Mitochondrial respiration controls the Prox1-Vegfr3 feedback loop during lymphatic endothelial cell fate specification and maintenance

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Recent findings indicate that mitochondrial respiration regulates blood endothelial cell proliferation; however, its role in differentiating lymphatic endothelial cells (LECs) is unknown. We hypothesized that mitochondria could work as a sensor of LECs’ metabolic specific needs by determining their functional requirements according to their differentiation status and local tissue microenvironment. Accordingly, we conditionally deleted the QPC subunit of mitochondrial complex III in differentiating LECs of mouse embryos. Unexpectedly, mutant mice were devoid of a lymphatic vasculature by mid-gestation, a consequence of the specific down-regulation of main LEC fate regulators, particularly Vegfr3, leading to the loss of LEC fate. Mechanistically, this is a result of reduced H3K4me3 and H3K27ac in the genomic loci of key LEC fate controllers (e.g., Vegfr3 and Prox1). Our findings indicate that by sensing the LEC differentiation status and microenvironmental metabolic conditions, mitochondrial complex III regulates the critical Prox1-Vegfr3 feedback loop and, therefore, LEC fate specification and maintenance.

INTRODUCTION
Recent discoveries have changed our conventional views about the roles of lymphatics in health and disease. In addition to its traditional role as a route for the transport of fluid and immune cells, increasing evidences suggest that lymphatics are important tissue-specific players in major physiological and pathophysiological processes. Work performed during the past few decades revealed the first molecular insights into lymphatic vascular development. The master transcription factor Prox1 and Vegfr3/Vegfr3 signaling are key regulators of early lymphatic endothelial cell (LEC) fate specification and developmental lymphangiogenesis (1–3). Our previous work identified a feedback loop between Prox1 and Vegfr3 critical for regulating the number of LEC progenitors and budding LECs produced in the murine cardinal vein (CV) during development and for maintaining LEC fate (4). However, how this feedback loop is turned on/off is currently unknown. Accordingly, identifying its control mechanisms will provide essential insights into this critical step in developmental lymphangiogenesis.

Recent studies suggested that cellular metabolism is a crucial regulator of endothelial cell (EC) function and (lymph)angiogenesis (5–13). For example, it was shown that Prox1-mediated transcriptional up-regulation of carnitine palmitoyltransferase 1a (CPT1a) promotes fatty acid oxidation (FAO); thus, it increases acetyl coenzyme A (acetyl-CoA) levels that are necessary for LEC differentiation (10). Acetyl-CoA also participates in the histone acetylation of various lymphangiogenic genes catalyzed by the lysine acetyltransferase P300 as part of the Prox1-P300 complex (10). In addition, ketone body oxidation by 3-oxoacid-CoA-transferase-1 (OXCT1) generates acetyl-CoA, which sustains the tricarboxylic acid (TCA) cycle for aspartate and deoxynucleotide triphosphate (dNTP) synthesis that is required for LEC proliferation (13). It was also shown that fibroblast growth factor (FGF) signaling in LECs stimulates c-MYC expression, which, in turn, increases hexokinase 2 (HK2) levels and promotes glycolytic flux (7). Inactivation of FGF receptors or c-MYC/HK2 impairs LEC proliferation and lymphatic development (7). To evaluate metabolic requirements during mammalian developmental lymphangiogenesis, we functionally inactivated the mitochondrial respiratory chain complex III QPC subunit. Mitochondrial respiration participates in three distinct mitochondria functions: oxidative phosphorylation for adenosine triphosphate (ATP) generation, oxidative TCA cycle flux to produce metabolites for macromolecule biosynthesis, and release of reactive oxygen species (ROS) and TCA cycle metabolites to determine cell fate and/or function. In blood endothelial cells (BECs), it was recently reported that mitochondrial complex III is required for their proliferation during angiogenesis (14). Using in vivo and in vitro approaches, we now demonstrate that functional inactivation of mitochondrial complex III impairs lymphatic development, resulting in mouse embryos fully devoid of a lymphatic vasculature. We show that this phenotype is because mitochondrial respiration–deficient LECs fail to maintain LEC fate, a consequence of the specific down-regulation of LEC markers, particularly that of Vegfr3, which, in turn, disrupts the maintenance of the Prox1-Vegfr3 feedback loop. We demonstrate that down-regulation in the expression of LEC genes is likely triggered by the reduction in H3K4me3 and H3K27ac histone modifications at specific target promoters, particularly those of Vegfr3 and Prox1.

RESULTS
Mitochondrial complex III is necessary for lymphatic vasculature formation
To investigate the role of the mitochondrial respiratory chain on LEC homeostasis, we took advantage of Uqcrq conditional knockout mice (QPC flox) (14, 15). Uqcrq encodes the QPC protein, a critical
subunit of mitochondrial respiratory chain complex III. QPC floxed mice were crossed to PdpnGFPCre mice (16) to generate QPC\textsuperscript{f/f}; PdpnGFPCre null mice (QPC mutants) and control littermates. In those mutant mice, QPC starts to be deleted in Pdpn-expressing budding LECs once outside the CV at around embryonic day 10.5 (E10.5). We were unable to obtain live born QPC\textsuperscript{f/f}; PdpnGFPCre pups, suggesting that mitochondrial respiration deficiency in LECs is embryonic lethal (table S1). Accordingly, QPC mutant embryos were collected at different stages to analyze their organ-associated lymphatic vasculature. At E14.5, QPC mutant embryos exhibit severe edema (Fig. 1, A and B). Whole-mount immunostaining of the embryonic dorsal skin using antibodies against VE-cadherin (VEcad) and Prox1 revealed that QPC mutants are largely devoid of a lymphatic network, although few scattered LECs are sometimes still detected at this stage (Fig. 1, C, D, and I). No obvious alterations were seen in the blood vasculature morphology or coverage (Fig. 1, D and J). We extended this analysis to other tissues and found that similar to the skin, at E16.5, no mesenteric or cardiac lymphatics are present in mutant embryos (Fig. 1, E to H and K and L). Therefore, we concluded that QPC deficiency in those differentiating LECs results in the complete absence of a lymphatic vasculature at mid-gestation.

To target LEC progenitors inside the CV, QPC floxed mice were also crossed with Prox1CreERT2 mice, and Cre recombination was induced by tamoxifen injection at E9.5 and E10.5. At E14.5, these mutant embryos also exhibit edema (fig. S1, A and B); however, different to QPC\textsuperscript{f/f}; PdpnGFPCre embryos, lymphatics are present, although their numbers were severely reduced (fig. S1, C, D, and I). Those lymphatics have less branch points such that they also fail to interconnect and form a normal-looking vascular network (fig. S1, C to J). In addition, Vegfr3 expression levels were reduced in the mutant lymphatics (fig. S1, G, H, and K). One possible explanation for the difference in the severity of the phenotypes when using the constitutive PdpnGFPCre or the inducible Prox1CreERT2 is that, in general, deletion is less efficient when using tamoxifen-inducible Cre strains. We found that deletion efficiency is about 75% in CD31\textsuperscript{+} Lyve1\textsuperscript{+} LECs that were fluorescence-activated cell sorting (FACS)-sorted from E16.5 QPC\textsuperscript{f/f}; Prox1CreERT2 embryos, in which Cre was induced by tamoxifen injections at E13.5 and E14.5 (fig. S1L). In addition, Prox1 drives Cre activity to early LEC progenitors inside

