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ORIGINAL ARTICLE

CHRNA5 belongs to the secondary estrogen signaling network exhibiting prognostic significance in breast cancer

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Abstract

Purpose Cholinergic signals can be important modulators of cellular signaling in cancer. We recently have shown that knock-down of nicotinic acetylcholine receptor subunit alpha 5, CHRNA5, diminishes the proliferative potential of breast cancer cells. However, modulation of *CHRNA5* expression in the context of estrogen signaling and its prognostic implications in breast cancer remained unexplored.

Methods Meta-analyses of large breast cancer microarray cohorts were used to evaluate the association of *CHRNA5* expression with estrogen (E2) treatment, estrogen receptor (ER) status and patient prognosis. The results were validated through RT-qPCR analyses of multiple E2 treated cell lines, CHRNA5 depleted MCF7 cells and across a breast cancer patient cDNA panel. We also calculated a predicted secondary (PS) score representing direct/indirect induction of gene expression by E2 based on a public dataset (GSE8597). Co-expression analysis was performed using a weighted gene co-expression network analysis (WGCNA) pipeline. Multiple other publicly available datasets such as CCLE, COSMIC and TCGA were also analyzed.

Results Herein we found that *CHRNA5* expression was induced by E2 in a dose- and time-dependent manner in breast cancer cell lines. ER^- breast tumors exhibited higher *CHRNA5* expression levels than ER^+ tumors. Independent meta-analysis for survival outcome revealed that higher *CHRNA5* expression was associated with a worse prognosis in untreated breast cancer patients. Furthermore, *CHRNA5* and its co-expressed gene network emerged as secondarily induced targets of E2 stimulation. These targets were largely downregulated by exposure to CHRNA5 siRNA in MCF7 cells while the response of primary ESR1 targets was dependent on the direction of the PS-score. Moreover, primary and secondary target genes were uncoupled and clustered distinctly based on multiple public datasets.

Conclusion Our findings strongly associate increased expression of *CHRNA5* and its co-expression network with secondary E2 signaling and a worse prognosis in breast cancer.

Keywords Breast cancer · CHRNA5 · Meta-analysis · Co-expression network · Estrogen receptor targets · Estrogen treatment · Prognosis

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1 Introduction

Cholinergic receptor nicotinic alpha 5 (CHRNA5) is encoded by one of the several nicotinic acetylcholine receptor (nAChR) subunit genes located on chromosome 15, i.e., *CHRNA5*, *CHRNA3* and *CHRNB4* [1]. Upregulated expression of *CHRNA5* [2], downregulation of *CHRNA3* [2] and epigenetic regulation of *CHRNB4* [3] in lung cancer implicate the involvement of these loci in cancer genetics. *CHRNA5* has various isoforms due to alternative splicing of the exon V [4, 5], and multiple isoforms are overexpressed in lung cancer in comparison to normal lung tissue [4]. Studies have revealed potential implications of several other cholinergic receptor subunits in estrogen (E2) signaling and breast cancer. For example, *CHRNA4* is regulated by E2 in a site-specific manner in brain [6]. Similarly, *CHRNA9* expression increases in response to E2 in the MCF7 cell line and patient data show a positive correlation between the ER status and *CHRNA9* expression [7]. In addition, *CHRNA9* overexpression has been associated with increased proliferative potential in non-tumor breast cell lines, whereas *CHRNA9* knockdown led to smaller tumor volumes in a breast cancer xenograft model in mice [8]. Recently, we have demonstrated that *CHRNA5* depletion in breast cancer cells by RNAi is associated with cell cycle inhibition, increased apoptosis and drug sensitivity [9]. Nevertheless, there is no comprehensive study yet analyzing changes in *CHRNA5* isoform expression in breast cancer cells in response to E2 or across ER^+/ER^- breast tumors. Moreover, *CHRNA5* expression along with its co-expression network has not been studied in the context of breast cancer prognosis.

E2 exerts its actions in genomic terms via ER alpha (α) (ESR1) and/or beta (β) (ESR2), or through non-genomic pathways mediated by G protein-coupled estrogen receptor 1 (GPER1) [10]. The E2-bound ER complex is targeted to specific sequences on DNA (estrogen response elements, EREs), upstream of target genes, altering their transcription [11]. Alternatively, the E2-bound ER complex may induce expression of various transcription factors (TF), exerting a global effect, secondarily [11]. Thus, E2 may regulate gene expression time dependently via interactions with ER primarily and/or secondarily by TFs which are directly induced/repressed by ER [12, 13]. ER status still remains one of the most significant determinants of therapy choice and outcome in breast cancer [14]. Therefore, it is important to investigate the differential contribution of primary and secondary target genes to ER^+ and ER^- breast cancer prognosis [15].

Combining gene expression signatures with clinical survival data enables prioritization of prognostic biomarkers for therapeutic targeting [16]. Hazard ratio (HR) is a widely used statistical measure to predict prognostic outcome in terms of risk of tumor relapse, metastasis or patient death [17]. Previous studies generally have focused on a small subset of genes and gene clusters while addressing the diagnostic/prognostic power of E2-regulated gene expression in cancer [18, 19]. Moreover, meta-analysis approaches via combining multiple similar-context transcriptomics studies to increase sample size and statistical power can be used to improve breast cancer prognostication [20–22]. However, a transcriptome-wide meta-correlation analysis between the expression level and clinical outcome for the primary or secondary targets of E2-signaling has not been performed.

In the present study, we employed meta-analyses as well as RT-qPCR to establish the association of *CHRNA5* expression response to E2 treatment and to ER status using in vitro cell line and in vivo patient tumor data, respectively. Our analysis revealed a negative correlation between *CHRNA5* mRNA expression and that of *ESR1* based on multiple independent datasets. Meta-survival analysis indicated that higher *CHRNA5* expression levels were associated with a worse prognosis in untreated breast cancer patients. We next found that genes positively co-expressed with *CHRNA5* were also associated with a worse prognosis, over-expressed in ER^- breast cancer patients and enriched with secondary E2 targets. Comparative transcriptomics analyses performed with the expression profile of CHRNA5 siRNA-treated MCF7 cells supported the newly discovered involvement of CHRNA5 in E2 signaling in breast cancer. Our study revealed an uncoupling between E2-modulated primary and secondary cellular signaling upon CHRNA5 depletion and a co-expression network of *CHRNA5* with significant prognostic implications in breast cancer.

2 Materials and methods

2.1 In vitro expression data

Microarray gene expression datasets (Affymetrix platform GPL570/96/571) were obtained from the GEO database [23, 24] for E2-treated ER^+ breast cancer cell line MCF7 (Supplementary Table 1) using keywords, i.e., MCF7 and/or estrogen (Supplementary Methods).

2.2 Breast cancer patient expression data

For TCGA dataset analyses, RNAseq normalized count data (RSEM) of breast cancer patients along with ER status of breast cancer patient data were downloaded from FireBrowse (http://firebrowse.org). Breast cancer patient microarray datasets used for meta-analysis were split in two cohorts: an *ER cohort* assembled from the GEO database [24] directly (Supplementary Table 2) and a *survival cohort* based on untreated patient data from the KM Plotter database (Supplementary Table 3) [25]. Retrieval and processing of meta-analysis cohorts are described in detail in Supplementary Methods.

2.3 Meta-differential expression analysis

Meta-analyses of E2 treated MCF7 datasets and ER^+/ER^- subtype patient datasets were conducted separately following PRISMA guidelines (Supplementary Figs. 1 and 2). For transcriptome-wide differential expression, *metaDE* [26] and for forest plots, *meta* [27] packages were used, respectively (details in Supplementary Methods).

2.4 Survival meta-analysis

Survival analysis of individual datasets of the *survival cohort* (Supplementary Table 3; Supplementary Fig. 3) was performed using the *survival* package along with the customized R code provided by KM Plotter using Cox proportional hazard model and *auto cut-off* selection (Supplementary Methods) [25]. Hazard Ratio (HR) scores obtained for each dataset were combined by *meta* [27] package to get meta-Hazard Ratios (meta-HRs) using the fixed and random effect models (FEM and REM, respectively).

2.5 Determination of secondarily induced targets of E2 signaling based on the GSE8597 dataset

The GSE8597 dataset [12], retrieved from the GEO database, consisted of four treatment groups in MCF7 cells, i.e., (1) E2, (2) EtOH, (3) CHX + E2 and (4) CHX treatments. A limma analysis was performed for each probeset between samples treated with E2 and with EtOH to identify E2-driven gene expression changes (Limma_E2). To analyze the secondary responses mediated by E2 exposure, expression data from E2 and CHX + E2 treated samples were first normalized to the mean expression value of EtOH and CHX control samples, respectively, before performing limma (Limma_PS: E2*i* - mean(EtOH) vs. CHX + E2*i* - mean(CHX), where *i* refers to probeset ID). LogFC values from Limma_PS analysis represented Predicted Secondary scores (PS-scores). E2 induced secondary targets were further determined upon filtering of: (1) E2 induced probesets (Limma_E2: LogFC > 0.5 and $p < 0.05$) and (2) probes showing secondary responses (Limma_PS: LogFC > 0.5 and $p < 0.05$). 367 probesets passed these filters. PS-scores were further used in correlation analyses.

2.6 Meta-correlation and co-expression network analysis

Correlation coefficients between each gene and *CHRNA5* for both the *ER cohort* and the *survival cohort* were calculated separately using DerSimonian-Laird (DSL) random-effect meta-analysis with the help of the *Metacor* bioconductor package [28]. On the other hand, the *CHRNA5* co-expression network was based only on *ER cohort* meta-correlation analysis (details in Supplementary Methods). Signed WGCNA (weighted gene co-expression network analysis) was used for the identification of *CHRNA5* associated co-expression modules [29]. Top positive and top negative co-expression modules and associated data were visualized in Cytoscape [30] using interactions from the STRING database [31]. iRegulon plugin [32] of Cytoscape was used to find the TF enrichments in each module. Functional annotation of co-expression modules for KEGG pathways was done using DAVID (version 6.8 Beta) (BH < 0.05) [33]. Additionally, the Harmonizome data portal [34] was used to retrieve transcription factor binding sites of the *CHRNA5* gene by selecting four datasets (CHEA, ENCODE, JASPAR and TRANSFAC transcription factors).

2.7 TCGA breast cancer data analysis

Log (base 2) transformed RSEM (+1) counts were used for principal component analysis (PCA) of gene lists specified in the results section (see figure legends for futher details). In case of multiple samples from the same patient, average RSEM was used (1212 total samples and 1093 unique samples). Correlation analysis was performed between the expression values of *CHRNA5* and those of every other gene using Spearman's correlation coefficient in MATLAB. Clustering was done based on linkage and distance parameters as described previously [9]. mRNA expression of *RB1* and *CHRNA5* along with copy number alteration (CNA) and ER status data were obtained from TCGA via cbioportal [35]. Patients with "indeterminate" ER status were removed prior to plotting ($n = 1026$). A two-way ANOVA was performed in R environment.

