A screening strategy for the discovery of drugs that reduce C/EBPβ-LIP translation with potential calorie restriction mimetic properties

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An important part of the beneficial effects of calorie restriction (CR) on healthspan and lifespan is mediated through regulation of protein synthesis that is under control of the mechanistic target of rapamycin complex 1 (mTORC1). As one of its activities, mTORC1 stimulates translation into the metabolic transcription factor CCAAT/Enhancer Binding Protein β (C/EBPβ) isoform Liver-specific Inhibitory Protein (LIP). Regulation of LIP expression strictly depends on a translation re-initiation event that requires a conserved cis-regulatory upstream open reading frame (uORF) in the C/EBPβ-mRNA. We showed before that suppression of LIP in mice, reflecting reduced mTORC1-signaling at the C/EBPβ level, results in CR-type of metabolic improvements. Hence, we aim to find possibilities to pharmacologically down-regulate LIP in order to induce CR-mimetic effects. We engineered a luciferase-based cellular reporter system that acts as a surrogate for C/EBPβ-mRNA translation, emulating uORF-dependent C/EBPβ-LIP expression under different translational conditions. By using the reporter system in a high-throughput screening (HTS) strategy we identified drugs that reduce LIP. The drug Adefovir Dipivoxil passed all counter assays and increases fatty acid β-oxidation in a hepatoma cell line in a LIP-dependent manner. Therefore, these drugs that suppress translation into LIP potentially exhibit CR-mimetic properties.

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Calorie restriction (CR; also called dietary restriction) without malnutrition increases health and lifespan in virtually all species studied1. Reduced signaling through the nutrient sensitive mechanistic target of rapamycin complex 1 (mTORC1) is thought to mediate many of the beneficial effects of CR2–4. In addition to CR, inhibiting mTORC1 either by pharmacological treatment, mutations, or low protein, high carbohydrate (LPHC) diets has similar beneficial effects4–8. A prominent result of CR or low mTORC1 signaling is a decrease in cancer incidence, which maybe related to the alterations in mTORC1-controlled metabolism2–9.

mTORC1 signaling coordinates the regulation of global protein synthesis and autophagy to adapt protein homeostasis to changing nutrient availability and growth factor signaling10. In addition, the expression of a subset of proteins is specifically regulated by mTORC1 through distinctive translation of their mRNAs involving cis-regulatory elements that make them responsive to regulators of translation. Previously, we presented that specific translation into the C/EBPβ protein isoform LIP (Liver-specific Inhibitory Protein) is under control of mTORC1 through regulation of the downstream eukaryotic translation initiation factor 4E (eIF-4E) binding proteins (4E-BPs), and that mTORC1-inhibition by rapamycin reduces LIP expression11,12. Translation of the C/EBPβ-mRNA involves two separate translation mechanisms, initiation and re-initiation: (1) Synthesis of the isoforms LAP and LAP* (Liver-specific Activating Protein) is the result of regular translation initiation where ribosomes scan the mRNA from the 5′-end to the first AUG-codon in a favorable Kozak sequence context to initiate translation (LAP* is often weakly expressed because it has no Kozak sequence) (Supplementary Fig. S1a and b);
(2) translation into LIP requires the initial translation of a cis-regulatory upstream open reading frame (uORF) in the mRNA leader sequence, followed by the continuation of mRNA scanning and translation re-initiation from the downstream LAP-AUG codon (Supplementary Fig. S1a)\(^{11}\). Activated mTORC1 signaling stimulates the latter re-initiation into LAP but only marginally affects the initiation into LIP. The dependence of LAP expression on the presence of the uORF can be explained by the finding that translation of the uORF prevents translation initiation at the LAP initiation codon. Mechanistically this is based on the fact that uORF-post-termination ribosomes have to be reloaded with new initiator tRNA (Met-tRNA\(_{\text{Met}}\)) in order to perform a second translation re-initiation at the same mRNA molecule. The LAP initiation codon is too close (4 nt) to the uORF for the post-termination ribosomes to be reloaded with new Met-tRNA\(_{\text{Met}}\) in time. Therefore, they omit the LAP initiation codon but can be reloaded with Met-tRNA\(_{\text{Met}}\) early enough to re-initiate translation at the downstream LAP initiation codon (Supplementary Fig. S1c). The sophisticated structure of the C/EBPβ-mRNA renders its translation responsive to changes in availability or activity of translation-regulatory factors.

Met-tRNA\(_{\text{Met}}\) is delivered to the ribosomes in a ternary complex with eIF2 and GTP and initiation is accompanied by GTP-hydrolysis followed by the release of the eIF2-GDP complex. The activity of eIF2 is restored by the guanine-nucleotide exchange factor eIF-2B, a process that is inhibited by eIF2α-kinases that phosphorylate eIF2α-subunit of eIF2-GDP and thereby limit translation under various stress conditions\(^{12,13}\). Since re-initiation at the LAP-AUG requires loading of a new initiator tRNA, re-initiation at the LAP-UAG is sensitive to eIF2α-kinases\(^{14}\).

