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Supplementary Materials for

Estrogen Alters the Splicing of Type 1 Corticotropin-Releasing Hormone Receptor in Breast Cancer Cells

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Published 2 July 2013, *Sci. Signal.* **6**, ra53 (2013)
DOI: 10.1126/scisignal.2003926

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SUPPLEMENTARY MATERIALS

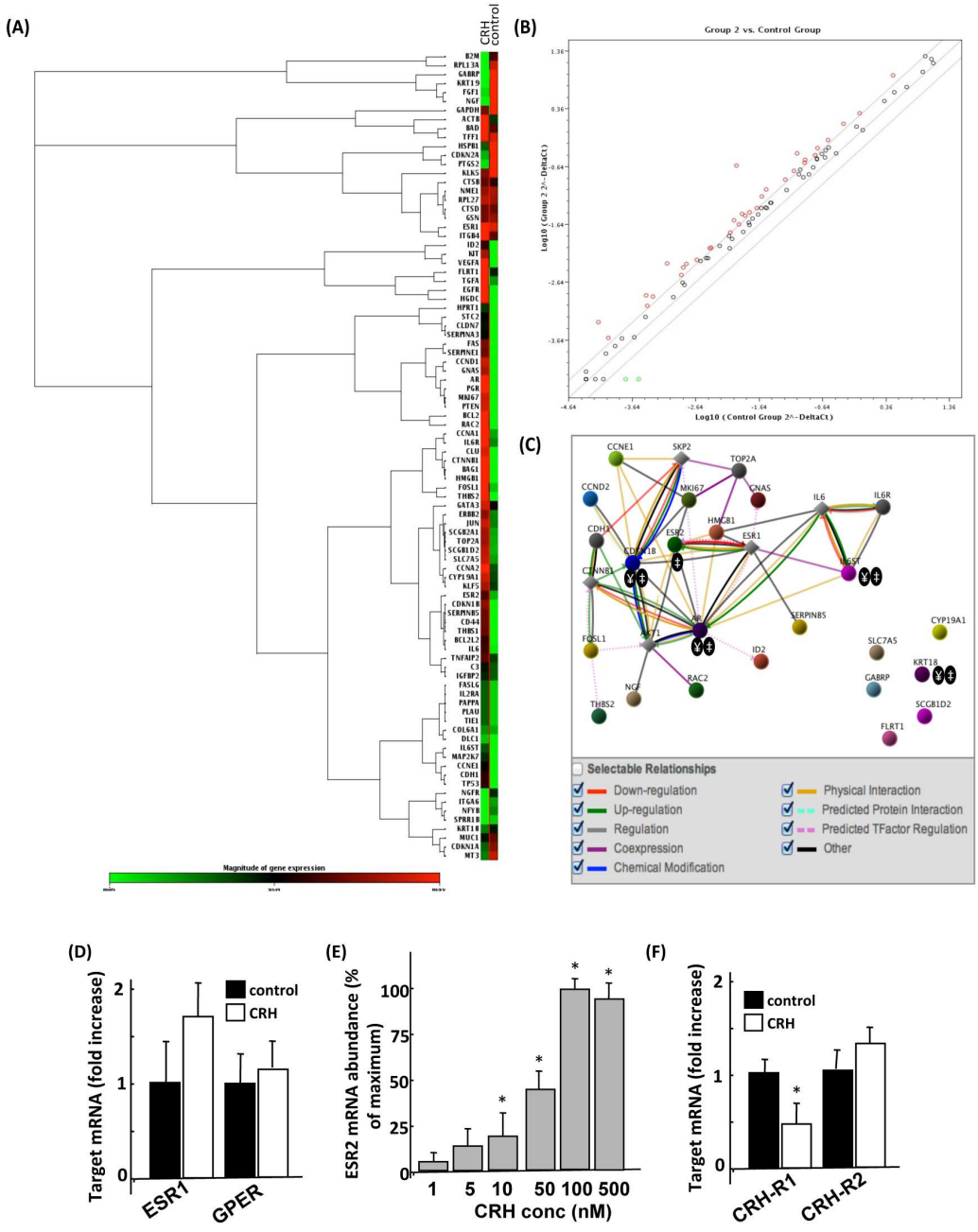

Figure S1: CRH-regulated genes involved in estrogen signaling in MCF7 cells and in silico–predicted integrated network. (A to B) Analysis using the Human Estrogen Receptor Signaling in Breast Cancer RT² Profiler™ PCR Array and target-specific real-time RT-PCR. **(A)** Heat map of genes altered 24 hours after treatment with 100nM CRH compared with 1% acetic acid (control vehicle). The maximum expression of any given gene is shown in red; minimum expression is shown in green. Representative results from

