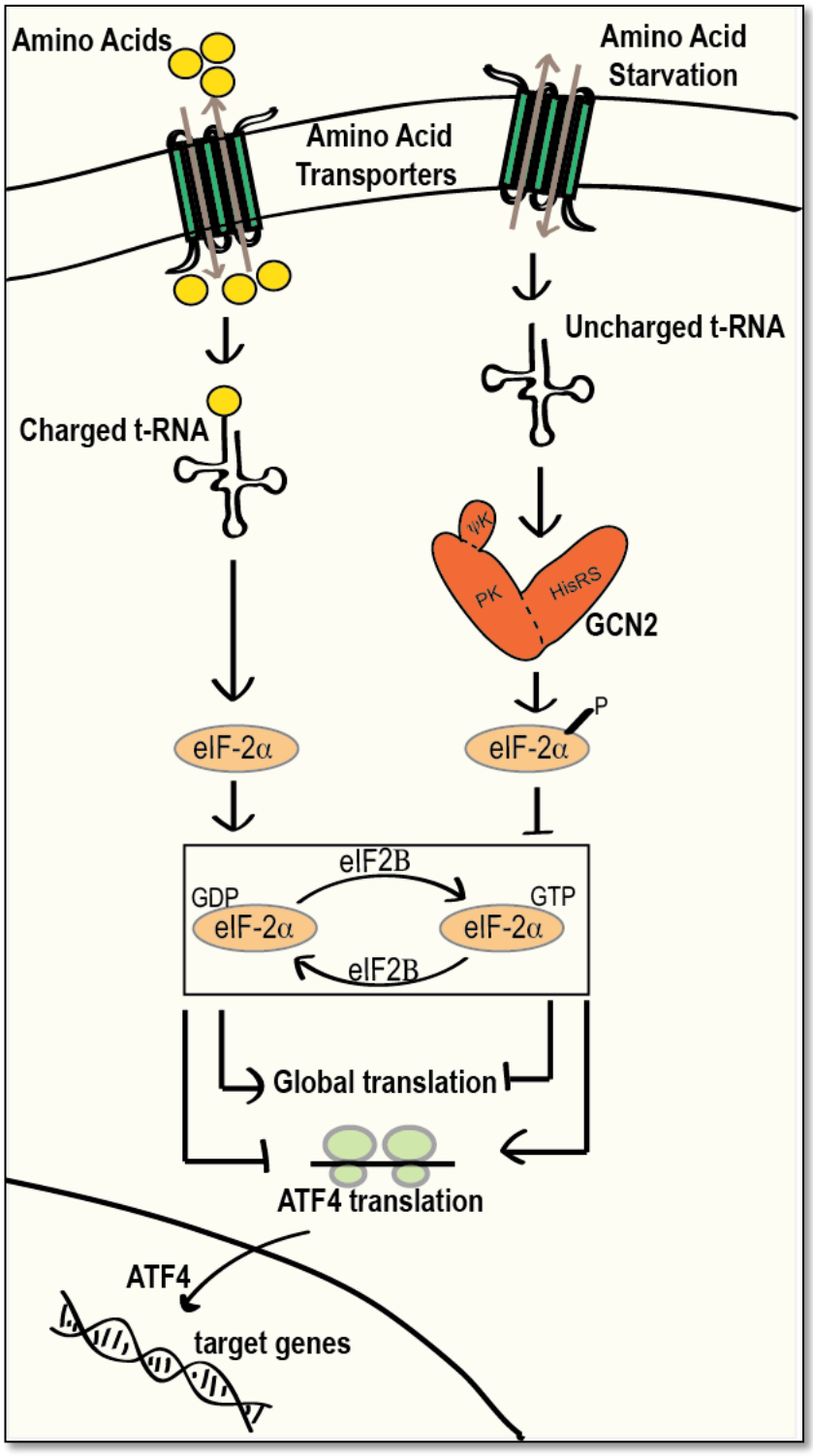


Figure 1.2: Amino acid starvation sensing by GCN2 kinase

Upon amino acid starvation, binding of uncharged t-RNAs to the HisRS-related domain activates GCN2 kinase activity. Activated GCN2 phosphorylates eIF2α, which inhibits eif2B activity resulting in reduced availability of GTP-eIF2α and inhibition of global translation. Concomitantly, translation of ATF4 is induced which activates transcription of genes to restore amino acid homeostasis.

1.3.4 Role of GCN2 in lipid metabolism

Dietary amino acid deficiency alters metabolism beyond protein homeostasis. For instance, a lysine and threonine deficient diet in rats induces expression of genes linked to cholesterol biosynthesis (Endo et al., 2002) and deprivation of any EAA inhibits expression of fatty acid synthase (FAS), a key component of lipid metabolism in HepG2 cells (Dudek and Semenkovich, 1995). Interestingly, Guo and Caver first showed that regulation of lipid metabolism upon deprivation of an EAA is mediated by the amino acid starvation sensor, GCN2 (Guo and Cavener, 2007). In response to leucine deprivation, a GCN2-dependent inhibition of lipogenic genes and FAS activity occurred in the liver and lipid mobilization was increased in the adipose tissue. In contrast, due to the combined effect of unrepressed lipid synthesis and reduced lipid mobilization, GCN2 deficient mice developed liver steatosis. Recently, the endocrine hormone, fibroblast growth factor 21 (FGF21), has been implicated as a downstream effector of GCN2 kinase for regulating lipid metabolism during amino acid starvation (De Sousa-Coelho et al., 2013; Laeger et al., 2014). Under leucine deprivation, GCN2-dependent phosphorylation of eIF2α and activation of ATF4 induces FGF21, which then represses hepatic lipogenic genes in mice.

An in-depth understanding of lipid metabolism and its link to protein deprivation can provide new insights into the treatment of obesity and other metabolic diseases. Particularly, it will be interesting to investigate the role of the central nervous system, which is implicated in the control of hunger and satiety, in mediating energy storage upon amino acid starvation.

1.3.5 Role of GCN2 in food uptake upon amino acid starvation

Multicellular organisms including flies, rodents and humans cannot synthesize almost half of the protein coding amino acids. Moreover, these EAAs cannot be stored (Munro, 1976). Therefore in order to survive, EAAs must be acquired through dietary means. Consequently, the ability to actively regulate feeding behavior based on the quality and quantity of amino acids in the diet is important in these organisms (Gietzen et al., 2007).

Studies in rodents have shown that wild type animals can rapidly identify and reject diets lacking an EAA (Koehnle et al., 2003; Maurin et al., 2005). Cell ablation experiments and direct injection of the limiting amino acid have implicated the anterior piriform cortex (APC) in this aversive response (Beverly et al., 1990; Leung and Rogers, 1971). Furthermore, eIF2α phosphorylation was reported to be increased in the APC of rats after

consumption of a threonine deficient diet, establisihing a molecular link to this aversive response (Gietzen et al., 2004). In 2005, two studies proposed that the rejection of the EAA-deficient diets is mediated by the amino acid starvation sensor GCN2 kinase. Maurin and colleagues showed that mice fed with a leucine or threonine deficient diet significantly reduced their food consumption within an hour of feeding (Maurin et al., 2005), while this aversive response was blunted in mice with brain specific deletion of GCN2. Interestingly, within 20 minutes of ingestion of an EAA deficient diet (Maurin et al., 2005) or being injected with amino alcohols (Hao, 2005) an increase in the phosphorylation of eIF2α was observed in the APC of wild type mice but was not in GCN2 knockout mice, indicating that recognition of uncharged t-RNAs and consequent eIF2α phosphorylation by GCN2 in APC neurons is critical for rejection of diets lacking an EAA. Further insights into the neural circuitary involved in GCN2 mediated rejection of EAA deficient diets comes from a recent study in *Drosophila* (Bjordal et al., 2014). Similar to rodents, fly larvae also rejected a maize-based diet deficient in trytophan and lysine. Using genetic interactions and calcium imaging they showed that this rejection response was a result of GCN2 activation in dopaminergic (DA) neurons and a consequent repression of GABA signalling. However, whether GCN2 activation in dopaminergic neurons also influences the feeding behaviour of adult flies, remains currently unanswered.

Strikingly, a very recent study by Leib and Knight has re-examined dietary amino acid sensing in mice and, contrary to the earlier reports, showed that mice cannot rapidly identify and reject diets lacking an EAA (Leib and Knight, 2015). In their study, wild type mice showed no difference in the consumption of control versus leucine- or threonine-deficient diets. Furthermore, no activation of GCN2 was observed in the brains of these mice following consumption of these EAA-deficient diets. The only condition under which mice attained the ability to rapidly and robustly sense the deficiency of an EAA in the diet was when they were previoulsy deprived of the same EAA, suggesting that mice reject an EAA-deficient diet if they have a physiologic need for that EAA. Most surprisingly, this need-based EAA sensing was independent of GCN2 and the authors suspected an involvement of some undescribed mechanism for amino acid sensing.

Although, substantial progress has been made in understanding how animals regulate their feeding behaviour in response to availability of amino acids, very important mechanistic aspects still have to be investigated. For instance, does individual deprivation of each

EAAs elicit similar rejection response or can some amino acids be preferred over others to influence food uptake? What is also presently unclear is, whether the mechanism behind the aversive response is similar for each of the EAAs or different pathways are involved for different amino acids. Most importantly, if dietary amino acid sensing is GCN2-independent then what is the underlying molecular mechanism that mediates it?

1.4 Activating transcription factor-4 (ATF4)

ATF4 is a member of the ATF/CREB (activating transcription factor/cyclic AMP response element binding protein) family of basic-region leucine zipper transcription factors (Ameri and Harris, 2008). It is considered to be an important downstream effector of GCN2 kinase, as it regulates expression and activity of several genes involved in a variety of adaptive functions, including amino acid import and metabolism (Harding et al., 2003). Besides being activated by GCN2, ATF4 is also activated by other eif2α kinases. In mammals each of the four eif2α kinases, including GCN2, in response to specific stress signals phosphorylates eif2α, which suppresses global translation but promotes increase in the translation of selected mRNAs including *Atf4* (Wek et al., 2006).

1.4.1 Regulation of ATF4 translation

The increase in the translational of ATF4 following eif2α phosphorylation is attributed to the two upstream open reading frames (uORF1 and uORF2) present within the 5' leader of the *Atf4* mRNA (Kilberg et al., 2009; Lu et al., 2004; Vattem and Wek, 2004). Under unstressed conditions, sufficient GTP bound eif2α makes it possible for ribosomes that had translated uORF1 to re-initiate translation at uORF2, an inhibitory element that overlaps with the coding sequence of ATF4 and blocks ATF4 translation. Conversely, under stressed conditions, phosphorylation of eif2α and the accompanying reduction in the availabilty of GTP bound eif2α delays ribosome scanning, consequently uORF2 is skipped and translation re-initiation starts at the ATG of the ATF4 ORF. Thus, ATF4 translation is selectively up regulated following eif2α phosphorylation under periods of celluar stress.

1.4.2 Transcriptional regulation by ATF4 under amino acid starvation

The translationally increased ATF4 initiates a transcriptional response involving a number of genes. A recent study has identified more than 450 ATF4 target genes using chromatin

immunoprecipitation sequencing (ChIP-seq) (Han et al., 2013). ATF4 triggers transcription of its target genes by binding to CCAAT enhancer-binding protein (C/EBP)-ATF response elements (CARE) (Fawcett et al., 1999; Kilberg et al., 2009). The CARE, which in context of amino acid starvation is called AARE, consists of a half-site for C/EBP family transcription factors and a half-site for the ATF family members. Consistent with the critical role of ATF4 in amino-acid stress response, the ATF half site is conserved, because ATF4 binds to all the known AARE sites and the half site of C/EBP is quite divergent (Kilberg et al., 2012). A microarray based analysis in cysteine-deprived HepG2/C3A cells observed a highly significant up-regulation of expression of genes that are known to contain AARE, including CCAAT/enhancer-binding protein homologous protein (CHOP) and asparagine synthetase (ASNS), and to respond to amino acid deprivation via the binding of ATF4 (Sikalidis et al., 2010). Hence, suggesting that transcriptional responses to amino acid deprivation is mediated by ATF4 binding to AARE sites (Sikalidis et al., 2010).

In yeast, under amino acid starvation GCN2 activates GCN4 (the functional homologue of mammalian ATF4), which induces expression of an array of genes that code for amino acid biosynthetic enzymes to replenish the supply of depleted amino acids (Hinnebusch and Natarajan, 2002). However, unlike yeast, higher organisms cannot synthesize EAAs and therefore upon amino acid starvation the array of genes induced by GCN2/ATF4 pathway in mammalian cells is different compared to those induced by the GCN2/GCN4 pathway in yeast. The ATF4 induced genes in mammalian cells under amino acid starvation are those involved in aminoacyl-tRNA synthesis, amino acid metabolism and assimilation and amino acid transport (Harding et al., 2003). One of the target genes of ATF4, FGF21, has recently been shown to be a key mediator of the physiological response to dietary protein restriction (Laeger et al., 2014). The study showed that the GCN2/ATF4 pathway mediated induction of the endocrine hormone FGF21 upon low protein conditions controls food intake, energy expenditure and growth in mice (Laegar et al., 2014).

Although the critical role of ATF4 in transcriptional control under amino acid starvation is well established, research over the past years has suggested that different basic leucine zipper (bZIP) transcription factor including ATF2, ATF3, ATF5, cJUN and CHOP might also influence transcription in response to amino acid starvation (Kilberg et al., 2012). Therefore it will be interesting to investigate whether deprivation of each of the EAAs

activates ATF4 or whether specific transcription factors get activated depending on which EAA is missing.

1.4.3 Regulation of transcriptional specificity of ATF4

GCN2 is one of the 4 kinases that phosphorylate eif2α in response to different cellular stresses. Another eif2α kinase is PEK, which is activated in response to ER stress and activates ATF4 translation and downstream transcriptional response (Harding et al., 2000). Array analysis in yeast and mouse revealed, that the transcriptional profile activated by ATF4 in response to ER stress (by PEK activation) mostly overlaps with amino acid starvation (by GCN2) and oxidative stress, suggesting that ATF4 integrates several upstream stress signals to orchestrate a common downstream transcriptional response (Harding et al., 2003). However, another study using mouse liver and hybridization array analysis demonstrated that, despite sharing a common downstream target, GCN2 and PERK differentially regulate mRNA transcription and translation (Dang Do et al., 2009). These findings raise an interesting question as to how ATF4 achieves its transcriptional specificity in response to different stresses. Some insight comes from a study on one of the ATF4 target gene, sodium-dependent neutral AA transporter 2 (SNAT2), in HepG2 human hepatoma cells (Gjymishka et al., 2008). ATF4 binds to the CARE in SNAT2 following amino acid starvation and activates a transcriptional response. However, upon ER stress, despite increased ATF4 binding to the *SNAT2* gene, transcription activity was not enhanced. Activating both the ER stress and amino acid stress response together showed that the ER stress generates a suppressive signal that blocks the amino acid stress induced *SNAT2* transcription activity downstream of ATF4 binding. Another possibiltiy could be that the transcriptional specifity of ATF4 might be a result of highly coordinated interaction of ATF4 with a precise set of transcription factors that can bind to the C/EBP half-site of the CARE sequences, however this remains to be investigated (Kilberg et al., 2009). Thus, it will be intruiging to decipher the mechanisms by which ATF4 triggeres an appropriate subset of genes on being activated by a specific eIF2α kinase.

1.5 Interplay between the two amino acid sensing pathways: GCN2 and TOR

In addition to GCN2, eukaryotes have a second evolutionarily conserved signal transduction pathway, TOR that is activated by the presence of amino acids (Gallinetti et al., 2013). The central component of the TOR pathway is TOR kinase, which functions in two different complexes: TORC1 and TORC2. TORC1 is activated under favourable, amino acid rich, conditions and is repressed by AMP-activated protein kinase (AMPK), a key sensor of cellular energy status (Johnson et al., 2013). Upon activation, TORC1 promotes mRNA translation and protein synthesis via two of its substrates, positive regulation by phosphorylated S6 kinase (S6K) (Fenton and Gout, 2011) and inhibitory phosphorylation of eukaryotic initiation factor 4E-binding protein (4E-BP) (Ma and Blenis, 2009). Genetic or pharmacological down regulation of TORC1 leads to increased lifespan from yeast to mammals (Kapahi et al., 2010). Rapamycin is the most specific pharmacological inhibitor of the TOR pathway and treatment with rapamycin extends lifespan in flies and mice (Bjedov et al., 2010; Guertin and Sabatini, 2009; Harrison et al., 2010).

Recent evidence suggests cross-talk between the GCN2 and the TOR pathways under amino acid deprivation. Human lymphocytic leukaemic cell lines when treated with L-asparaginase, an asparagine degrading enzyme that activates GCN2, inhibits TORC1 phosphorylation of its target substrates – S6K and 4E-BP1 (Iiboshi et al., 1999). A recent study in mouse embryonic fibroblast has reported that GCN2 sustains TORC1 suppression under amino acid starvation by inducing Sestrin2 (Ye et al., 2015). Consistent with these cell culture studies, in vivo evidence in mice also suggests that activation of GCN2 can result in TORC1 inhibition. Leucine deprivation caused decreased phosphorylation of S6K and 4E-BP1 in liver and pancreas dependent on GCN2 function (Anthony, 2004; Bunpo et al., 2009). Interestingly, a study in worms suggests that GCN2 activity is essential to mediate lifespan-extension conferred by down regulation of TOR (Rousakis et al., 2013). The long lifespan of TOR-deficient (LET-363) worms was abrogated in *Gcn2*-deficient worms. Despite growing evidence that the TOR and GCN2 pathways might act in a concerted manner under amino acid starvation, the direction and the mechanism of interaction still remains unclear. Moreover, the interplay between these two major nutrient-sensing pathways in longevity has yet to be established.

1.6 Aims of the Ph.D. Thesis

Dietary amino acids play a vital role in mediating longevity and health benefits in diverse species. However, the underlying molecular mechanisms through which amino acids regulate these benefits are presently unclear. As higher organisms must obtain EAAs through dietary means for survival, the sensing and signaling mechanisms that monitor and respond to EAA availability may play an important role in mediating amino-acid-dependent physiological responses. Thus far, the best understood mechanism for sensing intracellular amino acid availability is mediated by the evolutionarily conserved eIF2α kinase GCN2. Therefore, I aimed to investigate the role of GCN2 kinase in longevity and important physiological functions during nutritional deprivation of EAAs using *Drosophila melanogaster*, an established model organism for nutrition and lifespan research. In this study, I utilized two powerful tools, *Gcn2* null mutants and a fully defined chemical medium, to dissect the *in vivo* function of GCN2 kinase in response to the absence or limitation of each of the 10 EAAs.

Important questions addressed in my doctoral thesis are as follows:

1. How does GCN2 affect the development and lifespan in response to the deprivation of different individual EAAs?

2. How does GCN2 regulate feeding behavior, energy storage and stress resistance upon deprivation of each of the 10 EAAs?

3. Does ATF4 mediate the amino acid stress response as a downstream effector of GCN2 upon deprivation of each of the 10 EAAs?

4. What are the genes, biological processes and metabolic pathways differentially regulated by GCN2 under deprivation of specific amino acids?

5. Do the two amino acid sensing pathways, GCN2 and TOR, interact to modulate longevity and stress resistance conferred by TOR inhibition?

6. Does GCN2 modulate DR-mediated longevity in flies and does this regulation involve H_2S production by the transulfuration pathway (TSP)?

CHAPTER 2

Materials And Methods

2.1 Preparation of fly media

2.1.1 Holidic media

The holidic media, Yaa and HUNTaa, were prepared according to (Piper et al., 2013) (an open-access editable version of the protocol is available through Nature Protocol Exchange http://dx.doi.org/10.1038/protex.2013.082, also refer to Table 1 and Supplementary Table 1 of Piper et al., 2013). Briefly, sucrose, agar, amino acids with low solubility (L-isoleucine, L-leucine and L-tyrosine), metal ions and cholesterol were combined with milliQ water and autoclaved at 120 °C under constant stirring for 15 min in a mediaclave 10 media preparator (Integra Biosciences). After autoclaving, sterile-filtered stock solutions of buffer, amino acids, vitamins, nucleosides, choline, inositol and preservatives were added. RU486 (Mifepristone, Sigma) or rapamycin (LC Laboratories) at required concentrations (as specified in each experiment) were dissolved in ethanol was added to the food, where required. Control food contained the same volume of ethanol without addition of the drug. Diets with modified amino acid content were prepared similar to Yaa or HUNTaa media by only changing the content of the specified amino acid in the EAAs stock solution. Leucine and isoleucine were added as solid powder directly to the food and adjusted accordingly (Table 2.1 and 2.2).

Table 2.1: Amino acid content in the modified HUNTaa media. Amino acid changes are highlighted in red.

	HUNTaa	HUNTaa + R	HUNTaa + R + I
EAA stock solution	**(g/200 ml)**	**(g/200 ml)**	**(g/200 ml)**
F (L-phenylalanine)	2.60	2.60	3.03
H (L-histidine)	2.00	2.00	2.24
K (L-lysine)	3.80	3.80	5.74
M (L-methionine)	1.60	1.60	1.12
R (L-arginine)	1.60	4.70	4.70
T (L-threonine)	4.00	4.00	4.28
V (L-valine)	5.60	5.60	4.42
W (L-tryptophan)	1.00	1.00	1.45
EAAs added as solid	**(g/l of medium)**	**(g/l of medium)**	**(g/l of medium)**
I (L-isoleucine)	1.82	1.82	3.36
L (L-leucine)	1.21	1.21	1.64

- In HUNTaa+50% aa, all the EAAs and NEAAs were increased by 1.5 times of their amounts in the HUNTaa medium.

Table 2.2: Amino acid content in the modified Yaa media. * Only the amount of the specified AA was changed in the modified diets as indicated while the amounts of other AAs were kept the same as in the Yaa medium.

	AAs amount in Yaa	AAs amount in modified diets*
EAA stock solution	**(g/200 ml)**	**(g/200 ml)**
F (L-phenylalanine)	3.03	0.91 in Yaa-F (30%)
H (L-histidine)	2.24	0.67 in Yaa-H (30%)
K (L-lysine)	5.74	1.72 in Yaa-K (30%)
R (L-arginine)	4.70	1.41 in Yaa-R (30%)
T (L-threonine)	4.28	1.28 in Yaa-T (30%)
V (L-valine)	4.42	1.33 in Yaa-V (30%)
W (L-tryptophan)	1.45	0.44 in Yaa-W (30%) 0.29 in Yaa-W (20%) 0.15 in Yaa-W (10%)
M (L-methionine)	1.12	0.34 in Yaa-M (30%) 0.22 in Yaa-M (20%) 0.11 in Yaa-M (10%) 0.22 in 0.2xM Yaa 5.60 in 5xM Yaa
EAAs added as solid	**(g/l of medium)**	**(g/l of medium)**
I (L-isoleucine)	1.16	0.35 in Yaa-I (30%)
L (L-leucine)	1.64	0.49 in Yaa-L (30%)
Other AA stock solution	**(ml/l of medium)**	**(ml/l of medium)**
C (L-Cysteine) (50 mg/ml stock)	5.28	1.05 in 0.2xM-0.2xC Yaa

- In an EAA-deficient diet (Yaa-EAA), the particular EAA was completely removed
- In Yaa-all, all the EAAs and NEAAs were completely removed
- In Yaa-25% aa, all EAAs and NEAAs were decreased by 4 times of their amount in the Yaa medium

2.1.2 SYA media used for DR experiments

For DR experiments sugar/yeast/agar (SYA) medium was used and DR food was prepared according to the optimized protocol described in (Bass et al., 2007) (Table 2.3).

Table 2.3: SYA media used for DR experiments. Yeast (brewers yeast #903312, MP Biomedicals, London, UK); agar and propionic acid (Sigma, Dorset, UK); Nipagin M (methyl 4-hydroxybenzoate)

	Sugar	Yeast	Agar	Water	Propionic acid	Nipagin
	(g/l)	(g/l)	(g/l)	(ml)	(ml)	(ml)
0.1 SYA	50	10	15	917	3	30
0.5 SYA	50	50	15	912	3	30
0.75 SYA	50	75	15	899	3	30
1.0 SYA (standard)	50	100	15	886	3	30
1.5 SYA	50	150	15	860	3	30
2.0 SYA	50	200	15	834	3	30

2.2 Generation, maintenance and characterization of transgenic fly lines

2.2.1 Generation of *Gcn2* null mutants

$Gcn2^1$ and $Gcn2^2$ null mutants were generated by ends-out homologous recombination according to the methods described in (Gong, 2004; Gong and Golic, 2003; Huang et al., 2009) by Dr. Sebastian Grönke. In the $Gcn2^1$ allele the complete ORF of the *Gcn2* gene was replaced by a $white^{hs}$ *marker* gene, while in the $Gcn2^2$ allele the 5´part of the *Gcn2* gene including the part encoding the protein kinase domain were replaced by the $white^{hs}$ *marker* gene. Donor constructs used for targeting *Gcn2* were generated by amplifying approximately 4 kb of flanking sequences of the corresponding region of the *Gcn2* gene and subsequently cloned into the pGX attP vector (Huang et al., 2009) for $Gcn2^1$ and the pW25 vector for $Gcn2^2$ (Gong and Golic 2004). Long-range PCR was done using Takara LA Taq (Clontech) or Phusion polymerase (NEB) using primer combinations SOL310/311 and SOL312/313 for the 5´and 3´arm of the $Gcn2^1$ donor construct and SOL318/SOL319 and SOL320/321 for the 5´and 3´arm of the $Gcn2^2$ donor construct, respectively (sequence of primers are listed in Table 2.5). BAC clones covering the *Gcn2* locus used as PCR template for the $Gcn2^1$ donor construct were CH321-12O13 and RP98-2GL6 for the 5´and 3´arm, respectively and CH321-12O13 for both arms of the $Gcn2^2$

construct. BAC clones were obtained from the BACPAC Resource Center (Oakland, California, USA). PCR products were cut with the indicated restriction enzymes (Table 2.5) and subcloned into the corresponding vector (see above). *Gcn2* donor constructs were full length sequenced and checked for base pair substitutions in the coding region of neighbor genes before generation of transgenic fly lines. Transgenic fly lines were generated via P-element-mediated germ line transformation using the Best Gene *Drosophila* Embryo Injection Services, (Chino Hills, California, USA). Ends-out homologous recombination was done following the rapid targeting scheme (Rong and Golic, 2001) and homologous recombination events were identified by genetic mapping of the $white^{hs}$ marker gene and subsequent PCR analysis using primers SOL365/366.

2.2.2 Fly maintenance and stocks

All fly stocks were maintained at 25°C on a 12 h:12 h light:dark cycle at constant humidity (65%) on the standard SYA (1.0) medium (Table 2.4). $Gcn2^{1}$ mutants were backcrossed for at least ten generations into two different wild-type stocks, the outbred white Dahomey (w^{Dah}) strain and the inbred lab strain w^{1118} (a kind gift from Dr. Pierre Leopold). All other fly lines were only backcrossed into the w^{Dah} background.

Table 2.4: List of fly stocks used

Genotype	Chromosome	Background	Designed by/obtained from
w^{Dah}	-	w^{Dah} w+	Grönke et al., 2010
w^{Dah};;$Gcn2^{1}$	3	w^{Dah} w+	This study
w^{Dah};;$Gcn2^{2}$	3	w^{Dah} w+	This study
w^{1118}	-	w^{1118}	Bjordal et al., 2013
w^{1118};;$Gcn2^{1}$	3	w^{1118}	This study
w^{Dah};;PEK^{e01744}	3	w^{Dah} w+	Bloomington
w^{Dah};;da-Gal4	3	w^{Dah} w+	Bloomington
w^{Dah};;da-Gal4,$Gcn2^{1}$	3	w^{Dah} w+	This study
w^{Dah};daGS-Gal4	2	w^{Dah} w+	This study
w^{Dah};daGS-Gal4;$Gcn2^{1}$	2	w^{Dah} w+	This study
w^{Dah};;UAS-*Atf4-x3HA*	3	w^{Dah} w+	FlyORF, F000106
w^{Dah};;UAS-*Atf4-x3HA*, $Gcn2^{1}$	3	w^{Dah} w+	This study

2.2.3 Generation of experimental flies

All experimental flies were generated by transferring 20µl of eggs to SYA containing culture bottles (size) ensuring standard larval density. Newly emerged adults were

collected over a period of 24 h and transferred to fresh SYA food unless otherwise indicated and allowed to mate for 48 h (once-mated). Subsequently, males and females flies were sorted under brief CO_2 anesthesia and transferred to experimental vials (narrow plastic vials: 9.5 cm x 2.5 cm diameter, wide plastic vials: 9.5 cm x 2.85 cm or glass vials: 7.5 cm x 2.55 cm diameter were used as specified in each experiment). All experiments were performed at 25°C on a 12 h:12 h light:dark cycle at constant humidity (65%).

2.2.4 Development assay

For development assays, flies were allowed to lay eggs for a period of 4-5 h on grape juice plates. 250 eggs per genotype and diet were picked and transferred to vials containing experimental food at a density of 25 eggs per glass-vial. Upon eclosion adult flies were scored daily at 24-hour intervals and at the end of the experiment viability was calculated as the percentage of total number of eclosed adult flies per genotype and diet.

2.2.5 Lifespan and fecundity

For lifespan experiments, 100 to 200 once mated male or female flies per genotype and diet were maintained at a density of 10-15 flies per vial (narrow plastic vials for drug experiments and glass vials for all other lifespans) containing different experimental diets. Flies were transferred to fresh vials every 2–3 days and the number of dead flies was scored on the day of transfer. The sorting day was classified as day 0 of the lifespan experiment. For fecundity assay eggs were collected over 15-20 h periods at several time-points during the first 3-4 weeks of lifespan experiments. The number of eggs laid per vial at each time point was scored using a hand counter. The values are expressed as the mean number of eggs laid per female fly per 24 h ± SEM or as cumulative eggs laid per female fly.

2.2.6 Starvation stress assay

For starvation stress, 100 once mated female flies per genotype and diet were allocated at a density of 20 flies per vial (wide plastic vial). Flies were first kept on SYA, Yaa or Yaa-EAA diets for 7 days and transferred to fresh food vials every 2-3 days. After 7 days, flies were transferred to starvation medium (1% w/v agarose). Dead flies were scored 3-4 times per day.

2.2.7 Larvae food choice assay

Larvae food choice was performed based on the experimental setup described in (Schipanski et al., 2008). Two different diets (Yaa and HUNTaa, 5 ml each) were poured in each half of the petri dish (55 mm diameter), 3-4 h before the assay. Larvae were washed in PBS and starved for 4 h on 1% w/v agarose before the experiment. 15 aged matched third-instar larvae per genotype were placed in the middle of a petri dish containing two different diets in each half. Larvae on each side of the dish were scored hourly for a period of 5 h. Food choice is expressed as a preference index (PI), which was calculated as: PI for food A = (no. of larvae on food A – no. of larvae on food B) / (no. of larvae on food A + no. of larvae on food B) (Schipanski et al., 2008). The PI was calculated for each time-points and mean of these PI was taken as one biological replicate.

2.2.8 Adult food choice assay

For adult food choice assay once mated females flies were starved of amino acids for 3 days on Yaa-all diet. Subsequently, 40 flies per genotype were placed in the apparatus (Figure 5.1), specially designed for the food-choice assay, 14-15 h (overnight) before the start of assay. Measurements were started in the morning and readings were taken hourly for a period of ~8 h. Flies having a direct contact with the food surface were scored. Food choice is expressed as a preference index (PI), which was calculated similarly like larvae PI (see section 2.2.7). The PI was calculated for each time-points and mean of these PI was taken as one biological replicate.

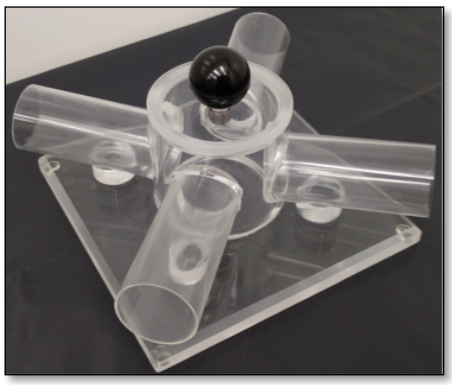


Figure 2.1: Description of the apparatus used for adult food choice assay
The apparatus used for the adult food choice assay is a modified version of that described in (Cooper, 1960). It contains a chamber in the middle connected to four vials. For each assay, choice between two diets were given by placing each set of two vials containing the same diet diagonally opposite to each other. To avoid any light-dependent behaviour or other environmental cues, the apparatus was enclosed in a polysterene container. The apparatus was designed and constructed by Wilfried Haider from the Max Planck Institute for Metabolic Research, Cologne.

2.2.9 Feeding rate: Proboscis-extension assay

For feeding assay newly emerged adults flies were allowed to mate on fresh Yaa medium for 48 h. Subsequently, female flies were sorted and kept at a density of 5 per vial (glass vials) on Yaa medium for a day. 15 h before the assay, flies were transferred to vials containing experimental diets. Next day in the morning (2 h after the lights are switched on), feeding rates were measured using a proboscis-extension assay in undisturbed conditions as previously described (Wong et al., 2009). The flies were observed on 7 consecutive days for a period of 90 minutes/day, with feeding events recorded every 10 min. Feeding data is expressed as a proportion by experimental group (sum of scored feeding events / total number of feeding opportunities, where total number of feeding opportunities = number of flies in vial × number of vials in the group × number of observations). For feeding assay with adult-onset of *ATF4* overexpression by da-GS driver, the entire set-up was the same except that all diets used during the assay were supplemented with 50 µM RU486 (Mifepristone).

