Anoikis resistance in mammary epithelial cells is mediated by semaphorin 7a

Semaphorin-7A and anoikis resistance

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ABSTRACT

Semaphorin-7a (SEMA7A), best known as a neuroimmune molecule, plays a diverse role in many cellular processes and pathologies. Here, we show that SEMA7A promotes anoikis resistance in cultured mammary epithelial cells through integrin mediated activation of pro-survival kinase AKT, which led us to investigate a role for SEMA7A during postpartum mammary gland involution—a normal process in development where cells die by anoikis. Our results reveal that SEMA7A is expressed on live mammary epithelial cells during involution, that SEMA7A expression is primarily observed in α6-integrin expressing cells, and that luminal progenitor cells, specifically, are decreased in mammary glands of SEMA7A-/- mice during involution. We further identify a SEMA7A-α6/β1-integrin dependent mechanism of cell survival that promotes anoikis resistance, mammosphere formation, and chemoresistance in mammary epithelial cells and suggest that this mechanism is relevant for recurrence in breast cancer patients.

INTRODUCTION

Identifying mechanisms of normal mammary epithelial cell (MEC) biology that contribute to tumorigenesis is critically important for furthering our understanding of breast cancer and extending the survival of breast cancer patients. It has been postulated that the functional plasticity of the adult mammary epithelium that is required for response to hormonal changes during pregnancy and lactation may make the tissue more susceptible to malignancy. Of particular interest are postpartum breast cancers (PPBCs), defined as breast cancers diagnosed in women within 10 years of most recent childbirth, as these cancers are nearly three times more likely to result in lethal metastasis(1).

The majority of mammary morphogenesis occurs postnatally beginning at puberty and culminating with lactation following pregnancy. As such, differentiation of the mammary epithelium is not complete until a full pregnancy-lactation cycle. Upon weaning, or after pregnancy in the absence of nursing, the mammary gland regresses to resemble the pre-pregnant architecture through a process termed postpartum involution. Postpartum involution occurs in two primary phases(2). In the first phase, milk proteins accumulate in the mammary ducts and alveoli resulting in activation of leukemia inhibitory factor (LIF), phosphorylation of signal transducer and activator of transcription (pSTAT3), and subsequent lysosomal-mediated cell death(3). In mice, if pups are returned within 48 hours of weaning, this process can be reversed, and lactation can continue. If pups are not returned, however, the irreversible second phase of involution begins. This phase is characterized by degradation of the mammary basement membrane by matrix metalloproteinases and anoikis-mediated cell death in the absence of laminin-mediated activation of integrin alpha-6/beta-1 ($\alpha6\beta1$) and downstream pro-survival signaling(4-6).

SEMA7A, or CD108w, is a member of the semaphorin protein family, named after the English verb “semaphore”, which means to transmit a signal across a long distance. SEMA7A was first identified for its expression on lymphocytes(7), but has since been shown to exert diverse roles in embryonic neuronal development(8), cell differentiation(9-11), ECM deposition(12, 13), various components of the immune system(14-17), and pathologies including fibrosis(12, 18, 19) and cancer(13, 16, 20-22). Though elevated SEMA7A is observed in breast cancer, where it has an established role in promoting tumor cell survival(13, 23), the role of SEMA7A in normal MECs has not previously been investigated. SEMA7A is known to signal through $\beta1$-integrin in immune cells, neurons, and cancer; $\beta1$-integrin also mediates MEC survival during development, in part, via phosphorylation and activation of downstream pro-survival signaling

mediated by PI3-kinase and protein kinase B, or Akt(24). Though it is known that redundant secretory epithelial cells die during postpartum involution, the factor(s) that allow some cells to survive and regenerate a lactation-competent gland for future pregnancies remain unknown. Herein, we have identified enrichment for SEMA7A expressing $\alpha6\beta1$ positive MECs during the matrix remodeling period of postpartum involution and a role for SEMA7A in specifically promoting the survival of α6-integrin+ luminal progenitor cells. We provide evidence that exogenous stimulation of immortalized MECs with purified SEMA7A protein promotes anoikis resistance via activation of α6 and β1-integrins and downstream activation of phosphorylated AKT (pAKT). Consistent with a role for SEMA7A in promoting anoikis resistance, SEMA7A knockout mice (SEMA7A$^{tmalk1/J}$) exhibit phenotypes associated with accelerated involution including early presence of apoptotic cell bodies in mammary lumens, increased pSTAT3, and premature adipocyte repopulation. α6-integrin also drives maintenance of tissue resident stem cells and therapy resistant cancer stem cells(25) and our findings reveal that SEMA7A drives stem cell phenotypes and chemoresistance in cultured MECs in a manner that is dependent on $\alpha6\beta1$. Finally, we provide evidence for SEMA7A in driving transformed phenotypes in normal MECs and reversing transformed phenotypes in genetically identical, ras-transformed, variants of the same cell line. Our results are the first to identify a role for SEMA7A in anoikis resistance and promotion of stem and cancer cell phenotypes via activation of α6 and β1-integrins.

METHODS

Cell culture:

MCF10A/MCF12A cells were obtained from, and cultured, according to ATCC. MCF10DCIS.com cells were obtained from K. Polyak (University of Harvard, Cambridge, MA). Cells were validated by the DNA sequencing core at the University of Colorado Anschutz Medical Campus and found to be pure populations of their respective cell lines. Cells were

regularly tested for mycoplasma throughout studies. Cells were sub-cultured as previously described (reference), or according to ATCC standards. Overexpression plasmid (SEMA7A-Fc) was a generous gift from R. Medzhitov (Yale University, New Haven, CT). Control plasmid (pcDNA3.1) was obtained from H. Ford (University of Colorado Anschutz Medical Campus, Denver, CO).

Animal Studies:

All animal procedures were approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee. Sema7a$^{tm1Alk/J}$ mice (a generous gift from Alex Kolodkin at John's Hopkins University) and C57Bl/6 (Jax) were housed and bred as previously described.(26) Briefly, C57Bl/6 SEMA7A^{tm1Alk} females were bred with WT C57Bl/6 males to induce pregnancy, lactation, and postpartum involution. Pup numbers were normalized to 6-8 per dam after birth to ensure for adequate lactation. Postpartum involution was initiated by forced weaning of pups at the peak of lactation (L10-L12). #4 right and left mammary glands were harvested from age-matched nulliparous animals and postpartum dams at involution days 1,2,4,6,8,10,14, and 28. For flow cytometry, inguinal lymph nodes were removed prior to tissue digestion.

In vitro survival assays:

MCF10A and MCF12A cells were cultured with 200ng/ml purified SEMA7A or PBS vehicle control in adherent (tissue culture plastic) or forced suspension conditions (ultra-low attachment plates (Corning, Corning, NY, USA: #3473)). Cells were seeded at a density of 1000 cells/well in a 96-well plate plus media only wells as controls. Cell death was measured 24 hours post seeding via luminescence using the Caspase Glo assay (Promega, Madison, WI, USA: #G8090). Annexin-V /7AAD staining (Biolegend, San Diego, CA, USA: #640930) and

pAKTS473 (ThermoFisher: Waltham, MA, USA, #17-9715-42) were also used to confirm cell viability by flow cytometry. See flow cytometry methods for staining protocol.

Label retention stem cell assay:

MCF10A and MCF12A cells were labeled with CellTrace Violet (ThermoFisher: #C34557) for 20 minutes at 37°C according to manufacturer's instructions. A sample of labeled cells was analyzed by flow cytometry to determine labeling efficiency and fluorescent intensity at time of seeding. Single cells were seeded at a density of 4000 cells/well in ultra-low attachment 24-well plates to induce mammosphere formation. Cells were cultured in normal culture media for 7 days and were subsequently counted, dissociated enzymatically in Accutase, (Stem Cell Technologies, Vancouver, Canada: #07922), and analyzed by flow cytometry. See flow cytometry methods for detailed information.

Paclitaxel resistance studies: MCF10DCIS.com cells were treated with 100nM paclitaxel (Millipore Sigma, Burling, MA, USA #T7402-5MG) for 24 hours prior to analysis by flow cytometry. Drug resistance was measured by label-retention and mammosphere formation as described above and %CD44+ (Biolegend: #103018) CD24- (Biolegend: #311104) of singlets was used as a molecular definition of drug-resistant stem cells.

