

## 1 Heat Shock Factor 1 (HSF1) as a new tethering factor for ESR1 supporting its action in breast cancer.

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32           **Abstract**

33           Heat shock factor 1 (HSF1), a key regulator of transcriptional responses to proteotoxic stress, was  
34           recently linked to estrogen (E2) signaling through ESR1. We found that an HSF1 deficiency could lead to the  
35           inhibition of the mitogenic action of E2 in breast cancer cells. The stimulatory effect of E2 on the transcriptome  
36           is weaker in HSF1-deficient cells, in part due to the higher basal expression of E2-dependent genes, which  
37           correlates with the enhanced binding of unliganded ESR1 to chromatin. HSF1 and ESR1 can cooperate directly  
38           in E2-stimulated regulation of transcription, and HSF1 potentiates the action of ESR1 through a mechanism  
39           involving chromatin reorganization. Analyses of data from the TCGA database indicate that HSF1 increases the  
40           transcriptome diversity in ER-positive breast cancer and can enhance the genomic action of ESR1. Moreover,  
41           *ESR1* and *HSF1* are only prognostic when analyzed together (the worst prognosis for ER-/HSF1<sup>high</sup> cancers).

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43           **Keywords**

44           Breast cancer; chromatin organization; estrogen receptor; genomic action; heat shock factor 1.

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46           **Introduction**

47           Breast cancer is the most common malignancy in women worldwide. Four clinically relevant molecular  
48           types are distinguished based on the expression of estrogen receptors (ERs) and HER2 (ERBB2). Among them,  
49           luminal adenocarcinomas, characterized by the expression of estrogen receptors, constitute about 70% of all  
50           breast cancer cases. There are two classical nuclear estrogen receptors, ER $\alpha$  (ESR1) and ER $\beta$  (ESR2), and  
51           structurally different GPR30 (GPER1), which is a member of the rhodopsin-like family of the G protein-coupled  
52           and seven-transmembrane receptors. ER $\alpha$  expression is most common in breast cancer and its evaluation is the  
53           basis for determining the ER status. Activity of estrogen receptors is modulated by steroid hormones, mainly  
54           estrogens, which are synthesized from cholesterol. According to epidemiological and experimental data,  
55           estrogens alongside the mutations in *BRCA1* and *BRCA2*, *CHEK2*, *TP53*, *STK11 (LKB1)*, *PIK3CA*, *PTEN*, and  
56           other genes, are key etiological factors of breast cancer development (Yaşar et al., 2017) (Verigos and Magklara,  
57           2015). The mechanism of estrogen-stimulated breast carcinogenesis is not clear. According to the widely  
58           accepted hypothesis, estrogens acting through ER $\alpha$ , stimulate cell proliferation and can support the growth of  
59           cells harboring mutations which then accumulate, ultimately resulting in cancer. Another hypothesis suggests the

60 ER $\alpha$ -independent action of estrogens via their metabolites, which can exert genotoxic effects, contributing to  
61 cancer development (Yager and Davidson, 2006) (Pescatori et al., 2021).

62 Previously, we have found that the major female sex hormone 17 $\beta$ -estradiol (E2) stimulates activation of  
63 the Heat Shock Factor 1 (HSF1) in estrogen-dependent breast cancer cells via MAPK signaling (Vydra et al.,  
64 2019). HSF1 is a well-known regulator of cellular stress response induced by various environmental stimuli. It  
65 mainly regulates the expression of the Heat Shock Proteins (HSPs), which function as molecular chaperones and  
66 regulate protein homeostasis (Ran et al., 2007). HSF1-regulated chaperones control, among other, the activity of  
67 estrogen receptors (Echeverria and Picard, 2010). ERs remain in an inactive state trapped in multimolecular  
68 chaperone complexes organized around HSP90, containing p23 (PTGES3), and immunophilins (FKBP4 or  
69 FKBP5) (Segnitz and Gehring, 1995). Upon binding to E2, ERs dissociate from the chaperone complexes and  
70 become competent to dimerize and regulate the transcription. ERs bind DNA directly, to the estrogen-response  
71 elements, EREs, or act indirectly through recruiting transcriptional co-activators (Heldring et al., 2007) (Renoir,  
72 2012). HSP90 is essential for ER $\alpha$  hormone binding (Fliss et al., 2000), dimer formation (Powell et al., 2010),  
73 and binding to the EREs (Inano et al., 1994). Also, the passage of the ER to the cell membrane requires  
74 association with the HSP27 (HSPB1) oligomers in the cytoplasm (Razandi et al., 2010). More than 20  
75 chaperones and co-chaperones associated with ER $\alpha$  in human cells have been identified through a quantitative  
76 proteomic approach (Dhamad et al., 2016), but their specific contribution in the receptor action still needs to be  
77 investigated. Moreover, HSF1 is involved in the regulation of a plethora of non-HSP genes, which support  
78 oncogenic processes: cell-cycle regulation, signaling, metabolism, adhesion, and translation (Mendillo et al.,  
79 2012). A high level of HSF1 expression was found in cancer cell lines and many human tumors (Vydra et al.,  
80 2014) (De Thonel et al., 2011) and was shown to be associated with increased mortality of ER-positive breast  
81 cancer patients (Santagata et al., 2011) (Gökmen-Polar and Badve, 2016).

82 E2-activated HSF1 is transcriptionally potent and takes part in the regulation of several genes essential for  
83 breast cancer cell growth and/or ER $\alpha$  action (Vydra et al., 2019). Thus, a hypothetical positive feedback loop  
84 between E2/ER $\alpha$  and HSF1 signaling may exist, which putatively supports the growth of estrogen-dependent  
85 tumors. Here, to study the cooperation of HSF1 and ESR1 in estrogen signaling and the influence of HSF1 on  
86 E2-stimulated transcription and cell growth, we created novel experimental models based on HSF1-deficient  
87 cells and performed an in-depth bioinformatics analysis of the relevant genomics data.

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90 **Results**

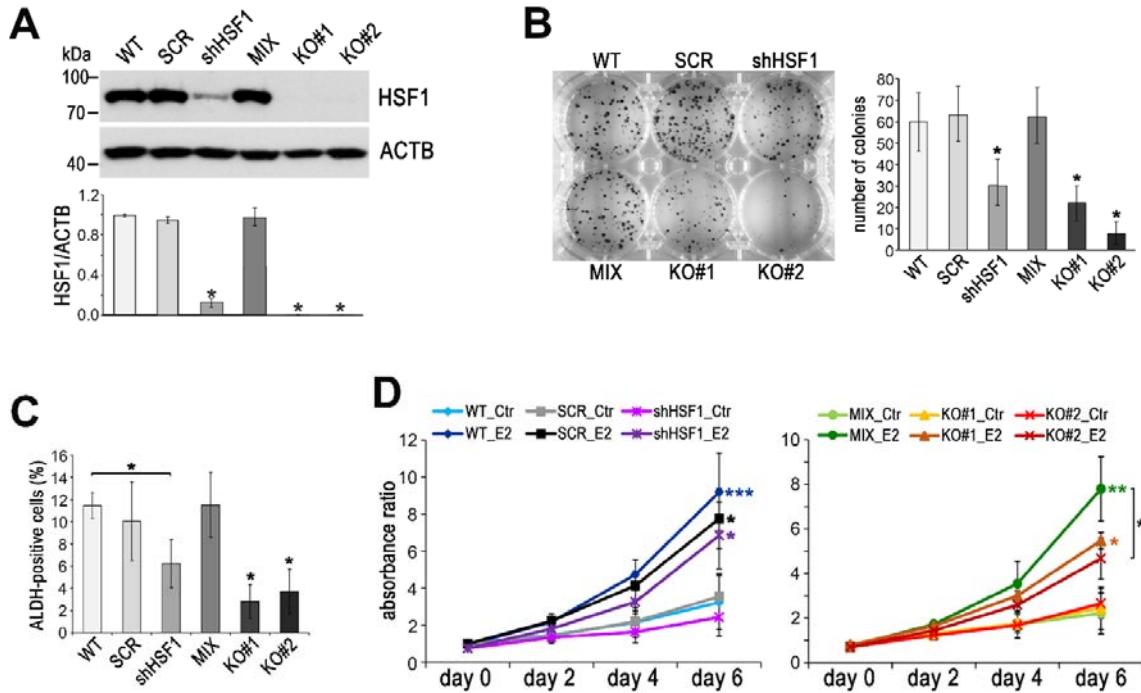
91 **HSF1 deficiency slows the estrogen-stimulated growth of ER $\alpha$ -positive MCF7 cells**

92 To study the contribution of HSF1 in E2 signaling, we established MCF7 cell lines with reduced HSF1  
93 expression. Firstly, we tested a few *HSF1*-targeting shRNAs (Fig. S1A). Then, the most potent variant that  
94 reduced HSF1 level about 10-fold (termed afterward shHSF1) was chosen for further studies. Although the heat  
95 shock response was significantly reduced, the expression of *HSP* genes (*HSPA1A*, *HSPH1*, *HSPB1*, and *HSPB8*)  
96 was still induced after this HSF1 knockdown (Fig. S1B). Thus, we additionally created MCF7 variants with  
97 HSF1 functional knockout using the CRISPR/Cas9 gene targeting approach (termed KO#1 and KO#2  
98 afterward). The complete elimination of HSF1 (Fig. 1A) was connected with a substantial loss of inducibility of  
99 *HSP* genes following hyperthermia (Fig. S1B). HSF1 knockdown did not affect the proliferation rate, while the  
100 functional HSF1 knockout led to a slight reduction in the proliferation rate under standard conditions (this effect  
101 was not visible under less favorable growing conditions, i.e. in 5% dextran-activated charcoal-stripped FBS; Fig.  
102 S1C). Also, the increased contribution of cells in the G1 phase was associated with the HSF1 knockout (Fig.  
103 S1D) while the ability of cells to form colonies in the clonogenic assay was reduced in both MCF7 experimental  
104 models of HSF1 depletion (using shRNA and sgRNA; Fig. 1B). Moreover, the population size of ALDH-  
105 positive (stem/progenitor) cells correlated with the HSF1 level and was reduced in HSF1-deficient cells (Fig.  
106 1C). To check if HSF1 deficiency would affect the growth of another ER $\alpha$ -positive cell line, we modified T47D  
107 cells using the CRISPR/Cas9 method (Fig. S1E). Under standard conditions, we did not observe differences  
108 between unstimulated HSF1+ and HSF1- T47D cell variants in the proliferation and clonogenic assay (not  
109 shown). Unlike MCF7 cells, HSF1- T47D cells grew slightly faster than HSF1+ cells but this difference was not  
110 statistically significant (Fig. S1F).

111 We have previously demonstrated that HSF1 was activated after E2 treatment of ER $\alpha$ -positive cells and it  
112 was able to bind to the regulatory sequences of several target genes, which correlated with the upregulation of  
113 their transcription (Vydra et al., 2019). Since most of these genes code for proteins involved in E2 signaling, we  
114 expected that HSF1 downregulation could affect E2-dependent processes, especially cell proliferation.  
115 Therefore, we compared E2-stimulated proliferation of HSF1-proficient (WT, SCR, MIX) and HSF1-deficient  
116 (shHSF1, KO#1, KO#2) MCF7 cells. The E2-stimulated growth was weaker in the HSF1 knockout cells than in  
117 the corresponding control cells but a statistically significant difference was only observed between stimulated  
118 KO#2 and MIX cells (Fig. 1D). A similar trend was observed in HSF1 knockdown cells (Fig. 1D). However, E2-  
119 stimulated proliferation was not significantly reduced in HSF1 knockout T47D cells (Fig. S1G). These results

120 indicate that HSF1 may influence the growth of ER-positive breast cancer cells, also stimulated by estrogen,  
121 although the effect also depends on other factors (differences between cells, culture conditions).

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124

125 **Fig.1. Effect of HSF1 depletion on MCF7 cell growth.** (A) Western blot analysis of HSF1 level in cell  
126 variants: unmodified (WT), stably transduced with non-specific shRNA (SCR), stably transduced with HSF1-  
127 specific shRNA (shHSF1), a combination of control clones arisen from single cells following CRISPR/Cas9  
128 gene targeting (MIX), two HSF1 negative clones obtained by CRISPR/Cas9 gene targeting (KO#1, KO#2).  
129 Actin (ACTB) was used as a protein loading control. The graph below shows the results of densitometric  
130 analysis of HSF1 immunodetection (n=3). \*p < 0.05. (B) The number of colonies formed by unstimulated cell  
131 variants in the clonogenic assay: representative images of single-cell clones stained with crystal violet and their  
132 quantification (mean±SD, n=5). \*p < 0.05. (C) Aldefluor assay of progenitor (ALDH-positive) cell variants  
133 assessed by flow cytometry (n=4). \*p < 0.05. (D) Growth curves of untreated (Ctr) and E2-stimulated cell  
134 variants in phenol red-free media with 5% charcoal-stripped FBS assessed using crystal violet staining. Mean  
135 and standard deviation from four independent experiments (each in six technical replicates) are shown. \*\*\*p <  
136 0.0001, \*\*p < 0.001, \*p < 0.05 (next to the curve – compared to the corresponding control, between curves –  
137 between cell variants).

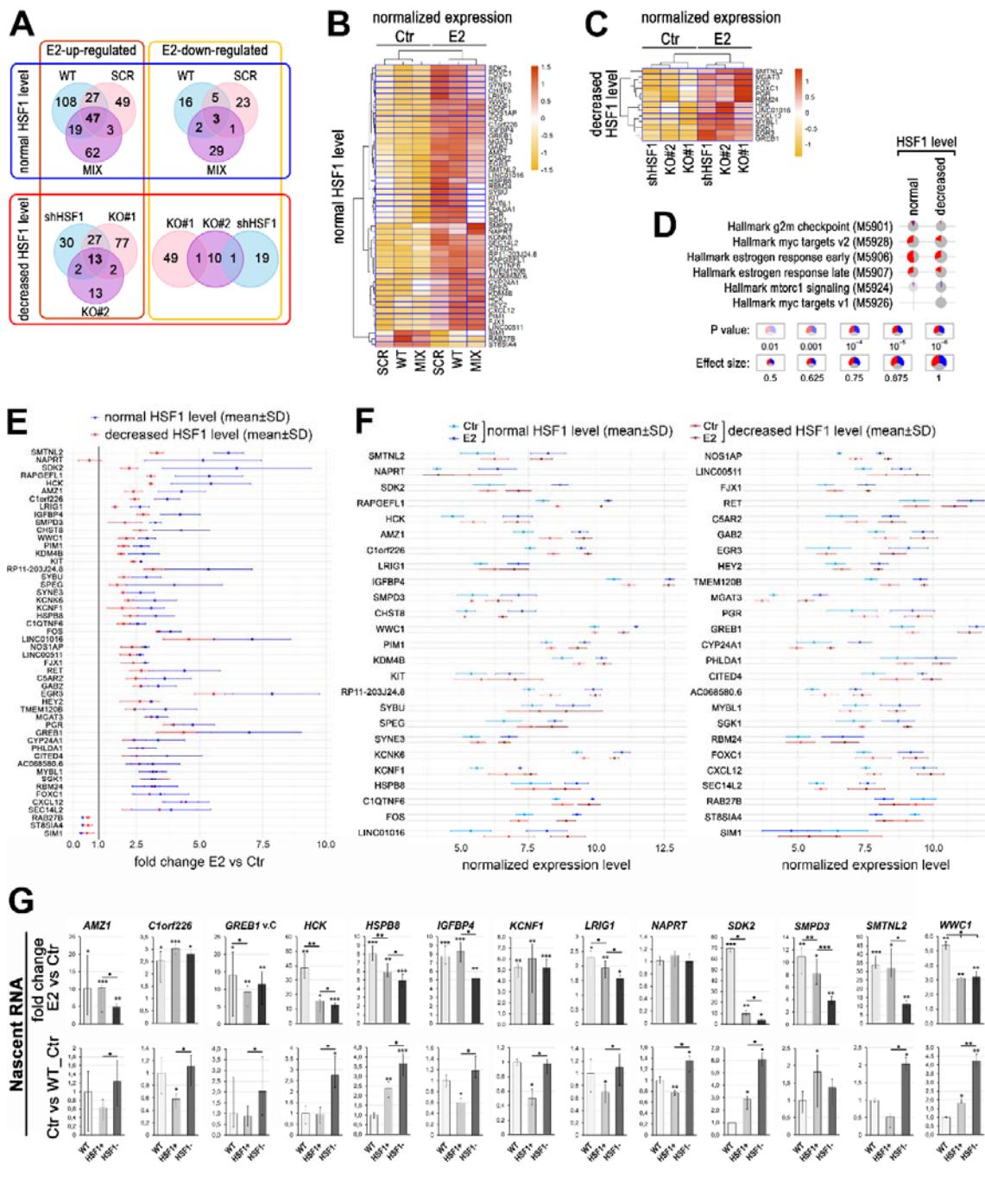
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### 139 Transcriptional response to estrogen is inhibited in HSF1-deficient cells

140 In a search for the mechanism responsible for a distinct response to estrogen in ER-positive cells with  
141 different levels of HSF1, we analyzed global gene expression profiles by RNA-seq in MCF7 cell variants

142 (Supplementary Dataset 1). At control conditions (no E2 stimulation), we found relatively few genes  
143 differentially expressed in HSF1-proficient (WT, SCR, and MIX) and HSF1-deficient (shHSF1, KO#1, and  
144 KO#2) cells that were common for different modes of HSF1 downregulation. These included mainly known  
145 HSF1 targets (e.g. *HSPH1*, *HSPE1*, *HSPD1*, *HSP90AA1*) slightly repressed in HSF1-deficient cells. After E2  
146 stimulation, there were 50 genes similarly regulated (47 upregulated and 3 down-regulated) in all HSF1-  
147 proficient MCF7 cell variants (Fig. 2A, B). On the other hand, only 13 genes were similarly upregulated after E2  
148 stimulation in all HSF1-deficient MCF7 cell variants (Fig. 2A, C). The geneset enrichment analyses indicated  
149 that HSF1 deficiency negatively affected the processes activated by estrogen, especially the early estrogen  
150 response (Fig. 2D; terms from other Molecular Signatures Database collections are shown in Fig. S2). Moreover,  
151 though almost all genes upregulated by E2 in HSF1-proficient cells were also upregulated in HSF1-deficient  
152 cells (except *NAPRT*), the degree of their activation (measured as a fold change E2 vs Ctr) was usually weaker in  
153 the latter cells (Fig. 2E), which indicated that the transcriptional response to estrogen was inhibited in the lack of  
154 HSF1. Interestingly, however, several E2-dependent genes revealed slightly higher basal expression (without E2  
155 stimulation) in HSF1-deficient cells (Fig. 2F), which suggested that in the absence of E2, HSF1 could be  
156 involved in the suppression of these genes.

