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Supplemental Information

A Functional Role for VEGFR1 Expressed in Peripheral Sensory Neurons in Cancer Pain

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Supplemental Data

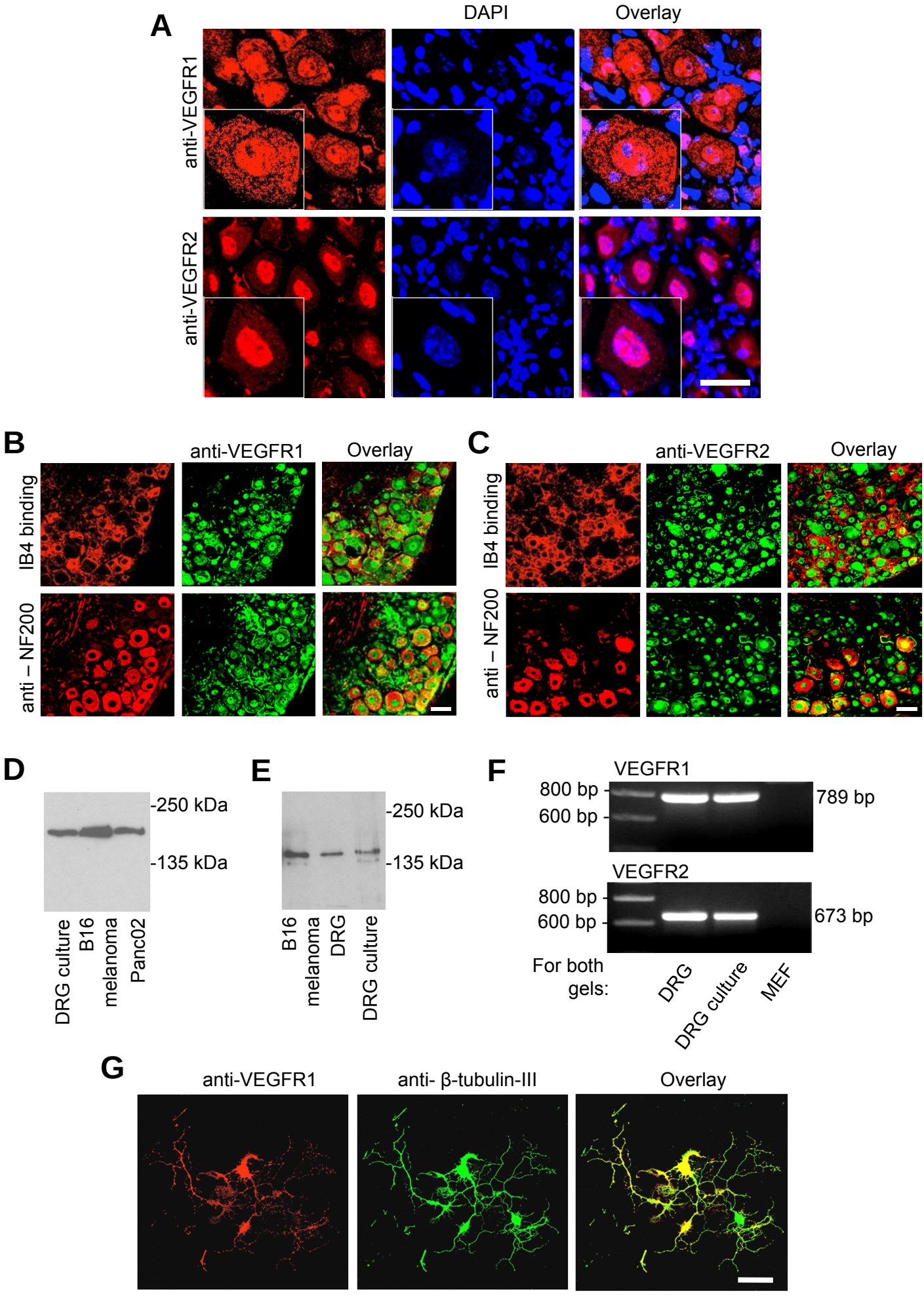


Figure S1 (related to main Figure 1): Expression analyses of VEGFR1 and VEGFR2 in sensory neurons of the dorsal root ganglia (DRG). (A) Immunofluorescence analysis with antibodies directed against VEGFR1 or VEGFR2 on DRG sections and counterstaining with the nuclear dye, DAPI. (B, C) Dual immunofluorescence analysis with antibodies directed against VEGFR1 (panel B) or VEGFR2 (panel C) with typical markers of distinct subtypes of sensory neurons on DRG sections. Biotinylated isolectin B4 (IB_4) was used to detect non-peptidergic nociceptive neurons and an anti-neurofilament 200 immunoreactivity was used to detect large-diameter (non-nociceptive) neurons. (D, E) Western blot analyses showing the expression of VEGF receptors, VEGFR1 (panel D) and VEGFR2 (panel E) with positive controls. (F) Reverse-transcriptase PCR analysis of expression of VEGFR1 mRNA and VEGFR2 mRNA in cDNAs derived from DRG and from neuron-enriched DRG cultures. cDNA derived from mouse embryonic fibroblasts (MEF) was included as a negative control. (G) Immunolabeling of VEGFR1 in cultured mouse DRG neurons identified via immunoreactivity for neuronal marker beta-tubulin-III. Scale bars represent 50 µm in panels A, B and C and G.

