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## Dynamic ER Interactomes Control the Estrogen-Responsive Trefoil Factor (TFF) Locus Cell-Specific Activities.

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1 **Dynamic ER Interactomes Control the Estrogen-Responsive Trefoil Factor (TFF) Locus Cell-Specific Activities**

2 **Running Title:** ER-mediated regulations of the TFF cluster in 4D

3 **Keywords:** Estrogen receptor; Transcription; Interactome; Triplex forming oligonucleotides.

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30  
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**32 ABSTRACT**

33 Estradiol signaling is ideally suited for analyzing molecular and functional linkages between the different layers of  
34 information directing transcriptional regulations: DNA sequence, chromatin modifications and the spatial  
35 organization of the genome. Hence, estrogen receptor (ER) can bind at a distance from its target genes and engages  
36 timely and spatially coordinated processes to regulate their expression. In the context of the coordinated  
37 regulations of co-linear genes, identifying which ER binding sites (ERBSs) regulate a given gene still remains a  
38 challenging question. Here, we investigated the coordination of such regulatory events at a 2 Mb genomic locus  
39 containing the estrogen-sensitive TFF cluster of genes in breast cancer cells. We demonstrated that this locus  
40 exhibits a hormone and cohesin-dependent reduction in the plasticity of its three-dimensional organization that  
41 allows multiple ERBSs to be dynamically brought to the vicinity of estrogen-sensitive genes. Additionally, by using  
42 triplex forming oligonucleotides, we could precisely document the functional links between ER engagement at  
43 given ERBSs and the regulation of particular genes. Hence, our data evidence a formerly suggested cooperation of  
44 enhancers towards gene regulations, and also show that redundancy between ERBSs can occur.

45

**46 INTRODUCTION**

47 In Mammals, gene transcription relies on complex and highly organized regulatory processes, which include  
48 binding of transcription factors to cognate DNA sequences (*cis* elements), chromatin structure and epigenetic  
49 information, the action of additional factors in trans (cofactors and RNA Polymerase II (Pol II) machinery) and the  
50 spatial organization of the genome (1-5). Signaling pathways initiated by steroid hormones, such as 17 $\beta$ -estradiol  
51 (E2), provide model systems to study these different layers of transcription regulation in mammalian cells. Indeed,  
52 exposure to estrogens leads to transcriptional changes of cell-specific gene repertoires, which are mediated by E2-  
53 bound Estrogen Receptors (ESR1 -ER throughout the manuscript- and ESR2) (6). On model gene promoters, such as  
54 *TFF1*, ER together with a number of its cofactors associate with cognate binding sites (BS) in a cyclic manner to  
55 direct their transcription (7, 8). The spatial organization of the genome also determines the coordinated expression  
56 of genes (9, 10). This is notably the case for ER, where the existence of clusters of co-regulated genes can originate  
57 from genetic and epigenetic information or from chromatin dynamics itself. In some instances, such coordinated  
58 regulation of co-linear genes depends on a single regulating unit [e.g., *HBB*, *Mrf4* and *Hox* clusters (11-13)].

59 Genome-wide analyses of ER binding sites (ERBSs) have demonstrated that ER binds only rarely to the proximal  
60 promoter of its target genes, but is mobilized onto intergenic and intronic sequences (14), which have been  
61 proposed to communicate with target genes via long-distance intrachromosomal interactions (15). Whether these  
62 distant elements are acting as global regulators for clustered E2-responsive genes is still an intriguing question. In  
63 addition, these genome-wide studies also showed that additional transcription factors are required for the accurate  
64 targeting of ER onto cognate sequences along the whole genome (16). These factors include FOXA1 (17), TFAP2C  
65 (19), and PBX1 (20). Among those, FOXA1 may act as an allosteric sensor for histone marks associated with active  
66 or poised chromatin (such as H3K4 mono/di-methylation), and it is therefore considered as a pioneer factor

67 preparing chromatin for subsequent binding of ER (21-23).

68 We aimed here to obtain functional and mechanistic evidence that distant ERBSs elements actually constitute  
69 global regulators for clustered E2-responsive genes. To do so, we engaged an extensive analysis of mechanisms  
70 involved in the coordination of the estrogenic response of one cluster of E2-sensitive genes in breast carcinoma  
71 cells. These studies were performed in different breast cancer cell lines: MCF-7 cells that constitutively express  
72 both ER and FOXA1, and in MDA-MB231 cells that were engineered to constitutively express ER [cells named  
73 MDA::ER; (24)] but not FOXA1. Comparative observations made in these two cell lines allowed us to interrogate  
74 whether the introduction of ER in MDA-MB231 cells is sufficient to recapitulate regulatory processes observed in  
75 MCF-7 at the TFF locus. The combination of chromosome conformation capture methods (3C/4C) with ChIP-chip  
76 experiments and the use of triple helix forming oligonucleotides (TFOs), which allows testing the functional  
77 importance of individual enhancers, defined key molecular features specifying the transcriptional response induced  
78 by E2. We show that, in both cell types, ER engages similar mechanisms to regulate transcription of co-regulated  
79 gene clusters, in particular through long-range and dynamic interactions between multiple ERBSs and its target  
80 genes. By interfering specifically with the association of ER with given ERBSs, we were also able to determine the  
81 relative importance of these different BSs in the regulation of corresponding E2-dependent genes.

82

### 83 MATERIALS AND METHODS

84 **Reagents.** All chemicals, restriction or modification enzymes were obtained from Sigma, Roche or New England  
85 Biolabs. All primers and siRNAs were purchased from Sigma. Antibodies were from from Abcam, Millipore or  
86 SantaCruz (Actin: sc-8432; CTCF: 07-729; ER: HC20 and ab10[TE111-SD1]; FOXA1: ab23738 and RAD21: ab992). The  
87 anti-Scc1/RAD21 was a gift from Dr. JM Peters and the anti-hCAPD2 Eg7.2 was previously published (25) BACs were  
88 purchased from Invitrogen (RP11-814F13, CTD-2337B13, RP11-35C4, CTD-260o11, RP11-113F1, CTD-1033M14).

89

90 **Triplex forming oligonucleotides (TFOs).** We developed a python algorithm (available upon request) following the  
91 rules defined in (26) to design putative TFOs targeting 15-30 bp long oligopyrimidine-oligopurine tracts included  
92 within ERBSs (Table 1), with one possible divergent base from a strict polyA/G sequence. Triplex formation was  
93 monitored *in vitro* by incubating increasing amounts of TFOs with DNA duplexes for 16h at 37°C in a buffer  
94 containing 10 mM MgCl<sub>2</sub>, 100 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10% glycerol and 0.5 mg/ml tRNA. Complexes  
95 were separated by native electrophoresis on polyacrylamide gel containing 10 mM MgCl<sub>2</sub> and 50 mM Tris-HCl (pH  
96 7.4) and visualized by methylene blue staining.

97

98 **Cell culture and reverse-transcription.** MCF-7, MDA-MB231 and MDA-MB231 cells stably expressing ER $\alpha$  [MDA::ER  
99 (24)] were maintained in DMEM (Gibco) containing 5% fetal calf serum (FCS, BioWest) and antibiotics (Roche) at  
100 37°C under 5% CO<sub>2</sub>. MDA::ER media was supplemented with 0.8 mg/ml hygromycin (Calbiochem). For experiments  
101 requiring treatment with E2, cells were cultivated for 2 days in DMEM without phenol red containing 2% charcoal-

102 stripped FCS (csFCS; BioWest) prior to the addition of E2 ( $10^{-8}$ M final concentration). Total RNAs from  $10^7$  cells were  
103 purified using Trizol™ reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Two  $\mu$ g of  
104 RNA served as template for M-MLV reverse transcriptase (Invitrogen) and Pd(N)6 random hexamers (Amersham  
105 Pharmacia Biosciences).

106

107 **Transfections.**  $2.5 \times 10^6$  cells were plated in 9cm dishes in DMEM/5%FCS for 16h and then grown for 24h in  
108 DMEM/2.5% csFCS. Media was then replaced with 4 ml of FCS and antibiotics free Opti-MEM (Sigma) and 1  $\mu$ mol  
109 siRNAs (sense: Luciferase, AACACUUACGCUGAGUACUUCGA; CTCF, GGAGCCUGCCGUAGAAAUU; RAD21,  
110 GGUGAAAUGGCAUUACGG) or 10  $\mu$ mol TFOs were then transfected using oligofectamine as recommended by the  
111 manufacturer (Invitrogen). Following 6h of incubation, the media was completed with 125  $\mu$ l of csFCS, and E2  
112 stimulation ( $10^{-8}$ M) was done 36h later.

113

114 **Western blotting.** Half cells from confluent 9cm-diameter dishes were directly lysed in sample buffer, and  
115 subjected to classical SDS-PAGE. Proteins were transferred onto Hybond nitrocellulose membrane (Amersham) for  
116 2h, which were subsequently blocked in PBS or TBS complemented with 0.1% Tween-20/4% dry milk for 1 h at 4°C.  
117 Membranes were then incubated overnight at 4°C with primary antibodies at appropriate concentrations (CTCF:  
118 1/2000; Scc1/RAD21: 1/1000; ER: 1/2500; FOXA1: 1/2500;  $\beta$ -Actin: 1/5000 and 1/2000 for anti-hCAPD2 Eg7.2).  
119 Following three successive washes, blots were further incubated for 1h at room temperature using appropriate  
120 peroxidase-coupled secondary antibodies diluted at 1/10,000 in PBS or TBS plus 0.1% Tween-20/4% dry milk.  
121 Western blots were revealed by the ECL detection kit (Amersham).

122

123 **DNA-FISH.** Probes were produced by direct labelling of BACs clones through random priming (Bioprime array CGH  
124 genomic labeling system, Invitrogen) using fluorochrome conjugated nucleotides (dUTP-alex fluor 488 from  
125 Invitrogen or dUTP-cyanine 3 from Perkin Elmer). Before use, probes were denatured 5min at 80°C and then 30min  
126 at 37°C. Cells were grown for two days on glass slides in DMEM without phenol red containing 2.5% csFCS. After  
127 addition of  $10^{-8}$ M E2 or ethanol (vehicle), slides were washed with PBS, and then fixed in 2% paraformaldehyde  
128 (PFA) for 10 min at 4°C. PFA fixed cells were permeabilized in 0.5% Triton X-100 and equilibrated in 1X SSC for 5  
129 min. Slides were incubated one hour with 20 $\mu$ g/mL RNaseA in 1X SSC at 37°C and then sequentially washed 3 times  
130 with PBS, incubated in 2% PFA for 10 min at room temperature, in HCl 100mM for 10 min and then in 0.5% Triton  
131 for 10 min, with 3 washings with PBS between each step. Slides were then subjected to denaturation through  
132 sequential heating at 73°C in 70% formamide/ 30% 2X SSC for 7 min and then 3 min in 50% formamide / 50% 2X  
133 SSC. Hybridization with 600ng of labelled denatured DNA probes was performed overnight at 42°C in hybridization  
134 buffer (per 800 $\mu$ l: 200 $\mu$ l 25% Dextran sulfate; 100 $\mu$ l 20X SSC; 500 $\mu$ l deionised formamid) containing 150 $\mu$ g of Cot-  
135 I (Invitrogen) and 150 $\mu$ g of Salmon Sperm DNA. Slides were rinsed three times in 2X SSC, in 50% formamide/50% 2X

136 SSC 20 min at 42°C and three times in 2X SCC again. Nuclei were stained with DAPI in 2X SSC for 5 min and then  
137 slides were mounted in ProLong Gold antifade reagent (Invitrogen) with a 22\*40 coverslip.

138

139 **Cytogenetic analysis.** MCF-7 and MDA cells were plated in Lab-Tek™ chamber slides (Nunc Thermo Scientific) and  
140 observed daily until they reached a stage of active division. Cells were then harvested using a MultiPrep Genie 205  
141 apparatus (Genial Genetics) according to the recommendations of the manufacturer. After R-banding, twenty  
142 metaphases were captured and analysed. Complementary analysis using fluorescence in situ hybridization (FISH)  
143 was carried out according to standard procedures as described in (27). Slides were analysed with an epifluorescent  
144 microscope Olympus BX61 and images were captured using Isis® software (MetaSystems).

145

146 **Microscopy and image analysis.** Stacks were obtained with a 63x oil immersion objective of a DMRXA (LEICA)  
147 microscope or a Zeiss apotome (63x objective). Measurements of nuclear area and distance between the centroid  
148 of each probe were performed under Image J (<http://rsbweb.nih.gov/ij/>). Distances were determined in 2D, since  
149 pilot experiments did not evidence any qualitative difference between 2D and 3D-FISH experiments (data not  
150 shown). Entire stacks were taken for all selected nuclei (non-mitotic and containing the expected 3 pairs of  
151 hybridization signals), and the three channels (red, green and blue) were isolated using the «DeInterleave» plugin.  
152 Pictures in z (distance of 0.3 µm) containing maximum red or green signal intensities were selected for all channels,  
153 merged and then thresholded to eliminate background from specific signals for distance measures. We used the  
154 DAPI (blue channel) pictures to consistently determine the nucleus area, calculated following the determination of  
155 a threshold fluorescence value corresponding to an entry transition into the nucleus. This value was manually  
156 determined as the inflection point of a profile plotting DAPI signal measured in a 10 pixel large longitudinal window  
157 crossing the nucleus against pixel distance. Images from up to 100 nuclei were analyzed in each experiment.  
158 Significant variations between experimental conditions were tested by a Fisher *t-test* comparison for unpaired data,  
159 with a significance threshold set for p-values ≤0.05. To calculate 3D volumes, we first segmented automatically the  
160 3-dimensional hybridization signals for each of the color channel using the triangle algorithm (28) implemented in  
161 ImageJ. After a cleaning step consisting of the successive application of an opening and closing filter, the 3D-  
162 volumes of the structures resulting from the union of the two segmentation masks were measured and expressed  
163 as voxels. To analyse the kinetic FISH experiment, we had to develop a custom Matlab (MathWorks, Natick,  
164 Massachusetts) image processing routine in order to quantitatively analyze the high number of images. For that,  
165 we used the maximum intensity projections of the 3-dimensional stacks acquired by fluorescence microscopy. The  
166 analysis steps were performed automatically to avoid potential bias associated to manual intervention. The nuclei  
167 were segmented on the DAPI channel using the Otsu approach (29) and a watershed algorithm (30) was applied to  
168 separate touching nuclei. For detecting the fluorescence spots on the images corresponding to each FISH probe, we  
169 used the algorithm developed by Sbalzarini and Koumoutsakos (31). A first filtering step was performed to remove  
170 the very dim spots and those located outside the nuclei. The intensities of the remaining spots were estimated and

171 subtracted to the local background. We only kept the spots whose intensity was exceeding the threshold  $T_I$ , which  
172 was calculated as follows:  $T_I = \langle I \rangle + n \cdot \sigma_I$ , with  $\langle I \rangle$  and  $\sigma_I$ , the mean and the standard deviation of the spot  
173 intensities, respectively, and  $n$  a user-defined integer. On a subset of images, we compared the spots detected by  
174 the automatic analysis and those selected by manual inspection. By setting  $n$  to 5, we optimized the matching  
175 between the automatic and manual selections. The intensity spots corresponding to the two FISH probes were  
176 paired based on a nearest neighbor criterion. FISH pair distances exceeding 20 pixels were not considered. We  
177 noted that our approach was robust towards the choice of the integer  $n$  as similar distance distributions were  
178 obtained for  $3 < n < 7$ .

