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Supplemental Information

Hypoxia Triggers the Intravasation of Clustered Circulating Tumor Cells

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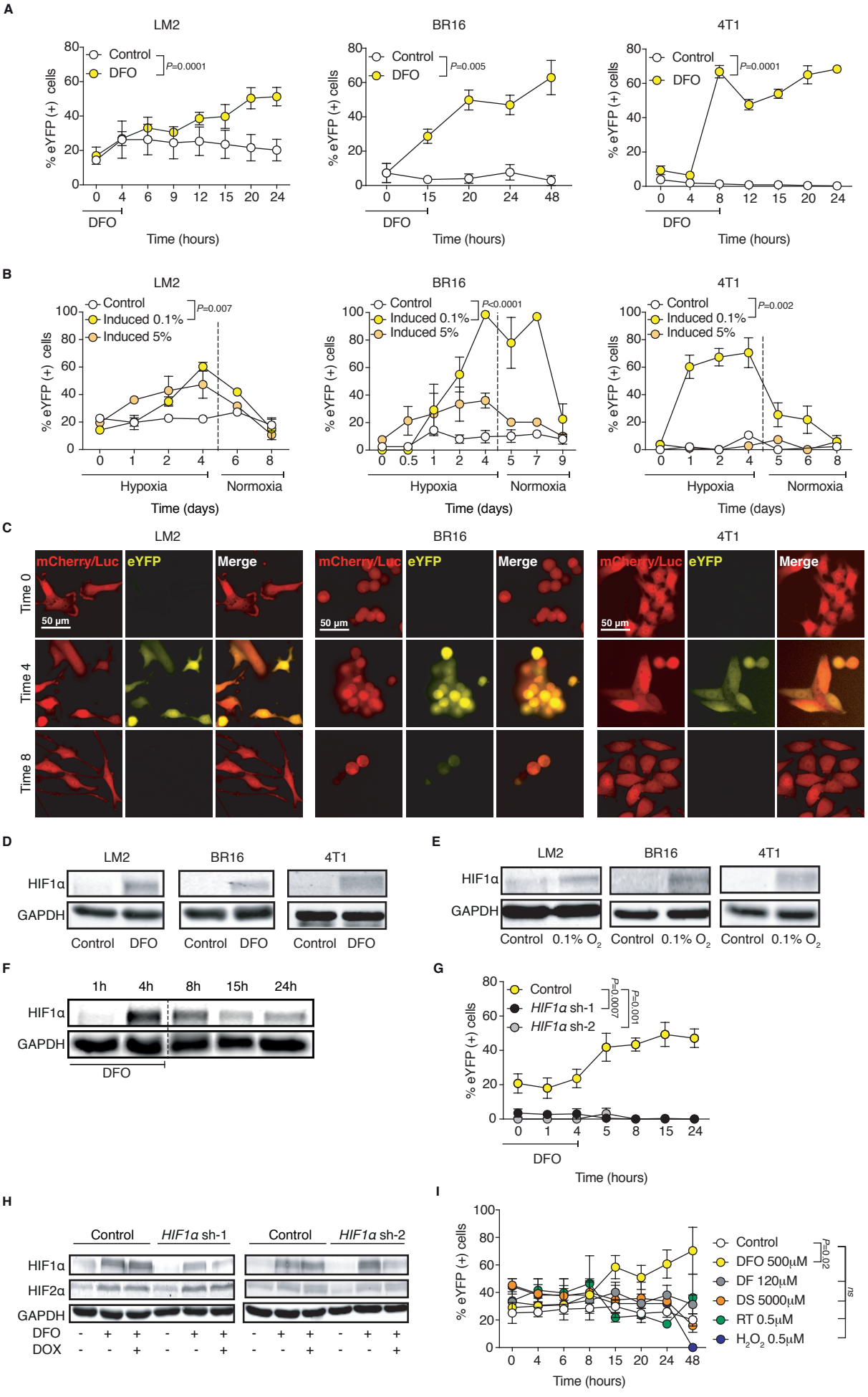

Figure S1. Donato et al.

Figure S1. *In vitro* characterization of the HIF1α reporter. Related to Figure 1.

(**A**) From left to right, the plots show the activation of the HIF1α reporter in LM2-mCherry/Luc, BR16-mCherry/Luc, and 4T1-mCherry/Luc cells upon Deferoxamine (DFO) induction (*n*=2). Error bars represent SEM; *P* value by linear regression is shown.

(**B**) From left to right, the plots show the activation of the HIF1α reporter in LM2-mCherry/Luc, BR16-mCherry/Luc, and 4T1-mCherry/Luc cells upon incubation in 0.1% O_2 (hypoxia) or 5% O_2 and subsequent treatment with 20% O_2 (normoxia) (*n*=2). Error bars represent SEM; *P* value by linear regression is shown.

(**C**) From left to right, representative pictures showing the induction of the HIF1α reporter upon incubation at 0.1% O_2 of LM2-mCherry/Luc, BR16-mCherry/Luc, and 4T1-mCherry/Luc cells.

(**D**) Representative western blot images showing HIF1α protein upon DFO induction of LM2-mCherry/Luc, BR16-mCherry/Luc and 4T1-mCherry/Luc cells for 4, 15 and 8 hours, respectively.

(**E**) Representative western blot images showing HIF1α protein upon 0.1% O_2 induction of LM2-mCherry/Luc, BR16-mCherry/Luc and 4T1-mCherry/Luc cells for 4, 15 and 8 hours, respectively.

(**F**) Representative western blot image showing HIF1α protein upon DFO induction for 4 hours in LM2-mCherry/Luc cells, and residual HIF1α protein post-DFO treatment.

(**G**) The plot shows the activation of the HIF1α reporter in LM2-mCherry/Luc cells expressing a control shRNA (control), h*HIF1α* shRNA-1 or h*HIF1α* shRNA-2 (sh-1 and sh-2) (*n*=2). Error bars represent SEM; *P* value by linear regression is shown.

(**H**) Representative western blot showing HIF1α protein and HIF2α protein in LM2-GFP/Luc cells expressing a control shRNA (control), *HIF1α* sh-1 or *HIF1α* sh-2 (sh-1 and sh-2).