157 Considering differences between KO#1 and KO#2 HSF1 knockout clones derived from individual cells  
158 (similarly, MIX was different from WT cells; Fig. S3), we created an additional experimental model to validate  
159 the results described above. Six new individual HSF1-negative (HSF1-) and six HSF1-positive (HSF1+) MCF7  
160 clones obtained using the DNA-free CRISPR/Cas9 system were pooled, characterized for the heat shock  
161 response (Fig. S4A), and used for validation analyses. Proliferation tests confirmed that both untreated and E2-  
162 stimulated HSF1- cells grew slower than corresponding HSF1+ cells, but the differences were statistically  
163 significant only under superior growing conditions (i.e. 10% FBS; Fig. S4B). Out of 13 genes selected for RT-  
164 qPCR-based validation using total or nascent RNA, all but *NAPRT* were estrogen-induced (Fig. 2G; Fig. S4C).  
165 In the case of 9 (total RNA) or 8 (nascent RNA) genes, the degree of activation was substantially lower in  
166 HSF1- than in HSF1+ cells. When the basal expression in E2-untreated cells was compared using the total  
167 RNA, 6 genes were expressed at a significantly higher and 1 at a lower level in HSF1- than HSF1+ cells (Fig.  
168 S4C). On the other hand, if the nascent RNA was analyzed, there were 12 genes expressed at a higher level in  
169 untreated HSF1- cells in comparison to HSF1+ cells (Fig. 2G). Hence, RT-qPCR-based validation analyses  
170 generally confirmed differences between HSF1-proficient and HSF1-deficient MCF7 cells revealed by the RNA-  
171 seq analyzes.



174 **Fig. 2. The deficiency of HSF1 reduces a transcriptional response to estrogen (E2) in ER-positive MCF7**  
175 **cells. (A)** Overlap of genes and **(B, C)** heatmaps with hierarchical clustering of normalized read counts from  
176 RNA-seq (row z-score) for genes stimulated or repressed after the E2 treatment in cells with different levels of  
177 HSF1: unmodified (WT), stably transduced with non-specific shRNA (SCR), stably transduced with HSF1-  
178 specific shRNA (shHSF1), the combination of control clones arisen from single cells following CRISPR/Cas9  
179 gene targeting (MIX), HSF1 negative single clones obtained by CRISPR/Cas9 gene targeting (KO#1, KO#2).  
180 Ctr, untreated cells; E2, 17 $\beta$ -estradiol treatment (10 nM, 4 h). **(D)** Geneset enrichment analysis showing  
181 differences between HSF1-proficient and HSF1-deficient cells in response to E2 stimulation. Only significant

182 terms from the hallmark gene sets collection are shown. Blue – a fraction of down-regulated genes, red – fraction  
183 of up-regulated genes. (E) Comparison of the response to E2 stimulation (E2 vs Ctr) in HSF1-proficient and  
184 HSF1-deficient cells. Genes shown in panel B are sorted from the highest to the lowest difference between  
185 average fold changes in both cell variants decreased by the standard deviation (SD). Up-regulation – fold change  
186 > 1.0, down-regulation – fold change < 1.0. (F) Comparison of the expression level (normalized RNA-seq read  
187 counts; mean  $\pm$  SD) of the same set of genes in unstimulated (Ctr) and E2-stimulated HSF1-proficient and HSF1-  
188 deficient cells. (G) Nascent RNA gene expression analyses by RT-qPCR in MCF7 cells created for validation  
189 using DNA-free CRISPR/Cas9 system (HSF1+, six clones with the normal HSF1 level; HSF1–, six HSF1-  
190 negative clones). The upper panel shows E2-stimulated changes (E2 vs Ctr fold change; E2 treatment: 10 nM, 4  
191 h), lower panel shows basal expression level represented as fold differences between untreated (Ctr) wild-type,  
192 HSF1+, and HSF1– cells. Total RNA analyzes are shown in Fig. S4C. \*\*\*p < 0.0001, \*\*p < 0.001, \*p < 0.05  
193 (above the bar – compared to the corresponding control, between the bars – between cell variants).

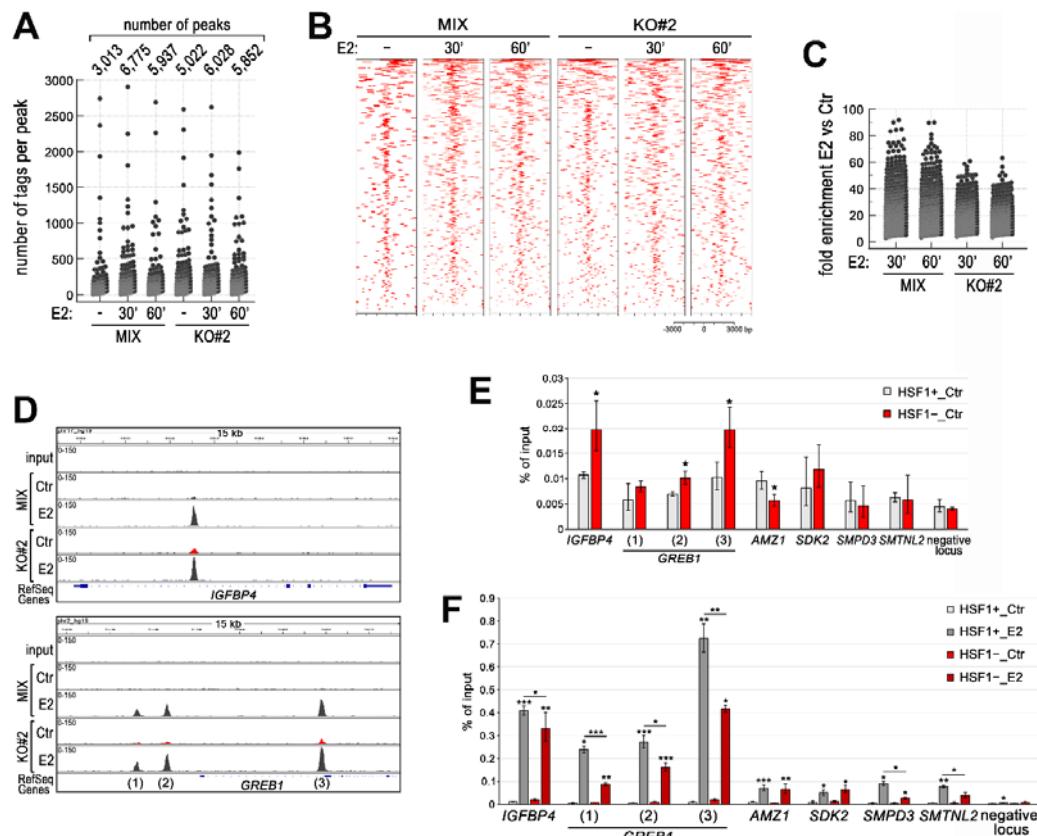
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195 **HSF1 influences the binding of ESR1 to chromatin**

196 To further study the influence of HSF1 on estrogen signaling, we analyzed ESR1 binding to chromatin  
197 in HSF1-proficient and HSF1-deficient MCF7 cells. A list of all ESR1 binding sites detected by ChIP-seq in  
198 unstimulated cells and after 30 or 60 minutes of E2 treatment is presented in Supplementary Dataset 2. These  
199 analyses revealed that in unstimulated cells, ESR1 binding was more efficient (more binding sites and increased  
200 number of tags per peak) in HSF1-deficient cell variant (KO#2) than in corresponding HSF1-proficient control  
201 (MIX cells) (Fig. 3A, B) (it is worth noting that the MIX cell variant was also different from wild type cells,  
202 indicating that the genome organization was affected by the CRISPR/Cas9 procedure itself, possibly due to off-  
203 targets). ESR1 target sequences in *IGFBP4* or *GREB1* are examples of such increased binding efficiency in  
204 unstimulated HSF1-deficient cells (Fig. 3D). Estrogen treatment for 30 or 60 minutes resulted in enhanced ESR1  
205 binding in all cell variants. However, fold enrichment (E2 versus Ctr) was lower in HSF1-deficient cells than in  
206 HSF1-proficient cells (Fig 3C). Moreover, the number of detected peaks in the E2-treated HSF1-deficient cells  
207 was only slightly higher than in unstimulated cells (Fig. 3A) and enhanced ESR1 binding was primarily  
208 manifested in sites already existing in unstimulated cells (Fig. 3C, D). We additionally searched for ESR1  
209 binding preferences in HSF1-proficient and HSF1-deficient cells. After estrogen treatment, ESR2 and ESR1  
210 motifs were centrally enriched in ESR1 binding regions in all cell variants (Fig. S5). Moreover, in untreated  
211 cells, the motif for PBX1 (not centrally enriched in peak regions), which is a pioneer factor known to bind to the  
212 chromatin before ESR1 recruitment (Magnani et al., 2011), was identified by MEME-ChIP analysis in all cell  
213 variants (not shown). This indicates that ESR1 chromatin binding preferences were not substantially changed in  
214 HSF1-deficient cells.

215 To validate ChIP-seq results, we analyzed the influence of HSF1 on the binding of ESR1 to selected  
216 target sites by ChIP-qPCR using the novel MCF7 CRISPR/Cas9 model. In the case of *IGFBP4* and *GREB1* (i.e.  
217 sequences highly enriched with ESR1 after E2 stimulation), the binding efficiency of ESR1 (shown as a percent  
218 of input) was higher in unstimulated HSF1- cells than in corresponding HSF1+ cells (Fig. 3E). On the other  
219 hand, although estrogen treatment strongly induced ESR1 binding, this induction was considerably lower in  
220 HSF1- cells (Fig. 3F). Therefore, we validated ChIP-seq results and confirmed that in strongly-responsive ESR1  
221 binding sites deficiency of HSF1 correlated with enhanced binding of unliganded ESR1 and weaker enrichment  
222 of ESR1 binding upon estrogen stimulation. However, other patterns of the response are also possible, especially  
223 in sequences that were weakly enriched in ESR1 after stimulation, as exemplified by *AMZ1*, *SDK2*, *SMPD3*, and  
224 *SMTNL2* (Fig. 3E, F).

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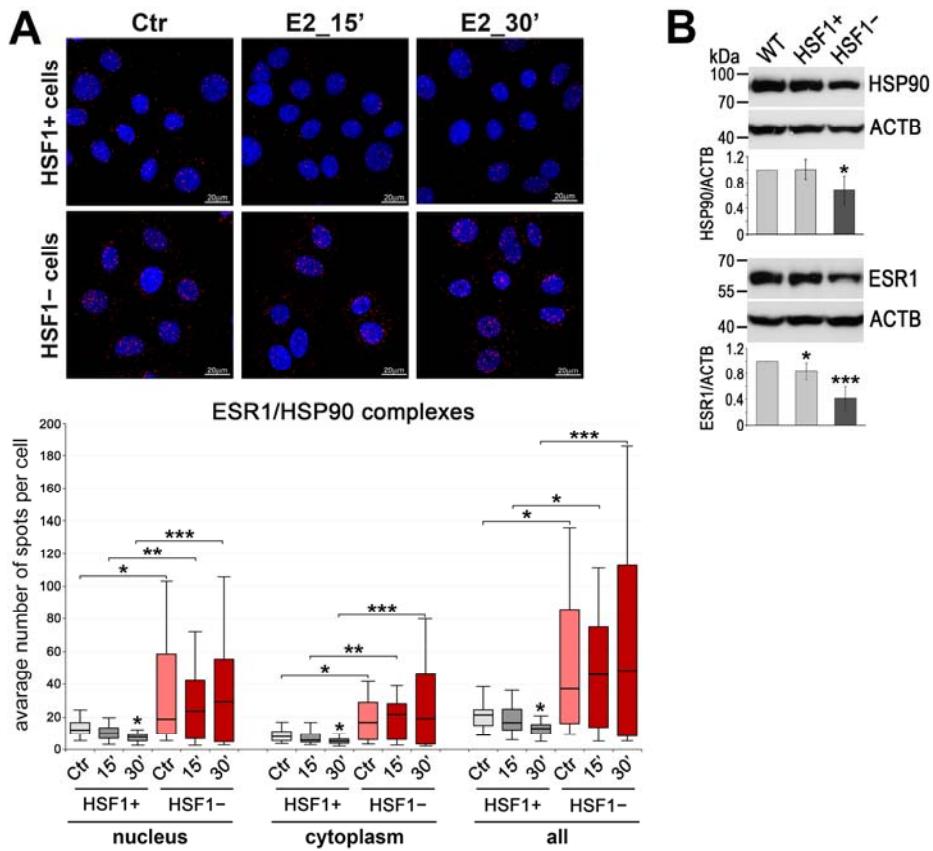
227 **Fig. 3. HSF1 deficiency influences the binding of ESR1 to chromatin in ER-positive MCF7 cells.** (A)  
228 Number of peaks and peak size distribution (number of tags per peak), (B) heatmap visualization of ESR1 ChIP-  
229 seq data (versus input), and (C) binding enrichment (fold enrichment E2 versus Ctr) after E2 stimulation (10 nM  
230 for 30 or 60 minutes) in HSF1-deficient cells (KO#2) and corresponding control (MIX, a combination of control  
231 clones arisen from single cells following CRISPR/Cas9 gene targeting). Heatmaps depict all ESR1 binding  
232 events centered on the peak region within a 3 kb window around the peak. Peaks in each sample were ranked on

233 intensity. **(D)** ESR1 peaks identified by MACS in ChIP-seq analyses and visualized by the IGV browser in  
234 unstimulated cells (Ctr) and after E2 treatment (10 nM, 30 min). The scale for each sample is shown in the left  
235 corner. Peaks showing more efficient local ESR1 binding in untreated HSF1-deficient cells are marked in red.  
236 **(E)** Comparison of ESR1 binding efficiency (by ChIP-qPCR) in selected sequences in untreated MCF7 cells  
237 created for validation using DNA-free CRISPR/Cas9 system: HSF1+, six clones with the normal HSF1 level;  
238 HSF1-, six HSF1-negative clones. **(F)** ESR1 binding after E2 stimulation: Ctr, untreated cells; E2, 17 $\beta$ -estradiol  
239 treatment (10 nM, 30 min). \*\*\*p < 0.0001, \*\*p < 0.001, \*p < 0.05 (above the bar – compared to the  
240 corresponding control, between the bars – between cell variants).

241

## 242 **HSF1 deficiency is associated with altered interactions between HSP90 and ESR1**

243 ESR1 is known to be kept in an inactive state by HSP90 (Pratt and Toft, 1997), in particular by  
244 HSP90AA1 (Dhamad et al., 2016) that is the HSF1 transcriptional target. Thus, looking for a reason for the  
245 dysregulated ESR1 binding in HSF1-deficient cells we focused on ESR1 and HSP90 interactions. Analyzes of  
246 the proximity of both proteins by PLA revealed that the number of ESR1/HSP90 complexes decreased after  
247 estrogen treatment in HSF1+ MCF7 cells (Fig. 4A). This indicates that liganded (and transcriptionally active)  
248 ESR1 is indeed released from the inhibitory complex with HSP90. *HSP90AA1* expression was substantially  
249 reduced in HSF1-deficient cells (RNA-seq analyses), which correlated with the reduced HSP90 protein level.  
250 Also, the ESR1 level was considerably decreased in most HSF1-deficient cell variants (except KO # 1 cells; not  
251 shown), especially in cells cultured in phenol-free media (Fig. 4B, Fig. S4A). Therefore, we hypothesized that  
252 the number of ESR1/HSP90 complexes could be reduced in HSF1-deficient cells, which would result in  
253 enhanced basal transcriptional activity of ESR1 in untreated cells. However, we observed an increased number  
254 of such complexes both in untreated and E2-stimulated HSF1-deficient cells when compared to HSF1-proficient  
255 cells (Fig. 4A). This indicates that the response to estrogen could be dysregulated in HSF1-deficient cells, also at  
256 the level of ESR1/HSP90 interactions, in a mechanism not related directly to the HSP90 and ESR1  
257 downregulation mediated by the HSF1 deficiency.



258

259

260 **Fig. 4. HSF1-deficiency is associated with the reduced HSP90 and ESR1 levels and altered HSP90/ESR1**  
261 **interactions.** (A) Interactions between ESR1 and HSP90 assessed by Proximity Ligation Assay (PLA; red spots)

262 in HSF1-positive (HSF1+) and HSF1-negative (HSF1-) MCF7 cells created using DNA-free CRISPR/Cas9  
263 system. Ctr, untreated cells; E2, 17 $\beta$ -estradiol treatment (10 nM). DNA was stained with DAPI. Scale bar, 20  
264  $\mu$ m. The mean number of spots per cell is shown in the boxplot below. The line dividing the box represents the  
265 median and the upper and lower side of the box shows the upper and lower quartiles, respectively. The whiskers  
266 show the highest and lowest values. \*\*\*p < 0.0001, \*\*p < 0.001, \*p < 0.05 (above the bar – compared to the  
267 corresponding control, between the bars – between cell variants). For detection of HSP90 and ESR1 by  
268 immunofluorescence see Fig. S6. (B) HSP90 and ESR1 levels assessed by western blot. Actin (ACTB) was used  
269 as a protein loading control. The graphs show the results of densitometric analyses (n=4). \*\*\*p < 0.0001, \*p <  
270 0.05.

