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Effects of Physiologic, Metabolic and Molecular Adaptations to Calorie Restriction on Biomarkers of Longevity

by

Matthew Douglas Bruss

A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Molecular and Biochemical Nutrition

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Marc K. Hellerstein, Chair

Professor Andreas Stahl

Professor George A. Brooks

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ABSTRACT

Effects of Physiologic, Metabolic and Molecular Adaptations to Calorie Restriction on Biomarkers of Longevity

by Matthew Douglas Bruss

Doctor of Philosophy in Molecular and Biochemical Nutrition

University of California, Berkeley Professor

Marc K. Hellerstein, Chair

Calorie restriction (CR), reducing caloric intake without malnutrition, increases lifespan and delays the onset of age-related diseases. Characterizing the underlying mechanisms that mediate the effects of calorie restriction on aging and lifespan will provide insight into the fundamental biology of aging, as well as guide research into the development of therapeutics for age-related diseases. It seems likely that some combination of physiologic, metabolic and molecular adaptations to CR lead to cellular responses that in-turn increase the longevity of the organism. Thus the goal of this thesis work was to combine a kinetic biomarker strategy with classic physiologic and molecular techniques to determine the role of physiologic adaptations, fat metabolism and molecular signaling on biomarkers of CR-induced longevity in mice. The data presented here demonstrate that CR leads to significant reductions in cell proliferation rates in keratinocytes, liver cells, mammary epithelial cells and splenic T-cells. These reductions in cell proliferation rates cannot be accounted for by reductions in food intake, energy expenditure, fat mass or body weight. In addition, the CR-induced reduction in cell proliferation is not dependent on Sirt1 expression, nor can it be mimicked by resveratrol treatment. However, reductions in cell proliferation rates were associated with a CR-induced increase in whole body fatty acid oxidation and have a strong negative correlation with circulating IGF-1 levels. Taken together these results suggest that increased reliance on fatty acid oxidation and reductions in IGF-1 signaling may be metabolic pathways that mediate the effects of CR on aging and longevity. These results also point to molecular mediators that can translate changes in substrate utilization to regulation of growth factor signaling as potential regulatory nodes necessary for the CR-induced effects on cell proliferation and longevity.

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I would like to acknowledge the help of mentors, colleagues, family and friends, for without their support this work would not be possible. My mentor throughout my PhD work, Marc Hellerstein, has been instrumental in guiding this research and making me a better scientist. His approach to conducting research will influence the rest of my career. My committee members, George Brooks and Andreas Stahl have challenged, guided and helped me excel. In addition, Sharon Fleming, Wally Wang and Hei Suk Sul have provided outstanding guidance.

Nearly all of the ideas and studies presented here were a direct result of discussions and experiments conducted with Cyrus Khambatta and Airlia Thompson. These two colleagues are excellent researchers and I am truly grateful for their contributions. In addition, Max Ruby and DJ Rhook have been critical in helping me develop as a scientist.

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I would also like to acknowledge Dr. Leonard Guarente for the generous contribution of the Sirt1 liver knockout mice.

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Overview:

Calorie restriction (CR), reducing caloric intake without malnutrition, increases lifespan and delays the onset of age-related diseases (Weindruch, 1988). Characterizing the underlying mechanisms that mediate the effects of calorie restriction on aging and lifespan will provide insight into the fundamental biology of aging, as well as guide research into the development of therapeutics for age-related diseases. It seems likely that some combination of physiologic, metabolic and molecular adaptations to CR lead to cellular responses that in-turn increase the longevity of the organism (Fig. 0.1).

Reductions in food intake (Harrison et al., 1984; Yu et al., 1985), energy expenditure (Sacher, 1977), fat mass (Berg and Simms, 1960; Bluher et al., 2003; Picard et al., 2004) and total body mass (Holehan and Merry, 1985; McCay et al., 1989; Miller et al., 2002) have all been suggested to mediate the effects of CR, however to date it is still not clear which if any of these physiologic adaptations is necessary for the CR effects. More recently several groups have suggested that alterations in fat metabolism are responsible for some of the longevity effects of CR (Guarente, 2008; Mobbs, 2007a), however fatty acid synthesis and oxidation have yet to be evaluated in response to CR. In addition, several molecular signals have also been associated with longevity, most notably Sirt1 (Donmez and Guarente) and IGF-1 (Berryman et al., 2008), however the mechanisms by which these molecules mediate longevity in response to CR are not known.

Fig. 0.1. Translation of CR to Longevity.

Progress in identifying the underlying mechanisms mediating the effects of CR has been slow, largely because longevity and aging studies, even in rodents, are time and resource-intensive, taking over 4 years and hundreds of mice (Nadon et al., 2008). Thus, to expedite investigation into CR and aging it would be useful to have biomarkers that could rapidly predict the outcome on health and longevity in response to physiologic, metabolic and molecular interventions. Recently, our lab has developed techniques for measuring fatty acid synthesis (Strawford et al., 2004) and cell proliferation rates *in vivo* (Busch et al., 2007). We believe that these kinetic measures serve as biomarkers that are mechanistically linked to health and longevity in response to CR.

Thus the goal of this thesis work was to combine a kinetic biomarker strategy with classic physiologic and molecular techniques to determine the role of physiologic adaptations, fat metabolism and molecular signaling on biomarkers of CR-induced longevity in mice. Each project of the thesis was designed to test adaptations to CR that have been suggested to mediate the effects on health and longevity, but have not been systematically evaluated in mammalian models.
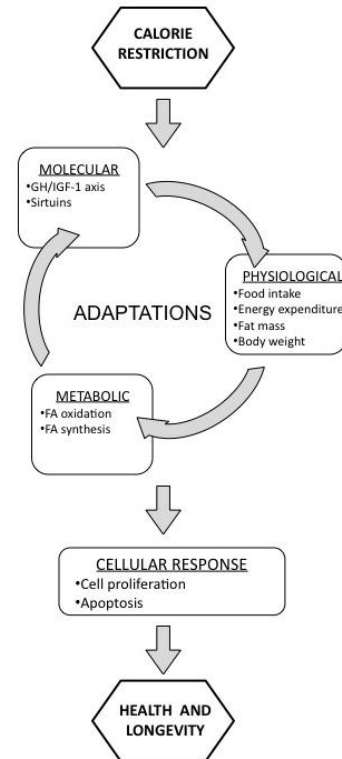
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Chapter 1

Background & Significance

Background & Significance:

CR & Maximal Lifespan

Increasing lifespan has been a human pursuit throughout our entire history. While no fountain of youth has been found, changes in hygiene and medical practices over the last century have increased average life expectancy (the age at which 50% of the population is still alive) by more than 30 years (Olshansky et al., 2002). However, during this same period the maximal lifespan (the age of the oldest individuals at death) has not changed, holding at approximately 120 years of life (Hayflick, 2007; Olshansky et al., 2002). This has led many to believe that the rate of aging in mammals is fixed and cannot be extended. In contrast to this view, a growing body of evidence in the field of dietary restriction suggests that maximal lifespan of mammals, and thus the rate of aging, can be extended, at least in laboratory models (Weindruch, 1988).

The first studies describing increased longevity by food restriction were performed by two Yale laboratories in 1915 and 1917 (Osborne et al., 1917). They showed that food restriction in rats led to both an increase in life expectancy and an increase in maximal lifespan, however these studies had some flaws due to premature deaths of animals from disease and did not garner much attention. In 1935 Clive McCay (McCay et al., 1989), working with rats in housing conditions that prevented premature death from disease, published what is considered the seminal work in food restriction and longevity. Confirming the results of Osborne and Mendel at Yale, Dr. McCay described increased life expectancy and increased maximal lifespan when growth was stunted by restricting food. Careful follow-up work by Dr. Masoro's group (Masoro et al., 1991) has shown that the effects of food restriction are due to restriction of total caloric intake and not by restriction of any particular micro or macronutrient. Thus, the feeding regimen is now known as calorie restriction (CR). Since the study by McCay the effects of CR on increased life expectancy and maximal lifespan have been repeated many times in laboratory rats and mice (Duffy et al., 2001; Goodrick et al., 1982; Merry and Holehan, 1985; Turturro et al., 1999; Weindruch et al., 1986; Yu et al., 1985) using several different feeding protocols. In addition, CR has been shown to increase longevity in yeast, worms, flies, dogs and, most recently, non-human primates, suggesting that the feeding regimen alters some basic aging mechanism that is common across many taxa.

The most common CR protocol in rodents, 30-40% CR provided once daily, leads to increased life expectancy of 15-50% and increased maximal lifespan of 10-30% as compared to ad libitum (AL) controls, with the variation coming from differences in species, strain and magnitude of restriction. While an increase in life expectancy could be achieved through a decrease in premature death, as in the human population through improved hygiene and vaccines, an increase in maximal lifespan is thought to be associated with a fundamental change in the rate of aging.

In addition to an increase in maximal lifespan, gompertzian analysis applied to CR survival curves has revealed a trend that is consistent with a decrease in the rate of aging (Merry, 2005). In the mid 1800s, Gompertz first identified that the mortality rate, the number of individuals dying during a given period of time, increases exponentially as

humans age (Gompertz, 1825). Since then, the same mortality trend has been validated in many animal species (Comfort, 1964) and widely used as a metric of population aging rate. The trend can be described mathematically by: $q_x = q_0e^{ax}$ where qx is the mortality rate at age x, qo is the initial death rate, and a is the mortality rate doubling time for a given population. While lifespan in CR rodents could be extended by either decreasing initial death rate or by increasing the mortality rate doubling time, it is only an increase in mortality rate doubling time that is associated with a fundamental change in the rate of aging. Holehan & Merry (Holehan and Merry, 1986) re-evaluated mortality characteristics from two independent CR longevity studies in Fisher 344 rats, each using a unique method of achieving CR. In the first study, calorie restricted rats were only allowed to eat enough to maintain 50% of the body weight of AL controls, while in the second study calorie restricted rats were given 60% of the calories consumed by AL controls. Both studies showed a remarkable similarity in the slowing of mortality rate doubling time in response to CR, 102 vs 203 days in the first study and 104 vs 189 days in the second study. Consistent with these findings, Finch (Finch, 1990) re-evaluated survival characteristics in six independent rat CR longevity studies and found that mortality rate doubling time was about 2-fold slower in CR rats as compared to AL fed controls. Taken together, these data suggest that CR not only prolongs lifespan, but fundamentally changes the rate of aging.

CR & Tumor Formation

Consistent with an attenuation in the rate of aging, CR also delays the onset and slows the progression of many age-related diseases, most notably cancer. In the early 1900s researchers began investigating the role of dietary restrictions on the growth of transplanted tumors in mice. The first observation, by Moreschi, reported that sarcoma grafts grew more slowly in malnourished mice (Moreschi, 1909). These observations were extended by Francis Peyton Rous, when he showed that tumor grafts grew more slowly in mice that lost or maintained weight on an energetically restricted diet (Rous, 1914). It was later shown that the reduction in transplanted tumor growth was due to a reduction in caloric intake, not the reduction in some other nutrient (Bischoff, 1935; Long, 1938). In long-term studies Flory et al. (C.M. Flory, 1943) showed that, not only did tumors grow more slowly, but CR mice outlived AL-fed mice after being inoculated with leukemia.

Tennanbaum (Tannenbaum, 1940) first began investigating the effect of various dietary restriction regimens on benzpyrene-induced skin tumors in mice and found that CR reduced tumor incidence. Since that time these findings have been expanded to various carcinogens and various tissues, all with the same result; CR reduces carcinogen-induced tumor incidence. Skin tumor growth in mice by induction with DMBA and topical application of 12-o-tetradecanoylphorbol-13-acetate is also inhibited by 40% CR (Pashko and Schwartz, 1992). In addition, CR inhibits mammary tumor growth in 7,12-dimethylbenzanthracene (DMBA)-induced rats (Heuson and Legros, 1972; Klurfeld et al., 1989; Kritchevsky et al., 1984; Sylvester et al., 1982), where body weight correlates inversely to tumor growth (Pariza, 1986). Expanding on the DMBA studies in rats, Ruth Lipman (Lipman, 2002) demonstrated that CR extends the lifespan of 8 different mouse

strains (A/J, BALB/c, C3H, C57BL/6, DBA/2J, NMRI, and 129/J) upon an oral dose of DMBA. The most common cause of death varied in each of these strains (hemangiosarcoma, lung adenoma, mammary gland hyperplasia and lymphoma), showing that CR delays death due to a number of different lesions. The effects of CR have also been shown in 1,2-dimethylhydrazine and azomethane-induced colon tumors (Klurfeld et al., 1987; Kritchevsky et al., 1986; Kumar et al., 1990; Reddy et al., 1987), asbestos-induced lung tumors (Koizumi et al., 1993), azaserine-induced pancreatic neoplasms (Roebuck et al., 1981) and diethylnitrosamine-induced liver tumors (Lagopoulos and Stalder, 1987).

Consistent with transplanted and carcinogen-induced tumors, a reduction in caloric intake leads to a reduction in spontaneous tumor growth (Ross and Bras, 1965; Ross et al., 1970). Since the initial experiments by Ross and Bras, there have been many reports confirming the effect of CR in reducing spontaneous tumors in rat and mouse models, showing repression of a number of different cancers, including spontaneous leukemia (J.A. Saxton, 1944), lymphoma (Blackwell et al., 1995; Cheney et al., 1980; Sheldon et al., 1995), pituitary tumors (Blackwell et al., 1995; Higami et al., 1995; Keenan et al., 1995; Tucker, 1979) and liver neoplasias (Blackwell et al., 1995; Tucker, 1979). More recently CR has also been shown to inhibit spontaneous tumor growth in several genetic models of cancer, including p53 knockout (Hursting et al., 1994), APC[min] (Mai et al., 2003) and wnt transgenic (Hursting et al., 2001). Taken together these data demonstrate that CR robustly inhibits transplanted, carcinogen-induced and spontaneous tumor growth in many tissues across a number of strains of rats and mice.

As alluded to earlier, the universal inhibition of tumor growth in rats and mice appears to be due to a reduction in caloric intake per se, rather than a reduction in any particular macro- or micronutrient. Reductions in protein (Ross et al., 1970), fat (Carroll and Khor, 1975) and minerals (Masoro, 2002) had all been suggested to be responsible for reduced tumor growth. However, in a comprehensive analysis in male F344 rats only a reduction in calories inhibited a wide range of tumors, with restriction of other components only having small effects (Masoro, 2002). Consistent with these findings, Albanes (Albanes, 1987) analyzed 82 independent CR and tumor studies, mostly of induced skin tumors and spontaneous mammary tumors, and showed a linear trend of increased CR and reduced tumor growth. In addition, he found that there was no association between fat intake and tumor incidence.

To better understand the mechanisms by which CR inhibits tumorigenesis, researchers began investigating the timeframe in which CR can affect tumor growth. While many of the early experiments began with rodents placed on CR soon after weaning, work soon moved to addressing the question of the effect of CR in limiting tumor growth in adult mammals. In both rats (Maeda et al., 1985) and mice (Tannenbaum, 1940; Weindruch and Walford, 1982) CR also delays the onset and inhibits the growth of tumors when started in young adults (3-6 months) and middle-aged (9-12 months) animals. Consistent with these data Dhahbi et al. (Dhahbi et al., 2004) demonstrated that CR started at 19 months of age, in C3H mice, delayed the onset of liver and lung carcinomas. Interestingly, further analysis of the mortality curves in this experiment revealed that the effects of CR on survival are

induced within 2 months, suggesting that CR rapidly initiates an active program of health rather than operating through passive prevention of aging.

According to the multi-stage model of carcinogenesis the first two steps in the appearance of tumors are initiation (i.e. irreversible genetic damage that confers a growth advantage) and promotion (i.e. the selective clonal expansion of initiated cells.) In theory CR could delay or prevent death from cancer by inhibiting either of these steps. While CR may have effects on tumor initiation in some models, the major action of CR appears to be inhibition of tumor promotion (Klurfeld et al., 1987; Klurfeld et al., 1989). Several groups have found that at death CR mice have as many tumors as AL fed mice, however the tumors grew slower in the CR mice, thus they lived longer (Keenan et al., 1995; Weindruch, 1992). In the 1940s Tannenbaum (Tannenbaum, 1944), addressed the initiation versus promotion question directly by evaluating skin tumor formation in DBA mice injected with benzyprene. Mice were divided into 4 dietary groups and all were injected repeatedly between 3-5.5 months of age. Mice that were fed ad libitum from 2-5.5 months of age and switched to CR from 5.5-20 months of age had 40% fewer tumors than mice who were calorie restricted from 2-5.5 months then switched to AL feeding from 5.5-20 months, suggesting that the promotional phase of tumor growth is more robustly inhibited by CR than the initiation phase.

Mechanisms of CR Action: Physiologic Adaptations

While the longevity benefits of CR have been well described, the mechanisms that lead to these effects are not fully understood. Decreased body weight, fat mass, energy expenditure and food intake are all physiologic adaptations to CR that have been suggested to mediate the longevity effects, however there is still no clear consensus on which, if any, adaptation is responsible for increased lifespan.

Growth retardation/Decreased body weight
The first mechanisms thought to mediate the longevity effects of CR, initially postulated by McCay (McCay et al., 1989) in his first longevity paper, was that decreased growth rate and body weight leads to increased lifespan. In fact, McCay's study (McCay et al., 1989) was designed to determine the role of growth on longevity and CR was used as a tool to achieve growth retardation. In the study McCay fed one group ad libitum, which grew at the normal rate. The second group was initially food restricted to inhibit growth completely, when rats began to look unhealthy they were given more food to allow them to grow slightly until they appeared healthy again. This regimen was repeated throughout the rat's life and this punctuated growth was associated with increased longevity. Thus, McCay confirmed his original hypothesis that decreased growth and decreased body weight lead to increased lifespan. This view was widely accepted for the next 30 years, with many longevity studies reducing food intake in order to limit body weight (Merry and Holehan, 1985). Consistent with this theory, studies in mice have shown that body weight at 8-16 weeks inversely correlates with lifespan (Miller et al., 2002), suggesting that controlling body weight during adolescence could be a mechanism by which CR extends longevity.

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More recently several studies have challenged the notion that decreased growth and body weight is sufficient to increase lifespan. Yu and Masoro (Yu et al., 1985) found that rats on CR after 26 weeks of age, when rats had reached full growth potential, still led to an increase in lifespan. Furthermore, when rats were placed on CR only during 6 to 26 weeks of age, during their rapid growth phase, there was not a significant increase in median or max lifespan. Studies investigating the effect of voluntary exercise, showed that while exercise dramatically reduced body weight in male rats, it had no effect on maximal lifespan (Holloszy, 1992). Thus, it is not clear if decreased growth rate and body weight are necessary and sufficient for increased longevity. It would be interesting to determine how the animal perceives a decrease in body weight once energy balance has been restored, and how this could be translated into increased longevity.

Body fat

In 1960 Berg and Simms (Berg and Simms, 1960) first suggested that decreased body fat could mediate the health benefits of CR. This hypothesis was based largely on the assumption that increased body fat is associated with premature death and that mice on CR have decreased body fat, which was later confirmed by Bertrand and Masoro (Bertrand et al., 1980). Since the first suggestion by Berg and Simms, several studies have tested the role of fat mass on longevity, however none have shown a direct role for fat mass. Bertrand et al. (Bertrand et al., 1980) showed no correlation between fat mass and longevity in AL fed F344 rats, and a positive correlation in those on CR. Testing the hypothesis more directly, Harrison et al. (Harrison et al., 1984) studied lean and OB/OB mice fed AL or CR. They reported that OB/OB mice on CR lived longer than lean AL despite having a much higher percentage body fat (48 vs 22%) and as long as lean CR despite a large difference in body fat (48 vs 13), thus showing a dissociation between fat mass and longevity. Exercise and longevity studies have also shown a dissociation between fat mass and longevity, where exercised rats have decreased fat mass, yet no increase in longevity (Holloszy, 1992).

While the majority of evidence suggests that body fat is not a critical factor in maximal longevity in response to CR, the theory has been revitalized by two intriguing molecular studies. First, Bluher et al. (Bluher et al., 2003) reported that mice with the insulin receptor knocked out specifically in adipose (FIRKO) have reduced adiposity and increased longevity. Although they did not place mice on CR, they concluded that decreased fat mass, not decreased caloric intake per se, mediates the longevity effects of CR. Second, Picard et al. (Picard et al., 2004) showed that Sirt1 deacetylates and increases fat mobilization in WAT. Since it has been hypothesized that Sirt1 activity is increased in response to CR, they concluded that the decrease in WAT could be a mechanism by which CR leads to longevity. It seems that further work is needed to determine if reduced fat mass as compared to lean controls is associated with increased longevity and, if so, what are the mechanisms.

Energy Expenditure

Another theory that has drawn some attention is that CR increases longevity by decreasing energy expenditure. This theory, originally proposed by George Sacher in 1977 (*Sacher*, 1977), was based on the "rate of living theory of aging". The basic principle of the rate of living theory of aging is that the lifespan of an organism is determined by its

metabolic rate. The higher the metabolic rate the shorter the lifespan. While Sacher's application of the theory to CR and aging is intriguing, it suffers from two major flaws. First, there is no strong evidence to suggest that energy expenditure is related to lifespan (support for the rate of living theory of aging came from the incorrect notion that an inverse relationship exists between metabolic rate and lifespan across all species). Second, Sacher developed his theory based on evidence that energy expenditure is decreased during CR (*Sacher*, 1977). However, while whole body energy expenditure does decrease in CR rodents (McCarter and Palmer, 1992), when corrected for body mass, metabolic rate is actually increased in CR rodents (Masoro et al., 1982; McCarter et al., 1985; McCarter and Palmer, 1992). Taken together it seems unlikely that CR leads to increased lifespan by reducing metabolic rate. However, the theory still exists and it would be interesting to examine the role of energy expenditure and aging using other models.

Food intake

Since it is not clear if any of the other major phenotypic adaptations to CR fully account for the longevity effects, several investigators have concluded that a decrease in food intake per se could be responsible for increased health and lifespan (Harrison et al., 1984; Masoro, 2002; Yu et al., 1985). Perhaps by initiating some neuroendocrine signaling mechanism (Yu, 1994). In contrast to this view Huffman et al. (Huffman et al., 2007) demonstrated that prostate tumor development in susceptible mice was reduced by CR in mice housed at 23c, but not reduced in mice kept at a higher ambient temperature (28c), despite the fact that they consumed the same amount of food as the CR group at 23c. While a decrease in food intake per se is an intriguing theory, there have been very few studies addressing the possible role in longevity. It will be interesting to further explore this theory and assess possible mechanisms by which decreased food intake could increase longevity.

Summary

While decreases in body weight, fat mass, metabolic rate and food intake have all been suggested to mediate the longevity effects of CR, none of these phenotypic adaptations seem to fully account for the CR-induced increase in lifespan. Thus some other adaptation to CR could be involved in initiating the signaling cascade that translates reduced caloric intake into a program of health and longevity.

Mechanisms of CR Action: Reactive Oxygen Species

Another mechanism suggested to mediate the longevity effects of CR is decreased damage from reactive oxygen species (ROS). Generation of the partially reduced oxygen molecules (O_2-, H_2O_2 and OH) from respiration can cause damage to DNA (base alterations and single strand breaks), lipids (peroxidation of polyunsaturated fatty acids) and proteins (carbonylation and loss of sulfydryls). This theory assumes that damage from ROS plays a role in aging and that CR attenuates this damage and extends lifespan. While it is not clear if this theory is correct, evidence exists to support both of the necessary assumptions.

The basic tenet of the oxidative damage theory of aging is that the accrual of ROS damage over time is associated with aging. Consistent with this principle, a number of studies have shown an age-associated increase in lipid peroxidation (Matsuo et al., 1993), oxidatively damaged proteins (Stadtman, 1992) and DNA base alterations (Sohal et al., 1994a) in laboratory rodents. More damage accrual in older animals, however, could be a consequence, not a cause of aging. To investigate the role of oxidative damage on aging Orr et al. (Orr and Sohal, 1994) overexpressed Cu,Zn-superoxide dismutase and catalase in Drosophila. Increased expression of the antioxidant enzymes resulted in attenuation of the age-associated increase in oxidative damage to DNA and proteins that was associated with a 33% increase in mean and maximal lifespan. Suggesting that oxidative damage could play a role in aging, and reducing damage could prolong lifespan.

Also, consistent with reduced ROS damage leading to increased longevity, CR dramatically attenuates the age-associated increase in ROS damage in DNA (Sohal et al., 1994a; Youngman, 1993), lipids (Baek et al., 1999; Cook and Yu, 1998) and protein (Aksenova et al., 1998; Dubey et al., 1996; Youngman, 1993). Sohal et al. (Sohal et al., 1994a) reported an age-associated increase in 8-hydroxydeoxyguanosine, a marker of DNA oxidative damage, in muscle, brain, heart, liver and kidney of 8 and 27 month old C57BL/6 mice. CR reduced the concentration of 8-hdG in both groups of mice and attenuated the age-associated increase from 8 to 27 months. Like DNA, oxidative damage to lipids increases with age. Baek et al. (Baek et al., 1999) reported an increase in lipid peroxidation in the cerebellum of rats as they aged from 6 to 24 months. CR severely blunted this age-associated increase. CR also reduces oxidative damage to proteins. Youngman et al. (Youngman et al., 1992) demonstrated decreased carbonylation of liver proteins in as little as 12 weeks of CR in F344 rats.