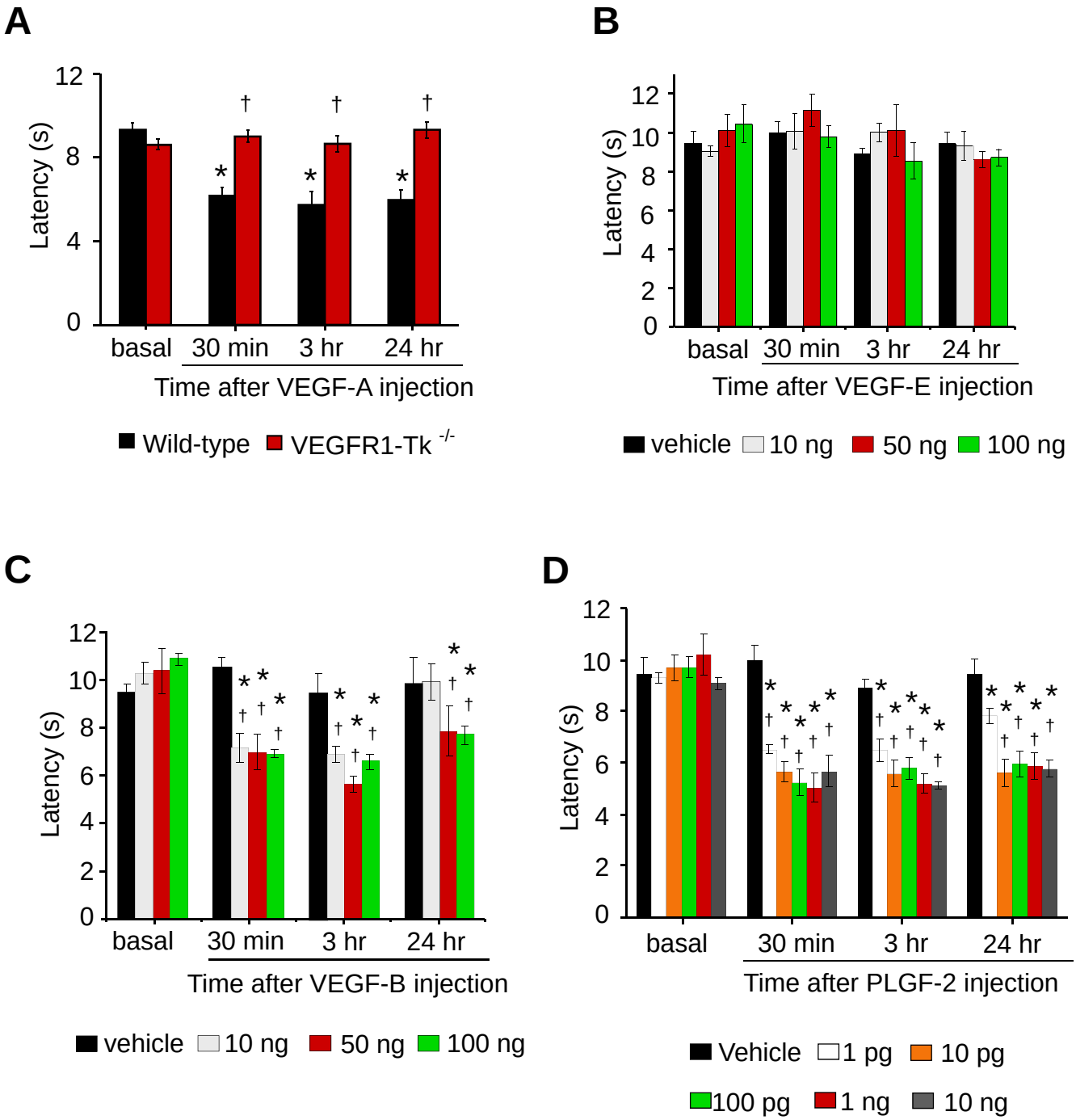
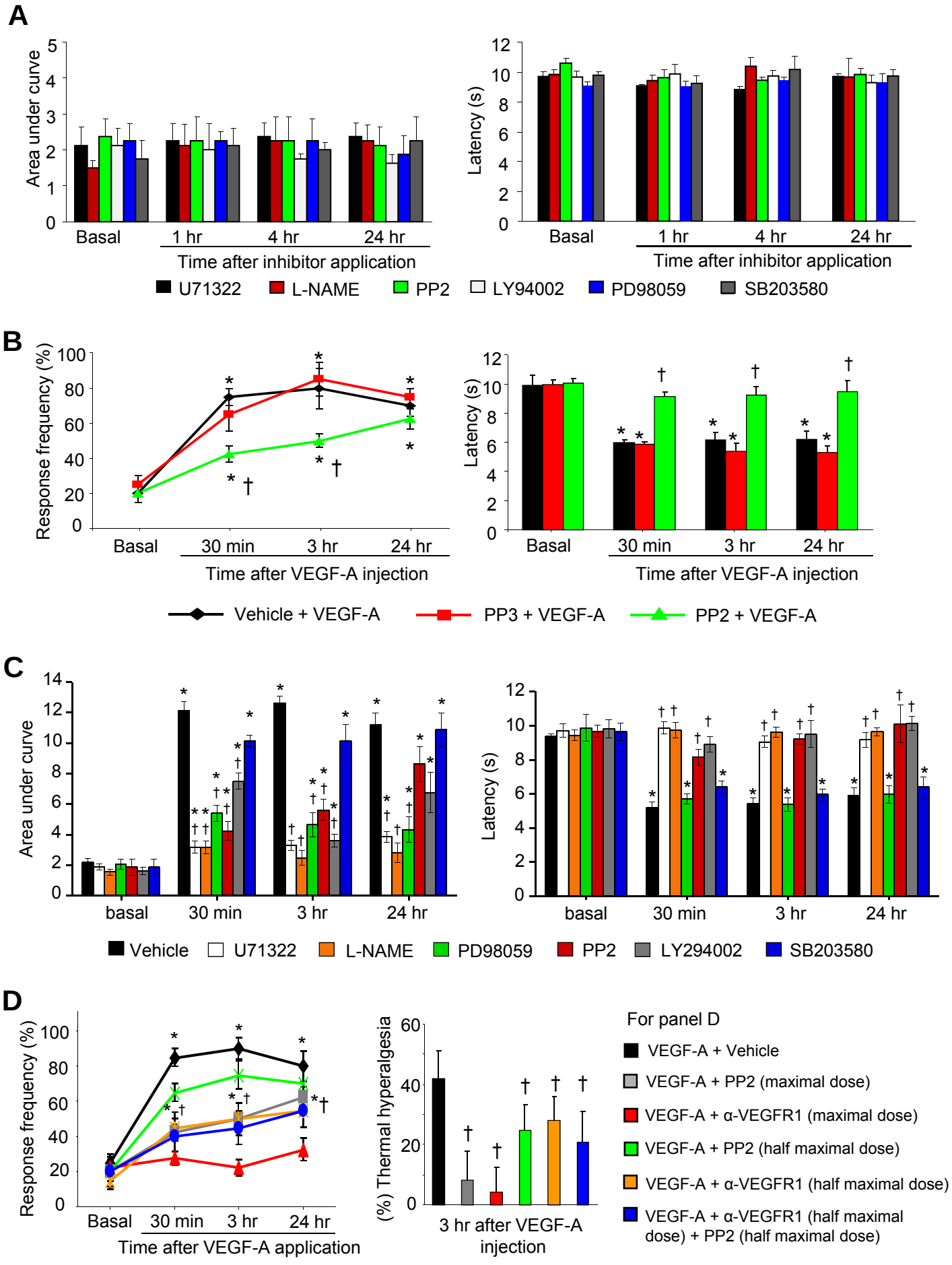
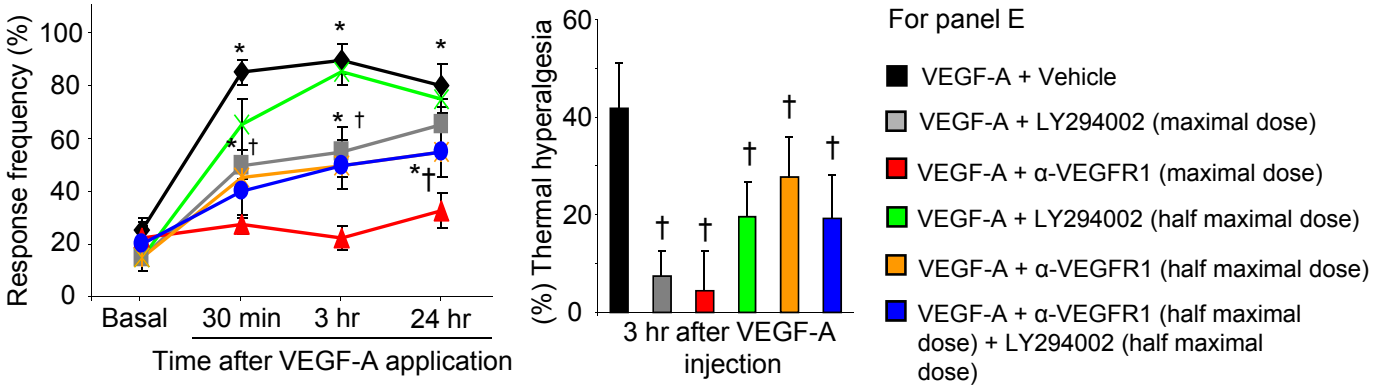

A
Latency (s)
basal
30 min
3 hr
24 hr
Time after VEGF-A injection
Wild-type
VEGFR1-Tk -/-
B
Time after VEGF-E injection
vehicle
10 ng
50 ng
100 ng
C
Time after VEGF-B injection
D
Time after PLGF-2 injection
Vehicle
1 pg
10 pg
100 pg
1 ng
10 ng

Figure S2 (related to main Figure 2): Agonist specificity of VEGF family ligands in the induction of thermal hyperalgesia. (A) Effect on thermal hypersensitivity upon intraplantar application of VEGF-A in VEGFR1-TK$^{-/-}$ as compared to wild type littermate controls (n = 8 mice per group). (B) Intraplantar application of a VEGFR2-specific agonist, VEGF- E up to a dose of 100 ng (n = 6 mice/group). (C, D) Dose-response relationships of the effects of two specific VEGFR1 agonists, VEGF-B (C) and PLGF-2 (D), on sensitivity to heat (n = 6 each). In all panels, *$p < 0.05$ as compared to basal; $^{\dagger}p < 0.05$ as compared to corresponding control group; ANOVA followed by post-hoc Fisher's test. Data are presented as mean +/- S.E.M.


