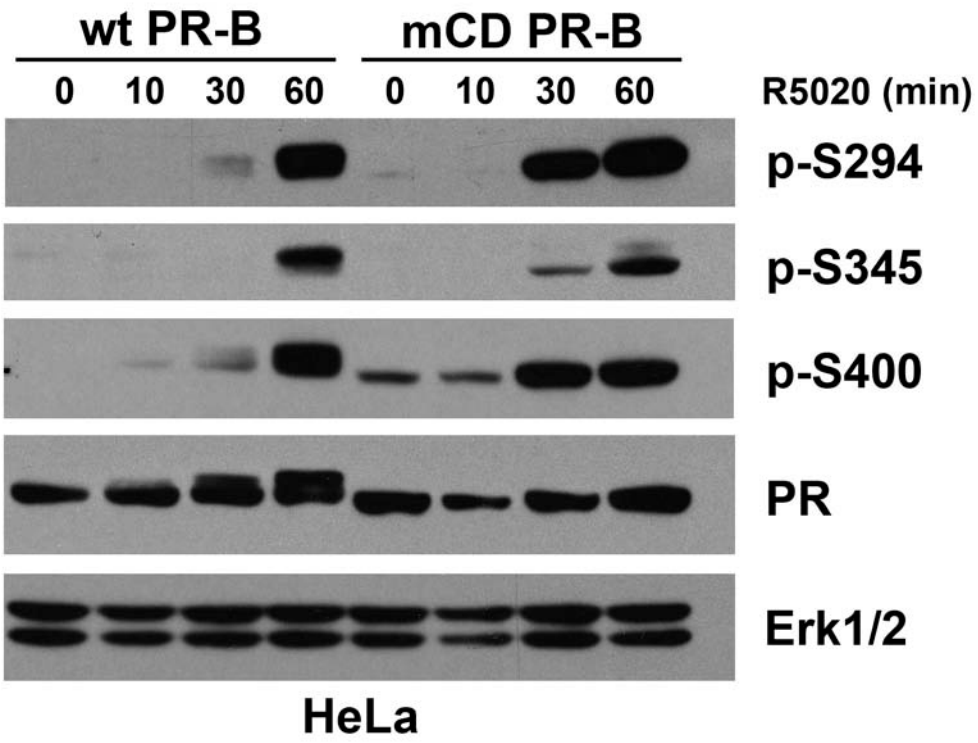
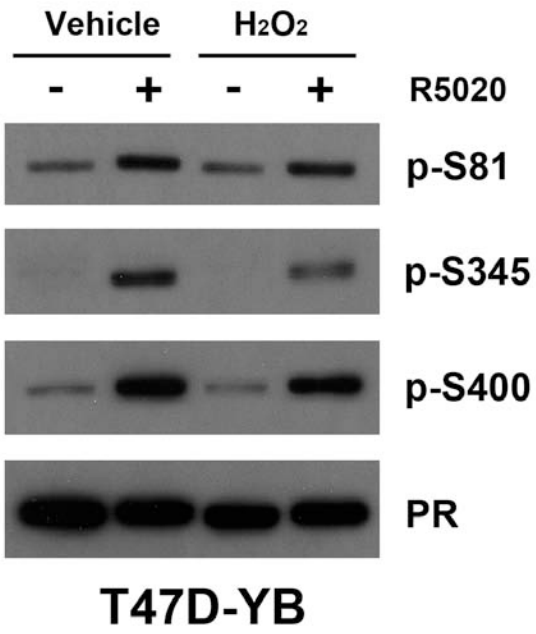
Supplementary Figure 1.



Supplementary Figure 1. PR-B phosphorylation is altered by mutation to the CD of PR-B.

PR-B Ser81 phosphorylation is altered in cells expressing mCD PR-B. HeLa cells were transiently transfected with wt PR-B, mCD PR-B, or vector only. 24hr following transfection, cells were starved for 18hr in serum-free media and then treated with 10nM R5020 or EtOH for 60min. Lysates were analyzed via Western blotting using p-S294, p-S345, p-S400, PR-B, and Erk1/2 antibodies.

