SUPPLEMENTARY FIGURES FOR

Circulating tumor cells exhibit metastatic tropism and reveal brain metastasis drivers

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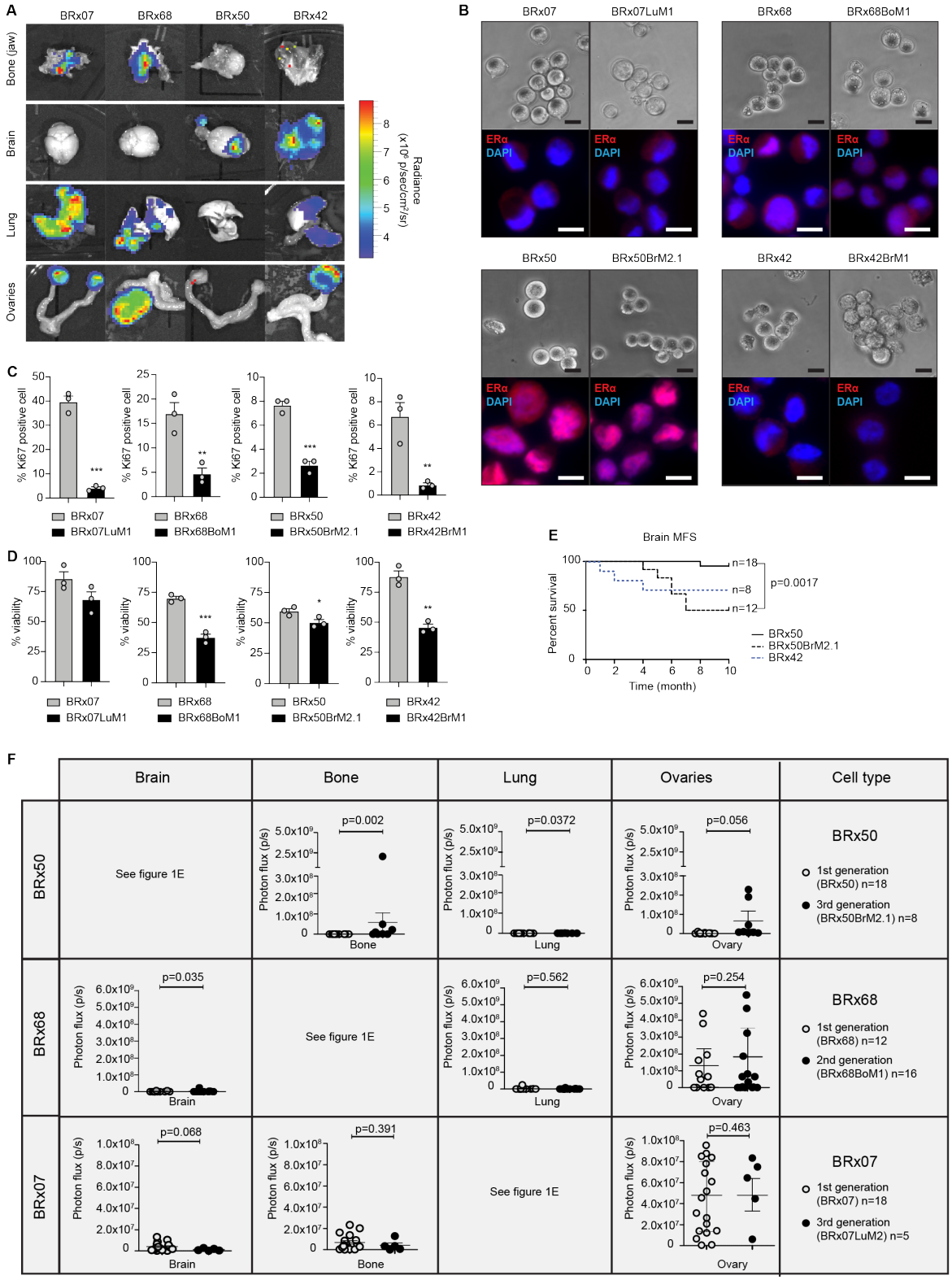
File includes supplementary figures S1 to S5

