Estrogen receptor alpha mutations regulate gene expression and cell growth in breast cancer through microRNAs

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Abstract: Estrogen receptor α (ER) mutations occur in up to 30% of metastatic ER-positive breast cancers. Recent data has shown that ER mutations impact the expression of thousands of genes not typically regulated by wildtype ER. While the majority of these altered genes can be explained by constant activity of mutant ER or genomic changes such as altered ER binding and chromatin accessibility, as much as 33% remain unexplained, indicating the potential for post-transcriptional effects. Here we explored the role of microRNAs in mutant ER-driven gene regulation and identified several microRNAs that are dysregulated in ER mutant cells. These differentially regulated microRNAs target a significant portion of mutant-specific genes involved in key cellular processes. When the activity of microRNAs is altered using mimics or inhibitors, significant changes are observed in gene expression and cellular proliferation related to mutant ER. An in-depth evaluation of miR-301b led us to discover an important role for PRKD3 in the proliferation of ER mutant cells. Our findings show that microRNAs contribute to mutant ER gene regulation and cellular effects in breast cancer cells.

Introduction

Estrogen receptor α (ER) is a steroid hormone receptor that is activated by estrogens and is expressed in 70% of breast cancers. ER plays an important role in driving the progression of these tumors by regulating genes to promote growth and proliferation. Due to the availability of effective anti-estrogen therapies, including selective estrogen receptor modulators and aromatase inhibitors (AI), ER-positive breast cancers generally have a good prognosis with a five-year survival rate of greater than 90% (1). However, many ER-positive breast cancer patients eventually develop resistance to hormone therapies and experience recurrent metastatic disease (2,3). Activating mutations in the ligand binding domain (LBD) of ER have been observed in approximately 30% of hormone therapy-resistant ER-positive breast cancers (4-6). Over the last decade, several studies have improved our understanding of ER mutations and the mechanisms by which these mutations lead to breast cancer recurrence (4-18). Structural changes in ER's LBD as a result of ER LBD mutations confer an active conformation and ligand-independent activity to unliganded mutant ER, which drives large transcriptional changes in the absence of estrogens (5,7,9,11-16). Several studies have shown that this dramatic change in gene expression can drive increased cellular proliferation, invasion, and metastasis in breast cancer cells expressing mutant ER (4,5,9,13,14,16-18). Recently, we showed that over half of these gene expression changes are driven directly by the constitutive activity of ER (16). Additionally,

changes in ER's genomic binding and widespread alterations to chromatin accessibility, accompanied by binding of other transcription-regulating factors such as CTCF, FOXA1, and OCT1, can explain an additional 20-30% of transcriptional changes observed in ER mutant breast cancer cells. However, the intermediate factors driving these transcriptional changes and the causes of the remaining unexplained gene expression changes are unknown.

Since ER has been shown to activate several microRNAs (19,20), we hypothesized that regulation of microRNAs(miRNAs) could contrubte to altered gene expression in ER mutant cells. miRNAs are short RNA molecules with an average length of 22bps that are transcribed by RNA polymerase II. miRNAs typically bind directly to the 3' untranslated region (UTR) of mature messenger RNAs (mRNAs), which usually leads to decreased translation and increased degradation of the target mRNAs (21,22). Many miRNAs have been described to have either oncogenic or tumor-suppressive roles, and the altered expression of these miRNAs often contributes to cancer-associated cellular processes, including increased proliferation, migration, and metastasis (23-26). These effects are often produced when miRNAs alter the activity of important signaling pathways in cancer cells (27). Altered activity of these pathways by miRNAs could provide therapeutic opportunities. In addition, miRNA mimics and inhibitors have recently been considered as a potential therapeutic approach for treating some diseases (26,28-30). The possible use of miRNA mimics and inhibitors as cancer treatments in the future makes understanding the role of miRNAs in cancer an important subject.

Activated ER homodimerizes and binds to genomic elements in the genome, termed estrogen response elements (ERE), where it recruits cofactors that drive the transcription of nearby genes via RNA Polymerase II. Because miRNAs are transcribed by RNA Polymerase II, they could be directly regulated by ER or by other transcription factors downstream of ER signaling. Indeed, some miRNAs have previously been shown to be regulated by ER activation (19,20). These ER-regulated miRNAs, along with many other miRNAs, have been shown to contribute to cancer progression in ER-positive breast cancers (27,31-38). Despite the clear role of miRNAs in contributing to cancer progression both broadly and in the specific case of ER-positive breast cancers, the role of miRNAs in recurrent mutant ER-positive breast cancers has thus far remained unexplored. The potential for ER to directly or indirectly regulate miRNA transcription suggests that differential expression of miRNAs could occur in ER mutant cells and could contribute to the large transcriptional changes we and others have observed in mutant ER-positive breast cancer cells.

Here, we investigate the role of miRNAs in regulating gene expression in breast cancer cell lines we developed previously, which express wildtype (WT) ER or heterozygously express either the Y537S or the D538G mutation, the two most common ER LBD mutations, from the endogenous ER locus (16). We identified many miRNAs that are differentially expressed in ER mutant cells compared to WT ER cells. We examined the effects of several of these miRNAs, including miR-301b, miR-181c, miR-331, and let-7f, on gene expression and found that they directly or indirectly alter the expression of hundreds of genes previously identified as being differentially expressed in ER mutant cells compared to WT cells (mutant-specific genes) (16). These miRNAs also impact the ability of ER mutant cells to proliferate, a process that is significantly increased in ER mutant

cells compared to their WT counterparts. In particular, we identify miR-301b as being significantly down-regulated in ER mutant cells, which led to altered expression of mutant-specific genes and increased proliferation. Decreased miR-301b expression increases the expression of PRKD3, a kinase regulator of several cellular signaling pathways, including the RAS/MEK/ERK pathway (39-42). We found that inhibiting PKD3 leads to significantly decreased proliferation in ER mutant cells. In summary, we discovered that several miRNAs, including miR-301b, play an important role in driving ER mutant gene regulation and contribute to increased cellular proliferation in ER mutant breast cancer cells. We show that these miRNAs and their targeted pathways could provide alternative therapeutic strategies for treating mutant ER-positive breast cancer.

Results

ER mutations alter the expression of miRNAs

Previously, we identified thousands of genes that are differentially expressed in D538G or Y537S ER mutant cells compared to WT ER cells (mutant-specific genes) regardless of the presence or absence of 17β-estradiol (E2). Post-transcriptional mechanisms, such as differential expression of miRNAs in ER mutant cells, may contribute to mutant-specific gene expression. To determine whether there is differential expression of miRNAs in ER mutant cells, we utilized T-47D breast cancer cells, developed previously, which heterozygously express either the D538G or Y537S mutant *ESR1* allele from the endogenous *ESR1* locus (16). ER mutant and WT cells were grown for 5 days in estrogen-deprived media followed by RNA extraction. Total RNA was profiled for miRNAs using the Nanostring Human v3 miRNA Assay, which examines the expression of nearly 800 miRNAs. We found that 85 miRNAs are differentially expressed in the D538G, Y537S, or both mutant cell lines compared to WT cells (fig. 1 and sup. table S1).

To identify which differentially expressed miRNAs likely contribute to mutant-specific gene expression, we utilized the DIANA-TarBase v8, an open-access reference database of experimentally supported miRNA targets (43). We compared the target genes for each differentially expressed miRNA with our previously identified mutant-specific genes. Because miRNAs effectively down-regulate the expression of their target genes by promoting their degradation, we compared the target genes of miRNAs that exhibited increased expression in ER mutant cells to mutant-specific down-regulated genes and the target genes of miRNAs that were down-regulated in ER mutant cells to mutant-specific up-regulated genes. Using this approach, we identified 15 differentially expressed miRNAs whose target genes are significantly enriched for mutant-specific genes (fig. 1 and sup. fig. 1a-f). Of these 15 miRNAs, 2 (miR-301b and miR-489) were differentially expressed in both the Y537S and the D538G mutant cells, while the remaining 13 miRNAs were differentially expressed in Y537S cells only (8 miRNAs) or in the D538G cells only (5 miRNAs). We found that genes that are both differentially expressed in ER mutant cells and are target genes of these 15 differentially expressed miRNAs are enriched for a number of cellular processes such as increased proliferation and cell migration (sup. fig. 1a-d). These processes were previously observed to be significantly altered in ER mutant cells. In particular, increased cell proliferation was a strong characteristic of both Y537S and D538G ER mutant cells (16).

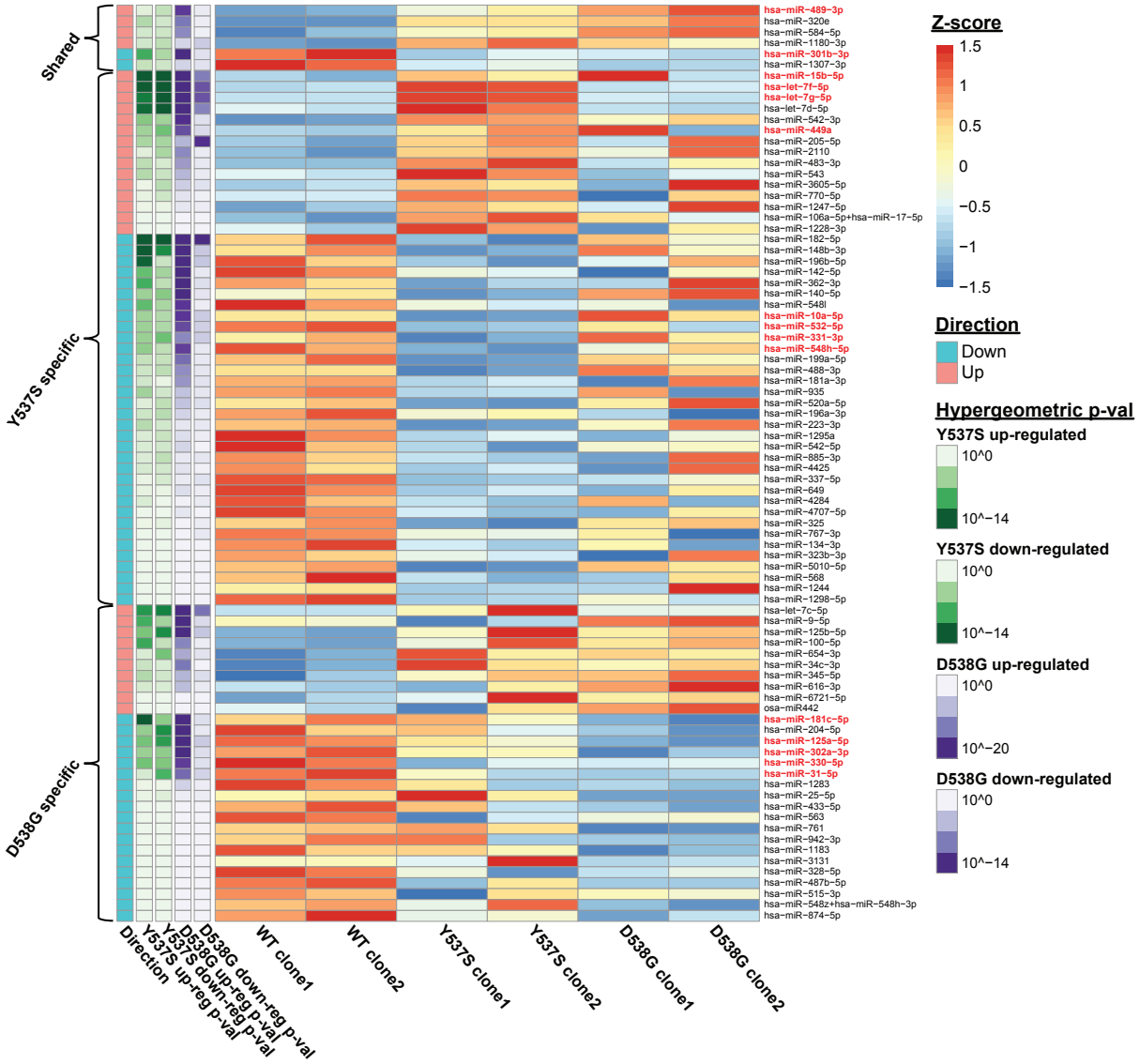
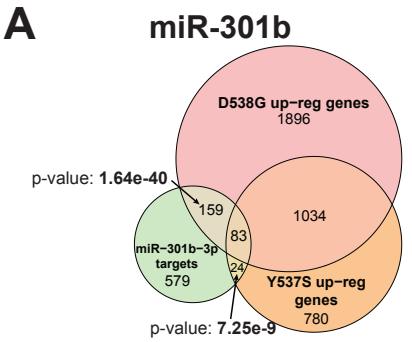
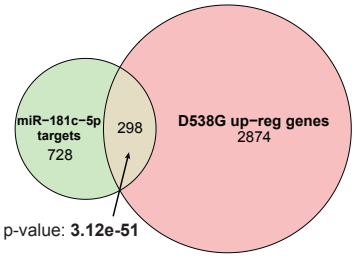


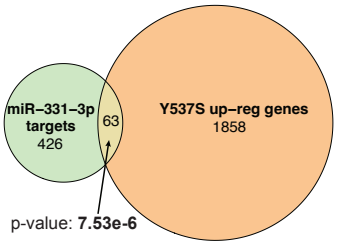
Figure 1. **ER mutations drive the differential expression of miRNAs.** Heatmap shows expression levels of 85 miRNAs identified as differentially expressed in ER mutant cells compared to WT cells. Hypergeometric p-values between each miRNA's respective target genes and previously defined mutant-specific genes are indicated on the left. Darker color indicates more significant hypergeometric p-values. 15 miRNAs selected for further investigation are highlighted in red.



