

# ESR1 mutations affect anti-proliferative responses to tamoxifen through enhanced cross-talk with IGF signaling

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**Abstract** The purpose of this study was to address the role of ESR1 hormone-binding mutations in breast cancer. Soft agar anchorage-independent growth assay, Western blot, ERE reporter transactivation assay, proximity ligation assay (PLA), coimmunoprecipitation assay, silencing assay, digital droplet PCR (ddPCR), Kaplan–Meier analysis, and statistical analysis. It is now generally accepted that estrogen receptor (ESR1) mutations occur frequently in metastatic breast cancers; however, we do not yet know how to best treat these patients. We have modeled the three most frequent hormone-binding ESR1 (HBD-ESR1) mutations (Y537N, Y537S, and D538G) using stable lentiviral transduction in human breast cancer cell lines. Effects on growth were examined in response to hormonal and targeted agents, and mutation-specific changes were studied using microarray and Western blot analysis. We determined that the HBD-ESR1 mutations alter anti-proliferative effects to tamoxifen (Tam), due to cell-intrinsic changes in activation of the insulin-like growth factor receptor (IGF1R) signaling pathway and levels of PIK3R1/PIK3R3. The selective estrogen receptor degrader, fulvestrant, significantly reduced the anchorage-independent growth of ESR1 mutant-

expressing cells, while combination treatments with the mTOR inhibitor everolimus, or an inhibitor blocking IGF1R, and the insulin receptor significantly enhanced anti-proliferative responses. Using digital drop (dd) PCR, we identified mutations at high frequencies ranging from 12 % for Y537N, 5 % for Y537S, and 2 % for D538G in archived primary breast tumors from women treated with adjuvant mono-tamoxifen therapy. The HBD-ESR1 mutations were not associated with recurrence-free or overall survival in response in this patient cohort and suggest that knowledge of other cell-intrinsic factors in combination with ESR1 mutation status will be needed determine anti-proliferative responses to Tam.

**Keywords** Estrogen Receptor · Mutations · IGF-1 · Fulvestrant · Everolimus

## Abbreviations

ESR1	Estrogen receptor
HBD-ESR1	Hormone-binding ESR1 mutation
TAM	Tamoxifen
IGF1R	Insulin-like growth factor receptor 1
AI	Aromatase inhibitor
SERD	Selective estrogen receptor degrader
PI3K	Phosphoinositide-3-kinase
SERM	Selective estrogen modulator
IGF-1	Insulin growth factor 1
EGF	Epidermal growth factor
IRS-1	Insulin receptor substrate 1

## Introduction

Adjuvant hormonal therapy targeting the estrogen receptor (ESR1) in human breast cancer has significantly improved overall survival for patients whose tumors express this

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receptor [1], but acquired resistance is a major problem. Several mechanisms of hormone resistance have been identified, including cross-talk with growth factor receptors such as HER2 and IGF1R [2]. Recently, it has been shown that mutations in the hormone-binding domain (HBD) of ESR1 occur frequently in metastatic breast tumor tissues [3–7] and are selected for during aromatase inhibitor (AI) treatment [8].

The first reported HBD-ESR1 mutation, a tyrosine to asparagine missense mutation at amino acid residue 537 (Y537N), was discovered in 1997 by cloning of cDNA from a metastatic lesion and displayed strong constitutive hormone-independent transcriptional activity [9]. A second mutation at lysine 303 (K303R) was reported in both atypical ductal hyperplasias and invasive breast cancers, and was hypersensitive to low levels of estrogen [10, 11]. It was therefore proposed that somatic ESR1 mutations might be acquired during resistance to therapy, which could play important roles in the metastatic spread of breast cancer [12]. However, this hypothesis remained controversial until a large number of metastatic tumors were interrogated using next generation sequencing [3–7]. Many ESR1 mutations appear to occur at a mutational “hot spot” surrounding residues 536–538 of the HBD, shifting the receptor into a constitutive agonist conformation [7] with stabilization of co-activator binding [13]. Investigators have consistently shown that these ESR1 mutations exhibit elevated estrogen-independent transcriptional activity [3–7]. Recently, it was suggested that the combination of constitutive recruitment of co-activators in the absence of hormone and reduced Tam binding affinity might underlie therapy resistance conferred by the Y537S and D538G ESR1 mutations [13]. HBD-ESR1 mutations predict reduced sensitivity to subsequent aromatase AI therapy in the metastatic setting [8].

The selective estrogen receptor degrader (SERD) fulvestrant is effective in ER-positive patients progressing on hormonal therapy [14]. FERG trial investigators reported that the presence of HBD-ESR1 mutations did not predict resistance to fulvestrant [15]. The BOLERO-2 trial demonstrated a 50 % reduction in progression when the mTOR inhibitor everolimus was combined with exemestane [16], and a subsequent correlative analysis reported that patients with certain of the ESR1 mutations might benefit from this combination as well [17].

Although the selective estrogen receptor modulator (SERM) Tam can block estrogen-stimulated ESR1 mutant transcriptional activity [3–7], it has not been shown whether Tam will block the growth of ESR1 mutant-expressing cells in patients. Herein, we report a preclinical study showing that sensitivity to the anti-proliferative effects of Tam in some ER-positive cells expressing the HBD hotspot

ESR1 mutations is dependent on cross-talk with the IGF1R signaling pathway.

