# Identification and Pharmacological Targeting of Treatment-Resistant, Stem-like Breast Cancer Cells for Combination Therapy

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## Abstract

Tumors frequently harbor isogenic yet epigenetically distinct subpopulations of multi-potent cells with high tumor-initiating potential—often called Cancer Stem-Like Cells (CSLCs). These can display preferential resistance to standard-of-care chemotherapy. Single-cell analyses can help elucidate Master Regulator (MR) proteins responsible for governing the transcriptional state of these cells, thus revealing complementary dependencies that may be leveraged via combination therapy. Interrogation of single-cell RNA sequencing profiles from seven metastatic breast cancer patients, using perturbational profiles of clinically relevant drugs, identified drugs predicted to invert the activity of MR proteins governing the transcriptional state of chemoresistant CSLCs, which were then validated by CROP-seq assays. The top drug, the anthelmintic albendazole, depleted this subpopulation *in vivo* without noticeable cytotoxicity. Moreover, sequential cycles of albendazole and paclitaxel—a commonly used chemotherapeutic —displayed significant synergy in a patient-derived xenograft (PDX) from a TNBC patient, suggesting that network-based approaches can help develop mechanism-based combinatorial therapies targeting complementary subpopulations.

## Introduction

Intratumor heterogeneity represents a major barrier in cancer treatment. Indeed, most tumors comprise co-existing, molecularly distinct subpopulations presenting non-overlapping drug sensitivities[1]. While some of the cells comprising them may represent genetically distinct subclones, a majority has emerged as representing the byproduct of pathophysiological epigenetic plasticity. In breast cancer (BRCA), for instance, there have been multiple reports of an isogenic Cancer Stem-like Cell (CSLC) subpopulation associated with differential expression of epigenetic regulators involved in controlling stemness programs, such as the BMI1, WNT, and NOTCH pathways[2-4]. CSLCs have been shown to display tumor-initiating capacity, expression of stem-cell markers, and resistance to common chemotherapeutics[5,6], such as paclitaxel—a microtubule inhibitor and antimitotic widely used in the treatment of multiple malignancies, including breast cancer. Indeed, while frequently leading to initial tumor shrinkage, treatment with this drug is often followed by relapse and resistance. Indeed, it has been suggested that chemotherapy resistant breast CSLCs may regenerate the full heterogeneity of the tumor, as confirmed by limiting dilution assays[7,8]. Multiple non-mutually exclusive mechanisms of chemotherapy resistance have been proposed for CSLCs in breast and other tumors, including upregulation of multi-drug transporters, increased DNA damage repair, and better scavenging of ROS[9-11]. Taken together, these data suggest that breast CSLCs pose a fundamental challenge to achieving durable remissions in BRCA, especially in Triple Negative Breast Cancer (TNBC), where chemotherapy remains a cornerstone of treatment.

To gain insight into the molecular heterogeneity of breast cancer and to predict the sensitivity of individual subpopulations to clinically relevant drugs, we generated single-cell RNA sequencing (scRNA-seq) profiles of malignant cells isolated from biopsies of seven metastatic breast cancer patients. To enrich for cells with a stem-like phenotype—or CSLCs for simplicity—which may include only a very small fraction of tumor cells, we used fluorescence-activated cell sorting (FACS), with antibodies selected to purify malignant cells with a phenotype analogous to that of stem/progenitor cells in the normal mammary epithelium[12]. See **Fig. 1A** for an illustrative graphical workflow of this process.

In previous studies, we have shown that highly sparse single scRNA-seq profiles, where >80% of the genes may produce no reads, can be transformed to fully populated protein activity profiles by the metaVIPER algorithm[13]—the single-cell adaptation of the extensively validated VIPER algorithm[14]. This is accomplished by measuring the activity of each regulatory and signaling

protein based on the expression of its entire repertoire of transcriptional targets, akin to using a highly multiplexed, tissue-specific gene reporter assay. As a result, the most differentially active VIPER-inferred proteins are also enriched for Master Regulator (MR) proteins representing mechanistic determinants, via their target genes, of the associated transcriptional state.

MetaVIPER analysis of single cells isolated from the seven metastatic breast cancer patients accrued to the study—including five hormone receptor-positive (HR+) and two triple-negative (TNBC) tumors—effectively separated cells with a more stem-like vs. more differentiated transcriptional state, using a *stemness score* (*SS*) based on both established breast cancer stemness markers and CytoTRACE analysis[15]. Consistent with expectations, cells with the highest score (i.e., most stem-like) emerged as the most resistant to *in vivo* treatment with paclitaxel, while those with the lowest score (*i.e.*, most differentiated-like) were significantly depleted by the drug. This provided the molecular basis to identify and genetically/pharmacologically target candidate Master Regulators (MRs) of CSLC transcriptional state(s) identified by metaVIPER analysis.

We thus performed patient-by-patient analysis, using the VIPER algorithm to identify candidate MR proteins controlling the transcriptomic state of cells with the highest vs. lowest Stemness Score. Candidate MRs identified by the analysis were highly conserved across virtually all patients, independent of hormone receptor (HR) status, thus supporting the notion of a common CLSC MR signature. Indeed, >80% of the most significant VIPER-inferred activated and inactivated MRs were able to statistically significantly reprogram cells to a more differentiated or CSLC state, respectively, following their CRISPR-mediated silencing in a pooled CROP-seq[16] assay in cell lines comprising both subtypes. We thus leveraged the OncoTreat algorithm[17], which assesses the activity of MR proteins in drug vs. vehicle control-treated cells, to identify small molecule compounds capable of inverting the activity of the CLSC MR signature (MR-inverter drugs), thus potentially inducing differentiation or selective ablation. For this purpose, we leveraged gene expression profiles of BRCA cells—selected to faithfully recapitulate the CSLC MR signature—treated with a repertoire of 91 clinically relevant drugs, see **Fig. 1B** for an illustrative graphical workflow of these steps. Notably, OncoTreat-predicted drugs from either bulk[17-19] or single-cell profiles[20,21] have been extensively validated *in vivo* in prior studies.

Albendazole, a well-tolerated anthelmintic drug, emerged as the most statistically significant MR-inverter drug, yet at a concentration that was approximately ten-fold lower than its clinically tolerated dose; this was especially surprising since albendazole is not considered an anti-tumor

drug. Based on these results, this drug was selected for experimental validation *in vivo*. Mice from a TNBC PDX model were treated with either albendazole or vehicle control for 14 days and compared to paclitaxel-treated animals. In contrast to paclitaxel, which caused highly significant increase of the CSLC to differentiated cell ratio, albendazole treatment induced equally dramatic yet opposite effects, suggesting that alternating treatment with the two drugs may abrogate the tumor-initiating potential of paclitaxel-resistant cells, while also preventing uncontrolled tumor growth. The strong rationale for combination-based, sequential therapy was confirmed by a preclinical study, where treatment with multiple cycles of albendazole and paclitaxel displayed superior anti-tumor activity compared to the corresponding monotherapies, resulting in a statistically significant synergistic effect.

## Results

**Intratumor heterogeneity in human breast carcinomas:** Since patient-derived breast cancer tissues vary widely in size, cellularity, necrotic fraction, stromal infiltration, and overall quality, we used FACS to purify malignant cells using appropriate antibody combinations. Single cells isolated from these tumors were then processed to generate plate-based scRNA-seq profiles using an approach that combines elements of Smart-seq2[22] and PLATE-seq[23] (see STAR methods). This procedure, which allows sorting individual cancer cells into separate wells filled with lysis buffer for RNA-seq profiling, is especially effective in enriching for relatively rare subpopulations from fresh tumor tissue, since it effectively supports FACS-based cell isolation while removing debris and dead cells that may otherwise degrade the performance of other platforms. It was thus preferred at the time, despite its higher cost and complexity.

Fresh samples were obtained from two metastatic TNBC and five metastatic $HR^+$ patients. To minimize post-resection transcriptional changes/drift, fresh samples were rapidly dissociated into a single-cell suspension (see STAR methods) and stained with DAPI, as well as $\alpha$-EpCAM, $\alpha$-CD49f, and $Lin^-$ antibodies. EPCAM effectively distinguishes epithelial breast cancer cells from stromal subpopulations, whereas CD49f is known to be expressed at the highest levels in a subset of mammary epithelial cells acting as mammary repopulating units (MRUs) in transplantation assays[12] and has been previously used to enrich for breast cancer cells with stem-like properties[24-26]. Starting from primary malignant tissues, we sorted live ($DAPI^-$) epithelial ($EpCAM^+$) cells into two distinct batches, including: (1) a first batch of unselected cancer cells ($EPCAM^+$), representative of the full heterogeneity of the epithelial compartment, contributing ~25% of the total cells in the analysis and (2) a second batch of epithelial cells with a phenotype characteristic

of MRUs in the mouse mammary gland (EPCAM$^{+}$, CD49f$^{high}$), expected to be CSLCs-enriched[12,24-26], contributing the remaining ~75% of analyzed cells (**Fig. 1A, Suppl. Fig. S1**).

**Copy Number Variation Analysis:** After NGS library generation and sequencing (see STAR methods), we performed several data pre-processing steps to ensure that subsequent analyses would be restricted to high-quality cancer cells. This included inference of somatic copy number alteration (CNA) assessment, using the Trinity CTAT Project inferCNV algorithm (https://github.com/broadinstitute/inferCNV) to exclude confounding effects from normal cells in the tumor microenvironment. Compared to cells representative of normal breast epithelium, most of the cells isolated from the seven patients presented clearly aberrant CNA structure, consistent with the high cellularity of metastatic samples (**Suppl. Fig. S2**). Interestingly, no intratumor CNA heterogeneity was detected by the analysis, suggesting that, at least from a copy number alteration perspective, the cells in these samples were clonally identical. However, as expected, the analysis showed significant inter-tumor CNA heterogeneity across the seven patients, especially between TNBC and HR+ samples.

**Protein activity-based analysis identifies a stem-like subpopulation:** In addition to biological variation between tumors from different patients, substantial batch and biology-related effects may also challenge the analysis of single cells isolated from different samples. Batch effects can arise due to technical artifacts, such as changes in temperature or reagents between samples processed on different days, or liquid handling drift in multi-well plate assays. In addition, inter-patient CNA differences may also contribute to significant gene expression heterogeneity, which may confound the analysis. Indeed, while only a handful of genes in CNAs play a functional role in tumorigenesis, most of the genes in these amplicons may still produce substantial inter-patient bias at the gene expression level, even though the activity of their encoded proteins is ultimately buffered by the post-transcriptional autoregulatory logic of the cell. When combined with the high *gene dropout* rate of scRNA-seq profiles—where >75% of the genes may fail to be detected by even a single read—this limits the ability to perform detailed, quantitative analyses using traditional gene expression-based methodologies.

Various approaches to reduce noise and minimize gene dropout effects have been proposed[27,28]—such as metaCells[29] and imputation-based[30] methods—as well as normalization methods aimed at reducing batch effects[31,32]. These methodologies, however, may introduce artifacts that affect subsequent analyses. For instance, using metaCells may prevent identification of rare subpopulations, whose gene expression profile would be averaged with cells from

molecularly distinct subpopulations, while normalization may reduce biologically relevant differences between samples. Most critically, generating a comprehensive repertoire of candidate molecular determinants of tumor cell state, potentially associated with differential expression of only a handful of genes, is quite challenging if the expression of most genes is undetectable in individual cells. Transcriptional regulators, which are critical in maintaining cell state/identity, are especially affected by such *gene dropout* issues because they can be functionally active even when expressed at very low levels.

To address these challenges, we leveraged the PISCES single-cell analysis pipeline[33], which provides a systematic framework for protein activity-based analysis of single-cell data—from raw counts quality control to construction of gene regulatory networks, to the identification of MR proteins (see STAR methods). Specifically, PISCES leverages the metaVIPER[13] algorithm to measure a protein's differential activity based on the differential expression of its transcriptional targets, as inferred by the ARACNe[34] algorithm. These algorithms have been extensively validated, showing low false positive rates (in the 20% – 30% range)[14,35,36] and almost complete elimination of technical (i.e., non-biologically-relevant) batch effects. In particular, we have recently shown that metaVIPER protein activity measurements significantly outperform gene expression and even antibody-based measurements in single cells[20,37,38], including based on large-scale CITE-seq assays[33].

We used metaVIPER to infer protein activity of single cells isolated from breast cancer biopsies from the two TNBC and five HR+ patients described in the previous section. The relative tumor purity of metastases, combined with EPCAM-based flow cytometry sorting produced single cells that were virtually all tumor related, as shown by the inferCNV analysis (**Suppl. Fig. S2**). As a result, we used metaVIPER to integrate results from both a bulk-level ARACNe network—generated from the TCGA breast cancer cohort—as well as a network generated from the scRNA-seq profiles captured in this study (see STAR methods). This approach allows optimal dissection of tumor cell-specific interactions (from single-cell profiles), while still providing adequate coverage (from bulk profiles) of the transcriptional targets of regulatory proteins that are undetectable in single-cell profiles.

MetaVIPER computes the normalized enrichment score (NES) of a protein's targets in genes differentially expressed between each individual cell and a reference state, typically the centroid of the entire single-cell population (see STAR methods). As a result, positive and negative NES scores indicate higher and lower protein activity compared to the average of the single-cell

population, respectively. While VIPER is most effective in assessing the activity of regulatory proteins, we have shown that it can quantitate the differential activity of signaling proteins[14,39] and surface markers[37] with similar accuracy. As a result, we included 339 cell surface markers and 3,407 signaling proteins in the analysis (see STAR methods for selection criteria).

Due to the large-scale CNA differences detected by the analysis, inter-patient heterogeneity was highly dominant at the gene expression level, with almost each patient contributing to an independent cluster in a Principal Component Analysis (PCA) representation, using the 5,000 genes with the highest standard deviation (**Fig. 2A**). In contrast, since VIPER-inferred protein activity is robust to noise and resilient to technical artifacts that are inconsistent with the underlying regulatory network[13] (**Fig. 2B**), protein activity-based PCA analysis virtually eliminated inter-patient variability, except when biologically relevant (**Fig. 2C**). For instance, differences linked to HR status were captured by the second principal PCA component (y-axis), which accounts for 15% of cross-cell variability. Yet, the most significant source of variance, accounting for 31% of cross-cell variability, was captured by the first PCA component (x-axis), which could be associated with high vs. low stemness (**Fig. 2D**).

Cell stemness was assessed using two complementary metrics, including (a) the global activity of established breast CSLC markers and (b) CytoTRACE[15], an experimentally validated algorithm designed to infer stemness based on gene count signature analysis (**Fig. 2D**, see STAR methods). CytoTRACE was previously validated within a hematopoietic lineage context and is based entirely on assessing expressed gene counts (a rough measure of cell entropy) rather than specific knowledge of stem cell biology. As a result, it has shown limitations, for instance, in differentiating quiescent stem cells from cycling progenitor cells[15]. To address this issue we complemented and compared the CytoTRACE analysis with biologically-relevant insights derived from the VIPER-measured activity of 14 previously reported CSLC markers, including CD44+/CD24-[40], ITGA6 (CD49f)[26], BMI1[4], SALL4[41], NOTCH1[42], NOTCH2[42], KLF4[43], CTNNB1[44], ITGB3 (CD61)[45,46], ITGB1[47], PROM1 (CD133)[48], POU5F1 (OCT4)[49], SOX2[50], and KIT[51], resulting in a *consensus Stemness Score*, ranging from $SS = 0$ (most differentiated) to $SS = 1$ (most CSLC), shown as a color gradient in **Fig. 2D** (see STAR methods). Supporting the use of such consensus metric, the CytoTRACE and CSLC marker-based scores were highly correlated despite being assessed by completely independent methodologies (Spearman's $\rho = 0.43$, $p \leq 2.2\times10^{-16}$) (**Suppl. Fig. S3**).

Despite the potential noisy nature of single-cell data, the PCA plot region comprising CD49f$^{high}$ cells was strongly associated with high activity of other established markers of stem-like function in mammary epithelial cells, such as BMI1[4] and NOTCH1/2[42], among several others, critically in both TNBC and HR+ derived cells (**Fig. 2E, Suppl. Fig. S4**). Consistent with the literature[48,52-54], activity of additional stemness markers such as PROM1, POU5F1, SOX2, and KIT was also more prominent in CD49f$^{high}$ cells from TNBC patients (**Suppl. Fig. S4**). Differential activity of metabolic CSLC markers, such as ALDH1[55], was not detectable, likely because these enzymes are less related to transcriptional regulation.

In sharp contrast to VIPER-based analyses—and fully consistent with prior studies, see[20,37,38] for instance—the expression of genes encoding for these markers was mostly uninformative and failed to provide insight into CSLC characterization, because of the drastic gene dropout effect associated with scRNA-seq profiles (**Suppl. Fig. S5**). For instance, despite having a clear readout at the protein activity level, CD44, ITGB3, and SOX2 generated virtually no reads, thus preventing meaningful assessment of their differential expression, while expression of most other markers could not be associated to specific regions of the PCA plots.

Differential activity of subtype-specific markers was also evident for cells isolated from HR+ vs. TNBC patients, especially within the differentiated cell compartment. For instance, the activity of luminal markers, such as GATA3[56,57], FOXA1[58], the estrogen (ESR1) and progesterone (PGR) receptors, was markedly higher in differentiated HR+ derived cells (**Fig. 2F, Suppl. Fig. S6A**), while the activity of TNBC markers, such as FOXC1[59,60] and BCL11A[61], as well as basal cytokeratin (KRT17), and vimentin (VIM)[62,63], was higher in differentiated TNBC derived cells (**Fig. 2F**, **Suppl. Fig. S6B**). To provide an objective baseline we leveraged KRT19, an established marker of luminal differentiation, whose NUMB-mediated interaction with WNT/NOTCH pathways is well documented[64,65] and whose differential protein activity and differential gene expression could be effectively assessed in single cells. Indeed, differential expression of *KRT19* was highly consistent with metaVIPER-measured KRT19 activity (**Suppl. Fig. S7A-C**), confirming VIPER-based identification of luminal vs. basal cells. Compared to other cancers, such as colon cancer[65], KRT19 holds special relevance in breast cancer, where its attenuated expression is strongly associated with poor prognosis and stemness[64,65]; consistent with these findings, KRT19 activity was also significantly lower in the PCA region associated with highest stemness (**Fig. 2F**).

**VIPER-inferred CSLCs are insensitive to paclitaxel:** Rather than assessing self-renewal and multipotency as characteristics of *bona fide* CSLC state—still a rather controversial topic—we

focused on the more pragmatic and objective assessment of the differential sensitivity to paclitaxel by cells identified as CSLC by our analysis, which presents critical relevance to patient treatment. For this purpose, we analyzed single cells dissociated from PDX models established by transplantation of a human primary TNBC in the mammary fat pad of immunodeficient NOD/SCID/IL2R$\gamma^{-/-}$ (NSG) mice, which were treated with either vehicle control or paclitaxel for 14 days after reaching a tumor volume of 100 $mm^3$ (**Fig. 3A**; see STAR methods).

First, we assessed the fidelity of PDX-derived, single-cell subpopulations to those dissociated from human samples. Single-cell analysis of a vehicle control-treated mouse confirmed prior findings from patient-derived samples. Specifically, based on protein activity analysis with metaVIPER, the 1st principal component (PC1) was again associated with cell differentiation and significantly correlated with both CytoTRACE score (Spearman's $\rho = 0.65$, $p \leq 2.2\times10^{-16}$, **Suppl. Fig. S8A-B**) and with overall activity of the 14 CSLC markers ($\rho = 0.90$, $p \leq 2.2\times10^{-16}$, **Suppl. Fig. S8C**). More importantly, there was a highly significant overlap of proteins differentially active in cells with the highest vs. lowest Stemness Score in PDX vs. human samples, as evaluated by GSEA analysis (OncoMatch algorithm[18]) (NES = 7.97, $p = 1.6\times10^{-15}$). Finally, based on GSEA analysis of MSigDB hallmarks[66], genes encoding for proteins associated with the 1st PC were highly enriched in hallmarks associated with cell developmental processes such as epithelial-mesenchymal transition and myogenesis ($p = 3.4\times10^{-4}$ and $p = 1.9\times10^{-3}$, respectively) as well as PI3K-AKT-mTOR[67] ($p = 9.8\times10^{-4}$), KRAS[68] ($p = 2.0\times10^{-3}$), and P53[69] ($p = 2.0\times10^{-3}$) pathways (**Suppl. Table 1**).

Consistent with data from primary tumor tissues, differential expression of most CSLC markers in single cells isolated from PDX tissue was not informative or undetectable (**Suppl. Fig. S9**). However, at the protein activity level, the PCA regions with the highest activity of different CSLC markers—including CD49f, BMI1, $CD44^+/CD24^-$ and NOTCH1/2—were largely overlapping in both human and mouse samples (**Fig. 3B**). Putative CSLCs from PDX samples (i.e., with highest Stemness Score) also presented high activity and expression of the established quiescent breast CSLC marker BIRC5[70] (Spearman's $\rho = 0.53$, $p \leq 2.2\times10^{-16}$, **Suppl. Fig. S10**) and lower activity and expression of E2F family proteins ($\rho = -0.69$, $p \leq 2.2\times10^{-16}$, **Suppl. Fig. S11**), which transactivate genes for G1/S transition[71]. These differences were likely more evident in PDX samples because of faster growth kinetics, as compared to primary human tumors. These data suggest that CSLC are more quiescent than differentiated cells, thus providing additional rationale

for their paclitaxel resistance. Taken together, these data characterize the PDX as a high-fidelity model to study CSLC vs. differentiated cells[18].

Changes in CSLC vs. differentiated cell density following drug treatment were then assessed by computing the normalized ratio between the number of cells with the highest (SS $\geq$ 0.8, most CSLC) and lowest (SS $\leq$ 0.2, most differentiated) Stemness Score in paclitaxel vs. vehicle control-treated samples, see STAR methods. Paclitaxel treatment induced striking depletion of differentiated cells vs. CSLCs (**Fig. 3C**) ($p = 2.6\times10^{-4}$, by Fisher's exact test), thus confirming the expected paclitaxel resistance of CSLC compartment cells identified by VIPER analysis.

Since the PDX was derived from a TNBC tumor, the 2$^{nd}$ PC could not be associated with HR status, as shown instead across the original 7 patient-derived samples. Rather, GSEA analysis revealed enrichment in two key categories, including cellular responses to DNA damage and oxidative stress, two hallmarks of paclitaxel mechanism of action ($p = 1.9\times10^{-11}$ and $p = 2.4\times10^{-12}$, respectively, by GSEA) (**Suppl. Table 1**)[72-74]. Indeed, the cells that were least affected by the drug were those presenting both high stemness score and a low proliferative potential (upper right quadrant on the PCA plot). Yet, for any given value of the PC2 metagene, predicted CSLC were always less sensitive to treatment than their differentiated counterpart. Indeed, the density of cells with the highest stemness score was virtually unaffected by treatment.