2.8 Comparative transcriptomics after CHRNA5 siRNA exposure in MCF7 cells

The primary target gene list in response to E2 exposure in MCF7 cells was obtained from a published ChIP-seq interaction study [36]. The expression profile of CHRNA5 siRNA-treated and that of scrambled siRNA-treated MCF7 cells have been previously analyzed (GSE89333; [9]). We used jetset best probesets to find the common probesets between the GPL96 and GPL570 platforms [37] and data from GSE89333 were compared with those from (a) GSE8597 [12] to determine the correlation of CHRNA5 siRNA treatment with E2 primary (ChIP-seq targets [36]) and secondary targets (predicted using GSE8597) [12] and (b) GSE4668 [38] to investigate the association of E2 stimulation and hormone starvation with the expression profile of CHRNA5 depletion in MCF7 cells.

2.9 Cell culturing after dose- and time-dependent E2 exposure

MCF7, MDA-MB-231, CAL-51, ZR-75-1 and T47D breast cancer cell lines were treated with β-estradiol (E2, 2 mM stock solution in EtOH; E2758, 32221, Sigma Aldrich, Missouri, USA) of 1 nM, 10 nM, and/or 100 nM as described below. For dose-dependent exposures, 1×10^6 MCF7 and MDA-MB-231 cells seeded into T75 flasks were incubated with 10% FBS (SH30021.01, SW30160.0, HyClone, Logan, USA) in phenol red supplemented DMEM growth medium with 1% penicillin/streptomycin solution and 1% non-essential amino acids for 24 h. Media were then replaced with phenol red-free DMEM with 5% charcoal dextran-treated FBS with supplements for 72 h. Synchronization performed for 24 h in phenol red-free Dulbecco's MEM (F0475, Biochrom AG, Berlin, Germany) with 0.1% charcoal/dextran-treated FBS (and with the rest of the additives from the 5% medium) was followed by β-estradiol (E2, 2 mM stock solution in EtOH; E2758, 32221, Sigma Aldrich, Missouri, USA) of 1 nM, 10 nM, and 100 nM for 24 h ($n = 2$ per dose). For the time-dependent exposure, MCF7 cells were seeded at a density of 1×10^6 cells into T75 flasks and the same procedure as above was applied (100 nM; 12 h and 24 h). E2 was dissolved in EtOH and the sample size per group equaled to two for all experiments. EtOH was used as control group. Similarly, 2×10^5 T47D, 2.5×10^5 ZR-75-1 and 1.5×10^5 CAL-51 cells were seeded in 6-well plates before being treated with 10 nM E2 for 24 h as above.

2.10 RNA isolation and cDNA synthesis

Total RNA was isolated using a RNeasy Mini kit (74,104, Qiagen, Hamburg, Germany) or QIAzol Lysis Reagent (cat. no. 79306) after which 1 μg total RNA was reverse transcribed using a RevertAid first strand cDNA synthesis kit (K1622, Fermentas, Ontario, Canada) according to the manufacturer's protocols. Primer details for CHRNA5 isoforms and reference genes are provided in Supplementary Table 4. A breast cancer tissue cDNA panel (BCRT501) was purchased from OriGene (Rockville, USA) and diluted in a ratio of 1:20 using ddH_2O before use.

2.11 RT-qPCR analysis and primers

A Roche Light Cycler (LC480 II; Roche, Basel, Switzerland) was used for RT-qPCR with an initial incubation at 95 °C for 5 min followed by 95 °C for 10 s and 20 s at 60 °C for 50 cycles in duplicates using a 10 μl reaction volume containing 5 μl SYBR green Master Mix (04707516001, Roche, Basel, Switzerland), 2 μl cDNA, 2 μl Forward and Reverse primer mix and 1 μl ddH_2O. Samples were checked for amplicon-specific melting temperatures (Tm) and those with duplicate Tm values as well as Ct readings with ±1 standard deviation away from the median plate Tm value were removed. Relative expression was calculated using *TPT1* as a reference gene [39, 40]. The *TFF1/PS2* gene was used as a positive control [41–43] to test for induction by E2 stimulation (Supplementary Table 4). Upon calculation of relative expression levels [44] logarithmically (base 2) transformed units were plotted as barplots using GraphPad software (California, USA). Primer pairs used for *CHRNA5* isoforms were previously reported (*CHRNA5_v1* and *CHRNA5_v2* from [9] and *CHRNA5_v3* from [5] and *iso2* and *iso3* from [4]; Supplementary Table 4). In addition, primers for selected primary and secondary targets of E2 signaling were designed and tested by RT-qPCR using cDNA samples obtained from 10 nM [9] as well as 20 nM and 50 nM treatments of CHRNA5 siRNA or scrambled oligo siRNA (Supplementary Table 4).

3 Results

3.1 Meta-analysis reveals that *CHRNA5* expression is responsive to E2 in ER^+ cell lines

Meta-analysis of E2 treatment in MCF7 cell line expression datasets showed a significant increase in the *CHRNA5* probeset "*206533_at*" (Supplementary Table 1; Fig. 1a) starting from the sixth hour of E2 treatment (*late E2 treatment cohort*; FEM: z-value = 5.04; $p = 4.5e\text{-}07$ REM: z-value = 3.28; $p = 0.001$) while no significant change in *CHRNA5* expression was observed during the initial hours of E2 treatment (Supplementary Fig. 4a). *CHRNA5* expression was also induced in T47D cells and to a lesser degree in ESR1 mutated MCF7- and T47D-derived cell lines (Supplementary Fig. 4b). The ESR1 mutations (D538G and Y537S) were presumed to be responsible for ligand-independent ER regulation [45]. These findings suggest that *CHRNA5* expression positively responds to E2 treatment in ER^+ cell lines in a time-dependent manner and could be a potential target gene upon E2 modulation.

Based on the meta-analysis results, two different time points (12 h and 24 h) were selected to evaluate E2-mediated *CHRNA5* isoform expression changes using RT-qPCR. 24 h of E2 treatment effectively modulated E2-dependent signaling, as shown by a significant increase in *TFF1/PS2* expression. All *CHRNA5* isoforms showed significant increases upon 24 h of E2 treatment in MCF7 cells, while *CHRNA5_iso2* also exhibited a milder but significant upregulation even at 12 h (Fig. 1b). The results were further validated in two other ER^+ cell lines (T47D and ZR-75-1) and a consistent pattern of significant upregulation of all *CHRNA5* isoforms was observed after 24 h of E2 treatment (Supplementary Fig. 4c). Our results confirm that *CHRNA5*

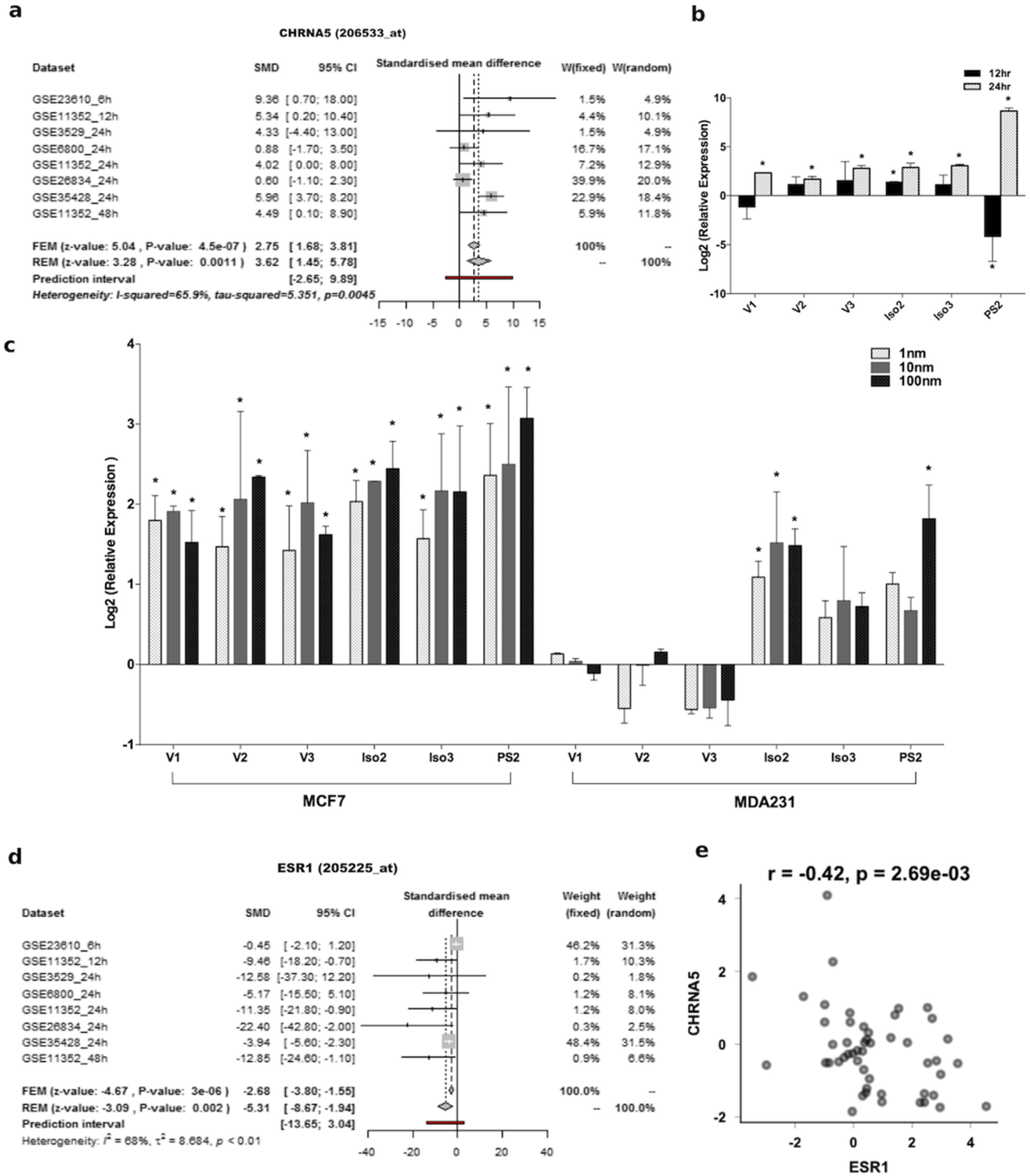


Fig. 1 CHRNA5 expression is modulated by E2 and varies with ER status. **a** Forest plot of *CHRNA5* expression in E2 treated samples compared to control samples (6–48 h E2 treatment). **b-c** Relative expression of *CHRNA5* isoforms (*V1*, *V2*, *V3*, *Iso2*, *Iso3*) along with a known E2 target gene, *PS2,* in a time-dependent manner in MCF7 cells (**b**), and in a dose-dependent manner in two different cell lines, MCF7 (ER^+) and MDA-MB-231 (ER^-) **(c). d** Meta-analysis of *ESR1* (jetset best probeset; 205225_at) expression modulation in late E2 treated (6–48 h) microarray data. **e** Correlation of *CHRNA5* and *ESR1* gene expression values in the COSMIC breast cancer cell line dataset ($n = 50$). *FEM: Fixed Effect Model; REM: Random Effect Model; SMD: Standardized Mean Difference; r: Correlation Coefficient. * Indicates $p < 0.05$*

is an E2 responsive gene and that the phenomenon is consistent among different ER^+ cell lines.

