Muscle-specific 4E-BP1 signaling activation improves metabolic parameters during aging and obesity

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Eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1) is a key downstream effector of mTOR complex 1 (mTORC1) that represses cap-dependent mRNA translation initiation by sequestering the translation initiation factor eIF4E. Reduced mTORC1 signaling is associated with life span extension and improved metabolic homeostasis, yet the downstream targets that mediate these benefits are unclear. Here, we demonstrated that enhanced 4E-BP1 activity in mouse skeletal muscle protects against age- and diet-induced insulin resistance and metabolic rate decline. Transgenic animals displayed increased energy expenditure; altered adipose tissue distribution, including reduced white adipose accumulation and preserved brown adipose mass; and were protected from hepatic steatosis. Skeletal muscle–specific 4E-BP1 mediated metabolic protection directly through increased translation of peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α) and enhanced respiratory function. Non–cell autonomous protection was through preservation of brown adipose tissue metabolism, which was increased in 4E-BP1 transgenic animals during normal aging and in a response to diet-induced type 2 diabetes. Adipose phenotypes may derive from enhanced skeletal muscle expression and secretion of the known myokine FGF21. Unlike skeletal muscle, enhanced adipose-specific 4E-BP1 activity was not protective but instead was deleterious in response to the same challenges. These findings indicate that regulation of 4E-BP1 in skeletal muscle may serve as an important conduit through which mTORC1 controls metabolism.

Introduction

mTOR complex 1 (mTORC1) is a principal cellular sensor of nutrient availability that, in turn, regulates cellular growth and proliferation. Activated mTORC1 signaling is associated with increased nutrient uptake and decreased autophagy, which are often seen in many cancers and metabolic disorders such as type 2 diabetes. Conversely, reduced mTORC1 activity, by caloric restriction, rapamycin treatment, or genetic manipulation, extends life span and likely health span in many model organisms (1–5). However, the relevant downstream substrates and key tissues by which reduced mTORC1 signaling provides these benefits remain to be determined.

Two key downstream substrates of the mTORC1 complex, S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein (4E-BP), have been linked to longevity in invertebrates (4). mTORC1 phosphorylation of S6K leads to its activation, whereas phosphorylation of 4E-BP inhibits its ability to sequester eIF4E (6–8). Although reduced S6K signaling increases energy expenditure and extends life span in mice (8–10), the fate of mice with activated 4E-BP is currently unknown.

In addition, the specific tissue(s) in which mTORC1 inactivation mediates longevity and improved metabolic homeostasis has not been determined. Adipose tissue is a prime candidate, since tissue-specific inactivation of the mTORC1 component Rptor in this tissue promotes reduced adiposity and subsequently protects against metabolic stress (11). Skeletal muscle is another candidate for mediating the metabolic protection afforded by reduced mTORC1 signaling, particularly given that activation of the Drosophila equivalent of 4E-BP (d4E-BP) specifically in muscle suppresses age-related tissue degeneration and is sufficient to enhance life span in the fly (12). mTORC1 activity is crucial during muscle development, as modulations that blunt mTORC1 activity cause muscular dystrophy, while enhanced mTORC1 activity is required for muscle hypertrophy and recovery from injury (13–17). However, sustained activation of mTORC1 in aging animals results in severe myopathy (18, 19), suggesting that the effects of mTORC1 activation are tightly regulated and coordinated in skeletal muscle, with proper levels of mTORC1 activity important for both response to injury and long-term tissue function in aging animals.

Skeletal muscle and adipose tissue share a number of intrinsic functions in the regulation of energy homeostasis. First, they are both derived from the same mesenchymal stem cells, with brown adipocytes in particular sharing a common precursor cell with skeletal muscle cells (20). Second, muscle and adipose tissue are both major sites of thermogenesis, oxidizing fatty acids and dissipating energy as heat via the action of uncoupling proteins (21). Third, both tissues exhibit plasticity, adapting to either oxidative or glycolytic metabolism as their main energy source, as needed (22–24). Finally, genetic reprogramming of transcription factors involved in mitochondrial biogenesis leads to muscle fiber transformation or a white fat–to–brown fat phenotypic switch, which is ultimately correlated with an alternation in energy expenditure (24–27).

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Mice lacking 4e-bp1 have a significant reduction of adipose tissue and an increase in whole-body energy expenditure, while mice lacking both 4e-bp1 and 4e-bp2 reverse this phenomenon (28), suggesting that the tissue-specific effect mediated by different 4E-BP family members in the regulation of metabolic homeostasis might be masked in whole-body knockout mice. To elucidate the factors downstream of mTORC1 signaling that systemically coordinate energy homeostasis in vivo, we generated transgenic mice overexpressing a constitutively active, mTORC1-nonresponsive form of 4E-BP1 (29) in either skeletal muscle or adipose tissue. We demonstrate that enhanced 4E-BP1 activity in skeletal muscle leads to increased oxidative metabolism and protects mice from diet- and age-induced whole-body metabolic dysfunction. In contrast, activated 4E-BP1 in adipose tissue sensitized mice to diet-induced obesity with insulin resistance.

