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Genetic Mechanisms Responsible for Dietary Restriction Dependent Lifespan Extension in Drosophila Melanogaster: A Role for Muscle Tissue

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Genetic Mechanisms Responsible for Dietary Restriction Dependent Lifespan Extension in Drosophila Melanogaster: A Role for Muscle Tissue

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CERTIFICATION OF APPROVAL

I certify that I have read *Title of Thesis* by Name of Student, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California and *(the Buck Institute of Aging or BioMarin Pharmaceutical Inc.)*.

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Abstract

Dietary Restriction (DR) is a robust intervention that is known to extend lifespan and increase spontaneous activity in multiple species. Whether activity increase plays a causal role in mediating the health protective benefits of DR remains unknown. To investigate this relationship, nutritional manipulations and laboratory selection for lifespan were simultaneously applied. Three physiological outputs were used for the screening and characterization of genes that may mediate the effects of DR: starvation resistance, spontaneous activity levels, and lifespan. The physiologic changes that occur are partially mediated by the nutrient sensing TOR pathway and its downstream signaling components, specifically the translational repressor, eukaryotic initiation factor eIF4E binding protein (4E-BP). Overexpression of constitutively active d4E-BP in the muscle tissue of *Drosophila melanogaster* led to starvation resistance and increased activity in flies fed a nutrient rich diet. However, the associated lifespan extension effect observed in previous studies was not reproduced. This may be due to the use of a different laboratory strain of d4E-BP, of which there are several. Three downstream targets of 4E-BP were identified from screening: Fumble, Nemo, and Nedd2-like Caspase. Both Fumble and Nemo extend lifespan upon DR when inhibited in the muscle tissue. While these candidate genes hold promise for future studies in healthy aging, sources of variation in results must be controlled. In order to truly understand the influence that a specific mutant gene has on lifespan, results need to be clearly interpretable, robust and repeatable. Only then will it be possible to start making conjectures about their relevance to human aging.

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Introduction

Nutrition has long been recognized as an important factor for influencing both the healthspan and lifespan in a variety of animals, including humans (Weindruch,1992; Walford, et al., 1996). Thus, within the field of aging research, use of known nutritional interventions that act as anti-aging therapies are employed as a tool to better understand the biology of aging. One such intervention is Dietary Restriction (DR), a robust method for extending lifespan and promoting vitality which is applicable through a broad range of taxa from yeast to mammals. In addition to extending lifespan, DR slows the progression of many age-related pathological conditions including cancer, diabetes, cardiovascular and neurodegenerative disease (Masoro, 2003). DR is defined as a reduction of particular or total nutrient intake without causing malnutrition (Weindruch, 1992; Bradley,et al. 1997; Katewa et al., 2010). DR is accomplished through a reduction in total caloric intake (CR), restriction of a particular class of nutrients, or temporal variation of food intake (Rogers et al., 2006). Understanding the underlying mechanism for how DR exerts its effects holds great promise for the development of drugs and preventative therapies which target age-related loss of function and disease.

Aging of Skeletal Muscles

Amid the plethora of age-associated pathologies, the decline of muscle's cellular structure and function is a hallmark of the aging organism. Skeletal muscle tissue is the most abundant tissue in the body. It is significantly affected by the degenerative aging process. After age 40, the average adult human loses 5% of muscle mass per decade (Lenk, et al, 2010). Sarcopenia, which means poverty of the flesh, is characterized by a decrease in muscle fiber size

and number. The etiology of sarcopenia is complex, with some known factors, and some which are only speculated to contribute. Known environmental factors are a lack of both exercise and proper nutrition. Other studies have emphasized muscle senescence (Nair, 2005), loss of protein homeostasis, (Demontis, 2010), altered hormonal status, increased apoptosis of myocytes, increased inflammatory cytokines, muscle mitochondrial damage, and oxidative stress (Flack, et al. 2011) as playing causal roles. Due to various etiological factors, an overall imbalance between muscle protein synthesis and degradation occurs. This alteration in skeletal muscle turnover causes higher rates of muscle protein degradation (Koopman, Van Loon, 2009). Muscle structural decline is accompanied by muscle functional decline. Muscular strength has a direct inverse relationship with risk of death from all causes and cancer (Ruiz, et al. 2012). Loss of muscular strength is associated with a significant reduction in quality of life and an increased risk of disability and functional dependence (Augustin, Partridge, 2009). A decline in muscle structure and function is the underlying cause behind slow gait speed, poor balance, frailty, and falls in the elderly (Janssen, et al. 2004), which ultimately leads to a greater risk of hospitalization and institutionalization.

As muscle mass is lost and muscle oxidative capacity declines, the risk of glucose intolerance increases. Glucose intolerance is the lack of ability for the body to efficiently metabolize blood sugar. This condition leads to Type II Diabetes and the metabolic syndrome, which includes excess weight, hypertension, and hyperlipidemia. Both Diabetes and Metabolic Syndrome put one at increased risk of cardiovascular comorbidities (Huang, 2009). In 2000, the health care costs for sarcopenia and its numerous repercussions were estimated to total $18.5 billion in the United States (Janssen, et al. 2004). As the aging population grows and average life expectancy continues to rise, the public health implications are staggering.

Muscle Aging Research with Drosophila melananogaster

Like humans, the fruit fly, *Drosophila melanogaster*, undergoes the age-associated decline in muscle structure and function (Augustin, Partridge, 2009). The striated muscles of the fruit fly resemble vertebrate skeletal muscles in structure, function, and protein composition. The fruit fly is small in size, matures rapidly, has a short lifespan, and is amenable to genetic manipulation, making it a useful model for the study of muscle aging. The molecular mechanisms that control lifespan in *Drosophila* are highly conserved in more complex organisms, including humans. Manipulation of these pathways using the UAS-Gal-4 system makes it possible to determine the role of a particular gene in biological function.

The UAS-Gal-4 System

The UAS-Gal-4 System is a well-established means of determining gene function in Drosophila by blocking or over-expressing the transcription of a particular gene (Phelps, Brand, 1998). It allows for the selective expression of a gene of interest both in a spatial and temporal manner. In this system, spatial control is accomplished with the selection of a driver line. The driver line directs expression of the yeast transcriptional activator protein, Gal-4, to a particular cell or tissue. In the Gal-4 driver line, the activator protein is present but has no effect. In the UAS responder line, the target gene is present but transcriptionally silent. Transcription of the target gene requires the presence of the Gal-4 protein. Crossing the Gal-4 driver line fly with a UAS responder line fly joins the activator protein to the target gene, and expression is effective in the progeny. To induce expression of a gene post-development, an inducible Gene Switch driver is selected. The Gal-4 protein becomes active only in the presence of the steroid hormone

RU486, thereby rendering two sample populations: one without the drug, which serves as a control, and one with the drug, which has gene silencing or over-expression.

Dietary Restriction in Drosophila melanogaster

In fly research, DR is accomplished through restriction of protein. Fly food media is composed of corn meal, sugar, yeast extract, and agar. By reducing yeast extract, the protein-rich component of the media, lifespan extension is consistently observed (Carvalho, et al. 2005; Min, Tatar, 2006; Mair, et al. 2005; Katewa, Kapahi, 2010). In the Kapahi laboratory, Stock Food, which mimics the fly's natural nutritional intake, is 1.5% live yeast. DR conditions are met by diluting the concentration of yeast extract to 0.5%. DR experimental conditions are contrasted to an enriched protein diet, termed Ad Libitum (AL), containing 5% yeast extract.

The Relationship Between Dietary Restriction and Activity

DR enhances spontaneous movement-related activity across a wide variety of species (Bross, et al., 2005). This phenomenon is hypothesized to result from the evolutionary theory that when nutrients are limited, a metabolic shift occurs from reproduction and growth towards somatic maintenance, thus facilitating foraging behavior (Katewa, et al, 2012). However, the genetic and biochemical mechanisms responsible for the increase in movement in response to DR are not well understood. DR may lead to increased activity levels by causing changes in myofibril proteins, enhancement of mitochondrial function (Zid et al., 2009), and/or enhancement of neuromuscular dynamics (Augustin et al., 2009). Or conversely, the increased activity levels associated with DR may be responsible for the changes in myofibril proteins, enhancement of mitochondrial function, and/or neuromuscular dynamics (Barres, et al. 2012). In

either case, elucidation of the genetic and biochemical mechanisms by which diet and activity ultimately influence lifespan begins with closer examination of muscle tissue metabolism.

DR alters translation of genes related to muscle structure and function. It has been suggested that these muscle-specific genes, in turn, may play a role in mediating healthy aging (Bauer, et al. 2010). In support of this hypothesis it has been found that enhanced fat metabolism in the muscle tissue is partially responsible for the protective effects of DR. In a recent study (Katewa, et al. 2012), wild type flies were subjected to DR and AL feeding conditions and global gene expression was measured via microarray, whereby it was determined that genes involved in fat metabolism, movement, and neuronal dynamics are major biological processes regulated by DR. Functional characterization of fat metabolism genes yielded the conclusion that enhanced fat turnover specifically in muscle tissue is required for the lifespan extension effect of DR. Additionally it was found that enhanced movement partially mediated lifespan extension upon DR. Both flies with genetically ablated wings and flies with clipped wings show a modest lifespan extension as compared to the control under DR conditions. Taken together, these data suggest that increased muscle activity is due to enhanced fat turnover in the muscle tissue and that this plays a causal role in the health protective effects of DR (Katewa, et al. 2012).

The Importance of Nutrient Sensing

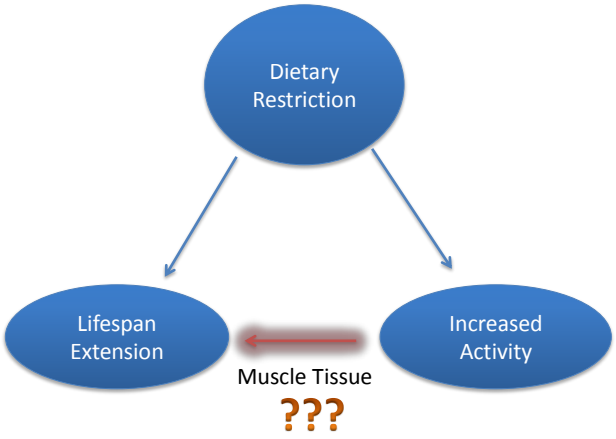
In further support of muscle tissue dynamics playing a pivotal role in aging, it has become apparent that evolutionarily conserved nutrient sensing pathways play a key role in mediating lifespan extension and that they do so through specific tissues, including muscle (Kapahi, et al. 2004, Demontis, Perrimon, 2010). There is strong evidence from research with Drosophila that the Target of Rapaymycin (TOR) pathway is one of these key pathways (Kapahi,

et al. 2004, Zid, et al. 2009). The TOR pathway integrates nutrient and environmental signals to mediate growth and metabolism. One of the strongest indications of an intervention-genetic or environmental- impacting organismal aging is to show that it extends lifespan. Overexpression of several genes in the TOR complex 1(TORC1) pathway extend lifespan in Drosophila, including the tuberous sclerosis complex proteins Tsc1, Tsc2 (Kapahi et al., 2004). The translational repressor, 4E-BP (eukaryotic initiation factor eIF4E binding protein), a downstream target of TORC1, has also been shown to mediate lifespan extension through DR. 4E-BP null mutant flies show a diminished DR response, while over-expression of 4E-BP extends lifespan in flies fed the AL diet (Zid, et al. 2009). Induction of mitochondrial processes upon DR was shown to be dependent on the activity of 4E-BP.

Furthermore, 4E-BP over-expression in muscles significantly extends lifespan and preserves muscle function (Demontis, et al, 2010). These data suggests that 4E-BP preserves muscle function by regulating muscle proteostasis via the autophagy/lysosome pathway of protein degradation (Demontis, et al, 2010). It is proposed that 4E-BP signaling in muscles plays a pivotal role in systemic metabolic regulation, regulating food intake and insulin release (Demontis, et al, 2010). These data provide more strong evidence for the idea that healthspan and lifespan are dependent on metabolic events in the muscle tissue. These events, in turn, depend on nutrient sensing pathways, and have a number of beneficial effects on distal tissues (Demontis, et al, 2010; Katewa, et al 2012).

