

AMPK promotes induction of the tumor suppressor FLCN through activation of TFEB independently of mTOR

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ABSTRACT: AMPK is a central regulator of energy homeostasis. AMPK not only elicits acute metabolic responses but also promotes metabolic reprogramming and adaptations in the long-term through regulation of specific transcription factors and coactivators. We performed a whole-genome transcriptome profiling in wild-type (WT) and AMPK-deficient mouse embryonic fibroblasts (MEFs) and primary hepatocytes that had been treated with 2 distinct classes of small-molecule AMPK activators. We identified unique compound-dependent gene expression signatures and several AMPK-regulated genes, including folliculin (*Fln*), which encodes the tumor suppressor FLCN. Bioinformatics analysis highlighted the lysosomal pathway and the associated transcription factor EB (TFEB) as a key transcriptional mediator responsible for AMPK responses. AMPK-induced *Fln* expression was abolished in MEFs lacking TFEB and transcription factor E3, 2 transcription factors with partially redundant function; additionally, the promoter activity of *Fln* was profoundly reduced when its putative TFEB-binding site was mutated. The AMPK-TFEB-FLCN axis is conserved across species; swimming exercise in WT zebrafish induced *Fln* expression in muscle, which was significantly reduced in AMPK-deficient zebrafish. Mechanistically, we have found that AMPK promotes dephosphorylation and nuclear localization of TFEB independently of mammalian target of rapamycin activity. Collectively, we identified the novel AMPK-TFEB-FLCN axis, which may function as a key cascade for cellular and metabolic adaptations.—Collodet, C., Foretz, M., Deak, M., Bultot, L., Metairon, S., Viollet, B., Lefebvre, G., Raymond, F., Parisi, A., Civiletto, G., Gut, P., Descombes, P., Sakamoto, K. AMPK promotes induction of the tumor suppressor FLCN through activation of TFEB independently of mTOR. *FASEB J.* 33, 12374–12391 (2019). www.fasebj.org

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AMP-activated protein kinase (AMPK) is an evolutionary conserved energy sensor that functions to maintain energy homeostasis through coordinating effective metabolic responses to reduced energy availability (1, 2). Low-energy

conditions under various physiological and pathophysiological settings (e.g., nutrient deprivation, intense physical activity, ischemia), characterized by elevated AMP:ATP or ADP:ATP ratios, trigger AMPK activation. Once activated,

ABBREVIATIONS: ACC, acetyl-CoA carboxylase; ACS2, acyl-CoA synthetase short-chain 2; ADaM, allosteric drug and metabolite; AICAR, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside; AMPK, AMP-activated protein kinase; C13, compound 13; Cas9, CRISPR associated protein 9; CoA, coenzyme A; *Crebrf*, CREB3 regulatory factor; DAVID, Database for Annotation, Visualization and Integrated Discovery; dpf, days postfertilization; FDR, false discovery rate; FLCN, folliculin; FNIP, folliculin interacting protein; *Hmgcr*, 3-hydroxy-3-methylglutaryl-CoA reductase; *Ift1*, IFN-induced protein with tetratricopeptide repeats 1; KO, knockout; *Ldlr*, LDL receptor; *Lpin1*, lipin 1; MEF, mouse embryonic fibroblast; *Mnt*, max binding protein; *Msmo1*, methylsterol monooxygenase 1; mTOR, mammalian target of rapamycin; NLS, nuclear localization signal; PCA, principal component analysis; *Pde4b*, phosphodiesterase 4B; *Ppia*, peptidylprolyl isomerase-a; qPCR, quantitative PCR; S6RP, ribosomal protein S6; SREBP, sterol regulatory element binding protein; TFEB, transcription factor EB; *Tollip*, toll interacting protein; *Txnip*, thioredoxin interacting protein; WT, wild type

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AMPK promotes ATP-producing, catabolic pathways and decreases ATP-consuming, anabolic pathways to restore and maintain cellular ATP at a constant level.

AMPK is a heterotrimeric complex composed of a catalytic α -subunit and 2 regulatory β and γ subunits. Two to three isoforms exist for each subunit ($\alpha 1$ and $\alpha 2$, $\beta 1$ and $\beta 2$, and $\gamma 1$, $\gamma 2$, and $\gamma 3$), giving rise to 12 distinct combinations of the heterotrimeric complexes. In general, $\alpha 1$, $\beta 1$, and $\gamma 1$ appear to be the ubiquitously expressed isoforms of AMPK. There are cell- and tissue-specific distributions of some isoforms [e.g., exclusive expression of $\gamma 3$ in skeletal muscle (3, 4)], and they may target AMPK complexes to particular subcellular locations to phosphorylate-specific substrates (5, 6). The γ subunits contain 4 tandem cystathionine β -synthase repeats that provide adenine nucleotide binding (7). AMPK activity increases >100-fold on phosphorylation of a conserved threonine residue within the activation loop (Thr172). Binding of ADP and AMP causes conformational changes that favor net Thr172 phosphorylation by the promotion of Thr172 phosphorylation and the inhibition of its dephosphorylation (8–10). In addition, the binding of AMP (but not ADP) further promotes AMPK activity by >10-fold by allosteric activation (8). The major upstream kinase catalyzing AMPK α (Thr172) phosphorylation in most mammalian cells and tissues, including skeletal muscle and liver, is the tumor suppressor kinase LKB1 (11). In some cell types, Thr172 can be phosphorylated in a Ca^{2+} -mediated process catalyzed by Ca^{2+} /calmodulin-dependent protein kinase kinases (12).

AMPK is considered an attractive therapeutic target for metabolic disorders because AMPK activation brings about metabolic responses anticipated to counteract the metabolic abnormalities associated with obesity, insulin resistance, and type 2 diabetes (13, 14). Indeed, several compounds, which can be divided into 3 categories (12), have been reported to activate AMPK and elicit metabolic effects in cellular and preclinical studies. The first class comprises indirect activators that, through inhibition of mitochondrial respiration and eventual suppression of ATP synthesis, increase cellular AMP:ATP or ADP:ATP ratios (e.g., metformin, resveratrol) (15). The second class includes prodrugs converted to AMP analogs inside the cells, the most well-characterized and commonly used compound being 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) (16, 17). The allosteric activators, binding to a site located between the α -subunit kinase domain and the β -subunit carbohydrate binding module termed “allosteric drug and metabolite (ADaM)” site, constitute the third class. The first compound identified through a high-throughput screen (18) as an allosteric AMPK activator is A769662, a thienopyridone that preferentially activates $\beta 1$ -containing complexes (19). More recently, 991 (a cyclic benzimidazole) was developed, which binds AMPK 10 times tighter than A769662 in cell-free assays (20) and activates both $\beta 1$ - and $\beta 2$ -containing complexes (with a higher affinity to the $\beta 1$ complexes) (4, 21). It has been reported that A769662 causes several off-target effects; for example, in isolated mouse skeletal muscle when used at high concentration (22) and also in other cells and tissues (23, 24) and was reported to have poor oral availability (18). Notably, an emerging new generation of ADaM site-binding compounds, including MK-8722 and PF-739, have been shown to

be effective in reversing elevated blood glucose concentrations in rodents and nonhuman primates through activation of AMPK *in vivo* (25, 26).

It is well established that AMPK elicits a plethora of acute metabolic responses through phosphorylation of serine residues surrounded by the well-characterized recognition motif (27). There has been much effort put into the identification of AMPK substrates, and several targeted and untargeted proteomics studies have been performed (28–33), which led to mechanistic understanding of AMPK-mediated metabolic responses and the discovery of new roles for AMPK (e.g., cell cycle, autophagy) beyond conventional metabolic regulation. Growing evidence suggests that in the long-term AMPK promotes metabolic reprogramming *via* effects on gene expression at least partly through regulation of specific transcription factors and transcriptional coactivators (1, 12).

In the current study, we initially performed a comprehensive transcriptome profiling in wild-type (WT) and AMPK-deficient [AMPK $\alpha 1/\alpha 2$ double knockout (KO)] mouse embryonic fibroblasts (MEFs) and primary hepatocytes treated with AICAR or 991, which led to the identification of distinct compound-dependent gene expression signatures and to the discovery of several AMPK-regulated genes. Pathway analyses and *in silico* transcription factor predictions prompted us to hypothesize that the transcription factor EB (TFEB) is a potential key transcription regulator responsible for AMPK-mediated gene expression changes. We found that expression of follistatin (*Fln*), a gene that encodes the tumor suppressor FLCN, was abolished in both AMPK KO and TFEB/TFE3 double KO MEFs and that the promoter activity of *Fln* was profoundly reduced when the putative TFEB-binding site was mutated. Finally, even though it has been reported that mammalian target of rapamycin (mTOR) plays a key role in regulating TFEB especially under nutrient deprived condition (34), we found that AMPK activates TFEB through promotion of dephosphorylation and nuclear translocation independently of mTOR signaling.

MATERIALS AND METHODS

Materials

The materials used comprise AICAR (OR1170T; Apollo Scientific, Bredbury, United Kingdom), 991 [5-[[6-chloro-5-(1-methylindol-5-yl)-1H-benzimidazol-2-yl]oxy]-2-methylbenzoic acid, CAS number 129739-36-2] (31). Protein G Sepharose (P3296), rapamycin (R0395), and Torin-2 (SML1224) were purchased from MilliporeSigma (Burlington, MA, USA). General and specific cell culture reagents were obtained from Thermo Fisher Scientific (Waltham, MA, USA). All other materials unless otherwise indicated were from MilliporeSigma.

Antibodies

Total FLCN antibody (11236-2-AP) was purchased from Proteintech (Rosemont, IL, USA). Flag (F7425), α -tubulin (T6074), and GAPDH (G8795) were obtained from MilliporeSigma. AMPK $\alpha 1$ (07-350) and AMPK $\alpha 2$ (07-363) antibodies were obtained from MilliporeSigma. Acetyl-coenzyme A (CoA) carboxylase (ACC; 3676), phospho-ACC1 (Ser79; 3661), AMPK α (2532), phospho-AMPK α (Thr172; 2535), AMPK $\beta 1$ (4178), lamin A/C

(4777), p70 S6 kinase (9202), phospho-p70 S6 kinase (Thr389; 9206), Raptor (2280), phospho-Raptor (Ser792; 2083), S6 ribosomal protein (5G10), phospho-S6 ribosomal protein (Ser235/236; 2211), and TFE3 (14779) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). TFE3 (#A303-673A) antibody was purchased from Bethyl Laboratories (Montgomery, TX, USA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). AMPK γ 1 (TA300519) antibody was from OriGene Technologies (Rockville, MD, USA). A site-specific rabbit polyclonal antibody against phospho-TFE3 (Ser142) was generated by YenZym Antibodies (South San Francisco, CA, USA) by immunization and affinity purification with a phosphorylated peptide of the sequence identical between human and mouse (*i.e.*, PN-*S-PMAMHLHGSNPC-amide, where the asterisk denotes the phosphorylated residue).

Cell culture and sample preparations

MEFs from WT and AMPK KO mice were generated as previously described by Laderoute *et al.* (35). TFE3/TFE3 double KO MEFs were a kind gift from Rosa Puertollano (National Institutes of Health, Bethesda, MD, USA). MEFs and COS1 cells were cultured in DMEM-Glutamax supplemented with 10% fetal calf serum and 1% penicillin streptomycin. Cells were seeded at ~80% confluence and treated the following day with the indicated treatments described in the figures. In some studies, COS1 cells were transfected with Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer's instruction, and cell lysates were generated 48 h post-transfection. For obtaining protein extracts, cells were washed with ice-cold PBS and scraped into lysis buffer [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 100 mM NaF, 10 mM Na-pyrophosphate, 5 mM EDTA, 250 mM sucrose, 1 mM DTT, 1% Triton X-100, 1 mM Na-orthovanadate, 0.5 mM PMSF, 1 mM benzamide HCl, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin-A, 1 mM microcystin-LR]. Preparation of nuclear and cytoplasmic fractions in MEFs was performed using the NE-PER Kit (Thermo Fisher Scientific) according to the manufacturer's instruction.