CR could lead to decreased oxidative damage by enhancing antioxidant defense, increasing turnover of damaged molecules or by simply reducing the production of ROS. The most heavily investigated mechanism has been the modulation of antioxidant defense in response to CR. Several early studies suggested that CR enhances antioxidant defenses (Heydari and Richardson, 1992; Koizumi et al., 1987; Laganiere and Yu, 1989), however more recent studies have been unable to show a consistent increase in antioxidant enzyme activity (Mura et al., 1996; Rojas et al., 1993; Sohal et al., 1994b). It is possible that age, sex, tissue specificity and circadian rhythm could be responsible for the seemingly inconsistent results. Increased turnover of damaged molecules may also play a role in minimizing oxidative damage accumulation under CR, however investigation into these mechanisms is just beginning. Perhaps the most consistent evidence shows that CR reduces the production of ROS in the mitochondria (Gredilla et al., 2001; Sohal and Dubey, 1994). Barja's group has taken this further to show that the reduction in ROS production appears to be occurring at complex I of the ETC (Gredilla et al., 2001). The question still remains as to how CR could lead to decreased ROS production in the mitochondria given that energy expenditure, and presumably electron flux through the ETC, is the same or higher for CR animals.

Mechanism of CR Action: Fat Metabolism

It has been suggested that CR leads to an increased reliance on fatty acid (FA) oxidation and that this in turn leads to decreased ROS production (Guarente, 2008; Mobbs, 2007b). The theory is based on the difference in reducing equivalents generated from glycolysis versus β-oxidation. Glucose oxidized through glycolysis and the krebs cycle generates NADH and FADH in a ratio of 5:1. In contrast, fatty acids oxidized through beta-oxidation and the krebs cycle generate NADH and FADH in a ratio of 2:1. Electrons from NADH are donated to complex I, whereas electrons from FADH are donated to complex II. Since complex I appears to be the major producer of ROS in the electron transport chain, bypassing this complex via increased reliance on FA oxidation could be responsible for decreased ROS production (Mobbs, 2007b).

While this theory is intriguing it had yet to be shown that CR mammals rely more heavily on FA oxidation for energy than their *ad libitum* fed counterparts (Project 2 of this thesis, addresses this question). Intuitively it is difficult to imagine how CR mice who are in energy balance and consume fewer total calories from fat than AL fed animals (same composition of macronutrients consumed, yet fewer total calories) could oxidize more fat. It appears to defy a basic tenet of metabolism that, in energy balance, FQ (food quotient; the composition of the diet) must equal RQ (respiratory quotient; the composition of oxidized substrates). If this is not balanced then there will be an accumulation of some substrate (fat, carbohydrate or protein) and the animal is no longer in energy balance. In fact, the one study that measured average daily RQ showed no difference between CR and AL fed rats (Masoro, 2002). However average daily RQ may not accurately reflect circadian patterns that influence absolute FA oxidation. Taken together, the interesting hypothesis postulated by Dr. Mobbs (Mobbs, 2007b) seemed unlikely based on basic metabolism, however as we demonstrate in Project 2, CR mice have a unique metabolic pattern that makes increased fat oxidation and energy balance mutually inclusive.

Mechanisms of CR action: Molecular & Hormonal Adaptations

GH/IGF-1 Axis

In addition to the physiologic and metabolic theories, several molecular and hormonal adaptations to CR have been suggested to mediate the longevity effects of CR. Modulation of the GH/IGF-1 axis was one of the first hormonal adaptations to CR that was implicated in longevity. Numerous studies have reported that circulating IGF-1 is reduced in CR rats and mice (Dunn et al., 1997; Fontana and Klein, 2007; Sonntag et al., 1999). With the obvious link between reduced IGF-1 and decreased growth it was immediately hypothesized that this could be the hormonal link to longevity. Consistent with this hypothesis two spontaneous dwarf mice, both with disruptions in the GH axis, have increased mean and maximal lifespans as compared to wild type littermates. The Ames and Snell Dwarf mice have loss-of-function mutations in the Prop1 and Pit1 genes, respectively. These disruptions inhibit the proper development of Pit1-positive cells that give rise to somatotrophs, lactotrophs, and thyrotrophs. Thus these mice do not secrete

GH, PRL, or TSH. In addition, they have dramatically reduced levels of circulating IGF-1. The Ames dwarf mice have been reported to live 35-69% longer than their wild-type littermates, while Snell dwarf mice have maximal lifespans 24-40% longer than wild-type mice (Bartke and Brown-Borg, 2004).

More recently several KO models have provided further support for a role of the GH/IGF-1 axis in aging. The GHR/BP KO mice, generated by Coschigano et al. (Coschigano et al., 2000), are characterized by increased GH, but drastically reduced levels of circulating IGF-1 (Coschigano et al., 2003). These mice have increased maximal lifespans of 26 & 16% for males and females, respectively (Coschigano et al., 2003). Another model of disrupted IGF-1 signaling, the IGF1 receptor heterozygous mice, have a 50% reduction in IGF1-R abundance and females have a 33% increase in maximal lifespan (Holzenberger et al., 2003). The final model of increased longevity, the β-klotho overexpressing mice, have a 20-31% increase in maximal lifespan as compared to wild-type controls (Masuda et al., 2005). While the levels of circulating IGF-1 are normal in β-klotho mice, the mice are resistant to IGF-1 action, suggesting a role for signals downstream of IGF-1 (Masuda et al., 2005).

In addition to the mammalian studies, IGF-1 seems to be important in regulating aging in flies and worms. Disruption of the insulin/IGF-1 receptors, DAF-2, in C. elegans and InR, in Drosophila leads to increased maximal lifespan (Berryman et al., 2008). Taken together it appears that IGF-1 could be a universal signal in the regulation of aging, and reducing circulating levels or cellular actions, as in CR, could be a mechanism for increasing maximal lifespan. However it is still not clear how a reduction in IGF-1 could lead to increased longevity or how CR could induce a decrease in circulating IGF-1 levels.

FGF21

Given that IGF-1 signaling appears to play a role in mammalian aging, identifying the upstream signals that modulate the GH/IGF-1 axis in response to CR, could provide insight into the mechanisms by which CR extends longevity. To date, no studies have directly addressed this question, however there are some data that could provide clues. FGF21 a recently characterized hormone, secreted by the liver, has been shown to disrupt GH signaling in the liver (Inagaki et al., 2008). Kliewer et al. reported that transgenic mice, overexpressing FGF21, are smaller, have GH resistance and decreased circulating IGF-1 (Inagaki et al., 2008). Interestingly, earlier work demonstrated that FGF21 expression and circulating protein are rapidly increased in response to fasting in a PPARα dependent manner (Badman et al., 2007; Inagaki et al., 2007). In addition, FGF21 is necessary for increased ketogenesis, increased fatty acid oxidation and decreased body temperature (torpor) which are all important adaptations to fasting in mammals (Reitman, 2007). Given the intermittent nature of feeding in calorie restricted mice (i.e. 1 hour of gorging, followed by 23 hours of fasting), it is possible that FGF21 expression is increased in a circadian fashion, and this hormonal signaling could lead to the decrease in circulating IGF-1 that is observed during CR.

Data from several recent papers support the connection between FGF21, IGF-1 signaling and longevity. As mentioned earlier, β-klotho overexpression leads to decreased

IGF-1 signaling and increased maximal lifespan. Interestingly, a recent report shows that β-klotho is necessary for FGF21 induced activation of its receptor (Kurosu et al., 2007). Perhaps overexpression of β-klotho leads to increased FGF21 signaling, decreased IGF-1 signaling and extended longevity, however these studies have not yet been performed. In addition to its connection to β-klotho, FGF21 has also been reported to be dependent on Sirt1, an enzyme implicated in longevity. Overexpression of Sirt1 in H2.35 hepatoma cells (Purushotham et al., 2009) and apparent activation of Sirt1 by SRT1720 in mouse livers (Feige et al., 2008) both lead to increased FGF21 expression.

While it appears that FGF21 can lead to decreased IGF-1 signaling, it must do so through an intracellular signaling network. As of yet, the intracellular signaling molecules that could regulate IGF-1 in response to CR have not been identified. However a recent report offers an intriguing possibility. Touvier et al. (Touvier et al., 2009) reported that transgenic mice overexpressing leptin receptor overlapping transcript (LEPROT), a small protein of approximately 130kD, have retarded growth and impaired GH sensitivity in the liver. Further analysis showed that GH-receptor was reduced in the livers of transgenic mice, while siRNA knockdown of LEPROT increased GH-receptor abundance at the cell membrane of H4IIE hepatocytes. Interestingly, Leprot expression is increased in response to fasting, suggesting a possible intracellular mechanism for fasting induced inhibition of the GH/IGF-1 axis (Touvier et al., 2009).

Sir2 & Sirt1: the intrigue

Over the last decade sirtuins have garnered considerable attention as molecular intermediates linking CR to increased longevity. Sir2, a member of the sirtuin family, was originally identified in yeast as a histone deacetylase that was responsible for limiting the replication of extrachromosomal circles (ERCs) (Gottlieb and Esposito, 1989; Kaeberlein et al., 1999). It was later demonstrated that accumulation of ERCs limited replicative lifespan (Sinclair and Guarente, 1997), the number of daughter cells produced by a mother prior to senescence, and that ablation of sir2 decreased lifespan, while overexpression increased lifespan (Kaeberlein et al., 1999). The mechanism leading to sir2-induced chromosomal silencing was shown to be through a unique NAD-consuming reaction that leads to the deacetylation of lysine residues and the production of 1-O-acetyl-ADP-ribose, and nicotinamide (Tanner et al., 2000). The role of NAD immediately suggested a link between lifespan and metabolism in yeast. The connection of sir2 to CR came when Lin et al. (Lin et al., 2000) reported that reducing the glucose concentration of yeast media, a form of CR, increased replicative lifespan and this increase was not evident in sir2 knockouts. This group and another went on to report that sir2 is activated in response to CR by initiating respiration which, depending on the report, increases NAD (Lin et al., 2002), decreases NADH (Lin et al., 2004) or decreases NAM (Anderson et al., 2003a).

Extending the findings from yeast, investigators have reported that the sir2 homologs in C. elegans (sir-2.1) and Drosophila (dSir2) appear to regulate longevity

and are necessary for the CR-induced increase in lifespan. Tissenbaum and Guarente (Tissenbaum and Guarente, 2001) reported that overexpression of sir-2.1 extends C. elegans lifespan by 50%. In addition, the lifespan extension of the C. elegans CR model, the eat-2 mutant, appears to be dependent on sir-2.1 (Wang and Tissenbaum, 2006). Wild-type C. elegans live 16 days and the eat-2 mutants, who have impaired food consumption, live 24 days. In contrast, the eat-2/sir-2.1 double mutants only live 18 days (Wang and Tissenbaum, 2006). As in yeast and C. elegans, increased transcription of dSir2 in Drosophila leads to increased mean and maximal lifespan (Rogina et al., 2002). Also consistent with yeast and c. elegans, dSir2 is necessary for CR-induced increase in longevity and CR does not increase longevity further in dsir2 overexpressing Drosophila (Rogina and Helfand, 2004).

With the intriguing discoveries in yeast, worms and flies, the question, obviously, turned toward the function of sirtuins in mammals. Seven sirtuins were identified in mammals (Sirt1-7), all with the same NAD-dependent deacetylase activity. Sirt1, found predominately in the nucleus, has the closest homology to yeast sir2 and has been the most actively studied. Sirt1 has been shown to deacetylate many non-histone proteins, including a host of transcription factors that regulate cell death and survival. The first to be reported was p53, which is negatively regulated by deacetylation (Langley et al., 2002; Luo et al., 2001; Vaziri et al., 2001), suggesting a role of Sirt1 in inhibiting apoptosis. Consistent with this role, Sirt1 has also been reported to downregulate FoxO-mediated apoptosis through deacetylation of FoxO 1, 3 & 4 (Brunet et al., 2004; Motta et al., 2004). However, Sirt1 appears to play a role in increased expression of GADD45, which requires positive regulation of FoxO targets (Guarente and Picard, 2005). In addition to GADD45, Sirt1 up-regulates DNA repair via deacteylation of Ku70 (Cohen et al., 2004). PGC-1a, a transcription factor coactivator involved in gluconeogenesis and mitochondrial biogenesis, also appears to be regulated by Sirt1 deacetylation (Lagouge et al., 2006; Rodgers et al., 2008), suggesting a role for Sirt1 in metabolic regulation.

Since Sirt1 seems to inhibit apoptosis through the downregulation of p53 and FoxO, it was hypothesized that Sirt1 could be oncogenic (Haigis and Sinclair). Several *in vitro* studies support Sirt1 as a stimulator of cell proliferation, while others demonstrate that Sirt1 activation inhibits cell proliferation. Two *in vivo* studies, are consistent with a tumor-suppressive action of Sirt1. Firestein et al. (Firestein et al., 2008) reported that overexpression of Sirt1 inhibits colon polyp formation in the APC[min] mice, while Wang et al. (Wang et al., 2008a) reported increased cancer burden in p53+/- mice when Sirt1 is partially ablated (Sirt1 +/+ vs Sirt1 +/-).

The connection of Sirt1 to CR in rodents was initially made from two studies demonstrating increased Sirt1 protein in several tissues in response to CR. Cohen et al. reported increased Sirt1 protein abundance in Brain, WAT, kidney and liver in rats subjected to 40% CR. Confirming the study in rats, Nisoli et al. (Nisoli et al., 2005), studying mice, reported increased Sirt1 protein abundance in WAT, Brain, Liver, heart and BAT in response to CR by alternate day fasting. The first CR phenotype reported to be dependent on Sirt1 was increased activity in response to CR (Chen et al., 2005). Chen et al. reported that CR mice have increased in-cage movement, as assessed by visual monitoring.

However, when Sirt1 knockout mice are calorie restricted there is no increase in in-cage movement (Chen et al., 2005). More recently, McBurney's group has used outbred Sirt1 whole body KOs to begin investigating lifespan in response to CR (Boily et al., 2008). They reported that Sirt1 KOs had no increase in lifespan on CR as compared to AL-fed controls (Boily et al., 2008). These experiments were performed with very few mice (n=5-11) and, thus, are difficult to interpret. Consistent with a role for Sirt1 in mediating mammalian longevity in response to CR, is a recent study by Guarente's group showing Sirt1 regulation of GH secretion. Cohen et al. (Cohen et al., 2009) report that brain specific knockout of Sirt1 results in smaller mice with decreased circulating GH and IGF-1 levels, suggesting a relationship between Sirt1, the somatotropic axis and longevity.

Further evidence seeming to support a role of Sirt1 in health and longevity came from the identification of small molecules that apparently activate Sirt1 and have effects on longevity and disease prevention in yeast, C. Elegans and mice. Dr. Sinclair's group reported that resveratrol, a naturally occurring, polyphenolic compound found in the skins of grapes, was a potent Sirt1 activator *in vitro* (Howitz et al., 2003). His group went on to report that RSV extends the lifespan of yeast, C. elegans and Drosophila in a sir2 dependent manner (Wood et al., 2004). Extending their findings to mice they showed that resveratrol protects mice from high-fat diet-induced fatty liver, improves glucose homeostasis and prevents premature death (Baur et al., 2006). They then synthesized compounds that were reported to be even more potent activators of Sirt1 than resveratrol (Milne et al., 2007). When they provided these synthetic Sirt1 activating compounds to Zucker fa/fa rats fed a high-fat diet, they reported improved whole-body glucose homeostasis and insulin sensitivity in adipose tissue, skeletal muscle and liver, suggesting an exciting therapeutic potential for treating metabolic syndrome (Milne et al., 2007).

Sir2 & Sirt1: the uncertainty

As reports were continuing to be published supporting the health benefits of Sirt1 in various mammalian models, follow-up work on the original studies in yeast and C. elegans was beginning to reveal inconsistencies and irreproducible results from some of the original studies. Dr. Kaeberlein's group put together an elegant re-evaluation of the original work in yeast, bringing up several arguments against sir2 mediating the effects of CR in yeast (Kaeberlein and Powers, 2007). In the original experiments by Guarente's group, yeast lifespan was limited by accumulation of extrachromosomal DNA circles and sir2 was necessary for the CR-mediated attenuation in their accumulation. In this strain of yeast Fob1p is necessary for the generation of the extrachromosomal DNA circles (Lin et al., 2002). When Fob1p and sir2 are both deleted in yeast, CR leads to a robust increase in replicative lifespan, suggesting that sir2 is only necessary for lifespan extension in a mechanism that is yeast specific (Kaeberlein et al., 2004). It also appears that the effect of sir2 on longevity and its role in CR may not be as robust as originally reported. Dr. Kaeberlein points out that studies showing increased lifespan in response to overexpression of sir2 were carried out in yeast strain W303AR5 (Defossez et al., 1999; Kaeberlein et al., 1999). In contrast, studies on the longevity effects of CR were carried out almost exclusively in the PSY316 strain (Anderson et al., 2003b; Bitterman et al., 2002; Lin et al., 2004; Lin et al., 2002). One possible explanation for this dichotomy offered by Dr.

Kaeberlein is that while CR increases lifespan in PSY316, overexpression of Sir2 does not (Kaeberlein et al., 2004). In addition, using several assays to determine sir2 activity, Kaeberlein's group showed that CR does not increase sir2 activity in yeast (Kaeberlein and Powers, 2007).

Inconsistencies, have also been revealed in the role of sir2 in CR and longevity in C. elegans. While the first report showed that sir2.1 was necessary for lifespan extension in a calorie restricted mutant (Wang and Tissenbaum, 2006). A recent report found that sir2.1 was not necessary for lifespan extension in the same mutant (Hansen et al., 2007). In addition, several other groups have reported that sir2.1 was not necessary for lifespan extension in response to another form of dietary restriction in C. elegans (Kaeberlein et al., 2006; Lee et al., 2006; Mair et al., 2009).

As in yeast and C. elegans, the relationship between CR and sirtuins in mammals is more complicated than originally reported. While the original reports described increased Sirt1 protein abundance in many tissues in response to CR (Cohen et al., 2004; Nisoli et al., 2005), subsequent papers have reported no change (Boily et al., 2008; Cohen et al., 2009; Mulligan et al., 2008), or even decreases (Chen et al., 2008) in Sirt1 in the same tissues in response to CR. Another potentially interesting result in mammals that has been difficult to reconcile is the role of Sirt1 on the somatotropic axis. As described above, Guarente's group has reported that Sirt1 brain-specific KO leads to decreased GH and circulating IGF-1 (Cohen et al., 2009), however in a previous paper the same group shows no change in circulating IGF-1 in a Sirt1 whole body knockout (Chen et al., 2005).

In addition to the questionable role of sirtuins in mediating the effects of CR, has been the questionable activity of putative Sirt1 activators. As mentioned earlier Dr. Sinclair's group reported extensively on the role of resveratrol and synthetic sirtuin activators (STACs) as direct Sirt1 activators with several health and longevity effects (Baur et al., 2006; Milne et al., 2007). Careful work by several independent labs has shown that neither resveratrol, nor the synthetic activators developed by Sinclair's group, are direct Sirt1 activators (Beher et al., 2009; Borra et al., 2005; Pacholec et al.). In a series of elegant studies these groups show that the reported effect of these compounds was due to an artifact inherent to the fluorescent based screening assay. When the fluorophore is removed from the acetylated peptide, resveratrol and STACs lose their apparent Sirt1 activating effect. In addition, one group attempted to repeat the phenotypic effects of SRT1720 on fa/fa rats that was previously reported, and they were unable to repeat any of the dietary, glucose homeostasis or insulin sensitizing effects (Pacholec et al.). Taken together, it appears that sirtuins have a conserved enzymatic function across many species and may play a role in regulating health and longevity, however many of the original studies have been irreproducible, suggesting the need for more work to evaluate the role of sir2 and Sirt1 in mediating the phenotypic effects of CR.

Biomarkers of longevity

As described above, many questions remain as to the mechanisms by which CR could lead to increased longevity. One reason for the lack of knowledge is that longevity studies, in rodents are time and resource intensive and are nearly impossible in humans. To identify signaling mechanisms and potential therapeutics in mice and to test these therapeutics in humans it would be useful to have a biological marker (biomarker) that changes rapidly in response to the intervention in a way that predicts the effect on maximum lifespan and rate of aging. Towards this end, in 1988, the NIA began a 10-year initiative aimed at defining a panel of biomarkers that would determine biological, rather than chronological age in rodents (Baker and Sprott, 1988). While the program led to valuable characterization of survival curves, hormonal changes and pathologies of aging, it failed to identify a single biomarker or panel of biomarkers that predict biological age or could be used for screening aging interventions. The question then becomes, why was the initiative unsuccessful in identifying biomarkers and has the environment changed so that new investigations could be successful.

We believe that the Biomarkers of Aging Program was unsuccessful for three main reasons. First, the expectation was that there is a panel of biomarkers that are characteristic for every biological age and that a change in these biomarkers represents the rate of aging. While this was a very noble pursuit with amazing clinical implications, it has proven very difficult, or even impossible as some suggest, to predict the biological age of an individual. However, we believe that a first step in aging research would be to identify interventions that on average increase lifespan in model systems, not necessarily predict remaining lifespan for an individual, for this a change in some biomarker that is consistent with increased lifespan in the population would suffice. Second, at the time of the initiative the only known intervention for increasing lifespan was calorie restriction. Thus, it is difficult to know if changes were specific to calorie restriction or represented some fundamental process of aging. Now with several other dietary, genetic and pharmacological interventions available it will be easier to cross-reference and identify common elements that may be central to aging. Lastly, many of the biomarkers tested were based on outcomes of aging, i.e. formations of neoplasms, or other pathological lesions. These, unfortunately, seem to be strain, species and individual dependent and overlap with diseases rather than underlying causes of aging. In addition, these outcomes can take a long time to manifest in a rodent and thus would not serve as a rapid biomarker to compare interventions. We believe that, a previously untested biomarker, cell proliferation is mechanistically linked to aging and could provide a rapid biomarker to test aging interventions.

Cell Proliferation & Lifespan

We hypothesize that cell proliferation is mechanistically linked to aging and may be a parameter that eventually limits maximal lifespan. Somatic cells are constantly exposed

to external and internal stresses that lead to damage of DNA, proteins and membrane lipids. This damage can, eventually, lead to apoptosis. In order to maintain tissue homeostasis, the apoptotic cells must be replaced with new functional cells. This requires division of stem/progenitor cells and clonal expansion. If there is a limit to the replicative capacity of stem cells, as Hayflick and others have suggested (Houck et al., 1971), then as an animal ages the capacity to replace apoptotic cells becomes compromised, leading to tissue dysfunction and ultimately death.   Thus, preserving replicative capacity by reducing the need for cell renewal will delay tissue dysfunction and prolong lifespan. In contrast, increasing the need for cell renewal or stimulating increased proliferation will lead to more rapid tissue dysfunction and a shorter lifespan. While, this hypothesis is difficult to test directly, there are many studies consistent with this model.

Reduced cell renewal, prolonged replicative capacity and extended longevity

The preservation of tissue function in response to CR in the skin (Reed et al., 1996), liver (Chou et al., 1995; Shaddock et al., 1996) and immune system (Chen et al., 1998; Weindruch et al., 1982) is associated with decreased cell proliferation early in life, prolonged replicative capacity, delayed age-related tissue dysfunction and increased lifespan. In the skin, wound healing is impaired in older animals, caused, in part, by impaired cell proliferation in response to injury. While CR leads to decreased skin cell proliferation early in life, cell proliferation in response to injury is preserved late in life leading to improved wound healing compared to age-matched AL fed mice (Reed et al., 1996). In the liver, regeneration and cell proliferation in response to hepatectomy is diminished in older mice (Chou et al., 1995), perhaps due to stem cell exhaustion. Calorie restriction leads to decreased cell proliferation in healthy livers, but maintains a more youthful regenerative capacity in older mice in response to hepatectomy (Chou et al., 1995; Shaddock et al., 1996). As for the immune system, aging leads to a decline in function, characterized by decreased number of circulating T cells (Chen et al., 1998; Weindruch et al., 1982) and a reduced lymphocytotoxic response (Goonewardene and Murasko, 1995). Like other tissues, CR reduces T cell proliferation early in life, but prevents the age-related decline in T cell number (Chen et al., 1998; Weindruch et al., 1982) and preserves the lymphocytotoxic response late in life (Goonewardene and Murasko, 1995). Taken together these data demonstrate that, in three separate mitotic tissues involved in aging, decreased cell proliferation is associated with delayed onset of age-related functional decline.

Further evidence relating cell proliferation to slower aging and increased lifespan comes from the genetic models of increased longevity (de Magalhaes and Faragher, 2008). There are at least 7 genetic mutations that lead to increased maximal lifespan in mice (de Magalhaes and Faragher, 2008). A common theme from these longevity models is diminished signaling through the GH/IGF-1 axis. While there has not been a systematic analysis of cell proliferation and regenerative capacity in these mice, reduced mitotic signaling through IGF-1 would be expected to reduce early life cell proliferation, potentially preserving replicative capacity in late life and delay aging. Several pieces of evidence are consistent with this hypothesis. First, most of the long-lived mutants have decreased body size (Berryman et al., 2008), suggesting decreased cellular proliferation. Second, as in calorie restriction, T-cells from Snell dwarf mice retain their proliferative capacity late in

life (Flurkey et al., 2001), consistent with delayed onset of immune system decline. Last, fibroblasts isolated from adult Snell and GHR-/- mice are resistant to stress induced apoptosis in culture (Salmon et al., 2005). Interestingly, stress resistance is not present in fibroblasts isolated from neonates, suggesting that it is an acquired process, not a direct effect of the genetic manipulation (Salmon et al., 2005). An intriguing possibility is that cell proliferation was reduced in the first few months in the dwarf mice giving the isolated fibroblasts a younger phenotype that is expressed as increased stress resistance in culture.