Hypothesis and Rationale

Based on research in the host laboratory and the other cited studies, it is clear that Dietary Restriction extends lifespan and also increases muscle activity in Drosophila. My hypothesis is that metabolic changes in muscle tissue play a causal role in mediating the lifespan extension effects of DR. The changes that occur are partially mediated by the nutrient sensing TOR pathway and its downstream signaling components. In this thesis, I ask what happens to the relationship between lifespan and activity when nutritional manipulations and laboratory selection for lifespan are simultaneously applied.



To address this inquiry, I systematically examined genes involved in muscle metabolism for influence on DR mediated lifespan extension. Candidate genes were selected based on their relationship to the structure and function of muscle tissue, or on their translational response to

dietary restriction. Initially, these genes were screened by conducting the lifespan assay. Genes that manifested a lifespan change upon high or low yeast diets were selected for further study. In a second screen, genes that exhibited starvation resistance and/or increased spontaneous activity were selected for further studies. In a third project, the translational repressor 4E-BP was over-expressed in the muscle tissue to better understand the role that it plays in lifespan and activity.

Results

A Screen to identify Genes that Modulate Lifespan in a Nutrient-Dependent Manner

In initial lifespan screening, inhibition of candidate genes was achieved using a ubiquitously expressing driver strain or a muscle-specific driver strain, depending on the gene. Dietary Restriction leads to an increase in lifespan. Lifespan extension or reduction compared to the control indicates that the gene of interest plays a role in mediating lifespan. If down-regulation of a target prevented DR mediated lifespan extension or extended AL lifespan, the lifespan assay was then repeated with the drug-inducible ubiquitous driver. As shown in Table 1, of the 13 genes related to muscle structure and function, two were selected for further studies based on lifespan results: Wings UpA with an AL increase of 41%; Nautilus with a DR increase of 36% and an AL increase of 24%.

Note: For MHC-Gal-4 crosses, the control was Mhc-Gal4 x W1118, for Da-Gal-4 crosses, the control was Da-Gal4 x W1118.

Table 1. Candidate Muscle Genes Lifespan Results

Gene	Function	Driver	Food	Average Lifespan	Median Survival	N	% change
****Wings UpA CG7178**	Tropomyosin binding Actin binding Muscle organ development	MHC	DR Control	69.8	71	146	-13%
			DR RNAi	59.9	62	132	
			AL Control	49	39	149	**41%
			AL RNAi	55.7	55	135	
UAS Mef2 Myocyte Enhancer Factor 2	Trancscription factor Muscle fiber development	MHC	DR Control	69.8	71	146	-39%
			DR RNAi	49.3	43	140	
			AL Control	49	39	149	-31%
			AL RNAi	33.9	27	141	
Lame Duck CG4677	Trancscription factor	Da	DR Control	63.5	58	126	22%
			DR RNAi	67.1	71	137	
			AL Control	36.3	33	138	30%
			AL RNAi	43.2	43	139	
Flightin CG7445	Myosin thick filament assembly Sarcomere organization	Da	DR Control	72	58	135	2%
			DR RNAi	58.9	59	103	
			AL Control	44	33	132	3%
			AL RNAi	39.9	34	117	
****Nautilus CG10250**	Transcription factor Muslce organ development	Da	DR Control	72	58	135	**36%
			DR RNAi	77.6	79	154	
			AL Control	42	33	132	**24%
			AL RNAi	46.1	41	138	

****Nautilus CG10250**	Transcription factor Muslce organ development	MHC	DR Control	69.8	71	146	**14%
			DR RNAi	72	81	147	
			AL Control	49	39	149	**54%
			AL RNAi	57	60	145	
Slouch CG6534	Transcription factor Muscle cell fate determination	Da	DR Control	63.5	58	126	26%
			DR RNAi	73.7	73	146	
			AL Control	36.3	33	138	24%
			AL RNAi	38.5	41	146	
Upheld CG7107	Calcium ion homeostasis Muscle cell homeostasis Thin filament assemby	MHC	DR Control	69.8	71	146	23%
			DR RNAi	73.4	87	135	
			AL Control	49	39	149	36%
			AL RNAi	50	53	144	
Muscle Protein 20 CG4696	Calcium ion binding Actin binding Regulation of cell shape	MHC	DR Control	69.8	71	146	1%
			DR RNAi	68.4	72	143	
			AL Control	49	39	149	3%
			AL RNAi	38.8	40	135	
Tropomyosin 2 CG4843	Actin binding	MHC	DR Control	69.8	71	146	23%
			DR RNAi	81.9	87	146	
			AL Control	49	39	149	56%
			AL RNAi	58.6	61	139	
Muscle Specific Protein 300 CG33715	Actin binding	MHC	DR Control	69.8	71	146	-3%
			DR RNAi	67.1	69	154	
			AL Control	49	39	149	-15%

			AL RNAi	34.6	33	115	
Myosin Heavy Chain CG17927	Myosin thick filament assemby Locomotion	MHC	DR Control	69.8	71	146	-10%
			DR RNAi	58.88	64	150	
			AL Control	49	39	149	-8%
			AL RNAi	38.5	36	142	
****Myosin Light Chain CG2184**	ATPase Muscle System Process	MHC	DR Control	69.8	71	146	28%
			DR RNAi	87.7	91	143	
			AL Control	49	39	149	**92%
			AL RNAi	68.9	75	126	
Troponin C at 73F CG7930	Calcium ion binding	MHC	DR Control	69.8	71	146	21%
			DR RNAi	82.5	86	149	
			AL Control	49	39	149	26%
			AL RNAi	48	49	135	
Myosin Heavy Chain CG17927	Myosin thick filament assembly Locomotion	DaGS	DR Control	43.8	45	154	-11%
			DR RNAi	39	40	155	
			AL Control	31.3	29	145	-17%
			AL RNAi	25.9	24	147	
Upheld CG7107	Calcium ion homeostasis Muscle cell homeostasis Thin filament assemby	DaGS	DR Control	55.3	55	118	0%
			DR RNAi	56	55	124	
			AL Control	30	32	96	-5%
			AL RNAi	29.8	30.5	106	
Wings UpA CG7178	Tropomyosin binding Actin binding Sarcomere	DaGS	DR Control	59.8	63	158	-2%
			DR RNAi	58.5	62	154	

	organization		AL Control	34.3	33	151	0%
			AL RNAi	33.1	33	165	
Wings UpA CG7178	Tropomyosin binding Actin binding Sarcomere organization	Act5c GS	DR Control	79.2	81	148	9%
			DR RNAi	84.7	88	159	
			AL Control	45.2	46	150	0%
			AL RNAi	45.7	46	153	
Nautilus CG10250	Transcription factor Muslce organ development	DaGS	DR Control	47.5	48	154	4%
			DR RNAi	48.2	50	150	
			AL Control	38.4	36	172	-11%
			AL RNAi	34.9	32	147	

As shown in Table 2, of the three DR-responsive candidate genes that were screened, two yielded significant results when crossed with the Da-Gal4 driver: Frizzled 2 with an AL increase of 42%, Wnt5 with a DR increase of 47% and an AL increase of 64%.

Table 2. Candidate Microarray Genes Lifespan Results

Gene	Function	Driver	Food	Average Lifespan	Median Survival	N	% change
****Frz2 CG9739**	Wnt recepter signalling pathway	Da	DR Control	63.5	58	126	-3%
			DR RNAi	52.4	56	144	
			AL Control	36.3	33	138	**42%
			AL RNAi	44.5	47	151	
Wishful Thinking CG10776	Neuromuscular junction development	Da	DR Control	63.5	58	126	5%
			DR RNAi	61.8	61	145	
			AL Control	36.3	33	138	6%
			AL RNAi	35.5	35	145	
****Wnt5 CG6407**	Wnt receptor signaling pathway	Da	DR Control	63.5	58	126	**47%
			DR RNAi	81.9	85	139	
			AL Control	36.3	33	138	**64%
			AL RNAi	53.4	54	139	
****Frz2 CG9739**	Wnt recepter signalling pathway	DaGS	DR Control	71.6	73	162	0%
			DR RNAi	69.8	73	130	
			AL Control	39.42	41	156	-17%
			AL RNAi	33.97	34	144	
Frz2 CG9739	Wnt recepter signalling pathway	Act5c GS	DR Control	83.4	85	139	0%
			DR RNAi	83.6	85	142	
			AL Control	54.6	53	133	0%
			AL RNAi	54.7	53	109	

Wnt5 CG6407	Wnt receptor signaling pathway	DaGS	DR Control	65	67	155	-7%
			DR RNAi	60.9	62	152	
			AL Control	41.2	41	159	2%
			AL RNAi	44	42	162	
Wnt5 CG6407	Wnt receptor signaling pathway	Act5c GS	DR Control	77	80	136	-5%
			DR RNAi	73	76	157	
			AL Control	39	41	132	0%
			AL RNAi	42	41	146	
Wishful Thinking CG10776	Neuromuscular junction development	DaGS	DR Control	60.5	61	188	-5%
			DR RNAi	56.1	58	196	
			AL Control	25.2	26	204	-12%
			AL RNAi	23.8	23	185	

Repeat of the Lifespan Assay Failed to Reproduce Significant Results

Using both the DaGS-Gal4 and the Act5c-GS-Gal4 drug-inducible ubiquitous drivers to repeat the lifespan assays did not yield the same results, however. For all four of the candidate genes, no significant effect was observed between the Control and the experimental (Figure 1).

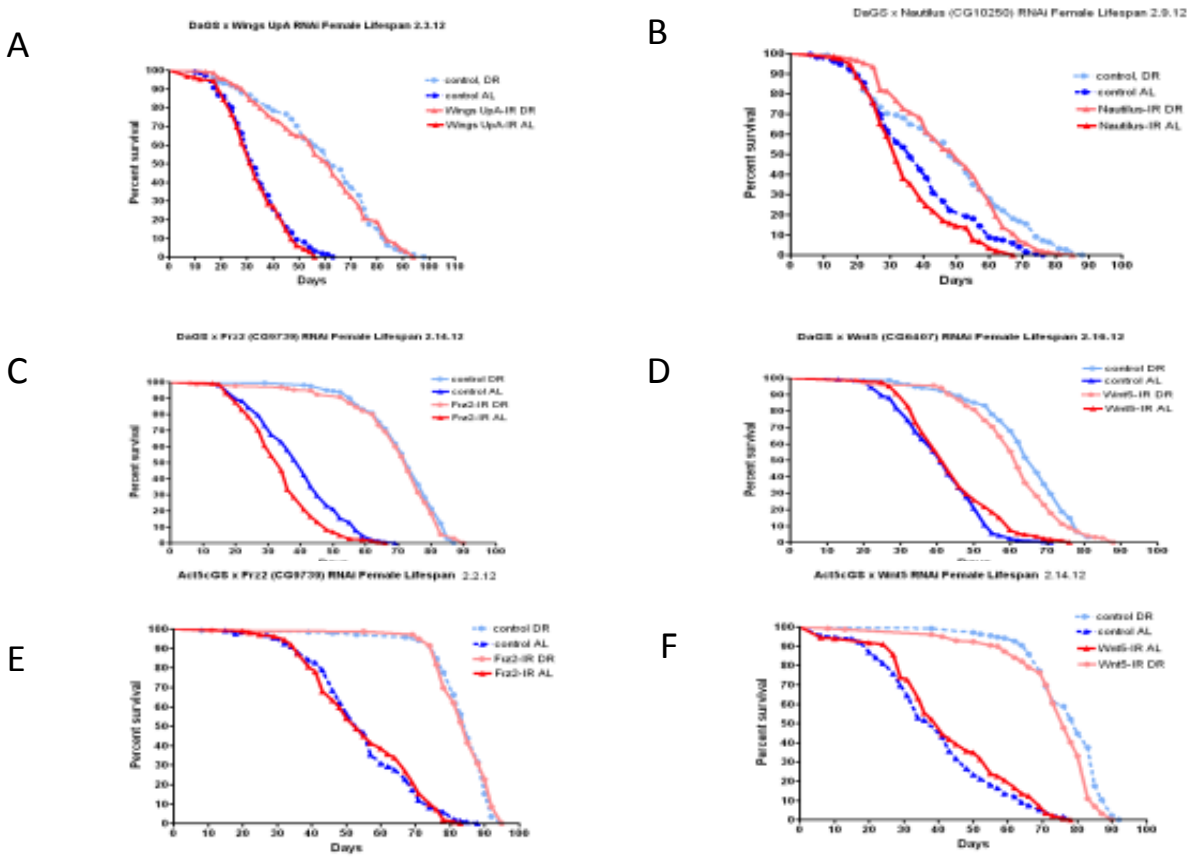


Figure 1. Repeat Lifespans on Candidate Genes Failed to Reproduce Significant Results. Median life span was calculated from Kaplan-Meier survival analysis of female flies upon RNAi of candidate genes in whole body under DR (light red) and AL (dark red) conditions

