

Supplementary material: Stefin B and Cystatin C Deficiency Suppresses Tumor Growth and Alters Tumor Microenvironment in a Breast Cancer Model

Supplementary methods

Histology

Histopathological examinations were performed on the liver, kidneys, spleen, and lungs to evaluate potential pathological changes in these organs. Tissue samples were fixed in 10% Neutral buffered Formalin, embedded in paraffin, sectioned at 5 µm thickness, and stained with hematoxylin and eosin (H&E) for microscopic evaluation. Sections were analyzed under a light microscope at 20x magnification, examining 20 fields of view (FOV) per sample. For each organ, tissue was collected from three distinct regions, with a 100 µm spacing between sampling areas, to ensure a comprehensive assessment of pathological alterations across different organ areas.

Analysis of DNA synthesis using the BrdU incorporation method

The BrdU cell proliferation kit was purchased from EMD Millipore Corporation (Billerica, MA, USA). Primary tumor cells were cultured in 96-well plates at the density of 10^5 cells/ml. Cells were labeled with BrdU for 2 h, and levels of BrdU incorporation were determined according to the manufacturer's instructions using a Tecan Safire (Tecan, Gröding, Austria) microplate reader at a wavelength of 450 nm and subtracting absorbance measured at 540 nm.

Immunofluorescence

Organs were cut into 4 µm thick sections and placed on the pre-cooled slides. The slides were incubated in 80% methanol at 4°C, followed by incubation in 100% acetone pre-cooled to -20°C. Finally, the slides were rinsed thoroughly in PBS to remove any residual fixatives. Sections were stained with the CD31 antibody (BD Pharmingen, San Diego, CA, USA; 550274, rabbit monoclonal, dilution 1:50) and were co-stained and mounted in SlowFade Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA) and examined with a fluorescence microscope (Olympus IX 81, Olympus, Tokyo, Japan) with Olympus CellSens Imaging software as described [52].