## Results

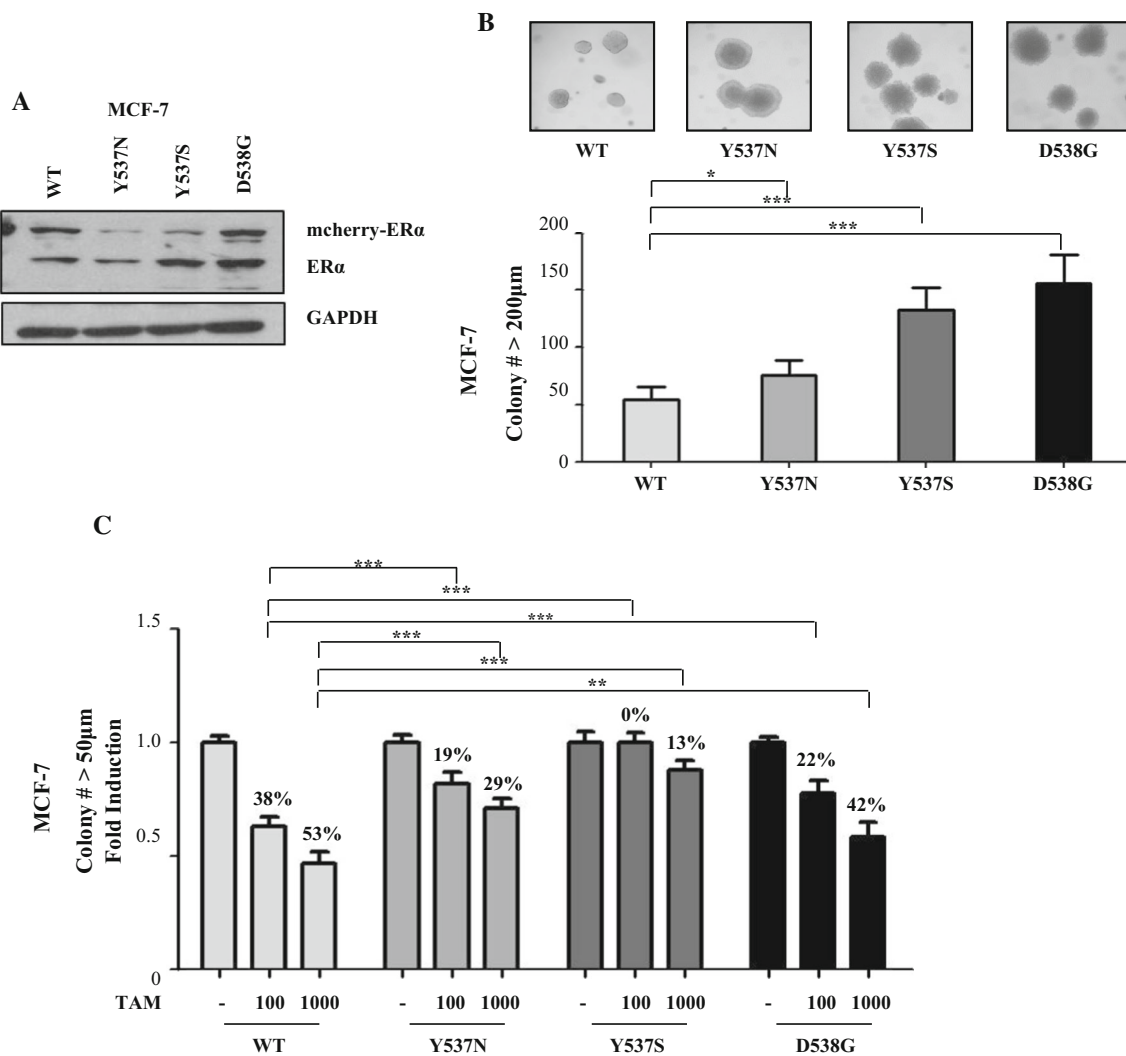
### HBD-ESR1 mutations modulate anti-proliferative responses to Tam depending on cellular background

We established stable pools of ER-positive MCF-7 cells expressing WT ESR1, or HBD mutants Y537N, Y537S, or D538G. Endogenous ER $\alpha$  (66 kDa) and an m-cherry-tagged exogenous receptor (~95 kDa) were visualized using immunoblot analysis (Fig. 1a), and growth responses were determined. The most noticeable difference was the change in shape and larger size of the ESR1 mutant colonies under control conditions (–). Control growth of all mutant-expressing colonies was significantly enhanced (Fig. 1b), and HBD-ESR1 mutant-expressing cells exhibited reduced anti-proliferative responses to Tam (Fig. 1c). MCF-7 Y537S ER-expressing cells consistently exhibited the least Tam-responsive phenotype. Similar data were obtained using another MCF-7 subline (designated BK) maintained separately in the laboratory for over 25 years (Fig. S1a, b) and in another ER-positive cell line (HCC1428) (Fig. S1c, d). Of note, the overall mutation rate (genomic divergence) between the MCF-7 sublines is substantial. Typically breast cancer tumors contain between 50 and 200 mutations, while the two sublines differ by 451 nonsynonymous variants, they may represent a common founder but have separately evolved during subclonal passaging (data not shown, manuscript in preparation).

We generated ESR1 mutant-expressing pools using two other ER-positive breast cancer cell lines, ZR-75B, and T47D (Fig. S1e–h). Both of these models express high mutant levels. ZR-75B and T47D mutant cells responded to Tam treatment equivalent to WT-expressing cells, and colony size was not affected (data not shown). These results indicate that individual anti-proliferative responses to Tam may depend on cellular background and that other tumor cell-intrinsic factors may be associated with response in cells expressing ESR1 mutants.

### Activation of IGF-1 signaling in HBD-ESR1 mutants is associated with Tam response

Since response to Tam requires many cell-specific effectors, we next employed expression microarray profiling, three-way ANOVA, and canonical pathway analysis using Ingenuity to identify pathways significantly associated with ESR1 mutant expression in MCF-7 compared to ZR-75B cells (Fig. 2a); darker orange bars are pathways most



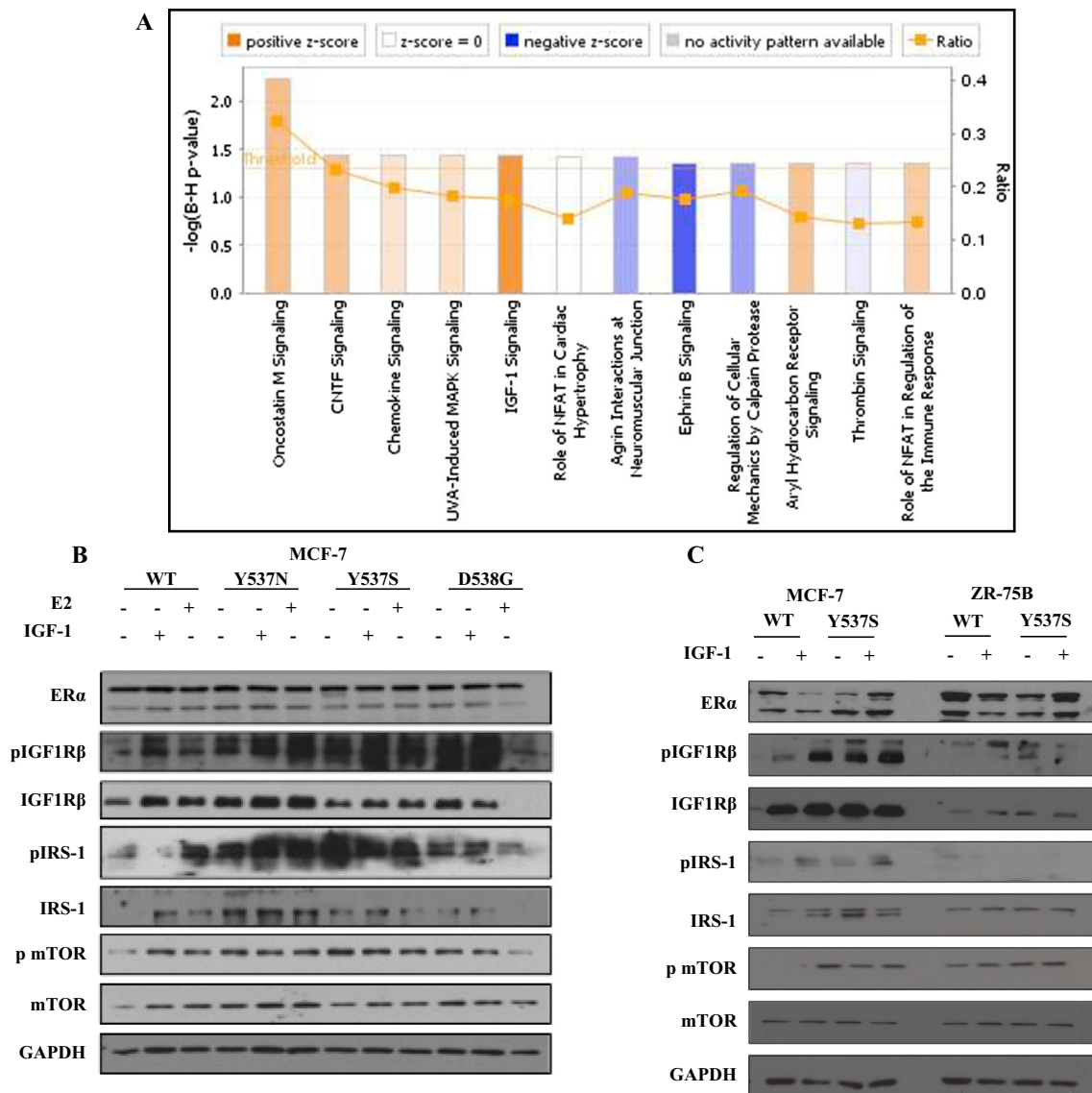
**Fig. 1** HBD-ESR1 mutations influence Tam response. **a** Immunoblot analysis to detect ER $\alpha$  expression in MCF-7 stable clones. GAPDH was used as loading control. **b** Cells were plated for soft agar assays. *Upper*, representative photographs of three different well plates captured from soft agar assays showing higher number of large colonies (200  $\mu$ m) in mutant-expressing cells, *lower*, graphs showing the number of the colonies bigger than 200  $\mu$ m. Experiments were

