Caveolin-1 regulates context-dependent signaling and survival in Ewing sarcoma.

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ABSTRACT:

Plasticity is a hallmark function of cancer cells, but many of the underlying mechanisms have yet to be discovered. In this study, we identify Caveolin-1, a scaffolding protein that organizes plasma membrane domains, as a context-dependent regulator of survival signaling in Ewing sarcoma (EwS). Single cell analyses reveal a distinct subpopulation of EwS cells, which highly express the surface marker CD99 as well as Caveolin-1. *CD99 High* cells exhibit distinct morphology, gene expression, and enhanced survival capabilities compared to *CD99 Low* cells,

both under chemotherapeutic challenge and *in vivo.* Mechanistically, we show that elevated Caveolin-1 expression in *CD99 High* cells orchestrates PI3K/AKT survival signaling by modulating the spatial organization of PI3K activity at the cell surface. Notably, CD99 itself is not directly involved in this pathway, making it a useful independent marker for identifying these subpopulations. We propose a model where the *CD99 High* state establishes a Cav-1-driven signaling network to support cell survival that is distinct from the survival mechanisms of *CD99 Low* cells. This work reveals a dynamic state transition in EwS cells and highlights Caveolin-1 as a key driver of context-specific survival signaling.

INTRODUCTION

Cellular adaptation is a hallmark function of cancer, driving intratumoral heterogeneity and contributing to tumor growth, metastasis, and therapeutic resistance [1]. Across many cancer types and under varying stresses, phenotypically distinct yet genetically similar subpopulations with different growth and metastatic capabilities have been identified [2]. However, the mechanisms underlying these adaptations remain poorly understood. This gap in knowledge is especially critical in pediatric cancers, which often exhibit a low mutational burden beyond a single driver, despite variability in patient outcome [3, 4]. In this work, we focus on Ewing sarcoma (EwS) as a prototype of pediatric cancer. EwS is the second most common malignant bone tumor occurring in children and young adults, characterized by a single chromosomal translocation leading to the formation of a chimeric fusion oncogene transcription factor, most commonly EWSR1::FLI1 [5, 6]. While much research has focused on EWSR1::FLI1 as the putative driver of distinct EwS cell states [7, 8], more recent works have identified EwS cell states with distinct gene signatures not classically associated with EWS::FLI1 gene expression [9-11]. In either case, the signaling mechanisms that drive segregation of cell states remain largely unknown.

One possible mechanism driving distinct cell states could be through regulation of signaling pathways on the plasma membrane, which integrates signals from the extracellular environment through targeted, localized signaling cascades. Differential organization of modified lipids or activated receptors on the cell surface into subcellular domains can affect downstream signaling[12, 13]. A prominent example of such domains can be observed in so-called caveolae, cave-like membrane invaginations that are rich in cholesterol and sphingolipids[14] and have roles in signal transduction [15-20]. Caveolae are considered specialized lipid domains, with effects on membrane fluidity and lipid species composition[21, 22]. These structures are prominently found in a subset of cell types, such as adipocytes and muscle cells, and have been implicated in many cancers[23, 24], including Ewing Sarcoma [25, 26]. Several studies have demonstrated that

perturbation of caveolae through mechanical means [27-29] or perturbations of the key structural protein Caveolin-1 affect canonical cancer signaling pathways, such as Src[16, 19, 20], Erk1/2[26, 30], and PI3K/Akt activation [31, 32]. Some of these studies have demonstrated differential subcellular organization of specific signaling molecules upon loss of caveolae [30, 33]. However, whether and how caveolae-based organization of signaling contributes to cellular plasticity in development and disease remains unclear.

In this study, we discovered an EwS cell population enriched for an epithelial-to-mesenchymal gene signature that is characterized by high expression of the surface receptor CD99. *CD99 High* cells show decreased proliferation but higher viability compared to *CD99 Low* cells, both *in vivo* and in response to chemotherapeutic drugs. We find that *CD99 High* cells associate with high endogenous Caveolin-1 expression levels and show an enrichment for caveolae. Mechanistic studies demonstrated that Caveolin-1 regulates the PI3K/AKT signaling pathway, associated with cell survival, through subcellular organization of PI3K on the cell surface. Notably, CD99 itself does not directly participate in this pathway, making it a useful independent marker for distinguishing this distinct population. Altogether, this work identifies a mechanism for context-dependent adaptation of survival signaling in EwS.

RESULTS

High CD99 marks a distinct Ewing Sarcoma cell state.

Nongenetic heterogeneity in cancer cells has increasingly been recognized as a critical driver of aggressive disease progression and treatment resistance [1, 2]. To study heterogeneity in EwS cell populations, we first used single cell transcriptomics to identify cell surface markers that would readily enable isolation of distinct subpopulations for further downstream characterization (Figure 1A). Given that cell surface receptors are sensitive to cleavage through standard enzymatic passaging techniques and inspired by classic studies in mesenchymal stem cells [34-36], we reasoned that cells passaged through mechanical means rather than enzymatic cleavage could be used to enable the discovery of distinct subpopulations of cells, as well as preserve surface receptors intact as future targets for flow cytometry. We applied this approach to the EwS cell line TC71, and uncovered a cluster of cells with a unique transcriptional profile (Figure S1A, Cluster 2), characterized by a distinct mesenchymal signature (Figure S1B). Interestingly, the differentially expressed genes showed little overlap with known EWSR1::FLI1-dependent gene signatures (Figure S1C). While previous literature has associated the mesenchymal gene signature state with low levels of EWSR1::FLI1 expression [8, 37-39], recent works have uncovered

a distinct mesenchymal cell state in the absence of the transcriptional repressor ETV6[10, 11]. that is distinct from the canonical EWSR1::FLI1 target gene signature. Consistent with this observation, ETV6 expression is decreased in cluster 2, while the ETV6-repressed gene SOX11 is increased (Figure S1D), suggesting this population represents a differentiation program distinct from the canonical EWSR1::FLI1 low cell state.

We next profiled the relative expression of transcripts of surface receptors between these two populations. Of 348 previously validated targets[38], seven surface receptors were enriched in cluster 2 (Figure 1B). Among those was the glycoprotein CD99, a commonly used biomarker for EwS[40], making it a natural target for flow cytometry (Figure 1B,C). To test the broader applicability of these findings, we also applied single-cell transcriptomics to cells isolated from two patient derived xenograft (PDX) lines, which have been passaged and subsequently extracted from subcutaneous xenografts in immunocompromised mice. These experiments also showed clusters with high CD99 expression and carried the mesenchymal gene signature (Figure 1D, S1B,E), suggesting CD99 could serve as a general marker for this particular cell state. Consistent with the single-cell transcriptomics results, immunolabeling of CD99 in TC71 cells followed by flow cytometric analysis uncovered a bimodality of CD99 distributions, representing intermediate and high levels of CD99 protein expression (Fig 1E). Of note, the CD99 High state appears to be reversible, since TC71 *CD99 High* cells that were isolated and subsequently grown for several passages again displayed a bimodal distribution of CD99 expression upon flow cytometric analysis (Fig 1F).

CD99 High* cells display differences in morphology, gene expression, and survival *in situ.

We subsequently utilized CD99 as a marker for flow cytometry, enabling isolation and further characterization of the two cell states. Differences in CD99 expression between *CD99 High* and *CD99 Low* cells were confirmed by western blot, while EWSR1::FLI1 protein expression levels remained consistent between the two groups (Fig S2A). Strikingly, the two subpopulations displayed distinct morphological phenotypes (Figure 2A, Figure S2B). The *CD99 High* cells were flat with thick actin bundles, while the *CD99 Low* cells appeared to be more rounded and in clusters, enriched with thin protrusions called filopodia. In addition, we observed that TC71 *CD99 High* cells displayed a marked enrichment of gene ontology terms related to neural differentiation by bulk transcriptomic analysis compared to *CD99 Low* cells (Figure 2B). Growth and viability assays revealed no differences between *CD99 High* and *CD99 Low* cells when grown in adherent flasks (Fig S2C,D). However, *CD99 High* cells displayed decreased growth capabilities through *in vitro* assays in soft environments (Fig S2E).

To test whether *CD99 High* cells show tumor growth or survival differences *in vivo,* we performed intratibial xenografts of isolated Luciferase-expressing *CD99 High* or *CD99 Low* TC71 cells, respectively, in immunocompromised mice. *CD99 High* cells showed dramatically lower tumor burden compared to *CD99 Low* cells (Figure 2C, S3A). A computed tomography scan also revealed that bony destruction, a phenomenon frequently found in EwS tumors and described as moth-eaten appearance of the bone[30], was decreased in *CD99 High* cells (Figure 2D, S3B).

To better determine sites of tumor growth we turned to analysis of histological sections. Tumor cells were identified morphologically through H&E staining and by CD99 immunohistochemistry (Figure 2E, S3C). Strikingly, most tumor growth of *CD99 Low* cells occurs through the local invasion of surrounding muscle, while both cell populations grow masses of limited size within the medullary cavity of the bone (Figure 2F, S3C). To test for survival and growth differences between groups, we combined fluorescent staining of the apoptotic marker TUNEL assay with immunostaining for the proliferation marker Ki67 to serially stained histological slides. For clusters of comparable size within the medullary cavity, the *CD99 Low* cells show comparable cell proliferation but strikingly more cell death compared to *CD99 High* cells (Figure 2E). Differences in cell death appear to hold true for local muscle invasions as well (Figure S3D). We observed that CD99 immunohistochemical staining appeared to be stronger in *CD99 High* tumors (Figure 2E, Figure S3C), suggesting distinct phenotypic cell states were sustained *in situ.* To determine whether morphological differences were also preserved *in situ*, we performed morphological feature classification on tumor nuclei in H&E-stained histological sections. *CD99 High* and *CD99 Low* cells occupied distinct regions in shape feature space (Figure S3E). Notably, *CD99 High* cells exhibited site-specific differences in shape features, whereas *CD99 Low* cells showed considerable overlap in feature space across seeding sites, suggesting that *CD99 High* cells may have a greater capacity to adapt to diverse microenvironments.

