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## Accepted Manuscript

Title: Identification of novel ER- $\alpha$  target genes in breast cancer cells: gene- and cell-selective co-regulator recruitment at target promoters determines the response to 17 $\beta$ -estradiol and tamoxifen

Authors: Andrea Romano, Michiel Adriaens, Sabine Kuenen, Bert Delvoux, Gerard Dunselman, Chris Evelo, Patrick Groothuis

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1 **Identification of novel ER- $\alpha$  target genes in breast cancer cells: gene- and cell-**  
2 **selective co-regulator recruitment at target promoters determines the response to**  
3 **17 $\beta$ -estradiol and tamoxifen**

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6 Andrea Romano<sup>1,2,a</sup>, Michiel Adriaens<sup>3</sup>, Sabine Kuenen<sup>1,2</sup>, Bert Delvoux<sup>1,2</sup>, Gerard  
7 Dunselman<sup>1,2</sup>, Chris Evelo<sup>3</sup>, Patrick Groothuis<sup>1,2,4</sup>

8  
9 <sup>1</sup> GROW, School for Oncology and Developmental Biology

10 <sup>2</sup> Department of Obstetrics and Gynaecology

11 <sup>3</sup> Department of Bioinformatics - BiGCaT

12  
13 Maastricht University Medical Centre, 6202 AZ Maastricht, The Netherlands.

14  
15 <sup>4</sup> *Present address:* Department of Pharmacology, Schering-Plough Corporation,  
16 Molenstraat 110, 5342 CC Oss, The Netherlands.

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<sup>a</sup> Contact person: Andrea Romano: a.romano@og.unimaas.nl  
Department of Obstetrics and Gynaecology,  
Maastricht University Medical Centre  
P. Debyelaan 25, 6202 AZ Maastricht, The Netherlands  
Phone: 0031 43 4331286/3881352  
fax: 0031 43 3874765

**1 Summary**

**2** Tamoxifen and  $17\beta$ -estradiol are capable of up-regulating the expression of some genes  
**3** and down-regulate the expression of others simultaneously in the same cell. In addition,  
**4** tamoxifen shows distinct transcriptional activities in different target tissues.

**5** To elucidate whether these events are determined by differences in the recruitment of co-  
**6** regulators by activated estrogen receptor- $\alpha$  (ER- $\alpha$ ) at target promoters, we applied  
**7** chromatin-immunoprecipitation (ChIP) with promoter microarray hybridisation in breast  
**8** cancer T47D cells and identified 904 ER- $\alpha$  targets genome-wide. On a selection of newly  
**9** identified targets, we show that  $17\beta$ -estradiol and tamoxifen stimulated up- or down-  
**10** regulation of transcription correlates with the selective recruitment of co-activators or co-  
**11** repressors, respectively. This is shown for both breast (T47D) and endometrial carcinoma  
**12** cells (ECC1). Moreover, differential co-regulator recruitment also explains that  
**13** tamoxifen regulates a number of genes in opposite direction in breast and endometrial  
**14** cancer cells. Over-expression of co-activator SRC-1 or co-repressor SMRT is sufficient  
**15** to alter the transcriptional action of tamoxifen on a number of targets. Our findings  
**16** support the notion that recruitment of co-regulator at target gene promoters and their  
**17** expression levels determine the effect of ER- $\alpha$  on gene expression to a large extent.

**18**  
**19** **Key words:**  $17\beta$ -estradiol / estrogen receptor- $\alpha$  / co-activators / co-repressors / tamoxifen  
**20**

## 1 Introduction

2 Upon ligand activation, estrogen receptor- $\alpha$  (ER- $\alpha$ ) binds to the promoters of responsive  
3 genes, interacting directly with estrogen response elements (EREs) or indirectly *via*  
4 associations with other transcription factors (reviewed in: Lonard and O'Malley B, 2007).  
5 Numerous mechanisms participate in the fine-tuning of estrogen regulatory actions in  
6 target cells. These mechanisms allow estrogens and selective estrogen receptor  
7 modulators (SERMs) to exert opposite transcriptional actions on different genes in the  
8 same cell type, or to act as agonists in one cell type and as antagonists in another cell  
9 type. However, they may also be responsible for the unwanted side effects that have been  
10 observed during the use of these compounds in medical treatments. The SERM  
11 tamoxifen, for instance, acts as an ER- $\alpha$  antagonist in breast cancer cells (Conzen, 2008;  
12 Riggs and Hartmann, 2003), but it is a partial agonist in the endometrium and increases  
13 the incidence of endometrial hyperplasia and cancer (Gielen et al., 2005; Shang, 2006). In  
14 addition, the same mechanisms may play a role in the resistance to tamoxifen of breast  
15 tumours (Conzen, 2008; Lonard et al., 2007) and in the patient-dependent therapeutic  
16 efficacy of tamoxifen to treat ovarian cancer (Perez-Gracia and Carrasco, 2002).

17 There is increasing evidence that the gene- and cell-specific actions of estrogens depend  
18 largely on the presence of co-regulators. These proteins either bridge the ER- $\alpha$  / target-  
19 promoter-complex with the transcriptional machinery (co-activators such as CBP, p300,  
20 SRC family) or impair it (co-repressors; SMRT, NCoR; Carroll and Brown, 2006; Lonard  
21 and O'Malley B, 2007). Several recent studies have indicated that the agonistic or  
22 antagonistic action of a SERM is determined by the cellular availability of co-regulators  
23 in different cell types. For instance, the agonistic action of tamoxifen in endometrial  
24 cancer cells is the consequence of high expression of the co-activator SRC-1 (Shang and  
25 Brown, 2002). In breast cancer cells, down-regulation of co-repressor NCoR turns  
26 tamoxifen into an inducer of proliferation and over-expression of co-activator SRC-3  
27 (AIB1) is predictive of resistance to tamoxifen in breast cancer patients and is associated  
28 with malignancies in the endometrium (Balmer et al., 2006; Conzen, 2008; Lonard et al.,  
29 2007).

30 Despite these evidences, the direct effect of co-regulators on ER- $\alpha$ -controlled gene  
31 transcription in distinct cell types has been demonstrated for a limited number of targets  
32 only (Shang and Brown, 2002; Shang et al., 2000; Stossi et al., 2006) or by means of  
33 reporter gene assays (Peterson et al., 2007; Smith et al., 1997). In addition, it remains  
34 difficult to understand how estrogens induce the expression of specific genes and repress  
35 others in the same cell type (Bourdeau et al., 2008; Carroll et al., 2006; Hodges et al.,  
36 2003; Kwon et al., 2007; Lin et al., 2004; Lin et al., 2007a; Lin et al., 2007b; Groothuis et  
37 al., 2007).

38 In the present study, we aimed at examining whether differential co-regulator recruitment  
39 (i) determines different transcriptional actions of one ligand on distinct target genes in the  
40 same cell type and (ii) determines the opposite transcriptional regulation of the same  
41 genes in different cell types treated with the same ligand. To this end, we applied  
42 chromatin immunoprecipitation (ChIP) together with promoter DNA array hybridisation  
43 (ChIP-chip) and identified 904 ER- $\alpha$  target promoters in T47D breast cancer cells. On a  
44 selection of newly identified target genes, we show that the transcriptional stimulatory or  
45 inhibitory effects of 17 $\beta$ -estradiol or OH-tamoxifen, the active metabolite of tamoxifen,  
46 closely correlate with the recruitment of co-activators or co-repressors, respectively.

1 Moreover, recruitment of distinct co-regulators correlates with the opposite  
2 transcriptional responses observed in T47D and endometrial cancer cells (ECC1). To  
3 further support this notion, we show that over-expression of co-activator SRC-1 or co-  
4 repressor SMRT is sufficient to change or to invert OH-tamoxifen response, irrespective  
5 to the cell context.  
6

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## 1 **Materials and Methods**

2  
3 *Cell lines and culture.* The human breast cancer cell line T47D and human endometrial  
4 cancer cell line ECC1 were purchased from the American Type Culture Collection  
5 (ATCC; Rockville, Md. USA) and maintained as described (Romano et al., 2007). For all  
6 experiments involving hormonal stimulation, cells were cultured for five days prior to,  
7 and during the experiment in RPMI without phenol-red (Invitrogen, Life Technologies,  
8 Inc., Carlsbad, CA) supplemented with 5% hormone-stripped serum (c.c.pro GmbH,  
9 Neustadt, Germany).

10  
11 *Steroid hormones.* 17 $\beta$ -estradiol and OH-tamoxifen were purchased from Sigma-Aldrich  
12 Chemie BV (Zwijndrecht, The Netherlands). ICI-164384 was a gift from Schering-  
13 Plough (Oss, The Netherlands).

14  
15 *RNA extraction and cDNA synthesis.* RNA was isolated using the Trizol reagent  
16 (Invitrogen, Life Technologies, Inc., Carlsbad, CA) as recommended by the  
17 manufacturer. Complementary DNA (cDNA) was synthesised using the M-MLV reverse  
18 transcriptase (Invitrogen, Life Technologies, Inc., Carlsbad, CA) as described earlier  
19 (Romano et al., 2007).

20  
21 *Oligonucleotides.* Oligonucleotides used for linear amplification of immunoprecipitated  
22 chromatin prior to ChIP-chip and used for PCR were purchased from MWG-Biotech AG  
23 (Ebersberg, Germany) and are listed in Supplemental Table S-III.

24  
25 *PCR and real time PCR (RT-PCR).* PCR was performed with the Taq DNA polymerase  
26 (Fermentas GMBH, St Leon-Rot, Germany) as recommended by the manufacturer. Semi-  
27 quantitative PCR was performed by stopping PCR reactions every three cycles and by  
28 evaluation of band intensity on an agarose gel. RT-PCR was performed using the Syber-  
29 green ABGene system (ABGene Limited, Epsom, United Kingdom), as recommended by  
30 the manufacturer and the BioRad MyIQ apparatus.

