Supplemental Materials and Methods

Cell culture.

Human Umbilical Vein Endothelial Cell (HUVEC) and HMVEC-dLyAd-Adult Human Dermal Lymphatic Microvascular Endothelial Cells (HDLEC) were purchased from Lonza. HUVEC were cultured in M199 medium supplemented with 20% FBS, 100μg/ml ECGS and 100μg/ml heparin (Sigma). (HDLEC) were purchased from Lonza. HDLEC were cultured in EBM-2MV medium (Lonza). HUVEC and HDLEC at passage 2 to 5 were used for experiment. Human Embryonic Kidney (HEK) 293 cells were cultured in DMEM with 10% FBS. All cells were cultured in 5% CO_2 at 37 degree.

Production and infection of lentiviruses and adenoviruses

Production of *pLVX-HA-RAF1 WT*, *-S259A* and *-mCherry* lentiviruses and constitutive active ERK adenoviruses were described previously[32]. Control lentiviruses were generated using pLVX-IRES-puro vector. For lentiviral infection, cells were incubated with the lentivirus in growth medium in the presence of 8 μg/ml polybrene (Sigma) for 8 hours. For adenoviral infection, cells were incubated with the adenovirus (MOI=100) in growth medium for overnight.

X-gal staining

X-gal staining was performed using a β-Gal Expression kit from Millipore followed the manufacturer's instruction.

RNAi

Human *SEMA6A*, *NTNG1* and *EFNB2* Flexitube siRNAs and AllStars Negative Control siRNA were purchased from QIAGEN, Inc. Cells cultured in 6-well plate at 50% confluence were transfected with 5nmol siRNA using TransPass R2 Transfection Reagent (NEB) following

the manufacturer's instructions. Forty eight hours post siRNA transfection, cells were used for experiments. Knocking down efficiency was determined by qPCR or Western blotting.

Supplemental figure legends

Figure S1. Endothelial-expression of *RAF1*^{S259A} blocks inhibition of RAF1 by AKT.

(**A**) RAF1-AKT crosstalk. Upon extracellular signal stimulation, AKT phosphorylates RAF1 at serine 259 and inhibits RAF1 activation. (**B**) Immunoblot of HUVECs infected with empty control, wild type HA-*RAF1* (WT) or HA-*RAF1*^{S259A} (S259A) lentiviruses. (**C**) Immunblot showing RAF1 expression in E14.5 yolk sac. (**D**) RAF1 protein levels shown in (C) were quantified by densitometry and are represented as ratio of RAF1 versus β-tubulin.

Figure S2. Whole mount immunofluorescent staining of E14.5 (A) and E15.5 (B) skin. The dorsal skin of S259A embryos and the control littermates were stained with indicated antibodies. CX40 and Endomucin stain arteries and veins respectively. Scale bars: 50 μm (A); 200 μm (B). a, artery; v, vein.

Figure S3. Decreased capillary bed size in S259A embryos.

(**A**) Whole mount immunofluorescent staining of E14.5 skin. (**B and C**) Quantitative analysis of branch points (**B**) and number of vessel segments (**C**) based on CD31 staining shown in (**A**). Control, n=6 embryos; S259A, n=4 embryos. Mean±SEM. (**D**) Wholemount immunofluorescent staining of E12.5 yolk sacs. (**E**) Quantitative analysis of vessels based on CD31 staining shown in (**D**). Control, n=5 embryos; S259A, n=5 embryos. Mean±SEM. For quantitative analysis of all of the above quantitative images, at least 6 random areas from each skin or yolk sac were quantified and averaged to represent that of one embryo.

Figure S4. cDNA microarray analysis of RAF1^{S259A}-induced gene expression.

(**A**) PCA map of cDNA microarray of HUVECs infected with empty control, wild type HA-*RAF1* (WT) or HA-*RAF1*^{S259A} (S259A) lentiviruses. (**B**) Overlapping of RAF1 WT and S259A-induced genes with expression values equal or more than 1.5-fold different compared to Control cells were considered significant. The numbers of shared or uniquely regulated are indicated. (**C**) A sub-list of differentially expressed genes between Control, RAF1 WT and S259A cells.

Figure S5. RAF1-AKT crosstalk controls VEGF-induced *DLL4* expression.

qPCR analysis of *DLL4* expression in U0126 and LY294002 pre-treated HUVECs (A) or HDLECs (B) upon VEGF-A or VEGF-C stimulation at indicated time points.