2.3 Biochemistry and molecular biology

2.3.1 Triacylglyceride (TAG) measurement

Once mated female flies were kept at the density of 20 per vial (wide plastic vial) on different food conditions for 7 days after which they were snap-frozen. Triacylglyceride (TAG) content quantification was performed according to (Grönke et al., 2003). Briefly, frozen flies were homogenized in 1 ml of 0.05% Tween 20 followed by a heat-inactivation step for 5 min at 70°C and centrifugation at 14000 rpm. 50 µl of the supernatant was incubated with 200 µl of Infinity^{TM} Triglyceride Reagent (ThermoScientific) at 37°C and absorbance was measured at 540 nm. Absolute TAG content was quantified using Triglyceride standards (Cayman Chemicals) and were normalised to the total protein content of the homogenate, determined by using the BCA protein assay reagent (Pierce).

2.3.2 H_2S measurements: Lead sulfide method

For H_2S measurements, 100 once mated female flies per genotype and diet were kept at a density of 20 per vial (wide plastic vial) on experimental diets for 15 days and transferred to fresh food every 2 to 3 days. H_2S measurements were done using lead-sulfide method as described in (Hine et al., 2015). Briefly, 15 days old flies were freshly homogenized in

300 ml of PBS supplemented with passive lysis buffer (Promega), 10 mM cysteine (Sigma) and 10 mM PLP (Sigma). Homogenates were transferred to a 96-well plate and a rectangular lead acetate H_2S detection strip (Fluka Analytical 37104-1EA) was placed over the wells. A layer of thick whatman paper was placed in between the H_2S strip and the cover of the 96-well plate to properly pack the system. The 96-well plate was incubated for 3-5 h at 37°C until spots appeared on the strip. H_2S strips were scanned on ChemiDoc^{TM} MP imaging system and intensity of the spots was quantified using ImageJ software (Scion Software). H_2S levels were normalised to total protein content of the homogenate, determined by BCA protein assay reagent (Pierce).

2.3.3 RNA extraction and qRT-PCR

Prior to RNA-extraction 100 once mated female flies per genotype and diet were kept at a density of 20 per vial (wide plastic vial) on experimental diets for 15 days and transferred to fresh food every 2 to 3 days. Total RNA was extracted from 20 adult flies using standard Trizol-Chloroform based protocol (Invitrogen) according to the manufacture's instructions. cDNA synthesis was done using oligod(T) primer and Superscript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed with TaqMan primers and probes (Applied Biosystems) in a 7900HT real-time PCR system (Applied Biosystems). Relative expression (fold induction) was determined using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) and *Rpl32* as normalization control. Four independent biological replicates per genotype and diet were analysed. TaqMan primer used: Dm01799764_g1 (for *cbs*) and Dm01827112_S1 (for *cgl*).

2.3.4 Genotyping

Genomic DNA was isolated from adult flies using the DNeasy kit (Qiagen). PCR reactions using primer combinations (SOL365/SOL366), (SOL636/SOL637), (SOL639/SOL640) and (SOL336/SOL315) were set up with HotStar Taq Plus master mix (Qiagen) according to the manufacturer's instructions (sequence of primers are listed in Table 2.5). PCR was performed for 35 cycles (30 sec 94°C, 30 sec 55°C and 60 sec 72°C) preceded by 5 min of initial denaturation at 95°C and followed by 10 min final elongation at 72°C. Gel electrophoresis was done with TAE buffered 1% agarose gels.

2.3.5 Northern blotting

2.3.5.1 Cloning of Probe Templates

Using the primer combinations (SOL368/403) for the *Gcn2* 5' region, (SOL404/405) for the *Gcn2* 3' region as well as (SOL326/402) for the upstream flanking gene, *CG31002* and (SOL336/315) for the downstream flanking gene, *CG11337*, PCR fragments of approx. 600 bp using BAC *Gcn2* 12O13 as template were generated. Subsequently those fragments were cloned into the pCRII vector using the TOPO TA Cloning Kit according to the manufacturer's instructions (sequence of primers are listed in Table 2.5).

2.3.5.2 Generation of Probes

Probes were generated by digesting 2 µg of plasmid containing the fragments described above with EcoRI. Probes (25 ng) were random primed using Klenow Fragment (5 U) and 10 µCi [α^{32}P]-dCTP (3000 Ci/mmol) per reaction.

2.3.5.3 RNA extraction and northern blotting

mRNA was extracted using the Dynabeads mRNA Direct Purification Kit from Trizol-Chloroform isolated total RNA (see section 5.3.3). Northern blots were performed using the Northern Max Kit (ThermoFisher Scientific) according to the manufacturer's instructions, using 2 µg mRNA per lane and DNA probes specific to the 5' and 3' region of *Gcn2* as well as for the flaking genes, *CG31002* and *CG11337*. Overnight hybridization was carried out at 42°C with 10^6 cpm/ml probe. For normalization, blots were re-hybridised with a probe detecting ribosomal protein RpL32 transcripts.

2.3.6 Total protein extraction

For protein extraction once mated 10-days old female flies were snap frozen in liquid nitrogen. 20 fly heads per biological replicate were homogenized in RIPA-1% SDS buffer supplemented with Complete mini protease inhibitor without EDTA (Roche) and PhosStop phosphatase inhibitors (Roche). Protein content was determined by the BCA protein assay reagent (Pierce). Before western blot analysis, 20 µg total protein was mixed with 4x SDS loading buffer (950 µl 4x Laemmli sample buffer with 50 µl β-mercaptoethanol as a reducing agent) and boiled for 5 min at 95°C.

2.3.7 Western blotting

Proteins were separated on 12% SDS-PAGE gels (Biorad) and transferred to PVDF membranes (GE Healthcare). After blocking nonspecific binding with 5% nonfat dry milk powder in TBST (0.1% Tween20), blots were incubated with primary antibodies (p-eIF2α - 1:1000 dilution, Abcam, ab32157 and t-eIF2α - 1:5000 dilution, Eurogentec, (Andersen and Leevers, 2007)), washed with TBST and incubated with HRP conjugated anti-mouse or anti-rabbit secondary antibodies (1:10,000 dilution, Invitrogen). Detection was done by chemiluminescence using ECL kits (GE Healthcare) and Hyperfilms (GE Healthcare). For normalization, blots were re-incubated with an antibody raised against β-actin (1:10000 dilution, Abcam, ab8224). Bands were quantified using the ImageJ software (Scion Software).

2.3.8 Mass-Spectrometry (MS)

2.3.8.1 MS Sample preparation

For mass spectrometry (MS) based measurements of TSP metabolites, 100 once mated female flies per genotype were kept at a density of 25 per vial (wide plastic vial) on Yaa medium for 15 days and transferred to fresh food every 2 to 3 days after which they were snap-frozen in liquid nitrogen. MS sample preparation was done using a modified version of the protocol described in (Jiang et al., 2009). Frozen flies were homogenised in 71 µl of 15 mg/ml Dithiothreitol (DTT) and 429 µl 50% methanol (for 25 flies total volume was kept 500 µl with 1 part DTT and 6 parts methanol). Homogenates were vortex-mixed for 5 min and centrifuged at 10,000 rpm at 4°C for 15 min. The supernatant was filtered (0.2 µm VWR centrifugal filters) at maximum speed at 4°C for 5 min. The filtrate was evaporated in a speedvac evaporator at 30°C for ~2h. The pellet was reconstituted into 100 µl of running buffer (5mM ammonium formate, 0.15% formic acid aqueous solution and 100 µg/ml DTT), filtered again and filtrate was immediately frozen at -20 C. Directly before analysis, samples were thawed. DTT was purchased from Biomol, all other chemicals were from Sigma.

2.3.8.2 Mass spectrometry (MS) analysis

For absolute quantification of metabolites (Sigma) in positive ESI MRM (multi reaction monitoring) mode an Acquitiy UPLC™ I-class System / Xevo™ TQ-S (Waters™) with MassLynx and absolute quantification TargetLynx™ (Waters™) was used. With settings for capillary kv 1.5, desolvation temp. 550°C, desolvation gas flow 800 l/h, Cone 150 l/h,

collision gas flow 0.15 ml/min. A SeQuant™ zic™-HILIC Column from Merck 5 µm x 2.1 mm x 100 mm was used at 40°C. Solvent A was 5 mM ammonium acetate (Sigma) + 0.1% formic acid (Biosolve) and B acetonitrile (Biosolve). A gradient from 100% A to 0% in 10 min at a flow rate of 0.35 ml/min and an equilibration step from 10 min to 17 min was used. The following MRM transitions were used as quantifier $(M+H^+)^+$ for L-Cystein hydrochloride 121 m/z to 75.93 m/z, DL-Homocystein 135.84 m/z to 56.05 m/z, L-Methionine 150.03 m/z to 55.99 m/z, Cys-Gly 178.85 m/z to 75.93, L-Cystationine 222.96 m/z to 87.90 m/z, γ-Glu-Cys 250.96 m/z to 83.94 m/z, L-Cystein 122.97 m/z to 107.13 m/z, L-Glutathione reduced 308.02 m/z to 75.92 m/z, SAH 385.10 m/z to 136.38 m/z, SAM 399.10 m/z to 250.03 m/z. Compounds were dissolved in 5 mM ammonium formate + 0.15% formic acid. For all compounds a calibration curve was calculated using following concentrations: 0.5, 1, 2, 4, 10, 20, 40, 60, 100, 150 ng/ml (prepared from stock solutions 100 µg/ml). Correlation coefficient: $r < 0.990$; response type: external standard. The peak integrations were corrected manually, if necessary. Quality control standards of each standard were used during sample analysis and showed between 0.5% and 40% deviation respectively. Blanks after the standards, quality control and sample batch proved to be sufficient. Mass spectrometry was done by Yvonne Hinze of the mass spectrometry core facility of the Max-Planck Institute for Biology of Ageing

2.3.9 RNA-Sequencing

2.3.9.1 Fly preparation and RNA extraction

For RNA-Seq, 3 replicates of 25 once mated female flies per genotype and diet were kept for 3 days or 7 days on the experimental diets and transferred to fresh food every 2 to 3 days after which they were snap-frozen in liquid nitrogen. For RNA-Seq, 4 different diets were used: Yaa, Yaa-M, Yaa-R and Yaa-all. Total RNA was extracted from head and thorax of 25 frozen flies using standard Trizol-Chloroform based protocol (Invitrogen, see section 5.3.3). DNase treatment was done with RNase free DNase kit (Qiagen). RNA quantification was done with the Eppendorf Biophotometer and quality control with the Bio-Rad Experion Automated Electrophoresis System using Eukaryote Total RNA StdSens chips. 2 µg RNA in a total of 20 µl was sent for sequencing.

2.3.9.2 Differential Gene Expression and Ontology Enrichment Analysis

Libraries for sequencing were generated using the polyA purification method. RNA sequencing with 100bp single-end reads and about 35 million reads per sample was

performed on the HiSeq 2500 platform at the Max Planck Genome Center, Cologne, Germany. Bioinformatic analysis was done by the bioinformatic core facility of the Max-Planck Institute for Biology of Ageing. Reads were trimmed for adapter sequences and quality filtered using Flexbar (1). Alignment and assembly was done using the software hisat (2) and StringTie (3). Differential expression analysis was carried out using Cuffdiff (4). Gene ontology enrichment analysis on gene sets of significantly differential regulated genes (adjusted $p < 0.05$) for each pairwise comparison was conducted with the Bioconductor R software package topGO using the elim algorithm (5) and Fisher test.

1. http://www.mdpi.com/2079-7737/1/3/895

2. http://www.nature.com/nmeth/journal/v12/n4/full/nmeth.3317.html

3. http://www.nature.com/nbt/journal/v33/n3/full/nbt.3122.html

4. http://www.nature.com/nbt/journal/v31/n1/abs/nbt.2450.html

5. http://bioinformatics.oxfordjournals.org/content/22/13/1600

2.3.10 Oligonucleotides used in this study

Table 2.5: Oligonucleotides used in this study. Restriction enzymes are specified in brackets.

Primer	Sequence
SOL310	TAGCGGCCGCCCAAGCCAATATACAAAACCATC (NotI)
SOL311	TAGGTACCTTCGGATTTCCCACCGGG (KpnI)
SOL312	TAACTAGTCCAACATCAAATGCATAGAATCGG (SpeI)
SOL313	TAGGCGCGCCGGTTGGAGGAGTGGCTTTTC (AscI
SOL315	TAGGCGCGCCTCTCCACGGATCCGCCCTATT
SOL318	TAGGCGCGCCCAAGCCAATATACAAAACCATC (AscI)
SOL319	TAGGCGCGCCTTCGGATTTCCCACCGGG (AscI)
SOL320	TAGGTACCACGATCCCGCCCAGAG (Acc65I)
SOL321	TAGCGGCCGCTCTCCACGGATCCGCCCTATT (NotI)
SOL336	GAGGAATTCCCGGGATCTTGAGG
SOL365	GCGGATGAAAAGGCCAAGGAAT
SOL366	CCTCCAGAGATATCTTAGGGGGC
SOL368	TGCGGCCAGTTGCTCTACATAA
SOL403	TGTCTACAAAGTCGCGCATCTCGT
SOL326	CGTTAGCAGCCGTATAATTGTAGACC
SOL402	GCTGAGCGGAAACCCTTGTC
SOL404	TCCAGAAGCAGGCGCAGAAGTT

SOL405	CGTTGGCTTGTCGTGGGTGAG
SOL636	GGGGCCCACTTTAGAGGAGGAC
SOL637	CCCGAAATAAGCGCCACAGACG
SOL639	GAGCATTGCGGGAACTTGAAACA
SOL640	CATGGCGGGATTAAACTTCTGCG

2.4 Statistical Analysis

Statistical significance for lifespan and stress assays was assessed by log-rank test performed in Excel (Microsoft). All the other data are presented as mean ± standard error of the mean (SEM). Differences between means were calculated either by two-tailed unpaired *Student's t-test* or one-way ANOVA followed by Tukey's post hoc test or Dunnett's test to compare to a control sample (specified in each experiment), using Graphpad Prism®. p-values <0.05 were considered as statistically significant.

CHAPTER 3

Role of GCN2 kinase in longevity and metabolism under amino acid starvation

Contents

3.1 INTRODUCTION

Accumulating evidence shows an important role of amino acids in mediating longevity and health benefits in diverse species (Fontana and Partridge, 2015). Restriction of dietary methionine and tryptophan extends lifespan in flies (Grandison et al., 2009, Lee et al., 2014a) and rodents (Miller et al., 2005; Orentreich et al., 1993). Restriction of branched-chain amino acids (leucine, isoleucine and valine) improves glucose tolerance and body composition (Fontana et al., 2016). However, the biological pathways through which amino acids mediate these beneficial effects are poorly understood.

The eIF2α kinase, GCN2, senses amino acid starvation and coordinates cellular translation with intracellular amino acid availability. GCN2 gets activated by binding of uncharged t-RNAs, which accumulates in response to amino acid starvation. Activated GCN2 phosphorylates eif2α which inhibits global translation initiation but concomitantly activates translation of specific mRNAs such as the transcription factor ATF4 (Lu et al., 2004; Vattem and Wek, 2004). ATF4 induces expression of its target genes involved in a variety of adaptive functions, including amino acid import and metabolism allowing cells to adapt to amino acid starvation (Harding et al., 2003). In mammals, besides regulating translation, GCN2 activation also initiates important physiological responses such as food intake inhibition (Hao et al., 2005; Maurin et al., 2005) and suppression of hepatic lipid metabolism (Guo and Cavener et al., 2007) during nutritional deprivation of an EAA. Furthermore, several studies suggest that GCN2 activation by pharmacological or dietary means can suppress TOR activity (Gallinetti et al., 2013). In particular, a worm study showed that GCN2 activity is essential to mediate lifespan-extension conferred by down regulation of TOR kinase (Rousakis et al., 2013).

With its function as an evolutionarily conserved amino acid starvation sensor, GCN2 is a potential candidate to be involved in regulation of amino-acid-mediated longevity and important physiological responses. Here, I have analyzed the *in vivo* functions of GCN2 kinase and its downstream effector, ATF4, in modulating longevity and metabolism under limitation or complete deprivation of each of the 10 EAAs. Furthermore, I have tested whether GCN2 and TOR kinase, interact to modulate longevity conferred by TOR-inhibition in flies.

3.2 RESULTS

3.2.1 Generation and characterization of *Drosophila Gcn2* knockout mutants

The *Drosophila Gcn2* gene consists of 10 exons and encodes for a protein of 1589 amino acids. The GCN2 protein contains a degenerate protein-kinase domain (ψK), a typical eukaryotic protein kinase domain (PD) and a histidyl-tRNA synthetase (HisRS)-related domain (DeAnne S Olsen et al., 1998; Santoyo et al., 1997). In order to analyse the *in vivo* function of *Gcn2*, we generated two *Drosophila Gcn2* mutants alleles by ends-out homologous recombination. In the $Gcn2^1$ mutant, the entire *Gcn2* ORF was replaced by a mini-white marker gene, while in the $Gcn2^2$ allele the genomic region covering exon 1 to 7 of the *Gcn2* gene was replaced by a mini-white marker gene (exon numbering according to Flybase data for *Gcn2*-RA transcript). Both mutant alleles lack the PD domain and therefore should be null alleles for *Gcn2* (Figure 3.1A). The knockout mutants were first validated at the DNA and mRNA level. PCR on genomic DNA using primer pairs targeting different regions of the *Gcn2* gene locus confirmed absence of the PD-domain-encoding region in both *Gcn2* alleles and verified the additional absence of the (HisRS)-related-domain-encoding region in the $Gcn2^1$ mutant allele (Figure 3.1B). Northern blot analysis showed no transcript in $Gcn2^1$ mutants but a shorter residual transcript using a probe targeting the 3' region of the *Gcn2* gene in $Gcn2^2$ mutants (Figure 3.1C). This additional band on the Northern blot in $Gcn2^2$ mutants is probably due to ectopic expression of exons 8-10. Expression of the adjacent genes *CG11337* and *CG31002* was not affected by the deletion of *Gcn2* (Figure 3.1C). To check whether the lack of GCN2 would affect phosphorylation of eiF-2α, the only known direct target of GCN2 kinase, we used western blot analysis. Both mutants showed a significant reduction in the phosphorylation level of eif-2α (Figure 3.1D) and there was no difference between the two *Gcn2* alleles. Of note, eiF2α phosphorylation was not completely eliminated in the *Gcn2* mutants, probably because this protein is also phosphorylated by a second *Drosophila* eiF2α kinase termed PEK (Malzer et al., 2013a). For most subsequent experiments I focused on the $Gcn2^1$ knockout mutant, as it is a clear transcript null allele. For control, I used wild type, outbred strain white Dahomey (w^{Dah}) and prior to experiments, *Gcn2* mutants were backcrossed for at least ten generations into w^{Dah} background.

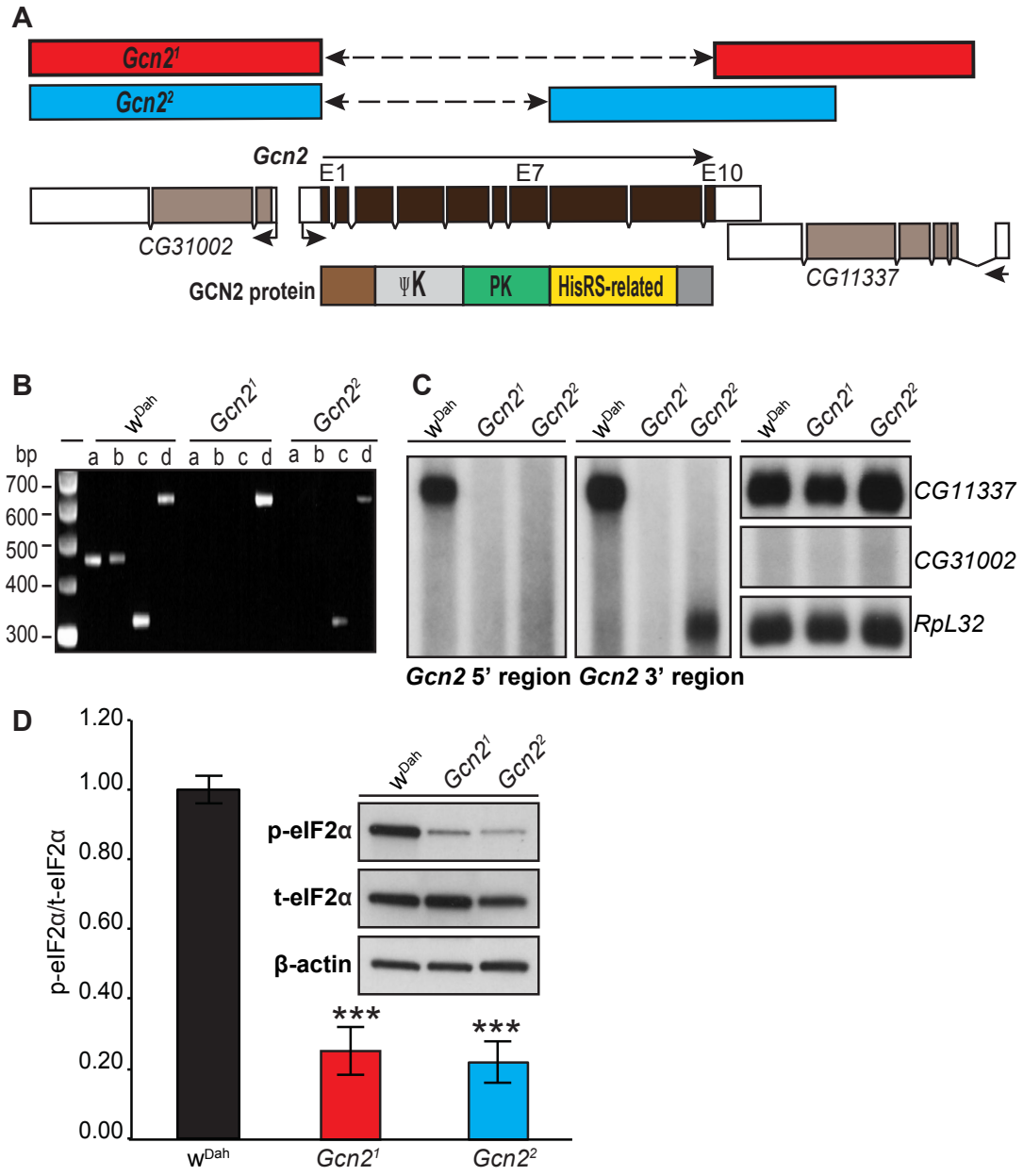


Figure 3.1: Generation of *Drosophila Gcn2* null mutants
(A) Simplified representation of the *Drosophila Gcn2* gene locus based on Flybase data for *Gcn2*-RA transcript and the neighboring genes (*CG31002* and *CG11337*). *Gcn2* mutants were generated by ends-out homologous recombination. In the $Gcn2^1$ mutant, the entire *Gcn2* ORF was replaced by a mini-white marker gene. In the $Gcn2^2$ mutant, the E1 to E7 region was replaced by a mini-white marker gene. **(B)** PCR on genomic DNA with gene-specific primer combinations. Primer pairs used are indicated as: a (putative homologous recombination event), b (exon 5-7), c (exon 8-9) and d (3'short arm). **(C)** Northern blot analysis was performed on the heads of w^{Dah} control flies and *Gcn2* mutants. No *Gcn2* transcript was observed in $Gcn2^1$ mutant. The $Gcn2^2$ mutant showed a shorter band at 3' probably due to the presence of three exons (E8-E10). *RpL32* was used as loading control. The transcripts of the neighboring genes *CG11337* and *CG31002* were unchanged in both mutant alleles. **(D)** Western blot analysis and quantification showed significantly reduced level of eiF2α phosphorylation in both mutant alleles. Results are normalised to both actin and total-eiF2α levels and are expressed relative to levels observed in the w^{Dah} control flies *(Student's t-test: ****p<0.0001, error bar shows SEM). Gcn2 null mutants were generated by Dr. Sebastian Grönke; PCR, Northern and Western Blotting were performed by Oliver Hendrich.*

3.2.2 Role of GCN2 kinase in fly development under EAA limitation

Availability of adequate nutrients has a profound effect during development of an organism. Starvation of EAAs inhibits larval growth and development in *Drosophila* (Britton and Edgar, 1998). We therefore assessed whether GCN2, a sensor of amino acid starvation, is involved in regulating fly development under limitation of EAAs.

3.2.2.1 GCN2 regulates fly development under arginine limitation

First, we studied the development of w^{Dah} control and *Gcn2*^{1} mutant flies under nutrient rich conditions. On our standard sugar-yeast-agarose (SYA) medium, *Gcn2*^{1} mutants showed normal viability, comparable to control w^{Dah} flies (Figure 3.2A). Consistently, using a chemically defined holidic medium, in which the amino acid composition was adjusted according to the SYA medium (referred to as Yaa, Piper et al., 2013), w^{Dah} flies and *Gcn2*^{1} mutants displayed comparable viability with ~80% of eggs developing into adult flies (Figure 3.2B). The developmental timing of *Gcn2*^{1} mutants on Yaa was also comparable to w^{Dah} flies (Figure 3.2C), suggesting that fly development is GCN2-independent under nutrient-rich conditions. To further assess this, we analyzed the development of w^{Dah} control and *Gcn2*^{1} mutant flies on a different holidic medium designed for fly growth and development (referred to as HUNTaa; Piper et al., 2013). On HUNTaa medium, w^{Dah} control flies took longer to develop but showed normal viability comparable to Yaa medium (Figure 3.2B & 3.2C). Surprisingly, viability of *Gcn2*^{1} mutants was dramatically reduced. Less than 10% of eggs developed into adult flies. In order to further confirm this phenotype, we analyzed the development of the *Gcn2*^{2} mutant and of trans-heterozygous *Gcn2*^{1}/*Gcn2*^{2} mutants on Yaa and HUNTaa medium. Similar to *Gcn2*^{1} mutants, viability of homozygous *Gcn2*^{2} mutants and of flies carrying both mutant alleles was normal on Yaa medium but significantly reduced on HUNTaa medium (Figure 3.2D), confirming that the observed developmental phenotype was likely due to the lack of GCN2.

Because flies have two eif2α kinases, GCN2 and (Malzer et al., 2013a) we next tested whether the observed developmental phenotype on HUNTaa medium was specific to GCN2 activation. To this end, we analyzed the development of *PEK* null mutants (generated by Luke Tain, unpublished) on Yaa and HUNTaa medium. *PEK* mutants showed lower viability compared to w^{Dah} flies on both diets (Figure 3.2E). However, viability of *PEK* mutants was not further affected on the HUNTaa medium, confirming

that specifically GCN2 kinase is required for development of flies on the HUNTaa medium.

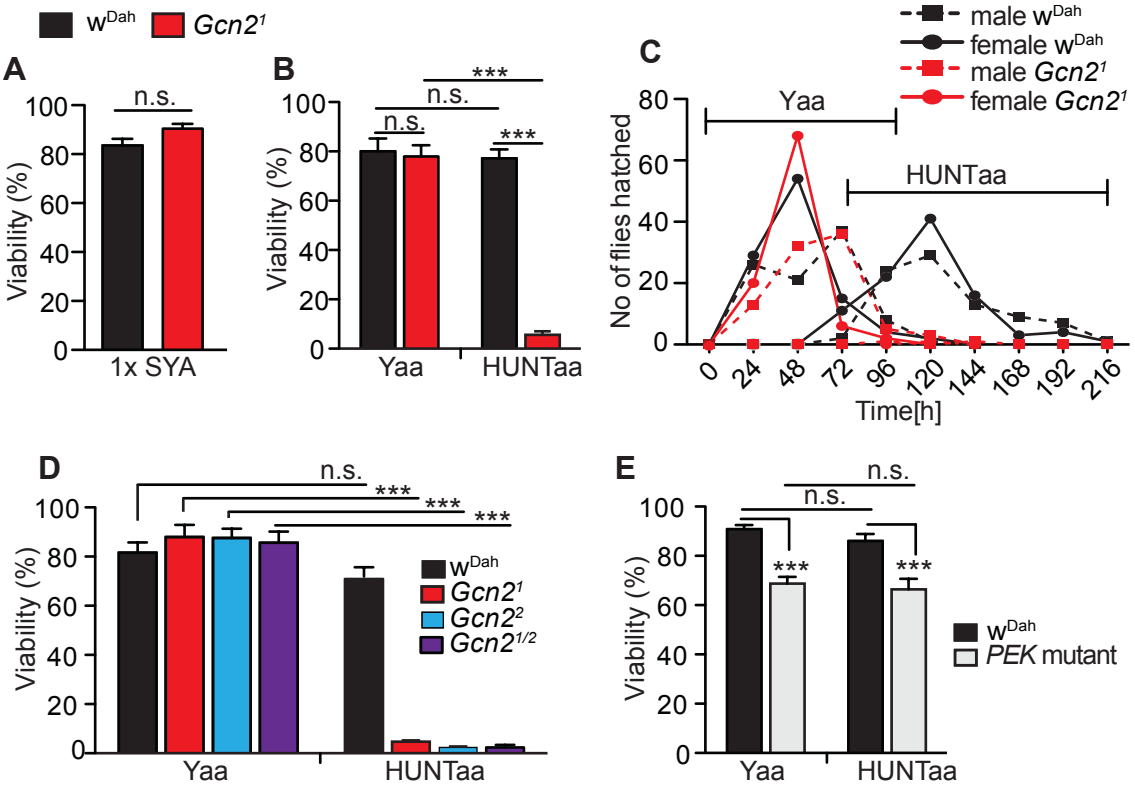


Figure 3.2: GCN2 function is essential for development on HUNTaa medium
(A) w^{Dah} control and *Gcn2*^{1} mutant flies showed comparable viability on SYA medium *(p>0.05, Student's t-test)*. **(B)** On Yaa medium, w^{Dah} control and *Gcn2*^{1} null mutant flies showed comparable viability. On HUNTaa medium, w^{Dah} flies showed normal viability while viability of *Gcn2*^{1} mutants was significantly reduced. **(C)** w^{Dah} control and *Gcn2*^{1} mutant flies were developmentally delayed on HUNTaa medium compared to Yaa medium. **(D)** *Gcn2*^{1} mutants, *Gcn2*^{2} mutants and flies trans-heterozygous for *Gcn2*^{1} and *Gcn2*^{2} (*Gcn2*$^{1/2}$) showed normal viability on Yaa but significantly reduced viability on HUNTaa medium. **(E)** Viability of *PEK* null mutants was comparable on Yaa and HUNtaa medium. Viability (%) represents percentage of eclosed adult flies on a given diet *($^{n.s.}p>0.5$, $^{***}p < 0.001$, one-way ANOVA followed by Tukey's post hoc test; n=10 replicates with 25 eggs each/genotype and diet; error bar shows SEM).*

In order to understand what could cause the difference in survival of *Gcn2*^{1} mutants on the two different holidic mediums, we compared the amino acid composition of Yaa and HUNTaa and found that HUNTaa contains ~70% less arginine (R) than Yaa (Figure 3.3A). We therefore hypothesized that the reduced viability of *Gcn2*^{1} mutants could be attributable to arginine limitation in HUNTaa medium. In order to test this hypothesis, we modified the Yaa diet by reducing the arginine content to 30% (Figure 3.3B). Indeed, viability of *Gcn2*^{1} mutants on YAA-R (30%) was severely reduced and comparable to the

lethality observed on the HUNTaa diet (Figure 3.3D). Consistent with HUNTaa medium, the reduction in arginine on the YAA-R (30%) diet did not affect the viability of w^{Dah} flies (Figure 3.3D). Furthermore, by increasing arginine in HUNTaa medium to the level of the Yaa diet (HUNTaa+R, Figure 3.3C), viability of *Gcn2*^{1} mutants was completely rescued (Figure 3.3D), confirming that arginine limitation was causal for decreased viability of *Gcn2*^{1} mutants.

Addition of arginine to the HUNTaa medium might disturb the amino acid proportion of the diet. Thus, to test whether arginine limitation per se or changes in the amino acid ratio were responsible for the reduced viability of *Gcn2* mutants, we generated a modified HUNTaa medium that had undisturbed amino acid proportion and also increased arginine, by increasing the total amino acid content of HUNTaa medium to 150% (designated as HUNTaa+50% aa). Interestingly, this did not rescue the viability of *Gcn2*^{1} mutants (Figure 3.3E), suggesting that, for GCN2-dependent regulation of fly development, the ratio of the limiting amino acid to the other amino acids in the diet is important rather than the absolute amount of the limiting amino acid. Moreover, we modified HUNTaa medium in order to keep the degree of the disproportion between the most limiting amino acid (arginine) and the most excess amino acid (isoleucine) constant. This was done by increasing isoleucine proportionally to the increase of arginine, which was to the level of Yaa (Figure 3.3F). On this diet viability of *Gcn2*^{1} mutants was not negatively affected (Figure 3.3G), suggesting that disproportion between isoleucine and arginine had no effect on GCN2-mediated viability.