31  
32 *Chromatin immunoprecipitation (ChIP).* ChIP was performed as described elsewhere  
33 (Romano et al., 2007). Briefly, T47D or ECC1 cells were grown to 80% confluence (165  
34 cm<sup>2</sup> culture flasks) treated with vehicle-only (ethanol) or with ligand for 50 minutes,  
35 fixed (1% formaldehyde, 10 minutes) and scraped in 1 ml of cold PBS supplemented  
36 with Complete<sup>TM</sup> protease inhibitor (Roche, Mannheim, Germany). After cell lysis,  
37 nuclei were pelleted, lysed and chromatin was sonicated. Chromatin-protein complexes  
38 were immunoprecipitated (IP) with protein-G/A magnetic beads (Dynal, Invitrogen Life  
39 Technologies, Inc., Carlsbad, CA) and 2  $\mu$ g of specific antibodies: HC-20 against ER- $\alpha$ ,  
40 H-224 against RNA-Pol-II, C-20, N-15 and A-22 against co-activators SRC-1, p300 and  
41 CBP, and antibodies sc-1609 and H-300 against co-repressors NCoR and SMRT (Santa  
42 Cruz Biotechnology, California, USA). After IP, bead washing and reverse crosslinking,  
43 DNA was purified using the Qiaquick reaction clean-up kit (Qiagen GmbH, Hilden,  
44 Germany). Binding of the RNA-Pol-II to the *GAPDH* promoter was used as positive  
45 control of the ChIP procedure and it was assessed using primers ChIP-positive  
46 (Supplemental Table S-III). ER- $\alpha$  binding to the *TFF1* promoter was used as a positive

1 control for ChIP with the ER- $\alpha$  antibody and it was assessed using primers indicated in  
2 Supplemental Table S-III. ChIP PCR signals were normalised with an unspecific negative  
3 control, using primers ChIP-negative (Supplemental Table S-III) that flank the  
4 cytogenetic location 12p13.3 where no transcription factors bind. All additional primers  
5 used to assess ER- $\alpha$  and co-regulatory protein binding are listed in Supplemental Table  
6 S-III.

7  
8 *ChIP-chip*. ChIP in T47D cells using the ER- $\alpha$  antibody was performed as described  
9 above. Successful ChIP was confirmed by assessing ER- $\alpha$  binding to the promoter of the  
10 estrogen responsive gene *TFF1*. Isolated DNA fragments were subsequently subjected to  
11 a linear-amplification as follows: a) 7.5  $\mu$ l of DNA were denatured, amplified with 1.5 U  
12 of Sequenase<sup>TM</sup> T7 DNA-polymerase (Invitrogen, Life Technologies, Inc., Carlsbad, CA)  
13 using primer LA-0 (Supplemental Table S-III) in the recommended buffer (1X) for 8  
14 minutes at 37 °C. This step was repeated once. b) 15  $\mu$ l of this reaction were amplified  
15 by Taq polymerase (Fermentas GMBH, St Leon-Rot, Germany) using primer LA-1  
16 (Supplemental Table S-III) in 0.1 mM dNTPs, 1X recommended buffer, 1.5 mM MgCl<sub>2</sub>  
17 in 100  $\mu$ l final volume. Aliquots (5 $\mu$ l) were taken at 25, 30, 35, 40 cycles to determine  
18 the number of cycles necessary to enter the exponential phase (which was determined  
19 based on the intensity of the smeared-DNA visualised on an agarose gel). A second round  
20 of amplification using the Taq polymerase was performed. Amplified DNA was purified  
21 using the Qiaquick reaction cleanup kit (Qiagen GmbH, Hilden, Germany). Enrichment  
22 of the *TFF1* promoter was confirmed at intermediate steps of the amplification and at the  
23 end of the amplification (Figure 1a). This quality control guaranteed that the  
24 amplification of signals in the ChIP-DNA did not reach saturation and therefore did not  
25 result in loss of enrichment of target promoters.

26 Samples were generated from three independent experiments (T1, T2 and T3). In each  
27 experiment, cells were treated with 17 $\beta$ -estradiol or vehicle for 50 minutes. In addition, a  
28 reference pool (P) was created by pooling equal amounts of the amplified DNA from the  
29 17 $\beta$ -estradiol and vehicle-treated samples of T1, T2 and T3. The ChIP-DNA fragment  
30 was labelled with Cy-5, while the input-DNA, the DNA purified from fragmented  
31 chromatin non-subjected to IP reaction and processed through the same linear-  
32 amplification as the ChIP-DNA, was labelled with Cy-3. Labelled ChIP- and input-DNA  
33 fractions from the eight samples (four treated and four untreated) were subsequently  
34 hybridised to the Nimblegen HGS17 genome build promoter microarray containing 1500  
35 bp of promoters from 24,134 human genes. Labelling and hybridisation were performed  
36 in-house by Nimblegen (Madison, USA). The promoter regions on the array are covered  
37 by 50- to 75-mer probes with approximately 100 bp spacing. The log-ratio of Cy-5 and  
38 Cy-3 intensities was subsequently calculated to assess enrichment of specific promoters  
39 of the ChIP-DNA compared to the input-DNA, suggesting binding of ER- $\alpha$ . The  
40 hybridisation efficiency of the samples from experiment T3 did not meet the quality  
41 criteria and these samples were excluded from further analysis.

42  
43 *Statistical analysis*. Two different methods were evaluated for the identification of ER- $\alpha$   
44 targets. Method (i), a within-array analysis, searches for four or more probes in each 1500  
45 bp promoter whose signals are above a specified cut-off value. This analysis was  
46 performed using the proprietary software of Nimblegen. Method (ii) is a between-array



1 analysis, employing positive (treated replicate samples) and negative controls (vehicle-  
2 treated samples) at probe level, which was performed in the statistical programming  
3 language R. This latter method is expected to produce a statistically more robust set of  
4 potential ER- $\alpha$  targets. First, the log-ratio between ChIP-DNA and input-DNA intensities  
5 is calculated separately for each array. Next, all probes are ordered according to genomic  
6 location and dichotomised using a threshold around twice the estimated standard  
7 deviation of the log-ratio. Probes with log-ratio values above this threshold are  
8 designated as positive, those below the threshold negative. Next, for each array, a sliding  
9 window of a variable number of base pairs is moved over all probes, calculating a p-value  
10 for each window with a Yates corrected chi-square test. To determine whether a promoter  
11 shows true significant enrichment, the promoter has to contain at least one window that  
12 shows significant enrichment in at least two treated samples (positive controls) and the  
13 same window or windows should not show significant enrichment in more than one  
14 untreated sample (negative controls). To minimise false positives, an adaptation of the  
15 Benjamini and Hochberg method (Benjamini and Hochberg, 1995) is applied to calculate  
16 false discovery rates (FDR).

17 Both methods showed over fifty percent consistency when a FDR threshold of 20 % was  
18 applied. We compared the list of target genes obtained with the two methods with a list of  
19 already known targets (O'Lone et al., 2004). Given that at the same FDR, method (ii)  
20 retrieved a larger number of known target promoters when compared to method (i) and  
21 considering the greater robustness of a between-array approach, method (ii) was used to  
22 generate the list of targets used for further analysis.

23 To identify our 904 promoters, we combined results using two FDR cut-off points. We  
24 first identified a suitable cut-off point able to retrieve as many previously found targets  
25 (O'Lone et al., 2004) as possible. Using a FDR cut-off of 20 % we identified most known  
26 targets (i.e. *CTSD*, *BRCA*, *c-Myc*, *ADORA1*, *AGT*, *HSPB1*, *LCN2*) and only few more  
27 (*TGFA*, *TERT*) were retrieved when cut-off points with lower stringency (FDR cut-offs  
28 higher than 20 %) were used. Therefore, 20 % FDR was fixed as the upper limit for the  
29 stringency of our statistics. Subsequently, a low stringency (FDR 20 %) was used to  
30 identify ER- $\alpha$  targets common in the arrays of the independent experiments (T1 and T2  
31 or T1, T2 and P). A high stringency (FDR 5 %) was used for targets that were common in  
32 one of the T arrays and the P array, as those are essentially technical replicates.

33 The promoter regions were scanned for occurrence of EREs using the Genomatix  
34 MatInspector software (Cartharius et al., 2005) and the Genomatix transcription factor  
35 motif database ([www.genomatix.de](http://www.genomatix.de)). We also scanned promoter sequences of a validated  
36 sub selection of ER- $\alpha$  targets for the presence of potential tethering domains for EREs  
37 (AP1, NF $\kappa$ B and SP1 binding sites), using the same approach.