(**I**) The plot shows the activation of the HIF1α reporter in LM2-mCherry/Luc measured by determining the expression of eYFP in living cells upon incubation with DFO, Diethyl fumarate (DF), Dimethyl succinate (DS), Rotenone (RT) or Hydrogen peroxide (H_2O_2); (*n*=2). Error bars represent SEM; *P* value by linear regression is shown.

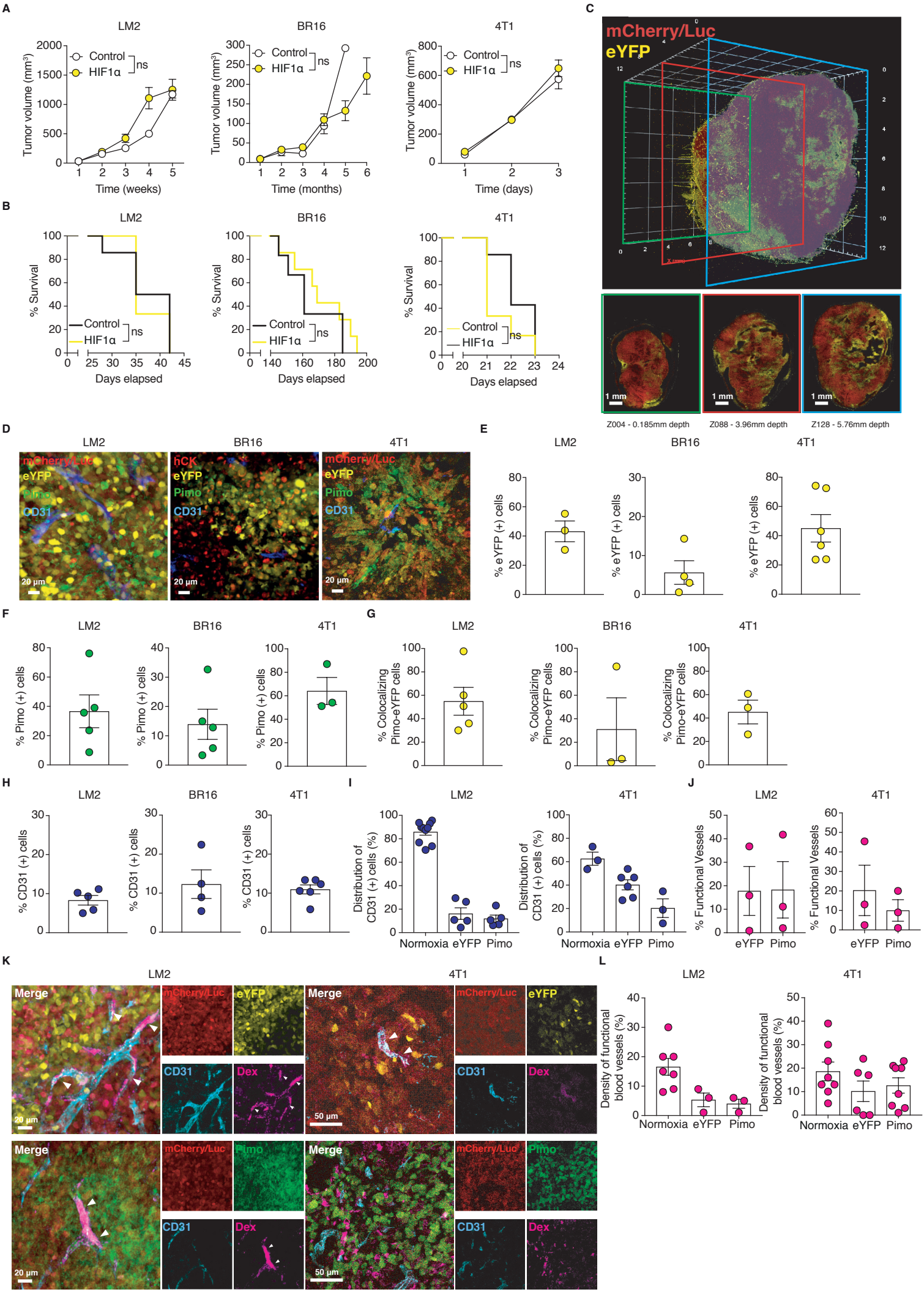

Figure S2. Donato et al.

Figure S2. *In vivo* analysis of vascularized hypoxic regions of breast tumors. Related to Figure 1.

(A) From left to right, the plots show the mean tumor volume of NSG-LM2-mCherry/Luc, NSG-BR16 and NSG-4T1-mCherry/Luc mice expressing either the HIF1α reporter (HIF1α; *n*=4 for LM2, *n*=7 for BR16, *n*=7 for 4T1) or empty vector (control; *n*=4 for LM2, *n*=6 for BR16, *n*=6 for 4T1). Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**B**) From left to right, overall survival rates of NSG-LM2-mCherry/Luc, NSG-BR16 and NSG-4T1-mCherry/Luc mice, expressing either the HIF1α reporter (HIF1α; *n*=4 for LM2, *n*=7 for BR16, *n*=6 for 4T1) or empty vector (control; *n*=4 for LM2, *n*=6 for BR16, *n*=7 for 4T1). Error bars represent SEM; *P* values by two-sided log-rank test are shown.

(**C**) 3D rendering of a whole NSG-LM2-mCherry/Luc tumor expressing the HIF1α reporter (eYFP; *left*), and three max intensity snapshots at 0.2 mm, 4 mm and 6 mm depth of the tumor volume (*right*).

(**D**) From left to right, representative images of NSG-LM2-HIF1α, NSG-BR16-HIF1α and NSG-4T1-HIF1α stained for human cytokeratin (hCK), CD31, dextran (Dex) and Pimonidazole (Pimo).

(**E**) Graphs showing the mean percentage of eYFP-positive (+) cells in NSG-LM2-HIF1α, (*n*=3), NSG-BR16-HIF1α (*n*=4) and NSG-4T1-HIF1α (*n*=6) tumor model. Error bars represent SEM.

(**F**) Graphs showing the mean percentage of Pimo-(+) cells in NSG-LM2-HIF1α, (*n*=5), NSG-BR16-HIF1α (*n*=5) and NSG-4T1-HIF1α (*n*=3) tumor model. Error bars represent SEM.