We wondered whether the putative adaptivity of *CD99 High* cells also confers greater resilience to drug challenges. To test this, we grew a mixed population of *CD99 High* and *CD99 Low* cells in the presence or absence of Doxorubicin (DOXO, 0.5uM), which serves as part of first-line therapy of EwS[40, 41]. Strikingly, by flow cytometry we found that DOXO treatment causes higher cell death in the *CD99 Low* cell population (Figure 2G). Closer examination of the surviving cells revealed a significant enrichment of *CD99 High* cells upon drug treatment, suggesting these cells have a survival advantage under these stress conditions (Figure 2H).

As an orthogonal approach to test distinctions in behavior between *CD99 High* and *CD99 Low* cells, we performed cancer cell xenografts in the hindbrain ventricle (HBV) of 2-day old zebrafish, which is permissive to high-resolution imaging[42]. Using cell morphology as a functional readout of cell state, we first examined whether distinctions in cell state were maintained in this *in vivo* environment. For this purpose, we injected a cell mixture consisting of 90% unsorted TC71 cells expressing the nuclear marker H2B-GFP, and 10% *CD99 High* or *CD99 Low* cells expressing the actin tag Ftractin-mRuby2 (Figure 3A-B). Following fixation in 4% paraformaldehyde (PFA) and 3D imaging of the zebrafish larvae at 1-day post-injection, we computationally extracted the cell volumes of individual *CD99 High* or *CD99 Low* cells for shape feature analysis to capture differences in cell morphotypes, as we previously described[42]. *CD99 High* cells displayed distinctly more protrusive, irregular shapes than *CD99 Low* cells (Figure 3C-D), suggesting that these EwS cells maintain distinctions in their cell state *in vivo*.

We next used xenografts in zebrafish to test differences in survival or proliferation between the two cell populations in the HBV. For this purpose, we injected *CD99 High* or CD99 *Low* cells expressing F-Tractin-mRuby2 into the HBV. Following chemical fixation at 2 days post-injection, we combined immunostaining of the proliferation marker Phospho-histone-H3 (PH3) with apoptotic TUNEL staining. Strikingly, *CD99 High* cells showed significantly less cell death and more proliferation than *CD99 Low* cells (Figure 3E-F). Taken together, the results presented thus far suggest that the *CD99 High* population represents a transcriptionally and functionally distinct cell state, potentially with survival advantages in certain tissues.

Caveolin-1 emerges as a molecular signature of *CD99 High* State.

Next we sought to identify potential molecular signature that would drive functionally distinct state of the *CD99 High* cell. Two initial lines of evidence converged around the protein Caveolin-1 (Cav-1) as a potential regulator of these functions. First, Cav-1 expression was highly enriched at the transcript level in the TC71 *CD99 High* cluster by single-cell transcriptomics (Figure 4A). Second, through a functional proteomics screen based on a Reverse Phase Protein Array (RPPA), we observed Cav-1 as the most differentially enriched protein in TC71 *CD99 High* cells compared to *CD99 Low* cells (Figure 4B). Protein measurement by western blot confirmed higher Cav-1 expression in *CD99 High* cells in EwS cell lines, TC71 and RDES (Figure S4A).

Cav-1 is the main structural protein responsible for cholesterol-rich membrane invaginations termed caveolae[14]. It is upregulated in metastases across several cancer types[43], including

EwS[25, 26], and has been implicated in diverse processes, including cell signaling, membrane trafficking, and stress response, making it a promising candidate for further investigation. To examine whether Cav-1-driven caveolae were enriched in *CD99 High* cells, we turned to ultrastructure analysis by electron microscopy. Indeed, we observed an abundance of caveolae in *CD99 High* cells but not in *CD99 low* cells (Figure 4C).

It is well established that Cav-1 can have both caveolar and non-caveolar localization patterns, with possibly distinct signaling functions [29, 33]. Therefore, we examined the colocalization of endogenously tagged Cav-1-mClover with another marker of caveolae, termed PTRF/Cavin-1[44], through immunofluorescence. While Cav1-mClover colocalized strongly with PTRF in *CD99 High* cells, the *CD99 Low* cells showed decoupled PTRF and Caveolin-1 localization (Figure 4D-E). The absence of caveolae and lack of colocalization with PTRF in *CD99 Low* cells is consistent with previous reports that PTRF expression is suppressed in EwS[45]. Quantitative analysis of diffusion dynamics of Cav-1 puncta showed slower diffusion in *CD99 High* cells (Figure S4B), another hallmark of stable caveolar structures.

To investigate whether Cav-1 drives the *CD99 High* state, we established a tetracycline (TET)-inducible shRNA knockdown system targeting Cav-1, combined with an RFP reporter, in TC71 cells (Figure S4C). TET-inducible scrambled shRNA had no effect on Cav-1 expression, confirming the absence of off-target effects (Figure S4D). In *CD99 High* cells, Cav-1 knockdown markedly reduced the density of membrane invaginations, demonstrating that changes in Cav-1 expression directly regulate the formation of caveolae (Figures 4C). However, Cav-1 knockdown had no major effect on CD99 protein expression (Figure S4C). We next used the previously established flow cytometry assay to test whether the proportion of the *CD99 High* population changed in response to Cav-1 KD. As an internal control, we co-cultured the TET-inducible Cav-1 shRNA, RFP+ cells with naïve TC71 cells with no drug induced Cav-1 knockdown. The TET-induced Cav1 shRNA expressing cells and naive cells could then be distinguished in flow cytometry by the presence or absence of the RFP reporter. Surprisingly, we observed not a decrease but an increase in *CD99 High* cells upon Cav-1 knockdown in TC71 cells (Figure S4E), with no change in the flat morphology compared to naïve *CD99 High* cells (Figure S2B, S4F).

Although Cav-1 knockdown does not alter CD99 expression levels or the morphology of *CD99 High* cells, we investigated whether *CD99 High* cells exhibit a Cav-1-dependent functional advantage. To address this, we assessed the viability of a mixed *CD99 High/CD99 Low* cell population in response to 10 μM DOXO, with or without Cav-1 knockdown. Cav-1 knockdown

did not confer an overall survival advantage under drug treatment (Figure S4G). However, closer analysis of the surviving CD99+ cell population revealed a dependency on Cav-1. While drug treatment enriched for *CD99 High* cells in populations with endogenous Cav-1 expression (Figures 2H, 4F), Cav-1 knockdown abolished this survival advantage, resulting in comparable levels of *CD99 High* and *CD99 Low* cells (Figure 4F). These findings demonstrate that Cav-1 is essential for the survival advantage of *CD99 High* cells during drug treatment, suggesting that Cav-1 drives survival-associated signaling in this cell state.

Caveolin-1 regulates PI3K/AKT signaling in *CD99 High* Cells

The change in viability upon drug treatment prompted us to examine changes in survival signaling pathways. By RPPA we noted in *CD99 High* cells an enrichment of Receptor Tyrosine Kinases that have been associated with the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, namely EphA2, VEGFR, c-Kit, and Axl (Figure 4B, Table S1)[46-49]. PI3K/Akt signaling is associated with cell survival in cancer, and is upregulated by Caveolin-1[31, 32]. Indeed, western blot showed increased Akt in its active form in *CD99 High* cells upon stimulation with Insulin Growth Factor (IGF, Figure S5A), or in the absence of Cav-1 knockdown (Figure S5B). Knockdown of Caveolin-1 in TC71 cells drastically reduced phosphorylated Akt levels in *CD99 High* but not *CD99 Low* cells, suggesting only *CD99 High* cells depend on Caveolin-1 for their Akt signaling. Notably, CD99 itself does not appear to be part of this pathway, since inducible knockdown of CD99 did not affect Cav-1 or phosphorylated Akt in *CD99 High* cells (Figure S5C).

PI3K/Akt signaling occurs specifically at the cell surface, and the downstream effects of PI3K/Akt signaling on diverse cell functions rely on the spatial organization of the active molecule [50]. We therefore applied a high-resolution 3D imaging approach to measure the levels and organization of PI3K activity at the surfaces of individual TC71 cells. We employed a GFP-tagged Akt-PH construct as a reporter of the local concentration of PI3K-activated lipid products[51, 52] in *CD99 High* cells*, CD99 Low* cells*,* and *CD99 High* cells with Cav-1 KD, embedded in 3D collagen (Figure 5A,B). Timelapse imaging with the pharmacological PI3K inhibitor GDC-0941 confirmed the specificity of active PI3K enrichment to the cell surface (Figure S5D). To compare differences in PI3K signaling between experimental groups, we leveraged the Cellular Harmonics framework [53] to assess the spatial scales of signal organization in a cell-morphology-invariant manner. Using this approach, we detected no differences in overall enrichment of active PI3K between *CD99 High* and *CD99 Low* cells

(Figure 5B), but major differences in the spatial organization of the signal (Figure 5A, 5C, S5E). Specifically, *CD99 High* cells displayed a more uniform distribution of active PI3K on the cell surface, represented by broad energy spectrum of GFP-Akt-PH localization. In contrast, *CD99 Low* cells displayed a more polarized distribution with a characteristic length scale, represented by a narrower band energy spectrum. Upon Cav-1 knockdown, *CD99 High* cells displayed higher overall enrichment of active PI3K on the cell surface (Figure 5B), but a strikingly more polarized distribution compared to *CD99 High* cells with Cav-1 expression (Figure 5C, S5E). Taken together, these results suggest that the presence of caveolae in in *CD99 High* cells promotes a uniform distribution of PI3K-activated lipid products that results in more effective signal transduction to AKT and downstream survival.