Primary hepatocytes were isolated from AMPK α 1/ α 2 liver-specific KO mice and control AMPK α 1^{lox/lox} α 2^{lox/lox} mice littermates (10-wk-old male mice) by collagenase perfusion and cultured as previously described by Foretz *et al.* (36). The experiments were performed in accordance with the European guidelines (approved by the French authorization to experiment on vertebrates, 75-886) and the Ethics Committee at University Paris Descartes (CEE434.BV.157.12). Briefly, the cells were plated in M199 medium containing Glutamax and supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 10% (v/v) fetal calf serum, 500 nM dexamethasone (MilliporeSigma), 100 nM triiodothyronine (MilliporeSigma), and 10 nM insulin (MilliporeSigma). The hepatocytes were allowed to attach for 4 h and were subsequently maintained in M199 medium with antibiotics and 100 nM dexamethasone for 16 h. Experiments were performed the following morning by treating hepatocytes with the indicated compounds (*e.g.*, AMPK activators, mTOR inhibitors) for 1 or 4 h. For viral infection, the hepatocytes were coinfecting (1:3 multiplicity of infection) with 3 individual adenovirus encoding AMPK subunits α 2, β 1, and γ 1 into AMPK α 1/ α 2 KO hepatocytes to restore expression of the AMPK trimeric complexes as previously described by Ducommun *et al.* (33). Sixteen hours after adenovirus infection, the cells were treated for 1 h with vehicle (DMSO) or 991 (10 μ M). Following the previously described treatments, culture media were aspirated and cells lysed on ice in cold lysis buffer. Lysates were snap frozen in liquid nitrogen and stored at -80°C for subsequent analyses. Lysates were clarified at 3500 g for 15 min at 4°C and protein concentration determined using Bradford reagent and BSA as standard.

For obtaining protein extracts from larvae for Western blot analysis, 3 d postfertilization (dpf) larvae were pooled and homogenized in 100 μ l of lysis buffer (described above) using a pestle motor mixer (Argos Technologies, Vernon Hills, IL, USA).

RNA extraction, microarray, and bioinformatic analysis

Total RNA was extracted from cells using RNeasy Tissue Kit (A32645; Beckman Coulter, Brea, CA, USA) and quantified by RiboGreen (R11490; Thermo Fisher Scientific). RNA integrity was determined by capillary electrophoresis on a Fragment Analyzer, (DNF-471; Agilent Technologies, Santa Clara, CA, USA) and an RNA Quality Number >8 was observed. Three hundred nanograms of total RNA were used as input to produce labeled cRNA targets with the TotalPre-96 RNA Amplification Kit following the manufacturer's instructions. cRNA quality was analyzed by Fragment Analyzer, subsequently 11 μ g of cRNA was fragmented and hybridized onto Affymetrix Gene Chip Mouse 430 2.0 following manufacturer's instructions (Thermo Fisher Scientific). Partek Genomics Suite software was used to analyze the data from CEL files. Values were normalized using the robust multichip average method (37). The removal batch effect was applied during the analysis of the hepatocyte samples. Based on the normal distribution of the data sets, the parametric Pearson's product moment correlation was applied for quality control. Two-way ANOVA with Benjamini and Hochberg multiple testing correction was applied to discriminate 991 *vs.* control and AICAR *vs.* control conditions. The moderated significance value was set at $P < 0.05$ for the interaction within genetic background and treatment as well as for the pairwise comparisons. In addition, a fold-change cutoff of 1.3 was applied. Considering the quality test results by principal component analysis (PCA), 2 samples emerged as outliers and therefore were excluded from the analysis (*i.e.*, MEF WT, 991-treated, technical replicate 1 and hepatocytes AMPK KO, vehicle-stimulated, technical replicate 4, biologic replicate 1). The gene ontology was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; <https://david.ncifcrf.gov/>) (38, 39), and the transcription factor prediction using the upstream regulator analysis available in Ingenuity Pathway Analysis (Qiagen, Germantown, MD, USA) (40).

Quantitative RT-PCR

For cDNA synthesis, 500 ng of total RNA was used as starting material for the PrimeScript RT Kit (RR037A; Takara Bio, Kusatsu, Japan). Quantitative RT-PCR reactions were performed on a LightCycler 480 with SYBR Green Assay (04707516001; Roche, Basel, Switzerland), with primers at a final concentration of 0.3 μ M/reaction. The following mouse primers were used: acyl-CoA synthetase short-chain 2 (*Acsc2*), 5'-ACTTGGCGACAAAGTTGCTT-3' (forward), 5'-GAATGCCCTGTTTACGGAGA-3' (reverse); β 2 microglobulin, 5'-GAGCCCAAGACCGTCTACTG-3' (forward), 5'-GCTATTTCTTTCTGCGTGCAT-3' (reverse); CREB3 regulatory factor (*Crebrf*), 5'-TGGGGCCTCAACACTGAATA-3' (forward), 5'-CCATGCTGGGTCTCTCTCT-3' (reverse); *Fln*, 5'-TGGATCGGATCTACCTCATCA-3' (forward), 5'-TGGACATCCAAACTGCTCTG-3' (reverse); *Fln* precursor (pre)-mRNA 5'-TGGACATCCAAACTGCTCTG-3' (forward), 5'-CCCTCTCTCCACCTTTGT-3' (reverse); folliculin interacting protein 1 (*Fnip1*), 5'-GATGCGTGTTCATGTCAAGG-3' (forward), 5'-GGAGAGTGGGTGCTTGCTAC-3' (reverse); 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), 5'-TGGTGGGACCAACCTTCTAC-3' (forward), 5'-GCCATCACAGTGCCACATAC-3' (reverse); IFN-induced protein with tetratricopeptide repeats 1 (*Ifit1*), 5'-GCTCTGCTGAAAACCCAGAG-3' (forward), 5'-GCTTCCATGTGAAGTGACATCT-3' (reverse); LDL receptor (*Ldlr*), 5'-CAATCGGAAAACCATTTTGG-3' (forward), 5'-GTGAGTCGATTGGCACTGAA-3' (reverse); lipin 1 (*Lpin1*), 5'-CATGCCAAGACCAACATCAG-3' (forward), 5'-CAGTGGCTCTCTCCAAAGG-3' (reverse); max binding protein (*Mnt*), 5'-GCAGCAACAACAGAGAGCAC-3' (forward), 5'-AGTGTTGGAGCCTCGATTTC-3' (reverse); methylsterol monooxygenase 1 (*Msmo1*), 5'-GGCTCCCTGATAGTTCACGA-3' (forward), 5'-AAACACTTCCACTGGCCTTC-3' (reverse); phosphodiesterase

4B (*Pde4b*), 5'-ATCAGGGAACCAGGTGTCTG-3' (forward), 5'-ATGGGATTTCACATCGTTC-3' (reverse); peptidylprolyl isomerase-a (*Ppia*), 5'-AGCATACAGGTCCTGGCATC-3' (forward), 5'-TTCACCTTCCAAAGACCAC-3' (reverse); TATA-box binding protein, 5'-TTGACCTAAAGACCATTGCACTTC-3' (forward), 5'-TTCTCATGATGACTGCAGCAAA-3' (reverse); transferrin receptor, 5'-GGAATCCCAGCAGTTTCTTTTG-3' (forward), 5'-CAATGCCTCATAGGTATCCAATCTAG-3' (reverse); toll interacting protein (*Tollip*), 5'-CGCTGGAACAAAGTCATTCA-3' (forward), 5'-GATGTGGGTCCTCAAGCTATGC-3' (reverse); thioredoxin interacting protein (*Txnip*), 5'-ACCAGTGTCTGCCAAAAGG-3' (forward), 5'-TCAAAGTCAGCATGGATGGA-3' (reverse). The following zebrafish primers were used: elongation factor 1- α , 5'-CTTCTCAGGCTGACTGTGC-3' (forward), 5'-CCGCTAGCATTACCCTCC-3' (reverse); *flcn*, 5'-AGTGCCAAACACA-CAACCAAC-3' (forward), 5'-AGTAGGCAGAGCGAGACCAC-3' (reverse); *fnip2*, 5'-CGAGAGAAGAGGACGACAGG-3' (forward), 5'-GGGGAGTTTGGTTTCATCA-3' (reverse); nuclear respiratory factor 1, 5'-TTAACTGGAGTTCCGGATGG-3' (forward), 5'-CTGTTCACAGGGTCACCACTT-3' (reverse).

$\beta 2$ microglobulin, *Ppia* and transferrin receptor, and elongation factor 1 α and nuclear respiratory factor 1 were used as normalization genes for mouse and zebrafish samples, respectively. Normalized values were calculated by dividing the mean expression value by a factor equal to the geometric mean of the normalization genes (41). The relative *Flcn* mRNA quantity of the samples was assessed using a Biomark gene expression 192.24 IFC δ gene assay (Fluidigm Biomark, South San Francisco, CA, USA) following the manufacturer's instruction. Ct values were calculated using the system's software (Biomark Real-Time PCR Analysis; Fluidigm Biomark). For the analysis, the *Ppia* and TATA-box binding protein genes were used for normalization, followed by fitting a 2-way ANOVA to the log-transformed data. For each analysis, the selection of the normalization genes resulted from an analysis of their stability using GeNorm (41, 42).

Generation of *actc1b:tfel-ZsGreen*; *actc1b:nls-mCherry* double transgenic fish and imaging

Adult AB zebrafish were raised at 28°C under standard husbandry conditions. All experimental procedures were carried out according to the Swiss and European Union ethical guidelines and were approved by the animal experimentation ethical committee of Canton of Vaud (permit VD3177). Transgenic zebrafish *Tg(actc1b:tfel-ZsGreen)^{nei08}* and *Tg(actc1b:nls-mCherry)^{nei09}* were independently generated using I-SCEI meganuclease-mediated transgenic insertion into 1-cell stage embryos as previously described by Thermes *et al.* (43). One founder for each transgenic line was selected, and subsequent generations were propagated and expanded. The 2 lines were crossed to generate double transgenic embryos, which have been raised at 28°C under standard laboratory conditions before treatment. Double transgenic embryos were selected at 3 dpf and treated with 991 (10 μ M) or vehicle (DMSO) in 96-well plates. After 24 h of treatment, embryos were anesthetized with 0.016% tricaine and imaged with ImageXpress confocal system at $\times 20$ magnification (Molecular Devices, San Jose, CA, USA). Z-stack images were captured for each embryo and maximal projection images were produced.

Generation of *prkaa1*^{-/-}; *prkaa2*^{-/-} double KO fish

The *prkaa1*^{-/-}; *prkaa2*^{-/-} double KO fish were generated by using the CRISPR/CRISPR associated protein 9 (Cas9) approach (unpublished results). gRNA targeting *prkaa1* exon 5 and *prkaa2* exon 6 were designed using the Chopchop online tool (<http://chopchop.cbu.uib.no/index.php>) and ordered as DNA gene strings. After PCR amplification and purification, they were used as input for *in vitro* transcription

with the MEGAshortscript T7 Kit (Thermo Fisher Scientific). Subsequently, the transcripts were purified with the RNA Clean and Concentrator Kit (Zymo Research, Irvine, CA, USA), and their concentration was determined by NanoDrop (Thermo Fisher Scientific). *Prkaa1*^{-/-} and *prkaa2*^{-/-} single mutants were generated by independent coinjection of single cell stage AB embryos with 50 pg of gRNA and 200 pg of the GeneArt Platinum Cas9 Nuclease (Thermo Fisher Scientific). Injected embryos were grown to adulthood and outcrossed with AB WT fish to identify individuals with mutant germline. Putative filial 1 mutants were fin clipped, their genotype was determined by high-resolution melting quantitative PCR (qPCR), and the mutation was identified by sequencing. We selected a 5 bp deletion for *prkaa1* and a 7 bp deletion for *prkaa2*, both resulting in frameshift and premature stop codons. Single heterozygous animals for the same gene were incrossed to obtain single *prkaa1*^{-/-} or *prkaa2*^{-/-}. The homozygotes were then crossed to obtain double heterozygous fish (*prkaa1*^{+/-}; *prkaa2*^{+/-}). Finally, double KO animals *prkaa1*^{-/-}; *prkaa2*^{-/-} were obtained by double heterozygous incross, and their genotype was determined by high-resolution melting qPCR.