(**G**) Graphs showing the mean percentage of colocalizing Pimo and eYFP signal (cells) in NSG-LM2-HIF1α, (*n*=5), NSG-BR16-HIF1α (*n*=3) and NSG-4T1-HIF1α (*n*=3) tumor model. Error bars represent SEM.

(**H**) Graphs showing the mean percentage of CD31-(+) cells within LM2 (*n*=5), BR16 (*n*=4) and 4T1 (*n*=6) tumor models. Error bars represent SEM.

(**I**) The plot shows the percentage of CD31-positive (+) cells within the normoxic and the hypoxic tumor areas of NSG-LM2-HIF1α (*n*=5) and NSG-4T1-HIF1α (*n*=5) tumor models, defined by HIF1α reporter (eYFP), Pimo staining, or the lack of both (Normoxia).

(**J**) Graphs showing the mean percentage of CD31-Dex(+) cells across the hypoxic tumor areas of NSG-LM2-HIF1α (*left; n*=3) and NSG-4T1-HIF1α (*right; n*=3) mice, respectively. Error bars represent SEM.

(**K**) Representative images of NSG-LM2 HIF1α (*left*) and NSG-4T1 HIF1α (*right*) tumors stained for CD31, Pimo and Dex. White triangles highlight the Dex-positive vessels.

(**L**) Plot showing the density of functional blood vessels in normoxic areas (*n*=7), eYFP (*n*=4) or Pimo-stained (*n*=4) areas of NSG-LM2-HIF1α (*left*) and NSG-4T1-HIF1α (*right*) reporter tumors. Error bars represent SEM.

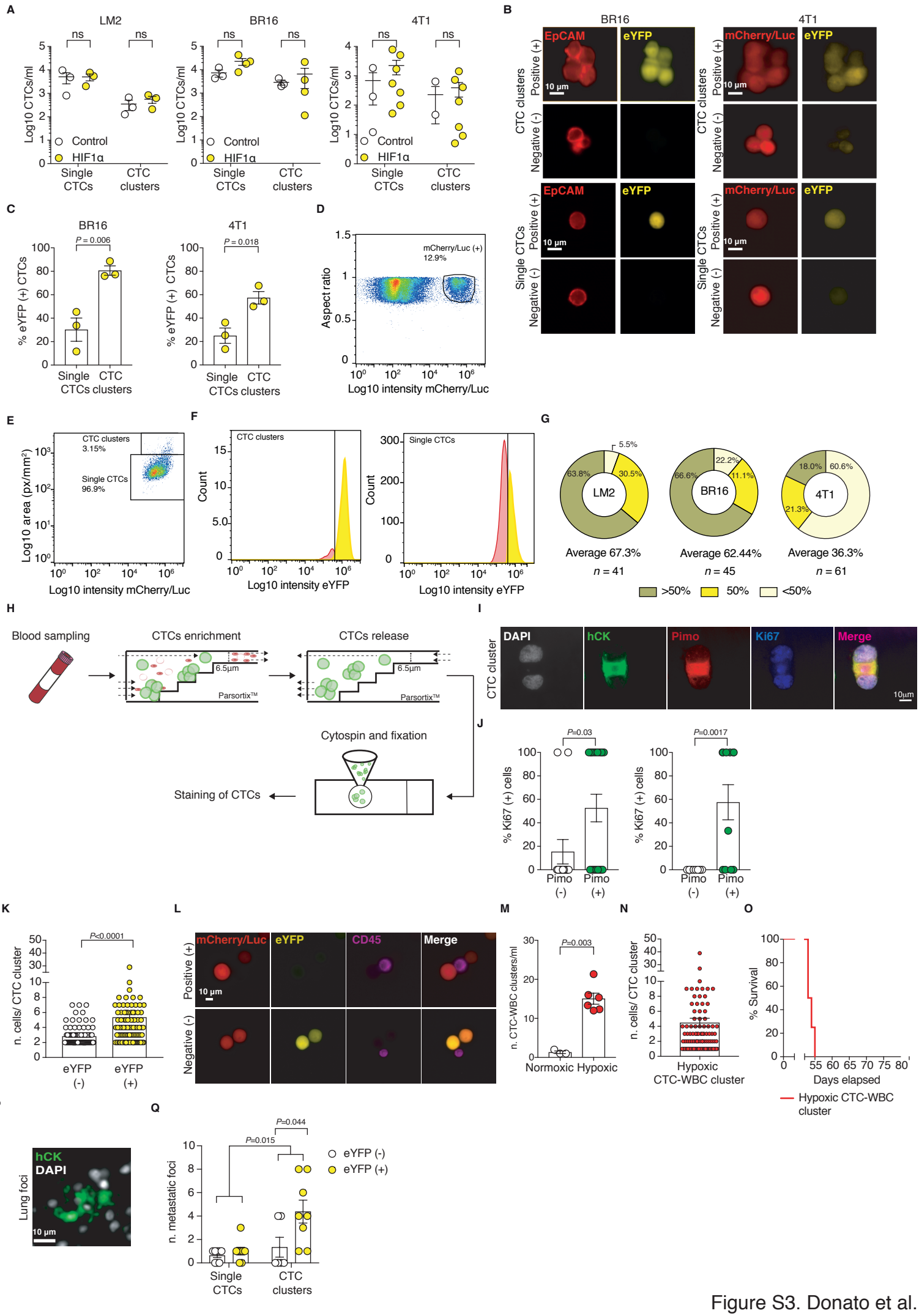

Figure S3. Donato et al.

Figure S3. Analysis of eYFP expression in single CTCs and CTC clusters. Related to Figure 2.

(**A**) From left to right, scatter plots showing the Log10 of total CTC counts per ml of blood from NSG-LM2-mCherry/Luc, NSG-BR16 or NSG-4T1-mCherry/Luc mice, expressing either the HIF1α reporter (HIF1α; *n*=3 for LM2, *n*=4 for BR16, *n*=7 for 4T1) or empty vector (control; *n*=3 for LM2 and BR16, *n*=7 for 4T1). Error bars represent SEM; *P* values by two-way Anova are shown.

(**B)** Representative pictures showing CTC clusters (*top*) and single CTCs (*bottom*) from NSG-BR16 mice (*left*; stained for EpCAM) or NSG-4T1-mCherry/Luc (*right*), positive or negative for the expression of the HIF1α reporter.