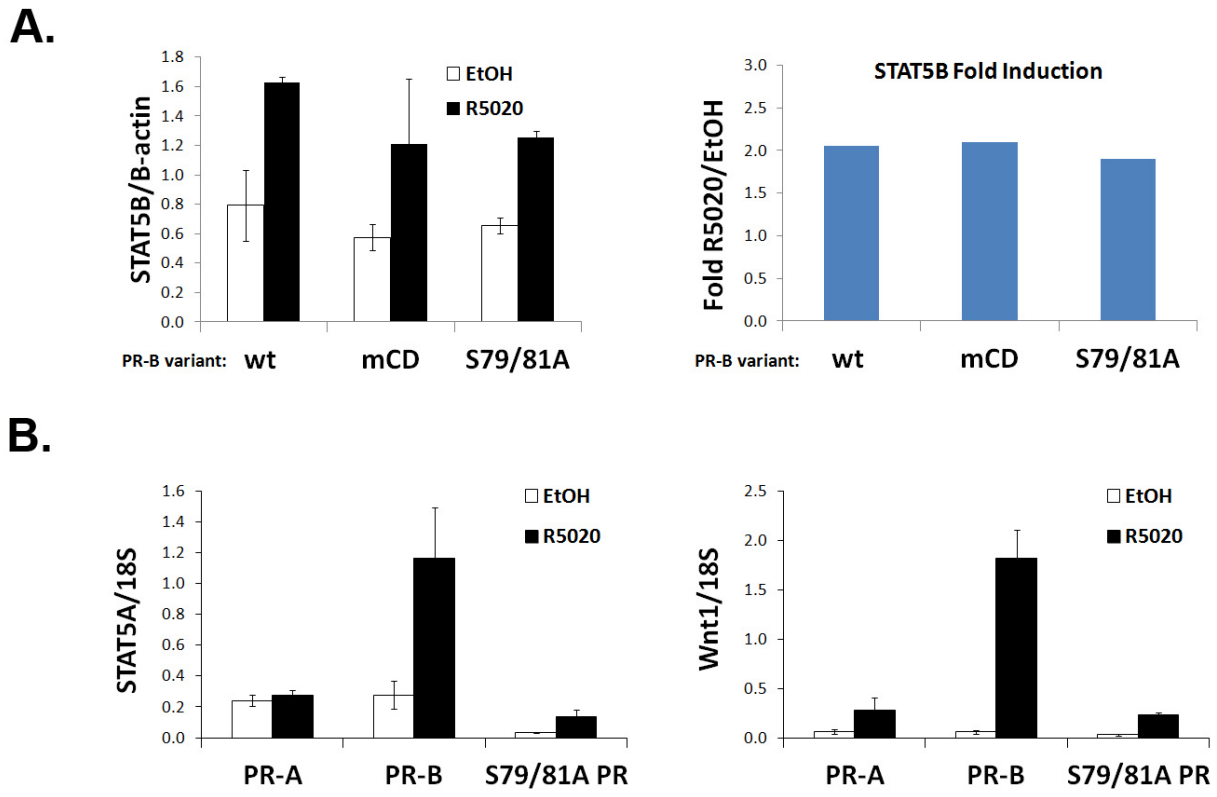
Supplementary Figure 2.



Supplementary Figure 2. PR phosphorylation is unaffected by high ROS levels.

T47D-YB cells were pre-treated with 1mM H_2O_2 for 20min, followed by 10nM R5020 (or EtOH) for 30 min. Lysates were analyzed via Western blotting using phospho-specific PR, total PR, p-Erk1/2 and Erk1/2 antibodies.

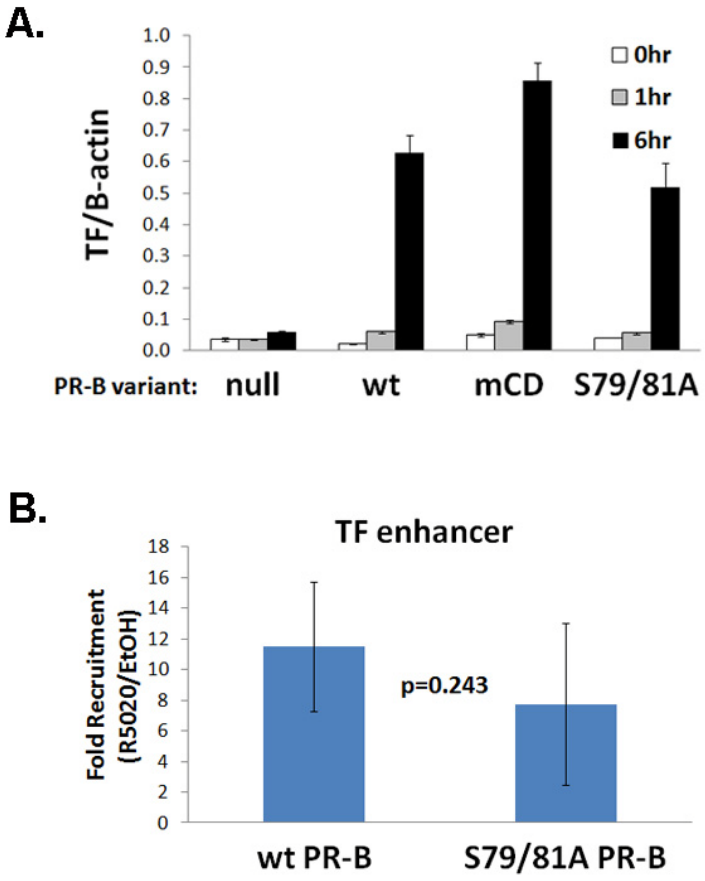
Supplementary Figure 3.



