Reconstruction of nuclear receptor network reveals that *NR2E3* is a novel upstream regulator of *ESR1* in breast cancer

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ESR1 is one of the most important transcription factors and therapeutic targets in breast cancer. By applying systems-level re-analysis of publicly available gene expression data, we uncovered a potential regulator of *ESR1*. We demonstrated that orphan nuclear receptor *NR2E3* regulates *ESR1* via direct binding to the *ESR1* promoter with concomitant recruitment of PIAS3 to the promoter in breast cancer cells, and is essential for physiological cellular activity of *ESR1* in estrogen receptor (ER)-positive breast cancer cells. Moreover, expression of *NR2E3* was significantly associated with recurrence-free survival and a favourable response to tamoxifen treatment in women with ER-positive breast cancer. Our results provide mechanistic insights on the regulation of ESR1 by *NR2E3* and the clinical relevance of *NR2E3* in breast cancer.

INTRODUCTION

Nuclear receptors (NRs) are one of the largest families of transcription factors in metazoans and govern expression of various genes involved in a wide range of reproductive, developmental, metabolic and immunological responses (Gronemeyer et al, 2004; Hegele, 2005; McKenna et al, 2009). Because they contribute to physiologic and pathologic conditions in many organs, NRs have been recognized as one of the most important and successful therapeutic targets for various human diseases, including cancer (Gronemeyer et al, 2004; Hegele, 2005). While much is known about molecular mechanisms of certain NRs, relatively little is known about the complex network of crosstalk among NRs that may play a key role in their regulation of many normal and pathologic conditions. The large number of NRs (48 in humans) makes it time-consuming and difficult to discover such a network using conventional molecular techniques alone (Bookout et al, 2006; Yang et al, 2006).

Genome-wide approaches using high-throughput technologies have been used to explore potential NR networks. In studies by Bookout et al (2006) and Yang et al (2006), expression patterns for all NRs were collected from all mouse organs to gain new insights on integrated NR networks in various organs (Bookout et al, 2006; Yang et al, 2006). In two different studies, the genome-wide chromatin-immunoprecipitation (ChIP) assay revealed *FOXA1* as a new estrogen receptor (ESR1) partner bound to the *ESR1* promoter region in breast cancer cells (Carroll et al, 2005, 2006). Wang et al (2009) used a similar approach and uncovered *UBEC2*, which functions as an androgen receptor (AR) downstream target gene in prostate cancer.

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In recent years, genome-wide analysis and the use of publicly available high-throughput data has facilitated identification of genes having an unexpected association with malignant diseases or with therapeutic targets of diseases (Carroll et al, 2006; Tomlins et al, 2008). Thus, to uncover a potential network of NRs in human cancer or novel interplay among NRs, we conducted a systems-level analysis of publicly available gene expression data from National Cancer Institute-60 (NCI-60) cell lines (Scherf et al, 2000). The NCI-60 cell lines consist of 60 human cancer cell lines derived from nine different tumour types including colorectal, renal, ovarian, breast, prostate, lung and central nervous system tumours, as well as leukemia and melanoma (Monks et al, 1991). NCI-60 cell lines are the most extensively characterized panel of cancer cell lines and are used for drug screening and many pilot experiments (Shoemaker, 2006).

Here we show for the first time that a systems-level analysis of publicly available data uncovered an unexpected relationship between two NRs, namely *NR2E3* and *ESR1*. Furthermore, we show that *NR2E3* is a novel regulator of *ESR1* expression in breast cancer cells and a potential predictive marker for response to tamoxifen for women with ESR1-positive and node-negative breast cancer.

RESULTS

Systems-level analysis of genome-wide gene expression data from NCI-60 cell lines uncovered novel interactions among nuclear receptors in breast cancer

To uncover potential interacting network of NR genes and to generate testable hypotheses, we have used publicly available gene expression data from NCI-60 cell lines that have been used extensively as an exploration data set (Amundson et al, 2008; Hsu et al, 2009; Park et al, 2010; Potti et al, 2006; Reinhold et al, 2010; Wang & Li, 2009). We first tried to uncover an NR network using direct correlation of expression patterns of NRs across NCI-60 cell lines but were not able to produce a recognizable network with a higher degree of interaction among NRs (Fig 1A). Since all NRs are transcription factors that regulate expression of many genes, we hypothesized that expression patterns of direct or indirect target genes regulated by NRs would be are well correlated with patterns of NR expression. Therefore, we identified genes whose expression was significantly correlated with those of NR genes in NCI-60 cell lines as potential downstream targets of NRs. After establishing a Pearson's correlation test p-value of less than 0.001 as being indicative of significance with expression patterns of the NR genes, we generated correlated gene lists of 45 NRs (Fig 1B). As expected, many of the identified correlated genes were previously identified as downstream targets of NR genes. For example, expression of *GATA3*, a well-known downstream target of *ESR1* (Eeckhoute et al, 2007), was highly correlated with expression of *ESR1* ($r = 0.76$, $p = 3.09 \times 10^{-12}$).

Using 45 NR-correlated gene lists comprised of 86–4580 genes, we investigated how many genes in each NR-correlated gene lists were shared in other NR-correlated gene lists, and then we generated the matrix of the shared gene number of each NR across all NRs. To generate a simple but comprehensive network of relationships among NRs, we applied hierarchical clustering to the shared gene number data (Fig 1C). Of interest, the highest number of correlated genes was shared among *ESR1*, *PPARA*, *NR2C2*, *THRA*, *ESRRA*, *NR2E3* and *HNF4A*. Out of 7 NRs, expression patterns of *HNF4A*, *NR2E3*, *THRA* and *PPARA* were directly correlated with *ESR1* expression in NCI-60 cell lines (Fig 1D), indicating that these NRs might be directly or indirectly involved in *ESR1*-signalling pathways.

Since biological and pathological roles of *ESR1* have been best characterized in breast cancer, next we performed correlation analysis using gene expression data from breast cancer patients [Netherands Cancer Institute (NKI) data set, $n = 295$] (van de Vijver et al, 2002). Of the four NRs selected from the NCI-60 cell lines, only the expression of *NR2E3* remained significant ($r = 0.69$, $p = 1.59 \times 10^{-9}$) and correlated positively with the expression of *ESR1* in the NKI breast cancer cohort (Fig 2A). A strong correlation with *ESR1* was observed in another large breast cancer cohort [University of North Carolina (UNC) cohort, $n = 380$, $r = 0.667$, $p = 2.2 \times 10^{-16}$] (Fig 2B; Hu et al, 2006; Oh et al, 2006; Parker et al, 2009). In addition, more than 50% of *NR2E3* correlated genes overlapped with those of *ESR1* in gene expression data from both the NKI and UNC cohorts (Fig 2C–H). Taken together, the concordant and significant association of *NR2E3* with *ESR1* in multiple data sets suggests that *NR2E3* may be involved in regulation of *ESR1*-mediated gene expression and pathways in breast cancer.

NR2E3 directly regulates expression of *ESR1*

NR2E3 was first identified as a photoreceptor-specific nuclear receptor (PNR; Kobayashi et al, 1999; Takezawa et al, 2007), that is necessary for proper eye development and maintenance by regulating the expression of cone-specific and rod-specific genes in retinal cells (Onishi et al, 2009). Mutations in *NR2E3* have been linked to many degenerative eye diseases including enhanced S-cone sensitivity syndrome, Goldmann-Favre syndrome and clumped pigmentary retinal degeneration (Schorderet & Escher, 2009).

Since little is known about the function of *NR2E3* in breast cancer, we investigated possible roles of *NR2E3* related to the *ESR1*-signalling pathway using *NR2E3*-specific small hairpin RNA (shRNA) in estrogen receptor (ER)-positive breast cancer MCF-7 cells (Fig 3A). Surprisingly, when expression of *NR2E3* was silenced by shRNA, expression of *ESR1* and its downstream targets (*GATA3*, *PGR*, *CCND1* and *TFF1*) were also significantly downregulated (Fig 3B); reduced expression of *ESR1* and its downstream targets was also validated at the protein level (Fig 3E). The effect of silencing *NR2E3* expression on *ESR1* and its downstream targets was also highly reproducible in another ER-positive breast cancer cell line: T47D (Fig 3C and D). It is interesting to point out that expression of FOXA1 was not altered after silencing NR2E3expression in MCF-7 cells while its expression was down-regulated in T47D cells, suggesting that additional regulatory mechanisms for expression of FOXA1 might exist in MCF-7 cells. Transcriptional activity of *ESR1* was also diminished by small hairpin NR2E3 (shNR2E3; Fig 3F).

