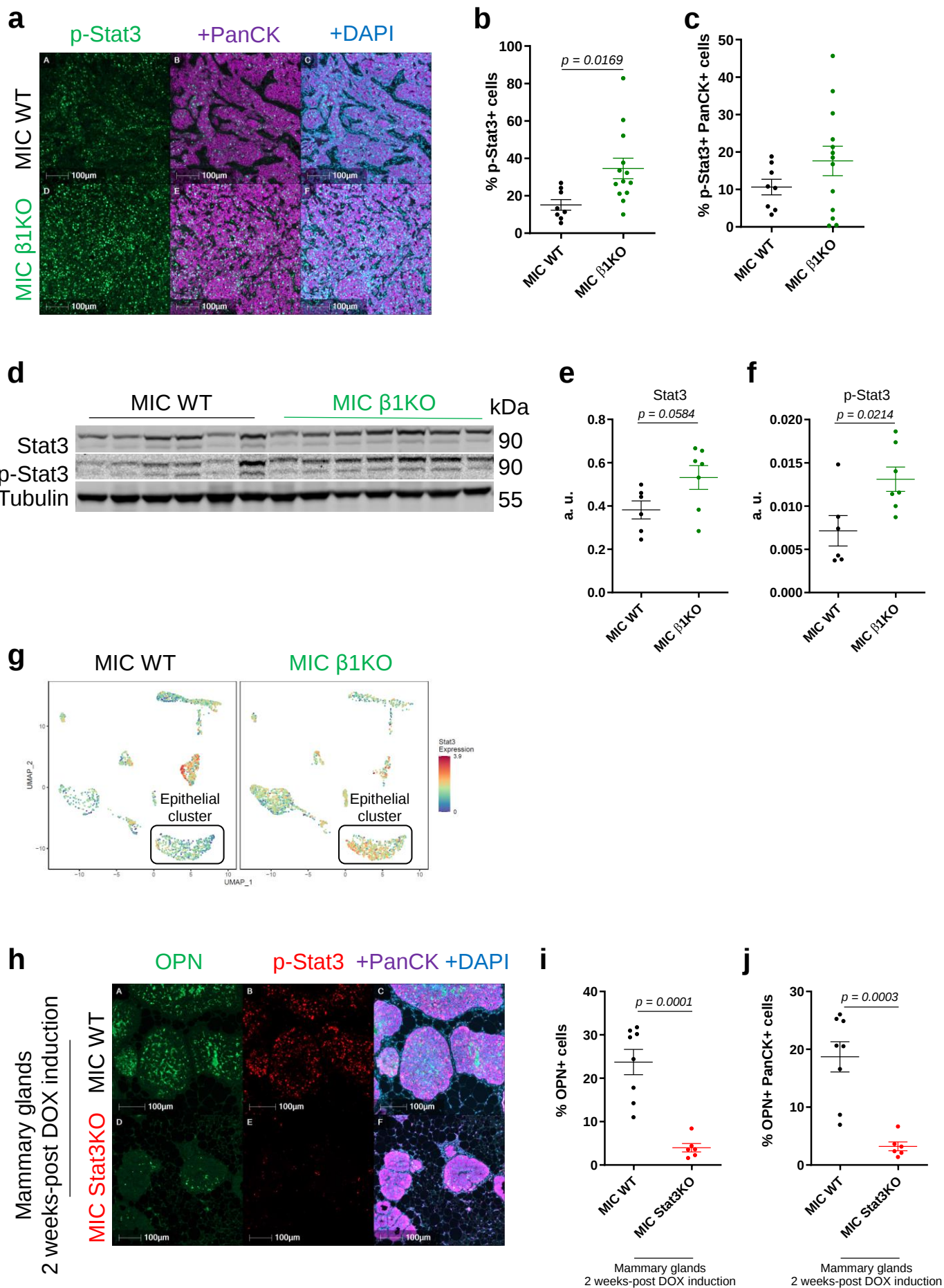


Supplementary Information

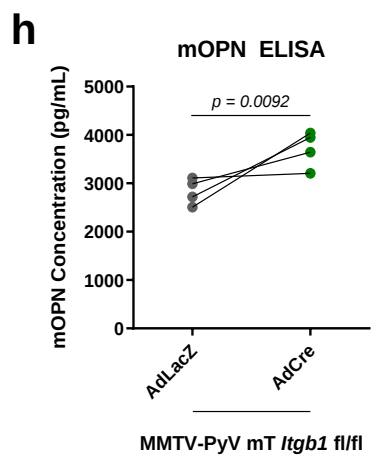
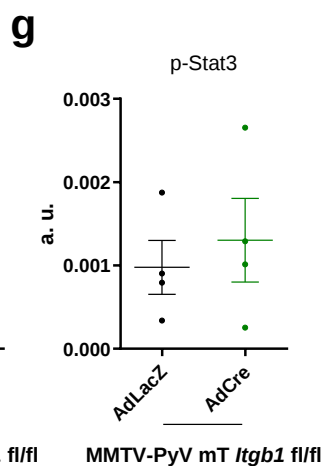
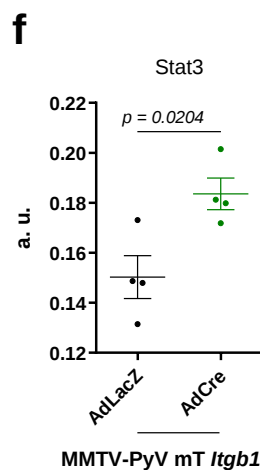
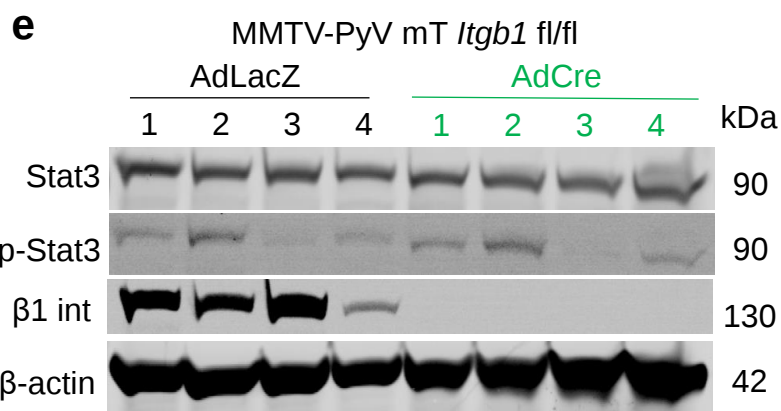
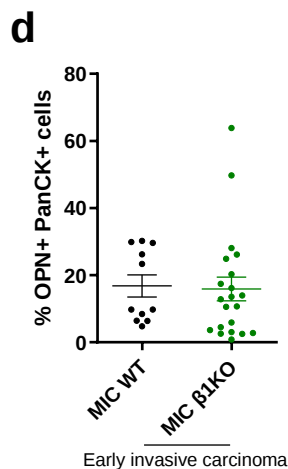
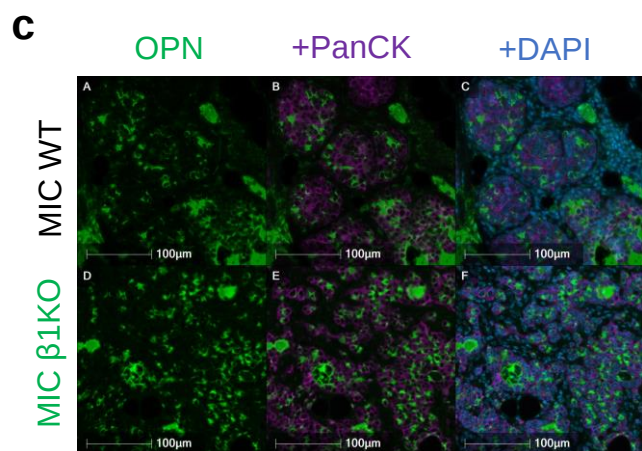
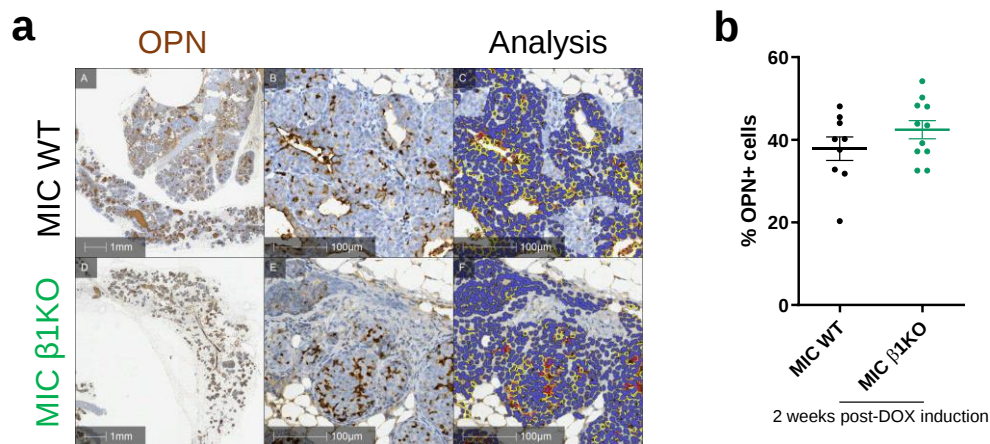
Title: Osteopontin is a therapeutic target that drives breast cancer recurrence

