Supplemental Information

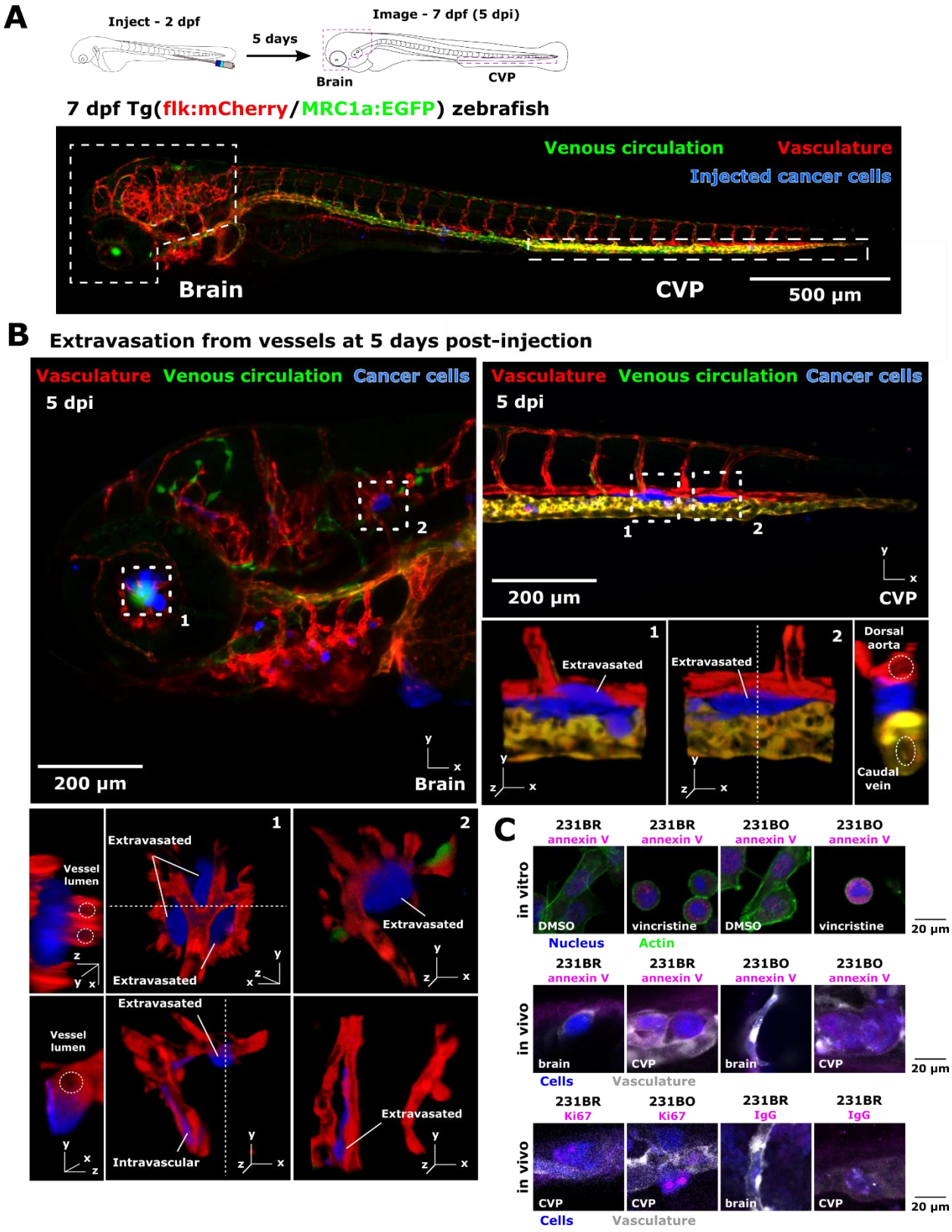

A
Inject - 2 dpf
5 days
Image - 7 dpf (5 dpi)
Brain
CVP
7 dpf Tg(flk:mCherry/MRC1a:EGFP) zebrafish
Venous circulation
Vasculature
Injected cancer cells
Brain
CVP
500 µm
B
Extravasation from vessels at 5 days post-injection
Vasculature Venous circulation Cancer cells
5 dpi
1
2
200 µm
Brain
Vasculature Venous circulation Cancer cells
5 dpi
1
2
200 µm
CVP
Extravasated
Dorsal aorta
Caudal vein
Vessel lumen
Intravascular
C
231BR annexin V
231BR annexin V
231BO annexin V
231BO annexin V
in vitro
DMSO
vincristine
DMSO
vincristine
20 µm
Nucleus
Actin
in vivo
brain
CVP
brain
CVP
Cells
Vasculature
231BR Ki67
231BO Ki67
231BR IgG
231BR IgG
in vivo
CVP
CVP
brain
CVP
Cells
Vasculature

Figure S1. Related to Figure 1. Injected cancer cells recapitulate key steps of the early metastatic cascade in a zebrafish xenograft model. (A) Schematic of experimental protocol. Cancer cells (MDA-MB-231 brain-tropic [231BR]) were injected into the circulation of transgenic Tg(flk:mCherry/MRC1a:EGF) zebrafish at 2 days post-fertilization (dpf) via the posterior cardinal vein. Fish were imaged at 5 days post-injection (dpi) at an age of 7 dpf. An overview (tiled average intensity projection from confocal z stack) of a 7 dpf zebrafish is shown for context. The vasculature is displayed in red, the venous circulation in green, and injected cancer cells in blue. The brain and caudal vein plexus (CVP) are outlined. Scale is indicated. (B) Detail of cancer cells in the zebrafish brain (left panels, 231BR cells) and CVP (right panels, MDA-MB-231 bone marrow-tropic [231BO] cells) at 5 dpi. Regions of interest (labeled 1,2) are indicated on overview images of the brain and CVP (average intensity projections from confocal z stacks). 3D reconstructions of these regions are shown in detail below. Two additional images of cells in the brain of a different zebrafish larva are also shown. For select images, orthogonal views of extravasated cells are presented, with the vessel lumen of neighboring vessels outlined. Image 2 in the CVP is reproduced from Figure 1. (C) Cell staining for apoptosis and proliferation markers. Top panels – 231BR or 231BO cells were treated with DMSO control or vincristine and fixed. Cells were stained using an antibody directed against annexin V (magenta). The nucleus is shown in blue and actin in green. Middle and bottom panels – 231BR or 231BO cells were injected into the circulatory system of Tg(flk:mCherry/MRC1a:EGFP) zebrafish at 2 dpf, and larvae were fixed at 5 dpi. Cells were stained for annexin V or Ki67 using whole-mount immunofluorescence, or incubated with a rabbit IgG control antibody. Stained zebrafish were imaged using confocal microscopy. The vasculature is shown in grayscale, cells in blue, and the indicated protein in magenta. In all panels, images are single confocal slices. Scales are indicated. See also Figure 1.