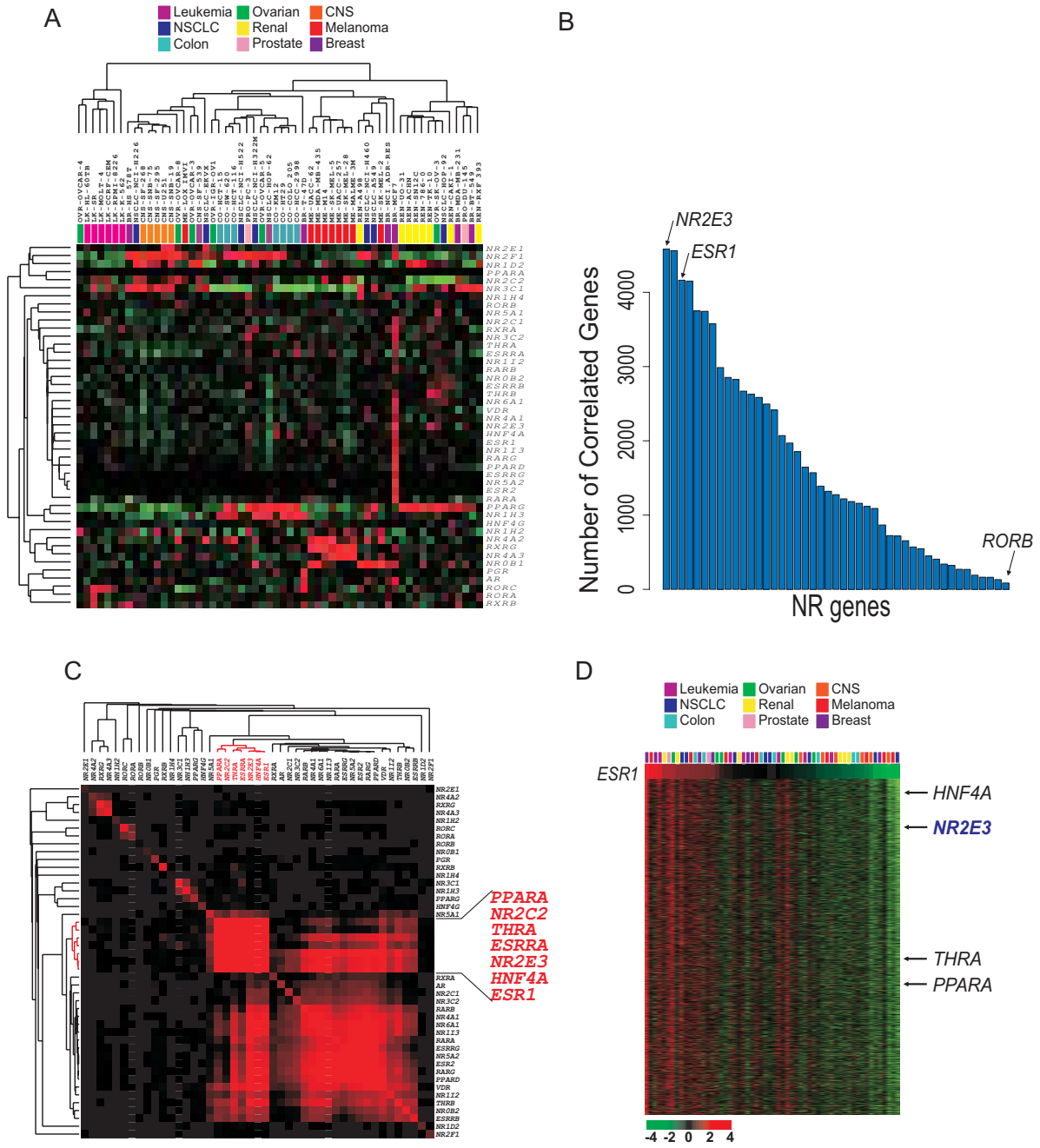

Figure 1. NR gene network in NCI-60 cell lines.

A. Out of 48 human NR genes, expression data of 45 NRs were available in publically available NCI-60 data set and used for hierarchical clustering analysis. The data are presented in matrix format in which rows represent individual gene and columns represent each cell lines. Each cell in the matrix represents the expression level of a gene feature in an individual cancer cell. The red and green colour in cells reflects relative high and low expression levels in log 2 transformed scale.

B. Establishing a Pearson's correlation test *p*-value of less than 0.001 as indicative of in trans significance with expression patterns of the potential downstream genes, we generated 45 gene sets of in trans correlated genes as putative targets genes for each NR gene; these gene sets were comprised of 86–4580 genes (median = 1275).

C. By cross-comparison of correlated genes in all 45-gene lists, we generated secondary lists reflecting overlap of correlated genes among NR genes. These secondary gene lists are presented in matrix format, and hierarchical clustering analysis was performed with the number of correlated genes overlapped between NR. Heat maps indicate the number of genes overlapped between NRs.

D. Only positively correlated genes are presented (2255 gene features). With a cut-off of Pearson's correlation test *p*-value of less than 0.001, expression of 2255 gene features was positively correlated with that of *ESR1*. Of six genes whose correlated genes significantly overlapped with those of *ESR1*, expression of four genes (*HNF4A*, *NR2E3*, *THRA* and *PPARA*) was significantly correlated with expression of *ESR1*.

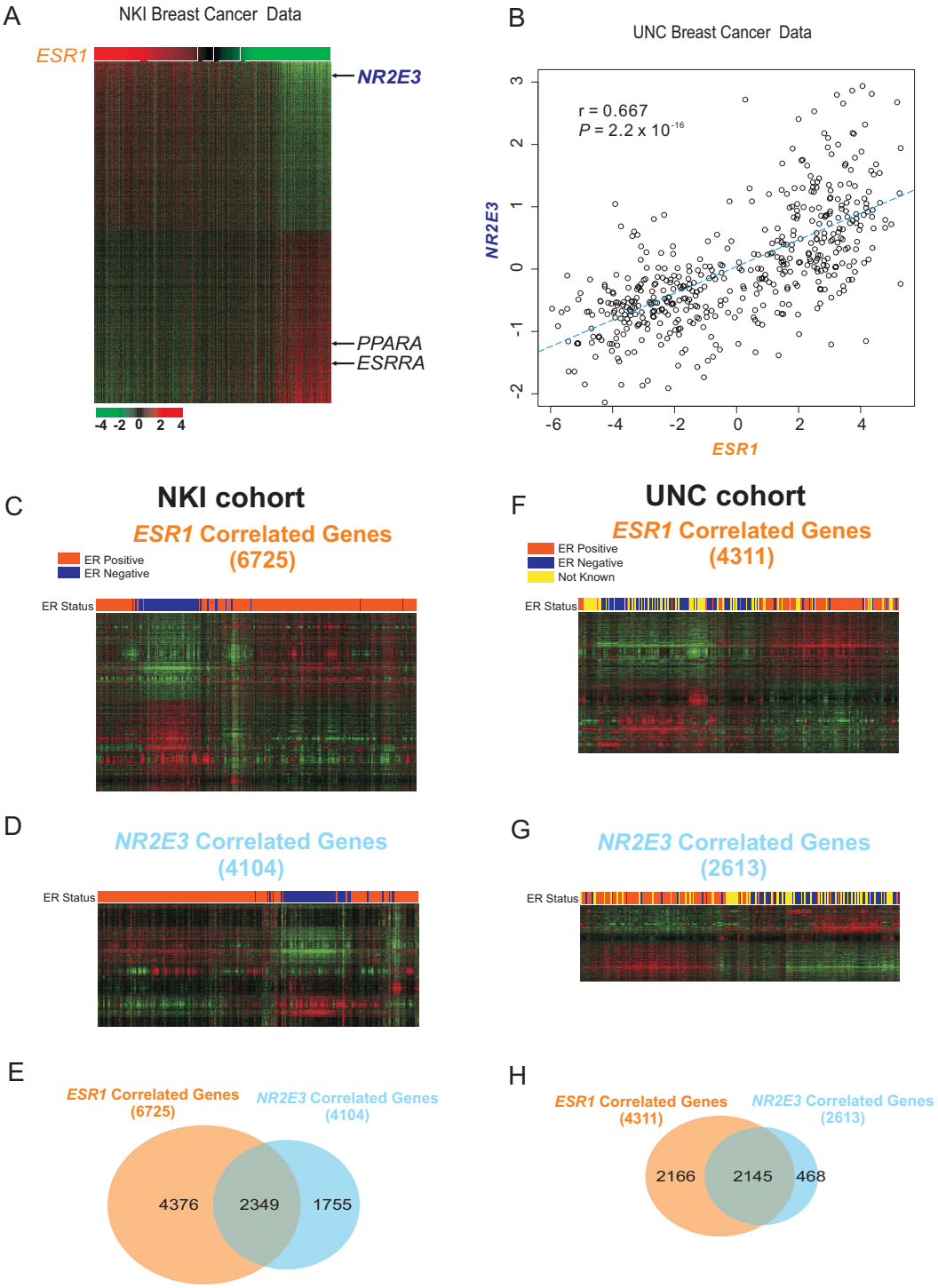

Figure 2. *NR2E3* is highly correlated with *ESR1* in breast cancer patients.

A. With a cut-off of Pearson's correlation test *p*-value of less than 0.001, expression of 6753 gene features were correlated with that of *ESR1* in the NKI breast cancer data set ($n = 295$). Of six genes whose correlated genes significantly overlapped with those of ESR1, expression of *NR2E3* was only positively correlated with ESR1 expression.

B. Correlation of ESR1 and NR2E3 expression in UNC breast cancer patient cohort. Scatter plots between ESR1 and NR2E3 in UNC cohort ($n = 380$).

C-D. ESR1-corelated genes (**C**) or NR2E3 correlated genes (**D**) in NKI cohort were clustered according to their expression patterns.

E. Venn diagram of comparison of two correlated genes in NKI cohort.

F-G. ESR1-corelated genes (**F**) or NR2E3 correlated genes (**G**) in UNC cohort were clustered according to their expression patterns.