38  
39 *Cell transfection, luciferase assay and immunocytochemistry.* Plasmids used for  
40 transfection were previously described: *ERE-TK-luciferase* (2X ERE-TK-LUC)  
41 containing the estrogen responsive promoter-luciferase reporter (Oehler et al., 2004), was  
42 gifted by Prof Schuele. The expression vector for co-activator SRC-1 (Smith et al., 1997)  
43 and the co-repressor SMRT (Chen and Evans, 1995) were gifts from Prof O'Malley and  
44 Prof Evans, respectively. The SMRT expression plasmid used in these experiments  
45 encodes for a truncated form of the human co-repressor SMRT (amino-acids 1032-2517)  
46 with a dominant co-repressing action (Peterson et al., 2007). Plasmid pCND3.1

1 (Invitrogen, Life Technologies, Inc., Carlsbad, CA) was used as empty vector (when  
2 indicated). All techniques were previously described (Romano et al., 2007). In short,  
3 transfection was performed using the jetPEI™ reagent (Q-Biogene, Heidelberg,  
4 Germany) as recommended by the manufacturer. Prior to luciferase assays, cells were  
5 cultured in two wells of a 12-well plate and were transfected (2 µg DNA plus 3 µl  
6 jetPEI™ per well). Sixteen hours after transfection, cells from the two wells were  
7 trypsinised, pooled and seeded into 12 wells of a 96 well-plate. Eight hours after plating,  
8 treatments were applied. Each treatment was performed in triplicate (the number of  
9 initially transfected wells was scaled up according to the number of stimulations needed).  
10 In case of RNA isolation, cells were transfected in two 25 cm<sup>2</sup> flasks (10 µg DNA plus  
11 15 µl jetPEI™ per flask) and subsequently cells were pooled and plated in 9 wells of a  
12 12-well plate. For immunocytofluorescence, cells were cultured on glass cover slips fixed  
13 in buffered formaldehyde (4% paraformaldehyde in PBS), permeabilised with 0.1%  
14 Triton-X-100 in PBS and stained with the following antibodies (as indicated in the  
15 figures): goat polyclonal C-20 against co-activator SRC-1 and sc-1609 against co-  
16 repressor NCoR (Santa Cruz Biotechnology, California, USA), followed by anti-goat  
17 FITC secondary antibody 705-095-147 (Jackson ImmunoResearch/Brunschwig chemie  
18 B.V., Amsterdam, The Netherlands); rabbit polyclonal H-300 against co-repressor SMRT  
19 (Santa Cruz Biotechnology, California, USA), followed by anti-rabbit FITC F005401  
20 (DAKO, Glostrup, Denmark). For western blot (Supplemental Figure S-1) ER-α was  
21 detected with monoclonal antibody F10 (Santa Cruz Biotechnology, California, USA),  
22 whereas p300 and CBP with rabbit A-22 and N-15 antibodies, respectively (Santa Cruz  
23 Biotechnology, California, USA). Mouse antibody AC-15 (Sigma-Aldrich Chemie BV,  
24 Zwijndrecht, The Netherlands) was used to detect β-actin. HRP-conjugated rabbit anti-  
25 mouse-antibodies (DAKO, Glostrup, Denmark) and goat-anti-rabbit-antibodies (Pierce,  
26 Aalst, Belgium) and the super signal-R West-Femto kit (Pierce, Aalst, Belgium) were  
27 used for primary antibody visualisation.  
28  
29 *URL.* Nimblegen: [www.nimblegen.com](http://www.nimblegen.com); Genomatix transcription factor database:  
30 [www.genomatix.de](http://www.genomatix.de).  
31

## 1 Results

2

### 3 Identification of genomic binding sites for ER- $\alpha$

4 ER- $\alpha$  binding sites in gene promoters were searched genome-wide using the estrogen-  
5 responsive T47D breast cancer cells. Estrogen-responsiveness was shown by the  
6 expression of ER- $\alpha$ , the induction of various known estrogen responsive genes (*TFF1*, *c-*  
7 *Myc*, *CCND1*) and by the induction of cell proliferation by 17 $\beta$ -estradiol (Supplemental  
8 Figures S-1 and S2). T47D cells were incubated with 1 nM 17 $\beta$ -estradiol for 50 minutes,  
9 which was shown to result in maximal ER- $\alpha$  binding to the *TFF1* promoter (Carroll et al.,  
10 2005; Carroll et al., 2006; this study, results not shown). After chromatin  
11 immunoprecipitation (ChIP) using an ER- $\alpha$  antibody, two rounds of nucleic acid  
12 amplification were performed to yield sufficient DNA for hybridisation to the Nimblegen  
13 promoter arrays. In order to assure adequate quality of the amplified DNA fragments,  
14 enrichment of the *TFF1* promoter was confirmed after each amplification round (Figure  
15 1a). Three independent experiments, each consisting of a 17 $\beta$ -estradiol and a vehicle  
16 treated sample, were performed (T1, T2 and T3). Given that the hybridisation  
17 performance of the T3 samples was poor, data from experiment T3 were not used for  
18 subsequent analyses. An additional sample was included (referred to as the pool, P)  
19 created by combining equal amounts of amplified DNA material from T1, T2 and T3.

20 We applied robust statistical procedures (see ‘Materials and Methods’), which allowed us  
21 to retrieve several previously known ER- $\alpha$  target promoters (i.e. *CTSD*, *BRCA*, *c-Myc*,  
22 *ADORA1*, *AGT*, *HSPB1*, *LCN2*; O’Lone et al., 2004). With this method, 904 potential  
23 ER- $\alpha$  binding sites were identified in total (Supplemental Table S-I), some of which are  
24 common to recent genome-wide screenings for ER- $\alpha$  targets (Supplemental Table S-II).  
25 The 904 binding sites are equally distributed over all chromosomes (Table I), excluding  
26 the Y chromosome, as the T47D line is derived from a woman. Only one site was found  
27 on chromosome Y and is not included in the list of 904 targets.

28

### 29 ChIP-chip validation and target promoter features

30 To validate the findings of the ChIP-chip, standard ChIP assays were performed using  
31 additional independent experiments (two or more) and ER- $\alpha$  binding was confirmed for a  
32 selection of 12 promoter regions (Figure 1b). Enrichments were not seen for three non-  
33 target locations (*PGR* gene exons 4 and 6 and chromosome region 12p13.3).

34 To demonstrate that ER- $\alpha$  binding to the promoter regions is functional, the effect on  
35 mRNA expression was studied with RT-PCR (Figure 1b). The expression of most genes  
36 is induced by 17 $\beta$ -estradiol, with the exception of *DKFZ p762E1312*, which is down-  
37 regulated, and *FANCM*, which does not respond despite ER- $\alpha$  binding to its promoter  
38 (Figure 1b). In addition, we evaluated the transcriptional response of six target genes for  
39 which ChIP reactions were not set-up, *CCNE2*, *IGF1-R*, *FBP-1*, *BCL2*, *MALL* and *CA2*  
40 (Supplemental Figure S-3). All genes, except *CA2*, are induced by 17 $\beta$ -estradiol. *MALL*  
41 and *CA2* are induced by OH-tamoxifen, whereas *BCL2* and *CCNE2* expression is reduced  
42 by OH-tamoxifen.

43 Binding sites for ER- $\alpha$  are present both upstream and downstream of the transcription  
44 start site (TSS) and are evenly distributed along the promoter regions with respect to the  
45 distance from the TSS (results not shown). Seventy four percent of the 904 target

1 promoters contain an estrogen-response element (ERE; Figure 1c), determined with the  
2 Genomatix MatInspector software.

#### 3 4 **Selective recruitment of co-regulators determines the ER- $\alpha$ mediated transcription**

5 Both 17 $\beta$ -estradiol and OH-tamoxifen can simultaneously up- and down-regulate the  
6 transcription of different genes in the same cell. To verify whether differential co-  
7 regulator recruitment (i.e. co-activators *versus* co-repressors) accounts for these opposite  
8 transcriptional responses in the same cells, we performed ChIP with antibodies directed  
9 against ER- $\alpha$ , co-activators p300, CBP and SRC-1 or co-repressors SMRT and NCoR  
10 after exposing T47D cells for 50 minutes to 1 nM 17 $\beta$ -estradiol or to 1  $\mu$ M OH-  
11 tamoxifen. These co-regulators were selected because they are expressed in T47D cells  
12 (Supplemental Figure S-1) and all three co-activators are efficiently recruited at the  
13 promoter of *TFF1* after 17 $\beta$ -estradiol induction (results not shown). It should be noted  
14 that we did not aim at identifying which specific co-regulator binds to one region, but  
15 rather whether co-activators or co-repressors are recruited. CBP / p300 are general  
16 mediators, which bridge the basal transcriptional machinery to the ER- $\alpha$  / co-activators  
17 complex, irrespective to which specific protein is present (SRC1, SRC2 or SRC3; Smith  
18 et al., 1996; Vo and Goodman, 2001). Therefore, in order to immunoprecipitate all DNA  
19 sequences interacting with co-activators simultaneously, we pooled the antibodies against  
20 p300, CBP and SRC-1. For the same reasons, we pooled co-repressors NCoR and SMRT  
21 antibodies.

22 The expression of *TFF1*, *DDX-27*, *ZNF-228* and *ZWINT* is up-regulated by 17 $\beta$ -estradiol  
23 and down-regulated by OH-tamoxifen (Figure 2), which correlates well with the  
24 recruitment of co-activators and co-repressors, respectively. In contrast, the expression of  
25 *FLNA*, *SYMPK*, *KGFLP1* and *BCL2L1* is induced by both 17 $\beta$ -estradiol and OH-  
26 tamoxifen (Figure 3). In these cases, predominant recruitment of co-activators is  
27 observed, although for some gene-promoters a non-significant recruitment of co-  
28 repressors can be seen as well (*BCL2L1* after 17 $\beta$ -estradiol treatment and *FLNA* after  
29 OH-tamoxifen treatment). Expression of *DKFZ p762E1312* is suppressed by both 17 $\beta$ -  
30 estradiol and OH-tamoxifen (Figure 4a). In the presence of 17 $\beta$ -estradiol, ER- $\alpha$  recruits  
31 co-repressors only; however, in the presence of OH-tamoxifen, co-activators are recruited  
32 as well (Figure 4a). This could be explained by the fact that OH-tamoxifen induces the  
33 transcription of *DKFZ p762E1312* at later time points (Supplemental Figure S-3). Also in  
34 case of the transcription up-regulation by 17 $\beta$ -estradiol of *EPHA4* (Figure 4b), ER- $\alpha$   
35 recruits co-activators at the *EPHA4* promoter. No recruitment of co-regulators is  
36 observed for this gene in response to OH-tamoxifen (Figure 4b) and its transcription is  
37 not altered, even though ER- $\alpha$  binds to the promoter.