(**C**) The scatter plot shows the mean percentage of eYFP-positive (+) single CTCs and CTC clusters from NSG-BR16 (*left*) or NSG-4T1-mCherry/Luc (*right*) mice (*n*=3). Error bars represent SEM; *P* value by two-tailed unpaired Student's *t*-test is shown.

(**D**) Flow cytometry analysis showing CTCs from NSG-LM2-HIF1α mice gated for the Log10 intensity of mCherry (*X axis*) versus the aspect ratio (*Y axis*).

(**E**) Flow cytometry analysis showing single CTCs and CTC clusters from NSG-LM2-HIF1α mice gated for the Log10 intensity of mCherry (*X axis*) versus the Log10 of the area in pixels per squared millimeter (px/mm2) (*Y axis*).

(**F**) Flow cytometry analysis showing CTC clusters (*left*) and single CTCs (*right*) from NSG-LM2-HIF1α mice gated for the Log10 intensity of eYFP (*X axis*).

(**G**) From left to right, pie charts showing the percentage of eYFP positive cells within clusters of NSG-LM2-HIF1α, NSG-BR16-HIF1α or NSG-4T1-HIF1α mice. The number of independent biological replicates (*n*) is shown.

(**H**) Schematic of the experimental design.

(**I**) Representative pictures of a LM2 CTC cluster stained for DAPI (nuclei), human Cytokeratin (hCK), Pimonidazole (Pimo) and Ki67 (proliferation).

(**J**) Scatter plots showing the percent of Ki67-(+) cells among Pimo-(-) and Pimo-(+) single CTCs (*left; n*=13 and *n*=19) and CTC clusters (*right; n*=10 and *n*=11) of NSG-LM2-HIF1α mice. Error bars represent SEM; *P* value by two-tailed unpaired Student's *t*-test is shown.

(**K**) The scatter plot shows the number of cells per normoxic (*n*=107) or hypoxic (*n*=112) CTC clusters from NSG-LM2-HIF1α reporter mice. On average, 2.82 and 5.34 cells are present in normoxic and hypoxic CTC clusters, respectively. Error bars represent SEM; *P* value by two-tailed unpaired Student's *t*-test is shown.

(**L**) Representative pictures of eYFP-(-) (*top*) and eYFP-(+) (*bottom*) CTC-White Blood Cell (WBC) clusters of NSG-LM2-HIF1α mice.

(**M**) Scatter plot showing the number of normoxic or hypoxic CTC-WBC clusters per ml of blood of NSG-LM2-HIF1α mice. Error bars represent SEM; *P* value by two-tailed unpaired Student's *t*-test is shown.

(**N**) The scatter plot shows the number of cells per hypoxic CTC-WBC clusters of NSG-LM2-HIF1α mice. Error bars represent SEM.

(**O**) Survival analysis of NSG mice injected with eYFP (+) CTC-WBC clusters (*n*=3).

(**P**) Representative picture of lung metastasis in NSG mice injected with eYFP-(+) CTC clusters of NSG-BR16-HIF1α mice. Lungs were stained for human Cytokeratin (hCK) and DAPI (nuclei).

(**Q**) Scatter plot showing the number of metastatic foci of NSG mice injected with eYFP-(+) or eYFP-(-), single CTCs or CTC clusters of NSG-BR16-HIF1α reporter mice (n=2). Error bars represent SEM; *P* value by two-tailed unpaired Student's *t*-test is shown.