Results

Muscle 4E-BP1 non–cell autonomously regulates adipose tissue homeostasis. To test the role of mTORC1-dependent regulation of 4E-BP1 in energy homeostasis, we generated 4E-BP1 transgenic mice with mTOR phosphorylation site mutations (threonine to alanine at amino acid positions 37 and 46; Tg-4EBP1mt) to yield a constitutively active form of the protein (ref. 29 and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI77361DS1). The expression of the transgene is repressed by a loxP-flanked STOP codon cassette. Tissue-specific expression from the potent CRE excises the repressive element and permits expression of the potent CAGGS promoter-enhancer. Therefore, we could study both cell-autonomous and non–cell autonomous mechanisms of 4E-BP1 activities regulated by mTORC1 dependency in whole-body energy homeostasis.

The CRE recombinase enzyme under the control of the mouse Fabp4 promoter (Fabp4-Cre) or the muscle creatine kinase promoter (Ckmm-Cre) was used to selectively induce transgenic 4E-BP1 expression in mouse adipose tissue or skeletal muscle (refs. 30, 31, and Supplemental Figure 1, B–D). For simplicity, we refer to these mice as Tg-4EBP1mt-fat and Tg-4EBP1mt-muscle, respectively. Both Fabp4-Cre and Ckmm-Cre are expressed at a late stage of adipose and skeletal muscle differentiation; thus, we could study the role of 4E-BP1 in the maintenance of mature adipocytes and skeletal muscle fibers and determine how the genetically modified target tissues regulate whole-body energy expenditure. Both mouse lines yielded viable and fertile mice.

In transgenic mice, in which 4E-BP1mt protein is overexpressed, 4E-BP1 migrated faster than endogenous mouse 4E-BP1, which we attribute to mTORC1 nonresponsiveness (Supplemental Figure 1, B and D). Transgenic 4E-BP1mt protein showed increased binding to eIF4E, which in turn reduced eIF4E association with eIF4G (Supplemental Figure 2, A–D). Global protein translation was not altered in 4E-BP1–activated skeletal muscle (Supplemental Figure 2, E–G), suggesting that cap-independent translation control is compensatory. This is consistent with prior observations in an independent 4E-BP1 transgenic mouse line in which acute activation of 4E-BP1 in normal thymocytes did not impede global protein translation (32). In contrast, acute activation of 4E-BP1 in AKT-induced T cell lymphoma markedly reduced protein translation (32).

Notably, endogenous 4E-BP1 expression was upregulated in Tg-4EBP1mt-muscle mouse skeletal muscle (Supplemental Figure 1, B and E). d4E-BP is a known dFOXO target in Drosophila (12, 33). In addition, we detected induction of FOXO1 expression at both the protein and mRNA levels (Supplemental Figure 3). This suggests positive feedback regulation, with 4E-BP1 promoting FOXO1 expression, which in turn leads to enhanced transcription of 4e-bp1 mRNA.