3.2 E2 affects *CHRNA5* expression differently in ER^+ and ER^- cell lines

The dose-dependent effect of E2 treatment on expression of *CHRNA5* isoforms was evaluated in MDA-MB-231, an ER^- cell line, in comparison to MCF7 cells (ER^+). Increased expression of *CHRNA5* was prominent for all isoforms even at 1 nM E2 exposure for 24 h in MCF7 cells. However, isoform expression was variably affected by E2 in MDA-MB-231 cells with a mild but significant induction of *CHRNA5_Iso2* for all three concentrations (Fig. 1c). Another ER^- cell line, CAL-51, showed no significant regulation of *CHRNA5* isoforms upon E2 treatment after 24 h of E2 treatment (Supplementary Fig. 4c). Our findings show that the effect of E2 on *CHRNA5* isoform expression varies with the ER status of the cell lines.

Meta-analysis of the *late E2 treatment cohort* in MCF7 cells confirmed that increased expression of *CHRNA5* in response to E2 (Fig. 1a) was indeed accompanied by significant *ESR1* downregulation, thus highlighting a negative association between the two genes at the transcriptional level (Fig. 1d; Supplementary Fig. 4d). Analysis of 50 different COSMIC breast cancer cell lines validated that the negative correlation between *CHRNA5* and *ESR1* levels was not specific to MCF7 ($r = -0.42$; $p = 0.003$; Fig. 1e). Furthermore, in CCLE breast cancer cells a negative association of *CHRNA5* (mRNA) with ESR1 could be observed at both the transcript and protein level (*ESR1* (mRNA): $r = -0.34$, $p = 2.11e\text{-}02$; Total ER-alpha (protein): $r = -0.37$, $p = 9.4e\text{-}03$; ER-alpha-pS118 (protein): $r = -0.41$, $p = 4.6e\text{-}03$) (Supplementary Fig. 5).

3.3 *CHRNA5* expression is upregulated in breast tumors

Using patient tumor data, differential expression analysis (meta-analysis) on the basis of IHC (immunohistological status) of ER (*ER cohort*: 31 datasets; 1255 ER^- and 1650 ER^+ breast cancers; details in Supplementary Table 2) indicated that the expression of *CHRNA5* was significantly higher in the ER^- breast tumors than in the ER^+ breast tumors, with high heterogeneity but no publication bias (I-squared = 72.6%; Z-score = 7.4; $p = 1.4e\text{-}13$; Fig. 2a; Egger's test $p = 0.99$; Supplementary Fig. 6). The inverse correlation between *CHRNA5* and ER status could also be observed at the transcript level in the same *ER cohort* datasets, indicating a high concordance between IHC- and microarray-based ER level results (Supplementary Fig. 7), as well as in the TCGA RNA-seq breast cancer data (1093 patients) (Fig. 2b). These findings indicate that although induced by E2, *CHRNA5* was overexpressed in ER^- tumors.

RT-qPCR validation using a breast cancer cDNA panel revealed that three of the isoforms (*V3*, *Iso2*, *Iso3*) were significantly overexpressed in ER^- compared to ER^+ samples (Fig. 2c). Comparison with the normal samples showed that all of the *CHRNA5* isoforms exhibited a higher expression in tumor samples than in normals. After splitting the normal/tumor analysis to subtypes on the basis of ER status, all isoforms were significantly overexpressed in ER^- tumors while only *CHRNA5_Iso3* and *CHRNA5_V1* showed significantly higher expression levels in ER^+ breast tumors compared to normals. These findings support our meta-analysis results indicating that *CHRNA5* exhibited a relatively higher expression in ER^- than in ER^+ patients while showing isoform specificity (Fig. 2c).

3.4 High *CHRNA5* expression indicates a poor prognosis for breast cancer patients

Using an independent patient cohort (*survival cohort*; 5 datasets; 894 patients; Supplementary Table 3), meta-survival analysis results showed that a higher *CHRNA5* expression was associated with a poor prognosis in untreated breast cancer patients (FEM z-value = 2.73; $p = 0.0063$; Fig. 3a). Both meta-analysis models (FEM and REM) showed similar and significant meta-hazard ratios, yet the fixed effect model (FEM) results may be more appropriate since the *survival cohort* exhibited no significant heterogeneity ($I^2 = 47.4\%$; $p = 0.1$).

3.5 *CHRNA5* co-expression network is associated with ER status and breast cancer prognosis

Transcriptome-wide differential expression of ER^- breast tumors with reference to ER^+ tumors (effect size, i.e., standardized mean difference from the *ER cohort*) showed a strong positive correlation with the *CHRNA5* meta-correlation profile (*survival cohort*; significant genes ($p < 0.05$), $r = 0.88$; Fig. 3b). Comparative analysis showed that *CHRNA5* itself and genes positively correlated with *CHRNA5* were overexpressed in ER^- patients, while the expression levels of negatively correlated genes were downregulated in ER^- patients (Fig. 3b). Similarly, genes positively co-expressed with *CHRNA5* exhibited high hazard ratios (meta-HR values) and were associated with a worse prognosis among untreated breast cancer patients as shown by the color spectrum on the graph (Fig. 3b and c).

3.6 *CHRNA5* co-expression modules show functional enrichment and differential HR

Classification of the *CHRNA5* co-expressed genes (obtained using WGCNA) revealed six distinct modules,

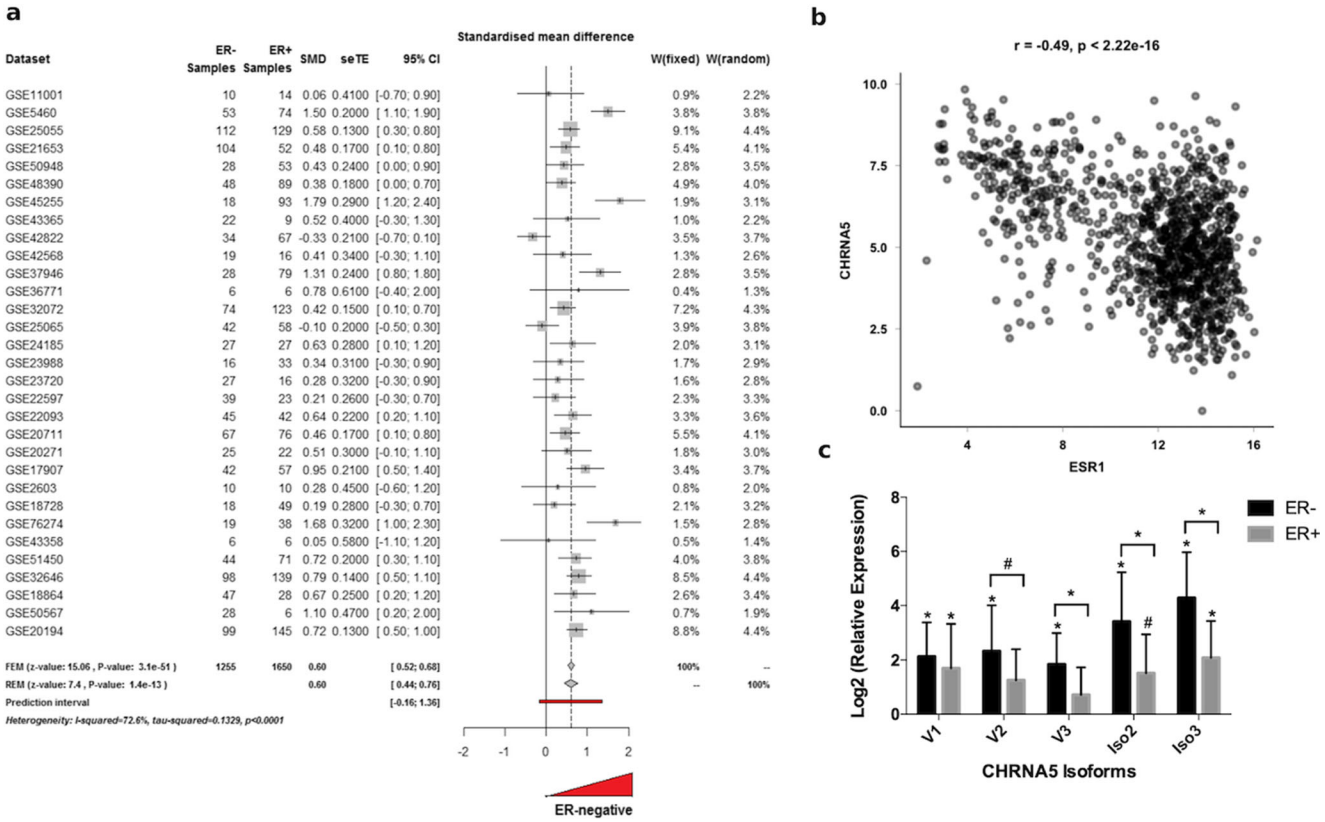


Fig. 2 *CHRNA5* is over-expressed in ER$^-$ breast cancer patients. **a** Meta-differential expression of *CHRNA5* in ER$^-$ breast cancer patients compared with ER$^+$ breast cancer patients (*ER cohort*; 31 datasets). **b** Correlation between *CHRNA5* and *ESR1* expressions using TCGA RNA-seq gene normalized data. **c** Validation of the expression of *CHRNA5* isoforms in the cDNA panel using RT-qPCR. Statistical analysis was performed using ANOVA and post-hoc Tukey HSD tests. *FEM: Fixed Effect Model; REM: Random Effect Model; SMD: Standardized Mean Difference; r: Correlation Coefficient.* * *Indicates* $p < 0.05$, # *Indicates* $p < 0.1$

three of which were positively and the other three were negatively co-expressed with *CHRNA5* (Fig. 4a). Functional annotation using KEGG pathways showed that positively co-expressed modules were involved in cell cycle, DNA replication and repair, and p53 signaling pathways, while negatively co-expressed modules were rather enriched with focal adhesion and extracellular matrix adhesion pathways (Table 1), supporting our previously reported findings [9]. In addition, the turquoise module (positively co-expressed) indicated the role of *CHRNA5* in addiction pathways including those of nicotine and morphine, as well as in retrograde endocannobinoid signaling, neuroactive ligand-receptor interaction and calcium signaling (Table 1). Overlaying modular information with our meta-analysis scores strengthened our previous finding that positively co-expressed modules were enriched with genes over-expressed in the ER$^-$ tumors (FDR < 0.05 and ER$^-$/ER$^+$ treatment effect > 0; Fig. 4b), while exhibiting a worse prognosis ($p < 0.05$ and meta-HR > 0; Fig. 4c) and vice-versa.