![Fig. 1. QPC\textsuperscript{f/f}; PdpnGFPCre embryos show edema and lack LECs. (A and B) E14.5 QPC\textsuperscript{f/f}; PdpnGFPCre mutant embryos exhibit severe dermal edema (white arrow) (n = 5). (C and D) Whole-mount immunostaining of the dermal lymphatics of those embryos using Prox1 and VEcad antibodies (n = 4) shows that lymphatic vessels (red) are missing from the skin of the mutant embryos. Whole-mount immunostaining of control and mutant E16.5 mesenteric (E and F) and cardiac (dorsal view) (G and H) lymphatics (n = 4) revealed that lymphatics were also absent in these organs. Dermal lymphatic vessel length (I), blood vessel density (J), mesenteric lymphatic vessel length (K), and cardiac lymphatic vessel length (L) are quantified by ImageJ and shown as a percentage of the mean of total vessel length in the control group. All results are presented as means ± SEM and analyzed with a two-tailed Student’s t test. Scale bars, 2 mm (A and B), 200 μm (C and D), 75 μm (E and F), and 500 μm (G and H).]
the CV; instead, as shown by green fluorescent protein (GFP) staining, PdpmGFPCre expression initiates later on in differentiating LECs outside the veins (fig. S2). Accordingly, as BECs and LECs have different transcriptional, epigenetic, and metabolic profiles (10, 17–19), it is likely that LEC progenitors inside the veins still retain much of their original underlying BEC metabolic profile; therefore, their mitochondrial respiration requirements are likely different than those from LECs outside the CV. However, once those LEC progenitors bud off outside the CV and start migrating, a consequence of their de novo expression of additional LEC markers (e.g., Pdpm), they differentiate further, acquiring a more definitive LEC fate. As LEC differentiation and migration progress, it is likely that their metabolic status changes accordingly such that, around mid-gestation, QPC becomes necessary for LEC fate maintenance. Hence, given the severity of the QPC<sup>−/−</sup>;PdpmGFPCre phenotype, for the rest of this study, we only used this mutant strain.

**LEC fate specification and migration take place in early-stage QPC<sup>−/−</sup>;PdpmGFPCre embryos**

To identify the mechanisms leading to the lack of LECs in QPC mutants, we examined earlier developmental stages. Immunostaining of the jugular region in E11.5 mutant embryos identified Prox1-expressing LECs budding from the CV into the surrounding mesenchyme (fig. S3, A to D), a result indicating that initial LEC fate specification and migration take place in mitochondrial respiration–deficient LECs. Nevertheless, quantification of the Prox1<sup>+</sup> LECs outside the CV revealed that their numbers are reduced in E11.5 QPC mutant embryos (fig. S3I). To evaluate whether this reduction is a consequence of reduced proliferation and/or increased apoptosis, we performed Ki67 and TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) analysis. The number of proliferating Ki67<sup>+</sup> Prox1<sup>+</sup> LECs is reduced in the mutant embryos at E11.5 and E12.5 (fig. S3, C, D, G, H, and J); however, TUNEL<sup>+</sup> Prox1<sup>+</sup> LECs are rarely observed in either control or mutant embryos (fig. S3, A, B, E, and F). We validated that the proliferating LECs were QPC nulls by costaining against the GFP reporter included into the PdpmGFPCre strain. As shown in fig. S4, A to H, the Ki67<sup>+</sup> Prox1<sup>+</sup> LECs were also GFP<sup>+</sup>, confirming that they are QPC null LECs. Their numbers were reduced when compared to controls (fig. S4). These data suggest that the lack of mitochondrial activity in LECs does not promote cell death, but similar to BECs (14), its activity is required for LEC proliferation.

**QPC deficiency down-regulates Vegfr3 expression resulting in loss of LEC fate**

Next, we aimed to identify the causes leading to the absence of LECs at mid-gestation. Prox1 expression level appears normal in migrating mutant LECs at E11.5, yet, unexpectedly, Vegfr3 expression level is severely reduced at this stage (Fig. 2, A, B, and K, and fig. S4, I to P). The expression of other typical LEC markers such as Pdpn and Lyve1 is also strongly reduced (Fig. 2, C to F and K). The severity in the down-regulation of those markers in the mutant LECs outside the CV varies among LECs, a result indicating that, at this stage, down-regulation in the expression of those LEC genes is an ongoing process (Fig. 2, A to F). We confirmed that those Prox1<sup>+</sup> LECs showing reduced levels of Vegfr3 are null for QPC according to their GFP<sup>+</sup> expression (fig. S4, I to P). Expression of the pan-EC markers CD31 and VEcad is normal in the mutant LECs (Fig. 2, G to J and L). Expression of the LEC markers Vegfr3, Lyve1, Pdpn, and Nrp2 is barely detected in the lymph sacs of E12.5 mutant embryos, whereas that of Proxl, although reduced, is still observed (Fig. 3, A to K) and that of CD31 appears normal (Fig. 3, J and K). In addition, the Proxl-expressing jugular lymph sac is markedly enlarged in the mutant embryos (Fig. 3, A to J and L), and this enlargement is likely a consequence of LEC migration defects. In wild-type (WT) embryos, at around this stage, LECs start to sprout from the lymph sacs to form an interconnected dermal lymphatic vascular network (Fig. 3E, E). By contrast, mutant LECs appear to be trapped in the lymph sacs failing to reach distant tissues and organs such as the dermis (Fig. 3F). This defect in LEC migration is likely a consequence of their loss of Vegfr3 expression required for LECs to migrate into the surrounding tissues, a process starting around E12.5 (Fig. 3B). The presence of lymph sacs in the mutant embryos confirms that Vegfr3 expression was not completely shut off in early budding mutant LECs (around E10.5 to E12.5), as lymph sac formation is mediated by Vegfr3-Vegfc signaling promoting LEC migration.

