Translational Control Mediates Lifespan Extension Due to Dietary Restriction in *Drosophila*

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Abstract

Aging is characterized by the declining ability of an organism to maintain homeostasis, which eventually leads to death. Dietary restriction (DR), the reduction of nutrients without malnutrition, extends lifespan in various organisms, yet its molecular underpinnings are poorly understood. We show that in *Drosophila,* DR upregulates the translational repressor 4EBP, the eukaryotic translation initiation factor 4E binding protein, and that this upregulation is necessary for the full lifespan extension upon DR and sufficient to extend lifespan on a nutrient rich diet. Investigation of the genome-wide translational changes upon DR using translation state array analysis (TSAA) found that translationally downregulated genes tend to have extensive 5' untranslated regions (UTR) secondary structures, while those that are upregulated have weakly structured 5'UTRs. Among the translationally upregulated genes, mitochondrial ribosomal proteins and electron transport chain components were overrepresented. Mitochondrial genes were found to have weakly structured 5'UTRs in *Drosophila,* and this was conserved in Humans. The 5'UTRs of mitochondrial genes were found to be sufficient to confer preferential translation during times of high 4EBP activity in a cap-independent manner to reporter constructs. Upregulation of mitochondrial function was verified and found to be *d4EBP* dependent, implicating a novel mechanism for regulating mitochondrial function upon DR. These results implicate mRNA translation initiation in modulating lifespan and mitochondrial function upon DR.

Contents

Background

Chapter 1 Aging

1.1 Introduction

Aging can be defined as the progressive and irreversible decline in physiological function, which leads to an increased susceptibility to death. While it would seem much simpler for an organism to maintain itself in a healthy adult state compared to the complex development of an adult organism from a single sperm and egg, it is apparent this is not the case. In fact, a human's chance of dying doubles every 8.9 years after reaching sexual maturity (Arking 1998). From an evolutionary perspective, aging may arise because of a lack of selective forces acting on organisms that have started reproducing. In most wild populations organisms do not survive to a time in which they have decreased physiological functions because of mortality from environmental factors (Medawar 1952). This leads to a lack of selective pressure against deleterious mutations that may be harmful late in life (Medawar 1952).

1.2 Dietary Restriction

While there is no known way to halt aging, there are ways to delay or slow the aging process. One of the most well studied ways of slowing aging is dietary restriction (DR), the reduction of nutrients without malnutrition. This was first shown to extend the lifespan of rats in the 1930s (McCay, Crowell et al. 1935). This procedure has since been shown to extend lifespan in yeast (Jiang, Jaruga et al. 2000), *C. elegans* (Klass 1977), *Drosophila* (Chapman and Partridge 1996), mice (Weindruch and Walford 1982), and is currently being tested in rhesus monkeys (Roth, Ingram et al. 2001). Along with extending lifespan, DR slows the progression of many age related diseases including

cancer, diabetes, and cardiovascular disease (Weindruch and Walford 1982; Hursting, Lavigne et al. 2003). Upon DR, organisms are thought to undergo a shift in resource allocation from reproduction to somatic maintenance, which would allow an organism to sustain itself until nutrients are abundant again (Holliday 1989).

In mammals, DR is usually performed by restricting total calories ingested (Masoro 2002). In *Drosophila* it has been found that the composition of the nutrients restricted is important. While many labs use whole food dilution, the main determinant in lifespan extension from DR in *Drosophila* is yeast (Mair, Piper et al. 2005), the major amino acid source for the fly. It has also been found that *Drosophila* compensate their food intake dependent on the food dilution (Carvalho, Kapahi et al. 2005), with the carbohydrate content being a major determinant of this compensation (Kapahi unpublished).

1.3 Genetics of Aging

1.31 Insulin Pathway

Along with DR, single gene mutations extend lifespan in a variety of organisms. The *Age-1* mutation in *C. elegans* was the first single gene mutation found to extend lifespan (Friedman and Johnson 1988). *Age-1* is a mutation in the *daf-23* gene, a dauer constitutive gene. The dauer is an alternative form of *C. elegans* larvae induced during times of stress (Riddle and Albert 1997). While normal larvae progress to adulthood in ~3 days, and live for a couple weeks, dauers can be maintained for more than 70 days and then live a normal adult life (Klass and Hirsh 1976). *Age-1* was later cloned and found to be the homolog of mammalian phosphatidylinositol-3-OH kinase (PI(3)K) catalytic subunit (Morris, Tissenbaum et al. 1996). It was found that another lifespan extension

mutant, *daf-2*, was the insulin/IGF-1 receptor ortholog (Kimura, Tissenbaum et al. 1997). The dauer constitutive and lifespan phenotypes of *daf-2* and *age-1* are suppressed by mutations in the dauer defective gene, *daf-16,* a FOXO family transcription factor (Lin, Dorman et al. 1997; Ogg, Paradis et al. 1997), important for heat and oxidative stress resistance, fat metabolism, fertility, and metabolism (Larsen 1993; Finch and Ruvkun 2001). These, along with mutations in *pdk-1*, *daf-18* (the worm homolog of human PTEN), *akt-1,* and *akt-2,* defined the insulin-like signaling pathway (ILSP) as a key regulator of lifespan in *C. elegans (Ogg and Ruvkun 1998; Paradis and Ruvkun 1998; Paradis, Ailion et al. 1999)*. Reduction of flux through the ILSP has been found to be a conserved lifespan extension pathway. In flies, mutants in the insulin receptor (*InR*) (Tatar, Kopelman et al. 2001) or the insulin receptor substrate (*chico*) (Clancy, Gems et al. 2001) extend lifespan, as well as overexpression of the *daf-16* homolog (*dFOXO*) (Giannakou, Goss et al. 2004; Hwangbo, Gershman et al. 2004). In mice, lifespan was extended by both a fat-specific insulin receptor knockout (FIRKO) (Bluher, Kahn et al. 2003) or heterozygote knockout mice for the insulin-like growth factor type 1 receptor (*Igfr1*) (Holzenberger, Dupont et al. 2003).

1.32 TOR Pathway

Another nutrient sensing growth pathway that is parallel and interacting with the ILSP is the target of rapamycin (TOR) pathway. The *Drosophila* homologs of human *Tsc1* (*Hamartin*) and *Tsc2* (*tuberin*) function *in vivo* as a complex that controls growth and size in a cell-autonomous manner (Ito and Rubin 1999; Potter, Huang et al. 2001). *Tsc2* acts as a GTPase-activating protein (GAP) for *Rheb* (*R*as *h*omolog *e*nriched in *b*rain), a small, highly conserved guanine triphosphatase (GTPase) (Inoki, Li et al. 2003). *Rheb* has

been demonstrated through genetic and biochemical analyses to function downstream of the tuberous sclerosis complex (*TSC1/TSC2*) and activate the kinase activity of TOR (Inoki, Li et al. 2003; Stocker, Radimerski et al. 2003). TOR regulates many processes important for cell growth including protein synthesis (Burnett, Barrow et al. 1998), ribosome biogenesis (Powers and Walter 1999), and autophagy (Noda and Ohsumi 1998). Two downstream effectors of TOR which affect protein synthesis are ribosomal S6 kinase (S6K) and the translational inhibitor eIF4E binding protein (4EBP). S6K was originally thought to affect translation by regulating the translation of 5'-tract of polypyrimidine (TOP) mRNAs, but this has since been found to be untrue (Pende, Um et al. 2004). It has also been seen that S6K1-deficient mice that have a growth defect also have normal phospho-S6 levels, implicating a pathway independent of S6 in S6K's role in growth regulation (Shima, Pende et al. 1998). A way in which S6K may exert its influence on growth is by phosphorylating the translation initiation factor eIF4B (Raught, Peiretti et al. 2004). Another downstream component of the TOR pathway is 4EBP, which is directly phosphorylated by TOR (Burnett, Barrow et al. 1998; Miron, Lasko et al. 2003). When TOR activity is low, 4EBP is hypophosphorylated and efficiently binds eIF4E, blocking cap-dependent translation (explained below). 4EBP is transcriptionally regulated by many types of stress including hypoxia (Liu, Roy et al. 2006), oxidative stress (Landis, Abdueva et al. 2004), starvation (Zinke, Schutz et al. 2002), and infection (Bernal and Kimbrell 2000). *dFOXO* is a transcriptional activator of *d4EBP* (Puig, Marr et al. 2003), and this activation is necessary for the transcriptional upregulation of *d4EBP* upon starvation and oxidative stress (Teleman, Chen et al. 2005). Increased *d4EBP*

expression is sufficient to rescue the oxidative stress sensitivity of *dFOXO* null flies (Tettweiler, Miron et al. 2005).

Similar to the ILSP, reduction of flux through the TOR pathway has been shown to extend lifespan in variety of organisms. Downregulation of the TOR pathway extends both replicative (the measure of how many buds, or daughter cells a single yeast produces) (Kaeberlein, Powers et al. 2005) and chronological lifespan (a measure of the time cells in a stationary phase culture remain viable) (Powers, Kaeberlein et al. 2006) in yeast. In *C. elegans,* downregulation of TOR, or raptor, a TOR-interacting protein, extended lifespan (Vellai, Takacs-Vellai et al. 2003; Jia, Chen et al. 2004). In *Drosophila,* overexpression of the negative regulators of TOR, *dTsc1, dTsc2*, or overexpression of dominant-negative *dTOR* or *dS6K* causes lifespan extension (Kapahi, Zid et al. 2004).

1.4 Genetics of Dietary Restriction

1.41 Sir2

There have been several genetic pathways which have been implicated in the lifespan extension due to DR. The first gene that was found to be necessary for DR was the *silent information regulator 2* (*Sir2*) gene in yeast, an NAD-dependent histone deacetylase (Imai, Armstrong et al. 2000; Lin, Defossez et al. 2000). A deletion of *Sir2* was unresponsive to DR, while overexpression of *Sir2* extended lifespan, and DR in this long lived strain gave no further benefit (Lin, Defossez et al. 2000). It was also found that increasing *Sir2* homologs in *C. elegans* (Tissenbaum and Guarente 2001) and *Drosophila* (Rogina and Helfand 2004) also extends lifespan. In *Drosophila,* the *Sir2* homolog *dSir2* is necessary for the lifespan extension due to a DR paradigm in which the food was

diluted (Rogina and Helfand 2004). Recently there has been contention about the role of Sir2 in DR. The Kennedy and Kaeberlein groups have shown that in yeast if you delete the *fob1* gene, *Sir2* is not necessary for the lifespan extension due to DR (Kaeberlein, Kirkland et al. 2004).

1.42 TOR Pathway

The TOR pathway has also been shown to interact with DR in multiple organisms. In *Drosophila,* downregulation of the TOR pathway extended lifespan on a rich nutrient diet but not upon DR (Kapahi, Zid et al. 2004). Similarly, in yeast DR failed to further increase the lifespan of the long lived tor1Δ line and sch9Δ (Kaeberlein, Powers et al. 2005). *Sch9*, a serine/threonine protein kinase involved in cell size and oxidative stress resistance (Fabrizio P 01, Tyers 02), has recently been shown to be a functional S6 kinase (Urban, Soulard et al. 2007).

1.43 Insulin Pathway

While the insulin pathway was originally postulated to be a molecular output for DR, as DR reduces insulin and IGF-1 levels in animals (Sonntag, Lynch et al. 1999; Roth, Lane et al. 2002; Heilbronn and Ravussin 2003), this has failed to be verified experimentally. In *Drosophila,* while the DR response of *chico* is shifted towards higher nutrient concentrations, it still responds to DR (Clancy, Gems et al. 2002). In *C. elegans daf-16* mutants, which completely suppress the lifespan extension due to downregulation of the ILSP, respond normally to DR by nutrient dilution or in combination with the *eat-2* mutant, a genetic means of DR (Lakowski and Hekimi 1998; Houthoofd, Braeckman et al. 2003).

1.5 Mitochondrial Respiration

One common, though still controversial proposal, on how DR may be working is by increased respiration. In yeast DR, decreasing the glucose concentration from 2% to 0.5% increases respiration and overexpression of *Hap4*, which switches the metabolism of the yeast from fermentation to respiration, extends lifespan (Lin, Kaeberlein et al. 2002). Inhibition of the TOR pathway by rapamycin treatment increases respiration by increasing the expression of the TCA cycle and oxidative phosphorylation genes (Hardwick, Kuruvilla et al. 1999). The *tor1Δ* also increases respiration by increasing the translation of mitochondrial-encoded oxidative phosphorylation subunits, and this was found to be a primary means by which *tor1Δ* extends the chronological lifespan of yeast (Bonawitz, Chatenay-Lapointe et al. 2007). Also in yeast, reduction of oxidative phosphorylation using the Complex III inhibitor antimycin A shortens lifespan, while adding 2,4- dinitrophenol, which uncouples ATP production from electron transport, increases chronological lifespan (Barros, Bandy et al. 2004). *C. elegans* respire more upon DR (Houthoofd, Braeckman et al. 2002; Bishop and Guarente 2007), and DR was found to upregulate *skn-1*, a transcription factor, that is necessary for the lifespan extension and increased respiration upon DR (Bishop and Guarente 2007). This upregulation of respiration was shown to be necessary for lifespan extension by administering two different Complex III inhibitiors, antimycin A and myoxithiazol, which reduced respiration and completely rescued the DR lifespan effect. These inhibitors were specific to DR, as there was no effect on the lifespan extension due to *daf-2* or on the normal lifespan of the control (Bishop and Guarente 2007). It has also recently been shown that DR increases mitochondrial biogenesis and respiration in mice

by upregulating the expression of eNOS (Nisoli, Tonello et al. 2005), though the necessity of this upregulation on lifespan is still unknown.

Conversely, there is also data that mitochondrial respiration plays no part in the lifespan extension of DR and even that inhibition of mitochondrial respiration can extend lifespan. In yeast replicative lifespan, it was found that in a more severe form of DR, functional mitochondria were not necessary for lifespan extension upon DR (Kaeberlein, Hu et al. 2005). In *C. elegans* there are many instances where decreasing mitochondrial function, by mutation and RNAi increase lifespan (Feng, Bussiere et al. 2001; Dillin, Hsu et al. 2002; Lee, Lee et al. 2003). It is interesting that this effect on lifespan is only seen if mitochondrial function is decreased while the worm is still developing (Dillin, Hsu et al. 2002; Rea, Ventura et al. 2007). This effect also depends on the level of inhibition, as high levels of inhibition are detrimental to the organism (Rea, Ventura et al. 2007).