Yu Gu ^{1, 2}, Tarek Taifour ^{1, 3}, Tung Bui ^{1, 2}, Dongmei Zuo ¹, Alain Pacis ^{1, 4}, Alexandre Poirier ^{1, 3}, Sherif Attalla ¹, Anne-Marie Fortier ¹, Virginie Sanguin-Gendreau ¹, Tien-Chi Pan ⁵, Vasilios Papavasiliou ¹, Nancy U Lin ⁶, Melissa E Hughes ⁶, Kalie Smith ⁶, Morag Park ^{1, 2, 7}, Michel L. Tremblay ^{1, 2, 3, 7}, Lewis A. Chodosh ⁵, Rinath Jeselsohn ⁶ and William J. Muller ^{1, 2, 7, *}



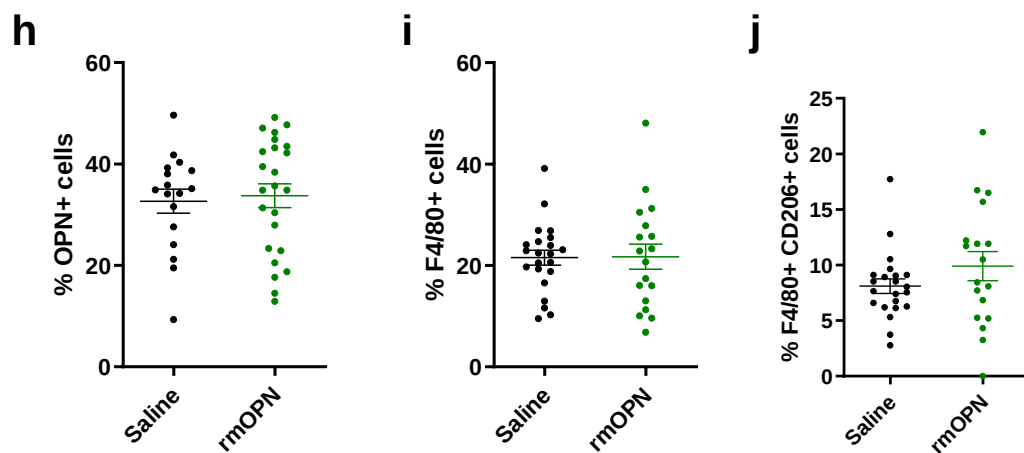
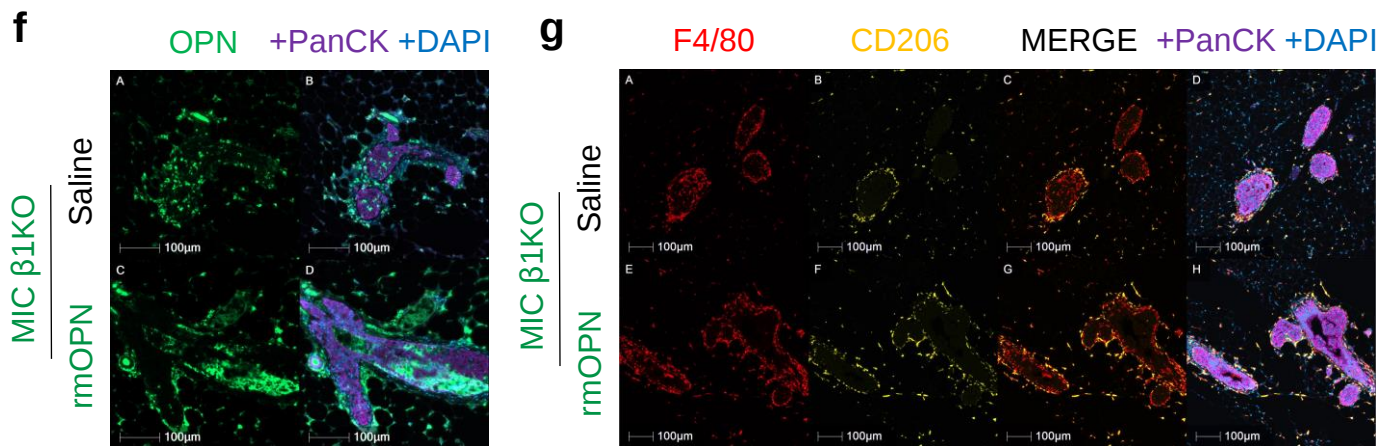
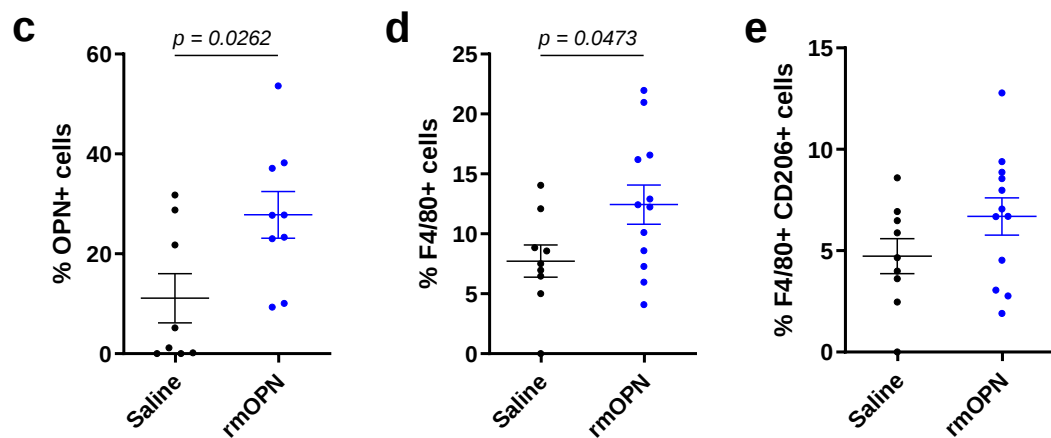
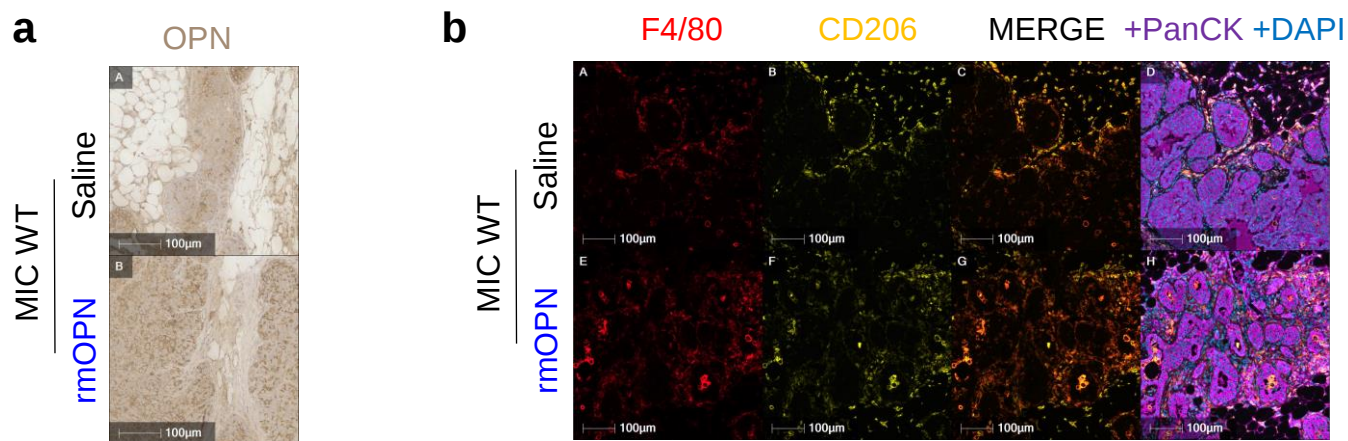
Supplementary Figure 1. $\beta 1$ integrin-deficient recurrent tumors have elevated levels of osteopontin transcription factor Stat3.

a Fluorescent immunohistochemistry (IHC) for p-Stat3, PanCK, and DAPI on MIC WT (n = 8) and MIC $\beta 1$ KO (n = 13) recurrent tumors. **b-c** Quantification of p-Stat3+ cells and p-Stat3+ PanCK+ cells in MIC WT (n = 8) and MIC $\beta 1$ KO (n = 13) recurrent tumors. **d** Immunoblot for Stat3 and p-Stat3 with loading control (tubulin) on MIC WT (n = 6) and MIC $\beta 1$ KO (n = 7) recurrent tumor lysates. **e-f** Quantification of immunoblot for Stat3 and p-Stat3 normalized to tubulin. **g** UMAP plots showing *Stat3* expression from single-cell RNA sequencing of early invasive carcinoma from MIC WT lesions (fast-growing, pooled lesions from n = 3 mice) or MIC $\beta 1$ KO lesions (dormant, pooled lesions from n = 6 mice), specifically in the epithelial tumor cell cluster. **h** Fluorescent IHC for OPN, p-Stat3, PanCK, and DAPI on MIC WT (n = 8) and MIC Stat3KO (n = 6) mammary glands 2 weeks post-DOX induction. **i-j** Quantification of OPN+ cells and OPN+ PanCK+ cells in MIC WT (n = 8) and MIC Stat3KO (n = 6) mammary glands 2 weeks post-DOX induction. Scale bars are as indicated on each image. Mean $\pm$ SEM for data calculated using two-tailed Student's t test. Each data point is representative of one biological sample for **(b)**, **(c)**, **(e)**, **(f)**, **(i)**, and **(j)**. Source data are provided as a Source Data file.