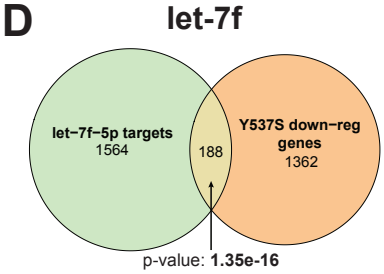
Pathway/GO term	Y537S Adj. p-value	D538G Adj. p-value
Cell Cycle	1.640e-8	3.830e-3
G2-M Checkpoint	1.994e-6	2.169e-5
E2F Targets	1.994e-6	4.734e-4
Mitotic Spindle	NS	2.169e-5
Retinoblastoma gene in cancer	3.859e-6	1.189e-2
FOXM1 transcription factor network	1.529e-5	2.760e-2
Regulation of Microtubule Cytoskeleton	2.677e-2	4.901e-3
Rac 1 cell motility signaling pathway	NS	5.663e-3
Myc Targets V1	4.882e-2	8.006e-3



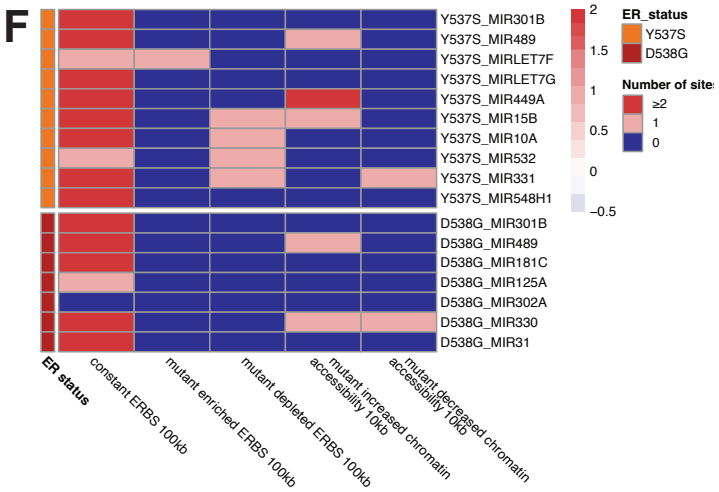
Pathway/Go term	Adj. p-value
Gene Expression	9.152e-5
ErbB1 downstream signaling	7.213e-5
Integrated breast cancer pathway	1.327e-4
E2F Targets	4.344e-4
Mitotic Spindle	6.971e-4
Epithelial Mesenchymal Transitioin	6.971e-4

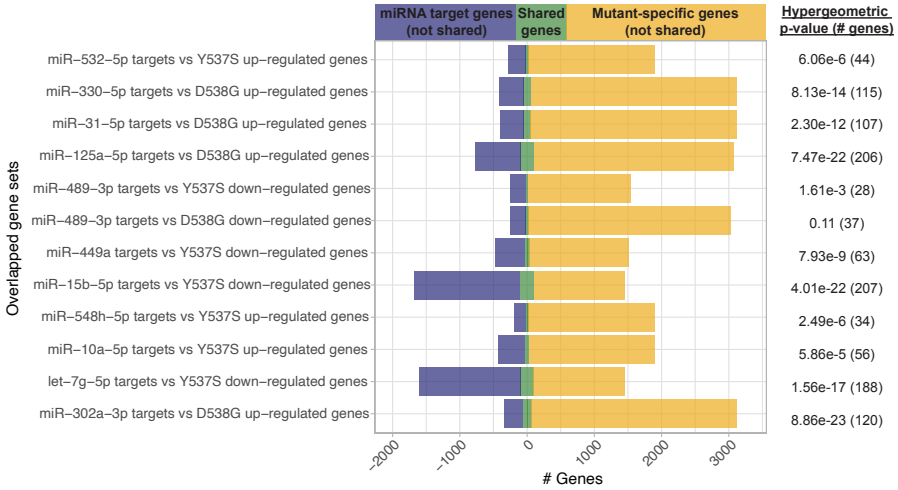


Pathway/Go term	Adj. p-value
Mitotic Prometaphase	1.080E-6
Cell Cycle	1.678E-6
RHO GTPases Activate Formins	2.975e-5
Organelle biogenesis and maintenance	7.251e-5
Signaling events mediated by HDAC class II	2.323e-4
Integrin-linked kinase signaling	3.846E-4
Protein Nuclear Import and Export	1.288E-3
Focal adhesion	3.583E-3



Pathway/GO terms	Adj. p-value
MAP kinase tyrosine/serine/threonine phosphatase activity	2.332e-4
Calnexin/calreticulin cycle	2.670e-3
VEGFA-VEGFR2 Signaling	3.575e-3
RAF-independent MAPK1/3 activation	6.532e-3
Nectin adhesion pathway	7.282e-3
RHO GTPases activate IQGAPs	8.947e-3
Cadherin binding	0.0107





Supplemental Figure 1. miRNA target genes are enriched for mutant-specific genes and differential expression of miRNAs can be explained by differential ER genomic binding and chromatin accessibility. Overlaps between Y537S, D538G, or both mutant-specific up- or down-regulated genes with predicted target genes for miR-301b (**A**), miR-181c (**B**), miR-331 (**C**), and let-7f (**D**) are shown. P-values indicating the significance of enrichment were calculated using the hypergeometric test. **E,** Overlaps between Y537S or D538G up- or down-regulated genes and predicted miRNA target genes are shown for the remaining miRNA selected for further analysis. Blue bars indicates predicted miRNA target genes that are not mutant-specific genes, yellow indicates mutant-specific genes that are not predicted miRNA target genes, and green indicates genes that are both predicted miRNA target genes and mutant-specific genes. P-values indicating the significance of enrichment were calculated using the

hypergeometric test. (F) Heatmap indicates the number of constant or mutant-enriched or -depleted ER-binding sites or altered chromatin accessibility regions within 100kb of the transcription start site of differentially expressed miRNAs.

The differential expression of these 15 miRNAs occurs as a result of mutant ER expression and could be directly regulated by ER. Using WT and mutant ER ChIP-seq data (16), we found that 14 of these 15 miRNAs had at least one ER-binding site (ERBS) within 100kb of their TSS while 11 had two or more ERBS within 100kb (sup. fig. 1f). Additionally, 5 of these miRNAs had ERBS within 100kb that exhibited significantly increased or decreased ER binding in ER mutant cells compared to WT cells. While not all nearby ERBS exhibit differential ER binding between mutant and WT cells, mutant ER may still be important in altering the expression of these miRNAs. We also utilized previously obtained ATAC-seq data and found that differential chromatin accessibility near differentially expressed miRNAs may also factor into the altered expression of 6 out of 15 miRNAs (sup. fig. 1f). These findings suggest that the differential expression of most, if not all, of these miRNAs is directly controlled by mutant ER and possibly downstream factors.

Differentially regulated miRNAs in ER mutant cells significantly impact cell proliferation

To more directly determine the effects of differential miRNA expression on cell proliferation in ER mutant cells, we used miRNA mimics to increase a miRNA's effect or miRNA inhibitors to decrease a miRNA's effect in both ER WT and ER mutant cells. WT or ER mutant cells were transfected with a single miRNA mimic or inhibitor for each of the 15 differentially expressed miRNAs that exhibited enrichment for mutant-specific genes in their respective target gene sets. Immediately following inhibitor or mimic introduction, cells were monitored for proliferation for 48 hours using the IncuCyte Zoom platform for live-cell imaging. Treatment with a miRNA mimic or inhibitor led to significant changes in proliferation rates in mutant and/or WT ER cells for several of the 15 miRNAs examined, with miR-301b-3p, let-7f-5p, miR-181c-5p, and miR-331-3p producing particularly notable effects (fig. 2a-d and sup. fig. 2a-b).

Treatment with a mimic for miR-301b in Y537S and D538G ER mutant cells, where miR-301b is significantly down-regulated, had a striking effect, completely abrogating proliferation in both ER mutant models (fig. 2a). Conversely, treatment with a miR-301b inhibitor in WT ER cells, recapitulating the loss of miR-301b in ER mutant cells, had a negligible effect on WT ER cell proliferation. miR-301b has been shown in other contexts to drive increased proliferation (44,45). However, these results suggest that the miR-301b-mediated effects on proliferation are dependent on the cells' ER mutation status and that loss of miR-301b contributes to increased proliferation rates specifically in ER mutant cells.

Treatment with mimics or inhibitors for the other three miRNAs of interest led to milder, yet still significant, effects on proliferation. Introduction of a mimic of miR-181c, which was down-regulated in D538G mutant cells, significantly decreased proliferation (fig. 2b). Decreased miR-181c activity in WT and Y537S mutant cells, recapitulating the loss of miR-181c in D538G cells, led to significantly increased proliferation, although these effects were minimal in WT cells (fig 2b). Increasing the activity of miR-331, which was down-regulated in the Y537S mutant, resulted in a significantly decreased proliferation rate in the Y537S mutant cells, while treatment of WT or D538G cells with a miR-331 inhibitor produced minimal effects on proliferation (fig 2c). Finally, reversing the high expression of let-7f observed in Y537S mutant cells, by treating with a let-7f

inhibitor, led to significantly decreased proliferation in Y537S cells, while let-7f mimic treatment in D538G and WT cells resulted in significant increases in proliferation rates (fig 2d). Let-7f is generally thought to function as a tumor suppressor (46-49). However, here it was found to drive increased proliferation, most notably in ER mutant cells, again suggesting that some miRNAs behave differently in the ER mutant context than in other contexts. The mimic or inhibitor treatments for the remaining 11 miRNAs, which were organized into 5 groups based on their expression patterns in WT and ER mutant cells, resulted overall in weaker proliferative effects (sup. fig. 2a-b). Together, these data show that multiple miRNAs differentially expressed in ER mutant cells contribute to the regulation of cell proliferation, a phenotype associated with ER mutant cells. In the ER mutant setting, these miRNAs changes drive increased proliferation with some miRNAs having a stronger effect on proliferation than others.

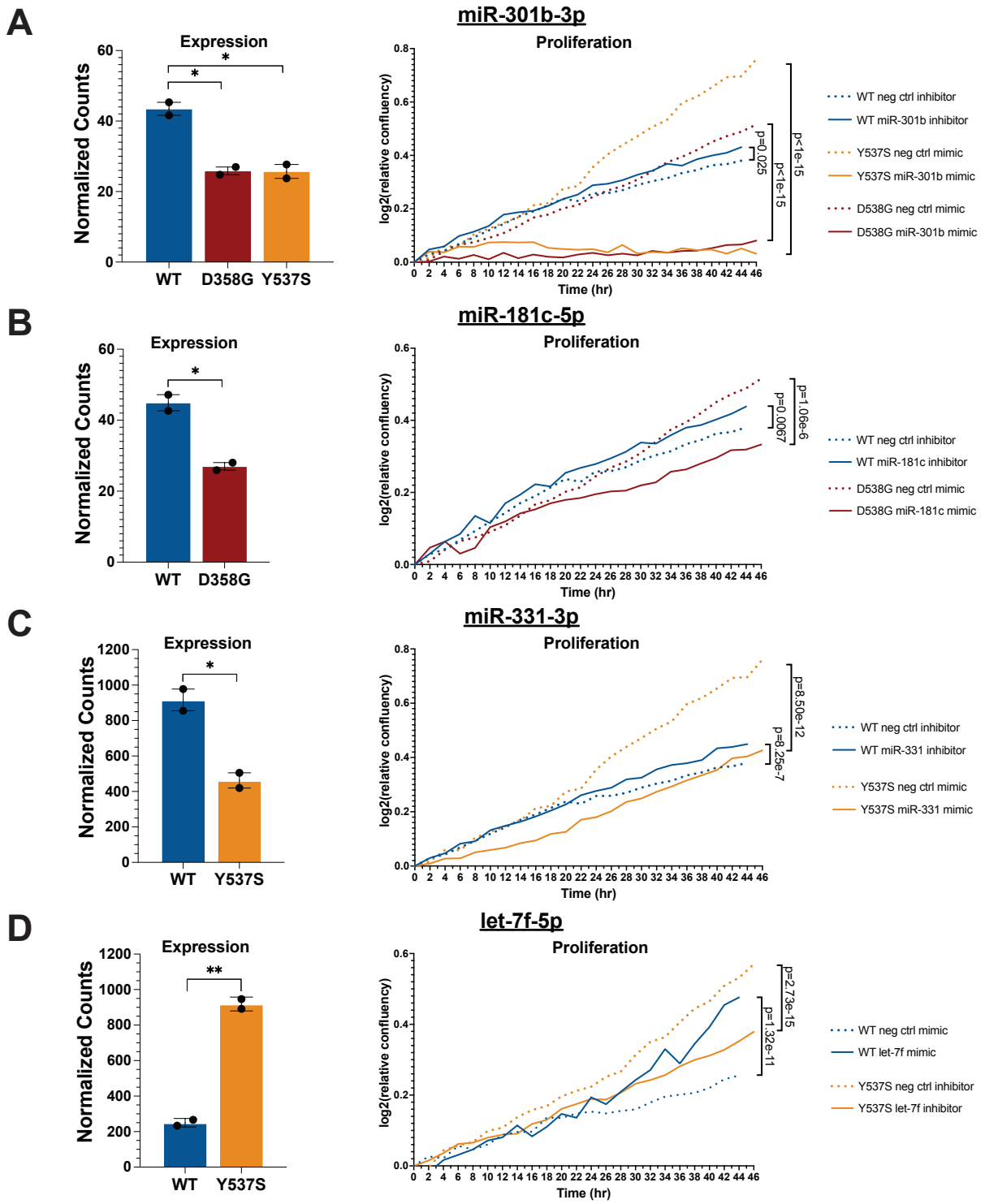
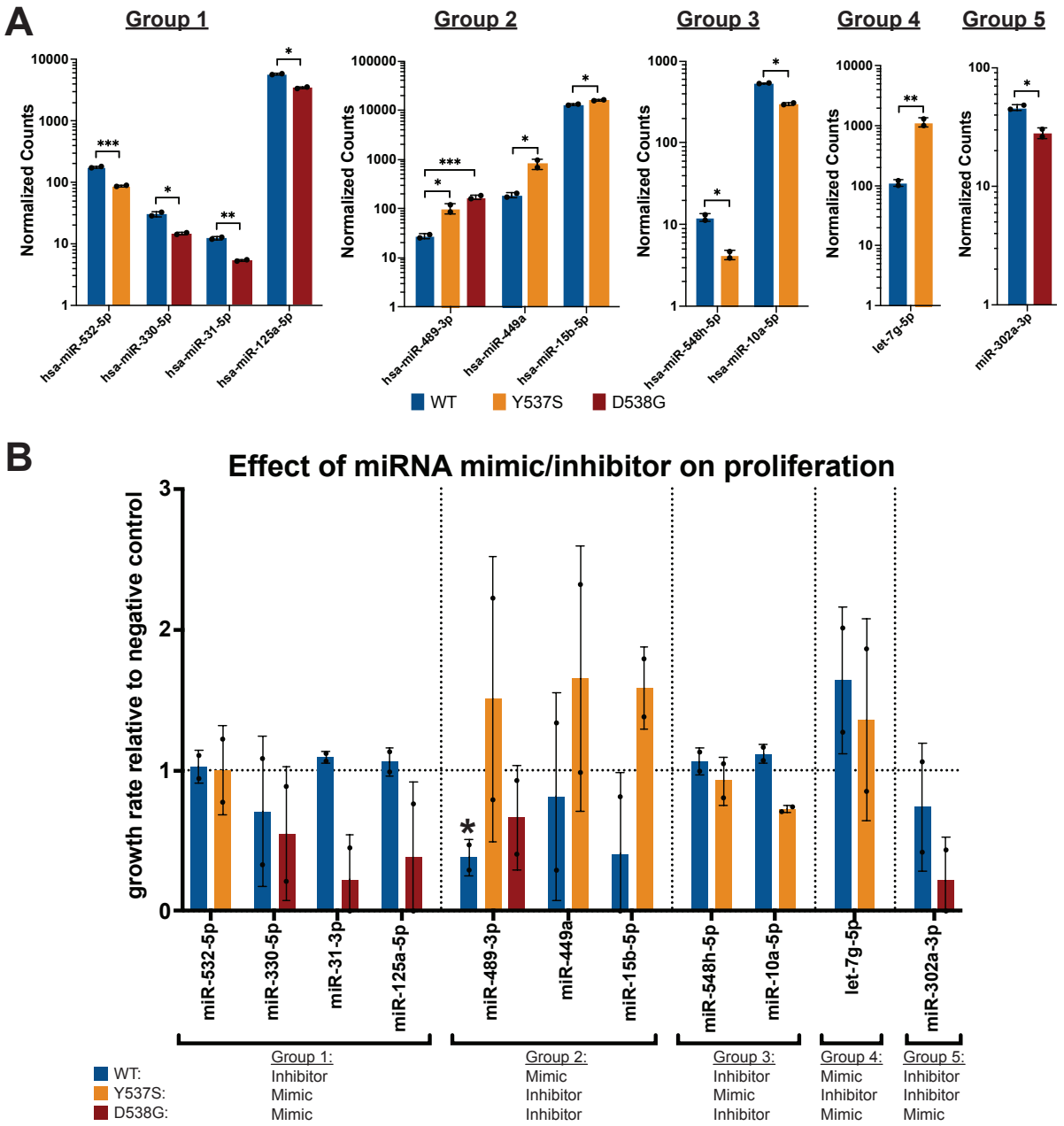


