

Reactivation of cAMP Pathway by PDE4D Inhibition Represents a Novel Druggable Axis for Overcoming Tamoxifen Resistance in ER-positive Breast Cancer



Rasmi R. Mishra¹, Nevin Belder¹, Suhail A. Ansari¹, Merve Kayhan¹, Hilal Bal¹, Umar Raza¹, Pelin G. Ersan¹, Ünal M. Tokat¹, Erol Eyüpoğlu¹, Özge Saatci¹, Pouria Jandaghi^{2,3}, Stefan Wiemann⁴, Ayşegül Üner⁵, Caglar Cekic¹, Yasser Riazalhosseini^{2,3}, and Özgür Şahin^{1,6}

Abstract

Purpose: Tamoxifen remains an important hormonal therapy for ER-positive breast cancer; however, development of resistance is a major obstacle in clinics. Here, we aimed to identify novel mechanisms of tamoxifen resistance and provide actionable drug targets overcoming resistance.

Experimental Design: Whole-transcriptome sequencing, downstream pathway analysis, and drug repositioning approaches were used to identify novel modulators [here: phosphodiesterase 4D (PDE4D)] of tamoxifen resistance. Clinical data involving tamoxifen-treated patients with ER-positive breast cancer were used to assess the impact of PDE4D in tamoxifen resistance. Tamoxifen sensitization role of PDE4D was tested *in vitro* and *in vivo*. Cytobiology, biochemistry, and functional genomics tools were used to elucidate the mechanisms of PDE4D-mediated tamoxifen resistance.

Results: PDE4D, which hydrolyzes cyclic AMP (cAMP), was significantly overexpressed in both MCF-7 and T47D tamoxifen-resistant (TamR) cells. Higher PDE4D expression pre-

dicted worse survival in tamoxifen-treated patients with breast cancer ($n = 469$, $P = 0.0036$ for DMFS; $n = 561$, $P = 0.0229$ for RFS) and remained an independent prognostic factor for RFS in multivariate analysis ($n = 132$, $P = 0.049$). Inhibition of PDE4D by either siRNAs or pharmacologic inhibitors (dipyridamole and Gebr-7b) restored tamoxifen sensitivity. Sensitization to tamoxifen is achieved via cAMP-mediated induction of unfolded protein response/ER stress pathway leading to activation of p38/JNK signaling and apoptosis. Remarkably, acetylsalicylic acid (aspirin) was predicted to be a tamoxifen sensitizer using a drug repositioning approach and was shown to reverse resistance by targeting PDE4D/cAMP/ER stress axis. Finally, combining PDE4D inhibitors and tamoxifen suppressed tumor growth better than individual groups *in vivo*.

Conclusions: PDE4D plays a pivotal role in acquired tamoxifen resistance via blocking cAMP/ER stress/p38-JNK signaling and apoptosis. *Clin Cancer Res*; 1–15. ©2018 AACR.

Introduction

More than 70% of breast tumors express estrogen receptor alpha (ER α) and consequently receive various endocrine therapies modulating ER α (1, 2). ER-targeted therapies include (i) selective ER modulators (SERMs) like tamoxifen and raloxifene,

(ii) aromatase inhibitors (AI) such as letrozole, anastrozole, and exemestane, and (iii) selective ER downregulators (SERDs) like fulvestrant. Tamoxifen is widely used as the standard first-line adjuvant therapy since its discovery in 1970 for treatment of patients with ER-positive breast cancer, particularly in premenopausal women (3, 4). Several clinical trials have showed that treatment with adjuvant tamoxifen for at least 5 years reduces the breast cancer recurrence and mortality rates by around half and one-third, respectively, throughout the first 15 years (3–5). However, approximately 20%–30% cases of high risk, advanced ER-positive breast cancer develop *de novo* or acquired resistance to tamoxifen, despite its clinical success (6).

The mechanism of tamoxifen resistance is partially understood and involves multiple factors. Most common mechanisms include the activation of several receptor tyrosine kinases (RTKs), for example, EGFR, HER2, fibroblast growth factor receptor 1 (FGFR1), and insulin-like growth factor-1 receptor (IGF-1R). This leads to enhanced activity of kinases downstream of these receptors such as extracellular-regulated kinase (ERK) 1/2, AKT, and p21-activated kinase-1 (PAK1), and promotes tumor growth and metastasis (7, 8). Furthermore, altered expressions of cell survival molecules such as c-Myc, Bad, and Bcl-2 are correlated with poor

¹Department of Molecular Biology and Genetics, Faculty of Science, Bilkent University, Ankara, Turkey. ²Department of Human Genetics, McGill University, Montreal, Quebec, Canada. ³McGill University and Genome Quebec Innovation Centre, Montreal, Quebec, Canada. ⁴Division of Molecular Genome Analysis, German Cancer Research Center (DKFZ), Heidelberg, Germany. ⁵Department of Pathology, Faculty of Medicine, Hacettepe University, Ankara, Turkey. ⁶National Nanotechnology Research Center (UNAM), Bilkent University, Ankara, Turkey.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Corresponding Author: Özgür Şahin, Bilkent University, Department of Molecular Biology and Genetics, Faculty of Science, B Building SB-247, Ankara 06800, Turkey. Phone: 90-312-290-2402; Fax: 90-312-266-5097; E-mail: sahinogzur@gmail.com

doi: 10.1158/1078-0432.CCR-17-2776

©2018 American Association for Cancer Research.

Translational Relevance

Tamoxifen remains an important hormonal therapy for patients with ER-positive breast cancer; however, development of resistance is a major obstacle for therapeutic success. Therefore, there is an urgent need for identification of novel druggable targets overcoming resistance. In this study, we identified phosphodiesterase 4D (PDE4D)/cAMP/ER stress axis as a novel tamoxifen resistance mediator in ER-positive breast cancer. Tamoxifen-treated patients with breast cancer with higher PDE4D expression showed poorer distant metastasis and relapse-free survival, and higher PDE4D expression remained an independent prognostic factor in multivariate analysis. Moreover, inhibition of PDE4D by either siRNAs or pharmacologic inhibitors restored tamoxifen sensitivity both *in vitro* and *in vivo*. Overall, our study uncovered a cAMP pathway, specifically PDE4D, as a critical modulator of tamoxifen resistance, and thus paves the way for testing cAMP inducers, some of which are already FDA approved such as dipyridamole and the over-the-counter drug acetylsalicylic acid (aspirin), in tamoxifen-refractory patients with ER-positive breast cancer.

survival of patients under endocrine therapy (9, 10). In addition, involvement of noncoding RNAs, such as microRNAs (miRNAs) and long noncoding RNAs (lncRNA), in tamoxifen resistance has been described. For example, several oncogenic miRNAs, for example, miR-519 and miR-221/222 confer tamoxifen resistance (11, 12), while reexpression of miR-375, let-7, or miR-342 induce tamoxifen sensitivity via their respective mRNA target genes (11, 12). Recently, upregulation of a few lncRNAs, for example, HOTAIR and BCAR4, has also been shown to confer tamoxifen resistance (11).

With nearly 1.2 million new ER-positive breast cancer cases diagnosed in 2012 worldwide (<http://www.wcrf.org/>) and significant number of cases developing inevitable drug resistance, there is an immediate need for identification of novel druggable targets to overcome tamoxifen resistance. Here, we aim to identify novel mechanisms of tamoxifen resistance and thus pave the way for testing new combinations to improve survival in tamoxifen refractory, ER-positive breast cancer.

Materials and Methods

Cell culture

Human breast cancer cell lines MCF-7 and T47D were purchased from ATCC and cultured in phenol red-free DMEM (Gibco) with 10% FBS, 0.1% insulin, 50 U/mL penicillin/streptomycin, 1% nonessential amino acids (Gibco). Tamoxifen-resistant MCF-7 cells (MCF-7 TamR) were generated as described previously (12). In brief, MCF-7 and T47D TamR cells were grown in the presence of 5 μ mol/L of 4-hydroxytamoxifen (Sigma-Aldrich) for 1 year. In parallel, parental MCF-7 and T47D cells were maintained under identical conditions without tamoxifen. Cells were routinely tested for mycoplasma contamination using MycoAlert detection kit (Lonza), and were authenticated by STR sequencing (Promega) at German Cancer Research Center (DKFZ). The cumulative culture length of the cells between thawing and use in the study was less than 20 passages.

Reagents and chemicals

Reagents and other chemicals including their catalog numbers are as following: tamoxifen (Sigma, T176), acetylsalicylic acid (Sigma, A5376), dipyridamole (Tocris, 691), Gebr-7b (Millipore/Calbiochem, 524748), 6-Bnz-cAMP sodium salt (Tocris, 5255), 8-pCPT-2-O-Me-cAMP-AM (Tocris, 4853), cAMPSp triethylammonium salt (Tocris, 1333) and thapsigargin (Sigma, T9033), and SQ22536 (Sigma, AB120642).

Whole-transcriptome sequencing (RNA-Seq) and data analysis

Ribosomal RNA (rRNA)-depleted libraries were generated for each sample, and these sequences were multiplexed. RNA sequencing was performed for each condition (MCF-7 parental and MCF-7 TamR) in triplicates using the Illumina HiSeq 2000 platform at McGill University and Genome Quebec Innovation Centre. Details are provided in Supplementary Data.

Generation of TamR gene signature and bioinformatics analysis

Tamoxifen resistance gene signature (TamR-GS) was generated comprising of the top 417 differentially expressed mRNAs (\log_2 FC ± 2 and $P \leq 0.05$) between MCF-7 TamR cells and their parental counterparts. For the calculation of the TamR-GS score, the sum of z-scores of the downregulated genes in the TamR-GS was subtracted from the sum of z-scores of the upregulated genes for each patient. This method of calculating a gene signature score by using the mRNA expression data has been reported previously (13). In survival analysis and GSEA, patients were separated either from the median or from 25th percentile, as low and high PDE4D or TamR-GS scorers based on their PDE4D expression or the TamR-GS score, respectively. Details of the bioinformatic analysis are provided in Supplementary Data.

qRT-PCR analysis

Total RNA was extracted from cultured cells using TRIzol (BioLine), and cDNAs were generated using RevertAid RT Reverse Transcription Kit (Life Technologies). qRT-PCR analysis was performed with gene-specific primers using LightCycler 480 SYBR Green I Master kit (Roche). *HPRT1*, *GAPDH*, and *ACTB* were used as housekeeping genes. The average C_t value was calculated from triplicates of each sample, and the relative mRNA expression was determined. Sequences of the qRT-PCR primers are listed in Supplementary Table S1.

Transient transfection with siRNAs and overexpression vectors

PDE4D-specific siRNAs were purchased from Dharmacon (Supplementary Table S2). Transfections were done with 20 nmol/L siRNA using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. PDE4D cDNA (NM_006203.4) present in pcDNA3.1+/C-(K) DYK mammalian expression vector (GenScript) was used for overexpression experiments. Briefly, 50 ng (for 96-well plate) or 500 ng (for 6-well plate) of PDE4D plasmid DNA was transiently transfected using Effectene transfection reagent (Qiagen).

Inhibitor treatments, cell proliferation and apoptosis assays

Details of the inhibitor treatments, cell proliferation, and apoptosis assays are provided in Supplementary Materials and Methods section in Supplementary Data.