Flow cytometry:

Single cell suspensions were generated from cultured cells via enzymatic harvest with Accutase. Mouse mammary tissues were minced with scalpels, digested in Click's media containing 500 units/mL Collagenase II (Worthington, Columbus, OH, USA; LS004174) and Collagenase IV (Worthington, LS004186) and 20ug/mL DNAse (Worthington, LS002004) for 1 hour at 37°C with occasional trituration and strained through 70um filters to generate single cell suspensions. Single cells were stained at 4°C for 30 minutes and washed with PBS+2%FBS. Cells were filtered through 30um filters, diluted to $<10^6$ cells/ml, and analyzed with the ZE5-YETI

flow cytometer. Fixation/Permeabilization kit (BD Biosciences, Franklin Lakes, NJ, USA: #554715) was used for intracellular antigen staining. Data were analyzed with Kaluza software. Single cells were lineage depleted using CD45- (BD Biosciences: #564279)/CD31- (BD Biosciences: #745436) gating.

Immunohistochemistry:

Tissues were formalin-fixed and paraffin-embedded as previously described(27). Hematoxylin and Eosin staining was used to define morphological features. Antigen retrieval was performed using target retrieval solution (Dako, Glostrup, Denmark; #S1699) for CC3(Cell Signaling Technologies, Danvers, MA, USA; #9661) and Perilipin(Cell Signaling: #3470) or (EDTA Dako cat# S2367) for pSTAT3 (Cell Signaling Technologies; #9145). ImmPRESS polymer anti-rabbit IgG secondary reagent (Vector Labs, San Francisco, CA, USA t#MP-7401-15) was used for secondary staining of CC3 and pSTAT3 stained tissues and anti-rabbit secondary (Dako #K4003) for perilipin stains. DAB (Vector Labs,; #SK-4105) and counterstaining were performed with hematoxylin (Vector Labs #H-3401). Alveolar area was measured on H&E-stained tissues in ImageJ on 10 alveoli/field; 5 fields/tissue. 10 fields/tissue were analyzed for positive CC3, pSTAT3, and perilipin stained tissues using CellSense Dimension software count and measure feature on regions of interest.

Statistical Analysis:

Unpaired *t*-test and one-way ANOVA were performed in GraphPad Prism. Nonparametric equivalent analyses were used for samples with uneven distribution or unequal standard deviations. *p* values of less than 0.05 were considered significant. Error bars represent mean +/- standard error of the mean. All in vivo studies were replicated in at least 3 mice and in vitro studies performed in technical and biological triplicate with representative data presented.

RESULTS

Semaphorin-7a promotes anoikis resistance in normal MECs

To identify whether SEMA7A promotes survival of normal MECs we utilized two methods: transfection of FC-tagged SEMA7A into immortalized human (MCF10A) cells and addition of exogenous SEMA7A to wild-type MCF10A and MCF12A cells—another non-transformed MEC line. We found that genetic SEMA7A overexpression resulted in resistance to cell death, as measured by cleaved caspase 3/7, in a forced suspension assay, but not in attached conditions suggesting that SEMA7A specifically mediates resistance to cell death by anoikis. Therefore, all subsequent analyses of cell death were performed in forced suspension. Stimulation with exogenous purified SEMA7A protein in both MCF10A and MCF12A cells also significantly decreased cell death **(Figure 1A, B)**. We independently confirmed decreased cell death in detached MECs cultured with exogenous SEMA7A by flow cytometry for Annexin-V/7AAD (**SFigure 1**). Inhibition of a known SEMA7A receptor, β1-integrin, with the function blocking antibody 9EG7 significantly abrogated SEMA7A-mediated anoikis resistance **(Figure 1B).** We also examined pAKT in MECs cultured with SEMA7A using insulin as a positive control as the IGF-1 receptor (and insulin) can activate pAKT via β1-integrin in the mammary gland(28, 29). We observed percentages of pAkt expressing cells similar to those observed with insulin when cells were exposed to SEMA7A *in vitro* **(Figure 1C)**.

To determine whether SEMA7A inhibits anoikis *in vivo*, we examined MECs harvested from age-matched nulliparous and force weaned WT animals for SEMA7A expression. We observed a low percentage of SEMA7A+EpCAM+ cells in the nulliparous mammary gland, which was enriched approximately 3-fold at involution day 3 when basement membrane degradation and extracellular matrix remodeling ensues. The percentage of SEMA7A+ epithelial cells declined sharply starting at involution day 4 and returned to levels comparable to those observed in

nulliparous glands at involution day 6 **(Figure 1D).** We also compared live epithelial cells by flow cytometry in age matched SEMA7A knockout mice (Sema7a$^{tm1Alk/J}$) and WT C57Bl/6 mice at days 1, 2, 3, & 6 post-wean and observed a modest, but significant, reduction in MECs as measured by the percentage of Aquazombie- EpCAM+ cells **(Figure 1E**). To test whether the decrease in live cells during involution in Sema7a$^{tm1Alk/J}$ animals was accompanied by changes to gland histology and programs normally activated during postpartum involution, we performed H&E and immunohistochemical analysis of whole mammary tissues isolated from Sema7a$^{tm1Alk/J}$ dams and WT controls. We observed accelerated glandular regression, as evidenced by decreased alveolar area, increased pSTAT3, precocious appearance of cleaved caspase 3 (CC3) positive cells, and increased adipocyte repopulation in glands harvested from Sema7a$^{tm1Alk/J}$ animals **(Figure 2 and Figure 3)** suggesting that loss of SEMA7A accelerates postpartum involution.

SEMA7A promotes anoikis-resistance via α6-integrin mediated pAKT activation.

MECs can be divided into four primary cell types: luminal, luminal progenitor (LPCs), basal, and basal progenitor cells (BPCs) and these populations can be resolved by flow cytometry based largely on their expression of epithelial cell adhesion molecule (EpCAM) and CD49f, also known as α6-integrin(30, 31). Since the magnitude of the changes observed in Figure 1E was relatively small, we used flow cytometry to resolve the four primary MEC populations to determine which cells are specifically affected by loss of SEMA7A during postpartum involution. We observed a significant decrease in EpCAM^{hi}CD49f+ LPCs at all timepoints examined, which were not consistently observed in the other SEMA7A expressing cell populations. Additionally, luminal cells remained unchanged **(Figure 4A, B and SFigure 2&3)**. We also found that more than 80% of the live LPCs express SEMA7A at involution days 2-4 **(Figure 4C)**. One factor that distinguishes the SEMA7A+ MEC populations from the SEMA7A- luminal cells is their expression of CD49f, or α6-integrin. β1-integrin is a known receptor that mediates SEMA7A

cellular signaling(8), but the preferred alpha binding partner has not been identified. We quantified cells that co-express α6 and β1 in WT and SEMA7A$^{tmalk1/J}$ animals and found a significant reduction during involution **(Figure 4D)**. Furthermore, we observed that over 90% of the α6 and β1 positive MECs express SEMA7A at the transition to the reversible phase of involution when basement membrane degradation and anoikis-induced cell death is initiated **(Figure 4E).** To determine whether SEMA7A confers anoikis-resistance via α6-integrin dependent activation of pAKT, we cultured MCF10A cells with SEMA7A in the presence of the α6-integrin blocking antibody (GoH3) in forced suspension cultures. We observed increased CC3/7+ cells in GoH3 conditions as well as a significant decrease in pAKT-expressing cells **(Figure 4F&G).** Taken together, our results suggest SEMA7A promotes anoikis-resistance during involution, in part, through α6β1-mediated activation of pAKT.