three independent biological replicates are shown. (B) Volcano plot of gene expression in (A), showing expression in response to CRH versus vehicle. Each circle in the plot represents one gene. Genes which are increased at least 2-fold are in red whereas the genes which are decreased at least 2-fold are in green; genes represented by black circles were not differentially regulated ($P<0.05$, paired Student's *t*-test $N=3$). (**C**) In silico predicted integrated network of genes with significantly altered expression in response to CRH. ¥: genes identified as increased in ERα(+) breast tumors (55). ‡: genes whose expression is weakly inhibited by E_2 in MCF7 cells (54). Solid lines denote distinct regulatory relationships as indicated, whereas dotted lines indicate predicted interactions. (**D to F)** Real-time RT-PCR for **(D)** ESR1 and GPER, **(E)** ESR2, or **(F)** CRH-R1 and CRH-R2 in MCF7 cells stimulated with CRH as in (A). Data are mean ± SEM % or fold change relative to control or basal conditions against *GAPDH* expression from at least three independent biological replicates (*$P<0.05$, Mann-Whitney U test; $N=3$).

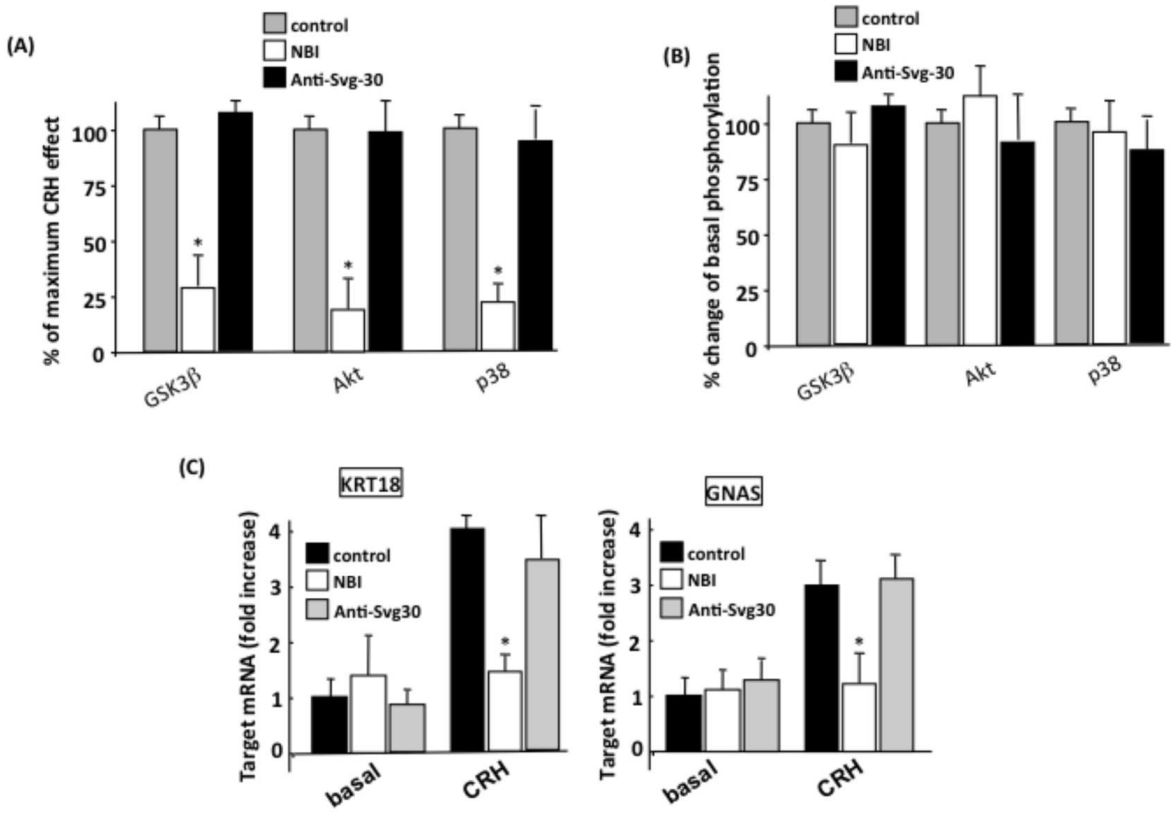

