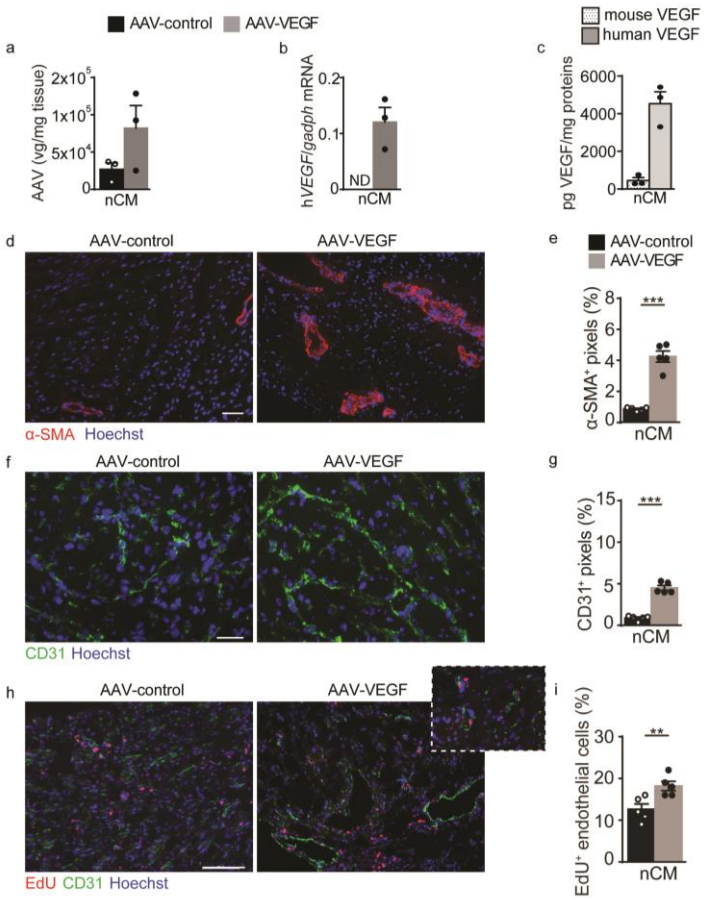
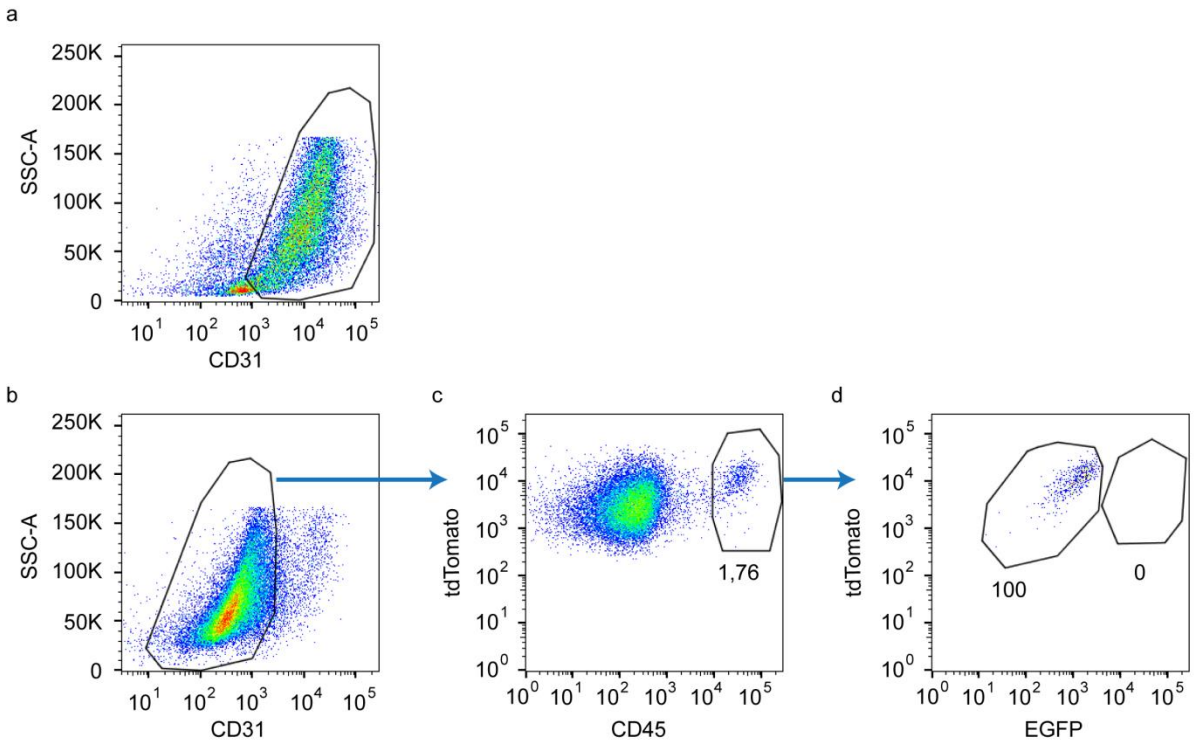
Genetic lineage tracing reveals poor angiogenic potential of cardiac endothelial cells