A
Area under curve
Latency (s)
Basal
1 hr
4 hr
24 hr
Time after inhibitor application
U71322
L-NAME
PP2
LY94002
PD98059
SB203580
B
Response frequency (%)
Latency (s)
Basal
30 min
3 hr
24 hr
Time after VEGF-A injection
Vehicle + VEGF-A
PP3 + VEGF-A
PP2 + VEGF-A
C
Area under curve
Latency (s)
basal
30 min
3 hr
24 hr
Vehicle
U71322
L-NAME
PD98059
PP2
LY294002
SB203580
D
Response frequency (%)
Basal
30 min
3 hr
24 hr
Time after VEGF-A application
(%) Thermal hyperalgesia
3 hr after VEGF-A injection
For panel D
VEGF-A + Vehicle
VEGF-A + PP2 (maximal dose)
VEGF-A + α-VEGFR1 (maximal dose)
VEGF-A + PP2 (half maximal dose)
VEGF-A + α-VEGFR1 (half maximal dose)
VEGF-A + α-VEGFR1 (half maximal dose) + PP2 (half maximal dose)

E


Response frequency (%)
100
80
60
40
20
0
Basal
30 min
3 hr
24 hr
Time after VEGF-A application
(%) Thermal hyperalgesia
60
40
20
0
3 hr after VEGF-A injection
For panel E
VEGF-A + Vehicle
VEGF-A + LY294002 (maximal dose)
VEGF-A + α-VEGFR1 (maximal dose)
VEGF-A + LY294002 (half maximal dose)
VEGF-A + α-VEGFR1 (half maximal dose)
VEGF-A + α-VEGFR1 (half maximal dose) + LY294002 (half maximal dose)

F

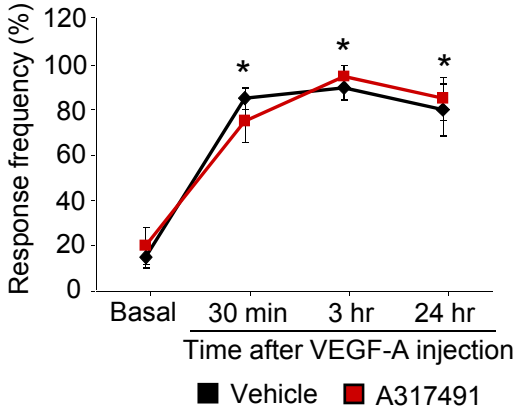

Response frequency (%)
120
100
80
60
40
20
0
Basal
30 min
3 hr
24 hr
Time after VEGF-A injection
Vehicle
A317491

Figure S3 (related to main Figure 3): Signaling pathways underlying nociceptive sensitization by VEGF-A or PLGF-2. (A) Effects of intraplantar delivery of inhibitors as a single dose at concentrations employed in experiments described in main Figure 3C in the absence of VEGF-A or PLGF-2 on mechanical sensitivity (left panel) and thermal sensitivity (right panel). (B) Effect of PP3 (200 pmoles), an inactive analog of the Src inhibitor, PP2 on mechanical (left panel) and thermal hypersensitivity (right panel) evoked by a single intraplantar injection of VEGF-A (1 ng) as compared to vehicle. (C) Effects of pretreatment with pharmacological inhibitors injected into the hind paw on the magnitude and time-course of mechanical hypersensitivity (left panel) and thermal sensitization (right panel) evoked by intraplantar injection of PLGF-2 (100 pg). Shown are effects of the following compounds at the indicated doses given as a single intraplantar injection: L-NAME (18.5 nmoles, NOS inhibitor), U71322 (20 pmoles, PLCγ inhibitor), LY294002 (1 nmole, PI3K inhibitor), PP2 (200 pmoles, Src Kinase inhibitor), PD98059 (18.7 nmoles, MEK inhibitor), SB203580 (30 nmoles, p38 MAPK inhibitor), vehicle (1% DMSO). (D, E) Experiments testing effects of intraplantar combinations of half-maximal doses of Src inhibitor (panel D) or of PI3K inhibitor (panel E) given in combination with a half-maximal dose of the anti-VEGFR1 antibody on mechanical hypersensitivity to 0.4 g von Frey force (left panels in D, E) and thermal hyperalgesia (right panels in D, E) evoked by intraplantar VEGF-A (1 ng) injection. (F) Effects on mechanical hypersensitivity upon intraplantar injection of VEGF-A in the presence of a P2X3 inhibitor, A317491 (n = at least 6 mice/group). In all panels, * $p < 0.05$ as compared to basal value; $^{\dagger}p < 0.05$ as compared to corresponding control; ANOVA followed by post-hoc Fisher's test. Data are presented as mean +/- S.E.M.

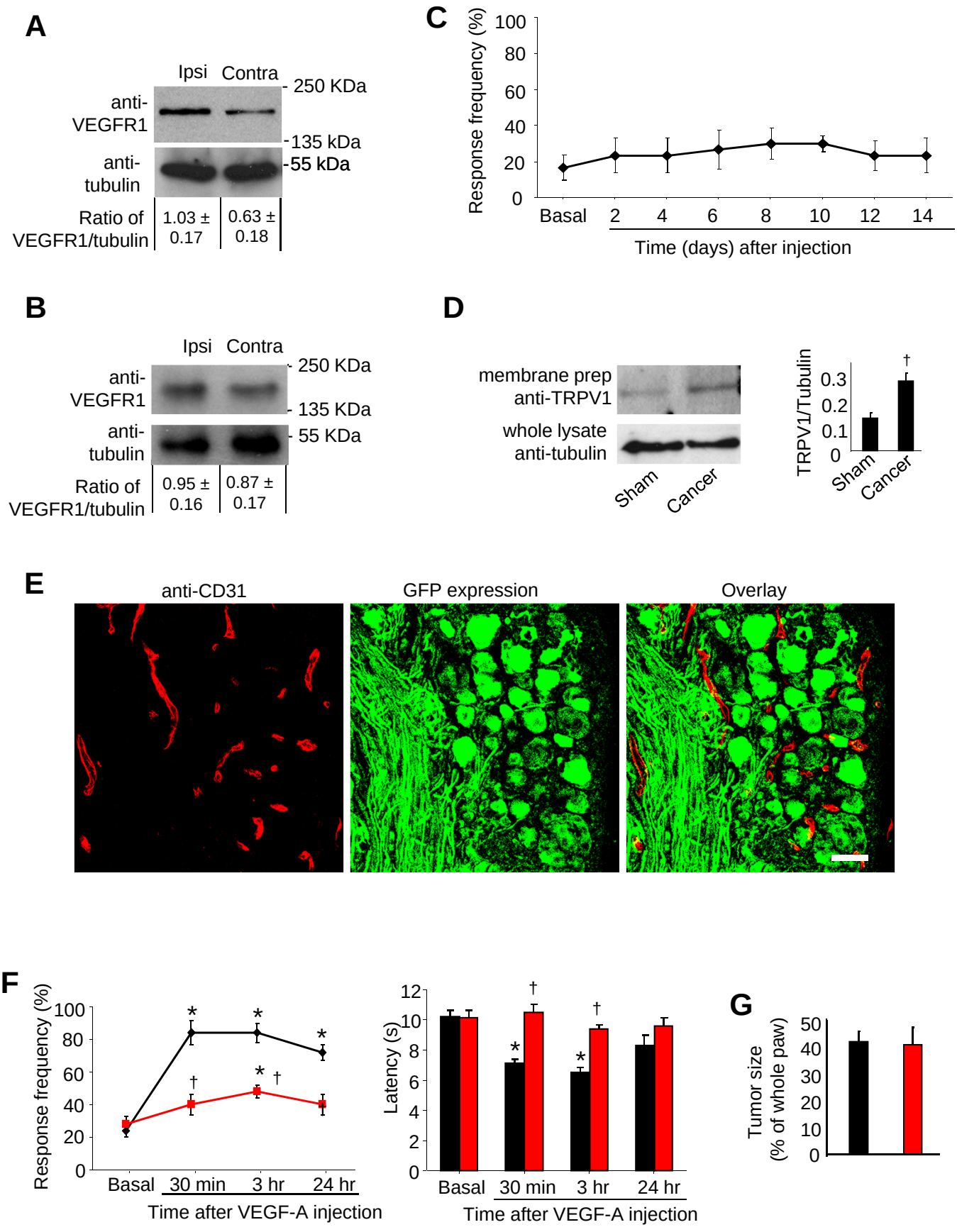

A
Ipsi
Contra
anti-VEGFR1
250 KDa
135 kDa
anti-tubulin
55 kDa
Ratio of VEGFR1/tubulin
1.03 ± 0.17
0.63 ± 0.18
B
Ipsi
Contra
anti-VEGFR1
250 KDa
135 KDa
anti-tubulin
55 KDa
Ratio of VEGFR1/tubulin
0.95 ± 0.16
0.87 ± 0.17
C
Response frequency (%)
100
80
60
40
20
0
Basal
2
4
6
8
10
12
14
Time (days) after injection
D
membrane prep anti-TRPV1
whole lysate anti-tubulin
Sham
Cancer
TRPV1/Tubulin
0.3
0.2
0.1
0
†
Sham
Cancer
E
anti-CD31
GFP expression
Overlay
F
Response frequency (%)
100
80
60
40
20
0
*
*
*
†
*
†
Basal
30 min
3 hr
24 hr
Time after VEGF-A injection
Latency (s)
12
10
8
6
4
2
0
†
†
*
*
Basal
30 min
3 hr
24 hr
Time after VEGF-A injection
G
Tumor size (% of whole paw)
50
40
30
20
10
0
For panels F & G: Lenti non-targeting shRNA Lenti VEGFR1-shRNA