SEMA7A promotes stem cell phenotypes, cellular transformation, and chemoresistance

Anoikis-resistance is rarely observed in normal epithelial cells; rather, it is a characteristic of stem cells and tumor cells. Given our findings that SEMA7A promotes anoikis-resistance in normal MECs, we tested whether it also confers features associated with stem cells and transformation. We used a label-retention and mammosphere assay to identify phenotypes associated with mammary stem cells *in vitro* and found that SEMA7A overexpression in MCF10A cells increases the quiescent stem cell population **(Figure 5A & SFigure 4)**. We also measured mammosphere formation as a readout for self-renewal in cells cultured with exogenous SEMA7A in the presence or absence of function blocking antibodies for α6 and β1 and found that increased sphere formation occurred with SEMA7A which was abrogated with either inhibitor **(Figure 5B&C)**. Secondary sphere formation was also increased in MCF10A and MCF12A cells cultured with SEMA7A **(SFigure 5).** We then tested the ability of SEMA7A to promote cell growth in anchorage-independent conditions using a soft agar assay—a classical

measurement of cellular transformation. We found that overexpression of SEMA7A in MCF10A cells is sufficient to increase growth in soft agar to levels similar to a Ras-transformed variant of these cells, MCF10DCIS.com. Moreover, we found that shRNA-mediated knockdown of SEMA7A in MCF10DCIS cells(13, 32) significantly decreased anchorage-independent growth, to levels more similar to the MCF10A non-transformed EV control and this effect was rescued by addition of exogenous SEMA7A **(Figure 5D).** Cancer stem cells, and tumor cells expressing pAkt, are also known to be chemoresistant(33). To test whether SEMA7A promotes chemoresistance in breast cancer cells, we treated SEMA7A-overexpressing and knockdown MCF10DCIS mammosphere cultures with the chemotherapeutic drug, paclitaxel. We found that SEMA7A overexpression increases mammosphere formation in treated MCF10DCIS cells, which can be partially blocked with 9EG7 and completely blocked with GoH3. We then confirmed an increase in tumor-initiating and drug-resistant cells in these cultures by increased label-retention and CD44+CD24- expression—markers frequently used to characterize drug resistant cancer stem cells **(Figure 5E-G).** Since patients with chemotherapy resistant disease often experience disease relapse, we examined whether co-expression of *SEMA7A* and α6-integrin (*ITGA6*) was associated with decreased relapse free survival (RFS) by Kaplan Meier analysis. We observed that expression of each molecule individually did not decrease RFS, but co-expression of *SEMA7A* in *ITGA6*^{high} tumors did (HR=1.5; 95% CI=1.18-1.9; p=0.00075) **(Figure 6)**. Taken together, these data suggest a clinically relevant mechanism by which SEMA7A and α6-integrin may promote cellular phenotypes associated with tumor progression and chemoresistance.

DISCUSSION

Despite published roles for SEMA7A in breast cancer progression, the current study is, to our knowledge, the first investigation into the role of SEMA7A in normal MEC survival during

postpartum mammary gland involution. In 2004, Clarkson and Watson published a comprehensive analysis of gene expression across mammary gland development in which they identified roles for both death receptors and immunomodulation during specific phases of postpartum development including the first and second phases of involution(34). In this highly impactful study, they identified seven basic profiles of gene expression across the lactation-involution cycle. *SEMA7A* was identified in the Inv1 cluster, which was upregulated at the transition between lactation and involution. In this study, we assessed cell surface SEMA7A protein expression by flow cytometry in recently weaned mice to show its expression during the initial phases of postpartum involution. We found that the proportion of MECs expressing SEMA7A peaks at involution day 3, marking the transition from the reversible to the irreversible phase of mammary gland remodeling. During the irreversible phase, MECs lose contact with the basement membrane, primarily laminin, and die by anoikis. Since anoikis is one of the hallmarks of the irreversible phase of mammary involution, and our lab has published that SEMA7A overexpression in multiple breast cancer cell lines decreases anoikis and, most recently, that SEMA7A expression increases over time when cells are detached from matrix, we predicted that expression of SEMA7A would promote anoikis resistance during the second phase of irreversible involution(13, 35). We now also show that treating cells with exogenous SEMA7A can confer resistance to anoikis in cultured human MECs suggesting that SEMA7A may signal to neighboring cells to promote resistance to cell death. This is consistent with recent data showing that, in human breast tissues, entire acini are lost during involution rather than a percentage of cells from each acini(36). We propose that SEMA7A promotes anoikis resistance, in part, by signaling to detached cells in a manner similar to matrix molecules, thereby mimicking attachment. Consistent with this hypothesis, we show that exogenous SEMA7A increases pAKT in cultured MECs in a manner that is dependent on α6 and β1-integrins, which is normally the receptor for pro-survival signaling via laminin in the mammary gland(6, 24). While β1-integrin is a known mediator of SEMA7A signaling in a variety of contexts, few studies

have addressed the alpha integrin that cooperates with β1 to mediate SEMA7A-activated signaling. Our results presented herein identify, for the first time, that SEMA7A may mimic the effects of laminin to promote anoikis resistance via α6β1 heterodimers in MECs.

Anoikis resistance is a hallmark of stem cells and breast cancer cells, particularly those that result in lethal metastasis as these cells must survive in the absence of extracellular matrix signaling as they travel to and throughout the vasculature to seed distant tumors(37). Since tumor cells are known to hijack normal developmental programs, we postulated that sustained expression of SEMA7A in MECs, particularly in postpartum women, could result in pro-tumor characteristics that are similar to those induced by Ras—a known oncogene. This hypothesis was based on our recent publication showing that SEMA7A expression is higher in normal adjacent breast tissue from postpartum women and our identification of a cut-point of expression that predicts for recurrence in women with PPBC(38). Our hypothesis is supported by our findings that SEMA7A mediates hallmarks of cellular transformation and stemness in non-transformed cultured MECs. While others have shown that SEMA7A is required for Ras dependent induction of EMT(39), we now show that growth in soft agar in Ras transformed variants of MCF10A cells is dependent on SEMA7A and that SEMA7A can promote growth of non-transformed cells in soft agar(32, 40, 41). Therefore, we predict that SEMA7A promotes cellular transformation and tumor initiation in breast cancer patients and, in particular, in PPBC patients(22, 41). A question that remains unanswered is whether sustained expression of SEMA7A is causative for malignancy or whether SEMA7A expression in the tumor influences normal adjacent cells to express SEMA7A thereby promoting tumor progression and metastasis. Evidence for the latter comes from our studies showing that SEMA7A can affect multiple aspects of the tumor microenvironment including collagen and fibronectin deposition, lymphangiogenesis and macrophage infiltration, as well as neighboring cells such as

fibroblasts(40, 41). Additionally, we provide evidence that SEMA7A expressing cells upregulate drug resistant cell-surface markers(23) and therefore may not respond to conventional chemotherapy; this, along with our analysis of RFS in a large patient dataset revealing that co-expression of *ITGA6* and *SEMA7A* significantly influences probability of relapse suggests that SEMA7A can not only initiate tumor formation, but also influence clinical outcomes. Future studies will investigate these important questions to identify how to best prevent and treat PPBC and all patients with α6-integrin+SEMA7A+ breast cancers.

To date, the majority of research into PPBC has focused on how the tissue microenvironment in the postpartum breast drives metastasis with lesser focus on cellular mechanisms at play in the MECs that may also support transformation(38). As more than half of all breast cancers in patients under 40 can be defined as PPBC, which are highly metastatic, understanding the mechanisms at play in postpartum breast tissue is, thus, critically important for identifying molecular drivers of PPBC. In this study, we have identified a known tumor-promotional protein, SEMA7A, as being temporally expressed during the irreversible phase of postpartum mammary gland involution where it promotes anoikis resistance and we have shown for the first time that SEMA7A can promote properties of transformation, stemness, and metastasis in non-cancerous cells.

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FIGURE LEGENDS

Figure 1: Semaphorin-7a promotes anoikis resistance in normal MECs. a. Cleaved caspase 3/7 expression in MCF10A EV or S7a-OE cells in adherent and forced suspension cultures, n=3 and **b.** in MCF12A cells +/- exogenous S7a protein and/or ITGB1 blocking antibody, 9eg7, n=3. **c.** Flow cytometry analysis of pAKT in insulin or S7a-treated MCF12A (left) with representative flow plots and MCF10A cells (right), n=3. **d.** Flow cytometry for %S7a+ mammary epithelial cells from tissues harvested from nulliparous (N) or recently weaned C57/BL6 mice (N: n=6, I1: n=5, I2: n=5, I3: n=5, I4: n=6, I6: n=4, I10: n=6, I14: n=4, I28: n=6 glands/group). **e.** Flow cytometry analysis of live epithelial cells as measured by Lin-Aquazombie-EpCAM+ in mammary tissues harvested from recently weaned WT (I1: n=5, I2: n=6, I3: n=5, I6: n=4 glands/group) or Sema7a$^{tm1Alk/J}$ (I1-I6: n=6 glands/group) mice . Error bars indicate mean +/- standard error of the mean. Significance denoted as: p<0.05=*, p<0.01=**, p<0.001=***, p<0.0001=****.