performed in triplicate and *error bars* indicate SD. \* $P < 0.05$ ; \*\*\* $P < 0.001$ . **c** Cells were plated for soft agar assays, and then treated with vehicle or two doses of Tam (100 nM or 1000 nM). Percentage of growth reduction with Tam treatment is shown. Experiments were performed in triplicate and *error bars* indicate SD. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

activated in MCF-7 mutant cells, and blue bars are deactivated pathways. The most significantly activated pathway was insulin growth factor-1 (IGF-1) signaling. A known mechanism of hormone resistance is enhanced cross-talk between ER and growth factor receptors, which can feedback and impact ER transcriptional activity. High levels of phosphorylated IGF1R $\beta$  and phosphorylated insulin receptor substrate-1 (pIRS-1) were seen after IGF-1 treatment of MCF-7 mutant cells (Fig. 2b). Estrogen activation of IGF signaling served as a positive control in this experiment [18]. We did not detect enhanced IGF1R $\beta$  or IRS-1 phosphorylation in ZR-75B Y537S cells (Fig. 2c). Thus, activated IGF-1 signaling was specifically associated with mutant expression in MCF-7 cells exhibiting reduced

anti-proliferative effects with Tam, implying that cross-talk with this signaling pathway may be involved as a proliferative escape mechanism in some cellular backgrounds.

We used the IGF1R $\beta$  inhibitor GSK1838705A in transcriptional assays. Mutant ER transcriptional activity was efficiently blocked by Tam treatment (Fig. 3a). The combination of Tam with GSK further enhanced the ability of Tam to block transcriptional activity, demonstrating functional ER cross-talk with IGF1R. We found that GSK treatment enhanced growth sensitivity to Tam in all cells (Fig. 3b). Of note, the growth of Y537S-ESR1 cells was significantly stimulated by Tam treatment in this experiment, and the IGF1R $\beta$  inhibitor was able to block this agonist growth.



**Fig. 2** IGF-1 signaling pathway activation in HBD-ESR1 mutants. **a** Ingenuity pathway analysis (IPA) to identify activation of signaling pathways in mutant MCF-7 versus ZR-75B. **b** Total cellular extracts were analyzed for phosphorylation and expression of ER, IGF1R $\beta$ , IRS-1, and mTOR; GAPDH was used as a loading control.

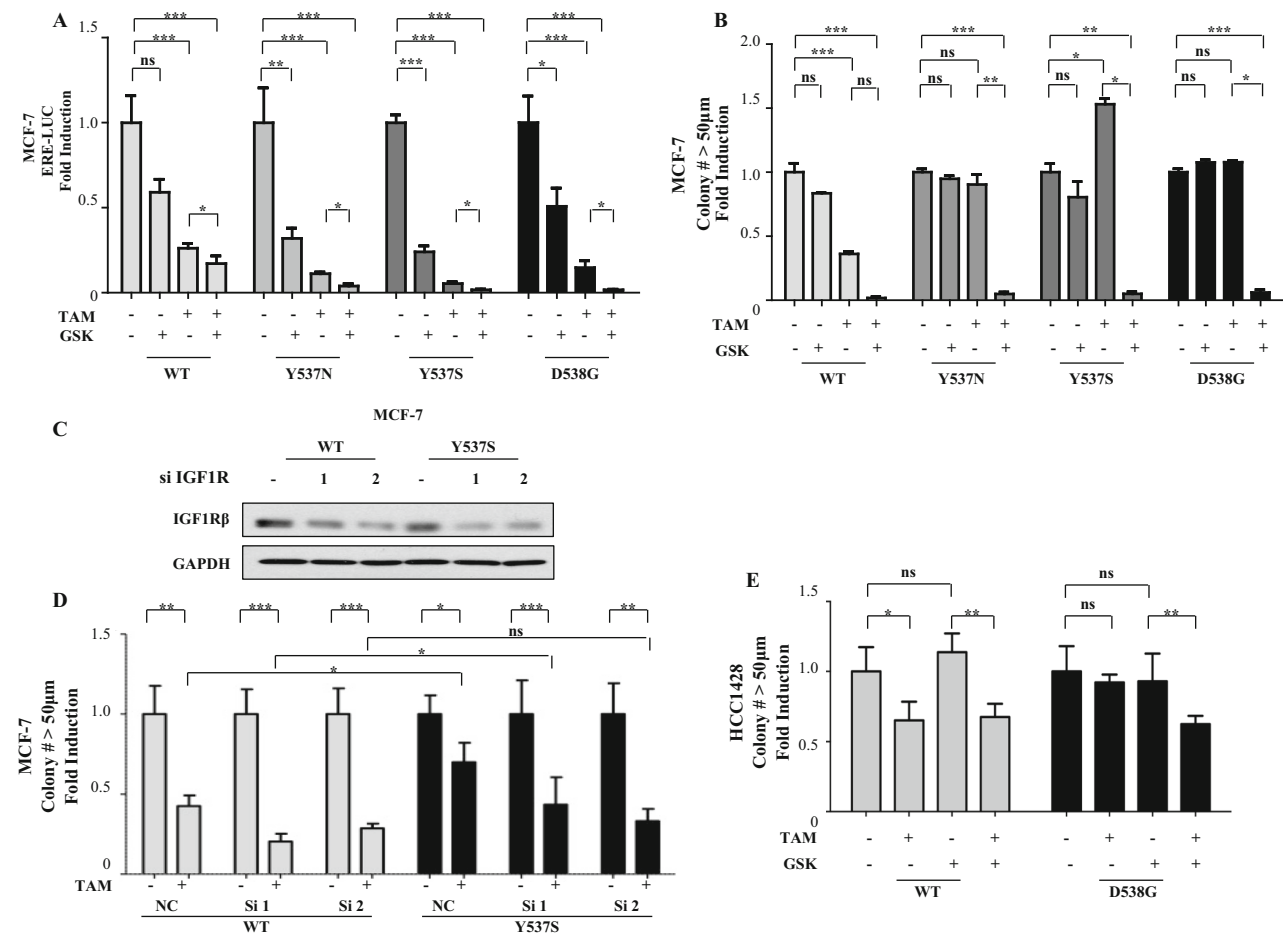
Immunoblots show a representative example of three experiments. **c** Total cellular extracts were analyzed for phosphorylation and expression of ER $\alpha$ , IGF1R $\beta$ , IRS-1, and mTOR; GAPDH was used as a loading control. Immunoblots show a representative example of three different experiments

We performed knockdown of IGF1R $\beta$  levels using two siRNAs to confirm these results, and knockdown enhanced Tam's anti-proliferative effects (Fig. 3c, d). We further confirmed these data in HCC1428 cells (Fig. 3e). These results suggest that specific blockade of IGF1R or other downstream signaling molecules is required, along with hormonal therapy, in some mutant-expressing tumors.