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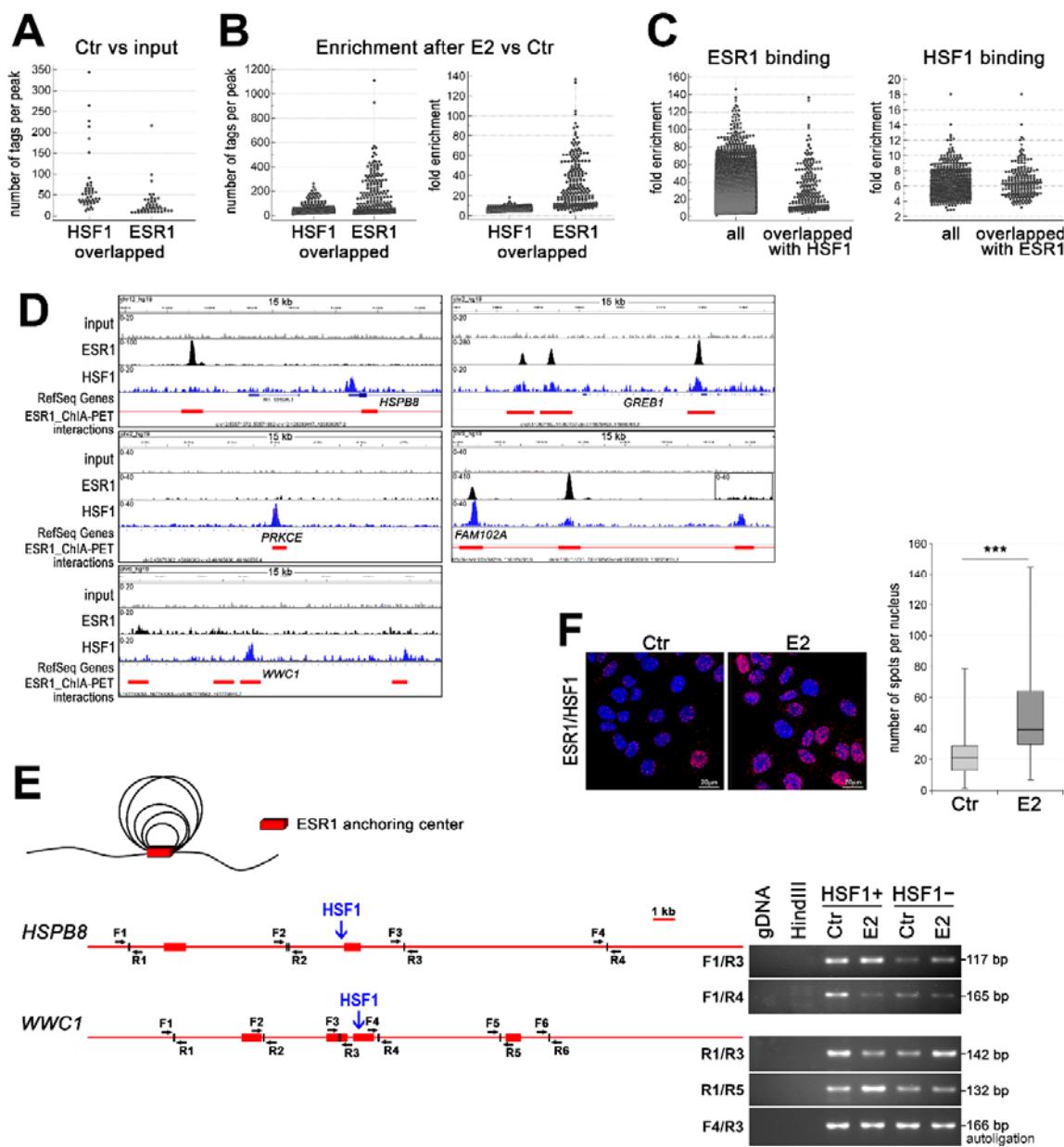
272 **HSF1 can cooperate with ESR1 in chromatin binding and participate in the spatial organization of**  
273 **chromatin loops**

274 Since estrogen-activated HSF1 has been shown to bind to chromatin, we compared the binding patterns  
275 of ESR1 and HSF1 in wild-type MCF7 cells (using our ChIP-seq data deposited in the NCBI GEO database; acc.  
276 no. GSE137558; (Vydra et al., 2019)). Although in untreated cells (Ctr) there were 1,535 and 2,248 annotated

277 peaks for ESR1 and HSF1 respectively (compared to the input), only a few (below 50) binding sites with  
278 overlapped peaks for both transcription factors were identified. Moreover, these common binding regions were  
279 characterized by a small number of tags (smaller in the case of ESR1) (Fig. 5A; Supplementary Dataset 3, sheet  
280 1). On the other hand, the search for ESR1 and HSF1 common binding regions created after estrogen treatment  
281 (E2 vs Ctr) returned more than 200 peaks (Supplementary Dataset 3, sheet 2). Numbers of tags per peak and fold  
282 enrichment increased after E2 stimulation for both factors yet more for ESR1 than HSF1 binding in such regions  
283 (Fig. 5B). Although distributions of the fold enrichment after E2 stimulation in all peaks for each transcription  
284 factor separately and the overlapped ones were similar, sites mapped to the common binding regions represented  
285 only a small fraction of the total number of ESR1 binding sites (~2.5% from 8,320 peaks; in the case of HSF1  
286 this represents 35% of 571 peaks) (Fig. 5C). These results suggest that co-binding of both factors in the same  
287 DNA region is not critical in the regulation of the ESR1 transcriptional activity. Instead, we postulate that HSF1  
288 may influence the organization of the chromatin loops created after estrogen stimulation. There were different  
289 patterns of estrogen-stimulated binding of ESR1 and HSF1 to chromatin (Fig. S7). Generally, more abundant  
290 ESR1 binding was observed at overlapped sites (e.g. in the case of *GREB1*; Fig. 5D and Fig. S7A), but HSF1  
291 binding could be stronger as well (Fig. S7B). When we combined ESR1 and HSF1 ChIP-seq peaks with data  
292 from chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) performed by (Fullwood et al.,  
293 2009), it was evident that the HSF1-binding sites mapped to ESR1-interacting loci (ESR1 anchor regions) even  
294 if actual ESR1 binding was not detected in the same locus (examples of such anchors in *FAM102A*, *HSPB8*,  
295 *PRKCE*, and *WWCI* regulatory sequences are shown in Fig 5D). HSF1 peaks unrelated to ESR1 anchoring were  
296 also existing (Fig. S7C). Further analyzes of the spatial organization of chromatin by chromosome conformation  
297 capture (3C) technique revealed that some interactions between different ESR1 anchor regions were dependent  
298 on the presence of HSF1. This is exemplified by *HSPB8* and *WWCI* loci analyzed in HSF1-proficient and HSF1-  
299 deficient cells (Fig. 5E), which confirms the role of HSF1 in the formation of ESR1-mediated chromatin loops.

300 Though the co-binding of HSF1 and ESR1 to DNA was rare and relatively weak, particularly in  
301 untreated cells, the proximity of both factors was easily detected. In general, both transcription factors co-  
302 localized in the nucleus when assessed by immunofluorescence (Fig. S6). Thus, PLA spots indicating putative  
303 HSF1/ESR1 interactions were mainly located in the nucleus and their number dramatically increased after E2  
304 treatment (Fig. 5F). However, large diversity was observed between individual cells, which suggests that also  
305 HSF1 binding to DNA may be differentiated at the single-cell level. Nevertheless, we concluded that the  
306 proximity of HSF1 and ESR1 putatively reflecting their interactions frequently happens in the cell nucleus.

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309

310 **Fig. 5. HSF1 may cooperate with ESR1 in chromatin binding and take a part in chromatin organization.**  
311 (A) Overlapped HSF1 and ESR1 ChIP-seq peaks in untreated wild-type MCF7 analyzed for peak size  
312 distribution (number of tags per peak). (B) Overlapped HSF1 and ESR1 ChIP-seq peaks in wild-type MCF7 after  
313 E2 stimulation analyzed for peak size distribution (number of tags per peak) and fold enrichment. (C)  
314 Comparison of the binding enrichment (E2 vs Ctr) of all ESR1 and HSF1 peaks and overlapped peaks identified  
315 after stimulation in wild-type MCF7 cells. (D) Examples of ESR1 and HSF1 peaks identified by MACS in ChIP-  
316 seq analyses in wild-type MCF7 cells after E2 treatment and corresponding ChIA-PET interactions (Fullwood et  
317 al., 2009) downloaded from ENCODE database and visualized by the IGV browser. The red bar shows the ESR1  
318 anchor region (interacting loci), red line – the intermediate genomic span between the two anchors forming a  
319 putative loop; the scale for each sample is shown in the left corner. (E) ESR1-mediated chromatin interactions

320 analyzed by chromosome conformation capture (3C) technique in *HSPB8* and *WWCI* loci. The scheme  
321 represents ESR1 anchor regions (red bars), HSF1 binding sites (blue arrows), and forward (F) and reverse (R)  
322 primers around subsequent *HindIII* cleavage sites. A model of chromatin loops resulting from interactions  
323 between ESR1 anchor regions is also illustrated above. Interactions between selected DNA regions were  
324 analyzed by PCR in untreated and E2-stimulated HSF1+ and HSF1- cells. (F) Interactions between ESR1 and  
325 HSF1 assessed by PLA (red spots) in wild-type MCF7 cells after E2 treatment. DNA was stained with DAPI.  
326 Scale bar, 20  $\mu$ m. The number of spots per nucleus is shown (boxplots represent the median, upper and lower  
327 quartiles, maximum and minimum. \*\*\*p < 0.0001. E2, 10 nM for 60 minutes (or 30 minutes for 3C).

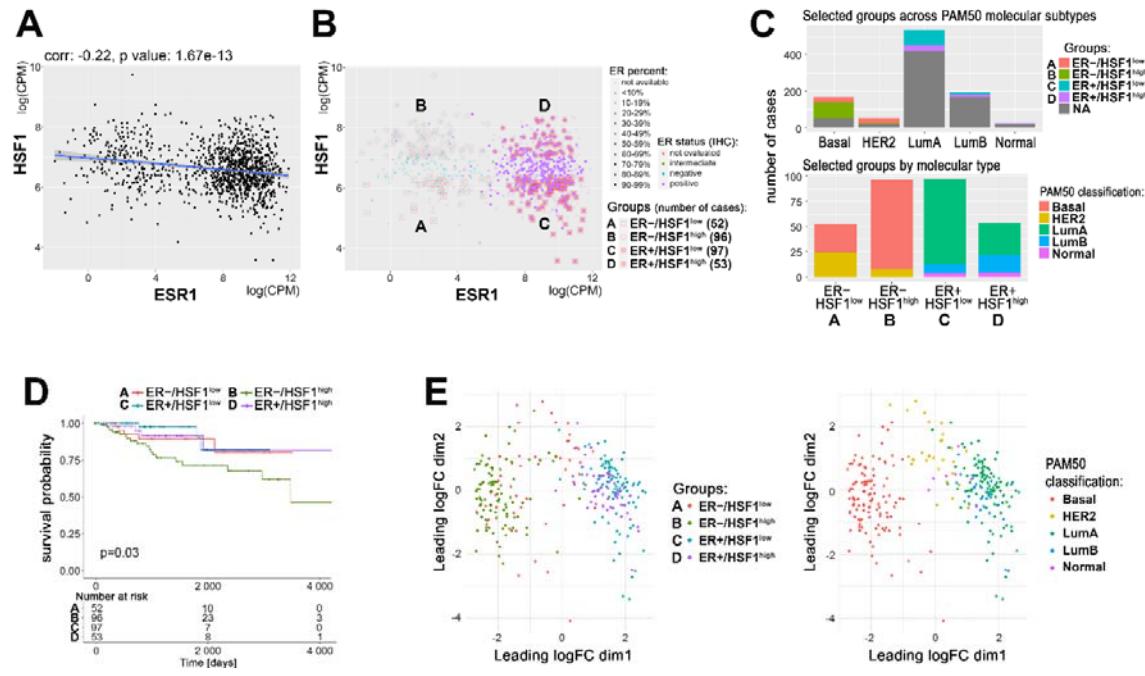
328

329 **The combined expression level of ESR1 and HSF1 can be used to predict the survival of breast cancer**  
330 **patients**

331 Our *in vitro* analyses indicated that HSF1 could support the transcriptional action of ESR1 upon  
332 estrogen treatment. On the other hand, HSF1-regulated chaperones are necessary to keep estrogen receptors in an  
333 inactive state in the absence of ligands, which collectively indicated important functional crosstalk between both  
334 factors. Therefore, to further study the significance of the interaction between ESR1 and HSF1 in actual breast  
335 cancer, we utilized RNA-seq data deposited in the TCGA database. The analysis revealed that the transcript level  
336 of *HSF1* negatively correlated with the *ESR1* transcript level, although this tendency was relatively weak (Fig.  
337 6A). Neither *ESR1* nor *HSF1* transcript levels analyzed separately had a significant prognostic value (Fig. S8).  
338 Therefore, out of all breast cancer cases, we selected four groups characterized by different levels of ESR1  
339 (mRNA and protein level) and *HSF1* (mRNA) expression: ER-/HSF1<sup>low</sup>, ER-/HSF1<sup>high</sup>, ER+/HSF1<sup>low</sup>, and  
340 ER+/HSF1<sup>high</sup> (Fig. 6B). These groups varied in molecular subtypes composition. In ER+ cancers (luminal A,  
341 luminal B, and normal-like), the HSF1<sup>low</sup> group was more homogenous (mostly luminal A) than the HSF1<sup>high</sup>  
342 group. In ER- cases (basal-like and HER2-enriched), the HSF1<sup>high</sup> group was more homogenous (mostly basal-  
343 like) (Fig. 6C). Analyses of the survival probability showed that a high *HSF1* expression had a greater negative  
344 effect on the survival of ER- than ER+ cancers. The most divergent groups were: ER+/HSF1<sup>low</sup> and  
345 ER-/HSF1<sup>high</sup> (better and worse prognosis, respectively; p=0.0044), which represented luminal A and basal-like  
346 enriched groups (Fig. 6D). These analyses indicate that *HSF1* and *ESR1* may have an additive effect on survival  
347 and have prognostic value only if analyzed together. The difference between ER+/HSF1<sup>low</sup> and ER-/HSF1<sup>high</sup>  
348 cancers was also clearly visible in the multidimensional scaling (MDS) plots where the cancer cases belonging to  
349 these groups were separated. MDS plotting generally separated ER+ cases from ER-/HSF1<sup>high</sup> cases, while  
350 ER-/HSF1<sup>low</sup> cases were scattered between them (Fig. 6E). On the other hand, HSF1<sup>high</sup> and HSF1<sup>low</sup> cases were  
351 not separated although they were slightly shifted against each other. When looking at molecular subtypes, it

352 became apparent that ER-/HER2-positive cancers were separated from ER-/basal-like cancers and slightly  
353 overlapped with ER+ cancers.

354



355

356

357 **Fig. 6. Relationship between ESR1 and HSF1 expression in breast cancer.** (A) Correlation of *HSF1* and  
358 *ESR1* transcript level in all TCGA breast cancers. Each spot represents one cancer case; log(CPM), log2-counts  
359 per million. (B) Groups with different mRNA levels of *HSF1* and *ESR1* selected for further analyses. In the case  
360 of *ESR1*, also the protein level assessed by immunohistochemistry (IHC) was taken into consideration. (C)  
361 Characteristics of selected groups in relation to the molecular subtypes of breast cancer. (D) Kaplan-Meier plots  
362 for all selected groups. (E) MDS plots of selected cases with marked: ER and HSF1 statuses (left) or molecular  
363 subtypes (right). ER+/-, estrogen receptor-positive/negative; HSF1<sup>high</sup>, high *HSF1* level, HSF1<sup>low</sup>, low *HSF1*  
364 level.

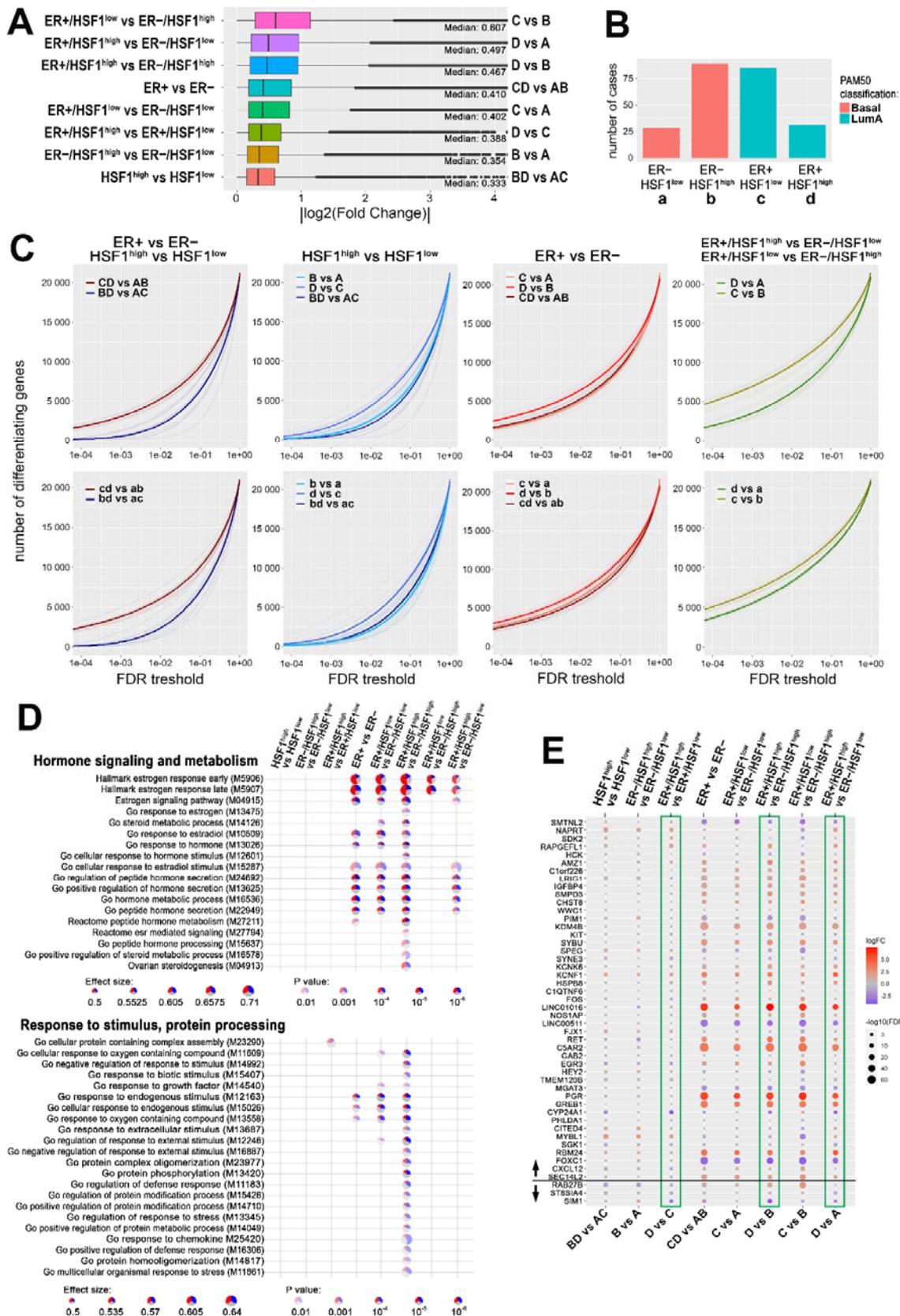
365

### 366 HSF1 increases the diversity of the transcriptome in ER-positive breast cancers

367 Furthermore, we analyzed global gene expression profiles in breast cancers with different ESR1 and  
368 HSF1 statuses. Differential expression tests between the above-selected groups of patients (Supplementary  
369 Dataset 4) revealed that generally, ESR1 had a much stronger influence on the transcriptome (i.e., ER+ versus  
370 ER-) than HSF1 (i.e., HSF1<sup>high</sup> versus HSF1<sup>low</sup>). Nevertheless, differences between ER+ and ER- cases were  
371 higher in the presence of high levels of HSF1, which implicates that HSF1 increases the diversity of the

372 transcriptome of ER+ cancers. Also, the differences in the transcript levels between HSF1<sup>high</sup> and HSF1<sup>low</sup>  
373 cancers were higher in ER+ than ER- cases (Fig. 7A). Remarkably, the most divergent were ER+/HSF1<sup>low</sup> and  
374 ER-/HSF1<sup>high</sup> cancers, which resembled the most significant differences in the survival probability (Fig. 6D).  
375 Then, we looked at differences in numbers of differently expressed genes (DEGs) between patients' groups. To  
376 eliminate the possible influence of the group size on DEGs, we repeated each test 10 times, randomly  
377 subsampling groups to an equal number of cases and averaging the number of DEGs. Furthermore, to check  
378 whether heterogeneity of selected groups regarding molecular subtypes could affect observed differences in gene  
379 expression profiles, only basal-like (ER-) and luminal A (ER+) cancers were included in these tests (Fig. 7B). In  
380 general, these analyses also revealed that the number of genes differentiating ER+ and ER- cases were higher in  
381 HSF1<sup>high</sup> cancers, while the number of genes differentiating HSF1<sup>high</sup> and HSF1<sup>low</sup> cases was higher in ER+  
382 cancers. The most divergent were again ER+/HSF1<sup>low</sup> and ER-/HSF1<sup>high</sup> cases while the most similar,  
383 ER-/HSF1<sup>low</sup> and ER-/HSF1<sup>high</sup> (Fig. 7C). This tendency was maintained when groups with mixed molecular  
384 subtypes composition were analyzed as well as more homogenous cancer groups (i.e., only basal-like and  
385 luminal A). Differences in gene expression profiles between pairwise compared groups of cancer are further  
386 illustrated on volcano plots that additionally separated upregulated and downregulated genes (Fig. S9). We  
387 further searched for the hypothetical influence of the HSF1 status on functions of ESR1-related genes in actual  
388 cancer tissue. The geneset enrichment analysis identified terms related to estrogen response among the most  
389 significant ones associated with transcripts differentiating between ER+ and ER- cancers (Fig. S10). The more  
390 detailed analysis focused on terms related to hormone signaling and metabolism showed differences between  
391 HSF1<sup>high</sup> and HSF1<sup>low</sup> cases when ER+ and ER- cancers were compared. These analyses indicate that HSF1 may  
392 enhance estrogen signaling. On the other hand, the analysis focused on terms related to response to stimulus and  
393 protein processing (i.e., functions presumed to be dependent on HSF1 action via the HSPs expression), revealed  
394 that most of them reached the statistical significance of differences between ER+/HSF1<sup>high</sup> and ER-/HSF1<sup>high</sup>  
395 cases (Fig. 7D).

