Deficiency in mTORC1-controlled C/EBPβ-mRNA translation improves metabolic health in mice

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Abstract

The mammalian target of rapamycin complex 1 (mTORC1) is a central regulator of physiological adaptations in response to changes in nutrient supply. Major downstream targets of mTORC1 signalling are the mRNA translation regulators p70 ribosomal protein S6 kinase 1 (S6K1/p70) and the 4E-binding proteins (4E-BPs). However, little is known about vertebrate mRNAs that are specifically controlled by mTORC1 signalling and are engaged in regulating mTORC1-associated physiology. Here, we show that translation of the CCAAT/enhancer binding protein beta (C/EBPβ) mRNA into the C/EBPβ-LIP isoform is suppressed in response to mTORC1 inhibition either through pharmacological treatment or through calorie restriction. Our data indicate that the function of 4E-BPs is required for suppression of LIP. Intriguingly, mice lacking the cis-regulatory upstream open reading frame (uORF) in the C/EBPβ-mRNA, which is required for mTORC1-stimulated translation into C/EBPβ-LIP, display an improved metabolic phenotype with features also found under calorie restriction. Thus, our data suggest that translational adjustment of C/EBPβ-isomorph expression is one of the key processes that direct metabolic adaptation in response to changes in mTORC1 activity.

Keywords C/EBPβ; calorie restriction; metabolism; mTORC1; translation

Introduction

C/EBPβ is a transcriptional regulator with a broad tissue expression including liver and adipose tissue (http://www.genecards.org). It controls genes related to glucose and fat metabolism as well as other cellular processes [1,2]. The Cebpb gene is intronless, and from its mRNA three different protein isoforms are expressed through usage of alternative translation initiation sites (Fig EV1A). The isoforms LAP* and LAP (liver activating protein) are transcriptional activators that consist of transactivation domains and a DNA-binding domain. The truncated isoform LIP (liver inhibitory protein) lacks the N-terminal transactivation domains but still possesses the DNA-binding domain. LIP can therefore act as a competitive inhibitor of LAP*/LAP function [3]. However, LIP may also have additional and distinct functions. Hence, the ratio between LAP and LIP is crucial for the biological functions elicited by C/EBPβ. Translation from both the LAP* and LAP AUG codons is achieved by regular translation initiation, although translation into LAP* is often weaker since this AUG codon lacks a Kozak consensus sequence required for efficient recognition by the ribosome [4,5]. Expression of LIP from a distal initiation codon depends on a cis-regulatory uORF located in the 5' UTR of the C/EBPβ-mRNA. The limited size of the uORF allows the small ribosomal subunit to remain attached to the mRNA after translation termination and to resume scanning along the mRNA. After reloading of the ribosomal complex with initiator tRNA, translation of LIP from the downstream initiation codon can be re-initiated. Mutation of the uORF consequently results in diminished LIP expression [4,5] (see also schematic representation in Fig EV1A).

Reducing signalling through mTORC1 by pharmacological treatment, mutations, restricted calorie intake or low protein:carbohydrate macronutrient ratio enhances metabolic health and increases life span in many species up to mammals. On the contrary, hyperactivation of mTORC1 is believed to promote metabolic disorders resulting from overfeeding such as diabetes [6–15]. Our earlier studies pointed to an involvement of mTORC1 signalling in the regulation of C/EBPβ-LIP expression, since mTORC1 inhibition by rapamycin reduced LIP expression in a uORF-dependent manner [4,16]. Here, we show that interventions that cause a reduction in mTORC1 signalling also decrease the translation of the C/EBPβ-LIP.
isofrom. Genetic elimination of the mTORC1-sensitive uORF of the C/EBPβ-mRNA in mice similarly results in a reduction of the LIP isofrom [5]. Our data show that mice with this mutation display an improved metabolic phenotype, including reduced fat accumulation and increased β-oxidation, improved insulin sensitivity and glucose tolerance as well as enhanced activity. Thus, pharmacological targeting of C/EBP-isoform expression may provide a promising strategy for the treatment of metabolic diseases such as obesity and type II diabetes, thereby extending health span.

Results

mTORC1 controls C/EBPβ-LIP expression

To clarify the regulation of C/EBPβ-isoform expression by mTORC1, we treated different cell lines with the catalytic pan-mTOR inhibitor pp242 or the allosteric mTOR inhibitor rapamycin. Rapamycin primarily acts on mTORC1 but was shown to also affect mTORC2 after prolonged treatment [17]. Treatment with pp242 resulted in a strong reduction in LIP levels without affecting LAP expression in all cell lines tested (Figs 1A and EV1B). pp242 treatment consistently resulted in strong de-phosphorylation of the mTORC1 targets S6K1 (Thr389) and 4E-BP1 (Thr37/46), which was analysed with phospho-specific antibodies. Reduced phosphorylation of 4E-BP1 is also visible in the pan-4E-BP1 immunoblots as a decrease in γ/β-phosphorylation signals and an increase in α-hypophosphorylation signals. Treatment with rapamycin strongly reduced S6K1 phosphorylation in all cases. However, the effect on 4E-BP1 phosphorylation and LIP levels was weaker and varied between different cell types (Figs 1A and EV1B). The correlation between 4E-BP1 phosphorylation state and LIP expression levels suggests that decreased phosphorylation of 4E-BP1 and the resulting restrain of eukaryotic initiation factor 4E (eIF4E) are important for reducing LIP expression through mTORC1 inhibition in these cells. To discriminate between effects on C/EBPβ-isoform expression by 4E-BPs or S6K1, we used 4E-BP1/4E-BP2 double knockout (4E-BP DKO) MEFs (mouse embryonic fibroblasts), or MEFs treated with either the S6K1 inhibitor DG2 or an sh-RNA targeting S6K1. It has been shown that 4E-BP DKO MEFs contain more accessible eIF4E [18,19]. In 4E-BP DKO cells, expression of LIP was strongly increased compared to control cells. This was concomitant with a lower LAP/LIP ratio, which could not be reversed by pp242 treatment (Fig 1B). Treatment with the S6K1 inhibitor DG2 also did not reduce LIP expression in wt MEFs, GH3 or 3T3-L1 cells although S6K1 activity was completely abolished as shown by the lack of S6 (Ser235/236) phosphorylation (Figs 1C and EV1C). On the contrary, LIP levels were enhanced by DG2 treatment. Similarly, S6K1 knockdown did not reduce but rather stimulated LIP expression (Fig EV1D) by a yet to be identified mechanism. Therefore, our data indicate that mTORC1 inhibition decreases LIP expression rather through 4E-BPs than through S6K1. Since pp242 inhibits both mTORC1 and mTORC2 [20], we employed a knockdown of either the mTORC1-specific component raptor or the mTORC2-specific component rictor to clarify their involvement in the regulation of LIP expression. Knockdown of raptor resulted in decreased LIP expression (higher LAP/LIP ratio) and a concomitant decrease in phosphorylation of S6K1 and 4E-BP1, while knockdown of rictor reduced the rictor-mTORC2-sensitive Ser473-Akt phosphorylation but did not change the LAP/LIP ratio, although we observed reduced expression levels of all protein isoforms (Fig 1D). These data demonstrate that only mTORC1 specifically regulates the ratio of LAP/LIP expression.