While LAP/LAP* is a transcriptional activator LIP lacks the transactivation domains and can therefore act as a competitive inhibitor of LAP function\(^{15}\). Consequently, the ratio between LAP and LIP is crucial for the biological functions elicited by C/EBPβ. Genetic elimination of the uORF in mice abrogates regulated translation into LIP\(^{12,16}\). These C/EBPβ\(_{\text{ΔuORF}}\) knockin mice display a metabolic profile, including reduced fat accumulation and increased fatty acid β-oxidation, improved insulin sensitivity and glucose tolerance, and enhanced activity\(^{12,17}\). Intriguingly, these metabolic improvements are achieved without reducing calorie intake. Furthermore, genetically modified mice with mono-allelic or bi-allelic overexpression of LIP display an increase in cancer incidence\(^{18}\). Thus, pharmacological targeting of C/EBPβ-LIP expression may provide a promising strategy to screen for drugs with CR-mimetic properties for the treatment of metabolic disease and cancer.

Other transcripts than C/EBPβ use the mechanism of uORF-dependent re-initiation for translationally controlled protein expression, including the well-studied yeast Gcn-4 or the eukaryotic ATF-4 mRNAs as well as other mRNAs involved in proliferation and differentiation\(^{19}\). It is believed that post-uORF re-initiation involves the same translation factors as regular initiation. There is, however, emerging evidence that uORF-containing transcripts are in addition coordinately regulated by non-canonical re-initiation factors. This model was recently strengthened by the discovery that the complex of density regulated protein (DENR) and Multiple Copies in T-cell Leukemia (MCT-1) is a selective regulator of re-initiation involved in tissue growth\(^{20}\). In addition, our recent work suggests that proper re-initiation is disturbed by mutations in the ribosomal maturation factor gene Shwachman-Bodian-Diamond Syndrome (SBDS) that cause Shwachman Diamond Syndrome (SDS)\(^{21}\). Therefore, beyond the regulation of C/EBPβ translation per se coordinated uORF-dependent regulation of subsets of transcripts may be involved in controlling physiological as well as pathological alterations.

Conditions that reduce LIP levels like CR or other means of mTORC1 inhibition are explored for their feasibility to improve metabolic health and to treat cancer. Long term CR, however, is not manageable for most people and current treatment with mTORC1 inhibitors are of limited success and come with serious side effects\(^{22-25}\). Transcription factor function is considered to be largely undruggable and in case of LIP it would almost inevitably mean that LAP is targeted too, since LAP includes all sequences that make up LIP. Fortunately, we know that the specific translation mechanism that is responsible for LIP translation is potentially druggable, as reduction of LAP through pharmacological inhibition of mTORC1 exemplifies. Therefore, we set out to develop a screening strategy for the identification of compounds that alter C/EBPβ-uORF dependent translation re-initiation into LAP and thereby ultimately identify compounds that have CR-mimetic and/or anti-cancer activities. Here we present a cellular system containing C/EBPβ-uORF controlled luciferase-reporters that measures simultaneously translation initiation and re-initiation and is suited for high-throughput screening strategies. The screening system was validated with pharmacological and genetic approaches and used to screen a library of FDA approved drugs. Hereby we identified drugs known to inhibit mTORC1 as well as other drugs previously unknown to affect re-initiation/initiation ratios. Finally, a single drug was identified that reduces translation re-initiation in different cell lines, reduces endogenous LIP expression and increases β-oxidation in a mouse hepatoma cell line. Therefore, with our reporter we present a reliable tool for the search for small molecule compounds that may mimic calorie restriction in which C/EBPβ and other uORF controlled genes are causally implicated.

Results

A reporter system to emulate (monitor) C/EBPβ-mRNA translation. In order to identify small molecule compounds that modulate C/EBPβ-uORF dependent and differential translation of the C/EBPβ-mRNA into the protein isoforms LAP and LIP we designed a reporter system as a surrogate for this process. A single dual reporter plasmid was constructed that expresses a renilla luciferase transcript for the simulation of translation initiation into LAP and a firefly luciferase transcript for the simulation of translation re-initiation into LIP, both under control of the C/EBPβ-5′-leader sequence (Fig. 1a, pCDNA-Firel\(_{\text{5′-leader}}\)-Renilla\(_{\text{Renilla}}\)). Comparable to translation into LAP from the genuine C/EBPβ-mRNA, regular translation initiation after leaky scanning over the uORF results in expression of renilla luciferase from the initiation cassette (Renilla\(_{\text{Renilla}}\)) (Fig. 1a, Initiation Cassette). This cassette does not contain the downstream LAP-UAG and therefore uORF translation and eventual subsequent downstream re-initiation events are not detected. The efficiency of uORF-dependent re-initiation, simulating translation into LIP, is measured as expression levels of firefly luciferase from the re-initiation cassette.
The mTORC1 inhibition was monitored by reduced phosphorylation of 4E-BP1 and S6 kinase (S6K). Finally, overexpression of eIF-4E that increases LIP expression accordingly increased TRI values (Fig. 2d). Therefore, a TRI > 1 indicates enhanced re-initiation, whereas a TRI < 1 indicates reduced re-initiation, respectively. TRI provides a more stringent normalized measurement for the validation of compounds identified by FireflyRe-ini/RenillaIni reporter system.

