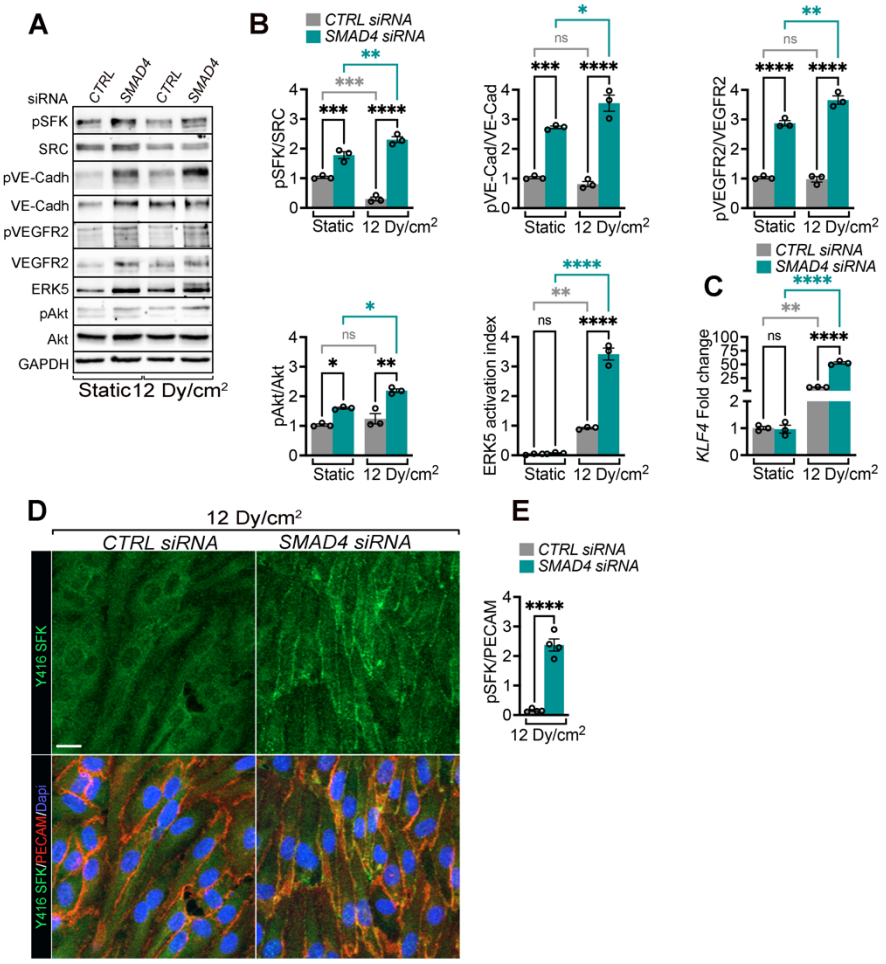
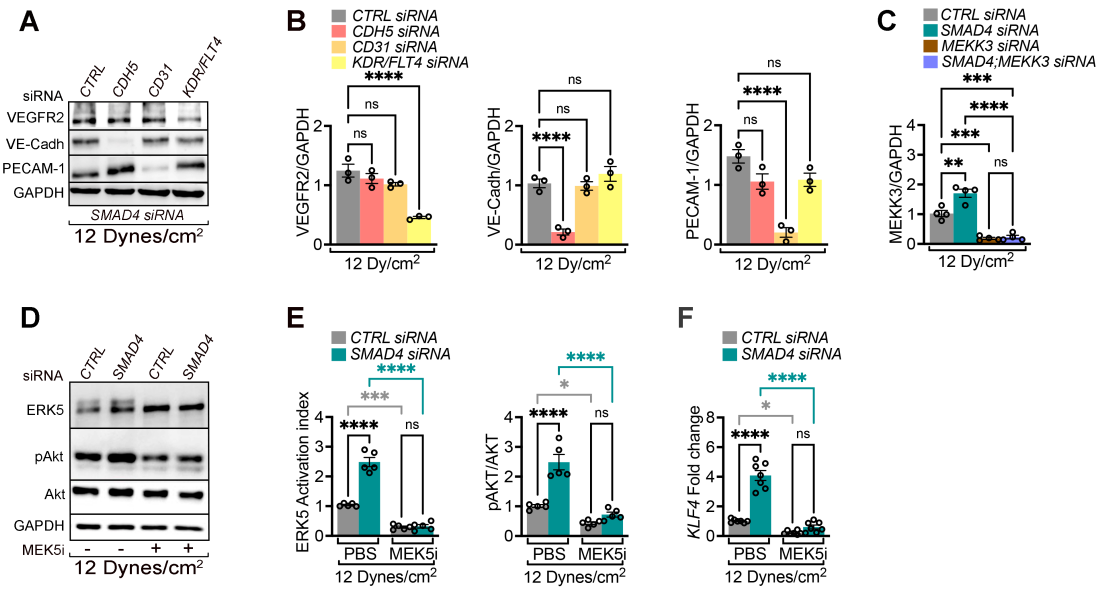
**SUPPLEMENTARY MATERIAL**