Acute exercise protocol

Acute exercise was performed using a 5 L swim tunnel (SW10050; Loligo Systems, Viborg, Denmark). The day before the acute exercise session, 6 WT and 6 *prkaa1*^{-/-}; *prkaa2*^{-/-} 4-mo-old siblings were subjected to an endurance test to determine their exercise speed. Baseline critical speed (Ucrit) was determined as the speed by which the weakest zebrafish fatigued (5 s at the rear of the tunnel). Based on this result, 6 WT and 6 *prkaa1*^{-/-}; *prkaa2*^{-/-} fish were let habituate for 20 min at a low current speed of 5 cm/s and then trained for 2 h 40 min at 60 cm/s (75% of baseline Ucrit). As the control group, the 6 WT and 6 *prkaa1*^{-/-}; *prkaa2*^{-/-} fish were placed in the swim tunnel for 3 h at a low swim speed of 5 cm/s. An electronic controller and a motor-driven propeller were used to adjust the water velocity to maintain the indicated speed. At the end of the 3-h session, fish were euthanized immediately and trunk muscle was isolated, flash frozen in liquid nitrogen, and processed for RNA and protein extraction.

Bicistronic luciferase assay

MEFs were transfected with Lipofectamine 3000 and 12-h post-transfection cells were treated with 30 μ M 991 for 12 h. After the treatment, cells were washed once with PBS and harvested in Glo Lysis Buffer (E2661; Promega, Madison, WI, USA). Cell lysates were centrifuged at 3500 g for 10 min at room temperature. Luciferase assay was performed using a Dual-Luciferase Reporter Assay System Stop & Glo Kit (E1910; Promega). Differences in the ratio of Firefly to NanoLuc luciferase signals were analyzed.

Immunoprecipitation and immunoblotting

For immunoprecipitation of FLCN, 200 μ g of lysates were incubated with 1 μ g of antibody and 5 μ l of Protein G Sepharose on a shaker (1000 rpm) overnight at 4°C. Immunoprecipitates or total lysates were denatured in SDS sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose membrane. Membranes were blocked for 1 h in 10 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% (v/v) Tween-20 (TBST) containing skimmed milk 5% (w/v). Membranes were incubated in primary antibody prepared in TBST containing 1% (w/v) BSA overnight at 4°C. Detection was performed using horseradish

peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagent.

Cloning and mutagenesis

All plasmid constructs were generated using standard molecular biology techniques. The promoter sequences and the TFEB/TFE3 binding site were identified from the EPD promoter database (<https://epd.vital-it.ch/index.php>). The promoter regions were amplified from mouse genomic DNA (Promega) and then ligated into a modified pNL 1.2 luciferase vector (Promega). The mouse TFEB cDNA (clone MR223016) was obtained from OriGene. Site-directed mutagenesis was carried out according to the Quick Change method (Agilent Technologies) using KOD polymerase (MilliporeSigma). The sequences of all constructs were verified in house utilizing the BigDyeR Terminator 3.1 Kit and the 3500XL Genetic Analyzer (Thermo Fisher Scientific).

Statistical analysis

For the statistical analysis, 2-way ANOVA with interaction was used to analyze the data and was fit to the log-transformed data, as specified in the figure legend. Data are expressed as box-and-whisker plot (minimum to maximum) and log₂ fold-change of the mean $\pm$ SEM or SD, as indicated. Differences between groups were considered statistically significant when $P < 0.05$.

Accession numbers

The microarray data were deposited in Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>), with GSE104170 as the associated accession code.

RESULTS

Whole-genome transcriptome profiling revealed distinct gene expression profiles in response to AMPK activators in MEFs and mouse primary hepatocytes

To identify genes and pathways regulated in an AMPK-dependent mechanism, we performed a whole-genome transcriptome profiling using microarray technology. Taking cell type-specific roles and isoform- or compound-selective response of AMPK into account, we used 2

different cell models and genotypes, namely AMPK WT and AMPK KO in both MEFs and mouse primary hepatocytes, and treated them with 2 AMPK activators (991 and AICAR) known to target distinct AMPK regulatory sites or mechanisms (27). MEFs and mouse primary hepatocytes were treated with vehicle, 991, or AICAR at the indicated concentrations for 4 h (Fig. 1). Following treatment, 1 set of the samples was subjected to transcriptome profiling and the other set was used for Western blot analysis to assess the effect of compounds on AMPK activity. We initially confirmed by Western blot analysis that treatment of WT MEFs with 991 (10 μ M) or AICAR (2 mM) resulted in the activation of AMPK, as confirmed by an increased phosphorylation of AMPK (Thr172) and its *bona fide* substrates ACC (Ser79) and Raptor (Ser792) (Fig. 1A). Similar results were obtained when WT mouse primary hepatocytes were treated with 991 (3 μ M) or AICAR (300 μ M) (Fig. 1B). Notably, although treatment with 991 or AICAR resulted in a comparable elevation of ACC phosphorylation in both MEFs and hepatocytes, 991-induced Raptor phosphorylation was higher in MEFs but lower in hepatocytes compared to AICAR (Fig. 1). As previously demonstrated (36, 44, 45), there was no detectable phosphorylation of AMPK, ACC, and Raptor in vehicle- and 991- or AICAR-treated AMPK KO MEFs and hepatocytes (Fig. 1). Taken together, we validated a complete ablation of AMPK activity in both AMPK KO MEFs and hepatocytes and observed differential responses in commonly used surrogate markers for cellular AMPK activity (*i.e.*, phosphorylation of ACC and Raptor) following the treatment with 991 or AICAR.

We next performed a whole-genome expression profiling using Affymetrix Mouse GeneChips 430 2.0. A PCA was conducted for initial evaluation of data quality and assessment of the effect of genotype and treatment on the transcriptome (Supplemental Fig. S1A, B). In MEFs, the PCA clarified that genotype was the primary factor responsible for variance, followed by treatment, and that globally AICAR caused a greater transcriptional response compared to that induced by 991, implying a potentially more specific transcriptional responses induced by 991 compared to AICAR. In contrast, in primary hepatocytes, treatment was the main cause of variance, followed by a

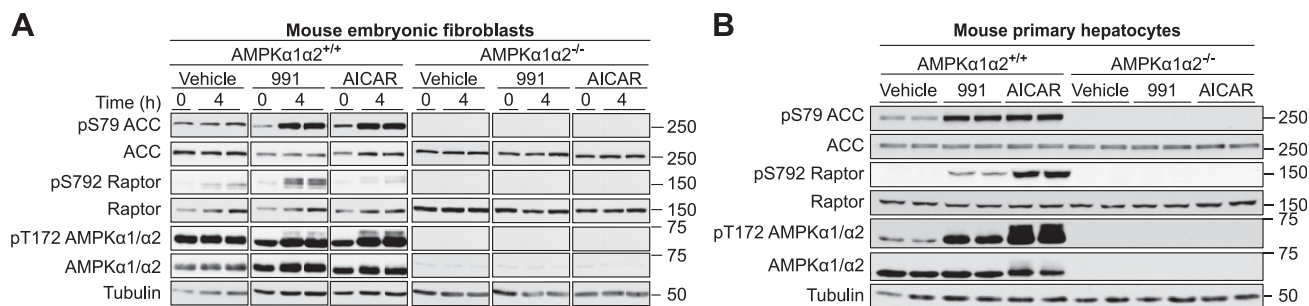


Figure 1. AMPK-activation profiles in samples used for transcriptome analyses. *A*) MEFs WT (AMPKα1α2^{+/+}) or KO (AMPKα1α2^{-/-}) were treated with vehicle (DMSO), 2 mM AICAR or 10 μ M 991 for 4 h. Cell lysates (20 μ g) were subjected to Western blot analysis with the indicated antibodies and a representative blot of $n = 3$ is shown. *B*) Hepatocytes were isolated from AMPKα1/α2 liver-specific KO (AMPKα1α2^{-/-}) mice and control AMPKα1^{lox/lox} α2^{lox/lox} mice littermates (AMPKα1α2^{+/+}). The plated hepatocytes were treated for 4 h with vehicle (DMSO), 3 μ M 991, or 300 μ M AICAR. Cell lysates (20 μ g) were subjected to Western blot analysis using the indicated antibodies. Images are representative of $n = 2$.

lower variance explained by genotype. AICAR consistently produced a much greater response than that driven by 991. These observations were well corroborated by the hierarchical clustering (Fig. 2). This latter analysis, based on transcripts having a false discovery rate (FDR) value of $P < 0.0001$, depicted a clear distinction between AICAR- and 991-treated samples. For instance, in both models, AICAR stimulation caused a separation that was independent of the genetic background. In contrast, 991 treatment led to more subtle transcriptional changes

compared to AICAR, which were detectable exclusively in AMPK WT cells. Finally, we also observed that the absence of functional AMPK led to greater differences as compared to WT cells in MEFs than in primary hepatocytes (Fig. 2).

Identification and validation of AMPK-dependent genes and pathways

To clarify genes specifically regulated following AMPK activation, we first selected genes by pairwise differential

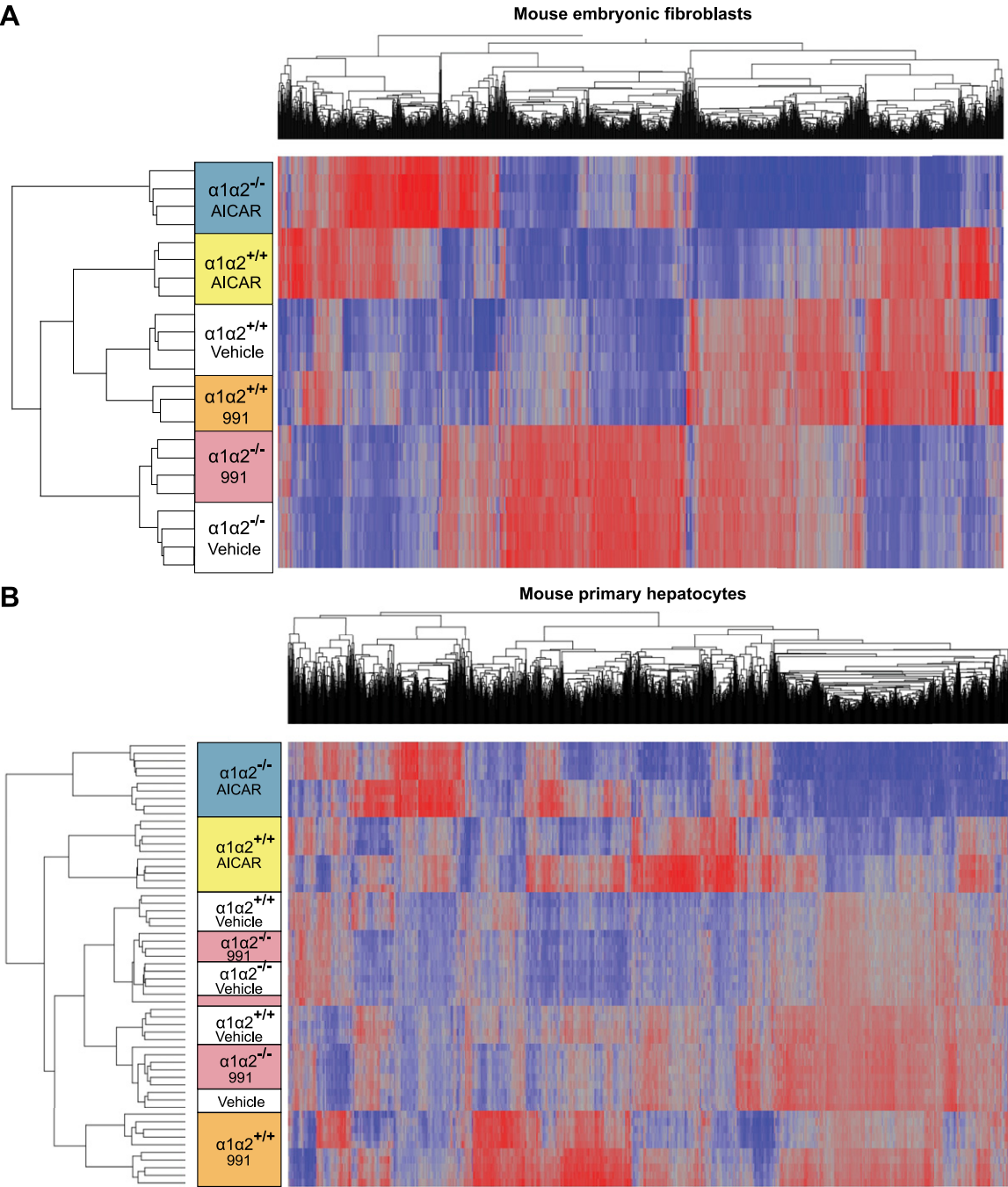


Figure 2. Hierarchical clustering of the transcriptome data. Overview of the hierarchical clustering analyses in MEFs (A) and mouse primary hepatocytes (B). Mean-centered gene expression ratios are depicted by a $\log_2$ pseudocolor scale; red indicates that the gene is overexpressed compared to the mean value, whereas blue specifies that the gene is less expressed. Data were analyzed by 2-way ANOVA with the factors of genetic background, treatment, and interaction. Only selected genes having a FDR of $P < 0.0001$ for the interaction are shown.

