

1 **Supplementary material**

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3 **Spheroid cultures reveal cancer stem cell enrichment and distinct chemotherapeutic**
4 **response in triple-negative breast cancer**

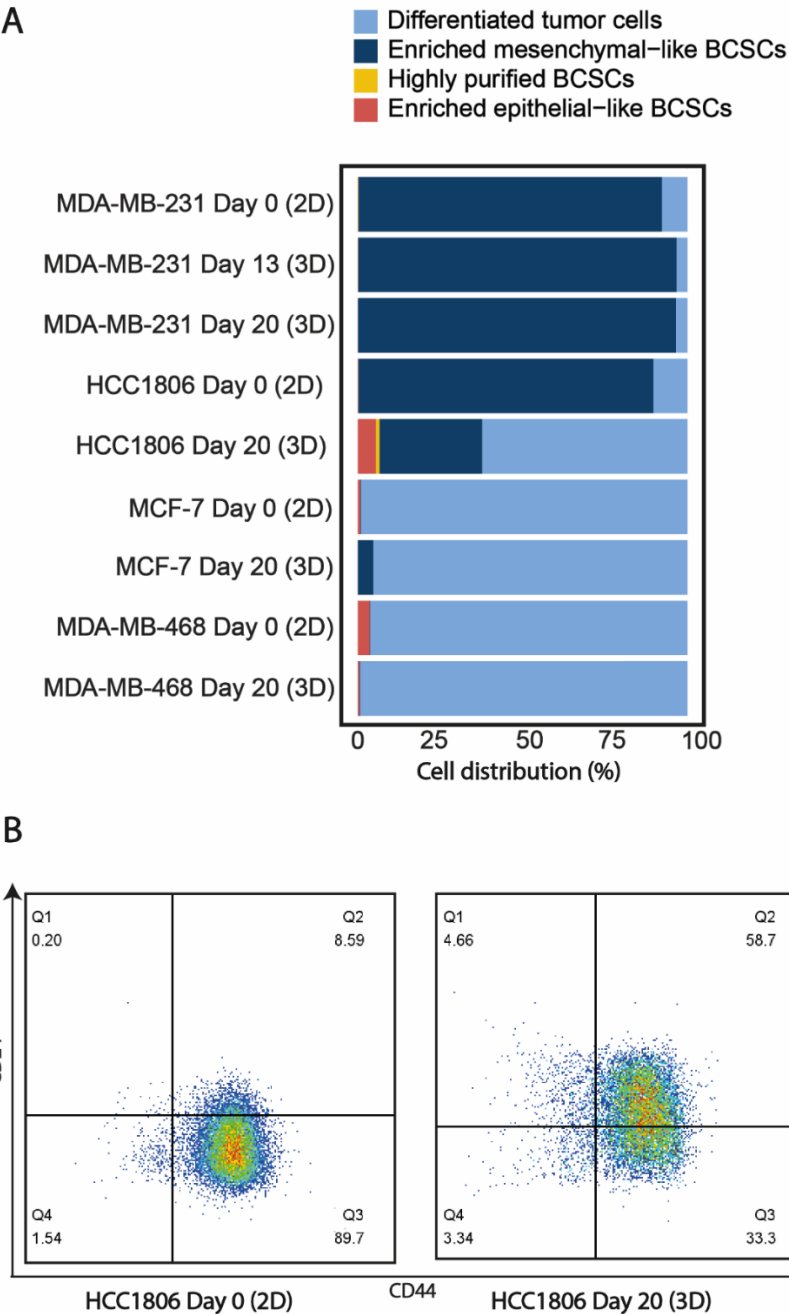
5 Sithumini Sarathchandra, Chiara Fittipaldi, Nanfizat Abiket Alamukii, Anikó Kovács, Toshima Z.
6 Parris

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30 **Supplementary Figure 1**



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32 **Supplementary Fig. 1 | Flow cytometry analysis of breast cancer cell lines with the ALDEFLUOR™**

33 **kit and antibodies for CD24, CD44, and 7-AAD. A** Distribution of different breast cancer stem cell

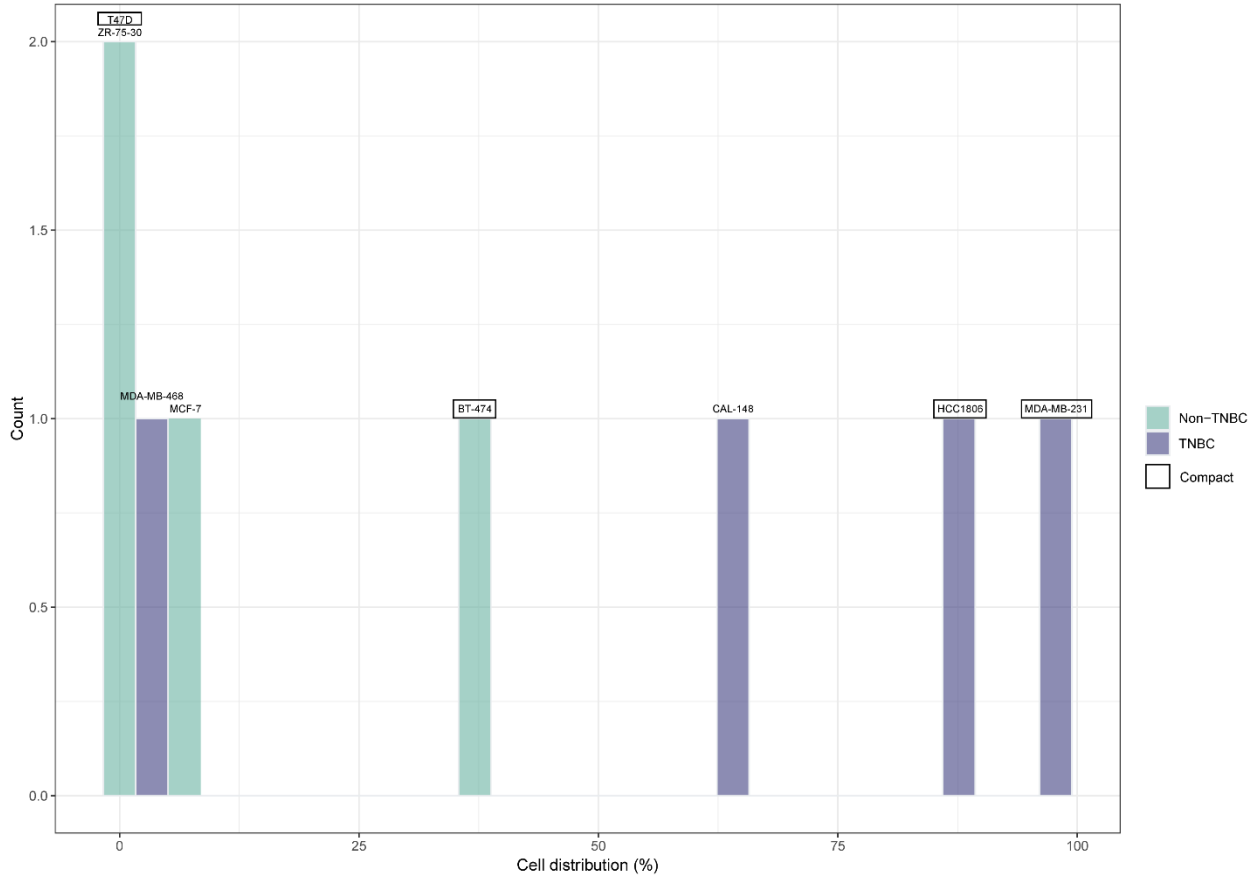
34 subpopulations identified by flow cytometry analysis in 2D and 3D samples. HCC1806 cells showed a

35 significant shift in BCSC subpopulations from enriched mesenchymal-like BCSCs in 2D monolayers to an

36 increase in differentiated tumor cells, highly purified BCSCs, and enriched epithelial-like BCSCs in 3D

spheroids; the other cell lines showed little difference between 2D and 3D cultures. **B** Different cell distribution patterns for ALDH- in the 2D monolayers and 3D spheroids for HCC1806 cells.