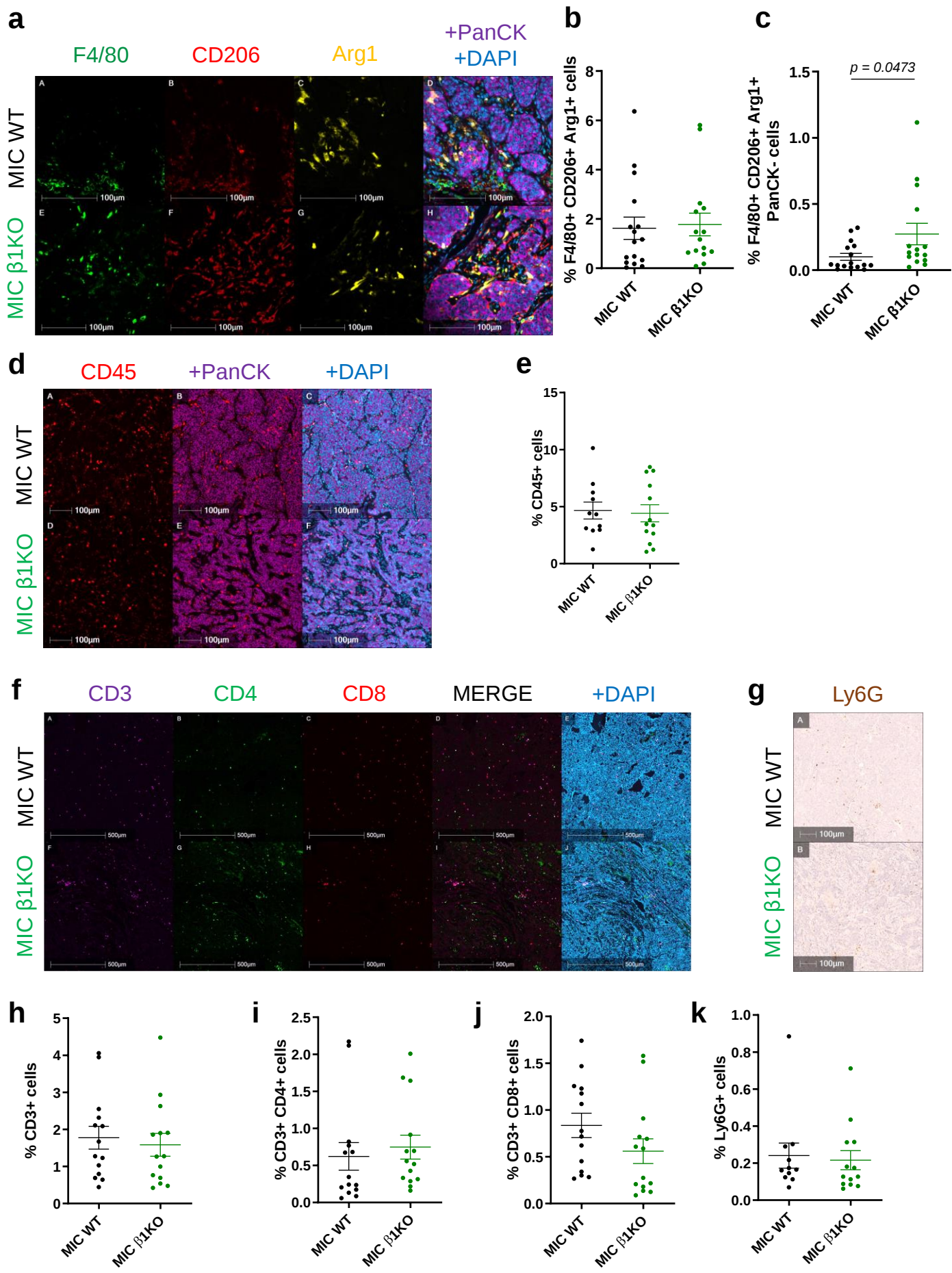
Supplementary Figure 2. OPN levels during different stages of MIC WT and MIC β 1KO tumor progression and Stat3 and OPN levels after acute β 1 integrin ablation.

a IHC staining for OPN on mammary glands of MIC WT (n = 9) and MIC β 1KO (n = 11) mice 2 weeks post-DOX induction (early tumor initiation stage). **b** Quantification of OPN+ cells in mammary glands of MIC WT (n = 9) and MIC β 1KO (n = 11) mice 2 weeks post-DOX induction. **c** Fluorescent IHC for OPN, PanCK, and DAPI on early invasive carcinoma from MIC WT lesions (fast-growing, n = 11) or MIC β 1KO lesions (dormant, n = 21). **d** Quantification of OPN+ PanCK+ cells in early invasive carcinoma from MIC WT lesions (fast-growing, n = 11) or MIC β 1KO lesions (dormant, n = 21). **e** Immunoblot for Stat3, p-Stat3, and β 1 integrin (β 1 int) with loading control (β -actin) on n = 4 MMTV-PyV mT cell lines carrying conditional floxed alleles (*Itgb1* fl/fl) at day 4 post-AdLacZ or AdCre viral infection. **f-g** Quantification of immunoblot for Stat3 and p-Stat3 normalized to β -actin (n = 4 cell lines). **h** Enzyme-linked immunosorbent assay (ELISA) for mouse OPN on cell culture media supernatant (diluted 1:20) of n = 4 MMTV-PyV mT cell lines carrying conditional floxed alleles (*Itgb1* fl/fl) at day 4 post-AdLacZ or AdCre viral infection. Scale bars are as indicated on each image. Mean $\pm$ SEM for data calculated using two-tailed Student's t test unless otherwise specified. Each data point is representative of one biological sample for (**b**) and (**d**) and one cell line for (**f-h**). Source data are provided as a Source Data file.



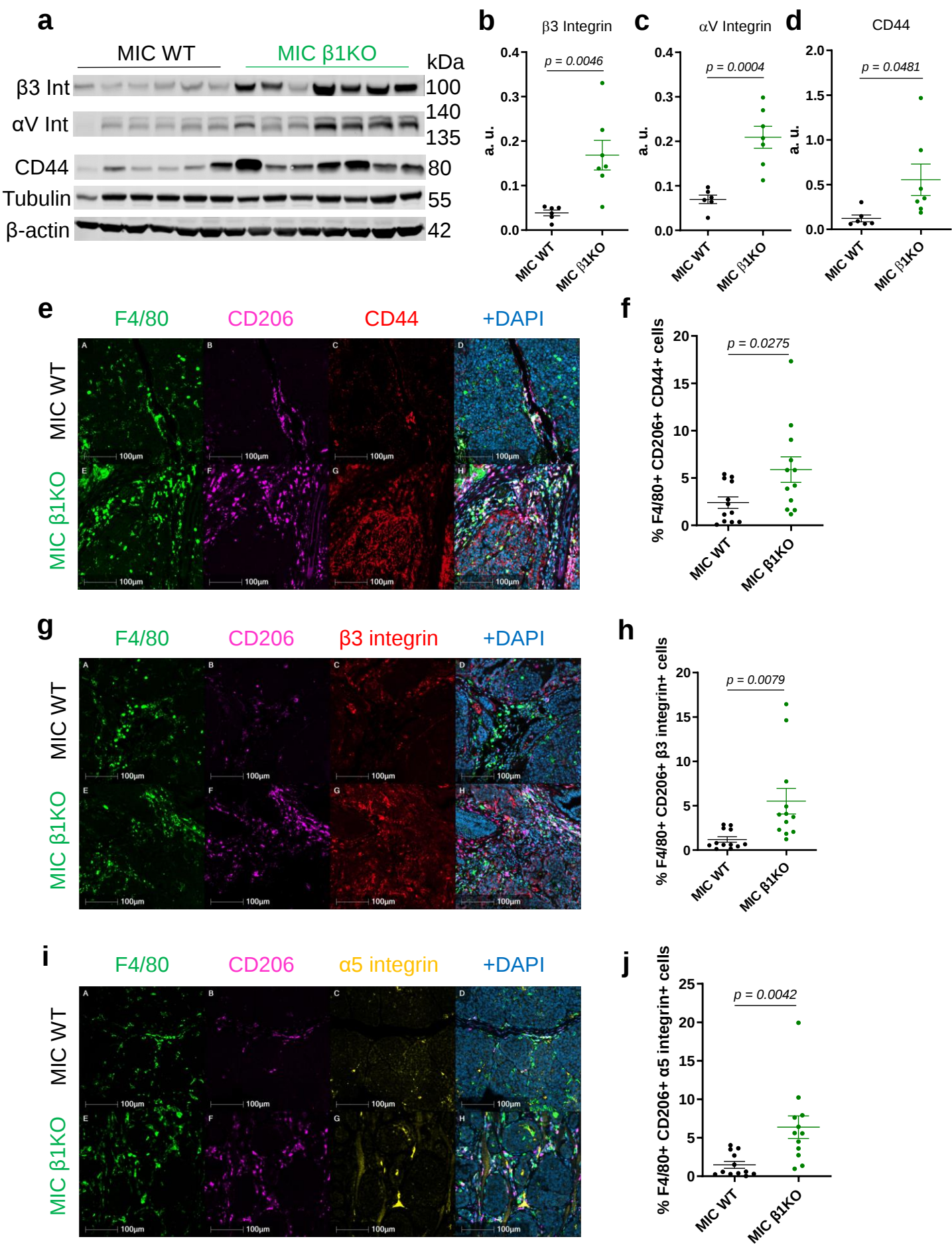
Supplementary Figure 3. Characterization of the mammary tissue microenvironments of MIC WT and β 1KO mice treated with recombinant mouse OPN.

