Supplementary figures for:

Breast cancer cells produce tenascin-C as a metastatic niche component to colonize the lungs

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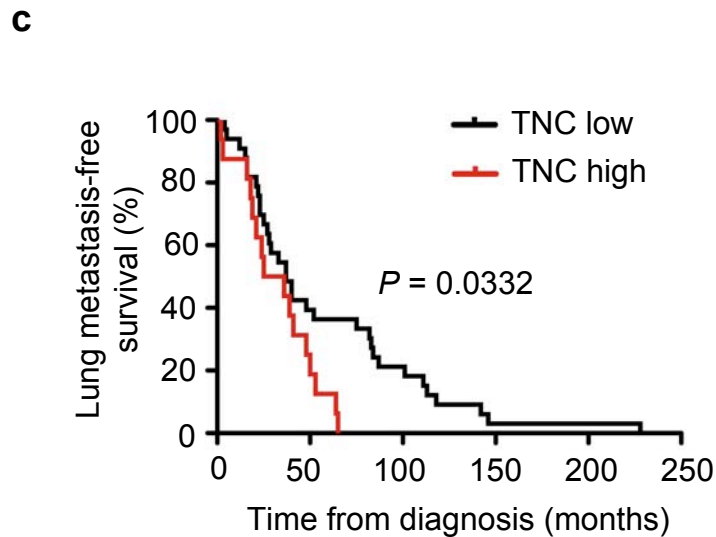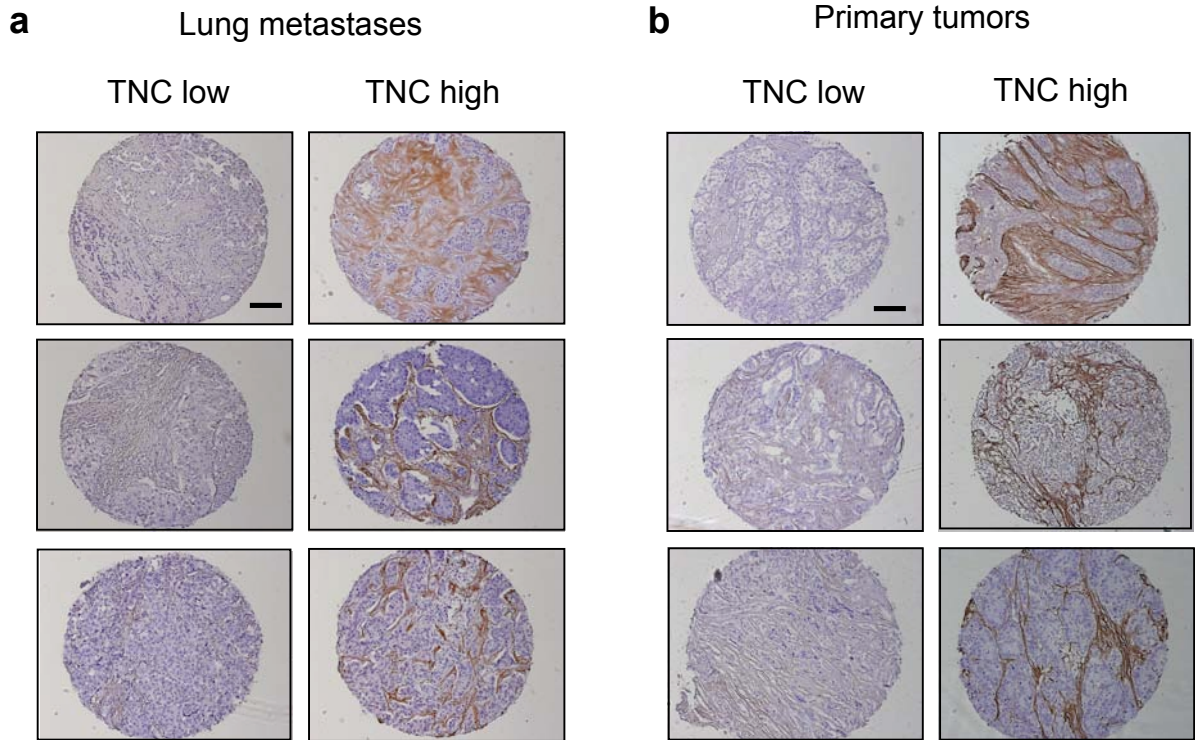
Supplementary Figure 1. TNC expression in primary tumor is associated with lung metastatic relapse. (**a**, **b**) Representative examples of tissue microarray (TMA) cores from lung metastases of breast cancer patients (**a**) and primary tumors (**b**), classified as TNC low or TNC high. Scale bar 50 µm. (**c**) Kaplan-Meier analysis of lung metastasis-free survival in patients classified according to TNC status of the primary tumor at the time of diagnosis. TNC low, n = 33; TNC high, n = 16. P value was calculated by log-rank test.
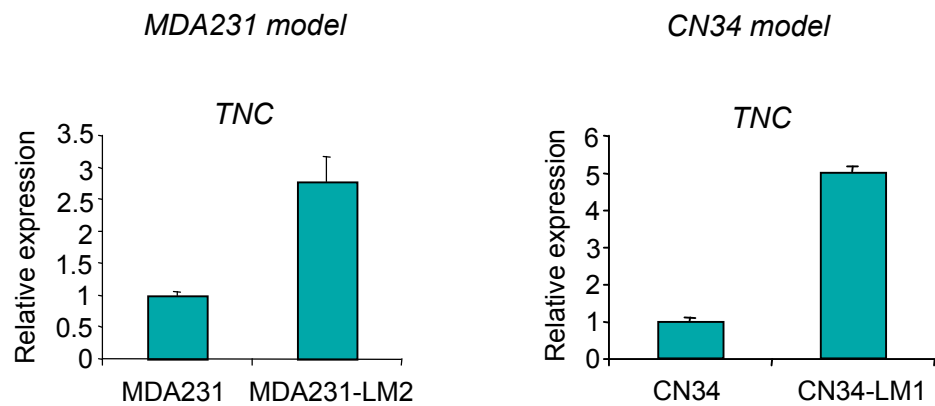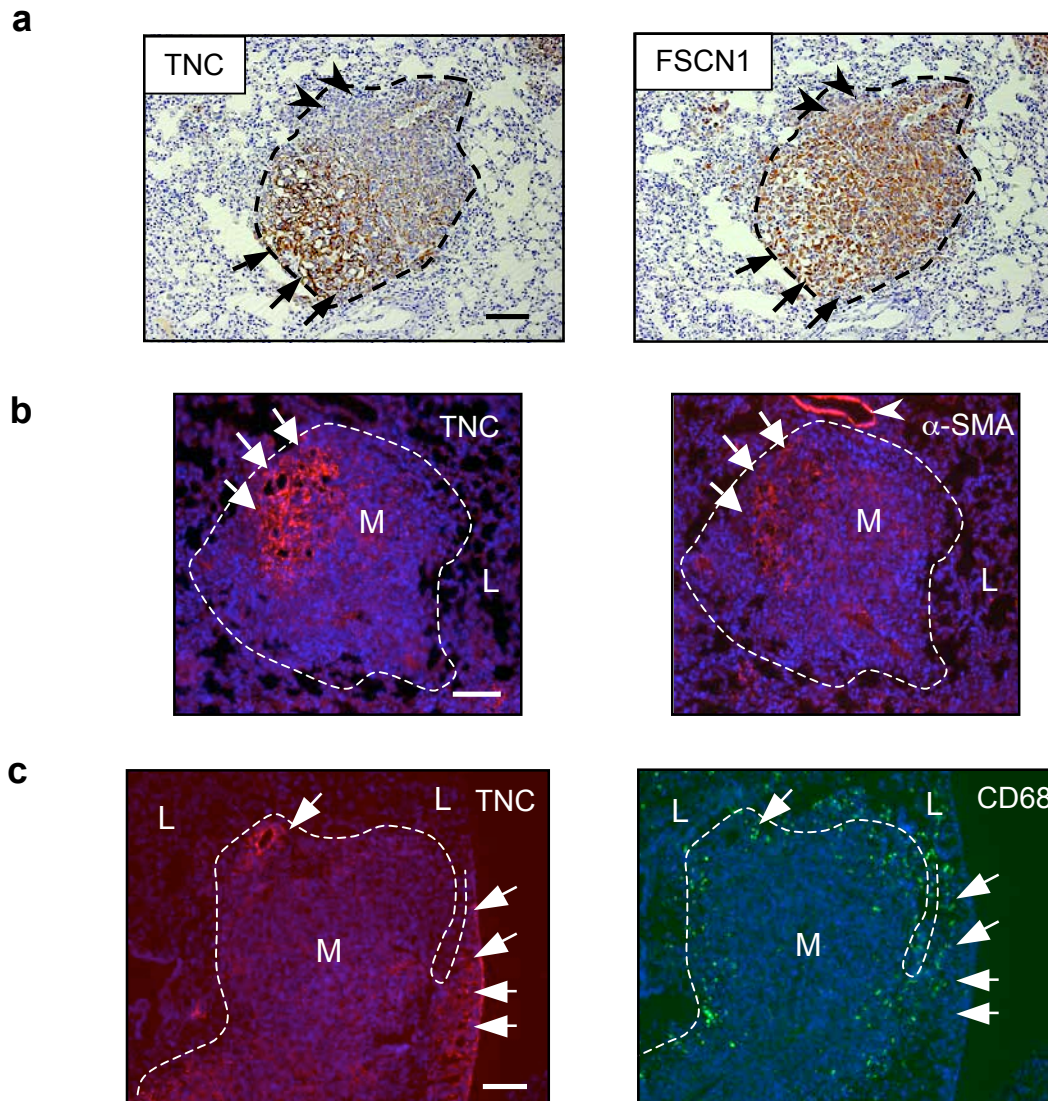