Western blotting

Total protein was extracted using RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris base pH 8.0, 1 mmol/L EDTA, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, 1 mmol/L DTT, and 1 mmol/L Na_3VO_4) supplemented with Complete Protease Inhibitor (Roche). Protein concentrations were measured using BCA Protein Assay (Thermo Scientific). Equal amounts of protein lysates (15–20 μg) were separated on a 10% SDS-PAGE, transferred onto polyvinylidene difluoride membrane (Bio-Rad) and incubated with primary antibodies (Supplementary Table S3). The blots were developed using enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences) after incubation with horseradish peroxidase-conjugated secondary antibody. β -Actin was used as a loading control.

Intracellular cAMP measurements

To measure intracellular cAMP levels, 10,000 cells were seeded per well in 96-well plates. Following day, cells were treated with PDE4D inhibitors (dipyridamole or Gebr-7b or aspirin) alone or vehicle-only or in combination with tamoxifen (5.0 $\mu\text{mol/L}$) for 20 minutes. After treatment, cells were washed with PBS and incubated with 100- μL ice-cold 0.6 mol/L perchloric acid followed by 15 μL 2.5 mol/L potassium carbonate for neutralization. Cells were incubated on ice for 30 minutes and then centrifuged at 13,000 rpm to collect the clear lysate. Twenty microliters of the clear lysate was used for measurement of cAMP concentration with the LANCE Ultra cAMP detection reagents (PerkinElmer) according to the manufacturer's protocol.

Mouse xenograft studies

All animal work was approved by the Institutional Animal Care and Use Committee of Bilkent University. In xenograft studies, female athymic nude mice were used. All mice were maintained under a temperature-controlled environment with a 12-hour light/dark cycle and received a standard diet and water *ad libitum*. Primary tumors with MCF-7 TamR cells were developed in 6- to 8-week-old mice. Slow release estradiol pellets (0.36 mg, 60 days; Innovative Research of America) were implanted subcutaneously (s.c) at the nape of the neck one day before injection. A total of 1×10^7 MCF-7 TamR cells were injected subcutaneously into both left and right sides of mammary fat pads. Tumor growth was regularly monitored, and size measurements were performed two times per week. Tumor volume was calculated using the formula $(\text{length} \times \text{width}^2)/2$. After tumor size reached 100 mm^3 , mice were randomly divided into 8 groups, with 5 tumors per group. Animals were treated with vehicle, tamoxifen (2 mg/kg in corn oil, by oral gavage), dipyridamole (15 mg/kg in PBS: PEG 400 (1: 1), daily intraperitoneally), Gebr-7b (3 $\mu\text{g/kg}$ in 0.5 % methyl 2-hydroxyethyl cellulose+0.005% DMSO, daily via intraperitoneally), aspirin [100 mg/kg in PBS: PEG 400 (1: 1), daily intraperitoneally] or combinations. The body weight and tumor size of each mouse were recorded twice a week. Mice were sacrificed 28 days after initiation of the treatment, and the tumors were collected and stored for subsequent analysis.

IHC

Tumor samples isolated from the MCF-7 TamR xenografts were fixed in 10% formalin and processed into paraffin blocks. Histologic sections were taken with 5- μm thickness and deparaffinized. All sections were prepared for Haematoxylin & Eosin, Ki-67 (Dako, catalog no: 7240) and ApopTag Fluorescein *In Situ* Apo-

ptosis Detection Kit (Millipore SiGMA), staining according to the standard procedures.

Statistical analysis

All statistical analyses were performed using either Student *t* test or one-way ANOVA (multiple comparisons) in GraphPad Prism 6 (GraphPad Software, Inc). All results were represented as mean \pm standard deviation (SD) and obtained from three independent experiments. The Kaplan–Meier survival plot, HR, and log-rank *P* values were calculated and plotted using GraphPad Prism. The differences were considered statistically significant if $P < 0.05$ and indicated by *, $P < 0.05$; **, $P < 0.01$.

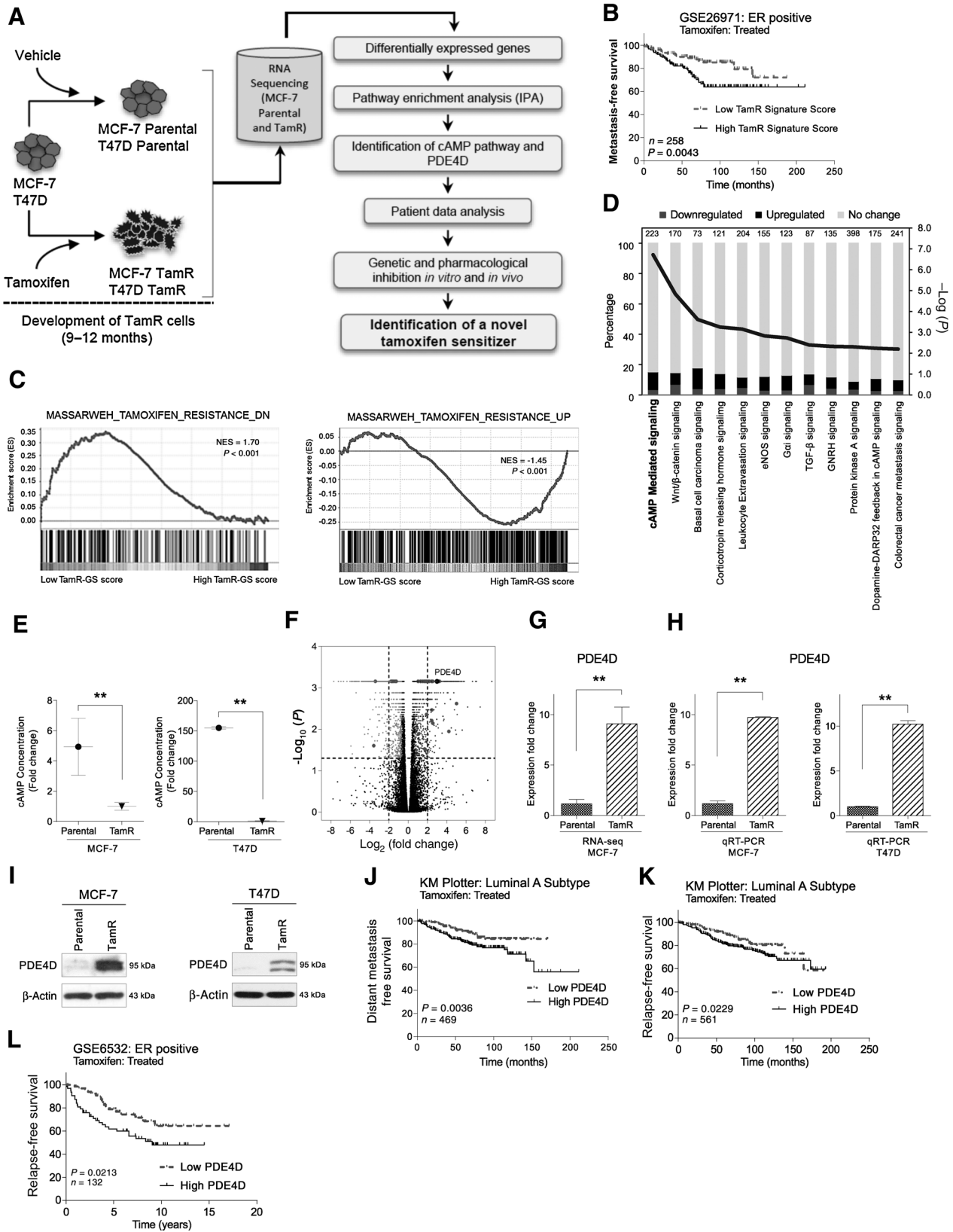
Results

Whole-transcriptome sequencing combined with pathway enrichment and patient data analyses identify PDE4D as a potential mediator of tamoxifen resistance.

We had previously developed, characterized, and reported an acquired tamoxifen-resistant model of MCF-7 cell line (MCF-7 TamR cells and their sensitive counterpart MCF-7 parental) to identify miRNA regulators of tamoxifen resistance (12, 14). To further understand the molecular underpinnings of tamoxifen resistance, and to identify novel druggable targets in ER-positive breast cancer, we performed whole-transcriptome sequencing in these cells, and derived a tamoxifen resistance gene signature (TamR-GS) comprising the most differentially expressed mRNAs ($\log_2\text{FC} \pm 2$ and $P \leq 0.05$; 417 genes; Supplementary Table S4) between sensitive and resistant cells (Fig. 1A). To examine the clinical relevance of our tamoxifen-resistant cell line and the TamR-GS, we applied this signature to a gene expression profiling data of tumors obtained from patients treated with tamoxifen (GEO dataset GSE26971), and assigned each patient a TamR-GS score by subtracting the sum of z-scores of the downregulated genes in the TamR-GS from the sum of z-scores of the upregulated genes (see Materials and Methods for details). We observed that the patients with high TamR-GS scores showed poor metastasis-free survival (MFS) as compared with their counterparts with low TamR-GS score (Fig. 1B), confirming the clinical relevance of our cell line. Furthermore, we performed gene-set enrichment analysis (GSEA) after separating tamoxifen-treated, ER-positive patients from GSE22220 as low or high TamR-GS scorers and found that genes downregulated in tamoxifen-resistant xenografts were significantly enriched in patients having low TamR-GS scores while genes upregulated in the same xenografts were significantly enriched in patients having high TamR-GS scores, further supporting the validity of our tamoxifen-resistant cells (Fig. 1C). Ingenuity Pathway Analysis (IPA) indicated that the most significantly enriched canonical pathway in MCF-7 TamR cells compared with their parental cells was cAMP-mediated signaling ($P < 8.77 \times 10^{-8}$; Fig. 1D). Therefore, we measured the cellular cAMP levels in sensitive and resistant derivatives of MCF-7 and of an additional cell line, T47D, which has recently been developed and characterized in our laboratory (Supplementary Fig. S1A–S1D). We observed that the TamR derivatives of both cell lines have markedly lower cellular cAMP levels compared with their sensitive counterparts (Fig. 1E). These observations confirmed our pathway analysis results, and suggest a possible involvement of cAMP pathway in acquired tamoxifen resistance.

Investigating the mechanisms responsible for the observed deregulation of cAMP pathway, we examined the expression of genes associated with this pathway and observed that majority of

Mishra et al.



these genes (25 of 33 with differential expression) are upregulated in TamR cells (big gray circles in Fig. 1F and PDE4D as black circle), and three of them were validated by qRT-PCR (Supplementary Fig. S2A). Two phosphodiesterases; PDE10A (32.4-fold; $P < 0.002$) and PDE4D (9.1-fold; $P < 0.0007$) were among the most significantly upregulated genes in MCF-7 TamR cells (Supplementary Fig. S2A; Fig. 1G), which are amenable for therapeutic intervention. PDE10A can hydrolyze both cAMP and cGMP, whereas PDE4D is a cAMP-specific phosphodiesterase and thus both are important regulators of cAMP signaling (15). However, siRNA-mediated silencing of PDE10A in MCF-7 TamR did not restore tamoxifen sensitivity (Supplementary Fig. S2B). On the other hand, qRT-PCR and Western blotting (WB) validated the substantial upregulation of PDE4D in both MCF-7 TamR and T47D TamR cells compared with their sensitive counterparts (Fig. 1H and I), and siRNA-mediated knockdown resulted in tamoxifen sensitization as described below. Therefore, we focused on the role of PDE4D, but not that of PDE10A, in tamoxifen resistance.