Figure S2: Use of receptor antagonists to identify CRH-R subtypes mediating CRH effects in MCF7 cells. (A and B) Western blots for the phosphorylation of GSK3β at Ser^{9}, Akt, and p38 in (A) MCF7 cells stimulated with CRH for 5 minutes and pre-treated with 1μM NBI (CRH-R1-specific antagonist), 1μM anti-sauvagine-30 (CRH-R2 antagonist) or vehicle (control) for 2 hours or (B) unstimulated cells. Data are mean ± SEM % change versus controls from at least three independent biological replicates (**P* <0.05, Mann-Whitney U test; *N=3*). (C) Real-time RT-PCR for *KRT18* and *GNAS* expression in MCF7 cells stimulated with 100nM CRH for 24hours and pre-treated with specific CRH-R antagonists as in (A) or vehicle. Data are mean ± SEM fold change relative to basal conditions, normalized to *GAPDH*, of at least three independent biological replicates (**P* <0.05, paired Student's *t*-test *N=3*).

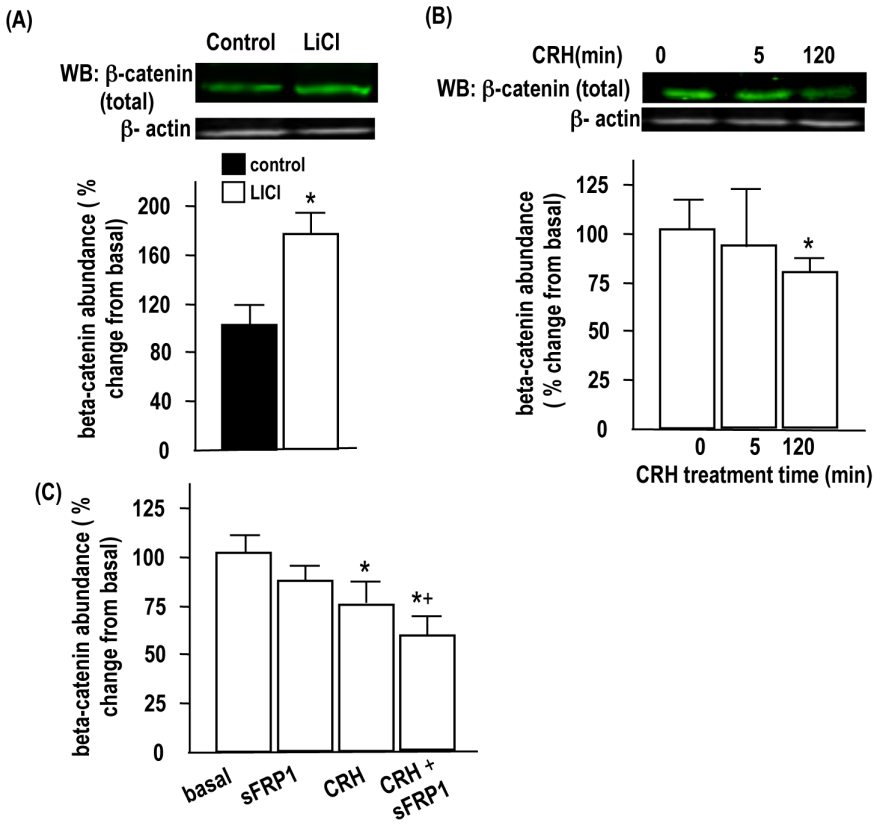

Figure S3: CRH-regulated β-catenin expression in MCF7 cells. (A – C) Western blots with lysates from MCF7 cells treated with (A) 40 nM LiCl, (B) 100nM CRH for up to 120 min, or (C) 300nM sFRP1, 100nM CRH, or combined for 3 hours,. Immunoblots shown are representative of three independent experiments. Bar graphs show means ± SEM of % change relative to basal (untreated) of at least three independent biological replicates. (*P <0.05, Mann-Whitney U test; $N=3$; ^{+}P<0.05, Kruskal-Wallis ANOVA followed by Bonferoni test, $N=3$).