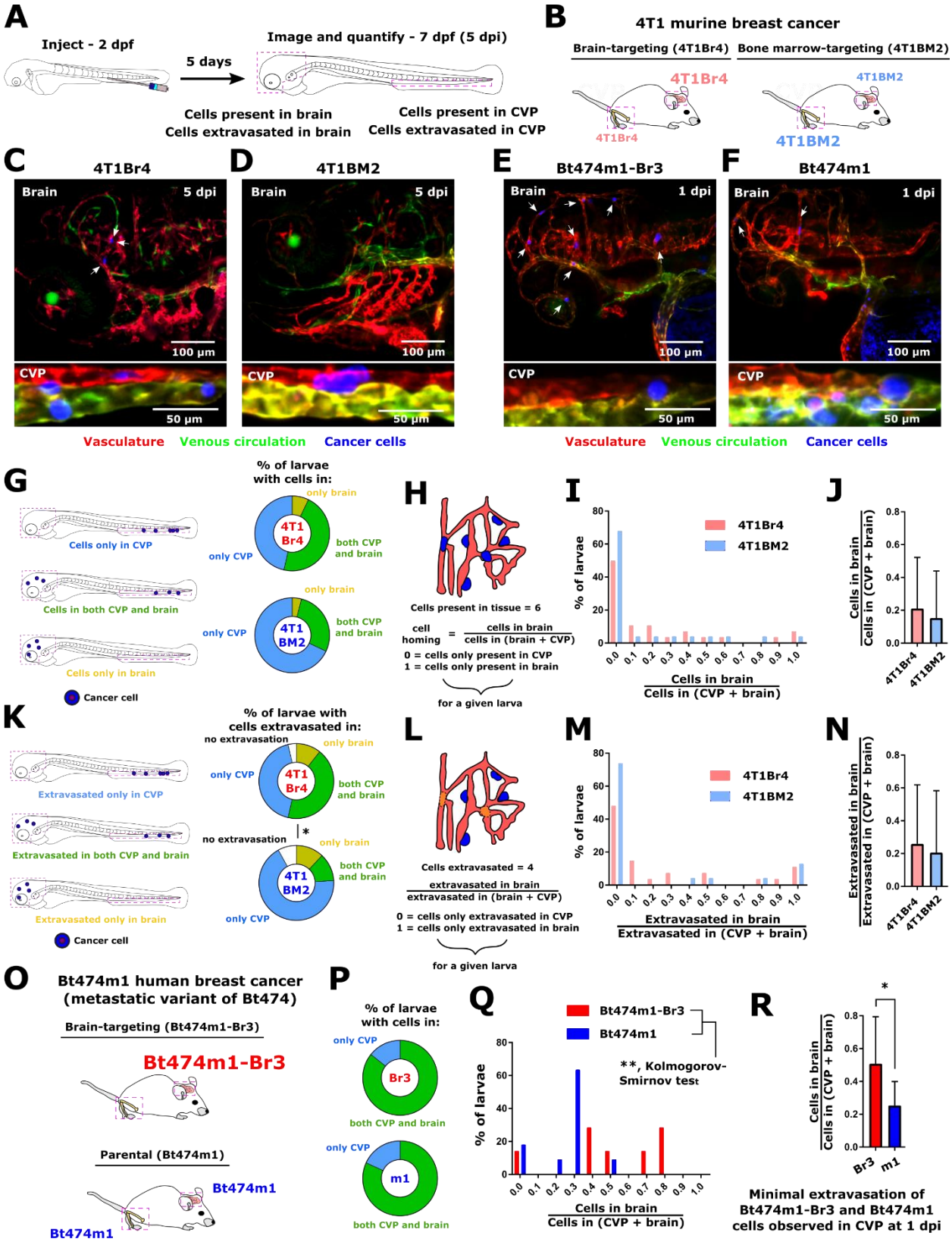

A
Inject - 2 dpf
5 days
Image and quantify - 7 dpf (5 dpi)
Cells present in brain
Cells extravasated in brain
Cells present in CVP
Cells extravasated in CVP
B
4T1 murine breast cancer
Brain-targeting (4T1Br4)
Bone marrow-targeting (4T1BM2)
4T1Br4
4T1BM2
C
4T1Br4
D
4T1BM2
E
Bt474m1-Br3
F
Bt474m1
Brain
5 dpi
1 dpi
100 µm
CVP
50 µm
Vasculature
Venous circulation
Cancer cells
G
% of larvae with cells in:
Cells only in CVP
Cells in both CVP and brain
Cells only in brain
Cancer cell
only brain
only CVP
both CVP and brain
4T1 Br4
4T1 BM2
H
Cells present in tissue = 6
cell homing = cells in brain / cells in (brain + CVP)
0 = cells only present in CVP
1 = cells only present in brain
for a given larva
I
% of larvae
4T1Br4
4T1BM2
Cells in brain / Cells in (CVP + brain)
J
Cells in brain / Cells in (CVP + brain)
K
% of larvae with cells extravasated in:
Extravasated only in CVP
Extravasated in both CVP and brain
Extravasated only in brain
Cancer cell
no extravasation
only brain
only CVP
both CVP and brain
4T1 Br4
4T1 BM2
*
L
Cells extravasated = 4
extravasated in brain / extravasated in (brain + CVP)
0 = cells only extravasated in CVP
1 = cells only extravasated in brain
for a given larva
M
% of larvae
4T1Br4
4T1BM2
Extravasated in brain / Extravasated in (CVP + brain)
N
Extravasated in brain / Extravasated in (CVP + brain)
O
Bt474m1 human breast cancer
(metastatic variant of Bt474)
Brain-targeting (Bt474m1-Br3)
Bt474m1-Br3
Parental (Bt474m1)
Bt474m1
Bt474m1
P
% of larvae with cells in:
only CVP
Br3
both CVP and brain
m1
Q
Bt474m1-Br3
Bt474m1
**, Kolmogorov-Smirnov test
% of larvae
Cells in brain / Cells in (CVP + brain)
R
*
Cells in brain / Cells in (CVP + brain)
Br3
m1
Minimal extravasation of Bt474m1-Br3 and Bt474m1 cells observed in CVP at 1 dpi

Figure S2. Related to Figure 1. Non-random targeting of organ-tropic cancer cell lines in a zebrafish metastasis model. (A) Schematic of experimental setup. Cancer cells were injected into the circulatory system of Tg(flk:mCherry/MRC1a:EGFP) zebrafish at 2 days post-fertilization (dpf) and imaged at 5 days post-injection (dpi; zebrafish age at imaging was 7 dpf). In each larva, both the total number of cells and the number of extravasated cells were counted in the brain and CVP. (B) Schematic of reported organ colonization of 4T1 brain-tropic (4T1Br4, pink) and bone marrow-tropic (4T1BM2, light blue) subclones in murine models. Font size indicates relative homing within a given organ. Representative images of 4T1 (C) brain-tropic subclone (4T1Br4) and (D) bone marrow-tropic subclone (4T1BM2) colonization in the zebrafish brain and CVP at 5 dpi. Arrows indicate extravasated cells. Representative images of Bt474m1 (E) brain-tropic subclone (Bt474m1-Br3) and (F) parental (Bt474m1) colonization in the zebrafish brain and CVP at 1 dpi. Arrows indicate cells. For panels C-F, images are average intensity projections from confocal z stacks. The vasculature is displayed in red, the venous circulation in green, and injected cancer cells in blue. Scales are indicated. (G) Percentage of larvae with 4T1 cells present only in the brain, only in the CVP, or in both tissues at 5 dpi. Schematic illustrates these outcomes. (H) Schematic illustrating assessment of all cells present in a tissue of interest (cell homing). For cell homing calculations, both intravascular and extravascular cells were considered. For a given larva, this metric could range from a value of 0 (indicating that no cells were present in the brain) to 1 (indicating that cells were present in the brain but not CVP). (I) Distribution of values for cell homing ratio of 4T1Br4 and 4T1BM2 cells at 5 dpi. (H) Average ± SD ratio across all larvae of 4T1Br4 and 4T1BM2 cells present in the brain at 5 dpi to the total number of cells present in the brain and CVP. For panels G, I, and J, values were calculated on a per larvae basis for N=28 larvae injected with 4T1Br4 cells (from 316 total cells) and N=25 larvae injected with 4T1BM2 cells (from 153 total cells). (K) Percentage of larvae with 4T1 cells extravasated only in the brain, only in the CVP, or in both tissues, or with no extravasated cells, at 5 dpi. Percentages were calculated from N=28 larvae injected with 4T1Br4 cells and N=25 larvae injected with 4T1BM2 cells. *, p=0.0138, chi-square test. (L) Schematic illustrating assessment of cell extravasation. For extravasation calculations, only extravascular cells were considered. For a given larva, this metric could range from a value of 0 (indicating that no cells were extravasated in the brain) to 1 (indicating that cells were extravasated in the brain but not CVP). Fish with no extravasated cells were excluded from analysis. (M) Distribution of values for the ratio of cells extravasated in the brain to total cells extravasated in the brain and CVP for 4T1Br4 and 4T1BM2 cells at 5 dpi. (N) Average ± SD across all larvae at 5 dpi of extravasated cells in the brain divided by total number of extravasated cells in the brain and CVP for 4T1Br4 and 4T1BM2 cells. For panels M and N, values were calculated on a per larva basis for N=27 larvae with extravasated 4T1Br4 cells (from 258 total extravasated cells) and N=23 larvae injected with 4T1BM2 cells (from 130 total extravasated cells). (O) Schematic of reported organ colonization of Bt474m1 cells (blue) and brain-tropic subclones (Bt474m1-Br3, red) in murine models. Font size indicates relative homing within a given organ. (P) Percentage of larvae with Bt474m1 cells present only in the brain, only in the CVP, or in both tissues at 1 dpi. (Q) Distribution of values for cell homing ratio of Bt474m1-

Br3 and Bt474m1 cells at 1 dpi. The distributions of cells between the brain and CVP were statistically different for the cell types (**, p=0.0049, Kolmogorov-Smirnov test). (R) Average ± SD ratio across all larvae of Bt474m1-Br3 and Bt474m1 cells present in the brain at 1 dpi to the total number of cells present in the brain and CVP. *, p=0.0236 by unpaired two-tailed t test. For panels R and S, values were calculated on a per larvae basis for N=7 larvae injected with Bt474m1-Br3 cells (from 53 total cells) and N=11 larvae injected with Bt474m1 cells (from 64 total cells). Minimal extravasation of these cells was observed at 1 dpi. See also Figure 1.