a IHC for OPN on tumors and mammary glands of MIC WT mice treated with saline (n = 8) or recombinant mouse OPN (rmOPN) (n = 9). **b** Fluorescent IHC for F4/80, CD206, PanCK, and DAPI on tumors and mammary glands of MIC WT mice treated with saline (n = 9) or rmOPN (n = 12). **c-e** Quantification of OPN+ cells, F4/80+ cells, and F4/80+ CD206+ cells in tumors and mammary glands of MIC WT mice treated with saline or rmOPN. **f** Fluorescent IHC for OPN, PanCK, and DAPI on tumors and mammary glands of MIC β 1KO mice treated with saline (n = 17) or rmOPN (n = 24). **g** Fluorescent IHC for F4/80, CD206, PanCK, and DAPI on tumors and mammary glands of MIC β 1KO mice treated with saline (n = 22) or rmOPN (n = 18). **h-j** Quantification of OPN+ cells, F4/80+ cells, and F4/80+ CD206+ cells in tumors and mammary glands of MIC β 1KO mice treated with saline or rmOPN. Scale bars are as indicated on each image. Mean $\pm$ SEM for data calculated using two-tailed Student's t test. Each data point is representative of one biological sample for (**c-e**) and (**h-j**). Source data are provided as a Source Data file.



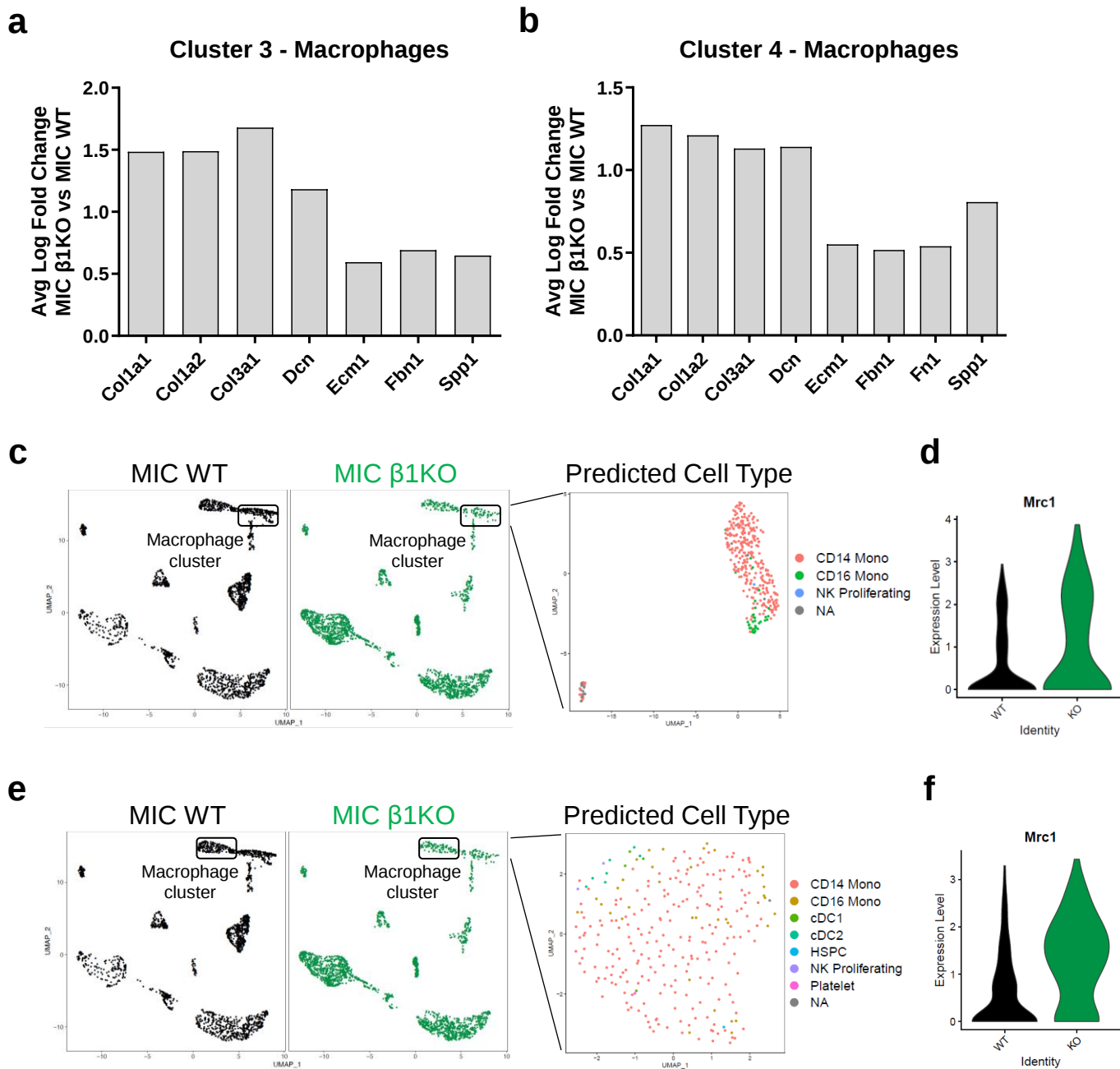
Supplementary Figure 4. β 1 integrin-deficient recurrent tumors have comparable levels of total immune cells, T cells, and neutrophils as wild-type tumors.

a Fluorescent IHC for F4/80, CD206, arginase 1 (Arg1), PanCK, and DAPI on MIC WT (n = 16) and MIC β 1KO (n = 15) recurrent tumors. **b-c** Quantification of F4/80+ CD206+ Arg1+ cells and F4/80+ CD206+ Arg1+ PanCK- cells in MIC WT (n = 16) and MIC β 1KO (n = 15) recurrent tumors. **d** Fluorescent IHC for CD45, PanCK, and DAPI on MIC WT (n = 11) and MIC β 1KO (n = 13) recurrent tumors. **e** Quantification of CD45+ cells (total immune cells) in MIC WT (n = 11) and MIC β 1KO (n = 13) recurrent tumors. **f** Fluorescent IHC for CD3, CD4, CD8, PanCK, and DAPI on MIC WT (n = 14) and MIC β 1KO (n = 14) recurrent tumors. **g** IHC for Ly6G on MIC WT (n = 11) and MIC β 1KO (n = 13) recurrent tumors. **h-k** Quantification of CD3+ cells (total T cells), CD3+ CD4+ cells (T helper cells), CD3+ CD8+ (cytotoxic T cells), and Ly6G+ (neutrophils) in the tumor in MIC WT and MIC β 1KO recurrent tumors. Scale bars are as indicated on each image. Each data point is representative of one biological sample for **(b-c)**, **(e)**, and **(h-k)**. Mean $\pm$ SEM for data calculated using two-tailed Student's t test. Source data are provided as a Source Data file.



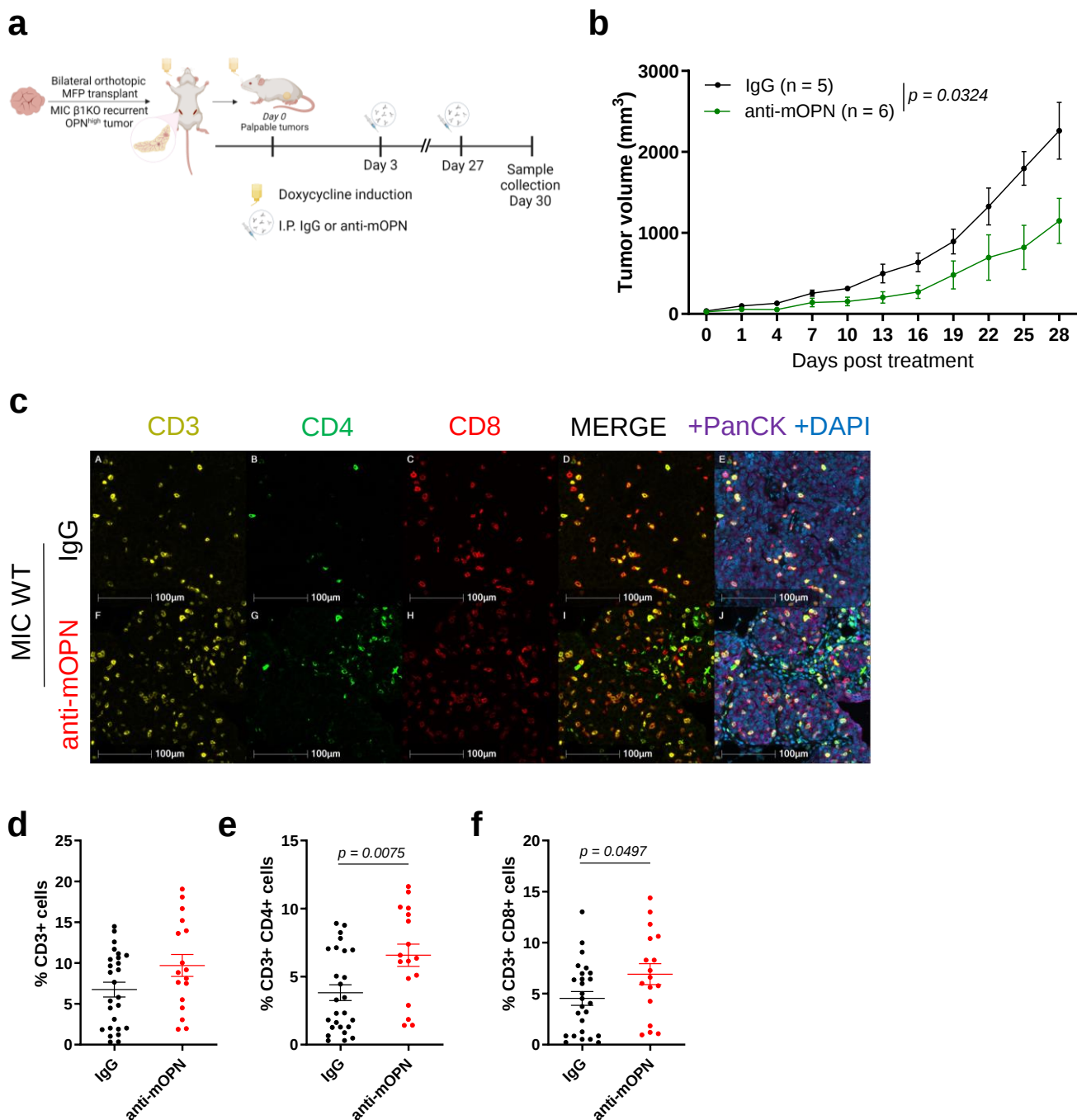
Supplementary Figure 5. Pro-tumorigenic macrophages in $\beta 1$ integrin-deficient recurrent tumors have higher levels of osteopontin receptors.

