Kinase Plasticity in Response to Vandetanib Enhances Sensitivity to Tamoxifen in Estrogen Receptor Positive Breast Cancer

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ABSTRACT

Resistance to endocrine therapy (ET) is common in estrogen receptor (ER) positive breast cancer. Multiple studies have demonstrated that upregulation of MAPK signaling pathways contributes to ET resistance. Herein we show that vandetanib treatment enhances sensitivity to ET in ET-sensitive and -resistant ER+ breast cancer models. Vandetanib treatment alters the gene expression program of ER+ breast cancer cells resulting in a less proliferative and more estrogen responsive Luminal-A like character. Tyrosine kinase network reprogramming was assessed using multiplexed kinase inhibitor beads-mass spectrometry (MIB/MS) assay to identify adaptive resistance mechanisms to vandetanib treatment, including upregulation of HER2 activity. Co-treatment to inhibit HER2 with lapatinib enhanced sensitivity to vandetanib, demonstrating biologic activity of HER2 upregulation. Using a CRISPR knockout model, we demonstrate that vandetanib effects are partially mediated by RET receptor tyrosine kinase. Finally, we use our operating room-to-laboratory assay that measures drug response in individual primary tumor cells in short term cultures to demonstrate conserved gene expression changes, including increased HER2 activity signatures, in vandetanib treated cells, and identify features associated with vandetanib response. These results support future investigation of RET targeting strategies considering reprogrammed networks, such as activated HER2, in patients with ET resistant ER+ breast cancer.

SIGNIFICANCE

Vandetanib enhances sensitivity to tamoxifen in ER+ breast cancer cells by reprograming kinase signaling networks, which can be used to select patients most likely to respond and develop more efficacious co-treatment strategies.

INTRODUCTION

Approximately 75% of breast cancers fall within the estrogen receptor (ER)+/luminal subtypes(1-3). Over 40,000 women are expected to die from breast cancer each year and most of these will have ER+ disease(4). Most patients with ER+ breast cancer will be treated with endocrine therapy (ET) targeting ER. However, resistance to ET occurs in about 1/3 of early cancers and develops over time in all metastatic ER+ cancers(5-7). Targeted inhibitors to PI3K, mTOR, and CDK4/6 have shown efficacy in patients with ET resistant disease(8-11). However, responses are sometimes short lived and there are a significant number of patients in need of durable, effective therapy.

Multiple studies have demonstrated that activation of ERK/MAPK pathways is an important mechanism by which ER+ breast tumors resist ET(12-18). Gain of function mutations in ERK/MAPK nodes or upstream receptor tyrosine kinase (RTK) are seen in ET-resistant tumors, although these are only present in about 13% of cases(12). The rearranged during transfection (RET) receptor tyrosine kinase is expressed in approximately 50% of ER+ breast tumors and is more highly expressed in ET resistant tumors(19-22). Expression of RET and co-expression of its activating ligand glial derived neurotropic factor (GDNF) is associated with transciptional signatures of ERK/MAPK activation and worse outcomes in human ER+ breast tumors.(23) Further, RET is overexpressed in ET-resistant breast cancer organoids that contain gain of function ESR1 (encoding the estrogen receptor) mutations and inhibiting RET suppresses tumor growth(24).

Our work has shown that tyrosine kinase inhibitors (TKIs), such as vandetanib and sunitinib, that inhibit RET reduce proliferation and enhance sensitivity to ET in ER+ breast cancer cell lines. Further, dual treatment with vandetanib and tamoxifen reduced breast tumor xenograft growth greater than either agent alone(25, 26). In RET+ primary breast tumors, we showed that *in* vitro treatment with sunitinib reduces phosho-ERK.(27) These findings support the use of vandetanib to reduce ERK/MAPK activation and improve response to endocrine therapy.

Despite promise in the pre-clinic, clinical trials with vandetanib have had disappointing results. However, the design of these trials failed to account for potentially predictive biomarkers including ER and RET or only evaluated vandetanib as a monotherapy. For example, a phase II trial of single-agent vandetanib in 46 patients with metastatic breast cancer did not demonstrate a benefit(28), but did not report receptor status, making it difficult to interpret the extent to which this trial included patients that mechanistically would be predicted to respond. Our work, including the findings presented herein, suggests that vandetinib may provide a clinical benefit for some patients. More recent TKI trials with more specific inclusion criteria have shown more promising results and have renewed interest in TKI for ET resistant ER+ breast cancer. A phase II trial of cabozantinib, in metastatic ER+/HER2- was positive by objective response rate(29), as was a phase Ib/II trial of lenvatinib with letrozole(30). More precise biomarkers are needed to identify patients most likely to respond to TKI therapy. To develop more precise biomarkers and elucidate bypass resistance

mechanisms, we sought to investigate how vandetanib alters kinase signaling pathways in ET-sensitive and ET-resistant models of ER+/HER2- breast cancer.

METHODS

Cell Lines

We obtained ER+ breast cancer cell lines parental MCF7, tamoxifen-resistant MCF7 (MCF7-TAM), exemestane-resistant MCF7 (MCF7-EXE), parental T47D, and tamoxifen-resistant T47D (T47D-TAM), from the American Type Culture Collection (ATCC). Cell lines were cultured using Dulbecco's Modified Eagle Medium (DMEM) (Gibco) with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Life Technologies) at 37°C and 5% CO2. MCF7-TAM and T47D-TAM were maintained by chronic treatment with 1 μM of tamoxifen and MCF7-EXE with 0.1 μM of exemestane.

Chemicals and Treatment

Vandetanib (SelleckChem) and z-4-Hydroxytamoxifen (4OH-Tam) (SelleckChem) was dissolved in dimethyl sulfoxide (DMSO). GDNF (R&D Systems) was dissolved in phosphate-buffered saline (PBS) and treatments were done at a final concentration of 25 ng/mL. Pralsetinib was obtained from Chemietek, and lapatinib from Medchem Express LLC. Treatments were performed for 24 hours unless otherwise specified.

Cell Viability Assays

Cells were plated on opaque-walled 96-well plate at a density of 4,000 cells per well in technical triplicates and allowed to adhere overnight prior to the addition of the specified inhibitors. Viability was assessed using the CellTiter-Glo (CTG) Luminescent Cell Viability Assay (Promega). Plates were read using a Synergy 2 microplate reader. Samples were averaged

over biological triplicates. Statistical analysis and plotting were performed using GraphPad Prism 9.

Western Blots

Cells were lysed in Pierce RIPA buffer containing Halt protease inhibitor cocktail and PhosStop phosphatase inhibitor (Thermo Scientific). Proteins were resolved by SDS polyacrylamide gel electrophoresis, transferred to PVDF membrane, and blocked with 10% BSA, then incubated overnight with the indicated primary antibody in blocking buffer. Membranes were washed three times in tris buffered saline containing 0.1% tween-20 then then incubated for 1 hour with HRP-conjugated secondary antibody. Blots were developed using a Chemidoc-touch digital imager and band intensity quantified using ImageJ (https://imagej.net/ij/index.html).The antibodies used for Western blot included: p44/42 MAPK (ERK1/2) (Cell Signaling Technologies), Phospho-p44/42 MAPK (Erk1/2) (Cell Signaling Technologies), RET (Abcam), and β-Actin (SantaCruz).