Figure 2: Loss of SEMA7A promotes histologic features of accelerated involution. Representative 20x images of #3 mammary glands from WT or SEMA7A $^{tm1Alk/J}$ mice at 1,2,3,&6 days post-wean. **a.** Hematoxylin and Eosin, **b.** pSTAT3, **c.** cleaved caspase-3 and **d.** perilipin staining.

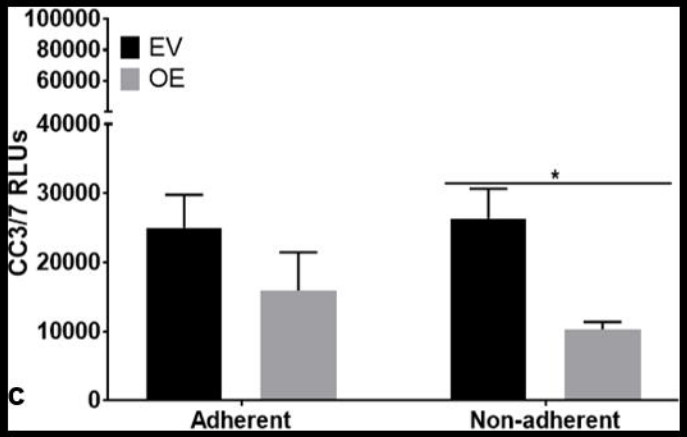
Figure 3: Loss of SEMA7A promotes alveolar collapse through alterations to cell survival and adipocyte repopulation. Immunohistochemical quantification of mammary glands from WT (I1: n=5, I2: n=6, I3: n=5, I6: n=4 glands/group) or SEMA7A $^{tm1Alk/J}$ (I1-I6: n=6 glands/group) mice at 1,2,3,&6 days post-wean. Quantification of **a.** alveolar area **b.** pSTAT3 **c.** cleaved caspase-3 and **d.** perilipin staining. Error bars indicate mean +/- standard error of the mean. Significance denoted as: p<0.05=*, p<0.01=**, p<0.001=***, p<0.0001=****.

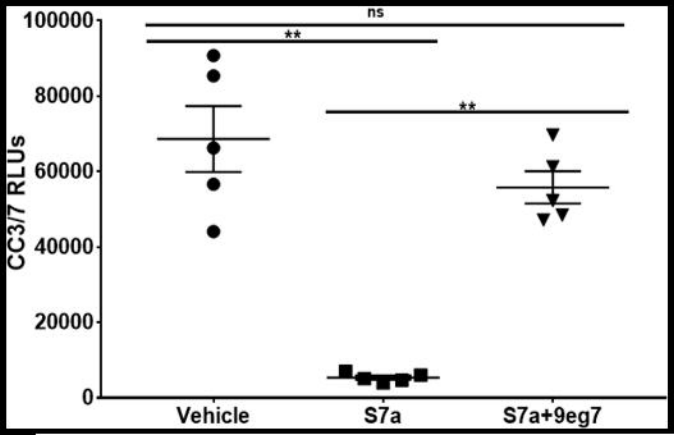
Figure 4: Semaphorin-7a promotes survival via integrin mediated activation of pAKT. a-e. Flow cytometry analysis of Lin- singlets from #4 mammary glands from WT (left, I1: n=4, I2: n=6, I3: n=5, I6: n=4 glands/group) and SEMA7A$^{tm1Alk/J}$ (right, I1-I6: n=6 glands/group) mice. **a.** %MEC subtype of total MECs by EpCAM/CD49f **b.** %EpCAMhiCD49f+ LPCs of Lin-EpCAM+. **c.** %SEMA7A+ of live (Aquazombie-) Lin-EpCAMhiCD49f+ LPCs. **d.** %ITGB1+ITGA6+ of Lin-EpCAM cells. **e.** %SEMA7A of ITGB1+ITGA6+ cells. **f.** CC3/7 expression in MCF12A cells +/- S7a and/or GoH3, n=3. **g.** %pAKT in MCF12A cells +/- S7a and/or GoH3, n=3. Error bars indicate mean +/- standard error of the mean. Significance denoted as: p<0.05=*, p<0.01=**, p<0.001=***, p<0.0001=****.

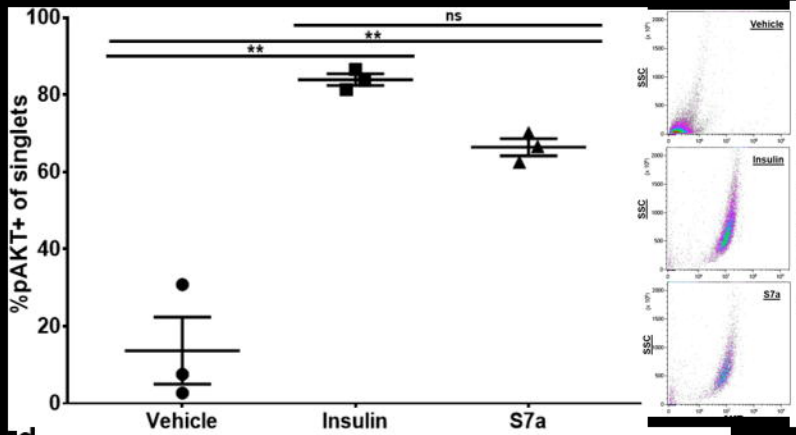
Figure 5: SEMA7A promotes features of transformation and chemoresistance via β1/α6-integrins. a. %Celltrace violet bright of singlets in MCF10A EV and OE cells, n=3. **b,c.** Number of mammospheres formed by MCF12A cells +/- S7a and GoH3 or 9eg7, n=4. **d.** Number of colonies formed in soft agar assay, n=3. **e,f,g.** DCIS KD or OE mammospheres cultured in

paclitaxel +/- 9eg7 or GoH3, n=3. **e**. Number of mammospheres. **f.** %CD44+CD24- of singlets by flow cytometry. **g.** Representative images, 20x magnification. Error bars indicate mean +/- standard error of the mean. Significance denoted as: p<0.05=*, p<0.01=**, p<0.001=***, p<0.0001=****.

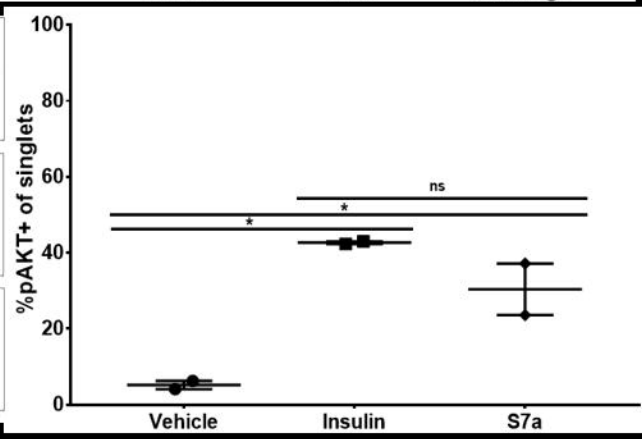
Figure 6: High expression of SEMA7A and α6-integrin in breast cancer correlates with decreased relapse-free survival. a-d. Kaplan-Meier survival curves of relapse-free survival over 5 years in breast cancer patients. **a.** SEMA7A **b.** α6-integrin **c.** SEMA7A in patients with low α6-integrin **d.** SEMA7A in patients with high α6-integrin.