Figure 2. **Altered miRNA activity significantly impacts proliferation in ER mutant cells.** Expression levels and relative confluence are shown over time for miR-301b (**A**), miR-181c (**B**), miR-331 (**C**), and let-7f (**D**). Bar graphs show normalized counts for each miRNA for ER WT, D538G, and Y537S clones. Error bars indicate average ± SEM for two clones for each genotype. Student two-sample *t* test was used: *, P< 0.05; **, P < 0.01; ns, not significant. Proliferation graphs show $\log_2$ transformed normalized confluence values over 48 hours. Significance between the slopes of the lines was determined using a generalized linear model and the Wald test.



Supplemental Figure 2. Altered miRNA actvity impacts proliferation in ER mutant cells. A, Expression levels are shown for 11 differentially expressed miRNAs in ER mutant cells. Normalized counts are shown on a log10 scale and miRNAs are organized into 5 groups based on their expression patterns and the mimic or inhibitor treatments used. Group 1 contains miRNAs that were down-regulated significantly or noticably in both ER mutants. Group 2 contains miRNAs that were significantly or noticably up-regulated in both ER mutants. Group 3 contains miRNAs that were down-regulated in the Y537S ER mutant cells only. Group 4 contained a miRNA that was up-regulated in the Y537S ER mutant cells only. Group 5 contained a miRNA that was down-regulated in the D538G ER mutant only. **B**, Growth rates relative to mimic or inhibitor negative control treatments are shown for each miRNA mimic or inhibitor treatment in WT (blue), Y537S (yellow), and D583G (red) cell lines. Significance was determined using a one-tailed Student's *t* test.

Differentially expressed miRNAs contribute to mutant-specific gene regulation

We investigated the effect of miR-301b, miR-181c, miR-331, and let-7f on gene expression in ER mutant cells by performing RNA-seq on total RNA collected from ER mutant or WT cells after treatment with a single miRNA mimic or inhibitor. miRNAs that were up-regulated in ER mutant cells were evaluated with a miRNA inhibitor (let-7f-5p), while miRNAs that were down-regulated were evaluated with a miRNA mimic (miR-301b-3p, miR-181c-5p, miR-331-3p). In this way, the activity of the each miRNA of interest was effectively reverted towards WT levels. RNA-seq was performed 24 hours post-transfection.

We found that altered expression of these miRNAs by mimic or inhibitor treatment resulted in the differential expression of tens to hundreds of genes (miRNA-altered genes) depending on the miRNA (fig. 3a and sup. table S2). To validate that the observed gene expression changes are a result of mimic or inhibitor treatment, we asked whether miRNA-altered genes exhibited enrichment for target genes of the corresponding miRNA. Mimic treatment should lead to enrichment of miRNA targets in down-regulated genes, while inhibitor treatment should exhibit enrichment of miRNA targets in up-regulated genes. As expected, we observed an enrichment for predicted miRNA target genes in down-regulated genes altered upon miR-301b, miR-181c, or miR-331 mimic treatments and in up-regulated genes after let-7f inhibitor treatment (sup. fig. 3a). The enrichment for miR-331 targets in down-regulated genes after miR-331 mimic treatment was relatively weak. However, more miR-331 target genes were observed in the group of down-regulated genes than in the group of up-regulated genes suggesting that the differential gene expression observed after mimic treatment may still be driven by altered miR-331 activity and that the target genes of miR-331 may not be well defined or could be highly cell type specific.

To determine whether our miRNAs of interest contribute to the mutant-specific gene expression patterns we observed previously (16), we asked whether miRNA-altered genes were significantly enriched for mutant-specific genes. Because mimic or inhibitor treatment reversed the activity of these miRNAs relative to their expression in ER mutant cells compared to WT cells, we expected to find significant overlaps between mimic- or inhibitor-regulated genes and mutant-specific genes regulated in the opposite direction. For example, we would expect genes down-regulated by miR-301b mimic treatment to be up-regulated in ER mutant compared to WT cells. As expected, we found that genes that were up-regulated upon mimic or inhibitor treatment were significantly over-represented in the corresponding mutant-specific down-regulated gene sets (fig. 3b). Conversely, genes that were down-regulated upon mimic or inhibitor treatment were significantly enriched for mutant-specific up-regulated genes. Comparison of mutant-specific genes regulated in the same direction as miRNA-altered genes (i.e. mutant-specific up-regulated vs miRNA mimic/inhibitor up-regulated) resulted in non-significant or very low enrichment between gene sets (fig. 3b). These results show that miRNAs regulate the expression of many mutant-specific genes in the expected direction and suggest that they impact gene regulation and proliferation in ER mutant cells.

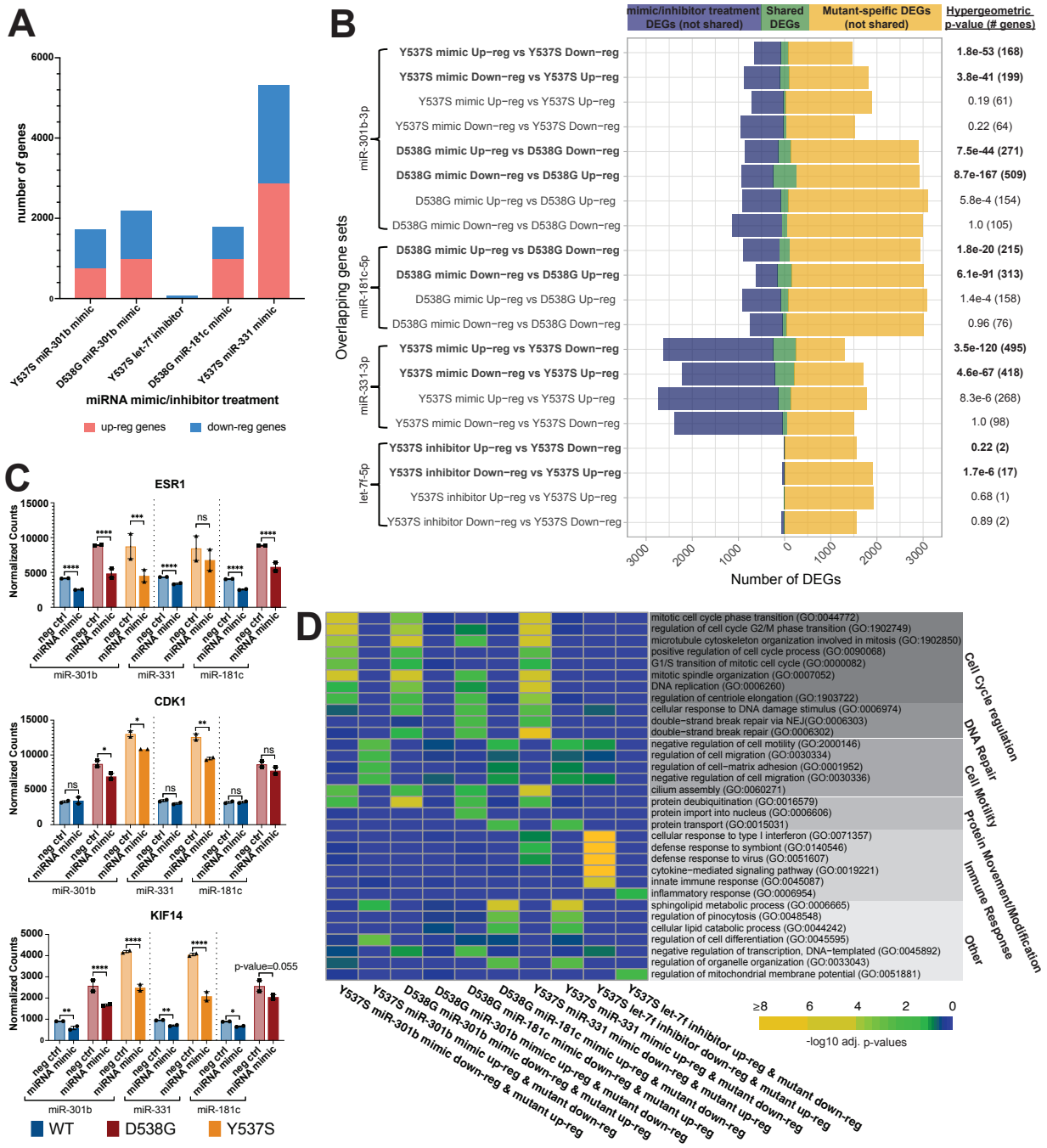
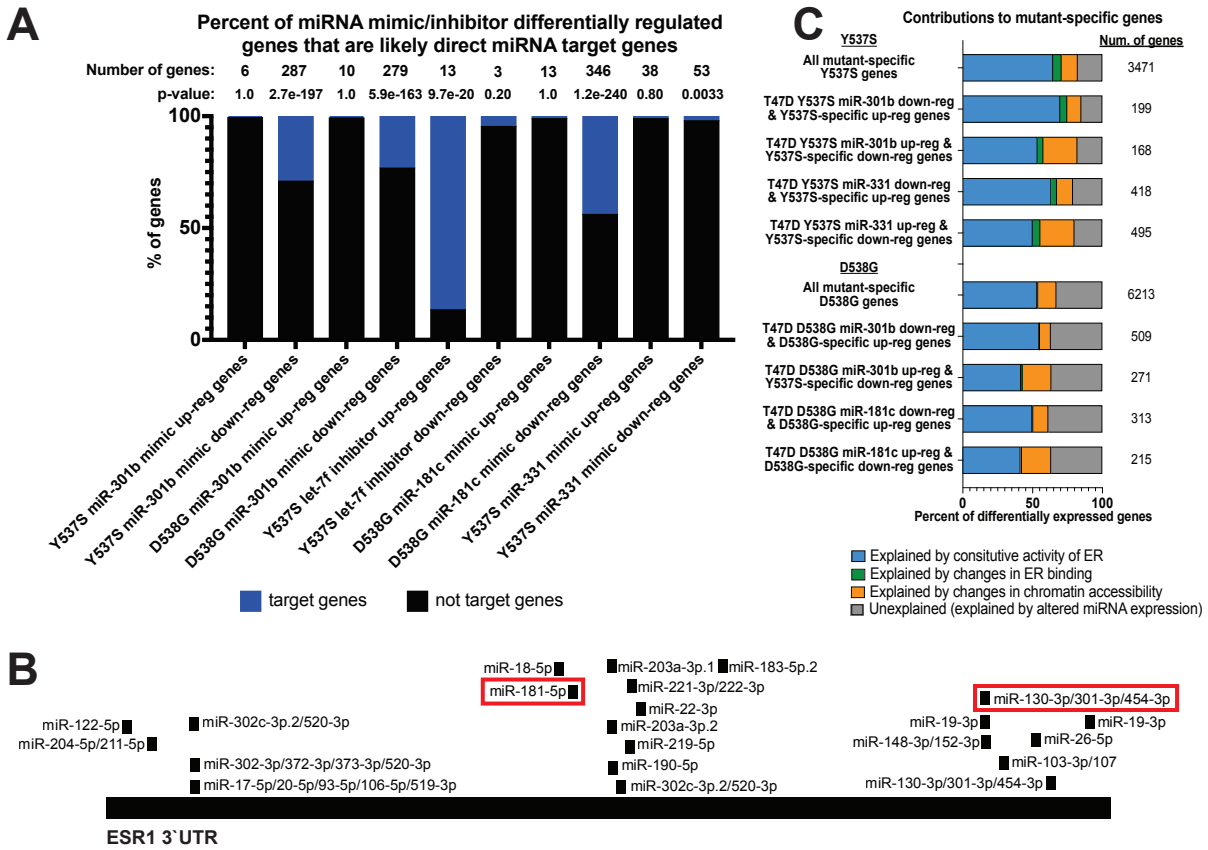


Figure 3. **Altered miRNA activity leads to differential expression of many mutant-specific genes involved with processes driving cancer-associated phenotypes. A,** Total number of genes altered upon miRNA mimic or inhibitor treatment is shown; red indicates up-regulated genes and blue indicates down-regulated genes. **B,** Overlap (green) of miRNA mimic- or inhibitor-treated differentially expressed genes (DEG) (blue) and mutant-specific DEG (yellow) is displayed for each gene set overlap. Significance of overlapping gene sets, indicated on the right, are hypergeometric p-values. **C,** Examples of miRNA-altered mutant-specific genes are shown. Error bars represent average ± SEM for two clones for each genotype. Adjusted p-values are indicated as calculated by DESeq2 (Wald test): *, P< 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant. **D,** Heatmap indicates the enrichment for miRNA-altered mutant-specific genes in the indicated gene ontology terms. Colors indicate the -$\log_{10}$ adjusted p-values.

