Limited survival and impaired hepatic fasting metabolism in mice with constitutive Rag GTPase signaling

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The mechanistic target of rapamycin complex 1 (mTORC1) integrates cellular nutrient signaling and hormonal cues to control metabolism. We have previously shown that constitutive nutrient signaling to mTORC1 by means of genetic activation of RagA (expression of GTP-locked RagA, or RagAGTP) in mice resulted in a fatal energetic crisis at birth. Herein, we rescue neonatal lethality in RagAGTP mice and find morphometric and metabolic alterations that span glucose, lipid, ketone, bile acid and amino acid homeostasis in adults, and a median lifespan of nine months. Proteomic and metabolomic analyses of livers from RagAGTP mice reveal a failed metabolic adaptation to fasting due to a global impairment in PPARα transcriptional program. These metabolic defects are partially recapitulated by restricting activation of RagA to hepatocytes, and revert by pharmacological inhibition of mTORC1.

Constitutive hepatic nutrient signaling does not cause hepatocellular damage and carcinomas, unlike genetic activation of growth factor signaling upstream of mTORC1. In summary, RagA signaling dictates dynamic responses to feeding-fasting cycles to tune metabolism so as to match the nutritional state.
Multicellular organisms adjust their metabolism to the fluctuations of internal and environmental nutrient levels. Insulin and other hormones act as second messengers of systemic nutrient sufficiency to ensure coordinated, coherent responses in different organs to uptake, consume and store during nutrient surpluses. At the cellular level, hormonal cues are integrated with those stemming from the detection of nutrients in the cell to adjust anabolism and catabolism accordingly to both systemic and local abundance. In response to cues from hormones and cellular nutrients, the mechanistic target of rapamycin (mTOR) kinase complex 1 (mTORC1) controls cellular anabolic responses including protein, RNA, nucleotide and lipid synthesis. The signal transduction cascade emanating from insulin binding its receptor at the plasma membrane involves the activation of PI3K and AKT, which leads to the inhibitory phosphorylation of the tuberous sclerosis complex (TSC), itself a GTPase activating protein (GAP) toward Ras homology enriched in brain (RHEB), responsible for direct binding and kinase activation of mTORC1. The involvement of the PI3K-AKT-TSC axis in human disease, including cancer and syndromes related to deregulated control of mTORC1, has fostered the development of mouse models with deregulated hormonal input to mTORC1, being the prime example TSCI/2 knockout mice. These mice die embryonically with supra-physiological mTORC1 activity, and tissue-specific models of deregulated hormonal signaling to mTORC1 have taught us its relevance in coordinating fasting-feeding responses, and in driving pathological growth.

The cellular nutrient signaling cascade that activates mTORC1 occurs independently of that of growth factors, and starts with the activation of sensors of different nutrients, including arginine, leucine, cholesterol, glucose, and other molecules. The signals are ultimately transduced to the Rag family of GTPases, which includes RagA, B, C and D, tethered to the outer lysosomal surface, where amino acid sensing occurs in an inside-out manner. The Rag GTPases operate as heterodimeric complexes formed by either RagA or RagB plus either RagC or RagD. Nutrient sufficiency results in loading of RagA with GTP and RagC with GDP, nucleotide configuration that drives recruitment of RagA to the outer lysosomal surface where the Rag and RHEB GTPases reside, and hence mTORC1 activation occurs. The detailed information regarding the molecular architecture of the regulation of mTORC1 by cellular nutrients contrasts with the fragmented information on its relevance in mammalian physiology. To interrogate on the physiological perturbations caused by systemic chronic elevation in nutrient signaling we have previously generated a gain-of-function model of nutrient signaling to mTORC1 with knock-in mice expressing a GTP-bound, constitutively-active form of RagA under endogenous control of RagA constitutive nutrient signaling to mTORC1 in adult mice. Adult RagA mice constitute a genetic model of chronically elevated nutrient signaling to mTORC1, without experimental perturbations in nutrient intake and without indirect effects associated to their caloric value or other signaling pathways. Adult RagA mice also allow for the interrogation of the consequences of full-body constitutive mTORC1 activity after development, as other genetic tools of activation of mTORC1 in mice (such as loss of the TSC complex) result in developmental lethality.

Results

Rescue of neonatal death of RagAΔGTP mice. We have previously engineered a murine genetic system (RagAΔGTP mice) to drive constitutive nutrient signaling to mTORC1 in all organs. This tool allows for determining the consequences of chronic nutrient-dependent activation on mTORC1 activity in vivo, without elevating nutrient levels and the consequences that this elevation may have on other signaling pathways. Generating adult mice with constitutive RagA signaling was not straightforward because homozygous expression of the RagAΔGTP allele in the mixed 75:25 C57BL/6:129 Sv background, in which the genetic system was engineered, albeit yielding expected Mendelian proportions up to birth, resulted in a fully-penetrant early postnatal lethality. In contrast, RagAΔGTP/+ cells and mice had normal regulation of mTORC1 activity and normal phenotype. We backcrossed the RagAΔGTP strain into a pure C57BL/6 genetic background and found sub-Mendelian frequency of RagAΔGTP/ΔGTP neonates (Supplementary Fig. 1a). Moreover, and in contrast to our previous findings in the original 75:25 C57BL/6:129 Sv background, a large fraction of RagAΔGTP/ΔGTP pups showed deformities and/or were visibly swollen with features inconsistent with fetal viability (Supplementary Fig. 1a, b). Hence, we reasoned that enriching the RagAΔGTP strain to the 129/Sv background could result in a milder phenotype that would allow partial neonatal survival. This rescue occurred, albeit marginally, as only 5% of RagAΔGTP/ΔGTP pups survived long enough to be weaned (3 Supplementary Fig. 1c).

The co-expression of wild-type and mutant alleles of certain GTPases, such as the K-Ras GTPase, suppresses the signaling activation elicited by active mutant variant, phenomenon likely to be at work in the RagAΔGTP/+ cells and mice, which showed no detectable increase in mTORC1 signaling. Hence, we restricted the expression of RagAΔGTP to one allele, in the absence of expression from the other allele, and indeed, survival of hemizygous RagAΔGTP mice (RagAΔGTP/ΔGTP) was observed in the original 75:25 C57BL/6:129 Sv background. No obvious, macroscopic deformities could be appreciated in RagAΔGTP/ΔGTP neonates, indicating an amelioration of the phenotype previously observed in pure C57BL/6 background. The differential outcomes of these genetic systems are summarized in Fig. 1a. The partial rescue of early lethality of RagAΔGTP/ΔGTP mice in 50:50 C57BL/6:129 Sv background occurred without noticeable normalization of mTORC1 activity in hemizygous RagAΔGTP/ΔGTP cells when compared to RagAΔGTP/ΔGTP in primary mouse embryonic fibroblasts (MEFs), revealed by phosphorylation of the mTORC1 target 4eBPs in threonine 389 and phosphorylation of 4EERP1 in threonines 37 and 46 (Fig. 1b). As reported, mTORC1 signaling in heterozygous RagAΔGTP/ΔGTP MEFs was indistinguishable from RagAΔGTP/ΔGTP counterparts (Fig. 1b), indicating that wild-type RagA, when present, suppresses the effect of a GTP-locked RagA.

Characterization of mice with constitutive nutrient signaling. To determine to what extent was mTORC1 activity increased in adult RagAΔGTP/ΔGTP mice, we subjected mice to overnight fasting followed by either 30 min or 2 h refeeding and obtained whole protein extracts from several organs. Fig. 1c shows increased mTORC1 pathway activity in organs from RagAΔGTP/ΔGTP mice during fasting periods, which was more noticeable in liver and...
gastrocnemius (Fig. 1c), and in kidney and heart (Supplementary Fig. 1e), but not as strong in white and brown adipose tissues (Supplementary Fig. 1f). Noteworthy, upon refeeding, the maximal phosphorylation of mTORC1 targets was similar in most organs from RagA<sup>−/−</sup> and RagA<sup>GTP/Δ</sup> mice (Fig. 1c and Supplementary Fig. 1e, f), suggesting that genetic activation of the nutrient signaling pathway results in resistance to inhibition by fasting, but not in overall supra-physiological activation of the mTORC1 pathway. This absence of increased maximal mTORC1 activity was also seen in RagA<sup>GTP/Δ</sup> MEFs (Fig. 1b). Overall, the results of these signal transduction experiments show that Rag<sup>GTP/Δ</sup> mice have increased, but not supra-physiological mTORC1 activity in the fasted state, consistently with a constitutively-active nutrient / Rag GTPase signaling input.
All weaned RagA<sup>GT/P</sup>Δ mice exhibited a fully-penetrant hypopigmented coat (Fig. 1d), a phenotype also observed in tuberous sclerosis patients and in other syndromes related with increased mTORC1 activity, and also in TSC1 floxed strain bred to a strain of transgenic mice that express the Cre recombinase in a melanocyte-specific promoter. Moreover, and although not appreciable at birth, visual examination of adult RagA<sup>GT/P</sup>Δ mice also revealed stereotypical craniofacial morphometric abnormalities, which were confirmed and quantified by computerized axial tomography scans. Three morphometric alterations were most prominent: aberrant nasal septum angle (Fig. 1e, left), decreased length and increased height of the skull (Fig. 1e, right), all of which occurred without significant changes in the total cranial capacity (Fig. 1e, middle) and without obvious behavioral abnormalities. Consistently with the deformities observed in adult RagA<sup>GT/P</sup>Δ mice (Fig. 1e) and in RagA<sup>GT/P</sup>Δ fetuses in pure C57BL/6 background (Supplementary Fig. 1a, b), skull and craniofacial deformities are also prevalent in patients with syndromes caused by mutations in components of the PI3K/mTORC1 pathway.