Because stable LEC identity requires constant Proxl expression (20), and Vegfr3 is required to maintain Proxl expression through a feedback loop (4), we reasoned that down-regulation of Vegfr3 in QPC mutant LECs will eventually affect Proxl expression levels leading to an abnormal stepwise LEC to BEC fate dedifferentiation process. It is worth noticing that in WT embryos, at around E11.5, the levels of Vegfr3 expression are generally higher in budding LECs outside the CV than in LEC progenitors inside the CV (Figs. 2A and 4A and fig. S5, A and B). At around E13.5 to 14.5 and as the process of LEC differentiation progresses and LECs migrate further away from the CV, their Vegfr3 levels get gradually reduced when compared with those at earlier stages (fig. S5, C to G). It was previously reported that in mice, dedifferentiating LECs abnormally express BEC markers (e.g., CD34 and endomucin) (20, 21). In WT E11.5 and E12.5 embryos, CD34 is not detected in LECs (Fig. 4, A and C). Expression of CD34 is also not detected in conditional null LECs at E11.5 (Fig. 4B); however, starting at around E12.5, once expression of Vegfr3 starts to get down-regulated, some Proxl<sup>(low)</sup> Vegfr3–QPC-null LECs begin to abnormally coexpress the BEC markers CD34 (Fig. 4, D and E) and endomucin (fig. S6, A to C). The panendothelial nuclear marker ERGI is normally coexpressed with Proxl in LECs, as seen in WT E12.5 lymph sacs (fig. S6D and S6F). In QPC-null littersmates, expression of Proxl is lost in some of the ERGI<sup>+</sup> ECs in the lymph sacs (fig. S6E and S6G). As shown in fig. S6H, there is no difference in the number of ERGI<sup>+</sup> versus Proxl<sup>+</sup> LECs on the lymph sacs of control embryos; however, in mutant littersmates, some ERGI<sup>+</sup> ECs are negative for Proxl. This result argues that as mutant LECs start to also down-regulate Proxl, they start to revert their phenotype toward their original BEC fate. To further validate the loss of LEC fate, we also performed lineage tracing analysis using QPC<sup>+/−</sup>;RosamTmG;ProxlCreERT2 mice. Pregnant females were injected with a low dosage of tamoxifen at E9.5 to lineage trace the QPC null LECs at E12.5. As expected, in WT embryos, the few GFP<sup>+</sup> cells all colocalize with Proxl; however, in mutant littersmates, some of the GFP<sup>+</sup> cells are negative for Proxl, confirming their loss of LEC fate (Fig. 5, A to C). Although we did not detect an increase in LEC death in QPC mutant embryos at early stages (fig. S3, A, B, E, and F), concomitant with Proxl down-regulation and the loss of LEC fate at later stages, colocalization of Proxl and active caspase 3 in mutant LECs increased at E14.5 (fig. S7). Together, these data indicate that as LECs bud off from the CV and fully differentiate, mitochondrial complex III deficiency is
responsible for the down-regulation in the expression of LEC markers, particularly that of Vegfr3. This eventually leads to the arrest in LEC migration from the lymph sacs to the different organs and tissues, loss of LEC fate, and increase in LEC apoptosis such that, by mid-gestation, mutant embryos are mostly devoid of a lymphatic vasculature.

**LEC proliferation and migration in vitro require QPC activity**

Because QPC conditional mutant embryos lack LECs, we used an alternative in vitro assay to better characterize the observed in vivo phenotypes. We treated human LECs with the mitochondrial complex III inhibitor antimycin A, a drug known to reduce the oxygen consumption rate (OCR); however, cells can still generate ATP by glycolysis. As expected, in antimycin A–treated LECs, basal and maximal OCR is inhibited and maximal glycolysis is used (fig. S8, A to C). In addition, blockage of mitochondrial electron transport results in a decreased NAD⁺ (nicotinamide adenine dinucleotide)/NADH (reduced form of nicotinamide adenine dinucleotide) ratio as previously reported (fig. S8D) (14, 15) but does not affect mitochondrial content as quantified by fluorescence intensity using Mitotracker Green (fig. S8, E to G). In agreement with the reduced LEC proliferation observed in vivo, cell numbers are significantly reduced in LEC cultures treated with antimycin A for 24 and 48 hours (fig. S8, H and I). This reduction is a direct consequence of decreased cell proliferation as measured by phospho–histone H3 staining (fig. S8, H to J), whereas no changes in the number of TUNEL-positive cells are observed (fig. S8, H and K). This effect of mitochondrial respiration on LEC proliferation is similar to what

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**Fig. 2. Expression of LEC markers is reduced in E11.5 QPCff/PdnGFPCre embryos.** (A and B) Transverse sections at the level of the anterior CV show that expression of Vegfr3 is severely reduced in differentiating Prox1-expressing LECs outside the CV. Similarly, other LEC markers such as Pdpn (C and D) and Lyve1 (E and F) are also down-regulated, whereas expression of the pan-endothelial markers VEcad (G and H) and CD31 (I and J) is normal. White box insets correspond to higher magnification of the dotted box region in each panel. Arrows indicate dorsal (D) and lateral (L) orientations. Mean fluorescence intensity of each staining was quantified and shown as percentage of mean of control group in (K) and (L). Each data point represents a biological replicate. All results are presented as means ± SEM and analyzed by multiple t test. Scale bar, 50 μm (n = 6).
was previously reported for BECs (14). Next, to distinguish alterations as a consequence of decreased cell proliferation from other ones resulting from defective mitochondrial respiration, we pretreated LEC cultures with mitomycin C to block proliferation and then treated these arrested cultures with antimycin A or vehicle control. As shown in fig. S9A, mitomycin C treatment efficiently inhibited LEC proliferation. Then, using a trans-well migration assay, we confirmed that LEC migration toward a vascular endothelial growth factor–C (VEGF-C) gradient is also reduced in the respiration-deficient LECs (fig. S9, B to D). In addition, we determined that respiration-deficient LECs migrate more slowly compared to controls in a wound scratch migration assay (fig. S9, E to I). Together, these results argue that mitochondrial respiration is required for LEC proliferation and migration in vitro.

**Mitochondrial complex III inhibition also down-regulates Vegfr3 expression in vitro**

To determine whether the lack of mitochondrial respiration also affects LEC fate in vitro, LECs were treated with antimycin A and expression of the lymphatic markers Prox1, Vegfr3, and Nrp2 and the pan-EC marker VEcad was analyzed by quantitative polymerase chain reaction (qPCR). As shown in Fig. 6A, expression of those LEC markers

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**Fig. 3. QPC<sup>Cre</sup>/PdpnGFPCre mutant embryos show severely enlarged lymph sacs.** (A to J) At E12.5, expression of Prox1 still remains in the mutant embryos; however, expression of most other LEC markers is barely detected or is severely down-regulated. Expression of the pan-endothelial marker CD31 seems normal. White arrows in control embryo (E) point to the dermal lymphatic vasculature; mutant LECs fail to sprout from the lymph sacs such that lymph sacs (LS) become abnormally enlarged and mutant embryos lack dermal lymphatics. White box insets correspond to higher magnifications of the regions inside the dotted boxes in each panel. Mean fluorescence intensity of each staining was quantified and shown as percentage of mean of control group in (K). Each data point represents a biological replicate. Area of the CV and lymph sac was quantified and shown as fold change of lymph sac area compared to CV in each genotype (L). All results are presented as means ± SEM and analyzed by multiple t test for (K) and two-tailed Student’s t test for (L). Scale bar, 50 μm (n = 3 to 8).
is reduced in the antimycin A–treated LECs, while that of VECad is increased. These results were validated further using myoxothiazol, another mitochondrial complex III inhibitor (Fig. 6B) (22). These qPCR results are also confirmed at the protein level using Western blotting analysis (Fig. 6, C and D). Similarly, short hairpin RNA (shRNA) knockdown of QPC also reduces Vegfr3 and Prox1, but not VECad, protein levels (fig. S10, A and B). Previous studies using cultured human LECs showed that Nrp1 and ICAM1 are up-regulated when LEC fate is lost upon Prox1 knockdown (19, 23, 24). We found that antimycin A or myoxothiazol treatment of cultured LECs increases Nrp1 and ICAM1 mRNA levels; however, those of CD34 are reduced in LECs treated with antimycin A (fig. S11, A and B). These results suggest that there are some environmental (in vivo/cultured) or species (human/mice) differences among LECs in their response to those assays. To evaluate such possibility, we performed RNA sequencing (RNA-seq) using LECs treated with vehicle or antimycin A and analyzed differences among previously reported BEC and LEC markers (25). We found that nearly all LEC genes included in that dataset were down-regulated, and half of the BEC genes were up-regulated, including Nrp1 and ICAM1 (Fig. 6, E and F). To further evaluate the specificity of mitochondrial complex III inhibitors on mitochondrial respiration in the observed phenotypes, we expressed the Ciona intestinalis alternative oxidase (AOX) in LECs. This enzyme is capable of accepting electrons, therefore bypassing complex III and IV functions and restoring normal electron transport chain activity in the presence of complex III inhibition (14, 26–28). To do this, LECs were transduced with lentiviral particles containing AOX-GFP or with an empty GFP vector control and GFP+ LECs were enriched by FACS. We found that antimycin A diminishes OCR and the NAD+/NADH ratio in cells transduced with the empty vector (EV), while AOX expression restores normal OCR and NAD+/NADH ratio in antimycin A–treated LECs (Fig. 7, A and B). Furthermore, AOX restores proliferative capacity (Fig. 7C) and the expression levels of Vegfr3, Prox1, and Nrp2 (Fig. 7D) in antimycin A–treated LECs. Together, these data demonstrate the specificity of antimycin A treatment, and that its effects on Vegfr3 expression are a consequence of complex III inhibition.