(A) Effect of WingsUpA RNAi on DR-dependent lifespan extension ([+/+; +/+; Da-GS/ CG7178])

(B) Effect of Nautilus RNAi on DR-dependent lifespan extension ([+/+; +/+; Da-GS/ CG10250])

(C) Effect of Frizzled2 RNAi on DR-dependent lifespan extension ([+/+; +/+; Da-GS/ CG9739])

(D) Effect of Wnt5 RNAi on DR-dependent lifespan extension ([+/+; +/+; Da-GS/ CG6407])

(E) Effect of Frizzled2 RNAi on DR-dependent lifespan extension ([+/+; +/+; Act5c-GS/ CG9739])

(F) Effect of Wnt5 RNAi on DR-dependent lifespan extension ([+/+; +/+; Act5c-GS/ CG6407

A New Screen to Identify Genes that Modulate Lifespan in a Nutrient Dependent Manner

In a second screen based on starvation resistance and activity levels, positive hits were determined based on responsiveness to both of these criteria as well as Translational State Array Analysis (TSAA) and known gene function. The Starvation Assay is used as a stress test and also may be thought of as an abbreviated representation of lifespan. Measurement of spontaneous activity is essential in making a correlation between a gene and activity levels. All genes were inhibited using the muscle specific driver, Mhc-Gal4. Of the 29 genes screened, three were selected for further studies: Fumble, Nemo, and Nedd2-like Caspase.

Table 3. Candidate Microarray Genes Starvation Results

Genotype	Biological Process	Food	Median Survival	N	Mutant DR/AL	Mutant DR/AL Relative to Control DR/AL
MHC x W1118	**Control**	DR RNAi	**66**	**89**	**1.5**	**1.5**
		AL RNAi	**43**	**98**		
****Fbl CG5725**	Triglyceride homeostasis Locomotion	DR RNAi	86	74	1.3	0.9
		AL RNAi	64	73		
Slob CG43756	Starvation response Neuromuscular dynamics	DR RNAi	135	72	2.1	1.4
		AL RNAi	65	71		
Fst CG9434	Cold response	DR RNAi	66	69	1.6	1.0
		AL RNAi	42	74		
****Nc CG8091**	Programmed cell death Biological	DR RNAi	119	96	2.6	1.7

	regulation	AL RNAi	46	89		
Eip7EF CG32180	Transcription factor Autophagy	DR RNAi	92	93	1.9	1.2
		AL RNAi	49	98		
Trp-like CG18345	Calcium ion transport Ca signaling	DR RNAi	71	93	1.5	1.0
		AL RNAi	46	94		
Loqs CG6866	Gene silencing Nervous sys development	DR RNAi	90	95	2.0	1.3
		AL RNAi	46	99		
CG34781	Unknown	DR RNAi	119	73	2.2	1.4
		AL RNAi	55	94		
Bif CG1822	Axon extension Axon guidance	DR RNAi	88	101	2.0	1.3
		AL RNAi	45	111		
Fax CG4609	Axonogenesis Neurogenesis	DR RNAi	90	102	2.0	1.3
		AL RNAi	45	102		
Lola CG10252	Unknown	DR RNAi	65	99	1.6	1.0
		AL RNAi	41	102		
CG10959	Zinc ion binding Nucleic acid binding	DR RNAi	90	97	2.1	1.4
		AL RNAi	42	86		
Dgrn CG10981	Regulation of protein binding	DR RNAi	90	101	2.1	1.4
		AL RNAi	42	98		
Adar CG12598	Locomotory process Response to stimulus	DR RNAi	93	99	2.2	1.4
		AL RNAi	42	100		
Hs3st-B CG7890	Post-embryonic organ development	DR RNAi	98	95	1.5	1.0
		AL RNAi	64	97		

CG9883	Unknown	DR RNAi	110	135	1.7	1.1
		AL RNAi	64	100		
CG10621	Unknown	DR RNAi	88	96	2.1	1.4
		AL RNAi	42	95		
CG31782	Unknown	DR RNAi	66	103	1.6	1.0
		AL RNAi	42	100		
U2A CG1406	Mitosis	DR RNAi	88	99	2.1	1.4
		AL RNAi	42	96		
CG3408	Muscle cell homeostasis	DR RNAi	90	104	1.4	0.9
		AL RNAi	66	73		
CG5352	Gonad development Mitotic spindle organization	DR RNAi	66	98	1.6	1.0
		AL RNAi	42	99		
CG6854	Neurogenesis	DR RNAi	90	100	2.0	1.3
		AL RNAi	46	106		
CG11398	Unknown	DR RNAi	114	99	2.6	1.7
		AL RNAi	44	100		
UK114 CG15261	Protein folidng	DR RNAi	66	104	1.6	1.0
		AL RNAi	42	95		
Loqs CG6866	Central nervous sys dev MiRNA metabolic proceses	DR RNAi	69	88	1.6	1.0
		AL RNAi	43	92		
Nbr CG9247	Production of miRNA	DR RNAi	89	98	1.3	0.9
		AL RNAi	66	99		
****Nmo CG7892**	Wnt receptor signaling Synaptic growth at NMJ	DR RNAi	114	89	2.5	1.7
		AL RNAi	45	96		
MHC (OC) x W1118	**Control**	**DR RNAi**	**89**	**79**	**1.3**	**1.3**
		AL RNAi	**66**	**96**		

4EBP UAS (OC)	Lifespan, mitochondrial translation	DR RNAi	88	104	1.3	1.0
		AL RNAi	66	106		
Fbl CG5725 (OC)	Triglyceride homeostasis Locomotion	DR RNAi	93	99	1.4	1.0
		AL RNAi	66	102		
Nc CG8091 (OC)	Programmed cell death Biological regulation	DR RNAi	115	72	1.7	1.3
		AL RNAi	66	97		
Nmo (OC)	Wnt receptor signaling Synaptic growth at NMJ	DR RNAi	89	100	1.3	1.0
		AL RNAi	66	106		
Slob CG43756 (OC)	Starvation response Neuromuscular dynamics	DR RNAi	119	108	2.5	1.8
		AL RNAi	48	91		
Dilp6	Regulation of organism growth	DR RNAi	113	100	1.7	1.3
		AL RNAi	66	103		
Hip14	Synaptic transmission	DR RNAi	80.5	98	1.2	0.9

Relative Change in Median Survival was Determined by the Ratio of Median Survival in the Mutant Compared to the Control

<1 = DR Reduction or AL Extension

>1 = DR Extension or AL Reduction

1 = No Effect

Three Candidate Genes Emerge with Dramatically Different Biological Functions

Fumble is a pantothenate kinase. This type of enzyme phosphorylates pantothenate, which is involved in the Coenzyme A biosynthetic pathway. It has known biological functions including involvement in locomotion (Wu, et al. 2009) and triglyceride homeostasis (Bosveld, et al., 2008).

Nedd2-like Caspase is an endopeptidase. This type of enzyme breaks peptide bonds of a nonterminal amino acid (Yan, et al., 2006). It has known biological functions including central nervous system development (Chew, et al. 2004), autophagic cell death (Gorski et al, 2003; Lee et al., 2011; Yang et al., 2010), and determination of adult lifespan (Bauer et al., 2005)

Nemo is a protein kinase. Protein kinases are enzymes that phosphorylate proteins. It has known biological functions including positive regulation of synaptic growth at the neuromuscular junction (Merino, et al. 2009).

To further characterize the genes of interest, I examined the changes in mRNA translation upon DR. Because initiation is the rate limiting step for the translation of most mRNA's, mRNA translational rate can be inferred from the number of ribosomes that are recruited (Sonenberg et al, 2001). For the gene, Nemo, 40, 60, 80S Ribosomes and Polysomes decreased relative to the total, suggesting that the translational rate of mRNA decreased under DR conditions (Figure 2A). For the gene, Fumble, 40, 60, 80S Ribosomes and Polysomes increased relative to the total, suggesting that mRNA increased in response to DR (Figure 2B). For the gene, Nedd2-like Caspase, the Polysomal fractions decreased relative to the total, suggesting that mRNA levels decreased in response to DR (Figure 3C).

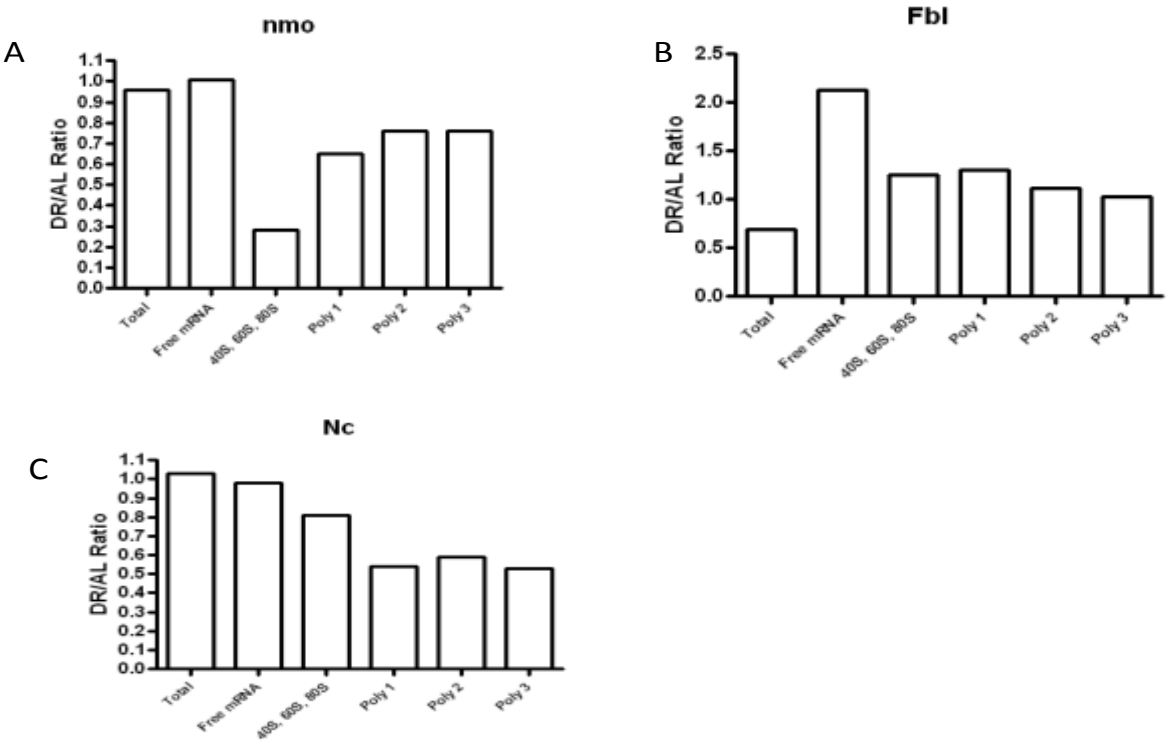


Figure 2. mRNA Translation-Associated Changes Upon DR of candidate genes.
Polysomal distribution of mRNAs of young adult female flies on 5% YE and 0.5% YE showing the DR/AL ratio of ribosomal subunits and polysomal fractions

(A)Nemo ribosomal and polysomal fractions decrease relative to the total

(B) Fumble ribosomal and polysomal fractions increase relative to the total

(C) Nedd2-like Caspase polysomal ractions decrease relative to the total

These data gave me some indication of what results I might expect from future assays. When RNAi is used against upregulated and downregulated targets, the expectation is that the diet will produce different outcomes. RNAi against upregulated proteins should prevent the DR-mediated increase in longevity, activity, and starvation resistance and would cause a similar decrease in AL-fed flies. Alternatively, RNAi against downregulated proteins should not alter

DR-mediated increase in lifespan, and should extend AL-fed lifespan, increase activity, and starvation resistance.