analysis of MEFs and primary hepatocytes treated with AICAR or 991 as compared to vehicle. Significance values were corrected for multiple testing using the FDR method of Benjamini and Hochberg (46), and we applied a conservative significance threshold of 5% FDR associated with a fold-change value of 1.3 or more, given that moderate fold changes were observed. Following 991 treatment, the vast majority of differentially expressed transcripts (>92%) required a functional AMPK, with 184 out of 199 for MEFs and 670 out of 684 for primary hepatocyte transcripts regulated in an AMPK-dependent fashion, respectively (Fig. 3 and Supplemental Table S1). This observation confirms the nearly exclusive specificity of 991 for targeting AMPK in MEFs and hepatocytes and is consistent with the *in vitro* (cell-free) observation in our previous study (4). In contrast, AICAR induced a much greater transcriptional response with a majority (~50%) of the transcripts differentially regulated in the absence of AMPK (1026 out of 2053 in MEFs and 754 out of 1718 in primary hepatocytes, respectively) (Fig. 3). Notably, despite having demonstrated similar profile of specificity across the models, the compounds affected different sets of genes in MEFs and primary hepatocytes. Altogether, these findings suggest that 991 elicits much more AMPK-specific transcriptional responses compared to AICAR, an observation that is corroborated by the results of PCA (Supplemental Fig. S1) and hierarchical clustering (Fig. 2).

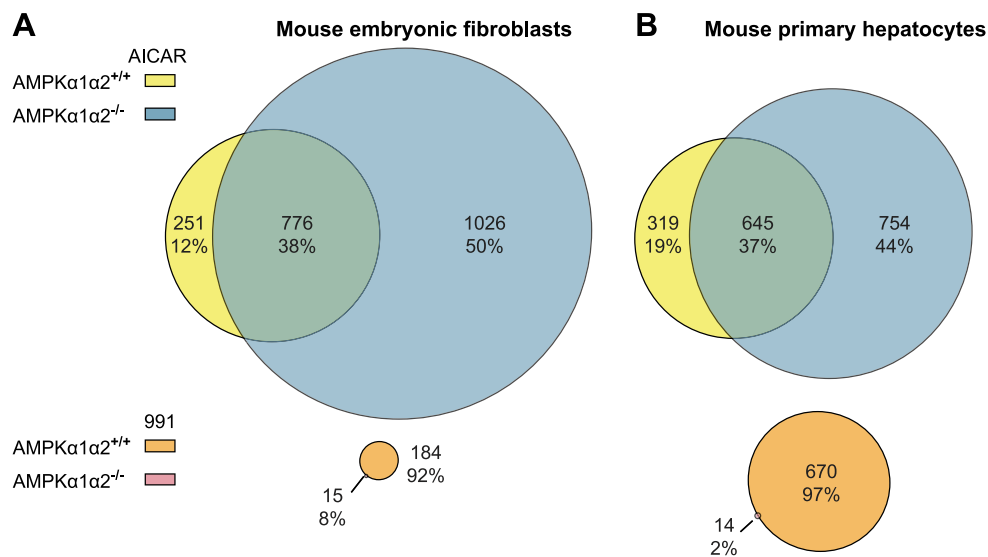
To illuminate the pathways regulated by AMPK, we performed a gene ontology analysis on the genes differentially expressed upon 991 treatment in primary hepatocytes and MEFs using the DAVID program (38, 39) (Fig. 4A, B). This analysis revealed a commonly shared signature of biological and metabolic pathways (e.g., lysosomes) observed in both cell types, as well as additional cell type-specific pathways such as ErbB signaling, sphingolipid metabolism and adipocytokine signaling for primary hepatocytes, and steroid biosynthesis and biosynthesis of antibiotics for MEFs, respectively (Fig. 4A, B). We

observed a higher number of significantly modulated pathways in primary hepatocytes compared to MEFs, among which we found the AMPK signaling pathway, predicted given the changes in expression for transcripts previously shown to be controlled by AMPK [i.e., *Hmgcr*, *G6pc*, and peroxisome proliferator-activated receptor γ coactivator 1 α (*Ppargc1a*)]. In order to validate the microarray data, we performed qPCR analyses on several genes that are known to be involved in lipid/cholesterol signaling and metabolism (*Acss2*, *Crebrf*, *Hmgcr*, *Ldlr*, *Lpin1*, *Msmo1*, *Pde4b*), glucose transport (*Txnip*), immunity (*Ifit1*, *Tollip*), and cell growth/cancer (*Flcn*, *Fnip1*, *Mnt*) (Fig. 4C, D). Overall, we observed a strong correlation between the microarray and qPCR data. Additionally, we also found cell type-specific and compound-specific responses. For example, AICAR had a more profound effect on gene expression compared to 991 in MEFs (*Acss2*, *Flcn*, *Ldlr*, *Lpin1*, *Mnt*, *Tollip*, *Txnip*), but this was not the case in primary hepatocytes (Fig. 4C, D). Interestingly, although AICAR exhibited significant and greater effects on expression of *Flcn* and *Fnip1* in MEFs compared to 991, it had no significant effect in primary hepatocytes. In addition, AICAR elicited an AMPK-dependent effect on *Hmgcr* and *Lpin1* expression in MEFs, which was absent in primary hepatocytes (while the changes were present also in AMPK KO cells). Collectively, we identified AMPK-dependent genes (involved in metabolism, immunity, and cell growth) that demonstrate cell type-specific and compound-specific responses in MEFs and mouse primary hepatocytes.

991-/AICAR-stimulated *Flcn* expression is AMPK-TFEB/TFE3 dependent

To shed light on the mechanism by which AMPK modulates expression of specific genes, we next conducted an *in silico* analysis to identify candidate transcription factors responsible for the AMPK-dependent responses upon

Figure 3. Transcriptome data analysis of the AMPK-activation response following treatment with 991 and AICAR. Venn diagrams showing the transcriptome profiling specificity of 991 and AICAR in MEFs (A) and mouse primary hepatocytes (B). Two-way ANOVA with Benjamini and Hochberg multiple testing correction was applied to discriminate 991 *vs.* control and AICAR *vs.* control conditions. The moderated significance value was set at $P < 0.05$ for the interaction between the genetic background and treatment, as well as for the pairwise comparisons, and a fold-change cutoff of 1.3 was applied. Each group of transcripts is shown with a color code, as specified in the figure. The numbers correspond to the numbers of transcripts altered and the percentage contribution to the total number of transcripts identified for each treatment, respectively. The surface of each circle is proportional to the total number of transcripts it contains.



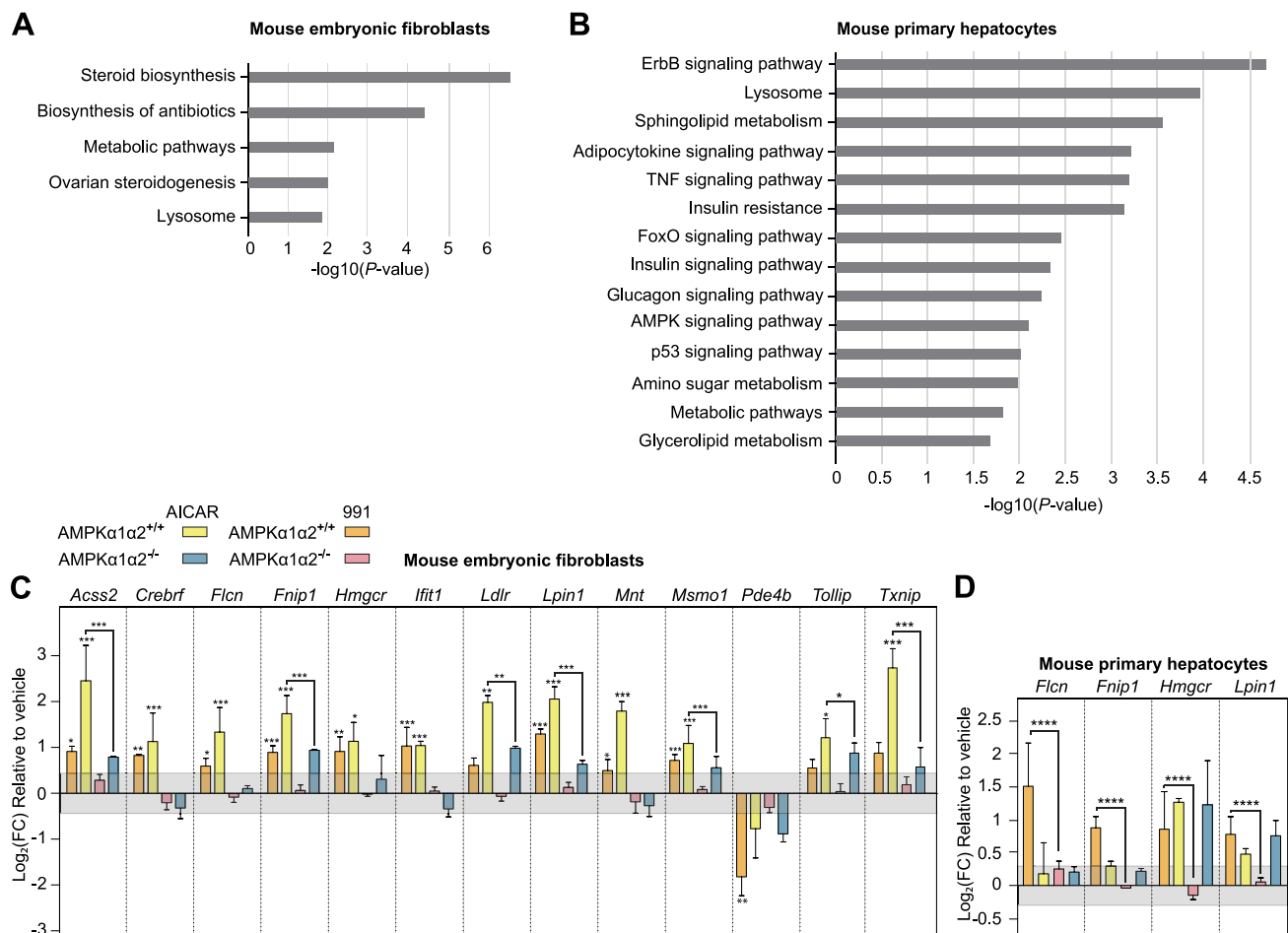


Figure 4. Identification of pathways and genes modulated by AMPK. *A, B*) Gene enrichment analysis of the 991-responsive signature in MEFs (*A*) and mouse primary hepatocytes (*B*). DAVID was used to explore the gene ontology terms associated to the AMPK-regulated genes. The bars represent the negative $\log_{10}$ (P value) of enriched terms, indicating the significance of association between the gene list and an indicated ontology term. *C, D*) Relative mRNA levels of the indicated genes are displayed following qPCR analysis in MEFs (*C*) and primary hepatocytes (*D*). AMPK $\alpha 1\alpha 2^{+/+}$ or AMPK $\alpha 1\alpha 2^{-/-}$ MEFs were stimulated with 10 μ M 991 or 2 mM AICAR for 4 h, and AMPK $\alpha 1\alpha 2^{+/+}$ or AMPK $\alpha 1\alpha 2^{-/-}$ hepatocytes were treated with 3 μ M 991 or 300 μ M AICAR for 4 h. The color corresponds to the 2 treatment conditions and 2 cellular models, as indicated in the figure. Values are represented as $\log_2$ fold-change of the mean $\pm$ SD ($n = 9$). The gray shaded area indicates the $\log_2$ fold-change threshold of ± 0.37 . For the analysis, a 2-way ANOVA with interaction was fit to log-transformed data. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