**MR Analysis of human breast cancer cells.** VIPER analysis has been effective in identifying candidate MR proteins representing mechanistic determinants of cell state[75,76], as well as clinically validated biomarkers [77-81], see[82] for a recent perspective. Critically, we have shown that VIPER-inferred MRs are highly enriched in tumor-essential genes[75,76,83], such that their pharmacologic targeting can abrogate tumor viability *in vivo*[17-19]. Equally important, we have shown that genetic or pharmacologic targeting of MRs that are differentially active in molecularly distinct transcriptional states can effectively reprogram cells between these states[20,84,85]. This suggests that elucidating candidate MRs of breast CSLC state may help identify drugs that either selectively ablate paclitaxel-resistant cells or reprogram them to a paclitaxel-sensitive state, thus providing a rationale for combination therapy.

To discover the most conserved CSLC MRs across the available metastatic samples, we first leveraged metaVIPER to identify proteins whose transcriptional targets were most differentially expressed in the 20 cells with the highest vs. the 20 with the lowest Stemness Score in each individual patient, as well as in the PDX model, on an individual sample basis (see STAR

methods). As discussed, the most differentially active proteins are also those expected to be most likely to mechanistically regulate the cell state of interest, via their transcriptional targets. As previously shown[18,19], the PDX model was included in the analysis to help prioritize MR-inverter drugs that are conserved in a model that may be leveraged for drug validation *in vivo*.

As discussed, CytoTRACE was originally developed and validated only in a hematopoietic linage context[15]. As a result, for MR elucidation purposes, we decided to rely only on the differential activity of the 14 CSLC markers, including CD44+/CD24-, ITGA6, BMI1, SALL4, NOTCH1, NOTCH2, KLF4, CTNNB1, ITGB3, ITGB1, PROM1, POU5F1, SOX2, and KIT (see STAR methods). Indeed, while the enrichment of breast CSLC and stem-related markers in differentially active protein was still significant when CSLC were predicted by CytoTRACE analysis (NES = 2.57, $p = 10^{-2}$), statistical significance increased substantially when relying only on the established CSLC markers (NES = 4.66, $p = 3.2\times10^{-6}$). Nevertheless, confirming that this choice has only minimal effects on MR analysis, statistically significantly MR proteins ($p \leq 10^{-3}$, Bonferroni corrected) were highly overlapping when CytoTRACE was included or excluded from the analysis ($p \leq 1.2\times10^{-44}$, by hypergeometric test).

Surprisingly, independent analysis of each patient and of the PDX model produced highly consistent MR predictions, including across HR+ and TNBC samples (**Fig. 4A, Fig. S12A-B**), suggesting that CSLC MR proteins are conserved independent of tumor HR status. This provided the rationale for the generation of a consensus CSLC MR signature, obtained by ranking all proteins by integrating their metaVIPER NES across all samples, using the weighted Stouffer's method (**Fig. 4B**, see STAR methods). Based on this analysis, in addition to the original 14 CSLC markers, other proteins broadly associated with stem cell processes—including ALDH family[86,87], ABC family[87], quiescent stem-cell markers (FGD5[88] and HOXB5[89]), embryonic diapause[90] and asymmetric cell division processes[91] (**Suppl. Table 2**)—also emerged as significantly enriched among the most differentially active proteins ($p = 2.0\times10^{-12}$) (**Suppl. Fig. S13**).

These results suggest that several of the most statistically significant differentially active proteins, not previously associated with breast CSLCs, may represent novel, *bona fide* MRs and potential biomarkers (**Fig. 4C and Fig. S14-18**, see also **Suppl. Table 3**), as later confirmed by CRISPR/Cas9-mediated KO (see next section). Among cell membrane-presented proteins, which may be leveraged for CSLC enrichment purposes, the analysis identified Integrin beta-8 (ITGB8) as the second most differentially active protein (after CD49f). ITGB8 was previously suggested as a marker of glioblastoma CSLCs[92] and was identified as a prime receptor binding a latent

complex of transforming growth factor beta 1 and beta 3 (TGF-β1/β3) in the extracellular matrix, responsible for activating TGF-β-associated signaling. Despite its role in tumor suppression in the early stages of tumorigenesis, TGF-β has been shown to prompt stem-like properties in advanced cancers and to increase chemotherapy resistance by promoting DNA damage response pathway activation[93-95].

**MR Modularity Analysis:** A key question in network-based analyses is whether—similar to what has been shown in other contexts[75,76,96]—candidate MRs may comprise hyper-connected, autoregulated modules providing coordinated, homeostatic cell state regulation. For this purpose, we assessed whether metaVIPER-inferred CSLC MRs were statistically significantly enriched in protein-protein and transcriptional interactions—as reported in PrePPI[97], STRING[98], and ARACNe-based networks—compared to an equivalent number of same-class proteins selected at random. The analysis revealed that the top 20 CSLC MRs form a highly hyperconnected module, with 67 MR-MR interactions, compared to only 13.2 detected on average in an equal size set of randomly selected proteins ($p = 6.6\times10^{-7}$). This supports the potential role of this module as a homeostatic On/Off switch controlling CSLC state (**Fig. 4D**), further suggesting that its inactivation may induce transition toward a more differentiated, paclitaxel-sensitive state.

**CSLC MR validation by pooled, CRISPR-KO-mediated CROP-seq analysis:** To validate the CSLC MRs inferred by these analyses, CRISPR droplet sequencing (CROP-seq) was used to assess whether KO of the 25 most significant MR of CSLC state ($MR_{CSLC}$, i.e., most active proteins in CSLC vs. differentiated cells) and 25 most significant MRs of differentiated state ($MR_{DIFF}$, i.e., most active proteins in differentiated cells vs. CSLCs) would induce reprogramming towards a more or less differentiated cell state, respectively. To optimally assess reprogramming, we selected two breast cancer cell lines that most effectively recapitulate the CSLC state, also assuming that all cell lines comprise differentiated cells. For this purpose, we assessed the enrichment of proteins in the consensus CSLC MR signature in proteins differentially active in each CCLE breast cancer cell line (based on bulk RNA-seq analysis), and ranked them from the one with the highest NES (HCC1143)—*i.e.*, most likely to be enriched in CSLCs—to the one with the most negative NES (VP229)—*i.e.*, most likely to be enriched in differentiated cells—(**Suppl. Fig. S19**). We then selected two of the most CSLC-enriched cell lines for CROP-seq assays, including HCC1143 (ranked No. 1) and HCC38 (ranked No. 3), which were also supported by literature evidence on CSLC content[99,100]. Single-cell analyses confirmed that both cell lines had substantial CSLC representation, compared to two of the most differentiated cell lines (MCF7 and

HCC2557), with HCC1143 presenting a greater fraction of differentiated cells compared to HCC38, potentially due to spontaneous differentiation in culture conditions (**Suppl. Fig. S20**).

The primary objective of CRISPR-Cas9-mediated gene knockout (CRISPR-KO) is to abrogate the function of the target protein. While it may reduce transcript copy number through mechanisms like nonsense-mediated decay, this effect is inconsistent and not generally detectable[101]. Therefore, we assessed KO efficiency based on VIPER-mediated analysis of the target protein in cells harboring the associated targeting guide RNAs (sgRNA) vs. non-targeting sgRNAs (see STAR methods). For each MR, we used 3 distinct sgRNAs and disregarded the effect of sgRNAs detected in < 10 cells. This allowed computing the effect of CRISPR/Cas9-mediated MR-KO on cell state, using the scRNA-seq profile of 10 or more cells containing the same targeting sgRNA, compared to cells harboring intergenic control sgRNAs. We then plotted the resulting effect on cell state reprogramming in HCC38 and HCC1143 cells by integrating across all positive and negative MRs of CSLC state (**Fig. 5A)**, as well as on an MR-by-MR basis (**Fig. 5B**). The expectation is that KO of positive and negative MRs will induce reprogramming towards a more or less differentiated state, respectively, as assessed by Stemness Score analysis. To avoid biasing the analysis, the MR directly targeted by a sgRNA in each cell was excluded from the Stemness Score assessment, such that only its downstream effectors were considered (see STAR methods).

Based on Stemness Score analysis and fully consistent with predictions, $MR_{CSLC}$ KO induced significant shift of HCC38 cell state towards a differentiated state ($p = 1.2\times10^{-3}$, by Mann Whitney U Test). Given the small fraction of differentiated cells in this cell line (**Suppl. Fig. S20**), however, $MR_{DIFF}$ KO did not induce significant shift towards a CSLC state. In contrast, both $MR_{DIFF}$ KO and $MR_{CSLC}$ KO induced significant reprogramming towards a CSLC ($p = 5.8\times10^{-4}$) and differentiated state ($p = 3.3\times10^{-2}$), respectively, in HCC1143 cells, which comprise a more balanced ratio of CSLC and differentiated cells (**Suppl. Fig. S20**). When enrichment in genes associated with stem cell process-related genes (i.e., not breast cancer-specific) was considered (see **Suppl. Table 3**) the same statistically significant trends were observed (**Suppl. Fig. S21**).

In summary, CROP-seq analysis produced highly consistent results in both cell lines, confirming the predicted role of most VIPER-inferred MRs. Note that the statistical significance of this analysis is quite underestimated, because both cell lines include a mixture of CSLC (low $MR_{DIFF}$ and high $MR_{CSLC}$) and differentiated cells (high $MR_{DIFF}$ and low $MR_{CSLC}$), while MR KO-mediated

effects can only be assessed in cells with high MR activity. As a result, the number of validated MRs is also likely to be underestimated.

The library-normalized differential abundance of sgRNA guides targeting positive MRs was not statistically significant compared to control sgRNAs (**Suppl. Fig. S22**), confirming that these MRs have no effect on cell viability or proliferation. In contrast, differential abundance of sgRNAs targeting negative MRs was significantly lower (**Suppl. Fig. S22**), suggesting that the latter—which includes cell proliferation and viability regulators—may include more essential proteins.

The contribution of each individual MR to cell state reprogramming was then analyzed and is shown in **Fig. 5B**. For the 25 $MR_{CSLC}$ and 25 $MR_{DIFF}$ tested in this assay, we only considered sgRNAs inducing effective MR KO, based on the above-described criteria. As a result, only 16 of 25 candidate $MR_{SCLC}$ (BMPR1A, MTDH, ZNF131, MAML3, GON4L, ZNF24, SMAD5, KLF3, UBP1, SMAD1, TMF1, XBP1, MIER1, VEZF1, ETV3, ZNF566, underlined are statistically significant at $p \leq 0.05$, FDR corrected) and 9 of 25 candidate $MR_{DIFF}$ (PCBD1, RUVBL2, HDGF, RPS3, RORC, ENY2, PEX14, THAP8, PARK7) could be evaluated in HCC38. Similarly, in HCC1143 cells, only 15 of 25 $MR_{SCLC}$ (STAT3, BMPR1A, MTDH, ZNF131, GON4L, MYBL1, SMAD5, UBP1, NCOA1, SMAD1, TMF1, XBP1, VEZF1, ETV3, ZNF566) and 11 of 25 $MR_{DIFF}$ (PCBD1, RUVBL2, HDGF, PRDX2, YBX1, RORC, LAMTOR5, ENY2, THAP8, HLX, PARK7) could be evaluated.

In summary, of 16 and 15 $MR_{CSLC}$ tested one or both cell lines, 15 (94%) and 10 (67%) were validated in at least one or both cell lines ($p \leq 0.05$, FDR corrected), respectively. Similarly of 9 and 11 $MR_{DIFF}$ tested one or both cell lines, 4 (44%) and 8 (73%) were validated in at least one or both cell lines ($p \leq 0.05$, FDR corrected), respectively.

CRISPR-mediated KO of the 5 most activated candidate $MR_{SCLS}$ proteins, by VIPER analysis, identified 2 (BMPR1A and ZNF141) capable of inducing highly significant ($p \leq 8.0\times10^{-24}$ and $p \leq 3.5\times10^{-7}$, respectively for HCC38 and $p \leq 2.1\times10^{-24}$ and $p \leq 4.2\times10^{-6}$, respectively for HCC1143 after FDR correction) Stemness Score decrease in both cell lines, confirming their mechanistic role in CSLC state regulation. Among these, ZNF131 was the only one previously associated with essentiality in these cell lines (gene dependence score = -1.76 for HCC38 and –2.16 for HCC1143 by CERES [102], a copy-number correction method for computing gene essentiality). Indeed, ZNF131 KD-mediated centrosome fragmentation and cell viability decrease were previously reported in GBM[103]. This raises an important question related to the potential role of ZNF131 as

a CSLC-specific essential gene in breast cancer. Similarly, CRISPR-mediated KO of the 5 most inactive candidate $MR_{DIFF}$ proteins, by VIPER analysis, identified PDBD1 capable of inducing statistically significant ($p \leq 0.022$ for HCC38 and $p \leq 7.4\times10^{-13}$ for HCC1143, FDR corrected) Stemness Score increase in both cell lines. Taken together, this confirms that VIPER-inferred MRs are highly enriched in mechanistic, causal determinants of CSLC state rather than pure gene/phenotype statistical associations.

**Identification of drugs able to invert stem-like MR programs.** The high validation rate of VIPER-inferred MRs in the CROP-seq analysis suggests that MR-inverter drugs capable of inhibiting and activating the most positive and negative MRs, respectively, should induce CSLC differentiation, thus increasing their sensitivity to chemotherapy. Indeed, MR-mediated reprogramming of cell state has already been validated in multiple contexts, from de-differentiation[84], to reprogramming[96,104] and trans-differentiation[85,105]. For this purpose, we leveraged the OncoTreat algorithm, which has proven highly effective in discovering MR-inverter drugs that were extensively validated *in vivo*, based on MR proteins inferred by VIPER analysis of both bulk[17-19] and single-cell profiles[20,21].

OncoTreat relies on perturbational RNA-seq profiles representing the response of cells—selected based on their ability to phenocopy the MR activity signature of interest—to treatment with multiple drugs and vehicle control. Perturbational profile analysis, using VIPER, allows measuring the differential activity of each MR in drug vs. vehicle control-treated cells thus providing a quantitative assessment of the activity inversion across the entire MR-signature. For this purpose, we used previously generated perturbational profiles in the BT20 BRCA cell line, which strongly recapitulates the consensus CSLC MR signature (6$^{th}$ most significant among 62 BRCA cell lines in CCLE, (NES = 7.3 by enrichment analysis), **Suppl. Fig. S22A-C**). Specifically, BT20 cells were profiled at 24h following treatment with 90 clinically relevant drugs, including FDA-approved, late-stage experimental oncology drugs (i.e., in Phase II and III clinical trials) and other selected drugs[23] (**Fig. 6A; Suppl. Tables 4,5**). Transcriptional profiles were generated using PLATE-seq[23]—a fully automated 96- and 384-well, microfluidic-based technology that is highly efficient and cost-effective—at an average depth of 2M reads. To optimize elucidation of drug mechanism of action (MoA), rather than activation of stress or death pathways, drugs were titrated at 1/10$^{th}$ of their $EC_{50}$ concentration, based on 10-point dose response curves[17].

Analysis of proteins that were differentially active in drug vs. vehicle control-treated cells identified five protein clusters (M1 – M5) that were consistently activated or inactivated in response to

different drug subsets. These were significantly enriched in five main Gene Ontology (GO) pathways, including RNA splicing/Ribosome biogenesis (M1), Epigenetic modification/DNA methylation (M2), Cell cycle/Apoptosis (M3), Cellular response to steroid hormone stimulus and Stem cell population maintenance (M4), and Cell differentiation/Development (M5), respectively (**Fig. 6A; Suppl. Table 4**). Notably, drugs inducing activation or inversion (*i.e.*, positive or negative NES) of breast CSLC MRs had opposite effects on the M4/M5 vs. M1/M2/M3 modules. Specifically, M5 proteins, which were associated with differentiation and developmental processes, were significantly activated by the drugs inducing strongest inversion of CSLC MR activity. In contrast, the drugs predicted to further activate the CSLC MR signature induced activation of M4 proteins, associated with stem cell population maintenance.

Among the 17 statistically significant MR-inverter drugs predicted by OncoTreat ($p \leq 0.05$, FDR corrected), the anthelmintic drug albendazole emerged as the most significant one ($p = 4.0\times10^{-4}$) (**Fig. 6B; Suppl. Table 6**).

***Albendazole validation in vivo:*** To experimentally validate albendazole's ability to deplete the CSLC compartment in breast cancer, we extended the protocol used to study paclitaxel in PDX models to assess the effect of 14-day treatment *in vivo* with albendazole vs. vehicle control treatment, at the single-cell level. For these *in vivo* studies, albendazole was used at 1/3$^{rd}$ of its maximum tolerated dose in mice, consistent with assessment of MR-inversion potential at low concentration. Although albendazole is not an oncology drug, it has been shown to inhibit growth of some cancer cell lines and of a murine carcinoma, reportedly by inducing oxidative stress[106-108]. Consistently, albendazole clustered separately from chemotherapeutic drugs (**Fig. 6A**), and its activity was associated with activation of cell differentiation pathways (**Fig. 6A**).

Consistent with the paclitaxel analysis, depletion of CSLC vs. differentiated cell compartment was computed by measuring the ratio between the number of cells with the highest ($SS \geq 0.8$) vs. lowest ($SS \leq 0.2$) stemness score in albendazole vs. vehicle control-treated samples, normalized to the subpopulations size (see STAR methods). Whereby paclitaxel had induced dramatic increase in this ratio, indicating relative depletion of the differentiated tumor cell compartment (**Fig. 3C**), albendazole had the opposite effect (**Fig. 7A**), producing equally significant relative depletion of the breast CSLC compartment ($p = 2.0\times10^{-4}$, by Fisher's exact test). When comparing albendazole to paclitaxel-treated tumors, relative changes in the density of the two compartments

were even more statistically significant ($p = 3.0\times10^{-12}$, by Fisher's exact test) (**Fig. 7B**), suggesting a highly complementary effect.

**Albendazole synergizes with paclitaxel in a TNBC PDX model.** Since albendazole and paclitaxel deplete complementary metastatic breast cancer cell compartments, it is reasonable to hypothesize that combining or alternating their administration may outperform either drug used as monotherapy. To test this hypothesis, we evaluated whether CSLC compartment depletion by repeated administration of albendazole would enhance the *in vivo* anti-tumor activity of paclitaxel.

A PDX line, established from a human primary TNBC, was implanted in the mammary fat pad of NSG mice. When tumors reached a volume of 100 $mm^3$, they were randomly enrolled to receive different treatments (paclitaxel monotherapy, albendazole monotherapy, albendazole + paclitaxel, and vehicle control) until six mice per arm were enrolled. Mice in the combination arms underwent two treatment cycles, separated by a 15-day drug holiday. Each cycle included albendazole-based sensitization for two weeks, starting at Day -13—defined as the day when a specific tumor reached a volume of 100 $mm^3$—followed by three paclitaxel treatments (Day 1, 8 and 15) (**Fig. 7C**). For monotherapy treatment, mice were treated for the same amount of time and on the same schedule with albendazole, paclitaxel, and vehicle control, independently.

Paclitaxel monotherapy significantly reduced relative tumor volume (TV), compared to vehicle control ($p = 0.0024$), while albendazole was indistinguishable from vehicle control ($p = 0.21$) (**Fig. 7D; Suppl. Fig. S23**). TV change was assessed from initiation of albendazole therapy (Day -13) through Day 49; during this period, the majority of vehicle control-treated animals (n = 5 of 6) required euthanasia, due to attaining the maximal allowed humane TV endpoint (median TV = 1543 $mm^3$). Additionally, compared to vehicle control, albendazole monotherapy showed no significant improvement in disease control ($p = 0.83$) or overall survival ($p = 0.63$) (**Suppl. Fig. S24**).

In sharp contrast, the albendazole + paclitaxel combination was associated with profound suppression of tumor growth, compared to both vehicle control ($p = 1.7\times10^{-4}$) and paclitaxel monotherapy ($p = 0.015$) (**Fig. 7E**). Drug synergy was further confirmed by Bliss independence analysis ($p = 9.0\times10^{-3}$) and translated into a statistically significant increase in overall survival ($p = 0.02$) (**Suppl. Fig. S24**).

## Discussion

Despite remarkable therapeutic advances, the prognosis for metastatic breast cancer patients remains dismal. Among the most critical obstacles to achieving a permanent eradication of the disease is the heterogeneity of tumor cell response to therapy. Indeed, while many chemotherapies and targeted therapies may be highly effective on subpopulations that contribute to the bulk of the malignant tissue, the presence of drug-resistant subpopulations within the same tumor mass inevitably leads to relapse and poor outcome. The cellular heterogeneity associated with pre-existing differential drug sensitivity can be of a genetic origin, for instance due to mutations in the active site of the target protein[109] or to the presence of clonally distinct subpopulations with bypass or alternative mutations[110]. However, it is more often associated with the presence of epigenetically distinct transcriptional states with differential drug sensitivity—either pre-existing[1] or induced by cell adaptation[111,112]—some of which can plastically regenerate the full heterogeneity of the tumor[113]. This is especially relevant in the metastatic context, where tumors have already reached a high degree of heterogeneity, due to paracrine interaction differences at distinct distal sites. Consistently, progression to metastatic breast cancer dramatically reduces the probability of achieving complete and durable responses. Indeed, most metastatic breast cancer patients rapidly progress through multiple lines of anti-tumor treatment, and eventually end up receiving conventional chemotherapy, which typically provides only short-term control of the disease.

