LETR1 is a lymphatic endothelial-specific lncRNA governing cell proliferation and migration through KLF4 and SEMA3C

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Recent studies have revealed the importance of long noncoding RNAs (lncRNAs) as tissue-specific regulators of gene expression. There is ample evidence that distinct types of vasculature undergo tight transcriptional control to preserve their structure, identity, and functions. We determine a comprehensive map of lineage-specific lncRNAs in human dermal lymphatic and blood vascular endothelial cells (LECs and BECs), combining RNA-Seq and CAGE-Seq. Subsequent antisense oligonucleotide-knockdown transcriptomic profiling of two LEC- and two BEC-specific lncRNAs identifies LETR1 as a critical gatekeeper of the global LEC transcriptome. Deep RNA-DNA, RNA-protein interaction studies, and phenotype rescue analyses reveal that LETR1 is a nuclear trans-acting lncRNA modulating, via key epigenetic factors, the expression of essential target genes, including KLF4 and SEMA3C, governing the growth and migratory ability of LECs. Together, our study provides several lines of evidence supporting the intriguing concept that every cell type expresses precise lncRNA signatures to control lineage-specific regulatory programs.
The blood and lymphatic vascular systems are essential for the efficient transport of oxygen, nutrients, signaling molecules, and leukocytes to and from peripheral tissues, the removal of waste products, and the preservation of fluid homeostasis. Increased activation or impaired function of these vascular networks represent a hallmark of many pathological conditions, including cancer progression, chronic inflammatory diseases, and diseases leading to blindness1–3.

During development, the blood vascular system arises from endothelial cell progenitors that differentiate from mesodermal cells, mostly through the expression of the transcription factor (TF) ETV2. Activation of the VEGFA/VEGFR2 signaling and expression of blood vascular endothelial cell (BEC) markers, such as NRPI and EphrinB2, further differentiate these precursor cells into BECs, which then form the hierarchical network of blood vessels4. In contrast, lymphatic vasculogenesis starts after the establishment of the blood circulatory system. Thereafter, a distinct subpopulation of endothelial cells lining the cardinal vein starts differentiating by expressing the TF PROX1, the master regulator of lymphatic endothelial cell (LEC) identity, via the TFs SOX18 and COUPTFII. Once exiting the veins, LECs start expressing other lymphatic-specific markers, such as podoplanin, VEGF3, and NRPII, and they migrate, in a vascular endothelial growth factor C (VEGF-C)-dependent manner, to form the primary lymph sacs from which the lymphatic vascular system further develops following sprouting, branching, proliferation, and remodeling processes5. However, a nonvaginal origin of LECs has also been described in the skin, mesenteries, and heart6–8. In adulthood, while the blood and lymphatic vasculature are generally quiescent, they can be readily activated in pathological conditions such as wound healing, inflammation, and cancer by disturbance of the natural balance of pro- and anti-(lymph)angiogenic factors3,9. Therefore, this complex regulatory network requires precise control of gene expression patterns at both transcriptional and post-transcriptional levels in order to ensure proper maturation, differentiation, and formation of blood and lymphatic vessels.

In this scenario, many studies have recently revealed the importance of a new member of the noncoding RNA clade, termed long-noncoding RNAs (lncRNAs), in the regulation of gene activity10,11. In particular, the FANTOM (Functional Annotation of the Mammalian Genome) consortium pioneered the discovery of the noncoding RNA world by providing, through Cap Analysis of Gene Expression (CAGE-Seq), the first evidence that large portions of our genome are transcribed, producing a multitude of sense and antisense transcripts12. In the latest genome annotation, lncRNAs, which are arbitrarily defined as noncoding RNAs longer than 200 nucleotides, constitute ~72% of the transcripted genome12, whereas mRNAs comprise only 19%, indicating the need for functional annotation of lncRNAs. Importantly, lncRNAs have recently been shown to display a higher tissue-specificity than mRNAs, suggesting them as new players in the regulation of cell-type-specific gene expression programs14.

As lncRNAs lack a protein-coding role, their primary categorization is based on their genomic location and orientation relative to protein-coding genes15. lncRNAs can reside either between protein-coding genes (intergenic, lincRNAs), between two exons of the same gene (intronic lncRNAs), antisense to protein-coding transcripts (antisense lncRNAs), or in promoters and enhancers (natural antisense transcripts or transcribed from bidirectional promoters)16–18. lncRNAs may regulate gene expression through a multitude of mechanisms depending on their subcellular localization. For instance, in the nucleus, lncRNAs can act as a scaffold for TFs, chromatin remodeling complexes, or ribonucleoprotein complexes, indicating a potential role in transcriptional regulation19. Nuclear lncRNAs can furthermore act in cis or trans to regulate gene expression by the recruitment of activating and repressive epigenetic modification complexes. cis-acting lncRNAs, such as the 17-kb X chromosome-specific transcript Xist, regulate gene expression of adjacent genes by directly targeting and tethering protein complexes20,21. On the other hand, trans-acting lncRNAs, such as the HOTAIR lncRNA, regulate gene expression at distinct genomic loci across the genome by serving as a scaffold that assists the assembly of unique functional complexes22,23.

In blood vessels, some lncRNAs have been reported to play a role in angiogenesis (MALAT-1, Inc-Ang362)24–26, tumor-induced angiogenesis (MVII, HOTAIR)27,28, and proliferation as well as cell function regulation of endothelial cells (MALAT-1, Tie-1AS)29,30. In contrast, although cancer cell expression of the anti-angiogenic lincRNA MALAT-1, also known as ‘angiostatin’ (MVIH, HOTAIR)26,27, and proliferation-associated lncRNAs, such as the 17-kb X chromosome-specific transcript Xist, regulate gene expression of adjacent or associated genes by directly targeting and tethering protein complexes20,21, lncRNAs associated with vascular diseases, and diseases leading to blindness1–3. In this context, the integration of the international FANTOM6 project, which aims to functionally annotate all lncRNAs present in our genome, we first determined lineage-specific lncRNAs associated with human primary dermal LECs and BECs by combining RNA-Seq and CAGE-Seq analyses. Genome-wide functional interrogation after antisense-oligonucleotide (ASO) knockdown of robustly selected LEC and BEC lncRNAs allowed us to identify LINC01197, which we renamed LETR1 (lymphatic endothelial transcriptional regulatory IncRNA 1), as a lymphatic endothelial-specific lncRNA that functions in the transcriptional regulation of LEC growth and migration. We demonstrated that LETR1 is a trans-acting lncRNA that acts as a protein scaffold in order to facilitate the assembly of unique functional epigenetic complexes involved in gene expression regulation. Through these interactions, LETR1 controls intrinsic transcriptional networks to fine-tune the expression, above all, of essential proliferation- and migration-related genes, including the tumor-suppressor TF KLF4 and the semaphorin guidance molecule SEMA3C.

Results
Identification of a core subset of vascular lineage-specific lncRNAs. To identify vascular lineage-specific lncRNAs, we performed both RNA-Seq and CAGE-Seq31 of total RNA isolated from neonatal human primary dermal LECs and BECs. Before sequencing, the LEC and BEC identity was confirmed by qPCR (Supplementary Figure 1a, b). Compared with RNA-Seq, CAGE-Seq allows mapping transcription start sites (TSSs) after quantification of the expression of 5′-capped RNAs32. To ensure endothelial cell specificity, we included RNA-Seq and CAGE-Seq data from neonatal human primary dermal fibroblasts (DFs)33. In a first step, we performed differential expression (DE) analysis of RNA-Seq and CAGE-Seq of LECs against BECs, LECs against DFs, and BECs against DFs using EdgeR34. From defined LEC- or BEC-specific genes (see Methods section), we selected genes annotated as lncRNAs in the recently published FANTOM CAT database12. Finally, we overlapped the RNA-Seq and CAGE-Seq results to select lncRNAs identified as differentially expressed using both techniques (Fig. 1a). RNA-Seq identified 832 LEC- and 845 BEC-associated lncRNAs, after the exclusion of 232 LEC and 672 BEC lncRNAs also expressed in DFs (Fig. 1b). In contrast, CAGE-Seq identified 277 LEC lncRNAs and 243 BEC lncRNAs, after the removal of 143 BEC and 282 LEC lncRNAs also expressed in DFs (Fig. 1c). The integration of DF data sets led us to determine a large fraction of lncRNAs differentially expressed in the two vascular cell types compared with DFs, suggesting them...
as endothelial-associated lncRNAs (Supplementary Data 1). The overlap between RNA-Seq and CAGE-Seq data sets revealed 142 LEC- and 160 BEC-specific lncRNAs to be reproducibly expressed in either LECs or BECs by both sequencing methods. We defined these subsets as LEC and BEC core lncRNAs (Fig. 1d and Supplementary Data 2). Remarkably, through this approach, we could select more abundant and more differentially expressed LEC- and BEC-associated lncRNAs compared to lncRNAs solely detected by RNA-Seq (Supplementary Figure 2). The overlap between TSSs and DHSs, selection for actively transcribed lncRNAs with overlap with predicted genomic evolutionary rate profiles (GERP), and filtering for expression levels in LEC and BEC RNA-Seq and CAGE-Seq data sets (Fig. 2a) led to the identification of 5 LEC and 12 BEC lncRNAs that are potentially conserved at the sequence level, actively transcribed, and robustly expressed in the respective endothelial cell types (Fig. 2b, c). Finally, we identified through qPCR 2 LEC (AL583785.1 and LETR1) and 2 BEC (LINC00973 and LINC00973) lncRNAs that were consistently differentially expressed between LECs and BECs derived from newborn and adult skin samples (Fig. 2d, e). We next analyzed the expression levels of the four lncRNA candidates and specific blood and lymphatic markers in freshly isolated LECs and BECs from human healthy skin biopsies, using flow cytometry followed by qPCR (Fig. 2f). We found that the two LEC and two BEC lncRNAs were also more highly expressed in the respective endothelial cell type after ex vivo isolation. Particularly interesting was that the LEC specificity of LETR1 was even more pronounced in freshly isolated ECs than in cultured ECs, similar to the LEC lineage-specific TF PROX1 (Fig. 2g).