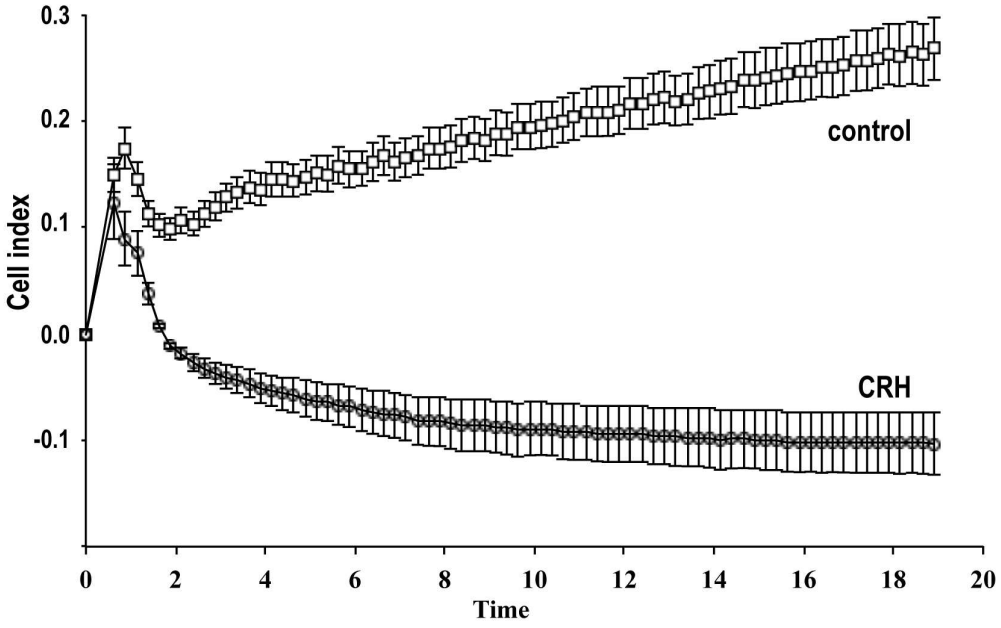

Figure S4: Kinetics of basal and CRH regulated MCF7 cell invasion. Kinetics of invasion was measured with the xCELLigence System. Cells were either treated with 1% acetic acid (control; squares) or treated with 100 nM CRH (circles), and invasion was measured over 19 hours at frequent time intervals. Results shown are means ± SEM of the Cell Index curves of at least three independent biological replicates.

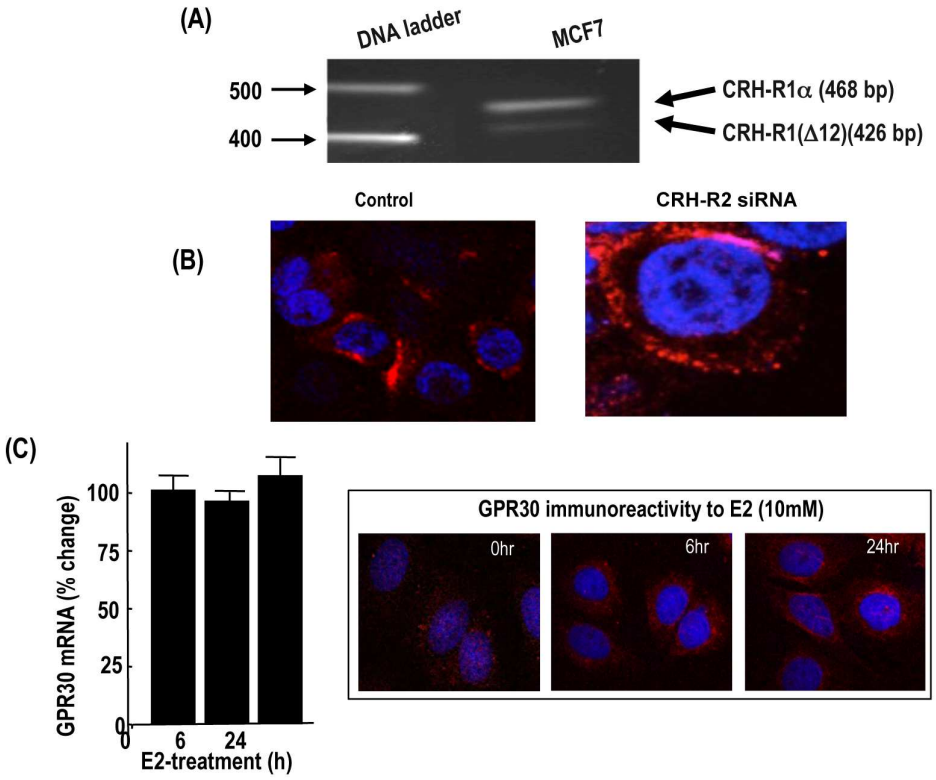

Figure S5: CRH-R1 and GPR30 mRNA and protein expression in MCF7 cells. (A) Conventional RT-PCR for CRH-R1α or CRH-R1(Δ12) in MCF7 cells. PCR products were subsequently electrophoresed and visualized by ethidium bromide. Photomicrographs shown are representative of three independent experiments. (B) Confocal microscopy images of CRH-R1 in MCF7 cells transfected with or without *CRH-R2* siRNA prior to stimulation with 100nM E_2 for 24 hours. Photomicrographs shown are representative of three independent experiments. **(C)** Real time PCR of GPR30 expression from RNA isolated from MCF7 cells stimulated with E_2. Data are mean ± SEM of % change versus control, normalized to*GAPDH* mRNA, from at least three independent biological replicates (**P*<0.05, Mann-Whitney U test, *N*=*3*). Representative confocal microscopy images of GPR30 localization in MCF7 cells treated with E_2 (inset, C). Photomicrographs shown are representative of three independent experiments.