H. Venn diagram of comparison of two correlated genes in UNC cohort.

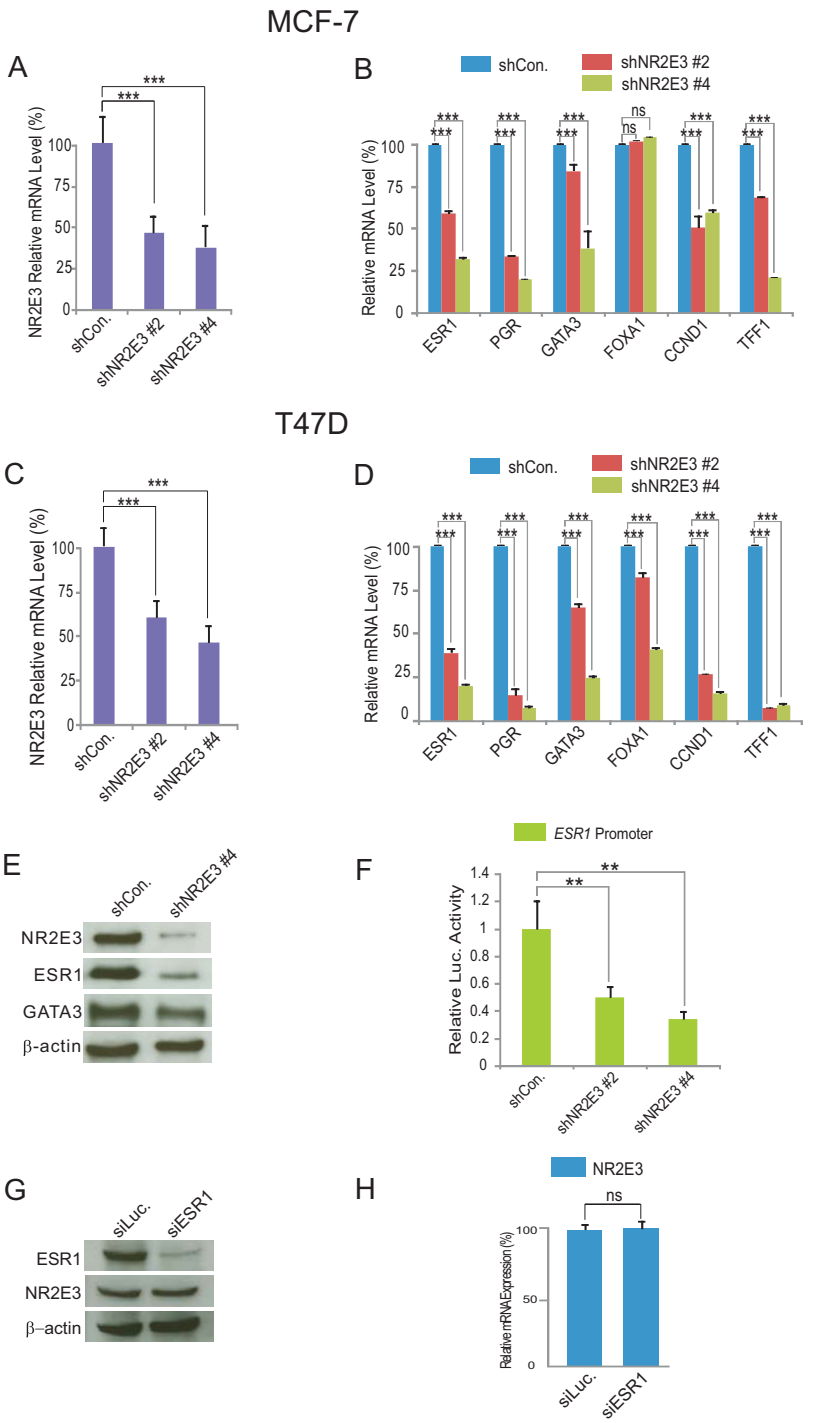

Figure 3. *NR2E3* regulates *ESR1* function in breast cancer cells.

A-B. MCF-7 cells were stably transfected with shNR2E3 or control shRNA (shCon). Total RNA from indicated cell extracted and analysed by qRT-PCR with indicated probe.

C-D. T47D cells were stably transfected with shNR2E3 or shCon. Total RNA and protein from indicated cell extracts were analysed by qRT-PCR with indicated probes.

E. MCF-7 cells were stably transfected with shNR2E3 or control shRNA (shCon). Total protein from indicated cell extracted and analysed by Western blot with indicated antibody.

F. Stably transfected MCF-7 with shNR2E3 or with shCon were transfected with *ESR1* promoter construct, and the cells were harvested for luciferase assay. Values indicated relatively normalized luciferase activity.

G-H. MCF-7 cells were transiently transfected with indicated siRNA, and cell lysates were used for Western blot (**G**) or for qRT-PCR (**H**). All results are shown as mean plus standard deviation (SD) from three-independent replicates ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.005$).

Furthermore, overexpression of exogenous *NR2E3* further increased expression of *ESR1* and its downstream targets as well as its transcriptional activity in MCF-7 cells (Fig S1 of Supporting Information), strongly demonstrating that *NR2E3* regulates *ESR1* expression and subsequent *ESR1*-mediated induction of target genes.

Since a previous report had shown that *GATA3* mutually regulates *ESR1* (Eeckhoute et al, 2007), we investigated whether *ESR1* could also regulate *NR2E3* through an auto-regulatory feedback loop. As shown in Fig 3G and H, silencing *ESR1* expression did not alter expression of *NR2E3* (mRNA and protein), indicating that *NR2E3* is not part of the feedback regulation loop in the *ESR1*-signalling pathway.

To test whether regulation of *ESR1* expression by *NR2E3* is due to direct binding of NR2E3 on the *ESR1* promoter region, we carried out the luciferase reporter assay to map the binding region of the *ESR1* promoter using three promoter constructs containing different lengths of the *ESR1* promoter as described (Fig 4A;

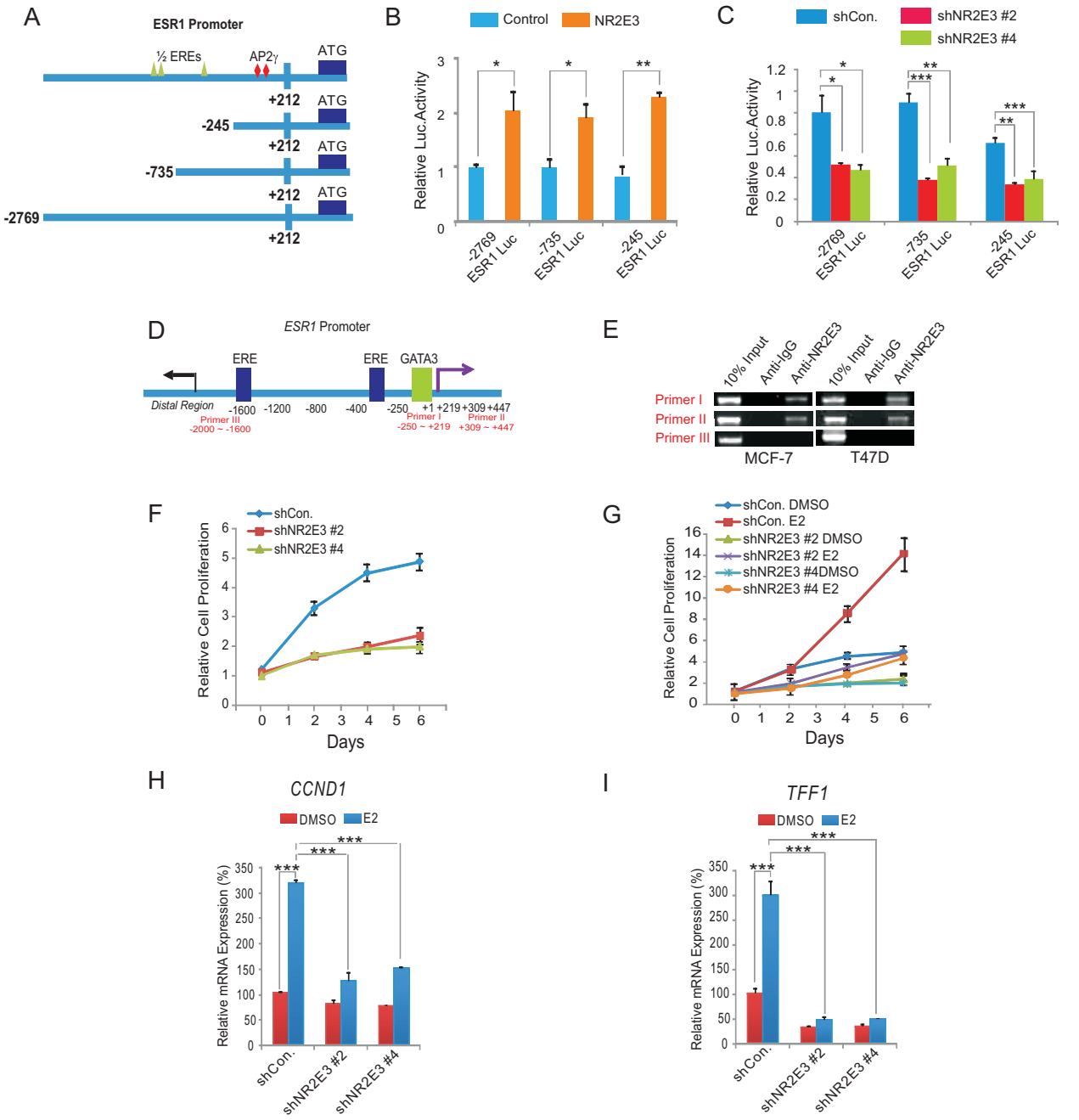

Figure 4. *NR2E3* maintains *ESR1* function via direct binding to *ESR1* promoter in breast cancer cells.