a Immunoblot for OPN receptors ($\beta 3$ integrin, αV integrin, and CD44) with loading controls (β -actin and tubulin) on MIC WT (n = 6) and MIC $\beta 1$ KO (n = 7) recurrent tumor lysates. **b-d** Quantification of immunoblots for $\beta 3$ integrin, αV integrin, and CD44 normalized to β -actin or to tubulin. **e** Fluorescent IHC for F4/80, CD206, CD44, and DAPI on MIC WT (n = 12) and MIC $\beta 1$ KO (n = 12) recurrent tumors. **f** Quantification of the percentage F4/80+ CD206+ CD44+ cells (OPN receptor-expressing pro-tumorigenic macrophages) in MIC WT (n = 12) and MIC $\beta 1$ KO (n = 12) recurrent tumors. **g** Fluorescent IHC for F4/80, CD206, $\beta 3$ integrin, and DAPI on MIC WT (n = 12) and MIC $\beta 1$ KO (n = 12) recurrent tumors. **h** Quantification of the percentage of F4/80+ CD206+ $\beta 3$ integrin+ cells (OPN receptor-expressing pro-tumorigenic macrophages) in MIC WT (n = 12) and MIC $\beta 1$ KO (n = 12) recurrent tumors. **i** Fluorescent IHC for F4/80, CD206, $\alpha 5$ integrin, and DAPI on MIC WT (n = 12) and MIC $\beta 1$ KO (n = 12) recurrent tumors. **j** Quantification of the percentage of F4/80+ CD206+ $\alpha 5$ integrin+ cells (OPN receptor-expressing pro-tumorigenic macrophages) in MIC WT (n = 12) and MIC $\beta 1$ KO (n = 12) recurrent tumors. Scale bars are as indicated on each image. Mean $\pm$ SEM for data calculated using two-tailed Student's t test. Each data point is representative of one biological sample for (**b-d**), (**f**), (**h**), and (**j**). Source data are provided as a Source Data file.



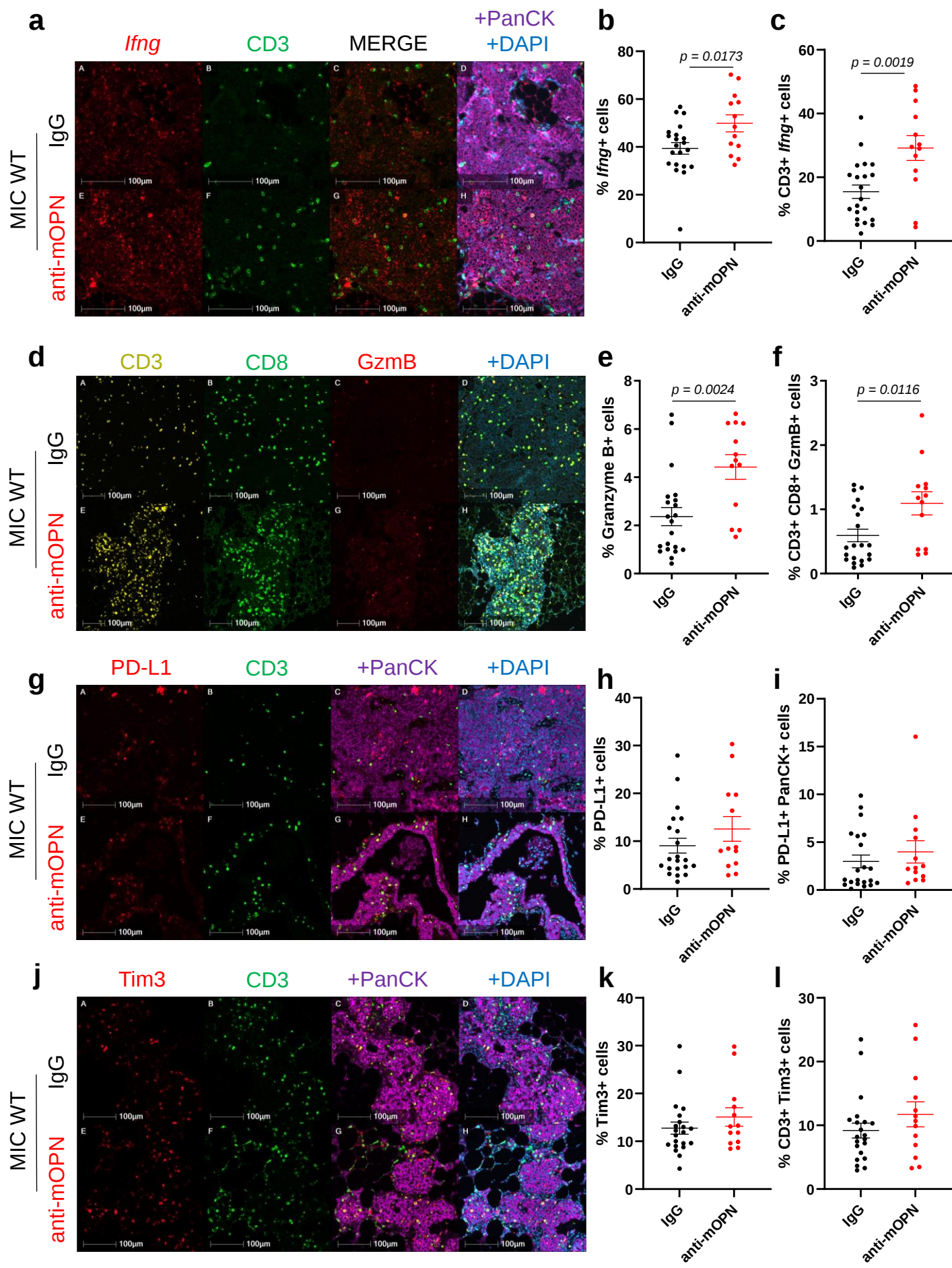
Supplementary Figure 6. Pro-tumorigenic macrophages upregulate extracellular matrix transcription contributing to tumor fibrosis during recurrence.

a-b Average log fold change of extracellular matrices in clusters 3 and 4 (macrophages) from single-cell RNA sequencing between MIC WT lesions (fast-growing) or MIC β 1KO lesions (dormant), where the fold change above 0 reflects higher expression in MIC β 1KO (dormant) lesions. **c** UMAP plots showing sub-clustering analysis of predicted cell types from the macrophage cluster 3 from single-cell RNA sequencing of early invasive tumors from MIC WT (fast-growing) or MIC β 1KO (dormant) mice. **d** Violin plot showing *Mrc1* (CD206) expression, specifically in the macrophage cluster from **c**. **e** UMAP plots showing sub-clustering analysis of predicted cell types from the macrophage cluster 4 from single-cell RNA sequencing of early invasive tumors from MIC WT (fast-growing) or MIC β 1KO (dormant) mice. **f** Violin plot showing *Mrc1* (CD206) expression, specifically in the macrophage cluster from **e**. Mean $\pm$ SEM for data calculated using two-tailed Student's t test. Source data are provided as a Source Data file.



