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14. ABSTRACT In breast cancer the androgen receptor (AR) is more widely expressed than estrogen receptor alpha (ER) or the progesterone receptor (PR), suggesting a potential role for AR in BC. To explore the function of AR in models of the three main subtypes of breast cancer (ER positive, ER negative and Her2+), we are using a new-generation AR inhibitor, enzalutamide (Enza), which impairs nuclear localization of AR. Our research seeks to determine whether Enza will be effective in breast cancer and utilize preclinical models to determine if and how it should be combined with standard treatments with the primary objective being to guide future clinical trials. In Dec 2015 Drs. Elias and Richer, demonstrate synergy between Enza and Tamoxifen or Fulvestrant <i>in vitro</i> and results of a Phase 1 study (NCT01597193) on pharmacokinetics and safety of Enza plus Fulvestrant in women with advanced ER+ disease. Regarding TNBC, we reported that AR is anti-apoptotic and facilitates anchorage independent growth and Enza decreased tumor viability <i>in vivo</i> . AR regulates secreted factors that support cancer stem cells and Enza significantly decreased tumor initiating capacity of TNBC <i>in vivo</i> . Enza administered to mice with chemotherapy greatly decreased TNBC tumor burden. These findings have guided future clinical trials with Enza in breast cancer. We report on the two trials in ER+ breast cancer.					
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1. INTRODUCTION:

The central thesis of this grant is to understand the role of AR signaling in breast cancer subtypes, and understand how to best use an inhibitor of AR signaling, enzalutamide (enza), as a therapeutic agent in breast cancer. In breast cancers, the androgen receptor (AR) is more widely expressed than estrogen receptor alpha (ER) or the progesterone receptor (PR), which are used as therapeutic targets and biomarkers, suggesting a potential role for AR in BC. We examined the primary tumors of women treated with tamoxifen or aromatase inhibitor therapy and found that a higher AR to ER protein ratio correlates with worse response to the anti-estrogen tamoxifen (Cochrane DR et al 2014). In this DOD Clinical Translational Grant we utilize a new-generation AR inhibitor, enzalutamide, which impairs nuclear localization of AR, to explore the function of AR in models of the three main subtypes of breast cancer (ER positive, ER negative and Her2+). This is a very different mode of action than previous generation anti-androgens such as bicalutamide (Casodex), a competitive inhibitor of endogenous androgens that allows ligand-mediated nuclear localization of AR. Enzalutamide has shown success in the clinic in patients with late stage prostate cancer refractory to bicalutamide and is now FDA approved as a prostate cancer therapy. The research in this proposal seeks to determine whether inhibition of AR with enzalutamide will be effective in breast cancer and utilize preclinical models to determine if and how it should be combined with currently used standard of care treatments in the three main types of breast cancer, with the primary objectives of guiding the design of future clinical trials with enzalutamide. The clinical portion of this grant serves to conduct a neoadjuvant trial to study fulvestrant (standard of care) to fulvestrant plus enzalutamide and another trial for women with metastatic ER+ breast cancer treating with fulvestrant plus enzalutamide and to obtain serial biopsies and perform molecular analyses to identify changes in the pre-versus post treatment biopsies in concert with the overall clinical analysis of the efficacy of enzalutamide in ER+ breast cancer. The preclinical portion of the grant is over, but Dr. Richer's lab and the molecular pathology core are performing the assays on biopsies from the clinical trials, which are nearing completion of enrollment.

2. **KEYWORDS:** Breast cancer, androgen receptor, estrogen receptor, growth factors, enzalutamide, endocrine resistance, targeted therapy.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The objective of Stage I of this proposal is to rapidly generate preclinical data in the laboratory of Jennifer Richer, Ph.D., testing Enza alone or in combination with standard of care therapeutics in different subtypes of BC to help guide the clinical trials described in **Stage II** (PI clinical partner Dr. Anthony Elias) and steer the rational design and focus on patients most likely to benefit from enzalutamide alone or in combination with currently used therapeutics. Below we describe for each task in the official statement of work the major activities; specific objectives; significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or other achievements. We include a discussion of stated goals not met or tasks not fully completed. We include pertinent data and graphs in sufficient detail to explain significant results achieved. Detailed description of the methodology used is provided in the methods section of two manuscripts in the appendix. The first manuscript was published in January of 2014 and was submitted with the first annual progress report. The second manuscript arising from this work investigated AR in the non-LAR subtype of TNBC was published in 2015 (Barton VN et al MOL CA THER 2015). A third primary manuscript on AR in ER+ breast cancer came out in 2016 (D'Amato NC MOL CA RES 2016). In July 2017 we published an additional two primary manuscripts, one on AR in HER2+ breast cancer and synergy between enzalutamide and everolimus or trastuzumab. Everolimus was found in clinical trials to be very effective in ER+ breast cancer, but less so in HER2+ and TNBC. Our studies show that everolimus increases the amount of AR and AR activity and therefore combining the anti-androgen enzalutamide with everolimus gives a synergistic effect over either drug alone (Gordon MA et al MOL CA THER 2017). The next study demonstrated that AR is anti-apoptotic, supports anchorage independent growth and androgens expand a cancer stem cell-like population in TNBC (Barton VN. And Christenson JL, CAN RES 2017). We reported that enzalutamide given either simultaneous with or sequential to chemotherapy and found that simultaneous treatment was more effective at

preventing recurrence after cessation of chemotherapy, a very important finding for future clinical trial design. A sixth manuscript was with a colleague in my department in which we determined that AR supported tumor progression in a preclinical model of obesity after the loss of ovarian function (Wellberg EA, et al HORMONES AND CANCER, 2017). While no DOD funds from the Richer lab were used for that study, we did use enzalutamide to show AR involvement and it is very pertinent to topic of this grant), so we mention it here. We also published 4 review articles on the topic of AR in breast cancer: The publications are all listed in the products section.

Currently we have just submitted 3 manuscripts on AR in breast cancer and one is almost ready to One focuses on genes regulated by AR in AR+ TNBC PDX and how AR positive TNBC may be targeted with CDK4/6 inhibitors (just submitted to *Molecular Cancer Therapeutics*). The second paper is under review at *Cancer Research* and is on AR in ER+ metastatic breast cancer with or without mutations in the ligand binding domain of ER and this paper has some preclinical and clinical results. In a third manuscript under review at *Endocrinology*, we studied AR crosstalk with the TGFbeta pathway. We are preparing a fourth paper on AR regulated secreted factors that might have utility as serum biomarkers in breast cancer patients to reflect tumor cell AR activity.

Finally as we finish the clinical trials, we will write up the results of the molecular analyses of gene mutations, expression levels and phospho proteins are altered in ER+ breast cancers treated either in the metastatic or neoadjuvant setting with fulvestrant versus fulvestrant plus Enza. Although we are not done with enrollment for the neoadjuvant trial, the metastatic breast cancer trial has completed and laboratory correlates are being integrated with clinical outcomes and the manuscript is in preparation. Two abstracts has been submitted to the San Antonio Breast Cancer Conference.

Preclinical Aim 1. To test enzalutamide (enza) in combination with currently approved therapies for breast cancer (BC) in the various subtypes of BC.

Task 1 – Evaluate enzalutamide in combination with anti-estrogen therapy in ER+/AR+ BC lines (MCF7, BCK4) and a ER+/AR+ patient derived xenograft.

Task 2. Test enza in three different tamoxifen resistance models *in vitro*.

Task 3. Test enzalutamide in combination with Her2 directed therapy in ER+ and ER- Her2+ models

Task 4. Examine enzalutamide in combination with an mTOR inhibitor (Afinitor/everolimus)

Task 5. In true TNBC cell lines and explants that retain AR, enzalutamide will be evaluated alone and in combination with chemotherapy and everolimus, *in vitro* and *in vivo*).

What was accomplished under these goals?

TASK1- Evaluate enzalutamide in combination with anti-estrogen therapy in ER+/AR+ BC lines (MCF7, BCK4) and a ER+/AR+ patient derived xenograft. Months 1-4. 100% completed. Results published summarized below and published in D'Amato NC *et al* Mol Ca Res 2016. Androgen receptor (AR) is expressed in 90% of estrogen receptor alpha positive (ER+) breast tumors, but its role in tumor growth and progression remains controversial. Use of two anti-androgens that inhibit AR nuclear localization, enzalutamide and MJC13, revealed that AR is required for maximum ER genomic binding. Here, a novel global examination of AR chromatin binding found that estradiol induced AR binding at unique sites compared to dihydrotestosterone (DHT). Estradiol-induced AR binding sites were enriched for estrogen response elements and had significant overlap with ER binding sites. Furthermore, AR inhibition reduced baseline and estradiol-mediated proliferation in multiple ER+/AR+ breast cancer cell lines, and synergized with tamoxifen and fulvestrant. *In vivo*, enzalutamide significantly reduced viability of tamoxifen-resistant MCF7 xenograft tumors and an ER+/AR+ patient derived model. Enzalutamide also reduced metastatic burden following cardiac injection. Lastly, in a comparison of ER+/AR+ primary tumors versus patient-matched local recurrences or distant metastases, AR expression was often maintained even when ER was reduced or absent. These data provide preclinical evidence that anti-androgens that inhibit AR nuclear localization affect both AR and ER, and are effective in combination with current breast cancer therapies. In addition, single agent efficacy may be possible in tumors resistant to

traditional endocrine therapy, since clinical specimens of recurrent disease demonstrate AR expression in tumors with absent or refractory ER.

The first therapy of choice to treat an ER+ tumor, at least at the current time is an anti-estrogen or aromatase inhibitor. We showed that the relative expression of AR to ER protein (percent cells positive) can predict a poor response to tamoxifen and poor overall survival (Cochrane DR et al 2014). We determined that the enzalutamide is efficacious in tamoxifen resistant MCF 7 cells *in vivo*. However, ER+ tumors will likely be treated first with tamoxifen (if the woman is premenopausal) or aromatase inhibitor (AI) if post-menopausal or having recurred while on tamoxifen, then if there is a recurrence of disease, with the ER degrader Fulvestrant. Therefore, we tested for synergy between these two drugs. Since Fulvestrant must be given IM in oil, being able to reduce the effective dose necessary would be clinically useful. We published a dose reduction index in D'Amato NC *et al* Mol Ca Res 2016 that demonstrated that combining Fulvestrant with Enzalutamide may allow a reduction in the amount of both drugs while achieving the same efficacy. This data (which was confirmed in other cell lines) helped to justify the two clinical trials described in detail in Dr. Elias's trial with Fulvestrant vs Fulvestrant plus Enza in both the neoadjuvant and metastatic setting.

Task 3. Test enzalutamide in combination with HER2-directed therapy in ER+ and ER- HER2+ models. (100% complete) published in (Gordon MA *et al* MOL CA THER 2017).

Task 4. Examine enzalutamide in combination with an mTOR inhibitor (Afinitor/everolimus). (100% complete) published in (Gordon MA *et al* MOL CA THER 2017).

Abstract: The androgen receptor (AR) is widely expressed in breast cancer, and evidence suggests dependence on AR signaling for growth and survival. AR antagonists such as enzalutamide and seviteronel have shown success in preclinical models and clinical trials of prostate cancer and are currently being evaluated in breast cancer. Reciprocal regulation between AR and the HER2/PI3K/mTOR pathway may contribute to resistance to HER2- and mTOR-targeted therapies; thus, dual inhibition of these pathways may synergistically inhibit breast cancer growth. HER2⁺ and triple-negative breast cancer cell lines were treated with AR antagonist plus anti-HER2 mAb trastuzumab or mTOR inhibitor everolimus. Apoptosis, cell proliferation, and drug synergy were measured *in vitro*. Pathway component genes and proteins were measured by qRT-PCR, Western blot, and reverse phase protein array. *In vivo*, HER2⁺ breast cancer xenografts were treated with enzalutamide, everolimus, trastuzumab, and combinations of these drugs. AR antagonists inhibited proliferation of both HER2⁺ and TNBC cell lines. Combining AR antagonist and either everolimus or trastuzumab resulted in synergistic inhibition of proliferation. Dihydrotestosterone caused increased phosphorylation of HER2 and/or HER3 that was attenuated by AR inhibition. Everolimus caused an increase in total AR, phosphorylation of HER2 and/or HER3, and these effects were abrogated by enzalutamide. Growth of trastuzumab-resistant HER2⁺ xenograft tumors was inhibited by enzalutamide, and combining enzalutamide with everolimus decreased tumor viability more than either single agent. AR antagonists synergize with FDA-approved breast cancer therapies such as everolimus and trastuzumab through distinct mechanisms. Treatment combinations are effective in trastuzumab-resistant HER2⁺ breast cancer cells *in vivo*.

Task 5. In true TNBC cell lines and explants that retain AR, enzalutamide will be evaluated alone and in combination with chemotherapy and everolimus, *in vitro* and *in vivo*. (100% completed. Published in Barton VN and Christenson JL *et al* CANCER RES 2017).

Abstract: Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype lacking estrogen and progesterone receptors, and human epidermal growth factor receptor 2 (HER2). While to date there are no approved targeted therapies for TNBC, preclinical and early clinical trials indicate that up to 50% express some degree of positivity for androgen receptors (AR) and are sensitive to AR targeted therapy. However, the function of AR in TNBC and the mechanisms by which AR targeted therapy reduces tumor burden in preclinical and clinical settings are unknown. We hypothesized that AR maintains a cancer stem cell-like (CSC) tumor initiating population and that it serves as an anti-apoptotic factor that facilitates anchorage independence.

Methods Anchorage independence/anoikis resistance was assessed on poly-Hema coated tissue culture plates used to achieve forced suspension culture and apoptosis was measured with cleaved caspase 3 antibody. AR was inhibited using the AR inhibitor Enzalutamide (Enza) or shRNAs targeting AR. CSC populations were assessed in vitro using ultra low attachment plates, CD44/CD24 staining, the ALDEFLUOR assay, and single cell mammosphere formation efficiency (MFE) assays in TNBC cell lines SUM159PT and MDA-MB-453.. In vivo, tumor-initiating capacity was assessed using a limiting dilution assay of SUM159PT cells pre-treated with or without Enza. Lastly, the efficacy of combination Enza and chemotherapy was assessed by caliper measurement and intravital imaging of TNBC xenografts in mice treated with Enza and paclitaxel. **Results** AR transcript ($P<0.05$), protein, and transcriptional activity ($P<0.01$) increased in tumor cells in suspension culture compared to attached conditions. Cells that expressed AR protein resisted detachment-induced apoptosis. The CSC population increased in suspension culture by ALDEFLUOR staining ($P<0.01$), CD44/CD24 staining ($P<0.001$), and MFE ($P<0.05$). AR inhibition decreased ADLH staining ($P<0.001$), increased CD24 staining ($P<0.05$), and decreased MFE ($P<0.01$). In vivo, pre-treatment with Enza decreased the tumor-initiating capacity of TNBC cells in a limiting dilution assay ($P<0.05$). Enza significantly decreased tumor volume and viability when administered during or after chemotherapy in vivo ($P<0.05$) and simultaneous treatment significantly reduced tumor recurrence.

Conclusions AR supports anchorage independence, maintenance of CSCs, tumor initiation and regrowth following chemotherapy in a TNBC preclinical model. Thus AR targeted therapies may enhance the efficacy of chemotherapy even in TNBC with very few cells positive for AR, perhaps by targeting a different cell population.

Preclinical Aim 2. Using samples collected from the xenograft studies, examine if and how the mechanism of action by which enzalutamide works in the various subtypes of breast cancer.

- **Task 1.** Perform IHC on xenograft tumors for AR, ER, Her3, BrdU, FOXA1, PSA SDF1, Cyr61. Months 12-18. We have performed IHC for all these proteins from xenograft experiments on ER+, HER2+ and TNBC where relevant (see published papers and AR+ TNBC PDX below in Figure 2. We also think the AR regulation of the EGFR ligand amphiregulin is very important (Barton V et al 2015).
- **Task 2.** Make RNA from xenografts, perform RNA sequencing analyze. Months 15-18 (100% completed) We analyzed RNA profiling from ER+ and TNBC cell line xenografts as reported in previous progress reports. Additionally we performed RNA-seq on ER+PDX (the PT12 PDX grown with E2 plus or minus Enza and reported the analysis in the D'Amato Mol Ca Res 2016 paper. In short, inhibiting AR with Enza, inhibits many classic E2/ER and AR regulated genes. We generated RNA-sequencing data from an AR+ TNBC PDX HCI-009 (submitted to GEO) that increases in size in response to DHT. We identified numerous known AR regulated genes such as prostate specific antigen (PSA), also called KLK3 that is used as a marker of disease burden in prostate cancer and we are comparing this to genes that are downregulated in the presence of the anti- androgen enzalutamide in this and other TNBC PDX. This is in the paper under review right now in Mol Ca Therapeutics (Sent Sept 15th).

Preclinical Aim 3. Identify mechanisms of resistance to enzalutamide in triple negative breast cancers to elucidate pathways that impinge on the AR pathway to potentially target in combination with enzalutamide.

- **Task 1.** Sequence 3 AR+ triple negative cell lines resistant and 3 that are sensitive. Months 18-24 Since the TNBC cell lines that we have studied so far are sensitive to enzalutamide and we have found it to particularly affect growth on soft agar (Barton V et al 2015), we have not performed sequencing of all of these yet because we are still trying to figure out the best conditions and timing. We are also exploring another approach, which is to chronically treat the cells with enza to generate resistant lines. We have taken this approach with the MDA-MB-453 TNBC line which we showed in Cochrane et al 2014 to be very responsive to enza in vitro and in vivo. We now have a resistant line that we did mutational analysis on the

resistant line and it does not have the F876L mutation that has been reported to confer resistance to enzalutamide in prostate cancer cells and patient tumors. It is likely that the cell line that we have rendered resistant is resistant via a different mechanism other than this AR mutation.

Summary for this aim:

- No completely resistant TNBC lines. Enza IC50s does not correlate with AR protein.
- No clearly “sensitive” versus “resistant” cell lines. Still looking at PDX. Do have MDA-MB-231 with extremely low AR and they express much more glucocorticoid receptor GR.

Our two clinical trials were approved by our local IRB (COMIRB 16-1042 and COMIRB 16-1001) to complete the clinical aims of this grant. The metastatic trial finished accruing and the data are now being analyzed.

Clinical Aim 4: Protocol 16-1042: a randomized phase II trial of fulvestrant with or without enzalutamide as preoperative treatment for women with ER+/AR+/Her2- breast cancer was activated for accrual on 08/30/2017 at the University of Colorado. Overall accrual slowed in recent months due to impacts of COVID-19. Currently 59 patients consented. 52 enrolled (3 screen failures and 4 withdrawals prior to enrollment), and 51 randomized and treated (1 patient withdrew prior to treatment). Stage I accrual was completed 3/2/20, with 22 evaluable subjects enrolled on the experimental (combination) arm. More than 4 patients on the combination arm have achieved a PEPI score = 0, therefore an additional 16 patients would need to be enrolled in stage 2. Currently we have the need to accrue 10 patients to complete the trial.

Clinical Aim 5: Protocol 16-1001: fulvestrant plus enzalutamide in ER+/AR+/Her2- metastatic breast cancer (with serial biopsies) was activated for accrual on 06/29/2017 at the University of Colorado. The 16-1001 trial completed accrual in November 2019 and results are being analyzed by Dr. Dexiang Gao and team. Clinical outcomes will be reported in the next quarterly report and at the 2020 SABC meeting along with the molecular analyses reported below and the RPPA protein analysis, which is now being analyzed.

Clinical Aims:

The tumor specimens from the IIT Phase II Fulvestrant +/- Enzalutamide trial in the metastatic adjuvant and neoadjuvant setting will be analyzed as follows in this diagram.

This analysis uses a custom Archer VariantPlex that evaluates 60 gene hot spots for commonly mutated genes in cancer with our Colorado Molecular Correlates Laboratory (CMOCO) custom version assay. We are particularly interested in mutations in the estrogen receptor alpha (ERalpha) and this assay covers detection of any mutation in exon 8 which covers N519 through the C-terminus V595 (based on refseq NM_000125) this includes the 3 most common ER mutations that arise in metastatic ER+ breast cancers treated with aromatase inhibitors (AI) and we also added the AR mutation hotspot that arises with enzalutamide during treatment of prostate cancer). The limit of detection for clinical cases is 10% allele frequency, but it often can detect 3.0%.

For the RPPA we are evaluating with Univ of CO sending Dr. Petricoin's lab the first set for phospho-protein analysis from frozen sections laser captured to enrich for tumor. When necessary, laser captured is performed to enrich for tumor. We have now finished sending all samples from the pre-and post-treatment biopsies of tumor from patients enrolled in the metastatic trial (16-001). We have just obtained the data from the RPPA and Dexiang Gao and team are performing the analyses to determine what phospho-proteins and pathways differ pre-versus post-treatment in responders versus non-responders based on progression free survival below or above 12 weeks. Since in the metastatic trial the patients were heavily pre-treated, there is a lot of heterogeneity, but we do have the power of having two biopsies from each patient so we can do paired comparisons. The neoadjuvant trial is still enrolling so we the RPPA will be done for that trial in one batch and it will be less heterogeneous since it is from primary untreated tumor (pre-treatment), then after treatment with either fulvestrant alone or fulvestrant plus enzalutamide.

We have performed mutation Archer VariantPlex assay to detect common mutations in the biopsies from the first 18 patients in the metastatic disease trial that had enough evaluable material in the core needle biopsy from

a pretreatment core. Results indicated that of 18 tumors assayed, about half had mutations in the ESR1 exon 8 mutations which result in a constitutively active ERalpha because the ligand binding domain is in a position similar to as if it was bound to ligand. Additional mutations were found in ATM, SMAD4, TP53, PIK3CA, CDH1, and PTEN. Mutation results are shown in Figure 1 (top) and immunohistochemistry representative figures for ER, PR and AR protein are shown at bottom. All slides were scored by pathologist Sharon Sams. While it has been published that PR is higher in ER mutated tumors and cell lines, AR had not yet been examined at the protein level in patients with metastatic breast cancer with or without ER mutations.

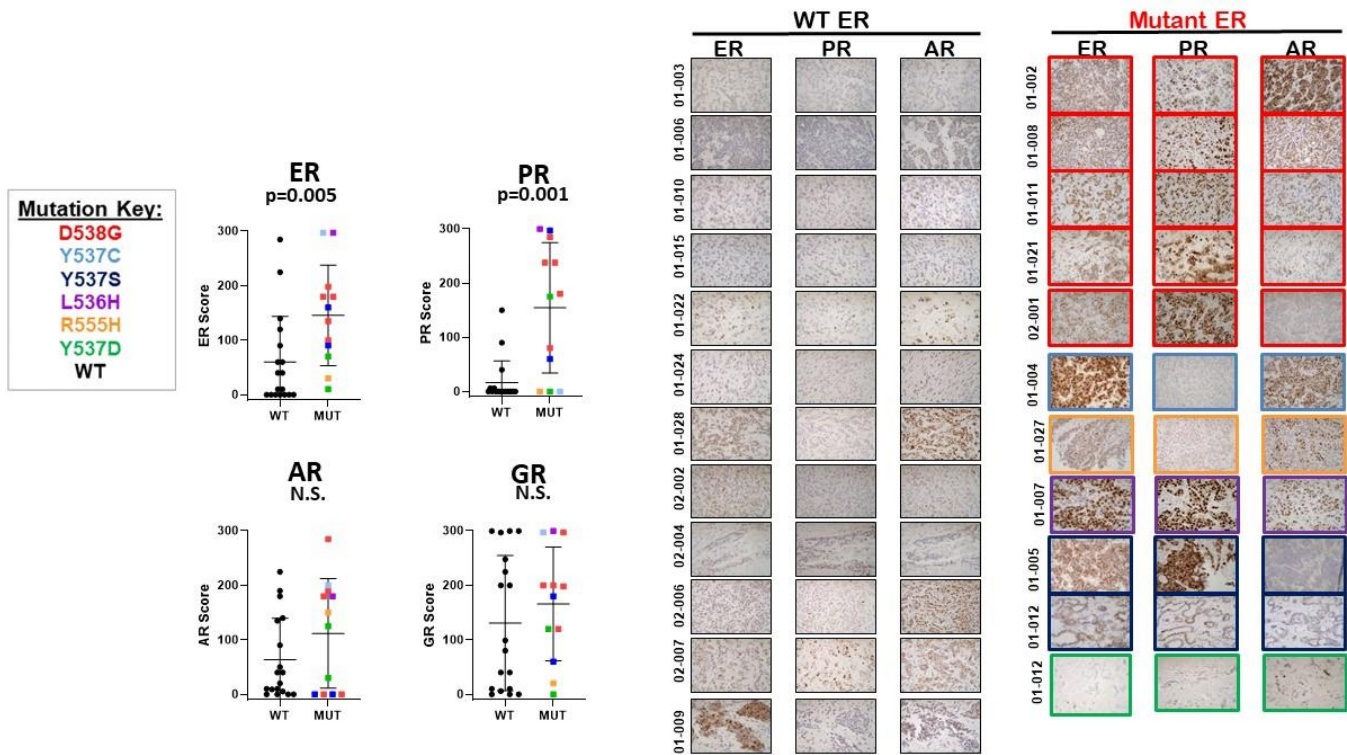
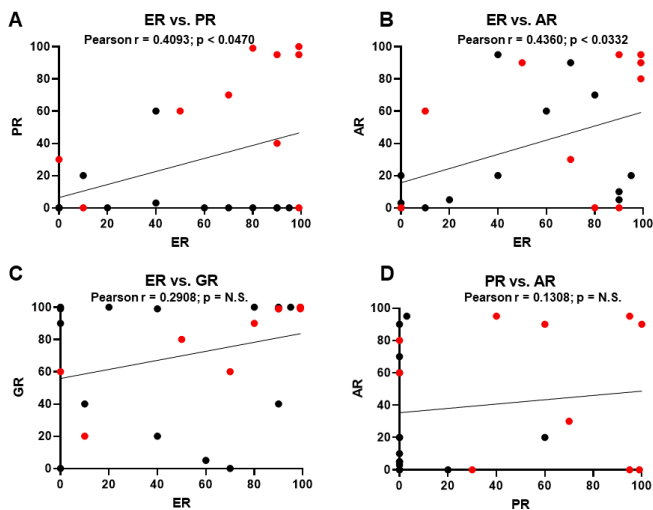


Figure 1. Steroid hormone receptor expression in biopsies of metastatic breast cancer with wild type or mutant ESR1. A. Formalin fixed paraffin embedded (FFPE) sections of core needle biopsies (N=18 WT ER, N=12 mutant ER) were stained by IHC for ER, PR, AR, and GR. Depicted are the mean scores (intensity x percent cells staining) ± SEM. Mann-Whitney tests were performed for each receptor stained. **B.** Representative images for all WT ER metastases (left) and all mutant ER metastases (right) stained for ER, PR, and AR and GR are shown at 400X. Ki67 or cleaved caspase 3 were not different between the two groups.

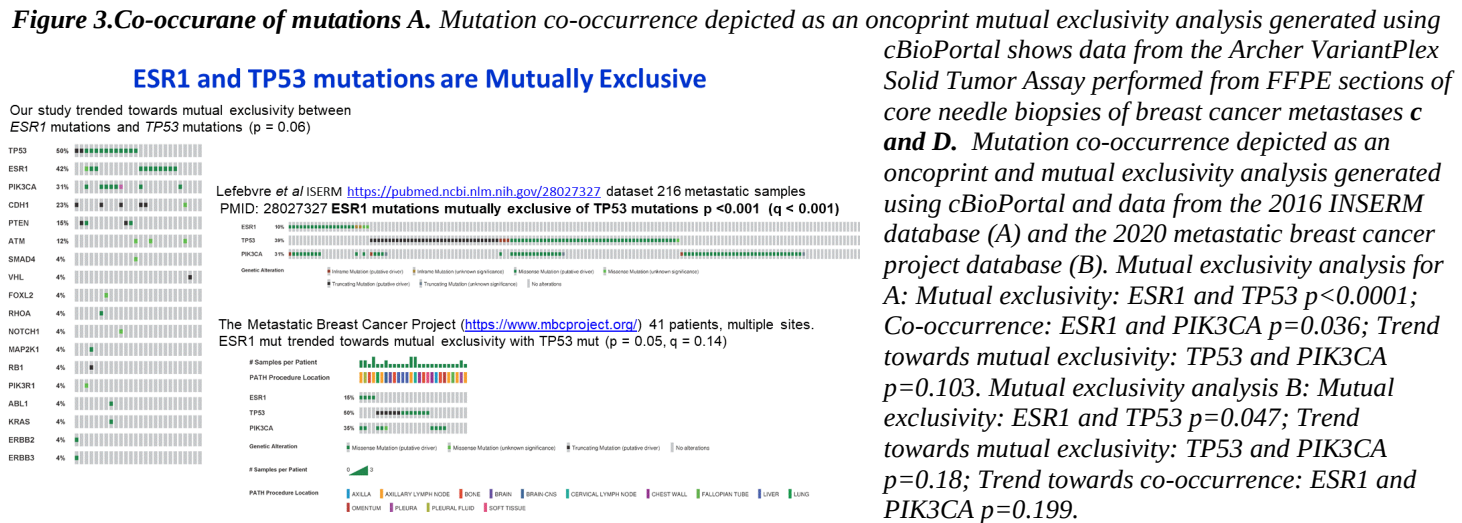


When looking at correlations between the levels of ER with PR and AR protein in the pretreatment biopsies, while both PR and AR significantly correlate with ER (Fig 2 A and B), it is obvious that the ESR1 mutant biopsies (Red) are high for both ER and PR, while the WT (black) have often completely lost PR (Fig 2A). In contrast, both ESR1 Mut biopsies (red) and WT (black) often retain some level of AR expression (Fig 2B) indicating that targeting AR may help in both cases. In contrast, ER and GR did not correlate, nor did AR and PR, although it is interesting to note that both ESR1 mut and WT that have lost PR often retain some degree of AR expression.

Figure 2. Immunohistochemical analysis of hormone receptor status of metastatic breast cancer specimens in Figure1 were correlated

for ER versus PR (A), ER versus AR (B), ER versus GR (C), and PR versus AR (D) using percent positive cells for each steroid hormone receptor (N=14 ER WT, N=10 with ER mutation), Pearson correlation (r); p-value represents significance of non-linear slope after simple linear regression.

The assay also detects mutations in 60 other genes that commonly occur in solid tumors. So far we have detected mutations in ATM, CDH1, FOXL2, MAP2K1, Notch1, PIK3CA, PTEN, RB1, RhoA, SMAD4, VHL and TP53, with the latter being the most common. We find that in our study and two other data bases ESR1 mutations are significantly mutually exclusive with TP53 mutations.



Graphs of the IHC scores for the four hormone receptors, Ki67, and Cleaved Caspase 3 in the archival, baseline (pre-treatment) and post-treatment (week 5) shows these results for all patients to date averaged (**Figure 4**).

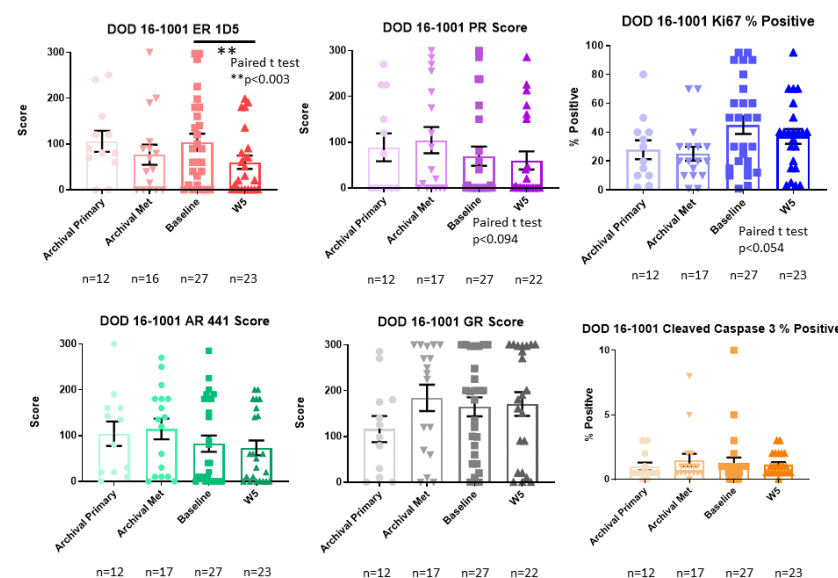
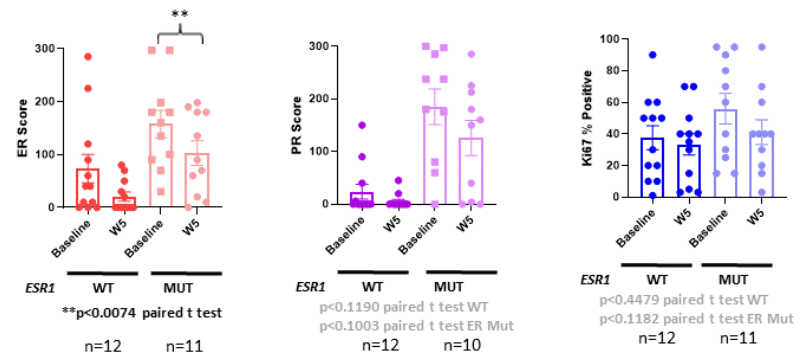


Figure 4. All patients for whom we have archival, baseline (pretreatment0 and week five of treatment biopsies stained for steroid hormone receptors ER, AR, PR GR and Ki67 for proliferation and Cleaved Caspase 3 (CC3) for apoptosis. So far none show significant change with treatment when averaged.

Interestingly, if you breakdown the change in ER expression by the presence or absence of ESR1 mutations, the significant decrease in ER protein with the Fulvestrant plus Enzalutamide treatment is only found in the ESR1 Mut biopsies and not in the WT when performing a paired t-test between the baseline pretreatment disease and the post-treatment week 5 biopsy. While the change in PR or Ki67 did not quite reach significance, they were closer to being significant in the ESR1 Mut than WT.



The finding that only the ESR1 Mut biopsies respond to the Fulv plus Enz is likely because they have so much more of the target (ER protein), but this finding is very impactful, because patients could be screened for ESR1 mutations in their circulating tumor cells and perhaps if they have them benefit more from fulvestrant than if they are not present.

We next stained the biopsies of metastases pre and post treatment to examine tumor infiltrating lymphocytes (TIL) by multiplex staining for T helper cells, T regulatory cells, cytotoxic T cells, macrophages and B cells. While TNBC is known to have a higher overall number of TIL than ER+, we find here that the tumors that harbor ESR1 mutations have a significantly higher number of FOXP3 positive T helper cells, macrophages and B cells (**Figure 5**). Results show that while these ER+ tumors have very low amounts of T cells, both ER mutants have more T regulator cells and T helper cells to begin with, but these do not change with treatment. On the other hand the macrophages are higher in the biopsies of ER mutants than ER WT. The macrophages increased more with treatment in the ESR1 mutants such that post-treatment the two groups were also significantly different. This could indicate that M2 immune suppressive macrophages are more present in tumors with ESR1 mutations.