While both groups of 4E-BP1 double-transgenic mice developed normally, we found that Tg-4EBP1mt-muscle mice exhibited reduced body weight (Figure 1A and Supplemental Figure 4A). At 2 months of age, Tg-4EBP1mt-muscle mice weighed significantly less than control mice (19% less for males and 17% less for females). The difference was not a result of feeding (Supplemental Figure 4, B and C), and we did not detect a difference in IG-F1 or growth hormone levels (Supplemental Figure 4D and data not shown), suggesting that changes in hormone levels do not underlie the small body size of Tg-4EBP1mt-muscle mice. Correlated with the decrease in body weight, there was a reduced lean body mass in Tg-4EBP1mt-muscle mice (Figure 1B). Moreover, male Tg-4EBP1mt-muscle mice had significantly lower plasma glucose levels than control mice (152 ± 18.0 mg/dl versus 173 ± 25.4 mg/dl, respectively) (Supplemental Figure 5A). Nonetheless, Tg-4EBP1mt-muscle mice exhibited comparable glucose homeostasis to control mice, including a normal insulin response and glucose clearance rate on a normal chow diet (Supplemental Figure 5, B–E). There was also a slight reduction in body weight for male Tg-4EBP1mt-fat mice, coupled to increased insulin sensitivity and glucose tolerance (Figure 1A, Supplemental Figure 4A, and Supplemental Figure 5). Next, we examined the quantity and quality of adipose tissue in 4E-BP1 double-transgenic mice. The major fat pads, including visceral fat surrounding reproductive organs, inguinal fat, and brown fat on the shoulder, all exhibited no difference in weight when normalized to body weight in both groups of 4E-BP1 double-transgenic mice (Supplemental Figure 6). Histological analysis of white adipose tissue from Tg-4EBP1mt-muscle mice revealed a marked reduction in cell size and a decrease of subcutaneous fat deposits under the skin, whereas there was no effect in Tg-4EBP1mt-fat mice (data not shown). The reduction of cell size in Tg-4EBP1mt-muscle mice was restricted to white adipose tissue, as brown adipose tissue was not affected (Figure 1, C and D; Supplemental Figure 7; and see below). Previously, transgenic mice with whole-body knockout of 4E-BP1 were reported to undergo a white-to-brown adipose transition, generating beige fat (24). Notably, we detected an induction of Pgc1a transcription in Tg-4EBP1mt-muscle mouse inguinal fat (Supplemental Figure 8A); however, we did not observe either multilocular adipocytes (Figure 1D) or enhanced UCP1 expression in visceral and inguinal fat pads (Supplemental Figure 8B). Moreover, expression of the peroxisome proliferator–activated receptor γ coactivator-1α (PGC-1α) target Fndc5, which is secreted from PGC-1α-overexpressing skeletal muscle and controls white adipose transformation (34), did not change in 4E-BP1–activated skeletal muscle (Supplemental Figure 17A). Of note, we observed increased expression of UCP1 and CPT1α in Tg-4EBP1mt-muscle mouse brown adipose tissue, which might contribute to their increased metabolic rate, as discussed below (Supplemental Figure 8C and Figure 2D).
The differences between skeletal muscle Rptor knockout mice and Tg-4EBP1mt-muscle mice are somewhat surprising, since (a) reduced mTORC1 signaling should lead to enhanced 4E-BP1 function and (b) both mutants have elevated FOXO1 expression. Mice with skeletal muscle lacking Rptor or overexpressing Foxo1 have both impaired insulin signaling and glucose homeostasis (17, 37). However, Tg-4EBP1mt-muscle mice had comparable glucose metabolism to that of control mice and upregulated AKT1 expression in skeletal muscle (Supplemental Figure 5 and Supplemental Figure 13A). Increased AKT1 levels may better attenuate the effects of enhanced FOXO1, thereby allowing mice to maintain normal glucose homeostasis. For instance, upon insulin activation, we observed a proportional increase of phosphorylated FOXO1 relative to total protein (data not shown). In addition, there was differential expression of FOXO1 target genes in skeletal muscle of Tg-4EBP1mt-muscle mice. While atrophy, autophagy, and gluconeogenesis-associated genes were induced, we did not detect enhanced expression of genes encoding cell cycle, oxidative stress, or DNA repair components (Supplemental Figure 13B). These results demonstrate that, unlike inactivation of Rptor in skeletal muscle, which causes progressive dystrophy and abnormal glycogen deposition (Supplemental Figure 10A), which was observed in the skeletal muscle–specific Rptor knockout mice (17). The differences between skeletal muscle Rptor knockout mice and Tg-4EBP1mt-muscle mice are somewhat surprising, since (a) reduced mTORC1 signaling should lead to enhanced 4E-BP1 function and (b) both mutants have elevated FOXO1 expression. Mice with skeletal muscle lacking Rptor or overexpressing Foxo1 have both impaired insulin signaling and glucose homeostasis (17, 37). 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also detected an increase in whole-body fat, which may be attributable to the skeletal muscle phenotype (Supplemental Figure 6B and Supplemental Figure 14). This is surprising since accumulation of intramuscular fat, at least coupled to increased white adipose tissue mass and higher serum-free fatty acid levels, is highly correlated with insulin resistance and the development of type 2 diabetes. However, calorie restriction and exercise also lead to an increase of intramuscular fat, with a reduction in white adipose tissue and normal levels of serum-free fatty acids (38–42). Furthermore, athletes or calorie-restricted model organisms are insulin sensitive, having higher metabolic rates and extended health span. Consistent with the latter scenario Tg-4EBP1mt-muscle mice had comparable basal leptin and glucose levels and serum lipid profiles and, more importantly, retained insulin sensitivity (Supplemental Figure 5, Supplemental Figure 14C, and Supplemental Figure 15).

There are 4 defined muscle types characterized in mammals. Type I, type IIa, and type IIx predominantly use oxidative respiration for energy production, while type IIb mainly uses glycolysis (43, 44). Overexpression of AKT1 in skeletal muscle causes muscle hypertrophy and increased type IIb muscle (glycolytic) fibers (15). Even though there was an induction of AKT1 expression in Tg-4EBP1mt-muscle mouse skeletal muscle, immunofluorescence revealed an increase of oxidative muscle, a finding confirmed by enhanced troponin I (slow) and MhcI expression (Figure 2A and Supplemental Figure 16). Moreover, protein levels of PGC-1α, which regulates mitochondrial biogenesis (45, 46), were enhanced even though mRNA levels were not altered (Figure 2, B and C). We speculate that activation of 4E-BP1 promotes PGC-1α translation. There was also a differential enhancement of PGC-1α target genes in 4E-BP1–activated skeletal muscle (Supplemental Figure 17). The fact that some but not all target genes were induced may reflect differential expression of transcription factors coactivated by PGC-1α.

There was a marked increase in mitochondrial DNA copy number as well as enhanced whole-body oxygen consumption and energy expenditure in Tg-4EBP1mt-muscle mice, which correlated with increased oxidative muscle fibers (Figure 2D, Supplemental Figure 16E, and Supplemental Figure 18A). Consistently, mitochondrial oxygen consumption rates (OCRs) from isolated skeletal muscle mitochondria in the presence of indicated substrates were measured in an XF24 Extracellular Flux Analyzer (n = 5 per genotype). (E) State III oxygen consumption measured in a 3-day/night period in 6-month-old male mice fed a normal chow diet (n = 8–12 per genotype). (F) RT-PCR and (G) Western blot analysis of SIRT4 expression in quadriceps. P values were assessed by a 2-way ANOVA. Bonferroni post-tests were used to compare replicate means by row. **P < 0.01; ***P < 0.001. RT-PCR samples were from n = 4 per genotype.
sion of SIRT4 in cancer cells (48). Therefore, we checked the expression of SIRT4 but did not find any change (Figure 2, F and G). Moreover, Tg-4EBP1mt-muscle mice have comparable respiratory quotient (RQ) values, as quantified by oxygen consumption and CO2 generation, and a similar circadian transition to those of control littermates (Supplemental Figure 18B). Taken together, these results indicate that activation of 4E-BP1 in skeletal muscle directly contributes to increased oxidative metabolism in mouse skeletal muscle and leads to white adipose tissue atrophy, which consequently enhances whole-body energy expenditure.