Short title: Poor angiogenic potential of the adult heart

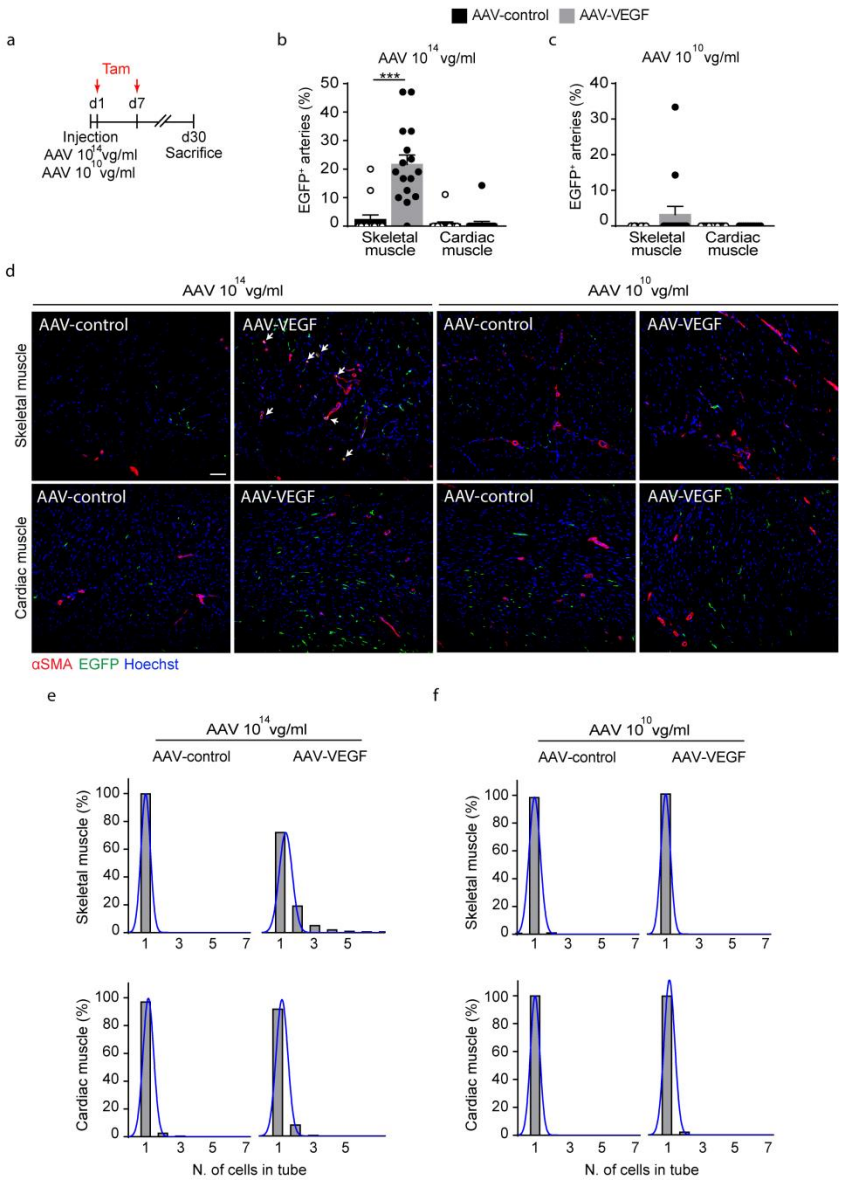
Supplementary figures



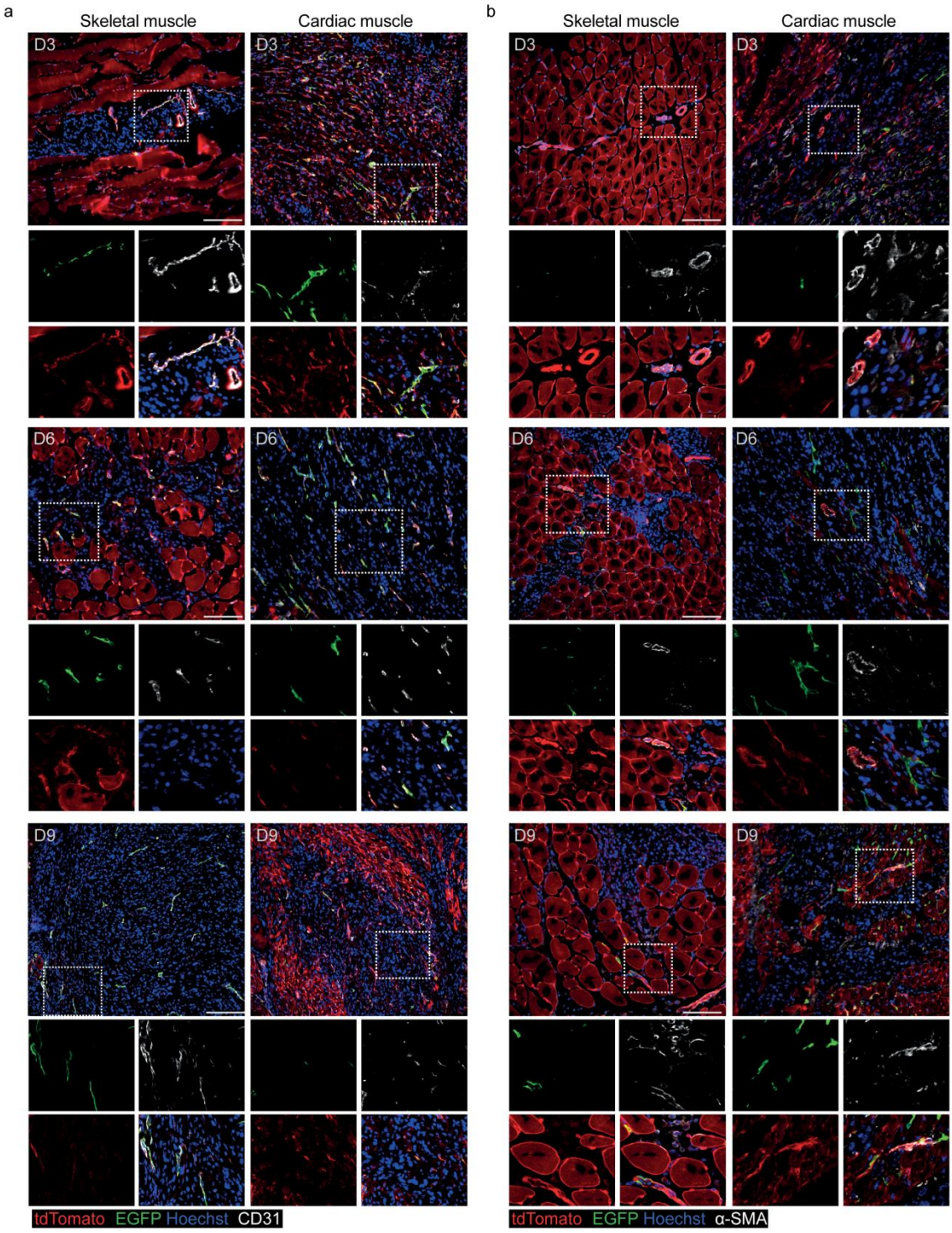
Supplementary Figure 1. VEGF induces angiogenesis in the neonatal heart. a. Real-time PCR quantification of the number of AAV viral genomes normalized on tissue weight in neonatal hearts. **b**. Real-time PCR quantification of human VEGF165 mRNA normalized on mouse *Gapdh* in neonatal hearts. **c**. ELISA quantification of mouse and human VEGF normalized on tissue weight. **d**. Representative immunofluorescence staining of neonatal hearts, injected with either AAV-control or AAV-VEGF, using anti-α-SMA antibodies. **e.** Quantification of the area covered by α-SMA$^+$ cells in neonatal cardiac muscle. **f**. Representative immunofluorescence staining of neonatal hearts, injected with either AAV-control or AAV-VEGF, using anti-CD31 antibodies. **g**. Quantification of the area covered by CD31$^+$ cells in neonatal cardiac