Taken together, our findings suggest that GCN2 is essential for ensuring development of flies under arginine limitation.

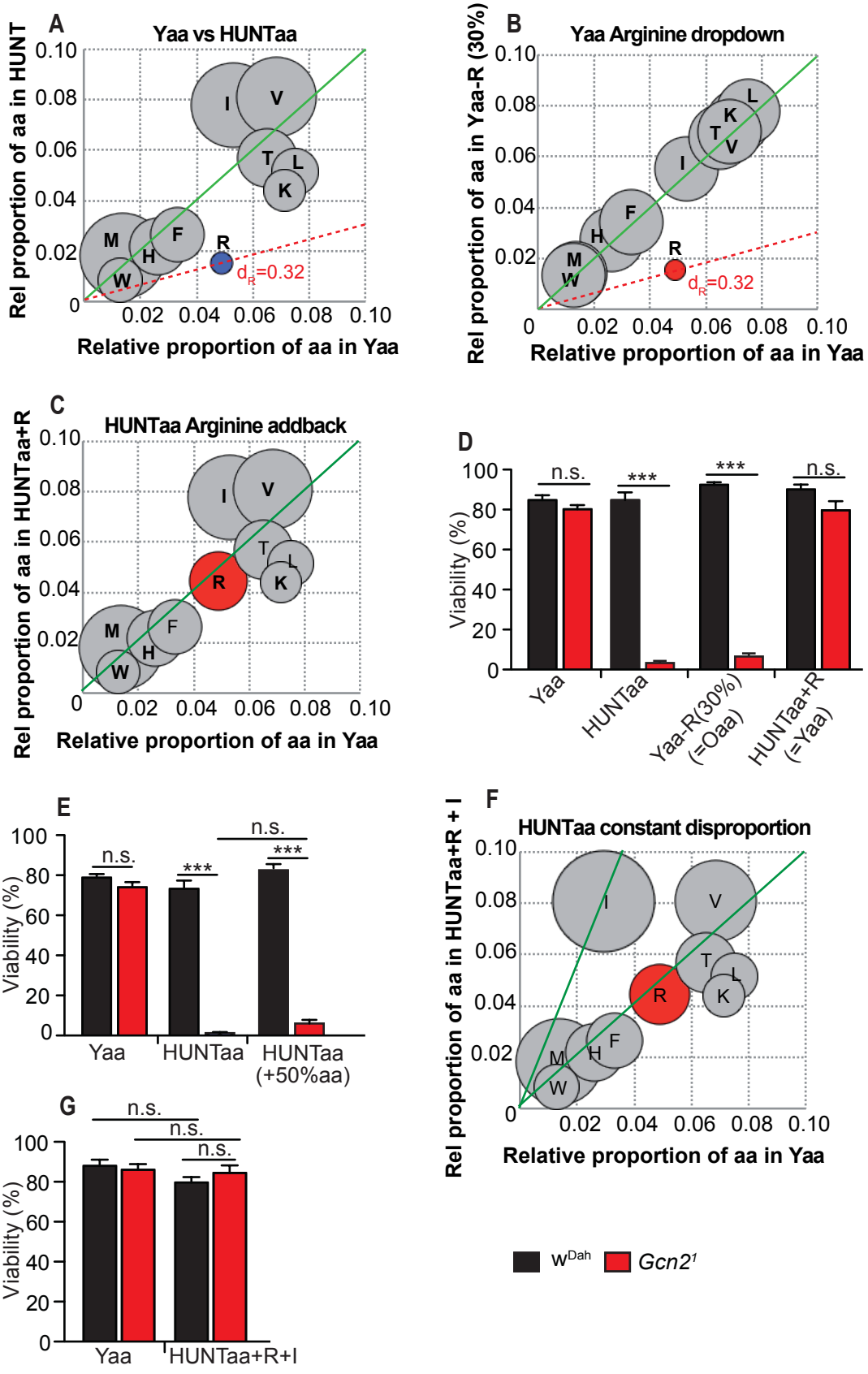

A
Yaa vs HUNTaa
Rel proportion of aa in HUNT
Relative proportion of aa in Yaa
d_R=0.32
B
Yaa Arginine dropdown
Rel proportion of aa in Yaa-R (30%)
Relative proportion of aa in Yaa
d_R=0.32
C
HUNTaa Arginine addback
Rel proportion of aa in HUNTaa+R
Relative proportion of aa in Yaa
D
Viability (%)
n.s.
***
***
n.s.
Yaa
HUNTaa
Yaa-R(30%) (=Oaa)
HUNTaa+R (=Yaa)
E
Viability (%)
n.s.
***
n.s.
***
Yaa
HUNTaa
HUNTaa (+50%aa)
F
HUNTaa constant disproportion
Rel proportion of aa in HUNTaa+R + I
Relative proportion of aa in Yaa
G
Viability (%)
n.s.
n.s.
n.s.
Yaa
HUNTaa+R+I
w^{Dah}
Gcn2^{1}

Figure 3.3: GCN2 function is essential for development under arginine limitation
(A) Relative proportion of EAAs in Yaa and HUNTaa medium. Arginine (R) is the most limiting EAA in HUNtaa medium, ~30% less than in Yaa medium. **(B)** Relative proportion of EAAs in Yaa and Yaa-R (30%). **(C)** Relative proportion of EAAs in Yaa and HUNTaa+R. **(D)** Reducing R to HUNTaa level, Yaa-R (30%), significantly reduced viability of *Gcn2*^{1} mutants. Adding back R to the level of the Yaa medium, Oaa+R, rescued the viability of *Gcn2*^{1} mutants. **(E)** Increasing all amino acids in HUNTaa medium by 50% did not rescue the viability of *Gcn2*^{1} mutants. **(F)** Relative proportion of EAAs in Yaa and HUNTaa+R+I. **(G)** Viability of *Gcn2*^{1} mutants were rescued back to Yaa level on HUNTaa+R+I. Viability (%) represents percentage of eclosed adult flies on a given diet *($^{n.s.}p>0.5$, $***p < 0.001$, one-way ANOVA followed by Tukey's post hoc test; n=10 replicates with 25 eggs each/genotype and diet; error bar shows SEM). Analysis of diets (Figure 3.3A, B, C and F) were done with the help of Dr. George Soultoukis.*

3.2.2.2 GCN2 is essential for fly development under limitation of individual EAAs

Our observation that GCN2 regulates development under arginine limitation, prompted us to investigate whether GCN2 also regulates development under limitation of other EAAs. In order to test this, we took advantage of the holidic medium Yaa, and generated ten different diets by dropping down individual EAAs to 30% of the level in the Yaa diet. Consistent with arginine limitation, individual 30% restriction of all ten EAAs did not affect the viability of w^{Dah} control flies (Figure 3.4A). In contrast, viability of *Gcn2*^{1} mutants was significantly reduced, although to different extents on the individual EAA-limited diets. The two exceptions were limitation of methionine and tryptophan, for which *Gcn2*^{1} mutants showed the same viability as the wild type control flies. However, restriction of methionine and tryptophan, similar to other amino acids, delayed the development of w^{Dah} control flies and *Gcn2*^{1} mutants to a similar extent compared to Yaa diet (Figure 3.4B-3.4L), suggesting that methionine and tryptophan were already limiting for developmental timing.

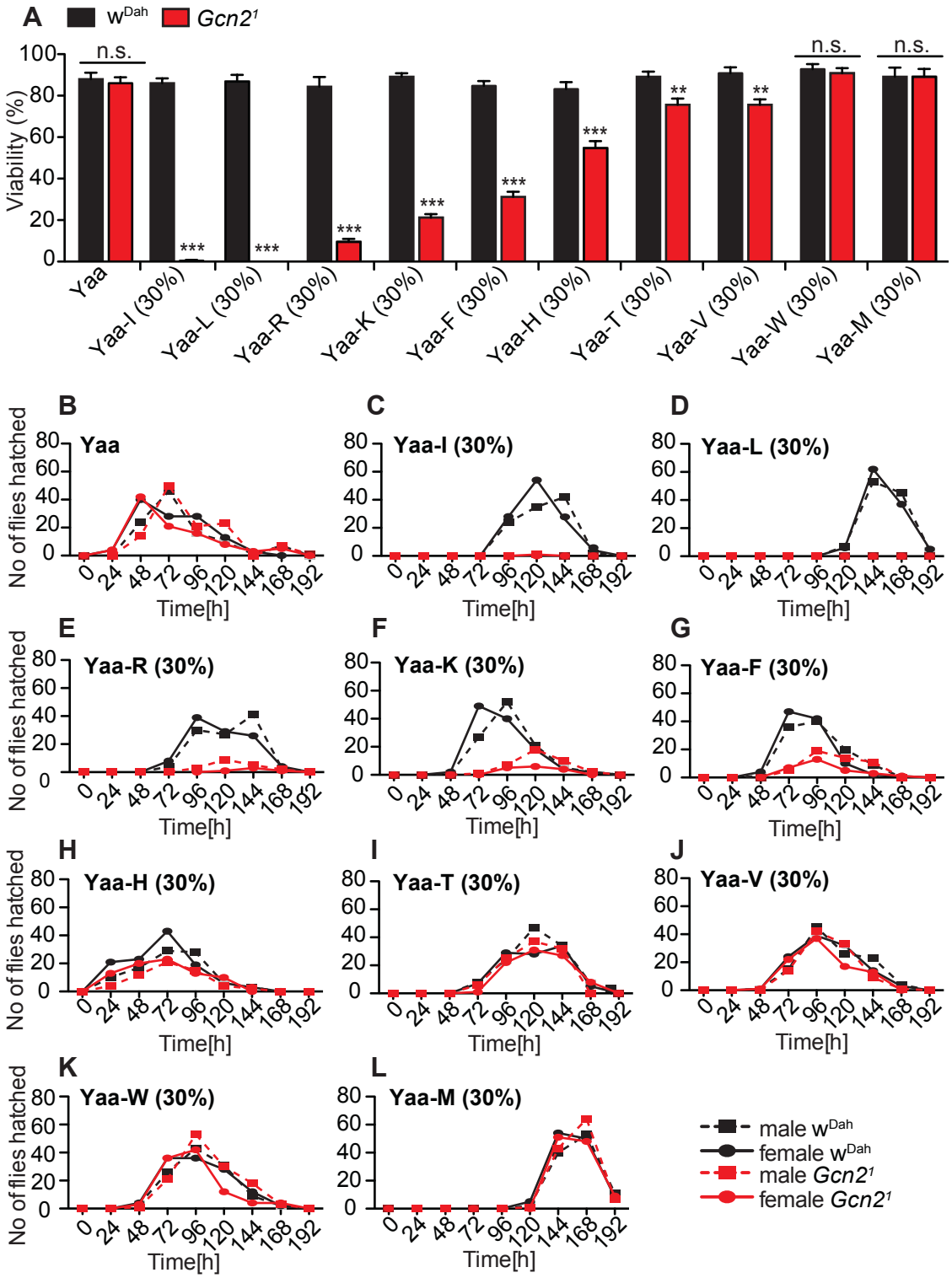


Figure 3.4: GCN2 function is essential for development under EAAs limitation
(A) Dropping down individual EAAs to 30% in Yaa medium did not affect the viability of w^{Dah} flies *(p>0.05, one-way ANOVA)*, however viability of *Gcn2*^{1} mutants was significantly reduced on all EAA drop down diets except on tryptophan (W) and methionine (M) drop-down diets *($^{n.s.}p>0.05$ **$p<0.01$ and ***$p<0.001$, one-way ANOVA followed by Dunnett's post hoc test taking viability of Gcn2^{1} mutant on Yaa as control group)*. **(B-L)** Development timing of w^{Dah} control and *Gcn2*^{1} mutant flies on diets having 30% drop-down of individual EAAs (*error bar shows SEM, n=10 replicates with 25 eggs each/genotype and diet).*

To test whether a stronger limitation of tryptophan and methionine would affect the viability of *Gcn2*^{1} mutants, we repeated the experiment by including diets in which tryptophan and methionine levels were dropped down to 30%, 20% and 10%. Interestingly, this titration experiment revealed that drop-down of tryptophan to 20% significantly decreased the viability of *Gcn2*^{1} mutants without affecting the viability of w^{Dah} controls (Figure 3.5A). Furthermore, drop-down of trytophan to 10% decreased the viability of w^{Dah} flies while *Gcn2*^{1} mutants even failed to develop into larvae. Intriguingly, at no level of methionine drop-down did *Gcn2*^{1} mutants show a reduced viability relative to the w^{Dah} controls (Figure 3.5A). Methionine drop-down to 20% reduced viability and induced developmental delay in both w^{Dah} controls and *Gcn2*^{1} mutants (Figure 3.5A and 3.5B). Methionine dropped down to 10% was lethal for w^{Dah} controls and *Gcn2*^{1} mutants as both of them failed to develop into larvae (Figure 3.5A). Taken together, these findings demonstrate that GCN2 is essential for fly development under limitation of individual EAAs, with the exception of methionine.

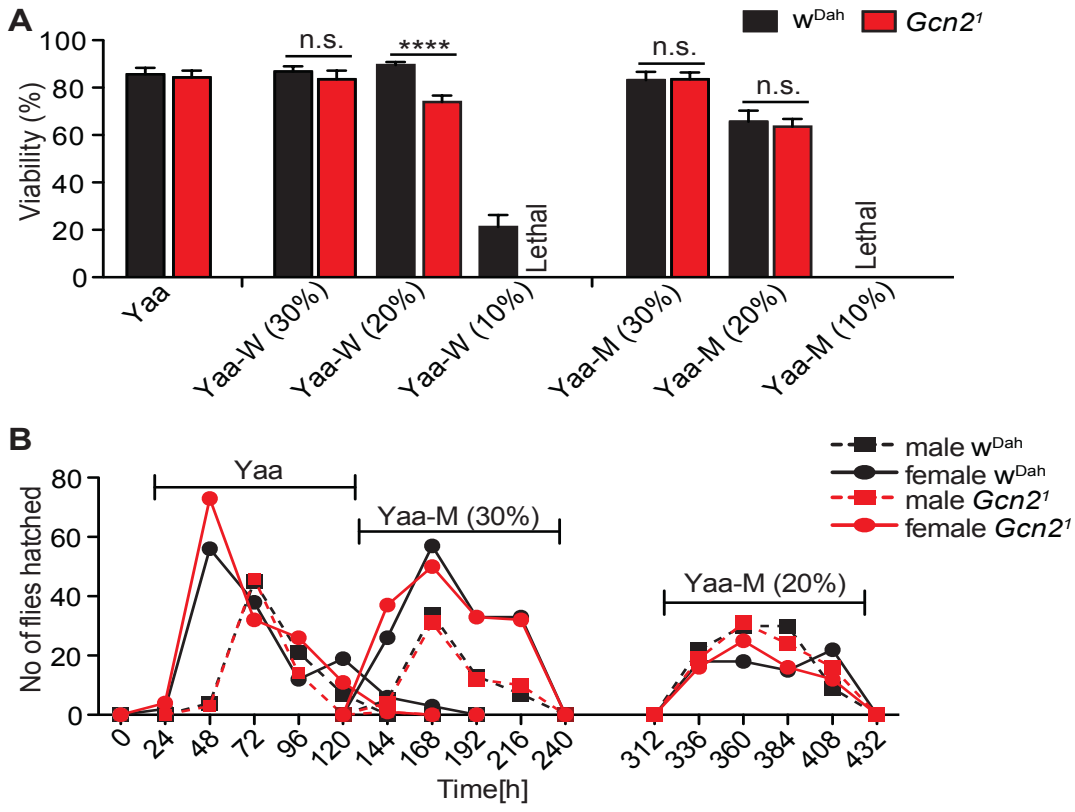


Figure 3.5: GCN2 function is not essential for development under methionine limitation
(A) Reduction of W to 20% significantly reduced the viability of *Gcn2*^{1} mutants. Drop-down of W to 10% was lethal for *Gcn2*^{1} mutants. Reducing M to 20% did not affect the viability of *Gcn2*^{1} mutants compared to control flies. Drop down of M to 10% was lethal for flies (****$p<0.0001$, *Student's t-test).* **(B)** Reduction of M to 30% and 20% delayed the development of w^{Dah} control and *Gcn2*^{1} mutant flies (*error bars show SEM, n=10 replicates with 25 eggs each/genotype and diet).*

3.2.3 Role of GCN2 kinase in lifespan regulation under EAAs deprivation

Reduction of dietary amino acids has been implicated in lifespan extension in diverse species (Fontana et al., 2010). However, the underlying molecular mechanisms and pathways of amino-acid-mediated lifespan-regulation are still not fully understood. Given that GCN2 kinase is a sensor of amino acid starvation we hypothesized that amino-acid-dependent lifespan-regulation could be mediated by this kinase. To this end we analyzed survival of w^{Dah} control and *Gcn2*^{1} mutant female flies in response to amino acid availability.

3.2.3.1 GCN2 modulates fly lifespan under arginine starvation

First, we studied the lifespan of w^{Dah} control female flies and *Gcn2*^{1} mutants under nutrient-rich conditions. Interestingly, *Gcn2*^{1} mutants showed a modest, but significant, increase in median lifespan compared to w^{Dah} flies, on the standard yeast based SYA diet (Figure 3.6A) and on the holidic medium, Yaa (Figure 3.6B, black dotted versus black solid lines). Next, we analyzed survival of w^{Dah} control and *Gcn2*^{1} mutant flies on HUNTaa medium. Survival of w^{Dah} control flies was comparable to that on Yaa medium (Figure 3.6B, orange solid line). However, survival of *Gcn2*^{1} mutants was significantly lower and *Gcn2*^{1} mutants displayed an unusual survival profile on HUNTaa medium (Figure 3.6B, orange dotted line). The mutants first showed an early phase of increased mortality after which they stabilized and stop dying, followed by a second mortality phase that started at about the same time as the control flies started to die on HUNTaa medium. Notably, the start of early mortality of *Gcn2*^{1} mutants on HUNTaa diet was extremely consistent among four independent trials. The first deaths always occurred at ~9 days of age and continued until ~20 days of age. Therefore, we tested whether early mortality could be rescued if flies were put on HUNTaa medium only after 9 days or 20 days of age. To this end, we first kept flies on Yaa medium and later switched them to HUNTaa medium at 10 and 21 days of age. Strikingly, we observed that, irrespective of the age at which *Gcn2*^{1} mutants were subjected to HUNTaa medium, the mortality started 9 days after the switch and continued for the next ~10 days (Figure 3.6C, light-green and purple dotted lines). Furthermore, fecundity of female flies was also affected on HUNTaa medium. w^{Dah} controls displayed significantly reduced egg-laying compared to that on Yaa medium while *Gcn2*^{1} mutants completely ceased their egg-production on HUNTaa medium (Figure 3.6D).

To address whether limitation of arginine in the HUNTaa medium was causal for the increased mortality of *Gcn2*^{1} mutants, we conducted a lifespan analysis on the Yaa-R (30%) diet. Interestingly, *Gcn2*^{1} mutants showed a similar survival profile on the Yaa-R (30%) diet as on the HUNTaa medium (Figure 3.6E, pink dotted line), suggesting that early mortality of *Gcn2*^{1} mutants was caused by arginine limitation. Residual arginine in the diet might explain why some of the *Gcn2*^{1} mutants recovered after early mortality on HUNTaa and Yaa-R (30%). In order to test this, we designed a diet that did not contain any arginine (Yaa-R), and hence should not be able to rescue the early mortality. Indeed, on the Yaa-R diet, *Gcn2*^{1} mutants displayed early mortality but did not show the stabilizing effect as was seen on HUNTaa and Yaa-R 30% diets (Figure 3.6E, dark-green dotted line). As a result *Gcn2*^{1} mutants were extremely short lived on the Yaa-R diet with a median lifespan of 13 days. In contrast, survival of w^{Dah} control flies was only slightly, although significantly, decreased by arginine restriction (Figure 3.6E, dark-green solid line).

Interestingly, when arginine along with other amino acids was restricted to 25% (referred to as Yaa-25%aa), *Gcn2*^{1} mutants showed normal survival, comparable to w^{Dah} control flies (Figure 3.6F), suggesting that the ratio of limiting amino acid to other amino acids is more important for survival of *Gcn2* mutants than the absolute amount of the limiting amino acid, consistent with the development data.

Taken together, our results suggest an important role of GCN2 for survival under arginine deprivation.

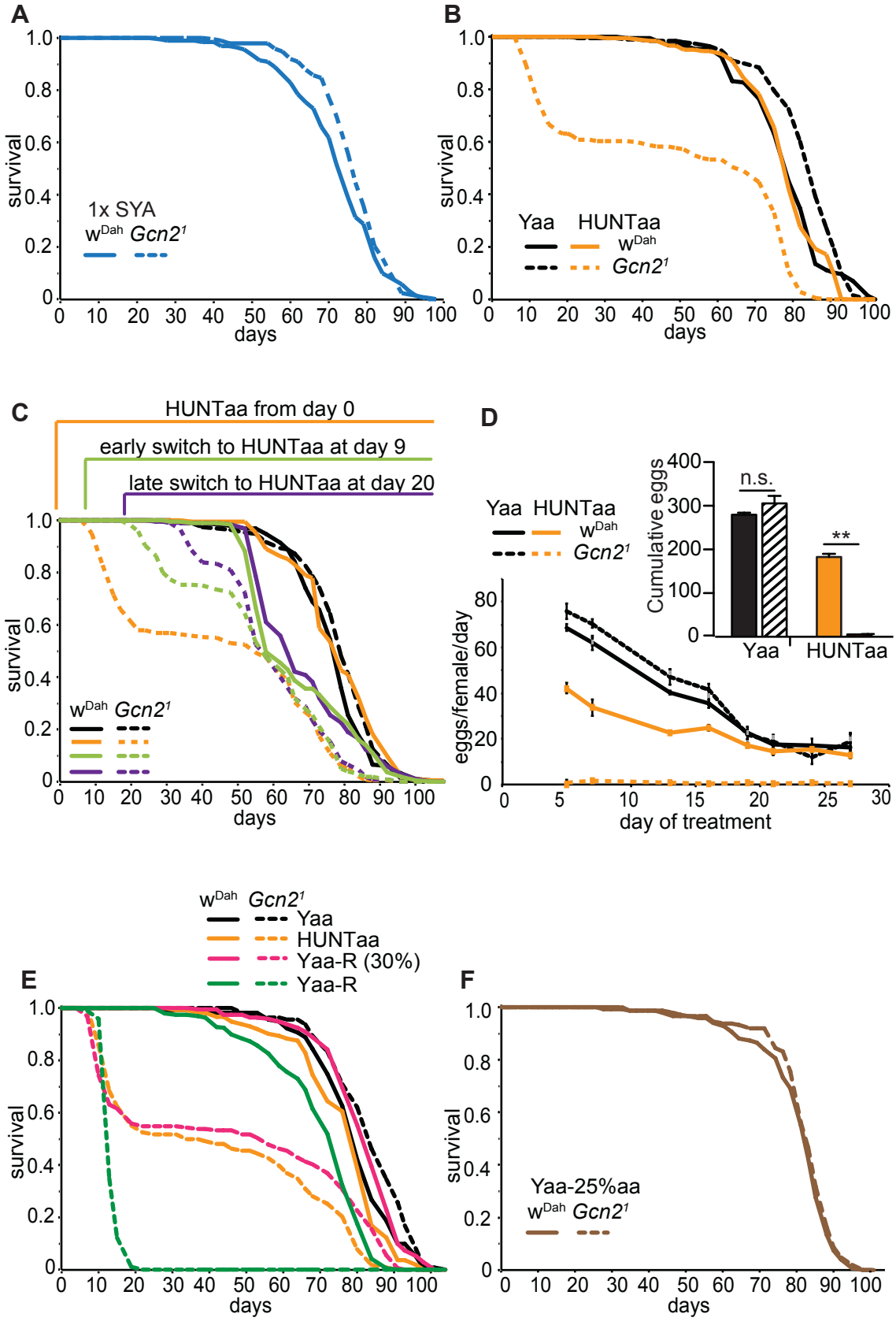

A
survival
1x SYA
wDah Gcn2¹
days
B
survival
Yaa HUNTaa
wDah
Gcn2¹
days
C
HUNTaa from day 0
early switch to HUNTaa at day 9
late switch to HUNTaa at day 20
survival
wDah Gcn2¹
days
D
Yaa HUNTaa
wDah
Gcn2¹
Cumulative eggs
n.s.
**
Yaa
HUNTaa
eggs/female/day
day of treatment
wDah Gcn2¹
Yaa
HUNTaa
Yaa-R (30%)
Yaa-R
E
survival
days
F
survival
Yaa-25%aa
wDah Gcn2¹
days

Figure 3.6: GCN2 function is essential for survival under arginine limitation
(A) *Gcn2*^{1} mutants showed significantly increased median lifespan compared to w^{Dah} control flies on SYA medium *(***p<0.001, log rank test)*. **(B)** *Gcn2*^{1} mutants showed significantly increased median lifespan compared to w^{Dah} control flies on Yaa medium *(***p<0.001, log rank test)*. On HUNTaa medium, the survival curve of w^{Dah} control flies was comparable to Yaa, however *Gcn2*^{1} mutants showed an unusual survival profile. **(C)** Survival curve of w^{Dah} control and *Gcn2*^{1} mutants switched from Yaa to HUNTaa at 9 and 20 days of age. **(D)** Fecundity of *Gcn2*^{1} mutants was comparable to w^{Dah} flies on Yaa medium. On HUNTaa medium *Gcn2*^{1} mutants had significantly reduced fecundity *(**p<0.01, Student's t-test)*. **(E)** On Yaa-R (30%) *Gcn2*^{1} mutants showed a survival profile similar to that on HUNTaa medium. Complete drop out of R (Yaa-R) significantly reduced the median lifespan of w^{Dah} flies compared to Yaa medium *(****p<0.001, log rank test)*. Survival of *Gcn2*^{1} mutants was severely reduced compared to w^{Dah} flies on Yaa-R *(****p<0.0001, log rank test)*. **(F)** w^{Dah} control and *Gcn2*^{1} mutant flies showed comparable lifespan when total amino acid content of Yaa medium was reduced to 25% *(p>0.05, log rank test) (n=200 flies/genotype and diet)*.

3.2.3.2 GCN2 mediates survival in response to deprivation of individual EAAs

Our data demonstrated that GCN2 regulates fly lifespan under arginine deprivation. Therefore, we next investigated whether GCN2 also mediates survival under deprivation of other EAAs. To this end, we modified the holidic medium, Yaa and generated ten different diets, each lacking an EAA. Compared to the control diet Yaa, deprivation of all single EAAs significantly decreased the survival of w^{Dah} flies (Figure 3.7A-3.7J, solid black line versus solid green/red line). However, deprivation of different EAAs decreased the survival of wild type control flies to different extents, e.g. deprivation of methionine caused a strong reduction in median lifespan (median of around 30 days, Figure 3.7J), while median lifespan of w^{Dah} flies on the arginine-deficient diet was about 70 days (Figure 3.7A). Interestingly, in comparison to w^{Dah} control flies, *Gcn2*^{1} mutant flies were significantly shorter lived on nine of the ten EAA-deficient diets (Figure 3.7A-3.7I, solid green line versus dotted green line). Strikingly, on the methionine deficient diet *Gcn2*^{1} mutants were significantly longer lived than w^{Dah} flies (Figure 3.7J and 3.7K), consistent with the results of the development assay.

In addition to single EAA starvation conditions, we also investigated how GCN2 regulate lifespan under complete deprivation of all the amino acids. To this end we designed a diet that was devoid of all the essential and non-EAAs (referred to as Yaa-all). Intriguingly, w^{Dah} control and *Gcn2*^{1} mutant flies behaved similarly and survived for a comparatively long time, with a median lifespan of ~30 days (Figure 3.7L), indicating an involvement of GCN2-independent regulatory mechanisms under total amino acid deprivation.

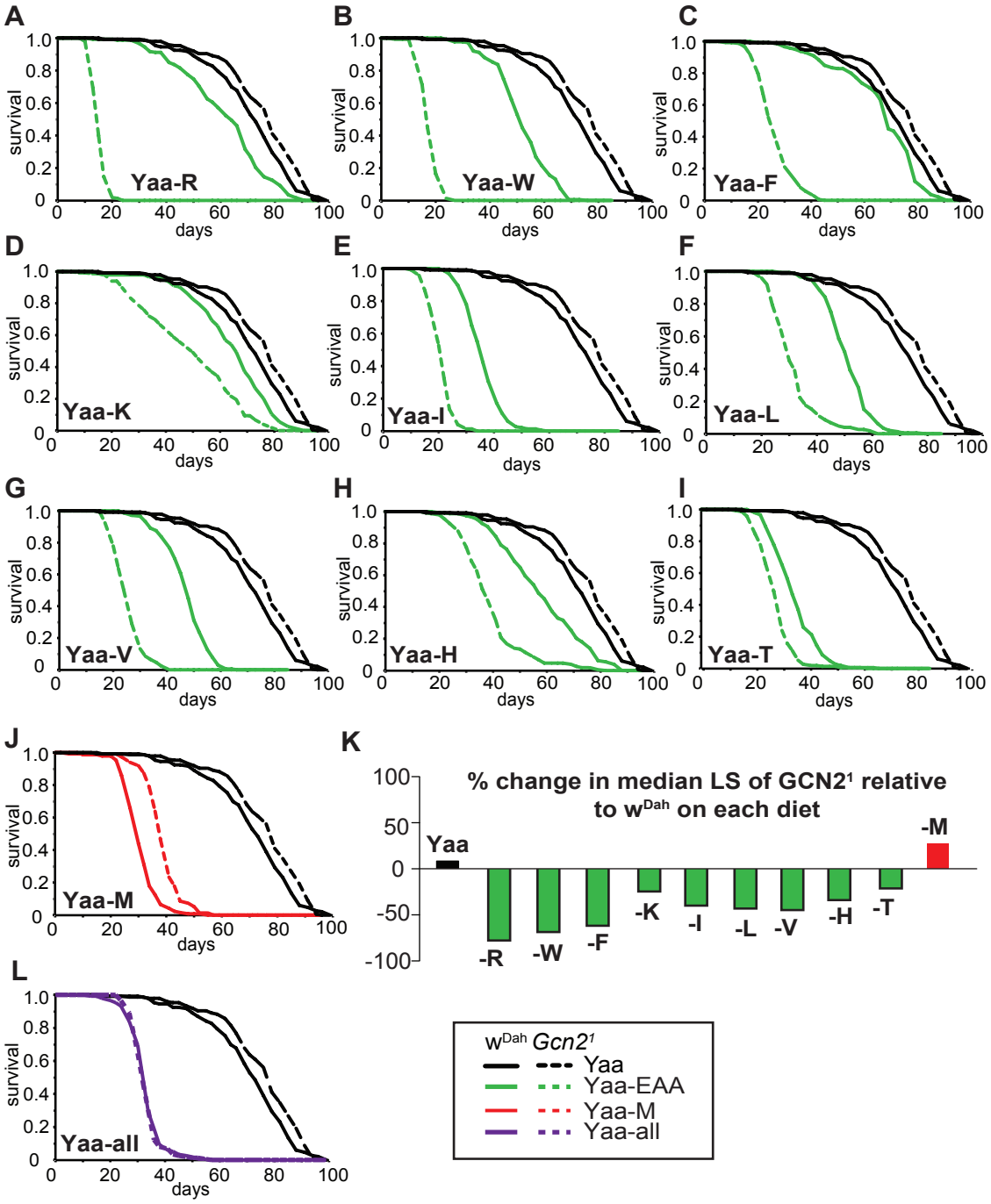


Figure 3.7: **GCN2 mediates lifespan under EAAs deprivation**
(A-J) Complete deprivation of individual EAAs from the control medium, Yaa significantly decreased the lifespan of w^{Dah} flies *(solid black versus solid green lines, ****p<0.0001 for Yaa-R, W, K, I, L, V, H, T, M and **p<0.001 for Yaa-F, log-rank test)* and of *Gcn2*^{1} mutants *(dotted black versus dotted green lines, ****p<0.0001 for all the Yaa-EAA diets, log-rank test)* compared to their respective survival on Yaa medium. **(A-I)** Compared to the survival of w^{Dah} flies on EAAs-deficient diets, survival of *Gcn2*^{1} mutants was severely reduced *(solid green versus dotted green lines, ****p<0.0001, log-rank test)*. **(J)** On Yaa–M diet, *Gcn2*^{1} mutants were significantly longer lived than w^{Dah} control flies *(dotted red versus solid red lines, ****p<0.0001, log-rank test)*. **(K)** Summary of lifespan analysis represented as percentage change in median lifespan of *Gcn2*^{1} mutants relative to w^{Dah} controls on each diet. **(L)** On Yaa-all diet, *Gcn2*^{1} mutants and w^{Dah} flies showed comparable survival *(p>0.05, log-rank test)*. *Lifespan analysis on all the diets was performed in the same lifespan experiment with 150 flies/genotype and diet.*

Flies markedly decrease their egg production if one of the EAAs is missing in the diet (Sang and King, 196). Therefore, we also analyzed fecundity of w^{Dah} control and *Gcn2*^{1} mutant flies on each of the individual EAA-deficient diets (Figure 3.8A and 3.8B). Fecundity of *w*^{Dah} control flies and *Gcn2*^{1} mutants was severely reduced on all the individual EAA dropout diets and egg-production almost stopped at ~8 days of age. While there was no difference in fecundity between *Gcn2*^{1} mutants and wild type control flies on the Yaa control medium, *Gcn2*^{1} mutants had reduced egg production on all individual EAA-deficient diets (Figure 3.8A). In contrast, under full amino acid starvation, fecundity of *w*^{Dah} and *Gcn2*^{1} mutants was also severely reduced but was comparable among each other.