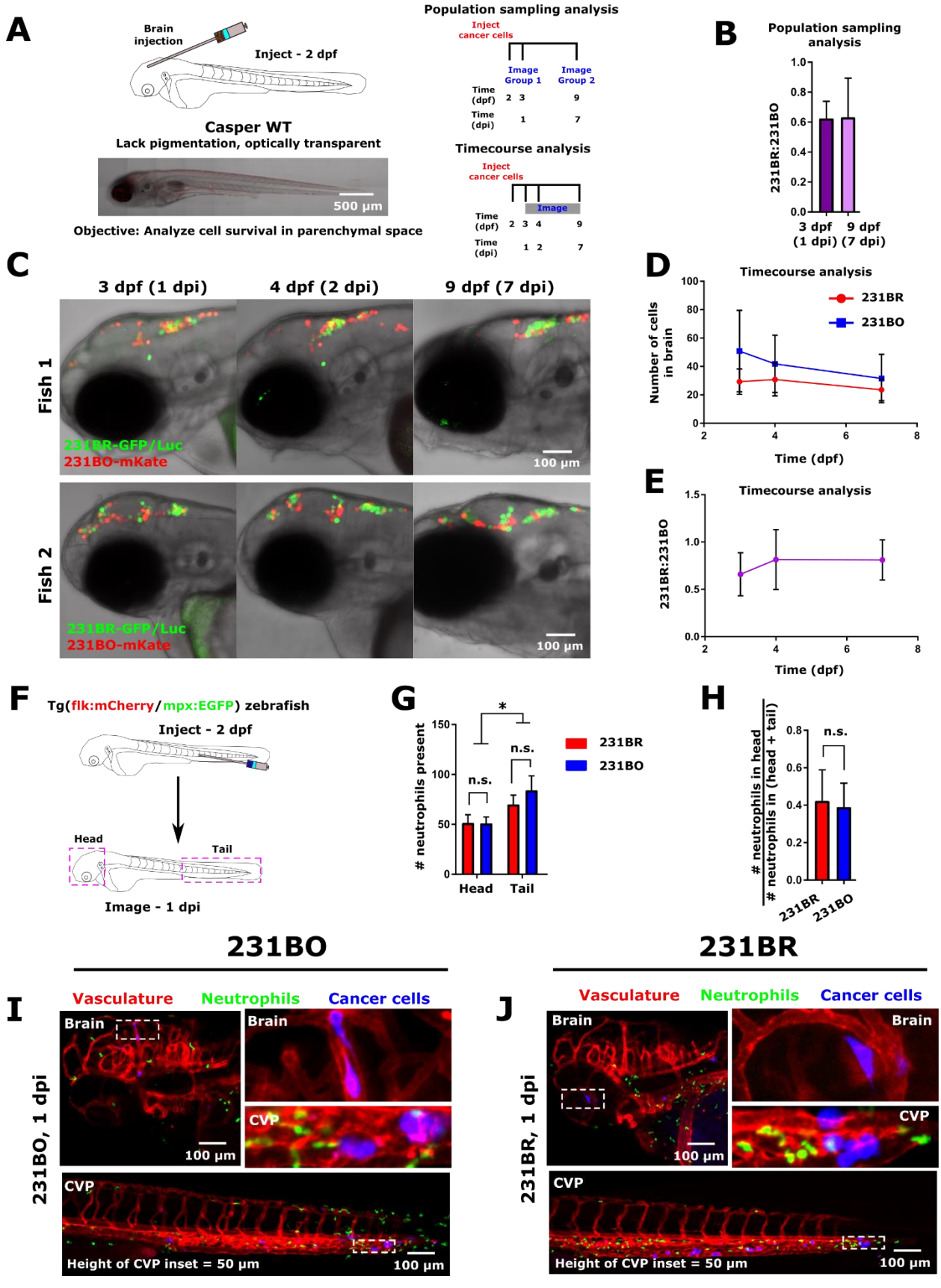

A
Brain injection
Inject - 2 dpf
Casper WT
Lack pigmentation, optically transparent
500 µm
Objective: Analyze cell survival in parenchymal space
Population sampling analysis
Inject cancer cells
Image Group 1
Image Group 2
Time (dpf) 2 3 9
Time (dpi) 1 7
Timecourse analysis
Inject cancer cells
Image
Time (dpf) 2 3 4 9
Time (dpi) 1 2 7
B
Population sampling analysis
231BR:231BO
3 dpf (1 dpi)
9 dpf (7 dpi)
C
3 dpf (1 dpi)
4 dpf (2 dpi)
9 dpf (7 dpi)
Fish 1
Fish 2
231BR-GFP/Luc
231BO-mKate
100 µm
D
Timecourse analysis
231BR
231BO
Number of cells in brain
Time (dpf)
E
Timecourse analysis
231BR:231BO
Time (dpf)
F
Tg(flk:mCherry/mpx:EGFP) zebrafish
Inject - 2 dpf
Head
Tail
Image - 1 dpi
G
neutrophils present
231BR
231BO
n.s.
n.s.
*
Head
Tail
H
neutrophils in head / # neutrophils in (head + tail)
n.s.
231BR
231BO
231BO
231BR
I
J
Vasculature
Neutrophils
Cancer cells
231BO, 1 dpi
231BR, 1 dpi
Brain
CVP
100 µm
Height of CVP inset = 50 µm

Figure S3. Related to Figure 1. MDA-MB-231 brain-tropic and bone marrow-tropic cell lines exhibit equal levels of survival following injection. (A) Schematic of direct brain injection experiments. Brain-tropic MDA-MB-231 cells stably expressing GFP and luciferase (231BR-GFP/Luc) and bone marrow-tropic MDA-MB-231 cells stably expressing mKate (231BO-mKate) were co-injected into the brain of non-pigmented Casper WT zebrafish at 2 days post-fertilization (dpf). A representative image of the fish at 7 days post-injection (dpi; zebrafish is 9 dpf) is shown for reference. Cell survival was assessed by two methods. For population sampling of co-injected cells, a subset of fish was imaged at 1 dpi prior to being euthanized. At 7 dpi, a second subsampling from the injected pool of fish was imaged. Alternatively, for timecourse analysis, injected larvae were imaged at 1, 2, and 7 dpi, and total cell numbers were counted on each day. (B) Quantification of the ratio of 231BR:231BO cells present from population subsamplings at 1 dpi and 7 dpi. Plot shows mean ± SD on a per larvae basis. N=10 fish at 1 dpi (520 total cells counted), N=6 fish at 7 dpi (142 total cells counted). Difference was not significant by unpaired two-tailed t test (p=0.9371). (C) Two representative examples of cell survival following direct injection into the brain parenchyma. Each zebrafish is imaged at 1, 2, and 7 dpi. 231BR-GFP/Luc cells are shown in green, and 231BO-mKate cells are displayed in red. Images are average intensity projections of confocal z stacks. Scales are indicated. (D) Numbers of 231BR-GFP/Luc and 231BO-mKate cells present over time following co-injection to the brain. Plot displays mean ± SD cells from N=4 injected zebrafish. (E) Quantification of the ratio of 231BR:231BO cells present over time following co-injection to the brain. Plot displays mean ± SD cells from N=4 injected zebrafish. (F) Schematic of neutrophil homing experiment. Brain-tropic MDA-MB-231 cells (231BR) or bone marrow-tropic MDA-MB-231 cells (231BO) were injected into the circulation of 2 days post-fertilization (dpf) Tg(flk:mCherry/mpx:EGFP) zebrafish. The zebrafish head and tail were imaged at 1 day post-injection (dpi), when zebrafish were 3 dpf. (G) Average ± SD of zebrafish neutrophil counts in the head and tail 1 day after injection with 231BR or 231BO cells. Values were calculated on a per larva basis. Tissue of interest but not cell type was a significant source of variation by two-way ANOVA (*, p=0.0168, F=6.813, DF=1). Neutrophil counts for each cell type were not significantly different by Sidak's multiple comparisons test. N=7 larvae injected with 231BR cells (total of 255 neutrophils counted in the head and 421 neutrophils counted in the tail), N=5 larvae injected with 231BO cells (total of 360 neutrophils counted in the head and 490 neutrophils counted in the tail). (H) Average ± SD of the ratio of neutrophils present in the head to neutrophils present in the head and tail. Values were calculated on a per larva basis. n.s., not significant by two-tailed t test, p=0.7176. N=7 larvae injected with 231BR cells (total of 255 neutrophils counted in the head and 421 neutrophils counted in the tail), N=5 larvae injected with 231BO cells (total of 360 neutrophils counted in the head and 490 neutrophils counted in the tail). Representative images of zebrafish injected with (I) 231BO and (J) 231BR cells. Images display average intensity projections from confocal z stacks. Blood vessels are displayed in red, zebrafish neutrophils in green, and human cancer cells in blue. Inset locations are indicated by the dashed white boxes. For scale of insets, the height of the caudal vein plexus (CVP) inset = 50 μm. See also Figure 1.