compound treatment. To this end, we performed a search for correlation in expression between the 991-responsive transcripts identified in MEFs (184 targets) and hepatocytes (670 targets), and the expression of potential upstream regulators using the Ingenuity Pathway Analysis tool (40) (Table 1). The analysis with Ingenuity Pathway Analysis revealed that there were ~ 10 candidates in both cell models, among which we identified transcription factors that have been reported to be regulated by AMPK, including FOXO3 (47) and TP53 (48) in MEFs as well as CREB1 (49) in primary hepatocytes. Of particular interest, only 2 transcription factors, sterol regulatory element binding protein (SREBP) 1 and TFEB, were commonly identified in both MEFs and hepatocytes (Table 1). SREBP1 is a master transcriptional regulator of lipid synthesis, and its activity is known to be regulated by AMPK-mediated phosphorylation (50). TFEB and TFE3 are members of the microphthalmia (MiT/TFE) family of HLH-leucine zipper transcription factors that play an important role in the control of cell and organismal homeostasis through

regulating lysosomal biogenesis and autophagy (51). TFEB is also known to be indirectly regulated by AMPK in the control of lineage specification (52). Interestingly, it was recently reported that TFEB/TFE3 regulate energy metabolism, although the underlying mechanism remains elusive (53, 54). Among the genes regulated specifically in an AMPK-dependent manner (Fig. 4C, D), we focused on *Flcn*, which encodes the tumor suppressor FLCN. FLCN and its binding partner FLCN-interacting protein (FNIP) are known to interact with AMPK and this FLCN-FNIP-AMPK interaction/complex has been proposed to control various metabolic functions (55–57). In addition, it has been suggested that starvation-induced *Flcn* expression is mediated through TFEB/TFE3 in adult retinal pigment epithelial (ARPE-19) cells (58). We first determined the kinetics of the effects of 991 and AICAR on the expression of *Flcn* in a time-course experiment over 24 h, followed by qPCR analysis. We observed that 991 and AICAR induced significant and prolonged expression of *Flcn* throughout the time points measured in an AMPK-dependent manner

TABLE 1. List of predicted transcription factors mediating AMPK transcriptional response

Mouse embryonic fibroblasts	<i>P</i>	Mouse primary hepatocytes	<i>P</i>
SREBP1	1E-14	NUPR1	2E-08
SREBP2	8E-12	CREB1	3E-08
STAT4	2E-08	TFEB	6E-08
SIRT2	7E-08	SREBP1	6E-07
SRC2	7E-06	Miz-1	1E-05
TFEB	2E-04	ATF-4	1E-04
FOXO3	3E-04	ATF-2	3E-04
TP53	4E-04	TCF-3	3E-04
NFATc2	9E-04	MITF	3E-03
IRF-7	3E-03	TAF7L	1E-02
IRF-3	4E-03	HOXD10	1E-01
		HNF-1B	2E-01

The 991-responsive genes in MEFs and primary hepatocytes were used to perform an upstream regulator analysis in Ingenuity Pathway Analysis. The table shows the top predicted transcription factors together with their associated *P* value. ATF-2/4, activating transcription factor 2/4; CREB1, cAMP-responsive element-binding protein 1; FOXO3, forkhead box O3; HNF-1B, hepatocyte nuclear factor-1β; HOXD10, hepatocyte nuclear factor-1β; IRF-3/7, interferon regulatory factor 3/7; MITF, microphthalmia-associated transcription factor; Miz-1, myc-interacting zinc finger protein 1; NFATc2, nuclear factor of activated T cells 2; NUPR1, nuclear protein 1; SIRT2, sirtuin 2; SRC2, steroid receptor coactivator 2; STAT4, signal transducer and activator of transcription 4; TAF7L, TATA box binding protein-associated factor 7-like; TCF-3, transcription factor 3; TP53, tumor protein p53.

compared to the vehicle control, except the later time points (*i.e.*, 8, 12, 24 h) where AICAR caused an increase in *Flcn* expression in AMPK KO MEFs (Fig. 5A). We do not know the mechanism by which AICAR induces *Flcn* expression independently of AMPK; however, this further highlights the necessity to have AMPK KO control to dissect AMPK-dependent from the AMPK-independent effects of AICAR. We confirmed that the increased levels of *Flcn* mRNA were due at least partly to enhanced gene transcription because premRNA levels of *Flcn* were also significantly elevated in response to 991 and AICAR treatment (Supplemental Fig. S2A, B). Moreover, we confirmed that the elevated levels of *Flcn* transcripts were translated into an increase in FLCN protein levels in a time- and AMPK-dependent manner in response to 991 or AICAR treatment (Fig. 5B). We hypothesized that TFEB regulates *Flcn* expression through enhancing its promoter activity, especially based on the fact that the *Flcn* promoter contains a putative TFEB-binding site, which is conserved between humans and mice. To test this hypothesis, we cloned and placed fragments of various length (8000, 1200, and 100 bp) of the mouse *Flcn* promoter region upstream of the luciferase gene and transfected the resulting reporter construct individually into WT or AMPK KO MEFs. 991 (30 μM) increased the promoter activity of all 3 constructs when introduced into WT MEFs, whereas in contrast no or only a marginal increase was seen when they were introduced into AMPK KO MEFs (Fig. 5C). We also observed a similar response using 10 μM 991, with a smaller increase in promoter activity (1.3-fold) in WT MEFs (unpublished results). Notably, when the putative TFEB-binding site was mutated in the 1200 and 100 bp fragments and the corresponding reporter constructs were introduced in WT MEFs, 991-induced promoter activity was significantly reduced (Fig. 5D). Finally, the 991-stimulated *Flcn* promoter activity for both 1200 and 200 bp fragments was ablated when the reporter constructs were introduced

and assayed in TFEB/TFE3 double KO MEFs (Fig. 5E). TFEB/TFE3 double KO MEFs were employed because TFEB and TFE3 have been proposed to be at least partially redundant (50). Finally, we have provided further genetic evidence that TFEB is required for mediating AMPK-induced expression of FLCN at both mRNA and protein levels using TFEB/TFE3 double KO MEFs (Fig. 5F, G). Taken together, these results demonstrate that AMPK promotes *Flcn* transcription through activation of TFEB/TFE3.

AMPK promotes dephosphorylation and nuclear translocation of TFEB independently of mTOR

It has been reported that the transcriptional activity of TFEB is coupled to its subcellular localization and is regulated by reversible phosphorylation. We thus wanted to determine if AMPK-induced activation of TFEB occurs as a consequence of its dephosphorylation and nuclear localization. To test this hypothesis, we treated WT and AMPK KO MEFs with 991 or AICAR for 4 h and prepared cytoplasmic and nuclear fractions followed by Western blot analysis (Fig. 6A). ACC was only detected in the cytoplasm as anticipated, and its phosphorylation was increased (~2 fold) after treatment with the compounds. In WT MEFs, both 991 and AICAR similarly decreased and increased TFEB levels in cytoplasmic and nuclear fractions, respectively, whereas in AMPK KO MEFs, the levels of TFEB were not altered in both fractions upon treatment with 991 or AICAR compared to control (vehicle). Notably, both compound treatments resulted in a marked increase in a faster-migrating form of TFEB, indicative of TFEB dephosphorylation, in the nuclear fraction in WT but not in AMPK KO MEFs (Fig. 6A). To examine if the faster migration of TFEB was associated with dephosphorylation, we generated a phospho-specific antibody against Ser (S)142, one of the key phosphorylation

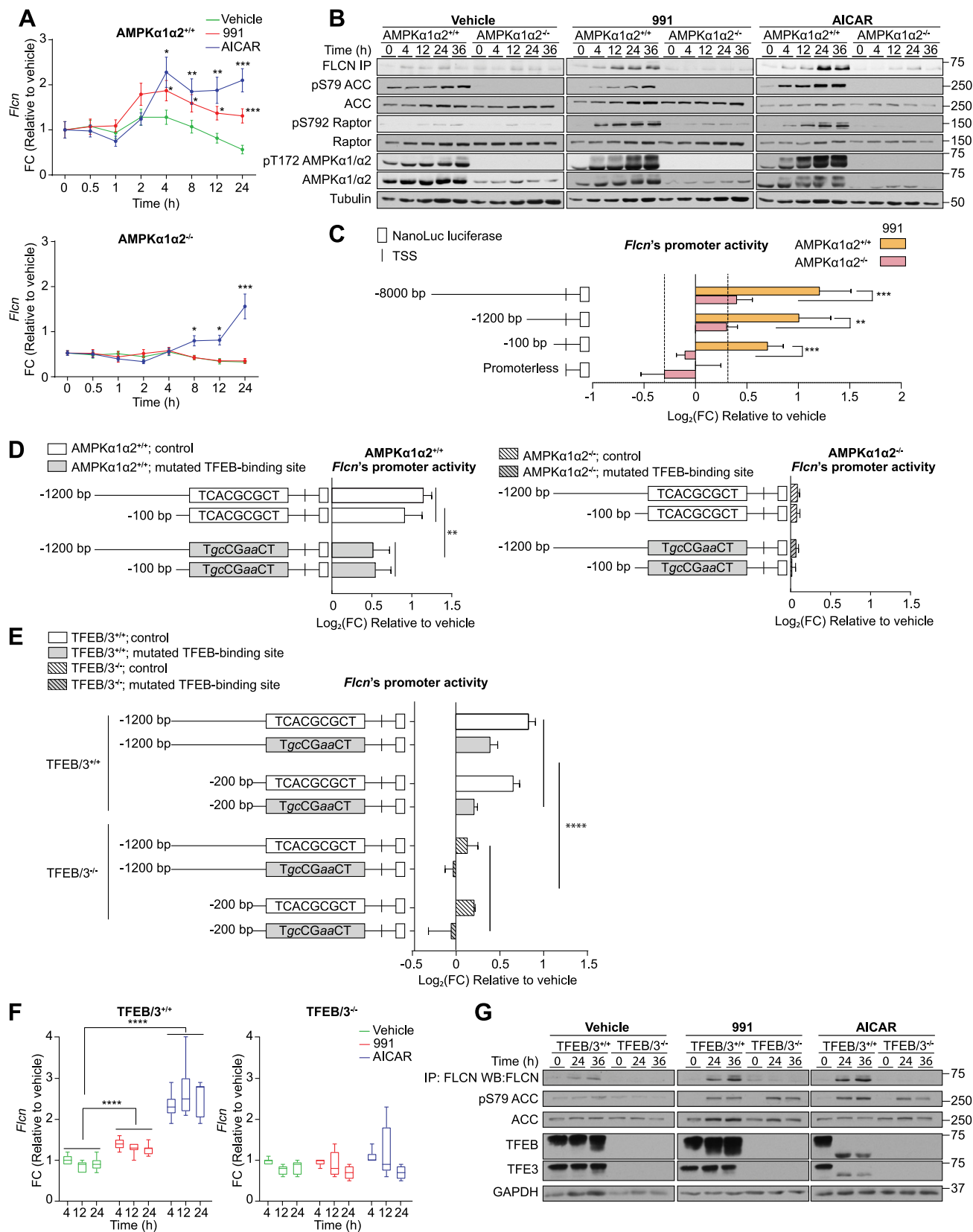


Figure 5. Identification of *Flcn* as an AMPK-regulated gene and TFEB as a transcription factor that mediates this response. **A)** Relative *Flcn* mRNA levels were assessed with a Biomark gene expression 192.24 IFC δ gene assay. AMPK α 1 α 2 $^{+/+}$ or AMPK α 1 α 2 $^{-/-}$ MEFs were treated with vehicle (DMSO), 10 μ M 991 or 2 mM AICAR, for 0, 0.5, 1, 2, 4, 8, 12, and 24 h. Two-way ANOVA with interaction was fit to log-transformed data. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Each data point represents the mean $\pm$ SEM ($n = 12$). **B)** AMPK α 1 α 2 $^{+/+}$ or AMPK α 1 α 2 $^{-/-}$ MEFs were treated with 10 μ M 991 or 2 mM AICAR for the indicated time points. FLCN was immunoprecipitated (IP) from cell lysates and subjected to Western blot analysis with the FLCN antibody. A representative

