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ORIGINAL ARTICLE

Crosstalk between PKCα and Notch-4 in endocrine-resistant breast cancer cells

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The Notch pathway is functionally important in breast cancer. Notch-1 has been reported to maintain an estrogen-independent phenotype in estrogen receptor α (ERα) + breast cancer cells. Notch-4 expression correlates with Ki67. Notch-4 also plays a key role in breast cancer stem-like cells. Estrogen-independent breast cancer cell lines have higher Notch activity than estrogen-dependent lines. Protein kinase Cα (PKCα) overexpression is common in endocrine-resistant breast cancers and promotes tamoxifen (TAM)-resistant growth in breast cancer cell lines. We tested whether PKCα overexpression affects Notch activity and whether Notch signaling contributes to endocrine resistance in PKCα-overexpressing breast cancer cells.Analysis of published microarray data from ERα + breast carcinomas shows that PKCα expression correlates strongly with Notch-4. Real-time reverse transcription PCR and immunohistochemistry on archival specimens confirmed this finding. In a PKCα-overexpressing, TAM-resistant T47D model, PKCα selectively increases Notch-4, but not Notch-1, expression *in vitro* and *in vivo*. This effect is mediated by activator protein-1 (AP-1) occupancy of the Notch-4 promoter. Notch-4 knockdown inhibits estrogen-independent growth of PKCα-overexpressing T47D cells, whereas Notch-4IC expression stimulates it. Gene expression profiling shows that multiple genes and pathways associated with endocrine resistance are induced in Notch-4IC- and PKCα-expressing T47D cells. In PKCα-overexpressing T47D xenografts, an orally active γ-secretase inhibitor at clinically relevant doses significantly decreased estrogen-independent tumor growth, alone and in combination with TAM. In conclusion, PKCα overexpression induces Notch-4 through AP-1. Notch-4 promotes estrogen-independent, TAM-resistant growth and activates multiple pathways connected with endocrine resistance and chemoresistance. Notch inhibitors should be clinically evaluated in PKCα- and Notch-4-overexpressing, endocrine-resistant breast cancers.

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INTRODUCTION

Tamoxifen (TAM) has been widely used to treat patients with hormone-responsive breast cancers. It remains the treatment of choice for premenopausal women, whereas aromatase inhibitors are preferred in postmenopausal patients.[1] Acquired TAM resistance is a serious clinical problem.[2] *De novo* resistance occurs in approximately 20% of estrogen receptor α (ERα)–progesterone receptor-positive and 60% of ERα-positive, progesterone receptor-negative cases.[3] Resistance to aromatase inhibitors is expected to become similarly common.[4] Recurrent ERα-positive breast cancers are generally resistant to endocrine therapy and poorly responsive to chemotherapy. Thus, understanding the molecular basis for acquired endocrine resistance is essential to develop novel therapeutic regimens for recurrent ERα-positive breast cancer.

Notch signaling is aberrantly active in a variety of breast cancers.[5] High expression of Notch-1 and Notch ligand Jagged-1 is associated with poor prognosis in breast cancer.[6,7] We have shown by immunohistochemistry that Notch-1 significantly correlated with node status and tumor grade, whereas Notch-4 and Jagged-1 significantly correlated with Ki67 status in breast cancer specimens.[8] Recent evidence indicates that Notch-1 inhibition maintains estrogen responsiveness in breast cancer cells.[9] Notch receptors (1–4) and ligands (delta-1, delta-3 and delta-4 and Jagged-1 and Jagged-2) are membrane proteins that regulate cell fate through cell–cell contact.[10] Mature Notch receptors consist of an extracellular and a transmembrane subunit. Ligand binding triggers subunit separation followed by sequential proteolytic processing of the transmembrane subunit by A Disintegrin And Metalloprotease 10 (ADAM10) and

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γ-secretase, generating an intracellular fragment. The intracellular fragment translocates into the nucleus and modulates transcription via CBF-1/Suppressor of Hairless/Lag1 (CSL) factors, also known as C-promoter binding factor 1 (CBF-1) in mammals and recombination signal binding protein of immunoglobulin kappa chain J region (RBP-Jk) in mice.[11] γ-Secretase inhibitors (GSIs) are being studied in several phase 1–2 trials in breast cancer. However, which patient subgroups are most likely to benefit from these agents is unknown, and rational combinations including Notch inhibitors with other agents need to be designed. We reported that ERα-positive breast cancer cells respond to estrogen deprivation or TAM by reactivating Notch signaling.[12] In T47D cells, Notch-1 or Notch-4 knockdown synergized with TAM, as did GSI *in vivo*.[12] These data suggested that GSIs may be useful to prevent or overcome antiestrogen resistance.[12] In the same study, we noticed that the estrogen-independent, ERα-negative T47D:C42 subclone, generated by selection in an estrogen-free medium,[13] has significantly higher Notch activity than its estrogen-dependent counterpart, T47D:A18. T47D:C42 cells spontaneously overexpress protein kinase Cα (PKCα), and enforced expression of PKCα in T47D:A18 cells recapitulates the estrogen-independent, TAM-resistant phenotype.[14] PKCα overexpression has been reported in 73% of breast cancers that recurred while on TAM.[15] Stable overexpression of PKCα in breast cancer cells produces a TAM-resistant phenotype *in vitro*[16] and *in vivo*.[17] Elevated expression of PKCα in clinical specimens predicts endocrine resistance.[18] We investigated the possibility of crosstalk between PKCα and Notch in TAM-resistant breast cancer.

RESULTS

Expression of PKCα in breast cancer gene expression profiles and cancer specimens correlates with that of Notch-4

We used publicly available gene expression data. The data were downloaded from Gene Expression Omnibus (GEO) under accession number GSE6532. The data included 327 ERα-positive breast cancer patients and were generated using the Human U133A Affymetrix platform as previously described.[19] We extracted gene expression values for the Notch genes (Notch-1–4) and PKCα from this data set. We performed correlation analysis between PCKα and the Notch genes. The strength of the estimated Pearson's correlation coefficient was determined by *P*-value ($P<0.05$). Expression levels of the Notch genes were significantly correlated with PKCα values ($P=0.005$ by Fisher's exact test). The highest correlation ($R=0.95$) was between Notch-4 and PKCα (Figure 1a). Tumors clearly clustered into two distinct groups, showing high and low expression of both PKCα and Notch-4, respectively. To confirm the correlation between PKCα and Notch-4, we analyzed RNAs from 19 ERα+ breast tumor archival samples by quantitative reverse transcription PCR (qRT–PCR). We found a highly significant correlation ($r=0.63$) between PKCα and Notch4 expression levels (Figure 1b), consistent with microarray data.

PKCα-overexpressing breast cancers have high levels of Notch-4 protein

To examine whether mRNA expression data correlate with PKCα and Notch-4 protein expression, we studied de-identified archival clinical specimens by immunohistochemistry. Cases with staining intensity scored 1–2+ were classified as 'low expression', and cases with staining intensity scored 3+ were classified as 'high expression'. We analyzed 10 PKCα-overexpressing (3+) and 10 PKCα-non-overexpressing tumors (negative). Notch-4 was positive in all cases, consistent with our previous findings.[12] However, 8/10 PKCα-overexpressing tumors had high expression (3+) for Notch-4 in virtually all cancer cells compared with 1/10 among PKCα-non-overexpressing tumors ($P=0.005$ by Fisher's exact test). Notch-1 signal in PKCα-overexpressing tumors was much weaker than Notch-4 and weaker than that in PKCα-non-overexpressing tumors (Figure 1c). All PKCα-non-overexpressing tumors were positive for Notch-1 (eight low and two high), consistent with our previous data.[8,12] Among PKCα-overexpressing tumors, five were Notch-1 negative and five low ($P=0.016$ by Fisher's exact test). These data suggest that PKCα increases the steady-state expression of Notch-4 protein but not necessarily Notch-1. However, we cannot rule out that PKCα may affect Notch-4 and/or Notch-1 protein processing via post-transcriptional mechanisms.[20]

PKCα overexpression results in upregulation of Notch-4 protein and mRNA

To test our hypothesis, we examined an established model of PKCα-mediated TAM resistance in T47D:A18 cells.[14,15,21] T47D:A18/PKCα cells *in vitro* had much higher Notch-4, but not Notch-1, protein levels than T47D:A18/neo controls (Figure 2a, top). To exclude clonal selection artifacts, we compared parental T47D:A18

