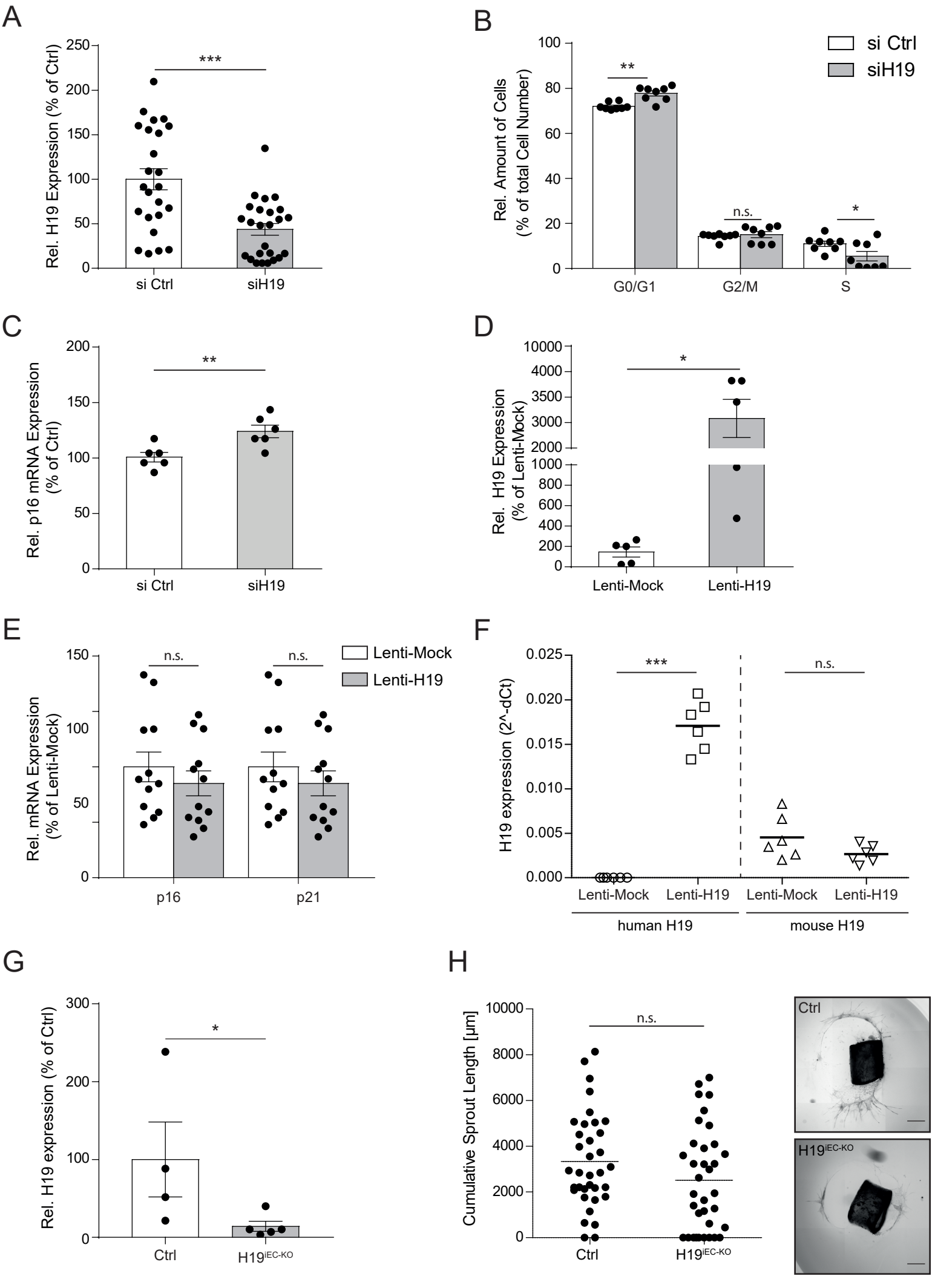
CVR-2018-725

Supplementary