Fatty acid carbon is essential for dNTP synthesis in endothelial cells

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The metabolism of endothelial cells during vessel sprouting remains poorly studied. Here we report that endothelial loss of CPT1A, a rate-limiting enzyme of fatty acid oxidation (FAO), causes vascular sprouting defects due to impaired proliferation, not migration, of human and murine endothelial cells. Reduction of FAO in endothelial cells did not cause energy depletion or disturb redox homeostasis, but impaired de novo nucleotide synthesis for DNA replication. Isotope labelling studies in control endothelial cells showed that fatty acid carbons substantially replenished the Krebs cycle, and were incorporated into aspartate (a nucleotide precursor), uridine monophosphate (a precursor of pyrimidine nucleoside triphosphates) and DNA. CPT1A silencing reduced these processes and depleted endothelial cell stores of aspartate and deoxyribonucleoside triphosphates. Acetate (metabolized to acetyl-CoA, thereby substituting for the depleted FAO-derived acetyl-CoA) or a nucleoside mix rescued the phenotype of CPT1A-silenced endothelial cells. Finally, CPT1 blockade inhibited pathological ocular angiogenesis in mice, suggesting a novel strategy for blocking angiogenesis.

Angiogenesis relies on the proliferation and migration of endothelial cells1. The importance of endothelial cell proliferation for expansive growth of the vascular network has long been recognized2. While numerous molecules regulating vessel sprouting have been identified3, little is known about the role of metabolism. We recently reported that endothelial cells generate 85% of their ATP for vessel sprouting via glycolysis4. Fatty acid oxidation (FAO) has been linked in various cell types to ATP production and to ROS scavenging during cellular stress, but apart from Fatty acid oxidation (FAO) has been linked in various cell types to ATP production and to ROS scavenging during cellular stress, but apart from FAO, the TCA cycle also provides precursors for macromolecule synthesis, necessary for proliferation. However, fatty acids have not yet been shown to function as carbon sources for biosynthetic processes. Here we elucidated the role of FAO in endothelial cells during angiogenesis in vivo and presented new evidence for a role of FAO in vessel sprouting. In EC spheroids, CPT1A silencing (CPT1A knockdown (CPT1AKD)) decreased vessel sprout length and numbers (Fig. 1a–c and Extended Data Fig. 1i). This defect was due to decreased EC proliferation, as CPT1AKD reduced proliferation and had only minimally additive effects on mitomycin-C-treated mitotically inactivated ECs (Fig. 1c–f and Extended Data Fig. 1i, j). By contrast, CPT1ATOE did not affect EC migration or motility (Fig. 1g–i and Extended Data Fig. 1k). Similar results were obtained when silencing long-chain acyl-CoA dehydrogenase (ACADVL), another FAO gene (Extended Data Fig. 1l–o). Additional evidence for a role of FAO in vessel sprouting was provided by overexpression of CPT1A (CPT1AOE), which yielded opposite results to those obtained by CPT1A silencing (Extended Data Fig. 1p–t). Thus, CPT1A-driven FAO regulates EC proliferation during vessel sprouting.

To study the effects of endothelial CPT1A deficiency on vessel formation in vivo, we used Cpt1alox/lox mice intercrossed with Vcadherin(PAC)-CreERT2 mice (CPT1AEC mice) (Extended Data Fig. 1u), Isletin-B4 staining of retinal vessels of transgenic pups at postnatal day (P) 5 revealed that EC loss of CPT1A diminished the number of vascular branch points, and reduced radial expansion of the vascular plexus (Fig. 2a–c). This was not caused by increased vessel regression (Fig. 2f–h) but by reduced EC proliferation (Fig. 2i–k). Furthermore, CPT1AEC mice had normal numbers of filopodia (Fig. 2l–n) and vessel maturation (Fig. 2o, p and Extended Data Fig. 1v). Thus, impaired angiogenesis in CPT1AEC mice was due to EC proliferation defects.

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**Figure 1 | FAO stimulates vessel sprouting via EC proliferation.**

a, b. Representative images (original magnification, 10×) of control (Ctrl) and CPT1A\(^{KD}\) EC spheroids. c. Total sprout length in control and CPT1A\(^{KD}\) EC spheroids treated with mitomycin C (MitoC) where indicated (n = 3). d. \(^{3}H\) thymidine incorporation in DNA in control and CPT1A\(^{KD}\) ECs (n = 5), d.p.m., disintegrations per minute. e, f. Representative images (original magnification, 10×) of MitoC-treated control and CPT1A KD EC spheroids. g. Number of MitoC-treated control and CPT1A KD ECs that traversed a Boyden chamber (n = 4; P = NS). h. Scratch wound assay using MitoC-treated control and CPT1A\(^{KD}\) ECs (n = 4; P = NS). i. Lamellipodia area in control and CPT1A\(^{KD}\) ECs (n = 4; P = NS). Data are mean ± s.e.m. of n independent experiments. Statistical test: mixed models (c, d, g-i). NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 2 | CPT1A gene deletion in ECs causes vascular defects in vivo.**

a, b. Representative images (original magnification, ×20) of retinal vessels of wild-type (WT; a) and CPT1A\(^{KD}\) (b) mice. c, d. Branch point quantification in WT and CPT1A\(^{AEc}\) mice in the front (c) and rear (d) of the retinal vasculature (5 litters, n = 11 pups for WT and CPT1A\(^{AEc}\)). e. Retinal vascular outgrowth in wild-type and CPT1A\(^{AEc}\) mice (6 litters, n = 13 pups for WT and 18 for CPT1A\(^{AEc}\)). f, g. Representative images (original magnification, 20×) of the retina stained for isoleictin-B4 (green) and collagen IV (red) in WT (f) and CPT1A\(^{AEc}\) mice (g). h. Quantification of isoleictin-B4− collagen IV− empty sleeves in WT and CPT1A\(^{AEc}\) pups (4 litters, n = 8 pups for WT and 14 for CPT1A\(^{AEc}\); P = NS). i, j. Representative images (original magnification, 10×) of retina stained for EdU (red) and isoleictin-B4 (green) in WT (i) and CPT1A\(^{AEc}\) (j) mice. k. Quantification of EdU\(^{+}\) ECs in WT and CPT1A\(^{AEc}\) mice (3 litters, n = 9 pups for WT and 6 for CPT1A\(^{AEc}\)). l. Quantification of filopodia in WT and CPT1A\(^{AEc}\) mice (6 litters, n = 20 pups for WT and 16 for CPT1A\(^{AEc}\); P = NS). m, n. Representative images of filopodia (original magnification, 63×) in WT (m) and CPT1A\(^{AEc}\) (n) mice. o, p. Representative images (original magnification, 20×) of the retinal vasculature of WT (o) and CPT1A\(^{AEc}\) mice (p) stained for isoleictin-B4 (blue) and the pericyte marker NG2 (pink). Data are mean ± s.e.m. of n individual mice. Statistical test: mixed models (c-e, h, k, l). NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001.
CPT1A KD does not cause ATP or redox imbalance

To explore by which mechanism FAO regulates EC proliferation, we studied whether CPT1A KD impaired EC proliferation by causing energy stress due to ATP depletion. However, CPT1A KD did not lower ATP levels, nor did it affect the energy charge or ADP/ATP ratio (Fig. 3a, b and Extended Data Fig. 2a). This was not surprising, as FAO generated only 5% of the total amount of ATP in ECs. Oxygen consumption (OCR) coupled to ATP synthesis was modestly reduced (Fig. 3c), but could not explain the sprouting defect, as blocking mitochondrial ATP production with oligomycin failed to affect sprouting of control and CPT1A KD ECs, suggesting that CPT1A KD impaired sprouting independently of mitochondrial ATP production (Fig. 3d and Extended Data Fig. 2b). Additional experiments confirmed that CPT1A KD ECs were not in energy stress (Extended Data Fig. 2c, d).