Reduced body weight, as observed in neonates, was maintained in young and old RagA<sup>GT/P</sup>Δ mice (Fig. 1f, i, and Supplementary Fig. 1g). This reduction correlated with a smaller nose-to-tail distance (Fig. 1g), and with smaller WAT and BAT depots (Supplementary Fig. 1h), also quantified as percentage of fat by means of densitometric analysis (Supplementary Fig. 1i). Decreased weight was not a consequence of abnormal food intake (Supplementary Fig. 1j) neither caused by increased voluntary movement in ad libitum conditions (Supplementary Fig. 1k).

Compromised survival of adult RagA<sup>GT/P</sup>Δ mice. In spite of the partial rescue of neonatal lethality upon hemizygous expression of RagA<sup>GT</sup> in a 50:50 background, survival of RagA<sup>GT/P</sup>Δ mice was also compromised in adulthood, with a mean survival of RagA<sup>GT/P</sup>Δ males and females around 9 months (Fig. 2a and Supplementary Fig. 2a). Necropsies from RagA<sup>GT/P</sup>Δ mice, euthanized due to a sharp decline in body condition, frequently revealed aspiration pneumonia (Fig. 2b). While the cause of death could not be unequivocally ascertained in all mice, fatal aspiration pneumonia in a large fraction of adult RagA<sup>GT/P</sup>Δ mice was most likely caused by the presence of fully penetrant megaesophagus (Fig. 2c), itself a possible cause for aspiration pneumonia. Upon regurgitation, and ultimately caused by a distended esophagus. To formally determine the potential existence of a functional defect in esophageal transit, we monitored the oral-cavity-to-stomach transit, by means of oral administration of iopamiro, an imaging contrast reagent, which showed a partial delay of about 2 h in the clearing of the oral contrast in the esophagus of RagA<sup>GT/P</sup>Δ mice (Fig. 2d). Of note, this modest 2 h transit delay was unlikely to affect the kinetics of fasting – refeeding cycles and experiments, as a short-term drop in glycaemia upon 12 h fasting was indistinguishable from that of RagA<sup>+</sup/+ mice (Supplementary Fig. 2b).

Glucose homeostasis defects in RagA<sup>GT/P</sup>Δ mice. To ascertain the consequences of constitutive nutrient signaling on the control of glucose homeostasis, compromised in other mouse models of deregulated mTORC1 activity, we first measured glycaemia in RagA<sup>+</sup/+ and RagA<sup>GT/P</sup>Δ mice in ad libitum and fasted states. In contrast to the profound hypoglycaemia of RagA<sup>GT/P</sup> mice, RagA<sup>GT/P</sup>Δ males (Fig. 3b) and females (Fig. 3c) had increased fasted insulin levels and the HOMA-IR ratio, indicative of systemically decreased insulin sensitivity (Fig. 3d, e and Supplementary Fig. 3d, e). Because mTORC1 activity unleashes a negative feedback loop that indirectly suppresses the activation of PI3K-Akt that can impair the positive effect of insulin in glucose uptake, we ascertainment the phosphorylation status of Akt and some Akt targets. Interestingly, the livers of RagA<sup>GT/P</sup>Δ mice did not show evidence of suppressed Akt signaling (Fig. 3f and Supplementary Fig. 3f), but the gastrocnemius muscle presented a slight, but significant decrease in the phosphorylated Akt, and in some of its targets, upon refeeding (Fig. 3f and Supplementary Fig. 3g). This differential effect in liver and skeletal muscle highlights the importance of this negative feedback loop for muscle-regulated physiology and in the onset of insulin resistance, but is unlikely to fully explain the mild systemic glucose intolerance. Despite this mild decrease in Akt signaling, an insulin tolerance test (ITT) revealed no alterations in insulin-induced decrease in glycaemia in RagA<sup>GT/P</sup>Δ mice (Fig. 3g and Supplementary Fig. 3h), and ex vivo glucose uptake measured in isolated extensor digitorum longus (EDL) and in soleus muscles derived from RagA<sup>GT/P</sup>Δ and wild-type mice was similar between genotypes (Supplementary Fig. 3i). Thus, the intolerance to glucose, and significant increase in fasting insulin levels of female RagA<sup>GT/P</sup>Δ mice do not appear to stem from an intrinsic impairment in glucose uptake. Because increased gluconeogenesis can lead to increased fasting hyperglycaemia, we conducted pyruvate tolerance test (PTT), and observed that pyruvate injection caused a similar increase in glycaemia in RagA<sup>+</sup/+ and RagA<sup>GT/P</sup>Δ mice (Fig. 3h and Supplementary Fig. 3j). In addition, and consistently with no differences in hepatic Akt signaling,
RagA<sup>GTP/Δ</sup> mice. a Kaplan-Meier survival curves of RagA<sup>+/+</sup> and RagA<sup>GTP/Δ</sup> males (n = 503 and n = 235) and females (n = 521 and n = 220). Mean survival depicted in red. Statistical significance was calculated with the log-rank (Mantel-Cox) test. b Representative H&E staining of a normal lung (RagA<sup>+/+</sup>) and a lung with areas of pneumonia (RagA<sup>GTP/Δ</sup>), black arrowheads indicate infiltration of inflammatory cells, red arrowheads indicate the presence of macrophages with hemosiderin and green arrowheads indicate areas of emphysema. This phenotype was observed in at least 32 mutant mice. c (Left) Maximum esophageal diameter was measured upon sacrifice of RagA<sup>+/+</sup> (n = 9) and RagA<sup>GTP/Δ</sup> (n = 9) mice. (Right) Representative CT image of an esophagus administered with an oral contrast (iopamiro). Statistical significance was calculated by using unpaired two-tailed t-test. d Quantification of remnant iopamiro in the esophagus following its administration in RagA<sup>+/+</sup> (n = 4) and RagA<sup>GTP/Δ</sup> (n = 3) mice. Half-life of iopamiro was calculated from the slope of each individual curves and analyzed by using an unpaired two-tailed t-test. For the intensity of iopamiro, statistical significance was calculated by using 2-way ANOVA (mixed-effects model RELM). In all panels, horizontal lines indicate the mean and error bars depict Standard deviation (SD).

**Proteomics analysis of livers from RagA<sup>GTP</sup> mice.** To identify cellular changes driven by constitutive nutrient signaling in vivo in an unbiased manner, we conducted whole-proteome analysis of livers obtained from overnight fasted and ad libitum fed wild-type and RagA<sup>GTP/Δ</sup> mice (Fig. 4a). From a total of 7175 proteins quantified in all samples, we observed that the levels of 250 proteins were significantly different in RagA<sup>GTP/Δ</sup> versus RagA<sup>+/+</sup> livers from mice fasted overnight (Fig. 4b, Supplementary Data. 1), and 144 were significantly different in RagA<sup>GTP/Δ</sup> versus RagA<sup>+/+</sup> livers in ad libitum conditions (Fig. 4c). Only 92 proteins were found significantly altered when comparing ad libitum vs. fasted conditions in RagA<sup>GTP/Δ</sup> livers (Supplementary Fig. 4a), almost half of the observed changes in RagA<sup>+/+</sup> mice (Supplementary Fig. 4b), indicating higher similarity between the two nutritional states when mTORC1 is constitutively active. Principal Component Analysis (PCA) scattered RagA<sup>+/+</sup> and RagA<sup>GTP/Δ</sup> samples in identifiable, different regions (Supplementary Fig. 4c). Interestingly, a significant fraction of the differentially abundant proteins in RagA<sup>GTP/Δ</sup> versus RagA<sup>+/+</sup> were either decreased or increased in both fasted and fed states (Supplementary Fig. 4d; p < 6.028 × 10⁻³⁴ for upregulated, and p < 1.119 × 10⁻³⁰ for downregulated proteins), indicating that certain changes caused by constitutive nutrient signaling do not depend on the nutritional status.