Chapter 2 Translation

2.1 Translation Initiation

Gene expression can be controlled at many levels, including transcription, translation, and protein turnover. The regulation of translation, the conversion of the mRNA to protein by the ribosome and many accessory factors, is a key process for cell growth and proliferation (Jorgensen, Rupes et al. 2004) as well as during times of stress, reviewed in (Holcik and Sonenberg 2005). There are three main steps to translational control; initiation, elongation, and termination.

2.11 Cap-dependent Translation Initiation

Translation initiation is the rate-limiting step for translation of most mRNAs (Sonenberg, Hershey et al. 2000). This is based on the fact that ribosomes are usually spaced along an mRNA at 80-100nt intervals. If elongation were limiting, they would spaced at 30nt intervals, the limit of putting ribosomes in tandem. Indeed, if you add a translational elongation inhibitor, such as cycloheximide, to make elongation limiting, that is what is seen (Sonenberg, Hershey et al. 2000). During normal conditions, most translation initiation begins with the interaction of the cap-binding complex (eIF4F) with the mRNA "cap" structure, m^7GpppN (where N is any nucleotide, p is phosphate and m is a methyl group), at the 5' terminus. eIF4F is composed of three initiation factors, eIF4E, the cap-binding protein, eIF4A, an RNA helicase, and eIF4G, a scaffolding protein. eIF4G has binding sites for eIF4E and eIF3, thereby bridging the mRNA to the pre-initiation complex through eIF3's direct contact with the 40S ribosomal subunit. The 43S pre-initiation complex is composed of the 40S ribosomal subunit which associates with eIF3 and eIF1A, and then is further bound by the ternary complex consisting of eIF2, methionyl-initiatior tRNA (Met-$tRNA_i^{Met}$), and GTP.

Once the 40S subunit is bound to the mRNA it scans the 5'untranslated region (UTR) from 5' to 3', an ATP dependent process, until it finds an initiation codon (AUG) in the right sequence context (Kozak 1980). Upon finding the initiation codon, the 60S ribosomal subunit joins the complex to form the 80S ribosome and translation elongation commences. Cap-dependent translation is enhanced by circularization of the mRNA and interaction with the poly(A) tail through eIF4G's interaction with the polyA binding protein (PABP) (Kahvejian, Svitkin et al. 2005).

2.12 Internal Ribosome Entry

An alternative means of translation initiation that is independent of the 5' cap and eIF4E is internal ribosome entry site (IRES)-mediated translation initiation. While the mechanism of IRES translation is still unclear, it is known that the cap and eIF4E are not necessary for the translation of a subset of mRNAs. This type of translation was first observed in Picornavirus infections, where the RNAs of the polio and encephalomyocarditis virus were translated in eukaryotic cells even though they were uncapped (Jang, Krausslich et al. 1988). Viral proteases were found to cleave eIF4G to a form that does not bind eIF4E (Gradi, Svitkin et al. 1998). Other mechanisms that reduce cap-dependent translation initiation include increasing or hypophosphorylating eIF4E binding proteins (4EBPs), which compete with eIF4E for the binding site on eIF4G, as well as hypophosphorylation of eIF4E, which reduces eIF4Es affinity for eIF4G. Along with viral infections, cap-dependent translation is reduced during mitosis (Pyronnet, Dostie et al. 2001) as well as during many types of stress (Holcik and Sonenberg 2005).

2.13 Cancer

Translation initiation plays a key role in growth control and cancer. This first became evident when eIF4E overexpression was shown to cause cellular transformation (Lazaris-Karatzas, Montine et al. 1990). eIF4E is also upregulated in a broad spectrum of cancers (Petroulakis, Mamane et al. 2006), as well promotes tumor formation *in vivo* (Ruggero, Montanaro et al. 2004). eIF4E is thought to promote carcinogenesis by differential translation of mRNAs. Transcripts with extensive secondary structure in their 5' untranslated regions (UTRs) are particularly sensitive to the activity of the cap binding complex (Koromilas, Lazaris-Karatzas et al. 1992). Many genes with high 5'UTR

secondary structure are enhanced translationally *in vivo* upon increased eIF4E expression (Mamane, Petroulakis et al. 2004), and many oncogenes, growth factors and regulatory proteins have 5'UTRs with high secondary structure (Kozak 1991). Recently 4E-BP1 has been postulated as a funnel factor for cancer, as its phosphorylation status is associated with malignant progression in a large variety of cancers regardless of the upstream oncogenic alterations (Armengol, Rojo et al. 2007).

2.2 Lifespan

Recently multiple labs have shown that inhibiting translation can extend lifespan. In yeast, deletion of ribosomal protein subunits are sufficient to extend replicative lifespan (Kaeberlein, Powers et al. 2005). In *C. elegans,* inhibition of multiple translation initiation and elongation factors as well as many ribosomal protein subunits extend lifespan (Henderson, Bonafe et al. 2006; Curran and Ruvkun 2007; Hansen, Taubert et al. 2007; Pan, Palter et al. 2007). This lifespan extension due to reducing protein synthesis was found to be independent of *daf-16* (Curran and Ruvkun 2007; Pan, Palter et al. 2007).

Results

Chapter 3 Introduction

Dietary restriction (DR), the reduction of nutrient intake without malnutrition, is a method of lifespan extension conserved from yeast to mammals (Masoro 2002). DR also slows the progression of many age-related diseases, including cancer (Hursting, Lavigne et al. 2003). It has been suggested that DR extends lifespan by inducing a shift from growth and reproduction towards somatic maintenance (Holliday 1989). It has become apparent in recent years that nutrient sensing growth pathways are key regulators of

lifespan (Longo and Finch 2003). Two parallel and interacting pathways which affect lifespan in diverse species are the insulin-like signaling pathway (ILSP) and the target of rapamycin (TOR) pathway (Kapahi and Zid 2004; Kenyon 2005). Both of these pathways integrate nutrient and other environmental signals to mediate growth, and have been implicated in lifespan extension by DR (Clancy, Gems et al. 2002; Kapahi, Zid et al. 2004; Kaeberlein, Powers et al. 2005).

One common downstream component of both the ILSP and the TOR pathways is the translational repressor 4EBP. Decreased ILSP signaling transcriptionally upregulates 4EBP via the forkhead transcription factor FOXO (Puig, Marr et al. 2003), while decreased TOR activity causes hypophosphorylation of 4EBP that increases its affinity for eIF4E (Beretta, Gingras et al. 1996). 4EBP disrupts the interaction between eIF4E and eIF4G, which are components of the eIF4F cap-binding complex that mediates the initiation of mRNA translation (Richter and Sonenberg 2005). Transcripts with extensive secondary structure in their 5' untranslated regions (UTRs) are particularly sensitive to the activity of the cap binding complex (Koromilas, Lazaris-Karatzas et al. 1992). Recently, it has been shown in *C. elegans* that downregulation of components of the cap binding complex extends lifespan (Henderson, Bonafe et al. 2006; Hansen, Taubert et al. 2007; Pan, Palter et al. 2007; Syntichaki, Troulinaki et al. 2007). In mammals, eIF4E overexpression has oncogenic properties (Lazaris-Karatzas, Montine et al. 1990; Ruggero, Montanaro et al. 2004), and upregulation of eIF4E is found in a broad spectrum of cancers (Petroulakis, Mamane et al. 2006). Since 4EBP and eIF4E are regulated by a

diverse array of nutrient, stress, and mitogenic signals, we investigated the role of 4EBP in lifespan extension by DR in *Drosophila*.

Chapter 4 4EBP and Dietary Restriction

Reducing the concentration of yeast or yeast extract in the fly diet has been shown to extend lifespan (Nusbaum and Rose 1999; Kapahi, Zid et al. 2004; Mair, Piper et al. 2005) (Fig. 1).

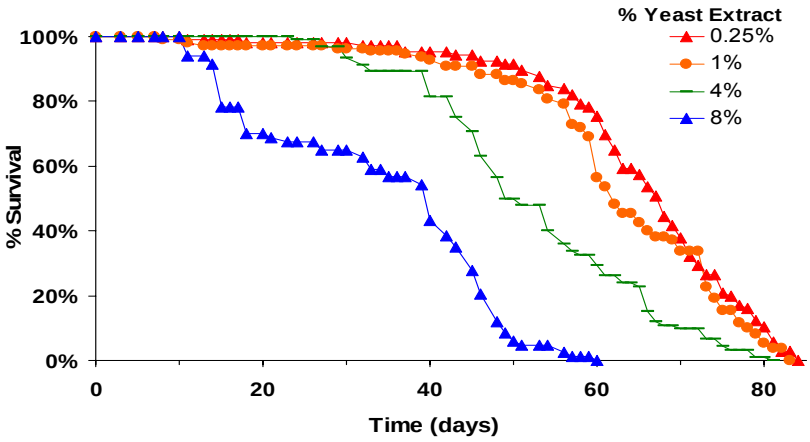


Figure 1 Lifespan of control male flies on diets with varying amounts of yeast extract and constant sucrose.

4.1 4EBP Protein Levels

We examined the levels of d4EBP under a paradigm of DR in which the yeast extract (YE) was varied while sucrose, the major carbohydrate source, was constant. As the YE concentration decreased from 4% to 0.25%, both male and female flies showed upregulation in d4EBP protein levels (Fig. 2). This upregulation was not dependent on FOXO, as *dFOXO* null flies showed a normal response (Fig. 2).

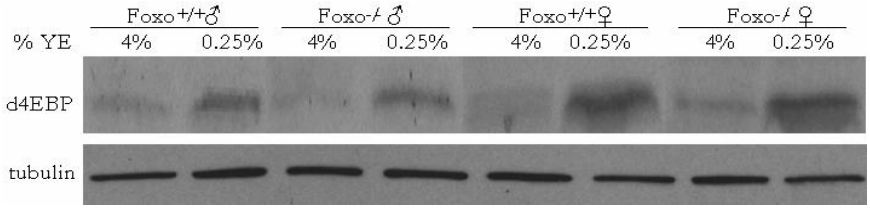


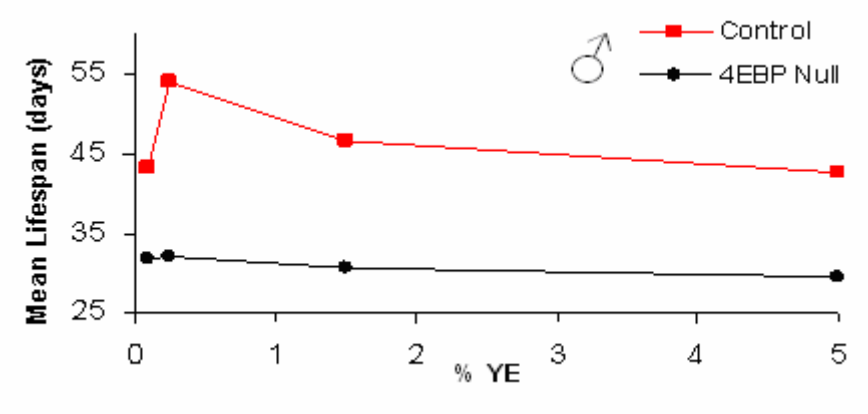
Figure 2 4EBP protein is induced upon DR. 4EBP protein is induced on 0.25% YE (DR) as measured by western blot using 30μg of protein probed with a polyclonal anti-d4EBP. This induction is not dependent on *dFOXO*, as *dFOXO* null flies show a normal response. β-tubulin levels were measured as a loading control.

4.2 Lifespan

4.21 4EBP Null

We next asked whether the upregulation of 4EBP is necessary for lifespan extension upon DR. To do this we used two strains, a *d4EBP* null line, created by imprecise excision of the P-element insertion *Thor*^{1}, which is inserted in *d4EBP* (Bernal and Kimbrell 2000), and a control strain created by precise excision of the same P-element line. Concentrations of YE were varied from 0.1% to 5%, with maximal average lifespan at 0.25% YE for males and 1.5% YE for females (Fig. 3a, b and Table S1). Control flies showed lifespan extension of 27% in males and 32% in females compared to 5% YE (Fig. 3a, 3b). In contrast, *d4EBP* null flies showed a diminished response across all nutrient concentrations (Fig. 3a, b and Table S1).

a



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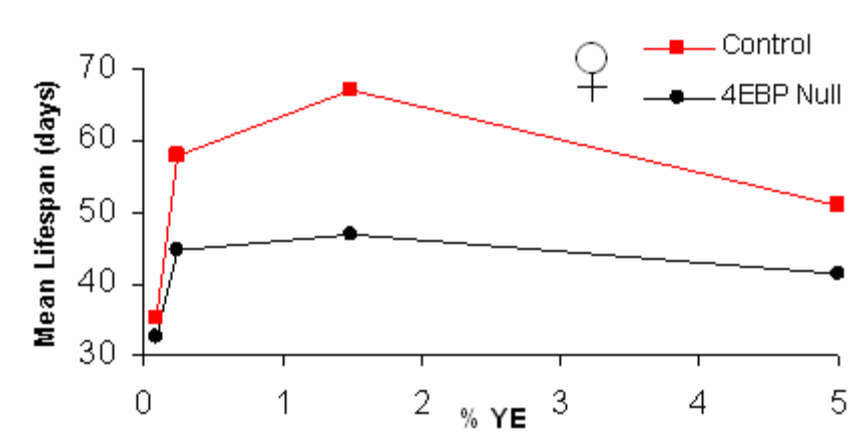


Figure 3 4EBP is necessary for full lifespan extension upon DR in *Drosophila.*

Lifespan of male and female revertant and *d4EBP* null flies on various YE concentrations. **a,** Male control and *d4EBP* null flies. **b,** Female control and *d4EBP* null flies.

To confirm that lack of d4EBP was causal for the diminished DR response, wild-type *d4EBP* was ubiquitously expressed with *daughterless-Gal4*, using the Gal4-UAS system (Brand and Perrimon 1993) in the *d4EBP* null background. First, the RNA levels of 4EBP were measured using qRT-PCR. Null flies contained very minimal amounts of 4EBP, by qRT-PCR criteria, while flies containing *UAS-4EBPwt* and the *da-Gal4* driver

returned the 4EBP levels to an amount similar to control flies in both males and females. This expression of 4EBP rescued the DR lifespan response from 8% in 4EBP null males to 37% and from 13% in 4EBP null females to 48%. This was similar to the 35% and 42% DR lifespan extension seen in control males and females, respectively (Fig. 5a, 5b and Table S2).