3.7 Top positive *CHRNA5* co-expression module is enriched with E2-induced secondary targets

Protein-protein interactions of top ranked positive (yellow) and negative (brown) modules from WGCNA revealed highly connected networks (Fig. 5 and Fig. 6, respectively). Expectedly, transcription factor (TF) enrichment analysis of the *CHRNA5* co-expressed module showed regulation by ER-alpha (ESR1) in the negatively co-expressed module (Fig. 6). Multiple members of the E2F family were among the enriched TFs in the top most positive *CHRNA5* co-expression module (Fig. 5). In addition, the promoter of the *CHRNA5* gene itself had binding sites for E2F members (E2F1, E2F4, E2F6) and multiple other enriched transcription factors including SIN3A, NFYB and ZIN143, but not for ESR1 (ENCODE database; Harmonizome portal; Supplementary Table 5). Enrichment of E2F1 in the *CHRNA5* promoter site and its co-expressed module highlighted that the *CHRNA5* co-expressed network could be a downstream target of E2F1 and/or indirectly regulated by the E2 response.

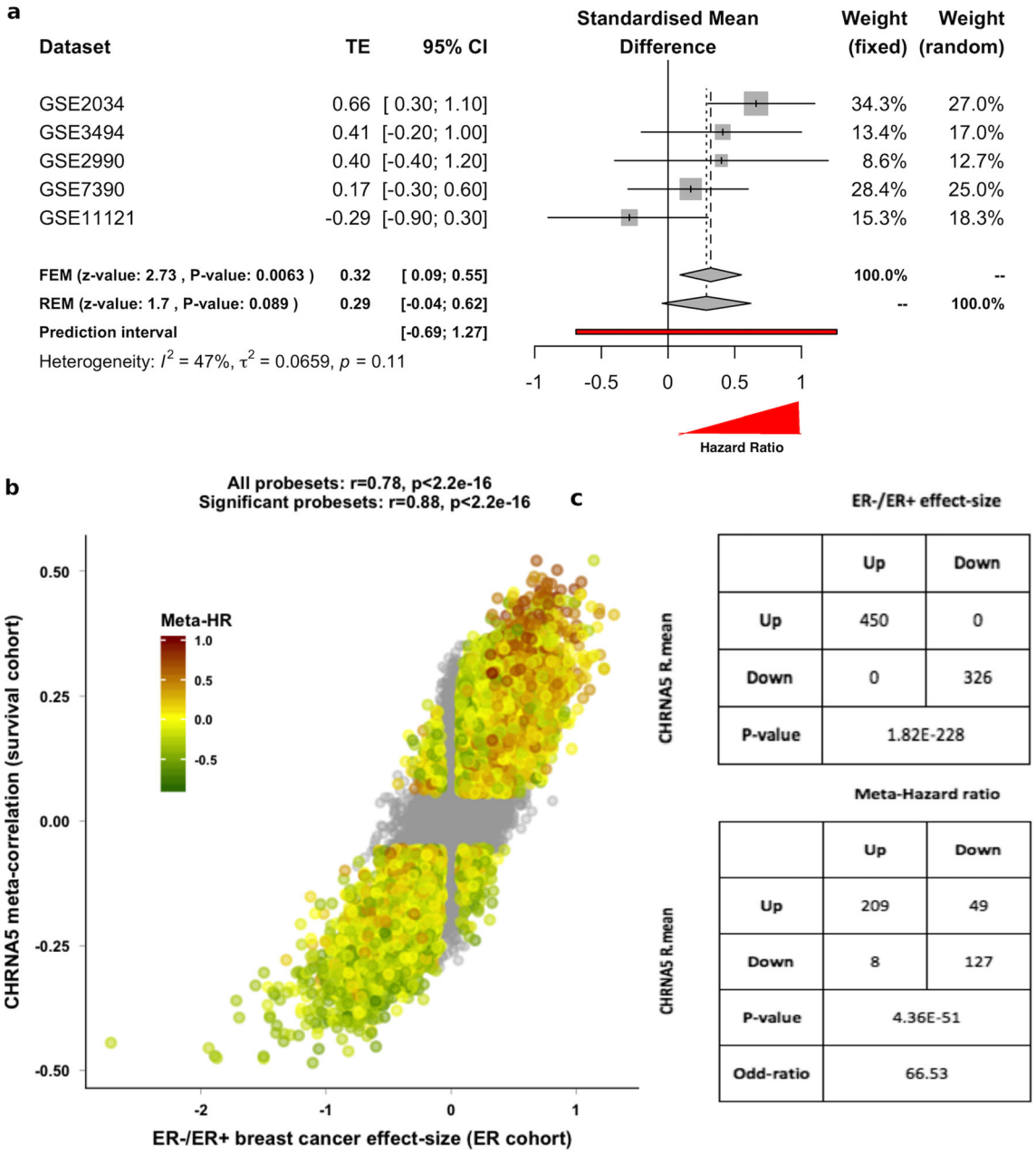


Fig. 3 *CHRNA5* and its co-expressed genes are associated with a worse prognosis and are over-expressed in the ER$^-$ breast cancer subtype. **a** Meta-survival analysis of *CHRNA5* in breast cancer untreated patient data, i.e., the *survival cohort* (datasets were obtained from KM plotter database). Positive effect size indicates a worse prognosis (high HR) and vice-versa. *FEM: Fixed Effect Model; REM: Random Effect Model; TE: Treatment Effect.* **b** Transcriptome-wide meta-correlation profile of *CHRNA5* obtained from the *survival cohort* (i.e., each dot represents a meta-correlation score between each gene and *CHRNA5* from all datasets of the *survival cohort*) along with the respective ER$^-$/ER$^+$ effect sizes obtained from the *ER cohort.* Meta-HRs (*survival cohort*) are shown by color code. **c** Association statistics of genes highly correlated with *CHRNA5* (absolute r.mean > 0.3) with ER$^-$/ER$^+$ effect size (FDR < 0.05) and meta-hazard ratio ($p < 0.05$) values indicated

Enrichment of the E2F family in the *CHRNA5* co-expressed module also points to the potential involvement of the E2F-RB pathway in the regulation of *CHRNA5* expression. Mutational and/or transcriptional loss of *RB1* has been reported in 20% of basal breast cancers [46, 47]. Using the *ER cohort*, we indeed found that *RB1* expression was downregulated in ER$^-$ breast cancer patients (REM: z-value: −6.04, p = 1.5e-09; Supplementary Fig. 8a) and negatively correlated with *CHRNA5* (r = −0.28, p = 2.2e-20; Supplementary Fig. 8b) as well as with *E2F1* (r = −0.43, p = 9.7e-63; Supplementary Fig. 8c). Consistently, TCGA CNA data analysis confirmed differential CNA frequencies of *RB1* between ER$^-$ and ER$^+$ breast tumors (χ^2 = 32.29, p < 1e-5). In accordance with these results, *CHRNA5* expression was higher in low *RB1* expressing samples (Supplementary Fig. 8d). Using an independent ChIP-seq dataset, we confirmed the presence

Table 1 Significantly regulated KEGG pathways of *CHRNA5* co-expressed modules.

	Term	Count	*P*-value	Benjamini
Yellow (positive)	hsa04110:Cell cycle	35	1.12E-29	1.49E-27
	hsa03030:DNA replication	17	3.05E-18	2.03E-16
	hsa04115:p53 signaling pathway	12	1.12E-07	4.94E-06
	hsa04114:Oocyte meiosis	13	2.53E-06	8.40E-05
	hsa03430:Mismatch repair	7	5.79E-06	1.54E-04
	hsa00240:Pyrimidine metabolism	12	9.96E-06	2.21E-04
	hsa04914:Progesterone-mediated oocyte maturation	11	1.22E-05	2.32E-04
	hsa03440:Homologous recombination	6	3.00E-04	4.98E-03
	hsa00230:Purine metabolism	12	1.12E-03	1.65E-02
	hsa03420:Nucleotide excision repair	6	2.87E-03	3.75E-02
Turquoise (positive)	hsa04080:Neuroactive ligand-receptor interaction	83	1.29E-23	3.51E-21
	hsa05033:Nicotine addiction	15	5.82E-06	7.88E-04
	hsa04060:Cytokine-cytokine receptor interaction	41	3.72E-05	3.35E-03
	hsa05032:Morphine addiction	22	4.95E-05	3.35E-03
	hsa04723:Retrograde endocannabinoid signaling	20	1.02E-03	5.38E-02
	hsa04020:Calcium signaling pathway	30	1.04E-03	4.60E-02
Green (negative)	hsa04510:Focal adhesion	16	5.44E-07	9.80E-05
	hsa04512:ECM-receptor interaction	11	7.87E-07	7.08E-05
	hsa04151:PI3K-Akt signaling pathway	17	7.37E-05	4.41E-03
	hsa05200:Pathways in cancer	18	1.02E-04	4.58E-03
	hsa04142:Lysosome	9	5.48E-04	1.95E-02
	hsa05146:Amoebiasis	8	1.23E-03	3.64E-02

of an E2F1 binding site in the *CHRNA5* promoter with a binding capacity that seemed to directly increase with *RB1* knockdown (Supplementary Fig. 8e). These analyses indicate that *CHRNA5* expression may be directly or indirectly regulated by E2F1 binding and/or loss of RB1.