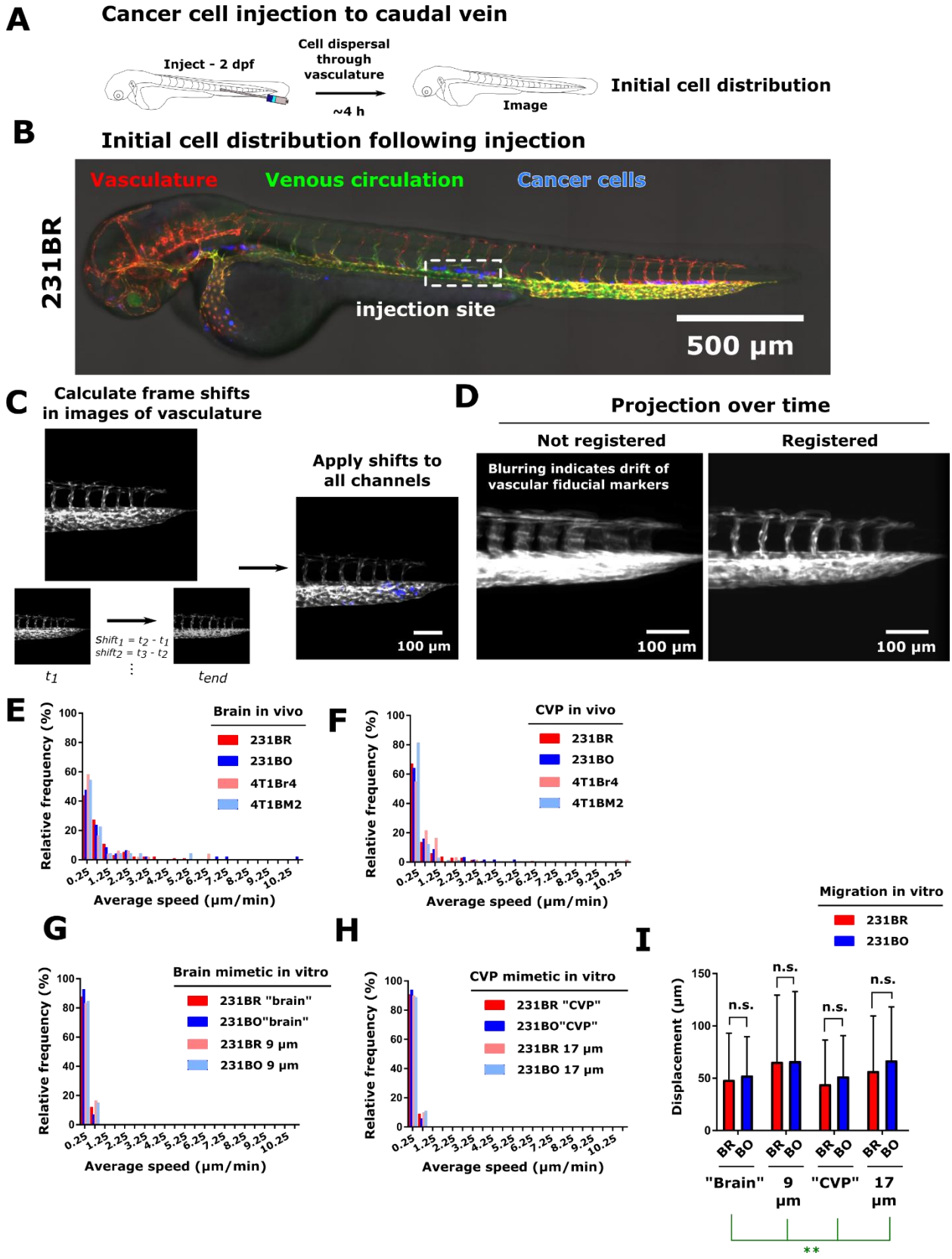

A
Cancer cell injection to caudal vein
Inject - 2 dpf
Cell dispersal through vasculature
~4 h
Image
Initial cell distribution
B
Initial cell distribution following injection
231BR
Vasculature
Venous circulation
Cancer cells
injection site
500 µm
C
Calculate frame shifts in images of vasculature
Shift1 = t2 - t1
shift2 = t3 - t2
t1
tend
Apply shifts to all channels
100 µm
D
Projection over time
Not registered
Registered
Blurring indicates drift of vascular fiducial markers
100 µm
100 µm
E
Brain in vivo
231BR
231BO
4T1Br4
4T1BM2
Relative frequency (%)
Average speed (µm/min)
F
CVP in vivo
231BR
231BO
4T1Br4
4T1BM2
Relative frequency (%)
Average speed (µm/min)
G
Brain mimetic in vitro
231BR "brain"
231BO"brain"
231BR 9 µm
231BO 9 µm
Relative frequency (%)
Average speed (µm/min)
H
CVP mimetic in vitro
231BR "CVP"
231BO"CVP"
231BR 17 µm
231BO 17 µm
Relative frequency (%)
Average speed (µm/min)
I
Migration in vitro
231BR
231BO
Displacement (µm)
n.s.
BR
BO
"Brain"
9 µm
"CVP"
17 µm
**

Figure S4. Related to Figures 2, 3. Cell speeds in vivo and in vitro do not distinguish organotropic cell clones. (A) Schematic of experiment to visualize initial cell dissemination throughout the vasculature. Cells were injected into the zebrafish circulation via the posterior cardinal vein. Fish were imaged ~4 h post-injection to determine initial cell distribution. (B) Representative image of MDA-MB-231 brain-tropic (231BR) cells following injection to a 2 days post-fertilization (dpf) Tg(flk:mCherry/MRC1a:EGFP) zebrafish. The vasculature is displayed in red, the venous circulation in green, and injected cancer cells in blue. Cells can be seen at the injection site and had also dispersed through the fish, as indicated by cells present in the brain, caudal vein plexus (CVP), and Duct of Cuvier. Image is average intensity projections of confocal z stacks. Scale is indicated. (C) Schematic of image registration process. A reference channel containing fiducial markers (here, the zebrafish vasculature, displayed in gray) was used to register images over time. For each time interval, frame shifts were calculated. These shifts were then applied to all channels of the image stacks to obtain a registered image. (D) Average intensity projection of the vasculature (shown in gray) over time illustrates drift in the non-registered image (left) that is corrected in the registered image (right). Projection was made over a 12-hour timelapse, with images obtained every 10 minutes. (E) Histogram of cell speeds in the zebrafish brain in vivo. (F) Histogram of cell speeds in the zebrafish CVP in vivo. For panels E, F, speeds in vivo were measured from: 231BR cells – N=91 cells in brain, N=131 cells in CVP, across 6 larvae; 231BO cells – N=46 cells in brain, N=56 cells in CVP, across 8 larvae; 4T1Br4 cells – N=48 cells in the brain, N=60 cells in the CVP, across 6 larvae; 4T1BM2 cells – N=22 cells in the brain, N=65 cells in the CVP, across 8 larvae. (G) Histogram of cell speeds in brain mimetic microchannels and corresponding 9 μm-wide straight microchannels in vitro. Channels were coated with 10 μg/ml fibronectin. (H) Histogram of cell speeds in CVP mimetic microchannels and corresponding 17 μm-wide straight microchannels in vitro. Channels were coated with 10 μg/ml fibronectin. (I) Displacements (mean ± SD) of cells in tissue mimetic devices and corresponding straight microchannels in vitro. Channels were coated with 10 μg/ml fibronectin. Microchannel topography but not cell type was a significant source of variation by two-way ANOVA (**, p=0.0038, F=4.530, DF=3), and displacements of 231BO and 231BR cells for a given environment were not significantly different (n.s.) by Sidak's multiple comparisons test. For panels G-I, speeds and displacements in vitro were measured from: 231BR cells – N=41 cells in brain mimetic channels, N=42 cells in 9 μm-wide channels, N=120 cells in CVP mimetic channels, N=90 cells in 17 μm-wide channels; 231BO cells – N=42 cells in brain mimetic channels, N=46 cells in 9 μm-wide channels, N=118 cells in CVP mimetic channels, N=81 cells in 17 μm-wide channels. Measurements were pooled from 4 biological replicates. See also Figures 2,3.