Figure S4 (related to main Figure 4): Control experiments for analysis of the expression and role of VEGFR1 expression in the bone metastatic pain. (A) Analysis of VEGFR1 expression by Western blot analyses on lysates of L3-L4 DRGs ipsilateral and contralateral to the tumor-bearing (NCTC cell-injected) hindpaw and corresponding quantitative densitometric analysis of the bands (n = 3 independent experiments; $^{\dagger}p < 0.05$; Student's t-test). (B, C) VEGFR1 expression in the L3-L4 ipsilateral and contralateral DRGs (B) and analysis of tumor-induced mechanical hypersensitivity (C) following injection of mouse embryonic fibroblasts (MEFs) in the calcaneus bone of mice (0.4 g shown here). (D) Analysis of TRPV1 expression in membrane preparations of distal branches of sciatic nerve in mice bearing cancer in the calcaneus bone and sham-treated mice (n = 3 mice/group); right panel represents quantitative summary. (E) Analysis of lentiviral expression in blood vessels of injected DRGs shown via expression of GFP and immunostaining for CD31, a blood vessel marker. (F) Effects on mechanical hypersensitivity (left panel) and thermal hyperalgesia (right panel) induced by intraplantar VEGF-A (1 ng) upon lentiviral knockdown of VEGFR1 in vivo (n = 5 mice/group). (G) Analysis of tumor size in mice expressing VEGFR1-shRNA and non-targeting control shRNA in the osteolytic calcaneus sarcoma model of cancer pain. In all panels, * $p < 0.05$ as compared to basal value; $^{\dagger}p < 0.05$ as compared to corresponding control; ANOVA followed by post-hoc Fisher's test. Data are presented as mean +/- S.E.M. Scale bars represents 50 μm in panel E.

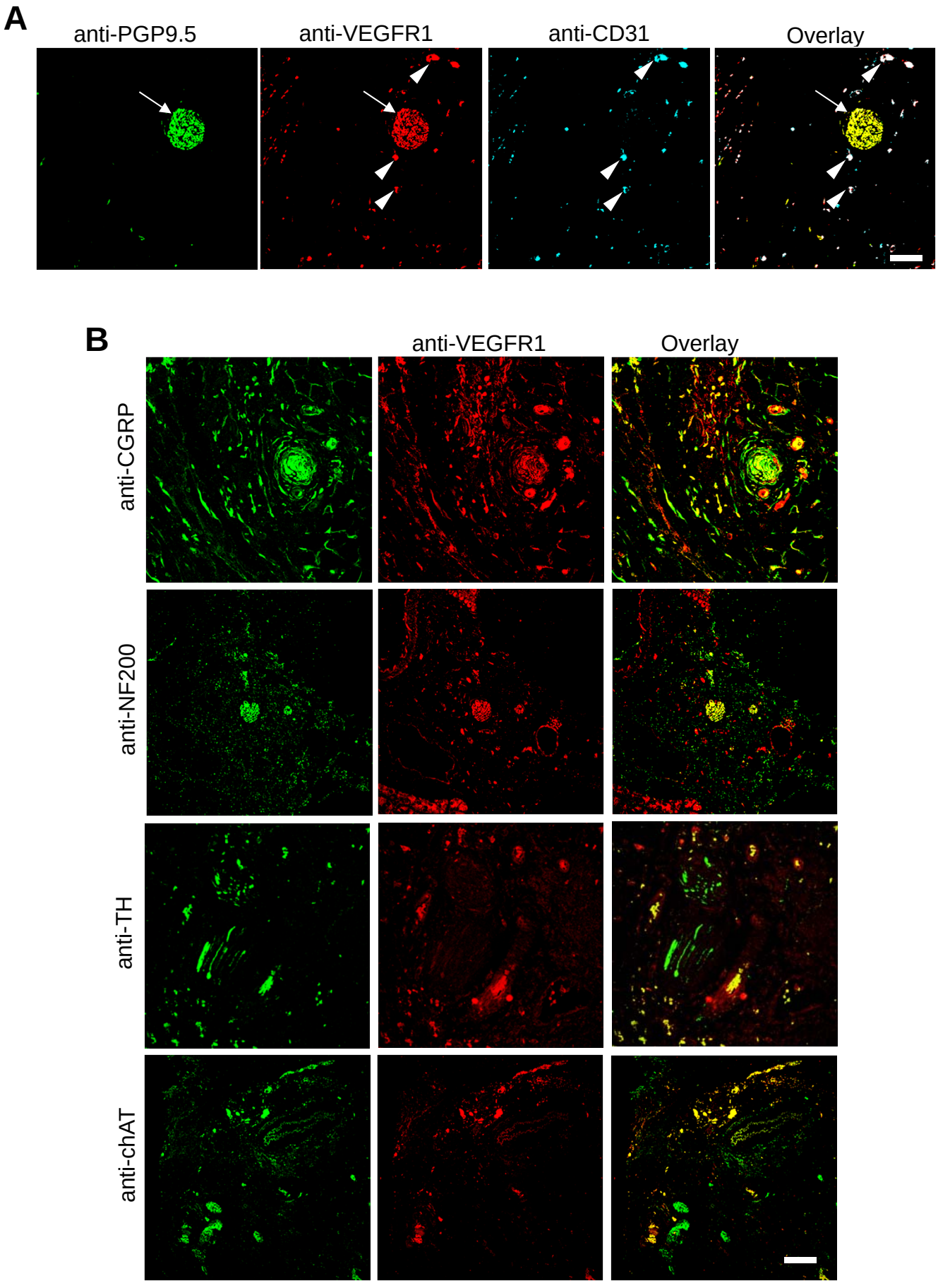

A
anti-PGP9.5
anti-VEGFR1
anti-CD31
Overlay
B
anti-VEGFR1
Overlay
anti-CGRP
anti-NF200
anti-TH
anti-chAT

Figure S5 (related to main Figure 6). Characterization of neuronal VEGFR1 expression in human pancreatic ductal adenocarcinoma (PDAC). (A) Triple co-immunostaining for VEGFR1, a peripheral nerve marker (PGP9.5) and a blood vessel marker (CD31). Co-localization of VEGFR1 and PGP9.5 is represented by arrows (yellow overlay) and co-localization of VEGFR1 and CD31 is denoted by arrowheads (white overlay). (B) Analysis of co-immunoreactivity or anti-VEGFR1 and various markers of specific types of nerves. Scale bars represent 100 μm in panel A and B.