Supplementary Figure 3. Gene expression characterization of mCD and S79/81A PR-expressing cells.

A. STAT5B is unaffected by PR Ser81 phosphorylation. Left: T47D-Y cells stably expressing either wt PR-B, mCD PR-B or S79/81A PR-B were starved for 18hr in serum-free media, followed by treatment with 10nM R5020 or EtOH for 6hr. mRNA levels were analyzed by qPCR. Error bars represent ±STD. Right: Fold Induction (R5020/EtOH) of STAT5B mRNA. **B.** S79/81A PR-B-expressing cells are phenotypically similar to PR-A-expressing cells. T47D-Y cells stably expressing either PR-A, PR-B or S79/81A PR-B were starved for 18hr in serum-free media, followed by treatment with 10nM R5020 or EtOH for 6hr. mRNA levels were analyzed by qPCR. Error bars represent ±STD.

Supplementary Figure 4.



Supplementary Figure 4. PR regulation of TF gene expression is independent of PR-B Ser81 phosphorylation.

A. TF expression is not regulated by PR-B Ser81 phosphorylation. T47D-Y cells stably expressing either wt PR-B, mCD PR-B, S79/81A PR-B, or unmodified (PR-null) cells were starved for 18hr in serum-free media, followed by treatment with 10nM R5020 or EtOH for 0-6hr. mRNA levels were analyzed by qPCR. Error bars represent ±STD. **B.** T47D-Y cells stably expressing wt PR-B or S79/81A PR-B were serum-starved for 18hr. Cells were then treated with EtOH or 10nM R5020 for 60min. Fixed lysates were subjected to ChIP with antibodies against PR (or species-specific IgG as a control), and qPCR was performed on the isolated DNA using primers designed to amplify a PRE in the TF enhancer. Fold recruitment of PR in R5020/EtOH is shown. Error bars represent ±STD of triplicate experiments.

Materials and Methods (Supplemental)

Cell Lines and Constructs

The estrogen-independent ER/PR positive T47Dco (T47D) variant cell line has been previously described (35), and is the parent cell line from which all T47D-variants used herein were created. T47D-Y (PR negative), T47D-YB (stably expressing wt PR-B) and T47D-YA (stably expressing wt PR-A) cells were characterized by Sartorius *et al* (36). T47D-S79/81A PR have been previously described (19). T47D-mCD PR cells were created by stable expression of pSG5-mCD PR and pSV-neo in T47D-Y cells using FuGene-HD (Roche). Individual colonies were selected in 500µg/ml G418 and maintained in 200µg/ml G418 after initial selection.

T47D-Y and HeLa cells were maintained at 37°C in 5% CO_2 in Minimum Essential Media (MEM; CellGro) supplemented with 5% FBS, 1% Penicillin/Streptomycin, 1% non-essential amino acids, and 6 ng/ml insulin (cMEM). T47D-YB, T47D-YA, T47D-S79/81A PR and T47D-mCD PR cells were maintained under the same conditions, with the addition of 200 µg/ml G418. COS cells were maintained in Dulbecco's Minimum Essential Media (DMEM; CellGro) supplemented with 10% FBS and 1% Penicillin/Streptomycin.

The pSG5-mCD PR plasmid was created as follows: DNA fragments encoding base pair changes (glutamic/aspartic acid to alanine mutations at D68, D71, E72 and D76) needed to achieve the mCD PR mutations were created using two-step PCR, as previously described (41). Fragments were then inserted into the pSG5 PR-B vector using restriction enzyme-digestion, replacing the respective wt PR-B sequence with mCD PR sequence.