Supplementary Figure 2. Lung metastatic cell lines MDA231-LM2 and CN34-LM1 show increased TNC expression compared to their respective parental lines. Expression of TNC in MDA231-LM2 compared to the parental line MDA231 (left) and CN34-LM1 compared to CN34 (right). TNC expression in lung metastatic cell lines determined by qRT-PCR. Means from triplicates, error bars indicate standard deviation.
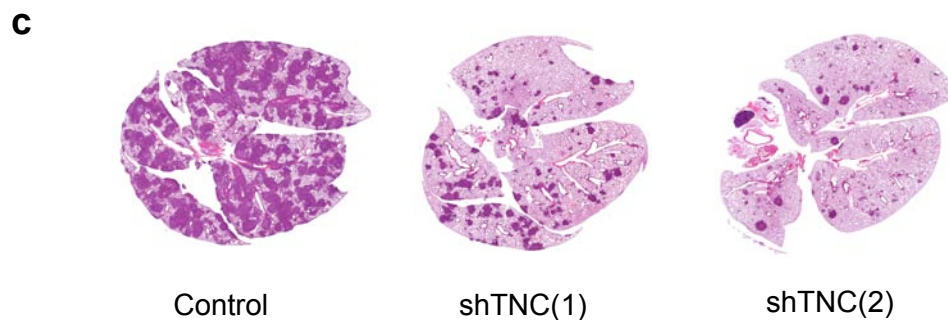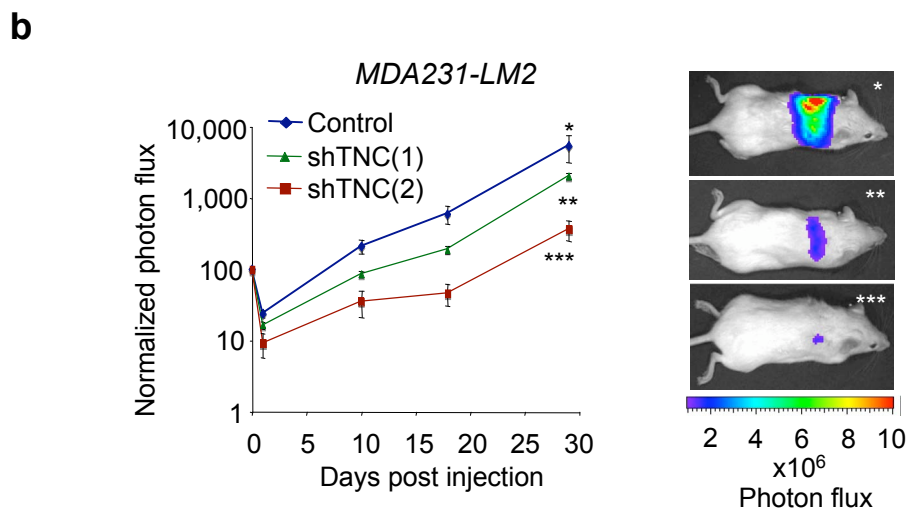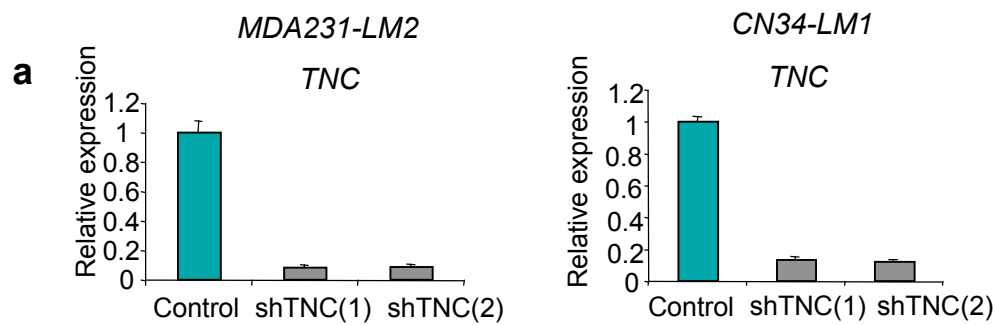
CN34-LM1
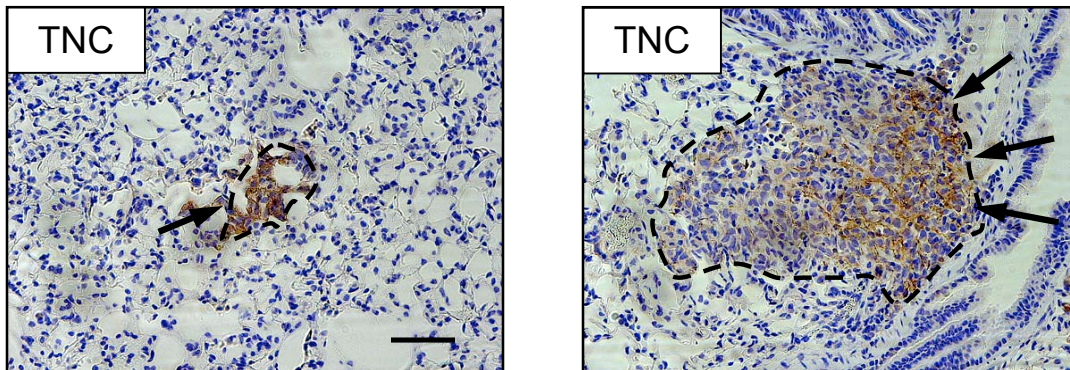
Supplementary Figure 3. TNC expression in CN34-LM1 metastatic foci in mouse lungs. Immunohistohemical analysis of TNC expression and localization (arrows) within metastatic foci derived from colonizing CN34-LM1 breast cancer cells. Micrometastasis (left) and macrometastasis (right). Scale bar 20 μm.