Identification of lncRNA candidates for functional characterization by ASOs. To further select lncRNA candidates for genome-wide functional screening, we relied on the FANTOM CAT annotations. First, we filtered for lncRNAs with a conserved transcription initiation region (TIR) and/or exon regions, based on overlap with predefined genomic evolutionary rate profiling elements. Second, we selected for actively transcribed lncRNAs with an overlap between TSSs and DNase hypersensitive sites (DHSs). Third, filtering for expression levels in LEC and BEC RNA-Seq and CAGE-Seq data sets (Fig. 2a) led to the identification of 5 LEC and 12 BEC lncRNAs that are potentially conserved at the sequence level, actively transcribed, and robustly expressed in the respective endothelial cell types (Fig. 2b, c). Finally, we identified through qPCR 2 LEC (AL583785.1 and LETR1) and 2 BEC (LINC00973 and LINC00973) lncRNAs that were consistently differentially expressed between LECs and BECs derived from newborn and adult skin samples (Fig. 2d, e). We next analyzed the expression levels of the four lncRNA candidates and specific blood and lymphatic markers in freshly isolated LECs and BECs from human healthy skin biopsies, using flow cytometry followed by qPCR (Fig. 2f). We found that the two LEC and two BEC lncRNAs were also more highly expressed in the respective endothelial cell type after ex vivo isolation. Particularly interesting was that the LEC specificity of LETR1 was even more pronounced in freshly isolated ECs than in cultured ECs, similar to the LEC lineage-specific TF PROX1 (Fig. 2g).
As ASO GapmeRs are more effective in reducing the expression of nuclear lncRNAs than short interference RNAs (siRNAs), we analyzed the subcellular localization of the two LEC and two BEC lncRNAs in LECs and BECs, using cellular fractionation followed by qPCR. For LEC lncRNAs, AL583785.1 was almost equally distributed between cytoplasm and nucleus, whereas LETR1 showed a higher nuclear distribution (Fig. 2h). Both LINC00973 and LINC01013 were mainly localized in the nucleus (Fig. 2i). Therefore, we next used the ASO-based approach to analyze the genome-wide transcriptional changes upon knockdown of the two LEC and two BEC lncRNAs. After testing their knockdown efficiencies, we selected three out of five ASOs for each lncRNA target (Supplementary Figure 2a–d and Supplementary Data 3).

Transcriptional profiling after LETR1-ASOKD indicates potential functions in cell growth, cell cycle progression, and migration of LECs. To investigate the potential functional relevance of the two LEC and two BEC lncRNAs, we first transfected LECs and BECs with three independent ASOs per target, followed by CAGE-Seq (Fig. 3a and Supplementary Figure 3a, b). Next, we
performed DE analysis by comparing the combined results of the three independently transfected ASOs per target with their scrambled controls, using EdgeR with a Generalized Linear Model (GLM). Finally, we defined DE genes by a false discovery rate (FDR) < 0.05 and a fold change (log2FC) > 0.5 (Supplementary Data 4). We found that ASO knockdown (ASOKD) of AL583785.1 in LECs and of LINC00973 and LINC01013 in BECs showed rather modest changes in gene expression. AL583785.1-ASOKD caused changes of only nine genes (four up and five down), LINC00973-ASOKD of 43 genes (6 up and 37 down), and LINC01013-ASOKD of 24 genes (2 up and 22 down) (Supplementary Figure 3c–g).

In contrast, ASOKD of LETR1 had a high impact on the global transcriptome of LECs, resulting in 133 up- and 122 down-regulated genes (Fig. 3b and Supplementary Figure 3f). Among these, several genes have previously been reported to play prominent roles in vascular development and differentiation pathways, including PTGS2, KLF4, VEGFA, and ANGPT2 among the upregulated genes, and PROX1, CCBEI, SEMA3C, and ROBO1 among the downregulated genes. GO analysis for biological processes using g:Profiler revealed that indeed both up- and downregulated genes were enriched (P value < 0.05) for terms related to vascular development. In addition, upregulated genes were mainly involved in cell death, inflammatory signaling, and response to external stimuli, whereas downregulated genes were primarily related to the regulation of cell migration and chemotaxis (Fig. 3c and Supplementary Data 5). Gene Set Enrichment Analysis (GSEA) also identified significant (FDR < 0.05) biological processes related to cell migration, chemotaxis, and response to external stimuli/virus. More importantly, several downregulated biological processes were associated with cell growth, cell cycle progression, and cytoskeleton organization (Fig. 3d and Supplementary Data 6).

To identify TFs potentially affected by LETR1-ASOKD, we performed Motif Activity Response Analysis (MARA) by analyzing the activity of 348 regulatory motifs in TF sites in the proximal promoters of highly expressed genes in knockdown and control samples (see Methods section). We found 19 upregulated and 7 downregulated motifs, among which were binding sites related to several TFs known to be essential for LEC biology, including STAT6, KLF4, NR2F2 (COUPTFII), and MAFB (P value < 0.05, Supplementary Data 7). Interestingly, KLF4 was the only TF to be also upregulated on the transcriptional level upon LETR1-ASOKD. Based on the MARA analysis, we next reconstructed a gene regulatory network with the 255 genes affected by LETR1-ASOKD. We identified modules of up and downregulated genes linked with the identified TF motifs. Among these modules, we found genes associated with endothelial cell proliferation and migration, such as VEGFA, MAFF, ANGPT2, RAD1, PROX1, SEMA3C, and ROBO1. Overall, these results suggest that the absence of LETR1 has a critical impact on the global transcriptome of LECs by affecting complex TF regulatory networks targeting essential genes largely involved in endothelial cell differentiation, proliferation, and migration.

**LET1R is a bona fide lncRNA expressing three main transcripts in LECs.** Several lines of evidence from our molecular phenotyping screen pinpointed LETR1 as a potential functional lncRNA in LECs. We next sought to investigate the potential coding property of LETR1 given its low presence in the cytoplasm (Fig. 2h) and the previous evidence showing that putative lncRNAs can function through translated micropeptides. According to the FANTOM CAT database, LETR1 transcribes 19 different exon combinations (Supplementary Figure 2b). Calculation of the protein-coding probabilities through the Coding Potential Assessment Tool (CPAT) and the Phylogenetic Codon Substitution Frequencies (PhyloCSF) algorithms confirmed the noncoding nature of these 19 transcript variants (Fig. 4a).

Subsequent characterization of LETR1 isoforms in LECs using 3′ Rapid Amplification of cDNA Ends (3′ RACE) identified three primary polyadenylated LETR1 transcripts that overlapped with the RNA-Seq signal in LECs (Fig. 4b–d and Supplementary Data 8). In vitro translation analysis confirmed that all three transcripts could not generate any micropeptides (Fig. 4e). Expression analysis of the three LETR1 transcripts by qPCR revealed LETR1-1 as the most represented isoform in LECs (Fig. 4f). Thus, we decided to focus our attention on this transcript variant and, for simplicity, we will refer to this isoform as LETR1.

**Knockdown of LETR1 reduces cell growth, cell cycle progression, and migration of LECs in vitro.** To investigate the potential effects of LETR1-ASOKD on LEC growth, we performed cell growth assays based on dynamic imaging analysis. We found that LETR1-ASOKD strongly reduced cell growth of LECs over time (Fig. 5a and Supplementary Figure 4a, b). To study whether the cell growth phenotype was not owing to off-target effects of the ASOs, we also performed cell growth assays after CRISPR interference (CRISPRi). Consistently, we found that CRISPRi-KD of LETR1 also significantly reduced the growth rate of LECs. However, owing to the lower knockdown efficiency, the effect was less prominent compared with ASOKD (Fig. 5b and Supplementary Figure 4c, d). Next, we analyzed the cell cycle progression of LECs upon LETR1-ASOKD, using flow cytometry. Double staining for Ki-67 (proliferation marker) and propidium iodide (PI, DNA content) showed that LETR1-ASOKD
significantly increased the percentage of LECs arrested in G0 (Fig. 5c, d and Supplementary Figure 4e). Although there was a slight increase of subG0 LECs in LETR1-ASOKD samples, analysis of cleaved caspase 3-positive cells showed inconsistent results where only LETR1-ASO2 caused a small but significant increase in apoptosis in LECs, suggesting that apoptosis is not a primary phenotype caused by the absence of LETR1 (Supplementary Figure 5a).

As the transcriptional studies also indicated a potential role of LETR1 in cell migration, we performed wound closure assays ("scratch assays") after LETR1-ASOKD in LECs pre-treated with the proliferation inhibitor mitomycin C. We observed a significant reduction of LEC migration compared with scrambled control ASO (Fig. 5e, f and Supplementary Figure 4f). Similarly, LETR1-ASOKD significantly inhibited LEC migration in a trans-well hapto-chemotactic assay (Supplementary Figure 5b, c).

We next studied whether ectopic overexpression of LETR1 could rescue the proliferation and migration phenotypes observed after knockdown of endogenous LETR1. We therefore overexpressed the most abundant transcript variant determined by 3′ RACE (Fig. 4b–f) using a lentiviral vector and analyzed the cell cycle progression and cell migration after LETR1-ASOKD. We performed both assays with the most effective LETR1-ASO2, which binds to the first intron recognizing exclusively the endogenous but not ectopically overexpressed LETR1 (Supplementary Figure 2b). The reintroduction of
ectopic LETR1 significantly ameliorated both phenotypes, further supporting the role of LETR1 in cell growth and migration regulation. Overexpression of LETR1 per se did not enhance both cellular functions compared with scrambled control ASO, implicating a possible saturation of the regulatory system (Fig. 5g, h and Supplementary Figure 4g–i).

As hinted by the GO analysis (Fig. 3c and Supplementary Data 5), we additionally investigated the role of LETR1 in driving (lymph)angiogenic processes in vitro. To do so, we performed tube formation and 3D-(lymph)angiogenic sprouting assays after LETR1-ASOKD in LECs. We found that knockdown of LETR1 significantly reduced the ability of LECs to form capillary-like structures as well
as the ability to form sprouts in collagen gel-based assays, thereby confirming the essential role of LETR1 in the regulation of essential LEC functions (Supplementary Figure 5d–g).

LETR1 is a nuclear lncRNA interacting in trans with DNA regions near a subset of differentially expressed genes. A first step to study the molecular mechanism of a lncRNA of interest is to analyze its subcellular distribution at the single-molecule level. To this end, we performed single-molecule RNA Fluorescence In Situ Hybridization (smRNA-FISH) in cultured LECs and human skin samples. Consistent with the cellular fractionation data (Fig. 2h), LETR1 was predominantly localized in the nucleus of cultured LECs, showing a broad nuclear distribution.