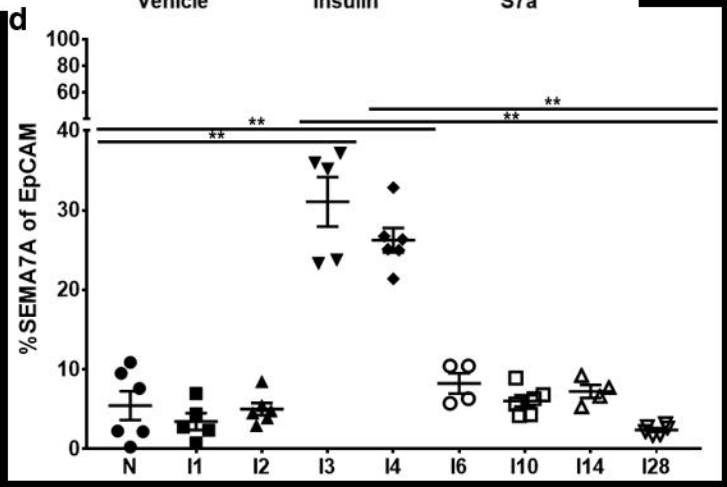

c
EV
OE
CC3/7 RLUs
100000
80000
60000
40000
30000
20000
10000
0
*
Adherent
Non-adherent