Table 4. Summary of TSAA Results and Expected Outcomes for Candidate Genes

Gene	TSAA upon DR	Interpretation	Expected Outcome
Nmo	**Ribosomes and Polysomes down relative to Total**	**Translation of mRNA decreasing**	**No effect on DR-mediated increase in lifespan. Extend AL-fed starvation, increase activity, lifespan.**
Fbl	**Ribosomes and Polysomes up relative to Total**	**Translation of mRNA increasing**	**Prevent DR-mediated increase in starvation, activity, lifespan. Same in AL**
Nc	**Polysomes down relative to Total**	**Translation of mRNA decreasing**	**No effect on DR-mediated increase in lifespan. Extend AL-fed starvation, increase activity, lifespan.**

In order to verify initial results, a second starvation assay was run on the three candidates. At the time of the initial starvation screening, the Mhc-Gal4 driver being used in the host laboratory was lacking genetic diversity. Inbred driver lines, though they still enable targeted gene expression, produce a phenotype that is problematic. For this reason, periodic out-crossing of the line is necessary. By out-crossing, the genetic background of a wild strain is bred into the driver line. When the out-crossed driver line became available for use in the laboratory, all crosses were then carried out with it. As shown in Figure 3, starvation resistance between the non-out-crossed and the out-crossed flies yielded very different results.

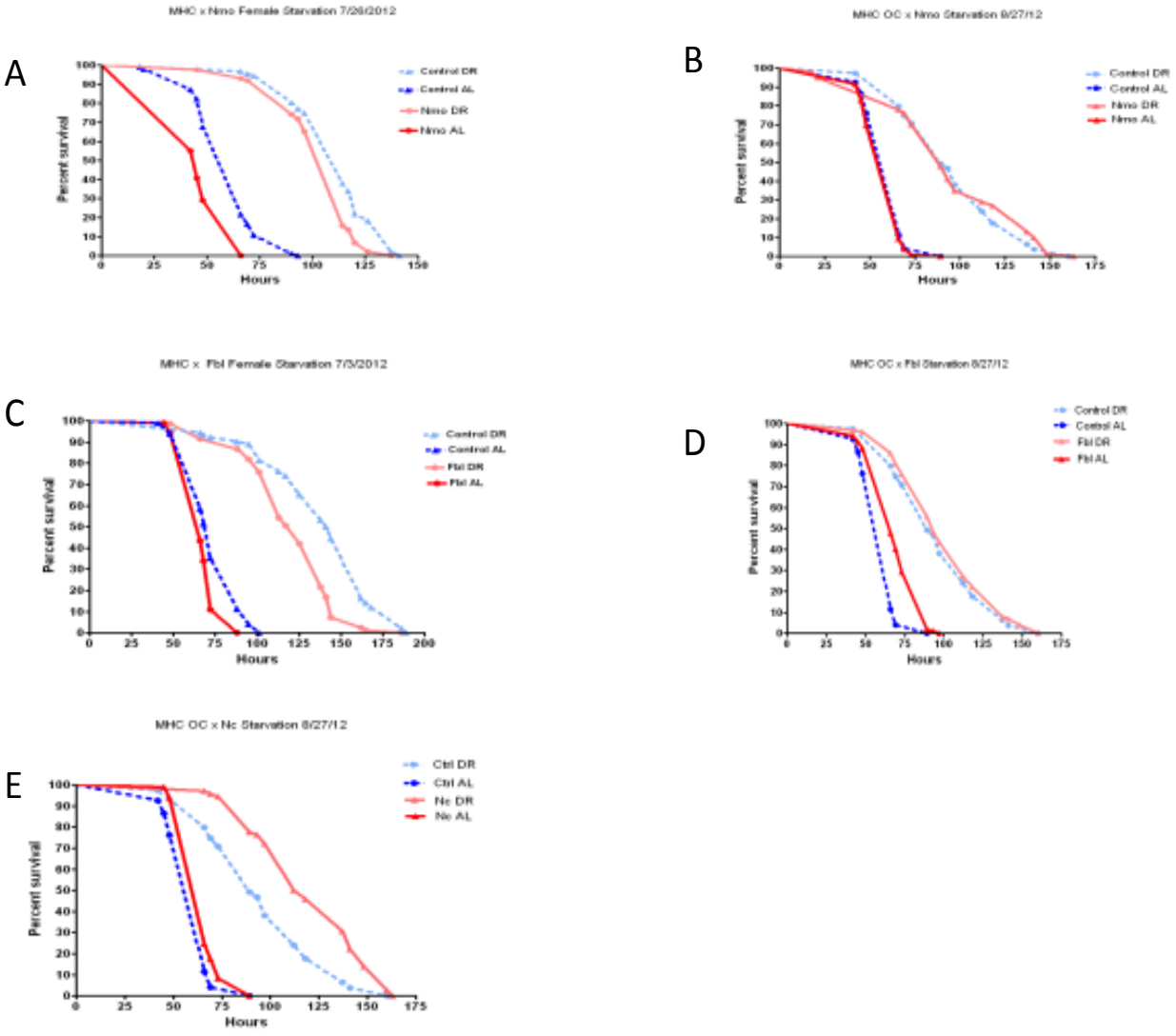


Figure 3. Muscle-Specific Inhibition of Candidate Genes Influences Starvation Resistance in a Nutrient-Dependent Manner in D. melanogaster Kaplan Meir survival analysis for starvation resistance in RNAi female flies (solid line) and control flies (dashed line). Control used was W1118 Mhc-Gal4/+ (A-E)

(A) Nemo (CG7892-RNAi/Mhc-Gal4)

(B) Nemo with out-crossed driver (CG7892-RNAi/Mhc-Gal4)

(C) Fumble (CG5725-RNAi/Mhc-Gal4)

(D) Fumble with out-crossed driver (CG5725-RNAi/Mhc-Gal4)

(E) Nedd2-like Caspase with out-crossed driver (CG8091-RNAi/Mhc-Gal4)

Inhibition of the gene Nemo in the non-out-crossed Mhc-Gal4 background resulted in both AL and DR reduction in starvation resistance (Figure 2A). Inhibition of Nemo in the out-crossed Mhc-Gal4 background resulted in no significant effect on starvation resistance (Figure 2B). Inhibition of Fumble resulted in both an AL and DR reduction in starvation resistance when expressed in the non-out-crossed Mhc-Gal4 background (Figure 2C). When inhibited in the out-crossed Mhc-Gal4 background, Fumble exhibited AL extension on starvation resistance (Figure 2D). Inhibition of the gene Nedd2-like Caspase was only carried out in the out-crossed Mhc-Gal4 background, and it exhibited a slight AL extension and a DR extension in starvation resistance.

Next, activity levels were measured in the three candidate genes. Two controls were used: Mhc-Gal4 crossed with the wild type fly (W1118), and W1118 crossed with the mutant. This was done to control for the Gal4 protein and the mutant, respectively. After out-crossing, both the Mhc-Gal4 driver and the mutant line should have had the same genetic background-that of a wild type fly. Therefore, in theory, there should have been very little phenotypic difference between Mhc-Gal4 x W1118 and Mhc-Gal4 x mutant because they should have had the same genetic background. However, due to decreased confidence in the genetic interactions between the Gal4 protein of the driver and the transgene, the third control was used. By crossing a wild type fly with a fly carrying the transgene, the progeny did not express the transgene but still had the transgene in their genotype. The third control was used to evaluate discrepancy in genetic interactions between the Gal4 protein of the driver and the transgene. Using this third control ensured that the phenotypic effects observed in crossing Mhc-Gal4 with each transgene were not just due to hybrid vigor.

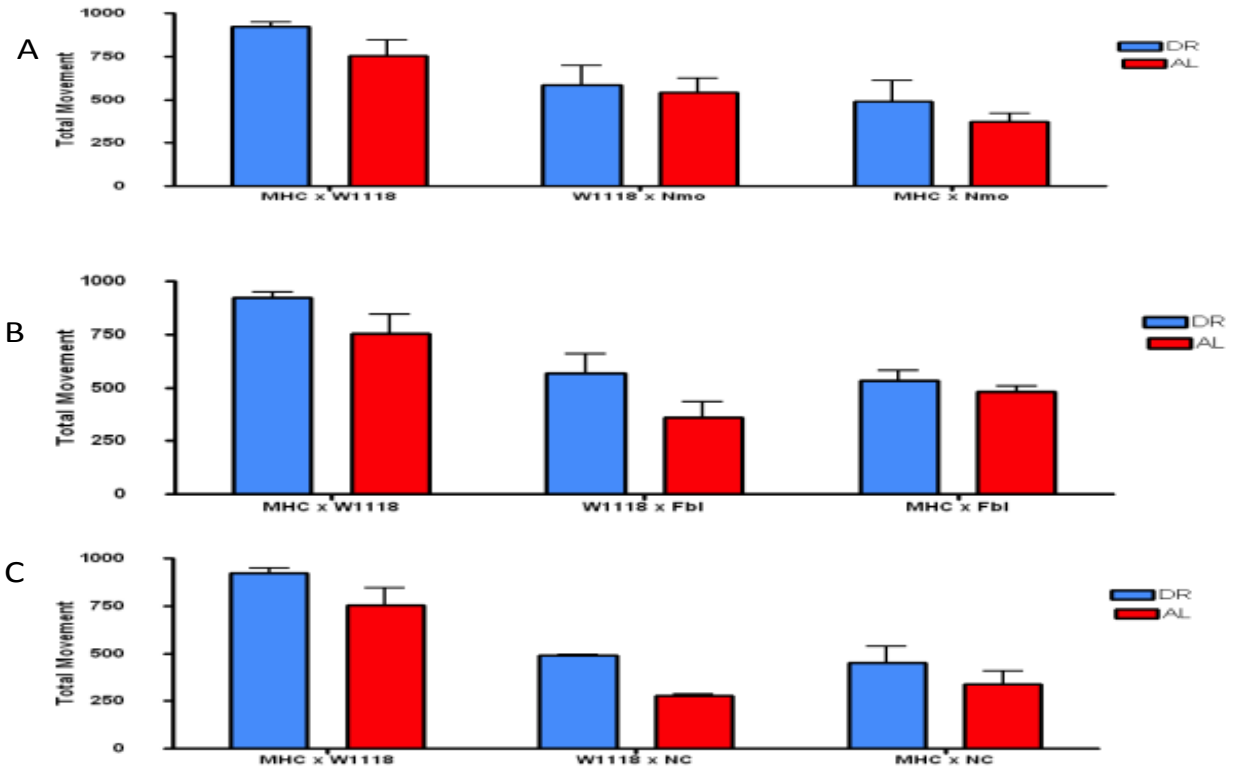


Figure 4. Muscle-specific Inhibition of Candidate Genes Influences Spontaneous Activity in a Nutrient-Dependent Manner in D. melanogaster. Controls used were w1118; Mhc-Gal4/+ (A-C) and w1118; CG7892/+ (A), w1118; CG5725/+ (B), w1118; CG8091/+ (C)

(A)Effect of Nmo (CG7892-RNAi/Mhc-Gal4) on spontaneous activity

(B)Effect of Fbl (CG5725-RNAi/Mhc-Gal4) on spontaneous activity

(C) Effect of Nc (CG8091-RNAi/Mhc-Gal4) on spontaneous activity

Activity data failed to show trends or statistical differences. Furthermore, interpretation of the experimental groups was ambiguous due to the differences in the activity of the two controls. Inhibition of Nemo led to an AL reduction in activity compared to both Controls and a DR reduction compared to the Mhc Control (Figure 3A). Fumble showed an AL increase in activity compared to W1118 Control, and an AL reduction in activity compared to the Mhc-Gal4

Control (Figure 3B). Fumble showed a DR decrease in activity compared to the Mhc-Gal4 Control, and no effect compared to the W1118 Control (Figure 3B). Inhibition of Nedd2-like Caspase showed an AL increase in activity compared to the W1118 Control, and a decrease in activity compared to the Mhc-Gal4 Control (Fig 3C). Nedd2-like Caspase showed a reduction in activity compared to the Mhc-Gal4 Control, and no effect upon DR compared to the W1118 Control (Figure 3C).