396



399 **Fig. 7. HSF1 increases the diversity of the transcriptome of ER-positive breast cancer.** (A) Boxplots of fold  
400 changes (logFC absolute values) illustrating differences in gene expression between groups characterized in  
401 Fig. 6. The line dividing the box represents the median of the data and the right and left side of the box shows the  
402 upper and lower quartiles respectively. The whiskers show the highest and lowest values, excluding outliers,  
403 which are shown as circles. (B) Composition of ER+ and ER- groups with different levels of HSF1 reduced to  
404 one molecular subtype. (C) The number of differently expressed genes (y-axis) plotted cumulatively against the  
405 False Discovery Rate value of differences (x-axis). Comparisons of ER+ and ER- cancer cases as well as  
406 HSF1<sup>high</sup> and HSF1<sup>low</sup> cases (upper graphs; all cases, for group indexes see Panel A) and cases from pre-selected  
407 cancer types (lower graphs; for group indexes see Panel B). (D) Geneset enrichment analyses showing  
408 differences between ER+ and ER- breast cancers with different HSF1 levels. Terms related to hormone  
409 signaling and metabolism and response to stimulus and protein processing in comparisons between groups  
410 selected in Fig. 6B. Blue – a fraction of down-regulated genes, red – fraction of up-regulated genes. (E)  
411 Differences in the expression of the E2-regulated gene set (as identified in MCF7 cells by RNA-seq; see Fig. 2)  
412 between breast cancers with different levels of ESR1 and HSF1 selected from the TCGA database and qualified  
413 to four groups as shown in Fig. 6B. FDR, False Discovery Rate; logFC, log fold change. Green boxes mark all  
414 possible comparisons between the ER+/HSF1<sup>high</sup> group to other groups. The black horizontal line separates genes  
415 up- and down-regulated after E2 treatment in MCF7 cells.  
416

417 We additionally compared the expression of E2-regulated genes (the set identified in MCF7 cells by  
418 RNA-seq, i.e. 47 up-regulated and 3 down-regulated genes; Fig. 2) in selected groups of breast cancers with  
419 different levels of ESR1 and HSF1. The analysis revealed the highest up-regulation of *PGR* and *LINC01016*  
420 genes in ER+ compared to ER- cancers (regardless of HSF1 status) (Fig. 7E). It is noteworthy, however, that not  
421 all genes up-regulated by E2 in MCF7 cells revealed an increased expression level in ER+ compared to ER-  
422 cancers. Especially, *FOXC1* and *LINC00511* were expressed at a higher level in ER- cancers. Moreover,  
423 regardless of ER status, cancers with high HSF1 levels revealed a higher expression of *MYBL1* and *NAPRT* than  
424 cancers with low HSF1 levels. Furthermore, expression of few genes systematically differentiated cancers with  
425 high levels of both factors (ER+/HSF1<sup>high</sup>) compared to cancers with the low level of at least one factor  
426 (including *RAPGEFL1*, *AMZ1*, *KCNFI*, *HSPB8* up-regulated, and *CYP24A1*, *SIM1* down-regulated in  
427 ER+/HSF1<sup>high</sup> cancers), which was consistent in all relevant comparisons (marked with green boxes in Fig. 7E).  
428 Nevertheless, the observed features of gene expression profiles confirmed collectively that HSF1 affects the  
429 genomic action of ESR1 in breast cancer.  
430

### 431 **Discussion**

432 The precise mechanisms by which estrogens stimulate the proliferation of breast cancer cells are still  
433 unclear. We found that HSF1-deficiency in ER-positive breast cancer cells could slow down the mitogenic

434 effects of estrogen. This may be a consequence of a reduced transcriptional response to estrogen in these cells  
435 and therefore implies that HSF1 may support estrogen action. Indeed, analyses of the transcriptome of breast  
436 cancers from the TCGA database showed higher transcriptome diversity in ER-positive cases with high  
437 expression of *HSF1* than with low *HSF1* levels. High HSF1 nuclear levels (estimated by immunohistochemistry  
438 in patients with invasive breast cancer at diagnosis; *in situ* carcinoma only and stage IV breast cancer were  
439 excluded from outcome analysis) were previously associated with decreased survival specifically in ER-positive  
440 breast cancer patients (Santagata et al., 2011). However, in another study performed on samples from patients  
441 with ER-positive tumors, only a weak association was found between the HSF1 protein expression and poor  
442 prognosis (Gökmen-Polar and Badve, 2016). Nevertheless, both studies showed a significant correlation between  
443 *HSF1* transcript levels and the survival in ER-positive breast cancer patients. In our analysis, using non-  
444 preselected data from the TCGA gene expression database, we found that although high *HSF1* levels slightly  
445 reduced the survival in ER-positive cancer patients, they had a greater negative outcome on survival in ER-  
446 negative patients. Therefore, we concluded that *HSF1* had prognostic value when analyzed together with *ESR1*  
447 transcript level.

448 The mechanism of supportive HSF1 activity in ER-positive cells was already proposed, by which upon  
449 E2 treatment, HSF1 is phosphorylated via ESR1/MAPK signaling, gains transcriptional competence, and  
450 activates several genes essential for breast cancer cell growth and/or ESR1 action (Vydra et al., 2019). Here we  
451 found that HSF1-deficiency results in a weaker response to estrogen stimulus of many estrogen-induced genes. It  
452 is noteworthy, that the reduced transcriptional response to estrogen could at least partially result from the  
453 enhanced binding of unliganded ESR1 to chromatin and higher basal expression of ESR1-regulated genes. This  
454 suggests that HSF1-dependent mechanisms may amplify ESR1 action upon estrogen stimulation while inhibiting  
455 it in the absence of ligands. The proper action of ERs depends on HSF1-regulated chaperones, especially HSP90.  
456 As expected, the number of HSP90/ESR1 complexes decreased after ligand (E2) binding in cells with the normal  
457 level of HSF1. However, although HSP90 was down-regulated in HSF1-deficient cells, more HSP90/ESR1  
458 complexes were found both in untreated and estrogen-stimulated cells. Hence, increased activity of ESR1 in  
459 HSF1-deficient cells could not be explained by reduced sequestration of unliganded ESR1 by HSP90.  
460 Accordingly, additional HSF1-dependent factors may influence formation of these complexes. Ligand-  
461 independent genomic actions of ESR1 are also regulated by growth factors that activate protein-kinase cascades,  
462 leading to phosphorylation and activation of nuclear ERs at EREs (Stellato et al., 2016). Involvement of HSF1  
463 in the repression of estrogen-dependent transcription was reported in MCF7 cells treated with heregulin (NRG1),

464 the ligand for the NEU/ERBB2 receptor tyrosine kinase (Khaleque et al., 2008). Interactions of HSF1 with the  
465 co-repressor metastasis-associated protein 1 (MTA1) and several additional chromatin-modulating proteins were  
466 implicated in that process. Therefore, it cannot be ruled out that HSF1 influences unliganded and liganded ESR1  
467 by various mechanisms that have to be further investigated.

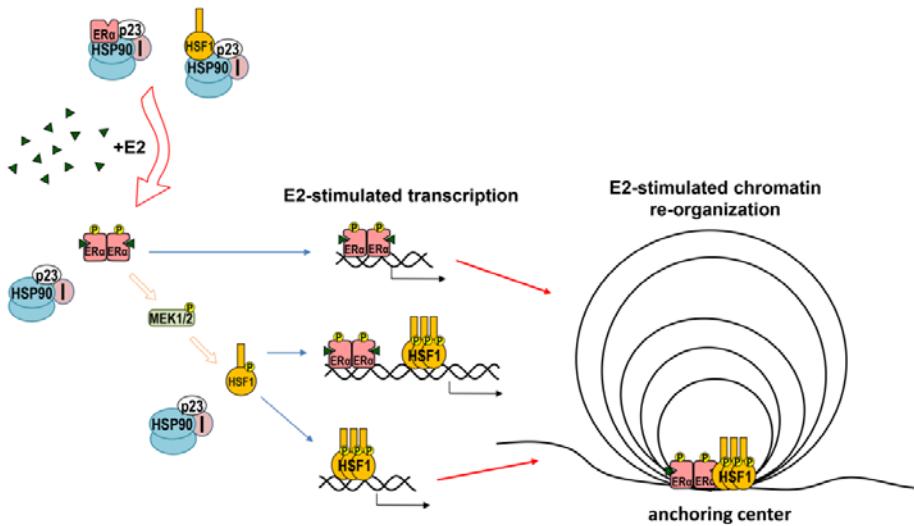
468 Transcriptional activation by ESR1 is a multistep process modulated by coactivators and corepressors.  
469 Cofactors interact with the receptor in a ligand-dependent manner and are often part of large multiprotein  
470 complexes that control transcription by recruiting components of the basal transcription machinery, regulating  
471 chromatin structure, and/or modifying histones (Welboren et al., 2009) (Kovács et al., 2020) (Pescatori et al.,  
472 2021). Liganded ESR1 may bind directly to DNA (to ERE), and indirectly via tethering to other transcription  
473 factors such as FOS/JUN (AP1), STATs, ATF2/JUN, SP1, NF $\kappa$ B (Björnström and Sjöberg, 2005) (Welboren et  
474 al., 2009) (Heldring et al., 2011). Here we found that HSF1 can potentially be an additional factor tethering  
475 liganded ESR1 to DNA. ESR1 has been shown to function via extensive chromatin looping to bring genes  
476 together for coordinated transcriptional regulation (Fullwood et al., 2009). Since ESR1 anchor sites were  
477 identified also in sites bound by HSF1 but not ESR1, we propose that HSF1 may be a part of this “looping”  
478 machinery (other components in the same anchoring center are also possible). In general, estrogen-induced  
479 HSF1 binding was weaker than ESR1 binding. However, PLA analyses indicated a large heterogeneity in a cell  
480 population regarding ESR1 and HSF1 interactions and showed individual cells in which such interactions  
481 induced by estrogen were very strong. The final transcriptional activity of ESR1 is modulated by interactions  
482 with various tethering factors, including HSF1. Therefore we hypothesize that it can be modulated differently at  
483 the single cell level by different cofactors and chromatin remodeling factors. Thus, the response measured on the  
484 whole cell population is heterogeneous and stochastic when a single cell is considered.

485 Estrogen-dependent cancers are treated with hormonal therapies, and high levels of HSF1 have been  
486 associated with antiestrogen resistance (Silveira et al., 2021). It was proposed that overexpression of HSF1 in  
487 ER-positive breast cancers was associated with a decreased dependency on the ER $\alpha$ -controlled transcriptional  
488 program for cancer growth. However, this conclusion was based on experiments performed without estrogen  
489 stimulation. Our *in vitro* studies indicate that the influence of HSF1 on ESR1 action depends on the presence of  
490 the estrogen and HSF1 may repress ER $\alpha$ -controlled transcriptional program only in the absence of the ligand.  
491 Enhanced resistance to hormonal therapies could be mediated by HSF1-regulated genes. Heat shock proteins  
492 themselves can be prognostic factors in breast cancer and especially oncogenic properties of HSP90AA1  
493 correlated with aggressive clinicopathological features and resistance to the treatment (Whitesell et al., 2014)

494 (Klimczak et al., 2019). Here we proposed a novel mechanism of the HSF1 action in ER-positive breast cancers,  
495 which is independent of typical HSF1-regulated genes. This mechanism assumes that HSF1 influences the  
496 transcriptional response to estrogen via the re-organization of chromatin structure in estrogen-responsive genes.  
497 This mode of HSF1 action may be important in all ESR1-expressing cells. ESR1 is for example a critical  
498 transcription factor that regulates epithelial cell proliferation and ductal morphogenesis during postnatal  
499 mammary gland development. It is noteworthy that HSF1 has been shown to promote mammary gland  
500 morphogenesis by protecting mammary epithelial cells from apoptosis and increasing their proliferative capacity  
501 (Xi et al., 2012).

502 In conclusion, HSF1 and ESR1 cooperate in response to estrogen stimulation. Estrogen via ESR1 and  
503 MAPK activates HSF1 (Vydra et al., 2019) which together with ESR1 forms new chromatin loops that enhance  
504 estrogen-stimulated transcription (Fig. 8). Moreover, HSF1 may be involved in repression of unliganded ESR1.  
505 Genes activated by ESR1 and HSF1 play an important role in regulating the growth of estrogen-dependent  
506 tumors and the combination of both factors has a prognostic value in breast cancer patients.

507



508

509

510 **Fig. 8. Model of cooperation between ESR1 (ER $\alpha$ ) and HSF1 in response to estrogen (E2) stimulation.**  
511 Both ESR1 and HSF1 are kept in an inactive state by the complexes of HSP90, p23, and immunophilins (I).  
512 Binding of E2 to ESR1 is connected with the release of the chaperone complex and activation of ESR1 leading  
513 to the phosphorylation of MEK1/2 followed by HSF1 activation. Oligomers of active transcription factors can  
514 bind to DNA and cooperate in the regulation of the transcription either directly or through chromatin re-  
515 organization.

516

517

518 **Materials and Methods**

519 **Cell lines and treatments:** Human MCF7 and T47D ER $\alpha$ -positive breast cancer cell lines were purchased from  
520 the American Type Culture Collection (ATCC, Manassas, VA, USA) and the European Collection of  
521 Authenticated Cell Cultures (ECACC, Porton Down, UK), respectively. Cells were cultured in DMEM/F12  
522 medium (Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS) (EURx,  
523 Gdansk, Poland). For heat shock, logarithmically growing cells were placed in a water bath at a temperature of  
524 43 °C for one hour. The cells were allowed to recover for the indicated time in a CO<sub>2</sub> incubator at 37 °C. For  
525 estrogen treatment, cells were seeded on plates and the next day the medium was replaced into a phenol-free  
526 medium supplemented with 5% or 10% dextran-activated charcoal-stripped FBS (PAN-Biotech GmbH,  
527 Aidenbach, Germany). 17 $\beta$ -estradiol (E2; Merck KGaA) was added 48 hours later to a final concentration of 10  
528 nM (an equal volume of ethanol was added as vehicle control) for the indicated time. For longer E2 treatments,  
529 the medium was changed every two days. The growth media were not replaced either before or after treatments.  
530 Cells were routinely tested for mycoplasma contamination.

531 **HSF1 down-regulation using shRNA.** The shRNA target sequence for human HSF1 (NM\_005526.4) was  
532 selected using the RNAi Target Sequence Selector (Clontech, Mountain View, CA, USA). The target sequences  
533 were: shHSF1 - 5'GCA GGT TGT TCA TAG TCA GAA-3' (1994-2013 in NM\_005526.4), shHSF1.2 - 5'CCT  
534 GAA GAG TGA AGA CAT A (526-544), and shHSF1.3 - 5' CAG TGA CCA CTT GGA TGC TAT (1306-  
535 1326). The negative control sequence was 5'-ATG TAG ATA GGC GTT ACG ACT. Sense and antisense  
536 oligonucleotides were annealed and inserted into the pLVX-shRNA vector (Clontech) at *Bam*HI/*Eco*RI site.  
537 Infectious lentiviruses were generated by transfecting DNA into HEK293T cells and virus-containing  
538 supernatant was collected. Human MCF7 cells were transduced with lentiviruses following the manufacturer's  
539 instructions and selected using a medium supplemented with 1  $\mu$ g/ml puromycin (Life Technologies / Thermo  
540 Fisher Scientific, Waltham, MA, USA).

541 **HSF1 functional knockout using the CRISPR/Cas9 editing system.** To remove the human *HSF1* gene, Edit-R  
542 Human HSF1 (3297) crRNA, Edit-R tracrRNA, and Edit-R hCMV-PuroR-Cas9 Expression Plasmid  
543 (Dharmacon, Lafayette, CO, USA) were introduced into MCF7 cells using DharmaFECT Duo (6  $\mu$ g/ml)  
544 (Dharmacon) according to producer's instruction. Transfected cells were enriched by puromycin (2  $\mu$ g/ml)  
545 selection for 4 days. Afterward, single clones were obtained by limiting dilution on a 96-well plate. The  
546 efficiency of the HSF1 knockout was monitored by Western blot. Out of 81 tested clones, two individual clones

547 with the *HSF1* knockout (KO#1 and KO#2) and six pooled control clones (MIX) were chosen for the next  
548 experiments. Among individually tested HSF1-targeting crRNAs, only two were effective (target sequences:  
549 GTGGTCCACATCGAGCAGGG and AAAGTGGTCCACATCGAGCA, both in exon 3 on the plus strand).  
550 For validation experiments, a new model was created: Edit-R Human HSF1 (3297) crRNAs  
551 (GGTGTCCGGGTCGCTCACGA in exon 1 on the minus strand and AAAGTGGTCCACATCGAGCA in exon  
552 3 on the plus strand), Edit-R tracrRNA (Dharmacon), and eSpCas9-GFP protein (#ECAS9GFPPR, Merck  
553 KGaA) were introduced into MCF7 and T47D cells using Viromer® CRISPR (Lipocalyx GmbH, Halle (Saale),  
554 Germany) according to the manual provided by the producer. Single clones were obtained by limiting dilution on  
555 a 96-well plate. The efficiency of the HSF1 knockout was monitored by western blot and confirmed by  
556 sequencing (Genomed, Warszawa, Poland). Five (T47D) or six (MCF7) individual unaffected clones (HSF1+) or  
557 with the HSF1 functional knockout (HSF1-) were pooled each time before analyzes.

558 **Protein extraction and Western blotting.** Whole-cell extracts were prepared using RIPA buffer supplemented  
559 with CompleteTM protease inhibitors cocktail (Roche) and phosphatase inhibitors PhosStopTM (Roche,  
560 Indianapolis, IN, USA). Proteins (20-30 µg) were separated on 10% SDS-PAGE gels and blotted to a 0.45-µm  
561 pore nitrocellulose filter (GE Healthcare, Europe GmbH, Freiburg, Germany) using Trans Blot Turbo system  
562 (Bio-Rad, Hercules, CA, USA) for 10 min. Primary antibodies against HSF1 (1:4,000, ADI-SPA-901, Enzo Life  
563 Sciences, Farmingdale, NY, USA), HSP90 (1:2,000, ADI-SPA-836, Enzo Life Sciences), anti-HSP70 (1:2,000,  
564 ADI-SPA-810, Enzo Life Sciences), anti-HSP105 (1:600, #3390-100, BioVision, Milpitas, CA, USA), ESR1  
565 (1:2,000, #8644, Cell Signaling Technology, Danvers, MA, USA), and ACTB (1:25,000, #A3854, Merck  
566 KGaA) were used. The primary antibody was detected by an appropriate secondary antibody conjugated with  
567 horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA, USA) and visualized by ECL kit (Thermo  
568 Fisher Scientific) or WesternBright Sirius kits (Advansta, Menlo Park, CA, USA). Imaging was performed on x-  
569 ray film or in a G:BOX chemiluminescence imaging system (Syngene, Frederick, MD, USA). The experiments  
570 were repeated in triplicate and blots were subjected to densitometric analyses using ImageJ software to calculate  
571 relative protein expression after normalization with loading controls (statistical significance of differences was  
572 calculated using T-test).