# Loss of *Smad4* drives vascular malformations via c-KIT-dependent high shear stress mimicry

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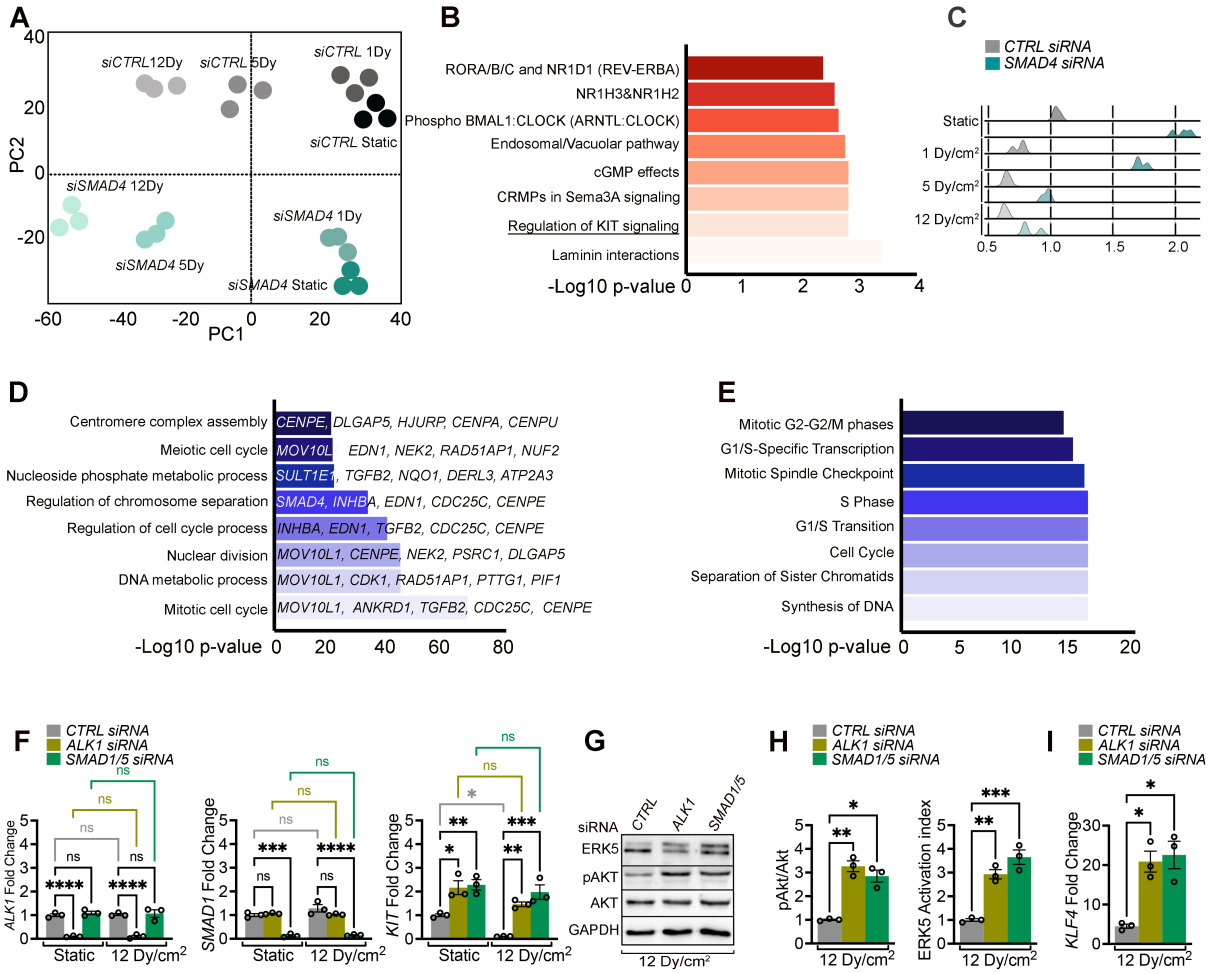