**Fig. 5 Knockdown of LETR1 reduces cell growth, cell cycle progression, and migration of LECs in vitro.**

- **a, b** Cell growth profiles and cell growth rates of LECs over 48 h after ASOKD a or CRISPRi-KD b of LETR1 using IncuCyte. Confluences were normalized to T0. Growth rates were calculated as the slope of linear regression and normalized to scrambled control ASO/sgRNA.
- **c** Representative flow cytometry plots of LECs after 24 h LETR1-ASOKD. Cells were firstly gated with live/dead Zombie staining (upper plots). Resulting living cells were further gated for non-proliferating stages subGo and Go, and proliferating stages G1, S, G2, and M, using propidium iodide (Pi) and Ki-67 (lower plots).
- **d** Quantification of the cell cycle progression analysis of LECs after 24 h LETR1-ASOKD. Bars represent percentages of gated living cells in subGo, G0, G1, S, G2, and M.
- **e** Representative images of the wound closure assay (9 h) in LECs after LETR1-ASOKD. Confluence mask is shown for all time points. Before scratch, cells were incubated for 2 h with 2 µg/mL Mitomycin C (proliferation inhibitor) at 37 °C. Scale bar represents 200 µm.
- **f** Quantification of the wound closure assay (up to 9 h) of LECs after LETR1-ASOKD. Quantification of the wound closure assay (up to 9 h) of LECs after LETR1-ASOKD. Data are displayed as mean values ± SD (n = 10 in a, f, and h; n = 5 in b; n = 3 in g; n = 2 in d). Percentages represent LETR1 knockdown efficiencies after the experiments. **P < 0.01, ****P < 0.0001 using ordinary one-way (for a, b, and g) and two-way (for a, b, f, and h) ANOVA with Dunnett’s multiple comparisons test against scrambled control ASO/sgRNA or LETR1-ASO2—scrambled control siRNA. In d, g, statistical analysis was performed on G0 populations. In f, h, percentages were determined for each time point using TScratch. All displayed in vitro assays were performed in neonatal LECs derived from the same donor.

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with distinct foci (Fig. 6a, b). This localization pattern was also observed in lymphatic vessels in human skin (Supplementary Figure 6a), suggesting that LETR1 might exert a chromatin-related function in vitro as well as in vivo.

To further elucidate the possible interactions between LETR1 and chromatin, we performed Chromatin Isolation by RNA Purification followed by DNA Sequencing (ChIRP-Seq)59. Cross-linked LECs were hybridized with two biotinylated probe sets (odd and even, internal controls) tiling LETR1 (Supplementary Data 9). Probes targeting LacZ were used as an additional control. After pull-down, the percentage of retrieved RNA was assessed (Supplementary Figure 6b), and DNA was subjected to sequencing. Using a previously published analysis pipeline60, we found 2258 binding sites of LETR1 to be at least threefold significantly enriched compared with input ($P$ value < 0.05; see Methods section), including a peak in the LETR1 exon one region as pull-down control (Supplementary Figure 6c and Supplementary Data 10).

To identify candidate genes directly regulated by LETR1, we first analyzed the genomic distribution of LETR1-binding sites. Out of 2258 binding sites, 1497 mapped within protein-coding genes (65.5%), with a large fraction residing in introns (1010 peaks, 68.3%) (Fig. 6c). Since only 19% of all annotated genes are categorized as protein-coding in the FANTOM CAT database13, these results suggested a preference of LETR1 to interact with regulatory regions near protein-coding genes (fold enrichment = 3.34, $P$ value < 0.05). Therefore, we focused on the identified 1607 protein-coding genes displaying at least one LETR1-binding site within their promoters, exons, or introns. From these, 1193 genes were expressed in LECs, and comparison with the 255 modulated genes upon LETR1-ASOD showed a significant overlap of 44 genes (12 upregulated and 32 downregulated) (fold enrichment = 1.9, $P$ value < 0.05) (Fig. 6d). Importantly, the vast majority of the 44 targets resided on different chromosomes, indicating a predominant trans-regulatory function of LETR1 (Fig. 6e and Supplementary Data 10). These included important lymphatic-related genes such as KLF4, ROBO1, SEMA3A, SEMA3C, and CCBE1. Interestingly, 29 of the 44 targets showed a congruent higher expression in LECs for downregulated genes or in BECs for upregulated genes, implicating these genes as potential downstream targets of LETR1 (Fig. 6f).

Motif analysis using Multiple Em for Motif Elicitation (MEME)60 of the 53 binding regions present in the 44 target gene bodies identified two significantly enriched motifs ($E$ value: $2.01 \times 10^{-6}$—motif 1; $E$ value: $7.90 \times 10^{-6}$—motif 2), suggesting that LETR1 interaction with the genomic DNA might happen through a distinct DNA motif (Supplementary Figure 6d, e). Comparison motif analysis using Tomtom61 revealed that LETR1 motifs displayed a significant similarity with several TF-binding sites previously identified by MARA (Supplementary Figure 6f, g). Additionally, triplex analysis using Triplexator62 identified 30 matching triplex-forming oligonucleotides (TFO)-triplex target sites (TTS), showing that LETR1 binding to the target genomic regions might involve triplex formation (Supplementary Data 11). Taken together, these data further support the conclusion that LETR1 is a critical gatekeeper of the LEC transcriptome via influencing complex TF regulatory networks.

**LETR1 regulates cell proliferation and migration through transcriptional regulation of KLF4 and SEMA3C.** Among the 44 potential downstream targets of LETR1, KLF4 caught our attention as a potential cell proliferation regulator given its well-established tumor-suppressor role63 and the previously observed upregulation at the RNA level as well as increased TF-binding activity upon LETR1 knockdown (Fig. 3). Among cell migration regulatory molecules, we focused on one member of the semaphorin protein family, SEMA3C, that was previously shown to enhance migration in endothelial cells64.

To functionally characterize the relationship between LETR1 and KLF4 as well as SEMA3C, we performed the experimental strategies represented in Fig. 7a. For KLF4, we analyzed the cell cycle progression of LECs after LETR1-ASO2 knockdown, followed by siRNA knockdown of KLF4. As expected, LETR1-ASO2 knockdown resulted in an upregulation of KLF4 as well as an increase of G0-arrested LECs. Consecutive knockdowns of LETR1 and KLF4 rescued this phenotype by significantly increasing the fraction of proliferating LECs. However, downregulation of KLF4 alone (by 70%) was not sufficient to consistently improve the proliferation activity of LECs (Fig. 7b, c and Supplementary Figure 7a). For SEMA3C, we first ectopically overexpressed the SEMA3C protein in LECs, using a lentiviral vector (Supplementary Figure 7b). Subsequently, we analyzed the migratory behavior of infected LECs after LETR1-ASO2 knockdown. Again, LETR1-ASO2 knockdown alone caused the expected downregulation of SEMA3C as well as reduced migration in the vector-control cells. In contrast, overexpression of SEMA3C in conjunction with LETR1-ASO2 knockdown showed a significant recovery of migration capability, as compared to LETR1-ASO2 knockdown alone. SEMA3C overexpression alone did not affect cell migration of LECs (Fig. 7d, e and Supplementary Figure 7c).

**LETR1 interacts with several protein complexes to exert its transcriptional regulatory function.** To identify proteins that are potential co-regulator of LETR1 target genes, we performed in vitro biotin-LETR1 pull-down assays65. Nuclear extracts of LECs were incubated with the biotinylated full-length LETR1 transcript and its antisense as negative control (Supplementary Figure 8a, b). After streptavidin bead separation, mass spectrometry was performed to identify possible interacting proteins. Initial analysis identified a total of 642 proteins. After filtering for proteins present in both replicates but absent in the antisense control, we found 59 proteins to interact with LETR1 (Supplementary Data 12). GO analysis for molecular functions and cellular compartments using gProfileR66 confirmed that the 59 identified proteins were significantly enriched for nuclear RNA-binding proteins (Supplementary Figure 8c, d). Protein–protein interaction analysis using Search Tool for Recurring Instances of Neighbouring Genes (STRING)67 revealed that a large fraction of these 59 proteins were associated with RNA-processing functions, such as RNA splicing, RNA polyadenylation, and RNA nuclear transport. Furthermore, six proteins were associated with chromatin remodeling and three with nuclear organization, suggesting that LETR1 may operate at several levels to regulate gene expression (Fig. 8a).

To screen for protein candidates, we analyzed the RNA expression of the 59 proteins interacting with LETR1 in LECs versus BECs. Four proteins (DDX39A, NUMA1, RBBP7, and DDX5) had a log2FC > 0.5 in LECs and a unique peptide detection greater than five (Fig. 8b). Among these proteins, we identified the histone-binding protein RBBP7, which has previously been reported to be involved in the regulation of many cellular functions, including proliferation and migration.76,68 Subsequent RNA immunoprecipitation assays in LECs validated the interaction between RBBP7 and LETR1, suggesting RBBP7 as a potential mediator of LETR1 gene regulatory functions (Fig. 8c, d).

To evaluate the extent to which the interaction with RBBP7 mediates the transcriptional regulatory function of LETR1, we performed a series of chromatin immunoprecipitation experiments followed by qPCR after LETR1-ASO2 knockdown (Fig. 8e, f). First,
LETIR1 is a nuclear lncRNA interacting in trans with DNA regions near a subset of differentially expressed genes. a Representative images of negative control dapB (bacterial gene), MALAT-1 (nuclear lncRNA), and LETIR1 expression using single-molecule RNA Fluorescence In Situ Hybridization (smRNA-FISH) in neonatal LECs derived from two donors. Immunostaining of endothelial cell marker CD31 was used to outline cell shape. Scale bars represent 20 µm. b Quantification of the nuclear (green) and cytoplasmic (black) smRNA-FISH signal of LETIR1 in neonatal LECs derived from two donors quantified with ImageJ108. Bars represent percentages displayed as mean values + SD (n = 2). c Pie chart showing the genomic localization of the 2258 LETIR1 peaks in protein-coding, overlap between protein-coding and noncoding, noncoding, and intergenic regions according to FANTOM CAT annotations using bedtools116. Magnification shows the distribution of LETIR1-binding sites within promoter, exon, or intron of protein-coding genes (1607 genes). LETIR1 peaks are listed in Supplementary Data 10. d Venn diagrams showing the overlap between total genes expressed in LECs (TPM and CPM > 0.5) and identified LETIR1-ChIRP genes, and the significant overlap between LETIR1-ChIRP genes and differentially expressed genes after LETIR1-ASOKD. e Circular plot showing genome-wide interactions of LETIR1 near the 44 targets generated by Circos117. Scaled chromosomes with their respective cytobands are placed in circle. Major and minor ticks: 50 Mb and 10 Mb; orange and purple lines: interactions between LETIR1 locus and its up- and downregulated targets; green line: genomic locus of LETIR1. f Heat maps based on expression levels (CAGE-Seq, CPM) in scrambled control ASO and LETIR1-ASOKD samples (left, two replicates), as well as in LECs and BECs (right, two replicates) of the 44 LETIR1 targets (orange: upregulated; purple: downregulated). Color code for row Z score values on a scale from −1 to +1. Genes were ordered by log2FC values of ASOKD data and according to their differential expression between LECs and BECs.
we analyzed in LETR1-deficient samples the recruitment of RBBP7 at the TSSs of KLF4 and SEMA3C, and at the identified LETR1-binding regions. Subsequently, we further evaluated the effects of LETR1 knockdown on the recruitment of RNA Pol II and on the enrichment of positive (H3K4me3) or negative (H3K27me3) histone marks at the transcription initiation region of these two target genes (Supplementary Figure 8e, f). We found that the lack of LETR1 significantly decreased RBBP7 and RNA Pol II localization at both KLF4 and SEMA3C promoters (Fig. 8g, h). Remarkably, we found that the absence of LETR1 significantly affected the recruitment of RBBP7 only at the identified LETR1-binding site present in the KLF4 genomic locus (Fig. 8g). It is intriguing since only the binding region of KLF4 displayed the predicted LETR1 motif as well as triplex-forming pairs, suggesting that LETR1 occupancy at the genomic loci might involve both direct and transient RNA:DNA interactions (Supplementary Figure 8g). At the histone modification level, knockdown of LETR1 impacted merely the H3K4me3 modification, especially at the TSS of SEMA3C (Fig. 8i). H3K27me3 levels, however, were unaltered after LETR1-ASO2 knockdown (Fig. 8i).