RET CRISPR Knockout

An sgRNA was designed to target exon 3 of RET (AGCACTCCCGCTCACGGTC), cloned into the pSpCas9(BB)-2A-Puro(PX459) V2.0 (Addgene) plasmid, and verified by Sanger sequencing. MCF-7 cells were cultured in antibiotic free media and grown to a confluency of 70%-90%. Cells were then transfected with sgRNA plasmid using FuGENE 4K Transfection Reagent according the manufacturer's recommendations. After 72 hours, cells were passaged onto 15 cm plates at a low density and selected with 1 μg/mL puromycin (Gibco) for 3 days. Cell colonies were then isolated by pipet, and DNA was isolated using the Quick-DNA Miniprep Kit

(Zymo Research). The target sequence was then amplified by PCR and then sequenced for verification of a frameshift mutation. Both RET alleles were found to have frameshift mutations resulting in the introduction of premature stop codons.

mRNA Sequencing and Data Analysis

Cells were treated with 10 µM vandetanib or vehicle for 24 hours in biological triplicates and total RNA was isolated (Qiagen). mRNA sequencing libraries were made using TruSeq RNA Library Prep Kit (Illumina) and sequenced using the Illumina NextSeq2000. Plots and statistical analyses were performed using RStudio 2022.07.1 Build 554.

Differential gene expression (DGE) was determined using the DESeq2 R package using raw mRNA-seq counts from STAR/Salmon. Genes with less than 10 counts across all samples were omitted before analysis and counts were filtered and normalized to the upper quartile using NOISeq. Significant differentially expressed genes were defined by using a Log2FC (log2 fold change) cutoff of 0.5 for upregulation and -0.5 for downregulation, with a false discovery rate (FDR) < 0.05. Volcano plots of all genes were plotted using the EnhancedVolcano R package.

Enrichment of gene sets was then investigated through hypergeometric analysis of an unranked list of upregulated or downregulated elements using the enricher function of the clusterProfiler package. Gene sets from MSigDB were first compiled and screened for keywords of interest pertaining to breast cancer, including estrogen and ER signaling.

METABRIC and SCAN-B databases were obtained for survival analysis and filtered for ER+/HER2- patients treated with ET. A median-centroid approach was implemented to assign a

gene set score per patient. Kaplan-Meier (KM) plots were then computed using the survminer and survival packages.

MIB/MS and Data Analysis

Cells were treated with vandetanib at its IC90 for 48 hours. Cells were harvested by scraping and cell pellets obtained for proteomic analysis using the MIB/MS technology(31, 32). Scaled label free quantification (LFQ) values were obtained and a correlation plot of samples was computed to assess homogeneity of triplicates and heterogeneity of treatment conditions for quality control using ggplot2. Differential protein expression (DPE) was analyzed using the limma R package using scaled LFQ values. Significant differentially expressed proteins were defined using a Log2FC (log2 fold change) cutoff of 0.5 for upregulation and -0.5 for downregulation, along with a p-value < 0.05.

Primary ER+/HER2- Human Tumor Cells

A primary tumor sample was obtained from a patient with ER+/HER2- invasive ductal carcinoma. The tumor was processed fresh as previously described(33). In brief, tumor tissue was transported immediately to the laboratory after surgical resection, dissociated into a single cell suspension, and treated with vandetanib (10 µM) or control media for 12 hours. After treatment, single cell RNA sequencing libraries were made using the 10x Genomics Chromium Next GEM Single Cell 3’ v3.1 and sequenced on the NextSeq2000 (Illumina) with pair-end sequencing.

Single cell RNA sequencing analysis

Reads were aligned and counted using Cellranger count function with default parameters. The counts and features matrixes were used to generate Seurat objects. The Seurat objects corresponding to each treatment group were merged with Seurat's function merge(), and low quality cells were removed. Doublets were removed using scDblFinder (https://pubmed.ncbi.nlm.nih.gov/35814628/) and cells were further filtered with the following parameters: nFeature_RNA > 200 & nFeature_RNA < 6000 & percent.mt < 10.

Cell types were annotated on the combined Seurat using SingleR using the cell type atlas HumanPrimaryCellAtlasData() accessed with the package celldex. InferCNV was used on the combined Seurat to predict copy number alterations, defining the immune cells ("T_cells","Macrophage","DC","Monocyte","NK_cell") as reference cells (cells without CNVs). The tumor cells were identified using the copy number heatmap per cell.

Differential analysis of gene expression was performed on the tumor cells to compare vandetanib-treated versus non-treated cells using findmarkers(). Gene Set Enrichment Analysis was then performed using the list of differentially expressed genes, ranking the genes according to log2(FC)/FDR, and the ranked gene lists were used in the GSEA software (https://pubmed.ncbi.nlm.nih.gov/16199517/) (https://pubmed.ncbi.nlm.nih.gov/12808457/). Gene signatures were scored using AddModuleScore_UCell() from the UCell package.

We used the package NMF to build a non-negative matrix factorization (NMF) model of the tumor cells using a list of genes differentially expressed upon vandetanib treatment in MCF7 cells from this study. The Seurat was subset to contain only the above-mentioned genes, and the NMF model was fitted to 8 factors. Heatmaps were built for each treatment group to assess

enrichment of cells to different NMF factors. Each cell was assigned to a unique NMF according to the NMF with maximum coefficient value for that cell, and the percentage of cells belonging to each NMF factor was calculated for each treatment group. DE analysis was performed comparing cells in certain NMF factors.

Study Approval

Patient samples were obtained after written informed consent under a protocol approved by the institutional review board at the University of North Carolina (IRB#22-1888) in accordance with the US Common Rule.

Sex as a Biologic Variable

Breast cancer occurs primarily in women and so primary tumor cells were obtained from a female patient with breast cancer.

Statistics

All experiments were performed with at least 3 biological or technical replicates. Small sample number comparisons were analyzed with an unpaired *t* test with Welch's correction for non-equal SD. Linear regressions and nonlinear regression curves were compared by statistical tests on the parameters of the curves using the extra sum of squares *F* test. Experiments with large sample numbers were analyzed by Wilcoxon test.

Data Availability

Single cell and bulk RNA sequencing data was made available at the Sequence Read Archive (SRA) under BioProject PRJNA1177387. Mass spectrometry protein sequencing results are included in this manuscript as Supplementary Table 1.

Code Availability

All original code has been deposited on the Github, and it is publicly available at the Github repository at https://github.com/santiagohaase/Spanheimer-lab-Kinase-Plasticity-in-Response-to-Vandetanib-Enhances-Sensitivity-to-Tamoxifen

RESULTS

Vandetanib Inhibits ERK Activation and Viability in ER+ Breast Cancer Cells

We previously showed that vandetanib treatment decreases ERK activation in MCF7 breast cancer cells. To investigate if vandetanib similarly decreases ERK activation in ET-resistant ER+ breast cancer cells we used MCF7 and two ET-resistant derivatives, MCF7-TAM (tamoxifen) and MCF7-EXE (exemestane) which had been cultured long-term under drug treatment to develop resistance. pERK/ERK was reduced in MCF7 with vandetanib treatment (-81%, $p<0.001$). Similarly, pERK/ERK was reduced in MCF-TAM with vandetanib treatment (-78%, $p=0.025$) and MCF7-EXE (-78%, $p=0.01$), Fig. 1A. Dose response curves demonstrated an IC50 for vandetanib of 8.5 μM in MCF7, 10.4 μM in MCF7-TAM, and 15.9 μM in MCF7-EXE (Fig. 1B). Similar vandetanib sensitivities were seen in T47D and T47D-TAM (Supplementary Fig. 1)