573 **Total and nascent RNA isolation, cDNA synthesis, and RT-qPCR.** For nascent RNA labeling, 500 µM of 4-  
574 thiouridine (Cayman Chemical, Ann Arbor, MI, USA) was added to control and E2-treated cells for the duration  
575 of the treatment (4h). Next, total RNA was isolated using the Direct-ZolTM RNA MiniPrep Kit (Zymo  
576 Research, Irvine, CA, USA), digested with DNase I (Worthington Biochemical Corporation, Lakewood, NJ,

577 USA), and cleaned with RNAClean XP beads (Beckman Coulter Life Science, Indianapolis, USA). Five  
578 micrograms of total RNA from each sample were taken for nascent RNA fraction isolation using methane  
579 tiosulfonate (MTS) chemistry according to (Duffy and Simon, 2016). After the biotinylation step using MTSEA-  
580 biotin-XX (Biotium, Fremont, CA, USA), s4U-RNA was cleaned with RNAClean XP beads and isolated using  
581 μMacs Streptavidin Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as described (Garibaldi et al., 2017).  
582 Total RNA (1 μg) and nascent RNA (isolated from 5 μg of total RNA) from each sample were converted into  
583 cDNA as described (Kus-Liskiewicz et al., 2013). Quantitative PCR was performed using a BioRad C1000  
584 Touch<sup>TM</sup> thermocycler connected to the head CFX-96. Each reaction was performed at least in triplicates using  
585 PCR Master Mix SYBRGreen (A&A Biotechnology, Gdynia, Poland). Expression levels were normalized  
586 against *GAPDH*, *ACTB*, *HNRNPK*, *HPRT1*, if not stated otherwise. The set of delta-Cq replicates (Cq values for  
587 each sample normalized against the geometric mean of four reference genes) for control and tested samples were  
588 used for statistical tests and estimation of the p-value. Shown are median, maximum, and minimum values of a  
589 fold-change vs untreated control. The primers used in these assays are described in Table S1.

590 **Clonogenic assay.** Cells were plated onto 6-well dishes ( $1 \times 10^3$  cells per well) and cultured for 14 days.  
591 Afterward, cells were washed with the phosphate-buffered solution (PBS) and fixed with methanol. Colonies  
592 were stained with 0.2% crystal violet, washed, and air-dried. Colonies were counted manually.

593 **Aldefluor assay.** The assay was performed using a kit from Stem Cell Technology (Vancouver, Canada,  
594 #01700) according to the protocol. Cells ( $6 \times 10^5$ ) were harvested by trypsinization and resuspended in 1 ml  
595 Aldefluor Buffer. After the addition of 5 μl of BODIPY-aminoacetaldehyd (BAAA), the substrate for aldehyde  
596 dehydrogenase (ALDH), and a brief mixing, 500 μl of the cell suspension ( $3 \times 10^5$ ) was immediately transferred  
597 to another tube supplemented with 5 μl of diethylaminobenzaldehyde (DEAB), a specific inhibitor of ALDH,  
598 and pipetted to mix evenly. Tubes were incubated at 5% CO<sub>2</sub>, 37 °C for 60 min. Cells were collected by  
599 centrifugation and resuspended in Aldefluor Buffer. Analyses were performed using the BD Canto III cytometer  
600 (Becton Dickinson, Franklin Lakes, NJ, USA).

601 **Proliferation test.** Cells ( $2 \times 10^4$  cells per well) were seeded and cultured in 12-well plates. At the indicated  
602 time, cells were washed with PBS, fixed in cold methanol, and rinsed with distilled water. Cells were stained  
603 with 0.1% crystal violet for 30 min, rinsed with distilled water extensively, and dried. Cell-associated dye was  
604 extracted with 1 ml of 10% acetic acid. Aliquots (200 μl) were transferred to a 96-well plate and the absorbance  
605 was measured at 595 nm (Synergy2 microtiter plate reader, BioTek Instruments). All values on day 0 were

606 normalized to the optical density of wild-type cells (shown as 1.0). Grow curves are shown as the ratio of the  
607 absorbance on days 2, 4, and 6 against day 0 and were calculated from three to six independent experiments,  
608 each in 2-3 technical replicates. For each dataset the normality of distribution was assessed by the Shapiro-Wilk  
609 test and depending on data distribution homogeneity of variances was verified by the Levene test or Brown-  
610 Forsythe test. For analysis of differences between compared groups with normal distribution, the quality of mean  
611 values was verified by the ANOVA test with a pairwise comparison done with the HSD Tukey test or Games-  
612 Howell test and Tamhane test depending on the homogeneity of variance. In the case of non-Gaussian  
613 distribution, the Kruskal-Wallis ANOVA was applied for the verification of the hypothesis on the equality of  
614 medians with Conover-Iman's test for pairwise comparisons.

615 **Proximity Ligation Assay.** To detect the ESR1/HSP90 and ESR1/HSF1 interactions, the DuoLink in situ  
616 Proximity Ligation Assay (PLA) (Merck KGaA) was used according to the manufacturer's protocol. Cells were  
617 plated onto Nunc® Lab-Tek® II chambered coverglass (#155383, Nalge Nunc International, Rochester, NY,  
618 USA) one day before the experiment. Cells were fixed for 15 minutes with 4% paraformaldehyde solution in  
619 PBS, washed in PBS, and treated with 0.1% Triton-X100 in PBS for 5 minutes. After washing, slides were  
620 incubated in Blocking Solution and immunolabeled (overnight, 4 °C) with primary antibodies diluted in the  
621 DuoLink® Antibody Diluent: rabbit anti-HSP90 (1:200; #ADI-SPA-836, Enzo Life Science) or rabbit anti-HSF1  
622 (1:300; ADI-SPA-901, Enzo Life Sciences) and mouse anti-ERalpha (1:200; # C15100066, Diagenode, Liège,  
623 Belgium); negative controls were proceeded without one primary antibody or both. Then the secondary  
624 antibodies with attached PLA probes were used. Signals of analyzed complexes were observed using Carl Zeiss  
625 LSM 710 confocal microscope with ZEN navigation software; red fluorescence signal indicated proximity (< 40  
626 nm) of proteins recognized by both antibodies (Fredriksson et al., 2002). Z-stacks images (12 slices; 5.5 µm)  
627 were taken at ×630 magnification. From each experimental condition, spots from 10 images were identified  
628 using Photoshop (*Red Channel → Select → Color Range*) and counted (*Picture → Analysis → Record the  
629 measurements*). Next, the mean number of spots per cell (nucleus, cytoplasm) in each image was calculated.  
630 Experiments were repeated three times. Outliers were determined using the Grubbs, Tuckey criterion, and QQ  
631 plot. For each dataset, the normality of distribution was assessed by the Shapiro-Wilk test. In the case of non-  
632 Gaussian distribution, the Kruskal-Wallis ANOVA was applied for the verification of the hypothesis on the  
633 equality of medians with Conover-Iman's test for pairwise comparisons.

634 **Global Gene Expression Profiling.** Total RNA was isolated from all MCF7 cell variants (untreated, treated  
635 with 10 nM E2 for 4 h, conditions based on (Vydra et al. 2019)) using the Direct-ZolTM RNA MiniPrep Kit

636 (Zymo Research) and digested with DNase I (Worthington Biochemical Corporation). For each experimental  
637 point, RNAs from three biological replicates were first tested by RT-qPCR for the efficiency of treatments, then  
638 pooled. cDNA libraries were sequenced by Illumina HighSeq 1500 (run type: paired-end, read length: 2 × 76  
639 bp). Raw RNA-seq reads were aligned to the human genome hg38 in a Bash environment using tophat2 (Kim et  
640 al., 2013) with Ensembl genes transcriptome reference. Aligned files were processed using Samtools (Li et al.,  
641 2009). Furthermore, reads aligned in the coding regions of the genome were counted using FeatureCounts (Liao  
642 et al., 2014). Further data analyses were carried out using the R software package v. 3.6.2 (R Foundation for  
643 Statistical Computing; <http://www.r-project.org>). Read counts were normalized using DESeq2 (Lowe et al.,  
644 2014), then normalized expression values were subjected to differential analysis using NOISeq package v. 3.12  
645 (Tarazona et al., 2015) (E2 versus Ctr in all cell variants separately). To find common genes between samples,  
646 lists of differentiating genes were compared and Venn diagrams were performed (package VennDiagram v  
647 1.6.20 from CRAN). Heatmaps of normalized read counts or log2 fold changes (E2 versus Ctr) for genes shared  
648 between samples were generated (package pheatmap v. 1.0.12 from CRAN). The hierarchical clustering of genes  
649 was based on Euclidean distance. Colors are scaled per row. Geneset enrichment analysis was performed as  
650 follows: From the count matrices, we filtered out all the genes with less than 10 reads in each of the libraries.  
651 Then, we analyzed the gene-level effects of E2 stimulation of cells with normal/decreased HSF1 levels,  
652 performing the DESeq2 test for paired samples, with pairs defined by the cell type (3 cell types with normal-  
653 HSF1 level: WT, SRC, MIX, and 3 cell types with decreased HSF1 level: shHSF1, KO#1 and KO#2). Finally,  
654 we performed the geneset enrichment analysis in the same way as for the TCGA data (see below for details) - for  
655 each test, genes were ranked according to their Minimum Significant Difference, CERNO test from tmmod  
656 package was used to find enriched terms, tmmodPanelPlot function was used to visualize the results. The raw  
657 RNA-seq data were deposited in the NCBI GEO database; acc. no. GSE159802.

658 **Chromatin Immunoprecipitation and ChIP-qPCR.** The ChIP assay was performed according to the protocol  
659 from the iDeal ChIP-seq Kit for Transcription Factors (Diagenode) as described in detail in (Vydra et al., 2019).  
660 For each IP reaction, 30 µg of chromatin and 4 µl of mouse anti-ERalpha monoclonal antibody (C15100066,  
661 Diagenode) was used. For negative controls, chromatin samples were processed without antibody (mock-IP).  
662 Obtained DNA fragments were used for global profiling of chromatin binding sites or gene-specific ChIP-qPCR  
663 analysis using specific primers covering the known estrogen response elements (ERE). The set of delta-Cq  
664 replicates (difference of Cq values for each ChIP-ed sample and corresponding input DNA) for control and test  
665 sample were used for ESR1 binding calculation (as a percent of input DNA) and estimation of the p-values. ERE

666 motifs in individual peaks were identified using MAST software from the MEME Suite package (Bailey, 2011).

667 The sequences of used primers are presented in Table S2.

668 **Global Profiling of Chromatin Binding Sites.** In each experimental point, four ChIP biological replicates (each  
669 from 30 µg of input chromatin) were collected and combined in one sample before DNA sequencing.  
670 Immunoprecipitated DNA fragments and input DNA were sequenced using the Illumina HiSeq 1500 system and  
671 QIAseq Ultralow Input Library Kit (run type: single read, read length: 1 × 65 bp). Raw sequencing reads were  
672 analyzed according to standards of ChIP-seq data analysis as described below. Quality control of reads was  
673 performed with FastQC software ([www.bioinformatics.babraham.ac.uk/projects/fastqc](http://www.bioinformatics.babraham.ac.uk/projects/fastqc)) and low-quality  
674 sequences (average phred < 30) were filtered out. Remained reads were aligned to the reference human genome  
675 sequence (hg19) using the Bowtie2.2.9 program (Langmead and Salzberg, 2012). Individual peaks (Ab-ChIPed  
676 samples versus input DNA) and differential peaks (17β-estradiol-treated versus untreated cells) were detected  
677 using MACS software (Feng et al., 2012), whereas the outcome was annotated with Homer package (Heinz et  
678 al., 2010). Peak intersections and their genomic coordinates were found using Bedtools software (Quinlan and  
679 Hall, 2010). The input DNA was used as a reference because no sequences were obtained using a mock-IP  
680 probe. The locations of identified ESR1-binding sites were compared to genomic coordinates of E2-induced  
681 HSF1 peaks from our previous ChIP-seq analysis (NCBI GEO database; acc. no. GSE137558). We defined  
682 ESR1/HSF1 binding sites as “common” if at least the center of one peak was within the corresponding peak. Dot  
683 plots showing peak size distribution were generated using MedCalc Statistical Software version 19.2.1 (MedCalc  
684 Software Ltd, Ostend, Belgium; <https://www.medcalc.org>; 2020). ChIP-Seq heatmaps were prepared using  
685 peakHeatmap function from ChIPseeker Bioconductor package (version 1.26.2), with margins of 3,000  
686 nucleotides upstream and downstream from the promoter. The raw ChIP-seq data were deposited in the NCBI  
687 GEO database; acc. no. GSE159724.

688 **Chromosome conformation capture assay (3C).** The procedure was carried out according to the protocol from  
689 (Deng and Blobel, 2017). In brief, 1 × 10<sup>7</sup> cells per sample were trypsinized and fixed with 1% formaldehyde in  
690 1x PBS. Crosslinking was quenched by 0.125 M glycine and cells were lysed (10 mM Tris pH 8.0, 10 mM NaCl,  
691 0.2% NP-40, protease inhibitors). Cell nuclei were resuspended in *HindIII* RE buffer (10 mM Tris pH 8.0, 50  
692 mM NaCl, 10 mM MgCl<sub>2</sub>, 100 µg/ml BSA) and incubated sequentially with 0.3% SDS (1.5 hours) and 1.8%  
693 Triton X-100 (1.5 hours) at 37 °C with rotation. Chromatin was cleaved using 450U *HindIII* restriction enzyme  
694 (BioLabs, Massachusetts, USA) at 37 °C overnight and diluted 15-fold in ligation buffer (50 mM Tris pH 7.5, 10  
695 mM MgCl<sub>2</sub>, 10 mM DTT, 1% Triton X-100, 100 µg/ml BSA). Ligation was carried out using 4,000U T4-DNA

696 ligase (EURx) at 16°C overnight, in the presence of 1 mM ATP. All samples were de-crosslinked (65 °C,  
697 overnight with mixing), RNase A and Proteinase K treated, and DNA was isolated using standard  
698 Phenol/Chloroform/Isoamyl alcohol purification method. Precipitated DNA was dissolved in 10 mM Tris pH 8.0  
699 and used as a template in PCR analyses. The primers used are listed in Table S3.

700 **Analysis of human patient TCGA data** (performed using R version 3.6.2.).

701 Data retrieval. Clinical and RNA-seq (HTSeq counts) data from the TCGA (The Cancer Genome Atlas) breast  
702 cancer (BRCA) project was downloaded (1102 total samples) and prepared using the TCGAbiolinks package  
703 (version 2.14) (Colaprico et al., 2016). An additional file with clinical data containing ER receptor status,  
704 ‘nationwidechildrens.org\_clinical\_patient\_brea.txt’, was downloaded directly from the GDC repository  
705 <https://portal.gdc.cancer.gov>. Molecular subtype classification (according to (Berger et al., 2018)) was retrieved  
706 through TCGAbiolinks.

707 Cases selection. Counts were log CPM normalized with the *cpm* function from the edgeR package (version  
708 3.28.1) (Robinson et al., 2010). Then we selected four groups (called A-D) of patients: ER-positive/negative  
709 with high/low HSF1 expression level using the following clinical and expression (log CPM) criteria: ER-positive  
710 if *er\_status\_by\_ihc*: “Positive”, *er\_status\_ihc\_Percent\_Positive*: “90-99%” and expression level of *ESR1* > 6,  
711 ER-negative if *er\_status\_by\_ihc*: “Negative” and expression level of *ESR1* < 6, HSF1-high (low) if the  
712 expression of *HSF1* was above 67 (below 33) percentile across all TCGA\_BRCA cases. We also excluded cases  
713 classified to HER2-enriched and basal-like subtypes from the ER-positive group and luminal A (LumA), luminal  
714 B (LumB), and normal-like subtypes from the ER-negative group. In reduced groups (called a-d) only the  
715 luminal A and basal-like cases were analyzed.

716 Survival analysis was performed using the *survfit* function from the *survival* package (version 3.1-8) and plotted  
717 with the *ggsurvplot* function from the *survminer* package (version 0.4.6) (Therneau and Grambsch, 2000).

718 MDS plots were used to visualize differences between patients. We performed multidimensional scaling (MDS)  
719 with *MDSplot* function from edgeR and plotted the results with ggplot2 (version 3.3.2) (Wickham, 2016).

720 Differential expression analysis was done with the edgeR package (Robinson et al., 2010). Lowly expressed  
721 genes were filtered out by *filterByExpr* function with default parameters, resulting in 24,696 genes kept for  
722 statistical analysis. Then we performed a quasi-likelihood F test for all groups’ combinations one-vs-one and two  
723 obvious two-vs-two cases: ER+ versus ER- and HSF1-high versus HSF1-low (using mean expression levels for

724 joined groups, with the weight of 0.5). P-values were corrected for multiple testing using the Benjamini and  
725 Hochberg method.

726 Comparison of the number of genes differentially expressed between groups. To compare the size of differences  
727 identified in each test we plotted the cumulative distributions of FDR (False Discovery Rate). Each test was  
728 repeated 10 times with the groups randomly subsampled to equal size of 30 (or 28 in case of reduced groups) to  
729 avoid the p-values being affected by the group size inequality. Results were averaged and plotted with ggplot2  
730 (Wickham, 2016).

731 Geneset enrichment analysis. For gene set enrichment analysis we selected hallmark, BioCarta, Reactome, and  
732 PID genesets from MSigDB (v7.0) (Subramanian et al., 2005) and merged it with the list of pathways  
733 downloaded from KEGG. DESeq2 was used to calculate log-fold changes with its standard error (Love et al.,  
734 2014). Then all genes were ordered according to their Minimum Significant Difference (MSD) calculated as  
735  $|\log FC| - 2 * \log FC\_standard\_error$  and tested for enrichment using the CERNO test (Zyla et al., 2019) from the  
736 tmod package (version 0.44) (Weiner 3rd and Domaszewska, 2016). The most significant results (effect size >  
737 0.65, p-value < 0.001 at least in one comparison) and results for genesets related to the biological processes of  
738 interest were visualized with the *tmodPanelPlot* function.