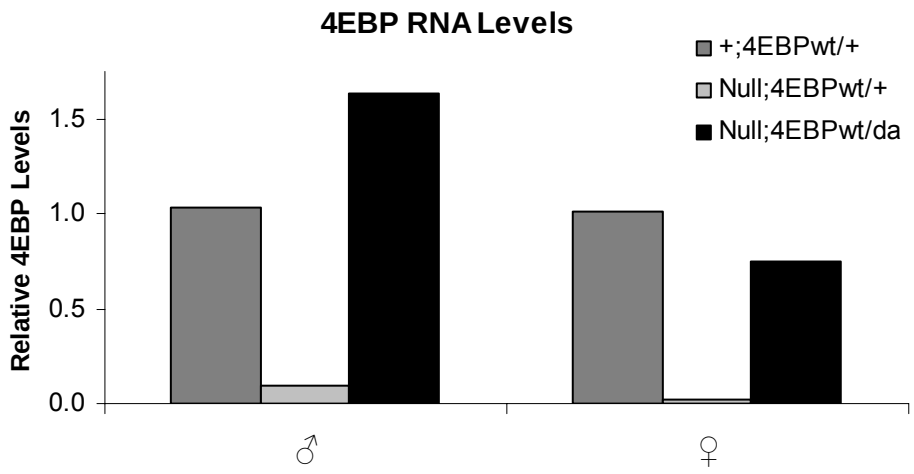
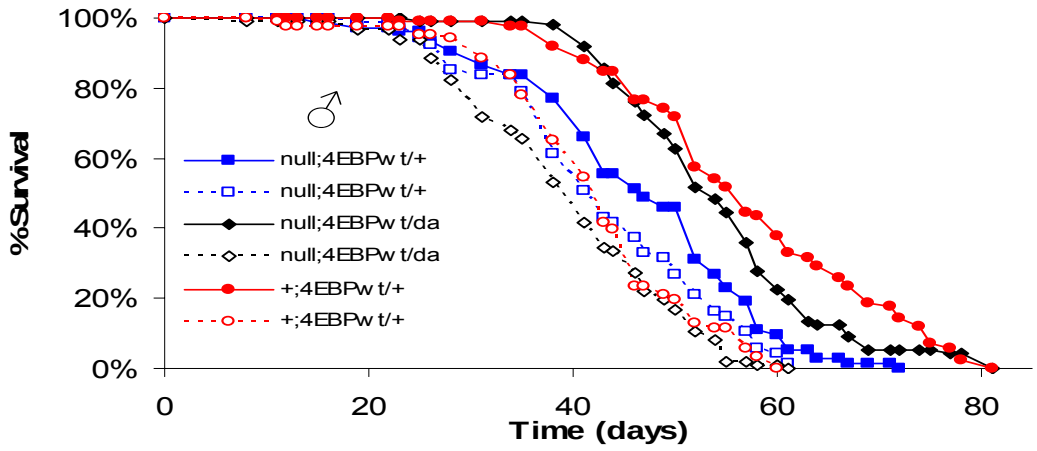


Figure 4 Rescue of 4EBP RNA levels using the UAS-Gal4 system. qRT-PCR of *d4EBP*, in male and females, with values normalized to *Actin5C,* and the background set as control flies with UAS-4EBPwt (n=2).

a



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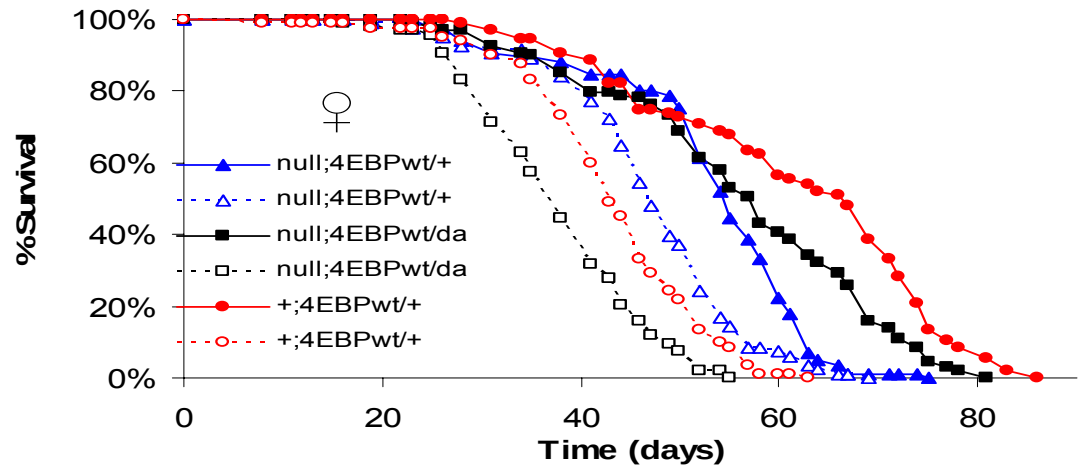
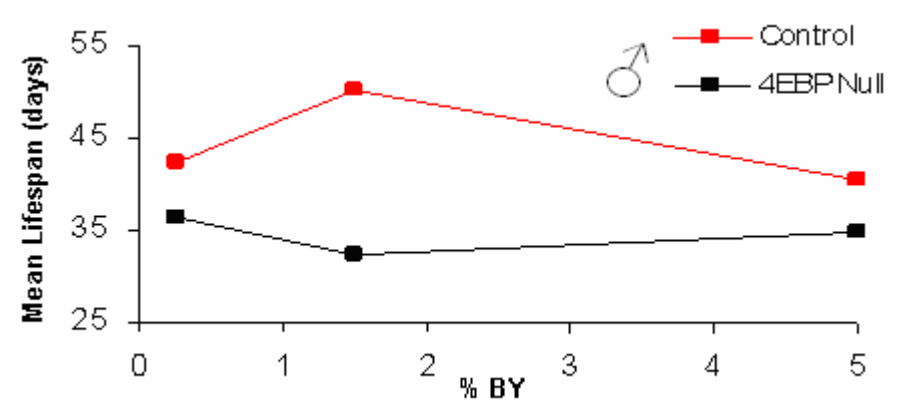


Figure 5 Replacing 4EBP rescues the 4EBP null DR lifespan effect. a,b Rescue of DR effect by ubiquitously expressing *d4EBP* using da-gal4 in the 4EBP-null background. Solid symbols DR (0.25% YE), empty symbols control (5% YE) **a**, Male 4EBP-null flies have an 8% lifespan extension upon DR. Control flies have a lifespan extension of 35%, and putting 4EBP back in the null line gives a lifespan extension of 37%. **b**, Female 4EBP-null flies have a 13% lifespan extension upon DR. Control flies have a lifespan

extension of 42%, and putting 4EBP back in the null line gives a lifespan extension of 48%.

As many labs use different yeast sources in fly media, we also investigated the lifespan effect due to DR using Brewer's Yeast (BY). Male and female control flies had a 24% and 15% lifespan increase upon DR with BY, while neither male nor female *d4EBP* nulls showed a significant change in lifespan upon DR (Fig. 6a, 6b and Table S3). While an inverse correlation between reproduction and lifespan extension has been noted (Partridge, Gems et al. 2005), recent experiments have shown that this link can be decoupled (Dillin, Crawford et al. 2002; Mair, Sgro et al. 2004). We found that both control and *d4EBP* null flies showed similar reductions in egg production upon YE restriction despite their differences in lifespan (Fig. 7). These observations suggest that *d4EBP* is necessary for the full lifespan extension upon DR, but not for reproductive changes.

a



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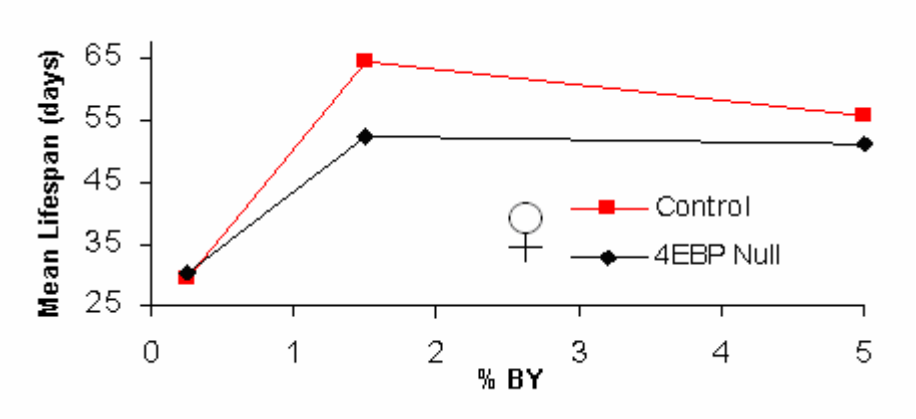


Figure 6 ***d4EBP*** **is necessary for the lifespan extension due to DR using Brewer's Yeast**. Lifespan of male and female revertant (control) and *d4EBP* null flies on various YE concentrations. **a,** Male control and *d4EBP* null flies. **b,** Female control and *d4EBP* null flies.

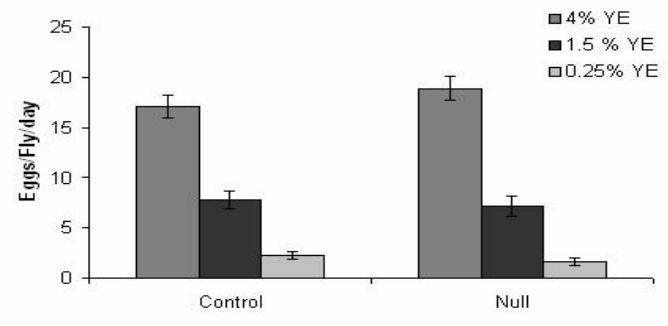


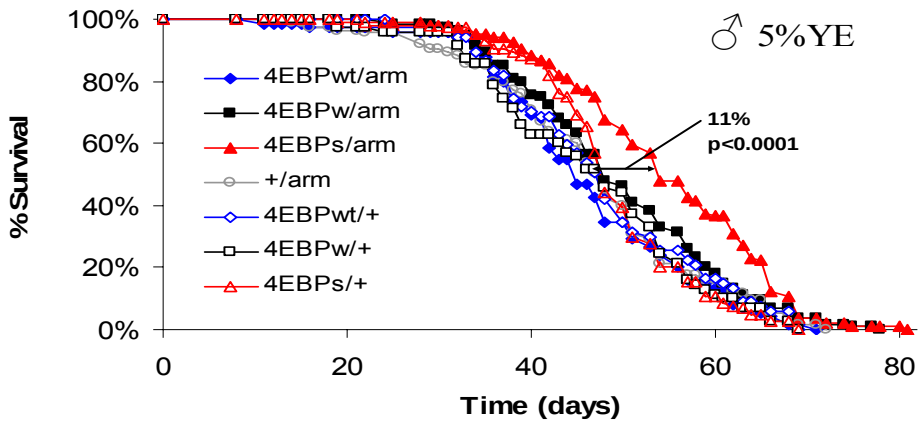
Figure 7. **4EBP null flies show normal fecundity response to variations in YE**. *d4EBP* revertant (control) and *d4EBP* null flies both show increased egg laying in a dose dependent manner when YE is increased. 5 females and 3 males were put into individual

vials within 24 hrs of eclosion. The number of eggs laid were measured daily from day 4 to day 8 and averaged per fly per day ±SEM (n=5).

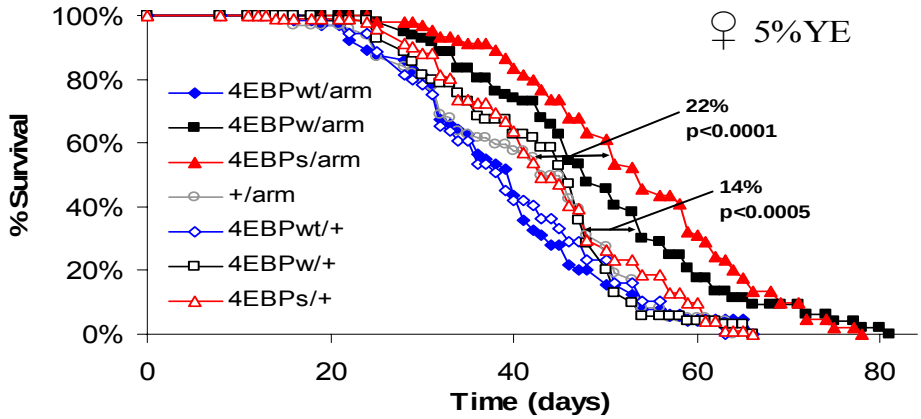
4.22 4EBP Overexpression

To ascertain whether elevated levels of 4EBP are sufficient to extend lifespan, a wild-type *d4EBP (d4EBP^{wt})*, as well as two activated alleles of *d4EBP*, which bind more strongly to eIF4E (Miron, Verdu et al. 2001), were overexpressed with the ubiquitously expressed driver, *armadillo-Gal4*. The two activated alleles were previously classified as strong (*d4EBP^{s}*) and weak (*d4EBP^{w}*) based on their growth inhibition properties. Overexpression of d*4EBP^{wt}* caused no change in lifespan on rich food in males or females, while overexpression of the weak activated allele extended mean lifespan of females on rich food, but no significant lifespan extension was observed in male flies. Induction of the strong allele extended both male and female lifespan on rich food (Fig. 8a, b and Table S4). In contrast, under DR (0.25% YE), there was no lifespan extension, beyond the effect of DR alone, in all of the 4EBP alleles tested (Fig. 8c, d and Table S4). These observations are consistent with the hypothesis that lifespan extension during DR is mediated by an increase in d4EBP activity.