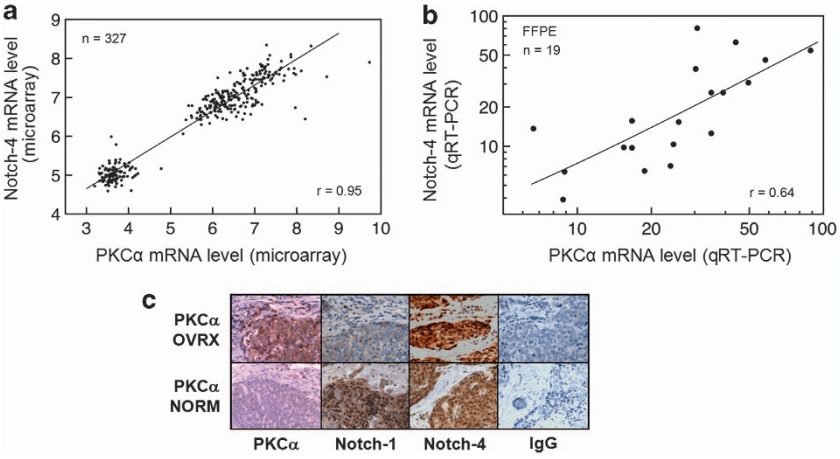


Figure 1. PKCα and Notch-4 expression levels show significant correlation in breast cancers. (**a**) Microarray gene expression data using 327 ER-positive breast cancer samples. mRNA levels are expressed as $\log_2$ of normalized values. (**b**) Correlation of PKCα and Notch-4 mRNA levels in 19 formalin-fixed, paraffin-embedded (FFPE) ERα+ clinical samples determined by qRT–PCR. 18*S* ribosomal RNA was used to normalize the values from each sample (see the Materials and methods section). (**c**) Immunohistochemistry showing that PKCα-overexpressing breast cancers have high levels of Notch-4, but not Notch-1, as shown in representative images (×400) of 10 cases with PKCα overexpression (PKCα OVRX) and 10 non-overexpressing cases (PKCα NORM).

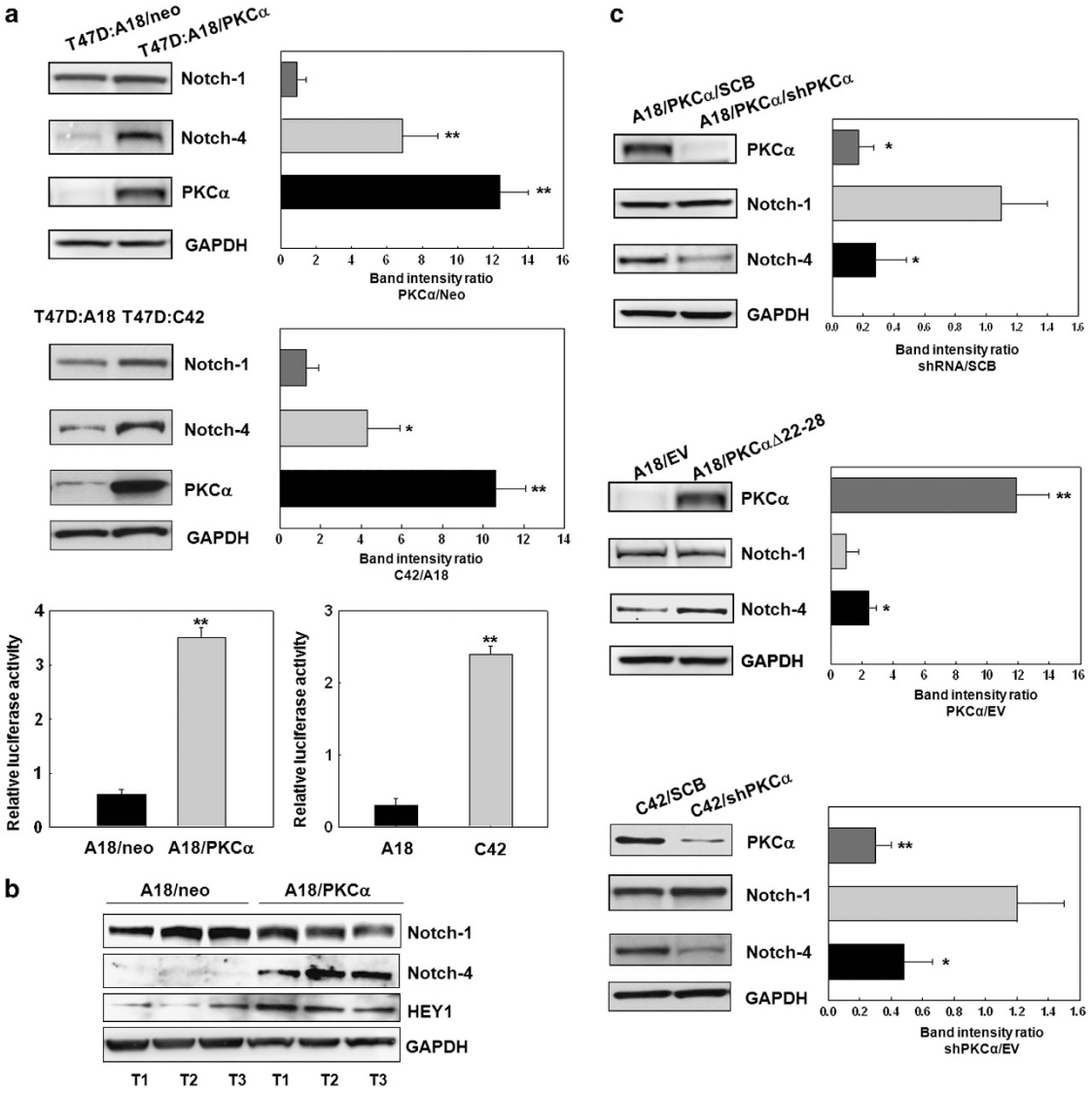


Figure 2. PKCα overexpression in breast cancer cell lines correlates to high level of Notch-4, but not Notch-1 protein. (**a**) Western blots of Notch-4 and Notch-1 (top two panels) and CSL luciferase assays (bottom panels) in T47D:A18/PKCα and T47D:C42 cells compared with T47D:A18/neo and T47D:A18 parental controls, respectively. (**b**) T47D:A18/PKCα xenograft tumors ($n = 3$, T1, T2 or T3) express higher levels of Notch-4, but not Notch-1, than T47D:A18/neo xenografts. (**c**) PKCα knockdown by retrovirally transduced shRNA (top panel) decreases Notch-4 compared with scrambled (SCB) shRNA in T47D:A18/PKCα. In the same cells, expression of constitutively active PKCαΔ22–28 increases Notch-4 compared with EV control (middle panel). PKCα knockdown by retrovirally transduced shRNA decreases Notch-4 compared with scrambled (SCB) shRNA in T47D:C42 (bottom panel). *$P < 0.05$, **$P < 0.005$.

cells to T47D:C42, which spontaneously overexpress PKCα, and obtained similar results (Figure 2a, middle). To measure Notch-dependent transcriptional activity, we used a CSL dual-luciferase assay. T47D:A18/PKCα cells had much higher basal Notch-dependent transcriptional activity than T47D:A18/neo control cells. Similarly, T47D:C42 cells have much higher Notch activity than T47D:A18 cells ($P < 0.005$) (Figure 2a, bottom).

Identical results were obtained from T47D:A18/neo and T47D:A18/PKCα xenograft tumors grown in the mammary fat pads of ovariectomized athymic mice. T47D:A18/neo xenograft-bearing mice were treated with estradiol (E2) to maintain tumor growth. T47D:A18/PKCα xenograft-bearing mice were not treated with E2 because these tumors were growth inhibited by estrogen *in vivo*.[17,22] Figure 2b shows that in three different tumor lysates analyzed per cell line (T1, T2 and T3), T47D:A18/PKCα xenografts had clearly higher levels of Notch-4 as well as downstream target protein HEY1 than T47D:A18/neo, whereas Notch-1 levels were similar in the two models. These data were consistent with the hypothesis that T47D:A18/PKCα cells upregulate Notch-4 protein expression independently of Notch-1. Also consistent with this hypothesis, we observed no difference in expression level of Notch-4 when silencing Notch-1 by small hairpin RNA (shRNA) and vice versa (data not shown). This is different than what we observed in parental T47D:A18 or MDA-MB231 cells, where Notch-1 is upstream of Notch-4.[12] We did not investigate whether Notch-2 or Notch-3 participates in Notch-4 regulation.