Next we analyzed the differential proteome of RagA<sup>+/+</sup> versus RagA<sup>GTP/Δ</sup> samples in fasting conditions, as this comparison would presumably show the impact of elevated nutrient signaling in the physiological setting in which it is normally suppressed. Consistently with the expected changes caused by genetic activation of Rag GTPase signaling, gene set enrichment analysis (GSEA) 32 showed the expected enrichment of a mTOR signaling signature in RagA<sup>GTP/Δ</sup> livers in fasted state (Supplementary Fig. 4e), but not in ad libitum fed RagA<sup>+/+</sup> versus RagA<sup>GTP/Δ</sup> mice. The proteomic dataset also showed significant enrichment of proteins encoded by classical TOP mRNAs (Supplementary Table 1) in RagA<sup>GTP/Δ</sup> samples from fasted mice (Supplementary
This association was expected because mTORC1 promotes global protein synthesis by driving the translation initiation of 5′ TOP mRNAs that encode most of the translation machinery, including ribosomal proteins and other known translation initiators. Surprisingly, targets of TFEB, a transcription factor inhibited by direct phosphorylation by mTORC1, were not down-regulated in RagA \( \text{GTP/Δ} \) samples (Supplementary Fig. 4g). This result suggests that, whereas activation of RagC strongly suppresses TFEB activity, the effect of genetic activation of RagA alone may have a more subtle effect on such inhibition.

Only three main functional categories stood out among the signatures significantly depleted from RagA \( \text{GTP/Δ} \) livers under fasting conditions: lipid metabolism, mitochondria, and...
Fig. 3 Constitutive activation of RagA compromises systemic glucose homeostasis. a Glycaemia of 5- to 23-week-old RagA+/+ and RagAGTP/Δ females in ad libitum conditions and after 16 h of fasting. RagA+/+ (ad libitum n = 20 and fasted n = 47). RagAGTP/Δ (ad libitum n = 20 and fasted n = 38). Statistical significance was calculated by using 2-way ANOVA with Sidak’s multiple comparison correction. b 24- to 33-week-old RagA+/+ (n = 10) and RagAGTP/Δ (n = 6) females were starved for 48 h. Glucose was monitored every 1 h except in between 8 pm and 8 am. c Glucose tolerance test (GTT) of 14- to 22-week-old RagA+/+ (n = 8) and RagAGTP/Δ (n = 7) females. d Insulin levels of 18- to 33-week-old RagA+/+ (n = 15) and RagAGTP/Δ (n = 14) females fasted 16 h. e HOMA-IR ratio calculated from the ratio between insulin and glucose levels obtained from 18- to 33-week-old RagA+/+ (n = 15) and RagAGTP/Δ (n = 14) females fasted for 16 h. For d and e statistical significance was calculated by using unpaired two-tailed t-test. f 25- to 36-week-old RagA+/+ and RagAGTP/Δ females were deprived from food for 16 h and sacrificed following a 30 min refeeding. Ad libitum (ad lib.) fed mice were also included. Protein lysates from the liver and gastrocnemius muscle (Gastroc.) were immunoblotted for the indicated proteins (top) and the quantification of the intensity relative to vinculin was calculated for the indicated antibodies (bottom). Statistical significance was calculated by using 2-way ANOVA with Sidak’s multiple comparison correction. g Insulin tolerance test (ITT) of 19- to 29-week-old RagA+/+ (n = 12) and RagAGTP/Δ (n = 12) females. h Pyruvate tolerance test (PTT) of 20- to 22-week-old RagA+/+ and RagAGTP/Δ (n = 6) females. i RT-qPCR of livers from 15- to 36-week-old RagA+/+ (fasted n = 5; fed n = 4) and RagAGTP/Δ (fasted n = 5; fed n = 4) females. Expression levels of the indicated genes relative to those of β-actin were relativized to the average level of RagA+/+ mice in fed state. Fast: Fasted. For b, c, g, and h, statistical significance was calculated by using 2-way ANOVA with Sidak’s multiple comparison correction. The area under the curve (AUC) was calculated and statistical significance was calculated by using unpaired two-tailed t-test. In all panels, horizontal lines indicate the mean and error bars depict Standard deviation (SD).

pexosomes (Fig. 4d, e and Supplementary Fig. 4h). These three categories are largely composed by proteins encoded by transcriptional target genes of peroxisome proliferator-activated receptor alpha (PPARα). Indeed, signatures of PPARα targets, including one list of 168 PPARα target genes curated by us (Supplementary Table 1), were also significantly depleted (Fig. 4f) from RagAGTP/Δ vs. RagA+/+ livers obtained from fasted mice (Fig. 4d-f, and Supplementary Fig. 4h). These data suggest that a major consequence of the inhibition of nutrient signaling during fasting is the induction of PPARα targets.

Constitutive nutrient signaling impairs the PPARα program. The PPAR family of nuclear receptors is composed of three isoforms (a, β/δ, and γ) that differ in tissue distribution, ligand specificity, and metabolic regulatory activities. mTOR controls PPARα, PPARγ, and transcriptional co-activators such as PGC1α. Hence, we evaluated the transcriptional levels of PPAR family members in RagA+/+ and RagAGTP/Δ livers. In agreement with our proteomics analysis, the mRNA levels of PPARα were significantly decreased upon fasting in RagAGTP/Δ mice. Interestingly, PPARγ levels were also decreased, while no changes were noticeable in the levels of PPARβ/δ and PGC1α (Fig. 5a). Because mTORC1 activity, together with mTORC1-independent actions of Akt, promotes a SREBF- and PPARγ-dependent lipogenic program, we measured the mRNA levels of the transcription factor Sreb1 and of some key lipogenic factors, such as Acaca, Acly, and Fasn, but found no experimental support for increased expression of these factors in livers from RagAGTP/Δ mice (Supplementary Fig. 5a) neither were they increased in the proteomics datasets (Supplementary Fig. 5b). In addition, the hepatic triglyceride content was not increased in RagA+/+ and RagAGTP/Δ mice (Supplementary Fig. 5c), consistently with other models of increased hepatic mTORC1 activity. These results are consistent with the strict requirement for increased Akt activation, in addition to constitutive nutrient signaling - mTORC1 activity, to drive lipogenesis in the liver. Interestingly, and in contrast to the findings in liver, expression of PPARγ was increased in subcutaneous WAT from RagAGTP/Δ in the fed state (Supplementary Fig. 5d), suggesting that the impact of nutrient signaling activation on the transcriptional program of lipogenesis is not identical in the liver and in WAT. In subcutaneous WAT from RagAGTP/Δ mice, neither the mRNA levels of PPARα nor its transcriptional targets involved in ketogenesis, β-oxidation or peroxisomal function were significantly decreased during fasting, as compared to RagA+/+ samples (Supplementary Fig. 5d). A similar picture was observed in skeletal muscle (Supplementary Fig. 5e). These results suggest that the PPARα program in the liver is more sensitive to constitutive nutrient signaling, compared to that of WAT and skeletal muscle.

A key role of PPARα is to promote a transcriptional program that elicits hepatic ketogenesis and fatty acid oxidation during fasting. The mRNA levels of PPARα targets important for ketogenesis were decreased in livers from fasted RagAGTP/Δ mice (Fig. 5b, c). Accordingly, fasting-induced ketogenesis was impaired in RagAGTP/Δ mice (Fig. 5d), as revealed by the decrease in the levels of the ketone bodies acetoacetate and β-hydroxybutyrate, a defect that has been previously observed in liver-specific TSC1 knockout mice. Additionally, mRNA levels of key enzymes involved in mitochondrial β-oxidation were also decreased in the livers from fasted RagAGTP/Δ mice as compared to RagA+/+ counterparts (Fig. 5b), differences that were also noticeable at the protein level (Fig. 5e and Supplementary Fig. 5f), pointing to a defect in lipid metabolism. An incomplete burning of lipids species can generate hepatic accumulation of lipid intermediates, a trend we observed for medium and long saturated and unsaturated fatty acids and acyl-carnitine species in liver extracts from fasted RagAGTP/Δ versus RagA+/+ mice (Fig. 5f). Interestingly, the accumulation of fatty acids in RagAGTP/Δ livers occurred without concomitant changes in the levels of the same fatty derivatives and of free fatty acids in circulation (Supplementary Fig. 5g, h).

To ascertain whether the proteomic, transcriptomic, and metabolomic findings translated into a physiological metabolic defect, we conducted indirect calorimetric analyses in ad libitum fed and fasted RagAGTP/Δ and RagA+/+ mice. RagAGTP/Δ mice had no alterations in energy expenditure (EE) nor in the respiratory quotient (RQ) in ad libitum conditions, but interestingly, presented an increase in both EE and RQ upon fasting (Fig. 5g and Supplementary Fig. 5i), supporting a defective systemic adaptation to fasting. In agreement with an impaired fed-to-fasted adaptation, bodyweight loss was significantly higher in RagAGTP/Δ mice than in RagA+/+ (Supplementary Fig. 5j). Finally, the upward shift in RQ under fasting conditions is consistent with increased metabolism of carbohydrates to detriment fat/ketone body metabolism, as the biochemical observations suggested. Collectively, RagAGTP/Δ mice show defects in energy balance that manifest only under a fasted state, driven by an impairment in decreasing mTORC1 activity in a Rag GTPase-dependent manner upon nutrient limitation.