**Supplementary Figure 1. *SMAD4*KD leads to sustained mechanotransduction in HUVECs**
(**A**) Representative immunoblots of the indicated proteins in *CTRL* and *SMAD4* siRNAs HUVECs grown in static versus 12 Dynes/cm$^2$ for 24 h (**B**) Quantifications of pSFK, pVE-Cadherin, pVEGFR2, and pAkt levels normalized to total SRC, VE-Cadherin, VEGFR2 and Akt, respectively and of ERK5 gel shift (n=3/group). (**C**) *KLF4* fold change in *CTRL* and *SMAD4* siRNAs HUVECs grown in static versus 12 Dynes/cm$^2$ FSS for 24 h (n=3/group). (**D**) Co-immunostaining for pSFK (green) and PECAM-1 (red) in *CTRL* and *SMAD4* siRNAs HUVECs subject to 12 Dynes/cm$^2$ conditions for 24 h. Scale bar: 50 µm. (**E**) Ratio of integrated intensity of junctional pSFK/PECAM (n=4 independent experiments). Data are represented as mean ± SEM. 2-way ANOVA in **B**,**C** and Student's t-test (**E**) were used to determine statistical significance. **P* <0.05, ***P* <0.01, ****P* <0.005, *****P* <0.001, ns=non-significant.