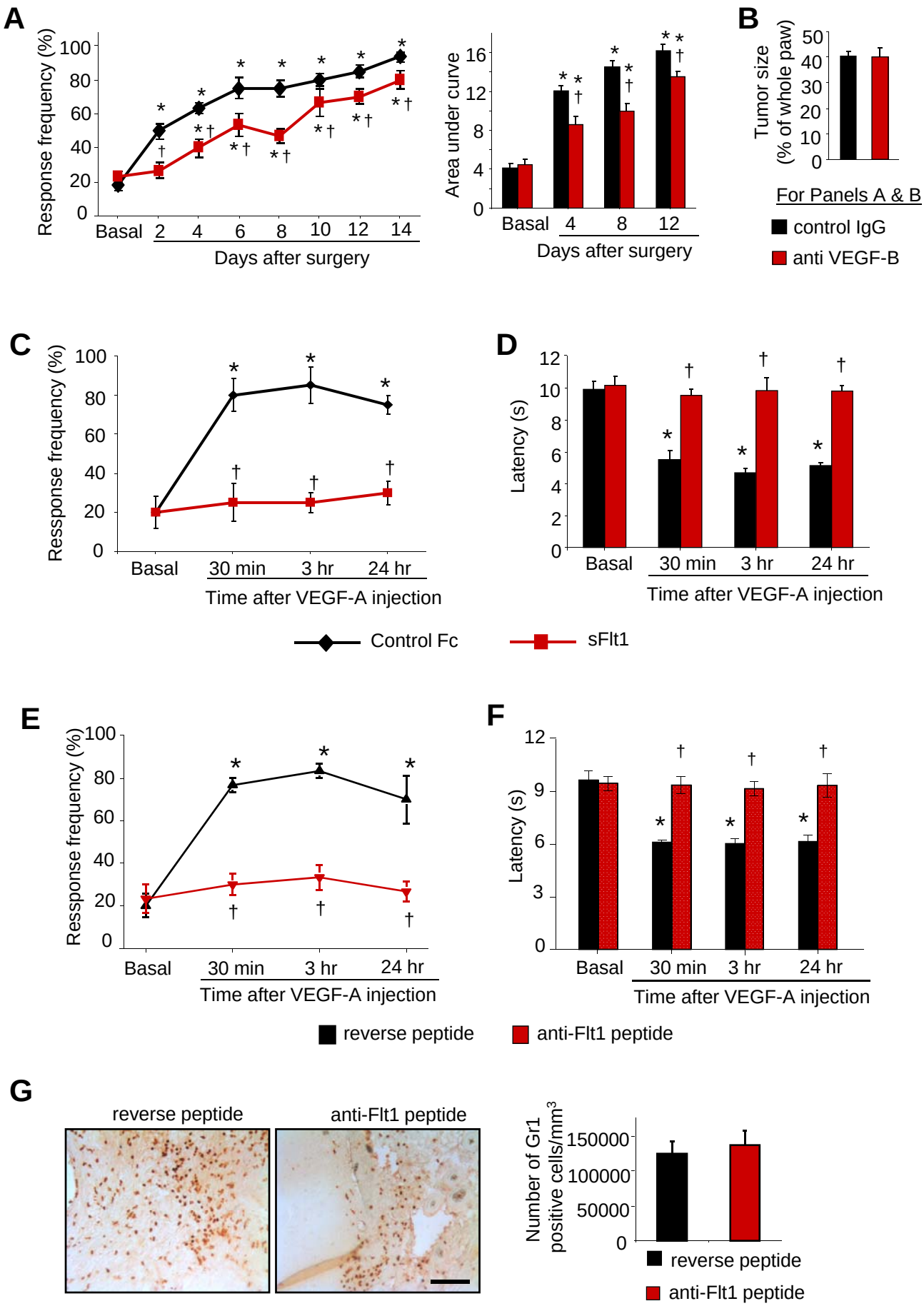

A
Response frequency (%)
100
80
60
40
20
0
Basal
2
4
6
8
10
12
14
Days after surgery
Area under curve
16
12
8
4
0
Basal
4
8
12
Days after surgery
B
Tumor size (% of whole paw)
50
40
30
20
10
0
For Panels A & B
control IgG
anti VEGF-B
C
Ressponse frequency (%)
100
80
60
40
20
0
Basal
30 min
3 hr
24 hr
Time after VEGF-A injection
Control Fc
sFlt1
D
Latency (s)
12
10
8
6
4
2
0
Basal
30 min
3 hr
24 hr
Time after VEGF-A injection
E
Ressponse frequency (%)
100
80
60
40
20
0
Basal
30 min
3 hr
24 hr
Time after VEGF-A injection
reverse peptide
anti-Flt1 peptide
F
Latency (s)
12
9
6
3
0
Basal
30 min
3 hr
24 hr
Time after VEGF-A injection
G
reverse peptide
anti-Flt1 peptide
Number of Gr1 positive cells/mm3
150000
100000
50000
0
reverse peptide
anti-Flt1 peptide

Figure S6 (related to main Figure 7): Validation of agents sequestering VEGF-family ligands. (A) Calcaneus tumor-induced mechanical hypersensitivity to von Frey filament 0.4 g (left panel) and area under curve over von Frey forces 0.07- 1.0 g (right panel) in mice receiving neutralizing antibodies against VEGF-B (n = 6 mice/group). (B) Effect of VEGF-B antibody on tumor growth in the calcaneus bone. (C-F) Effect of local injections of sFlt1 or anti-Flt1 peptide in vivo or their corresponding controls on mechanical hypersensitivity (panels C and E) and thermal hyperalgesia (panels D and F) induced by intraplantar VEGF-A (1 ng) upon (n = at least 6 mice/group). (G) Typical examples (left panel) and quantitative summary (right panel) of macrophages identified via anti-Gr1 immunostaining in the skin adjoining the osteolytic tumor. In all panels, *$p < 0.05$ as compared to basal value; $^{\dagger}p < 0.05$ as compared to corresponding control; ANOVA followed by post-hoc Fisher's test. Data are presented as mean +/- S.E.M. Scale bars represents 100 μm in panel G.