Information

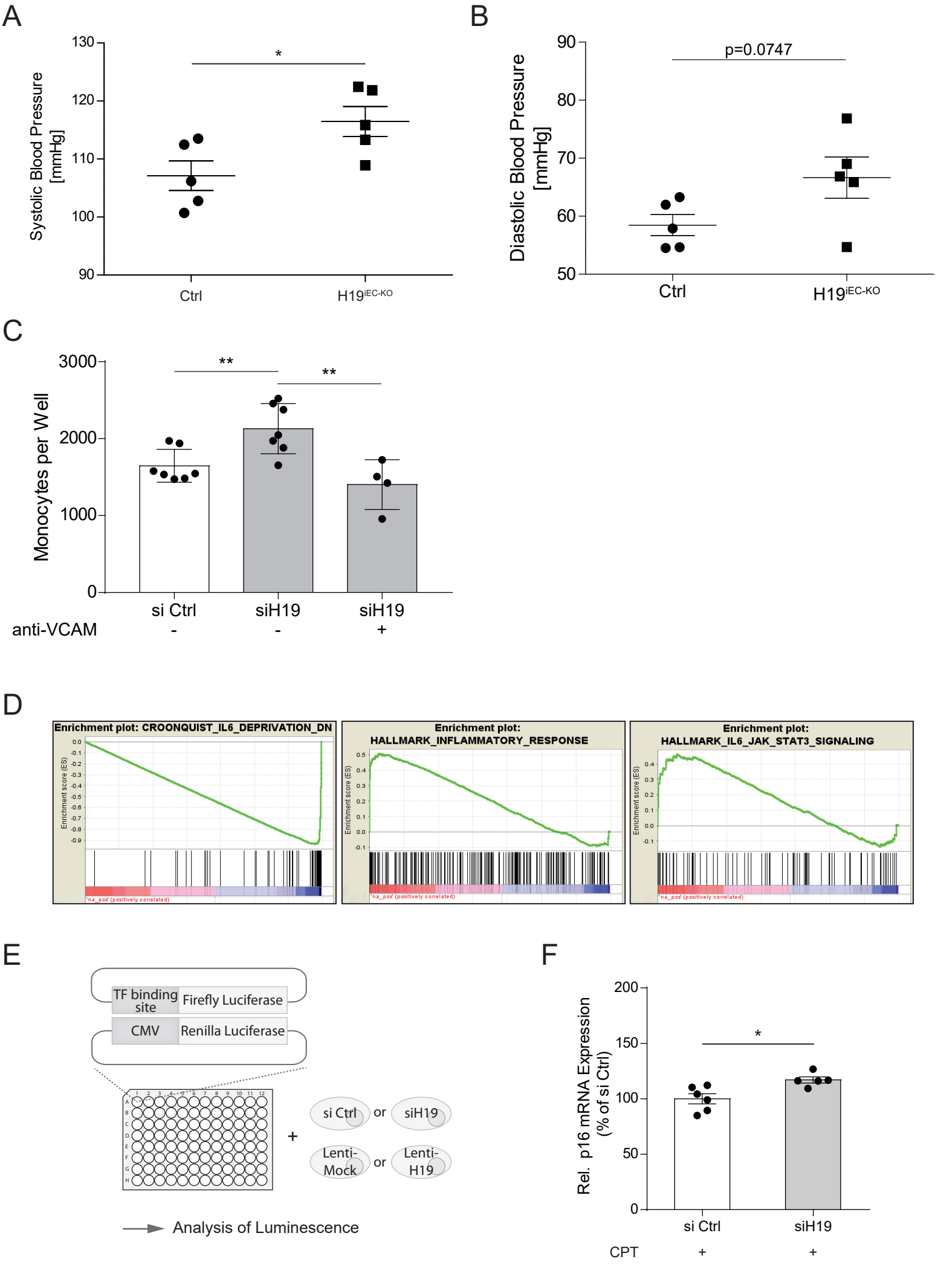
Hofmann et al.