A growing body of evidence suggests that less differentiated breast cancer cells may be chemotherapy resistant, while retaining the ability to further differentiate and reconstitute the full heterogeneity of the tumor. These cells may thus play a key role in relapse to drug-resistant disease. Tumor cells with stem-like properties (CSLCs) and tumor initiating potential were first discovered in leukemia[114,115] and later reported also in solid tumors, such as gliomas[116,117], breast[118], and colon cancer[119]. As a result, the identification of novel therapeutic approaches to specifically target the CSLC compartment represents a potentially impactful area of investigation[120-122] and may help identify drugs that synergize with chemotherapy. Network-based, single-cell analysis of cells dissociated from metastatic breast cancer patients identified a well-defined transcriptional state controlled by an exceedingly conserved repertoire of MR proteins—including transcription factors and co-factors previously associated with mammary repopulation units and breast cancer stem cells—whose sensitivity to chemotherapy is dramatically reduced compared to differentiated breast cancer cells. Indeed—based on a consensus Stemness Score that combines both the CytoTRACE metric and the activity of 14 established BRCA stemness

marker proteins—there was highly significant association between cell stemness and chemotherapy resistance. This helped us identify a molecularly distinct subpopulation of chemotherapy resistant, poorly differentiated cells (CSLC for simplicity), based on the highly conserved repertoire of MR proteins that control their transcriptional state, across virtually all patients in the study. While this definition may encompass previously reported breast cancer stem cells, we use the term CSLC more broadly as it may also include an additional repertoire of undifferentiated, chemotherapy resistant progenitors. Thus, we make no claims that the CSLCs identified by our analysis represent *bona fide* tumor stem cells; rather, we show that they are chemotherapy resistant and would thus benefit from complementary therapeutic options. To enrich for CSLCs, we leveraged CD49f-based flow cytometry-based sorting of single cells dissociated from patient-derived samples. While CD49f is considered a marker of basal cells and is most highly expressed in a subset of cells from TNBC samples, previous results[24-26] and our analysis confirmed that CD49f is also differentially expressed in CSLCs from HR+ patients. Indeed, its expression gradient was significantly correlated with the activity of 14 previously reported BRCA CLSC markers across all patients in the study, independent of HR status, thus justifying its use in our study. Confirming the value and accuracy of the proposed protein activity assessment methodology, CD49f was identified as significantly differentially active by metaVIPER in cells dissociated from human samples (**Fig. 2E**), even though its encoding gene, *ITGA6*, could not be identified as differentially expressed (**Suppl. Fig. S5**). This is fully consistent with the fact that these cells were FACS sorted with and without the associated antibody and highlights the limitations introduced by gene dropout effects in scRNA-seq profiles.

Targeting the CSLC compartment may be accomplished by developing drugs that either preferentially kill these cells or reprogram them toward treatment-sensitive states. The latter strategy is supported by recent results in fields ranging from hematopoiesis, cancer, and diabetes[84,85,105,123] where genetic or pharmacologic targeting of MR proteins—as identified by network-based VIPER/metaVIPER analyses—effectively reprogrammed the cell's transcriptional state towards a different target state, thus also confirming their nature as mechanistic determinants of cellular state transitions. An additional advantage of these approaches is that metaVIPER analysis effectively removes technical artifacts (batch effects) and non-functional gene expression differences, for instance due to inter-tumor CNA heterogeneity[13,38], thus resulting in highly reproducible identification of MR proteins across samples from different patients.

To confirm mechanistic control of the CSLC state by metaVIPER-inferred MRs we performed pooled CRISPR/Cas9-mediated KO of candidate MRs in two cell lines, followed by scRNA-seq

profiling, using the CROP-seq methodology. As shown, following CRISPR/Cas9-mediated KO, the vast majority of positive and negative CSLC MRs identified by metaVIPER analysis induced statistically significant reprogramming towards either a more differentiated or a more CSLC state, respectively, thus confirming the algorithm's predictions. This includes four of the top five candidate MRs that had been previously nominated as potential players in CSLC biology but had not been experimentally validated, including STAT3[124,125], MTDH[126], ARID1A[127], BMPR1A[128], and ZNF131[103], the first two of which had been proposed as key (co-)regulators of breast CSLCs, through the JAK/STAT3 and NF-kB pathways, respectively[124-126]. These two pathways are not only crucial in immune and inflammatory response but also pivotal for crosstalk between tumor and immune cells, especially in tumor microenvironment[129]. Moreover, the downstream effectors of these signaling pathways are often linked to cell survival and self-renewal as well as tumor proliferation, invasion, and metastasis[130]. Of these five metaVIPER-nominated MRs, only MTDH failed to induce statistically significant reprogramming in HCC38 and HCC1143 cells.

With the possible exception of ZNF131, CRISPR/Cas9-mediated KO of positive CSLC MRs had virtually no effect on cell viability, confirming that cells were reprogrammed to a chemotherapy sensitive state and not selectively ablated. This supports the identification of the MR-inverter drugs via the OncoTreat algorithm, leading to the selection and *in vivo* experimental validation of the anthelmintic albendazole as a highly efficient mediator of CSLC reprogramming. Consistent with these findings, combination therapy with albendazole and paclitaxel resulted in more profound and durable responses, as compared to either monotherapy, leading to a statistically significant increase in overall survival of preclinical models.

Remarkably, since metaVIPER identified a CSLC transcriptional state (and associated MR signature) that was virtually identical across all the tissues and models in this study, irrespective of hormone receptor status, we anticipate that the synergy between albendazole and paclitaxel in a PDX model from a metastatic TNBC patient may also be conserved in HR+ tumors, potentially in combination with hormonal blockade therapy, and may thus be relevant to a large fraction of metastatic breast cancer patients, especially since albendazole is well tolerated.

In parasites, albendazole's mechanism of action is mediated by high-affinity binding to beta tubulin. While the binding is quite selective for parasite tubulin, the drug retains some tubulin-disrupting activity in cancer cells, even though no cytotoxicity is observed at clinically relevant concentrations. Consistently, there are a few tubulin-binding antineoplastic drugs in clinical trials—such as PTC596—that do not present the anti-mitotic cytotoxic effects of drugs such as

paclitaxel, which induce harmful myelosuppression. Indeed, no cytotoxic effects of albendazole were detected in this study, either *in vitro* or *in vivo*. While It has been hypothesized that drugs like PTC596 may work by modulating trafficking of CSLC proteins, like BMI-1, and DNA repair proteins, which may provide a partial rationale for albendazole's effect in CSLCs, and despite its highly reproducible effects *in vitro* and *in vivo*, the precise mechanism of action by which albendazole inverts the activity of CSLC MRs remains to be elucidated and will be the subject of future research. Notably, even though the study was limited to 90 drugs, it identified 17 as statistically significant candidates to reprogram CSLCs to a paclitaxel-sensitive state. As a result, we expect that extending this highly cost-effective approach to much larger drug/compound libraries may reveal even more potent agents.

Taken together, the data presented in this manuscript show that drugs targeting heterogeneous, drug-resistant subpopulations can be effectively identified by single-cell, network-based analyses and that non-oncology drugs may be effectively repurposed to enhance the therapeutic activity of anti-tumor agents, including chemotherapy.

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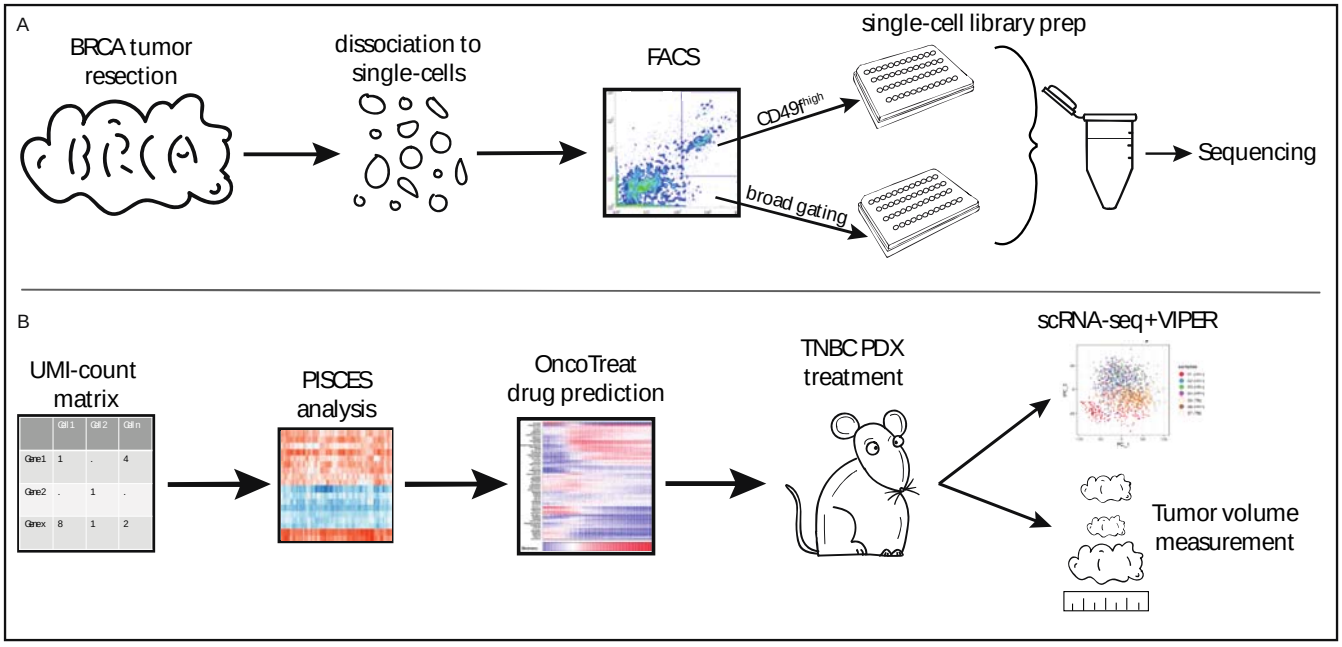



**Figure 1.** Overview of the workflow. **A.** The experimental workflow for generating scRNA-seq data from breast cancer cells from patient samples. FACS was used to enrich CSLCs. **B.** A systems biology approach to identifying a candidate drug targeting the CSLCs and subsequent experimental validations.

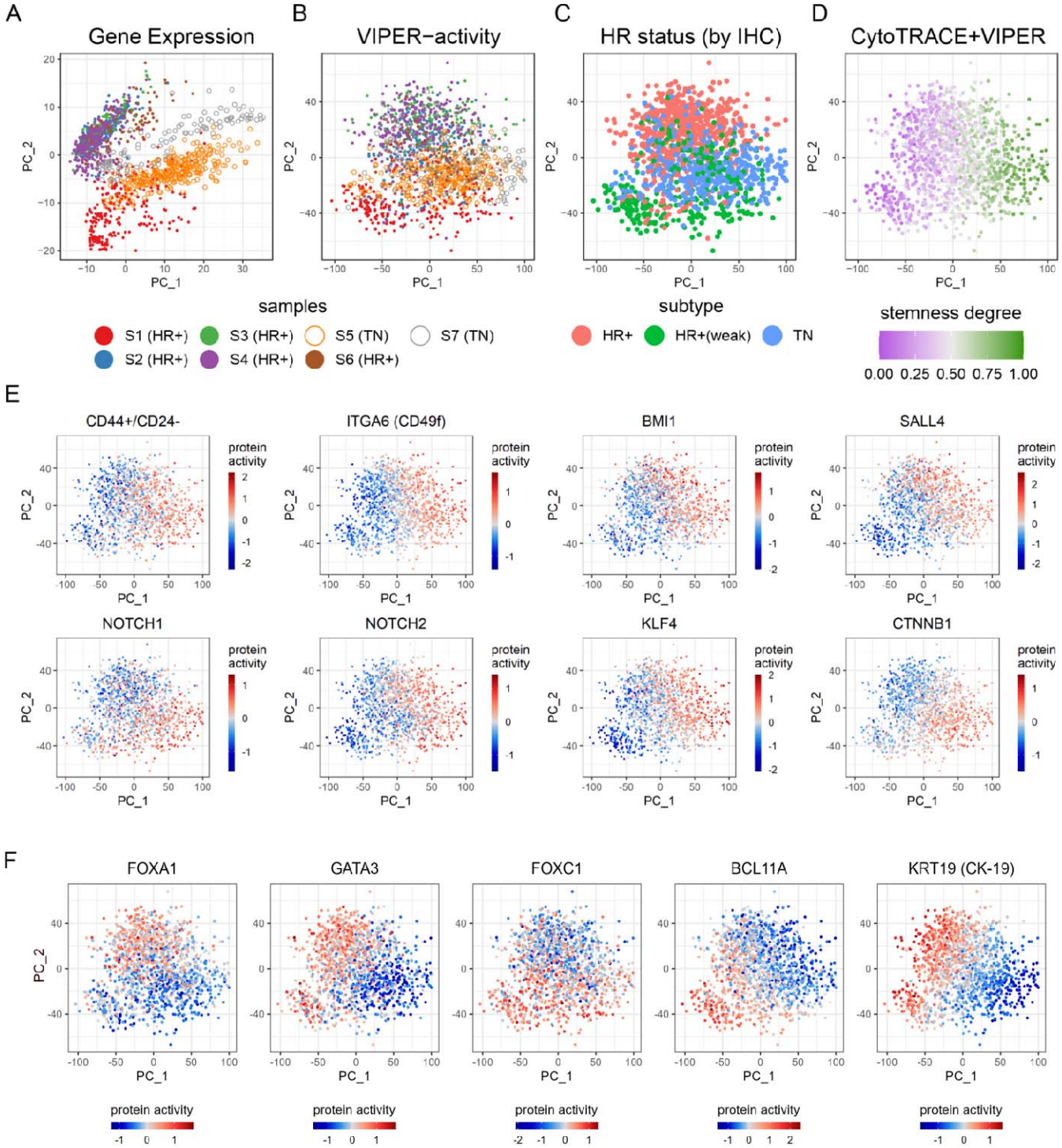



**Figure 2.** Analysis of scRNA-seq data for 7 breast cancer patient samples. Cells were clustered based on the first two principal components of the cell's gene expression (**A**) and the protein activity inferred by VIPER (**B**-**F**). In **A** and **B,** cells are colored according to the patient they came from. **C.** The breast cancer subclasses (HR+, weakly HR+, and TN) are shown. **D**. The degree of each cell's stemness is indicated using a green-grey-purple color gradient, corresponding to the degree of stemness from one (most stem-like) to zero (most differentiated). The stemness degree was estimated based on the combination of the CytoTRACE score and the protein activities of well-known stemness markers: CD44+/CD24-, ITGA6, BMI1, SALL4, NOTCH1, NOTCH2, KLF4, CTNNB1, ITGB3, ITGB1, PROM1, POU5F1, SOX2, and KIT (see Methods). This stemness degree score was re-scaled to the range between 0 and 1. **E**. The VIPER-inferred protein activity (centered) of individual breast CLSC markers. From the highest to the lowest, activity is shown with a red-white-blue color gradient (white = mean). **F**. The VIPER-inferred protein activity of HR+ markers (FOXA1 and GATA3), TNBC makers (FOXC1 and BCL11A), and a differentiated-cell marker (KRT19).

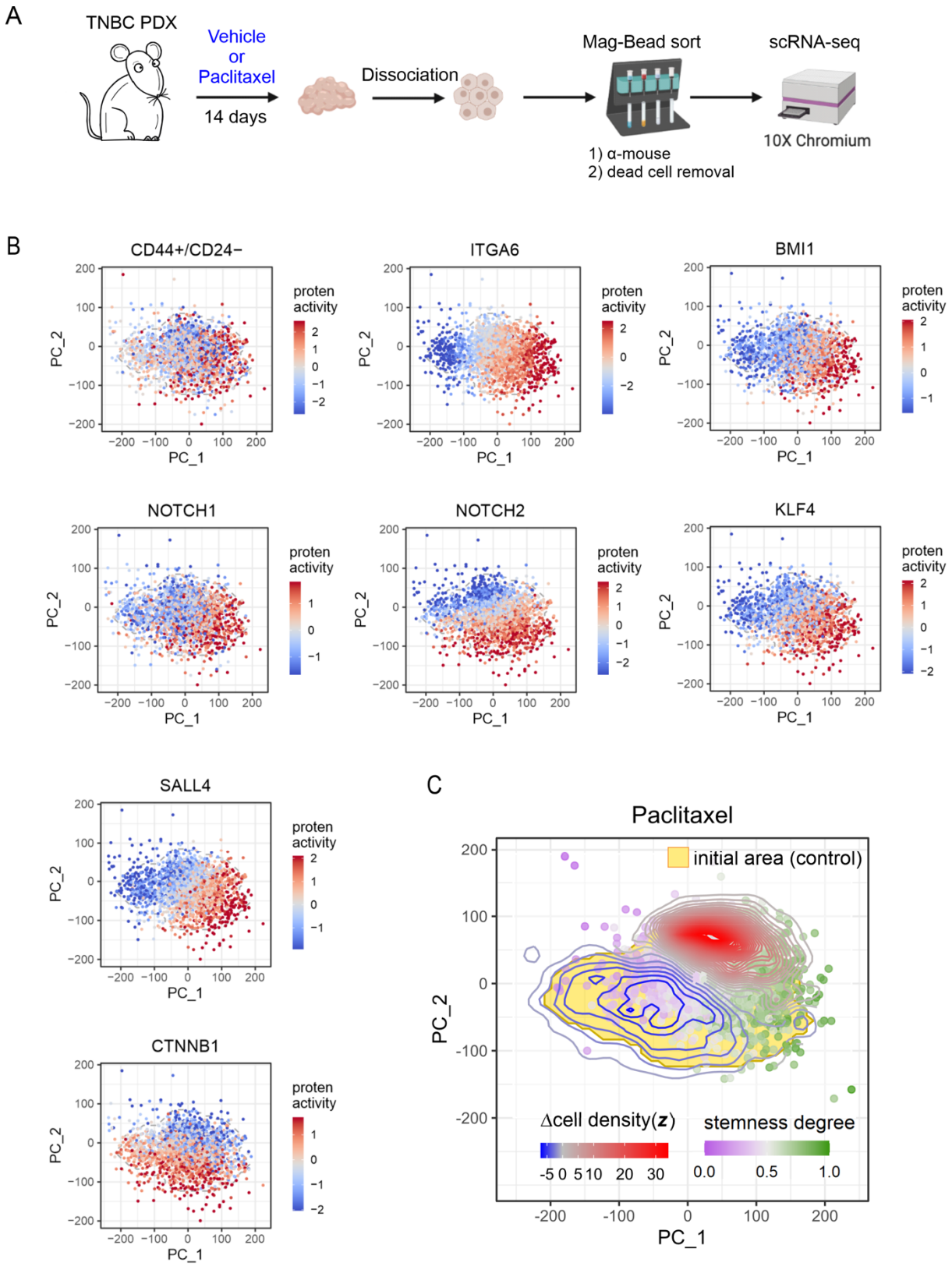



**Figure 3. A.** Workflow for scRNA-seq analysis of a TNBC PDX model. **B.** The VIPER-inferred activities of established breast CSLC markers in the vehicle control. **C**. Effect of Paclitaxel on the TNBC PDX cells. The cells were clustered based on the first two principal components of VIPER-inferred protein activity profiles under vehicle- and drug-treated conditions (see Methods). Based on the degree of stemness, cells were colored in a green-grey-purple color scheme (green: more stem-like cells, purple: more differentiated cells). The stemness degree was estimated by the combination of the CytoTRACE score and the protein activities of stemness markers (CD44+/CD24-, ITGA6, BMI1, SALL4, NOTCH1, NOTCH2, KLF4, CTNNB1, ITGB3, ITGB1, PROM1, POU5F1, SOX2, and KIT) (see Methods). The estimated stemness degree score was rescaled to the range of 0-1. The area in yellow indicates a boundary of the cell cluster which 95% of cells in the control fall into. Cell density change (z-score) is shown with contour lines in the PDX sample treated with paclitaxel. The red and blue contour lines denote an increase or decrease, respectively, in cell densities under drug treatment compared to the control.

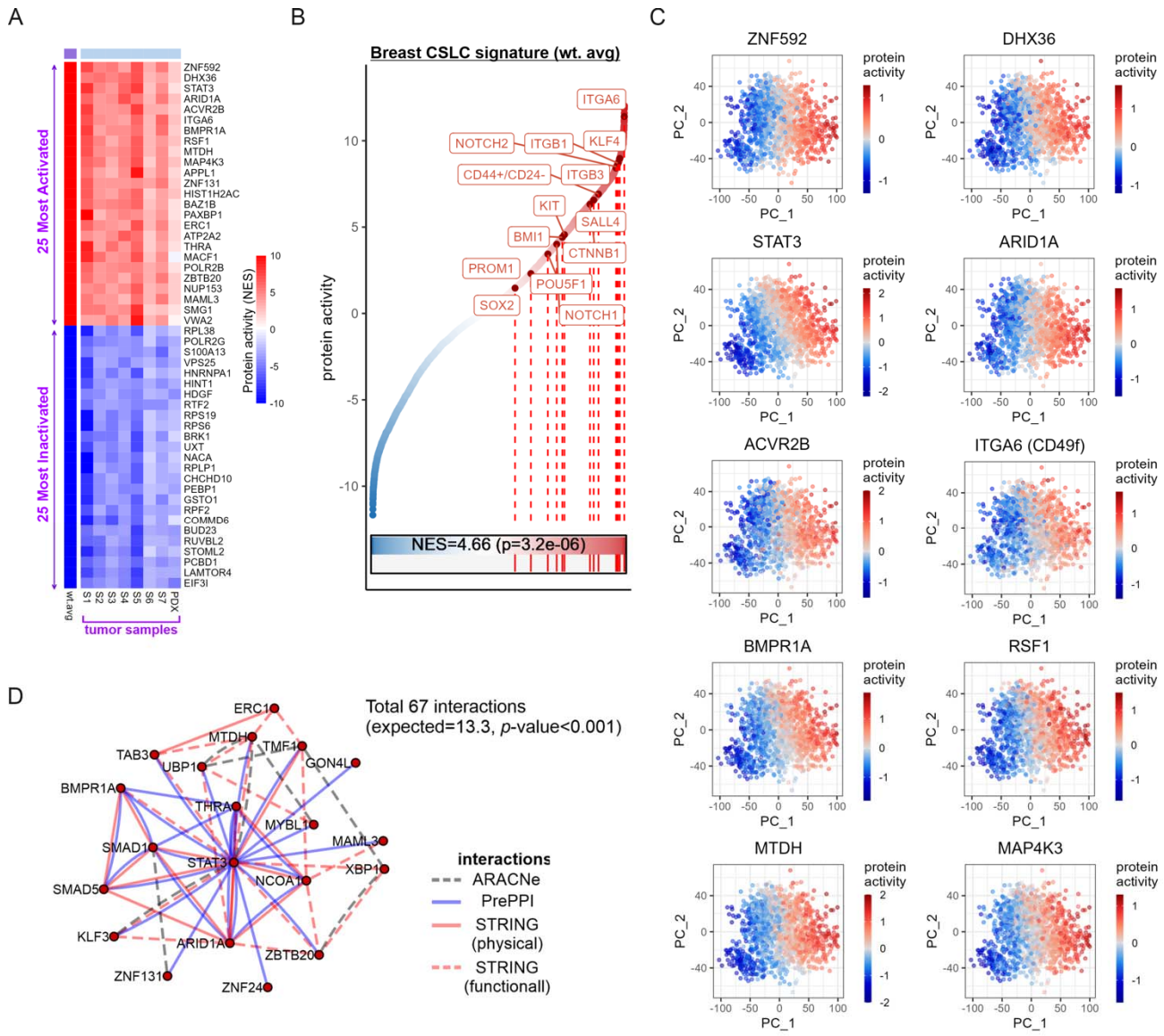



**Figure 4. A.** A heatmap showing the VIPER-inferred protein activity of the 25 most activated and the 25 most inactivated proteins in the breast CSLC signature and their activities in individual samples (7 patient samples and the PDX vehicle-treated sample). For each sample, differential protein activity from non-CSLCs to CSLC was computed using metaVIPER. The overall CSLC signature was obtained by the weighted average of the protein activities across samples. A larger positive (or negative) value in the signature means that the protein was more (or less) activated in CSLCs than in non-CSLCs. If there is little change in protein activity between non-CSLCs and CSLCs, the value approaches zero. Note that CSLCs and non-CSLCs were identified based on the average activity of the following CSLC markers in the sample: CD44+/CD24-, ITGA6, BMI1, SALL4, NOTCH1, NOTCH2, KLF4, CTNNB1, ITGB3, ITGB1, PROM1, POU5F1, SOX2, and KIT. **B.** A waterfall plot displaying the sorted protein activities in the breast CSLC signature, in which the signatures of individual samples were integrated using weighted Stouffer's method. In this plot, the NES of the 14 breast CSLC markers is shown. **C.** Top 10 activated proteins in the identified signature and their protein activities in the patient data. **D.** Top 20 transcriptional regulators in the identified breast CSLC signature and their interactions identified by ARACNe, PrePPI, and STRING tools.