Vandetanib Sensitizes ER+ Breast Cancer Cells to Tamoxifen

Because we had previously shown interaction between vandetanib and ER downstream signaling, we tested if co-treatment with vandetanib enhanced antiproliferative effects of tamoxifen. The IC50 of the active metabolite of tamoxifen, 4OH-Tam, was 10.7 μM in MCF7, 15.8 μM in MCF7-TAM, and 14.1 μM in MCF7-EXE (Fig. 1C) demonstrating decreased

sensitivity to tamoxifen with chronic ET treatment.Treatment with 10 μM vandetanib resulted in left shift of the 4OH-Tam sensitivity curve in all 3 cell lines with the IC50 for 4OH-Tam of 3.8 μM in MCF7, 4.8 μM in MCF7-TAM, and 4.8 μM in MCF7-EXE. This demonstrates that cells are more sensitive to tamoxifen in the presence of vandetanib. To confirm synergy, we calculated the combination index (CI) of a combined treatment of 5 μM vandetanib with 2 μM 4OH-Tam (Fig. 1D). When compared to the individual treatments (i.e., Tamoxifen or vandetanib alone) the combination index was 0.47 for MCF7-TAM, 0.55 for MCF7-EXE and 0.59 for MCF7, indicating increased synergy in ET-chronically treated cells.

Vandetanib Regulated Genes in ET Sensitive and Resistant ER+ Breast Cancer

To delineate the molecular basis for how ER+ breast cancer cells respond to vandetanib treatment resulting in increased sensitivity to tamoxifen, we performed mRNA sequencing after treatment with vandetanib (10 μM) compared to vehicle. In MCF7, downregulated genes were enriched for MAPK signaling and proliferative signatures, Fig. 2A, consistent with loss of pERK observed in Fig. 1A. Recently derived KRAS/ERK activity signatures were also depleted in treated cells (34). Upregulated genes were enriched for signatures of estrogen response and sensitivity to tamoxifen consistent with sensitization of MCF7 cells to tamoxifen (Fig. 1D). Up-regulated and down-regulated gene sets in MCF7-TAM (Fig. 2B) and MCF7-EXE (Fig. 2C) showed similar depletion of proliferative and MAPK associated signatures and enrichment of estrogen and tamoxifen response signatures. Similar transcriptional effects of vandetanib were seen in T47D and T47D-TAM (Supplementary Fig. 2).

Because estrogen response signatures were enriched and proliferative signatures reduced with vandetanib treatment, which are hallmark features of the indolent and ET responsive Luminal A molecular subtype, we analyzed the effect of vandetanib on correlation to genes that define the LumA subtype on the PAM50 assay (LumA centroid). This analysis demonstrated vandetanib treatment resulted in a stronger LumA correlation supporting that treatment is inducing a more indolent and ET responsive transcriptional phenotype (Fig. 2D).

To elucidate common and unique vandetanib regulated genes we determined overlap across the parental and ET resistant MCF7 cell lines (Fig. 2E). Notably, significant commonly upregulated genes included *SELENBP1* and *BAG1*, which are associated with reduced proliferation, improved survival, and increased ER transcriptional activity in ER+ breast tumors(35-37). In the common downregulated genes, *AURKA* is associated with poor survival in ER+ breast cancer patients(38). Reduced expression of *ECT2* is associated with abrogation of MAPK signaling(39). Finally, several key regulators of cell cycle progression associated with higher grade, worse outcomes, and poor response to endocrine therapy in ER+ breast cancer are commonly downregulated including *CCND1*, *TOP2A*, and *CCNB1*.(40-43)

To test if signatures of vandetanib regulated genes are associated with prognosis in patients with ER+/HER2- breast cancer treated with endocrine therapy, we obtained survival annotated transcriptional data from METABRIC(44) and SCAN-B(45). Tumors were stratified by median gene set score using median thresholding. METABRIC patients with low expression of genes that were downregulated by vandetanib had significantly improved OS (HR=0.75, $p<0.0001$) and RFS (HR=0.60, $p<0.0001$). Similarly, SCAN-B ER+ breast cancer patients with

low expression of vandetanib downregulated genes demonstrated significantly lower mortality compared to high signature score (HR=0.87, p=0.034), Fig. 2F. These findings support that downregulation of genes that are repressed by vnadetanib is associated with better outcomes across datasets. To determine whether vandetanib treatment is reducing expression of genes that drive an ET- resistant phenotype, we obtained gene expression data from patients with ER+/HER2- breast cancer profiled on ET and clinically annotated as sensitive or resistant(13). Resistant tumors had significantly higher expression of the vandetanib downregulated signature compared to sensitive tumors (4.3 vs 3.8, p<0.001), Fig. 2G. Cumulatively, these findings support that in ER+ breast cancer, vandetanib treatment promotes gene expression changes associated with lower proliferation, increased ET response, and improved survival.

Functional Signatures of Vandetanib Response

Vandetanib alters kinase signaling which is regulated at the protein level. We used multiplex inhibitor bead enrichment coupled to mass spectrometry (MIB/MS) to investigate reprogramming of kinase signaling networks in response to vandatinib (31). In this technique, kinases are enriched by incubating cell extracts with type 1 kinase inhibitors immobilized on Sepharose beads. Each cell line was treated with the IC90 of vandetanib or vehicle for 48 hours, Fig. 3A. We first investigated the effects of vandetanib on enrichment and depletion of known vandetanib targets(46). RET, EGFR, ABL1, RIP2K, PTK6, and DDR1 had similar magnitude of depletion in all 3 models, despite large differences in baseline enrichment between MCF7 and the ET-resistant derivatives, Fig. 3B. Similar results were seen for T47D and T47D-TAM (Supplementary Fig. 3). These results demonstrate that several distinct target proteins may

be mediating vandetanib effects in ER+ breast cancer models, that effects of treatment do not appear to be proportional to baseline expression, and that the relative level of expression of a target alone may not be a good biomarker of response.

Enriched and depleted kinase proteins by cell line are shown in Fig. 3C. Overlap analysis (Fig. 3D) showed that similar to transcriptional findings, kinome dynamics were heterogenous across cell lines. There were 12 common upregulated and 22 downregulated kinase proteins. Common upregulated genes included AURKA, PLK1, PIP4K2A, which promote proliferation and aggressive tumors(47-49), as well as JAK1 and TYK2 which are associated with immune infiltrates and are upstream of AKT signaling(50, 51). Common depleted proteins with vandetanib treatment included proliferation promoting cell cycle regulators CDK1, CKD2, and PKMYT1. Depletion of MAP2K2 (MEK2) as well as upstream receptor tyrosine kinases EPHA1, EPHB4, DDR1, and EGFR suggest downregulation of the MAPK signaling pathway in response to vandetanib treatment consistent with Western blots (Fig. 1A). Gene set enrichment analysis demonstrated a common upregulation of the PI3K/AKT pathway and downregulation of the ERK cascade including transciprtional and phosphoproteomic signatures (52), Fig. 3E.

Pharmacologic Inhibition of Adaptive Resistance Kinases Enhances Vandetanib Efficacy

Upregulation of functional kinases may identify common mechanisms of adaptive bypass resistance so activity levels of druggable kinases(24) were assessed by treatment (Fig. 4A). To determine whether enriched druggable targets may be mediating adaptive resistance to vandetanib we performed co-treatment assays on the principle that inhibiting mediators of adaptive resistance should enhance sensitivity to vandetanib. Because ERBB2/HER2 was

enriched in all 3 MCF7 cell lines, we co-treated cells with vandetanib and the FDA approved HER2 small molecule inhibitor lapatinib. Treatment with lapatinib resulted in significantly augmented vandetanib reduction in cell viability in all 3 cell lines, (Fig. 4B), demonstrating the phenotypic significance of upregulated protein activity.