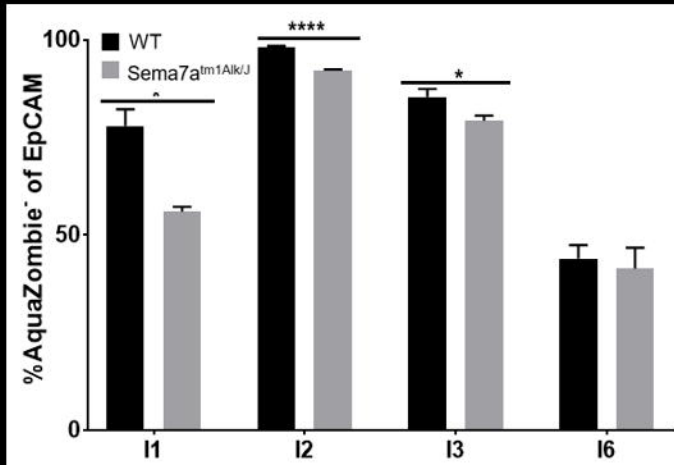

ns
**
**
CC3/7 RLUs
100000
80000
60000
40000
20000
0
Vehicle
S7a
S7a+9eg7

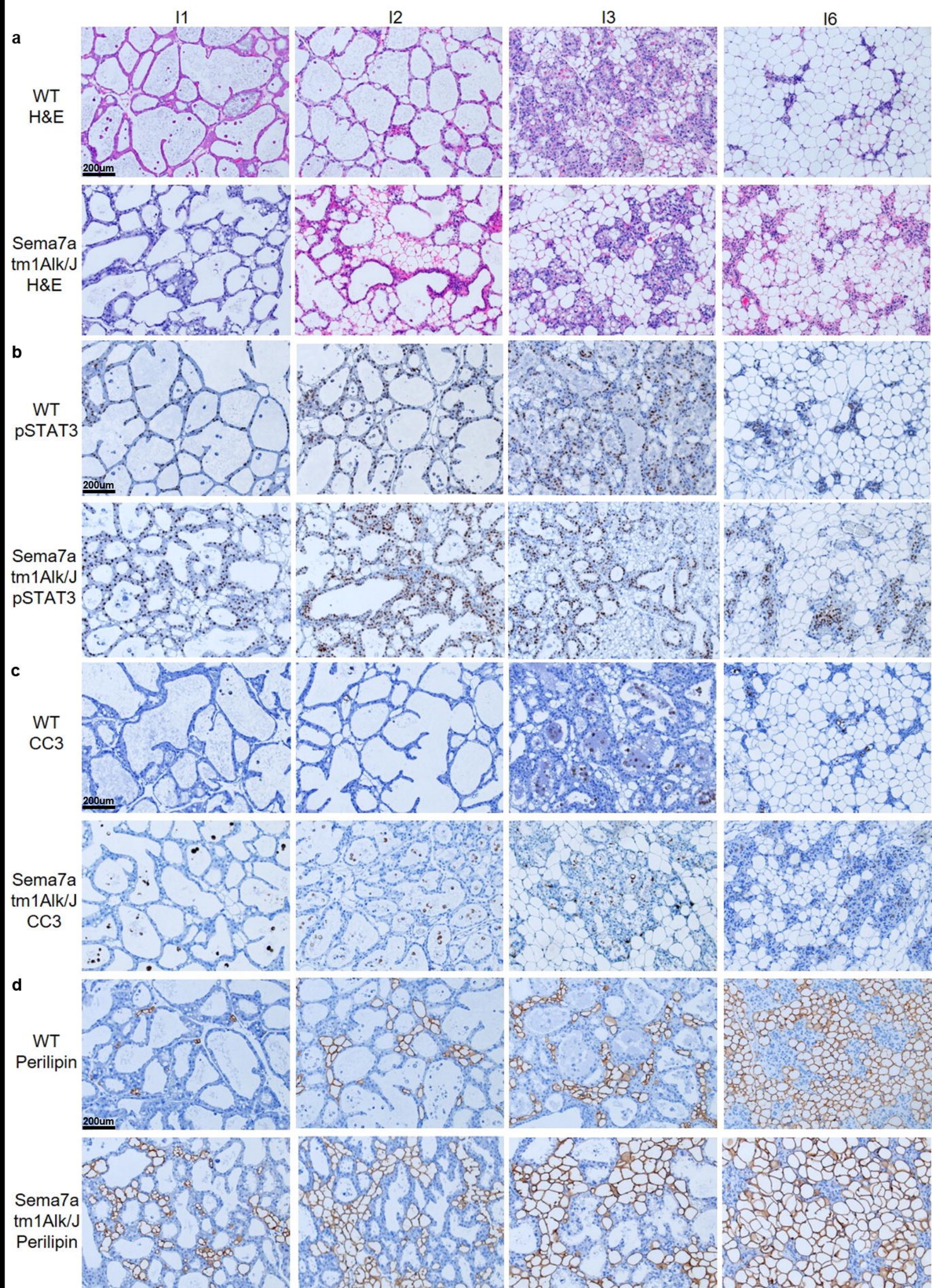

ns
**
**
%pAKT+ of singlets
100
80
60
40
20
0
Vehicle
Insulin
S7a

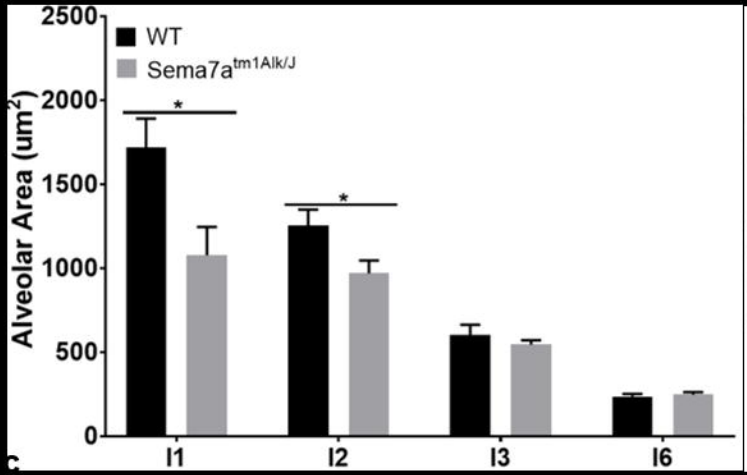
Vehicle
Insulin
S7a
SSC

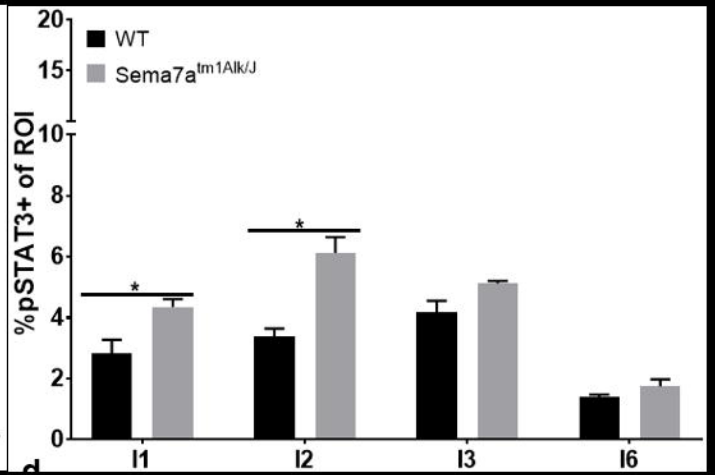

ns
*
*
%pAKT+ of singlets
100
80
60
40
20
0
Vehicle
Insulin
S7a

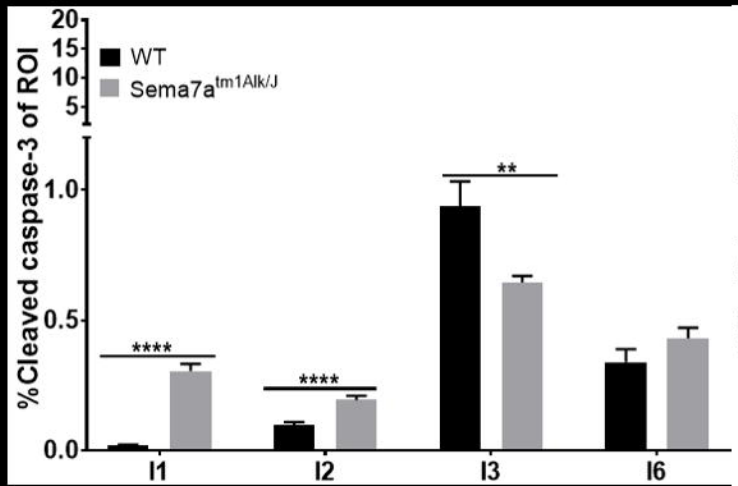

d
**
**
**
**
%SEMA7A of EpCAM
100
80
60
40
30
20
10
0
N
I1
I2
I3
I4
I6
I10
I14
I28

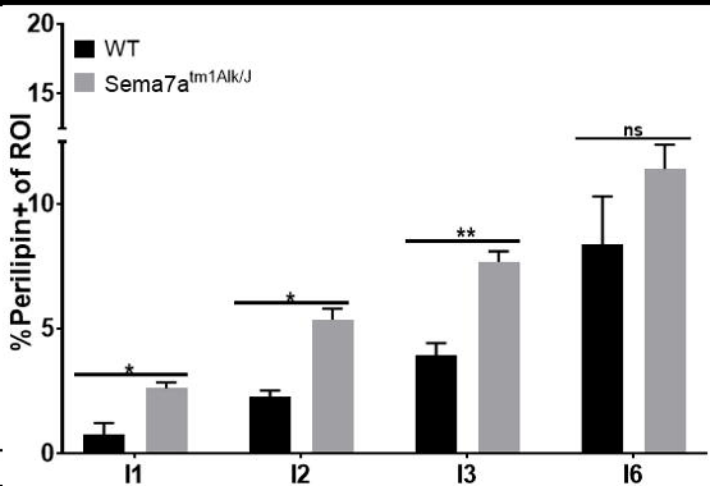

WT
Sema7a$^{tm1Alk/J}$
****
*
%AquaZombie$^-$ of EpCAM
100
50
0
I1
I2
I3
I6

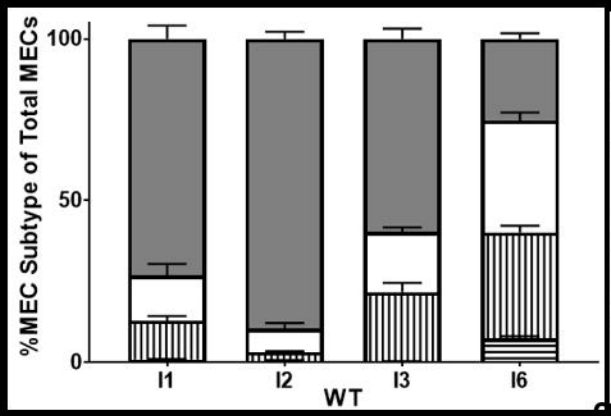

I1
I2
I3
I6
a
WT
H&E
200um
Sema7a
tm1Alk/J
H&E
b
WT
pSTAT3
200um
Sema7a
tm1Alk/J
pSTAT3
c
WT
CC3
200um
Sema7a
tm1Alk/J
CC3
d
WT
Perilipin
200um
Sema7a
tm1Alk/J
Perilipin


WT
Sema7a$^{tm1Alk/J}$
Alveolar Area (um^2)
2500
2000
1500
1000
500
0
*
*
I1
I2
I3
I6


WT
Sema7a$^{tm1Alk/J}$
%pSTAT3+ of ROI
20
15
10
8
6
4
2
0
*
*
I1
I2
I3
I6


WT
Sema7a$^{tm1Alk/J}$
%Cleaved caspase-3 of ROI
20
15
10
5
1.0
0.5
0.0
****
****
**
I1
I2
I3
I6


WT
Sema7a$^{tm1Alk/J}$
%Perilipin+ of ROI
20
15
10
5
0
*
*
**
ns
I1
I2
I3
I6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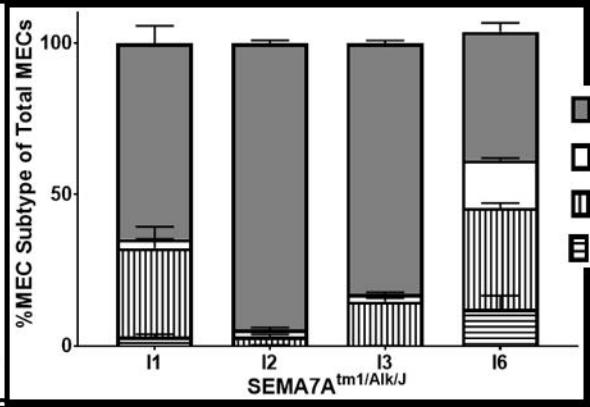
100
50
0
I1
I2
I3
I6
WT


%MEC Subtype of Total MECs
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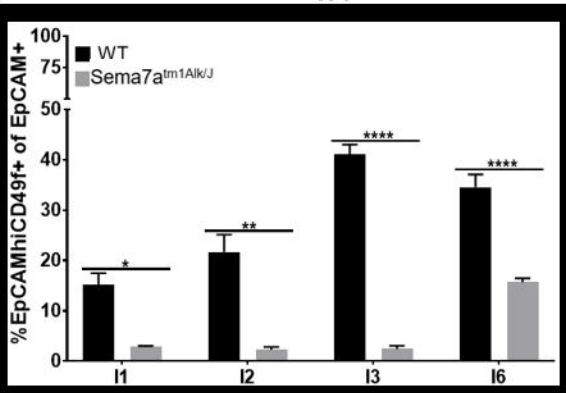
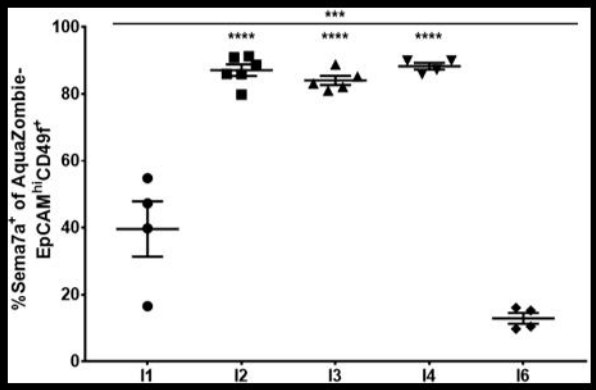
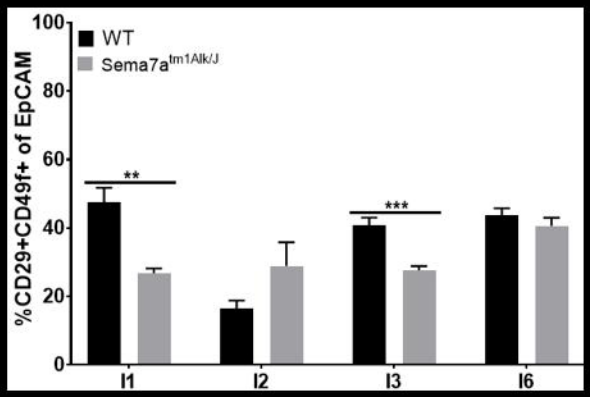
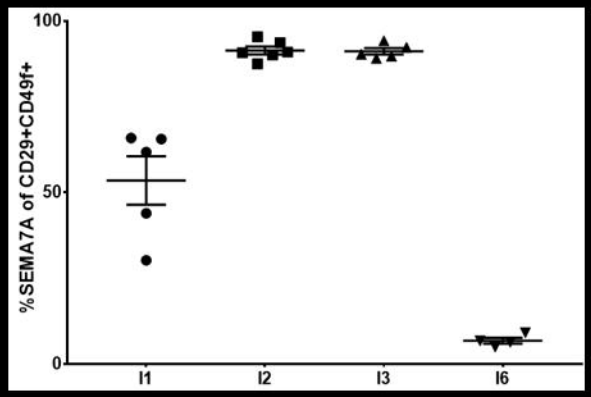
50
0
I1
I2
I3
I6
SEMA7A tm1/Alk/J
Luminal
LPCs
Basal
BPCs