Tg-4EBP1mt-muscle mice are protected from diet-induced insulin resistance and lipid dysfunction. Since there is altered fat distribution in Tg-4EBP1mt-muscle mice on normal chow, we next examined their behavior in response to a high-fat diet (HFD) challenge. Although the weight accumulation in Tg-4EBP1mt-muscle mice was similar to that in littermate controls in response to overnutrition (Supplemental Figure 19), the basal glucose levels were significantly lower and the glucose clearance rate was improved (basal level of glucose: 200 ± 29.8 mg/dl versus 221 ± 27.1 mg/dl in males and 155 ± 20 mg/dl versus 172 ± 23.5 mg/dl in females) (Figure 3A and Supplemental Figure 20). Moreover, Tg-4EBP1mt-muscle mice maintained insulin sensitivity (Figure 3, B and C; Supplemental Figure 20). Moreover, Tg-4EBP1mt-muscle mice have comparable insulin levels to control mice on a HFD (Supplemental Figure 22). However, upon HFD feeding, littermate control mice exhibited elevated serum cholesterol and triglycerides, whereas Tg-4EBP1mt-muscle mice maintained their lipids at lower levels that were comparable to those of animals on normal chow (Figure 4, A and B). Moreover, mice placed on a HFD exhibit white adipocyte infiltration into brown adipose tissue (49, 50). We found that brown adipose tissue of Tg-4EBP1mt-muscle mice was preserved and that the expression of brown adipose Ucp1 was comparably higher than that in controls on the HFD regimen (Figure 4C and Supplemental Figure 23).

As described above, Tg-4EBP1mt-muscle mice have improved serum lipid profiles on a HFD. The HFD often induces abnormal accumulation of triglyceride in the liver (steatosis), which further deteriorates fat metabolism. We examined the livers of 4E-BP1 double-transgenic and control mice by Oil red O staining to detect lipid accumulation. Histological analysis of Tg-4EBP1mt-muscle mouse liver clearly revealed marked protection against hepatic steatosis (Figure 4D). In contrast, activation of 4E-BP1 in adipose tissue did not prevent brown adipose tissue loss or alter the development of hepatic steatosis on a HFD (Figure 4, C and D, and Supplemental Figure 23). These data from HFD-fed mice with induced obesity and insulin resistance reinforce the concept that muscle-specific 4E-BP1 activation non–cell autonomously regulates fat metabolism.

Activation of 4E-BP1 in skeletal muscles preserved metabolic parameters in mice on HFD. Consistent with observations that Tg-4EBP1mt-muscle mice maintained insulin sensitivity and glucose homeostasis on a HFD, their metabolic rate was also maintained at a higher level (Supplemental Figure 24A). Moreover, HFD usually blocks RQ switch during the circadian cycle. Tg-4EBP1mt-muscle mice were protected from this (Supplemental Figure 24B). The PGC-1α targets associated with uncoupling, Ucp2 and Ucp3, were further induced in the skeletal muscle of Tg-4EBP1mt-muscle mice, a finding that correlated with an enhanced metabolic rate (Supplemental Figure 25). Interestingly, we observed gender-dependent behaviors in Tg-4EBP1mt-muscle mice, as male Tg-4EBP1mt-muscle mice tended to eat less and female Tg-4EBP1mt-muscle mice tended to be more active (Supplemental Figure 26).
Figure 4. Tg-4EBP1mt-muscle mice have improved fat metabolism on HFD. (A) Cholesterol plasma values and (B) triglyceride plasma values from 6-month-old HFD-fed mice (the number of mice analyzed is indicated in bars). Representative images of (C) hematoxylin and eosin–stained liver sections from 6-month-old male brown adipose tissue sections and (D) Oil red O–stained liver sections from 6-month-old male mice. Scale bar: 100 μm. *P values were assessed by a 2-way ANOVA. Bonferroni post-tests were used to compare replicate means by row. **P < 0.01; ***P < 0.001. NCD, normal chow diet.