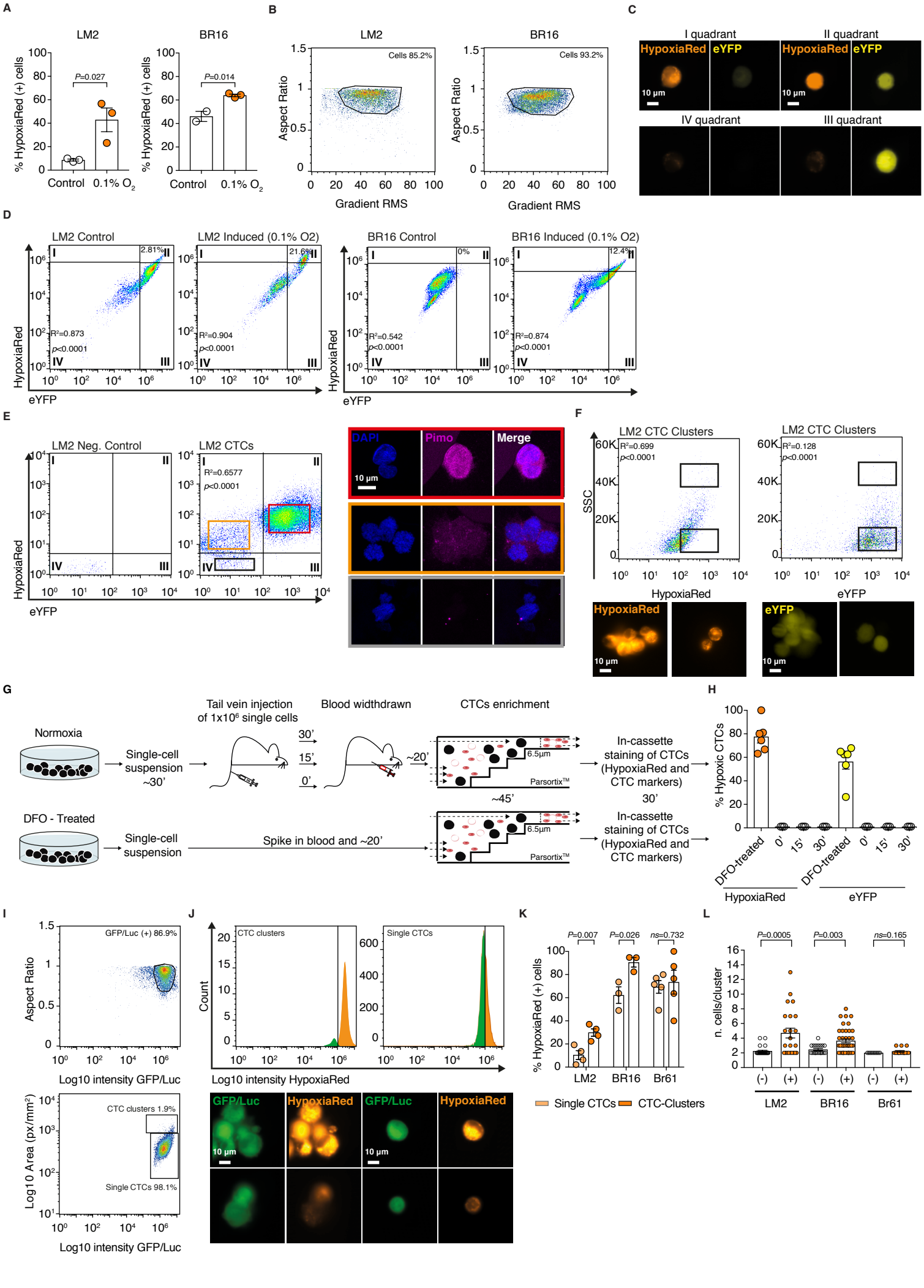

Figure S4. Donato et al.

Figure S4. HypoxiaRed correlates with HIF1α reporter and reacts with live hypoxic CTC clusters. Related to Figure 2.

(**A**) The plots show the percentage of HypoxiaRed-positive (+) cells from LM2-GFP/Luc or BR16-GFP/Luc cells incubated in hypoxic conditions (0.1% O_2) for 8 or 15 hours, respectively (*n*=3). Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**B**) Flow cytometric analysis showing LM2-HIF1α cells gated for the gradient RMS (*X axis*) versus the aspect ratio (*Y axis*).

(**C**) Representative pictures of LM2 cells expressing the HIF1α reporter and stained for HypoxiaRed.

(**D**) Flow cytometric analysis showing the correlation between the expression of eYFP (in Log10 intensity; *X axis*) and HypoxiaRed staining intensity (in Log10 intensity; *Y axis*) in LM2-HIF1α (*left*) and BR16-HIF1α (*right*) cells. Controls (uninduced and induced with hypoxia 0.1% O_2 for 4 days) are shown for both models.

(**E**) On the left panels, flow cytometric analysis showing the correlation between the expression of eYFP (in Log10 intensity; *X axis*) and HypoxiaRed staining intensity (in Log10 intensity; *Y axis*) in CTCs from NSG-LM2-HIF1α mice. Control represents a normoxic *in vitro* sample stained with HypoxiaRed. On the right panels, representative pictures of CTCs from NSG-LM2-HIF1α sorted from the highlighted quadrants and stained for Pimonidazole.

(**F**) On the top panels, flow cytometric analysis showing the correlation between the expression of HypoxiaRed (in Log10 intensity; *left*; *X axis)* or eYFP (in Log10 intensity; *right*; *X axis*) and size (SSC; *Y axis*) of CTC clusters from NSG-LM2-HIF1α mice. On the bottom panel, representative pictures of CTC clusters sorted from the highlighted quadrants with same fluorophore intensity but different cluster size.

(**G**) Schematic of the experimental design.

(**H**) Plot showing the percentage of HypoxiaRed-(+) or eYFP-(+) CTCs. DFO-treated samples represent the positive control. Error bars represent SEM; (*n*=6).

(**I**) On the top panel, flow cytometric analysis showing CTCs from NSG-LM2-HIF1α mice gated for the Log10 intensity of GFP (*X axis*) versus the aspect ratio (*Y axis*). On the bottom panel, flow cytometric analysis showing single CTCs and CTC clusters from NSG-LM2-HIF1α mice gated for the Log10 intensity of GFP (*X axis*) versus the Log10 of the area in pixels per squared millimeter (px/mm_2) (*Y axis*).

(**J**) On the top panel, flow cytometric analysis showing CTC clusters (*left*) and single CTCs (*right*) from NSG-LM2-GFP/Luc mice gated for the Log10 intensity of HypoxiaRed (*X axis*). On the bottom panel, representative pictures showing HypoxiaRed-(+) or –(-) CTC clusters (*left*) and single CTCs (*right*).

(**K**) The plot shows the mean percentage of HypoxiaRed-(+) single CTCs and CTC clusters from NSG-LM2-GFP/Luc (*n*=4) and NSG-BR16-GFP/Luc (*n*=3) mice, as well as from BR61 patient (*n*=5). Error bars represent SEM; *P* value by two-tailed unpaired Student's *t*-test is shown.

(**L**) The plot shows the number of cells per CTC cluster stained HypoxiaRed-(+) or –(-) in NSG-LM2-GFP/Luc (*n*=3) and NSG-BR16-GFP/Luc (*n*=4) mice as well as in BR61 patient (*n*=3). Error bars represent SEM; *P* value by two-tailed unpaired Student's *t*-test is shown.