179

180 **Microarray and mRNA profiling data analyses.** RNAs for microarray analysis were isolated from  $20^7$  MCF7 or  
181 MDA::ER cells treated with E2 or ethanol as vehicle control using the RNeasy Plus Mini Kit (Qiagen) with  
182 homogenization through QIAshredders (Qiagen). Integrity and purity of the RNAs were controlled on an Agilent  
183 Bioanalyzer using the RNA 6000 Nano Assay (Agilent). Ten  $\mu\text{g}$  of selected samples exhibiting a RIN  $>9.8$  were then  
184 subjected to cDNA synthesis using the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen) and a mix of 50  
185 pmol random hexamers and 50 pmol of Oligo-dT. cDNAs were then treated for 10 min at  $37^\circ\text{C}$  with 5  $\mu\text{g}$  RNaseA  
186 (Invitrogen), purified through a phenol:chloroform:isoamyl alcohol extraction on Phase Lock light gels (Fisher  
187 Scientific) and then precipitated. Following a quality control on agarose/BET gels, all subsequent steps (labeling of  
188 cDNA, hybridization and scanning of the arrays) were performed at the NimbleGen service facilities (Rejkyavic,  
189 Island). For each experimental condition, three arrays (NimbleGen Homo sapiens 385K Array) were hybridized with  
190 independently prepared pools of cDNAs synthesized from experimental triplicates (independent experimental and  
191 biological triplicates). Quantile normalization of the data through the RMA algorithm and all primary analyses were  
192 performed using the ArrayStar software suite (DNASTar, Inc.). Data were filtered according to two criteria: i)  
193 expression values greater than the first quartile in all samples of at least one triplicate; and ii) triplicate standard  
194 deviations lower than the third quartile in all triplicates. Experimental groups were compared by analysis of  
195 variances ( $t$ -test) and  $p$ -values were adjusted by the Benjamini and Hochberg method. Genes were considered as  
196 differentially expressed between two experimental conditions when their adjusted  $p$ -value was lower than 0.05  
197 and their fold change greater than 1.8. Estrogen-sensitive clusters were then defined by sliding a window of  
198 variable sizes and counting the number of E2-regulated genes within these windows. The best empirically defined  
199 parameters were to define clusters as regions comprising at least 3 regulated genes within a window of 7 genes.

200

201 **FAIRE assays.** Formaldehyde-assisted isolation of regulatory elements [FAIRE; (32)] methodology was conducted as  
202 previously (23).

203

204 **Chromatin immunoprecipitation.** Cells were washed twice with PBS, and cross-linked during 10 min at room  
205 temperature using 1.5% formaldehyde (Sigma) diluted in PBS. Following a subsequent incubation with 0.125 M

206 glycin for 2 min, the cells were collected in 1 ml collection buffer [100 mM Tris-HCl (pH 9.4) and 100 mM DTT]. Cells  
207 were then washed in 1 ml PBS, lysed for 15 min at room temperature in 300  $\mu$ l of lysis buffer [10 mM EDTA, 50 mM  
208 Tris-HCl (pH 8.0), 1% SDS, 0.5% Empigen BB (Sigma)], and chromatin sonicated during 14 min using a BioRuptor  
209 apparatus (Diagenode), with 30 sec on/off duty cycles. Chromatin was then cleared through a 10 min centrifugation  
210 at 10,000 x g. ChIP experiments were then conducted with some modifications from previous protocol (2) using a  
211 tenth of the chromatin samples (30  $\mu$ l) of the supernatants as inputs, and the remainder diluted 5-fold in IP buffer  
212 [2 mM EDTA, 100 mM NaCl, 20 mM Tris-HCl (pH 8.1), and 0.5% Triton X-100]. A 1/4<sup>th</sup> of this fraction was subjected  
213 to immunoprecipitations overnight after a 3 hr preclearing at 4°C with 10  $\mu$ g yeast tRNA and 80  $\mu$ l of a 50% protein  
214 A-Sepharose bead (Amersham Pharmacia Biosciences) slurry. Complexes were recovered after 3 hr incubation at  
215 4°C with 5  $\mu$ g yeast tRNA and 40  $\mu$ l of protein A-Sepharose. Precipitates were then serially washed, using 300  $\mu$ l of  
216 Washing Buffers (WB) I [2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 0.1% SDS, 1% Triton X-100, 150 mM NaCl], WB II [2  
217 mM EDTA, 20 mM Tris-HCl (pH 8.1), 0.1% SDS, 1% Triton X-100, 500 mM NaCl], WB III [1 mM EDTA, 10 mM Tris-HCl  
218 (pH 8.1), 1% NP-40, 1% Deoxycholate, 0.25 M LiCl] and then twice with 1 mM EDTA, 10 mM Tris-HCl (pH 8.1).  
219 Precipitated complexes were removed from the beads through two sequential incubations with 50  $\mu$ l of 1% SDS,  
220 0.1 M NaHCO<sub>3</sub>. Cross-linking was reversed by an overnight incubation at 65°C. DNA was purified on NucleoSpin™  
221 columns (Macherey-Nagel) using 50  $\mu$ l NTB buffer. Subsequent qPCR analysis used 1  $\mu$ l of input material and 3  $\mu$ l of  
222 ChIP samples.

223

224 **ChIP-on-chip assays and analysis of published datasets.** The ChIP procedure conducted on chromatin prepared  
225 from 15.10<sup>6</sup> cells was similar to the one described above with the following modifications for final steps.  
226 Crosslinking was reversed by an overnight incubation at 65°C with 10  $\mu$ g of Proteinase K (Sigma). Following a  
227 subsequent incubation of the samples with 2.5  $\mu$ g RNAse (Sigma) for 1h at 37°C, the DNA was then purified on  
228 NucleoSpin™ columns (Macherey-Nagel) using NTB buffer and eluted in 40  $\mu$ l of elution buffer. Efficiency of the  
229 ChIP assay was then evaluated using qPCR positive and negative controls. Experimental input and ChIP triplicates  
230 were then pooled by precipitation, resuspended in 25  $\mu$ l H<sub>2</sub>O, and divided in two for amplification using the WGA  
231 whole genome amplification kit (Sigma). Following a quality control step, the amplified material was pooled and  
232 sent to NimbleGen services (Rejkyavic, Island) for hybridization on custom 385K arrays. These arrays were  
233 conceived by spotting genomic regions containing clusters of E2-regulated genes as defined from MDA::ER and  
234 MCF-7 estrogen-sensitive transcriptomes, as well as regions containing individual control cell-specific estrogen-  
235 sensitive genes (*cf.* Table 2). ChIP-chip signals normalization and peak calling steps were performed using the MA2C  
236 algorithm (33) on raw data obtained from two arrays hybridized with DNA prepared in two independent  
237 experiments. MA2C parameters were: robust normalization (C=1) and peak calling for a minimum of 4 probes  
238 (maximum gap set at 400 bp) in a sliding window of 300 bp half-width. All binding sites determined in our ChIP-chip  
239 experiments were confirmed by independent ChIP-qPCR assays, except the CTCFBS identified in the TFF1 promoter  
240 which was found to be antibody- and experiment-dependent. This site was therefore not included in the statistical

241 examinations of our data. Analysis of published Affymetrix tiling arrays data (ER and Pol II ChIP-chips performed in  
242 MCF-7 cells) were analyzed under MAT (34). All genomic annotations were performed using algorithms present  
243 within the cistrome web-platform [http://cistrome.dfci.harvard.edu/ap; (35)]. MCF-7 ChIP-seq data for CTCF and  
244 RAD21 (concatenation of fastq obtained in duplicate experiments) and corresponding input were extracted from  
245 the GSE25710 series and aligned onto indexed chromosomes of genome hg18 using bowtie-0.12.7 (36) with  
246 parameters -p 7, --best, -m 1, -n 1, --sam and -l 28. The .sam files were then converted to .wig files, using samtools-  
247 0.1.12a (37) and MACS-1.3.7.1 (38). To compare RAD21 ChIP-seq datasets obtained in vehicle and estradiol-treated  
248 cells, we adjusted the bias of diverging sequencing depths through a linear normalization (factor of 2.1) of signal  
249 intensities of a given .wig so as to be comparable to the .wig file with the highest sequencing depth. Peak calling  
250 was then performed as previously described (39).

251

252 **Triplex capture experiments.** Triplex capture assays were performed on transfected cells which were subsequently  
253 cross-linked by 2% formaldehyde and lysed by sonication as described above. Sonicated chromatin was then  
254 incubated for 4h with streptavidin-coated magnetic beads (Dyna) that were blocked with 10 µg/ml BSA and 10  
255 µg/ml yeast tRNA for 1h. Captured DNA was eluted by two rounds of elution in 0.1% SDS, purified following  
256 digestion with proteinase K and RNaseA and analyzed by qPCR.

257

258 **Chromosome conformation capture (3C) and circular 3C (4C).** Methods were adapted from (40), and used the  
259 *DpnII* 4-base cutter as an enzyme of choice. Following a 5 min centrifugation at 2,000 x g, aliquots of 2.10<sup>6</sup> cross-  
260 linked cells were washed with 200 µl of 1X *DpnII* buffer, and then lysed overnight at 37°C in 200 µl of 1X *DpnII*  
261 buffer containing 0.3 % SDS with shaking. Following 2 passages through a syringe needle, 400 µl of 1X *DpnII* buffer  
262 were sequentially added in 4 times, and SDS was sequestered with 67 µl of 20 % Triton X-100 at 37°C for 1h. 50 µl  
263 of the reaction mixture were then kept as input fraction for digestion efficiency controls. 550 µl of the chromatin  
264 preparation were then digested overnight with 400 U *DpnII* at 37°C with shaking in a total volume of 500 µl of  
265 digestion buffer containing protease inhibitors (Roche). An additional step of 6h digestion with 150 U *DpnII* was  
266 then performed. 50 µl of the digested chromatin was then kept for digestion efficiency controls, whilst the  
267 remaining was kept at 4°C during this step. To control the digestion efficiency, both input and digested aliquots  
268 were incubated with 9.5% SDS for 20 min at 65°C. Cell fragments were then eliminated by centrifugation at 12,000  
269 x g for 5 min. 117 µl of TE buffer were then added together with 5 µl of 10 µg/µl RNase A (Sigma) and the mixture  
270 was incubated at 37°C for 30 min before the addition of 8 µl of 5M NaCl and 10 µl of 10 µg/µl of Proteinase K  
271 (Sigma). Cross- linking was then reversed overnight at 65°C, and DNA purified on Macherey-Nagel columns. qPCR  
272 were then performed on input and digested fractions to calculate the digestion efficiency as follows: E%=  
273  $[1.9^{(Ct_{input}-Ct_{sample})^{test\ region}}/1.9^{(Ct_{input}-Ct_{sample})^{control\ region}}]*df*100$ , where the dilution factor (df) was =0.98360  
274  $[(50/610)/(50/600)]$ , and the control region a region that contains no *DpnII* fragment. If this % was > 85%, the  
275 remaining digested chromatin was subjected to a final lysis step by addition of 108 µl of 10% SDS and incubation at

276 65°C with shaking for 20 min. Two µg of digested chromatin, as evaluated from the amounts of DNA recovered in  
 277 the digested fraction using a NanoDrop were then incubated for 1h at 37°C with 40 µl of 20 % Triton X-100 in a  
 278 total volume of 694 µl of Ligation buffer (10 mM Tris-HCl pH=7.8; 0.1 µg/µl BSA and protease inhibitors). Ligations  
 279 for 3C or 4C experiments were then performed at 16 °C with gentle agitation for 4h or 5 days respectively. For 3C,  
 280 10 µl of T4 DNA ligase were added in the reaction mixture together with 80 µl of its buffer, 8 µl of 10 µg/µl BSA and  
 281 8 µl of 100 mM ATP (total volume of 800 µl/DNA concentration=2.5 ng/µl). For the 4C samples, requiring a more  
 282 diluted concentration of DNA, 906 µl of H<sub>2</sub>O were first added and then 160 µl of 10X T4 DNA ligase buffer, 16 µl of  
 283 10 µg/µl BSA, 16 µl of 100 mM ATP and 15 µl of T4 DNA ligase (total volume of approx. 1800 µl). The ligation mix  
 284 was replenished at days 2 and 4 with ATP (20 µl of 100 mM ATP). Following these 5 days of ligation, 4C samples  
 285 were further incubated for 1.5h with 1 µl of T4 DNA ligase in order to ensure that the ligase fills any nicks in the  
 286 circularized 3C products. The cross-linking of either 3C or 4C DNA products was then reversed overnight at 65°C  
 287 following the addition of EDTA (1 mM final), NaCl (0.2 M final) and 10 or 20 µl of 10µg/µl proteinase K. Samples  
 288 were then subjected to three successive phenol/chloroform/isoamylalcohol (25:24:1) extractions followed by a  
 289 chloroform washing step, diluted 4 times in water and precipitated at -20°C for 2h by 2 volumes of isopropanol.  
 290 Following centrifugation at 13, 000 x g, samples were then washed 3 times with 1 volume of 75% EtOH and  
 291 resuspended in 50 µl of TE. 3C samples were then processed for analysis. In contrast, the circularized 3C products  
 292 (4C) generated by the 5 day ligation were then purified from linear DNA by a combined digestion with 7 µl of  
 293 exonuclease I and 2 µl of exonuclease III (New England BioLabs) in a total volume of 100 µl of 1X exonuclease I  
 294 buffer. Circular DNA was then purified on Macherey-Nagel columns following a 25 min heating step at 85°C to  
 295 inactivate the enzymes. Elution step was modified, by incubating the DNA bound on the columns with 50 µl of Tris-  
 296 HCl (pH=7.8) containing 20 ng of yRNA for 2h. 4C libraries were then amplified on a thermocycler using High-  
 297 fidelity Taq polymerase (Invitrogen) using the following primers: TMPRSS2 5'-AACATAGTCCTCTTTGGCACA-3' and 5'-  
 298 GTCAGTCTCGGGAGGGACT-3'; RIPK4 5'-TTGGGAGCTCCATCAAGAC-3' and 5'-GCTCCTTCATGGGTTTCATTC-3'; TFF3  
 299 5'-GACCAGGGTGTGGTGTCC-3' and 5'-CAGCTCTGCTGAGGAGTACG-3'; TFF2 5'-CAGACCCTCATCCTCCAGAC-3' and  
 300 5'TATAAAGGCATCTGGCAATGTG-3'; TFF1 5'-GCTACATGGAAGGATTGCTG-3' and 5'-CAGTGGAGATTATTGTCTCAGA-  
 301 3'; TMPRSS3 5'-CATGGCTGCTCTGGGAAC-3' and 5'CCTCGGCTAAGGAGGTAGAG-3'; UBASH3A 5'-  
 302 GTACGGCTTCCTGCCAAA CCGCTGCCATCTTCTCT-3'. Amplification was made following a 30 sec denaturation step  
 303 at 98°C as follows: (98°C 10 sec, 60°C 4 min, 68°C 5 min)x4 and (98°C 5 sec, 60°C 2 min, 68°C 5 min)x34 with a final  
 304 incubation at 68°C for 10 min. In parallel of samples subjected to the whole procedure, additional aliquots of cells  
 305 or of chromatin were processed to generate additional controls: minus cross-linking (entire procedure on cells not  
 306 incubated with formaldehyde), minus ligase (entire procedure but with no ligase in the mix). These samples served  
 307 to control the specificity of the ligation (opportunistic ligation by background proximity: minus cross-linking) and of  
 308 the PCR (minus ligase). The relative frequencies of interactions detected by 3C were calculated as follows:

309 
$$\text{Freq} = [1.9^{(Ct_{\text{control region}} - Ct_{\text{test}})} / \min(E\%_{\text{test}5'}; E\%_{\text{test}3'})] / [1.9^{(Ct_{\text{control region}} - Ct_{\text{pos3Cct}})} / \min(E\%_{\text{control}}; E\%_{\text{tpos3Cct}3'})]$$

310 where the control region amplification (same as for the digestion controls) served to normalize over the inter-  
311 samples variations of DNA amounts, the pos3Ctl a region that was always ligating under any condition (ligation of  
312 two adjacent regions). All values were normalized for their availability for ligation, by taking into account the  
313 minimal (min) digestion efficiency measured for either extremities of the ligated product. As negative controls, we  
314 tested the minus ligase samples and assessed for two negative control interactions which were 1- the ligation of  
315 each fragments of interest with a fragment located within a *GAPDH* exon and 2- a negative region taken from our  
316 4C screenings. We did not normalize the values obtained on test regions over those obtained in these negative  
317 controls as they were generally not producing any amplification signal. However, whenever this had to occur, the  
318 interaction detected with the test region was not taken into account. The 4C interactions were considered as being  
319 only qualitative, due to the amplification step present in the procedure. Calculations made to determine interacting  
320 regions were the same as for the 3C, except that in this case, the amounts of DNA were sufficient to allow further  
321 normalization over negative controls. In this case, the  $Ct_{\text{control region}}$  used was the lower one (maximum interaction)  
322 obtained in the whole set of tested ligation-produced fragments. We considered values which were at least 2-fold  
323 higher than negative control regions as significant. Networks of long-distance chromatin interactions were  
324 generated under Cytoscape (41).

325

326 **Quantitative-PCRs (qPCR) and statistics.** All qPCRs used 1  $\mu\text{M}$  of specific oligonucleotides (Sigma; sequences in  
327 Table S1 in the supplemental material) and were performed on BioRad MyiQ and BioRad CFX96 machines using  
328 BioRad iQ SYBR Green supermix with 50 rounds of amplification followed by determination of melting curves.  
329 Primers for RT-PCR were designed using the QuantPrime design tool (<http://www.quantprime.de/>) (42).  
330 Oligonucleotides for all other type of samples were designed under Primer3 (<http://frodo.wi.mit.edu/primer3/>).  
331 ChIP sample values were normalized in three steps: to inputs ( $\Delta Ct$ ), then to control ChIP samples (beads alone or  
332 anti-H3 ChIP;  $\Delta\Delta Ct$ ) and then to  $\Delta\Delta Ct$  values obtained on control DNA regions. FAIRE values were normalized to a  
333 positive control region (promoter of the *Rplp0* gene). Heatmaps of qPCR data were all generated under Mev (43),  
334 with values that were declared as significant from the control by Wilcoxon or Student t-tests (depending upon the  
335 number of values). To facilitate their reading, only values significantly differing from the control ones were included  
336 within the heatmaps.

337

#### 338 **Microarray data**

339 Dataset were deposited at the NCBI's Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>, (44)) under  
340 the GSE23850 and GSE32132 accession numbers (expression and ChIP-chip data, respectively).

341

## 342 **RESULTS**

343 **Cell-specific transcriptional regulations of the TFF cluster and ERBSs.** To consider processes governing the co-  
344 regulation of co-linear genes by E2, we first characterized the MDA::ER and MCF-7 repertoires of E2-sensitive genes

345 by microarray analyzes. As shown within the Venn diagram in Fig. 1A, the estrogen-sensitive transcriptome of both  
346 cell-types was extremely divergent with only 19 genes in common after 4h of E2 treatment. This is consistent with  
347 previous studies (45-47). We next identified clusters of E2-regulated genes defined as regions comprising at least 3  
348 regulated genes within a window of 7 genes. As a paradigm, we focused here on one cluster located within locus  
349 21q22.3, previously identified in MCF-7 cells (48) and termed TFF (Fig. 1). This choice was guided by the facts that i)  
350 this region includes the prototypical estrogen-responsive gene *TFF1* and that ii) our transcriptome analysis  
351 indicated that this E2-sensitive region include different E2-sensitive genes in MCF-7 and MDA::ER cells, with only 4  
352 genes regulated by E2 in MCF-7 and up to 7 in MDA::ER. This indicated that specific events may influence the  
353 transcriptional response of genes included in this genomic region, and thereby provided the opportunity to address  
354 these mechanisms. Note that we included the *TMPRSS2* gene into our definition of the estrogen-responsive TFF  
355 cluster, since it was found to be regulated by E2 in MDA::ER cells. We first performed RT-qPCR experiments to  
356 confirm these regulations. The results of these assays, illustrated within Fig. 1B showed i) a timely coordinated  
357 regulation of expected genes by E2; and ii) that ER is the main transcription factor responsible for their E2-  
358 responsiveness, since a 24h pre-treatment of the cells with the ER-targeting antiestrogen ICI abrogated E2 actions  
359 (Fig. 1B). Moreover, none of the tested genes were regulated in naïve MDA-MB231 cells (Fig. 1B), further  
360 confirming that the observed genes regulations by E2 strictly relied on the presence of ER.

361 These differing subsets of estrogen-sensitive genes between MCF-7 and MDA::ER cells might reflect the  
362 existence of different ER cistromes in the two cell lines. Hence, we characterized ER binding sites on this genomic  
363 region in MDA::ER cells. ER chromatin immunoprecipitation (ChIP) experiments were performed on chromatin  
364 prepared from MDA::ER cells treated for 50 min with  $10^{-8}$ M E2, with resulting samples hybridized on custom tiling  
365 DNA arrays designed to cover genomic regions containing E2-sensitive clusters (Table 2). These assays identified 17  
366 ERBS within the TFF cluster in MDA::ER cells at a FDR<5% (Fig. 1C). When comparing these data to the ERBSs  
367 determined in MCF-7 cells by ChIP-chip technology [data from Carroll et al. (17)], only three binding sites were  
368 common to both cell lines (in green in Fig. 1C), including the comBS2 located within the *TFF1* promoter. This low  
369 overlap between MDA::ER and MCF-7 ERBSs is consistent with analyses made on the entirety of the genomic  
370 regions spotted on the arrays (Fig. 1D).

371 Independent anti-ER ChIP-qPCR assays confirmed the cell-specificity of these ERBSs. FAIRE (formaldehyde  
372 assisted isolation of regulatory elements) experiments further showed that MDA::ER specific ERBSs exhibited a  
373 condensed chromatin state in MCF-7 cells, and *vice-versa* (Fig. 1E). This observation was also made when  
374 evaluating the enrichment of these regions in canonical histone marks for enhancers (not shown). Thus, the  
375 chromatin condensation state of these genomic sequences confirmed their cell-specific function. Exception to this  
376 observation was made for the MCF-7 ERBS5 which also exhibited an “opened” conformation in MDA::ER and MDA-  
377 MB231 cells. This might illustrate the vicinity of this sequence with an annotated transcript. Recent data has shown  
378 that transcription can occur at enhancers (49-51). This prompted us to assess by ChIP-chip whether the RNA  
379 polymerase II (Pol II) was present on MDA::ER ERBSs. As shown within the Fig. 1F, we indeed found a general

380 enrichment of MDA::ER ERBs in Pol II that further exhibited cell-specificity since it was not observed on sequences  
381 corresponding to MCF7 ERBs. Concordantly, using MCF7 Pol II ChIP chip data from (17) in these analyzes also  
382 showed that the polymerase was enriched on MCF7 ERBs but not MDA::ER ERBs sequences (Fig. 1F).

383 In conclusion, these results altogether show that the studied 2 Mb genomic region that covers the TFF cluster  
384 includes different sets of estrogen-responsive genes and ERBs in MCF-7 and MDA::ER cells. Interestingly, the cell-  
385 specific ERBs were not predominantly found at the proximity of cell-specific E2-regulated genes. In accordance  
386 with the fact that chromatin loops may place the promoter of these cell-specific genes in the vicinity of cell-specific  
387 ERBs (14), this latter observation suggested that the three-dimensional organization of the TFF loci may differ  
388 between the two cell lines.

389

390 **Dynamic spatial reorganization of the TFF cluster upon E2 treatment.** We next envisioned that the coordinated  
391 regulations occurring at the level of the studied genomic region in both MCF-7 and MDA::ER cells (Fig. 1) may  
392 involve an E2-dependent spatial reorganization of this locus. To test this hypothesis, we sought to perform DNA-  
393 FISH experiments. Noteworthy, MDA::ER cells present three fluorescent TFF loci and there were a higher number of  
394 loci (at least 6) in our MCF-7 cell line. This originates from the complex hypertriploid karyotype of these MCF-7 cells  
395 harboring 3 chromosomes 21 and multiple non assignable chromosome parts that contain at least 4 TFF loci (see  
396 Fig. S1 in the supplemental material). As this high number of loci hindered the correct evaluation of the  
397 experimental results, we focused on MDA::ER cells for these specific analyzes. We first conducted experiments  
398 aiming to evaluate the spatial volume occupied by the 2 Mb genomic region encompassing the TFF cluster and thus  
399 performed DNA-FISH with a mix of fluorescent probes generated from multiple BACs covering a large part of the  
400 TFF region (Fig. 2A). The results of these experiments led us to evidence a compaction of the chromatin domain  
401 containing the TFF cluster after a 50 min treatment with E2 (Fig. 2B). This could be assigned to a smaller dispersion  
402 of the distances separating central (B5) and 5' (B1) probes following exposure to E2 (Fig. 2C). Interestingly, whilst  
403 there were no changes in the distribution of distances between probes generated using the central and the 3' BACs  
404 (B6), the distribution of the distances separating probes located at both extremities of the TFF region were again  
405 significantly different. Furthermore, quantile-quantile representations of data (Q-Q plots, Fig. 2C, bottom) indicated  
406 that observed changes mostly reflected a disappearance of large distances separating paired probes. Importantly,  
407 these variations were not an indirect consequence of a global reduction in nucleus volume upon E2 exposure (not  
408 shown). The 2 Mb region containing the TFF cluster thus undergoes spatial rearrangements under E2 treatment in  
409 MDA::ER cells, reflected by a more constrained three-dimensional conformation.

410 To gain further insights into how the cell-specific transcriptional activity of the TFF cluster was spatially  
411 organized, we characterized the spatial proximity of ERBs with the promoters of E2-regulated genes. We conducted  
412 4C-qPCR on chromatin prepared from MCF-7, MDA::ER as well as MDA-MB231 cells all treated for 50 min with E2.  
413 Results of these experiments (Fig. S2 and Fig.S3) are schematized in Fig. 3A. All determined spatial proximities are  
414 given within the Table S2. These 4C assays recovered all but one of the interactions previously determined by ChiA-

415 PET (15) as linking ERBSs to regulated promoters in MCF-7 cells, and further uncovered 38 new interactions (Fig.  
416 3B), in agreement with the differing outcome of 4C and ChiA-PET techniques. These experiments indicated that  
417 major spatial constraints of this chromatin domain involve interactions between central and distant regions mainly  
418 located 5' of the TFF cluster (approx. 70% of the ERBS-promoters interactions) in both MCF-7 and MDA::ER cells.  
419 This is consistent with results obtained in DNA-FISH experiments. Interestingly, the *RIPK4* promoter was situated in  
420 the spatial vicinity of more ERBSs in MDA::ER than in MCF-7 cells (Fig. 3A and Fig. 3C). This might be correlated with  
421 the estrogenic regulation of this gene in the former cell line but not in the latter (Fig. 1). However, there was no  
422 strict correlation between the numbers of ERBS-promoter interactions and the amplitude of the estrogenic  
423 regulation of the gene, as evaluated by the RT-qPCR results from Fig. 1 (maximum  $R^2$  of 0.22 and 0.51 observed  
424 following 2h of E2 treatment, in MCF-7 and MDA::ER cells, respectively). Importantly, 27% of the interactions  
425 detected in MDA::ER were also detected in ER negative MDA-MB231. This indicates that some of the MDA::ER  
426 ERBSs may be pre-existing enhancers that recruit additional transcription factors regulating the activity of all  
427 considered genes, with the exception of *TFF3*, as it did not establish any contact with the tested regions in MDA-  
428 MB231 cells (Fig. 3A and Fig. 3C). Conversely, since the remaining 73% of the interactions detected in MDA::ER  
429 were not detected in MDA-MB231, these results also demonstrate that the expression of ER in this system is  
430 sufficient, either directly or not, to remodel the spatial organization of this genomic region.

431 As illustrated within Fig. 3D and Fig. 3E, the MCF-7 and MDA::ER network of interactions between ERBSs and  
432 promoters of regulated genes is complex. One striking difference between these interactomes detected by 4C is  
433 that there are more singleton interactions in MCF-7 than in MDA::ER. Indeed, while 6 ERBSs were interacting with  
434 one single gene in MCF-7, there was only one of these exclusive contacts in MDA::ER cells (the BS15/*TFF3*  
435 association). Finally, this interactome study also showed that the ERBS located within the *TFF1* promoter (comBS2)  
436 directs more interactions than the others in both cell lines, suggesting that it plays a crucial role in the chromatin  
437 re-organization of the TFF cluster in response to E2.