Figure S6. Summary of guidance molecule expression in HUVECs transduced with control, wild type *RAF1* or *RAF1*^{S259A} lentiviruses.

Expression of repulsion molecules such as the *Semaphorins*, the *Netrins*, the *Slits*, the *Ephrins* and their receptors was determined by cDNA microarray of HUVECs transduced with control, wild type *RAF1* or *RAF1*^{S259A} lentiviruses. Data represents Mean ±SEM of the cDNA array score of four independent cDNA microarrays.

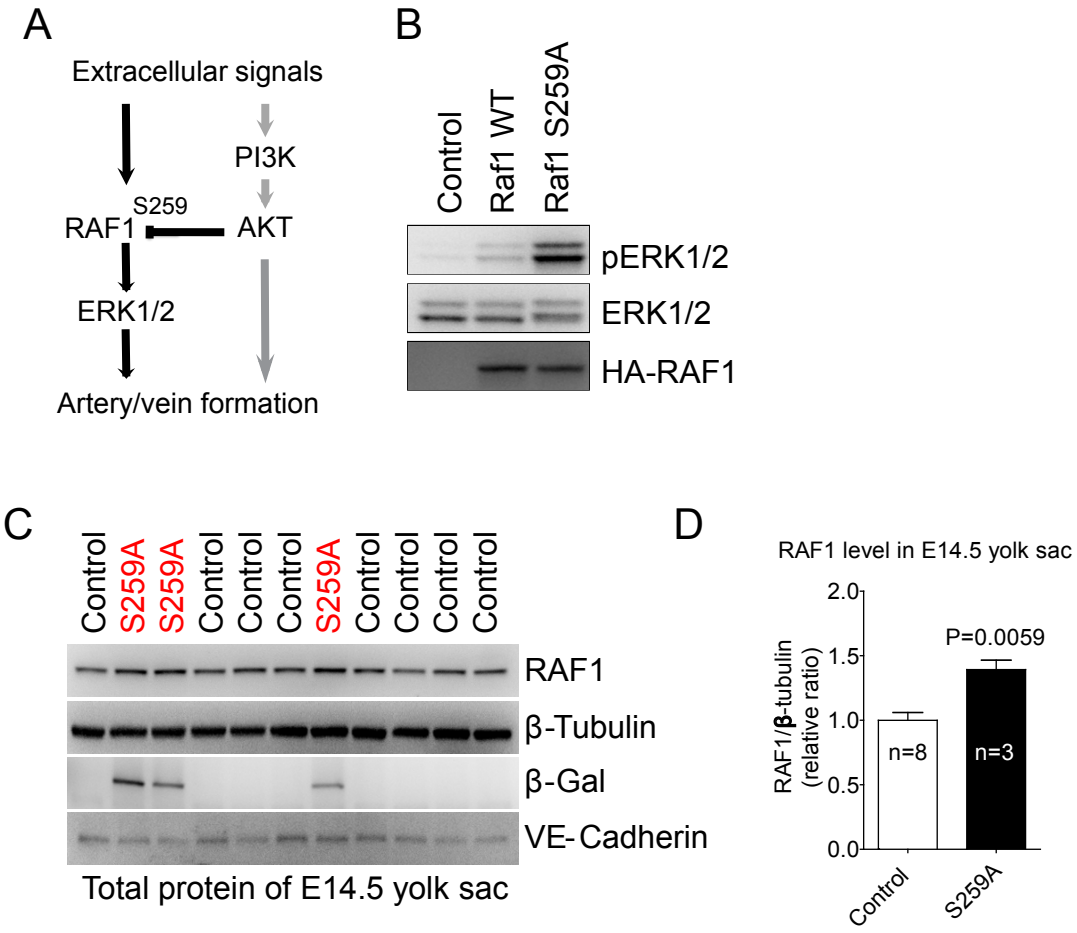
A
Extracellular signals
PI3K
S259
RAF1
AKT
ERK1/2
Artery/vein formation
B
Control
Raf1 WT
Raf1 S259A
pERK1/2
ERK1/2
HA-RAF1
C
Control
S259A
S259A
Control
Control
Control
S259A
Control
Control
Control
Control
RAF1
β-Tubulin
β-Gal
VE-Cadherin
Total protein of E14.5 yolk sac
D
RAF1 level in E14.5 yolk sac
RAF1/β-tubulin (relative ratio)
2.0
1.5
1.0
0.5
0.0
P=0.0059
n=8
n=3
Control
S259A