### Enhanced interactions between IGF1R and the Y537S ESR1 mutant

The MCF-7 Y537S model was examined for protein interactions between IGF1R $\beta$  and ER using immunoprecipitation

coupled with immunoblot analysis. Input extracts confirmed higher levels of IGF1R $\beta$  protein in MCF-7 mutant, but not in ZR-75B mutant cells; all cells were able to induce pIRS-1 with IGF-1 treatment (Fig. 4a). Higher constitutive binding was observed between the Y537S mutant and IGF1R $\beta$  in MCF-7 (Fig. 4b), but not in ZR-75B cells (Fig. 4c). This suggests that although all cells can express pIRS-1, there were diminished anti-proliferative effects in MCF-7 cells with elevated constitutive pIGF1R $\beta$  levels. We also performed proximity ligation assays in MCF-7 and ZR-75B cells (Fig. 4d). Prominent interactions (red speckled staining, arrows) were seen in mutant-expressing cells compared to WT, but only in the MCF-7 cells. We conclude that the role



**Fig. 3** Specific blockade of the IGF1R pathway restores anti-proliferative effects of Tam in mutant-expressing cells. **a** ERE-luc assays in MCF-7 cells transiently transfected with WT-, Y537N-, Y537S-, or D538G-ER plasmids. Experiments were performed in triplicate and error bars indicate SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . **b** MCF-7 cells were plated for soft agar assays, and then treated with vehicle or Tam (100 nM) or GSK (1  $\mu$ M). Experiments were performed in triplicate and error bars indicate SD. ns, not significant. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . **c**, **d**

immunoblot analysis to detect IGF1R $\beta$  protein expression after transfection with two different IGF1R $\beta$  siRNAs; GAPDH was used as a loading control (**c**), same cells were plated for soft agar assay and then treated with vehicle (–) or with Tam (100 nM) (**d**). Experiments were performed in triplicate and error bars indicate SD. ns, not significant, \* $P < 0.05$ . **e** HCC1428 cells were plated for soft agar assays, and then treated with vehicle or Tam (100 nM) or GSK (1  $\mu$ M). Experiments were performed in triplicate and error bars indicate SD. ns, not significant. \* $P < 0.05$ ; \*\* $P < 0.01$

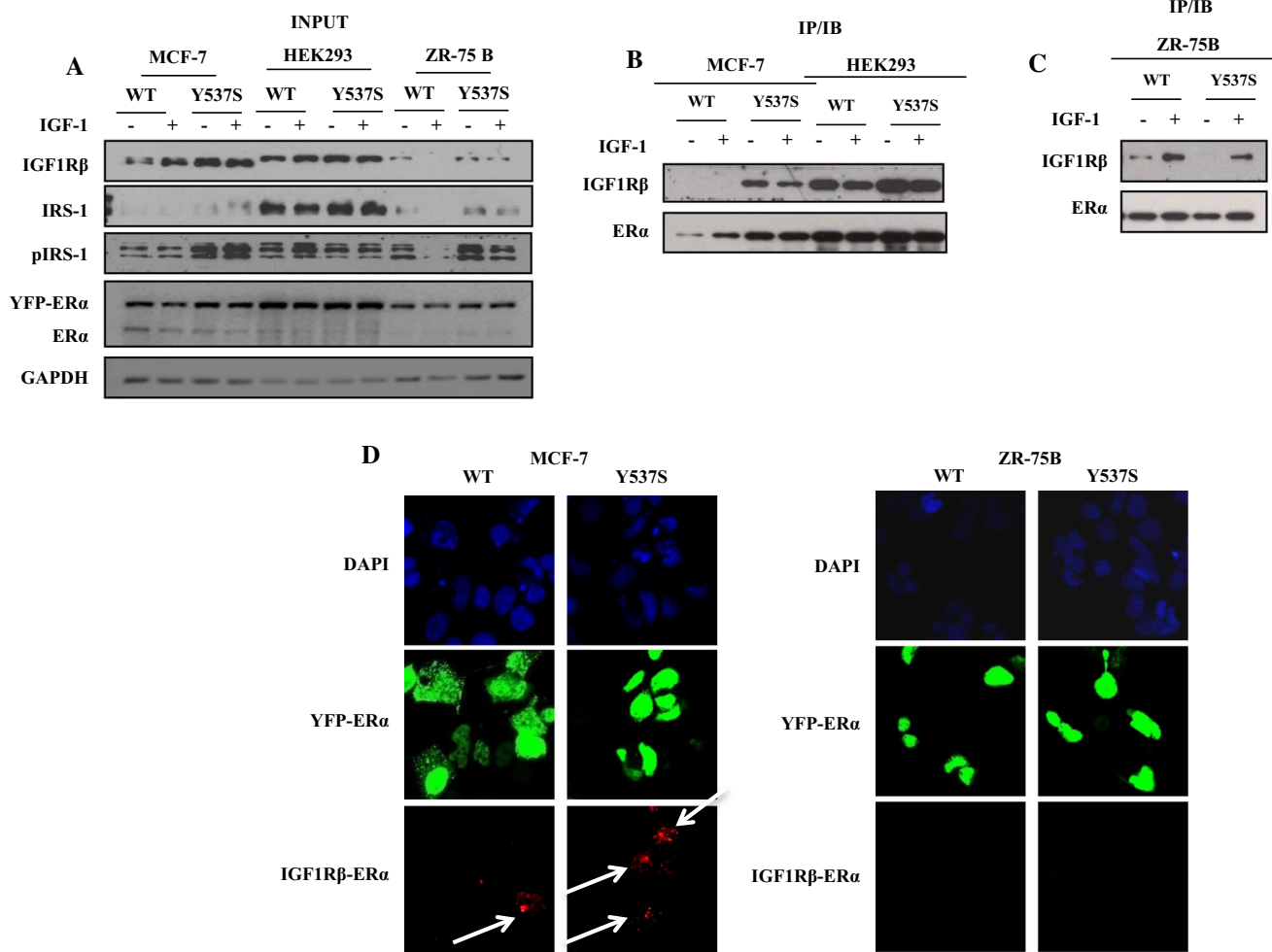
of ESR1 mutations in Tam response may be dependent on a cellular background with elevated pIGF1R.