muscle. **h**. Representative images of neonatal hearts, injected with either AAV-control or AAV-VEGF, showing EdU$^+$ nuclei and CD31$^+$ endothelial cells. The inset shows a higher magnification to visualize EdU$^+$CD31$^+$ endothelial cells. **i.** Quantification of the number of EdU$^+$ nuclei in CD31$^+$ cells in neonatal cardiac muscle**.** Scale bar in **d**, **f**, 100 µm, in **h** 200 µm. ** $P < 0.01$, *** $P < 0.001$. n=3-6 per group.



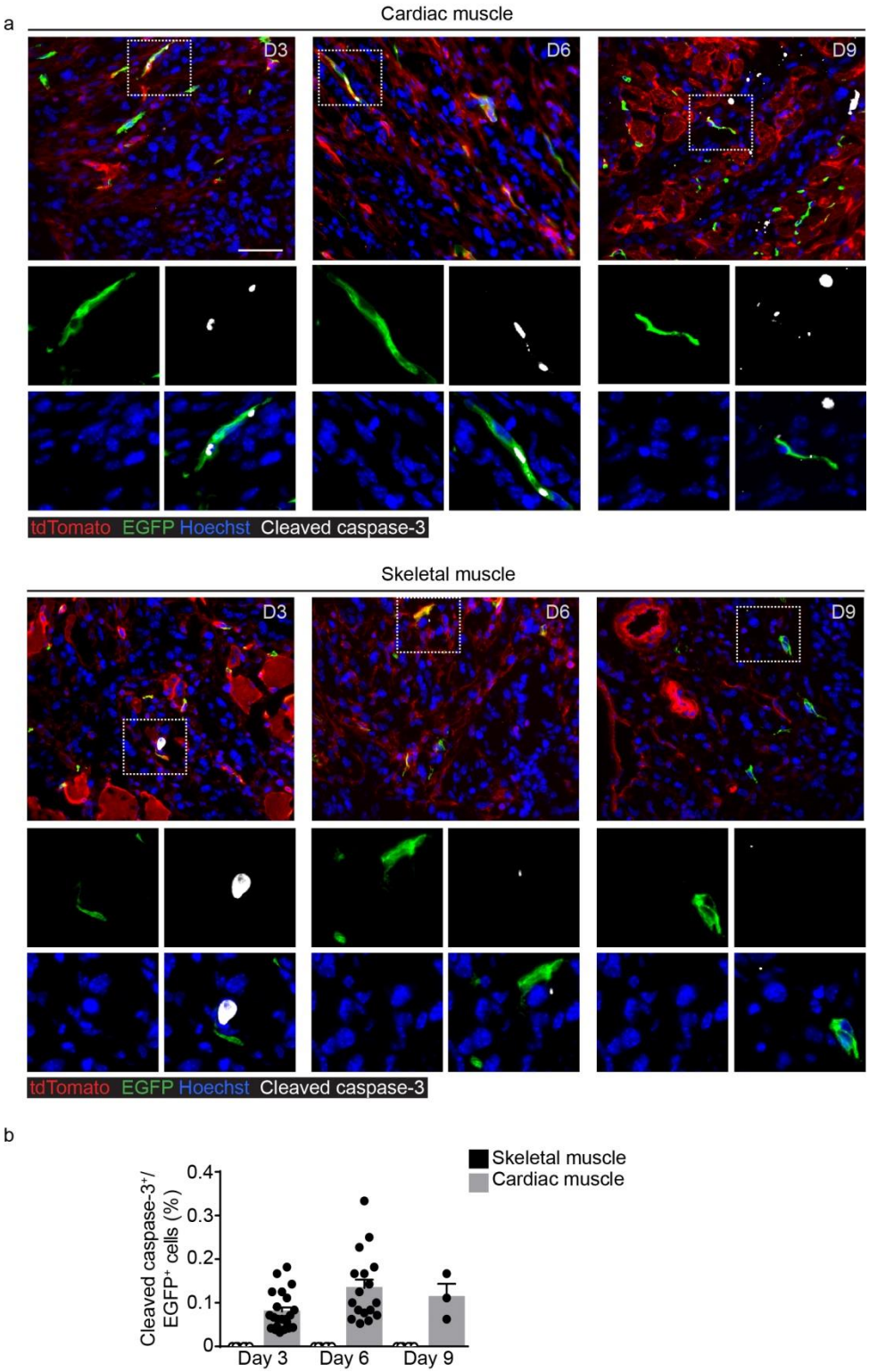
Supplementary Figure 2. EGFP$^+$ cells from Apln-CreER;R26mT/mG mice do not express CD45. Flow cytometry was performed on cells purified from the cardiac and skeletal muscle of from Apln-CreER;R26mT/mG mice using anti-CD31 magnetic beads, as well as on the flow through. Purified cells were almost totally positive for CD31 (a), whereas the flow through essential contained CD31$^-$ cells (b). Of these, about 2% was positive for the pan-leukocytic marker CD45 (c). The CD31$^-$CD45$^+$ cells did not express EGFP$^+$.