**QPC-deficient LECs have reduced nucleotide synthesis**

Mitochondrial complex I oxidizes NADH to NAD⁺ and passes two electrons to ubiquinone within the mitochondrial respiratory chain. Blockage of electron transfer at mitochondrial complex III impairs the cells’ capacity to regenerate NAD⁺ and decreases TCA cycle flux (14, 15, 26). The TCA cycle metabolites succinate, fumarate, and 2-hydroxylglutarate (2HG) competitively inhibit α-ketoglutarate (α-KG)–dependent dioxygenases, such as the Jumonji C domain–containing histone lysine demethylases (JmJC) and the ten-eleven translocation (TET) family of 5-methylcytosine hydroxylases (29). Accumulation of these metabolites in mitochondrial respiration–deficient hematopoietic stem cells impairs their differentiation, a phenotype associated with hypermethylation of DNA and histones (30). In addition, the TCA cycle intermediate acetyl-CoA is the acetyl donor in histone acetyltransferase reactions. Loss of mitochondrial respiratory functions leads to hypoacetylation of histone 3 markers in cancer cells (26). Acetyl-CoA derived from FAO and ketone body oxidation is important for lymphangiogenic gene regulation and LEC proliferation (10, 13). Therefore, we performed metabolic profiling of antimycin A– or control vehicle–treated LECs. We found that respiration-deficient LECs exhibit reduced citrate, fumarate, and malate levels, which is consistent with metabolic profiling.  

**Fig. 4. QPC-deficient LECs gradually up-regulate the expression of BEC markers.** (A and B) At E11.5, QPC-f/f;PdpnGFPCre LECs show severely reduced Vegfr3 levels, but similar to controls, Prox1+ LECs do not coexpress BEC markers such as CD34 (n = 3). (C and D) At around E12.5, Vegfr3 expression becomes undetectable in mutant LECs and Prox1+ (low) LECs abnormally up-regulate the expression of CD34, an indication that they are losing LEC fate and regaining BEC fate. White arrows indicate Prox1+ CD34+ LECs. Blood cells are abnormally seen inside the mutant lymph sacs (D), most likely a consequence of defective lympho-venous valves. White box insets correspond to higher magnification of the regions in dotted boxes in each panel. CD34+ Prox1+ cells were quantified and shown as percentage of Prox1+ in each group (E). Each data point represents a biological replicate. All results are presented as means ± SEM and analyzed by two-tailed Student’s t test. Scale bar, 50 μm (n = 6).
but the succinate (Fig. 8A) and 2HG levels are increased (Fig. 8B). These changes could potentially influence histone methylation and acetylation. Next, we treated LECs with cell-permeable 1 mM dimethyl succinate or octyl-L2HG and found that octyl-L2HG, but not dimethyl succinate, reduces Prox1 and Vegfr3 mRNA and protein levels (fig. S12, A to F). We also treated the cultured LECs with 1 mM dimethyl \( \alpha \)-KG with and without antimycin A. Although the Vegfr3 and Prox1 levels increased in the presence of antimycin A, the effect was not statistically significant (fig. S12, G and H).

We also found that the levels of most amino acids are not significantly changed after antimycin A treatment (fig. S13A). Glutamine metabolism in human umbilical vein endothelial cells (HUVECs) fuels the TCA cycle of ECs to support EC proliferation (9). Accordingly, we noticed that glutamate levels are diminished after antimycin A treatment of LECs (fig. S13A), a result that might be associated with the observed decrease in cell proliferation. We also found a general reduction in nucleotides (fig. S13, B and C). These data suggest that, in LECs, mitochondrial complex III mainly supports nucleotide synthesis.

Mitochondrial complex III is required for H3K4me3 and H3K27ac function in the Vegfr3 loci

Our results suggest that the reduced levels of Vegfr3 in mutant LECs could be a consequence of alterations in metabolites associated to epigenetic modifications. Therefore, we first performed quantitative proteomics for a panel of histone posttranslational modifications in LECs treated with antimycin A or vehicle control. Our results indicated that most histone modifications in LECs were unchanged (fig. S14, A and B). We found minimal alterations in H3K79, H4K8, H4K12, and H4K16 acetylation and in H3K36me3 and H1K25me1 methylation after antimycin A treatment (fig. S14, C to X). This suggests that the lack of QPC does not cause broad alterations in histone modifications in LECs. Next, to evaluate possible local chromatin changes at selected LEC (Vegfr3 and Prox1) and BEC genes (Nrp1 and ICAM1), we performed chromatin immunoprecipitation sequencing (ChIP-seq) for H3K27ac, H3K4me3, and H3K4me1, all histone modifications associated with active gene expression. When using LECs, we found a marked reduction in the H3K4me3 and H3K27ac peaks nearby the Vegfr3 loci, as well as a reduction in

Fig. 5. LEC fate is lost in QPC null embryos. (A to B'') Lineage tracing analysis using Prox1CreERT2 mice crossed to QPC\(^{+/-}\);RosamTmG. A low dose of tamoxifen is injected at E9.5, and embryos are harvested at E12.5. As expected, in QPC\(^{+/-}\) embryos, few GFP\(^{+}\) cells colocalize with Prox1 (A to A''); however, in QPC\(^{+-}\) mutant littermates, some of the GFP\(^{+}\) cells were negative for Prox1 (arrows), confirming their loss of LEC fate (B to B''). GFP\(^{+}\) Prox1\(^{-}\) cells were quantified and shown as percentage of GFP\(^{+}\) cells in each group (C). Each data point represents a biological replicate. All results are presented as means ± SEM and analyzed by two-tailed Student's t test. Scale bar, 50 \( \mu \)m (n = 3).
H3K4me3 in the Prox1 promoter region (Fig. 8C). It was previously shown that Prox1 regulates LEC fate by increasing expression of LEC genes and repressing that of BEC genes such as Nrp1 and ICAM1 (19, 23, 24). Accordingly, we also detected an increase in H3K27ac in the Nrp1 and ICAM1 loci after antimycin A treatment (Fig. 8C). To determine whether those histone landscapes were relevant, we next performed the same ChIP-seq analysis using LECs and BECs and compared the same genomic regions. We found that H3K27ac and H3K4me3 peaks were elevated in the Prox1 and Vegfr3 loci in LECs compared to BECs; instead, H3K27ac peaks were reduced in the Nrp1 and ICAM1 loci in LECs compared to BECs (Fig. 8D). Therefore, we believe that those identified histone modifications are likely responsible for maintaining LEC gene expression and LEC fate, and blockage of mitochondrial respiration alters the histone landscape leading to the dedifferentiation of LECs into BECs.