We then asked whether PKCα causes Notch-4 overexpression. Knockdown of PKCα by retrovirally transduced shRNA in T47D:A18/PKCα and T47D:C42 cells inhibited Notch-4 expression (Figure 2c, top). Conversely, transient expression of constitutively active PKCα (PKCαΔ22–28, carrying a deletion of the pseudosubstrate domain) in parental T47D:A18 cells upregulated Notch-4

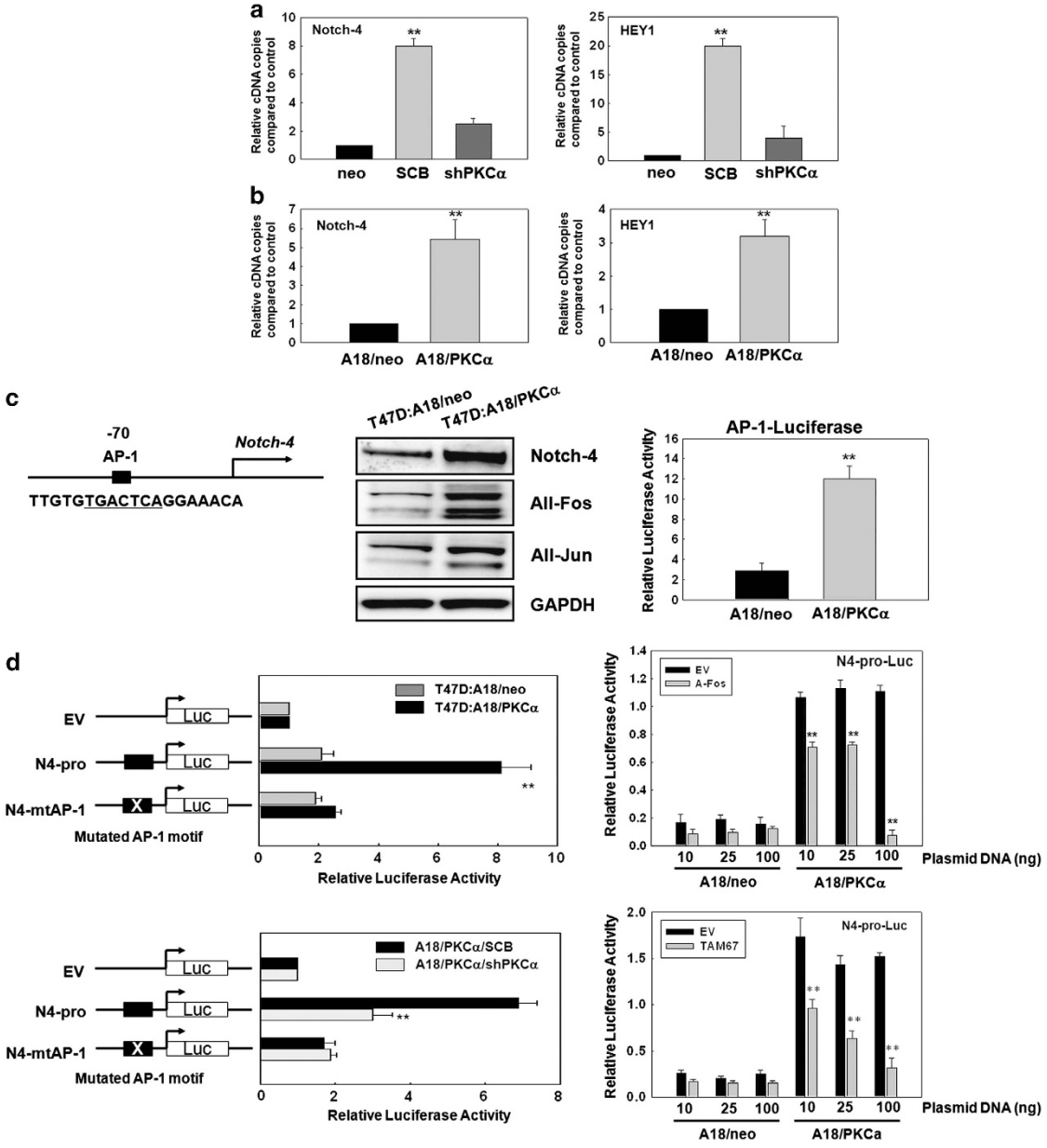


Figure 3. PKCα regulates Notch-4 mRNA levels, and AP-1 mediates increased Notch-4 promoter activity in PKCα-overexpressing cells. (**a**) qRT–PCR of Notch-4 and HEY1 mRNAs from T47D:A18/neo (Neo), T47D:A18/PKCα/SCB (SCB, transduced with scrambled shRNA) and T47D:A18/PKCα/shPKCα (transduced with PKCα shRNA) cells. (**b**) qRT–PCR of Notch-4 and HEY1 mRNAs from T47D:A18/neo (Neo) and T47D:A18/PKCα (A18/PKCα) xenograft tumors. (**c**) Left: diagram representing the human Notch-4 promoter. Middle, western blots: T47D:A18/PKCα cells express higher AP-1 protein levels than T47D:A18/neo controls. Right: AP-1 reporter assays showing higher AP-1 activity in T47D:A18/PKCα cells. (**d**) Left: reporter assays showing higher Notch-4 promoter activity in PKCα-overexpressing cells than that in T47D:A18/neo controls (top panel), which was downregulated by PKCα knockdown via shRNA (shPKCα) compared with scrambled shRNA (SCB, bottom panel). Right: expression of dominant-negative c-Fos (A-Fos, top panel) or a dominant-negative c-Jun (TAM67, bottom panel) reduced Notch-4 promoter activity in a dose-dependent manner. $^{**}P<0.005$.

(Figure 2c, bottom). Notch-1 levels were unaffected by either silencing PKCα or overexpressing constitutively active PKCα.

We examined the mechanism of Notch-4 upregulation by PKCα. Notch-4 and Notch target HEY1 mRNA levels, detected by qRT–PCR, were higher ($P<0.005$) in T47D:A18/PKCα cells than in T47D:A18/neo controls and were suppressed by silencing PKCα (Figure 3a). Xenograft tumors showed the same pattern, with T47D:A18/PKCα tumors containing significantly higher levels of Notch-4 and HEY1 mRNAs (Figure 3b). Thus, PKCα regulates Notch-4 mRNA levels *in vitro* and *in vivo*, and Notch signaling is active on endogenous target promoters in PKCα-overexpressing cells.

Activator protein-1 mediates higher level of Notch-4 promoter activity in T47D:A18/PKCα cells

To determine whether the elevated Notch-4 mRNA levels are due to transcriptional regulation, we studied the activity of the Notch-4 promoter in T47D:A18/PKCα cells. The Notch-4 promoter has activator protein-1 (AP-1)-binding sites (Figure 3c) and is regulated by AP-1 subunits in other systems.[23,24] Elevated AP-1 activity in T47D:A18/PKCα cells has been reported.[14] T47D:A18/PKCα cells express higher levels of AP-1 subunits and have higher basal AP-1 reporter activity than controls (Figure 3c). Using a Notch-4 promoter–luciferase reporter construct,[23,24] we found higher

level of Notch-4 promoter activity in T47D:A18/PKCα cells than in T47D:A18/neo controls (Figure 3d, top left). Knockdown of PKCα downregulated Notch-4 promoter activity (Figure 3d, bottom left). Mutation of the AP-1 motif strongly decreased reporter activity (Figure 3d).

To further probe whether the Notch-4 promoter is regulated by AP-1 subunits, we used two dominant-negative proteins that antagonize endogenous AP-1: dominant-negative cFos (A-Fos)[25] and dominant-negative c-Jun (TAM67).[26] Transient expression of A-Fos reduced Notch-4 promoter activity in T47D:A18/PKCα cells but had no effect on the already low Notch-4 promoter activity in T47D:A18/neo cells (Figure 3d, top right) ($P<0.005$). Dominant-negative c-Jun (TAM67) gave similar results (Figure 3d, bottom right).