Supplementary figure 3. Injection of a high dose of AAV-VEGF does not improve the angiogenic response. a. Schematic of AAV (different doses) and tamoxifen (Tam) injection in Apln-CreER;R26mT/mG mice. **b-c**. Quantification of the number of arteries containing EGFP$^+$ endothelial cells (% of total arteries) in response to the injection of AAV vectors at the indicated titers (10^{10} vg/ml and 10^{14} vg/ml). **d**. Representative immunofluorescence staining of skeletal and cardiac muscle of Apln-CreER;R26mT/mG mice injected with either AAV-control or AAV-VEGF at the indicated titers, labelled with anti-α-SMA antibodies. **e-f**. Histograms showing the number of EGFP$^+$ cells lining in a vascular tube in either skeletal or cardiac muscle injected with AAV-control (left) and AAV-VEGF (right) at the indicated titers. Superimposed to each histogram is the density plot (in blue). Data in **b** and **c** are shown as mean±s.e.m. Statistical significance was determined using Student's t-test and one-way ANOVA followed by Tukey's multiple comparison test, *** $P < 0.001$, n=3 per group. Scale bar in **d** ,100 μm.

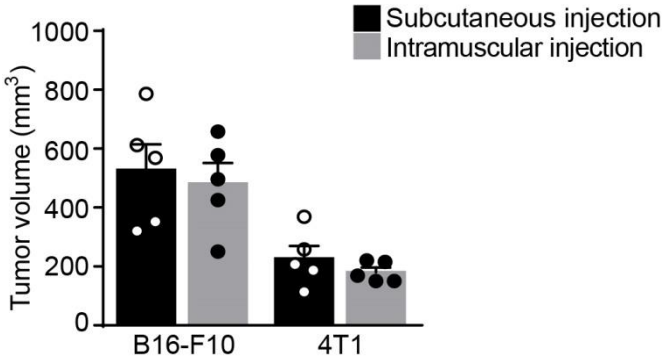


Supplementary Figure 4. Time-course analysis of the incorporation of Apelin^{+} cells in cancer-associated vessels. Representative immunofluorescence staining of skeletal and cardiac muscle of Apln-CreER;R26mT/mG mice injected with LG cells, labelled with anti-CD31 (**a**) and anti-α-SMA (**b**) antibodies at days 3 (D3), 6 (D6) and 9 (D9) after cancer cell injection. Nuclei are stained in blue with Hoechst. Boxed regions are magnified in the lower panels to show split channels. Scale bar, 200 µm.

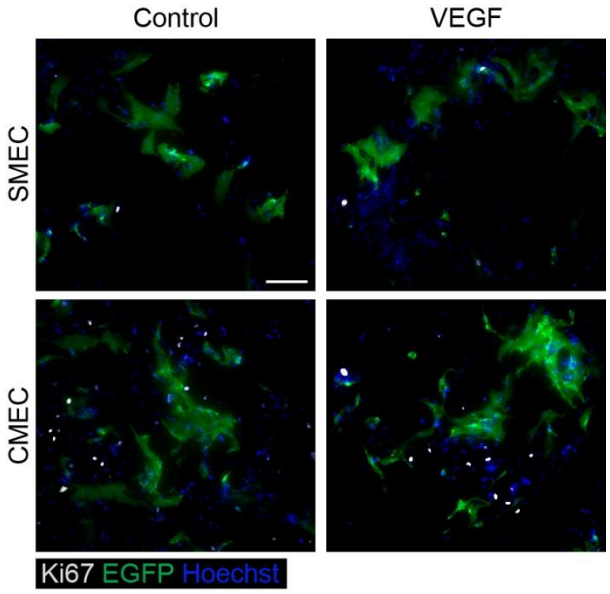


Supplementary Figure 5. Time-course analysis of apoptosis in cancer-associated Apelin⁺ cells. a. Representative immunofluorescence staining of skeletal and cardiac muscle of Apln-CreER;R26mT/mG mice injected with LG cells, labelled with anti-cleaved caspase-3 (white) antibodies at days 3 (D3), 6 (D6) and 9 (D9) after cancer cell injection. Nuclei are stained in blue with Hoechst. Boxed regions are magnified in the lower panels to