438

439 **CTCF and cohesin are required for appropriate regulations and organization of the TFF cluster.** ER has been  
440 proven to modulate the frequencies of interactions between distant ERBSs and promoters of either up- or down-  
441 regulated genes (15, 52), in concert with CTCF and/or the cohesin complex (53). Hence, to better understand how  
442 E2 signaling impacts on chromatin organization, we mapped CTCF and RAD21 (a subunit of the cohesin complex)  
443 binding sites in MDA::ER through ChIP-chip analysis (Fig. 4A). The comparison of all the MDA::ER CTCFBSs and  
444 RAD21BSs identified on all the genomic regions spotted on our arrays with those previously determined in MCF-7  
445 cells (53; restricted here to BSs contained within the spotted regions) indicated that most CTCFBSs were conserved  
446 between the two cell types on the contrary to RAD21BSs (Fig. 4B). This cell-specific RAD21 cistrome may represent  
447 a major source for the different E2-responses of the clustered TFF genes. On the other hand, the overlap between  
448 the BSs for ER, CTCF and RAD21 was much more important in MCF-7 cells than in MDA::ER within the TFF cluster  
449 (Fig. 4C). These differing ER/CTCF/RAD21 BS overlaps between MCF-7 and MDA::ER were reflected in the

450 proportions of established contacts between ERBS and genes promoters (Fig. 4D). These data also indicated that  
451 the presence of CTCF and/or RAD21 at ERBSs does not correlate with the number of interactions made with gene  
452 promoters in the context of the TFF cluster (Chi2 and Fisher tests  $p$ -values>0.4). In agreement with this observation,  
453 we did not observe any significant increase in CTCF and RAD21 binding on respective ERBSs following the addition  
454 of E2 (Fig. 4E). This may signify that the dynamic modulations of the TFF three-dimensional organization that occurs  
455 upon E2 treatment involve other RAD21/CTCF sites. Alternatively, the physical contacts established between the  
456 ERBSs and the promoters may be directed by ER and occurring within a structurally fixed frame imposed by CTCF  
457 and/or RAD21. In favor of the latter hypothesis, the alignment of RAD21 ChIP-seq reads obtained in MCF-7 [data  
458 from (53)] on ER, CTCF and RAD21 shared binding sites within the TFF cluster did not evidence any significant  
459 change in RAD21 mobilization on ER/CTCF/RAD21, the sole ER/RAD21 BS and on CTCF/RAD21 BSs ( $p$ -value 0.0676  
460 for this latter category of sites) (Fig. 4F).

461 The putative role of CTCF and RAD21 in establishing the spatial conformation of the TFF cluster genomic domain  
462 was next examined following the transfection of siRNAs targeting their expression. Control RT-qPCR and Western  
463 blots performed in MDA::ER cells are shown in Fig. 5A and 5B, with similar reductions observed in MCF-7 cells (not  
464 shown). We first performed 3C-qPCR experiments to evaluate the impact of these siRNA-mediated reductions in  
465 CTCF and RAD21 intracellular amounts on the frequencies of interaction between RAD21 and/or CTCF positive  
466 ERBSs and the promoters of E2-regulated genes. These experiments, summarized in Fig. 5C showed that the  
467 silencing of RAD21 diminished the frequency of interactions between ERBSs and their target gene promoters in  
468 both MDA::ER and MCF-7 cells. This was also observed following the transfection of siRNAs targeting CTCF,  
469 although to a lesser extent (Fig. 5C). DNA-FISH experiments further showed that RAD21 is essential for the global  
470 E2-induced constraints exerted on the TFF three-dimensional conformation (Fig. 5D). Unfortunately, the  
471 involvement of CTCF could not be addressed here due to its inefficient depletion ( $\approx$ 25%) in this particular  
472 experimental setup. Finally, disrupting CTCF and RAD21 expression by siRNAs drastically reduced both basal and  
473 induced transcriptional activity of E2-regulated genes in both cell lines (Fig. 5E). This ultimately led to a strong  
474 decrease of their fold inductions by E2 except for *TFF3* and *RIPK4* in MDA::ER cells and *UBASH3A* in MCF-7 cells.

475 Altogether, these data indicate that cohesin and CTCF organize the E2-responsiveness of the genes included in  
476 the TFF cluster, in both cell lines, possibly by promoting a three-dimensional organization of the studied genomic  
477 locus which is propitious for the interaction between distant ERBS and promoters of activated genes.

478

479 **Dynamic 3D organization of the TFF cluster.** We questioned next whether one or a limited number of ERBSs within  
480 the TFF cluster could orchestrate the observed coordinated genes regulations through long-range interactions. This  
481 hypothesis would imply that one -or a few - ERBS is brought nearby promoters in a dynamic manner compatible  
482 with these transcriptional responses to E2. Hence, we evaluated by 3C-qPCR the dynamics of the interactions  
483 between ERBSs and genes promoters following treatment of the cells with E2. These data are summarized within  
484 Fig. 6 and Fig. 7; with circle areas being directly proportional to the fold changes in the relative frequency of

485 interactions as compared to the initial situation (t0) (MCF-7 data are presented in Fig. S4). In x are the coordinates  
 486 for the promoters of the genes and in y-axis are the coordinates for the ERBSs, whose positions are indicated.  
 487 Importantly, all of the ERBS-promoter interactions were detected at t0 (MCF-7 controls in Fig. S4). In both cell  
 488 types, a spatial reorganization was apparent as soon as 10 min following E2 addition with both increases and  
 489 decreases in frequencies of interaction (Fig. 6 and Fig. 7). The existence of several TFF loci in both cell types may  
 490 influence the interpretation of these kinetic 3C data, since we cannot ascertain that all of the interactions are  
 491 occurring on the same genomic fragment. However, supporting the existence of dynamic variations in the three-  
 492 dimensional organization of the TFF genomic region occur, we were able to further evidence such processes by  
 493 DNA-FISH time-course experiments in MDA::ER cells (see Fig. S5). Some of the reorganizations evidenced by 3C-  
 494 qPCR exhibited a relatively dynamic or even cyclical nature, such as those highlighted in orange within Fig. 6 and  
 495 Fig. 7 subpanels. Interestingly, the dynamics of these spatial reorganizations differed between MDA::ER and MCF-7  
 496 cells. In MCF-7 cells, these variations were restrained to short-range interactions except for *TFF1/BS1*, *TFF1/BS18*  
 497 and *TMPRSS3/BS8* contacts (Fig. 6). In contrast, the dynamic MDA::ER interactome highlighted important variations  
 498 in long-range interactions between ERBSs and the promoters of regulated genes. This is notably illustrated by the  
 499 interactions made by the ERBSs located in the 5' region of the cluster that climax at 50 min following the addition  
 500 of E2 (Fig. 7). Furthermore, in MDA::ER cells, an apparent combination of local (BS2 and comBS1) and long-range  
 501 interactions (comBS2 and even comBS3 located in the far 3' of the genomic region) could be correlated with the E2-  
 502 mediated regulations of *TMPRSS2* and *RIPK4*. In contrast to what happens in MCF-7, the regulations of core TFF  
 503 genes would thus be more influenced by distal than by local ERBSs in MDA::ER cells. Indeed, variations of the  
 504 interactions made between ERBSs located within the *TFF3* to *TMPRSS3* (core TFF cluster) region appear less  
 505 important than what was observed in the case of MCF-7 cells.

506 In conclusion, these data indicate that there is no single major ERBS that organizes E2 responsiveness within this  
 507 genomic region. Keeping in mind that 3C-based assays have the intrinsic limitation to be unable to ascertain the co-  
 508 occurrence of detected interactions in the same cell, we propose that the coordination of the transcriptional  
 509 response of the TFF cluster to E2 mainly originates from a combinatorial engagement of ERBSs located within the  
 510 *TFF1* promoter in MCF-7 cells with two nearby ones (BS14 or BS16), and with those located in the distant 5' region  
 511 of the cluster in MDA::ER cells.

512

513 **E2 regulation of a given gene is driven by different ERBSs.** Although highly informative, the above 3C data did not  
 514 allow us to establish the exact contribution of each ERBS toward the specific regulation of the genes that they  
 515 contact. To investigate at the molecular level the contribution of given ERBS in regulating specific genes, we used  
 516 small triplex forming oligonucleotides (TFOs) to interfere with ER binding at a given BS (Fig. 8A). Such  
 517 oligonucleotides that form Hoogsteen or reverse Hoogsteen hydrogen bonds with the purine-rich strand of DNA  
 518 have already been used to inhibit the transcription of genes such as *ets2* (54) or *c-myc* (55) [reviewed in (56, 57)].  
 519 We characterized 11 TFOs (Table 1) which were able *i*) to form DNA triplex *in vitro* (TFO anti-MCF-7 BS1 as example

520 in Fig. 8B, otherwise see Fig. S6); *ii*) to specifically bind to target sequences but not *PKNOX1* promoter used here as  
521 a control (Fig. 8C and Fig. S6) and *iii*) to significantly disrupt ER binding on the corresponding ERBS (Fig. 8D and Fig.  
522 S6). Although all of the designed TFOs did not precisely target the center of each ERBS defined here as from the  
523 ChIP-chip profiles, we observed that their relative efficiency in disrupting ER binding was only mildly correlated to  
524 the distance separating the TFO target sequence to the center of the ERBS peak (Fig. 8E). The ability of TFOs to bind  
525 to their target sequence seemed also relatively independent of their chromatin status as evaluated from their  
526 relative enrichment by FAIRE (Fig. 8F).

527 Subsequent RT-qPCR experiments showed that in a majority of tested cases, decreasing ER binding on one ERBS  
528 did impact the transcriptional status of the genes to which it was spatially close (Fig. 8G), but not on control genes  
529 (Fig. S7). However, the converse was also observed, with no observable impact of ER binding disruption, as for  
530 instance the associations between the MCF-7 BS4 and *UBASH3A*, BS6 and *TMPRSS3* or MDA::ER BS10 and  
531 *UBASH3A*. This seems to imply that the binding of ER on some distant sites might not be essential for the regulation  
532 of the analyzed genes. Alternatively, this could also indicate a functional redundancy between the enhancers  
533 controlling the activity of the tested E2-sensitive genes. Abrogating ER binding on such site would be compensated  
534 for by the activity of the others. Additionally, the reduced ER mobilization provoked by TFOs diminished the  
535 frequencies of interactions linking the targeted ERBS with the promoters of their target genes (data not shown).  
536 Interestingly, these experiments also evidenced that decreasing ER binding on some sites affected the  
537 transcriptional status of genes that they did not contact. This was for instance observed in MCF-7 cells for the BS1  
538 on *TFF3* levels, comBS1 on *TMPRSS2*, BS10 on *TFF2*, BS14 on *TMPRSS2* and *TFF3*. And this was also true in MDA::ER  
539 for the BS10 on *RIPK4* and BS12 on *TMPRSS2*, *RIPK4* and *TFF3* amounts in MDA::ER. As shown by Fullwood et al.  
540 (15) in ChiA-PET assays, the three-dimensional organization of chromatin can place distant ERBSs in spatial vicinity.  
541 We therefore evaluated whether targeting the recruitment of ER on one ERBS by a specific TFO could reduce its  
542 mobilization on another ERBS (Fig. S8). Results from these experiments indicated that the observed transcriptional  
543 “collateral” effects were due -at least in part- to the establishment of additional interactions between ERBSs  
544 themselves. For instance, the reduction of *RIPK4* and *TFF3* expressions by the TFO targeting the ERBS12 in MDA::ER  
545 cells could reflect a contact made between this ERBS12 and the ERBS16 that controls these genes.

546 The use of TFOs therefore allowed us to demonstrate the functional relevance of the interactions linking ERBSs  
547 to E2-regulated promoters we have characterized. Although limited to the ERBSs on which TFOs were able to  
548 disrupt ER recruitment, these data illustrate that the comERBS2 located in the close vicinity of the *TFF1* promoter  
549 plays a central regulating role in both cell types. They also clearly indicate that the MDA::ER ERBS1 and MCF-7  
550 ERBS6 play prominent roles in the transcriptional activity of the genes included in the TFF locus.

551

## 552 DISCUSSION

553 We investigated here molecular processes allowing estradiol to co-regulate the transcriptional activity of genes  
554 clustered within a 2 Mb genomic region. Using a naturally E2-responsive breast cancer cell line (MCF-7) and a

555 cellular system with a forced E2-sensitivity (MDA::ER) we interrogated whether these mechanisms could be triggered  
556 by ER on its own. Despite different chromatin contexts, ER was found to drive tight regulations of the TFF cluster in  
557 both cell types, relayed by its mobilization on distinct genomic regions. Interestingly, the chromatin status of  
558 MDA::ER ERBSs in native MDA-MB231 cells indicated that a number of ERBSs were already exhibiting an opened  
559 conformation and characteristics of functional enhancers: enrichment in FAIRE experiments (Fig. 1), presence of  
560 marks for poised or active chromatin (unpublished observations) and spatial vicinity with the promoters of  
561 considered genes (Fig. 3). Preparation of chromatin to ER binding in MCF-7 cells involves the actions of the pioneer  
562 factor FOXA1 (17, 18) which is not expressed in MDA::ER cells. Accordingly, MCF-7 specific ERBSs are all but four  
563 FOXA1 positive. By contrast, ERBSs in MDA::ER were principally not located at sites bound by FOXA1 in MCF-7,  
564 except the common ERBS2 and ERBS3 and ERBS13. Hence, it may be that other factors act in a similar way than  
565 FOXA1 in the MDA system, or that ER acts on its own. Motifs analysis performed on the entire set of MDA::ER  
566 ERBSs identified in our ChIP-chip data indicated that in addition to ERE motifs, GATA sites were also significantly  
567 enriched (not shown). This is consistent with reports showing that factors of this family, and in particular GATA3  
568 (47,58,59), are controlling ER activity.

569 4C and 3C assays allowed the description of dynamic interactomes linking ERBSs to promoters of genes. Some  
570 of the detected interactions, however, engaged promoters of genes that were not regulated by E2 in our RT-qPCR  
571 or transcriptomic data, such as *TMPRSS2*, *RIPK4* or *TFF3* in MCF-7 cells. It is possible that these genes actually  
572 exhibit rapid transcriptional responses to estrogen as those evidenced by global run-on assays [GRO-seq (49-51)]  
573 that we would have missed in our analyses for sensitivity and timeliness reasons. In contrast with what would have  
574 been expected from an artificial cellular model as compared to a more “natural” one like MCF-7 cells, we found  
575 that the ER-mediated three-dimensional re-organization of the TFF cluster response to E2 is more important and  
576 more intricate in MDA::ER cells. Indeed, there were more singleton interactions in MCF-7 cells and the dynamics of  
577 the MCF-7 interactome following treatment with E2 was apparently lower than in MDA::ER cells. It could be  
578 hypothesized that the chromatin three-dimensional structure of this whole genomic region is already prepared for  
579 a response to E2 in MCF-7 cells, in contrast to the reconstituted E2-sensitive cellular model provided by MDA::ER  
580 cells. If true, this implies that, in MDA::ER cells, ER on its own is able to provoke important three-dimensional  
581 remodeling of the TFF locus to finely tune the transcription of target genes. Alternatively, the differing level of  
582 ploidy of our model cells (3 TFF loci in MDA::ER cells vs. 6 in MCF-7) may also impact the interpretation of the  
583 differences observed between both cell types. For instance, the presence of inactive or E2-insensitive loci may  
584 hamper and reduce the variations observed in either cell line. Unfortunately, we were unable to ascertain by RNA-  
585 FISH that all loci were transcribed and regulated in these cells, presumably due to the small size of the TFF genes.  
586 However, E2-induced variations of the three-dimensional organization of the studied genomic region were  
587 observed in each of the 3 loci in MDA::ER cells. This suggests that all 3 loci may transcriptionally respond to E2 in  
588 this cell type.