Taken together, these results suggest that LETR1 acts as a transcriptional regulator by mediating, through RBBP7, the recruitment of RNA Pol II and, to some extent, the chromatin organization at the site of transcription of target genes. In summary, our multilayered mode of action analysis demonstrates that LETR1 is a nuclear lncRNA that interacts with essential epigenetic partners, especially RBBP7, to regulate cell growth and cell migration of LECs by tuning the expression of distinct target genes, in particular KLF4 and SEMA3C (Fig. 8k).

Discussion
Precise regulation of proliferation, migration, and maintenance of cellular identity is not only essential to ensure proper development and integrity of the vascular systems, but also to guarantee that LECs and BECs are able to perform their necessary functions. In this study, we characterized a comprehensive map of lineage-specific lncRNAs in LECs and BECs, and analyzed the transcriptional impacts after ASO-mediated knockdown of LEC- and BEC-specific lncRNAs followed by CAGE-Seq. Importantly, we identified LETR1, originally annotated as LINC01197, as a...
lymphatic-specific lncRNA that is essential in the regulation of LEC growth and migration.

By integrating RNA-Seq and CAGE-Seq transcriptome profiling, we showed that LECs and BECs express a specific cohort of lncRNAs, mainly residing near vascular-related protein-coding genes. These results are in accordance with the intriguing concept that cells might display a set of lncRNAs explicitly expressed to function in the fine-tuning of cell type-specific gene expression programs\(^1\). Most notably, our selection strategy highlighted two LEC (ALS83785.1 and LETR1) and two BEC (LINC00973 and LINC01013) lncRNAs that are robustly and differentially expressed in the respective endothelial lineage. These candidates therefore represent the first set of lineage-specific LEC and BEC lncRNA markers.

The nuclear localization of our lncRNA candidates coincides, to some extent, with previous findings, demonstrated by RNA in situ hybridization, that lncRNAs are commonly located in the nucleus\(^6\). To investigate the biological functions of these lncRNA
candidates, we used the ASO GapmecR knockdown approach, given its higher efficiency in targeting nuclear RNA transcripts over siRNA. In addition, our ASO design strategy proved to be very successful, resulting in a very high knockdown efficiency of all four targets. In the general experimental design, we have not taken into consideration the use of CRISPRi owing to several practical limitations to study lncRNAs. For instance, as opposed to ASOKD, CRISPRi may also interfere with the transcription of overlapping or neighboring transcriptional units, and it is not able to distinguish the cis- and trans-acting functions of lncRNAs. However, we are aware that CRISPRi provides less pronounced off-target effects compared with other loss-of-function methods.

Although we confirmed the lncRNA function by rescue experiments, we are aware that ASO-mediated knockdown approaches might not exclusively study the function related to a lncRNA transcript over a transcription-based mechanism. In fact, recent studies have found that ASO-mediated knockdown could interfere with the transcription of the lncRNA, causing its premature transcription termination. In this context, among the TFs with altered binding activities, we have identified COUPTFII, a crucial TF involved in early lymphatic development. The synergy of the LETR1 and COUPTFII genomic loci (Supplementary Figure 2b) suggests a possible regulatory connection between these two factors. However, neither ASO nor CRISPRi knockdown of LETR1-affected COUPTFII expression in LECs (Supplementary Figure 8h), indicating that LETR1 does not possess a transcription-based mode of action and that the regulation of COUPTFII binding activity may happen indirectly.

Importantly, our study identified the first LEC lncRNA with specific biological functions. Through a multilayered analysis, including DE analysis, GO, GSEA, and MARA, we found that LETR1 is a critical gatekeeper of the global transcriptome of LECs by influencing complex TF regulatory networks regulating essential targets largely involved in the control of LEC growth and migration. These “molecular phenotypes” observed after LETR1 knockdown were confirmed in vitro by analyzing the cell growth profile, cell cycle progression, and wound closure ability of primary human LECs. As shown in a previous study, we thus were able to distinguish LETR1 as a bona fide functional lncRNA in LECs by combining sequencing data analyses and cellular phenotype assays.

The nuclear localization of LETR1, as shown by subcellular fractionation and smRNA-FISH, hinted at a chromatin-related function. Indeed, as confirmed by RNA-DNA interaction assay, we revealed that LETR1 interacts, predominantly in trans, with DNA regions near a subset of differentially expressed genes. In addition, RNA–protein interaction assays indicated a potential scaffold function of LETR1 in recruiting proteins involved in several levels of gene expression regulation, including chromatin organization. These results are in line with the general model in which lncRNAs are crucial for the assembly of unique protein complexes and for guiding them to specific target sites. Specifically, we found that LETR1 interacts with RBBP7, a protein previously reported to be part of several multi-protein complexes that are involved in chromatin remodeling, histone post-translational modification, and gene expression regulation. Intriguingly, RBBP7 is a relevant constituent of the polycomb repressive complex 2 (PRC2) complex, which was also previously shown to interact with 20% of the lncRNAs in human cells. It is conceivable that LETR1 might act as an epigenetic regulator to recruit or guide protein partners to influence the three-dimensional structure of the genome. In this setting, the differential CAGE-Seq peak intensities at the target TSSs after LETR1-ASOKD might provide a hint on the function of LETR1 in mediating the transcriptional machinery access at the site of transcription. In fact, significant correlations at TSS regions between RNA Pol II occupancy and CAGE-Seq signal have been reported. Our multilayered chromatin immunoprecipitation approach evidenced that LETR1 indeed influences the recruitment of RNA Pol II at TSSs of target genes via the interaction with the epigenetic factor RBBP7. Moreover, we observed that this function could be achieved either through direct (KLF4) or transient (SEMA3C) RNA:DNA interaction mechanisms. We also showed that this interaction plays, to a certain degree, a role in manipulating chromatin states at the target gene transcription initiation site.

We showed that LETR1 exerts its effects on LEC functions, at least in part, via the modulation of KLF4 and SEMA3C, as knockdown of KLF4 or overexpression of SEMA3C partially restored the cellular phenotypes observed upon LETR1-ASOKD. Previous studies have reported that KLF4 is a tumor-suppressor TF that, once upregulated, inhibits cell growth and induces cell cycle arrest. A primary mechanism by which KLF4 regulates cell growth is via the induction of CDKN1A expression, a gene encoding a cyclin-dependent kinase (CDK) inhibitor. Consistently, we found that CDKN1A was also upregulated upon knockdown of LETR1 (Supplementary Data 4), suggesting that suppression of the KLF4-CDKN1A axis through LETR1 is required for the maintenance of a proliferative state of LECs. Moreover, MARA analysis highlighted that the trans-acting activity of LETR1 has a general inhibitory effect on the binding activity of KLF4, suggesting an additional genome-wide interplay between KLF4 and LETR1 to modulate sensitive targets indispensable for LEC function.
A recent study reported that viral ectopic expression of FOXO1 in ducal pancreatic adenocarcinoma cells upregulates LINCO1197 (LETR1), resulting in the inhibition of cell proliferation. Interestingly, FOXO1 has previously been shown to promote LEC migration and to participate in the regulation of lymphatic development. Moreover, in our sequencing data, FOXO1 was significantly more highly expressed in LECs than BECs.

SEMA3C belongs to the semaphorin class 3 guidance cue molecules, which mainly bind to a receptor complex composed of neuropilins (NRP1 or NR2P2) and plexins (PLXNA1-A4 and PLXND1). Our findings that overexpression of SEMA3C partially rescued the LETR1 inhibition of LEC migration are consistent with previous reports showing that SEMA3C has pro-migratory activities in several cell types, including endothelial cells. In support of this claim, our sequencing data revealed that LECs expressed the two neuropilins, NRPI (at a low level) and NR2P2, as well as the plexins A1-A4 and D1. In addition, knockdown of LETR1 also significantly reduced the expression of plexin A4 (Supplementary Data 4), pinpointing LETR1 as an important player in semaphorin signal transduction.

Although we initially identified LETR1 as a LEC-specific lncRNA by sequencing of cultured human LECs, smRNA-FISH, and Fluorescence-Activated Cell Sorting (FACS) validated its lymphatic specificity in human skin in situ. Future studies are needed to investigate its expression pattern and mechanistic role in pathological conditions associated with impaired lymphatic function (e.g., lymphedema) or active lymphangiogenesis (e.g., tumor metastasis and wound healing).

It is of interest that the knockdown of three other lncRNA candidates showed merely a minor or no impact on the transcriptome of LECs and BECs after ASO-mediated knockdown. Likely, these could be owing to four potential reasons. First, they may have alternative functions unrelated to transcriptional regulation, such as ribozymes or riboswitches and translation initiation regulators. Second, the act of transcription, rather than the lncRNA product of this transcription, may be functional by having, for instance, an enhancer-like function. Third, they may function as molecular signals at a specific time and place in response, for example, to unique stimuli. Finally, although clearly differentially expressed, all three lncRNA candidates might not be functional and might just be part of transcriptional noise.

Therefore, future research is needed to elucidate the biological role and function of these lncRNAs in LECs or BECs. Taken together, our study enumerates the collection of lncRNAs explicitly expressed in LECs and BECs and highlights, through the functional characterization of LETR1, the importance of those lncRNAs in the regulation of lineage-specific endothelial cell functions.