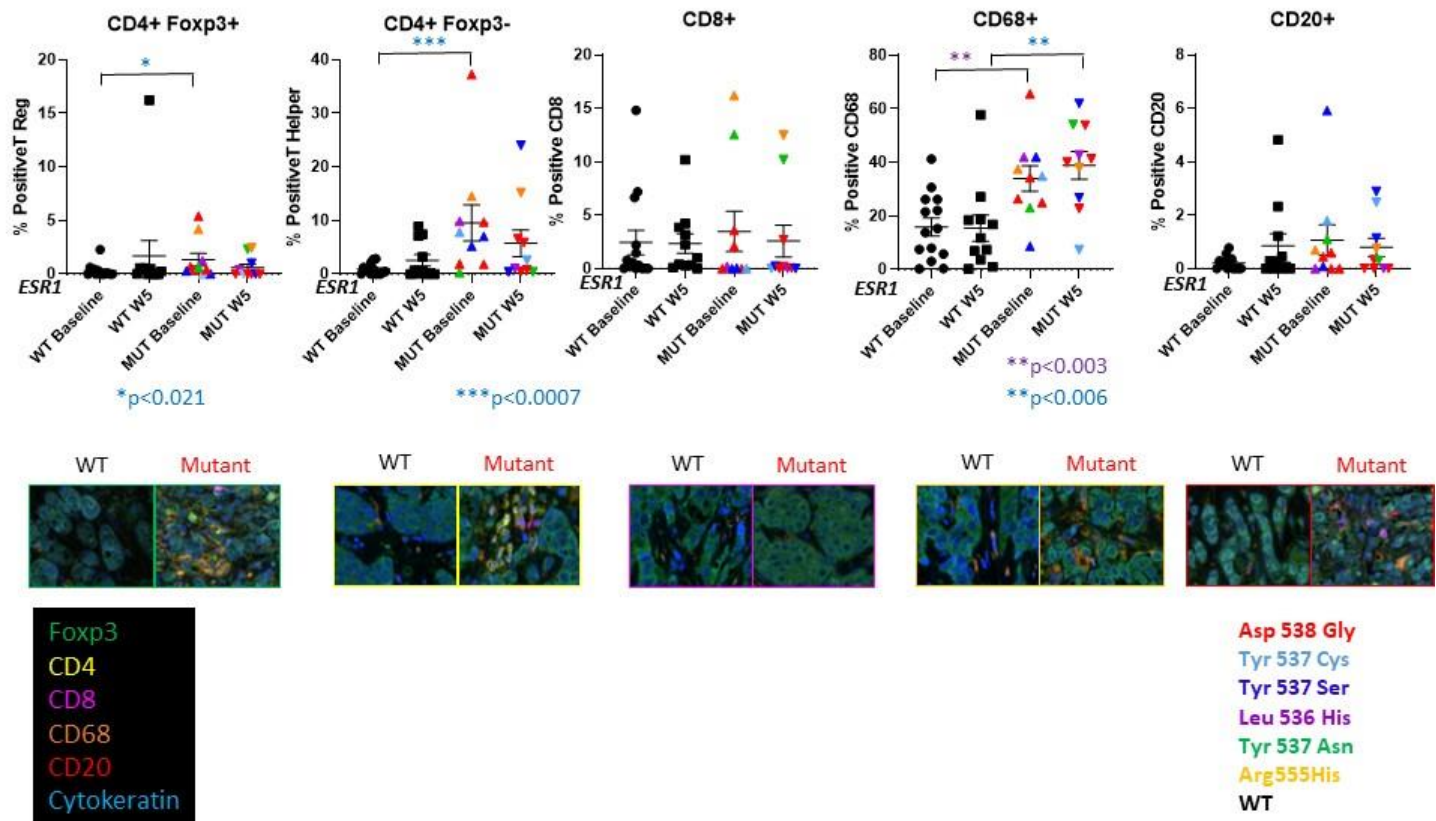


Figure 5. Tumor infiltrating lymphocytes were stained by multiplex. Patients with metastatic breast cancer (n=14 ER WT, n=10 ER mutation) were treated with fulvestrant and enzalutamide for 5 weeks. Baseline and week 5 biopsies were taken at the metastatic site and stained for CD4, Foxp3, CD8, CD68, CD20 and cytokeratin using Opal™ TSA technology (Akoya Biosciences).

Slides were scanned using a Vectra 3 Automated Quantitative Pathology Imaging System (Perkin Elmer), and 3 to 5 representative fields/tumor were analyzed for percent positive cells. Error Bars = Mean with SEM, Mann-Whitney test.

To determine if there was more PD-L1 on these macrophages we performed a dual staining for CD68 and PD-L1 (Fig 6). We found that indeed PD-L1 positive macrophages are higher pre-treatment in biopsies with *ESR1* mutations. This indicates that these tumors might respond to PD-L1+ checkpoint inhibitor therapy. Interestingly, PD-L1 increases even more with

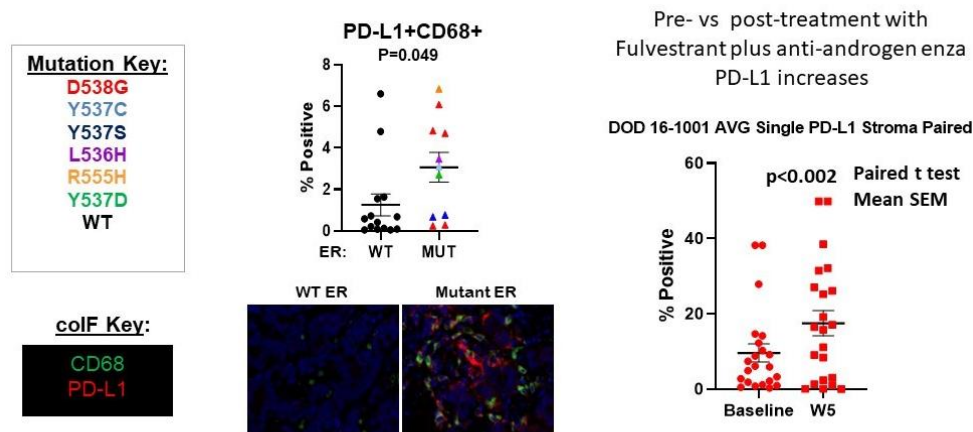


Figure 6. CD4+ T cells and PD-L1+ macrophages were significantly higher in biopsies of metastatic breast cancer harboring mutant ER as compared to WT ER. Biopsies of the same metastases characterized in Figure 1 (N=14 with ER WT, N=10 with mutant ER) were stained for tumor infiltrating immune cells using Opal™ TSA technology (Akoya Biosciences) with the following colors indicating respective immune cell markers: CD4 (yellow), Foxp3 (green), CD8 (magenta), CD68 (orange), CD20 (red), and cytokeratin (cyan). Slides were scanned using Vectra 3 Automated Quantitative Pathology Imaging

System (Perkin Elmer) technology and 3 to 5 representative 20x fields/tumor were analyzed using inform software (Perkin Elmer) and a pixel-based algorithm for percent positivity, mean percent positive cells $\pm$ SEM, Mann-Whitney test (top); representative images (20x, bottom) for: **A.** T regulatory cells (CD4+ FoxP3+), T helper cells (CD4+ FoxP3-), and cytotoxic T cells (CD8+), **B.** B cells (CD20+), and **C.** macrophages (CD68+). **D.** The same biopsies were stained for PD-L1 and CD68 using Opal™ TSA technology (Akoya Biosciences). Slides were scanned using a Vectra 3 Automated Quantitative Pathology Imaging System (Perkin Elmer), and up to 5 representative fields/tumor were analyzed for either total PD-L1 or dual expression of PD-L1 and CD68 using a cell phenotype-based algorithm for percent positive cells, mean positive cells $\pm$ SEM, Mann-Whitney test.

The next set of data pertains to the **neoadjuvant trial (16-1042)** in which patients are treated with fulvestrant with or without enzalutamide. Figure 6 depicts staining for the 4 steroid hormone receptors as well as Ki67 and Cleaved Caspase 3 in the first patients in the neoadjuvant trial. It is evident that ER, PR and Ki67 proteins are significantly decreased between the baseline pretreatment biopsy and the week 5 biopsy by t-test. We are blinded to which patients received fulvestrant only versus fulvestrant plus enzalutamide, so we have not divided the patient samples by treatment at this time, but this will be interesting!

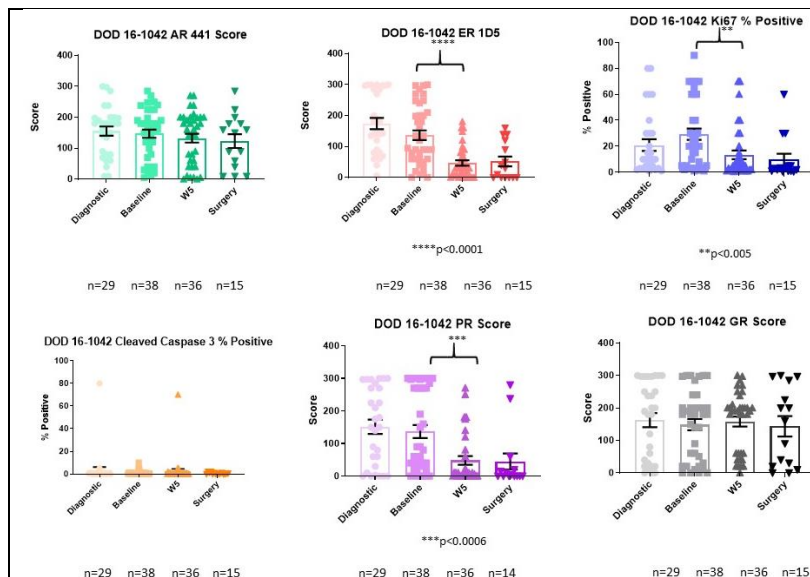


Figure 6. IHC for AR, ER, Ki67, Cleaved Caspase 3, PR, and GR in tumor biopsy and surgical resection sections of tumors from patients in the neoadjuvant trial 16-1042 treated with Fulvestrant only or Fulvestrant plus Enzalutamide. Staining score (intensity x percent cells staining) in the diagnostic core needle biopsy, baseline (pre-treatment), week 5, and at time of surgery. Statistical analysis (SEM, student's t-test) was performed between the baseline (pre-treatment) tumors and post-treatment at week 5. We will do ANOVA at a later date when we have all the IHC done from all enrolled patients as well.

What opportunities for training and professional development has the project provided?

The main graduate student that worked on this project was Valerie Barton who then obtained an NIH NRSA on work that stemmed from this grant. She defended her dissertation in April 2016 and obtained her doctorate from the University of Colorado Cancer Biology Graduate Program.

Postdoctoral fellows Drs. Nicholas D'Amato and Michael Gordon also completed their tenures in the lab and have taken positions at AstraZeneca and Abbvie respectively.

Professional research assistant Nicole Spoelstra has been integral to this project and performed all of the IHC on the clinical specimens she passes them to pathologist Sharon Sams to read in a blinded fashion. Nicole Spoelstra also performed the TSA multiplex staining for the tumor infiltrating lymphocytes and learned this technique at a week long workshop in Boston when it first started.

Postdoctoral fellow Michelle M Williams has been working on preclinical aspects of AR in the ESR1 mutant ER+ disease and is preparing the manuscript on that topic now. She is supported on our Cancer Biology T32 and obtained an F32 NCI Fellowship on a different topic.

How were the results disseminated to communities of interest?

During the last reporting period Dr. Richer gave the following lectures at national meetings. However, no grant funds were used to support the travel.

- April 2018 **AACR** Invited “Meet the Expert” session “Update on Potential for Targeting Androgen Receptors in Breast Cancer.”
- Aug 2018 **Endocrine Society of Australia** Two invited symposium lectures on the role of androgen receptors in breast cancer. Adelaide, AU
- Aug 2019 **Gordon Research Conference Hormones and Cancer**, Sunday River, Maine, “Hormone Deprivation Influences Breast Cancer Immune Suppression

Invited lectures:

- Aug 2018 **The Westmead Institute for Medical Research, Sydney, AU** “Targeting AR in BC resistant to anti-estrogen therapy and TNBC”
- Aug 2018 **University of Adelaide, Adelaide AU Cancer Biology and Reproductive Sciences** "Triple Negative Breast Cancer Hijacks a Trophoblast-Like Program of Immune Suppression"

Local lectures at the University of Colorado:

- 2019 Feb 22 Pathology Grand Rounds “Androgen Receptors in Breast Cancer-what have we learned?”
- 2019 April Endocrine Division Research Conference- “Estrogen Receptor Mutations in “Castrate Resistant Breast Cancer” – a role for androgen receptors?”

Dr. Richer gave the following presentations in 2019-2020

- April 2019 International Association for Breast Cancer Research Conference Egmond aan Zee, The Netherlands “*Clinical biopsies and preclinical models reveal new therapeutic targets in estrogen receptor mutant metastatic breast cancer*”
- Aug 2019 Gordon Research Conference Hormones and Cancer, Sunday River, Maine, “Hormone Deprivation Influences Breast Cancer Immune Suppression”
- Feb 2019 Pathology Grand Rounds “Androgen Receptors in Breast Cancer-what have we learned?”
- April 2019 Endocrine Division Research Conference- “Estrogen Receptor Mutations in “Castrate Resistant Breast Cancer” – a role for androgen receptors?”
- Feb 2020 Advances in Breast Cancer Updates from San Antonio- Horizon CME – Endocrine Resistance

▪ What do you plan to do during the next reporting period to accomplish the goals?

We will continue to analyze patient samples from the two clinical trials as they are completed this year. We will continue to learn more about basic mechanisms when we start to get results from the frozen sections of the biopsies from both clinical trials analyzed by RPPA. We are analyzing RPPA data now. We are also preparing FFPE sections for gene expression analyses.

4. IMPACT:

▪ **What was the impact on the development of the principal discipline(s) of the project?**

These studies are helping to determine the role of androgen receptors in breast cancer and whether new anti-androgens might be utilized as therapy for breast cancers that fail to respond or reoccur while women are on current therapies such as anti-estrogens, trastuzumab or chemotherapy. These studies have provided preclinical evidence that the anti-androgen enzalutamide could serve as the first effective targeted therapy for a subset of triple negative breast cancers (TNBC). TNBC is the most aggressive type of breast cancer and there is currently no effective treatment for TNBCs with de novo or acquired resistance to chemotherapy. Our studies regarding timing (concurrent enzalutamide treatment with chemotherapy and enzalutamide versus sequential) provided valuable information for upcoming clinical trials- in fact, a trial like this is underway at MD Anderson for TNBC. Our studies on the ESR1 mutations in metastatic ER+ breast cancer will also inform clinical trial design. Already it is evident that if the receptor does not mutate to become constitutively active in metastatic disease in the absence of ligand (most all patients were on aromatase inhibitors prior to going on this trial), the tumors have likely survived the estrogen deprivation by losing the hormone receptors completely and may no longer be candidates for fulvestrant. Indeed the treatment decreased Ki67 significantly only in the ESR1 mutant biopsies. The other important finding is that the amount of PD-L1 staining increases with treatment significantly, which could mean that anti-PD-L1 checkpoint inhibitor therapy may work in ER+ metastatic disease following a new endocrine therapy approach. This type of therapy is only currently FDA approved in TNBC.

▪ **What was the impact on other disciplines?** Our studies of how steroid hormone receptors affect each other is pertinent to other cancers and development.

▪ **What was the impact on technology transfer?**

- Transfer of results to entities in government or industry: The results of this project are also reported to our clinical industry partners Medivation Inc and Astellas Pharma who are running the clinical trials of enzalutamide in prostate and breast cancer. They are very interested in our preclinical results combining enzalutamide with other therapeutics currently being utilized in breast cancer since these results will guide the design of further industry or investigator initiated clinical trials. We filed a patent on the idea of looking at the AR to ER ratio in breast cancer and the company Ventana signed an agreement to pay the filing fees in Europe and to contract some additional sponsored research to design a clinical test to examine the ratio of these two receptors using their antibodies potentially simultaneously on the same section of tumor. We may file a patent on the technology of staining for all of the hormone receptors on the same section and also on the results of the staining for all receptors in the mutant versus non-mutant disease.

▪ **What was the impact on society beyond science and technology?**

- Since we have given reports of our research to several lay audiences in various community settings, we believe we are improving public knowledge regarding how hormones typically thought of as male hormones (such as androgens) are made by women and do affect women's health.

5. CHANGES/PROBLEMS: Nothing to Report

Changes in approach and reasons for change

- Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.
-
- **Actual or anticipated problems or delays and actions or plans to resolve them**

We found that AR protein expression in TNBC PDX models was decreasing even after two passages. However, we have now found that with androgen treatment, AR is re-expressed. We also recently found that ER+ PDX recapitulate the clinical samples in that those with ESR1 mutations express higher AR.

- **Changes that had a significant impact on expenditures.** Nothing to report.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents** No changes
Significant changes in use or care of human subjects. None
- **Significant changes in use or care of vertebrate animals. None**
- **Significant changes in use of biohazards and/or select agents. None**

6. PRODUCTS:

Journal publications. Although we did not publish any new manuscripts this year, we have 3 that we are currently working on that are listed below in red.

- Cochrane DR, Bernales S, Jacobsen BM., Cittelly DM, Howe EN, D'Amato NC, Spoelstra NS, Jean A, Jedlicka P, Torkko KC, Protter A, Elias AD and **JK Richer**. Role of the Androgen Receptor in Breast Cancer and Preclinical Analysis of Enzalutamide. BREAST CANCER RESEARCH 2014 Jan 22;16(1). PMID: 24451109 D'Amato NC, Jacobsen BM, Gordon MA, Babbs BL, Spoelstra NS, Carson Butterfield KT, Barton VN, Rogers TJ, Sartorius CA, Elias AD, Gertz, J and **JK Richer**. Cooperative Dynamics of AR and ER Activity in Breast Cancer. MOLECULAR CANCER RESEARCH 2016 Nov;14(11):1054-1067. PMID: 27565181
- Barton VN, D'Amato NC, Gordon MA, Lind HT, Spoelstra NS, Babbs B, Heinz RE, Elias A, Jedlicka P, Jacobsen BM and **JK Richer**. Multiple molecular subtypes of triple negative breast cancer critically rely on androgen receptor and respond to Enzalutamide *in vivo*. MOL CANCER THER. 2015 Mar: 14(3):769-78 PMID: 25713333* Top most highly cited for this journal in 2015.
- Christenson JL, Butterfield KT, Spoelstra NS, Norris JD, Josan JS, Pollock JA, McDonnell DP, Katzenellenbogen BS, Katzenellenbogen JA, and **JK Richer**. MMTV-PyMT and Derived Met-1 Mouse Mammary Tumor Cells as Models for Studying the Role of the Androgen Receptor in Triple-Negative Breast Cancer Progression. HORMONES AND CANCER. 2017 Apr;8(2):69-77.PMID: 28194662
- Gordon MA, D'Amato NC, Gu H, Babbs B, Wulfkühle JD, Petricoin EF, Gallagher RI, Dong T, Torkko KC, Liu B, Elias A and **JK Richer**. Synergy between androgen receptor antagonism and inhibition of mTOR and HER2 in breast cancer. MOLECULAR CANCER THERAPEUTICS. 2017 Jul;16(7):1389-1400. PMID: 28468774
- Barton VN., Christenson JL, Rogers TJ, Butterfield K, Babbs B, Spoelstra NS, D'Amato NC, Elias A, and **JK Richer**. Androgen receptor supports an anchorage independent, cancer stem cell like population in triple negative breast cancer. CANCER RESEARCH. 2017 Jul 1;77(13):3455-3466. PMID: 28512248

These papers are under review and are included in the appendix as merged files.

Williams MM, Michelle Williams, Nicole Spoelstra, Jordan Reese, Jessica Christenson, Kathleen O'Neill, Kathleen Torkko, Toru Hanamura, Britta Jacobsen Sharon Sams, Anthony Elias, Jason Gertz, and Jennifer Richer. Adaptations of ESR1 mutant metastatic breast cancers in the estrogen-depleted environment include increased androgen receptor activity and tumor infiltrating lymphocytes. In preparation. Will submit within two months.

Christenson JL, Kathleen O'Neill, Kathleen Torkko, Toru Hanamura, and **JK Richer**. Androgen Receptor positive triple negative breast cancer responds to CDK4 inhibition. In preparation.

Rosas, M., Christenson, JL, Spoelstra, NS, Hanamura T and JK Richer. Crosstalk between the TGFbeta pathway and androgen receptors in triple negative breast cancer. In preparation

Reviews:

- Barton VN, Gordon MA, Christenson J, D'Amato N, and **JK Richer**. Androgen receptor biology in triple negative breast cancer: A case for AR+ and quadruple negative disease subtypes. *HORMONES AND CANCER* 2015 Dec 6(5-6):206-13. PMID: 26201402
- Barton VN, Gordon MA, **Richer JK**, Elias A. Anti-androgen therapy in triple-negative breast cancer. *Ther Adv Med Oncol*. 2016 Jul;8(4):305-8. PMID: 27482289
- Gordon MA, Harrison B, **Richer JK**, Elias A. Anti-androgen therapy in breast cancer. *American Journal of Hematology/Oncology*. 2016.
- Christenson JL, Trepel JB, Ali HY, Lee S, Eisner JR, Baskin-Bey ES, Elias AD, **Richer JK**. Harnessing a Different Dependency: How to Identify and Target Androgen Receptor-Positive Versus Quadruple-Negative Breast Cancer. *HORMONES AND CANCER*. 2018 Apr;9(2):82-94. doi: 10.1007/s12672-017-0314-5. Epub 2018 Jan 16. Review. PMID: 29340907.

- **Books or other non-periodical, one-time publications.** Nothing to report.
- **Other publications, conference papers, and presentations.**

Dr Richer gave the following lectures:

- Oct 2017 **Breast Cancer Research Foundation** Think Tank for Androgen Receptor in Breast Cancer
- Dec 2017 **San Antonio Breast Cancer Symposium**. Invited Educational Session presentation “Androgen Receptors in Breast Cancer” Symposium on Androgen, Progesterone and Glucocorticoid Receptors: Reprogramming of Steroid Receptors during Breast Tumor Progression.
- April 2018 **AACR** Invited “Meet the Expert” session “Update on Potential for Targeting Androgen Receptors in Breast Cancer.”
- Aug 2018 **Endocrine Society of Australia** Two invited symposium lectures on the role of androgen receptors in breast cancer. Adelaide, AU
- Aug 2019 **Gordon Research Conference Hormones and Cancer**, Sunday River, Maine, “Hormone Deprivation Influences Breast Cancer Immune Suppression

- **Website(s) or other Internet site(s):**

AACR/SABC video of 2017 December meeting lecture in the educational section by Dr. Jennifer Richer- video

- **Technologies or techniques.** None
- **Inventions, patent applications, and/or licenses**

Richer *et. al.*, PCT Patent Application WO 2014/031164 filed March 15, 2013, “Methods for Determining Breast Cancer Treatment.” U.S. Patent Application No. 14/423,133, filed February 22, 2015 Issued in European Countries [European Patent 28888594](#), published August 15, 2018 and in the US allowed by the United States Patent and Trademark Office on August 27, 2018.

Protter and JK Richer, PCT Patent Application PCT/US2012/48471 Serial No. 14/236,036 filed on January 29, 2014 “Treatment of Breast Cancer.”

- **Other Products**

data or databases- we now have databases of genes expression data from the following experiments.

ER+ MCF7 breast cancer cells treated in vitro with vehicle, enzalutamide alone, estradiol alone (E2), E2 plus enzalutamide for 48 hrs.

ER+ MCF7 breast cancer cells grown as xenografts in nude mice treated with E2, E2 plus tamoxifen, or E2 plus enzalutamide.

HCC1806 TNBC breast cancer line treated in vitro with either vehicle, DHT, enzalutamide alone, DHT plus enzalutamide.

SUM159 treated in vivo.

MDA-453 treated with CDK4/6 inhibitor

TNBC PDX HCI-009 treated in vivo with or without DHT and gene changes list from RNAseq

- **biospecimen collections;**

Formalin fixed paraffin embedded xenograft tumors from the following experiments:

MCF7 tumors grown in nude mice and treated with either E2, E2 plus tamoxifen, E2 plus enzalutamide or in a separate experiment, the same treatments plus the combination of E2 plus enzalutamide and tamoxifen.

Triple negative breast cancer (TNBC) cell line SUM159PT grown as xenograft tumors in mice treated with control rodent chow or enzalutamide containing chow.

TNBC cell line HCC1806 grown as xenograft tumors in mice treated with control rodent chow or enzalutamide containing chow.

TNBC PDX-009 tumors grown in mice with or without DHT and gene expression change RNA-seq data

- research material (e.g., Germplasm; cell lines, DNA probes, animal models); We have generated luciferase labelled breast cancer cell lines to image by IVIS and put nuclear red and green expression vectors in these lines to utilize the Incucyte machine to count the number of red or green nuclei to do real time proliferation assays with enzalutamide alone or in combination with standard therapies for breast cancer.
- Biopsies (frozen and FFPE) cores obtained from the clinical trial are stored with our Pathology Biobank.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Dr. Richer had no more salary support for this project in the last reporting period. Dr. Richer is helping to pay Dr. Richer's technician Nicole Spoelstra, who does all of the immunostaining of biopsies from the clinical aims.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** No changes in active support for the PD/PI(s) or senior/key personnel.
- **What other organizations were involved as partners?** *Nothing to Report*

SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** Partnering PI, Dr. Anthony Elias has sent a separate report on the clinical progress.
- **APPENDICES:** The Appendix contains three new manuscripts on AR in breast cancer from this project that are currently under review. The first contains some of the pretreatment data from the clinical trial. Another manuscript (not shown) is in preparation that contains the pre versus post-treatment analyses for the completed metastatic trial.

Therapeutic vulnerabilities of *ESR1* mutant breast cancer revealed by steroid hormone receptor and infiltrating immune cell status.

Michelle M. Williams¹, Nicole S. Spoelstra¹, Spencer Arnesen², Kathleen I. O'Neill¹, Jessica L. Christenson¹, Jordan Reese¹, Kathleen C. Torkko¹, Andrew Goodspeed³, Emmanuel Rosas¹, Toru Hanamura¹, Sharon B. Sams¹, Zheqi Li^{4,5}, Steffi Oesterreich^{4,5}, Rebecca B. Riggins⁶, Britta M. Jacobsen¹, Anthony Elias⁷, Jason Gertz², and Jennifer K. Richer¹

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Running title: *ESR1* mutant breast cancer therapeutic vulnerabilities

Key words: breast cancer, steroid hormone receptor, androgen receptor, estrogen receptor, *ESR1* mutant, type-1 interferon, CHI3L1, IFITM3, IFITM1

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Disclosure of potential conflicts of interest: none

Word count: 4,762 (+1,637 Methods); Seven main figures, one table, and ten supplementary figures

Abstract (225 of 250 words)

Mutations in *ESR1* that confer constitutive estrogen receptor alpha (ER) activity in the absence of ligand are acquired by $\geq 40\%$ of metastatic breast cancers (MBC) resistant to adjuvant aromatase inhibitor (AI) therapy. To identifying targetable vulnerabilities in MBC, we examined biopsies of metastases with or without ER mutations for steroid hormone receptors and tumor infiltrating immune cells. We observed significantly lower expression of ER and progesterone receptor (PR) in metastases with wild type (WT) ER compared to those with mutant ER. This finding suggests that metastases that evade AI therapy by mechanism(s) other than acquiring ER mutations may lose dependency on ER and PR. Metastases with mutant ER also had significantly more T regulatory, T helper cells, total macrophages, and PD-L1 positive immunosuppressive macrophages than those with WT ER. Using BC cells with CRISPR Cas9-edited ER (D538G, Y537S, or WT) and patient derived xenografts (PDX) harboring mutant or WT ER, we identified estrogen-independent genes/proteins elevated in mutant ER cells including androgen receptor (AR), Chitinase-3-Like Protein 1 (CHI3L1), and Interferon Stimulated Genes (ISGs). Targeting these proteins blunted the selective advantage of ER mutant tumor cells to survive estrogen deprivation, anchorage independence, and invasion. Thus, patients with mutant ER MBC might respond to standard of care fulvestrant or other selective estrogen receptor degrader (SERD) when combined with AR or CHI3L1 inhibitors, perhaps with the addition of immunotherapy.

Significance statement: Targetable estrogen-independent alterations in MBC, including AR, CHI3L1, and ISGs arise following estrogen-deprivation, and ER mutant metastases may respond to immunotherapies due to elevated PD-L1+ macrophages.

Introduction

Two-thirds of all early (non-metastatic) primary breast cancers (BC) express estrogen receptor alpha (ER). First line adjuvant treatment for the majority of patients with ER+ disease is aromatase inhibitor (AI) therapy, which blocks conversion of androgens to estrogens by the enzyme aromatase and significantly extends time to recurrence (reviewed in (1)). However, all patients who recur will eventually develop AI resistant metastatic breast cancer (MBC) and acquired point mutations in the *ESR1* gene often emerge under the selective pressure of this estrogen deprivation therapy. While other known mechanisms of endocrine therapy resistance exist, including amplification and fusions of *ESR1* (2), point mutations in exon eight encoding the ER ligand binding domain (LBD) are the most prevalent. Indeed, ER mutations are detectable in circulating tumor cells or metastatic lesions in up to 40% of women with AI resistant MBC (reviewed in (3)). Specific mutations in this region result in amino acid alterations that confer constitutive receptor activity in the absence of estrogen (4-10) and consequent estrogen-independent gene regulation (11-16). In preclinical models, BC cells with mutant ER exhibit increased proliferation *in vitro* (13,14) and *in vivo* (11,12) compared to cells with wild type (WT) ER and have a greater propensity to metastasize with or without 17beta-estradiol (E2) (11,12).

Despite ER being constitutively active in the absence of E2, BC cells harboring mutant ER retain sensitivity to E2. Fulvestrant (Fulv), the selective ER degrader that serves as the current second line therapy for AI resistant disease, binds to mutant ER at lower affinity than to WT ER (reviewed in (17)). The poor bioavailability of Fulv necessitates intramuscular injections and higher doses are problematic. Hence, combination strategies to lower the effective dose of Fulv or alternative therapies are desirable. Cyclin dependent kinase-4/6 (CDK 4/6) inhibitors, such as Palbociclib (Palbo), improve survival for patients with ER+ MBC and Palbo+Fulv slightly improved survival in patients harboring mutant ER when compared to Fulv alone; however, CDK 4/6 inhibitors are not without side effects (18). Other therapies being tested include combination with Fulv include the CDK7 inhibitor THZ1 that showed enhanced efficacy in preclinical models (12) and second-generation selective ER degraders (SERDs),

such as AZD9496 (19). However, there is clearly a need for novel therapeutic approaches for patients with AI refractory ER+ MBC.

Although both WT and mutant ER arise from ER+ BC that escapes adjuvant AI therapy, it is possible that due to differing biological properties, alternative therapeutic strategies may be required to treat these two forms of AI refractory MBC (11,20,21). In the companion manuscript, Arnesen *et al* molecularly characterize WT versus mutant ER BC cells, studying the most commonly occurring D538G and Y537S mutants associated with poor patient outcomes (4-7,22). Interestingly, concurrent analyses of new data from Arnesen *et al* and two other studies (12,13) indicated that many genes differentially expressed by mutant ER BC cells compared to WT are not E2 regulated. These results suggested that the selective advantages conferred by mutant ER are not solely attributable to constitutive ER activity. Since ER mutations represent an acquired adaptation that facilitates BC survival and recurrence as metastatic disease in women on AI therapy, we postulated that the selective advantages conferred by mutant ER might become more evident by modeling the clinical situation in which the mutations arise (E2-deprivation). Therefore, we examined specific proteins from mutant-enhanced pathways identified by genomics in Arnesen *et al*, under conditions that mimic the altered hormonal milieu that ensues upon AI therapy and anchorage independence, to simulate the selective pressure of treatment and early metastasis. This analysis led to identification of innate immune cell activating signatures that were confirmed by multiplex analysis of tumor infiltrating immune cells in MBC specimens. We also hypothesized that the long-term E2-deprivation induced by AI therapy blocking conversion of androgens to estrogens might cause tumor cells to shift to reliance on androgens/AR. The complement of steroid hormone receptor proteins including ER, progesterone receptor (PR), androgen receptor (AR), and glucocorticoid receptor (GR), had not previously been examined together in MBC with or without *ESR1* mutations. Our approach combining data from preclinical models and biopsies of MBC, identified proteins that confer survival advantages relevant to AI refractory MBC that may serve as vulnerabilities and lead to new therapeutic approaches for this fatal form of the disease.

Materials and Methods

Metastatic Breast Cancer Patient Samples

Mutation analysis. Core needle biopsies were acquired from consented patients with ER+/Her2- measurable or evaluable metastatic breast cancer (MBC) without CNS disease enrolled in clinical trial NCT02953860 (COMIRB 16-1001). Median age of patients was 61 years (46-87); PS 1 (0-1); a median of 2 prior chemotherapy and 2 prior hormonal therapies for metastatic disease (including 7 with prior fulvestrant), and 90% had visceral disease. An additional three biopsies were from NCT01597193 (COMIRB 12-0970). Formalin fixed paraffin embedded sections were analyzed for mutations in *ESR1* exon 8 as well as 67 other gene hotspots frequently altered in cancer using a modified Archer VariantPlex Solid Tumor Assay through the CMOCO Laboratory (University of Colorado Department of Pathology). Mutation mutual exclusivity analyses were performed for commonly occurring mutations using cBioPortal for our data set and data from the 2016 INSERM database and the 2020 Metastatic Breast Cancer Project database. The majority (67%) of biopsies were from the liver. The other sites included skin (3.7%) and the rest equally divided across lymph node, other soft tissue, breast and bone (at 7.4% each). Invasive ductal carcinomas represented 63% of the biopsies, invasive lobular carcinoma, 18.5%, and invasive mammary carcinoma (IMC) 14.8% and 3.7% unknown.

Immunohistochemistry (IHC). IHC to detect ER (Dako 1D5), PR (Dako 1294), AR (Dako 441), GR (Cell Signaling #3660), Ki67 (Dako M7240), cleaved caspase 3 (Cell Signaling #9661), and IFITM3 (GeneTex GTX115407) was performed on FFPE sections of the MBC biopsies described above. Antigen retrieval and detection were optimized for each antibody. IHC stained slides were scored for intensity and percent cells positive by breast pathologist Sharon B Sams, MD, who was blinded to ER mutation status. Pearson correlation values were calculated between the different receptors in MBC biopsies using percent positive cells.

Multiplex panel for tumor infiltrating immune cells. FFPE sections were stained for immune markers using multiplex Opal™ TSA technology (Akoya Biosciences) along with the Vectra 3 Automated Quantitative Pathology Imaging System. TIL antibodies used were: CD4 (Dako 4B12), Foxp3 (Abcam 236A/E7), CD8 (Dako C8/144B), CD20

(Abcam L26), and CD68 (Dako KP1), and pan cytokeratin (Dako AE1/AE3) was used to identify tumor epithelium. PD-L1/CD68 Co-IF antibodies used were: PD-L1 (Abcam #ab228462) and CD68 (Dako KP1). Dapi (Akoya) was used as a counterstain for each core needle biopsy, and positive cells in three to five 669um x 500um fields were scored using InForm software (Perkin Elmer) using either a pixel or cell-based algorithm including both tissue and cell segmentation.

Cell Culture

Cell culture conditions. BC cell lines (MCF-7 and T-47D) were obtained from Dr. Kathryn Horwitz at the University of Colorado Anschutz Medical Campus. Cell lines were authenticated by Short Tandem Repeat DNA Profiling (Promega) and tested for mycoplasma in the University of Colorado Cancer Center (UCCC) Cell Technologies shared resource. WT and mutant ER CRISPR cell lines were created by Dr. Jay Gertz at the Huntsman Cancer Institute using CETCH-seq to endogenously flag-tag WT or mutant (Y537S or D538G) ER using Cas9 mediated homologous recombination (see companion paper and (23)). MCF-7 cells were maintained in MEM with 5% FBS, 1% non-essential amino acids, 6.0 µg/mL insulin, and 1% penicillin/streptomycin. T-47D cells were cultured in RPMI-1640 media supplemented with 5% FBS and 1% penicillin/streptomycin. To mimic conditions resulting from AI therapy, where the ER mutations arise in patients, cells were also cultured in phenol red-free media (MEM for MCF-7 and RPMI-1640 for T-47D) supplemented with 5% dextran charcoal (DCC) stripped FBS for 6 months to generate long-term estrogen-deprived (LTED) cells.

Immunoblotting. Cells were grown in full media under attached or forced-suspension conditions (on poly 2-hydroxyethyl methacrylate, poly-HEMA, plates) for 24, 48, or 72 hours prior to harvest. Cells were then lysed in RIPA buffer (150 nM NaCl, 1% IPEGAL, 0.5% Na-Deoxycholate, 0.1% SDS, 50mM Tris, and 1.0mM EDTA) containing protease and phosphatase inhibitors. Whole cell protein lysates were separated by 10-12% SDS-PAGE gels, transferred to PVDF membranes, blocked in 3% BSA in Tris-buffered saline-Tween (TBST), and incubated with antibody overnight at 4°C. Primary antibodies used include: AR (EMD Millipore PG-21), Flag (Sigma), ERalpha (Thermo SP-1), GR (Cell signaling D8H2), PR (Dako), Tubulin (Sigma), IFITM3 (GeneTex), and GAPDH

(Sigma). Incubation in secondary antibody was followed by detection using an Odyssey CLx Imager (Licor, Inc). Densitometry was performed using the ImageJ software and reported as a ratio normalized to respective loading control (tubulin or GAPDH).