3.8 *CHRNA5* co-expressed network is indirectly/secondarily regulated by E2 signaling

An independent microarray dataset of E2 treatment in the presence or absence of CHX (GSE8597) was used to predict indirectly or secondarily induced targets by E2 using the PS-score such that the higher the PS-score value, the more secondarily induced the transcription was (algorithm details in methods). Including *CHRNA5*, we predicted a total of 367 such targets with PS-scores greater than 0.5 ($p < 0.05$). *CHRNA5* having a high PS-score (0.87) suggested that it was indeed an E2-induced secondary target (Fig. 7a; Supplementary Table 6). Additionally, the top positively co-expressed module (yellow) was found to be highly enriched with these predicted secondary targets of E2 signaling (180 probesets from a total of 348 probesets in the module; Fig. 7b). In addition, these predicted secondarily induced targets were over-expressed in ER^- breast cancers (Fig. 7c, e) and exhibited a worse prognosis (Fig. 7d, f). Our findings revealed that the more a gene was induced secondarily, i.e., PS-score > 0.5, by E2 signaling and overexpressed in ER^- breast tumors, the more hazardous it was as a prognostic biomarker ($r = 0.62$; $p = 4.9e\text{-}41$; Fig. 7d, f).

3.9 *CHRNA5* expression negatively correlates with that of primary ER targets

We next mapped the ChIP-seq based list of primary ER interacting genes [36] onto our meta-analysis results (145 genes corresponding to jetset best probesets (GPL96)) and confirmed that unlike the predicted secondarily induced targets, more than half of the primary targets (76; 52%) were significantly overexpressed (FDR < 0.05) in ER^+ tumors while only 38 (26%) were enriched in the ER^- subtype (Supplementary Fig. 9a). In accordance with our previous findings, the expression of primary target genes enriched in ER^+ samples was negatively correlated with that of *CHRNA5* (Supplementary Fig. 9a). Additionally, only nine genes out of 145 (including *GREB1*) overlapped between the predicted secondary targets and ChIP-seq retrieved primary targets (Supplementary Fig. 9b), demonstrating a low false positive rate and/or both primary as well as secondary targeting. We also investigated expression of these primary and secondary targets based on the TCGA breast cancer RNA-seq dataset. A

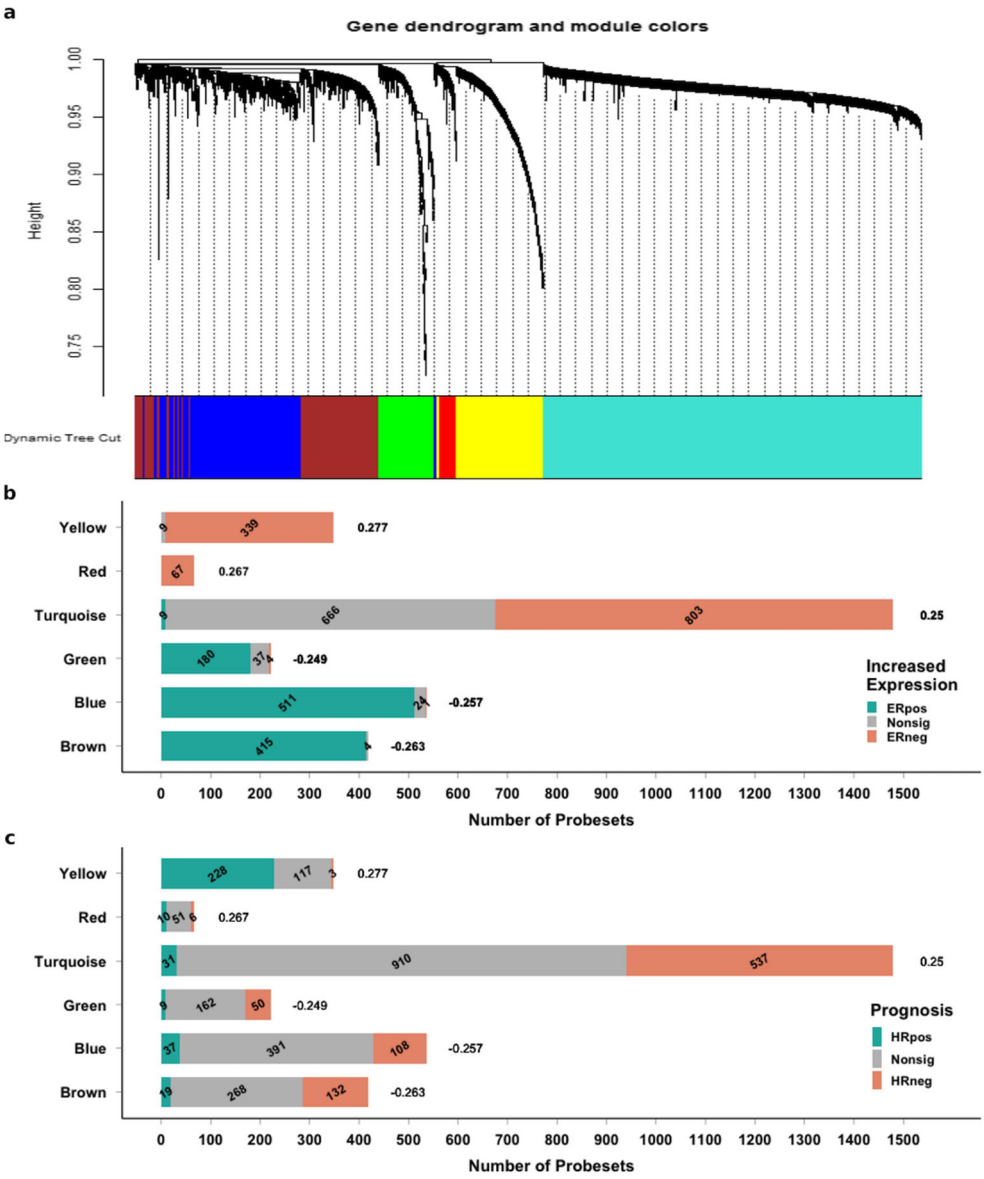


Fig. 4 Distribution of meta-analysis scores in WGCNA *CHRNA5* co-expression modules. **a** Six co-expression modules obtained from WGCNA using meta-correlated genes of the *ER cohort* (absolute r.mean > 0.2 and $p < 0.05$). **b** Distribution of ER^- (effect-size > 0 and FDR < 0.05; *ER cohort*), ER^+ (effect-size < 0 and FDR < 0.05; *ER cohort*) enriched and non-significant genes in *CHRNA5* co-expressed modules. **c** Distribution of worse prognostic markers (Meta-HR > 0 and $p < 0.05$; *survival cohort*), good prognostic markers (Meta-HR < 0 and $p < 0.05$; *survival cohort*) and non-significant markers in *CHRNA5* co-expression modules. After each bar, the mean *CHRNA5* meta-correlation score of each module is shown to indicate positively and negatively correlated modules

principal component analysis (PCA) plot of expression of primary targets and targets secondarily induced by E2 revealed the presence of two distinct clusters, with targets in the latter being more variably expressed (Fig. 8a). Primary and secondary targets were generally negatively correlated with each other based on a clustergram of TCGA data (Fig. 8b). Overlaying of the meta-HR values on the PCA plot made the association between a worse prognosis and the secondary nature of E2 signaling more prominent. Retrospectively, higher expression of many primary targets was found to be associated with better survival outcomes (Fig. 8c).

3.10 Comparison of CHRNA5 siRNA profile with E2-stimulation and hormone-starvation profiles

We have previsouly shown that downregulation of *CHRNA5* by RNAi in MCF7 cells results in increased apoptosis and reduced cell proliferation (GSE89333) [9]. However, the similarity between CHRNA5 depletion and E2-stimulation/starvation has not been studied yet. First, we confirmed that hormone starvation inversely modulated, while E2 exposure stimulated, the expression of *CHRNA5* (Fig. 9a; GSE4668 [38]). Comparative transcriptomics performed between the

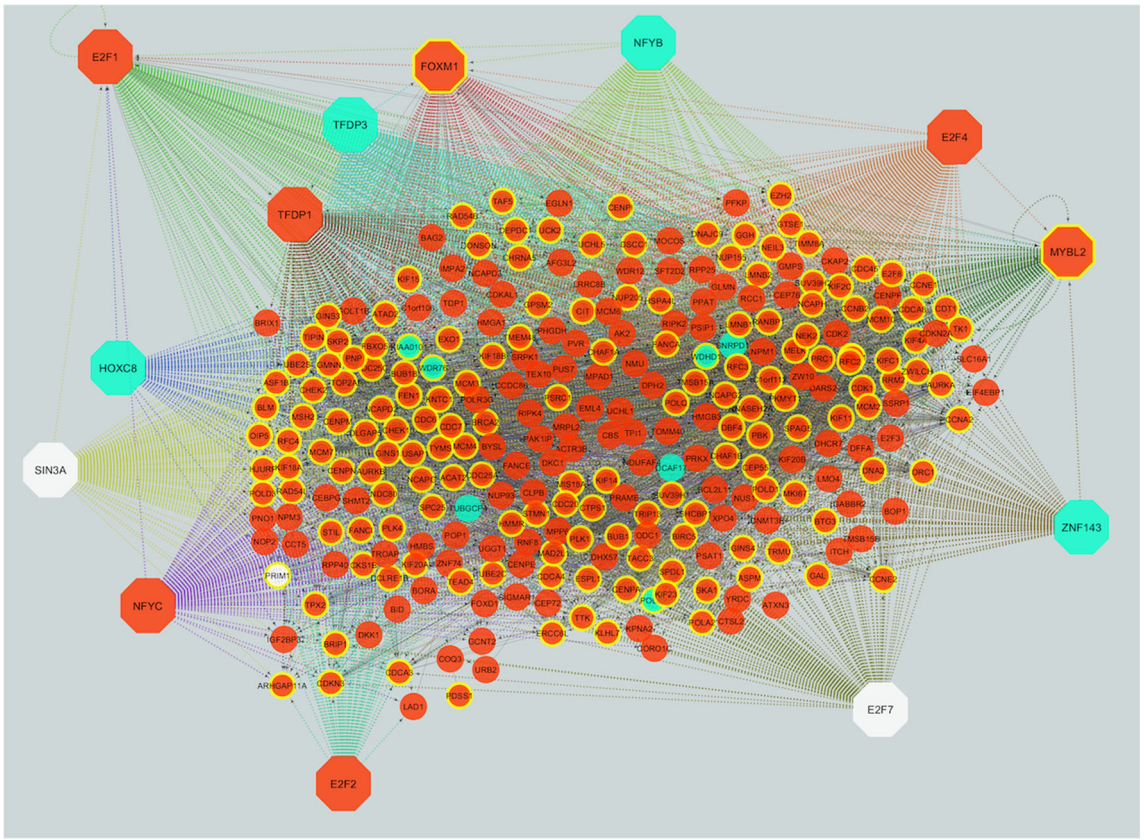


Fig. 5 Interaction network of the top positive *CHRNA5* co-expressed network module (yellow) reveals targets of TF enrichment analysis. Octagonal nodes represent enriched TFs from IRegulon. Brown color indicates gene upregulation in ER^- breast cancer patients and turquiuse color of nodes indicates gene upregulation in ER^+ breast cancer patients. White nodes represent either non-significant changes or unavailable probesets for genes. Yellow node borders indicate genes predicted to be E2-induced secondary targets based on PS-scores

expression profiles showed a significantly negative correlation between CHRNA5 depletion and E2-stimulation response, while a positive one was observed between CHRNA5 depletion and hormone starvation (Fig. 9b-c) with highly significant Odds Ratios (ORs) (Fig. 9d). The results showed that knockdown of CHRNA5 in MCF7 cells partially mimicked hormone starvation-induced changes and negatively influenced E2-mediated transcriptomic regulation. Venn-diagrams and KEGG enrichment of genes shared by all three conditions prioritized several pathways related with proliferation (Fig. 9e), as well as viral carcinogenesis and FoxO signaling (Fig. 9f).