The CD-PR-A and mCD-PR-A constructs were created as follows: wt and mCD oligos (sequence included in Supplementary Table 2; both forward and reverse oligos were created) were commercially synthesized (IDT), ligated and cloned into pSG5-wt PR. The restriction sites were designed such that cloning of the ligated oligos replaces the BUS (first 165 amino acids) in wt PR-B with the synthetic wt or mCD domain, with translation in frame following the methionine start site of PR-A. Synthetic CD domain oligos contain the translational start site (methionine) followed by the CD domain from wt PR (flanked on either side by two amino acids from the wt PR sequence), finishing with the first four amino acids from PR-A (flanked on both ends with restriction enzyme sites for cloning). Both CD-PR-A and mCD-PR-A were capable of activating PRE-luciferase transcription.

The MKP3 construct (pcDNA3.1(-)Myc-His) was the gift of Stefanie Dimmeler, University of Frankfurt.

Gene expression profiling

T47D cells stably expressing pSG5 empty vector, wt PR or mCD PR were serum starved in modified IMEM (Gibco) for 1 day, treated with R5020 (10 nM) or vehicle control for 6 hr. Total RNA was extracted using a RNeasy kit (QIAgen) with on-column DNase I treatment (QIAgen). Triplicate RNA samples were labeled and hybridized to the Illumina HT-12v4 bead chip platform according to manufacturer's protocols. Chip scanning within Genome Studio software produced raw expression values were analyzed within R software using the Bioconductor (37) package called lumi where raw intensities were log2 transformed and quantile normalized. Differentially expressed genes were analyzed using the limma package, where empirical Bayes was used to better estimate the variance of the genes. Gene expression data presented contain log2 normalized intensities and biological comparisons presented (e.g. R5020/vehicle) contain log2 fold change with the Benjamini and Hochberg (BH) adjusted *P* value) (38). Heat maps were generated through unsupervised hierarchical clustering of probes by the heatmap.2 function in the gplots R software package. Clustering was performed using Euclidean distance and complete linkage. Rows were scaled to have mean zero and standard deviation equal to one. Gene expression data is available in the NCBI Gene Expression Omnibus (GEO) database (accession number: GSE46850; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=phcjjiycasueqza&acc=GSE46850)

Pathway and Gene Set Analysis

Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com) was used to compare biological functions or network pathways in cells expressing wt or mCD PR after progestin treatment (wt+R5020/ wt–R5020 or mCD+R5020/ mCD–R5020). Default IPA settings for Core analyses were used to evaluate upregulated genes (fold change >1.5, P <0.05). IPA Comparison analyses were used to reveal whether or not cells expressing wt or mCD PR regulated functionally distinct pathways. Analyses were scored based on significance (the BH adjusted *P* value, corrected for multiple hypothesis testing) and the threshold for a gene list to be significantly involved in a particular biological function was P <0.05 (i.e. $-\log_{10}$(BH adjusted *P* value) >1.30).

Gene set enrichment (GSEA) and Leading Edge analysis (39,40) was performed using the javaGSEA desktop software; all five gene set collections (c1-c5) from the Molecular Signatures Database (MSigDB) version 3.1 were queried. Dataset files were developed based on normalized Illumina expression intensities from cells that constitutively express PR. Specifically, the log2 fold change values were compared for two phenotypes in our ligand-dependent analysis: (wt +R5020/–R5020) vs. (mCD +R5020/–R5020). GSEA was executed using the default settings, except the permutation type was set to Gene_set with 1000 permutations, and the metric for ranking genes was set to Diff_of_Classes, because normalized expression data was log2 transformed. Each MSigDB collection was analyzed individually in multiple GSEA runs.

Immunoblotting

For the majority of immunoblotting presented here (exceptions noted in figure legends), cells were starved for 18hr in serum-free iMEM media. Following 18hr starvation, cells were treated, if applicable. Whole cell lysates were isolated using a modified radioimmune precipitation assay (RIPA) buffer (0.15M NaCl, 6mM Na_2HPO_4, 4mM NaH_2PO_4, 2mM EDTA, 1% Triton-X, 0.1M NaF; in H_2O) supplemented with protease and phosphatase inhibitors. Lysates containing equal protein levels (between 25 and 30µg protein was loaded per lane on each gel) were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore) for subsequent immunoblotting analysis. Membranes were probed with primary antibodies recognizing total PR (ThermoScientific #MS-298-P), phospho-Ser294 (Lab Vision Corp. #MS-1332), phospho-Ser81, 345 and 400 (custom-created antibodies previously published in (15,19,42)), Erk1/2 (Cell Signaling #9102), phospho-Erk1/2 (Cell Signaling #9101), myc-tag (Cell Signaling #2276) and MKP3-M (Cell Signaling #3058 or Santa Cruz Biotechnology #sc-137245 or sc-100374). Mouse and rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from BioRad, and chemiluminescence was visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Company). All Western blotting experiments were performed at a minimum in triplicate, and representative experiments are shown in each respective figure. Densitometry (when applicable) was determined using Image J analysis.