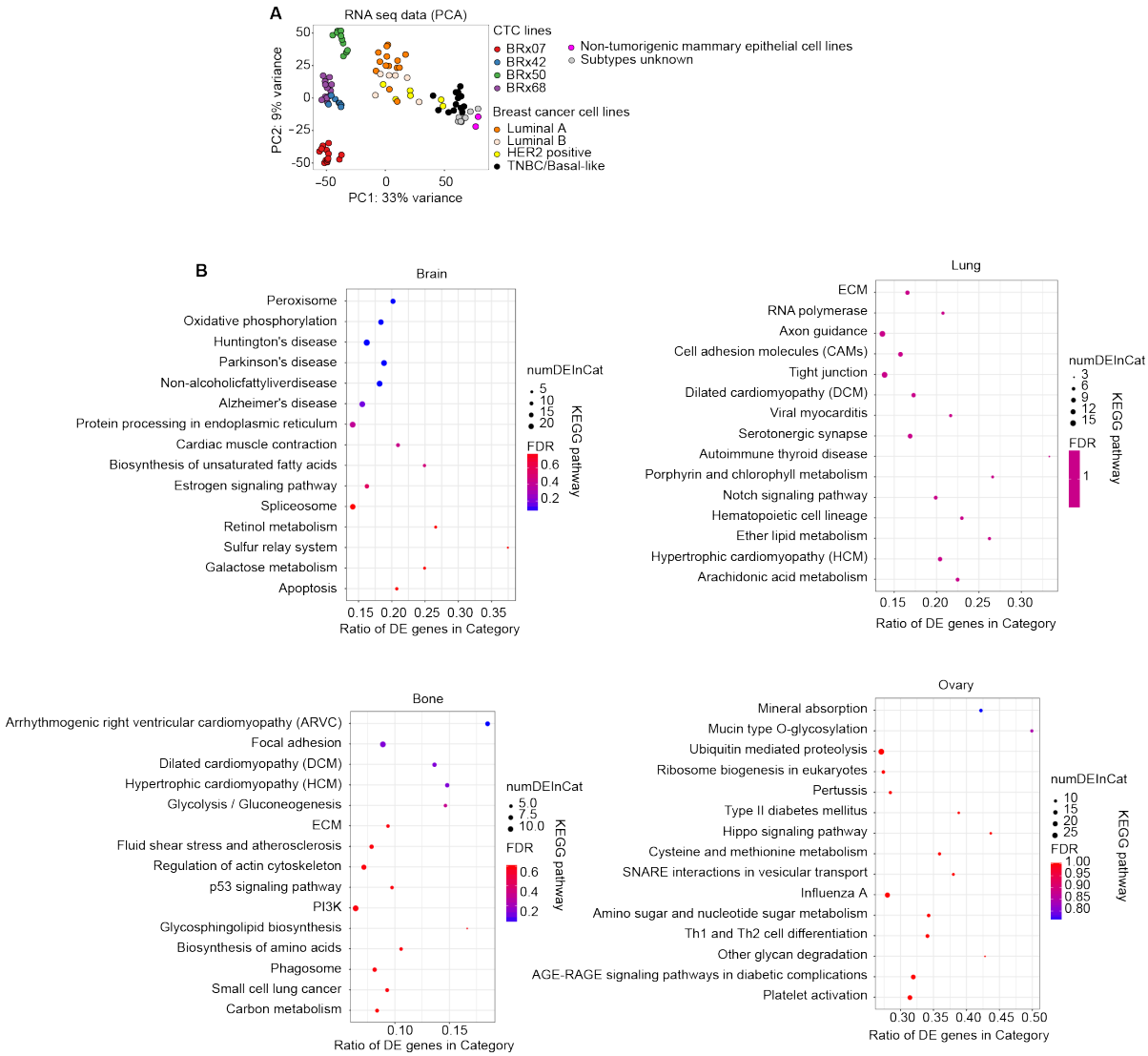
Supplementary Figure S1 (legend on next page)



Supplementary Figure S1 (legend)