Limiting mTORC1 activity through inhibition by rapamycin or caloric restriction inhibits C/EBPβ-LIP expression in vivo

To verify mTORC1-dependent C/EBPβ-isoform expression in vivo, mice were injected intraperitoneally with rapamycin and analysed for expression of C/EBPβ on protein level. In liver (Fig 2A) and epididymal white adipose tissue (eWAT) (Fig 2B), treatment with rapamycin led to higher LAP/LIP ratios mainly resulting from decreased LIP expression. The efficacy of mTORC1 inhibition through treatment with rapamycin was shown by reduced phosphorylation of S6K1, S6 or 4E-BP1.

The mTORC1 signalling pathway is an important regulator of metabolic adaptations in response to nutritional changes. Therefore, we examined mTORC1-mediated C/EBPβ regulation in adult wt mice fed either ad libitum (AL) or caloric restricted (CR) for 4 weeks. As described by [21], mice on a CR regime consume their daily single food allotment immediately followed by a prolonged period of absence of food (mice fed AL spread their food intake over the day). This results in a pronounced change in whole body fuel selection with an initial nutrition phase of high carbohydrate utilisation and a prolonged starvation phase of primarily fat utilisation that lasts until the next feeding [21]. We verified the dynamics of fuel selection in AL and CR mice by calculating the respiratory exchange ratio (RER) between the amount of CO2 exhaled and O2 inhaled from mice kept individually in metabolic chambers (RER = VCO2/VO2 = 1.0 for pure carbohydrate usage; RER = 0.7 for pure fat usage) (see Fig EV2A and legend for further explanation). This analysis allows covering these accentuated nutritional states for analysing mTORC1 activity and C/EBPβ-isoform expression. We chose 3 and 14 h after feeding as time points of analysis since they represent the maximal usage of carbohydrate or fat in CR fed mice, respectively. At 3 h post-feeding, mTORC1 activity was slightly reduced in livers from CR compared to mice fed AL as reflected by the levels of phosphorylated S6 and 4E-BP1 (Fig 2C). This correlated with a moderate reduction in LIP levels and resulted in a slight change in the LAP/LIP ratio in the CR fed mice. However, in the starvation phase at 14 h, both mTORC1 activity and LIP expression were reduced to a higher extent in the CR fed mice compared to mice fed AL (Fig 2C). Mice fed AL display more moderate diurnal cycles of fuel selection with a relative high mTORC1 activity and a LAP/LIP ratio that stays constant at 3 and 14 h (Fig 2C). These data show that mTORC1 signalling alternates between the activated and suppressed state in liver during the diurnal cycle of fuel selection under CR. Furthermore, these data demonstrate that LIP expression levels follow the changes in mTORC1 activity also under these physiologically induced conditions.

Taken together, our data show that expression of the C/EBPβ-LIP isofrom correlates with mTORC1/4E-BP1 signalling in vitro and in vivo and follows mTORC1 activity upon changes in calorie supply or pharmacological inhibition of mTORC1.
To investigate whether a permanently altered C/EBPβ isoform ratio affects metabolic performance, we analysed mice deficient in the cis-regulatory uORF that is required for LIP expression (C/EBPβ\textsuperscript{AuORF/BL6} mice; based on mice described in [5], but back-crossed in C57BL/6J background, see also Fig EV1A). These mice display a diminished LIP expression that was not further reduced by rapamycin treatment (Figs 2D and EV2B) or through caloric restriction (Fig 2E). Thus, the C/EBPβ\textsuperscript{AuORF/BL6} mice with their invariably low LIP expression mimic reduced mTORC1 activity
Figure 2.

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at the level of C/EBPβ translation. mTORC1 activity itself does not seem to be influenced by the C/EBPβuORF/BL6 mutation or the reduced LIP levels since neither 56 phosphorylation nor 4E-BP1 phosphorylation was noticeably altered in livers from C/EBPβuORF/BL6 mice compared to wt mice (3 h post-feeding) (Fig EV2C).