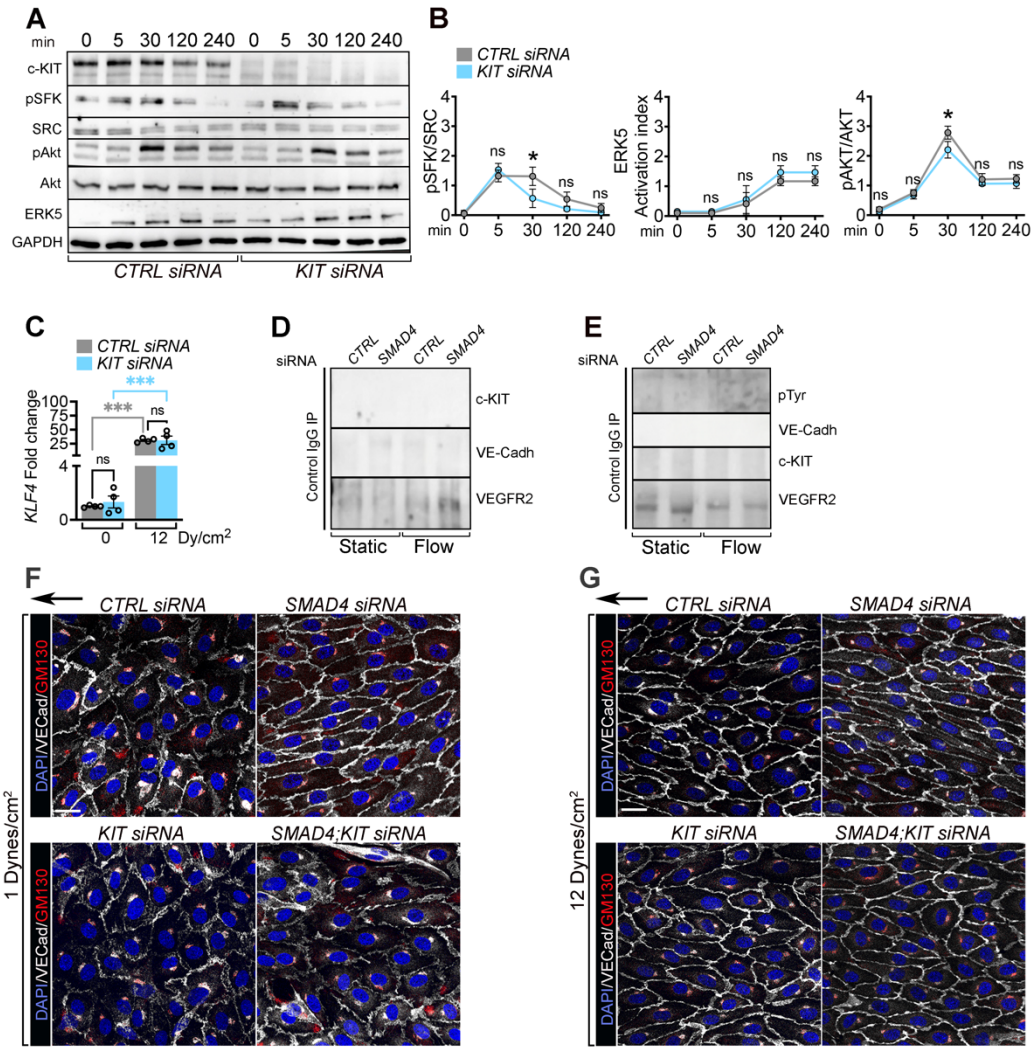

**Supplementary Figure 2. Junctional mechanosensing apparatus acts upstream of MEKK3-MEK5-KLF4**

(**A**) WB for the indicated proteins in *SMAD4* siRNA HUVECs subject to 12 Dynes/cm$^2$ for 2 h, transfected additionally with *CD31*, *CDH5* and *KDR/FLT4* siRNAs. (**B**) Quantifications of total levels of PECAM-1, VE-Cadherin and VEGFR2 normalized to GAPDH (n=3/group). (**C**) Quantifications of total levels of MEKK3 normalized to GAPDH (n=4/group). (**D**) WB for the indicated proteins in *CTRL* and *SMAD4* siRNAs HUVECs subjected to 12 Dynes/cm$^2$ for 2 h in the presence of PBS or MEK5 inhibitor (MEK5i). (**E**) Quantifications of ERK5 gel shift and Akt (S473) levels normalized to total Akt (n=5/group). (**F**) *KLF4* fold change in *CTRL* and *SMAD4* siRNAs HUVECs grown in static versus 12 Dynes/cm$^2$ FSS for 2 h treated with PBS versus MEK5i (n=7/group). Data are represented as mean ± SEM. 1-way ANOVA (**B**,**C**) and 2-way ANOVA (**E**,**F**) were used to determine statistical significance. **P* <0.05, ***P* <0.01, ****P* <0.005, *****P* <0.001, ns=non-significant.