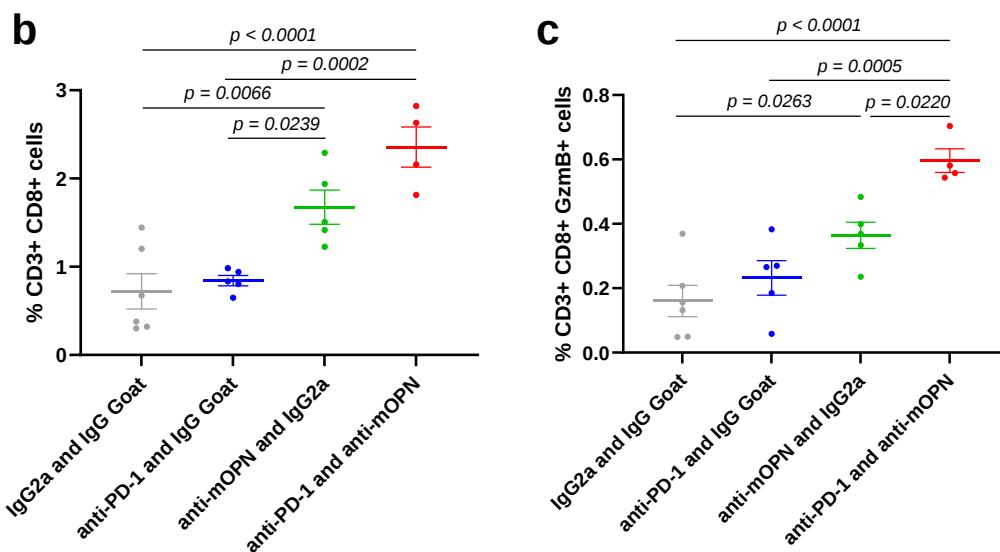
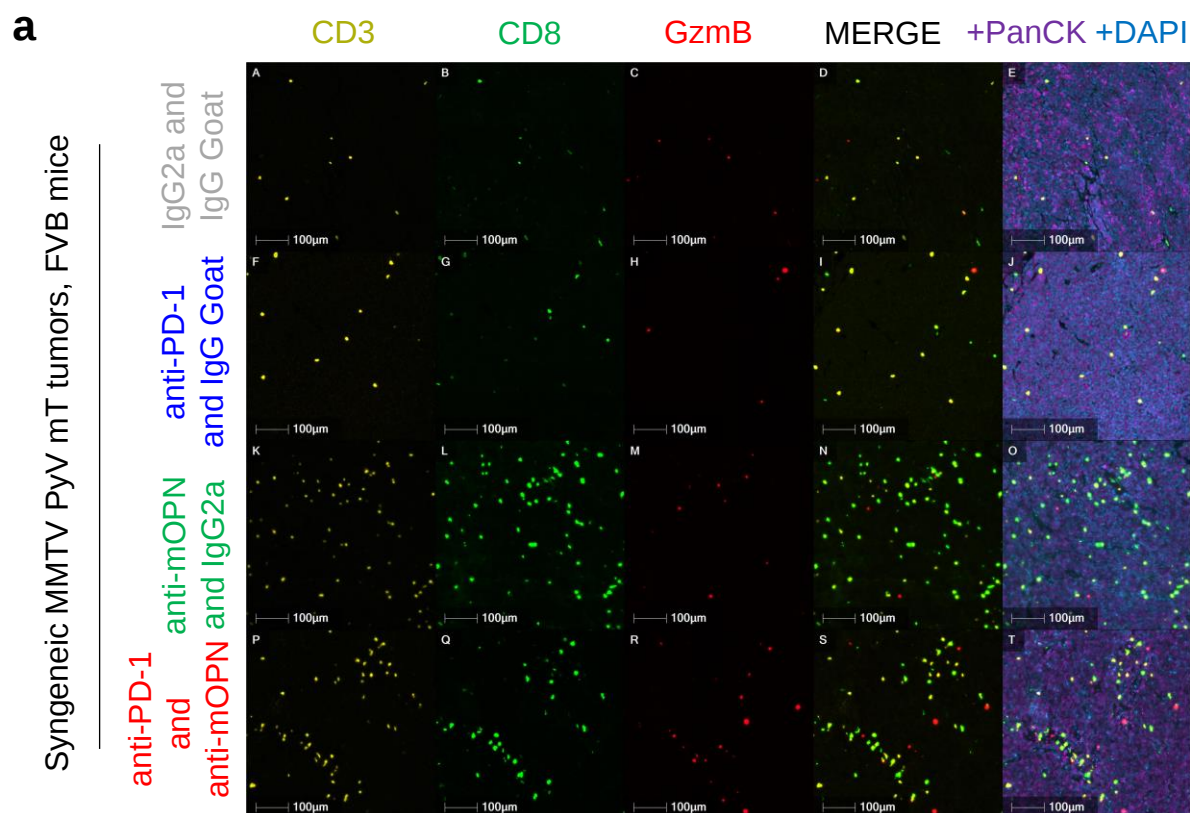
Supplementary Figure 7. Targeting osteopontin permits T cell-mediated tumor clearance.

a Schematic representation of experimental design for FVB mice induced on doxycycline with mammary fat pad (MFP)-transplanted MIC $\beta 1$ KO recurrent tumors and treated for intraperitoneal injection of control antibody IgG or anti-mOPN. Created in BioRender. Muller, W. (2024) BioRender.com/x91n317. **b** Tumor volume measured from weekly palpations of FVB mice induced on doxycycline with MFP-transplanted MIC $\beta 1$ KO recurrent tumors treated with IgG or anti-mOPN. Two-tailed Student's t-test was performed at endpoint and n denotes number of tumors per treatment arm. **c** Fluorescent IHC for CD3, CD4, CD8, PanCK, and DAPI on tumors and mammary glands of MIC WT mice treated with IgG (n = 26) or anti-mOPN (n = 17). **d-f** Quantification of CD3+ cells, CD3+ CD4+ cells, and CD3+ CD8+ cells in tumors and mammary glands of MIC WT mice treated with IgG (n = 26) or anti-mOPN (n = 17). Scale bars are as indicated on each image. Mean $\pm$ SEM for data calculated using two-tailed Student's t test unless otherwise specified. Each data point is representative of one biological sample for (**d-f**). Source data are provided as a Source Data file.



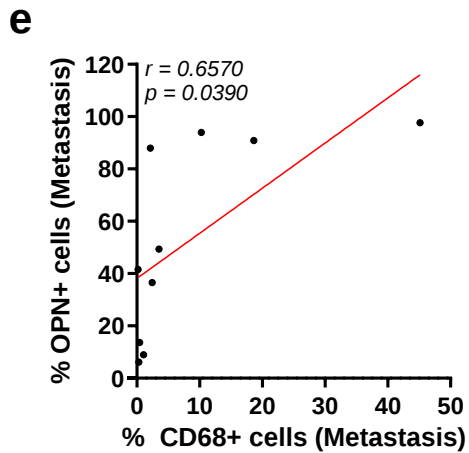
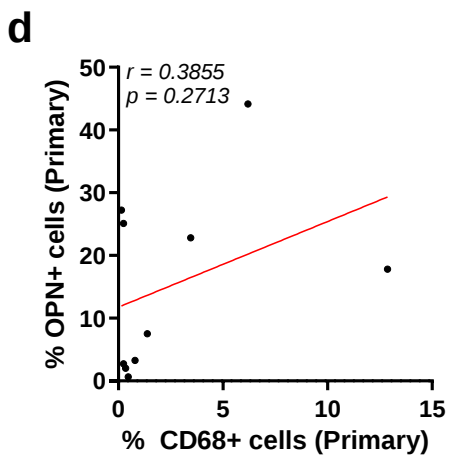
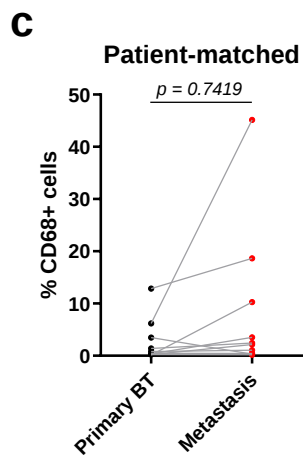
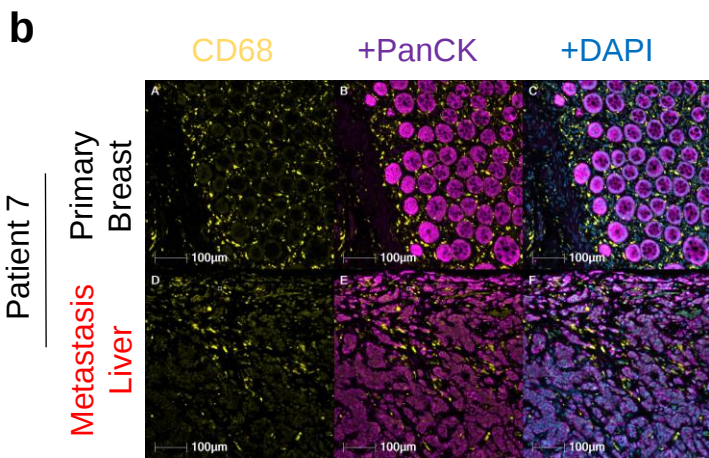
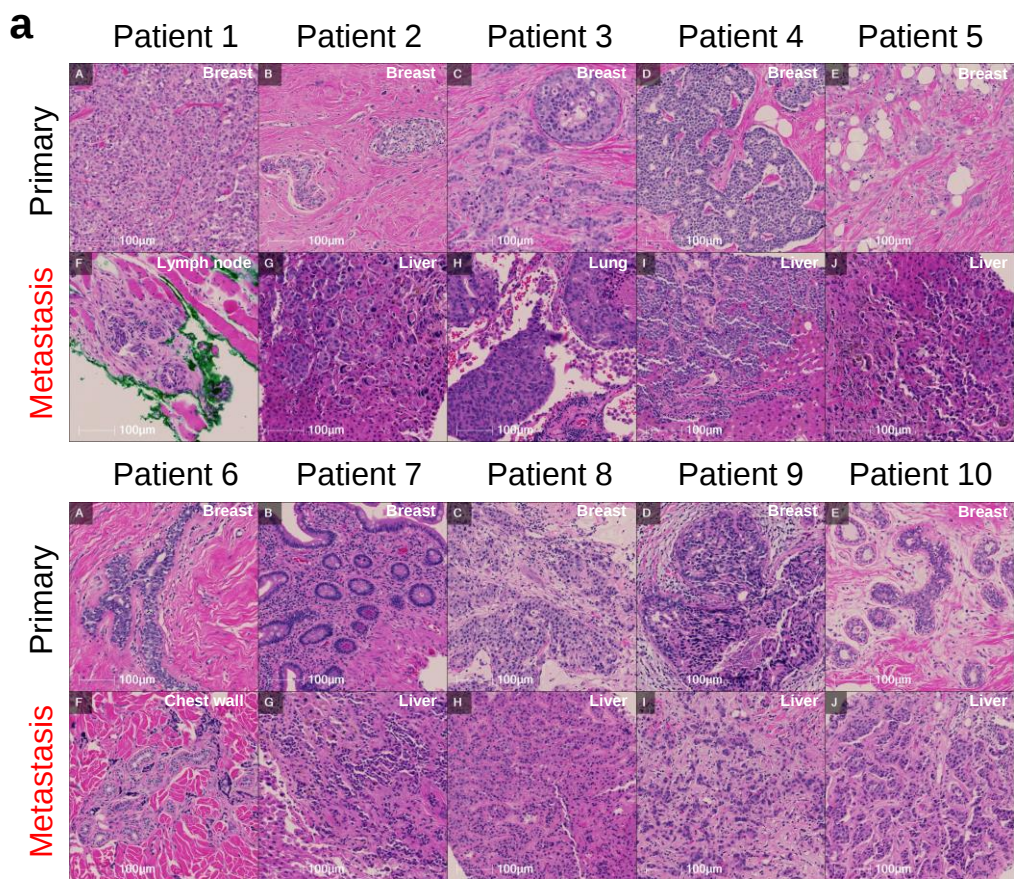
Supplementary Figure 8. T cell activation and exhaustion characterization anti-mOPN-treated mice.