Supplementary Figure 4. TNC, myofibroblasts and macrophages at the invasive front of lung metastatic foci. MDA231-LM2 derived lung metastases analyzed by immunostaining (consecutive sections). (**a**) Immunohistochemical analysis showing TNC and FSCN1 expression. (**b**) Immunofluorescence analysis of TNC expression and myofibroblast marker alpha-smooth muscle actin (α-SMA). Left ,TNC: red. Right, α-SMA: red. DAPI: blue. Arrowhead, α-SMA positive vessel. (**c**) Immunofluorescence showing TNC expression (red) and presence of macrophages determined by CD68 (green). DAPI: blue.
M, metastatic foci. L, lung parenchyma. Scale bars a–c, 50 μm.

Supplementary Figure 5. TNC knockdown inhibits lung colonization of MDA231-LM2 cells. (**a**) TNC expression in MDA231-LM2 and CN34-LM1 compared to respective TNC knockdown lines. (**b**) Kinetics of lung colonization by control and shTNC MDA231-LM2 cells determined by normalized lung luminescence (means ± SEM). Shown are representative luminescence images, one for each group indicated by asterisks. (**c**) Representative H&E stained lung sections from mice injected with control and shTNC MDA231-LM2 cells.

a Migration

b Matrigel invasion
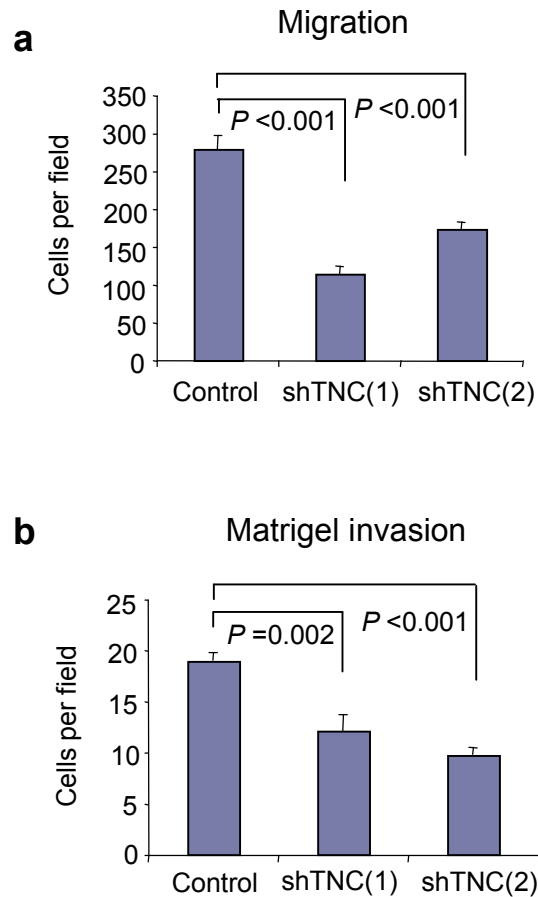
Supplementary Figure 6. Invasion and migratory activities are reduced upon TNC depletion. (**a**) Migration of control and TNC-depleted MDA231-LM2 cells through transwell membranes (3 μm pore size). Histogram shows average cell number migrating through the filter in 6 h. (**b**) Matrigel invasion assay of TNC knockdown MDA231-LM2 cells. Graph shows averages of invading cells after 24 h period. Means for a and b are from triplicates, error bars SEM. *P* values were obtained by two tailed Student's t test.