#### 38 39 **Differential recruitment of co-regulators determines cell-specific transcriptional** 40 **activities of ER- $\alpha$**

41 Next we examined whether co-activators and co-repressors are recruited to selected ER- $\alpha$   
42 target genes in accordance with their opposite transcriptional responses to estrogens in  
43 T47D breast cells *versus* ECC1 endometrial cancer cells (ECC1 cells are ER- $\alpha$  / co-  
44 regulator positive - Supplemental Figure S-1 - and estrogen-responsive - Supplemental  
45 Figure S-2). In ECC1 cells, *KGFLP1*, *DDX-27* and *FLNA* are induced by 17 $\beta$ -estradiol  
46 and OH-tamoxifen, whereas *TFF1* is induced by 17 $\beta$ -estradiol only. ER- $\alpha$  preferentially

1 recruits co-activators to up-regulate these genes (Figure 5). In contrast, the transcriptional  
2 inhibitory effects of 17 $\beta$ -estradiol (for *BCL2L1*) or OH-tamoxifen (for *TFF1*, *BCL2L1*  
3 and *EPHA4*) are associated with the recruitment of co-repressors after ER- $\alpha$  binding  
4 (Figure 5).

5 Interestingly, OH-tamoxifen and 17 $\beta$ -estradiol reduce the expression of *BCL2L1* in  
6 ECC1, but induce it in T47D cells (Figures 5b and 3, respectively). In contrast, the  
7 expression of *DDX-27* is induced in ECC1 and reduced in T47D cells by OH-tamoxifen  
8 (Figures 5b and 2, respectively). These opposite transcriptional effects are clearly related  
9 to the recruitment of different co-regulatory proteins in the two cell contexts: co-  
10 activators in case of induction and co-repressors in the case of inhibition of transcription.  
11 Analogue results are observed for *EPHA4*. This gene is induced by 17 $\beta$ -estradiol in T47D  
12 cells, under which condition ER- $\alpha$  recruits co-activators (Figure 4b). However, *EPHA4* is  
13 not responsive to 17 $\beta$ -estradiol in ECC1 cells, and in this cell context, binding of ER- $\alpha$  to  
14 the corresponding promoter is not accompanied by further co-regulator recruitment  
15 (Figure 5b). The opposite is observed with OH-tamoxifen, which inhibits *EPHA4*  
16 expression in ECC1 cells but has no effect T47D cells. In T47D cells, no co-regulators  
17 are recruited by ER- $\alpha$  (Figure 4b), whereas in ECC-1 cells, binding of ER- $\alpha$  is followed  
18 by recruitment of co-repressors (Figure 5b). The recruitment of distinct co-regulators at  
19 the promoters of *DDX-27* and *BCL2L1* in T47D and ECC1 after induction with OH-  
20 tamoxifen was confirmed by real-time PCR (Figure 5d).

## 21 22 **Over-expression of SRC-1 and SMRT alters the response of target genes to OH- 23 tamoxifen**

24 If the regulation of the aforementioned genes is truly dependent on co-regulators, it  
25 should be expected that, as previously shown (Peterson et al., 2007; Smith et al., 1997),  
26 modification in the level of some of these proteins modifies the response of the target  
27 genes. Therefore, to confirm the association between up- or down-regulation and  
28 recruitment of co-activators or repressors, we over-expressed co-activator SRC-1 or co-  
29 repressor SMRT by transient transfections in T47D and ECC1 cells (Figure 6a). To proof  
30 that these transfections had significant and measurable effects, we assessed the activity of  
31 the estrogen-responsive construct *ERE-TK-luciferase* after co-transfection with SRC-1 or  
32 with SMRT. As expected, SRC-1 over-expression enhances the 17 $\beta$ -estradiol-induced  
33 luciferase activity, whereas SMRT reduces it (Figure 6b). Moreover, to confirm the  
34 transfectability of T47D and ECC1 cells we also measured GFP expression after transient  
35 transfection with a GFP expression plasmid (Supplemental Figure S-4).

36 Figure 6c shows the effect of SRC-1 or SMRT over-expression on a number of identified  
37 target genes. In T47D cells, *BCL2L1* transcription is normally up-regulated by OH-  
38 tamoxifen. Over-expression of the co-activator SRC-1 enhances this effect, whereas over-  
39 expression of the co-repressor SMRT changes OH-tamoxifen into an inhibitor of  
40 transcription (Figure 6c). In ECC1, *BCL2L1* is normally repressed by OH-tamoxifen, but  
41 over-expression of SRC-1 changes OH-tamoxifen into an inducer of transcription.

42 With regard to the expression of *EPHA4*, over-expression of SRC-1 in T47D cells turns  
43 OH-tamoxifen into an inducer of transcription, whereas this gene is unresponsive under  
44 normal conditions. In ECC1 cells, *EPHA4* transcription is inhibited by OH-tamoxifen and  
45 SRC-1 over-expression impairs this repressive activity. Also in case of the transcriptional  
46 activation of *KGFLPI* in both T47D and ECC1 cells, SMRT over-expression is sufficient

1 to revert (in T47D cells; OH-tamoxifen becomes a repressor of transcription) or impair  
2 (in ECC1 cells; OH-tamoxifen does not change gene transcription) this response (Figure  
3 6c).  
4 Transcription of *DDX-27* is suppressed by OH-tamoxifen in T47D and induced in ECC1  
5 cells. Over-expression of SRC-1 does not affect the inhibitory action of OH-tamoxifen in  
6 T47D, but over-expression of SMRT in ECC1 cells turns OH-tamoxifen into a repressor  
7 of transcription (Figure 6c).  
8 The response to OH-tamoxifen of other validated genes (*TFF1*, *FLNA*, *SYMPK*, *DDFZ*  
9 *p762E1312*, *ZWINT* and *ZNF-228*) and the responses to 17 $\beta$ -estradiol in general, were  
10 not significantly influenced by modifications of the level of SRC-1 and SMRT (data not  
11 shown). This suggests that co-regulators, themselves or as a consequence cell-specific be  
12 post-translational modifications, are promoter-specific.  
13

## 1 Discussion

2 The aim of the present study was to elucidate the role of co-regulators in (i) the opposite  
3 transcriptional actions mediated by ER- $\alpha$  on different target genes and (ii) the tissue  
4 specific actions of OH-tamoxifen (and 17 $\beta$ -estradiol) in breast and endometrial cancer  
5 cells. To this end, we first identified ER- $\alpha$  target promoters genome-wide by ChIP-chip  
6 and subsequently we examined whether co-activators or co-repressors are recruited by  
7 activated ER- $\alpha$  at the promoters of a number of newly-identified targets.

8 Though some past studies have focussed on the genome-wide identification of ER- $\alpha$   
9 binding sites in breast cancer cell lines (Bourdeau et al., 2008; Carroll et al., 2005;  
10 Carroll et al., 2006; Cheng et al., 2006; Jin et al., 2004; Kwon et al., 2007; Laganieri et  
11 al., 2005; Lin et al., 2004; Lin et al., 2007a; Lin et al., 2007b), none have further  
12 considered the role of co-regulators on the transcriptional regulation of these ER- $\alpha$   
13 targets. Up to now, this knowledge has been generated by means of reporter gene assays  
14 (Peterson et al., 2007; Smith et al., 1997) or by studying a low number of estrogen  
15 responsive genes only (Shang and Brown, 2002; Shang et al., 2000).

16 In the present study, we identified 904 promoters targeted by ER- $\alpha$  using ChIP-chip.  
17 These results were validated by standard ChIP, by the estrogen responsiveness of the  
18 corresponding genes at the mRNA level, and by the high prevalence of EREs among  
19 target promoters (Figure 1).

### 20 21 Co-regulator recruitment at target promoters determines gene- and cell-specific 22 responses to ER- $\alpha$ ligands

23 In line with previous studies (Shang and Brown, 2002; Shang et al., 2000; Stossi et al.,  
24 2006), activated ER- $\alpha$  binds to gene promoters, recruits co-activators or co-repressors,  
25 which determine the subsequent transcriptional up- or down-regulation, respectively  
26 (Figures 2, 3, 4 and 5). In one cell type, all determinants of the ER- $\alpha$  action (like ligand  
27 concentration, level and activation of ER- $\alpha$  and co-regulators) are identical, except for  
28 the promoter, which therefore must be responsible for the recruitment of different co-  
29 regulators. A number of studies have already shed light on the role of ERE-motifs and  
30 additional cis-regulatory elements (AP1, Sp1, NF $\kappa$ B binding sites) in the cell- and ligand-  
31 specific regulation of ER- $\alpha$  and ER- $\beta$  (Klinge, 2001; Ramsey et al., 2004; Schultz et al.,  
32 2005). The main features of the genes analysed in the present study (EREs and binding  
33 sites for additional transcription factors) are given in Supplemental Table S-IV.  
34 Alternatively, it is possible that co-regulators are modified post-translationally in a cell-  
35 specific manner, resulting in altered interactions at gene promoters in the distinct cell  
36 contexts.

37 In one case only (*DKFZ p762E1312*), transcription repression by OH-tamoxifen was  
38 associated with recruitment of both co-repressors and co-activators. We explained this  
39 effect with the ability of OH-tamoxifen to induce *DKFZ p762E131* transcription at later  
40 time points. However, it should also be noted that the dynamics, the sequential and  
41 combinatorial assembly of co-activators and co-repressors at target promoters have not  
42 been addressed in the present investigation. Nevertheless, these events are important for  
43 the action of nuclear receptors (Metivier et al., 2004; Metivier et al., 2003).

44  
45 Differential co-regulator recruitment also explains the opposite transcriptional response  
46 observed at a number of target genes in response to OH-tamoxifen (*DDX-27*, *BCL2L1*

1 and *EPHA4*) or 17 $\beta$ -estradiol (*BCL2L1* and *EPHA4*) in breast cancer (T47D; Figures 2, 3  
2 and 4) and endometrial cancer cells (ECC1; Figure 5). These results confirm a previous  
3 finding based on a number of known estrogen responsive genes (*c-Myc*, *IGF-I*, *EBAG9*  
4 and *CTSD*; Shang and Brown, 2002). The present study extends this mechanism of action  
5 to potentially all ER- $\alpha$  target genes.