%EpCAMhiCD49f+ of EpCAM+
WT
Sema7a tm1Alk/J
100
75
50
40
30
20
10
0
*
**
****
****
I1
I2
I3
I6


%Sema7a+ of AquaZombie-
EpCAMhiCD49f+
***
****
****
****
100
80
60
40
20
0
I1
I2
I3
I4
I6


%CD29+CD49f+ of EpCAM
WT
Sema7a tm1Alk/J
100
80
60
40
20
0
**
***
I1
I2
I3
I6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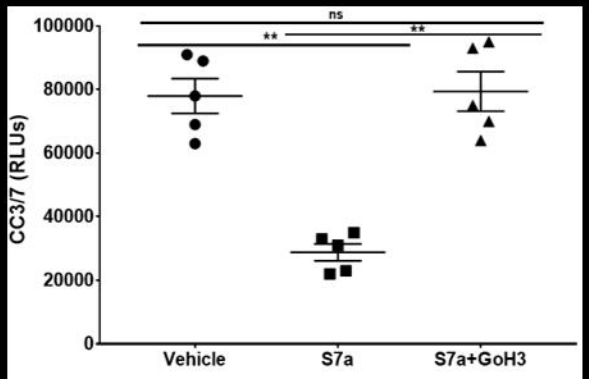
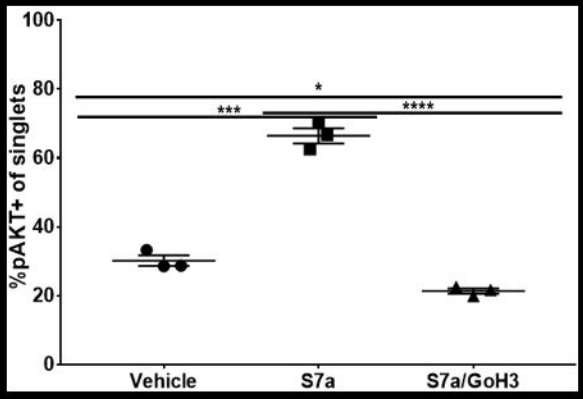
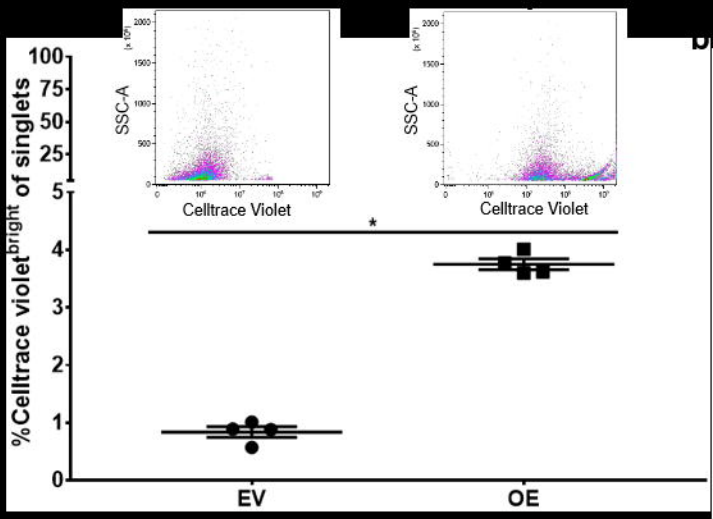
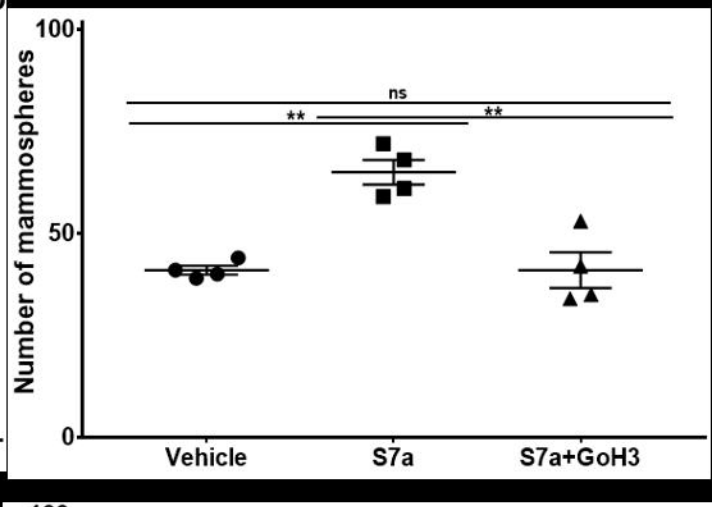
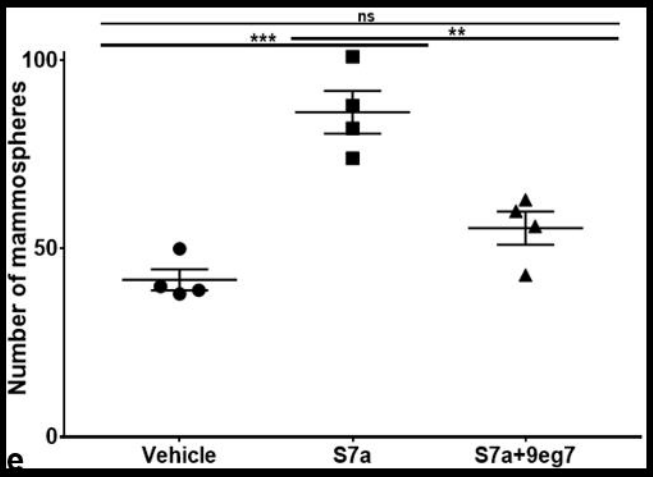
100
50
0
I1
I2
I3
I6