**Validation of the reporter system.** The differential translation of the C/EBPβ-mRNA into LAP and LIP protein isoforms is under control of the mTORC1/4E-BP/eIF4E pathway. To examine the performance of the reporter in response to mTORC1 hyperactivation we separately transfected the pcDNA3-FireflyRe-ini/RenillaIni and pcDNA-FireflyRef/RenillaRef reporter plasmids in mouse embryonic fibroblasts (MEFs) that are deficient in 4E-BP1/2 (4E-BP1/2-DKO) or in wt MEFs and calculated the TRI. Compared to the wt MEFs the TRI was increased in the TSC1-KO MEFs, revealing enhanced translation re-initiation, which also resulted in enhanced expression of LIP (Fig. 2a). A significant part of the mTORC1-dependent LIP regulation is mediated through the inhibitory phosphorylation of 4E-binding proteins (4E-BPs) by mTORC1, resulting in release of the eukaryotic translation initiation factor 4E (eIF-4E). In accordance, we found higher TRI values in TSC1-KO MEFs and 4E-BP1/2-DKO MEFs comparing to wt MEFs (Fig. 2b). mTORC1 signaling can be pharmacologically inhibited by the allosteric inhibitor rapamycin or the catalytic inhibitor PP242. To examine the performance of the reporter under pharmacological conditions we generated HEK293T based cell lines with stable integrated pcDNA3-FireflyRe-ini/RenillaIni or pcDNA-FireflyRef/RenillaRef reporters. Treatment of the reporter cell lines with 200 nM rapamycin or 10 nM PP242 decreased LIP expression and lowered TRI values, revealing suppression of re-initiation under inhibited mTORC1 conditions (Fig. 2c).

The mTORC1 inhibition was monitored by reduced phosphorylation of 4E-BP1 and S6 kinase (S6K). Finally, overexpression of eIF-4E that increases LIP expression accordingly increased TRI values (Fig. 2d). Therefore, the reporter system reliably reflects differential translation of the C/EBPβ-mRNA under changing mTORC1 signaling.
Figure 2. Reporter system validation. (a) Mouse embryonic fibroblasts (MEFs) that are deficient in the mTORC1-inhibitor protein TSC1 (TSC1-KO) or wt MEFs transiently transfected with pcDNA3-Firefly\textsubscript{Re-ini}/Renilla\textsubscript{Ref} and pcDNA-Firefly\textsubscript{Re-ini}/Renilla\textsubscript{Ref} reporter plasmids. The bar graphs show the calculated TRI mean values (n = 5). Immunoblots show the levels of C/EBP\textbeta, TSC1, phospho-S6K, S6K and the $\beta$-actin loading control. (b) 4E-BP1/2 double knockout (DKO) MEFs and wt MEFs transiently transfected with reporter constructs as described in (a). The bar graphs show the calculated TRI mean values (n = 5). Immunoblots show the levels of C/EBP\textbeta, 4E-BP1, 4E-BP2 and the $\beta$-actin loading control. (c) HEK293T cells stably expressing pcDNA3-Firefly\textsubscript{Re-ini}/Renilla\textsubscript{Ref} or pcDNA-Firefly\textsubscript{Re-ini}/Renilla\textsubscript{Ref} reporters treated with 200 nM rapamycin or 10 nM PP242 for 8 h. The bar graphs show the calculated TRI mean values (n = 5). The immunoblots show mTORC1 inhibition by reduced phosphorylation of 4E-BP1 and S6K in addition to $\beta$-actin as loading control. (d) HEK293T cells described in (c) transfected with eIF expression vectors. The bar graphs show the calculated TRI mean values (n = 3). Immunoblots show levels of eIF4, eIF2\alpha, PKR and the $\beta$-actin loading control. (e) HEK293T cells described in (c) treated with the PKR inhibitor (C16) for 8 h. The bar graphs show the reduced phosphorylation of eIF2\alpha and the $\beta$-actin loading control. (f) DENR-KD (+ Dox) and DENR-Control (−Dox) HEK293T cells transiently transfected with reporter constructs as described in (a). The bar graphs show the calculated TRI mean values (n = 6). Immunoblots show levels of DENR and the $\beta$-actin loading control. (g) C33A cells with stable SBDS knockdown (shSBDS) or control cells (shCTRL) transiently transfected with reporter constructs as described in (a). The bar graphs show the calculated TRI mean values (n = 6). Immunoblots show levels of SBDS and the $\beta$-actin loading control. Statistical differences were analyzed by Student’s t-tests. Error bars represent ± SD, *P < 0.05, **P < 0.01, ***P < 0.001. Full scans of the immunoblots are presented in Supplementary Fig. S3.
The eIF2α-kinases suppress post-uORF translation re-initiation through inhibitory phosphorylation of eIF2α in response to various stresses\textsuperscript{13,14}, which results in reduced expression of LIP\textsuperscript{11}. Augmenting eIF2 activity by treatment with the eIF2α-kinase inhibitor C16, which reduces eIF2α-phosphorylation, resulted in elevated translation re-initiation measured as higher TRI values and an increase in LIP expression (Fig. 2e). Similarly, activation of eIF2α by overexpression of the dominant negative mutant of the eIF2α-kinase PKR (PKRA6) or the non-phosphorylatable eIF2αS51A mutant but not the phosphorylation mimicking eIF2αS51D mutant resulted higher TRI values (Fig. 2d). Thus, the alterations in eIF2α-kinase signaling can be measured using the TRI reporter system.