6  
7 To further substantiate the association between transcriptional regulation and co-regulator  
8 recruitment, we over-expressed either co-activator SRC-1 or co-repressor SMRT. In a  
9 number of cases, the transcriptional response to OH-tamoxifen in T47D or ECC1 cells  
10 could be modified or inverted by over-expression of these co-regulators (*BCL2L1*,  
11 *KGFLP1*, *EPHA4*; Figure 6).

12 The transcription of other genes in response to OH-tamoxifen was not influenced by  
13 SRC-1 or SMRT over-expression (*TFF1*, *FLNA*, *SYMPK*, *DDX-27*, *DFPZ p762E1312*, *ZWINT* and  
14 *ZNF-228*). In some cases, as observed for *DDX-27*, the inducing action of OH-tamoxifen  
15 could be impaired in ECC1 after over-expression of SMRT, but the opposite inhibitory  
16 action of OH-tamoxifen observed in T47D cells could not be changed by SRC-1 over-  
17 expression. As shown by others (Peterson et al., 2007; Yahata et al., 2001), each  
18 promoter interacts with a limited number of co-regulators only and therefore each co-  
19 regulator modulates the expression of a limited number of genes. These events explain  
20 why co-regulators have distinct physiological functions (Kuang et al., 2005; Smith and  
21 O'Malley, 2004; Wang et al., 2009; Yu et al., 2007). In our case, it is entirely possible  
22 that SRC-1 cannot be efficiently recruited at the *DDX-27* promoter, whereas neither  
23 SRC-1 nor SMRT can be efficiently recruited at the promoter of other target genes,  
24 whose transcription was not influenced by these two co-regulators.

## 25 26 **Conclusions**

27 Complex events determine the action of ER- $\alpha$ , including histone modifications (Krum et  
28 al., 2008), distal and proximal cis-regulatory elements (Carroll et al., 2006, Klinge, 2001;  
29 Ramsey et al., 2004; Schultz et al., 2005), ligand independent signalling and indirect  
30 DNA binding mediated by additional transcription factors. Our results suggest that, at  
31 least for direct ER- $\alpha$  targets, distinct co-regulator recruitment is one of the key  
32 modulators of hormonal response.

33 In case of important drugs like tamoxifen, ER- $\alpha$  is necessary but not sufficient to mediate  
34 its actions. The direction of the hormonal response is for a large part dependent on co-  
35 regulators. Aberrations in the functions mediated by these proteins may lead to endocrine  
36 related cancers, to innate and developed drug-resistance in breast tumours (Balmer et al.,  
37 2006; Conzen, 2008; Lonard et al., 2007) or to poor therapeutic response observed, for  
38 instance, in case of ovarian tumours (Perez-Gracia and Carrasco, 2002). Unravelling the  
39 expression and activation patterns of co-regulators in estrogen-dependent tumours may be  
40 the next step in predicting drug response and personalise endocrine.

41



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7 *Author contribution:* this study was designed by AR, GD and PG; the experimental  
8 procedures were performed by AR with assistance from SK and BD; microarray,  
9 statistical and additional bioinformatics analyses were performed by MA and CE.

10

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11

1 **Figure Legends**

2

3 **Figure 1. ChIP-chip: quality control, validation and prevalence of EREs**

4 A. Prior to ChIP-chip hybridisation, immunoprecipitated (IP) DNA fragments were  
5 amplified (linear amplification). As a quality check, binding of ER- $\alpha$  to the *TFF1*  
6 promoter was confirmed after each amplification round (shown for each experiment at  
7 the end of the amplification, just prior to labelling and hybridisation). ChIP-DNA = IP  
8 DNA. Input-DNA = non-IP- chromatin amplified similarly to the ChIP-DNA.

9 B. ER- $\alpha$  targets identified by ChIP-chip and validated by standard ChIP. For all ChIP  
10 experiments, cells were treated for 50 minutes; control = vehicle treated cells; E2 or 17 $\beta$ -  
11 estradiol: 1 nM. IgG = ChIP with non-specific antibodies; ER- $\alpha$  = ChIP with the ER- $\alpha$   
12 antibody. Column on the right: mRNA level of the corresponding gene after 17 $\beta$ -estradiol  
13 (1 nM) induction. mRNA was assessed (RT-PCR or semi-quantitative PCR – semiQ-  
14 PCR) after different periods of hormone stimulation (up to 24 hours) in triplicate. Results  
15 in column signify that the considered mRNA is significantly regulated in the indicated  
16 direction ( $p < 0.05$  compared to time point zero) at one time point at least (results not  
17 shown). ND: not determined.

18 C. Prevalence of EREs in the promoters of the entire group ( $n = 904$ ) of ER- $\alpha$  target  
19 genes as determined by Genomatix MatInspector (<http://www.genomatix.de>). Promoters  
20 were scanned using a family of ERE consensus matrices (Cartharius et al., 2005).

21

22 **Figure 2. Co-regulator recruitment at targets induced by 17 $\beta$ -estradiol and repressed  
23 by OH-tamoxifen in T47D cells**

24 A. Transcriptional responses of the indicated target genes (RT-PCR) after treatment with  
25 17 $\beta$ -estradiol, OH-tamoxifen (1 nM and 1  $\mu$ M, respectively) in T47D. Mean  $\pm$  standard  
26 deviation (SD),  $n = 3$ . Asterisks:  $p < 0.05$  compared to time point zero. Expression data  
27 were reconfirmed in at least one extra independent experiment.

28 B. ChIP assessing binding of ER- $\alpha$ , co-activators (p300, CBP and SRC-1) or co-  
29 repressors (SMRT and NCoR) to the corresponding promoter (E2 = 17 $\beta$ -estradiol. Tam =  
30 OH-tamoxifen. No treatment: treatment with vehicle only - ethanol). Cells were treated  
31 for 50 minutes before ChIP.

32 C. Quantitative evaluation (estimated by agarose-gel band intensities) of chromatin  
33 enrichments after ChIP with ER- $\alpha$ , co-activator (ACT) or co-repressor (REP) antibodies.  
34 Mean  $\pm$  SD;  $n = 2$  or 3. Asterisks:  $p < 0.05$  compared to the IgG control. ChIP  
35 experiments were reconfirmed in at least one additional independent experiment. The  
36 ChIP negative control for these assays is shown in Figure 4c.

37

38 **Figure 3. Co-activators are recruited at genes induced by both 17 $\beta$ -estradiol and OH-  
39 tamoxifen in T47D cells**

40 A. Transcriptional responses in T47D to 1 nM 17 $\beta$ -estradiol or 1  $\mu$ M OH-tamoxifen (RT-  
41 PCR and semiQ-PCR for *KGFLP1*). Mean  $\pm$  SD,  $n = 3$ . Asterisks:  $p < 0.05$  compared to  
42 time point zero. Expression data were reconfirmed in at least one independent  
43 experiment.

44 B. ChIP assessing binding to the corresponding promoter of ER- $\alpha$ , co-activators or co-  
45 repressors (50 minutes after induction start: E2 = 17 $\beta$ -estradiol. Tam = OH-tamoxifen.  
46 No treatment: induction with vehicle only).

1 C. Quantitative evaluation of chromatin enrichments after ChIP with ER- $\alpha$ , co-activator  
 2 (ACT) or co-repressor (REP) antibodies. Mean  $\pm$  SD; n = 2 or 3. Asterisks: p < 0.05  
 3 compared to IgG control. ChIP experiments were reconfirmed in at least one additional  
 4 independent experiment (ChIP negative in Figure 4c).

5  
 6 **Figure 4. Co-regulator recruitment by activated ER- $\alpha$  at DKFZ p762E1312 and**  
 7 **EPHA4 in T47D cells**

8 A. On the left: transcriptional responses of the *DKFZ p762E1312* gene (repressed by both  
 9 17 $\beta$ -estradiol - 1 nM - and OH-tamoxifen - 1  $\mu$ M) in T47D (RT-PCR). Mean  $\pm$  SD, n = 3.  
 10 Asterisks: p < 0.05 *versus* time point zero. RNA data were reconfirmed in at least one  
 11 extra independent experiment. Middle: ChIP assessing binding to the *DKFZ p762E1312*  
 12 promoter of ER- $\alpha$ , co-activators or co-repressors. ChIP was performed 50 minutes after  
 13 induction start: E2 = 17 $\beta$ -estradiol. Tam = OH-tamoxifen. No treatment: vehicle only.  
 14 Right: quantitative evaluation of chromatin enrichments after ChIP with ER- $\alpha$ , co-  
 15 activator (ACT) or co-repressor (REP) antibodies. Mean  $\pm$  SD. Asterisks: p < 0.05 *versus*  
 16 IgG control, n = 2 or 3.

17 B. *EPHA4* gene is induced by 17 $\beta$ -estradiol (1 nM) but is not influenced by 1  $\mu$ M OH-  
 18 tamoxifen (on the left; mean  $\pm$  SD, n = 3. Asterisks: p < 0.05 *versus* time point zero).  
 19 RNA data were reconfirmed in at least one extra independent experiment. Middle and  
 20 right: ChIP assay and quantitative evaluation of the ChIP experiments (mean  $\pm$  SD based  
 21 on at least two independent experiments. Asterisks: p < 0.05 *versus* IgG control).