a
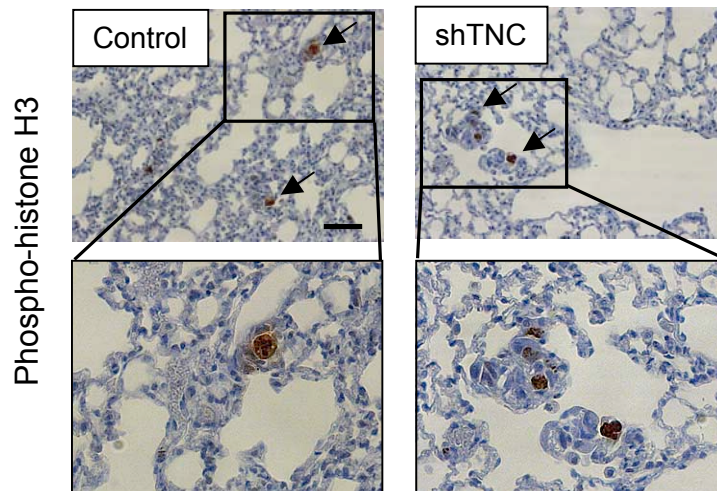
b
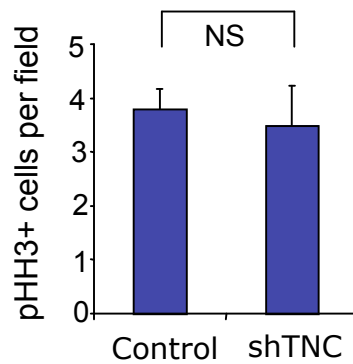
Supplementary Figure 7. Cell proliferation in TNC knockdown lung metastatic foci. (**a**) Proliferation in MDA231-LM2 lung metastatic foci, determined by immunostaining of phospho-histone H3. Scale bar 20 μm. (**b**) Quantification of phospho-histone H3 positive cells per field in control and TNC knockdown lung foci. The graph shows averages from three mice for each condition. Error bars depict SEM. Statistical significance was determined by two tailed Student's t test.
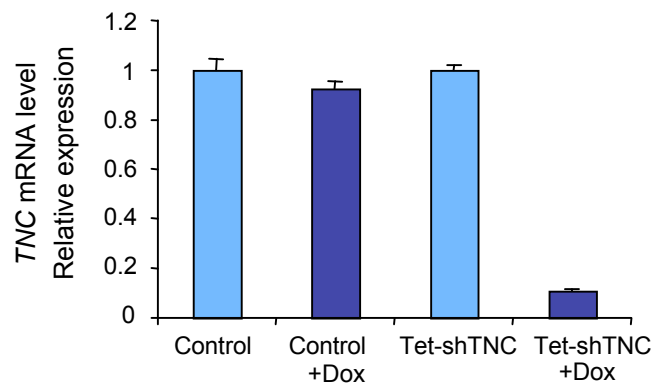
Supplementary Figure 8. Conditional TNC knockdown. TNC expression determined by qRT-PCR in doxycycline inducible knockdown MDA231-LM2 cells. Knockdown efficiency, in vitro, after 72 h incubation with doxycycline.
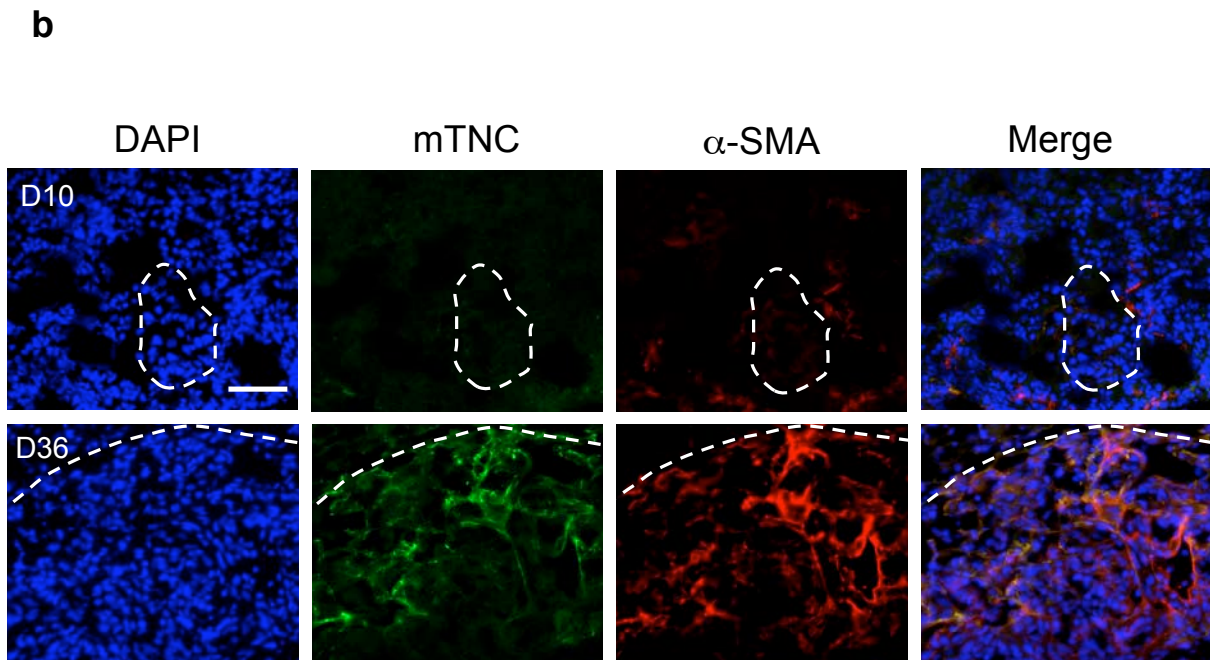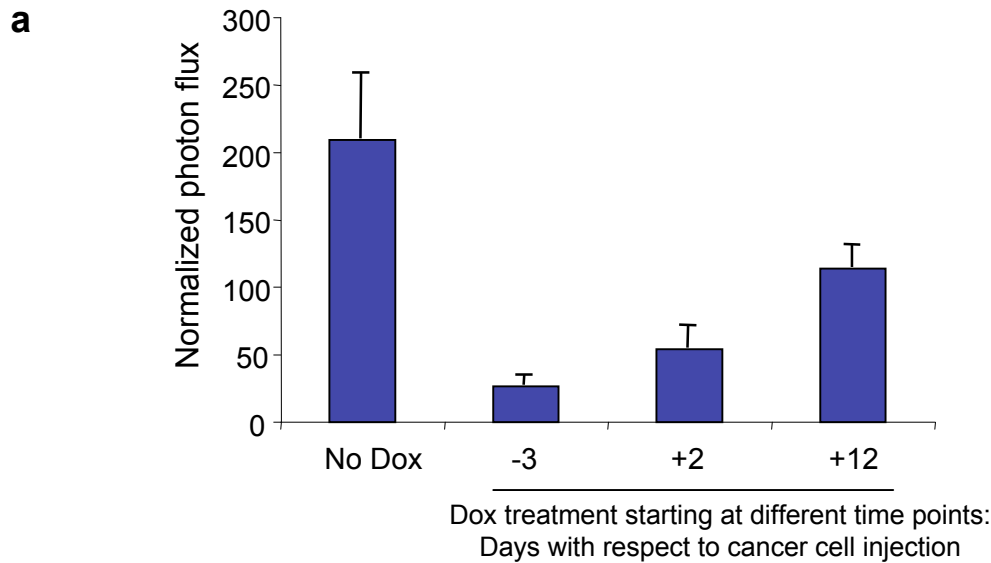
**Supplementary Figure 9. TNC compensation by myofibroblasts in late metastatic foci.**
(**a**) Lung luminescence two weeks after mice were injected with MDA231-LM2 Tet-shTNC. The TNC knockdown was induced at different time points as indicated. (**b**) Immunofluorescence analysis of lung metastatic foci collected at day 10 or day 36 after tail vein injection of cancer cells. Sections were analyzed for mouse TNC expression (mTNC) and α-SMA, a marker of myofibroblasts. DAPI was used to stain nuclei. Scale bar 20 μm.