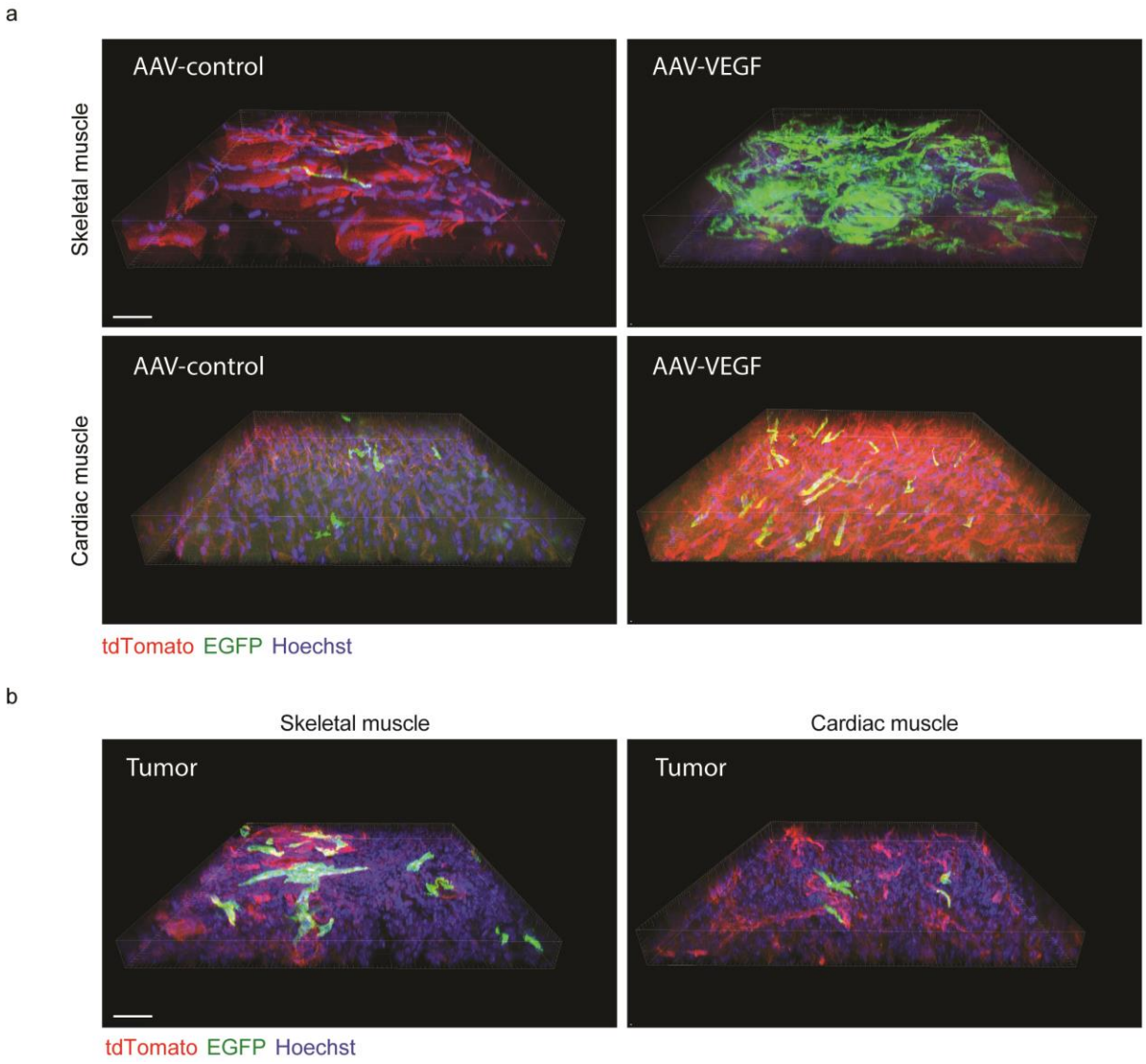
show split channels. Scale bar, 100 µm. b. Quantification of the number of cleaved caspase-3^{+} cells, expressed as percentage of EGFP^{+} cells. Data are shown as mean±s.e.m. n≥8 per group.