Immunohistochemistry on BC cell lines and patient-derived xenografts. Cells were plated as described above and then were pelleted, fixed in 10% formalin, and paraffin-embedded by the University of Colorado Denver Tissue Biobanking and Processing Core. Slides were deparaffinized in xylene and ethanol and heat induced epitope retrieval was performed. Primary antibodies used were: AR (Cell Marque SP107), ER (Thermofisher SP1), PR (DAKO 1294), IFITM3 (GeneTex GTX115407), MUC1 (Cell Signaling 4538), and CHI3L1 (Abcam). Representative images were taken at 200x, 400x, or 1000x magnification. Quantification of nuclear AR was completed using the Aperio microscope and ImageScope, to calculate percent nuclei positive and staining intensities of 0, 1+, 2+, or 3+. Data represents the nuclear intensity of all cells in each cell pellet (N>100 cells/pellet). For CHI3L1, IFITM3, and MUC1, ImageJ software (NIH) was used to determine the average number of cells with 3+ staining in three representative images taken at 400x.

Soft Agar Colony Formation. Soft agar colony formation assays were used to assay for anchorage-independent survival in WT and mutant ER MCF-7 and T-47D cells treated with vehicle (ethanol), 20 μ M Seviteronel (Sevi), or 20 μ M Enzalutamide (Enza). Experiments were performed in 6-well plates containing the following layers: 0.5% bottom agar, 0.3% top agar containing 20,000cells/well, and covered with the appropriate media. Cells were grown for approximately 21 days with bi-weekly media changes then fixed and stained with nitro blue tetrazolium at the time of harvest. Experiments were performed in triplicate (N=3) and the average colony number was quantified using ImageJ software.

Invasion Assays. Cells were plated at 50,000 cells/well in 1% FBS on the top of a Matrigel-coated invasion chamber with 8.0 micron holes (Corning) and normal growth media (with 5% FBS) was used as a chemoattractant in the bottom of each well. The following day 10 μ g/mL anti-CHI3L1 blocking antibody or IgG control was added to the top of each well. 72 hours post-treatment, cells were fixed in 10% formalin and stained with 1% crystal violet. Cell invasion was determined by analyzing the average area of

crystal violet staining on the bottom of each insert using ImageJ software. Presented is the average area of two separate experiments (N=3/experiment).

Identification of mutant specific and AR regulated genes.

D538G and Y537S ER mutant-specific genes were identified by analyzing RNA-seq data from WT and mutant ER T-47D and MCF-7 cell lines generated by the Gertz lab (Arnesen et al, co-submitted), the Oesterreich lab [UPMC Hillman Cancer Center (13)], and the Brown lab [Dana Farber Cancer Institute groups (12)] using an adjusted p-value cutoff of <0.05. Pathway analysis of mutant-specific genes identified through the multivariate/multilab RNA-seq analysis (Arnesen *et al*) were analyzed using Illumina's BaseSpace Correlation Engine to identify pathways and molecular and cellular processes that are significantly associated with mutant-specific gene expression changes. Gene sets were ranked by log2 fold change and p-value. Gene ontology enrichment p-values were calculated by Illumina's BaseSpace Correlation Engine. A list of AR regulated genes was compiled from multiple sources including prostate and breast cancer, and cell lines derived from both types of cancer (24-26). This curated list of AR-regulated genes was overlapped with mutant-specific genes identified in our multi-lab RNA-seq analysis described above. Significant overlap between mutant specific genes and AR regulated genes was identified using a hypergeometric p-value cut off of <0.05.

Animal Studies

All animal experiments were performed in accordance with protocols approved by the University of Colorado Institutional Animal Care and Use Committee (IACUC) using humane procedures. 6 week old ovariectomized NOD *scid* gamma (NSG) mice (Jackson Laboratories) were implanted subcutaneously at base of neck with pellets containing cellulose or pellets containing 2mg 17beta-estradiol (E2) in cellulose (N=10/group). Mice were injected via the tail vein with T-47D^{GFP/Luc} cells containing either WT or D538G ER to generate 4 experimental groups of mice: cellulose pellets plus WT T47D cells, cellulose pellets plus D538G T-47D cells, E2 pellets plus WT T-47D cells, and E2 pellets plus D538G T-47D cells (N=5/group). Mice were imaged weekly by IVIS beginning the day after tumor cell injection. Six weeks post cell injection,

mice were imaged by IVIS, then were sacrificed, and excised lungs were imaged by IVIS ex vivo. Tumor burden at endpoint (6 weeks) is presented as total flux (photons/second) of luminescence from IVIS imaging. Patient derived xenografts (PDX) were kindly provided by Dr. Alana Welm, Huntsman Institute and Dr. Carol Sartorius, University of Colorado. PDX were passaged in cycling female NSG mice supplemented with E2 with the exception of HCI-013-EI tumors passaged in un-supplemented mice.

Statistical Analysis

Data are presented as the mean $\pm$ Standard Error of the Mean (SEM). When comparing two groups of data, an unpaired Student's t-test was used. When heteroscedasticity was present or data were skewed, the non-parametric Mann-Whitney test was used. For statistical analysis between more than two groups a One way ANOVA or Two way ANOVA with a Tukey's multiple comparison test was used. In the case of heteroscedasticity or skewed data, non-parametric Kruskal-Wallis or Friedman's tests were used. When data were presented on a log-scale (Figure 4) data analysis using parametric tests was conducted on log transformed data to account for heteroscedasticity between groups. Statistical significance was defined as $p < 0.05$ and all tests were two-sided. Analyses were done using GraphPad Prism (ver 8.3, GraphPad Software, La Jolla, CA). Power calculations for the animal study were conducted under the supervision of a collaborating biostatistician Dr. Kathleen Torkko. Animal numbers were calculated at 80% power to the expected difference at $P < 0.05$ (two-tailed).

Results

Steroid hormone receptor status of metastases harboring ER mutations

Core needle biopsies from accessible metastases were collected from consented patients primarily enrolled in NCT02953860 and 3 from NCT01597193. All patients had ER+HER2- BC at time of diagnosis. The steroid hormone receptor proteins, estrogen receptor alpha (ER), progesterone receptor (PR), androgen receptor (AR), and glucocorticoid receptor (GR), had not previously been examined together in the same cases of MBC harboring WT versus mutant ER. FFPE sections from core needle

biopsies were stained for ER, PR, AR, and GR (Figure 1A-B), and Ki67 and cleaved caspase-3 (Sup Figure S1A-B). IHC was scored by breast pathologist (SBS) and graphed according to ER mutation status. ER and PR protein were significantly higher in biopsies harboring mutant ER as compared to metastases with no detectable mutations in exon 8 (designated as WT ER, Figure 1A). In contrast AR and GR were not significantly different in biopsies with WT ER compared to those with mutant ER (Figure 1A). In WT ER metastases, where ER and PR were often low, AR was frequently maintained (patients: 01-006, 01-010, 01-0144, 01-022, 01-028, 02-006, 02-007: Figure 1B) and was therefore not significantly different between WT and mutant biopsies. Interestingly, some mutant ER cancers had a substantially higher AR than PR (patients: 01-002, 01-004 and 01-027: Figure 1B), and mutant ER metastases with high PR had low AR. Ki67 and cleaved caspase-3 were not significantly different between the two groups, although there was a trend towards higher Ki67 in metastases with mutant ER compared to WT ER (Sup Figure S1A-B). We also examined correlations between hormone receptors and as would be expected since ER regulates PR, these two receptors are positively correlated. ER is correlated with AR as well. It is evident that while many ER mutant containing biopsies (red) are high for ER and PR, the ER WT (black) have predominately lost PR, but often retain AR expression. ER does not correlate with GR and PR and AR do not correlate (Sup Figure 10).

Mutant ER breast cancer cells upregulate AR expression and activity

To study mutant ER expressed from its native locus in MCF-7 and T-47D human BC cell lines, FLAG-tagged ER mutant models were engineered using CRISPR to introduce one allele of FLAG-tagged *ESR1* containing the D538G or Y537S mutation or FLAG-tagged WT *ESR1* (Arnesen *et al*, co-submitted). Immunoblot for FLAG confirmed CRISPR construct expression in two clones for each cell line (Sup Figure S2A-B). Further experiments were conducted using two clones pooled for each line.

Steroid hormone receptor expression was assessed by immunoblot of WT, D538G, and Y537S MCF-7 and T-47D cells grown in culture media containing full serum (containing steroid hormones). However, to mimic conditions resulting from AI therapy, where the ER mutations arise in patients, the cells were also cultured in phenol

red free media containing DCC stripped serum for 6 months to generate long-term estrogen-deprived (LTED) cells. While AR was slightly elevated in the MCF7 ER mutant compared to WT cells cultured in full serum, in the LTED conditions ER, PR, AR and GR were all increased. Interestingly AR and PR were dramatically increased in the LTED mutants compared to LTED WT. (Figure 2A). After LTED, mutant ER MCF-7 lines had dramatically increased ER, PR, AR and GR when compared to WT ER cells. In T-47D LTED resulted in increased ER, PR AR and GR compared to the cells cultured in full serum. AR and GR increased with LTED in both WT and Mutants, indicating that WT cells that survive LTED can also upregulate AR and GR. PR did not increase as a result of LTED in the WT (Sup Figure S3).

Since AR was upregulated in ER mutant versus WT cells and by LTED, we evaluated AR by IHC in multiple ER+ patient-derived xenografts (PDX) (Figure 2B). Upon knowledge of the ER mutational status of the PDX, AR was found to be higher in PDX with ER mutations as compared to those with WT ER. Further, the Y537S mutant HCI-013-EI (estrogen independent) PDX, which was derived from the parental HCI-013 PDX passaged in mice without E2 supplementation (to mimic the E2 deprivation therapy in BC patients), exhibited elevated AR expression compared to parental HCI-013 tumors passaged in E2 supplemented mice. Human AR was also maintained in spontaneous metastases from the HCI-013-EI primary tumors to lung (Figure 2B).

Cross-referencing known AR target genes with genes differentially expressed in mutant ER BC cells revealed statistically significant enrichment (hypergeometric test p-values $< 6 \times 10^{-5}$) of AR target genes in mutant ER cells compared to WT (Figure 2C, genes identified in Sup Table S1). In this experiment, cells were cultured in phenol red free media containing DCC stripped serum for 5 days, then supplemented $\pm$ E2 for 8 hours. AR target genes were upregulated in mutant ER cell lines with or without E2, indicating that the mutant-specific alterations in known AR target genes occurred in an E2 independent manner.

AR inhibition abrogates the selective advantage of mutant ER BC cells for anchorage independent survival

We previously reported that AR mRNA, protein, and activity were increased in triple negative breast cancer (TNBC) cells that survive under anchorage independent conditions as compared to adherent cells, and AR protected against caspase-mediated cell death (27). To determine whether AR also increased in anchorage independent ER+ BC lines, parental MCF-7 cells were grown for 24-72 hours under adherent conditions (att) or forced suspension conditions on poly-HEMA coated plates (susp), which prevent cell adherence. AR was elevated at 24, 48, and 72 hours of anchorage independent growth as compared to adherent cells, while ER and GR were not (Sup Figure S4A). PR was slightly elevated at 24 hours of anchorage independent culture as compared to attached cells, but to a much lesser extent than AR. IHC on cell pellets harvested at 24, 48, and 72 hours demonstrated a striking increase in AR at each time point in the anchorage independent cells compared to attached cells, while ER and PR were not altered (Sup Figure S4B).

To determine if AR protein increased in mutant ER cells under anchorage independent conditions, AR was analyzed by IHC on FFPE sections of cell pellets generated from WT, D538G, and Y537S MCF-7 cells grown in attached or forced suspension conditions for 24 hours. AR protein increased in all three MCF-7 cell lines following suspension culture for 24 hours as compared to adherent cells and AR expression decreased with addition of the CYP17A1 lyase inhibitor Seviteronel (Sevi) that also acts as an AR antagonist (Figure 3A) (28). Quantification showed that strong (3+) nuclear AR staining in D538G cells was 5-fold higher at baseline than in WT MCF-7 cells (Figure 3B). The number of nuclei strongly positive for AR was increased by 19-fold for WT, 7.5-fold for D538G, and 21-fold for Y537S MCF-7 cells grown in suspension as compared to attached conditions. Sevi dramatically reduced the number of AR+ nuclei and the staining intensity (Figure 3A and B). Similar results were observed for WT, D538G, and Y537S T-47D cells (Sup Figure S5A-B). Furthermore, when grown on soft agar, D538G MCF-7 cells, which at baseline expressed the highest level of AR, formed significantly more colonies than WT counterpart (1.6-fold higher, Figure 3C-D). To determine if increased AR was indicative of a dependency on AR for survival in anchorage independent conditions, as in TNBC lines (27), MCF-7 cells were treated with Sevi, which completely

abolished growth on soft agar of all MCF-7 lines. Since the AR inhibitor enzalutamide (Enza) showed clinical benefit in AR+ TNBC (as did bicalutamide) (29,30), we also tested Enza, which significantly inhibited D538G cell growth in soft agar. Importantly, Enza negated the survival advantage of D538G cells compared to WT MCF-7 in soft agar. Similar results were seen in D538G T-47D cells, where both Sevi and Enza inhibited growth on soft agar (Sup Figure S5C). WT and mutant ER MCF-7 and T-47D cells generated in the Oesterreich lab (13) were also studied on two-dimensional standard culture as compared to three-dimensional culture using ultra-low adhesion plates and three days of hormone deprivation (Sup Figure S4C). AR was higher in both D538G and Y537S mutant ER MCF-7 lines compared to WT (in both 2D and 3D). While AR decreased in 3D compared to 2D in the WT cells, it remained elevated in 3D in the mutant ER lines, again demonstrating a potential role for AR in the ER mutants. Results in T47D mutants versus WT were similar.

To determine whether mutant ER cells have a selective advantage for anchorage independent survival or metastatic outgrowth in vivo under conditions modeling the E2-depleted state, we introduced labeled WT or D538G T-47D cells, in which AR was elevated (Figure 4A), by tail vein injection into ovariectomized mice that were supplemented with either cellulose control or E2 pellets. Whole mouse IVIS imaging at the end of study showed significantly higher metastatic burden in mice injected with D538G T-47D cells compared to those with WT cells, both with and without E2 (Figure 4B-C). Ex vivo imaging of lungs completed immediately after necropsy also showed significantly higher metastatic burden in lungs harboring D538G cells as compared to those injected with WT T-47D with or without E2 (Figure 4D-E). In non-E2 supplemented mice, WT tumor cells were nearly undetectable when imaged in vivo or ex vivo.

Mutant ER BC have elevated expression of pro-metastatic *CHI3L1*

Analysis of RNA-seq data from three independent laboratories using mutant ER cell lines generated by different methods showed that *CHI3L1* (also known as *YKL-40*) was elevated in Y537S MCF-7 and T-47D compared to WT (S6A-B and Arnesen *et al*, co-submitted). *CHI3L1* is a well-established androgen/AR regulated gene in prostate

cancer where it is detectable in tumor and patient serum and correlates with disease progression (31-33). Since we previously reported that *CHI3L1* increased in TNBC lines under anchorage independent conditions (27), we evaluated CHI3L1 protein in WT, D538G, and Y537S MCF-7 cells in attached versus suspension culture for 24 hours (Figure 5A). CHI3L1 was significantly higher in both D538G (5-fold) and Y537S (3-fold) MCF-7 cells than WT; however, CHI3L1 was not further enhanced by 24 hours of anchorage independent culture. Since CHI3L1 promotes invasion in other cancer types, we compared the invasive capacity of D538G and Y537S MCF-7 to WT and found that D538G MCF-7, which had the highest expression of CHI3L1, were significantly more invasive than WT MCF-7 in Matrigel coated Boyden chamber assays (Figure 5B). Importantly, a CHI3L1 blocking antibody negated this significant invasive advantage of D538G MCF-7 cells compared to WT, suggesting that mutant ER BC cells can utilize CHI3L1 to promote invasion.

We also examined CHI3L1 expression in HCI-013-EI (estrogen independent) PDX tumors, which harbor the Y537S mutant and were grown in the absence of E2, as compared to parental HCI-013 PDX tumors, which were grown in the presence of E2 (Figure 5C). HCI-013-EI tumors had an >3-fold increase in CHI3L1 expression compared to HCI-013 tumors, demonstrating that CHI3L1 is enhanced by propagating this PDX under conditions similar to the selective pressure of E2-depletion that selects for ER mutations in BC patients.

Mutant ER BC cells upregulated the immunomodulatory type-1 interferon pathway

To identify additional mutant ER-specific vulnerabilities, we conducted pathway analysis of mutant-specific genes that revealed innate immune, viral recognition, and type-1 interferon among the top pathways preferentially upregulated in mutant versus WT ER MCF-7s (Figure 6A-C Arnesen *et al*, co-submitted). The interferon-induced transmembrane (IFITM) gene *IFITM3* was consistently increased in D538G and Y537S MCF-7 cells in data from multiple laboratories (Sup Fig 6C). Both D538G and Y537S MCF-7 cells demonstrated enhanced levels of a number of interferon stimulated genes (ISGs) such as OAS1/2, IFIT1/2/3/5, IFITM1/3, and ISG15

compared to WT MCF-7 (Figure 6B-C and Sup Figure S6), demonstrating robust activation of this pathway in mutant ER BC cells as compared to WT. Since IFITM proteins have been reported to play a role in AI-resistance (34,35), we explored the expression of IFITM3 in WT, D538G, and Y537S MCF-7 cells $\pm$ interferon-beta (IFN β) stimulation, a key cytokine and activator of the type-1 interferon signaling pathway. Baseline IFITM3 levels were 2-4 fold higher in D538G and Y537S MCF-7 cells compared to WT and were further enhanced by IFN β (Figure 6D). IHC quantification showed that IFITM3 was higher in D538G MCF-7 than WT (Figure 6E). Given that MUC1 drives IFITM1 overexpression in AI-resistant BC cells (36) and *MUC1* was also on the list of genes significantly elevated in cells containing mutant versus WT ER, we also examined MUC1 protein by IHC and found it was elevated in D538G MCF-7 cells compared to WT (Figure 6E). Lastly, we performed IHC for IFITM3 in biopsies of five patients with MBC harboring the D538G ER mutation as compared to all of the WT biopsies presented in Figure 1 and found IFITM3 protein was significantly upregulated in the ER mutant versus WT biopsies (Figure 6F).

Metastases harboring mutant ER have significantly more CD4+ T cell and macrophage infiltrates

Given that mutant ER cell lines showed enhanced innate immune and interferon pathway signatures compared to WT ER, we tested biopsies of MBC with mutant versus WT ER for differences in tumor infiltrating immune cells. A multiplex panel consisting of antibodies that distinguish T cells (FoxP3+CD4+ T regulatory cells; FoxP3-CD4+ T helper cells; or CD8+ T effector cells), macrophages (CD68+), B cells (CD20+) and tumor cells (cytokeratin+) was utilized. InForm Software analysis demonstrated that the number of T regulatory (CD4+FoxP3+) and T helper cells (CD4+FoxP3-) were significantly increased in mutant ER BC metastases when compared to WT ER, although no difference in CD8+ T effector cells was observed (Figure 7A). Interestingly, in both our data (Sup Fig 8) and data from two publicly available studies (Sup Fig 9A-B), *ESR1* mutations were mutually exclusive with *TP53* mutations. Further, patients with either *TP53* or *PIK3CA* mutations contained significantly higher CD8+ cytotoxic T effector cells (Sup Figure S8B-C). There was no significant change in CD20+ B cells in

mutant ER metastases compared to WT (Figure 7B). However, the number of CD68+ macrophages in mutant ER metastases was significantly increased when compared to biopsies with WT ER (Figure 7C). Finally, Programmed Death Ligand-1 (PD-L1) expression was analyzed in the same biopsies due to the recent testing of PD-L1 inhibitors in ER+ BC (Figure 7D) (37,38) and FDA approval in TNBC (39). Interestingly, while the overall level of PD-L1 did not differ between mutant and WT ER biopsies, PD-L1+ macrophages were significantly higher in mutant ER biopsies compared to WT, suggesting that macrophages infiltrating mutant ER metastases are more immunosuppressive. Interestingly, this finding was more significant when examining macrophages intimately associated with the tumor cells as compared to those found predominately in the intra-tumoral stroma (Sup Figure 7B).

Discussion

Mutations in *ESR1* that render ER constitutively active in the absence of ligand are a common mechanism of acquired resistance to AI therapy. While increased PR resulting from constitutively active mutant ER is well-documented, the four main steroid hormone receptor proteins (ER, PR, AR, and GR) had not been evaluated together in MBC biopsies harboring WT versus mutant ER. While E2 is extremely low in postmenopausal women with BC on AI, circulating and intra-tumoral androgens are increased (40). This, combined with the predominantly nuclear localization of AR in clinical samples (indicating ligand bound receptors), led us to postulate that long-term E2-deprivation via AI therapy, which blocks conversion of androgens to estrogens, might cause tumor cells to shift to reliance on androgens and AR. Indeed, in TNBC, where ER is absent, AR binds chromatin in a manner more similar to ER in ER+ BC cells than to AR in prostate cancer cells, suggesting that AR can replace ER in some TNBC (41) and confer the “luminal AR” designation (42). Our analysis of steroid hormone receptors suggests that ER+ MBC can become resistant to AI treatment by either 1) acquiring mutations that render ER constitutively active (retaining robust ER and PR positivity) or 2) by expressing little to no ER and PR in an apparent “subtype switch” to resemble TNBC, which often retain AR and/or GR.

Cross-referencing known AR regulated genes with genes differentially expressed by mutant ER versus WT ER BC cells revealed significant enrichment for AR regulated genes. Although AR protein had not specifically been examined in WT versus mutant ER MBC, it was elevated in both primary and MBC resistant to AI therapy when compared to responsive disease (43-48). AR was often maintained or elevated in MBC compared to patient-matched primary tumors, whereas ER was decreased (49). BC patient CTCs have been found to be AR+ in several studies and AR+ CTC associate with bone metastases (50) and AR mRNA was higher in CTC from BC PDX compared to primary tumors in mice (51). Furthermore, AR inhibition decreased metastatic burden of BC cells from an ER+/AR+ PDX (49) and conversely, ligand-activated AR potentiated metastasis in ER+/AR+ BC cell lines (52). These data, together with our analysis herein of hormone receptor status in clinical biopsies of AI resistant MBC and other data presented in the present study suggest that AR could be active in a subset of ER+ MBC, and might facilitate survival under conditions of E2 deprivation or anchorage independent survival. Indeed, MCF-7 and T-47D ER mutant BC lines, had higher ER, PR, and AR compared to WT, similar to results from the clinical biopsies of MBC. While all steroid receptors increased under long-term E2 deprivation (LTED) conditions, PR and AR were the most strikingly different in mutant compared to WT LTED cells. Historically, ER+ PDX were propagated in mice supplemented with E2 to increase take rate and growth rate for research studies regardless of ER mutational status. We now know that many of these ER+ PDX, derived from MBC in postmenopausal women on AI therapy (a long-term E2-deprived context) do harbor ER mutations. Placing these into mice supplemented with E2 could result in tumors that are “re-addicted” to E2 and therefore do not accurately model the selective pressure of long term E2-deprivation under which the mutations arise. We stained ER+ PDX for AR and found that PDX harboring ER mutations had higher AR than those with WT ER. Furthermore, in support of the theory that E2-deprivation enhances AR expression, the E2-independent HCI-013-EI PDX (with Y537S ER mutation) propagated in E2-deprived mice, expressed more AR protein than its parental counterpart propagated in mice supplemented with E2. Importantly, HCI-013-EI lung metastases originating from the orthotopic site still expressed AR. Thus, maintaining models of mutant ER disease under conditions similar

to those in which they arise in the clinic (E2-deprived) may reveal important, clinically relevant molecular vulnerabilities.

In addition to E2-deprivation, we used anchorage independence as a model of early metastasis. Although AR significantly increased in both ER mutant and WT BC cells surviving anchorage independent conditions, anti-androgens abrogated the selective advantage of mutant ER cells for anchorage independent survival. This is consistent with previous studies in TNBC cells that demonstrated increased AR in anchorage independent cells, where it protected against apoptosis (27), rendering anchorage independent cells highly sensitive to AR inhibition (53). Therefore, AR targeting may limit AI-resistant MBC recurrence, perhaps for both patients with either WT or mutant ER, since some WT ER biopsies retained AR but had low ER and PR.

CHI3L1, encoding Chitinase 3-like 1, was preferentially expressed in BC cells harboring mutant ER. *CHI3L1*/YKL-40 is linked to chronic inflammation and cancer and is upregulated in the mammary gland during involution, where it contributes to a pro-tumorigenic wound-healing like environment via enhanced macrophage infiltration (54). In prostate cancer, *CHI3L1* is upregulated by androgens/AR (33), and in breast cancer it is immune-suppressive (55), and correlates with M2 macrophage infiltration (56), enhanced metastasis (57,58), and poor prognosis (59). While *CHI3L1* was noted previously as increased in mutant ER BC cells (13), it was not further explored. In the present study, *CHI3L1* protein was higher in mutant ER MCF-7 compared to WT. Since *CHI3L1* is associated with decreased E-cadherin and enhanced MMP-9 activity in the involuting mammary gland (54), we investigated its role in invasion and found that the selective advantage of D538G mutant ER MCF-7 cells for invasion was abolished by a *CHI3L1* neutralizing antibody. While a gene signature suggestive of increased migration/invasive capacity was linked to mutant ER (12,13), to our knowledge this is the first demonstration that a specific protein can be targeted in mutant ER cells to decrease invasiveness.

Pathway analysis of genes significantly differentially expressed by mutant versus WT ER in data from three studies demonstrated that genes in the type-1 interferon (IFN) signaling and innate immune pathways were enhanced in mutant ER lines. In normal tissues, infectious pathogens are recognized by Pattern or Damage Recognition

Receptors (PAMPs or DAMPs) that activate type-1 IFN production. IFN α/β signal through IFN receptors and STAT1 activate transcription of Interferon Stimulated Genes (ISGs) to facilitate an innate immune response against the pathogen. The cyclic GMP-AMP synthase (cGAS) is a known stimulator of ISGs via the STING signaling pathway that can be upregulated in cancer cells in response to cytoplasmic DNA or mitochondrial DNA (reviewed in (60)). We focused on the ISGs *IFITM3* and *M1* because they were elevated in mutant ER cells compared to WT in our mRNA profiling, correlated with AR positivity in TNBC (25), elevated in AI resistant BC cells (35), induced in therapy resistant prostate cancer models (61,62), and upregulated by MUC1 in BC cells (63). *MUC1* also emerged as a mutant ER-specific gene increased in mutant ER cells at the protein level. We confirmed that both D538G and Y537S MCF-7 cells have elevated IFITM3 protein at baseline that was further elevated by IFN β . Importantly, clinical biopsies harboring the D538G mutation had significantly more IFITM3 protein compared to metastatic lesions with WT ER.

Given the enhanced expression of the type-1 IFN pathway protein IFITM3 in mutant ER MBC specimens, we sought to determine if there was a difference in immune cell infiltrate in WT versus mutant ER biopsies. We found significantly elevated T helper and T regulatory cells, as well as enhanced macrophage infiltration in MBC with mutant compared WT ER. Androgens stimulated alternative activation (M2 pro-tumor polarization) of macrophages in an AR dependent manner in an allergic asthma model (64). Further, CHI3L1 stimulated by inflammation leads to M2 macrophage polarization during allergic reactions (65). Thus, enhanced macrophage infiltration in mutant ER MBC may be the result of elevated innate immune signaling pathways/proteins like type-1 IFN, active AR, and/or CHI3L1.

Although ER+ BC is more “immunologically cold” than TNBC, the recent efficacy of anti-PD-L1 antibody in high risk patients with ER+ disease (37), confirms that some ER+ BC may respond to immunotherapy. Indeed, we found that PD-L1 positive macrophages were significantly higher in mutant ER metastatic lesions than those with WT ER. Interestingly, the other two genes most commonly mutated in BC, *TP53* and *PIK3CA*, did not correlate with altered macrophages, but did correlate with increased CD8+ cytotoxic T cells. These data suggest that enhanced immune suppression via PD-

L1+ macrophages may be uniquely acquired by ER mutant MBC. Since expression of PD-L1 in the tumor microenvironment, primarily its expression on myeloid cells, predicts BC response to checkpoint inhibitors (39), future studies will explore checkpoint inhibition in WT versus mutant ER MBC.

Evidence presented here, and in Arnesen *et al*, suggest that mutant ER containing cells may be poised to survive the stress of E2-deprivation and anchorage independence by the constitutive activity of mutant ER and by changes in non-ER regulated genes. Our studies in BC cells, PDX, and patient biopsies revealed proteins/pathways that may serve as targetable vulnerabilities in ER mutant disease, including AR, CHI3L1, and ISGs. On the other hand, metastases without *ESR1* exon 8 mutations expressed significantly less ER and PR than those with mutant ER, but clearly adapt to the same selective pressure of E2-deprivation (AI therapy) and the metastatic cascade. Adaptations may occur through shared targetable mechanisms like activated AR, or unique mechanisms that remain to be determined via preclinical modeling under clinically relevant conditions that mimic the selective pressures under which they evolve in patients. The differences in hormone receptor protein expression and tumor infiltrating immune cells in biopsies with *ESR1* mutations versus *ESR1* WT suggest that AI resistant MBC should be examined in light of the different pathways and adaptations that lead to resistance, which in turn result in new vulnerabilities to guide novel therapeutic strategies.

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Author Contributions

Dr. Michelle M. Williams was the lead researcher on this project and generated much of the data with the assistance Nicole S. Spoelstra, Spencer Arnesen, Kathleen I. O'Neill, Dr. Jessica L. Christenson, Dr. Jordan Reese, Dr. Toru Hanamura, Dr. Zheqi Li, and Dr. Britta M. Jacobsen. Dr. Williams and Dr. Jennifer K. Richer contributed significantly to the intellectual development and manuscript preparation with the assistance of Dr. Steffi Oesterreich, Dr. Rebecca B. Riggins, Dr. Britta M. Jacobsen, Dr. Anthony Elias, and Dr. Jason Gertz. Dr. Elias is the principal investigator on the clinical trial from which the patient biopsies of metastatic breast cancer were obtained. Dr. Kathleen C Torkko was consulted for power number calculations and data analysis for all mouse experiments. Dr. Sharon B Sams completed all pathological analyses of patient biopsies in a blinded manner.

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Figure Legends

Figure 1. Steroid hormone receptor expression in biopsies of metastatic breast cancer with wild type or mutant *ESR1*. **A.** Formalin fixed paraffin embedded (FFPE) sections of core needle biopsies (N=18 WT ER, N=12 mutant ER) were stained by IHC for ER, PR, AR, and GR. Depicted are the mean scores (intensity x percent cells staining) $\pm$ SEM. Mann-Whitney tests were performed for each receptor stained. **B.** Representative images for all WT ER metastases (left) and all mutant ER metastases (right) stained for ER, PR, and AR and GR are shown at 400X.

Figure 2. AR protein and gene regulation differs in WT versus mutant ER expressing MCF-7 breast cancer cells and patient derived xenografts (PDX), particularly after long-term estrogen deprivation (LTED). **A.** Western blot of whole cell lysates from WT, D538G, and Y537S MCF7 cells grown in full serum media (left) or charcoal stripped serum containing media (LTED, right) were probed for AR, ER, GR, PR-A/B, and tubulin. **B.** ER+ patient-derived xenografts (PDX) tissue microarray from Dr. Alana Welm, Huntsman Cancer Institute (HCI) and ER+ PDX from University of Colorado (UCD) with *ESR1* mutation status indicated, were stained for AR by IHC with representative images at 100X (left) and 400X (right). For the estrogen independent version of HCI-013, HCI-013-EI, an image representative of primary tumor (PT) and lung metastasis (Met) are shown. **C.** Heatmap of known AR regulated genes in MCF7 (right) and T47D (left) depicting relative expression of genes displayed as a Z-score across WT and mutant cells after 5 days of growth in hormone-depleted media followed by treatment with either 10nM E2 or vehicle (DMSO) for 8 hours. Significant overlap was determined using hypergeometric tests with a p-value cut off of <0.05 : AR genes vs D538G up-regulated genes = 5.5×10^{-05} ; AR genes vs D538G down-regulated genes = 1.0×10^{-08} ; AR genes vs Y537S up-regulated genes = 3.2×10^{-08} ; AR genes vs Y537S down-regulated genes = 9.3×10^{-11} .

Figure 3. AR increased in breast cancer cells grown in soft agar and AR inhibition abolished the selective advantage of mutant ER cells for anchorage-independent survival. **A.** WT, D538G, and Y537S mutant MCF7 cells were grown in full serum media under attached (Att) or suspension conditions (on poly-HEMA plates, Susp) and treated $\pm$ 20 μ M Seviteronel (Sevi) for 24 hours. Cells were pelleted, FFPE, and AR IHC performed. Representative images are shown at 400x. **B.** Quantification of A with ImageScope software for percent positive nuclei for staining intensities of 0-3+ for all conditions attached (Att), suspended (Susp), and $\pm$ Sevi. **C.** WT, D538G, and Y537S cells plated in 0.3% agar and treated with 20 μ M Sevi, 20 μ M enzalutamide (Enza) or EtOH control and grown for three weeks with bi-weekly media and drug changes. Representative images of anchorage-independent growth are shown. **D.** Colony number quantified using ImageJ software for assay in C (N=3). Mean $\pm$ SEM, One way ANOVA with Tukey's multiple comparison test is depicted. Two way ANOVA, interaction between cell line and treatment p = 0.003.

Figure 4. Breast cancer cells harboring D538G ER mutation thrive at metastatic sites in an experimental metastasis model in estrogen-deprived mice, while WT ER cells do not. **A.** WT and D538G ER T47D^{GFP/Luc} cells were stained for AR and

representative images shown at 400x. **B.** The same lines were delivered via tail vein injection into 6 week old ovariectomized NSG mice (N=5/group) with or without E2 or cellulose and six weeks post cell injection, mice were imaged by IVIS. **C.** Quantification of whole mouse luminescence signal (total flux), mean $\pm$ SEM. Raw data are presented with p-values from a Student's unpaired two-tailed t test conducted on the log transformed data due to heteroscedasticity, one way ANOVA (p=0.006). **D.** At end of study mice were sacrificed, lungs excised and imaged ex vivo by IVIS. **E.** Quantification of luminescence in lungs ex vivo mean $\pm$ SEM. Raw data are presented with p-values from a Student's unpaired two-tailed t test conducted on log transformed data due to heteroscedasticity, one way ANOVA (p=0.0003).

Figure 5. MCF-7 BC cells with mutant ER express increased CHI3L1 and blocking CHI3L1 decreased mutant ER BC cell invasion. **A.** IHC staining for CHI3L1 protein in WT, D538G, and Y537S MCF-7 cells grown in full serum media under attached (Att) or suspension conditions (on poly-HEMA plates, Susp) for 24 hours. Representative images are shown at 1000x (left) and quantification of percent cells strongly positive (3+) for CHI3L1 is shown (right), mean $\pm$ SEM, N=3 photos/pellet, One way ANOVA with Tukey's multiple comparison test is depicted. Two way ANOVA, interaction between cell line and treatment is N.S. **B.** Invasion through Matrigel was determined for WT, D538G, and Y537S MCF7 cells grown in full serum media. Cells were treated with 10 μ g/mL anti-CHI3L1 blocking antibody or IgG control 24 hours after plating and harvested 72 hours post-treatment. After crystal violet staining and imaging, the relative amount of invasion was determined using ImageJ software, mean $\pm$ SEM of two separate experiments, N=3 One way ANOVA with Tukey's multiple comparison test is depicted. Two way ANOVA, interaction between cell line and treatment is N.S. **C.** CHI3L1 IHC was conducted on Y537S mutant HCI-013 grown in E2 supplemented mice and HCI-013-EI grown in ovariectomized mice. Representative images are shown at 400x (left) and quantification of strongly positive (3+) cells was conducted using the ImageScope Software (right), mean $\pm$ SEM, N=6-7, Mann-Whitney test.