In addition to the canonical translation initiation pathways we examined the effects of two proteins that were shown to determine the efficiency of translation re-initiation by different mechanisms. The density-regulated protein (DENR) has been identified as a key regulator of eukaryotic re-initiation downstream of uORFs, and knockdown of DENR impairs the post-uORF re-initiation of translation\textsuperscript{20}. To examine the effect of DENR expression we generated HEK293T cells with a doxycycline-inducible DENR-shRNA expression vector. Induction of DENR-KD (+Dox) resulted in lower TRI comparing to control cells (Fig. 2f), confirming the crucial role of DENR in uORF-dependent translation re-initiation. Recently, we have shown that deficiency in the Shwachman-Bodian-Diamond Syndrome (SBDS) protein impairs translation re-initiation into the C/EBPβ-LIP isoform\textsuperscript{12}. In accordance, we measured lower TRI values in cells with shSBDS mediated knockdown of SBDS (Fig. 2g).

Taken together, the TRI reporter system reliably detects changes in the ratio of initiation versus re-initiation by known translationally active drugs or alterations in key translation initiation factors and their regulatory kinases or non-canonical regulators of re-initiation.

**Assay development and execution of a high-throughput screening (HTS).** To establish a reporter cell line suitable for high-throughput screening (HTS) we generated HEK293T cells with stably integrated pcDNA-FireflyRe-ini/RenillaRef reporter plasmid. Single clones were tested for renilla and firefly expression and the highly expressing cell clones were in addition tested for stability of expression over repeated cell passages. Cell line clones that met these criteria were selected for further experiments. Similarly, a cell line with stably integrated pcDNA-FireflyRef/RenillaRef plasmid was generated. To determine an optimal assay window using reference compounds we performed kinetic experiments with the mTORC1 inhibitors rapamycin and PP242 over 24 hours. Both drugs showed the strongest decrease in TRI value at 8 hours, which was chosen as a suitable time point to perform the HTS (Supplementary Fig. S2a).

We screened a library consisting of 780 FDA approved drugs (ENZO #BML-2841) using the FireflyRe-ini/RenillaRef HEK293T reporter cell line. 1.5 × 10\textsuperscript{4} cells per well (50 μl volume) were seeded in a 384-well format and the FireflyRe-ini/RenillaRef system was exposed to 10 μM of a single drug per well for 8h (three assay plates in total). Each assay plate contained 6 wells with 0.5% v/v DMSO added as reference (Fig. 3a,b and c; bar graph at the right). Rapamycin was included as positive control on each plate (n = 3, ±0.04; plate 3, 0.9 ± 0.028; Fig. 3a,b and c; bar graph at the right). 45 drugs were found to decrease the translation re-initiation/initiation ratio (FireflyRe-ini/RenillaRef) below the threshold of three times the standard deviation from the mean value of DMSO treated cells (hit rate 5.7%) (Fig. 3a, b and c). The ENZO library contains three mTORC1 inhibitors that are derived from rapamycin (the rapalogues everolimus, sirolimus and temsirolimus) and two of them, sirolimus and everolimus, decreased re-initiation/initiation ratio (Fig. 3a and b). The temsirolimus signal was neglected because of aberrantly low luciferase signals (technical failure).

**Validation of the drugs that decrease translation re-initiation/initiation ratio.** Four drugs with the strongest effect on decreasing re-initiation/initiation ratio were chosen for further validation using counter assays (Fig. 3a–c, numbered values #1–4; Fig. 3d, drug names and structural formulas). Treatment of the pcDNA-FireflyRef/RenillaRef or pcDNA3-FireflyRe-ini/RenillaRef containing HEK293T cell line using 10 μM of each drug for 8h in a 96-well format revealed for drug #3 and #4 enhancement of the renilla reference signal compared to DMSO control (Supplementary Fig. S2b and c). The calculated TRIs (see formula Fig. 1b) revealed that similar to the result of the HTS screen drug #4 showed the strongest effect compared to drug #2 and #3, however, we could not confirm an effect of drug #1 on the TRI (Fig. 4a). Only drug #4 (Adefovir Dipivoxil) significantly lowered TRI values in two additional different cell lines, the mouse hepatoma cell line Hepa1-6 and the human breast cancer cell line MCF-7 (Fig. 4b and c). Drugs #2 did significantly lower the TRI in Hepa1-6 but not in MCF7 cells, and drug #3 did not alter the TRI in Hepa1-6 and even increased the TRI in MCF7 cells (Fig. 4b and c).