To examine whether AP-1 subunits occupy the Notch-4 promoter, we performed quantitative chromatin immunoprecipitation analysis with AP-1 antibodies against multiple Jun and Fos family members. We found that AP-1 subunits occupy the Notch-4 promoter in PKCα-overexpressing cells (Figure 4a, left) ($P<0.005$). PKCα knockdown downregulated AP-1 occupancy at the Notch-4 promoter in T47D:A18/PKCα cells (Figure 4a, right). No occupancy was detected at the negative control β-globin promoter (Figure 4a, bottom). Our data indicate that AP-1 complexes, including Jun/Fos heterodimers, specifically occupy the Notch-4 promoter in T47D:A18/PKCα cells, but not in T47D:A18/neo controls, and PKCα is required for AP-1's presence at the Notch-4 promoter.

Notch-4 is necessary and sufficient for endocrine-independent, TAM-resistant proliferation of T47D:A18 cells

To elucidate the possible role of Notch-4 downstream of PKCα in TAM resistance, we generated T47D:A18 cells stably transfected with the intracellular form of Notch-4 (Notch-4IC) (T47D:A18/N4IC) and the corresponding empty vector (T47D:A18/puro). As shown in Figure 4b, T47D:A18/N4IC could grow in an estrogen-depleted medium and in the presence of 4-hydroxytamoxifen (4-OH-TAM), although at a slower rate compared with T47D:A18/PKCα cells. Levels of Notch-4IC protein in T47D:A18/N4IC cells were higher than in controls but still lower than in T47D:A18/PKCα cells (Figure 4b, top). To rule out selection artifacts, we performed transient transfection experiments in T47D:A18 cells. Crystal violet cell mass assays showed that transient expression of Notch-4IC increases proliferation of these cells in the presence of vehicle ($P<0.005$) and 1 μM 4-OH-TAM ($P=0.0018$) (Figure 4b, bottom). In the presence of E2, we obtained identical results (Figure 4b). Measurements of DNA content after 3 days in culture confirmed that Notch-4IC increased DNA synthesis ($P<0.005$) compared with the empty vector (EV) in the absence of E2 (Figure 4b). These data indicate that overexpression of Notch-4IC in T47D:A18 cells can stimulate proliferation via estrogen-independent, TAM-resistant mechanisms.

We then tested whether knockdown of endogenous Notch-4 inhibits proliferation of T47D:A18/PKCα cells. Figure 4c shows that Notch-4 knockdown by shRNA inhibited proliferation of T47D:A18/PKCα cells in the presence or absence of 4-OH-TAM. Knockdown of Notch-4 in T47D:A18/PKCα cells had a virtually identical effect as 4-OH-TAM had in parental cells. Silencing Notch-4 also decreased DNA replication in PKCα-overexpressing cells compared with control scrambled shRNA vector (SCB, Figure 4c). These data indicate that endogenous Notch-4 is at least in part responsible for TAM-resistant growth of PKCα-overexpressing cells. To determine whether PKCα knockdown restores TAM sensitivity to T47D:A18/PKCα cells, we performed proliferation assays with T47D:A18/PKCα/shPKCα cells. T47D:A18/PKCα cells grew in the absence of E2 and showed TAM resistance, as previously reported.[14] Conversely, T47D:A18/PKCα/shPKCα cells showed reversal of the TAM-resistant and partially hormone-dependent phenotype (Figure 4c).

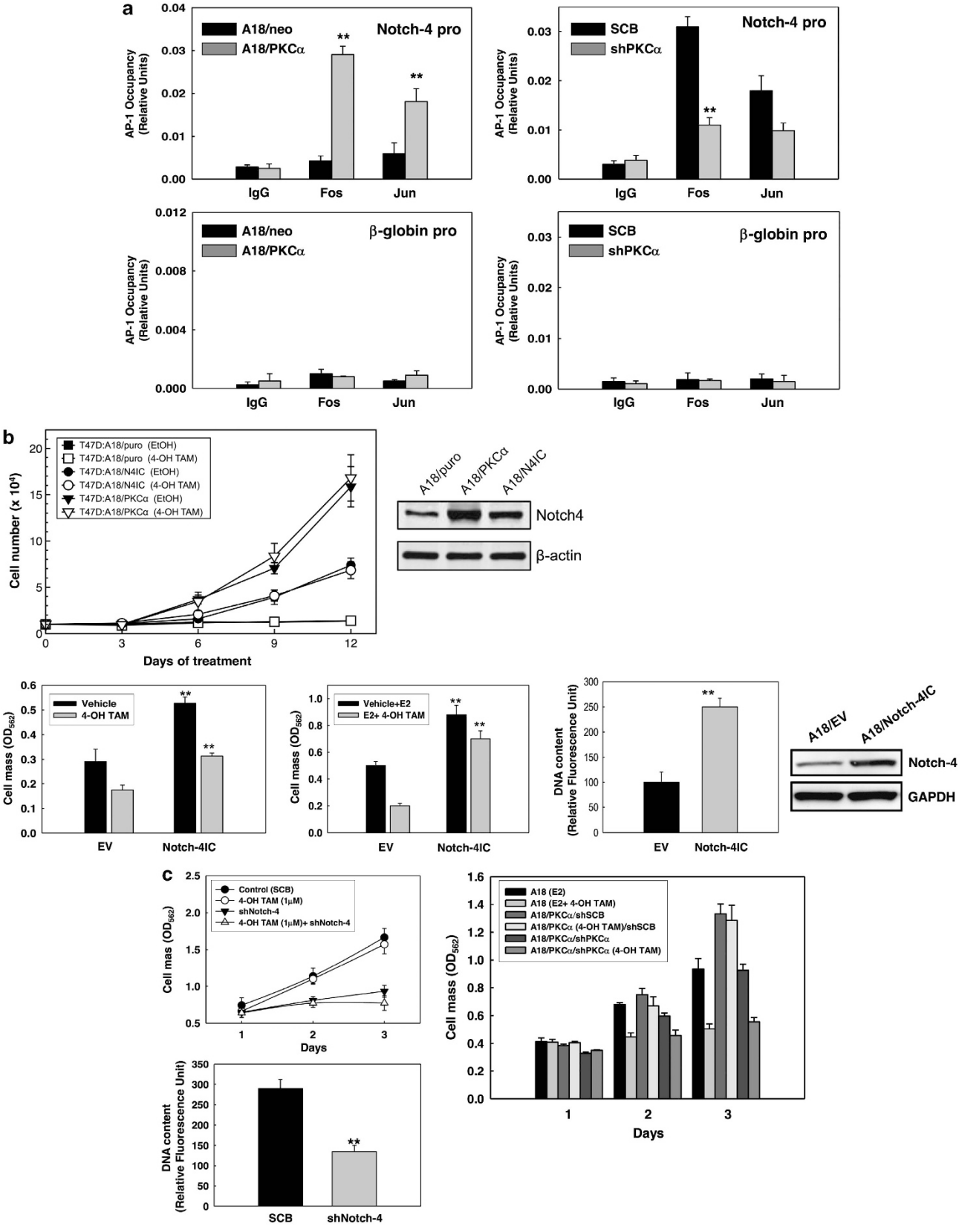
Gene expression profiling in T47D:A18/neo and TAM-resistant cell lines

We explored the possible mechanisms whereby Notch-4 induces endocrine resistance in our model compared with Notch-1 and PKCα. To that end, we compared gene expression profiles of T47D:A18/neo and T47D:A18/PKCα cells with those of T47D:A18 cells stably expressing Notch-4IC or Notch-1IC (T47D:A18/N4IC or T47D:A18/N1IC) grown in a complete (estrogen-containing) medium and in a charcoal-stripped (estrogen-depleted) medium. Like Notch-4IC, also Notch-1IC expression enabled endocrine-independent growth in T47D cells (data not shown). This is consistent with our previous observation in MCF-7 cells, where Notch-1 can stimulate ERα-dependent transcription in the absence of estrogen,[27] and with the data of Haughian *et al.*[9] However, the transcriptional profiles induced by stable expression of Notch-1IC or Notch-4IC were considerably different. Unsupervised cluster analysis (Figure 5) revealed that Notch-4IC cells clustered most closely with PKCα cells, whereas Notch-1IC cells clustered most closely with parental T47D:A18/neo cells grown in a complete (estrogen-containing) medium. This is consistent with the hypothesis that Notch-4 plays a key role in PKCα-induced endocrine resistance, whereas Notch-1 can mimic some of the effects of estrogen. Gene expression profiles revealed several mechanistic clues that we are currently investigating (see the Discussion section).