A. Diagram of *ESR1* gene promoter spanning from −245 to +212 bp, from −735 to +212 bp and from −2769 to +212 bp.

B. MCF-7 cells transiently transfected with indicated *ESR1* deletion reporters and NR2E3 construct.

C. MCF-7 cells-stably knocked down by NR2E3 were transiently transfected with indicated *ESR1* deletion constructs. Cell lysates were used for measuring the luciferase activity.

D. Schematic representation of *ESR1* promoter region for ChIP assay. Estrogen receptor response element (ERE).

E. ChIP assay was done in MCF-7 or in T47D with NR2E3 antibody. Recruitment of NR2E3 to the *ESR1* promoter was analysed using primers specific to the *ESR1* promoter. IgG was used as an internal control.

F-G. After stably transfecting control and NR2E3-specific shRNA in MCF-7 cells, the total viable cell numbers were determined by Coulter Z1 counter (Beckman Coulter, Brea, CA). Following stable transfection of shRNAs, the total viable cell numbers were determined after vehicle or E2 treatment with the indicated dose.

H-I. Expression of *CCND1* and *TFF1* in shRNA transfected MCF-7 cells after vehicle or E2 treatment for 24 h. Student's *t* test (two-tailed) was applied to estimate the significance of gene expression changes. All results are shown as mean plus standard deviation (SD) from three-independent replicates ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.005$). Significant differences in cells were compared with controls.

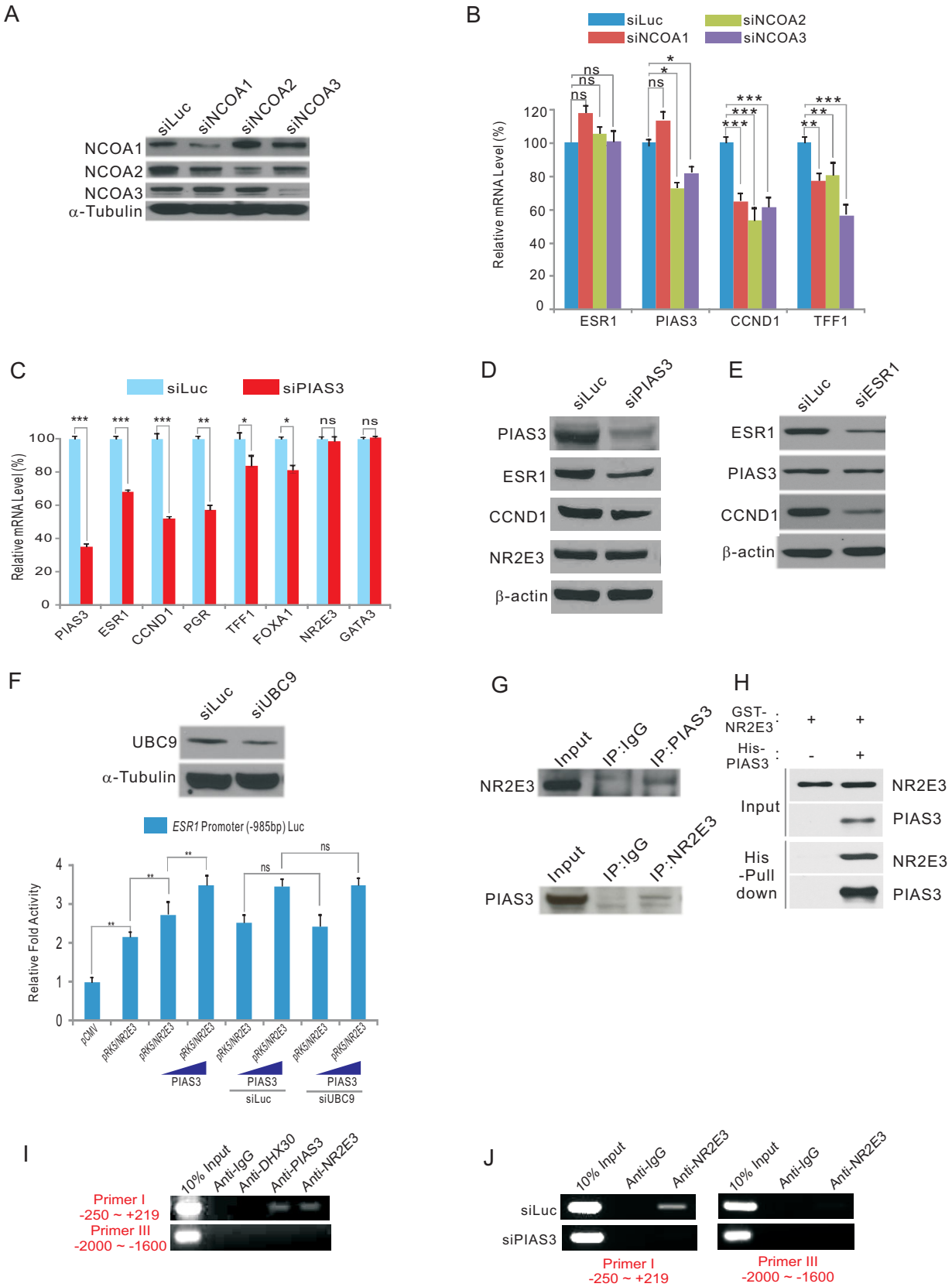

Figure 5.

deGraffenried et al, 2002). Transcription activities remained same in all three constructs of *ESR1* promoter (Fig 4B). In addition, transcription activity in shortest construct (−245 *ESR1* Luc.) was diminished when expression of NR2E3 was silenced by shNR2E3 (Fig 4C). These results suggest that the binding sites reside near and/or inside of the −245 promoter regions. The outcome of the reporter gene assay was supported by a subsequent chromatin immunoprecipitation (ChIP) assay. In agreement with reporter assay, NR2E3 only interact with *ESR1* promoter between the −250 and +447 bp regions (Fig 4D and E). Taken together, our results strongly indicate that *NR2E3* regulates *ESR1* at the transcriptional level via direct binding to the *ESR1* promoter.

Proliferation of ER-positive breast cancer cells largely depends on *ESR1* responding to estrogen (Ali & Coombes, 2002; Cosman & Lindsay, 1999; Kanavos, 2006). Thus, we investigated whether *NR2E3* is necessary for proliferation of ER-positive breast cancer cells. As expected, basal-level and estradiol (E2) induced proliferation were dramatically decreased after silencing *NR2E3* expression in MCF-7 cells (Fig 4F and G). In addition, expression of *ESR1*-dependent genes such as *CCND1* and *TFF1* was also decreased by shNR2E3 (Fig 4H and I).

Coregulator PIAS3 is required for regulation of *ESR1* expression by *NR2E3*

Many biochemical and genetic studies have demonstrated that coregulators are critically important for the function of NRs and the induction of NR-dependent genes (Xu et al, 2009). Since NR coactivators (NCOAs, also known as steroid receptor coactivators) are the best-known coregulators of NRs and interact with diverse NRs in human cancer (Xu et al, 2009), we first investigated their possible role in regulating *ESR1* expression by NR2E3. After silencing *NCOA1*, *2* and *3* by their specific siRNA (SMART Pool) in MCF-7 cells, we measured gene expression of *ESR1* and its downstream target genes. While silencing the expression of the *NCOA* family genes significantly downregulated expression of *ESR1* downstream target genes, expression of *ESR1* itself was not altered (Fig 5A and B). When we used different siRNAs to knock down NCOA family members, the result were significant as shown in Fig S2B and C of Supporting Information. This suggests that the NCOAs are only involved in regulation of *ESR1*'s transcriptional activity and do not influence *NR2E3*-mediated regulation of *ESR1* expression in breast cancer cells.

In retinal cells, the transcriptional activity of *NR2E3* is tightly controlled through interactions with protein inhibitor of activated STAT3 (PIAS3), which is a coregulator of many transcription factors (Chung et al, 1997; Jimenez-Lara et al, 2002; Junicho et al, 2000; Onishi et al, 2009). PIAS3, the main inhibitor of STAT3, is a multifunctional protein that plays a significant role in the modulation of several key factors such as NFκB, SMAD and MITF involved in the immune response pathways (Chung et al, 1997; Jimenez-Lara et al, 2002; Junicho et al, 2000).

Thus, we next tested whether *PIAS3* is essential for regulation of *ESR1* expression by *NR2E3* in breast cancer cells. Silencing the expression of *PIAS3* significantly downregulated the expression of *ESR1* and its downstream targets in both MCF-7 and T47D cells (Fig 5C and D and Fig S3 of Supporting Information). When different siRNA (SMART Pool) was used to knock down PIAS3 in MCF-7 cells, the result was significant as shown in Fig S4 of Supporting Information.