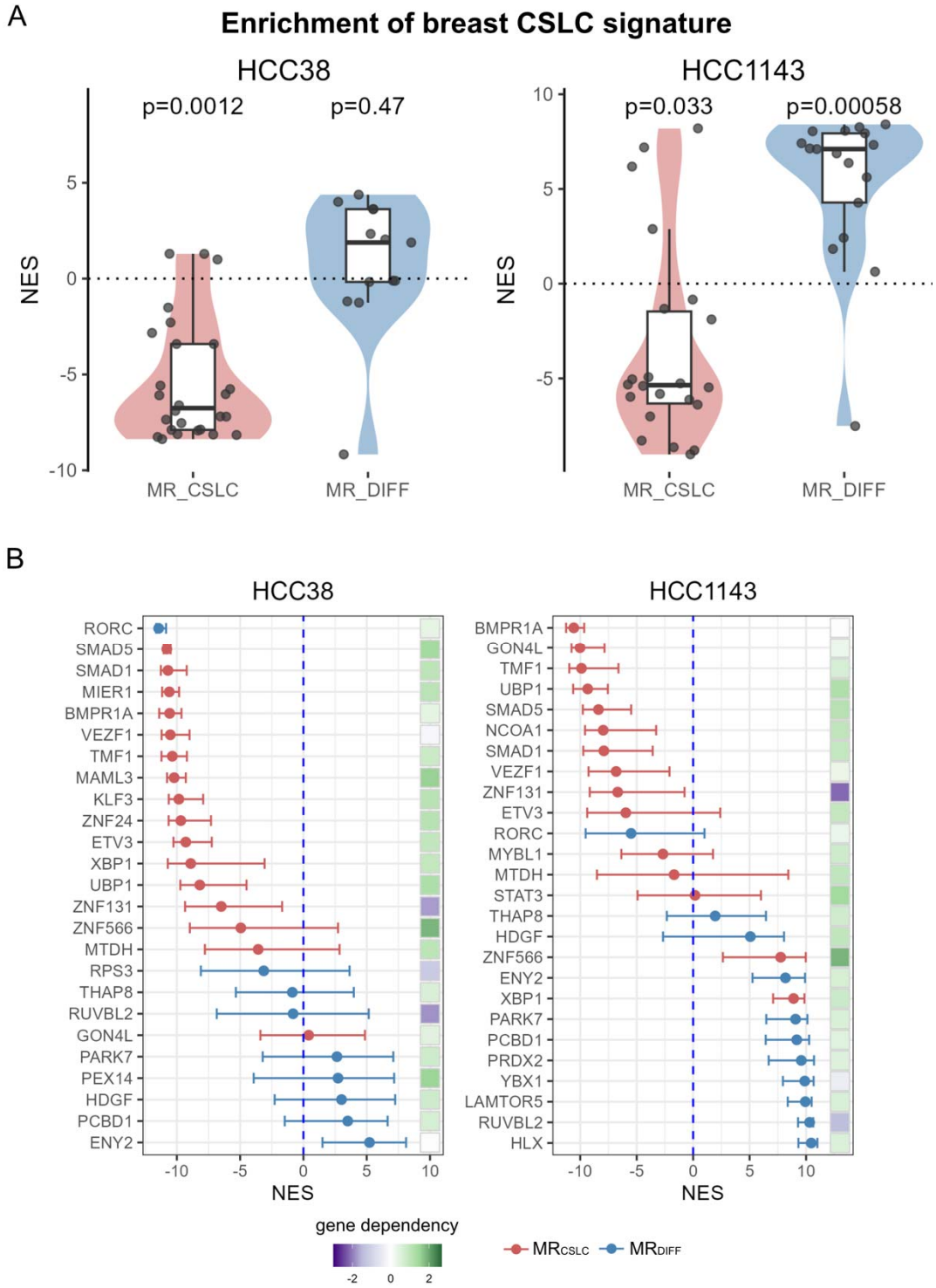



**Figure 5. A.** Cellular reprogramming after knocking out the top 25 most activated MRs ($MR_{CSLC}$) and the 25 most inactivated MRs ($MR_{DIFF}$) of breast CSLC signature, compared to the effects in the control sgRNA group for the breast cancer cell lines HCC38 and HCC1143. For each sgRNA, the VIPER-inferred protein activity profiles were generated from the pseudo-bulk expression of cells detected with the same sgRNA. The knockout (KO) efficiency was determined based on the threshold of one standard deviation below the target gene's mean protein activity. The enrichment score of the 50-MR set ($MR_{CSLC}$ and $MR_{DIFF}$) was investigated in the protein activity profiles for each group of $MR_{CSLC}$ and $MR_{DIFF}$ (**A**) and for each sgRNA (**B**) to assess the effects of cellular reprogramming for both HCC38 and HCC1143 cell lines. To assess the reprogramming effects of each sgRNA, pseudo-bulk expressions were bootstrapped by resampling cells with the same sgRNA with replacement. Lower NES signifies greater differentiation. Error bars indicate the 1st and 3rd quartiles of NES for the reprogramming effects of multiple sgRNAs targeting the same MR in B. The effect of each MR on cell proliferation in the CROP-seq experiment is indicated by the gene dependency score, using a green-white-purple gradient where darker purple = greater dependence. White/green indicates no significant reduction in the proliferation rate when that MR is knocked out.

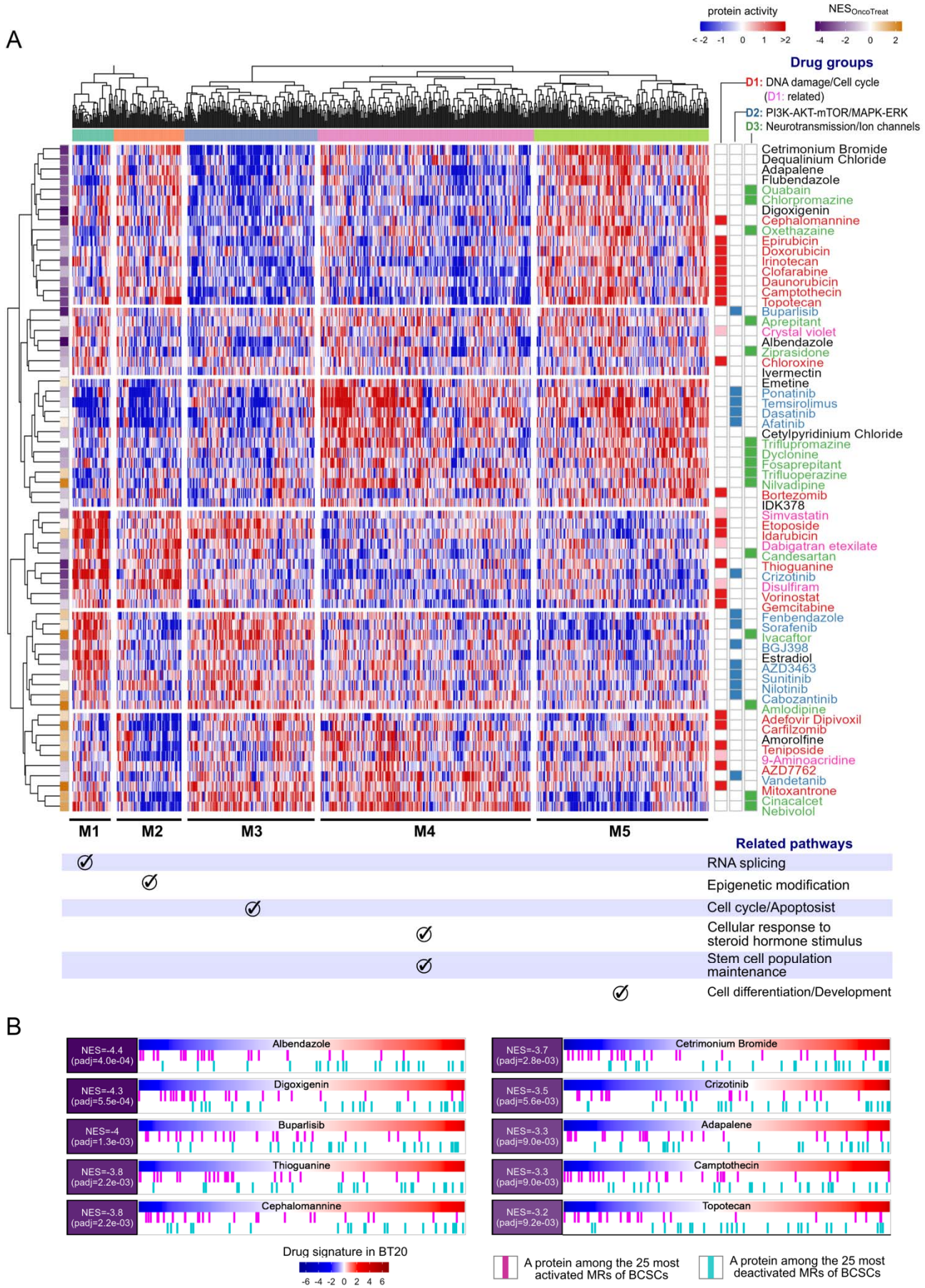



**Figure 6 A.** Bi-clustered drug perturbation profiles for the breast cancer cell line BT20. In the heatmap, the rows and columns are drug samples (24h, 1/10$^{th}$ EC20) and master regulator proteins ($FDR_{BCSC}<1\times10^{-5}$), respectively. The activated and inactivated proteins are shown in red and blue, and the protein activities with no change are shown in white. **B.** The enrichment plot of the top 10 drugs, predicted from the perturbation profiles with 24h treatment at 1/10$^{th}$ the drug's EC20 in BT20 using OncoTreat analysis. The magenta and turquoise bars denote the top 25 most activated proteins and the top 25 most inactivated proteins in the breast CSLC signature, respectively, which were derived from the 7 patient samples. In each plot, these 50 proteins (i.e. magenta and turquoise bars) were mapped to their corresponding activity in a drug sample.

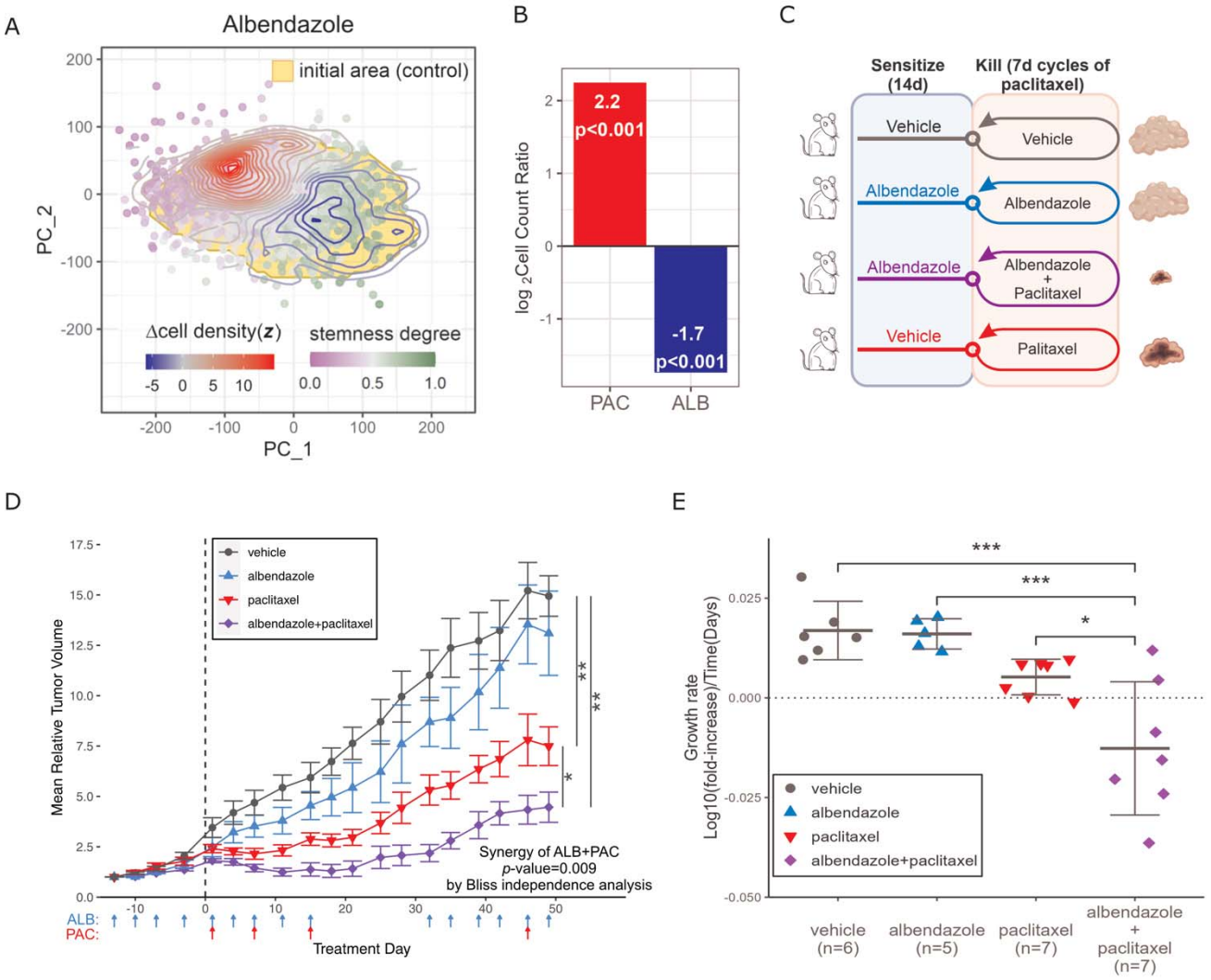



**Figure 7. A.** Analysis of scRNA-seq data showing the effect of albendazole on cells taken from a TNBC PDX model. The red and blue contour lines denote an increase or decrease, respectively, in cell densities under drug treatment compared to the vehicle control. The area in yellow indicates a boundary of the cell cluster into which 95% of cells in the control fall. **B.** The cell-count ratios between the stem-like (stemness score > 0.8) and differentiated (stemness score < 0.2) cells under the treatments of paclitaxel and albendazole compared to vehicle control. Based on Fisher's exact tests, the differences between the treatments are statistically significant (*p*-value < 0.001) compared to the ratio in the control. **C.** Schematic view of combination therapies used in the preclinical tests. **D.** Mean relative tumor volumes over time under individual therapeutic strategies. Biological replicates were averaged, and the error bar indicates one standard error of the mean. Mice were treated with albendazole 3 times weekly for two weeks (Day -13 to Day 0) before the start date of the combined drug therapy with paclitaxel to sensitize the tumor cells. Mice with albendazole monotherapy were treated for the same amount of time as those in the combination therapy (Day 0 to Day 49). **E.** A comparison of the tumor growth rates during the 1st cycle of drug treatments. Differences between mean growth rates were tested for statistical significance using Tukey's honest significance test (*p<0.05, **p<0.01, ***p<0.001). Tumor growth rates were calculated assuming exponential kinetics from 1 to 18 days. The error bar indicates one standard deviation from the mean growth rates.

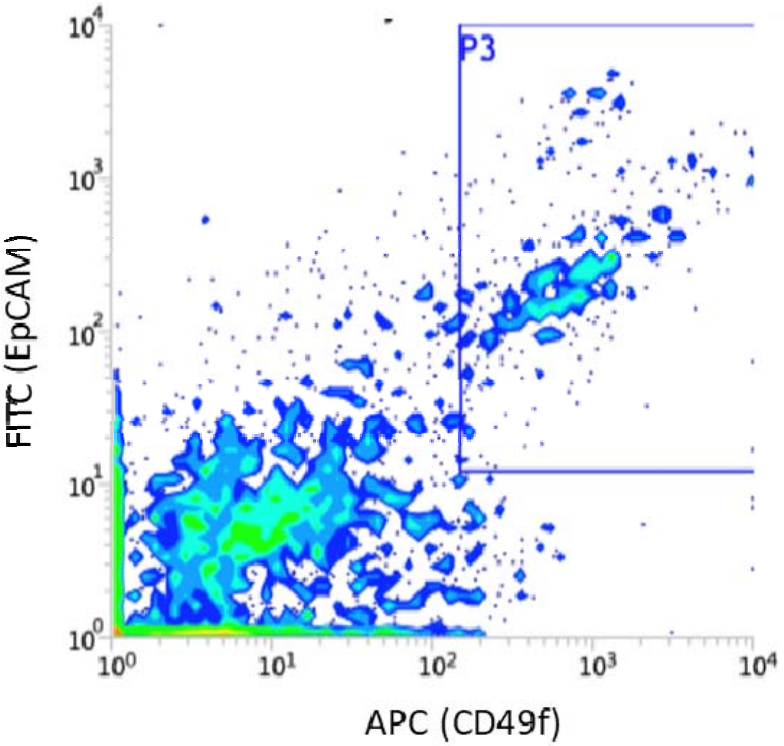



**Figure S1.** FACS of a human metastatic breast cancer tissue (patient S1). Malignant epithelial cells were isolated based on EPCAM$^+$ and CD49f$^{high}$ and subjected to scRNA-seq.

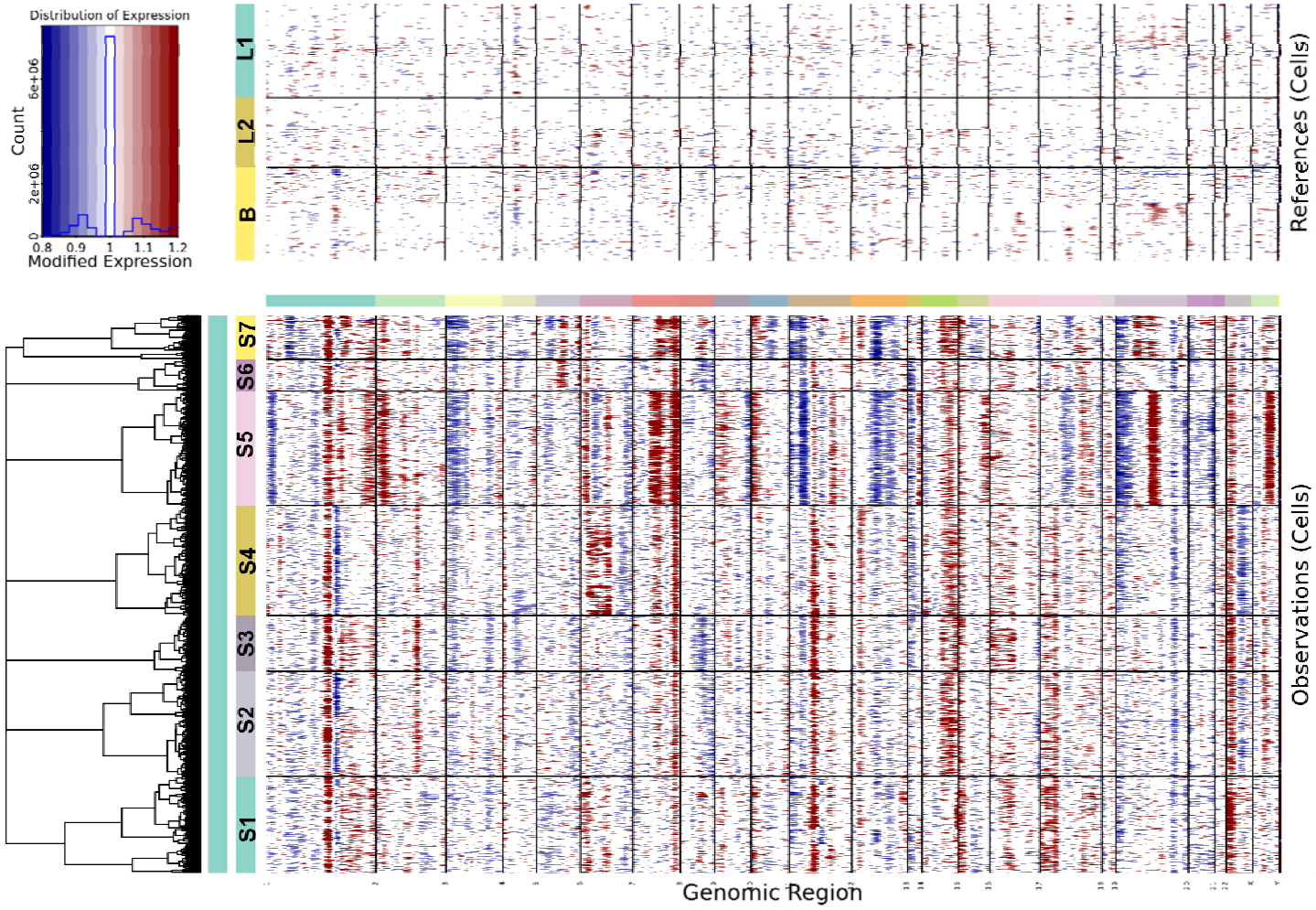



**Figure S2.** Inferred copy number variations in breast cancer patient samples (S1-S7) by inferCNV. Normal breast epithelial cells containing one basal (B) and two luminal (L1 and L2) subtypes from 4 individuals (GSE113196) were used as reference. In the patient samples, S1 and S2 were weakly HR+, S2, S3, and S4 were strongly HR+, and S5 and S7 were TNBCs, according to immunohistochemistry (IHC).