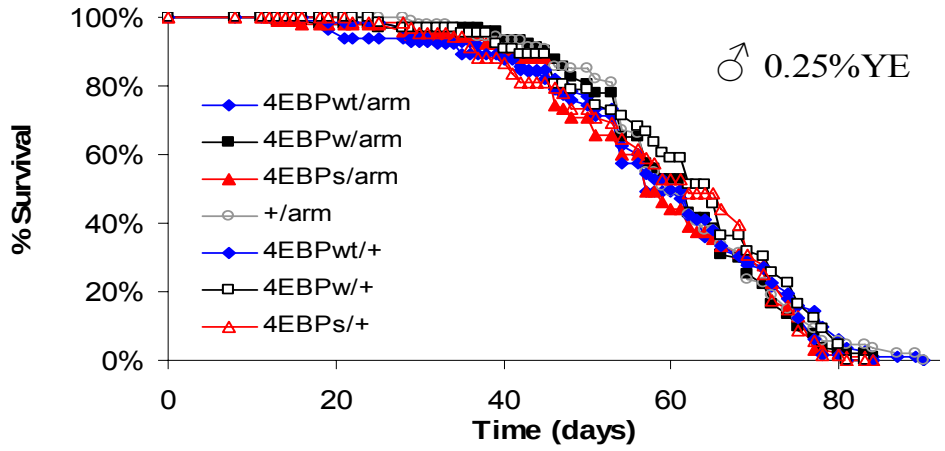
a


♂ 5%YE
100%
80%
60%
40%
20%
0%
%Survival
0
20
40
60
80
Time (days)
4EBPwt/arm
4EBPw/arm
4EBPs/arm
+/arm
4EBPwt/+
4EBPw/+
4EBPs/+
11%
p<0.0001

b


♀ 5%YE
100%
80%
60%
40%
20%
0%
%Survival
0
20
40
60
80
Time (days)
4EBPwt/arm
4EBPw/arm
4EBPs/arm
+/arm
4EBPwt/+
4EBPw/+
4EBPs/+
22%
p<0.0001
14%
p<0.0005

c



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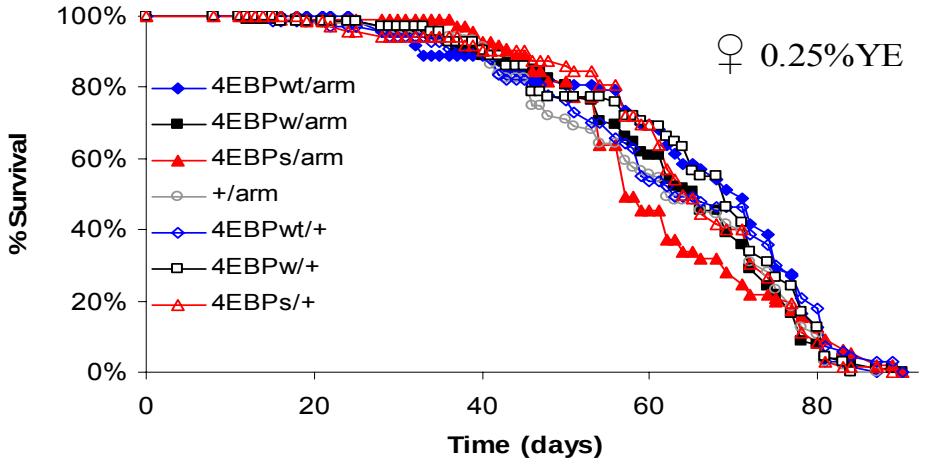


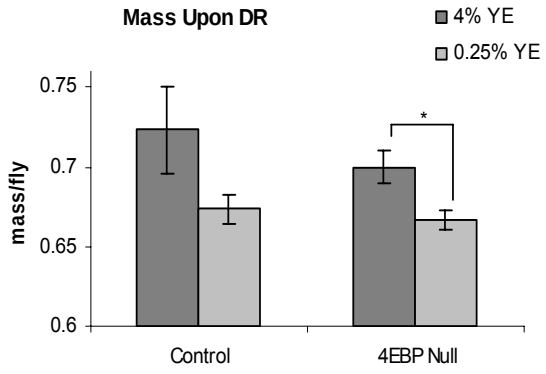
Figure 8 Overexpression of activated *d4EBP* extends lifespan in a nutrient-dependent manner in *Drosophila*. Male flies overexpressing $d4EBP^{LLs}$ and female flies overexpressing $d4EBP^{w}$ or $d4EBP^{s}$ extend lifespan on high nutrition (5%YE) but not under DR (0.25% YE). **a,** Survival of male flies on high nutrition. **b,** Female flies on high nutrition **c,** Male flies under DR. **d,** Female flies on high nutrition. "+" is the Benzer Lab w1118 strain, into which each of these lines was outcrossed 6x. P values

were obtained by comparing the survival curves with GraphPad Prism Software using the longest lived control.

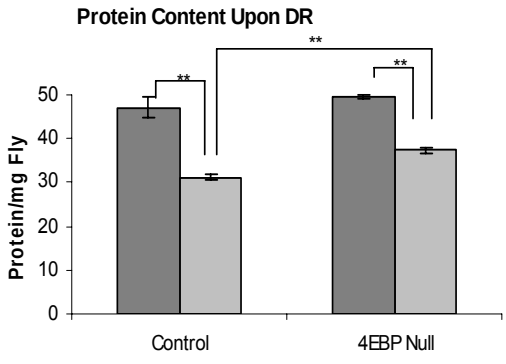
4.3 Metabolic Changes

To understand how 4EBP may be modulating the organism under DR, we investigated the composition of male flies. Upon DR there is a similar trend towards mass reduction in both the control and 4EBP null (Fig. 9a). The levels of total protein per mg of fly were also reduced in both lines upon DR, though 4EBP null flies had 20% more protein per mg of fly on DR compared to the control (Fig. 9b). Next, the storage metabolites, triglycerides, and glycogen were measured. Upon DR there is an increase in triglycerides in both control and null flies, while the null flies have lower levels of triglycerides on both diets compared to the control (Fig. 9c). Glycogen levels were also found to increase upon DR in control and null flies, yet while 4EBP played no role in glycogen levels upon high nutrition, there was an almost 70% decrease in glycogen in flies missing 4EBP on DR (Fig. 9d).

a



b



c **d**

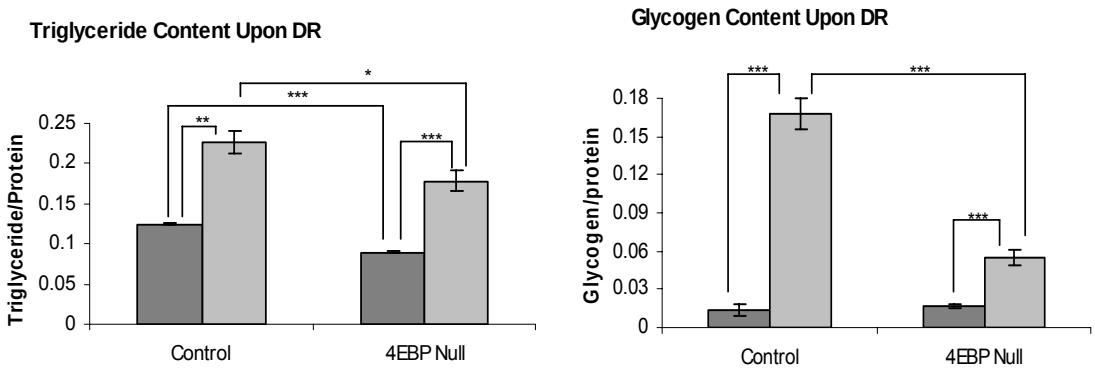


Figure 9 DR induces a shift towards increased storage metabolites that is altered in male flies lacking 4EBP. a, Control and 4EBP null flies have a trend towards decreased mass upon DR. **b,** DR decreases the protein content of the fly upon DR, though 4EBP null flies have more protein on DR compared to control flies. **c,** DR increases the triglyceride content of the control and 4EBP null flies. 4EBP null flies have decreased triglyceride contents on both food concentrations. **d,** On high nutrition control and 4EBP null flies have the same levels of glycogen. Upon DR controls have a 9 fold increase in glycogen levels, while 4EBP null flies increase only 3 fold.

4.4 Stress Resistance

It has previously been seen that 4EBP null flies are sensitive to starvation (Teleman, Chen et al. 2005; Tettweiler, Miron et al. 2005). As 4EBP null flies have altered composition that is diet-dependent, the starvation resistance of control and 4EBP null flies after 6 days on high nutrition or DR was investigated. While control flies have a 26% increase in starvation resistance upon DR, 4EBP null flies are sensitive to starvation and have no benefit from DR (Fig 10). This data implicates a shift in metabolism towards storage metabolites upon DR, which 4EBP nulls are deficient in.

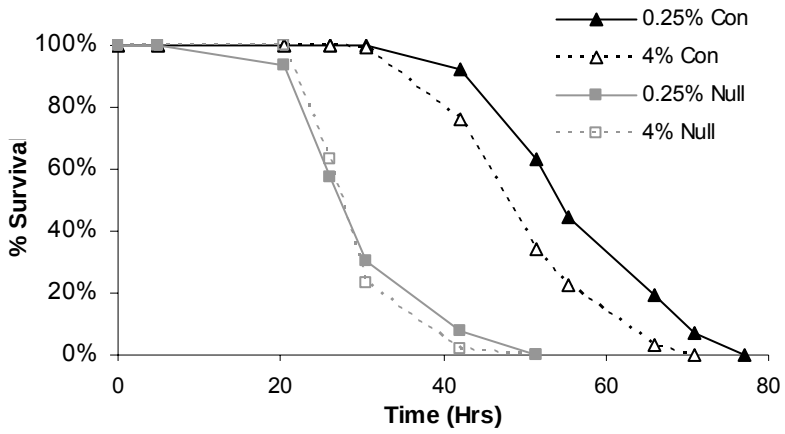


Figure 10 Starvation resistance increases upon DR in a 4EBP dependent manner. Starvation resistance of 6 day old male flies was measured on 1% agarose. Control flies show increased stress resistance in 0.25% YE, while 4EBP null flies are sensitive on all nutrient conditions and show no benefit from DR. Con 4% - 43.7 hrs (n=118), 0.25% - 55.1 hrs (n=106), 4EBP Null 4% - 25.2 hrs (n=103), 0.25% - 24.9 hrs (n=105).

Chapter 5 Genome Wide Translational Changes

5.1 Translation state array analysis

Given the role of 4EBP in regulating the translation initiation factor eIF4E, we investigated the translational changes occurring under DR. Initiation, the binding of the ribosome to mRNA, is the rate-limiting step for the translation of most mRNAs (Sonenberg, Hershey et al. 2000). Hence, the relative translation rate of an mRNA can be inferred from the number of ribosomes (polysomes) bound to it. To analyze the translation profile of *Drosophila*, polysomes were fractionated according to size over sucrose gradients. Under DR, there was an overall reduction in the number of polysomes and ribosomal subunits (Fig. 11). Though overall translation was reduced upon DR,

translation state array analysis (TSAA) (Zong, Schummer et al. 1999; Arava, Wang et al. 2003) was performed to investigate if individual mRNAs might be comparatively up and downregulated upon DR. Sucrose gradients containing resolved polysomes were fractionated to contain low (1 to 4) or high (5 or more) numbers of ribosomes bound per transcript. Each fraction was then hybridized to an Affymetrix microarray chip to determine the relative abundance in the high and low fractions.

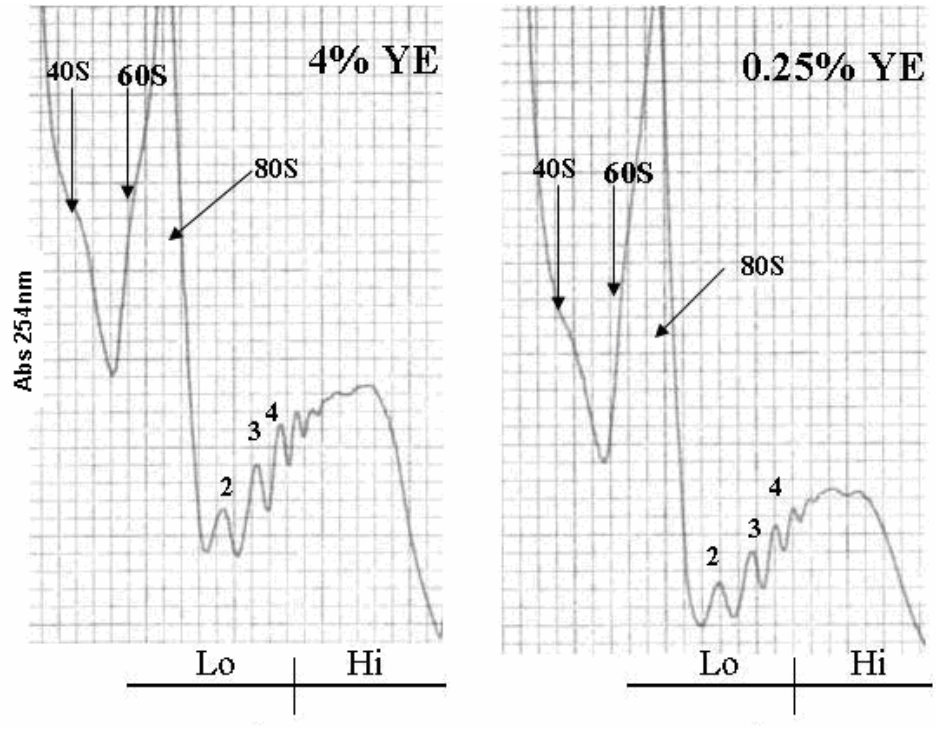


Figure 11 Polysomal distribution of mRNAs of male flies on 4% YE and 0.25% YE
Individual ribosomal subunits and the polysome peaks are noted, normalized to body weight of flies. RNA was prepared from the low (Lo) and high (Hi) translation fractions for microarray analysis performed in triplicate.

Using a false discovery rate (FDR) of less than 5%, 55 genes were translationally downregulated and 201 upregulated upon DR. We used Gene Ontology (GO)

classification (Ashburner, Ball et al. 2000) to identify biological themes overrepresented in the differentially translated genes. Categories enriched among the downregulated genes were carboxylic acid, cellular biosynthesis, and carbohydrate metabolism genes, while increased translation genes were enriched for mitochondrial ATP generation, oxidative phosphorylation, and protein folding genes (Table 1). Components of the mitochondria overrepresented were nuclear encoded Complex I and IV subunits of the electron transport chain and mitochondrial ribosomal proteins. Mitochondrial ribosomal proteins are necessary for the translation of mitochondrially encoded electron transport chain subunits.