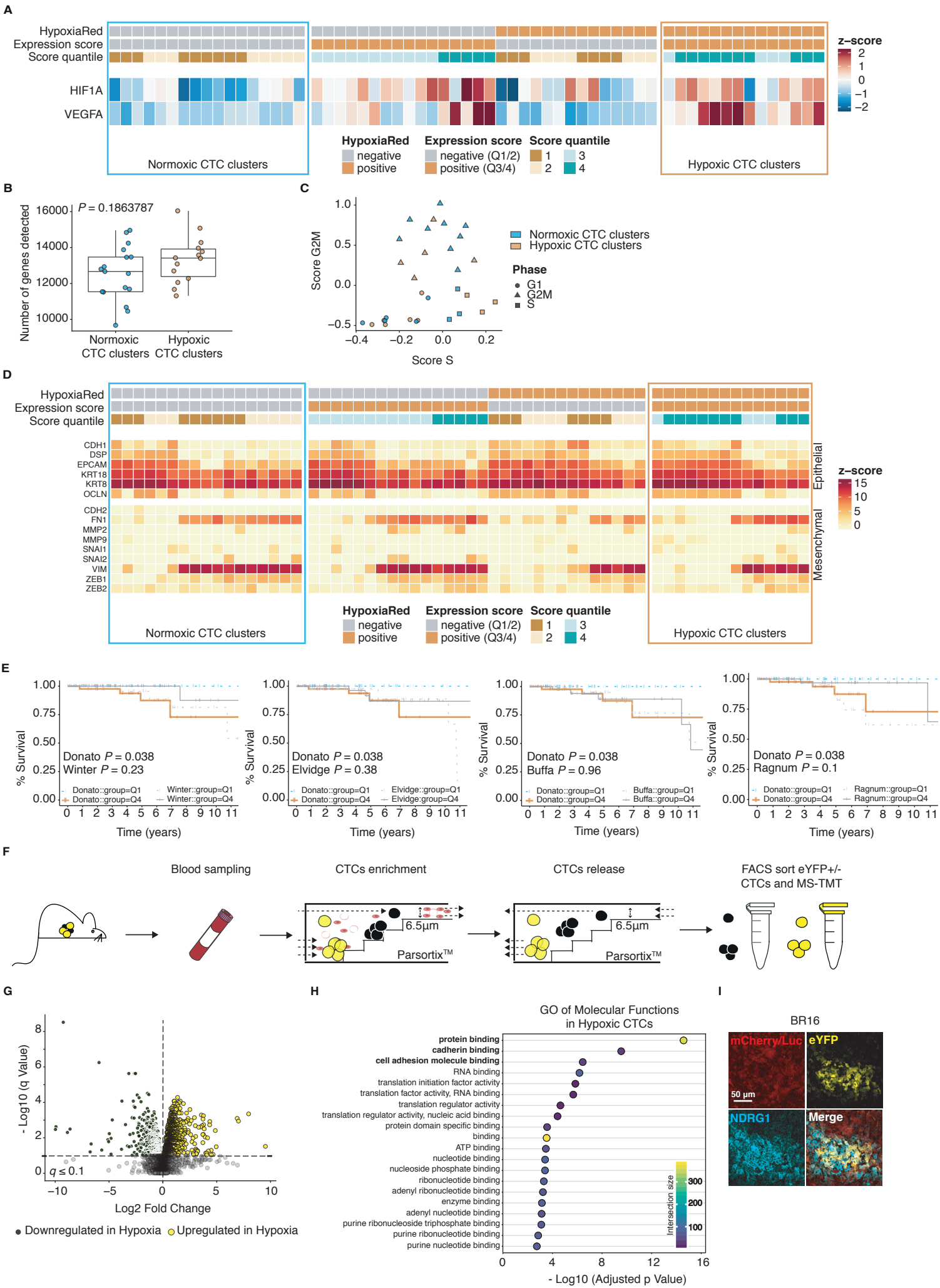

Figure S5. Donato et al.

Figure S5. Single-cell resolution RNA-sequencing analysis and proteomics analysis. Related to Figure 3 and 4.

(**A**) Heatmap showing the sample categories based on HypoxiaRed staining, expression score (based on HIF1α and VEGFA expression: negative for quantiles Q1 and Q2, positive for quantiles Q3 and Q4) and quantile score.

(**B**) Scatter plot showing the number of detected genes in normoxic versus hypoxic CTC clusters. The lower and upper hinges of the boxplot correspond to the 25th and 75th percentiles, respectively, and whiskers are extended to the most extreme data points. *P* value by two-sided Wilcoxon rank sum test is shown.

(**C**) Scatter plot showing the cell cycle phase of normoxic and hypoxic CTC clusters.

(**D**) Heatmap showing the expression of Epithelial-to-Mesenchymal transition (EMT) genes in normoxic and hypoxic CTC clusters. Colors are based on normalized length-scaled transcript per million (TPM) values.

(**E**) Kaplan-Meier curves showing overall survival rates of Stage I breast cancer patients expressing in their primary tumor high (quantile 4; Q4) or low (quantile 1; Q1) levels of genes upregulated in the hypoxic CTC clusters signature (Donato; *orange*) versus four other hypoxia signatures (*grey*). *P* value by two-sided log-rank test is shown.

(**F**) Schematic of the experimental design. From left to right, mice bearing LM2- HIF1α reporter tumors are sacrificed and the blood is processed for CTCs enrichment. Released CTCs are FACS-sorted into eYFP-(+) or eYFP-(-) single CTCs and CTC clusters, and analyzed via mass spectrometry using tandem mass tags-labeling (MS-TMT).

(**G**) Volcano plot showing all proteins detected with mass spectrometry analysis. Proteins that are enriched or depleted in eYFP-positive cells ($q<0.1$) are shown in yellow or black, respectively.

(**H**) Gene ontology (GO) analysis of molecular function pathways upregulated in hypoxic CTCs and ranked by adjusted *P* value. Intersection size bar shows the number of proteins detected in each GO term.

(**I**) Representative pictures of BR16-HIF1α reporter tumor expressing mCherry/Luc and stained for Pimonidazole (Pimo) and NDRG1.