Next, the question was asked whether changes in starvation resistance and spontaneous movement could be correlated with lifespan in the candidate genes.

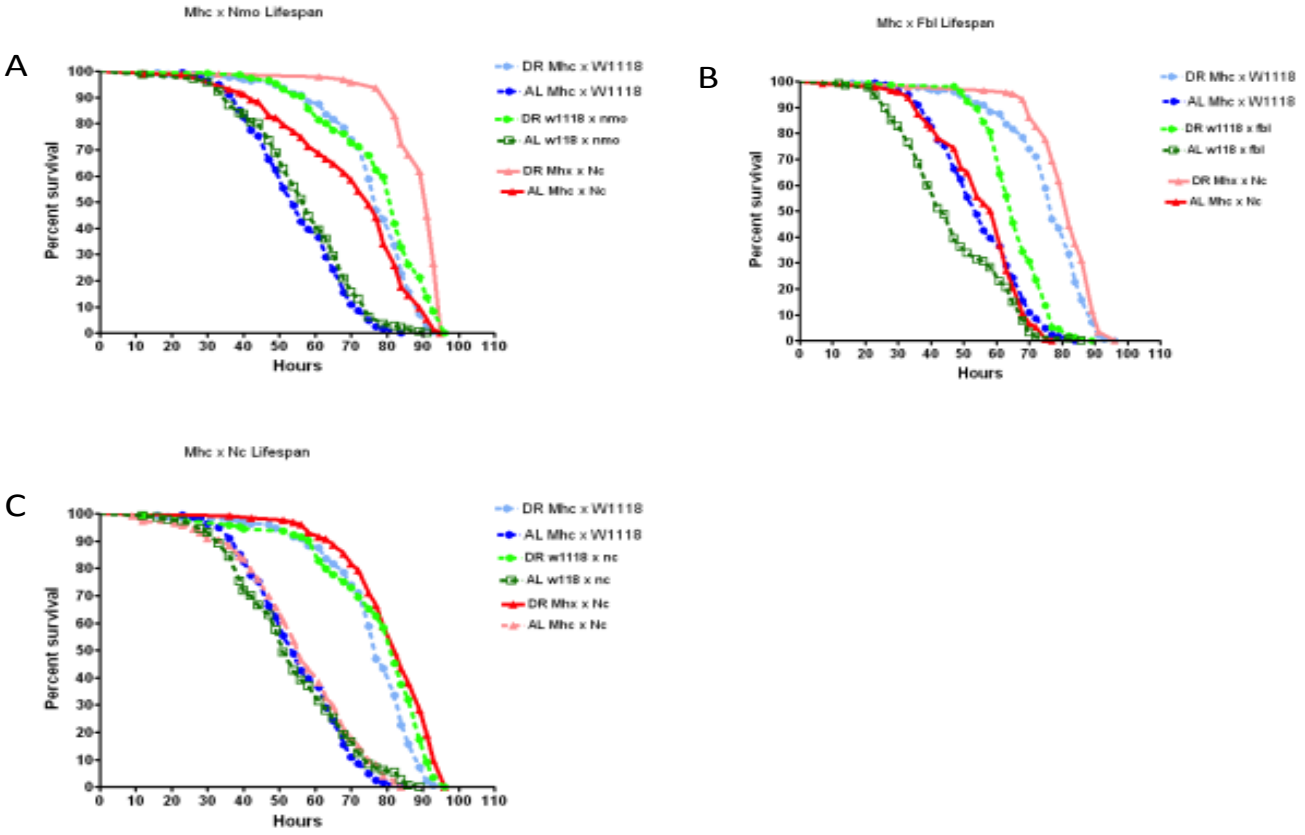


Figure 5. Inhibition of Two Candidate Genes Extends Lifespan upon DR in *D. melanogaster* . Median life span was calculated from Kaplan-Meier survival analysis of female flies upon RNAi of candidate genes in muscle tissue under DR (light red) and AL (dark red) conditions**.** Controls used were w1118; Mhc-Gal4/+ (blue, dashed lines) (A-C) and w1118; CG7892/+ (A), w1118; CG5725/+ (B), w1118; CG8091/+ (C) (green, dashed lines)

(A)The effects of Nmo (CG7892-RNAi/Mhc-Gal4) on lifespan

(B)The effects of Fbl (CG5725-RNAi/Mhc-Gal4) on lifespan

(C)The effect of Nc (CG8091-RNAi/Mhc-Gal4) on lifespan

The gene, Nemo exhibited lifespan extension both in DR and AL conditions. The percentage increase in lifespan upon DR was 13% and 21% compared to W1118 and Mhc Control, respectively. The percentage increase in lifespan in the nutrient-rich condition was 29% and 39% compared to W1118 and Mhc Control, respectively.

The gene, Fumble exhibited lifespan extension in both DR and AL conditions. The percentage increase in lifespan upon DR was 26% and 6% compared to W1118 and Mhc Control, respectively. The percentage increase in lifespan in the nutrient-rich condition was 39% and 13% compared to the W1118 and Mhc Control, respectively.

The gene, Nedd2-like Caspase exhibited a negligible lifespan effect compared to both Controls. The percentage increase in lifespan upon DR was 2% and 9% compared to the W1118 and Mhc Control, respectively. The percentage increase in the lifespan in the nutrient-rich condition was 10% and 4% compared to W1118 and Mhc Control, respectively.

To investigate whether the candidate genes would influence the age-related decline in muscle function, activity measurements were taken over the lifespan.

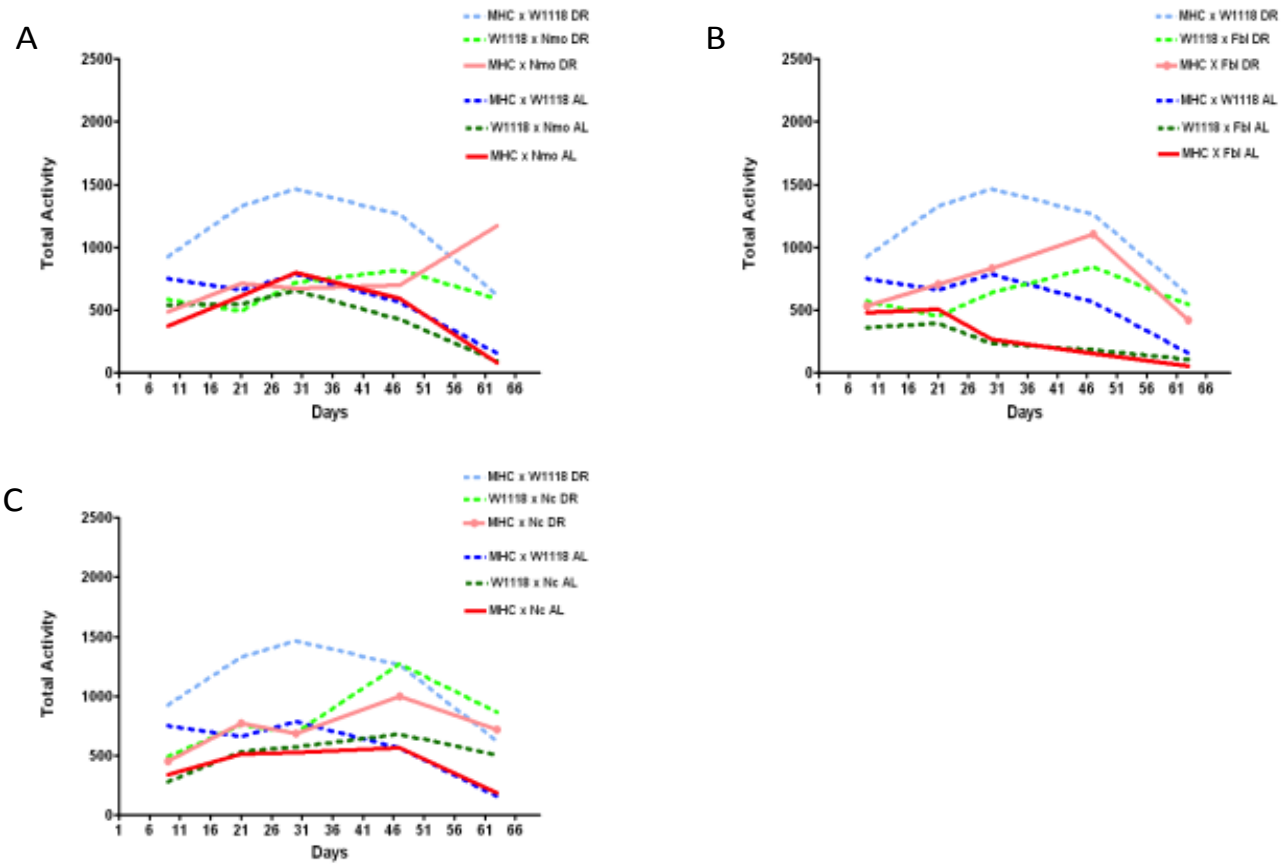


Figure 6. Inhibition of Candidate Genes Influences Age-Related Decline in Muscle Function Age-dependent measurement of total activity in female flies. Daily activity was measured in the Drosophila activity monitors. Controls used were w1118; Mhc-Gal4/+ (blue, dashed lines) (A-C) and w1118; CG7892/+ (A), w1118; CG5725/+ (B), w1118; CG8091/+ (C) (green, dashed lines)

(A)Total activity in Nmo RNAi (CG7892-RNAi/Mhc-Gal4)

(B)Total activity in Fbl RNAi (CG5725-RNAi/Mhc-Gal4)

(C)Total activity in Nc RNAi (CG8091-RNAi/Mhc-Gal4)

Similar to Day 10, interpretation of activity over the lifespan was ambiguous. The gene, Nmo decreased activity in the DR condition over the lifespan compared to the Mhc Control, and caused no effect when compared to the W1118 Control. Of note, however, is that activity increased starting at Day 46 and increased compared to both Controls as the flies aged. In the AL condition, there was no effect. The gene, Fumble decreased in the DR condition compared to the Mhc Control, and increased in activity compared to the W1118 Control. Of note, activity steadily increased until Day 51, at which point it rapidly decreased. In the AL condition, activity decreased compared to the Mhc Control, and caused no effect compared to the W1118 Control. The gene, Nedd2-like Caspase decreased in the DR condition relative to the Mhc Control, and caused a slight decrease relative to the W1118 Control. In the AL condition, there was no effect.

Overexpression of 4E-BP in Muscle Tissue Enhances Starvation Resistance and Increases Spontaneous Activity in a nutrient-dependent manner

In a third project, the effect of over-expressing d4E-BP in the muscle tissue on starvation resistance and activity over the lifespan was examined.