Tg-4EBP1mt-muscle mice are protected from age-induced metabolic decline. We have shown that activation of 4E-BP1 in skeletal muscle, instead of adipose tissue, suppresses fat metabolic dysfunction associated with HFD. Next, we investigated whether activation of 4E-BP1 in skeletal muscle also protects mice from age-induced metabolic dysfunction. The loss of skeletal muscle mass (sarcopenia) with age is associated with higher mortality in the elderly (51–53). Sarcopenia often leads to frailty, resulting in a decreased metabolic rate and increased risk of insulin resistance (54). We found that Tg-4EBP1mt-muscle mice, unlike their littermate control counterparts, preserved their muscle fiber size with advanced age (Figure 5, A and B; Supplemental Figure 27; and Supplemental Figure 28, B and C). In addition, whereas whole-body oxygen consumption declined in mice with age, Tg-4EBP1mt-muscle mice maintained higher levels of whole-body oxygen consumption (Figure 5C). RQ is usually reduced during aging in control mice (55), whereas 24-month-old aged Tg-4EBP1mt-muscle mice exhibited RQ levels comparable to those observed at 6 months of age (Supplemental Figure 29). Decreasing metabolic rate is usually associated with an increase in adiposity. We observed age-induced obesity in the control aging cohort, while Tg-4EBP1mt-muscle mice maintained body weight and adiposity during aging (Supplemental Figure 28). Because Ucp1 expression was increased modestly in the brown fat pads of Tg-4EBP1mt-muscle mice (Supplemental Figure 8, B and C), we investigated the fate of brown fat in more detail. Loss of “browning” in brown adipose tissue has been correlated with increasing age (56, 57). At 17 months of age, we found marked white adipocyte infiltration in the brown fat pads of control mice. Strikingly, this infiltration was not apparent in Tg-4EBP1mt-muscle mouse brown adipose tissue at the same age (Figure 5D). Moreover, Tg-4EBP1mt-muscle mice have improved insulin sensitivity compared with that of aged control mice (Figure 5E). There was a reduced glucose tolerance, mainly due to increased insulin serum level, in aged male control mice (Figure 5, F and G), similar to previously reported observations (58). In contrast, Tg-4EBP1mt-muscle mice had insulin serum levels comparable to those of their younger counterparts and maintained improved glucose tolerance compared with that of aged controls. Thus, Tg-4EBP1mt-muscle mice are protected against age-induced metabolic decline both cell autonomously and non-cell autonomously.

Increased FGF21 expression from 4E-BP1-activated skeletal muscle. Activation of 4E-BP1 in skeletal muscle protects mice from either diet- or age-induced white adipose tissue accumulation and metabolic rate decline. When we screened for secreted myokines that might contribute to increased energy expenditure in 4E-BP1-activated skeletal muscle, we discovered that expression of Fgf21 was significantly increased (Figure 6, A–C, and Supplemental Figure 30A). Correlated with this observation, the serum level of FGF21 was also higher in Tg-4EBP1mt-muscle mice (Figure 6D). In adipose tissue, FGF21 has been shown to regulate PPARγ activity, which contributes to enhanced metabolic activity in adipocytes (59). We observed increased expression of PPARγ in both white and brown adipose tissue (Figure 6E). As described above, white adipose tissue size was decreased and brown adipose tissue was preserved during HFD feeding or aging in Tg-4EBP1mt-muscle mice. We also observed decreased expression of genes involved in fatty acid and transgenic synthesis in white adipose tissue (Figure 6F), while there was increased expression in thermogenesis genes in brown adipose tissue (Figure 6G) on normal chow. A similar phenomenon was observed in Tg-4EBP1mt-muscle mouse liver, in which there was a reduction of SREBP1c expression, which regulated de novo lipogenesis and its target gene expression (Supplemental Figure 30, B...
and C). Administration of FGF21 in obese or diabetic mice has been reported to increase β-oxidation in both adipose and liver tissue (59–62). There was no significant differential expression of β-oxidation genes in normal chow–fed Tg-4EBP1mt-muscle mice (Supplemental Figure 30C and Supplemental Figure 31, A and B); however, after 4 months of HFD feeding, we detected increased Fgf21 expression in Tg-4EBP1mt-muscle mice (Supplemental Figure 31C) and markedly increased expression of β-oxidation genes in the skeletal muscle and white adipose tissue but not in liver (Supplemental Figure 31, D–F).

**Induced 4E-BP1 activation in adult skeletal muscle retains increased metabolic rate.** Since there is rapid skeletal muscle growth during the first week of postnatal development (63), activation of 4E-BP1 at this early stage might impair skeletal muscle growth and lead to a compromised metabolic phenotype. This raises the question of whether the phenotypes detected in the Tg-4EBP1mt-muscle mice were a result of differences during postnatal development or adulthood. To determine whether activation of 4E-BP1 in adult skeletal muscle directly triggers an enhanced metabolic rate without interfering with skeletal muscle development, we crossed Tg-4EBP1mt...
Taken together, these results provide a comprehensive assessment of the consequences of enhanced mTORC1-nonresponsive 4E-BP1 activity in skeletal muscle and adipose tissue, leading us to conclude that activation of 4E-BP1 in skeletal muscle is a potent regulator of systemic whole-body metabolism. Muscle 4E-BP1 is also a promising therapeutic target to treat diet- and age-induced metabolic decline, since the acute activation of 4E-BP1 is sufficient to increase energy expenditure.

Discussion

Our data provide robust genetic evidence that the beneficial effects of mTOR-dependent regulation of 4E-BP1 activity are associated with skeletal muscle, rather than adipose tissue. Enhanced respiratory activity in Tg-4EBP1mt-muscle mice was apparent both in skeletal muscle and brown fat. While the former is likely due, at least in part, to enhanced PGC-1α activity, the non–cell autonomous effects on brown fat likely derive from increased FGF21 expression and secretion from skeletal muscle. While we cannot rule out a model where brown fat levels are enhanced due to secondary effects of reduced subcutaneous white fat to maintain body temperature, this seems unlikely since inactivation of Rptor in skeletal muscle also leads to white adipose tissue atrophy, without an increase in brown fat or any changes in body temperature regulation (17). Instead, we favor a model whereby (a) a myokine signal is transmitted from the skeletal muscle of Tg-4EBP1mt-muscle mice to brown fat or (b)
altered metabolism of skeletal muscle leads to metabolic changes that are interpreted differently by the heat-producing tissue.