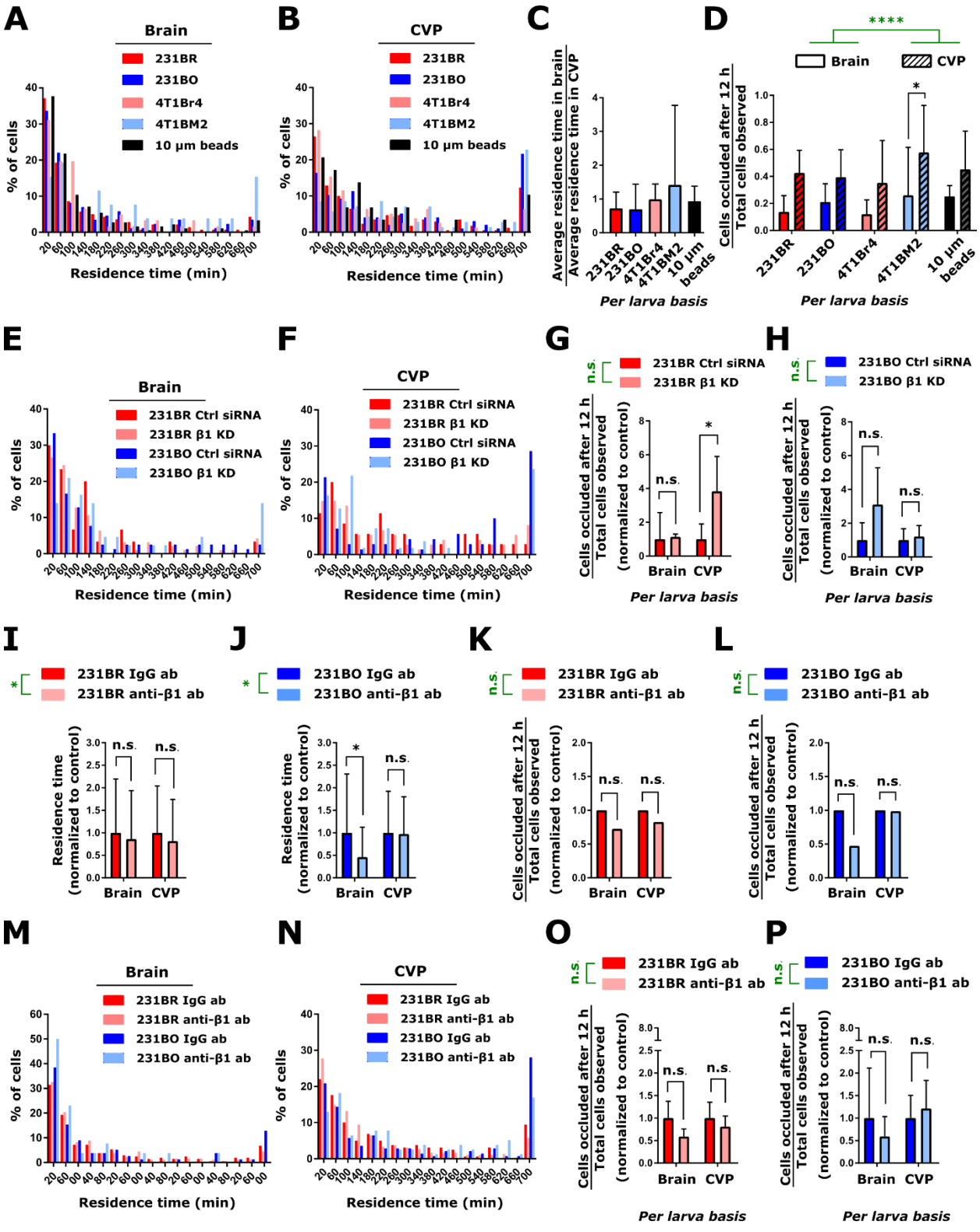


Figure S5. Related to Figure 5. Cell residence time details and occlusion fractions following calculations on a per larva basis. Two days post-fertilization (dpf) zebrafish were injected with

MDA-MB-231 brain-tropic (231BR) or bone marrow-tropic (231BO) cells, 4T1 brain-tropic (4T1Br4) or bone marrow-tropic (4T1BM2) cells, 10 μm-diameter polystyrene beads (beads), or MDA-MB-231 cells with silenced β1 integrin expression or blocked β1 integrin function, in the posterior cardinal vein. Following dispersal through the vasculature, the brain and caudal vein plexus (CVP) of each larva were imaged for 12 h via confocal microscopy, and cell residence times were tracked. (A) Histogram of cell and bead residence times in the brain. (B) Histogram of cell and bead residence times in the CVP. Histograms were generated using data from 7 larvae for 231BR cells (N=140 cells in the brain, N=170 cells in the CVP), 10 larvae for 231BO cells (N=86 cells in the brain, N=97 cells in the CVP), 6 larvae for 4T1Br4 cells (N=61 cells in the brain, N=78 cells in the CVP), 8 larvae for 4T1BM2 cells (N=26 cells in the brain, N=70 cells in the CVP), and 6 larvae for polystyrene beads (N=183 beads in the brain, N=29 beads in the CVP). (C) Ratio (mean ± SD) of the average residence time in the brain to the average residence time in the CVP on a per larva basis. Averages were calculated across N=7 larvae for 231BR cells, N=10 larvae for 231BO cells, N=6 larvae for 4T1Br4 cells, N=8 larvae for 4T1BM2 cells, and N=6 larvae for polystyrene beads. Differences were not significant by Kruskal-Wallis test (p=0.2670) and Dunn's multiple comparisons test between values for all conditions. (D) Mean ± SD of the fraction of cells occluded in the brain or CVP at the end of the 12 h imaging period to the total number of cells imaged in each organ over that period, averaged across larvae for a given organ. Tissue of observation but not cell type was a significant source of variation by two-way ANOVA (****, $p<0.0001$, F=19.38, DF=1), and the fraction of cells occluded in the CVP was significantly greater than the fraction of cells occluded in the brain for 4T1BM2 cells (*, p=0.0417, Sidak's multiple comparisons test). For panels A-D, measurements were made in 2 days post-fertilization (dpf) Tg(flk:mCherry/MRC1a:EGFP) zebrafish. (E) Histogram of 231BR and 231BO control and β1 integrin knockdown cell residence times in the brain. (F) Histogram of 231BR and 231BO control and β1 integrin knockdown cell residence times in the CVP. Histograms were generated from: 231BR Ctrl – 3 larvae, N=30 cells in brain, N=35 cells in CVP; 231BR integrin β1 KD – 4 larvae, N=94 cells in brain, N=74 cells in CVP; 231BO Ctrl – 3 larvae, N=78 cells in brain, N=70 cells in CVP; 231BO integrin β1 KD – 4 larvae, N=43 cells in head, N=55 cells in CVP. Fraction of (G) 231BR or (H) 231BO β1 integrin knockdown cells passing through a given organ that became occluded over the 12 h imaging period tracking initial cell dissemination, normalized to the fraction in the same organ for control cells. Fractions were calculated on a per larvae basis in a given tissue across N=3 larvae for 231BR Ctrl cells, N=4 larvae for 231BR integrin β1 KD cells, N=3 larvae for 231BO Ctrl cells, and N=4 larvae for 231BO integrin β1 KD cells. For both cell types, integrin β1 silencing was not a significant source of variation by two-way ANOVA (231BR – p=0.0766, F=3.898, DF=1; 231BO – p=0.1437, F=2.517, DF=1). The fraction of 231BR integrin β1 KD cells arrest in the CVP was significantly greater than that for 231BR Ctrl cells (*, p=0.0471, Sidak's multiple comparisons test). For panels E-H, measurements were made in 2 days post-fertilization (dpf) Tg(flk:mCherry/MRC1a:EGFP) zebrafish. (I) Residence time (average ± SD) of 231BR cells in the brain and CVP upon treatment with function-blocking antibody against β1 integrin, normalized to the average residence time in the same organ for cells treated with a control

