Angiogenesis in Matrigel plugs in vivo

Sterile liquid Matrigel (500µl, BD Bioscience, San Diego, CA) supplemented with VEGF 165 (500 ng/ml) (R&D Systems, Minneapolis, MN) and heparin (60 U/ml) (Sigma, St. Louis, MO) was injected subcutaneously into mice. Eight days after injection, mice were sacrificed and Matrigel plugs were removed, homogenized in PBS and hemoglobin (Hb) content was measured using Drabkin's reagent (Sigma, St. Louis, MO) according to the manufacturer's instructions. Alternatively, excised Matrigel implants were snap-frozen in OCT medium, and 8 µm sections were processed for immunohistochemical analyses using the same antibodies as for RM1 tumor sections.

Antibodies used for immunohistochemical analysis of tumor and matrigel sections.

(biotin-conjugated rat anti-mouse CD31 (BD Biosciences, San Jose, CA), rabbit anti-mouse Laminin (AbD Serotec, Oxford, UK), rabbit anti-Smooth Muscle Cell Actin (SMA, Abcam, Cambridge, MA), rat anti-mouse CD105 (BD Biosciences, San Jose, CA), rabbit anti-human Desmin (Abcam, Cambridge, MA), goat anti-Fibrin II (Accurate Chemical,Westbury,NY), rabbit anti-NG2 (Milipore, Temecula, CA).

Transfection of kindlin-2 $^{+/-}$ EC.

Kindlin-2$^{+/-}$ EC cells were transiently transfected using the Nucleofector system from Amaxa Biosystems. EC (passage 2) were grown to ~70—80% confluency in complete EC medium. After centrifugation, 5x 10^5 cells were suspended in 100 µl of prewarmed Nucleofector solution (Endothelial cell kit) containing 2 µg of vectors encoding EGFP alone, EGFP-tagged WT kindlin-2 or EGFP-tagged QW^{615}/AA kindlin-2 {Ma, 2008 8888 /id}. The samples were transferred into an electroporation cuvette, and transfections were performed with the electrical setting A-20. After nucleofection, the cells were immediately transferred into prewarmed complete endothelial cell medium and cultured at 37°C in a humidified atmosphere of 5% CO_2. Cells, 48 hr after nucleofection, were spread on vitronectin (2.5 µg/ml) for 1h in serum-free DMEM/F12 and analyzed for

spreading as described above. Transfected cells were identified by their EGFP fluorescence.

Bone marrow transplantation

Two months old C57BL/6 mice were lethally X-irradiated with a total dose of 9 Gy and reconstituted intravenous injection of 10^7 BM cells isolated from the femurs of donor mice. Mice were used 6-8 weeks after BMT.

Tube formation assay

To compare tube formation by WT and kindlin-$2^{+/-}$ ECs, 12-well tissue culture plates were coated with 500 μl of Matrigel (BD Bioscience, San Diego, CA) and incubated at 37°C for 30 min. When the Matrigel solidified, 2×10^5 cells were added to each well in 1 ml of DMEM F-12 medium containing 10% FBS, 90 μg/ml Endothelial Cell Growth Supplement (Milipore, Temecula, CA), 5 ng/ml VEGF and 90 μg/ml heparin. Live time-lapse photography was performed for 12 h, using 5 min intervals on Leica DMIRB Inverted Microscope equipped with Roper Scientific CoolSNAP HQ Cooled CCD camera, temperature Controller and CO2 incubation Chamber. Snapshots were taken using MetaMorph Software.

Figure S1. Bone marrow-derived cells do not contribute to impaired angiogenesis and leakiness in kindlin-$2^{+/-}$ mice

(A) Representative images of tumor sections stained with CD31 antibody (brown) (left panel). Scale bars, 50 μm. Image analysis shows similar areas of CD31-stained vasculature in tumors grown in WT-WT and kindlin-$2^{+/-}$ -WT mice (right panel). Data are representative of two independent experiments including 6 mice per group. **(B)** Fibrin content (brown) does not differ in prostate tumors developed in WT-WT and kindlin-$2^{+/-}$-WT mice. Scale bars, 30 μm. (left panel) Quantification of fibrin-positive area is shown in the right panel. Data are representative of two independent experiments with 5 mice per group. **(C)** Similar prostate tumor growth in WT-WT and kindlin-$2^{+/-}$-WT mice. Data are means ± SEM from n=5 mice per group, two independent experiments were performed. **(D, E)** Comparison of leakage from ear **(D)** or dorsal skin **(E)** blood vessels in

recipient WT mice after BMT from WT or the kindlin-2$^{+/-}$ donors. Quantitative analysis showed similar vascular permeability in both groups either at baseline or upon stimulation with mustard oil or VEGF. Data are means ± SEM from n=5 mice per group, two independent experiments were performed.