We found that C/EBPβuORF/BL6 mice fed AL displayed a reduced body weight compared to wt mice accumulating to a difference of 8% at 26 weeks of age (Fig 3A). This was not due to changes in body length, food intake or caloric utilisation (Fig 3B–D). C/EBPβuORF/BL6 mice even showed a slightly increased food intake. To investigate why the body weight was reduced, we performed an abdominal computed tomography (CT) to analyse body composition. The data demonstrated that both visceral and subcutaneous fat volumes were clearly reduced in C/EBPβuORF/BL6 mice compared to age- and sex-matched littermate controls (Fig 3E), while there was no significant change in the lean body mass (Fig 3F). Histological analyses of epididymal fat pads revealed an average reduction of white adipocyte cell size of 30% in C/EBPβuORF/BL6 male mice compared to wt control tissue (Fig 3G). C/EBPβ is a known regulator of adipogenesis and induces transcription of the adipogenic transcription factors C/EBPα and PPARγ. mRNA expression levels of C/EBPα and PPARγ were similar in visceral adipose tissue of C/EBPβuORF/BL6 mice compared to control littersmates. This indicates that the reduced fat accumulation in WAT is not caused by deficiencies of key adipogenic transcription factors (Fig EV3A). Notably, MEFs derived from C/EBPβuORF/BL6 mice displayed increased differentiation into adipocytes in cell culture compared to MEFs derived from wt mice, as was revealed by Oil Red O staining of lipid droplets (Fig EV3B). Vice versa, experimental induction of LIP in 3T3-L1 adipocytes resulted in less efficient adipogenic differentiation and a reduction in fat accumulation (Fig EV3C) reminiscent of what has been described before [4]. In accordance with the lower fat content of C/EBPβuORF/BL6 adipocytes, adiponectin levels were increased (Fig 3H), while leptin levels were unchanged (Fig EV3D) in the blood plasma of C/EBPβuORF/BL6 mice compared to wt controls. High levels of the adipocyte-derived hormone adiponectin correlate with increased fatty acid oxidation, reduced lipid accumulation in non-adipose tissues and increased insulin sensitivity [22–24]. This prompted us to examine whether whole body fatty acid oxidation is increased in the C/EBPβuORF/BL6 mice by determination of the RER using metabolic cages. The RER curves of both C/EBPβuORF/BL6 and wt mice fed ad libitum reflected the diurnal rhythm with a higher RER in the active (dark) phase representing mostly carbohydrate usage and a lower RER in the resting (light) phase in which more fatty acids are oxidised. As shown in Fig 4A, we measured continuously lower RER values both over the active and over the resting phases for C/EBPβuORF/BL6 mice compared to wt mice. Thus, C/EBPβuORF/BL6 mice have a moderate but significant daily increase in fatty acid oxidation over carbohydrate oxidation under normal feeding conditions. Furthermore, oxygen consumption of the C/EBPβuORF/BL6 mice was increased compared to wt mice, indicating that the C/EBPβuORF/BL6 mice have a higher energy expenditure (Fig 4B). To examine whether altering the LIP/LAP ratio results in a cell intrinsic shift in β-oxidation, we ectopically expressed LIP in the mouse hepatoma cell line Hepa 1-6 and studied palmitate-substrate fatty acid oxidation (FAO) using the Seahorse FX extracellular flux analyser. Ectopic expression of LIP (low LAP/LIP ratio) resulted in a reduced FAO compared to FAO in the parent cells (high LAP/LIP ratio) (Fig 4C). Hence, the LAP/-LIP-associated β-oxidation changes found in cell culture support the phenotype found in C/EBPβuORF/BL6 mice.

Increased fatty acid oxidation is known to improve the health status by lowering the concentration of free fatty acids in the serum and counteracting lipid accumulation in non-adipose tissues [25]. In the serum of C/EBPβuORF/BL6 mice, the concentration of free fatty acids (FFA) and triglycerides (TG) was reduced compared to wt mice, while the levels of cholesterol/high-density lipoprotein (HDL)/low-density lipoprotein (LDL) were similar (Fig 4D). Furthermore, lipid accumulation in liver and heart was strongly reduced in 8-month-old C/EBPβuORF/BL6 mice compared to wt littersmates as revealed by Sudan III staining (Fig 4E).

C/EBPβuORF/BL6 mice display a CR-like metabolic gene expression profile

Next, we analysed mRNA expression of genes involved in fat metabolism that could be involved in the metabolic phenotype of C/EBPβuORF/BL6 mice. We measured a moderate but consistent shift
towards higher transcript levels of genes that are involved in glucose/fat transport, lipogenesis and lipolysis in WAT of C/EBPΔuORF mice compared to littermate controls using quantitative real-time PCR (Fig 5A). This points towards an increased fat turnover in adipose tissue. We found elevated transcript levels of the insulin-dependent glucose transporter GLUT4 (1.6-fold, \( P = 0.027 \)) required for glucose uptake for \textit{de novo} lipogenesis. Furthermore, we detected increased mRNA levels of fatty acid translocase (FAT)/cluster of differentiation 36 (CD36) (1.5-fold, \( P = 0.007 \)) that is involved in fatty acid uptake. We observed a small but not significant increase for lipoprotein lipase (LPL) (1.4-fold, \( P = 0.052 \)) or the intracellular fatty acid binding protein aP2 (1.2-fold, \( P = 0.376 \)). In addition, transcripts that stimulate lipogenesis were elevated: the sterol regulatory element-binding protein 1c (SREBP1c) (1.5-fold, \( P = 0.002 \)), acetyl-CoA carboxylase (ACC), which is the flux-determining enzyme of the lipogenic pathway (1.4-fold, \( P = 0.021 \)), the key enzyme in fatty acid synthesis fatty acid synthase (FAS) (1.7-fold, \( P = 0.002 \)) and stearoyl-coenzyme A desaturase 1 (SCD1) (1.6-fold, \( P = 0.015 \)), which is important for the synthesis and regulation of unsaturated fatty acids. Finally, among the transcripts that stimulate lipolysis, the hormone-sensitive lipase (HSL) mRNA was elevated (1.4-fold, \( P = 0.007 \)), while upregulation of the adipose triglyceride lipase (ATGL) transcript was not significant (1.3-fold, \( P = 0.142 \)). Immunoblot
analyses showed that the moderate increases in mRNA levels correlate with significantly increased protein levels for FAS, ACC and GLUT4. Smaller increases were observed for CD36 or HSL (Fig EV4A). To investigate whether expression of genes involved in β-oxidation is altered in WAT, we examined mRNA expression of the short-chain (SCAD), medium-chain (MCAD), long-chain (LCAD) and very long-chain (VLCAD) acyl-CoA dehydrogenases (Fig 5A). Although we observed a general tendency towards enhanced expression, only the MCAD transcript was found to be significantly upregulated (1.7-fold, \( P = 0.013 \)), which is also reflected on the protein levels (Fig EV4A).

