

Supplemental Materials

Supplemental methods

Exclusion and inclusion criteria

Carotid patients underwent detailed neurological examination within two days before and after the procedure by a neurologist. All clinical data available were recorded for each patient, including age, sex, neurological symptom, hypertension, hyperlipidemia, smoking status, accompanying diseases and medication. The study was performed according to the Guidelines of the World Medical Association Declaration of Helsinki. The local ethics committee of Klinikum rechts der Isar, Technische Universitaet Muenchen, approved the study and written informed consent was given by all patients.

Inclusion criteria:

- Carotid artery stenosis of $\geq 70\%$ following ultrasound criteria
- Stenosis treatable with CEA (carotid endarterectomy)
- Written informed consent from the patient
- Availability of patient medical history and clinical data
- Sufficient quality of plaque tissue for morphological characterisation

Exclusion criteria:

- Non-atherosclerotic origin of stenosis (dissection, dysplasia, floating thrombus)
- Known intracranial angioma or aneurysm
- Autoimmune disease
- Cancer disease

Figure S1

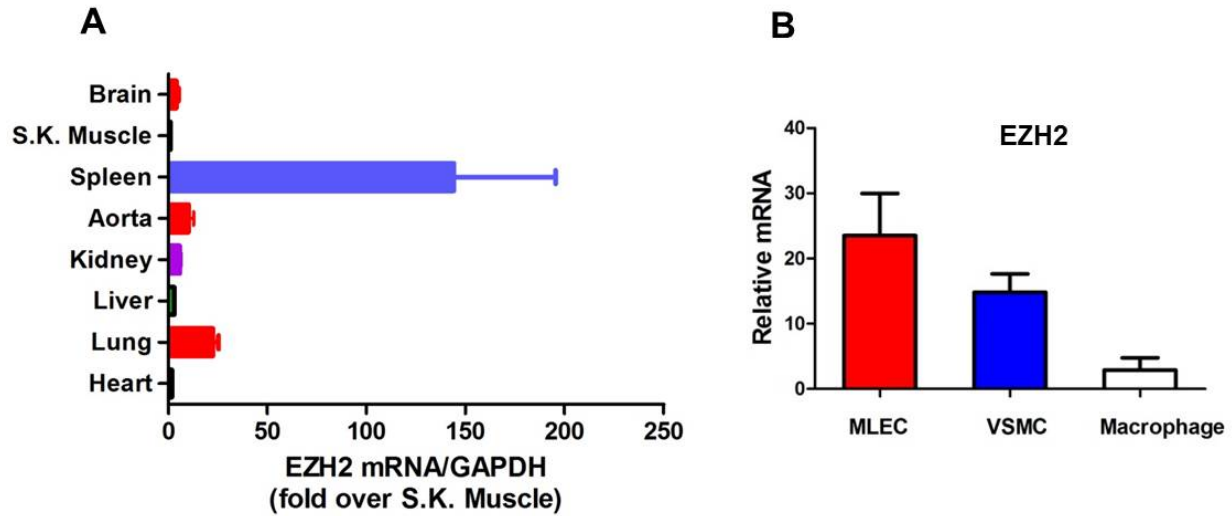


Figure S1. Tissue and cellular expression of EZH2 mRNA. (A) EZH2 expression in mice tissues. **A**, Indicated tissues were collected from 3-month-old C57Bl/6J mice (n=3). Then, total RNA was isolated using TRIzol Reagent from Thermo Fisher Scientific (Cat# 15596026), and homogenates were prepared by using Precellys® Minilys Bead Homogenizer (Bertin Technologies, France). Real time PCR was performed to determine relative EZH2 gene expression using GAPDH as normalization control.

B, EZH2 gene expression in vascular cells from mice. Total RNAs from mouse lung endothelial cells (MLEC), mouse vascular smooth muscle cells (VSMC), and mouse peritoneal macrophages were extracted and EZH2 gene expression was determined by real-time PCR using GAPDH as normalization control. Data were obtained from primary cells isolated from 2-3 mice.