### Everolimus and fulvestrant are effective at reducing growth of stable HBD-ESR1 mutant-expressing cells

Although a number of inhibitors targeting the IGF1R growth pathway have entered clinical trials, they have proven disappointing in randomized clinical trials [19]. IGF1R activates the PI3K/AKT/mTOR (mammalian target of rapamycin) signaling transduction pathways, which can drive resistance in ER-positive patients [20]. mTOR regulates proteins involved in growth and proliferation [21], and the mTORC1 inhibitor everolimus is approved for the

treatment of hormone-resistant breast cancer [16]. Since there is correlative data suggesting that patients with some of the ESR1 mutants can indeed respond to fulvestrant or exemestane [15, 17], we next tested these combinations. Everolimus treatment reduced downstream pS6 levels, and combination treatments with Tam reduced both mTOR and pS6 phosphorylation in Y537S mutant cells (Fig. 5a). The IGF1R inhibitor GSK in combination with Tam effectively reduced IGF1R $\beta$  and p-mTOR levels.

Fulvestrant, alone or in combination with a steroidal AI, is used after treatment has failed with a nonsteroidal aromatase inhibitor [22]. Everolimus in combination with fulvestrant has shown significant efficacy in Phase II studies in heavily pretreated metastatic ER-positive breast cancer patients



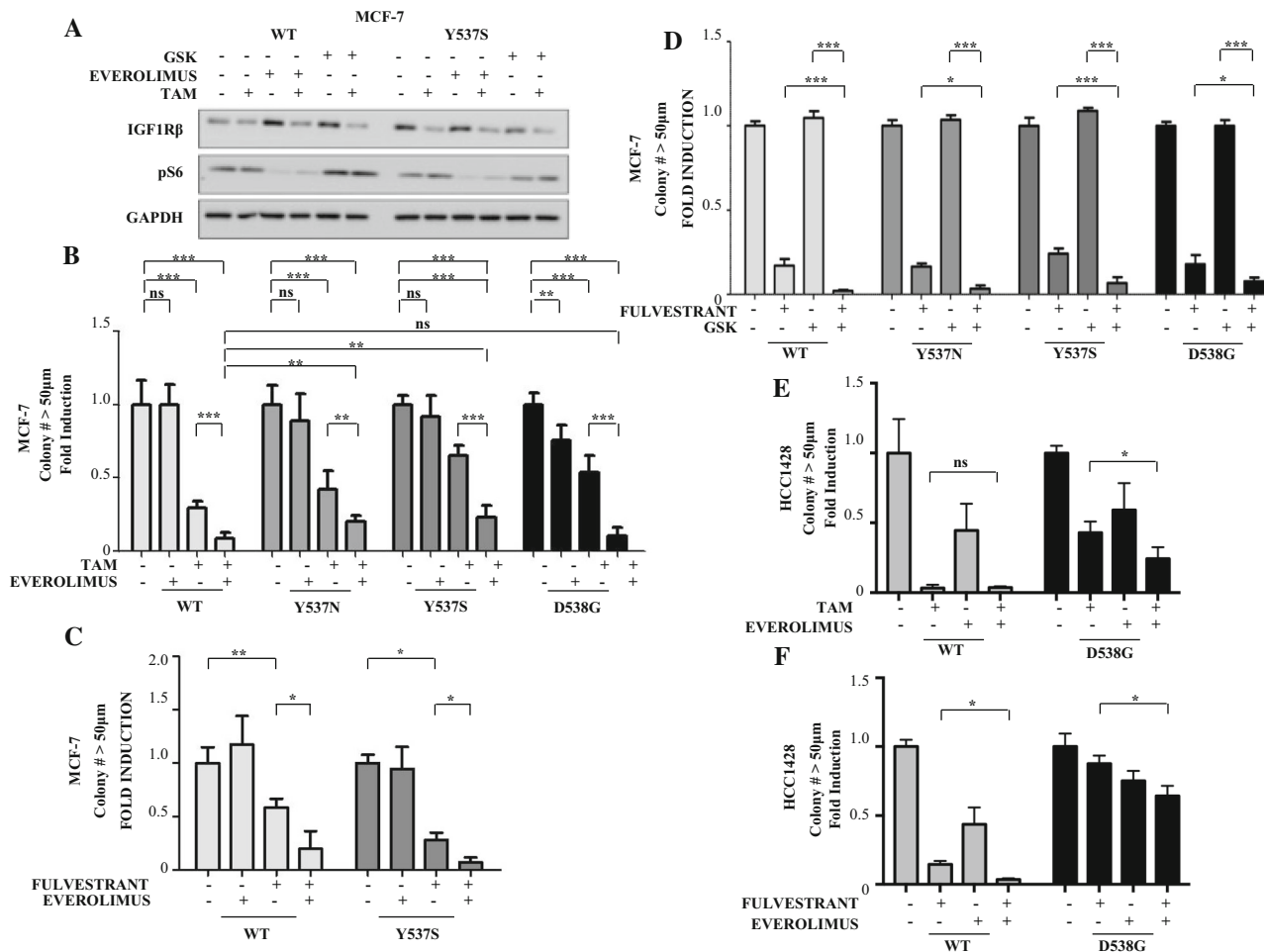
**Fig. 4** MCF-7 Y537S cells show the lowest anti-proliferative response to Tam through increased interaction between IGF1R/ER. **a–c** immunoblot analysis to detect expression of IGF1Rβ, pIRS-1, IRS-1, and ERα in input extracts; GAPDH was used as a loading

control (**a**). Coimmunoprecipitation assays of IGF1Rβ/ERα interactions (**b**, **c**). **d** duolink staining (*red*) to detect IGF1Rβ and ERα interaction in MCF-7 and ZR-75B cells. Images are representative of three different experiments. (Color figure online)

[23]. Everolimus treatment alone exhibited minimal effects on cellular proliferation, but in combination with Tam enhanced anti-proliferative effects in mutant-expressing cells (Fig. 5b). Fulvestrant treatment alone blocked growth and in combination with everolimus, inhibited growth of the Y537S ESR1 mutant (Fig. 5c). Fulvestrant plus GSK was effective at reducing anchorage-independent growth of ESR1 mutations (Fig. 5d). Similar results were seen in the HCC1428 cells (Fig. 5e, f) and in the MCF-7 BK subline (Fig. S2). Y537S and D538G-expressing cells demonstrated enhanced response to IGF treatment in wound-healing scratch motility assays, and GSK blocked IGF-induced motility (Fig. S3). Collectively these data demonstrate that everolimus/fulvestrant is an effective combination for the Y537S mutant and suggest that fulvestrant plus strategies to completely block IGF1R signaling [24] should be explored in the metastatic setting in ESR1 mutant patients.