a RNA Scope for mouse *Ifng* and fluorescent IHC for CD3 and DAPI on tumors and mammary glands of MIC WT mice treated with IgG or anti-mOPN. **b-c** Quantification of total *Ifng*⁺ cells and CD3⁺ *Ifng*⁺ cells in tumors and mammary glands of MIC WT mice treated with IgG or anti-mOPN. **d** Fluorescent IHC for CD3, CD8, Granzyme B (GzmB), and DAPI on tumors and mammary glands of MIC WT mice treated with IgG or anti-mOPN. **e-f** Quantification of GzmB⁺ cells and CD3⁺ GzmB⁺ cells in tumors and mammary glands of MIC WT mice treated with IgG or anti-mOPN. **g** Fluorescent IHC for PD-L1, CD3, PanCK, and DAPI on tumors and mammary glands of MIC WT mice treated with IgG or anti-mOPN. **h-i** Quantification of PD-L1⁺ cells and of PD-L1⁺ PanCK⁺ cells in tumors and mammary glands of MIC WT mice treated with IgG or anti-mOPN. **j** Fluorescent IHC for Tim3, CD3, PanCK, and DAPI on tumors and mammary glands of MIC WT mice treated with IgG or anti-mOPN. **k-l** Quantification of Tim3⁺ cells and CD3⁺ Tim3⁺ cells in tumors and mammary glands of MIC WT mice treated with IgG or anti-mOPN. Scale bars are as indicated on each image. Mean $\pm$ SEM for data calculated using two-tailed Student's t test. Each data point is representative of one biological sample for **(b-c)**, **(e-f)**, **(h-i)**, and **(k-l)** with IgG (n = 21) and anti-mOPN (n = 13) for all. Source data are provided as a Source Data file.



Supplementary Figure 9. Anti-mOPN and anti-PD-1 combinational treatment increases T cell infiltration and activation.

a Fluorescent IHC for CD3, CD8, granzyme B (GzmB) PanCK, and DAPI on syngeneic MMTV PyV mT tumors treated with IgG2a and IgG Goat ($n = 6$), anti-PD-1 and IgG Goat ($n = 5$), anti-mOPN and IgG2a ($n = 5$), or anti-PD-1 and anti-mOPN ($n = 4$). **b-c** Quantification of CD3+ CD8+ cells and CD3+ CD8+ GzmB+ cells in syngeneic MMTV PyV mT tumors treated with IgG2a and IgG Goat ($n = 6$), anti-PD-1 and IgG Goat ($n = 5$), anti-mOPN and IgG2a ($n = 5$), or anti-PD-1 and anti-mOPN ($n = 4$). Scale bars are as indicated on each image. Mean $\pm$ SEM for data calculated using Ordinary One Way ANOVA with Tukey's post hoc test. Each data point is representative of one biological sample for (**b-c**). Source data are provided as a Source Data file.



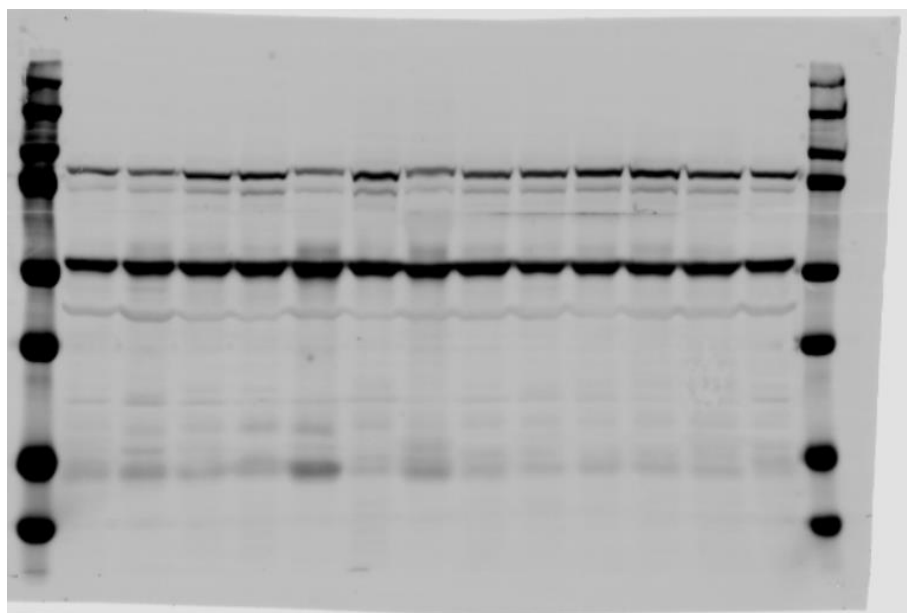
Supplementary Figure 10. Osteopontin positivity correlates with macrophage infiltration in patient-matched recurrent metastatic tumors.

a H&E image of patient-matched primary breast tumors and recurrent metastatic tumors. **b** Fluorescent IHC for CD68, PanCK, and DAPI on patient-matched primary breast tumors and recurrent metastatic samples. **c** Quantification of CD68+ cells in patient-matched primary breast tumors (n = 10) and recurrent metastatic tumors (n = 10). **d-e** Correlation between OPN+ cells and CD68+ cells in patient-matched primary breast tumors (n = 10) and recurrent metastatic tumors (n = 10). Statistical significance was calculated using the Spearman rank correlation test. Scale bars are as indicated on each image. Mean $\pm$ SEM for data calculated using two-tailed Student's t test unless otherwise specified. Each data point is representative of one biological sample for (**c-e**). Source data are provided as a Source Data file.