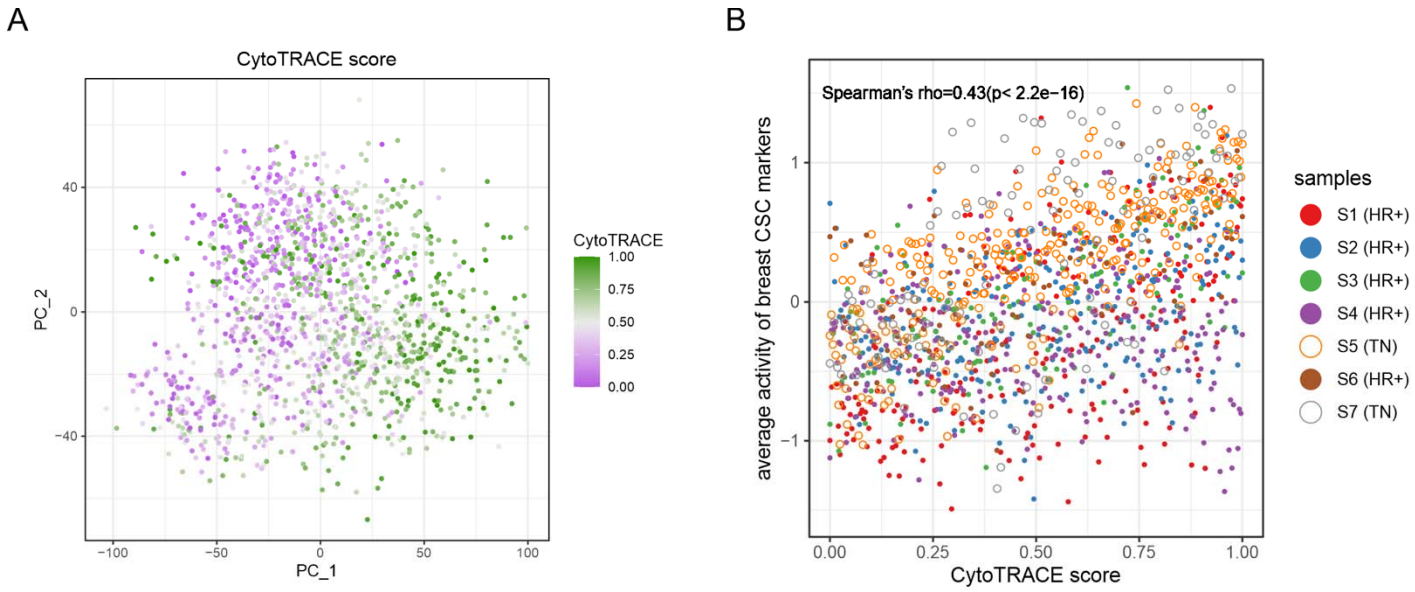



**Figure S3. A.** The CytoTRACE prediction in the patient scRNA-seq data. Cells were colored with a green-grey-purple gradient corresponding to the CytoTRACE score from one (least differentiated) to zero (most differentiated). **B.** The correlation between the CytoTRACE score and the average activity of breast CSC markers in the patient samples. The following proteins were considered as breast CSC markers in the patient data: CD44+/CD24-, ITGA6, BMI1, SALL4, NOTCH1, NOTCH2, KLF4, CTNNB1, ITGB3, ITGB1, PROM1, POU5F1, SOX2, and KIT. The correlation between CytoTRACE and the maker's activity was statistically significant (Spearman's rho=0.43, *p*-value<0.001).

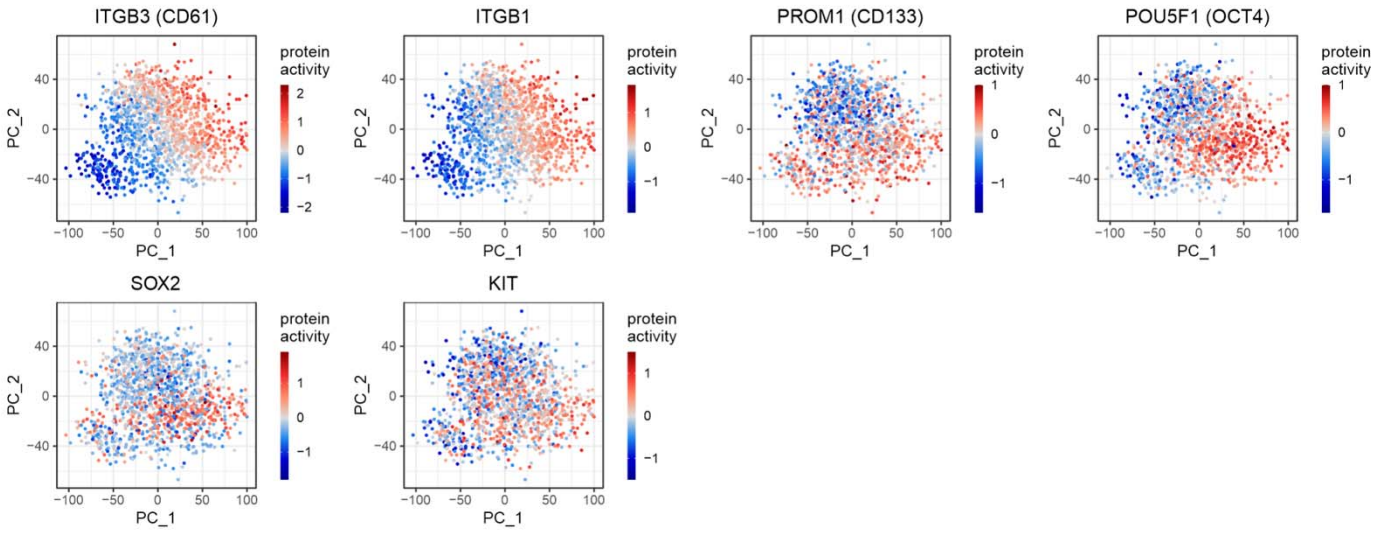



**Figure S4**. The inferred activity of several breast CSC markers, namely ITGB3, ITGB1, PROM1, POU5F1, SOX2, and KIT. Note that the VIPER-inferred activities were centered in this visualization.

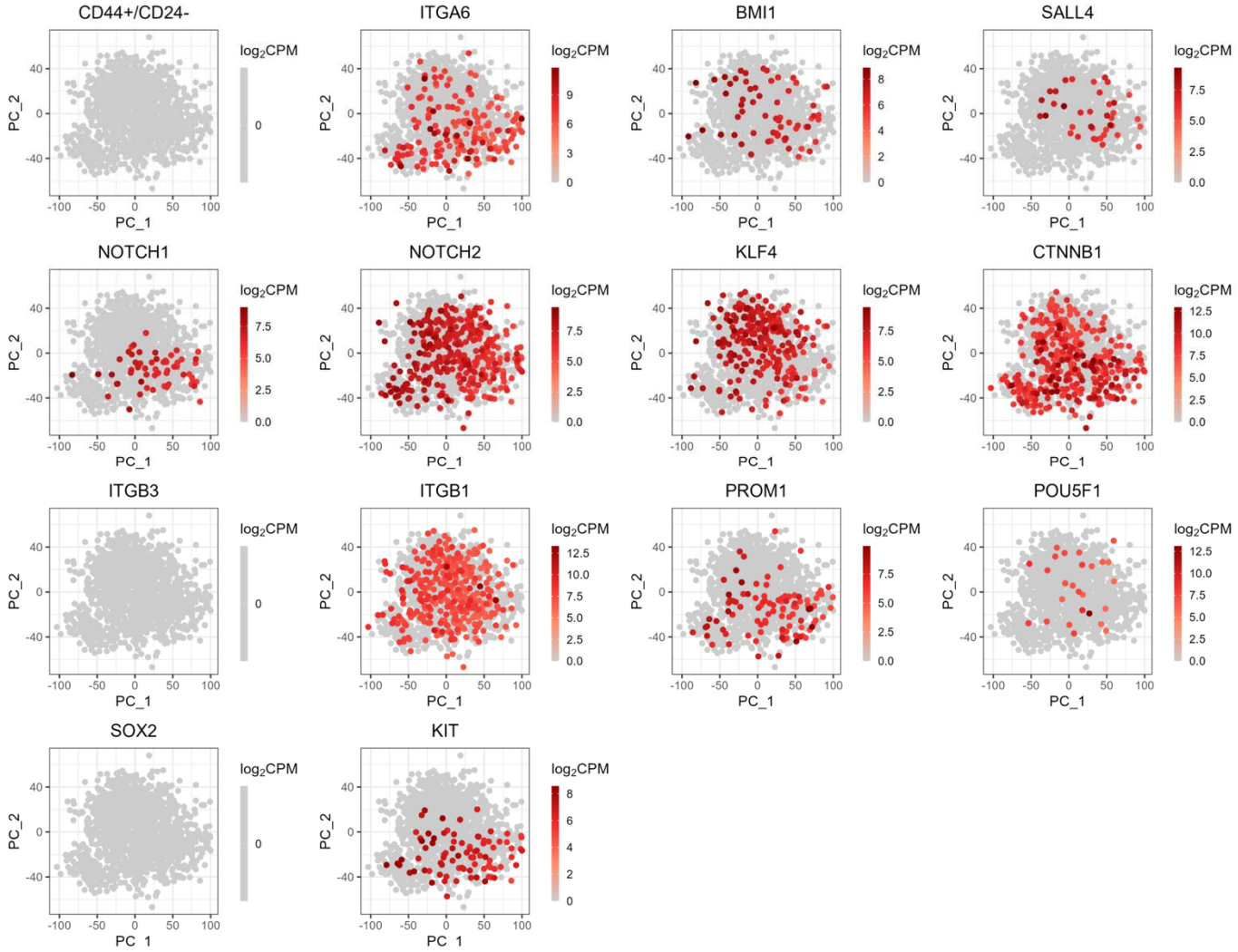



**Figure S5**. Gene expression ($log_2$CPM) of known breast CSLC markers. The same PCA mapping based on the protein activity was used for the comparison. Note that only the gene expression greater than the upper quantile is shown for visualization purposes.

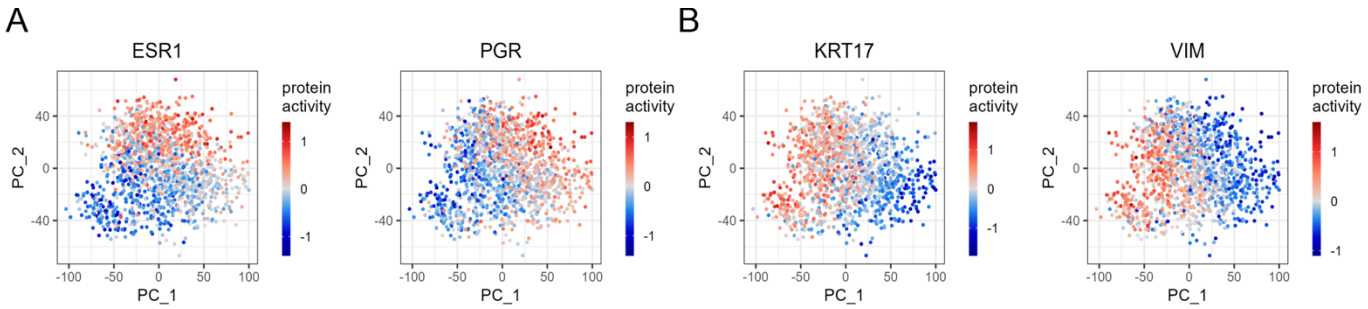



**Figure S6**. The inferred activity of breast cancer subtype markers. **A.** Two HRs (ESR1 and PGR). **B.** A basal-type marker (KRT17) and non-HR+ marker (VIM). While ESR1 and PGR activities are greater in HR+ patient samples, KRT17 and VIM activities are greater in TNBC samples. Note that the VIPER-inferred activities are centered in this visualization.

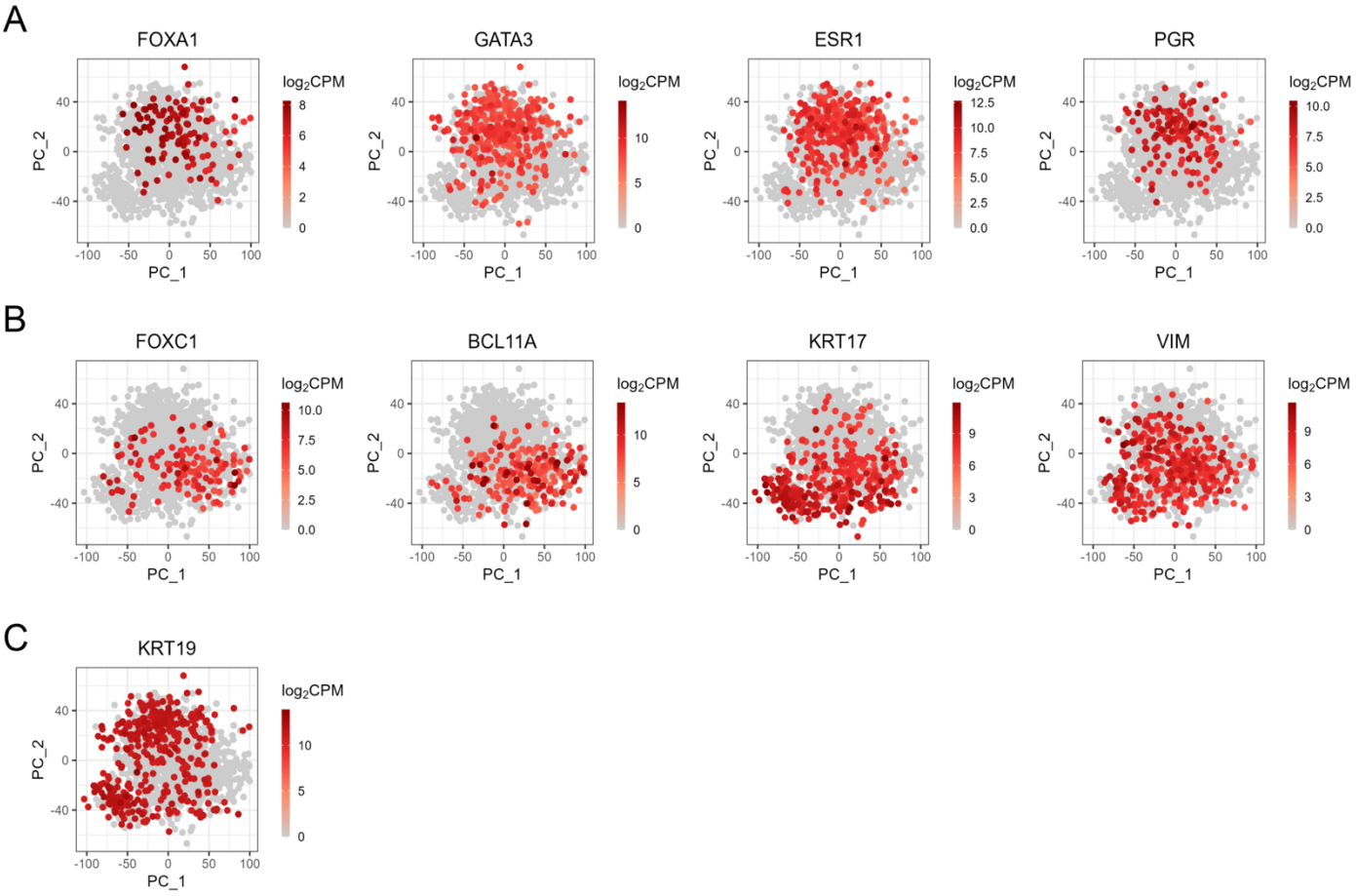



**Figure S7**. Gene expression ($\log_2$CPM) of known breast cancer subtype markers in the patient samples: HR+ markers (**A**), TNBC-specific markers (**B**), and a differentiated cell marker (**C**). The same PCA-mapping based on the protein activity was used for the comparison. Note that only the gene expression greater than the upper quantile is shown for visualization purposes.

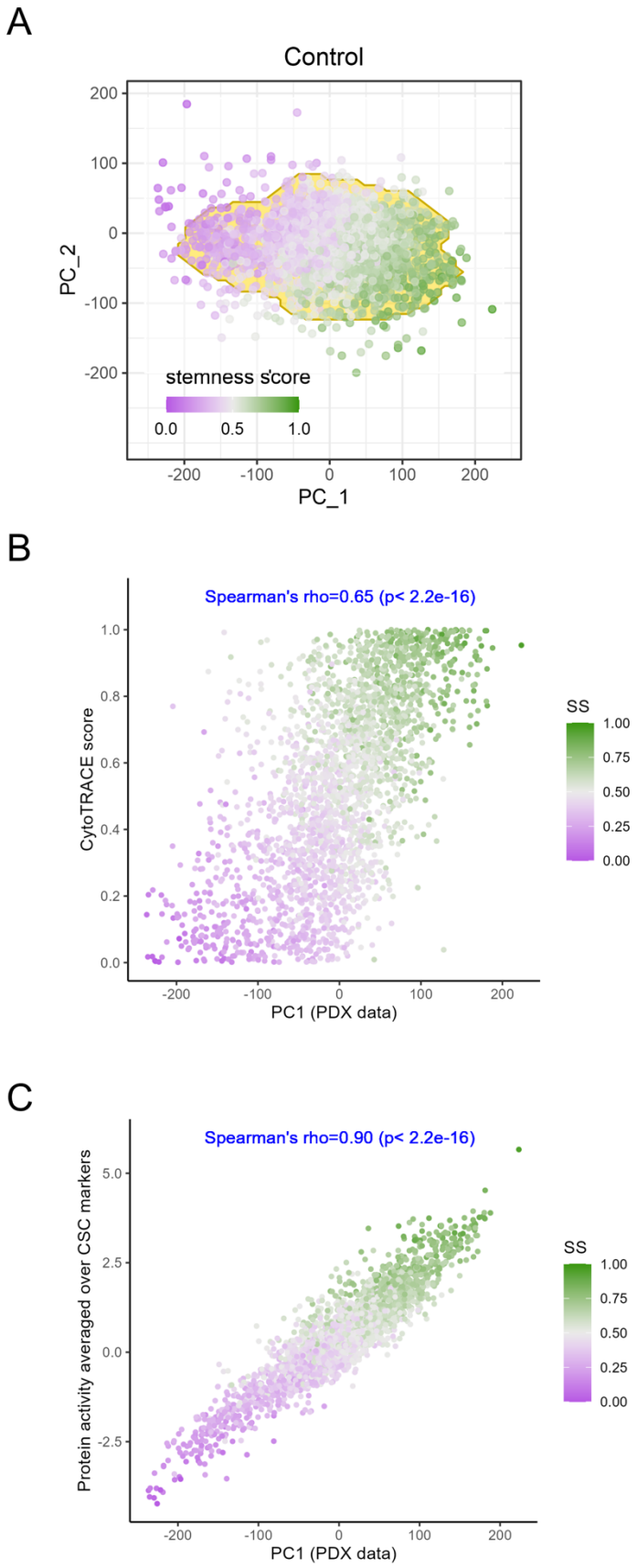



**Figure S8**. Protein activity-based cell clustering in the PDX control sample (i.e. vehicle treatment). The color of cells indicates the stemness score (SS) as described in Methods. **A.** The first two principal components (PCs) of the protein activity of cells. **B.** The correlation between the 1st PC and the CytoTRACE. **C.** The correlation between the 1st PC and the averaged activity of the 14 CSC markers.

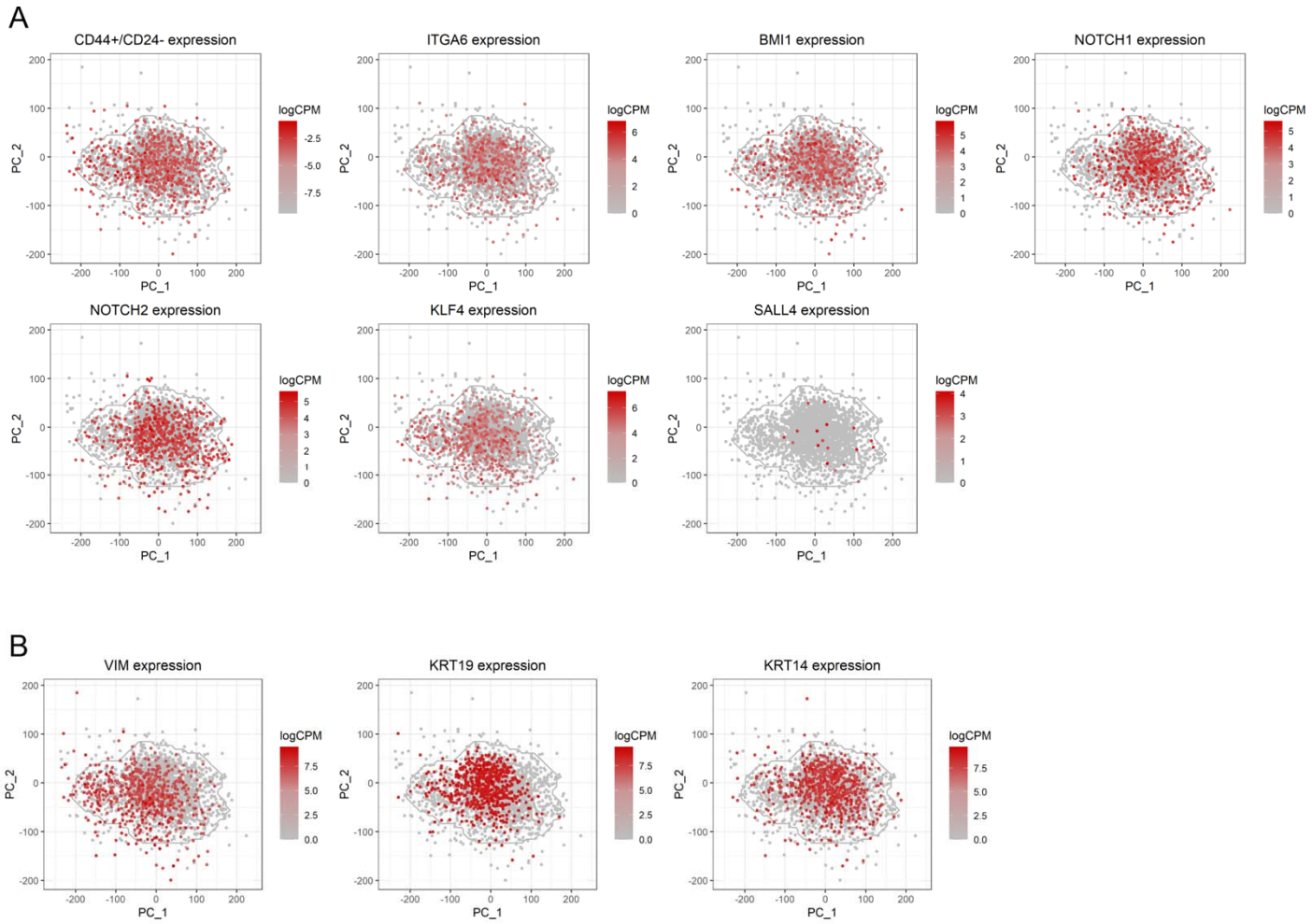



**Figure S9**. Gene expression ($log_2$CPM) of known breast CSC markers (**A**) and differentiated markers (**B**) in the PDX control sample. Protein activity-based PCA-mapping was used for the comparison. Note that only the gene expression greater than the upper quantile was shown for a visualization purpose.