We also explored whether CPT1A KD impaired vessel sprouting by altering redox balance. CPT1A KD increased ROS levels by only 20%, an increase reported to enhance EC proliferation, without affecting the percentage of oxidized glutathione or disturbing redox homeostasis (Fig. 3e, f). Furthermore, lowering ROS levels by using N-acetyl-cysteine (NAC) did not restore vessel sprouting upon CPT1A KD silencing (Fig. 3g and Extended Data Fig. 2e). Finally, CPT1A KD did not compromise EC survival and did not increase levels of oxidative DNA damage markers (Extended Data Fig. 2f–j). Thus, CPT1A KD did not impair vessel sprouting by inducing toxic ROS levels.

FAO is used for de novo synthesis of nucleotides

We thus considered a novel role for FAO in EC proliferation and explored whether FAO regulated the production of biomass building blocks. Supplementing EC monolayers with [U-13C]palmitate or an algal [U-14C]fatty acid mix revealed that carbons from fatty acids provided a substantial fraction of the total carbon fuelling the TCA cycle intermediates and TCA-cycle-derived amino acids; this fraction was similar to the contribution of carbons from glutamine and glycolytic carbon (Fig. 4a–c). This was unexpected as many cancer cell types rely almost exclusively on glucose and glutamine to fuel the TCA cycle. CPT1A KD also lowered the cellular pool size of citrate, aspartate and glutamate (Fig. 4d).

Since TCA intermediates are used for the synthesis of biomass precursors, and inhibition of FAO limited the supply of these TCA intermediates, we explored whether CPT1A KD impaired protein and/or nucleotide synthesis. CPT1A KD did not, however, impair de novo protein synthesis (Fig. 4e) and did not consistently alter intracellular amino acid levels (Fig. 4d). Also, CPT1A KD still lowered EC proliferation when protein synthesis was reduced by cycloheximide (Fig. 4f), suggesting that a decrease of de novo protein synthesis did not prevent CPT1A silencing to establish its proliferation defect.

Given that aspartate is a precursor of nucleotides and its levels were reduced upon CPT1A KD, we explored whether fatty-acid-derived carbons were used for de novo ribonucleotide synthesis. Label from [U-14C]palmitate was incorporated into RNA in control ECs, and this process was decreased upon CPT1A KD (Fig. 4g). However, despite this, CPT1A KD ECs did not have reduced RNA levels and pools of ribonucleotides (rNTPs) (Fig. 4h, i), presumably because of compensatory production by salvage pathways. Thus, insufficient RNA synthesis did not cause the proliferation defect of CPT1A KD ECs.

We therefore assessed whether impaired de novo deoxyribonucleotide synthesis caused the proliferation defect of CPT1A KD ECs. Label from [U-14C]palmitate was incorporated into DNA, and this was reduced upon CPT1A silencing (Fig. 4j). As CPT1A KD also decreased de novo DNA synthesis (Fig. 1d), we hypothesized that FAO blockade reduced aspartate levels and thereby compromised de novo deoxyribonucleotide biosynthesis. Indeed, labelled carbons from [U-13C]palmitate were incorporated in the pyrimidine precursors uridine monophosphate (UMP) and uridine-5’-triphosphate (UTP), and this incorporation was reduced upon CPT1A silencing (Fig. 4k, l and Extended Data Fig. 3a). Moreover, quantification of deoxyribonucleotide (dNTP) levels confirmed that CPT1A KD lowered the intracellular levels of all pyrimidine (dCTP, dTTP) and purine deoxyribonucleotides (dATP, dGTP) needed for DNA replication (Fig. 4m). Thus, in contrast to the retained rNTP levels, CPT1A KD depleted the pool of dNTPs.

To functionally confirm that the effect of CPT1A KD depends on reduced de novo nucleotide synthesis, we inhibited de novo synthesis of nucleotides by methotrexate (MTX) and 5-fluorouracil (5FU), which reduced vessel sprouting in control but did not further reduce vessel sprouting in CPT1A KD cells (Fig. 4n, o and Extended Data Fig. 3b, c). Thus, CPT1A KD reduced sprouting mainly via inhibition of de novo deoxyribonucleotide synthesis.

To confirm the role of fatty-acid-derived carbons for nucleotide synthesis, we supplemented CPT1A KD cells with acetate (which is converted to acetyl-CoA that can fuel the TCA cycle). Treatment of CPT1A KD ECs

Figure 3 | CPT1A silencing does not cause ATP depletion or redox imbalance. a, Intracellular ATP levels in control and CPT1A KD ECs (n = 4). b, Energy charge measurement (ratio) in control and CPT1A KD ECs (n = 3; P = NS). c, ATP coupled oxygen consumption rate (OCR ATP, expressed relative to control) in control and CPT1A KD ECs (n = 3). d, Total sprout length upon oligomycin (oligo) treatment in control and CPT1A KD EC spheroids (n = 3). e, Intracellular ROS levels in control and CPT1A KD ECs as measured using the fluorescent ROS detection reagent chloromethyl-2’,7’-dichlorofluorescin diacetate, acetyl ester (CM-DCF) (n = 6). f, Oxidized glutathione levels as per cent of total glutathione, in control and CPT1A KD ECs (% of total glutathione) (n = 3; P = NS). g, Total sprout length upon N-acetyl-cysteine (NAC) treatment in control and CPT1A KD EC spheroids (n = 3). Data are mean ± s.e.m. of n independent experiments. Statistical test: mixed models (a–g). NS, not significant. *P < 0.05, ***P < 0.001, ****P < 0.0001.
with acetate completely restored the levels of aspartate and dNTPs (Fig. 5a, b). Furthermore, CPT1AKD and control cells incorporated comparable amounts of [U-13C]acetate into UMP and UTP (Fig. 5c, d). Functionally, acetate rescued the sprouting defect of CPT1AKD ECs (Fig. 5e and Extended Data Fig. 3d–g). Also, blockade of the ATP synthase did not prevent this rescue, indicating that acetate was not used for the production of ATP but instead for the synthesis of biomass (Extended Data Fig. 3h, i).