DISCUSSION

In this study, we uncover a subpopulation of EwS cells characterized by distinctions in transcriptional signature, cell morphology, and growth *in vivo.* These cells can be identified and isolated through flow cytometry based on their high expression of the cell surface receptor CD99, a glycoprotein often used as a diagnostic marker in EwS[54]. Functional characterization demonstrates a survival advantage of *CD99 High* cells in certain tissues *in vivo* and in response to a chemotherapeutic drug. We identify the protein Cav-1 as a key regulator of this context-dependent cell survival in *CD99 High* cells through regulation of PI3K signaling.

Heterogeneity in cancer cell states is a major factor in driving drug resistance and metastasis in cancer [1], creating an urgent clinical need to better understand the underlying mechanisms. In EwS, as in many pediatric cancers[4], mutational tumor burden beyond a single driver mutation is low, suggesting that non-genetic mechanisms are at play in modulating state heterogeneity. Several previous works have identified variability in expression levels of the oncogene EWSR1::FLI1 or its transcriptional signature as a driver of variable EwS cell states [8, 37-39], mostly through targeted knockdown of the oncogene and its targets. Notably, both CD99 and Cav-1 are known direct targets of EWS:FLI1[55]. However, the differences between *CD99 Low and CD99 High* cells described in this work appear to be agnostic to the oncogene level, but rather rely on Cav-1 as the modulator of cell state. Several recent studies have identified other potential drivers of EwS plasticity aside from EWSR1::FLI1. Some examples include depletion of the transcriptional repressor ETV6, which drives a mesenchymal gene signature with upregulation of neural developmental genes[10, 11], or discovery of a cancer-associated fibroblast (CAF)-like tumor cell state identified by higher presence of the surface receptor CD73[9]. The *CD99 High* state could be comparable to both these cell states since it too exhibits mesenchymal and

neural gene signatures, as well as low ETV6 expression and high expression of the neural marker Sox11, compared to *CD99 Low* cells. CD99 served as a useful independent marker of the distinct cell states, since our results demonstrated no clear connection with the Cav-1 regulatory pathway. This contrasts with previous studies that showed CD99 can induce Caveolin-1 expression [56] and inhibit neural differentiation [54, 57] in other contexts.

Several studies have demonstrated that mechanical and biochemical cues, such as substrate detachment, matrix stiffness, and hypoxia affect activity of Cav-1[27-29], suggesting Cav-1 could potentially respond to cell-extrinsic cues to influence cell state. Cav-1 has also been implicated in many cancers[23, 24], including EwS [25, 26]. We chose to pursue Cav-1 as a potential driver of distinct cell states in EwS cells due to the high differential expression levels of Cav-1 and striking difference in presence of Cav-1-driven caveolae in the *CD99 High* cells. Depletion of Cav-1 affected activation of downstream signaling pathways, and the ability of cells to survive under drug challenge. Since *CD99 Low* cells do not display caveolae, have different growth and survival phenotypes *in vitro* and *in situ*, and show distinct organization and expression of downstream signaling pathways, we presume this population of cells relies on alternative, Cav-1 independent mechanisms to regulate its cell survival.

Cav-1 is known to regulate key cellular signaling pathways, such as Src- family kinases, receptor tyrosine kinases, and RAS signaling[16, 19, 20, 20, 31, 32]. Whether these effects are through direct binding of Cav-1 to components of the signaling pathway, or through alternate mechanisms, remains unclear. One possibility is that Cav-1 can indirectly influence downstream signaling through changes in the composition and biophysical properties of the plasma membrane[21, 22]. This is evidenced by the protein's ability to selectively recruit specific lipids, such as cholesterol and PtdIns(4,5)P2, to caveolae [33], as well as its role in RAS nanoclustering[30]. Some studies have demonstrated flattening of caveolae and subsequent release of Cav-1 in response to mechanical stress[29, 58], suggesting that these structures can also serve as passive buffers of mechanical stress by acting as membrane reservoirs. Our work demonstrates Cav-1 dependent organization of active PI3K on the cell surface. Overall, our results lead us to a model whereby Cav-1 regulates PI3K organization in a specific subpopulation of EwS cells through its effect on membrane composition on the cell surface, driving context-dependent cell survival (Figure 5D). Specifically, *CD99 High* cells depend on high expression of Cav-1 and high caveolae density to organize the membrane through stabilization of lipid microdomains or decreased membrane fluidity. These make docking sites promiscuously available to PI3K localization, enhancing opportunities for stable signaling across

the entire cell surface. *CD99 Low* cells, or *CD99 High* cells with reduced Cav-1 expression, have dynamic, less stable microdomains unable to widely spread PI3K's signaling power. Future work will explore the cell extrinsic cues that drive enrichment of *CD99 High* cells with Cav-1-dependent cell states in EwS, with the hopes of uncovering novel insights in EwS tumor biology that will guide treatment.

METHODS

Cell culture and cell line generation

Cells were cultured at 5% CO2 and 21% O2. TC71 and RDES cells were cultured using RPMI (Gibco) supplemented with 10% FBS and 1% antibiotic antimycotic. For mechanical passaging, cells were grown in T125 cell-culture treated flasks (Corning 430825), and iteratively washed with PBS by tapping of flask to detach cells. Cells were then reseeded in the same flask for 2-6 weeks until mixed cell morphologies of the *CD99 High* and *CD99 Low* cell states were readily observed. IGF stimulation was done using 1nM Human IGF-1 recombinant protein (Peprotech) for 15 minutes, following 4 hours serum starvation.

TC71 Cav1, EF1, and CD99 shRNA lines cells were generated using the pZIP shRNA Promoter selection kit (https://www.transomic.com/cms/home.aspx). shRNA knockdown was induced by continuous 1 µg/ml tetracycline in cell culture, and shRNA knockdown induction was verified by RFP reporter expression and Western blotting.

Fluorescent constructs were introduced into TC71 cells using the pLVX lentiviral system (Clontech) and selected using antibiotic resistance to either puromycin or neomycin. The GFP-FTractin and mRuby2-FTractin constructs contain residues 9–52 of the enzyme IPTKA30 fused to eGFP or mRuby2, respectively. pLenti6-H2B-GFP was acquired from Addgene (generated by Fuchs lab, New York University, plasmid # 25999; Addgene*).* The GFP-AktPH construct was obtained from the laboratory of Jason Haugh (North Carolina State University, Raleigh NC)[59] and cloned into the pLVX-IRES-puro vector (Clontech).

Endogenously tagged TC71 Cav-1-mClover was generated through CRISPR-based knock-in strategy. Briefly, two guides were generated using CRISPOR[60] (gRNA1: GCATCCCGATGGCACTCATC and gRNA2: GACCCACTCTTTGAAGCTGT), and were cloned into plasmid pSpCas9(BB)-2A-Puro (Feng Zhang lab, plasmid # 62988, Addgene). The donor vector was generated using plasmid pMA-Tia1L which was a gift from Tilmann Bürckstümmer [61].

RPPA

Functional Proteomics Reverse Phase Protein Array (RPPA) was performed by the RPPA core at MD Anderson Cancer Center. Cellular proteins were denatured in a 1% SDS + 2-mercaptoethanol buffer solution and diluted in five 2- fold serial dilutions in dilution lysis buffer. Serially diluted lysates were arrayed on nitrocellulose-coated slides (Grace Bio-Labs) by the

Quanterix (Aushon) 2470 Arrayer (Quanterix Corporation). A total of 5808 spots were arrayed on each slide including spots corresponding to serially diluted (1) standard lysates, and (2) positive and negative controls prepared from mixed cell lysates or dilution buffer, respectively. Each slide was probed with a validated primary antibody plus a biotin-conjugated secondary antibody. Antibody validation for RPPA is described in the RPPA Core website: https://www.mdanderson.org/research/research-resources/core-facilities/functional-proteomics-rppacore/antibody-information-and-protocols.html. Signal detection was amplified using an Agilent GenPoint staining platform (Agilent Technologies) and visualized by DAB colorimetric reaction. The slides were scanned (Huron TissueScope, Huron Digital Pathology) and quantified using customized software (Array-Pro Analyzer, Media Cybernetics) to generate spot intensity. Relative protein level for each sample was determined by RPPA SPACE (developed by MD Anderson Department of Bioinformatics and Computational Biology, https://bioinformatics.mdanderson.org/public-software/rppaspace/)[62] by which each dilution curve was fitted with a logistic model. RPPA SPACE fits a single curve using all the samples (i.e., dilution series) on a slide with the signal intensity as the response variable and the dilution steps as the independent variable. The fitted curve is plotted with the signal intensities, both observed and fitted, on the y-axis and the log2 concentration of proteins on the x-axis for diagnostic purposes. The protein concentrations of each set of slides were then normalized for protein loading. Correction factor was calculated by (1) median-centering across samples of all antibody experiments; and (2) median-centering across antibodies for each sample. Results were then normalized across RPPA sets by replicates-based normalization as described[63]. Details of the RPPA platform as performed by the RPPA Core are described in[64].