RET Mediates Vandetanib Sensitivity

Functional kinases that are downregulated after vandetanib treatment may be mediating the effects of vandetanib. Based on our prior work showing a role for RET in mediating vandetanib response and in driving poor outcomes and ET resistance in ER+ breast cancer we explored the role of RET signaling in vandetanib sensitivity. As expected from the MIB/MS data, transient RET antagonism with the selective RET inhibitor BLU667 resulted in reduced sensitivity to vandetanib in MCF7 in dose dependent fashion. BLU667 also reduced vandetanib sensitivity in MCF7-TAM, but not in the low RET expressing MCF7-EXE (Fig. 5A). These findings demonstrate that baseline activity, as well redcuced activity with treatment, are important for mediators of response.

We next sought to clearly elucidate the role of RET In ER+ breast cancer cells by generating a CRISPR knockout in MCF7 cells (RET KO). Western blot confirmed knockout of RET protein (Fig. 5B). We used our CRISPR KO model to determine the impact of RET KO on sensitivity to vandetanib. Supporting a role for RET as a mediator of vandetanib response, RET KO cells were significantly less sensitive to vandetanib compared to WT (Fig. 5C). Based on the vandetanib synergy assay we hypothesized that RET KO would sensitize cells to tamoxifen, but unexpectedly observed that KO cells were less sensitive (Fig. 5D).

Because decreased sensitivity to tamoxifen with RET KO was observed we next sought to explore the molecular basis for this unexpected finding by performing mRNA sequencing and comparing RET KO and WT cells. We assessed ESR1 (encoding ER) expression in the mRNA data and found that RET KO cells had reduced expression of ESR1 (log2FC -1.27, padj <0.001), Fig. 5E. Reduced ER expression could account for reduced sensitivity to tamoxifen in cells that have stable KD of RET. We next performed unbiased analysis of differentially regulated genes with RET KO (Fig. 5F). Hallmark gene sets for estrogen response and KRAS signaling were depleted with RET KO, as were genes previously associated with RET knockout. RET KO resulted in lower proliferation score compared to WT. Supportive reduced proliferative signature, RET KO cells had significantly reduced growth at 48 and 72 hours relative to WT cells (Fig. 5G). Cumulatively these findings support that RET is a mediator of vandetanib response and drives a proliferative phenotype, but that complex RET-ER interactions may evolve over time, and this may impact sensitivity to endocrine therapy.

Vandetanib Response at Single Cell Resolution in Primary ER+ Breast Cancer

We used our previously validated *ex vivo* short term drug treatment platform coupled to single cell RNA sequencing(33) to assess how primary breast tumor cells respond to vandetanib treatment. Specifically, we obtained a primary ER+/HER2- breast cancer, dissociated into single cells, treated with control of vandetanib containing media for 12 hours and assayed by 10x scRNAseq. Uniform manifold projection (UMAP) was performed using Seurat v3(53). Cell populations were annotated using known transcriptional profiles and canonical markers (Fig. 6A) and by treatment group (Fig. 6B). Malignant (tumor) cells were distinguished from non-tumor

epithelial cells as cells having significant inferred copy number alterations by InferCNV(54). Differentially expressed genes were identified using a false discovery rate (FDR) cutoff of < 0.05 in the iCNV+ tumor cell compartment with vandetanib treatment compared to control, Fig. 6C. Enriched and depleted pathways in vandetanib treated tumor cells relative to control treated were determined, Fig. 6D. Of note, as seen in cell lines, ESR1 targets and ERBB2 pathway signaling were enriched in vandetanib treated cells (Fig. 6E).

We tested the overlapping downregulated genes after vandetanib treatment across the 3 MCF7 cell lines (Fig. 3) and these genes were also depleted in vandetanib treated primary tumor cells (Fig. 6E). Next LumA correlation score was assigned to each tumor cell using a validated single cell PAM50 classifier(55). Treated tumor cells had a stronger correlation to the LumA centroid (median score treated 0.259 vs median score untreated 0.244, p= 0.001, Wilcoxon rank-sum test). Thus, as with cell lines, short term vandetanib treatment alters the transcriptional profile of ER+/HER2- primary tumor cells towards a more indolent and estrogen responsive phenotype.

To explore features of vandetanib response within primary tumor cells we used non-negative matrix factorization (NMF) to elucidate features that were enriched and depleted in vandetanib treated cells (Supplementary Fig. 4A). This analysis yielded metagene modules that were enriched in vandetanib treated samples (module 1) and depleted in vandetanib treated cells (Module 6) (Supplementary Fig. 4B). We next identified features that may be associated with vandetanib response by comparing module 1 and 6 gene expression profiles. Module 1 (vandetanib resistant) had expected high expression of genes that increase with vandetanib

treatment, consistent with reduced response. Module 6 was enriched for MYC targets, which is known to support tamoxifen resistance, but may support a transcriptional profile of vandetanib sensitivity (Supplementary Fig. 4C). Module 1 was also enriched for MTORC signaling which we identified in cells lines as an adaptive vandetanib resistance mechanism. Interestingly, module 1 was enriched for hallmark estrogen response genes and our previously generated single cell tamoxifen response score(33) supporting that cells with poor vandetanib response have enhanced tamoxifen sensitivity (Supplementary Fig. 4D). Lastly, we looked at our human tumor derived signatures of high GDNF/RET expressing human ER+-breast tumors(23). Genes that were depleted in GDNF/RET high human tumors were depleted in vandetanib sensitive cells, supporting that signature as a biomarker of response (Supplementary Fig. 4E).

DISCUSSION

Resistance to endocrine therapy remains a common problem and a significant driver of poor patient outcomes despite advances in understanding of how ER+ breast tumors respond to therapy. The identification of activation in MAPK pathways as a driver of resistance to ET has generated significant interest in targeting this pathway to enhance ET response. However, clinical trials have demonstrated inconsistent and disappointing results regarding response to tyrosine kinase inhibitors for non HER2-amplified tumors. These findings demonstrate the need for improved understanding of how intracellular signaling pathways are altered by treatment to determine biomarkers of patients most likely to respond and clinically actionable kinase targeting strategies in MAPK-activated ET resistant breast tumors.

We found that response to vandetanib, measured by IC50 or by depletion of RET activity, was largely the same across the cell lines tested, despite large differences in baseline RET expression, and this was also true for the other direct vandetanib targets. These findings demonstrate that single protein expression may not be an effective biomarker of response and signatures of activity may be more informative of response than single gene expression. This concept has been shown in HER2 targeting therapies where expression of the HER2E gene expression pattern is more predictive of response than HER2 expression alone(56). We recently generated a signature of RET active ER+ breast tumors(23) using treatment naïve samples from The Cancer Genome Atlas (TCGA), and that low expression of genes that were depleted in GDNF/RET high tumors was associated with vandetanib sensitivity, suggesting that this may be a biomarker to select patients most likely to respond.