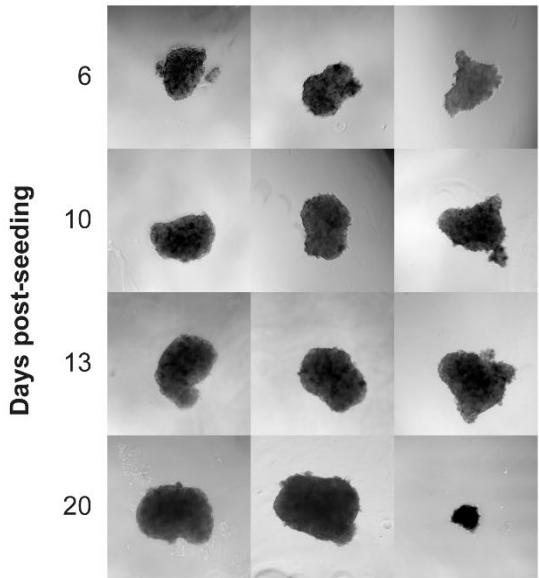
Supplementary Figure 2



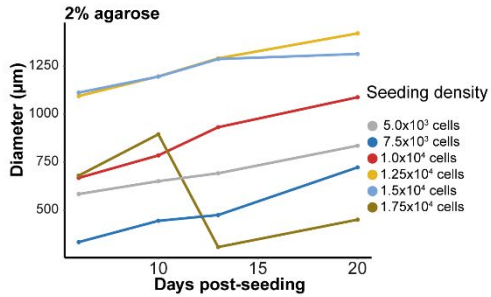
Supplementary Fig. 2 | Histogram of the percentage of breast cancer stem cells (BCSCs) in the 8 breast cancer cell lines stratified by subtype (triple-negative breast cancer [TNBC] vs non-TNBC). In total, 3/4 TNBC cell lines contained >50% BCSCs, while all non-TNBC cells contained <50% BCSCs; MDA-MB-468 (TNBC BL1 subtype) was the only TNBC cell line to contain <50% BCSCs. Both TNBC cell lines classified as BL2 subtype (HCC1806 and MDA-MB-231) contained >50% BCSCs. Cell lines forming compact spheroids are outlined in black.

Supplementary Figure 3

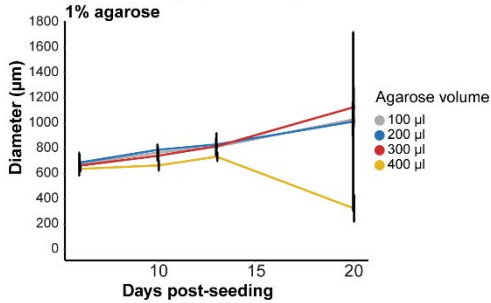
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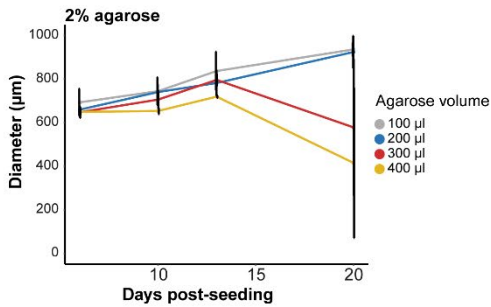
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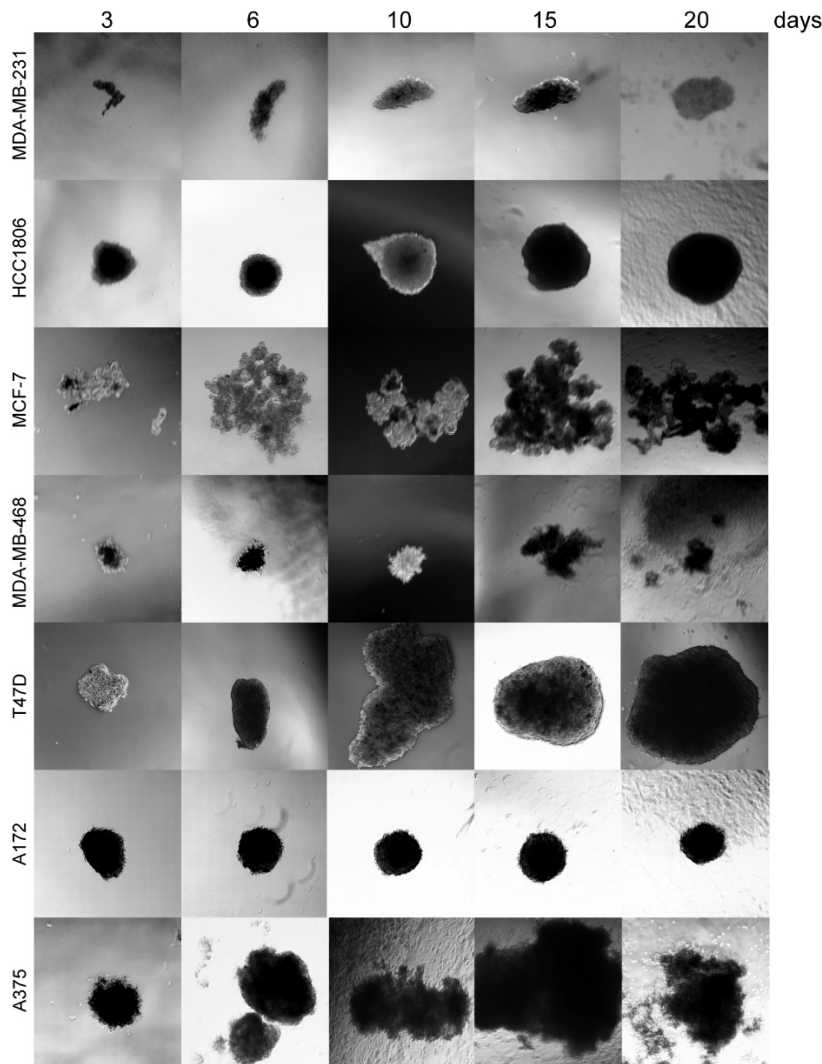
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Supplementary Fig. 3 | Optimization of the experimental parameters for the liquid overlay method.

A Phase-contrast images of MDA-MB-231 spheroids starting with 1.25×10^4 cells in 100 μl of 2% agarose-coated wells and grown for up to 20 days post-seeding. Replicate 3 on Day 20 is smaller, likely due to spheroid disruption during medium changes. Replicate wells are shown for each time point. Growth of the MDA-MB-231 spheroids with different **B** seeding densities (5.0×10^3 to 1.75×10^4 cells per 24-well), **C-D** agarose concentrations (1-2%) and volumes (100-400 μl). The error bars depict the standard deviation.