Long non-coding RNA H19 regulates endothelial cell aging via inhibition of Stat3 signaling

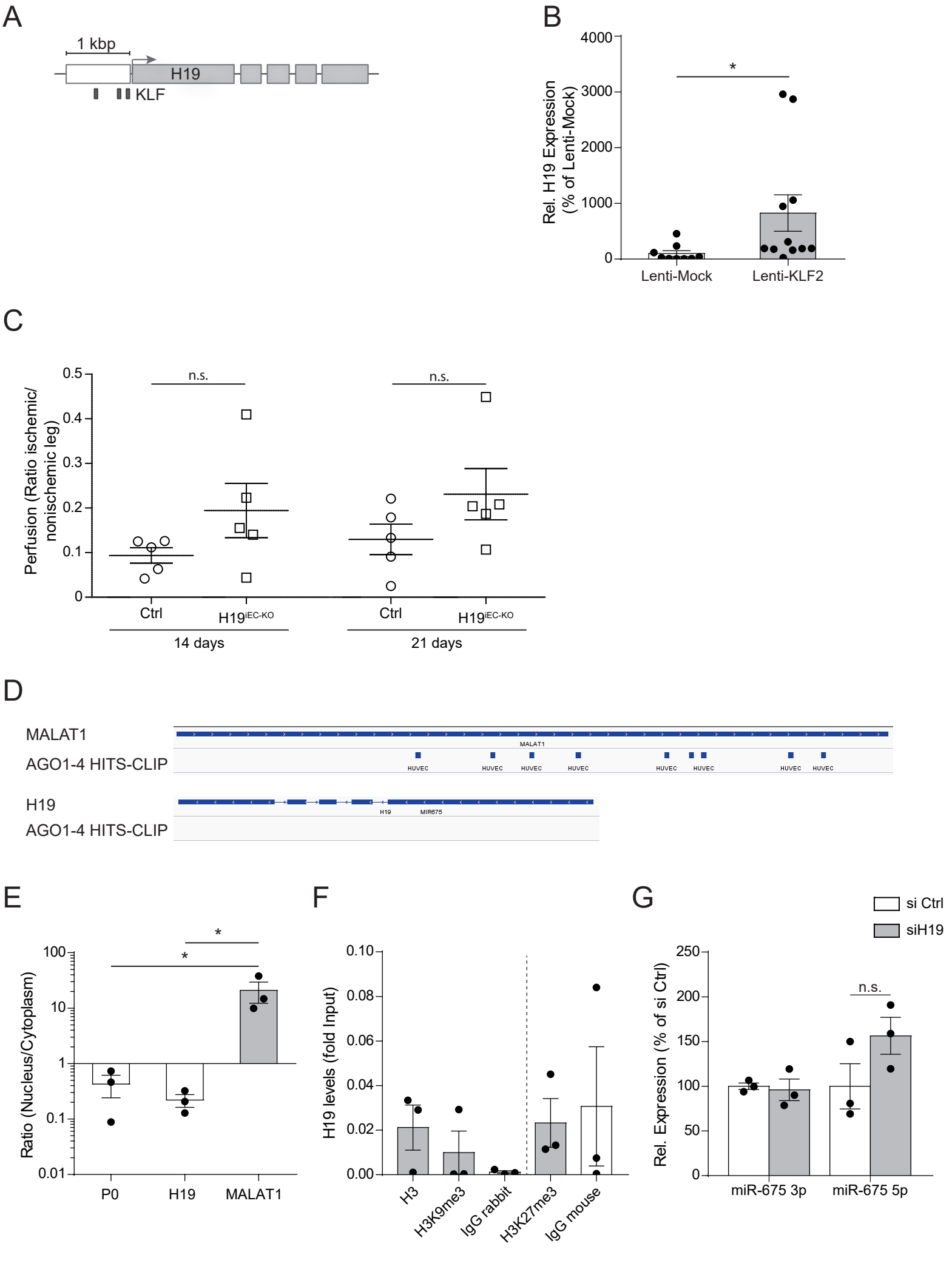
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Table 1