To determine whether expression of PDE4D is associated with tamoxifen resistance in breast cancer, we first examined PDE4D expression in tamoxifen-treated luminal A breast cancer patients ($n = 469$) using KM plotter tool (16). We found a significant association between higher PDE4D expression and poorer distant metastasis-free survival (DMFS; $P = 0.0036$; $n = 469$) in tamoxifen-treated patients with luminal A breast cancer (Fig. 1J). We also found a significant association between higher PDE4D expression and poorer relapse-free survival (RFS; $P = 0.029$; $n = 561$) in tamoxifen-treated patients (Fig. 1K and L). Particularly, prognostic effect of PDE4D expression was specific to tamoxifen treatment, as high or low PDE4D expression alone could not significantly predict survival in systemically untreated (no treatment received) luminal A patients (Supplementary Fig. S3A and S3B) or patients who did not receive endocrine therapy, but may have received chemotherapy (Supplementary Fig. S3C and D). Notably, PDE4D expression remained an independent prognostic factor in the multivariate analysis by using an expression profiling dataset of tamoxifen-treated patients with breast cancer, GSE6532 (Supplementary Table S5). Overall, our results demonstrate that there is an inverse correlation between cellular cAMP and PDE4D levels, and PDE4D expression has an independent prognostic role in tamoxifen-treated patients with luminal A breast cancer. These results suggested that PDE4D might regulate tamoxifen resistance in breast cancer.

Genetic or pharmacologic inhibition of PDE4D resensitizes tamoxifen-resistant breast cancer cells *in vitro*

To test whether modulating PDE4D expression can overcome tamoxifen resistance, siRNA-mediated knockdown of PDE4D was

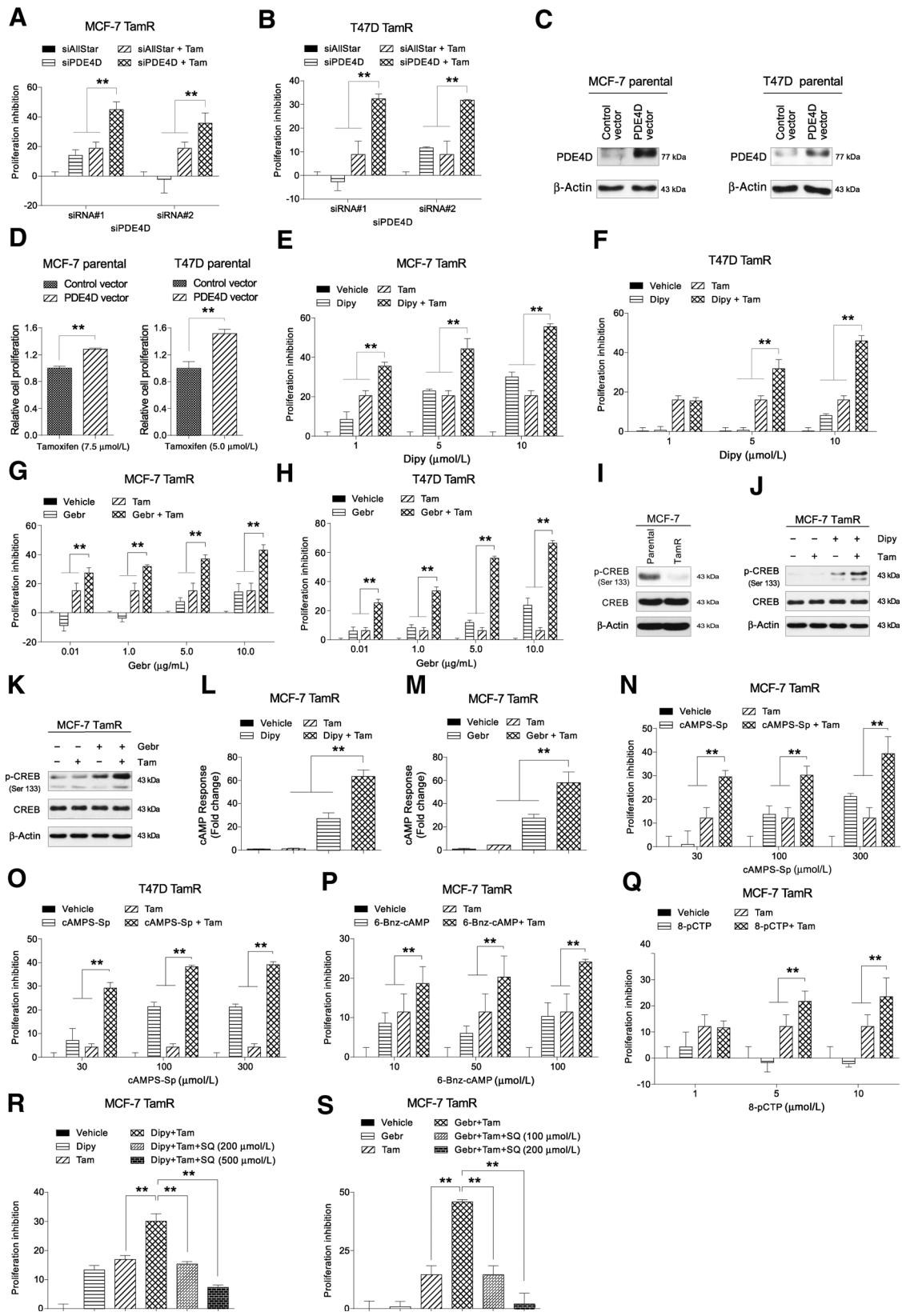
carried out in both MCF-7 TamR and T47D TamR cells using two different siRNA sequences, and cell proliferation was assessed. The efficiency of PDE4D knockdown was validated by qRT-PCR and Western blot analysis (Supplementary Fig. S4A and S4B). Silencing of endogenous PDE4D restored sensitivity to tamoxifen in both TamR cells (Fig. 2A and B). To further test whether PDE4D could drive tamoxifen resistance *in vitro*, a PDE4D overexpression construct was transiently transfected into MCF-7 and T47D parental cells (Fig. 2C), and these cells were subjected to a cell proliferation assay, which showed significantly increased cell proliferation in PDE4D-transfected cells as compared with control cells (empty vector) in the presence of tamoxifen (Fig. 2D). Next, we sought to examine the effects of pharmacologic inhibition of PDE4D via PDE4D inhibitors (PDE4Is) on tamoxifen sensitization by using Gebr-7b, a highly potent and specific PDE4D inhibitor (17), and dipyridamole (Dipy), a nonselective PDE inhibitor predicted to be a PDE4D inhibitor in our Ingenuity Pathway Analysis (IPA). To examine the effects of pharmacologic inhibition of PDE4D on tamoxifen sensitization, MCF-7 TamR, and T47D TamR cells were treated with dipyridamole or Gebr-7b in the absence and presence of tamoxifen. Combinatorial treatment (tamoxifen with either of the examined PDE4Is) inhibited cell growth in a dose-dependent manner for both dipyridamole (Fig. 2E and F) and Gebr-7b (Fig. 2G and H) in both TamR cell lines. Notably, among the two PDE4Is, Gebr-7b was more potent and showed stronger proliferation inhibitory effect together with tamoxifen at doses as low as 10 ng/mL, which is in agreement with the selective inhibitory effect of Gebr-7b on PDE4D. Altogether, our results demonstrate that inhibition of PDE4D activity acts as a tamoxifen sensitizer in tamoxifen-resistant breast cancer cells.

Elevation of cAMP levels overcomes tamoxifen resistance and is necessary for PDE4D inhibition-mediated tamoxifen sensitization

cAMP binds to and activates cAMP-dependent protein kinase A (PKA) and Rap guanine nucleotide-exchange factor 3 (RAP-GEF3), more commonly known as EPAC (exchange protein directly activated by cAMP; refs. 18, 19). Furthermore, cAMP induction and thus activated PKA and EPAC induce phosphorylation of the transcription factor cAMP response element-binding protein (CREB) at Ser133 (20). In line with these reports, we observed significantly higher levels of phosphorylated CREB (p-CREB) in parental cells compared with TamR cells (Fig. 2I) corresponding to higher cAMP levels (Fig. 1E). As PDE4D is a cAMP-specific phosphodiesterase, we tested whether pharmacologic inhibition of PDE4D increases cellular cAMP levels and increases the phosphorylation of CREB. We observed an increase

Figure 1.

Whole-transcriptome sequencing combined with pathway enrichment and patient data analyses identify PDE4D as a potential mediator of tamoxifen resistance. **A**, Workflow of the identification and validation of PDE4D as a novel modulator of tamoxifen resistance. Whole-transcriptome analysis followed by pathway enrichment, *in vitro*, and *in vivo* assays and analyses of clinical data identify cAMP pathway and its component PDE4D as novel regulator of tamoxifen resistant in MCF-7 cells. **B**, Association of high and low TamR signature score with metastasis-free survival (MFS) in patients with breast cancer treated with tamoxifen validating the clinical relevance of our TamR cell line. **C**, Enrichment plots of tamoxifen-treated ER-positive patient tumors from GSE22226 with low and high TamR-GS scores. **D**, Ingenuity Pathway Analysis (IPA) of significantly enriched pathways (z -score ≥ 1) in MCF-7 TamR cells compared with the parental MCF-7 cells. **E**, Intracellular levels of cAMP in parental MCF-7 and T47D cells compared with MCF-7 TamR and T47D TamR cells, respectively. **F**, Volcano plot showing significantly differentially expressed genes in MCF-7 TamR cells compared with parental ones. The genes belonging to cAMP signaling are shown as big gray circles and PDE4D is highlighted with a big black circle. **G**, PDE4D expression in MCF-7 TamR cells compared with parental MCF-7 cells in RNA-Seq data. FPKM values were used to calculate expression fold change in RNA-Seq data. **H**, qRT-PCR validations of PDE4D upregulation in TamR cells. **I**, Western blot analysis of PDE4D expression in parental and TamR MCF-7 and T47D cells. β -Actin was used as a loading control. **J–L**, DMFS (**J**), and RFS (**K**), and RFS (**L**; univariate analysis) of patients with luminal A breast cancer who underwent tamoxifen therapy with respect to the PDE4D expression.



in the phosphorylated CREB (Fig. 2J and K) and intracellular cAMP levels (Fig. 2L and M; Supplementary Fig. S5A and S5B) in both MCF-7 and T47D TamR cells when treated with dipyrindamole or Gebr-7b. Interestingly addition of tamoxifen along with dipyrindamole or Gebr-7b led to further increase in p-CREB and cAMP levels (Fig. 2J–M).

Next, we tested whether elevated cAMP levels by PDE4D inhibition will sensitize resistant cells to tamoxifen by using stable cell-permeable cAMP analogues (21). Treatment with general cAMP analogue, cAMPSp, triethylammonium salt (cAMPSp) resulted in sensitization of both MCF-7 TamR and T47D TamR cells to tamoxifen in a dose-dependent manner (Fig. 2N and O). Treatment with PKA-specific analogue (6-Bnz-cAMP sodium salt) or EPAC-specific analogue (8-pCPT-2-O-Me-cAMP-AM) also sensitized cells to tamoxifen in a dose-dependent manner (Fig. 2P and Q). To further prove that the sensitizer role of PDE4D in tamoxifen resistance is due to its ability to increase cAMP levels, MCF-7 TamR cells were pretreated with SQ22536, a specific adenylyl cyclase inhibitor, to prevent cAMP accumulation even in the presence of PDE4D inhibitors and tamoxifen. SQ22536 completely reversed the sensitization of resistant cells by both dipyrindamole (Fig. 2R) and Gebr-7b (Fig. 2S) in a dose-dependent manner. Overall, these results suggest that PDE4D reduces intracellular cAMP to cause tamoxifen resistance.