589 The regulatory unit that integrates the TFF cluster is of ~1 and ~2 Mb in size in MCF-7 and MDA::ER cells,

590 respectively. These dimensions are coherent with those defined for Topologically Associating Domains (TADs) from  
591 Hi-C and 5C data (60-62). Hence, the different number of estrogen-sensitive genes between MDA::ER and MCF-7  
592 cells could characterize the existence of cell-specific TAD geometries and differing boundaries. CTCF and  
593 RAD21/cohesin have been proposed to delineate regions of correlated transcriptional regulations (63-65), even if  
594 their presence might not systematically reflect a demarcation between insulated gene domains (66). Our data  
595 extend observations made in MCF-7 cells regarding the involvement of CTCF and RAD21 in the establishment of key  
596 connections between distant ERBSs and regulated promoters of the TFF cluster (53, 67). We further showed that  
597 RAD21 is required for the proper folding of this genomic region and its response to estrogen. Studies in MCF-7 or  
598 mice liver (53, 68) suggested that the main part of RAD21 actions on tissue-specific expression or estrogenic  
599 regulations would be CTCF independent. Accordingly, the limited overlap of RAD21 binding sites in MCF-7 and  
600 MDA::ER cells indicates that the MDA::ER specific cistrome of RAD21 engages cell-specific functions. Whether the  
601 cell-specific interactomes between ERBSs and gene promoters and the cell-specific size of the putative TFF TAD are  
602 directly linked to the differing RAD21 cistromes between MCF-7 and MDA::ER cells still remains an open question.  
603 One possible way to address this problematic would be to define the chromatin loops established between RAD21  
604 BSs by ChiA-PET experiments in both cell lines.

605         Contrasting with the slight increase reported at the genome-scale by others (50, 51, 53), RAD21 recruitment to  
606 ERBSs in the TFF cluster was not significantly affected by E2. Whether this situation reflects an exception or a  
607 general behavior for clustered genes may constitute an interesting point to pursue the analysis of RAD21 role in  
608 organizing chromatin domains. On the other hand, our observations are coherent with a model in which active  
609 chromatin compartments are organized through constitutive loci (63-65). The characterization of physical contacts  
610 between RAD21 BSs through ChiA-PET may help in defining the cohesin complexes that organize such  
611 compartments where dynamic contacts between enhancers and genes would occur [as proposed in (69)]. The  
612 nature of the mechanisms that underlie the E2-mediated remodeling of the TFF domain may therefore be directly  
613 or indirectly under the sole control of ER or of the estrogenic response of the genes. For instance, Mediator, a  
614 protein complex loaded on active promoters can establish physical contacts between gene and promoters (70). In  
615 accordance with models of proximity ligation proposed by Gavrilov and coworkers (69), the recruitment of multiple  
616 proteins provoked by ER on its sites and its affinity with components of the transcriptional machinery would  
617 stabilize interactions that occur otherwise. It can also be inferred from this hypothesis that mobilization dynamics  
618 of the proteins recruited by ER on chromatin (7, 8) may at least partly be responsible for the dynamic property of  
619 ERBS-promoters physical contacts. Such a process was evidenced in the case of the *CDKN1A* gene promoter placed  
620 under the transcriptional control of another nuclear receptor, VDR (vitamin D3 receptor) (71). Accordingly, our  
621 kinetic 3C dissection of the three-dimensional reorganization of the TFF cluster indicates that all interactions  
622 between ERBSs and gene promoters do exist already in the absence of E2. Hormone and ER binding would then  
623 have to be considered as signals that remodel pre-existing conformations; a conclusion that seems to emerge from  
624 recent Hi-C data, which compared the global organization of MCF-7 chromatin in the absence of hormone and

625 following 50 min of treatment (72).

626 A general problematic that emerged from genome-wide studies is the evidence that the number of interactions  
627 made between the BSs of a transcription factor and genes promoters are not systematically reflected at the  
628 transcriptional level (73). The use of TFOs allowed us to give further indications on how binding sites for a TF are  
629 mobilized in space and time in order to regulate the transcription of its target genes. Although already  
630 hypothesized (14, 17, 74), we demonstrate here the validity of the concept of functional redundancy between ER-  
631 bound enhancers. Indeed, a single promoter can establish contacts with several ERBSs, and we demonstrated that  
632 the resulting buildout might be in some cases insensitive to a particular ERBS inactivation, providing robustness to  
633 the regulatory system. In other cases, the mobilization of ER on distinct master regulatory regions appears  
634 sufficient to provoke the transcriptional response of the gene. Comparing the enrichments of main vs. secondary  
635 ERBSs in particular histone modifications, ER, Pol II, CTCF or RAD21 proteins, or even their relative frequencies of  
636 interaction with their target promoters, did not evidence a particular segregating characteristic. The differential  
637 recruitment of FOXA1, PBX1, GATA proteins or yet uncharacterized cofactors may account for the specific use of a  
638 given ERBS or the collaborative use of several ERBSs. Alternatively, the existence of a particular master regulatory  
639 region may originate from a coincident propitious folding of local chromatin due to high-order organization and the  
640 presence of cognate DNA sequences mobilizing ER. Finally, our dynamic 3C experimental data taken together with  
641 results obtained following transfection of TFOs also indicate that the role of one redundant enhancer towards that  
642 of another one may depend or shift over time or upon experimental condition. The latter was for instance  
643 suggested from data comparing the recruitment of ER when activated by EGF and E2 (75).

644 Although limited by the target sequence requirements, the nucleic acid composition of 2 to 7% of the ERBSs  
645 identified by ChIP-seq appears compatible for the binding of TFOs. Extending the experimental workflow used in  
646 this study to this whole sub-population of ERBSs would give rise to a functionalized partial interactome that could  
647 greatly enhance our knowledge of the links existing between the functions of enhancers and the organization of  
648 the genome. Furthermore, as for ER, BSs for many transcription factors have been shown to be grouped around  
649 responsive genes in clusters of enhancers (76). Hence TFOs or modified TFOs with increased efficiencies such as  
650 PNAs (peptide nucleic acids) (77), LNAs (locked nucleic acids) (78) or bisLNAs (79) may constitute powerful  
651 molecular tools to assess for the generality of enhancer redundancy.

652

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 851

852 **FIGURE LEGENDS**

853 **FIG 1** Cell-specific E2-sensitive genes and ER binding sites in a 2 Mb genomic region including the TFF locus. (A)  
 854 Venn diagram illustrating the overlap of identified E2-sensitive genes in MDA::ER and MCF-7 cells. (B) Heatmap  
 855 representation of RT-qPCR results obtained on RNAs prepared from MCF-7, MDA::ER and MDA-MB231 cells treated  
 856 for the indicated times with  $10^{-8}$  M E2 and pre-treated for 36h with  $10^{-6}$  M ICI164,384 where precised. Results are  
 857 the log2 of the fold inductions of gene expression levels by E2 obtained in two independent triplicate experiments.  
 858 (C) Integrated genome browser [IGB; (80)] illustration of the studied genomic region with RefSeq genes indicated.  
 859 ER binding signal obtained in an ER ChIP-chip analysis performed using chromatin of MDA::ER cells treated for 50  
 860 min with E2 is depicted in gray. MCF-7 data were obtained from published dataset (17). For the sake of clarity, only  
 861 the highest 5% signals are shown. Grey and red boxes delineate cell-specific ER binding sites (ERBSs) whilst  
 862 common ERBSs are in green. (D) Anti ER ChIP and FAIRE assays were conducted using chromatin prepared from  
 863 MCF-7, MDA-MB231 or MDA::ER cells treated with E2 or ethanol (EtOH) as vehicle control for 50 min. Results  
 864 shown within heatmaps are means from 6 to 9 values obtained in independent triplicate experiments. Values are  
 865 fold enrichments over control samples and a negative control region (promoter of the transcriptionally active *Rplp0*  
 866 gene). (E) Overlap of MDA::ER ERBSs with MCF-7 ones on regions spotted on the arrays. (F) Enrichment signals  
 867 obtained for anti-RNA Polymerase II (Pol II) ChIP-chip experiments performed in MDA::ER cells treated for 50 min  
 868 with E2 [MCF-7 data from (17)] were aligned on MCF-7 or MDA::ER ERBS identified within the regions spotted on  
 869 the arrays and located more than 10 Kbp away from the TSS of any annotated gene.

870

871 **FIG 2** E2 provokes a three-dimensional reorganization of the TFF cluster in MDA::ER cells. (A) Locations of the BACs  
 872 (B1 to B6 referring respectively to RP11-814F13, CTD-2337B13, RP11-35C4, CTD-260o11, RP11-113F1 and CTD-  
 873 1033M14) used to generate fluorescent probes for FISH experiments all along the genomic region of interest,  
 874 illustrated as in Figure 1. Positions of the ERBSs are also indicated. (B) Analysis of the 3D volume of the TFF loci by  
 875 DNA-FISH using a mix of all generated probes in cells treated with  $10^{-8}$  M E2 or ethanol (EtOH) for 50 min.  
 876 Representative pictures of these assays are on the left side of the panel, with magnified views of the three TFF loci  
 877 present in MDA:ER cells. Quantitative measurements (n=416) of the 3D volume of the loci is shown at the right side  
 878 of the panel with indicated Fisher *t*-test *p*-value. (C) Quantitative analysis of the distribution of distances separating  
 879 indicated paired FISH probes. Values are shown within boxplots (top of the panel) or within quantile-quantile (Q-Q)  
 880 plots representation of conditionally ranked measured distances (bottom). The normal distribution expected from  
 881 non-variating distances is illustrated by the straight black line in each Q-Q plots. Non-parametric Fisher test *p*-  
 882 values are indicated when determined as significant, as well as the number of measurements made in at least three  
 883 independent experiments.

884

885 **FIG 3** ERBS-promoters interactomes. (A) IGB visualization of MCF-7, MDA-MB231 and MDA::ER interactomes linking  
 886 *DpnII* fragments encompassing ERBS or promoter regions of indicated genes, as detected by 4C-qPCR on chromatin

887 prepared from cells treated for 50 min with  $10^{-8}$  M E2. Shown are RefSeq genes coordinates along chr21, as well as  
888 the positions of ERBS with grey, red and green boxes delineating MDA::ER, MCF-7 or common ERBS. 4C data are  
889 represented as lines linking one ERBS to its target promoters. MCF-7 interactions are in red, MDA::ER ones in grey  
890 and those shared between MDA::ER and MDA-MB231 in orange. (B) Venn diagrams depicting the overlapping  
891 interactions characterized in this study and to those identified in published ER ChiA-PET dataset (15) restricted to  
892 loops involving the gene promoters that served here as 4C baits. (C) Stacked histograms illustrating the overlap of  
893 the 4C-detected interactions for each tested promoters in the different cell lines. (D and E) Cytoscape (41) circular  
894 layouts of the networks of interactions that link E2-regulated genes to ERBS in MCF-7 (D) and in MDA::ER (E) cells.  
895 The sizes of the nodes are directly related to the number of interactions they direct.

896

897 **FIG 4** CTCF and cohesin recruitment on ERBSs. (A) MDA::ER anti-ER, CTCF or RAD21 ChIP-Seq or ChIP-chip signals  
898 visualized under IGB as in Figure 1. All data were obtained from cells treated with  $10^{-8}$  M E2 for 50 min. (B) Overlaps  
899 on array-spotted regions between RAD21 or CTCF binding sites determined in this study in MDA::ER cells and those  
900 previously determined in MCF-7 (53). (C) Overlap of RAD21 and CTCF binding sites with ERBSs. (D) Repartition of  
901 CTCF and RAD21 BS within the interactions between ERBS and gene promoters identified by 4C. (E) CTCF and  
902 RAD21 ChIP-qPCR were performed on chromatin prepared from MCF-7 or MDA::ER cells treated for 4h with  $10^{-8}$ M  
903 E2 or ethanol (EtOH) as vehicle control. Fold enrichment of the precipitated proteins on tested sequences was  
904 normalized over control ChIP and negative region (*PKNOX1* promoter) values. Data shown are mean values  $\pm$  SD  
905 obtained in three independent triplicate experiments. The line depicts the significance threshold applied (Fold  
906 enrichment>2). (F) Boxplots of MACS normalized reads of RAD21 ChIP-seq experiments performed in the absence  
907 or presence of E2 in MCF-7 cells [dataset from (53)]. Values shown are mean counts measured in a 500 bp window  
908 centered on indicated binding sites located within the 2 Mb genome region studied. Calculations were also made  
909 on random sites of similar mean size and in equal number than the shared CTCF/RAD21 BSs.

910

911 **FIG 5** CTCF and cohesin organize the E2-responsiveness of the TFF cluster. (A) Amounts of indicated mRNA were  
912 evaluated in untreated MDA::ER cells following 72h of transfection with either control siRNA (directed against the  
913 Luciferase gene, siLuc) or siRNAs targeting mRNAs of interest. Results shown are mean data  $\pm$  SEM of values  
914 obtained in at least 5 different experiments (n ranges from 15 to 18), normalized to the expression of control  
915 *PKNOX1* gene. Wilcoxon test was used to identify statistically relevant variations from control siLuc/EtOH condition:  
916 p-value <0.001 (\*\*\*), <0.01 (\*\*). (B) Western blots assaying the expression of RAD21, CTCF, ER and hCAPD2  
917 (loading control) in cells transfected with the indicated siRNAs. (C) 3C-qPCR experiments following the impact of  
918 the reduction in CTCF and RAD21 contents on the interactions between indicated ERBS and gene promoters.  
919 Results shown originate from one representative experiment out of two and are expressed as the log<sub>2</sub> of values  
920 normalized to those obtained in the control siLuc condition. (D) Quantitative DNA-FISH analysis of the distribution  
921 of distances separating paired fluorescent probes in transfected MDA::ER cells. Results are illustrated as in Figure 3.