Next, we studied cell intrinsic effects of LAP/LIP ratio on adipogenic gene expression in primary MEFs or the adipoblast cell line 3T3-L1 in cell culture. Briefly, we transduced confluent C/EBP\( \beta^{uORF}/BL6 \) MEFs with a lentivirus expressing either C/EBP\( \beta \)-LIP or an empty control vector, and induced the adipogenic programme. mRNA expression of all examined adipogenic genes was generally downregulated by ectopic expression of LIP (low LAP/LIP ratio) compared to control C/EBP\( \beta^{uORF}/BL6 \) MEFs (high LAP/LIP ratio) (Fig EV4B). In 3T3-L1 cells containing an inducible LIP expression cassette (cumate-inducible system), adipogenesis was induced simultaneously with LIP induction (+ cumate) or without ectopic LIP induction (− cumate, solvent treatment) as control. Induction of LIP resulted in reduced expression of adipogenic transcripts measured at day 6 of differentiation (Fig EV4C). Cumate treatment of the empty vector control cells had no or a rather stimulatory effect on the expression of adipogenic genes, ruling out that cumate itself acts anti-adipogenic. Therefore, LAP-/LIP-associated regulation of adipogenic transcripts found in cell culture supports the observations we made in mice.

In the liver of C/EBP\( \beta^{uORF}/BL6 \) mice, the expression of the following transcripts involved in fatty acid β-oxidation was upregulated compared to wt mice: the peroxisome acyl coenzyme...
A oxidase (AOX) (1.6-fold, \( P = 0.007 \)), SCAD (1.3-fold, \( P = 0.016 \)), MCAD (1.4-fold, \( P = 0.003 \)), LCAD (1.3-fold, \( P = 0.009 \)) and VLCAD (1.3-fold, \( P = 0.009 \)). Only expression of the FAT/CD36 mRNA for fatty acid uptake was not significantly increased (1.7-fold, \( P = 0.057 \)) (Fig 5B). On the contrary, transcript levels of factors fostering lipogenesis were unchanged (GLUT2, SREBP1c, ACC and SCD1) or even decreased (FAS, \( P = 0.041 \)). Furthermore, immunoblot analyses showed that expression of FAS and ACC proteins (lipogenesis) is decreased, while expression of the MCAD, AOX and CD36 (\( \beta \)-oxidation) was not significantly different (Fig EV4D). To further support the direct regulatory role of C/EBP\( \beta \) in the examined gene regulation, we analysed the ENCODE database (http://genome.ucsc.edu/ENCODE/) for promoter occupation by chromatin immunoprecipitation sequencing (ChIP-Seq). This analysis revealed that all genes analysed are associated with C/EBP\( \beta \) at regions that are in most cases associated with the histone H3 lysine 27 acetylation (H3K27Ac) mark characterising active enhancers (Table EV1).

**C/EBP\( \beta^{uORF/BL6} \) mice are physically more active**

An intriguing aspect of the C/EBP\( \beta^{uORF/BL6} \) phenotype is the lower body weight in spite of similar food intake and caloric utilisation. This points to an increased energy expenditure in C/EBP\( \beta^{uORF/BL6} \) mice, which is supported by the observed increase in oxygen consumption. Possible causes for higher energy use are respiratory uncoupling in BAT, browning of WAT or an increase in physical activity. To examine the potential involvement of altered BAT or WAT function,
we analysed mRNA expression levels of genes involved in uncoupling and thermogenesis (UCP1, PGC-1α, Dio2, PRDM16) in BAT, inguinal (i) WAT or epididymal (e) WAT (Fig 5C). The expression of those genes was very low in both iWAT and eWAT and not increased in C/EBPβ<sup>uORF/BL6</sup> mice excluding a critical involvement of uncoupling or browning of WAT as a cause of the higher energy expenditure.

To examine if an increase in physical activity could be the cause for this phenotype, we determined physical activity of C/EBPβ<sup>uORF/BL6</sup> mice compared to age- and sex-matched littermate controls using the Oxymax/CLAMS animal motion detection system. As shown in Fig SD, the total activity (Xtot; total counts of fine movements (e.g. grooming) and ambulatory activity), as well as the ambulatory activity (Xamb; “distance”) and vertical activity (Ztot; “rearing”) of the C/EBPβ<sup>uORF/BL6</sup> mice, were higher compared to wt mice during their active period at night. Thus, the reduced body weight of the C/EBPβ<sup>uORF/BL6</sup> mice might be caused by higher energy expenditure due to their increased physical activity.

**C/EBPβ<sup>uORF/BL6</sup> mice have improved glucose clearance and insulin sensitivity**

Fat metabolism is highly interconnected with glucose metabolism and particularly with insulin responsiveness. Therefore, we analysed glucose metabolism in C/EBPβ<sup>uORF/BL6</sup> mice. The intraperitoneal (i.p.) glucose tolerance test (IPGTT) revealed an enhanced glucose clearance in C/EBPβ<sup>uORF/BL6</sup> mice, which is reflected by a smaller area under the curve (AUC) (Fig 6A). The i.p. insulin sensitivity test (IPIST) demonstrated that C/EBPβ<sup>uORF/BL6</sup> mice also display an increase in insulin sensitivity compared to wt control mice (Fig 6B). Accordingly, tissue insulin sensitivity was higher in C/EBPβ<sup>uORF/BL6</sup> mice, as revealed by enhanced induction of Akt-Ser473 phosphorylation in muscle and liver after intravenous (i.v.) insulin injection (Figs 6C and EV5A). Improved insulin sensitivity is usually accompanied by reduced levels of circulating glucose and insulin, which is also found under caloric restriction [26]. In C/EBPβ<sup>uORF/BL6</sup> mice, fasting insulin and fed glucose levels were lower compared to wt mice, while fasting glucose and fed insulin levels showed no significant reduction (Fig 6D and E). The significantly lower HOMA-IR (homeostatic model assessment of insulin resistance) supports the conclusion that C/EBPβ<sup>uORF/BL6</sup> mice show improvements in insulin sensitivity (Fig 6F). To exclude that the lower insulin levels were due to aberrantly reduced pancreatic β-cell mass, insulin production in the pancreas was examined by quantitative immunofluorescence analysis. We observed no difference in β-cell mass but a decreased insulin production in C/EBPβ<sup>uORF/BL6</sup> mice compared to wt mice (Fig EV5B). This indicates that the improved tissue insulin sensitivity in C/EBPβ<sup>uORF/BL6</sup> mice requires less insulin production for efficient function.