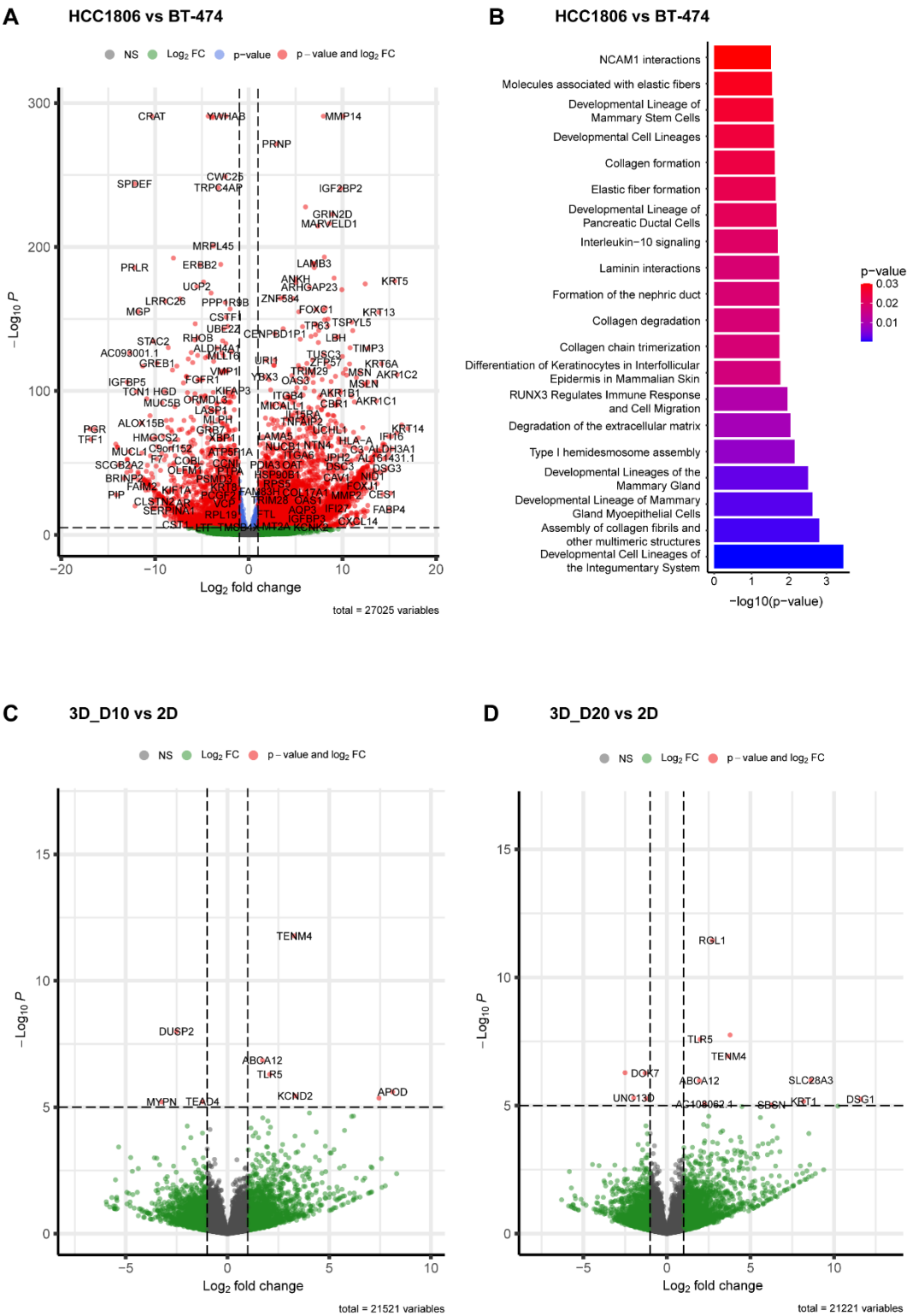
Supplementary Figure 4



Supplementary Fig. 4 | Spheroid formation in cancer cell lines using the liquid overlay method.

Representative phase-contrast (left; 5x objective) images of cancer cell lines for the growth of compact (A172 glioblastoma, as well as HCC1806, MDA-MB-231, and T47D breast cancer cells) spheroids and loose spheroids/aggregates (A375 malignant melanoma cells, MCF-7 and MDA-MB-468 breast cancer cells). Note images from alternative days after seeding are shown for T47D: Days 2 and 7 instead of days 3 and 6; A172 and A375: Days 7 and 14 instead of days 6 and 15.

70 **Supplementary Figure 5**



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72 **Supplementary Fig. 5 | Differential gene expression and pathway analysis.** Using the R package
73 DESeq2, differentially expressed genes were identified and visualized using the Enhanced Volcano

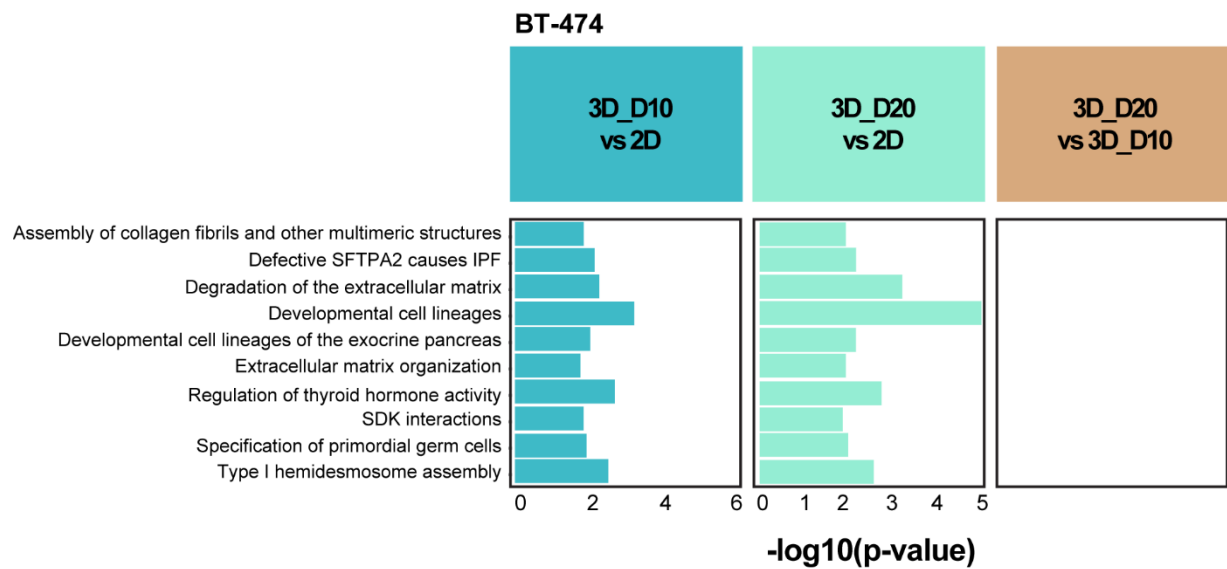
74 package, where the x-axis represents the $\log_2$ fold change and the y-axis corresponds to the $-\log_{10}$ p-value.
75 **A-B** Differentially expressed genes (DEGs highlighted in red; $|\log_2\text{FoldChange}| > 1.5$ and Benjamini-
76 Hochberg $\text{padj} < 0.05$) and Reactome pathway analysis for HCC1806 and BT-474 cells. **C-D** DEGs enriched
77 in day 10 (3D_D10) or day 20 (3D_D20) spheroids versus 2D cells.