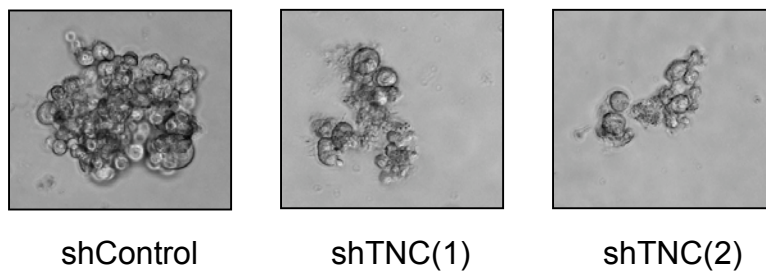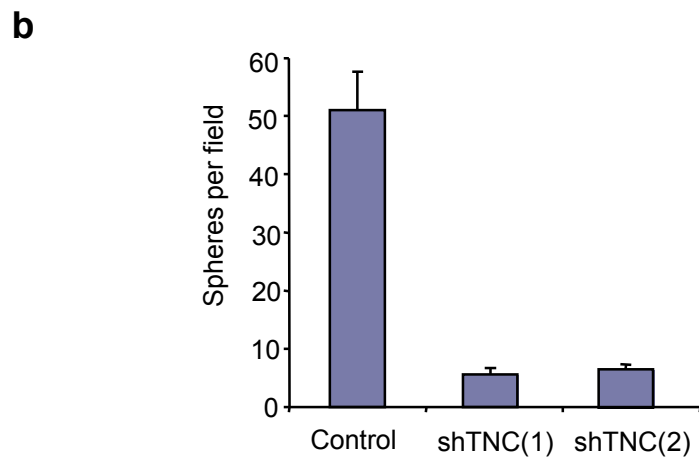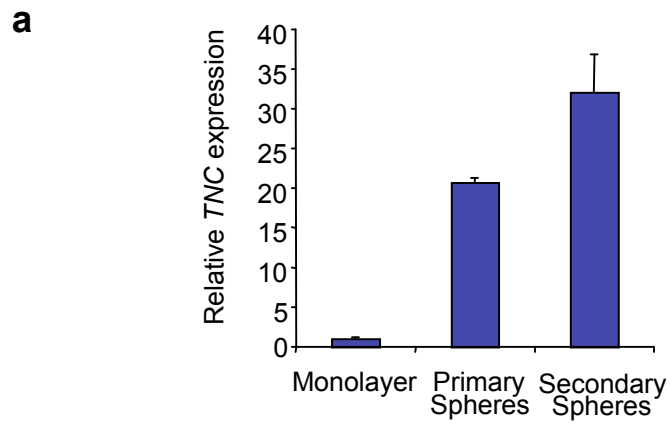
Supplementary Figure 10. TNC in oncospheres. (**a**) TNC expression in primary and secondary oncospheres derived from pleural effusion sample CN109. (**b**) Analysis of primary oncospheres formation in TNC deficient CN34-LM1 cells cultivated in the absence of supplemental factors. Quantitation (top) and representative examples of spheres (bottom).
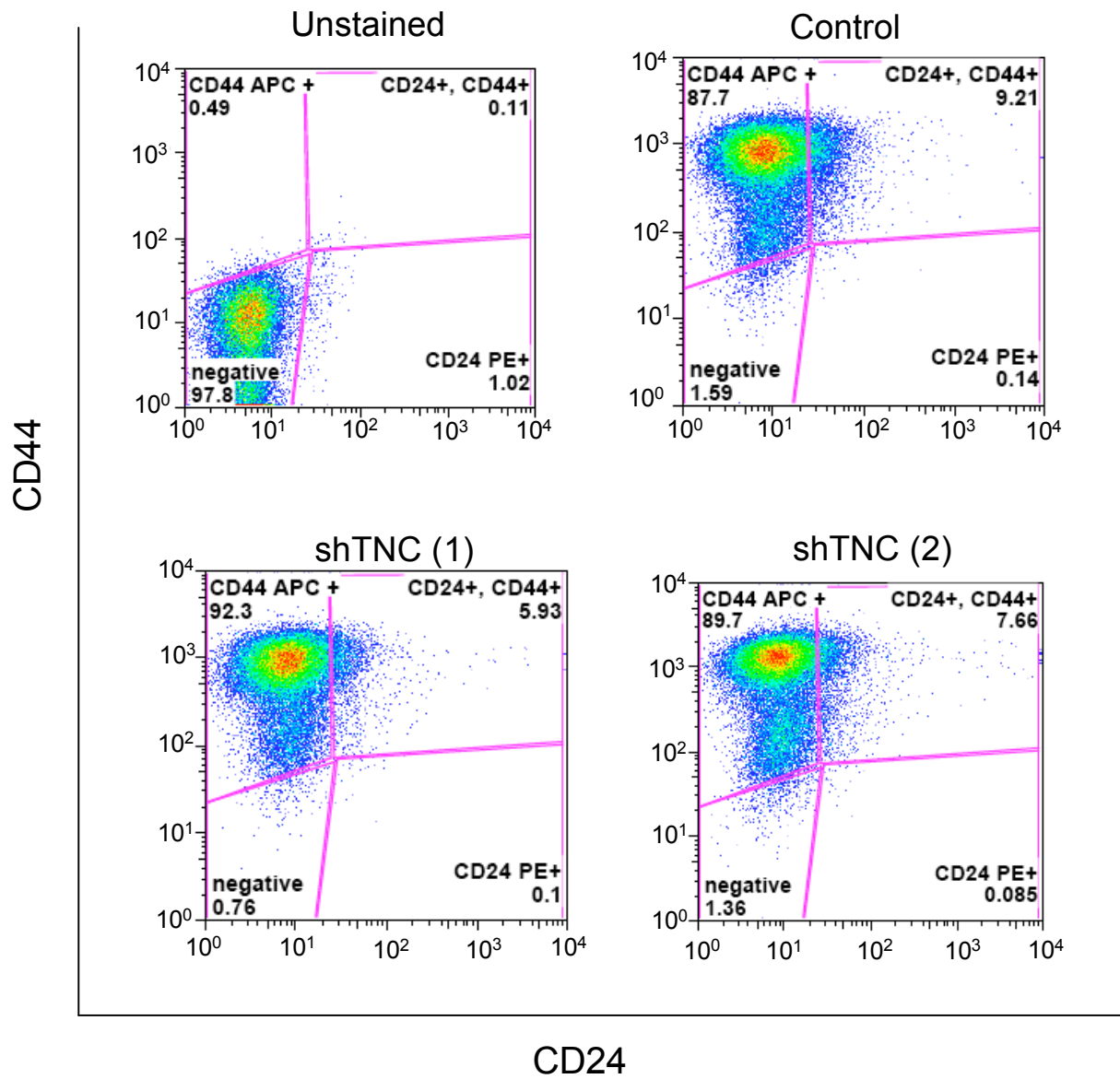
Supplementary Figure 11. TNC deficiency does not affect CD44⁺CD24⁻ population.
FACS analysis showing CD44 and CD24 expression in CN34-LM1 control and TNC knockdown oncospheres.
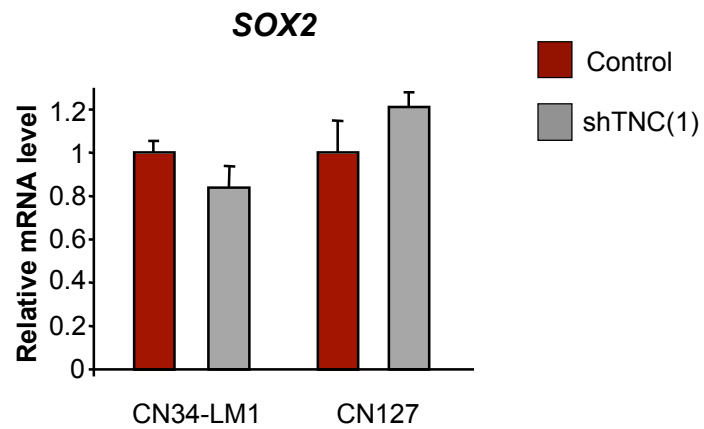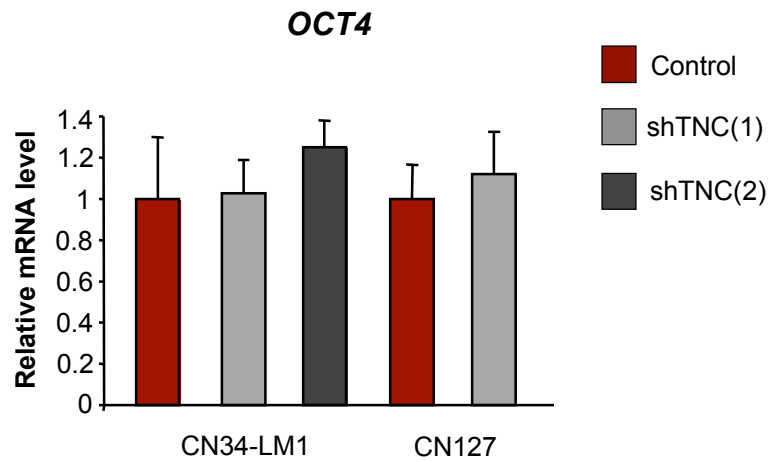
Supplementary Figure 12. Expression of *OCT4* and *SOX2* in TNC deficient oncospheres. Expression of *OCT4* and *SOX2* in oncospheres derived from TNC depleted CN34-LM1 and CN127 breast cancer cells. mRNA was quantified by qRT-PCR; values are mean ± s.d. of triplicate experiments.
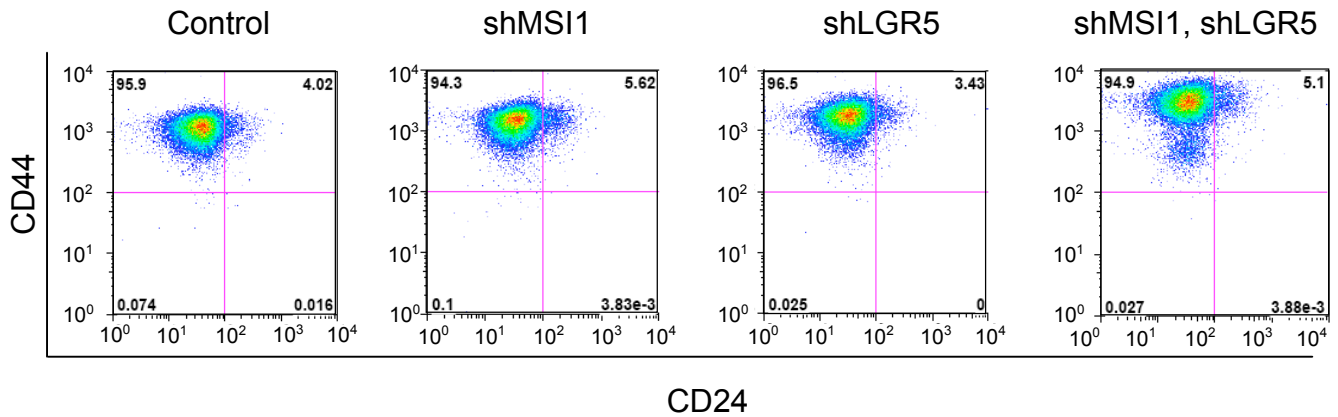
MDA231-LM2