**Discussion**

Reduced mTORC1 signalling is thought to be responsible for many of the metabolic improvements under caloric restriction (CR) [7] and is believed to attenuate the development of the metabolic syndrome [15]. Here, we show that the C/EBPβ<sup>uORF/BL6</sup> mice display a range of metabolic improvements that are remarkably similar to what has been described for CR, however, without reducing calorie (food) intake. Loss of the C/EBPβ uORF leads to diminished expression of the C/EBPβ-LIP isofrom in vitro and in vivo [4,5]. This mimics reduced mTORC1 activity at the level of C/EBPβ translation and is sufficient to improve a whole set of metabolic health parameters.

We observed a gradual difference in body weight for the C/EBPβ<sup>uORF/BL6</sup> mice compared to wt mice, which becomes apparent after maturity (week 12) and accumulates to a reduction of 8% in adult C/EBPβ<sup>uORF/BL6</sup> mice (week 26) (Fig 3A). The reduced body weight of the C/EBPβ<sup>uORF/BL6</sup> mice is largely due to reduced fat accumulation in WAT. This is accompanied by increased levels of the adipocyte-specific hormone adiponectin and a metabolic shift in whole body energy utilisation towards more fatty acid oxidation (lower RER) (Figs 3E and H, 4A). In addition, we found increased expression of lipogenesis and lipolysis genes in WAT and increased expression of β-oxidation genes in liver (Fig 5A and B). Together with the enhanced adipocyte differentiation of C/EBPβ<sup>uORF/BL6</sup> MEFs (Fig EV3B) in cell culture, the increased expression of lipogenesis genes in WAT may seem to be inconsistent with the lower fat accumulation and leanness in the C/EBPβ<sup>uORF/BL6</sup> mice. However, increased expression of lipogenesis and lipolysis genes in WAT and increased expression of β-oxidation genes in liver are also observed in mice on CR [21,27–30]. Intriguingly, in calorie-restricted mice, fatty acid oxidation exceeds fat intake [21]. Thus, additionally required amounts of fat are generated from ingested carbohydrates by de novo lipogenesis in WAT followed by lipolysis to meet the increased requirements of fatty acids for energy production. The C/EBPβ<sup>uORF/BL6</sup> mutation may induce a similar metabolic round-about of enhanced WAT function and fat turnover coupled to increased fatty acid oxidation.

The number of fat cells in WAT of the C/EBPβ<sup>uORF/BL6</sup> mice, as can be calculated from the fat volume (CT analysis) and cell size (histology), does not seem to be altered, indicating that the reduction of fat mass observed in C/EBPβ<sup>uORF/BL6</sup> mice results from less fat storage. This suggests that the enhanced adipogenic differentiation potential observed in MEFs derived from C/EBPβ<sup>uORF/BL6</sup> mice in vitro does not lead to increased number of adipocytes in vivo.

The prolonged period of increased fatty acid oxidation is thought to contribute to the healthy phenotype induced by CR [21]. This is also supported by a study in flies, which shows that the reduction in fatty acid oxidation limits CR-induced life span extension [31]. Therefore, we hypothesise that C/EBPβ is an important factor in regulating the CR type of fat metabolism. Moreover, we postulate that the increased LAP/LIP ratio as a result of the ΔuORF mutation retains this metabolic state in C/EBPβ<sup>uORF/BL6</sup> mice without reduction in food intake. A high rate of fatty acid oxidation prevents the accumulation of lipids in the liver and in other non-adipose organs [25]. Reduction in liver fat has been shown to increase insulin sensitivity [32]. We found decreased levels of free fatty acids in the serum and strongly reduced lipid accumulation in liver and heart of C/EBPβ<sup>uORF/BL6</sup> mice compared to wt littermates. Moreover, we found lower glucose and insulin levels in the serum with concomitantly increased glucose tolerance and insulin sensitivity [29]. Together with the increased fatty acid oxidation,
these findings underscore the healthy metabolic condition of the mice.

The C/EBP\textsuperscript{\textDelta uORF/BL\textsuperscript{6}} mice have a similar food intake compared to wt mice but are leaner most probably due to higher energy expenditure as indicated by their increased oxygen consumption. An important question deals with the underlying cause of the higher energy expenditure. Our data indicate that neither increased respiratory uncoupling in BAT nor browning of WAT are causative factors for the higher energy expenditure. However, the C/EBP\textsuperscript{\textDelta uORF/BL\textsuperscript{6}} mice display an increased physical activity, suggesting that the associated higher energy expenditure contributes to the lower body weight (Fig 5D). Elevated physical activity is also associated with rapamycin treatment [33,34] or CR [35–37]. This suggests that the C/EBP\textsuperscript{\textDelta uORF/BL\textsuperscript{6}} mutation might mimic effects of mTORC1 inhibition on physical activity. Intriguingly, a study using flies demonstrated that increased activity is not only associated with CR but is also at least partially required to induce the beneficial effects of CR [31].

The transcriptional effects we observed in the C/EBP\textsuperscript{\textDelta uORF/BL\textsuperscript{6}} mice are moderate (Fig 5A and B), although they translate into
changes on protein levels in most cases. The more subtle changes in gene expression may support the improved overall metabolic phenotype: a small and consistent shift in gene regulation resulting in a continuous shift in metabolism as suggested by the continuously lower RER (Fig 4A) that is still in a physiological range. Stronger transcriptional effects may result in a fully unbalanced metabolism with detrimental metabolic effects. In addition to its direct involvement in gene regulation, the C/EBP\textsuperscript{aORF/BL6} mutation most likely results in systemic (hormonal) alterations such as increased serum concentration of adiponectin that contributes to the improved metabolic parameters.