22 C. ChIP negative control (cytogenetic location 12p13.3).

23  
 24 **Figure 5. mRNA level and co-regulator recruitment in ECC1 cells**

25 A and B. Transcriptional responses (RT-PCR and semiQ-PCR for *KGFLP1*) after 17 $\beta$ -  
 26 estradiol or OH-tamoxifen stimulation (1 nM and 1  $\mu$ M, respectively) in ECC1 (left side  
 27 of panels A and B). Mean  $\pm$  SD, n = 3. Asterisks: p < 0.05 *versus* time point zero. RNA  
 28 expression data were reconfirmed in at least one additional independent experiment. ChIP  
 29 assays (50 minutes of induction) showing binding of ER- $\alpha$ , co-activators (SRC-1, CBP  
 30 and p300) and co-repressors (NCoR and SMRT) to the corresponding promoter are  
 31 shown on the right of each A and B panels (E2 = 17 $\beta$ -estradiol. Tam = OH-tamoxifen.  
 32 No treatment: vehicle only). ChIP experiments were reconfirmed in at least one  
 33 additional independent experiment.

34 A. The transcriptional response of these genes in ECC1 (shown in panel) is similar to the  
 35 response observed in T47D cells (shown in Figures 2a and 3a) and ChIP indicates that the  
 36 same kind of co-regulators are recruited at gene promoters in the two cell lines (ECC1,  
 37 shown in this figure, and T47D cells, Figures 2 and 3).

38 B. The transcriptional response of these genes in ECC1 (shown in panel) is opposite  
 39 compared to the response observed in T47D cells (shown in Figures 2, 3 and 4) and ChIP  
 40 indicates that the distinct co-regulators are recruited at gene promoters in ECC1 (shown  
 41 in panel) and T47D cells (Figures 2, 3 and 4).

42 C. ChIP negative control (cytogenetic location 12p13.3).

43 D. Relative enrichments of ER- $\alpha$ , co-activators (ACT) or co-repressors (REP) at the  
 44 promoters of *DDX-27* and *BCL2L1* in T47D and ECC1 cells after OH-tamoxifen  
 45 induction (50 minutes). OH-tamoxifen induces *DDX-27* and *BCL2L1* in opposite  
 46 directions in T47D and ECC1 cells. The direction of the mRNA regulation is indicated by

1 the arrows. ChIP reactions were measured by real-time PCR (mean  $\pm$  SD based on two  
2 replicates. Asterisks:  $p < 0.05$  versus IgG control).

3  
4 **Figure 6. Over-expression of SRC-1 and SMRT modifies OH-tamoxifen responses**

5 A. Over-expression of co-activator SRC-1 and co-repressor SMRT in T47D and ECC1  
6 cells after transient transfection (immunocytofluorescence). Empty arrow-heads:  
7 endogenous expression level. Solid arrow-heads: over-expressing cells.

8 B. Induction of the ERE-TK promoter after co-transfection of ECC1 cells with the 2X  
9 ERE-TK-LUC construct (containing the luciferase reporter) along with either the  
10 expression plasmid for co-activator SRC-1 (increasing amounts of plasmids used for  
11 transfection) or the plasmid expressing co-repressor SMRT. Cells were transfected as  
12 described in material and methods in 12-well plates using 2  $\mu$ g of total plasmid DNA: 1  
13  $\mu$ g of 2X ERE-TK-LUC combined with variable amounts (0 – 1  $\mu$ g of SRC-1). Total  
14 amount of transfected DNA was kept constant using the empty vector. For induction ( $n =$   
15 3 per treatment  $\pm$  SD) and luciferase assay, transfected cells were re-plated on a 96 well-  
16 plate. Similar results are obtained in T47D cells (not shown).

17 C. Transcriptional responses of *BCL2L1*, *DDX-27*, *EPHA4* (RT-PCR) and *KGFLPI*  
18 (semiQ-PCR) after stimulation with 1  $\mu$ M OH-tamoxifen or with vehicle only (no  
19 treatment) for 5 hours in T47D and ECC1 cells transiently transfected with the empty  
20 vector, SRC-1 expression plasmid or SMRT expression plasmid. Cells were transfected  
21 as described in material and methods in 25 cm<sup>2</sup> flask (10  $\mu$ g DNA) and re-plated for  
22 induction and RNA isolation in 12-well plates. Bars indicate mean  $\pm$  SD,  $n = 3$ . Asterisks  
23 indicate a significant difference ( $p$  value  $< 0.05$ : t-test) between transfection experiments  
24 in the mRNA fold-change after OH-tamoxifen induction.

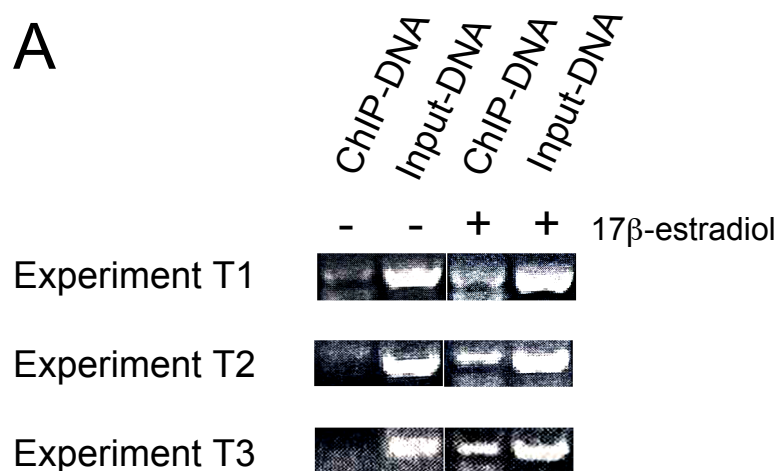


FIGURE 1. *Romano et al.*

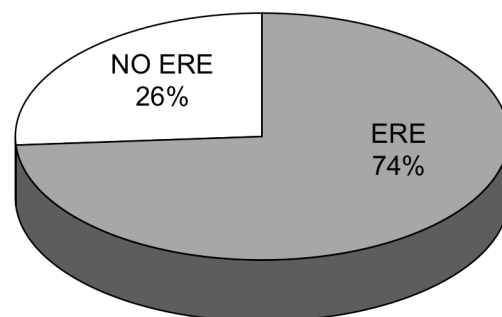
B

Gene	Description	BINDING (Standard CHIP)				mRNA level after 17 $\beta$ -estradiol treatment	
		control (c)		17 $\beta$ -estradiol (E2)			
targets identified by CHIP-chip		IgG	ER- $\alpha$	IgG	ER- $\alpha$	input c E2	
<i>TFF1</i> (positive control)	Trefoil factor 1						up
<i>ZNF-228</i>	zinc finger protein 228						up
<i>ZWINT</i>	ZW10 interactor						up
<i>DDX-27</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27						up
<i>FLNA</i>	filamin A						up
<i>SYMPK</i>	symplekin						up
<i>BCL2L1</i>	BCL2-like 1						up
<i>KGFLP1</i>	keratinocyte growth factor-like protein 1						up
<i>DKFZ p762E1312</i>	hypothetical protein DKFZ p762E1312						down
<i>OGG1</i>	8-oxoguanine DNA glycosylase						up
<i>EPHA4</i>	EPH receptor A4						up
<i>FANCM</i>	Fanconi anemia, complementation group M						no regulat.
<i>FKBP3</i>	FK506 binding protein 3, 25kDa						ND
	NEGATIVE CHIP						

A



C

EREs AMONG THE 904 ER- $\alpha$  TARGETS

P &lt; 0.0001

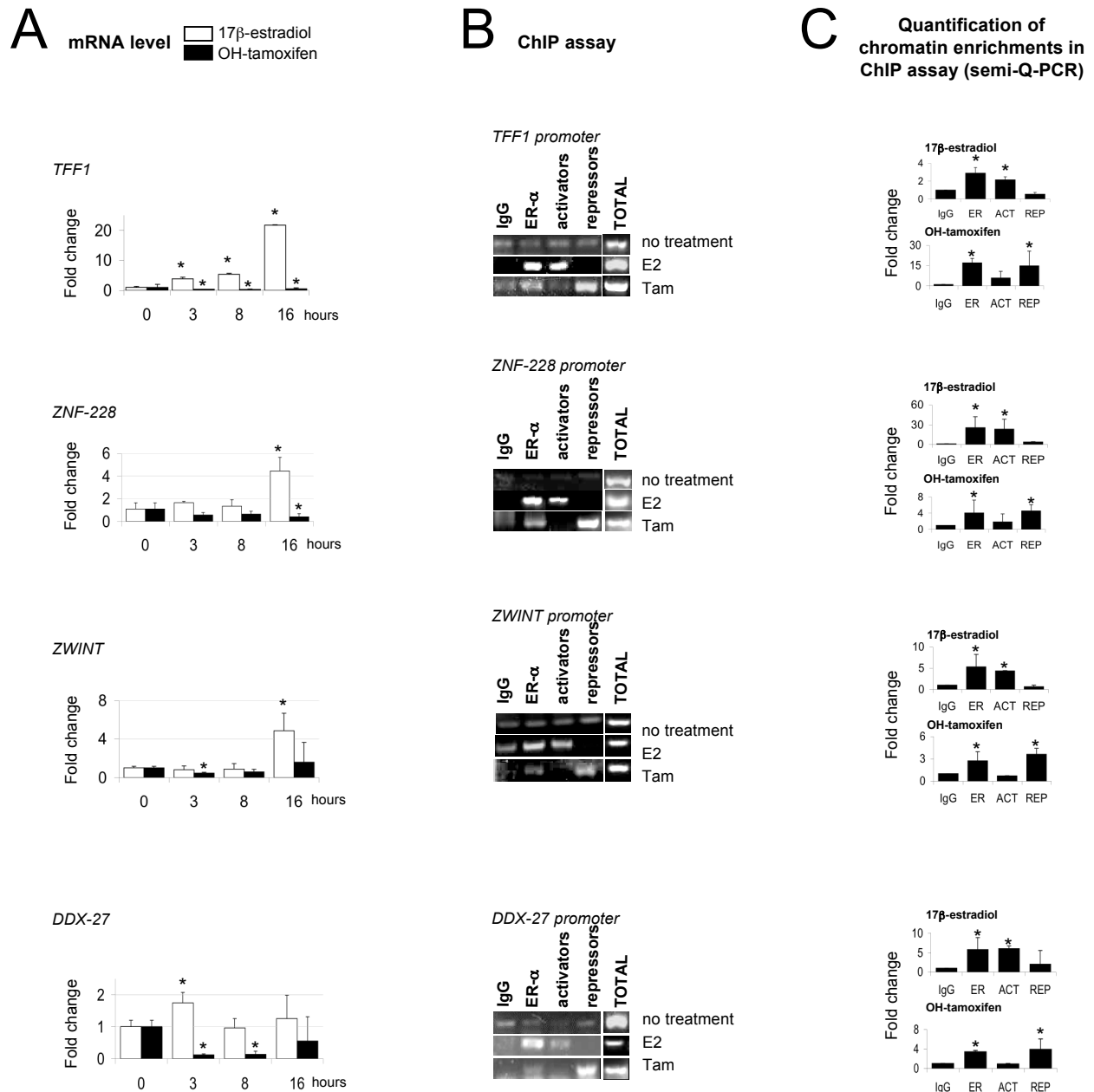
FIGURE 2. *Romano et al.*Genes up-regulated by 17 $\beta$ -estradiol and repressed by OH-tamoxifen