Figure S1

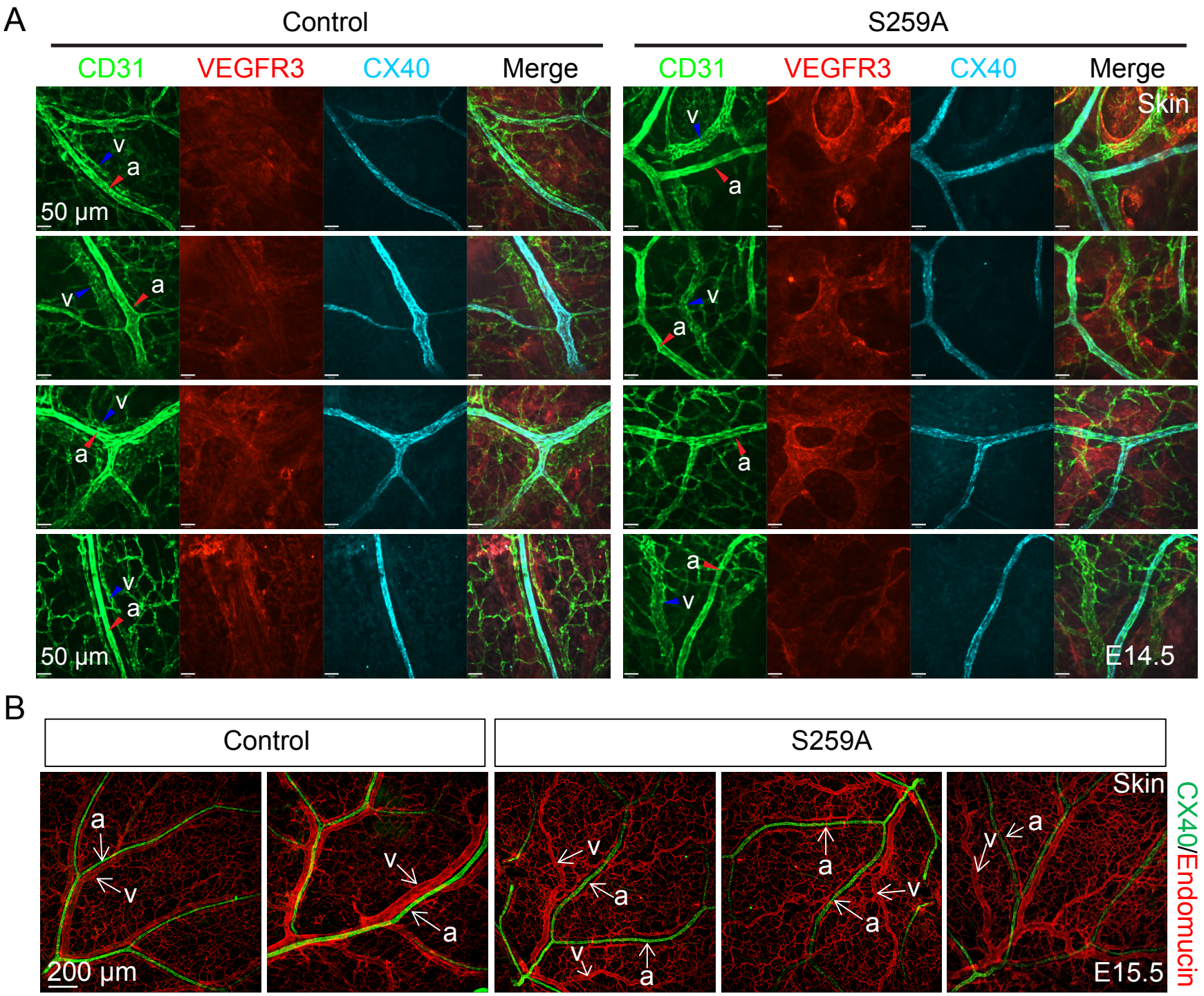
A
Control
S259A
CD31
VEGFR3
CX40
Merge
CD31
VEGFR3
CX40
Merge
Skin
v
a
50 μm
v
a
v
a
v
a
v
a
v
a
v
a
50 μm
a
v
E14.5
B
Control
S259A
Skin
a
v
200 μm
v
a
v
a
v
a
a
v
a
v
a
E15.5
CX40/Endomucin