The mammalian target of rapamycin (mTor) inhibitor, rapamycin, is the only pharmacological intervention known to extend lifespan in mammals (Harrison et al., 2009). Interestingly, activation of the mTor pathway is a robust stimulator of cell growth and proliferation, via several signaling pathways, including SGK and p27 (Toker, 2008). While the effects of rapamycin on cell proliferation have not been described *in vivo*, several *in vitro* studies have shown that rapamycin reduces proliferation in T-cells (Breslin et al., 2005), hepatocytes (Varma and Khandelwal, 2007) and keratinocytes (Javier et al., 1997). As mentioned earlier, aging-related cellular senescence in these three tissues is related to a diminished replicative capacity and inability to respond to stress. In CR, decreased early life cell proliferation in these tissues is correlated to preservation of replicative capacity in late life. Perhaps rapamycin mimics the longevity effects of CR through a common reduction in early life cell proliferation accompanied by late life retention of replicative capacity.

It should be noted that the reductions in cell proliferation rates that are consistent with longevity take place in pathogen and predator free environments. These experiments allow for the assessment of aging while minimizing premature death due to disease or predation. It is unclear how these interventions would benefit an animal in the wild.

Increased cell renewal, diminished replicative capacity and shortened lifespan
In contrast to the long-lived models, increased cell proliferation early in life is associated with diminished replicative capacity and shortened lifespan. There are at least two mouse progeroid models that suggest that increased cell proliferation is related to premature aging. First, mice overexpressing bovine growth hormone have increased postnatal growth and grow to about twice the size of wild-type mice, but their lifespan is cut in half (Bartke et al., 1994). Overexpression of growth hormone also leads to premature aging phenotypes including early decline in memory and reproductive competence (Bartke et al., 1994). Interestingly, while growth hormone overexpression is characterized by early hyperproliferation, fibroblasts and epithelial cells of older mice overexpressing growth hormone have a decreased replicative potential, perhaps due to stem/progenitor cell exhaustion (Bartke et al., 2002). The second mouse model consistent with overproliferation exhausting regenerative capacity, is the deletion of ATR, a gene involved in DNA damage repair (Ruzankina et al., 2007). ATR deletion leads to acute loss of 80-90% of proliferating cells and a rapid decrease in cellular mass in mitotic tissues, followed by a subsequent repopulation (Ruzankina et al., 2007). Like GH overexpression, ATR deletion leads to premature aging phenotype in several tissues including bone marrow, intestines, spleen and thymus (Ruzankina et al., 2007). A likely hypothesis for the

phenotype of ATR deletion is that increased proliferation necessary to repopulate the depleted tissues leads to cellular senescence, reduced regenerative capacity, premature aging and ultimately reduced lifespan.

The mechanisms leading to some human progeroid diseases may also be related to early cellular hyperproliferation. Hutchinson-Gilford progeria syndrome (HGPS), a rare genetic disease, is characterized by accelerated and premature aging (Bridger and Kill, 2004; Halaschek-Wiener and Brooks-Wilson, 2007). Biological aging seems to be accelerated nearly 7-fold in HGPS patients, with hair loss, lypodystrophy and stiffened joints occurring by two years of age and death from heart failure typically around 13 years of age (Bridger and Kill, 2004; Halaschek-Wiener and Brooks-Wilson, 2007). HGPS fibroblasts are hyperproliferative, hyper-apoptotic and reach senescence more rapidly than wild-type fibroblasts (Bridger and Kill, 2004; Halaschek-Wiener and Brooks-Wilson, 2007). Halaschek-Wiener & Brooks-Wilson conclude that mutations in the lamin A gene in HGPS patients leads to hyperproliferation, premature accumulation of senescent cells and early decline of tissue function that is normally seen in elderly individuals (Halaschek-Wiener and Brooks-Wilson, 2007). Interestingly, like HGPS, fibroblasts derived from patients with Werner's syndrome, a progeroid disease characterized by premature accumulation of senescent cells and tissue dysfunction, are also hyperproliferative and have accelerated senescence (Faragher et al., 1993; Kill et al., 1994). Thus, it appears that in both rodent models and human diseases, increased cell proliferation in early life precedes a premature aging phenotype and decreased lifespan.

Effects of CR: Artifact of laboratory conditions?

It should be noted that there is a sentiment among some biologists that the effects of CR are simply an artifact of imposed laboratory conditions. They argue that mice under *ad libitum* fed laboratory conditions "overeat" and that calorie restricting the mice brings them back to their "normal" eating patterns in the wild (Austad and Kristan, 2003). Thus, these biologists believe that the previous work in CR is really just comparing overeating to normal feeding conditions. This argument is based on two fundamental principles. First, that mice in laboratory conditions eat more than mice in the wild. And second, that there is no additional benefit of restricting calories below what animals consume in the wild. However, it appears that both of these principles are false.

To address the first principle, Austad et al. (Austad and Kristan, 2003) analyzed the data from three separate studies that used doubly labeled water to assess energy expenditure of wild mice. They assumed that these mice were relatively close to energy balance over the 2-3 day monitoring period and thus, energy expenditure would reflect energy intake. This data was then compared to mice kept under laboratory housing conditions. When corrected for body mass, there was no difference in food intake between wild and laboratory mice.

To address the second principle that there is no additional benefit of restricting calories below what animals consume in the wild, Weindruch et al. (Weindruch et al., 1986)

found that restricting calories to 25% less than ad libitum fed mice increased average lifespan from 27 to 33 months, and restricting calories to 65% below ad libitum fed mice increased average lifespan to 45 months. This study combined with similar studies led these researchers to conclude that, until starvation, increased CR leads to increased lifespan. More evidence comes from experiments in spiders (Austad, 1989). Wild spiders eat on average 8 flies/day and live a maximum of 30 days. When these spiders were fed 5 flies or 3 flies/day they lived a maximum of 99 days and 139 days respectively. Suggesting that, at least in spiders, underfeeding below what is naturally consumed leads to increased maximal lifespan. Consistent with these data, many female mice under CR conditions have energy intake reduced such that the estrous cycle ceases. It is unlikely that this condition could persist in the wild without a serious threat to the species.

Taken together it appears that, while the sentiment that the effects of CR are simply due to imposed laboratory conditions still persists, it is not founded on any experimental evidence. Indeed, the experiments done to test this theory contradict this sentiment and demonstrate that the longevity effects of CR in the laboratory are due to reduced caloric intake beyond what the animal would normally consume, even in the wild.

Chapter 2

Experimental Design & Methods

Experimental Design & Methods:

Project 1
Mice and diets

12 week-old C57BL/6 mice (Charles River Breeding, Wilmington, MA, USA) were housed individually and maintained under temperature- and light-controlled conditions (12-h light/dark cycle, lights on at 0700 h and off at 1900 h). For all experiments mice were provided semi-purified AIN-93M diet (Bio-Serv, Frenchtown, NJ, USA) and free access to water. Mice on restricted diets were provided with food daily at approximately 1200h. Food intake was recorded for each individual mouse every other day. All protocols and procedures were approved by the University of California Berkeley Animal Use Committee.

Experiment 1 design:

After 1 week of adaptation to their environments and AIN-93M diet, female C57BL/6 mice were randomly assigned to one of three groups (n=3 to 5); *ad libitum*-fed and housed at 22°C in a temperature-controlled room (AL/22), *ad libitum*-fed and housed at 27°C in a temperature-controlled room (AL/27) or pair-fed to the AL/27 group but housed at 22°C in a temperature-controlled room (PF/22) (each mouse in the PF/22 group was provided food equal to the average food intake of the AL/27 group from the previous day). The groups were designed to modulate two of the physiological adaptations to CR, reduced food intake and reduced energy expenditure, independent of CR itself. Mice were maintained on the feeding regimen for 4 weeks and labeled with ^2H_2O for the final 3 weeks.

Experiment 2 design:

After 1 week of adaptation to their environments and AIN-93M diet, female C57BL/6 mice were assigned randomly to one of four groups (n=15); *ad libitum*-fed and sedentary (AL/SED), *ad libitum*-fed and provided 24h access to a voluntary running wheel (AL/EX), pair-fed to the AL/SED group and provided 24h access to a voluntary running wheel (PF/EX) or calorie restricted to body weight match the PF/EX group and sedentary (CR/SED). The groups were designed to modulate two of the physiological adaptations to calorie restriction, reduced percent body fat and reduced body weight, independent of CR itself. Mice were maintained on the feeding regimen for 5 weeks and labeled with ^2H_2O for the final 3 weeks.

Experiment 3 design:

The group design for Experiment 3 was identical to Experiment 2, except 12 week-old male C57BL/6 mice were used instead of female mice. Male C57BL/6 mice were used in this experiment as they have elsewhere been shown to compensate incompletely to voluntary exercise through *ad libitum* food intake and, therefore, weigh less than *ad libitum*-fed sedentary controls (Holloszy et al., 1985). The groups were designed to modulate one of the physiological adaptations to calorie restriction, reduced body weight, independent of CR itself. Mice were maintained on the feeding regimen for 5 weeks and labeled with ^2H_2O for the final 3 weeks.

Voluntary exercise

AL/EX and PF/EX mice in Experiments 2 and 3 were provided 24hr access to a 24cm-running wheel (mini-Mitter) attached to a digital counter. Revolutions were recorded daily.

Body weight measurement, ^2H_2O labeling, and blood and tissue collection

The body weight of each mouse was measured one to three times per week. Mice were labeled with an intraperitoneal injection of 100% ^2H_2O (0.35ml/ 10g body weight) 3 weeks prior to the end of the study and were then provided 8% ^2H_2O as drinking water for the remainder of the study, as described previously (Busch et al., 2007). Upon completion of each experiment, mice were anesthetized under 3% isoflurane and blood was collected via cardiac puncture, followed by cervical dislocation and tissue collection.

Keratinocyte isolation

After euthanasia, the back of each mouse was shaved followed by an application of Nair for complete hair removal (Carter Products, New York, NY, USA). A small piece of the dorsal skin was dissected, washed with phosphate buffered saline solution (PBS; Gibco, Grand Island, NY, USA), cut into three small sections, and placed in 5mL PBS with 10 units of dispase II (Roche, Indianapolis, IN, USA). Dorsal skins were incubated for 3.5 hour with shaking at 100rpm at 37°C. The epidermis was then peeled from the dermis and collected for DNA isolation.

Liver cell isolation

A section of the liver (~30mg) was dissected and homogenized and total DNA from all liver cells was isolated.

Mammary epithelial cell isolation

Mammary epithelial cells were isolated using a protocol adapted from Fata et al. (Fata et al., 2007). Briefly, the 4L and 4R inguinal mammary glands were removed, placed in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) and minced. The minced tissue was incubated in 20mL collagenase/trypsin solution (0.2% Collagenase A (Worthington Biochemical, Lakewood, NJ, USA), 0.2% Trypsin, 5% fetal bovine serum in DMEM) for 20min, shaking at 100 rpm at 37°C. Following digestion, the tissue suspension was centrifuged at 1500 rpm for 10min and the collagenase/trypsin solution and upper fat layer were discarded and the pellet was resuspended in 10mL DMEM. The suspension was pelleted via centrifugation at 1500 rpm for 10min, resuspended in 4mL DMEM containing 5ul DNase (≥500U/mL; Sigma, St. Louis, MO, USA), shaken vigorously for 2min and then incubated at room temperature for 5min. 6mL of DMEM were added to the suspension, which was then pelleted via centrifugation at 1500 rpm for 10min. The pellet was resuspended in 10mL DMEM and the suspension was briefly centrifuged at 1500 rpm and the supernatant discarded. The pellet was subjected to a total of three rounds of this brief differential centrifugation at 1500 rpm. The resulting pellet containing the mammary epithelial cells was then collected for DNA isolation.

Splenic T-cell isolation

Upon dissection, the spleen was homogenized and passed through a 40μM nylon cell strainer. T-cells were isolated from the single cell suspension using mouse anti-CD90.2 microbeads and the MACS cell separation column following the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA), pelleted and then collected for DNA isolation.

Bone marrow cell isolation

The femur was dissected for isolation of bone marrow cells. Bone marrow cells were flushed from the femur with 2mL of PBS and were then pelleted and collected for DNA isolation.

DNA isolation

DNA was extracted using DNeasy kits (Qiagen, Valencia, CA, USA). Briefly, isolated keratinocytes, liver, MECs, T-cells, and bone marrow cells were digested overnight at 37 °C in proteinase K solution followed by DNA isolation and elution into 200ul water.

DNA synthesis measurement

Determination of ^{2}H incorporation into purine deoxyribose (dR) of DNA was performed as described previously (Busch et al., 2007). Briefly, isolated DNA was hydrolyzed overnight at 37 °C with nuclease S1 and potato acid phosphatase. Hydrolyzates were reacted with pentafluorobenzyl hydroxylamine and acetic acid, then acetylated with acetic anhydride and 1-methylimidazole. Dichloromethane extracts were dried, resuspended in ethyl acetate, and analyzed by GC/MS on a DB-17 column with negative chemical ionization, using He as carrier and CH_4 as reagent gas. The fractional molar isotope abundances at m/z 435 (M_0 mass isotopomer) and 436 (M_1) of the pentafluorobenzyl triacetyl derivative of purine dR were quantified using ChemStation software. Excess fractional M_{+1} enrichment (EM$_1$) was calculated as:

$$EM1 = \frac{(abundance\, m/z\, 436)_{sample}}{(abundance\, m/z\, 435+436)_{sample}} - \frac{(abundance\, m/z\, 436)_{std}}{(abundance\, m/z\, 435+436)_{std}}$$

where sample and standard (std) refer to the analyzed sample and an unenriched pentafluorobenzyl triacetyl purine dR derivative standard, respectively. The fractional synthesis rate (f) of keratinocytes, liver, MECs and T-cells was calculated by a comparison to bone marrow cells in the same animal, which represents an essentially fully turned over population of cells.

$$f\left(\right) = \frac{(E\quad 1)_s M}{(E\quad 1)_b M_m} \times \, 1^m$$

Body Composition Analysis

Percent body fat was determined via chemical extraction (Bell and Stern, 1977). Briefly, mouse carcasses where weighed before and after freeze drying to determine percent water. Dried carcasses were placed in a Soxhlet extraction apparatuses and extracted in ether for 7 days and in acetone for 5 days. The carcasses were then removed and placed in a fume hood for 3 days to allow complete solvent evaporation and then weighed to determine percent body fat.

<u>Serum Analyte Measurements</u>

Mice were anesthetized under 3% isoflurane and blood was collected via cardiac puncture. Following centrifugation, serum was collected and stored at -20°C. Serum concentrations of insulin-like growth factor-1 (R&D Systems, Minneapolis,MN, USA), insulin-like growth factor binding protein-3 (R&D Systems, Minneapolis,MN, USA), triiodothyronine (T_3) (Calbiotech,Spring Valley, CA, USA) and thyroxine (T_4) (Calbiotech,Spring Valley, CA, USA) were measured via ELISA following the manufacturer's instructions.

<u>Statistical Analysis</u>

All results are presented as mean ± SEM. A repeated-measures ANOVA followed by Bonferroni *post hoc* test was used to analyze change in body weight over time. Following a significant result on repeated-measures ANOVA, single time point comparisons were made using one-way ANOVA followed by a Tukey *post hoc* test. For cell proliferation, differences between groups were analyzed by one-way ANOVA with Tukey *post hoc* test. Data were analyzed by Prism Graphpad software (version 5.0a).

Project 2

<u>Mice and diets</u>

Nine-week-old C57BL/6J male mice were obtained from Charles River Breeding Laboratories, housed individually, and maintained under temperature and light controlled conditions (12 hour light/dark cycle: lights on at 7 AM and off at 7 PM) for 1 week. During this acclimation period, mice were given free access to water and a semipurified AIN-93M diet (Bio-Serv), and the daily amount of food consumed by each mouse was recorded.

At 10 weeks of age, mice were randomly assigned to either the CR or the AL control group.   Mice in the AL group were provided free access to food throughout the day, while mice in the CR group were provided 70% of the calories consumed by the AL group. Food was provided to the CR group daily at 6 PM. Mice were maintained on CR or AL for at least 5 weeks before any experimental studies were carried out. Upon completion of each experiment, mice were anesthetized under 3% isoflurane and blood was collected via cardiac puncture, followed by cervical dislocation. All procedures and protocols received approval from the University of California Berkeley Animal Use Committee.

<u>Food Consumption</u>

Food consumption was determined by 24-hour automatic food monitoring system in Environment Controlled CLAMS metabolic cages (Columbus Scientific). Total food consumption was measured six times per hour and averaged to determine hourly food consumption.

Respiratory Exchange Ratio (RER), Energy Expenditure (EE), FA Oxidation and Fuel Selection

We determined RER, EE, FA oxidation and fuel selection in environment controlled CLAMS metabolic cages, equipped with an indirect open circuit calorimeter (Oxymax Equal Flow System). The system measures carbon dioxide produced and oxygen consumed over a one-minute period, six times per hour. These values were averaged to determine the rate of carbon dioxide produced (VCO_2) and oxygen consumed (VO_2) in milliliters per hour. EE, RER and FA oxidation were calculated using the following equations (Lusk, 1924):

$$RER = VCO_2 / VO_2$$

$$EE(kcal/h) = \left[(3.815 + 1.232 \times RER) \times VO2\right] \times 1000$$

$$FA\ oxidation\ (kcal/h) = EE \times \left(1 - RER / 0.3\right)$$

Daily FA oxidation was calculated from the 24-hour area under the curve (AUC) of hourly FA oxidation. Daily carbohydrate plus protein oxidation was calculated from total daily EE, determined from the AUC of hourly EE, minus daily FA oxidation.

FA Synthesis

FA synthesis was measured by stable isotope incorporation, with analysis by gas chromatography-mass spectrometry (GC-MS). Mice were labeled with an intraperitoneal injection of 100% ^2H_2O (0.35ml/ 10g body weight) and then provided 8% ^2H_2O as drinking water for 6–24 hours, as described previously (Varady et al., 2009). Upon completion of labeling, mice were euthanized and tissue or serum was collected and homogenized in 2:1 chloroform/methanol solution. Pentadecanoic acid (10mg/mL) was added to solutions as an internal control. Lipid was extracted in methanol/chloroform overnight and methylated with 5% methanolic acid with heating at 50°C for 1 hour. The FA methyl esters were extracted via the Folch technique with hexane after the addition of water.

Total FA concentrations were measured via gas chromatography-flame ionization detection using an Agilent 6890N GC (Agilent Technologies, Palo Alto, CA) and a DB-225MS column. Hydrogen was used as carrier gas at a constant flow rate of 40mL/min. The temperature of the GC oven was set to 110°C for 2 minutes, increased to 220°C for 8min and held at 240°C for 5 minutes. HP Chemstation software was used for data analysis.

The fraction of newly synthesized non-essential saturated FA formed during the ^2H_2O labeling period was assessed using a combinatorial model of polymerization biosynthesis, as described previously (Strawford et al., 2004; Turner et al., 2003). Briefly, mass isotopomer distribution analysis (MIDA) was used to determine the number (n) of hydrogen atoms in FA C–H bonds that were derived from cellular water during endogenous synthesis of FA. Enrichments, or excess fractional molar abundances (EM_x) above baseline, were measured for the parent (EM_0), single-labeled (EM_1) and double-labeled (EM_2) mass isotopomers in the FA by GC-MS. Specifically, the fractional abundances of molecular anions m/z 270, 271 and 272 (for 16:0, palmitate), and m/z 298, 299 and 300 (for 18:0, stearate) were determined in natural abundance (baseline) samples and in ^2H_2O labeled samples. The measured body ^2H_2O enrichment was then used to represent the isotopic

enrichment of hydrogen atoms entering C-H bonds in the FA synthetic pathway, i.e., the true precursor pool (p) (Strawford et al., 2004; Turner et al., 2003), and n was calculated from EM_2/EM_1 ratios, as described previously (Lee et al., 1994). The maximal or asymptotic ^2H excess mass +1 isotopomeric enrichment (A^*_1) possible in the FA at this p and n, representing the EM_1 value if 100% of the FA were newly synthesized from this body water pool was then calculated (Strawford et al., 2004; Turner et al., 2003). The measured EM_1 value was divided by the asymptotic value to calculate the fraction (f) of FA that was endogenously synthesized during the labeling period.

$$Fraction\ endogenously\ synthesized\ FA\ (f) = EM_1/A_1{}^*$$

This approach was modified to determine the fractional synthesis of palmitoleate (16:1) and oleate (18:1). The double bonds were saturated by reacting 10mg of FA methyl esters with 5mg of bromine in 400uL of carbon tetrachloride for 30 minutes. The resultant dibromo methyl ester-FAs were analyzed by positive chemical ionization and, after loss of bromine, anions m/z 347 and 348 (for 16:1) and m/z 377, 378 and 379 (for 18:1) were compared to determine EM_1 and EM_2 values.

Whole-body fat mass, used to calculate total adipose FA synthesis, was estimated at 15% body weight for CR and 25% body weight for AL, based on literature values (Berrigan et al., 2005; Brochmann et al., 2003; Colman et al., 2007; Hamrick et al., 2008; Hong et al., 2009; Mai et al., 2003).

Gene Expression

RNA was isolated from snap frozen inguinal and epididymal adipose tissue and liver tissue using RNeasy kit (Qiagen) and reverse transcribed with M-MulV reverse transcriptase (New England Biolabs). Next, 25ng of diluted cDNA was run on an ABI 7500 Fast Real-Time PCR System, using TaqMan gene expression master mix and probed with Fasn (Mm01253300_g1), Acaca (Mm01304289_m1), B2m (Mm00437762_m1) and GAPDH (Pre-developed TaqMan assay reagent) according to manufacturers instructions (Applied Biosystems). The amount of FAS and ACC1 mRNA was compared relative to the average of GAPDH and B2-microglobin. Data from all groups were expressed as means ± SEM, relative to AL control animals at 6 PM.

Statistical Analysis

All results are presented as mean ± SEM. The area under the curve for Fig. 3 was calculated with a baseline of 0 and from x =0 to x= 24. Differences between groups were analyzed by two-way ANOVA with Bonferroni *post hoc* test or T-test. Data were analyzed by Prism Graphpad software (version 5.0a).

Project 3

Mice and Diets

For SIRT1 liver knockout experiments, SIRT1 liver-specific knockout mice were generated by crossing mice expressing a floxed allele of SIRT1 exon 4 (Cheng et al. 2003) with Cre-expressing mice driven by the liver-specific albumin promoter (generously provided by Dr. Leonard Guarente). All mice were housed individually, and maintained under temperature and light controlled conditions (12 hour light/dark cycle: lights on at 7

AM and off at 7 PM). Wild-type and liver knockout littermates were randomly divided into 4 groups (n=5/group); wild-type and fed AIN-93m ad libitum (WT/AL), Sirt1 liver knockout and fed AIN-93m ad libitum (LKO/AL), wild-type and fed AIN-93m at 70% of ad libitum intake (WT/CR), Sirt1 liver knockout and fed AIN-93m at 70% of ad libitum intake (LKO/CR). CR mice were provided food daily at 16:00. Mice were maintained on diets for 5 weeks and labeled with ^2H_2O during the last 3 weeks of the study. Mice were euthanized, tissues were collected and liver cell proliferation was assessed as described in Project 1.

For the resveratrol experiments, twelve-week-old C57BL/6J male mice were obtained from Charles River Breeding Laboratories, housed individually, and maintained under temperature and light controlled conditions (12 hour light/dark cycle: lights on at 7 AM and off at 7 PM) for 1 week. During this acclimation period, mice were given free access to water and a semipurified AIN-93M diet (Bio-Serv), and the daily amount of food consumed by each mouse was recorded. At 13 weeks of age, mice were randomly divided into 3 groups (n=6/group); provided AIN-93M ad libitum (AL), provided AIN-93M at 70% of ad libitum intake (CR), provided AIN-93M supplemented with 1g resveratrol/1kg diet (RSV). Mice were maintained on diets for 6 months and labeled with ^2H_2O for the final 8 weeks. Mice were euthanized, tissues were collected and liver cell proliferation was assessed as described in Project 1.

For the time course study, twelve-week-old C57BL/6J male mice were obtained from Charles River Breeding Laboratories, housed individually, and maintained under temperature and light controlled conditions (12 hour light/dark cycle: lights on at 7 AM and off at 7 PM) for 1 week. During this acclimation period, mice were given free access to water and a semipurified AIN-93M diet (Bio-Serv), and the daily amount of food consumed by each mouse was recorded. At 13 weeks of age, mice were randomly divided into ad libitum fed or CR (70% of AL intake) groups (n=15/group). CR mice were provided food daily at 16:00. Mice were maintained on diets for 5 weeks then euthanized at 09:00, 16:00, or 21:00. Tissues were collected and snap frozen.