Supplementation of a mix of nucleosides or dNTPs also fully rescued the sprouting defect of CPT1AKD vessels, indicating that FAO blockade impaired nucleoside synthesis (Fig. 5f and Extended Data Fig. 3j, k). Notably, acetate or the nucleoside mixture were unable to rescue the CPT1AKD sprouting defect upon mitotic inactivation of CPT1AKD ECs, indicating that their effect relied on stimulating EC proliferation (Extended Data Fig. 3l; not shown). Thus, CPT1A-driven FAO is critical for de novo deoxyribonucleotide synthesis to ensure EC proliferation during sprouting.

**Glucose partly compensates for FAO loss**

Nucleotides can also be synthesized from glucose and glutamine, so we asked whether ECs would compensate for the loss of FAO using these nutrients. CPT1AKD increased the oxidation of glucose but not of glutamine (Extended Data Fig. 3m, n), and increased glycolytic carbon incorporation into citrate and aspartate, whereas glutamine incorporation into citrate and aspartate was not (citrate or aspartate), or not negligibly (aspartate), altered (Fig. 5g and Extended Data Fig. 3o). The relative activity of pyruvate dehydrogenase and pyruvate carboxylase was increased in CPT1AKD ECs (Fig. 5h–j), which can contribute to the increase in glucose oxidation. Nonetheless, this compensation was insufficient to rescue the proliferation defect of CPT1AKD ECs, indicating that fatty acids are irreplaceable for DNA replication in ECs.

**Most cells do not use fatty acids for DNA synthesis**

To assess the broader relevance of the role of FAO in deoxyribonucleotide synthesis, we studied [U-13C]palmitate contribution to citrate in a panel of normal and malignant proliferating cells. Fibroblasts (and to a certain extent pericytes) but no other primary cells incorporated substantial amounts of fatty-acid-derived carbon into citrate (Fig. 6a). Except for breast cancer (T47D, MCF7) and lung cancer (A549) cell lines (known to rely on FAO for ATP and NADPH production), fatty acids contributed minimally to citrate production (Fig. 6a). Notably, even when these cancer cells incorporated fatty-acid-derived carbons into citrate, they incorporated them to a much lesser extent into DNA (Fig. 6b), implying that fatty-acid-derived carbons entering the TCA

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**Figure 4 | CPT1A silencing reduces TCA replenishment and FAO is used for nucleotide synthesis.** a. Total contribution of palmitate (Palm), glycolytic carbon (Glyc) and glutamine (Gln) to citrate (n = 3). b, c. Total contribution of [U-13C]palmitate (b) or [U-13C]algal fatty acid (FA) mix (c) to citrate (Cit), α-ketoglutarate (αKG), fumarate (Fum), malate (Mal), aspartate (Asp), glutamine (Gln), isoleucine (Ile), methionine (Met), tyrosine (Tyr), asparagine (Asn), alanine (Ala), glycine (Gly), serine (Ser), proline (Pro), valine (Val), leucine (Leu) and phenylalanine (Phe) in control and CPT1AKD ECs (n = 7 for TCA intermediates, n = 3 for Asn and n = 5 for all other amino acids in b; n = 3 in c). d. Intracellular content of citrate (Cit), glutamate (Glu), aspartate (Asp), glutamine (Gln), isoleucine (Ile), methionine (Met), tyrosine (Tyr), asparagine (Asn), alanine (Ala), glycine (Gly), serine (Ser), proline (Pro), valine (Val), leucine (Leu) and phenylalanine (Phe) in control and CPT1AKD ECs (n = 8 for Cit and Asp, n = 6 for Glu, n = 4 for Asn and Ser, n = 5 for Ala, Gly, Gln, Pro, Val, Leu, Ile, Met, Phe and Tyr). e. De novo protein synthesis in control and CPT1AKD ECs (n = 3). f. Incorporation of [U-14C]palmitate into DNA in control and CPT1AKD ECs (n = 3; P = NS). g. Incorporation of [U-13C]palmitate into RNA in control and CPT1AKD ECs (n = 3; P = NS). h. De novo RNA synthesis in control and CPT1AKD ECs (n = 4; NS). i. Total contribution of [U-13C]palmitate incorporation into DNA in control and CPT1AKD ECs (n = 5). j. Percentage: 5 + 1 and M + 2 labelling from [U-13C]palmitate in UMP (k) and UTP (l) in control and CPT1AKD ECs (n = 5). M denotes mass of the unlabelled metabolite. m. Reduction of intracellular dNTP levels in CPT1A KD versus control ECs (n = 5 for dATP and dCTP, n = 4 for dTTP and dGTP). n, o, Total sprout length upon 5-fluourouracil (5FU) (n) or methotrexate (MTX) (o) treatment in control and CPT1AKD ECs (n = 4). Data are mean ± s.e.m. of n independent experiments. Statistical test: mixed models (a–o). NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Inhibition of CPT1A impairs angiogenesis

Pharmacological agents with FAO-blocking activity are being used clinically\(^1\). To explore whether pharmacological blockade of CPT1 reduced pathological angiogenesis, we used etomoxir, an irreversible inhibitor of mitochondrial long-chain FAO. Etomoxir reduced FAO and EC proliferation, but not migration, of ECs, which caused vessel sprouting (Fig. 5). The novelty of this study relates to several observations. First, FAO is derived carbons into DNA to ECs (Fig. 6b). In fact, only fibroblasts incorporated similar amounts of fatty-acid-derived carbons into DNA to ECs (Fig. 6b).

**Discussion**

The novelty of this study relates to several observations. First, FAO is critical for vessel sprouting \textit{in vivo}, and the extent of its function is greater than expected based on earlier reports\(^5\). Second, FAO affects proliferation, not migration, of ECs, which caused vessel sprouting defects. Unlike PFKFB3-driven glycolysis, which affects EC proliferation and migration\(^4\), FAO selectively regulates EC proliferation, indicating that distinct metabolic pathways control distinct EC functions during vessel sprouting. Third, we discovered an unknown role of fatty-acid-derived carbons in \textit{de novo} nucleotide synthesis for DNA replication in ECs. Our results indicate that FAO promotes \textit{de novo} deoxyribonucleotide synthesis by providing carbons for the production of aspartate and glutamate. The fact that aspartate is a direct carbon donor for the synthesis of the pyrimidine nucleoside (Extended Data Fig. 3a) and that the pool of aspartate was reduced in CPT1A \textit{Knockdown} (CPT1A \textit{KD}) ECs explains why pyrimidine dNTP levels were reduced. But aspartate and glutamate also serve as a nitrogen source for the production of the nucleobase of pyrimidines and purines. This explains why the synthesis of purine dNTPs was also impaired upon FAO blockade. Interestingly, of other primary and malignant cell types tested, only ECs and fibroblasts used substantial amounts of fatty acid carbons for DNA synthesis, even though certain cancer cell lines incorporated fatty-acid-derived carbons in the TCA cycle.