Analysis of primary (direct) and secondary (indirect) E2-induced targets in the context of these three expression profiles showed that the expression changes stratified according to the direction of PS-scores for the primary targets (Fig.10a). Interestingly, most ChIP-seq based gene probes [36] exhibiting positive PS-scores also had lower logFC values, indicating downregulation both in the CHRNA5 depletion and E2-starvation profiles (Fig. 10a). Most of the predicted secondarily induced targets (PS-scores > 0.5 by definition) were also significantly downregulated in both the CHRNA5 depleted and E2 starved MCF7 cells while being upregulated by E2 stimulation in general (Fig. 10b). LogFC values of the primary targets along with those of the secondarily induced targets could predict a significant portion of the variance across the meta-HR values (*survival cohort*) ($R^2 = 0.37$; Fig. 10c). We also overlayed the expression changes mediated by CHRNA5 depletion on the TCGA breast cancer dataset (Fig. 10d), further underscoring the degree of association between primary and secondary E2 signaling and CHRNA5 depletion. We were able to confirm the microarray-based expression changes for selected primary and secondary targets by RT-qPCR using CHRNA5 siRNA-treated MCF7 cells (Supplementary Table 4; Fig. 10e). In addition, we showed that there was dose as well as time dependency for CHRNA5 depletion, since the primary target effects decreased over time (72 h vs. 120 h) and with increasing doses (lower vs. 50 nM) (Fig. 10e).

4 Discussion

Previously, the importance of the *CHRNB4/A3/A5* locus in nicotine dependence and lung cancer has been reported [48]. Subsequently, several members of cholinergic receptors other

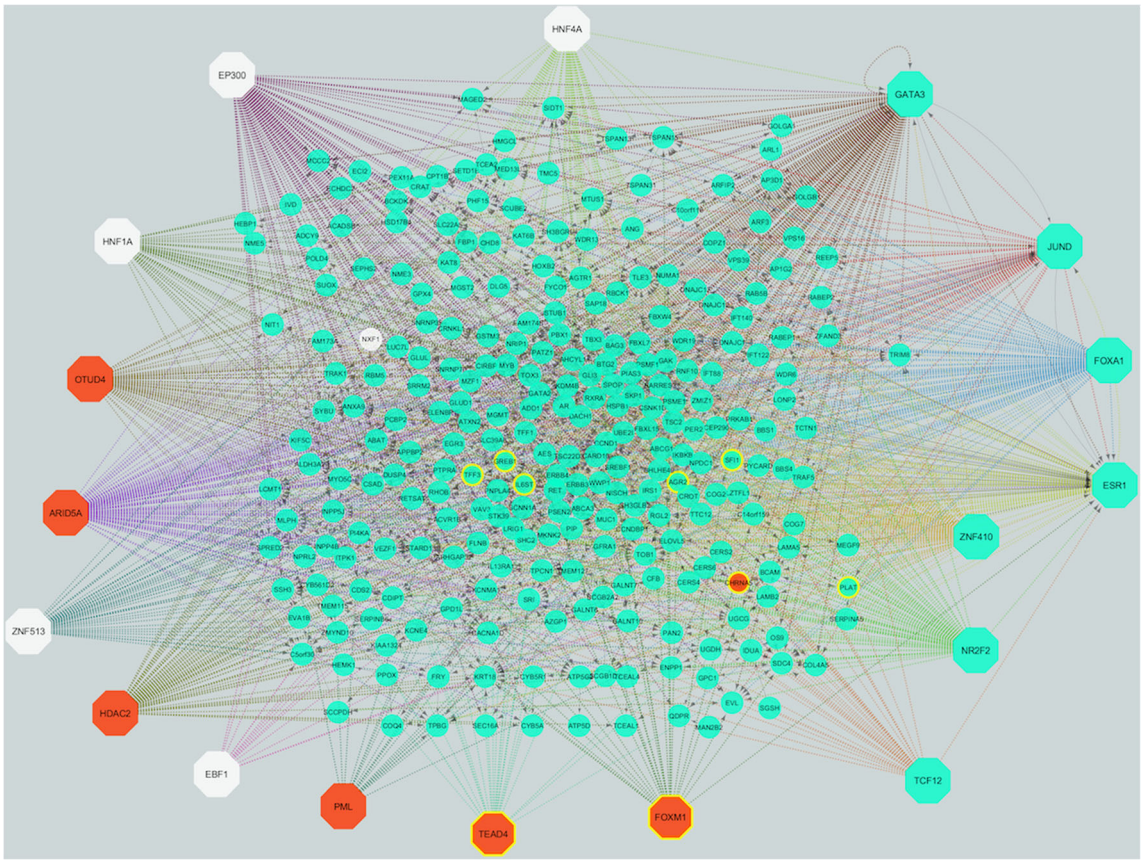
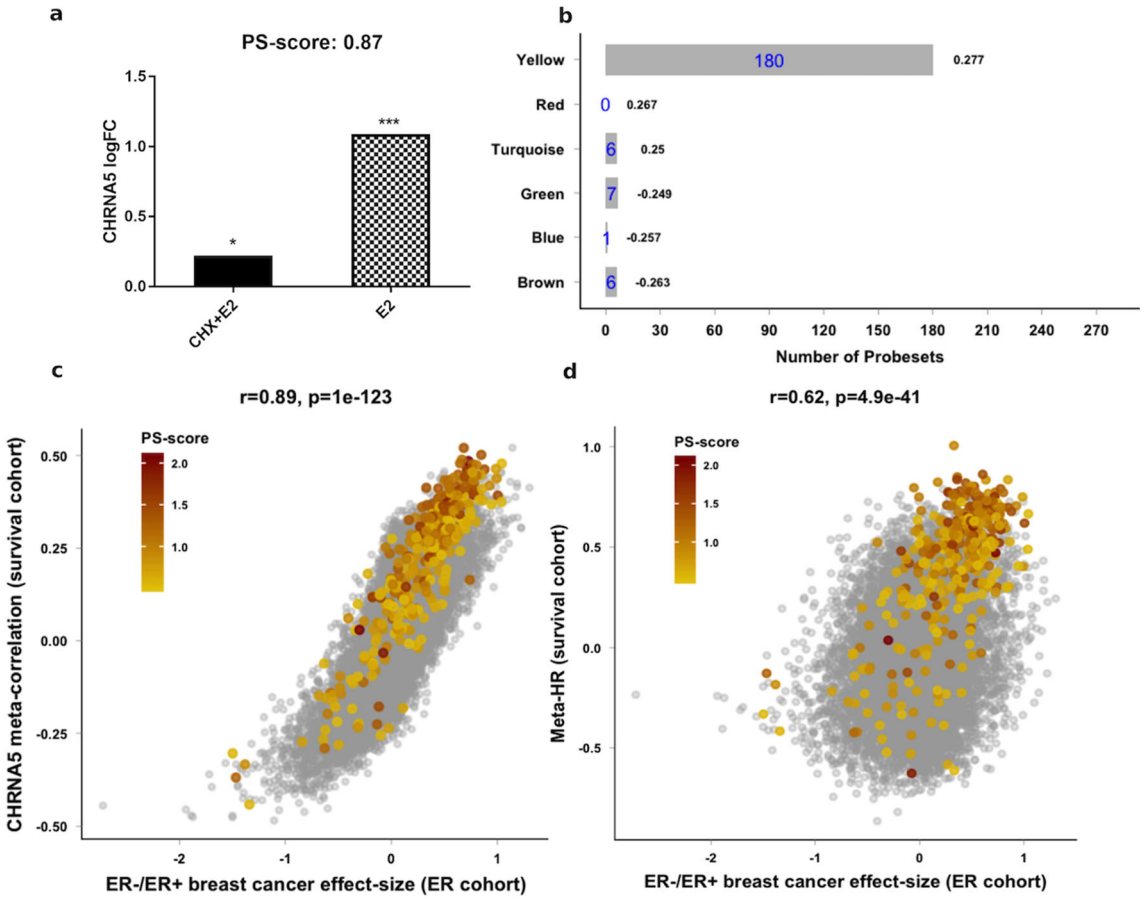


Fig. 6 Interaction network of the top negative *CHRNA5* co-expression network module (brown) reveals targets of TF enrichment analysis. Octagonal nodes represent enriched TFs from IRegulon. Brown nodes indicate gene upregulation in ER$^-$ breast cancer patients and turquiuse nodes indicate gene upregulation in ER$^+$ breast cancer patients. White nodes represent either non-significant changes or unavailable data for genes. Yellow node borders indicate genes predicted to be secondary targets of E2 signaling according to PS-scores

than CHRNA5 have been implicated in breast cancer and E2 signaling [6–8]. We previously revealed a role of CHRNA5 depletion by siRNA in cell cycle modulation, apoptosis and drug sensitivity in breast cancer cells [9]. Here, we provide a link between *CHRNA5* expression and E2-mediated secondary signaling in breast cancer using meta-analysis, co-expression network and survival analyses, and RT-qPCR validation based on ex vivo and in vitro studies. Our results show that the expression of *CHRNA5* may be modulated in breast cancer by E2 in cell line- and isoform-specific manners, while it exhibits a negative correlation with the expression of *ESR1* in both cell lines and primary tumor samples. The observed decreased *ESR1* expression upon E2 exposure is in accordance with the literature, since E2 exposure in breast cancer cells is known to lead to a feedback inhibition of *ESR1* mRNA levels [48], which may be associated with increased CHRNA5 levels [49].