Methods

Isolation of adult primary skin LECs and BECs from biopsies. LECs and BECs were obtained from the abdominal or breast skin of healthy adult subjects admitted for plastic surgery at the University Hospital Zurich in accordance with the principles of the Helsinki declaration. Written informed consent was obtained from each donor/tissue collection, as approved by the Ethics Committee of the Canton of Zurich (2017-0093). Skin samples were washed in Hank’s balanced solution supplemented with 5% fetal bovine serum (FBS, Gibco), 2% antibiotic and antimycotic solution (AA, Gibco), and 20 mM HEPES (Gibco), and subsequently digested was stopped by washing the tissues with RPMI basal medium supplemented with 10% FBS, 2% AA, and 20 mM HEPES. After removal of the epidermal sheets, the dermis was finely minced and enzymatically digested (RPMI basal medium, 1000 U/mL collagenase type 1 (Worthington), and 1000 U/mL DNase I (Roche)) for 1 h at 37 °C under constant agitation. Digested tissues were then filtered through a 100 μm cell strainer (Falcon), washed with RPMI basal medium, and centrifuged at 850 x g for 6 min at 4 °C. Cells were seeded into fibronectin (Roche) coated plates and were cultured in EGM-2-MV complete medium (Lonza). After 7–10 days, cells were trypsinized, and endothelial cells were selected based on CD31 positivity with Dynabeads CD31 endothelial cell magnetic beads (Thermo Fisher Scientific) and cultured in EBM-2 medium supplemented with 20% FBS, 10 µg/mL hydrocortisone (Sigma), washed with FACS buffer (DPBS with 2% FBS and 1 mM ethylenediaminetetraacetic acid (EDTA)), and stained with Alexa647-conjugated mouse anti-human podoplanin antibody (I:70, clone 18HS, Novus Biologicals) and PE-conjugated mouse anti-human CD31 antibody (1:20, clone WM59, BD Pharmingen) in FACS buffer for 30 min at 4 °C. After a wash with FACS buffer, endothelial cells were finally sorted on a FACSAria II (BD Biosciences) with a 70 μm nozzle, using FACSDivide software (ver. 6.1.3). LECs were defined as CD31- and podoplanin-positive cells, whereas BECs were defined as CD31-positive and podoplanin-negative cells.

Cell culture. Primary human dermal LECs and BECs were isolated from neonatal human foreskin as described previously. The collection and use of dermal LECs and BECs from neonatal foreskin were approved by the Human Research Committee of the Massachusetts General Hospital, Boston, MA (IRB protocol number 1999-P-006905/9) and were compliant with the declaration of Helsinki. Written informed consent was obtained from the parents. Both cell types were cultured in venous peripheral blood endothelial basal medium (EBM, Lonza) supplemented with 20% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (Pen–Strep, Gibco), 2 mM l-glutamine (Gibco), 10 µg/mL hydrocortisone (Sigma) on sterile dishes/plates (TPP) pre-coated with 50 µg/mL purecol type I bovine collagen solution (AdvancedBioMatrix) in DPBS at 37 °C in a 5% CO2 incubator. LECs were additionally cultured in endothelial basal medium (EBM, Lonza) supplemented with 5% fetal bovine serum (FBS, Gibco), 2% antibiotic and antimycotic solution (AA, Gibco), and 20 mM HEPES (Gibco) in a 5% CO2 incubator. Cells were used between passages 2 and 6. HEK293T cells were cultured in DMEM with glutamax (Gibco) supplemented with 10% FBS (Gibco) and Pen–Strep at 37 °C in a 5% CO2 incubator. All cells were routinely tested for mycoplasma contamination using the MycoScope PCR Mycoplasma Detection Kit (Genlantis).

RNA isolation, reverse transcription, and qPCR. If not differently specified, total RNA was isolated using the QuiagenNucleoSpin RNA kit (Machery Nagel) according to the manufacturer’s instructions and quantified by NanoDrop ND-1000 (Witeg AG). Equal amounts of total RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer’s instructions. In all, 10 ng RNA per reaction was then subjected to qPCR using PowerUp SYBR Green Master Mix (Applied Biosystems) on a QuantStudio 7 Flex Real-Time PCR system (Applied Biosystems). If not specified differently, for qPCR analysis, cycle threshold (Ct) values were normalized to the housekeeping gene GAPDH. Relative expression was calculated according to the comparative Ct method. Primers for qPCR are listed in Supplementary Data 13.

Western blot analysis. To perform western blot analysis, the protein concentration of lysates was first determined using the Microplate BCA protein assay kit—reducing agent compatible (Thermo Fisher Scientific), according to the manufacturer’s instructions. To 5–30 µg of total protein, SDS sample buffer and reducing agent (Thermo Fisher Scientific) were added to a 1× final concentration. Then, samples were heated for 5 min at 95 °C and subjected to sodium dodecylsulfate disc-gel electrophoresis (SDS-PAGE) (4–12%) and blotted to nitrocellulose membranes (Merck Millipore) for 1 h at 200 V. Protein loading was checked by staining membranes with Ponceau staining solution (Sigma) for 2 min at room temperature. Membranes were blocked with 5% milk powder in TBST (50 mM Tris Base, 150 mM NaCl, 0.1% Tween 20, pH 8.4) for 1.5 h at room temperature. Membranes were then stained overnight at 4 °C with primary antibodies (see below) diluted in TBST. Blots were washed three times with TBST for 15 min at room temperature and subsequently incubated for 2 h at RT (37 °C). Dyes (goat anti-rabbit, rabbit anti-mouse, and mouse anti-strep) at a dilution of 1:1000–1:5000 in TBST were used at 4 °C, washed, then incubated for 30 min at room temperature with avidin–biotin complex (ABC, Vector Laboratories) at a dilution of 1:200, washed, and finally stained with diaminobenzidine (DAB) as substrate. Membranes were washed, air-dried, and finally exposed to autoradiographic film (Kodak). Densitometry was performed using GelPro Vision software (Media Cybernetics) in a 1.5 ratio to total DNA content. Each band was quantified by densitometry using Image J software and normalized to the corresponding housekeeping gene expression. All bands are analyzed in Supplementary Data 13.

Lentivus production. For the production of lentiviruses, 2.5 x 106 HEK293T cells were seeded into 10 cm dishes and cultured overnight. One hour before transfection, the medium was replaced with an antibiotic-free medium containing 25 µM chloroquine (Sigma). The transfection mixture was subsequently prepared as follows. In a first tube, 1.3 pmol psPAX2 (12260, Addgene), 0.72 pmol pMD2.G (12259, Addgene), and 1.64 pmol of target vector were mixed in 500 µL Opti-MEM (Gibco). In another tube, polyethyleneimine (PEI, Sigma) was added to 500 µL Opti-MEM in a 1:3 ratio to total DNA content. PEI-containing Opti-MEM was transfected dropwise to the plasmid-containing Opti-MEM, and the mixture was...
incubated for 20 min at room temperature. Finally, the transfection mixture was transferred dropwise to the HEK293T cells. In all, 24 h post-transfection, the medium was changed with 8 mL complete containing cell type-specific conditions. Genes with expression $>5$ CPM in at least two CAGE libraries (targeted lncRNA ASOs $+\pm$ scrambled control ASO (NC_A) CAGE libraries) were compared for downstream analysis.

**GO analysis of ASOKD data.** GO analysis was performed separately on upregulated and downregulated genes, using gProfiler (ver. 0.6.7)\(^3\), as described above. All the significant GO terms ($P < 0.05$) were used for further analysis and are listed in Supplementary Data 5.

**GSEA of ASOKD data.** GSEA was performed individually for each targeted lncRNA using tool xtoolsgsea (ver 0.4.3.0). For each targeted lncRNA, DE analysis was performed using the RNASeq-Free DNase set (Qiagen). The identity of BECs and LECs was checked by qPCR (Supplementary Figure 1a, b). LEC and BEC total RNA were then subjected to ribosomal-RNA depletion and RNA-sequencing (RNA-Seq) and nAnT-iCAGE sequencing (CAGE-Seq) protocols as previously described\(^{44}\). For RNA-Seq, the Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Human/Mouse/Rat (RS-122-2201) was used.

**Differential gene expression analysis of CAGE-Seq and RNA-Seq data.** For both RNA-Seq and CAGE-Seq data, read alignment was performed, and expression tables were generated as described previously\(^{34}\). Next, we performed DE analysis of RNA-Seq and CAGE-Seq of LECs against BECs, LECs against DFs, and BECs against DFs using EdgeR (ver. 3.12.1)\(^{34,99,100}\). DE-omics genes were identified using the following criteria: $|\log_{2} (\text{FC})| > 0.5$ and FDR $< 0.05$ were defined as differentially expressed genes and used for the downstream analysis.

**GO analysis of ASOKD data.** GO analysis was performed separately on upregulated and downregulated genes, using gProfiler (ver. 0.6.7)\(^3\), as described above. All the significant GO terms ($P < 0.05$) were used for further analysis and are listed in Supplementary Data 5.

**GSEA of ASOKD data.** GSEA was performed individually for each targeted lncRNA using tool xtoolsgsea (ver 0.4.3.0). For each targeted lncRNA, DE analysis was performed using the RNASeq-Free DNase set (Qiagen). The identity of BECs and LECs was checked by qPCR (Supplementary Figure 1a, b). LEC and BEC total RNA were then subjected to ribosomal-RNA depletion and RNA-sequencing (RNA-Seq) and nAnT-iCAGE sequencing (CAGE-Seq) protocols as previously described\(^{44}\). For RNA-Seq, the Illumina TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero Human/Mouse/Rat (RS-122-2201) was used.

**Differential gene expression analysis of ASOKD data.** All ASOs for each targeted lncRNA were compared against scrambled control ASO (NC-A) libraries from corresponding cell types. Genes with expression $>5$ CPM in at least two CAGE libraries were included in the input table for the analysis. Genes sets for GO (Biological Process, Molecular Function, Cellular Component), Hallmark, KEGG, Reactome, BioCarta, and Canonical pathways from MSigDB (ver. 6.0) were used for the analysis. The parameters used for each run were: -norm meandiv -nperm 500 -permute gene_set -rdtype no_balance -scoring_scheme weighted -metric Signal2Noise -rnd_seed -set_max 1000 -set_min 5. Enriched GO biological processes were selected and organized in a network using Cytoscape (ver. 3.11.1)\(^{101}\). Gene-set filtering was set as follows: FDR q value cutoff $< 0.05$ and P value cutoff $< 0.001$. Gene-set similarity cutoff was set as $< 0.5$ with an Overlap Metric. Genes were filtered by expression. Terms were then organized manually according to their biological meaning using the Cytoscape plugin Wordcloud\(^{102}\). Significant GO biological processes after GSEA are listed in Supplementary Data 6.