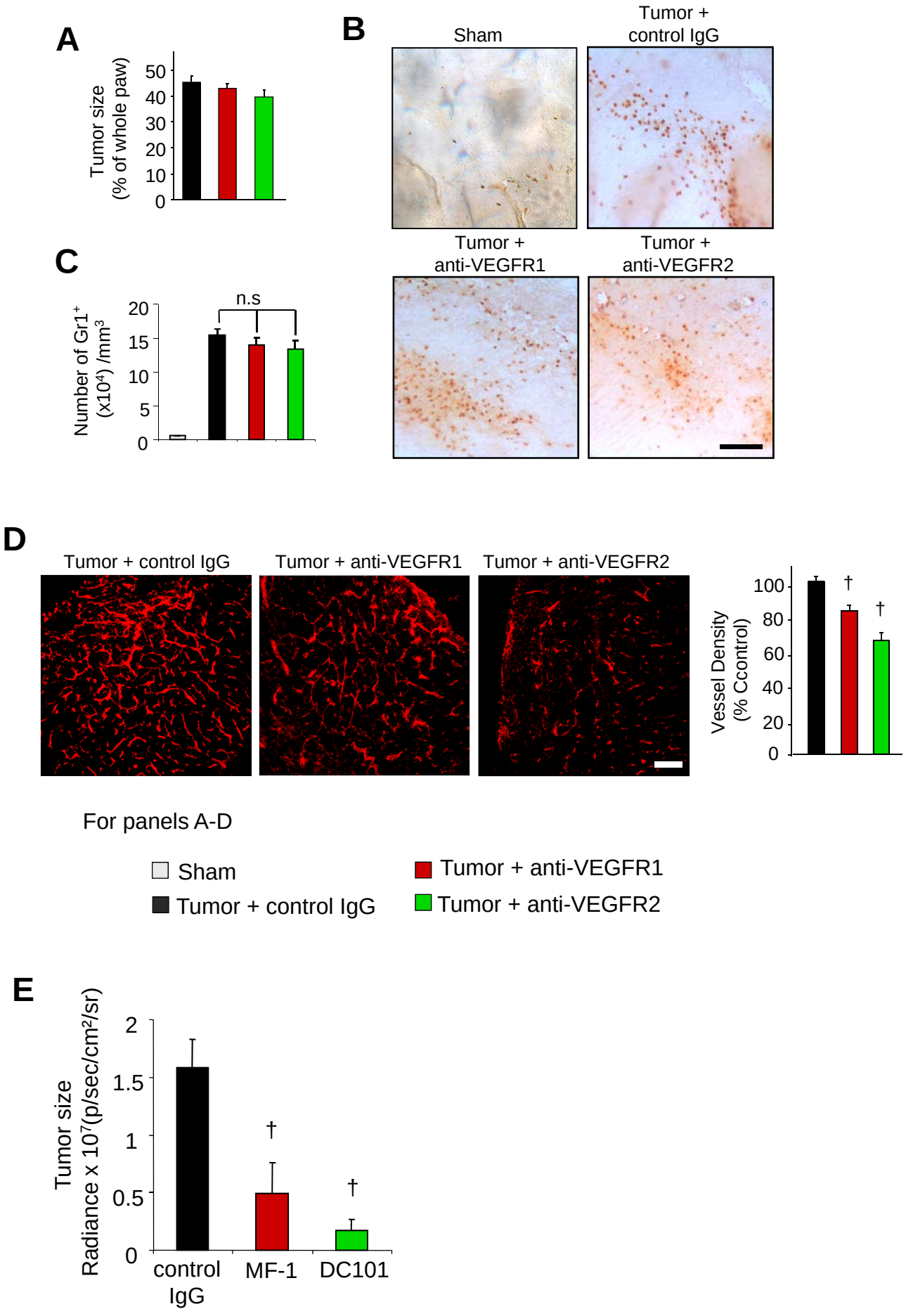

A
Tumor size
(% of whole paw)
50
40
30
20
10
0
B
Sham
Tumor +
control IgG
Tumor +
anti-VEGFR1
Tumor +
anti-VEGFR2
C
Number of Gr1+
(x10⁴) /mm³
n.s
20
15
10
5
0
D
Tumor + control IgG
Tumor + anti-VEGFR1
Tumor + anti-VEGFR2
Vessel Density
(% Ccontrol)
100
80
60
40
20
0
†
†
For panels A-D
Sham
Tumor + control IgG
Tumor + anti-VEGFR1
Tumor + anti-VEGFR2
E
Tumor size
Radiance x 10⁷(p/sec/cm²/sr)
2
1.5
1
0.5
0
†
†
control
IgG
MF-1
DC101

Figure S7 (related to main Figure 8): Effects of immunologicals targeting VEGFR on tumor growth, tumor-induced angiogenesis and inflammation. (A) Analysis of tumor size in mice treated locally with antibodies against VEGFR1 or VEGFR2 or control IgG. (B, C) Typical examples (B) and quantitative summary (C) of macrophages identified via anti-Gr1 immunostaining in the skin adjoining the osteolytic tumor in the calcaneus bone metastatic model of cancer pain; n = 5 mice/group. (D) Typical examples and quantitative analysis (right panel) of the density of blood vessel identified via CD31 immunoreactivity in the above groups of mice. (E) Analysis of tumor size in mice receiving systemic injections of MF-1, DC101 or control IgG estimated in vivo via measurements of the activity of luciferase expressed by the murine breast cancer cells, 4T1-Luc, in the femur bone. $^{\dagger}p < 0.05$ as compared to control IgG; ANOVA followed by post-hoc Fisher's test. Data are presented as mean +/- S.E.M. Scale bars represents 100 μm in panel B and D.

Movie S1 (related to main Figure 8): This movie depicts the nature and extent of nocifensive behavior and weight bearing in mice 30 days after sham surgery.

Movie S2 (related to main Figure 8): This movie depicts the nature and extent of nocifensive behavior, such as guarding of the tumor-bearing limb and lifting of the affected paw above the wire-mesh bottom, reduction in weight-bearing of the affected paw, sporadic hopping behavior during ambulation in tumor-bearing mice 30 days after implantation of breast cancer cells unilaterally in the femur bone cavity; in addition the mouse shown in the video received intraperitoneal injections of control IgG.

Movie S3 (related to main Figure 8): This movie depicts the nature and extent of nocifensive behavior in tumor-bearing mice 30 days after implantation of breast cancer cells unilaterally in the femur bone cavity; in addition the mouse shown in the video received intraperitoneal injections of an anti-VEGFR1 antibody (MF1 clone).

Movie S4 (related to main Figure 8): This movie depicts the nature and extent of nocifensive behavior in tumor-bearing mice 30 days after implantation of breast cancer cells unilaterally in the femur bone cavity; in addition the mouse shown in the video received intraperitoneal injections of an anti-VEGFR2 antibody (DC101 clone).

Supplemental Experimental Procedures

Materials from human tissues: Archived tissues from patients who had undergone resections for pancreatic ductal adenocarcinoma were obtained retrospectively from the tissue database of University clinics Heidelberg, Germany. The use of archived tissues has been approved by the institutional review board and written informed consent had been obtained from the patients prior to the surgical procedure (ethics committee, University of Heidelberg, Germany; #301/2001). Detailed clinical and histopathological data were available for all patients. Pain ratings in patients were recorded in our database according to responses to a questionnaire asking for a description of the intensity of pain on a short scale: 0 = no pain; 1 = mild pain; 2 = moderate pain ("abdominal discomfort or pain that is non disabling but requires analgesics"); 3 = severe pain ("pain that is disabling and controlled only by narcotics"), as previously described (Michalski et al., 2007). Due to the infrequent availability of the groups of patients without pain or suffering from mild pain, they were combined for analyses. Thus, patients without/with mild pain were compared to patients with moderate pain and to patients with strong pain. Normal pancreas tissues were obtained through an organ donor program from previously healthy individuals which was approved by the local ethics committee as indicated above.

Immunohistochemistry on pancreas, paw biopsy punches and DRG: Punch biopsies of the plantar surface were taken on day 14 to determine the nerve innervation of the skin overlaying the tumor, both in the NCTC and LL2 induced bone metastases model as described previously (Schweizerhof et al., 2009). After perfusion of the animals with 4% paraformaldehyde, 4 mm punches were taken and post fixed in 4% paraformaldehyde for 24 h at 4°C and cryoprotected in 30% sucrose in 0.05 M PBS

at 4 °C. Punches were cut at 25 µm using a cryotome (CM3050 S, Leica Microsystems). The sections were treated with 50 mM glycine in 0.05 M PBS for 15 min followed by permeabilization with 0.2% Triton-X-100 for 15 min. After blocking for an hour with 10% normal horse serum in 0.1 M PBS, sections were incubated with an antibody recognizing PGP9.5 (1:1000, Ultraclone), which is used as a phenotypic marker for peripheral neurons, or anti-VEGFR1 (1:100; MAB471, R&D Systems) or VEGFR2 antibody (1:100; sc-505, SCBT), in 0.1% normal horse serum in 0.1 M PBS, overnight. After 3 washes for 15 min each with 10% normal horse serum in PBS, sections were incubated with FITC-labeled anti-rabbit-antibody (1:200; Dianova) for 1 hour at room temperature. After 3 washes for 15 min each with 10% normal horse serum in PBS, sections were rinsed with 0.1 M PBS thrice for 10 min each and then treated with 10 mM Tris pH 8.0 and mounted with mowiol and stored in dark in 4°C. Immunofluorescence for PGP9.5 in paw sections was imaged on a confocal laser scanning microscope (TCS SP2 AOBS, Leica) and maximal projections were created.

Human paraffin embedded pancreatic tissue was cut in 3 µm sections using a microtome (HM 350 S, Microm). The sections were de-paraffinized and then treated with Roticlear (Roth) for 30 min, rehydrated using 100%, 95%, 70%, 50% Ethanol for 5 min sub sequentially and kept in warm citrate buffer (10 mM citric acid, pH 6.0) for 20 min. After washing with H2O and PBS for 20 min each, sections were quenched for endogenous peroxidase activity using 3% H2O2 in Methanol for 20 min, washed again with PBS for 10 min and blocked with 10% normal horse serum and 0.05% Triton X-100 in PBS for 30 min. Primary antibodies were applied overnight in 10% normal horse serum, 0.05% Triton X-100 in PBS at 4°C. After treatment with 10% normal horse serum, 0.05% Triton X-100 in PBS for 30 min, the respective secondary antibody, FITC-labeled anti-rabbit antibody and TRITC-labeled anti-mouse and anti-

goat antibody (1:200, Dianova) in 10% normal goat serum in PBS was applied for 1 hour. After washing with PBS thrice 15 min each, the sections were then treated with 10 mM Tris pH 8 and embedded with mowiol.