Sirt1 liver knockout genotyping

Tail snips from all mice in Sirt1 colony were assessed for loxp sites flanking exon 4 of Sirt1 and Cre recombinase expression using REDExtract-N-Amp™ Tissue PCR Kit (Sigma) following the manufacturer's instructions. The following primer pairs were used:

Floxed Sirt1 gene: F-5' CCC CAT TAA AGC AGT ATG GTG 3'; R-5' CAT GTA ATC TCA ACC TTG AG 3'. Conditional knockout mice have a PCR product of 1 kb, while wild-type mice have a product of 700bp. Cre Recombinase gene F-5' GCG GCA TGC TGC AAG TTG AAT 3'; R-5' CGT TCA CCG GCA TCA ACG TTT-3'. Transgenic mice express a product at 232bp.

SDS-PAGE/Western Blotting

Livers from mice were rapidly dissected and snap frozen in liquid nitrogen. Livers were homogenized in a TissueLyzer (Qiagen) in RIPA buffer with protease inhibitors at a concentration of 100mg liver/1mL RIPA. Homogenates were then sonicated and protein concentration was assessed by BCA assay (Pierce). 20ug of protein was loaded per well on

a 4-12% acrylamide gradient gel. Sirt1 was identified by staining with anti-Sirt1 antibody (Cell signaling)

<u>Statistical Analysis</u>
All results are presented as mean ± SEM. Differences between groups were analyzed by two-way ANOVA with Bonferroni *post hoc* test or T-test. Data were analyzed by Prism Graphpad software (version 5.0a).

Chapter 3

Project 1: The Effects of Physiological Adaptations to Calorie Restriction on Global Cell Proliferation Rates

Project 1: Rationale

Reductions in food intake (Harrison et al., 1984; Yu et al., 1985), energy expenditure (*Sacher*, 1977), percent body fat (Berg and Simms, 1960; Bluher et al., 2003; Picard et al., 2004) and body weight (Holehan and Merry, 1985; McCay et al., 1989; Miller et al., 2002) have all been suggested to mediate the effects of CR. To date, however, it is still not clear which, if any, of these physiological adaptations are sufficient to increase longevity. In addition, the hormonal mediators translating these physiological adaptations to cellular responses have not been fully characterized. Progress in this field has been limited by both the difficulty in dissociating the individual physiological effects of CR, as well as the time and resources required for classical longevity studies in mice, which take up to 3 years to complete (Harrison et al., 2009).

Cell proliferation is mechanistically linked to aging and may be a parameter that eventually limits maximal lifespan (de Magalhaes and Faragher, 2008). Interventions, such as CR, that reduce early life cell proliferation are associated with preserved tissue function in old age and increased longevity (Reed et al., 1996; Chou et al., 1995; Shaddock et al., 1996; Chen et al., 1998; Weindruch et al., 1982). CR leads to reductions in proliferation rates of liver cells, keratinocytes, mammary epithelial cells and splenic T-cells in mice within several weeks and the effect persists as long as the mice remain on the diet. Thus, cell proliferation rate is a component phenotype of aging in several tissues and should be predictive of interventions that effect longevity. However, the individual effects of food intake, energy expenditure, percent body fat and body weight on cell proliferation rates are not known.

Previous studies have established that changes in ambient housing temperature and voluntary wheel running can alter food intake, energy expenditure, percent body fat, and body weight (Holloszy, 1993; Huffman et al., 2007) independent of CR. Therefore, our hypothesis is that changes in ambient housing temperature and voluntary wheel running will lead to changes in food intake, energy expenditure, percent body fat and body weight independent of CR and that by measuring changes in cell proliferation rates in response to these physiologic adaptations we will gain insight into their individual effects on longevity. Accordingly, the goal of this study was to determine the effects of food intake, energy expenditure, percent body fat and body weight on cell proliferation rates, independent of CR in young C57BL/6 mice. In addition, molecular mediators involved in the growth factor response to CR were investigated.

Project 1: Results

Experiment 1: *The effect of mimicking physiological adaptations to CR via changes in housing temperature on cell proliferation rates*

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To determine the effects of reduced food intake and energy expenditure on cell proliferation rates, independent of classical CR, female C57BL/6 mice were randomized into one of three groups: ad libitum-fed and housed at the standard 22°C (AL/22), ad libitum-fed and housed at 27°C (AL/27), or pair-fed to the AL/27 group and housed at the standard 22°C (PF/22). The rationale for the group design in Experiment 1 was based on two primary assumptions: i) AL/27 mice are closer to thermoneutrality than AL/22 mice and will, therefore, expend less energy to maintain body temperature, and ii) AL/27 mice will voluntarily decrease their food intake to maintain energy balance, due to decreased energy expenditure and will, therefore, have comparable body weights to AL/22 mice, despite reduced food intake.

The average daily food intake of both the AL/27 and PF/22 mice was significantly lower than that of AL/22 mice (AL/22: 3.3 ± 0.05, AL/27 2.6 ± 0.06, PF/22: 2.6 ± 0 g/day) (Fig 1.1A). There was no difference in average body weight between AL/22 and AL/27 mice while PF/22 mice weighed significantly less than both AL groups at the end of the experiment (AL/22: 22.0 ± 0.7, AL/27 22.5 ± 0.7, PF/22: 18.0 ± 0.3g) (Fig 1.1B). The maintenance of body weight in the face of decreased food intake in AL/27 mice relative to AL/22 mice was presumably due to a decrease in energy expenditure.
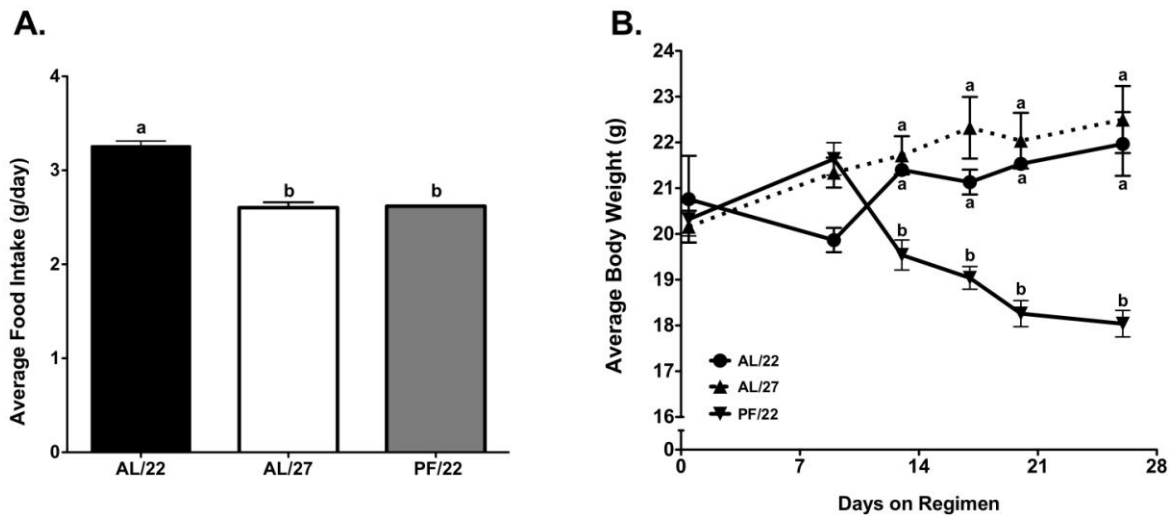
Fig. 1.1 Effect of housing temperature on food intake and body weight (Ex 1). Female mice were divided into 3 groups; *ad libitum*-fed and housed at 22°C (AL/22), *ad libitum*-fed and housed at 27°C (AL/27) and pair-fed to the AL/27 group and housed at 22°C (PF/22). Food was provided once daily at 1200 hours. (A) Food consumption was recorded every other day. Data represent the mean food intakes over the 4-week experiment. (B) Body weights were recorded once per week. Data represent the mean weekly body weights. All values are expressed as means ± SEM, n=3 to 5 for each group. One-way ANOVA with Tukey's post hoc test was used for all between–group analyses; means not sharing a common letter are significantly different ($p < 0.05$).

All mice remained on their regimens for a total of four weeks at which point they were euthanized and their tissues were collected for cell proliferation analysis. Cell proliferation rates were significantly lower in all cell types analyzed in PF/22 mice compared to AL/22 mice (24%, 71%, 51% and 32% lower in keratinocytes, liver, mammary epithelial and splenic T-cells, respectively) (Fig. 1.2A-D). Interestingly, despite a

significantly lower average daily food intake and energy expenditure compared to AL/22 mice, there were no differences in cell proliferation rates in any of the cell types analyzed in AL/27 mice compared to AL/22 mice (Fig 1.2A-D). These data indicate that reduced food intake and energy expenditure are not sufficient to account for the cell proliferation rate-lowering effects of CR.
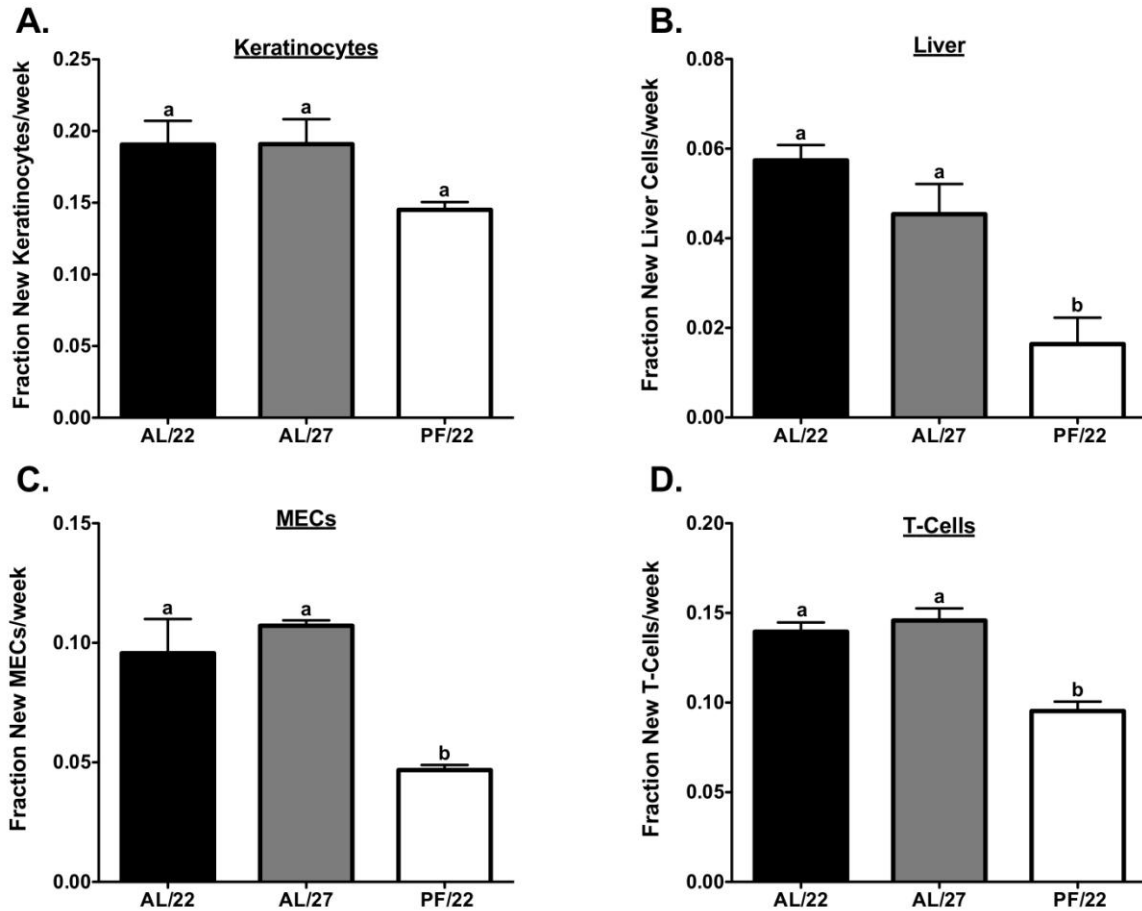

Fig. 1.2 Effect of housing temperature on cell proliferation rates (Ex 1). Cell proliferation rates in (A) keratinocytes, (B) liver, (C) mammary epithelial and (D) splenic T-cells. Values are expressed as means ± SEM, n=3 to 5 for each group. One-way ANOVA with Tukey's post hoc test was used for all between–group analyses; means not sharing a common letter are significantly different (p < 0.05).

Experiment 2: *The effect of mimicking physiological adaptations to CR via voluntary wheel running on cell proliferation rates (female mice)*

To determine the effects of reductions in percent body fat and body weight on cell proliferation rates, independent of classical CR, female C57BL/6 mice were randomized into one of four groups: ad libitum-fed and sedentary (AL/SED), ad libitum-fed with free access to a voluntary running wheel (AL/EX), pair-fed to the AL/SED group with free access to a voluntary running wheel (PF/EX) or calorie restricted to body weight match PF/EX mice and sedentary (CR/SED). The rationale for the group design in Experiment 2

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was based on three primary assumptions: i) female AL/EX mice, while expending more energy than AL/SED mice, will voluntarily increase their food intake to compensate for an increase in energy expenditure and will, therefore, have comparable body weights to AL/SED mice, ii) PF/EX mice will lose body weight compared to AL/SED and iii) exercising mice in either dietary setting will have decreased percent body fat compared to AL/SED mice.
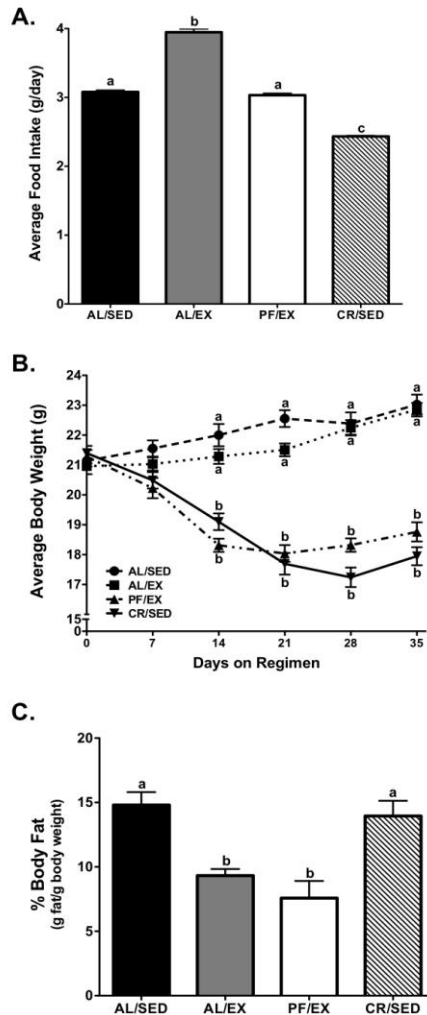

The average daily food intake of the AL/EX mice was significantly elevated compared to both AL/SED and PF/EX mice (AL/SED: 3.1 ± 0.02, AL/EX: 3.9 ± 0.05, PF/EX 3.0 ± 0.03, CR/SED 2.4 ± 0.01 g/day) (Fig 1.3A). There was no difference in average body weight between AL/SED and AL/EX mice at the end of the experiment. Similarly, there was no difference in average body weight between PF/EX and CR/SED mice, while mice in these two groups did have significantly lower body weights than both AL/SED and AL/EX mice (AL/SED: 23.0 ± 0.3, AL/EX: 22.9 ± 0.2, PF/EX 18.8 ± 0.3, CR/SED 18.0 ± 0.3 g) (Fig 1.3B). Body composition analyses confirmed that percent body fat was significantly reduced in AL/EX and PF/EX mice compared to AL/SED and CR/SED mice, which did not differ significantly from one another (AL/SED: 14.8 ± 1.0, AL/EX: 9.3 ± 0.5, PF/EX 7.6 ± 1.3, CR/SED 14.0 ± 1.2 g fat/g body weight) (Fig 1.3C).

All mice remained on their regimens for a total of five weeks at which point they were euthanized and their tissues were collected for cell proliferation analysis. Despite significantly lower percent body fat, there were no differences in cell proliferation rates in any of the cell types analyzed in AL/EX compared to AL/SED mice. Cell proliferation rates were significantly lower in all cell types analyzed in CR/SED mice compared to AL/SED mice (31%, 75%, 62% and 55% lower in keratinocytes, liver, mammary epithelial and splenic T-cells, respectively). Cell proliferation rates were also significantly lower in all cell types analyzed in PF/EX mice compared to AL/SED mice (15%, 36%, 42% and 31% lower in keratinocytes, liver, mammary epithelial and splenic T-cells, respectively). Interestingly, cell

Fig. 1.3 Effect of voluntary wheel running on food intake, percent body fat and body weight in female mice (Ex 2). Female mice were divided into 4 groups; *ad libitum*-fed and sedentary (AL/SED), *ad libitum*-fed and provided free access to a running wheel (AL/EX), pair-fed to AL/SED and provided free access to a running wheel (PF/EX) and calorie restricted to body weight match the PF/EX group and sedentary (CR/SED). Food was provided once daily at 1200 hours. (A) Food intake was recorded every other day. Data represent the mean food intakes over the 5-week experiment. (B) Body weights were recorded approximately 3 times per week. Data represent the mean weekly body weights. (C) Following euthanization, percent body fat was determined via chemical extraction from the carcass. Values are expressed as means ± SEM, n=15 for each group for food intake and body weight and n=5 to 14 for each group for percent body fat. One-way ANOVA with Tukey's post hoc test was used for all between–group analyses; means not sharing a common letter are significantly different (p < 0.05).

proliferation rates in CR/SED mice were significantly lower than in PF/EX mice, to which they were body weight matched (19%, 60%, 34% and 35% lower in keratinocytes, liver, mammary epithelial and splenic T-cells, respectively) (Fig 1.4A-D).
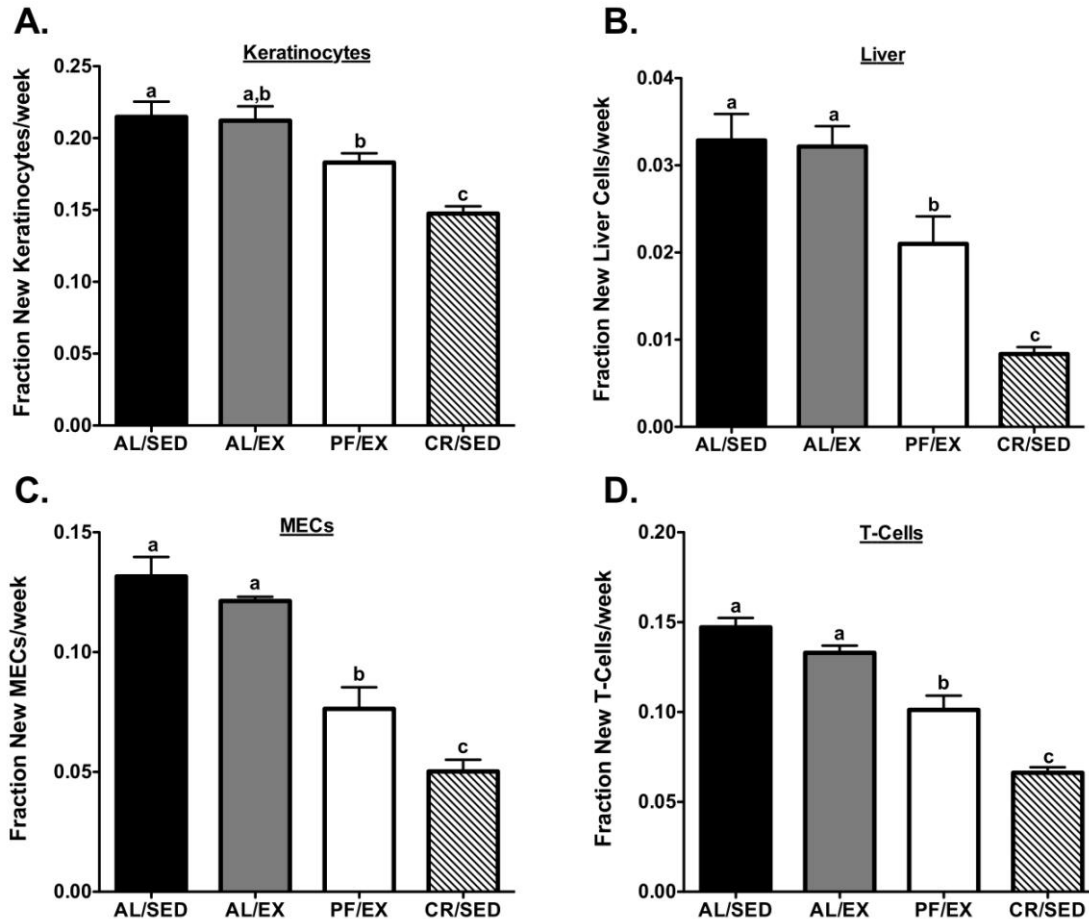

Fig. 1.4 Effect of voluntary wheel running on cell proliferation rates in female mice (Ex 2). Cell proliferation rates in (A) keratinocytes, (B) liver, (C) mammary epithelial and (D) splenic T-cells. Values are expressed as means ± SEM, n=10 to 15 for each group. One-way ANOVA with Tukey's post hoc test was used for all between–group analyses; means not sharing a common letter are significantly different (p < 0.05).

These data suggest that reductions in percent body fat are not sufficient to account for the cell proliferation rate lowering effects of CR, whereas reductions in body weight may be associated with lower rates of cell proliferation. Interestingly, both groups of mice in which we observed an effect of reduced body weight on cell proliferation rates (PF/EX and CR/SED) were food restricted relative to what they would have eaten if fed ad libitum. As exemplified by the increased food intake of AL/EX mice, PF/EX mice would have consumed more food had they been given free access and not restricted to the food intake of AL/SED mice. These results suggest that the effects on cell proliferation rates could be

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due to a "perceived" reduction in energy intake rather than to reduced body weight per se. Accordingly, we designed Experiment 3 with the intention of dissociating reduced body weight from CR.

Experiment 3: *The effect of mimicking physiological adaptations to CR via voluntary wheel running on cell proliferation rates (male mice)*

To determine the effect of reduced body weight on cell proliferation rates in the context of ad libitum food intake, male C57BL/6 mice were randomized into one of four groups: ad libitum-fed and sedentary (AL/SED), ad libitum-fed with free access to a voluntary running wheel (AL/EX), pair-fed to the AL/SED group with free access to a voluntary running wheel (PF/EX) or calorie restricted to body weight match PF/EX mice and sedentary (CR/SED). The rationale for the group design in Experiment 3 was based on the assumption that male AL/EX mice will voluntarily increase their food intake to counter their increased energy expenditure, but will fail to fully compensate energetically and will, therefore, have reduced body weight compared to AL/SED mice. Therefore, Experiments 2 and 3 had identical designs, but utilized differences in food intake and energy balance in response to exercise between female (Experiment 2) and male (Experiment 3) mice.
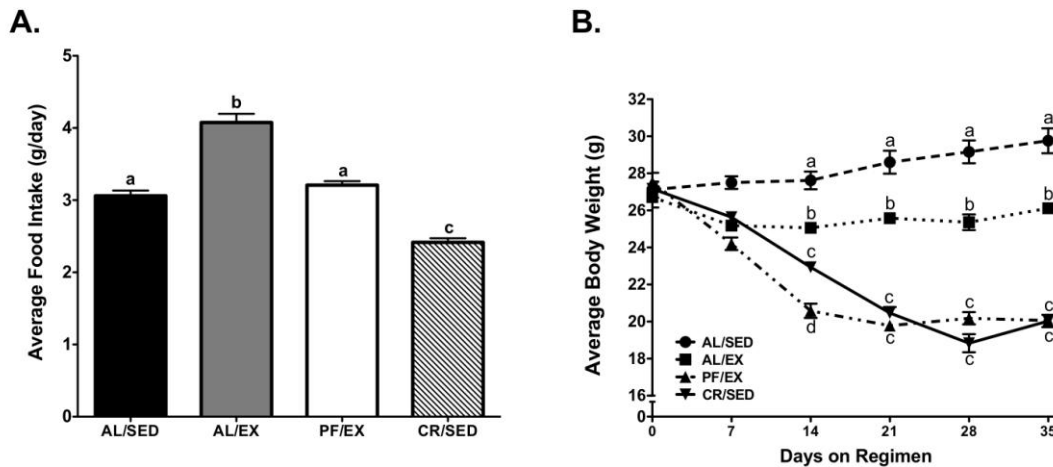
Fig. 1.5 Effect of voluntary wheel running on food intake and body weight in male mice (Ex 3). Male mice were divided into 4 groups; *ad libitum*-fed and sedentary (AL/SED), *ad libitum*-fed and provided free access to a running wheel (AL/EX), pair-fed to AL/SED and provided free access to a running wheel (PF/EX) and calorie restricted to body weight match the PF/EX group and sedentary (CR/SED). Food was provided once daily at 1200 hours. (A) Food intake was recorded every other day. Data represent the mean food intakes over the 5-week experiment. (B) Body weights were recorded approximately 3 times per week. Data represent the mean weekly body weights. Values are expressed as means ± SEM, n=3 to 5 for each group. One-way ANOVA with Tukey's post hoc test was used for all between–group analyses; means not sharing a common letter are significantly different ($p < 0.05$).

