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SUPPLEMENTARY DISCUSSION

In the supplementary discussion, we provide additional rationale, description and discussion of experiments, which we could not include into the main text due to space restrictions. These data are shown in the Extended Data Figures.

SUPPLEMENTARY DISCUSSION 1 – *Residual CPT1A protein precludes analysis of early lymphatic development*

We could not study the role of complete loss of CPT1A in early lymphatic development, because of the following reasons: (i) use of the *Prox1-cre*^{ERT2}, *Prox1*^{FK}*-cre* and *Flt4-cre*^{ERT2} lines all possess the inevitable limitation that *Cpt1a* becomes inactivated only after PROX1 initiates lymphatic development and not all CPT1A protein is immediately and sufficiently rapidly cleared in LEC precursors and LECs (Extended Data Fig. 2f-j), despite detection of a CPT1A excision band as early as E9.5 (Extended Data Fig. 2k), thereby still allowing residual lymphatic formation at E14.5 (data not shown); and (ii) CPT1A protein stability precluded rapid clearance of CPT1A; even despite a very efficient lowering of *CPT1A* mRNA levels (Extended Data Fig. 2l), CPT1A protein levels remained much higher, resulting in 35-40% residual CPT1A protein levels 3 days after *CPT1A* silencing (Extended Data Fig. 2m).

We considered using *VE-cadherin(PAC)-cre*^{ERT2} mice (also known as *Cdh5(PAC)-cre*^{ERT2}), as they express Cre^{ERT2} in VECs of the cardinal vein prior to LEC precursor specification[13] in order to obtain earlier *Cpt1a* excision, but these mice exhibited blood vascular defects (as expected, based on our previously characterized role of CPT1A in blood vascular endothelial cells (BECs)[11]), which precluded us from studying lymphatic development (Extended Data Fig. 2n).

SUPPLEMENTARY DISCUSSION 2 – *VLCAD* SILENCING PHENOCOPIES *CPT1A* SILENCING AND *CPT1A* OVEREXPRESSION IS NOT SUFFICIENT TO INDUCE VEC-TO-LEC DIFFERENTIATION

Silencing of very long chain acyl-CoA dehydrogenase ($VLCAD^{KD}$), a gene directly involved in the FAO pathway, phenocopied the effects of $CPT1A^{KD}$ in pLECs, reducing FAO flux and *VEGFR3* mRNA expression (Extended Data Fig. 5l-n). However, while *CPT1A* overexpression in VECs increased FAO flux, it was unable to induce VEC-to-LEC differentiation (Extended Data Fig. 5o-r), implying that FAO is essential but not sufficient for the PROX1-mediated induction of LEC differentiation, and may require the coordinated activity of PROX1. In line, $CPT1A^{KD}$ in VECs did not impair the expression of blood vascular EC (BEC) markers (*KDR*, *EFNB2*, *EPHB4*) (Extended Data Fig. 5s-v). These data also suggest that FAO is downstream of PROX1.

SUPPLEMENTARY DISCUSSION 3 – ADDITIONAL METABOLIC PARAMETERS OF FAO IN LECS

FAO regulates mediators of redox homeostasis

$CPT1A^{KD}$ trended to slightly elevate oxidized glutathione levels (GSSG levels, percent of the total glutathione (GSSG+GSH) levels), although this increase was not statistically significant (Extended Data Fig. 6b). The $NADP^+$ / NADPH ratio was also elevated upon $CPT1A^{KD}$ in pLECs (Extended Data Fig. 6c), but did not cause significant changes in cellular ROS or H_2O_2 levels in pLECs (Extended Data Fig. 6d,e). We speculate that the reduction in ROS scavenging capacity is balanced by a reduction in ROS generation upon $CPT1A^{KD}$.

FAO-derived acetyl-CoA contributes to Krebs cycle intermediates and nucleotide precursors necessary for nucleotide synthesis

We recently documented another metabolic role of FAO in VECs, namely that FAO regulates proliferation of these BECs by generating fatty acid-derived carbons for incorporation into deoxyribonucleotide during DNA replication [11]. Stable isotopomer

analysis with [U-^{13}C]palmitate revealed that CPT1A^{KD} in pLECs reduced the incorporation of labeled carbons in Krebs cycle metabolites (citrate, α-ketoglutarate, fumarate, malate; Extended Data Fig. 6f) and reduced total pools of these metabolites (Extended Data Fig. 6g), phenocopying VECs [11]. This mechanism also regulated, at least in part, the proliferation of LECs, since CPT1A^{KD} reduced [U-^{14}C]palmitate incorporation into DNA in LECs (Extended Data Fig. 6h), similar as in VECs [11]. Use of [U-^{13}C]palmitate further revealed that CPT1A^{KD} in pLECs decreased tracer incorporation into the nucleotide precursors aspartate, glutamate, UTP and CTP (Extended Data Fig. 6i), and also resulted in a reduction in the total pools of aspartate, glutamate, UTP, CTP, ATP and GTP (Extended Data Fig. 6j). Further, supplementation of a nucleotide mix to CPT1A^{KD} pLEC spheroids completely rescued sprouting to baseline levels (Extended Data Fig. 6k). These results show that LECs, like BECs, use FAO to generate carbons for incorporation into dNTPs during DNA synthesis.

SUPPLEMENTARY DISCUSSION 4 – CHARACTERIZATION OF THE ACLY^{KD} PHENOTYPE AND STATEMENT ON HISTONE DEACETYLATION

We assessed whether the phenocopy of the CPT1A^{KD} phenotype by ACLY^{KD} was due to reduced CPT1A expression in pLECs. ACLY^{KD} lowered *CPT1A* mRNA expression (Extended Data Fig. 7o,p) and decreased *VEGFR3* mRNA expression (Extended Data Fig. 7q), however, without significantly altering FAO flux (Extended Data Fig. 7r), likely due to decreased pools of malonyl-CoA, an inhibitor of FAO (Extended Data Fig. 7s).

Histone acetylation can also be inversely regulated by histone deacetylases. For instance, butyrate, a fatty acid metabolite produced by colonic bacteria, increases histone acetylation either by inhibiting histone deacetylation through inhibition of histone deacetylases (HDACs) or by providing acetyl-CoA through FAO [48]. We did not specifically focus on the role of HDACs in the regulation of lymphangiogenesis, as we discovered a novel interaction between PROX1 and p300, and PROX1-mediated FAO-driven acetyl-CoA production promotes the expression of lymphangiogenic genes.

Whether histone deacetylation also regulates lymphangiogenesis in this context merits further study.

SUPPLEMENTARY REFERENCES

48 Donohoe, D. R. *et al.* The Warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. *Mol Cell* **48**, 612-626 (2012).