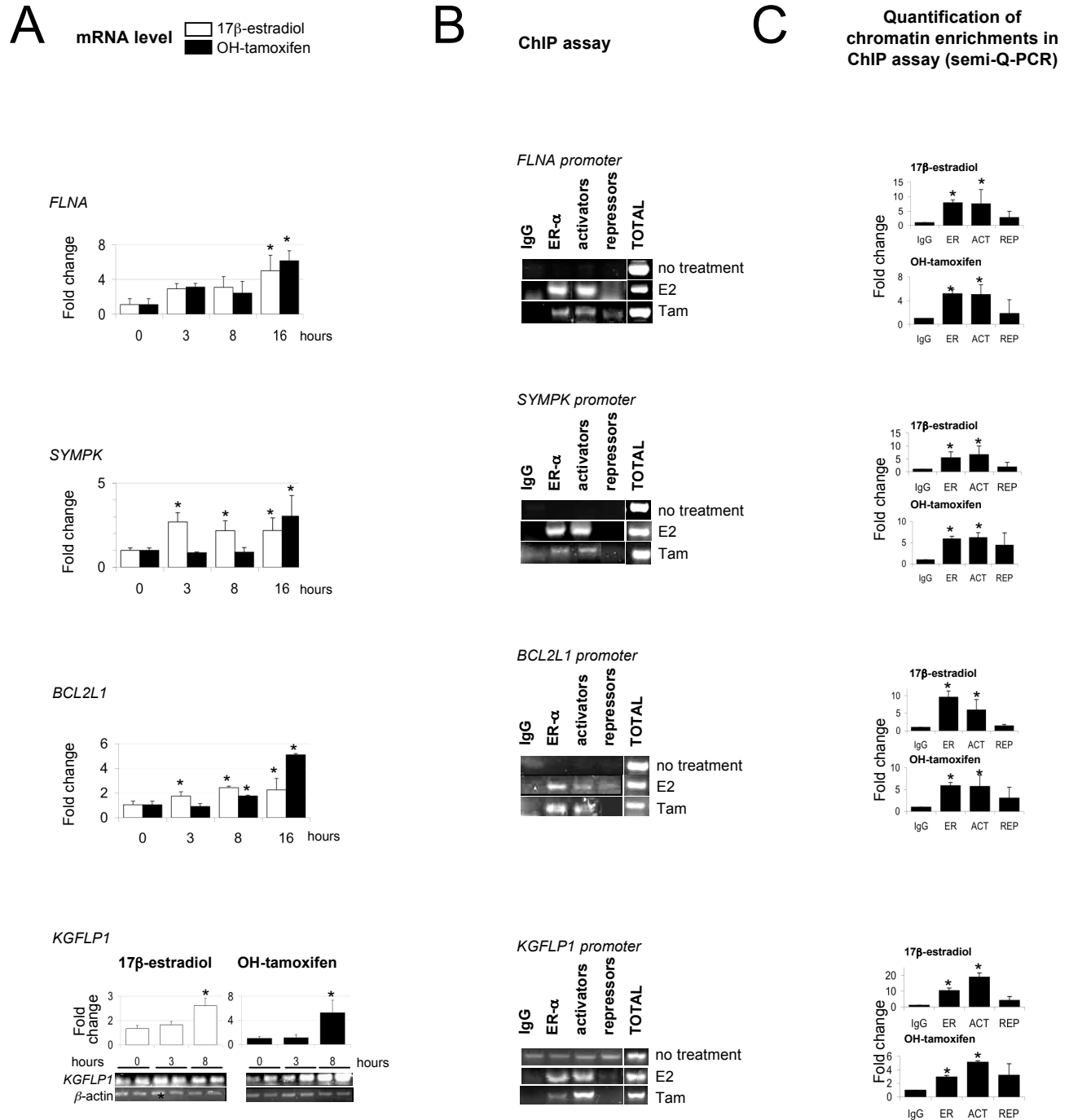
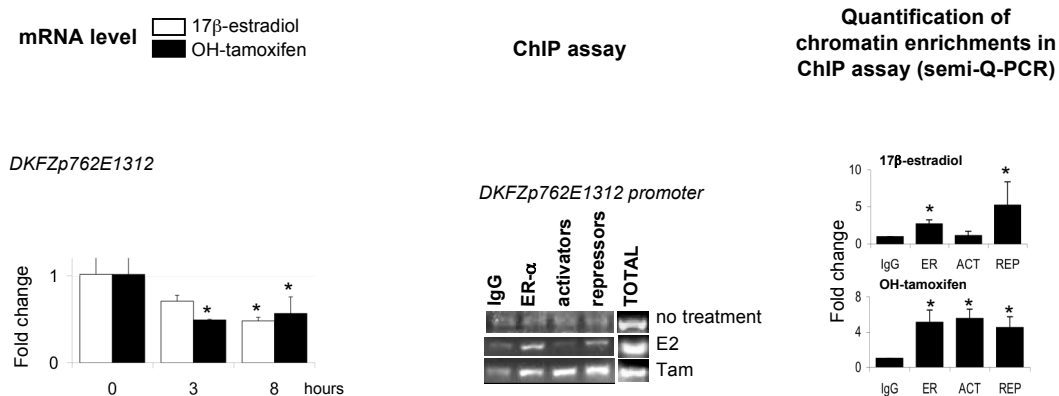
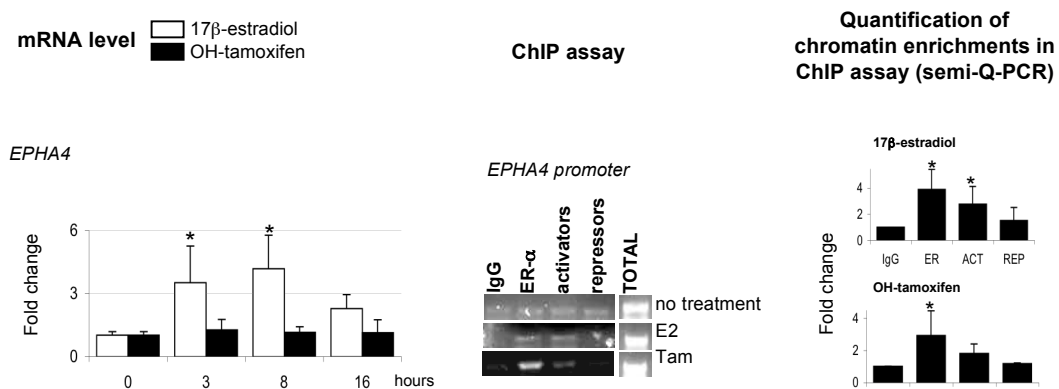
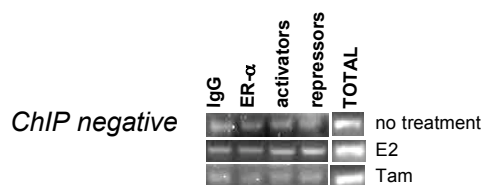
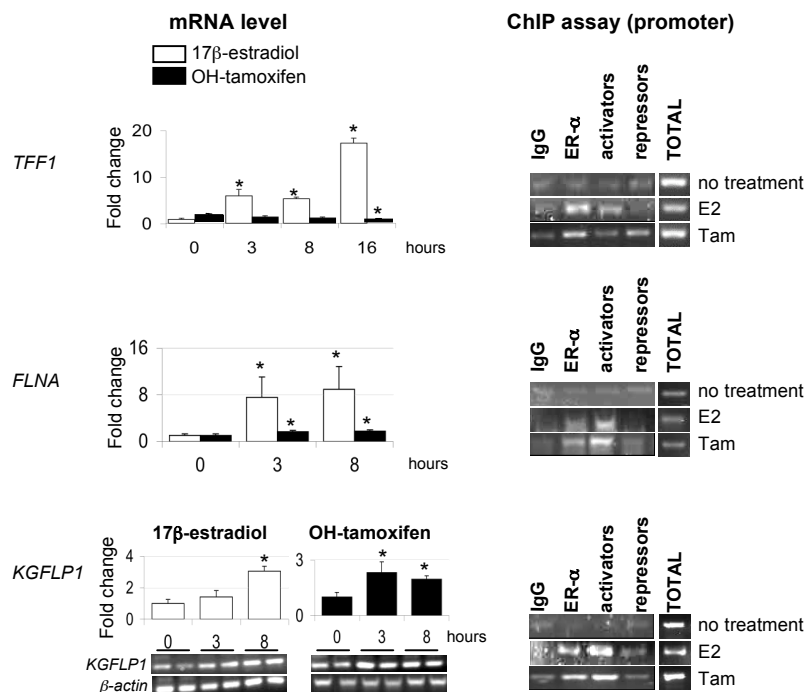
FIGURE 3. *Romano et al.*Genes up-regulated by both 17 $\beta$ -estradiol and OH-tamoxifen