Taken together, these findings demonstrate that GCN2 is an important regulator of survival under deprivation of individual EAAs, although the mechanism by which it regulates survival under methionine-deprivation might be different than for the other EAAs. Furthermore, these results also suggest that full amino acid starvation might act via different mechanisms than deprivation of individual amino acids and is probably independent of GCN2 function.

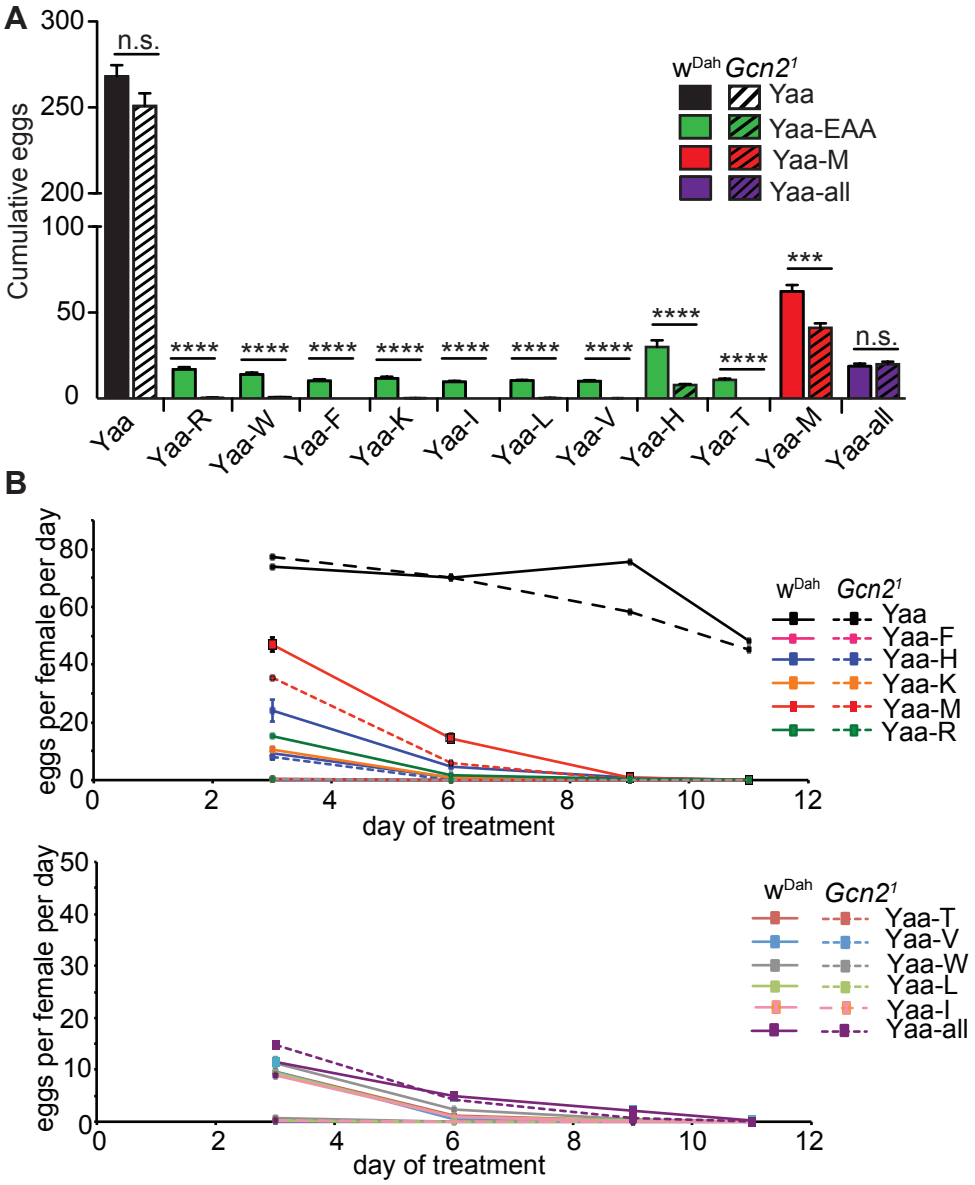


Figure 3.8: **Fecundity of w^{Dah} and *Gcn2*^{1} mutant females on EAA-deficient diets**
(A) Comparison of cumulative egg production of w^{Dah} control and *Gcn2*^{1} mutant female on individual EAA-deficient diets and on Yaa-all. **(B)** Complete deprivation of individual or all EAAs severely reduced the fecundity of w^{Dah} flies and *Gcn2*^{1} mutants to almost zero by ~8 days of age.

3.1.3.3 GCN2 regulates lifespan under EAA deprivation in both genders

All adult survival data presented up to here were done using female flies. To test whether GCN2 is also required in males for survival in response to amino acid availability, we performed lifespan analysis under amino acid starvation using w^{Dah} control and *Gcn2*^{1} mutant males. Consistent with survival of female flies, *Gcn2*^{1} mutant males showed a modest but significant lifespan-extension compared to w^{Dah} males on Yaa medium (Figure 3.9A, black solid versus black dotted lines). Deprivation of arginine on the Yaa-R diet,

which was chosen as a representative diet, resulted in short-lived *Gcn2*^{1} mutant males when compared to w^{Dah} control flies (Figure 3.9A, green solid line versus green dotted line). Interestingly, *Gcn2*^{1} mutant males were longer lived than w^{Dah} male flies under methionine deprivation, in line with survival of female flies (Figure 3.9B). Furthermore, w^{Dah} control and *Gcn2*^{1} mutant males had comparable survival under full amino acid starvation with a comparatively long median lifespan of more than 30 days (Figure 3.9C), again consistent with the survival of female flies.

Thus, GCN2 regulates survival in both male and female flies under deprivation of EAAs. Furthermore, also in male flies survival under full amino acid starvation is GCN2-independent.

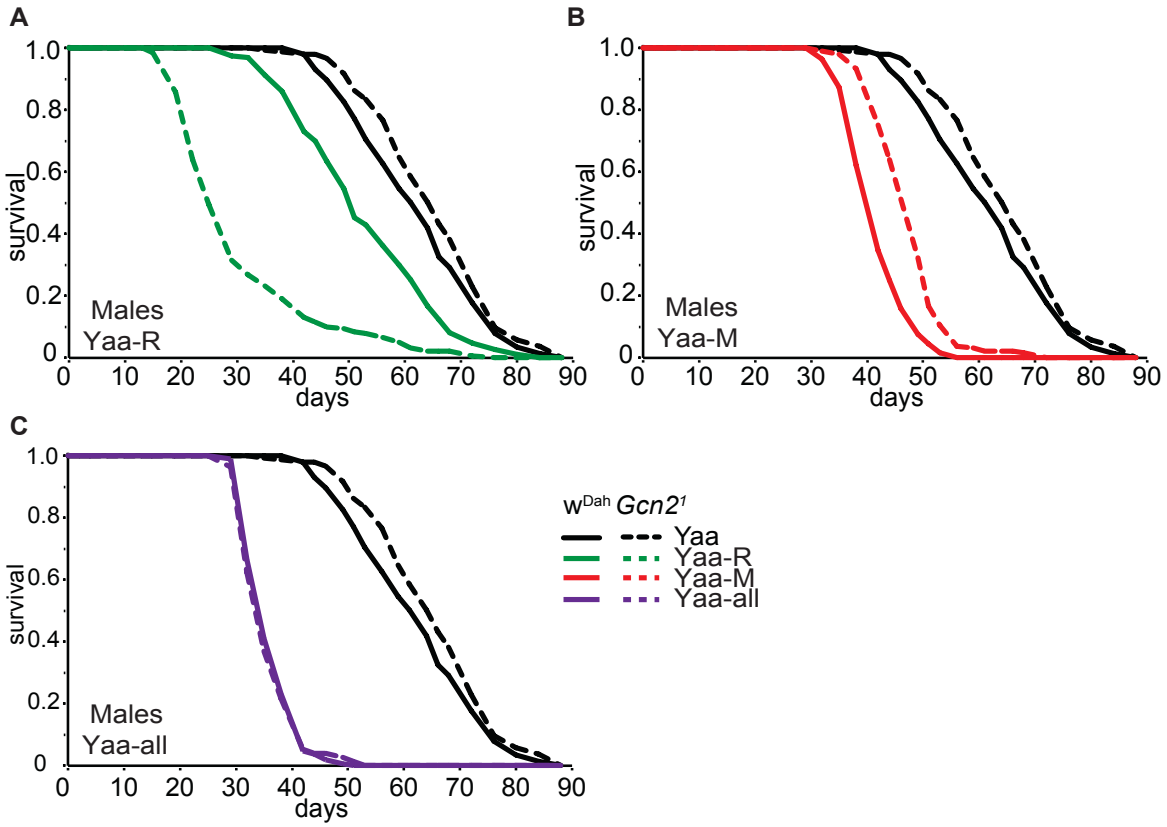


Figure 3.9: **GCN2 is essential for survival under EAAs deprivation in male flies**
(A-C) *Gcn2*^{1} mutants (males) showed significantly increased median lifespan on Yaa compared to w^{Dah} control males *(*p<0.05, log rank test)*. On Yaa-R, Yaa-M and Yaa-all, w^{Dah} control and *Gcn2*^{1} mutant flies showed significantly decreased survival compared to their survival on Yaa *(****p<0.0001, log-rank test)*. **(A)** On Yaa–R, *Gcn2*^{1} mutant males were significantly shorter lived than w^{Dah} males flies *(****p<0.0001, log-rank test)*. **(B)** On Yaa–M, *Gcn2*^{1} mutant males were significantly longer lived than w^{Dah} males flies *(****p<0.0001, log-rank test)*. **(C)** On Yaa-all diet, *Gcn2*^{1} mutant males and w^{Dah} male flies showed comparable survival *(p>0.05, log-rank test)*. *n=150-200 flies/genotype and diet; lifespan analysis of all the diets was performed in the same lifespan experiment.*

3.2.4. GCN2 mediates starvation resistance

A study in mammal reported that GCN2 is involved in the adaptive response of lipid metabolism to an EAA-deficient diet (Guo and Cavener, 2007). We therefore evaluated starvation resistance as a potential indicator of impaired lipid metabolism. First, we tested starvation survival of *Gcn2*^{1} mutants and w^{Dah} female flies after exposure to the yeast-based SYA medium (Figure 3.10A). *Gcn2*^{1} mutants were significantly short lived on the full starvation medium (1% agarose) compared to the wild type control flies. Next we tested starvation survival after a 7-day preconditioning phase on individual EAA deprivation (for details of the experimental set up see Figure 3.10B). The duration of preconditioning on EAA-deficient diets was restricted to 7 days, because at ~9 days *Gcn2*^{1} mutants started dying on the arginine-deficient diet (see Figure 3.7A). *Gcn2*^{1} mutants preconditioned on Yaa medium were mildly, but significantly, starvation sensitive compared to w^{Dah} control flies, consistent with the effect on the SYA diet (Figure 3.10C, black dotted line versus black solid line). Preconditioning on EAA-deprived diets affected the starvation resistance of *w*^{Dah} control flies and *Gcn2*^{1} mutants in an opposite manner. Wild type flies raised on EAA-deficient diets, when transferred to full starvation medium, displayed significantly increased starvation resistance compared to w^{Dah} flies raised on the control Yaa diet (Figure 3.10C-3.10L, solid green line versus solid black line). In contrast, EAA deprived *Gcn2*^{1} mutants showed significantly decreased starvation resistance (Figure 3.10C-3.10K, dotted green line versus dotted black line). Interestingly, methionine was again an exception, because *Gcn2*^{1} mutants preconditioned on a methionine-deprived diet were significantly more starvation resistant, similar to w^{Dah} flies (Figure 3.10L and 3.10M).

Taken together, our findings demonstrate that prior deprivation of an EAA aids in better survival under conditions of full starvation, and this effect seems to be mediated by GCN2 kinase.

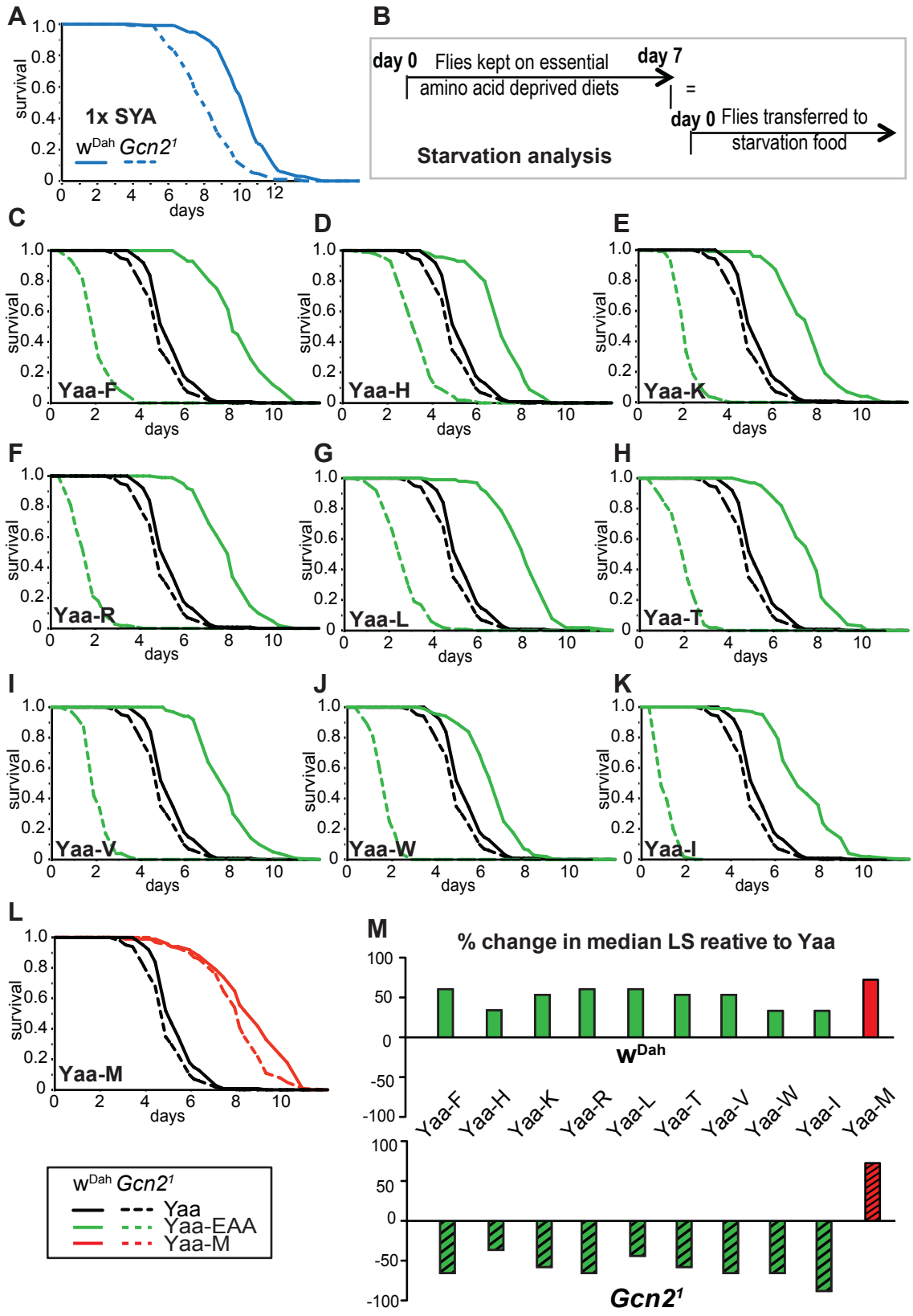

A
survival
1x SYA
w^Dah Gcn2^1
days
B
day 0 Flies kept on essential amino acid deprived diets
day 7
=
day 0 Flies transferred to starvation food
Starvation analysis
C
Yaa-F
D
Yaa-H
E
Yaa-K
F
Yaa-R
G
Yaa-L
H
Yaa-T
I
Yaa-V
J
Yaa-W
K
Yaa-I
L
Yaa-M
w^Dah Gcn2^1
Yaa
Yaa-EAA
Yaa-M
M
% change in median LS reative to Yaa
w^Dah
Gcn2^1
Yaa-F
Yaa-H
Yaa-K
Yaa-R
Yaa-L
Yaa-T
Yaa-V
Yaa-W
Yaa-I
Yaa-M

Figure 3.10: GCN2 increases starvation resistance under normal and individual EAA deprived conditions
(A) *Gcn2*^{1} mutants raised on SYA diet for 7 days were starvation sensitive compared to w^{Dah} control flies *(**p<0.001, log-rank test).* **(B)** Schematic representation of the experimental set up for the starvation-stress assay. Survival under full starvation was measured on *Gcn2*^{1} mutants and w^{Dah} flies, preconditioned for 7 days on diets lacking an EAA. **(C-L)** *Gcn2*^{1} mutants raised on Yaa diet were starvation sensitive compared to w^{Dah} control flies *(solid black versus dotted black lines, **p<0.001, log-rank test).* **(C-L)** w^{Dah} control flies preconditioned on an EAA-deficient diet were significantly starvation resistant in comparison to the control flies preconditioned on Yaa diet *(solid green/red versus solid black lines, ****p<0.0001, log-rank test).* **(C-K)** *Gcn2*^{1} mutants preconditioned on EAA-deficient diets were significantly starvation sensitive in comparison to mutants preconditioned on Yaa diet *(dotted black versus dotted green lines, ****p<0.0001, log-rank test),* **(L)** with the only exception of methionine-deprived mutants, which showed increased starvation resistance *(dotted black versus dotted red lines ****p<0.0001, log-rank test).* **(M)** Summary of starvation stress represented as percentage change in median lifespan under full starvation of w^{Dah} control and *Gcn2*^{1} mutant flies preconditioned on a Yaa-EAA diet relative to their median lifespan under full starvation when preconditioned on Yaa diet *(n = 100 flies/genotype and diet).*

3.2.5 GCN2 regulates lipid storage in response to EAA deprivation

GCN2 suppresses hepatic lipid synthesis in response to leucine deprivation in mice (Guo and Cavener, 2007). Therefore, we hypothesized that GCN2 might affect energy storage under EAA starvation, which could underlie the increased resistance of w^{Dah} control flies under full starvation medium. To this end, we measured triacylglycerol (TAG) storage in w^{Dah} and *Gcn2*^{1} mutant flies after 7 days of preconditioning on the EAA-deficient diets (Figure 3.11A). Interestingly, the starvation resistance of w^{Dah} control and *Gcn2*^{1} mutant flies on EAA-deficient diets correlated well with the TAG levels on EAA-deficient diets. On each of the EAA-deficient diets, *Gcn2*^{1} mutants showed significantly lower TAG levels as compared to w^{Dah} flies (Figure 3.11B), indicating that activated GCN2 in w^{Dah} flies is required for increased TAG levels and thus increased starvation resistance. Furthermore, we analyzed how TAG levels on EAA-deficient diets changed relative to TAG levels on Yaa diet for w^{Dah} control and *Gcn2*^{1} mutant flies. In w^{Dah} flies, TAG levels on EAA-deficient diets were higher compared to those on the Yaa diet (Figure 3.11D). This finding is consistent with the increased survival of w^{Dah} flies under full starvation when previously deprived of an EAA (see Figure 3.10C-3.10L). In contrast, *Gcn2*^{1} mutants had lower TAG levels on EAA-deficient diets compared to the Yaa diet (Figure 3.11D), in line with their decreased survival on full starvation when previously fed an EAA-deficient diet (see Figure 3.10C-3.10K). Remarkably, under methionine deprivation, TAG levels of *Gcn2*^{1} mutants were higher than TAG levels on the Yaa diet (Figure

3.11D), consistent with the increased resistance of Yaa-M fed *Gcn2*^{1} mutants under full starvation (see Figure 3.10L).

Thus, our results indicate that upon starvation of an EAA, GCN2 function results in increased TAG levels in flies, which is probably causal for the increased resistance under full starvation.

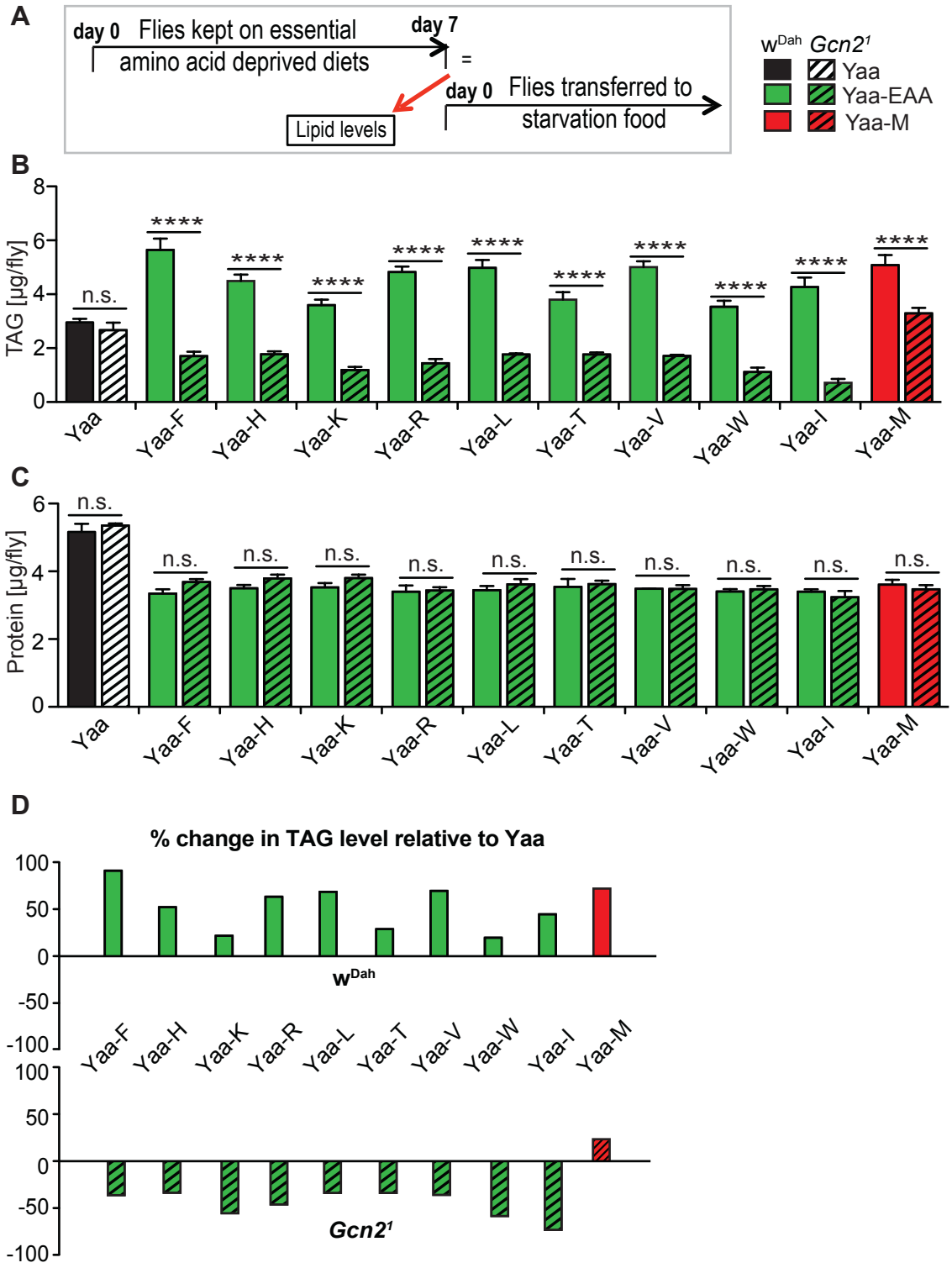


Figure 3.11: GCN2 regulates lipid levels in response to an EAA starvation
(A) Triacylglycerol (TAG) level was measured at the start of full starvation in *Gcn2*^{1} mutants and w^{Dah} flies, preconditioned for 7 days on diets lacking an EAA. **(B)** TAG levels of w^{Dah} and *Gcn2*^{1} mutants on Yaa and Yaa-EAA diets *(****p<0.0001, Student's t-test)*. **(C)** Protein level of w^{Dah} control and *Gcn2*^{1} mutant flies on Yaa and Yaa-EAA diets. On each diet, *Gcn2*^{1} mutants and w^{Dah} flies had comparable protein content *($^{n.s.}$p>0.05, Student's t-test)*. **(D)** Percentage change in TAG level on Yaa-EAA relative to Yaa *(n=4 replicates/ genotype and diet with 5 flies per replicate; error bar shows SEM)*.

3.2.6 GCN2 regulates feeding behavior in response to EAA deprivation

Manipulations of dietary amino acids can alter feeding behavior (Morrison et al., 2012; Toshima and Tanimura, 2012). However, the role of GCN2 in such responses is presently debatable (Leib and Knight, 2015; Maurin et al., 2005). Therefore, we assessed how deprivation of individual EAAs affects feeding in w^{Dah} control flies and *Gcn2*^{1} mutants. We speculated that an altered feeding behavior could be causal for the changed lipid levels under EAA starvation.

We first performed a two-choice preference assay with larvae, by presenting a choice between Yaa (control diet) and HUNTaa medium (limited for developmental timing and egg-laying). Both w^{Dah} control and *Gcn2*^{1} mutant larvae displayed a significantly stronger preference for Yaa over HUNTaa (Figure 3.12A). Next we performed a food preference assay on adult flies. Both w^{Dah} flies and *Gcn2*^{1} mutants preferred Yaa to HUNTaa medium (Figure 3.12B and 3.12C), consistent with the observation in larvae. We further tested the preference of adult flies for an EAA-deficient diet (Yaa-T). w^{Dah} control flies as well as *Gcn2*^{1} mutants preferred Yaa over Yaa-T (Figure 3.12B and 3.12C). Surprisingly, a methionine deficient diet appeared more attractive to w^{Dah} control and *Gcn2*^{1} mutant flies, as they preferred it to Yaa medium (Figure 3.12B and 3.12C). In summary, *Gcn2*^{1} mutants are able to distinguish an amino acid deprived medium from a control diet and show similar preferences to wild type flies. These findings suggest that GCN2 is not required for short-term sensing of the amino acid composition of the food.

A two-choice preference assay evaluates the comparative preferences between the presented diets. We next measured the amount of food intake under no-choice conditions. We assessed the feeding rate on the EAA-deficient diets by performing proboscis extension assays over a period of 7 days (we first tested arginine-, leucine- and methionine-deprived diets). Flies were transferred to the test diet 15 hours prior to the first observation (represented as day 1, Figure 3.12D). On the control diet Yaa, w^{Dah} control and *Gcn2*^{1} mutants showed comparable feeding rates over the whole observation period of seven days (Figure 3.12D, black solid versus black dotted lines). Interestingly, on EAA-deficient diets, w^{Dah} flies significantly increased their feeding rate from day 3 onwards. Strikingly, *Gcn2*^{1} mutants massively reduced their feeding rate on Yaa-R and Yaa-L diets. However, on a methionine deficient diet, *Gcn2*^{1} mutants significantly increased their feeding rate, similar to w^{Dah} flies.

Importantly, based on our observations, feeding response under EAA starvation could be divided into two phases: 1) Aversion phase: the first 15 hours (day 1) during which w^{Dah} control and *Gcn2*^{1} mutants both had reduced feeding under amino acid deprived conditions compared to Yaa diet. 2) Adaptation phase: starting from day 2 onwards during which w^{Dah} flies increased their feeding rate and *Gcn2*^{1} mutants further decreased their feeding rate. Because differences in feeding behavior during adaptation phase were evident from day 2 onwards, for further analysis we chose one time point (day 4) as a representative of this phase. Next we examined the feeding rate of w^{Dah} control and *Gcn2*^{1} mutant flies on the other individual EAA-deficient diets at day 4 (Figure 3.12E). Consistent with our observations on arginine- and leucine-deficient diets, *Gcn2*^{1} mutants showed a significant decrease in proboscis extension, while w^{Dah} flies showed a significant increase on most of the EAA-deficient diets. The only exception was the tryptophan-deficient diet (Yaa-W), on which feeding rate of w^{Dah} flies were comparable to Yaa but not increased. Interestingly, increased feeding rate of w^{Dah} flies and reduced feeding rate of *Gcn2*^{1} mutants on EAA-deficient diets was in line with their observed lipid levels (see Figure 3.11B). Additionally, we also examined the feeding rate under complete amino acid deprivation (Yaa-all). Feeding rate was significantly reduced for both w^{Dah} control and *Gcn2*^{1} mutant flies throughout the 7 days, and not different from each other (Figure 3.12D, purple solid and purple dotted lines).