**Cloning sgRNA targeting LETR1 and establishment of dCas9-expressing LECs.** sgRNAs targeting LETR1 were designed using the online CRISPRdesign tool from the Zhang lab, MIT (https://crispr.mit.edu/). 250 bp upstream of the highest CAGE-Seq peak were used as the design region. We then selected three then three sgRNAs to be cloned into Lentiguide-Puro (52963, Addgene) as previously described\(^{103}\). Briefly, each pair of oligos was first annealed and phosphorylated using T4 PNK (New England BioLabs) using the following program: 30 min at 37 °C, 5 min at 95 °C, and then tranformed down to 25 °C at 5 °C/min. Annealed oligos were then ligated into OptiMAX previously mixed in 20 µL Opti-MEM according to the manufacturer’s instructions. Plasmids were isolated using the Nucleospin Plasmid kit (Machery Nagel), as described in the manufacturer’s protocol. Sequences of inserted sgRNAs were checked by Sanger sequencing (Microsynth). Sequences of sgRNAs are listed in Supplementary Data 13. Lentiviruses containing pHAGE EF1a dCas9-KRAB (50919, Addgene, with custom blasticidin cassette), scrambled control sgRNA, or each of the sgRNA targeting LETR1 were produced as described above.

To establish dCas9-overexpressing LECs, $1.2 \times 10^5$ LECs were seeded into pre-coated six-well plates (TPP) and infected with medium containing dCas9-KRAB lentiviruses diluted at a 10 multiplicity of infection (MOI) and 5 µg/mL polybrene (hexamidine bromide, Sigma). Plates were then sealed with paraffin and centrifuged at 340 g for 1.5 h at room temperature. The next day, the medium was changed, and positively infected cells were selected with 10 µg/mL blasticidin (InvivoGen). Once confluent, at least 5 $\times 10^5$ dCas9-KRAB-expressing LECs were split to pre-coated 10 cm dishes and cultured under antibiotic selection until confluency. After checking RNA and protein levels of dCas9-KRAB as described previously\(^{105}\), LECs were then used in the cell growth profile experiment.
For cell growth profiling after CRISPRi, 3000 dcCas9-expressing LECs per well were seeded into a 96-well plate and grown overnight. LECs were then infected with 500 ng/ul of LETR1-ASOKD-containing scrambled EGFP or sgRNA or three sgRNAs targeting LETR1 diluted in complete growth EB medium supplemented with 5 µg/mL polybrene. After 24 h, the virus-containing medium was changed.

In both experiments, LECs were continuously imaged every 3 h over three days with 4 fields per well using the IncuCyte ZOOM live-cell imaging system (Essen Bioscience). Confluence in each well was determined using IncuCyte ZOOM software (ver. 2016B). The normalized growth rate was calculated as the slope of linear regression and normalized to control. To check knockdown efficiency, LECs were homogenized with pestle type B for 25 min and rocked again for 2 min at room temperature. These last two steps were repeated for a total of four times. Finally, the volume was adjusted to 500 µL, and the cells were incubated at 37°C for 24 h. After 24 h, the transfection of scrambled control ASO or three ASOs targeting LETR1 was performed as described above. In all, 24 h post transfection, 50,000 LECs were seeded into 48-well plate (Costar) and cultured overnight in complete EB medium. The next day, the cells were washed with DPBS, and medium was replaced with starvation medium for 6 h. Cells were then stained in starvation medium containing 0.25 µM CMFDA (Thermo) for 30 min at 37°C. The staining medium was then replaced with starvation medium for an additional 30 min. The meantime, 1 µg/mL (ph 7.4) PureCol collagen type I solution was prepared in starvation medium. Finally, medium was replaced with 200 µL collagen solution, and cells were incubated for 14 h at 37°C. Images of capillary-like structures were taken at 5x magnification using a bright field microscope (Zeiss Axiovert 200 M). Images were analyzed using AutoTube (ver. 1.0). To check knockdown efficiency, an aliquot of LECs for each condition was lysed before being seeded into the 48-well plate. Total RNA, cDNA synthesis, and qPCR were then performed as described above.

### Trans-well migration assay after LETR1-ASOKD

In all, 2.5 × 10⁵ LECs were seeded into 6 cm dishes and cultured for at least 6 h. Transfection of scrambled control ASO or three ASOs targeting LETR1 was performed as described above. In all, 24 h post transfection, 50,000 LECs were seeded into 48-well plate (Costar) and cultured overnight in complete EB medium. The next day, the cells were washed with DPBS, and medium was replaced with starvation medium for 6 h. Cells were then stained in starvation medium containing 0.25 µM CMFDA (Thermo) for 30 min at 37°C. The staining medium was then replaced with starvation medium for an additional 30 min. The meantime, 1 µg/mL (ph 7.4) PureCol collagen type I solution was prepared in starvation medium. Finally, medium was replaced with 200 µL collagen solution, and cells were incubated for 14 h at 37°C. Images of capillary-like structures were taken at 5x magnification using a bright field microscope (Zeiss Axiovert 200 M). Images were analyzed using AutoTube (ver. 1.0). To check knockdown efficiency, an aliquot of LECs for each condition was lysed before being seeded into the 48-well plate. Total RNA, cDNA synthesis, and qPCR were then performed as described above.

### Sprouting assay after LETR1-ASOKD

In all, 2.5 × 10⁵ LECs were distributed into a non-coated low-adhesive 24-well plate (Costar). Approximately 5000 Cytodex-3 gelatin-coated microcarrier beads (Sigma) to reach a ratio of 1:40 (beads:cells) were added to each well. To allow the cells to cover the beads, the plate was first rocked at room temperature for 30 min. The plate was then incubated at 37°C for 30 min and rocked again for 2 min at room temperature. These last two steps were repeated for a total of four times. Finally, the volume was adjusted to 500 µL, and the cells were incubated at 37°C for 24 h. After 24 h, the transfection of scrambled control ASO or three ASOs targeting LETR1 was performed as described above. Of note, to apply a similar number of ASO molecules per cell as used in previous in vitro experiments, we scaled up the concentration to 100 nM. In all, 24 h post transfection, 50,000 LECs were seeded into 48-well plate (Costar) and cultured overnight in complete EB medium. The next day, the cells were washed with DPBS, and medium was replaced with starvation medium for 6 h. Cells were then stained in starvation medium containing 0.25 µM CMFDA (Thermo) for 30 min at 37°C. The staining medium was then replaced with starvation medium for an additional 30 min. The meantime, 1 µg/mL (ph 7.4) PureCol collagen type I solution was prepared in starvation medium. Finally, medium was replaced with 200 µL collagen solution, and cells were incubated for 14 h at 37°C. Images of capillary-like structures were taken at 5x magnification using a bright field microscope (Zeiss Axiovert 200 M). Images were analyzed using AutoTube (ver. 1.0). To check knockdown efficiency, an aliquot of LECs for each condition was lysed before being seeded into the 48-well plate. Total RNA, cDNA synthesis, and qPCR were then performed as described above.

### Wound closure assay after LETR1-ASOKD

In all, 2.5 × 10⁵ LECs were seeded into 6 cm dishes and cultured for at least 6 h. Transfection of scrambled control ASO or three ASOs targeting LETR1 was performed as described above. In all, 24 h post transfection, 50,000 LECs were seeded into 48-well plate (Costar) and cultured overnight in complete EB medium. The next day, the cells were washed with DPBS, and medium was replaced with starvation medium for 6 h. Cells were then stained in starvation medium containing 0.25 µM CMFDA (Thermo) for 30 min at 37°C. The staining medium was then replaced with starvation medium for an additional 30 min. The meantime, 1 µg/mL (ph 7.4) PureCol collagen type I solution was prepared in starvation medium. Finally, medium was replaced with 200 µL collagen solution, and cells were incubated for 14 h at 37°C. Images of capillary-like structures were taken at 5x magnification using a bright field microscope (Zeiss Axiovert 200 M). Images were analyzed using AutoTube (ver. 1.0). To check knockdown efficiency, an aliquot of LECs for each condition was lysed before being seeded into the 48-well plate. Total RNA, cDNA synthesis, and qPCR were then performed as described above.

### Subcellular fractionation followed by qPCR

Fractionation of LECs or BECs was adapted from [1]. After trypsinization, 1 × 10⁵ LECs or BECs were collected in 15 mL Falcon tubes and washed once with DPBS. LECs or BECs were then resuspended in 1 mL cold cell disruption buffer (10 mM KCl, 1.5 mM MgCl₂, 20 mM Tris-Cl pH 7.5, 1 mM DTT) and incubated for 10 min on ice. At this point, LECs or BECs were transferred into a 7 mL Dounce homogenizer (Kimble) and homogenized 30 times with pestle type B for 30 s. The cell nuclei were then observed under the microscope (Zeiss Axiovert 200 M). LEC and BEC nuclei were subsequently transferred to a fresh tube, and Triton X-100 was added to a final
and ethanol washes. Once dried for 5 min at 60 °C, slides were incubated with ImageJ (ver. 2.0.0-rc-69/1.52i)108 was used to quantify the nuclear versus (positive control, Type 1 Probe, VA1-11317, Thermo Fisher Scientific), Simultaneous smRNA-FISH and immunostaining were performed using the RNAScope Multiplex Fluorescent Reagent kit v2 (Advanced Cell Diagnostics), concentration of 0.1%. After mixing four times by inverting the tube, LEC and BEC nuclei were pelleted, and the supernatant was recovered as cytoplasmic fraction. To isolation of total RNA, the nucleo pellet was lysed in 1% Tri-reagent (Biotec). After 5 min incubation at room temperature, 200 µL chloroform were added to the homogenized nuclear fraction. After vortexing for 10 s, the nuclear fraction was spun down at 16,000 × g for 15 min at 4 °C. The upper aqueous phase was transferred into a new tube. For cytoplasmic RNA, on the other hand, two volumes of absolute alcohol were added and mixed several times before RNA precipitation at −20 °C. After RNA precipitation, the temperature, the tubes were centrifuged at 16,000 × g for 10 min at 4 °C. RNA pellets were subsequently washed with 75% ethanol and dried for 10 min at room temperature. Dried RNA pellets were resuspended in RNAse-free water and incubated for 15 min at 58 °C on a heating block. Finally, both samples were subjected to cDNA synthesis, and qPCR was performed as described above.