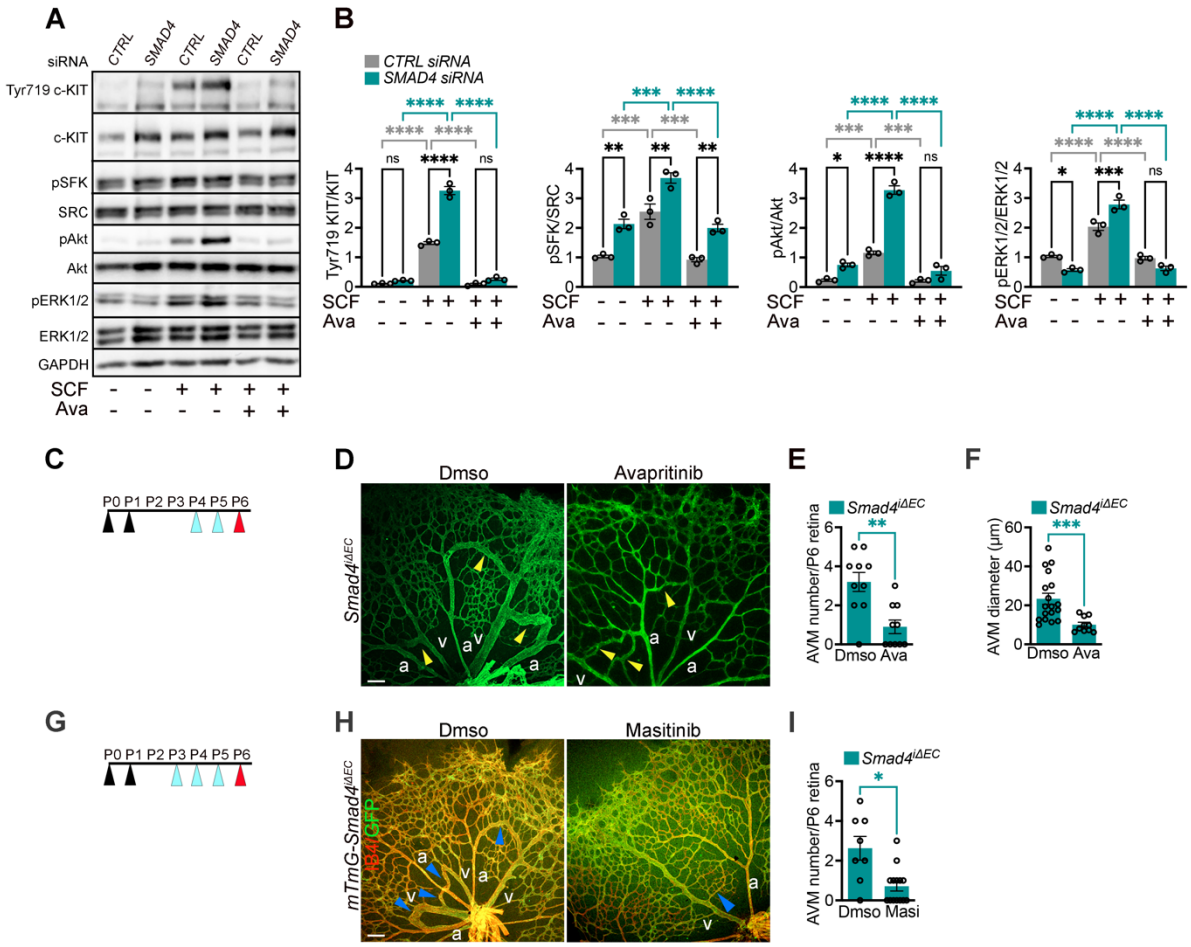

**Supplementary Figure 3. ALK1-SMAD1/5 upstream of SMAD4 regulate c-KIT-mediated flow signaling**

(**A**) Principal component analysis (PCA) of RNA seq. groups (n=3). (**B**) Reactome pathway analysis in *SMAD4* versus *CTRL* siRNA HUVECs in all conditions. (**C**) Expression of *KIT* in RNA seq. (Ridgeplot) (n = 3/group). (**D**,**E**) Significant downregulated GO terms (**D**) and reactome pathway (**E**) analysis in *CTRL* versus *SMAD4* siRNA conditions in RNA seq. (**F**) qPCR analysis for *ALK1*, *SMAD1* and *KIT* in HUVECs transfected with *CTRL, ALK1* and *SMAD1/5* siRNAs and grown in static versus 12 Dynes/cm$^2$ conditions for 2 h (n=3/group). (**G**) WB for the indicated proteins in *CTRL, ALK1* and *SMAD1/5* siRNAs HUVECs subject to 12 Dynes/cm$^2$ for 2 h. (**H**) Quantifications of ERK5 gel shift and activated Akt (S473) levels normalized to total Akt (n=3/group). (**I**) qPCR for *KLF4* in HUVECs transfected with *CTRL* versus *ALK1* and *SMAD1/5* siRNAs subject to 12 Dynes/cm$^2$ for 2 h. (n=3/group). Data are represented as mean ± SEM. 1-way ANOVA (**H**,**I**) and 2-way ANOVA (**F**) were used to determine statistical significance. **P* <0.05, ***P* <0.01, ****P* <0.005, *****P* <0.001, ns=non-significant.




**Supplementary Figure 4. c-KIT is dispensable for FSS mediated mechanotransduction**

(**A**) WB for the indicated proteins in *CTRL* and *KIT* siRNAs HUVECs grown in static (0) or 12 Dynes/cm$^2$ for 5-30-120-240 min. (**B**) Quantifications of phosphorylated SFK (Y416), Akt (S473) levels normalized to total SRC and Akt, respectively and of ERK5 gel shift (n=3/group). (**C**) *KLF4* fold change in *CTRL* and *KIT* siRNAs HUVECs grown in static (0) versus 12 Dynes/cm$^2$ FSS for 2 h. (**D**,**E**) Rabbit immunoglobulin G (IgG) immunoprecipitation used as IP control for c-KIT (**D**) and VE-Cadherin (**E**). (**F**,**G**) Co-labeling for VE-Cadherin (white), GM130 (red), and DAPI (blue) of *CTRL*, *SMAD4, KIT* and *SMAD4;KIT* siRNAs HUVECs subject to 48 h 1 Dyne/cm$^2$ (**F**) or 12 Dynes/cm$^2$ (**G**). Black arrow indicates flow direction from right to left. Scale bars: 50 μm. Data are represented as mean ± SEM. Student's t-test (**B**) and 2-way ANOVA (**C**) were used to determine statistical significance. **P* <0.05, ****P* <0.005, *****P* <0.001, ns=non-significant.