We found that one of the mutant-specific genes regulated by miRNAs is *ESR1*, the gene encoding ER. miR-301b and miR-181c were both found to regulate ER, which has been shown to have complementary sequence in its 3`UTR to the seed sequences for both of these miRNAs (fig. 3c, sub fig. 3b) (50,51). ER is significantly up-regulated in ER mutant cells, where ER is constitutively active, compared to WT cells (fig. 3c). This is counter to some reports that show that ER, in the mammary gland, regulates its own expression through a negative feedback mechanism in which ER activity leads to decreased transcript levels of *ESR1* (52-54). This suggests that ER mutant cells are able to evade the negative regulation typically exerted by active ER and potentially take advantage of the resulting sustained or even heightened levels of ER to promote increased cell growth and proliferation. The observation that multiple miRNAs targeting *ESR1* are significantly down-regulated in ER mutant cells indicates an ER mutant-driven down-regulation of miRNAs as a key mechanism for evading normal negative feedback regulation by active ER on *ESR1* expression.



Supplemental Figure 3. miRNAs regulate mutant-specific genes. A, Bar graph shows the percent of miRNA mimic/inhibitor differentially regulated genes that are predicted direct targets for the corresponding miRNA. Hypergeometric tests were used to determine p-values. **B,** Figure represents *ESR1* 3'UTR and the regions targeted by miRNAs with the miR-301b and miR-181c target sites are boxed in red. **C,** miRNA-altered mutant-specific genes overlap with different classes of mutant-specific genes including those explained by mutant ER constitutive activity (blue), mutant ER driven altered chromatin accessibility (orange), mutant ER driven differential genomic binding (green), and unexplained genes (gray). Gene set is indicated on the left and the total number of genes for the corresponding gene set is listed on the right.

To examine the overall effects of miRNAs in regulating ER mutant phenotypes, we performed gene ontology analysis on miRNA-altered mutant-specific genes. This analysis revealed that these genes play roles in several important biological processes that are altered in mutant cells. miR-301b and miR-331 altered mutant-specific up-regulated genes were enriched for processes related to cell cycle progression (fig. 3d). Many of these genes, such as *CDK1* and *KIF14*, play important roles in mediating cell cycle progression, indicating again the role of these miRNAs in driving increased proliferation (fig. 3c). miRNA-altered mutant-specific up-regulated genes resulting from miR-181c mimic treatment in D538G cells were also involved in cell cycle regulation, although these genes were more particularly involved in DNA damage repair (fig. 3d). Additional gene ontology terms associated with miRNA-altered mutant-specific genes include cell-matrix adhesion and negative regulation of cell motility as well as increased expression of immune response-related genes (fig. 3d). In the case of cell-matrix adhesion and negative regulation of cell motility, these genes were down-regulated in ER mutant cells and up-regulated in cells treated with either a miR-301b, miR-181c, or miR-331 mimics, suggesting that these miRNAs restrict cell motility. The decreased expression of these miRNAs in ER mutant cell lines may contribute to increased cell motility and migration observed in ER mutant cells (9,14,17,55,56).

To understand the direct effects of miRNAs in driving these broad phenotypic effects, we asked which miRNA-altered mutant-specific genes are predicted direct miRNA targets based on the miRDB database for miRNA target genes, which uses an algorithm trained on large RNA-seq and CLIP-ligation data sets to predict miRNA targets (51). We found that miRNA-altered mutant specific genes were enriched for miRNA target genes in the expected direction. Of the genes overlapping mutant-specific up-regulated genes, 23% of miR-301b mimic (p-value = 1.7e-26 (Y537S) and 1.2e-64 (D538G); hypergeometric test) and 48% of miR-181c mimic (p-value = 2.1e-108; hypergeometric test) down-regulated genes were direct miR-301b or miR-181c target genes (fig. 4a, sup. fig. 4a). All of the let-7f inhibitor (p-value = 1.0; hypergeometric test) down-regulated genes shared with mutant-specific up-regulated genes were let-7f direct targets (fig. 4a, sup. fig. 4a). However, only 1.5% (pvalue = 0.45; hypergeometric test) of miR-331 mimic down-regulated mutant-specific up-regulated genes were direct miR-331 target genes, similar to the lack of miR-331 target gene enrichment described above (sup. fig. 4a). We found that direct target genes of these miRNAs were enriched for several key cell signaling pathways including PI3K signaling, nuclear receptor signaling, and EGF receptor signaling, specifically through the MAPK signaling cascade (fig. 4b, sup. fig. 4b).

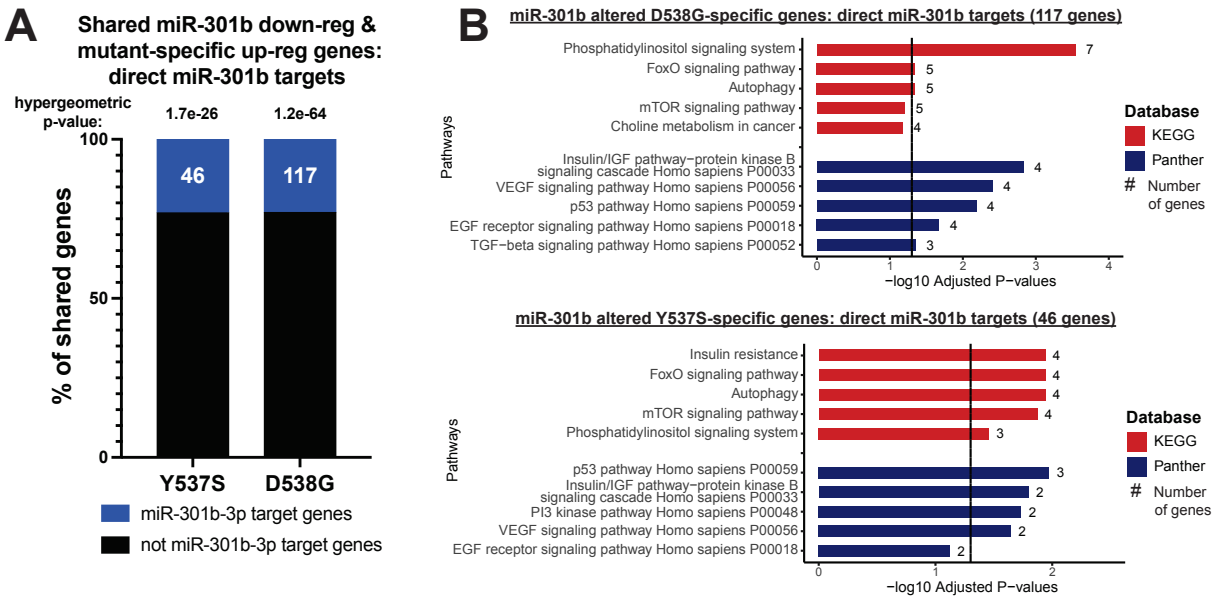
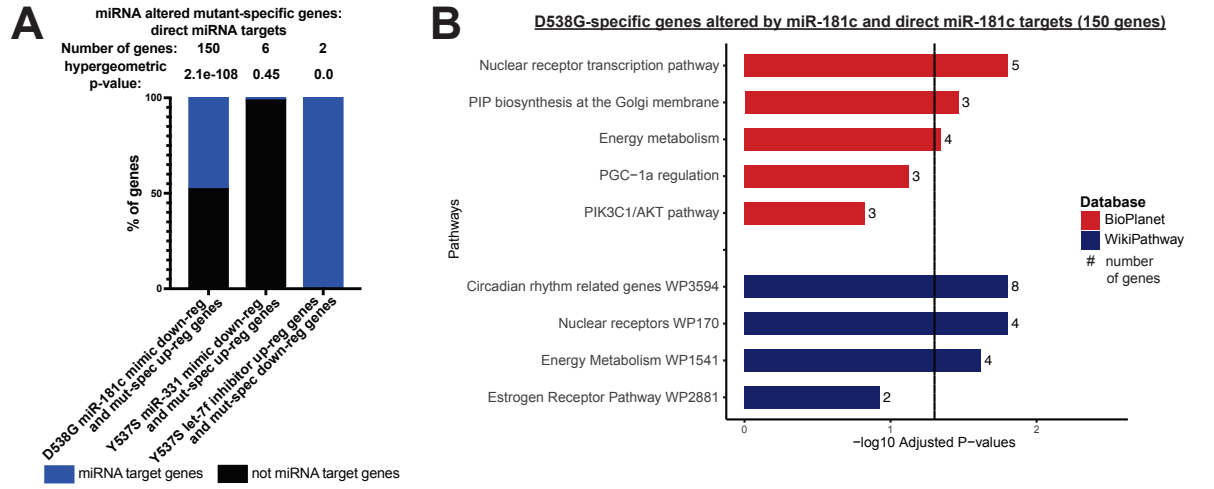


Figure 4. miRNAs in ER mutant cells directly target mutant-specific genes involved in important cell signaling pathways. A, Percent of miR-301b mimic down-regulated mutant-specific up-regulated genes that are predicted direct targets of miR-301b is shown. Hypergeometric tests were used to calculate p-values. **B,** Bar graphs show pathways found to be enriched in miRNA-altered mutant-specific genes. -$\log_{10}$ adjusted p-values are indicated and the number of genes in each pathway gene set are shown.



Supplemental Figure 4. miRNAs in ER mutant cells directly target mutant-specific genes involved in important cell signaling pathways. A, Percent of miR-181c, miR-331, or let-7f altered genes mutant-specific genes that are predicted direct targets of miR-181c, miR-331, or let-7f, respectively. Hypergeometric tests were used for p-value determination. **B,** Bar graphs show pathways found to be enriched in miRNA-altered mutant-specific genes. -$\log_{10}$ adjusted p-values are indicated and the number of genes in each pathway gene set are shown.

miR-301b target PRKD3 contributes to increased proliferation in ER mutant cells

Analysis of miR-301b direct target genes that were also mutant-specific genes found that many of these shared genes are key components of major cell signaling pathways including MAPK signaling, PI3K signaling, and ER signaling. Of note, miR-301b targets *ERK2*, a central component of MAPK signaling which is known to drive cell proliferation, differentiation, and survival (fig. 5a), reviewed in (57). miR-301b also targets PIK3CB (P110-β), the catalytic subunit of PI3Kβ, which has been shown to enhance cell proliferation and promote tumorigenesis in

breast cancer (fig. 5a), reviewed in (58). The regulation of these pathway components by differentially expressed miRNAs in ER mutant cells indicate that altered miRNA expression in ER mutant cells directly alters important cellular pathways with roles in regulating cell growth and proliferation.

miR-301b also regulates PRKD3 (protein kinase D3) which regulates activity of several signaling pathways by phosphorylating key pathway components leading to increased proliferation and cell migration (39-42). PRKD3 is significantly up-regulated in both Y537S and D538G ER mutant cells but is significantly down-regulated upon treatment with a miR-301b mimic (fig. 5a). Additionally, PRKD3 contains a recognition sequence for miR-301b in its 3'UTR indicating that it is likely a miR-301b target gene (fig. 5b) (50). To validate that miR-301b regulation of PRKD3 leads to altered PKD3 protein levels, we performed Western blot analysis on protein samples collected from ER mutant or WT cells treated for 48 hours with miR-301b mimic or inhibitor, respectively. We found that PKD3 levels were increased with miR-301b inhibitor treatment in ER WT cells and decreased with miR-301b mimic treatment in ER mutant cells, providing further evidence that miR-301b down-regulates PRKD3 (fig. 5c).