Activation of stress-related kinases downstream of cAMP leads to apoptosis and tamoxifen sensitization

Elevated cAMP levels are known to activate the stress-related kinases (p38 and JNK) and subsequently lead to phosphorylation and activation of CREB (18, 22). Importantly, PI3K pathway crosstalks with cAMP signaling (23), and activated AKT was shown to play a critical role in endocrine resistance (14). Therefore, we initially examined the basal expression and phosphorylation levels of JNK, p38/SAPK and AKT in parental and tamoxifen-resistant cells. Western blot analysis showed that phosphorylation levels of JNK and p38 were significantly reduced while those of AKT were upregulated in TamR cells compared with the parental ones in both MCF-7 and T47D cells (Fig. 3A; Supplementary Fig. S6A). To test the effects of elevated cAMP on the phosphorylation status of JNK and p38 proteins, MCF-7 TamR cells were treated with PDE4D siRNA or cAMP analogue (cAMPSp). Elevated levels of cAMP resulted in activation of JNK and p38 proteins. Interestingly, p-JNK and p-p38 signals were further enhanced in the treatment groups combined with tamoxifen (Fig. 3B and C). Inhibition of PDE4D via treatment with dipyrindamole or Gebr-7b also resulted in similar increase in p-JNK and p-p38 as

in case of cAMP-Sp or PDE4D siRNA (Fig. 3D and E). Significant decrease in phosphorylated AKT (both Thr-308 and Ser-473) levels was observed upon treatment with PDE4D siRNA, cAMPSp, dipyrindamole, and Gebr-7b, especially when combined with tamoxifen (Fig. 3B–E). These results suggest that elevated cAMP levels upon PDE4D inhibition activates JNK and p38 signaling and inhibits AKT activation.

As AKT pathway is a major regulator of cell survival, and PDE4D is a potential oncogenic protein regulating cancer cell proliferation and apoptosis (24), apoptosis levels were evaluated where MCF-7 TamR cells were treated with dipyrindamole or Gebr-7b alone or in combination with tamoxifen for 72 hours. Pharmacologic inhibition of PDE4D in combination with tamoxifen activated cleavage of caspase-7 and PARP proteins and induced apoptosis (Fig. 3F–I). Finally, treatment with JNK inhibitor SP600125 or p38 inhibitor SB203580 completely reversed the measurable effects of PDE4D on proliferation inhibition and sensitization to tamoxifen, confirming the mediatory roles of JNK or p38 pathways in this context (Fig. 3J and K).

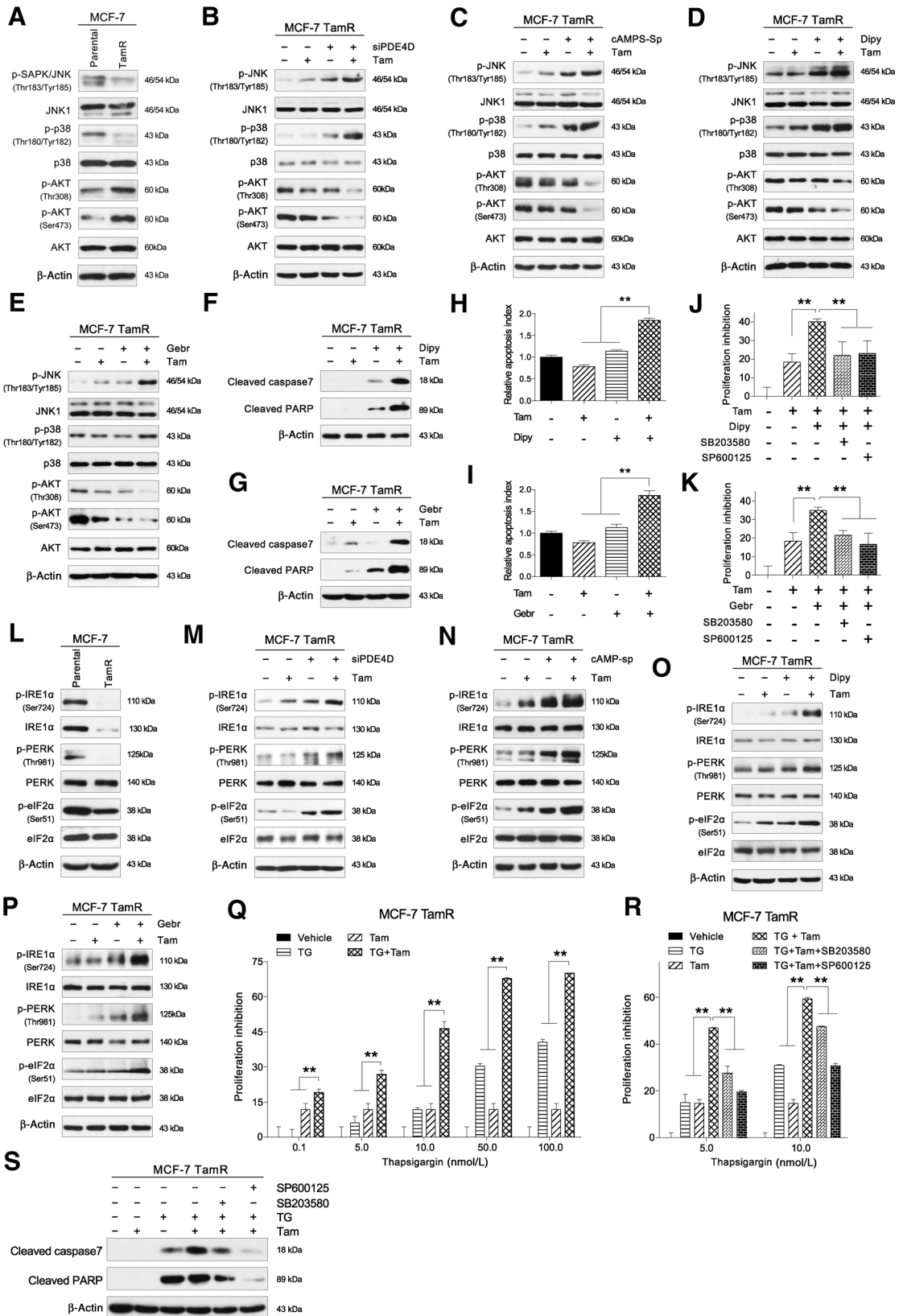
PDE4D inhibition-mediated cAMP induction leads to ER stress conferring tamoxifen sensitivity

One of the well-known mediators of p38/JNK-induced apoptosis is activation of unfolded protein response (UPR)/ER stress pathway (25). Therefore, we examined whether elevated PDE4D activity regulates ER stress response to cause tamoxifen resistance. We observed that activated inositol-requiring enzyme 1 α (IRE1 α), protein kinase RNA-like ER kinase (PERK), and eukaryotic translation initiator factor 2 α (eIF2 α) levels were significantly reduced in both MCF-7 TamR and T47D TamR cells compared with their parental counterparts (Fig. 3L; Supplementary Fig. S6B). We then examined possible consequences of PDE4D inhibition on ER stress pathway. Notably, treatment with PDE4D siRNAs or with a cell-permeable cAMP analogue (cAMPSp) resulted in activation of IRE1 α , PERK and eIF2 α proteins, especially when combined with tamoxifen (Fig. 3M and N). Similar results were obtained with pharmacologic inhibition of PDE4D with dipyrindamole or Gebr-7b (Fig. 3O and P).

To further prove that induction of ER stress is directly involved in tamoxifen sensitization, we investigated the effects of a well-known ER stress inducer thapsigargin (TG) (26) on tamoxifen sensitization in MCF-7 TamR cells. While a certain level of proliferation inhibition was observed with thapsigargin alone, combination of thapsigargin with tamoxifen further inhibited cell proliferation (Fig. 3Q). Importantly, the blockade of JNK or p38 pathways by SB203580 or SP600125 reversed the combinatorial

Figure 2.

Inhibition of PDE4D overcomes tamoxifen resistance and increasing cAMP levels is necessary for PDE4D inhibition-mediated tamoxifen sensitization. **A** and **B**, Cell proliferation of tamoxifen-resistant cells MCF-7 TamR (**A**) and T47D TamR (**B**) transfected with two different siRNA sequences against PDE4D in the absence and presence of tamoxifen. The concentrations of tamoxifen used were 7.5 μ M/L and 5.0 μ M/L for MCF-7 TamR and T47D TamR cells, respectively. **C**, PDE4D overexpression was detected using DYKDDDDK tagged antibody in parental MCF-7 and T47D cells. Predicted molecular weight of overexpressed protein is 77 kDa (**D**) Cell proliferation after ectopic expression of PDE4D in parental MCF-7 and T47D cells. **E–H**, Tamoxifen sensitization with pharmacologic PDE4D inhibition with different doses of PDE inhibitor (dipyrindamole) and PDE4D-specific inhibitor (Gebr-7b) in MCF-7 TamR (**E** and **G**) and T-47D TamR (**F** and **H**) cells. **I**, Western blot analysis showing reduced p-CREB in MCF-7 TamR cells relative to parental cells. β -Actin was used as a loading control. **J** and **K**, Western blot analysis of p-CREB in MCF-7-TamR cells treated with tamoxifen (5.0 μ M/L) in combination with PDE inhibitor (dipyrindamole; **J**) and PDE4D inhibitor (Gebr-7b; **K**), respectively. **L–M**, Intracellular cAMP levels in MCF-7 TamR cells upon treatment with tamoxifen (5.0 μ M/L) in combination with PDE inhibitor (dipyrindamole) (**L**) and PDE4D inhibitor (Gebr-7b; **M**), respectively. **N–Q**, Tamoxifen sensitization with different doses of general cAMP analogue (cAMP-Sp; **N** and **O**), PKA-specific analogue (6-Benz-cAMP; **P**) and EPAC-specific analogue (8-pCPT-2-O-Me-cAMP; **Q**) in the presence of 7.5 μ M/L tamoxifen. **R** and **S**, Cell proliferation of the resistant MCF-7 cells pretreated with SQ22536, a specific cAMP inhibitor, in combination with tamoxifen and PDE inhibitor (dipyrindamole; **R**) and PDE4D inhibitor (Gebr-7b; **S**). Dipy: dipyrindamole, Gebr: Gebr-7b, 8-pCPT: 8-pCPT-2-O-Me-cAMP.



effect of thapsigargin and tamoxifen on the proliferation inhibition (Fig. 3R) and apoptosis (Fig. 3S), establishing JNK/p38 signaling as the mediator of ER stress-induced tamoxifen sensitization. Taken together, our results demonstrate that pharmacologic blockade of PDE4D elevates cAMP levels, which results in cAMP-dependent activation of ER stress downstream kinases, leading to apoptosis in tamoxifen-resistant cells.