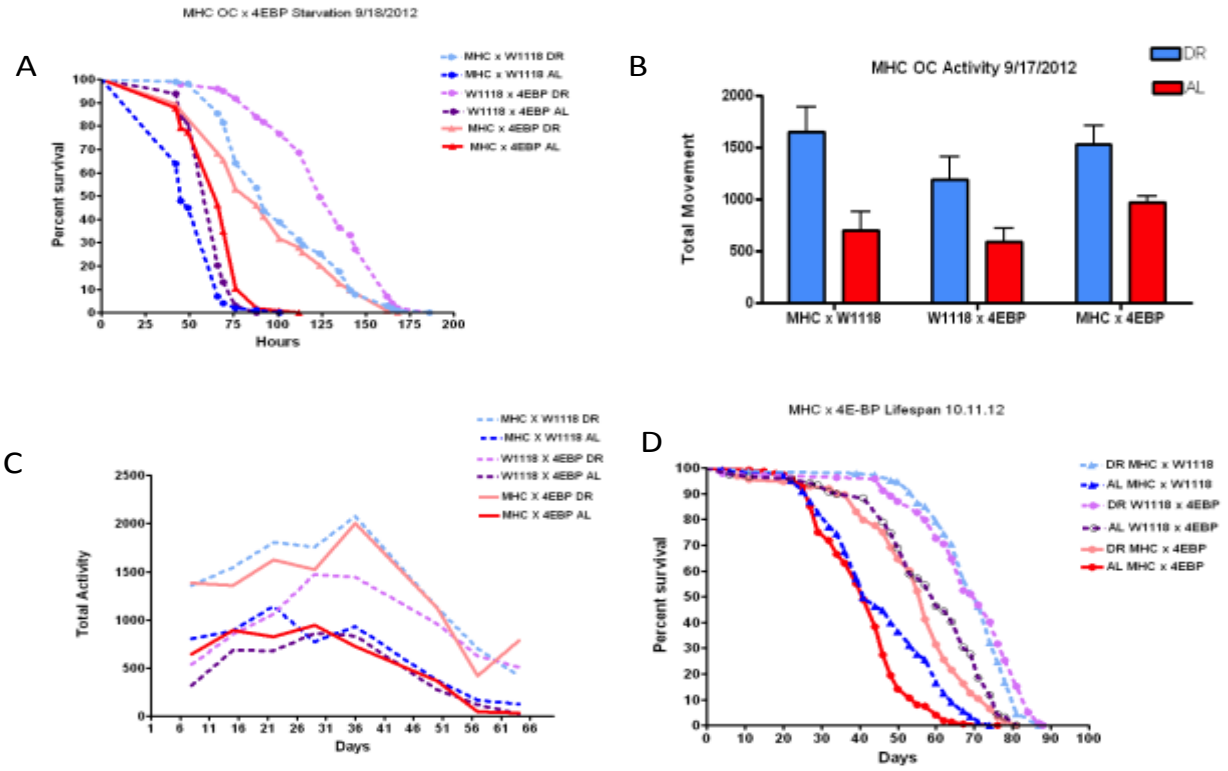


Figure 7. d4E-BP Overexpression in Muscle Tissue Enhances Starvation Resistance and Spontaneous Activity,Yet Does Not Extend Lifespan in a Nutrient-Dependent Manner *D. melanogaster* Controls used were w1118; Mhc-Gal4/+(blue, dashed lines) and w1118; 4E-BP/+ (purple, dashed lines) (A-D)

(A) Starvation Resistance was enhanced in a Nutrient-Dependant Manner. Kaplan Meir survival analysis for starvation resistance in RNAi female flies (solid line) and control flies (dashed line).

(B)Spontaneous Activity taken on Day 10 of the lifespan, Activity was measured in the Drosophila activity monitors. Activity increased in a Nutrient-Dependant Manner.

(C) Age-dependent measurement of total activity. Spontaneous Activity over the lifespan increased in a Nutrient-Dependent Manner.

(D) Median life span was calculated from Kaplan-Meier survival analysis of female flies upon DR . (light red) and AL (dark red) conditions**.** Life span was abrogated in both AL and DR flies.

Starvation and activity data from flies overexpressing activated d4E-BP in the muscle tissue indicated *enhanced* starvation resistance and *increased* activity in a nutrient-dependant manner in AL relative to the W1118 x 4E-BP Control. Or alternately, activity *decreased* in flies on both AL and DR relative to the MHC x 4EBP Control. Flies over-expressing d4E-BP increased in spontaneous activity on Day 9 following eclosion and over the lifespan in a nutrient-dependant manner but the interpretation, again, is moot depending on which Control is used. Interestingly, lifespan was decreased in both AL (32%, 0%) and DR (20%, 20%) flies compared to the W1118 x 4E-BP and Mhc x 4E-BP controls, respectively. This was an unexpected result because earlier studies (Zid, et al. 2009) with flies of the same genotype demonstrated lifespan extension in a nutrient dependent manner.

4E-BP may play a causal role in increased movement upon DR

In a previous study done in the host lab, enhanced muscle activity was shown to play a causal role in the lifespan extension effects upon DR (Katewa, et al, 2012). To demonstrate this, flies with reduced movement due to clipped wings showed only a 33% extension in lifespan while control flies showed a 97% extension in lifespan. Based on this finding, the question was asked whether overexpression of d4E-BP might influence the increased activity levels in the DR condition and whether this might ultimately play a causal role in lifespan extension. To address this inquiry, activity, activity over lifespan, and lifespan in flies overexpressing d4E-BP with reduced mobility due to having their wings clipped was examined. The hypothesis was that if d4E-BP plays a causal role in the increased movement upon DR, then flies overexpressing d4E-BP with clipped wings would exhibit higher activity and also live longer than the controls.

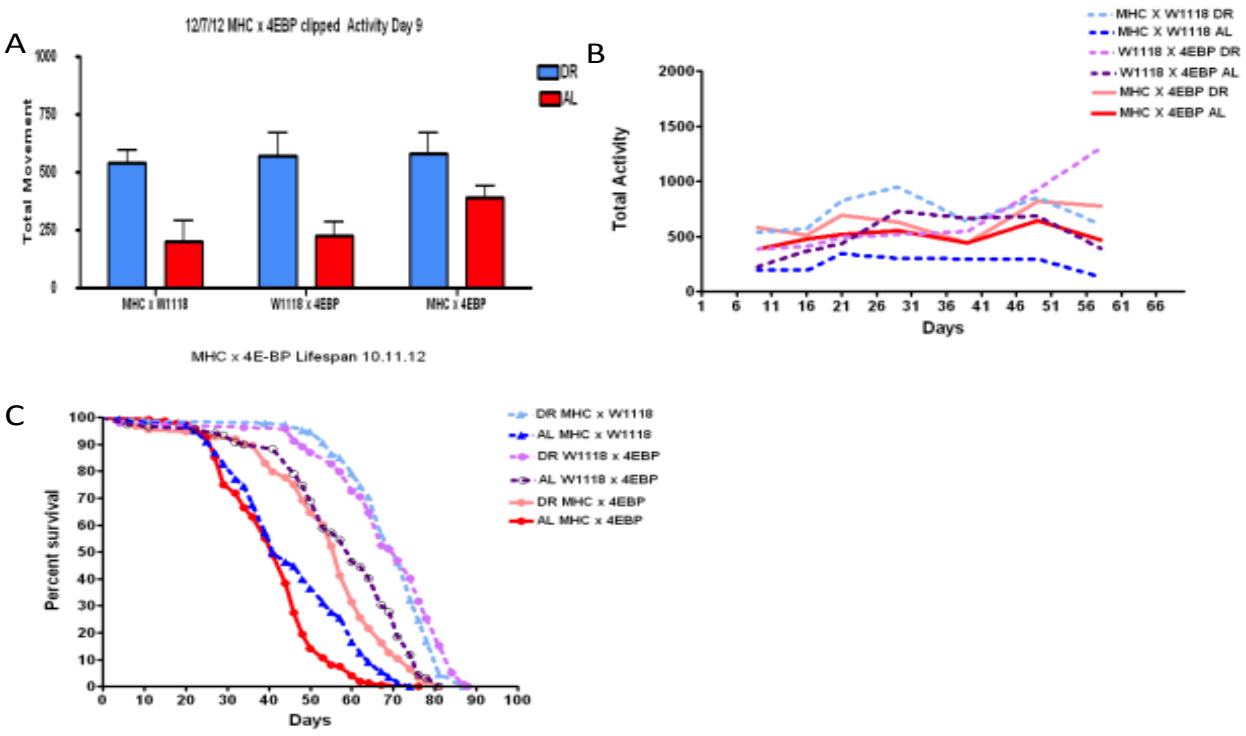


Figure 8. Muscle-specific overexpression of 4E-BP in Flies with Clipped Wings Increases Activity in a Nutrient-Dependent Manner But Does Not Correlate with Lifespan Extension Controls used were w1118; Mhc-Gal4/+ (blue, dashed lines) and w1118; 4E-BP/+ (purple, dashed lines) (A-C)

(A) Spontaneous Activity taken on Day 9 of the lifespan. Activity was measured in the Drosophila activity monitors. Activity increased in a Nutrient-Dependant Manner.

(B) Age-dependent measurement of total activity in female flies. Spontaneous Activity over the Lifespan increased in a nutrient-dependent manner

(C) Effect on nutrient-dependent increase in lifespan. Median life span was calculated from Kaplan-Meier survival analysis of female flies upon DR (light red) and AL (dark red) conditions

On Day 9, flies overexpressing d4E-BP with clipped wings showed an increase in activity in the AL condition (Figure 6A). This trend continued throughout lifespan compared to Mhc-Gal4 Control, but not compared to W1118 x 4E-BP Control (Figure 6B). These changes in

activity correlated with a slight increase in lifespan in AL (19%) condition when compared to W1118 x 4E-BP Control (Figure 6C).

Discussion

While it is undeniable that a relationship exists between diet, activity, and lifespan, this relationship remains only partially understood. In D. *melanogaster*, the TOR pathway and its downstream signaling components have proved to play a function in ageing and its effects appear to be mediated through muscle tissue. By using starvation resistance, activity measurement, and lifespan assays, this relationship was investigated. Some of the results hold promise, yet there are several caveats to the research.

Lifespan results differ between the Da-Gal4 driver and the Da-GS-Gal4 driver

In the initial lifespan screen, genes of interest were first inhibited using the Daughterless-Gal-4 ubiquitous driver or the Mhc-Gal-4 muscle tissue specific driver. There were several shortcomings to using these drivers:

1) Gene knockdown occurred at conception, and therefore may have had an effect on the development of the progeny

2) The corresponding Controls (Mhc-Gal4 x W1118 or Da-Gal4 x W1118) differ in genetic background from the experimental flies, and therefore, results are difficult to interpret

3) Laboratory strains lacked genetic diversity and therefore yielded phenotypic results that were the result of inbreeding depression; therefore not truly representing the characteristics of the gene of interest

For these reasons, positive hits were then repeated with the drug-inducible driver, Da-GS-Gal4 driver and in some cases, Act5c-GS-Gal4 driver. Using Gene Switch drivers, repeated lifespan assays failed to produce the same results as initially observed. The lack of repeatability with Gene Switch drivers indicates that there is an issue or multiple issues with the functioning of the drivers that is not yet completely understood. Theoretically, Da-Gal4 driver and Da-GS-Gal4 driver should yield the same effect on the phenotype. The defining difference between the two is that Da-GS-Gal4 driver is used to inhibit the expression of a gene post-development. This is advantageous in some cases because it enables the study of genes which are essential to the development of the organism. Secondly, and of extreme importance, this system provides a control which is identical in genetic background. Two populations of flies with the same genetic background are used. One group receives the drug, achieving gene knockdown, and the other does not receive the drug, therefore retaining gene function. A caveat of using this system, however, is that the effects of RNAi are less pronounced. The drug-inducible driver is less robust in its effect on gene knockdown for a variety of reasons that are only partially understood (Clark, Pazpernik, 2012).