### HBD-ESR1 mutations in primary tumors do not significantly impact outcomes in Tam-treated patients

Based on the low frequency of ESR1 mutations using next generation sequencing [7], it was concluded that they must develop as a consequence of treatment selection, perhaps via emergence from an undetectable subclonal mutant population [8]. Using sensitive ddPCR assays, Takeshita et al. reported that 2.5 % of primary breast cancer specimens contained ESR1 mutations, with the Y537S mutation being the most frequent [25]. To address, whether ESR1 mutations might predict response to Tam in primary breast cancers, we used ddPCR sequencing of archived DNAs from 203 primary tumors treated with Tam monotherapy [11]. Positive ESR1 mutation status was associated with smaller tumor size and progesterone receptor (PR) negativity (Supplementary Table 1). Mutations were found at



**Fig. 5** Fulvestrant in combination with everolimus or GSK enhances anti-proliferative effects in mutant-expressing cells. **a** immunoblot analysis to detect IGF1R $\beta$  and pS6 levels; GAPDH was used as a loading control. Immunoblots show a representative example of three different experiments. **b** MCF-7 cells were plated in soft agar assay and then treated with vehicle (–), Tam (100 nM), or everolimus (1 nM). Experiments were performed in triplicate and *error bars* indicate SD. ns, not significant. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . **c** MCF-7 cells were plated in soft agar assay and then treated with vehicle (–) or with fulvestrant (100 nM) and everolimus (1 nM). Experiments were performed in triplicate and

*error bars* indicate SD. \* $P < 0.05$ . **d** MCF-7 cells were plated in soft agar assay and treated with vehicle (–), fulvestrant (100 nM), and/or GSK (1  $\mu$ M). Experiments were performed in triplicate and *error bars* indicate SD. \* $P < 0.05$ , \*\*\* $P < 0.001$ . **e** HCC1428 cells were plated in soft agar assay and then treated with vehicle (–), Tam (100 nM), or everolimus (1 nM). Experiments were performed in triplicate and *error bars* indicate SD. ns, not significant. \* $P < 0.05$ . **f** HCC1428 cells were plated in soft agar assay and treated with vehicle (–), fulvestrant (100 nM), and/or GSK (1  $\mu$ M). Experiments were performed in triplicate and *error bars* indicate SD. \* $P < 0.05$

relatively high frequencies using a sensitive cut-off in this cohort, ranging from 12 % for Y537N, 5 % for Y537S, and 2 % for D538G (Table 1). However, allele frequencies were low in primary tumors and are shown as the percent of mutant compared to WT ESR1 (Fig. 6a), suggesting that they indeed represent a minor subpopulation within the primary tumor. We assessed the clinical impact of ESR1 mutations on outcomes in this cohort, and for patients with any (Y537N/S or D538G) mutation, patients with an ESR1 mutant exhibited slightly improved recurrence-free survival [(RFS), Fig. 6b,  $P = 0.0118$ ], but no difference was seen in overall survival (OS, Fig. 6c). Effects of individual mutations on RFS or OS are shown in Supplementary

Fig. S4. Thus the HBD-ESR1 mutations were not significantly associated with resistance to Tam as predicted by preclinical studies. These results suggest that measurement of ESR1 mutation status as a single biomarker may not provide significant information in primary breast cancers treated with Tam.

#### Knockdown of PI3K regulatory units PIK3R1 and PIK3R3 enhances anti-proliferative effects of Tam

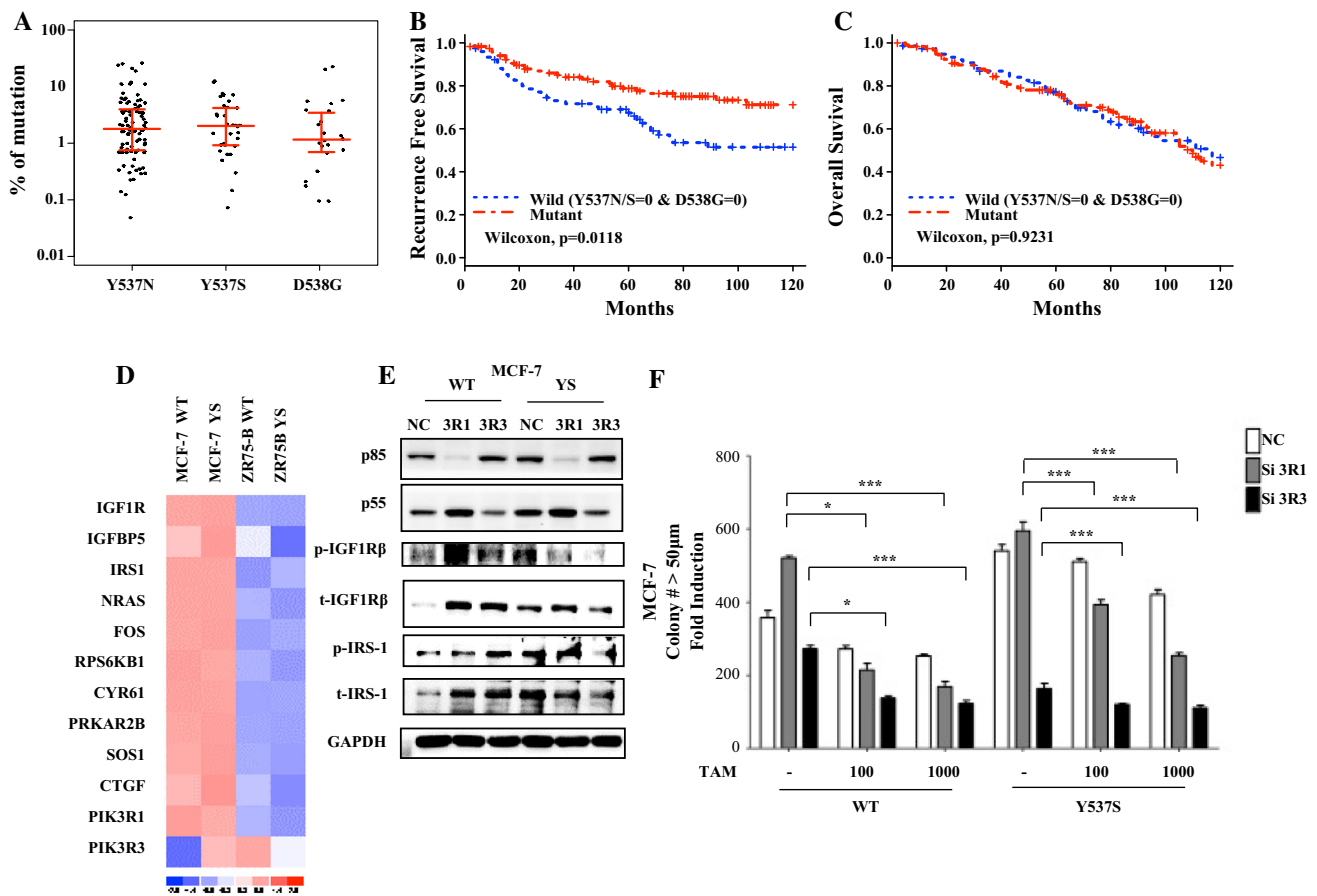
Undoubtedly, primary breast cancers are heterogeneous, and a combination of molecular alterations will define the