Previously, the mTORC1 pathway was implicated in the regulation of adipogenesis, as inactivation of mTORC1 by RNAi knockdown of Rptor or treatment with rapamycin was found to reduce adipocyte differentiation (11). Conversely, preadipocytes lacking both 4E-BP1 and 4E-BP2 exhibit increased differentiation in vitro (28). Data from mice lacking 4-e-bps are conflicting, perhaps due to genetic background differences or tissue-specific effects of 4E-BP family members. Knockout of 4-e-bps results in a lean phenotype in the C57BL/6J background and, in contrast, leads to an obese phenotype in the BALB/c background (28, 65). In addition, 4e-bp1 single-knockout mice are lean (66), while 4e-bp1/2 double-knockout mice are obese (28). These differences may stem from different tissue-specific responses to 4E-BP family members in the regulation of metabolism. A similar phenomenon has been observed in mice chronically treated with rapamycin, which leads to hepatic inactivation of mTORC2, resulting in impaired fat metabolism and increased adiposity (67). Here, we show that 4E-BP1 activation is only beneficial in skeletal muscle, whereas the activation of 4E-BP1 in adipose tissue is deleterious upon HFD feeding. Thus, whole-body knockout of 4-e-bps or treatment with rapamycin may mask mTORC1-dependent regulation of adipogenesis and energy expenditure.

We performed our 4E-BP1 studies in transgenic mouse lines on the C57BL/6J background, as in previously published studies of genetically modified Rptor or S6ki−/− mice and in the rapamycin studies (5, 10, 11, 17, 68) to permit a direct comparison of metabolic phenotypes. Our data indicate that mTORC1-dependent regulation of 4E-BP1 activity in skeletal muscle, instead of adipose tissue, non–cell autonomously regulates fat metabolism in the context of aging and overnutrition.

Activation of 4E-BP1 in skeletal muscle induces autophagy, which in turn results in muscle atrophy. Similarly, whole-body deletion of S6ki also leads to muscle atrophy (69–71), perhaps secondary to AMPK activation. Whether autophagy genes are upregulated in S6ki knockout mice was not addressed. Both Tg-4EBP1mt-muscle and S6ki knockout mice are lean and have high energy expenditure. Autophagy has been shown to be essential for maintaining favorable metabolic parameters in aging. For example, suppression of autophagy by knockout of Atg7 (72) or activation of mTORC1 signaling (19) leads to an impaired metabolic rate and age-dependent myopathy, whereas transgenic activation of Atg5 protects mice from age-induced metabolic decline and extends life span (73). It is possible that mild blunting of mTORC1 signaling in skeletal muscle leads to a sustained activation of autophagy. Our findings from the Tg-4EBP1mt-muscle mice thus indicate that elevated autophagy causes muscle atrophy and activates muscle metabolism, consequently stimulating fat metabolism and whole-body energy expenditure in the context of overnutrition and aging. However, a complete block of mTORC1 signaling leads to skeletal muscle deterioration and metabolic defects (16, 17).

Emerging evidence has identified 4E-BPs as key translational regulators mediating mTORC1-dependent signaling. Ribosome-profiling studies indicate that the translational targets of mTORC1 signaling are mainly mediated by 4E-BPs (74–76). Consistently, of the targets identified from previous studies (74, 75), Foxol and Akt1 were both increased in translation in skeletal muscle from Tg-4EBP1mt-muscle mice. Even though the previous ribosome-profiling studies were performed in cell lines, our data suggest similar results in vivo. Moreover, calorie restriction induces d4E-BP activation in the fly, which causes the selective translation of TOP mRNAs, indicating that enhanced mitochondrial biogenesis is a key underlying mechanism of 4E-BP-dependent life span extension (77). Here, we also found enhanced mitochondrial respiration with increased PGC-1α translation in 4E-BP1-activated skeletal muscle, suggesting that the beneficial effects of 4E-BP-regulated metabolic homeostasis are evolutionarily conserved.

Enhanced mTORC1 activity has been associated with glutamine addiction in cancer cells, as either reduced mTORC1 signaling or a depleted glutamine supply suppress tumor growth (48,
78–80). A recent study in a mouse model of Leigh syndrome found that repression of mTORC1 by rapamycin treatment in wild-type mice suppresses mitochondrial state III respiration rates in the presence of glutamate (47). Consistent with the current view that mTORC1 positively regulates glutamine metabolism, we observed that blunted mTORC1 activity via 4E-BP1 activation specifically suppresses glutamine metabolism, while enhancing the utilization of other TCA cycle substrates (81). Hence, 4E-BP1 activation may have therapeutic potential as a cancer treatment.

Reduced mTOR signaling mediated by rapamycin leads to a significant extension in life span and health span (5). The downstream effectors of these exciting phenotypes are yet to be fully resolved. Deletion of one mTORC1 target, S6K1, leads to life span extension, but to a lesser extent (10). The metabolic phenotypes reported here for 4E-BP1mt-mice suggest mTORC1-dependent regulation of 4E-BP1 in skeletal muscle as another potential conduit through which these beneficial effects might be mediated upon aging.