antibody. The presence of the antibody was a significant source of variation by two-way ANOVA (*, p=0.0298, F=4.735, DF=1). Differences between treated and control cells within a given organ were not significant by Sidak's multiple comparisons test (n.s.). (J) Residence time (average ± SD) of 231BO cells in the brain and CVP upon treatment with function-blocking antibody against β1 integrin, normalized to the average residence time in the same organ of cells treated with a control antibody. The presence of the antibody was a significant source of variation by two-way ANOVA (*, p=0.0344, F=4.513, DF=1). Additionally, cell residence time was significantly reduced in the brain upon antibody treatment (*, p=0.0346, Sidak's multiple comparisons test. (K) Fraction of 231BR cells treated with function-blocking antibody against β1 integrin passing through a given organ that became occluded, normalized to the fraction in the same organ for cells treated with a control antibody. Differences were not significant (n.s.) by two-way ANOVA with Sidak's multiple comparisons test. (L) Fraction of 231BO cells treated with function-blocking antibody against β1 integrin passing through a given organ that became occluded, normalized to the fraction in the same organ for cells treated with a control antibody. Differences were not significant (n.s.) by two-way ANOVA with Sidak's multiple comparisons test. (M) Histogram of 231BR and 231BO cell residence times in the brain following treatment with a β1 integrin function blocking antibody or control IgG antibody. (N) Histogram of 231BR and 231BO cell residence times in the CVP following treatment with a β1 integrin function blocking antibody or control IgG antibody. For panels I-N, values were calculated from: 231BR IgG – 5 larvae, N=207 cells in head, N=159 cells in CVP; 231BR anti-β1 – 4 larvae, N=181 cells in head, N=296 cells in CVP; 231BO IgG – 5 larvae, N=78 cells in head, N=139 cells in CVP; 231BO anti-β1 – 3 larvae, N=26 cells in head, N=77 cells in CVP. Fraction of (O) 231BR or (P) 231BO cells passing through a given organ that became arrested over the 12 h imaging period tracking initial cell dissemination following treatment with a function blocking β1 integrin antibody, normalized to the fraction in the same organ for cells treated with a control IgG antibody. Fractions were calculated on a per larvae basis in a given tissue across N=5 larvae for 231BR IgG cells, N=4 larvae for 231BR anti-β1 cells, N=5 larvae for 231BO IgG cells, and N=3 larvae for 231BO anti-β1 cells. For both cell types, integrin β1 blocking was not a significant source of variation (n.s.) by two-way ANOVA (231BR – p=0.4573, F=0.5843, DF=1; 231BO – p=0.8106, F=0.06030, DF=1). Additionally, cell occlusion fractions in a given organ were not significantly different for both cell types upon integrin blocking (n.s., Sidak's multiple comparisons test). For panels I-P, measurements were made in 2 days post-fertilization (dpf) Tg(fli1:EGFP) zebrafish. See also Figure 5.

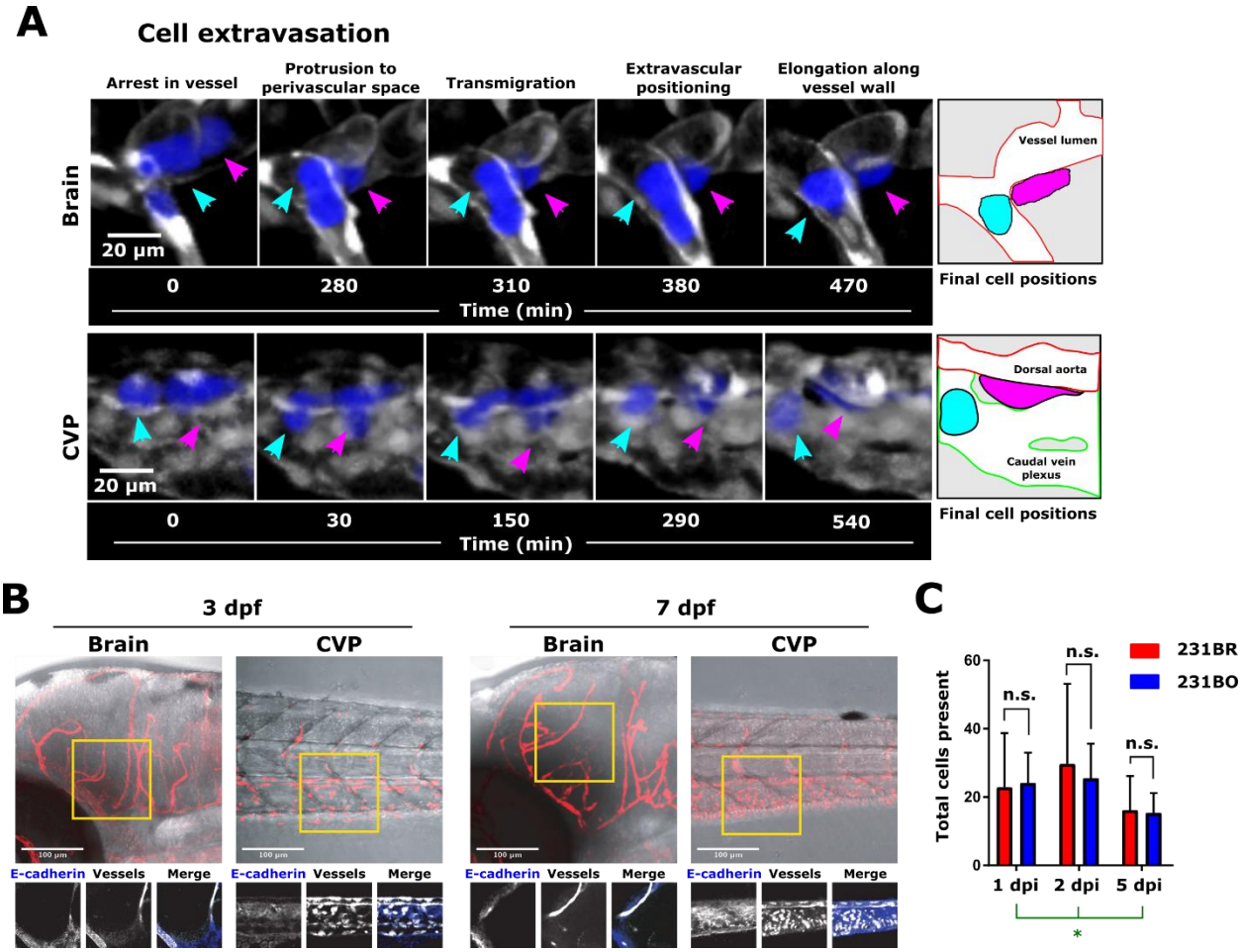


Figure S6. Related to Figure 6. Real-time imaging of cell extravasation and summary of 231BR and 231BO cell survival over time after dissemination through the circulatory system. (A) Time-lapse images of cells extravasating in the brain (MDA-MB-231 brain-tropic cell [231BR]) and caudal vein plexus (CVP; images shows MDA-MB-231 bone marrow-tropic cell [231BO]) of 2.5 days post-fertilization (dpf) Tg(flk:mCherry/MRC1a:EGFP) zebrafish. Cells are displayed in blue. Zebrafish vasculature and venous circulation are shown in grayscale to facilitate visualization of cells. Magenta arrows indicate extravasating cells. Cyan arrows indicate cells that remain intravascular. Schematic illustrates final positions of cells. Scale bar = 20 µm. (B) Tg(flk:mCherry/MRC1a:EGFP) zebrafish were fixed at 3 dpf or 7 dpf and stained with an antibody directed against zebrafish E-cadherin via whole mount immunohistochemistry. Confocal z stacks of the brain and CVP were acquired. Top panels show brightfield and mCherry channels to illustrate regions from which images were acquired. The vasculature is displayed in red. Boxed regions show positions of insets in bottom panels. Insets are maximum intensity projections of six confocal slices centered around the vasculature. Separate inset images display E-cadherin staining, the location of the vasculature (by endogenous mCherry expression), and a merged image. (C) Average ± SD number of 231BR and 231BO cells present in the head plus CVP of Tg(flk:mCherry/MRC1a:EGFP) zebrafish at 1,2, and 5 days post-injection (dpi) zebrafish.

Averages were calculated across N=10 larvae for 231BR cells and N=8 larvae for 231BO cells and were calculated independently on each day. Time but not cell type was a significant source of variation by two-way ANOVA (*, p=0.0493, F=3.206, DF=2). The number of cells present on a given day was not significantly different (n.s.) between 231BR and 231BO cells by Sidak's multiple comparisons test. See also Figure 6.