RET partially mediated effects of vandetanib, but stable knockout of RET unexpectedly resulted in reduced, rather than augmented, sensitivity to tamoxifen. ESR1 expression was found to be reduced after RET knockout and this could account for decreased sensitivity to tamoxifen. While ER has 2 binding sites in the RET promoter that activate RET expression (57), no reciprocal mechanism by which RET regulates ER is known. Prior work has shown that TKI treatment reprograms transcriptional networks in HER2-amplified (58) and triple negative breast cancer(32) and similar remodeling over time may reduce ESR1 expression with loss of RET activity. Alternatively, because RET expression and ER-activity are correlated, RET KO may select for cells that are less dependent on RET signaling, which may also be cells that have lower baseline ER-activity. This finding could help explain for negative clinical trials of RET inhibitors with anti-ER therapy if tumor cell subpopulations that evolve resistance to RET inhibitors are also less dependent on ER-activty, and therfore less responsive to ET. Further study is needed to model emerging resistance to RET and ER inhibitors and track relative dependencies on RET and ER within emerging resistant tumor cell subpopulations.

Kinase signaling networks are plastic and inhibiting one node can lead to adaptive bypass through another pathway. We were able to identify mediators of adaptive resistance to vandetanib treatment and augment response with dual treatment. Up-regulation of HER2 activity was common to all cell lines and primary tumor cells and targeting HER2 with lapatinib enhanced sensitivity to vandetanib. Activating HER2 signaling could open a number of efficacious therapeutic options for patients. While HER2 activity was increased, there was no

change in HER2 RNA expression, and this highlights the need for assessment at the level of protein activity to determine adaptive bypass mechanisms.

While HER2 upregulation was a common adaptive bypass pathway, we also identified cell line specific enriched proteins. This could be a result of distinct adaptations necessary to resist exemestane compared to tamoxifen or could represent distinct alternative paths to resist vandetanib treatment. How these adaptive mechanisms are conserved or unique in human tumors will be essential to understand the therapeutic implications of these co-treatment targets. Specific adaptive bypass pathways may be predictable from pretreatment gene expression or chromatin accessibility states, or tumor level assessment of response to therapy may be needed to identify tumor specific bypass mechanisms and cotreatment vulnerabilities. To this end, our group has recently developed a method for whole kinome characterization on limited sample (such as a core needle biopsy), which could make on treatment assessment of adaptive response and targeted co-treatment strategies feasible.(59)

In summary, we demonstrate that ER+ breast cancer cells become more sensitive to ET with vandetanib treatment. Vandetanib results in broad reprogramming of gene expression and kinase signaling which results in a lower proliferative, more estrogen responsive luminal A-like state. Early adaptive response, including upregulation of HER2, to vandetanib identified co-treatment strategies to enhance response. Future trials will need to utilize signature-based biomarkers of response with attention to tumor specific upregulated adaptive bypass pathways as cotreatment strategies to improve efficacy.

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FIGURES

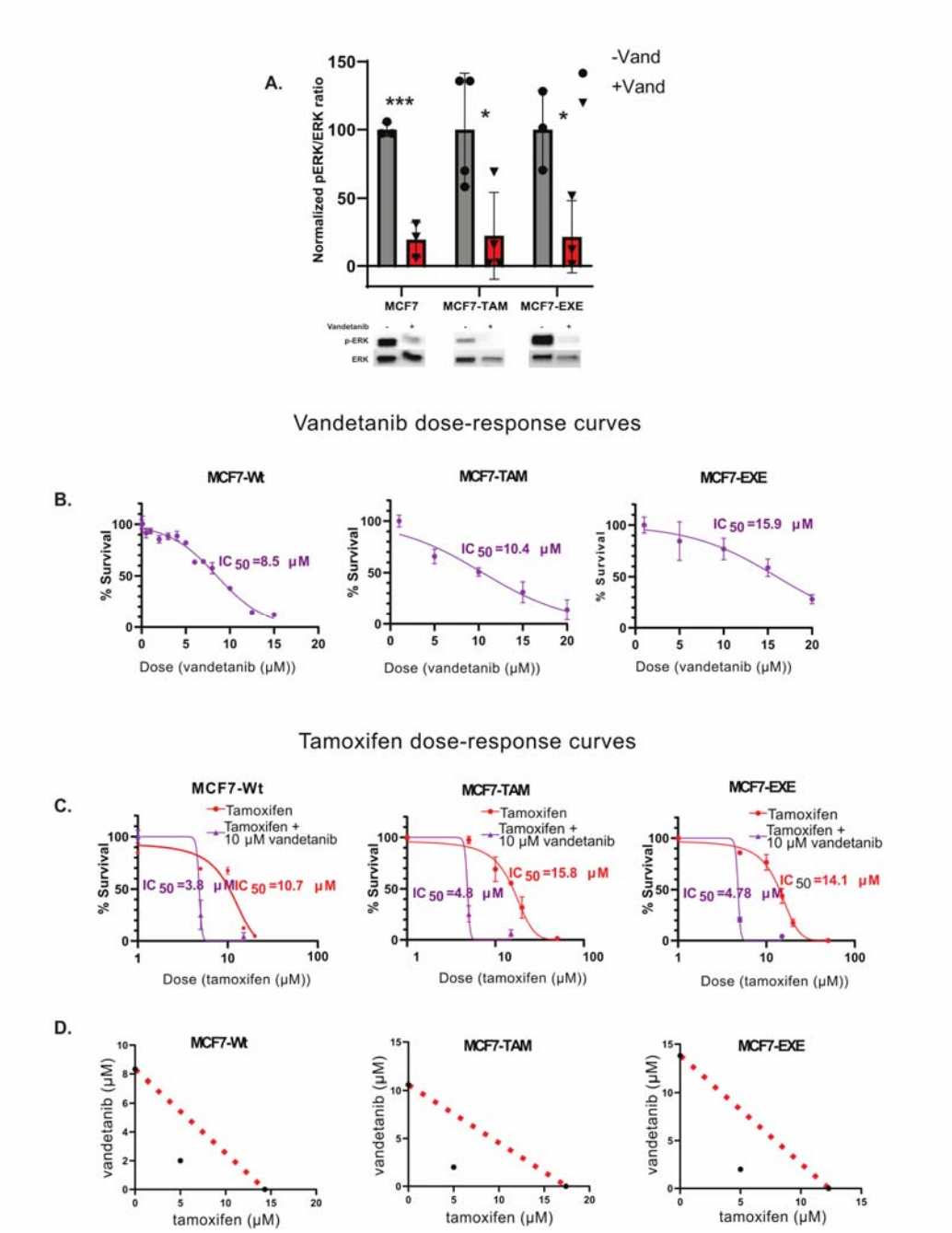

Figure 1. (A) Treatment of MCF-7 and tamoxifen resistant MCF7 (MCF7-TAM) and exemestane resistant MCF7 (MCF7-EXE) reduces pERK/ERK in all 3 cell lines. (B) Vandetanib dose dependent reduction in cell viablilty in MCF7, MCF7-TAM, and MCF7-EXE. (C) Treatment with Vandetanib results in a left shift of the tamoxifen sensitivity curve and reduction in IC50 in all 3 cell lines. (D) Synergy plots demonstrate synergistic activity of tamoxifen and vandetanib.*$p<0.05$, ***$p<0.01$ by t-test with Welch correction and analysis of the slope difference in the nonlinear regression model.