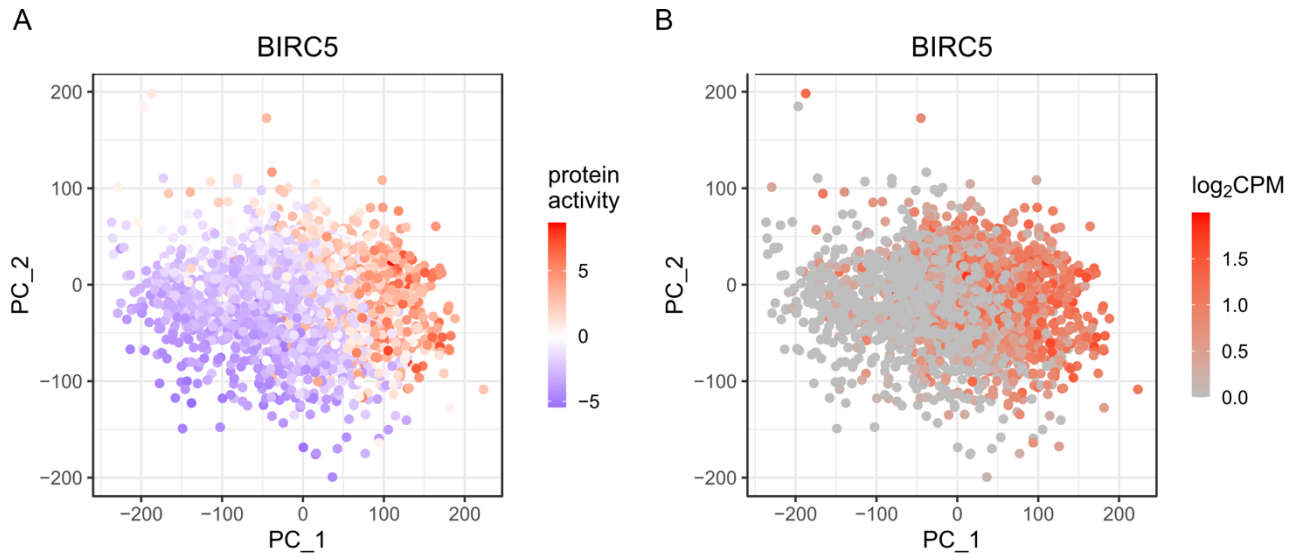



**Figure S10**. Protein activity (**A**) and gene expression ($log_2$CPM) (**B**) of BIRC5, a marker of quiescent-breast CSCs.

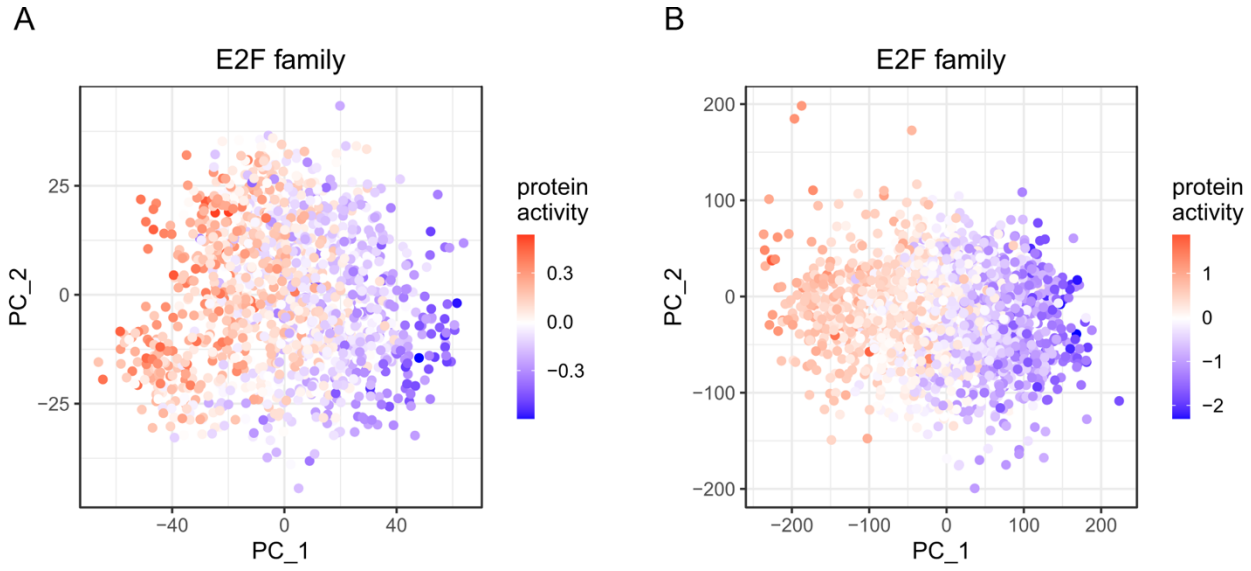



**Figure S11**. Protein activity of E2F family, a proliferative cell marker in the patient (**A**) and PDX control (**B**) data.

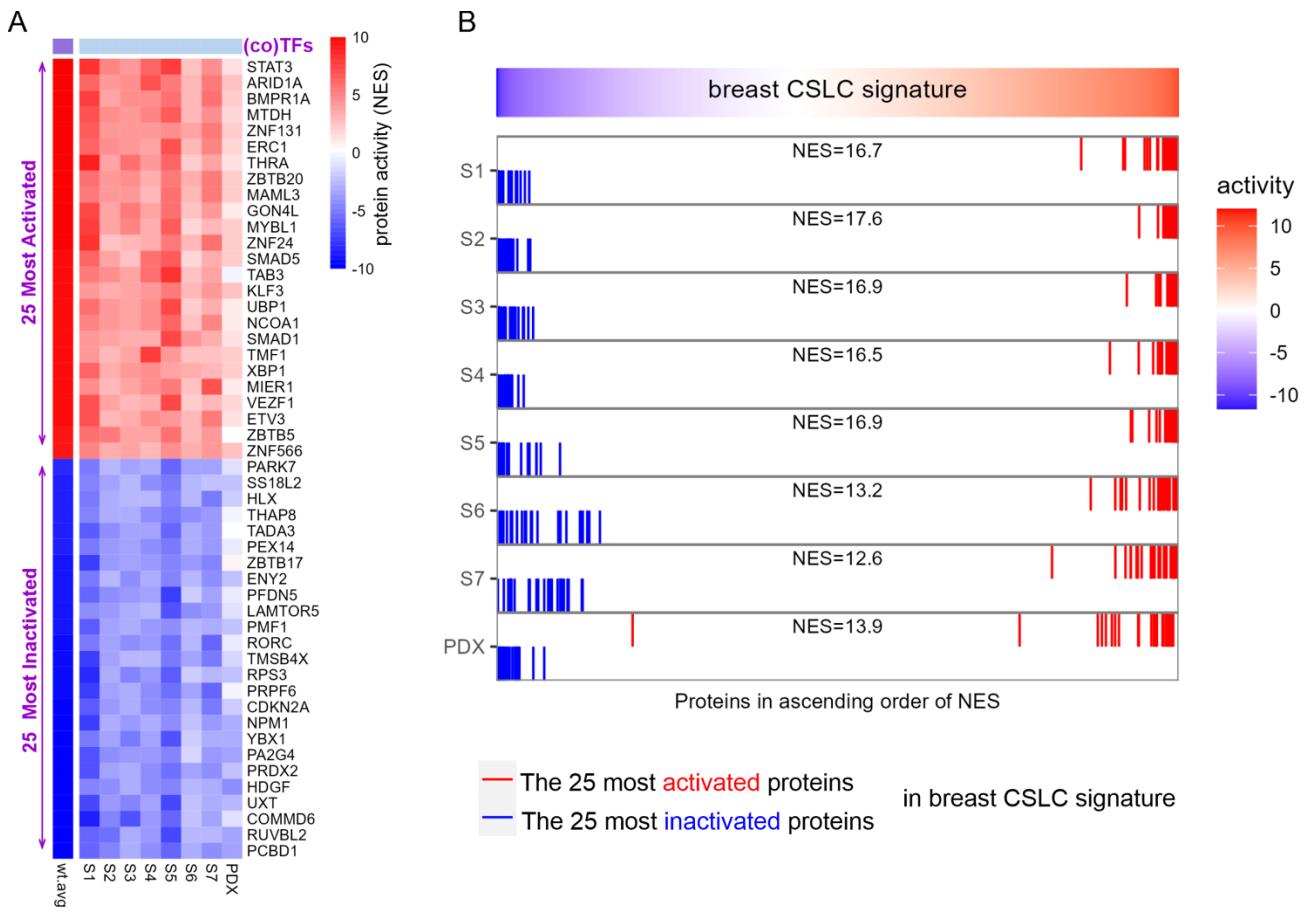



**Figure S12. A.** A heatmap exhibiting the activity of the 25 most activated and 25 most deactivated TFs/coTFs in the breast CSLC signature and their activities in individual samples (7 patient samples and the PDX vehicle-treated sample). **B.** An enrichment analysis plot of the 25 most activated and 25 most inactivated proteins of the CSLC signature in each individual sample. The consistency of the activity of these 50 proteins is observed in all samples, showing a significant enrichment (p-value< $1.0\times10^{-16}$).

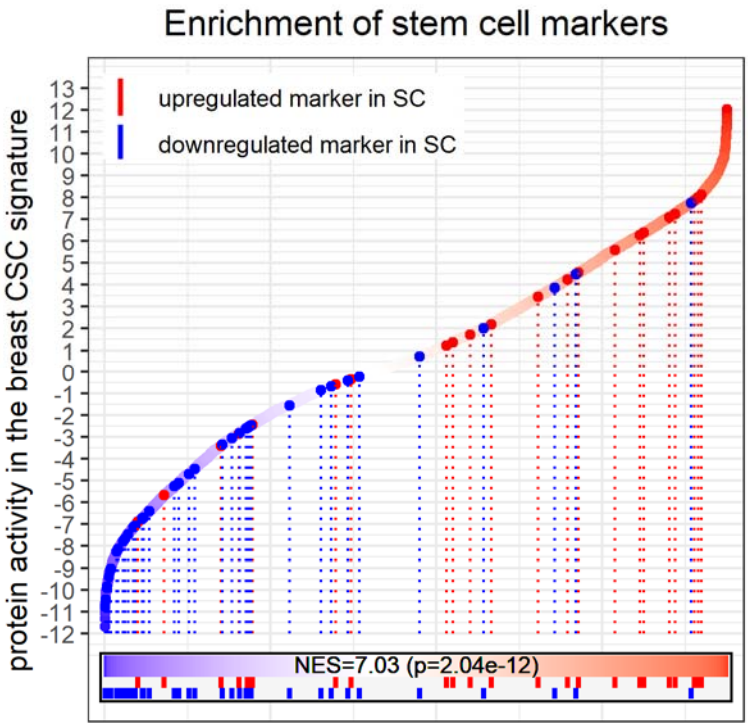



**Figure S13.** An waterfall plot of sorted protein activities in the breast CSLC signature, identified using both the patient and PDX control data. The NES of genes involved in general stem cell processes (supplementary data) is shown on the CSC signature.

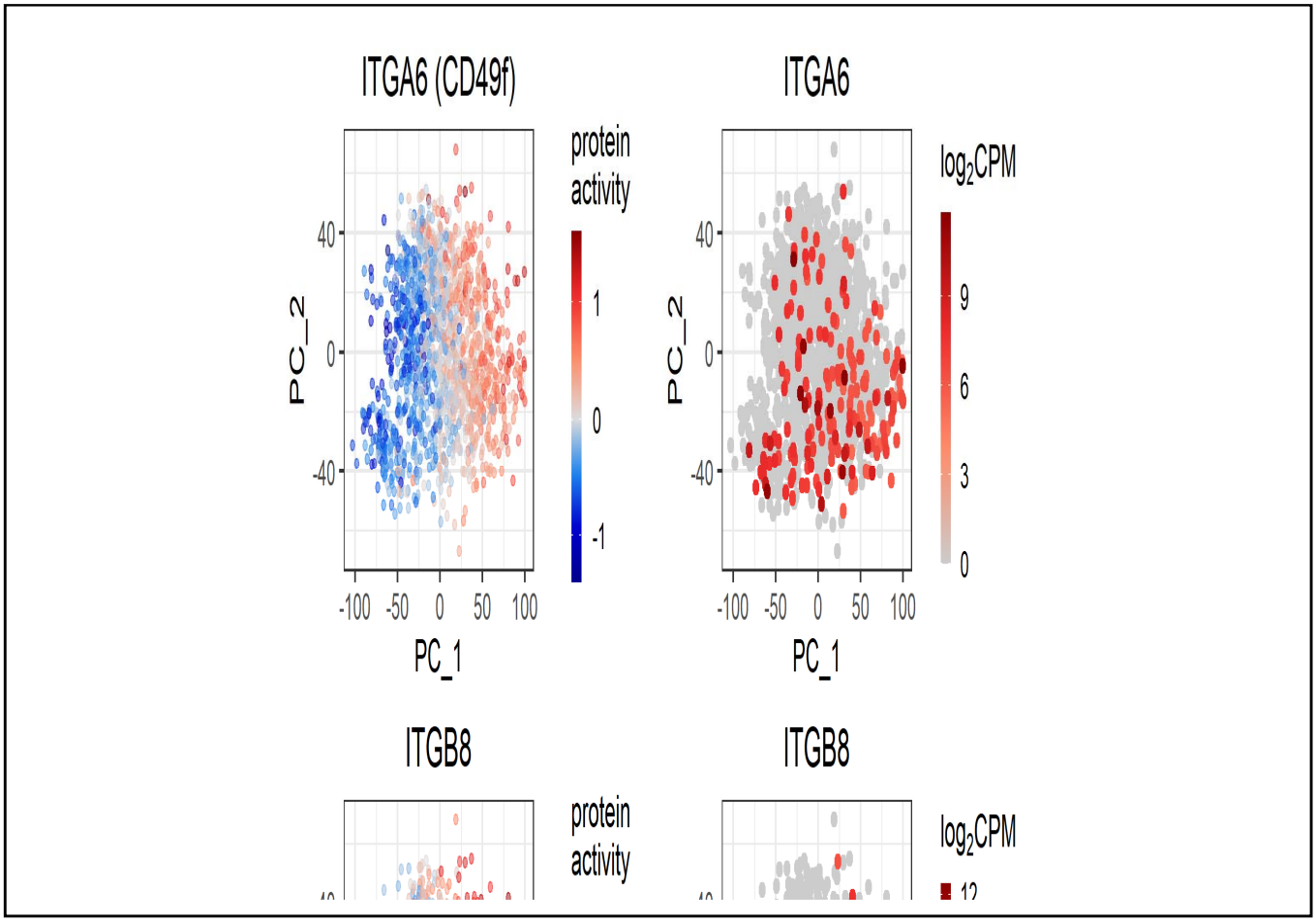



**Figure S14.** The VIPER-inferred activity (centered) and the gene expression ($log_2$CPM) of cell surface proteins among the top 50 most activated proteins in the breast CSCs. For gene expression, only cells with expression greater than the upper quantile are shown for visualization purposes.

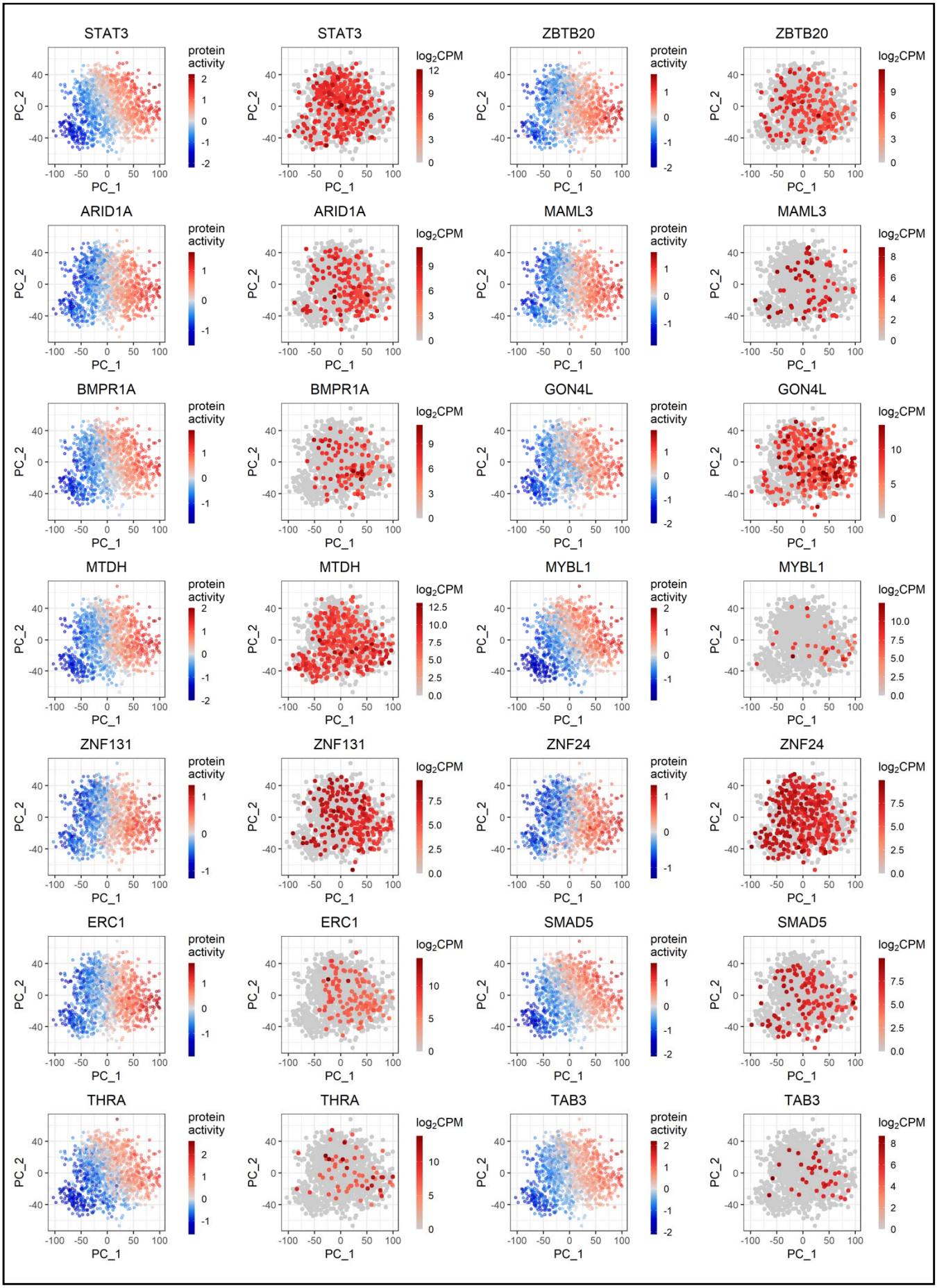



**Figure S15-A.** The VIPER-inferred activity (centered) and the gene expression ($\log_2$CPM) of transcription (co-)factors among the top 50 most activated proteins in the breast CSCs. For gene expression, only cells with expression greater than the upper quantile were shown for visualization purposes.

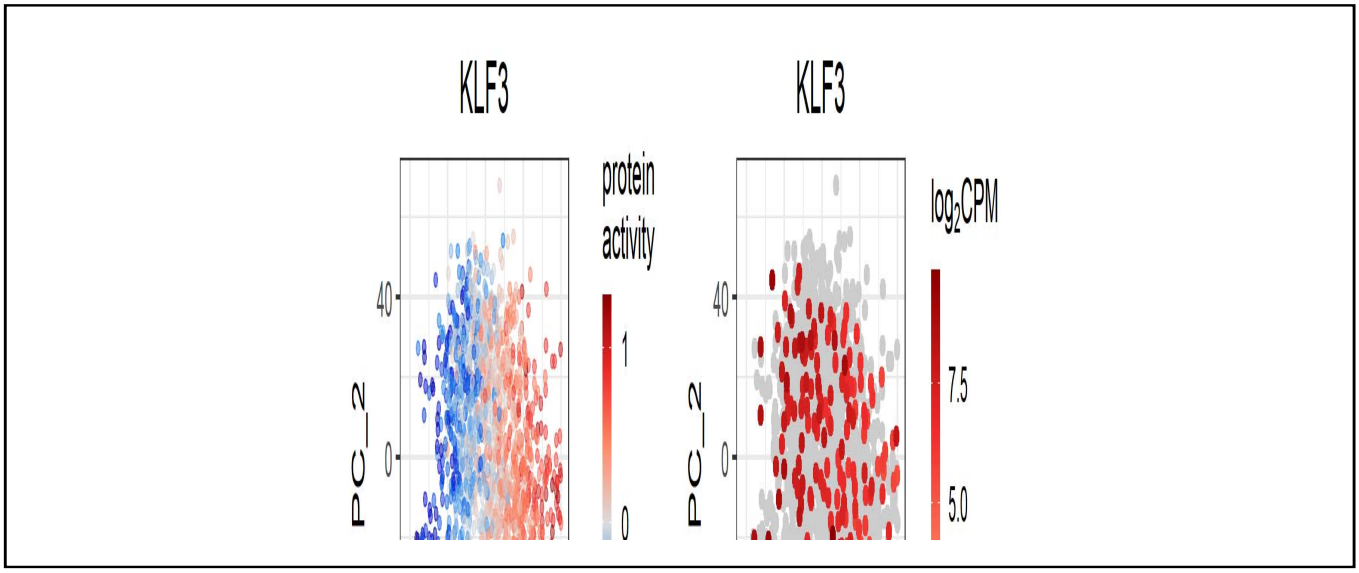



**Figure S15-B (continued).** The VIPER-inferred activity (centered) and the gene expression ($log_2$CPM) of transcription (co-)factors among the top 50 most activated proteins in the breast CSCs. For gene expression, only cells with expression greater than the upper quantile were shown for visualization purposes.