γ-Secretase inhibition strongly inhibits T47D:A18/PKCα growth and is synergistic with 4-OH-TAM

We tested the effects of GSI MRK003 in our models. GSI already at 2.5 μM caused nearly complete growth inhibition in T47D:A18/PKCα cells. After 8 days in 2.5 μM GSI, T47D:A18/PKCα cell proliferation plateaued (Figure 6a), whereas T47D:A18/PKCα cells expressing Notch-4IC or Notch-1IC continued growing vigorously. Stable expression of Notch-4IC or Notch-1IC significantly rescued the antiproliferative effects of GSI, indicating that this effect is caused at least in large part by Notch inhibition (Figure 6a). We cannot rule out that both Notch-4 and Notch-1 inhibition contribute to the activity of MRK003 in T47D:A18/PKCα cells. GSI concentrations >5 μM were cytotoxic, and off-target effects most likely contributed to cytotoxicity. Combination index values and isobologram analysis[28] demonstrated strong synergism over a broad range of GSI concentrations (0.8–50 μM) and 4-OH-TAM concentrations (0.5–5 μM) (Figure 6b and Supplementary Table 1). Western blots and densitometric analysis from T47D:A18/PKCα cells showed that GSI treatment decreased the amount of Notch-1IC, whereas 4-OH-TAM had no effect. Combined treatment had the same effect as GSI alone (Figure 6c and Supplementary Figures 1A and B). This ruled out an effect of 4-OH-TAM on γ-secretase. Notch-4 has been reported to be virtually insensitive to GSIs N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and (S)-2-[2-(3,5-difluoro-phenyl)-acetylamino]-N-((S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl)-propionamide (DBZ).[29] Therefore, we determined whether MRK003 inhibits Notch-4 cleavage. This task was complicated by the lack of specific antibodies to Notch-4IC and contradictory reports on its apparent molecular mass. When we transiently transfected increasing amounts of authentic Notch-4IC cDNA (residues 1470–2003 plus an N-terminal Flag) into T47D:A18 cells, this generated two bands: a more abundant band with apparent molecular mass of 64.3 kDa and a less abundant one with apparent molecular mass of 73 kDa (Supplementary Figure 1C). The theoretical molecular mass of Notch-4IC calculated from its amino-acid sequence is 56.8 kDa, suggesting post-translational modifications. Ongoing studies that will be reported elsewhere indicate that the 73 kDa band is phosphorylated. Endogenous Notch-4 in T47D:A18/PKCα cells and in stably transfected T47D:A18/N4IC cells showed predominantly the 64 kDa band (Supplementary Figure 1D). The same band was

suppressed by a specific Notch-4 small interfering RNA (Supplementary Figure 1E). Treatment of T47D:A18/PKCα cells with 5 μM MRK003 drastically reduced the 64 kDa band (Supplementary Figures 1F–H). When we compared the effects of 5 μM DAPT and MRK003 in T47D:A18/PKCα cells, DAPT did not decrease the 64 kDa Notch-4IC band and even appeared to increase it, consistent with the results of Harrison *et al.*,[29] whereas MRK003 decreased it (Supplementary Figures 1I and J).



γ-Secretase inhibition strongly decreases T47D:A18/PKCα xenograft tumor growth

The isobologram analysis suggested that pharmacological Notch inhibition may at least partially reverse the TAM resistance of T47D:A18/PKCα cells. We tested this hypothesis in xenograft experiments. In T47D:A18/PKCα xenograft experiments, mice with established tumors were randomized into four treatment groups: vehicle, TAM (1.5 mg/day TAM), GSI (100 mg/kg MRK003) and TAM plus GSI. Tumor volume was monitored for up to 4 weeks.

T47D:A18/PKCα cells are TAM resistant *in vivo* and are inhibited by E2. They readily form tumors in ovariectomized mice that grow exponentially once established,[17] as seen in the vehicle-treated group. We found that TAM alone was nearly ineffective, as expected, whereas oral GSI decreased tumor volume compared with the control group ($P = 0.0002$ by analysis of variance). GSI and GSI/TAM combination further decreased tumor growth (Figure 6d). Average body weight was not significantly affected by treatment, and the animals appeared healthy.

At the end of the experiment, both GSI alone ($P = 0.0002$) and GSI/TAM ($P = 0.0001$) decreased tumor growth, and the combination provided greater antitumor effect than GSI alone ($P = 0.0018$). Western blots on tumor extracts confirmed inhibition of Notch signaling by GSI (Figure 6d). Both GSI and TAM/GSI caused the appearance of a Notch-1 doublet band, suggestive of unprocessed ADAM-cleaved transmembrane subunit. The 64 kDa Notch-4IC band was virtually undetectable in one GSI-treated tumor and decreased in another and was undetectable in both TAM/GSI-treated tumors tested. These data indicate that pharmacological suppression of Notch signaling significantly inhibits the growth of T47D:A18/PKCα xenografts. The addition of TAM appears to further improve the efficacy of MRK003, although most of the therapeutic effect can be attributed to the latter. Thus, inhibition of γ-secretase is therapeutically active in T47D:A18/PKCα tumors and either circumvents or partially reverses TAM resistance.

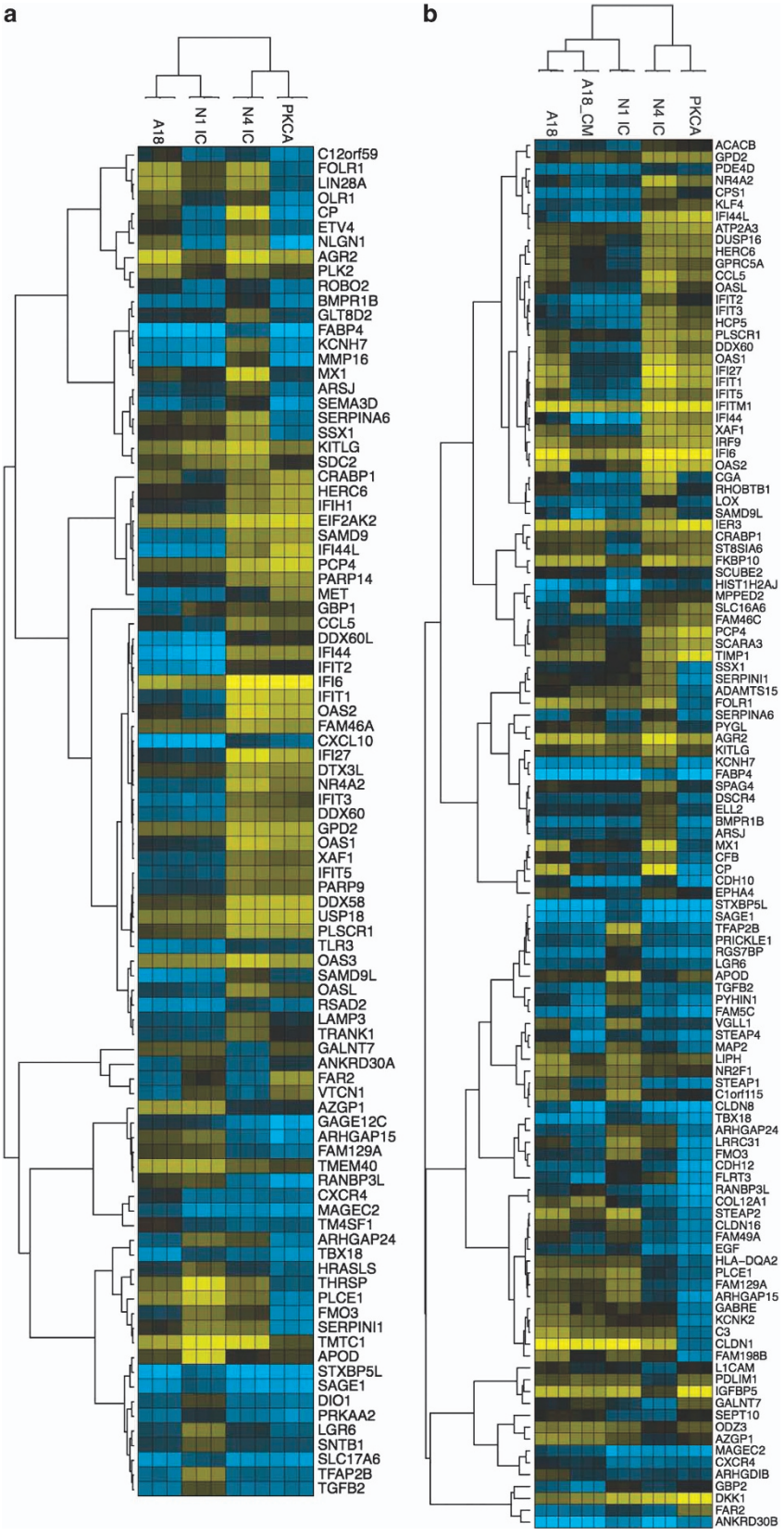
Identical experiments were performed in parental T47D:A18/neo xenografts. Ovariectomized mice with a subcutaneously implanted E2-releasing capsule were treated as described above. T47D:A18/neo tumors were highly sensitive to TAM, as expected. The combination TAM/GSI was more effective than TAM alone, causing virtually complete tumor regression (Supplementary Figure 2). At the end of the experiment, only the combination group had zero mortality. However, GSI alone in this model caused intolerable intestinal toxicity, diarrhea and death (Supplementary Figure 3). As the only difference between the two models was the presence of E2 capsules in mice carrying T47D:A18/neo xenografts, we conducted a further experiment in which xenograft-carrying mice were treated for 1 week with vehicle, GSI, TAM and TAM/GSI. Intestinal histology confirmed massive goblet metaplasia and epithelial loss in GSI-treated mice but not in vehicle- or TAM-treated mice. TAM/GSI combination treatment greatly alleviated GSI toxicity while causing widespread cell death in the tumors (Supplementary Figures 4 and 5). This may be due to inhibition of Notch activity by estrogen,[12] which would enhance the toxicity of GSI.