The average daily food intake of the AL/EX mice was significantly elevated compared to both AL/SED and PF/EX mice (AL/SED: 3.1 ± 0.07, AL/EX: 4.1 ± 0.1, PF/EX 3.2 ± 0.06, CR/SED 2.4 ± 0.01 g/day) (Fig 1.5A). However, AL/EX mice had significantly lower body weight compared to AL/SED mice at the end of the experiment (reduced by 12.4%; AL/SED: 29.8 ± 0.7, AL/EX: 26.1 ± 0.2, PF/EX 20.1 ± 0.2, CR/SED 20.0 ± 0.3 g) (Fig 1.5B).

There was no difference in average body weight between PF/EX and CR/SED mice, while mice in these two groups did have significantly lower body weights than both AL/SED and AL/EX mice.

All mice remained on their regimens for a total of five weeks at which point they were euthanized and their tissues were collected for cell proliferation analysis. Interestingly, despite a significant reduction in body weight as compared to AL/SED, AL/EX male mice did not have significant differences in cell proliferation rates in any of the cell types analyzed. Cell proliferation rates were significantly lower in all cell types analyzed in CR/SED mice compared to AL/SED mice (37%, 71% and 51% lower in keratinocytes, liver and splenic T-cells, respectively) (Fig 1.6A-D).
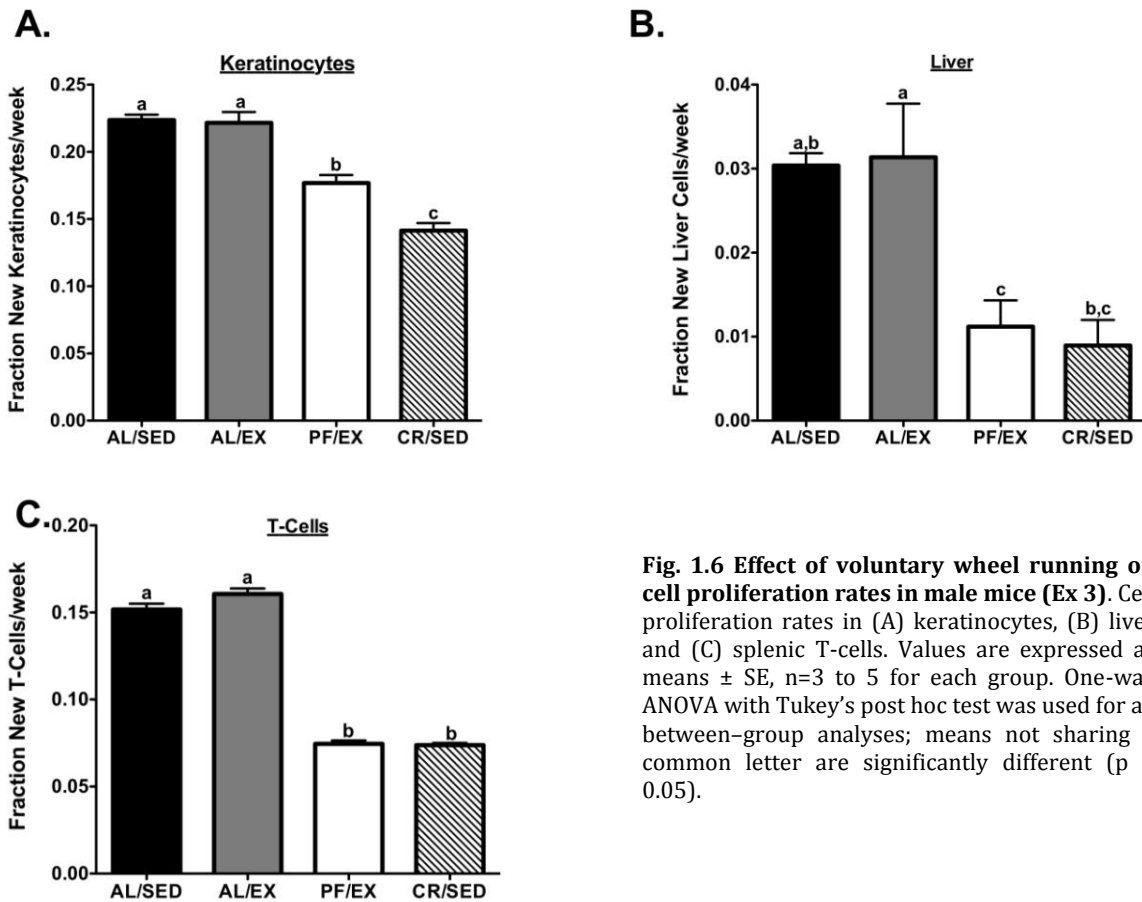
Fig. 1.6 Effect of voluntary wheel running on cell proliferation rates in male mice (Ex 3). Cell proliferation rates in (A) keratinocytes, (B) liver and (C) splenic T-cells. Values are expressed as means ± SE, n=3 to 5 for each group. One-way ANOVA with Tukey's post hoc test was used for all between–group analyses; means not sharing a common letter are significantly different (p < 0.05).

These data suggest that in the context of ad libitum food intake, reduced body weight relative to sedentary controls is not sufficient to account for the cell proliferation rate lowering effects of CR. Together, the results from Experiments 1, 2 and 3 (Fig 1.7A-C) suggest that none of the classic adaptations to CR (reductions in food intake, energy expenditure, percent body fat and body weight) are sufficient to account for the reductions in "global" cell proliferation rates observed in CR mice.

Circulating factors and cell proliferation

To identify circulating factors that may mediate global reductions in cell proliferation rates, we measured the concentrations of several circulating growth factors in mice from Experiments 2 and 3. Insulin-like growth factor binding protein-3 (IGFBP-3), triiodothyronine (T3) and thyroxine (T4) serum levels were not different in any group in Experiment 2 (Table 1). In contrast, serum insulin-like growth factor-1 (IGF-1) levels were significantly lower in CR/SED compared to both AL groups (Table 1). In addition, IGF-1 serum levels correlated positively with cell proliferation rates in all cell types analyzed in all mice in Experiments 2 and 3 (Fig 1.8A-D).
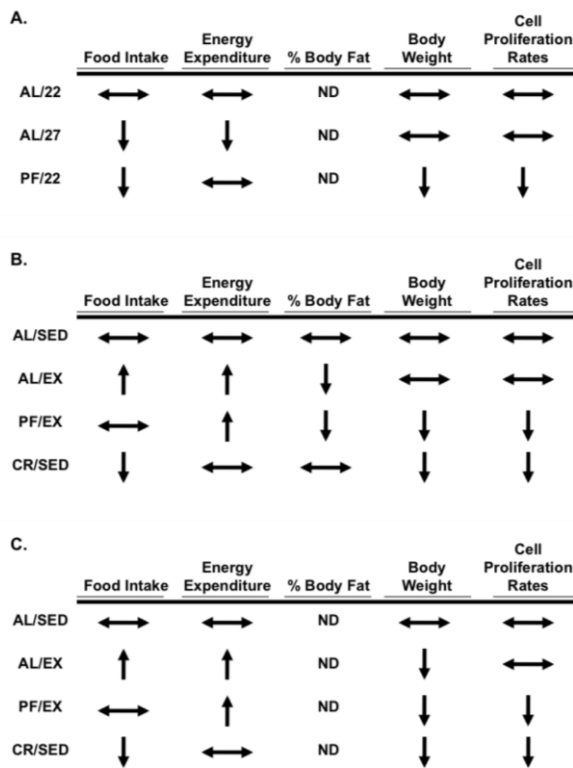
Fig. 1.7 Summary of the effects of the physiological adaptations to CR on cell proliferation rates (Ex 1,2 and 3). Cell proliferation rates were measured under experimental conditions in which (A) food intake and energy expenditure (Ex 1), (B) percent body fat (Ex 2) or (C) body weight were reduced, relative to *ad libitum*-fed controls, through changes in ambient housing temperature (Ex 1) or voluntary wheel running (Ex 2 and 3), independent of classic CR. Indicated changes in food intake, energy expenditure, percent body fat, body weight and cell proliferation rates are relative to AL/22 (Ex 1) or AL/SED (Ex 2 and 3) controls. ↔ = no change, ↑= increased and ↓= decreased. ND = not determined. Changes in energy expenditure were assumed based on changes in food intake and body weight.

Table 1 Circulating factors (Ex 2).

Group	Circulating Factor IGF-1	IGFBP-3	T_3	T_4
AL/SED	$339.2 \pm 30.6^{a,b}$	2446 ± 143^a	$1301 \pm 68^{a,b}$	47284 ± 5264^a
AL/EX	346.5 ± 18.9^a	2508 ± 243^a	1444 ± 66^a	44541 ± 3909^a
PF/EX	$267.9 \pm 19.2^{b,c}$	2083 ± 131^a	$1177 \pm 98^{a,b}$	41783 ± 3374^a
CR/SED	232.9 ± 8.9^c	2446 ± 102^a	1137 ± 86^b	44490 ± 4311^a

Serum insulin-like growth factor-1 (IGF-1), insulin-like growth factor binding protein-3 (IGFBP-3), triiodothyronine (T_3) and thyroxine (T_4) levels were determined at the end of the 5-week experiment. Values are expressed as means ± SEM (pg/mL). n=20 (IGF-1), n=15 (IGFBP-3) and n=10 (T_3 and T_4) for each group. One-way ANOVA with Tukey's post hoc test was used for all between–group analyses; means not sharing a common letter are significantly different ($p < 0.05$).

A. Keratinocytes
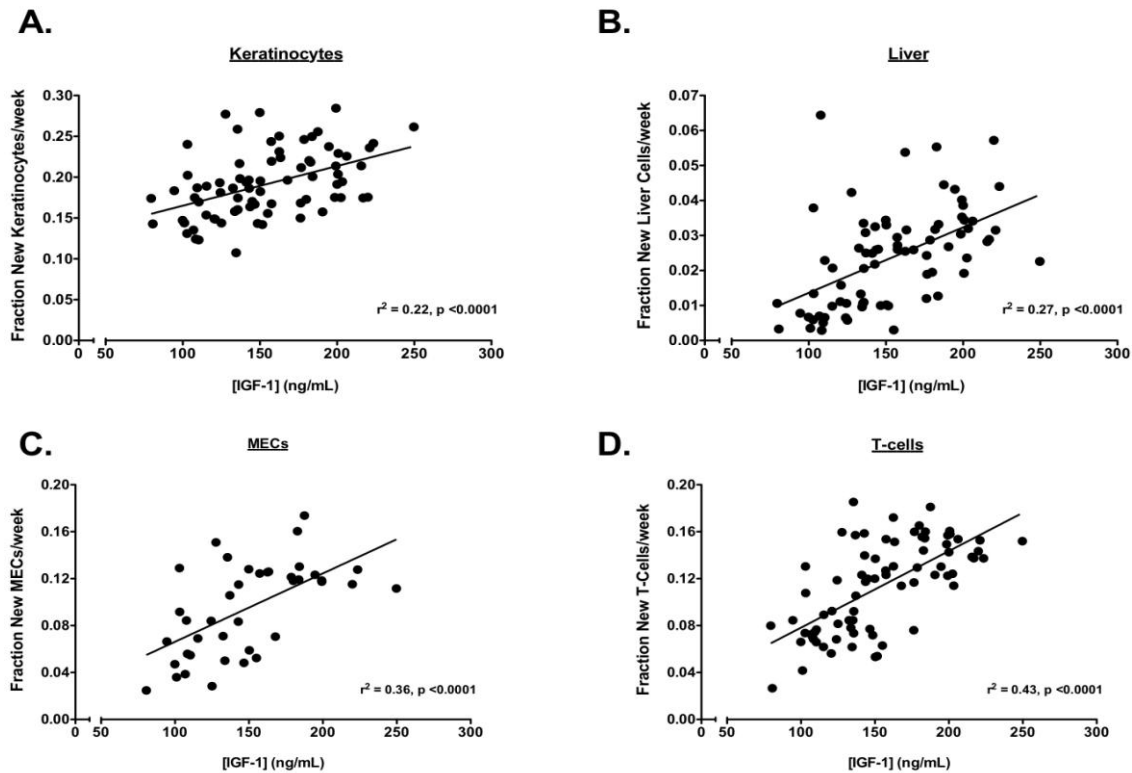
B. Liver

C. MECs

D. T-cells

Fig. 1.8 Correlation of serum IGF-1 and cell proliferation (Ex 2 and 3). Serum IGF-1 levels were determined at the end of the 5-week experiments and correlated to (A) keratinocyte, (B) liver, (C) mammary epithelial (Ex 2 only) and (D) splenic T-cell proliferation rates. Linear regression analyses were performed and coefficient of determination values (r^2) were determined. n = 40 to 80 mice per correlation.

In addition to circulating IGF-1 levels, IGF-1 signaling can be regulated by circulating IGFBP-1, which binds IGF-1 and inhibits its binding to IGF-1 receptors. In CR mice we observed an increase in circulating IGFBP-1 levels (Fig 1.9), which may be another hormonal mechanism for limiting IGF-1 signaling and reducing cell proliferation rates.
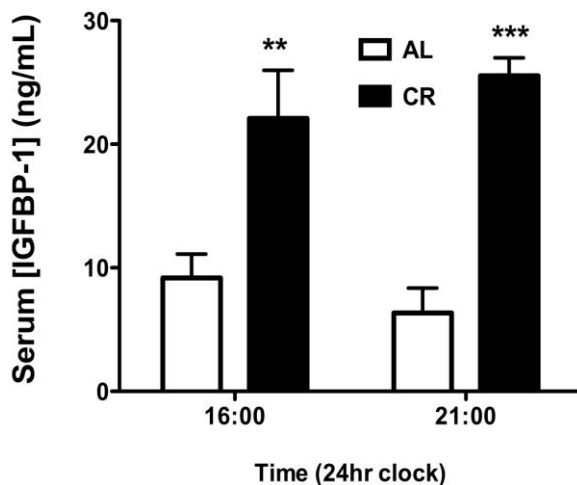
Fig. 1.9 Effect of calorie restriction on circulating IGFBP-1 concentration.
Male mice were fed ad libitum (AL) or calorie restricted by 30% (CR). Food was provided to CR mice once daily at 17:00. Mice were euthanized at 16:00, just before food was provided, or at 21:00, immediately post-prandial, blood was collected via cardiac puncture. Serum IGFBP-1 was assessed by ELISA. Values are expressed as means +/- SEM; n=10/group in two independent experiments. Two-way ANOVA with Bonferroni post hoc test was used for all between group analyses; ** ($P<0.01$), ***($P<0.001$) significantly different than time-matched AL fed controls.

The question then becomes, what are the mechanisms by which CR leads to reductions in IGF-1 and increases in IGFBP-1 levels. To begin to investigate this question we measured expression levels of IGF-1 and IGFBP-1 in the livers of AL fed and CR mice (Fig 1.10). Consistent with the circulating data, mRNA expression of IGFBP-1 was increased in CR mice livers at 21:00, while there was a trend for reduced IGF-1 mRNA expression in livers of CR mice at 16:00 as compared to AL fed controls.
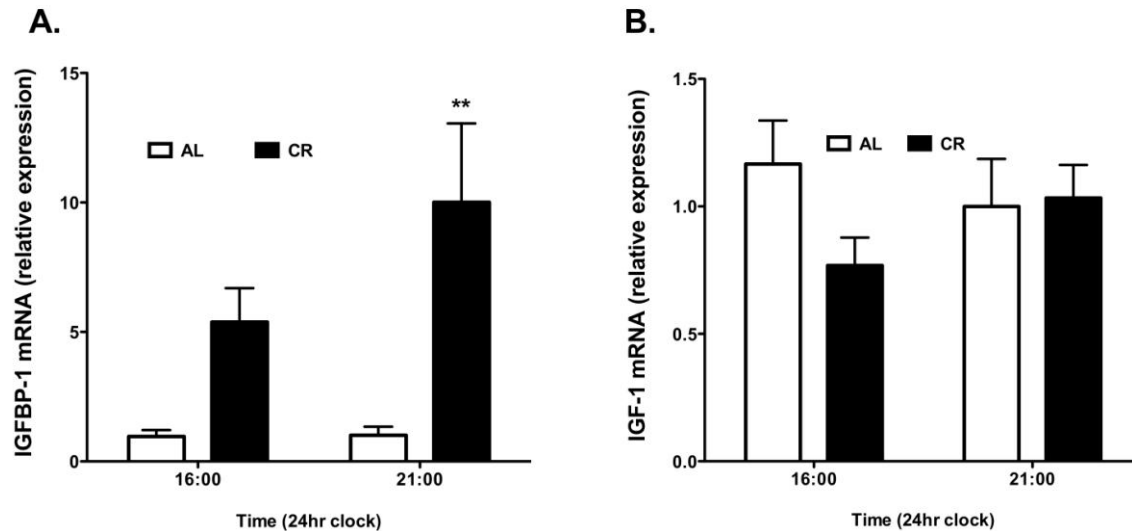

Fig. 1.10 Effect of calorie restriction on liver IGFBP-1 and IGF-1 mRNA expression. IGFBP-1 (A) and IGF-1 (B) gene expression was measured in livers of ad libitum fed (AL) and calorie restricted (CR) male mice at 16:00 or 21:00. Expression was normalized to 18sRNA and expressed relative to AL 21:00. Values are means +/- SEM (n=10/group) in two independent experiments. Two-way ANOVA with Bonferroni post hoc test was used for all between group analyses; ** (P<0.01) significantly different than time-matched AL fed controls.

Transcription of IGF-1 and IGFBP-1 is known to be regulated through the canonical GH/JAK2/STAT5 pathway, whereby growth hormone binding to GH receptors in the liver leads to JAK2 phosphorylation, which in-turn leads to STAT5 phosphorylation and translocation to the nucleus. In the nucleus STAT5 stimulates IGF-1 and represses IGFBP-1 transcription. Thus, we sought to determine if this signaling pathway was altered in CR mice. Previous work has shown that fasting and CR do not lead to decreases in GH secretion and, in fact, may induce increased GH secretion, however the downstream GH signaling has not been evaluated in CR mammals. Consistent with the GH data, there was no difference in JAK2 phosphorylation between CR and AL fed mice (Fig 1.11A), suggesting that CR-induced alterations in IGF-1 and IGFBP-1 transcription must be downstream of GH-receptor and JAK2. Next, we investigated the effect of CR on STAT5 phosphorylation. In contrast to JAK2 phosphorylation, CR led to a reduction in STAT5 phosphorylation that persisted throughout much of the day (Fig 1.11B). Thus it appears that the CR-induced regulation of IGF-1/IGFBP-1 transcription exists downstream of JAK2 and upstream of STAT5.

Interestingly, recent evidence suggests that FGF21 can be induced in response to fasting and can regulate IGF-1 and IGFBP-1 transcription via a SOCS mediated inhibition of STAT5, however the effect of CR on FGF21 levels has not been previously investigated.

Consistent with the fasting data, CR leads to a daily transient increase in FGF21 mRNA and circulating protein levels beginning immediately post-prandial (Fig 1.12). While further work needs to be done, these data suggest that CR may lead to reduced IGF-1 signaling, reduced cell proliferation rates and increased lifespan through FGF21 signaling.
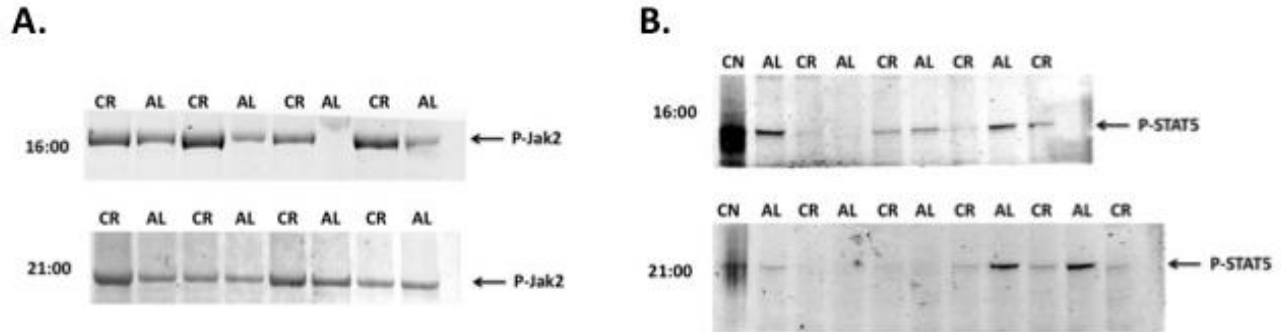

Fig. 1.11 Effect of calorie restriction on JAK2 and STAT5 phosphorylation. JAK2 (A) and STAT5 (B) protein phosphorylation was measured via SDS-PAGE/Western blot in livers of ad libitum fed (AL) and calorie restricted (CR) male mice at 16:00 or 21:00.
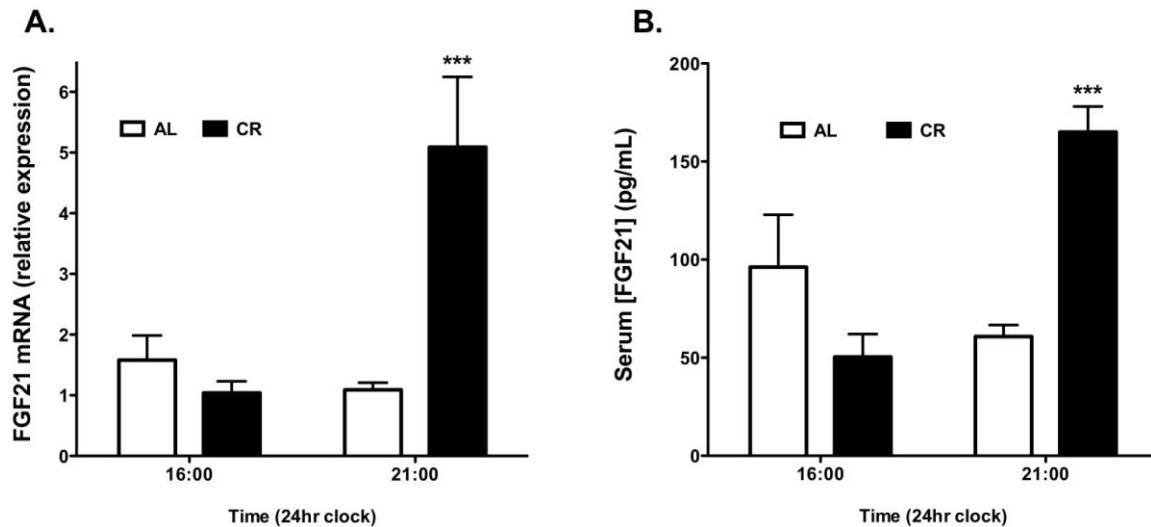
Fig. 1.12 Effect of calorie restriction on FGF21 liver mRNA expression and circulating serum concentration. Male mice were fed ad libitum (AL) or calorie restricted by 30% (CR). Food was provided to CR mice once daily at 17:00. Mice were euthanized at 16:00, just before food was provided, or at 21:00, immediately post-prandial, blood was collected via cardiac puncture and liver was dissected and snap frozen. Liver FGF21 mRNA expression was normalized to 18sRNA and expressed relative to AL 21:00 (A). Serum FGF21 was assessed by ELISA (B). Values are expressed as means +/- SEM; n=10/group in two independent experiments. Two-way ANOVA with Bonferroni post hoc test was used for all between group analyses; ** (P<0.01), ***(P<0.001) significantly different than time-matched AL fed controls.

Chapter 4

Project 2: Calorie restriction increases fatty acid synthesis and whole body fat oxidation rates

Project 2: Rationale

Mice on CR regimens have been reported to exhibit increased expression of genes for fatty acid (FA) oxidation and decreased expression of genes for FA synthesis compared to *ad libitum* fed (AL) controls (Cao et al., 2001; Chen et al., 2008; Mulligan et al., 2008; Tsuchiya et al., 2004). Due to differential entry points into the electron transport chain, a metabolic shift from carbohydrate to FA oxidation may reduce the production of reactive oxygen species (ROS) (Guarente, 2008). A shift to FA oxidation thereby represents a potential mechanism for reduced oxidative damage, which has been proposed as a potential explanation for the health benefits of CR (Gredilla and Barja, 2005; Guarente, 2008; Merry, 2002; Sohal and Weindruch, 1996). It has also been proposed that reduced rates of FA synthesis may inhibit tumor formation (Mulligan et al., 2008; Tsuchiya et al., 2004). Thus, changes in macronutrient metabolism – specifically, FA oxidation or synthesis - may be an important metabolic mediator of the health benefits of CR.

A problem with the FA oxidation hypothesis, however, relates to a basic principle of energetics and macronutrient balance in whole organisms. At metabolic steady state, i.e., when body composition is stable, fuel selection must match dietary macronutrient composition (Flatt, 1995; Westerterp, 1993). In other words, the respiratory quotient (RQ) over each 24-hour period is usually identical to the net daily "food quotient" (FQ). Since the macronutrient composition of the diets fed to CR and AL mice are typically identical (FQ = 0.94) and total energy intake is lower on CR, neither relative nor absolute FA intake is elevated in CR animals. On the surface, these considerations argue against a role for increased FA oxidation rates as a signal mediating health benefits in CR mice.

Thus the goal of this study was to determine whole body FA oxidation and synthesis rates in CR and AL mice, using a combination of indirect calorimetry and stable isotope labeling, and to compare these metabolic changes to gene expression in white adipose tissue and liver.