To evaluate whether the hepatocytes of RagAGTP/Δ mice had an intrinsic defect in mitochondrial activity, we measured ex vivo cellular respiration in primary isolated hepatocytes. Indeed, RagAGTP/Δ hepatocytes showed a striking reduction in basal and maximal respiration (Fig. 5h), while the suppression of fatty
acid oxidation (FAO) by etomoxir was subtle in hepatocytes from both genotypes (Fig.5h).

PPARα also regulates amino acid degradation and controls the expression of peroxisomal proteins, including those involved in peroxisomal β-oxidation. Like PPARα−/− mice, RagA GTP/Δ mice showed elevated plasma urea levels upon fasting (Fig. 6a) and a striking reduction in the circulating levels of several amino acids (Fig. 6b and Supplementary Fig. 6a). These changes occurred with a similar increase in protein and mRNA levels of hepatic enzymes involved in the urea cycle (Fig. 6c, d, and Supplementary Fig. 6a), which are transcriptionally repressed by PPARα.45,46 Moreover, and in addition to the decrease in peroxin proteins detected by mass spectrometry (Supplementary Fig. 6d), mRNA levels of several structural peroxisomal genes and proteins detected by western blot were significantly decreased in livers of RagA GTP/Δ mice (Fig. 6e, f), strongly suggesting a consistent decrease in peroxisomes. Peroxisomes are involved in several physiologically relevant metabolic reactions, including long chain fatty acid oxidation and bile acid synthesis. Interestingly, both protein and mRNA levels of specific enzymes and transporters involved in peroxisomal β-oxidation were diminished in the livers of RagA GTP/Δ mice (Fig. 6c–g and Supplementary Fig. 6e). This decrease was also observed in primary hepatocytes isolated from RagA GTP/Δ mice, as compared to those from RagA+/+ mice (Supplementary Fig. 6f). Importantly, the levels of bile acids, controlled by peroxisomal activity, were abnormally high in sera from RagA GTP/Δ mice (Fig. 6h). Altogether, these defects underlie a complete, global impairment in the PPARα-dependent fasting metabolic program in mice with constitutive nutrient signaling.

Reversion of metabolic defects by acute rapamycin treatment. Next, we tested whether impaired PPARα transactivation and its
physiological consequences observed in RagA\textsuperscript{GTP/Δ} mice could be reverted by short-time inhibition of mTORC1 with rapamycin. Administration of rapamycin partially reverted the impaired transcription of PPAR\textalpha{} and PPARγ in RagA\textsuperscript{GTP/Δ} mice, and it also increased their levels in RagA\textsuperscript{+/+} mice (Fig. 6i). Rapamycin also restored the expression of PPAR\textalpha{} target genes involved in ketogenesis in livers from RagA\textsuperscript{GTP/Δ} mice (Supplementary Fig. 6g) and fasting-induced ketogenesis (Fig. 6j). Although the expression of the PPAR\textalpha{} target genes Cpt\textalpha{}, Acadm and Acadl, involved in β-oxidation, was minimally affected by rapamycin (Supplementary Fig. 6g), the abnormally high levels of fatty-acid intermediates in the liver of RagA\textsuperscript{GTP/Δ} mice were reduced (Supplementary Fig. 6h). Similarly, acute pharmacological inhibition of mTORC1 corrected the differences in peroxin protein
levels (Supplementary Fig. 6i, j). Finally, the aberrantly high levels of circulating bile acids in RagAGTP/Δ mice were normalized by rapamycin (Fig. 6k). Hence, acute correction of mTORC1-signaling under fasting is efficacious to boost the defective PPARα activity caused by constitutive nutrient signaling.

Metabolic phenotypes in liver-specific RagAGTP mice. To assess whether Rag GTPase signaling in the liver was responsible for the metabolic phenotypes observed in full-body RagAGTP mice, we generated mice with constitutive activation of RagA restricted to hepatocytes (RagAGTP/lox; Albumin-CreTg mice, hereafter referred to as Li-RagAGTP/Δ mice) (Supplementary Fig. 7a). Li-RagAGTP/Δ mice were born at expected mendelian ratios without abnormalities (Supplementary Fig. 7b). As expected, livers from Li-RagAGTP/Δ mice showed increased mTORC1 activity upon fasting (Fig. 7a), but not other organs, such as gastrocnemius muscle (Supplementary Fig. 7c). Unlike full-body RagAGTP/Δ mice, Li-RagAGTP/Δ mice had no differences in body weight (Supplementary Fig. 7d), nor in the percentage of fat (Supplementary Fig. 7e).

In contrast to full-body RagAGTP/Δ mice, Li-RagAGTP/Δ mice had normal glycemia during long-term fasting (Supplementary Fig. 7f). However, like full-body RagAGTP/Δ mice, both male and female Li-RagAGTP/Δ mice exhibit a mild but reproducible decrease in glucose tolerance (Fig. 7b). This alteration in glycemia occurred without a concomitant increase in fasting insulin levels nor in the HOMA-IR ratio (Supplementary Fig. 7g). Indeed, Li-RagAGTP/Δ mice responded as wild-type counterparts when subjected to an ITT (Supplementary Fig. 7h). In agreement with the signaling results of livers from full-body RagAGTP/Δ mice (Fig. 3f and Supplementary Fig. 3f), Akt signaling in vivo was normal in livers from Li-RagAGTP/Δ mice (Supplementary Fig. 7i), and was only mildly decreased in the gastrocnemius muscle of Li-RagAGTP/Δ mice (Supplementary Fig. 7j). In addition, and like full-body RagAGTP/Δ mice, the results of the PTT were similar in Li-RagAGTP/Δ and wild-type mice (Supplementary Fig. 7k), and also similar was the expression of hepatic gluconeogenic enzymes and PYPGL, enzyme involved in glycojen degradation (Supplementary Fig. 7l). Glycogen was mobilized with similar kinetics in Li-RagAGTP/Δ and wild-type animals both in the liver and in the gastrocnemius muscle (Supplementary Fig. 7m). Thus, deregulated hepatic Rag GTPase signaling suffices to deteriorate glucose homeostasis without detectable effects on gluconeogenesis or glycogen mobilization.

Proteomics analysis from Li-RagAGTP mice. We next performed whole-proteome analysis of livers from overnight fasted-refed Li-RagAGTP/Δ and control mice (Fig. 7c, Supplementary Data. 2), as done with full-body RagAGTP/Δ mice (Fig. 4). From a total of 6553 detected proteins found in all samples, the levels of 212 proteins were significantly different in Li-RagAGTP/Δ vs. control livers from fasted mice (Supplementary Fig. 7n), while the levels of only 127 proteins were significantly different in Li-RagAGTP/Δ vs. control livers in refed conditions (Supplementary Fig. 7n). As seen in the proteomics analysis from full-body RagAGTP/Δ mice, unbiased whole-proteome comparisons revealed that the most striking differences observed in Li-RagAGTP/Δ vs. control livers from fasted mice were the downregulation of proteins related to fatty acid metabolism (Fig. 7d), and PPARα targets, but not TFEB targets (Fig. 7d and Supplementary Fig. 7o). The similarities of the proteomic analyses from full-body and liver-specific RagA activation (Supplementary Fig. 7p), showing a non-random overlap of differentially abundant proteins in the two systems, together with the assays with primary hepatocyte cultures, indicate that the restricted activation of Rag GTPase signaling in the liver cell-autonomously impairs PPARα-dependent transactivation and affects hepatic lipid homeostasis. Consistently, the mRNA levels of PPARα and several of its target genes, including those involved in ketogenesis and peroxisomes, were reduced in livers from fasted Li-RagAGTP/Δ mice (Fig. 7e), and a similar reduction in the levels of proteins involved in mitochondrial and peroxisomal β-oxidation was found specifically in fasting conditions (Supplementary Fig. 7q, r), as observed in full-body RagAGTP/Δ mice. Finally, ketogenesis was impaired in Li-RagAGTP/Δ mice upon fasting (Fig. 7f), together with significantly increased levels of bile acids in circulation (Fig. 7g). Altogether, we conclude that the sole activation of Rag GTPase signaling in hepatocytes recapitulates metabolic defects and impaired adaptation to fasting observed in mice with systemic activation of RagA, dissecting a prominent role for hepatic nutrient signaling upstream of mTORC1 in the systemic control of fasting metabolism.
Side-by-side comparison of Li-RagA^{GTP} and Li-TSC1^{KO} mice. Constitutive activation of RagA in the liver did not lead to increased liver size (Fig. 8a), neither it increased the levels of transaminases indicative of liver damage, such as alanine amino transferase (ALT) and alkaline phosphatase (ALP) (Fig. 8b), nor resulted in the development of spontaneous liver tumors and shortened lifespan (Fig. 8c, d). These results contrast with those observed in models of deregulated growth factor-dependent activation of mTORC1 in the liver: liver damage, necrosis, fibrosis, and liver tumors^8,9.