These two drugs were excluded from further examination. To examine dose-dependency of treatment the TRI was measured for serially diluted concentrations between 1 nM and 100 μM for drugs #4 compared to drug #1 (Supplementary Fig. S2d). Drug #4 showed a dose-response with a half maximal effective concentration (EC\textsubscript{50}) of 1.1 μM, while drug #1 showed a much higher EC\textsubscript{50} of 70.4 μM. Cytotoxicity over 8 hours treatment was examined using fluorescent cell viability assay (CellTiter-Fluor, Promega) and revealed for both drugs #1 and #4 only serious cytotoxicity at 100 μM concentrations (Supplementary Fig. S2e). In addition, treatment with drug #4 lowered endogenous LIP/LAP ratio comparable to treatment with rapamycin in HEK293T, Hepa 1-6 and MCF-7 cells (Fig. 4d,e and f). Taken together, the counter assays revealed that drug #4 is best in affect re-initiation measured as reduced TRI and lower LIP levels in cells that originate from three different tissues.

We have recently shown that suppression of LIP expression results in a metabolic shift towards more fatty acid β-oxidation (FAO) in cell culture experiments and in a mouse model that is deficient in LIP expression (C/EBPβ\textsuperscript{ΔuORF} mice)\textsuperscript{12}. To examine an eventual similar effect of drug #4 that also lowers LIP levels we measured FAO of exogenously added palmitate-BSA in Hepa1-6 cells using the Seahorse XF extracellular flux analyzer. Treatment with drug #4 resulted in an enhanced FAO-related oxygen consumption rate (OCR) that can be reverted by
The enhanced FAO phenotype of the C/EBPβΔuORF mice is associated with upregulation of genes involved in FAO and unchanged or downregulated expression of lipogenesis genes in the liver. Treatment of Hepa1-6 cells with drug #4 resulted in the upregulation of the FAO-related genes.

Figure 3. High throughput screening of a drug library. (a–c) Scatter plots for three different plates with measured translation re-initiation/initiation ratio (FireflyRe-ini/RenillaIni) of 780 FDA approved drugs. Each plate contained 6 wells treated with DMSO as a solvent and rapamycin as a positive control (bar graph at the right, error bars represent SD). The threshold of three times the standard deviation from the DMSO mean value (3 × SD) is indicated. Drugs that decreased the translation re-initiation/initiation ratio more than 3xSD are indicated in red. Drugs indicated by numbers (#1, 2, 3 and 4) were selected for further evaluation. The arrows point to Sirolimus in plate 1 and Everolimus in plate 2. (d) Names and structural formulas of the drug #1–4 (retrieved from the ChemSpider database, www.chemspider.com). Statistical differences were analyzed by Student’s t-tests. Error bars represent ± SD (n = 6), **P < 0.01, ***P < 0.001.

ectopic expression of LIP (Fig. 5a). The enhanced FAO phenotype of the C/EBPβΔuORF mice is associated with upregulation of genes involved in FAO and unchanged or downregulated expression of lipogenesis genes in the liver. Treatment of Hepa1-6 cells with drug #4 resulted in the upregulation of the FAO-related genes short-chain
(SCAD), medium-chain (MCAD) and very long-chain (VLCAD) acyl-CoA dehydrogenases as well as acyl coenzyme A oxidase (AOX), but not of the long-chain acyl-CoA dehydrogenase (LCAD) gene (upregulated in C/EBPβΔuORF mice) (Fig. 5b). In addition, the lipogenesis-related genes stearoyl-coenzyme A desaturase 1 (SCD1) and sterol regulatory element-binding protein 1c (SREBP1c) were modestly downregulated, fatty acid synthesis (FAS) was unchanged (downregulated in C/EBPβΔuORF mice) and acetyl-CoA carboxylase (ACC) upregulated (unchanged in C/EBPβΔuORF mice). Overall the effects of drug#4 on the examined genes show characteristics of enhanced FAO with a similar but not identical pattern compared to the C/EBPβΔuORF mouse liver.