Fatty acids were irreplaceable for DNA synthesis, but not for protein and RNA synthesis. A possibility is that CPT1A \textit{KD} ECs maintained RNA synthesis primarily on nucleotide salvage rather than \textit{de novo} synthesis. However, the notion that ECs use nucleotide salvage primarily for RNA synthesis is not supported by the data. By contrast, proliferating cells switch on \textit{de novo} synthesis of dNTPs to duplicate their DNA genome\(^13\). Support for the notion that ECs use nucleotide salvage primarily for RNA synthesis is provided by evidence that incorporation of carbons from hypoxanthine (a nucleotide salvage intermediate) was higher in RNA than DNA (Extended Data Fig. 3p). This may explain why FAO blockade affected dNTP levels but not rNTP levels. Recent studies in pyruvate kinase isoenzymes documented that PKM1 expression reduced incorporation of \(^13\)C label from [U-\(^{13}\)C]glucose and [U-\(^{13}\)C]glutamine in UMP and decreased dNTP levels and cell proliferation, yet did not alter rNTP levels\(^14\), illustrating that the production of rNTPs and dNTPs is regulated differently.

Fourth, we identified that fatty-acid-derived carbons entering the TCA cycle are used for biomass production. This was unexpected since evidence in rapidly proliferating cancer cells indicated that glucose and glutamine are used as the primary carbon source for \textit{de novo} deoxyribonucleotide synthesis\(^{15,16}\). The utilization of fatty acids for biomass production is, however, not unprofitable. Indeed, given that fatty acids are a much richer carbon source than glucose or glutamine, use of fatty acids is an efficient mechanism to generate nucleotides. Finally, pharmacological CPT1 blockade reduced pathological angiogenesis, which

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**Figure 5** | Acetate or nucleosides rescue the CPT1A \textit{Knockdown} EC sprouting defect. 
**a.** Intracellular aspartate levels upon acetate supplementation in control and CPT1A \textit{KD} ECs \((n=3)\). **b.** Intracellular levels of the indicated dNTPs upon acetate supplementation in control and CPT1A \textit{KD} ECs \((n=3)\). **c.** Total sprout length upon acetate supplementation in control and CPT1A \textit{KD} ECs \((n=3)\). **d.** Percentage M+1 and M+2 labelling from [U-\(^{13}\)C]acetate in UMP (c) and UTP (d) in control and CPT1A \textit{KD} ECs \((n=5); P=NS\). *M* denotes mass of the unlabelled metabolite. **e.** Total sprout length upon acetate supplementation in control and CPT1A \textit{KD} ECs \((n=3)\). **f.** Total sprout length upon nucleic acid supplementation in control and CPT1A \textit{KD} EC spheroids \((n=4)\). **g.** Total contribution of glutamine and glycolytic carbon to citrate in control and CPT1A \textit{KD} ECs \((n=3)\). **h.** Percentage M+2 labelled citrate from [U-\(^{13}\)C]glucose in control and CPT1A \textit{KD} ECs \((n=3)\). *M* denotes mass of the unlabelled metabolite. **i.** Percentage M+3 labelled malate (i) and aspartate (j) from [U-\(^{13}\)C]glucose in control and CPT1A \textit{KD} ECs \((n=3)\). *M* denotes mass of the unlabelled metabolite. Data are mean ± s.e.m. of \(n\) independent experiments. Statistical test: mixed models (**a–j**). NS, not significant. **P** < 0.001, **PPP** < 0.0001.
Figure 6 | Most other cell types do not use fatty acid carbons for dNTP synthesis, and inhibition of CPT1A impairs angiogenesis. **a,** Total contribution of [U-13C]palmitate to citrate in various primary cells and cancer cell lines, expressed relative to the value in ECs (n = 9 for ECs, n = 8 for pericytes (Per), n = 6 for T cells, B16, A549 and 143B, n = 5 for PC-3, n = 4 for DU145, T47D and MDA, n = 3 for fibroblasts (Fib), ES cells, HCT, CT2A, U87 and MCF7). **b,** Contribution of [U-13C]palmitate to DNA, expressed relative to the value in ECs (n = 14 for ECs, n = 6 for pericytes (Per), n = 5 for B16, Panc-2, A549, n = 4 for fibroblasts (Fib), T47D, 143B and T cells, n = 3 for HCT, MDA, DU145, U87, CT2A, MCF7 and ES cells). **c, d,** Representative images (original magnification, 10×) of retinal flat-mounts of retinopathy of prematurity (ROP) mice treated with vehicle (c) or etomoxir (Eto) (d). **e,** Vascular tuft area in control and etomoxir-treated pups (n = 13 for WT and 9 for etomoxir). **f,** Mechanistic model. Top, control ECs: uptake of palmitate and FAO in ECs are not essential for the production of ATP and NADPH (indicated in the shaded box), but fatty acid-derived carbons are incorporated in amino acids, and in precursors of rNTPs and dNTPs; how critically these pathways regulate EC proliferation cannot be assessed in control cells (denoted by question mark). Bottom, CPT1A-deficient ECs: silencing of CPT1A reveals, however, that decreasing FAO depletes dNTP pools, without affecting rNTP and protein synthesis, implying that fatty acids are irreplaceable for DNA synthesis; since de novo synthesis of dNTPs is critical for DNA replication, CPT1A silencing impairs EC proliferation. Data are mean ± s.e.m. of n independent experiments (a, b) or the total number of mice (of 4 litters) (e). Statistical test: two-sided t-test (a, b); mixed models (e). NS, not significant. **P < 0.05, ***P < 0.001, ****P < 0.0001.

may hint at an underappreciated therapeutic potential of lowering FAO for the inhibition of pathological angiogenesis.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Cell culture. Primary cells human umbilical vein endothelial cells (HUVECs) were freshly isolated from different donors as described19 with approval of medical ethical committee KU Leuven/UZ Leuven and informed consent obtained from all subjects, regularly tested for mycoplasma and used between passage 1 and 5. Murine fibroblasts were freshly isolated from lungs of FVB mice and cultured and supplemented in media containing 5% fetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin. CT2A murine glialblastoma cells and U87 human glioblastoma cells were cultured in DMEM containing 10% FBS and 2 mM glutamine (Invitrogen, Life Technologies, Ghent, Belgium) supplemented with conditioned medium obtained from CT2A or U87 cells.

Knock-down and overexpression strategies. RNA interference: CPT1A silencing was achieved using a validated pool of siRNA duplexes directed against human CPT1A (Trifacta Kit, IDT) and lipofectamine RNAi Max transfection reagent (Invitrogen, Belgium) according to the manufacturer’s instructions. A scrambled siRNA was used as a negative control. Lentiviral transfections: for overexpression of murine CPT1A, the cDNA was cloned in the pPRKlSinPPT. CMV-MCS MM W vector20. Of note, this increased FAO in some but not all EC isolations, suggesting that in such cases CPT1A abundance was not limiting the maximal capacity of FAO. However, whenever FAO was enhanced, sprout numbers and length of EC spheroids were increased. To generate shRNA vectors against CPT1A, CPTIC, or ACADVL, oligonucleotides were cloned into the pLKO-shRNA2 vector (No. PT4052; Clontech, Westburg BV, Leusden, The Netherlands) for RNA sequences, see below. A nonsense scrambled shRNA sequence was used as a negative control. Production of lentiviruses by transfection into 293T cells was performed as described21. For transfections, a multiplicity of infection (MOI) of 20 was used in all shCPT1A experiments. For transfections with shACADVL and shCPTIC and for fluorescent ubiquitination assays (see below) and for overexpression, a MOI of 10 was used. Cells were transduced overnight and re-fed with fresh medium the next day. Transduced cells were used in functional assays at least 3 to 4 days after transduction. Of note, similar data were obtained using siRNA and two non-overlapping shRNAs directed against CPT1A.