a

b

CN34-LM1

c
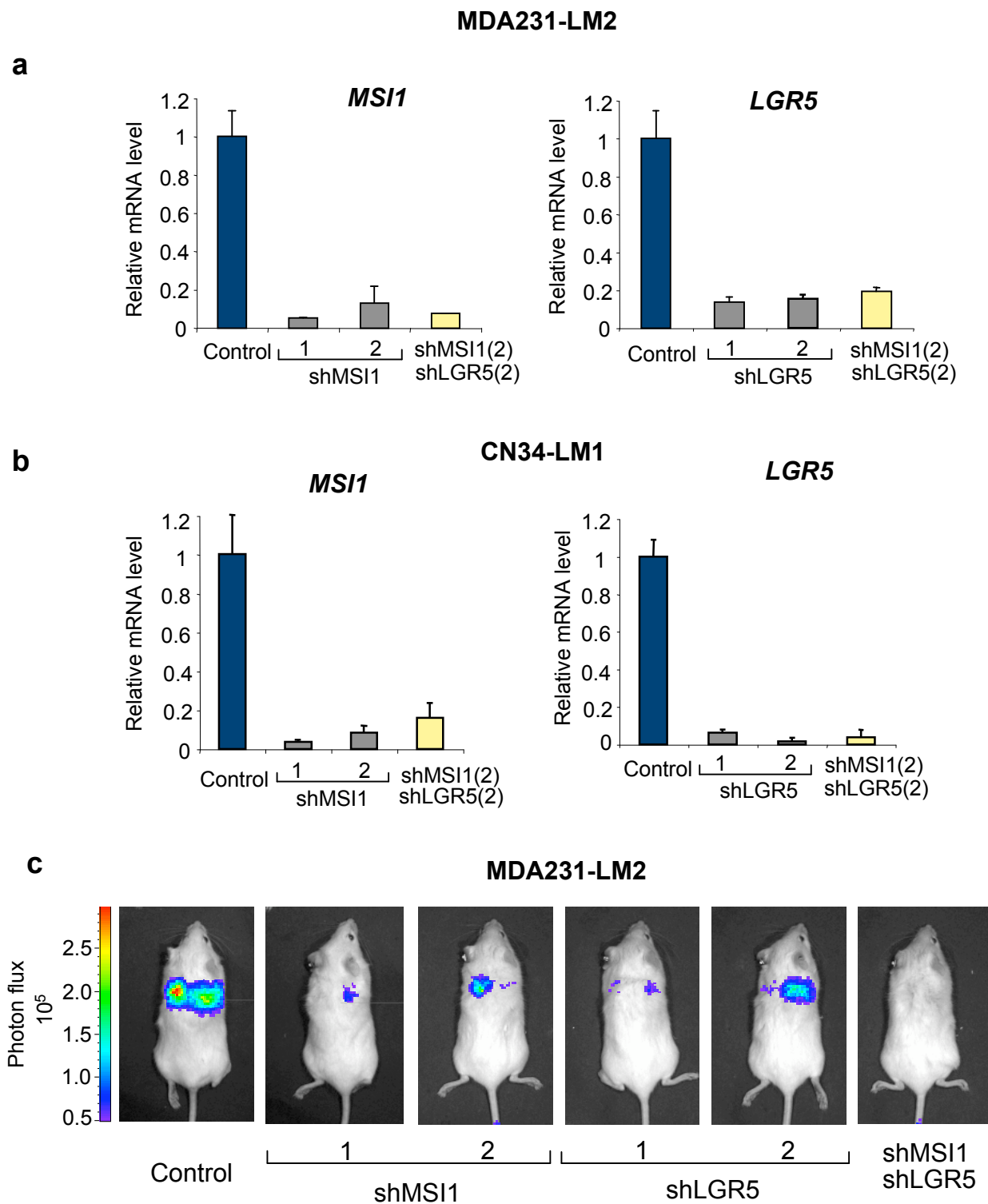
MDA231-LM2

Supplementary Figure 13. MSI1 and LGR5 knockdown recapitulates TNC deficiency.
(**a–b**) Knockdown analysis of MSI1 and LGR5 individually and together in MDA231-LM2
cells (**a**) and CN34-LM1 cells (**b**). (**c**) Representative luminescence images of mice
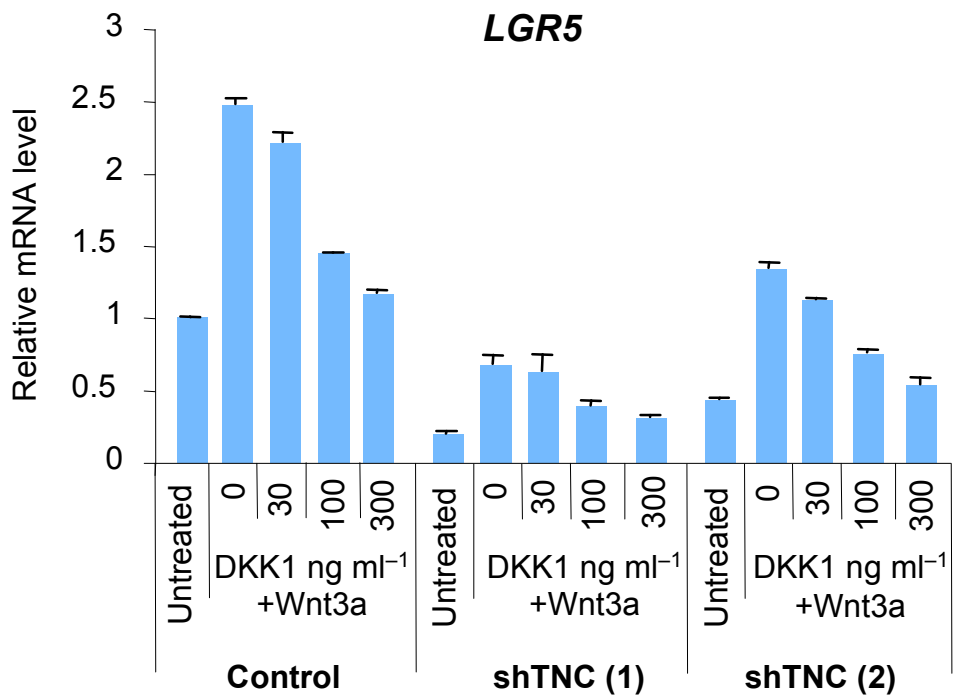injected with MDA231-LM2 control, shMSI1, shLGR5 and double knockdown cells.