Figure S2. VEGF induction fails to rescue the aberrant angiogenesis observed in zebrafish kindlin-2 morphants

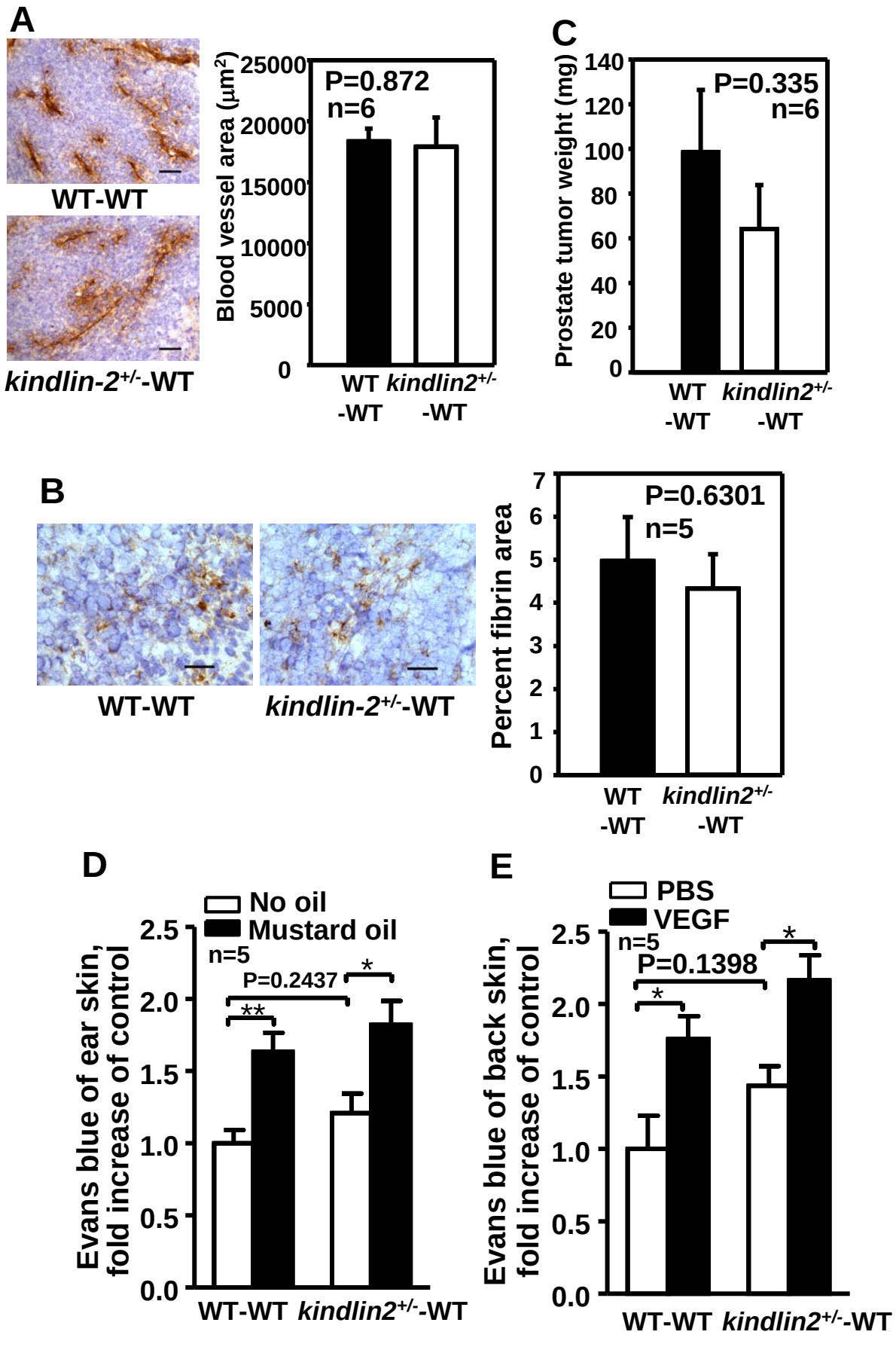
Embryos were injected with morpholino at the 1-2 cell stage and then treated with the VEGF inducer GS4012 as previously described (Peterson et al., 2004). Embryos were evaluated at 48 hpf. No qualitative change in blood vessels was apparent between sham treated and GS4012 treated embryos. Quantitation of ISV length demonstrated no statistical change. Lengths (in pixels): Control + DMSO (CTL DMSO) = 33.4 +/- 0.4; CTL + GS4012 (CTL VEGF) = 33.9 +/- 0.4; kindlin-2 + DMSO (K2 DMSO) = 28.8 +/- 0.5; kindlin-2 + GS4012 (K2 VEGF) = 28.5 +/- 0.4. Data are means ± SEM. 5 vessels were counted per fish and n=20 fish were analyzed per condition. Two independent experiments were performed.