Table 1 Biological Processes Translationally Changed Upon DR

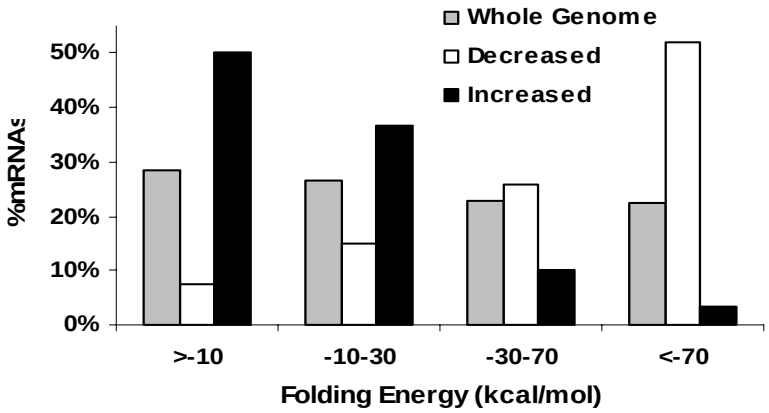
Translationally upregulated (201 genes)		
GO Category	Genes	P-Value
ATP synthesis coupled electron transport	14	1.3E-12
oxidative phosphorylation	16	4.5E-10
respiratory chain complex I	8	4.3E-09
protein folding	12	1.1E-06
generation of precursor metabolites and energy	22	3.9E-06
calcium-mediated signaling	8	6.3E-06
mitochondrial ribosome	6	6.8E-05
Phosphorylation	18	8.9E-05

Translationally downregulated (55 genes)		
GO Category	Genes	P-Value
carboxylic acid metabolism	12	1.1E-06
hydrogen-transporting ATPase V1 domain	4	1.7E-06
cellular biosynthesis	17	1.1E-05
generation of precursor metabolites and energy	12	3.7E-05
carbohydrate metabolism	11	1.1E-04

5.2 5'UTR Analysis

eIF4E regulates the translation of mRNAs with extensive 5'UTR secondary structure (Koromilas, Lazaris-Karatzas et al. 1992). To assess the 5'UTR secondary structures of the differentially translated genes, theoretical folding free energies (ΔG) were calculated using DAMBE Software (Xia and Xie 2001). While exact folding energies would need to be experimentally calculated, DAMBE was used to estimate the folding energies for the individual mRNAs. The mean ΔG for the 5'UTRs of all mRNAs in the *Drosophila* genome is -47 kcal/mol. Under DR, translationally downregulated mRNAs have highly structured 5'UTRs with an average ΔG of -69 kcal/mol, whereas upregulated mRNAs have significantly less secondary structure, averaging -20 kcal/mol (Fig.12a, Table 2). Since many genes from Complexes I and IV, and mitochondrial ribosomal proteins, were translationally upregulated, we examined the 5'UTR secondary structures of all subunits of these complexes and observed that most possess low secondary structure (Fig. 12b, Table 2). Upon further analysis, it was also found that translationally downregulated genes have, on average, longer 5'UTRs then the rest of the genome. Translationally upregulated and mitochondrial 5'UTRs were found to be shorter, with lower GC content then the rest of the genome (Table 2).

a



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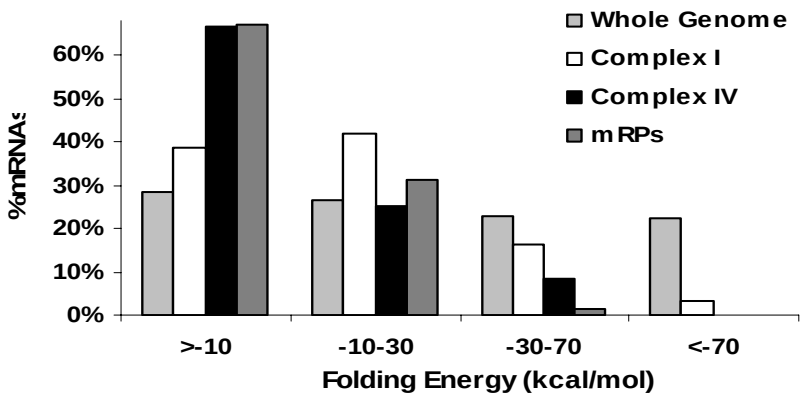


Figure 12 5'UTR secondary structure analysis of differentially translated mRNAs upon DR and mitochondrial genes. a, Theoretical 5'UTR folding free energies, ΔG, were calculated for mRNAs which showed higher or lower translation ratios and the whole genome. The distribution of ΔG's are shown for each class of translation regulation. **b,** 5'UTR folding free energies of genes in Complex I, Complex IV and ribosomal proteins of the mitochondria. P-values were calculated by generating sampling distributions for each experimental distribution, as described in the methods.

Table 2 Structural properties of the 5'UTRs of translationally regulated genes

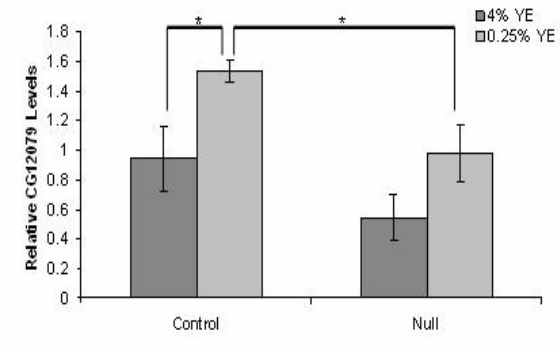
	N	ΔG(kcal/mol) ±SEM	P-value	Length (bp) ±SEM	P-value	%GC ±SEM	P-value
Whole Genome	9544	-46.7 ± 0.6		232 ± 3		42%±0.1%	
Down	49	-69 ± 10	<0.01	342 ± 44	<0.01	44%±1%	0.122
Up	146	-20 ± 3	<0.0001	130 ± 21	<0.0001	37%±1%	<0.0001
ComplexI	31	-21 ± 5	<0.01	129 ± 19	<0.01	39%±1%	<0.01
ComplexIV	12	-7 ± 1	<0.05	74 ± 8	<0.01	36%±2%	<0.01
mRP	64	-8 ± 1	<0.0001	75 ± 4	<0.0001	34%±1%	<0.0001

5.3 Mitochondrial Measurements

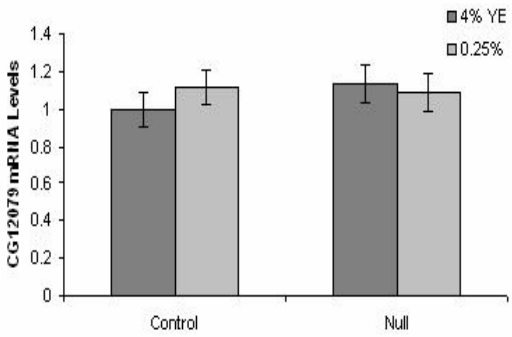
These findings suggest coordinate translational upregulation of the mitochondrial electron transport chain. To validate and further investigate this finding, we examined protein levels or activity for mitochondrial Complexes I and IV under normal and DR conditions and in the presence and absence of *d4EBP*. Protein levels of CG12079, a Complex I subunit with low 5'UTR secondary structure, were measured by western blot. Under DR, CG12079 protein levels increased 65% in control flies, while *d4EBP* nulls showed a smaller, non-significant increase upon DR and appeared to have reduced levels on both nutrient conditions (Fig. 13a). There were no significant changes in transcript levels of CG12079 upon manipulation of nutrients or *d4EBP* (Fig. 13b). The coordinate upregulation of Complex IV subunits was investigated by measuring cytochrome C oxidase (COX) activity. Upon DR, control flies showed a 60% increase in COX activity, while in *d4EBP* mutants COX activity was unchanged (Fig. 13c). Microarray analysis of total RNA from control flies upon DR shows no overall transcriptional changes in Complex IV subunits (Fig. 13d). The respiration of flies upon DR was also investigated. While control flies have a small but significant increase in CO_2 output, 4EBP null flies show no significant difference in respiration upon DR and have reduced respiration on

low nutrition compared to control flies (Fig. 14). Similar to the lifespan experiments, where a small, but consistent DR response was seen in *d4EBP* nulls, both the Complex I subunit levels and COX activity showed a trend towards upregulation upon DR in *d4EBP* nulls. This may be because of 4EBP independent regulation of eIF4E, such as phosphorylation (Arquier, Bourouis et al. 2005; Reiling, Doepfner et al. 2005), or alternatively there may be 4EBP/eIF4E independent pathways which also affect lifespan and mitochondrial function upon DR. Regardless, these data clearly show a post-transcriptional upregulation of the mitochondria under DR that is partially *d4EBP* dependent and support our TSAA results that mitochondrial mRNAs are translationally upregulated upon DR.

a



b



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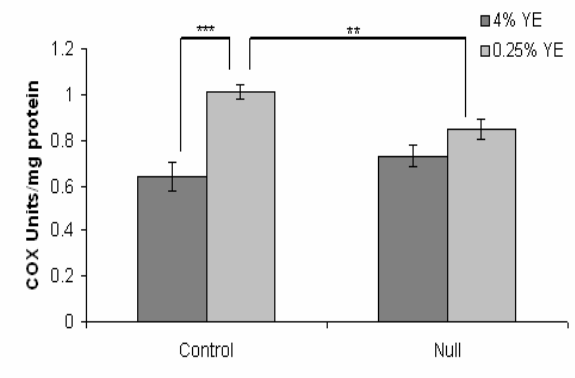


d

The ratio of total RNA between 0.25%YE and 4%YE of Complex IV subunits

Gene	Ratio	P - value
CG9065	**1.20**	0.024
CG10396	**1.02**	0.999
CG11015	**1.12**	0.013
CG14724	**1.07**	0.279
CG10664	**1.04**	0.430
CG11043	**1.09**	0.974
CG17280	**1.09**	0.389
CG18809	**1.00**	0.748
CG14028	**1.04**	0.928
CG9603	**1.01**	0.918
CG18193	**1.08**	0.932
CG2249	**1.08**	0.216

Figure 13 DR induces the mitochondrial electron transport chain without affecting transcription a, Western blot of mitochondrial Complex I subunit, CG12079, in male revertant (control) and *d4EBP* null flies upon DR, with values normalized to tubulin (±SEM) (n=4). **b,** qRT-PCR of CG12079, in male control and *d4EBP* null flies upon DR, with values normalized to *Actin5C* (±SEM) (n=4). **c,** Cytochrome C oxidase (COX) activity measured on crude homogenates in male control and *d4EBP* null flies upon DR, normalized to protein content (±SEM) (n=7). **d,** Lack of transcriptional changes in genes of Complex IV upon DR. The ratio of total RNA on 0.25% YE vs 4% YE in control flies using Affymetrix microarrays. Complex IV gene list from the MitoDrome database (www2.ba.itb.cnr.it/MitoDrome) (*p<0.05, ** p<0.01, *** p<0.001).

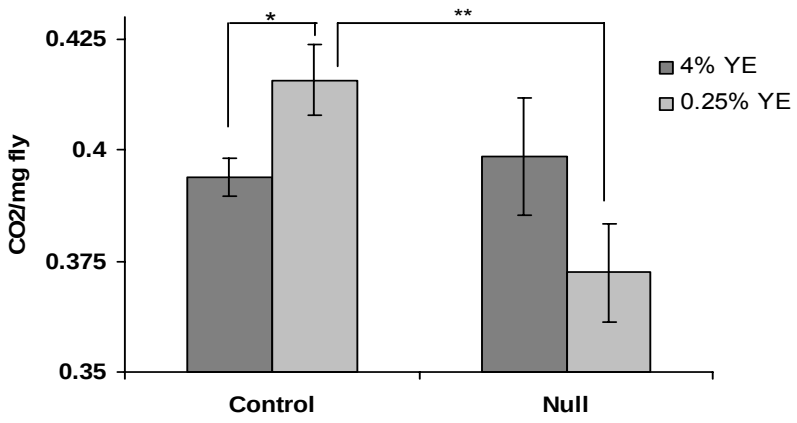


Figure 14 Respiration increases upon DR in a 4EBP dependent manner. CO2 output of control and 4EBP null flies on 4% and 0.25% YE normalized to fly mass. (n=5) (*p<0.05, ** p<0.01).

Chapter 6 5'UTR Analysis

6.1 Conserved Gene Ontology Categories

To further understand the biological role that secondary structure of 5'UTRs may play in *Drosophila*, we investigated the overrepresented biological processes of mRNAs which had strong predicted secondary structure (ΔG > -120 kcal/mol) and weak predicted 5'UTR secondary structures (ΔG > -5 kcal/mol). Previously it has been seen that in mammals signal transduction and growth genes have strong 5'UTR secondary structure (Kozak 1991). Similarly, upon investigation of the *Drosophila* genome, gene classes overrepresented for strong 5'UTR secondary structure include development, signal transduction, regulation of metabolism, growth, and transcription factors (Table 4). As the original mammalian 5'UTR analysis used a small subset of mRNAs, we analyzed the current annotation of 5'UTRs for the Human Genome (Table 4). There was strong

conservation in the overrepresentation of gene classes between *Drosophila* and Human including developmental, protein kinases, and regulation of metabolism in the human genome. Upon analysis of *Drosophila* weakly structured 5'UTRs, the immune response, electron transport, and proteolysis were all biological processes that were overrepresented (Table 5). In Human 5'UTRs, immune response, proteolysis, aerobic respiration, and the TCA cycle were all processes that were overrepresented for low structured 5'UTRs (Table 5).

Table 4

GO of Drosophila 5'UTRs with ΔG < -120 kcal/mol

Term	Count (874)	PValue
development	279	2.6E-30
morphogenesis	156	1.1E-27
regulation of biological process	258	2.9E-24
cell communication	243	1.2E-23
signal transduction	212	5.2E-22
regulation of metabolism	184	3.7E-19
metamorphosis	79	7.1E-19
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	164	3.7E-18
regulation of transcription, DNA-dependent	147	7.3E-18
cell differentiation	114	1.0E-14
oogenesis	75	5.1E-13
sexual reproduction	97	3.9E-12
protein amino acid phosphorylation	65	7.6E-12
transmembrane receptor protein tyrosine kinase signaling pathway	35	1.7E-08
phosphate metabolism	99	2.2E-08
transmembrane receptor protein serine/threonine kinase signaling	18	1.3E-06
MAPKKK cascade	29	1.6E-06
transforming growth factor beta receptor signaling pathway	13	1.4E-05
neurogenesis	39	1.4E-05
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	214	6.0E-05
behavior	39	8.7E-05
JNK cascade	16	1.7E-04
regulation of growth	17	1.9E-04
actin cytoskeleton organization and biogenesis	24	2.0E-04
programmed cell death	42	9.2E-04
Wnt receptor signaling pathway	16	9.6E-04

GO of Human 5'UTRs with ΔG < -250 kcal/mol

Term	Count (4178)	PValue
protein modification	331	9.3E-15
regulation of biological process	605	7.9E-09
ubiquitin cycle	113	4.3E-08
development	321	6.0E-08
nervous system development	99	3.8E-06
protein amino acid phosphorylation	125	4.4E-06
phosphate metabolism	174	5.5E-06
transmembrane receptor protein tyrosine kinase signaling pathway	38	7.4E-06
morphogenesis	116	2.1E-05
cell cycle	131	9.1E-05
regulation of metabolism	399	1.1E-04
actin cytoskeleton organization and biogenesis	39	1.7E-04
insulin receptor signaling pathway	10	3.1E-04
regulation of transcription, DNA-dependent	339	3.8E-04
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	535	5.1E-04
neurogenesis	27	8.9E-04

Table 5

GO of Drosophila 5'UTRs with ΔG > -5 kcal/mol			GO of Human 5'UTRs with ΔG > -10 kcal/mol		
Term	Count (1630)	PValue	Term	Count (3370)	PValue
biological process unknown	96	4.3E-07	antigen presentation	29	8.1E-12
humoral immune response	22	4.5E-05	defense response	191	3.7E-08
electron transport	69	5.5E-05	immune response	167	1.3E-06
defense response to bacteria	20	6.6E-05	proteolysis	133	2.9E-06
response to pest, pathogen or parasite	25	3.2E-04	acetyl-CoA metabolism	16	4.0E-06
vitamin biosynthesis	10	4.6E-04	macromolecule biosynthesis	138	5.8E-06
proteolysis	107	6.4E-04	humoral immune response	43	2.5E-04
protein biosynthesis	90	8.2E-04	aerobic respiration	14	2.9E-04
immune response	27	9.1E-04	tricarboxylic acid cycle	12	3.0E-04

As we have previously seen that mitochondrial genes were translationally upregulated upon DR in *Drosophila* and aerobic respiration was one of the classes overrepresented among human mRNAs, all the 5'UTRs for Complex I, IV, and the mitochondrial ribosomal proteins were further analyzed. Similar to *Drosophila,* we found that each of these complexes had a shift towards reduced 5'UTR secondary structure compared to the rest of the genome (Fig. 15).