Fig. 6. Mitochondrial complex III respiration is required for Vegfr3 and Prox1 expression maintenance in cultured LECs. (A) Antimycin A (Anti) treatment reduces Prox1 (n = 9), Vegfr3 (n = 9), and Nrp2 (n = 5) mRNA levels but not those of VECad (n = 3). (B) Myxothiazol (Myx) treatment also reduces Prox1 (n = 4), Vegfr3 (n = 6), and Nrp2 (n = 3) mRNA levels but not those of VECad (n = 3). (C) Representative Western blot shows that antimycin A and myxothiazol treatment reduces VEGFR3 (n = 6) and PROX1 (n = 6) levels but not those of VECAD (n = 5 to 6). (D) Quantification of the densitometry of each protein normalized to GAPDH and shown as fold change compared to controls. Each data point represents a biological replicate. All results are presented as means ± SEM and analyzed by multiple t test in (A) and (B) or two-way analysis of variance (ANOVA) in (D). (E and F) RNA-seq of LECs treated with vehicle or antimycin A shows that expression of most LEC genes in the dataset is reduced, while almost half of the BEC genes are up-regulated, including Nrp1 and ICAM1 (n = 3).
DISCUSSION

A critical step in developmental lymphangiogenesis is how to become and remain a LEC. Recent work provided valuable clues about key players and processes regulating LEC fate specification (31–33). LEC specification is a stepwise process, where initiation of Prox1 expression is the critical event that converts venous ECs into LEC progenitors in the CV (1). As embryonic development progresses, LEC progenitors bud off from the CV and express additional lymphatic markers such that their balance between BEC and LEC fates becomes more biased toward that of LECs. Then, at ~E13.5, the newly specified LEC fate is on its way to differentiate further in those LECs outside the CV (32). LEC fate is plastic, as ablating Prox1 after LEC specification promotes a dedifferentiation reprogramming cascade that causes cells to reacquire their original BEC fate (20). Another critical step is the regulation of the number of LEC progenitors and budding LECs produced during early lymphangiogenesis. Although it is known that Prox1 and the Vegf-c/Vegfr3 feedback signaling loop are critical to maintain the identity and the number of LEC progenitors (4), there is still a gap in our understanding regarding the instructive cues that cause progenitor cells to commit to becoming and remaining LECs.

Our results indicate that mitochondria, a dominant regulator of cellular metabolism, are key players in the process of developmental lymphangiogenesis by controlling and maintaining an active Prox1-Vegfr3 feedback loop during early embryonic stages (around E9.5 to E13.5). Around mid-gestation, changes in the metabolic status of differentiating LECs (e.g., changes in FAO levels) are likely responsible for the down-regulation of Vegfr3 expression and eventually for disrupting the Prox1-Vegfr3 feedback loop such that the generation and budding of LEC progenitors eventually stops. Supporting this proposal, it was recently shown that Vegfr3 inhibition decreased FAO in LECs but not in BECs (10). Sensing when to end the process of LEC budding is likely very important, as LEC progenitors inside the CV barely proliferate, and their constant budding could compromise the CV physical integrity due to the reduction in the number of ECs in the wall of the CV.

Recent studies added some clues as to how metabolism regulates lymphangiogenesis (Fig. 9) (7, 10, 34). Results from our work now provide a comprehensive characterization of the developmental defects leading to the total lack of LECs in QPC conditional null embryos and reveal how mitochondrial respiration participates in that process. Similar to what was previously shown for BECs (14), mitochondrial complex III is also necessary for LEC proliferation. However, functional impairment of mitochondrial complex III activity in vivo and in vitro also leads to the reduction in the expression of LEC markers through the epigenetic regulation of Vegfr3 and Prox1. This down-regulation eventually disrupts the critical Prox1-Vegfr3 autoregulatory feedback loop required to promote and maintain LEC fate specification and maintenance (4). Our results provide a better understanding of how, by directly controlling nucleotide synthesis and the epigenetic modification of Vegfr3, mitochondrial complex III function is a critical player in developmental lymphangiogenesis.

We argue that by sensing the cellular metabolic status, mitochondrial complex III is able to integrate all key players, such as CPT1α, to properly maintain or shut off the Prox1-Vegfr3 autoregulatory feedback loop required to promote and maintain LEC fate.
and regulate the number of budding LECs. It is likely that during normal development, at around E14.5, certain changes in the local microenvironment of migrating LECs lead to changes in their metabolic status. This, in turn, results in the epigenetic silencing of Vegfr3, the shutoff of the Prox1-Vegfr3 feedback loop, and the stop in the generation and budding of additional LEC progenitors from the CV.

In QPC mutant embryos, those metabolic changes leading to the down-regulation of Prox1 will promote a LEC to BEC fate transition, cell death, and lack of LECs (Fig. 9). It was recently shown that different to BECs, LECs do not exhibit organ-specific transcriptional signatures (35). However, according to our results, it could be argued that LECs are heterogeneous in their metabolic profiling depending on their developmental stage and tissue and organ microenvironment. In turn, those differences will affect the transcriptional levels of some key gene regulators in the different organs. This is likely to be a common feature for most differentiating cell types.

Fig. 8. Mitochondrial respiration is required for epigenetic regulation of Vegfr3 and Prox1. Mitochondrial complex III inhibition with antimycin A alters TCA cycle metabolites (A) and 2HG levels (B). Values are normalized to mean of control group. (C) ChIP-seq analysis of H3K27ac, H3K4me3, and H3K4me1 histone modifications in LEC cultures treated with vehicle control (Ctrl) or antimycin A (Anti) for 48 hours. Track examples for LEC-specific genes (Prox1 and Vegfr3) and BEC genes (Nrp1 and ICAM1) are shown. Antimycin A–treated LECs show a marked reduction in the H3K4me3 and H3K27ac signal at the Vegfr3 locus and a reduction in H3K4me3 in the Prox1 locus, while H3K27ac peaks are increased in the Nrp1 and ICAM1 locus. (D) ChIP-seq analysis of the same modifications in (C) in LECs and BECs reveals that H3K27ac and H3K4me3 peaks are elevated in the Prox1 and Vegfr3 loci in LECs, whereas those of Nrp1 and ICAM1 are reduced in LECs and BECs. Scale bar, 5 kb. Each data point represents a biological replicate. All results are presented as means ± SEM and analyzed by multiple t test or two-tailed Student’s t test.
We found that multiple metabolites and metabolic pathways are changed in respiration-deficient LECs. For example, the levels of succinate and 2HG are increased in the mitochondrial respiration–deficient LECs (Fig. 8, A and B). Succinate and 2HG are competitive inhibitors of α-KG–dependent dioxygenases and are linked to hypermethylation of DNA and histones in mitochondrial complex III null hematopoietic stem cells (30). Furthermore, we found that L2HG, but not succinate, is sufficient to reduce Prox1 and Vegfr3 expression levels in cultured LECs (fig. S12, A to F) and that Vegfr3 and Prox1 levels increased when supplemented with α-KG in the presence of antimycin A, but this effect was not statistically significant (fig. S12, G and H). These results argue that L2HG could influence LEC fate through epigenetic regulation. It was shown that BECs and LECs have different DNA and histone methylation profiles, and
treatment with epigenetic modifying drugs promotes the expression of BEC markers in LECs (17, 18). Furthermore, BECs and LECs show different H3K4me3 and H3K27me3 landmarks such that multiple BEC genes show repressive histone marks in LECs (18). Similar to what we observed, H3K4me3 was shown to be enriched in the Prox1 and Vegfr3 promoters in LECs (18). Collectively, these data support the idea that epigenetics regulates LEC’s plasticity and fate. In addition, acetyl-CoA, which can derive from citrate, is the substrate of histone acetylation, while NAD+ is the substrate for deacetylation. A potential explanation for the reduced levels of Prox1 and Vegfr3 in QPC-deficient LECs is hypoaoyctilation, a consequence of reduced citrate generated in the mitochondria. Accordingly, other studies suggested that the epigenetic changes caused by metabolites, especially acetyl-CoA, are locus specific and reversible (10, 28). It was recently shown that ketone-rich diet and supplementary acetate promote lymphatic vessel growth in injury-induced cornea and tail wound lymphangiogenesis (10, 13), further highlighting the importance of acetylation as a mechanism to control LEC function and identity.