Figure S3. Morpholino knockdown of kindlin-3 does not affect developmental angiogenesis

Embryos injected with control (CTL MO), kindlin-2 (KIND2 MO), and kindlin-3 (KIND3 MO) morpholinos at the 1-2 cell stage were analyzed at 48 hpf. In contrast to kindlin-2 morphants (Dowling et al., 2008a), no obvious morphologic abnormalities were observed in kindlin-3 morphants. In addition, as determined using knockdown in the VEGFR promoter-GFP zebrafish line, no changes were apparent in overall appearance of the vasculature with kindlin-3 knockdown. Changes as described in Figure 6 are depicted for the kindlin-2 morphants. At least 100 embryos per group were qualitatively examined for morphologic abnormalities (5 independent injections) and at least 50 embryos per group were qualitatively examined for abnormalities in vasculature (4 independent experiments).

Figure S4. Kindlin-2 overexpression restores spreading of kindlin-2$^{+/-}$ ECs on vitronectin. EC were nucleofected with vectors encoding EGFP alone, EGFP-

tagged WT kindlin-2 or EGFP-tagged QW^{615}/AA. After 48 hr cells were detached and spread on 2.5 µg/ml vitronectin for 1 h. The cells were fixed and stained with Alexa 568-phalloidin. Transfected cells were identified with EGFP fluorescence and cell surface was measured using Image J. 40-50 cells were measured for each construct. P=<0.001. Data are means ± SEM from 40-50 cells measured for each construct and are representative of two independent experiments.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WT-WT
kindlin-2+/--WT
Blood vessel area (μm2)
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20000
15000
10000
5000
0
P=0.872
n=6
WT -WT
kindlin2+/- -WT
C
Prostate tumor weight (mg)
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120
100
80
60
40
20
0
P=0.335
n=6
WT -WT
kindlin2+/- -WT
B
WT-WT
kindlin-2+/--WT
Percent fibrin area
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6
5
4
3
2
1
0
P=0.6301
n=5
WT -WT
kindlin2+/- -WT
D
No oil
Mustard oil
n=5
P=0.2437
**
*
Evans blue of ear skin, fold increase of control
2.5
2.0
1.5
1.0
0.5
0.0
WT-WT
kindlin2+/--WT
E
PBS
VEGF
n=5
P=0.1398
*
*
Evans blue of back skin, fold increase of control
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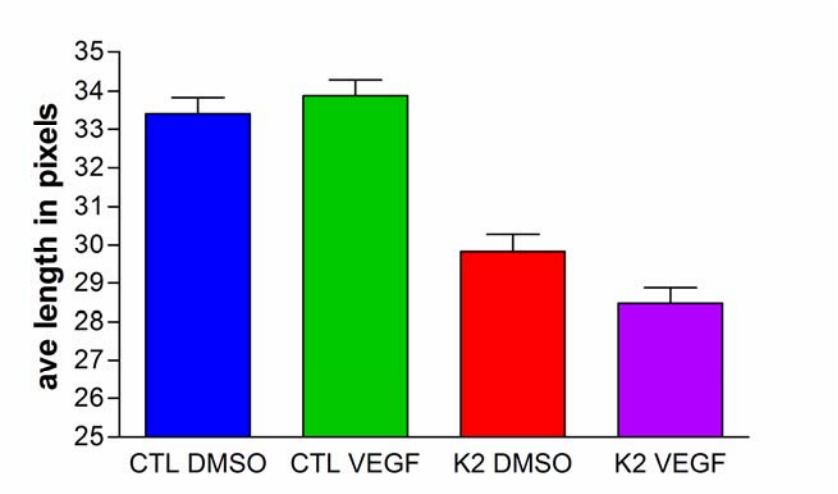
2.0
1.5
1.0
0.5
0.0
WT-WT
kindlin2+/--WT

Figure S1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33
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31
30
29
28
27
26
25
CTL DMSO
CTL VEGF
K2 DMSO
K2 VEGF