These differences beg the question whether the results emanated from a developmental issue or a difference in the robustness of the inducible drivers. To test whether it is a developmental issue, a future experiment would be to perform gene knockdown with the Da-Gal4-GS driver at a time point earlier in development. This would be carried out by exposing the driver line and the mutant line to the drug when setting up the cross. Effectively, the progeny should have the gene knocked down from conception, thereby yielding lifespan results similar to that obtained with the Da-Gal4 driver. If they do, then this would serve as support for the hypothesis that lifespan effects of the gene are dependent on developmental interactions. If

application of the inducible system earlier in development still does not elicit lifespan effects, then this would suggest that the Da-Gal4 GS driver is less robust or somehow different in its function from the Da-Gal4 driver.

Starvation and Activity Results differ between the non-outcrossed Mhc driver and the outcrossed Mhc driver

Similar to the first screen, examining starvation resistance and activity of positive hits in the second screen yielded differing results in follow-up assays. The fundamental issue lies again with the driver, but in this case it can be explained by different genetic backgrounds between the non-outcrossed Mhc-Gal4 driver used initially and the outcrossed Mhc-Gal4 driver used in later assays.

The process of outcrossing is carried out to increase hybrid vigor in the laboratory strains used for targeted gene expression. Hybrid vigor is often discussed as the opposite of inbreeding depression (Birchler, et al, 2006). Over time, strains kept in laboratory stocks tend towards genetic divergence because low numbers of animals lead to inbreeding. In this environment, harmful genetic variants become more common as the associated recessive alleles accumulate in the homozygous state. When inbred strains are crossed with each other, resulting lifespans are often longer in the offspring simply due to hybrid vigor; thus yielding results that potentially misrepresent the function of the gene of interest (Partridge, et al 2007).

Out-crossing is also necessary to establish a genetic background that is similar between experimental flies and their respective controls. For initial screening, the out-crossed muscle-specific driver line was crossed with a mutant line which had the same genetic background-that of a wild type fly. The progeny was compared to two controls which also had the wild type

genetic background. Theoretically, the two controls should have exhibited similar phenotypes, allowing for detection of the mutant genes' effect. However, one of the Controls (Mhc-Gal4 x W1118) was unusually active, creating ambiguity and making interpretation difficult. This suggests that the genetic background between the strains is not identical or that there is some other dynamic in the genetic background of this fly that is not understood yet. This could be due to the possibility that the backcrossing was not effectively carried out in the Mhc-Gal4 driver. Another explanation is that the mutant lines have not been outcrossed. Therefore, the second control (W1118 x Gene X) is less active. To eliminate ambiguity in future studies, backcrossing of all strains should be done regularly prior to conducting assays. To further explore muscle-specific gene function, a drug-inducible muscle-specific driver could be used. This would circumvent the issues that arise with the driver and the controls. At this point, an Mhc-Gal4-GS driver is not available in the host laboratory.

Of note, although the secondary screens with the out-crossed Mhc-Gal4 driver yielded less significant and in some cases entirely opposite results from the first screen, the targets still hold promise because they were selected by several additional criteria: known gene function and TSAA analysis. Additionally, knockdown of the genes Fumble and Nemo showed significant lifespan extension effects in both AL and DR (Figure 5). These data provide further evidence that these genes hold promise in lifespan and healthspan studies.

Starvation and Activity Results were incongruent with expectations based on Translational State Array Analysis (TSAA)

Previously published (Zid, et al. 2009) and unpublished data from the host laboratory suggest that the benefits of dietary restriction are mediated through 4E-BP. The candidate genes for the second screen were selected based on data suggesting that they are translationally 4E-BP dependent (Figure 2). While RNAi was used against both upregulated and downregulated targets, different outcomes were expected depending on the diet.

In examining the results from the TSAA, the gene, Fumble showed that polysomal fractions were increased relative to the total, indicating that it is translationally upregulated in response to DR. Therefore the expectation was a decrease in lifespan, activity, and starvation resistance in both DR and AL-fed flies. In the initial starvation screen using the non-outcrossed Mhc-Gal-4 driver, this expectation held true. However, in the following starvation assay using the outcrossed Mhc-Gal-4 driver, AL extension and no DR effect was observed. Interpretation of the activity data is similarly confounding depending on which control is used for comparison. Relative to the Mhc-Gal4 x W1118 control, both DR and AL-fed flies decreased in activity. But relative to the W1118 x Fbl control, AL-fed flies increased in activity and DR-fed flies showed no effect.

In examining the results from the TSAA, the gene, Nemo showed that polysomal fractions decreased relative to the total, as well as the 40S, 60S, 80S ribosomal fractions, indicating that it is translationally down-regulated in response to DR. Therefore no difference was expected in DR-fed flies, and lifespan extension, activity increase, and starvation resistance in AL fed flies. In the initial starvation screen using the non-outcrossed Mhc-Gal4 driver, the opposite occurred.

Flies on both DR and AL fed diets showed a decrease in starvation resistance. Interestingly, in the following starvation assay using the outcrossed Mhc-Gal4 driver, the expectation held true and mutant flies on AL diet showed starvation resistance. Interpretation of the activity data is mootable depending on which control is used. Relative to the Mhc-Gal4 x W1118 control, both DR and AL fed flies decreased in activity. But relative to the W1118 x Nmo Control just AL fed-flies decreased in activity. In either case, the result is counter to the expectation.

In examining the results from the TSAA, the gene, Nedd2-like caspase showed that polysomal fractions were decreased relative to total, indicating that it was translationally down-regulated in response to DR. Therefore the expectation was for there to be no difference DR fed flies, and for there to be lifespan extension, activity increase, and starvation resistance in AL fed flies. In the starvation screen using the outcrossed Mhc-Gal4 driver, only a slight increase in starvation resistance occurred in AL-fed flies, and an unexpected increase occurred in DR fed flies. Interpretation of the activity data is again mootable depending on which control is used. Relative to the Mhc-Gal4 x W1118 control, both DR and AL fed flies decreased in activity. But relative to the W1118 x Nc control, AL flies did exhibit the expected increase in activity.

Table 5. Summary of Results from Candidate Genes

Gene	Biological Process	Condition	mRNA Translation	Median Survival	Starvation: Mutant DR/AL Relative to Control DR/AL	Activity	Lifespan Extension: Relative to Mhc x W1118 Control, W1118 x Mutant Control
Nmo	Wnt receptor signaling Growth at NMJ	DR	Decrease	89	1	Decrease or no effect*	21%, 13%
		AL		66		Decrease	39%, 29%
Fbl	Triglyceride homeostasis Locomotion	DR	Increase	93	1.04494382	Decrease or no effect*	6%, 26%
		AL		66		Decrease or Increase*	13%, 39%
Nc	Programmed cell death Biological regulation	DR	Decrease	115	1.292134831	Decrease or no effect*	9%, 2%
		AL		66		Decrease or no effect*	4%, 10%

*Interpretation is based on the control

At this time, the only apparent explanation for these inconsistencies is that correlation does not prove causation. It is true that in some genotypes, Dietary Restriction appears to play a causal role in the three distinct outputs measured in this thesis: starvation resistance, increased spontaneous activity, and lifespan extension (Katewa, et al. 2012). However, the TOR pathway is highly pleiotropic in its effects on metabolism. We are only just beginning to understand the complexities of these effects. By manipulating single genes downstream of TOR, one can only deduce that compensatory changes in physiology related to energy allocation, storage, and utilization are impacted, but will vary depending on the gene.

Future experiments to further understand these relationships in candidates should include measurement of both triglyceride levels and glucose tolerance. It has been shown that flies under DR that exhibit higher activity levels also show higher steady-state triglyceride levels (Katewa, et al. 2012). In flies, insulin signaling has also been found to effect locomotor function and lifespan (Jones, et al. 2009).

Overexpression of d4E-BP in the muscle tissue shows differing results from previous studies carried out in the host laboratory.

In a previous study, muscle-specific overexpression of d4E-BP was shown to increase lifespan and prevent the age-related decline in muscle function (Demontis, Perrimon, 2010). However, attempts to repeat this failed to demonstrate an increase in lifespan. One possible explanation for this is that organisms of the same strain, but from different laboratories, sometimes differ in lifespan (Partridge, Gems, 2007). This could be due to genetic divergence within strains, or due to use of different isoforms of the same gene. There is a wild type d4E-BP, as well as two activated alleles of d4E-BP, classified as “weak” and “strong”. It has been found

that overexpression of these different forms produces variable effects on the lifespan (Zid, et al. 2009). In order to reproduce the lifespan effects observed by other laboratories, the same strain of flies needs to be acquired directly from that laboratory. Another explanation may be due to variance in the laboratory environment. For instance, small differences in light, heat, and humidity can all account for effects observed in lifespan. Although these variables are usually standardized, it is difficult to control them absolutely.

Conclusion

Aging is a complex process which is inevitable and ostensibly irreversible. One distinct intervention, Dietary Restriction, has been shown to slow aging in D. melanogaster and improve biomarkers associated with healthy aging. In this thesis, the relationship between Dietary Restriction and activity, a known biomarker of healthy aging, was investigated. To explore this relationship, nutritional manipulations and laboratory selection for lifespan were simultaneously applied. The hypothesis was that metabolic changes in muscle tissue would play a causal role in mediating the lifespan extension effects of DR. To address this inquiry, components of the nutrient sensing TOR pathway were systematically examined for their effect on lifespan and activity by conducting RNAi in muscle tissue. It was found that muscle-specific overexpression of eukaryotic translation initiation factor 4E binding protein (4E-BP), a direct target of TOR, enhanced both starvation resistance and activity levels in a nutrient dependent manner. Additionally, three downstream targets of 4E-BP were identified that may play a role in mediating the lifespan extension effects of DR through eliciting an increase in activity. Two of the three, Nemo and Fumble, are very promising candidates for future lifespan studies due to their significant lifespan extension effects. Yet a caveat of this research is the presence of

uncontrolled genetic differences between strains under study. It is difficult to decipher the results of assays when using controls that are not genetically identical to the mutant strain. In order to truly understand the influence that a specific mutant gene has on lifespan, results need to be clearly interpretable, robust and repeatable. To maximize the potential for this type of result, future studies on the candidate genes will utilize backcrossed strains and make use of inducible drivers so that differences in genetic backgrounds are eliminated. These candidate genes hold promise for future studies in healthy aging. Once sources of variation in results are controlled, it will then be possible to start making conjectures about their relevance to human health.

Methods

Fly husbandry and lifespan analysis

Fly husbandry was carried out per Kapahi Laboratory protocols (Zid, et al. 2009). In the first screen, males from the RNAi lines were crossed to virgin females carrying the ubiquitous Da-Gal4 driver and muscle specific Mhc-Gal4 driver (from VDRC). For repeat lifespans, males from the RNAi lines were crossed to virgin females carrying the ubiquitously expressing RU486 inducible Act4C-GS-Gal4 driver or the Da-GS-Gal4 driver. In the second screen, males from the RNAi lines were crossed to virgin females carrying the muscle specific Mhc-Gal4 driver (from VDRC).

Genotype of the fly strains used for candidate genes in the second screen:

(+/+; Mhc-Gal4/+; UAS-4E-BP), (+/+; Mhc-Gal4/+; CG5725 (fbl)), (+/+; Mhc-Gal4/+; CG8091 (nc)), (+/+; Mhc-Gal4/+; CG7892 (nmo))

Flies were developed on standard lab food, containing 1.5% live yeast. On day 14 after crossing, progeny were sorted on light CO2 and females were transferred to the yeast extract (YE) diet. The AL diet contained 5% yeast extract and the DR diet contained 0.5% yeast extract.

Lifespan Assay

The assay commences on Day 6 after sorting. Flies are placed on DR/AL media. Deaths are recorded every other day for the duration of life and plotted as a graph of Percent Survival over Time. Kaplan Meier Survival Analysis was used to assess the effect of genes on lifespan.

Starvation Assay

The assay commences on Day 10 after sorting. Females were placed on starvation media, composed of 1% agar. Deaths were recorded every 2-4 hours for the duration of life and plotted as a graph of Percent Survival over Time.