**Supplementary Figure 5. Inhibition of c-KIT kinase activity improves AVM outcome**
(**A**) WB for the indicated proteins in *CTRL* and *SMAD4* siRNAs HUVECs grown in static and stimulated with SCF (50ng/ml) for 5 min ± 1 µM Avapritinib (Ava). (**B**) Quantifications of phosphorylated c-KIT (Tyr719), SFK (Y416), Akt (S473) levels normalized to total c-KIT, SRC and Akt, respectively and of ERK5 gel shift (n=3/group). (**C**) Schematic of Tx administration (black arrowheads), Dmso or Ava treatment (blue arrowheads) and harvesting (red arrowheads) in *Smad4 fl/fl* and *Smad4*$^{i\Delta EC}$ P6 neonates. (**D**) IB4 labeling (green) of P6 retinas from *Smad4*$^{i\Delta EC}$ Dmso versus Avapritinib treated neonates at the indicated time points in **C**. (**E**,**F**) Quantification of AVMs' number per retina (n=6 retinas/genotype) and of capillary diameter in *Smad4*$^{i\Delta EC}$ P6 retinas (n=10-18 AVMs in 3 retinas/genotype) harvested from Dmso versus Ava treated neonates. (**G**) Schematic representation of Tx administration (black arrowheads), Dmso or Masitinib (Masi) treatment (blue arrowheads) and harvesting (red arrowheads) in *Smad4 fl/fl* and *Smad4*$^{i\Delta EC}$ P6 neonates. (**H**) Labeling for IB4 (red) and GFP (green) of P6 retinas *Smad4*$^{i\Delta EC}$ from Dmso versus Masitinib treated neonates. (**I**) Quantification of the number of AVMs per retina (n=8-14 retinas/genotype). Scale bar: 50 µm. Data are represented as mean ± SEM. 2-way ANOVA (**B**) and Student's t-test (**E**,**F**,**I**) were used to determine statistical significance. **P* <0.05, ****P* <0.005, *****P* <0.001, ns=non-significant

**Supplemental Table 1. List of human primers**

| Primers | Forward Sequence | Reverse Sequence |
|---|---|---|
| *KLF4* | CCCACATGAAGCGACTTCCC | CAGGTCCAGGAGATCGTTGAA |
| *KIT* | CGTTCTGCTCCTACTGCTTCG | CCCACGCGGACTATTAAGTCT |
| *KITL* | AATCCTCTCGTCAAAACTGAAGG | CCATCTCGCTTATCCAACAATGA |
| *GAPDH* | CTGGGCTACACTGAGCACC | AAGTGGTCGTTGAGGGCAATG |
| *SMAD4* | QT00013174 | QIAGEN |
| *SMAD1* | QT00103824 | QIAGEN |
| *ALK1* | QT00050351 | QIAGEN |

**Supplemental Table 2. List of primary antibodies used for immunoblotting**

| Primary Antibody | Dilution (IF) | Company | Cat. Number/Clone number |
|---|---|---|---|
| Isolectin GS-IB4 568 | 10 μg/ml | Thermo Fisher | I21412 |
| Isolectin GS-IB4 488 | 10 μg/ml | Thermo Fisher | I21411 |
| Isolectin GS-IB4 647 | 10 μg/ml | Thermo Fisher | I32450 |
| c-Kit | 1:100 | Agilent | A450229-2 / Polyclonal |
| pErk5 (Thr218/Tyr220) | 1:100 | Cell Signaling | 3371 / Polyclonal |
| PECAM-1 | 1:50 | Santa Cruz | sc-376764/ H-3 |
| Dapi | 1 μg/mL | Sigma-Aldrich | 5087410001 |
| ENG | 1:50 | R&D Systems | AF1097 / Polyclonal |
| α-SMA | 1:100 | Agilent | M0851 / 1A4 |
| KLF4 | 1:100 | R&D Systems | AF3158 / Polyclonal |
| KI67 (eFluor™ 660) | 1:100 | Thermo Fisher | 50-5698-82 / SolA15 |
| ERG | 1:100 | Abcam | AB92513 / EPR3864 |
| GOLPH4 | 1:200 | Abcam | Ab28049 / Polyclonal |
| GM130 | 1:100 | BD Pharmingen™ | 610823 / 35/GM130 |
| VE-Cadherin | 1:100 | Cell Signaling | 2500S / D87F2 |
| Phospho-S6 | 1:100 | Cell Signaling | 5364S / D68F8 |