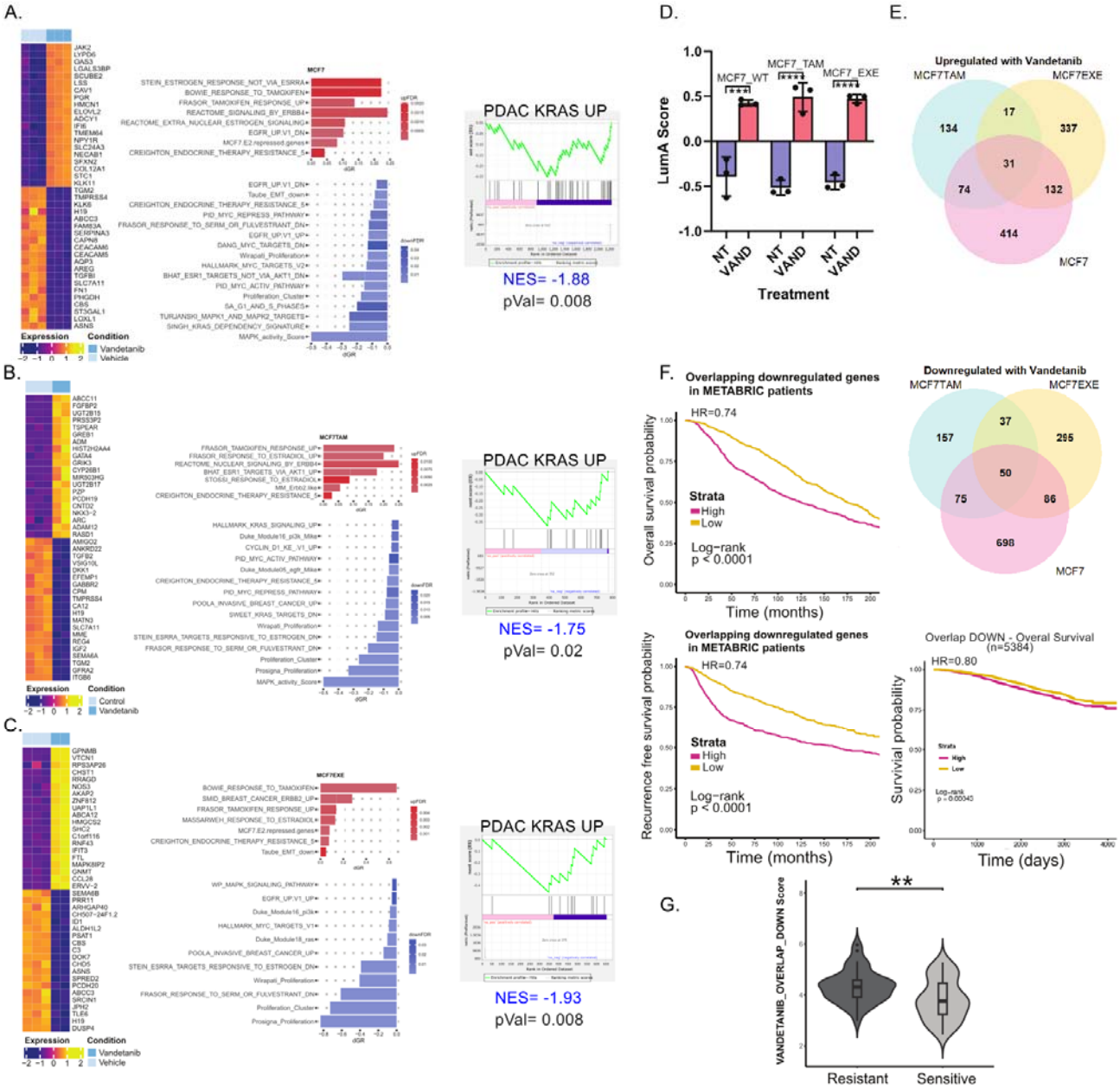

Figure 2. Heatmap and enrichment analysis of differentially expressed genes in (A) MCF7, (B) MCF7-TAM, (C) MCF7-EXE. KRAS activity signatures were depleted in all 3 cell lines by vandetanib treatment. (D) Vandetanib treatment increases association with the more indolent, endocrine therapy responsive Luminal A subtype, measured by correlation to Luminal A defining genes (centroid). (E) Overlap of up- and down- regulated genes by cell line. (F) Signatures of overlapping up-and down regulated genes are associated with recurrence free and overall survival in ER+ breast cancer patients in METARIC and SCAN-B studies and (G) downregulated genes are enriched in clinically ET-resistant ER+ tumors. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by t-test with Welch correction for Wilcoxon test.