In summary, we propose that the mitochondria-regulated Prox1-Vegfr3 feedback loop is a simple but necessary sensing mechanism of the metabolic microenvironment required to maintain LEC fate and to regulate the number of generated LEC progenitors and, subsequently, the number of budding LECs (4). Given the roles of Vegfr3 in lymphangiogenesis in diseases, targeting mitochondria respiration could potentially offer new treatment strategies.

MATERIALS AND METHODS

Mice
RosamTmG mice were purchased from The Jackson Laboratory (36); Prox1CreERT2 (37) and PdpnGFPCre (16) mice have previously been described and are maintained in the NMRI background. The PdpnGFPCre strain was originally generated using part of the 5’ regulatory region of the mouse Pdpn gene fused to an eGFP:Cre cassette as previously described (16). QPCα/β mice were previously reported and are maintained on a C57BL/6 background (14, 15). Tamoxifen stock was prepared by dissolving tamoxifen (Sigma-Aldrich) in 95% ethanol and 5% acetic acid at a concentration of 20 mg/mL. The following secondary antibodies were used: Alexa 488–conjugated donkey anti-mouse (A-21208, Invitrogen), Alexa 488–conjugated donkey anti-rabbit (A-21206, Invitrogen), Alexa 488–conjugated donkey anti-goat (A-11055, Invitrogen), Alexa 568–conjugated donkey anti-rabbit (A-11056, Invitrogen), Alexa 568–conjugated donkey anti-goat (A-11055, Invitrogen), Alexa 488–conjugated donkey anti-rabbit (A-21208, Invitrogen), goat anti–phospho–histone H3 (06-570, Upstate), rabbit anti–histone H3 (ab8580, Abcam), and rabbit anti-H3K27ac (9649S, Cell Signaling). The following primary antibodies were used: rabbit anti-Prox1 (11002, AngioBio), goat anti-Prox1 (AF2727, R&D Systems), goat anti-Vegfr3 (AF743, R&D Systems), goat anti-PDpn (AF3244, R&D Systems), goat anti-Lyve1 (AF2125, R&D Systems), goat anti–NRP2 (AF567, R&D Systems), rat anti-CD31 (553370, BD Pharmingen), rat anti-CD34 (09431D, BD Pharmingen), rabbit anti–VE-cad (ab33168, Abcam), rabbit anti–QPC (10756-1-AP, Proteintech), rabbit anti–Ki67 (MA5-14520, Invitrogen), rabbit anti–active caspase 3 (9664, Cell Signaling), rabbit anti–ERG1 (ab133695, Abcam), rat anti–ENDOMUCIN (14-5851-82, Ebioscience), rabbit anti–GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (sc-32232, Santa Cruz Biotechnology), mouse anti–β–ACTIN (A5316, Sigma-Aldrich), rabbit anti–phospho–histone H3 (06-570, Upstate), rabbit anti–H3K4me1 (8895, Abcam), rabbit anti–H3K4me3 (ab8580, Abcam), and rabbit anti–H3K27ac (9649S, Cell Signaling). The following secondary antibodies were used: Alexa 488–conjugated donkey anti-rabbit (A-21206, Invitrogen), Alexa 488–conjugated donkey anti-rabbit (A-21208, Invitrogen), Cy3-conjugated donkey anti-rabbit (711-165-151, Jackson ImmunoResearch), Cy3-conjugated donkey anti-mouse (715-165-151, Jackson ImmunoResearch), Cy3-conjugated donkey anti-goat (705-165-147, Jackson ImmunoResearch), Cy5-conjugated donkey anti-rabbit (711-165-152, Jackson ImmunoResearch), Cy5-conjugated donkey anti-mouse (715-165-151, Jackson ImmunoResearch), Cy3-conjugated donkey anti-goat (705-165-147, Jackson ImmunoResearch), Cy5-conjugated donkey anti-goat (705-495-147, Jackson ImmunoResearch), Cy5-conjugated donkey anti-rabbit (711-495-152, Jackson ImmunoResearch), horseradish peroxidase (HRP) anti-mouse (32430, Thermo Fisher Scientific), HRP anti-rabbit (31460, Thermo Fisher Scientific), and HRP anti-goat (705-035-147, Jackson ImmunoResearch). TUNEL staining was performed in sections using the In Situ Cell Death Detection Kit, Fluorescein (Sigma-Aldrich).

Immunofluorescent staining
Cryosections
Embryos were washed with ice-cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) at 4°C overnight and then dehydrated in 30% sucrose and embedded in Tissue-Tek O.C.T (Sakura). Cryosections were washed with PBS and blocked with blocking buffer (5% normal donkey serum, 1% bovine serum albumin, 0.1% Triton X-100, and 0.05% sodium azide) for 1 hour at room temperature. Primary antibodies were incubated at 4°C overnight. Secondary antibodies were incubated for 2 hours at room temperature. Slides were washed with TBST (tris-buffered saline + 1% Tween 20) and mounted using antifade-mounting medium (Vector Laboratories, Burlingame, CA).

Whole-mount staining
Tissues were dissected and fixed with 4% PFA and blocked with blocking buffer (5% normal donkey serum, 1% bovine serum albumin, 0.1% Triton X-100, and 0.05% sodium azide) for 1 hour at room temperature, followed by overnight primary antibody incubation at 4°C on a rotator. The next day, tissues were extensively washed three times with 0.1% Triton X-100–PBS for 45 min at room temperature and incubated in the corresponding secondary antibodies for 2 hours at room temperature. Samples were washed again with 0.1% Triton X-100–PBS and mounted using a Vectashield antifade-mounting medium (Vector Laboratories, Burlingame, CA). Images were acquired with a Zeiss A2 imager and a Leica TCS SPE DM2500 confocal microscope. For quantification of fluorescence intensity among images, the same laser intensity and exposure settings were used, images were acquired the same day, and the mean fluorescence intensity of the selected areas was measured using ImageJ. For quantification, multiple images were acquired per biological sample, and mean values were quantified for each image. Representative images were selected to reflect the mean value in the quantitative data. The numbers of samples in each group and the statistical analysis are listed in the individual figure legends.