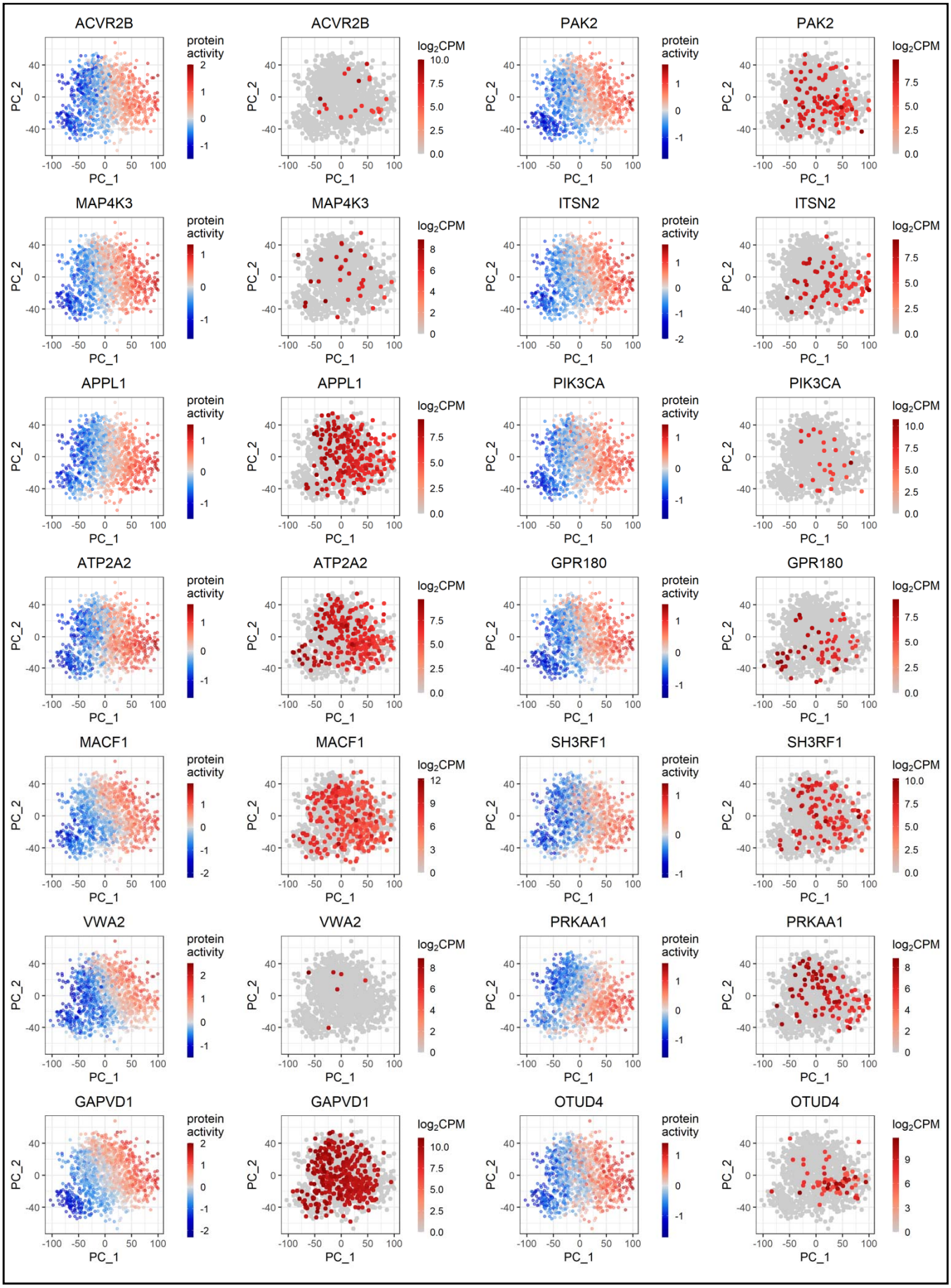



**Figure S16-A.** The VIPER-inferred activity (centered) and the gene expression ($\log_2$CPM) of signaling molecules among the top 50 most activated proteins in the breast CSCs. For gene expression, only cells with expression greater than the upper quantile were shown for visualization purposes.

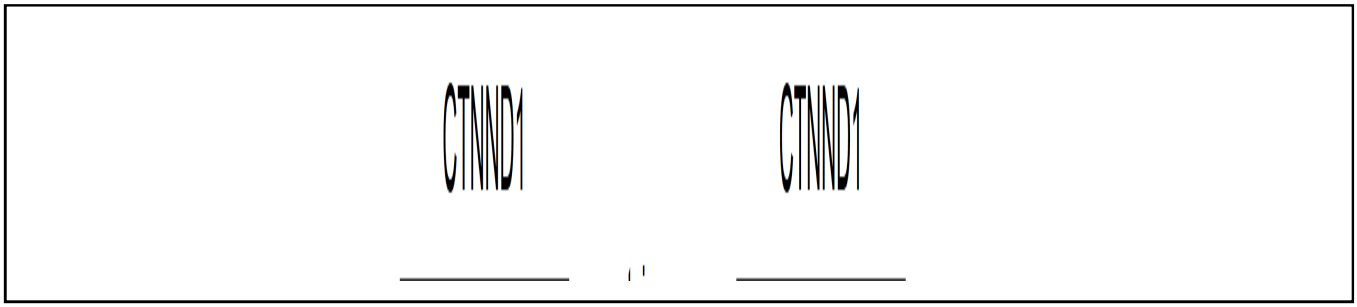



**Figure S16-B (continued).** The VIPER-inferred activity (centered) and the gene expression ($log_2$CPM) of signaling molecules among the top 50 most activated proteins in the breast CSCs. For gene expression, only cells with expression greater than the upper quantile were shown for visualization purposes.

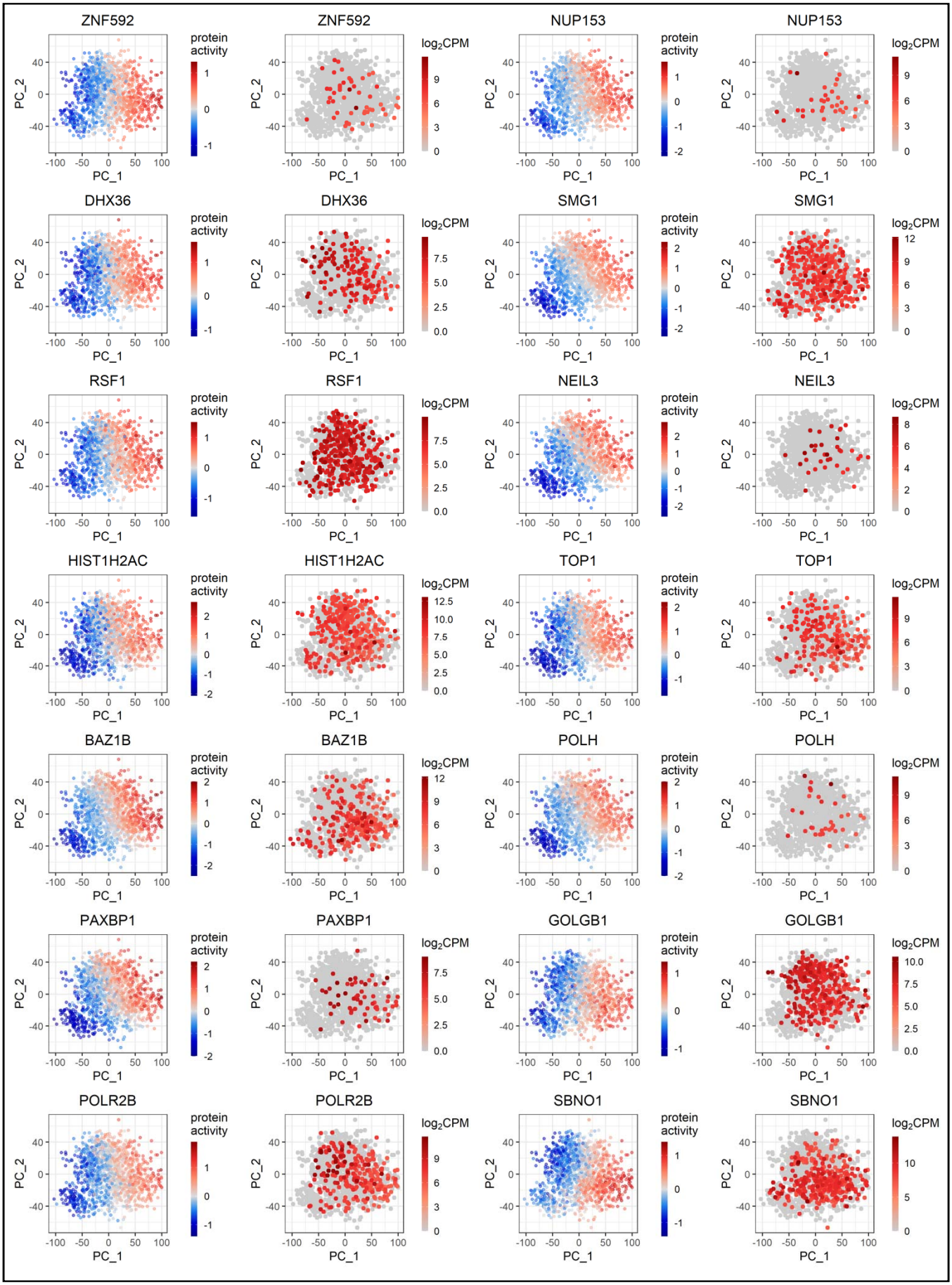



**Figure S17-A.** The VIPER-inferred activity (centered) and the gene expression ($log_2CPM$) of other proteins involved in transcriptional programs among the top 50 most activated proteins in the breast CSCs. For gene expression, only cells with expression greater than the upper quantile were shown for visualization purposes.

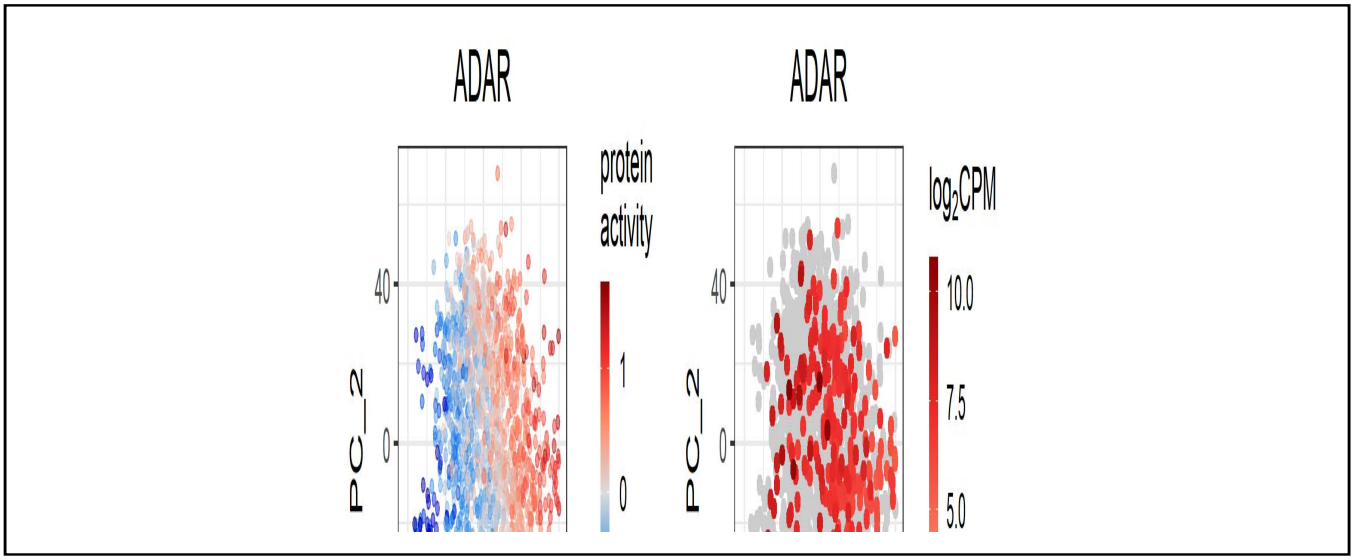



**Figure S17-B (continued).** The VIPER-inferred activity (centered) and the gene expression ($log_2$CPM) of other proteins involved in transcriptional programs among the top 50 most activated proteins in the breast CSCs. For gene expression, only cells with expression greater than the upper quantile were shown for visualization purposes.

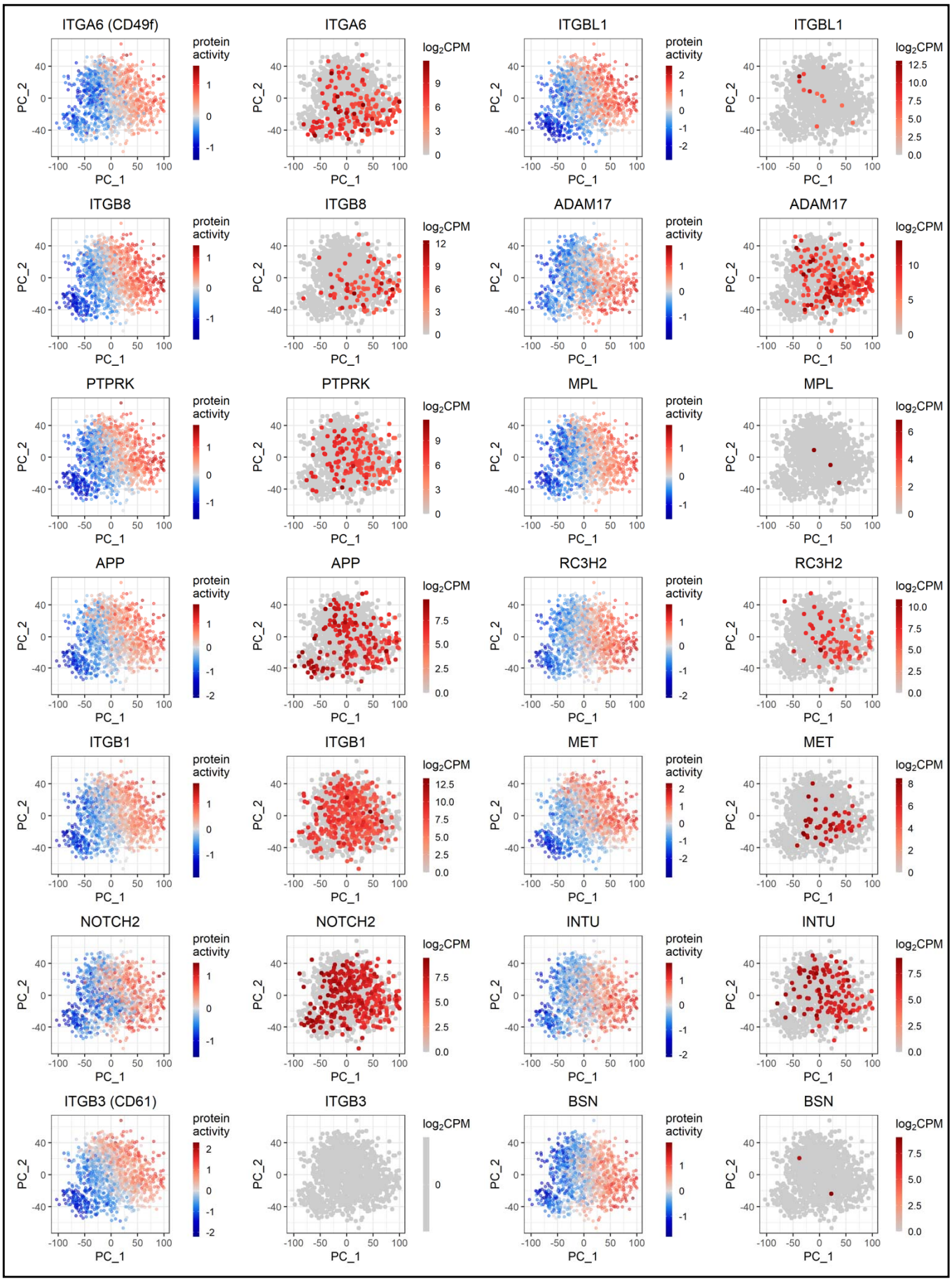



**Figure S18-A.** The VIPER-inferred activity (centered) and the gene expression ($\log_2$CPM) of cell-surface proteins among significantly activated proteins in the breast CSCs (Bonferroni adjusted *p*-value < 0.001). For gene expression, only cells with expression greater than the upper quantile were shown for visualization purposes.

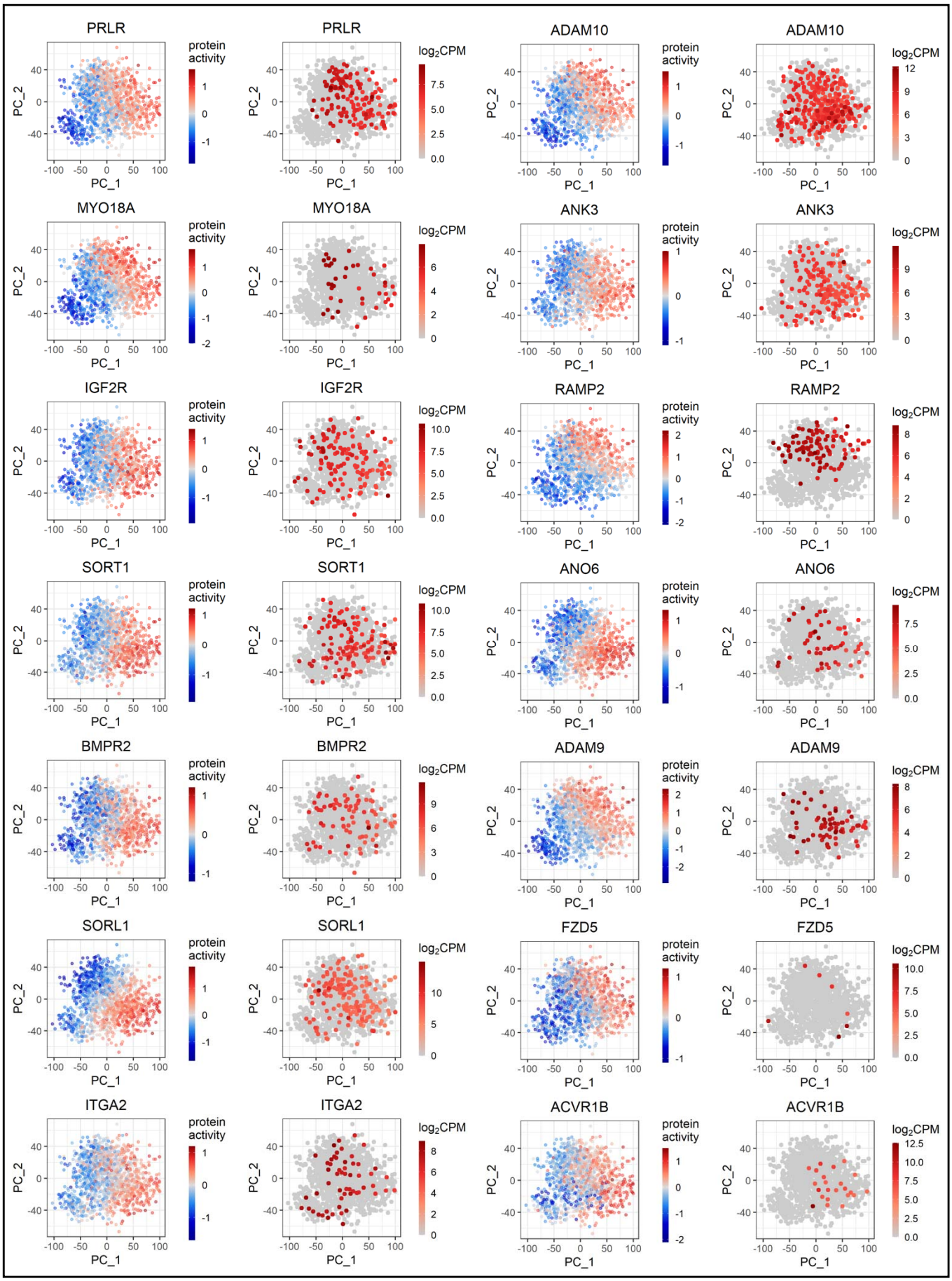



**Figure S18-B (continued).** The VIPER-inferred activity (centered) and the gene expression ($\log_2$CPM) of cell-surface proteins among significantly activated proteins in the breast CSCs (Bonferroni adjusted *p*-value < 0.001). For gene expression, only cells with expression greater than the upper quantile were shown for visualization purposes.

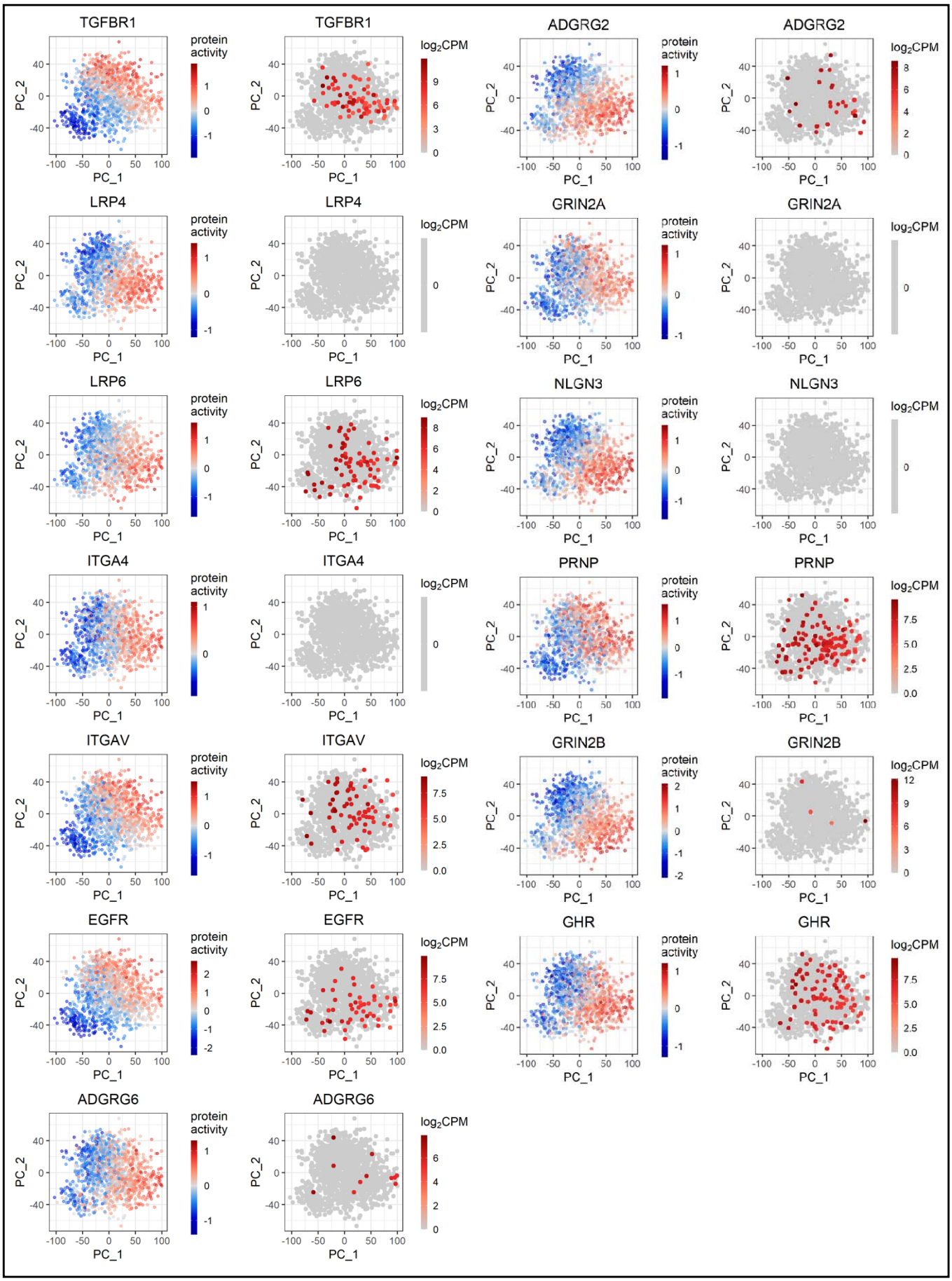



**Figure S18-C (continued).** The VIPER-inferred activity (centered) and the gene expression ($\log_2$CPM) of cell-surface proteins among significantly activated proteins in the breast CSCs (Bonferroni adjusted *p*-value < 0.001). For gene expression, only cells with expression greater than the upper quantile were shown for visualization purposes.