Figure S2

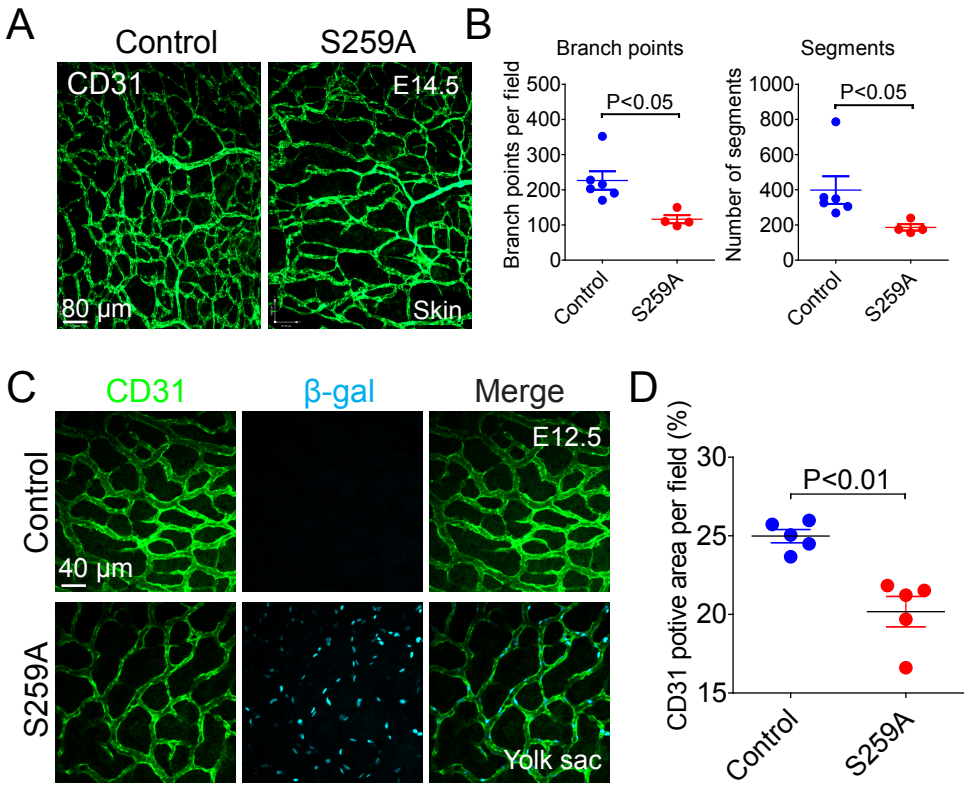
A
Control
S259A
CD31
E14.5
80 μm
Skin
B
Branch points
Branch points per field
500
400
300
200
100
0
P<0.05
Control
S259A
Segments
Number of segments
1000
800
600
400
200
0
P<0.05
Control
S259A
C
CD31
β-gal
Merge
Control
S259A
E12.5
40 μm
Yolk sac
D
CD31 potive area per field (%)
30
25
20
15
P<0.01
Control
S259A