Figure S2

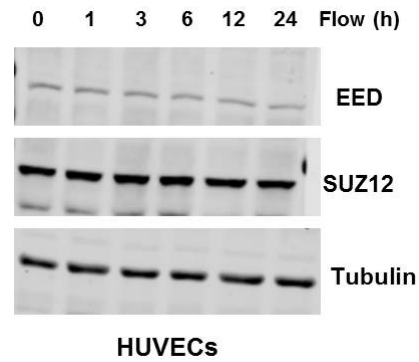


Figure S2. Laminar flow does not affect protein expression of EED and SUZ12. HUVECs were exposed to laminar flow for indicated time, and then whole cell lysates were collected for Western blot analysis. α -tubulin was used as loading control. Representative images from three independent experiments were shown.

Figure S3

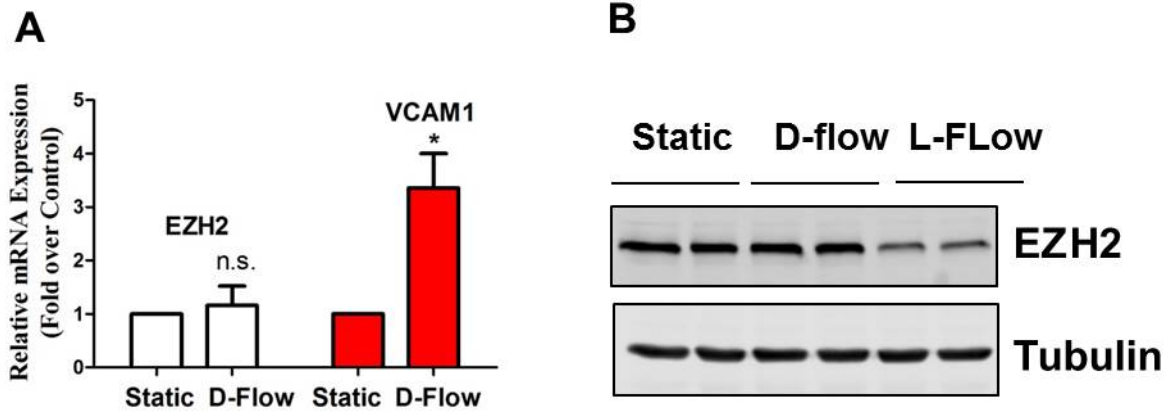


Figure S3. No significant effect of disturbed flow on EZH2 mRNA and protein expression.

A, HUVECs were untreated (static), or exposed to disturbed flow (D-flow) for 24 h, and then total RNA were collected for real-time PCR (n=3) to detect EZH2 and VCAM1 gene expression (using GAPDH as loading control), n.s. indicates not statistically significant.

B, HUVECs were untreated (static), or exposed either to laminar flow (L-flow) or disturbed flow (D-flow) for 24 h, and then, whole cell lysates was collected for Western blot analysis (using α -tubulin as loading control). Representative images from three independent experiments were shown.

Figure S4

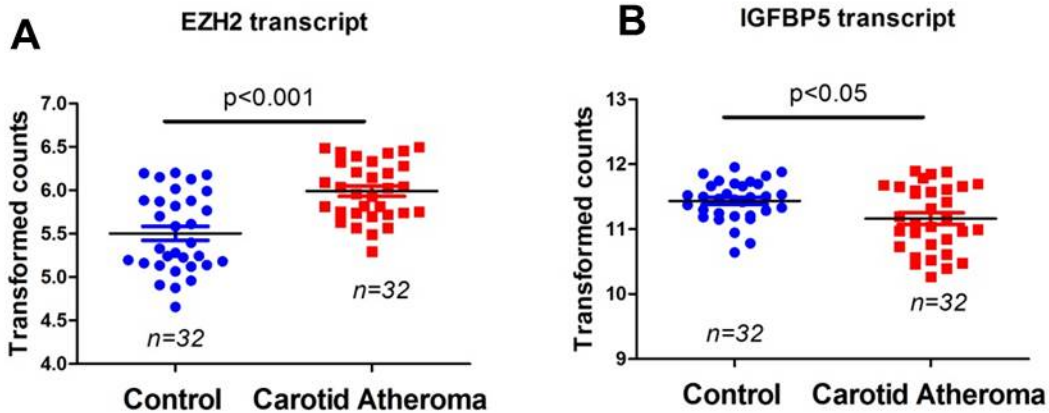


Figure S4. EZH2 and IGFBP5 expression in human atherosclerotic plaques areas compared with adjacent plaque-free areas. The expression levels (transformed counts) of EZH2 (A) and IGFBP5 (B) are mined from NCBI GEO database with accession number of GDS5083¹.