Figure S2

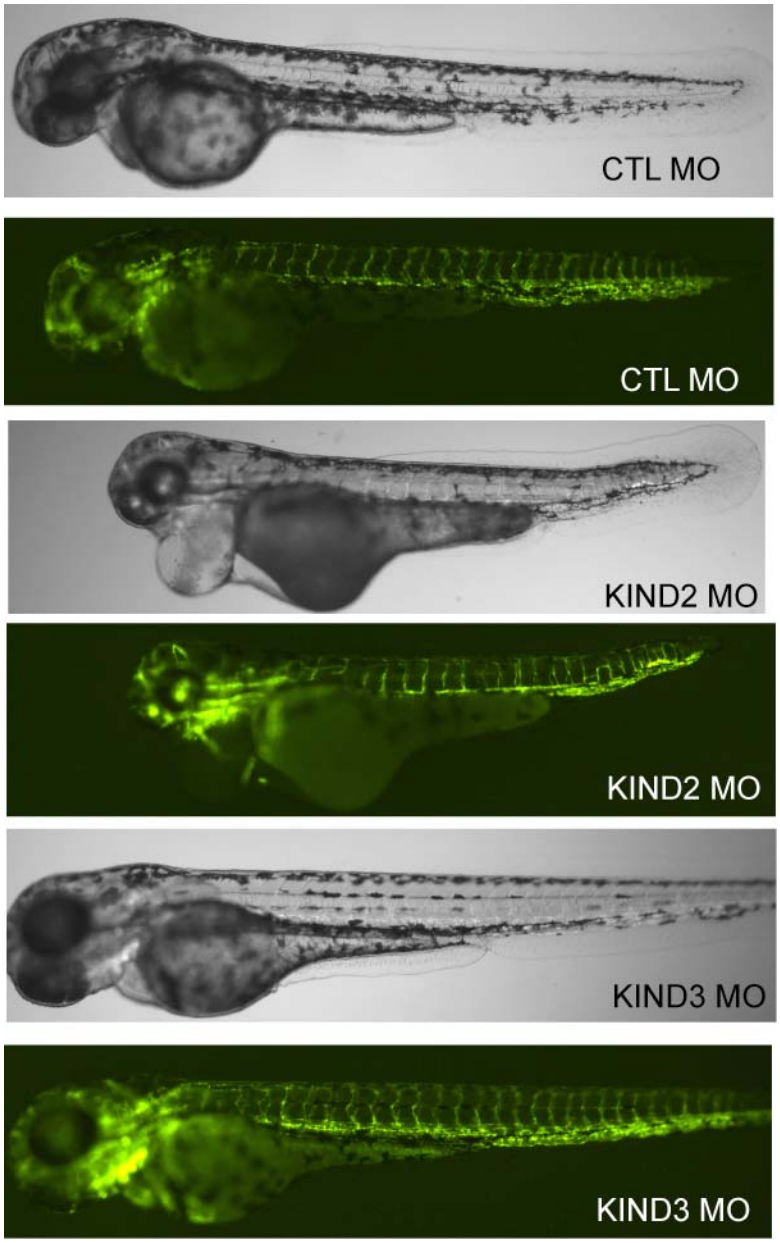

Figure S3

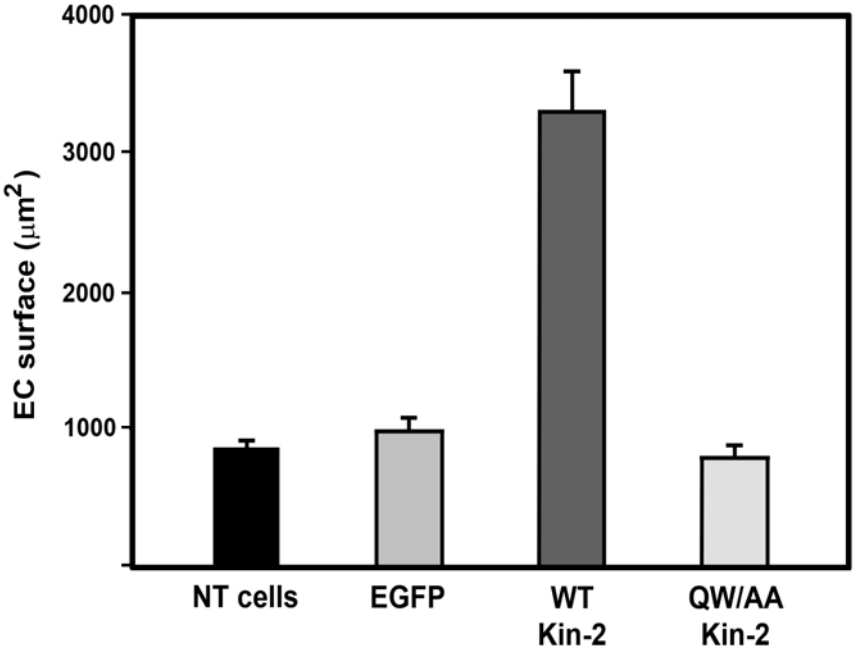
4000
3000
2000
1000
EC surface (μm²)
NT cells
EGFP
WT Kin-2
QW/AA Kin-2

Figure S4