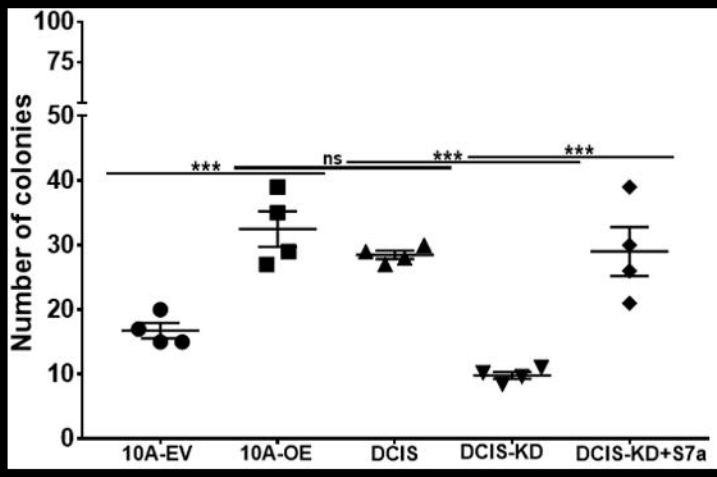

CC3/7 (RLUs)
100000
80000
60000
40000
20000
0
ns
**
**
Vehicle
S7a
S7a+GoH3

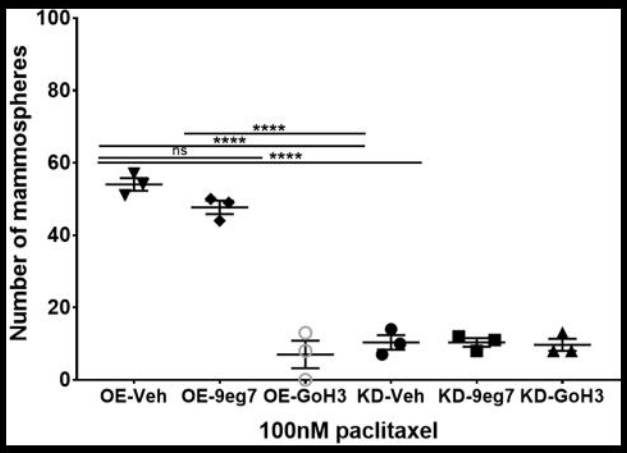

%pAKT+ of singlets
100
80
60
40
20
0
*
***
****
Vehicle
S7a
S7a/GoH3

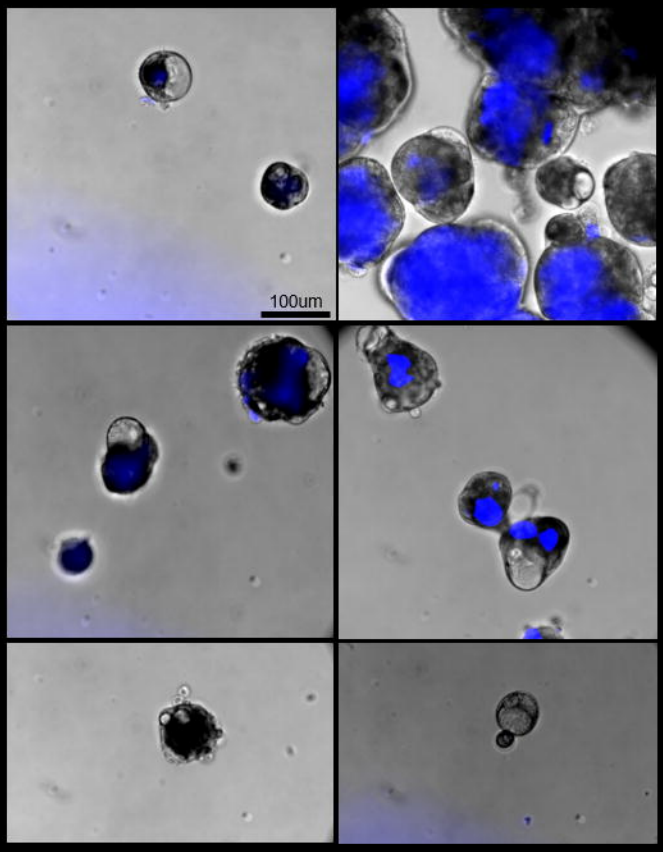

SSC-A
Celltrace Violet
SSC-A
Celltrace Violet
%Celltrace violet bright of singlets
*
EV
OE


b
Number of mammospheres
ns
**
**
Vehicle
S7a
S7a+GoH3


Number of mammospheres
ns
***
**
Vehicle
S7a
S7a+9eg7


Number of colonies
***
ns
***
***
10A-EV
10A-OE
DCIS
DCIS-KD
DCIS-KD+S7a


e
Number of mammospheres
****
****
ns
****
OE-Veh
OE-9eg7
OE-GoH3
KD-Veh
KD-9eg7
KD-GoH3
100nM paclitaxel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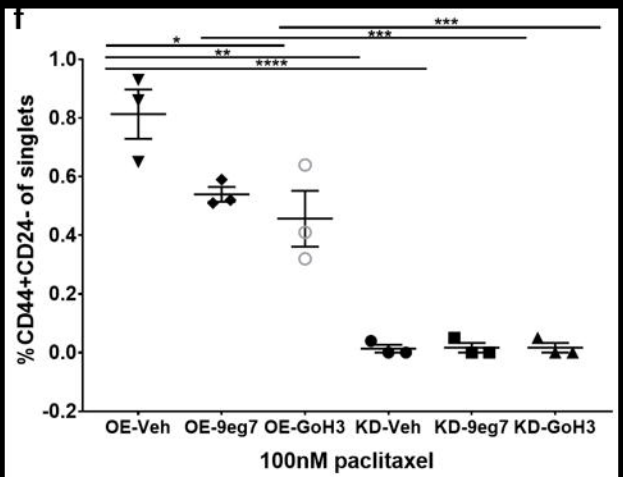
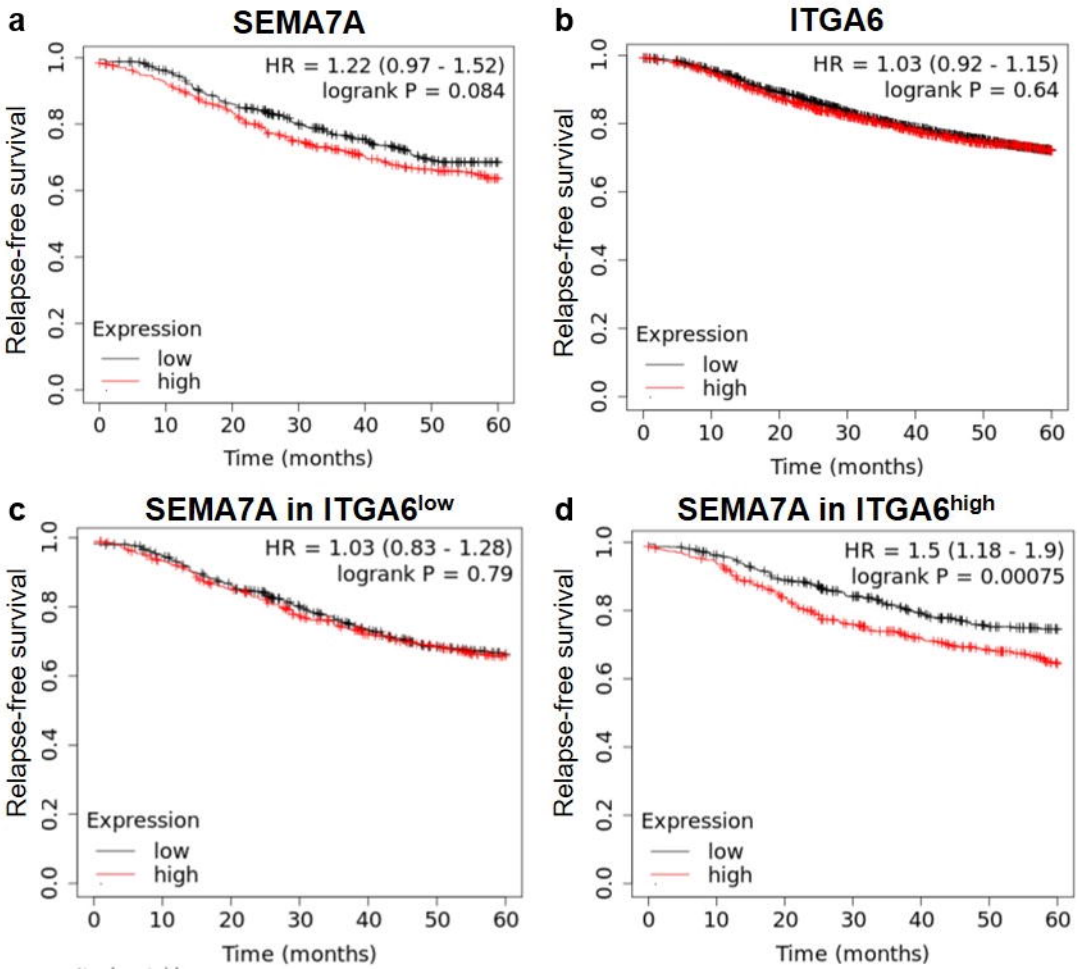

f
% CD44+CD24- of singlets
*
**
****
***
***
OE-Veh
OE-9eg7
OE-GoH3
KD-Veh
KD-9eg7
KD-GoH3
100nM paclitaxel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SEMA7A
HR = 1.22 (0.97 - 1.52)
logrank P = 0.084
Relapse-free survival
Expression
low
high
Time (months)
b
ITGA6
HR = 1.03 (0.92 - 1.15)
logrank P = 0.64
Relapse-free survival
Expression
low
high
Time (months)
c
SEMA7A in ITGA6low
HR = 1.03 (0.83 - 1.28)
logrank P = 0.79
Relapse-free survival
Expression
low
high
Time (months)
d
SEMA7A in ITGA6high
HR = 1.5 (1.18 - 1.9)
logrank P = 0.00075
Relapse-free survival
Expression
low
high
Time (months)