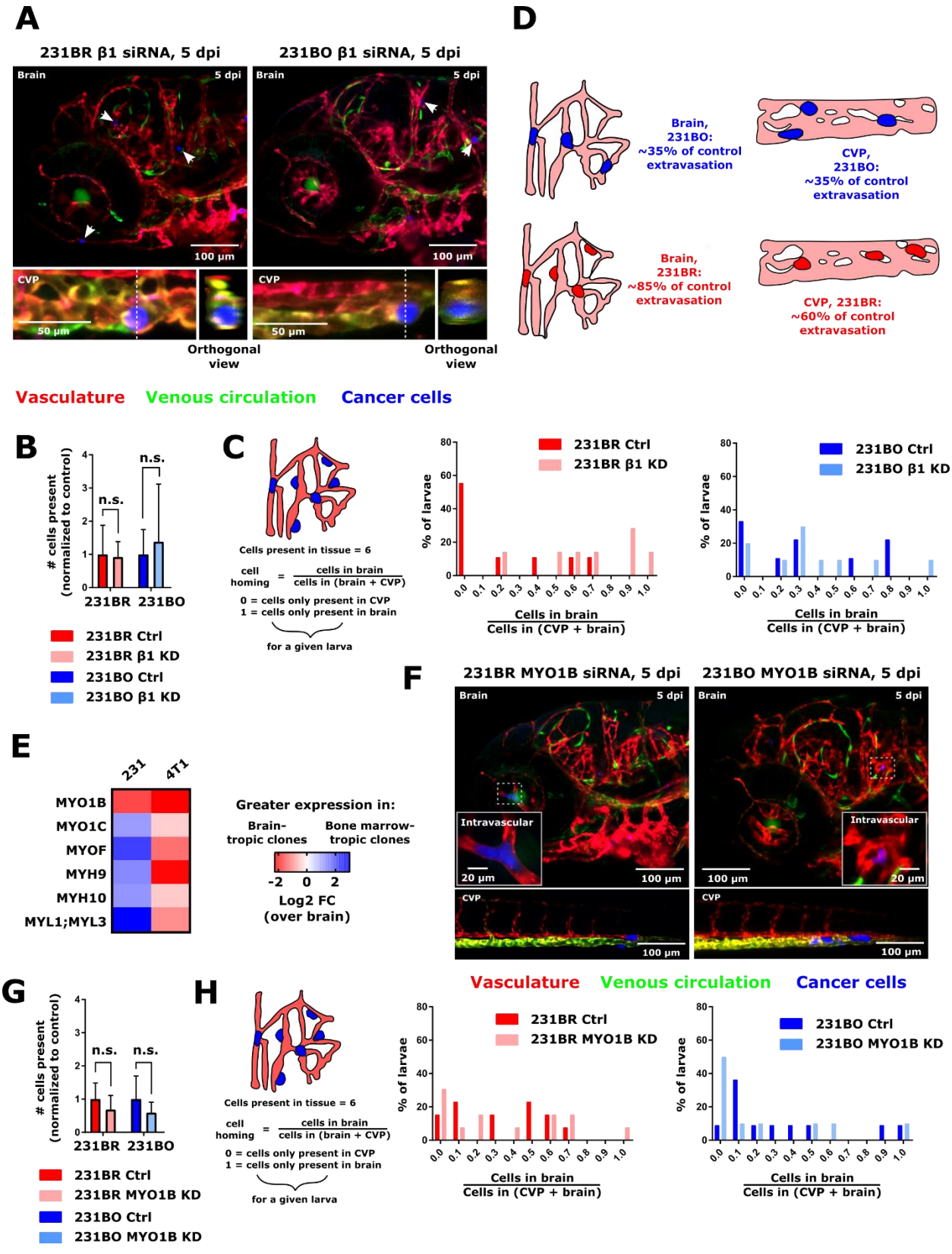

A
231BR β1 siRNA, 5 dpi
231BO β1 siRNA, 5 dpi
Brain
5 dpi
100 µm
CVP
50 µm
Orthogonal view
D
Brain, 231BO: ~35% of control extravasation
CVP, 231BO: ~35% of control extravasation
Brain, 231BR: ~85% of control extravasation
CVP, 231BR: ~60% of control extravasation
Vasculature
Venous circulation
Cancer cells
B
cells present (normalized to control)
n.s.
231BR
231BO
231BR Ctrl
231BR β1 KD
231BO Ctrl
231BO β1 KD
C
Cells present in tissue = 6
cell homing = cells in brain / cells in (brain + CVP)
0 = cells only present in CVP
1 = cells only present in brain
for a given larva
% of larvae
Cells in brain / Cells in (CVP + brain)
231BR Ctrl
231BR β1 KD
231BO Ctrl
231BO β1 KD
E
231
4T1
MYO1B
MYO1C
MYOF
MYH9
MYH10
MYL1;MYL3
Greater expression in:
Brain-tropic clones
Bone marrow-tropic clones
-2 0 2
Log2 FC (over brain)
F
231BR MYO1B siRNA, 5 dpi
231BO MYO1B siRNA, 5 dpi
Intravascular
20 µm
G
cells present (normalized to control)
231BR MYO1B KD
231BO MYO1B KD
H
Vasculature
Venous circulation
Cancer cells

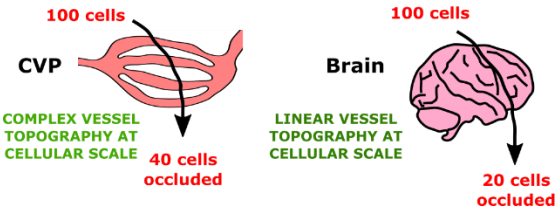
Figure S7. Related to Figure 7. Cell survival and localization following integrin β1 or myosin 1B silencing. (A) Representative images of MDA-MB-231 brain-tropic subclone (231BR) and bone marrow-tropic subclone (231BO) colonization in the zebrafish brain and CVP at 5 dpi following β1 integrin silencing prior to cell injection. Arrows indicate cell positions in the brain. Images are average intensity projections from confocal z stacks. The vasculature is displayed in red, the venous circulation in green, and injected cancer cells in blue. Scales are indicated. In the CVP, dashed line shows position of orthogonal view shown in inset. (B) Average ± SD of cells present in the brain plus caudal vein plexus (CVP) of Tg(flk:mCherry/MRC1a:EGFP) zebrafish at 5 days post-injection (dpi), when zebrafish were 7 days post-fertilization (dpf), upon injection with control cells or cells in which integrin β1 was silenced. Averages were calculated across all larvae injected with a given cell type. The averages for larvae injected with cells transfected with siRNA targeting β1 integrin for a given cell type were then normalized to averages for larvae injected with cells transfected with non-targeting control (Ctrl) siRNA for the same cell type. Knockdown was not a significant source of variation by two-way ANOVA ($p=0.6869$, $F=0.1656$, $DF=1$), and differences between knockdown and Ctrl cells for a given cell type were not significant by Sidak's multiple comparisons test. (C) Schematic illustrating assessment of all cells present in a tissue of interest (cell homing) and cell homing results upon integrin β1 knockdown. For cell homing calculations, both intravascular and extravascular cells were considered. For a given larva, this metric could range from a value of 0 (indicating that no cells were present in the brain) to 1 (indicating that cells were present in the brain but not CVP). Panels show distributions of values for cell homing ratio of 231BR Ctrl and integrin β1 knockdown cells (red) and 231BO Ctrl and integrin β1 knockdown cells (blue) at 5 dpi. For panels B, C, values were calculated from N=9 larvae injected with 231BR Ctrl cells (68 total cells counted), N=7 larvae injected with 231BR β1 integrin knockdown cells (49 total cells counted), N=9 larvae injected with 231BO Ctrl cells (52 total cells counted), and N=10 larvae injected with 231BO β1 knockdown cells (80 total cells counted). (D) Schematic illustrating effect of β1 integrin knockdown on 231BR and 231BO cells. Of the larvae with extravasated 231BO cells, the percentage of cells extravasated was reduced to ~35% of control levels in both the brain and CVP upon knockdown. For 231BR cells, knockdown did not affect extravasation in the brain (~85% of control levels) and moderately decreased extravasation in the CVP (~60% of control levels), shifting the distribution of extravasated cells to favor the brain. (E) Relative expression of myosins in 231 and 4T1 bone marrow-targeting cell clones, relative to expression in brain-targeting clone of each cell line. Heat map color indicates log2 fold change compared to brain-targeting clone. Protein expression levels were from whole-lysate mass spectrometry, from lysates taken from three independent experiments. Significance levels are not indicated. (F) Representative images of 231BR and 231BO colonization in the zebrafish brain and CVP at 5 dpi following myosin 1B knockdown. For both cell types, myosin 1B was knocked down prior to cell injection. Images are average intensity projections from confocal z stacks. The vasculature is displayed in red, the venous circulation in green, and injected cancer cells in blue. Scales are indicated. Insets show intravascular cells in the brain. (G) Average ± SD of cells present in the brain plus caudal vein plexus (CVP) of

Tg(flk:mCherry/MRC1a:EGFP) zebrafish at 5 dpi, when zebrafish were 7 dpf, following injection of control cells or cells in which myosin 1B was silenced. Averages were calculated across all larvae injected with a given cell type. The averages for larvae injected with cells transfected with siRNA targeting myosin 1B for a given cell type were then normalized to averages for larvae injected with cells transfected with non-targeting Ctrl siRNA for the same cell type. Knockdown was a significant source of variation by two-way ANOVA ($p=0.0188$, $F=5.962$, $DF=1$), but differences between knockdown and Ctrl cells for a given cell type were not significant by Sidak's multiple comparisons test. (H) Schematic illustrating assessment of all cells present in a tissue of interest (cell homing) and cell homing results upon myosin 1B knockdown. Panels show distributions of values for cell homing ratio of 231BR Ctrl and myosin 1B knockdown cells (red) and 231BO Ctrl and integrin β1 knockdown cells (blue) at 5 dpi. For panels G, H, values were calculated from N=13 larvae injected with 231BR Ctrl cells (196 total cells counted), N=13 larvae injected with 231BR myosin 1B knockdown cells (135 total cells counted), N=11 larvae injected with 231BO Ctrl cells (238 total cells counted), and N=10 larvae injected with 231BO myosin 1B cells (129 total cells counted). See also Figure 7.