DISCUSSION

PKCα expression is associated with resistance to endocrine therapy in experimental models[14–16,21] and is a marker of poor response to endocrine therapy in patients.[15,18] Our data provide a functional link between PKCα and Notch-4 in endocrine resistance. Gene expression profiling studies, qRT–PCR and immunohistochemistry indicate that PKCα correlates strongly with the expression of Notch-4 in breast cancers. In our model, PKCα overexpression causes AP-1-mediated induction of Notch-4, promoting TAM resistance. Stable expression of Notch-4IC is sufficient to promote estrogen-independent, TAM-resistant growth and produces a transcriptional profile more similar to that of T47:A18/PKCα cells than stable expression of Notch-1IC. Among the genes affected by Notch-4IC and PKCα expression in an estrogen-depleted medium (Figure 5 and Supplementary Table 2) are several genes that have been associated with endocrine resistance. IRS1 (insulin receptor substrate 1) is an adaptor for the insulin-like growth factor type I receptor that mediates among other effects AKT activation. IRS1 is induced by estrogen,[30] and its knockdown potentiates the effects of TAM in MCF-7 cells.[31] IRS1 can associate with ErbB3 in ERα-positive breast cancer cells, where TAM resistance induced by IRS1 overexpression/activation may be due to AKT-mediated ERα activation.[32] CD44, a family of transmembrane glycoproteins derived from alternate splicing of a common gene, modulates intracellular signaling through interaction with hyaluronan and through formation of coreceptor complexes with various receptor tyrosine kinases. Enhancement of ErbB receptor activity by CD44 has been proposed as a mechanism of TAM resistance.[33,34] One downstream target of CD44 activity is Rac1, a member of the Rho family of GTPases. ARHGDIB (Rho GDIβ), a negative regulator of RhoA and Rac1, is downregulated in Notch-4IC, PKCα and Notch-1IC cells in an estrogen-depleted medium compared with in parental T47D:A18 cells. Loss of the paralog ARHGDIA (Rho GDIα) enhances metastasis and TAM resistance in models of ERα-positive breast cancer through ERα activation.[35] Forkhead box protein M1 (FoxM1) is an ERα transcriptional target and a key mediator of ERα mitogenic effects that plays a critical role in TAM resistance.[36] FoxM1 induces a gene signature characteristic of cell cycle progression and mitosis.[37] Numerous such genes are induced in Notch-4IC and PKCα cells, including cyclin B1, which we previously reported to respond to Notch-4 small interfering RNA,[12] cyclin A2, cyclin E2, E2F1, AURKB and others. Moreover, several of the genes

Figure 4. AP-1 occupies the Notch-4 promoter in T47D:A18/PKCα cells; Notch-4 promotes endocrine-independent and TAM-resistant growth. (**a**) Quantitative chromatin immunoprecipitation. Fos and Jun family proteins occupy the Notch-4 promoter (Notch-4 pro). Left: Notch-4 promoter occupancy is significantly higher in PKCα-overexpressing cells than in neo controls. Right: silencing PKCα by shRNA (shPKCα) significantly downregulates Fos family occupancy at the Notch-4 promoter compared with scrambled shRNA (SCB). No occupancy was observed at the negative control β-globin promoter (β-globin pro). (**b**) Top: T47D:A18 cells (indicated as 'A18' in figure panels for clarity) stably expressing Notch-4IC (A18/N4IC) grow in an estrogen-free medium containing vehicle (ethanol, EtOH) and in the presence of 4-OH-TAM (1 μM). Vector-transfected cells (A18/puro) do not grow in these conditions, whereas PKCα-overexpressing cells (A18/PKCα) grow faster than A18/N4IC. Western blots (right) showed that A18/N4IC cells express intermediate levels of Notch-4IC (64 kDa) between controls and A18/PKCα cells. Bottom: transient expression of Notch-4IC (Notch-4IC) in T47D:A18 cells increases cell mass in the presence or absence of 4-OH-TAM (1 μM) in an estrogen-free medium (left) or in the presence of E2 (1 nM, center). Right: Notch-4IC-transfected cells also show higher DNA content compared with EV in the absence of E2, indicating that increased total cell mass is paralleled by DNA replication and indicating proliferation rather than cell size increase. Western blots confirmed expression of Notch-4IC. (**c**) Top left: Notch-4 knockdown via shRNA inhibits T47D:A18/PKCα cell proliferation compared with control in the presence or absence of 4-OH-TAM (1 μM). Bottom left: Notch-4 shRNA-transfected cells show lower DNA content compared with scrambled vector (SCB). Right: PKCα knockdown (shPKCα) decreased the proliferation rate of T47D:A18/PKCα cells and restored 4-OH-TAM sensitivity compared with scrambled control (shSCB). T47D:A18 parental cells were grown in the presence of E2 (1 nM), whereas T47D:A18/PKCα cells were grown in an estrogen-free medium. $^{**}P < 0.005$.

induced by Notch-4IC and PKCα have important functions in DNA repair and survival (e.g., BRCA2, CHECK2, BRCA1 and BIRC5). BIRC5 (survivin) is a putative Notch target.[38] MKI67 (Ki67) correlates with Notch-4 expression by immunohistochemistry in breast cancer specimens.[8] Many cell cycle-related genes were regulated in the same direction by Notch-1IC and Notch-4IC. However, a striking difference in expression profiles between Notch-1IC on one hand and Notch-4IC and PKCα on the other was the strong induction of



a type I interferon signature, including STAT1 and multiple STAT1 targets, in Notch-4IC and PKCα cells. These genes (Figure 5 and Supplementary Table 3) were mostly suppressed by an estrogen-containing medium and Notch-1IC. A type I interferon signature has been associated with a distinct subgroup of breast cancers with poor outcome.[39] It has been proposed that constitutive, prolonged STAT1 activation, unlike transient activation, induces a pro-survival phenotype.[40] A type I interferon/STAT1 gene signature has been associated with chemoresistance, radioresistance and metastasis in breast cancer and melanoma cells.[41–43] Notch-4IC induces mammary tumors in mice[44–49] and transforms human immortalized MCF-10A mammary cells *in vitro*.[50] Importantly, Notch-4IC can cause mammary tumors in the absence of canonical Notch-regulated transcription factor RBP-Jκ (CSL).[51] This raises the possibility that at least some of the effects we observed (e.g., the strong induction of STAT1 targets) are mediated by non-canonical signaling. This may explain the remarkable difference in gene expression profiles induced by Notch-4IC and Notch-1IC. We[8,12] have shown that Notch-4 is commonly expressed at variable levels in breast cancer specimens and that high Notch-4 expression strongly correlates with Ki67. Harrison *et al.*[29] have shown that Notch-4 is essential for breast cancer 'stem-like' cells. Our data are consistent with a model in which Notch-4 is necessary and sufficient for estrogen-independent, TAM-resistant growth of PKCα-overexpressing ERα+ breast cancer cells. In most unselected breast cancer specimens we studied,[8,12] Notch-4 was coexpressed with Notch-1. In breast cancer cell lines, Notch-4 expression was controlled by Notch-1. Our data indicate that PKCα can upregulate Notch-4 independently of Notch-1 via AP-1 and that a subset of breast cancers co-overexpress PKCα and Notch-4 irrespective of Notch-1 protein.