Pharmacologic blockade of PDE4D by dipyrindamole or Gebr-7b overcomes tamoxifen resistance *in vivo*

To examine whether targeting PDE4D overcomes tamoxifen resistance *in vivo*, xenografts using tamoxifen resistant MCF-7 TamR cells were generated. The dipyrindamole or Gebr-7b combination with tamoxifen inhibited the growth of the MCF-7 TamR tumors in nude mice compared to the vehicle or single-agent treatments (Fig. 4A and B). Tumor weights were also significantly less in combination-treated groups as compared with vehicle or individual treatments (Fig. 4C). While the expression level of Ki-67, a marker of cell proliferation, was markedly reduced, fluorescence-based TUNEL assay showed an increased apoptosis in combination treatments with tamoxifen and PDE4Ds (Fig. 4D and E). These results demonstrate that pharmacologic inhibition of PDE4D results in tamoxifen sensitization in resistant tumors *in vivo*.

Aspirin overcomes tamoxifen resistance via increasing cAMP levels and activation of ER stress-mediated p38/JNK signaling leading to apoptosis

Although there are several resistance mechanisms put forward for tamoxifen resistance, one of the major challenges is to identify druggable targets which may help rapid translation into clinic. Our IPA results predicted that acetylsalicylic acid (ASA), commonly known as aspirin, could be a potential PDE4D inhibitor. Analysis of disease-associated and drug-induced phenotypes in terms of their differential gene expression profiles can predict the cytotoxic effect of a drug on the model system being used. Therefore, we analyzed our TamR-GS based on its correlation with the aspirin-responsive genes (provided in Connectivity map analysis; ref. 27). As shown in Fig. 5A, the genes upregulated in our TamR-GS are significantly downregulated and vice versa by aspirin treatment in MCF-7 cells ($P = 0.0004$; details are given in Methods). This suggests that resistance phenotype can be reversed by aspirin treatment, that is, aspirin might be repositioned to treat

tamoxifen resistance. Importantly, such a negative correlation was not observed with other COX inhibitors and lapatinib, an EGFR/HER2 inhibitor, ($P = 0.053$, $P = 0.655$, $P = 0.12$ for piroxicam, diclofenac, and lapatinib, respectively), suggesting that the therapeutic effect of aspirin on tamoxifen resistance might not rely on its inhibitory action on COX pathway. Rather, it could be due to COX-independent activities, one of which could be PDE4D inhibition as predicted by IPA. We further tested potential clinical effects of aspirin treatment by evaluating the correlation between TamR-GS and the expression of aspirin-responsive genes generated from GSE76583 (28) in patients treated with tamoxifen. We observed a significant negative correlation between the expression of TamR-GS and aspirin-responsive genes in tamoxifen-treated patients ($r = -0.21$, $P = 0.0004$; Fig. 5B). Our analysis suggests that tamoxifen-resistant patients could potentially be responsive to aspirin treatment by restoring aspirin-responsive genes which are expressed at low levels. All these results prompted us to test whether aspirin can act as tamoxifen sensitizer, as seen with PDE4D inhibition. Therefore, we treated both MCF-7 TamR and T47D TamR cells with increasing concentration of aspirin alone or in combination with tamoxifen for 72 hours. Aspirin significantly resensitized both MCF-7 TamR and T47D TamR cells to tamoxifen (Fig. 5C and D). We also observed that cellular cAMP (Fig. 5E; Supplementary Fig. S7) and p-CREB levels (Fig. 5F) were increased substantially upon treatment with aspirin in combination with tamoxifen in both MCF-7 and T47D TamR cells. Remarkably, treatment with SQ22536 completely abrogated the sensitizer effects of aspirin (Fig. 5G), confirming that tamoxifen sensitization effects of aspirin is mediated by cAMP pathway.

To test whether increased cAMP upon tamoxifen and aspirin combination induces the activation of stress-related kinases, we treated the MCF-7 TamR cells with aspirin alone or in combination with tamoxifen. While aspirin treatment alone significantly increased p-JNK and p-p38 levels, phosphorylation of AKT (Thr-308) was decreased. Importantly, the combination of aspirin and tamoxifen had a stronger effect on the phosphorylation status of all the proteins tested (Fig. 5H), phenocopying the effects of dipyrindamole and Gebr-7b in combination with tamoxifen. Specific inhibition of JNK by SP600125 or p38 by SB203580 completely reversed the effects of aspirin and tamoxifen combination on the proliferation inhibition and sensitization in MCF-7 TamR cells (Fig. 5I). Treatment with aspirin also increased the levels of p-IRE1 α , p-PERK, and p-eIF2 α , and they were further

Figure 3.

Induction of cAMP activates stress-related kinases downstream of ER stress pathway and resensitizes tamoxifen-resistant breast cancer cells. **A**, Western blot analysis of stress-related kinases (JNK and p38) and AKT pathway in parental and tamoxifen-resistant MCF-7 cells. β -Actin was used as a loading control. **B–E**, Western blot analysis of stress-related kinases and AKT pathway in MCF-7 TamR cells upon knockdown of PDE4D with PDE4D specific siRNA (**B**), treatment with cAMP analogue (cAMPS-Sp; **C**), treatment with PDE inhibitor (dipyrindamole; **D**), and treatment with PDE4D-specific inhibitor (Gebr-7b; **E**) in combination with tamoxifen (5.0 μ mol/L). **F** and **G**, Western blot analysis for apoptosis markers in MCF-7 TamR cells after treatment with dipyrindamole (**F**) and Gebr-7b (**G**) in combination with tamoxifen (5.0 μ mol/L). **H** and **I**, Luminescence-based caspase-3/7 assay in MCF-7 TamR cells upon treatment with PDE inhibitor (dipyrindamole; **H**) and PDE4D-specific inhibitor (Gebr-7b; **I**) in combination with tamoxifen (7.5 μ mol/L). **J** and **K**, Cell proliferation of the resistant MCF-7 cells treated with PDE inhibitor (dipyrindamole; **J**) and PDE4D-specific inhibitor (Gebr-7b; **K**) together with p38 inhibitor (SB203580) or JNK inhibitor (SP600125) in presence of tamoxifen (7.5 μ mol/L). **L**, Western blot analysis of ER stress-related proteins in parental and tamoxifen-resistant MCF-7 cells. β -Actin was used as a loading control. **M–P**, Western blot analysis of ER stress-related proteins in MCF-7 TamR cells upon knockdown of PDE4D (**M**), treatment with cAMP analogue (cAMPS-Sp; **N**), treatment with PDE inhibitor (dipyrindamole; **O**), and treatment with PDE4D-specific inhibitor (Gebr-7b; **P**) in combination with tamoxifen (5.0 μ mol/L). **Q**, Cell proliferation of the resistant MCF-7 cells treated with ER stress inducer (thapsigargin) in the absence and presence of tamoxifen (7.5 μ mol/L). **R**, Cell proliferation of the resistant MCF-7 cells treated with thapsigargin in the absence and presence of tamoxifen (7.5 μ mol/L) or together with p38 inhibitor (SB203580) or JNK inhibitor (SP600125) in presence of tamoxifen. **S**, Western blot analysis for apoptosis markers for combination therapies in MCF-7 TamR cells after treatment with thapsigargin alone or in combination with tamoxifen (5.0 μ mol/L) or together with p38-inhibitor (SB203580) or JNK inhibitor (SP600125) in presence of tamoxifen (5.0 μ mol/L). TG, thapsigargin.

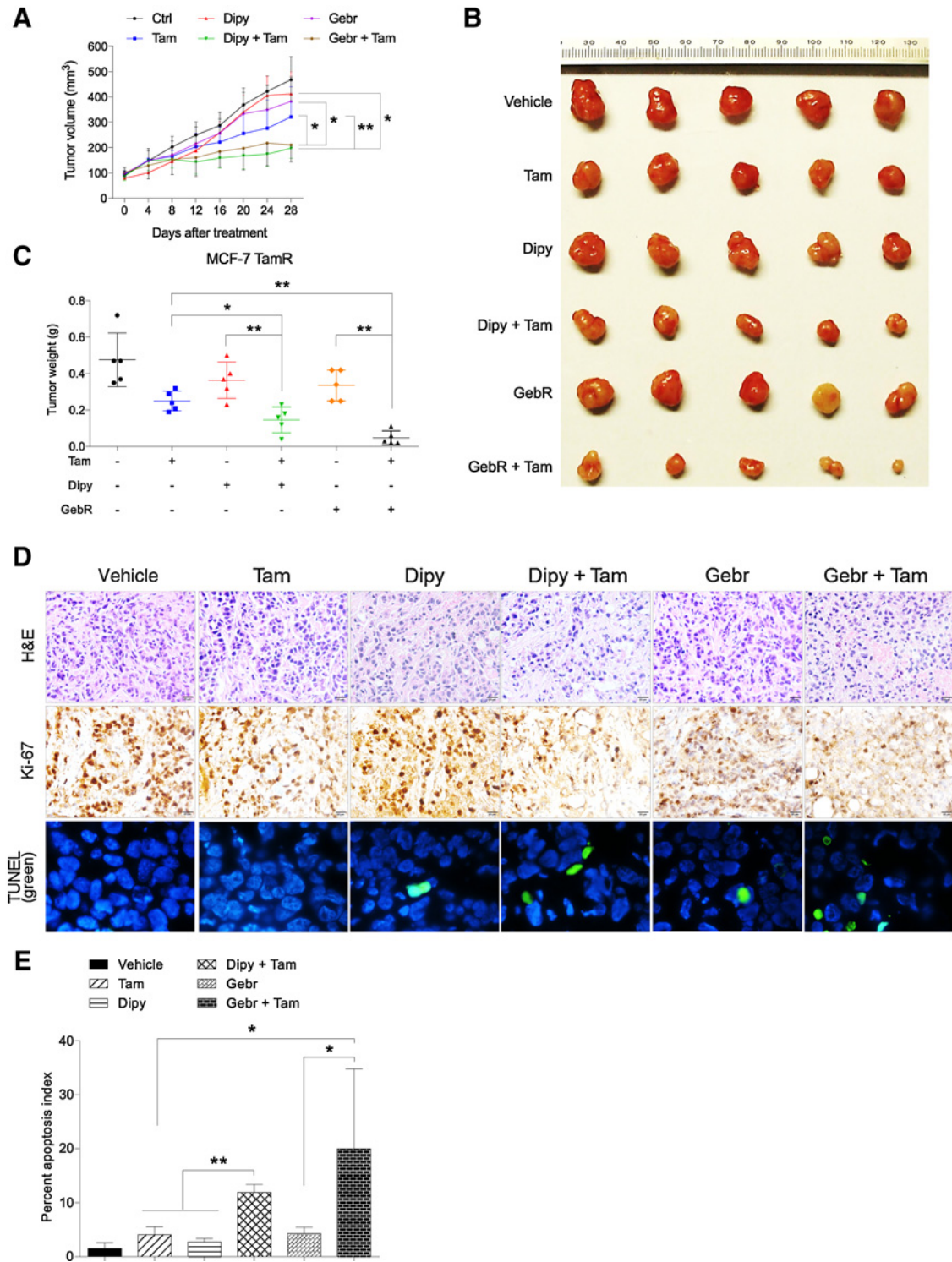
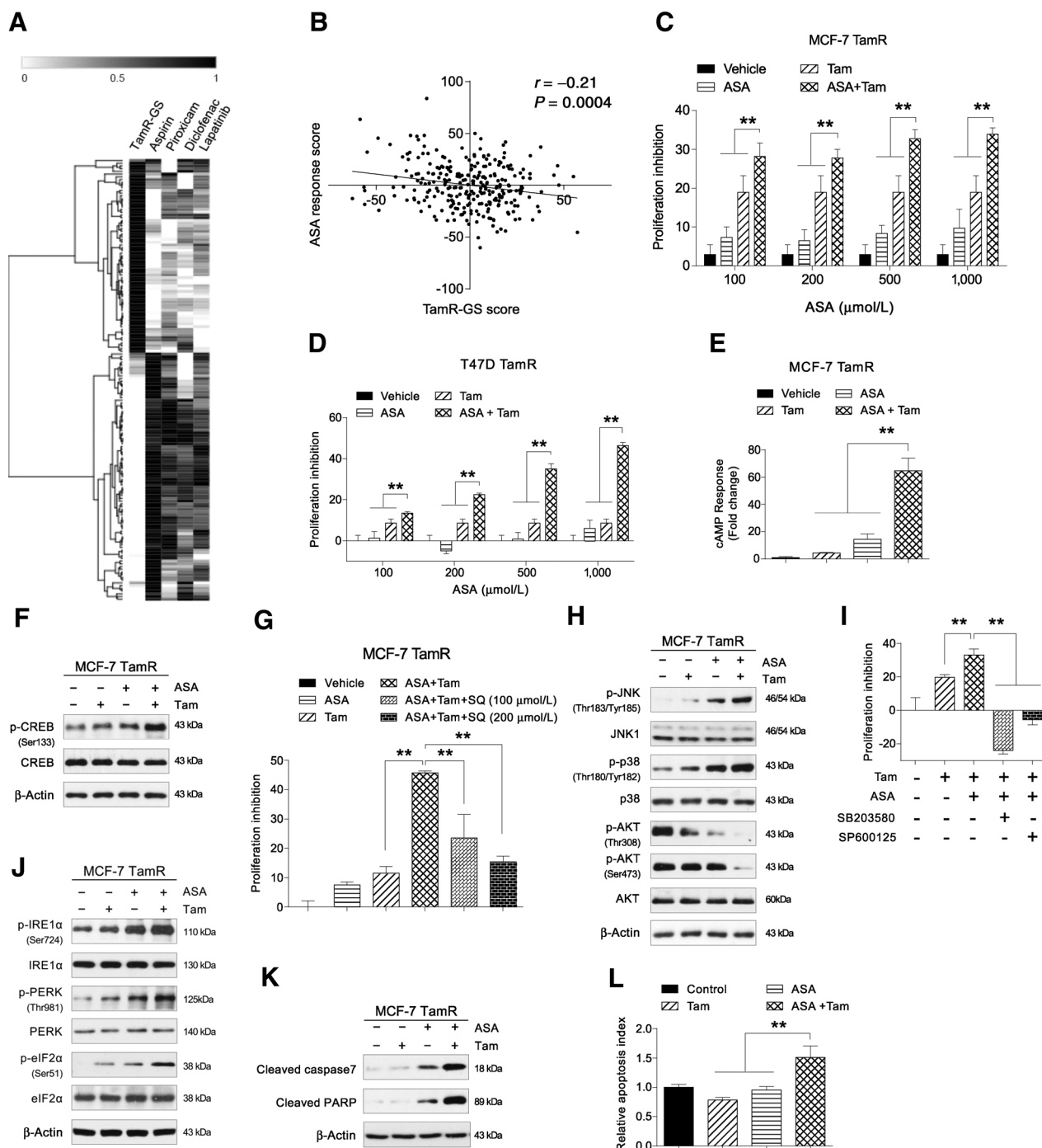