Antibodies
The following primary antibodies were used: rabbit anti-Prox1 (11002, AngioBio), goat anti-Prox1 (AF2727, R&D Systems), goat anti-Vegfr3 (AF743, R&D Systems), goat anti-PDpn (AF3244, R&D Systems), goat anti-Lyve1 (AF2125, R&D Systems), goat anti–NRP2 (AF567, R&D Systems), rat anti-CD31 (553370, BD Pharmingen), rat anti-CD34 (09431D, BD Pharmingen), rabbit anti–VE-cad (ab33168, Abcam), rabbit anti–QPC (10756-1-AP, Proteintech), rabbit anti–Ki67 (MA5-14520, Invitrogen), rabbit anti–active caspase 3 (9664, Cell Signaling), rabbit anti–ERG1 (ab133695, Abcam), rat anti–ENDOMUCIN (14-5851-82, Ebioscience), rabbit anti–GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (sc-32232, Santa Cruz Biotechnology), mouse anti–β–ACTIN (A5316, Sigma-Aldrich), rabbit anti–phospho–histone H3 (06-570, Upstate), rabbit anti–H3K4me1 (8895, Abcam), rabbit anti–H3K4me3 (ab8580, Abcam), and rabbit anti–H3K27ac (9649S, Cell Signaling). The following secondary antibodies were used: Alexa 488–conjugated donkey anti-rabbit (A-21206, Invitrogen), Alexa 488–conjugated donkey anti-rabbit (A-21208, Invitrogen), Cy3-conjugated donkey anti-rabbit (711-165-152, Jackson ImmunoResearch), Cy3-conjugated donkey anti-mouse (715-165-151, Jackson ImmunoResearch), Cy3-conjugated donkey anti-goat (705-165-147, Jackson ImmunoResearch), Cy5-conjugated donkey anti-rabbit (711-165-147, Jackson ImmunoResearch), Cy5-conjugated donkey anti-goat (705-495-147, Jackson ImmunoResearch), Cy5-conjugated donkey anti-rabbit (711-495-152, Jackson ImmunoResearch), horseradish peroxidase (HRP) anti-mouse (32430, Thermo Fisher Scientific), HRP anti-rabbit (31460, Thermo Fisher Scientific), and HRP anti-goat (705-035-147, Jackson ImmunoResearch). TUNEL staining was performed in sections using the In Situ Cell Death Detection Kit, Fluorescein (Sigma-Aldrich).

Cell culture and drug treatment
Primary human dermal microvascular LECs and HUVECs were purchased from Lonza. LECs were cultured in Endothelial Cell Basal Medium-2 (EBM-2, Lonza) supplemented with Microvascular Endothelial Cell Growth Medium-2 SingleQuots (Lonza). HUVECs were cultured in Endothelial Cell Growth Supplement (Lonza). EGM-2 with supplements (Lonza) as described previously (19, 20).
were cultured in Endothelial Cell Basal Medium-2 (EBM-2, Lonza) supplemented with Endothelial Cell Growth Medium-2 SingleQuots (Lonza). Cells were maintained at 37°C with 5% CO₂. P3 to P5 cells were used for experiments. Cells were treated with vehicle, 25 μM antimycin A (Sigma-Aldrich), 15 μM myxothiazol (Sigma-Aldrich), 1 mM octyl-β-D-Hex (Cayman), 1 mM dimethyl succinate (Sigma-Aldrich), or 1 mM dimethyl α-KG (Sigma-Aldrich) for 48 hours.

**Oxidative and extraacellular acidification rate measurements**

Forty thousand LECs (Lonza) per well were plated onto XF96 cell culture plates in MCDB 131 (US Biological) supplemented with Microvascular Endothelial Cell Growth Medium SingleQuots. Once attached, cells were treated with antimycin A for 2 hours and OCR was measured using an XF96 extracellular flux analyzer (Seahorse Bioscience). After three initial readings, 2.5 μM oligomycin (Sigma-Aldrich) was injected into each well of the plate to determine coupled respiration. Maximal respiration was established after injection of 10 μM carbonyl cyanide m-chlorophenyl hydrazone (Sigma-Aldrich). Then, 2 μM antimycin A and 2 μM rotenone (Sigma-Aldrich) were injected to determine nonmitochondrial respiration. Basal respiration was measured by subtracting the OCR values after treatment with 2 μM antimycin A (Sigma-Aldrich) and 2 μM rotenone (Sigma-Aldrich). To measure extracellular acidification rate (ECAR), cells were treated in MCDB 131 medium without sodium bicarbonate. Basal ECAR was measured by subtracting the ECAR rate after treatment with 20 mM 2-deoxyglucose (Sigma-Aldrich). Maximum ECAR rate was measured by subtracting the rate after 2-deoxyglucose treatment from the rate after treatment with 2.5 μM oligomycin A.

**NAD⁺/NADH ratio**

The NAD⁺/NADH ratio was measured using the NAD/NADH-Glo Assay (Promega) by following the manufacturer’s protocol. The result is read on a BioTek synergy 2 microplate reader.

**Measurement of mitochondrial content**

LECs were plated and treated with antimycin A as described above. To measure mitochondrial content, cells were stained with 100 nM MitoTracker Green (Molecular Probes) in starvation medium for 20 min at 37°C. Images were acquired in an EVOS microscope, and fluorescence intensity was calculated using ImageJ.

**Transwell migration assay**

LECs were treated with mitomycin C (5 μg/ml) for 2 hours before the experiment. Forty thousand LECs per well were seeded in culture medium onto the transwell insert (Millipore) with and without antimycin A. The bottom chamber was filled with medium containing VEGf (100 ng/ml), and cells were incubated in the 24-well plate inserts in a humidified incubator (37°C, 5% CO₂) for 6 hours. The insert was washed in PBS twice and fixed with cold methanol on ice for 20 min; last, the nonmigrated cells on the upper side of the chamber were carefully removed and the migrated cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and counted using ImageJ.

**Cell proliferation and viability assays**

LECs were plated in supplemented EBM-2 medium and allowed to attach, and then the medium was replaced with growth medium with vehicle or antimycin A. Cells were then fixed at the indicated time points and stained with DAPI. Images were acquired in an EVOS microscope, and the nuclei were counted using ImageJ. Viability was assessed at 48 hours after treatment using In Situ Cell Death Detection Kit, Fluorescin (Roche).

**Scratch wound cell migration assay**

Cells were plated onto 96-well Image Lock tissue culture plates (Essen BioScience, 4379) at a density of 30,000 cells per well and allowed to form a monolayer overnight. Cells were then treated with mitomycin C (5 μg/ml) for 2 hours to inhibit proliferation. Monolayers were then wounded using the 96-well WoundMaker (Essen BioScience) following the manufacturer’s protocol. Cells are washed twice with PBS to remove any debris. Medium was then replaced with supplemented EBM-2 with or without drug treatments, and images of wounds were taken every 4 hours until closure.

**Lentiviral constructs**

EV and AOX pWPI GFP lentiviral constructs were transfected into 293T cells using Lipofectamine 2000 transfection reagent (Invitrogen), along with pMD2.G and psPAX2 packaging vectors. Cells were transduced with each lentivirus, and GFP⁺ cells were sorted using FACS Melody Cell Sorter (BD). LECs were infected using pKO.1 control shRNA and QPC shRNA lentiviral particles (Sigma-Aldrich) followed by puromycin selection.