To understand why growth factor- versus nutrient-dependent activation of mTORC1 differentially impact hepatocellular homeostasis, while mirroring several metabolic alterations, we looked side-by-side at the extent of mTORC1 activation in Li-RagA^{GTP/Δ}
RagA with Sidakulating nutrient intake or hormonal signaling. These efforts were interrogation how chronic elevation of nutrients impacts mammalian physiology by deregulating mTORC1 and without manipulating nutrient intake or hormonal signaling. The i indicates a significant difference between RagA/+/− and RagAGTP/Δ in ad libitum fed conditions. The schematic representation of the Urea Cycle reactions, metabolites, and key enzymes. e RT-qPCR of livers from 15- to 36-week-old RagA/+/− (fasted n = 8; fed n = 4) and RagAGTP/Δ (fasted n = 8; fed n = 4) females. Expression levels of the indicated genes relative to the average level in fed RagAGTP/Δ mice. β-actin was used as a housekeeping gene. f Livers were obtained from 18- to 28-week-old RagA/+/− and RagAGTP/Δ mice in ad libitum fed conditions or deprived from food for 16 h. Whole-cell protein lysates from liver were immunoblotted for the indicated proteins. For each protein, the levels in each lane are relative to vinculin levels and presented normalized to the average level of fasted RagA/+/− mice (n = 5). Gels/blots were processed in parallel. g Heat map from all proteins involved in peroxisomal β-oxidation detected in the proteomics experiment from Fig. 4. RagAGTP/Δ (fasted n = 3; ad libitum fed n = 2) and RagAGTP/Δ (fasted n = 3; ad libitum fed n = 3). Statistical significance was calculated using the LIMMA program as indicated in Materials and Methods. The * indicates a significant difference between RagAGTP/Δ and RagA/+/− in fasted conditions. h Bile acid levels were measured in blood from 6- to 23-week-old RagAGTP/Δ (fasted n = 25; ad libitum n = 29) and RagAGTP/Δ (fasted n = 19; ad libitum n = 30) males and females. i 11- to 21-week-old RagA/+/− and RagAGTP/Δ females were treated with rapamycin encapsulated in food for 2 weeks and fasted 16 h before sacrifice. mRNA levels of the indicated genes from liver extracts were quantified by RT-qPCR, relative to β-actin levels, and then normalized to the average levels of samples from non-treated RagA/+/− mice. RagAGTP/Δ (no rapa n = 8; rapa n = 10) and RagAGTP/Δ (no rapa n = 8; rapa n = 9). j Mice treated or not treated with Rapamycin for 1 week were bleed after 16 h of fasting, then the levels of β-hydroxybutyrate (β-HB) were addressed. 7- to 36-week-old RagAGTP/Δ (no rapa n = 7; rapa n = 8) and RagAGTP/Δ (no rapa n = 7; rapa n = 6). k Bile acid levels were measured from blood samples from 6- to 23-week-old RagAGTP/Δ (no rapa n = 53 and rapa n = 6) and RagAGTP/Δ (no rapa n = 49 and rapa n = 8). For a, b, e, f, i, and j statistical significance was calculated for individual metabolites by using 2-way ANOVA with Sidak’s multiple comparison correction. For h and k statistical significance was calculated by using Chi-square test. In all panels, horizontal lines indicate the mean and error bars depict Standard deviation (SD).

To determine, at the whole proteome level, the similarities between Li-RagAGTP/Δ and Li-TSC1−/− mice, we analyzed the proteome of fasted Li-TSC1−/− mice (Fig. 8f and Supplementary Data 3). A total of 7992 proteins were detected, and, consistently with previous reports40,47, and in striking overlap with Li-RagAGTP/Δ mice, fatty acid metabolism, peroxisome, bile-acid metabolism, oxidative phosphorylation, PPPRs target signatures were significantly depleted from fasted Li-TSC1−/− livers and validated by qPCR (Fig. 8g, h, and Supplementary Fig. 8c–f). Finally, the reduction of bile acids and ketone bodies upon fasting was comparable in Li-RagAGTP/Δ and Li-TSC1−/− mice (Fig. 8i, j). Altogether, the striking overlap of metabolic alterations observed in Li-RagAGTP/Δ and Li-TSC1−/− mice indicates that suppression of mTORC1 by inhibitory cues from nutrient and hormonal signaling during fasting are sufficient to coordinate the metabolism of the fasted state (Fig. 8k). However, while chronically deregulated growth factor signaling to mTORC1 drives hepatocellular damage and liver cancer, deregulated nutrient signaling upstream of mTORC1 does not overtly affect hepatocyte homeostasis, it does not result in hepatocellular tumorigenesis, neither it compromises long-term survival.

**Discussion**

This work constitutes the first report of mice with constitutive RagA signaling to mTORC1 in all adult cells, a genetic system to interrogate how chronic elevation of nutrients impacts mammalian physiology by deregulating mTORC1 and without manipulating nutrient intake or hormonal signaling. These efforts were challenged in the past because constitutive expression of the RagA_Q66L allele (RagAGTP) in homozygosity lead to neonatal lethality, and heterozygous expression of this mutant allele caused no signaling activation15. The restriction of the expression of the RagAGTP variant to one allele, in the absence of expression of a wild-type RagA from the other allele, together with a different genetic background, allowed the rescue of the aforementioned neonatal lethality (Fig. 1a). This rescue shows that subtle changes in the extent of increased nutrient signaling have dramatic consequences in mice: i.e. developmental aberrations and fully-penetrant neonatal lethality versus a 60% survival with mild and stereotypical morphometric alterations.

Genetically engineered mice for studying the consequences of systemic activation of mTORC1 have frequently faced the issue of embryonic lethality, as in TSC1/2 KO mice4 and Pten KO mice48,49. Conditional, tissue-specific activation has provided substantial pathophysiological insight on the in vivo functions of mTORC1 and its deregulation by hormonal cues. The rescue of neonatal lethality in RagAGTP mice allowed the study of systemic activation of nutrient signaling in adult mice. These mice exhibited obvious phenotypic alterations, including stereotypical cranial abnormalities and hypopigmentation, two clinical features present in syndromes of deregulated mTORC1 activity19,23, and interestingly, in a familial hypomyorphic variant of a Regulator component50. Thus, both gain- and loss-of-function alterations in nutrient signaling can impair skin and hair pigmentation.

The signaling perturbation on mTORC1 generated by constitutive activation of nutrient signaling is seen in starved cells and in fasted mice (Fig. 1) but does not increase maximal activity of mTORC1 in nutrient replete conditions. Thus, the consequences of constitutive nutrient signaling are likely to affect only physiological responses linked to fasted state. In addition, the extent of signaling activation was not identical in every organ from RagAGTP mice: while liver and skeletal muscle showed a comparable state of mTORC1 activation in fasted vs. fed states, kidney, heart, WAT, and BAT from RagAGTP mice show a partial inhibition of mTORC1 in the fasted state (Fig. 1c and Supplementary Fig. 1e, f). The differential impact on mTORC1 by the same genetic perturbation is unlikely to be caused by a compensatory effect of very low levels of RagB, and most likely reflects the dependence on a normal regulation of hormonal signaling, not genetically manipulated in RagAGTP mice.
While RagA<sub>GTP</sub>GTP neonates experience a lethal metabolic crisis during the first episode of nutrient limitation occurring immediately after the interruption of transplacental supply of nutrients that occurs with birth, the metabolic consequences of endogenous expression of RagA<sub>GTP/Δ</sub> in adult mice are comparatively milder. Constitutive nutrient signaling in adult mice affects the adaptation to fasting, but mice do not succumb or experience a severe hypoglycaemic state. In contrast, the levels of circulating glucose under fasting resulted abnormally high and RagA<sub>GTP</sub> mice exhibited a moderate glucose intolerance with an elevated HOMA-IR index (Fig. 3). The Framingham epidemiology study showed that elevated levels of circulating branched chain amino acids are the best predictors of future development of type 2 diabetes in healthy, lean individuals. Our results provide experimental support, by genetic means in mice, for an emerging idea that systemically high branched chain amino acids, via Rag GTPase - mTORC1 activation, may perturb glucose homeostasis. This moderate loss of glycemic control did not correlate with an
obvious inhibition of hepatic PI3K-Akt signaling, decreased glucose uptake or overt changes in hepatic glucose output. We cannot exclude the provocative possibility of increased gluconeogenesis in RagA<sup>GTP/Δ</sup> mice from amino acids such as glutamine (the most abundant amino acid in circulation), whose levels are reduced in RagA<sup>GTP/Δ</sup> mice.