The phenotypes observed in C/EBP\textsuperscript{aORF/BL6} mice are similar to those described for C/EBP\textsuperscript{b/-} complete knockout mice with dietary or genetically induced obesity. C/EBP\textsuperscript{b/-} mice on a high fat diet display a reduced accumulation of body fat, are resistant to steatosis and have enhanced fatty acid oxidation compared to wt mice that develop an obese phenotype [38]. Lepr\textsuperscript{b/-} mice are homozygous for a loss of function mutation in the Leptin receptor and become obese at ~4 weeks of age. Complete C/EBP\textsuperscript{b} deficiency in Lepr\textsuperscript{b/-} mice results in a general healthier metabolic phenotype with reduced total body fat and weight gain, less steatosis, enhanced fatty acid oxidation and better glucose homeostasis [39]. The fact that the reduced total body fat and weight gain, less steatosis, enhanced fatty acid oxidation and better glucose homeostasis [39]. The fact that the metabolic phenotype of C/EBP\textsuperscript{aORF/BL6} mice (normal expression levels of LAP) resembles the one of C/EBP\textsuperscript{b/-} knockout mice suggests that the lack of the LAP isoform and not the lack of LAP is decisive for the phenotype. Therefore, the complete C/EBP\textsuperscript{b} knockout may display the beneficial metabolic phenotypes because of its deficiency for the metabolic “harmful” LAP isoform that is under control of mTORC1.

Many open questions remain how CR and the associated metabolic changes influence health span. However, it is intriguing that mutation of a single mRNA-translation cis-regulatory element in a single gene results in a phenotype that (at least partially) resembles the phenotype induced by CR. This implies that pharmacological alteration of the C/EBP\textsuperscript{b}-isoform ratio may provide a promising therapeutic strategy to intervene with metabolism-related disorders, thereby increasing health span.

**Materials and Methods**

**Cell culture**

HEK293T, Fao, 3T3-L1 (all obtained from ATCC), Hepa 1-6 cells, p53\textsuperscript{-/-} MEFs, 4E-BP wt and DKO MEFs [19], immortalised C/EBP\textsuperscript{aORF/BL6} and C/EBP\textsuperscript{b} KO MEFs and freshly isolated C/EBP\textsuperscript{aORF/BL6} and C/EBP\textsuperscript{b} KO MEFs and the corresponding wt MEFs were maintained in DMEM supplemented with 10% FCS, 1% HEPES and penicillin/streptomycin. GH3 cells were maintained in F12K medium supplemented with 15% horse serum, 2.5% FCS, 1% HEPES and penicillin/streptomycin. For mTOR repression, cells were incubated with pp242 (1 \textmu M) or rapamycin (1 \textmu M or 200 nM for GH3 cells) for different time periods (6 h for Fao, 12 h for MEFs, 24 h for 3T3-L1 and GH3). For S6K1 inhibition, cells were incubated with DG2 (20 \textmu M) for different time periods (12 h for MEFs, 24 h for 3T3-L1 and GH3). For inhibitor treatment, 3T3-L1 cells had been differentiated for 4 days (as described for primary MEFs but in the absence of troglitazone).

**DNA constructs**

The mouse S6 kinase 1 shRNA expression vector was generated by annealing the oligonucleotides sh-a 5\textsuperscript{'}-CGG AAG TGC ATT GTA CAC CCA GTA TCT GTA GAT ACT GCC TGT GTA ACA ATG TTT TTT-3\textsuperscript{'} and sh-b 5\textsuperscript{'}-SAA TAA AAA ACA TGG TTA CAC ACC CAG TAT CTC GAG ATA CTC GCT GTG TAA CAA TGT-3\textsuperscript{'} and ligating them into the Tet-pLKO-puro vector (Addgene plasmid 21915, described in [40]). For generating the C/EBP\textsuperscript{a} LAP or C/EBP\textsuperscript{b} LAP expression vectors, the rat C/EBP\textsuperscript{a} mutants AD and C, respectively, that are described in [4] were cloned into pCDNA3 or pZeoSV2 vector (both from Invitrogen). A FLAG-tagged version of rat C/EBP\textsuperscript{b} LAP [16] was cloned into the lentiviral pLVX-ires-neo expression vector (Clontech), and for the generation of the cumate-inducible C/EBP\textsuperscript{b} LAP-FLAG lentiviral construct, it was cloned into the pCDH-CuO-MCS-ires-GFP-ef1-cym-R-T2A-Puro-All-in-one vector (System Biosciences).

**Isolation and differentiation of primary MEFs**

Mouse embryonic fibroblasts (MEFs) were isolated from embryos at embryonic day 14.5 following standard protocols. Cells from passage 3 were seeded into 10-cm dishes for differentiation assays. Adipogenic differentiation was induced 2 days after cells reached confluency by replacing the medium with differentiation medium (DMEM containing 1 \mu M dexamethasone, 0.5 mM methylisobutyxanthine, 10 \mu g/ml insulin, 10 \mu M troglitazone and 10% FCS). After 2 days of incubation, the medium was replaced by DMEM supplemented with 10 \mu g/ml insulin and 10% FCS and then replaced every second day. At different days of the differentiation protocol, cells were fixed with 4% PFA and stained with Oil Red O.

**Immortalisation of primary MEFs**

Primary MEFs of passage 2 were retrovirally infected with a pSUPER-retro-based shRNA construct targeting p19ARF using the PhoenixE producer cell line as described in [4] and selected with puromycin (1.5 \mu g/ml).

**Transfection**

For lentivirus production, HEK293T cells were seeded to a density of 3 \times 10^6 cells in 10-cm culture dishes. Twenty-four hours later, transfection was carried out using the calcium phosphate method. For stable C/EBP\textsuperscript{b} LAP expression, a pCDNA3-based LIP expression vector was transfected into Hepa 1-6 cells using the Fugene HD transfection reagent (Promega) according to the protocol of the manufacturer. Transfected cells were selected with 0.9 mg/ml G418. For stable expression of C/EBP\textsuperscript{b} LAP\*, C/EBP\textsuperscript{b} LAP and C/EBP\textsuperscript{b} LAP immortarlised C/EBP\textsuperscript{b} KO MEFs were transfected with pZeoSV2-based expression vectors using the Fugene transfection reagent and selected with zeocin (0.1 mg/ml).