922 (E) Heatmaps summarizing RT-qPCR results obtained in at least 3 independent triplicate experiments following the  
 923 transfection of control siLuc or siRNAs directed against CTCF or RAD21 expression and a 4h treatment of the cells  
 924 with  $10^{-8}$  M E2 or EtOH as vehicle control. Expression levels were normalized to the *PKNOX1* internal control and  
 925 reported to those calculated for the siLuc EtOH condition. The fold inductions of these genes expression by E2 are  
 926 also shown on the right side of the panel as relative to those measured in cells transfected by the siLuc. Results  
 927 originate from at least 4 independent triplicate experiments.

928

929 **FIG 6** Dynamic three-dimensional reorganization of the studied genomic region in MCF-7 cells. Summary of one 3C  
 930 experiment representative from two, performed on chromatin sampled from MCF-7 cells treated from 0 to 80  
 931 minutes with  $10^{-8}$  M E2. As indicated, the size of the bubble that corresponds to one interaction is proportional to  
 932 the fold changes in frequencies of interaction as compared to the basal (t0) situation. The location of the gene  
 933 promoters that served as anchors is illustrated on the top of each subpanel and the ERBSs on the left. Distance  
 934 scale is accurate (2 Mb between ticks) but had to be broken in some instances for sake of figure size and clarity.  
 935 Bubbles highlighted in orange are those commented in the main text and those in yellow correspond to  
 936 interactions made by the ERBS located within the *TFF1* promoter (comERBS2).

937

938 **FIG 7** Dynamic three-dimensional reorganization of the studied genomic region in MDA::ER cells. Summary of one  
 939 3C experiment representative from two, performed on chromatin sampled from MDA::ER cells treated from 0 to 80  
 940 minutes with  $10^{-8}$  M E2. Results are illustrated as in Figure 6.

941

942 **FIG 8** Functionalization of MDA::ER and MCF-7 interactomes. (A) Triplex forming oligonucleotides (TFOs) were  
 943 designed to interfere with ER binding and thus to identify the roles of ER on specific BSs for the regulation of E2-  
 944 sensitive genes. (B) Formation of DNA triplex as analyzed by gel-shift. Increasing amounts of TFO (25 to 1,500 pmol)  
 945 were added to 25 pmoles of target DNA duplexes and incubated for 16 h at 37 °C. Control was made using an  
 946 unspecific TFO (Ctrl TFO) at the highest concentration. Complexes were separated by electrophoresis and stained  
 947 with methylene blue. (C) MCF-7 cells were transfected for 36 h with 10  $\mu$ mol of TFO or biotinylated (Biot-) TFO  
 948 directed against the ERBS1, subjected to cross-linking and sonicated chromatin was then incubated with  
 949 streptavidin-coated beads. Amounts of captured DNA were analyzed by qPCR. Values are mean  $\pm$  SD of two  
 950 independent duplicates, and are expressed as % of captured DNA relative to input DNA normalized to the amounts  
 951 of recovered negative control region (*Rplp0* promoter). (D) Anti-ER ChIP-qPCR performed on MCF-7 cells  
 952 transfected as previously and treated for 50 min with  $10^{-8}$  M E2. Results are mean values  $\pm$  SD of three independent  
 953 triplicates expressed as relative enrichment towards the *PKNOX1* promoter. (E) Fold changes in ER mobilization  
 954 measured by ChIP-qPCR on each tested ERBS following the transfection of corresponding TFO was plotted against  
 955 the distance separating the sequence targeted by the TFO from the center of the ERBS defined from ChIP-chip data.  
 956 (F) Amounts of streptavidin-captured DNA following the transfection of Biot-TFOs are plotted against the relative

957 chromatin accessibility of their target regions as measured by FAIRE experiments in control conditions (ethanol  
958 vehicle control, EtOH). (G) RT-qPCR experiments performed on MCF-7 and MDA::ER cells transfected with the  
959 indicated TFOs. Boxes at the left of each heatmap indicate identified interactions by 4C. Experimental values were  
960 normalized to those obtained in untransfected cells and expressed as log<sub>2</sub>. Data originate from at least three  
961 independent duplicate experiments.

**TABLE 1.** Sequences and characteristics of TFOs.

Target	sequence	Distance from ERBS center	Off-target vs. specific e-values <sup>a</sup>	35 bp probe sequence <sup>b</sup>
MCF-7 BS1	UGGUGTUUGGUUUGGUUGG	276	-1.95 [1]	CACAGACGTGG <b><i>AAGGAAAGGAATGAGG</i></b> ATGATATT
MCF-7 BS4	UGGGUGUGUUGGGUCUUGGUUU	479	-5.74 [1]	TGACCCT <b><i>AAAGGAACAGGGAAGAGAGGG</i></b> ATTCAGC
MCF-7 BS6	UUUUGUUUGGUUGGUCUG	2	-1.95 [1]	TGGGCACT <b><i>GACAGGAAGGGAAGAAA</i></b> CAGCCTGC
MCF-7 BS10	GUUGGUUCGUUGGUGUUU	248	-1.98 [1]	GATCTTCT <b><i>AAAGAGGAAGCAAGGAAGCC</i></b> AGCCTC
MCF-7 BS14	UUGUUCGGUUUGUGGUGG	511	-2.02 [2]	GATCTGAT <b><i>GGAGGAGAAAGGCAAGA</i></b> ACATGTGCGA
MCF-7 BS16	GGGGTUGUGGUGGUGUUU	355	-5.28 [2]	AGGTGTCC <b><i>AAAGAGGAGGAGGAGT</i></b> AGGGCAACAG
MDA::ER BS1	UGUUGUUUUGUGGTGGGUG	371	-1.98 [1]	CTAGGATGT <b><i>GAGGGTGGAGAAA</i></b> GAAGACGTGAGG
MDA::ER BS10	UUUGGGUCGUGUUGGUGG	273	-1 [1]	GCAGGAGATGG <b><i>AGGAAGAGCAGGGA</i></b> AATAGAAGCT
MDA::ER BS12	UGGGUGGTUUUGUGGUGGGUU	301	-5.74 [1]	AGGTGACCA <b><i>AGGGGAGGAGAAATG</i></b> AGGGACATTC
Common BS1	UGGUUUGUUGGGUGUGUGUG	185	-1.74 [2]	GGAGTTA <b><i>GAGAGAGAGGGAAGAAA</i></b> AGGAGGGAGGGA
Common BS2	UUUUUUUGUGGGUGGUCGGG	450	-3.93 [1]	GGCTGGGG <b><i>CAGGAGGGAGAAAAA</i></b> AATAGTATATA

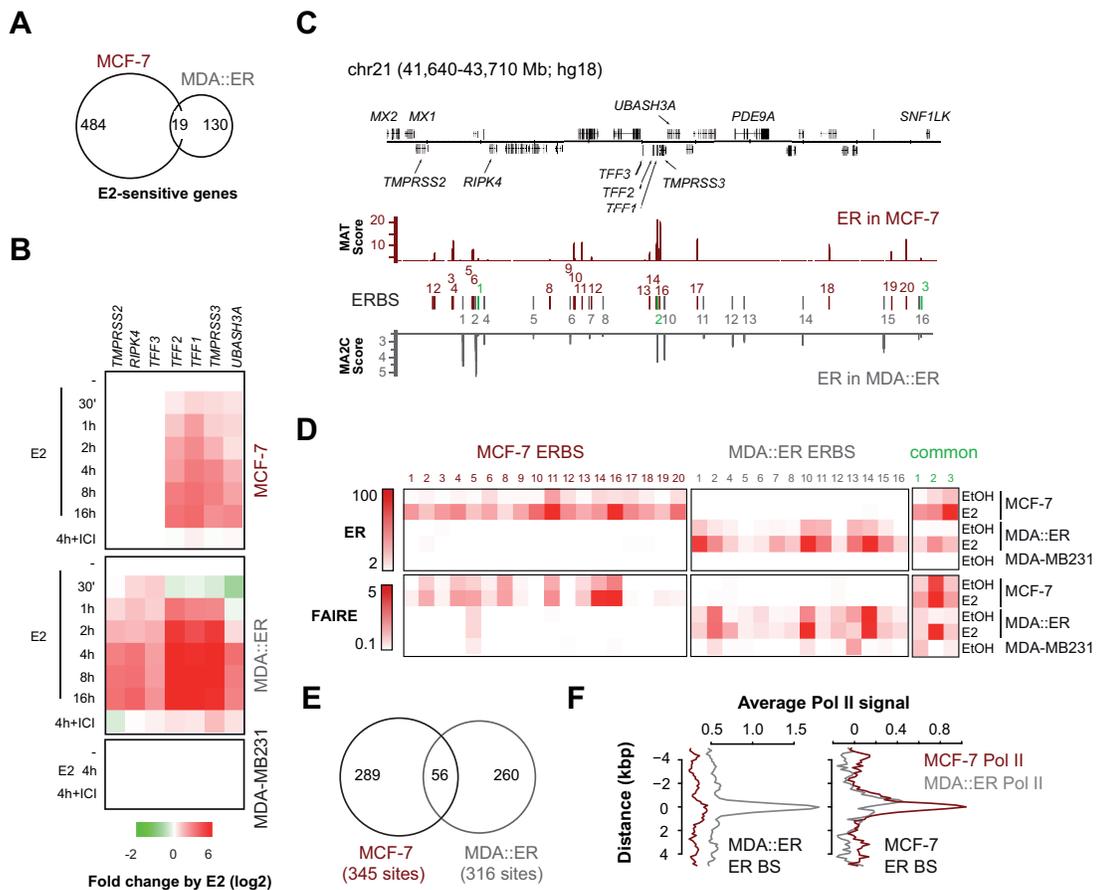
<sup>a</sup> Expressed as  $\log(\text{Inv}(\text{off target}/\text{specific target}))$ . The number in brackets indicates the number of off-targets in the top5 hits determined from BLAST (<http://blast.ncbi.nlm.nih.gov>). <sup>b</sup> Nucleotides targeted by the TFOs are in bold italic. Only the sense oligonucleotide is indicated.

**TABLE 2.** Genomic regions spotted on microarrays.

Chromosome	Start <sup>a</sup>	Stop <sup>a</sup>	Cluster	Regulated in	Top gene <sup>a</sup>
1	16,920,000	18,300,000	Yes	MDA::ER	<i>PADI1</i>
1	150,450,000	151,200,000	No	MCF-7	<i>LCE3B</i>
2	118,600,000	123,280,000	Yes	MDA::ER	<i>INHBB</i>
3	49,890,000	51,600,000	Yes	Both	<i>SEMA3B</i>
7	72,600,000	73,200,000	Yes	MDA::ER	<i>CLDN4</i>
8	67,060,000	68,460,000	Yes	MCF-7	<i>MYBL1</i>
9	138,882,000	139,620,000	Yes	MCF-7	<i>ENTPD2</i>
10	43,500,000	44,600,000	No	MCF-7	<i>CXCL12</i>
10	99,800,000	100,130,000	No	MDA::ER	<i>LOXL4</i>
11	1,500,000	3,300,000	Yes	MDA::ER	<i>TH3</i>
11	93,800,000	94,930,000	Yes	MCF-7	<i>FUT4</i>
12	9,600,000	10,920,000	Yes	MDA::ER	<i>KLRC3</i>
12	14,100,000	16,300,000	Yes	MCF-7	<i>ART4</i>
14	91,800,000	96,600,000	Not in MCF-7	Both	<i>Serp1A1</i>
16	21,500,000	24,420,000	Yes	MCF-7	<i>SCCN1C1</i>
16	54,600,000	55,900,000	Yes	MDA::ER	<i>MT1X</i>
16	65,600,000	66,240,000	Yes	MDA::ER	<i>HSF4</i>
17	35,530,000	37,440,000	Yes	Both	<i>KRT9</i>
19	54,150,000	54,480,000	Yes	MCF-7	<i>LHB</i>
20	21,000,000	26,800,000	Yes	MDA::ER	<i>ACSS1</i>
21	36,200,000	47,000,000	Yes	Both	<i>TFE1</i>
22	48,720,000	49,380,000	Yes	MDA::ER	<i>MAPK12</i>
X	36,600,000	39,600,000	Yes	Both	<i>SYTL5</i>

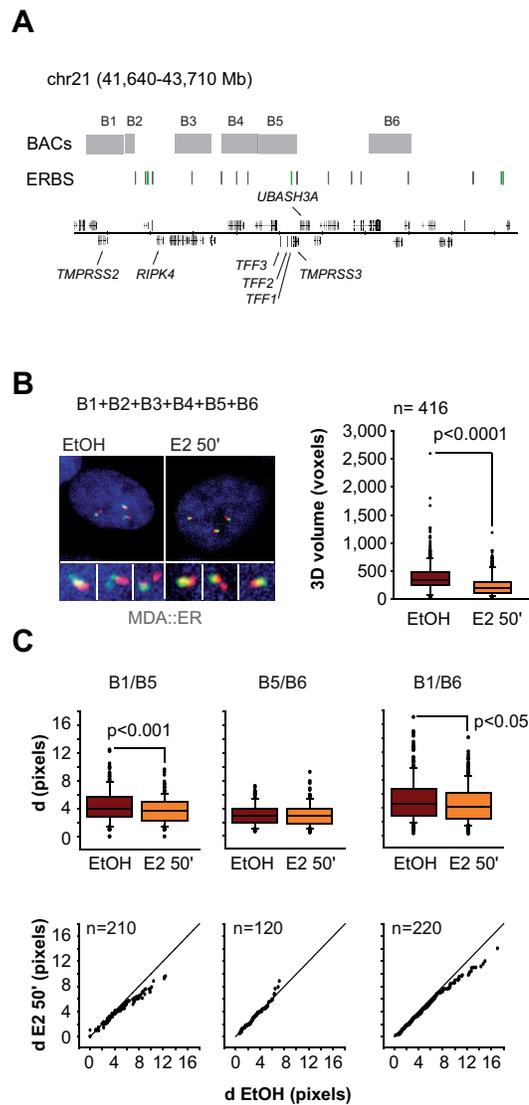
<sup>a</sup> Genomic coordinates are given from the hg18 assembly of the human genome. <sup>b</sup> Gene exhibiting the most important fold-change in expression upon E2 treatment in either MDA::ER or MCF-7 cells.