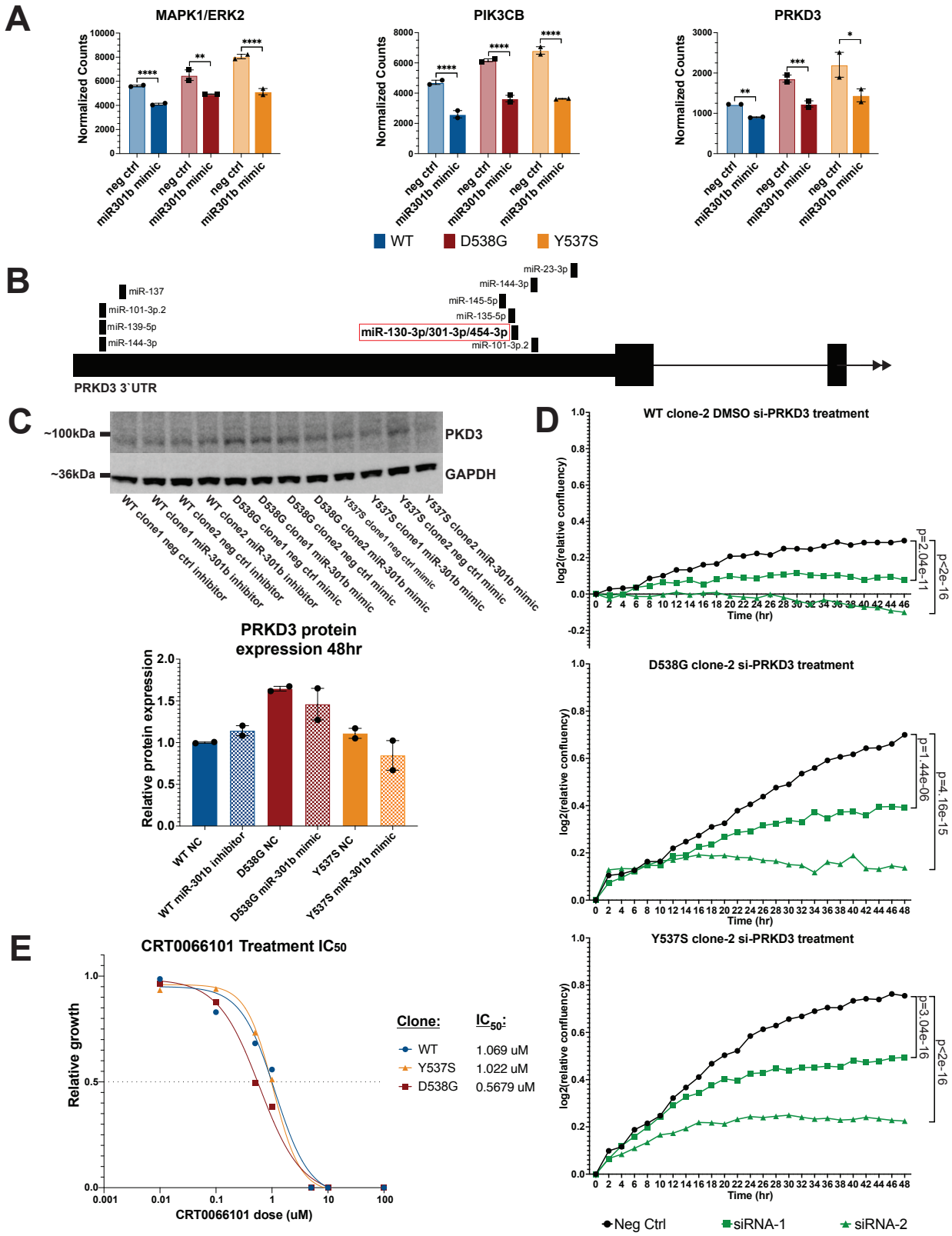
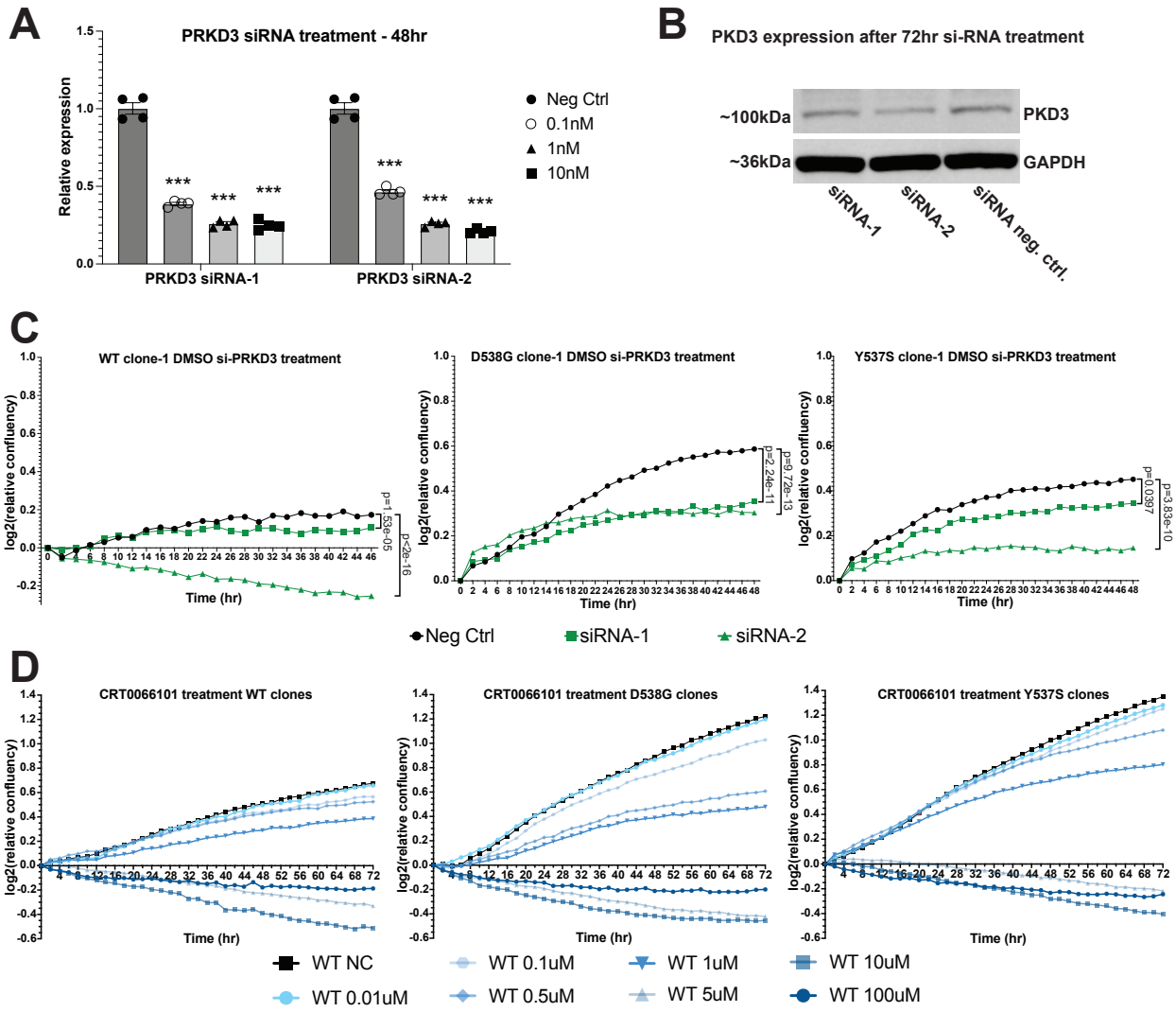


Figure 5. Inhibition of *PRKD3* (PKD3) reduces proliferation in ER WT and mutant cells. A, Bar graphs show three examples of miR-301b altered mutant-specific genes that are direct miR-301b target genes, including *PRKD3*.

Error bars represent average ± SEM for two clones for each genotype. Adjusted p-values are indicated as calculated by DESeq2 (Wald test). **B,** Diagram of *PRKD3* 3'UTR indicates the regions with miRNA target sequences. The miR-301b target site is shown in the red box. **C,** Immunoblot analysis shows PKD3 expression after miR-301b inhibitor treatment in ER WT cells and miR-301b mimic in ER mutant cells. Bar graph shows quantification of PKD3 signal for two clones for each ER genotype. Error bars indicate average signal ± SEM. **D,** Line graphs show $\log_2$ transformed relative confluence over time of WT or mutant ER cells treated with anti-*PRKD3* siRNAs or negative control. Significance between the slopes of the lines was determined using a generalized linear model and the Wald test. **E,** Relative growth is shown for WT, Y537S, and D538G ER clones after treatment with CRT0066101 (pan-PKD inhibitor).



Supplemental Figure 5. Inhibition of *PRKD3* (PKD3) reduces proliferation in ER WT and mutant cells. A, Bar graph shows qPCR analysis of *PRKD3* expression in T-47D cells after treatment with 0.1nM, 1nM, or 10nM siRNA against *PRKD3* or a negative control. Bars represent average ± SEM of four replicates for each treatment. **B,** Immunoblot analysis shows PKD3 protein expression after siRNA treatment against PRKD3 in WT ER cells. **C,** Line graphs show $\log_2$ transformed relative confluence over time of WT or mutant ER cells treated with anti-*PRKD3* siRNAs or negative control. Significance between the slopes of the lines was determined using a generalized linear model and the Wald test. **D,** Line graphs show $\log_2$ transformed relative confluence over time of WT or mutant ER cells treated with pan-PKD inhibitor CRT0066101.

Due to its function in regulating proliferation, we assessed whether PRKD3 is important for proliferation of ER mutant cells in a similar manner to miR-301b mimic treatment. We knocked down PRKD3 expression, using siRNAs, and found that treatment with either of two anti-PRKD3 siRNAs significantly reduced PRKD3 expression and cell proliferation in ER mutant and WT cells (Fig. 5d and Sup. Fig. 5a-c). Because knockdown of PRKD3 via siRNA reduced proliferation, we investigated whether pharmacological inhibition of PKD3 with CRT0066101 could similarly reduce cell proliferation. CRT0066101 is a pan-PKD inhibitor that has been shown to reduce proliferation in a number of tumor types, including ER-negative breast cancer, pancreatic cancer, and colon cancer (59-62). We treated ER mutant and WT cells with a CRT0066101 at a range of doses and found that CRT0066101 treatment reduced cell proliferation in all cell lines in a dose dependent fashion (Fig. 5e and Sup Fig. 5d). Significant reduction in proliferation rates were seen at concentrations as low as 0.1 uM, with IC_{50} values between 0.5 uM and 1.1 uM depending on ER mutation status, similar to IC_{50} values observed in other successful treatment settings (59,61). Significant loss of proliferation upon knock-down or inhibition of PRKD3 points to PRKD3 as a promising therapeutic target in ER+ breast cancers, including those containing ER mutations.

Differential expression of miRNAs in mutant ER cell lines is recapitulated in patient-derived xenograft models

To determine if the differential expression of miRNAs seen in the cell line models is observed in additional models, we used patient-derived xenograft (PDX) breast cancer models (63), harboring either WT ER or naturally-occurring heterozygous Y537S or L536P ER mutations. PDX models included two WT ER lines, a series of three L536P ER mutant lines from the same patient, and two Y537S ER mutant lines from the same patient. Results of miRNA profiling revealed that over 400 miRNAs were significantly differentially expressed between the Y537S and WT PDX lines, while nearly 60 miRNAs were significantly differentially expressed in the L536P PDX lines vs WT (fig. 6a and sup. table S1). When compared to miRNAs that are differentially expressed in the T-47D ER mutant cell lines, only one up-regulated miRNA was shared between the Y537S PDX lines and the T-47D Y537S cell lines. However, half of the down-regulated miRNAs identified in T-47D Y537S mutant ER cell lines were also significantly down-regulated in Y537S PDX lines (fig. 6b). Of the 15 miRNAs that were differentially expressed in T-47D cells and exhibited significant overlap of target genes with mutant-specific genes, 10 were differentially expressed in the T-47D Y537S mutant clones. Of these, 5 exhibited significant up- or down-regulation in the Y537S PDX lines in the same direction as that observed in the T-47D Y537S mutant clones (fig. 6c), including miR-301b and miR-331, while an additional 3 were trending in the same direction but were below the significance threshold. miRNA let-7f-5p was up-regulated with a nearly significant p-value, while miR-181c was not differentially regulated in T-47D Y537S mutant cell lines and therefore was not expected to be differentially regulated in Y537S PDX lines. Of the 5 miRNAs that were differentially regulated in the T-47D D538G mutant cell lines only, none were differentially regulated in the PDX Y537S lines, indicating consistent ER allele-specific effects. These miRNA expression results in WT and mutant ER PDX lines show that miRNAs are significantly altered in patient-derived models harboring mutant ER and validate many of our findings in T-47D cells.