**Table 1** Mutation frequency in invasive breast tumors treated with Tam

Mutation	Frequency (%)	Number of tumors
Y537N	12	74/195
Y537S	5	29/199
D538G	2	4/195

functional consequences determining therapeutic sensitivity to hormone therapy. To explore other cell-intrinsic effectors of Tam response in mutant cells, we examined the biology of two of the differentially expressed IGF-1 pathway genes identified using comparative microarray analysis of WT and the Y537S mutant in MCF-7 versus ZR-75B cells (Fig. 6d). The data showed up-regulated IGF-1 pathway members, the PI3K regulatory subunits 1 and 3 (PIK3R1 and PIK3R3). PIK3R1 and 3 can also serve as adaptors for cellular signaling. PIK3R3 physically interacts

with IGF1R and impact signaling [26], and naturally occurring mutations in PIK3R1 activate PI3K/MAPK signaling and dictate sensitivity to MAPK inhibitors [27]. Since RNA levels of both PIK3R1 and 3 were elevated in MCF-7 but not ZR-75B Y537S mutant-expressing cells (Fig. 6d), we used siRNAs for selective knockdown of the 3R1 (p85) and 3R3 (p55) subunits and examined effects on signaling and Tam response. Knockdown of PIK3R3 exhibited the greatest effect on reducing pIGF1R $\beta$  levels in mutant cells and affected proliferation of both WT and mutant cells (Fig. 6e, f). Knockdown of both regulatory subunits potentiated the anti-proliferative effects of Tam, especially in mutant-expressing cells. Thus, altered PI3K regulatory subunit expression may be cell-intrinsic factors associated with Tam response in breast cancer, especially in patients expressing ESR1 mutants. These observations will require validation in clinical material from ESR1 mutant-positive patients.



**Fig. 6** ESR1 mutations do not predict outcomes in patient treated with adjuvant Tam. **a** ddPCR analysis showing allele frequencies of three HBD-ESR1 mutations in primary breast cancers. **b**, **c** Kaplan–Meier analysis of RFS (**b**), and OS (**c**). **d** heatmaps of mRNA levels of genes involved in IGF-1 signaling pathway comparing MCF-7 and ZR-75B stable clones. **e** Immunoblot analysis to detect

phosphorylation and total protein expression of IGF1R $\beta$ , pIRS-1, p85, and p55; GAPDH was used as a loading control. **f** Cells were also plated in soft agar assays and treated with vehicle (–) or with Tam (10 and 100 nM). Experiments were performed in triplicate and error bars indicate SD. ns, not significant. \* $P < 0.05$ ; \*\*\* $P < 0.001$



## Discussion

The “rediscovery” of ESR1 mutations in metastatic breast cancer has reopened a number of important clinical questions about their role in acquired resistance to hormonal agents [28]. The most important question of course is whether the mutations are targetable? It is well demonstrated that ER transcriptional activity associated with mutations in the HBD is hormone-independent and that the mutations display different transcriptional responses to Tam and fulvestrant [4–7]. Molecular modeling of the Y537S and D538G mutations show that they shift the receptor into an agonist conformation, which may account for their elevated basal transcriptional activity [7], and recently, it has been shown these two mutations stabilize binding to co-activators and reduce binding affinity for Tam [13]. Using stable expression, we demonstrate that the mutations reduce the absolute anti-proliferative effects of Tam depending on the cellular background, and Tam is an effective agent to reduce anchorage-independent growth.

We employed microarray analysis to identify cell-intrinsic mediators associated with reduced Tam effects in MCF-7 compared to ZR-75B cells, and activation of the IGF-1 pathway was the most significant differentially expressed pathway in ingenuity analyses. Our data suggest that the activation of IGF1R $\beta$  was a key determinant of Tam response and was driven by cross-talk with the HBD-ESR1 mutations since enhanced binding between the Y537S ESR1 mutant and IGF1R $\beta$  was detected. Similarly, increased binding and cross-talk between IGF1R $\beta$ /HER2 and the K303R ESR1 mutation has previously been reported [29, 30]. Enhanced cross-talk between WT ER and these growth factor receptors is a well-studied mechanism of hormone resistance in breast cancer [31, 32]. In ZR-75B cells expressing the HBD-ESR1 mutations Y537 N, Y537S, and D538G where differences in anti-proliferative effects to Tam were not observed, IGF-1 signaling was similarly not engaged. Thus, the ESR1 mutations may employ a common mechanism via cross-talk with the IGF1R pathway to escape hormone therapy in some cellular backgrounds.

Although previous preclinical studies have suggested that HBD-ESR1 mutants may be relatively resistant to the SERD fulvestrant [4, 5, 33], in this study, fulvestrant was very effective alone, or in combination with either an IGF1R or mTOR-targeting agent. It has been shown that the oral SERD AZD9496 inhibited the growth of a patient-derived xenograft (PDX) harboring the D538G mutation [34], and the SERM/SERD hybrid piperidoxifen inhibited the growth of a PDX with the Y537S mutation [35]. An early report suggests that ESR1 mutations are not associated with resistance to fulvestrant [34]. This strongly

supports the use of fulvestrant in metastatic patients harboring ESR1 mutations.

Another important question is which component of the activated signaling cascade observed in ESR1 mutant tumors might be the optimal target for combination therapy with fulvestrant? Fulvestrant or exemestane combined with IGF1R inhibition was unsuccessful in a phase II trial, which effectively halted the use of hormonal therapies combined with an IGF1R inhibitor [36]. Our results showed fulvestrant combined with the GSK inhibitor was effective in ESR1 mutant cells with activated IGF-1 signaling. The assessment of an IGF-1 molecular signature and alternate IGF1R modulators, such as PIK3R3 also mediating IGF1R escape, within the context of a clinical trial, might improve patient selection for combined hormonal/IGF1R strategies in ESR1 mutant metastatic patients.

Preclinical studies have shown that inhibition of the PI3K/AKT/mTOR pathway can restore hormone sensitivity [37–39]. We show that this pathway is activated in ESR1 mutant-expressing cells, and everolimus in combination with either fulvestrant or Tam exhibited significant anti-proliferative effects in cells expressing the ESR1 mutations. Preliminary results from the BOLERO-2 trial suggest that metastatic patients harboring the D538G, but not the Y537S mutation respond to exemestane plus everolimus [17]. A small phase II study of fulvestrant and everolimus in metastatic patients after AI treatment failure showed some efficacy in delaying fulvestrant resistance, and another phase II study of everolimus in combination with Tam also showed clinical benefit in AI-resistant metastatic patients [40], warranting study of these combination in patients with ESR1 mutations. Elevated expression of PIK3R3 is reported to be a biomarker predicting for response to everolimus [41]. An important challenge of mTOR inhibition, however, is signaling feedback via elevated activity of growth factor receptors, providing alternative survival pathways to evade therapy [42]. We speculate that this could become problematic in the treatment of ESR1 mutant tumors with constitutively activated pIGF1R.

A recent report from Takeshita et al. using ddPCR reported a frequency of 2.5 % in primary breast cancer [43]. Analysis of cell-free tumor DNA reports ESR1 mutant frequencies at almost 40 % in metastatic patient [17]. We used a sensitive ddPCR technique to detect the three HBD-ESR1 mutations and report high frequencies in primary tumors. The low allele frequency of the ESR1 mutations in our cohort suggests that the mutations exist in only a minor subpopulation of the primary tumor. Using single cell sequencing, it was demonstrated that mutations in breast cancer occur at low frequencies and evolve

gradually over time [44]. In a small series of patients monitored for circulating tumor DNA, ESR1 mutations are rarely selected during adjuvant AI therapy, but are selected for during AI therapy for metastatic disease. Since AI therapy might provide a selective advantage to the HBD-ESR1 mutations, our data suggest that the use of targeted ESR1 mutation sequencing might be warranted earlier in patients with advanced breast cancer. Since the ESR1 mutations might also confer enhanced metastatic potential and/or aggressive biological attributes, testing of this possibility in archived material from adjuvant studies is warranted.