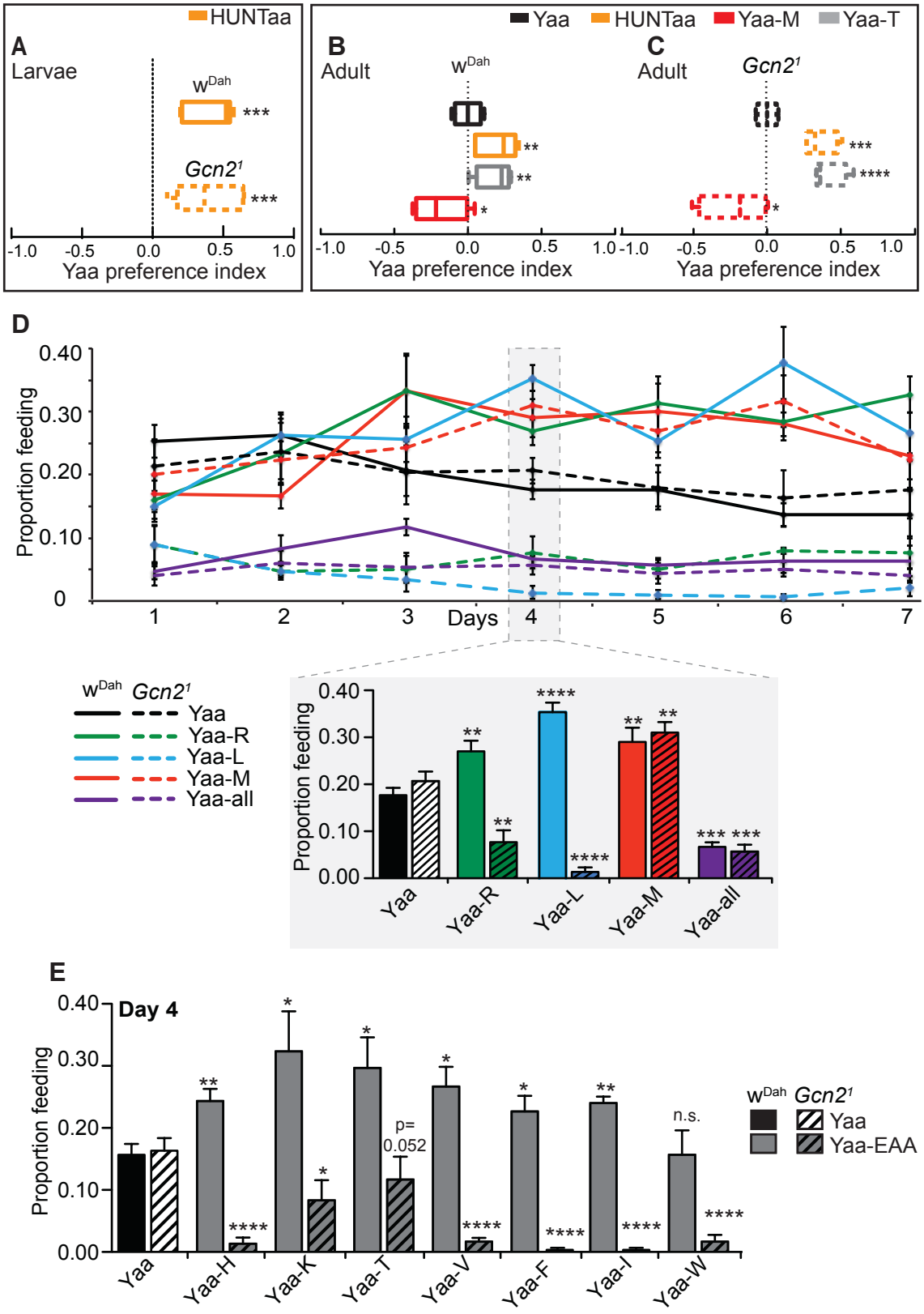
Taken together our findings suggest that:

a) The mechanisms involved in the perception of and preference for amino acids in the food are GCN2-independent in flies.

b) GCN2 regulates feeding rate of flies in response to a physiological deficit of EAAs as a result of the consumption of EAA-deficient diets for more than 24 hours.

c) Feeding rates on a methionine-deficient diet are GCN2-independent

d) Feeding rates under full amino acid starvation are GCN2-independent.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Larvae
HUNTaa
wDah
Gcn21
Yaa preference index
B
Adult
Yaa
HUNTaa
Yaa-M
Yaa-T
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D
Proportion feeding
Days
wDah Gcn21
Yaa
Yaa-R
Yaa-L
Yaa-M
Yaa-all
E
Day 4
Yaa-H
Yaa-K
Yaa-T
Yaa-V
Yaa-F
Yaa-I
Yaa-W
p= 0.052
n.s.
Yaa-EAA

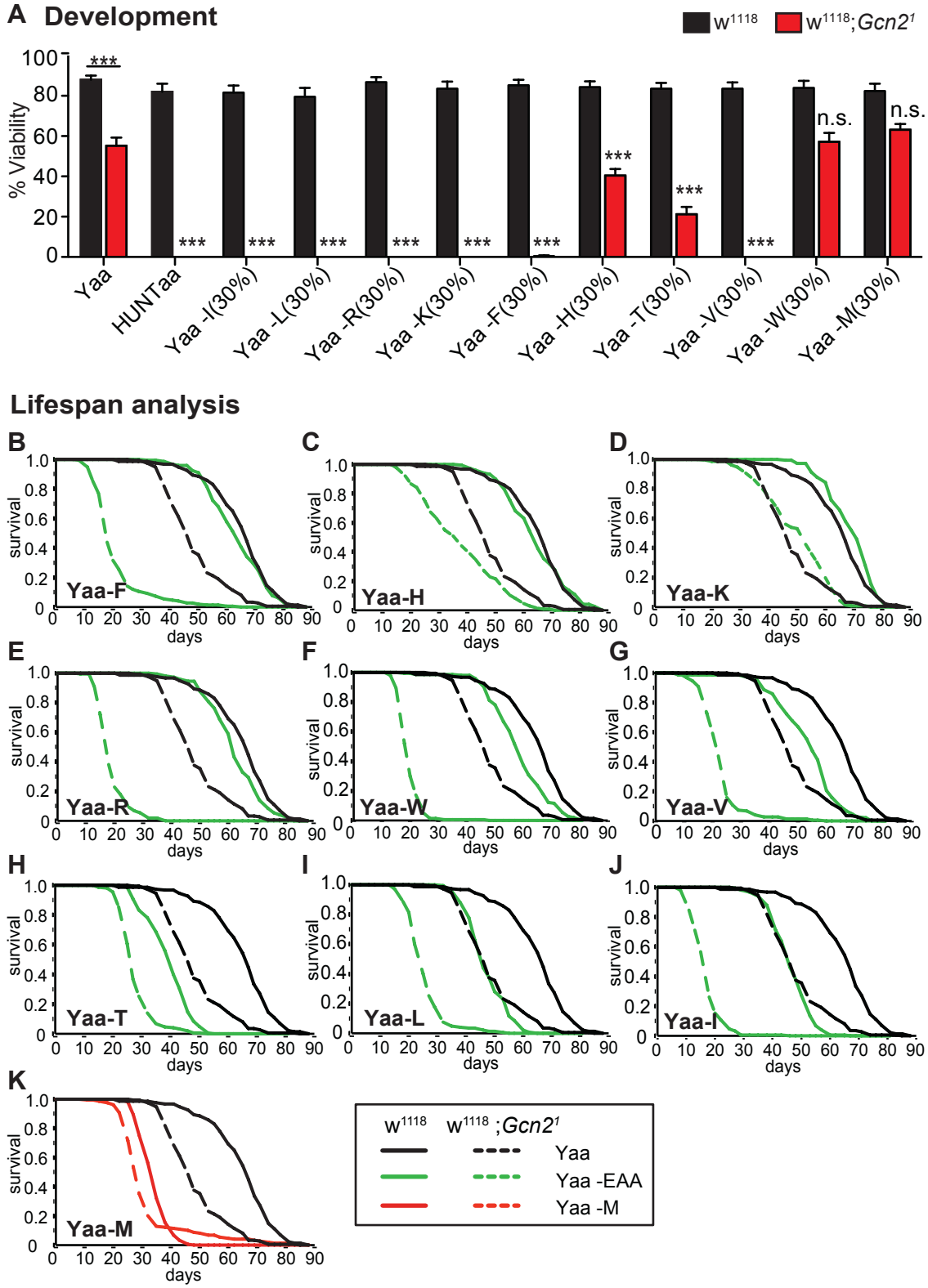
Figure 3.12: GCN2 regulates feeding rate under EAA deprivation
(A-C) Food choice assay was performed on w^{Dah} control and *Gcn2*^{1} mutant **(A)** larvae and **(B & C)** adult flies. Color of the box represents the specific diet offered against Yaa. Food choice is depicted as preference index for Yaa over the presented diet. Each bar represents 3-6 biological replicates, with 40 females per replicate *(*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, Student's t-test, n for larvae = 3 biological replicates with 15 larvae/genotype, for adult flies each bar represents 3-6 biological replicates, with 40 females per replicate).* **(D)** Feeding rates of w^{Dah} control and *Gcn2*^{1} mutant flies observed over a period of 7 days on individual and all EAAs-deficient diets. On Yaa, the feeding rate of w^{Dah} control and *Gcn2*^{1} mutant flies was comparable (*solid black versus dotted black lines)*. On Yaa-all diet, both w^{Dah} control and *Gcn2*^{1} mutant flies showed severely reduced feeding rate (*solid purple versus dotted purple lines)*. On Yaa-R and Yaa-L, w^{Dah} flies showed an increase in feeding rates from day 2 onwards whereas feeding rate of *Gcn2*^{1} mutants was severely reduced (*solid green and blue versus dotted green and blue lines respectively)*. On Yaa-M diet, both w^{Dah} control and *Gcn2*^{1} mutant flies showed an increase in feeding rates (*solid red versus dotted red lines)*. Inset shows the feeding rate at day 4. **(E)** Feeding rates of w^{Dah} control and *Gcn2*^{1} mutant flies on day 4 for individual EAAs deficient diets. w^{Dah} flies significantly increased their feeding rate on EAA-deficient diets (except on Yaa-W). *Gcn2*^{1} mutants significantly decreased their feeding rate on EAA-deficient diets *(*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, Student's t-test; n=6 replicates/genotype and diet with 5 flies per replicate).*

3.2.7 GCN2 function under amino acid deprivation in an independent genetic background

Genetic background can have profound effects on physiological phenotypes like development, starvation and lifespan, and phenotypes of specific mutants might only be restricted to one genetic background (Toivonen et al., 2007). All experiments presented up to now were done in the outbred w^{Dah} background. To test whether *Gcn2* mutants would show similar phenotypes in response to amino acid deprivation in an independent genetic background, we backcrossed *Gcn2*^{1} mutants into a frequently used inbred w^{1118} strain. We first tested development of w^{1118} control and w^{1118};*Gcn2*^{1} mutant flies under limitation of single EAAs (Figure 3.13A). Interestingly, viability of w^{1118};*Gcn2*^{1} mutants was significantly reduced compared to w^{1118} flies on the Yaa control diet, suggesting that lack of GCN2 has more severe consequences in the w^{1118} background. w^{1118} control flies on HUNTaa medium and on individual EAAs drop-down diets showed normal viability comparable to Yaa medium (Figure 3.13A). w^{1118};*Gcn2*^{1} mutants displayed significantly reduced viability on HUNTaa medium compared to Yaa and on most of the EAAs drop-down diets with the exception of tryptophan and methionine drop-down diets (Figure 3.13A). Thus, as in the w^{Dah} background, limitation of methionine did not negatively affect the viability of *Gcn2*^{1} mutant flies in the w^{1118} background (see Figure 3.4A). For most EAA drop down diets the effect on viability was more severe than in the w^{Dah} background (compare Figure 3.13A with 3.4A), consistent with the hypothesis that lack of

GCN2 has more severe consequences in the w^{1118} background compared to the w^{Dah} background.

Next, we analyzed lifespan of w^{1118} control and w^{1118};*Gcn2*^{1} mutant flies under deprivation of single EAAs. w^{1118};*Gcn2*^{1} mutants were significantly shorter lived on the Yaa control diet compared to w^{1118} control flies (Figure 3.13B, black dotted line versus black solid line). Thus, as with viability during development, mutation of GCN2 has detrimental consequences for survival in the w^{1118} background. Furthermore, unlike w^{Dah} control flies, whose survival was significantly reduced on all of the EAA-deficient diets (see Figure 3.7A-3.7J), w^{1118} control flies displayed reduced survival compared to Yaa diet only on 7 of the 10 EAA-deficient diets including –R, -W, -V, -T, -L -I and -M (Figure 3.13E-3.13K). In general, EAA deprivation affected the survival of w^{1118} flies to a lesser extent than survival of w^{Dah} flies. However, consistent with the survival of *Gcn2*^{1} mutants in the w^{Dah} background (see Figure 3.7A-3.7I), survival of w^{1118};*Gcn2*^{1} mutants compared to w^{1118} flies was significantly decreased on all individual EAA-deficient diets (Figure 3.13B-3.13K, dotted green line versus solid green line). Noteworthy, under methionine deprivation, median lifespan of w^{1118};*Gcn2*^{1} mutants was significantly reduced compared to w^{1118} flies, but maximum lifespan was increased (Figure 3.13K).


A Development
w1118
w1118;Gcn2¹
% Viability
100
80
60
40
20
0
***
n.s.
Yaa
HUNTaa
Yaa -I(30%)
Yaa -L(30%)
Yaa -R(30%)
Yaa -K(30%)
Yaa -F(30%)
Yaa -H(30%)
Yaa -T(30%)
Yaa -V(30%)
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Lifespan analysis
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Yaa-H
Yaa-K
Yaa-R
Yaa-W
Yaa-V
Yaa-T
Yaa-L
Yaa-I
Yaa-M
w1118
w1118 ;Gcn2¹
Yaa
Yaa -EAA
Yaa -M

Figure 3.13: GCN2 mediates development and lifespan in response to amino acid availability in w^{1118} background
(A) w^{1118};*Gcn2*^{1} mutants compared to w^{1118} control flies showed significantly reduced viability on Yaa medium *(***p < 0.001, Student's t-test)*. Compared to Yaa medium, HUNTaa medium and individual EAAs drop-down diets did not affect the viability of w^{1118} flies *(p>0.05, one-way ANOVA)*, however viability of w^{1118};*Gcn2*^{1} mutants was significantly reduced on HUNTaa medium and on all the EAAs drop down diets with the exception of W and M drop-down diets *($^{n.s.}$p >0.05 and ***p < 0.001, one-way ANOVA followed by Dunnett's post hoc test taking viability of w^{1118};*Gcn2*^{1} mutant on Yaa as control group; error bar shows SEM; n=10 replicates with 25 eggs each/genotype and diet)*. **(B)** w^{1118};*Gcn2*^{1} mutants were significantly shorter lived on the Yaa diet compared to w^{1118} control flies *(dotted black versus solid black lines, ****p<0.0001, log-rank test)*. **(E-K)** Only 7 of the 10 Yaa-EAA diets significantly decreased the lifespan of w^{1118} flies compared to Yaa diet *(solid green versus solid black lines, **p<0.01 for Yaa-R, ****p<0.0001 for Yaa-W, -V, -T, -L, -I, -M, log-rank test)*. **(B-K)** All of the Yaa-EAA diets significantly decreased the lifespan of w^{1118};*Gcn2*^{1} mutants compared to Yaa diet *(dotted green versus dotted black lines ****p<0.0001, log-rank test)*. **(B-K)** Compared to the survival of w^{1118} flies on Yaa-EAA diets, survival of w^{1118};*Gcn2*^{1} mutants was severely reduced *(solid green/red versus dotted green/red lines ****p<0.0001, log-rank test). n=150 flies/genotype and diet; lifespan analysis of all the diets was performed in the same lifespan experiment.*

Next, we tested starvation resistance using the same experimental setup of preconditioning for 7 days on Yaa and single EAA-deficient diets before being transferred to full starvation food (see Figure 3.10B). We observed that w^{1118};*Gcn2*^{1} mutants preconditioned on the Yaa control diet were significantly starvation sensitive compared to w^{1118} control flies (Figure 3.14A and 3.14B). w^{1118} control flies preconditioned on Yaa-EAA diets were significantly starvation resistant compared to the control flies preconditioned on Yaa diet (Figure 3.14A and 3.14B). In contrast, w^{1118};*Gcn2*^{1} mutants preconditioned on Yaa-EAA diets showed a significantly increased starvation sensitivity compared to the mutant flies preconditioned on Yaa diet (Figure 3.14A and 3.14B). The only exception was again deprivation of methionine, which resulted in significantly increased starvation resistance in w^{1118};*Gcn2*^{1} mutants (Figure 3.14A and 3.14B). These observations are in line with starvation resistance of *Gcn2*^{1} mutant flies in the w^{Dah} background (see Figure 3.10C-3.10L).

Thus, using an independent genetic background, we could confirm most of the phenotypes observed with *Gcn2*^{1} mutants in the w^{Dah} background. However, the results also show that the genetic background has effects on the strength of the phenotype, suggesting an interaction of genetic and dietary factors in the regulation of survival under amino acid deprivation.

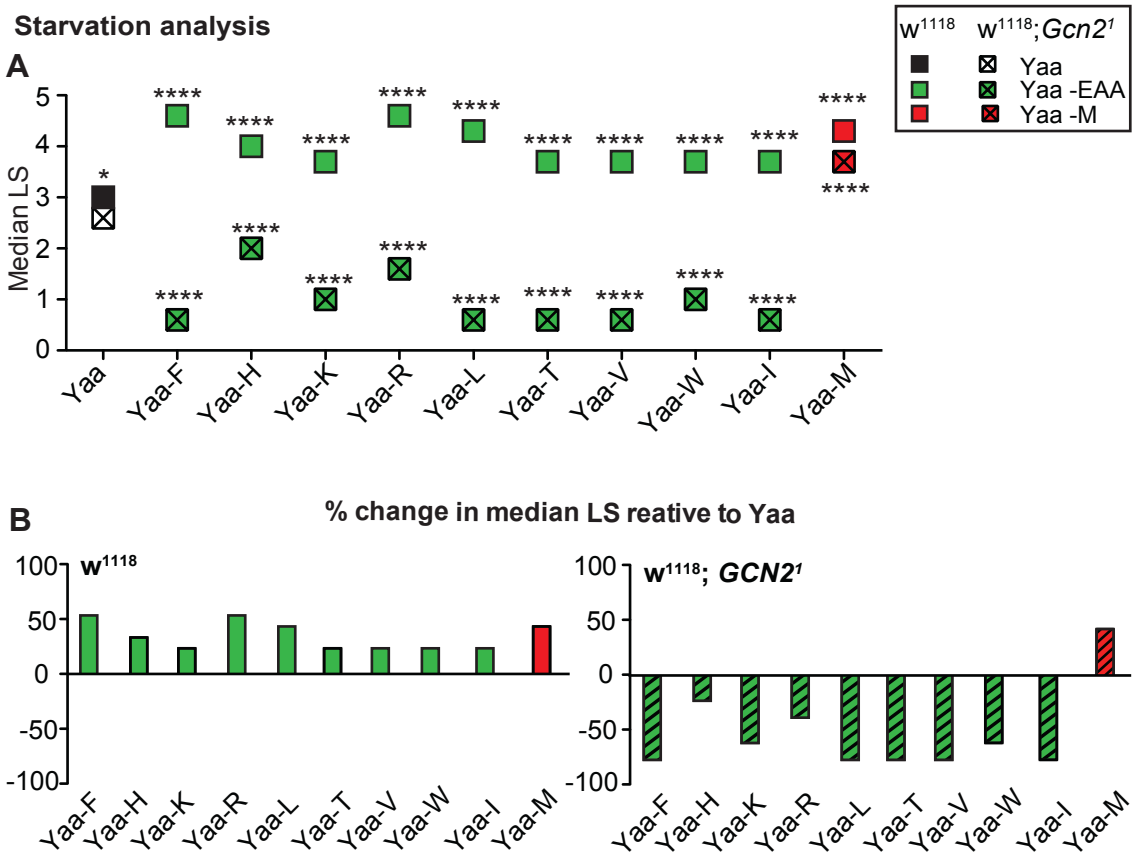

Figure 3.14: GCN2 mediates starvation resistance under EAA deprivation in w^{1118} background

(A) w^{1118};*Gcn2*^{1} mutants raised on Yaa diet were starvation sensitive compared to w^{1118} control flies *(striped black versus solid black box, *p<0.05, log-rank test)*. w^{1118} control flies preconditioned on Yaa-EAA diets were significantly starvation resistant in comparison to the control flies preconditioned on Yaa diet (*solid green boxes versus solid black box, ****p<0.0001, log-rank test)*. w^{1118};*Gcn2*^{1} mutants preconditioned on Yaa-EAA diets were significantly starvation sensitive in comparison to mutants preconditioned on Yaa diet *(striped green boxes versus striped black box, ****p<0.0001, log-rank test),* with the only exception of M deprived mutants which showed increased starvation resistance *(striped red boxes versus striped red box, ****p<0.0001, log-rank test)*. **(B)** Summary of starvation stress represented as percentage change in median lifespan under full starvation of w^{1118} control and w^{1118};*Gcn2*^{1} mutant flies preconditioned on a Yaa-EAA diet relative to their median lifespan under full starvation when preconditioned on Yaa diet *(n = 100 flies/genotype and diet)*.

3.2.8 Analysis of ATF4 as a downstream effector of GCN2 Kinase

In response to amino acid starvation GCN2 suppresses global translational but concomitantly activates translation of specific mRNAs with open reading frames in their 5' UTRs, such as the transcription factor ATF4 (Lu et al., 2004; Vattem and Wek, 2004). ATF4 induces expression of target genes involved in a variety of adaptive functions, including amino acid import and metabolism (Harding et al., 2003). We therefore tested whether enhanced *Atf4* expression in the absence of GCN2 is sufficient to rescue the phenotypes of $Gcn2^{1}$ mutants under EAA starvation. To this end, we used a UAS-*Atf4* fly line (FlyORF) and ubiquitous GAL4 driver lines, which were introduced into the w^{Dah}; $Gcn2^{1}$ mutant background.

3.2.8.1 Rescue of starvation resistance by ubiquitous overexpression of *Atf4*

First, we tested if ubiquitous over-expression of *Atf4* (driven by the da-GAL4 driver) in absence of GCN2 could rescue the reduced starvation resistance displayed by $Gcn2^{1}$ mutants. We used the same experimental setup as described above (see Figure 3.10B). We used three representative amino acid deprived diets: Yaa-L, Yaa-I and Yaa-W. Starvation resistance was completely rescued to wild-type level upon *Atf4* over-expression if previously fed on Yaa diet (Figure 3.15A, rescue of mean survival by *Atf4* over-expression was calculated compared to both driver control and UAS-*Atf4* control and relative to w^{Dah} flies). Interestingly, rescue of starvation resistance by enhanced *Atf4* expression was dependent on which amino acid was deprived prior to full starvation. *Atf4* over-expression resulted in the strongest rescue of starvation resistance of $Gcn2^{1}$ mutants when previously deprived of tryptophan (Figure 3.15B, 82% compared to UAS-*Atf4* control and 83% compared to driver control). Starvation resistance of $Gcn2^{1}$ mutants was rescued by more than 35% (36% compared to UAS-*Atf4* control and 46% compared to the Gal4 driver control) when preconditioned on a leucine-deprived diet (Figure 3.15C). In contrast, starvation resistance of $Gcn2^{1}$ mutants was very mildly rescued (14% compared to UAS-*Atf4* control and 11% compared to driver control) if fed on an isoleucine-deprived diet (Figure 3.15D).

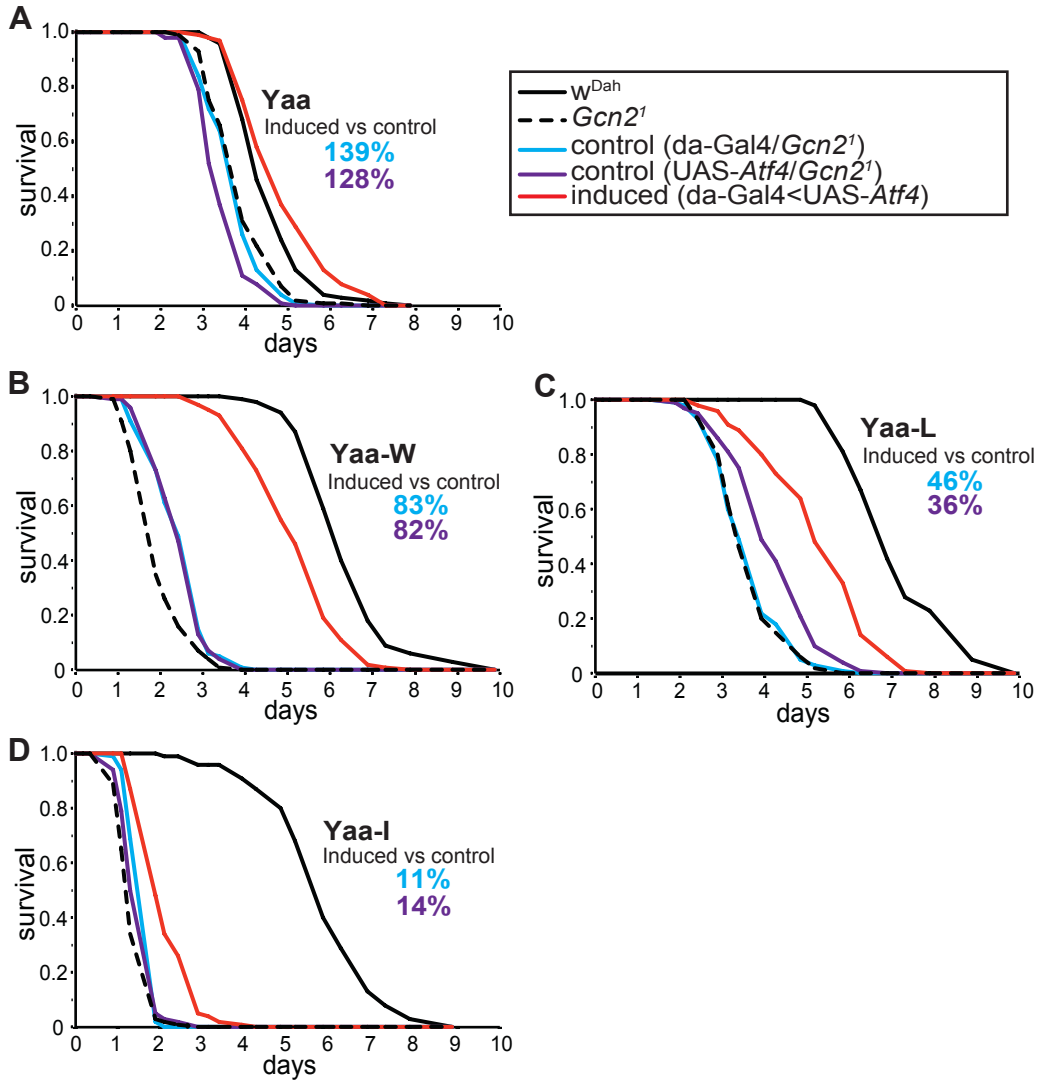

Figure 3.15: Ubiquitous overexpression of *Atf4* partially rescues starvation sensitivity of *Gcn2*^{1} mutants
(A-D) Ubiquitous overexpression of *Atf4* in the *Gcn2*^{1} mutant background (red lines) partially rescued the starvation sensitivity of *Gcn2* mutants. Rescue is shown as percentage change in mean survival of induced line compared to the driver control (da-Gal4/*Gcn2*^{1} – light blue) and UAS control (UAS-*Atf4*/*Gcn2*^{1} – dark blue) relative to w^{Dah} flies. All fly lines were preconditioned for 7 days on diets lacking single EAAs before being transferred to full starvation food *(n=100 flies/genotype and diet).*

3.2.8.2 Adult-onset, ubiquitous over-expression of *Atf4* is sufficient to rescue GCN2-dependent starvation sensitivity

The da-GAL4 driver is active during development and adulthood. Thus, it is not clear whether *Atf4* over-expression rescues GCN2 function during adulthood or through an effect on developmental processes. In order to evaluate whether adult-onset, ubiquitous over-expression of *Atf4* can rescue the amino-acid-deprivation-induced starvation sensitivity of *Gcn2*^{1} mutants, the inducible da-GeneSwitch (GS) driver (Osterwalder et al.,

2001) was used. The experimental set up was modified to ensure similar induction of *Atf4* expression at the start of the preconditioning period. Newly eclosed adult flies were allowed to mate for 48 hours on the control diet, Yaa supplemented with 50 µm of the GS inducer RU486. Subsequently, flies were transferred to RU486 (50 µm)-supplemented single EAA-deficient diets and Yaa control diet for a period of 7 days after which they were transferred to full starvation food (not supplemented with RU486).

Interestingly, adult-specific over-expression of *Atf4* was not sufficient to rescue the starvation sensitivity of $Gcn2^{1}$ mutants on the Yaa control diet. This might argue for a developmental origin of this phenotype (Figure 3.16A, 0% compared to the UAS-*Atf4* control and 35% compared to the driver control). Starvation sensitivity of $Gcn2^{1}$ mutants could be partly rescued by adult onset *Atf4* over-expression on the leucine-deprived diet (Figure 3.16B, 34% compared to UAS-*Atf4* control and 48% compared to driver control) and on the tryptophan-deprived diet (Figure 3.16C, 22% compared to UAS- *Atf4* control and 31% compared to driver control). Rescue efficiency was slightly higher on the Yaa-L than on the Yaa-W diet. In contrast, no rescue was observed on flies preconditioned on an isoleucine-deprived diet (Figure 3.16D), suggesting that the rescue of GCN2 function by *Atf4* over-expression is amino-acid-specific. Despite differences in the degree of rescue of starvation resistance between constitutive (da-GAL4) and adult onset of *Atf4* over-expression (da-GS), the overall tendency was comparable. In both setups, prior deprivation of tryptophan and leucine showed stronger rescue of starvation sensitivity whereas prior deprivation of isoleucine displayed mild or no rescue (compare Figure 3.15 with Figure 3.16).

Rescue efficiency might depend on the level of ATF4 and therefore on the concentration of RU486 in the diet. Therefore in order to test whether one of the reasons for the partial rescue of starvation sensitivity was insufficient ATF4 induction by the 50 µm concentration of RU486 used in our set up, we repeated the experiment with a two times higher RU486 concentration (100 µm) and tested all the ten single EAA-deficient diets. Doubling RU486 concentration mildly increased the degree of rescue, however did not result in full rescue on any diet (Figure 3.17A-3.17K), suggesting that 50 µm concentration of RU486 was not the cause for partial rescue of starvation resistance. Consistent with previous observations, preconditioning with three amino acids (leucine, tryptophan and threonine) displayed a more than 40% rescue whereas prior starvation of

most amino acids showed mild/no rescue (isoleucine, methionine, arginine, valine, lysine, histidine and phenylalanine).

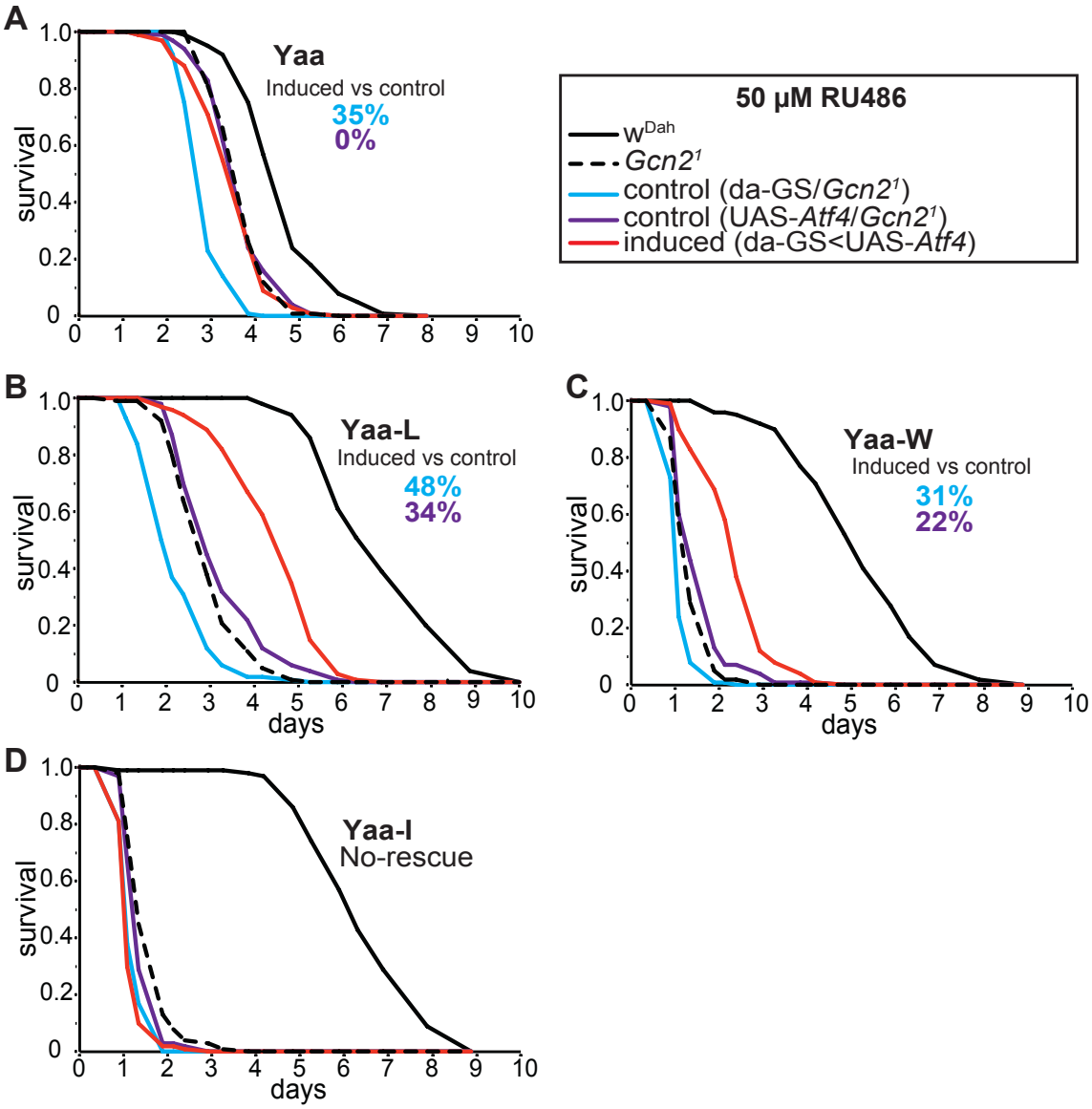

Figure 3.16: Ubiquitous adult-onset overexpression of *Atf4* partially rescues starvation sensitivity of *Gcn2*^{1} mutants
(A-D) Ubiquitous adult-onset overexpression of *Atf4* in *Gcn2*^{1} mutant background (50 µM RU486 - red lines) partially rescued the starvation sensitivity. Rescue is shown as percentage change in mean survival of induced line compared to the driver control (da-GS/*Gcn2*^{1} – light blue) and UAS control (UAS-*Atf4/Gcn2*^{1} – dark blue) relative to w^{Dah} flies. All the fly lines were preconditioned for 7 days on diets lacking single EAA as depicted before being transferred to full starvation food *(n=100 flies/genotype and diet).*

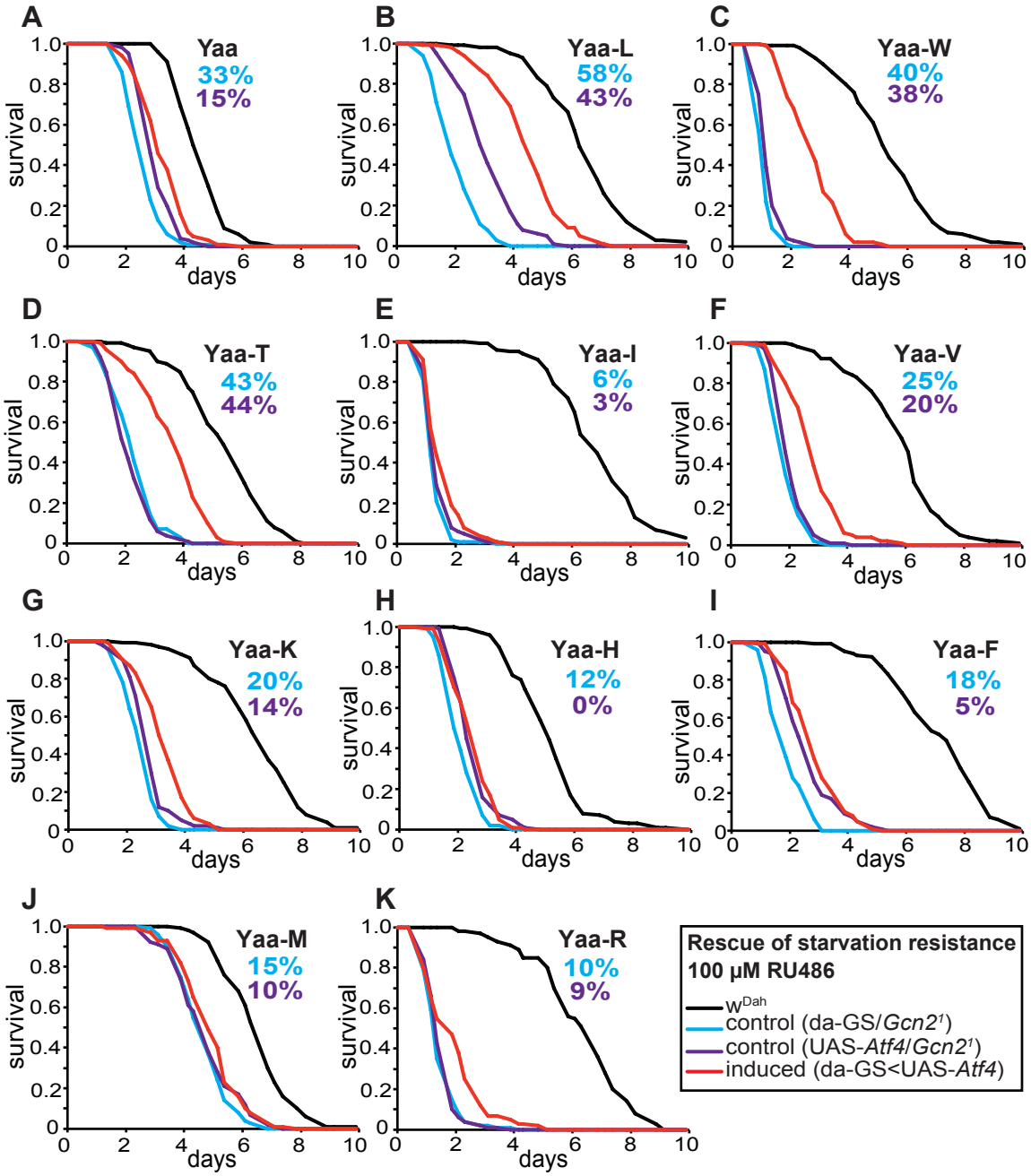

Figure 3.17: Ubiquitous adult-onset overexpression of *Atf4* partially rescues the starvation resistance of *Gcn2*^{1} mutants

(**A-K**) Ubiquitous adult-onset overexpression of *Atf4* in *Gcn2*^{1} mutant background (100 µM RU486-red lines) partially rescued the starvation resistance. Rescue is shown as percentage change in mean survival of induced line compared to the driver control (da-GS/*Gcn2*^{1} – light blue) and UAS control (UAS-*Atf4*/*Gcn2*^{1} – dark blue) relative to w^{Dah} flies. All the fly-lines were preconditioned for 7 days on diets lacking single EAA as depicted, before being transferred to full starvation food *(n=100 flies/genotype and diet).*

3.2.8.3 Adult-onset, ubiquitous over-expression of *Atf4* rescues food intake of *Gcn2*^{1} mutants on EAA-deficient diets

Our data suggested that reduced feeding rates of *Gcn2*^{1} mutants on individual EAA-deficient diets (with the exception of methionine) decreased lipid levels and thereby resulted in reduced starvation resistance. Since over-expression of *Atf4* in the absence of GCN2 partially rescued starvation sensitivity in an amino-acid-dependent manner, we hypothesized that this might be a result of rescued feeding rates. Indeed, feeding rates upon *Atf4* over-expression in the *Gcn2*^{1} mutant background correlated well with the rescue of starvation sensitivity of *Gcn2*^{1} mutants (compare Figure 3.18 with Figure 3.16). Adult onset, ubiquitous expression of *Atf4* significantly rescued the feeding rate of *Gcn2*^{1} mutants on leucine and tryptophan-deficient diets, although not to the level of the wild type control (Figure 3.18). Furthermore, feeding rate on the isoleucine-deficient diet was not rescued by *Atf4* over-expression, consistent with the lack of rescue of starvation sensitivity (compare Figure 3.18 with Figure 3.16D).