DRGs were dissected from wild-type mice and cut at 16 µm using a cryotome. Sections were washed with 50 mM glycine in PBS and PBST for 10 min each, blocked with 10% normal horse serum in PBS for 40 min and incubated with primary antibody against either VEGFR1 (1:100; MAB471, R and D systems) or VEGFR2 (1:100; sc-505, Santa Cruz Biotechnology SCBT, CA, USA) combined with either Isolectin B4, IB4 (1:200; B-1205, Vector) or an antibody recognizing the Calcitonin Gene Related Peptide, CGRP (1:300; 24112, Immunostar, Germany) or neurofilament-200 (1:500, # N2912, # N4142, Sigma Aldrich) or anti-CD31, a marker for endothelial cells (1:500, #550274, BD Pharmingen, USA) overnight. After washing with 10% normal horse serum in PBS, the secondary antibodies anti-rabbit-FITC (1:200), anti-goat-TRITC (1:200), anti-rat TRITC (1:400) and Streptavidin-TRITC respectively (1:200; against the biotinylated IB4 antibody) were applied for 60 min. After washing with PBS for 20 min, and treatment with 10 mM TRIS-HCl for 10 min, sections were embedded with mowiol and stored at 4°C in the dark. For the quantification of cell types expressing VEGFR1 and VEGFR2, stereological counting was performed on multiple sections and expressed as % of overlay to the total positive cells.

Behavioral testing: Mechanical hyperalgesia was measured using a von Frey monofilament with the bending forces 0.07 g, 0.16 g, 0.4 g, 0.6 g and 1.0 g. The filament was applied 5 times onto the plantar skin overlying the calcaneus bone. Mice were acclimatized to the experimental setups several times before the analysis. The experimenter was blinded to the identity of the animals being analyzed. There are

multiple ways of representing these data: e.g. one could represent a response frequency values for each filament over the whole course of tumor growth in days. Alternatively, one could show a stimulus-response function for all filaments at one particular time point in a graph. Both ways of representation would require showing 5 number of curves per group, which would take up a lot of space. We have therefore chosen to show a few examples of responses evoked by particular filaments and represented the data from the all filaments in form of 'area under curve'. Area under curve was calculated as an integral of a stimulus-response function for all filaments per time point tested, thus allowing a more compact representation of sensory sensitivity over the entire course of tumor growth as represented by (Gangadharan et al., 2011). The behavioral analyses were performed in a blinded manner.

Analysis of responses to noxious heat and mechanical pressure: Mice were acclimatized and the latency in response to heat and the threshold to mechanical pressure applied via von Frey filaments were measured as described before (Hartmann et al., 2004). Withdrawal latency to infrared heat was measured prior to and at 30 min, 3 h and 24 h according to the Hargreaves method using a Plantar test apparatus (Ugo Basile Inc.). Mechanical hypersensitivity was measured using von Frey filaments and paw withdrawal frequency was calculated from 5 independent applications. The following VEGF ligands were injected into the plantar surface dissolved in saline (0.9% NaCl): murine VEGF-A (1 pg-10 ng per 20 µl, CYT-336, Prospec-Tany, Ness-Ziona, Israel), PLGF-2 (1 pg to 10 ng per 20 µl, # 465-PL-010/CF, R&D systems, Germany), VEGF-B (10 ng to 100 ng per 20 µl, # 767-VE-010/CF, R&D systems) or VEGF-E (10 ng to 100 ng, # CYT-263, Prospec-Tany, Ness-Ziona, Israel). The corresponding vehicle was given to control animals, which mostly comprised saline. In some experiments, mice were pretreated with

neutralizing antibodies against VEGFR1 (5 µg diluted in 25 µl, #AF471; R&D systems, Minneapolis, MN, USA) or against VEGFR2 (5 µg diluted in 25 µl, AF644; R&D systems) or control IgG (5 µg diluted in 25 µl, AB-108-,C; R&D systems), against NRP-1 (5 µg diluted in 25 µl, AF566, R&D systems), against NRP-2 (5 µg diluted in 25 µl, AF567, R&D systems), soluble VEGFR1 (SFC-M06; 10 µg diluted in 25µl, Reliatech, Germany) or the corresponding Fc control (4460-MG, R and D systems) an anti-FLT1 peptide (GNQWFI, 25 µg diluted in 25 µl, RB-PP-0245, Raybiotech, GA, USA) or the corresponding reverse peptide (IFWQNG, 25 µg diluted in 25 µl, Raybiotech, USA). In pharmacological experiments in vivo, mice were pretreated with the following inhibitors; U71322, PLCγ inhibitor (20 pmoles/20 µL, # 662035, Calbiochem, Germany); L-NAME, NOS inhibitor (18.5 pmoles/20 µl, #N5751, Sigma Aldrich, Germany); PP2, Src kinase inhibitor (200 pmoles/20 µl, # 529573, MERCK, Germany); PD98059, MEK inhibitor (18.7 µg/20 µl, PHZ1164, Invitrogen, Germany) ; LY942002, PI3 kinase inhibitor (1 nmole/20 µl, # 9901, Cell signaling technology, Germany); SB203580 (30 nmoles /20 µl, #PHZ1253, Invitrogen, Germany); P2X3 inhibitor (A-317491, 10 nmoles/ 20 µl, #A2979, Sigma Aldrich, Germany). Mice were analyzed for thermal and mechanical sensitivity at the indicated time points and returned to their cages between measurements. All behavioral analyses were performed in a blinded manner.

Spontaneous nocifensive behavior: Mice were placed on an open-wire mesh bottom and were allowed to habituate for 20 minutes. Following acclimatization, time spent in nocifensive behavior was recorded over a period of 5 minutes (Mantyh et al., 2011). The mice were then returned back to their cages. Nocifensive behavior was defined as: (i) spontaneous guarding (lifting the affected limb and holding it against the body), (ii) flinching, (iii) sporadic hopping or limping (intermittent jumps without using the

affected limb while moving). Spontaneous nocifensive behavior was assessed in mice on days 10, 20 and 30 following surgery. The experimenter was blinded to the experimental conditions of the mice.

Lentiviral injection into the DRG: Readily made high titer lentivirions particles expressing shRNA against murine VEGFR1 mRNA was obtained from Open Biosystems Inc. (GIPZ Lentiviral shRNAmir, that are tagged with eGFP), represented as sense-loop-antisense in the 5'-3'direction shRNA#1 GAGCGACGGAATCTTCAATCTACATATTAGTGAAGCCACAGATGTAATATGTAGATTGAAGATTCCGCTGCC(Catalog #VGM5520-98979496)or shRNA #2 TGCTGTTGACAGTGAGCGCGCGGAATCTTCAATCTACATATAGTGAAGCCACAGATGTATATGTAGATTGAAGATTCCGCTTGCCTACTGCCTCGGA (Catalog # VGM5520-99435948) or a GIPZ non-silencing control viral particles were obtained from Source (Catalog # RHS4348). Lentiviral injections into the DRGs *in vivo* were performed as described previously (Schweizerhof et al. 2009). Briefly, lentivirions (approx. $9X10^9$ transfection units per ml) were diluted 1:2 with 20% Mannitol and injected unilaterally into L3 and L4 DRGs (2 µl per DRG, or $6X10^6$ transfection units per DRG) using a 35G needle with a microinjection pump (WPI) at a rate of 500 nl per minute in adult mice, deeply anesthetized using fentanyl/domitor/dormicum (4:6:16 vol/vol/vol; 0.7 ml/g, intraperitoneally). At 3 weeks after viral infection, mice were subjected to tumor induction in the calcaneus bone as described above. Mice were killed 14 days after tumor induction and the injected L3-L4 DRGs were rapidly isolated and subjected to western blot analysis for VEGFR1 and tubulin (control).

Vascular permeability assay: Mice were anaesthetized with 50 mg/kg pentobarbital sodium and injected i.v. with 1% Evans blue in PBS (50 µl). After 1 min, intraplantar

injections of either saline (20 µl), histamine (1 µg in 20 µl, Sigma Aldrich) or VEGF-A (10 ng in 20 µl) were performed unilaterally while the contralateral paw served as control. Mice were killed 10 min after injections and tissue samples were collected using a paw biopsy punch that retains 12.5 mm^2 of paw skin. The skin punches were incubated in 200 µl of formamide at 55°C for 48 hours. Evans blue content was determined by absorption at 595 nm and expressed in ng/mm^2 of the skin (Korhonen et al., 2009).