Spontaneous activity measurements

The Drosophila Activity Monitor (Tri kinetics Inc.) is used to measure total movement activity over a period of 24 hours. Movement of flies is measured in the vertical direction and a three equidistant points over the length of the vial (approximately 2 cm, 5 cm, and 8 cm about food surface). The flies were transferred to fresh food in the morning by 12:00 pm and then moved to the monitors. Measurements for a 24 hour period began at 4:00 pm. Time points are taken throughout the course of the lifespan and plotted as Total Fly Activity/Day.

Outcrossing

To outcross the Mhc-Gal4 driver line, males from Mhc-Gal4 stocks were mated to W1118 females. From the progeny, heterozygous mutant males were then backcrossed to females with the W1118 genetic background 5 times.

Wing Clipping

For wing clipping, females were sorted on Day 10 after eclosion. Following brief anesthetization with CO2, the wings were clipped to approximately 1/3 of their original length. Activity measurements began that same day.

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References

Augustin, H., Partridge, L., Invertebrate models of age-related muscle degeneration. BBA - General Subjects Oct 2009, 1790 (10), 1084-1094

Barres, R, Yan, J, Egan, B, Treebak, JT, Rasmussen, M, Fritz, T, Caidahl, K, Krook, A, O'Gorman, DJ, Zierath, JR. Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle. Cell Metabolism 2012 405-411

Bauer, J, Antosh, M, Chang, C, Schorl, C, Kolli, S, Neretti, N, Helfand, SL. Comparative transcriptionial profiling identifies takeout as a gene that regulates lifespan. Aging, 2010, Volume 2 Number 5 298-310

Bauer, J, Poon, PC, Glatt-Deeley, H., Abrams, JM., Helfand, SL. 2005. Neuronal expression of p53 dominant-negative proteins in adult Drosophila melanogaster extends lifespan. Curr. Biol. 15(22): 2063-2068

Bosveld, F., Rana, A., van der Wouden, PE. Lemstra, W. Ritsema, M., Kampinga, HH., Sibon, OCM. 2008. De novo CoA biosynthesis is required to maintain DNA integrity during development of the Drosophila nervous system. Hum. Mol. Genet. 17(13): 2058-2069

Birchler JA, Yao H, Chudalayandi S, Unraveling the genetic basis of hybrid vigor, PNAS, August 29, 2006 , vol. 103 no. 35.

Bradley, TJ, Simmons, FH. An analysis of resource allocation in response to dietary yeast in Drosophila Melanogaster. J. Insect Physiology. 43. 779-788

Bross, T.G., Rogina, B., Helfand, S.L. Behavioral, Physical, and Demographic Changes in Drosophila Populations Through Dietary Restriction. Aging Cell, 2005

Carvalho, GB, Kapahi, P, Benzer, S. Compensatory ingestion upon dietary restriction in Drosophila melanogaster. 2005. Nature Methods. 2(11)

Chew, SK., Akdemir, F., Chen, P., Lu, WJ., Mills, K., Daish, T., Kumar, S., Rodriguez, A., Abrams, JM. 2004. The apical caspase Dronc governs programmed and unprogrammed cell death in Drosophila. Dev. Cell 7(6): 897:907

Clark, D, Pazpernik, N, Biotechnology-Academic Cell Update, Academic Press, 2012, 453

Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng YH, Doria A, Kolodny GM, Kahn CR. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 2009 Apr

Demontis, F, Perrimon, N. FOXO/4E-BP Signaling in Drosophila Muscles Regulates Organism-wide Proteostasis during Aging. Cell Nov. 2010. 813-825

Faulkner, JA., Larkin, LM, Claflin, DR, Brooks, SV. Age-Related Changes in the Structure and Function of Skeletal Muscles. Clinical and Experimental Pharmacology and Physiology Nov. 2007 Volume 34, Issue 11, pg. 1091–1096

Flack, KD, Davy, KP, Hulver, MW, Winett, RA, Frisard, MI, Davy, BM. Aging, Resistance Training, and Diabetes Prevention. Journal of Aging Research. Vol. 2011 Article ID 127315, 12 pages

Gorski, SM., Chittaranjan, S., Pleasance, ED., Freeman, JD., Anderson, CL., Varhol, RJ., Coughlin, SM., Zuyderduyn, SD., Jones, SJM., Marra, MA. 2003. A SAGE approach to discovery of genes involved in autophagic cell death. Curr. Biol. 13(4): 358-363

Huang, P. A comprehensive definition for metabolic syndrome**.** *Dis. Model. Mech.* May/June 2009

Hwang, J. H., Pan, J. W., Heydari, S., Hetherington, H. P. & Stein, D. T. Regional differences in intramyocellular lipids in humans observed by in vivo 1H-MR spectroscopic imaging. *J. Appl.Physiol.* 90, 1267–1274 (2001)

Hurt, R., Kulisek, C., Buchanan, L., and McClave, S., The Obesity Epidemic: Challenges, Health Initiatives, and Implications for Gastroenterologists. Gastroenterology and Hepatology(N Y). 2010 December; 6(12): 780–792.

James PT, Rigby N, Leach R. The Obesity Epidemic, Metabolic Syndrome and Future Prevention Strategies. European Journal of Preventive Cardiology **Feb 2004** 11(1) **3-8**

Janssen, I. Shepard, DS. Katzmarzyk, PT. Roubenoff, R. The healthcare costs of sarcopenia in the United States. J Am Geriatr Soc. 2004 Jan;52(1):80-5.

Johannsen DL, Ravussin E Aging health. Obesity in the elderly: Is faulty metabolism to blame? 2010 Apr 1;6(2):159-167Exp Gerontol. 2009 Aug;44(8):532-40

Jones MA, Gargano JW, Rhodenizer D, Martin I, Bhandari P, Grotewiel M. A forward genetic screen in Drosophila implicates insulin signaling in age-related locomotor impairment

Kapahi, P., Chen, D., Rogers, A., Katewa, S., Wai-Lun Li, P., Thomas, E., Kockel, L. With TOR, Less is More: A Key Role for the Conserved Nutrient-Sensing TOR Pathway in Aging. Cell Metabolism 11, 2010 June. Pg. 453

Karakelides, H., Irving, B., Short, K., O'Brien, P., Sreekumaran Nair, K. Age, Obesity, and Sex Effects on Insulin Sensitivity and Skeletal Muscle Mitochondrial Function. Diabetes. 2010 January; 59(1): 89–97

Katewa SD, Kapahi, P. Dietary Restriction and Aging, 2009. Aging Cell 2010(9): 105-112

Katewa SD, Demontis F, Kolipinski M, Hubbard A, Gill MS, Melov S, Kapahi P. Intra-myocellular Triglyceride Turnover Plays a Critical Role in Mediating Responses to Dietary Restriction in Drosophila Melanogaster. Article In Review, 2012

Kerndt, PR, Naughton, JL, Driscoll, CE, Loxterkamp, DA Fasting: The History, Pathophysiology and Complications**.** West J Med. 1982 November; 137(5): 379–399.

Koopman R, van Loon LJ. Aging, exercise, and muscle protein metabolism. *J Appl Physiol* 106: 2040–2048, 2009

Lenk, K, Schuler, G, Adams, V. Skeletal muscle wasting in cachexia and sarcopenia: molecular pathophysiology and impact of exercise training. Journal of Cachexia, Sarcopenia and Muscle. September 2010, Volume 1, Issue 1, pp 9-21

Mair, W., Piper, M., Partridge, L. Calories do not explain extension of lifespan by dietary restriction in Drosophila. 2005. PLoS Biol 3(7): e22

Masoro, E.J. Subfield History: Caloric Restriction, Slowing Aging, and Extending Life ci. Aging Knowl. Environ., 26 February 2003 Vol. 2003, Issue 8

Merino, C, Penney, J, Gonzalez, M, Tsurudome, K, Moujahidine, M, O'Connor, M, Verheyen, E, Haghighi, P. Nemo kinase interacts with Mad to coordinate synaptic growth at the Drosophila neuromuscular junction. 2009. Journal of Cell Biology. Vol. 185 no. 4 713-725

Min, K. Tatar, M. Drosophila diet restriction in practice: Do flies consume fewer nutrients? Mech of Ageing and Dev. 2006. 127: 93-96

Nair, KS. Aging Muscle. American J. Clinical Nutrition. 2005. 81; 953-963

Nicholson L, Singh GK, Osterwalder T, Roman GW, Davis RL, Keshishian H. Spatial and temporal control of gene expression in Drosophila using the inducible GeneSwitch GAL4 system. I. Screen for larval nervous system drivers. Genetics. 2008 Jan;178(1):215-34.

Nusbaum TJ, Rose MR. The effects of nutritional manipulation and laboratory selection on lifespan in Drosophila melanogaster. J. Gerontol. A Biol. Sci. Med. Sci 1999;54:B192–B198

Partridge, L, Gems, D. Commentary: Benchmarks for ageing studies. Nature. Nov. 2007. Vol 450 (8). 165-167

Penedo FJ, Dahn JR. Exercise and Well-Being: A Review of Mental and Physical Health Benefits Associated with Physical Activity. Current Opinion in Psychiatry.March 2005 18 (2). 189-193

Phelps CB, Brand AH. Ectopic gene expression in Drosophila using GAL4 system.Methods. 1998 Apr;14(4):367-79.

Rogers, A.N., Kapahi, P. Genetic Mechanisms of Lifespan Extension by Dietary Restriction. Drug Discovery Today: Disease Mechanisms. Vol 3. No 1 2006

Rimbert, V.., Boirie, Y., Bedu, M., Hocquette, J., Ritz, P., Morio, B. Muscle Fat Oxidative Capacity Is Not Impaired By Physical Inactivity: Association with Insulin Senstitivity. The FASEB Journal. 2004; 18:737-739

Ruiz, JR, Sui, X, Lobelo, F, Morrow, JR, Jackson, AW, Sjostrum, M, Blair, SN. Association between muscular strength and mortality in men: prospective cohort study. British Medical Journal. 2012; 337:a439

Sinclair, DA._Toward a unified theory of caloric restriction and longevity regulation. Mech Ageing Dev. 2005 Sep;126(9):987-1002

Stathopoulou, G., Powers, M. B., Berry, A. C., Smits, J. A. J. and Otto, M. W. 2006, Exercise Interventions for Mental Health: A Quantitative and Qualitative Review. Clinical Psychology: Science and Practice, 13: 179–193.

Sonenberg, N, Gingras, A, Raught, B. 2001. Regulation of translation initiation by FRAP/mTOR. Genes & Dev.. 15: 807-826

Walford, RL, Haris, SB, Gunion, MW. The calorically restricted low-fat nutrient-dense diet in Biosphere 2 significantly lowers blood glucose, total leukocyte count, cholesterol, and blood pressure in humans. Proc. Natl. Acad. Sci. USA 1992, 89, 11533-11537

Weindruch, R. The Retardation of Aging by Caloric Restriction: Studies in Rodents and Primates. *Toxicol Pathol* 1996 24: 742

Wu, Z., Li, C., Lv, S., Zhou, B. 2009. Pantothenate kinase-associated neurodegeneration: insights from a Drosophila model. Hum. Mol. Genet. 18(19): 3659-3672

Yan, N., Huh, JR., Schirf, V., Demeler, B., Hay, BA., Shi, Y. 2006. Structure and activation mechanism of the Drosophila initiator caspase Dronc. J. Biol. Chem. 281(13): 8667-8674

Yang, CS., Thomenius, MJ., Gan, EC., Tang, W., Freel, CD., Merritt, TJ., Nutt, LK. Kornbluth, S. 2010. Metabolic regulation of Drosophila apoptosis through inhibitory phosphorylation of Dronc. EMBO J. 29(18): 3196-3207

Zid B, Rogers A, Katewa S, Vargas M, Kolipinski M, Au Lu T, Benzer S, Kapahi P. 4E-BP Extends Lifespan Upon Dietary Restriction By Enhancing Mitochondrial Activity in Drosophila. Cell Oct 2009 139(1) pg. 149-160