Transcriptomics

For single cell RNA-seq, RDES and TC71 cell lines were dissociated in Tryp-LE express enzyme (GIBCO), resuspended in 0.04% BSA in PBS, and passed through a 70um filter before diluting to 1000 cells/uL. NCH- EWS1 and SJEWS001321_X1 PDX lines were kindly provided by Peter Houghton (University of Texas Health Sciences center San Antonio), and St. Jude Children's Research Hospital Childhood Solid Tumor Network (SJEWS001321_X1), respectively. For PDXs, tumor chunks >5 grams were dissociated using Trypsin and Collagenase, treated with a combination of DNAse and Magnesium Chloride to dissolve DNA, and passed through a 40um filter and treated with a Red Blood Cell lysis buffer before resuspending in 0.04% BSA in PBS and diluting to 1000 cells/uL. Samples were processed as

in 10× Genomics Chromium 3’ V3.1 with Cell Multiplexing kit and pooled together evenly for a final concentration of 1500 cells/μL in 0.04% BSA in PBS. Single-cell RNA-seq libraries were prepared using 10× Genomics Chromium 3′ V3.1 kit, following the standard protocol.

Single-cell RNA sequencing (scRNA-seq) data were generated for three samples: NCH-NCH-EWS1 PDX, SJEWS001321_X1 PDX, and TC71 cell line using the 10x Genomics platform. The data were processed using the Cell Ranger pipeline (version 6.0.0) to generate gene-barcode matrices, which were subsequently analyzed in Seurat (version 5.0.2). For each sample, raw count matrices, barcodes, and feature files were loaded into Seurat to create individual Seurat objects. Quality control was meticulously performed to exclude cells with extreme total RNA counts (nCount_RNA) and detected features (nFeature_RNA), with thresholds set based on the 5th and 95th percentiles for RNA counts, and the 10th and 90th percentiles for detected features. Additionally, cells with high mitochondrial content, indicative of potential cell stress or apoptosis, were filtered out by excluding cells with mitochondrial transcript percentages exceeding 15%. Data normalization was conducted using the "LogNormalize" method with a scale factor of 10,000, and highly variable features were identified using the "vst" method with 2,000 features selected for downstream analysis. Principal component analysis (PCA) was applied for dimensionality reduction, followed by clustering using the Louvain algorithm and visualization using Uniform Manifold Approximation and Projection (UMAP). Cell cycle scoring was performed by classifying cells into G1, S, and G2/M phases based on established markers. Specifically, cells in the G1 phase were subsetted for more focused analysis, ensuring that cell cycle effects were minimized in the downstream analyses. Gene Set Enrichment Analysis (GSEA) was conducted on the differentially expressed genes, utilizing pathways relevant to the EWSR1::FLI1 fusion as described by Siligan and Torchia. Furthermore, marker gene expression, such as CD99, was profiled across the different clusters, and volcano plots were generated to highlight significant differentially expressed genes.

Flow Cytometry

For flow cytometry, TC71 cells were dissociated in Tryp-LE express enzyme, resuspended in 5% FBS in PBS, and passed through a 70um filter before diluting to 1X10^6 cells/mL. For live cell staining of CD99, cell suspension was incubated with mouse anti-human PE-CD99 antibody (BD Pharmingen 555689, 1:6). For shRNA expressing cell lines expressing RFP, cell suspension was incubated with rabbit monoclonal CD99 antibody (Abcam EPR3096 1:50) followed by secondary anti-rabbit antibody conjugated to AlexaFlour 405 (Abcam ab175652,

1:1000). For viability staining in Figure S4D, cells were additionally stained with 1:1000 Live-Dead fixable Far-Red stain (ThermoFisher L34973) prior to flow cytometry. Samples were analyzed or sorted based on CD99 detected fluorophore intensity using BD FACSAria Zelda (BD BioSciences) flow cytometers. Flow cytometry plots were visualized using FloJo Software (BD Bioscences).

Western Blots

Primary antibodies used for western blotting were anti-CD99 (Abcam; EPR3096), Fli1 (Abcam; ab133485), Tubulin, Cav-1 (Cell Signaling; #3267), pAKT (Cell Signaling; #4060), AKT (Cell Signaling; #2920), Histone H3 (Cell Signaling: #9715), Phospho-PI3K (Invitrogen, #17387), PI3K (Cell Signaling, #42925).

Zebrafish husbandry and xenografts

Danio rerio were maintained in an Aquaneering aquatics facility according to industry standards. Vertebrate animal work was carried out by protocols approved by the institutional animal care and use committee at UT Southwestern, an Association for Assessment and Accreditation of Laboratory Animal Care accredited institution. Wild Indian Karyotype (WIK) wild-type lines used were obtained from the Zebrafish International Resource Center (zebrafish.org).

For xenografts of TC71-Ftractin-mRuby2 cells, cells were sorted for *CD99 High* and *CD99 Low* populations, then expanded prior to injection. Preparation of cell mixture consisted of 90% mRuby2-Ftractin labeled cells and 10% unsorted H2B-GFP labeled cells, or 100% mRuby2-Ftractin labeled cells, as indicated. Cell mixtures (2×10^6 cells) were run through a 70-µm cell strainer and resuspended in 5% FBS in PBS mixture for optimal cell viability.

Zebrafish embryos were collected, dechorionated, and treated with 0.1 mM phenylthiourea starting at 24 h post fertilization to prevent pigmentation. Xenografts were generated by microinjection of cell mixture into zebrafish larvae (2 days post fertilization [dpf]) with glass capillary needles into HBV. For each cell xenograft group, cells were injected into 3-aminobenzoic acid ethyl ester (Tricaine)–anesthetized embryos. Injected zebrafish larvae were incubated for 1–2 d in 34°C before imaging.

For immunostaining of zebrafish larvae, anesthetized embryos were fixed in 4% paraformaldehyde/1XPBS for 2 h in scintillation vials, rinsed in PBS +0.5% Tween, and stored in PBS up to 2 wk. Immunostaining was carried out as previously described (Miller et al., 2015). Primary antibodies were rabbit anti-GFP (ab290; Abcam) and mouse anti-Phospho-Histone H3

(9706S; Cell Signaling). TUNEL assay was performed using the ApopTag Green In-Situ Apoptosis Detection Kit (Millipore).

Mouse xenografts

All experiments involving animals were reviewed, approved, and monitored for compliance by the UT Southwestern Institutional Animal Care and Use Committee.

Lenti-luciferase-P2A-Neo was acquired from Addgene (Vakoc lab, Cold Spring Harbor Laboratory, plasmid: #105621; Addgene), and introduced into TC71 cells, which were subsequently sorted for *CD99 High and CD99 Low* populations and expanded prior to xenografts. Maintenance of isolated cell states was validated by distinct cell morphology in cell culture. Orthotopic intratibial xenografts were performed in NOD *scid* gamma chain knockout (NSG) mice, following a previously established protocol[65]. Briefly, the top of the right tibia was punctured with a 25 gauge needle, followed by injection of $5x10^5$ cells suspended in 5ul media into the medullar cavity of the bone using a 27 gauge needle from a tuberculine syringe (Cat 26040, Exelint, Int. Co, Redondo Beach, CA) coupled to a 0.1 ml Hamilton syringe (Cat 8001, Hamilton Co, Reno, NV).

Functional imaging to monitor tumor growth in live mice was performed using the IVIS Spectrum System (PerkinElmer). At each imaging session, 80 µL of a Luciferin working solution (40 mg/mL in PBS without calcium or magnesium) was administered. Mice were imaged at 0, 7, 14, 21, 28, 35, and 42 days post-injection. Regions of interest (ROIs) around the injection site were manually delineated for quantitative analysis of total flux [photons/second]. Luciferin signals observed in the liver were excluded from the analysis. At the end of the experiment, tumor-bearing Grossly trimmed tibia samples and their encapsulating musculature and non-tumor bearing counter-parts from the same mouse were harvested for further evaluation.

For micro-Computer Tomography (microCT) scans, formaline-fixed samples were scanned using a Scanco Medical µCT 35. Tibias were scanned at an isotropic voxel size of 3.5µm, with peak tube voltage of 55 kV and current of 0.145 mA (µCT 35; Scanco). A Gaussian filter (s = 0.8) with a limited, finite filter support of one was used to suppress noise. Mineralized bone was segmented from air and soft tissues by intensity thresholding. Scanco Medical software was used to determine bone volume.

Mouse tumor histology

Grossly trimmed tibia samples and their encapsulating musculature from each condition were formalin-fixed, decalcified 14% ethylenediaminetetraacetic acid disodium salt dihydrate in paraffin-embedded, and sectioned at 4 µm thickness. Serial sections were used for Hematoxylin and Eosin staining (H&E), immunohistochemistry (IHC), immunofluorescence (IF), and TUNEL assay. All histology samples were evaluated by an expert pathologist.

Confirmation of proliferating cells and cell death were assessed by IF of Ki-67 (Leica, Cat# KI67-MM1-L-CE, 1:300) and DeadEnd Fluorometric TUNEL assay (Promega; Cat #: G3250), respectively, and counterstained with propidium iodide. IHC of CD99 staining was done with rabbit monoclonal CD99 antibody (Abcam EPR3096 1:50), using the SignalStain Boost IHC detection reagent (Cell Signaling Cat #8114), as directed.