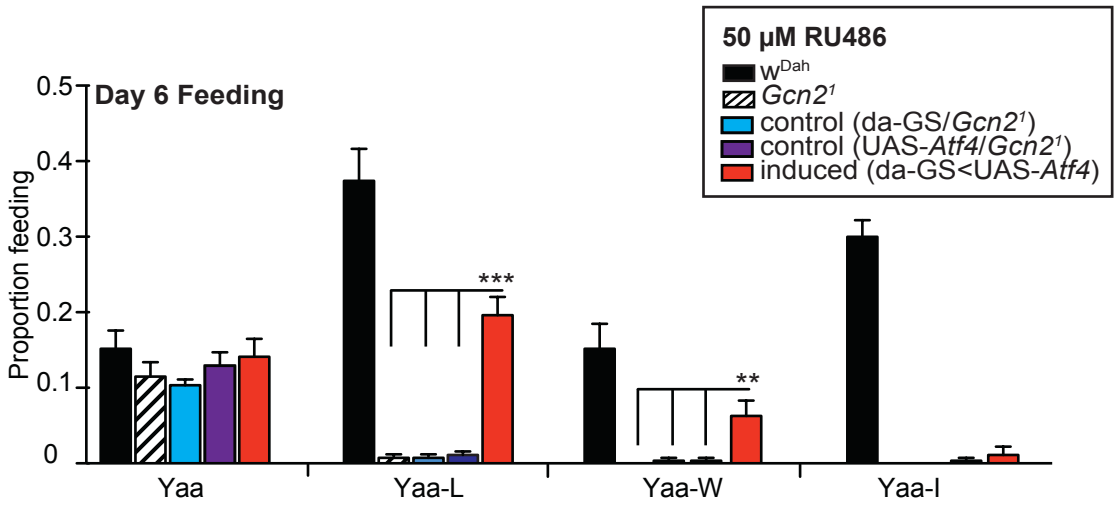

Figure 3.18: Ubiquitous adult-onset overexpression of *Atf4* partially rescues feeding of *Gcn2*^{1} mutants on EAA-deficient diets
Feeding rate was partially rescued by ubiquitous adult-onset overexpression of *Atf4* in *Gcn2*^{1} mutant background (red bar) on Yaa-L and Yaa-W but not on Yaa-I *(**p<0.01 and ***p<0.001, one-way ANOVA followed by Tukey's post hoc test; error bar shows SEM).*

3.2.8.4 Adult-onset, ubiquitous over-expression of *Atf4* rescues lifespan of *Gcn2*^{1} mutants on EAA-deficient diets

Since adult onset, ubiquitous *Atf4* over-expression partially rescued starvation resistance and feeding on EAA-deficient diets, we tested whether reduced survival due to the absence of GCN2 under EAA deprivation could also be rescued.

On Yaa medium, lifespans of the control lines (da-GS and UAS-*ATF4*) and of the induced line were comparable to that of w^{Dah} flies (Figure 3.19A). Interestingly, adult onset, ubiquitous *Atf4* over-expression fully rescued the reduced survival of *Gcn2*^{1} mutants under leucine deprivation (Figure 3.19B). Furthermore, consistent with starvation sensitivity and feeding rateno rescue of survival was observed under isoleucine deprivation (compare Figure 3.19C with Figure 3.16D and Figure 3.18). As lifespan of *Gcn2*^{1} mutants was significantly increased on a Yaa-M diet (see Figure 3.7J), we also tested if this could be rescued by *Atf4* over-expression. Indeed, lifespan of *Gcn2*^{1} mutants under methionine deprivation was rescued back to wild type level (Figure 3.19D).

Taken together our findings show that activation of ATF4 is able to partially compensate for the lack of GCN2 function, dependent on the dietary context.

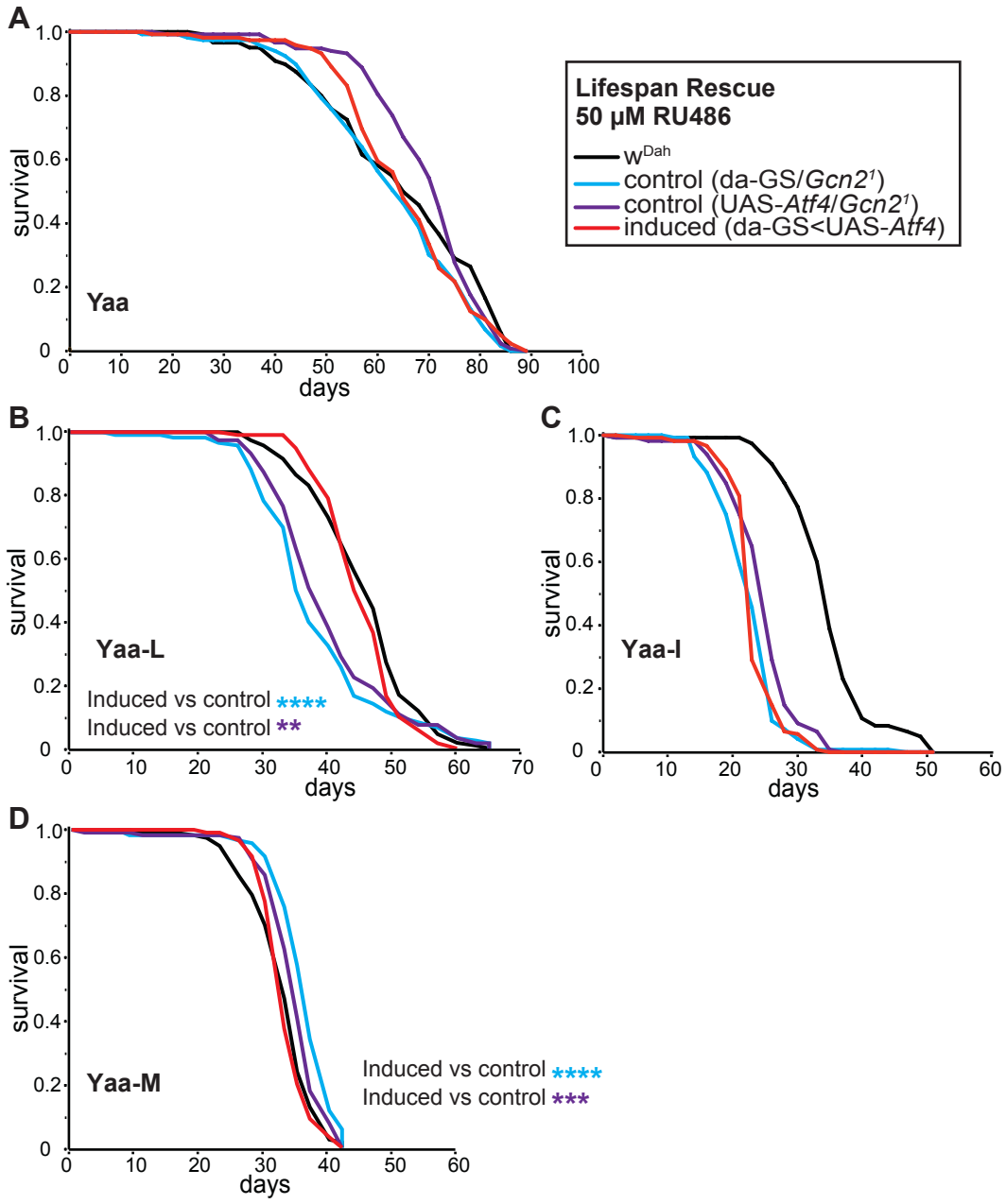

Figure 3.19: Ubiquitous adult-onset overexpression of *Atf4* rescues lifespan of *Gcn2*^{1} mutants under deprivation of selective EAA

(**A-D**) Ubiquitous adult-onset overexpression of *Atf4* in the *Gcn2*^{1} mutant background (50 µM RU486-red lines) completely rescued survival under **(B)** Yaa-L and **(D)** Yaa-M but not on **(C)** Yaa-I (*RU486 induced da-GS<UAS-Atf4 vs. da-GS/Gcn2*^{1} *– light blue asterisk and RU486 induced da-GS<UAS-Atf4 vs. UAS-Atf4/Gcn2*^{1} *– dark blue asterisk; **p<0.01, ***p<0.001 and ****p<0.0001, log-rank test).*

3.2.9 Genome wide transcriptional profiling: GCN2 dependent and independent transcriptional response upon amino acid starvation

Amino acid limitation or total deprivation alters gene expression in different species and this response, at least in part, is mediated by GCN2 kinase (Deval et al., 2008; Kilberg et al., 2009). However, several aspects of amino acid regulated gene expression are still not fully understood. For instance, it is unclear whether starvation of different individual amino acids triggers similar or distinct set of genes, which signaling pathways are activated upon starvation of different amino acids, and whether full protein deprivation manifests similar transcriptional changes as single amino acid deprivation. Therefore, this part of the study was aimed to gain more insights into control of gene expression and signaling pathways under individual and complete amino acid deprivation and to distinguish GCN2-dependent and -independent amino acid responsive genes and biological processes. To this end, gene expression profiles of w^{Dah} control flies and *Gcn2*^{1} mutants were analyzed under methionine, arginine and full amino acid starvation using RNA-seq analysis (for experimental set up see Figure 3.20A). We chose methionine and arginine deprivation for studying changes in gene expression in response to individual amino acid starvation because of the different response of *Gcn2*^{1} mutants towards methionine deprivation and the deprivation of other EAAs (represented by arginine deprivation). Initially, the experiment was designed to study the effects of long-term amino acid deprivation (for a period of 7 days) and short-term amino acid deprivation (for a period of 3 days). However, the sequencing quality of day 7 data was poor with same treatments not clustering together, therefore only the day 3 data set was used for the subsequent bioinformatics analysis. Bioinformatics analysis was done in collaboration with the bioinformatics core facility at the Max-Planck Institute for Biology of Ageing. At the moment, the analysis is still ongoing therefore only preliminary results are shown here.

3.2.9.1 GCN2 regulates similar as well as distinct set of gene in response to individual and complete amino acid deprivation

Gene expression profiles under methionine, arginine and full amino acid starvation were analyzed in comparison to the control diet (Yaa). The number of differentially regulated genes in w^{Dah} control flies and *Gcn2*^{1} mutants under methionine, arginine and protein deprivation is shown in Figure 3.20B. Preliminary bioinformatics analysis revealed some interesting observations: 1) A significant heterogeneity in the transcriptional responses to

the deprivation of methionine, arginine and full amino acid starvation was observed (Figure 3.20B). (2) A substantial number of genes were commonly regulated under methionine, arginine and protein deprivation in w^{Dah} control flies, suggesting that all the three conditions also triggers a general amino acid deprivation response (Figure 3.20B).

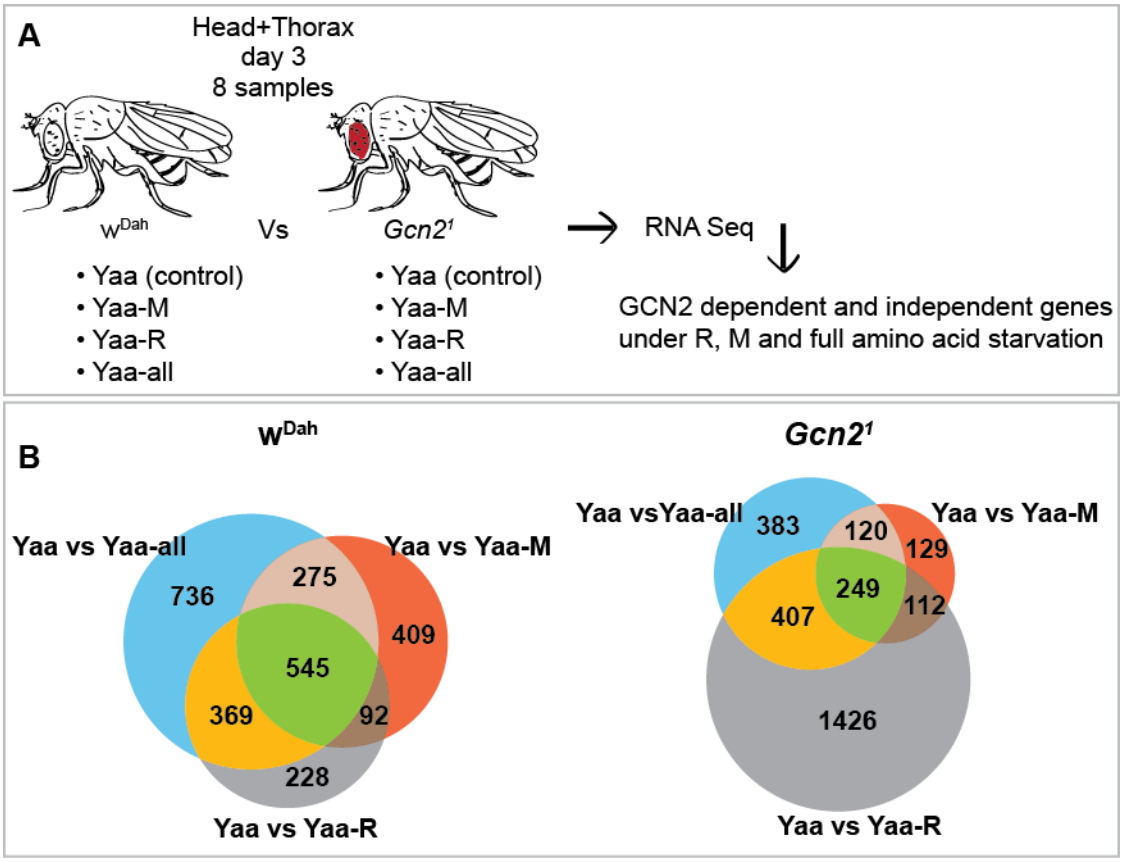

Figure 3.20: Transcriptional profiling of w^{Dah} control and *Gcn2*^{1} mutant flies
A) Differential gene regulation by GCN2 under EAA starvation (Yaa-R, Yaa-M and Yaa-all) was studied using RNA-Seq. **(B)** Venn-diagrams showing the overlap of genes differentially changed under Yaa-M, Yaa-R and Yaa-all in w^{Dah} control and *Gcn2*^{1} mutant flies.

3.2.9.2 GCN2 regulates distinct biological processes in response to individual or complete amino acid deprivation

To identify GCN2 dependent biological processes specifically regulated under methionine, arginine or protein deprivation, a GO enrichment analysis using Bioconductor R software package, topGO, was performed. Preliminary data showed that processes related to amino acid metabolism and protein translation such as t-RNA aminoacylation and amino acid transport were among the top 10 GCN2-dependent GO categories under

methionine deprivation (Figure 3.21A). Furthermore, down-regulation of translation emerged as the top most enriched GCN2-dependent GO category under arginine deprivation, in line with previous studies showing that GCN2 modulates translation in response to amino acid deprivation (Figure 3.21B). GO enrichment analysis for full amino acid starvation revealed non-canonical GCN2-dependent biological processes such as potassium ion transport and cellular response to carbon dioxide (Figure 3.21C).

As mentioned before, this is a preliminary analysis of our RNA-seq data and in future we aim to decipher whether deprivation of any single EAA leads to similar or different changes compared to the full amino acid starvation or to other single amino acid deprivation.

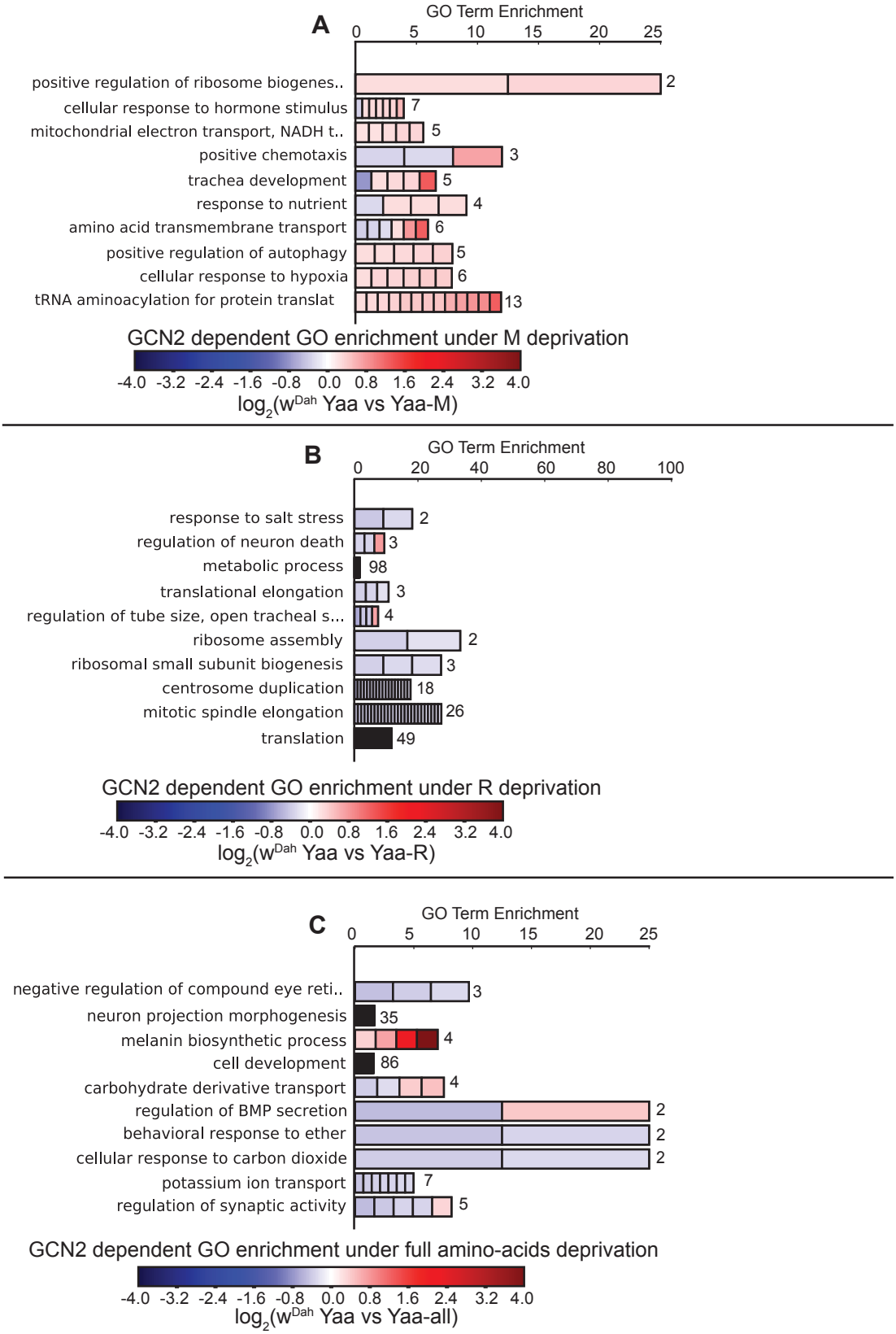

Figure 3.21: GO enrichment analysis of GCN2-dependent gene expression
Cell plots of GO-enrichments for GCN2-dependent GO categories on **(A)** Yaa-M **(B)** Yaa-R and **(C)** Yaa-all. Horizontal axis represents GO-term enrichment (ElimFisher), colouration represents specific $\log_2$ fold change, and the gene count is shown at the end of each bar. The ten most significantly regulated GO categories (biological process) are shown.

3.2.10. Cross-talk between GCN2 kinase and TOR kinase

Cells have evolved at least two different pathways to monitor intracellular amino acid levels: GCN2 to sense absence of amino acids and TOR to sense presence of amino acids. Studies in different model organisms have shown that crosstalk between these two nutrient-sensing pathways exists; however the mechanism and the direction of interaction are not clearly understood (Gallinetti et al., 2013). We therefore tested whether these two pathways interact in the regulation of organismal traits such as lifespan and stress resistance.

3.2.10.1 Longevity and starvation resistance conferred by rapamycin-mediated TOR inhibition is GCN2 independent.

Genetic or pharmacological down regulation of TORC1 leads to increased lifespan from yeast to mammals (Kapahi et al., 2010). Interestingly, a recent study in worms suggests that GCN2 activity is essential to mediate lifespan-extension conferred by down regulation of TOR (Rousakis et al., 2013).

Therefore, we investigated whether GCN2 mediates lifespan extension conferred by inhibition of the TOR pathway in flies. To this end we used rapamycin to inhibit TORC1 activity in w^{Dah} control and *Gcn2*^{1} mutant flies and performed lifespan analysis. Interestingly, rapamycin treatment significantly increased the median lifespan of both genotypes as compared to the respective EtOH control (Figure 3.22A), suggesting that longevity conferred by rapamycin-mediated inhibition of TOR is GCN2 independent. Furthermore, we analyzed survival of rapamycin treated *Gcn2*^{1} mutants and w^{Dah} flies under full starvation conditions. Aged matched flies were raised on rapamycin supplemented Yaa diet (5µm, 10µm, and 20µm) for ten days and then transferred to starvation food (1% agarose). Consistent with published data (Bjedov et al., 2010) we observed a dose-dependent increase in survival of rapamycin-treated w^{Dah} control flies (Figure 3.22B). Interestingly, rapamycin treatment also resulted in a dose-dependent increase in survival of *Gcn2*^{1} mutants (Figure 3.22C). Thus, our results show that longevity and starvation resistance conferred by rapamycin-mediated TOR inhibition is GCN2-independent.

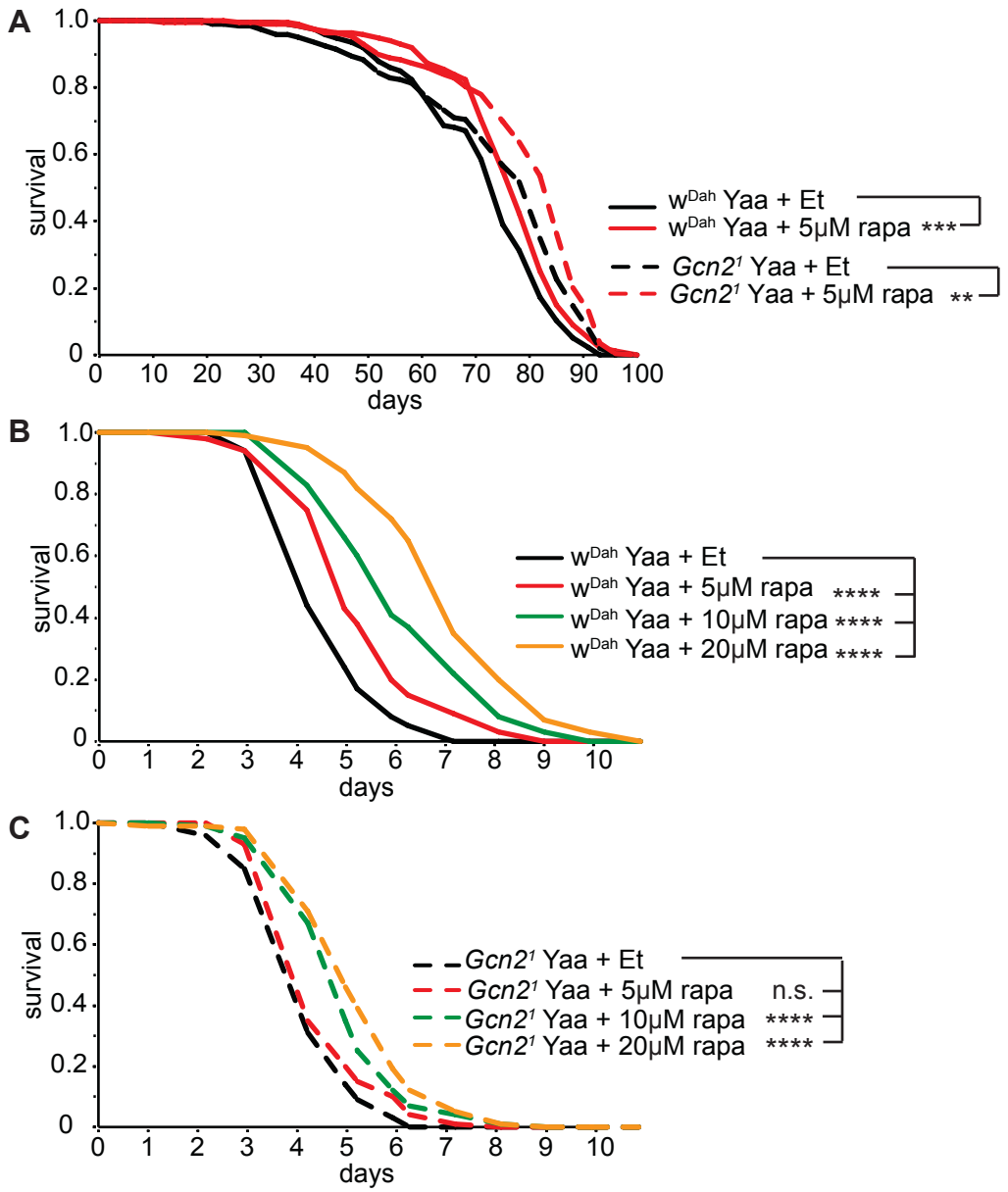

Figure 3.22: Longevity and starvation resistance conferred by rapamycin-mediated TOR inhibition is GCN2 independent

(A) Rapamycin significantly extended the median lifespan of w^{Dah} control and *Gcn2*^{1} mutant flies. Survival analysis of rapamycin treated **(B)** w^{Dah} control and **(C)** *Gcn2*^{1} mutant flies under full starvation showed a significant dose dependent increase in the starvation resistance of w^{Dah} control and *Gcn2*^{1} mutant flies *(****p<0.0001, ***p<0.001 and **p<0.01, log-rank test; n for lifespan analysis =200 flies/genotype and diet, n for starvation stress =100 flies/genotype and diet).*

3.3 DISCUSSION

The role of nutrient-sensing pathways, such as the TOR or insulin/ IGF-1 signaling (IIS) pathways, in lifespan and other age-related phenotypes is well documented in diverse species (Fontana et al., 2010). In contrast, the role of the evolutionary conserved amino acid sensing pathway mediated via GCN2 kinase in ageing is not yet fully understood. Using *Drosophila melanogaster* as a model organism, we show that GCN2 kinase plays a vital role in modulating longevity and important physiological functions during nutritional deprivation of EAAs.

3.3.1 GCN2 ensures fly development under limitation of an EAA

Starvation of EAAs inhibits larval growth and development in *Drosophila* (Britton and Edgar, 1998), however, the underlying molecular mechanisms remain unclear. Here, we show that GCN2 kinase ensures fly development under limitation of all EAAs (except methionine, discussed in section 3.3.6). Our findings are consistent with studies suggesting a role of GCN2 kinase in growth and development in response to amino acid availability (Dever et al., 1993; Zhang et al., 2002). In yeast, GCN2 is required for growth in the absence of amino acids or in the presence of inhibitors of amino acid biosynthesis (Dever et al., 1993) and in mice loss of GCN2 function negatively impacts fetal development when leucine is missing in the maternal diet (Zhang et al., 2002).

One of the possible mechanisms of GCN2-dependent regulation of fly development under EAAs limitation might involve control of translation by GCN2. Studies in flies and mice have shown that regulation of translation through changes in eIF2α phosphorylation status plays a critical role in development (Harding et al., 2009; Malzer et al., 2013b). De-phosphorylation of eif2α is performed by eif2α phosphatase, PPP1R15, whose function is antagonistic to GCN2. In *Drosophila*, knockdown of both GCN2 and PPP1R15 results in strongly reduced hatching rate, indicating that precise regulation of eIF2α phosphorylation by GCN2 and PPP1R15 is necessary for efficient embryogenesis (Malzer et al., 2013b). In mice combined deletion of both PPP1R15 paralogues, PPP1R15a and PPP1R15b, leads to early embryonic lethality (Harding et al., 2009).

GCN2-mediated feeding response might also influence fly development under EAA limitation. Activation of GCN2 on a tryptophan and lysine deficient diet reduces food intake of *Drosophila* L3 larvae and prolonged expression of a constitutively active form of fly GCN2 throughout development results in anorexic larvae that die from starvation

(Bjordal et al., 2014). However, in our study, development of wild type larvae on an EAA limited diet was not affected, suggesting that activation of GCN2 might not lead to inhibition of food-intake in larvae (GCN2-mediated feeding response is discussed in detail in section 3.3.3).

Our study clearly shows that GCN2 is vital for ensuring fly development under limitation of EAAs and we speculate that GCN2-mediated control of translation and/or feeding might be responsible for this effect. However, feeding and translation rate in *Gcn2*-knockout and wild-type *Drosophila* larvae under EAA limitation remain to be tested.

3.3.2 GCN2 function is essential for survival under EAAs deprivation

Previous studies suggest an important role of amino acids in modulating longevity in diverse species (Fontana and Partridge, 2015). In yeast, supplementation with serine, threonine, or valine decreases chronological lifespan (Mirisola et al., 2014) while selective restriction of asparagine, glutamate or methionine extends lifespan (Wu et al., 2013a; 2013b). In flies, adding back EAAs, in particular methionine (or to a lesser degree tryptophan) to the diet of DR flies abrogates lifespan extension (Grandison et al., 2009) and restricting methionine content under low protein status increases lifespan (Lee et al., 2014a). In rodents, methionine and tryptophan restriction increases lifespan (McIsaac et al., 2016; Miller et al., 2005; Orentreich et al., 1993) and supplementation of branched chain amino acids (BCAAs), leucine, isoleucine and valine, increases average lifespan of male mice (D'Antona et al., 2010). Although the role of EAAs in modulating longevity is well established, the underlying molecular mechanisms still remain poorly understood.