Figure S3

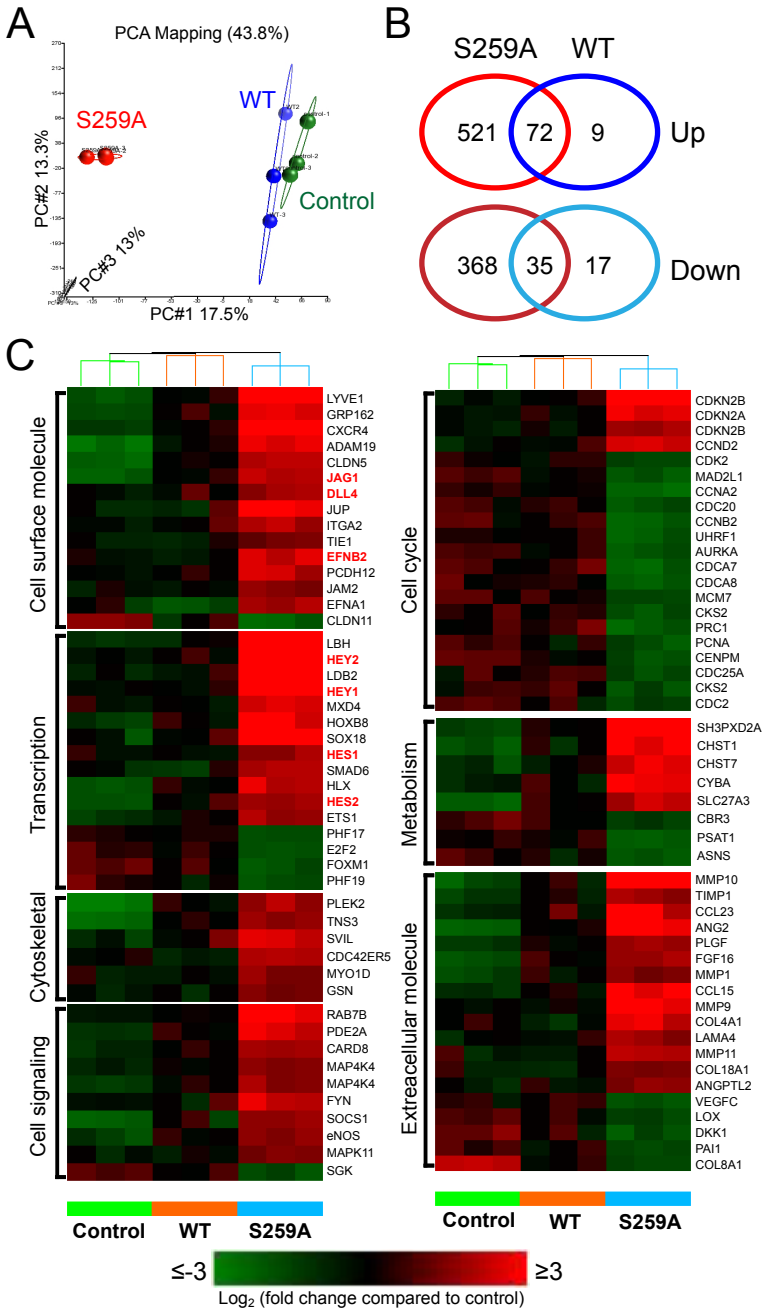
A
PCA Mapping (43.8%)
S259A
WT
Control
PC#2 13.3%
PC#3 13%
PC#1 17.5%
B
S259A
WT
521
72
9
Up
368
35
17
Down
C
Cell surface molecule
LYVE1
GRP162
CXCR4
ADAM19
CLDN5
JAG1
DLL4
JUP
ITGA2
TIE1
EFNB2
PCDH12
JAM2
EFNA1
CLDN11
Transcription
LBH
HEY2
LDB2
HEY1
MXD4
HOXB8
SOX18
HES1
SMAD6
HLX
HES2
ETS1
PHF17
E2F2
FOXM1
PHF19
Cytoskeletal
PLEK2
TNS3
SVIL
CDC42ER5
MYO1D
GSN
Cell signaling
RAB7B
PDE2A
CARD8
MAP4K4
MAP4K4
FYN
SOCS1
eNOS
MAPK11
SGK
Control
WT
S259A
Cell cycle
CDKN2B
CDKN2A
CDKN2B
CCND2
CDK2
MAD2L1
CCNA2
CDC20
CCNB2
UHRF1
AURKA
CDCA7
CDCA8
MCM7
CKS2
PRC1
PCNA
CENPM
CDC25A
CKS2
CDC2
Metabolism
SH3PXD2A
CHST1
CHST7
CYBA
SLC27A3
CBR3
PSAT1
ASNS
Extreacellular molecule
MMP10
TIMP1
CCL23
ANG2
PLGF
FGF16
MMP1
CCL15
MMP9
COL4A1
LAMA4
MMP11
COL18A1
ANGPTL2
VEGFC
LOX
DKK1
PAI1
COL8A1
Control
WT
S259A
≤-3
≥3
Log2 (fold change compared to control)