## Materials and methods

### Reagents, hormones, and antibodies

4-OH-tamoxifen (Tam), 17 $\beta$ -estradiol, insulin growth factor-1 (IGF-1) and puromycin were purchased from Sigma (St. Louis, MO). GSK1838705A (GSK), and everolimus were obtained from Selleck Chemicals (Houston, TX). MEM, RPMI 1640, DMEM, L-glutamine, penicillin/streptomycin, MEM non-essential amino acids, and SeaPlaque<sup>TM</sup> Agarose were from Lonza (Walkersville, MD). Fetal bovine serum was obtained from Gemini Bio Products (West Sacramento, CA). SuperScript III reverse Transcriptase, qPCR probes (IGF1R $\beta$  and GAPDH), and lipofectamine LTX was provided by Life Technologies (Grand Island, NY). Antibodies were ER $\alpha$  (Vector Laboratories, Burlingame, CA); total IGF1R, IRS-1, mTOR, phosphorylated IGF1R<sup>(Tyr1131)</sup>, mTOR<sup>(Ser2448)</sup>, pS6<sup>(Ser240, 244)</sup>, p85 and p55 (Cell signaling Technology, Beverly, MA); IRS-1<sup>(Tyr612)</sup> (Invitrogen, Carlsbad, CA); and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Goat anti-mouse and anti-rabbit secondary antibodies were from Amersham Biosciences (Piscataway, NJ). The Renilla Luciferase assay kit was from Promega (Madison, WI). GFP-nAB beads were from Allele Biotechnology (San Diego, CA).

### Plasmids

The ER constructs were generated using QuickChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA) in LV111-m-Cherry-ER $\alpha$  (purchased from Genecopoeia, Rockville, MD) and in pYFP-ER $\alpha$ . The primer sequences were Y537N, 5'-cgtggtgccctcaatgacctgctgctggag-3'; Y537S, 5'-cgtggtgccctctctgacctgctgctggag-3'; D538G, 5'-cgtggtgccctctatggcctgctgctggag-3'. Sequences were verified using Sanger Sequencing. Empty vector (EV), IGF1R siRNAs, PIK3R1, and PIK3R3 siRNAs were from Addgene (Cambridge, MA).

### Cell culture and stable transfection

MCF-7 and MCF-7 BK cells were grown in MEM; T47D, HCC1428, and ZR-75B cells (generously obtained from Dr. Marc Lippman) in RPMI-1640, HEK293 in DMEM without sodium pyruvate. All stable clones were maintained in puromycin (1  $\mu$ g/ $\mu$ l). Transduced cells were generated as previously described [45]. A growth disadvantage was observed during selection of mutant ER $\alpha$ , especially with the Y537N and Y537S clones, and only the D538G stable clone could be obtained in HCC1428 cells.

### Transfection assays

Transfection for ERE-luciferase assays, siRNAs, immunoblots, coimmunoprecipitations, and PLA assays was performed using the lipofectamine reagent as recommended by the manufacturer.

### Proximity ligation assays

Proximity ligation assays was performed using Duolink detection kits (Sigma-Aldrich, St. Louis, MO) as recommended by the manufacturer. Fluorescence was detected using a Leica TCS SP5 (Leica Microsystems, Buffalo Grove, IL) and Leica Suite Software (LAS, Wetzlar, Germany).

### Cell extraction, immunoblot, and coimmunoprecipitation analysis

Cells were cultured in regular media or starved in 5 % charcoal-stripped serum media for 24 h and treated with IGF-1 50 ng/ml for 15' and E2 100 nM for 2 h, GSK 1  $\mu$ M, everolimus 100 nM and Tam 100 nM for 5 days, or IGF-1 50 ng/ml for 2 h for coimmunoprecipitation analysis. Immunoblot analyses were performed as previously described [45]. Coimmunoprecipitation analyses were performed using GFP-nAb beads as recommended by the manufacturer.

### Cell proliferation assays

Cell proliferation was measured using a soft agar anchorage-independent assay as previously described [45].

### Expression microarray analysis

Cells were plated in media for 24 h and then treated for 24 h with Tam 100 nM. RNA was extracted using the RNeasy micro-kit (Qiagen, Valencia, CA). Labeled cRNA was hybridized onto Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix Inc. Santa Clara,

CA) in triplicate. Expression values were estimated using RMA with Partek software (<http://partek.com/>). Three-way ANOVA with contrasts were run using Partek. Differentially expressed genes with FDR (Fold Discovery Rate) = 0.01 and fold = 3 were used for Ingenuity Pathway Analysis (<http://www.ingenuity.com/>).

### Wound-healing scratch assays

Cell monolayers were scraped and wound closure monitored over 12–24 h, cells fixed, and stained with Coomassie brilliant blue. Photomicrographs were at  $\times 10$  magnification using phase-contrast. The rate of wound healing was quantified using Scion Image Program.

### Mutation detection using ddPCR

DNA was isolated from archived, formalin fixed paraffin-embedded patient samples, and amplified using a QX100 ddPCR system. We used invasive breast cancers obtained from women in the United States and maintained in an archived tumor bank. Patients were diagnosed between 1973 and 1993 and treated with mastectomy or lumpectomy plus axillary dissection, with or without postoperative radiation therapy. All patients had been treated with adjuvant Tamoxifen monotherapy [11]. The fraction of positive droplets determined the concentration of the target molecules in the sample; any drop was considered positive. Samples were analyzed by fractional abundance (molecules of mutation/molecules of WT). Specific assays for ESR1 Y537N, Y537S, or D538G were designed and optimized using MCF-7 stable expressing cells. FAM was used for Y537S, Y537N, and D538G mutations, and HEX for wild type.

Sequences for primers:

Y537 N WT: TACAGCATGAAGTGCAAGAACGTG GTGCCCTCTATGACCTGCTGCTGGAGATGCTGGACG; Y537 N Mutant: GTACAGCATGAAGTGCAAGAA CGTGGTGCCCTCAATGACCTGCTGCTGGAGATGCTGGACG; Y537S WT: GTACAGCATGAAGTGCAAGA ACGTGGTGCCCTCTATGACCTGCTGCTGGAGATGCTGGACGCCC; Y537S Mutant: TACAGCATGAAGTG CAAGAACGTGGTGCCCTCTCTGACCTGCTGCTGG AGATGCTGGACGCCC; D538G WT: GTACAGCATGA AGTGCAAGAACGTGGTGCCCTCTATGACCTGCTGCTGGAGATGCTGGACGCCC; D538G Mutant: GTA CAGCATGAAGTGCAAGAACGTGGTGCCCTCTAT GGCCTGCTGCTGGAGATGCTGGACGCCC.

### Statistical analyses

Data were analyzed by student's *t* test using GraphPAD Prism5 software (GraphPad Software, Inc. San Diego, CA). Standard deviations (SD) are shown.

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### Compliance with ethical standards

**Conflict of interest** Authors have nothing to disclose.

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