All slides were digitally scanned on a Nanozoomer S60 (Hamamatsu) or Axioscan 7 (Zeiss) systems. Measurements of area were made through manual annotations of ROIs on images using NDP.view2 (Hamamatsu).

Electron Microscopy

For each treatment, duplicate cell monolayers were grown on MatTek dishes. After an initial wash with PBS, the monolayers were washed with 0.1 M sodium cacodylate and then with 2.5% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer. After fixation, the monolayers were rinsed five times in 0.1 M sodium cacodylate buffer, then were post-fixed in 1% osmium tetroxide and 0.8 % K3[Fe(CN)6] in 0.1 M sodium cacodylate buffer for 1 hour at 4 degrees C. Cells were rinsed with water and en bloc stained with 2% aqueous uranyl acetate overnight at 4 degrees C. After five rinses with water, specimens were dehydrated with increasing concentration of ethanol at 4 degrees C, infiltrated with Embed-812 resin and polymerized in a 60°C oven overnight. Embed-812 disc was removed from MatTek plastic housing by submerging the dish in liquid nitrogen. Two discs were sandwiched together side to cell side by putting a drop of Embed-812 and polymerized in a 60°C oven overnight. Sandwiched discs were sectioned perpendicular with a diamond knife (Diatome) on a Leica Ultracut UCT (7) ultramicrotome (Leica Microsystems) and collected onto copper grids, post stained with 2% Uranyl acetate in water and lead citrate. TEM images were acquired on a JEOL-1400 Plus transmission electron microscope equipped with a LaB6 source operated at 120 kV using an AMT-BioSprint 16M CCD camera.

Cells Embedded in 3D Collagen

Collagen gels were created by mixing bovine collagen I (Advanced Biomatrix 5005 and 5026) with concentrated phosphate buffered saline (PBS) and water for a final concentration of 2 mg/mL collagen. This collagen solution was then brought to pH 7 with 1N NaOH and mixed with cells just prior to incubation at 37°C to induce collagen polymerization. Cells were suspended using trypsin/EDTA (Gibco), centrifuged to remove media, and then mixed with collagen just prior to incubation at 37°C to initiate collagen polymerization. Collagen mixed with cells was allowed to polymerize in custom 3D printed mounts for imaging in light-sheet microscopes.

Imaging

Light-sheet fluorescence microscopes were used to image the zebrafish larvae or TC71-AktPH-GFP cells embedded in 3D soft collagen within a custom-made mount. Fixed zebrafish larvae were embedded in 1% low-melting agarose into sonicated, ethanol-washed fluorinated ethylene propylene tubes (Zeus, HS 029EXP/ .018 REC). In this case, the tube is held by a custom-made mount, which allows 3D translation and rotation in our light-sheet microscopes. A conventional mSPIM[66] was used as previously described[42]. Briefly, a 40× NA 0.8 water-dipping objective (CFI Apo NIR 40XW; Nikon Instruments) was used for detection, at room temperature in PBS. A 200-mm focal length tube lens (58-520; Edmund Optics) that formed the image on an sCMOS camera (ORCA-Flash4.0; Hamamatsu Photonics). These conditions provide a resolution of 380 nm laterally and about 1.4 μm axially. A 10× NA 0.28 objective (M Plan Apo 10×, 378-803-3; Mitutoyo) was used for light-sheet illumination. The thickness and the length of the light-sheet were controlled by a variable slit (VA100C; Thorlabs) placed at the conjugate plane of the back focal plane of the illumination objective.

For live imaging on glass, F-Tractin-GFP cells were mounted on a glass-bottom 35 mm cover dish (MatTek). Mounted cells were subsequently imaged using a custom-built Oblique Plane Microscope (OPM), as previously described[67]. Briefly, a tilted light sheet was launched after a primary objective (25x NA 1.1, Nikon MRD77220) to illuminate the cells in a glass-bottom dish; the fluorescence was collected by the same objective and formed the image on the camera after passing through the optical train with the magnification of around 43.3. By scanning the light sheet using Galvo mirrors in the optical train, we can rapidly acquire the 3D image of the cells without any mechanical movement of either sample or objective. Also, using a single objective for both illumination and fluorescence collection frees the whole sample space above the objective and makes the system compatible with the standard sample mounting methods. The resolution of the system across the extended FOV was around 0.39, 0.43, and 1.22 μm in the x, y, and z directions, respectively.

To image dynamics of Cav-1 puncta, TC71 Cav1-mClover cells were imaged on a SoRA spinning disc confocal microscope (Nikon) at 100X magnification, NA1.45, 200ms exposure, at 2 second time intervals. PTRF and Cav1-mClover localization was imaged on LSM780 scanning confocal microscope (Zeiss) at 40X magnification, NA1.4.

Image Analysis

For quantification of Cav-1 puncta dynamics, particle detection and tracking were performed using u-track (version 2.4; https://github.com/DanuserLab/u-track)[68]. The detection algorithm used was Gaussian-mixture model fitting, using default parameters. The mean localization precision per movie was 8.5-12.1 nm. The resulting detections were then tracked over time, using default tracking parameters. The detection and tracking results were verified using visual inspection as well as using the performance diagnostics included in u-track. Tracks lasting for at least 10 frames were then analyzed to obtain each track's diffusion coefficient from its frame to frame displacements[69, 70].

For 3D morphology classifications in zebrafish, images were segmented using the previously established 3D morphology pipeline[71] to segment cell volumes and calculate local membrane curvature from single cell images. For the segmentation process, we smoothed the deconvoluted images with a 3D Gaussian kernel (σ = 3 pixels) and applied a gamma correction (Υ = 0.7), then employed an Otsu threshold, followed by a 3D grayscale flood-fill operation to fill possible holes. The binary images of all cells were visually inspected for segmentation artifacts. Miss-segmented cells were excluded from the data set. Aspect Ratio was calculated on segmented volumes using "regionprops3" in MATLAB. 3D rendering were generated using ChimeraX[72].

To quantify differences in tissue morphology between *CD99 High* and *CD99 Low* cells xenografted in mice, we compared various aspects of nuclear morphology in Hematoxylin and Eosin (H&E) stained histological slides. First, tumor regions to be analyzed were manually annotated using QuPath[73] on tissue images from 3 slides in each experimental group, scanned at 40X magnification (~0.25 microns/pixel). Second, nuclei in these regions were segmented using the PanNuke version of the HoverNet model as implemented in the TIAToolbox[74]. Third, these images were downsampled to 20X magnification and their color intensities we normalized

using the Macenko algorithm[75] against a single (randomly selected) reference slide. Finally, we extracted 37 morphological features relating to size, shape, color, and texture of each individual nucleus, as previously described[76]. Then for each annotated tumor region we randomly selected neighborhoods of 25 nuclei: the number of neighborhoods was proportional to the area of the region but capped at 500 neighborhoods. Each neighborhood was then represented by a feature vector of the median value of individual features across constituent cells, which were then pooled across slides, z-score normalized, and visualized using T-distributed Stochastic Neighbor Embedding (t-SNE) .

For analysis of active PI3K organization on the cell surface, we applied the Cellular Harmonics framework (https://github.com/DanuserLab/u-signal3D) [53]. 3D images underwent Richardson-Lucy deconvolution, with apodization (height parameter -.06) to reduce deconvolution artifacts, smoothing (3D Gaussian kernel, σ=1.5 pixels), and gamma correction (0.7). Surface segmentation and generation of a triangle mesh were performed using the u-shape3D analytical framework[71]. For intensity measurement, we measured the fluorescence intensity local to each mesh vertex from the raw image. The background noise was first removed by subtracting the median pixel intensity outside the cell. Then, at each vertex, the average pixel intensity was computed within a sampling radius of 1 μm over the pixels inside the cell. Finally, we normalized each cell's surface intensity to a mean of one. The calculated values were projected onto the triangular mesh to visualize normalized intensity on the cell surface and rendered using ChimeraX[72]. To calculate spectral decomposition values for each cell, we applied the Laplace-Beltrami operator to the extracted cell surface, as previously described[53], creating a curve of Eigenvalues ordered by increasing frequency. Since the overall intensity of the fluorescence signal often varies between cells due to the concentration of the probes, we first normalized the fluorescence intensity and further calculated an energy density across levels. The energy density spectrum defines the length scale of signal organization, with broad spectra representing more uniform signals and narrower spectra more polarized or patchy signals. Heatmaps in Figure 5C were generated using MATLAB to visualize and compare the energy density curves of individual cells between experimental groups. Statistical differences were calculated using a permutation test comparing the Mean Absolute Deviation between rows in the heatmaps.

Plots and statistical tests were visualized and preformed using GraphPad Prism.