Table S1. Information of atherosclerotic patients and healthy controls

Advanced Atherosclerotic Carotid Plaques	Sample#	Age (yr)	Gender (M/F)	Classif. AHA *	Stability
	AC1	72	M	VI	unstable
	AC2	67	F	VI	unstable
	AC3	58	M	VI	unstable
	AC4	57	M	VI	unstable
	AC5	62	M	VI	unstable
	AC6	75	M	VI	unstable
	AC7	75	M	VI	unstable
	AC8	67	M	VI	unstable
	AC9	75	M	VI	unstable
	AC10	72	F	VI	unstable

Healthy Carotid Artery	Sample#	Age (yr)	Gender (M/F)
	HC1	57	M
	HC2	55	F
	HC3	68	M
	HC4	58	M
	HC5	66	F
	HC6	49	M
	HC7	51	F
	HC8	59	M
	HC9	58	M
	HC10	62	M

Table S2. Detailed patient characteristics

	Unstable Carotid Plaques	Healthy Carotid Vessels*
Number of patients	10	10
Sex: female/male	2 / 8	3 / 7
Age (years)	68.7 ± 7.0	58.3 ± 6.0
Symptoms	7 (70%)	0
Hypertension	8 (80 %)	-*
Smokers	5 (50 %)	-
Hyperlipidaemia	1 (10 %)	-
Coronary heart disease	1 (10 %)	-
Peripheral arterial disease	0	-
Chronic kidney disease	2 (20 %)	-
Diabetes mellitus	1 (10 %)	-
ASA / Clopidogrel	10 (100%)	-
Beta-blocker	2 (20%)	-
ACE	2 (20%)	-
Statins	9 (90%)	-
Diuretics	3 (30%)	-

* No information available is due to the confidentiality policy of the hospital we do not possess any other information about the donors of the healthy tissue vessels. Detailed information is provided in the table below.

	Age	Sex	Sympt.	Klassif.	Stab.	Rup.	Hypertony	Diabetes	Hypercholesterinemia	Hyperlipidemie	Nikotinabusus	Chronic kidney disease	coronary heart disease	Peripheral arterial disease	ASS/Clopid	Beta-Blocker	ACE	Statins	Diuretics	Hamstoff (7-18)	Kreatinin (0,7-1,3)	Creatinkinase (<174)	Leukozyten (4-9)	Erythrozyten (4-6)	Thrombozyten (150-450)	Hb (14-18)	Hämatokrit (40-48)	MCH (27-32)	MCV (82-92)	MCHC (32-36)	Natrium (135-145)	Kalium (3,5-5,0)	Kalzium (2,2-2,6)		
AC1	62	1	1	VI	0	1	1	0	0	1	0	0	0	0	1	1	1	1	1	25	1.3	69	6.69	4.9	158	14.4	41.3	30	85	34.9	142	4.2	-		
AC2	75	1	0	VI	0	1	1	0	0	1	0	0	0	0	1	0	0	1	0	13	1	-	6.2	5.2	166	16.3	48.1	31	93	33.9	140	4.7	-		
AC3	75	1	0	VI	0	1	1	0	0	1	1	0	1	0	1	1	1	1	1	0	19	1.1	129	7.31	4.6	319	14.3	43.2	31	95	33.1	138	4.8	-	
AC4	67	1	1	VI/VII	0	1	1	0	0	0	0	1	0	0	1	0	0	0	1	22	1.2	-	7.77	5.2	153	15.8	45.1	30	86	35	141	4.1	-		
AC5	75	1	1	VI	0	1	1	0	0	1	0	1	0	0	1	0	0	1	1	20	1.1	177	7.36	4.5	136	14	40.3	31	90	34.7	145	4.3	-		
AC6	72	0	1	VI	0	1	0	0	0	1	1	0	0	0	1	0	0	1	0	18	0.9	64	4.72	3.9	225	13.7	41.2	35	105	33.3	142	4.5	-		
AC7	72	1	1	VI	0	1	1	0	0	1	1	0	0	0	1	0	0	1	0	17	1.1	-	5.28	3.8	240	12.8	36	34	96	35.6	133	4.7	-		
AC8	67	0	1	VI/VII	0	1	0	1	0	1	0	0	0	0	1	0	0	1	0	19	0.5	134	6.81	4.4	268	13.8	40.2	31	91	34.4	141	4.2	-		
AC9	57	1	0	VI	0	1	1	0	0	1	1	0	0	0	1	0	0	1	0	14	0.8	434	5.53	4	258	13.8	38.4	34	95	35.9	128	4.9	-		
AC10	58	1	1	VI	0	1	1	0	1	0	1	0	0	0	1	0	0	1	0	23	0.9	118	8.17	4.7	197	14.7	41.6	31	88	35.3	142	4.2	-		
HC1	57	1																																	
HC2	55	0																																	
HC3	68	1																																	
HC4	58	1																																	
HC5	66	0																																	
HC6	49	1																																	
HC7	51	0																																	
HC8	59	1																																	
HC9	58	1																																	
HC10	62	1																																	