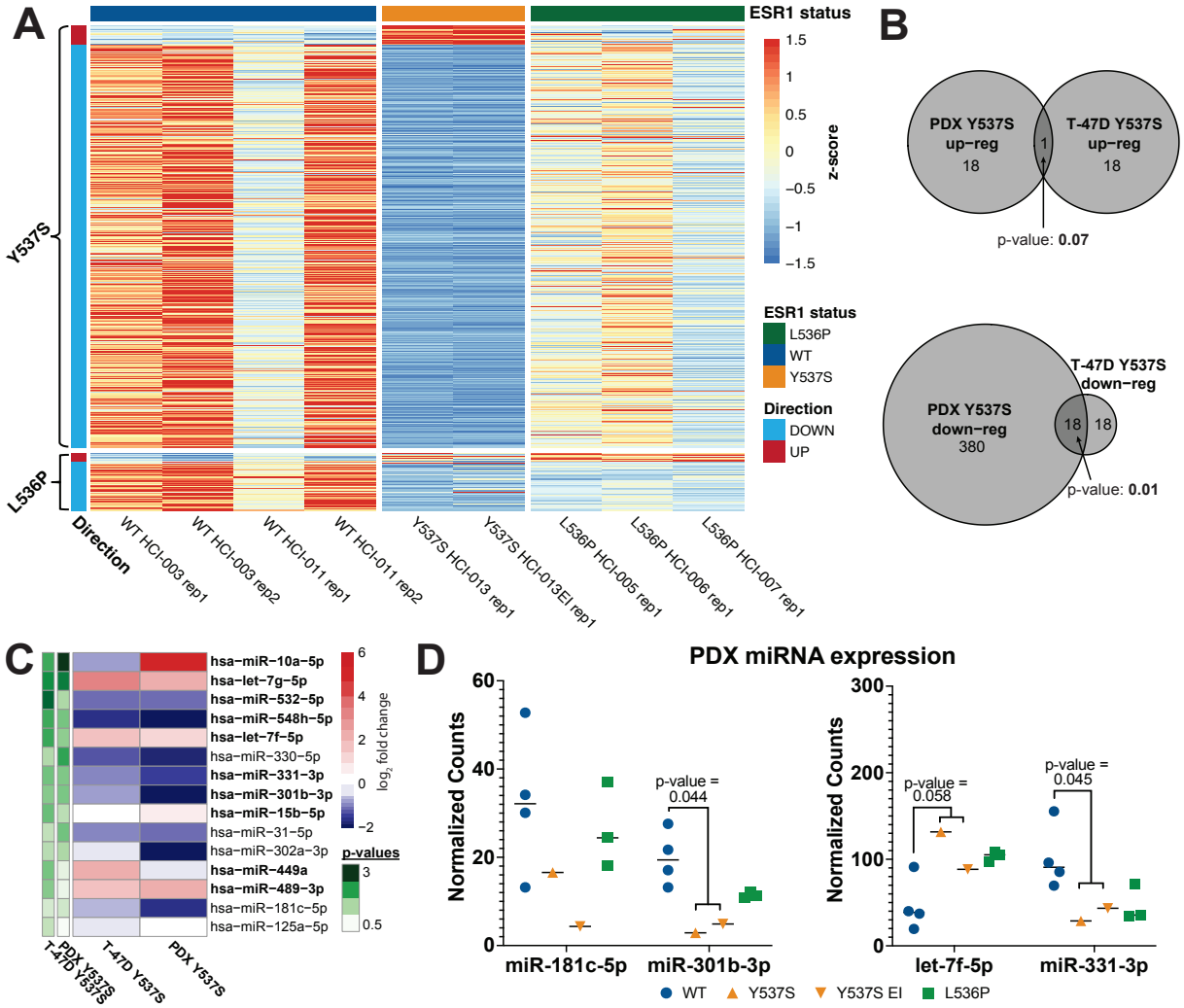


Figure 6. miRNAs are altered in PDX models similar to changes observed in cell line models. A, Heatmap representing differentially expressed miRNAs in L536P or Y537S ER mutant PDX models compared to WT ER models. **B,** Overlap between PDX and T-47D differentially expressed miRNAs. Significance was determined using a hypergeometric test. **C,** Heatmap of the 15 differentially expressed miRNAs analyzed in T-47D clones for proliferation effects. Heatmap shows the $\log_2$ fold change values for these miRNAs in T-47D and PDX Y537S models. Bolded miRNAs were differentially expressed in T-47D Y537S models. -$\log_2$ p-values are indicated in green. **D,** Expression of miR-181c, miR-301b, let-7f, and miR-331 in L536P and Y537S PDX models. P-values were calculated using a Student's two sample *t* test.

Discussion

The frequent occurrence of ER mutations in hormone therapy-resistant ER-positive metastatic breast cancer has led to intense research efforts to elucidate the molecular and phenotypic effects of ER mutations and to identify vulnerabilities in ER mutant cancers that could be exploited to develop effective therapies for hormone therapy-resistant tumors (4-18). In a previous study, we used isogenic T-47D models expressing the Y537S or D538G ER mutations from the endogenous ER locus to show that ER mutations regulated the differential expression of thousands of genes and that these transcriptional changes were accompanied by phenotypic changes, including increased migration, invasion, and proliferation (16,17). Although our understanding of ER

mutations and their effects has improved significantly, the contribution of miRNAs to ER mutant gene regulation and downstream effects was unknown. In humans, over 2000 miRNAs regulate the expression of roughly 60% of protein coding genes (64,65), indicating a potential for miRNAs to aid mutant ER in alterating gene expression. In this study, we aimed to determine the role of miRNAs in regulating transcriptional and phenotypic changes in mutant ER breast cancer.

Using the isogenic cell line models introduced previously (16), we found that 85 miRNA (over 10% of those profiled) were differentially expressed in the Y537S, D538G, or both mutant cell lines. Mutant-specific genes identified in our previous work were over-represented in the target genes of many of these miRNAs. In several cases, namely miR-301b, miR-181c, miR-331, and let-7f, these miRNA targets were enriched for genes with important roles in driving cell growth, proliferation, or motility, indicating a possible role for these miRNAs in driving cancer-associated phenotypes that we observed previously in ER mutant cells. When we directly tested the effect of these miRNA on ER mutant-specific gene expression by altering miRNA activity through mimic or inhibitor treatment followed by RNA-seq, we found that these miRNAs did indeed alter the expression of many mutant-specific genes. Furthermore, these miRNA-altered mutant-specific genes were enriched for cell cycle-related genes and therefore altered activity of these miRNAs in ER mutant cells can partially explain the observed increased proliferation.

Altered expression of miRNAs in ER mutant cells can lead to broad gene expression changes both through direct miRNA targeting or by downstream effects in which miRNAs directly alter transcriptional regulators which in turn alter the expression of their target genes. We found that for three of the four miRNAs we investigated in depth (miR-301b, miR-181c, and let-7f), mutant-specific genes that were up- or down-regulated upon miRNA inhibitor or mimic treatment, respectively, were highly enriched for predicted direct target genes of the respective miRNA. For miR-301b and miR-181c, many direct target genes were components or regulators of important cell signaling pathways. Altered expression of these pathways could lead to additional gene expression changes, potentially explaining the remaining 50-75% of miRNA-altered mutant-specific genes. Only 2 of the 17 genes up-regulated by let-7f inhibitor treatment were also mutant-specific down-regulated genes, but both of these genes (TUSC2 and PLAGL2) were predicted let-7f direct target genes. These findings indicate that miRNA-mediated mutant-specific gene expression changes occur as a result of both direct miRNA-mediated mRNA degradation and through secondary effects driven by altered expression or activity of genes that are direct miRNA targets.

The majority of mutant-specific gene expression changes can be explained by ER mutant's constitutive activity, altered ER genomic binding, or altered chromatin accessibility (16); however, the mechanism behind the differential expression of the remaining mutant-specific genes was unexplained. Our findings showed that miRNA-altered mutant-specific genes are represented in each of these categories at a distribution similar to that observed for all mutant-specific genes (sup. fig. 3c). miRNAs contribute considerably to these previously-unexplained mutant-specific gene expression effects. We found that 4 miRNAs (miR-301b, miR-181c, miR-331, and let-7f) could explain an overlapping 32% of mutant-specific genes that were unexplained from our previous findings (sup. table S3). Additionaly, we found that these miRNAs contribute to an

overlapping 27% of mutant-specific genes that are explained by constitutive mutant ER activity, indicating that miRNAs are a likely mechanism by which constant ER activity leads to the altered expression of genes that are not ER regulated during a transient E2 induction.

One major question regarding differentially expressed miRNAs is what drives their altered expression. Altered miRNA expression could be driven directly by ER, by other transcription factors downstream of ER signaling, or by other transcription factors that have improved or decreased access to genomic regions due to mutant ER driven changes in chromatin accessibility (16). Utilizing mutant ER ChIP-seq data and Assay for Transposase Accessible Chromatin (ATAC-seq) data from our previous study, we found altered ER binding at the TSSs for let-7f, miR-301b, and miR-331 indicating direct regulation of these miRNAs by mutant ER. miR-181c had little to no ER binding near its TSS, but altered chromatin accessibility between mutant and WT ER cells indicated that mutant ER drives decreased accessibility at this site and therefore could regulate the expression of this miRNA indirectly.

Not only does mutant ER likely regulate the expression of these miRNAs, but conversely, at least two differentially expressed miRNAs (miR-301b and miR-181c) regulate the expression of *ESR1*, the gene encoding ER. miR-301b and miR-181c are down-regulated in ER mutant cells which correlates with increased expression of ER. We found that reversing the low expression of these miRNAs to higher levels, such as those observed in WT cells, through miRNA mimic treatment, resulted in significantly decreased *ESR1* expression. In addition, *ESR1* contains recognition sequences for both miR-301b and miR-181c in its 3'UTR, indicating that *ESR1* is a direct target of these miRNAs. In the normal mammary gland, ER activity results in a negative feedback loop that reduces ER expression (53). In T-47D cells, initial ER activity increases ER expression, but with long-term ER activity, ER expression plateaus and drops back towards starting levels. Mutant ER-driven down-regulation of miR-301b and miR-181c represents a mechanism by which mutant ER evades this normal negative feedback on ER expression and instead achieves higher ER levels.

In addition to understanding the role of miRNAs in regulating mutant-specific gene expression, we also aimed to understand how miRNAs contribute to cancer-associated phenotypes in ER mutant cells. One major phenotype observed in ER mutant cells is their increased proliferative capacity (16) and miRNAs could contribute to the positive effect mutant ER has on proliferation. When we tested the effect of 15 differentially expressed miRNAs on proliferation by individually modulating their activity with miRNA mimics or inhibitors, we found that several miRNAs significantly altered proliferation. In particular, miR-301b, miR-181c, miR-331, and let-7f significantly altered ER mutant cell growth. This effect is explained by the finding that the mutant-specific genes that are altered by these miRNAs include many cell cycle-related genes and genes involved in pathways that promote cell proliferation. Thus, altered expression of miRNAs driven by ER mutations likely represents an important mechanism by which ER mutant cells achieve increased proliferation.

Another possible explanation for the increased proliferation in ER mutant cells is that altered miRNA expression impacts the activity of important signaling pathways that promote proliferation.

miR-301b, which is down-regulated in ER mutant cells, directly targets the *PRKD3* gene, which codes for protein kinase D3 (PKD3), a regulator of cell signaling pathways that promote cell proliferation. *PRKD3* is up-regulated in a mutant-specific fashion and altering the activity of miR-301b significantly altered the expression of *PRKD3*. PKD3 promotes phosphorylation of several components of cell signaling pathways which drive proliferation in breast and other cancer types (39-42). We found that increased proliferation in ER mutant cells can be significantly reduced when PKD3 is inhibited either genetically by siRNA knockdown, or pharmacologically by treatment with the PKD inhibitor CRT0066101. Overall, these findings suggest that miR-301b repression contributes to mutant ER phenotypes, such as increased proliferation, by driving increased expression of *PRKD3*, as well as increased levels of *ESR1*. In addition, these findings also show that PKD3 inhibition is a promising option for future therapeutics, with CRT0066101 having already been shown to inhibit tumor growth both *in vitro* and *in vivo* in several other cancer types (59-62).

miRNAs that are differentially expressed in ER mutant cells could produce varying effects that are dependent on ER mutant status. miR-301b, for example, has been shown in other settings, including triple negative breast cancer, to promote proliferation (44,45). However, we found that it has the opposite effect in ER mutant cells where increasing its activity completely abolished proliferation. These findings not only provide evidence of a significant role for miRNAs in establishing ER mutant effects, but also indicate that some miRNAs are utilized in unique fashion in ER mutant cells. The effect of miRNAs is highly cell context-dependent and this observation could be explained by the altered transcriptional profile seen in ER mutant cells compared to WT cells. The differential effects could result in a different set of miR-301b target genes expressed in ER mutant cells than are expressed in WT cells, leading to a different overall effect of miR-301b in these cells.

We validated the mutant ER-driven differential expression of miRNAs by identifying differentially expressed miRNAs in PDX models of mutant or WT ER-positive breast cancer. We found that many of the same, as well as many additional, miRNAs are differentially expressed in Y537S PDX models as those identified in our T-47D cell line models. Of note, miR-301b, miR-331, and let-7f were all differentially expressed in the same direction in PDX Y537S models as in T-47D cells. This consistency between cell line and PDX models demonstrates that differential expression of miRNAs has the potential to play a significant role in mutant ER pathology in patients.

Overall our findings show that certain miRNAs exhibit consistent differential expression in mutant ER breast cancer models and are capable of regulating key genes and pathways that can be targeted to successfully inhibit breast cancer growth. These results also help to identifying additional factors and signaling pathways that could be effective drug targets to treat hormone therapy-resistant ER mutant breast cancers. Additionally, the use of miRNA mimics and inhibitors to directly alter the activity of a specific miRNA has recently become an exciting new therapeutic option currently being explored. Our finding that many miRNAs are differentially regulated in the ER mutant breast cancer setting suggests that the direct targeting of these miRNAs through mimics and inhibitors could also represent a promising therapeutic approach in treating this hormone therepy-resistant disease.

Methods

Cell culture

The generation of T-47D ER mutant and WT clones was described in Arnesen et al (16). Cells were cultured in RPMI1640 Media (ThermoFisher Scientific) supplemented with 10% FBS (Sigma-Aldrich) and 1% penicillin-streptomycin (ThermoFisher Scientific). Five days prior to all experiments, except those otherwise specified, cells were moved to growth in hormone-deprived media which was composed of phenol red-free RPMI1640 Media (ThermoFisher Scientific) supplemented with 10% charcoal-stripped FBS (ThermoFisher Scientific) and 1% penicillin-streptomycin.

miRNA expression analysis

ER mutant or WT T-47D cells were plated in 100mm dishes and grown in estrogen-deprived media for five days prior to treatment with DMSO (0.1%) for 8 hours. Cells were washed with phosphate-buffered saline (PBS; ThermoFisher Scientific) and treated with RLT plus (Qiagen) lysis buffer with 1% beta-mercaptoethanol (BME; Sigma-Aldrich) for cell lysis. Cells were passed through a 21-gauge needle (Sigma-Aldrich) to facilitate genomic DNA lysis. For PDX lines, tissue samples were used from seven PDX lines (WT: HCI-003 and HCI-011; L536P: HCI-005, HCI-006, HCI-007; Y537S: HCI-013, HCI-013EI). PDX samples were surgically removed from mice and flash frozen. PDX samples were not treated. Approximately 20mg of tissue per sample was homogenized in RLT plus lysis buffer with 1% beta-mercaptoethanol using gentleMACS M-tubes and a gentleMACS Octo Dissociator (Miltenyi Biotec). RNA extraction for both T-47D and PDX samples was performed using a Quick-RNA RNA Miniprep kit (Zymo Research), and RNA was measured using a Qubit fluorometer. For each sample, 200ng of total RNA was submitted for miRNA expression analysis using NanoString's nCounter Human v3 miRNA expression assay. NanoString miRNA expression assay results in the form of raw counts were analyzed using NanoString's nSolver software. Raw counts were normalized using two approaches. The first approach used the geometric mean for each sample of the top 100 highest expressed miRNAs across all samples to determine a normalization factor for each sample. The second approach used the geometric mean for each sample of the five spike-in miRNAs profiled for the assay to generate the normalization factors. Normalized counts were then log-scaled and used to perform *t* test to identify significantly differentially expressed miRNAs. Because this was a hypothesis generating step, we considered differentially expressed miRNAs from both normalization approaches in our study. The DIANA TarBase database (43) was used to compare putative target genes of differentially expressed miRNAs with mutant-specific genes described in Arnesen et al (16). Significant enrichments were defined using a hypergeometric test as implemented in R using phyper.