**Lentiviral transduction**

p53\textsuperscript{-/-} MEFs were infected following a standard protocol with pLKO.1 lentiviral constructs containing shRNAs against mouse
C57BL/6 genetic background (C/EBPβD°raptor: sh 5'-CCG GCC TCA TCG TCA AGT CCT TCA ACT CGA GTT GAA GGA CTT GAC GAT GAG GTT TTT G-3' (Addgene plasmid 21339); mouse raptor: 5'-CCG GCC CAG TAA GAT GGG AAT CAT TCT CGA GAA TGG TTC CCA TCT TAC TGG CTT TTT G-3' (Addgene plasmid 21341), both described in [41], mouse S6 kinase 1: 5'-CCG GAC ATT GTT ACA CAG CCA GTA TCT CGA GAT ACT GGC TGT GTA ACA ATG TTT TTT-3' [42].

Feeding experiments

Male mice were caged individually, and daily food consumption was measured for 7 days. In the subsequent 4 weeks, the mice received every day 70% of their normal food consumption at 6 p.m. (caloric restriction).

Rapamycin treatment

Mice were injected i.p. with 8 μg rapamycin per gram body weight (1.2 mg/ml rapamycin, 0.25% (w/v) PEG, 0.25% (v/v) Tween-20 in H2O) or solvent. Twelve or twenty-four hours after injection, mice were sacrificed and tissue samples were taken for immunoblot analyses.

Body composition

The abdominal region from lumbar vertebrae 5 to 6 of anaesthetised mice was analysed with an Aloka LaTheta Laboratory Computed Tomograph LCT-100A (Zinsser Analytic). Scans were performed with a resolution of 1.00 mm with high X-ray voltage and fast speed settings. Visceral and subcutaneous fat was discriminated with the supplied software (for visceral fat measurement).

Caloric utilisation

Faeces and food samples were collected and dried in a speed vacuum drier for 5 h at 60°C and then grinded and pressed into tablets. Energy content of food and faeces was determined by bomb calorimetry (IKA-Calorimeter C 5000). Energy efficiency was calculated by subtracting energy loss in faeces from consumed energy.

Energy expenditure

O2 consumption and CO2 output were measured simultaneously through indirect calorimetry with an Oxymax Comprehensive Lab Animal Monitoring System CLAMS open circuit system (Columbus Instruments). Mice were placed in individual metabolic cages with free access to water and food. Measurements were performed every 18 min for 48 h starting at 6 p.m. For graphical presentation, the average of four measurements was taken.

Animal activity measurement

Activity was monitored with the Oxymax CLAMS open circuit system (Columbus Instruments) over 18 h starting at 10 a.m. Movements were recorded for 18-min intervals for the x-axis and z-axis and total movements and ambulatory movements counted. For graphical presentation and analyses, average values for day and night (6 p.m.–6 a.m.) phases were used.

Blood tests

Mice were anaesthetised with isoflurane, and whole blood was collected from the suborbital node with heparinised capillaries into heparin blood collection tubes. Plasma was separated from cells by centrifugation at 5,000 × g for 10 min at 4°C. Levels of insulin, adiponectin and leptin were measured by enzyme-linked
immuno-sorbent assay (ELISA) according to the instructions of the manufacturer (Biocat). Lipids in serum were determined by standard clinical laboratory techniques. Free fatty acids (FFA) were determined by enzymatic conversion to \( \text{H}_2\text{O}_2 \) followed by a colorimetric peroxidase assay. Cholesterol, LDL, HDL and triglycerides were analysed with the Architect System from Abbott. For measuring LDL and HDL, all non-LDL and non-HDL were removed prior to analysis, respectively. The procedure for the determination of triglycerides is described in [42,43]; however, 4-chlorophenol was used instead of 2-hydroxy-3,5-dichlorobenzene sulphonate.

**Glucose tolerance and insulin sensitivity**

For the i.p. glucose tolerance test, mice were fasted overnight (16 h) and injected i.p. with 10 \( \mu \)l of a 20% (w/v) glucose solution per gram body weight. Blood glucose concentration was measured with a glucometer (Accu Chek Aviva, Roche). For the i.p. insulin sensitivity test, mice that had free access to food before, but not during the experiment, were i.p. injected with 0.5 IU/kg insulin (0.05 IU/ml insulin in 1× PBS with 0.08% BSA fatty acid-free) and blood glucose concentration was measured as described before. For determination of insulin sensitivity in muscle, mice were fasted for 6 h and injected i.v. with 0.75 IU/kg insulin (0.21 IU/ml insulin in 1× PBS) or solvent. Ten minutes after injection, mice were sacrificed and tissue samples were collected and analysed in an immunoblot using Akt- and phospho-Akt-specific antibodies (see below). HOMA2-IR was calculated using the HOMA2 Calculator v2.2.3 from the Diabetes Trials Unit, University of Oxford (http://www.dtu.ox.ac.uk/homa).

**Immunoblot analyses**

For protein extraction, mouse tissue was homogenised in 500 \( \mu \)l tissue lysis buffer (60 mM Tris pH 6.8; 1% SDS, supplemented with protease and phosphatase inhibitors) with a glass douncer on ice. Protein extracts were sonicated and centrifuged for 5 min at 10,000 \( \times \) g at 4°C, and the fatty layer and cell debris were removed. For protein extraction from cells, these were washed twice with ice-cold 1× PBS and lysed in 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, supplemented with protease and phosphatase inhibitors followed by sonication. Equal amounts of total protein were separated by SDS-PAGE, transferred to a PVDF or nitrocellulose membrane and analysed using the following antibodies: C/EBP\( \beta \) (C-19), 4E-BP1 (C-19) and \( \alpha \)-tubulin (TU-02) from Santa Cruz; phospho-p70S6K (Thr389) (108D2 or 1A5) and p70S6K (9202) phospho-S6 ribosomal protein (54D2), phospho-Akt (Ser473) (D9E) and (Thr308) (L32A4), Akt (9272), phospho-4E-BP1 (Thr473) (D9E) and (Thr308) (L32A4), Akt (9272), phospho-EF-2 (Ser253/256) (2F9), S6 ribosomal protein (54D2), and N-terminal ACT (Ser235/236) (2F9), S6 ribosomal protein (54D2), phospho-4E-BP1 (Ser201) (D9E), phospho-4E-BP1 (Ser204) (D9E), and phospho-4E-BP1 (Ser206) (D9E). After incubation for 3 h at RT, washed with 1× PBS, sections were incubated with the primary antibody (rabbit IgG insulin (H-86) antibody, Santa Cruz) at 4°C over night in a humidified chamber. After washing with 1× PBS, slides were incubated with Alexa Fluor 488-conjugated secondary antibody (Invitrogen) for 2 h at RT. After washing with PBS, sections were sealed with cover slips using DAPI containing mounting medium. All microscopic analyses were done with the Imager ApoTome Axiouvert 200 and Axiosvision (Zeiss) software.