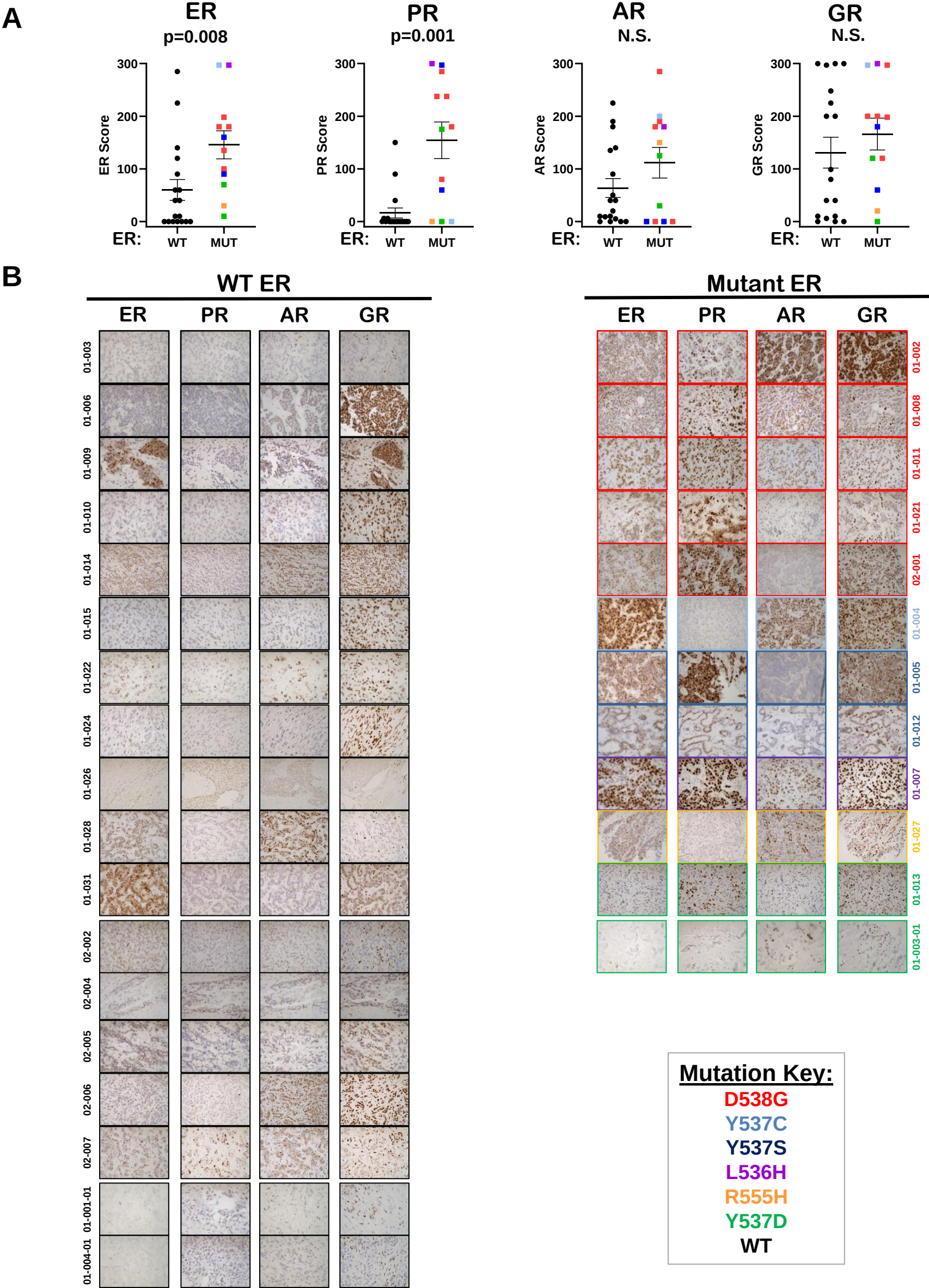
Figure 6. Mutant ER BC cells and specimens have increased type-1 interferon signaling pathway signature and increased IFITM3 protein compared to those with WT ER. **A.** Illumina's BaseSpace Correlation Engine was used to identify mutant-specific pathways from MCF7 mutant-specific genes identified through the multivariate/multilab RNA-seq analysis (Arnesen *et al*). **B-C.** Heatmap depicts relative expression, displayed as a Z-score, of genes associated with Innate Immunity (B) and Type I Interferon (C) identified by two different pathway analyses (David and ENRICH) in ER WT and mutant MCF7 cells grown in hormone-depleted media without E2 for 3-5 days depending on the laboratory. **D.** WT, D538G, and Y537S MCF7 cells were treated with 1000 units of IFN β for 24 hours, whole cell lysates were generated and analyzed by western blot for IFITM3. **E.** IHC for IFITM3 and MUC1 was performed on FFPE pellets generated from WT, D538G, and Y537S MCF-7 cells grown in media containing full serum, mean $\pm$ SEM, N=3 photos/pellet, One way ANOVA with Tukey's multiple comparison test was conducted for each staining (right). Representative images are shown at 1000x (left). **F.** Formalin fixed paraffin embedded (FFPE) sections of core needle biopsies (N=14 WT ER, N=5 D538G mutant ER) were stained by IHC for

IFITM3. Depicted are the mean scores (intensity x percent cells staining) $\pm$ SEM, Student's unpaired two-tailed t test.

Figure 7. CD4+ T cells and PD-L1+ macrophages were significantly higher in biopsies of metastatic breast cancer harboring mutant ER as compared to WT ER.

Biopsies of the same metastases characterized in Figure 1 (N=14 with ER WT, N=10 with mutant ER) were stained for tumor infiltrating immune cells using Opal™ TSA technology (Akoya Biosciences) with the following colors indicating respective immune cell markers: CD4 (yellow), Foxp3 (green), CD8 (magenta), CD68 (orange), CD20 (red), and cytokeratin (cyan). Slides were scanned using Vectra 3 Automated Quantitative Pathology Imaging System (Perkin Elmer) technology and 3 to 5 representative 20x fields/tumor were analyzed using inform software (Perkin Elmer) and a pixel-based algorithm for percent positivity, mean percent positive cells $\pm$ SEM, Mann–Whitney test (top); representative images (20x, bottom) for: **A.** T regulatory cells (CD4+ FoxP3+), T helper cells (CD4+ FoxP3-), and cytotoxic T cells (CD8+), **B.** B cells (CD20+), and **C.** macrophages (CD68+). **D.** The same biopsies were stained for PD-L1 and CD68 using Opal™ TSA technology (Akoya Biosciences). Slides were scanned using a Vectra 3 Automated Quantitative Pathology Imaging System (Perkin Elmer), and up to 5 representative fields/tumor were analyzed for either total PD-L1 or dual expression of PD-L1 and CD68 using a cell phenotype-based algorithm for percent positive cells, mean positive cells $\pm$ SEM, Mann–Whitney test.

Figure 1



A

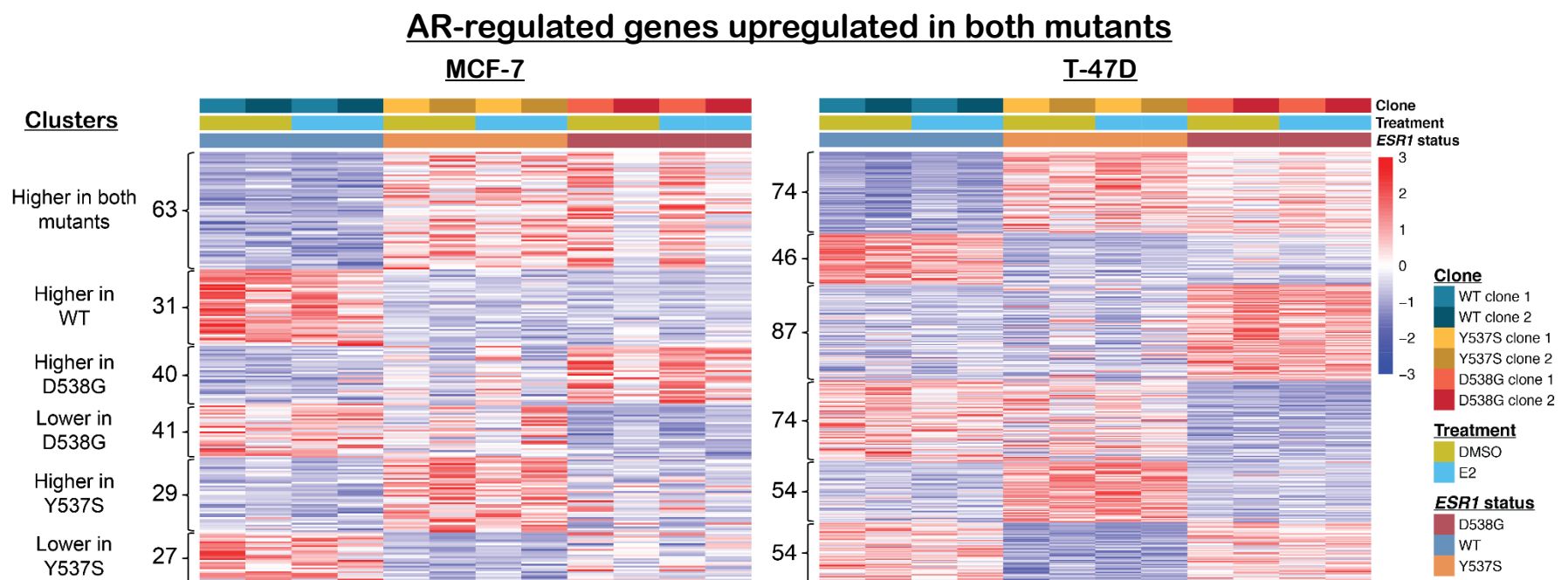


Figure 3

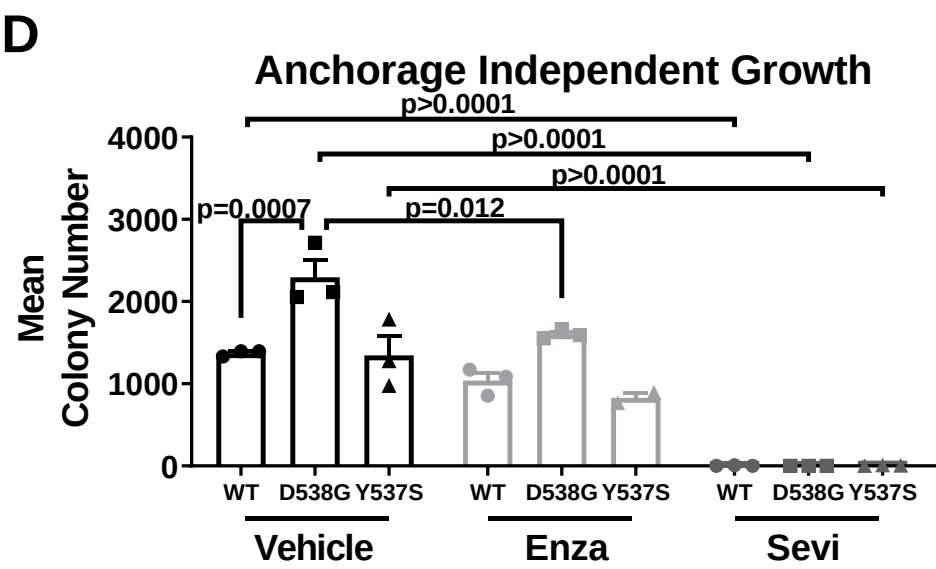
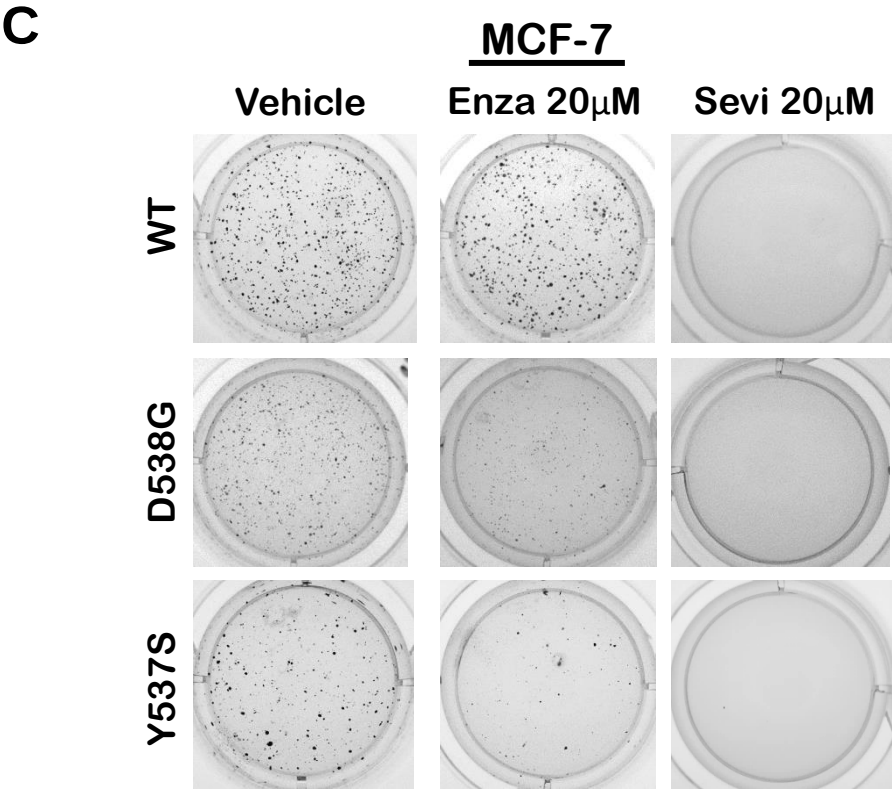
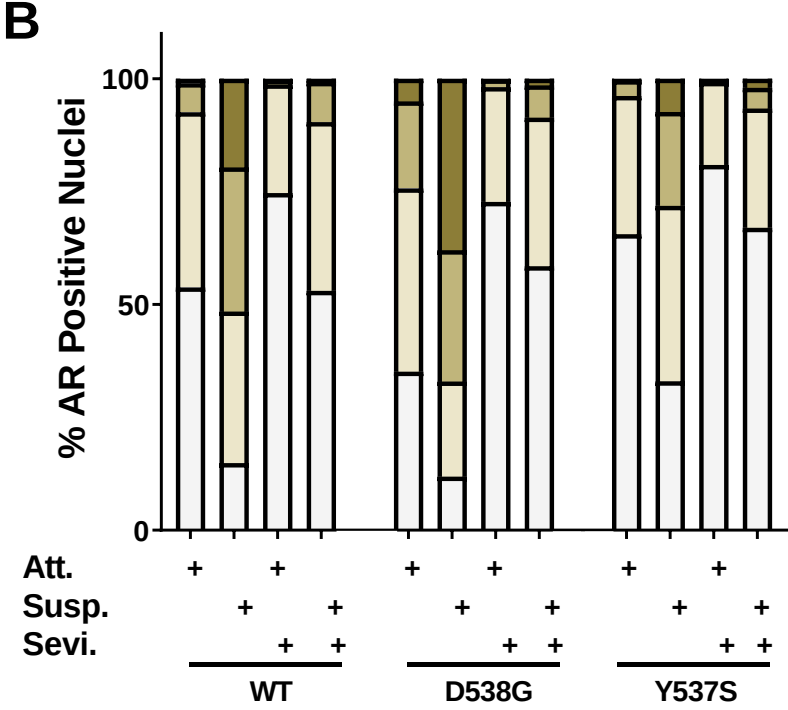
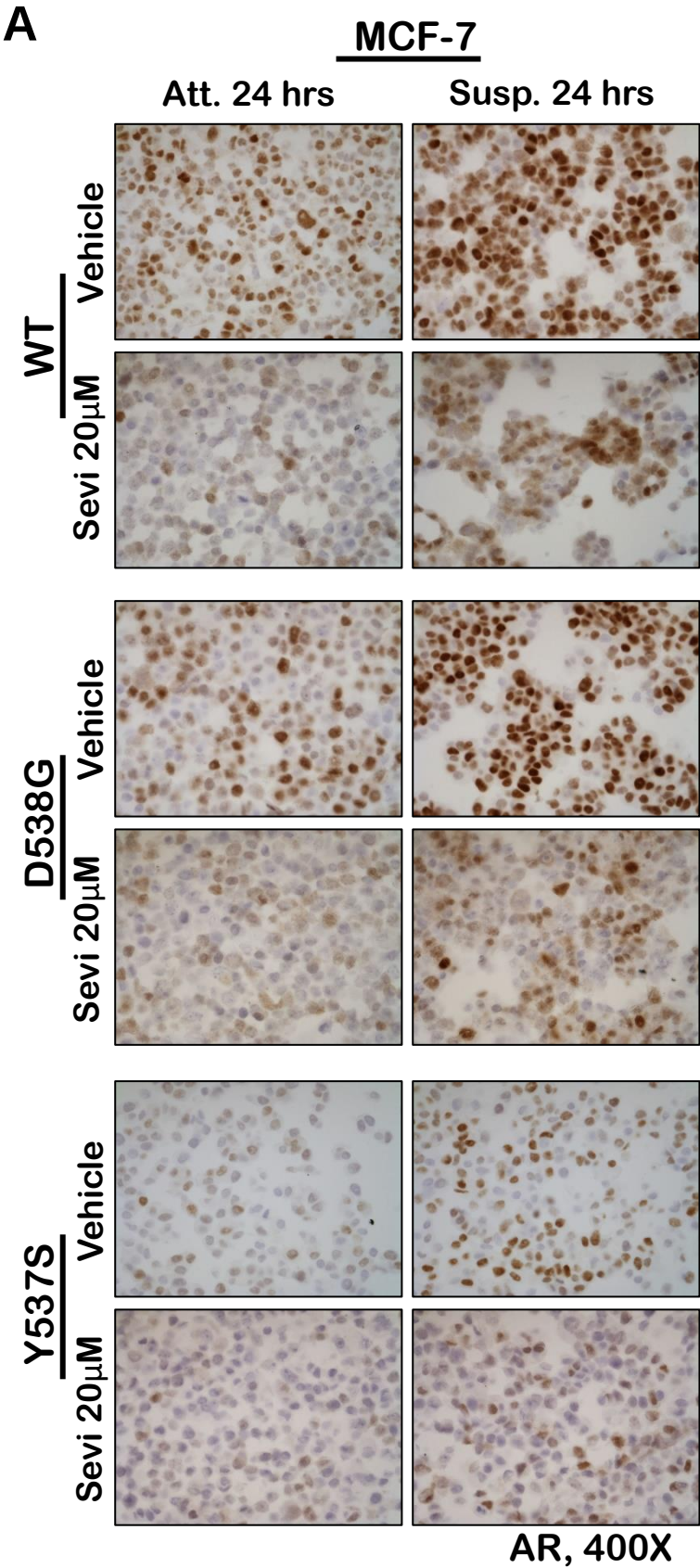


Figure 4

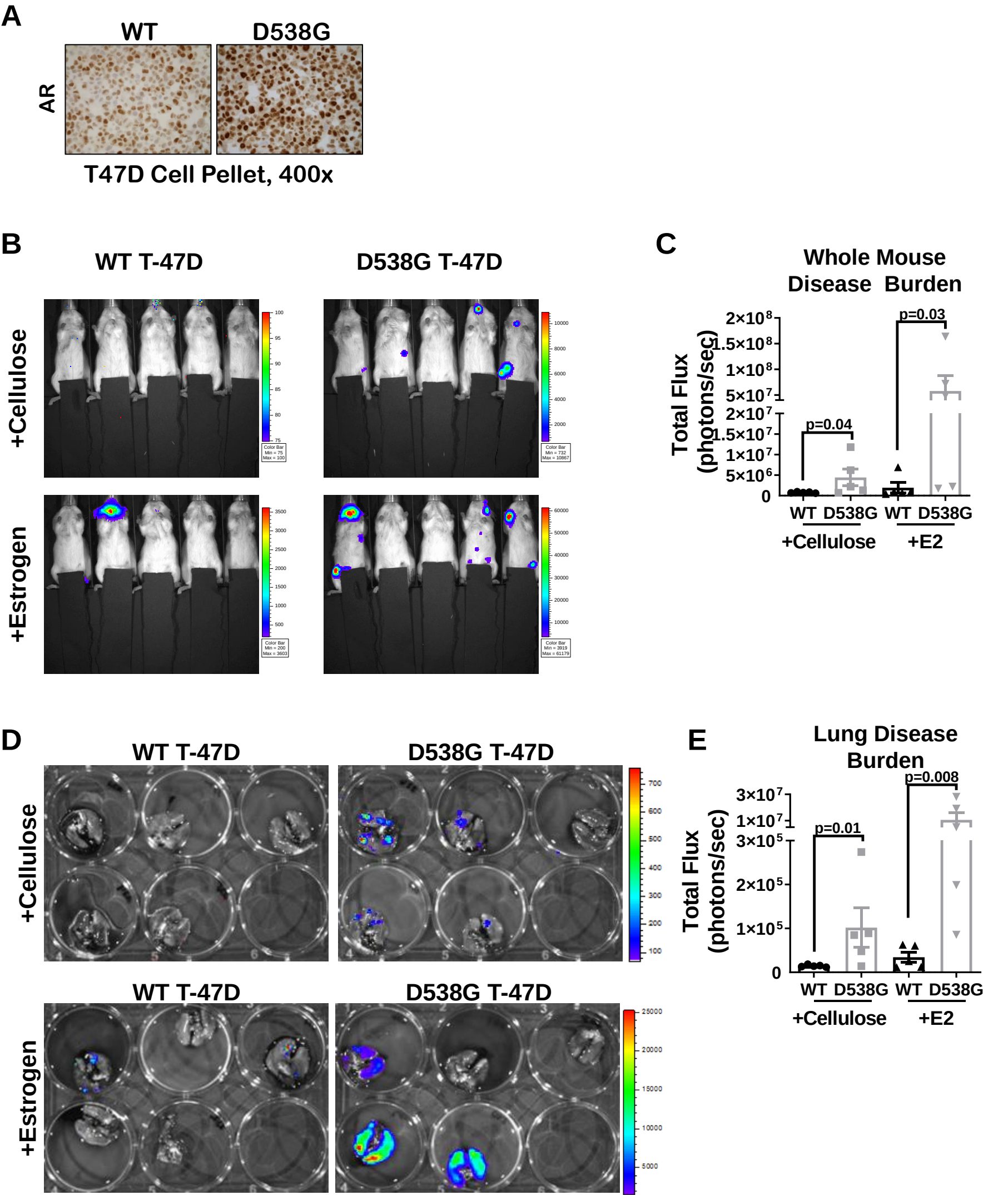
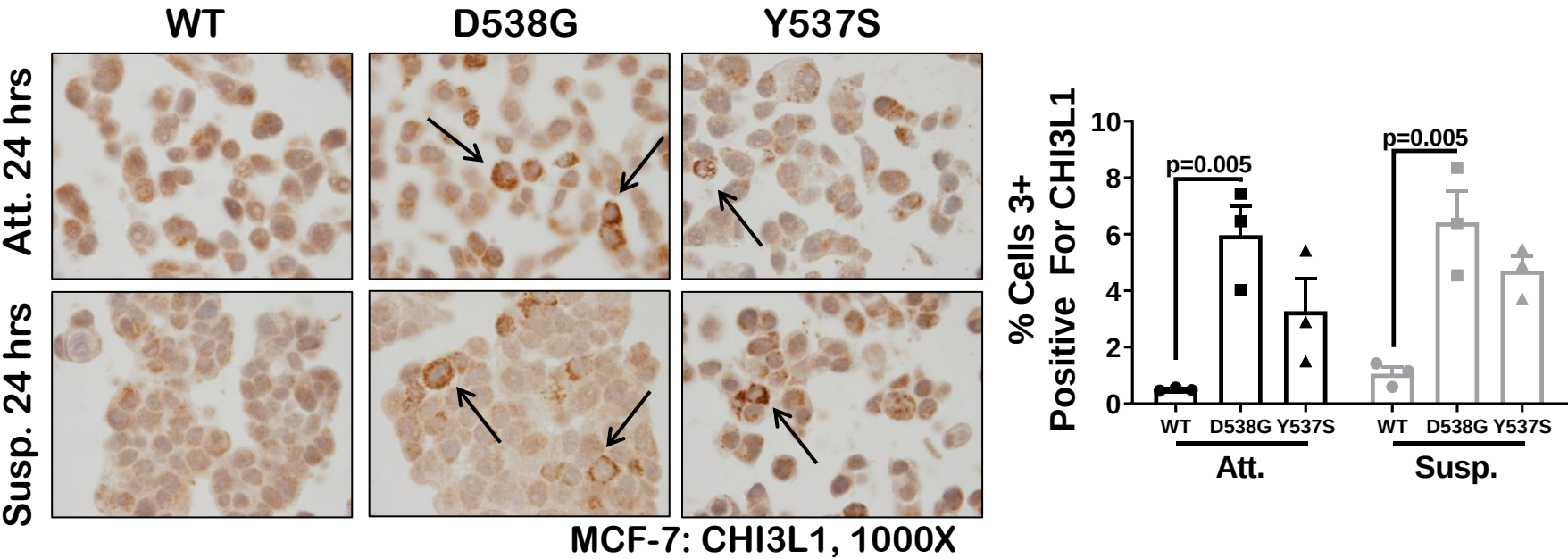
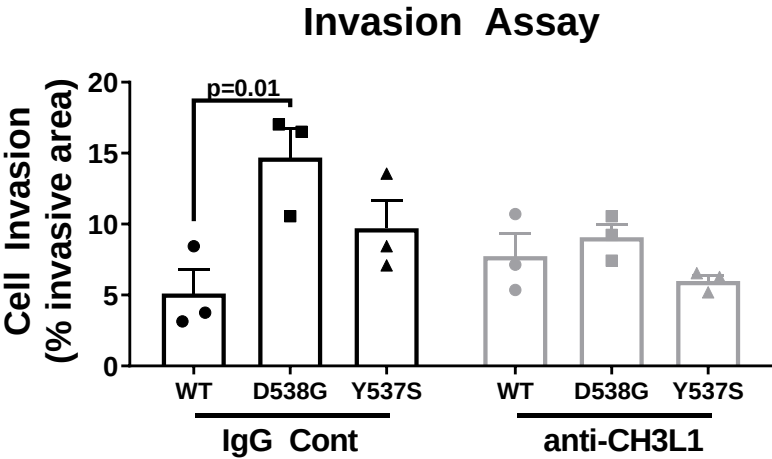


Figure 5

A



B



C

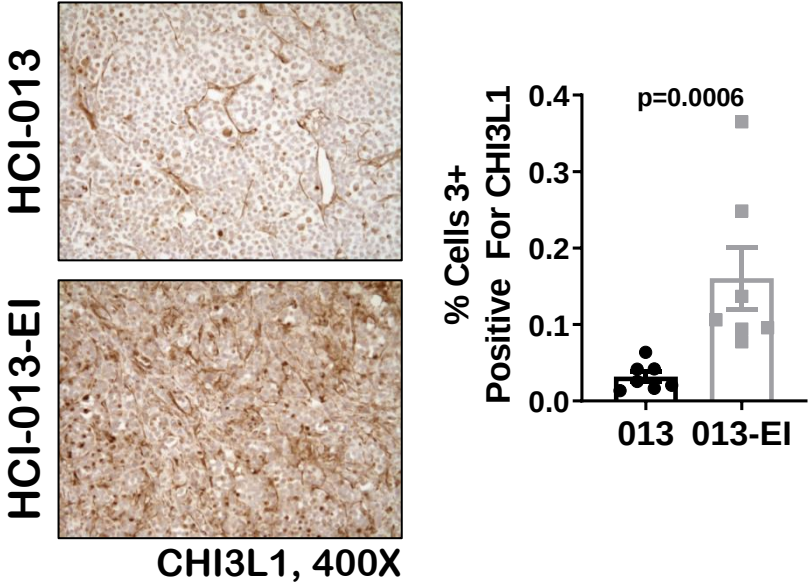


Figure 6

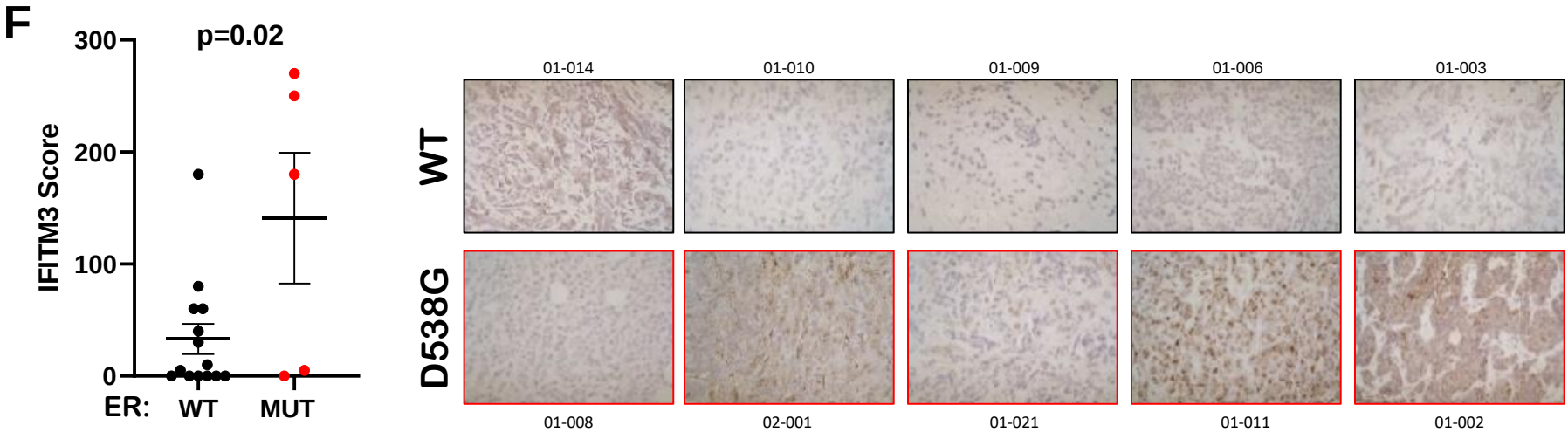
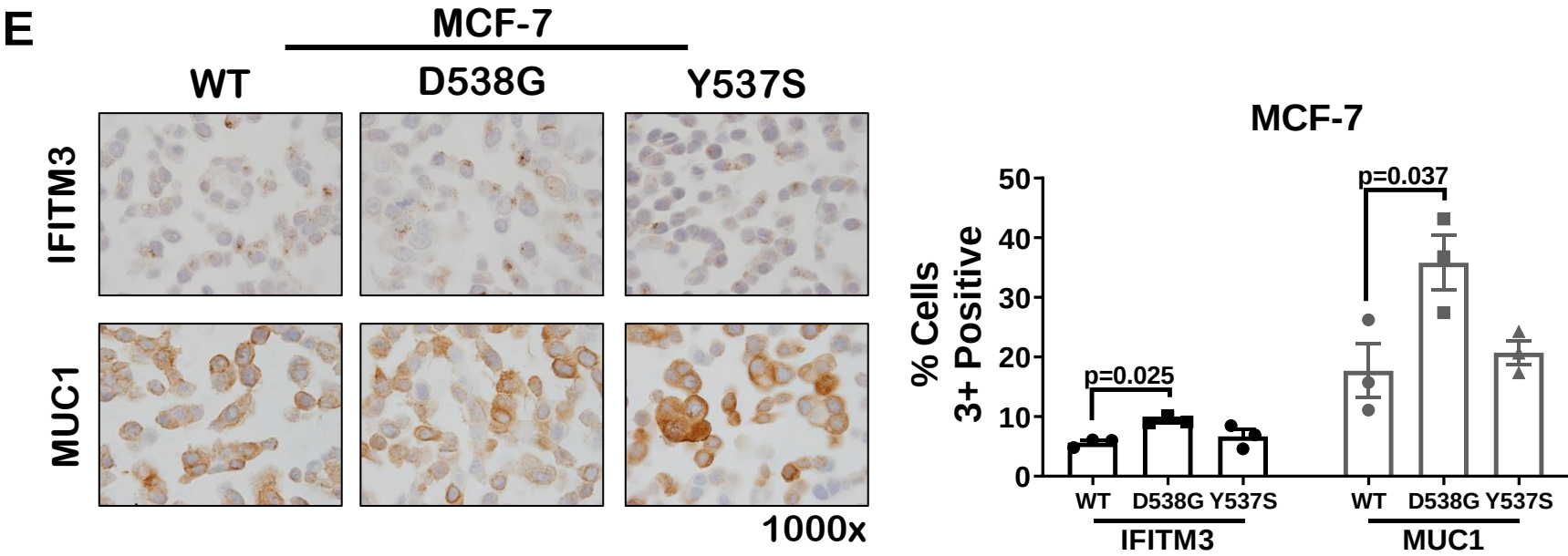
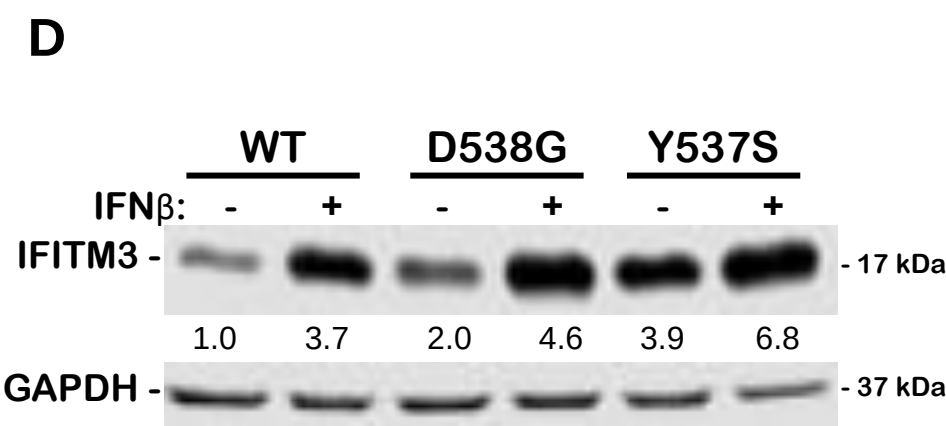
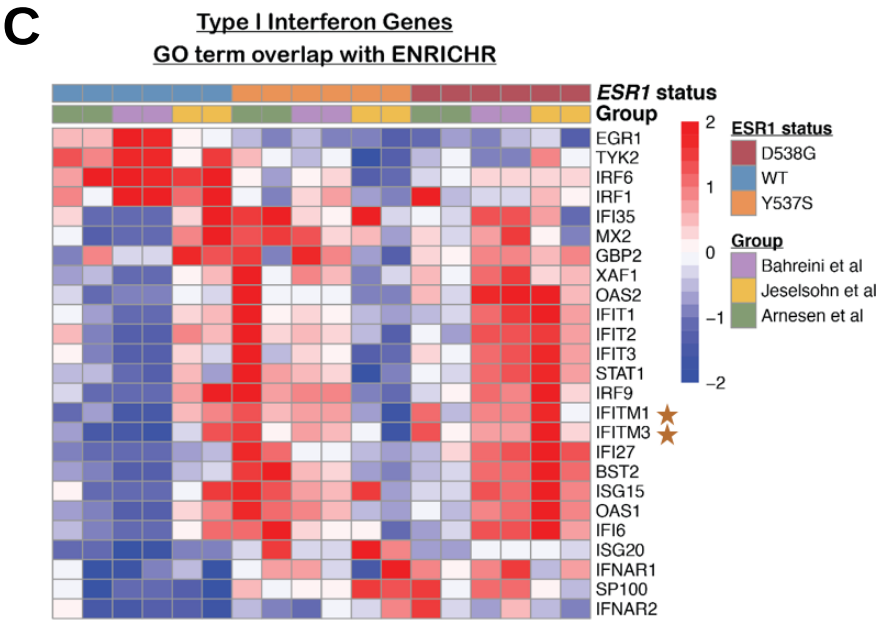
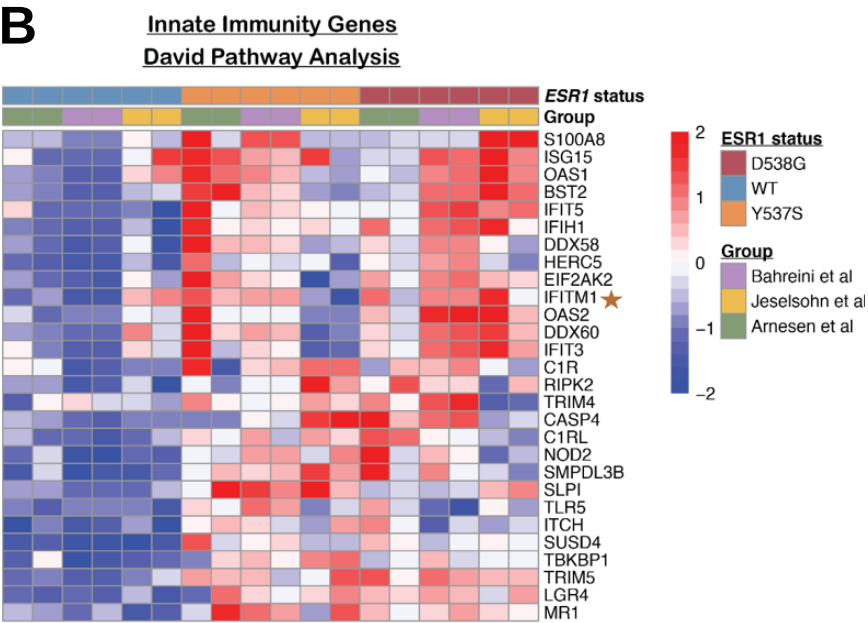
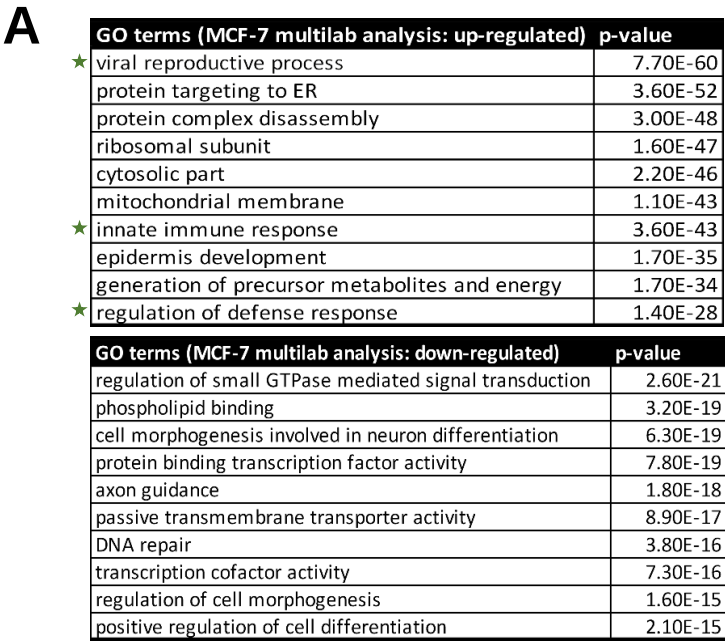
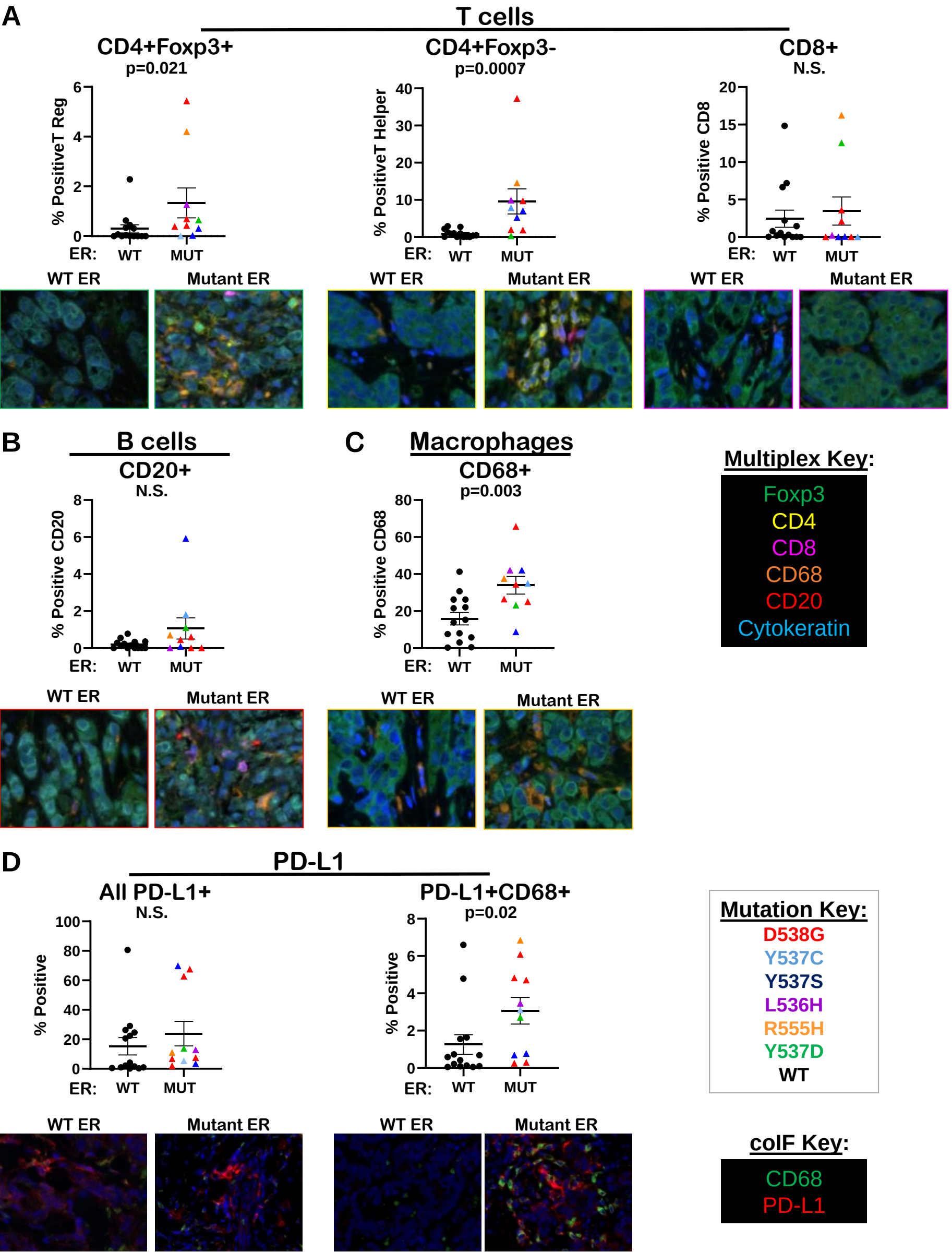