Supplementary figures

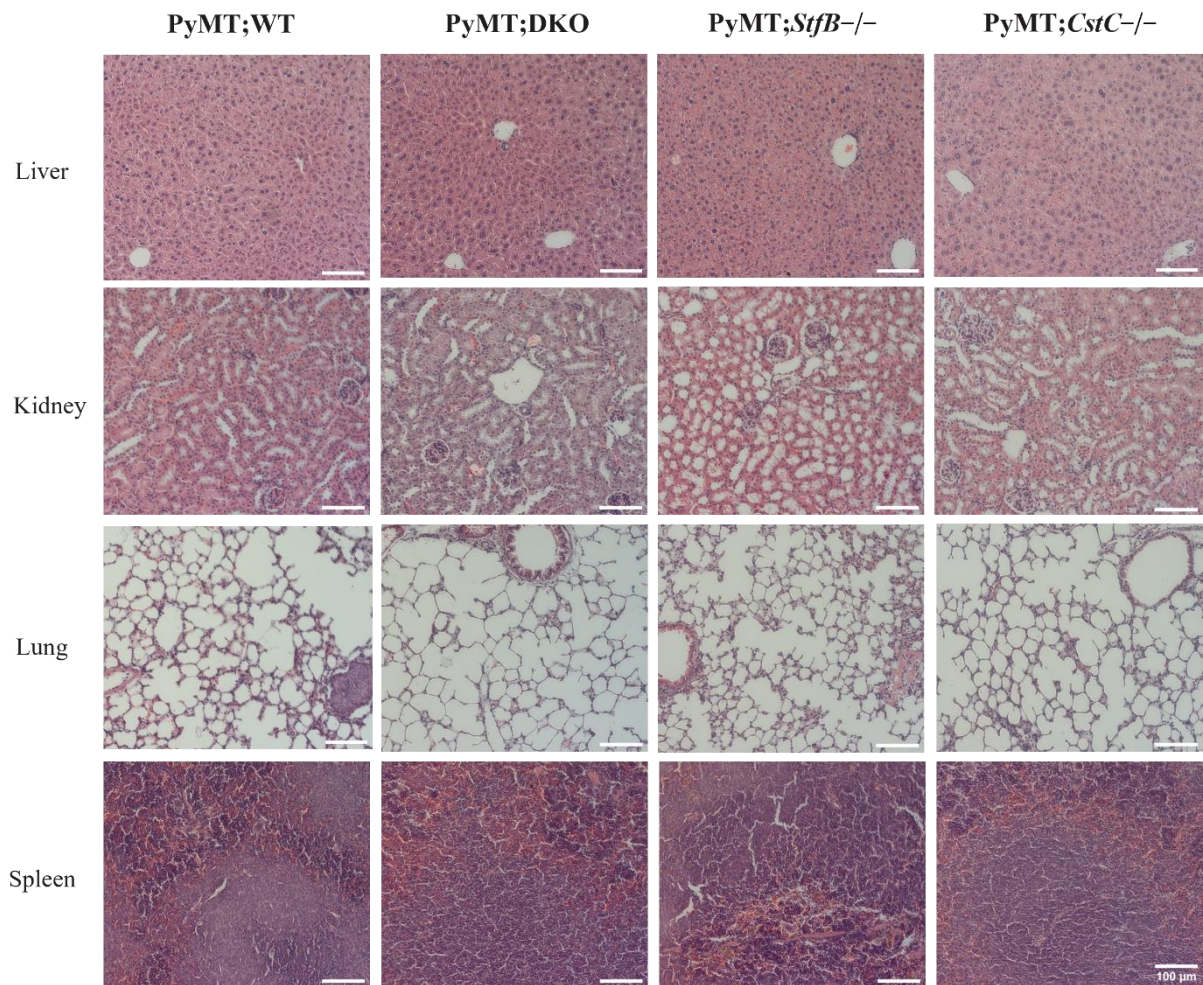


Figure S1. Comparative histological assessment of tissues from mice with different genotypes. Hematoxylin and eosin staining of liver, kidney, lung, and spleen from PyMT;WT, PyMT;DKO, PyMT;*StfB*^{-/-} and PyMT;*CstC*^{-/-} mice. Organs were collected, dehydrated, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. A pathohistological examination of the organs was performed. Scale bar: 100 μm.

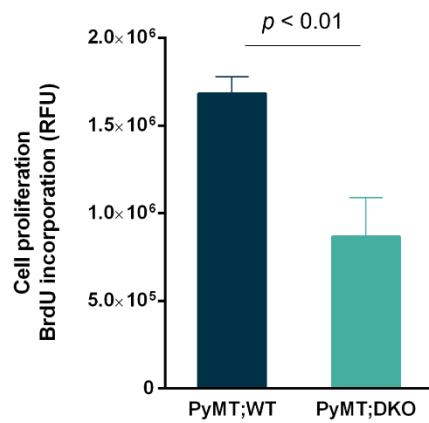


Figure S2. Cell proliferation of PyMT;WT (n = 6) and PyMT;DKO (n = 6) primary tumor cells determined by BrdU incorporation assay. Cell proliferation was assessed by BrdU cell proliferation assay HTS kit. PyMT;WT and PyMT;DKO cells were cultured and treated as described in the assay kit.