Global proteomic analysis of liver samples from wt and RagAGTP mice revealed the expected features consistent with increased nutrient signaling. These include mTORC1 activation itself, proteins encoded by TOP mRNAs (mostly involved in protein synthesis), PPARG targets; but surprisingly failed to identify a strong suppression of TFEB activity<sup>31,32</sup>. A potential technical explanation for this is the enrichment for transcriptional targets of TFEB translate to membrane-bound proteins with lysosomal localization, which tend to be under-represented using standard proteomic approaches. A biological explanation is that inhibitory phosphorylation of TFEB, a target of mTORC1 with unique sensitivity to Rag GTPase complex activity<sup>37,53</sup>, may be more strongly controlled by the nucleotide state of RagC and less so on that of RagA, used herein. Collectively, our whole proteomic analysis revealed a striking inability of RagAGTP mice to unleash the fasting responses executed by PPARGα, which normally include ketogenesis, fatty acid oxidation with increased respiratory capacity in the mitochondria, amino acid catabolism, and peroxisomal abundance. Hence, under fasting conditions, the metabolic alterations in mice with constitutive nutrient signaling to mTORC1 include glucose, lipid, ketone, and amino acid metabolism, a wide range of metabolic defects that highlight the central role of the dynamic regulation of mTORC1 for metabolic homeostasis. In addition, the abnormal systemic metabolic state of fasted RagAGTP mice is reflected with increased energy expenditure and increased weight loss, and a respiratory quotient indicative of a shift to sugar metabolism to detriment of fat/ketone metabolism (Fig. 5g). While a deficient PPARGα axis largely explains the aberrant fasting metabolism of mice with constitutive nutrient signaling, the fasting hyperglycaemia contrasts with the hypoglycaemia that is observed in PPARGα KO mice<sup>42</sup>, pointing to PPARGα-independent metabolic defects at work in RagAGTP mice.

An unmet expectation for an outcome of constitutive nutrient signaling systemically was a premature aging phenotype, as seen in other model organisms<sup>54,55</sup> but which did not seem to occur in RagAGTP mice, as more severe pathologies precipitate before a moderate acceleration of aging can manifest (Fig. 2). This difference, which is not unprecedented as other models of increased mTORC1 activity also succumb before physiological longevity can be scored<sup>39</sup>, precludes still pending efforts to understand the underpinnings of the connections between increased mTORC1 activity and aging.

Importantly, liver-specific activation of Rag GTPase signaling did not recapitulate developmental and morphometric alterations, but partially mirrored (Fig. 7) the metabolic alterations observed in full-body RagAGTP mice. Li-RagAGTP/Δ mice exhibited loss of glucose tolerance, but not the fasting hyperglycaemia observed in full-body RagAGTP mice. Moreover, whole proteome analysis confirmed the outstanding suppression of the PPARGα transcriptional program, including mitochondrial, peroxisomal and lipid metabolism, and the systemic defects in ketogenesis and increased bile acids levels.

Li-TSC1<sup>−/−</sup> mice and Li-RagAGTP/Δ largely shared metabolic alterations, but Li-RagAGTP/Δ mice did not suffer the overt, aberrant hepatocellular damage and spontaneous tumor development seen in Li-TSC1<sup>−/−</sup> mice (Fig. 8). This milder phenotype correlated with the smaller extent of activation of mTORC1 in Li-RagAGTP/Δ versus Li-TSC1<sup>−/−</sup> samples compared side-by-side, and also correlated with the extent of indirect inhibition of Akt, which is stronger in TSC1/2-deficient cells and mice. Thus, these results suggest that deregulated growth factor signaling more profoundly affects hepatocellular homeostasis, and that the degree of chronic overactivation of mTORC1 may result in either benign or pathological outcomes. Although speculative, these differences may underlie the existence of frequent genetic alterations in growth factor signaling upstream of mTORC1 in human cancer, being mutations in the nutrient signaling cascade more infrequent and found mostly in B cell lymphomas<sup>36</sup>.

Finally, by putting together the phenotypes of Li-RagAGTP mice, Li-TSC1 KO mice and those of hepatic deletion of Deptor, a negative Regulator of mTOR, the involvement of mTORC1 inhibition in the control fasting metabolism is likely biphasic: (1) an early post-absorptive state (6 h to 12 h after food intake) where mTORC1 is inhibited in a Deptor-dependent manner<sup>57</sup>, which contributes to modulate an rapid drop in glycaemia; followed by (2) a complete fasting response driven by overlapping nutrient/Rag GTPase- and growth-factor/TSC-mediated inhibition of mTORC1, in turn enabling the PPARGα transcriptional program and metabolic adaptation (Fig. 8k).

**Methods**

*Generation of RagAGTP/GTP and RagAGTP/Δ mice.* All animal procedures carried out at the CNIO were performed according to protocols approved by the CNIO-ISCIII Ethics Committee for Research and Animal Welfare (CEIyBA) and the Autonomous Community of Madrid (CAM). Protocol numbers PROEX285/15 and PROEX18/18. Mice were housed under specific pathogen free conditions at 22 °C and with 12 h light/dark cycles. RagAGTP/Δ<sup>Q66L</sup> mice were generated in the David M Sabatini laboratory at the Whitehead Institute for Biomedical Research at Massachusetts Institute of Technology<sup>37</sup>. Expression of the RagAGTP/Δ<sup>Q66L</sup> allele (also referred to as RagAGTP) only occurs upon Cre-dependent elimination of the transcriptional
STOP cassette on the null RagA<sub>LSL-Q66L</sub> locus. Hemizygous RagA<sub>Q66L/LSL-Q66L</sub> (referred to as RagA<sub>GTP/Δ</sub>) mice were generated by crossing RagA<sub>Q66L/+</sub> mice with mice carrying the null allele RagA<sub>LSL-Q66L</sub>. To minimize genetic drift and to maintain maximum genetic representation in the mixed background in which neonatal viability occurred, experimental cohorts were always from F2 generation.

For hepatocyte-specific activation of RagA, we produced mice that expressed the RagA<sub>Q66L</sub> variant, in the absence of wild-type RagA, exclusively in the liver. Thus, we crossed RagA<sub>GTP/+</sub> mice with RagA<sub>floxed/floxed</sub>; Albumin-Cre transgenic mice<sup>58</sup> to generate RagA<sub>floxed/GTP</sub>; Albumin-Cre<sup>Tg</sup> mice. Mice with normal Rag GTPase signaling used as controls were RagA<sub>GTP/floxed</sub> without Albumin-Cre allele, and RagA<sub>GTP/+</sub>; Albumin-Cre<sup>Tg</sup> mice.

**Animal procedures.** For hepatocyte-specific deletion of TSC1, we crossed TSC1<sub>floxed/floxed</sub> mice<sup>5</sup> with Albumin-Cre transgenic mouse to generate Li-TSC1<sup>−/−</sup> mice. Mice were housed under specific pathogen-free (SPF) conditions, at 22°C,
Survival curve of controls (males and females) and Li- RagA KD (n = 11) and Li- RagA KD (n = 4) females were deprived from food for 24 h and sacrificed following a 2 h refeeding. Protein lysates from the liver were immunoblotted for the indicated proteins. Schematic summary of the proteomics experimental setting. Protein lysates from livers of 17- to 25-week-old control (n = 4) and Li- RagA KD (n = 4) females, fasted for 24 h were extracted, processed and labelled as indicated. Graphical representation of the false discover rates (FDRs) from the indicated hallmark genes sets from Li- RagA KD (n = 4) versus control (n = 4) mice. RT-qPCR of livers from 17- to 26-week-old control (n = 7) and Li- RagA KD (n = 7) females fasted for 24 h. Expression levels of the indicated genes relative to the average level in fed control mice. β-actin was used as housekeeping gene. Statistical significance was calculated by using unpaired two-tailed t-test corrected for multiple comparisons (Holm-Sidak).

with 12-hr dark/light cycles. Mice were fed with a standard chow diet (Harlan Teklad 2018). For fasting experiments of overnight or 24 h, mice were placed in a clean cage without access to food from 4 pm to 8 am or from 8 am to 8 am next day, respectively. In each cage a maximum of two animals were placed. Unless otherwise stated in the legends, samples were obtained from fasted mice as described above with and without ad libitum feeding with chow diet for 30 min or 2 h. For the 48 h fasting, the same protocol was used, and fasting was started at 7:30 am. Body weight was monitored every 6 h to avoid a weight loss higher than 20%. Glucose was monitored with Accu-check Aviva glucometer by making a small incision at the end of the tail to obtain a drop of blood. For survival experiments, mice were observed weekly by trained personnel until they presented signs of distress. Rapamycin was administered in food, encapsulated in chow diet at 42 ppm (Rapamycin Holdings and Purina Lab Diet). Before the sacrifice, the mice were fasted overnight and injected intra-peritoneally (IP) with 2 mg/kg of Rapamycin (Alpha Aesar J62473) dissolved in 5% Tween 80 (Sigma P7949) + 5% PEG400 (Fluka S1172).

Glucose tolerance test. Mice were fasted for 4 pm to 8 am as previously indicated. Before injecting the mice, around 20 µL of blood was extracted from the tail to measure the insulin level and calculate the HOMA-IR. Following fasting, D-Glucose 2 g/Kg (Sigma Aldrich G7528) was injected intra-peritoneally and glucose levels were measured at 15, 30, 45, 60, 90 and 120 min after injection.