Methods

**Generation of tissue-specific 4E-BP1 transgenic mice and animals studies.** A constitutively active mutant form (T37A and T46A) of human 4E-BP1 cDNA was placed downstream of a loxP-GFP-3xstop-loxP cassette under the control of a chicken β-actin promoter. The GFP-stop cassette prevents expression of the 4E-BP1 transgenic allele. Transgenic mice were crossed with mice expressing a transgenic allele. Transgenic cDNA was placed downstream of a 4E-BP1 cassette, to selectively induced expression of a mutant 4E-BP1 transgene under the control of the anti-estrogen tamoxifen administration. Control mice (single-transgenic mice: HSA-Cre-ER<sup>2</sup> mice and Tg-4EBP1mt mice) and Tg-4EBP1mt-ER mice were treated with 0.1 mg tamoxifen dissolved in corn oil for 5 consecutive days at 8 weeks of age. All mice used in this study were backcrossed to the C57BL6/J background for 5 generations, and littermates were used in the study. For the HFD-induced type 2 diabetes study, mice at 8 weeks of age were placed on either a standard laboratory rodent chow (2018, Harlan Teklad) or HFD (D12492, Research Diets) as indicated and monitored for 24 weeks.

**Indirect calorimetric studies.** Oxygen consumption, CO₂ production, volunteer home cage activity, and food and water intake were measured by open-flow respirometry (Sable systems).

**Histological analysis and immunofluorescence.** Tissue samples were either fixed in 4% paraformaldehyde solution in PBS before being embedded in paraffin or directly mounted in OCT before being stored at -80°C. After harvesting, muscle tissues were equilibrated in 30% sucrose (in PBS) for 2 to 3 hours and flash-frozen in liquid nitrogen-cooled isopentane. Morphology was examined in the hematoyxin and eosin-stained section. Oil red O staining was performed as previously described (83). Periodic acid–Schiff and Masson’s trichrome staining were performed per the manufacturer’s instructions (PAS Staining System, Sigma-Aldrich, and Masson’s Trichrome Stain Kit, Dako). The size of muscle fibers and adipose cells was quantified in ImageJ. Results are shown as mean ± SEM of independent animals (n ≥ 4). Muscle types were distinguished and quantified by immunofluorescence staining using antibodies against specific muscle type on snap frozen muscle sections (43). Primary antibodies were purchased from the Developmental Studies Hybridoma Bank (University of Iowa): BF-F8, SC-71, and BF-F3. Secondary antibodies were purchased from Invitrogen: Alexa Fluor 350 mulG2b, Alexa Fluor 488 mulG1, and Alexa Fluor 555 mulGM.

**Treadmill endurance test.** Control and transgenic mice were trained on a treadmill (18 meters per minute for 15 minutes) every other day for 1 week before the final test. For the treadmill running test, mice were run on a treadmill at a 5-degree inclination at a speed of 18 meters per minute. Endurance was measured by the total distance mice ran before exhaustion (n = 3-10 mice in each genetic group).

**Blood lipids, glucose, and hormone analyses.** Mouse blood was collected using the submandibular pouch technique. Sera were separated using Serum Separator Tubes (365956, BD). IGF-1 and growth hormone assays were performed in nonfasted mouse serum. The analysis of lipids, leptin, FGF21, and insulin profile was performed in sera collected from mice that were fasted for 6 hours. Triglyceride, free fatty acid, and cholesterol tests were purchased from Stanbio. The insulin test was purchased from Mercodia. The FGF21 ELISA was purchased from R&D Systems. All the assays followed the manufacturer’s instructions. Leptin, IGF-1, and growth hormone tests were analyzed at the Diabetes, Endocrinology, and Metabolism Hormone Assay and Analytical Services Core at Vanderbilt University (n = 8–25 for each group).

**Glucose and insulin tolerance assays.** Glucose and insulin tolerance tests were performed on mice that were fasted for 6 hours. Glucose concentrations were determined with an Accu-Chek Advantage glucometer (Roche) in blood collected from the tail vein at the indicated time points. Insulin (0.75 U/kg for mice in the HFD group and 0.375 U/kg for mice in the normal chow diet group) or glucose (2 g/kg) was intraperitoneally injected into mice (n = 8 for each group).

**RNA analyses.** Total RNA was extracted from tissue using Trizol (Life Technologies) following the manufacturer’s instructions (n = 4 for each group; 6-month-old male mice). An amount of 2 μg total RNA was reversely transcribed into cDNA by using the SuperScript III Reverse Transcription Kit (Invitrogen). Real-time PCR was performed in the Roche 480 iCycler PCR machine. Reactions were performed in triplicate, and relative amounts of cDNA were normalized to cyclophilin A (Ppia) or hypoxanthine guanine phosphoribosyl transferase (Hprt).