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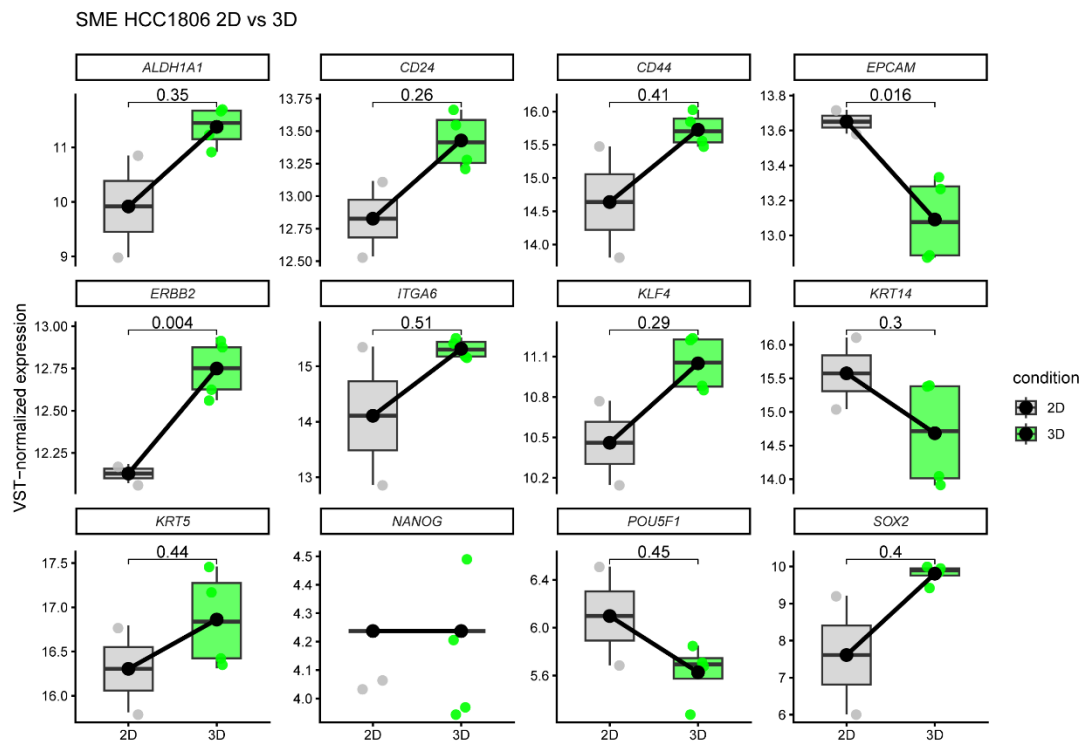
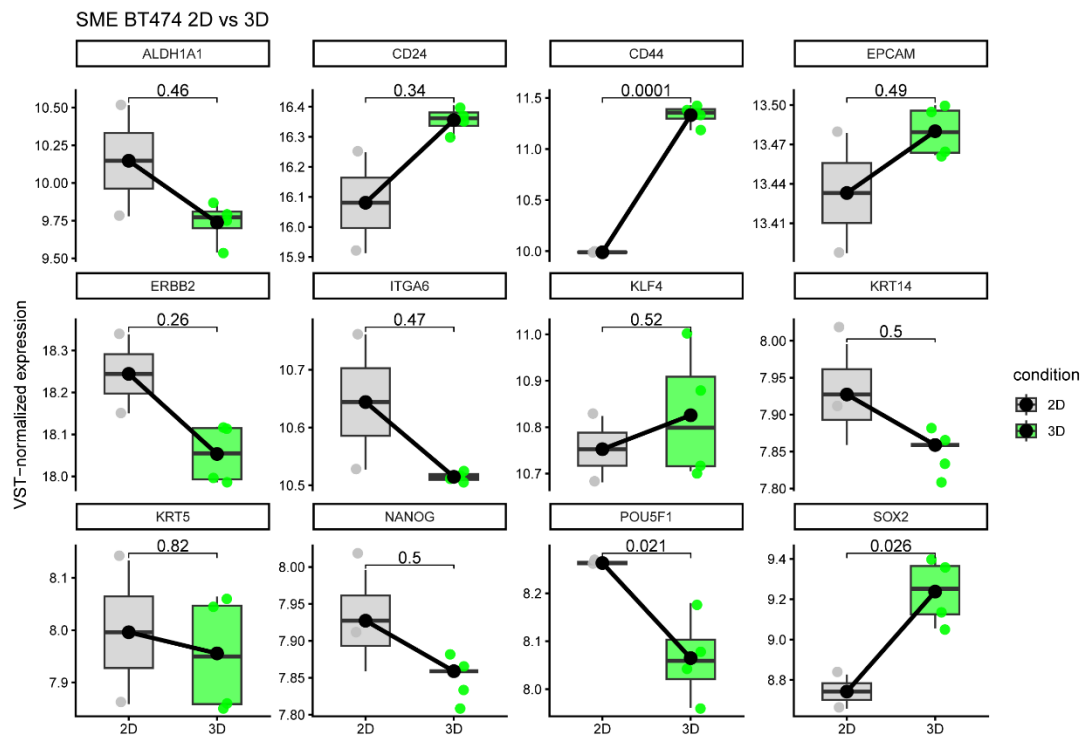
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Supplementary Figure 6



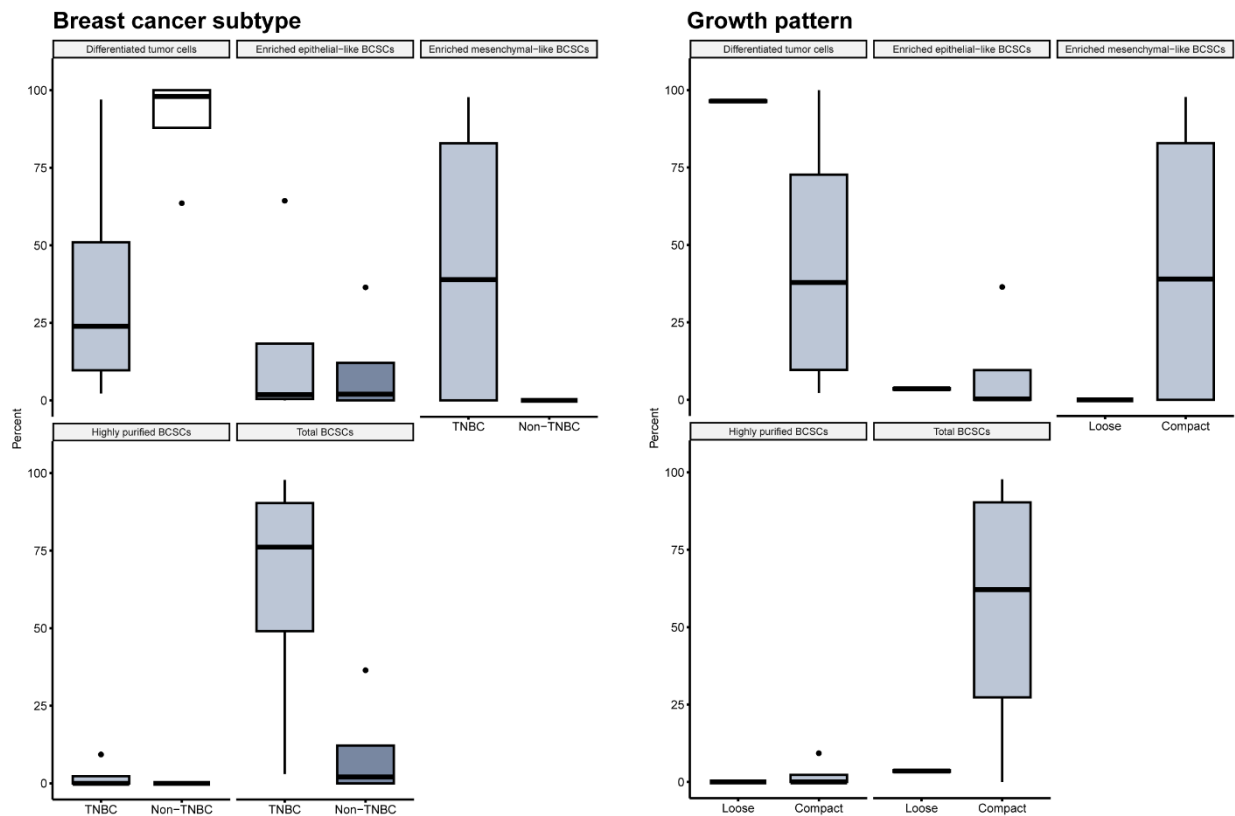
Supplementary Fig. 6 | Pathway analysis for BT-474 spheroids vs. 2D cells. Significant pathways (identified in at least two group comparisons) enriched in BT-474 day 10 (3D_D10) or day 20 (3D_D20) spheroids.