Supplementary Figure S1. The assessment of metastatic potential in breast cancer patient-derived CTC lines. A, Representative *ex vivo* BLI images of bone, brain, lung and ovary after intracardiac injection with CTC lines in mice. **B**, Representative images of CTC culture. Phase contrast (top) and immunofluorescent staining for ERα (Red, Bottom). Scale bar, 10 μm. **C**, Quantification of Ki67 positive cells by immunofluorescence in CTC lines and metastatic variants with at least 3 independent fields per sample (mean ± s.e.m., two-tailed unpaired *t*-test). **D**, Histograms representing the percent of viability in CTC lines by staining with trypan blue. (mean ± s.e.m., two-tailed unpaired *t*-test). **E**, Comparison of brain MFS between first generation of mice inoculated with BRx50 or BRx42 cells and third generation of mice inoculated with BRx50BrM2.1. n = number of mice, and *P* value was determined with log-rank test. **F**, Quantification of BLI intensity in brain, bone, lung and ovary at 5 months after intracardiac inoculation of CTC or CTC-derived metastatic variants in mice. Circles represent individual mouse, horizontal lines represent the mean ± s.e.m. n = number of mice. *P* values were obtained with Wilcoxon rank sum test. * *P* value < 0.05, ** *P* value < 0.01 and *** *P* value < 0.001.

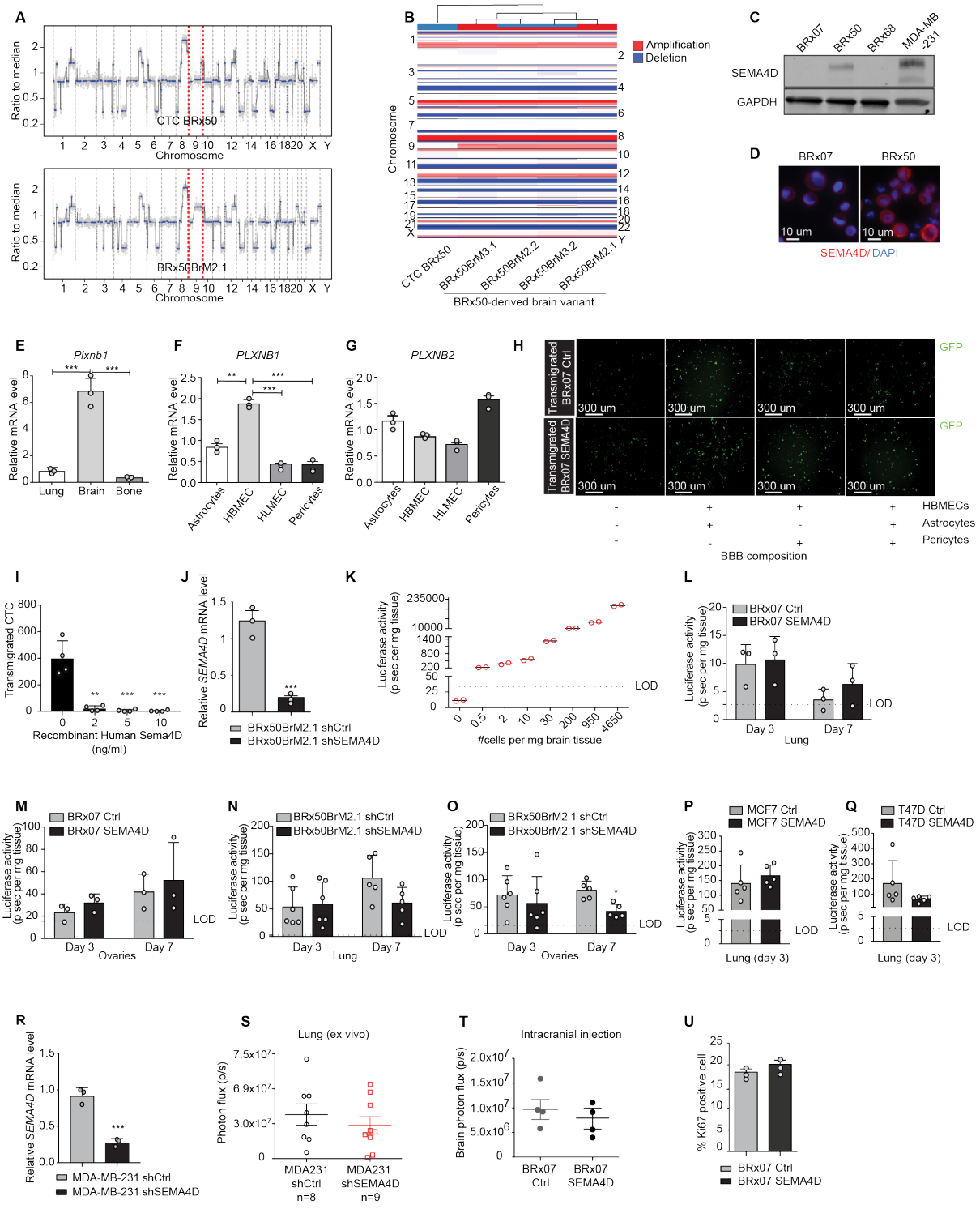
Supplementary Figure S2 (legend on next page)