Identification of transcripts variants of LETR1 in LECs. The TSS of LETR1 was determined by examining the CAGE-Seq signal (Supplementary Figure 2). Tran-

In vitro translation of the three LETR1 transcript variants. The in vitro translation analysis was performed using the TnT quick coupled transcription/translation system (Promega), following the manufacturer’s instructions. In brief, a T7 promoter was attached to the 5′-end of the three LETR1 transcripts and the luciferase ORF (positive control) by PCR amplification using Phusion high-fidelity DNA Polymerase (New England BioLabs). After gel purification, PCR fragments were used as templates to perform the in vitro translation reaction for 90 min at 30 °C. Transfected biotin-labeled RNA (Promega) was used to label the newly synthesized proteins. Next, western blotting was performed largely as described above, with the exception of the usage of Bolt 12% Bis-Tris gels to be able to separate and keep small-sized proteins (microproteins). Detection of biotinylated proteins was performed using the Transcend chemiluminescence non-radioactive translation detection system (Promega), following the manufacturer’s instructions. Blots were imaged on a ChemiDoc imaging system (Bio-Rad).

Simultaneous smRNA-FISH and immunostaining in cultured LECs. In all, 5000 LECs per well were seeded into a 96-well glass-bottom imaging plate (Greiner bio-one). Once confluence was reached, smRNA-FISH was performed using the viewRNA Cell Plus Assay kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. LECs were stained with probes designed to target human LETR1 (Type 1 Probe, VA1-3018146, Thermo Fisher Scientific), human Malat-1 (positive control, Type 1 Probe, VA1-11317, Thermo Fisher Scientific), and bacterial DapB (negative control, Type 1 Probe, VF1-11712, Thermo Fisher Scientific). LECs were additionally co-stained with mouse anti-human CD34 antibody (clone JC70A, Dako) at a dilution of 1:50, followed by donkey anti-mouse Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific) at a dilution of 1:200. Z-stacks of fluorescent images spanning over the entire tissue section were acquired using an inverted confocal microscope (Zeiss LSM 880). Z-projection of acquired images was done using ImageJ (ver. 2.0.0-r69/1.52i).

RNAsequencing hydrogen peroxide for 10 min at room temperature. Target retrieval was then performed for 10 min in RNAscope target retrieval solution using a steamer and 95 °C heat for 10 min. Sections were air-dried and deparaffinized with a series of xylene and ethanol washes. Once dried for 5 min at 60 °C, slides were incubated with

FACS sorting of primary BECs and LECs followed by qPCR. Single-cell suspensions from human skin samples of adult subjects obtained as described in the section “Isolation of adult primary skin LECs and BECs from biopsies” were prepared as described above. Subsequently, isolated single cells were stained with primary antibodies (CD34 and CD45) and then washed using the TnT quick coupled transcription/translation system (Promega), following the manufacturer’s instructions. In brief, ChIRP probes (37 × 19–20 nucleotides) targeting LETR1 were designed using the Stellaris Probe Designer (LOC Biosearch Technologies). As non-specific control, 17 probes targeting the bacterial gene LacZ were selected from ref.59. Probes were LNA-modified using the Phototope Labelling Reaction kit (Vector Lab), according to the manufacturer’s instructions. Probe concentrations were determined by NanoDrop. Probes targeting LETR1 were divided into odd and even sets to be used as an additional internal control. Probe sequences are listed in Supplementary Data 4. A total of 30 million LECs per sample were cross-linked with 1% glutaraldehyde (Sigma) for 10 min at room temperature. Crosslinking was quenched with 0.125 M glycine (Sigma) for 5 min at RT. LECs were rinsed with PBS (Ambion) twice and pelleted for 5 min at 850 × g. Between 60 and 80 mg, pellets were lysed in 1 mL lysis buffer (50mMTris-CI pH 7.8, 10 mM EDTA, 1% SDS, 1 mM PMSF, complete protease inhibitor cocktail (Roche), 0.5 U/mL Ribonuclease inhibitor (Thermo Fisher Scientific)) and the cell suspension was sonicated for 1.5–2 h until DNA was in the size range of 100–500 bp using the Covaris S220 system (Covaris, Peak Power: 140, Duty Factor: 50%, Cycles per Burst: 200) with the following on-off intervals: 5 × 4 min, 1 × 10 min, and 4 × 15 min. At this point, the sonicated chromatin was focused into a single sample from one replicate. A total of 100,000 LECs (3 LetR1-Odd, Even, and LacZ), and each sample was diluted with 2 mL hybridization buffer (750 mM NaCl, 1% SDS, 50 mM Tris-Ci pH 7.0, 1 mM EDTA, 15% formamide, 1 mM PMSF, complete protease inhibitor cocktail, 0.1 U/mL Ribonuclease inhibitor). Two aliquots of 20 µL cDNA were used as input RNA and DNA. Diluted cDNA samples were then incubated with 100 pmol probes and mixed with rotation at 37 °C overnight. After hybridization overnight, the three samples (LETR1-Odd, LETR1-Even, and LacZ) were pulled down using 120 µL Dynabeads M-270 streptavidin magnetic beads (Thermo Fisher Scientific) for 30 min at 37 °C with rotation. After five washes with washing buffer (2 x SSC, 0.5% SDS, 1 mM PMSF), beads were used for RNA isolation and 900 µL for DNA isolation. RNA aliquots were incubated in 80 µL RNA proteinase K solution (100 mM NaCl, 10 mM Tris-Ci pH 7.0, 1 mM EDTA, 0.5% SDS, 0.2 U/µL proteinase K (Ambion)) for 45 min at 50 °C with rotation and boiled for 10 min at 95 °C. 500 µL Qiazol (Qiagen) were added to each sample, and RNA was extracted according to the manufacturer’s instructions. Individual small aliquots of isolated RNA (10 ng) were subjected to one-step real-time PCR using the One-Step SYBR PrimeScript RT-PCR kit (Takeda Bio) on an HT7900 system in order to
determine the percentage of RNA retrieval. DNA, on the other hand, was eluted twice using 150 µL DNA elution buffer (50 mM NaHCO3, 1% SDS, 200 mM NaCl, 1 mM PMSF, 0.1% Triton X-100) in a thermomixer at room temperature and stored at -80 °C. The RNA and DNA were then washed twice with 70% ethanol (v/v) and dried under vacuum at room temperature for 20 min. DNA concentration was determined on a Qubit fluorometer (Thermo Scientific) and DNase I (Invitrogen) treatment and RNase-free water were used to prepare libraries using the TruSeq DNA-Seq kit (Rubicon), and sequencing was performed according to the manufacturer’s protocol on a HiSeq system (Illumina).

**ChiRP-Seq analysis.** Analysis including alignment and peak calling was performed following the published ChiRP-Seq pipeline from Chang’s Lab at Stanford University with minor modifications. In brief, raw reads were firstly trimmed using SolexaQA (ver. 3.1.7.1) with -dynamictrim and -lengthsoft default settings. Trimmed reads were then uniquely mapped to human reference genome hg38 using bowtie (ver. 1.1.1)\(^{114}\). Mapping parameters were -m 1 -chunkmbs 1024 -p 6. Peaks against input were called using MACS 2.0 (ver. 2.1.1)\(^{115}\) with following settings: callpeak -f SAM --SPMR -g hs-bw 200 -m 10 -q 0.01. Finally, peaks were filtered based on fold enrichment against input lane > 3, Pearson correlation > 0.2, and average coverage > 1.25. ChiRP peaks are listed in Supplementary Data 10.

**Genomic location analysis of significant peaks was performed using bedtools (ver. 2.2.7.1)\(^{116}\) and FANTOM CAT annotations\(^{13}\) for gene body, exon, and promoter. Enrichment analyses of protein-coding genes, as well as of common genes between LETR1-ASOKD and ChiRP-Seq, were performed using SuperExactTest (ver. 1.0.0)\(^{101}\). Annotation of genes annotated in the FANTOM CAT database \((n = 124,047)\) and total genes from ChiRP and ASOKD data \((n = 13,127)\) were, respectively, used. The circular plot of the identified gene targets was generated using Circos (ver. 0.69-7)\(^{117}\). Motif analysis of the 53 binding sites of the 44 targets was performed using MEME (ver. 5.1.0)\(^{118}\) with the following settings -mod an -nmotifs 3 -minw 6 -maxw 50 -objfun classic -revcomp -markov_order 0 -brief -nmotifs 3 -minw 6 -maxw 50 -objfun classic -revcomp -markov_order 0 -brief.

**VirtualBox software (ver. 6.1.0)\(^{62}\), using the parameters -l 10 -e 20 -g 40 -fr off by the manufacturer. Second, RNA secondary structure probability was calculated using the RNAfold function from the ViennaRNA package 2.0\(^{119}\) with a pairing probability cutoff of 0.95. Second, RNA regions with the highest probability were masked in the LER1-ASOKD and ChiRP-Seq, were performed using SuperExactTest (ver. 1.0.0)\(^{101}\). Annotation of genes annotated in the FANTOM CAT database \((n = 124,047)\) and total genes from ChiRP and ASOKD data \((n = 13,127)\) were, respectively, used. The circular plot of the identified gene targets was generated using Circos (ver. 0.69-7)\(^{117}\). Motif analysis of the 53 binding sites of the 44 targets was performed using MEME (ver. 5.1.0)\(^{118}\) with the following settings -mod an -nmotifs 3 -minw 6 -maxw 50 -objfun classic -revcomp -markov_order 0 -brief -nmotifs 3 -minw 6 -maxw 50 -objfun classic -revcomp -markov_order 0 -brief.

**Cloning of LETR1 and SEMA3C, and lentivirus production.** For cloning, full-length LETR1 transcripts were first amplified from cell line RNA by reverse transcription (RT)-PCR and then cloned into pCDH-5′ (addiction, vector) and pCDH-3′ (addiction gene). Subsequently, the plasmid was transformed into E. coli DH5α. The resulting plasmid was identified by restriction enzyme digestion and sequenced to confirm the authenticity of the insert. The plasmid was then shipped and transfected into HEK-293 cells using Lipofectamine 2000 (Thermo Scientific) according to the manufacturer’s protocol. The transfected cells were selected with puromycin (1 µg/mL) to resist the plasmid. The plasmid was then used to transduce 2.27.1 cell line with puromycin (1 µg/mL) selection for 4 days.