Figure 4.

Dipyridamole or Gebr-7b overcomes tamoxifen resistance *in vivo*. **A**, Tumor growth in MCF-7 TamR xenografts treated with tamoxifen and dipyrindamole or Gebr-7b, individually or in combination. Treatments were started when tumor volumes reached around 100 mm³. Mice were treated daily with tamoxifen (2 mg/kg), dipyrindamole (15 mg/kg), Gebr-7b (3 µg/kg), or combinations for 28 days. **B**, Pictures of the tumors from mice treated with vehicle, tamoxifen, dipyrindamole, Gebr-7b or the combination from the treatment groups. **C**, Tumor weights of the treatment groups. Statistical significance was determined with an unpaired, two-tailed Student *t* test. **D**, H&E, Ki-67, and fluorescence-based TUNEL stainings of the tumors. Pictures of H&E and Ki-67 stainings were taken at 40× while TUNEL was taken at 100× magnification. **E**, Quantification of the apoptotic cells from TUNEL staining.

**Figure 5.**

Aspirin overcomes tamoxifen resistance via modulating cAMP levels and activation of ER stress-mediated p38/JNK signaling leading to apoptosis. **A**, The heatmap showing the genes in TamR-GS that are reversed by aspirin treatment, but not with other COX inhibitors (piroxicam, diclofenac) and lapatinib, an EGFR/Her2 inhibitor. The positions are normalized, and black shows the upregulated genes while white shows the downregulated genes. **B**, The correlation between TamR-GS and aspirin response score in patients from GSE26971. **C** and **D**, Tamoxifen sensitization with aspirin at different doses in MCF-7 TamR (**C**) and T-47D TamR (**D**) cells. The concentrations of tamoxifen used were 7.5 $\mu\text{mol/L}$ and 5.0 $\mu\text{mol/L}$ for MCF-7 TamR and T-47D TamR cells, respectively. **E**, Intracellular cAMP levels in MCF-7 TamR cells upon treatment with aspirin in combination with tamoxifen (5.0 $\mu\text{mol/L}$). **F**, Western blot analysis of p-CREB in MCF-7 TamR cells treated with aspirin in combination with tamoxifen (5.0 $\mu\text{mol/L}$). β -Actin was used as a loading control. **G**, Cell proliferation of the MCF-7 TamR cells pretreated with SQ22536, a specific cAMP inhibitor, in combination with aspirin and tamoxifen (7.5 $\mu\text{mol/L}$). **H**, Western blot analysis of stress-related kinases and AKT pathway in MCF-7 TamR treated with aspirin in the absence and presence of tamoxifen (5.0 $\mu\text{mol/L}$). **I**, Cell proliferation of the MCF-7 TamR cells treated with aspirin together with p38 inhibitor (SB203580) or JNK inhibitor (SP600125) in the presence of tamoxifen (7.5 $\mu\text{mol/L}$). **J**, Western blot analysis of ER stress-related proteins in MCF-7 TamR cells upon treatment with aspirin in combination with tamoxifen (5.0 $\mu\text{mol/L}$). **K**, Western blot analysis for apoptosis markers for combination therapies in MCF-7 TamR cells. **L**, Luminescence-based caspase-3/7 assay in MCF-7 TamR upon treatment with aspirin in combination with tamoxifen (7.5 $\mu\text{mol/L}$).

increased in aspirin and tamoxifen combination (Fig. 5J). These effects were further reflected in apoptosis where aspirin in combination with tamoxifen resulted in apoptosis as evidenced by the cleavage of caspase-7 and PARP and increase in relative apoptosis index (Fig. 5K and L).

To examine whether aspirin overcomes tamoxifen resistance *in vivo*, we generated xenografts using tamoxifen resistant MCF-7 TamR cells. The aspirin and tamoxifen combination inhibited the growth of the MCF-7 TamR tumors in nude mice as compared with the vehicle or single agents (Fig. 6A and B). Tumor weights were also significantly lower in the combination-treated group as compared with vehicle or individual treatment (Fig. 6C). We observed a reduced cell proliferation shown by Ki-67 staining and an enhanced apoptosis shown by fluorescence-based TUNEL assay in combination therapy (Fig. 6D and E). Overall, our results indicate a novel mechanism where aspirin overcomes tamoxifen resistance by modulating the PDE4D activity and increasing cAMP levels both *in vitro* and *in vivo*.