739

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753

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914 **Supplementary Files:**

915 **Supplementary Figure legends**

916 **Fig. S1. Characterization of HSF1-deficient cell variants.** (A) Western blot analysis of HSF1 level in  
917 unmodified MCF7 cells (WT) and variants stably transduced with non-specific shRNA (SCR) or with three  
918 different HSF1-specific shRNAs (shHSF1). Actin (ACTB) was used as a protein loading control. The graph  
919 below shows the results of densitometric analyses of HSF1 immunodetection (n=3). \*p < 0.05. (B) Expression of  
920 indicated *HSP* genes analyzed by RT-qPCR in MCF7 cell variants exposed to elevated temperature (HS: 43  
921 °C/1h + recovery 37 °C/4h) in relation to untreated control (Ctr), \*\*\*p < 0.0001, \*\*p < 0.001, \*p < 0.05. (C)  
922 Cell growth curves under standard conditions (10% FBS) and 5% dextran-activated charcoal-stripped FBS  
923 assessed using crystal violet staining. Mean and standard deviation from three to four independent experiments  
924 (each in two technical replicates) are shown. \*p < 0.05. (D) Cell cycle phases and sub-G1 distribution in sub-  
925 confluent cells at 72 hours after plating presented as 100% stacked column plots (mean±SD, n=3). \*p < 0.05,  
926 significantly different to WT. (E) Western blot analysis of HSF1 level in T47D cell variants: unmodified (WT),  
927 and a combination of five control (HSF1+) and five HSF1-negative (HSF1-) clones arisen from single cells  
928 following CRISPR/Cas9 gene targeting. Actin (ACTB) was used as a protein loading control. (F) Cell growth  
929 curves of untreated (Ctr) and E2-treated T47D cell variants in phenol red-free media with 10% charcoal-stripped  
930 FBS assessed using crystal violet staining. Mean and standard deviation from three independent experiments  
931 (each in six technical replicates) are shown. \*\*p < 0.001, \*p < 0.05.

932 **Fig. S2. Geneset enrichment analysis showing differences between HSF1-proficient and HSF1-deficient**  
933 **MCF7 cells in response to E2 stimulation.** Significant terms from the REACTOME, PID, and BIOCARTA  
934 subsets of the canonical pathways collection are shown. Blue – a fraction of down-regulated genes, red – fraction  
935 of up-regulated genes.

936 **Fig. S3. Principal component analysis (PCA) of normalized RNA-seq read counts.** Gene expression changes  
937 were investigated in untreated (Ctr) and estrogen-treated (10 nM, 4 hours) MCF7 cell variants: unmodified  
938 (WT), stably transduced with non-specific shRNA (SCR), stably transduced with HSF1-specific shRNA  
939 (shHSF1); a combination of control clones arisen from single cells following CRISPR/Cas9 gene targeting  
940 (MIX), two HSF1 negative clones obtained by CRISPR/Cas9 gene targeting (KO#1, KO#2).

941 **Fig. S4. Characterization of MCF7 cell model created for validation using DNA-free CRISPR/Cas9**  
942 **system. (A)** Heat shock response assessed by western blot in unmodified (WT), HSF1+ (six HSF1-positive  
943 clones), and HSF1- (six HSF1-negative clones) cells. Actin (ACTB) was used as a protein loading control. Heat  
944 shock: 43 °C/1h + recovery 37 °C/6h. **(B)** Cell growth curves of untreated (Ctr) and E2-treated cells in phenol  
945 red-free media with 5 or 10% charcoal-stripped FBS assessed using crystal violet staining. Mean and standard  
946 deviation from three independent experiments (each in six technical replicates) are shown. \*\*p < 0.001, \*p <  
947 0.05 (next to the curve – compared to the corresponding control, between curves – between cell variants). **(C)**  
948 Gene expression analyses by RT-qPCR using total RNA. The upper panel shows E2-stimulated changes, lower  
949 panel shows differences between untreated WT, HSF1+, and HSF1- cells. Ctr, untreated cells; E2, 17 $\beta$ -estradiol  
950 treatment (10 nM, 4 h). \*\*\*p < 0.0001, \*\*p < 0.001, \*p < 0.05 (above the bar – compared to the corresponding  
951 control, between the bars – between cell variants).

952 **Fig. S5. Top enriched motifs in ESR1 ChIP-seq peak regions.** The CentriMo plots show the distribution of  
953 the given motifs in peaks from untreated (Ctr) and estrogen (E2)-treated (10 nM for 30 minutes) MCF7 cell  
954 variants. JASPAR motif names, IDs, and the p-value of the motif's central enrichment in peaks are shown in the  
955 legend of each plot. MCF7 cell variants: wild type (WT), a combination of control clones arisen from single cells  
956 following CRISPR/Cas9 gene targeting (MIX), HSF1 negative clone obtained by CRISPR/Cas9 gene targeting  
957 (KO#2).

958 **Fig. S6. HSF1, HSP90, and ESR1 localization assessed by immunofluorescence in MCF7 cells.** DNA was  
959 stained with DAPI. Scale bar, 20  $\mu$ m.

960 **Fig. S7.** Examples of different patterns of ESR1 and HSF1 binding to chromatin. **(A)** Stronger binding of ESR1  
961 than HSF1. **(B)** Stronger binding of HSF1 than ESR1. **(C)** HSF1 binding unrelated to ESR1 anchoring. Peaks  
962 identified by MACS in ChIP-seq analyses in wild-type MCF7 cells after E2 treatment (10 nM, 60 min) and  
963 corresponding ChIA-PET interactions (Fullwood et al., 2009) downloaded from ENCODE database and  
964 visualized by the IGV browser. The red bar shows the ESR1 anchor region (interacting loci), red line – a loop  
965 (the intermediate genomic span between the two anchors). The scale for each sample is shown in the left corner.

966 **Fig. S8.** Effect of *ESR1* **(A)** and *HSF1* **(B)** transcript levels on survival in TCGA breast cancer patients analyzed  
967 using The Kaplan Meier Plotter.

968 **Fig. S9. (A)** Volcano plots visualizing differential expression patterns between two distinct groups of breast  
969 cancers with different levels of ESR1 and HSF1 expression (the same scale is kept). **(B)** Volcano plots with gene  
970 labels. The red points in the plots represent genes with statistically significant increased expression in the first  
971 group, the blue points - in the second group in the given comparison (adjusted  $p < 0.05$ ).

972 **Fig. S10. Geneset enrichment analyses showing the most significant terms differentiating ER+ and ER-**  
973 **breast cancers with different HSF1 levels** (in comparisons between groups selected in Fig. 6B). Blue – down-  
974 regulated genes, red – up-regulated genes. Terms related to estrogen response are marked with the green  
975 rectangle.

976

977 **Supplementary Dataset 1.** Summary table of RNA-seq results (normalized signals and expression fold changes  
978 after E2 treatment) in MCF7 cell variants with different levels of HSF1. xlsx document.

979 **Supplementary Dataset 2.** Summary tables of ChIP-seq results: characteristics of ESR1 binding in wild-type,  
980 HSF1-proficient (MIX), and HSF1-deficient (KO#2) MCF7 cells, untreated (Ctr) and after E2-stimulation. xlsx  
981 document.

982 **Supplementary Dataset 3.** Summary tables of ChIP-seq results: characteristics of ESR1 and HSF1 common  
983 binding regions in wild-type MCF7 cells, untreated (Ctr) and after E2-stimulation. xlsx document.

984 **Supplementary Dataset 4.** Differential expression tests between selected groups of breast cancer patients with  
985 different ESR1 and HSF1 statuses based on RNA-seq data deposited in the TCGA database. xlsx document.

986

987 **Supplementary methods:** Cell-cycle distribution. MEME-ChIP analyses. Immunofluorescence (IF).

988

989 **Supplementary Tables:**

990 **Table S1.** RT-qPCR primers for gene expression analyses.

991 **Table S2.** ChIP-qPCR primers for ESR1 binding analyses.

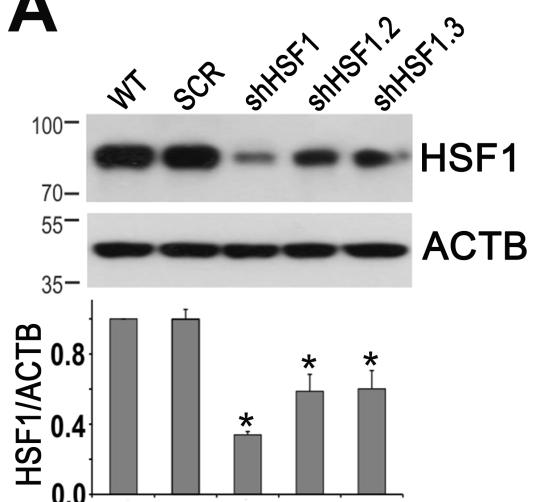
992 **Table S3.** PCR primers for chromosome conformation capture assay.

993     **Source Data**

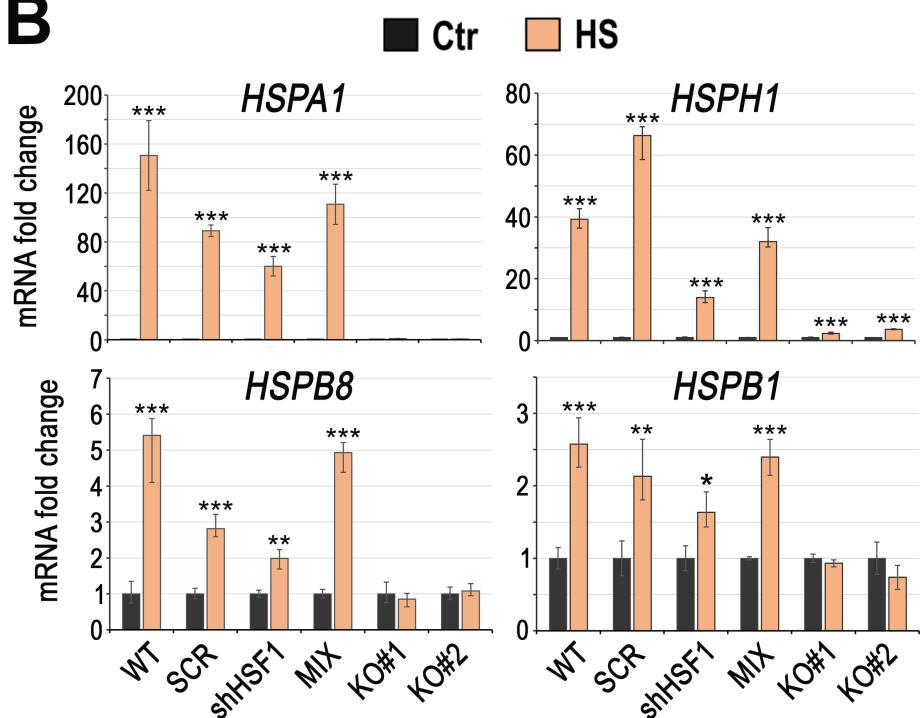
994     **Figs 1A, 4B, 5E - source data** (unedited gels and blots): the original files of the full raw unedited blots and gels

995     and figures with the uncropped blots and gels with the relevant bands labelled.

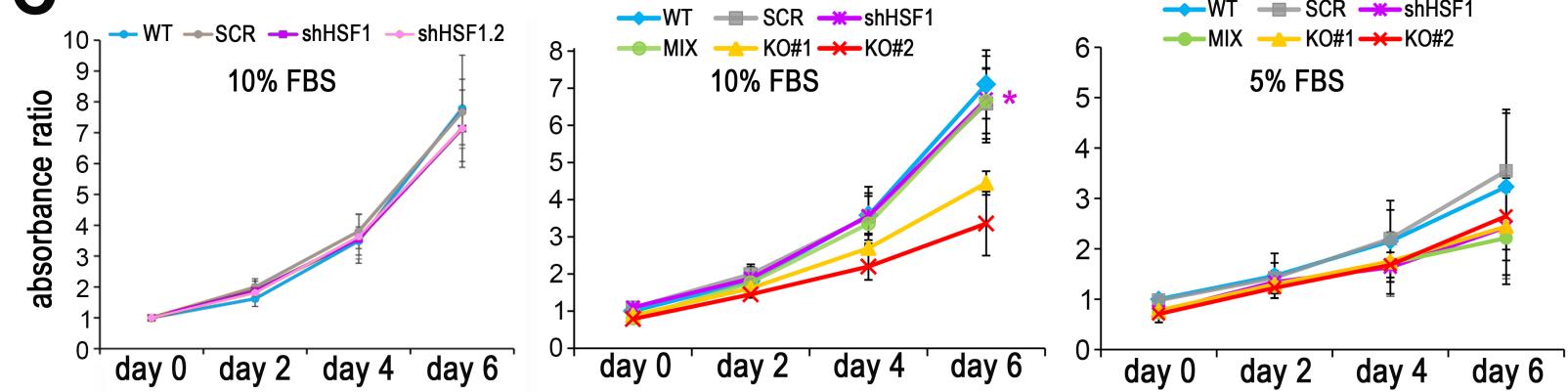
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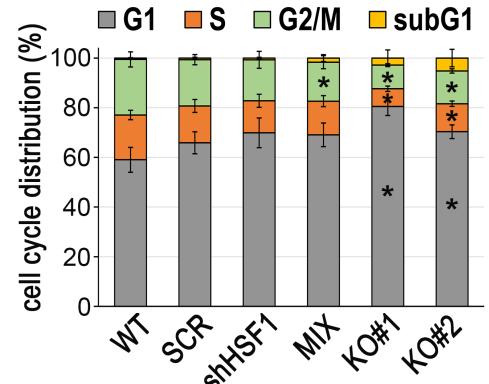
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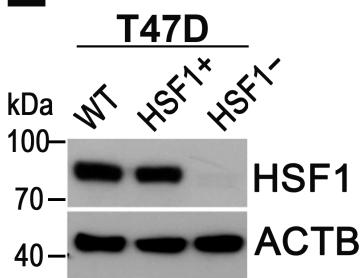
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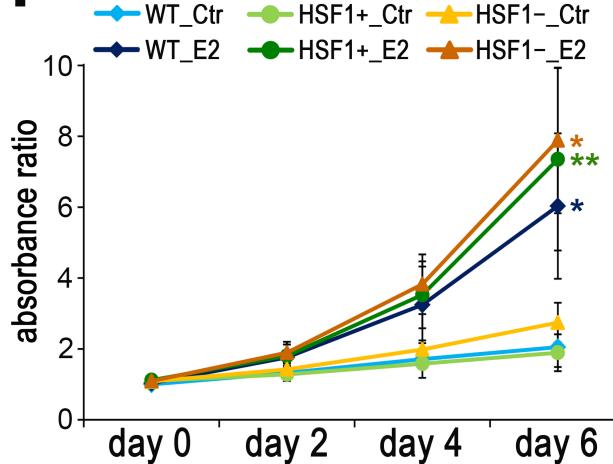
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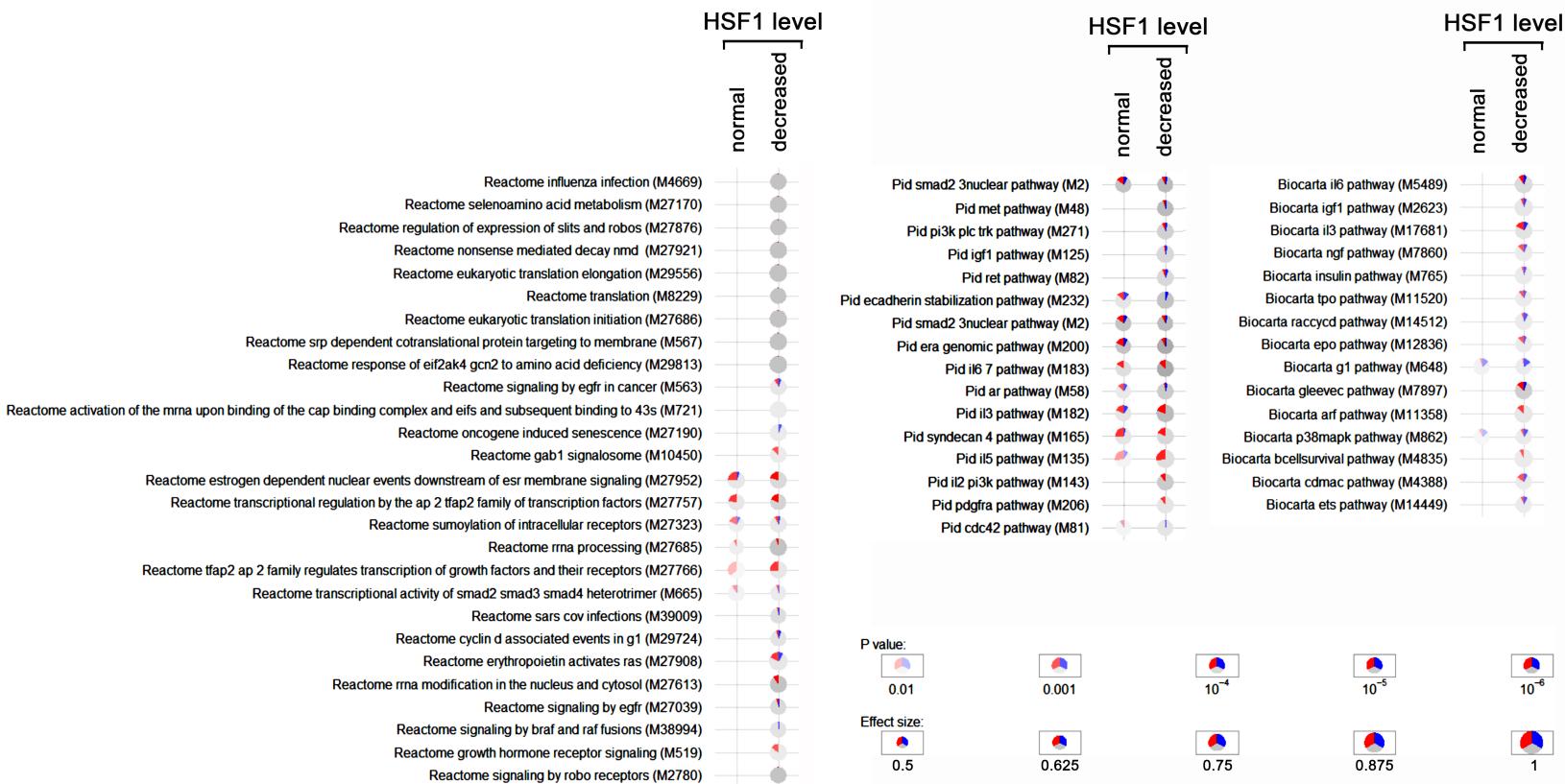