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FIGURES

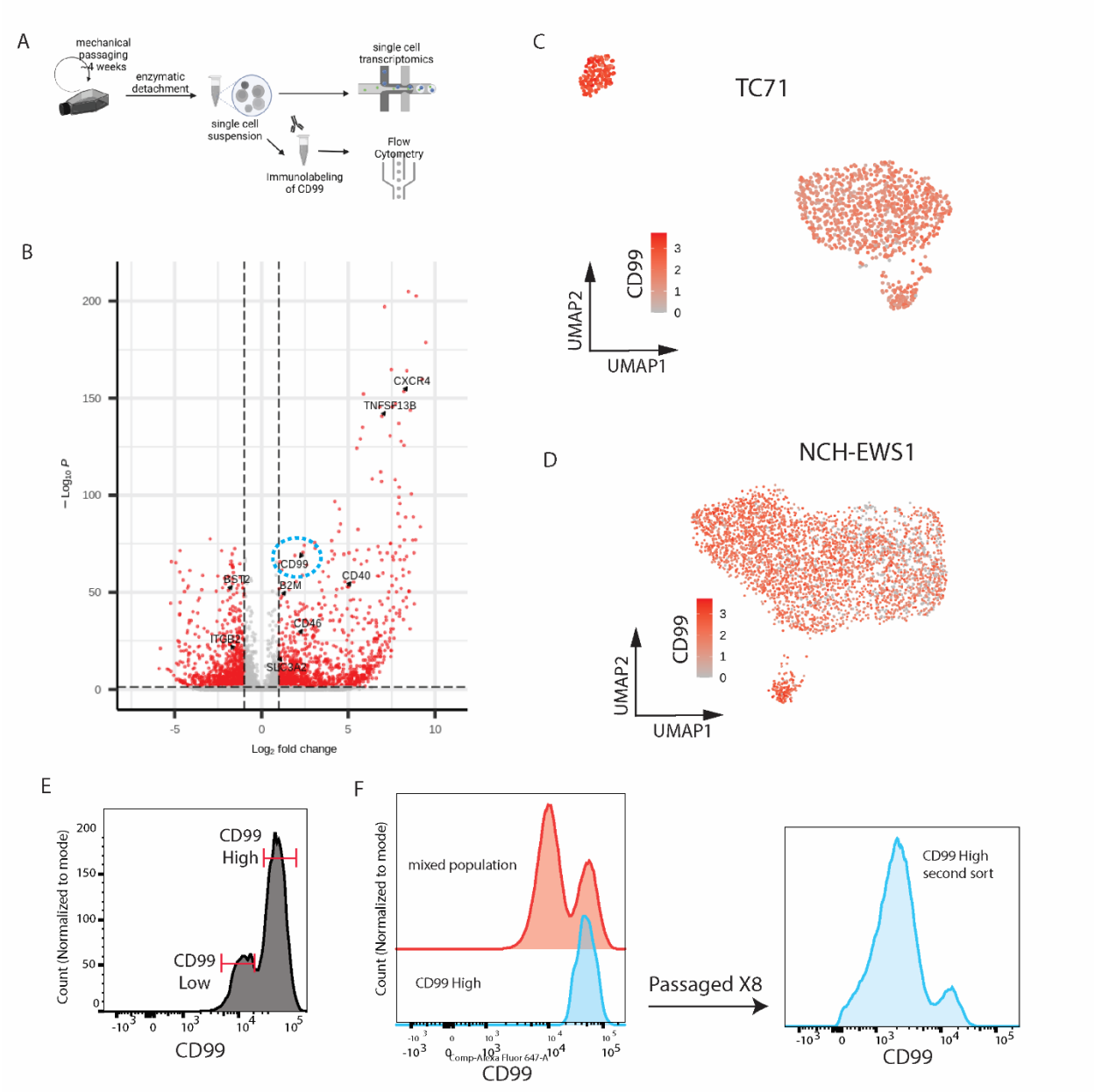

Figure 1: High CD99 marks a distinct Ewing Sarcoma cell state. (A) Experimental workflow. After 6-10 cycles of mechanical passaging to preserve surface receptors, cells are enzymatically detached from the dish and prepared in a single cell suspension for single cell RNA seq (scRNA-seq) to profile receptor expression, or immunolabeling of target receptor for flow cytometry. **(B)** Volcano plot depicting differential enrichment of transcripts expression in highlighted cluster (see cluster 2 in Figure S1A) compared to all other cells. Differentially expressed cell surface receptors are indicated; CD99 is highlighted (dashed circle). **(C-D)** UMAP visualization with log normalized expression of CD99 color-coded in TC71 cells **(C)**, or in NCH-EWS1 patient-derived xenograft (PDX) cells **(D)**, analyzed by scRNA-seq. **(E)** Flow cytometry histogram depicting relative intensity of CD99 as measured by immunolabeling, displaying a bimodal distribution of *CD99 High* and *CD99 Low* cell populations. Distinct

populations are highlighted by red bars. **(F)** *CD99 High* cells (blue, left) were isolated from a mixed population of *CD99 Low* and *CD99 High* cells (red). The isolated *CD99 High* cells were reevaluated by the same assay (blue, right) after expansion and several rounds of passaging. The recurrent bimodal distribution of CD99 expression suggests that *CD99 High* cells can shift between *CD99 High* and *CD99 Low* cell states.

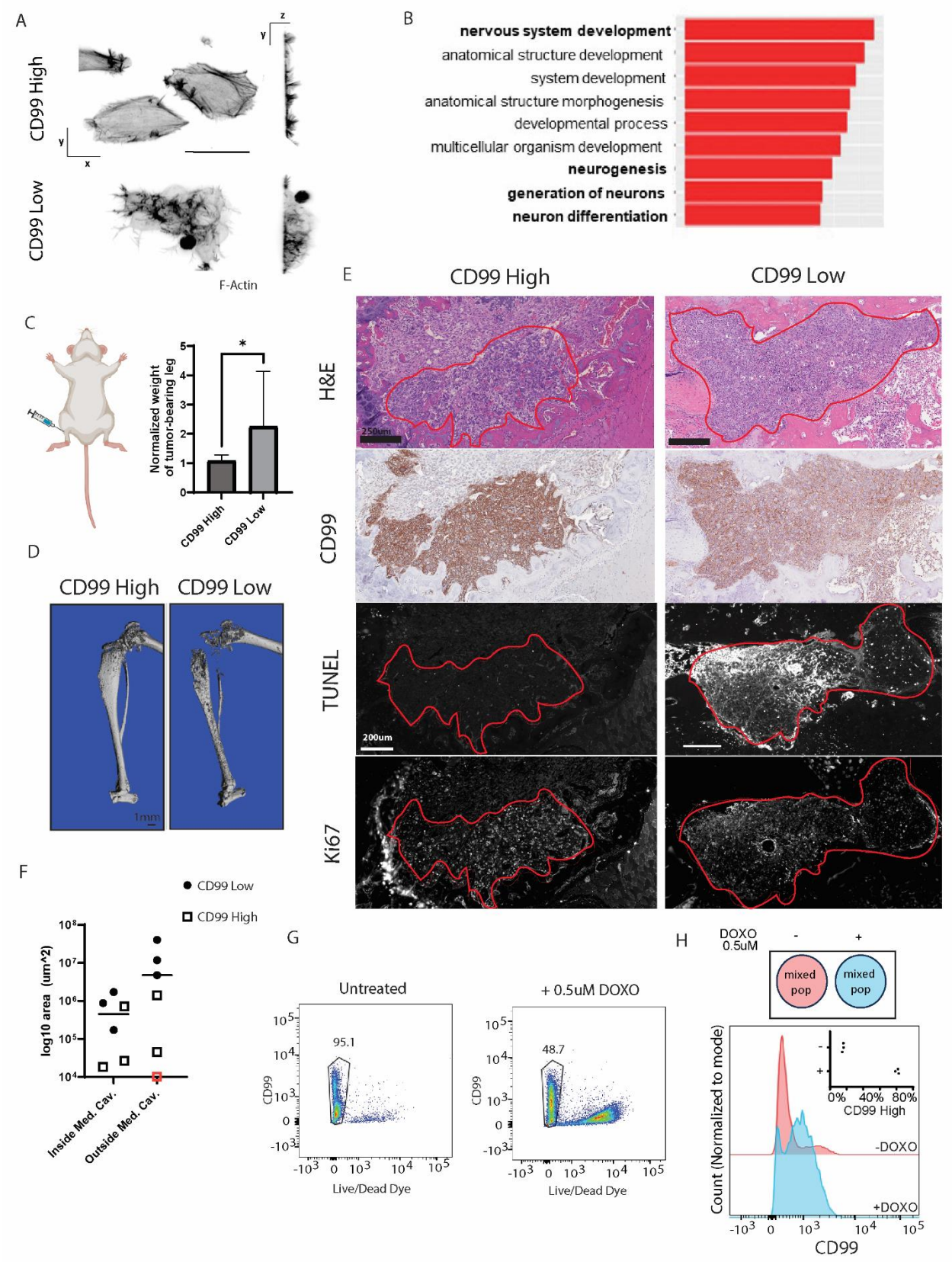
A
CD99 High
CD99 Low
F-Actin
B
nervous system development
anatomical structure development
system development
anatomical structure morphogenesis
developmental process
multicellular organism development
neurogenesis
generation of neurons
neuron differentiation
C
Normalized weight of tumor-bearing leg
CD99 High
CD99 Low
D
CD99 High
CD99 Low
1mm
E
CD99 High
CD99 Low
H&E
CD99
TUNEL
Ki67
250um
200um
F
CD99 Low
CD99 High
log10 area (um^2)
Inside Med. Cav.
Outside Med. Cav.
G
Untreated
+ 0.5uM DOXO
95.1
48.7
CD99
Live/Dead Dye
H
DOXO 0.5uM
mixed pop
mixed pop
Count (Normalized to mode)
-DOXO
+DOXO
CD99 High
CD99