Supplementary Figure S2 (legend)

Supplementary Figure S2. Molecular pathways differentially enriched in CTC-derived metastases. A, PCA of RNA-seq data in CTC lines, CTC-derived metastatic cells and publicly available breast cancer cell lines (11) (GSE48213). **B**, Pathway analyses with differentially upregulated genes (FDR≤0.05) in each organ metastasis. Shown are KEGG pathways enriched in CTC-derived brain, lung, bone and ovary metastatic cells.

Supplementary Figure S3 (legend on next page)



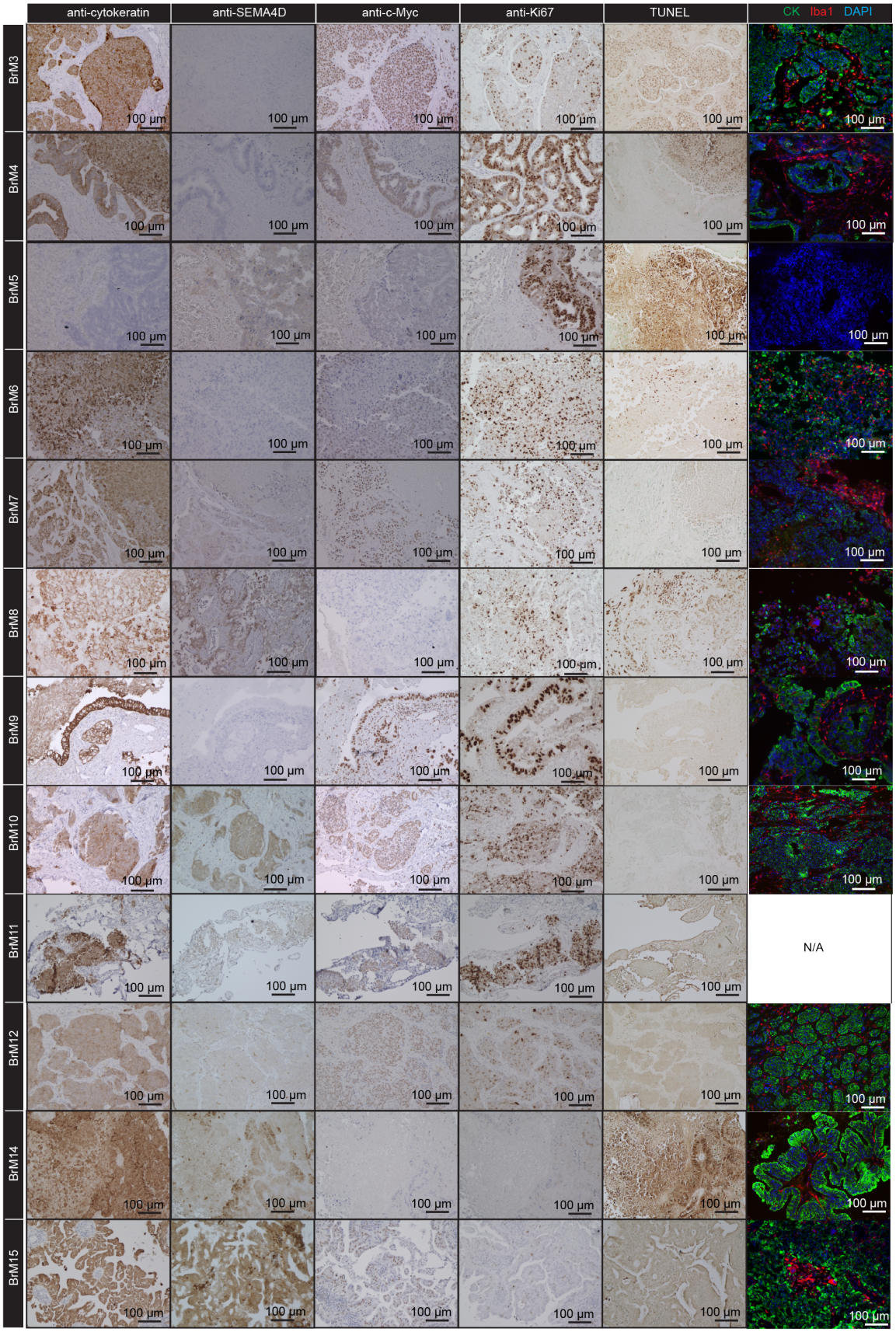
Supplementary Figure S3 (legend)