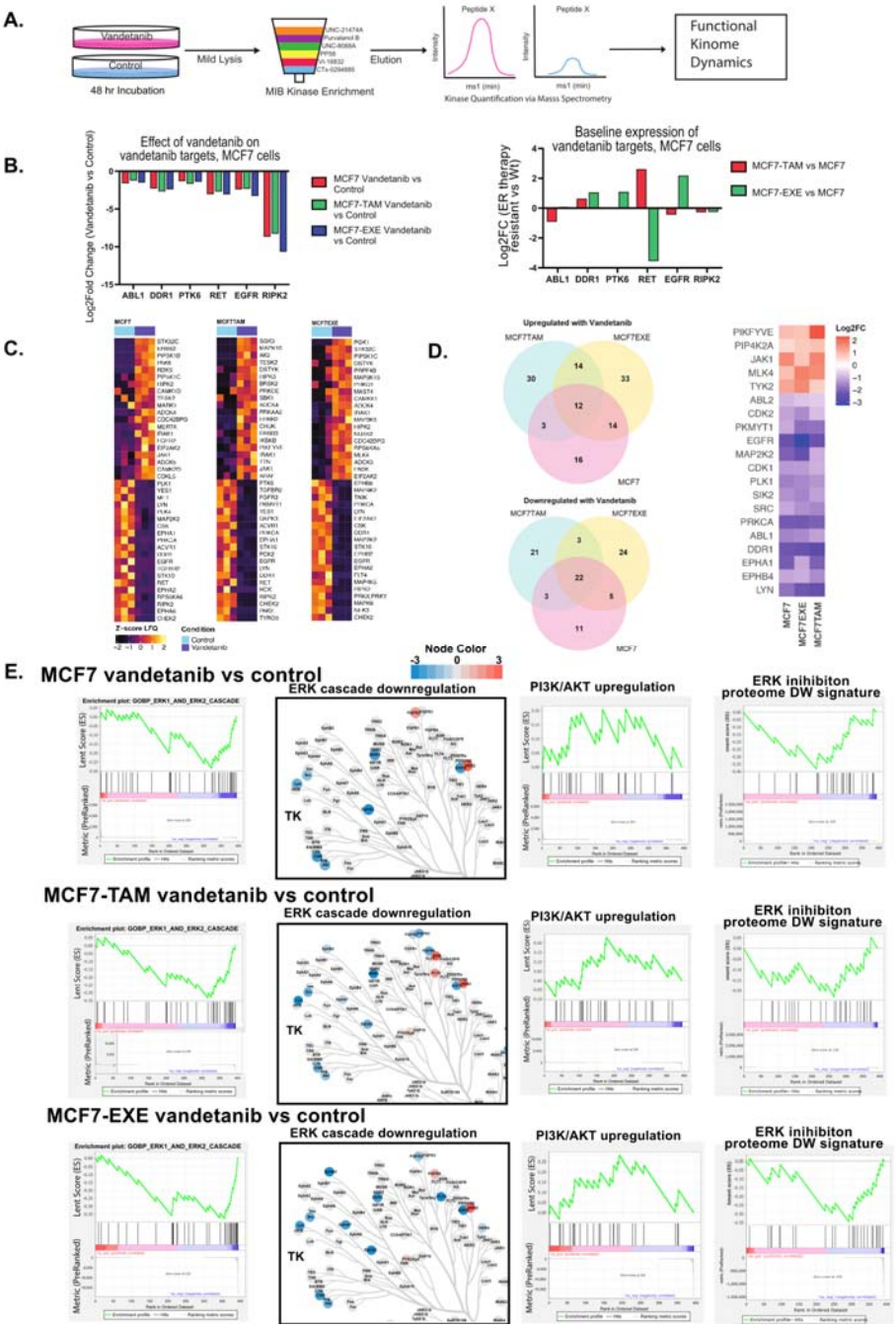

Figure 3. (A) Multiplex inhibitor bead mass spectrometry assay work flow. (B) Effects of vandetanib on enrichment and depletion of known vandetanib targets. Baseline expression of vandetanib targets in tamoxifen and exemestane resistant cell lines compared to WT MCF7. (C) Heatmap showing enriched and depleted proteins after vandetanib treatment. (D) Overlap analysis of up- and downregulated proteins between the 3 cell lines when treated with vandetanib. (E) Pathway analysis of the downregulation of the ERK cascade and upregulation of the PI3K/AKT pathway.

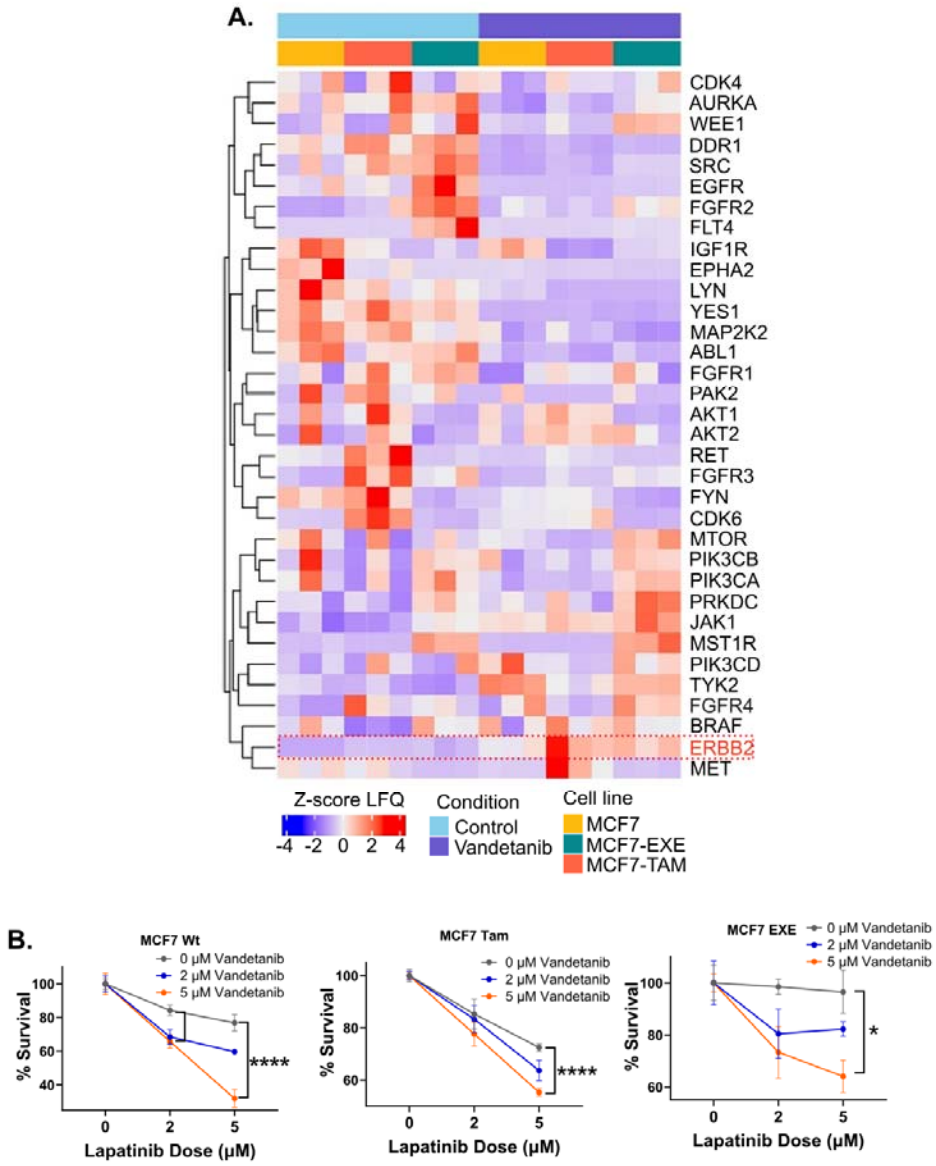

Figure 4. (A) Heatmap showing enrichment levels of druggable kinases of sensitive and resistant MCF7 cell lines before and after vandetanib treatment. Of note, ERBB2 (encoding HER2) was enriched in all 3 cell lines after treatment and RET was dpeleted in MCF7 and MCF7-TAM. (B) Sensitivity plots of co-treated cells with vandetanib and HER2 inhibitor lapatinib demonstrating enhanced lapatinib sensitivity with vandetanib treatment. *p<0.05, ****p<0.0001 by t-test with Welch correction.