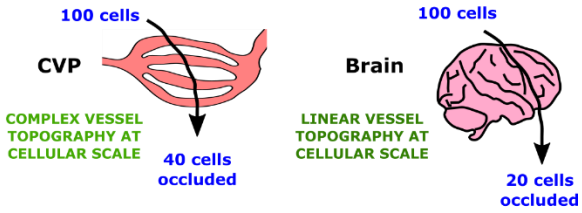
Phase 1: Initial organ distribution driven by local topography

0-12 h post-injection

231BR, 4T1Br4, 10 µm beads



231BO, 4T1BM2, 10 µm beads



Phase 2: Organ tropism driven by differential extravasation at site of arrest

1-5 days post-injection

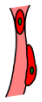
231BR

CVP

~20% of initially occluded cells extravasated by 5 dpi

8 of 40 cells extravasated

Brain

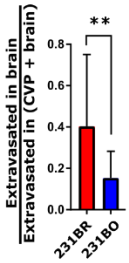


~30% of initially occluded cells extravasated by 5 dpi

6 of 20 cells extravasated

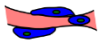
REDUCED EXTRAVASATION IN CVP

$$\frac{\text{Extravasated in brain}}{\text{Extravasated in CVP + brain}} = \frac{6}{(6+8)} = 0.43$$



231BO

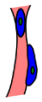
CVP



~40% of initially occluded cells extravasated by 5 dpi

16 of 40 cells extravasated

Brain



~30% of initially occluded cells extravasated by 5 dpi

6 of 20 cells extravasated

EFFICIENT EXTRAVSATION IN CVP

$$\frac{\text{Extravasated in brain}}{\text{Extravasated in CVP + brain}} = \frac{6}{(16+8)} = 0.25$$

Effect of β1 integrin knockdown

231BR

CVP

~60% of control extravasation by 5 dpi

5 of 40 cells extravasated

Brain

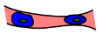
~85% of control extravasation by 5 dpi

5 of 20 cells extravasated

REDUCED EXTRAVASATION IN CVP BUT NOT BRAIN; REDISTRIBUTION OF CELLS TOWARD BRAIN

231BO

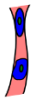
CVP



~35% of control extravasation by 5 dpi

5 of 40 cells extravasated

Brain



~35% of control extravasation by 5 dpi

2 of 20 cells extravasated

SIGNIFICANT REDUCTION IN EXTRAVASATION THROUGHOUT LARVA

Effect of myosin 1B knockdown

231BR

CVP

~85% of control extravasation by 5 dpi

7 of 40 cells extravasated

Brain

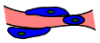
~55% of control extravasation by 5 dpi

3 of 20 cells extravasated

REDUCED EXTRAVASATION IN BRAIN BUT NOT CVP; REDISTRIBUTION OF CELLS TOWARD CVP

231BO

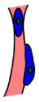
CVP



~140% of control extravasation by 5 dpi

22 of 40 cells extravasated

Brain



~90% of control extravasation by 5 dpi

5 of 20 cells extravasated

INCREASED EXTRAVASATION IN CVP BUT NOT BRAIN; REDISTRIBUTION OF CELLS TOWARD CVP

Figure S8. Model of MDA-MB-231 brain-tropic (231BR) and bone marrow-tropic (231BO) organ selectivity in larval zebrafish xenograft system. Tracking the early metastatic organ colonization of circulating tumor cells in a zebrafish xenograft model revealed two phases of non-random targeting. In the first phase (~0-12 h after injection), cells disseminated to the brain and caudal vein plexus (CVP). Dissemination was tissue but not cell type specific, with ~40% of cells observed becoming occluded in the CVP and ~20% of cells observed becoming occluded in the brain. This pattern was observed across multiple organotropic cell lines (231BR, 231BO, 4T1Br4, and 4T1BM2), along with 10 μm-diameter polystyrene beads. Extravasation of cells from 1-5 days post-injection (dpi) drove differences in organ targeting between 231BR and 231BO cells. Of the cells arrested in the brain, ~30% were able to extravasate for both cell types. Of the cells arrested in the CVP, ~40% of 231BO cells were able to extravasate, while this percentage was reduced to ~20% for 231BR cells. On the basis of 100 cells initially passing through each organ (during dissemination), this led to cell-type dependent differences in the distribution of extravasated cells by 5 dpi (plot reproduced from Figure 1 to illustrate how this model could explain the experimentally observed distributions of extravasated 231BR and 231BO cells). Knockdown of β1 integrin moderately reduced extravasation of 231BR cells in the CVP but did not affect extravasation in the brain, leading to a redistribution of extravasated 231BR cells toward the brain upon knockdown. In contrast, β1 integrin knockdown significantly reduced the extravasation of 231BO cells in both the larval brain and CVP. Knockdown of myosin 1B significantly reduced extravasation of 231BR cells in the brain but did not significantly affect 231BR cell extravasation in the CVP. Myosin 1B knockdown moderately increased the extravasation of 231BO cells in the CVP. In summary, knockdown of myosin 1B redirected early metastatic targeting toward the CVP.

Table S1. Related to Figure 5, Figure S5. Occlusion fractions of cancer cells and polystyrene beads in zebrafish.

Cell type	Tissue	Total cells tracked	Cells occluded	Occlusion fraction
231BR	Brain	140	28	0.200
231BR	CVP	170	69	0.406
231BO	Brain	86	20	0.233
231BO	CVP	97	42	0.433
4T1Br4	Brain	61	12	0.197
4T1Br4	CVP	78	27	0.346
4T1BM2	Brain	26	7	0.269
4T1BM2	CVP	70	32	0.457
10 μm beads	Brain	183	32	0.175
10 μm beads	CVP	29	12	0.414

Table S2. Related to Figure 5, Figure S5. Occlusion fractions of cancer cells in zebrafish upon inhibition of integrin β1.

Cell type	Tissue	Total cells tracked	Cells occluded	Occlusion fraction	Normalized occlusion fraction
231BR Ctrl siRNA	Brain	30	5	0.167	1
231BR integrin $\beta 1$ siRNA	Brain	94	24	0.255	1.532
231BR Ctrl siRNA	CVP	35	8	0.229	1
231BR integrin $\beta 1$ siRNA	CVP	74	29	0.392	1.715
231BO Ctrl siRNA	Brain	78	18	0.231	1
231BO integrin $\beta 1$ siRNA	Brain	43	17	0.395	1.713
231BO Ctrl siRNA	CVP	70	38	0.543	1

231BO integrin $\beta 1$ siRNA	CVP	55	33	0.600	1.105
231BR IgG	Brain	207	52	0.251	1
231BR anti-$\beta 1$	Brain	181	33	0.182	0.726
231BR IgG	CVP	159	48	0.302	1
231BR anti-$\beta 1$	CVP	296	74	0.250	0.828
231BO IgG	Brain	78	19	0.213	1
231BO anti-$\beta 1$	Brain	26	3	0.115	0.474
231BO IgG	CVP	139	57	0.410	1
231BO anti-$\beta 1$	CVP	77	32	0.416	1.013

Table S3. Related to Figure 3, Figure S4. Summary of cells tracked in confined migration experiments with varying microchannel geometry.

Cell type	Microchannel geometry	Biological replicates	Total number of cells tracked (N)
231BR	Brain mimetic	4	41
231BO	Brain mimetic	4	42
231BR	9 μm	4	42
231BO	9 μm	4	46
231BR	CVP mimetic	4	120
231BO	CVP mimetic	4	118
231BR	17 μm	4	90
231BO	17 μm	4	81

Table S4. Related to Figure 3, Figure S4. Summary of cells tracked in confined migration experiments with varying microchannel protein coating.

Cell type	Protein coating	Biological replicates	Total number of cells tracked (N)
231BR	Collagen type I	2	60
231BO	Collagen type I	2	60
231BR	Fibronectin	2	60
231BO	Fibronectin	2	60
231BR	BSA	3	52
231BO	BSA	3	57

Table S5. Related to Figure 4. Summary of red blood cell tracking in 2 dpf larvae.

Fish	Strain	Tissue	Number of cells tracked
1	Tg(flk:mCherry/MRC1a:EGFP)	Brain	40
1	Tg(flk:mCherry/MRC1a:EGFP)	CVP	40
2	Tg(flk:mCherry/MRC1a:EGFP)	Brain	40
2	Tg(flk:mCherry/MRC1a:EGFP)	CVP	40
3	Tg(flk:mCherry/MRC1a:EGFP)	Brain	9
3	Tg(flk:mCherry/MRC1a:EGFP)	CVP	40
4	Tg(flk:mCherry/mpx:EGFP)	Brain	13
4	Tg(flk:mCherry/mpx:EGFP)	CVP	40