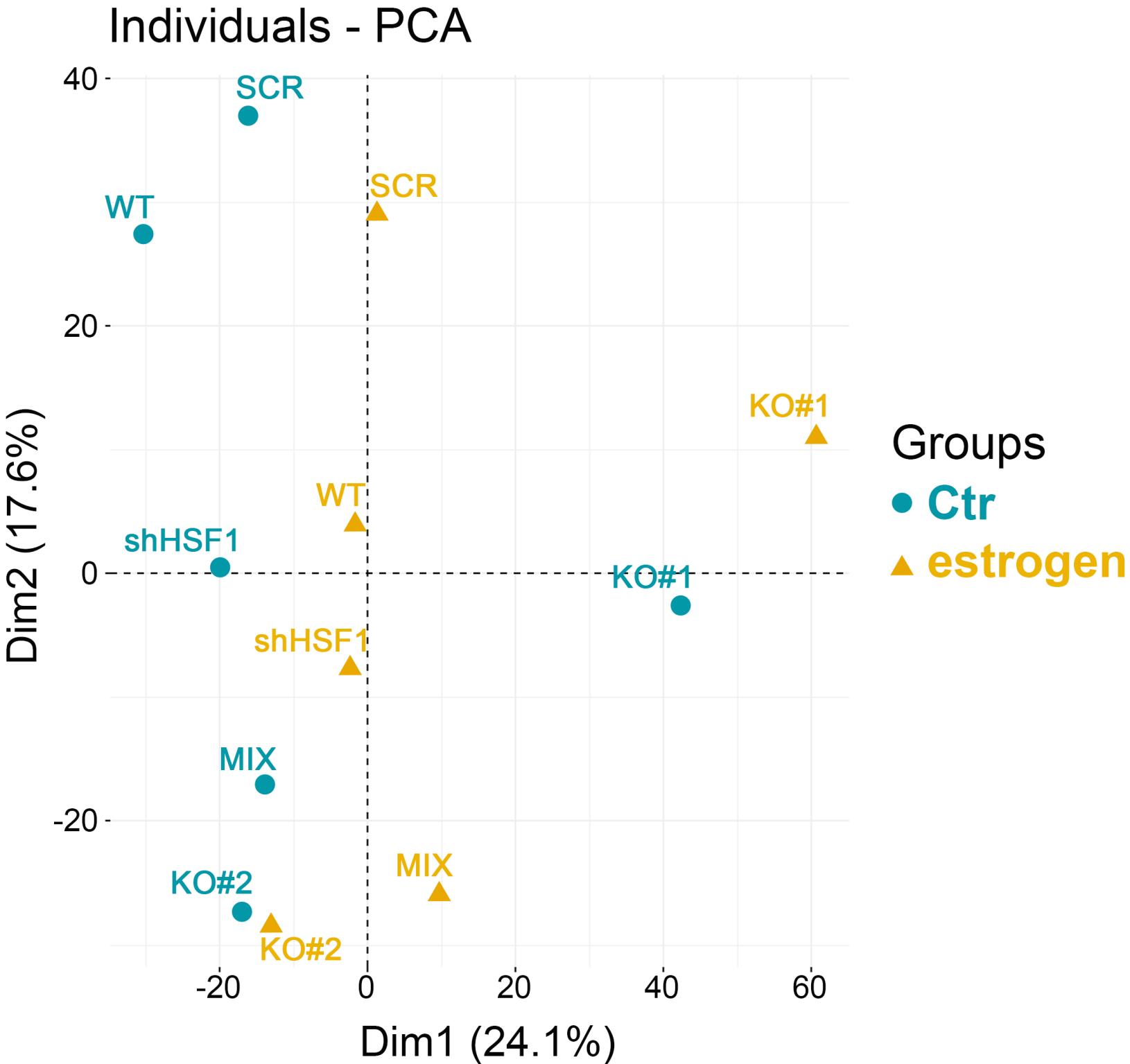
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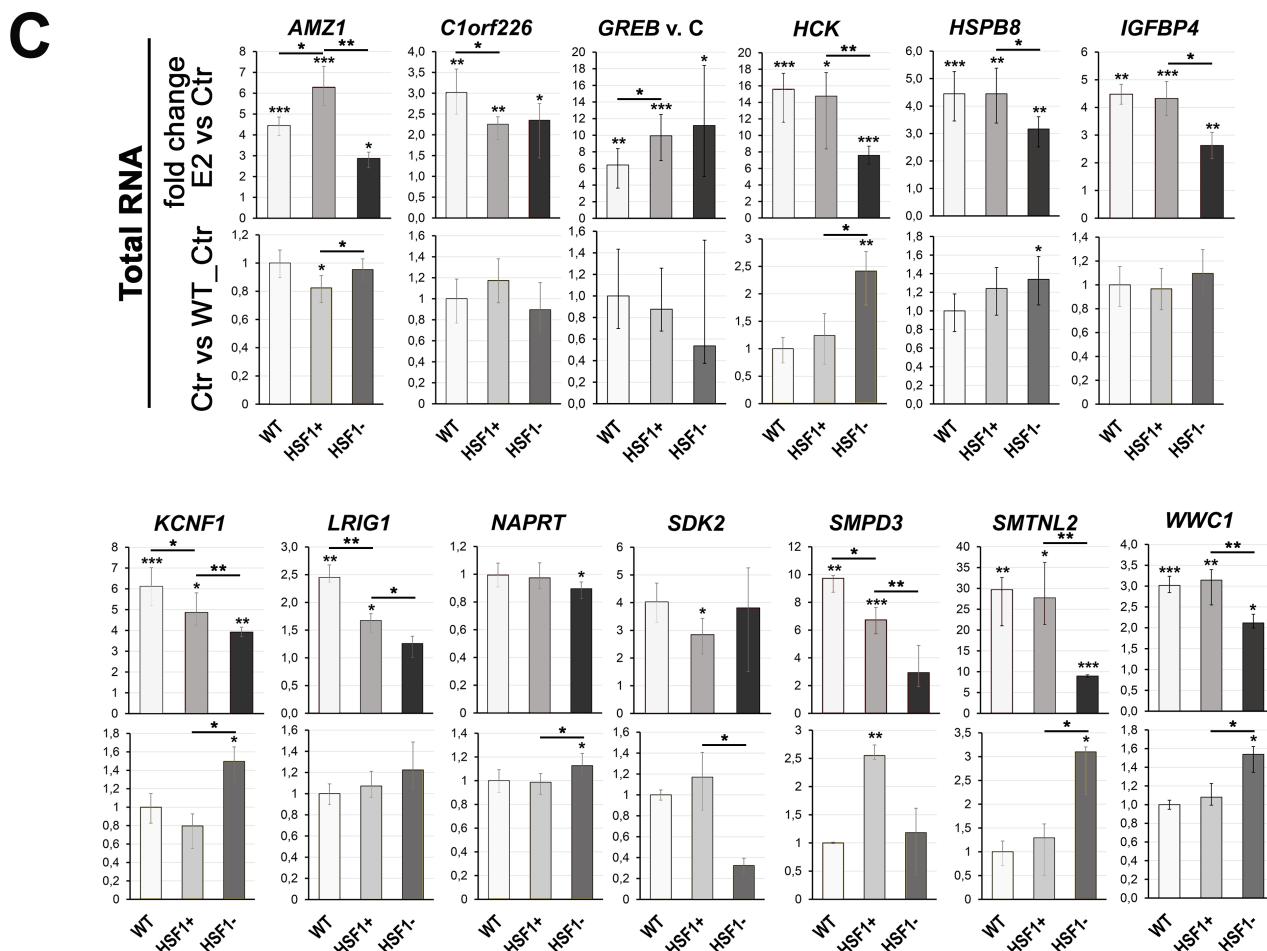
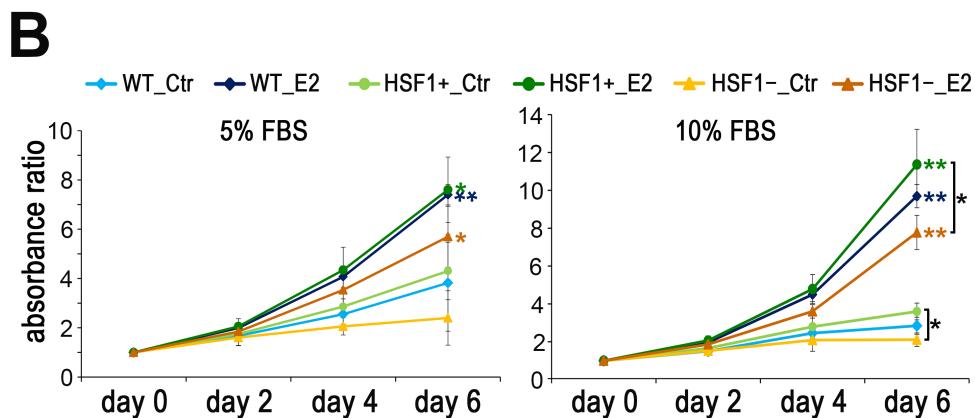
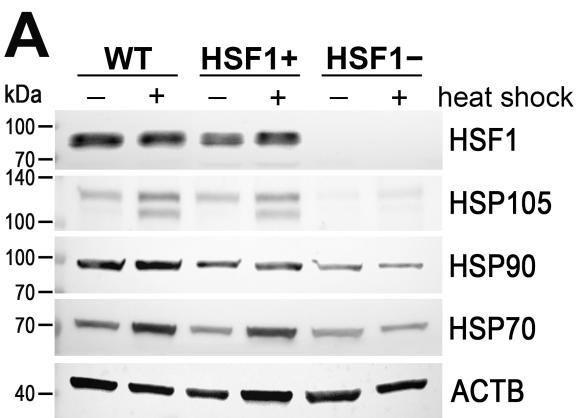


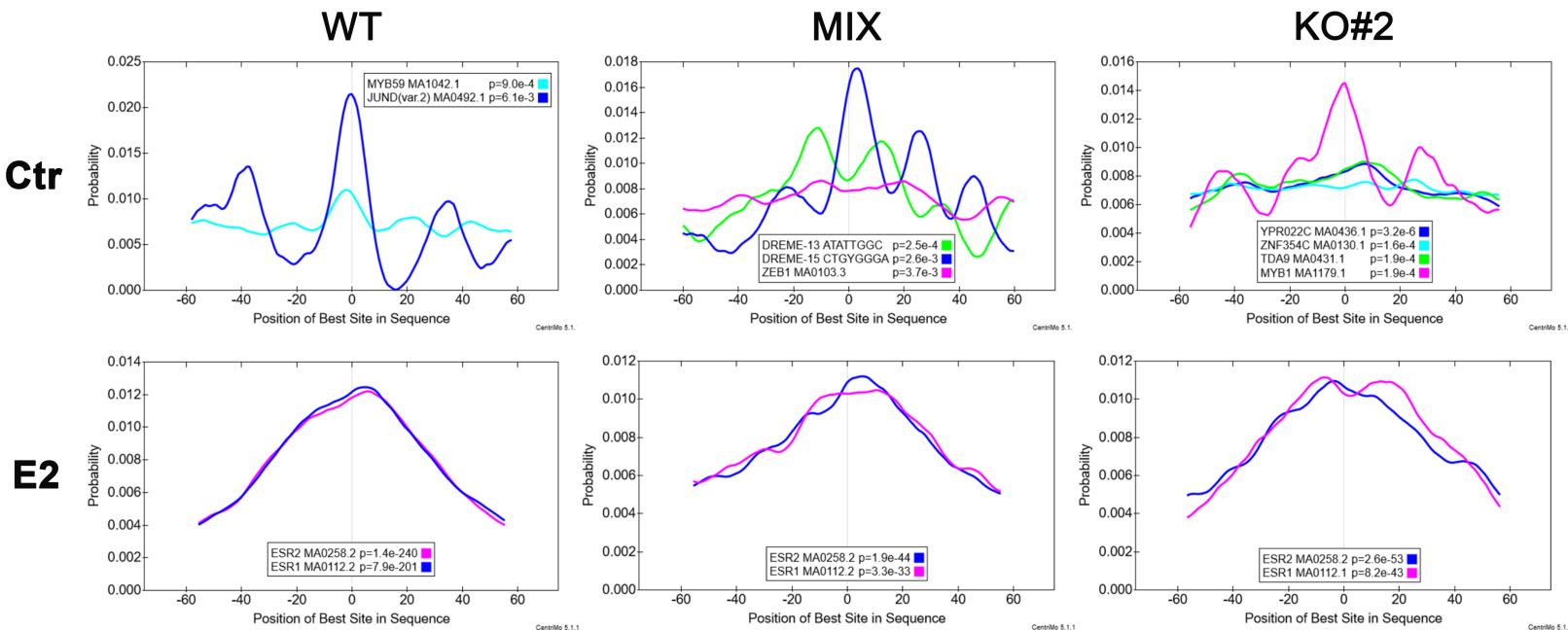
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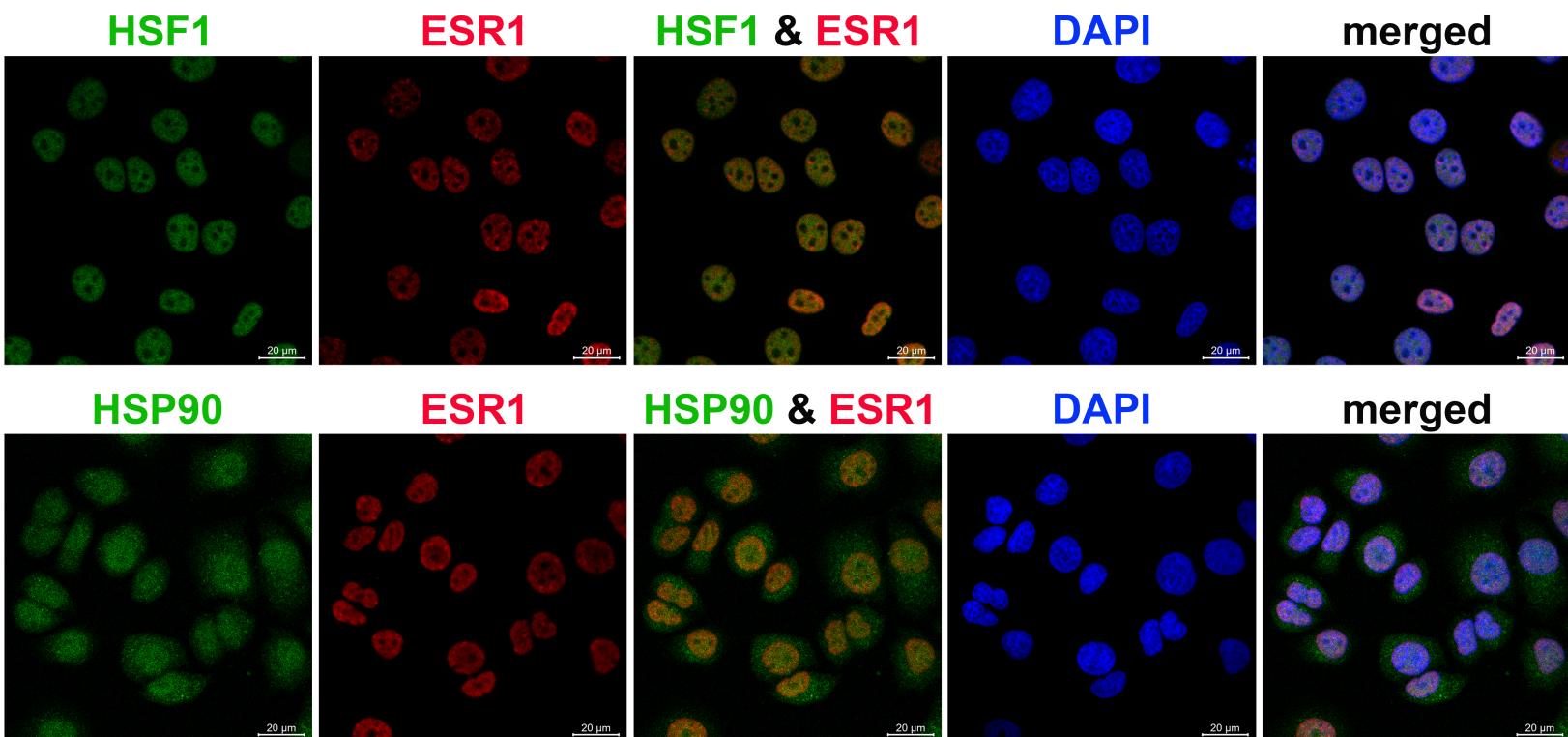






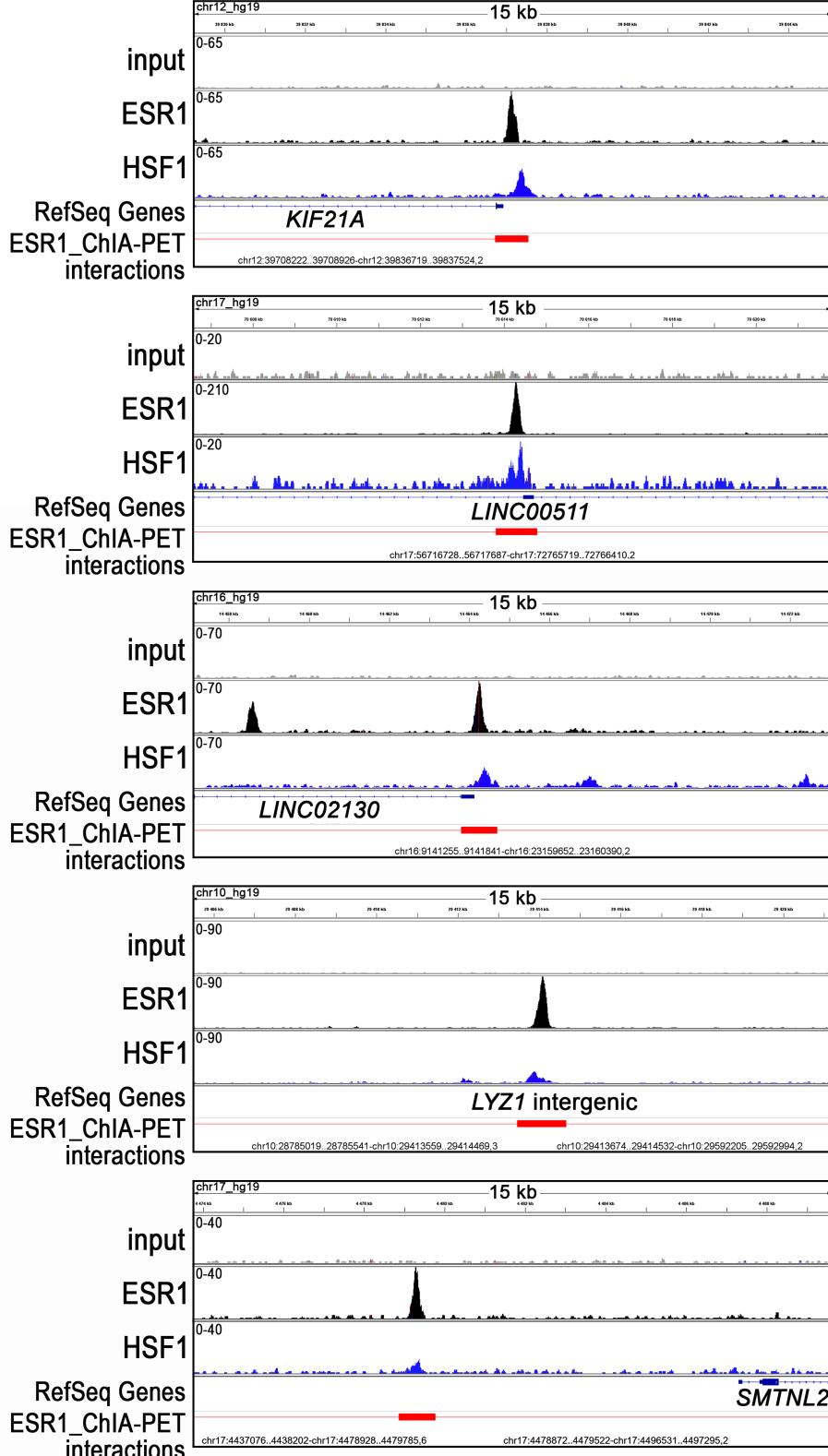
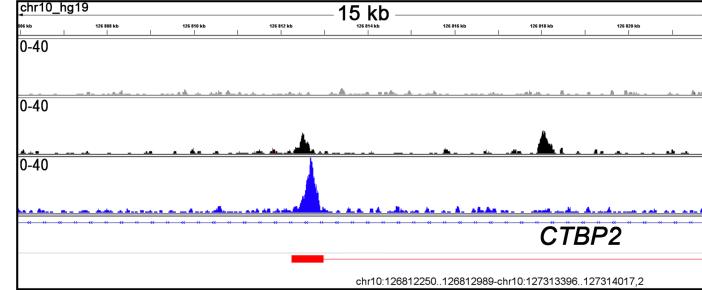
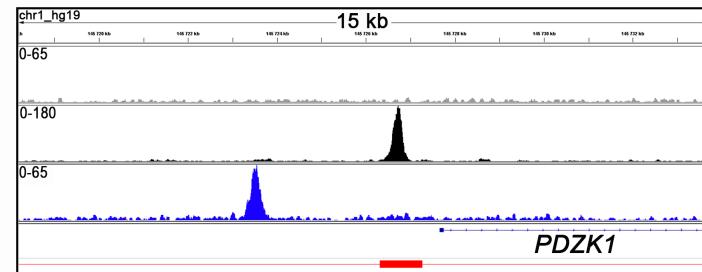
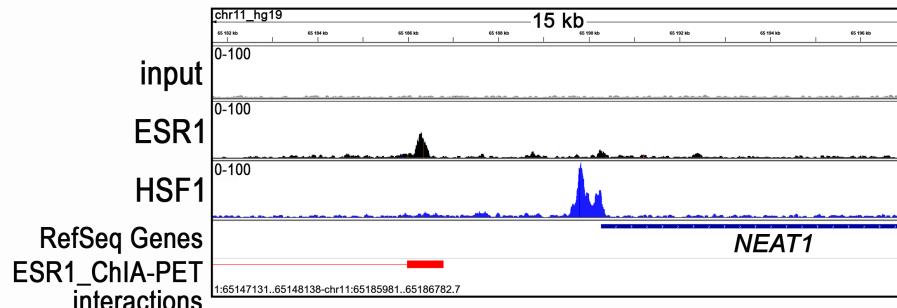