Wu *et al.* and Wu and Bresnick reported that Fos species occupy the Notch-4 promoter in human umbilical vein endothelial cells, upregulating Notch-4 promoter activity.[23,24] Our data are consistent with their model and support a role for AP-1 in stimulating Notch-4 expression. PKCα activates the AP-1 pathway in many experimental models.[52] PKCα induces cFos and cJun mRNA via Raf/MEK/ERK.[53] The same pathway can modulate the activity of JunD and FosB by phosphorylation.[54] Our data raise the possibility that Notch-4 activation could be associated with the development of TAM resistance in PKCα-overexpressing breast cancers. It was reported that 50% reduction of PKCα in T47D:A18/PKCα cells is associated with a partial reversal of the TAM resistance *in vivo*.[21] Frankel *et al.*[16] showed that PKCα knockdown restored TAM sensitivity in TAM-resistant MCF-7 cells that overexpress PKCα. Most of our data were obtained using a well-characterized T47D-based model for TAM resistance.[14,15] This is a potential limitation of our study. The spontaneously selected, TAM-resistant T47D:C42 cells are ERα negative, which is not a common occurrence in human endocrine-resistant ERα+ disease. Therefore, we also examined an independently derived, spontaneously TAM-resistant MCF-7 cell line.[55] These cells, which retain ERα, overexpress Notch-4IC and are sensitive to Notch-4 knockdown (Supplementary Figure 6).

Taken together, our data indicate that Notch-4 is an important mediator of PKCα-induced endocrine resistance and that PKCα induces Notch-4 via AP-1. Therefore, inhibition of Notch signaling via GSI or selective inhibition of Notch-4 may be effective strategies to treat or prevent endocrine resistance in breast cancers that overexpress PKCα or have increased AP-1 activity. AP-1 inhibitors such as synthetic retinoids may be an alternative strategy.[56]

Gene expression profiles indicate that ERα+ tumor samples break down into two clearly distinct groups: low PKCα/low Notch-4 and high PKCα/high Notch-4. The latter group would be a candidate for a trial of GSI-including rational combinations. Our data indicate that GSIs are safe and may be effective in combination with TAM in endocrine-resistant, PKCα-overexpressing tumors. We cannot rule out that combined inhibition of multiple Notch paralogs or GSI effects on tumor stroma and angiogenesis may participate in the *in vivo* therapeutic activity of GSI. However, our data indicate that GSIs to be used in PKCα-overexpressing breast cancers should be tested for Notch-4 cleavage inhibition, as not all GSIs inhibit Notch-4.[29] MRK003 inhibited the cleavage of Notch-4 in our system, whereas DAPT did not. The mechanistic reasons for this difference are unclear. They may include differences in drug stability, intracellular concentration/distribution or affinity for a specific isoform of γ-secretase. The dose of MRK003 we used was nontoxic in ovariectomized animals and is considerably lower than its maximum tolerated dose. Thus, significant antitumor effects were observed with clinically achievable doses of an orally active GSI. We recently completed a pilot clinical trial of TAM or letrozole plus MK0752, the clinical counterpart of MRK003,[57] in the presurgical setting. This combination resulted in no intestinal toxicity while suppressing Ki67 in tumors in 17 out of 20 patients, consistent with our preclinical data reported here. Another phase 1b clinical trial of GSI R04929097 plus exemestane in recurrent, endocrine-resistant breast cancers showed that this combination was safe and produced clinical responses in 7 out of 14 patients. Three of the patients had stable disease for >6 months.[58] Our results suggest that combinations including a GSI plus TAM or an aromatase inhibitor may be effective in PKCα/Notch-4-overexpressing, endocrine-resistant breast cancers and that endocrine therapy may decrease the toxicity of GSIs.

MATERIALS AND METHODS

Cell culture

T47D:A18/neo, T47D:A18/PKCα and T47D:C42 breast cancer cell lines have been previously described.[14] TAM-sensitive MCF-7 (MCF-7/Tam-S) and TAM-resistant MCF-7 (MCF-7/Tam-R) cells have been previously described.[55] See Supplementary Materials and methods for additional detail.

Antibodies and chemicals

See Supplementary Materials and methods.

Immunohistochemistry

We studied archival de-identified routine formalin-fixed, paraffin-embedded blocks from Dr John Coon at Rush University, Chicago, IL, USA. L-16 Notch-4 antibody was used for immunohistochemistry as previously described,[8,12] as it generated the cleanest background.

Figure 5. Microarray analysis. T47D:A18/Notch-4IC cells cluster with T47D:A18/PKCα cells and show modulation of multiple genes linked to endocrine resistance. Heat maps of expression values for genes significantly modulated in T47D:A18 cells stably expressing Notch-4IC (N4IC), Notch-1IC (N1IC) or PKCα. From the gene expression values determined by microarray analysis, transcripts were selected from A18/N1IC and A18/N4IC cells showing a $\log_2$ ratio $\geqslant 1.5$ and $\leqslant -1.5$ ($P \leqslant 0.0005$) compared with the T47D:A18/neo (parental) line, when cells were either grown in a complete medium (**a**) or an estrogen-depleted medium (**b**). Expression values for this subset of genes from four cell lines, T47D:A18/neo, T47D:A18/N1IC, T47D:A18/N4IC and T47D:A18/PKCα (each sample measured in triplicate), are shown in the figure. The values shown in panel **b** were from cells incubated in an estrogen-depleted medium for 3 days before the RNA extraction. As T47D:A18/neo cells, contrary to the other three cell lines, undergo growth arrest in the absence of estrogen, T47D:A18/neo cells grown in a complete medium were included in the analysis (indicated as A18_CM). Regardless of the estrogen content in the medium, there was limited overlap between Notch-1IC- and Notch-4IC-overexpressing cells, whereas Notch-4IC- and PKCα-overexpressing cells showed much more extensive overlap and clustered together. High, medium and low values are represented as yellow, black and cyan.