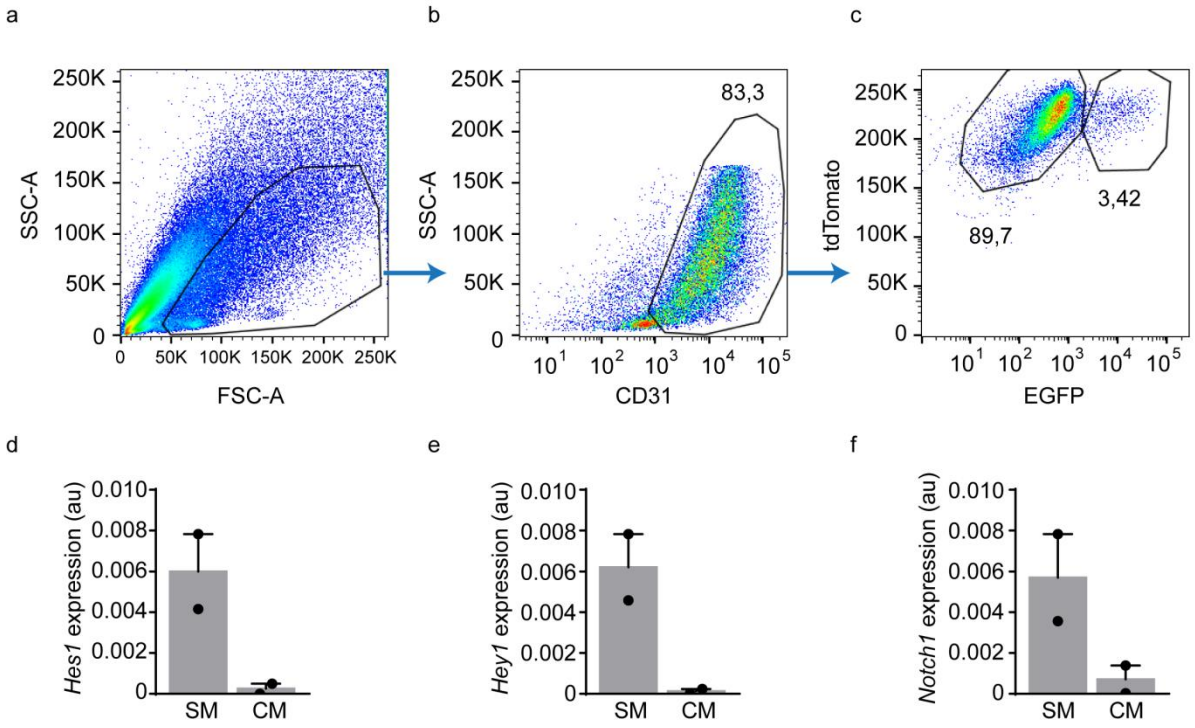
Supplementary Figure 6. Comparison of tumor growth at different anatomical sites. Quantification of B16-F10 and 4T1 tumor volume at 10 days after implantation of 100,000 cells into either the skeletal muscle or subcutaneous tissue. Data are shown as mean±s.e.m. Statistical significance was determined using a two-way ANOVA followed by Tukey's multiple comparison test, n=5 per group.



Supplementary Figure 7. Apelin^{+} cells are poorly proliferative. Representative images of cultured skeletal muscle and cardiac endothelial cells (SMECs and CECs, respectively) isolated from Apln-CreER;R26mT/mG mice, treated with tamoxifen, either in the presence or in the absence of VEGF (100 ng/ml) for 48 hours. Proliferating cells are labelled with anti-Ki67 antibodies (white) and nuclei are stained in blue with Hoechst. Scale bar, 50 µm.



Supplementary Figure 8. Three-dimensional organization of the vascular network in Apln-CreER;R26mT/mG mice. a. Representative three-dimensional images of 100 µm thick sections of skeletal and cardiac muscle from Apln-CreER;R26mT/mG mice injected with either AAV-control or AAV-VEGF. **b.** Representative three-dimensional images of 100 µm thick sections of skeletal and cardiac muscle from Apln-CreER;R26mT/mG mice injected with LG cells. Scale bar, 50 µm.



Supplementary Figure 9. Notch1 signaling is more active in sprouting endothelial cells in the skeletal muscle than in the heart

a-c. Gating strategy used to purify EGFP$^+$ and tdTomato$^+$ endothelial cells from Apln-CreER;R26mT/mG mice. Living cells were gated based on forward- and side-scattering (**a**) and endothelial cells labelled using anti-CD31 antibodies (**b**). The CD31$^+$ population was then sorted according to EGFP and tdTomato fluorescence (**c**) for further molecular analysis. **d-f.** Real-time PCR quantification of the expression of the Notch1 target genes, *Hes1* (**d**), *Hey1* (**e**) and *Notch1* (**f**) itself in tdTomato$^+$ endothelial cells sorted from either the skeletal or the cardiac muscle of Apln-CreER;R26mT/mG mice. Expression levels were normalized on total RNA (au = arbitrary units).

Extended materials and methods

AAV vector production and administration

The rAAV vectors used in this study were produced by the AAV Vector Unit at ICGEB Trieste (http://www.icgeb.org/RESEARCH/TS/COREFACILITIES/AVU.htm), according to the protocol previously described [1]. All the vectors used in this study either expressed the cDNA of human VEGF165 under the control of the constitutive CMV immediate early promoter or contained an empty multiple cloning site (MCS) cassette. All the viral stocks used in this study had a titer higher than $1x10^{12}$ viral genomes/ml. The proper expression of the transgene was tested in vitro by western blotting using specific antibodies after transduction of 293T cells. Vectors (30 µl corresponding to 3×10^{10} viral genomes) were injected into either the tibialis anterior skeletal muscle or the left ventricular wall of the adult heart using a 30 G syringe, as described [1, 2]. Neonatal hearts received 5 µl of the same vectors corresponding to 5×10^9 viral genomes.