Supplementary Fig. 7 | Differences in expression for 12 canonical breast cancer stem cell markers

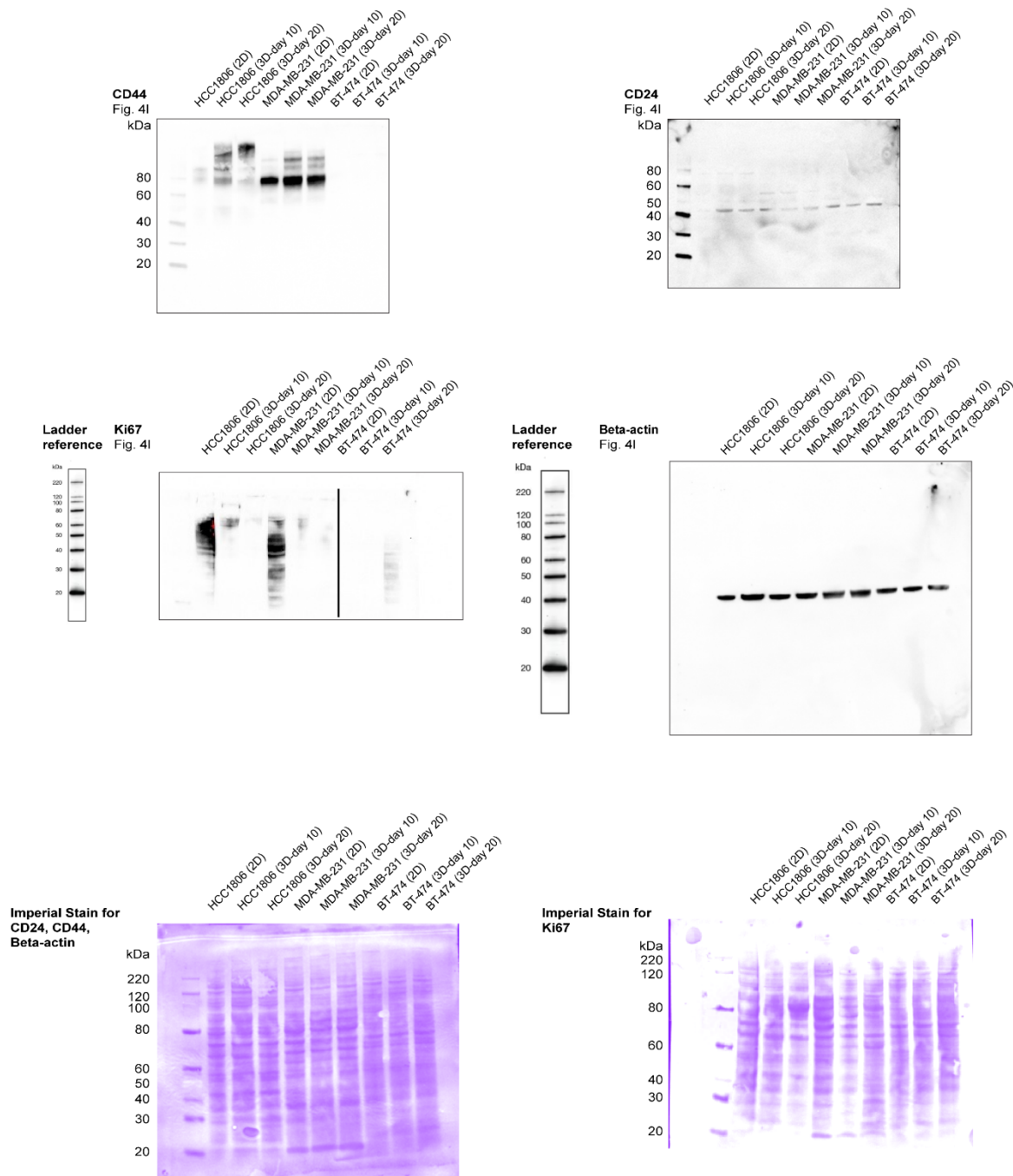
for BT-474 and HCC1806 spheroids vs. 2D cells. Normalized expression values were transformed using variance-stabilizing transformation (VST) and compared between groups with t-test. In BT-474 cells, *CD44* and *SOX2* were upregulated, while *POU5F1* was downregulated in 3D spheroids relative to 2D monolayers. In HCC1806 cells, *ERBB2* was upregulated, while *EPCAM* was downregulated in spheroids relative to 2D cells.

98 **Supplementary Figure 8**



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100 **Supplementary Fig. 8 | Proportion of differentiated tumor cells and breast cancer stem cell (BCSC)**
101 **subpopulations in the 8 breast cancer cell lines stratified by subtype (triple-negative breast cancer**
102 **[TNBC] vs non-TNBC) and spheroid growth pattern (compact vs. loose). A higher proportion of total**
103 **BCSCs (highly purified, enriched epithelial-like, and enriched mesenchymal-like BCSCs) and enriched**
104 **mesenchymal-like BCSCs, though non-statistically significant, were found in cell lines classified as TNBC**
105 **(n = 4) compared to non-TNBC (n = 4) and those forming compact spheroids (n = 4) compared to loose**
106 **spheroids (n = 2). The Wilcoxon rank-sum test was used to calculate statistically significant differences**
107 **(Benjamini-Hochberg adjusted p-values) between groups.**