Supplementary Figure S3. **SEMA4D promotes tumor cell infiltrating the brain**. **A**, Copy number profiles of BRx50 and BRx50BrM2. Chromosome 9 is highlighted in red. **B**, Heatmap of copy number alterations for BRx50 and 4 BRx50-derived brain metastatic variants. **C**, Immunoblot analysis of SEMA4D and GAPDH levels in CTC lines and MDA-MB-231 cells. **D**, Representative fluorescent images of SEMA4D level (red) in BRx07 (non-brain tropic) and BRx50 (brain tropic). Nuclei are stained with DAPI (blue). Scale bar = 10 μm. **E**, Histogram representing mouse *Plxnb1* mRNA relative expression level in lung, brain and bone from NSG female mice (mean ± s.e.m., two-tailed unpaired *t*-test). **F-G:** Histograms representing human *PLXNB1* (**F**) and *PLXNB2* (**G**) mRNA relative expression levels in human astrocytes, HBMEC, HLMEC and pericytes (mean ± s.e.m., two-tailed unpaired *t*-test). **H**, Representative images of GFP-positive tumor cells in basolateral side of the transwell membrane. Scale bar = 300 μm. **I**, Bar graph showing quantification of transmigrated GFP-positive BRx07 SEMA4D cells through BBB pre-incubated with increasing concentration of human recombinant SEMA4D (ng/ml). Error bars represent s.e.m. *P* values were obtained with two-tailed unpaired *t*-test. **J**, Histogram representing *SEMA4D* mRNA relative expression level in BRx50BrM2.1 control (Ctrl) and BRx50BrM2.1 knockdown for SEMA4D (shSEMA4D) (mean ± s.e.m., two-tailed unpaired *t*-test). **K**, Luciferase activity measured after spiking increasing numbers of luciferase-positive CTCs in mouse brain lysate. n=2 per condition. Limit of detection (LOD) was used as threshold to detect micrometastases in brain. *Ex vivo* luciferase activity measured from mice lung or ovaries extracted at day 3 and 7 (**L-O**) or day 3 (**P-Q**) after intracardiac injection of BRx07 control and BRx07 SEMA4D (**L-M**), BRx50BrM shControl and shSEMA4D (**N-O**), MCF7 control and MCF7 SEMA4D (**P**) or T47D control and T47D SEMA4D cells (**Q**). Limit of detection (LOD) was used as threshold to detect micrometastases in each organ. Error bars represent s.e.m. *P* values were obtained with two-tailed Wilcoxon Rank Sum Test. **R**,

Supplementary Figure S3 (legend)