We also investigated whether *ESR1* regulates *PIAS3* via feedback loop. As shown in Fig 5E, silencing *ESR1* expression did not alter expression of *PIAS3* protein, indicating that *PIAS3* is not also part of the feedback regulation loop in the *ESR1*-signalling pathway. Because PIAS3 has E3 SUMO ligase activity, we next investigated whether E3 SUMO ligase activity of PIAS3 is important for regulation of *ESR1* expression by silencing expression of UBC9 E2 SUMO conjugating enzyme, essential protein for E3 ligase activity (Sakaguchi et al, 2007). Silencing of UBC9 expression did not alter transcriptional activity of *ESR1* promoter (Fig 5F), suggesting that it is unlikely that E3 ligase activity in PIAS3 plays important roles in regulation of *ESR1* expression via NR2E3.

Since PIAS3 is best known as a suppressor of many transcription factors via direct interactions (Chung et al, 1997), we tested whether PIAS3 directly regulates expression of *ESR1* through interaction with NR2E3 or indirectly regulates its expression by inhibiting a repressor of *NR2E3*. Co-

Figure 5. PIAS3 association with ESR1 via NR2E3.

A-B. MCF-7 breast cancer cells were transiently transfected with NCOA1, 2 and 3 specific siRNA Smart Pool or control siLuc. Protein lysates were isolated from indicated samples and used for Western blot with indicated antibodies (**A**) and 30 ng of total RNA from transfected cell lines were analysed by qRT-PCR using gene-specific primers as indicated (**B**). Student *t*-test (two-tailed) was applied to estimate the significance of gene expression changes: $^{***}p < 0.005$, $^{**}p < 0.01$ and $^{*}p < 0.05$.

C-D. MCF-7 cells were transiently transfected with siPIAS3or siLuc. Total RNA or protein from indicated cell extracts was analysed by qRT-PCR (**C**) or Western blot (**D**) to detect the indicated mRNA or protein expression levels.

E. MCF-7 cells were transiently transfected with siESR1 or siLuc. Protein from indicated cell extracts was analysed by Western blot to detect the indicated protein expression levels.

F. *ESR1* promoter construct was transfected with indicated constructs after indicated siRNA was transfected with MCF-7 cells, and the cells were harvested for luciferase assay. Values indicated relatively normalized luciferase activity. Western blot shows silencing efficiency of UBC9.

G. Co-IP of NR2E3 with PIAS3 analysed by Western blotting from MCF-7 cells.

H. GST-NR2E3 was incubated with His-PIAS3 for 2 h at 4°C and then isolated from reaction mixture by an immobilized nickel resin. The resulting precipitates were subjected to immunoblot analysis to NR2E3 or PIAS3.

I. ChIP assay was done in MCF-7 with PIAS3 or NR2E3 antibodies. Recruitment of PIAS3 to the *ESR1* promoter was analysed using a primer specific to the *ESR1* promoter. IgG was used as an internal control.

J. After MCF-7 cells were transiently transfected with siPIAS3 or siLuc., cells were used for ChIP assay.

immunoprecipitation (IP) experiments from MCF-7 cell lysates clearly demonstrated direct physical interaction between PIAS3 and NR2E3 (Fig 5G), which are also confirmed by *in vitro* association between recombinant NR2E3 and PIAS3 proteins (Fig 5H).

In addition, the ChIP assay illustrated in Fig 5I showed that PIAS3 is directly or indirectly recruited to NR2E3 on the *ESR1* promoter region. It is interesting to point out that interaction with PIAS3 was necessary for binding of NR2E3 to the *ESR1* promoter; silencing of PIAS3 expression in MCF-7 cells abolished the binding of NR2E3 to the *ESR1* promoter (Fig 5J). Of note, interaction of NR2E3 with PIAS3 is specific for *ESR1* expression in breast cancer cells, since DHX30/RetCoR, another NR2E3-interacting coregulator in retina cells, failed to show interaction with NR2E3 on the *ESR1* promoter (Fig 5I).

Clinical relevance of *NR2E3* in human breast cancer

Since *ESR1* is known to be an important prognostic marker in breast cancer management (Fisher et al, 1988; Hilsenbeck et al, 1998; Loi et al, 2008; Oh et al, 2006), we tested whether *NR2E3* (an upstream regulator of *ESR1*) is also significantly associated with prognosis in breast cancer patients. For our analysis, we used a public database (Gene Expression Omnibus in the National Center for Biotechnology Information) to retrieve gene expression data of breast cancer patients. Patients in the NKI cohort ($n = 295$; van de Vijver et al, 2002) were first dichotomized according to expression levels of *ESR1*. As expected, two groups of breast cancer patients showed a significant difference in recurrence-free survival (RFS; Fig 6A). When the patients were dichotomized according to expression level of *NR2E3*, RFSs of patients with higher expression of *NR2E3* were significantly better than that of those with lower expression of *NR2E3* (Fig 6B). Furthermore, patients with a higher expression of both *ESR1* and *NR2E3* had the best clinical outcomes, while patients with a lower expression of both ESR1 and NR2E3 had the worst clinical outcomes (Fig 6C). The association of *NR2E3* expression with prognosis also remained significant in a large independent breast cancer cohort (UNC cohort, $n = 380$; Hu et al, 2006; Oh et al, 2006; Parker et al, 2009; Fig 6D–F).

When a patient cohort of all ER-positive breast cancer [Institut Jules Bordet (IJB) cohort, $n = 349$; Loi et al, 2007, 2008] was dichotomized by expression levels of *ESR1*, it was no longer associated with RFS (Fig 7A), whereas,expression of *NR2E3* was still a significant predictor of recurrence in this cohort (Fig 7B). Since *ESR1* is also the best-known predictive marker for adjuvant therapy with tamoxifen (Kanavos, 2006; Loi et al, 2007), we assessed clinical relevance of *NR2E3* expression in patients with ER-positive breast cancer who received systemic tamoxifen treatment (subset of IJB cohort, $n = 263$). Unlike *ESR1*, which lacks predictive value in ER-positive patients, expression of *NR2E3* was significantly associated with RFS of patients (Fig 7C and D). This association remained significant even when only patients with lymph node-negative breast cancer were considered for analysis (subset of IJB cohort, $n = 114$; Fig 7E and F), indicating that expression level of *NR2E3* might be useful in predicting the response of ER-positive and node-negative patients to tamoxifen treatment.

To further validate the association of *NR2E3* with prognosis observed in publicly available data sets, we next carried out reverse-phase protein array (RPPA) experiments using the NR2E3-specific antibody in a new breast cancer cohort [M. D. Anderson Cancer Center (MDACC) cohort, $n = 575$] (Fig S5 of Supporting Information). As observed in three previous cohorts, expression of NR2E3 or ESR1 proteins were significantly associated with prognosis of breast cancer patients, and use of both ESR1 and NR2E3 expression together greatly improved the predictability of prognosis (Fig 7G and H and Fig S6 of Supporting Information). These results strongly indicated that *NR2E3* not only regulates the expression of *ESR1* but also may dictate the clinical behaviour of breast cancer patients expressing *ESR1*. Because our previous experiments suggested that PIAS3 and NR2E3 co-regulates *ESR1* expression in breast cancer cells (Fig 5), we next assessed correlation of expression patterns among three genes in ER-positive breast cancer patients (IJB cohort). Expression of three genes is significantly correlated to each other among patients with breast cancer (Fig S7 of Supporting Information), suggesting that significant association of three genes in functional and molecular levels remains same in clinical data.

We next examined gene networks shared between *NR2E3* and *ESR1* by comparing gene expression signatures specific to silencing expression of each gene in MCF-7 cells. The Venn diagram (Fig 8A) shows that a substantial number of gene features were identified as downstream targets of both *NR2E3* and *ESR1*, suggesting that a significant part of *NR2E3*-mediated biological activity is dependent on *ESR1*. We next tested the clinical relevance of the shared signature by applying a previously established prediction strategy that employs multiple different algorithms (Fig 8B and Method 1 of Supporting Information; Lee et al, 2004, 2006). As expected, the shared gene expression signature was significantly associated with disease recurrence in breast cancer patients (Figs S8 and S9 of Supporting Information) when judged by predicted outcomes of various classifiers. Of interest, the *NR2E3*-specific gene expression signature (1847 gene features; Gene list on Table 1 of Supporting Information) that was independent of *ESR1* was also significantly associated with disease recurrence (Fig 8C), suggesting that *NR2E3* activity, that is independent of ESR1 might have important functional roles and prognostic significance in breast cancer. Gene network analysis revealed several interesting features that may contribute to prognostic features of *NR2E3* in breast cancer (Fig S10 of Supporting Information).