Figure 1



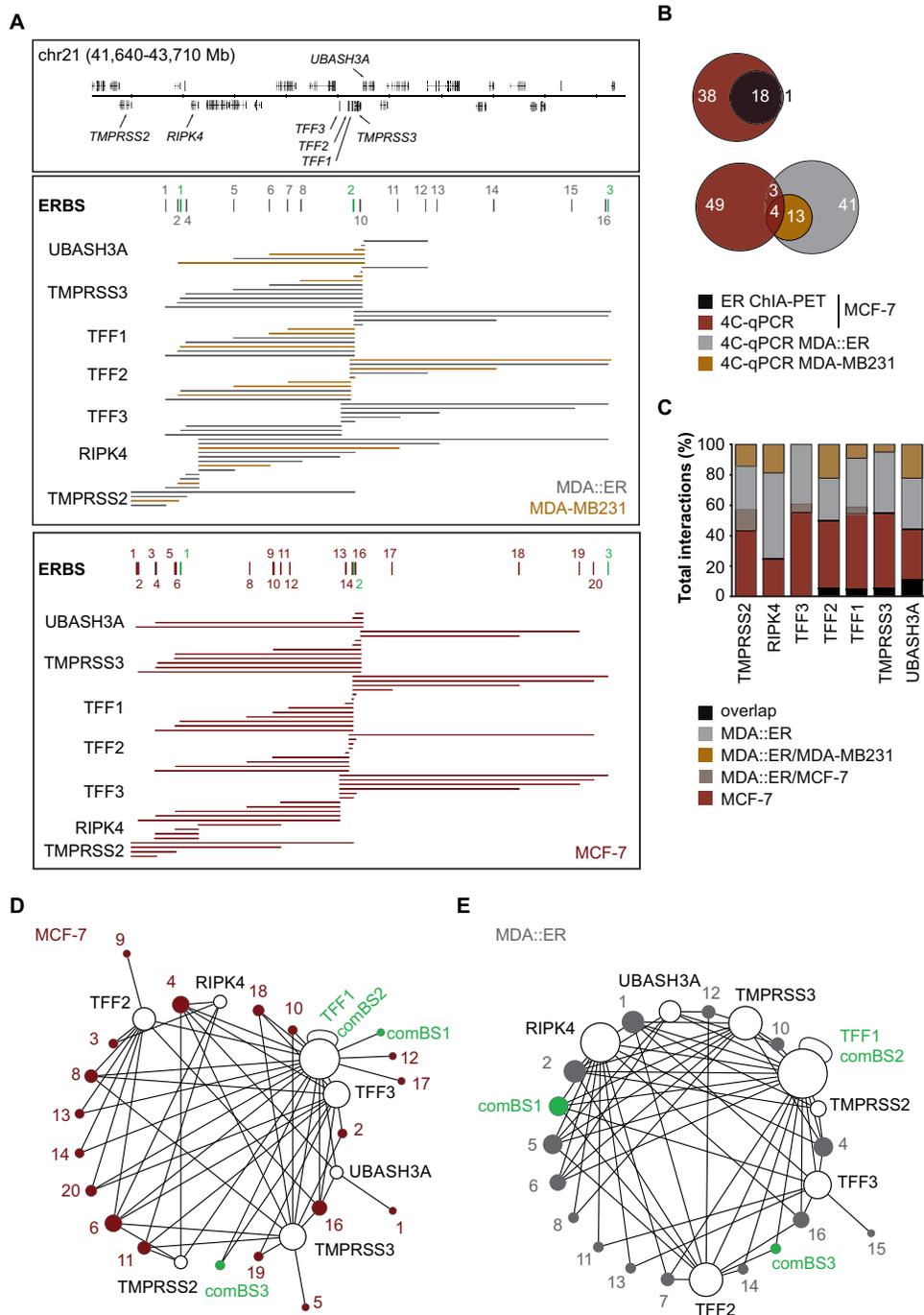
**FIG 1** Cell-specific E2-sensitive genes and ER binding sites in a 2 Mb genomic region including the TFF locus. (A) Venn diagram illustrating the overlap of identified E2-sensitive genes in MDA::ER and MCF-7 cells. (B) Heatmap representation of RT-qPCR results obtained on RNAs prepared from MCF-7, MDA::ER and MDA-MB231 cells treated for the indicated times with 10-8 M E2 and pre-treated for 36h with 10-6 M ICI164,384 where precised. Results are the log<sub>2</sub> of the fold inductions of gene expression levels by E2 obtained in two independent triplicate experiments. (C) Integrated genome browser [IGB; (80)] illustration of the studied genomic region with RefSeq genes indicated. ER binding signal obtained in an ER ChIP-chip analysis performed using chromatin of MDA::ER cells treated for 50 min with E2 is depicted in gray. MCF-7 data were obtained from published dataset (17). For the sake of clarity, only the highest 5% signals are shown. Grey and red boxes delineate cell-specific ER binding sites (ERBSs) whilst common ERBSs are in green. (D) Anti ER ChIP and FAIRE assays were conducted using chromatin prepared from MCF-7, MDA-MB231 or MDA::ER cells treated with E2 or ethanol (EtOH) as vehicle control for 50 min. Results shown within heatmaps are means from 6 to 9 values obtained in independent triplicate experiments. Values are fold enrichments over control samples and a negative control region (promoter of the transcriptionally active *Rplp0* gene). (E) Overlap of MDA::ER ERBSs with MCF-7 ones on regions spotted on the arrays. (F) Enrichment signals obtained for anti-RNA Polymerase II (Pol II) ChIP-chip experiments performed in MDA::ER cells treated for 50 min with E2 [MCF-7 data from (17)] were aligned on MCF-7 or MDA::ER ERBS identified within the regions spotted on the arrays and located more than 10 Kbp away from the TSS of any annotated gene.

Figure 2



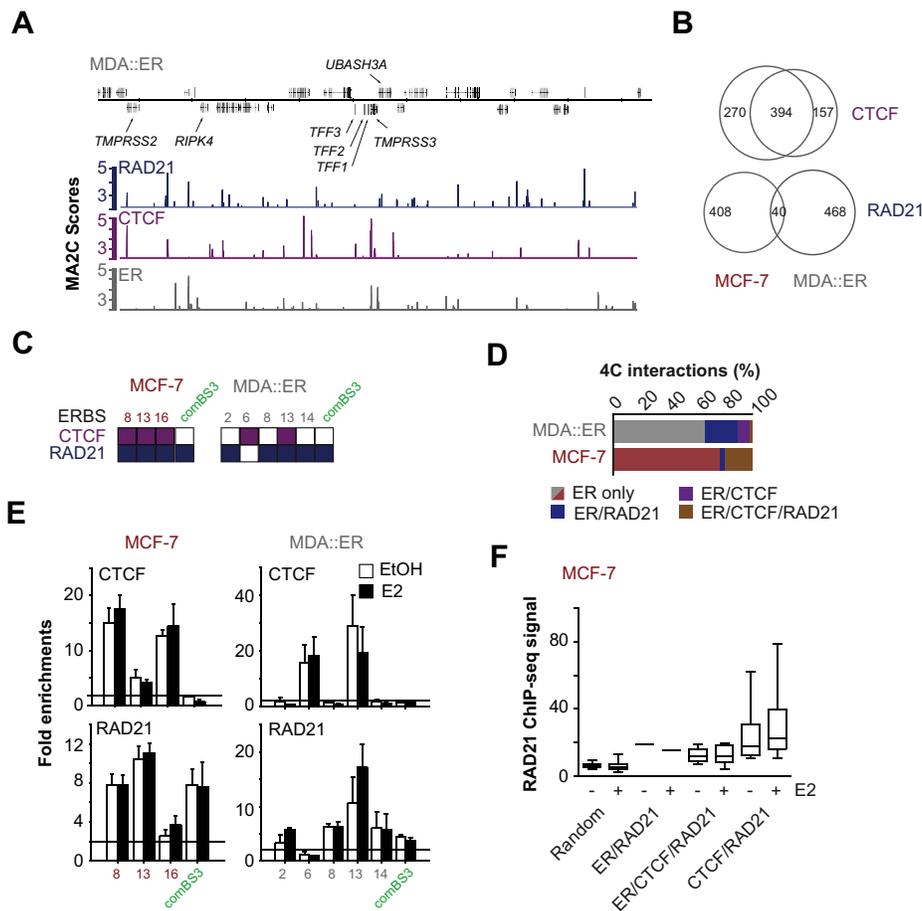
**FIG 2** E2 provokes a three-dimensional reorganization of the TFF cluster in MDA:ER cells. (A) Locations of the BACs (B1 to B6 referring respectively to RP11-814F13, CTD-2337B13, RP11-35C4, CTD-260o11, RP11-113F1 and CTD-1033M14) used to generate fluorescent probes for FISH experiments all along the genomic region of interest, illustrated as in Figure 1. Positions of the ERBSs are also indicated. (B) Analysis of the 3D volume of the TFF loci by DNA-FISH using a mix of all generated probes in cells treated with 10-8 M E2 or ethanol (EtOH) for 50 min. Representative pictures of these assays are on the left side of the panel, with magnified views of the three TFF loci present in MDA:ER cells. Quantitative measurements (n=416) of the 3D volume of the loci is shown at the right side of the panel with indicated Fisher t-test p-value. (C) Quantitative analysis of the distribution of distances separating indicated paired FISH probes. Values are shown within boxplots (top of the panel) or within quantile-quantile (Q-Q) plots representation of conditionally ranked measured distances (bottom). The normal distribution expected from non-varying distances is illustrated by the straight black line in each Q-Q plots. Non-parametric Fisher test p-values are indicated when determined as significant, as well as the number of measurements made in at least three independent experiments.

Figure 3



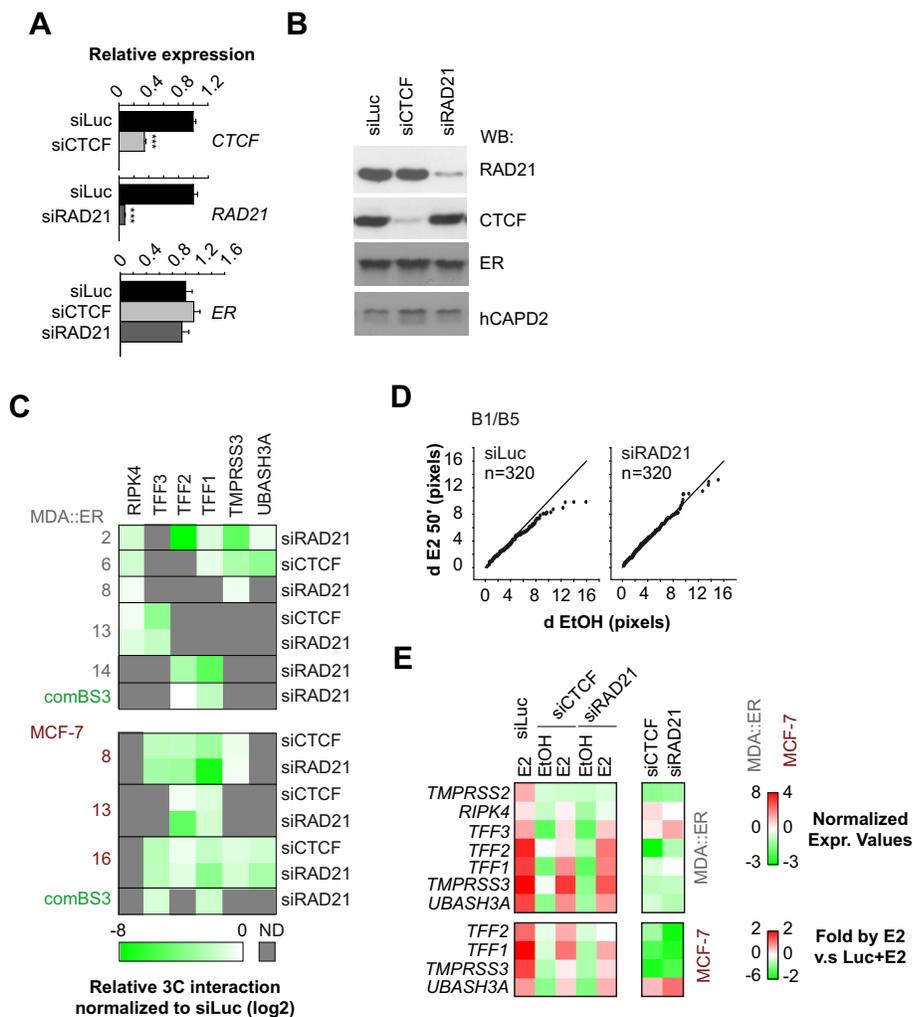
**FIG 3** ERBS-promoters interactomes. (A) IGB visualization of MCF-7, MDA-MB231 and MDA::ER interactomes linking *DpnII* fragments encompassing ERBS or promoter regions of indicated genes, as detected by 4C-qPCR on chromatin prepared from cells treated for 50 min with 10-8 M E2. Shown are RefSeq genes coordinates along chr21, as well as the positions of ERBS with grey, red and green boxes delineating MDA::ER, MCF-7 or common ERBS. 4C data are represented as lines linking one ERBS to its target promoters. MCF-7 interactions are in red, MDA::ER ones in grey and those shared between MDA::ER and MDA-MB231 in orange. (B) Venn diagrams depicting the overlapping interactions characterized in this study and to those identified in published ER ChIA-PET dataset (14) restricted to loops involving the gene promoters that served here as 4C baits. (C) Stacked histograms illustrating the overlap of the 4C-detected interactions for each tested promoters in the different cell lines. (D and E) Cytoscape (41) circular layouts of the networks of interactions that link E2-regulated genes to ERBS in MCF-7 (D) and in MDA::ER (E) cells. The sizes of the nodes are directly related to the number of interactions they direct.