**Polymerase chain reaction**

To measure gene expression, RNA was isolated using the RNaseasy Micro Kit (Qiagen). Complementary DNAs (cDNAs) were synthesized following the Advantage RT-for-PCR protocol (Takara), and reactions were prepared using SYBR Green Master Mix (Thermo Fisher Scientific) and run on an Applied Biosystem 7500 Real-Time PCR machine. The following primers were used: β-actin, 5’-TCACCT-TCCACGACATGTG-3’ (forward) and 5’-GCATTTCGCTGAGAT-3’ (reverse); Gapdh, 5’-GAAGTGAAGGGTCGGAGTCTC-3’ (forward) and 5’-GAAGATGGTGTAGGGGATTTTCC-3’ (reverse); Proxl, 5’-AAAGTCGAATGTACTCGCAAGC-3’ (forward) and 5’-CTGGGAAATTATGTTGGTCTTCT-3’ (reverse); Vegfr3, 5’-GGTTCCTCCAGAGTAGAAC-3’ (forward) and 5’-CAAGCAAGCAGTGCAGTG-3’ (reverse); Nrp2, 5’-GTCTCCTCATA GCCCTAACGGGCA-3’ (forward) and 5’-GGGTCAACCCCTG GATGTCA-3’ (reverse); QPC, 5’-GTGTGGAGCCACACAGTAG TA-3’ (forward) and 5’-GCCACACAGCAATGACTT-3’ (reverse); VEcad, 5’-CTCTGCATCCATCCACCATC-3’ (forward) and 5’-CTCCAGCGAATCTTCTGAT-3’ (reverse); ICAMI, 5’-AGCCGGCTACGAGTGCAGTAG-3’ (forward) and 5’-TCAGGCCTCTGCCTGAGTA-3’ (reverse); CD34, 5’-CCTCATG TCTACCTGCTGTCTC-3’ (forward) and 5’-GGAATAGCTCTGG GCCCTGCA-3’ (reverse); Nrp1, 5’-TTCAGGCGCATCTTTATTTACT-3’ (forward) and 5’-GGAACATCTGCGACCTCTCGTG-3’ (reverse).

**Protein extraction and Western blot**

LECs were washed with cold PBS and lysed with SDS lysis buffer or radioimmunoprecipitation assay buffer (Sigma-Aldrich) containing Complete protease inhibitor cocktail (Millipore). The cell lysate protein extract was quantified using a Pierce Protein assay kit. Samples were mixed with 4× Laemmli sample buffer (Bio-Rad, Hercules, CA) and boiled for min at 95°C. Lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Billerica, MA), and incubated with primary antibodies. After three washes in TBST buffer, membranes were incubated with HRP-conjugated secondary antibodies and visualized using ECL reagents and Image Quant software (GE Healthcare).
antibodies, anti-rabbit immunoglobulin G (IgG) (Sigma-Aldrich), anti-mouse IgG (Jackson ImmunoResearch Laboratories), and anti-goat IgG (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature. Membranes were washed three additional times with TBST. Proteins were detected using the HRP-detecting SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer’s instructions (Thermo Fisher Scientific). Blots were developed using a ChemiDoc MP imaging system (Bio-Rad). Densitometric quantification of bands was done using National Institutes of Health (NIH) ImageJ software.

**Metabolite analysis**

LEC were plated in supplemented EBM-2 medium with or without antimycin A for 48 hours. Then, cells were washed with ice-cold saline twice and collected in 80% methanol at −80°C. The suspension was transferred to cryovials and subjected to three freeze-thaw cycles from liquid nitrogen to a 37°C water bath. Next, samples were centrifuged at 20,000g for 10 min at 4°C. Supernatants were transferred to fresh tubes and dried. The protein concentration in each sample was measured using the BCA kit (Thermo Fisher Scientific). Samples were analyzed by high-performance liquid chromatography and high-resolution mass spectrometry and tandem mass spectrometry (HPLC-MS/MS). Specifically, the system consisted of Thermo Q-Exactive in line with an electrospray source and an ultimate 3000 (Thermo Fisher Scientific) series HPLC consisting of a binary pump, degasser, and autosampler outfitted with an XBridge Amide column (Waters; dimensions of 4.6 mm by 100 mm and a 3.5-μm particle size). Mobile phase A contained 95% (v/v) water, 5% (v/v) acetonitrile, 20 mM ammonium hydroxide, and 20 mM ammonium acetate (pH 9.0); B was 100% acetonitrile. The gradient was as follows: 0 to 1 min, 15% A; 15 min, 76% A; 20.4 to 20.5 min, 15% A; 20.5 to 28 min, 15% A with a flow rate of 400 μl min⁻¹. The capillary of the electron spray ionization source was set to 275°C, with sheath gas at 45 arbitrary units, auxiliary gas at 5 arbitrary units, and the spray voltage at 4.0 kV. In positive/negative polarity switching mode, a mass/charge ratio (m/z) scan range from 70 to 850 was chosen, and MS1 data were collected at a resolution of 70,000. The automatic gain control target was set at 1 × 10⁶, and the maximum injection time was 200 ms. The top five precursor ions were subsequently fragmented in a data-dependent manner, using the higher-energy collisional dissociation cell set to 30% normalized collision energy in MS2 at a resolution power of 17,500. The sample volumes of 10 μl containing 200,000 cells were injected. Data acquisition and analysis were carried out by Xcalibur 4.0 software and TraceFinder 2.1 software, respectively (both from Thermo Fisher Scientific).

**RNA-seq analysis**

LCs were treated with vehicle or antimycin A for 48 hours, and then RNA was extracted using the RNeasy Micro Kit (Qiagen). RNA quality and quantity were measured using Agilent 4200 TapeStation using the High Sensitivity RNA ScreenTape System (Agilent Technologies). TruSeq mRNA-Seq Library Prep was used for library preparation. Libraries were sequenced on a HiSeq 4000 instrument. The analysis was performed at the Northwestern University NUSeq Core facility. The cutoff for determining significantly differentially expressed genes was a false discovery rate (FDR)-adjusted P value less than 0.05 using the Benjamini-Hochberg method.

**ChiP-seq**

In brief, 4 × 10⁷ to 5 × 10⁷ cells were treated with control or antimycin A, crosslinked with 1% formaldehyde for 20 min at room temperature, and quenched by glycine. After washing, fixed chromatin was sonicated with a Covaris focused-ultrasonicator and immunoprecipitated with the indicated antibody. ChiP-seq libraries were prepared with a TruSeq ChIP-Seq Library preparation kit (Illumina) and sequenced on a HiSeq 4000 instrument using the 50-nucleotide single-read configuration at the Northwestern University NUSeq Core facility. Genome browser snapshots were generated using Integrative Genomics Viewer.

**Statistical analysis**

No statistical methods were used to predetermine sample size. The significance of differences in the experimental data was determined using GraphPad Prism 8 software. All data involving statistics are presented as means ± SEM. D’Agostino-Pearson test was used to evaluate normality of data distribution. Student’s t test or one-way analysis of variance (ANOVA) test was used for data with a normal distribution. Differences between two groups were determined by multiple or two-tailed unpaired t test, and differences between multiple groups were calculated using one-way or two-way ANOVA. No corrections for multiple testing were applied. P < 0.05 was determined to be statistically significant.

**SUPPLEMENTARY MATERIALS**

Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/18/eabe7359/DC1

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