ELISA for VEGF family ligands: The levels of VEGF-A, PLGF-2 and VEGF-B were determined using ELISA kits according to manufacturer's instructions (Ray biotech, Inc) on lysates derived the paw heel tissue overlying the ipsilateral calcaneus bone of tumor-bearing or sham mice.

Single nerve electrophysiological recordings in the skin-nerve preparation: An in vitro skin nerve preparation was used to study the properties of the afferent fibers in the saphenous nerve under control conditions and at 30 minutes after the application of 1, 10, 100 and 200 ng/ml VEGF-A or the vehicle (0.9% saline). Animals were killed under CO_2; the saphenous nerve was dissected with the innervated skin attached and placed in organ bath chorium side up. The skin was placed in the oxygen-saturated modified synthetic interstitial fluid solution (123 NaCl, 3.5 KCl, 0.7 $MgSO_4$, 1.7 NaH_2PO_4, 2.0 $CaCl_2$, 9.5 sodium gluconate, 5.5 glucose, 7.5 sucrose, and 10 HEPES, in mM) at temperature of 32 ± 1°C and pH 7.4 ± 0.05, and the nerve was de-sheathed and teased to enable single-unit recording. Units were classified according to their conduction velocities, von Frey thresholds, and firing properties. Electrical stimulation of the nerve trunk was employed to calculate conduction velocities of individual nerve fibers. Fibers which conducted > 10 m/s and fibers conducting

between 1-10 m/s were considered to be myelinated A-β fibers and A-δ fibers, respectively. Receptive fields were found using mechanical stimulation with a glass rod. A computer-controlled linear stepping motor (Nanomotor Kleindiek Nanotechnik) was used to apply standardized mechanical stimuli. A hollow metal cylinder was placed above the unit and the unit was tested with ascending series mechanical stimuli ranging from 6 to 96 µm of displacement. The same test was used after the application of either VEGF (10ng/ml to 200 ng/ml) or saline applied directly to the receptive field within the metal cylinder. Electrophysiological data were collected with Powerlab 4.0 system and analyzed off-line with the spike histogram extension of the software (Milenkovic et al., 2008).

Culture and analysis of DRG neurons: Cultures of DRG neurons were prepared from C57BL/6 mice using standard protocols as described previously (Owen and Egerton, 2012; Schweizerhof et al., 2009). Cells were treated with media containing 5 ng/ml murine VEGF-A, lysed at given time points and Western blotting was performed using antibodies recognizing either phosphorylated ERK1 and ERK2 (1:1500; #4377, Cell Signalling, Danvers, MA, USA), or total ERK1 and ERK2 (1:1500; #4695, Cell Signalling, Danvers, MA, USA) or Src antibody, phospho-Src antibody (1:1000; #2208; #2101, cell signaling technologies). Neuron enriched DRG cultures were also plated on poly-l-lysine coated cover slips and used for immunostaining with antibody against VEGFR1 (1:100; MAB471, R&D Systems) and neuronal marker beta-tubulin-III (#T8660, Sigma Aldrich) as described previously.

Membrane Preparation: Mice 8 weeks-old were injected intraplantar with VEGF-A 10 ng/ 20 µl or vehicle in the paw. After 1 and 3 hours of injection, mice were sacrificed and DRGs and sciatic nerve tissue were rapidly extracted and immediately frozen or

lysed in low salt buffer. After lysis, the samples were centrifuged at 800 g 10 min and then ultra-centrifuged at 100000 g for 1 h. The pellet were resuspended and lysed in RIPA buffer. Lysates were than used in western blot as previously described with an anti-TRPV1 antibody (sc-12498, 1:500, Santa Cruz).

Histology and Tumor size analysis:

Decalcification of tumor bearing mouse hind paws: After the injection of NCTC 2472 fibrosarcoma cells into the calcaneus bone of mouse paw, to analyze the tumor size the mice were transcardially perfused with PBS and then with 4% PFA. The whole hind paw was cut above the calcaneus bone in order to ensure the spread of tumor can also be taken into measuring the tumor growth. Before the paws can be cut for sectioning, the paws were decalcified as the bone will interfere with sectioning. The paws were immersed in decalcifying buffer (10% EDTA in 0.3 M Tris buffer) and incubated at 37°C for at least 21 days. The amount of decalcification was estimated by mixing the remaining solution with 3% ammonium (Alers et al., 1999). If the solution turned turbid, then the paws were incubated longer than 3 weeks to enable complete decalcification. The decalcified paws were dehydrated in different concentrations of alcohol (30%, 50%, 70%, 90% and 100%; 2- 3 hours in each). After treating with xylene for an hour and in xylene-paraffin mixture for 2 hours at 60° C, the tissues were immersed in paraffin at 60° C overnight. Following day, the paws were embedded in paraffin and cut with a microtome in a sagittal plane. Hematoxylin and Eosin staining was performed as previously (Cain et al., 2001; Schweizerhof et al., 2009) on microtome sections (8 µm) obtained for every 50 µm throughout the entire thickness of the paw and averaged. Tumor area was evaluated in microscopic images using Cell explorer software (BioSciTec) and normalized to the total paw area measured in the same sections. For measuring tumor inflammation, the sections

were stained with anti-GR-1 antibody (1:500, #560454, BD Biosciences, USA) a marker for infiltrating neutrophils and a thorough stereological analysis was performed to count the immunopositive cells (Gangadharan et al., 2011). For the tumors isolated from mice with PDAC, tumor volume was calculated using the formula: Volume = length x width x height x π/6 (Partecke et al., 2011).

Femur Histology: Mice were transcardially perfused with 4% PFA and the femoral bone was isolated. The femurs were decalcified with 10% EDTA for two weeks. The decalcified femurs were cryoprotected for 48 hours in 30% sucrose and were cut into 25 µm thick sections for analyzing peptidergic fiber sprouting in the periosteum of the bone (Bloom et al., 2011; Chartier et al., 2014; Ghilardi et al., 2012) using an antibody recognizing CGRP (1:300; 24112, Immunostar, Germany).

In vivo bioluminescence imaging: Tumor size from mice having femoral tumors was measured using in vivo IVIS bioluminescence imaging systems since the murine 4T1-Luc cells stably express Luciferase. Mice were briefly anesthetized and 100 µl of Luciferin substrate (# 122796, Perkin Elmer, Germany) was administered intraperitoneally. Mice were imaged using IVIS systems five minutes after luciferin injection to ensure consistent photon flux. IVIS acquires a photographic image of the animal under white light and a quantitative bioluminescent signal is overlaid on the image. The bioluminescent signal is expressed in photons per second and is displayed as an intensity map. Photon flux from the tumor is proportional to the number of live cells expressing luciferase so bioluminescence correlates directly with tumor size (Srivastava et al., 2014).

RT-PCR: Total RNA was isolated from enriched neuronal DRG cultures and in vivo DRG lysates using the Trizol method (Invitrogen) and purification steps using Turbo DNAse (Ambion) and RNAse out (Invitrogen) were employed as per manufacturer's instructions. RNA was reverse transcribed into cDNA, which then served as a template for PCR reactions. cDNA synthesis was performed according to the following protocol: 10 µg RNA and 0.2 µg Hexa nucleotide Mix in a volume of 20µl were incubated for 10 min at 70° C. A premix was made consisting of 6 µl of 5x First strand buffer (Invitrogen), 1.5 µl 0.1 M DTT, 1 µl RNasin (RNAse inhibitor, 40 U/µl), 1.5 µl 10 mM dNTP-Mix (containing equal amounts of dATP, dCTP, dGTP and dTTP) and added on ice to the RNA mixture. 5 µl from the total reaction volume were separated and processed in parallel without enzyme as negative control. The RT reaction was performed for 90 min at 42° C. After 2 min pre incubation, 1 µl SuperScript III Reverse Transcriptase (200 U/ml, Invitrogen) was added.

Primer sequences:

Murine VEGFR1: GGGACTATACGATCTTGCTGGGCA (forward) and GCAGGTGTGGCGCTTCCGAAT (reverse) amplicon size 789 bp.

Murine VEGFR2: TCGCCTCTGTCAGTGACCAGCATGG (forward) and GCCCACTGTGGCTTCCACCAAAGAT (reverse) amplicon size 673 bp.

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