A

Antibodies and Staining Reagents	
STAT3	Cell Signaling #4904
pSTAT3 TYR705	Cell Signaling #9145
p21	Cell Signaling #2947
Tubulin α	Thermo Fisher RB-9281-04
Actin	Dianova DLN-07274
H3	Abcam ab1791
H3K9me3	Abcam ab 8898
H3K27me3	Abcam ab6002
Rabbit IgG	Merck Millipore PP64B
Mouse IgG	Abcam 18394 mouse IgG3κ
CD45	Abcam ab25386
Smooth Muscle Actin	Sigma Aldrich C6198
Laminin	Abcam ab 11575
Isolectin B4	Vector Laboratories B-1205
Wheat Germ Agglutinin	Thermo Fisher W32466

B

Human Primers		
	forward	reverse
RPLP0	TCGACAATGGCAGCATCTAC	ATCCGTCTCCACAGACAAGG
GAPDH	ATGGAAATCCCATCACCATCTT	CGCCCCACTTGATTTTGG
H19	TCAAGCCTGGGCCTTTGAAT	GGCTGATGAGGTCTGGTTCC
IL-6	GCAGAAAAAGGCAAAGAATC	CTACATTTGCCGAAGAGC
KLF2	CAAGACCTACACCAAGAGTTCG	CATGTGCCGTTTCATGTGC
ICAM-1	ATGCCCAGACATCTGTGTCC	TCCTTTTTAGGCAACGGGGT
VCAM-1	GGGAAGCCGATCACAGTCAA	CTCCAGCCTGTCAAATGGGT
p21	AGTCAGTTCCTTGTGGAGCC	CATTAGCGCATCACAGTCGC
STAT3	ACCATTGACCTGCCGATGTC	AAGGTGAGGGACTCAAACTGC
Mouse Primers		
RPLP0	GCGTCCTGGCATTGTCTGT	GAAGGCCTTGACCTTTTCAGTAAG
H19	CAGAGCAAAGGCATCGCAAAG	GCCACGTCCTGTAACCAAAA

C

Human siRNAs	Sequence	Supplier
si Ctrl	CGUACGCGGAAUACUUCGA	Sigma Aldrich
siH19	GCACUACCUGACUCAGGAATT	Qiagen

Fig. S1: (*A*) H19 levels were analyzed upon siRNA-mediated H19 depletion 48 h after transfection in HUVECs by qRT-PCR. Results are represented as mean ± SEM from n = 25 biological replicates per group. Mann-Whitney test was used to determine statistical significance. (*B*) BrdU flow cytometry assay was performed to analyze EC proliferation upon H19 depletion 48 h after transfection in hCoAECs. Results are represented as mean ± SEM from n = 8 biological replicates per group. Unpaired t-test was used to determine statistical significance. (*C*) mRNA expression of p16 was analyzed upon H19 depletion in HUVECs 48 h after transfection by qRT-PCR. Results are represented as mean ± SEM from n = 6 biological replicates per group. Unpaired t-test was used to determine statistical significance. (*D*) Expression of H19 upon lentivirus-mediated H19 overexpression in HUVECs was analyzed ≥ 7 d after transduction by qRT-PCR. Results are represented as mean ± SEM from n= 5 biological replicates per group. Unpaired t-test was used to determine statistical significance. (*E*) mRNA expression of p16 and p21 was analyzed upon lentiviral-mediated overexpression of H19 in HUVECs ≥ 7 d after transduction by qRT-PCR. Results are represented as mean ± SEM from n = 12 biological replicates per group. Unpaired t-test was used to determine statistical significance. (*F*) Human and mouse H19 levels were analyzed in mouse aortic rings upon lentiviral-mediated overexpression of human H19 ≥ 7 d after transduction by qRT-PCR. Results are represented as mean ± SEM from 6 mice per group. Unpaired t-test was used to determine statistical significance. (*G*) H19 expression was analyzed in liver ECs from $H19^{iEC\text{-}KO}$ mice and Ctrls. Results are represented as mean ± SEM from 5 mice per group. Mann-Whitney test was used to determine statistical significance. (*H*) The cumulative sprout length from aortic rings from Ctrl and $H19^{iEC\text{-}KO}$ animals was analyzed. ECs were stained with Isolectin B4 and pictures from immunofluorescence microscopy analysis are represented inverted. Results are represented as mean ± SEM from 6 mice per group. Unpaired t-test was used to determine statistical significance, 6 rings per animal were analyzed. The scale bar denotes 500 µm. (*p<0.05, **p<0.01, ***p<0.001, n.s. = not significant).