In vitro assays. Proliferation was quantified by incubating cells for 2–24 h with 1 μM [3H]thymidine. Thereafter, cells were fixed with 10% ethanol at 4°C, precipitated with 10% trichloroacetic acid and lysed with 0.1 N NaOH. The amount of [3H]thymidine incorporated into DNA was measured by scintillation counting. Scratch wound migration assay: a scratch wound was applied on confluent EC monolayers (pretreated with MitoC where indicated) using a 200 μl tip, 24 h after seeding (100,000 cells per well in 24-well plates). After scratch wound and photography (T0), the cultures were further incubated in fully supplemented EB2 medium for 6–8 h (until near closure was reached in the control condition), and photographed again (T1). Migration distance (gap area at T0 minus gap area at T1) was measured with NIH Image J software package and is expressed in arbitrary units. Modified Boyden chamber migration assays were performed using MitoC-treated (24 h treatment with 2 μM MitoC) ECs. Therefore, 50,000 cells were seeded in fully supplemented EB2 medium on 0.1% gelatin-coated transwells. Upon adherence, the transwells were washed and re-fed with medium containing only 0.1% FBS and transferred to bottom wells containing medium with 0.1% FBS (baseline) or 5% FGS (migration stimulus). Transwells were incubated for 16 h and processed and analysed for migrated cells as described24.

Lamellipodia formation assay: the number and area of lamellipodia were measured on still photos of time-lapse recordings of sparsely seeded ECs, using the Axiovision morphometric analysis software (Carl Zeiss, Munich, Germany) with in-house developed macros. Lamellipodia area is expressed in per cent of total cell area. Directionality of migration was assessed by analysis of the total length of the migration track of a cell. The directionality value was then calculated by dividing the distance between the start and end location of the cell by the total track length; a value of 1 represents pure directionality. Expression experiments were performed with or without genetic shRNA CPT1A silencing. Spheroid capillary sprouting assay: ECs were incubated overnight in hanging drops in EB2 medium containing methylcellulose (20 volume% of a 1.2% solution of methylcellulose in DMEM). Therefore, 50,000 cells were seeded on 0.1% gelatin coated 24-well plates. After 1 day, ECs were treated with 2 μM MitoC. The following day, adherent ECs were stained for α-smooth muscle actin (αSMA) and analyzed for sprouting using an IX-81 epifluorescence microscope (Olympus) in three independent experiments. The sprouting activity was quantified as the number of sprouts per microbead. Lamellipodia formation was assessed by measuring the area and number of lamellipodia using NIH Image J software package. The microbead assay was performed on at least six independent experiments.

Molsheim Cedex, France) and Panco2 murine pancreatic carcinoma cells (provided by B. Wiedenmann, Charité®, Berlin, Germany) were cultured in RPMI containing 10% FBS, MCF7 (human breast cancer), HCT116 (human colon carcinoma), MDA-MB-468 (human breast carcinoma cells), T47D (human ductal breast epithelial tumour cells), DU145 (human prostate cancer cells), 143B (human osteosarcoma cells) and A549 (human lung carcinoma cells) were cultured in DMEM containing 10% FBS, 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin. CT2A murine glialblastoma cells and U87 human glioblastoma cells were cultured in DMEM containing 10% FBS and 2 mM glutamine (Invitrogen, Life Technologies, Ghent, Belgium) supplemented with conditioned medium obtained from CT2A or U87 cells.
per spheroid) was done on phase contrast images using the NIH Image J software package. To assess the number of viable versus dead cells, unfixed spheroids were stained with 500 µl of 1/500 dilution of TO-PRO-3 (Invitrogen, Life Technologies, Ghent, Belgium) solution at 37 °C for 10 min, washed with PBS and imaged by confocal microscopy Zeiss LSM 510 META NLO or Zeiss LSM 780 confocal microscope (objectives: ×10 with NA 0.3, ×20 with NA 0.4) (Carl Zeiss, Munich, Germany). ATP measurement: analysis of total ATP levels was performed using a commercially available kit (ATPite, PerkinElmer). Apoptosis: analysis of apoptosis was done by fluorescent staining for cleaved caspase 3 (Cell Signaling Technology, Bioke, Leiden, The Netherlands) in PFA-fixed monolayers.

RNA analysis. RNA expression analysis was performed by Taqman quantitative RT–PCR as described using in-house-designed primers and probes or pre-made primer sets (Applied Biosystems, Carlsbad, CA and IDT, Belgium). Sequences or pre-made primer set ID numbers are available upon request. For comparison of gene expression between conditions, expression (normalized to HPRT endogenous control) is expressed relative to control condition. For comparison between different genes, absolute expression levels were determined based on respective cDNA standard curves, and levels are expressed as copies mRNA per 10^6 copies HPRT mRNA.

Immunoblot analysis. Protein extraction and immunoblot analysis were performed using a modified Laemmli sample buffer (125 mM Tris-HCl, pH 6.8 buffer containing 2% SDS and 10% glycerol) or cell lysis buffer (Cell Signaling Technology) in the presence of protease and phosphatase inhibitors (Roche, Vilvoorde, Belgium). Lysates were separated by SDS–PAGE under reducing conditions, transferred to a nitrocellulose or PVDF membrane, and analysed by immunoblotting. Primary antibodies used were rabbit anti-CPT1A (No. 12252), rabbit anti-lamin A/C (No. 2032), rabbit anti-β-actin (No. 4970), anti-total AMPKζ (No. 2531), rabbit anti-phospho-AMPKζ Thr 172 (No. 2531), rabbit anti-p21 (No. 2947), anti-phospho-ATM (No. 5883), anti-total ATM (No. 2873) (Cell Signaling Technology, Bioke, Leiden, The Netherlands), and anti-total β-actin (FL 393, Santa Cruz Biotechnology, The Netherlands). Autophagy was analysed via western blot determination of LC3 II/I ratio (No. 3868, Cell Signaling Technology) to reveal the on-state of autophagy (autophagic flux). Equal loading was verified by ponceau Red staining and loading controls. Appropriate secondary antibodies were from Dako (Enschede, The Netherlands). Signal was detected using the ECL system (Amersham Biosciences, GE Healthcare, Diegem, Belgium) according to the manufacturer's instructions. Densitometric quantifications of bands were done with NIH Image J software.