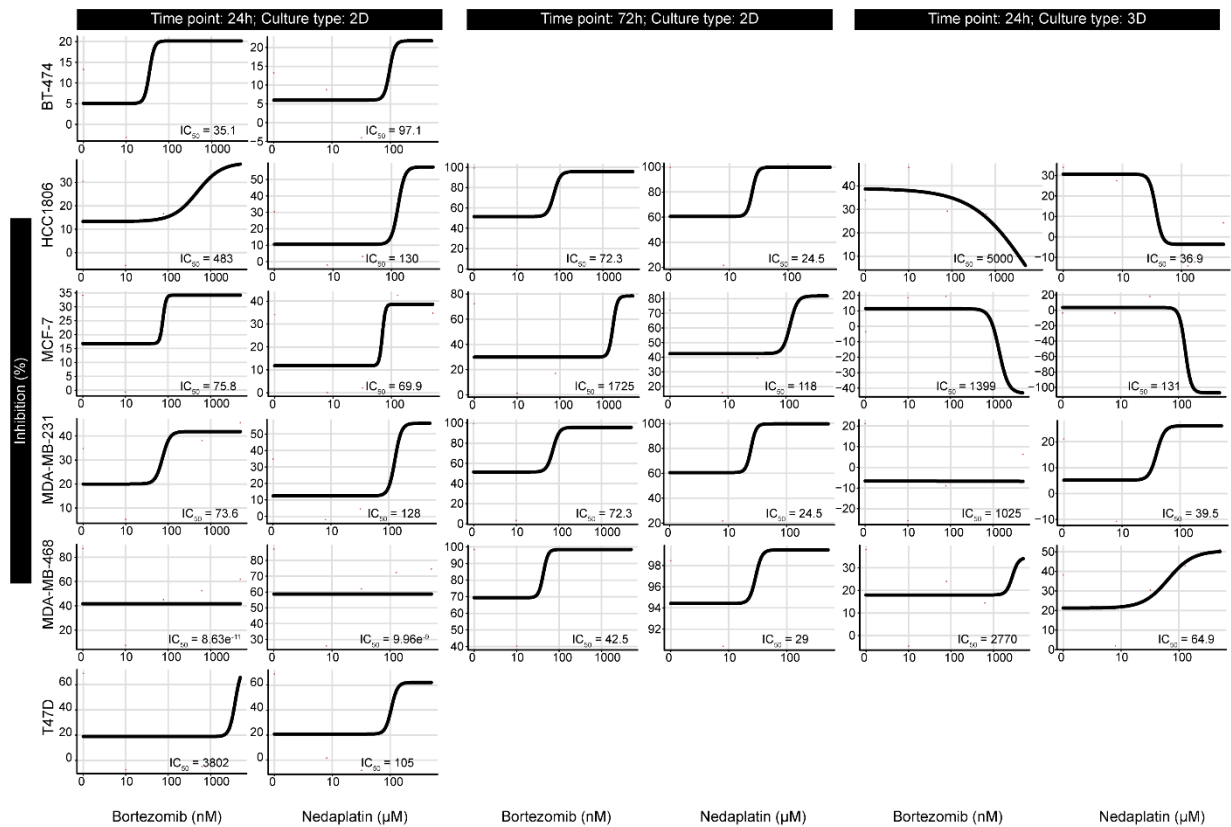
110 **Supplementary Figure 9**



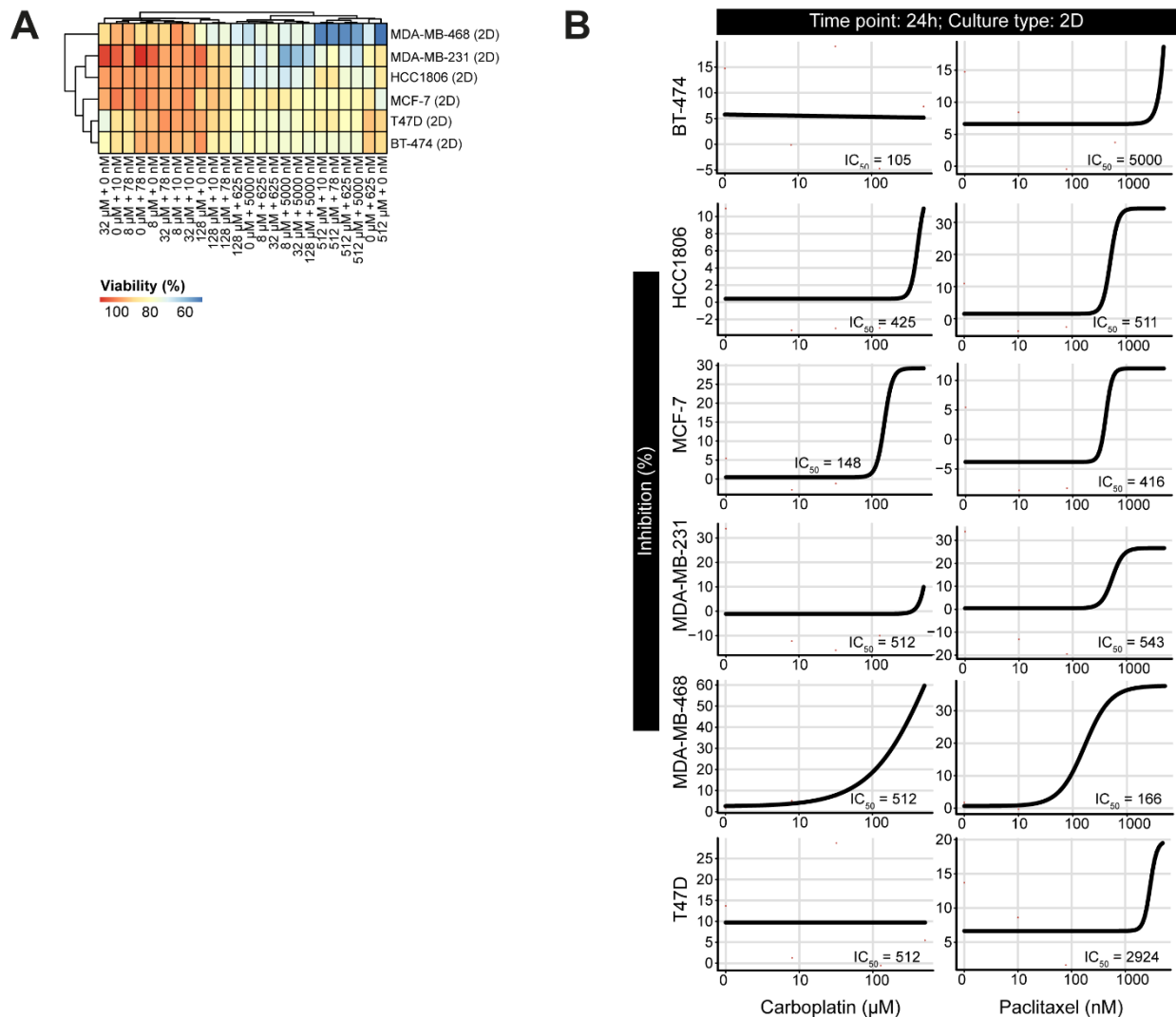
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112 **Supplementary Fig. 9 | Full-length Western blots for CD24, CD44, Ki67, and Beta-actin.** Western blot
113 was performed using primary antibodies (1:2000 dilution mouse Anti-beta actin [Abcam, Cat. ab6276], 1:500
114 dilution rabbit Anti-CD24 [Nordic Biosite, Cat. 10600-1-AP-150], 1:1000 rabbit Anti-CD44 [Abcam, Cat.
115 ab189524] or 1:1000 rabbit Anti-Ki67 [Abcam, Cat. ab16667]), followed by secondary antibodies (1:2000

dilution ECL anti-mouse IgG HRP-linked Whole Ab [Amersham, Cat. NA931V] or 1:2000 dilution ECL anti-rabbit IgG HRP-linked Whole Ab [Amersham, Cat. NA934V]). Since Beta actin, CD24, and CD44 were detected on the same membrane, the corresponding Imperial Protein Stain image to visualize total protein is identical for these three proteins and is shown once for clarity. Ki67 was detected on a separate membrane, and its corresponding Imperial Protein Stain image is shown separately. The molecular weight (kDa) for the MagicMark XP Western Protein Standard marker bands are indicated for each membrane. Since the marker bands for Beta actin and Ki67 are faint in the original blot images, the MagicMark XP standard is shown separately as a reference for molecular weight assignment. Boxes indicate the boundaries of the original membranes.