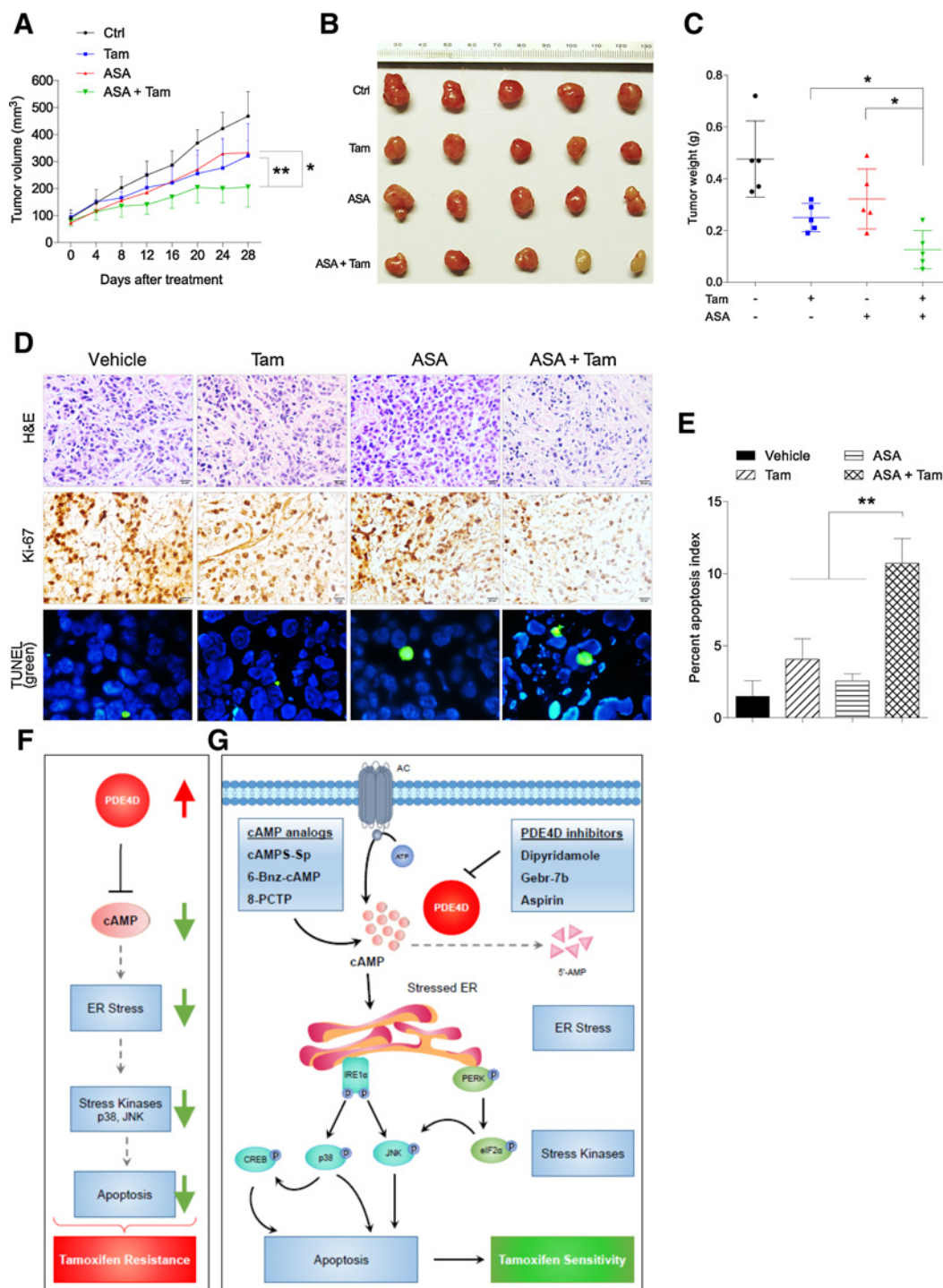
Discussion

Endocrine therapy is the most effective way to treat patients with early-stage, hormone-positive breast cancer, and tamoxifen is the preferred therapy, especially for premenopausal women. Despite its favorable efficacy, 20%–30% of patients acquire resistance during endocrine therapy, including tamoxifen (6). Multiple factors are responsible for resistance to tamoxifen, and various targeted therapies in combination with tamoxifen can be employed, depending on the pretreatment menopausal status or the change in menopausal status during the course of therapy, as well as on the risk of developing metastatic disease (29, 30). Major strategies to overcome tamoxifen resistance in breast cancer include use of an EGFR inhibitor (gefitinib), a HER2 inhibitor (trastuzumab), a dual kinase inhibitor (lapatinib), or an mTOR inhibitor (everolimus), depending upon the causal factor (31, 32). Recently, FDA has approved CDK4/6 inhibitor (palbociclib) to be used in combination with SERDs (fulvestrant) or AI (letrozole) for advanced ER-positive cancers, including those who progressed on tamoxifen (33, 34). Furthermore, fulvestrant has demonstrated clinical efficacy for patients with a second relapse after responding to tamoxifen and AIs (35). Another randomized trial demonstrated an improved median progression-free survival (PFS) in ER-positive, premenopausal metastatic patients when treated with tamoxifen plus ovarian function suppressor (36). Although early-stage disease is manageable owing to use of more potent endocrine agents, resistance inevitably develops in advance, metastatic disease (37). Therefore, identification of new targeted therapies for improving patient outcome is desirable. Here, we employed a systematic functional genomics approach to decipher the molecular mechanisms of tamoxifen resistance through combining whole-transcriptome sequencing, pathway enrichments, *in vitro* and *in vivo* assays and clinical data. This led us to identify PDE4D as a novel druggable target for overcoming tamoxifen resistance in breast cancer. PDE4D inhibition via specific siRNAs or pharmacologic inhibitors (dipyridamole and Gebr-7b) resulted in an increase in cAMP levels, induction of ER stress and increased JNK/p38 phosphorylation followed by apoptosis, overcoming resistance. Strikingly, we identified a novel mechanism by which aspirin through modulation of PDE4D and intracellular cAMP levels (similar to the effects of both PDE4D-specific inhibitor and PDE general inhibitor) overcomes tamox-

ifen resistance both *in vitro* and *in vivo*, highlighting the possibility of drug repositioning to expedite clinical application of our findings (Fig. 6F and G).

In the last few years, the potential role of PDEs as a target for treating inflammatory diseases, including asthma, depression, chronic obstructive pulmonary disorder (COPD) etc., has been studied. For example, roflumilast, a nonselective PDE4 inhibitor has been approved by FDA for treatment of erectile dysfunction and COPD (38). Importantly, recent studies have shown PDE4D as proliferation promoting factor in lung cancer cells (24). Inhibition of PDE4 by rolipram was shown to have antitumor effect in medulloblastoma, glioblastoma and in several hematologic malignancies making PDEs attractive targets for cancer therapy (39, 40). However, the role of PDE4D as a mediator of drug resistance has not been reported yet. We observed that blockade of PDE4D by siRNAs or specific inhibitors, for example, dipyridamole and Gebr-7b, has an antiproliferative effect on the tamoxifen-resistant breast cancer cells. Recent studies demonstrated that PDE4 inhibitors (e.g., rolipram) induce augmentation of intracellular cAMP levels leading to release of cytochrome c, reduction of antiapoptotic proteins, for example, Bcl-2 and Bcl-xL levels and activation of proapoptotic proteins, e.g., BAD, resulting in apoptosis (39). It has also been demonstrated that induction of cAMP by activation of PKA signaling induces apoptosis by activating PP2A, which dephosphorylates BAD and allowing apoptosis to commence through mitochondrial pathway. PP2A is an abundant phosphatase, which negatively regulates various signaling pathways through dephosphorylation of AKT (41). Our result also demonstrate that dephosphorylation of AKT is achieved through sustained activation of cAMP levels through cAMP analogs (cAMP-Sp) or by knockdown of PDE4D or by PDE4Is (dipyridamole and Gebr-7b) in combination with tamoxifen. Our results not only further support these findings with respect to increased apoptosis upon cAMP induction (here: upon combination of PDE4D inhibitors with tamoxifen), but also suggest that one of the mechanisms of cAMP-induced apoptosis is via inducing ER stress/p38/JNK pathways.

Under chronic ER stress, cells become incompetent to take care of unfolded protein load, and apoptosis is therefore triggered. There are three classes of ER stress-induced cell death sensors in mammals: IRE1 α , PERK, and ATF6 (42). Activated IRE1 α cleaves XBP1 mRNA and activates ASK1. Similarly, activated PERK phosphorylates eIF2 α , which leads to the inhibition of protein synthesis and induction of ATF4, which in turn regulates genes involved in apoptosis, growth arrest, and DNA damage response (25, 26). UPR is an adaptive cellular response that evolves to regulate protein folding homeostasis, and if the UPR fails to resolve the misfolding condition, then cells undergo apoptosis (43). Hence, UPR is becoming an attractive target for cancer therapy. In response to various extracellular stimuli, G-protein coupled receptors (GPCR) are responsible for cAMP accumulation in cells. Conversion of cAMP from ATP is mediated by adenylate cyclases (AC) which, in turn, are activated via GPCRs. In response to cellular stress, both JNK and p38 MAPKs are activated by GPCRs. It has been known that cAMP and cAMP-elevating agents activate JNK and p38 MAP kinases (19, 20, 44). As evident from the literature, there is a connection between cAMP, p38/JNK signaling and apoptosis. However, it has not been known that cAMP-induced activation of p38 MAPK and JNK signaling is preceded by induction of ER stress. Our study shows that elevated levels of cAMP can potentially lead to the activation

**Figure 6.**

Aspirin overcomes tamoxifen resistance *in vivo* and schematic summary of findings. **A**, Tumor growth in MCF-7 TamR xenografts treated with tamoxifen and aspirin individually or in combination. Treatments were started when tumor volumes reached around 100 mm³. Mice were treated daily with tamoxifen (2 mg/kg), aspirin (100 mg/kg) or their combination for 28 days. Note that the vehicle control and Tam groups are the same as in Fig. 4. ASA; aspirin. **B**, Pictures of the tumors from mice treated with vehicle, tamoxifen, aspirin, or the combination from the treatment groups. **C**, Tumor weights of the treatment groups. Statistical significance was determined with an unpaired, two-tailed Student *t* test. **D**, H&E, Ki-67, and fluorescence-based TUNEL stainings of the tumors. Pictures of H&E and Ki-67 stainings were taken at 40 \times while TUNEL was taken at 100 \times magnification. **E**, Quantification of the apoptotic cells from TUNEL staining. **F**, Elevated PDE4D levels in TamR cells leads to a decreased cAMP level, subsequently giving rise to a decrease in ER stress, activation of stress kinases and apoptosis conferring tamoxifen resistance. **G**, Inhibition of PDE4D (with dipyridamole, Gebr-7b or aspirin) or inducing cAMP directly (with cAMPS-Sp, 6-Bnz-cAMP, or 8-PCTP) together with tamoxifen leads to elevated cAMP levels, induction of ER stress and increased JNK/p38 phosphorylation leading to activation and phosphorylation of CREB followed by apoptosis and tamoxifen sensitization.

of ER stress pathway that necessitates stress-related kinases to induce apoptosis. In the context of tamoxifen resistance, we showed, for the first time, that modulation of cAMP levels through cAMP analogues (cAMPS-Sp) or by knockdown of PDE4D or by PDE4Is (dipyridamole and Gebr-7b) in combination with tamoxifen results in stepwise induction of cAMP, activation of ER stress, elevated phosphorylation of JNK and p38 MAPK and apoptosis to overcome tamoxifen resistance in breast cancer. Thus, targeting PDE4D, or induction of cAMP in general, can be used as a sensitizer for overcoming tamoxifen resistance in breast cancer cells.

Aspirin, a more than a century old drug, has been termed as a "wonder drug" by many clinicians (45). Aspirin belongs to the group of nonsteroidal anti-inflammatory drugs (NSAID), and is one of the most widely used drugs over a century. In several meta-analyses, regular use of aspirin was associated with a reduced cancer incidence, decreased distant metastases, and improvement of overall and disease-free survival (DFS) in patients with breast cancer who had acquired chemoresistance (46, 47). However, mechanisms underlying these protective effects are still poorly known. Aspirin has been known to exert anticancer effects, most of which are attributed to its COX-2 inhibitory function (46, 47). However, here we showed, for the first time, that aspirin can be repositioned to treat tamoxifen resistance by utilizing a different mechanism other than COX-2 inhibition to cause antitumoral effects *in vitro* and *in vivo* by targeting PDE4D and elevating cAMP. While dipyridamole has been used as an anticoagulant over a half-decade, and aspirin is in use as an anti-inflammatory drug over a decade, Gebr-7b is a newly developed cell-permeable and the most specific PDE4D inhibitor of all PDE4Is available. Emesis associated with PDE4Is is an apparent translational concern; however, Gebr-7b has been shown to be 100–3,000 times less emetic than other PDE4D inhibitors in three different species (*Suncus murinus*, beagle dog, and cynomolgus monkey; ref. 48); thus showing its promising potential for clinics. Although Gebr-7b has not been tested in a clinical trial yet, dipyridamole and aspirin could be used in combination with tamoxifen for overcoming tamoxifen resistance in future.

In summary, we have uncovered a novel molecular mechanism of tamoxifen resistance by an in-depth characterization of two

different tamoxifen resistant cells and identified PDE4D as a novel drug target in tamoxifen-refractory ER α -positive breast cancer. We believe that our results are the first, to the best of our knowledge, in providing cAMP induction as a novel concept for tamoxifen sensitization. As all three drugs which we showed to induce cAMP levels are readily available, and some of them are FDA approved (e.g., aspirin or dipyridamole), these drugs in combination with tamoxifen may offer new ways to overcome resistance in patients with ER-positive breast cancer.

Disclosure of Potential Conflicts of Interest

R. R. Mishra and Ö. Şahin have a pending patent (patent number: 14790/4) with Turkish Patent and Trademark Office on the use of cAMP inducers for the treatment of tamoxifen resistant breast cancer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: C. Cekic, Ö. Şahin

Development of methodology: R.R. Mishra, H. Bal, P. Jandaghi, C. Cekic

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.R. Mishra, N. Belder, S.A. Ansari, M. Kayhan, U. Raza, P.G. Ersan, S. Wiemann, Y. Riazalhosseini, Ö. Şahin

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.R. Mishra, Ü.M. Tokat, Ö. Saatci, S. Wiemann, A. Üner, C. Cekic, Y. Riazalhosseini, Ö. Şahin, E. Eyüpoğlu

Writing, review, and/or revision of the manuscript: R.R. Mishra, A. Üner, C. Cekic, Ö. Şahin

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Wiemann, A. Üner, Ö. Şahin

Study supervision: Ö. Şahin

Acknowledgments

This project was supported by EMBO Installation Grant 2791 (to Ö. Şahin). Ü.M. Tokat is a scholar of TÜBİTAK BİDEB 2211/A Scholarship Program. We thank Gamze Aykut for helping us with mouse xenografts studies.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 24, 2017; revised December 6, 2017; accepted January 25, 2018; published OnlineFirst January 31, 2018.