DNA and RNA extraction and real-time PCR quantification

Total DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions and used as a template to detect and quantify vector DNA by real time PCR. Total RNA was isolated using TRIzol Reagent (Invitrogen). Reverse transcription was performed on 800 ng of total RNA using First Strand cDNA Synthesis Kit (Thermo Scientific) with random hexameric primers. Primers and TaqMan® probes were used to amplify the CMV promoter driving *VEGF* expression [2]. The expression of human *VEGF* mRNA transcripts were quantified and normalized on the expression of *Gapdh* using gene-specific primers and TaqMan® probe (Thermo Scientific, Assay ID: Hs00173626_m1 for *VEGFa* and Mm99999915_g1 for *Gapdh*). *Hes1*, *Hey1* and *Notch1* expression was quantified using SYBR Green and the following primers: mNotch1 FW: GATGGCCTCAATGGGTACAAG; mNotch1 RV: TCGTTGTTGTTGATGTCACAGT; mHes1 FW: GCACCTCCGGAACCTGCAGCG; mHes1 RV: GCAGCCGAGTGCGCACCTCGG; mHey1 FW: AAAGACGGAGAGGCATCATCG; mHey1 RV: GCAGTGTGAAGCATTTTCAGG.

Protein extraction and ELISA for VEGF quantification

Total protein were isolated from cardiac and skeletal muscle. Briefly, mice were sacrificed and perfused with PBS to remove excess of blood. Tissues were extracted, immediately frozen in liquid nitrogen and minced in a fine powder. To lyse tissues, 0.5 ml SDS-free RIPA buffer (100 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1%Triton X-100, 0.5% sodium deoxycholate) were added to 50 mg of tissue, prior to mechanical dissociation using Lysing Matrix D tubes (MP biomedicals). Lysates were sonicated and spin down at 14.000 rcf at 4°C for 10 minutes to remove debris. To quantify mouse and human VEGF, Quantikine ELISA Mouse VEGF (MMV00, R&D) and VEGF Human ELISA (ab100663, Abcam) kits were used following manufacturer instructions, adding 50 and 20 µg of total proteins for mouse and human VEGF quantification, respectively. Data were normalized on protein content.

Mice

All animal experiments were conducted in accordance with guidelines from the Directive 2010/63/EU of the European Parliament on animal experimentation in compliance with

European guidelines and International Laws and Policies (EC Council Directive 86/609, OJL 34, 12 December 1987), and were approved by the ICGEB Animal Welfare Board, the Ethical Committee and the Italian Ministry of Health. CD1, Balb/c and C57BL/6 mice were purchased from Harlan Laboratories. Adult Apln-CreER[3] mice were crossed with Rosa26-mT/mG[4] mice to obtain Apln-CreER;Rosa26-mT/mG animals, which were maintained on a C57BL/6 background. To activate Cre-mediated recombination, 4-hydroxytamoxifen (Sigma, H6278) was dissolved in corn oil (20 mg/ml) and injected intraperitoneally (20 mg/Kg) at days 1 and 7 following AAV-VEGF injection, or at days 1 and 4 after cancer cell injection. To label proliferating cells, 5-ethynyl-2'-deoxyuridine (EdU, Life Technologies) was administered intraperitoneally (500 µg per animal) every 2 days, for a period of 14 days. All treatments were performed in 2 month-old mice. For intramuscular injection, mice were anesthetized with isoflurane 1% with constant monitoring of heart and breath rate for the minimum necessary time required for the solution to be completely absorbed and avoid its spilling out upon muscle contraction (about 1 minute). A 0.3 ml insulin syringe with a 30- gauge needle was used. For intracardiac injections, mice were anesthetized by intraperitoneal injection of ketamine-xylazine (40 mg/kg-100 mg/kg, Imalgene 1000 and Sigma respectively), at a dosage of 1.2-1.3 µl/g. When sleeping, mice were taken out from the cage and laid down in supine position on a dedicated pad at 37°C, fixed to the plate and intubated. To reach the anterior wall of the heart, a xifoaxillar incision was made, exposing the underlying muscles; the pectoralis major muscle was lifted up and fixed with a retractor, while then the underlying pectoralis minor was cut to expose the ribs. The 5th intercostal space was pierced and enlarged with a retractor opening the thorax. The pericardium was stripped exposing heart anterior wall and either AAV vectors or cancer cells were injected into the left ventricle anterior wall using a 0.3 ml insulin syringe with a 30-gauge needle. All anatomical structures were visualized with a stereomicroscope (Leica). After injection, intercostal space, muscles and skin were sutured and mice were extubated to re-establish normal breathing. Mice were then laid in prone position and kept on the warmed pad until awakening and later transferred to a new cage.

Tumor models

B16-F10 melanoma (American Type Culture Collection, ATCC, CRL-6475) and LG1233 lung adenocarcinoma cells[5] (derived from lung tumors of K-rasLSL-G12D/$^{+}$;p53fl/fl mice 30, kindly provided by Dr. Tyler Jacks, Massachusetts Institute of Technology, Cambridge, MA) were injected into syngeneic C57BL/6 mice either into the tibialis anterior skeletal muscle or the left ventricular wall of the heart (100,000 cells per mouse). Breast cancer cells 4T1cells (CRL-2539; ATCC) were injected into syngeneic Balb/c mice either into the tibialis anterior skeletal muscle or the left ventricular wall of the heart (100,000 cells per mouse). B16-F10 and 4T1 cells were also injected subcutaneously into the right flank of mice (100,000 cells per mouse). To analyze tumor-associated sprouting angiogenesis, 10,000 LG cells were injected either into the tibialis anterior skeletal muscle or the left ventricular wall of the heart of AplnCreER;Rosa26-mT/mG, followed by 4-hydroxytamoxifen, as indicated above. Tumor volume was evaluated using calipers and expressed in mm3 using the formula: V = p/6*(dmax2 * dmin/2)[6].