Insulin Tolerance test (ITT). Pure C57BL/6 mice were fasted from 9 am to 3 pm; mixed C57BL/6:129 Sv background mice were fasted for 3 h starting at 12 pm, due to an intrinsic increase in insulin sensitivity. After the fasting, mice were injected intra-peritoneally with 0.75 U/Kg of mice of insulin (Humulin R U-100 Lilly) in PBS and tail blood glucose levels were measured at 15, 30, 45, 60, 90 and 120 min after injection.

Pyruvate tolerance test (PTT). Mice were fasted from 4 pm to 8 am as previously indicated. Sodium pyruvate (1.5 g/Kg body weight; Sigma Aldrich G7528) was injected intra-peritoneally, and tail blood glucose levels were measured at 15, 30, 45, 60, 90, and 120 min after injection.

Indirect calorimetry. EE, RQ, food intake, voluntary movement (rearing), was measured with Oxylet device from Panlab. Briefer, mice were fasted for 12 h. Weight and levels were calculated before and after the ad libitum and the fasting conditions. Lean mass, obtained from densitometric analysis before the experiment, was used instead of the total body weight for normalization of values.

Animal imaging. For densitometry analysis, body composition measurements (body weights, fat mass, and lean muscle mass) was measured using dual-energy X-ray absorptiometry (DEXA) PIXImus, Mouse Densitometer (GE Lunar co, Madison, WI, USA), software version 1.46. Mice were anesthetized with isoflurane during recording. Quality control was performed using a calibrated phantom before imaging. For microCT scans mice were anesthetized and the area of interest was imaged with the explore Vista micro-CT scanner (GE Healthcare, London, Canada). The isotropic resolution of this instrument is 45 µm. The micro-CT image acquisition consisted of 400 projections collected in one full rotation of the gantry in approximately 10 min. The X-ray tube settings were 80kV and 450 µA. For skull imaging analysis, CT gantry was centered at the skull and the quantification was performed using Microview 2.5 software (GEHC; London, Canada). For ex novo imaging, the image acquisition was performed immediately after intraperitoneal administration of a contrast agent iopamiro 0.28 ml per mice (Bracco, Milano Italy) at a concentration 0.612 mg/ml by oral gavage. The gantry was centered in the thorax and upper part of the abdomen. The reconstructed images were viewed and analyzed using 3D Slicer 4.10.1 software [https://www.slicer.org].

Ex vivo muscle [3H]-2-deoxy-d-glucose uptake. For ex vivo muscle [3H]-2-deoxy-d-glucose uptake, mice were fasted overnight and rapidly after cervical dislocation solutox solen and extensor digitorum longus (EDL) muscles from both legs were dissected out and incubated for 60 min at 37°C in Krebs-Ringer bicarbonate (KRB) solution containing the following (in mmol/L): 117 NaCl, 4.7 KCl, 2.5 CaCl2, 2H2O, 1.2 KH2PO4, 1.2 MgSO4·7H2O, and 24.6 NaHCO3, and supplemented with 2 mmol/L pyruvate and 0.1% of BSA. Glucose transport was assessed during 10 min at 37°C using 6.5 mM 2-DG containing 1.5 µCi/ml 2-deoxy-D-[3H]-glucose (Perkin Elmer NET5249250UC) and 7 µm mannitol as standard. Before incubation, muscles were washed once in cold PBS and dried on Whatman paper. Then, flash-frozen in liquid nitrogen, weighed, and solubilized for 30 min at 65°C in 300 µL of 1 M NaOH. After centrifugation at 10,000 g for 10 min, 100 µL aliquots were taken for scintillation counting of the [3H] and [14C] labels using a Perkin Elmer Wall Ac 1414 Liquid Scintillation counter. [14C]-mannitol was used to correct the amount of extracellular [3H]-2-DG that was non-specifically uptaken. Counts were normalized to muscle weight.

Hepatic glycogen content. Glycogen was measured in liver samples. Briefly, glycogen was extracted from livers in 30% KOH saturated with Na2SO4, precipitated in 95% ethanol and re-suspended in double-distilled H2O. After the addition of phenol and H2SO4, absorbance at 490 nm was measured in duplicates.
High pH reverse phase chromatography for proteomics analysis. Peptides were pre-fractionated offline by means of high pH reverse phase chromatography using an Ultimate 3000 HPLC system equipped with a sample collector. Briefly, peptides were dissolved in 100 μl of phase A (10 mM NH₄OH) and loaded onto a XBridge BEH130 C18 column (3.5 μm, 150 mm length and 1 mm ID) (Waters). Phase B was 10 mM NH₄OH in 90% CH₂CN. The following gradient (flow rate of 100 μl/min) was used: 0–50 min 0–25% B, 50–56 min 25–60% B, 56–57 min 60–90% B. One minute fractions from minute 15 to 65 were collected, neutralized with 10 % of 10% formic acid and immediately vacuum dried. Based on the UV absorbance at 280 nm, 35 fractions were selected for LC-MS/MS analysis.

Proteomic data analysis. LC-MS/MS was done by coupling an Ultimate 3000 RSLCnano LC system to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). Five microliters of peptides were loaded into a trap column (Acclaim" PepMap" 100 C18 LC Columns 5 μm, 200 mm long) for 3 min at a flow rate of 10 μl/min in 0.1% FA. Peptides were then transferred to an EASY-Spray PepMap RSLC C18 column (Thermo) (2 μm, 75 μm × 50 cm) operated at 55 C. and separated using a 60 min effective gradient (buffer A: 0.1% FA; buffer B: 100% ACN, 0.1% FA) at a flow rate of 250 nL/min. The gradient used was, from 4 to 6% of buffer B in 2 min, from 6 to 33% B in 58 min, plus 10 additional minutes at 98% B. The mass spectrometer was operated in a data-dependent mode, with an automatic switch between MS and MS/MS scans using a top 12 method. (Intensity threshold: 1 × 10⁶ ions; exclusion of 20% for 60 s). The collision energy NCE of 35. MS/MS spectra were acquired with a Proton transfer (PTR) collision energy of 20 eV. The isolation window for precursor ions was 20 m/z. MS/MS spectra were filtered at 0.01 FDR (peptide and protein level). Proteins with a false discovery rate (FDR) > 17% were filtered out. Global normalization of log2-transformed intensities across samples was performed using the LOESS function. Differential analysis was done using the empirical Bayes statistics Limma 3.6.0. Proteins with a p-value < 0.05 and a log2 ratio > 0.3 or < -0.3 were defined as regulated. The FDR was estimated to be below 5% by Benjamini–Hochberg. GSEA Pre-rank test was performed with the gene-set enrichment of the described gene signatures on a pre-ranked gene list, setting 1000 gene-set permutations. GSEA Pre-ranked calculates an enrichment score for each gene set using the Kolmogorov–Smirnov test. The nominal P-value estimates the statistical significance of the enrichment score for a single gene set and it was corrected for gene-set size and multiple hypothesis testing.

Immunoblotting. For cell experiments, cells were rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer (50 mM HEpes (pH 7.4), 40 mM NaCl, 2 mM EDTA, 1.5 mM sodium orthovanadate, 50 mM NaF, 10 mM pyrophosphate, 10 mM glycrophosphate and 1% Triton X-100) and one tablet of EDTA-free protease inhibitors (Roche) per 25 ml). Cell lysates were cleared by 10,000 g for 5 min. Liver samples were immediately fixed in 10% neutral buffered formalin for 3–5 days, followed by being dehydrated using a series of ethanol, and then embedded in paraffin and cut at 3 μm. Formalin-fixed tissues were deparaffinized in xylene and rehydrated through a series of graded concentrations of ethanol in water, then paraffin sections were stained with hematoxylin and eosin (H&E). Periodic acid–Schiff (PAS) was used for detection of glycogen and other carbohydrates, and Sirius red for fibrosis were also applied. Positive control sections known to be primary antibody positive were included for each staining run. Whole slides were acquired with a slide scanner (AxioScan Z1, Zeiss), images captured with the Zen 2.5 Blue edition Software (Zeiss) and quantified with ImageJ (1.52a).

Assessment of liver damage. Liver necrosis was diagnosed by the observation of single-cell necrosis or small groups of hepatocytes presenting an increased and pale cytoplasm, with a small or fragmented hyperchromatic nucleus (pyknosis), sometimes surrounded by inflammatory cells. The diagnosis of hepatitis was done by the identification of mononuclear or polymorphonuclear cells including, lymphocytes, plasmocytes, macrophages, neutrophils, or eosinophils in multi-focal areas of the liver. In these cases, could or could not be accompanied by derangement or necrosis of the hepatocytes, with or without fibrosis. Liver fibrosis was determined by the observation of excess fibrous connective tissue between contiguous periportal spaces in the hematoxylin and eosin (H&E) staining. Fibrosis was corroborated, when needed, by the observation of collagen fibers in red color in the Sirius red staining specifically distributed surrounding the hepatocytes located between the perportal space or in the middle of the hepatic parenchyma. Hepatocellular carcinomas were diagnosed upon the observation of a disorganized proliferation of cells with different degrees of cellular atypia forming nodules or lesions in which the normal structure of the liver was lost and portal spaces and centrilobular veins were no longer observed.