Since gene expression data from gene-silencing experiments suggested that NR2E3 might have ESR1-independent functional roles in breast cancer, we next investigated whether NR2E3 would have clinical relevance in ER-negative breast cancer. Expression of NR2E3 was not associated with prognosis of patients with ER-negative breast cancer (Fig 11S of Supporting Information). In addition, ESR1-independent NR2E3-specific gene signatures (1847 genes) was not associated with prognosis of patients with ER-negative breast cancer (Fig 12S of Supporting Information) when prognostic significance of the signature was

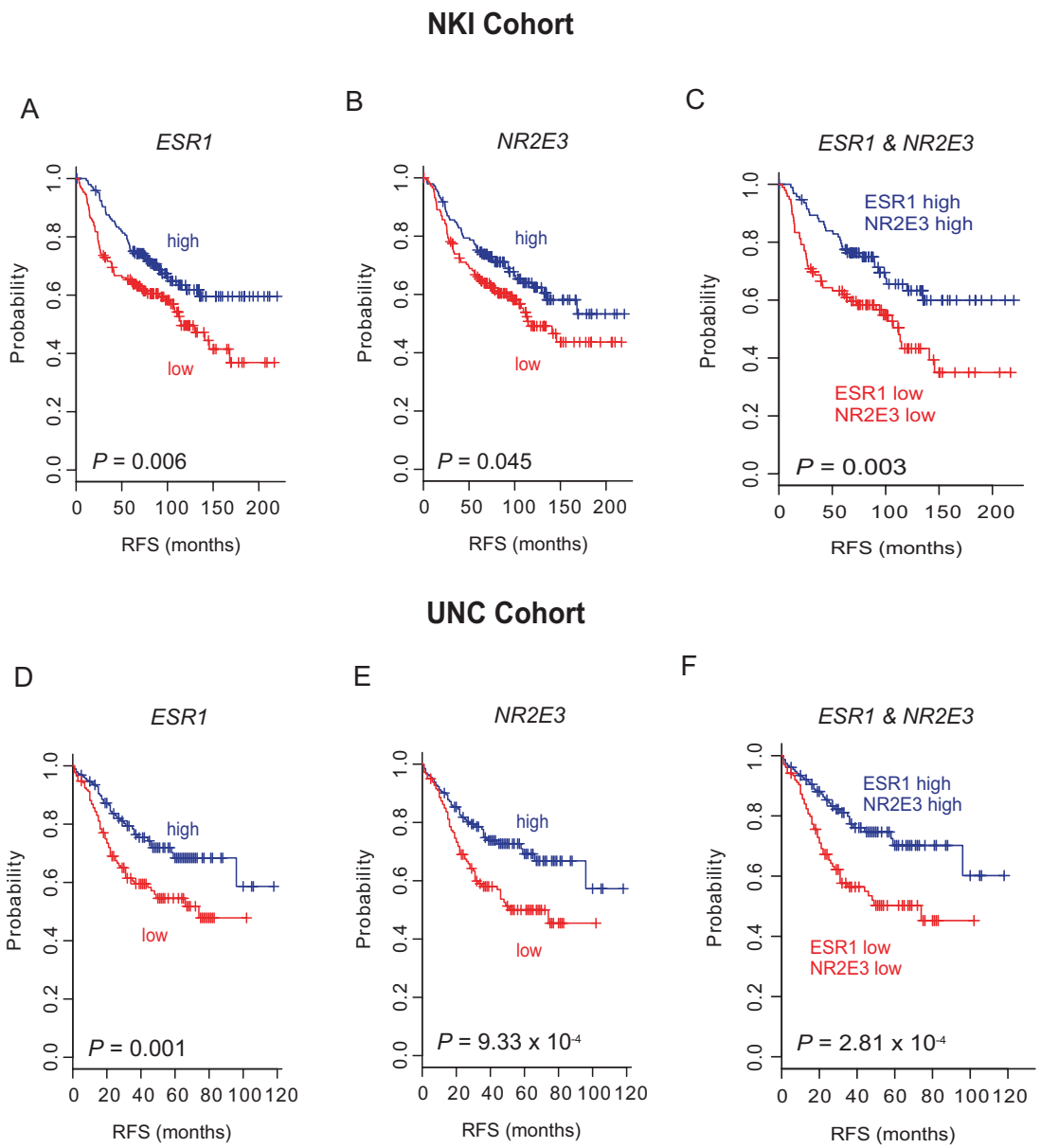

Figure 6. Expression of NR2E3 is significantly associated with recurrence of breast cancer.

A-C. Breast cancer patients in the NKI cohort ($n = 295$) were dichotomized by expression of *ESR1* (**A**) or *NR2E3* (**B**) and patient with relative high expression of both ESR1 and NR2E3 or relative low expression of both ESR1 and NR2E3 were considered for plotting (**C**).

D-F. Patients in the UNC cohort ($n = 380$) were dichotomized by expression of *ESR1* (**D**) or *NR2E3* (**E**) and patient with relative high expression of both ESR1 and NR2E3 or relative low expression of both ESR1 and NR2E3 were considered for plotting (**F**).

assessed to only those with ER-negative breast cancer. In contrast, the signature was significantly associated with those with ER-positive breast cancer (Fig 13S of Supporting Information).

DISCUSSION

Molecular mechanisms responsible for *ESR1*-mediated regulation of its downstream target genes (*i.e.*, *FOXA1*, *GATA3*, *CCND1* and *TFF1*) in normal and/or pathological conditions have been well characterized (Carroll et al, 2005; Eeckhoute et al, 2007; Krum et al, 2008), however, upstream regulators of *ESR1* and molecular mechanisms for regulating this gene are poorly understood (Hosey et al, 2007). Our systems-level exploration of the NR network using publicly available data uncovered an unexpected interaction between *NR2E3* and *ESR1*, and our subsequent experiments validated that *NR2E3* is novel upstream regulator of *ESR1* and may dictate the clinical behaviour of ER-positive breast cancer.

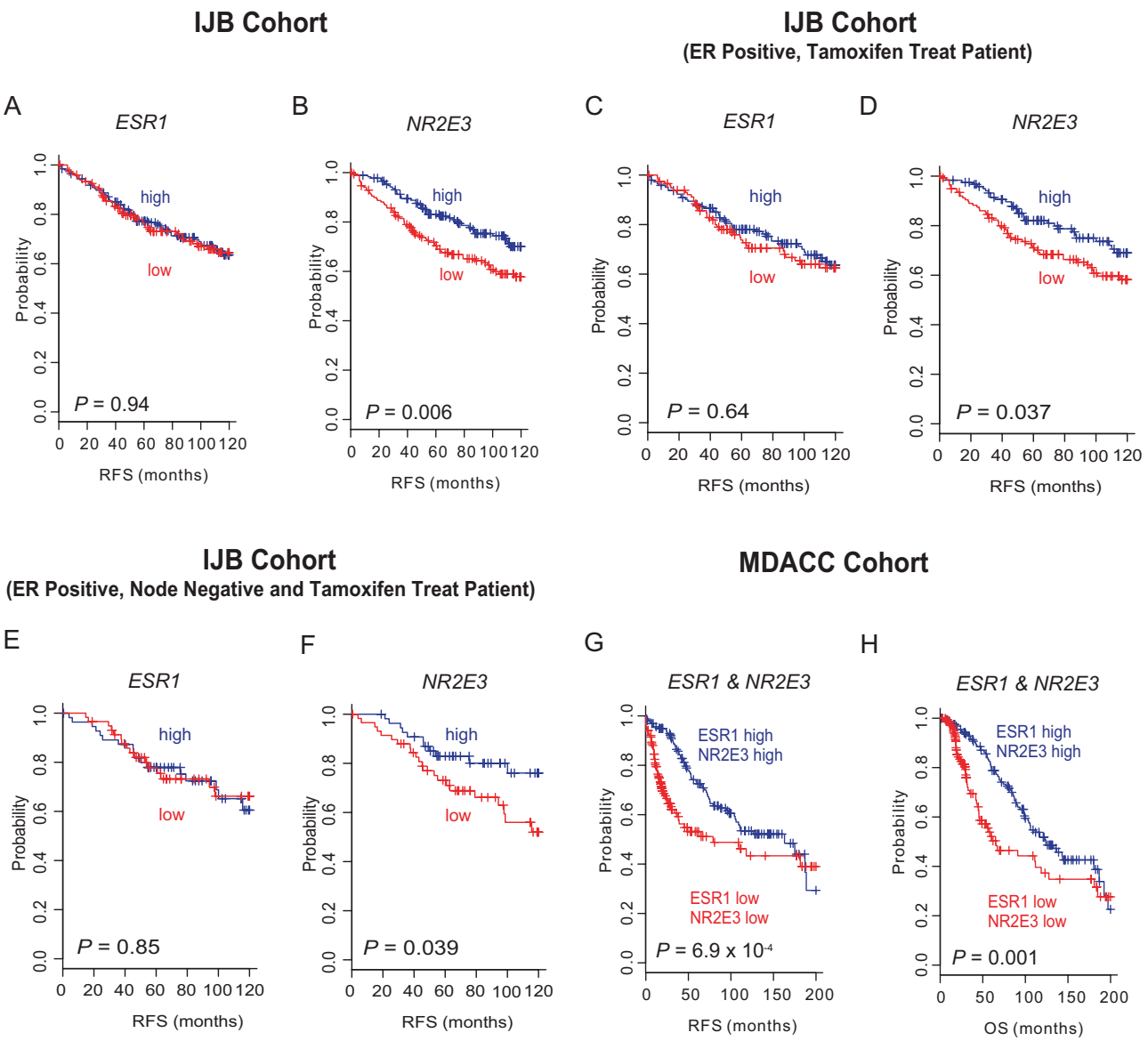

Figure 7. NR2E3 is a prognostic factor in ER-positive patients.

A-B. ER-positive patients in the IJB cohort ($n = 349$) were dichotomized by expression of *ESR1* (**A**) or *NR2E3* (**B**).