Co-immunoprecipitation (CoIP) experiments

For Co-IP experiments, cell lysates were collected in RIPA (as supplemented as above) and incubated on ice for 60min. Cell lysates containing equivalent protein concentrations (1000µg) were incubated overnight at 4˚C with 2µg appropriate antibody or control IgG. Protein G agarose (Roche Diagnostics, Indianapolis, IN) was added for the final 1hr of incubation time. Immune complexes were washed three times with supplemented RIPA buffer, resuspended in Laemmli sample buffer containing dithiothreitol (DTT) and β-mercaptoethanol, boiled for 5 min, and subjected to Western blotting analysis.

Transient transfections

24hr after cell plating, HeLa cells were transfected with pSG5-vector, pSG5-wt PR or pSG5-mCD PR using FuGene6 (Roche). 24hr following transfection, cells were starved for 18hr in serum-free iMEM (Modified Improved MEM). Following starvation, cells were treated as noted in the respective figure legend and total cell lysates were isolated as described below.

H_2O_2 assays

Following an overnight starve in serum-fee iMEM, T47D-wt PR cells were pre-treated with 1mM H_2O_2 for 20min, followed by 30min 10nM R5020. Protein lysates was isolated and analyzed as described above.

Luciferase Transcription Assays

Luciferase assays were performed as previously described (41) using the Dual Luciferase Reporter Assay (Promega). Relative luciferase units (RLU) were normalized to *Renilla* ± standard deviation (SD).

siRNA

ON-TARGETplus SMARTpool designed to target Human MKP3 (DUSP6) was purchased from Dharmacon, as were non-silencing siRNA controls. For siRNA experiments, 24hr following cell plating, T47D-YB cells were transfected with 50nM non-silencing or MKP3 siRNA. 72hr later, cells were treated with EtOH or 10nM R5020 for 60min. Protein lysates were isolated and analyzed as described above.

Reagents

Cells were treated with the following reagents (when applicable): R5020 (10nM; Sigma), AG490 (50μM; CalBioChem), H_2O_2 (1mM; from Cell Biolabs OxiSelect™ Intracellular ROS Assay Kit).

Cell cycle analysis/Flow cytometry

Flow cytometry for cell cycle analysis was performed as previously described (42).

Real-Time Quantitative PCR (qPCR)

Cells were plated at 5 x 10^5 cells/well in triplicate wells of a 6-well plate. Following 18hr starvation in serum-free iMEM, cells were treated for 1-6hr with 10nM R5020 or EtOH (if applicable; see respective figure legend). In experiments were AG490 was used, cells were pre-treated with 50μM AG490 for 1hr, followed by 6hr of R5020 treatment. Total RNA was isolated using Trizol (Invitrogen); cDNA was created using the QScript cDNA Supermix (VWR) following manufacturer's recommendations. qPCR was performed on equal amounts of cDNA using the Light Cycler 480 SYBR Green1 Master Mix on a Roche 480 Light Cycler. Results in triplicate for each gene of interest were normalized to either β-actin, 18S or GAPDH (as indicated in each

respective graph) ± SD. Primer sets used for qPCR are listed in Supplementary Table 2. Relevant genomic sequence information and enhancer positioning for Wnt1 genomic sequence is based on GR37 Release 57 (March 2010).

ChIP assays

ChIP was performed using the ChIP-IT Express Kit (Active Motif) according to manufacturer's instructions using sonication as the method for chromatin shearing. Lysates were immunoprecipitated (IP) overnight (18hr) with the following antibodies: PR (ThermoScientific #MS-298-P), STAT5 (Santa Cruz Biotechnology sc-1081 and sc-836), ck2α (Santa Cruz sc-12738), MKP3 (Santa Cruz Biotechnology sc-100374) or an equal amount of mouse or rabbit IgG. Resulting DNA was analyzed using qPCR as described above, and data is represented as a percentage of input DNA. *In silico* analysis using MatInspector (Genomatix) identified potential PRE-binding sites using the following consensus sequence: RGNACANRNTGTNCY. Primer sets used for ChIP-qPCR are listed in Supplementary Table 2.

CEAS

Web-based CEAS analysis (http://ceas.cbi.pku.edu.cn/index.html) was performed on a publically available PR ChIP-Chip dataset (http://cistrome.org/NR_Cistrome/Cistrome.html). TRANSFAC and JASPAR motifs were used to determine putative transcription factor binding sites.

Statistics

Statistical significance for all experiments was determined using an unpaired Student's *t* test, unless otherwise specified.