CHRNA5 isoforms, whose increased expression levels have been reported in the context of lung cancer [4], were also higher in breast tumors than normal tissues. *CHRNA5* isoforms differ on the basis of alternative splicing in exon 5 (the trans-membrane domain of the protein) of the gene. The observed variability between *CHRNA5* isoform expression in response to E2 in breast cancer cell lines and patient data may be explained by differential roles of the isoforms, since some (e.g., *CHRNA5_V3*) are implicated in impairment of signal transduction due to instability of the truncated trans-membrane domain. The isoform-specific effects need to be further explored mechanistically in breast and other E2 responsive tissues. Use of an immunofluorescent antibody to track spatial distribution of different isoforms can help to distinguish between isoform-specific signal transduction patterns in cancer and normal cells.

In the present study we have also shown that *CHRNA5* is induced by E2, secondarily. This novel finding is supported by (1) dependence of *CHRNA5* expression changes on the duration of E2 exposure as well as the presence/absence of new protein synthesis [12] and (2) enrichment of the *CHRNA5* co-expressed network with E2F transcription factors known to be directly or indirectly regulated by ESR1. Previously, E2F1-induced genes have also been found to be more pronounced in ER$^-$ breast cancers and to be characterized by a worse prognostic potential, validating our findings [50, 51]. Moreover, a recent study showed a positive association between E2F



e

ER-/ER+ effect-size

CHRNA5 R.mean

	Up	Down
Up	259	5
Down	0	34
P-value	9.06E-40	

f

ER-/ER+ effect-size

Meta-HR

	Up	Down
Up	202	10
Down	4	9
Odd ratio	43.15	
P-value	1.61E-08	

Fig. 7 *CHRNA5* co-expression profile is enriched with secondary targets of E2 signaling. **a** *CHRNA5* expression induced by E2 in the presence or absence of CHX (GSE8597). PS-scores were calculated as described in Materials and Methods. **b** Distribution of predicted E2-induced secondary targets in the *CHRNA5* associated co-expression modules identified by WGCNA. **c-d** Mapping of the 366 other predicted E2-induced secondary targets (by color code) on top of transcriptome-wide *CHRNA5* correlations (from the *survival cohort*) and the differential ER^-/ER^+ meta-analysis effect sizes (*ER cohort*) (**c**), and on top of transcriptome-wide HRs (*survival cohort*) and the differential ER meta-analysis (*ER cohort*) (**d**). **e** Contingency table and association statistics of **c**. **f** Contingency table and association statistics of **d**

signaling and *CHRNA5* expression in oral squamous cell carcinoma, while increased expression of the *E2F* geneset and *CHRNA5* was associated with a worse recurrence-free survival [52]. In addition to an E2F binding site enrichment, an inverse correlation between *CRHNA5* and *RB1* as well as downregulation of *RB1* in ER^- breast cancers highlighted that dysregulation of RB1-E2F signaling may be responsible for *CHRNA5* induction in ER^- breast cancer. It is also known that ER-alpha cooperates with multiple TFs including SP1, NF-Y and c-FOS to mediate *E2F1* expresion in breast cancer cells [53, 54], and that *E2F1* expression is reduced by E2 starvation [38, 55]. However, further studies are needed to confirm any direct or indirect impact of the RB1-E2F1 signaling pathway on the modulation of *CHRNA5* expression. Our findings indicate the importance of uncoupling cellular effects of primary and secondary targets of E2 signaling in breast tumors and implicate CHRNA5 depletion as a potential uncoupler of E2-driven ESR1 signaling (e.g., induction of primary target PGR [56], and depletion of secondary target CDC6 [57]).

The prominent expression of some E2-induced targets in the ER^- breast cancers may also point to alternative mechanisms driven by factors other than ESR1. Indeed, close to

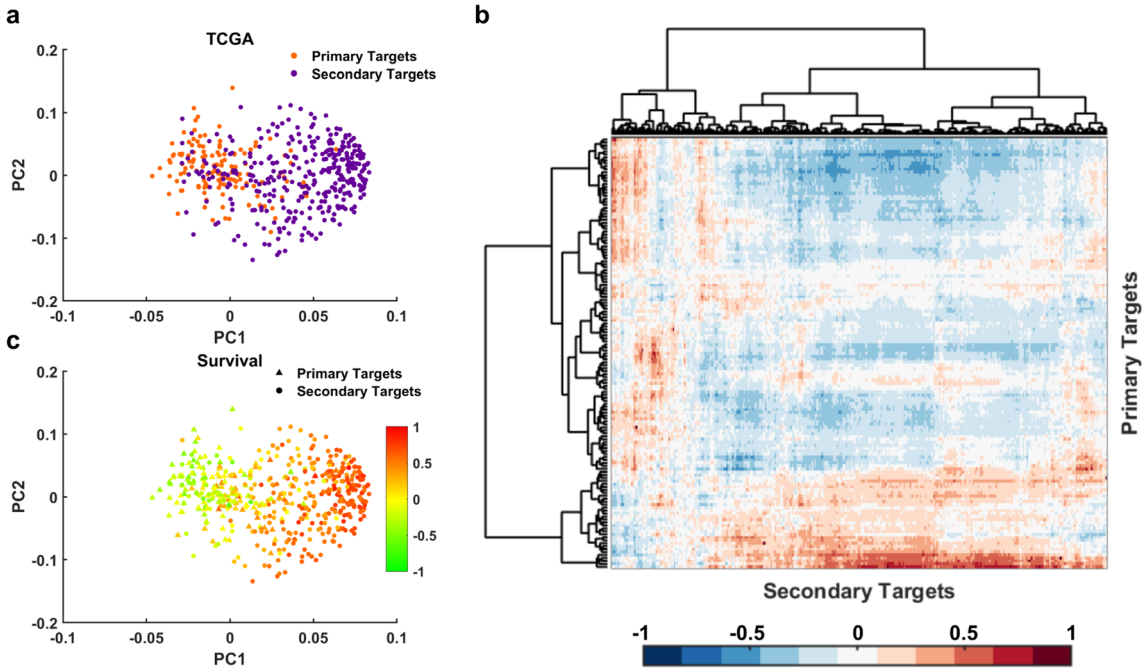


Fig. 8 ChIP-defined ESR1 primary targets and E2-induced secondary targets are clustered distinctly based on TCGA breast cancer RNA-seq data. **a** Principal component analysis (PCA) plot of primary (orange) and secondary (purple) target gene expression data from TCGA. **b** Clustergram of correlation between primary and secondary target gene expression from TCGA breast cancer dataset. **c** PCA plot from Fig. 8a overlayed with meta-HR color codes

half of breast cancer patients express ESR1, but almost 60% of the ER^- cases are reported to be positive for ESR2 (ER-beta receptor). ESR2 may exert its effects ligand-independently, for example, via microRNAs [58, 59]. ESR2 has also been shown to mediate responses to tamoxifen in ER^- breast cancer patients by interacting with TP53 [60–62]. GPER1, known to interact with E2, is yet another candidate receptor that may be highly expressed in ER^- breast cancer patients [63, 64]. Additionally, unliganded ESR1 can be constitutionally active due to mutations that can be found in the E2 binding site [43, 65, 66]. Steroid receptor coactivators have also been shown to mediate E2-dependent effects in breast cancer [67]. Hence, E2 may act through these alternative pathways and modify the genomic and non-genomic actions of ESR1. Moreover, CHRNA9, which is a cholinergic receptor with a higher expression in ER^+ tumors and known to form heteromeres with CHRNA5 subunits, can play modulatory roles in E2-mediated actions of ERs through phosphorylation events [7]. On the other hand, when the expression of *CHRNA9* was silenced along with that of *CHRNA5* in ER^- MDA-MB-231 cells, they could not survive, suggesting a complex interplay between these two receptors [8]. Therefore, future studies should focus on isogenic cell lines with and without receptors interacting with E2 and/or acetylcholine to mechanistically decipher the crosstalk between ERs and cholinergic receptor subunits.

Nicotinic acetylcholine receptor (nAChR) modulators have shown potential in reducing tumor growth by targeting different subunits of the pentameric complex. Alpha-neurotoxins (from snake venom) are potential anti-tumor drugs that affect cell proliferation by regulating the expression of *CHRNA7* in non-small cell lung carcinoma [68]. Natural anti-nicotinic ligands (SLURP-1/SLURP-2) in combination with nicotinic receptor antagonists (mecamylamine, luteoline or methyllycaconitine) have been found to exhibit effects on cell viability and cancer therapeutics by regulating the expression of *CHRNA7* or *CHRNA9* [69]. Conotoxins and α-cobratoxin (MII, PnIA, RgIA and ArIB11L16D) have been reported to potentially target and inhibit different nAChR subunits (α6, α3β2, α9α10 and α7 nAChRs) and, consequently, slow down tumor progression and inflammation in a mouse model [70, 71]. To the best of our knowledge, however, no specific CHRNA5 inhibitor has been studied for its anti-tumor activity. This, therefore, warrants further development and exploration.

Our study revealed a significantly linear and positive correlation between the values of prognostic indicators and PS-scores describing the direction and magnitude of primary and secondary expression modulations by E2. Accordingly, the majority of primary targets are associated with a better prognosis (meta-HR values < 0) while secondary targets exhibit higher meta-HRs. The magnitude and direction of PS-scores can help to predict the prognostic role of a gene in breast