Figure 7



Activity of combined androgen receptor antagonism and cell cycle inhibition in androgen receptor-positive triple-negative breast cancer

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ABSTRACT

Triple-negative breast cancer (TNBC) is an aggressive subtype, with a peak recurrence rate within the first few years post diagnosis. Few targeted therapies are available to treat this breast cancer (BC) subtype, defined by the lack of estrogen and progesterone receptors (ER and PR) or amplification of human epidermal growth factor receptor 2 (HER2). While cell cycle cyclin dependent kinase (CDK) 4/6 inhibitors are approved for treatment of ER-positive BC, they have not proven effective as monotherapy in patients with TNBC. The androgen receptor (AR) has emerged as a therapeutic target in a subset of TNBC and with significant clinical benefit observed in multiple trials. The purpose of this study was to investigate the pre-clinical activity of the CDK4/6 inhibitor abemaciclib in combination with an agent that targets both androgen biosynthesis and AR activity, seviteronel, using TNBC cell lines expressing high AR, cell line xenografts and an AR positive, androgen-responsive TNBC patient-derived xenograft (PDX). Single cell RNA-sequencing demonstrated heterogeneity in AR levels, even in a highly AR+ cell line, and identified cell cycle pathway activation in AR^{High} versus AR^{Low} expressing cells. Combination treatment with the cell cycle CDK4/6 inhibitor abemaciclib and seviteronel showed synergy in an AR+ TNBC model compared to each drug alone.

Implications. While cell cycle inhibitors are FDA-approved for use in ER-positive breast cancer, our studies suggest that they may also be effective in AR+ TNBC, perhaps combined with AR targeted agents.

INTRODUCTION

Breast cancer (BC) is the most common cancer among women in the United States, and approximately 1 in 8 women will be diagnosed with breast cancer within their lifetime [1]. Breast cancers can be divided into three main subtypes: estrogen receptor alpha (ER α)-positive, human epidermal growth factor receptor 2 (HER2)-positive or triple-negative (TNBC); and treatment regimens are determined based on the subtype at diagnosis [2]. TNBCs, which account for approximately 15-20% of diagnosed BC cases [2], lack expression of ER α , the progesterone receptor (PR) and do not have amplified HER2. TNBC is notoriously difficult to treat, particularly once chemoresistant metastatic disease develops, and few targeted therapies are available for the majority of patients. Additionally, TNBC is the most aggressive BC subtype with a high rate of metastases in the first three years and a low 5-year survival rate [3]. Thus, development of additional therapeutic strategies for TNBC is an important avenue of study.

The androgen receptor (AR) has emerged as a potential therapeutic target in BC. More than 80% of all BCs and up to 50% of TNBCs overexpress AR [4-9]. In TNBC, AR supports cancer stem cell-like properties including anchorage-independent survival,

mammosphere formation and tumor initiation [10]. AR activation also promotes cell survival through regulation of the cell cycle [11]. Androgen withdrawal results in G1 arrest [12], and AR-dependent gene expression is highest during G1 and decreases throughout the cell cycle [13]. In fact, AR+ TNBC cells are highly sensitive to cyclin dependent kinase (CDK) 4/6 inhibition due to their dependence on these proteins to progress through the cell cycle [14]. Thus, combining standard of care chemotherapy with AR antagonists and/or CDK4/6 inhibitors may prove effective in this patient population and this strategy is being employed in clinical trials.

Anti-androgens have been under investigation in BC for over a decade [15, 16]. First generation anti-androgens, such as bicalutamide (Bic), function as competitive inhibitors that block AR transcriptional activity. The second generation anti-androgen enzalutamide (Enza) is a competitive inhibitor that also blocks nuclear localization of AR, and consequently has less partial agonist activity than first generation anti-androgens [17, 18]. Both of these AR antagonists are well tolerated in patients and have shown beneficial clinical activity in TNBC, with subsets of patients showing prolonged stable disease [19-21].

In order to maximize response rates of AR+ TNBC to anti-androgens, studies are ongoing to determine the best diagnostic cutoff levels of AR expression, identify gene expression signatures predictive of AR dependency [22], and evaluate newer generation AR targeted therapies that block AR activity through the inhibition of androgen biosynthesis. One such therapy is abiraterone acetate, a CYP17 hydroxylase inhibitor that blocks androgen synthesis and has shown promise in the clinical setting against AR+ TNBC [23]. However, a consequence of inhibiting CYP17 hydroxylase is the interruption of cortisol production, which necessitates supplementation with the corticosteroid prednisone [23, 24]. Seviteronel (Sevi; INO-464) is a dual CYP17 lyase inhibitor and AR antagonist (Supplemental Fig. 1), with preclinical activity in multiple tumor types including castration-resistant prostate cancer (CRPC), ER+ BC, TNBC and glioblastoma (GBM) [25-30]. It has undergone clinical investigation in CRPC, BC [31, 32] and GBM (NCT03600467). Since Sevi preferentially inhibits CYP17 lyase over hydroxylase, there is less impact on cortisol production compared to hydroxylase inhibitors such as abiraterone [33]. Thus, Sevi's dual mechanism of action of CYP17 lyase inhibition and AR antagonism combines the actions of Enza and abiraterone, potentially providing increased efficacy in treating AR+ TNBCs. The purpose of this study was to investigate the pre-clinical activity of Sevi in AR+ TNBC and further explore the efficacy of targeting both androgen biosynthesis and AR activity in combination with CDK4/6 inhibition [34].

METHODS

Cell culture and reagents. Human TNBC cell lines were cultured in 5% carbon dioxide (CO₂). Luminal AR MDA-MB-453 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in DMEM with 10% FBS. SUM159PT cells, expressing moderate levels of AR [35], were obtained in 2013 from the University of Colorado Cancer Center (UCCC) Tissue Culture Core (Aurora, CO) and maintained in Ham's/F-12 with 5% FBS, 1% HEPES, 1 µg/mL hydrocortisone and 5 µg/mL insulin. Only cells of under ten passages were used in this study. All cell lines were routinely tested for mycoplasma contamination, and human cell lines were authenticated in 2017 by short tandem repeat analysis in the UCCC Tissue Culture Core. The androgen dihydrotestosterone (DHT; Sigma-Aldrich Corporation, St. Louis, MO) used for in vitro experiments was diluted in ethanol (EtOH). The AR and CYP17 lyase inhibitor seviteronel (Sevi) was provided by Innocrin Pharmaceuticals, Inc. (Durham, NC). The CDK4/6 inhibitor Abemaciclib (Abem) was purchased from Selleck Chemical LLC (Houston, TX). All drugs were diluted in dimethyl sulfoxide (DMSO).

Drug sensitivity assays. Cells were plated in 96-well plates in quadruplicate or quintuplicate, and treated with either DMSO or increasing concentrations of Sevi or Abem. After 5 days of drug treatment cells were fixed with 10% formalin, stained with 0.1% crystal violet dye, and dye was then solubilized with 10% acetic acid. Absorbance was measured at 570 nm. Data are presented as percent cell growth and normalized to the mean absorbance of DMSO-treated cells. Synergy was calculated with CalcuSyn Software (Biosoft Inc.) using mean percent growth inhibition values from crystal violet cell viability assays. CalcuSyn software uses the Median Effect method to determine synergy where a combination index (CI) < 0.9 indicates synergy, CI = 0.9–1.1 indicates additivity, and CI > 1.1 indicates antagonism [36].

In vivo preclinical models. All in vivo experiments were performed in accordance with NIH Guidelines of Care and Use of Laboratory Animals. Mice were euthanized by CO₂ inhalation followed by cervical dislocation. Tissue was immediately frozen whole in liquid nitrogen for RNA analysis or fixed in 10% buffered formalin for histological analysis. Xenograft studies. 1x10⁶ MDA-MB-453 cells were bilaterally, orthotopically injected into the mammary fat pads of cycling female nu/nu mice. There was no hormone supplementation in this experiment. When tumors reached a size of approximately 100 mm³, mice were randomized and matched into treatment groups (n=5-12 mice per group). Varying doses of Sevi and/or Abem were administered by oral gavage daily for 4 weeks. Tumors were measured weekly by caliper. Patient-derived xenograft (PDX) studies. HCl-009 is a TNBC PDX originally developed in the laboratory of Dr. Alana Whelm [37] and propagated in immunodeficient NOD/SCID/gamma (NSG) mice. In the DHT experiment (n=8 mice per group), cycling female NSG mice were

implanted with cellulose control or slow release 8mg DHT pellets, prepared in Dow Corning silastic tubing (Fisher Scientific, Hampton, NH). PDX tumor portions were then bilaterally, orthotopically injected into the mammary fat pads, and tumors were measured weekly by caliper for 9 weeks. In the Sevi experiment, PDX tumor portions were bilaterally, orthotopically injected into the mammary fat pads of cycling female NSG mice. When tumors reached a size of approximately 100 mm³, mice were randomized and matched into treatment groups (n=4-5 mice per group). 150mg/kg/day Sevi was administered daily by oral gavage for 2 weeks. Tumors were measured weekly by caliper.

Histology. For the analysis of MDA-MB-453 cultured cells, cells were fixed in 10% buffered formalin and pelleted in Histogel from ThermoFisher Scientific Inc. (Waltham, MA). The UC Denver Tissue Biobanking and Processing Core performed all tissue and cell processing and paraffin embedding. Five-micrometer sections of formalin-fixed paraffin-embedded (FFPE) samples were used for analyses. Immunohistochemistry (IHC). Sections were deparaffinized in a series of xylenes and ethanols, and antigens were heat-retrieved in either 10mM citrate buffer pH 6.0 or 10mM Tris, 1mM EDTA pH 9.0. Antibodies used included the following: rabbit monoclonal antibody specific for AR (#200R clone SP107, Cell Marque), rabbit polyclonal antibody specific for FKBP5 (#8245, Cell Signaling Technology), rabbit polyclonal antibody specific for PSA (#A0562, Agilent Technologies Inc., Santa Clara, CA), goat polyclonal antibody specific for GDF15 (#AF957, R&D Systems) and rabbit polyclonal antibody specific for CHI3L1 (#ab77528, Abcam). Tris-buffered saline with 0.05% Tween 20 was used for all washes. AR, FKBP5, PSA, and CHI3L1 antibodies were detected with EnVision-HRP anti-rabbit polymer (#K4003, Agilent), while the GDF15 antibody was detected with a biotinylated donkey anti-goat secondary antibody (#705-065-147, Jackson ImmunoResearch Laboratories, Inc.) followed by streptavidin-HRP (#P0397, Agilent). Representative images were taken using a BX40 microscope (Olympus, Center Valley, PA) with a SPOT Insight Mosaic 4.2 camera and software (Diagnostic Instruments, Inc., Sterling Heights, MI). Expression levels were scored visually by an experienced histotechnician for the entire sample and presented as an IHC score, calculated by multiplying the average staining intensity by the percentage of positive cells. Tumor necrosis. Mammary tumor sections were H&E stained and analyzed for necrosis by quantifying by a board-certified veterinary pathologist. Data was presented as the percentage of each tumor that was composed of necrotic dead cells.

Bioinformatics. Bulk RNA-sequencing. RNA isolated from frozen HCI-009 PDX mammary tumors was used to prepare Illumina HiSeq libraries according to manufacturer's instructions for the TruSeq Stranded RNA kit (Illumina Inc, San Diego, CA). The mRNA template libraries were then sequenced as single pass 50bp reads on

the Illumina HiSeq4000 platform at the University of Colorado's Genomics and Sequencing Core Facility. Derived sequences were analyzed by applying a custom computational pipeline consisting of the open-source gSNAP, Cufflinks, and R for sequence alignment and ascertainment of differential gene expression [38]. In short, reads generated were mapped to the human genome (GRCh38) by gSNAP [39], expression (FPKM) derived by Cufflinks [40], and differential expression analyzed with ANOVA in R. Differentially expressed genes (FDR < 0.05 for DHT and $p < 0.05$ for Sevi, due to a lack of statistical power) with a minimum expression ratio of 1.15 were used for downstream analyses. These data are available in the Gene Expression Omnibus (GEO) database as GSE152246 for the DHT experiment (Supplemental Fig. 2A) and GSE152318 for the Sevi experiment (Supplemental Fig. 2B). Single cell RNA-sequencing. 500,000 MDA-MB-453 cells were plated in two T25 flasks. After 48 hours cells were trypsinized, washed and prepared for scRNAseq analysis via the 10X Genomics platform and Illumina NovSeq 6000 platforms [41] at the University of Colorado's Genomics and Sequencing Core Facility using the 10X Genomics Sample Preparation Protocol. 3000 cells were sequenced with a minimum read-depth of 75,000 reads/cell. Read mapping and expression quantification was performed using a combination of the 10X Cellranger pipeline and custom analytic scripts. Briefly, single-cell reads were mapped to the human genome (GRCh38) and assigned to genes using the standard CellRanger pipeline. The R packages Monocle [42-44] and Seurat [45] were used for differential expression. The cell cycle heatmap, using 10 cell aggregates, was generated using the Morpheus Software Package. These data are available in the GEO database as GSE152315. Downstream RNAseq analyses were performed using Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany) and gene set enrichment analyses (GSEA) [46, 47]. Overlap between significantly differentially expressed genes from the MDA-MB-453 scRNAseq and HCI-009 Sevi experiments were compared using the web application BioVenn [48].

Statistical analysis. Statistically significant differences (p values < 0.05) were calculated using the GraphPad Prism 7.0 statistical program. Single variable comparisons were made with two-tailed unpaired t-tests. A repeated measure two-way ANOVA was used for in vivo tumor data analysis.

RESULTS

Seviteronel effectively limits AR activity in TNBC.

Sevi has been shown to target AR transcriptional activity in prostate and BC with preclinical activity similar to previously approved AR-targeted therapies [25-27, 49], and a recent publication found that Sevi sensitizes TNBC cells to radiation [50]. To determine the effect of Sevi on TNBC proliferation, AR+ MDA-MB-453 cells were

244 treated with increasing concentrations of Sevi, resulting in the inhibition of cell
245 proliferation at concentrations greater than 10 μ M (Fig. 1A). This was further confirmed
246 in the TNBC cell line SUM159PT (Fig. 1A), which express moderate levels of AR [10].
247 Given the ability of Sevi to inhibit androgen synthesis in addition to inhibiting AR directly
248 [49], as compared to other AR antagonists, we examined its effectiveness against
249 TNBC tumor growth in vivo using a xenograft model. Normal cycling female mice were
250 used as a more clinically relevant model of female patients with low levels of androgen
251 synthesis. Sevi significantly inhibited tumor volume and growth rate in a dose-
252 dependent manner (Fig. 1B-C). Together these data indicate that Sevi effectively
253 targets the pro-proliferative activities of AR in TNBC cell lines in culture and in vivo.

254
255 Patient-derived xenografts (PDXs) are preclinical models that more closely mimic
256 patient tumor heterogeneity and clinical responses to therapeutic agents than traditional
257 cultured cell lines [51]. HCI-009 is an AR+ TNBC PDX with a distinctly heterogeneous
258 AR staining pattern (Fig. 2A). In mice supplemented with AR agonist
259 dihydrotestosterone (DHT) HCI-009 PDX tumor growth was significantly increased (Fig.
260 2B) and AR protein levels were upregulated (Fig. 2C), indicating that this PDX model is
261 androgen-responsive. To evaluate the transcriptional activity of AR in these tumors,
262 known AR-regulated genes were examined for upregulation at the protein level by
263 immunohistochemistry (IHC). Expression of the AR-regulated proteins FKBP prolyl
264 isomerase 5 (FKBP5), prostate specific antigen (PSA), growth differentiation factor 15
265 (GDF15) and chitinase 3-like 1 (CHI3L1) was significantly upregulated in HCI-009 PDX
266 tumors from mice supplemented with DHT (Fig. 2D). Similarly, RNA-sequencing
267 (RNAseq) of HCI-009 tumors from mice supplemented with DHT (Supplemental Fig. 2A)
268 showed upregulation of known AR-regulated genes, such as *KLK3*, the gene that
269 encodes PSA (Fig. 2E, Supplemental Table 1) and many others.

270
271 After confirming that HCI-009 PDX tumors have active AR and are growth stimulated by
272 exogenous androgen, we tested responsiveness to Sevi in mice that did not have
273 exogenous androgen supplementation. Mice with HCI-009 PDX mammary tumors were
274 treated with 150mg/kg/day Sevi, and tumor volume and rate of growth were significantly
275 decreased by Sevi treatment (Fig. 3A-B). Sevi increased tumor necrosis (Fig. 3C) and
276 significantly decreased AR protein levels (Fig. 3D). RNAseq showed significant changes
277 in gene expression following Sevi treatment, inducing a distinctive Sevi gene signature
278 that included known AR-regulated genes (Fig. 3E, Supplemental Fig. 2B, Supplemental
279 Table 2). Pathway analysis performed on RNAseq data further demonstrated that while
280 Sevi affects several biological pathways, the Androgen Response pathway (normalized
281 enrichment score, NES=1.69) was one of the top altered pathways (Fig. 3F). Overall,
282 these data support the function of Sevi as a potent inhibitor of AR and tumor growth in
283 this AR+ TNBC PDX.

AR is a positive regulator of the cell cycle

While MDA-MB-453 cells are highly AR+, IHC demonstrated heterogeneity in cell-specific AR levels within this cell line (Fig. 2A). To examine gene expression associated with varying levels of AR, single-cell RNAseq (scRNAseq) was performed on MDA-MB-453 cells cultured in 10% serum containing endogenous levels of androgens (Supplemental Fig. 3A). MDA-MB-453 cells were divided into AR^{High} (1,205 cells) and AR^{Low} (948 cells) populations based on the degree of AR mRNA expression (Fig. 4A, Supplemental Tables 3 and 4 for gene lists). Pathway analysis on genes associated with AR^{High} versus AR^{Low} levels indicated that the Cell Cycle ($z=4.12$, $p=1.13E-45$) was one of the top pathways associated with high AR expression (Fig. 4B, Supplemental Fig. 4B). A large percentage (54%) of cell cycle related genes were upregulated in high AR expressing MDA-MB-453 cells (Fig. 4C, Supplemental Table 4 highlighted genes). Interestingly, when the genes associated with AR^{High} MDA-MB-453 cells (Fig. 3) were compared to the genes altered in HCI-009 PDX tumors treated with Sevi approximately 65% of genes altered by Sevi overlap with the MDA-MB-453 AR^{High} related gene signature (Supplemental Fig. 4, Supplemental Table 5), providing further evidence to support the AR-directed effects of Sevi in TNBC, although non-AR-directed Sevi effects cannot be ruled out.

Combination targeting of AR and CDK4/6 is effective in AR+ TNBC

Based on the link between AR levels and cell cycle activation in TNBC cells (Fig. 4), we aimed to evaluate dual targeting of AR and the cell cycle as a potential treatment strategy for AR+ TNBC. A previous investigation of combination therapy, using different early generation inhibitors, showed some promise clinically (NCT02605486; pablociclib with bicalutamide in AR+ metastatic BC patients) [52-54]. We tested combination treatment with Sevi and the CDK4/6 inhibitor abemaciclib (Abem, [55]) in a preclinical TNBC model. In MDA-MB-453 cells dual treatment showed synergy at multiple concentration combinations, analyzed by the Chou Talalay method [36], indicated by combination indexes less than 0.9 (Fig. 5A). While Abem is currently FDA approved for use in ER-positive breast cancer patients [56], our pre-clinical analysis showed that it is effective in inhibiting MDA-MB-453 tumor growth as well (Fig. 5B). These data led us to examine how dual treatment with Sevi and Abem affected MDA-MB-453 tumor growth in vivo. Combination treatment significantly inhibited tumor growth when compared to vehicle alone and was more effective than Abem treatment alone (Fig. 5C). Interestingly, the anti-tumor effects of combined treatment primarily occurred during the final week of treatment (weeks 3-4) where we observed the most significant reduction in tumor growth (Fig. 5D-E). Moreover, the combination treatment was well tolerated with no negative effects on mouse weight gain (Supplemental Fig. 5). These data suggest

that the combination of AR target therapies with cell cycle inhibitors may prove to be an effective treatment for patients with AR+ TNBC.

DISCUSSION

AR signaling has emerged as an effective targetable pathway in a subset of TNBCs, and first- and second-generation anti-androgens such as bicalutamide and enzalutamide have shown promising clinical activity in patients with AR+ TNBC. Sevi is unique in its ability to directly antagonize AR as well as inhibit the androgen-synthesizing enzyme CYP17 lyase, with less interference on corticosteroid production than earlier generations of anti-androgens [27]. Similar to what has previously been reported in prostate cancer [57], Sevi is as effective as Enza at inhibiting AR activity in TNBC cells [49]. Due to its mechanistic differences from earlier generation AR anti-androgens, Sevi's effectiveness was predicted to be particularly pronounced in vivo because of its ability to decrease ligand availability [25, 49]. Our data demonstrate that Sevi significantly inhibited an AR+ TNBC cell line xenograft in a dose-dependent manner (Fig. 1). Our data is the first evidence that Sevi is able to inhibit proliferation of a AR+ TNBC PDX tumor model as well (Fig. 3). Thus, Sevi shows promising anti-tumor activity in both cell line and PDX preclinical models of TNBC.

While the activity of Sevi as an inhibitor of both AR activity and ligand synthesis has been demonstrated [49, 57], there are likely other mechanisms of action contributing to its effect on TNBC growth. However, our data indicates that a proportion of the anti-tumor effects of Sevi are AR-directed, as shown by the "androgen response pathway" being one of the top biological pathways associated with Sevi treatment in vivo (Fig. 3F) and by the large overlap between genes altered by Sevi treatment and upregulated in AR^{High} MDA-MB-453 cells (Supplemental Fig. 4). Future studies are needed to confirm the anti-tumor contribution of other pathways affected by Sevi.

In addition to targeting AR directly and inhibiting ligand synthesis, targeting additional points within the AR signaling cascade maybe therapeutically beneficial since clinical data from prostate cancer show that acquired resistance to AR targeted therapies can develop over time [58]. While this has yet to be shown for BC, being mindful of acquired resistance from the outset could lead to more effective combination therapies for patients prior to their clinical need. Our data indicate that blocking the cell cycle may be an effective cooperative therapeutic approach (Fig. 4).

During the cell cycle, CDK4/6 binds to cyclin D to mediate phosphorylation and deactivation of Retinoblastoma (Rb) protein, which allows for G0/G1 to S phase progression through the cell cycle. ER α -positive BCs upregulate the Rb pathway

making CDK4/6 targeted therapies an attractive therapeutic option. The FDA (Food and Drug Administration) approved multiple CDK4/6 inhibitors for use in the treatment of ER α -positive BC, including palbociclib, ribociclib and Abem between 2015-2017 [59]. While a portion of TNBCs have RB1 mutations and/or loss, making CDK4/6 inhibition impractical, AR and Rb expression are positively associated suggesting the AR+ TNBCs may benefit from CDK4/6 blockade [59]. In fact our data indicate that not only do AR+ TNBC xenografts respond well to the CDK4/6 inhibitor Abem, but that the combination of Abem and Sevi was even more effective than Abem alone (Fig. 5). While CDK4/6 inhibitors are not currently approved for use in TNBC, our pre-clinical data suggest that this may be an effective treatment strategy in AR+ TNBC, especially when combined with AR targeted therapies.

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FIGURE LEGENDS

Figure 1. TNBC sensitivity to Sevi in a preclinical models. (A) TNBC cell lines were cultured in full serum media. Cell viability was determined by crystal violet assay after a 5-day exposure to DMSO (0) or increasing concentrations of Sevi. Data was normalized to the mean absorbance of DMSO-treated cells. Mean $\pm$ standard deviation. **(B)** MDA-MB-453 cells were injected into the mammary fat pads of cycling female nu/nu mice. Supplemental DHT (dihydrotestosterone) was not given in this experiment. Sevi was administered daily. Tumor volume was measured by calipers and **(C)** the rate of tumor growth per day over four weeks was calculated. Mean $\pm$ standard error of the mean; * $p < 0.05$, **** $p < 0.0001$.

Figure 2. Androgen responsive TNBC PDX model. (A) TNBC PDX HCI-009 was propagated in NSG mice. Tumor tissue was stained for AR by IHC. AR staining in MDA-MB-453 cells is shown for comparison. **(B-E)** HCI-009 PDX tumor portions were injected into the mammary fat pads of female NSG mice supplemented with cellulose, as a control, or DHT (dihydrotestosterone; 8mg/pellet). **(B)** Tumor volume was measured with calipers. **(C)** AR was stained by IHC. **(D)** IHC staining of known AR-regulated genes. Quantification of staining is presented as an IHC score, combining cell positivity and staining intensity. **(E)** Shown are the top 30 up- and downregulated genes identified by RNAseq in HCI-009 PDX tumors from mice treated with DHT. Mean $\pm$ standard error of the mean; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; scale bars = 50 μ m.

Figure 3. AR-positive TNBC PDX sensitivity to AR-targeted therapy. HCI-009 PDX tumor portions were injected into the mammary fat pads of female NSG mice. Mice were treated daily with vehicle or 150mg/kg/day Sevi (seviteronel). **(A)** Tumor volume was measured with calipers and **(B)** the rate of tumor growth per day over two weeks was calculated. **(C)** The percentage of tumor necrosis and **(D)** AR positive cells per tumor were quantified; scale bar = 50µm. **(E)** Shown are the top 30 up- and downregulated genes and **(F)** most highly correlated biological pathways identified by Gene Set Enrichment (GSEA) in RNAseq data from HCI-009 PDX tumors from mice treated with Sevi. Mean ± standard error of the mean; * $p < 0.05$, ** $p < 0.01$.

Figure 4. The association between high AR expression and cell cycle pathway activation. scRNAseq was performed on untreated MDA-MB-453 cells cultured in full serum media. Cells were divided into groups based on their expression of AR, AR^{Low} and AR^{High}. **(A)** Histogram of the distribution of AR expression in single MDA-MB-453 cells including AR level group determination. **(B)** Ingenuity pathway analysis was performed on genes differentially expressed between AR^{Low} and AR^{High} groups. Shown are the top 15 most significantly altered disease and function pathways. **(C)** Differential expression of genes involved in cell cycle indicating cell cycle activation associated with high AR expression, shown as 10 cell aggregates.