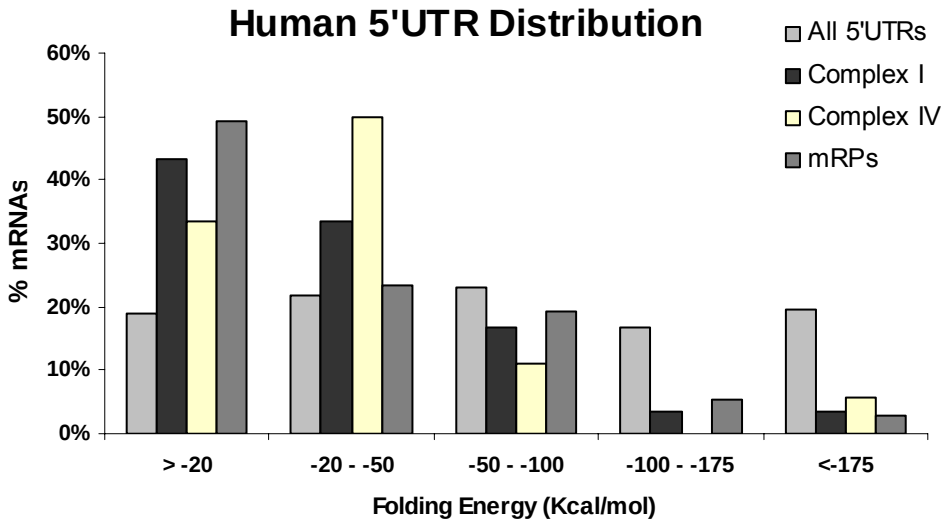


Figure 15 5'UTR secondary structure analysis of human mitochondrial genes a, Theoretical 5'UTR folding free energies, ΔG, for genes in Complex I, Complex IV, and ribosomal proteins of the mitochondria.

6.2 *In vivo* Analysis of 5'UTR Function

The importance of the various 5'UTRs on translational control were investigated using luciferase reporter constructs. 5'UTRs were cloned upstream of the *Firefly* Luciferase (FLuc) gene under the control of the *Act5C* promoter (Fig. 16a). These constructs were then transfected into S2 cells with an internal control *Renilla* Luciferase (RLuc) vector as well as either an activated allele of 4EBP ($4EBP^{LLAA}$) or a control, GFP construct, both under the *Act5C* promoter. Using various UTRs which had either strong, medium, or weak secondary structure (Table 6), we investigated their translational control upon expression of $4EBP^{LLAA}$ using monocistronic constructs.

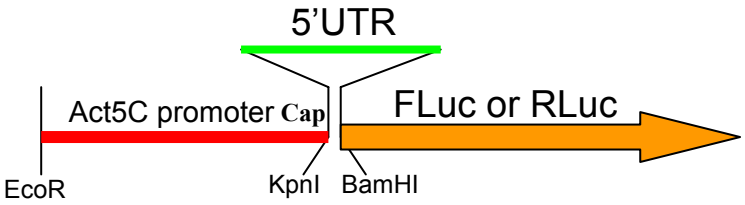
Table 6: 5'UTRs Analysed with reporter constructs

Gene	Description	ΔG	Length	%GC	TSAA	P Value
Tub	αTub84B	-32.6	204	45%	**0.67**	0.30
GPDH	Glycerol 3 phosphate dehydrogenase	-89.9	428	43%	**0.56**	0.0061
CG1213	GlucoseTrans	-149.9	661	41%	**0.71**	0.0093
mRPL30	mito Ribosomal Protein	-6.1	90	33%	**1.79**	0.002
mRPL14	mito Ribosomal Protein	-4.72	102	27%	**1.67**	0.062
CG3621	ComplexI Subunit	-14.4	116	34%	**1.66**	0.0081
CG6463	ComplexI Subunit	-15.4	89	42%	**1.64**	0.0059

6.21 Monocistronic Reporter

$4EBP^{LLAA}$ expression caused no significant expression change with the GPDH 5'UTR compared to the tubulin 5'UTR, which was used as a baseline control, while the glucose transporter, CG1213, had a comparative two fold decrease in reporter activity. $4EBP^{LLAA}$ expression caused 2 to 4 fold increases when the mitochondrial 5'UTRs for two mitochondrial ribosomal proteins and a Complex I subunit (CG3621) were present in the reporter construct (Fig 16b). The 5'UTR for CG6463, a Complex I subunit, showed no significant difference in reporter activity upon $4EBP^{LLAA}$ expression, though there was a trend towards increased reporter activity (Fig. 16b). To further confirm that these changes in FLuc expression are due to translational control, the levels of FLuc mRNA were measured using qRT-PCR. Compared to tubulin, GPDH was translated greater than 20 fold more efficiently in control cells or upon $4EBP^{LLAA}$ expression (Table 7). The two mRP 5'UTR constructs were inefficiently translated in control conditions compared to the tubulin 5'UTR construct (Table 7). While still inefficient compared to tubulin, $4EBP^{LLAA}$ expression enhanced the translation of mRP 5'UTR reporters between 4-5 fold compared to control conditions (Table 7).

a



b

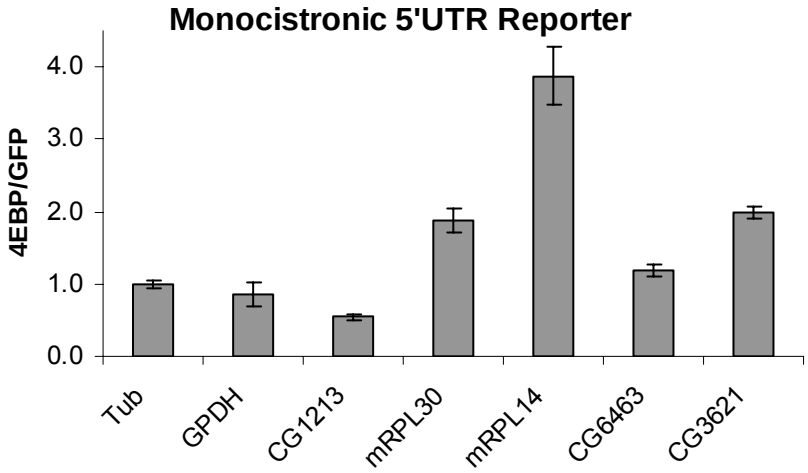


Figure 16 ***In vivo*** **analysis of 5'UTR function using a monocistronic reporter. a,** Schematic of construct used in analysis. **b,** The data are presented as the ratio of activity in the presence of $4EBP^{LLAA}$ versus the presence of GFP, with all data normalized to the RLuc transfection control plasmid.

Table 4 Translational efficiency of various 5'UTRs.

FLuc Protein/FLuc mRNA Levels normalized to Tubulin

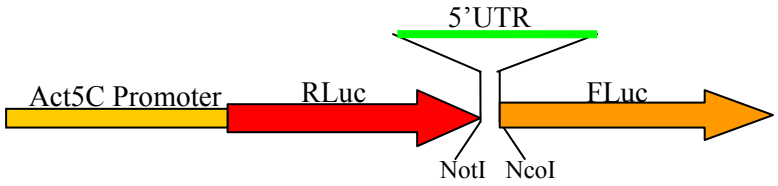
5'UTR	Plasmid GFP	Plasmid $4EBP^{LLAA}$	$4EBP^{LLAA}$ enhancement
Tub	1	1	
GPDH	25	29	1.2
mRPL30	0.03	0.15	5.7
mRP14	0.03	0.13	4.2

6.22 Bicistronic Reporter

It is known that during times of high 4EBP activity there is a shift from cap-dependent translation to IRES translation. To investigate if mitochondrial 5'UTRs have IRES activity, they were inserted into a bicistronic construct (Fig. 17a). As the RLuc ORF is the first in the mRNA, it should be more sensitive to changes in cap-dependent translation, while the FLuc should be dependent on internal ribosome entry. Therefore, the FLuc/RLuc ratio gives a measure of cap-independent vs cap-dependent translation initiation. Using this construct, Marr et al. have previously shown that the 5'UTR of the *Drosophila* insulin receptor (*InR*) has IRES activity when $4EBP^{LLAA}$ is expressed (Marr, D'Alessio et al. 2007). In our hands, the positive control *InR* 5'UTR had a 2-fold increase in cap-independent vs. cap-dependent translation (FLuc/RLuc) upon $4EBP^{LLAA}$ expression, while the Tubulin and CG1213 5'UTRs showed no change in IRES activity upon $4EBP^{LLAA}$ expression (Fig. 17b). The mitochondrial 5'UTRs showed increased IRES activity upon $4EBP^{LLAA}$ expression (Fig. 17b). Interestingly, the 4EBP dependent increase in IRES activity was much higher in the mitochondrial 5'UTRs than the previously published *InR* 5'UTR. While the trends for the mitochondrial 5'UTRs are similar between the monocistronic and bicistronic reporters, with mRPL14 and CG3621

5'UTRs conferring the greatest increase upon 4EBP^{LLAA} expression, the bicistronic constructs had much larger increases in reporter activity (Fig. 16b,17b).

a



b

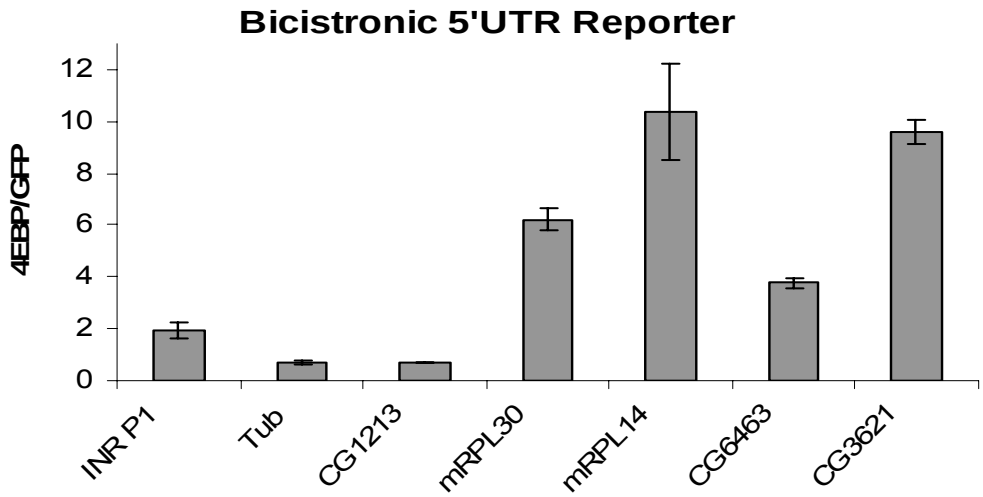


Figure 17. Assessing IRES activity using a bicistronic construct . a Schematic of construct used in analysis. **b** The data are presented as the ratio of activity in the presence of 4EBP^{LLAA} versus the presence of GFP, with all data FLuc activity normalized to the RLuc activity from the first cistron.

Concluding Remarks

Chapter 7 Summary of Results

We have shown that, in *Drosophila,* 4EBP is upregulated upon DR in a *dFOXO*-independent manner. While it has been shown that *dFOXO* is necessary for the

transcriptional induction of *d4EBP* under starvation (Teleman, Chen et al. 2005), we found that *dFOXO* is not necessary for the induction of the d4EBP protein in the DR conditions where YE is limited but sucrose is kept constant. We found that d4EBP is necessary for full lifespan extension upon DR and that upregulation of activated *d4EBP* is sufficient to extend the lifespan of the fly on a rich diet. This lifespan extension, from 4EBP overexpression, was correlated with the previously characterized growth inhibition properties of the various 4EBP insertions. Investigation of the metabolic changes upon DR found that there is a shift in metabolism towards metabolite storage that is partially dependent upon *d4EBP*. Given the role of 4EBP in inhibiting the translation initiation factor eIF4E, these data suggested that modulation of mRNA translation upon DR may alter the lifespan and metabolism of the fly. Investigation of genome-wide translation changes upon DR identified a correlation between mRNA translation changes and 5'UTR secondary structure. Control of mRNA translation by gross 5'UTR secondary structure may represent a novel means of regulating gene expression under nutrient limitation. Such a regulatory mechanism would have the advantage of being faster than transcriptional regulation, yet more energy-efficient than post-translational control. Though global translation is decreased upon DR, mitochondrial genes are one class of genes which is comparatively, translationally upregulated. We show that Complex I and IV subunits of the electron transport chain and mitochondrial ribosomal proteins have weakly folded 5'UTR secondary structures.

Upon investigation of genome-wide 5'UTRs we found a high conservation in gene classes overrepresented for weak and strong 5'UTR secondary structure between

Drosophila and Humans. This included conservation of low 5'UTR secondary structure among mitochondrial mRNAs in Humans. *Drosophila* mitochondrial 5'UTRs were found to confer inefficient translation to reporter constructs during conditions of high cap-dependent translation, yet were translationally upregulated upon increased 4EBP activity. This translational upregulation conferred by mitochondrial 5'UTRs was found to be through increase IRES dependent translation.