MDA231-LM2
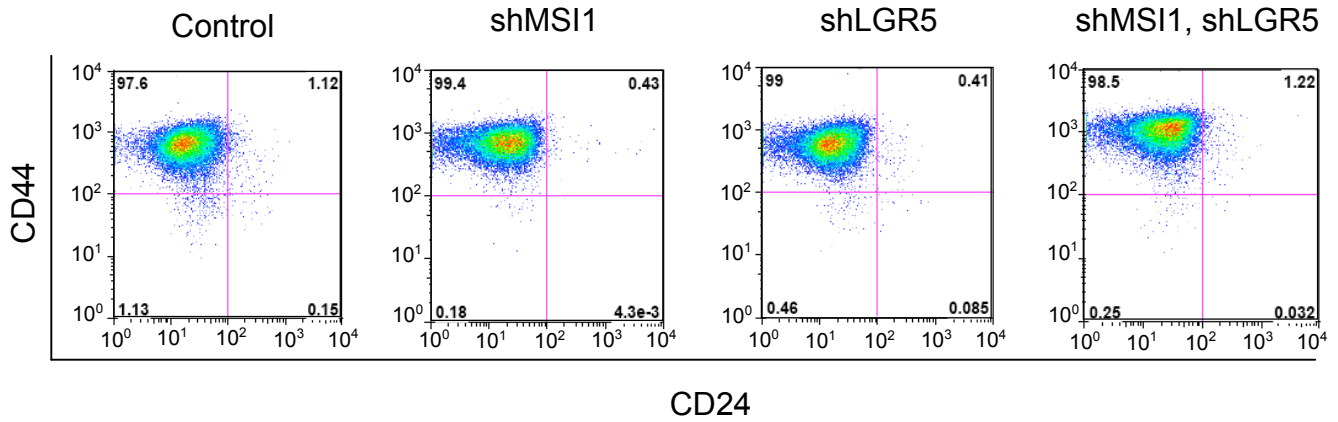
CN34-LM1

Supplementary Figure 14. CD44⁺CD24⁻ population in MSI1 and LGR5 knockdown breast cancer cells. FACS analysis showing CD44⁺CD24⁻ population in MDA231-LM2 and CN34-LM1 cells transduced with shMSI1, shLGR5 individually and in combination.
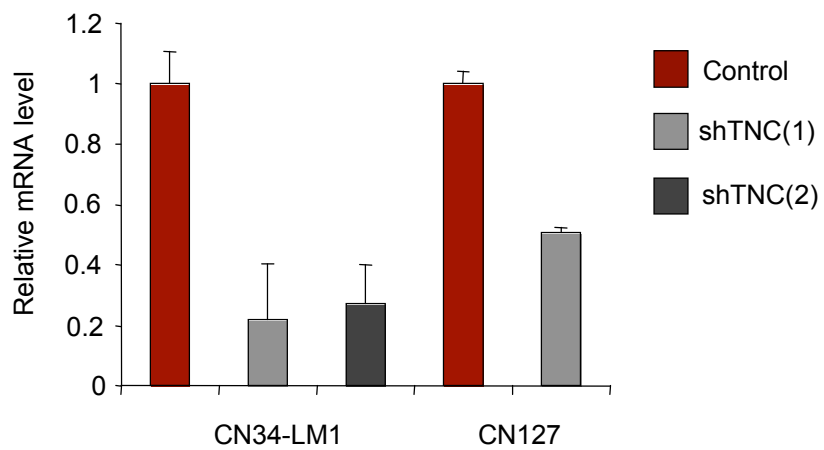
Supplementary Figure 15. *LGR5* **expression in response to Wnt3a and DKK1.** *LGR5* mRNA analysis in CN34-LM1 cells treated with Wnt3a (50 ng ml^{-1}) and incremental concentrations of Wnt antagonist DKK1 (0–300 ng ml^{-1}).
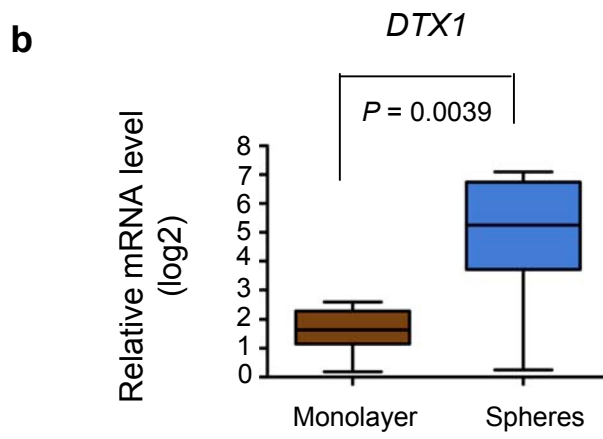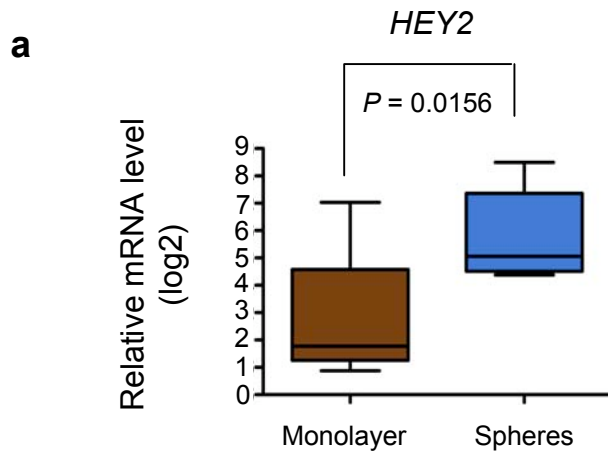
Supplementary Figure 16. NOTCH target gene expression in oncospheres.
(**a-b**) Expression of NOTCH target genes *HEY2* (**a**) and *DTX1* (**b**) in oncospheres derived from pleural effusion samples. *HEY2 n* = 8, *DTX1 n* = 10. *P* values were determined by paired Wilcoxon signed rank test (two tailed).
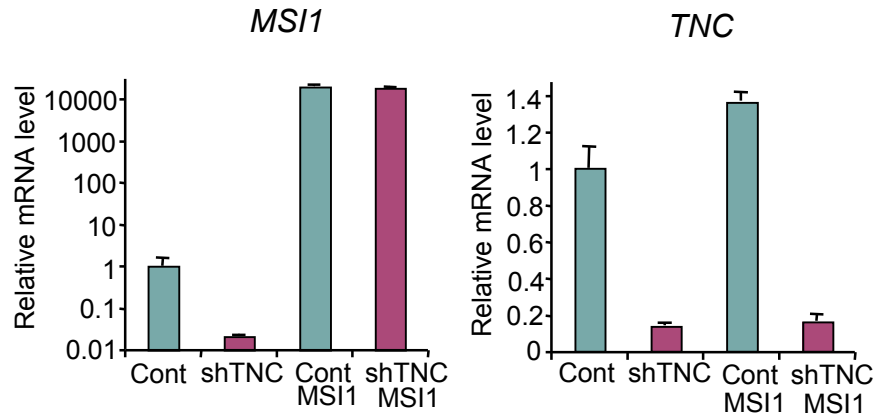
Supplementary Figure 17. *DTX1* **expression in oncospheres.**
DTX1 expression determined by qRT-PCR in TNC knockdown
oncospheres. Knockdown spheres were derived from CN34-LM1
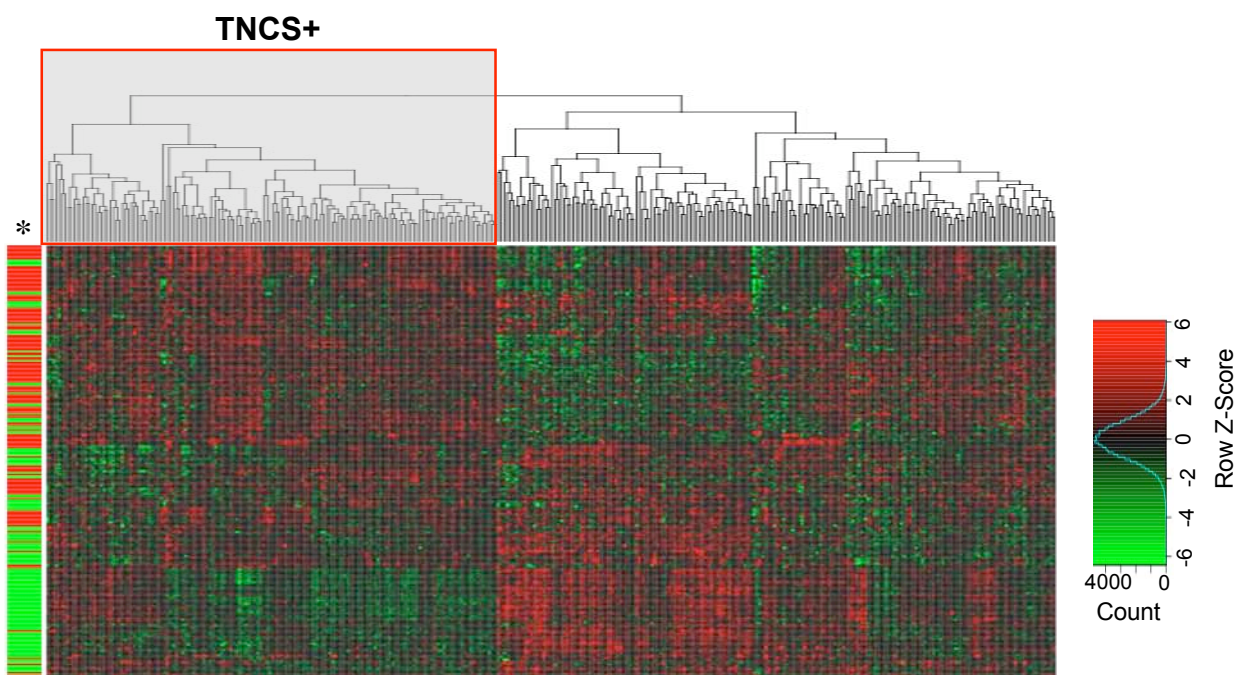and CN127 breast cancer cells.

MDA231-LM2
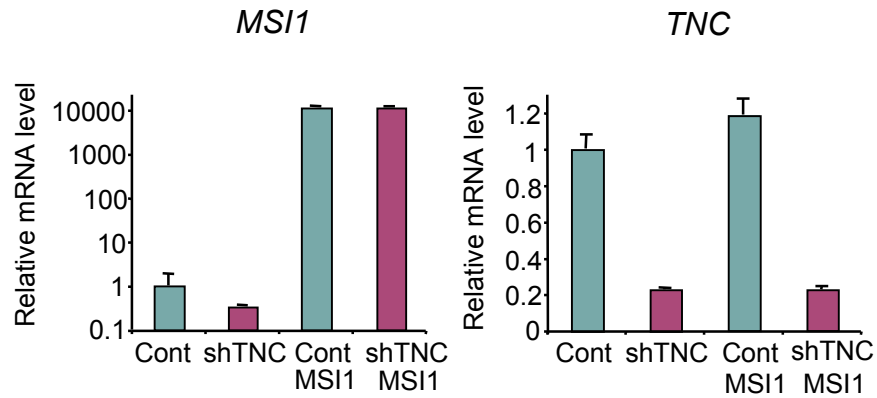
CN34-LM1

Supplementary Figure 18. *MSI1* **and** *TNC* **expression in MSI1 rescue cells.** qRT-PCR analysis of *MSI1* and *TNC* mRNA in MDA231-LM2 and CN34-LM1 cells (control and shTNC) transduced with *MSI1* expressing retroviral vector.