Figure 5. Combination treatment of TNBC cells with an anti-androgen and cell cycle inhibitor. **(A)** MDA-MB-453 cells were cultured in full serum media. Cell viability was determined by crystal violet assay after a 5-day exposure to DMSO (0) or increasing concentrations of Sevi (seviteronel) and Abem (abemaciclib; CDK4/6 inhibitor). Data was normalized to the mean absorbance of DMSO-treated cells and presented as mean percent growth inhibition. CalcuSyn software was used to calculate the degree of synergy between combination treatments, represented as the combination index. Combination indexes < 0.9 indicate synergy. **(B)** MDA-MB-453 cells were bilaterally injected into the mammary fat pads of cycling female nu/nu mice. Abem was administered daily. Tumor volume was measured with calipers. **(C-E)** MDA-MB-453 cells were injected into the mammary fat pads of cycling female nu/nu mice. Abem and/or Sevi were administered daily. **(C)** Tumor volume was measured with calipers and **(D)** the rate of tumor growth per day over four weeks was calculated. Rate of tumor growth was broken down to illustrate the differences in tumor growth over the first 3 weeks of treatment compared to the final week alone. **(E)** Change in tumor volume, either growth or reduction, during the final week of treatment is depicted for each individual mouse. Mean ± standard error of the mean; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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628

Figure 1

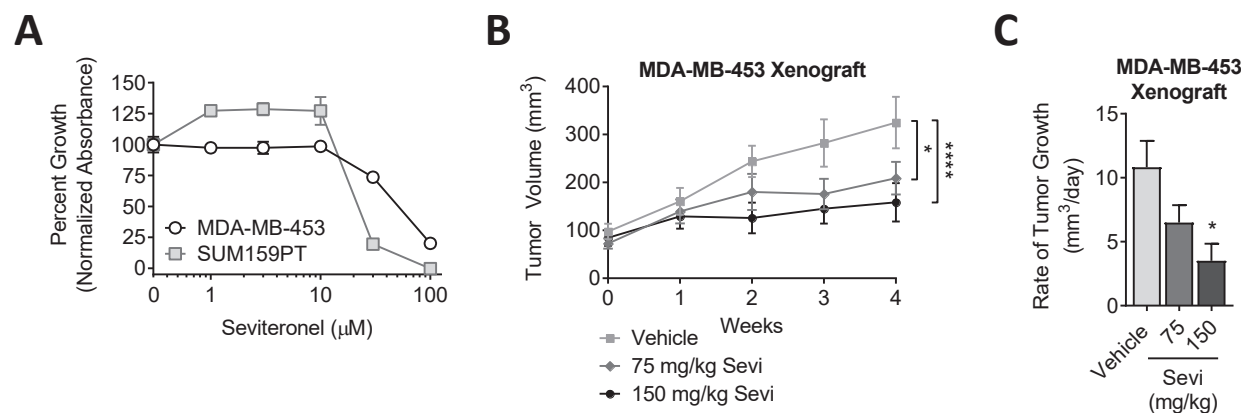


Figure 2

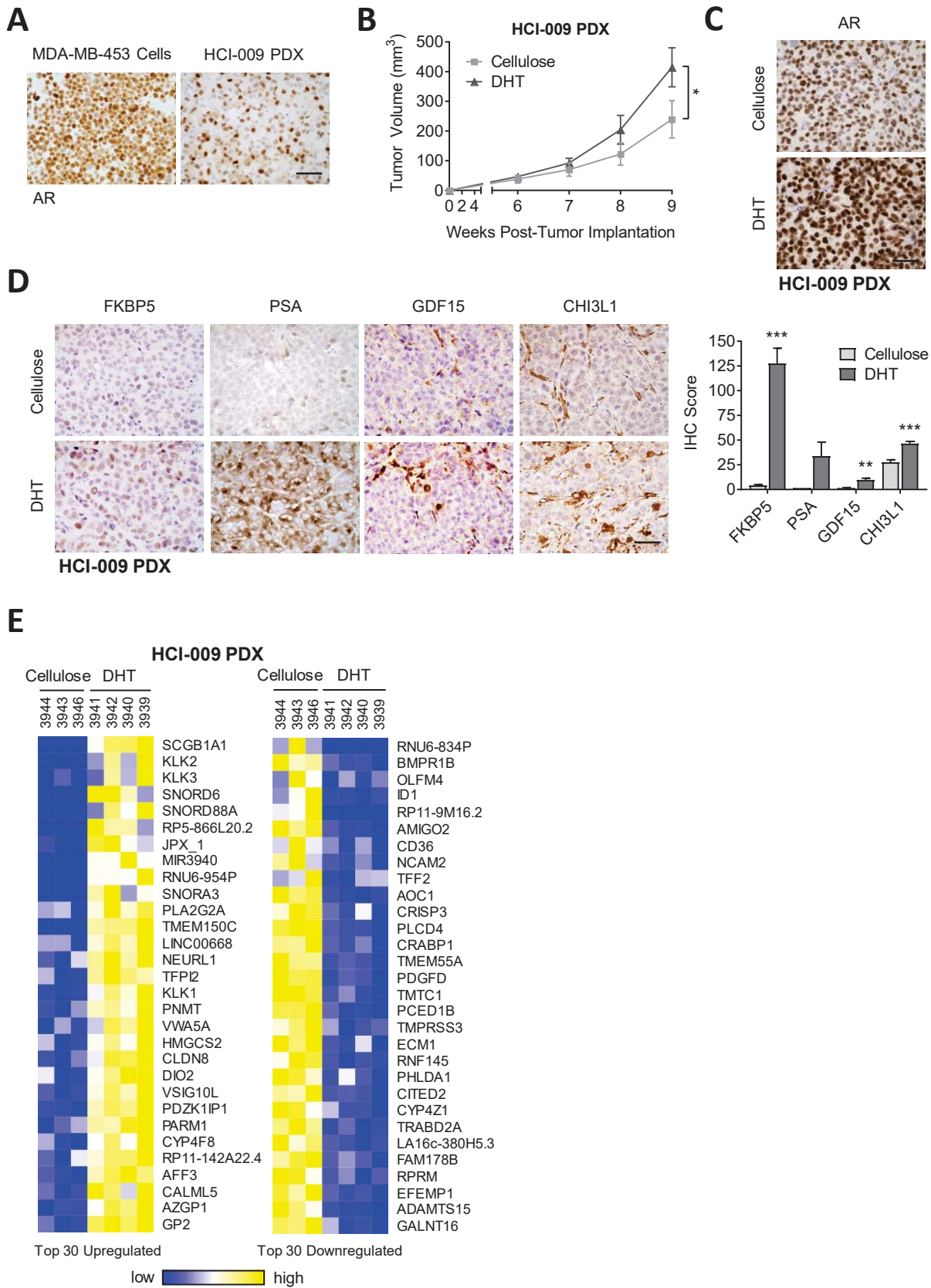


Figure 3

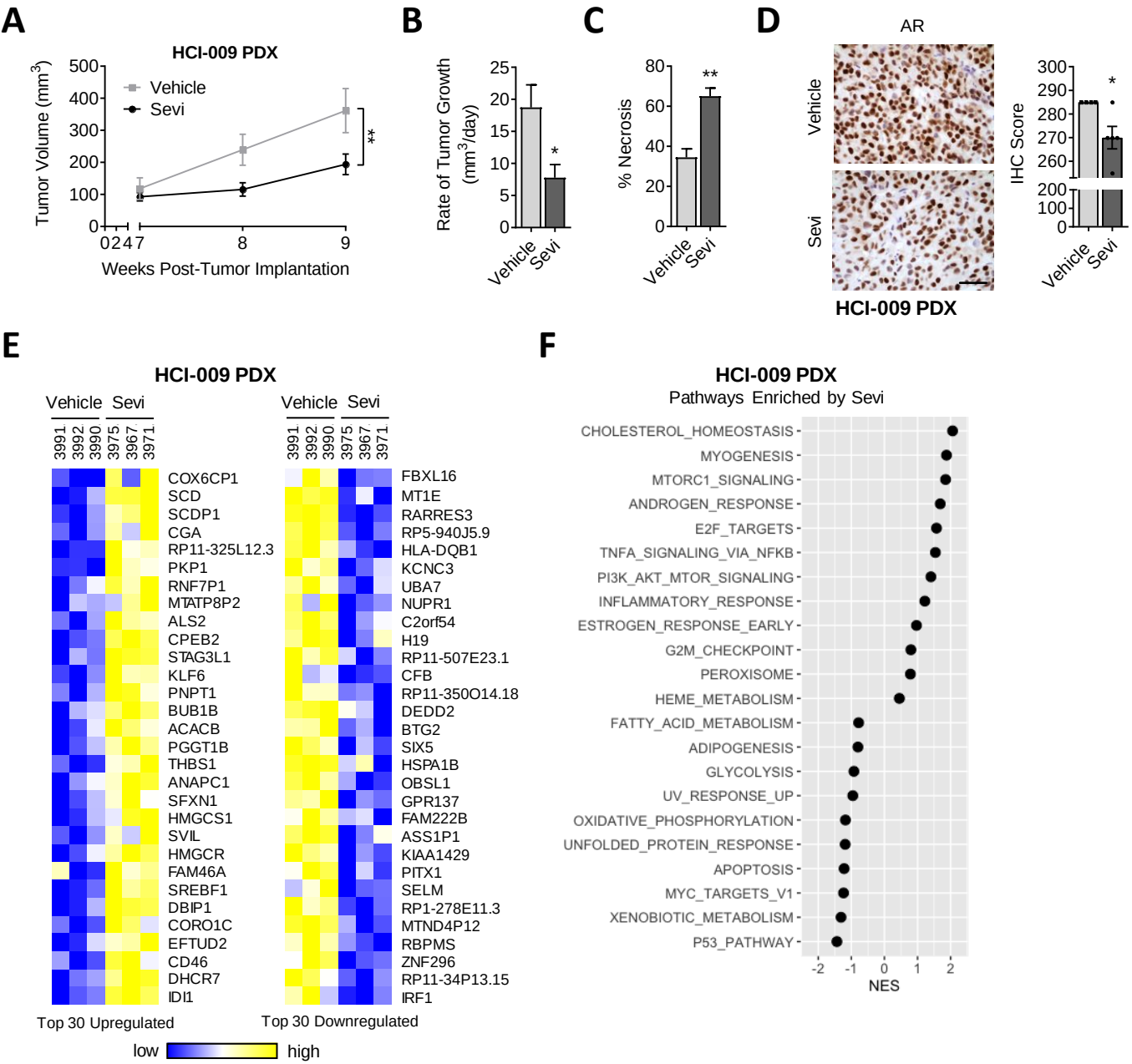


Figure 4

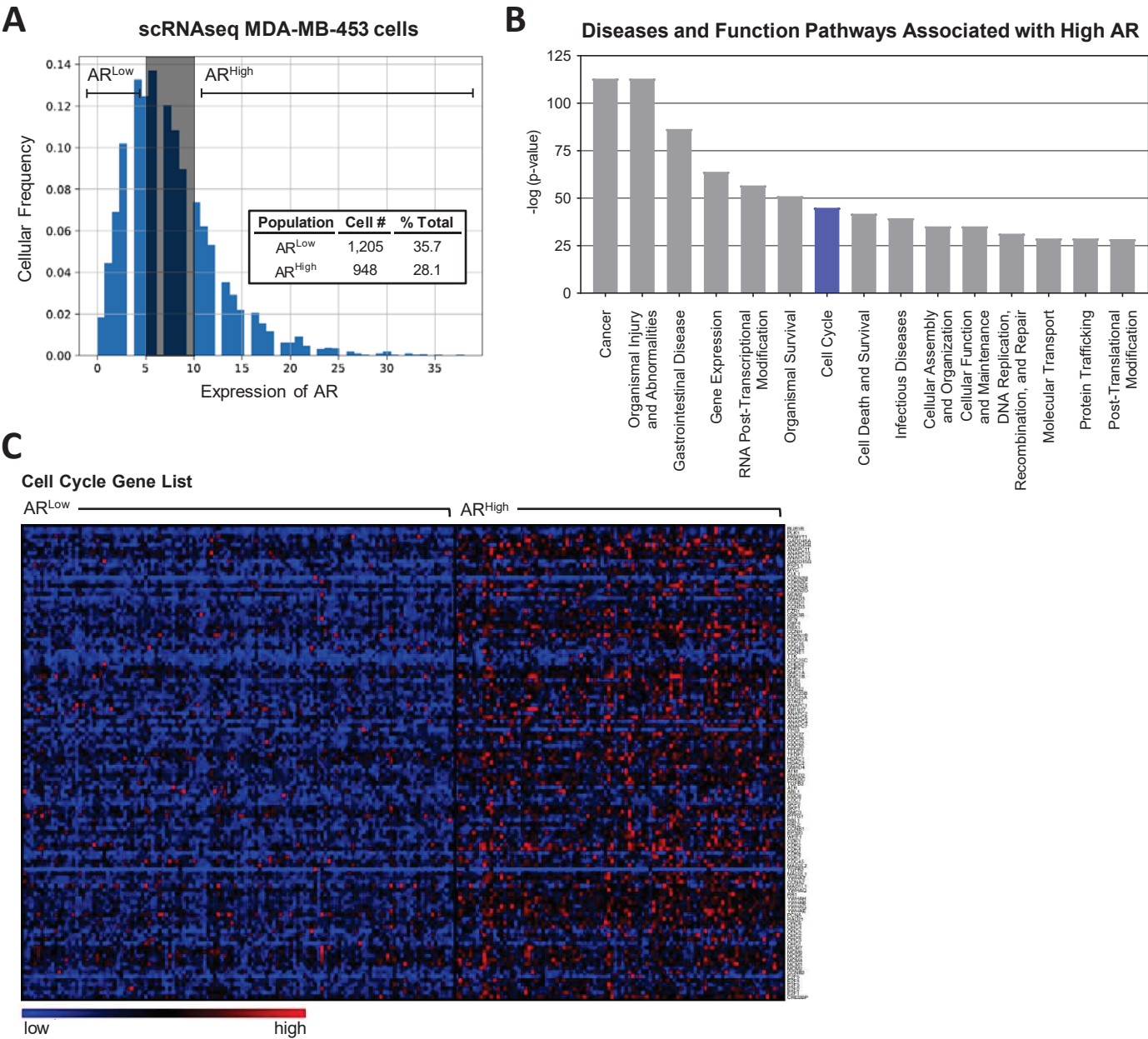
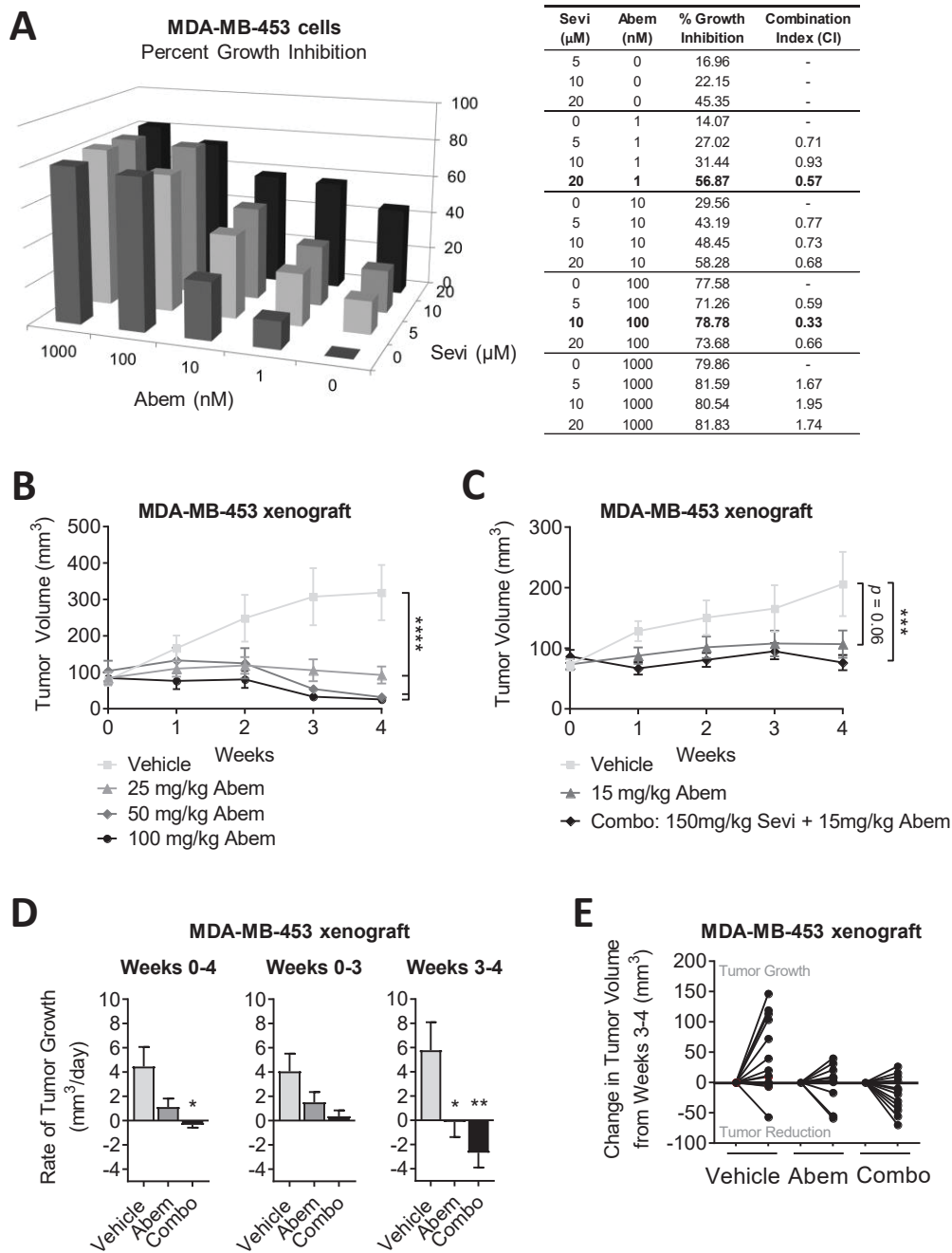


Figure 5



Endocrinology

A Positive Feedback Loop Between TGF β and Androgen Receptor Supports Triple-Negative Breast Cancer Anoikis Resistance

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Working Title: A Positive Feedback Loop Between TGF β and Androgen Receptor Supports Triple-Negative Breast Cancer Anoikis Resistance

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Abstract:

Triple-negative breast cancer (TNBC) is an aggressive subtype with peak recurrence as metastatic disease within the first few years of diagnosis. Androgen receptor (AR) expression is increased in anchorage independent cells in TNBC pre-clinical models. Both AR knockdown and inhibition lead to reduced TNBC invasion *in vitro*, and reduced tumorigenicity and less recurrence *in vivo* in pre-clinical models. Transforming growth factor β (TGF β) pathway gene signatures also increased during anchorage independent survival both *in vitro* and *in vivo* in pre-clinical models and in circulating tumor cells (CTC) from patients during emergence of chemo resistant disease. We hypothesized that a positive loop between AR and TGF β signaling facilitates TNBC anchorage independent survival. We find that multiple components of the TGF β pathway, including TGF β 1 and 3, as well as pathway activity measured by nuclear localization and transcriptional activity of phosphorylated Smad3 (pSmad3), are enhanced in anchorage independent conditions. Further, exogenous TGF β increased AR protein while TGF β inhibition decreased AR and TNBC viability, particularly under anchorage independent culture conditions. ChIP-seq experiments revealed AR binding to *TGFB1* and *SMAD3* regulatory regions. In clinical datasets, *TGFB3* and *AR* were positively correlated and co-expression corresponded to significantly worse recurrence-free and overall survival in both ER negative and basal-like breast cancer. Finally, inhibiting both AR and TGF β decreased cell survival, particularly under anchorage independent conditions where the effect was more than additive. These findings warrant further investigations into whether combined inhibition of AR and TGF β pathways might decrease metastatic recurrence rates and mortality from TNBC.

Introduction:

Triple-negative breast cancer (TNBC), characterized by the lack of three important biomarkers, estrogen receptor alpha (ER), progesterone receptor (PR), and amplification of human epidermal growth factor receptor 2 (HER2), is an aggressive breast cancer (BC) subtype with few FDA-approved targeted therapies compared to other subtypes [1-3]. Cytotoxic chemotherapeutic agents and radiation remain the standard of care [4]. However, the peak risk of recurrence for TNBC is between 1-3 years following surgery, and 70% of deaths occur within the first 5 years post-diagnosis [5], demonstrating a persistent need for novel therapeutic interventions or new combinations of targeted therapeutics.

Although TNBC lack ER and PR, between 20-50% of primary tumors express nuclear androgen receptor (AR) protein, depending on the study, antibody used, and the criteria for positivity [6-9]. Studies investigating AR action in TNBC point to a role in tumor cell survival, growth, cancer stem cell (CSC) properties, and specific steps in the metastatic cascade, such as anchorage independent survival or “anoikis resistance” [10, 11]. In 2005, Farmer *et al* coined the term “molecular apocrine” to describe an ER negative (ER-) subtype of BC with high AR and an increased AR gene signature, laying the groundwork for establishing AR as a potential driver and target in a subset of TNBC [12]. In 2006, Doane *et al* identified an ER-/PR- BC subset with a gene expression profile similar to that of ER+ BC that responded to androgens [13]. TNBCs with high expression of AR mRNA, later termed the “luminal AR-subtype” (LAR) [14, 15], have a gene expression profile more similar to ER+ BC than the other TNBC subtypes, because in AR regulates many of the same genes as ER, that are associated with the “luminal” gene signature [7, 14, 15]. However, other TNBC subtypes often express AR, albeit at lower levels, and also rely on AR for cell survival, including cell lines representative of the TNBC mesenchymal (M) or

mesenchymal stem-like/claudin-low (MSL/CL) subtypes [10, 11, 14-17]. Further, when TNBC cells are cultured under anchorage independent conditions, AR transcript levels, protein levels, and transcriptional activity increase, and inhibition or knockdown of AR dramatically decreases growth on soft agar, mammosphere formation, invasion, and tumorigenicity [10, 11]. AR is mutually exclusive with cleaved caspase 3 in TNBC in anchorage independent cells, indicating that AR protects against cell death due to detachment, a process called anoikis [11].

Transforming growth factor β (TGF β) signaling can promote epithelial-to-mesenchymal transition (EMT) and the early stages of metastasis [18]. TNBC M or MSL/CL cell lines have high TGF β gene signatures and high enrichment scores for TGF β signaling [14, 15, 17]. In addition, TGF β ligand promotes proliferation of M and MSL/CL TNBC cell lines [17]. Patient circulating tumor cells (CTCs) isolated by microfluidic antibody capture have higher characteristics of EMT and the most significantly upregulated gene signature in the more mesenchymal CTCs was the TGF β pathway [19]. Both AR and TGF β pathway transcripts increased in CTCs from TNBC patient derived xenografts (PDXs) and in micrometastases as compared to primary tumors [20].

AR and the TGF β pathway have been linked in prostate cancer. In chromatin immunoprecipitation (ChIP) assays in prostate cancer cells, following Activin A (a TGF β superfamily ligand) treatment, phosphorylated Smad3 (pSmad3) bound to the AR promoter at Smad-binding elements (SBEs). pSmad3 has also been described as an AR co-regulator [21-23]. Furthermore, using promoter deletion constructs in prostate cancer cell lines, multiple functional androgen response elements (AREs) upstream of the *TGFB1* start site establish AR as a direct regulator of *TGFB1* [24, 25]. Collectively, these studies suggested a positive feedback loop

between the AR and TGF β pathway in prostate cancer, but this relationship, or its role in supporting anchorage independent survival, has not been investigated in TNBC.

We hypothesized that a positive feedback loop between AR and TGF β signaling supports anoikis resistance in AR+ TNBC and the ability to rapidly deploy this feed-forward loop contributes to the early risk of metastatic recurrence in TNBC. Identification of both TGF β and AR as drivers of anchorage independent survival and determining how these two pathways intersect may provide rationale towards a combinatorial therapeutic strategy to reduce and or treat metastatic TNBC.

Methods:

Cell lines

Only cells under 10 passages were used in this study. MDA-MB-453 (LAR) cells (purchased from ATCC in 2012), were grown in DMEM with 10% fetal bovine serum (FBS). SUM159PT (Mesenchymal Stem-Like/Claudin-low; MSL/CL) cells (purchased from the UCCC Tissue Culture Core in 2013) were grown in Ham's F-12 with 5% FBS, hydrocortisone, insulin, and HEPES. BT549 (Mesenchymal; M) cells (ATCC in 2008), were grown in RPMI 1640 with 10% FBS and insulin. All cell lines were authenticated by short tandem repeat analysis (Promega) at the University of Colorado Cancer Center (UCCC) Tissue Culture Core and tested negative for Mycoplasma in 2019. Cells were maintained at 37°C in a humidified incubator containing 95% air and 5% CO₂.

Reagents

Cells were treated with TGF β receptor 1 inhibitors, 10 μ M LY2109761 or 10 μ M LY2197299 (Selleckchem #S2704 and #S2230, respectively), which came pre-dissolved in Dimethyl

sulfoxide (DMSO), 10nM of dihydrotestosterone (DHT) in ethanol, 40μM of Enzalutamide (Medivation) in DMSO.

Gene expression array analysis

BT549 cells were grown in attached or forced suspended culture (on poly-Hema coated plates) in quadruplicate for 24 hours and harvested for RNA using TRIzol as previously reported [11]. The array data are available in the Gene Expression Omnibus (GEO) database as GSE95472. Gene expression values from this study were subjected to Gene Set Enrichment Analysis (GSEA) and changes in certain pathways were examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) gene list. Normalized enrichment scores (NES) and p-values were calculated using GSEA software [26]. Microarray and MetaCore Pathway Analysis (Thomson Reuters) were performed as previously described [11].

Forced suspension (Anchorage independent) culture

Poly-2-hydroxyethyl methacrylate (poly-HEMA, Sigma) was reconstituted in 95% ethanol to a final concentration of 11 mg/mL. Cell/tissue culture plates were coated with reconstituted poly-HEMA and incubated overnight to allow ethanol evaporation. Plates were washed with PBS prior to use.

Quantitative RT-PCR

RNA was isolated by TRIzol (Invitrogen), and cDNA was synthesized from 1μg total RNA using qScript cDNA Supermix (QuantaBio). SYBR green quantitative gene expression analysis was performed on an ABI 7500 Fast Real Time PCR System using the primer sequences shown in Supplemental Table S1. Relative mRNA levels were calculated using the comparative Ct ($\Delta\Delta C_t$) method. Reported values are the means and standard deviation of three technical replicates and two biological replicates (n=6).

Reverse phase protein array

Reverse phase protein array (RPPA) printing and analysis was performed in the laboratory of Dr. Emmanuel Petricoin as previously described with antibody specificity confirmed by immunoblot and analysis [27-29]. BT549 cells were grown in technical triplicate in attached or suspended (on poly-Hema coated plates) culture for 24, 48, and 72 hours and cell pellets were lysed as described previously [30].

Enzyme-linked immunosorbent assays (ELISAs)

The TGF β 1 Human DuoSet ELISA Kits (R&D Systems) were used to measure extracellular TGF β 1 concentrations per the manufacturer's protocol. Results are representative of three technical triplicates from conditioned medium from MDA-MB-453, SUM159PT, and BT549 cell lines grown in attached or suspended (on poly-Hema coated plates) culture for 24 and 48 hours.

Immunohistochemistry

Cells were fixed in 10% formalin and pelleted in histogel (Thermo Fisher). Paraffin embedding was performed by the UC Denver Tissue Biobanking and Processing Core. Slides were deparaffinized using xylenes and ethanols, and antigens were heat-retrieved in 10 mM citrate buffer pH 6.0. Antibody pSmad3 (ab52903; Abcam) was used. The ImmPRESS® HRP Anti-Rabbit IgG (Peroxidase) Polymer Detection Kit (Vector Laboratories Inc.) was used for detection, and Tris-buffered saline with 0.05% Tween 20 was used for all washes. Representative images were taken using a BX40 microscope (Olympus) with a SPOT Insight Mosaic 4.2 camera and software (Diagnostic Instruments, Inc.). Quantifications for 3 fields at 40x magnification from 1 slide performed using IHC Profiler [31].

Luciferase assays

Transfections were performed using Lipofectamine 3000 (Thermo-Fisher) as per the manufacturer's protocol. Firefly luciferase activity from the p3TP-lux plasmid (as described in [32]) was normalized to renilla luciferase expressed from the pRL-SV40 plasmid. Cells were harvested and assayed for luciferase activity using the Dual Luciferase Assay System (Promega # E1910) as described by the manufacturer and measured using a Biotek Synergy 2 multimode microplate reader.

Western Blot Assays

Whole-cell protein extracts were prepared using radioimmunoprecipitation (RIPA) lysis buffer. Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Scientific). Equal amounts of cell protein lysates (10–50 µg total protein) were combined with SDS-PAGE loading buffer containing 0.6M DTT and resolved by gel electrophoresis, then transferred to Immobilon-FL PVDF transfer membranes (Millipore) or nitrocellulose membranes (Fischer Scientific). Membranes were blocked with either 5% BSA, 5% milk in TBS-Tween, or Odyssey Blocking Buffer (Li-Cor) for 1 hour. Blots were probed with primary antibodies shown in Supplemental Table S2. Secondary antibodies used were goat anti-rabbit IRDye® 800CW and goat anti-mouse IRDye® 680RD (Li-Cor). Following secondary antibody incubation, results were detected using the Odyssey® CLx Imaging System and analyzed using the Image Studio Ver 5.2 software (Li-Cor) and ImageJ (NIH). Cells were treated with 10ng/mL of recombinant human TGFβ1 (R&D Systems). All experiments were repeated at least twice and representative blots shown.

Trypan Blue Exclusion Assays

Cells were treated with either 10µM LY2109761 or 10µM LY2197299. Cell counts were assessed using trypan blue exclusion. Cell pellets were harvested and washed in 1ml of

phosphate-buffered saline (PBS). 10 μ l of 0.4% trypan blue was mixed with 10 μ l cell suspension. Trypan blue-positive and -negative cells were counted using the Countess™ II Automated Cell Counter. Reported values are the means and standard deviation of three technical replicates and repeated in three biological replicates (n=9).

Kaplan-Meier Plots

Kaplan Meier plotter (KM plotter) [33] was used to generate a series of Kaplan-Meier plots to interrogate relapse-free survival (RFS), overall survival (OS), and distant metastasis-free survival (DMFS) in Basal and ER- BC [33]. Using the multiple genes option, TGFB3 (Affymetrix ID: 209747_at) was entered into the database and patient data was filtered using high or low median expression of AR (Affymetrix ID: 201272_at) in either Basal or ER- cases using auto best cut-off.

Chromatin Immunoprecipitation

MDA-MB-453 cells were grown in charcoal-stripped serum (CSS) media for a total of 72 hrs before treatment. 24 hrs prior to treatment, cells were trypsinized and equal cell numbers were plated on control tissue culture dishes (attached) or polyHEMA-coated dishes (suspended). Cells were treated with DMSO (vehicle control), DHT (10 nM), or DHT+Enza (10 μ M) for 4hrs, followed by fixation in 1% formaldehyde. ChIP-seq was performed as previously described [34]. Chromatin was sonicated using an Epishear Probe Sonicator (Active Motif) for 4 minutes (cycles of 30 seconds with 30 seconds of rest inbetween) at 40% power. AR antibody H-280 (Santa Cruz) was utilized for immunoprecipitation. Libraries were sequenced on an Illumina HiSeq 2500 as single-end 50 bp reads to a minimum depth of 18 million reads per sample. Reads were aligned to the hg19 build of the human genome with bowtie [35] using the following parameters: -m 1 -t --best -q -S -l 32 -e 80 -n 2. Peaks were called with MACS2 [36] using a p-value cutoff

of 1e-10, the mfold parameter bounded between 15 and 100, and the -SPMR option for normalization. Read depth at each peak was calculated by BEDTools [37] using the coverage sub-command. Visualization was performed on the IGV browser [38] using the normalized bedgraphs output by MACS2.

Crystal Violet Assays

SUM159PT cells were treated with 10 μ M of LY2109761 or 40 μ M of Enza (Medivation). 40 μ M of Enza approximates the IC₅₀ of the SUM159PT cell line as described previously [39]. 10 μ M of LY2109761 has been used previously as a toxic dose in SUM159PT cells [40]. Cells were fixed in 10% formalin, rinsed in PBS, and stained with 1% crystal violet. Crystal violet was then dissolved in 10% acetic acid and measured at 540nm using a Biotek Synergy 2 multimode microplate reader (n = 3).

Statistical significance

Statistical significance was evaluated using GraphPad Prism software to assess either two-tailed unpaired Student *t* tests, or one-way ANOVA followed by post-hoc tests. Spearman's rank correlation coefficient (Spearman *r*) was used for correlation analysis. $P \leq 0.05$ was considered statistically significant with *P* values indicated in figures as * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Error bars represent standard deviation (SD) unless otherwise noted.

Results:

TGF β signature is enhanced in anchorage independent TNBC cells

MetaCore pathway analysis of our gene expression profiling of BT549 TNBC cells [11] indicated that *SMAD3* was one of the top predicted upstream regulators of gene changes after 24 hours of culture in attached versus anchorage independent culture (Fig. 1A). GSEA analysis confirmed that the TGF β pathway was altered in TNBC cells surviving in anchorage independent

versus attached conditions as this pathway was significantly upregulated in cells from the anchorage independent culture (NES = -1.60, nominal p-value $\leq$ 0.001) (Fig. 1B). 35 TGF β -associated genes were significantly differentially expressed between attached and suspended conditions (Fig. 1C), including 5 involved in the canonical TGF β signaling pathway as indicated by red stars). Changes in canonical TGF β signaling genes (*TGFB1*, *TGFB3*, *SMAD3*, *TGFBR1*) were independently verified by qPCR (Fig. 1D), where the majority of TGF β pathway genes interrogated in SUM159PT and BT549 TNBC cell lines were significantly increased by 48 hours in anchorage independent culture compared to attached culture, indicating that multiple transcripts encoding components of the TGF β pathway are significantly upregulated in TNBC cells in response to anchorage independent conditions.

Anchorage independent culture increases TGF β ligand secretion and pathway activation

Since our transcriptome profiling and qPCR showed increased expression of genes encoding TGF β ligands by TNBC cells cultured in anchorage independent conditions, we hypothesized that anchorage independent culture would increase ligand-dependent TGF β pathway activation. To investigate this hypothesis, we performed RPPA analysis on BT549 lysates from attached and anchorage independent culture for 24-72 hrs. These results confirmed our prior finding that anchorage independent culture increased AR protein levels [10, 11] and indicated a significant increase in TGF β 1, TGF β 3, and pSmad2 by RPPA within the first 24 hours in anchorage independent culture, indicating pathway activation, possibly via enhanced ligand production and/or secretion (Fig 2A, Supplementary Fig S1A). To confirm this, we performed an ELISA for TGF β 1 in conditioned medium from TNBC cells cultured in attached or suspended conditions for 24 or 48 hours. Secreted TGF β 1 significantly increased in conditioned medium from all cell lines following 48 hours in anchorage independent culture (Fig. 2B). TGF β

pathway activation was confirmed by staining for pSmad3 by IHC, which demonstrated increased expression and nuclear translocation of pSmad3 in TNBC cells under anchorage independent conditions (Fig 2C). pSmad3 transcriptional activity was further examined using the p3TP-lux reporter, a luciferase reporter under the control of a portion of the pSmad3-regulated *PAI1* promoter, where luciferase activity was significantly increased in TNBC cells in anchorage independent culture when compared to cells cultured in attached conditions (Fig. 2D, Supplementary Fig. S1B). Together, these results demonstrate that TGF β ligand production and secretion is increased by TNBC cells under anchorage independent conditions leading to TGF β pathway activation.

Manipulating the TGF β pathway affects AR expression and cell viability in TNBC cell lines

Since TNBC cells in anchorage independent culture upregulated production of TGF β ligand and AR protein, and AR increases via binding of pSmad3 to the AR promoter in prostate cancer, we hypothesized that recombinant TGF β would increase AR protein levels even in attached TNBC cells. We conducted a time-course to monitor AR protein levels at various timepoints post-treatment with exogenous TGF β 1. pSmad2 and ID1, the known TGF β target gene, increased within the first 30 minutes after treatment, indicating the TGF β pathway was activated (Fig. 3A). ID1 peaked in expression at 2 hours with a subsequent expression decreased by 6 hrs. Interestingly, AR protein levels also increased and peaked between 2-4 hours post-treatment. To examine whether TGF β 1 affected other hormone receptor levels we also examined GR protein, but its levels remained constant. Since AR and pSmad3 were upregulated in anchorage independent conditions, and recombinant TGF β increased AR protein (Fig. 3A), we hypothesized that TGF β inhibitor (TGF β i) would decrease AR protein that might be induced by the TGF β ligand secreted by anchorage independent tumor cells. LY2109761 (LY1) treatment

caused a significant downregulation of AR gene expression (Fig. 3B). In fact, TGFβi LY1 blocked the anchorage independent-induction of AR observed in the vehicle control in anchorage independent culture in three TNBC lines, MDA-MB-453, SUM159PT, and BT549 after anchorage independence (Fig. 3C). To account for potential LY1 off-target effects, we repeated and confirmed our results using an additional inhibitor LY2157299 (Galunisertib) (LY2) that also targets the kinase domain of TGFβ receptor 1 (TβRI) [41, 42]. LY1 and LY2 decreased AR in all three cell lines in anchorage independent culture for 48 hours relative to vehicle control (Supplementary Fig. S2A). Since TGFβi decreased AR levels, and TNBC cells in anchorage independent conditions rely on AR for survival [11], we hypothesized that TGFβi would increase cell death in anchorage independent culture. Indeed, SUM159PT and BT549 cell lines displayed increased apoptosis in anchorage independent culture when treated with TGFβi, as measured by cleaved PARP (Fig. 3D). Both the SUM159PT and BT549 lines, which have dramatically upregulated AR in under anchorage independent culture [11], showed increased cell death in the presence of LY1 or LY2 (Fig 3E). These results demonstrate that the TGFβ signaling pathway affects AR expression and cell viability in anchorage independent culture in TNBC cells. In contrast, in the MDA-MB-453 cell line, which has high baseline levels of AR [11], LY1 or LY2 treatment did not affect cleaved PARP levels (Supplementary Fig. S2B) and trypan blue exclusion assays on cells grown in under anchorage independent culture conditions in the presence of TGFβi showed that cell viability in the MDA-MB-453 cell line was not affected by LY1 nor LY2 in either attached or suspended culture (Supplementary Fig. S3C).