Supplementary Figure 10

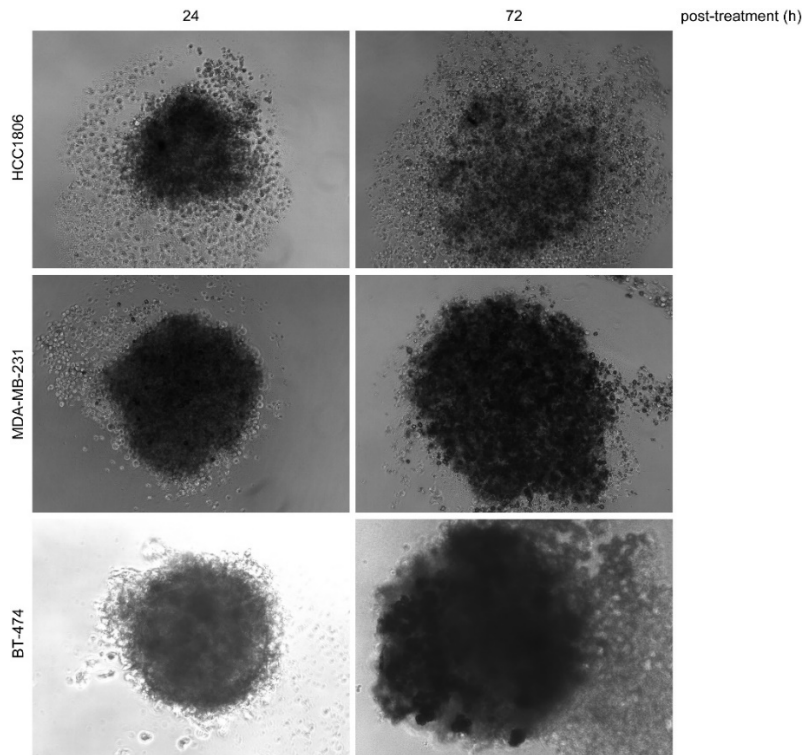


Supplementary Fig. 10 | Effect of bortezomib and nedaplatin treatment on 2D (24 and 72 hours) and 3D (24 hours) cell cultures. Inhibition (%) and half-maximal inhibitory concentration (IC₅₀) was determined after treatment using the SynergyFinder algorithm.



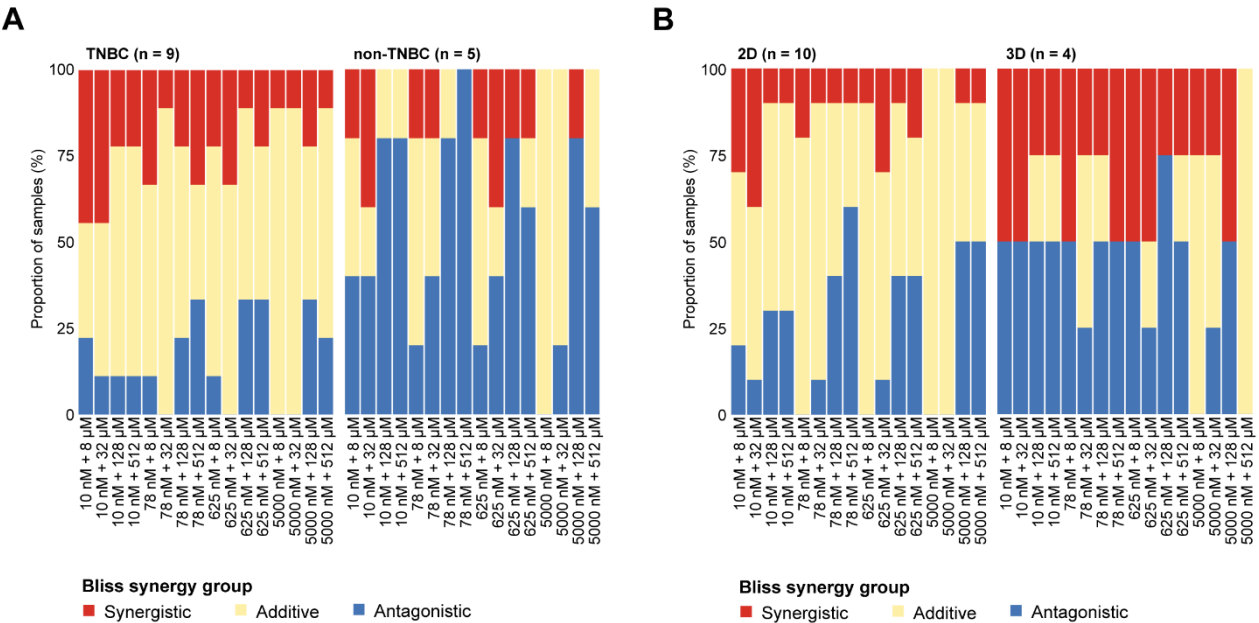
Supplementary Fig. 11 | Effect of Carboplatin+Paclitaxel (C+P) combination treatment on 2D cell cultures. A Heatmap showing viability (%) for cells treated with various doses of C+P. Doses for carboplatin ranged from 0 to 512 μM , while doses for paclitaxel ranged from 0 to 5000 nM. **B** Inhibition (%) and half-maximal inhibitory concentration (IC_{50}) was determined after treatment using the SynergyFinder algorithm.

Supplementary Figure 12



Supplementary Fig. 12 | Dissociation of cells from 3D spheroids after treatment. Phase-contrast images (10x objective) for HCC1806, MDA-MB-231, and BT-474 3D spheroids after 24- and 72-hours treatment with the highest Bortezomib+Nedaplatin (B+N) dose (5000 nM B + 512 μ M N). Spheroid dissociation is shown after treatment.

Supplementary Figure 13



Supplementary Fig. 13 | Stacked bar charts for the proportion of samples displaying Bliss synergy scores indicative of synergistic, additive or antagonistic based on subtype (TNBC vs non-TNBC) and culture type (2D vs 3D). Bliss synergy score ≥ 10 were classified as synergistic (red), $-10 > x < 10$ as additive (yellow), and ≤ -10 were considered as antagonistic (blue).

Supplementary Table 1. Cancer cell lines

	Cell line	Medium	Breast cancer subtype	TNBC subtype	Spheroid formation	BCSC content ¹
1	A172	DMEM, 10% FBS	-	-	Compact	Not tested
2	A375	DMEM, 1% sodium pyruvate, 10% FBS	-	-	Loose	Not tested
3	BT-474	DMEM, 10% FBS	Luminal B	-	Compact	<50% BCSCs
4	CAL-148	DMEM, 10% FBS	TNBC	LAR	None	>50% BCSCs
5	HCC1806	RPMI 1640, 10% FBS	TNBC	BL2	Compact	>50% BCSCs*
6	MCF-7	DMEM, 10% FBS	Luminal A	-	Loose	<50% BCSCs*
7	MDA-MB-231	DMEM, 10% FBS	TNBC	BL2	Compact	>50% BCSCs*
8	MDA-MB-468	RPMI 1640, 1% sodium pyruvate, 10% FBS	TNBC	BL1	Loose	<50% BCSCs*
9	T47D	DMEM, 10% FBS	Luminal A	-	Compact	0%
10	ZR-75-30	RPMI 1640, 10% FBS	HER2amp	-	None	0%

BCSC, breast cancer stem cell; BL1, basal-like 1; BL2, basal-like 2; DMEM, Dulbecco Modified Eagle's Medium high glucose; FBS, Fetal Bovine Serum; LAR, luminal androgen receptor; RPMI, Roswell Park Memorial Institute; TNBC, triple-negative; ¹DOI: <https://doi.org/10.3389/fcell.2023.1237673>. *BCSC content confirmed with flow cytometry using a new biological replicate with 2D monolayers and 3D spheroids.