Figure 4. Validation of the drugs that decrease translation re-initiation/initiation ratio. (a) TRI mean values of HEK293T cells transiently transfected with pcDNA3-FireflyRe-ini/RenillaIni and pcDNA3-FireflyRef/RenillaRef reporter plasmids and treated with drugs #1, 2, 3 and 4 (n = 4). (b,c) TRI mean values of Hepa1-6 or MCF7 cells transiently transfected with the constructs described in (a) and treated with drugs #2, 3 and 4 (n = 4). (d) Treatment with drug #4 (10μM for 8 h) decreases C/EBPβ-LIP/LAP ratio in HEK293T cells as analyzed by immunoblotting. β-actin was used as a loading control. Bar chart at the right shows quantification of the relative changes in LIP/LAP-isoform ratio by drug #4 and rapamycin compared to DMSO solvent (n = 3). (e,f) Same experiment as described in (d) with Hepa1-6 or MCF7 cells, respectively (n = 3). C/EBPβ-LIP/LAP isoform ratios were quantified by chemiluminescence and digital imaging. Statistical differences were analyzed by Student’s t-tests. Error bars represent ± SD, *P < 0.05, **P < 0.01, ***P < 0.001. Full scans of the immunoblots are presented in Supplementary Fig. S4.
Taken together, our experiments show that the TRI reporter system can be successfully applied in HTS campaigns to identify compounds that alter the ratio between translation initiation and re-initiation depending on the C/EBPβ-uORF, which potentially display CR-mimetic properties.

Discussion
We developed a cellular reporter system as a surrogate for measuring the ratio between translation initiation and re-initiation as it is used as a mechanism for differential translation of the C/EBPβ-mRNA into the protein isoforms LAP and LIP. We have used reporter systems in the past based on C/EBPα-uORF regulation, however, these systems are not suitable for HTS27,28. Our main motivation to generate a C/EBPβ-based system came from our recent studies where we showed that experimental prevention of uORF-dependent translation re-initiation and the resulting decrease in LIP expression (decrease in LIP/LAP ratio) leads to CR-like metabolic improvements 12,17,29. The uORF-dependent mechanism of LIP regulation seems reminiscent to the uORF-dependent induction of the nutrient-responsive yeast transcription factor Gcn4 in response to amino acid starvation and glucose limitation (dietary restriction). Gcn4 induction in yeast is required for full replicative lifespan extension in response to dietary restriction or depletion of 60S ribosomes by deletion of ribosomal protein genes or treatment with the drug diazaborine30. Ribosomal re-initiation and expression of LIP is controlled by the mTORC1 pathway, linking the expression of this transcription factor to a crucial signaling network that determines metabolic health. Several studies have reported that LIP levels are reduced under conditions that promote metabolic health like CR whereas increased LIP levels are associated with detrimental health conditions like

Figure 5. Drug #4 augments fatty acid β-oxidation. (a) Oxygen consumption rate (OCR) with palmitate as exogenous energy source for fatty acid β-oxidation in Hepa1-6 cells expressing either empty pcDNA3-vector control (E.V., left bar graph) or overexpressing LIP (right bar graph) and treated (overnight) with drug #4 or DMSO solvent (n = 5). (b) Relative mRNA expression levels (qRT-PCR) of the β-oxidation genes SCAD, MCAD, LCAD, VLCAD, AOX, and lipogenesis genes ACC, FAS, SCD1 SREBP1c in HEPA1-6 cells treated with drug #4 or solvent DMSO (n = 3). Statistical differences were analyzed by Student’s t-tests. Error bars represent ±SD, *P < 0.05, **P < 0.01, ***P < 0.001. Full scans of the immunoblots are presented in Supplementary Fig. S5.

Taken together, our experiments show that the TRI reporter system can be successfully applied in HTS campaigns to identify compounds that alter the ratio between translation initiation and re-initiation depending on the C/EBPβ-uORF, which potentially display CR-mimetic properties.
high fat diets, aging and cancer. Therefore, we reasoned that suppression of LIP function by translational downregulation might have therapeutic value since it mimics effects of CR. The specific uORF-dependent translation into LIP can be targeted since we know that pharmacological inhibition of mTORC1 or genetic alterations in certain translation initiation factors results in suppression of LIP translation. Reducing LIP levels through the well-known inhibition of mTORC1 by rapamycin and derivatives (rapalogs) is not optimal since the response varies widely between cell types and treatment comes with a diversity of serious side effects. Since other mechanisms that may impact on LIP translation are not well understood and partially obscure we set out to develop a phenotypic screening system for the identification of small molecule compounds that reduce translation re-initiation that is under control of the C/EBPβ-uORF, in an unbiased way. To validate the functionality of the screening system we chose several established genetic alterations and pharmacological treatments that alter LIP translation via different pathways to show that the cellular FireflyRe-ini/RenillaIni luciferase reporter reliably emulates the two different translation events from the C/EBPβ-mRNA; firefly expression through re-initiation and renilla expression through initiation.

The screening of novel targets against known drugs offers the potential for drug repurposing with reduced cost and time scale for evaluation against alternative diseases. For an initial screen we therefore chose a small molecule library of 780 FDA approved drugs (ENZO #BML-2841) using a HEK293T-based clonal cell line with stably integrated pcDNA3-FireflyRe-ini/RenillaIni reporter plasmid. Hits were classified as those compounds that resulted in a reduced FireflyRe-ini/RenillaIni signal compared to the DMSO control with values larger than three times standard deviation (3xSD), in total 45 drugs (5.7% hit rate). A selection of four drugs that showed the strongest reduction in FireflyRe-ini/RenillaIni signal was subjected to counter assays. Drug #4 was the sole drug to pass all tests and reducing the TRI and LIP levels in three different cell lines in comparison with rapamycin that showed similar effects. It is of course possible that additional drugs that also fulfill these criteria are among the other 41 identified but not yet re-tested drugs.