Quantification of metabolites and insulin from plasma. Plasma metabolites were measured using the following commercial kits according to manufacturer’s instructions: Blood Urea Nitrogen (BUN), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bile acids (VetScan Abaxis Model 200 6-91995, 270). Insulin levels (CRYSTAL CHEM INC. 90080). HOMA-IR was calculated according to the formula: fasting insulin (μIU/L) x fasting glucose (mg/dL)/405. The normal structure of the liver was lost and portal spaces and centrilobular veins were no longer observed.

Targeted LC-MS metabolites analyses. For metabolomic experiments, mice were fasted for 16 h and maintained fasted or allowed to fast access ad libitum for 2 h. For serum analysis, mice were bled before the fasting period and when euthanized. Blood samples were obtained in EDTA-free tubes. Serum was obtained by centrifugation of the blood at 10,000 g for 5 min. Serum samples were immediately snap-frozen in liquid nitrogen upon euthanasia. For the LC-MS analyses metabolites were extracted as follows61. Extraction solution was composed of 50% methanol and 50% water. Metabolite identification was performed by using a combination of a high-resolution and accurate mass spectrometry and database searching.
methanol, 30% ACN, and 20% water. The volume of extraction solution added was adjusted to mass of the tissue (1 ml per 40 mg) or serum volume (200µl per 10ul of serum). All vials of extraction solution samples were vortexed for 5 min at 4 C and then centrifuged at 16,000 g for 15 min at 4 C. The supernatants were collected and stored at −80°C until analyses. LC/MS analyses were conducted using QExactive Plus Orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe and coupled to a Dionex UltiMate 3000 UPLC system (Thermo). External mass calibration was performed using the standard calibration mixture every 7 d as recommended by the manufacturer. 5 μl of each sample was injected onto Zic-pHILIC (150 mm × 2.1 mm i.d. 5 μm) with the guard column (20 mm × 2.1 mm i.d. 5 μm) (Millipore) for the liquid chromatography separation. Buffer A was 20 mM ammonium carbonate, 0.1% ammonium hydroxide (pH 9.2); buffer B was acetonitrile. The chromatographic gradient was run at a flow rate of 0.200 μl/min as follows: 0–20 min; linear gradient from 80 to 20% B; 20–20.5 min; linear gradient from 20 to 80% B; 20.5–28 min; hold at 80% B1. The mass spectrometer was operated in full scan, polarity switching mode with the spray voltage set to 2.5 kV, the heated capillary held at 320°C. The sheath gas flow was set to 20 units, the auxiliary gas flow was set to 5 units, and the sweep gas flow was set to 0 unit. The metabolites were detected across a mass range of 75–1000 m/z at a resolution of 35,000 (at 200 m/z) with the AGC target at 106, and the maximum injection time at 250 ms. Lock masses were used to ensure mass accuracy below 5 ppm. Data were acquired using Thermo Scientific Xcalibur 4.0. The peak areas of metabolites were determined using Thermo TraceFinder 3.3 SP1 software (Thermo), identified by the exact mass of each singly charged ion and by known retention time on the HPLC column. For heatmap representation, http://www. heatmap.ca/expression/346 was used.

Mouse embryonic fibroblasts (MEFs). MEFs from E13.5 were prepared by chemical digestion with trypsin (Gibco, 25300-034) for 15 min, followed by mechanical disaggregation with a rubber policeman. The liver was minced, centrifuged in a gradient, 50% v/v/l of Buffer A and 50% v/v/l of Buffer B, and the supernatant was removed twice and incubated in RPMI without amino acids, and supplemented with 10% dialyzed FBS.24 Stimulus with amino acids (with concentration as in RPMI) was performed for 10 min.

Primary hepatocytes. Primary hepatocytes were isolated by perfusion (Masterflex C/L 77120-62 60RPM) with collagenase type I (Sigma-Aldrich C3867). Briefly, mice were anesthetized with Ketamine/Xylazine (Merial laboratory, SA: Imalgene 100 mg/ml injectable solution; Bayer: Rompun 20 mg/ml injectable solution) in saline solution, 100 mg/kg and 10 mg/kg respectively, the abdomen was incised and the liver was removed. The aorta, the cava vein and the portal vein were cut. Subsequently, liver was perfused with 25 mL of Buffer A at 37°C and the mouse was placed under heating red-light lamp. At the same time the abdominal knot was tightened and the animal was kept under anesthesia. After perfusion, the liver was placed in a 50 mL falcon containing 25 mL of Buffer C and once under the hood the liver was disaggregated and filtered through 100 μm sterile cell strainer. Hepatocytes were centrifuged at 50 g for 5 min, supernatant was removed, and the pellet was resuspended in an enzyme solution (50% v/v l of Buffer C and 50% v/v l of Buffer D). After centrifugation at 50 g for 10 min, the supernatant was removed, and the remaining pellet was washed once with Buffer C and centrifuged 5 min at 50 g. Hepatocytes were resuspended in fresh Buffer C and cells were counted with trypan blue to assess viability. For immunoblotting experiments cells were plated at 3 × 105 cells/well in collagen-coated (Sigma-Aldrich C3867) multi six-well plate. After 6 h the media was changed and hepatocytes were cultured overnight before any experimental procedure. The next day, cells were washed 3 times and then incubated in DMEM:F12 without amino acids (USBiological Life Science D9807-10) supplemented with 6 mM of NaHCO3 (Sigma 1063290100), 18 mM of Heps pH 7.4 (Lonza L7-735E), 25 mM of glucose (Sigma G8769) and 10% dialyzed fetal bovine serum and let sit for 16 h after which cells were collected for protein extraction. Buffer A: Hank Balanced Salt Solution (HBSS) 1x w/o Ca2+ / Mg2+ (Life Technologies 14175-053), with 10 mM of Heps pH 7.4 (Lonza L7-737E) and 0.2 mM of EGTA (Sigma-Aldrich E3849). Buffer B: Williams E media (gibco 22531012) with 10 mM of Heps pH 7.4 (Lonza L7-737E). Buffer C: DMEM-F12 media (Thermo Fisher G1965039 and Thermo Fisher 21765029) with 3 μM of BSA and 20 units, the sweep gas flow rate of 0.200 – 20.5 min; linear gradient from 20 to 80% B; 20.5–28 min; hold at 80% B1. The mass spectrometer was operated in full scan, polarity switching mode with the spray voltage set to 2.5 kV, the heated capillary held at 320°C. The sheath gas flow was set to 20 units, the auxiliary gas flow was set to 5 units, and the sweep gas flow was set to 0 unit. The metabolites were detected across a mass range of 75–1000 m/z at a resolution of 35,000 (at 200 m/z) with the AGC target at 106, and the maximum injection time at 250 ms. Lock masses were used to ensure mass accuracy below 5 ppm. Data were acquired using Thermo Scientific Xcalibur 4.0. The peak areas of metabolites were determined using Thermo TraceFinder 3.3 SP1 software (Thermo), identified by the exact mass of each singly charged ion and by known retention time on the HPLC column. For heatmap representation, http://www.heatmap.ca/expression/346 was used.

Statistics. Statistical analysis was carried out with Prism 8 (GraphPad). All experiments that included a second variable (e.g. time or nutritional status) were analyzed using 2-way ANOVA with Sidak's multiple comparison post-test. Correction for repetitive measures was performed where appropriate, and the area under the curve (AUC) was calculated. Unpaired Student’s t-test was used for single comparisons. Chi-square test (http://vassarstats.net/news.html) was used for comparing categorical parameters, Log-rank (Mantel-Cox) test was used for survival curves, 1-way ANOVA (Dunnett’s multiple comparison test) was used when more than one genotype was analysed. For proportional diagram representation and statistic calculation the online tools https://www.biovenn.nl/index.php and http://nemates.org/MA/progs/overlap_stats.html were used. All error bars in all panels depict Standard deviation (SD).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Metabolomics data have been deposited to the EMBL-EBI MetaboLights database (https://www.ebi.ac.uk/metabolights/) with the identifier MTBLS2397. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomeexchange.org/cgi/GetDataset?ID=PXD002733). The authors declare that other source data supporting the findings of this study within the article and its Supplementary information files are available upon reasonable request to the corresponding author. Source data are provided with this paper.

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Author contributions
C.C.A. performed most experiments, contributed to experimental design, data analysis, writing of the manuscript. A.B.P.G. provided substantial support and performed most experiments with the TSC1 knock-out mice, N.D.S., A.O.M., J.A.G., L.T., M.L. provided help with experimentation and analysis. F.G.M. and J.M. performed proteomics, E.C. and A.M. diagnosed the histology and pathology. R.C.O. performed NMR. FM performed mouse live imaging, I.N. and M.P. conducted metabolomics analyses, E.R. and G.S. contributed primary hepatocytes cultures and expertise, D.M.S. conceived the study and supervised the study, analyzed the data, wrote the manuscript and secured funding. All authors read and commented on the manuscript and figures.

Competing interests
The authors declare no competing interests.

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