Cell transfections

After cells were grown in hormone-deprived media for five days, cells were transfected with a miRNA mimic, miRNA inhibitor, or an siRNA. mirVana miRNA mimics and inhibitors were used for 15 miRNAs (ThermoFisher Scientific). Mimics and inhibitors were transfected at a 10nM final concentration using the RNAiMAX transfection reagent (ThermoFisher Scientific) per the manufacturer's instructions. siRNAs against PRKD3 from Integrated DNA Technologies'

TriFECTa RNAi kit were used to knock down PRKD3 transcript levels (sup. table S4). To test the efficacy of these siRNAs, WT cells were transfected with 0.1nM, 1nM, or 10nM concentrations of three anti-PRKD3 siRNAs using the RNAiMAX transfection reagent and knock-down or PRKD3 was measured using qPCR as described above. The two siRNAs achieving the best knock-down of PRKD3 were used at a 10nM concentration in subsequent siRNA transfection experiments.

Proliferation assays

Cells were plated in 100mm dishes and grown in hormone-deprived media for four days prior to plating in 96-well plates at approximately 12,500 cells per well and allowed to adhere for another day in hormone-deprived media. 24 hours after 96-well plating, cells were treated with 1% DMSO and a miRNA mimic/inhibitor, siRNA, or drug, depending on the experiment. Cells were then placed on the IncuCyte Zoom Live Cell Imaging Platform (Sartorius) and proliferation was monitored for 48 hours with 10x magnification images obtained at 2-hour intervals. Confluence was measured for three or four replicates, depending on the experiment, and percent confluence values were normalized to the initial confluence and then $\log_2$ transformed. Linear regression analysis using a general linear Wald test, as implemented by glm() in R, was used to compare the slopes of transformed confluence values over time and to determine significant differences in slopes.

RNA-seq

T-47D cells were grown in 100mm plates in hormone-deprived media for four days then plated into 12-well plates at approximately 50% confluence and allowed to adhere for 24 hours. Cells were then transfected with a miRNA mimic or inhibitor for a given miRNA or with a mimic or inhibitor negative control at a 10nM final concentration. 24 hours post-transfection, cells were collected, washed with PBS, then treated with RLT Plus lysis buffer supplemented with 1% BME. RNA was extracted using a Quick-RNA RNA Miniprep kit (Zymo Research), and RNA was measured using a Qubit fluorometer. Poly-A selected RNA-seq libraries were generated using a KAPA Stranded mRNA-Seq kit (KAPA Biosystems) and 500ng RNA per sample as starting material. Libraries were sequenced using the Illumina NovaSeq 6000 platform. The Hisat2 spliced aligner was used to align the resulting fastq files to the hg19 human genome build (66). SAM files were then converted to BAM files using SAMtools (67). Genes included in the University of California Santa Cruz's (UCSC) Known Genes table were assigned counts using the Subread package's featureCounts program using BAM files as inputs (68). Counts were then normalized and analyzed for differential expression using the DESeq2 package in R (69). Two clones for each ER genotype (Y537S, or D538G) were used for RNA-seq and clones of the same genotype were used as biological duplicates in our differential expression analysis. Significantly up- or down-regulated genes (adjusted p-value <0.05) were identified by comparing results from miRNA mimic or inhibitor treatment to the appropriate negative control treatment in clones of the same ER genotype. Genes that were identified as differentially expressed were compared to mutant-specific genes as defined previously (16). miRNA-altered mutant-specific genes were compared with putative miRNA target genes for the corresponding miRNA using the miRDB microRNA Target Prediction Database (51). Significant enrichments were determined using a hypergeometric test as implemented in R using phyper with a p-value cutoff of <0.05. Gene ontology and pathway analysis was performed by submitting genes on interest to Ma'ayan lab's

Enrichr online tool (70). Adjusted p-values were used to identify significantly enriched ontologies and pathways. Venn diagrams were made using the eulerr package.

Quantitative PCR

WT T-47D cells were grown in regular media. Cells were lysed using RLT plus (Qiagen) plus 1% beta-mercaptoethanol (Sigma-Aldrich) and total RNA was extracted using a Quick RNA Miniprep kit (Zymo Research). qPCR was performed using a Power SYBR Green RNA-to-CT 1-Step Kit (ThermoFisher Scientific) and 50ng of RNA per sample as input. A CFX Connect Real-Time light cycler (Bio-Rad) was used for thermocycling and light detection. A $\Delta\Delta C_t$ method was used to calculate expression levels with TBP used as the control. Duplicates were measured for each sample. Primer sequences for PRKD3 and TBP primers are listed in Supplemental Table S4. Significant changes in relative expression were determined using a one-tailed Student's *t* test using t.test in R, with a p-value cutoff of <0.05.

Immunoblot analysis

ER mutant or WT cells were grown in 6-well format in RPMI1640 media. For siRNA treatment, cells were grown in regular media and for miRNA mimic/inhibitor treatment, cells were grown in hormone-deprived media for five days before treatment. Cells were transfected with an siRNA or a miRNA mimic/inhibitor and then collected and lysed 48 or 72 hours post transfection using RIPA buffer (1x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease and phosphatase inhibitor (ThermoFisher Scientific, A32959). Total protein concentration was determined using the Bradford quantification assay (Bio-Rad). For electrophoresis, 30 µg of total protein was diluted with 4X NuPAGE™ LDS Sample Buffer (ThermoFisher Scientific, NP0007) and run on a NuPAGE™ 4-12% Bis-Tris polyacrylamide gel (ThermoFisher Scientific, NP0336BOX) at 150V. Protein was then transferred to an iBlot2 PVDF membrane (ThermoFisher Scientific, IB24002) using the iBlot2 transfer apparatus (ThermoFisher, IB21001). The blot was blocked with Odyssey Blocking Buffer (LI-COR Biosciences, 927-60001) for 1 hour at 4°C and probed with a 1:500 dilution of 0.5 mg/mL PRKD3 antibody (Abnova, H00023683-M01) and a 1:2000 dilution of 2 mg/mL GAPDH antibody (Abcam, [6C5] ab8245) overnight with shaking at 4°C. The blot was then washed three times with 1X TBST buffer at room temperature and probed with a 1:10000 dilution of mouse secondary antibody (LI-COR Biosciences IRDye® 800CW Goat anti-mouse IgG, 926-32210) for 1 hour at room temperature. Following incubation with secondary antibody, the blot was washed three times with 1X TBST buffer, visualized using an Azure™ Biosystems 600 imaging system, and PRKD3 band intensity was quantified using Image Studio Lite software (Image Studio Lite Software, LI-COR, Lincoln NE). Statistical analysis of PRKD3 abundance was performed using a one-tailed Student's *t* test using t.test in R, with a p-value cutoff of <0.05.

Drug treatments

Cells were grown for a single clone for each ER genotype (WT, Y537S, and D538G) in 100mm dishes in hormone-deprived media for 4 days and then plated in 96-well plates at approximately 12,500 cells per well and allowed to adhere in hormone-deprived media for 24 hours. Cells were then treated with varying doses (0.01 uM, 0.1 uM, 0.5 uM, 1 uM, 5 uM, 10 uM, or 100 uM) of the PKD inhibitor CRT0066101 (Selleck Chemicals, S8366) or with a vehicle control (H_2O) and

analyzed for proliferation. Experiments were performed in triplicate. Proliferation rates were calculated as described above and IC_{50} values were calculated using Prism 9 software.

Statistical analysis and packages

All statistical analyses, except those for gene ontology and pathways analysis, were performed using R version 3.5.2 or 3.5.3.

Data Access

All raw and processed sequencing data generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under the following accession number: GSE214580.

Supplemental Table S3. Percent overlap between mimic or inhibitor differentially expressed genes and all mutant-specific genes, unexplained mutant-specific genes, or mutant-specific genes explained by constitutive mutant ER activity

miRNA	mimic/inhibitor genes vs mutant-specific genes	% mimic or inhibitor genes	% mutant-specific genes	Hypergeometric p-value
miR-301b	Y537S mimic up-regulated vs Y537S mutant-specific down-regulated genes	22.46%	10.84%	1.82e-53
	Y537S mimic down-regulated vs Y537S mutant-specific up-regulated	20.22%	10.36%	3.80e-41
	D538G mimic up-regulated vs D538G mutant-specific down-regulated	27.24%	8.91%	7.51e-44
	D538G mimic down-regulated vs D538G mutant-specific up-regulated	42.77%	16.05%	8.67e-167
miR-181c	D538G mimic up-regulated vs D538G mutant-specific down-regulated	21.61%	7.07%	1.78e-20
	D538G mimic down-regulated vs D538G mutant-specific up-regulated	39.87%	8.87%	6.14e-91
let-7f	Y537S inhibitor up-regulated vs Y537S mutant-specific down-regulated	13.33%	0.13%	0.22
	Y537S inhibitor down-regulated vs Y537S mutant-specific up-regulated	26.98%	0.88%	1.72e-6
miR-331	Y537S mimic up-regulated vs Y537S mutant-specific down-regulated	17.25%	31.94%	3.54e-120
	Y537S mimic down-regulated vs Y537S mutant-specific up-regulated	17.15%	21.76%	4.63e-67
miRNA	**mimic/inhibitor genes vs unexplained mutant-specific genes**	**% mimic or inhibitor genes**	**% mutant-specific genes**	**Hypergeometric p-value**
miR-301b	Y537S mimic up-regulated vs Y537S down-regulated genes	4.01%	10.91%	3.42e-10
	Y537S mimic down-regulated vs Y537S up-regulated	3.05%	8.75%	1.58e-5
	D538G mimic up-regulated vs D538G down-regulated	9.95%	11.67%	1.06e-23

	D538G mimic down-regulated vs D538G up-regulated	15.80%	15.71%	5.38e-53
miR-181c	D538G mimic up-regulated vs D538G down-regulated	7.94%	9.32%	1.46e-13
	D538G mimic down-regulated vs D538G up-regulated	15.41%	10.11%	8.25e-33
let-7f	Y537S inhibitor up-regulated vs Y537S down-regulated	0.0	0.0	1.0
	Y537S inhibitor down-regulated vs Y537S up-regulated	3.17%	0.58%	0.20
miR-331	Y537S mimic up-regulated vs Y537S down-regulated	3.45%	36.0%	1.83e-28
	Y537S mimic down-regulated vs Y537S up-regulated	3.61%	25.66%	4.0e-19
miRNA	**mimic/inhibitor genes vs mutant-specific genes explained by constitutive ER activity**	**% mimic or inhibitor genes**	**% mutant-specific genes**	**Hypergeometric p-value**
miR-301b	Y537S mimic up-regulated vs Y537S down-regulated genes	12.03%	9.38%	1.29e-23
	Y537S mimic down-regulated vs Y537S up-regulated	14.13%	10.82%	3.00e-30
	D538G mimic up-regulated vs D538G down-regulated	11.36%	6.67%	2.23e-9
	D538G mimic down-regulated vs D538G up-regulated	23.45%	17.24%	1.46e-90
miR-181c	D538G mimic up-regulated vs D538G down-regulated	8.94%	5.26%	9.68e-4
	D538G mimic down-regulated vs D538G up-regulated	19.87%	9.64%	3.60e-40
let-7f	Y537S inhibitor up-regulated vs Y537S down-regulated	13.33%	0.21%	0.10
	Y537S inhibitor down-regulated vs Y537S up-regulated	20.63%	1.01%	8.85e-6
miR-331	Y537S mimic up-regulated vs Y537S down-regulated	8.64%	25.86%	2.52e-40

	Y537S mimic down-regulated vs Y537S up-regulated	10.87%	20.62%	2.50e-37

Supplemental Table S4. Sequence for qPCR analyses primers and siRNA knockdowns

Name	Sequence
PRKD3_qPCR_F	CTCACACGGGAGAGTGTTACC
PRKD3_qPCR_R	TTCATGTCATGGCGAAAGAGAA
TBP_qPCR_F	CCACTCACAGACTCTCACAAC
TBP_qPCR_R	CTGCGGTACAATCCCAGAACT
PRKD3_siRNA1_sense	rGrArArGrCrArArUrUrGrArUrCrUrGrArUrArArArCrArATC
PRKD3_siRNA1_antisense	rGrArUrUrGrUrUrUrArUrCrArGrArUrCrArArUrUrGrCrUrUrCrArC
PRKD3_siRNA2_sense	rArGrUrGrUrUrGrArCrArArArUrCrUrCrUrUrArGrUrCrATC
PRKD3_siRNA2_antisense	rGrArUrGrArCrUrArArGrArGrArUrUrUrGrUrCrArArCrArCrUrGrU