Supplementary Table 2. Pharmaceutical compounds

Drug	Drug class	Solvent	Stock concentration	Working concentration	Supplier	Catalog no.
Bortezomib	Proteasome inhibitor	DMSO	1 mM	0, 10, 78, 625, 5000 nM	Selleckchem	S1013
Paclitaxel	Mitosis inhibitor	DMSO	1 mM	0, 10, 78, 625, 5000 nM	Selleckchem	S1150
Carboplatin	Platinum agent	Milli-Q water	25 mM	0, 8, 32, 128, 512 μ M	Selleckchem	S1215
Nedaplatin	Platinum agent	Milli-Q water	25 mM	0, 8, 32, 128, 512 μ M	Selleckchem	S1826

Working concentrations were prepared using 1x PBS. PBS was also used as the vehicle control for the 0 nM (bortezomib and paclitaxel) and 0 μ M (carboplatin and nedaplatin) conditions. DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline

Supplementary Table 3. Drug dose combinations

Drug combination	Plate number	Drug dose 1	Drug dose 2
Bortezomib + nedaplatin	Plate 1	10 nM	8 μ M
		78 nM	8 μ M
		625 nM	8 μ M
		5000 nM	8 μ M
		10 nM	32 μ M
		78 nM	32 μ M
		625 nM	32 μ M
		5000 nM	32 μ M
	Plate 2	10 nM	128 μ M
		78 nM	128 μ M
		625 nM	128 μ M
		5000 nM	128 μ M
		10 nM	512 μ M
		78 nM	512 μ M
		625 nM	512 μ M
		5000 nM	512 μ M
	Plate 3	0 nM	8 μ M
		0 nM	32 μ M
		0 nM	128 μ M
		0 nM	512 μ M
		10 nM	0 μ M
		78 nM	0 μ M
		625 nM	0 μ M
		5000 nM	0 μ M
Carboplatin + paclitaxel	Plate 1	8 μ M	10 nM
		32 μ M	10 nM
		128 μ M	10 nM
		512 μ M	10 nM
		8 μ M	78 nM
		32 μ M	78 nM
		128 μ M	78 nM
		512 μ M	78 nM
	Plate 2	8 μ M	625 nM
		32 μ M	625 nM
		128 μ M	625 nM
		512 μ M	625 nM
		8 μ M	5000 nM
		32 μ M	5000 nM
		128 μ M	5000 nM
		512 μ M	5000 nM
	Plate 3	0 μ M	10 nM
		0 μ M	78 nM
		0 μ M	625 nM
		0 μ M	5000 nM
		8 μ M	0 nM
		32 μ M	0 nM
		128 μ M	0 nM
		512 μ M	0 nM

170 **Supplementary Table 4. Reactome signaling pathways for HCC1806 spheroids (day 10**171 **[D10] and day 20 [D20]) vs 2D cells**

Pathway	HCC1806-3D_D10 vs HCC1806-2D	HCC1806-3D_D20 vs HCC1806-2D	HCC1806-3D_D20 vs HCC1806-3D_D10
Activation of Matrix Metalloproteinases			x
Acyl chain remodelling of PI			x
Adipogenesis			x
Anchoring fibril formation		x	
Assembly of collagen fibrils and other multimeric structures		x	x
Collagen chain trimerization		x	
Collagen degradation		x	x
Collagen formation		x	x
Crosslinking of collagen fibrils			x
Defective SLC40A1 causes hemochromatosis 4 (HFE4) (macrophages)	x		
Degradation of the extracellular matrix			x
Developmental Biology		x	x
Developmental Cell Lineages		x	x
Developmental Cell Lineages of the Integumentary System	x	x	x
Differentiation of Keratinocytes in Interfollicular Epidermis in Mammalian Skin	x	x	x
Downstream signaling of activated FGFR1	x		
Elastic fibre formation			x
Epigenetic regulation of adipogenesis genes by MLL3 and MLL4 complexes		x	
Epigenetic regulation of gene expression by MLL3 and MLL4 complexes		x	
Extracellular matrix organization			x
FGFR1 ligand binding and activation	x		
FGFR1c and Klotho ligand binding and activation	x	x	
Fibronectin matrix formation		x	x
Formation of the cornified envelope	x	x	x
FRS-mediated FGFR1 signaling	x		
IGF1R signaling cascade	x		
Interleukin-4 and Interleukin-13 signaling	x		
IRS-mediated signaling	x		
IRS-related events triggered by IGF1R	x		

Keratinization	x	x	x
MLL4 and MLL3 complexes regulate expression of PPARG target genes in adipogenesis and hepatic steatosis		x	
NCAM signaling for neurite out-growth		x	
NCAM1 interactions		x	
Negative regulation of FGFR1 signaling	x		
Phospholipase C-mediated cascade: FGFR1	x		
PI3K Cascade	x		
PI-3K cascade:FGFR1	x		
RUNX2 regulates genes involved in cell migration	x	x	
SHC-mediated cascade:FGFR1	x		
Signaling by FGFR1	x		
Signaling by NOTCH3			x
Transcriptional regulation of white adipocyte differentiation		x	
Triglyceride catabolism			x
Triglyceride metabolism			x

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174 **Supplementary Table 5. Reactome signaling pathways for BT-474 spheroids (day 10 [D10]**

175 **and day 20 [D20]) vs 2D cells**

Pathway	BT474-3D_D10 vs BT474-2D	BT474-3D_D20 vs BT474-2D	BT474-3D_D20 vs BT474-3D_D10
Assembly of collagen fibrils and other multimeric structures	x	x	
Attachment and Entry		x	
Collagen formation	x		
Defective SFTPA2 causes IPF	x	x	
Degradation of the extracellular matrix	x	x	
Developmental Cell Lineages	x	x	
Developmental Cell Lineages of the Exocrine Pancreas	x	x	
Developmental Cell Lineages of the Integumentary System		x	
Developmental Lineage of Mammary Gland Myoepithelial Cells		x	
Developmental Lineage of Pancreatic Ductal Cells		x	
Differentiation of Keratinocytes in Interfollicular Epidermis in Mammalian Skin		x	
Extracellular matrix organization	x	x	

Miscellaneous transport and binding events		x
Neurophilin interactions with VEGF and VEGFR		x
NFE2L2 regulating MDR associated enzymes	x	
NGF-stimulated transcription	x	
Nuclear Events (kinase and transcription factor activation)	x	
Reactions specific to the hybrid N-glycan synthesis pathway		x
Regulation of thyroid hormone activity	x	x
SDK interactions	x	x
Sensory perception of salty taste	x	
Signal transduction by L1		x
Signaling by NTRK1 (TRKA)	x	
Signaling by NTRKs	x	
Specification of primordial germ cells	x	x
Stimuli-sensing channels	x	
Thyroxine biosynthesis		x
TP53 Regulates Transcription of Death Receptors and Ligands	x	
Transport of RCbl within the body	x	
Type I hemidesmosome assembly	x	x

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