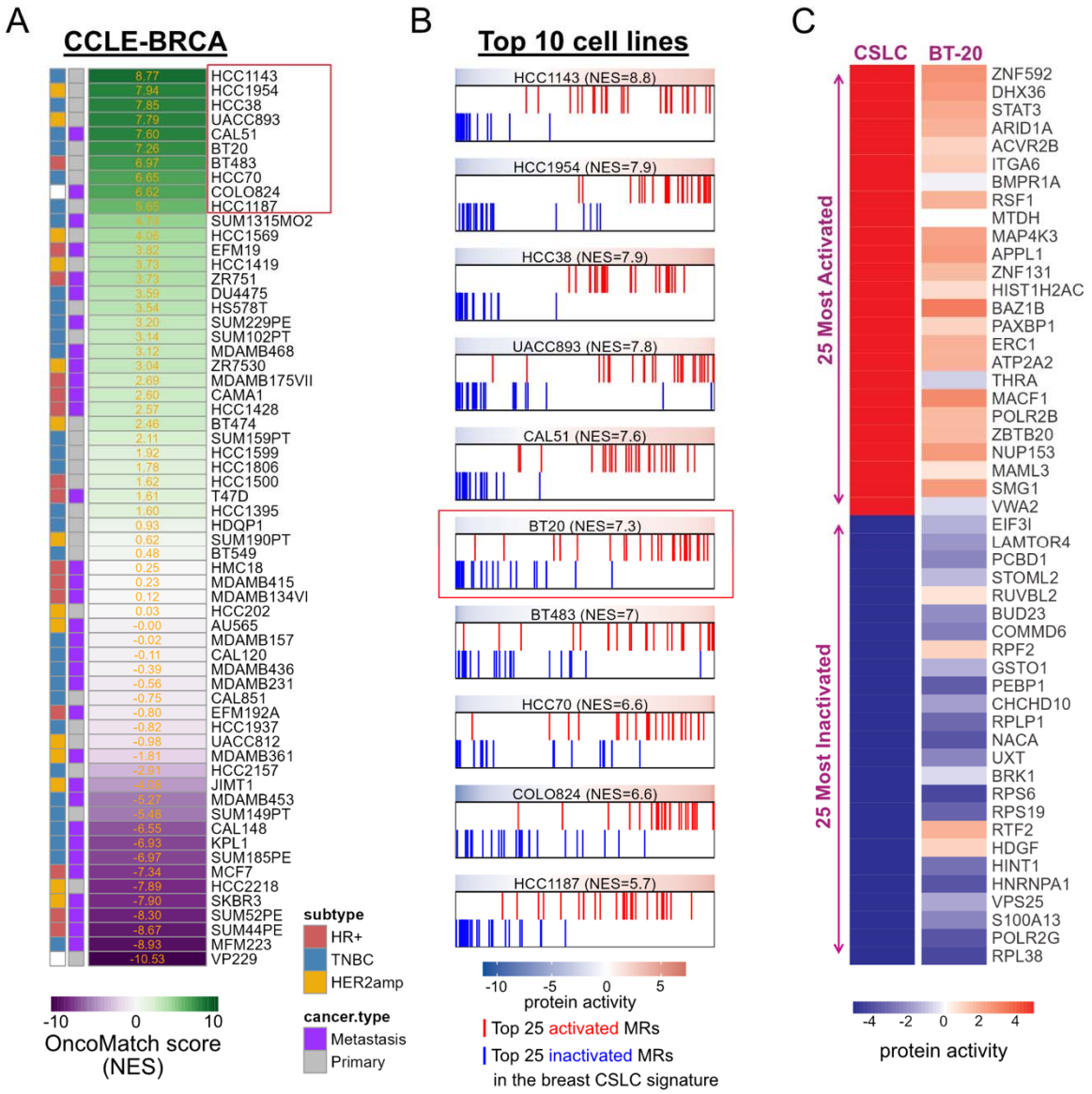



**Figure S19. A.** A heatmap illustrating the ranked list of breast cancer cell lines in CCLE, in a decreasing order of their OncoMatch score, which indicates the similarity score of the protein activity profiles between a cell line and the CSLC signature. Thus, the greater the OncoMatch score, the more stem-like cell properties. **B.** Enrichment analysis plots of the 25 most activated and the 25 most inactivated proteins in the breast CSLC signature for top 10 breast cancer cell lines by OncoMatch. The plot demonstrated a statistically significant enrichment (p-value< $1\times10^{-16}$), between BT20 and the CSLC signature. **C.** A heatmap of the activity of the 25 most activated and the 25 most inactivated proteins in the breast CSLC signature and their activities in BT20.

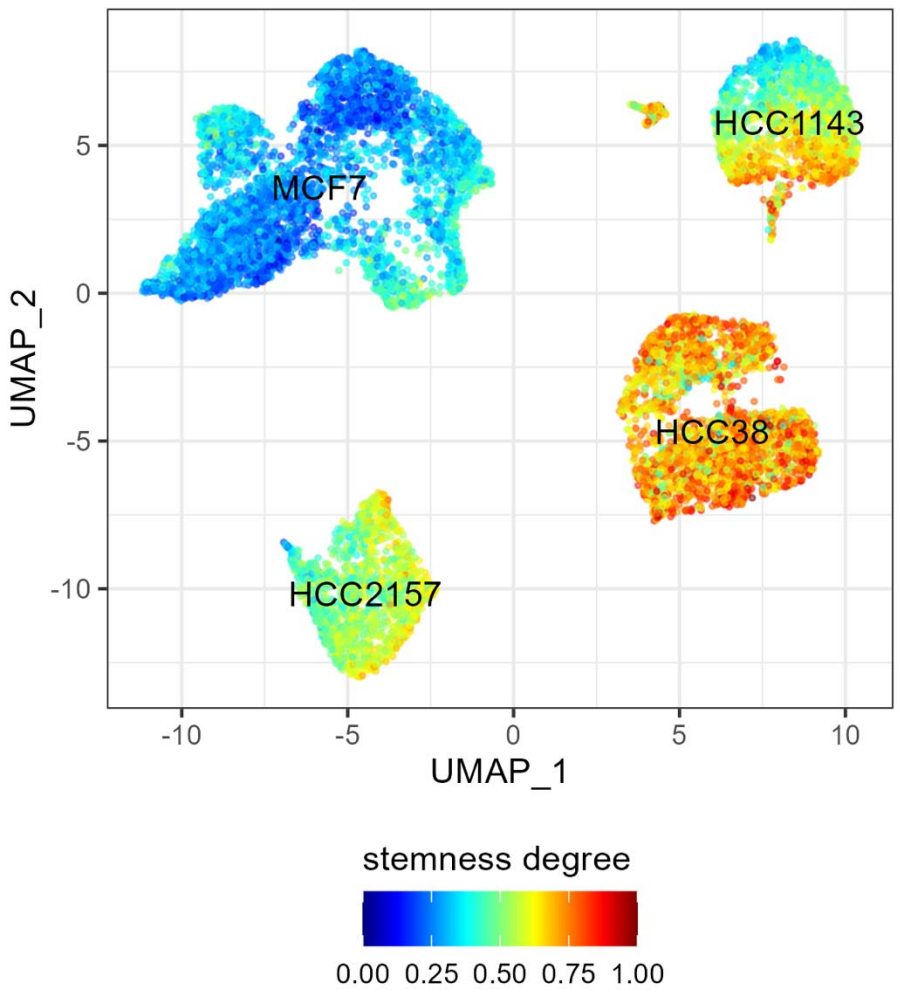



**Figure S20.** The UMAP projection of protein activity profiles of single cells for four breast cancer cell lines. HCC38 and HCC1143 are chosen for representing CSLC-rich cell lines, while HCC2157 and MCF7 are selected as well-differentiated cell lines (negative controls), based on our OncoMatch prediction as well as literature. The color of cells indicates the stemness degree calculated in the same manner as the previous (i.e. the weighted average of stemness marker activities and the CytoTRACE score). Thus, the greater score, the higher stemness in cells.

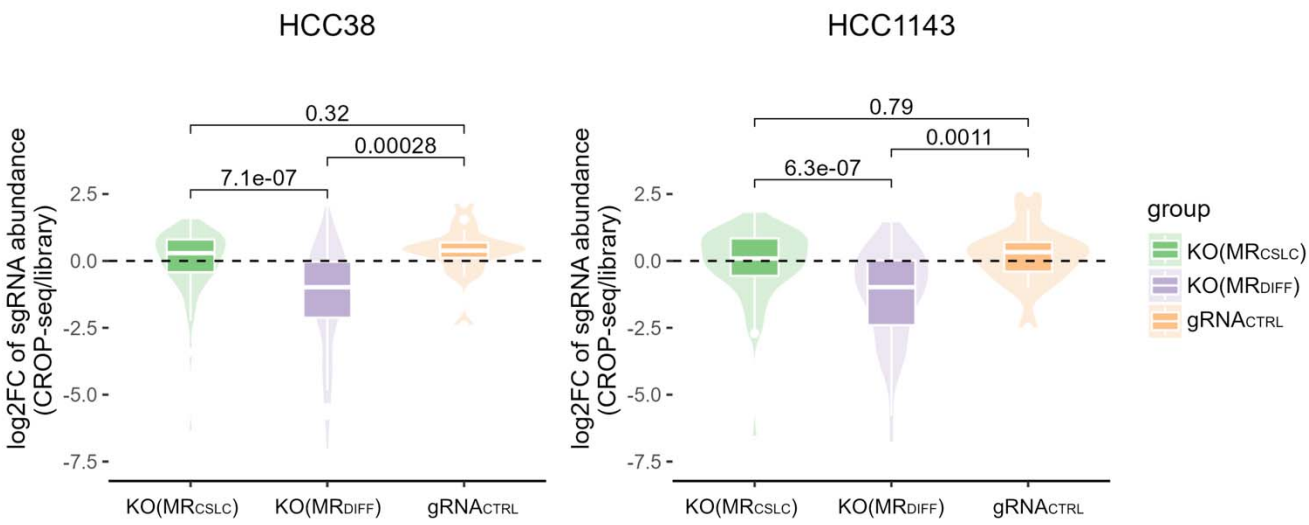



**Figure S21.** The log2 Fold Change (log2FC) of sgRNA abundance between CROP-seq and CRISPR library. sgRNA counts were normalized by dividing them by total sgRNA counts in each CROP-seq and CRISPR library. Unlike $MR_{CSLC}$, log2FC after $MR_{DIFF}$ KOs is significantly diminished, implying many of $MR_{DIFF}$ are responsible for cell fitness/proliferation.

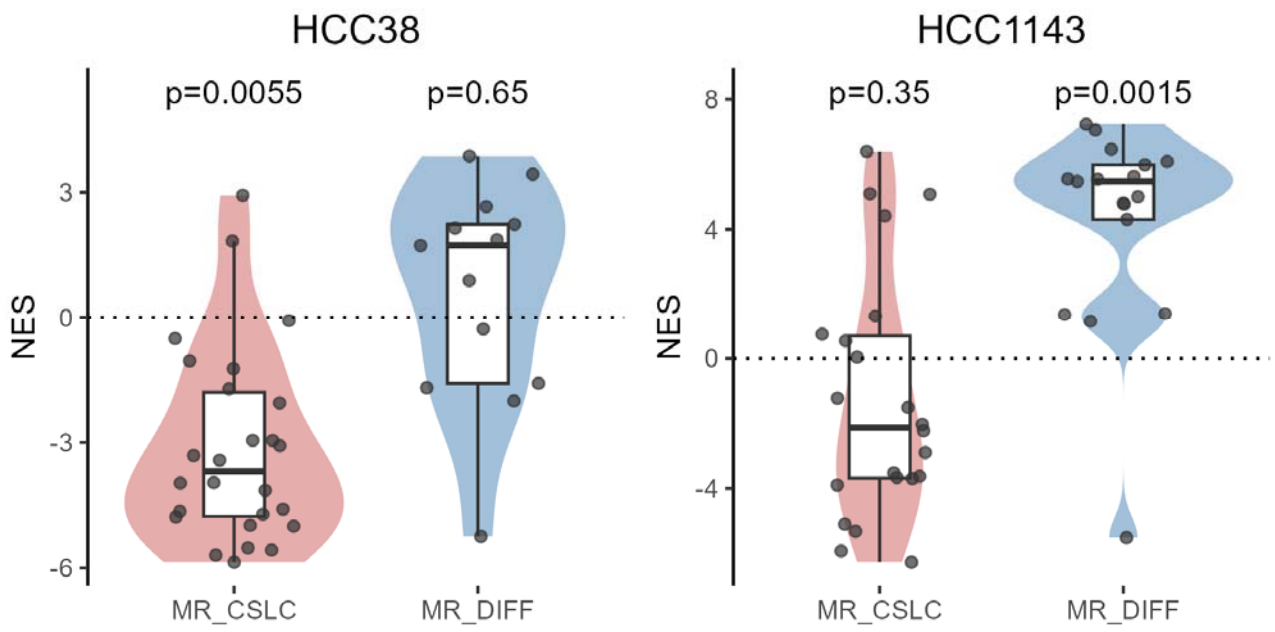
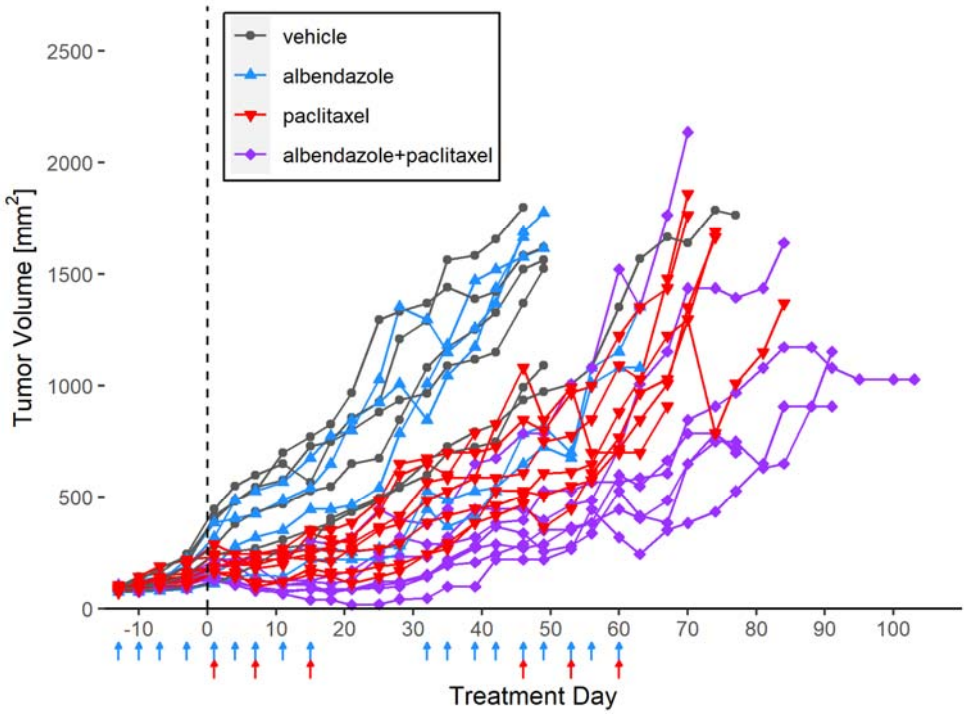



**Figure S22.** The enrichment of the gene set of stem cell process (supplementary table 2) between two groups ($MR_{CSLC}$ and $MR_{DIFF}$). Similar to Fig.5A, the enrichment score (NES) of stem cell process genes were significantly diminished ($p$=5.5×10$^{-3}$) after $MR_{CSLC}$ KO for HCC38, while NES was not significantly increased after $MR_{DIFF}$ KO for the same cell line. On the contrary, for HCC1143, the increase of NES was more striking ($p$=1.5×10$^{-3}$) after $MR_{DIFF}$ KO, while the NES change was not significant for the group of $MR_{CSLC}$ KO.

A




B

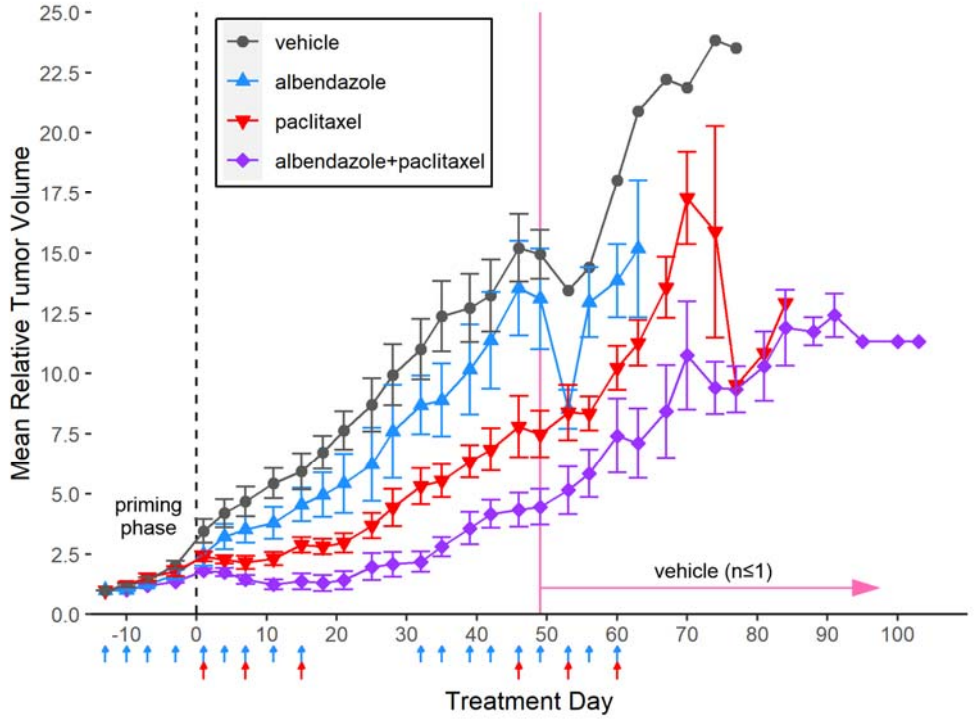



**Figure S23. A.** Spider plot of tumor volume measurements over time for individual mice. During a priming phase, mice were treated with albendazole 3 times weekly for two weeks before the start date of the combined drug therapy with paclitaxel, in order to sensitize the tumor cells. Mice with albendazole monotherapy were treated for the same amount of time as in the combination therapy. **B.** Mean relative tumor volumes over time. Tumor volumes are normalized by their volumes at Day-13. The error bar indicates one standard error of the mean. 5 out of 6 control mice (i.e. vehicle treatment) were euthanized before reaching Day 50.

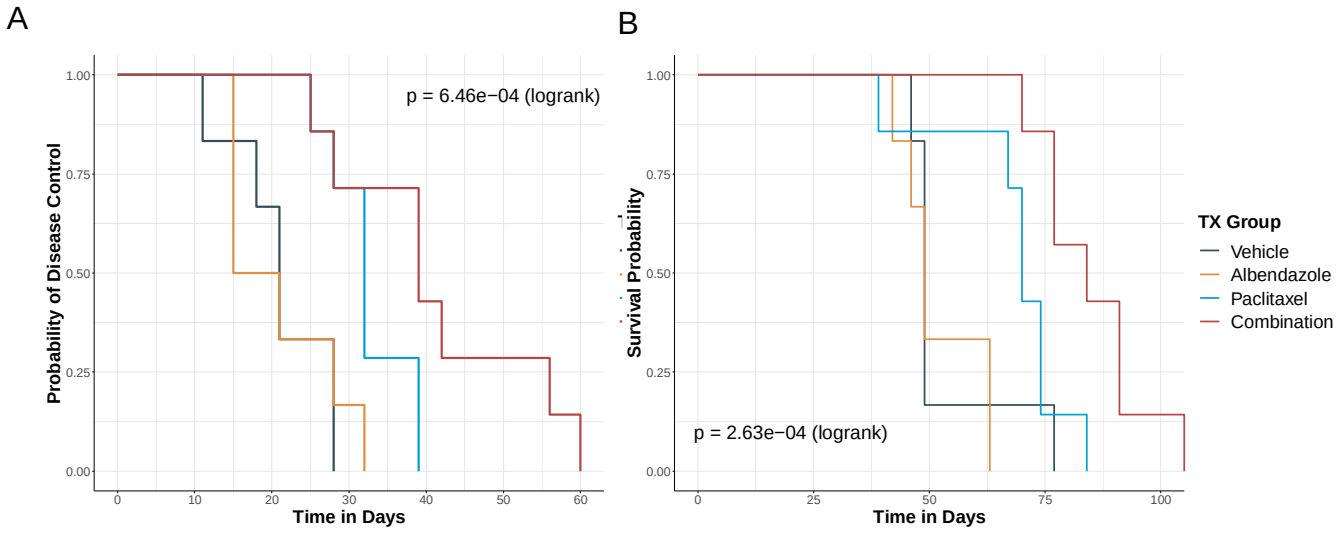



**Figure S24**. Kaplan-Meier analysis of the preclinical measurements for Disease Control (**A**) and survival (**B**) following treatment with albendazole and paclitaxel monotherapy vs. the combination.