a

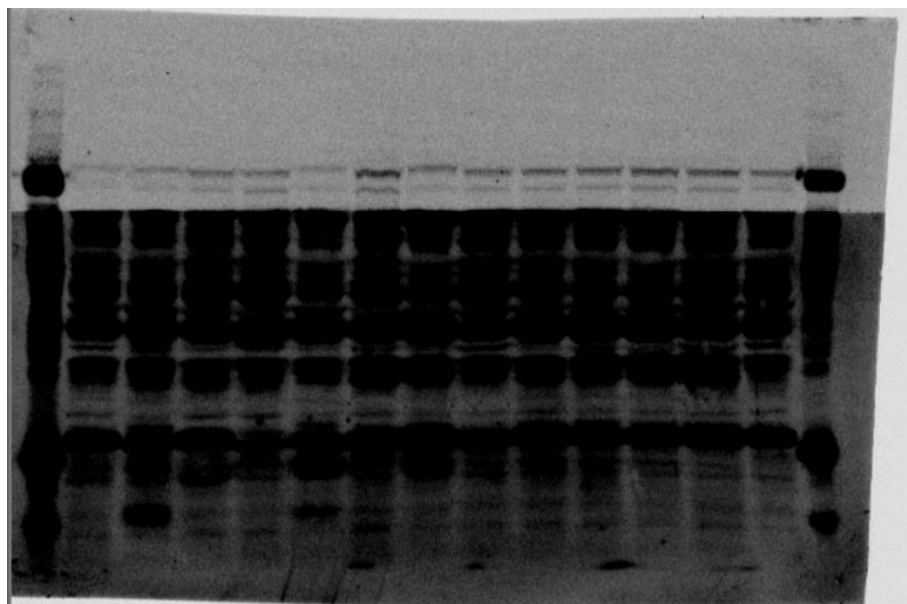
MIC WT

MIC β 1KO



Stat3, 90 kDa

Tubulin control; 55 kDa



p-Stat3, 90 kDa

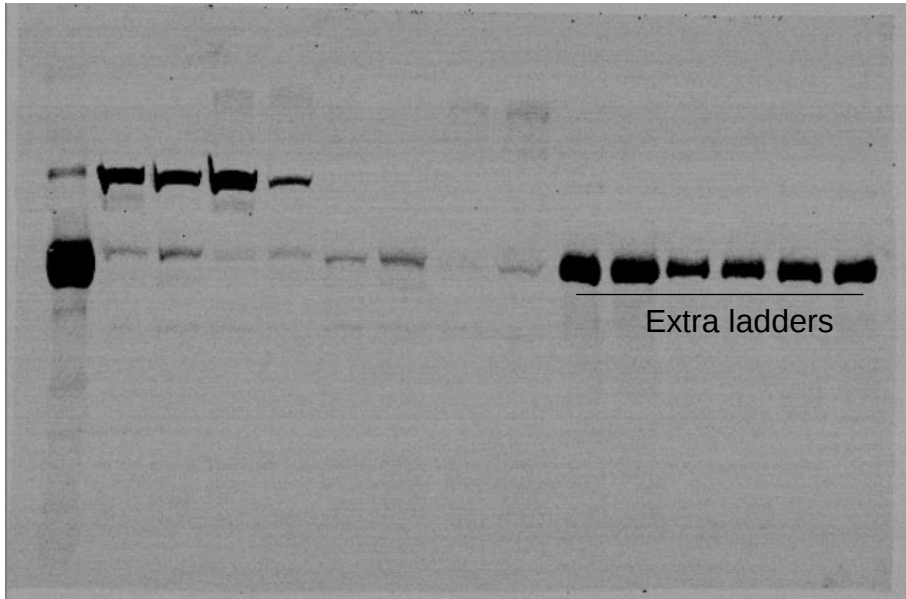
Supplementary Figure 11. Uncropped immunoblots for Supplementary Figure 1d.

a Immunoblot for Stat3, tubulin control, and p-Stat3 on MIC WT and MIC β 1KO recurrent tumor lysates. The same immunoblot is presented twice for separate channels.

aMMTV-PyV mT *Itgb1* fl/fl

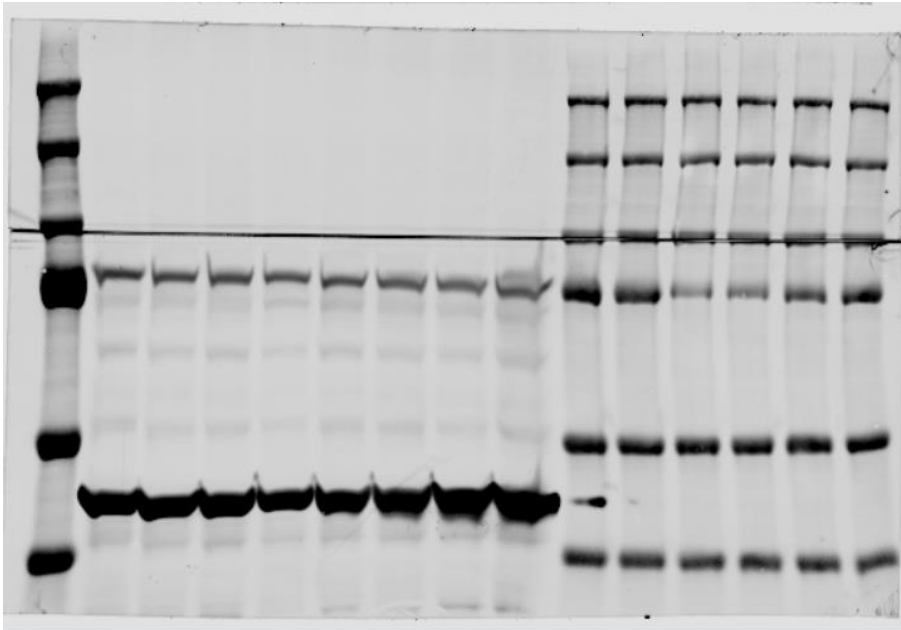
AdLacZ

AdCre

 $\beta 1$ integrin, 130 kDa

p-Stat3, 90 kDa

Extra ladders



Stat3, 90 kDa

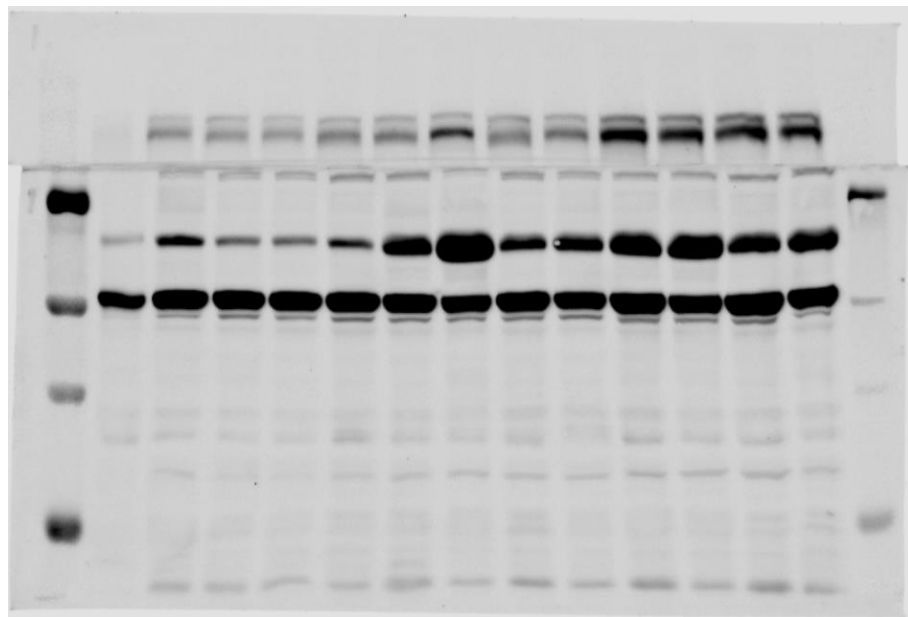
 β -actin control; 42 kDa**Supplementary Figure 12. Uncropped immunoblots for Supplementary Figure 2e.**

a Immunoblot for $\beta 1$ integrin and p-Stat3 and immunoblot for Stat3 and β -actin control on four MMTV-PyV mT cell lines carrying conditional floxed alleles (*Itgb1* fl/fl) at day 4 post-AdLacZ or AdCre viral infection. The same immunoblot is presented twice for separate channels.

a

MIC WT

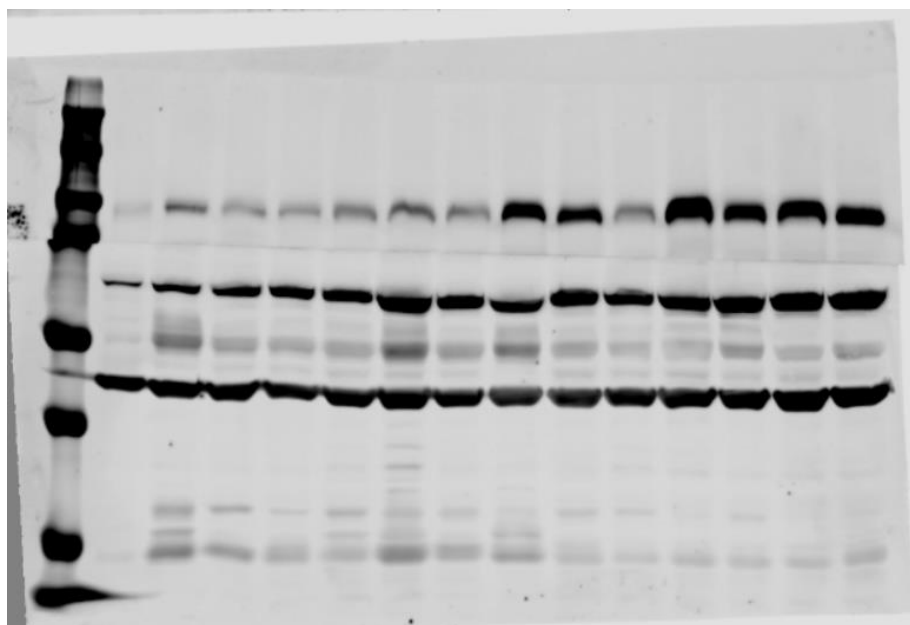
MIC β 1KO



α V Int; 140/135 kDa

CD44; 80 kDa

Tubulin control; 55 kDa



β 3 Int; 100 kDa

β -actin control; 42 kDa

Supplementary Figure 13. Uncropped immunoblots for Supplementary Figure 5a.

a Immunoblot for OPN receptors α V integrin, CD44 and tubulin control and immunoblot for OPN receptors β 3 integrin and β -actin control on MIC WT and MIC β 1KO recurrent tumor lysates.