Flow cytometry. Protein/RNA synthesis; protein synthesis was determined by incubation of cells with a methionine analogue (HPG, Invitrogen) for 24 h in bulk medium. RNA synthesis was determined by incubation of cells with a uridine analogue (5'-ethynyl uridine, EU, molecular probes). Cells were trypsinized, PFA fixed and incorporated HPG or 5'-ethynyl uridine was detected by a Click-IT reaction with Alexa flour 647 according to the manufacturer's instructions. Alexa flour 647 was excited with a 604-nm red laser and emission was recorded at 660 nm using a BD FACs Canto flow cytometer (Becton Dickinson Benelux NV, Erembodegem, Belgium). Intracellular ROS analysis: intracellular ROS levels were measured using the CM-DCF dye according to the manufacturer's instructions (Invitrogen, Belgium). For flow cytometry analysis, the BD FACs Canto was operated in targeted SIM mode following the ions m/z 311.11456 and 308.59499 (GSH and GSSG, respectively) using the ion 445.1203 as lock mass. The mass spectrometer ran in positive polarity, the source voltage was 3.0 kV, and the capillary temperature was set at 300 °C. Additional sheath gas flow was put at 35 and auxiliary gas flow rate at 10. Auxiliary gas heater temperature was put at 60 °C. ACG target was put at 100,000 ions with a maximum ion injection time of 200 ms, acquired at a resolution of 70,000. For the data analyses we manually integrated the peaks representing GSH and GSSG using the Thermo Xcalibur Qual Browser software (Thermo Scientific) and data are represented as area of the respective GSH and GSSG peaks. Determination of 13C-palmitate, glucose and glutamine incorporation in metabolites and total metabolite levels; for 13-Carbon incorporation from palmitate to metabolites, cells were incubated for 48 h with labelled substrates (confirmation of steady state at that time was confirmed, see Extended Data Fig. 5). For ECs, [U-13C]palmitate labelling was done in two ways: (1) '100% labelling', whereby all cold palmitate in M199 culture medium (120 µM) was replaced by 120µM [U-13C]palmitate using M199 medium, containing charcoal stripped serum (which does not contain any fatty acids); and (2) '50/50% labelling', whereby 50 µM [U-13C] palmitate was added to the EG2 medium culture containing 100 µM cold palmitate. Both types of labelling yielded similar data and were thus merged. For flow cytometry analysis, the BD FACs Canto was operated in targeted SIM mode following the ions m/z 311.11456 and 308.59499 (GSH and GSSG, respectively) using the ion 445.1203 as lock mass. The mass spectrometer ran in positive polarity, the source voltage was 3.0 kV, and the capillary temperature was set at 300 °C. Additional sheath gas flow was put at 35 and auxiliary gas flow rate at 10. Auxiliary gas heater temperature was put at 60 °C. ACG target was put at 100,000 ions with a maximum ion injection time of 200 ms, acquired at a resolution of 70,000. For the data analyses we manually integrated the peaks representing GSH and GSSG using the Thermo Xcalibur Qual Browser software (Thermo Scientific) and data are represented as area of the respective GSH and GSSG peaks. Determination of 13C-palmitate, glucose and glutamine incorporation in metabolites and total metabolite levels; for 13-Carbon incorporation from palmitate to metabolites, cells were incubated for 48h with labelled substrates (confirmation of steady state at that time was confirmed, see Extended Data Fig. 5).
content\textsuperscript{26–29}. To correct for enrichment dilution, we used previously reported methods\textsuperscript{22,23}; that is, we divided the fractional contribution of a labelled metabolite of interest by the fractional contribution of its precursor (calculated by the formula below).

The total contribution of carbon was calculated using the following equation\textsuperscript{26}:

\[
\text{total contribution of carbon} = \sum_{i=0}^{n} \frac{t_i \times m_i}{\sum_{i=0}^{n} m_i}
\]

where \( n \) is the number of C atoms in the metabolite, \( i \) represents the different mass isotopomers and \( m \) refers to the abundance of a certain mass. Glycortic carbon contribution was calculated based on [U-\(^{13}\)C]glucose labelling and label dilution in pyruvate\textsuperscript{7}. For total metabolite levels, arbitrary units of the metabolite of interest were determined by using a fluorescence-based PCR assay\textsuperscript{23} using G1 sorted ECs, identified as CherryRed\textsuperscript{+} Venus\textsuperscript{+} cells upon transduction with a fluorescent, ubiquitination-based cell cycle indicator (Fucci) construct\textsuperscript{1}\textsuperscript{9}.

Determination of \( ^{13} \)C-palmitate or \( ^{13} \)C-acetate incorporation in UMP and UTP: cells were labelled with [U-\(^{13}\)C]palmitate (100% labelling with 100 \( \mu \)M [U-\(^{13}\)C]palmitate; see above) or [U-\(^{13}\)C]acetate (20 mM supplementation with [U-\(^{13}\)C]acetate) for 48 h and were then collected in 500 \( \mu \)l ice-cold acetonitrile buffer (50% methanol, 30% acetic acid and 20% water). Samples were spun for 5 min and supernatants were dried down and were then reconstituted in 50 \( \mu \)l of HPLC-grade water, vortexed, centrifuged, and transferred into HPLC vials. LC-MS/MS analysis was done on a Waters Xevo TQ-S mass spectrometry system coupled to an H-Class UPLC system. Metabolites were separated by polarity using a Supelco Ascentis Express C18 column (2.7 \( \mu \)m particle size, 5 \( \times \) 2.1 mm). LC parameters are as follows: autosampler temperature, 5 \( ^\circ \)C; injection volume, 5 \( \mu \)l; column temperature, 50 \( ^\circ \)C; flow rate over 11 min: \( t = 0, 0.4 \text{ min}^{-1}; t = 2, 0.3 \text{ min}^{-1}; t = 5, 0.25 \text{ min}^{-1} \); \( t = 5, 0.15 \text{ min}^{-1}; t = 9, 0.4 \text{ min}^{-1}; t = 11, 0.4 \text{ min}^{-1} \). The LC solvents were solvent A: 10 mM tributylamine and 15 mM acetic acid in 97:3 water:methanol (pH 4.95); and solvent B: methanol. Elution from the column was performed over 11 min with the following gradient: \( t = 0, 0\% \text{ B}; t = 1, 0\% \text{ B}; t = 2, 20\% \text{ B}; t = 3, 20\% \text{ B}; t = 5, 55\% \text{ B}; t = 8, 85\% \text{ B}; t = 9, 0\% \text{ B}; t = 11, 0\% \text{ B}. Mass spectra were acquired using negative-mode electrospray ionization operating in multiple reaction monitoring (MRM) mode. The capillary voltage was 3,000 V, and cone voltage was 50 V. Nitrogen was used as cone gas and desolvation gas, with flow rates of 1500 \( \mu \)l \( \text{min}^{-1} \) and 6000 \( \mu \)l \( \text{min}^{-1} \), respectively. The source temperature was 150 \( ^\circ \)C, and desolvation temperature was 500 \( ^\circ \)C. Argon was used as collision gas at a manifold pressure of 4.3 \( \times \) 10\(^{-3}\) mbar. Collision energies and source cone potentials were optimized for each transition using Waters QuanOptimize software. Data were acquired and analysed using Masslynx 4.1 and Quanlynx software. Isootope labelling data was corrected for the natural abundance of different isotopes using IsoCor\textsuperscript{12}.