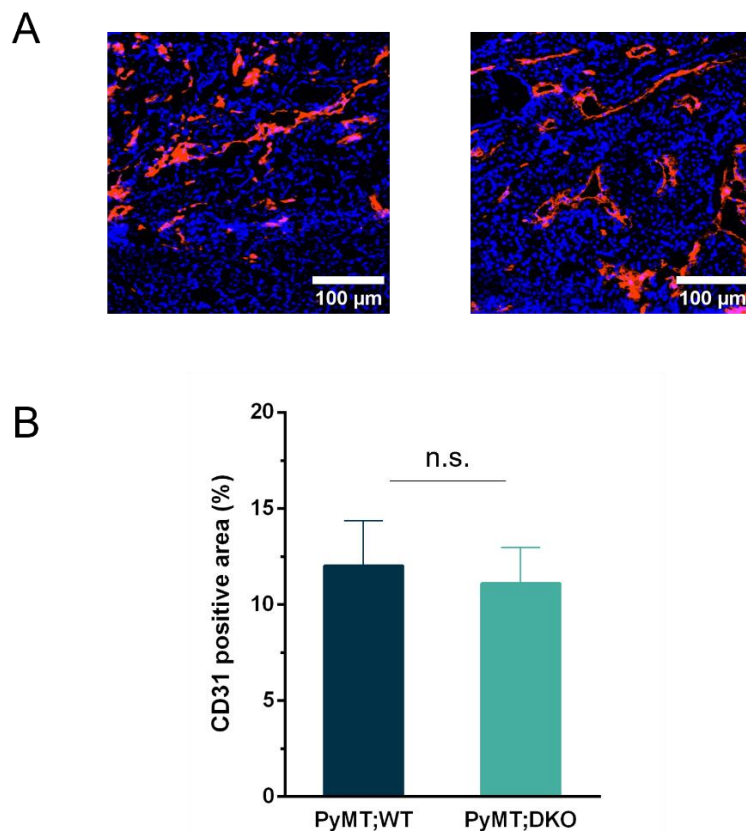


Figure S3. Immunofluorescence analysis of CD31 expression in PyMT;WT and PyMT;DKO mammary tumors. (A) Representative images of immunofluorescence staining of the endothelial cell-specific marker CD31 (red staining) on PyMT;WT and PyMT;DKO tumors. Scale bar: 100μM. (B) Quantification of CD31 positive area as percentage of all tumor area in PyMT;WT (n = 8) and PyMT;DKO (n = 9) tumors. Statistics were analyzed using Student's *t*-test.