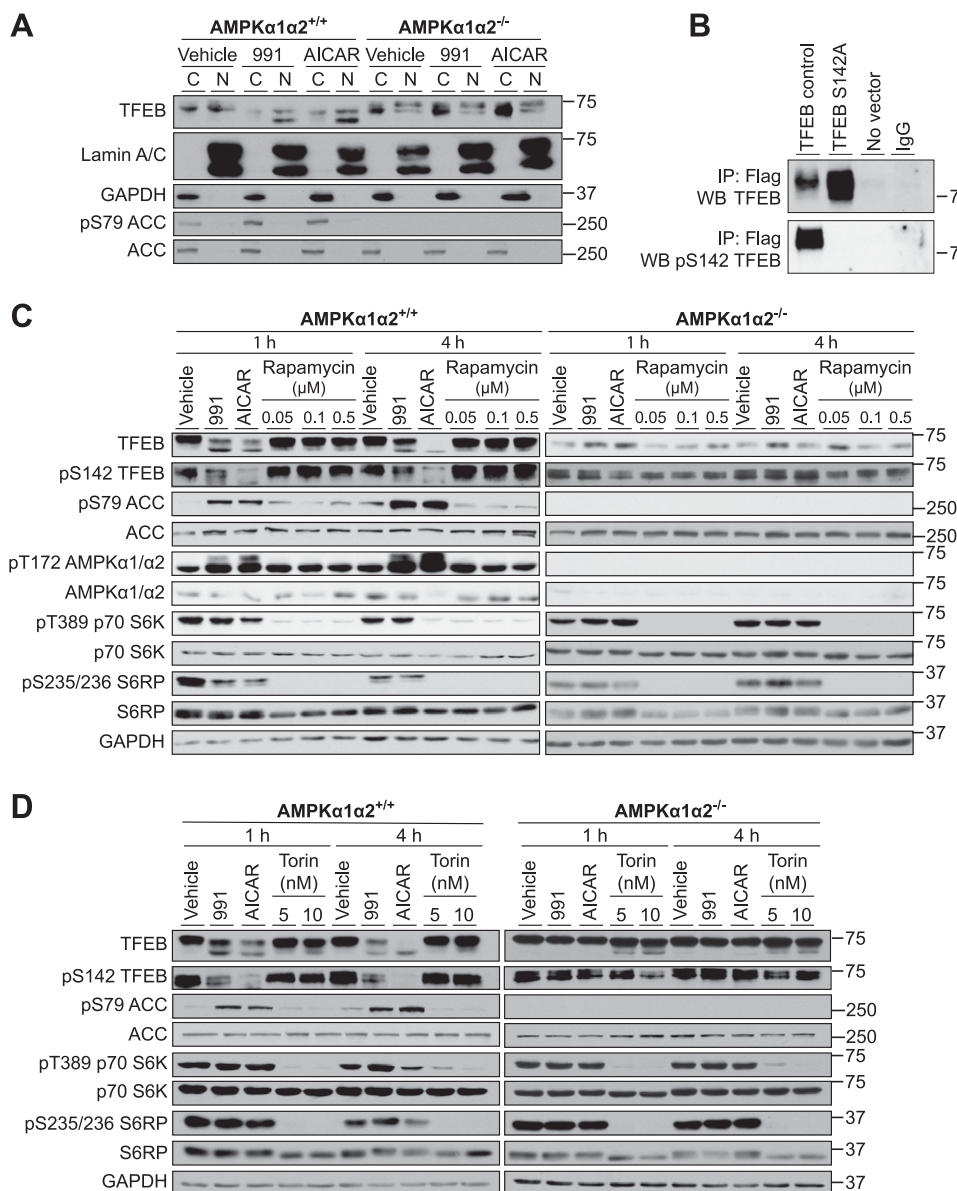
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sites impacting cellular localization and activity of TFEB (34). We verified and confirmed the specificity of the phospho-S142 TFEB antibody by Western blot analysis using recombinant WT and nonphosphorylatable Ala (A) mutant form (S142A) of N terminus Flag-tagged mouse TFEB (Fig. 6B). Given that mTOR has been demonstrated to act as a key upstream regulator of TFEB and that AMPK is known to modulate mTOR activity, we also wanted to address if AMPK-mediated dephosphorylation and nuclear localization of TFEB were dependent on mTOR (Fig. 6C). Consistent with the results shown in Fig. 6A, both 991 and AICAR treatment (for 1 and 4 h) resulted in a downward band shift of total TFEB in WT, which was associated with a decrease in S142 phosphorylation. In contrast, compound treatment had no effect on both band shift and S142 phosphorylation in AMPK KO MEFs (Fig. 6C). AICAR induced a robust decrease in mTOR activity as evidenced by a decrease in phosphorylation of p70 S6K (Thr389, a known mTOR target site) and its downstream substrate ribosomal protein S6 (S6RP) in WT (notably at 4 h posttreatment) but not in AMPK KO MEFs. 991 treatment showed only a modest decrease in S6RP phosphorylation in WT but not in AMPK KO MEFs. Treatment with rapamycin, an mTOR complex 1 inhibitor, for 1 or 4 h at 3 doses (0.05, 0.1, and 0.5 μ M) caused a robust decrease in phosphorylation of p70 S6K and S6RP in both WT and AMPK KO MEFs. In sharp contrast to this observation, rapamycin treatment did not lead to any notable band shift or dephosphorylation (S142) of TFEB in both WT and AMPK KO MEFs (Fig. 6C). It has previously been demonstrated that nutrient-induced S142 phosphorylation was resistant to rapamycin but sensitive to a novel class of mTOR inhibitor (Torin) targeting catalytic site of mTOR (34). Torin (5 or 10 nM for 1 or 4 h) inhibited mTOR activity (*i.e.*, ablated phosphorylation of p70 S6K and S6RP) to a similar degree compared to rapamycin. Of note, Torin

treatment (10 nM, 4 h) caused a modest downward band shift and dephosphorylation (S142) of TFEB, although this was not AMPK-dependent because it was observed in both WT and AMPK KO MEFs (Fig. 6D). To determine whether the presence of active mTOR is required for the AMPK-dependent TFEB dephosphorylation/translocation, we inhibited the mTOR prior to stimulation of AMPK (Supplemental Fig. S3). We pretreated MEFs AMPK WT and AMPK KO with vehicle, Rapamycin or Torin for 1 h and subsequently added either vehicle or 991 for an additional hour. Supplemental Fig. S3 illustrates the inhibition of mTOR complex 1 pathway in cells pretreated with Rapamycin or Torin, as well as the activation of AMPK in 991-stimulated WT cells. We observed that the pretreatment did not affect TFEB band shift pattern, suggesting that mTOR activity is not a prerequisite element for AMPK-mediated dephosphorylation of TFEB.

We next wanted to determine if the AMPK-dependent and mTOR-independent dephosphorylation and nuclear enrichment of TFEB in response to AMPK activators could also be observed in mouse primary hepatocytes (Fig. 7). As anticipated, treatment of hepatocytes with AMPK activators [991, AICAR, and also compound 13 (C13), a recently identified α 1-selective AMPK activator (45, 59)] for 1 or 4 h robustly increased phosphorylation of AMPK and ACC in WT, which was totally ablated in AMPK KO hepatocytes (Fig. 7A). AICAR and C13, but not 991, potentially reduced phosphorylation of p70 S6K and S6RP in an AMPK-dependent mechanism, except that C13 (4 h) displayed an AMPK-independent inhibition of mTOR. In control (vehicle-treated) hepatocytes, total TFEB appeared as doublets in WT, whereas in AMPK KO hepatocytes, TFEB band was fainter and smeary compared to WT. Consistent with our observations using MEFs (Fig. 6A, C, D), AMPK activators resulted in disappearance of the upper band and increased the amount of the lower- or

blot of 3 independent experiments is shown. C) Schematic representation of *Fln*-NanoLuciferase reporter plasmid and the correspondent luciferase activity profile. Different lengths of *Fln*'s promoter region (−8000, −1200, and −100 bp) were inserted upstream of the NanoLuciferase reporter gene. The constructs, along with a firefly luciferase plasmid, which served as internal control, were transiently transfected into AMPK α 1 α 2 $^{+/+}$ or AMPK α 1 α 2 $^{-/-}$ MEFs. Twelve hours post-transfection, cells were treated with vehicle (DMSO) or 30 μ M 991 for 12 h, then harvested and luciferase assay was performed. Values were first normalized by transfection efficiency, and then represented as log₂ fold-change $\pm$ SD, relative to control (vehicle-treated cells) ($n = 3$). The dotted lines indicate the log₂ fold-change threshold of ± 0.37 . Data were analyzed by 2-way ANOVA with the factors of genetic background and promoter length, plus the interaction between the 2 factors. Significance of the genetic background is indicated. *** $P < 0.001$, ** $P < 0.01$. TSS, transcription start site. D) AMPK α 1 α 2 $^{+/+}$ or AMPK α 1 α 2 $^{-/-}$ MEFs were transiently transfected with *Fln*-NanoLuciferase (−1200 or −100 bp) either control or with a mutation on TFEB-binding site (position −40 bp from the TSS), together with a firefly luciferase plasmid for control, and treated as above. Values were first normalized by transfection efficiency, and then represented as log₂ fold-change $\pm$ SD, relative to control (vehicle-treated cells) ($n = 3$). Data were analyzed by a 2-way ANOVA with the factors of promoter length and TFEB-binding site status (control or TFEB-mutated), plus the interaction between these 2 factors. Significance of the TFEB-binding site status is indicated. ** $P < 0.01$. E) TFEB/TFE3 control (TFEB/3 $^{+/+}$) and double KO (TFEB/3 $^{-/-}$) MEFs were transiently transfected with *Fln*-NanoLuciferase (−1200 or −200 bp) control or with a mutation on TFEB-binding site (position −40 bp), together with a firefly luciferase plasmid. Cells were then treated with vehicle (DMSO) or 30 μ M 991. Values were normalized and represented as described in D ($n = 4$). Data were analyzed by 2-way ANOVA with the factors of genetic background, TFEB-binding site status (control or mutated) and interaction. Significance of the genetic background is indicated, **** $P < 0.0001$. F) mRNA level of *Fln* in TFEB/3 $^{+/+}$ or TFEB/3 $^{-/-}$ MEFs treated with 10 μ M 991 or 2 mM AICAR for 4, 12, and 24 h. Data are presented as a box-and-whisker plots (minimum to maximum) of values normalized to control (vehicle-treated cells, 4 h). Data were analyzed by a 2-way ANOVA with the factors of time and treatment, plus the interaction between these 2 factors ($n = 9$). Significance of the treatment factor is indicated, **** $P < 0.0001$. G) TFEB/3 $^{+/+}$ and TFEB/3 $^{-/-}$ MEFs were lysed after 0, 24, and 36 h of treatment with 10 μ M 991 or 2 mM AICAR. Cell lysates (20 μ g) were subjected to Western blot analysis using the indicated antibodies. FLCN was IP from 500 μ g of cell lysate with 1 μ g of anti-FLCN antibody. Images are representative of $n = 2$.



faster-migrating form of TFEB in WT, whereas no apparent change or band shift in TFEB was observed in AMPK KO hepatocytes. We also observed that treatment of WT hepatocytes with the AMPK activators decreased and increased TFEB levels in cytoplasmic and nuclear fractions, respectively (unpublished results). Of note, we attempted to assess TFEB S142 phosphorylation, which turned out to be not detectable, most likely due to the low abundance of total TFEB in mouse primary hepatocytes (unpublished results). In line with the observations in MEFs (Fig. 6C), rapamycin had no apparent effect on TFEB band shift or migration (Fig. 7A). To demonstrate that a loss of band shift or dephosphorylation of TFEB observed in AMPK KO hepatocytes is intrinsic to AMPK deficiency, we reintroduced AMPK subunits (Flag- $\alpha 2$, $\beta 1$, $\gamma 1$) back in AMPK KO cells using adenovirus (Fig. 7B). Genetic deletion of AMPK $\alpha 1$ and $\alpha 2$ catalytic subunit (AMPK KO) resulted in profound reductions in other regulatory subunits ($\beta 1$ and $\gamma 1$). When AMPK $\alpha 2$, $\beta 1$, $\gamma 1$ were introduced/expressed in the AMPK KO hepatocytes, the effect of 991 (following

both 1 and 4 h treatments) on ACC phosphorylation, as well as a downward band shift of TFEB, was restored. Collectively, we demonstrate that AMPK activators promote dephosphorylation and nuclear localization of TFEB in an AMPK-dependent and mTOR-independent mechanism both in MEFs and mouse primary hepatocytes.