Figure S4

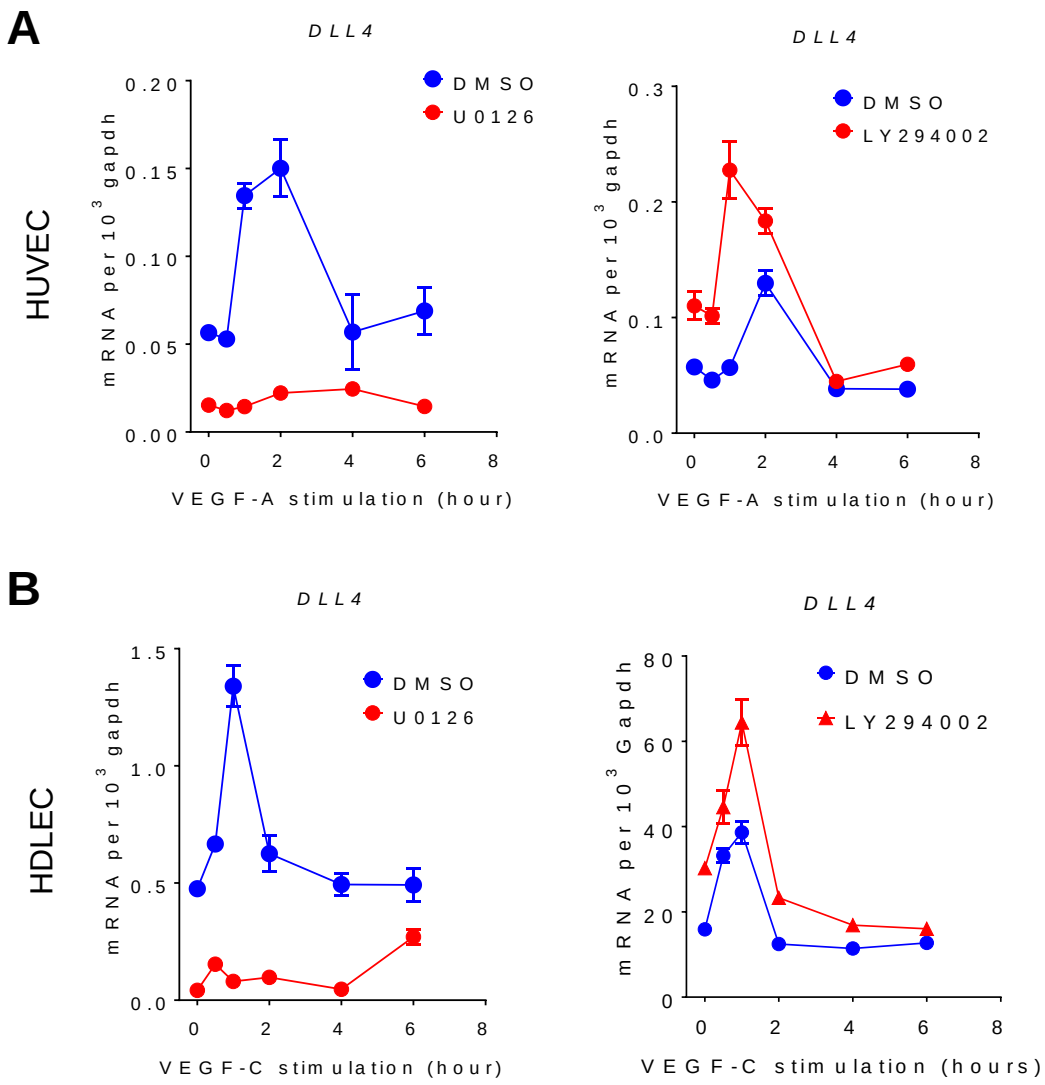
A
HUVEC
DLL4
DMSO
U0126
mRNA per 10^3 gapdh
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
VEGF-A stimulation (hour)
DLL4
DMSO
LY294002
mRNA per 10^3 gapdh
0.3
0.2
0.1
0.0
0
2
4
6
8
VEGF-A stimulation (hour)
B
HDLEC
DLL4
DMSO
U0126
mRNA per 10^3 gapdh
1.5
1.0
0.5
0.0
0
2
4
6
8
VEGF-C stimulation (hour)
DLL4
DMSO
LY294002
mRNA per 10^3 Gapdh
80
60
40
20
0
0
2
4
6
8
VEGF-C stimulation (hours)