High TGFβ Levels Positively Correlate with AR in Patients and is Associated with Poor Outcomes

Due to the observed reciprocal effects of AR and TGF β *in vitro*, we examined how AR correlates with TGF β ligands and receptors in publicly available clinical data. We hypothesized that TGF β pathway-associated gene expression would correlate with AR expression in TNBC patients. To explore this, we examined microarray data from TNBC patients in The Cancer Genome Atlas (TCGA)-BRCA and RNA expression data from the Sweden Cancerome Analysis Network - Breast (SCAN-B) cohorts [43, 44] to determine if AR expression correlated with *TGFB1*, *TGFB3*, *TGFBR1*, or *TGFBR2*. Although all TGF β genes were significantly correlated ($P \leq 0.05$; Supplementary Fig. S3), *TGFB3* showed the highest correlation with AR in both the TCGA and SCAN-B cohorts (Fig. 4A). Next, we examined how the different TGF β pathway genes correlate with a known AR-response gene signature. GSEA analysis was performed, using an androgen-responsive gene set generated from DHT-treated HCI-009 PDX tumors (unpublished), and we observed correlation between androgen-responsive genes and TGF β signaling genes (Fig. 4B). In the TCGA cohort, *TGFB1*, *TGFB3*, and *TGFBR1* were significantly correlated with an AR gene signature (Fig. 4B, Supplementary Fig. S4A). In the SCAN-B cohort, only *TGFB3* and *TGFBR1* were significantly correlated with an active AR gene signature (Fig 4B; Supplementary Fig. S4B).

Using KMplotter [33], we generated a series of Kaplan-Meier curves investigating how *TGFB3* levels correlate with survival in basal BC (classified as ER-, HER2-) after first separating patients with AR expression (high: AR^{high} or low: AR^{low}) expression of AR. Survival outcomes examined included relapse-free survival (RFS), overall survival (OS), and distant metastasis-free survival (DMFS). In AR^{high} basal BC, high *TGFB3* was associated overall shorter RFS, OS, and DMFS compared to tumors with low *TGFB3* (Fig. 4C, Supplementary Fig. S5). However, in AR^{low} basal BC, high *TGFB3* showed the opposite result and did not correlate with

OS or DMFS (Fig. 4C, Supplementary Fig. S5). Similar results were obtained in ER- BC. AR^{high} ER- BC cases with high *TGFB3* expression were associated with shorter RFS and DMFS compared to cases with low *TGFB3* (Supplementary Fig. S6), and there was no correlation with OS. In AR^{low} , ER- BC, *TGFB3* expression did not correlate with RFS, OS, or DMFS. Overall, these results demonstrate that *TGFB3* correlates with *AR* expression and pathway activation, and basal and ER- BCs that exhibit both high *AR* and high *TGFB3* have overall worse outcome than those where either or both are low.

AR binds to genomic regions proximal to TGFB1 and SMAD3

Since *AR* has been reported to bind to multiple AREs in the *TGFB1* promoter to increase its expression in prostate cancer [25, 45] and was a predicted upstream regulator of genes increased in anchorage independent culture of TNBC lines [11], we investigated what other genomic regions *AR* binds to in under anchorage independent culture compared to attached conditions. We performed ChIP-seq for *AR* in the MDA-MB-453 cell line grown in attached versus anchorage independent conditions and treated with either vehicle control, DHT, or DHT+Enza. DHT-induced *AR* bound to a similar number of sites in attached and suspended conditions, and Enza decreased *AR* binding to levels comparable with vehicle control (Fig. 5A). Many DHT-induced *AR* binding sites show 2-fold or greater changes in signal in all culture conditions (Fig. 5B), suggesting that *AR* binding is altered in under anchorage independent cells. The GREAT analysis software [46] was used to identify genes, and subsequently gene ontology biological processes, found near *AR* bound sites that are more than two-fold enriched in all culture conditions. Regulation of cell junction assembly and focal adhesion assembly were two of the main enriched biological processes upregulated by DHT-induced *AR* activation (Fig. 5C). 31 genes involved in focal adhesion assembly were identified as being more than 2-fold enriched

in under anchorage independent versus attached culture by AR ChIP-Seq, including *SMAD3* (Fig. 5D). For *SMAD3*, we identified 6 AR binding regions (4 upstream of the canonical transcriptional start site (TSS) and 2 within the *SMAD3* gene itself) that exhibited 2-fold enrichment of AR ChIP-seq binding in suspended compared to attached culture (Fig. 5E). Two peaks located ~300kb upstream of the *SMAD3* TSS are within the gene locus of another SMAD family member, *SMAD6*. Interestingly, we also found differential peak calls in 2 annotated lncRNAs, LINC02206 (~134kb upstream) and LOC102723493 (~40kb upstream) that could putatively play additional roles in AR-mediated *SMAD3* regulation. The two peaks following the TSS are within the *SMAD3* loci itself and occur in the intronic region of the canonical isoform that also serves as the promoter region of multiple alternate *SMAD3* isoforms. For *TGFB1*, an AR ChIP-seq signal was found to encompass a 2000bp window, consisting of 500bp upstream of the transcription start site, and 1500bp into the 5'UTR and 1st exon (Fig. 5F). Together, these results may explain the increases in transcript levels detected by qRT-PCR in under anchorage independent cultured cells (Fig. 2B). Future functional analyses will determine the relative functional contributions of these regions.

Combined inhibition of TGF β and AR Inhibit TNBC Cell Growth

Our *in vitro* and clinical data suggest TGF β and AR play an important role in TNBC progression. Therefore, we hypothesized that inhibiting both pathways together might inhibit tumor cell survival, particularly in the anchorage independent condition where evidence of signaling from both pathways increases. Crystal violet assays on TNBC cells in attached conditions with either vehicle control, 10 μ M of LY1, 40 μ M of Enza, or both for 72 hours, showed significantly less growth than vehicle control (P = 0.0375, P = 0.0197, P = 0.0005,

respectively), with the combination treatment demonstrating an additive effect significantly different from LY1 ($P = 0.0260$) or Enza ($P = 0.0498$) (Fig 6A).

To assess the capacity for regrowth following treatment, cells were treated with the same drugs for 72 hours, followed by washout of the drug and an additional 72 hours of growth in regular media. LY1-treated cells grew significantly less than vehicle-treated cells ($P = 0.0230$) (Fig 6B). As before, cells treated with the combination grew significantly less than vehicle-treated cells ($P = 0.0003$). Compared to LY1- or Enza-treated cells, the combination-treated cells grew significantly less than either treatment ($P = 0.0272$; $P = 0.0023$, respectively), recapitulating the additive effect on cell growth (Fig 6A). Lastly, to test the effects of combination drug treatment on the capacity for cell regrowth following treatment in anchorage independent conditions, SUM159PT cells were grown for 48 hours with treatments in under anchorage independent culture and were then re-plated with regular media in attached conditions for an additional 48 hours. While cells treated with LY1 or Enza in under anchorage independent culture showed modest, but significantly, reduced growth in attached conditions ($P < 0.0001$), cells treated with the combination in under anchorage independent culture showed a significantly reduced regrowth capability ($P < 0.0001$) (Fig. 6C). Together, these results suggest that inhibiting AR and TGF β signaling simultaneously has a greater effect on cell viability under anchorage dependent and independent conditions than either treatment alone.

Discussion:

Our data demonstrate the importance of AR and TGF β signaling in anoikis resistance in TNBC. The findings support a novel role for a positive feedback loop between AR and TGF β that supports TNBC anchorage independent survival. TGF β gene signature, pathway components, and activation increased in anchorage independent conditions. Further, TGF β i

prevented the increased AR protein in anchorage independent conditions that supports anoikis resistance even in non-LAR TNBC cell lines. TGF β inhibition decreased AR protein levels cell viability in anchorage independent culture; however, further studies are needed to investigate whether TGF β i-induced cell death is directly caused by decreased AR protein levels. In support of the previously unexplored role of this positive feedback loop during anchorage independent survival, we find that exogenous TGF β increased AR levels and also that AR binds to regulatory regions of genes involved in the canonical TGF β signaling pathway by AR-ChIP. Studies in prostate cancer also show that AR binds to multiple functional AREs within the *TGFB1* promoter [25]. The increased TGF β ligand production that we observed under anchorage independent conditions stimulates pSmad3 nuclear localization, supporting the reciprocal relationship (Fig. 7). This connection has been previously demonstrated in dermal papilla cells, where recombinant TGF β increases AR transcriptional activity [47]. AR in developing testis also correlates with Smad3 expression [48]. Altogether, the literature and our data further support an AR-TGF β connection.

Our previous findings show that even TNBC with low AR expression (represented by the SUM159PT and BT549) may benefit from AR-targeted therapies [10, 11]. AR mRNA, protein, and transcriptional activity in TNBC increased in under anchorage independent culture *in vitro* [10, 11] and *in vivo* [19, 20], and Enza has significantly decreased soft agar colony growth, another assay of anchorage independent growth, in TNBC cell lines [11]. Furthermore, results from Phase II clinical study assessing the efficacy of the AR antagonist Enzalutamide in metastatic, heavily pre-treated AR+ TNBC demonstrated clinical benefit, as did an earlier study with the anti-androgen bicalutamide [49, 50].

TGF β inhibitor (TGF β i) monotherapies have not been as promising in clinical studies [51-54], but TGF β i combined with chemotherapeutics produced favorable results [3, 55-58]. TGF β i has several major drawbacks including 1) TGF β has paradoxical roles as both tumor suppressor and tumor promoter; generally, TGF β causes cell cycle arrest in normal and premalignant cells, but has tumor promoting properties in certain cells and late-stage metastases [59-63], 2) tumors can develop resistance to long-term TGF β i and eventual progression [64], and 3) clinical trials with TGF β i monotherapy yielded mixed results [52-54, 65]. However, these experiences taught us that short-term TGF β i and combination with chemotherapeutics can address these issues [37] and numerous studies have effectively attenuated metastasis in preclinical models using TGF β i [61, 66-71]. LY2109761 (LY), a small-molecule dual inhibitor of TGF β receptor I (T β RI) [71], reduces breast, pancreatic, and liver metastasis, prolonging survival in preclinical models [60, 68, 69]. While clinical trials have investigated the efficacy of TGF β i and AR inhibitors separately in BC, our study suggests that inhibiting both pathways might be a logical therapeutic strategy for TNBC even in subtypes with low AR, since increased AR levels are critical for anchorage independence [10, 11, 20].

The TCGA-BRCA and SCAN-B cohorts both indicate that *TGFB3* positively correlates with both AR expression and an AR-regulated gene signature, and data from KmPlot also suggest that AR^{high}/TGFB3^{high} basal BC cases have an overall worse outcome. The same trend of AR^{high}/TGFB3^{high} cases having a worse outcome was also observed in all ER- BC. Clinical trials for various forms of TGF β i (neutralizing antibodies, soluble receptors, small molecules, etc) in BC have shown benefit [72] with more underway [NCT02672475, NCT03620201, NCT03834662]. While clinical trials have investigated the efficacy of TGF β i and AR inhibitors separately in BC, this study suggests that inhibiting both pathways might be a logical therapeutic

strategy for TNBC progression, and even subtypes with low AR since AR levels increase and are critical for anchorage independence [10, 11, 20].

Our results showed that TGFβi combined with Enza had greater effect on cell viability, particularly under anchorage independent culture, than either treatment alone, modeling viability of CTCs. The TCGA-BRCA and SCAN-B cohorts both indicate that *TGFB3* positively correlates with both AR expression and an AR-regulated gene signature, and data from KM Plotter also suggest that *AR*^{high}/*TGFB3*^{high} basal and ER- BC cases have an overall worse outcome. While the combination treatment of LY1+Enza in under anchorage independent culture severely impacted regrowth in attached conditions, future tests to optimize dose combination and dose reduction, and tests of true synergy *in vitro* and *in vivo* in metastatic models, as completed previously in the prior synergy experiments from our lab using Enza and the mTOR inhibitor everolimus [30] and paclitaxel [11], are needed. The latter preclinical results [11, 30] have led to trials such as the Artemis trial at MD Anderson treating patients with AR+ TNBC with Enza plus paclitaxel (NCT02276443).

In summary, this study indicates that a positive feedback loop between AR and TGFβ is present in TNBC that supports anoikis resistance. It further provides rationale for a combinatorial therapeutic strategy using both AR and TGFβ inhibitors to slow TNBC progression. Future studies are needed to evaluate the efficacy of AR/TGFβ dual inhibition therapy *in vivo* through use of metastatic TNBC PDXs [20] and transgenic mouse models of mammary cancer that retain AR during metastatic progression [39]. Additionally, while clinical trials studying the efficacy of AR and TGFβ inhibitors individually have produced favorable results, the proposed combinatorial strategy may further decrease TNBC metastasis and reduce patient mortality.

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Figures and Tables:

Figure 1:

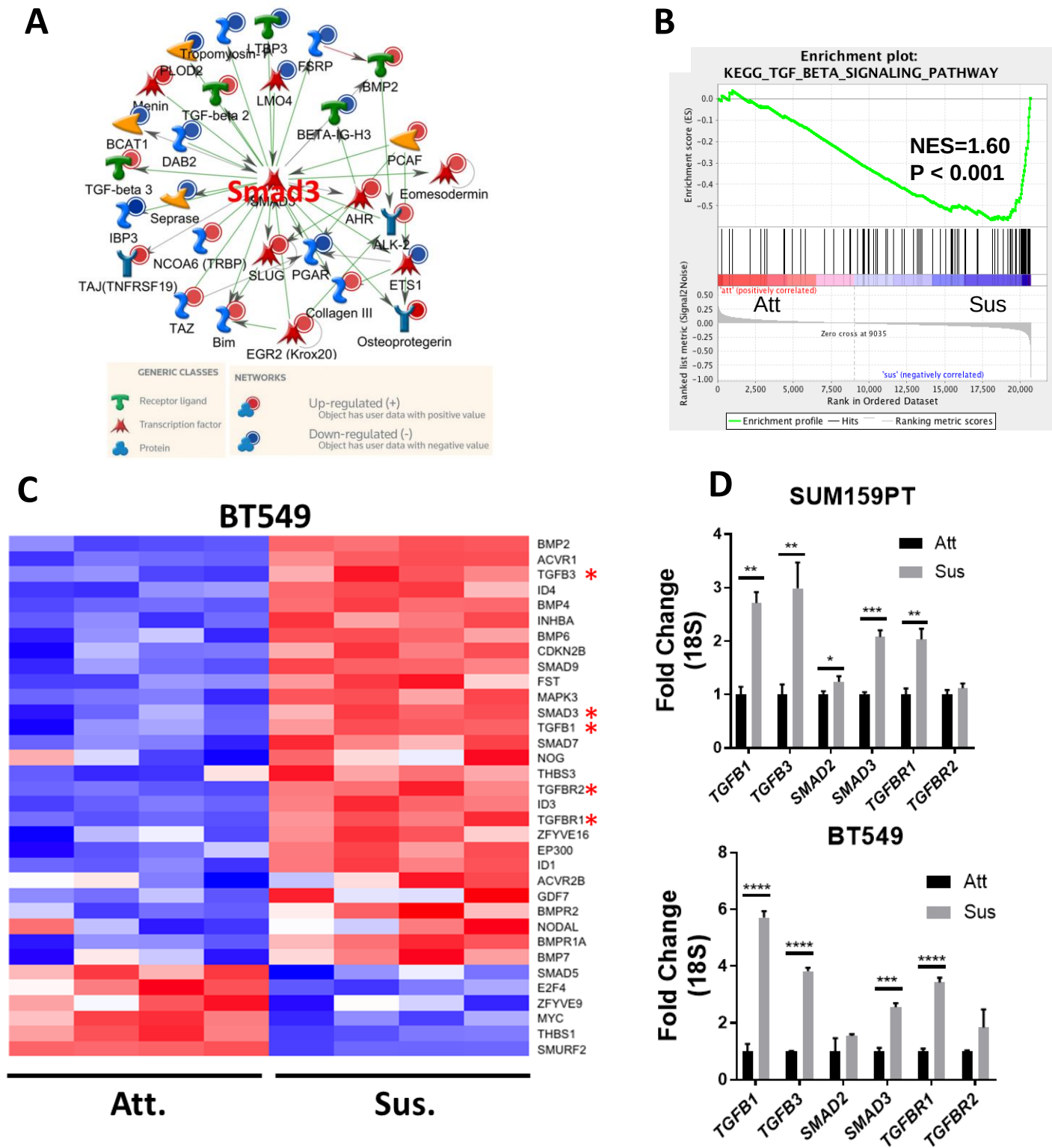
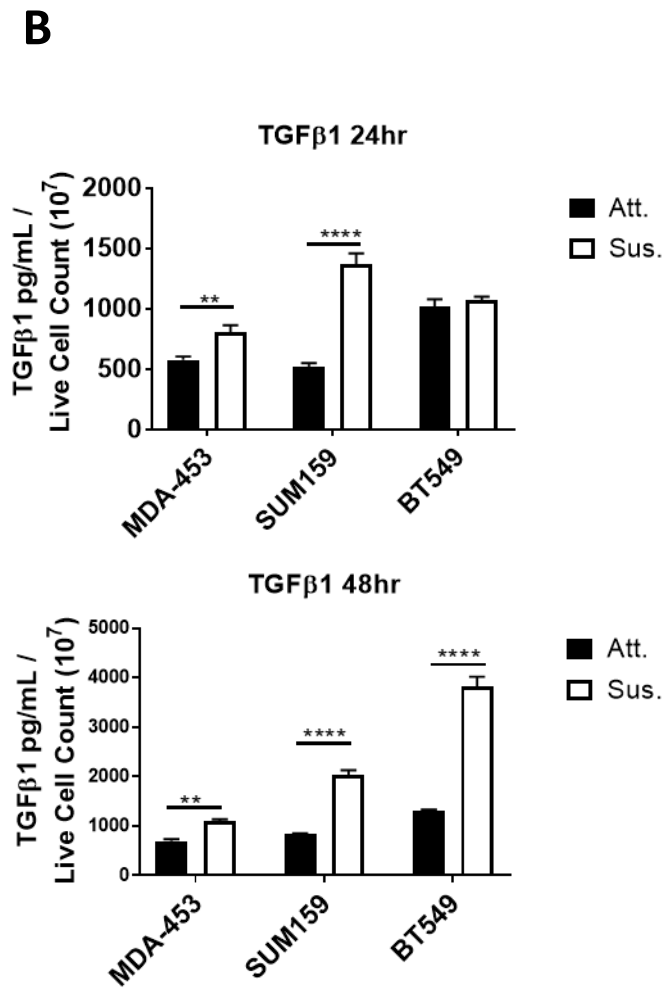
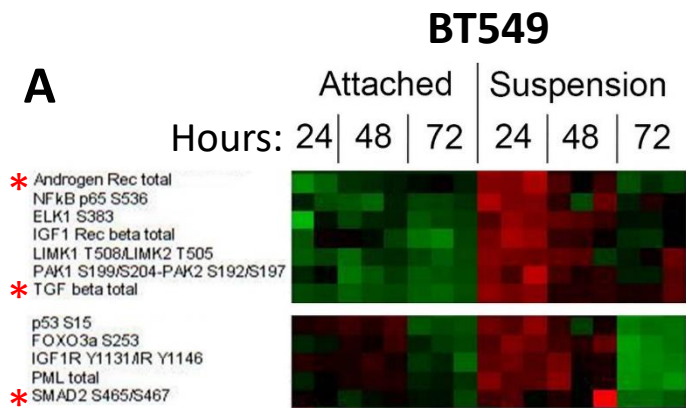


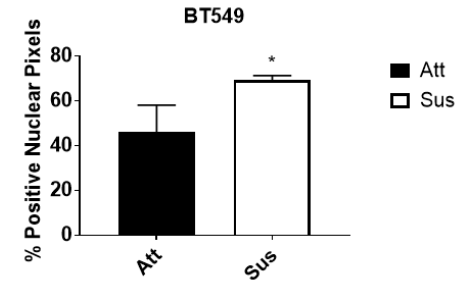
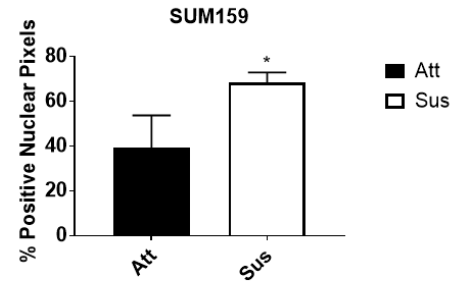
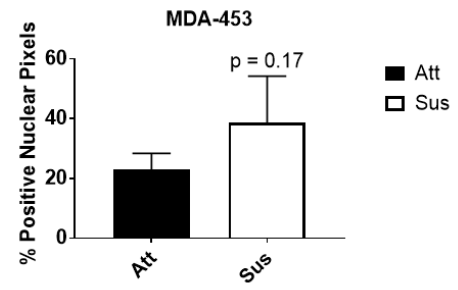
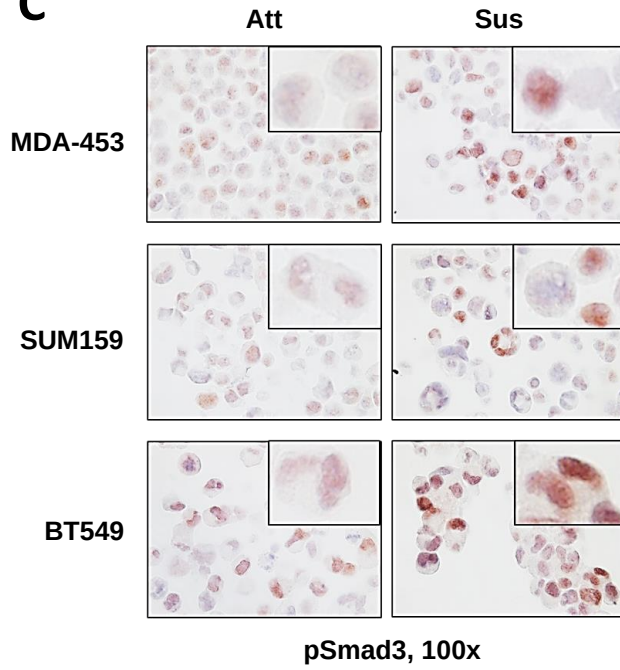
Figure 1: TGFβ pathway signature increases in TNBC cells upon anchorage independent culture and androgen receptor and SMAD3 are predicted upstream regulators. A)

Metacore pathway analysis of BT549 microarray data showing SMAD3 connecting with other genes altered in under anchorage independent culture for 24 hours. Original data set from Barton et al (2015) (GEO record GSE95472). **B)** GSEA pathway enrichment analysis showing changes in the TGFβ pathway in BT549 cells cultured in attached or under anchorage independent culture for 24 hours. **C)** Heatmap of significantly altered genes associated with the TGFβ pathway in BT549 cells grown in attached versus suspended culture conditions for 24 hours (n = 4). Gene list from KEGG database on TGFβ Signaling Pathway. Red asterisk *: genes associated with the canonical TGFβ signaling pathway. **D)** qRT-PCR for canonical TGFβ signaling pathway gene expression in attached versus under anchorage independent culture conditions at 48 hours in SUM159PT and BT549 cell lines (n = 6). Mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001. ****, P < 0.0001.

Figure 2:



C



D

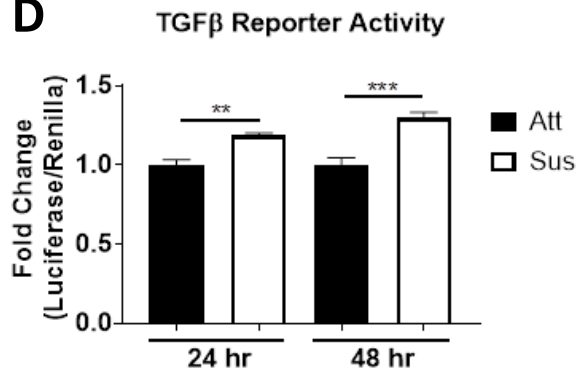


Figure 2: Anchorage independent conditions increase TGFβ1 expression and secretion and nuclear pSmad3 localization and activity. A) RPPA data showing changes in protein in BT549 cells grown in attached versus under anchorage independent culture conditions for 24, 48, and 72 hours with biological triplicates at each condition and timepoint shown. Full panel of RPPA data in supplemental. **B)** ELISA measuring changes in TGFβ1 in conditioned medium from three TNBC lines cultured in attached versus suspended conditions for 24 and 48 hours normalized to total live cell count as determined by trypan blue. **C)** IHC and quantification of percentage of pixels corresponding to nuclear DAB staining for pSmad3 in MDA-MB-453, SUM159PT, and BT549 cells in attached versus suspended culture conditions for 24 hours. **D)** 3TP-lux TGFβ reporter-linked luciferase activity normalized to renilla in cells grown in attached versus suspended culture. Mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001. ****, P < 0.0001.

Figure 3:

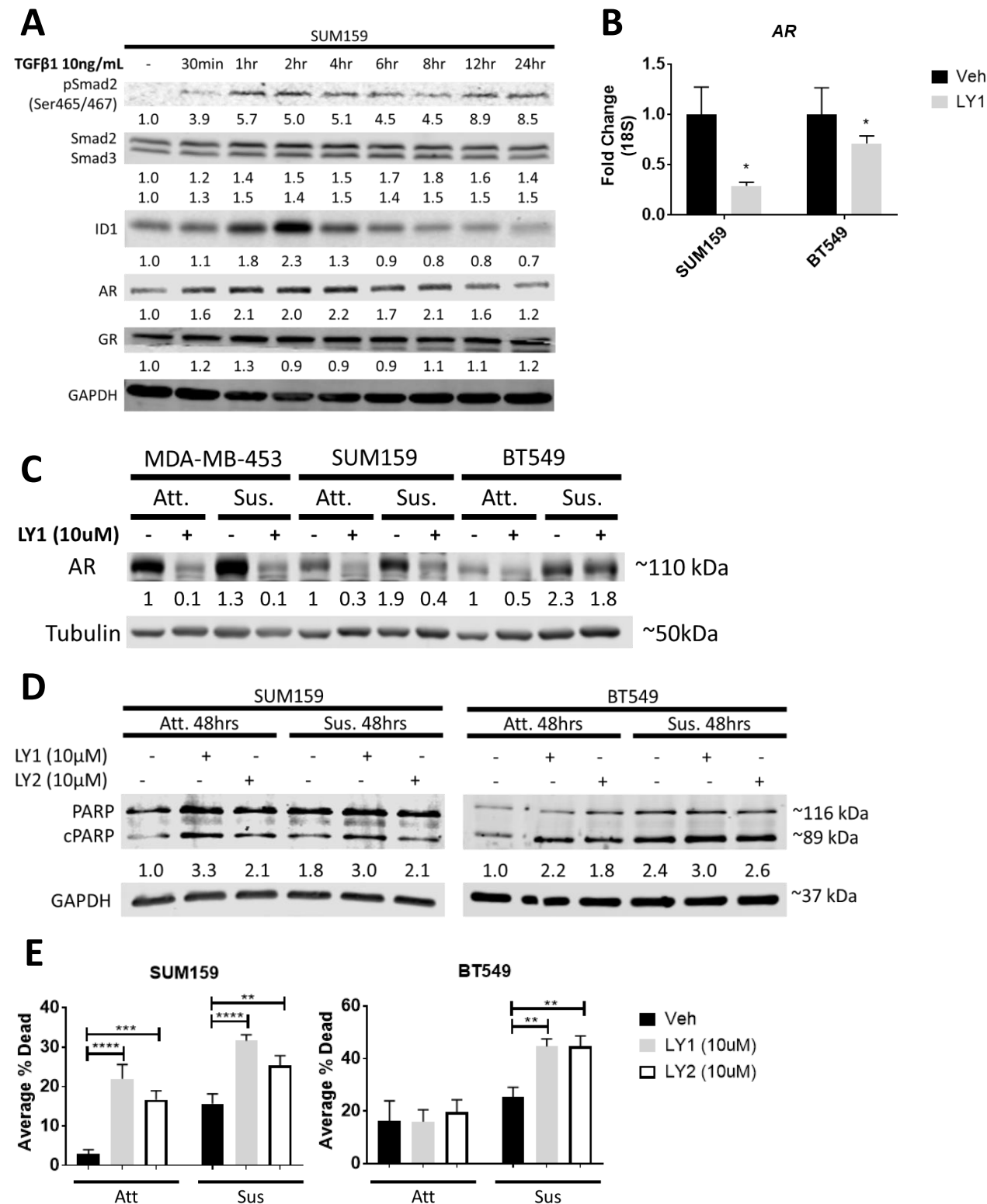


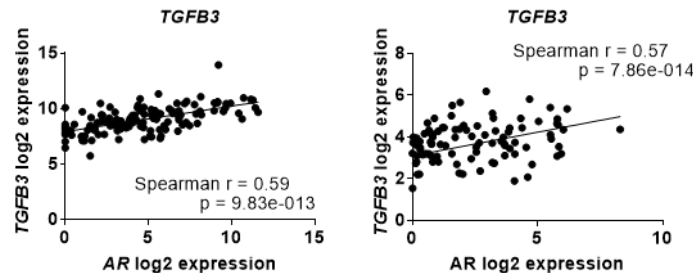
Figure 3: Exogenous TGF β increases AR protein levels and TGF β inhibition decreases AR and cell viability in anchorage independent conditions. A) Western blot for pSmad2, Smad2, Smad3, ID1, AR, GR, and GAPDH in the SUM159PT cell line treated with recombinant TGF β 1 (10ng/mL) over a time course of 24 hours. **B)** qRT-PCR for AR in SUM159 and BT549 cells grown in attached conditions for 48 hours +/- LY2109761 (10uM). **C)** Western blot for AR was performed on MDA-MB-453, SUM159PT, and BT549 cell lines grown in attached or suspended conditions for 48 hours +/- LY2109761 (10uM). **D)** Western Blot to analyze the apoptosis marker cleaved-PARP in SUM159PT and BT549 cells in attached or suspended conditions for 48 hours +/- LY2109761 or LY2157299 (10uM). **E)** Trypan blue assay for percentage of dead cells in SUM159PT and BT549 cells in attached or suspended conditions for 48 hours +/- LY2109761 or LY2157299 (10uM). Western blot quantifications performed by ratio of protein of interest to loading control and normalized to control. Mean $\pm$ SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001. ****, P < 0.0001.

Figure 4:

A

TCGA Cohort

SCAN-B Cohort



B

TCGA Cohort

SCAN-B Cohort

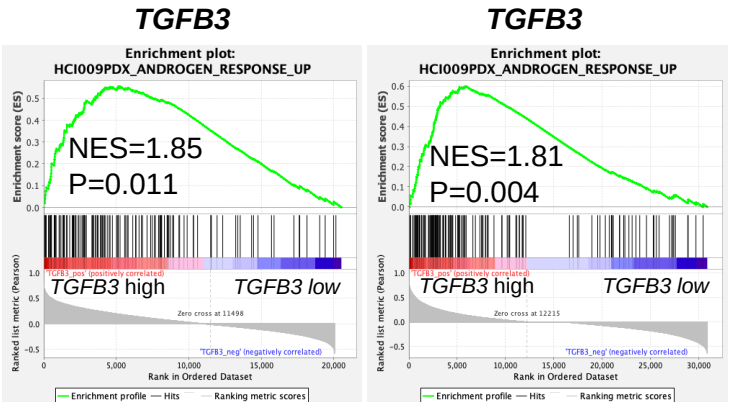


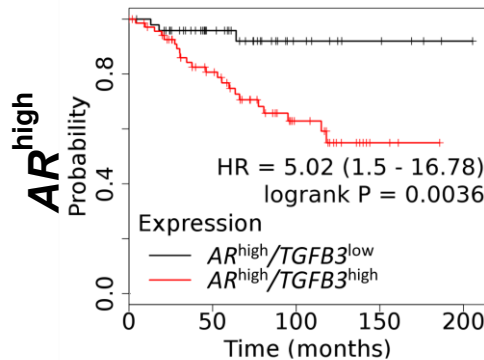
Figure 4 (cont):

C

Basal Breast Cancer

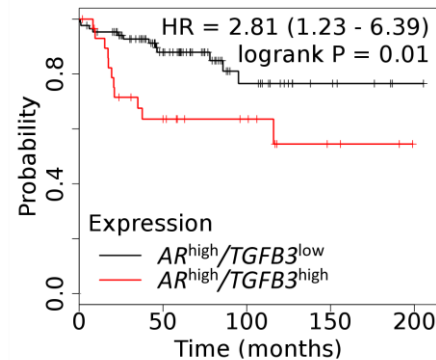
Overall Survival

**Distant Metastasis
Free Survival**



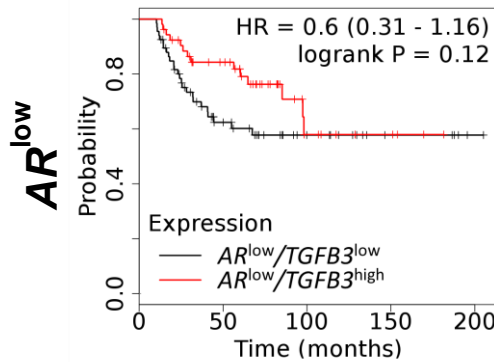
Number at risk

low	49	30	10	5	1
high	71	43	18	3	0



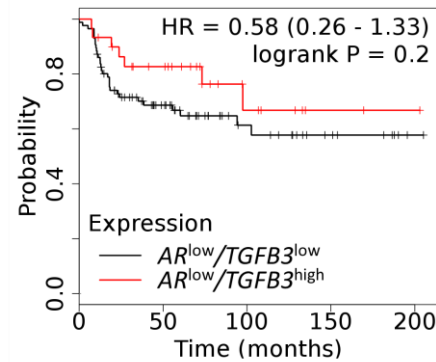
Number at risk

low	86	51	17	6	1
high	30	16	9	3	0



Number at risk

low	67	30	13	5	1
high	53	36	8	4	0



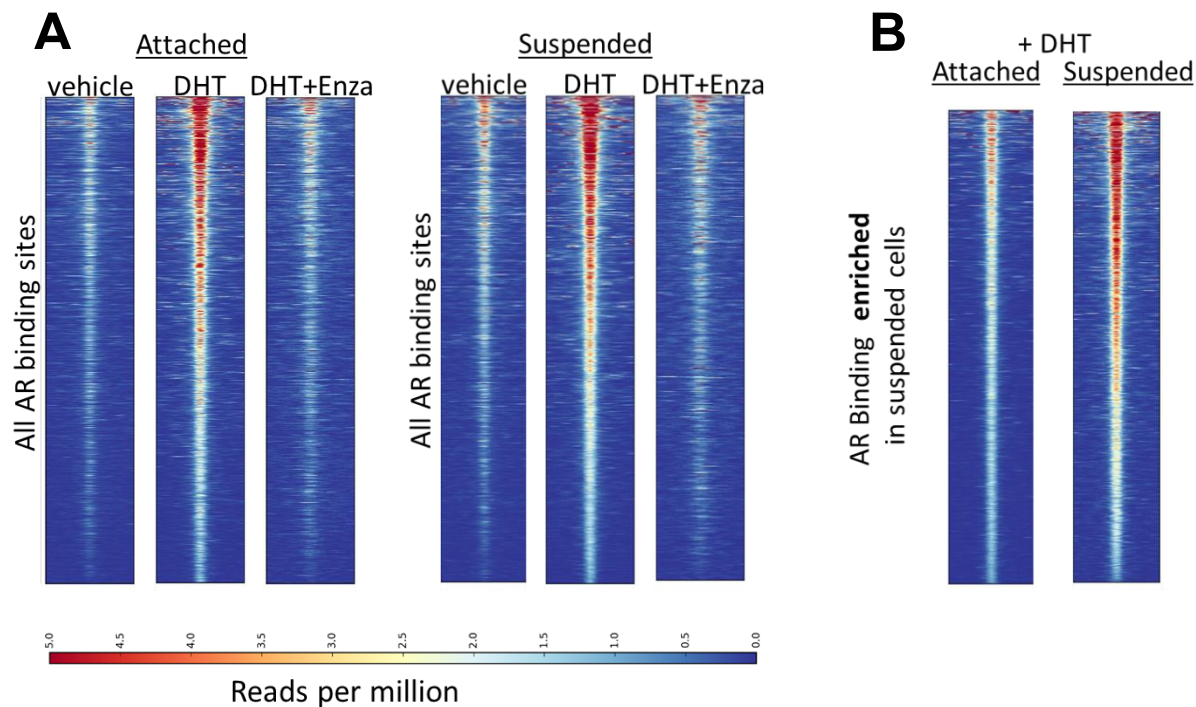
Number at risk

low	86	41	17	8	1
high	30	20	7	2	1

Figure 4: Clinical data suggests a high AR and *TGFB3* is associated with worse outcome. A)