***In vivo* evidence that AMPK activation promotes nuclear localization of TFEB and *Fln* expression using zebrafish**

Zebrafish (*Danio rerio*) represents a powerful vertebrate model in biomedical research, given its genetic similarities with humans together with its high fecundity, rapid development, and the optical transparency of embryos and larvae. Taking advantage of the zebrafish model, we sought to address if AMPK activation promotes nuclear localization of TFEB at organismal/tissue levels and across species. To this end, we employed zebrafish transgenically

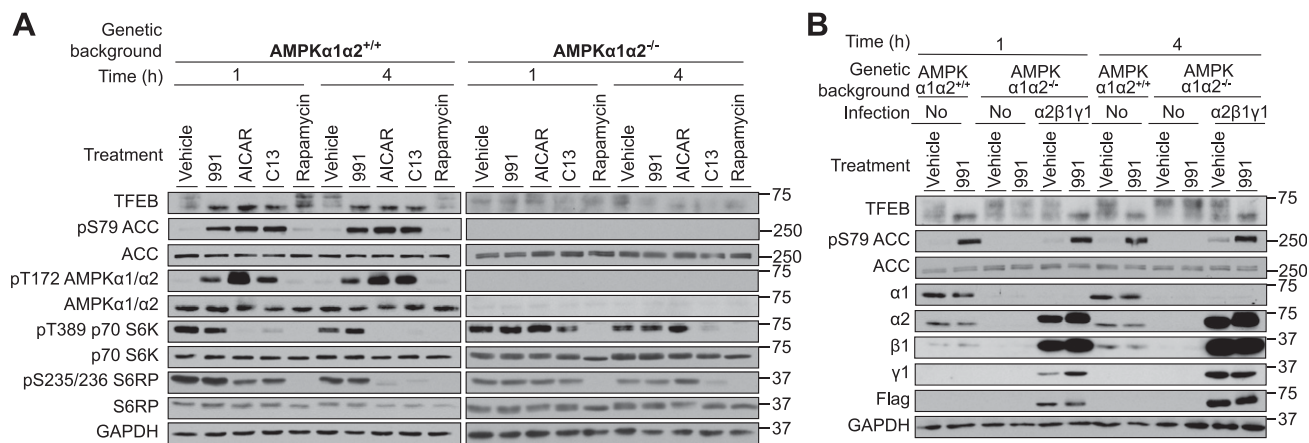


Figure 7. TFEB translocates to the nucleus upon AMPK stimulation independently of mTOR in mouse primary hepatocytes. A) AMPK $\alpha1\alpha2^{+/+}$ or AMPK $\alpha1\alpha2^{-/-}$ mouse primary hepatocytes were isolated and cells were treated with 10 μ M 991, 300 μ M AICAR, 30 μ M C13, or rapamycin 0.05 μ M for 1 or 4 h. Western blot analysis of the indicated proteins was performed on cell lysates. B) Primary hepatocytes were isolated from AMPK $\alpha1\alpha2^{-/-}$ mice and were untreated (No) or coinfecting with 3 adenoviruses (1:3 multiplicity of infection) in order to rescue the expression of the 3 subunits of AMPK (Flag- $\alpha2$, $\beta1$, and $\gamma1$). Sixteen hours postinfection, the primary hepatocytes were treated for 1 h with vehicle (DMSO) or 10 μ M 991. Cell lysates (20 μ g) were used for Western blot analysis with the indicated antibodies. Images are representative of $n = 3$.

expressing Tfeb fused to ZsGreen (ZsGreen-Tfeb), as well as mCherry fused to a nuclear localization signal (NLS) under the actin α cardiac muscle 1b promoter (*act1b*), which drives gene expression in skeletal muscle. We verified by Western blot analysis that 991 (10 μ M for 24 h) increases phosphorylation of AMPK and ACC using larvae protein extracts (Fig. 8A). Fluorescence-based confocal microscopy was performed on embryos 3 dpf following treatment with vehicle (DMSO) or 991 (10 μ M) for 24 h (Fig. 8B). Under vehicle-treated condition, the ZsGreen-Tfeb was distributed throughout the entire cell likely due to cytosolic localization of Tfeb. In support of this, merging the ZsGreen-Tfeb with the mCherry-NLS images showed that there was no apparent nuclear localization of Tfeb (Fig. 8B). Upon 991 treatment, we observed a change in the distribution pattern of ZsGreen-Tfeb going from a diffuse pattern to a forming puncta within the cell. Merging this image with the mCherry-NLS shows that these puncta superimpose with the signal from the mCherry-NLS image. This demonstrates that treatment of zebrafish larvae with 991 leads to translocation of ZsGreen-Tfeb to the nucleus, which is consistent with results obtained with cell fractionation and Western blot analysis in MEFs (Fig. 6A). Altogether, these results compellingly demonstrate that AMPK plays an important role in the regulation and activation of TFEB across species.

Having demonstrated that pharmacological activation of AMPK promotes nuclear localization of TFEB and its transcriptional activation, leading to induced *Flcn* expression, we wanted to study if a physiological activation of AMPK, such as physical exercise, would also increase *Flcn* expression *in vivo*. For this purpose, we generated loss of function of *prkaa1/prkaa2* (which encodes the paralogue of human/mouse AMPK $\alpha1$ and $\alpha2$, respectively) double KO zebrafish using CRISPR-Cas9 genome editing (unpublished data). Using this model, we investigated whether the expression of *Flcn* and *Fnip2*, the paralogue of *Fnip1*, alters in response to an acute bout of swimming exercise. We initially established an acute exercise training

protocol that stimulates AMPK in zebrafish (as summarized in Fig. 8C). The control and exercise groups comprised a mixed population of WT and KO fish, to ensure consistency between the experimental groups. The control group was placed in the swim tunnel and subjected to a low flow speed (5 cm/s) for 3 h. For the exercise group, an acclimatization period of 20 min at a low flow speed (5 cm/s) was followed by a gradual increase of water velocity up to 80% of maximum speed capacity (*i.e.*, 60 cm/s for this cohort of fish), which was maintained for ~ 2 h. Notably, the maximum speed of the fish, defined as the maximum sustainable swimming speed, was determined in a previous grouped endurance test following the existing guidelines (60). Subsequently, the skeletal muscles were collected, and both RNA and proteins were rapidly extracted to avoid AMPK activation due to cellular stress (*e.g.*, hypoxia). The loss of expression/function of AMPK in the KO fish was assessed by Western blot analysis, which confirmed the absence of expression and activity of the AMPK catalytic subunits (Fig. 8D). We confirmed that an acute bout of exercise increased AMPK activity as judged by an increase in phosphorylation of AMPK and ACC in WT muscle (Fig. 8D). Finally, we compared by qPCR the levels of *flcn* and *fnp2*, the paralogue of *fnp1*. The results showed an increased level of expression of both genes after physical exercise in WT zebrafish, and that this increase was significantly decreased in *prkaa1/prkaa2*-deficient zebrafish (Fig. 8E). Taken together, these data suggest that exercise increases the activity of AMPK in zebrafish, which is associated with an increase in *flcn* and *fnp2* expression that is at least in part mediated through AMPK.

DISCUSSION

It is well documented that AMPK not only elicits a plethora of acute metabolic responses but also promotes metabolic reprogramming by modulating gene

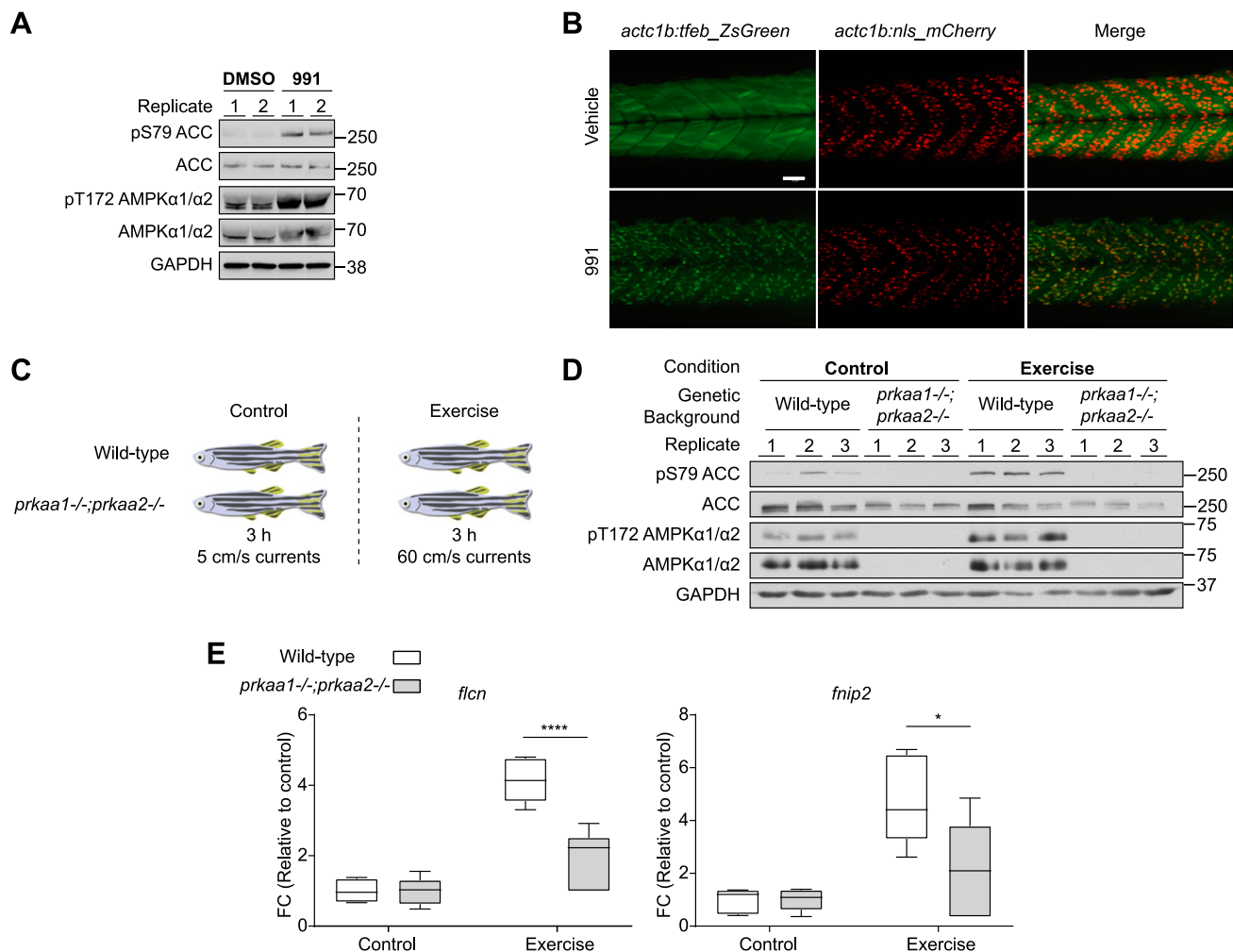


Figure 8. Activation of AMPK leads to translocation of TFEB to the nucleus and increased expression of *flcn* and *fnip2* in zebrafish. **A)** Embryos at 3 dpf were treated with 991 10 μ M or vehicle (DMSO) for 16 h. Tissue lysates (20 μ g) were resolved by SDS-PAGE and Western blot analysis was performed using the indicated antibodies. **B)** *Tg(actc1b:tfeb-ZsGreen);Tg(actc1b:nls-mCherry)* embryos at 3 dpf were treated with 991 10 μ M or vehicle (DMSO) for 24 h. Embryos were mounted live in water containing 0.016% tricaine and imaged with ImageXpress confocal system at original magnification, $\times 20$, the scale bar corresponds to 50 μ m ($n = 12$). **C)** Schematic summary of the acute exercise study design using WT and *prkaa1^{-/-};prkaa2^{-/-}* zebrafish. **D)** Immediately after the 3-h training session, muscle samples were collected from the zebrafish. Tissue lysates (20 μ g) were resolved by SDS-PAGE and Western blot analysis was performed using the indicated antibodies. **E)** Relative mRNA transcript levels of *flcn* and *fnip2* were determined by qPCR. Data are shown as box-and-whisker plots (minimum to maximum) of values normalized to control WT ($n = 6$). Two-way ANOVA with the factors of genetic background and exercise, plus the interaction between these 2 factors, was performed. The graph shows the significance of the interaction. * $P < 0.05$, **** $P < 0.0001$.

expression through regulation of specific transcription factors and transcriptional coactivators (12, 27, 61). In the current study, we performed whole-genome transcriptome profiling in AMPK-intact or -deficient MEFs and hepatocytes. We identified several new AMPK-dependent or -regulated genes and pathways that are differentially regulated in a cell type-specific and a compound-specific manner. The major finding of this study was that we found TFEB, a transcription factor and regulator of lysosomal biogenesis/autophagy (62), as one of the key mediators of AMPK-dependent transcriptional responses.