Here, we have systematically analyzed the role of each of the 10 EAAs in modulating fly longevity and demonstrate that the amino acid starvation sensor, GCN2 kinase, plays a vital role in regulating survival under deprivation of individual EAAs. Our data show that *Gcn2* knockout flies were significantly shorter lived when any of the EAAs (except methionine, discussed in section 3.3.6) was absent from the diet. Our findings are consistent with a recent study in worms suggesting a role of GCN2 in modulating longevity in response to amino acid availability (Edwards et al., 2015; Rousakis et al., 2013). Supplementation of histidine and tryptophan increased the lifespan of wild-type worms but failed to do so in *Gcn2*-deficient worms (Edwards et al., 2015). GCN2 kinase participates in amino acid starvation stress management by activating the key transcription factor ATF4 (Wek et al., 2006). This stress-induced reprogramming could also determine lifespan. In yeast, constitutive expression of GCN4 (homologue of mammalian ATF4)

suppresses lifespan extension conferred by BCAAs supplementation (Alvers et al., 2009). Our results showed that reduced survival of *Gcn2*-knockout flies was rescued by *Atf4* overexpression under leucine and methionine deprivation but not under isoleucine deprivation. This may suggest that to regulate survival under EAA deprivation, GCN2 selectively activates ATF4 or additional transcription factors (e.g. NF-κB, Jiang et al., 2003) depending on which EAA is being deprived (discussed in detail in section 3.3.5). One of the possible mechanisms by which GCN2/ATF4 pathway regulates survival under EAAs deprivation could involve recycling of amino acids through activation of autophagy. Enhanced autophagy has been linked to increased lifespan in diverse species including worms (Edwards et al., 2015), mice (Zheng et al., 2010) and flies (Juhasz et al., 2007) and cell culture studies have shown that the GCN2/ATF4 pathway enhances the transcription of autophagy-genes involved in the synthesis (Atg5, Atg12), maturation (LC3) and turnover of autophagosomes in response to amino acid starvation (B'chir et al., 2013; Carroll et al., 2015).

Decreased rate of translation is associated with increase in lifespan in diverse species including yeast, worms and flies (Kaeberlein and Kennedy, 2008). It has been suggested that GCN2 induced suppression of translation mediates longevity of eat-2 mutants, a genetic model of DR, in worms (Rousakis et al., 2013). Hence, it could be argued that failure to repress translation upon amino acid starvation might be responsible for reduced survival of *Gcn2*-knockout flies. However, our fecundity data suggest that *Gcn2*-knockout flies are able to down-regulate translation in response to deprivation of an EAA since they severely reduced their egg production on each of the EAA-deficient diets, similar to wild type flies. We speculate that, since protein synthesis is central to organisms' survival, cells might have feedback mechanisms to keep translation going under long term EAA deprivation.

Given that determination of lifespan is multifactorial in nature, it might be possible that GCN2 interacts with several other metabolic pathways and processes to regulate lifespan in response to amino acid availability. Furthermore, the pathways and processes involved might vary from one amino acid to the next. We suspect that GCN2-mediated feeding response (discussed in section 3.3.3) and lipid metabolism (discussed in section 3.3.4) might also contribute to determination of lifespan during nutritional deprivation of an EAA.

3.3.3 GCN2 mediates food-intake in response to long-term deprivation of an EAA

GCN2 is involved in the adaptive response of food intake in response to EAA deprivation (Bjordal et al., 2014, Hao et al., 2005, Maurin et al., 2005). In mice, GCN2 activation leads to rapid rejection of a leucine or threonine deficient diet within an hour of feeding (Maurin et al., 2005). Similarly, fly larvae rapidly reject a sugar/corn-based diet deficient in trytophan and lysine (Bjordal et al., 2014). However, another study in mice reported that, under long-term leucine deprivation (7 days and 17 days), both wild-type and *Gcn2*-deficient mice show reduced food-intake (Guo and Cavener, 2007). Furthermore, a recent study has called the rapid rejection of EAA-deficient diets into question, and showed that mice cannot rapidly sense and reject leucine or threonine deficient diets (Leib and Knight, 2015). Thus, the rapid sensing of dietary EAA deprivation via GCN2 and short-term GCN2-mediated food rejection is highly controversial.

In this thesis I have performed a comprehensive analysis of GCN2-mediated food intake in response to short-term and long-term deprivation of each of the 10 EAAs in adult *Drosophila* females. There are four main findings from this analysis: (1) Short-term (0-24 hours) single deprivation of all the EAAs triggers a GCN2-independent aversion response towards EAA-deficient diets. (2) Long-term (2-7 days) single deprivation of all the EAAs except for methionine induces a GCN2-dependent compensatory feeding response in wild type flies. (3) Full amino acid deprivation does not cause a compensatory feeding response and is sensed by a GCN2 independent mechanism and (4) long-term methionine deprivation is sensed by a GCN2 independent mechanism, suggesting the presence of at least two independent amino acid sensing systems.

In contrast to fly larvae (Bjordal et al., 2014) we did not observe a short-term GCN2-dependent food rejection in adult flies. There are several possible explanations for the observed discrepancy, including differences between larval and adult behavior, different base food composition and differences in genetic background (w^{Dah} in this study, w^{1118} in Bjordal et al., 2014). With respect to larval adult differences in food uptake behaviors we could show that *Gcn2* mutant larvae can sense the difference between Yaa and the arginine deprived HUNTaa diet, suggesting that the discrepancy between the studies is probably not caused by differences in the observed developmental stage. Furthermore, the larval data were generated by using *Gcn2*-RNAi expression specifically in DA neurons (Bjordal et al., 2014), while in the current study we used a *Gcn2* null mutant. Thus, the feeding response observed in the larval study might be the effect of some unspecific off-

target effect of the *Gcn2*-RNAi construct on the DA neurons and therefore the phenotype is not apparent with the *Gcn2* null mutant. One notable aspect of the proposed rejection response in the previously discussed studies is that the activation of GCN2 occurs within minutes after ingestion of an EAA-deficient diet (within 2 mins in DA neurons of flies or within 20 mins in the APC of mice) (Bjordal et al., 2014; Hao, 2005; Maurin et al., 2005). This rapid activation is surprising because GCN2 is supposed to sense the presence of uncharged t-RNAs as a potential indicator of amino acid deprivation, and it would imply that within minutes of EAA deprivation of the whole organism, specific cells already run out of charged t-RNAs. In fly larvae the transcription factor ATF4 has been suggested to be involved in the rapid food rejection response (Bjordal et al., 2014). However, it seems unlikely that transcriptional changes modulated by the GCN2/ATF4 pathway in response to amino acid deprivation will be effective within this short time frame. In line with these arguments, our data show that GCN2 may not be involved in the first 24 hours of an EAA deprivation but gets activated later in response to a physiological deficit of EAAs as a result of the consumption of EAA-deficient diets for more than 24 hours. Furthermore, mice attain the ability to identify leucine or threonine deficient diets following 2 days of EAA deprivation but surprisingly, this need-based EAA sensing is independent of GCN2 (Leib and Knight, 2015). Based on our data in flies, we would argue that the duration needed to create a physiological deficit for a particular EAA in order to activate GCN2 is probably longer than 2 days in mice. Taken together, our data show that the mechanisms involved in the perception of and preference for amino acids in the food are GCN2-independent and GCN2 mediates feeding behavior as a function of intracellular deprivation of EAAs. We propose that feeding behavior in response to an EAA-deficient diet is highly dependent on the duration of the amino acid starvation and is mediated, at least in parts by GCN2 kinase. Short-term deprivation of EAAs is sensed by a currently unknown mechanism that elicits a rejection response. However, when EAA deprivation continues over a longer period GCN2 kinase gets activated and might receive feedback signals from the short-term sensing pathway to trigger a transcriptional response, which induces compensatory feeding in flies in order to cope with the nutritional stress.

An important aspect of the nutrient sensing mechanism is the identification of the tissue(s) involved in the GCN2-dependent feeding response. Presently, the brain region most strongly implicated in feeding response is the APC, however several studies in mice also suggest an involvement of the hypothalamus (Hao et al., 2010) and other peripheral

tissues, including liver. In future we aim to identify the fly tissue(s) involved in the GCN2-dependent control of feeding behavior.

3.3.4 GCN2-mediated food-intake controls lipid synthesis and starvation resistance during nutritional deprivation of an EAA

In response to EAA deprivation, GCN2 activation regulates different physiological responses including food intake and lipid metabolism. In mice, GCN2 suppresses hepatic lipid synthesis upon 7 days of leucine deprivation (Guo and Cavener, 2007). In contrast to this finding, our data show that wild type flies had increased lipid levels under deprivation of any of the EAAs while *Gcn2* mutant flies had reduced lipid levels in response to deprivation of EAAs except for methionine. Consistent with the literature that increased lipid storage enhances survival under starvation stress (Rion and Kawecki, 2007), lipid levels in w^{Dah} control and $Gcn2^{1}$ mutant flies under deprivation of EAAs co-related well with their starvation resistance. Furthermore, given that GCN2 alters food intake in response to deprivation of EAAs, our data suggests that altered feeding behavior is causal for the changed lipid levels under EAA starvation. The causal link between GCN2-mediatd food intake, lipid levels and starvation resistance is further demonstrated by our finding that rescue of feeding rates upon *Atf4* overexpression correlates with the rescue of starvation sensitivity of *Gcn2* mutants.

Taken together, we propose that GCN2 mediated regulation of food intake under long-term nutritional deprivation of EAA controls lipid levels. Because, lipids are the main source of energy during prolonged starvation, changes in lipid synthesis thereby affects starvation resistance of flies.

3.3.5 ATF4 activation partially compensates for GCN2-mediated functions under deprivation of EAAs

ATF4 is suggested to be an important downstream effector of GCN2 kinase as it regulates expression and activity of several genes involved in amino acid transport, assimilation and metabolism under amino acid starvation (Harding et al., 2003; Kilberg et al., 2009). Here, we show that (1) increased *Atf4* expression is necessary but not sufficient to compensate for GCN2 mediated functions under deprivation of EAAs and (2) the rescue efficiency of *Atf4* over-expression depends on which EAA is being deprived from the diet. One way to account for our observations is that GCN2 induces *Atf4* translation to different degrees in response to starvation of different EAAs. This possibility is in accord with the study in

HepG2 human hepatoma cells that shows valine results in the highest ATF4 protein content whereas deprivation of isoleucine results in very weak induction of ATF4 protein content (Palii et al., 2008). This might suggest that under isoleucine deprivation GCN2 activates transcription factor(s) other than ATF4 and that might explain our observation of no rescue of GCN2-dependent functions upon *Atf4* over-expression in *Gcn2*-knockout flies when isoleucine was absent from the diet. Studies have shown that GCN2 activates a different transcription factor, nuclear-factor κB (NF-κB), via phosphorylation of eIF2α upon leucine deprivation in mouse embryonic fibroblasts (Jiang et al., 2003). Furthermore, in HepG2-C3A hepatoma cells GCN2/ATF4 target genes, including CHOP and ASNS, were induced to a much greater degree by leucine deprivation in comparison to cysteine limitation (Lee et al., 2008). These data suggest that degree of ATF4 induction as well as of its target genes depend on which EAA is being deprived.

We have shown that increased *Atf4* expression in *Gcn2*-knockout flies under deprivation of certain EAAs (tryptophan or leucine) resulted in partial rescue of GCN2-dependent functions. This could imply that certain EAAs may trigger distinct transcription factors and co-activators, which may act in concert with ATF4 to fully facilitate its function. This idea is further reinforced by a cell culture based study, which indicates that, to achieve same degree of transcriptional activation, the absolute amount of ectopically expressed ATF4 required is greater than that needed during histidine deprivation, suggesting that other factors may serve to enhance ATF4 functions (Shan et al., 2009). Several recent studies have documented that ATF4 and other transcription factors including ATF2, ATF3, ATF5 and cJUN interact together to regulate amino acid stress response (Bruhat et al., 2009; Kilberg et al., 2012). Data from human cell lines demonstrate that, in response to leucine deprivation, ATF4 induction along with ATF2 phosphorylation is necessary to induce expression of its target gene, *CHOP* (Averous et al., 2004). Moreover, another study in MEFs suggests that elevated levels of ATF3 is also essential for triggering expression of *CHOP* under leucine deprivation (Jiang et al., 2004). Furthermore, a tandem affinity purification tag approach identified p300/CBP-associated factor (PCAF) as a novel interaction partner of ATF4 in leucine-starved cells (Cherasse et al., 2007).

Thus, although it is well established that ATF4 is a primary component of amino acid stress response, our data suggest that, depending on which EAA is being deprived, GCN2 might activate additional transcription factors that work alone or in concert with ATF4 to regulate amino acid stress response. Therefore for an accurate understanding of the role of

ATF4 in amino acid stress response, the GCN2/ATF4 pathway should be studied in response to deprivation of individual amino acids. The mechanism by which deprivation of different amino acids selectively triggers ATF4 and the additional transcription factors are presently unknown. Moreover, it will be interesting to investigate the tissue specificity of the ATF4 dependent transcriptional response in future.

3.3.6 Methionine deprivation is sensed by a GCN2-independent mechanism

One of the important findings of our study is that GCN2 function is not essential under methionine deprivation. Our results show that *Gcn2*-knockout and w^{Dah} control flies exhibit similar physiological responses upon methionine deprivation, suggesting that methionine deprivation is sensed by a GCN2-independent mechanism. Our findings are in line with a recent study in mice that showed that GCN2 is not required for methionine-restriction-dependent physiological responses including increased food intake, induction of hepatic FGF21, increased energy expenditure, or enhancement of insulin sensitivity. They instead suggest that methionine restriction might go via PEK, the other eif2α kinase (Wanders et al., 2016). Methionine participates in multiple cellular metabolic pathways, including the salvage pathway, the SAM recycling pathway, the transsulfuration pathway, polyamine synthesis, and creatine biosynthesis (Drabkin and Rajbhandary, 1998; Stipanuk and Ueki, 2010; Tang et al., 2015) and hence methionine deprivation could affect a variety of pathways. Along the same lines, a comprehensive microarray analysis in a mammalian cell line demonstrated that, unlike deprivation of other EAAs, methionine deprivation triggers a unique and dramatic gene expression response through a reduction of both histone methylation and ornithine-mediated signaling (Tang et al., 2015). In future, we aim to decipher GCN2-dependent and -independent pathways and biological processes involved in sensing of methionine deprivation using our RNA-Seq data.

3.3.7 Deprivation of individual EAAs might differently affect GCN2 activation

In yeast, GCN2 is activated in response to starvation of a range of different amino acids including histidine, tryptophan, leucine, isoleucine, valine, lysine, arginine and serine (Wek et al., 1995). The majority of the work on GCN2 in multicellular organisms has been done on one or two prototype EAA-deficient diet(s) and the findings are assumed to be applicable to deprivation of the rest of the EAAs. For instance, a role of GCN2 in (1) inhibition of hepatic lipid synthesis is reported under leucine deprivation (Guo and Cavener, 2007), (2) inhibition of food intake in mice is tested on either leucine or

threonine deficient diets (Maurin et al., 2005, Hao et al., 2005) and (3) rejection of amino acid deficient diet in fly larvae is tested using corn based diet, which is deficient in tryptophan and lysine (Bjordal et al., 2013). Moreover, evidence for cross-talk between GCN2 and TOR pathways upon amino acid starvation is reported under leucine deprivation in mice (Anthony et al., 2004) or under leucine and arginine deprivation in mouse cell lines (Ye et al., 2015). Here we show that GCN2-mediated responses vary in degree under deprivation of different EAAs, suggesting that GCN2 is not comparably activated by each of the EAAs. This hypothesis is consistent with cell culture based studies that show in breast cancer cell line MCF7, 24 h deprivation of individual EAAs increases phosphorylation of eif2α but to varying degrees (Tang et al., 2015) and in HepG2 human hepatoma cells 2 h deprivation of leucine and threonine result in the largest increase in p-eIF2α, whereas isoleucine, lysine, methionine, and tryptophan elicit the weakest responses (Palii et al., 2009). In addition, previous work with leucine deprivation (100%) and leucine restriction (85%) shows that the physiological responses to them are fundamentally different, thereby questioning the assumption that GCN2 is comparably activated by any degree of restriction of an EAA (Anthony et al., 2013). Considered together, these findings indicate that both the EAA being deprived and the degree of restriction play important role in the activation of GCN2 kinase. Therefore, a precise way to dissect GCN2 functions is to study its responses under deprivation of individual amino acids.

Moreover, our study also highlights that full amino acid starvation poses different stress than an EAA deprivation and is dealt by GCN2 independent mechanisms. Consistently, data from HEK293 cells suggest that protein synthesis under full amino acid starvation is controlled by phosphorylation of eIF2B and is independent of changes in GCN2/eIF2α phosphorylation (Wang and Proud, 2008). Furthermore, in HepG2 total amino acid starvation decreases 4E-BP phosphorylation, suggesting that full amino acid starvation might involve the TOR pathway (Palii et al., 2009). However, more *in vivo* work is needed to understand the mechanisms by which cells sense and response to single and total amino acid starvation.

3.3.8 Effect of genetic background on GCN2 mutation

Physiological phenotypes including development, lifespan and stress resistance are highly sensitive to genetic background. A study with 41 recombinant inbred mouse strains shows that the response to DR-mediated longevity vary greatly between the different genetic strains (Liao et al., 2010). Furthermore, single gene mutations can interact epistatically with the genetic background to influence specific traits (Spencer et al., 2003). Previous work in flies has reported that phenotypes of specific mutants might only be restricted to one genetic background. No Influence of I'm-not-dead-yet (Indy) mutations on lifespan in *Drosophila* was observed after correction for genetic and cytoplasmic background effects (Toivonen et al., 2007). Superoxide dismutase over-expression in shorter-lived fly strains had a more dramatic increase in longevity than in the long-lived fly strain (Sun and Tower, 1999). We have shown that most of the phenotypes controlled by *Gcn2* mutation were independent of genetic background effects. However, the genetic background had strong effects on the strength of the phenotype, suggesting a strong interaction of genetic and dietary factors in the regulation of survival under amino acid deprivation. Furthermore, our observation that removal of some of the EAAs from the diet has no effect on the survival of the w^{1118} inbred strain is counter-intuitive given that EAAs are critical for survival. This argues in favor of using a healthy long-lived outbred genetic background like w^{Dah} to study the effect of single gene mutations on lifespan.

3.3.9 Lifespan extension and starvation resistance conferred by rapamycin mediated TOR-inhibition is GCN2 independent in flies

Genetic or pharmacological down regulation of TOR extends lifespan from yeast to mammals (Kapahi et al., 2010). A recent study shows that *Gcn2* deletion decreases the long lifespan of TOR-deficient (LET-363) worms (Rousakis et al., 2013) suggesting that GCN2 is required as a downstream effector to modulate longevity benefits conferred by TOR inhibition. In contrast our data show that in flies rapamycin-induced inhibition of TOR increased lifespan and starvation resistance in both wild type and *Gcn2*-deficient flies, indicating that GCN2 is not essential in modulating longevity benefits conferred by rapamycin-induced TOR repression. Our observation is in accordance with a gene expression analysis study (Deval et al., 2008) that shows rapamycin and amino acid deprivation do not regulate the same pattern of genes, indicating that TOR and GCN2 pathways are not involved in regulation of similar functions. However, since rapamycin directly inhibits the TOR pathway, we cannot exclude the possibility that GCN2 activation

might occur upstream of TOR repression. This direction of interaction between the two pathways is supported by some findings in cell culture and rodents showing that GCN2 activation is necessary to inhibit TOR signaling in response to amino acid deprivation (Averous et al., 2016; Gallinetti et al., 2013; Ye et al., 2015). A diet devoid of leucine fails to inhibit TOR activity in the liver of *Gcn2*-deficient mice (Anthony, 2004). Furthermore, a recent study in mouse embryonic fibroblast suggests that transcriptional activation of sestrin2, a target gene of GCN2/ATF4 pathway, is required to sustain TOR inhibition upon leucine deprivation (Ye et al., 2015). Together these studies suggest that GCN2 act upstream of TOR kinase, however by what mechanisms GCN2 inhibits TOR kinase remains poorly understood.

CHAPTER 4

Role of GCN2 in dietary restriction and the transsulfuration pathway

Contents

4.1 INTRODUCTION

DR, classically defined as reduced food intake without malnutrition, extends lifespan in a broad range of organisms ranging from worms, flies to mammals (Fontana et al., 2010). Accumulating evidence suggests a causal role of amino acids, particularly methionine, in mediating longevity benefits of DR (Fontana and Partridge, 2015). In flies, adding back methionine to the diet of DR flies abrogates lifespan extension (Grandison et al., 2009). In rodents, methionine restriction increases lifespan (McIsaac et al., 2016; Miller et al., 2005; Orentreich et al., 1993). However, the underlying molecular mechanisms of DR-mediated longevity are only poorly understood. Recent studies suggest a role of the highly conserved transulfuration pathway (TSP) in mediating DR-dependent longevity in yeast, worms and flies (Hine et al., 2015; Kabil et al., 2011).

4.1.1 The Transsulfuration Pathway

The evolutionary conserved transsulfuration pathway (TSP) is responsible for the metabolism of sulfur containing amino acids (SAAs) methionine and cysteine (Figure 4.1). Dietary methionine, is converted to homocysteine via the intermediates S-AdoMet (SAM) and S-AdoHcy (SAH). Homocysteine can either be re-methylated back to methionine by the process of transmethylation under conditions of methionine limitation or directed to the TSP to form cysteine under conditions of methionine sufficiency (Figure 4.1) (Stipanuk and Ueki, 2010). Conversion of homocysteine to cysteine via the TSP occurs in two steps, which are catalyzed by the tandem activity of the two pyridoxal-phosphate (PLP) dependent TSP-enzymes. The first step is catalyzed by cysthathionine-beta-synthase (CBS), which converts homocysteine to cystathionine. In the second step, cystathionine is converted to cysteine by cysthathionine-gamma-synthase (CGL). Further, cysteine is used for production of taurine and glutathione (GSH) (McBean, 2011; McIsaac et al., 2016). Besides, the canonical role of homocysteine conversion to cysteine, the two regulatory enzymes of the TSP, CBS and CGL, also catalyze alternative reactions utilizing cysteine or homocysteine to produce hydrogen sulfide (H_2S) gas (Figure 4.1) (Singh et al., 2009).

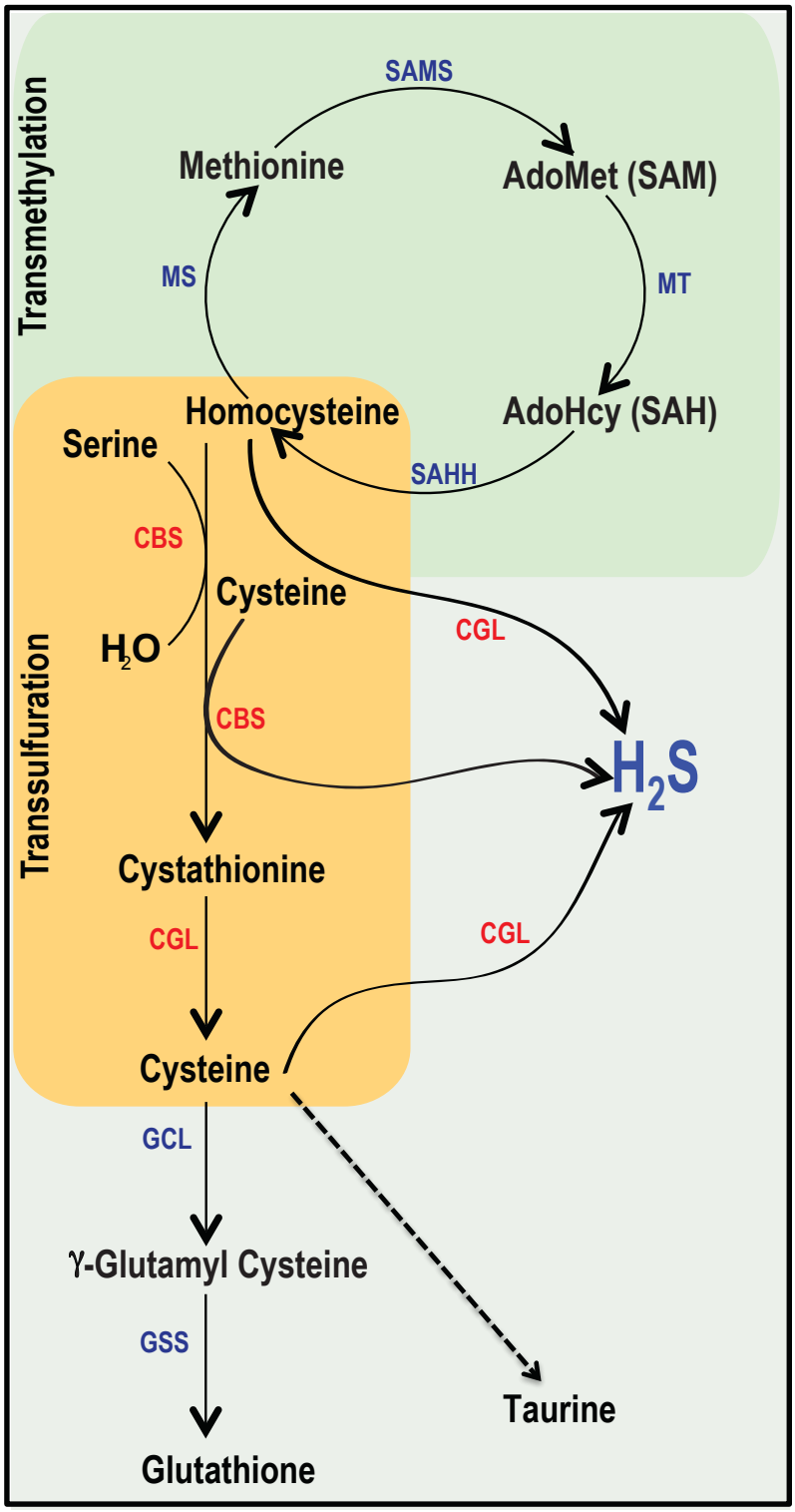

Figure 4.1: The Transsulfuration pathway (TSP)
Key metabolites involved in the transmethylation and transsulfuration pathway. Both CBS and CSE enzymes can produce H_2S using a number of reactions (red). Enzymes: SAMS (S-adenosyl methionine synthase), MT (methyl transferase), SAHH (S-adenosyl homocysteine hydrolase), CBS (cystathionine beta synthase), CGL (cystathionine gamma lyase), GCL (glutamyl cysteine synthase), GS (glutathione synthase), MS (methionine synthase).

4.1.2 Linking H_2S and DR-mediated longevity

H_2S, a water and fat soluble gas has been implicated in a variety of physiological functions and has emerged as a potential therapeutic target in a growing list of maladies including atherosclerosis, type1 diabetes and angiogenesis (Guangdong Yang et al., 2008; Zhang et al., 2013). Evidence that H_2S could also affect organismal lifespan came from a study that showed that exposure to low levels of exogenous H_2S extends lifespan in worms (Miller et al., 2005). Recently, another study in worms has suggested a role of the TSP and H_2S in promoting lifespan extension of germline-deficient animals (Wei and Kenyon, 2016).

A link between the TSP and DR-mediated lifespan extension was first suggested in flies (Kabil et al., 2011) Increased gene expression and protein level of the TSP enzyme, CBS was sufficient to increase fly lifespan, while inhibition of the TSP abrogated lifespan extension in DR animals (Kabil et al., 2011). More recently, it has been suggested that the TSP controls DR-mediated longevity through one of its metabolite, H_2S (Hine et al., 2015). Increased TSP-mediated H_2S production has been suggested to be causative for DR benefits including extended lifespan, in yeast, worms, and flies. However the underlying mechanism by which endogenous H_2S would control DR-mediated longevity and other benefits are still unknown. Moreover, some other aspects remain unanswered, including whether increased H_2S is an essential requirement for different DR regimens, how the TSP enzymes and H_2S production are regulated in response to SAAs availabity and whether major amino acid sensing pathways (TOR or GCN2) could potentially be involved in this pathway? Thus, although progress in understanding TSP-dependent control of DR-longevity has been initiated, several important mechanistic questions remain to be investigated.

4.2 RESULTS

In *eat-2* worms, a *C. elegans* model for DR, GCN2 is essential for DR-mediated lifespan benefits (Rousakis et al., 2013). Our data from previous chapter furthermore show that GCN2 is required for survival under single amino acid deprivation. Therefore, we tested whether GCN2 function is also essential for the beneficial effects of DR on survival in flies. In addition we tested whether GCN2 plays a role in the transulfuration pathway (TSP), which has recently been suggested to confer DR benefits via production of H_2S gas (Hine et al., 2015).

4.2.1 GCN2 function is not essential for DR mediated lifespan extension

Traditionally DR in *Drosophila* is achieved by diluting the yeast content, the major protein source, in the food. Thus, we first tested the effect of dietary protein restriction via yeast dilution on the survival of w^{Dah} control and $Gcn2^{1}$ mutant flies. As expected, wDah control flies displayed a typical tent-shaped DR response, with increased median lifespan on intermediate yeast concentrations (0.5-1.0SYA) and a reduced lifespan on the high yeast 2.0SYA food (Figure 4.2A and 4.2B). Interestingly, $Gcn2^{1}$ mutant flies showed a very similar DR response with higher median lifespan on intermediate yeast concentrations (Figure 4.2A and 4.2B). Noteworthy, on both intermediate yeast concentrations, $Gcn2^{1}$ mutants were significantly longer lived than w^{Dah} flies. Thus, in summary our data show that *Gcn2* mutants are able to respond to DR and suggest that GCN2 function is not essential for DR-mediated lifespan extension in flies.

Methionine and/or cysteine restriction (M/C-R) can also extend lifespan in mice and flies (Grandison et al., 2009; Lee et al., 2014a; Miller et al., 2005). We therefore tested whether GCN2 is required for the beneficial effects of methionine restriction on lifespan, employing the holidic medium. A 5-fold reduction in methionine content (0.2xM Yaa) and a combined methionine and cysteine restriction (0.2xM-0.2xC Yaa) significantly increased the survival of w^{Dah} flies compared to the control diet Yaa (Figure 4.2C and 4.2D). $Gcn2^{1}$ mutants also displayed significantly increased lifespan on the M/C-R diets, compared to Yaa medium, suggesting that GCN2 function is not essential for lifespan extension under M/C-R (Figure 4.2C and 4.2D). In addition, we tested the survival of w^{Dah} flies and $Gcn2^{1}$ mutants under high methionine conditions (five fold higher (5xM Yaa)). Interestingly, $Gcn2^{1}$ mutants showed a significantly reduced median lifespan on the 5xM Yaa medium compared to Yaa medium. In contrast, w^{Dah} control flies remained unaffected by excess methionine (Figure 4.2C and 4.2D), suggesting that GCN2 function is important

in the organismal response to high methionine levels. In summary our results show that in contrast to *C.elegans*, GCN2 function is not essential for DR- or methionine-restriction-mediated lifespan extension in flies.