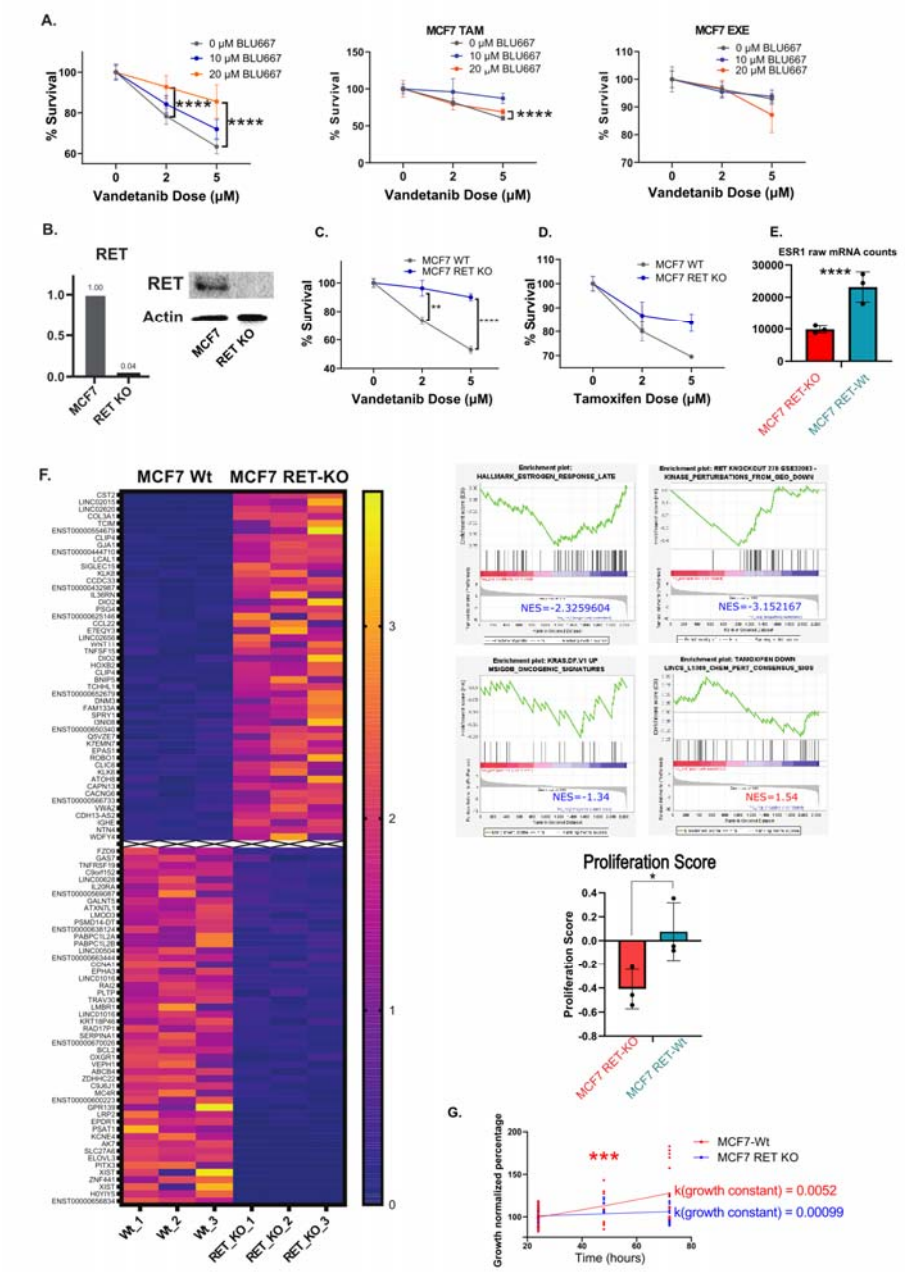

Figure 5. (A) Co-treatment with vandetanib and the selective RET inhibitor BLU667 reduced vandetanib sensitivitiy in MCF7 and MCF7-TAM, but not MCF7-EXE with RET inhibition consistent with activity depletion in Fig4. (B) Western blot demonstrating CRISPR knockout of RET resulting in >90% reduction in RET protein levels. (C) RET knockout cells have reduced sensitivity to vandetanib. (D) RET knockout cells have reduced sensitivity to tamoxifen. (E) ESR1 RNA levels encoding the estrogen receptor are decreased in RET KO cells, consistent with reduced sensitivity to tamoxifen. (F) Heatmap demonstrating differentially expressed genes with RET KO in MCF7. Enrichment analysis demonstrates reduction in hallmark estrogen response and RAS signaling in RET KO cells. RET KO cells have lower proliferation score. (G) RET knockout cells have reduced proliferation compared to WT.

(. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by t-test with Welch correction for the bar graphs, and Wilcoxon test.

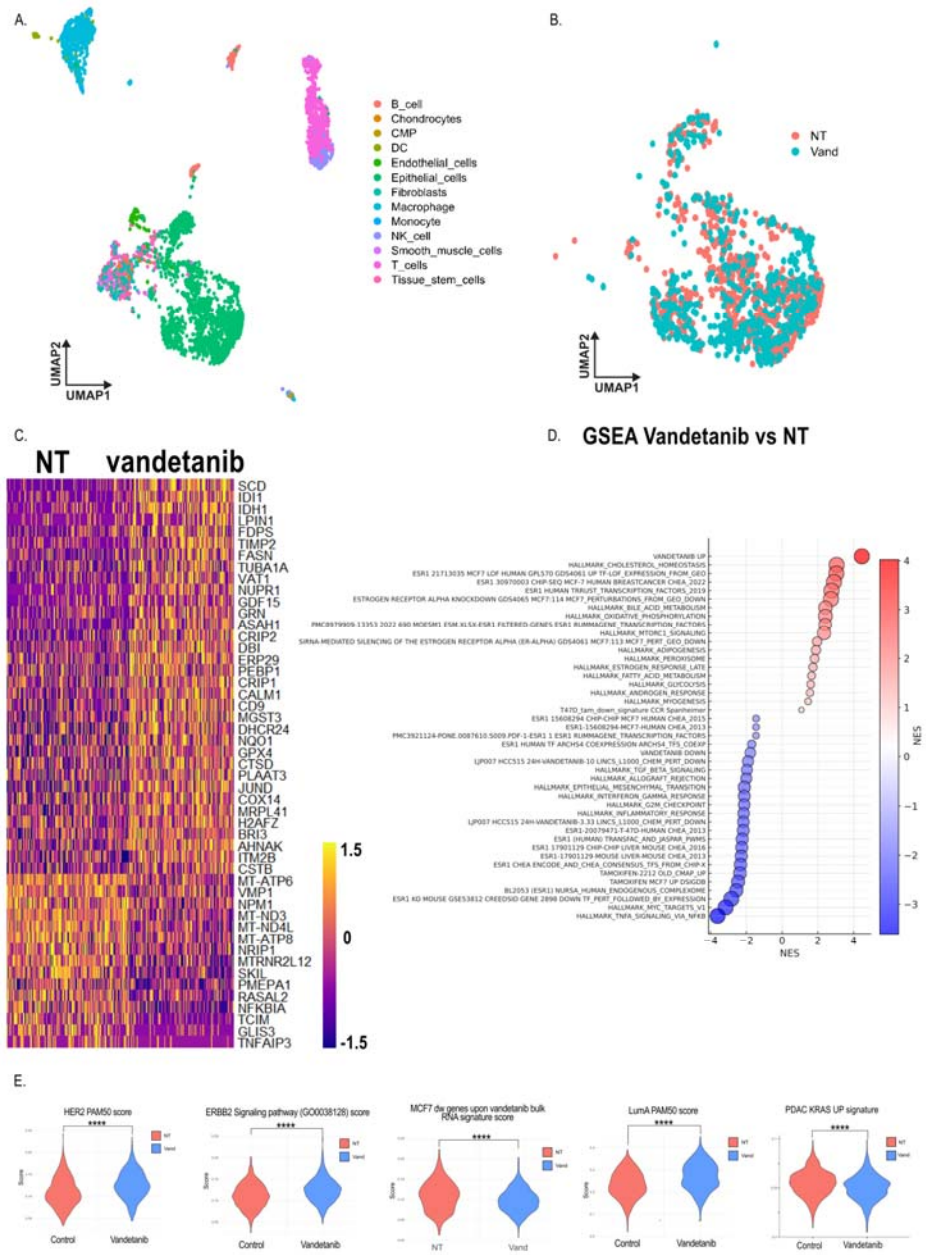

Figure 6. (A) Single-cell RNA sequencing uniform manifold projection (UMAP) annotated by cell type within a primary ER+ human breast tumor. (B) Tumor cells were computationally isolated as having significant inferred copy number changes and plotted by treatment condition. (C) Heatmap showing differentially expressed genes compared between vandetanib treated vs non-treated tumor cells. (D) Enriched and depleted pathways of vandetanib treated vs non-treated tumor cells. (E) ERBB2 activity signature score was increased in vandetanib treated tumor cells as seen in cell lines. Down regulated genes in vandetanib treated cell lines are depleted in vandetanib treated primary tumor cells, and LumA correlation increased in vandetanib treated tumor cells as was seen in cell lines, and depleted KRAS activity signature demonstrates on target effects. Significant signatures filtered by < 0.1 FDR on GSEA analysis and significant genes filtered by adjusted p value <0.05 (Wilcoxon test).

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