Figure 4



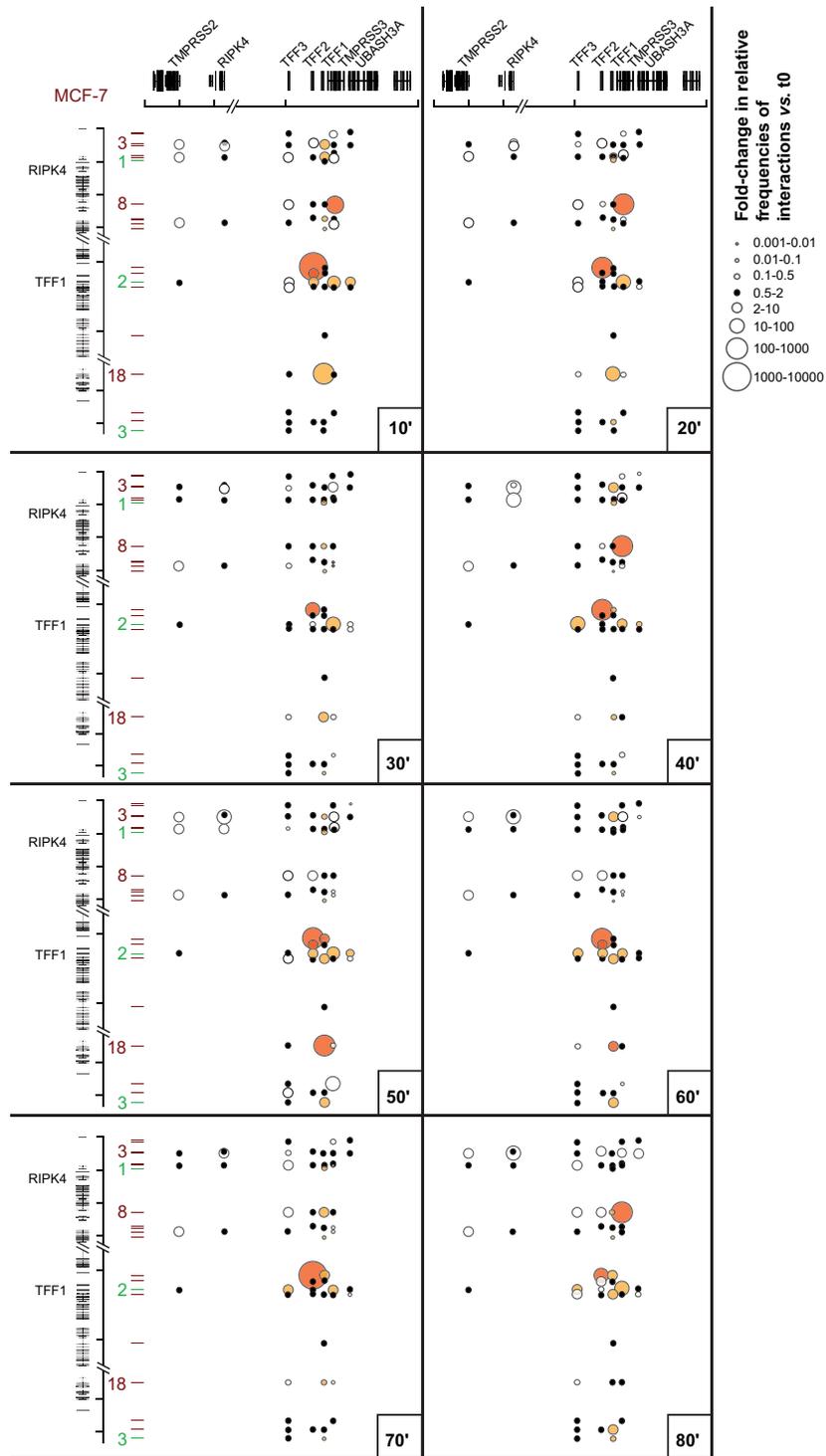
**FIG 4** CTCF and cohesin recruitment on ERBSs. (A) MDA::ER anti-ER, CTCF or RAD21 ChIP-Seq or ChIP-chip signals visualized under IGB as in Figure 1. All data were obtained from cells treated with 10-8 M E2 for 50 min. (B) Overlaps on array-spotted regions between RAD21 or CTCF binding sites determined in this study in MDA::ER cells and those previously determined in MCF-7 (53). (C) Overlap of RAD21 and CTCF binding sites with ERBSs. (D) Repartition of CTCF and RAD21 BS within the interactions between ERBS and gene promoters identified by 4C. (E) CTCF and RAD21 ChIP-qPCR were performed on chromatin prepared from MCF-7 or MDA::ER cells treated for 4h with 10-8M E2 or ethanol (EtOH) as vehicle control. Fold enrichment of the precipitated proteins on tested sequences was normalized over control ChIP and negative region (*PKNOX1* promoter) values. Data shown are mean values  $\pm$  SD obtained in three independent triplicate experiments. The line depicts the significance threshold applied (Fold enrichment > 2). (F) Boxplots of MACS normalized reads of RAD21 ChIP-seq experiments performed in the absence or presence of E2 in MCF-7 cells [dataset from (53)]. Values shown are mean counts measured in a 500 bp window centered on indicated binding sites located within the 2 Mb genome region studied. Calculations were also made on random sites of similar mean size and in equal number than the shared CTCF/RAD21 BSs.

Figure 5



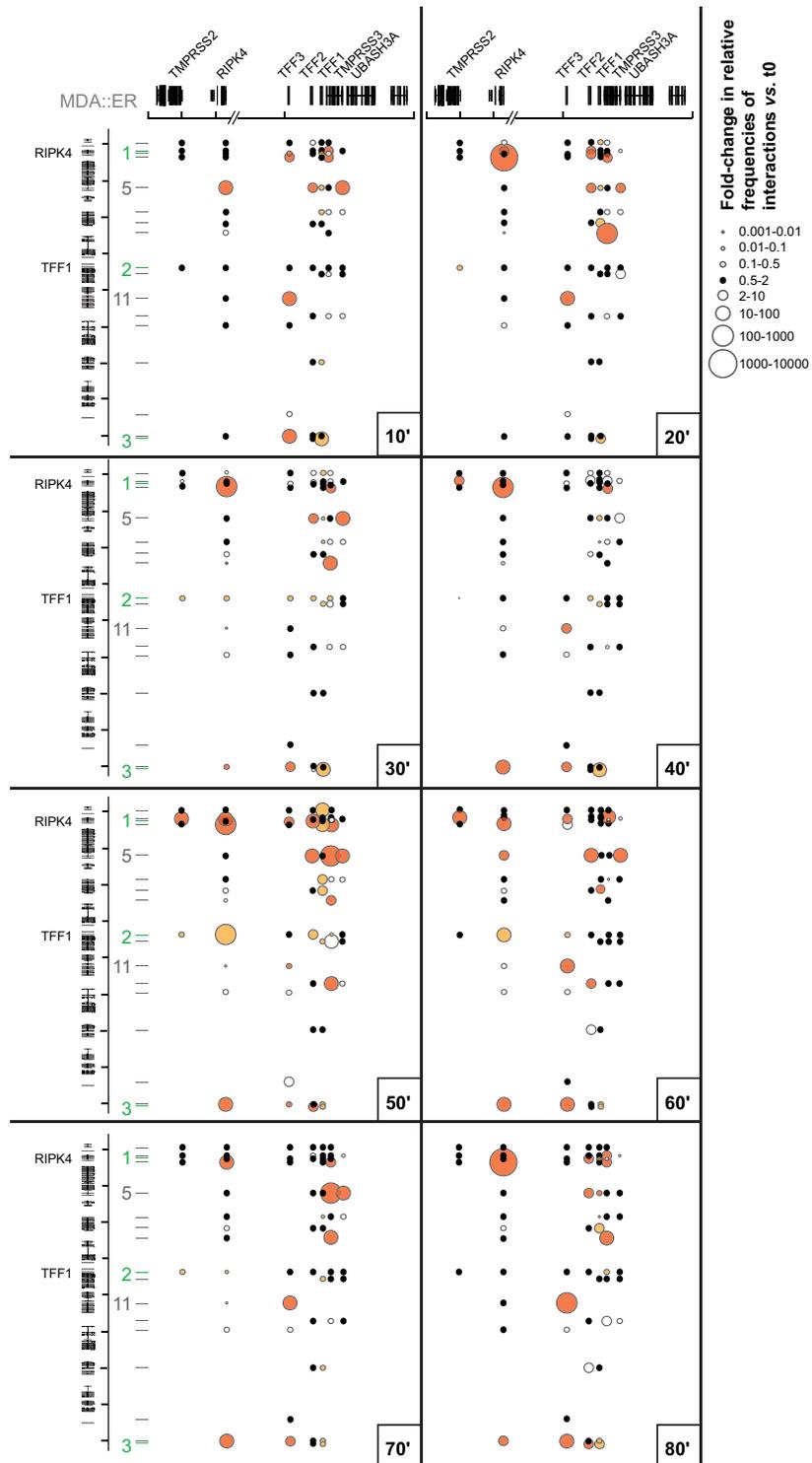
**FIG 5** CTCF and cohesin organize the E2-responsiveness of the TFF cluster. (A) Amounts of indicated mRNA were evaluated in untreated MDA::ER cells following 72h of transfection with either control siRNA (directed against the Luciferase gene, siLuc) or siRNAs targeting mRNAs of interest. Results shown are mean data  $\pm$  SEM of values obtained in at least 5 different experiments (n ranges from 15 to 18), normalized to the expression of control PKNOX1 gene. Wilcoxon test was used to identify statistically relevant variations from control siLuc/EtOH condition: p-value <0.001 (\*\*\*), <0.01 (\*\*). (B) Western blots assaying the expression of RAD21, CTCF, ER and hCAPD2 (loading control) in cells transfected with the indicated siRNAs. (C) 3C-qPCR experiments following the impact of the reduction in CTCF and RAD21 contents on the interactions between indicated ERBS and gene promoters. Results shown originate from one representative experiment out of two and are expressed as the log2 of values normalized to those obtained in the control siLuc condition. (D) Quantitative DNA-FISH analysis of the distribution of distances separating paired fluorescent probes in transfected MDA::ER cells. Results are illustrated as in Figure 3. (E) Heatmaps summarizing RT-qPCR results obtained in at least 3 independent triplicate experiments following the transfection of control siLuc or siRNAs directed against CTCF or RAD21 expression and a 4h treatment of the cells with 10-8 M E2 or EtOH as vehicle control. Expression levels were normalized to the *PKNOX1* internal control and reported to those calculated for the siLuc EtOH condition. The fold inductions of these genes expression by E2 are also shown on the right side of the panel as relative to those measured in cells transfected by the siLuc. Results originate from at least 4 independent triplicate experiments.

Figure 6



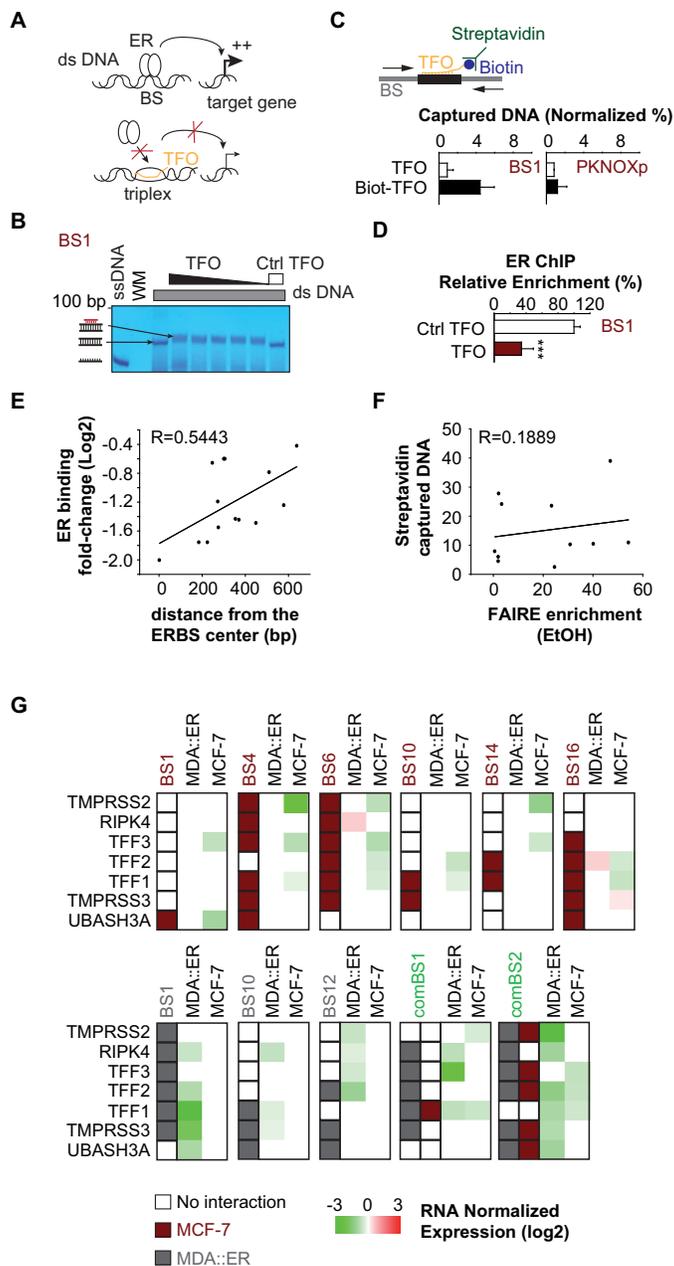
**FIG 6** Dynamic three-dimensional reorganization of the studied genomic region in MCF-7 cells. Summary of one 3C experiment representative from two, performed on chromatin sampled from MCF-7 cells treated from 0 to 80 minutes with 10-8 M E2. As indicated, the size of the bubble that corresponds to one interaction is proportional to the fold changes in frequencies of interaction as compared to the basal (t0) situation. The location of the gene promoters that served as anchors is illustrated on the top of each subpanel and the ERBSs on the left. Distance scale is accurate (2 Mb between ticks) but had to be broken in some instances for sake of figure size and clarity. Bubbles highlighted in orange are those commented in the main text and those in yellow correspond to interactions made by the ERBS located within the *TFF1* promoter (comERBS2).

Figure 7



**FIG 7** Dynamic three-dimensional reorganization of the studied genomic region in MDA::ER cells. Summary of one 3C experiment representative from two, performed on chromatin sampled from MDA::ER cells treated from 0 to 80 minutes with  $10^{-8}$  M E2. Results are illustrated as in Figure 6.

Figure 8



**FIG 8** Functionalization of MDA::ER and MCF-7 interactomes. (A) Triplex forming oligonucleotides (TFOs) were designed to interfere with ER binding and thus to identify the roles of ER on specific BSs for the regulation of E2-sensitive genes. (B) Formation of DNA triplex as analyzed by gel-shift. Increasing amounts of TFO (25 to 1,500 pmol) were added to 25 pmoles of target DNA duplexes and incubated for 16 h at 37 °C. Control was made using an unspecific TFO (Ctrl TFO) at the highest concentration. Complexes were separated by electrophoresis and stained with methylene blue. (C) MCF-7 cells were transfected for 36 h with 10 μmol of TFO or biotinylated (Biot-) TFO directed against the ERBS1, subjected to cross-linking and sonicated chromatin was then incubated with streptavidin-coated beads. Amounts of captured DNA were analyzed by qPCR. Values are mean ± SD of two independent duplicates, and are expressed as % of captured DNA relative to input DNA normalized to the amounts of recovered negative control region (*Rplp0* promoter). (D) Anti-ER ChIP-qPCR performed on MCF-7 cells transfected as previously and treated for 50 min with 10-8 M E2. Results are mean values ± SD of three independent triplicates expressed as relative enrichment towards the PKNOX1 promoter. (E) Fold changes in ER mobilization measured by ChIP-qPCR on each tested ERBS following the transfection of corresponding TFO was plotted against the distance separating the sequence targeted by the TFO from the center of the ERBS defined from ChIP-chip data. (F) Amounts of streptavidin-captured DNA following the transfection of Biot-TFOs are plotted against the relative chromatin accessibility of their target regions as measured by FAIRE experiments in control conditions (ethanol vehicle control, EtOH). (G) RT-qPCR experiments performed on MCF-7 and MDA::ER cells transfected with the indicated TFOs. Boxes at the left of each heatmap indicate identified interactions by 4C. Experimental values were normalized to those obtained in untransfected cells and expressed as log2. Data originate from at least three independent duplicate experiments.