Determination of rNTP levels by LC-MS: rNTP was extracted with the same solvent. rNTP concentrations were determined with the same LC-MS method as described for UTP and UMP. Additionally, \( ^{13} \)C-internal standards (generated based on fully labelled yeast extracts\textsuperscript{33}) were spiked into the extraction sample. rNTP concentrations were determined with the same LC-MS method as described for UTP and UMP. All samples were normalized to the \( ^{13} \)C-internal standard and protein content.

Mouse models of ocular angiogenesis. Generation of EC-specific CPT1A conditional knockout mice: for generation of the Cpt1a\textsuperscript{lox/lox} m mice,loxP sites were introduced flanking a segment comprising exons 11 and 12 via homologous recombination in embryonic stem (ES) cells. The targeting vector contained from 5’ to 3’: a 3.5–kb 5’ homology flanking the 2-kb floxed mid-flank consisting of the loxp flanked exon 11–12 segment, a FRT flanked neomycin resistance (NEO) cassette, a 2.5–kb 3’ homology flanking. Correctly targeted ES clones were identified by appropriate Southern blot and PCR analysis, and subjected to transient transfection with a Flp recombinase expression plasmid to remove the FRT-flanked NEO cassette. Correctly excised clones were used for morula aggregation, and resulting chimeric animals were bred for germline transmission, yielding Cpt1a\textsuperscript{lox/lox} m mice. For postnatal EC-specific loss of function studies, at least three technical replicates were used, except for the [U-\(^{13}\)C]palmitate incorporation analysis in cell lines (at least two technical replicates) (Fig. 6a).

Statistical. Data represent mean ± s.e.m. of pooled experiments unless otherwise stated. \( n \) values represent the number of independent experiments performed or the number of individual mice phenotyped. For each independent in vitro experiment, at least three technical replicates were used, except for the [U-\(^{13}\)C]palmitate incorporation analysis in cell lines (at least two technical replicates) (Fig. 6a).

Mouse models of ocular angiogenesis: genetic endothelial-cell-specific inactivation of CPT1A in neonatal mice was achieved by intraperinatal injection of 100 mg kg\(^{-1}\) tamoxifen solution (Sigma T5648, dissolved in 1:10 EtOHCorn oil) once daily from postnatal day (P) 1 to P4 in CPT1A\textsuperscript{12c} mice. Pharamaco-logical blockade of FAO in neonatal C57BL/6 WT mice (animal facility KU Leuven) was achieved by treatment with 30–35 mg kg\(^{-1}\) etomoxir from P2 to P4. For detection of cell proliferation, EdU (Invitrogen) was injected 2 h before dissection. At P5, pups were killed and eyes were enucleated, fixed with 4% PFA for 2 h at 4\( ^\circ \)C and prepared for vascular analysis. As all animal treatments were done in baseline conditions, no randomization was required. Retinal whole mounts were prepared for vascular analysis as described\textsuperscript{30}. Only litters for which the WT littermates (for CPT1A\textsuperscript{12c} mice) or vehicle-treated littermates (for etomoxir treated mice) reached normal outgrowth and body weight at P5 were included for analysis. Radial vascular area, branching points, and number of filopodia were analysed on isolecitin GS-IB4 stained retinas (see below) using the NIH Image J software package and Leica LASAF-MMAF morphometric analysis software (MetaMorph) (Leica Microsystems, Mannheim, Germany) with in-house developed macros. No statistical method was used to predetermine the sample size. Oxygen-induced retinopathy: oxygen-induced retinopathy (ROP) was induced by exposing C57BL/6 pups with their mother to 70% oxygen from P7 to P12 (refer. 35). Pups were then returned to normoxia and injected daily with 30 mg kg\(^{-1}\) etomoxir or vehicle. At P17, pups were killed and eyes were enucleated, fixed in 4% PFA and retinal flat-mounts were stained for isolecitin B4, using previously published methods\textsuperscript{31}. Mosaic images were captured using the inverted Leica DM6000B epifluorescence microscope (Leica, Mainhalb, Germany). Confocal analysis of the vascular tunica was performed using NIH Image J software. For all mouse experiments, data analysis was done by experimenters blinded to the group allocation. All experimental animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the University of Leuven.

Immunohistochemistry. PFA-fixed cell cultures (4% PFA except for CPT1A staining where 2% PFA was used), spheroids (4% PFA) or whole-mount retinas (4% PFA) were subjected to immunofluorescence staining using the following isolecitin conjugates or primary antibodies: isolecitin GS-IB4-Alexa 488, isolecitin GS-IB4-Alexa 568, isolecitin GS-IB4-Alexa 647 (Molecular Probes), anti-CPT1A (Cell Signaling), anti-Ng2 chondroitin sulfate proteoglycan (Chemicon), anti-Tomm20 (Abcam) and anti-collagen IV (Southern Biotech). Alexa-488, -568 or -633 conjugated secondary antibodies were used (Molecular Probes). EdU, EU and HPG staining was performed using a Click-IT assay with Alexa flour 555 according to manufacturer’s instructions.
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Extended Data Figure 1 | FAO regulates vessel sprouting. a, mRNA expression of CPT1 isoforms (n = 3). b, CPT1A mRNA levels upon CPT1A silencing (CPT1AKD) (n = 11). c, Representative immunoblot of CPT1A for control and CPT1AKD ECs. d, FAO flux upon CPT1A silencing in venous (HUV) and arterial (HA) ECs, or upon small interference RNA transfection in venous ECs (siRNA) (n = 6 for HUV shRNA, n = 3 for HA shRNA and HUV siRNA). e, Schematic representation of FAO measurement using [9,10-3H]palmitate (reprinted from Immunity 35, Wang, R. et al. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation, 871–882 (2011), with permission from Elsevier; ref. 24). f, Representative immunoblot for CPT1A upon genetic silencing of CPT1A using siRNA (siCPT1A). g, FAO flux upon silencing of CPT1C (CPT1CKD) (n = 3; P = NS). h, FAO flux levels in venous (HUV), arterial (HA) and microvascular (HMV) ECs (n = 4 for HUV versus HA and n = 3 for HUV versus HMV). i, Sprout number in control and CPT1AKD EC spheroids with mitomycin C (MitoC) treatment as indicated (n = 3). j, Flow cytometry counting of viable control and CPT1AKD ECs (n = 4; P = NS). k, FAO flux and proliferation upon silencing of ACADVL (ACADVLKD) (n = 3 for each). m, Wound closure in control and ACADVLKD ECs (n = 3; P = NS). n, o, Quantification of vessel sprouting in control and ACADVLKD EC spheroids with MitoC treatment as indicated, total sprout length (n) and sprout numbers per spheroid (o) (n = 5). p, Sprout number in control and CPT1A OE EC spheroids with MitoC treatment as indicated (n = 3). q, Total sprout length in control and CPT1A OE EC spheroids treated with MitoC as indicated (n = 5). r, s, Representative phase (original magnification, 10× magnification) contrast images of control (r) and CPT1A CKD (s) EC spheroids. t, Scratch wound assay in control and CPT1A OE ECs treated with MitoC as indicated (n = 3; P = NS). u, PCR analysis of genomic DNA from WT and CPT1A D EC pups, confirming Cre-mediated recombination of the floxed Cpt1a allele as shown by the appearance of a 300-bp band. v, NG2+ area in neonatal vascular plexus of WT and CPT1A D EC mice (3 litters, n = 8 pups for WT and 7 pups for CPT1A D EC; P = NS). Data are mean ± s.e.m. of n independent experiments (a, b, d, g–q, t) or the total number of mice (v). Statistical test: mixed models. NS, not significant. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Extended Data Figure 2 | CPT1A silencing does not cause cellular distress. 