Figure 2: *CD99 High and Low* cells display differences in morphology, gene expression, and survival. (A) Maximum Intensity Projections in XY view (left) or YZ view (right) of *CD99 High* and *CD99 Low* TC71 cells expressing F-Tractin-GFP and imaged using oblique plane microscopy in adherent culture display distinct cell morphologies (scale bar = 20um). **(B)** Gene Ontology terms for *CD99 High* cells show enrichment of neurally differentiated gene signatures. **(C)** Schematic depicting intratibial xenografts of *CD99 High* or *CD99 Low* TC71 cells into immunocompromised mice (left). Weight of tumor-bearing leg normalized to the weight of non-tumor bearing leg of the same mouse in *CD99 High* (n=6) or *CD99 Low* (n=6) xenografts (right, asterisk depicts p>0.05, t-test). **(D)** Representative computer tomography scan of tumor bearing legs depicting differences in presence of bony destruction caused by tumor cells. **(E)** Histological sections of intratibial xenografts in immunocompromised mice, showing tumor-bearing areas (outlined in red) within the medullary cavity (Med. Cav.) of either *CD99 High* or *CD99 Low* cells, labeled with H&E, chemically immunostained for CD99, and fluorescently stained for the apoptotic marker TUNEL or the proliferation marker Ki67 in serial sections. Note the decreased TUNEL staining observed in the *CD99 High* tumor compared to *CD99 Low* tumor. **(F)** Area inside the Med. Cav. of the bone, or outside of the Med. Cav., such as within the surrounding muscle. Note that while *CD99 Low* cells have larger clusters outside the Med. Cav., *CD99 High* cells have comparably smaller clusters in both areas (n = 3 mice). Red square indicates *CD99 High* mouse with no tumor-bearing area found outside Med. Cav. Bars, mean value. **(G)** Flow cytometry of a mixed population of *CD99 High* and *CD99 Low* cells stained with Live/Dead viability dye vs. CD99 immunolabeling, with or without Doxorubicin treatment (DOXO, 0.5uM, 48hrs). **(H)** Histograms depicting outlined regions from the dot plot in (G), which mark viable, CD99+ cells and show relative distributions of *CD99 Low* and *CD99 High* populations grown with (+, blue) or without (-, magenta) DOXO (n=3 independent experiments). Inset shows percentage enrichment of *CD99 High* cells in each group. *CD99 High* cells are systematically enriched upon drug treatment.

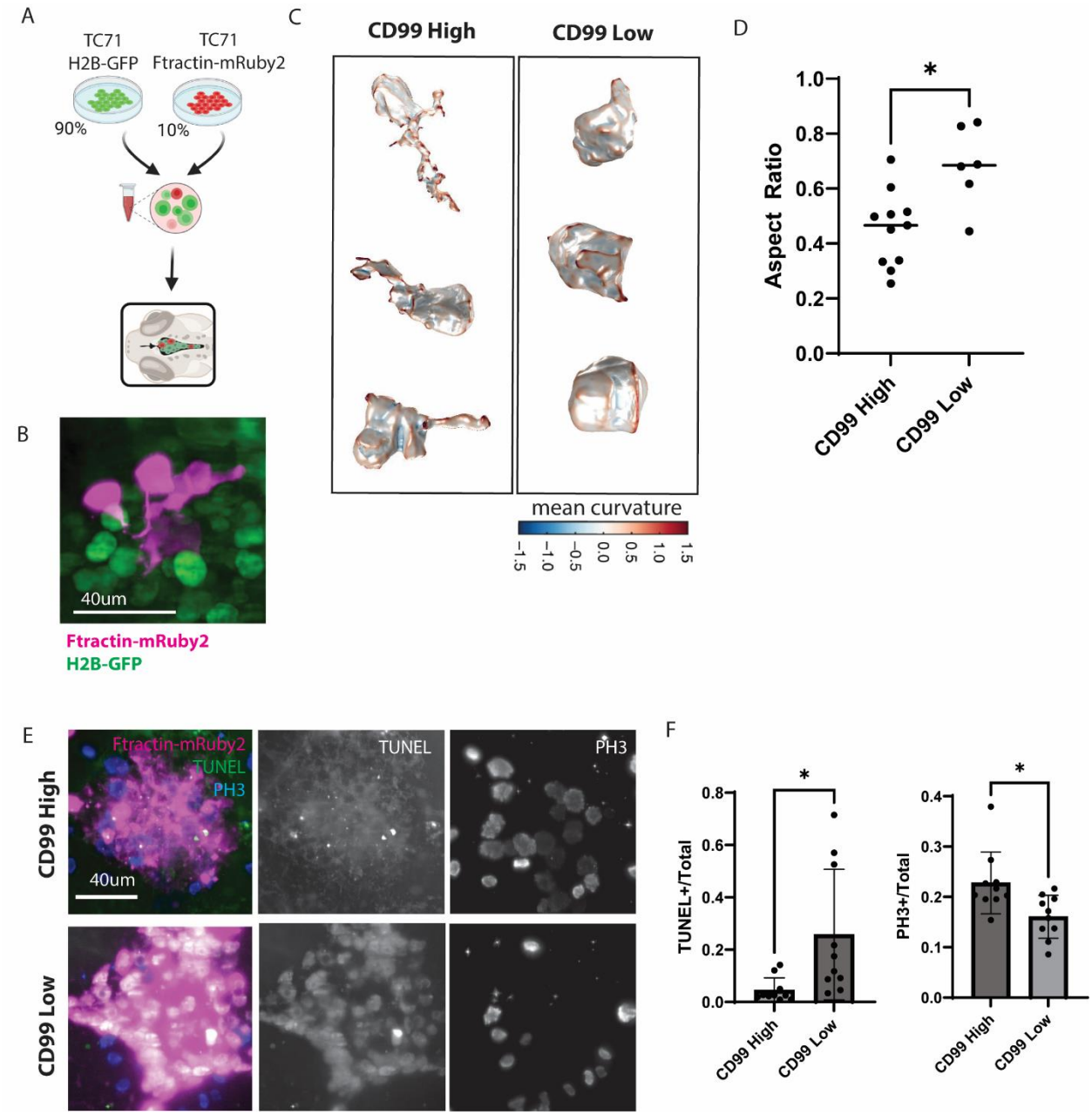

Figure 3: *CD99 High* and *Low* cells display differences in morphology and survival in zebrafish xenografts. (A) Experimental set-up to identify single-cell morphologies in xenografted cells within the hindbrain ventricle (HBV) of zebrafish larvae. In order to distinguish single-cell morphologies of individual cells, we prepared a mosaic cell mixture consisting of 90% of TC71 unsorted cells expressing the fluorescently-tagged nuclear marker H2B-GFP, and 10% of TC71 *CD99 High* or *CD99 Low* cells expressing F-Tractin-mRuby2. **(B)** Maximum Intensity Projection of TC71 cells within the HBV expressing F-Tractin-mRuby2 (magenta) or H2B-GFP (green). **(C)** Volume renderings of 3 representative single cells from each class, pseudocolored by local membrane curvature. Note the highly elongated and protrusive shapes observed in *CD99 High* cells. **(D)** Differences in aspect ratio of individual cells, as a metric of differences in cell shape in *CD99 High* (n = 11 cells, 9 fish) and *CD99 Low* cells (n=6 cells, 3 fish, p-value<0.5, t-test). **(E)** TC71 *CD99 High* or *CD99* Low cells expressing F-tractin-mRuby2 (magenta) were

injected into the HBV of zebrafish larvae, and stained for apoptotic marker TUNEL (green (left), grey (middle)) and proliferative marker Phospho-histone H3 (PH3, blue (left), grey (right)) 48hrs post-injection. Maximum Intensity Projections show increased TUNEL staining in *CD99 Low* cells. **(F)** Proportion of measured area of TUNEL-positive (left) or PH3-positive (right) signal overlapping with F-Tractin-mRub2 expressing cells (n=10 fish). Note decreased cell death and higher proliferation in *CD99 High* cells.