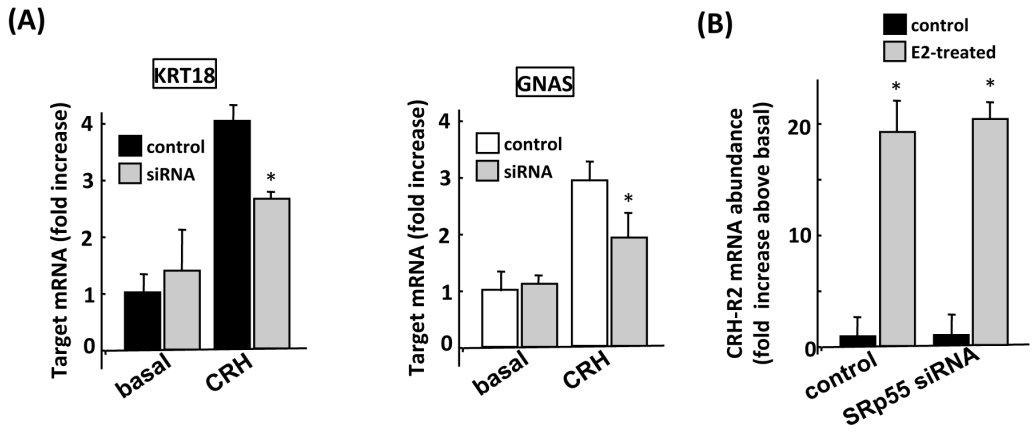

Figure S6: Effect of knockdown of SRp55 on CRH- and E_2-induced transcriptional effects in MCF7 cells. (A-B) Real time PCR of (A) *KRT18* and *GNAS* or (B) *CRH-R2* expression in MCF7 cells transfected with or without *SRSF6* siRNA prior to stimulation with either 100nM CRH for 24 hours (A) or 100nM E_2 for 24 hours (B). Data are mean ± SEM fold change relative to control from at least three independent biological replicates, normalized to *GAPDH* expression. (**P*<0.05, Mann-Whitney U test, *N=3*).

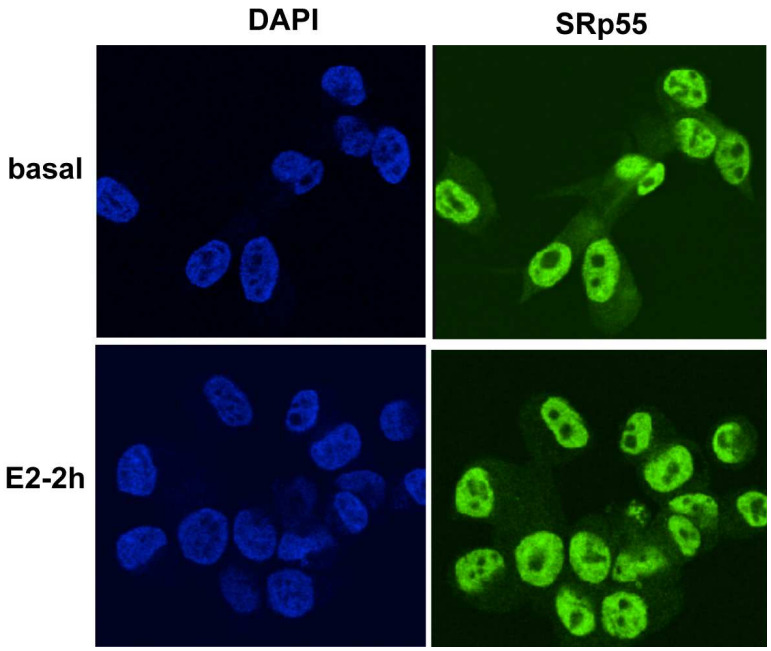

Figure S7: Subcellular localization of SRp55 expression in E_2-treated cells. Representative confocal microscopy images of immunofluorescent-labelled SRp55 localization in MCF7 cells treated with or without 100nM E_2 for 2 hours. Photomicrographs shown are representative of three independent experiments.