We performed transcriptome profiling using 2 different AMPK activators (AICAR and 991), known to target distinct regulatory sites or mechanisms. AICAR is a classic and the most commonly used pharmacological AMPK

activator, which significantly contributed to uncover critical metabolic functions of AMPK for decades (63–65). In recent years, with the development and application of genetic technologies or tools (e.g., AMPK KO models), as well as highly specific AMPK activators targeting ADaM site, the specificity of AICAR has been questioned. Several studies have reported AICAR's off-target effects (17), for example, on AMP-regulated enzymes such as fructose 1,6-bisphosphatase, a key regulator for hepatic gluconeogenesis (66, 67). To our knowledge, this is the first study investigating the effect of AICAR on genome-wide gene expression employing AMPK-deficient cell models to dissect AMPK-dependent or -independent effects. It was striking to find out that the vast majority of the transcripts were significantly altered following AICAR treatment, in the absence of functional AMPK. This implied that previously

claimed signaling, cellular, as well as physiologic effects induced by AICAR may not be mediated through AMPK (unless its off-target effects were ruled out using AMPK KO models as a control). In contrast, 991 exhibited nearly exclusive specificity for targeting AMPK in both MEFs and hepatocytes consistent with the specificity of this compound that we showed *in vitro* (cell-free) in our previous study (4). 991 activates both β 1- and β 2-containing complexes (with higher affinity to the β 1 complexes), thus it acts as a pan/total AMPK activator. Given that isoform-specific AMPK activators have recently been identified (45, 68), it would be of interest to elucidate isoform-specific gene responses and metabolic programming.

The cellular localization and activity of TFEB are primarily regulated by its phosphorylation status. Two serine residues, S142 and S211, in TFEB have mainly been proposed to play a key role in determining its subcellular localization (34, 62). When both sites are phosphorylated, TFEB is kept inactive in the cytosol, whereas mutation of either S142 or S211 to nonphosphorylatable alanine residue rendered TFEB constitutively active through keeping it within the nucleus. mTOR and MAPK (also indicated as ERK2) are the main protein kinases known to phosphorylate TFEB under nutrient-rich conditions, in most cell types supplemented with conventional cell culture medium. AMPK inhibits mTOR through multiple mechanisms (69–71), and it has recently been reported that AMPK controls endolysosomal function through suppression of mTOR and its subsequent transcriptional regulation of TFEB (52). Therefore, we initially hypothesized that 991- or AICAR-induced activation of TFEB, *via* dephosphorylation and nuclear localization, is mediated through the AMPK-dependent inhibition of mTOR. Contrary to our hypothesis, we observed that acute treatment with the specific AMPK activator 991 (4, 20), promoted TFEB dephosphorylation in the absence of detectable inhibition of mTOR (as judged by phosphorylation of p70 S6K and S6RP) in primary hepatocytes. It has recently been reported by El-Houjeiri *et al.* (72) that AMPK induces TFEB/TE3-dependent proinflammatory cytokine expression in macrophages and also shown that pharmacological (GSK621) activation of AMPK promotes TFEB/TE3 nuclear localization without apparent changes in downstream phosphorylation of mTOR (p70S6K and 4EBP) in MEFs.

In a previous study, it has been shown that phosphorylation of TFEB at S142 represents a rapamycin-resistant but Torin-sensitive site (34). In line with this, we observed that the S142 phosphorylation was not affected by rapamycin. However, in contrast to the previous observation, Torin displayed only a marginal effect on TFEB dephosphorylation (*i.e.*, appearance of a minor faster-migrating form of the total TFEB and reduced S142 phosphorylation), and notably this was not only observed in WT but also in AMPK KO MEFs. Collectively, in MEFs and primary hepatocytes, 1) we have demonstrated that acute pharmacological inhibition of mTOR (*i.e.*, 1–4 h) does not modulate TFEB phosphorylation, and 2) 991-induced dephosphorylation of TFEB is unlikely to be mediated through AMPK-dependent suppression of mTOR. However, it would be important to assess if other

phosphorylation sites (*e.g.*, S211, S138), which are proposed to play key roles for cellular distribution of TFEB (73), are regulated upon AMPK activation.

The mechanism by which AMPK dephosphorylates and activates TFEB is unknown. It has been demonstrated that nutrient deprivation induces the release of lysosomal Ca^{2+} through Ca^{2+} channel mucolipin 1. This leads to activation of calcium/calmodulin-activated serine/threonine phosphatase calcineurin (also known as protein phosphatase 2B), which binds to and dephosphorylates TFEB, thus promoting its nuclear localization and autophagy induction (74). It would be interesting to determine if AMPK-mediated activation of TFEB regulates the catalytic activity of calcineurin or interaction between TFEB and calcineurin. It has also been shown that nutrient/glucose deprivation-induced AMPK activation regulates lysosomal and autophagy gene expression through phosphorylation at S659 and nuclear localization of ACSS2 (75). Phosphorylated ACSS2 forms a complex with TFEB, which modulates lysosomal and autophagosomal genes by locally producing acetyl-CoA for histone [^3H] acetylation in the promoter regions of these genes. Therefore, AMPK indirectly modulates the transcriptional activity of TFEB after its nuclear translocation *via* inhibition of mTOR in response to energy stress. Nonetheless, in the current study, we found *Acss2* as one of the AMPK-dependent genes up-regulated at mRNA levels in response to both 991 and AICAR. Whether this up-regulation of *Acss2* is linked to an increase in its protein/phosphorylation levels needs to be determined.

Although TFEB is an established master regulator of lysosomal biogenesis (76, 77), emerging evidence suggests that it also acts as key controller for various other cellular and metabolic responses, including lipid metabolism in liver (78), mitochondrial biogenesis in muscle (54), and modulation of the immune response (79). In support of this, we identified genes that are involved in lipid/cholesterol signaling and metabolism (*Acss2*, *Crebrf*, *Hmgcr*, *Ldlr*, *Lpin1*, *Msmo1*, *Pde4b*) and immunity (*Ifit1*, *Tollip*). It would be of interest to determine if these genes are regulated through the AMPK-TFEB axis, as we have shown for *Flcn*. The tumor suppressor FLCN, responsible for the Birt-Hogg Dubé renal neoplasia syndrome, is an AMPK-interacting partner that has recently been proposed to function as a negative regulator of AMPK (56, 57). It has been reported that ablation of FLCN expression or loss of FLCN binding to AMPK causes constitutive activation of AMPK, which was associated with enhanced osmotic stress resistance and metabolic transformation. Notably, genetic inactivation of FLCN in adipose tissue led to a metabolic reprogramming characterized by enhanced mitochondrial biogenesis and browning of white adipose tissue (55). Mechanistically, adipose-specific deletion of FLCN results in induction of the Pparg1 (PGC-1) transcriptional coactivator through relieving mTOR-dependent cytoplasmic retention of TFE3 (80) or activation of AMPK (55). It has been shown that exercise promotes TFEB translocation into the myonuclei, which regulates glucose/glycogen metabolism by controlling expression of glucose transporters, glycolytic enzymes, as well as pathways linked to glucose homeostasis (54).

Moreover, muscle-specific overexpression of TFEB mimics the effects of exercise training and promotes metabolic reprogramming through induction of gene expression involved in mitochondrial biogenesis and function. We showed *in vivo* using zebrafish that exercise induces *Flcn* and *Fnip2* expression at least partially through an AMPK-dependent mechanism. Moreover, we proved that activation of AMPK by 991 induces Tfeb translocation to the nucleus, suggesting that the increased gene transcription of *Flcn* and *Fnip2* observed after exercise are likely mediated through the ability of AMPK to promote Tfeb translocation in skeletal muscle. Whether FLCN mediates part of metabolic responses downstream of TFEB/TFE3 or increased expression of FLCN functions as negative feedback loop to suppress AMPK to avoid its prolonged activation of AMPK is unknown.

Coincidentally, we noticed that in the data set generated by Cokorinos *et al.* (25), the mRNA levels of *Flcn* and *Fnip1/2* were elevated in response to acute or chronic treatment with the ADaM site-binding allosteric AMPK activator PF-739 in mouse skeletal muscle. This strongly supports the findings from our current study that activation of AMPK leads to changes in *Flcn* and *Fnip1/2* *in vivo* across multiple species and tissues. Our study suggests that the ability of PF-739 to increase transcription of *Flcn* and *Fnip1/2* are likely mediated by its ability to regulate TFEB.

In summary, we demonstrated in fibroblasts, hepatocytes, as well as at whole organism levels, *in vivo* using zebrafish, that pharmacological or physiological activation of AMPK promoted nuclear translocation of TFEB. This appeared to be through an apparent effect on dephosphorylation of TFEB, independent of mTOR, and was associated with induction of a tumor suppressor FLCN through activation of its promoter activity. Future studies using gain of function models of FLCN in skeletal muscle and other tissues (*e.g.*, liver) could reveal the physiological significance of the AMPK-TFEB-FLCN pathway. **[F]**

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AUTHOR CONTRIBUTIONS

C. Collodet, P. Descombes, and K. Sakamoto designed the study; C. Collodet performed all the treatments in MEFs and the promoter activity assay; C. Collodet also prepared and analyzed MEFs and zebrafish and hepatocyte samples, generated the figures, and contributed to analysis and data interpretation; M. Foretz performed the hepatocyte isolation, viral infection, compound treatments, cell collecting, and subcellular fractionation; M. Deak performed molecular

cloning and mutagenesis on TFEB and FLCN promoter; L. Bultot supervised C. Collodet during the setup of the gene expression study; S. Metairon executed the microarray and contributed to mRNA preparation; M. Foretz and B. Viollet generated liver-specific AMPK KO mouse model and AMPK KO MEFs; B. Viollet assisted the design of the hepatocyte study; G. Lefebvre contributed to the statistical analysis performing ANOVA analysis fit to log-transformed data; F. Raymond analyzed with C. Collodet the microarray data; A. Parisi generated the AMPK *prkaa1*^{-/-}; *prkaa2*^{-/-} zebrafish line and performed with C. Collodet the fish exercise; G. Civileto generated and performed experiments on the *act1b:tfeb-ZsGreen*; *act1b:nls-mCherry* double transgenic zebrafish; P. Gut supervised A. Parisi and G. Civileto and contributed study design and interpretation of zebrafish studies; P. Descombes supervised the microarray and qPCR experiment design, execution and analysis; P. Descombes and K. Sakamoto supervised C. Collodet; C. Collodet and K. Sakamoto drafted the manuscript; and all the authors contributed to writing and editing the manuscript.

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