A prominent effect of reducing LIP levels at both cellular and organismal levels is an enhanced fatty acid oxidation, which beneficially influences metabolic health. Drug #4 induced a similar increase in fatty acid oxidation in a liver cell line, which could be reversed by overexpression of LIP. Taken together, we identified drug #4 from a collection of 780 FDA approved drugs, which reduces C/EBPβ-uORF-dependent translation re-initiation and enhanced generation of the protein isoform LIP with concomitant associated enhancement of fatty acid oxidation. Treatment of drug #4 will have to clarify whether candidate drugs alter systemic metabolism comparable to the effects found in the C/EBPβ-uORF mouse. It is beyond the scope of this manuscript to identify the underlying molecular target(s) or mechanism of action of drug #4 (or one of the other candidates), or to address the potential for drug repurposing. In addition, all 45 hits should be subject of further validation and characterization. Nonetheless, the data do show that the system can be used for HTS campaigns to identify small molecule compounds that reduce LIP levels and potentially have CR-mimetic properties. This is important, since the pharmacological options to utilize the beneficial effects of CR-type metabolic alterations and its related cancer reducing properties are limited.

The system we present here belongs to the type of phenotypic drug discovery (forward pharmacology) where compounds are identified that cause a desirable change in phenotype, here reduction of C/EBPβ-uORF-dependent translation re-initiation. The system, however, is not purely phenotypic and target-agnostic (without having prior knowledge of their biological activity or mode of action against a specific molecular target or targets) and is better described as a translation re-initiation and initiation mechanism-informed phenotypic screen. Phenotypic screens potentially can identify compounds that modify a (disease) phenotype by acting on a yet undiscovered target or targets. The successive identification of the target(s) using for example techniques as chemical proteomics could give novel insights into translation control mechanisms and involved upstream signaling pathways and might thereby also broaden our knowledge of potential players in metabolic diseases, aging and cancer.

Methods
DNA constructs. For generation of pcDNA-FireflyRe-ini/RenillaIni, we first cloned the rat C/EBPβ-5′-leader sequence until the LAP initiation codon in frame with the renilla sequence (from pGL4) in pcDNA3, and separately cloned C/EBPβ-sequences spanning the 5′-leader and sequences until the LIP initiation codon with a +1 frame shift (7 nt upstream of the AUG) with the firefly sequences (from pGL3) in pcDNA3. A fragment of the pcDNA3-LAP-Renilla between the BglII restriction site and spanning the renilla sequences was amplified by PCR using the following primers: forward: 5′-CGG AAA TGT TGA ATA CTC ATA CTC-3′ and reverse: 5′-GCT CAG ATC TCC TCA GAA GCC ATA GAG C-′. The amplified fragment was sequenced and cloned in the pcDNA-FireflyRef/RenillaRef. For the reference construct pcDNA-FireflyRe-ini/RenillaIni, a similar cloning strategy was employed using solely renilla and firefly coding regions (from pGL4 and pGL3 respectively). Details of the cloning strategy will be provided upon request. elF4E, elF2α -5′1D, elF2α-5′1A and PKRΔ26 pcDNA3-based expression vectors are described in ref. 11. The human DENR shRNA expression vector was generated by annealing the oligonucleotides shDENR forward: 5′-CGG GCA AGA GTA TGT GGC CTT GCA ACT CGA GTT GCA AGG CCA CAT ACT CCT GTT TTT T-3′ and shDENR reverse: 5′-AAT TAA AAA ACA AGA AGA GTA TGT GGC CTT GCA ACT CGA GTT GCA AGG CCA CAT ACT CCT G-′ and ligating them into the Tet-pLKO-puro vector (Addgene plasmid #21915, described in ref. 42).

Cell culture. HEK293T and Hepa1-6 cells were maintained in DMEM and MCf7 cells in RPMI (Gibco) supplemented with 10% fetal calf serum (FCS) and 1% Penicillin/Streptomycin at 37 °C with 5% CO2. TSC1-KO and 4E-BP1/2-DKO MEFs were previously described. SBDS-KD C33A cells were used before. Hepa1-6 cells ectopically expressing C/EBPβ-LIP from a pcDNA3 expression vector were described at ref. 12. Cell number and viability was determined using the TC20™ automated cell counter (Bio-Rad) following the manufacturer's instruction. For generation of cells stably expressing the reporter constructs, pcDNA-FireflyRe-ini/RenillaIni.
or pcDNA-FireflyRef/RenillaRef were transfected into HEK293T cells using FUGENE (Roche). Cell clones were selected by addition of G418 to the medium (1 mg/ml). For knockdown of DENR in HEK293T cells, the cells were transduced with the inducible lentiviral Tet-PL.KO-puro-shDENR followed by selection of the transduced cells with puromycin (1.5 μg/ml). Then these cells were treated with doxycycline (100 ng/ml) for 36 h. Rapamycin (R-5000, LC Laboratories), PP242 (#P0073, Sigma) and eIF-2α-kinase PKR inhibitor (C16) (#I9785, Sigma) were used as chemical inhibitors.