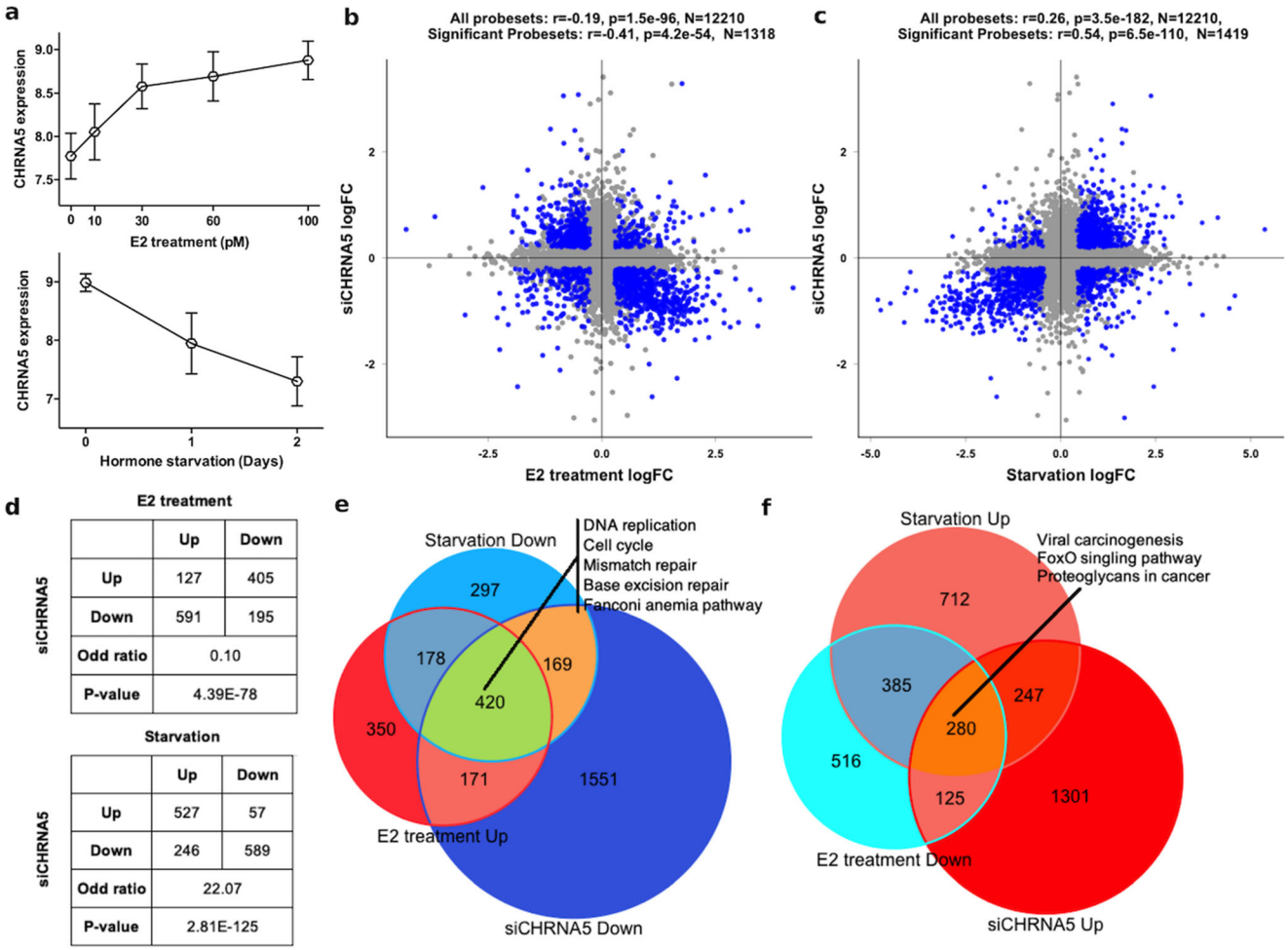
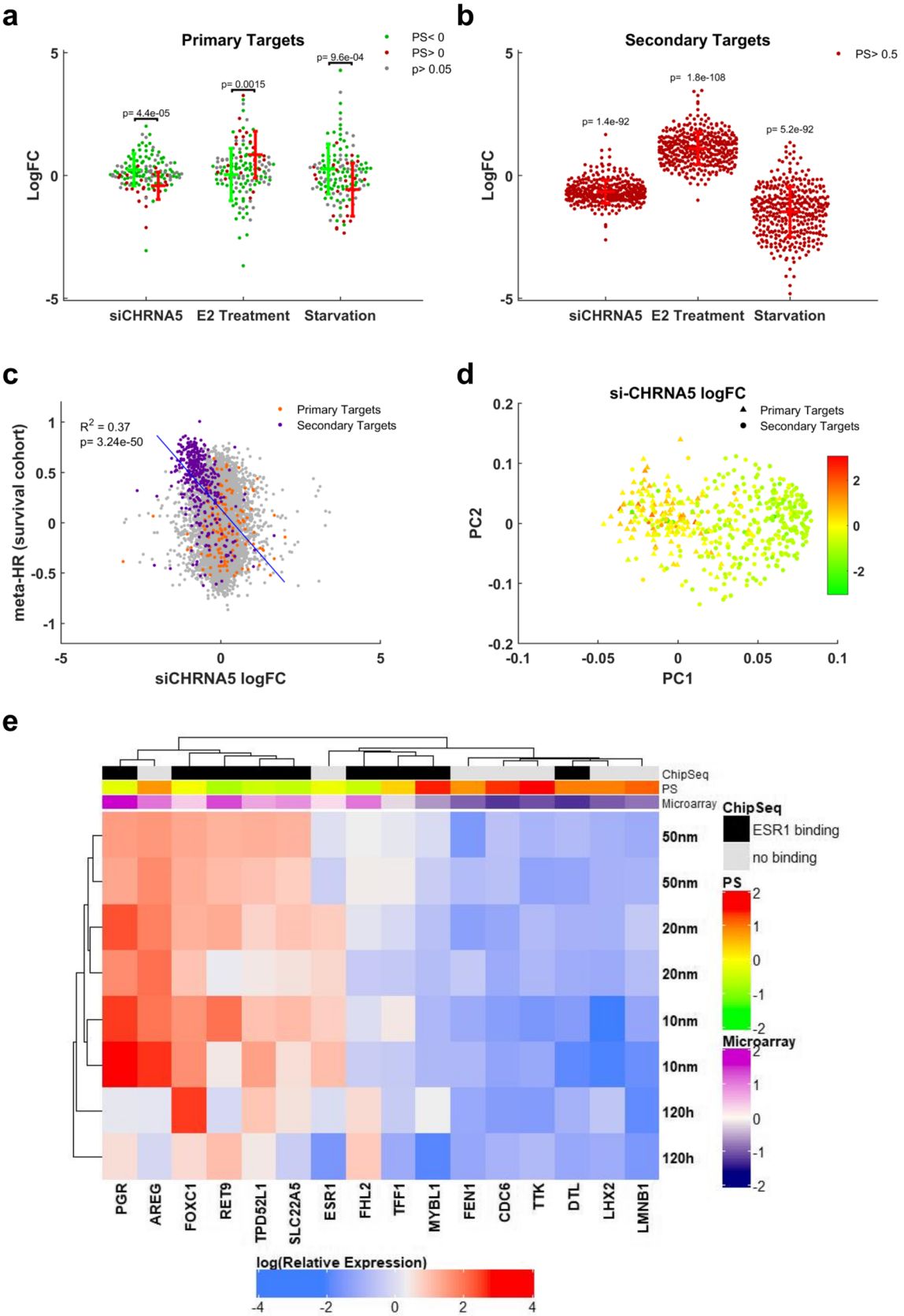


Fig. 9 CHRNA5 depletion profile resembles hormone starvation profile and downregulates proliferative pathways. **a** Change in *CHRNA5* probeset expression in E2-treated (across increasing doses) and -starved MCF7 microarray datasets (across time). **b-c** Comparison of CHRNA5 knockdown mediated transcriptomic changes with E2-treated (**b**), and hormone-starved log fold changes (**c**) where significant genes in both datasets ($p < 0.05$) are labeled blue while non-significant regulation in either of the dataset is shown in grey. **d** Contingency table and association statistics of significantly regulated genes shown in **b** and **c**. **e-f** Shared regulated genes downregulated by CHRNA5 knockdown and E2-starvation profile while upregulated by E2 treatment (**e**), and vice-versa (**f**). Top 5 pathways from KEGG pathway enrichment analysis with FDR < 0.05 are marked

cancer, and in the future these can be tested in other cancers where E2 plays a role. As a result, *CHRNA5* depletion downregulates most of the targets induced secondarily by E2 stimulation while the primary targets of ESR1, identified by ChIP-seq, can be induced or repressed, as partially predicted by their PS-scores. Our analysis of the TCGA breast cancer data supports this uncoupling via demonstrating a negative correlation between primary and secondary target expression profiles. However, it is important to note that some targets bound by ESR1 upon E2 stimulation (e.g., MYLB1 and DTL, Fig. 10e) can also be targeted secondarily. In such cases, PS-scores help to predict the prognostic significance better than ChIP-seq results alone.

Previously, microarray-based meta-analyses have focused on either expression profiles of E2 treated cells [13, 72], differential gene expression in breast cancer subtypes [73–75], or the prognostic importance of a gene or geneset in breast cancer [25]. In the present study, we have combined all three types of meta-analyses to address the involvement of *CHRNA5* and its co-expression network in breast cancer prognosis. We have also confirmed our predictions using different independent cohorts, e.g., COSMIC and CCLE data in case of cell lines and TCGA RNA-seq data in case of patient samples, validation by RT-qPCR using five different breast cancer cell lines, CHRNA5 siRNA or control siRNA treated MCF7 cells, and across independent patient cDNA samples. Our study presents CHRNA5 as a potential biomarker, whose expression can be targeted in breast cancer in a subtype-specific manner to obtain a better breast cancer prognostication. Our study has highlighted a novel association between *CHRNA5* expression and the RB1-E2F1 pathway. Future studies are needed to identify the underlying mechanisms and to open up novel


a
Primary Targets
PS< 0
PS> 0
p> 0.05
p= 4.4e-05
p= 0.0015
p= 9.6e-04
LogFC
siCHRNA5
E2 Treatment
Starvation
b
Secondary Targets
PS> 0.5
p= 1.4e-92
p= 1.8e-108
p= 5.2e-92
LogFC
siCHRNA5
E2 Treatment
Starvation
c
Primary Targets
Secondary Targets
R2 = 0.37
p= 3.24e-50
meta-HR (survival cohort)
siCHRNA5 logFC
d
si-CHRNA5 logFC
Primary Targets
Secondary Targets
PC2
PC1
e
ChipSeq
PS
Microarray
ChipSeq
ESR1 binding
no binding
PS
Microarray
50nm
50nm
20nm
20nm
10nm
10nm
120h
120h
PGR
AREG
FOXC1
RET9
TPD52L1
SLC22A5
ESR1
FHL2
TFF1
MYBL1
FEN1
CDC6
TTK
DTL
LHX2
LMNB1
log(Relative Expression)

◀ **Fig. 10** Primary and secondary targets are differentially modulated by CHRNA5 siRNA, E2-treatment and hormone starvation. **a-b** Change in the ESR1 primary targets **(a)** and predicted secondary targets (PS > 0.5 and $p < 0.05$) **(b)**, in response to CHRNA5 siRNA treatment, E2-treatment and -starvation; *p*-values from student's t test are reported on top of each comparison. **c** Mapping of the primary (orange) and predicted secondary (purple) targets on top of mHR scores (from the *survival cohort*) and the siCHRNA5 transcriptome. **d** Mapping of logFC values from si-CHRNA5 profile on top of principal component analysis (PCA) plot of primary and secondary target gene expression data from TCGA. **e** RT-qPCR validation of expression changes in selected primary and secondary targets in si-CHRNA5 treated MCF7 cells in a time- and dose-dependent manner

therapeutic avenues. The list of E2-induced secondary targets found in the *CHRNA5* co-expression network, and the significant association with PS-score, CHRNA5 depletion response and prognostic outcome may be of help to better understand the outcome of E2 signaling in breast tumors. Finally, our findings suggest that precise stratification of expression of primary and secondary targets of E2 signaling may help to better predict the prognostic outcome.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13402-020-00581-x.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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