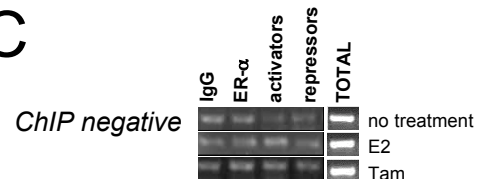
FIGURE 4. *Romano et al.***A** Gene repressed by 17 $\beta$ -estradiol and repressed by OH-tamoxifen**B** Gene induced by 17 $\beta$ -estradiol and non-responsive to by OH-tamoxifen**C**

# FIGURE 5. Romano et al.

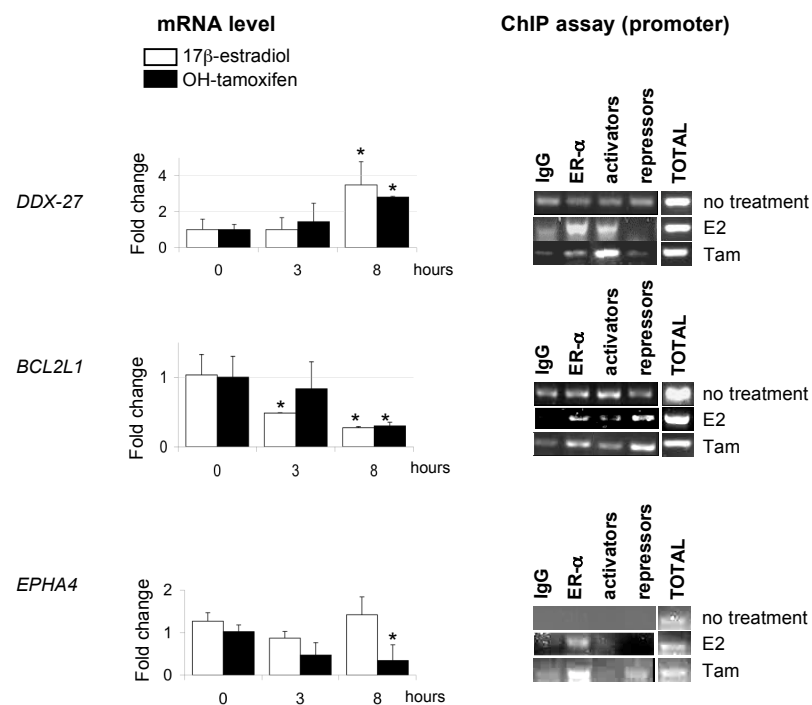
**A** mRNA level and ChIP in ECC1 cells. These genes are regulated in the same direction in T47D cells



**C**

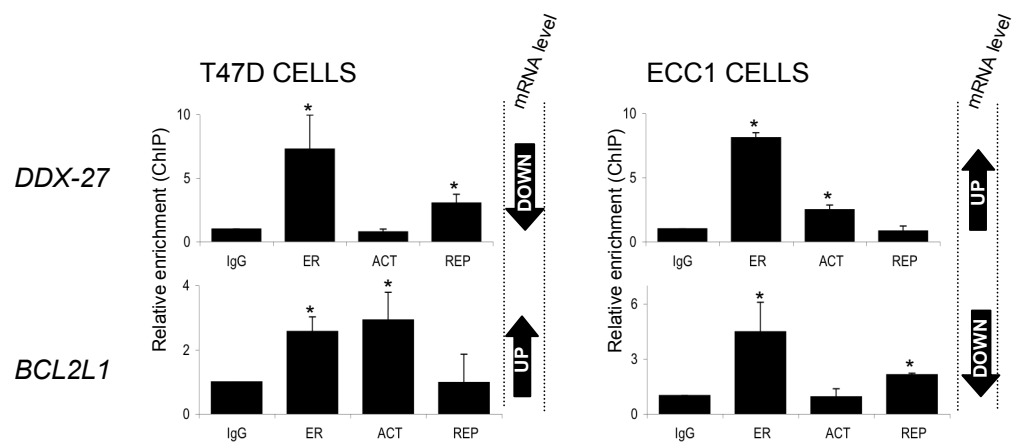


**B** mRNA level and ChIP in ECC1 cells. These genes are regulated in an opposite direction in T47D cells



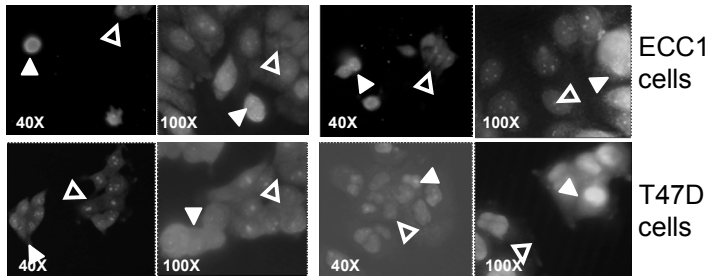
**D**

ChIP on *DDX-27* and *BCL2L1* (real time PCR) in T47D and ECC1 cells stimulated with OH-tamoxifen

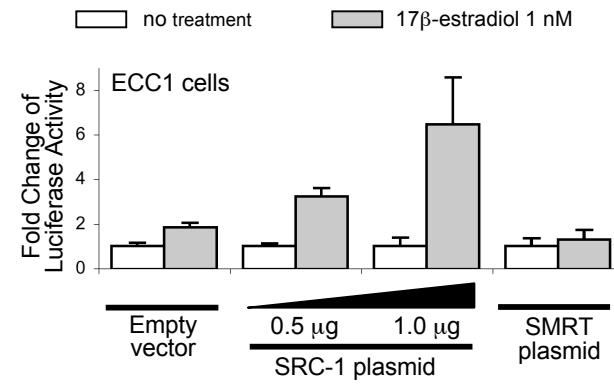


# FIGURE 6. *Romano et al.*

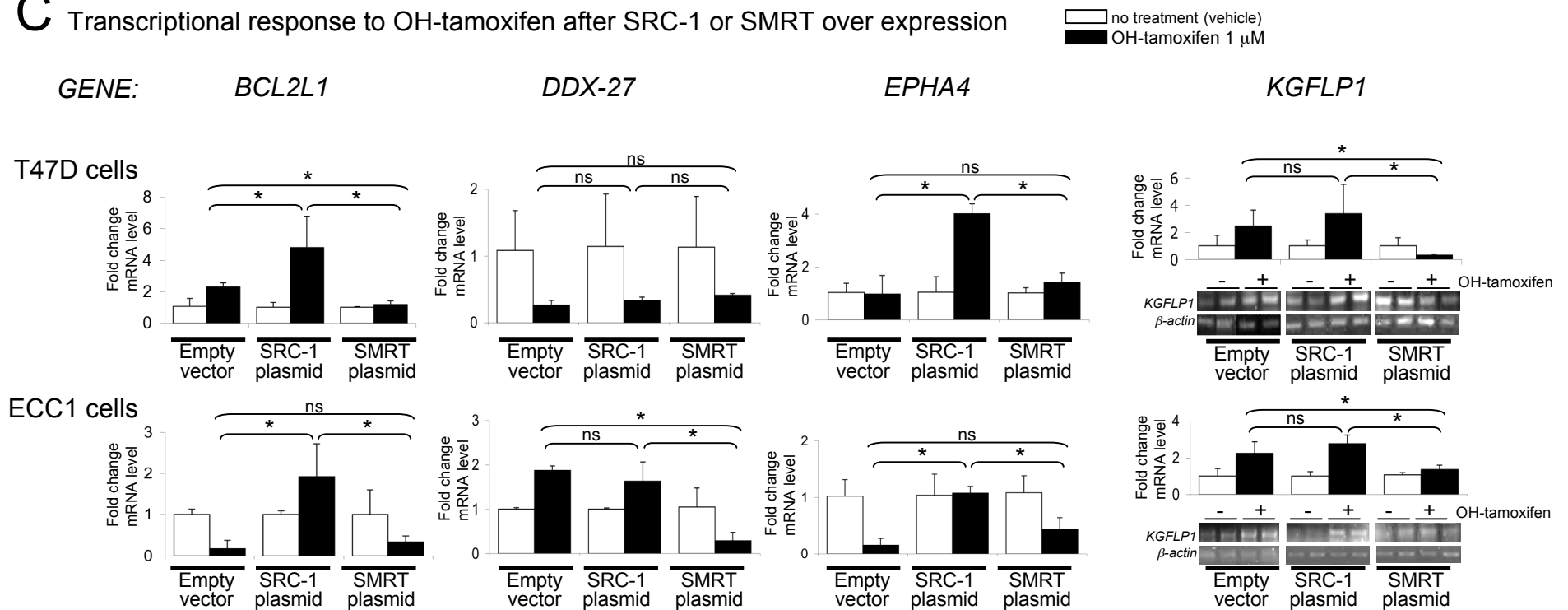
## A SRC-1 over expression SMRT over expression



## B *ERE-TK-Luciferase* activity after SRC-1 or SMRT over expression



## C Transcriptional response to OH-tamoxifen after SRC-1 or SMRT over expression



*Table I. Number of ER- $\alpha$  binding sites per chromosome.*

Chromosome	number of sites
1	79
2	51
3	45
4	36
5	36
6	61
7	41
8	35
9	43
10	25
11	83
12	56
13	17
14	33
15	31
16	27
17	54
18	12
19	41
20	31
21	12
22	21
X	34