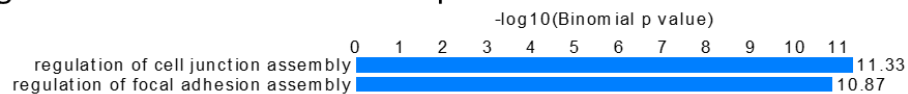
Correlation analysis on the co-expression of AR and the TGF β ligand *TGFB3* TNBC patients in the TCGA-BRCA and SCAN-B cohorts. **B)** GSEA analysis on *TGFB3* relating to the androgen response gene set from the HCI-009 PDX. **C)** KMplotter was used to stratify basal BC patients stratified into *AR*^{high} (top) or *AR*^{low} (bottom) populations. *TGFB3* expression was then interrogated based on overall survival (OS, n = 241) and Distant Metastasis Free Survival (DMFS, n = 232)

Figure 5:



C

Category of genes near AR enriched in suspension:

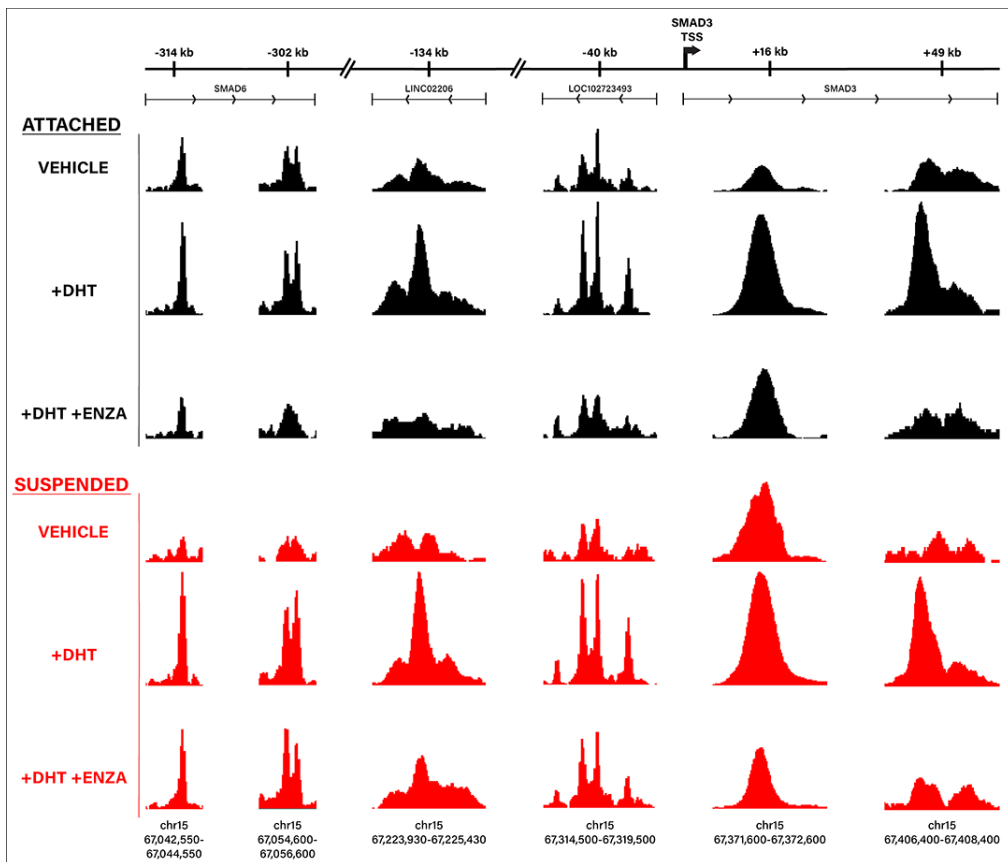


D

Genes enriched more than 2 fold in suspension = Focal adhesion

<i>APOD</i>	<i>ARHGAP6</i>	<i>EPB41L5</i>	<i>EFNA5</i>	<i>GJA1</i>	<i>NEDD4L</i>
<i>VEGFR</i>	<i>WNT4</i>	<i>PRKCH</i>	<i>SFRP1</i>	<i>ROCK1</i>	<i>SFRP1</i>
<i>SMAD3</i>	<i>PTEN</i>	<i>PPM1F</i>	<i>SRC</i>	<i>ROCK2</i>	<i>PTK2</i>
<i>SNAI1</i>	<i>MYO1C</i>	<i>RAPGEF1</i>	<i>ITGB1BP1</i>	<i>SDC4</i>	<i>PTPRJ</i>
<i>SNAI2</i>	<i>GREM1</i>	<i>THBS1</i>	<i>MACF1</i>	<i>RAPGEF2</i>	<i>VEGFA</i>
<i>EPHA3</i>					

E



F

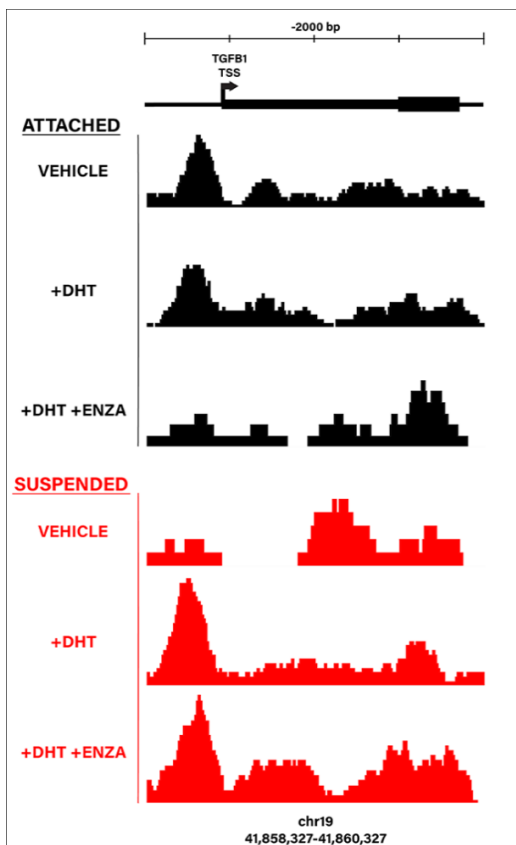


Figure 5: AR ChIP-seq peaks are identified near *SMAD3* and *TGFB1*. **A)** Heatmaps show that differential AR genome binding in attached and suspended conditions with vehicle, DHT, and DHT+Enza treatments. **B)** Heatmaps display the signal at differential DHT-induced AR binding sites that show a 2-fold or greater enrichment in the suspended condition. **C)** Gene ontology biological processes found nearby AR bound sites that are more than two-fold enriched in suspended cells using GREAT [46]. **D)** Genes involved in focal adhesion assembly that were identified as being 2-fold enriched in under anchorage independent culture by AR ChIP-seq. **E)** AR ChIP-seq peaks identified near *SMAD3* are shown. The scale bar at the top is labeled for each loci to indicate the relative distance from the canonical *SMAD3* TSS along with the respective gene annotations. For each region, the AR ChIP-seq signal for each treatment condition in attached (black) and suspended (red) are shown. The peaks at each loci are scaled to the same height for accurate comparison but are not directly comparable between loci due to differences in ChIP-seq signal intensity. The chromosomal coordinates of the area covered by each peak are shown below each region. **F)** Browser tracks show AR binding near the *TGFβ1* promoter. AR ChIP-seq signal within a 2000bp window covering 500bp upstream of the *TGFβ1* TSS and 1500bp into the 5'UTR and 1st exon is shown. ChIP-seq signal is scaled to the same height for comparison and represent each treatment condition in attached (black) and suspended (red), with the chromosomal coordinates included at the bottom for reference.

Figure 6:

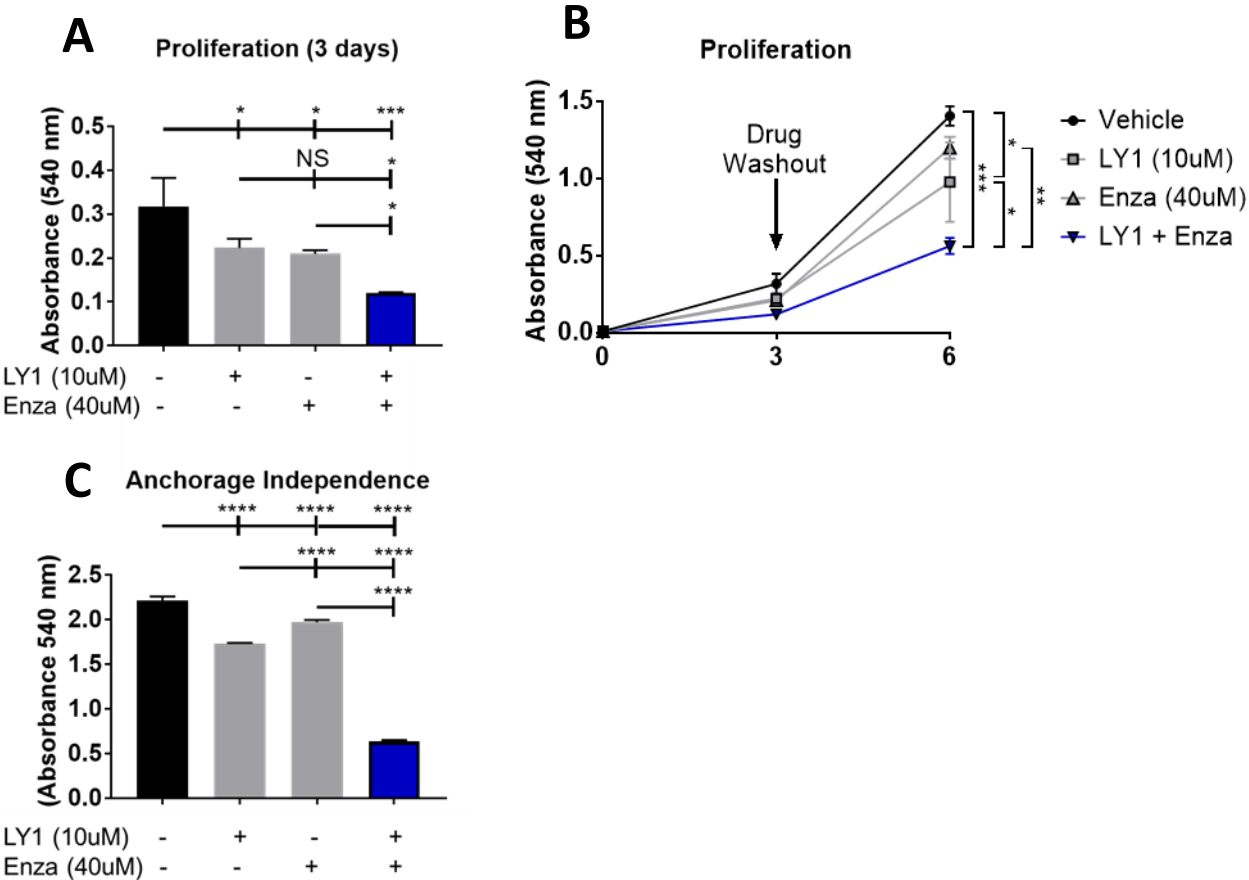


Figure 6: SUM159PT cells are more sensitive to a combined treatment with Enza and TGFβ inhibitor than either drug alone, particularly after anchorage independent culture.

A) SUM159PT cells were grown in triplicate in 6-well plates in attached conditions for 72 hours with either vehicle, LY1 (10μM), Enza (40μM), or both, followed by crystal violet assay. **B)** SUM159PT cells were grown in triplicate in 6-well plates in attached conditions for 72 hours with the same treatments, followed by washout of drug and an additional 72 hours in culture, then crystal violet assay was performed. **C)** SUM159PT cells were grown in under anchorage independent culture on poly-hema coated plates for 48 hours with the treatments mentioned above, followed by replating of cells in attached conditions for an additional 48 hours. Crystal violet assay was performed after. Mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001. ****, P < 0.0001.

953 **Figure 7:**

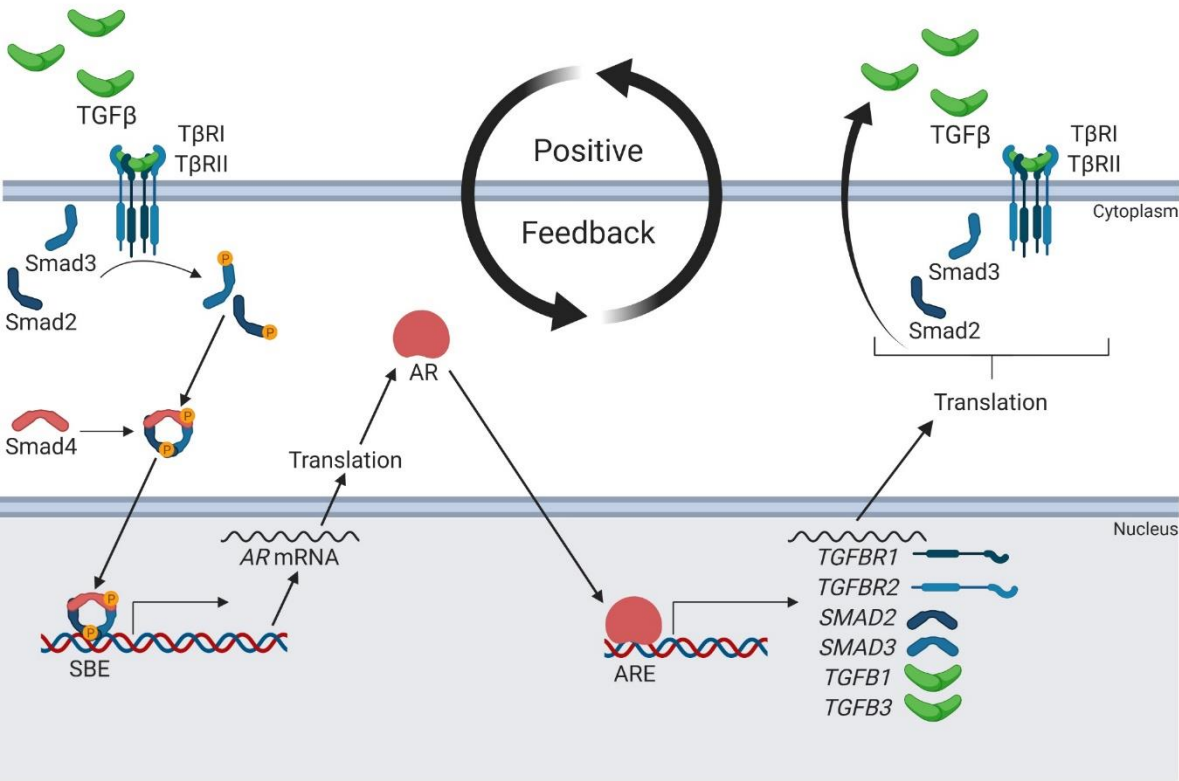


Figure 7: Proposed model of a positive feedback loop whereby TGF β signaling increases AR expression, and AR increases core TGF β pathway components.

Supplementary Table 1: Sequences of SYBR Green primers used for qRT-PCR analysis

SYBR Green Primers (5' to 3') (Integrated DNA Technologies, Coralville, IA)		
Gene Name	Forward Primer, 5'-3'	Reverse Primer, 5'-3'
<i>TGFB1</i>	GCCACAGATCCCCTATTCAA	GTCTCAGTATCCCACGGAAA
<i>TGFB3</i>	TGAGTGGCTGTTGAGAAGAGA	ATTGTCCACGCCTTTGAATTTGAT
<i>SMAD2</i>	CAATCGCCCATCCCCCTCTT	AGTCTCTTCACAACTGGCGG
<i>SMAD3</i>	GTCTGCAAGATCCCACCAGG	CTTGTCAAGCCACTGCAAAG
<i>TGFBR1</i>	CCTGGGATTTATAGCAGCAGAC	TGACACCAACCAGAGCTGAG
<i>TGFBR2</i>	GGACAACGTGTTGAGAGATCGA	TCAACGTCTCACACACCATCTG
<i>AR</i>	CCTGGCTTCCGCAACTTACAC	GGACTTGTGCATGCGGTACTC
<i>GAPDH</i>	TGGCATTGTGGAAGGGCTCATG	TTACTCCTTGGAGGCCATGTAGG

1001 **Supplementary Table 2:** Antibodies used for immunoblotting analysis

Antibodies used for Western Blotting			
Epitope	Dilution	Vendor	Catalog No.
AR	1:500	Millipore Sigma	#06-680
GR	1:1000	Cell Signaling Technologies	#3660
pSmad2	1:1000	Cell Signaling Technologies	#3108
pSmad3	1:5000	Abcam	ab52903
Smad2/3	1:1000	Cell Signaling Technologies	#8685
PARP	1:1000	Cell Signaling Technologies	#9542
ID1	1:1000	Santa-Cruz	#sc-488
GAPDH	1:20000	Millipore Sigma	#G8795

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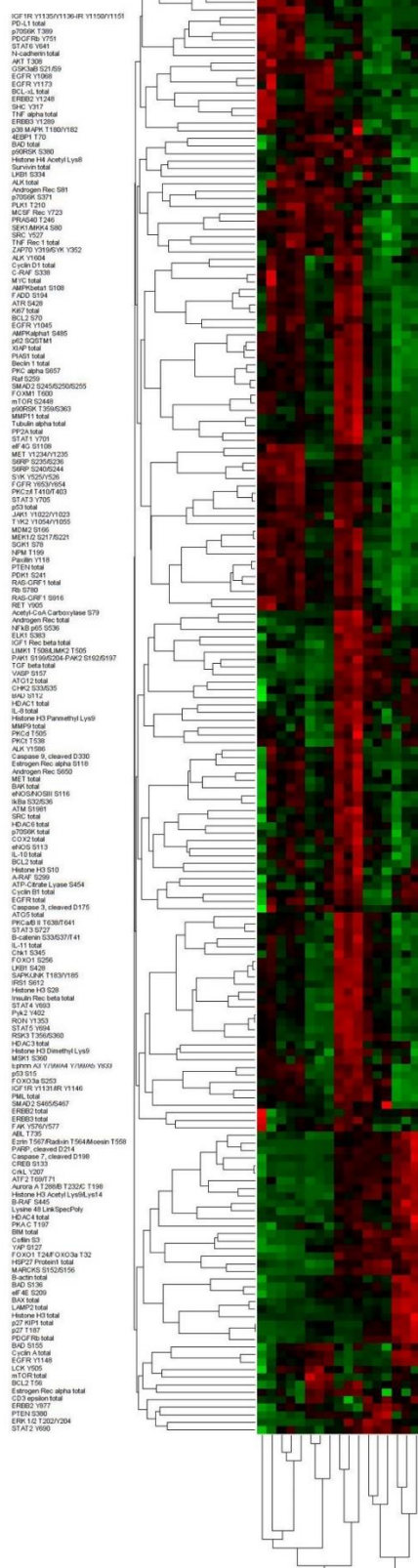
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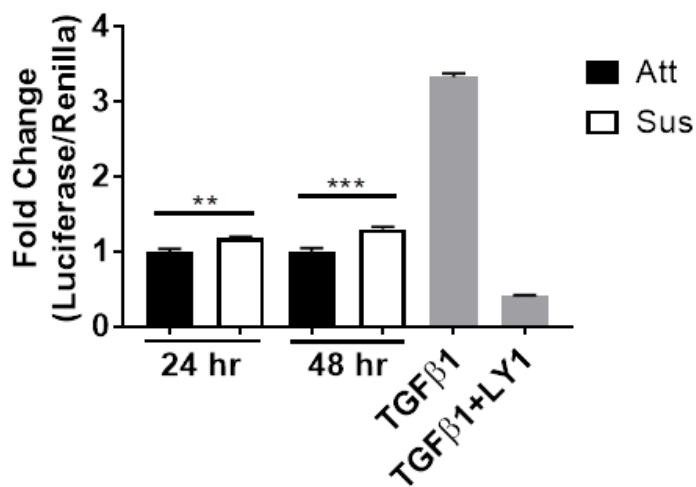
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Attached			Suspension		
24	48	72	24	48	72



S1B TGF β Reporter Activity

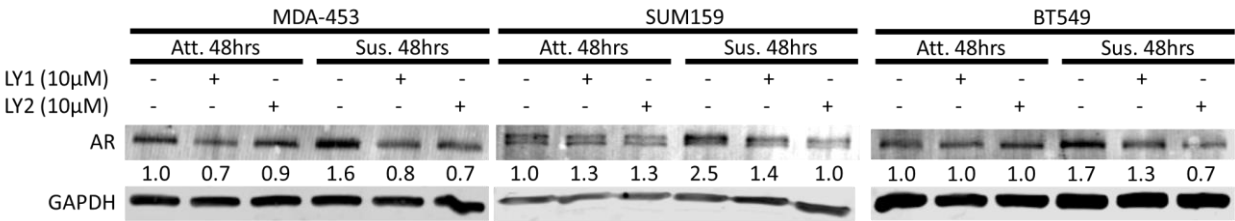


Supplementary Figure 1: S1A: RPPA data showing changes in protein expression and phosphorylation in BT549 cells grown in attached versus suspended culture conditions for 24, 48, and 72 hours. Biological triplicate samples of each treatment group are shown.

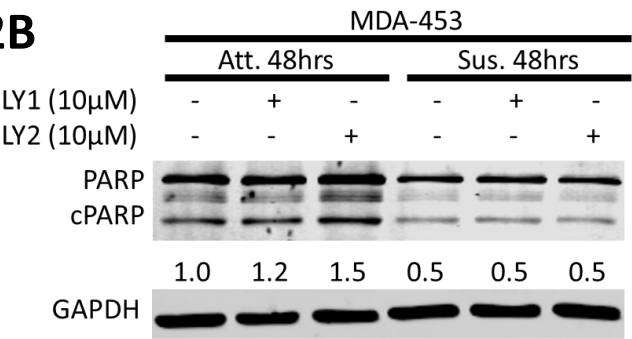
Supplementary Figure S1B: 3TP-lux TGF β reporter-linked luciferase activity normalized to renilla in cells grown in attached versus suspended culture. Treatment of 10ng/mL of TGF β used as positive control, 10ng/mL of TGF β + 10 μ M of LY2109761 used as negative control. Mean $\pm$ SD; **, P < 0.01; ***, P < 0.001.

Supplementary Figure S2

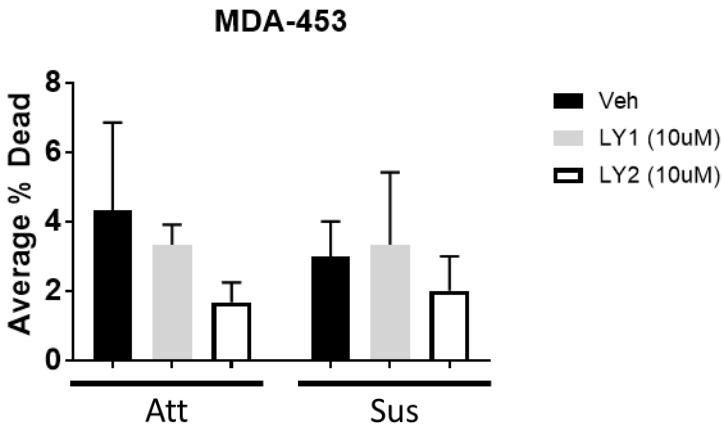
S2A



S2B



S2C



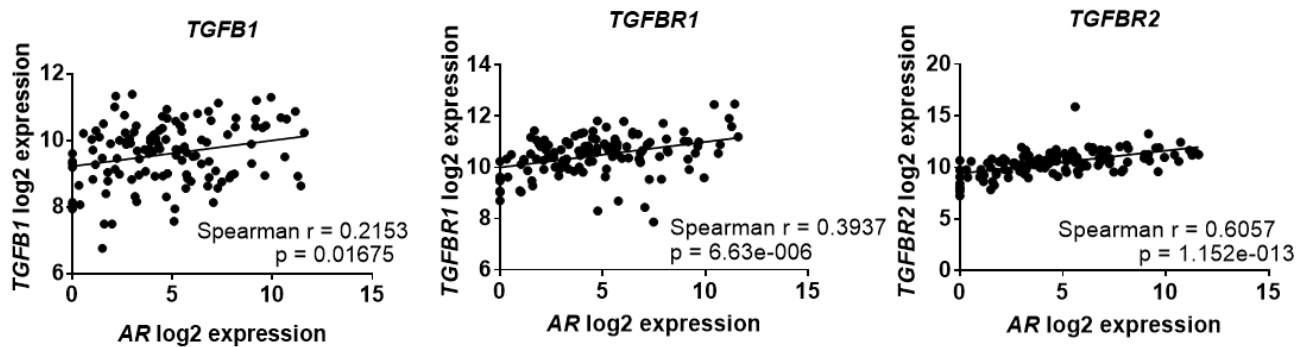
Supplementary Figure 2A: Western Blot to analyze AR in MDA-MB-453, SUM159, and BT549 cell lines in attached or suspended conditions for 48 hours +/- LY2109761 or LY2157299.

Supplementary Figure 2B: Western Blot to analyze the apoptosis marker cleaved-PARP in MDA-MB-453 cells in attached or suspended conditions for 48 hours +/- LY2109761 or LY2157299.

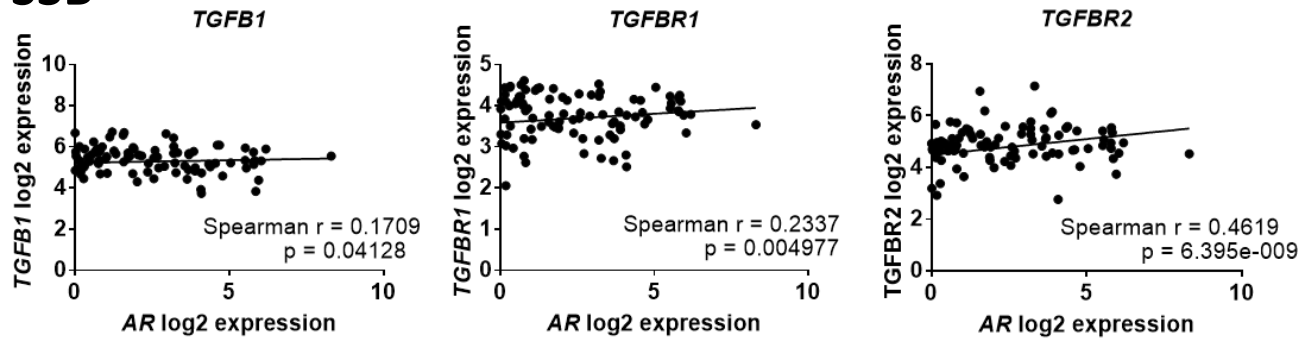
Supplementary Figure 2C: Trypan blue assay for percentage of dead cells count in MDA-MB-453 cells in attached or suspended conditions for 48 hours +/- LY2109761 or LY2157299.

Supplementary Figure S3

S3A



S3B



1091 **Supplementary Figure 3A)** TGF β signaling pathway genes from clinical data sets from the
1092 TCGA-BRCA cohort and their correlation with AR.

1093 **Supplementary Figure 3B)** TGF β signaling pathway genes from clinical data sets from the
1094 SCAN-B cohort and their correlation with AR.

1095 Significance at $P < 0.05$

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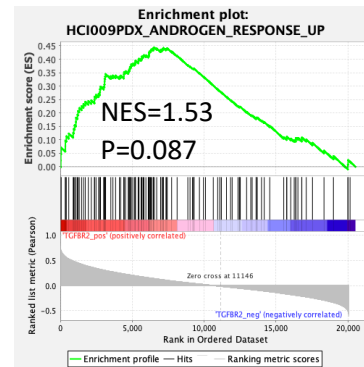
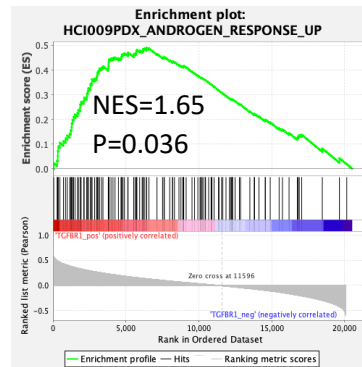
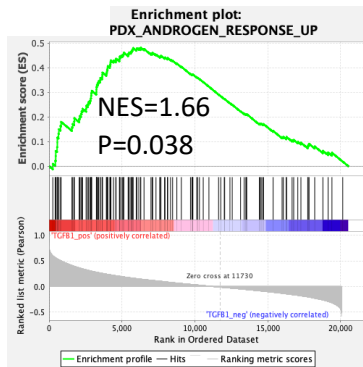
Supplementary Figure S4

S4A

TGFB1

TGFB1

TGFB2

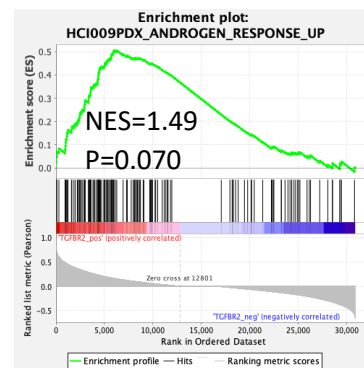
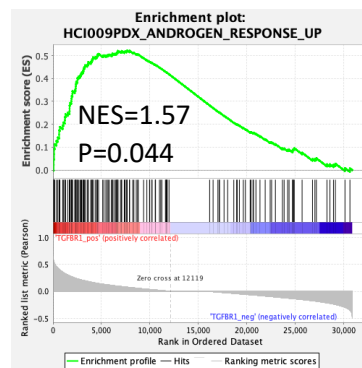
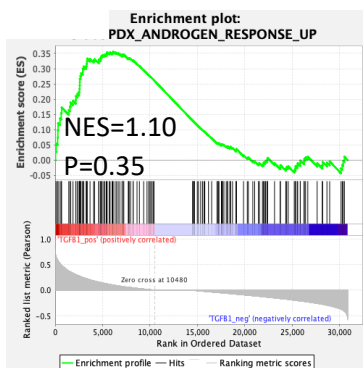


S4B

TGFB1

TGFB1

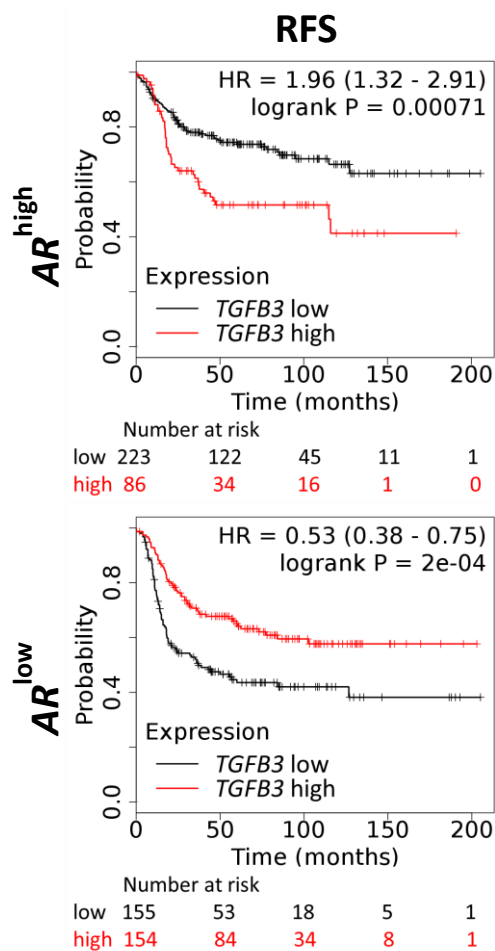
TGFB2



Supplementary Figure 4A) GSEA analysis on the same TGF β related genes relating to the androgen response gene set from the HCI-009 PDX in the TCGA data set

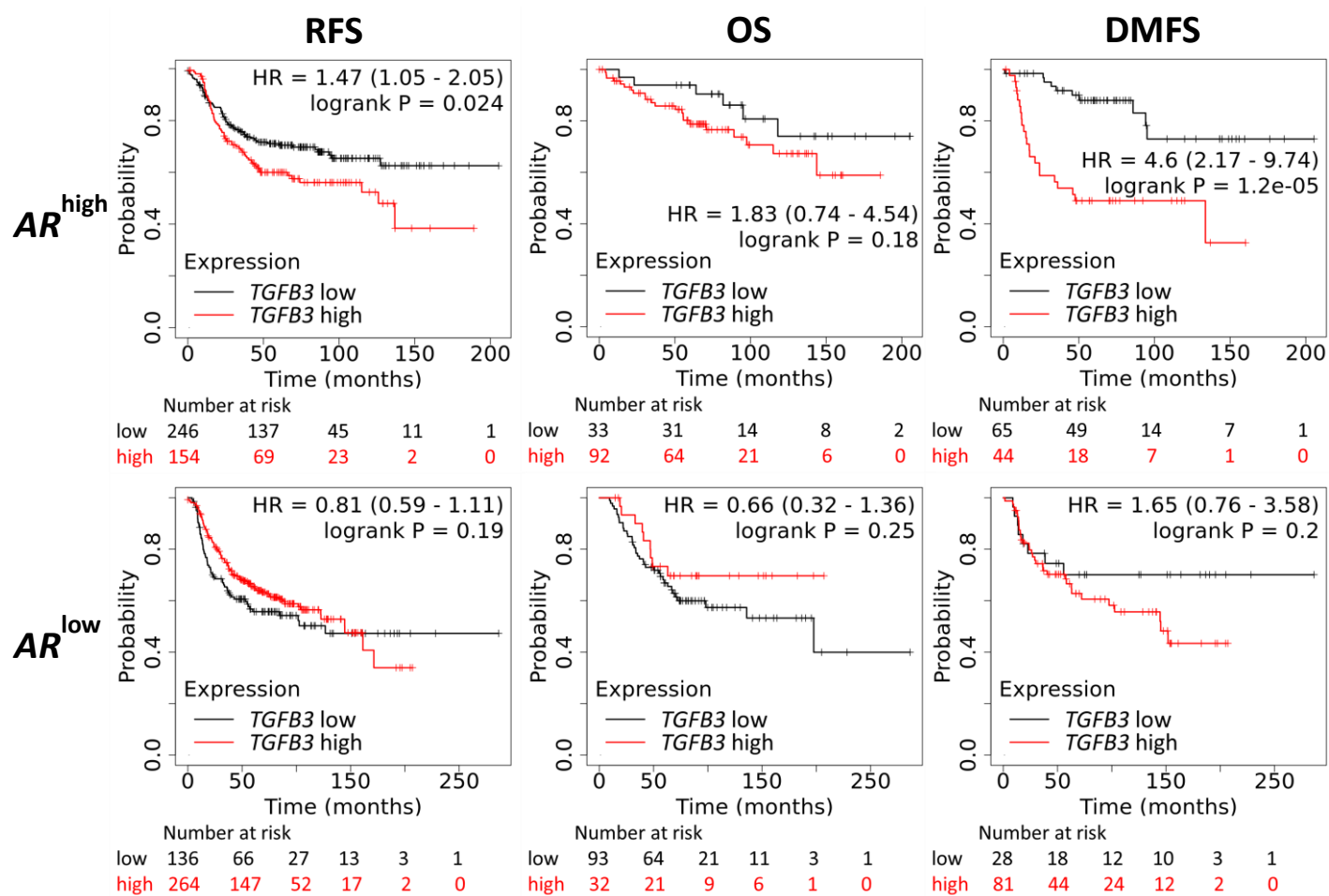
Supplementary Figure 4B) GSEA analysis on the same TGF β related genes relating to the androgen response gene set from the HCI-009 PDX in the SCAN-B data set

Supplementary Figure S5



Supplementary Figure S5: KMplotter was used to stratify basal BC patients stratified into AR^{high} (top) or AR^{low} (bottom) populations. *TGFB3* expression was then interrogated based on relapse-free survival (RFS, n = 618) and distant metastasis-free survival (DMFS, n = 232).

Supplementary Figure S6



1229 **Supplementary Figure S6:** KMplotter was used to stratify ER- patients stratified into AR^{high}
1230 (top) or AR^{low} (bottom) populations. *TGFB3* expression was then interrogated based on relapse-
1231 free survival (RFS, n = 801), overall survival (OS, n = 251), and distant metastasis-free survival
1232 (DMFS, n = 218).