Project 2: Results

Since the classic physiologic adaptations do not seem to fully account for the effects of CR on cell proliferation and longevity, we began to explore other adaptations that may influence cell proliferation, health and longevity. In addition to physiologic changes one of the most robust adaptations to CR is a dramatic change in feeding pattern. Mice fed AL maintained relatively constant energy intake throughout the day (0.69 ± 0.07 kcal/h; Fig. 2.1A). In contrast, CR mice given food each day at 6 PM consumed their entire daily allotment of food in approximately one hour, at an average rate of 8.7 kcal/h, followed by a nearly twenty-three hour daily absence of food energy intake (Fig. 2.1A). We were interested in how this pattern could influence fat metabolism, as increased fatty acid (FA) oxidation and decreased FA synthesis have been suggested to mediate reductions in ROS production and tumor formation, respectively. However, the effect of CR on FA oxidation and synthesis has not been systematically evaluated in mammals.
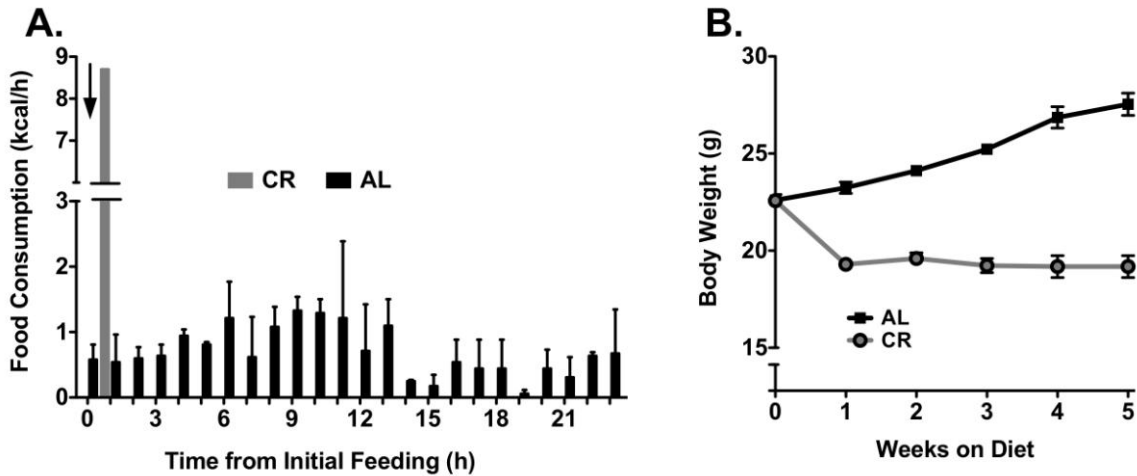
Figure 2.1. Altered feeding pattern and body weight in response to calorie restriction. Food was provided to CR and AL mice at 6 PM. Food consumption was recorded hourly, using food balance in metabolic cages, over the next twenty-four hours (A). Mice were weighed weekly (B). Values are means ± SEM for 6 mice per group. ↓ indicates time when daily food was provided to CR mice.

Fuel selection

To determine whether this pattern of feeding and fasting influences fuel selection throughout the day and to determine the duration of CR needed to induce a potential change, the RER was calculated after various durations of CR. We observed that CR immediately led to a dramatic change in the daily pattern of fuel selection (Fig. 2.2). While AL mice maintained an RER of approximately 0.9 - 1.0, representing primarily carbohydrate oxidation (Fig. 2.2A), as expected from the predominance of carbohydrate energy in the diet, CR mice exhibited two distinct phases of fuel selection each day (Fig. 2.2B-F).
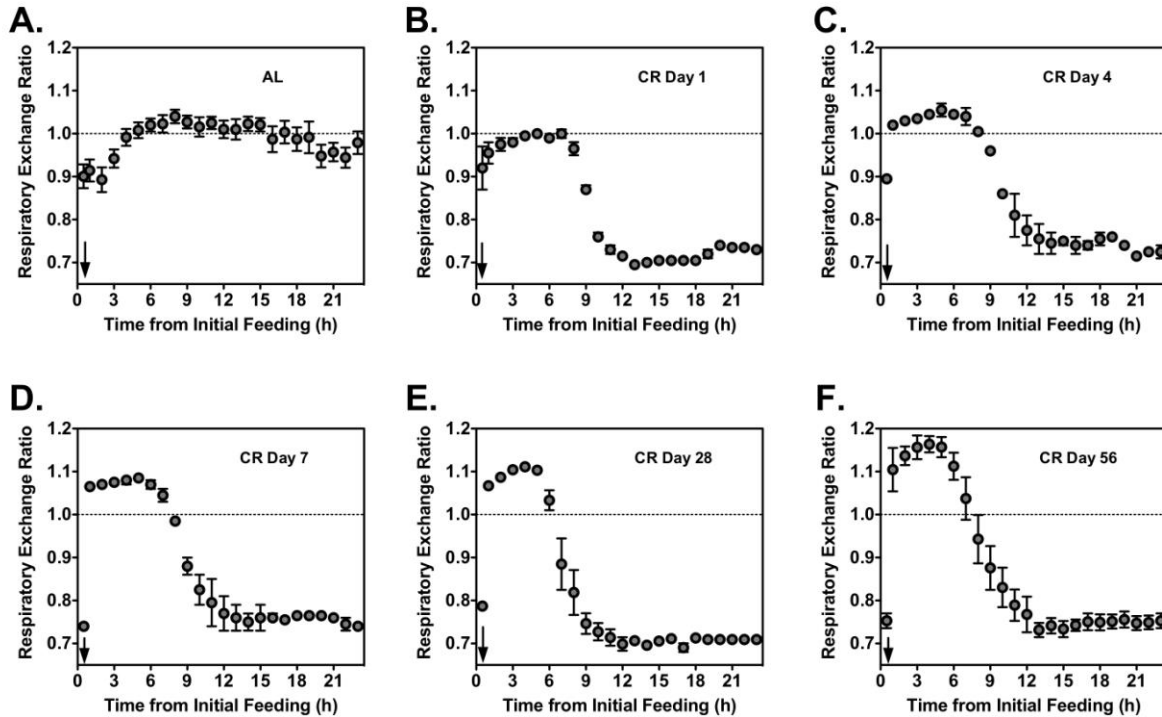
Figure 2.2. Time course of effect of CR on fuel selection pattern. AL fed mice were placed in metabolic cages before starting a CR diet (A) and again at day 1 (B), 4 (C), 7 (D), 28 (E) and 56 (F) of calorie restriction. Gas exchange was monitored for twenty-four hours each time mice were placed in metabolic cages. Respiratory exchange ratios (RER) were recorded six times per hour, then averaged to give RER for each hour. Values are means ± SEM for 4 mice per diet. ↓ indicates time when daily food was provided to CR mice.

In the first phase, immediately following provision of food, the CR mice exhibited RER values significantly greater than 1.0, which is generally taken to represent both carbohydrate oxidation and endogenous FA synthesis (33). In the second phase, beginning approximately six hours after food is presented each day, RER values rapidly fell to ~0.7, representing exclusively FA oxidation. This pattern became more pronounced over the course of the first 7 days of CR (Fig. 2.2A-D) and the circadian pattern continued for the remainder of the CR feeding regimen (Fig. 2.2F). Thus, it appears that CR rapidly and significantly alters whole body fuel selection, leading to a metabolically unusual pattern of both increased FA oxidation and increased FA synthesis, on the whole-body level.

Total fat oxidation in the whole body

The energy derived from FA oxidation was calculated from a combination of RER (Fig. 2.3A) and energy expenditure (Fig. 2.3B). Both the hourly and daily FA oxidation rates are shown (Fig. 2.3C & 2.3D; see calculations in "Methods"). CR mice oxidized almost four times as much fat as AL mice per day (3.3 ± 0.17 vs. 0.87 ± 0.13 kcal/d, or 367 ± 19 and 97 ± 14 mg/d, respectively; Fig. 2.3D). CR mice derived 37% of their daily energy needs from fat oxidation, compared to only 7% for AL mice (Fig. 2.3D). Fat content of the diet fed to both groups was identical (9.7% of metabolizable energy). These data demonstrate that CR dramatically increases the total energy derived directly from FA oxidation compared to AL controls.
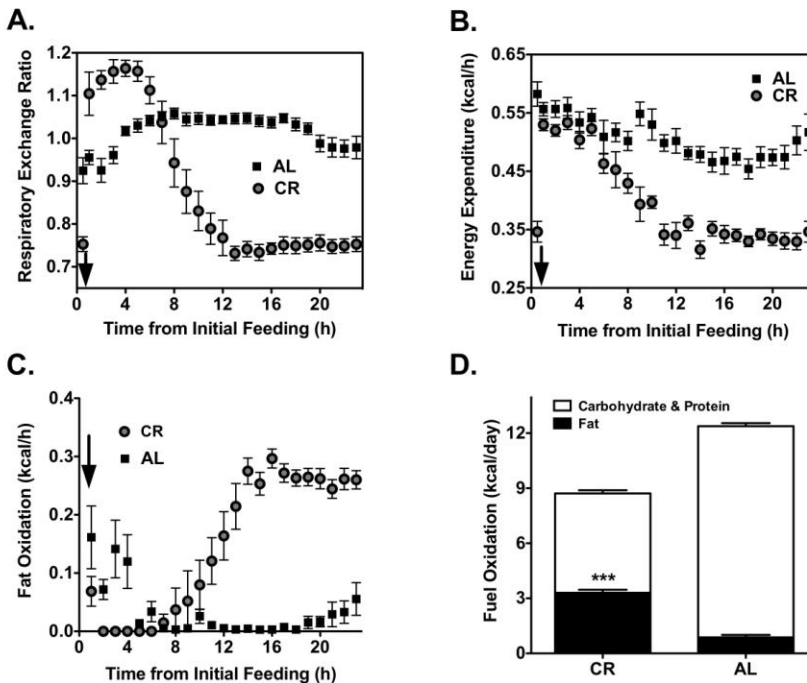

Figure 2.3. Higher rates of whole body fat oxidation in response to CR. Mice were adapted to CR or AL feeding for at least five weeks and were then placed in metabolic cages for twenty-four hours to determine respiratory exchange ratio (A) and energy expenditure (B) at each hour of the day. Hourly fat oxidation (C) and summed daily macronutrient oxidation (D) were calculated as described in "Methods". Values are means ± SEM (n=4-8 mice per group). ***P<0.001 difference from AL values, by ANOVA and Bonferroni *post hoc* test. ↓ indicates time when daily food was provided to CR mice.

CR mice oxidized over 300mg of fat per day, but they only consumed 92 mg of fat per day. Over the last 28 days of the 35 day CR regimen, CR mice were in neutral or positive energy balance (Fig. 2.1B). To establish whether increased FA synthesis can be detected biochemically and whether this synthesis can account for the higher FA oxidation rates in the whole body, we measured endogenous FA synthesis from ^2H incorporation into FAs in adipose and liver tissue after endogenous labeling with ^2H_2O.

Endogenous fatty acid synthesis

CR led to a 15.8-, 26.8-, 30- and 3.2-fold increase in the daily accumulation of endogenously synthesized palmitate, palmitoleate, stearate and oleate (mg/g adipose tissue), respectively, in adipose tissue relative to AL controls (Fig. 2.4A). In contrast, CR did not alter the accumulation of any endogenously synthesized FA in the liver (Fig. 2.4B). When extrapolated to total fat mass, CR mice synthesized and retained in adipose tissue and liver a total of 212 ± 13 mg FA/day as compared to 91 ± 9 mg/day for AL controls (Fig. 2.4C). Thus, endogenous FA synthesis that was retained in the adipose tissue and liver (212 mg/day) accounts for a substantial proportion of the whole body FA oxidation that was in excess of food intake (~270 mg/day). Endogenous FAs that were synthesized and directly oxidized, rather than mixing into the general adipose TG pool, would not accrue or be measured in adipose depots, and could account for the remainder of FA oxidation in excess of intake.
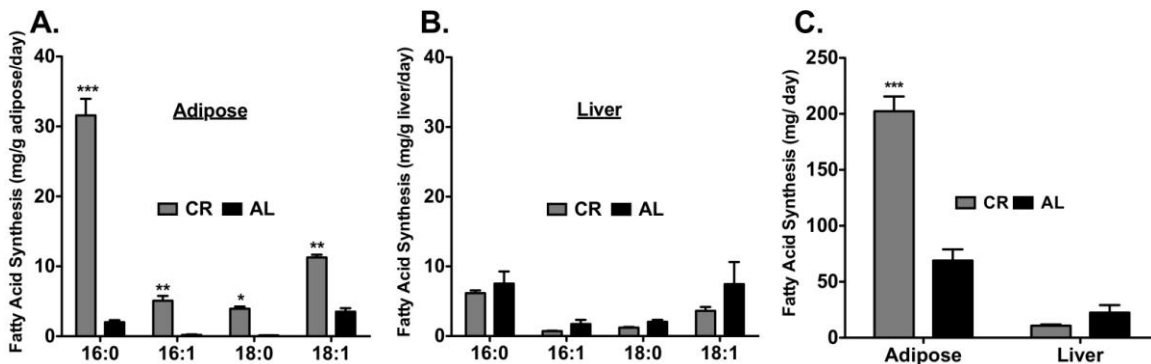

Figure 2.4. Effect of CR on FA synthesis in adipose tissue and liver. Daily FA synthesis was calculated for the 4 most abundant fatty acids: palmitate (16:0), palmitoleate (16:1), stearate (18:0) and oleate (18:1) in the subcutaneous adipose depot (A) and the liver (B) in CR and AL mice, values are expressed as milligrams per gram of tissue. Total adipose and liver FA that was synthesized during the period of ^2H_2O exposure and remained in the tissue was calculated from values in (A) and (B) and adjusted for estimated whole-body fat mass (as described in "Methods") and measured liver weights, respectively (C). Values are means ± SEM (n=8-14 mice per dietary group). ***$P<0.001$, **$P<0.01$, *$P<0.05$ difference from AL values, by ANOVA and Bonferroni *post hoc* test.

To determine whether the endogenous synthesis of FAs occurred in the adipose tissue directly or were transported to the adipose following synthesis in the liver, we measured ^2H incorporation into palmitate in adipose (subcutaneous and epididymal depots), liver and VLDL throughout the first day after exposure to ^2H_2O (Fig. 2.5). In the first 3 hours after food was provided, a 5-fold increase was observed in the accumulation of newly synthesized palmitate in the subcutaneous depot (14.1 ± 1.2 vs 2.7 ± 0.2 mg/depot) and a 2-fold increase was observed in the epididymal depot (2.9 ± 0.1 vs 1.5 ± 0.1 mg/depot) in CR mice relative to AL controls, after which accumulation occurred at a much slower rate (Fig. 2.5A & B). This circadian pattern of FA synthesis parallels the pattern seen in the RER data (Fig. 2.2). In contrast, there was no increase in the accumulation of endogenously synthesized palmitate in the liver at any time point (Fig. 2.5C) and only a small increase in plasma VLDL of CR mice six hours after feeding (Fig. 2.5D). The magnitude and timing of the accumulation of endogenously synthesized palmitate in the

adipose tissue relative to the liver suggests that adipose tissue is the major site of FA synthesis in response to CR.
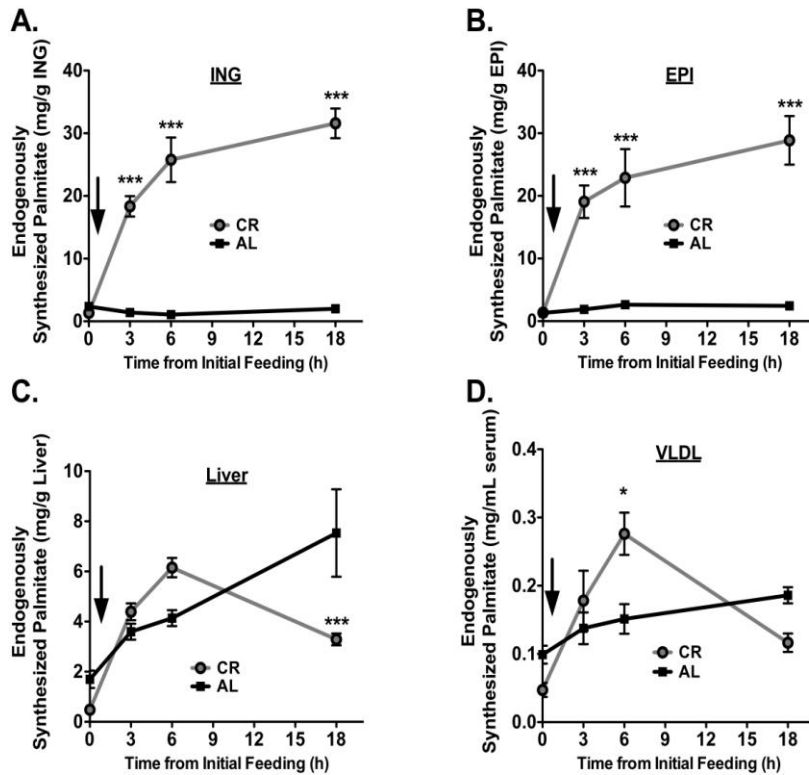
To more directly determine the site of endogenous FA synthesis, we measured the accumulation of endogenously synthesized FAs in the adipose in AL and CR mice in the presence and absence of the lipoprotein lipase inhibitor, Polaxamer 407 (P-407). Our hypothesis was that if FAs were synthesized in the liver and then exported to the adipose, then a lipoprotein lipase inhibitor would block the entry of FAs into the adipose and we would observe no increase in endogenously synthesized FAs in the adipose. On the other hand, if FAs were synthesized predominately in the adipose, during CR, then the lipoprotein lipase inhibitor would have no effect on accumulation of endogenously synthesized FAs in the adipose.

Consistent with our previous data, treatment with P-407 had no effect on the accumulation of endogenously synthesized FAs in the adipose. The fraction of newly synthesized FAs was increased equally in response to CR, in mice treated with saline and P-407 (Fig. 2.6A). Combined with our previous results, these data strongly suggest that the adipose tissue is the primary site of endogenous FA synthesis in calorie restricted mice.
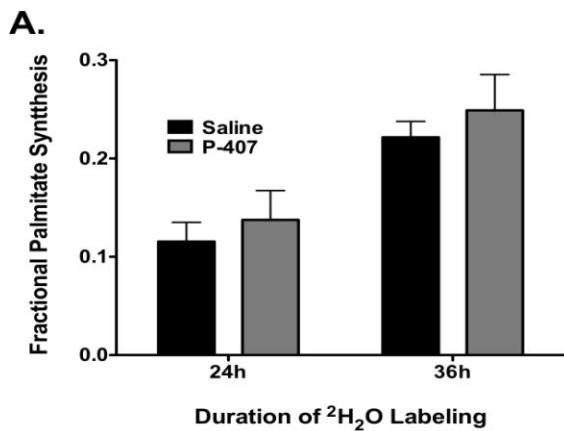
A.

Gene expression

To determine whether changes in gene expression reflected these striking changes in flux through FA synthetic pathways, we measured FAS, ACC1, SREBP-1 and PPARɣ mRNA levels in liver and adipose tissue at several time points throughout the day (Fig. 2.7 & Fig. 2.8). In the liver, FAS and ACC1 expression were widely variable throughout the day in CR mice, exhibiting 51- and 16-fold differences, respectively, between 6 PM and 9 PM (0.05 vs 2.55 RU and 0.09 vs 1.44 RU, respectively; Fig. 2.7A & B). In contrast, the expression of these genes varied little in AL controls over the same time period (1.00 vs 0.87 and 1.00 vs 0.72 RU; Fig. 6A & B). SREBP-1 followed a similar, but less dramatic, pattern between 6 PM and 9 PM in livers of CR mice (0.42 vs. 1.46 RU, respectively; Fig. 2.7C), and again, there was very little change in control values (1.00 vs 0.97 RU). There was a trend for decreased PPARɣ expression in CR livers at all time points (Fig. 2.7D).

Figure 2.7. Effect of CR on lipogenic gene expression in liver. FAS (A), ACC1 (B), SREBP-1 (C) and PPARɣ (D) gene expression was measured in the liver of CR and AL mice at 6 PM, 9 PM or 12 AM. Expression was normalized first to both GAPDH and b2m and then these normalized values were averaged. Values are shown relative to AL values measured at 6 PM (Relative Units; RU). Values are means ± SEM (n=6 mice per dietary group). ***$P<0.001$, **$P<0.01$, *$P<0.05$ difference from time-matched AL values, by ANOVA and Bonferroni *post hoc* test. ↓ indicates time when daily food was provided to CR mice.

In the adipose tissue, FAS and ACC1 expression were 4 and 3-fold higher, respectively, in CR than AL mice at 6 PM (3.97 vs 1.00 RU; 2.92 vs 1.00 RU; Fig. 2.8A & B), and this difference was maintained throughout the day. While there was no difference in SREBP-1 expression in adipose tissue of CR mice at 6 PM, there was a 2-fold increase at 9 PM (2.02 vs 1.03 RU; Fig. 2.8C). There was no difference in PPARY expression in the adipose between CR and AL mice at any time point (Fig. 2.8D).
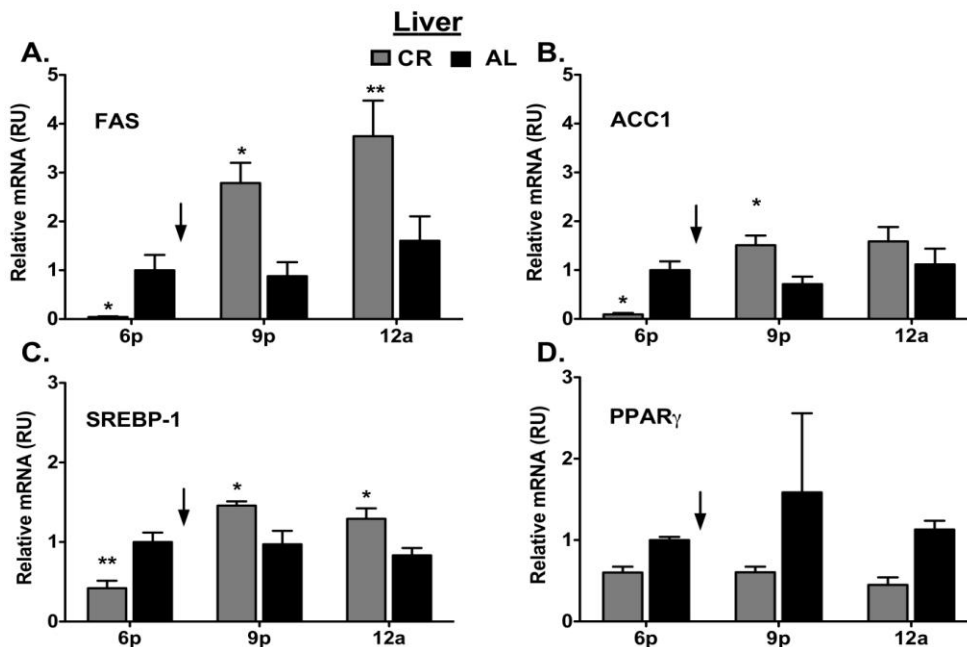
Figure 2.8.   Effect of CR on lipogenic gene expression in adipose tissue. FAS (A), ACC1 (B), SREBP-1 (C) and PPARγ (D) gene expression was measured in the adipose tissue of CR and AL mice at 6 PM, 9 PM or 12 AM. Expression was normalized first to both GAPDH and b2m and then these normalized values were averaged. Values are shown relative to AL values measured at 6 PM (Relative Units; RU). Values are means ± SEM (n=6 mice per dietary group). ***P<0.001 difference from time-matched AL values, by ANOVA and Bonferroni *post hoc* test. ↓ indicates time when daily food was provided to CR mice.
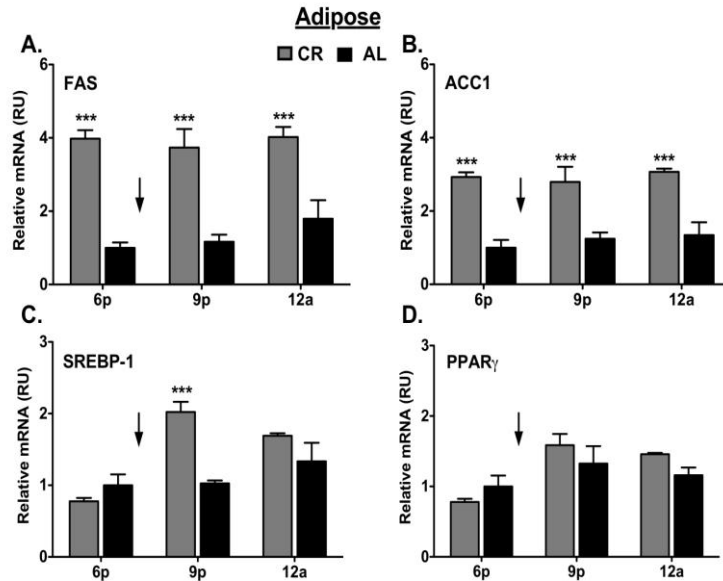
Palmitoleate levels in CR mice

It has recently been suggested that increased rates of FA synthesis in adipose tissue result in a particular accumulation of palmitoleate, which is an insulin sensitizing FA (5, 11), in adipose tissue as well as in serum (5, 19, 20, 32, 42, 43). To determine if there was an increase in palmitoleate accumulation in our CR mice, we used gas chromatography coupled to flame ionization detection to measure FA composition in the inguinal and epididymal adipose depots and in the liver. We found a significant increase in palmitate concentration in both adipose depots, but no increase in palmitoleate concentration in adipose tissue or the liver (Table 2.1).