Figure S5

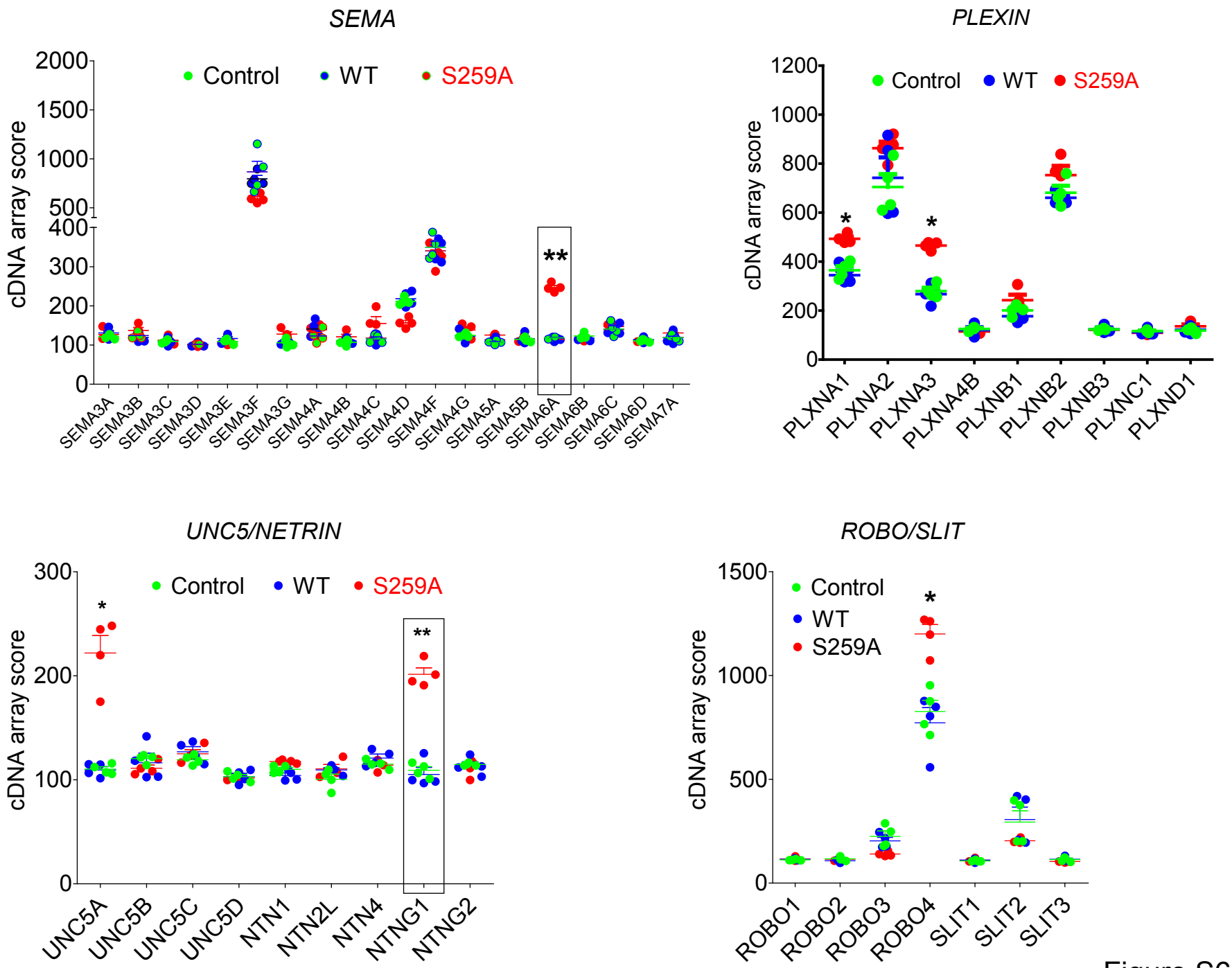
SEMA
Control
WT
S259A
cDNA array score
2000
1500
1000
500
400
300
200
100
0
**
SEMA3A
SEMA3B
SEMA3C
SEMA3D
SEMA3E
SEMA3F
SEMA3G
SEMA4A
SEMA4B
SEMA4C
SEMA4D
SEMA4F
SEMA4G
SEMA5A
SEMA5B
SEMA6A
SEMA6B
SEMA6C
SEMA6D
SEMA7A
PLEXIN
Control
WT
S259A
cDNA array score
1200
1000
800
600
400
200
0
*
*
PLXNA1
PLXNA2
PLXNA3
PLXNA4B
PLXNB1
PLXNB2
PLXNB3
PLXNC1
PLXND1
UNC5/NETRIN
Control
WT
S259A
cDNA array score
300
200
100
0
*
**
UNC5A
UNC5B
UNC5C
UNC5D
NTN1
NTN2L
NTN4
NTNG1
NTNG2
ROBO/SLIT
Control
WT
S259A
cDNA array score
1500
1000
500
0
*
ROBO1
ROBO2
ROBO3
ROBO4
SLIT1
SLIT2
SLIT3

Figure S6