**qRT–PCR analyses**

Tissue pieces were homogenised with the Precellys 24 system (Peqlab) in the presence of 1 ml QIAzol reagent (QIAGEN), and RNA was isolated from tissue samples using the RNeasy Lipid Tissue Mini Kit (QIAGEN). After incubation for 30 min at 37°C with RQ1 RNase-Free DNase (Promega), the RNA was further purified with the RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer’s protocol starting at step 4. For cDNA synthesis, 1 μg RNA was reverse transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche) using Oligo(d)T primers. qRT–PCR was performed using the LightCycler® 480 SYBR Green I Master Mix (Roche). The following primer pairs were used: \( \beta \)-actin: 5'-AGA GGG AAA TCG TGC GTG AC-3' and 5'-CAA TAG TGA TGA CCT GAA CCT GGT C-3'; H:\text{CT}G: 5'-TTT CTT CTC GCT GCT GAT CAT C-3' and 5'-ATC TTC TCG CAG CTC CTG CA-3'; UCP1: 5'-CAG GGA GTC AGC GGA G-3' and 5'-CAG GGA GTC AGC GGA G-3'; GH: 5'-CAG GGA GTC AGC GGA G-3' and 5'-CAG GGA GTC AGC GGA G-3'; AOX: 5'-CAG GGA GTC AGC GGA G-3' and 5'-CAG GGA GTC AGC GGA G-3'; LPL: 5'-CAG GGA GTC AGC GGA G-3' and 5'-CAG GGA GTC AGC GGA G-3'; GLUT4: 5'-CAT CAG GAG GAC GGC AAA TAG-3' and 5'-CAA TCA AGG TGC-3'.

**Histology and immunofluorescence**

Pieces of tissue were fixed with 4% paraformaldehyde for 24 h and embedded in paraffin. 5-μm-thick sections were stained with haematoxylin and eosin (H&E) using the Autostainer XL (Leica). For Sudan III staining, 100× Sudan III in 10% ethanol and 90% acetic acid. For immunohistochemistry, paraffin sections were treated with 10 mM citrate buffer pH 6.0 for 10 min at sub-boiling point for antigen retrieval. After washing with 1× PBS, slides were incubated with Alexa Fluor 488-conjugated secondary antibody (Invitrogen) for 2 h at RT. After washing with PBS, sections were sealed with cover slips using DAPI containing mounting medium. All microscopic analyses were done with the Imager ApoTome Axiouvert 200 and Axiosvision (Zeiss) software.

**Metabolic improvements in C/EBP\( \beta \)uORF mice**

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Stripping buffer (Perbio). Quantification of the protein bands was performed using the FluorChemE Imager (ProteinSimple/Biozym) or the ImageQuant LAS 4000 Mini Imager (GR Healthcare) or the supplied software or the ImageJ software [44] in case of films.
S′-CTT GGA CAG ACT CTG AGC TGC-3′, ACC (liver): S′- GGG ACT TCA TGA ATT TGC TGA TTC TCA GTT-3′ and S′-GTC ATT ACC TTC ACC TCA TAC TC-3′, ACC (WAT): 5′-TCC AGC AAA AGA GCT GTT-3′ and 5′-ACT AAG GAT GCT CCC CAC CT-3′, SC1: 5′-CCG GAG ACC CCT AGA TCG-3′ and 5′-TAG CCT GTA AAA GAT TTC TGC AAA CC-3′, αP2: 5′-GGA TGG AAA ATA GAC CAC AAC-3′ and 5′-GCT CAT GCC CTT TCA TAA ACT C-3′, GLUT2: 5′-GCA ACT GGG TCT GCA ATT TT-3′ and 5′-CCA GCG AAG AAG AAC AC-3′, VLCAD: 5′-TTG TCA ACG AGT AGC TTC TG-3′ and 5′-AGC CTC AAT GCA GCA CCT AT-3′, LCAD: 5′-GCT GCC CTC CTC CGG ATG TT-3′ and 5′-ATG TTT TTC TCT TGG GAT GAT G-3′, MCAD: 5′-GTT TGG GCT TTT GGA CAA TG-3′ and 5′-TGA CGT GTC CAA TCT ACC ACA A-3′, SCAD: 5′-CCT GCA ACC GAG AAG AAA TC-3′ and 5′-CCT GTC TGT TCC TGT TTG-3′, PGC1α: 5′-GTA AAT CTG CGG GAT GG-3′ and 5′-GTT GGA AGC AGG GTC AAA A-3′, PRDM16: 5′-GAC ATT CCA ATC CCA CCA GA-3′ and 5′-CAG CTC TGT ATC CCT GAT CA-3′, Dio2: 5′-CAG TGT GGT GCA CTG CTC CAA TC-3′ and 5′-TGA ACC AAA GTT GAC CAC CAG-3′.

**Data analyses**

Sample size was chosen based on our previous experiments and published studies in which the same experimental procedures were used. Animals were randomly assigned for measurements or treatments. Experimental groups were created concerning similarity in age and body weight. Animals that became sick or died during the experiment and those with which the experimental performance was not successful were excluded from analyses. For all data, normal distribution was assumed and the unpaired, two-tailed Student’s t-test was used to calculate statistical significance of results. All graphs show average ± standard error of the mean (s.e.m.). *P < 0.05; **P < 0.01; ***P < 0.005. No blinding of investigators was done.

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