Chapter 8 Discussion

8.1 IRES Translation

Upregulation of 4EBP in *Drosophila* has previously been shown to induce a shift in translational control from cap-dependent translation to cap-independent, internal ribosome entry site (IRES) translation for InR 5UTRs (Marr, D'Alessio et al. 2007). While there have been many IRES genes found, conserved sequences between various UTRs have not been found. While it has been postulated that IRESs may posses secondary structures recognized by the ribosome or transacting factors, recently Xia observed a positive correlation between low 5'UTR secondary structure and increased IRES activity (Xia 2007). This correlation was found by reanalyzing data from yeast genes upregulated upon invasive growth which have IRESs (Gilbert, Zhou et al. 2007). Gilbert et al. found that the IRES activity was dependent on eIF4G levels. The gene they focused on, YMR181c, had tracts of polyAs in its 5'UTRs, and its IRES activity was dependent on the PABP (Gilbert, Zhou et al. 2007). It is unclear if the IRES activity of the other genes were dependent on the PABP, but as eIF4G interacts with PABP, one could postulate that PABP may be able to bind the 5'UTR of unstructured genes, thereby

bringing the mRNA in contact with the 40S ribosome through eIF4Gs contact with eIF3. Whether the IRES activity of mitochondrial 5'UTRs in *Drosophila* is regulated in a similar manner has yet to be investigated.

To my knowledge this is the first proposal that mitochondrial genes contain IRESs. The majority of cellular IRES genes have been implicated in stress response. A lot of these have focused on upregulation under hypoxia, due to their relevance to cancer. This would seem to be contradictory that mitochondrial genes would be translationally induced upon hypoxia or in cancer, in which mitochondrial metabolism is severely reduced (Ristow 2006). 4EBP activity is upregulated in many conditions that reduce cap-dependent translation such as hypoxia, nutrient deprivation, heat stress, irradiation, infection, and mitosis. While many general stress proteins would need to be upregulated in a variety of these conditions, there would probably also be specific genes necessary for each condition. What differentiates which IRES genes are upregulated in each condition? It will be interesting to investigate what trans-acting factors may be necessary for the induction of the various IRES genes under different cap-dependent repressed translational conditions.

One class of genes which may have similar trans-acting IRES factors to mitochondrial genes are immunity genes. They were one of the conserved classes of genes with weak 5'UTR secondary structure in *Drosophila* and Humans. The *Drosophila* 4EBP mutant, *Thor1*, was originally isolated as a gene induced upon infection. It was subsequently found that *4EBP* null flies were sensitive to infection, yet the mechanism for this

sensitivity is unknown (Bernal and Kimbrell 2000). It is intriguing to postulate that this sensitivity may arise because in flies lacking 4EBP, during infection, a higher proportion of the translation machinery is devoted to cap-dependent translation, thereby reducing the ability to translate from IRESs.

8.2 Growth and Lifespan

8.21 Multicellular Organisms

What appears to be a common underlying theme across many lifespan extension mechanisms is the reduction of growth pathways. DR, the best characterized lifespan extending mechanism in a variety of organisms, directly inhibits growth. The two most robust genetic means of extending lifespan, the ILSP and TOR pathways, are both nutrient sensing growth pathways. While seemingly at the opposite extremes, as DR and the TOR pathway appear to increase mitochondrial function, reducing the function of the mitochondria also reduces growth. In *C. elegans,* the lifespan extension from inhibiting the mitochondria correlates well with the growth inhibition when titrating the levels of RNAi (Rea, Ventura et al. 2007). Similarly, there is a direct correlation between the growth suppression of the overexpressed 4EBP alleles and the lifespan extension in *Drosophila* (Zid et al).

Genome-wide RNAi screens in *C. elegans* have identified many genes which extend lifespan. From two screens in which RNAi was administered throughout development, it was found that 89 and 23 genes from approximately 16,000 genes screened extended lifespan (Hamilton, Dong et al. 2005; Hansen, Hsu et al. 2005). The administration of the

RNAi in the genome wide screens was performed throughout development, thereby excluding all developmentally lethal genes. The Ruvkun lab subsequently investigated the lifespan effects of 2700 developmentally lethal genes, which are five times more likely to be highly conserved across species, administered in adulthood (Curran and Ruvkun 2007). This gave a large enrichment in lifespan genes, as 64 genes were found to extend the worm's lifespan (Curran and Ruvkun 2007). Recently, the Kapahi lab analyzed the lifespan of 57 genes found to cause late larval arrest, a subclass of developmentally lethal genes, from a genome wide RNAi screen in *C. elegans* (Kamath, Fraser et al. 2003), which arrest growth in L3 larvae stage. It was found that 40% (24) of these growth arrest genes extended lifespan when reduced during adulthood (Chen, Pan et al. 2007). Comparatively, a primary screen for paraquat resistance in *C. elegans* isolated 608 positive RNAi clones, upon rescreening these clones for lifespan, 84 were found to extend lifespan (Kim and Sun 2007). This exemplifies the importance that reducing growth has in lifespan extension, which gives an even larger enrichment for lifespan genes then screening for stress resistance.

8.22 Yeast Growth Changes

Why does reducing growth pathways extend lifespan? In a mitotic organism, an easy answer would be reduced cancer incidence, and while this may be true for DR in mice, this is unlikely to be the case in worms and flies, two predominantly post-mitotic organisms. The changes that happen upon various growth conditions have been studied in yeast. At the transcriptional level, there is a large overlap between genes that are growth regulated, independent of limiting nutrient, and the stress response, independent of the stress (Brauer, Huttenhower et al. 2007; Castrillo, Zeef et al. 2007). This is

somewhat misleading, though, as any stress will reduce the growth rate of the organism. To this end, some canonical stress genes, such as chaperones, are not upregulated upon limiting nutrients. One class of genes transcriptionally upregulated upon reduced growth is oxidative phosphorylation (Brauer, Huttenhower et al. 2007). These genes are also upregulated translationally under conditions of nutrient deprivation, which would also reduce growth (Tuller, Kupiec et al. 2007). While this is not surprising under reduced growth conditions caused by limiting glucose, oxidative phosphorylation is also upregulated under conditions of limiting ammonia, phosphate, or sulfate. Under these conditions, yeast shift from fermentation to respiration even though there is excess glucose in the media (Brauer, Huttenhower et al. 2007). This is very similar to our DR conditions where the yeast extract is limited, there is an ample source of carbohydrates, and there is an upregulation in oxidative phosphorylation. It has previously been found that batch cultures, which have limiting amounts of carbon or nitrogen and upregulate oxidative phosphorylation, are more stress resistant (Elliott and Futcher 1993).

8.23 Mitochondrial Respiration

Our functional mitochondrial measurements of respiration, Complex I levels, and COX activity along with our measurements of 5'UTR translational control support the possibility of 4EBP dependent coordinate translational upregulation of mitochondrial components upon DR in *Drosophila*. Mitochondrial biogenesis and efficiency increase under DR in mammals (Nisoli, Tonello et al. 2005; Lopez-Lluch, Hunt et al. 2006). It has also been shown that respiration increases under DR in both yeast (Lin, Kaeberlein et al. 2002) and *C. elegans* (Houthoofd, Braeckman et al. 2002; Bishop and Guarente 2007). Interestingly, in yeast it was recently shown that mitochondrial genes are predominantly

upregulated under nutrient deprivation at the level of translation (Tuller, Kupiec et al. 2007). Our data showing increased translation of mitochondrial genes upon DR in *Drosophila* support the link between enhanced mitochondrial function and DR. *d4EBP* has been postulated to function as a general metabolic brake during times of starvation (Teleman, Chen et al. 2005; Tettweiler, Miron et al. 2005). Our results instead suggest that *d4EBP* modulates mRNA translation to induce a metabolic shift towards efficiently using the fuel present and allocating resources towards storage metabolites, which may prolong lifespan during times of nutrient limitation.

Increased reliance on ATP generation by mitochondrial respiration during times of slow growth may be advantageous. While glycolysis is 100 times faster in ATP generation, there is a trade-off of lower efficiency compared to mitochondrial respiration (Pfeiffer, Schuster et al. 2001). This phenomenon of relying on fermentation on higher growth rates is similar to the Warburg effect in cancer, where cancer cells rely much more on glycolysis than oxidative phosphorylation for ATP generation, even in the presence of O_2 (Plas DR 2005). There is an ongoing debate on what role the Warburg effect has in the genesis of cancer. One conserved transcriptional program across many species is the downregulation of oxidative phosphorylation with age (McCarroll, Murphy et al. 2004; Zahn, Sonu et al. 2006; Zahn and Kim 2007). It may be that as an organism ages there is a shift away from oxidative phosphorylation, which increases the propensity for aging and cancer.

How may a metabolic shift mechanistically extend lifespan? One possibility is that the compartmentalization of oxidative reactions to mitochondria can restrict the redox reactions from interacting with, and thereby possibly damaging, the rest of the cell. Also, with the increased efficiency of oxidative phosphorylation compared to glycolysis, less overall reactions need to take place. Another possibility is that reducing glycolytic flux reduces glycolytic intermediates, such as methylglyoxal, which can be harmful to the cell (Ramasamy, Yan et al. 2006).

Interestingly, in Warburg's 1956 Science paper, he quotes Louis Pasteur who saw that yeast lost their structure when they completely shifted their metabolism from respiration to fermentation (Warburg 1956).

"I should not be surprised if there should arise in the mind of the attentive hearer a presentiment about the causes of those great mysteries of life we conceal under the words youth and age of cells."

It will be interesting to investigate in the coming years how growth pathways exert there effects on lifespan, and if Pasteur was right 130 years ago, that a shift in metabolism may be a key link in understanding aging.

Methods

Strains – Thor1 ^{revertant} (*4EBP control), Thor1*^{null} (4EBP Null), strains were provided by Debra Kimbrell, *Foxo21, Foxo25* were provided by Ernst Hafen. 2475, *UAS-4EBP*^{wt}, *da-Gal4* and *arm-Gal4* was obtained from the Bloomington stock center. *UAS-4EBP*^{LL} strains were a gift from Nahum Sonenberg, which have their Met 59 and Lys 60 mutated to Leu, which increases 4EBPs binding to deIF4E 3.4 fold (Miron, Verdu et al. 2001).

Generation of 4EBP Null Rescue Strain and Controls

CyO; Sb/Xa = 2475: Δ = Thor1^{Null}: da = da-GAL4

CyO; Sb/Xa **x** da/da → CyO/+; Sb/da

CyO/+; Sb/da **x** Δ/Δ → CyO/Δ; da/+ & CyO/Δ; Sb/+

CyO/Δ; da/+ **x** CyO/Δ; Sb/+ → CyO/Δ; Sb/da (da followed by eye color)

Repeated for Thor1^{revertant} and UAS-4EBP^{wt}

Lifespan Analysis - Flies were developed on Standard Caltech food (8.6% Cornmeal, 1.6% Yeast, 5% Sucrose 10% Dextrose, 0.46% Agar, 1% Acid mix) (Lewis 1960), and adults transferred within 24 hr of eclosion toYE food (8.6% Cornmeal 5% Sucrose, 0.46% Agar, 1% Acid mix, and yeast extract (#212750 Bacto™ Yeast Extract, B.D. Diagnostic Systems, Sparks,MD) or Brewer's Yeast (#903312 MP Biomedicals.LLC, Solon, OH) At 3-4 days male and females were separated under light CO_2 anesthesia to ~30 flies/vial. Flies were transferred every 3 days into fresh food vials and deaths recorded. UAS-*4EBP* lines were crossed to *arm-Gal4* at 20°C, (as developmental arrest was observed at 25°C with the activated 4EBP strains), and adults transferred to food with varying concentrations of YE and lifespan measured at 25°C. Fecundity was

measured by placing 5 females and 3 males into a vial and eggs laid recorded daily from day 4 to day 8. The accumulated eggs were averaged per female per day. For starvation tests, flies were prepped in the same way as for DR, at 6 days, flies were put into vials containing 1% agarose and deaths were subsequently recorded.

Metabolic Measurements – Groups of 10 flies were isolated under light CO2 anesthesia, and then mass recorded on a Sartorius ME215S analytical balance (Sartorius, Bradford, MA). Flies were then ground in homogenization buffer (0.01 M KH_2PO_4, 1 mM EDTA, pH 7.4), centrifuged at 2,000 rpm for 2 min, and the supernatant isolated. Protein concentration was assayed using Pierce BCA protein assay kit (Pierce, Rockford, IL). Triglyceride measurements were performed using the Triglyceride LiquiColor® test (Enzymatic) (Stanbio laboratory, Boerne, TX). Glycogen measurements were performed by adding 0.1 U/mL of amyloglucosidase for 30min, to break the glycogen in to glucose. Then 5U/mL glucose oxidase, 1 U/mL peroxidase, and 0.04 mg/mL o-dianisidine dihydrochloride (Sigma, St. Louis, MO) were added, and the absorbance was measured at 450nm using a Benchmark Plus Spectrophotometer (Biorad, Hercules, CA). Using glycogen standards, the concentration of glycogen was determined.

Polysome profiling - Profiles were generated via optimization of previously used methods (Dinkova, Keiper et al. 2005). 100 6-day-old male flies were homogenized on ice in 350 mL of solublization buffer (300 mM NaCl, 50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 1 mM EGTA, 200 mg heparin/ml, 400 U RNAsin/mL, 1.0 mM phenylmethylsulfonyl fluoride, 0.2 mg cycloheximide/ml, 1% Triton X-100, 0.1%

Sodium Deoxycholate) by 60 strokes with a Teflon homogenizer. 850 mL additional solublization buffer was added, vortexed for no longer than 1 second, and placed back on ice for 10 minutes. Samples were spun at 20,000g for 15 minutes at 4°C, and 0.9 ml of the supernatant was applied to the top of a 10-50% sucrose gradient in high salt resolving buffer (140 mM NaCl, 25 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$). Polysomes and ribosomal subunits were separated via centrifugation in a Beckman SW41Ti rotor at 38,000 rpm for 90 min at 4°C. Gradients were fractionated using a Teledyne density gradient fractionator with continuous monitoring of absorbance at 252 nm. Fractions containing low (1 to 4) or high(5 or more) numbers of ribosomes per transcript were isolated for RNA isolation.

RNA isolation - RNA was extracted from fractions by mixing with 2X volume of TRIzol reagent (Invitrogen, Carlsbad, CA) and 1/15X volume of 2.5M Sodium acetate, pH 4.5. Samples were mixed thoroughly and allowed to sit for 5 minutes to allow ribonucleoproteins to dissociate from transcripts. 0.4X volume of chloroform was used to separate the organic phase. RNA from the aqueous phase was precipitated and washed using standard methodology. All samples were assayed for quality control prior to microarray hybridization, and each manipulation was performed in triplicate on different biological samples.