References

- Davies C, Pan H, Godwin J, Gray R, Arriagada R, Raina V, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. *Lancet* 2013;381:805–16.
- Viedma-Rodriguez R, Baiza-Gutman L, Salamanca-Gomez F, Diaz-Zaragoza M, Martinez-Hernandez G, Ruiz Esparza-Garrido R, et al. Mechanisms associated with resistance to tamoxifen in estrogen receptor-positive breast cancer (review). *Oncol Rep* 2014;32:3–15.
- Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, et al. Tamoxifen for prevention of breast cancer: report of the national surgical adjuvant breast and bowel project P-1 study. *J Natl Cancer Inst* 1998;90:1371–88.
- Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, et al. Update of the national surgical adjuvant breast and bowel project study of tamoxifen and raloxifene (STAR) P-2 trial: preventing breast cancer. *Cancer Prev Res* 2010;3:696–706.
- 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.
- Nass N, Kalinski T. Tamoxifen resistance: from cell culture experiments towards novel biomarkers. *Pathol Res Pract* 2015;211:189–97.
- Gutierrez MC, Detre S, Johnston S, Mohsin SK, Shou J, Allred DC, et al. Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. *J Clin Oncol* 2005;23:2469–76.
- Holm C, Rayala S, Jirstrom K, Stal O, Kumar R, Landberg G. Association between Pak1 expression and subcellular localization and tamoxifen resistance in breast cancer patients. *J Natl Cancer Inst* 2006;98:671–80.
- Cannings E, Kirkegaard T, Tovey SM, Dunne B, Cooke TC, Bartlett JM. Bad expression predicts outcome in patients treated with tamoxifen. *Breast Cancer Res Treat* 2007;102:173–9.
- Planas-Silva MD, Bruggeman RD, Grenko RT, Smith JS. Overexpression of c-Myc and Bcl-2 during progression and distant metastasis of hormone-treated breast cancer. *Exp Mol Pathol* 2007;82:85–90.

11. Rondon-Lagos M, Villegas VE, Rangel N, Sanchez MC, Zaphiropoulos PG. Tamoxifen resistance: emerging molecular targets. *Int J Mol Sci* 2016;17: pii: E1357.
12. Ward A, Shukla K, Balwierz A, Soons Z, Konig R, Sahin O, et al. MicroRNA-519a is a novel oncomir conferring tamoxifen resistance by targeting a network of tumour-suppressor genes in ER+ breast cancer. *J Pathol* 2014;233:368–79.
13. Dancik GM, Owens CR, Iczkowski KA, Theodorescu D. A cell of origin gene signature indicates human bladder cancer has distinct cellular progenitors. *Stem Cells* 2014;32:974–82.
14. Ward A, Balwierz A, Zhang JD, Kublbeck M, Pawitan Y, Hielscher T, et al. Re-expression of microRNA-375 reverses both tamoxifen resistance and accompanying EMT-like properties in breast cancer. *Oncogene* 2013; 32:1173–82.
15. Azevedo MF, Faucz FR, Bimpaki E, Horvath A, Levy I, de Alexandre RB, et al. Clinical and molecular genetics of the phosphodiesterases (PDEs). *Endocr Rev* 2014;35:195–233.
16. Gyorffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res Treat* 2010;123:725–31.
17. Bruno O, Romussi A, Spallarossa A, Brullo C, Schenone S, Bondavalli F, et al. New selective phosphodiesterase 4D inhibitors differently acting on long, short, and supershort isoforms. *J Med Chem* 2009;52:6546–57.
18. Delghandi MP, Johannessen M, Moens U. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. *Cell Signal* 2005;17:1343–51.
19. He J, Wang C, Sun Y, Lu B, Cui J, Dong N, et al. Exendin-4 protects bone marrow-derived mesenchymal stem cells against oxygen/glucose and serum deprivation-induced apoptosis through the activation of the cAMP/PKA signaling pathway and the attenuation of ER stress. *Int J Mol Med* 2016;37:889–900.
20. Cuadrado A, Nebreda AR. Mechanisms and functions of p38 MAPK signalling. *Biochem J* 2010;429:403–17.
21. Dostmann WR, Taylor SS, Genieser HG, Jastorff B, Dorskeland SO, Ogreid D. Probing the cyclic nucleotide binding sites of cAMP-dependent protein kinases I and II with analogs of adenosine 3',5'-cyclic phosphorothioates. *J Biol Chem* 1990;265:10484–91.
22. Hochbaum D, Tanos T, Ribeiro-Neto F, Altschuler D, Coso OA. Activation of JNK by Epac is independent of its activity as a Rap guanine nucleotide exchanger. *J Biol Chem* 2003;278:33738–46.
23. Rovida E, Stecca B. Mitogen-activated protein kinases and Hedgehog-Gli signaling in cancer: a crosstalk providing therapeutic opportunities? *Semin Cancer Biol* 2015;35:154–67.
24. Pullamsetti SS, Banat GA, Schmall A, Szibor M, Pomagruk D, Hanze J, et al. Phosphodiesterase-4 promotes proliferation and angiogenesis of lung cancer by crosstalk with HIF. *Oncogene* 2013;32:1121–34.
25. Darling NJ, Cook SJ. The role of MAPK signalling pathways in the response to endoplasmic reticulum stress. *Biochim Biophys Acta* 2014;1843:2150–63.
26. Lytton J, Westlin M, Hanley MR. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *J Biol Chem* 1991;266:17067–71.
27. Lamb J, Crawford ED, Peck D, Modell JW, Blat IC, Wrobel MJ, et al. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science* 2006;313:1929–35.
28. Guo H, Liu J, Ben Q, Qu Y, Li M, Wang Y, et al. The aspirin-induced long non-coding RNA OLA1P2 blocks phosphorylated STAT3 homodimer formation. *Genome Biol* 2016;17:24.
29. Klijn JG, Beex LV, Mauriac L, van Zijl JA, Veyret C, Wildiers J, et al. Combined treatment with buserelin and tamoxifen in premenopausal metastatic breast cancer: a randomized study. *J Natl Cancer Inst* 2000;92: 903–11.
30. Tevaarwerk AJ, Wisinski KB, O'Regan RM. Endocrine therapy in premenopausal hormone receptor-positive breast cancer. *J Oncol Pract* 2016;12: 1148–56.
31. Huober J, Fasching PA, Barsoum M, Petruzelka L, Wallwiener D, Thomssen C, et al. Higher efficacy of letrozole in combination with trastuzumab compared to letrozole monotherapy as first-line treatment in patients with HER2-positive, hormone-receptor-positive metastatic breast cancer - results of the eLEcTRA trial. *Breast* 2012;21:27–33.
32. Osborne CK, Neven P, Dirix LY, Mackey JR, Robert J, Underhill C, et al. Gefitinib or placebo in combination with tamoxifen in patients with hormone receptor-positive metastatic breast cancer: a randomized phase II study. *Clin Cancer Res* 2011;17:1147–59.
33. Cristofanilli M, Turner NC, Bondarenko I, Ro J, Im SA, Masuda N, et al. Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. *Lancet Oncol* 2016;17:425–39.
34. Finn RS, Crown JP, Ettl J, Schmidt M, Bondarenko IM, Lang I, et al. Efficacy and safety of palbociclib in combination with letrozole as first-line treatment of ER-positive, HER2-negative, advanced breast cancer: expanded analyses of subgroups from the randomized pivotal trial PALOMA-1/TRIO-18. *Breast Cancer Res* 2016;18:67.
35. Robertson JF, Osborne CK, Howell A, Jones SE, Mauriac L, Ellis M, et al. Fulvestrant versus anastrozole for the treatment of advanced breast carcinoma in postmenopausal women: a prospective combined analysis of two multicenter trials. *Cancer* 2003;98:229–38.
36. Kim HA, Ahn SH, Nam SJ, Park S, Ro J, Im SA, et al. The role of the addition of ovarian suppression to tamoxifen in young women with hormone-sensitive breast cancer who remain premenopausal or regain menstruation after chemotherapy (ASTRRA): study protocol for a randomized controlled trial and progress. *BMC Cancer* 2016;16:319.
37. Garcia-Becerra R, Santos N, Diaz L, Camacho J. Mechanisms of resistance to endocrine therapy in breast cancer: focus on signaling pathways, miRNAs and genetically based resistance. *Int J Mol Sci* 2012;14:108–45.
38. Rabe KF. Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease. *Br J Pharmacol* 2011; 163:53–67.
39. Lerner A, Epstein PM. Cyclic nucleotide phosphodiesterases as targets for treatment of haematological malignancies. *Biochem J* 2006;393: 21–41.
40. Goldhoff P, Warrington NM, Limbrick DD Jr., Hope A, Woerner BM, Jackson E, et al. Targeted inhibition of cyclic AMP phosphodiesterase-4 promotes brain tumor regression. *Clin Cancer Res* 2008;14:7717–25.
41. Moon EY, Lerner A. PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. *Blood* 2003;101:4122–30.
42. Maly DJ, Papa FR. Druggable sensors of the unfolded protein response. *Nat Chem Biol* 2014;10:892–901.
43. Han J, Back SH, Hur J, Lin YH, Gildersleeve R, Shan J, et al. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nat Cell Biol* 2013;15:481–90.
44. Li J, Dou X, Li S, Zhang X, Zeng Y, Song Z. Nicotinamide ameliorates palmitate-induced ER stress in hepatocytes via cAMP/PKA/CREB pathway-dependent Sirt1 upregulation. *Biochim Biophys Acta* 2015;1853: 2929–36.
45. Desborough MJ, Keeling DM. The aspirin story - from willow to wonder drug. *Br J Haematol* 2017;177:674–83.
46. Algra AM, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncol* 2012;13: 518–27.
47. Saha S, Mukherjee S, Khan P, Kajal K, Mazumdar M, Manna A, et al. Aspirin suppresses the acquisition of chemoresistance in breast cancer by disrupting an NFkappaB-IL6 signaling axis responsible for the generation of cancer stem cells. *Cancer Res* 2016;76:2000–12.
48. Bruno O, Fedele E, Prickaerts J, Parker LA, Canepa E, Brullo C, et al. GEBR-7b, a novel PDE4D selective inhibitor that improves memory in rodents at non-emetic doses. *Br J Pharmacol* 2011;164:2054–63.

Clinical Cancer Research

Reactivation of cAMP Pathway by PDE4D Inhibition Represents a Novel Druggable Axis for Overcoming Tamoxifen Resistance in ER-positive Breast Cancer

Rasmi R. Mishra, Nevin Belder, Suhail A. Ansari, et al.

Clin Cancer Res Published OnlineFirst January 31, 2018.

Updated version	Access the most recent version of this article at: doi: 10.1158/1078-0432.CCR-17-2776
Supplementary Material	Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2018/01/31/1078-0432.CCR-17-2776.DC1

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link <http://clincancerres.aacrjournals.org/content/early/2018/03/07/1078-0432.CCR-17-2776>. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.