**Supplemental Table 3. List of human siRNAs**

| siRNA | Company | Cat. Number |
|---|---|---|
| *Control* siRNA | Dharmacon/Horizon | D-001810-01 |
| *SMAD4* siRNA | Dharmacon/Horizon | L-003902-00 |
| *KIT* siRNA | Dharmacon/Horizon | L-003150-00 |
| *MEKK3* siRNA | Dharmacon/Horizon | L-003301-00 |
| *CDH5* siRNA | Dharmacon/Horizon | L-003641-00 |
| *CD31* siRNA | Sigma | GGCCCCAAUACACUUCACA AACCACUGCAGAGUACCAG |
| *KDR* siRNA | Dharmacon/Horizon | L-003148-00 |
| *FLT4* siRNA | Sigma | GAAGCCCAAUCAAUAACUGUU UAAGCACUCUGGCAGAGAAUU |
| *SMAD1* siRNA | Dharmacon/Horizon | L-012723-00 |
| *SMAD5* siRNA | Dharmacon/Horizon | L-015791-00 |
| *ACVRL1* siRNA | Dharmacon/Horizon | L-005302-02 |

**Supplemental Table 4. List of chemicals**

| Inhibitor/recombinant Protein | Company | Cat. Number |
|---|---|---|
| PP2 | Sigma-Aldrich | 529576 |
| BIX02189 | Selleck Chemicals | S1531 |
| Masitinib mesylate | MedChemExpress | HY-10209A |
| Avapritinib (BLU-285) | MedChemExpress | S8553 |
| Human recombinant SCF | Thermo Fisher | AF-300-07 |
| Human SCF blocking antibody | R&D Systems | AF-255-NA |

**Supplemental Table 5. List of primary antibodies used in WB**

| Primary Antibody | Dilution (WB) | Company | Cat. Number/Clone number |
|---|---|---|---|
| pSFK (Tyr416) | 1:2000 | Cell Signaling | 6943 / D49G4 |
| SRC | 1:2000 | Cell Signaling | 2109 / 36D10 |
| pVE-Cadherin (Tyr658) | 1:1000 | Abcam | ab119785 / Polyclonal |
| VE-Cadherin | 1:1000 | Cell Signaling | 2500S / D87F2 |
| pVEGFR2 (Tyr1054) | 1:1000 | Thermo Fisher | 44-1047G / Polyclonal |
| VEGFR2 | 1:1000 | Cell Signaling | 2479 / 55B11 |
| ERK5 | 1:1000 | Cell Signaling | 3372 / Polyclonal |
| pAKT (S473) | 1:2000 | Cell Signaling | 4060 / D9E |
| AKT | 1:2000 | Cell Signaling | 4685 / 11E7 |
| GAPDH | 1:5000 | Cell Signaling | 5174 / D16H11 |
| MEKK3 | 1:1000 | Cell Signaling | 5727 / Polyclonal |
| c-KIT | 1:1000 | Agilent | A450229-2 / Polyclonal |
| c-KIT (Tyr719) | 1:1000 | Cell Signaling | 3391 / Polyclonal |
| SMAD4 | 1:1000 | Cell Signaling | 38454 / D3M6U |
| p44/42 (ERK1/2) | 1:1000 | Cell Signaling | 4695 / 137F5 |
| Phospho-p44/42 (P-ERK1/2) | 1:1000 | Cell Signaling | 4370S / D13.14.4E |

**Supplemental Table 6. List of secondary antibodies used in WB and immunostaining**

| Secondary Antibody | Dilution | Company | Cat. Number |
|---|---|---|---|
| anti-rabbit HRP | 1:5000 | Vector Laboratories | PI-1000-1 |
| anti-mouse HRP | 1:5000 | Vector Laboratories | PI-2000 |
| anti-Rabbit 488 | 1:250 | Thermo Fisher | A-21206 |
| anti-Goat 488 | 1:250 | Thermo Fisher | A-11055 |
| anti-Mouse 568 | 1:250 | Thermo Fisher | A-10037 |
| anti-Rabbit 568 | 1:250 | Thermo Fisher | A-10042 |
| anti-Mouse 647 | 1:250 | Thermo Fisher | A-31571 |
| anti-Rabbit 647 | 1:250 | Thermo Fisher | A-31573 |