C-D. ER-positive patients who received tamoxifen treatment in IJB cohort ($n = 263$) were dichotomized by expression of *ESR1* (**C**) or *NR2E3* (**D**).

E-F. ER-positive and node-negative patients who received tamoxifen treatment in IJB cohort ($n = 114$) were dichotomized by expression of *ESR1* (**E**) or *NR2E3* (**F**).

G-H. Patients in the M. D. Anderson Cancer Center (MDACC) cohort ($n = 575$) were dichotomized by expression of both ESR1 and NR2E3 proteins. Log-rank test was applied to estimate the significance of difference.

In the current study, we have demonstrated that *NR2E3* is essential for expression of *ESR1* in ER-positive breast cancer cells by binding directly to the proximal region of the *ESR1* promoter. While searching for transcription coactivators that interact with *NR2E3*, we found that PIAS3 [a transcription coregulator with E3 small ubiquitin-like modifier (SUMO) ligase activity] is required for both binding of *NR2E3* to the *ESR1* promoter and expression of *ESR1*, as revealed by siRNA-mediated gene-silencing experiments (Fig 5). Additional gene-silencing experiments indicated this regulation is independent of other canonical coactivators of NRs *NCOA1*, *2* and *3* (Fig 5B).

The mechanism of action on the *ESR1* promoter is similar to regulation of rod-specific genes by *NR2E3* in retinal cells (Onishi et al, 2009), where *PIAS3* enhances the transcriptional activity of NR2E3. It is currently unknown whether SUMOylation of NR2E3 by PIAS3 is necessary for its transcriptional activity in breast cancer cells. However, a previous study has shown that PIAS3-mediated activation of NR2E3 in retinal cells is independent of E3 SUMO ligase activity in PIAS3 (Onishi et al, 2009), suggesting

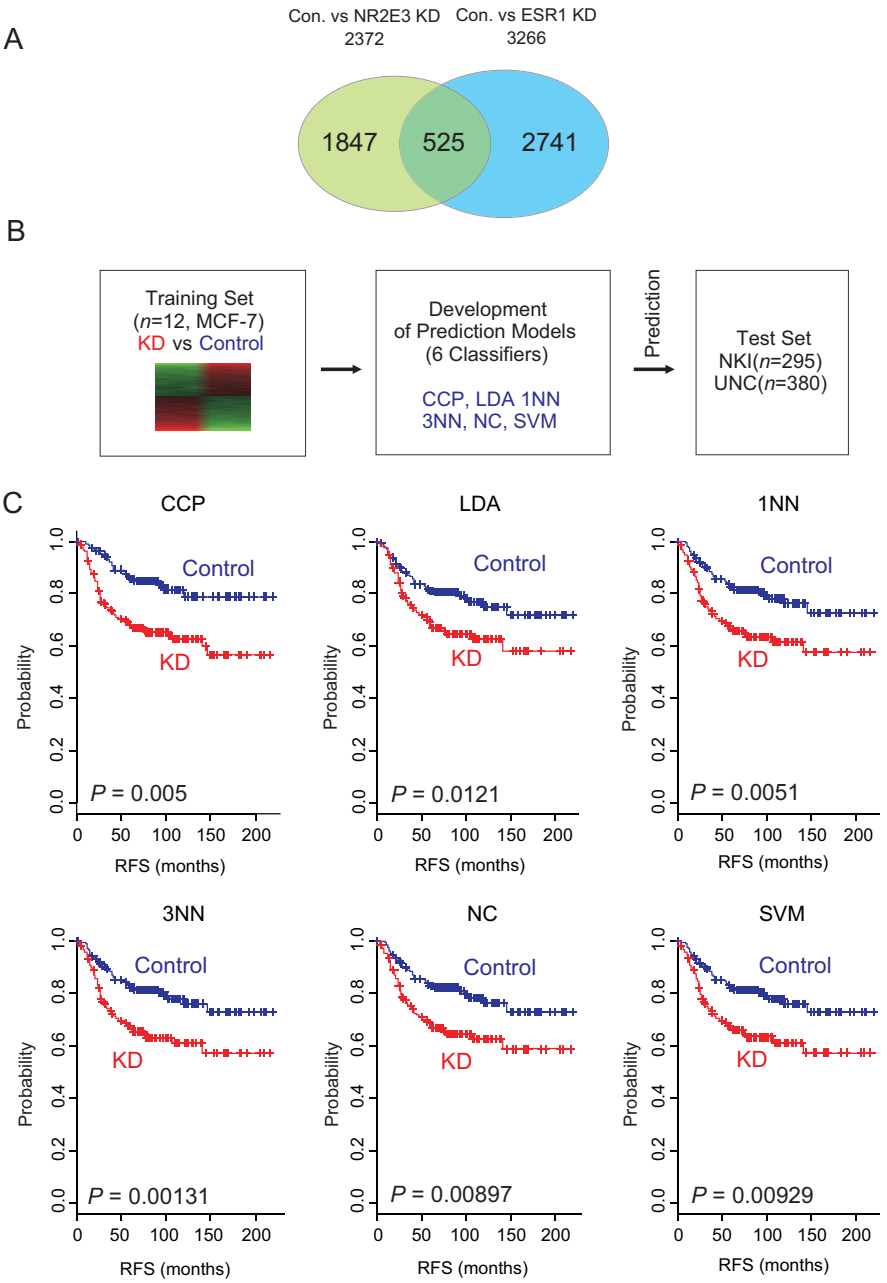

Figure 8. NR2E3-specific gene expression signatures.

A. Gene expression signature specific to loss of *NR2E3* or *ESR1* expression by shRNA or siRNA in MCF-7 cells. Genes in the Venn diagram were selected by applying a two-sample Student's *t*-test ($p < 0.005$). The green and blue circles represent genes whose expression patterns are significantly associated with loss of *NR2E3* or *ESR1*, respectively.

B. Overall scheme of generation of prediction models and evaluation of predicted outcome based on shared gene expression signature of NR2E3 and ESR1 in MCF-7. A shared gene expression signature was used to form a series of classifiers that estimated the probability of how much the expression pattern of a particular patient with breast cancer was similar to the shared signature; control (Con.) vs. knock down (KD).

C. Kaplan–Meier plots of RFS of breast cancer patients in the NKI cohort were predicted by using the *ESR1*-independent *NR2E3* gene expression signature as a classifier. The differences between groups were significant as indicated (log-rank test). CCP, compound covariate predictor; 1NN, one nearest neighbor; 3NN, three nearest neighbors; NC, nearest centroid; SVM, support vector machines; and LDA, linear discriminator analysis.

that SUMOylation of NR2E3 may not be necessary for expression of *ESR1* in breast cancer cells. While previous studies have identified Sp1 and *TP53* as upstream regulators for *ESR1* expression (Safe & Kim, 2004; Shirley et al, 2009), their roles in breast cancer are not well understood. NR2E3 may interact with them to regulate *ESR1* expression, however, additional studies are required to investigate the role of *TP53* and Sp1 on regulation of *ESR1* expression by *NR2E3* and *PIAS3*.

ESR1 has been used as a molecular marker for prognosis of breast cancer and more importantly, as a predictive marker for the benefits of anti-estrogen therapy with drug such as tamoxifen treatment (Ali & Coombes, 2002; Loi et al, 2007). However, not all ER-positive breast cancers respond to anti-estrogen therapy, indicating that the clinical behaviour of ER-positive breast cancer is heterogeneous despite detectable levels of expression of *ESR1* (Daidone et al, 2003; Loi, 2008; Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2005). Figure 7C and E shows that the expression level of the *ESR1* alone does not solely explain the considerable differences in clinical outcomes for patients with tumours that have apparently similar histopathological features. Our current study provides significant insight into clinical heterogeneity of ER-positive breast cancer associated with response to tamoxifen treatment (Fig 7D and F). The molecular mechanism associated with the favourable response of ER-positive patients with a higher expression of *NR2E3* to tamoxifen therapy is currently

unclear. However, the requirement of *NR2E3* for *ESR1* expression suggests that high expression of *NR2E3* might reflect a strong addiction of breast cancer cells to the *ESR1* pathway for survival and proliferation. Thus, ER-positive breast cancer cells with high expression of *NR2E3* might be more sensitive to inhibition of ESR1 by anti-estrogen therapy.

To better understand existing interactions or to discover new interaction among genes involved in malignant disease, it will be necessary to take a systemic view of gene networks and to develop new approaches to visualize such interactions. In our current study, we demonstrated that systems-level reanalysis of publicly available gene expression data uncovered unexpected interactions of NRs and generated a new hypothesis that has been tested by subsequent experiments. Moreover, our data suggest that NR2E3 may be the 'master' regulator of *ESR1* and may dictate the clinical outcome of ER-positive breast cancer treated with anti-estrogen therapies. Our current studies are focused on NR2E3-mediated downstream genes and pathways that are ESR1-dependent and -independent prognostic and functional roles in breast cancer.