Table 1. Fatty Acid Composition

TISSUE	DIET	Fatty Acids (% of total)				
		16:0	16:1	18:0	18:1	18:2
ING	CR	** 29 ± 2	** 7 ± 1	** 8 ± 1	** 42 ± 2	** 14 ± 1
	AL	22 ± 1	11 ± 1	2 ± 0.3	40 ± 1	24 ± 1
EPI	CR	** 31 ± 2	** 9 ± 1	** 7 ± 1	38 ± 1	** 16 ± 3
	AL	22 ± 1	10 ± 1	2 ± 0.1	39 ± 1	27 ± 1
LIVER	CR	32 ± 4	** 5 ± 1	10 ± 1	38 ± 3	15 ± 3
	AL	31 ± 2	6 ± 1	9 ± 2	38 ± 6	15 ± 2

Values are means ± SD. ING, inguinal fat depot; EPI, epididymal fat depot; CR, calorie restricted; AL, ad libitum fed; 16:0, palmitate; 16:1, palmitoleate; 18:0, stearate; 18:1, oleate; 18:2, linoleate. ** Significantly different than AL values ($P < 0.001$), by t-test.

Serum lipids in CR mice

To determine if changes in serum lipids reflect the pattern of feeding and fasting in CR mice, we measured serum NEFA and TG in CR and AL mice at 6 PM, before food was provided to CR mice, and 9 PM, 3h after food was provided. We found a significant increase in serum NEFA of CR mice compared to AL mice at 6 PM (0.87 ± .10 vs 0.45 ± .02 mEq/L; Table 2.2), but no difference at 9 PM. In contrast, for serum TG, we found a significant increase in CR mice at 9 PM (81.0 ± 3.8 vs 39.3 ± 1.9 mg/dL; Table 2.2), but no difference at 6 PM. These data are consistent with a prolonged fasting period in CR mice until 6 PM when food was provided and then a rapid feeding phase immediately following.

Table 2. Serum Lipids

	CR		AL	
	6 PM	9 PM	6 PM	9 PM
Serum NEFA (mEq/L)	0.87 ± .10 ***	0.33 ± .01	0.45 ± .02	0.47 ± .07
Serum TG (mg/dL)	34.5 ± 0.9 **	81.0 ± 3.8 ***	49.3 ± 1.5	39.3 ± 1.9

Values are means ± SEM. CR, calorie restricted; AL, ad libitum fed; NEFA, non-esterified fatty acids; TG, triglyceride. **P<0.01, ***P<0.001 difference from time – matched AL values.

Effect of feeding pattern

   From our data it appeared that the once daily feeding pattern was responsible for the increased FA oxidation and synthesis that we observed and may be responsible for some of the health and longevity effects reported under CR. However, it has been shown that CR leads to many of the same health benefits, even when animals are fed several times per day, raising questions about the role of the feeding pattern and FA metabolism on longevity.

   To determine the effect of feeding pattern on fuel selection and FA metabolism, we repeated our indirect calorimetry and stable isotope measures in CR mice that were fed 8 times per day (pellet-fed CR). Pellet-fed CR mice exhibited a remarkable metabolic flexibility with RER values rising to over 1.0 within 1.5 hours of food provision and then quickly returning to 0.7 in the next 1.5 hours (Fig. 2.9).
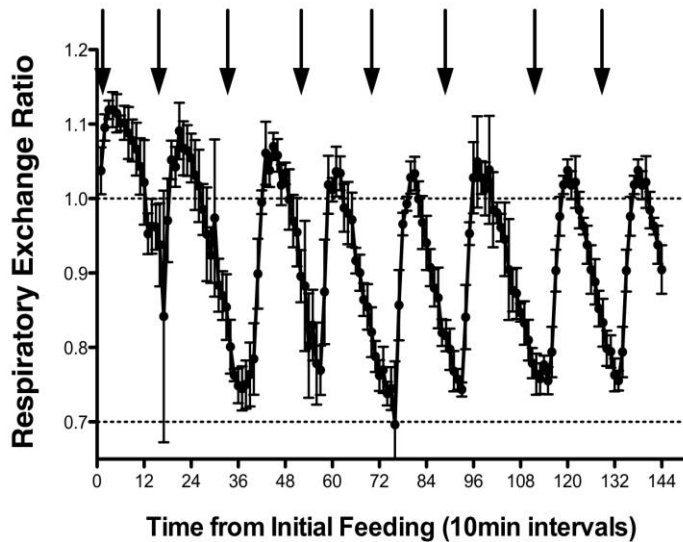
Figure 2.9. RER response to pellet feeding. Food was provided to CR mice every 3 hours (8 times/day). Values are means ± SEM for 6 mice per group. ↓ indicates time when daily food was provided to CR mice.

Despite the altered feeding pattern, pellet-fed CR had an equal increase in FA oxidation compared to once daily fed CR mice (Fig. 2.10A). In addition, this increase in FA oxidation was accompanied by a large increase in endogenous FA synthesis (Fig. 2.10B). Taken together these data suggest that calorie restriction, even when meal-fed, leads to increased FA oxidation and FA synthesis, that is associated with increased longevity.
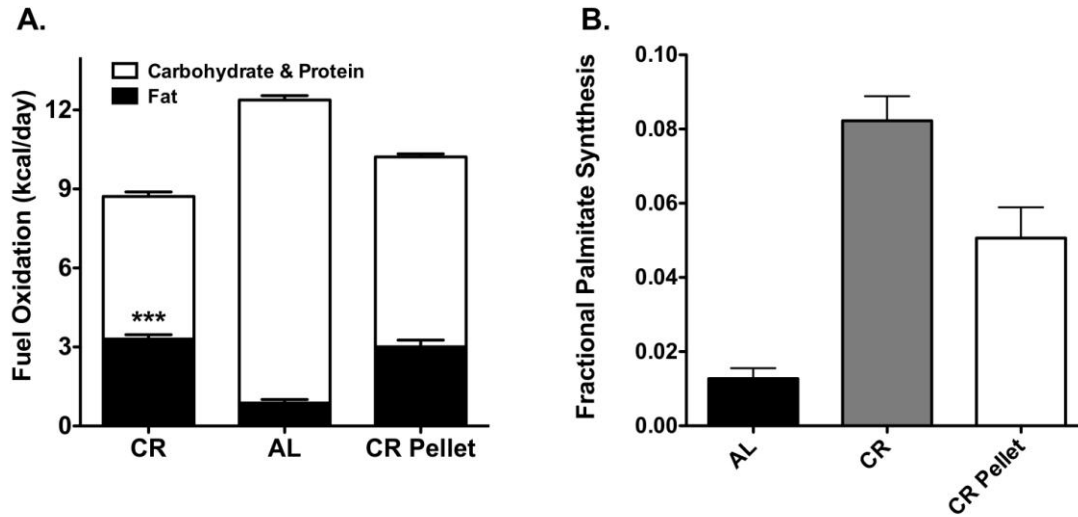
Fig. 2.10. Effect of pellet-feeding on FA oxidation and synthesis. Mice were fed AL, CR once daily (CR), or CR eight times per day (CR pellet) for 8 weeks. FA oxidation was calculated from 24h metabolic caging monitoring as previously described. At the end of 8 weeks of feeding, mice were labeled with 2H2O for 24h, inguinal fat was collected and palmitate synthesis was measured. Values are means +/- SEM (n=8/group). ***P<0.001 difference from AL-fed controls, by ANOVA and Bonferroni *post hoc* test.

Chapter 5

Project 3: The Effect of Sirt1 on the CR-Induced Reduction in Liver Cell Proliferation

Project 3: Rationale

The results from Project 1 suggest that CR leads to a reduction in "global" cell proliferation, in addition this effect is specific to interventions that are known to increase longevity. Recently, we have shown that "global" cell proliferation rates are reduced in Snell dwarf mice, another mouse model of increased longevity (Airlia Thompson, unpublished data). Taken together, these results suggest that reductions in cell proliferation rate, may be a biomarker of interventions that extend longevity. Thus, identifying molecular mediators that are activated in response to CR and mediate the effects on cell proliferation may provide both insights into the basic biology of aging, and therapeutic targets for age-related diseases.

Sir2, a class III histone deacetylase, has been suggested to be activated in response to CR and mediate its effects on longevity in yeast (Lin et al., 2000), flies (Rogina and Helfand, 2004) and worms (Wang and Tissenbaum, 2006). Sirt1, the mammalian homolog of the yeast sir2 gene, appears to be activated in response to CR in many tissues (Nisoli et al., 2005). Activation of Sirt1 leads to the modulation of activity of a number of transcription factors mediating cell proliferation, cell survival and fatty acid metabolism, including p53, FoxO, pgc-1α & PPARα (Saunders and Verdin, 2009). Indeed, overexpression of Sirt1 has been shown to inhibit cell proliferation and tumor growth in some *in vitro* systems (Fu et al., 2006) and *in vivo* models (Firestein et al., 2008). Thus it appears that Sirt1 could mediate the longevity effects of CR by modulating cell proliferation. However, the role of Sirt1 in mediating the CR-induced effects on cell proliferation *in vivo* have not been evaluated.

Accordingly the objective of this study was to determine if Sirt1 is necessary for the CR-induced reduction in liver cell proliferation in C57BL/6 mice. While whole body knockouts of Sirt1 can survive on an outbred background they are small, sterile and have severe abnormalities, making interpretation of studies difficult (Boily et al., 2009). In contrast, tissue specific knockouts seem to fair much better and can be used as models to study the role of Sirt1 on cell proliferation *in vivo* (Chen et al., 2008). Thus, we used liver specific Sirt1 knockout mice to determine the effect of local Sirt1 expression on cell proliferation. In addition, mice were treated with the putative Sirt1 activator, resveratrol, to determine the effect of Sirt1 activation on liver cell proliferation.

Project 3: Results

The effect of CR on Sirt1 protein expression in liver of rodents is not entirely clear. Several studies report increases in Sirt1 in rats and mice, while one study reports no change in mice, and another study reported a decrease in Sirt1 protein abundance in the liver in response to CR. It has been suggested that the disparate results could be due to different sampling times in the feeding cycle of CR mice. As described in Chapter 4, CR leads to a dramatic shift in feeding pattern. While AL fed mice nibble their food throughout the day, CR mice finish their entire daily allowance of food within one hour. This change is associated with altered expression of metabolic genes at different time points throughout

the day. Thus it is possible that Sirt1 protein abundance in a diurnal pattern in response to CR.

To determine if Sirt1 protein abundance in the liver is altered in a diurnal pattern in CR mice, we placed mice on AL or CR diets for 5wks. CR mice were fed once daily, at 17:00. On the last day of the study five AL and five CR mice were euthanized at 3 different time points; 21:00 (4 hrs after feeding), 9:00 (16 hours after feeding) and 16:00 (24 hours after feeding). While there was no significant difference in Sirt1 protein abundance in CR mice at any time during the day, there was a strong trend for increased Sirt1 at all time-points in CR mice (Fig 3.1).
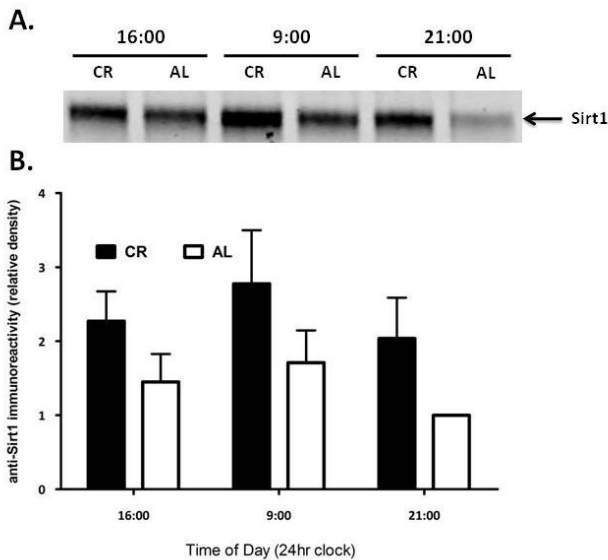
Fig 3.1. Effect of CR on Sirt1 protein abundance. Male mice were calorie restricted (CR) or fed ad libitum (AL) for 5 weeks. Food was provided daily at 17:00. Mice were euthanized at 16:00, 9:00 or 21:00 hours and liver abundance of Sirt1 protein in liver tissue was analyzed by western blot (A; representative blot). All blots were quantified by densitometry, normalized to beta-actin abundance and expressed relative to AL mice at 21:00. n=5/group.

After assessing the effect of CR on Sirt1 abundance in mouse liver, we wanted to assess the role of liver Sirt1 in mediating the CR-induced reduction in cell proliferation rate. To do this we used a liver specific Sirt1 knockout mouse model (LKO). These mice had loxp sites flanking the, catalytically necessary, exon 4 of the Sirt1 gene. Removal of this fragment was mediated by Cre recombinase, driven by the albumin promoter. We

Fig 3.2. Characterization of Sirt1LKO. Sirt1 mRNA was measured in livers of wild-type (WT) or Sirt1 liver-specific KOs (LKO) (A; n = 6 mice/group). Sirt1 protein in liver tissue was analyzed by western blot (B; representative blot).

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confirmed that mice expressing cre had dramatically reduced Sirt1 mRNA and reduced Sirt1 protein expression (Fig 3.2). We also observed that the small amount of Sirt1 protein migrated through the SDS-PAGE more quickly, indicating removal of the 5kD portion of exon 4.

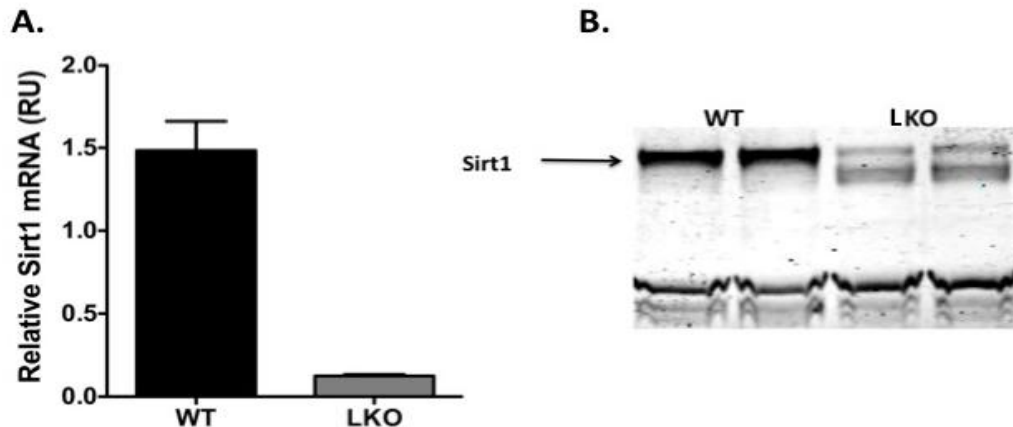**Fig 3.3**. **Effect of Sirt1 on CR-induced reduction in cell proliferation**. Mice were maintained on diets for 5 weeks and liver cell proliferation rates were measured during the last 3 weeks. Values are means ± SEM (n=4-6 mice/group). Two-way ANOVA with Bonferroni's post hoc was used for all between group analyses; means not sharing a common letter are significantly different (P< 0.001).
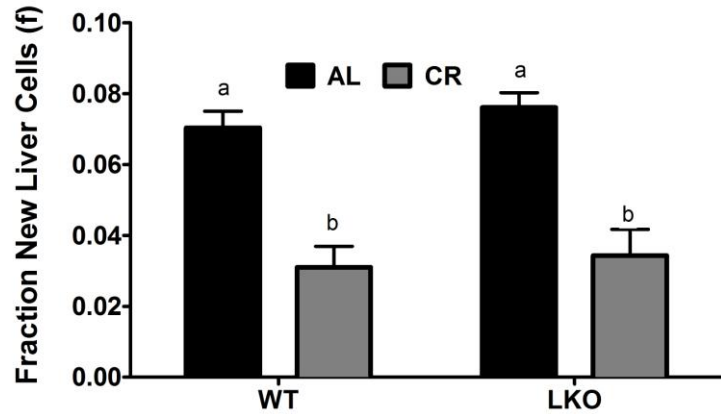
To determine if Sirt1 expression in the liver is necessary for the CR-induced reduction in liver cell proliferation we divided wild-type and Sirt1LKO mice into four groups; AL-fed wild-type (AL/WT), CR wild-type (CR/WT), AL-fed Sirt1LKO (AL/LKO), and CR Sirt1LKO (CR/LKO). Mice were fed their respective diets for 5 weeks and liver cell proliferation was assessed during the last 3 weeks. Consistent with previous studies, CR led to a significant reduction in liver cell proliferation in wild-type mice, however liver deletion of Sirt1 had no effect on the response to CR (Fig 3.3). Thus it appears that Sirt1 expression in the liver is not necessary for the CR-induced reduction in liver cell proliferation.
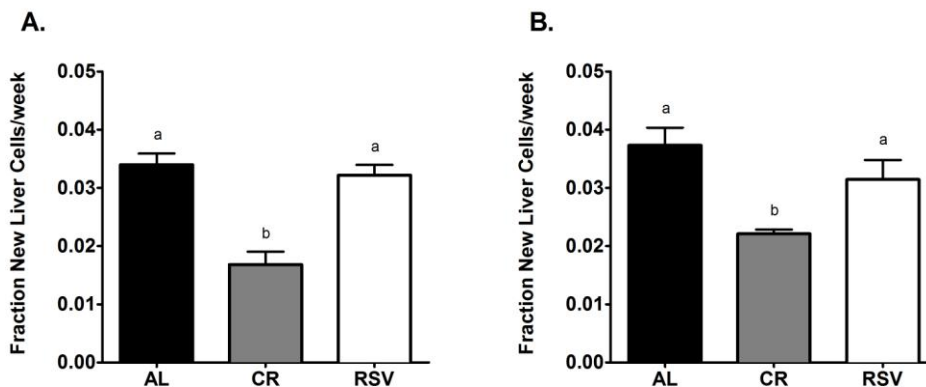
Fig 3.4. **Effect of RSV on CR-induced reduction in cell proliferation**. Mice were fed ad libitum (AL), calorie restriction (CR), or ad libitum + 0.1% resveratrol (w/w) (RSV). Mice were maintained on diets for 8 weeks (A) or 6 months (B) and liver cell proliferation rates were measured during the last 8 weeks. Values are means ± SE for 6 mice/group. One-way ANOVA with Tukey's post hoc test was used for all between-group analyses; means not sharing a common letter are significantly different (P< 0.05).

While Sirt1 expression in the liver is not necessary for the CR-induced cell proliferation effects, perhaps activation of Sirt1 signaling pathways could be sufficient to mimic the CR effects. Resveratrol, while apparently not a direct Sirt1 activator, has been suggested to mimic CR effects in a Sirt1-dependent manner. To determine if resveratrol could mimic the CR-induced reduction in liver cell proliferation we dived 12wk old male C57BL/6 mice into 3 groups; AL-fed AIN-93M, AL-fed AIN-93M + 1g/kg RSV and CR AIN-93M. Mice were placed on respective diet for 8 weeks or 6 months and cell proliferation was assessed the final 8 weeks of each group. We found that resveratrol was unable to mimic the effects of CR. While CR led to a significant reduction in liver cell proliferation at both time points compared to AL controls, resveratrol treatment had no effect on liver cell proliferation at either time point (Fig 3.4).

Chapter 6
Discussion

Discussion:

The goal of this thesis work was to determine the role of physiologic, metabolic and molecular adaptations to CR on biomarkers of longevity. The guiding hypothesis was that this work would provide insight into the mechanisms by which reduced caloric intake is translated into a program of delayed onset of age-related diseases and increased maximal lifespan. The data presented here demonstrate that CR leads to significant reductions in cell proliferation rates in keratinocytes, liver cells, MECs and splenic T-cells. These reductions in cell proliferation rates cannot be accounted for by reductions in food intake, energy expenditure, fat mass or body weight. In addition, the CR-induced reduction in cell proliferation is not dependent on Sirt1 expression, nor can it be mimicked by resveratrol treatment. However, reductions in cell proliferation rates were associated with a CR-induced increase in whole body FA oxidation and have a strong negative correlation with circulating IGF-1 levels. Taken together these results suggest that increased reliance on FA oxidation and reductions in IGF-1 signaling may be metabolic pathways that mediate the effects of CR on aging and longevity. These results also point to molecular mediators that can translate changes in substrate utilization to regulation of growth factor signaling as potential regulatory nodes necessary for the CR-induced effects on cell proliferation and longevity.

The work was divided into three projects. The aim of the first project was to mimic physiological adaptations to CR through changes in ambient housing temperature and voluntary wheel running in order to determine the effects of food intake, energy expenditure, percent body fat and body weight on "global" cell proliferation rates (keratinocyte, liver cell, MEC and splenic T-cell proliferation) in young C57BL/6 mice. The aim of the second project was to determine whole body FA oxidation and synthesis rates in CR and AL mice, using a combination of indirect calorimetry and stable isotope labeling, and to compare these metabolic changes to gene expression in white adipose tissue and liver. The aim of the third project was to determine if Sirt1 activation is necessary or sufficient for the CR-induced reduction in liver cell proliferation in C57BL/6 mice.

Project 1

The experiments presented in Project 1 demonstrate that reductions in food intake, energy expenditure, percent body fat or body weight cannot account for the decrease in cell proliferation rates in response to CR (results summarized in Fig 1.7). The unifying feature associated with lower cell proliferation rates among all experiments was restriction of food intake below the level that the animals would have selected if given ad libitum access to food. Potential signals mediating these effects were explored and reductions in serum IGF-1 were associated with decreased cell proliferation rates. These data are also consistent with the hypothesis that reduced cell proliferation is a biomarker that correlates with interventions that increase longevity.

In all experiments, a ~20% reduction in caloric intake led to reduced cell proliferation rates in keratinocytes, liver cells, MECs and splenic T-cells, as compared to ad

libitum-fed controls (Figs 1.2, 1.4 & 1.6). These data are consistent with previous work showing CR-induced reductions in cell proliferation rates in many tissues. Previous work using ^{3}H-thymidine incorporation, demonstrated decreased cell proliferation rates in the small intestine, colon, bladder, dermis, mammary tissue and esophagus in response to 25% CR in Swiss Webster mice (Lok et al., 1988) and decreased cell proliferation of primary hepatocytes from calorie restricted F344 rats (Shaddock et al., 1996). Work from our laboratory has confirmed these results in several tissues, based on ^{2}H incorporation from heavy water into the deoxyribose moiety of purine deoxyribonucleosides in DNA, and extended the findings to include lower proliferation rates of keratinocytes and splenic T-cells in CR mice (25, 41). Taken together with the present findings, it is clear that 20-40% CR leads to a robust decrease in cell proliferation rates across many tissues. However, until now, the physiological adaptations to CR that initiate this reduction in cell proliferation rate had not been systematically evaluated.

The overall goal of this work was to determine the effects of the physiological adaptations associated with CR on cell proliferation rates, independent of classical CR. The first adaptation that we examined was food intake. In two separate experiments we found that decreased food intake (Experiment 1) and increased food intake (Experiment 2), while maintaining the same relative body weight as ad libitum-fed sedentary controls, had no effect on cell proliferation rates.

While food intake has been previously suggested to mediate the longevity effects of CR (18, 29, 43), our data on cell proliferation rates are consistent with previous work on spontaneous tumor formation. Huffman et al. (26) demonstrated that prostate tumor development was reduced in CR mice housed at 23°C, but not reduced in ad libitum-fed mice housed at 28°C, despite the fact that the latter group consumed the same amount of food as the CR group at 23°C. In conjunction with the present data, it appears that CR-induced reductions in cell proliferation rates and tumor promotion are not mediated by decreased food intake per se.

The second CR-associated physiological adaptation that we examined was whole body energy expenditure. In two separate experiments we demonstrated that neither decreases (Experiment 1) nor increases (Experiment 2) in energy expenditure had any effect on cell proliferation rates. It should be noted that energy expenditure was estimated by energy balance, not directly measured through oxygen consumption. In both Experiment 1 and 2, there were significant changes in food intake, yet no change in body weight, implying that there were also changes in energy expenditure or food absorption. Neither increased housing temperature nor increased wheel running have been reported to affect food absorption, thus we believe changes in energy expenditure are most likely to account for body weight maintenance in these settings of altered food intake.

While reduced energy expenditure has been proposed to mediate the longevity effects of CR, our data on cell proliferation rates are consistent with the current views of energy expenditure and longevity. The "rate of living theory" proposed to explain CR-induced longevity (*Sacher*, 1977) stated that CR animals have reduced metabolic rates and thus longer lifespan. More recent work has suggested that while whole body energy expenditure does decrease in CR rodents (McCarter and Palmer, 1992), metabolic rate is

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actually increased in CR rodents when corrected for body weight (30, 32, 33). Our data here show that decreases in whole body energy expenditure cannot reproduce the cell proliferation rate lowering effects of CR. Thus, it appears that decreased energy expenditure, either at the level of the whole body or corrected for lean body weight, does not mediate either the cell proliferation or longevity effects of CR.