Histogram representing *SEMA4D* mRNA relative expression level in MDA-MB-231 control (shcontrol) and knockdown for SEMA4D (shSEMA4D) (mean ± s.e.m., two-tailed unpaired *t*-test). **S,** Quantification of BLI intensity in lung at 7 weeks after intracardiac inoculation of MDA-MB-231 control and knockdown for SEMA4D in mice. (mean ± s.e.m., two-tailed Wilcoxon Rank Sum Test.) n = number of mice. **T,** Quantification of brain *ex vivo* BLI intensity at 4 months after intracranial inoculation of BRx07 control of SEMA4D-overexpresing cells. n = 4 (mean ± s.e.m). **U**, Histogram representing percentage of Ki67 positive cells in BRx07 control and SEMA4D-overexpressing cells (n = 3 independent experiments, mean ± s.e.m.). * P value < 0.05, ** *P* value < 0.01 and *** *P* value < 0.001.

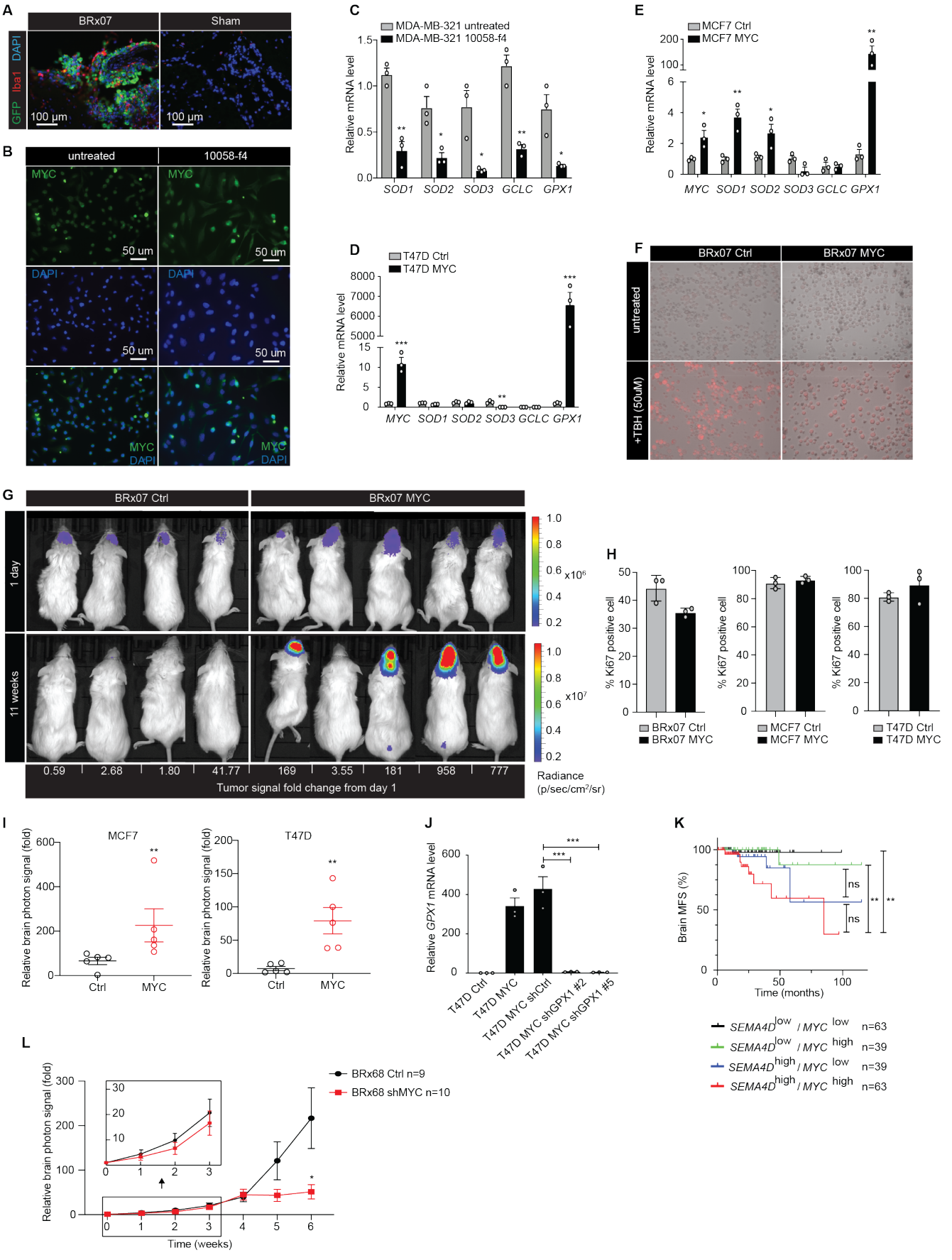
Supplementary Figure S4 (legend on next page)