**a**, ADP/ATP ratio in control and CPT1A<sup>KD</sup> ECs (n = 3; P = NS). **b**, Sprout number upon oligomycin treatment (oligo) in control and CPT1A<sup>KD</sup> EC spheroids (n = 3). **c**, Glycolysis measurement in control and CPT1A<sup>KD</sup> ECs (n = 3; P = NS). **d**, Representative immunoblot for phosphorylated AMPK (pAMPK) and total AMPK (AMPK) and for LC3b I and II in control and CPT1A<sup>KD</sup> ECs. The ratio of the densitometrically quantified bands of pAMPK/AMPK and LC3b II/I is shown below the blots (n = 3; P = NS). **e**, Sprout number upon N-acetyl-cysteine (NAC) treatment in control and CPT1A<sup>KD</sup> EC spheroids (n = 3). **f, g**, Representative images (original magnification, 10×) of EC spheroids upon staining for TO-PRO3 in control (f) and CPT1A<sup>KD</sup> spheroids (g). **h, i**, Representative pictures (original magnification, 10×) of Hoechst/cleaved-caspase-3-stained control (h) and CPT1A<sup>KD</sup> (i) ECs. **j**, Representative immunoblots showing the ratio of phosphorylated (pATM)/total-ATM (ATM), p21/lamin and p53/lamin in control and CPT1A<sup>KD</sup> ECs. The ratios of the densitometrically quantified bands are shown below the blots (n = 3; P = NS). Data are mean ± s.e.m. of n independent experiments (a–e, j). Statistical test: mixed models. NS, not significant. ****P < 0.0001.
Extended Data Figure 3 | FAO is used for de novo nucleotide synthesis.

**a**| Schematic representation of the different carbon sources used for de novo synthesis of UMP. Note that palmitate contributes three carbons to the nine carbon skeleton of UMP. PRPP, 5-phosphoribosyl-1-pyrophosphate.

**b**| Sprout number upon 5-fluorouracil (5FU) treatment in control and CPT1A KD EC spheroids (n = 4).

**c**| Sprout number upon methotrexate (MTX) treatment in control and CPT1A KD EC spheroids (n = 4).

**d**| Sprout number upon acetate treatment in control and CPT1A KD EC spheroids (n = 3).

**e-g**| Representative images (original magnification, 10×) of EC spheroids upon acetate treatment.

**h**| Rescue of the sprouting defect of CPT1A KD spheroids by acetate was not affected by oligomycin treatment; panel **h**, total sprout length; panel **i**, sprout numbers/spheroid (n = 3; P = NS).

**j**| Quantification of vessel sprouting using the EC spheroid model, showing that the reduction of total sprout length (j) and number of sprouts per spheroid (k) upon CPT1A silencing (CPT1A KD) was rescued by supplementation with a dNTP mix (n = 3). I. Quantification of MitoC-treated EC spheroid sprouting upon acetate or nucleoside mix supplementation (n = 3; P = NS).

**m**| Glucose oxidation in ECs, measured by 14CO2 formation from [6-14C]glucose in control and CPT1A KD ECs (n = 4).

**n**| Glutamine oxidation in ECs, measured by 14CO2 formation from [U-14C]glutamine in control and CPT1A KD ECs (n = 4; P = NS).

**o**| Total contribution of [U-13C]glucose and [U-13C]glutamine to aspartate in control and CPT1A KD ECs (n = 3).

**p**| [8-14C]hypoxanthine incorporation in RNA and DNA in control ECs (n = 3). Data are mean ± s.e.m. of n independent experiments (b-d, h-p). Statistical test: mixed models. NS, not significant. *P < 0.05, **P < 0.01, ****P < 0.0001.
Extended Data Figure 4 | Etomoxir reduces vessel sprouting. a, FAO flux upon etomoxir (Eto) treatment (n = 6). b, [3H]thymidine incorporation upon etomoxir treatment (n = 5). c, Scratch wound assay using MitoC-treated ECs upon etomoxir (Eto) treatment (n = 4; P = NS). d, Branch point quantification in the retinal vasculature of control (Ctrl) and etomoxir-treated (Eto) pups (8 litters, n = 24 pups for control and 16 for etomoxir treatment).

e, f, Representative confocal images (original magnification, 10×) of retinal vessels stained for isolectin-B4 in control (e) and etomoxir (f) treated pups. 
g, Filopodia quantification in the retinal vascular front of control and etomoxir (Eto) treated pups (4 litters, n = 11 for WT and 9 for etomoxir; P = NS). Data are mean ± s.e.m. of n independent experiments (a–d, g) or the total number of mice (d, g). Statistical test: mixed models. NS, not significant. ****P < 0.0001.
Extended Data Figure 5 | Analysis of steady state. Percentage M+2 or M+4 citrate and aspartate over different time points (24, 36, 48 and 52 h) after labelling with [U-13C]glucose (a), [U-13C]glutamine (b), or [U-13C]palmitate (c). Data are mean ± s.d. of n = 3 independent experiments.
Corrigendum: Fatty acid carbon is essential for dNTP synthesis in endothelial cells

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We thank our colleagues from the metabolism community (Emile Van Schaftingen and Guido Bommer, University of Louvain, Belgium, and Frans Schuit, University of Leuven, Belgium), who alerted us to a possible confusion arising from our Article. In particular, the statement in the abstract that “Isotope labelling studies in control endothelial cells showed that fatty acid carbons substantially replenished the Krebs cycle” and similar phrases later in the text could be misunderstood as implying anaplerosis. By no means did we intend to suggest that the Krebs cycle is replenished by the net contribution of fatty acid carbons in the traditional sense of anaplerosis. Indeed, even though labelled acetyl-CoA from fatty acids enters the Krebs cycle and labels oxaloacetate, we did not want to imply net formation of oxaloacetate from acetyl-CoA, derived from fatty acids. Rather, our data suggest that under conditions of adequate availability of anaplerotic substrates (glucose, glutamine), fatty acid oxidation is important to produce sufficient amounts of dNTPs. Fatty acids provide acetyl-CoA, which helps to sustain the TCA cycle and dNTP synthesis for proliferation in conjunction with an anaplerotic substrate. Why the anaplerotic nutrients glucose and glutamine do not sustain sufficient aspartate and nucleotide synthesis in the CPT1A-silenced endothelial cells is an intriguing but outstanding question.