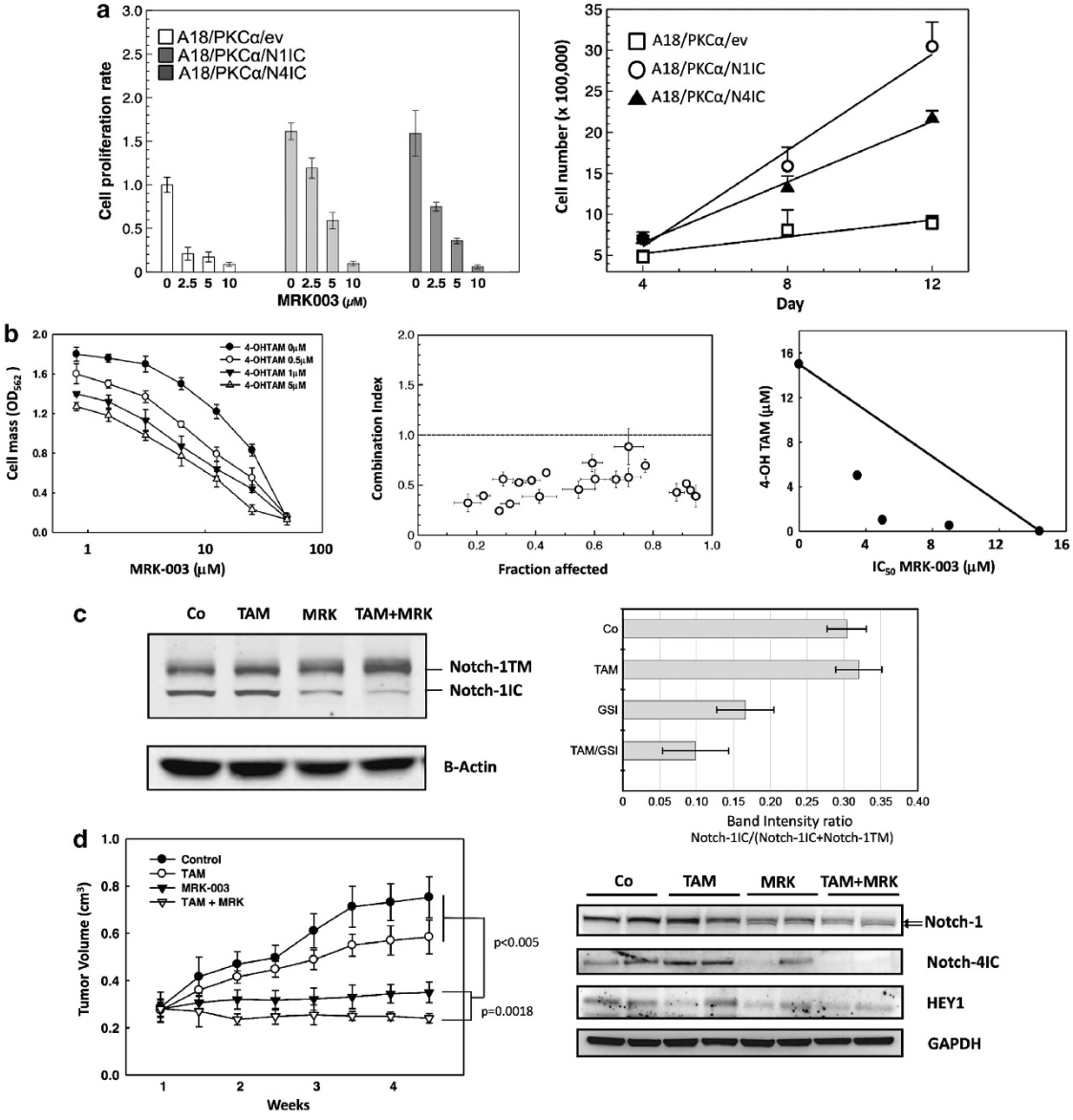


Figure 6. MRK003 GSI inhibits T47D:A18/PKCα cell proliferation and is synergistic with 4-OH-TAM *in vitro*, and it decreases tumor growth of T47D:A18/PKCα xenografts *in vivo*. (**a**) Cell proliferation assays. Left panel: T47D:A18/PKCα cells stably transduced with EV, Notch-1IC (N1IC) or Notch-4IC (N4IC) were cultured in an estrogen-depleted medium for 4 days and then treated with GSI MRK003 at different concentrations. A medium containing the proper concentration of GSI was replaced every 2 days. Linear growth rates were determined by measuring cell numbers from triplicate samples with a hemocytometer chamber every 4 days over 12 days from the start of treatment. The histograms show cell proliferation rates in the absence of GSI and in the presence of three concentrations of GSI MRK003. Right panel: growth curves between days 4 and 12 of T47D:A18/PKCα cells stably transduced with EV, Notch-1IC (N1IC) or Notch-4IC (N4IC) cultured in an estrogen-depleted medium containing 2.5 μM GSI MRK003. (**b**) Cell mass assays. Left: T47D:A18/PKCα cells were treated with GSI and 4-OH-TAM at the indicated concentrations for 48 h, and cell mass was determined by crystal violet assay. Middle: combination index values at each fraction affected by GSI and 4-OH-TAM treatment show strong synergistic effect at most of the concentrations tested. Right: isobologram plot showing synergistic effect of GSI and 4-OH-TAM in T47D:A18/PKCα cells. (**c**, left) Western blot of T47D:A18/PKCα cells treated with GSI, 4-OH-TAM and combination. The antibody sc-6014 (Notch-1 C-20, Santa Cruz Biotechnology, Dallas, TX, USA) detects transmembrane Notch-1 isoforms plus cleaved Notch-1 (Notch-1IC). (**c**, right) Densitometry measurements provide an assessment of the effect of GSI, TAM and combination treatments on the fraction of Notch-1IC with respect to total Notch. The Notch-1IC band identity was validated with two-color dual-antibody western blots (Supplementary Figures 1A and B). (**d**, left) Palpable tumors (average volume $0.28 \pm 0.05\ cm^3$) developed in 13 days after injection of cells into the axillary fat pad. On day 14, animals were randomized into control and test groups ($n = 8$) and treatment was started as follows: control (vehicle by gavage), TAM (TAM, 1.5 mg/day), GSI (100 mg/kg MRK003 by gavage) and TAM/GSI (1.5 mg TAM and 100 mg/kg MRK003). TAM was administered 5 days a week, whereas MRK003 was administered 3 days on, 4 days off to minimize intestinal toxicity. Tumors were measured twice a week using a Vernier caliper. One-way analysis of variance was used to examine tumor growth. A Tukey honestly significant difference test was conducted to examine pairwise comparisons of drug combinations. Western blots for Notch-1, HEY1 and Notch-4IC (64 kDa band, detected by Santa Cruz H-225 antibody) on tumors excised post treatment. Two tumors per treatment were examined. GSI treatment caused the appearance of a second Notch-1TM band (lower arrow), consistent with accumulation of S2 ADAM-cleaved intermediate. Notch-1IC was not visible. Notch-4IC was eliminated by MRK003 in one tumor and decreased in another, possibly due to individual differences in drug absorption between animals or different normal tissue contents in extracts. TAM/GSI eliminated Notch-4IC in both tumors examined, whereas TAM alone increased it, consistent with our previous observations.[12] HEY1 was decreased by GSI, especially in combination with TAM. Note that the tumor in which Notch-4IC was not completely eliminated also shows a smaller effect on HEY1.

Real-time qRT–PCR

Real-time qRT–PCR was performed as previously described.[27] See Supplementary Materials and methods for detail.

Quantitative chromatin immunoprecipitation analysis

Quantitative chromatin immunoprecipitation analysis was performed as previously described.[23,27]

Proliferation assays

Long-term proliferation assays were performed by manually counting viable cells using triplicate samples with a hemocytometer chamber and Trypan blue over 12 days from the start of treatment.

Cytotoxicity assays

Cytotoxicity and short-term proliferation in transiently transfected cells were assessed by measuring total cell mass using the crystal violet assay.[59]

DNA content measurement

Cellular DNA content was measured fluorometrically as previously described.[60]

RNA isolation, Affymetrix GeneChip Human Gene 1.0 ST Array, microarray analysis and bioinformatics

See Supplementary Materials and methods.

Xenograft tumor models and treatment

Four- to five-week-old nu/nu BALB/c ovariectomized female nude mice were from Harlan (Indianapolis, IN, USA). T47D:A18/PKCα or T47D:A18/neo tumors were established in mammary fat pad. Treatment began when average tumor volume was $0.28 \pm 0.05\,cm^3$. Mice were randomized into four groups ($n = 8$): (1) vehicle control; (2) TAM (1.5 mg TAM orally 5 days/week); (3) GSI (100 mg/kg MRK003 orally 3 days on, 4 days off); (4) TAM plus GSI (1.5 mg TAM and 100 mg/kg MRK003). Tumor volume was determined twice weekly. See Supplementary Materials and methods.

Statistical analysis

For pairwise comparisons, two-tailed unpaired Student's *t*-tests were used with $\alpha = 0.05$. When more than two samples were compared, one-way analysis of variance was used with $\alpha = 0.05$. A Tukey honestly significant difference post-hoc test was conducted to examine pairwise comparisons and determine statistical value. SPSS software (version 12.0 for Windows; SPSS Inc., Chicago, IL, USA) was used. For correlation analysis of public gene expression databases, we used the SAS software package (SAS version 9.1; SAS, Cary, NC, USA) to compute Pearson correlation coefficients. The authors declare no conflict of interest.

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