Luciferase assay. For the Luciferase assay, 25000 cells per well were seeded in 96-well plates. After 24 h, cells were cotransfected with the plasmids as indicated using FUGENE HD (Promega), or stably transfected cells were used. The next day, luciferase activity was measured in cells treated with 8 h of drug treatment or DMSO solvent control by Dual-Glo Luciferase Assay System (#2920, Promega) following the manufacturer’s protocol using a GloMax-Multi Detection System (Promega).

Viability Assay. For the viability assay, 40000 HEK293T cells were seeded in 96-well plates. After 24 h, cells were treated with the different drugs for 8 h and cell viability was measured by Cell-Titer Flour cell viability assay system (#G6081, Promega) following the manufacturer’s protocol using a GloMax-Multi Detection System (Promega).

Immunoblotting. For protein extraction, the cells 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 (#456-1094, BIO-RAD), transferred to a PVDF membrane using Trans-Blot Turbo System (#170-4273, BIO-RAD) following the manufacturer’s protocol. The following antibodies were used: C/EBPβ (Clone C19) and SBDS (S-15) from Santa Cruz Biotechnology; phospho-p70S6K (Thr389) (109D2), p70S6K (#9202), Hamartin/TSC1 (D43E2) (#6935), phospho-4E-BP1 (Thr493, 4E-BP1 (#9452), 4E-BP2 (#2845), eIF4E (#9742), Phospho-eIF2α (clone C4) (#691001) from MP Biomedicals. HRP-conjugated secondary antibodies were purchased from Amersham Life Technologies. The bands were visualized by chemiluminescence (ECL, Amersham Life Technologies) using ImageQuant LAS 4000 mini imaging machine (GE Healthcare Bioscience) and the supplied software was used for the quantification of the bands.

Library screening. HEK293T cells stably expressing FireflyRe-Inf/RenillaInf vector were seeded at a density of 1.5 × 10^6 in 30 μl media per well of a 384-well plate (Greiner CELLSTAR® 384 well plates, white) and grown overnight. Next day, the cells were treated for eight hours with 780 FDA approved drugs library (BML-2843-0100, Enzo Lifescience) (final concentration 10 μM), 0.5% v/v DMSO and rapamycin (final concentration 200 nM) using an Echo® 555 Liquid Handler (Labcyte) transferring up to 150 nl to each well. After lysis of the cells, luciferase activity was measured.

Measurements of cellular oxygen consumption rate and fatty acid oxidation. Oxygen consumption rate and fatty acid oxidation were determined using a Seahorse XF96 Extracellular Flux analyzer (Seahorse Bioscience). For fatty acid oxidation assay, 2 × 10^4 Hepa 1–6 cells per well were seeded into a 96-well XF cell culture microplate 24 h prior to the assay and cultured in the presence of 0.5 mM carnitine. Sixteen hours before the assay, the cells were treated with the drugs. One hour before the assay, the cells were washed twice with FAO assay buffer (Seahorse Bioscience), and 15 min before the assay, the fatty acid oxidation substrate palmitate-BSA or a BSA control (Seahorse Bioscience) was added and the oxygen consumption rate (OCR) with or without palmitate-BSA was measured.

Quantitative Real-Time PCR analysis. Total RNA was isolated using the RNeasy Kit (QIAGEN). 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: β-actin (normalization): 5′-aga ggg aaa tac tgc tgg ac tc-3′ and 5′-caa tag tga cct ggc gct ctc-3′, SCAD: 5′-gtc cag gct gtg GTG ACT ctg ctg ctg tct acc aca-3′, MCAD: 5′-gtg ttg ttg gtt gaa ccg tga aga aag aaa tc-3′ and 5′-tgt gct gtc caa tct acc aca-3′, LCAD: 5′-gtc ggc ctc ctc ctc ctc agt ttg tc-3′ and 5′-atg tgt ttc ttc ttc ttc ttc gat gat g-3′, VLCAD: 5′-ttg tca aag cag aat tgc tgg tgc ctg tgc tgg tc-3′, AOX: 5′-agg acg tca ttc tca aca gca ccc c-3′ and 5′-ctt gga cag act ctg ctc ctc ctc-3′, ACC: 5′-ggg act tca tta agg tca ttc tca acc ctc tc-3′ and 5′-tgt gaa aag tca ttc tca aca gca ccc c-3′.

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Author Contributions
M.A.Z., C.M., T.A., J.R. and G.K. performed the experiments; O.P., C.M. and C.F.C. supervised the project; M.A.Z., C.M., O.P. and C.F.C. wrote the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: C.F. Calkhoven has filed a patent pertaining the screening system and the identified drugs described in this study.

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