References

1. Early Breast Cancer Trialists' Collaborative G, Davies C, Godwin J, Gray R, Clarke M, Cutter D*, et al.* Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet **2011**;378:771-84
2. Group EBCTC. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet **2005**;365:1687-717
3. Group EBCTC. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet **1998**;351:1451-67
4. Toy W, Weir H, Razavi P, Lawson M, Goeppert AU, Mazzola AM*, et al.* Activating ESR1 Mutations Differentially Affect the Efficacy of ER Antagonists. Cancer Discov **2017**;7:277-87
5. Toy W, Shen Y, Won H, Green B, Sakr RA, Will M*, et al.* ESR1 ligand-binding domain mutations in hormone-resistant breast cancer. Nat Genet **2013**;45:1439-45
6. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X*, et al.* Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nat Genet **2013**;45:1446-51
7. Weis KE, Ekena K, Thomas JA, Lazennec G, Katzenellenbogen BS. Constitutively active human estrogen receptors containing amino acid substitutions for tyrosine 537 in the receptor protein. Mol Endocrinol **1996**;10:1388-98
8. Zhang QX, Borg A, Wolf DM, Oesterreich S, Fuqua SA. An estrogen receptor mutant with strong hormone-independent activity from a metastatic breast cancer. Cancer research **1997**;57:1244-9
9. Merenbakh-Lamin K, Ben-Baruch N, Yeheskel A, Dvir A, Soussan-Gutman L, Jeselsohn R*, et al.* D538G mutation in estrogen receptor-alpha: A novel mechanism for acquired endocrine resistance in breast cancer. Cancer research **2013**;73:6856-64
10. Jeselsohn R, Yelensky R, Buchwalter G, Frampton G, Meric-Bernstam F, Gonzalez-Angulo AM*, et al.* Emergence of constitutively active estrogen receptor-alpha mutations in pretreated advanced estrogen receptor-positive breast cancer. Clin Cancer Res **2014**;20:1757-67
11. Fanning SW, Mayne CG, Dharmarajan V, Carlson KE, Martin TA, Novick SJ*, et al.* Estrogen receptor alpha somatic mutations Y537S and D538G confer breast cancer endocrine resistance by stabilizing the activating function-2 binding conformation. Elife **2016**;5
12. Bahreini A, Li Z, Wang P, Levine KM, Tasdemir N, Cao L*, et al.* Mutation site and context dependent effects of ESR1 mutation in genome-edited breast cancer cell models. Breast Cancer Res **2017**;19:60
13. Harrod A, Fulton J, Nguyen VTM, Periyasamy M, Ramos-Garcia L, Lai CF*, et al.* Genomic modelling of the ESR1 Y537S mutation for evaluating function and new therapeutic approaches for metastatic breast cancer. Oncogene **2017**;36:2286-96
14. Jeselsohn R, Bergholz JS, Pun M, Cornwell M, Liu W, Nardone A*, et al.* Allele-Specific Chromatin Recruitment and Therapeutic Vulnerabilities of ESR1 Activating Mutations. Cancer Cell **2018**;33:173-86 e5
15. Katzenellenbogen JA, Mayne CG, Katzenellenbogen BS, Greene GL, Chandarlapaty S. Structural underpinnings of oestrogen receptor mutations in endocrine therapy resistance. Nat Rev Cancer **2018**;18:377-88
16. Arnesen S, Blanchard Z, Williams MM, Berrett KC, Li Z, Oesterreich S*, et al.* Estrogen Receptor Alpha Mutations in Breast Cancer Cells Cause Gene Expression Changes through Constant Activity and Secondary Effects. Cancer research **2021**;81:539-51

17. Williams MM, Spoelstra NS, Arnesen S, O'Neill KI, Christenson JL, Reese J*, et al.* Steroid Hormone Receptor and Infiltrating Immune Cell Status Reveals Therapeutic Vulnerabilities of ESR1-Mutant Breast Cancer. Cancer research **2021**;81:732-46
18. Li Z, Wu Y, Yates ME, Tasdemir N, Bahreini A, Chen J*, et al.* Hotspot ESR1 Mutations Are Multimodal and Contextual Modulators of Breast Cancer Metastasis. Cancer research **2022**;82:1321-39
19. Klinge CM. Estrogen Regulation of MicroRNA Expression. Curr Genomics **2009**;10:169-83
20. Castellano L, Giamas G, Jacob J, Coombes RC, Lucchesi W, Thiruchelvam P*, et al.* The estrogen receptor-alpha-induced microRNA signature regulates itself and its transcriptional response. Proc Natl Acad Sci U S A **2009**;106:15732-7
21. Huntzinger E, Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet **2011**;12:99-110
22. Eichhorn SW, Guo H, McGeary SE, Rodriguez-Mias RA, Shin C, Baek D*, et al.* mRNA destabilization is the dominant effect of mammalian microRNAs by the time substantial repression ensues. Mol Cell **2014**;56:104-15
23. Garzon R, Calin GA, Croce CM. MicroRNAs in Cancer. Annu Rev Med **2009**;60:167-79
24. Lee YS, Dutta A. MicroRNAs in cancer. Annu Rev Pathol **2009**;4:199-227
25. Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. Annu Rev Pathol **2014**;9:287-314
26. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol Med **2014**;20:460-9
27. Abolghasemi M, Tehrani SS, Yousefi T, Karimian A, Mahmoodpoor A, Ghamari A*, et al.* MicroRNAs in breast cancer: Roles, functions, and mechanism of actions. J Cell Physiol **2020**;235:5008-29
28. Hanna J, Hossain GS, Kocerha J. The Potential for microRNA Therapeutics and Clinical Research. Front Genet **2019**;10:478
29. Mollaei H, Safaralizadeh R, Rostami Z. MicroRNA replacement therapy in cancer. J Cell Physiol **2019**;234:12369-84
30. Ratti M, Lampis A, Ghidini M, Salati M, Mirchev MB, Valeri N*, et al.* MicroRNAs (miRNAs) and Long Non-Coding RNAs (lncRNAs) as New Tools for Cancer Therapy: First Steps from Bench to Bedside. Target Oncol **2020**;15:261-78
31. Kondo N, Toyama T, Sugiura H, Fujii Y, Yamashita H. miR-206 Expression is down-regulated in estrogen receptor alpha-positive human breast cancer. Cancer research **2008**;68:5004-8
32. Smith AL, Iwanaga R, Drasin DJ, Micalizzi DS, Vartuli RL, Tan AC*, et al.* The miR-106b-25 cluster targets Smad7, activates TGF-beta signaling, and induces EMT and tumor initiating cell characteristics downstream of Six1 in human breast cancer. Oncogene **2012**;31:5162-71
33. Tanic M, Yanowsky K, Rodriguez-Antona C, Andres R, Marquez-Rodas I, Osorio A*, et al.* Deregulated miRNAs in hereditary breast cancer revealed a role for miR-30c in regulating KRAS oncogene. PLoS One **2012**;7:e38847
34. Wang B, Wang H, Yang Z. MiR-122 inhibits cell proliferation and tumorigenesis of breast cancer by targeting IGF1R. PLoS One **2012**;7:e47053
35. Jiang Q, He M, Ma MT, Wu HZ, Yu ZJ, Guan S*, et al.* MicroRNA-148a inhibits breast cancer migration and invasion by directly targeting WNT-1. Oncol Rep **2016**;35:1425-32
36. Ma F, Li W, Liu C, Li W, Yu H, Lei B*, et al.* MiR-23a promotes TGF-beta1-induced EMT and tumor metastasis in breast cancer cells by directly targeting CDH1 and activating Wnt/beta-catenin signaling. Oncotarget **2017**;8:69538-50

37. Miao Y, Zheng W, Li N, Su Z, Zhao L, Zhou H*, et al.* MicroRNA-130b targets PTEN to mediate drug resistance and proliferation of breast cancer cells via the PI3K/Akt signaling pathway. Sci Rep **2017**;7:41942
38. Long X, Shi Y, Ye P, Guo J, Zhou Q, Tang Y. MicroRNA-99a Suppresses Breast Cancer Progression by Targeting FGFR3. Front Oncol **2019**;9:1473
39. Chen J, Deng F, Singh SV, Wang QJ. Protein kinase D3 (PKD3) contributes to prostate cancer cell growth and survival through a PKCepsilon/PKD3 pathway downstream of Akt and ERK 1/2. Cancer research **2008**;68:3844-53
40. Huck B, Duss S, Hausser A, Olayioye MA. Elevated protein kinase D3 (PKD3) expression supports proliferation of triple-negative breast cancer cells and contributes to mTORC1-S6K1 pathway activation. J Biol Chem **2014**;289:3138-47
41. Liu Y, Song H, Yu S, Huang KH, Ma X, Zhou Y*, et al.* Protein Kinase D3 promotes the cell proliferation by activating the ERK1/c-MYC axis in breast cancer. J Cell Mol Med **2020**;24:2135-44
42. Cui B, Chen J, Luo M, Liu Y, Chen H, Lu D*, et al.* PKD3 promotes metastasis and growth of oral squamous cell carcinoma through positive feedback regulation with PD-L1 and activation of ERK-STAT1/3-EMT signalling. Int J Oral Sci **2021**;13:8
43. Karagkouni D, DIANA-Lab DoECE, University of Thessaly, 382 21 Volos, Greece, DIANA-Lab HPI, 127 Vasilissis Sofias Avenue, 11521 Athens, Greece, Paraskevopoulou MD, DIANA-Lab DoECE, University of Thessaly, 382 21 Volos, Greece, Chatzopoulos S*, et al.* DIANA-TarBase v8: a decade-long collection of experimentally supported miRNA–gene interactions. Nucleic Acids Research **2022**;46
44. Shi W, Gerster K, Alajez NM, Tsang J, Waldron L, Pintilie M*, et al.* MicroRNA-301 mediates proliferation and invasion in human breast cancer. Cancer research **2011**;71:2926-37
45. Peng Y, Xi X, Li J, Ni J, Yang H, Wen C*, et al.* miR-301b and NR3C2 co-regulate cells malignant properties and have the potential to be independent prognostic factors in breast cancer. J Biochem Mol Toxicol **2021**;35:e22650
46. Lee YS, Dutta A. The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. Genes Dev **2007**;21:1025-30
47. Sun X, Qin S, Fan C, Xu C, Du N, Ren H. Let-7: a regulator of the ERalpha signaling pathway in human breast tumors and breast cancer stem cells. Oncol Rep **2013**;29:2079-87
48. Liu K, Zhang C, Li T, Ding Y, Tu T, Zhou F*, et al.* Let-7a inhibits growth and migration of breast cancer cells by targeting HMGA1. Int J Oncol **2015**;46:2526-34
49. Sun X, Xu C, Tang SC, Wang J, Wang H, Wang P*, et al.* Let-7c blocks estrogen-activated Wnt signaling in induction of self-renewal of breast cancer stem cells. Cancer Gene Ther **2016**;23:83-9
50. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. Elife **2015**;4
51. Chen Y, Wang X. miRDB: an online database for prediction of functional microRNA targets. Nucleic Acids Res **2020**;48:D127-D31
52. Borras M, Hardy L, Lempereur F, el Khissiin AH, Legros N, Gol-Winkler R*, et al.* Estradiol-induced down-regulation of estrogen receptor. Effect of various modulators of protein synthesis and expression. J Steroid Biochem Mol Biol **1994**;48:325-36
53. Hatsumi T, Yamamuro Y. Downregulation of estrogen receptor gene expression by exogenous 17beta-estradiol in the mammary glands of lactating mice. Exp Biol Med (Maywood) **2006**;231:311-6
54. Stoica GE, Franke TF, Moroni M, Mueller S, Morgan E, Iann MC*, et al.* Effect of estradiol on estrogen receptor-alpha gene expression and activity can be modulated by the ErbB2/PI 3-K/Akt pathway. Oncogene **2003**;22:7998-8011

55. Blanchard Z, Vahrenkamp JM, Berrett KC, Arnesen S, Gertz J. Estrogen-independent molecular actions of mutant estrogen receptor 1 in endometrial cancer. Genome Res **2019**;29:1429-41
56. Blanchard Z, Arnesen S, Vahrenkamp JM, Rodriguez AC, Jarboe EA, Brown C*, et al.* Allele-specific gene regulation, phenotypes, and therapeutic vulnerabilities in estrogen receptor alpha mutant endometrial cancer. bioRxiv **2022**:2022.06.13.495977
57. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F*, et al.* Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta **2007**;1773:1263-84
58. Bresnick AR, Backer JM. PI3Kbeta-A Versatile Transducer for GPCR, RTK, and Small GTPase Signaling. Endocrinology **2019**;160:536-55
59. Wei N, Chu E, Wipf P, Schmitz JC. Protein kinase d as a potential chemotherapeutic target for colorectal cancer. Mol Cancer Ther **2014**;13:1130-41
60. Borges S, Perez EA, Thompson EA, Radisky DC, Geiger XJ, Storz P. Effective Targeting of Estrogen Receptor-Negative Breast Cancers with the Protein Kinase D Inhibitor CRT0066101. Mol Cancer Ther **2015**;14:1306-16
61. Li QQ, Hsu I, Sanford T, Railkar R, Balaji N, Sourbier C*, et al.* Protein kinase D inhibitor CRT0066101 suppresses bladder cancer growth in vitro and xenografts via blockade of the cell cycle at G2/M. Cell Mol Life Sci **2018**;75:939-63
62. Liu Y, Wang Y, Yu S, Zhou Y, Ma X, Su Q*, et al.* The Role and Mechanism of CRT0066101 as an Effective Drug for Treatment of Triple-Negative Breast Cancer. Cell Physiol Biochem **2019**;52:382-96
63. Sun H, Cao S, Mashl RJ, Mo CK, Zaccaria S, Wendl MC*, et al.* Comprehensive characterization of 536 patient-derived xenograft models prioritizes candidatesfor targeted treatment. Nat Commun **2021**;12:5086
64. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res **2009**;19:92-105
65. Kozomara A, School of Biological Sciences FoB, Medicine and Health, University of Manchester, Manchester M13 9PT, UK, Birgaoanu M, School of Biological Sciences FoB, Medicine and Health, University of Manchester, Manchester M13 9PT, UK, Griffiths-Jones S, School of Biological Sciences FoB, Medicine and Health, University of Manchester, Manchester M13 9PT, UK. miRBase: from microRNA sequences to function. Nucleic Acids Research **2022**;47
66. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods **2015**;12:357-60
67. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N*, et al.* The Sequence Alignment/Map format and SAMtools. Bioinformatics **2009**;25:2078-9
68. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics **2014**;30:923-30
69. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol **2014**;15:550
70. Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV*, et al.* Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics **2013**;14:128