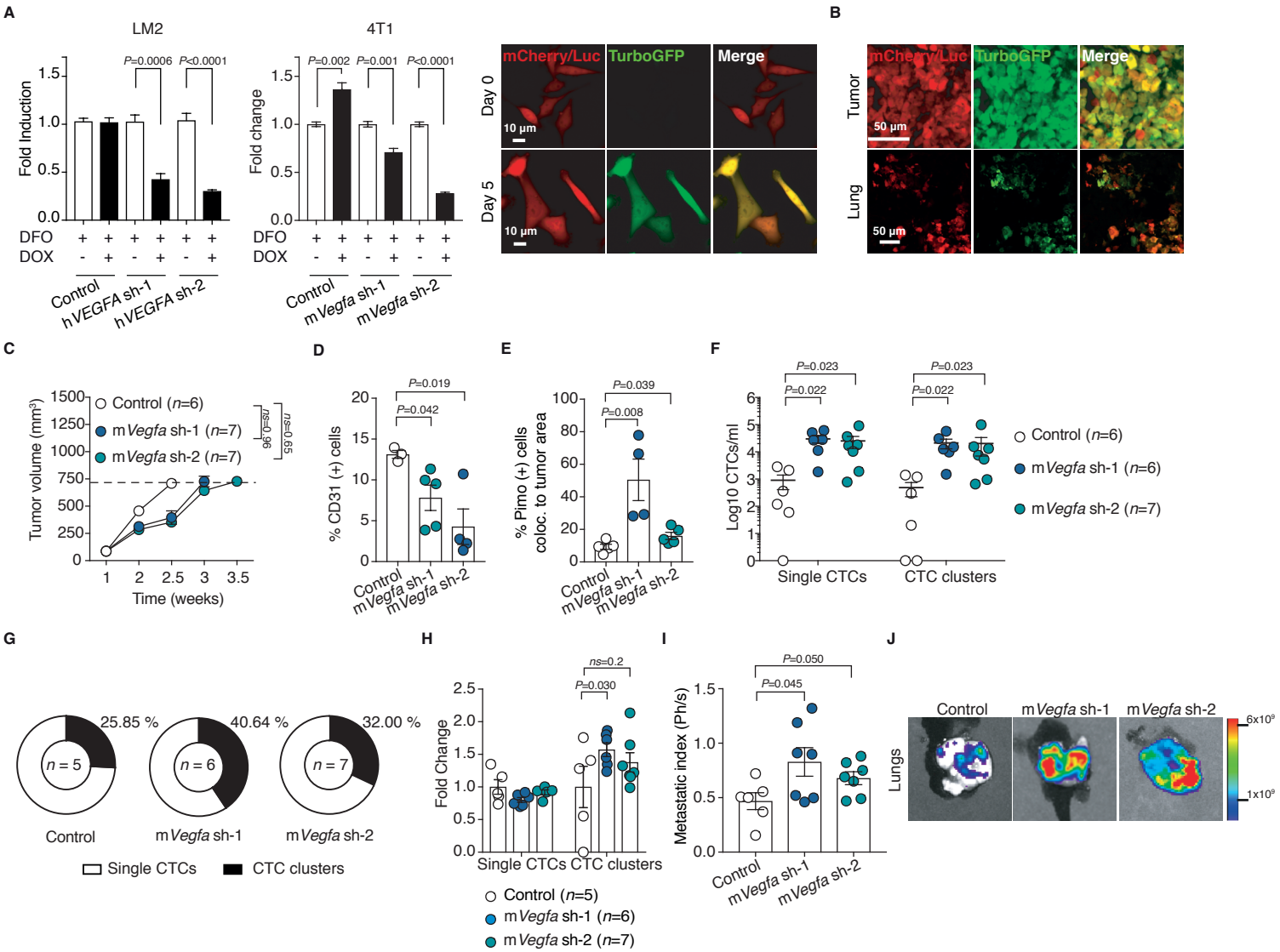

Figure S6. Donato et al.

Figure S6. Knockdown of VEGFA increases CTC cluster shedding and metastasis formation. Related to Figure 6.

(**A**) On the left side, the plot shows the mean fold change of expression (qPCR) of h*VEGFA* or m*Vegfa* in LM2-mCherry/Luc (*left*) or 4T1-mCherry/Luc (*right*) cells, respectively (*n*=3). Error bars represent SEM; *P* value by two-tailed unpaired Student's *t*-test is shown. On the right side, representative pictures of LM2-mCherry/Luc cells upon knockdown.

(**B**) Representative pictures of the primary tumor (*top*) and metastatic lungs (*bottom*) of NSG-LM2-mCherry/Luc mice expressing h*HIF1α* shRNAs.

(**C**) The plots show the mean tumor volume of NSG mice injected with 4T1-mCherry/Luc cells expressing a control shRNA (control) or m*Vegfa* shRNAs (m*Vegfa* sh-1 and sh-2). The number of independent biological replicates (*n*) is shown. Error bars represent SEM; *P* values by two-tailed paired Student's *t*-test are shown.

(**D**) The plot shows the mean percentage of CD31-(+) cells within the primary tumor of NSG-4T1 mice expressing a control (*n*=3) or VEGFA knockdown (*n*=5 for sh-1 and *n*=4 sh-2). Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**E**) The plot shows the mean percentage of Pimonidazole-(+) cells colocalizing with primary tumor cells of NSG-4T1 mice expressing a control (*n*=5) or VEGFA knockdown (*n*=4 for sh-1 and *n*=5 sh-2). Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**F**) Plot showing the Log10 of total CTC counts per ml of blood in NSG-4T1 mice expressing a control or VEGFA knockdown. The number of independent biological replicates (*n*) is shown. Error bars represent SEM; *P* values by two-way Anova are shown.

(**G**) Pie charts displaying the mean percentage of single CTCs and CTC clusters in NSG-4T1 mice expressing a control or VEGFA knockdown. The number of independent biological replicates (*n*) is shown.

(**H**) The plot shows the mean fold change of CTC ratios in NSG-4T1 mice expressing a control or VEGFA knockdown. The number of independent biological replicates (*n*) is shown. Error bars represent SEM; *P* values by two-way Anova are shown.

(**I**) The plot shows the metastatic index of NSG-4T1 mice expressing a control (*n*=6) or VEGFA knockdown (*n*=7 for sh-1 and sh-2). Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**J**) Representative bioluminescence images of metastatic lungs of NSG-4T1 mice expressing a control or VEGFA knockdown.

(**K**) Representative western blot showing hHIF1α protein and hHIF2α protein in LM2-mCherry/Luc cells expressing a dox-inducible control shRNA (control), h*VEGFA* sh-1 or h*VEGFA* sh-2 (sh-1 and sh-2).