Supplementary Figure S4 (legend)

Supplementary Figure S4. Characterization of a cohort of 12 brain metastasis tumor samples. Immunohistochemical staining on serial sections of cancer patient brain metastasis (BrM) for cytokeratin (CAM 5.2), SEMA4D, MYC, Ki67 and for *in situ* cell death detection (TUNEL). Immunofluorescence staining (right panel) of cytokeratin (CK) and Iba1 (red) for detection of tumor cells and activated microglia, respectively, with nuclei counterstained with DAPI (blue).

Supplementary Figure S5 (legend on next page)



Supplementary Figure S5 (legend)

Supplementary Figure S5. MYC mitigates oxidative stress and promotes tumor cell colonization in the brain. A, Immunofluorescence staining in mouse brain tissue sections, for GFP protein (human tumor cell, green), Iba1(activated microglia, red) at 4 months after intracranial inoculation with BRx07 cells (left) or sham injection (right). Nuclei are stained with DAPI (blue). Scale bar = 100 μm. **B**, Representative fluorescent images of MYC (green) in MDA-MB-231 untreated or treated with MYC inhibitor-10058-F4 (100 μM) for 24 hours. Nuclei are stained with DAPI (blue). Merged MYC and DAPI images are displayed on the bottom. **C-E**, Histogram representing *SOD1*, *SOD2*, *SOD3*, *GCLC* and *GPX1* mRNA relative expression levels in (**C**) MDA-MB-321 cells untreated and treated with 100 μM of MYC inhibitor (10058-f4), in (**D**) T47D and (**E**) MCF7 control or MYC-overexpressing cells (mean ± s.e.m., two-tailed unpaired *t*-test). **F**, Representative fluorescent images of intracellular ROS level detected by CellRox-Orange in absence or with 50 μM tert-Butyl hydroperoxide (TBH). **G**, Examples of BLI data of mice intracranially injected with BRx07 control (BRx07 Ctrl) or overexpressing MYC (BRx07 MYC) cells at 1 day (top) and 11 weeks (bottom) after inoculation. Top and bottom pictures correspond to the same mice with same order. Tumor signal fold change from day 1 is indicated for each mouse. **H**, Histogram representing percentage of Ki67 positive cells in control and MYC-overexpressing cells (n = 3 independent experiments, mean ± s.e.m.). **I**, Quantification of BLI intensity in brain at 3 weeks after intracranial inoculation of MCF7 or T47D control (Ctrl) and MYC-overexpressing cells (MYC) in mice. For each individual animal, BLI signal was normalized by signal from day 1 (mean ± s.e.m., two-tailed Wilcoxon Rank Sum Test). **J**, Histogram representing *GPX1* mRNA relative expression (mean ± s.e.m., two-tailed unpaired *t*-test). **K**, Kaplan-Meier curves showing brain metastasis free survival analysis in 204 breast cancer patients (GSE12276) based on *SEMA4D* and *MYC* expression in primary tumors. *P* values were determined with log-rank test. **L**, Quantification of BLI intensity in brain for 6 weeks after

Supplementary Figure S5 (legend)

intracranial inoculation of BRx68 control (Ctrl) and knockdown for MYC (shMYC) in mice. For each individual animal, BLI signal was normalized by signal from day 1. Black arrow shows magnified view of brain signal from week 0 to 3. (mean ± s.e.m., Two-way ANOVA) n = number of mice. * P value < 0.05, ** *P* value < 0.01 and *** *P* value < 0.001.