**Protein isolation and immunoblotting studies.** Tissues were homogenized in cold SDS lysis buffer (50 mM Tris, pH 7.5, 70 mM urea, 250 mM sucrose, and 2% SDS) with Protease Inhibitor Cocktail (40693124001, Roche) and Phosphatase Inhibitor Cocktail (P5726 and P0044, Sigma-Aldrich). 30 μg total protein was separated in 4% to 12% BT Precast Gel (NP0315, Invitrogen) or TGX Precast Gel (456-9033, Bio-Rad) following the manufacturer’s instructions. Antibodies were from Cell Signaling (α-4E-BP1 [9452], α-eIF4E [2492], α-AKT1 [4691], α-PPAR [4058], α-UCP2 [U6382], α-UCP3 [ab3477], and α-SIRT4 [ab124521]), Santa Cruz Biotechnology Inc. (α-SREBP1 [SC-8984], and Sigma-Aldrich (α-UCP1 [U6382]).

**Cap-binding assay and immunoprecipitation.** Tissues were homogenized in cold buffer A (10 mM Tris, pH7.5, 150 mM KCl, 4 mM MgCl₂, and 1 mM EDTA and 1% NP-40) with Protease Inhibitor Cocktail (04693124001, Roche) and Phosphatase Inhibitor Cocktail II and III (P5726 and P0044, Sigma-Aldrich). For cap-binding assay, 1.2 mg tissue sections were prepared from the Developmental Studies Hybridoma Bank (University of Iowa): BF-F8, SC-71, and BF-F3. Secondary antibodies were purchased from Invitrogen: Alexa Fluor 350 mulG2b, Alexa Fluor 488 mulG1, and Alexa Fluor 555 mulGM.
sue lysate was incubated overnight at 4°C with 50 μl of the mRNA cap analogue m7GTP-agarose (Jena Bioscience) in buffer A. The complex was washed once with buffer A. For immunoprecipitation, 1.2 mg tissue lysate was incubated overnight at 4°C with 5 μg eIF4E-4E antibody or rabbit IgG in buffer A. 60 μl of the protein A/G plus-agarose (Santa Cruz) was added the next day and incubated at 4°C for 2 hours. The agarose beads were once washed in buffer A. Protein complex was eluted using 2× sample buffer and evaluated in Western blot.

Protein synthesis assay. A nonisotopetabeled protein synthesis assay was performed as previously described using the SUnSET method (84, 85). Puromycin (0.04 μg/ml puromycin) was injected 30 minutes prior to harvesting. Protein was extracted and analyzed as stated above. Total protein was assessed with Coomassie blue staining, and puromycin-incorporated newly synthesized protein was detected by puromycin antibody (gift from Philippe Pierre, INSERM).

Isolation of intact mitochondria and oxygen consumption measurement. Isolation of mitochondria was performed using standard methods (86). Male mouse skeletal muscle mitochondria was isolated. Briefly, mouse skeletal muscles were dissected out and then rinsed twice in ice-cold CP1 buffer (100 mM KCl, 50 mM Tris-HCl, and 2 mM EGTA, pH 7.2, at 25°C). Tissues were quickly minced on an ice-cutting board and digested with 250 U/ 0.11 protease type VIII, 0.5% BSA, 5 mM MgCl₂, and 1 mM ATP in CP1 buffer for 2.5 minutes at 4°C. After digestion, tissues were homogenized using a Dounce homogenizer, followed by an additional 3-minute incubation on ice, and centrifuged at 500 g at 4°C for 10 minutes. The supernatant was filtered through the 2 layers of muslin into the new centrifuge tube and centrifuged at 10,000 g at 4°C for 10 minutes, and the pellet was washed with ice-cold CP1 buffer twice. After final centrifugation at 10,000 g at 4°C for 10 minutes, the mitochondrial pellet was resuspended in CP1 buffer and immediately used for the oxygen consumption assay in XF24 Extracellular Flux Analyzer (Seahorse Bioscience) according to the manufacture’s instructions (87). Isolated muscle mitochondria (total 1.5 μg) was diluted in the mitochondrial assay solution (70 mM sucrose, 220 mM mannitol, 10 mM KH₂PO⁴, 5 mM MgCl₂, 5 mM HEPES, 2 mM EGTA, and 0.2% BSA). State II respiration rate was measured in the presence of substrate (60 μM palmitoyl carnitine with 0.5 mM malate, 10 mM pyruvate with 0.5 mM malate, 5 mM glutamate with 0.5 mM malate, or 5 mM succinate with 0.002 mM rotenone). State III respiration rate was measured by adding 6 mM ADP in the presence of substrates. State IV(0) uncoupling rate was measured by adding 2.5 μg/ml oligomycin. The maximal respiration capacity was measured by adding 4 μM FCCP.

Mitochondrial DNA copy number measurement. Total DNA was extracted from tissue using lysis buffer with 100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 0.1 mg/ml proteinase K. An amount of 0.2 μg total DNA was used for real-time PCR detection in the Roche 480 iCycler PCR machine. Reactions were performed in triplicate. Mitochondrial DNA was quantitated using CoxII gene and nuclear DNA was quantitated by β-actin.

Statistics. Unless otherwise stated, all results are expressed as mean ± SEM of n observations, and statistical differences among the groups were assessed by 2-way ANOVA. Bonferroni post-tests were used to compare replicate means by row. A unpaired and 1-tailed Student’s t test was also used. A P value of more than 0.05 was considered significant.

Study approval. All experimental procedures involving animals in this study were reviewed and approved by the IACUC of Buck Institute for Research on Aging.

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