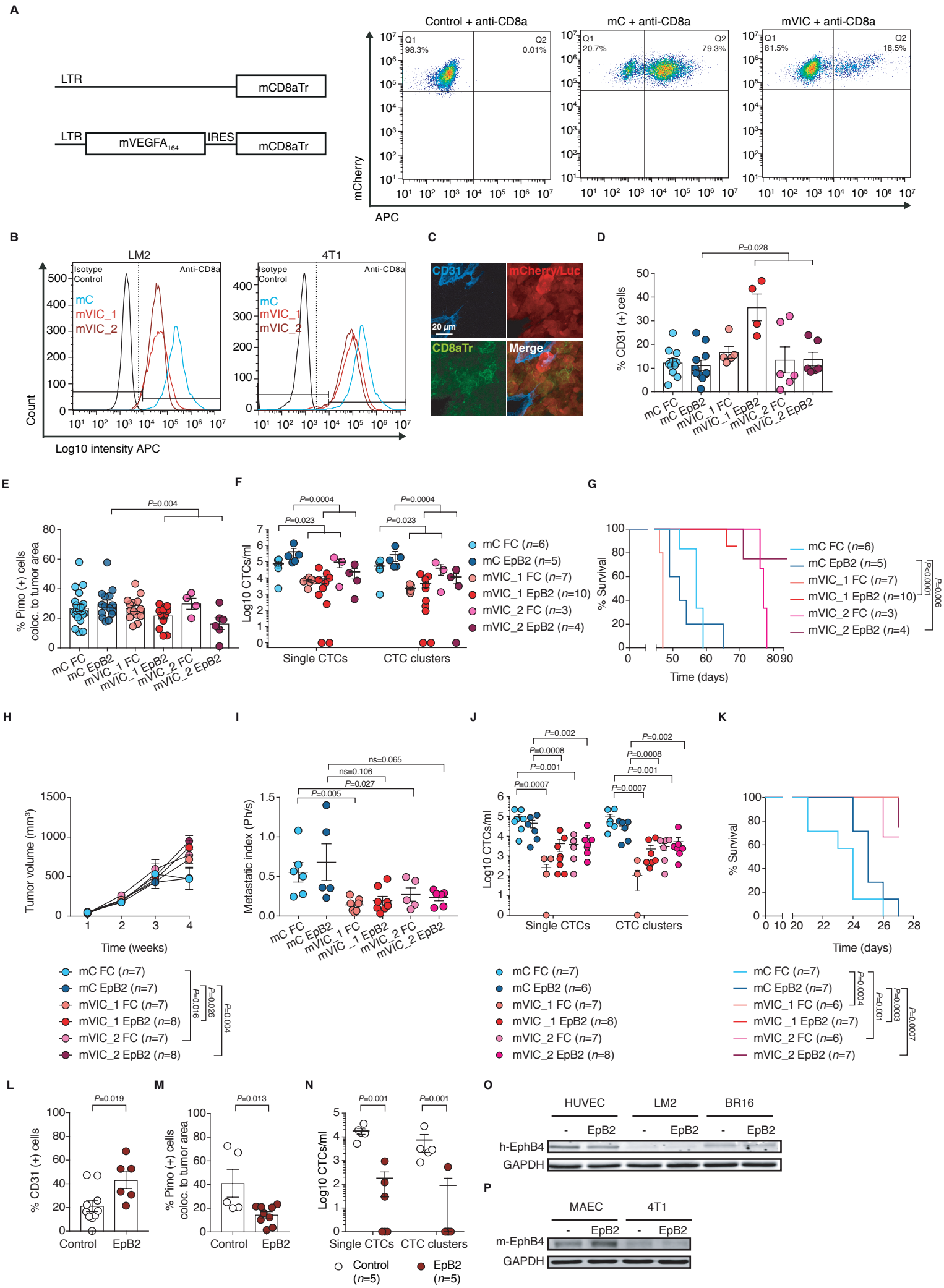

Figure S7. Donato et al.

Figure S7. Pro-angiogenic therapy reduces CTC clusters shedding and metastasis formation. Related to Figure 7.

(**A**) On the left, schematic representation of the mCD8aTr vector (mC; *top*) and the $_{m}$VEGFA$_{164}$-IRES-mCD8aTr vector (mVIC; *bottom*). On the right, representative flow cytometry analysis showing CD8a expression visualized through staining with anti-CD8a-APC antibodies in control, mC and mVIC 4T1-mCherry/Luc cells (quadrant Q2).

(**B**) Representative flow cytometry analysis showing selected mC and mVIC clones from LM2 (*left*) or 4T1 (*right*) cells expressing CD8a (*X axis*).

(**C**) Representative pictures of the *in vivo* expression of mVIC *via* staining of CD8aTr in LM2-mCherry/Luc mVIC tumors.

(**D**) The plot shows the mean percentage of CD31-positive (+) cells within the primary tumor of NSG-LM2 mice expressing mVIC or mC, treated with either control FC fragments (FC) or EphrinB2 (EpB2) (*n*=2). Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**E**) The plot shows the mean percentage of Pimo-(+) cells colocalizing within the primary tumor of NSG-LM2 mice expressing mVIC or mC, treated with either FC or EpB2 (*n*=2). Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**F**) Plot showing the Log10 of total CTC counts per ml of blood of NSG-LM2 mice expressing mVIC or mC, and treated with either FC or EpB2. The number of independent biological replicates (*n*) is shown. Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**G**) Overall survival rates of NSG-LM2 mice expressing mVIC or mC, and treated with either FC or EpB2. The number of independent biological replicates (*n*) is shown. *P* value by two-sided log-rank test is shown.

(**H**) Plots showing the mean tumor volume of NSG mice injected with 4T1 cells expressing mVIC or mC, and treated with either FC or EpB2. The number of independent biological replicates (*n*) is shown. Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**I**) The plot shows the metastatic index of NSG-4T1-mVIC or 4T1-mC mice, treated with either FC or EpB2 (*n*=5). Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**J**) Plot showing the Log10 of total CTC counts per ml of blood of NSG-4T1 mice expressing mVIC or mC, and treated with either FC or EpB2. The number of independent biological replicates (*n*) is shown. Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**K**) Overall survival rates of NSG-4T1 mice expressing mVIC or mC, and treated with either FC or EpB2. The number of independent biological replicates (*n*) is shown. *P* value by two-sided log-rank test is shown.

(**L**) The plot shows the mean percentage of CD31-(+) cells within the primary tumor of NSG-BR16-mCherry/Luc mice treated with either control FC or EpB2 (*n*=2). Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**M**) The plot shows the mean percentage of Pimo-(+) cells within the primary tumor of NSG-BR16-mCherry/Luc mice treated with either control FC or EpB2 (*n*=2). Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.

(**N**) Plots showing the Log10 of total CTCs counts per ml of blood of NSG-BR16 mice treated with either control FC or EpB2. The number of independent biological replicates (*n*) is shown. Error bars represent SEM; *P* values by two-tailed unpaired Student's *t*-test are shown.
(**O**) Representative western blot showing the expression of human EphB4 in HUVEC, LM2 and BR16 cells treated with either FC (-) or EpB2.
(**P**) Representative western blot showing the expression of mouse EphB4 in MAEC and 4T1 cells treated with either FC (-) or EpB2.