MATERIALS AND METHODS

Cell lines

The MCF-7 and T47D human breast cancer cell lines were obtained from American Type Culture Collection and maintained as described previously (Zhang et al, 2005). To measure proliferation rate of breast cancer cells, cells were stimulated with E2 or vehicle at the indicated time in the presence of charcoal-stripped serum.

shRNA and siRNA

shNR2E3 (SHCLND-NM_014249) and shControl (SHC002) clones were purchased from Sigma (St. Louis, MO). To express shRNA, we transfected shRNA expression vectors into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours later, transfected cells were selected for 10 days with 2 μg/ml puromycin. Sequences of siPIAS3 (Iwasaki et al, 2007), siESR1 (Li et al, 2003; Zhang et al, 2005), siNCOA1 (Li et al, 2003) and siNCOA3 (Zhou et al, 2003) were described previously. siNCOA2 sequence is 5′-CCU GGA AGG CAA CGU UGU GUU-3′. siRNA SMART Pool was purchased from Dharmacon. siRNA was transfected with the cells using Oligofectamine (Invitrogen). Briefly, 30–50% confluent cells were used for transfection. We transiently tranfected cells with siRNA (20 nM) for 2days and the cells were used for extraction of RNA or protein.

qRT-PCR

To measure expression level of genes in cells, total RNA was extracted from the indicated cell lines according to the manufacturer's instruction (mirVana RNA Isolation Kit; Ambion, Inc. Austin, TX), and Polymerase Chain Reaction (after reverse transcription) (RT-PCR) was assayed using real-time qRT-PCR with TaqMan primers specific to each gene (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the 7700HT Real-Time PCR System with a 96-well block module (Applied Biosystems). Cycling conditions were 45°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Relative amounts of mRNA were calculated from the threshold cycle (C_T) number using expression of cyclophilin A (PPIA) as an endogenous control. All experiments were performed in triplicate and the values were averaged.

Western blotting

To measure expression levels of proteins, cells were maintained and Western blot was performed as described previously with an anti-NR2E3 antibody (ARP39069; Aviva, San Diego, CA), PIAS3 antibody (SC-46682; Santa Cruz Biotech, Santa Cruz, CA) and anti-ESR1 antibody (RM-9101-S1; Neomarkers), NCOA1 (05-522; Upstate), NCOA2 (610984; BD Bioscience) and NCOA3 (612378; BD Bioscience), UBC9 (4918; Cell Signaling Technology) and β-actin (A5441) from Sigma.

Co-immunoprecipitation (IP)

Co-IP experiments were carried out as described previously (Peng et al, 2009). In brief, whole-cell extracts from MCF-7 were prepared in NP-40 buffer and precleaned with Protein A/G plus-agarose beads (Santa Cruz Biotech). Cell extracts were then subjected to incubation for 2 h with antibodies against NR2E3 (from Dr. Chen) or PIAS3 (Santa Cruz; sc-46482 (or normal IgG; 5 μg), followed by incubation overnight with protein A/G-agarose beads at 4°C. The immunocomplex was eluted in loading buffer by being boiled for 5 min at 95°C and loaded on the SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Expression and purification of recombinant proteins

Human-NR2E3 cDNA was inserted by using *Eco*RI and *Xho*I sites of pGEX4T1 and pET21-PIAS3 was described previously (Ban et al, 2011). *Escherichia coli* BL21 (DE3) carrying the plasmid encoding GST-NR2E3 was cultured at 25°C in LB medium supplemented with ampicillin (100 μg/ml). Isopropyl-1-thio-β-D-galactopyranoside (0.1 mM) was added and the culture was incubated for 5 h at 25°C. GST-NR2E3 proteins were purified using glutathione–Sepharose (Amersham Pharmacia). The His_6-tagged PIAS3 was expressed in *E. coli* and were purified with the use of an immobilized nickel resin (5Prime).

In vitro biding assay

The bead-immobilized His_6-PIAS3 was incubated for 4 h at 4°C with GST-NR2E3 in 1 ml of binding buffer consisting of 50 mM Tris–HCl (pH 7.6), 150 mM NaCl and 10 mM imidazole. The beads were then washed four times with binding buffer and subjected to immunoblot analysis with antibodies to the NR2E3 or PIAS3.

Plasmids and luciferase assay

pRK5-human NR2E3 and *ESR1* promoters were described previously (Chen et al, 2005; Sundar et al, 2008). Indicated cells were transfected with reporter genes and indicated plasmids using Lipofectamine 2000 ((Invitrogen) according to the manufacturer's instruction. After 48 h, cells were harvested to measure the luciferase activity, which was normalized with β-gal.

Cell counting

Stably transfected cells were stimulated with 100 nM estrogen for 24 h or in time course experiments for the time periods given in the Fig 4E and F. Total cells were harvested for automated cell counting using a Coulter Z1 counter.

The paper explained

PROBLEM:

ESR1, a pivotal transcription factor regulating cell proliferation, is one of the most well-known biomarker and an important therapeutic target in breast cancer. While much is understood about the molecular mechanisms underlying ESR1-downstream target genes regulation, relatively little is known about how expression of ESR1 is regulated in breast cancer.

RESULTS:

After generating a testable hypothesis by using a computational approach to publicly available genomic and clinical data, the authors have used biochemical and molecular biology techniques to validate their theory and uncover possible clinically relevant new findings. This strategy led to discover that orphan nuclearreceptor NR2E3 is a novel upstream regulator of ESR1 in breast cancer, and a potential biomarker for predicting a positive prognosis to anti-hormone therapy.

IMPACT:

These findings provide novel insight into the mechanism of ESR1 regulation in breast cancer and open up new avenues for developing novel predictive biomarker assay in response to anti-hormone therapy.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed as described previously using the Upstate EZ ChIP kit (Park et al, 2005; Peng et al, 2009; Millipore, Billerica, MA). Anti-NR2E3 (received from Dr. Chen (Onishi et al, 2009). Anti-DHX-30 (Bethyl; a302-218A), Anti-PIAS3 (Santa Cruz; sc-46682) and control IgG (Santa Cruz; sc-2763) were used for ChIP experiments. Primer sequences used (governing *ESR1* promoter region) were primer I (forward) 5′-GGGCCACCTTTAGCAGATC-3′ (reverse) 5′-CAGGGTGCA-GACCGTGTC-3′; primer II (forward) 5′-GCTGGAGCCCCTGAACCGTCCGC-3′ (reverse) 5′- GGCCCAGACTCCGACGCCGCA-3′; and primer III (forward) 5′-CCCTGTGAGCAGACAGCAAGTC-3′ (reverse) 5′-AGAACAG-CAATCCTCATCTCCCTGC-3′.

Reverse phase protein array (RPPA)

Human breast tumours were obtained from Tumour Banks following pathologist review under the auspices of Institutional Review Board-approved protocols at the M. D. Anderson Cancer Center (Stemke-Hale et al, 2008). Protein extracts from breast cancer patients and RPPA were performed as described previously (Stemke-Hale et al, 2008). To quantify NR2E3 and ESR1 expression as a ratio to the total expression of each protein, antibodies from Aviva (ARP39069) and Neomarkers (RM-9101-S1, Sigma) were used (Fig S5 of Supporting Information).

Microarray

We transfected shRNA expression vectors (shCon. or shNR2E3) into the cells using Lipofectamine 2000 (Invitrogen). Twenty-four hours later, transfected cells were selected for 10 days with 1 μg/ml puromycin. Total RNA from these cells was extracted from the indicated cell lines using a mirVana RNA Isolation Labeling kit (Ambion, Inc.). Five hundred nanograms of total RNA were used for labelling and hybridization according to the manufacturer's protocols (Illumina Inc., San Diego, CA). After the bead chips (Sentrix Human v.3 HT-12) were scanned with an Illumina BeadArray Reader (Illumina Inc.), the microarray data were normalized using the quantile normalization method in the Linear Models for Microarray Data (LIMMA) package in the R language environment (Wright & Simon, 2003). The expression level of each gene was transformed into a log 2 base before additional analysis was performed (Lee et al, 2006). All microarray data are available in NCBI's Gene Expression Omnibus public database (GSE18431). This microarray data was used for analysis in Fig 8.

Gene expression data of breast cancer patients

Gene expression data from three-independent breast cancer patient cohorts were used for analysis. Normalized gene expression data from NKI and UNC cohort were obtained from pubic Merck website (http://www.rii.com/publications/2002/nejm.html) and UNC microarray database (https://genome.unc.edu), respectively. Gene expression data from IJB cohort were obtained from Gene Expression Omnibus (accession number GSE2990 and GSE6532) and normalized using robust multi-array average methods (Irizarry et al, 2003).

Statistical analysis of microarray data and survival analysis

The random-variance *t*-test was applied to identify genes differentially expressed between the two classes using Biometric Research Branch (BRB) ArrayTools (National Cancer Institute, Bethesda, MD; Simon et al, 2007). Gene expression differences were considered statistically significant if the *p*-value was less than 0.005. Cluster analysis was performed with Cluster and Treeview (Eisen et al, 1998). Kaplan–Meier plots and log-rank test were used to estimate patient prognosis.

Author contributions

YYP, KK, SS and JSL conceived and designed this study; YYP and KK performed the experiments; YYP and JSL analysed the microarray data sets; SBK, ESP, SMK, JYL, WJ and GBM contributed to the interpretation of the data; BTH, JL, YL and AMGA performed the RPPA; YYP and JSL wrote the manuscript; All authors approved the final version of this manuscript.

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Shiming Chen for providing the NR2E3 antibody (Onishi et al, 2009). This work was supported by Bio R&D Program Grant M10642040002-07N4204-00210, the Grant R01-2008-000-11321-0 and the National Core Research Center Program Grant R15-2006-020 funded by the National Research Foundation of Korea (to W.J.).

Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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