Table S3. Primers sets for real-time PCR and ChIP assay
Real time PCR

QuantiTect Primer assays for human KLF2 (Hs_KLF2_1_SG, # QT00204729), NOS3 (Hs_NOS3_1_SG, #QT00089033), β -actin (Hs_ACTB_1_SG, # QT00095431) and GAPDH (Hs_GAPDH_1_SG, # QT00079247) were obtained from QIAGEN.

Gene name	Sequence (5'-3')
hEZH2	Forward: GCTGACCATTGGGACAGTAA Reverse: CAGATGGTGCCAGCAATAGA
mEZH2	Forward: GGAGTTTGCTGCTGCTCTTA Reverse: GCTGGGTCTGCTACTGTTATTC
hIGFBP5	Forward: TGAGATGAGACAGGAGTCTGAG Reverse: GTCACAATTGGGCAGGTACA
mGAPDH	Forward: AACAGCAACTCCCCTCTTC Reverse: CCTGTTGCTGTAGCCGTATT
m β -actin	Forward: CCGTAAAGACCTCTATGCCAAC Reverse: AGGAGCCAGAGCAGTAATCT

ChIP assay

Gene	Sequence
hIGFBP5-promoter	Forward: GGCCCTGCCCAATAGAAATA Reverse: GGGTTCTTAGAGGGAAGAAAGG

Table S4. Sources of antibodies used in this study

Antibodies	Supplier, Cat. No., Applications
EZH2	BD, #612666 (WB, IF) Cell Signal, #5246P (WB, IF), #3147S (WB)
H3K27me3	Active Motif, #39155 (IF, WB, ChIP-qPCR) Cell Signal Tech, #9733P (WB)
H3K9me3	Abcam, #Ab8898 (WB)
H3K9Ac	Sigma, SAB5100007 (WB)
Histone H3	Active Motif, #39763 (WB)
SUZ12	EMD, #04-046 (WB)
EED	EMD, #05-1320 (WB)
eNOS	BD, #610297 (WB)
α-Tubulin	Sigma, #T5168 (WB)
GAPDH	EMD, #AB2302 (WB)
ICAM1	Santa Cruz Biotechnology, #sc-8439 (WB)
VCAM1	Santa Cruz Biotechnology, #sc-1504 (WB)
IgG	Santa Cruz Biotechnology, #sc-2027 (ChIP)

WB, Western blot; IF, Immunofluorescence

Table S5. Flow induced endothelial transcriptome by RNA-sequencing, please refer to spreadsheet in Excel File

Table S6. TargetScan_7.0_ENST00000478654.1_predicted_targeting_details, please refer to spreadsheet in Excel File

Table S7. List of upregulated genes by EZH2 depletion and laminar flow, commonly regulated genes were mapped using Venn Diagram, please refer to spreadsheet in Excel File

Supplemental references

1. Ayari H, Bricca G. Identification of two genes potentially associated in iron-heme homeostasis in human carotid plaque using microarray analysis. *J Biosci.* 2013; 38: 311-5.