Fig. S2: (*A/B*) Blood pressure was analyzed in $H19^{iEC\text{-}KO}$ animals and Ctrl littermates 2 weeks after induction of H19 deletion for 10 days. Means from days 5-8 are displayed. Results are represented as mean ± SEM from 5 mice per group. Unpaired t-test was used to determine statistical significance (*C*) Adhesion of monocytes to HUVECs was measured upon siRNA-mediated depletion of H19 and stimulation with 100 ng/mL IL-6 and sIL6-Rα for 16 h in HUVECs, 48 h after transfection. ICAM-1 was blocked with a specific antibody. Results are represented as mean ± SEM from n = 7 biological replicates per group. One-way ANOVA followed by Tukey's post-test was used to determine statistical significance. (*D*) Enrichment plots of pathway analysis of microarray data upon siRNA-mediated H19 depletion in HUVECs. (Croonquist_IL6_Deprivation: GSEA M18506, [64]). *(E)* A high-throughput transcription factor activity assay was performed, where HeLa cells were reverse transfected with reporter plasmids carrying a binding site for a specific transcription factor upstream of a firefly luciferase and a plasmid with renilla luciferase under the control of a CMV promoter. Luminescence was measured 48 h after H19 depletion or 7 days after H19 overexpression and 24 h after reporter plasmid transfection (*F*) Expression of p16 mRNA upon depletion of H19 and inhibition of STAT3 activation by CPT treatment was analyzed by qRT-PCR 48 h after transfection in HUVECs. Cells were stimulated with 100 ng/µL IL-6 and sIL-6Rα for 16 h and 20 µM CPT for 17 h before the start of the experiment. Results are represented as mean ± SEM from n = 6 biological replicates per group. Unpaired t-test was used to determine statistical significance. (*p<0.05).

Fig. S3: (*A*) Schematic representation of the genetic locus of human H19, showing conserved transcription factor binding sites. (B) HUVECs were transduced with lentivirus for 24 h to overexpress KLF2 or with mock lentivirus. At least seven days after transduction, H19 expression was measured qRT-PCR. Results are represented as mean ± SEM from n = 11 biological replicates per group. Unpaired t-test was used to determine statistical significance. (*C*) Toe perfusion of endothelial specific inducible H19 KO mice ($H19^{iEC\text{-}KO}$) and Ctrl littermates was analyzed 14 and 21 days after hindlimb ischemia surgery by Laser Doppler

perfusion imaging. Results are represented as mean ± SEM from 5 mice per group. Unpaired t-test was used to determine statistical significance (*D*) AGO1-4 HITS-CLIP data from HUVECs were analyzed and binding sites in the MALAT1 and H19 transcripts are depicted. (*E*) The intracellular localization of different transcripts in HUVECs was analyzed by qPCR. Results are represented as mean ± SEM from n = 3 biological replicates per group. Unpaired t-test was used to determine statistical significance. *(F)* H19 interaction with histones in HUVECs was analyzed in a RNA immunoprecipitation. Results are represented as mean ± SEM from n = 3 biological replicates per group. Unpaired t-test was used to determine statistical significance. (*G*) The expression of miR-675 3p and 5p in HUVECs was analyzed upon siRNA mediated H19 depletion by 48 h after transfection. Results are represented as mean ± SEM from n = 11 biological replicates per group. Unpaired t-test was used to determine statistical significance. (*$p<0.05$).

Table S1: (*A*) List of used antibodies and staining reagents. (*B*) List of primers for qPCR. (*C*) List of siRNAs and LNA GapmeRs.