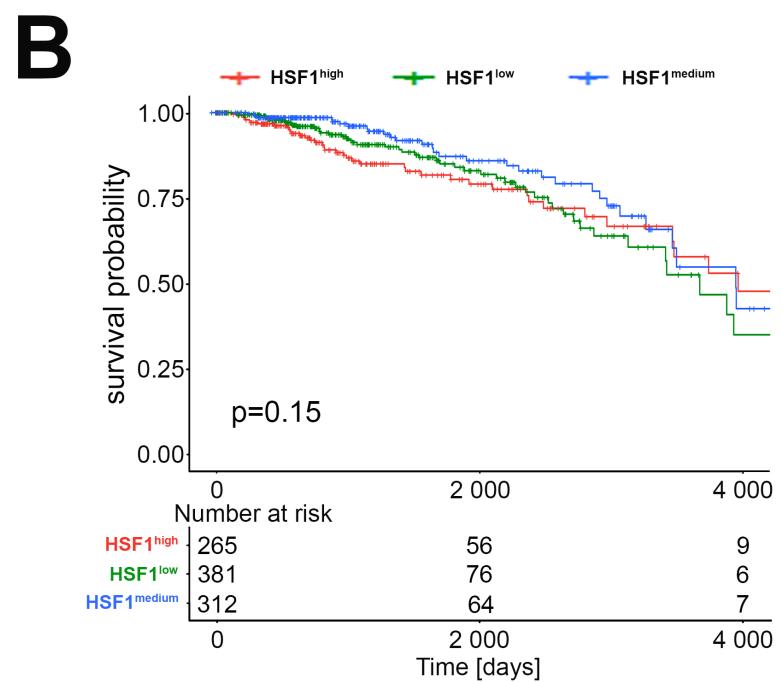
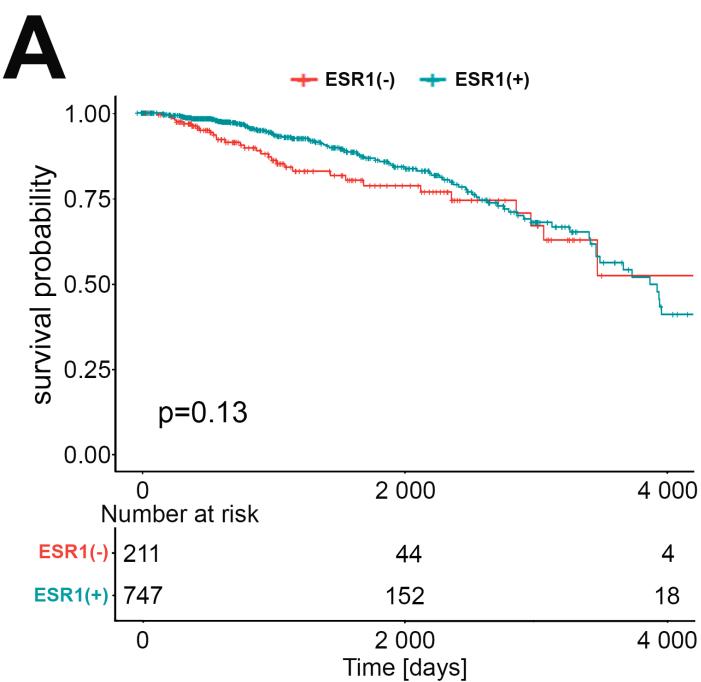




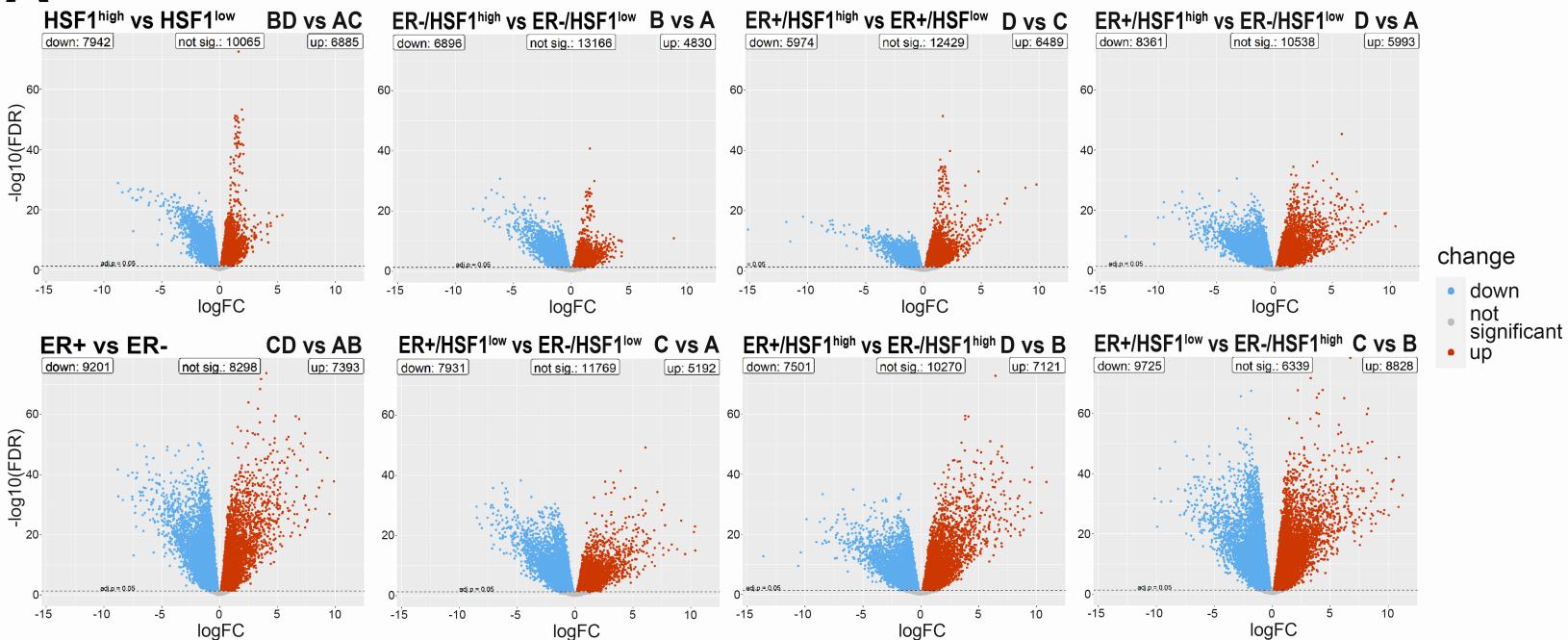
**A**

bioRxiv preprint doi: <https://doi.org/10.1101/2021.05.06.442900>; this version posted May 7, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

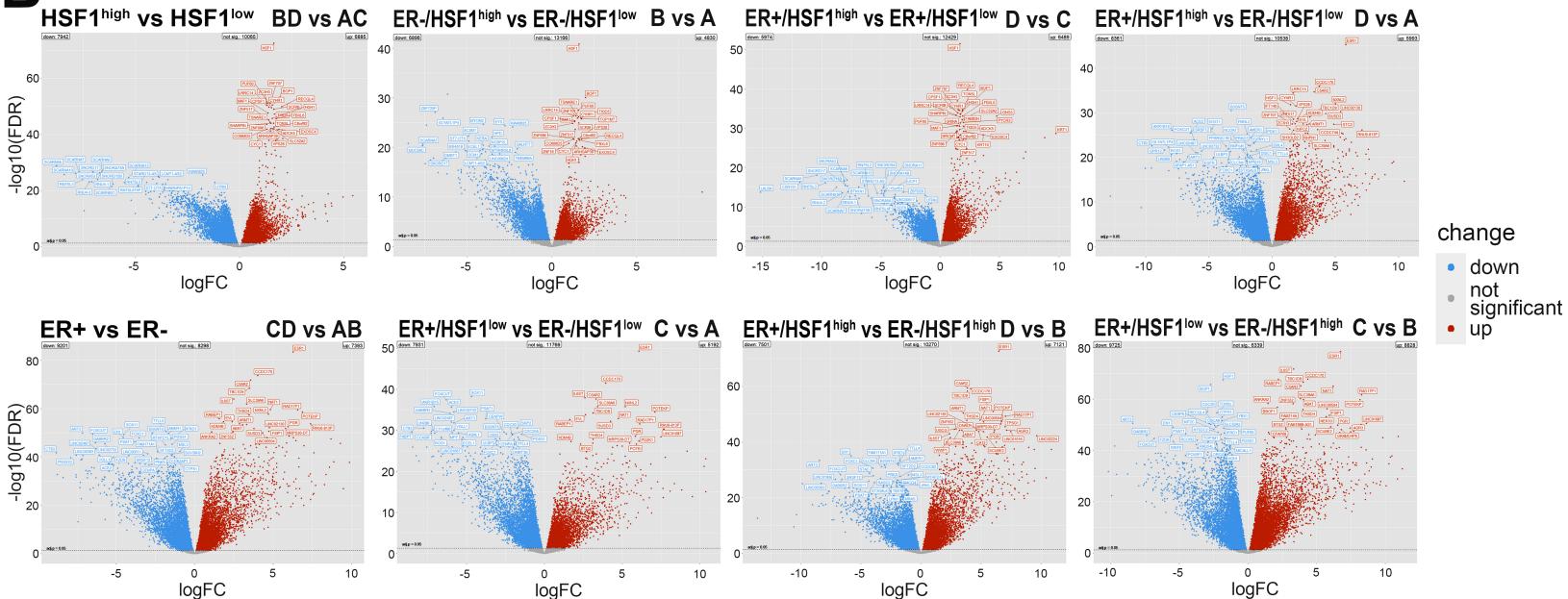
**ESR1>HSF1****HSF1>ESR1****B****C****unrelated to ESR1 anchoring**



**A**



**B**



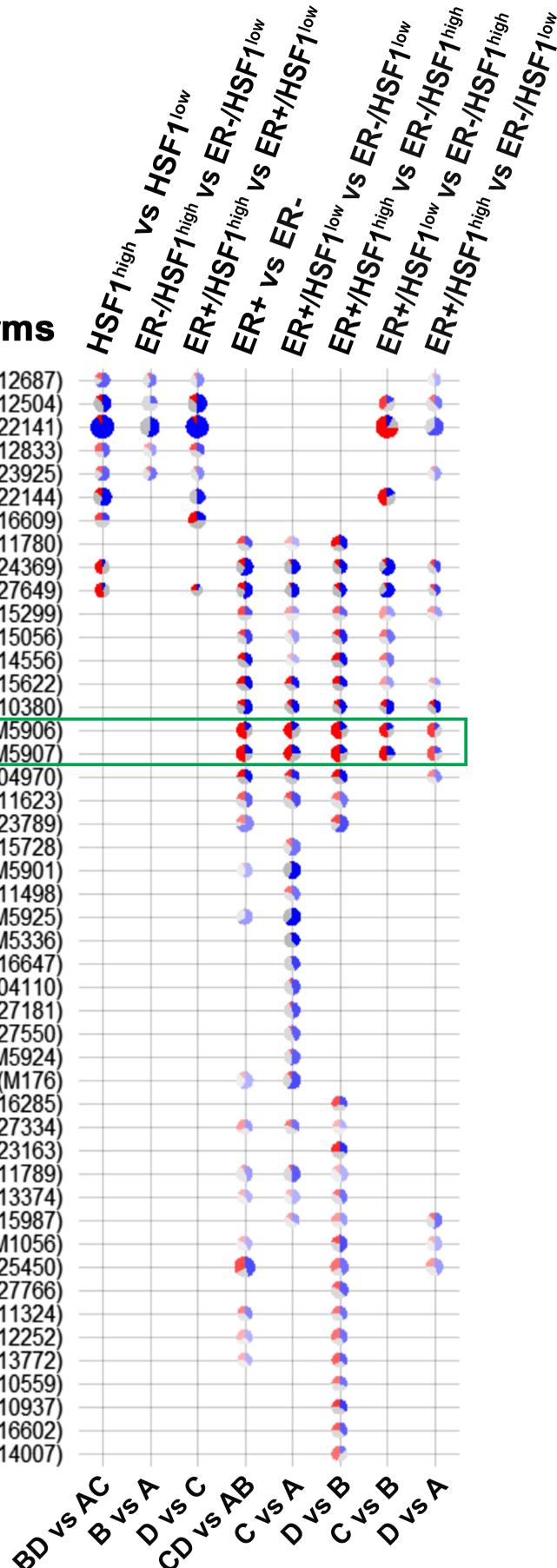
## The most significant terms

Go detection of chemical stimulus involved in sensory perception of taste (M12687)  
 Go spliceosomal tri snrnp complex assembly (M12504)  
 Go formation of quadruple sl u4 u5 u6 snrnp (M22141)  
 Go sensory perception of taste (M12833)  
 Go sensory perception of bitter taste (M23925)  
 Go mma 5 splice site recognition (M22144)  
 Go spliceosomal snrnp assembly (M16609)  
 Go dorsal ventral pattern formation (M11780)  
 Go cornification (M24369)  
 Reactome formation of the cornified envelope (M27649)  
 Go retina homeostasis (M15299)  
 Go odontogenesis of dentin containing tooth (M15056)  
 Go diencephalon development (M14556)  
 Go feeding behavior (M15622)  
 Go antimicrobial humoral response (M10380)  
 Hallmark estrogen response early (M5906)  
 Hallmark estrogen response late (M5907)  
 Salivary secretion (04970)  
 Go cell proliferation in forebrain (M11623)  
 Go saliva secretion (M23789)  
 Go calcium independent cell cell adhesion via plasma membrane cell adhesion molecules (M15728)  
 Hallmark g2m checkpoint (M5901)  
 Go drug transmembrane transport (M11498)  
 Hallmark e2f targets (M5925)  
 Reactome cell cycle mitotic (M5336)  
 Reactome cell cycle checkpoints (M16647)  
 Cell cycle (04110)  
 Reactome resolution of sister chromatid cohesion (M27181)  
 Reactome rho gtpases activate formins (M27550)  
 Hallmark mtorc1 signaling (M5924)  
 Pid foxm1 pathway (M176)  
 Go mesonephros development (M16285)  
 Reactome transport of bile salts and organic acids metal ions and amine compounds (M27334)  
 Go secretion by tissue (M23163)  
 Go sequestering of metal ion (M11789)  
 Go positive regulation of neural precursor cell proliferation (M13374)  
 Go o glycan processing (M15987)  
 Reactome voltage gated potassium channels (M1056)  
 Go negative regulation of feeding behavior (M25450)  
 Reactome tfap2 ap 2 family regulates transcription of growth factors and their receptors (M27766)  
 Go pituitary gland development (M11324)  
 Go positive regulation of muscle contraction (M12252)  
 Go water homeostasis (M13772)  
 Go positive regulation of cation channel activity (M10559)  
 Go positive regulation of transporter activity (M10937)  
 Go neural crest cell differentiation (M16602)  
 Go microtubule bundle formation (M14007)

Effect size:



P value:



## Supplementary methods

**Cell-cycle distribution.** Cells ( $3 \times 10^5$  per well) were plated onto 6-well plates. The next day medium was replaced and cells were grown for an additional 48 hours. Afterward, cells were harvested by trypsinization, rinsed with PBS, fixed with ice-cold 70% ethanol at -20 °C overnight. Cells were collected by centrifugation, resuspended in PBS containing RNase A (100 µg/ml), and stained with 100 µg/ml propidium iodide solution. DNA content was analyzed using flow cytometry to monitor the cell cycle changes.

**MEME-ChIP analyses.** The consensus DNA sequences for ESR1 binding were identified *in silico* by Motif Analysis of Large Nucleotide Datasets (MEME-ChIP, version 5.1.1) (Bailey et al., 2009) using a 150-bp region centered on the summit point and visualized by CentriMo (Local Motif Enrichment Analysis) (Bailey and Machanick, 2012).

**Immunofluorescence (IF).** Cells were plated onto Nunc® Lab-Tek® II chambered coverglass (#155383, Nalge Nunc International, Rochester, NY, USA) and fixed for 15 minutes with 4% PFA paraformaldehyde solution in PBS, washed, treated with 0.1% Triton-X100 in PBS for 5 minutes, and washed again in PBS (3 x 5 minutes). IF imaging was performed using primary antibodies: anti-HSP90 (1:200; ADI-SPA-836, Enzo Life Science), anti-HSF1 (1:300; ADI-SPA-901, Enzo Life Sciences) or anti-ESR1 (1:200; C15100066, Diagenode) and secondary Alexa Fluor (488 or 594) conjugated antibodies (Abcam). Finally, the DNA was stained with DAPI. Images were taken using Carl Zeiss LSM 710 confocal microscope with ZEN navigation software.

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Bailey TL, Machanick P. 2012. Inferring direct DNA binding from ChIP-seq. *Nucleic Acids Res* **40**:e128. doi:10.1093/nar/gks433

Fullwood MJ, Liu MH, Pan YF, Liu J, Xu H, Mohamed YB, Orlov YL, Velkov S, Ho A, Mei PH, Chew EGY, Huang PYH, Welboren W-J, Han Y, Ooi HS, Ariyaratne PN, Vega VB, Luo Y, Tan PY, Choy PY, Wansa KDSA, Zhao B, Lim KS, Leow SC, Yow JS, Joseph R, Li H, Desai KV, Thomsen JS, Lee YK, Karuturi RKM, Herve T, Bourque G, Stunnenberg HG, Ruan X, Cacheux-Rataboul V, Sung W-K, Liu ET, Wei C-L, Cheung E, Ruan Y. 2009. An oestrogen-receptor-alpha-bound human chromatin interactome. *Nature* **462**:58–64. doi:10.1038/nature08497