Supplementary Figure 19. Primary breast tumors classified according to TNC response signature. Unsupervised hierarchical clustering of 344 primary breast tumors based on 300 probes that constitute the TNC signature (TNCS, See Methods). The directionality of the expression of these probes in the TNCS is indicated by red (upregulated) and green (downregulated) bars in the heat map on the left (*). Tumors defined as TNCS+ are in a red box.
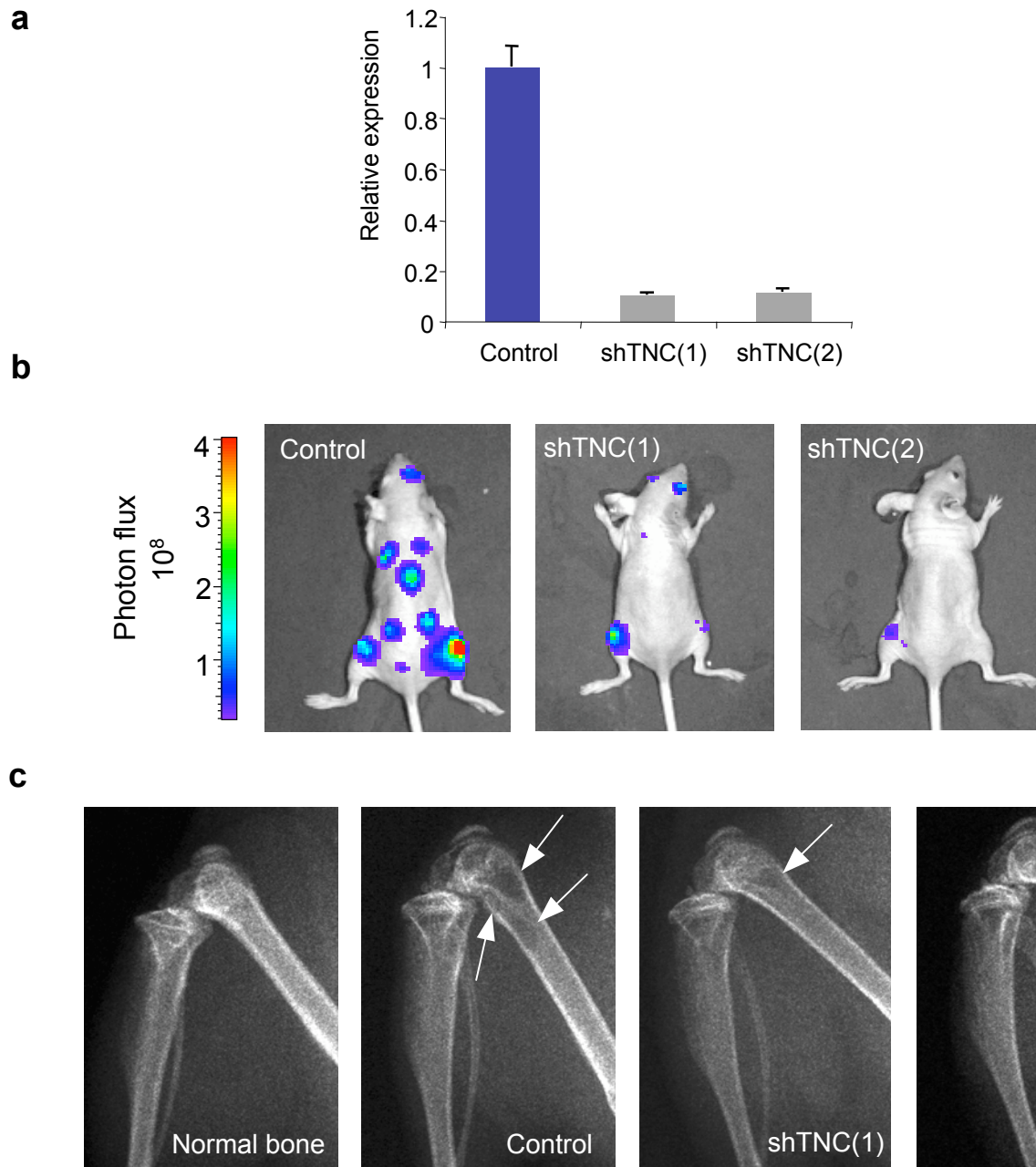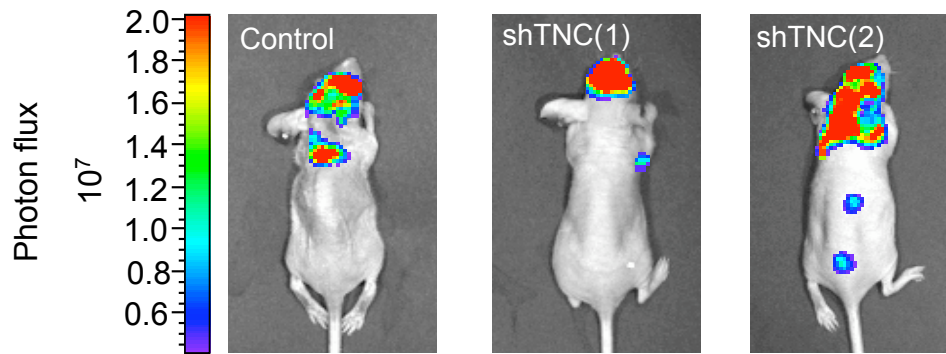
Supplementary Figure 20. TNC is required for exprerimental bone metastasis.
(**a**) TNC knockdown in MDA231-BoM1 cells. (**b-c**) Representative images of bioluminescence (**b**) and X-Ray analysis (**c**).
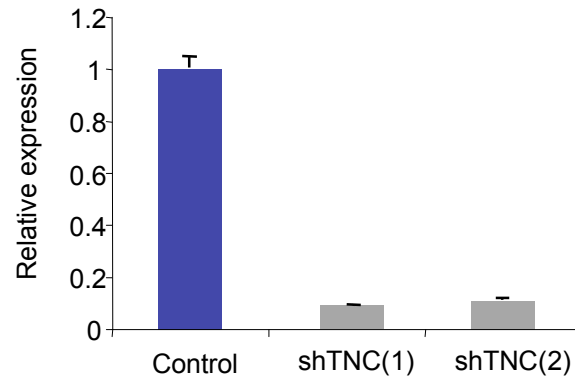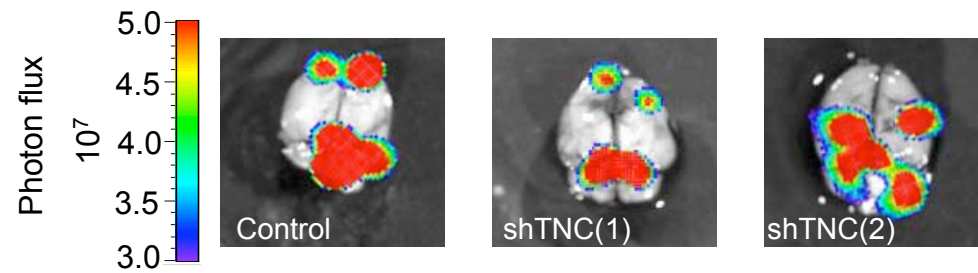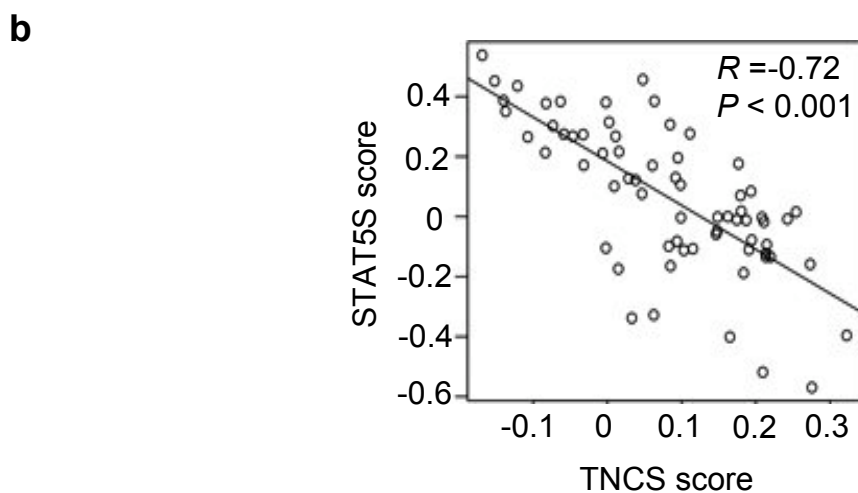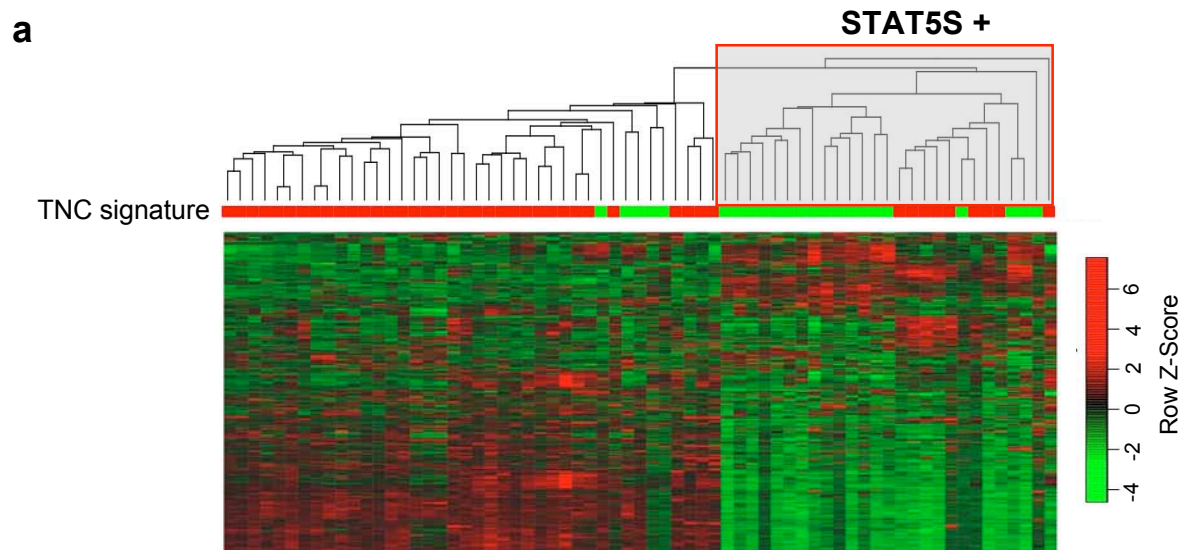
Supplementary Figure 21. TNC deficiency does not affect brain metastasis.
(**a**) TNC knockdown in MDA231-BrM2 cells. (**b-c**) Representative images of *in vivo* luminescence (**b**) and *ex vivo* brain luminescence (**c**).

Supplementary Figure 22. Human metastases classified according to STAT5 signature. (**a**) 67 human metastases classified according to STAT5 signature, using unsupervised hierarchical clustering. Status of TNCS is depicted by a colored bar above the STAT5 heatmap, red:TNCS positive and green: TNCS negative. (**b**) Correlation analysis of the 67 metastases samples according to their STAT5S score and TNCS score. *P* value was acquired by Student's t test.