Microarray Analysis – Data was normalized and analyzed using the Rosetta Resolver gene expression data analysis system. Translation ratios were calculated by dividing the high translation intensity by the low translation intensity. The translation changes under

DR were calculated by dividing the translation ratios from 0.25% YE by the translation ratio for 4% YE. From the translation ratios, P-values were assigned using the Benjamini-Hochberg (FDR) test for multiple correction. Setting a FDR of <5% significantly regulated genes were inputed in the DAVID bioinformatics resource (niaid.abcc.ncifcrf.gov) to identify overrepresented categories by GO classification.

5'UTR analysis – 5'UTRs were downloaded from Flybase, annotation 5.1. 5'UTRs which were shorter then 10bp were excluded from our analysis. For any gene that had more than one annotated 5'UTR, the longest 5'UTR was analyzed, and any gene without an annotated 5'UTR was omitted from further analysis. The sequences were analyzed with DAMBE (data analysis in molecular biology and evolution) software(Xia and Xie 2001), with settings of 37°C, with no lonely pairs and no GU pairs at the end of helices. DAMBE uses the Vienna RNA folding package, which gives similar folding energies to mFold (www.bioinfo.rpi.edu/applications/hybrid/quikfold.php), yet unlike mFold, it returns only non-positive folding energies. Mitochondrial oxidative phosphorylation genes were classified based on Mitodrome annotation (www2.ba.itb.cnr.it/MitoDrome/) mitochondrial ribosomal proteins were classified by Affy annotation. To determine the probability that the 5'UTR folding energies of our experimentally derived subgroups (downregulated n=49, upregulated n=146, Complex I n=31, Complex IV n=12 and mRPs n=61) would be expected by chance, we compared the mean of each subgroup to the mean of an appropriate sampling distribution. Sampling distributions were generated by randomly sampling 5'UTR folding energies from the pool of all 5'UTR's for each sample size. This was repeated 10,000 times, and a sampling distribution was constructed from

the sample means. The mean of each subgroup was then compared to the mean and standard deviation of their respective sampling distribution and p-values obtained. For mammalian UTR analysis, the 5'UTR length and ΔG were downloaded from UCSC Genome Browser. Human 5'UTR sequences and ΔGs were downloaded from the UCSC Genome Table Browser.

Quantitative Reverse Transcriptase (RT)-PCR - Total RNA was extracted from 30 flies by using TRIzol reagent (Invitrogen, Carlsbad, CA), concentration was measured by using a Nanodrop spectrophotometer, and sample concentrations were normalized. The Transcriptor Reverse Transcriptase (Roche, Penzberg, Germany) was used according to the manufacturer's instructions, with the oligo(dT) primers provided. Triplicate PCR reactions were carried out with the intercalating dye SybrGreen. Each sample (20 μl) contained 10.0 μl iQ™ SybrGreenI SuperMix (Bio-Rad), 0.4 μM each primer, and 1/60 of reverse transcriptase product. PCR cycles were programmed on an iCycler iQ™ Real Time PCR detection system (Bio-Rad). The cycle number at which CG12079 was detectable (CT) was compared to that of Actin, referred to as ΔCT. The gene level was relative to 4% YE control flies. The relative gene level was expressed as $2^{-P(\Delta\Delta CT)}$, in which ΔΔCT equals ΔCT of the flies of interest minus ΔCT of the 4% control flies.

Western Blot - Six to eight day old flies were ground in PBST, centrifuged at 10,000g for 5 min, and the supernatant was isolated. Equal amounts of protein, measured using Pierce BCA protein assay kit, were run on SDS-PAGE under reducing conditions. The proteins were then transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene,

NH). Proteins of interest were probed with specific primary antibodies; rabbit anti-d4EBP (Lasko and Sonenberg labs McGill Univ, Montreal Quebec Canada) at 1:2250, mouse anti-NDUFS3 (Mitosciences, Eugene, OR) at 0.75μg/mL, anti-β-tubulin E7 (DSHB, Iowa City, IA) at 1:1000. Blots were incubated in appropriate horseradish peroxidase conjugated secondary antibody; HRP Goat Anti-rabbit IgG (Vector Laboratories, Burlingame, CA) 1:22,500, HRP Goat Anti-mouse IgG (BioRad, Hercules, CA)1:2000, and then detected using ECL plus (Amersham Biosciences). Westerns were imaged using Storm 860 Scanner (Amersham Biosciences) and quantified using the Image Quant TL software (Amersham Biosciences).

Cytochrome C Oxidase Activity – 10 flies were ground in 400μL PBST, centrifuged at 8000g 5min 4°C, and supernatant isolated. Cytochrome C 0.22mM was made fresh and reduced with 0.1M dTT. Assay Buffer contained 0.01M TrisCl, 2.5mM $MgCl_2$, and 10μM Cyto C. 10μL of extract was added to 165uL Assay buffer and monitered at 550nm over 10s intervals for 1min. COX activity was normalized to total protein quantified using Pierce BCA Protein Assay.

Respiration – 10 flies were weighed with a Sartorius ME215S analytical balance. Then flies were placed in 2.2-ml glass vials and assayed by using a TR-2 CO_2 gas respirometer (model LI-6251; Sable Systems International, Henderson, NV). The vial was flushed with CO2-free air and placed in the respirometer for ~1 h at 25°C. CO_2 production was calculated by using DATACAN software (Sable Systems International) and normalized to the mass of the flies.

UTR cloning and Cell Culture - The parent vector was a promoter-less vector, pGLUC (Promega, Madison, WI). To this the minimal distal Act5 promoter, bases -335bp to +3bp from the transcriptional start site for exon 1(Chung and Keller 1990), was cloned using PCR from pMALF, provided by Tjian Lab. The PCR primers included EcoRI and KpnI restriction sites which allowed insertion into the pGLUC vector. This vector (pUTRminimal) was made to give a minimal vector 5'UTR contribution, as only 3bp were extra after the transcriptional start site to the 5'UTR insertion site. The firefly luciferase (FLuc) and *Renilla* luciferase (RLuc) genes were PCRed from vectors obtained from the DGRC (Drosophila Genome Resource Center) and cloned using BamHI and NotI sites for pUTRminimal or NcoI or BamHI for pMALF added to the end of the primers. 5'UTRs from differentially translated genes were PCRed from cDNAs with primers containing KpnI and BamHI restrictions sites and then inserted into the pUTRminimalFLuc vector. The control RLuc plasmid was made by cloning RLuc into the pMALF vector, as the RLuc in the pUTRminimal vector gave very low luciferase readings. Act-4EBP^{LLAA} or Act-GFP was transfected with FLuc and RLuc plasmids in a ratio of 1.2:0.3:0.3ug 4EBP:FLuc:RLuc with 3uL of Fugene Transfection reagent (Roche, Penzberg, Germany) in 600uL of cells at ~70% confluency. Luciferase activity was measured 48hrs after transfection using the Dual Glo Luciferase Assay (Promega, Madison, WI). The bicistronic construct, pMARL, was obtained from the Tjian Lab. 5'UTRs were amplified by PCR from cDNAs and cloned using NotI and NcoI sites added to the PCR primers. Act-4EBP^{LLAA} or Act-GFP was transfected with bicistronic plasmid in a ratio of 1.2:0.3ug 4EBP:pMARL with 3uL of Fugene Transfection Reagent. S2 cells were maintained using Gibco's Schneider's Drosophila Media (Gibco, Grand

Island, NY) supplemented with penicillin/streptomycin antibiotics (Gibco) and 10% heat inactivated fetal bovine serum(Gibco).

Supplemental Tables

Table S1 – Lifespans of Control and 4EBP Nulls on YE

% YE	5/19/2007	Mean LS	n	DR effect	Chi Square	P Value
0.1 ♂	Control	43.3	131	2%	0.01	0.9099
0.25		54.1	131	**27%**	31.0	<0.0001
1.5		46.7	132	10%	4.1	0.0439
5		42.5	149			
0.1	Null	31.8	160	8%	3.4	0.0654
0.25		32.2	143	9%	4.2	0.0399
1.5		30.6	127	3%	0.1	0.7497
5		29.6	136			
0.1 ♀	Control	35.0	162	**-31%**	19.9	0.0002
0.25		57.9	143	**14%**	33.7	<0.0001
1.5		67.2	120	**32%**	92.7	<0.0001
5		50.8	147			
0.1	Null	32.6	147	-21%	6.2	0.0128
0.25		44.6	130	8%	5.5	0.0191
1.5		46.7	132	**13%**	6.7	0.0094
5		41.3	125			
% YE	12/6/2006	Mean LS	n	DR effect	Chi Square	P Value
0.25 ♂	Control	50.5	125	**34%**	58.0	<0.0001
1.5		44.8	114	**19%**	23.9	<0.0001
4		37.7	136			
0.25	Null	38.4	79	**18%**	13.7	0.0002
1.5		35.5	81	9%	6.3	0.012
4		32.6	71			
0.25 ♀	Control	54.6	190	**52%**	127.6	<0.0001
1.5		45.7	195	**27%**	74.9	<0.0001
4		36.0	155			
0.25	Null	38.0	145	8%	4.4	0.036
1.5		40.3	126	**14%**	18.0	<0.0001
4		35.3	101			

Table S2 – Rescue of 4EBP Null Lifespan

% YE	7/27/2007	Mean LS	n	DR effect	Chi Square	P Value
0.25 ♂	+; 4EBPwt/+	57.2	85	**35%**	80.3	<0.0001
5		42.5	86			
0.25	Null;4EBPwt/+	46.6	74	8%	6.0	0.0139
5		43.1	67			
0.25	Null; 4EBPwt/da	54.5	97	**37%**	68.1	<0.0001
5		39.7	96			
0.25 ♀	+; wt/+	61.8	96	**42%**	114.7	<0.0001
5		43.5	82			
0.25	Null; 4EBPwt/+	53.2	85	**13%**	42.7	<0.0001
5		47.2	83			
0.25	Null; 4EBPwt/da	56.3	109	**48%**	88.5	<0.0001
5		37.9	94			

Table S3 – Lifespasn of Control and 4EBP Nulls on BY

% BY	5/19/2007	Mean LS	n	DR effect	Chi Square	P Value
0.25 ♂	Control	42.4	146	5%	4.5	0.033
1.5		50.1	128	**24%**	33.4	<0.0001
5		40.5	127			
0.25 ♂	Null	36.4	161	5%	2.7	0.098
1.5		32.2	143	-7%	2.8	0.097
5		34.7	130			
0.25 ♀	Contrrol	29.6	160	**-42%**	56.5	<0.0001
1.5		64.5	147	**15%**	8.9	0.003
5		50.8	147			
0.25 ♀	Null	30.3	136	**-41%**	38.6	<0.0001
1.5		52.7	119	2%	0.52	0.473
5		51.4	101			

%BY	11/1/2006	Mean LS	n	DR effect	Chi Square	P Value
1 ♂	Control	52.7	135	**24%**	43.0	<0.0001
2.5		46.0	136	8%	6.3	0.012
8		42.5	136			
1 ♂	Null	34.5	133	0%	0.3	0.559
2.5		33.2	98	-4%	2.1	0.145
8		34.4	98			
1 ♀	Contrrol	60.6	132	**39%**	89.7	<0.0001
2.5		60.5	109	**38%**	89.5	<0.0001
8		43.8	120			
1 ♀	Null	49.2	118	12%	3.1	0.078
2.5		49.4	91	13%	3.4	0.065
8		43.8	90			

Table S4. Lifespans of 4EBP Overexpression

% YE	6/6/2007	Mean LS	n	4EBP OE	Chi Square	P Value	DR Effect	Chi Square	P Value
5 ♂	4EBPwt/arm	46.3	75	-3%	0.7	0.408			
5	4EBPw/arm	49.4	114	6%	2.5	0.112			
5	4EBPs/arm	54.3	103	**11%**	21.9	<0.0001			
5	+/arm	46.9	113						
5	4EBPwt/+	47.7	67						
5	4EBPw/+	46.7	70						
5	4EBPs/+	49.0	104						
5 ♀	4EBPwt/arm	39.4	64	-1%	0.002	0.96			
5	4EBPw/arm	48.8	94	**14%**	14.4	1E-04			
5	4EBPs/arm	53.5	90	**22%**	30.8	<0.0001			
5	+/arm	41.6	94						
5	4EBPwt/+	39.7	69						
5	4EBPw/+	42.9	70						
5	4EBPs/+	43.9	91						
0.25 ♂	4EBPwt/arm	58.8	83	0%	1.5	0.21	**27%**	46.8	<0.0001
0.25	4EBPw/arm	60.2	104	-2%	1.5	0.21	**22%**	42.7	<0.0001
0.25	4EBPs/arm	58.2	102	-2%	0.2	0.62	**7%**	11.3	8E-04
0.25	+/arm	60.7	106				**29%**	61.4	<0.0001
0.25	4EBPwt/+	58.8	66				**23%**	35.7	<0.0001
0.25	4EBPw/+	61.7	66				**32%**	48.1	<0.0001
0.25	4EBPs/+	59.5	68				**22%**	46.5	<0.0001
0.25 ♀	4EBPwt/arm	64.7	72	4%	0.6	0.44	**64%**	92.1	<0.0001
0.25	4EBPw/arm	62.3	128	-3%	3.3	0.07	**28%**	41.7	<0.0001
0.25	4EBPs/arm	61.0	97	-4%	0.5	0.49	**14%**	15.9	<0.0001
0.25	+/arm	61.3	104				**47%**	85.9	<0.0001
0.25	4EBPwt/+	62.5	67				**57%**	69.5	<0.0001
0.25	4EBPw/+	64.5	71				**50%**	80.9	<0.0001
0.25	4EBPs/+	63.7	72				**45%**	88.4	<0.0001

% YE	7/19/2006	Mean LS	n	4EBP OE	Chi Square	P Value	DR Effect	Chi Square	P Value
4 ♂	4EBPw/arm	52.2	139	**14%**	20.0	<0.0001			
4	4EBPw/+	46.0	143						
4	+/arm	43.6	125						
4 ♀	4EBPw/arm	55.6	71	**40%**	56.8	<0.0001			
4	4EBPw/+	39.8	130						
4	+/arm	38.0	65						
0.25 ♂	4EBPw/arm	55.1	168	-8%	3.2	0.073	**6%**	13.6	2E-04
0.25	4EBPw/+	59.6	125				**30%**	73.4	<0.0001
0.25	+/arm	54.5	139				**25%**	65.2	<0.0001
0.25 ♀	4EBPw/arm	75.4	88	2%	0.31	0.57	**36%**	84.0	<0.0001
0.25	4EBPw/+	73.7	95				**86%**	191.4	<0.0001
0.25	+/arm	66.9	64				**76%**	120.2	<0.0001

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