Primary endothelial cell cultures and spheroids

Skeletal muscle and adult heart from AplnCreER;Rosa26-mT/mG mice were digested using Skeletal Muscle Dissociation kit (Miltenyi, 130-098-305). Endothelial cells were positively

selected using anti-CD31 conjugated magnetic beads (Miltenyi, 130-097-418) and MACS columns (Miltenyi, 130-042-401) for magnetic separation. For quantification of Apelin expression and proliferation rate, 2 x 10^4 endothelial cells per well were plated in primary 96-well plates coated with 1% fibronectin/gelatin and cultured for 72 hours in EGM-2 medium (Lonza, CC-3162) before fixation. For Apelin expression assay, cells were cultured in the presence of both 1 µM (Z)-4-Hydroxytamoxifen (Sigma, H7904) and 100 ng/ml recombinant human VEGF-A (R&D, 293-VE) for 48 hours. For endothelial spheroid formation, 5 x 10^4 isolated cells were seeded into 1% agarose-coated round bottom plates and left to aggregate for 48 hours in EMG-2 medium. Spheroids were transferred on top of a 2 mg/ml collagen layer (1.3% NaHCO3, 50% EGM-2, 2 mg/ml rat tail collagen) and cultured for 48 hours in EGM-2 medium supplemented with 1 µM (Z)- 4-Hydroxytamoxifen to allow endothelial cell sprouting and EGFP expression. Spheroids were fixed in 4% PFA and imaged.

Immunofluorescence, image acquisition and analysis

Hearts and skeletal muscles were collected in PBS and fixed in 2% PFA (Chem Cruz) overnight at 4°C. Tissues were then equilibrated in 30% sucrose overnight at 4°C before embedding in OCT (Bio-Optica) and cryosectioning. The 3 and 100 µm sections were transferred on Superfrost glass microslides (Thermo Scientific), permeabilized for 15 minutes in 0.5% Triton X-100, blocked in 5% BSA for 30 minutes and incubated with primary antibodies diluted in blocking solution overnight at 4°C. Primary cells were fixed in 4% PFA (Chem Cruz), permeabilized for 15 minutes in 0.1% Triton X-100, blocked in 5% BSA for 30 minutes and incubated with primary antibodies diluted in blocking solution overnight at 4°C. The following primary antibodies were used: anti-CD31 (R&D Systems, AF3628, 1:200), antiERG (Abcam, ab92513, 1:200), anti-α-SMA (Dako, M0851, 1:200), anti-cleaved caspase 3 (Cell Signalling Technologies, 9661S, 1:200) and anti-Ki67 (Cell Signalling Technology, D3B5, 1:200). Alexa Fluor-conjugated secondary antibodies (Invitrogen) were used to detect primary antibodies, while nuclei were counterstained with Hoechst (Invitrogen). Visualization of EdU incorporation by proliferating cells was performed using the EdU Click-iT assay (Invitrogen) following manufacturer's instructions. Images were acquired using a Nikon Eclipse Ti-E inverted fluorescent microscope equipped with an Andor's Neo sCMOS camera (DC-152Q-C00-FI) and a Zeiss LSM 880 confocal microscope. Images were analysed using ImageJ2 software[7]. The tumor border zone was defined as the region extending for 200 µm in both directions starting from the interface between tumor and healthy tissue. 3D rendering of EGFP signal from AplnCreER;Rosa26-mT/mG mice 100 µm thick slices has been obtained using Surface function of Imaris (Bitplane). Detection of nuclei inside $EGFP^+$ cells have been performed using Cells function of the same software.

Flow cytometry and cell sorting

Cells were resuspended in buffer solution (PBS, 1% BSA) and stained. First, samples were blocked with TruStain Fc™ CD16/32 antibody (1:100, BioLegend, 93) for 10 minutes at room temperature, followed by an incubation with anti-CD31 BV421 (1:200, BD Horizon™, MEC 13.3), Delta-like-4 APC (1:100, BioLegend, HMD4-1), VEGFR1/Flt1 APC (1:100, R&Dsystems), and CD45 APC-Fire (1:200, BioLegend, 30-F11) antibodies for one hour at 4°C. Samples were rinsed in PBS and stained with Live/Dead™ Fixable Aqua Dead Cell Stain kit in PBS (1:1000, Invitrogen) for 45 minutes at 4°C. Cells were then rinsed in buffer and fixed in 1% paraformaldehyde for 30 minutes at 4°C, washed and resuspended in

buffer. Cell acquisition was performed using a FACSCelesta (BD) and sorting was performed using a FACS Aria II (BD).

Data were eventually expressed as the difference between AAV-control and AAV-injected samples.

Statistical analysis

At least four animals per condition were analysed, with at least 15 images acquired per sample. Statistical analysis was performed using Excel and GraphPad Prism 7.0. Mean and standard error were calculated for all the datasets. Unpaired student's T-test and Two-Way ANOVA followed by Tukey's multiple comparison test were used to determine statistical significance.

References

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