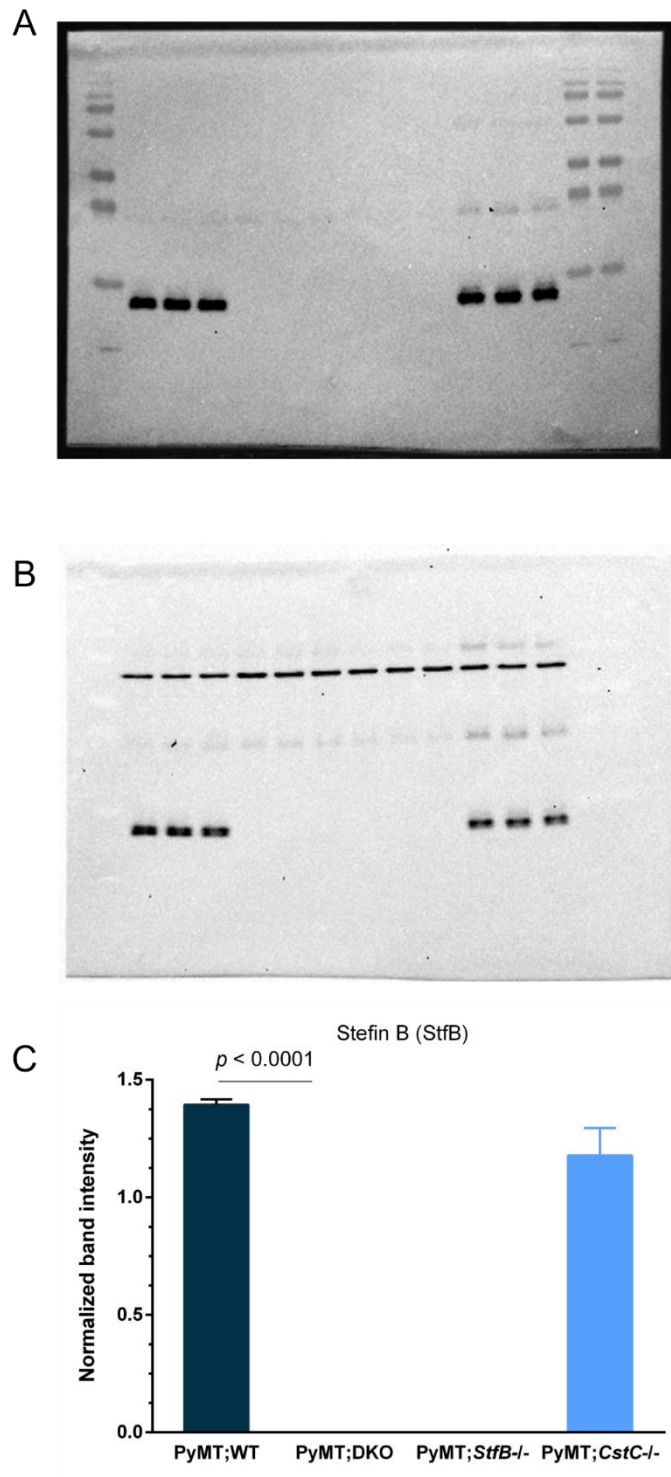


Figure S4: Original Western blot images and densitometric analysis for Figure 1A. (A) Original membrane probed for stefin B. (B) Original membrane probed for β -actin (loading control). (C) Bar graph showing densitometric quantification of stefin B expression normalized to β -actin. Bars represent mean values $\pm$ standard deviation (SD). Densitometric analysis was performed using ImageJ software. Statistical significance was determined using Student's *t*-test.

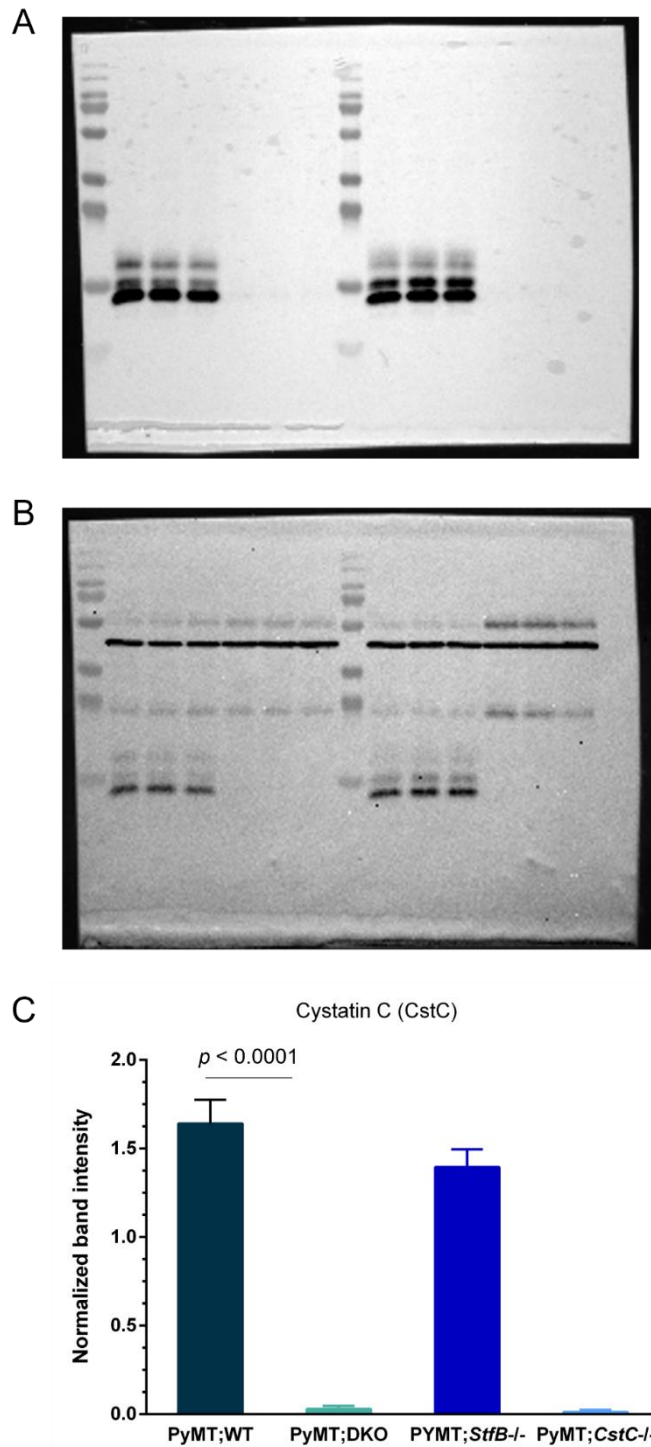


Figure S5. Original Western blot images and densitometric analysis for Figure 1B. (A) Original membrane probed for cystatin C. (B) Original membrane probed for β -actin (loading control). (C) Bar graph showing densitometric quantification of cystatin C expression normalized to β -actin. Bars represent mean values $\pm$ standard deviation (SD). Densitometric analysis was performed using ImageJ software. Statistical significance was determined using Student's *t*-test.