**Establishment of LECs overexpressing LETR1 and SEMA3C.** 1.2 × 10⁵ LECs per well were seeded into six-well plates and cultured overnight. LECs were then infected with medium containing viruses overexpressing LETR1 and SEMA3C diluted in 0.5 µL pseudovirus in 4 mL DMEM/F12 medium. LECs were then sealed with paraffin and centrifuged at 340 × g for 1.5 h at room temperature. After 16–24 h, the virus-containing medium was changed. 24 h later, infected LECs were subjected to antibiotic selection using puromycin at 1 µg/mL for at least 6 h. Infected LECs were then transfected with 20 nM of scrambled control ASO or LETR1-ASO2 to determine the percentage of RNA retrieval. DNA, on the other hand, was eluted twice using 150 µL DNA elution buffer (50 mM NaHCO3, 1% SDS, 200 mM NaCl, 1 mM PMSF, 0.1% Triton X-100) in a thermomixer at room temperature and stored at -80 °C. The RNA and DNA were then washed twice with 70% ethanol (v/v) and dried under vacuum at room temperature for 20 min. DNA concentration was determined on a Qubit fluorometer (Thermo Scientific) and DNase I (Invitrogen) treatment and RNase-free water were used to prepare libraries using the TruSeq DNA-Seq kit (Rubicon), and sequencing was performed according to the manufacturer’s protocol on a HiSeq system (Illumina).

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Pre-cleared nuclear extract was further diluted to 2 mL using RNA pull-down buffer and incubated with 100 pmol biotinylated RNA for 1 h at 4 °C on a rotary shaker. The streptavidin magnetic beads were washed twice with 20 mL standard wash buffer and incubated for 45 min at 4 °C. Beads were carefully washed five times in RNA pull-down buffer. Bound proteins were finally eluted twice by adding 3 mM biotin in PBS (Ambion) to the beads and incubating them for 20 min at room temperature and for 10 min at 65 °C. Eluted proteins were subjected to protein identification by mass spectrometry at the Functional Genomics Center Zurich (FGCZ). For the analysis performed by the FGCZ, samples were precipitated with an equal volume of 20% trichloroacetic acid (Sigma-Aldrich) and washed twice with cold acetone. The dry pellets were dissolved in 45 µl buffer (10 mM Tris, 2 mM CaCl₂, pH 8.2) and 100 mg/ml proteinase K (10-min HCl) for digestion, which was carried out in a microwave instrument (Discover System, CEM) for 30 min at 5 W and 60 °C. Samples were dried in a SpeedVac (Savant) and dissolved in 20 µl of 0.1% formic acid (Rothil) for liquid chromatography mass spectrometry analysis. For each sample, 1 µl was injected on a nanoAcquity UPLC (Waters Inc.) connected to a Q Exactive mass spectrometer (Thermo Scientific) equipped with a Digital PicoView emitter (New Objective). Spectra were searched against a swissprot network for the proteins identified in the analysis of RNA-pull-down data. Spectra were searched against a swissprot proteome database using the Mascot software (ver. 2.5.1.3, Matrix Science, https://www.matrixscience.com). The human PPI database was used for the analysis, while default values were used for the rest of the parameters. The identified proteins are listed in Supplementary Data 12.

**Native RNA immunoprecipitation followed by qPCR.** RIP experiments were performed as previously described with minor modifications. To prepare nuclear lysate, 40 million LECs per replicate were collected and washed once with DPBS. The cell pellet was resuspended in 40 mL consisting of 8 mL DPBS, 24 mL RNase-free H₂O, and 8 mL nuclear isolation buffer (1.28 M Sucrose, 100 mM Tris-HCl pH 7.5, 20 mM MgCl₂, 4% Triton X-100). After mixing by inversion, LECS were let stand on ice for 20 min with occasional mixing. Cells were then centrifuged at 600 × g for 15 min at 4 °C. Nuclear pellet was resuspended in 2 mL of RIP buffer (130 mM KCl, 25 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8, 0.5% NP-40, 0.5 mM DTT, 0.5% Nonidet P-40, 0.1% Triton X-100, 1 mM Na butyrate). Nuclei were sonicated using a Covaris S220 system (Covaris, peak power: 140, duty factor: 50%, cycles per burst: 200) for twelve ON/OFF cycles of 1 min. Sonicated lysate was finally transferred to a 1.5 mL Eppendorf tube and centrifuged at full speed for 10 min to collect cell debris. In the meantime, antibody pre-binding was performed as follows. First, 1.25 mg Dynabeads protein A or G magnetic beads (Thermo Fisher Scientific) were washed twice with 1 mL blocking solution (0.5% bovine serum albumin (BSA) in DPBS). Then, Dynabeads were incubated with 2 µg mouse anti-RNA Pol II (Sigma), 2 µg rabbit anti-H3K4me3 (Diagenode), 2 µg rabbit anti-H3K27me3 (Diagenode), or 2.5 µg rabbit anti-RBBP7 (Cell Signaling) antibodies for 3 h at 4 °C on a rotating platform. As control antibodies, rabbit or mouse IgG controls were used (Sigma). 200 µl of the nuclear lysate (corresponding to 1 × 10⁶ cells) was diluted to a final volume of 600 µL with lysis buffer 3 and the addition of Triton X-100 (final concentration: 1%) and 100 U/mL RNase inhibitors (Thermo Fisher Scientific). After washing once with 1 mL blocking solution, antibody-bound beads were added to 600 µL LEC nuclear lysate and incubated overnight at 4 °C with gentle rotation. Two aliquots of 6 µL nuclear lysate were used as input DNA controls. The next day, antibody-bound beads were washed twice with 1 mL RIPA buffer (50 mM HEPES-KOH pH 7.5, 500 mM LCl, 1 mM EDTA pH 8, 1% i geal CA-630, 0.7% sodium deoxycholate, 0.5% N-laurylsarcosine) for each 1 × 10⁶ cells. Sonication of LEC nuclei was performed using a Covaria S220 system (Covaria, peak power: 140, duty factor: 50%, cycles per burst: 200) for twelve ON/OFF cycles of 1 min. Sonicated lysate was finally transferred to a 1.5 mL Eppendorf tube and centrifuged at full speed for 10 min to collect cell debris. In the meantime, antibody pre-binding was performed as follows. First, 1.25 mg Dynabeads protein A or G magnetic beads (Thermo Fisher Scientific) were washed twice with 1 mL blocking solution (0.5% bovine serum albumin (BSA) in DPBS). 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The next day, antibody-bound beads were washed twice with 1 mL RIPA buffer (50 mM HEPES-KOH pH 7.5, 500 mM LCl, 1 mM EDTA pH 8, 1% i geal CA-630, 0.7% sodium deoxycholate and once with TBS (20 mM Tris-HCl pH 8, 150 mM NaCl). To elute the DNA, antibody-bound beads and DNA samples were incubated with a total of 200 µL elution buffer (30 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 1% SDS) at 65 °C overnight with slow rotation. Eluate was collected in a new Eppendorf tube, and 100 µL TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) was added to dilute SDNA present in the elution buffer. Diluted eluate was incubated with 27 µg RIPA buffer A (Thermo Fisher Scientific) at 30 min at 37 °C to overnight, followed by 20 µg Dynabeads Protein A (Thermo Fisher Scientific) for 1 h at 55 °C. DNA was isolated using the MiniElute PCR purification kit (Qiagen). DNA was eluted in 26 µL RNase/DNase-free water. For protein detection, protein input and antibody-bound beads were diluted to a final volume of 20 µL with lysis buffer 3, 4 × LDS sample buffer (final conc. 1×), and 10 × reducing agent (final conc. 1×). Then, samples were incubated for 15 min at 70 °C and western blot was performed as described above. In the case of RBBP7, we used a mouse anti-RBBP7 antibody (Origene) at a dilution of 1:1000 given the very high marking of IgG heavy chain in IP samples (Fig. 8f). Before performing qPCR, eluted DNA was diluted 1/10 with RNase/DNase-free water. qPCR was performed as described above. Target region Ct values were normalized to DNA input, and then enrichment against IgG isotype control was calculated. Primers targeting KIF4 and SEMA3C genomic loci were designed at the LETRI-binding regions as determined by ChIP-Seq (Supplementary Figure 8e, f and Supplementary Data 10) and at the TSSs as determined by CAGE-Seq (Supplementary Figure 8e, f). GAPDH, MYT122, and UNTR43 were used as controls. Primers for ChIP-qPCR and RNA-Seq in Supplemental Data.

**Statistical analysis.** All statistical analyses were performed using GraphPad Prism software (version 7.0.0). P values were calculated after performing ordinary and RM two- or one-way analysis of variance with Dunnett’s correction, paired or unpaired Student’s t test as indicated. Statistical significance was determined when P < 0.05. If not alternatively specified, each bar represents mean values with SD.

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

**Data availability**. All unprocessed sequencing data are deposited in the DDBJ DNA public repository with the following accession numbers: DRA009940, DRA009941, and DRA009942 for CAGE-Seq, ChIP-Seq, and RNA-Seq, respectively. LC/MS data are available at the ProteomXchange.
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Author contributions
L.D. design the project, performed the in silico analyses and wet-lab experiments, and wrote the manuscript. S.A. performed in silico analysis of ASO-mediated knockdown CAGE sequencing data, helped analyze the RNA immunoprecipitation data, discussed and interpreted the results, and strongly contributed to figures, general discussion, and writing of the manuscript. E.S. helped perform in vitro experiments, established the cell cycle progression method, and strongly contributed to general discussions and writing of the manuscript. C.T. isolated the adult LECs and BECs, performed the FACS sorting of ex vivo LECs and BECs, and strongly contributed to experimental design, general discussion, and comments on the manuscript. T.K. helped with the ChIRP-Seq experiment by performing the library preparation and sequencing, contributed to general discussions and comments for the manuscript. C.H. helped analyze the RNA-Seq and CAGE-Seq data of LECs and BECs, and contributed to general discussions and comments for the manuscript. S.D.B. helped perform in vitro studies and contributed to general discussions as well as added comments to the manuscript. D.M. performed the subcellular fractionation of lncRNA targets in LECs and BECs and provided comments on the manuscript. Y.H. supported L.D. in the ChIRP-Seq analysis and isolation of adult LECs and BECs, contributed to general bioinformatics discussions, helped to interpret the results, and provided comments for the manuscript. J.K. performed the gel-based sprouting assay, supported L.D. in the isolation of adult LECs and BECs, and provided comment to the manuscript. M.Da. performed lineage check of LECs and BECs and isolated RNA to be subjected to both RNA-Seq and CAGE-Seq, and provided comments for the manuscript. L.Di. made crucial contributions to in vitro as well as general experimental design, helped interpret the results, and contributed to writing the manuscript. P.C. and M.J.L.dH. discussed and interpreted the results, provided crucial comments for the progress of the project, and revised the manuscript. J.W.S. and M.D. supervised and guided the entire project, provided resources for all the experiments, helped in interpreting the results and writing the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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