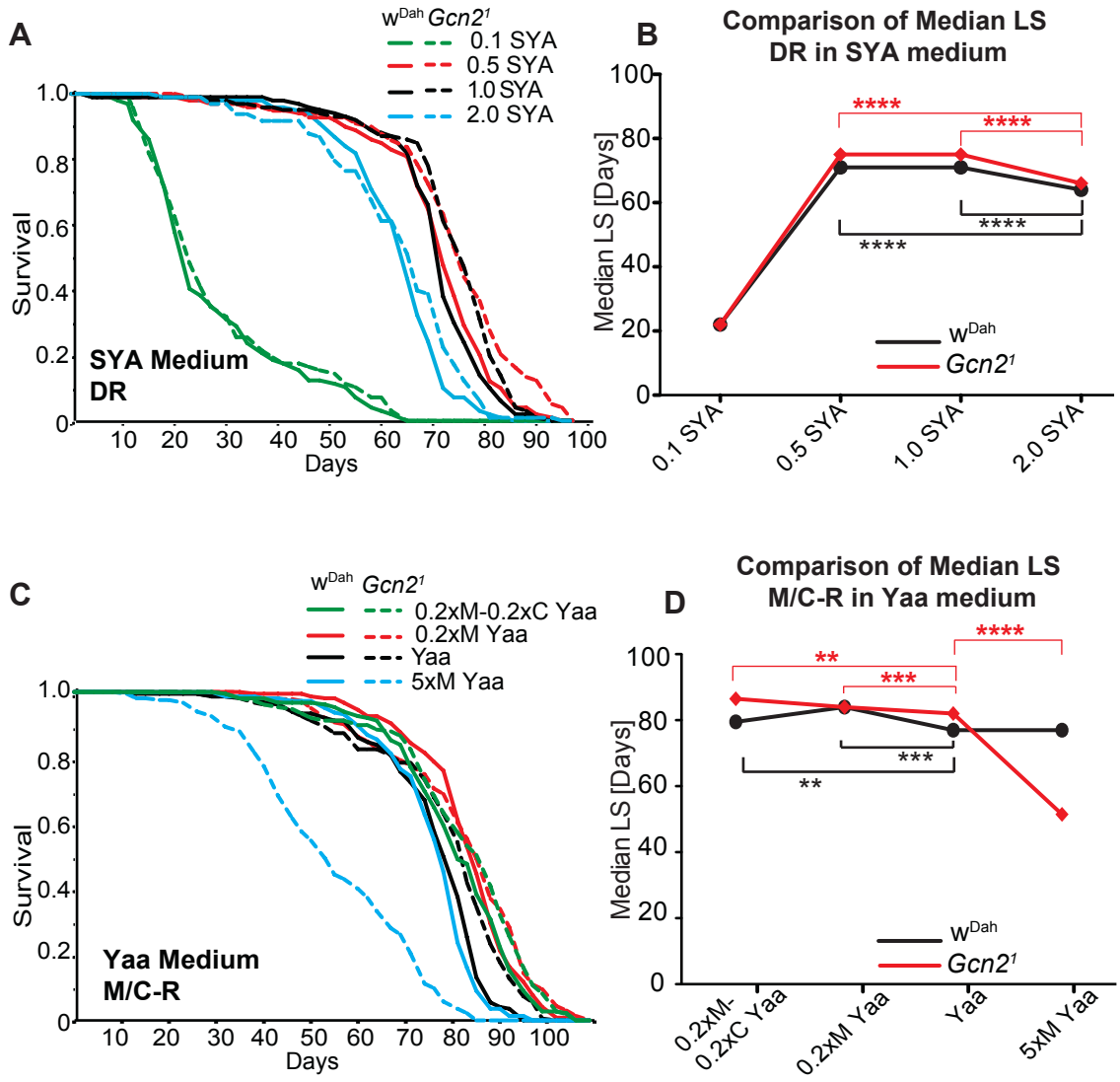

Figure 4.2: GCN2 is not essential for DR-mediated lifespan extension in *Drosophila*
(A & C) Survival curve of w^{Dah} flies and *Gcn2*^{1} mutants under DR and M/C-R respectively. **(B)** w^{Dah} flies and *Gcn2*^{1} mutants showed significantly increased median lifespans under DR (yeast dilution: 0.5 SYA and 1.0 SYA) compared to 2.0 SYA. **(D)** w^{Dah} flies and *Gcn2*^{1} mutants showed significantly increased median lifespans under M/C-R (0.2xM Yaa and 0.2xM-0.2xC Yaa) compared to the control diet Yaa. *Gcn2*^{1} mutants were significantly shorter lived under high methionine condition (5xM) compared to Yaa. *(****p < 0.0001. ***p<0.001, **p<0.01, log-rank test; n=150 flies/diet). Lifespan analysis under DR (Figure 4.2A) was done by Dr. Sebastian Grönke.*

4.2.2 GCN2 is required for the TSP mediated H_2S production in flies

The sensitivity of *Gcn2* mutants to high methionine concentrations might implicate GCN2 function in regulation of methionine metabolism. Thus, we analyzed H_2S production capacity as a proxy for TSP activity (Hine et al., 2015). The enzymatic based assay required exogenous addition of substrate (cysteine or homocysteine) and the cofactor pyridoxal-5′-phosphate (Hine et al., 2015). Because enzymatic activity is affected by the concentration and type of substrate used, we measured H_2S production at different concentrations of cysteine as well as homocysteine.

Interestingly, we observed that at low exogenous cysteine concentrations (5mM and 7.5mM), $Gcn2^{1}$ mutants had significantly reduced H_2S production compared to w^{Dah} control flies (Figure 4.3A). In contrast, at high cysteine concentrations (10mM and 15mM) $Gcn2^{1}$ mutants and w^{Dah} control flies had comparable H_2S production (Figure 4.3A). We next measured H_2S production by adding homocysteine as a substrate (Figure 4.3B). Strikingly, no H_2S production was observed in $Gcn2^{1}$ mutants at any of the tested homocysteine concentrations. In contrast, w^{Dah} flies showed normal H_2S production.

Notably, H_2S measurements were done on flies that were kept on the Yaa medium for 15 days. Therefore, to rule out any possible diet-induced effect on H_2S production and to confirm our observations on an independent fly food medium, we repeated H_2S measurements on standard yeast based SYA medium. Consistent with our observation on Yaa medium, H_2S production was significantly reduced in $Gcn2^{1}$ mutants compared to w^{Dah} flies at low exogenous cysteine concentrations but was comparable at high cysteine concentrations (Figure 4.3C). Remarkably, and in line with our observations on Yaa medium, $Gcn2^{1}$ mutants did not produce any H_2S when homocysteine was added as a substrate (Figure 4.3D). Thus, using two independent food conditions, we showed that GCN2 kinase affects TSP-mediated H_2S production in flies.

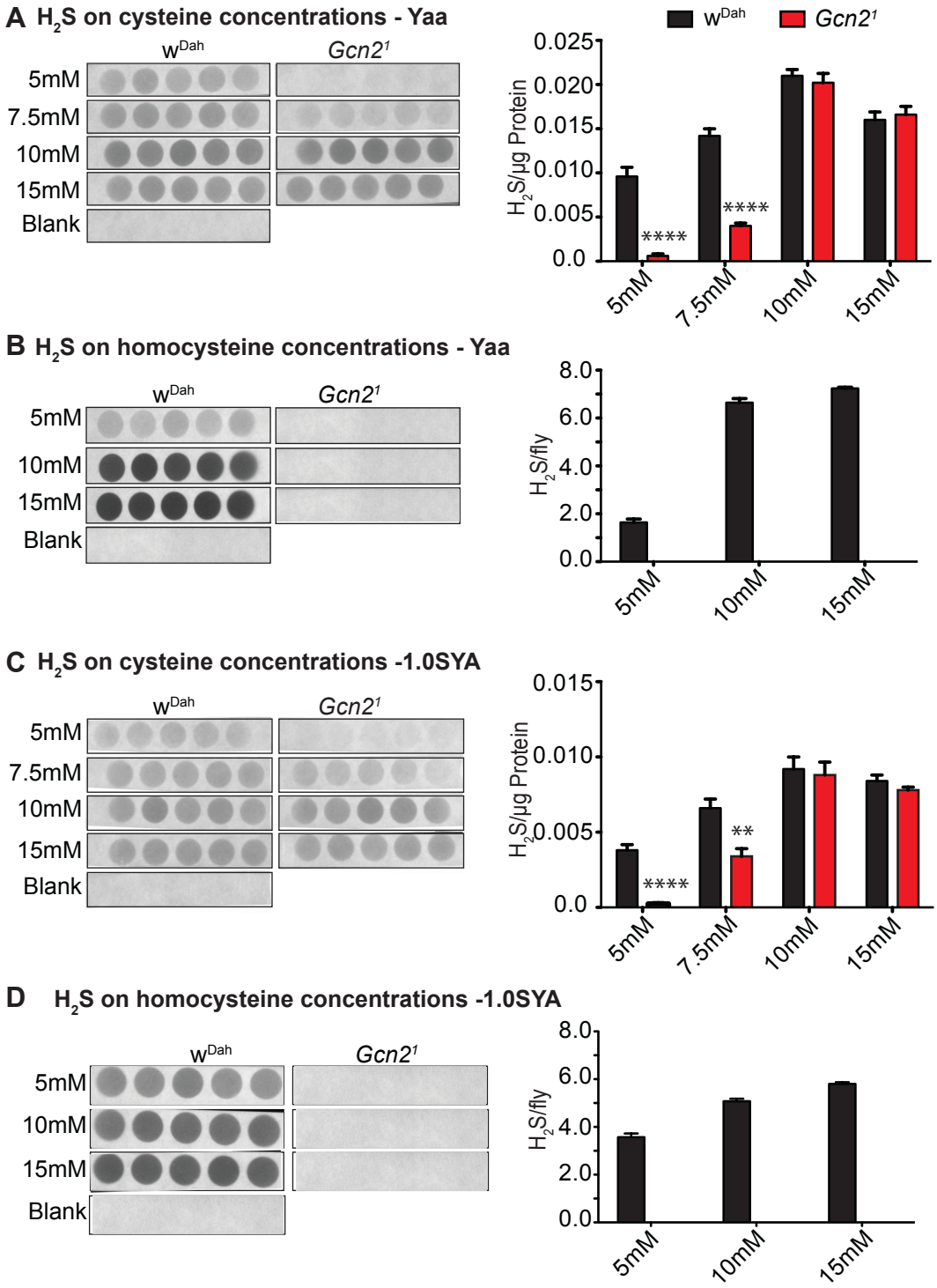

Figure 4.3: **GCN2 affects TSP-mediated H_2S production in flies**
H_2S production was measured at different concentrations of cysteine and homocysteine on Yaa and SYA medium. Image of H_2S blot is shown right, corresponding quantification is shown left. On **(A)** Yaa and **(C)** SYA medium H_2S production in *Gcn2*^{1} mutant flies was significantly reduced compared to w^{Dah} flies on lower cysteine concentrations (5mM and 7.5mM) but was similar on higher cysteine concentrations (10mM and 15mM), (*****p<0.0001, **p<0.01, Student's t-test).* On **(B)** Yaa and **(D)** SYA medium, no H_2S was detected in *Gcn2*^{1} mutant flies at any of the tested concentrations of homocysteine *(n=5 replicates/treatment/genotype; error bar shows SEM).*

4.2.3 TSP mediated H_2S production is not essential for DR-mediated longevity in flies.

As previously mentioned, increased TSP-dependent H_2S is essential for DR-mediated longevity in yeast, worm, fruit fly, and rodents (Hine et al., 2015). Interestingly, our data showed that DR-induced longevity is GCN2 independent but TSP mediated H_2S production, at least in part, is dependent on GCN2. Therefore, we decided to re-examine the link between DR-mediated longevity and H_2S production in flies by measuring H_2S production of w^{Dah} control flies and *Gcn2*^{1} mutants under DR.

We first measured H_2S production under M/C-R using cysteine (7.5mM) as a substrate. To our surprise, we found that H_2S production in w^{Dah} flies as well as in *Gcn2*^{1} mutants was lower on M/C restricted diets (0.2xM Yaa and 0.2xM-0.2xC Yaa), which resulted in significant lifespan extension, compared to control and high methionine diets (Yaa and 5xM Yaa), in contrast with the published results (Figure 4.4A). Similar observations for H_2S production under M/C-R were seen when 10mM cysteine was used (Figure 4.5A). Notably, H_2S production in *Gcn2*^{1} mutants compared to w^{Dah} flies on all diets was significantly reduced when 7.5 mM cysteine was used (Figure 4.4A) and comparable when 10mM cysteine was used (Figure 4.5A).

Next we repeated H_2S measurements under M/C-R using homocysteine as a substrate. We observed the same trend in w^{Dah} flies i.e. low H_2S production under methionine (0.2xM) and methionine-cysteine (0.2xM-0.2xC) restriction compared to high methionine concentrations (Yaa and 5xM Yaa) (Figure 4.4B). Notably, *Gcn2*^{1} mutants did not produce any H_2S with homocysteine as substrate on any of the diets, in line with previous observation (see also Figure 4.3B). Furthermore, we also measured the expression of the two main H_2S producing TSP enzymes, CBS and CGL, on 0.2xM and 1xM Yaa. Consistent with the H_2S production, relative expression of both the enzymes in w^{Dah} flies and *Gcn2*^{1} mutants was significantly reduced under methionine restriction (Figure 4.4C and 4.4D).

To further verify our results we measured H_2S production under DR using yeast dilution (SYA) and 7.5mM cysteine as exogenous substrate. Consistent with our observations under M/C-R and contrary to the published results, we observed that flies kept on the protein restricted diet (0.5 SYA) which resulted in lifespan extension, had significantly less H_2S production compared to the protein rich diet (2.0 SYA) on which survival was reduced (Figure 4.4E). Similar observations for H_2S production under DR were seen when

10mM cysteine was used (Figure 4.5B). Notably, H_2S production in *Gcn2*^{1} mutants compared to w^{Dah} flies on all diets was significantly reduced when 7.5 mM cysteine was used (Figure 4.4E) and comparable when 10mM cysteine was used (Figure 4.5B).

In summary, using two different lifespan extending dietary interventions, M/C-R and DR our results show that in flies increased lifespan is not correlated with increased H_2S production capacity. Furthermore, we show that in *Gcn2*^{1} mutants, a genetic model of reduced H_2S production capacity, lifespan is also uncoupled from H_2S production capacity. In contrast to published data our results rather suggest that H_2S production is correlated with methionine concentration in the diet and not with lifespan.

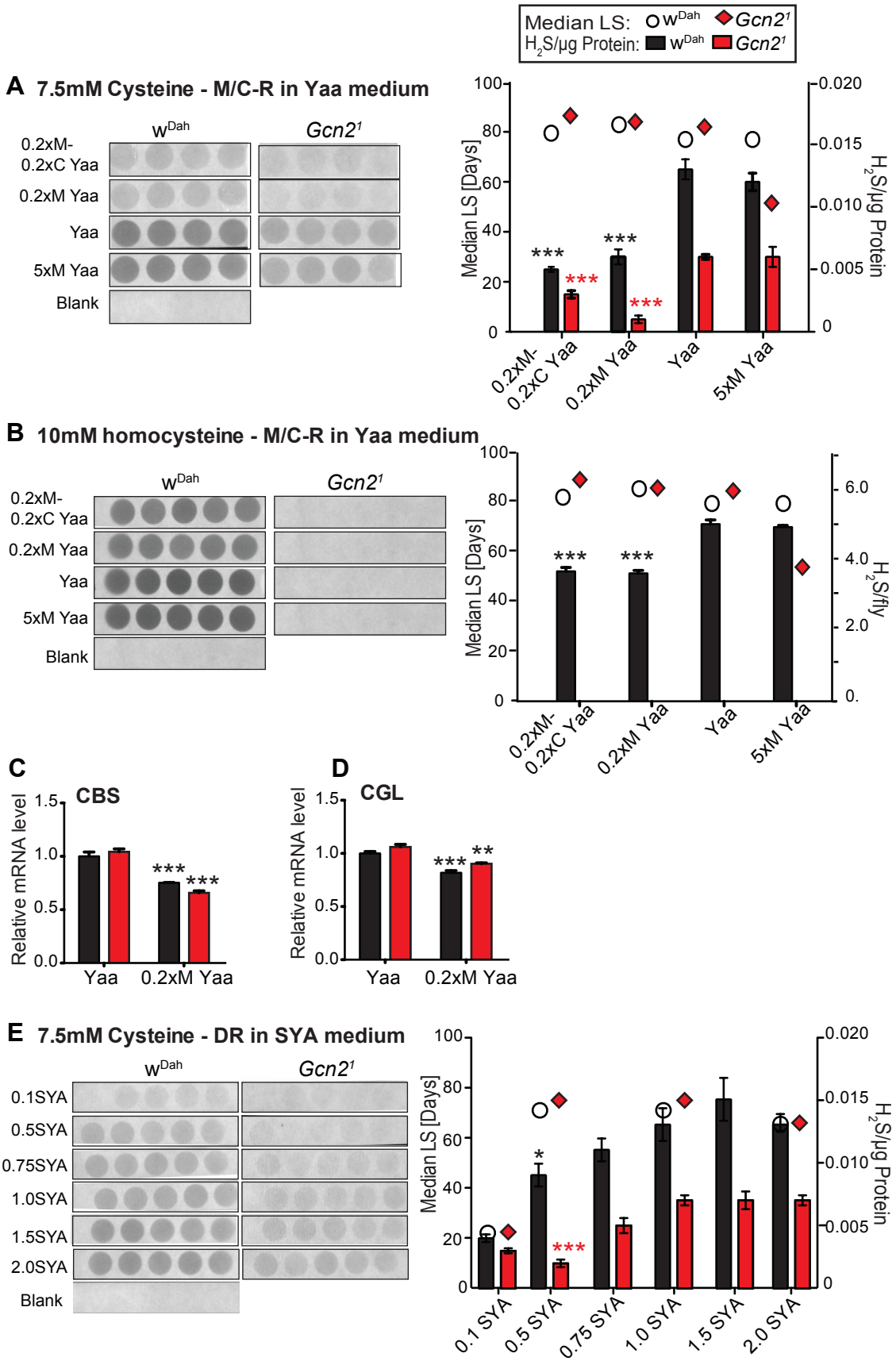
Median LS: wDah Gcn2¹
H2S/µg Protein: wDah Gcn2¹
A 7.5mM Cysteine - M/C-R in Yaa medium
wDah
Gcn2¹
0.2xM-0.2xC Yaa
0.2xM Yaa
Yaa
5xM Yaa
Blank
Median LS [Days]
H2S/µg Protein
B 10mM homocysteine - M/C-R in Yaa medium
H2S/fly
C
CBS
Relative mRNA level
D
CGL
E 7.5mM Cysteine - DR in SYA medium
0.1SYA
0.5SYA
0.75SYA
1.0SYA
1.5SYA
2.0SYA
0.1 SYA
0.5 SYA
0.75 SYA
1.0 SYA
1.5 SYA
2.0 SYA

Figure 4.4: TSP mediated H_2S production capacity does not correlate with longevity in flies
Image of H_2S blot is shown right, corresponding quantification is shown left. Median lifespans are plotted against H_2S measurement on each diet **(A)** H_2S measured under M/C-R using 7.5mM cysteine as a substrate. w^{Dah} control and *Gcn2*^{1} mutant flies produced significantly less H_2S on diets restricted for methionine and/or cysteine (0.2xM-0.2xC Yaa and 0.2xM Yaa) compared to control diet, Yaa *(One way ANOVA followed by Tukey's multiple comparison test, ***p<0.001)* **(B)** H_2S measured under M/C-R using 10mM homocysteine as a substrate. w^{Dah} flies showed significantly reduced H_2S on 0.2xM Yaa and 0.2xM-0.2xC Yaa compared to the control diet Yaa *(One way ANOVA followed by Tukey's multiple comparison test, ***p<0.001)*. No H_2S production was observed in *Gcn2*^{1} mutants. **(C & D)** Relative mRNA expression of CBS and CGL was significantly reduced on 0.2xM Yaa compared to Yaa diet in w^{Dah} flies and *Gcn2*^{1} mutants *(Student's t-test: ***p<0.001, **p<0.01)*. **(E)** H_2S measured under DR using 7.5mM cysteine as a substrate. w^{Dah} and *Gcn2*^{1} mutants produced significantly less H_2S on the protein restricted diet (0.5 SYA) compared to 2.0 SYA *(One way ANOVA followed by Tukey's multiple comparison test, ***p<0.001, *p<0.05)*. **(A, B & E)** Compared to w^{Dah} flies H_2S production in *Gcn2*^{1} mutants was significantly reduced on each diet *(p<0.001, Student's t-test, n=5 replicates/treatment/genotype; error bar shows SEM)*.

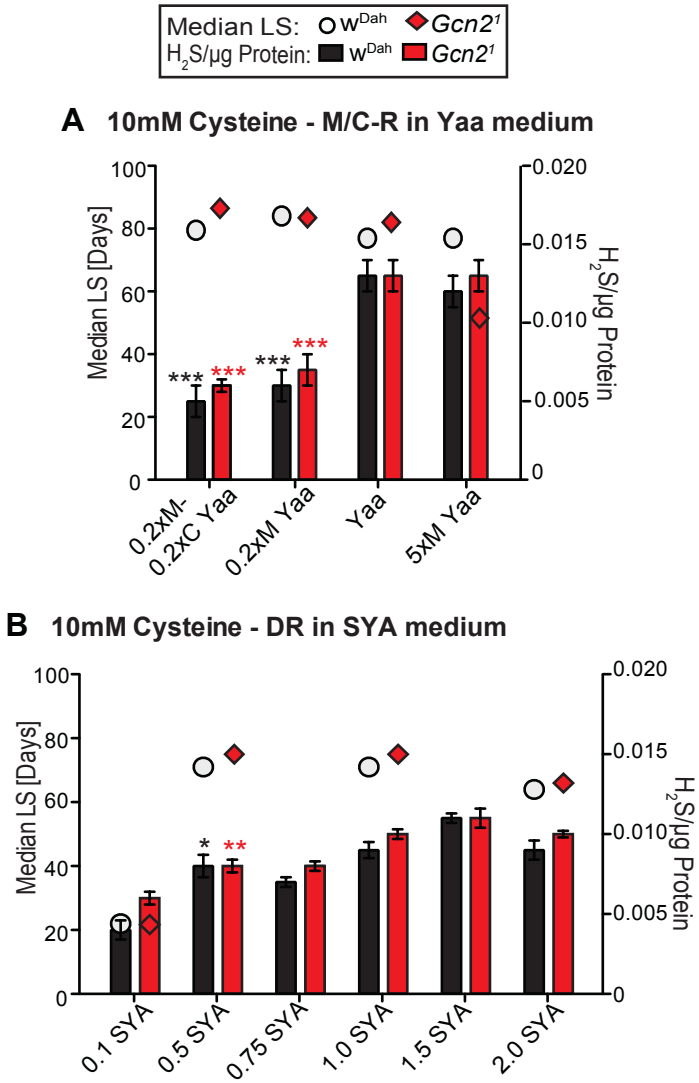

Figure 4.5: TSP mediated H_2S on DR diets using 10 mM cysteine
H_2S measured under M/C-R ad DR using 10mM cysteine as a substrate. Median lifespans are plotted against H_2S measurement on each diet. **(A)** w^{Dah} control and *Gcn2*^{1} mutant flies produced significantly less H_2S on diets restricted for methionine and/or cysteine (0.2xM-0.2xC Yaa and 0.2xM Yaa) compared to control diet, Yaa. **(B)** w^{Dah} control and *Gcn2*^{1} mutant flies produced significantly less H_2S on protein restricted diet (0.5 SYA) compared to 2.0 SYA *(One way ANOVA followed by Tukey's multiple comparison test, ***p,0.001, **p<0.01, n=5 replicates/treatment/genotype; error bar shows SEM).*

4.2.4 GCN2 affects the status of some of the TSP metabolites

Upon observing that the TSP-mediated H_2S production was lower or absent in *Gcn2*^{1} mutants when cysteine or homocysteine was used as a substrate, respectively, we speculated that limitation in intermediate TSP metabolites might be causal for the lower H_2S production in *Gcn2*^{1} mutants. In order to address this possibility, we conducted a mass spectrometry-based metabolomics analysis using w^{Dah} control and *Gcn2*^{1} mutants to measure different TSP metabolites. Mass-spectrometry was done in collaboration with the

mass-spectrometry core unit at the Max-Planck Institute for Biology of Ageing. Most TSP metabolites were unchanged between w^{Dah} control and *Gcn2*^{1} mutant flies including methionine, homocysteine, reduced glutathione and cysteine (Figure 4.5A-4.5I), suggesting that differences in TSP metabolite availability are probably not sufficient to explain the differences in H_2S production capacity in *Gcn2*^{1} mutants. Interestingly, we observed statistically significant differences in S-adenosyl methionine (SAM) and cystathionine levels between *Gcn2*^{1} mutants and w^{Dah} control flies (Figure 4.6B and 4.6E), suggesting that TSP activity are altered in *Gcn2*^{1} mutants, however, the exact mechanisms at work are still unclear.

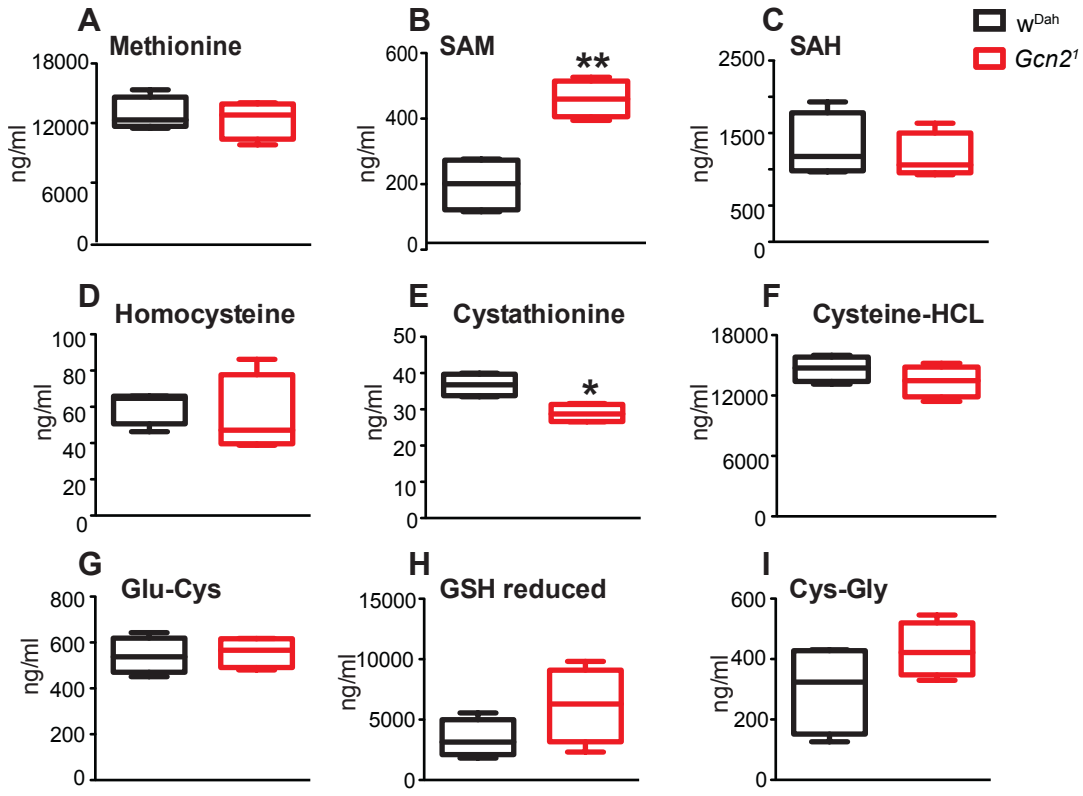

Figure 4.6: UPLC-MS/MS analysis of TSP metabolites in w^{Dah} control and *Gcn2*^{1} mutant flies
(A-I) UPLC-MS/MS analysis of TSP metabolites in 15-days old w^{Dah} control and *Gcn2*^{1} null mutant flies. SAM and cystathionine were differentially regulated in *Gcn2*^{1} mutants. *(Student's t-test: **p<0.01, *p<0.05; error bar shows SEM for 4 biological replicates*).

4.3 DISCUSSION

4.3.1 Role of GCN2 in DR-mediated longevity and the TSP

DR, classically defined as reduced food intake without malnutrition, extends lifespan in diverse species (Fontana et al., 2010). Amino acids, in particular methionine restriction, have been shown to play a causal role in mediating longevity benefits of DR in yeast (Wu et al., 2013a; 2013b), flies (Grandison et al., 2009; Lee et al., 2014) and rodents (McIsaac et al., 2016; Miller et al., 2005; Orentreich et al., 1993), however the underlying molecular mechanisms remain elusive. In *eat*-2 worms, a *C. elegans* model for DR, GCN2 is essential for DR-mediated lifespan benefits (Rousakis et al., 2013). In contrast, our finding shows that GCN2 function is not essential for DR- or methionine-restriction-mediated lifespan extension in flies, suggesting that methionine restriction might not be sensed by GCN2. This hypothesis is consistent with a recent finding that shows that physiological responses to methionine restriction are independent of GCN2 in mice (Wanders et al., 2016). However, our data indicate that GCN2 function is important for organismal survival under high methionine condition. A high methionine content could be detrimental due to its conversion to homocysteine (Pamplona and Barja, 2006), which could imply that GCN2 function is required for the clearance of homocysteine that occur via the TSP. This hypothesis is consistent with our finding that GCN2, at least in parts, regulates the TSP. In addition, our observation that no H_2S is produced in the absence of GCN2 when homocysteine is used as a substrate also argues for a role of GCN2 in regulating homocysteine levels through the TSP, although the exact mechanisms at work are still unclear. Interestingly, microarray data in cell culture suggest that cysteine deprivation induces expression of the TSP gene, *cgl*, most likely via the GCN2/ATF4 pathway (Harding et al., 2003; Lee et al., 2008). However, our qRT-PCR data indicate that, at least under methionine restriction, *cbs* and *cgl* expression is not dependent on GCN2. Nonetheless, GCN2 function could affect the activity of these enzymes at the protein level, which remains to be tested.

4.3.2 Uncoupling the link between TSP-dependent H_2S production and DR-mediated longevity in flies

Increased H_2S production via the TSP is reported to be an evolutionary conserved response to DR in yeast, worm, fruit fly, and rodent (Hine and Mitchell, 2015; Hine et al., 2015). Here, we have re-examined the connection between DR-induced longevity and

TSP-mediated H_2S production and surprisingly found no evidence to support this link in flies. Using two different lifespan extending dietary interventions, M/C-R and DR, and a genetic model of reduced H_2S production capacity, *Gcn2* mutants, we have shown that increased lifespan is not correlated with increased H_2S production capacity. We cannot exclude the possibility that the discrepancy between our results and the published report might reflect differences in the food conditions or use of different genetic background (w^{Dah} in this study and w^{1118} in the Hine et al., 2015). Nevertheless, our findings clearly show that this phenomenon is not nearly as robust or universal as is implied by the recent findings.

In contrast to the published data, our results rather suggest that H_2S production is positively correlated with methionine concentration in the diet and not with lifespan. Our observations are consistent with the available literature about the TSP and its regulation in response to methionine availability (Stipanuk and Ueki, 2010). Prudova and colleagues have shown that under conditions of methionine restriction, CBS protein levels are reduced by >10 fold, suggesting that the TSP activity is down regulated under limitation of methionine (Prudova et al., 2006). Furthermore, under high methionine conditions CBS is allosterically activated by SAM and methionine is directed towards the TSP to form cysteine. Conversely, when methionine levels are low, CBS is destabilized and flux through the TSP is down-regulated to conserve cellular methionine (Martinov et al., 2000). Therefore, the idea that TSP-mediated H_2S production is high under limitation of methionine and cysteine is counter-intuitive.

CHAPTER 5

Conclusion And Future Perspectives

In this study, I have dissected the *in vivo* functions of the amino-acid-starvation-sensor, GCN2 kinase, in response to the absence/limitation of each of the 10 EAAs, using a *Drosophila Gcn2* null mutant and a fully defined chemical diet. Findings of the study demonstrate that GCN2 kinase plays a vital role in regulating several important physiological responses during nutritional deprivation of EAAs and that GCN2-mediated responses are not influenced by genetic variability.

I have shown that GCN2 is essential for development and longevity under deprivation of an EAA. Furthermore, this study suggests that GCN2 induces a compensatory feeding response in flies under long-term nutritional deprivation of an EAA, leading to increased lipid level, which is probably causal for the increased resistance of flies under full starvation. Notably, these GCN2-mediated physiological responses vary in degree under deprivation of different EAAs, suggesting that absence of each of the 10 EAAs might not activate GCN2 to similar extent. However, given that GCN2 uses the presence of uncharged t-RNAs as a potential indicator of EAAs starvation, it will be interesting to understand how GCN2 activation depends on the EAA being deprived. Is it the ratio of uncharged to charged t-RNAs that decides the extent of GCN2 activation or is GCN2 more sensitive to specific uncharged t-RNAs? Answers to these questions will enhance our understanding of how GCN2 senses absence of EAAs. Interestingly, our data indicates that GCN2 function is not essential for sensing methionine deprivation, suggesting that there might be other amino acid sensors or pathways to be discovered. In addition, this study shows that an EAA starvation is different from full amino acid starvation and probably dealt with GCN2-independent mechanisms.

ATF4 is considered as a primary downstream effector of GCN2-induced amino acid stress response. This study shows that increased *Atf4* expression is necessary but not sufficient to compensate for GCN2 mediated functions under deprivation of EAAs and the rescue efficiency of *Atf4* over-expression is different under deprivation of different EAAs, suggesting that, depending on which EAA is being deprived, GCN2 might activate additional transcription factors that work alone or in concert with ATF4 to regulate amino acid stress response. However we still have to understand how the cellular concentration of different EAAs differentially trigger the GCN2/ATF4 pathway. In future I plan to use our RNA-seq data to decipher genes, biological processes and pathways differentially regulated by GCN2 under deprivation of different EAAs.

Furthermore, in contrast to worms, GCN2 function in flies is not essential for lifespan extension conferred by rapamycin-mediated TOR inhibition or by DR and M/C-R. However our data suggest that GCN2 function is important in the organismal response to high methionine levels and implicates GCN2 in regulation of the TSP, which is responsible for methionine metabolism. Finally, in contrast to the recent finding that increased TSP-mediated H_2S production is an evolutionary conserved response to DR-mediated longevity in yeast, worm and fruit fly, we have shown that increased lifespan is not correlated with increased H_2S production capacity in flies. However, the molecular mechanisms by which GCN2 mediates the TSP and H_2S production still remain unclear.

Thus, given the crucial role of GCN2 in regulating survival and other important physiological responses under EAAs deprivation, an in-depth understanding of how GCN2 senses absence of an amino acid and transform it to behavioural and physiological changes will provide important insights into the biology of metabolic age-related diseases.

CHAPTER 6

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CONTRIBUTIONS

The *Gcn2*^{1} and *Gcn2*^{2} mutants were designed and generated by Dr. Sebastian Grönke. Microinjection of the constructs for transgenic flies was performed by Jacqueline Eßer. Experiments in Figure 3.1 (B, C and D) were performed by Oliver Hendrich. Analysis of diets in Figure 3.3 (A, B, C and F) was performed together with Dr. George *Soultoukis*. Lifespan analysis in Figure 4.2A was performed by Dr. Sebastian Grönke. Bioinformatic analysis for RNA-Sequencing was done in collaboration with the bioinformatic core facility at the Max-Plack institute for Biology of Ageing. Mass-spectrometry was done in collaboration with the mass-spectrometry core unit at the Max-Planck Institute for Biology of Ageing.

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Anchal Srivastava

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