The third CR-associated physiological adaptation that we examined was percent body fat. In Experiment 2, a reduction in percent body fat had no effect on cell proliferation rates. These findings on cell proliferation rates are consistent with direct work on percent body fat and longevity. Since the first suggestion that decreased body fat could mediate the health benefits of CR (Berg and Simms, 1960), several studies have tested the role of percent body fat on longevity, however none have shown a direct role. Bertrand et al. (Bertrand et al., 1980) showed no correlation between percent body fat and longevity in ad libitum-fed F344 rats, and a positive correlation in those on CR. Testing the hypothesis more directly, Harrison et al. (Harrison et al., 1984) studied lean and ob/ob mice fed ad libitum or CR. They reported that ob/ob mice on CR lived longer than lean ad libitum-fed mice and as long as lean CR mice, despite having a much higher percent body fat than either lean ad libitum-fed or CR mice, again dissociating percent body fat and longevity. Exercise and longevity studies have also shown a dissociation between percent body fat and longevity, where exercised rats have decreased percent body fat, yet no increase in maximal lifespan (Holloszy, 1992). Accordingly, it appears that the effects of CR on cell proliferation rates and longevity are not mediated by reductions in percent body fat.

The final CR-associated physiological adaptation that we examined was body weight. From our first two experiments, it appeared that reduced body weight relative to ad libitum-fed sedentary controls was associated with lower cell proliferation rates. To determine the effect of reduced body weight relative to ad libitum-fed sedentary controls, independent of food restriction, we used male mice provided free access to food and a running wheel. In Experiment 3, AL/EX male mice weighed significantly (12.4%) less than AL/SED control mice (Fig 1.5A), yet there were no differences in cell proliferation rates between these two groups for any cell type analyzed (Fig 1.6). We have previously found that 15% CR results in a 13% reduction in body weight and decreased cell proliferation rates relative to ad libitum-fed controls (unpublished data). Therefore, the lack of change in cell proliferation rates observed in AL/EX male mice cannot be attributed to their reduced body weight relative to AL/SED controls. Moreover, in Experiment 2, CR/SED mice had lower cell proliferation rates than PF/EX mice in all cell types analyzed despite being matched for body weight (Fig 1.3B and Fig 1.4), suggesting that body weight could not fully account for the lower rates of cell proliferation observed in CR/SED mice. These data suggest that lower body weight, relative to ad libitum-fed sedentary controls, is not sufficient to account for the lower rates of cell proliferation in CR animals.

Our data on cell proliferation rates and body weight are consistent with previous work evaluating longevity in exercised rats. Since the original CR and longevity work by McCay (McCay et al., 1989), there has been the notion that decreased body weight, relative to ad libitum-fed controls, mediates the longevity effects of CR. Consistent with this hypothesis, several studies have shown that exercise leads to decreased tumor formation

and markers of cell proliferation in the context of restricted feeding (Zhu et al., 2008; Zhu et al., 2009). However, studies investigating the effect of voluntary exercise have shown that while exercise dramatically reduces body weight in rats, it has no effect on maximal lifespan (Holloszy, 1992). Thus, it appears that reduced body weight per se is not sufficient to account for the lower rates of cell proliferation or increased longevity in CR animals. Exercise in the setting of food restriction, however, may lead to improved health.

In contrast to these physiological adaptations to CR, reductions in circulating IGF-1 were associated with decreased cell proliferation rates. In Experiment 2, circulating IGF-1 levels were lower in both CR/SED and PF/EX mice as compared to AL/SED controls (Table 1.1), and in Experiments 2 and 3, IGF-1 levels correlated strongly with cell proliferation rates across all mice in all cell types (Fig 1.8). These data are consistent with many other studies reporting decreased IGF-1 in CR mice (Baur et al., 2006; Berryman et al., 2008, Comfort, 1964). Interestingly, many of the genetic mouse models of increased longevity have disruptions in the GH/IGF-1 axis, with reduced IGF-1 concentration or signaling (de Magalhaes and Faragher, 2008). The mechanisms connecting IGF-1 and longevity, however, have not been established experimentally. One hypothesis is that reduced IGF-1 signaling could increase longevity by reducing cell proliferation rates in younger animals, which would both preserve the replicative capacity of cells and inhibit tumor growth as animals age. Consistent with this, recent work from our lab has shown that Snell mice, which are long-lived, have reduced cell proliferation rates compared to their wild-type littermates (Unpublished data; Airlia Thompson). In future studies, it will be informative to assess cell proliferation rates in other models of altered longevity and to identify adaptations to CR that reduce IGF-1 signaling.

These results are consistent with the hypothesis that reductions in cell proliferation rates could serve as a rapidly responsive biomarker of interventions that extend lifespan. Cell proliferation rates have been suggested to mediate the longevity effects of CR, by preserving replicative capacity and reducing tumor promotion (de Magalhaes and Faragher, 2008). Cell proliferation rates are reduced rapidly and persistently in many tissues in response to CR (Hsieh et al., 2005; Lok et al., 1988; Shaddock et al., 1996) and alternate day fasting (Varady et al., 2007), two dietary regimens that extend maximal lifespan. In contrast, several progeroid diseases are associated with increased rates of cell proliferation in early life (Bridger and Kill, 2004; Faragher et al., 1993; Halaschek-Wiener and Brooks-Wilson, 2007; Kill et al., 1994). In the present study, we show that the cell proliferation rate response is specific to interventions that have been elsewhere demonstrated to extend maximal lifespan. Indeed, there were no changes in cell proliferation rates in response to increased housing temperature and voluntary wheel running, two interventions that mimic physiological adaptations to CR, but cannot reproduce its health and longevity effects in isolation (Holloszy, 1992; Huffman et al., 2007). We also confirmed the rapid response of cell proliferation rates to CR and identified a correlation between cell proliferation rates and circulating IGF-1.

If reductions in food intake, energy expenditure, percent body fat and body weight are insufficient to account for the decreased cell proliferation rates and the increased longevity in CR animals, what might be the adaptations that signal or mediate these effects?

A unifying feature of the interventions that reduced cell proliferation rates here was that food intake was restricted below levels that would have been selected by the animals if allowed ad libitum access to food. It is not clear how restriction below ad libitum intake could serve as a signal for cellular or biochemical events. However, one possibility is the striking alteration in the pattern of food intake that is observed as an adaptation to CR (Bruss et al., 2010). In contrast to the relatively constant nibbling pattern of ad libitum-fed mice, CR mice consume their total daily allotment of food within several hours (Bruss et al., 2010). We hypothesized that this pattern of food intake could lead to a dramatic change in fatty acid metabolism and may reduce the production of reactive oxygen species (ROS) (Guarente, 2008) . Thus, changes in macronutrient metabolism – specifically, FA oxidation or synthesis - may be an important metabolic mediator of the health benefits of CR. Thus the goal of Project 2 was to determine whole body FA oxidation and synthesis rates in CR and AL mice.

Project 2

The experiments presented in Project 2 demonstrate that CR, administered by daily feeding, leads to a unique pattern of fuel selection in mice, characterized by a brief period of markedly increased endogenous FA synthesis in adipose tissue followed by a prolonged period of elevated whole body FA oxidation. This pattern is consistent with previous reports demonstrating diurnal changes in RER in calorie restricted rats (Duffy et al., 1989; Masoro et al., 1992). Here we confirm these metabolic effects with indirect calorimetry, stable isotope labeling, FA composition and to some extent gene expression, and demonstrate that the pattern is induced rapidly and persists as long as the mouse remains calorie restricted.

The metabolic adaptations to CR described here have implications for current hypotheses about the mechanisms mediating the effects of CR. In addition, our data provide insight into a time dependence of experimental designs for studying genetic adaptations to CR.

The data confirm the view that the absolute amount of whole-body FA oxidation is higher in CR mice than AL controls (Guarente, 2008; Lopez-Lluch et al., 2008; Tsuchiya et al., 2004). This is, in fact, a remarkable result, in context of classic principles of whole-body macronutrient balances (Flatt, 1995; Westerterp, 1993). After an initial 1-2 week period of weight loss, CR mice reestablish a state of energy balance in which fat mass is preserved or even increased. The energy intake from fat in CR mice is ~92 mg/day (2.3g food/day x 4% fat in diet by weight), yet we measured by indirect calorimetry 367mg fat oxidized/day in CR mice. The majority of fat oxidation occurred during the 12 hours after feeding when mice were oxidizing exclusively fat (RER= 0.7, Fig. 3). While there are limitations to estimating fat oxidation from indirect calorimetry (Wolfe, 2005), given that it only accounts for net changes, in this case, where RER values are 0.7 for so long, alternate interpretations are unlikely. Thus, the question remains, if CR mice oxidize a much greater quantity of FAs per day than dietary intake but remain weight stable, where does the FA substrate come from?

Quantitatively, we demonstrate that the increase in FA oxidation is almost entirely accounted for by an increase in endogenous FA synthesis in CR mice. Some previous reports had suggested that CR reduces endogenous FA synthesis, based on FAS and/or ACC1 expression in the liver (Cao et al., 2001; Chen et al., 2008; Mulligan et al., 2008; Tsuchiya et al., 2004). Our stable isotope based measurements of flux through the FA synthetic pathway support the opposite conclusion. It should be noted that our calculations for endogenous FA synthesis are based on literature values for percent body fat in CR and AL from similarly aged and restricted C57BL/6 male mice. The literature values for percent body fat range from 15-30% for CR mice and 22-42% for AL mice. For a conservative estimate of FA synthesis in CR mice we used the lower value of 15%. For the estimation of AL FA synthesis we used the median value of 32%. In addition, any endogenously synthesized FAs that are oxidized rather than being stored in adipose tissue will not be detected by measurements on adipose tissue triglycerides. Thus, our measurements represent a lower bound estimate of FA synthesis in CR mice, yet accounted for the great majority of whole body FA oxidation in excess of dietary fat intake.

Adipose tissue, as opposed to the liver, may be the primary site for whole body endogenous FA synthesis in CR mice. Within three hours of feeding there was an accumulation of 14mg of endogenously synthesized palmitate in the subcutaneous depot and another 3mg in the epididymal tissue. In contrast, only 4mg of endogenously synthesized palmitate accumulated in the liver over the same time period. In addition, in the first three hours post-feeding, endogenously synthesized palmitate accumulated in the subcutaneous depot five times faster in CR mice than AL controls, whereas in the liver and VLDL there was no difference in newly synthesized FA accumulation between these groups (Fig 2.5C & D). It is possible, though quantitatively unlikely, that a dramatic increase in VLDL-TG delivery to adipose tissue in CR mice could account for the accumulation of endogenously synthesized fatty acids in this tissue. This possibility may warrant further investigation. Glucose is likely the predominant substrate for FA synthesis in the adipose tissue. Consistent with adipose playing a role in FA synthesis, Wetter et al. (Wetter et al., 1999) demonstrated that glucose uptake is increased in adipose tissue of calorie restricted rats.

Several recent reports (Cao et al., 2008; Kunesova et al., 2006; Kunesova et al., 2002; Parks et al., 2008; Volek et al., 2009; Watkins et al., 2002) have proposed that palmitoleate (16:1) accumulates in adipose tissue or serum during times of increased FA synthesis, and that increased palmitoleate content is a marker of FA synthesis in adipose tissue. While we saw an increase in palmitate (16:0) content in adipose of CR mice, we did not observe an increase in palmitoleate content. Consistent with these data, the rate of palmitate synthesis was six times higher than the rate of palmitoleate in adipose tissue of CR mice (Fig. 4A). To our knowledge, this is the first report to compare palmitate and palmitoleate synthesis rates under conditions of increased FA synthesis. Palmitoleate accumulation does not appear to be a universal marker of endogenous FA synthesis. It is possible that the increase in palmitoleate accumulation observed in previous reports was specifically related to a change in diet, since animals were switched from a high fat diet to a high carbohydrate diet, which may alter SCD-1 activity (Bassilian et al., 2002; Chong et al., 2008).

Liver FAS, ACC1 gene expression at a single time point did not necessarily reflect the expression pattern in CR mice throughout the day. This previously unreported circadian pattern may have implications for future genomic studies in CR mice. We observed that at 6 PM, just before food was provided, FAS and ACC1 expression were 20- & 10-fold lower in CR than AL mice (Fig 2.6A & B). This is the same trend reported in several previous publications (Cao et al., 2001; Chen et al., 2008; Mulligan et al., 2008; Tsuchiya et al., 2004) and has led to the conclusion that FA synthesis is decreased in CR. However, immediately after food was provided, FAS expression increased 50-fold in CR mice, leading to values nearly 3-fold higher than AL controls (Fig 2.6A). In the same period, ACC1 expression increased 16-fold in CR mice, leading to values 2-fold higher than the AL group (Fig 2.6B). This increase in FAS and ACC1 expression correlated with a relatively small, but statistically significant increase in FA synthesis in the livers of CR mice. Unfortunately, most studies investigating the changes in gene expression patterns in response to CR have measured expression at one time point, and in some cases animals were fasted overnight (Cao et al., 2001; Mulligan et al., 2008), masking the time-dependent gene expression pattern. Furthermore, it is likely that the expression of other metabolic genes are influenced by this unique circadian pattern. We believe that future studies into the physiologic, metabolic and genomic adaptations to CR must take into account this cyclical metabolic pattern.

A larger question is whether this diurnal fuel selection pattern plays a role in mediating the effects of CR on health and longevity. The pattern of food intake observed in response to CR is, in fact, a form of intermittent fasting. Other intermittent fasting protocols, most notably alternate day fasting, have been shown to slow tumor growth, decrease cell proliferation, improve insulin sensitivity and increase longevity, perhaps without a concomitant decrease in body weight (Varady and Hellerstein, 2007). It should be noted that Nelson & Halberg (Nelson and Halberg, 1986) demonstrated that CR provided as six meals per day increased longevity to an equal extent as CR provided as one meal per day. Yet, it is possible that some of the effects of CR may be mediated by intermittent energy intake rather than or in addition to altered body composition.

One potential effect of CR that may be mediated by intermittent fasting is a reduced production of ROS. It has been hypothesized that an increased reliance on FA oxidation could lead to decreased ROS production, because FA oxidation increases the FADH/NADH ratio compared to carbohydrate oxidation (Guarente, 2008). While NADH donates electrons to complex I of the electron transport chain, FADH donates electrons directly to complex II, the electron transfer flavoprotein dehydrogenase (ETF), which then passes the electrons to complex III via ubiquinone. FADH oxidation therefore bypasses complex I, which is a major contributor to cellular ROS production (Kushnareva et al., 2002; Lopez-Torres et al., 2002). An interesting question for future research is whether effects of CR on ROS production depend upon the cyclic stimulation of FA synthesis.

In conclusion, we have characterized a unique feeding and macronutrient metabolic pattern in CR mice associated with a dramatic increase in whole body FA oxidation and a marked increase in adipose tissue FA synthesis. This pattern is rapidly induced, influences

the daily pattern of gene expression, and may provide a metabolic switch that translates the dietary changes of CR into a program of health and survival.

The results of Projects 1 & 2 suggested that molecular signals that are activated in response to fasting and have effects on cell survival and proliferation could mediate the effects of CR on aging and longevity. Sirt1, the mammalian homolog of the yeast sir2 gene, appears to be activated in response to CR in many tissues. Activation of Sirt1 leads to the modulation of activity of a number of transcription factors mediating cell proliferation, cell survival, including p53, FoxO, pgc-1α & PPARα. Thus it appeared that Sirt1 could mediate the longevity effects of CR by modulating cell proliferation. However, the role of Sirt1 in mediating the CR-induced effects on cell proliferation *in vivo* had not been evaluated. Thus the objective of Project 3 was to determine if Sirt1 activation was necessary or sufficient for the CR-induced reduction in liver cell proliferation in C57BL/6 mice.

Project 3

In this study we found that calorie restriction leads to a trend for increased Sirt1 protein abundance in the liver at all time points in C57BL/6 mice. However, Sirt1 protein expression in the liver was not necessary for the CR-induced reduction in liver cell proliferation. In addition, resveratrol fed in the diet did not mimic the effects of CR on liver cell proliferation. These results were surprising in light of recent findings suggesting roles of Sirt1 in regulating cell proliferation through deacetylation of relevant transcription factors, including p53, FoxO, PPARα and PGC1a (Saunders and Verdin, 2009), as well as reports of resveratrol mimicking the effects of CR through Sirt1-dependent mechanisms (Lin et al.). Since reduced cell proliferation rate is thought to be associated with longevity (de Magalhaes and Faragher, 2008), our results suggest that peripheral Sirt1 expression does not mediate the longevity effects of CR, at least not through this potential mechanism. Thus, this study adds to the growing list of evidence questioning the role of Sirt1 in mediating the effects of CR in aging (Kaeberlein and Powers, 2007).

In the first experiment of this study, we sought to reconcile discrepancies in the literature as to the role of CR on Sirt1 protein expression in the liver. The first study assessing the role of CR on Sirt1 protein in mammals reported an increase in Sirt1 abundance in the liver of calorie-restricted rats (Cohen et al., 2004). Consistent with this result, Nisoli et al. (Nisoli et al., 2005) reported increased Sirt1 mRNA in liver of calorie restricted mice. However two subsequent studies reported no increase in Sirt1 protein (Boily et al., 2008; Mulligan et al., 2008), and one other study even reported a decrease in Sirt1 protein in the livers of CR mice (Chen et al., 2008). It had been hypothesized that differences in Sirt1 protein abundance may be due to the point in the feeding cycle of CR mice at which the liver tissue was collected (Chen et al., 2008). Indeed, we have previously seen dramatic differences in expression of metabolic genes in livers of CR mice depending on the time of day (Bruss et al.). Here we saw a trend for an increase in Sirt1 protein in the liver of CR mice that was not influenced by diurnal cycle, thus suggesting that diurnal cycle cannot explain the discrepancies existing in the literature.

Examining the existing studies that have assessed the role of CR on Sirt1 protein abundance in the liver, there are some possibilities that may explain the disparate results

in the literature. One of the studies reporting no difference in Sirt1 abundance in CR livers may be explained by differences in controls. In their study Saupe et al. used 10% CR for their controls and compared them to 30% CR. It is possible that the threshold for CR-induced increases in Sirt1 is very low, such that mild CR leads to increased Sirt1 expression with no further increase with more severe CR. The other study reporting no difference in Sirt1 expression in liver of CR mice did mention a small increase that was not significant. Interestingly, here we also show a small, insignificant, change in Sirt1 of CR mice. Perhaps the influence of CR on Sirt1 protein is very small in the liver, and with larger sample size we, and McBurney et al., may have found a significant difference. The study by Chen et al. is the most difficult to reconcile. Of six studies in rodents, they are the only group to see a decrease in Sirt1 in the livers of CR rodents. Taken together, the preponderance of evidence suggests that CR increases Sirt1 protein abundance moderately in the livers of CR rodents, however the discrepancies in the literature cannot be fully reconciled.

Regardless of the effect of CR on Sirt1 protein abundance we expected that Sirt1 activity would be modulated (either higher or lower) in response to CR and that this would play some role in mediating effects on cell proliferation. Two studies have shown that Sirt1 overexpression inhibits cell proliferation of tumor cell lines *in vitro* (Fu et al., 2006; Wang et al., 2008b), and two studies have demonstrated reduced tumor formation when Sirt1 is overexpressed *in vivo* (Boily et al., 2009). Since reduced cell proliferation is thought to be one mechanism by which CR leads to decreased tumorigenesis, we hypothesized that ablation of Sirt1 would increase cell proliferation in the liver and attenuate the effect of CR. However, the absence of Sirt1 had no effect on CR-induced reduction in liver cell proliferation, nor did Sirt1 ablation in the liver have any effect on liver cell proliferation in AL-fed mice.

The reasons for the absence of an effect are not immediately clear. To our knowledge no prior studies have investigated the effects of Sirt1 in mediating the anti-proliferative and anti-tumorigenic effects of CR. However, there are a number of possibilities that could be investigated. First, perhaps overexpression Sirt1 leads to non-phsyiologic effects that cannot be mimicked by endogenous activation or ablation of Sirt1. Consistent with this, overexpression of Sirt1 inhibits intestinal tumor formation in APC[min] mice (Firestein et al., 2008), while Sirt1 null mice do not have increased susceptibility (Boily et al., 2009). Another possibility is that Sirt1 acts in a tissue specific manner, playing a role in intestinal, mammary and T-lymphocytes, but not in liver hepatocytes. It is also possible that increased Sirt1 activity affects tumor cells specifically, yet has no effect on normal cell proliferation, perhaps due to different energetic needs of these cell types.

While Sirt1 does not appear to be necessary for the CR-induced reduction in cell proliferation, we had hypothesized that perhaps induction of Sirt1 activity may be sufficient to mimic the CR effects on cell proliferation. However, in this study, high doses of resveratrol provided in the diet had no effect on liver cell proliferation.

While this study was conceived and executed, resveratrol was widely purported to be a Sirt1 activator, however, as discussed in the Chapter 1, it now appears that resveratrol is not a direct Sirt1 activator. Regardless of resveratrol's affect on Sirt1, the results of this study were still surprising, given the large number of studies showing reduced cell

proliferation *in vitro* in response to resveratrol (Dong, 2003; Zhang, 2006). Other studies have shown that resveratrol also reduces tumor growth *in vivo* (Boily et al., 2009). Perhaps the effects of resveratrol on tumor growth have more to do with tumor initiation than tumor promotion, however, to our knowledge no studies have directly addressed this question.

A recent report shows that resveratrol treatment throughout life has no effect on maximal lifespan in mice (Miller et al., 2011). This is consistent with a previous report demonstrating that resveratrol treatment had no effect on lifespan in mice fed a standard high carbohydrate diet (Baur et al., 2006). However, resveratrol does seem to prevent premature death in mice fed a high-fat diet (Baur et al., 2006). We believe cell proliferation is mechanistically linked to and predictive of interventions that extend maximal lifespan and not necessarily related to premature death due to metabolic disease caused by diet induced obesity. Thus, the lifespan data related to resveratrol treatment are consistent with the cell proliferation data we found in Project 3.

Taken together it appears that effects of CR on Sirt1 expression in the liver and the role of Sirt1 on cell proliferation are not as robust as originally thought. A number of irreconcilable inconsistencies have arisen in the literature that make it difficult to determine the role of Sirt1 in mediating this critical physiological adaptation. Interestingly, recent work has called into question many of the original findings reporting a role for Sirt1 in mediating the longevity effects of CR in yeast and worms. Thus it is important to pursue future work to rigorously test assumptions about Sirt1 and its role in mammalian biology.

Summary

The results of this thesis help create a working model for one mechanism by which CR may lead to increased longevity in rodents (Fig 4.1). Calorie restriction leads to an altered feeding pattern of gorging and fasting that changes lipid metabolism towards increased FA synthesis immediately post-prandial, followed by prolonged periods of increased FA oxidation. The net result is an increase in reliance on FA oxidation for energy. This switch in substrate utilization could lead to both decreased ROS production and activation of PPARα. Both of these pathways may then converge on a

Calorie Restriction
↓
Altered Feeding Pattern
↓
↑Endogenous FA Synthesis
↓
↑Reliance on FA Oxidation
↓   ↓
↑Expression of PPARα Genes   ↓ROS Production
↓   ↓
↑FGF21 Signaling   ↓Cell Damage
↓   ↓
↓IGF-1 & ↑IGFBP   ↓Cell Death
↘   ↙
↓Cell Proliferation
↓
Delayed Cellular
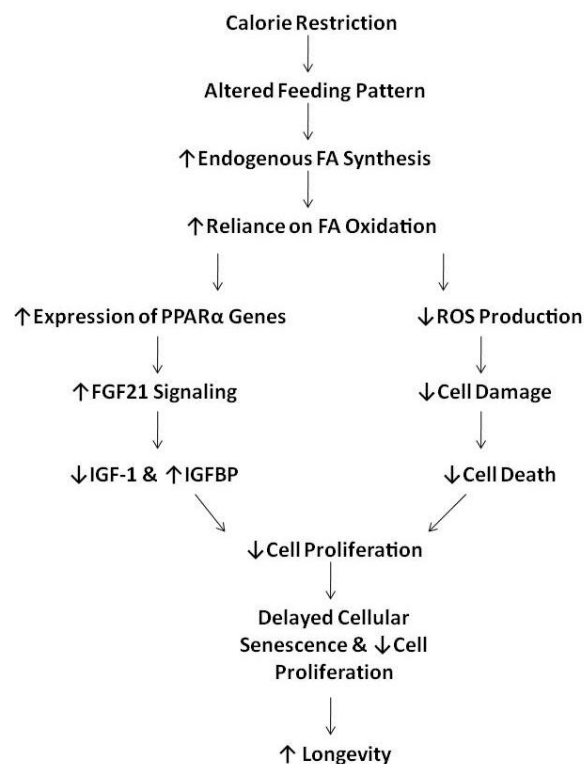Senescence & ↓Cell
Proliferation
↓
↑ Longevity

Fig 4.1. Working model of CR-induced longevity

reduction in rates of cellular proliferation in early to middle life. Decreased ROS leads to reduced cellular damage and reduced cell death, while increased PPARα activity could lead to reduced cell proliferation through FGF21 mediated reduction of IGF-1 signaling. As discussed in Chapter 2, reduced cell proliferation in early and middle life may lead to delayed cellular senescence, preserved replicative capacity and reduced tumor promotion, all of which can lead to increased longevity. In future work it will be interesting to determine the roles of FA synthesis and oxidation on CR-induced reductions in cell proliferation. In addition, it will be informative to explore the mechanisms by which CR leads to reductions in IGF-1 signaling and to determine if these mechanisms play a role in cellular proliferation rates. And finally, it will be important for the field of aging to confirm the role of cell proliferation as a biomarker of longevity in other aging models.

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