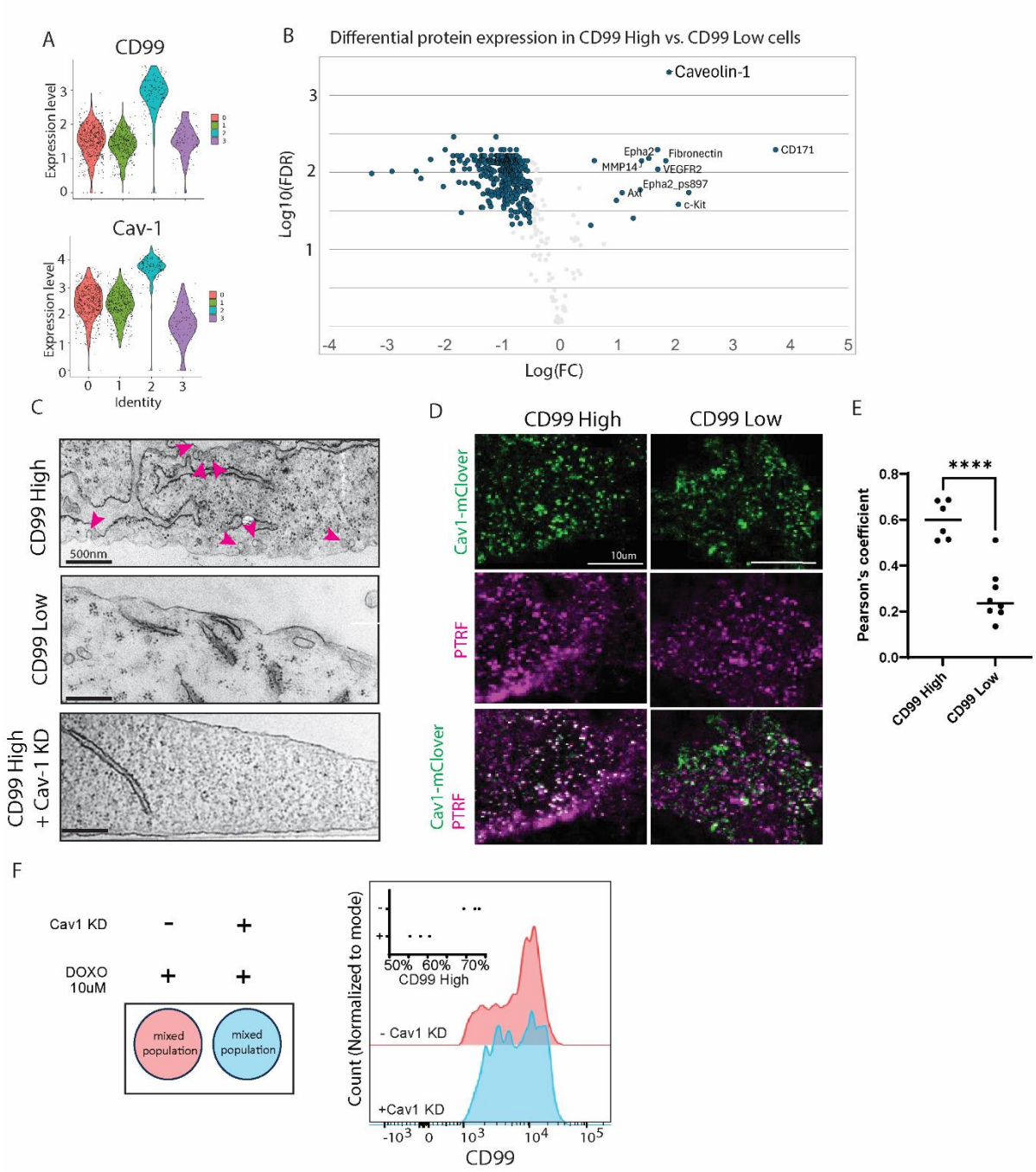

Figure 4: Caveolin-1 emerges as a potential driver of functions associated with the *CD99 High* State. (A) RNA expression of CD99 and Cav1 in different clusters, analyzed from scRNA-seq data (see Fig S1). **(B)** Volcano plot of differential protein expression between *CD99 High* vs. *CD99 Low* cells, analyzed by Reverse Phase Protein Array (RPPA). **(C)** Representative scanning electron microscope images of *CD99 High*, *CD99 Low*, and *CD99 High* cells expressing inducible Cav-1 shRNA (Cav1 KD). Magenta arrowheads highlight observed caveolae in *CD99 High* cells. **(D-E)** Representative images (D), and quantification of colocalization by Pearson's coefficient (E) of TC71 *CD99 High* or *CD99 Low* cells with endogenous expression for Cav1-mClover, and immunostained for PTRF show colocalization of Cav1 and PTRF in *CD99 High* but not *CD99 Low* cells (n=6,8 cells, respectively, t-test, ****p-value<0.0001). **(F)** Experimental setup for a flow cytometry assay of a mixture of TC71 *CD99 High* and *CD99 Low* cells, with (blue) or without (red) knockdown of Cav-1, grown with Doxorubicin (DOXO, 10uM, 48hrs). Histogram of *CD99 High* and *CD99 Low* populations. Representative of n=3 experiments. Inset, percentage enrichment of *CD99 High* cells in each group (n=3). Note that enrichment of *CD99 High* peak under DOXO treatment is lost upon Cav1 KD.

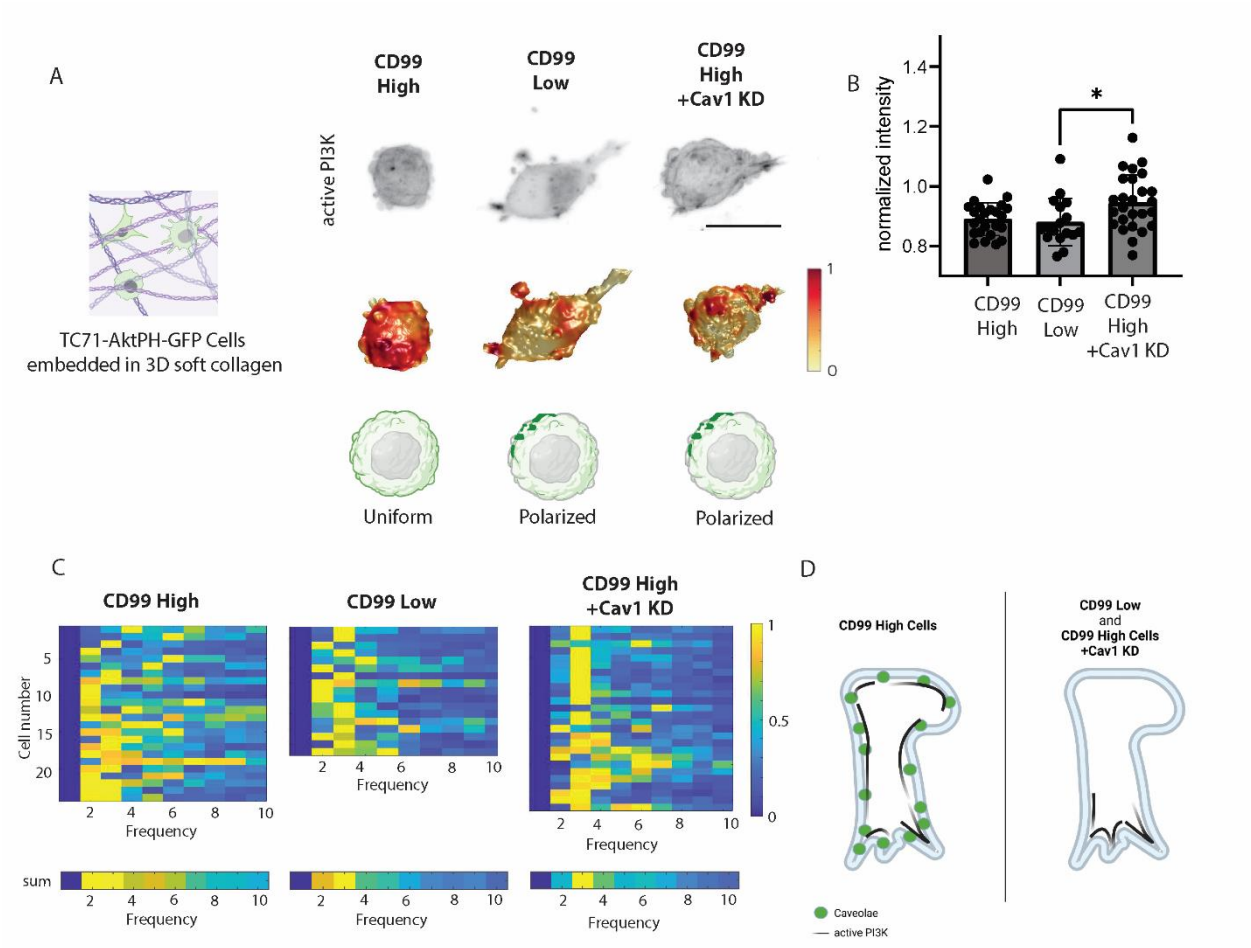

Figure 5: Caveolin-1 regulates PI3K/AKT signaling in *CD99 High* Cells. (A) TC71 *CD99 High* and *CD99 Low* cells*, and CD99 High* cells +Cav1 KD, expressing PI3K activation sensor AktPH-GFP. Cells were embedded in soft collagen. (Top) Maximum Intensity Projection of raw

data (scale bar 20um). (Middle) surface rendering of the same cell, with localization of PI3K activity projected onto the 3D cell surface. Red surface regions indicate high local PI3K activity. (Bottom) Schematics showing uniform or polarized PI3K activity distribution observed in each experimental group. **(B)** PI3K activity measured as total sum intensity on the cell surface normalized to mean intensity of cell volume. Note the slight increase of total PI3K activity in *CD99 High* + Cav1 KD (n = 24, 17, and 26 cells, for *CD99 High*, *CD99 Low*, and *CD99 High* +Cav1 KD, respectively. * p-value<0.05, KS test). **(C)** *(top)* Heatmaps depicting normalized spatial energy spectra of PI3K signaling pattern on the cell surface. Continuous spectra are binned and normalized to the highest bin. Each row represents a single cell. Low bins encode signaling variations over long distances, high bins short variations over short distances. Thus, cells with a polarized signaling distribution display a primary high energy bin (yellow) at lower frequencies, while cells with a more uniform signal distribution display several smaller bins with similar energy levels. *(bottom)* Sum of binned and normalized energy values across all cells. Note that *CD99 High* cells show a more uniform distribution of active PI3K compared to *CD99 Low* cells. *CD99 High* cells +Cav1 KD show more polarized signal distributions, similar to *CD99 Low* cells and significantly different from *CD99 High* cells (p-value =.02, permutation test of Mean Absolute Deviation). **(D)** Summary of proposed mechanism. *CD99 High* cells have high expression of Cav-1 and dense caveolae to organize the membrane, possibly through stabilization of lipid rafts. These make docking sites promiscuously available to PI3K localization, enhancing opportunities for stable signaling across the entire cell surface. *CD99 Low* cells, or *CD99 High* cells with reduced Cav-1 expression lack caveolae, causing PI3K localization to be concentrated to areas of high local curvature such as protrusions, as described in other works.

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