Supplemental Materials and Methods

Antibodies. An antibody against p-c-Jun KM-1 was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p65ser276, p65ser536, p-p38, p-JNK and p-MEK1/2 were all from Cell Signaling Technology (Danvers, MA). GRIP1 Ab-1 was from Thermo Scientific (Fremont, CA). The anti-mouse-HRP, anti-rabbit-HRP and anti-goat-HRP secondary antibody conjugates were from Amersham Biosciences (Buckinghamshire, UK).

Plasmids. 2XPRE-tk-Luc, 5X-Gal4-TATA-Luc, the mammalian two-hybrid plasmids pVP16-hPR-A, pVP16-hPR-B, pVP16-ERα, pVP-16-ERβ, and pVP16-GR were described previously (1). The normalization vector pCMV-ß-gal was obtained from Clontech (Palo Alto, CA).

Subcloning of pcDNA3-hPR-B plasmid was described previously (2). pENTR-hPR-B was subcloned from pcDNA3-hPR-B into pENTR-1A (Invitrogen, Carlsbad, CA) with KpnI and EcoRI. The hPR-B dimerization mutants hPR-B-A604T and hPR-B-3D (A604T/R606D/D608C) were created by site-directed mutagenesis, using the sense primer CACAACTACTTATGT**A**CTGGAAGAAATGAC and the antisense primer GTCATT TCTTCCAG**T**ACATAAGTAGTTGTG for hPR-B-A604T, the sense primer TTATGT**A**CTGGA**GAT**AAT**TG**CTGCATCGTTGAT and the antisense primer ATCAACGATGCAG**CA**ATT**ATC**TCCAG**T**ACATAA for hPR-B-3D.

The pMSCV-GFP-hPR-B, pMSCV-GFP-hPR-B-A604T, pMSCV-GFP-hPR-B-3D or pMSCV-Gal4-DBD (no ATG, empty vector control) construct was created using the Invitrogen Gateway recombinase subcloning system according to the manufacturer's

instructions. To do this, hPR-B, hPR-B-A604T, hPR-B-3D or Gal4-DBD was shuttled from pENTR-Gal4DBD, pENTR-hPR-B, hPR-B-A604T or pENTR-Gal4-DBD to pMSCV-IRES-EGFP that was converted to a Gateway destination vector.

pM-LX23 was constructed as follows: the peptide LX23 was obtained by phage display as described below. The Gal4-DBD-peptide construct (pM-LX23) was made by digesting the phage DNA with XhoI and XbaI and subcloning the resulting fragment encoding the peptide into pMs/x that had been digested with SalI and XbaI. To make pM-LXAA, oligonucleotides with mutations, TCGAGAATTCATGGGTATTCGCCGATG**GCT**AGGGCT**GCTGCT**CTTGAGGAGG AGGCGCCGAAG and the antisense primer CTAGCTTCGGCGCCTCCTCCTCAAG**AGCAGC**AGCCCT**AGC**CATCGGCGAATA CCCATGAATTC, were ligated into pMsx vector (1) digested with SalI and XbaI. The fragment containing Gal4-DBD-LX23 or Gal4-DBD-LXAA was amplified by PCR using the sense primer CACCATGAAGCTACTGTCTTCTATCGAAC and the antisense primer CTACTTCGG CGCCTCCTCCTC using pM-LX23 or pM-LXAA as a template respectively, then subcloned into pENTR/D-Topo vector (Invitrogen).

pVP16-hPR-B-E907A/E911A was created as follows: the fragment of hPR between HindIII restriction site and 3' end was amplified by PCR using pCMV-PRB-E907A/E911A (3) as a template, and subcloned into pVP16-hPR-B at HindIII site.

Cell Culture. HepG2 cells and Hela cells were purchased from ATCC (Manassas, VA). HepG2 cells or Hela cells were maintained in MEM supplemented with 8% FBS, 0.1 mM NEAA and 1 mM NaPyr.

Transient transfection Assays. For mammalian two hybrid assays, HepG2 cells or Hela cells were seeded in 24-well plates 24 hr before transfection. DNA was introduced into the cells using Lipofectin (Invitrogen)-mediated transfection as described by the manufacturer. Briefly, triplicate transfections were performed using 3 μg of total DNA, and cells were incubated with the DNA-Lipofectin mixture for 24 hr. Next, the transfection mix was replaced with fresh media containing the appropriate ligands. Following overnight treatment, luciferase and β-gal activities were assayed on a Fusion Alpha-FP HT Universal Microplate Reader (Perkin Elmer, Danvers Grove, IL).

Microarray data analysis. Microarray gene profiling experiments were performed with the Human Genome U133 Plus 2.0 Array chips (Affymetrix, Santa Clara, CA). Target preparation, hybridization to the Affymetrix HG-U133 plus 2.0 arrays, and scanning and analysis were performed by the Duke Microarray Center. The entire experiment was repeated ten times. Analysis of global gene expression was performed in JMP Genomics 4.0 (SAS Institute, Cary, NC). The expression data were log-2 transformed and subject to RMA background correction with quantile normalization and median polish summarization of probesets. Significant changes in gene expression were identified by one-way ANOVA. Family-wise error rate was controlled by Holm step-down procedure at alpha level of 0.05. Gene Ontology overrepresentation analysis (GO ORA) was

performed using GeneTrail (4). All H. sapiens genes were used as a background set; statistical significance was determined by hypergeometric test with FDR correction. Overrepresentation analysis of binding sites for known transcription sites was performed using oPOSSUM (5) at minimum conservation score of 70% and matrix match score of 85%. Both upstream and downstream 5,000 base pairs of transcription initiation sites were considered. Correlations of expression of select genes with relapse-free survival was performed by ranking clinical samples by the expression level of the genes of interest, separating them into equal-sized groups by median expression level and comparing the relapse-free survival by Kaplan-Meyer analysis. Significance was measured by log-rank test. Clinical datasets were RMA-normalized prior to analysis and batch adjustment was performed when necessary using distance-weighted discrimination (6). Raw microarray data have been deposited to NCBI GEO, accession number GSE24468.

Creation of T47D:C42 stable cell lines. T47D:C42 cells were infected with retrovirus expressing either pMSCV-IRES-GFP- hPR-B, pMSCV-IRES-GFP-hPR-B-A604T, pMSCV-IRES-GFP -hPR-B-3D or pMSCV-IRES-GFP-Gal4-DBD. Enhanced green fluorescent protein (EGFP)-positive cells were selected through two rounds of cell sorting using flow cytometry.

M13 phage display of LX23 peptide. Baculovirus-expressed hPR-A, purified in the presence of R5020, was used as a target for the phage display screens. Phage display screening was performed essentially as described previously (1, 7) using the LxxLL peptide library (1). Briefly, for library pannings, immulon-4 96 well plates (Dynex

Technologies) were coated with streptavidin and annealed oligos containing biotinylated 2X PRE (progesterone response elements) were bound to wells at 4°C overnight. 4 pmol of R5020-bound hPR-A diluted in 100mM NaHCO3 (pH8.5) was added to wells and allowed to incubate at room temperature for 3 hours. Wells were then blocked with 1mg/ml BSA in 100mM NaHCO3 (pH8.5) for 1 hour and washed 3 times with phosphate buffered saline plus 0.1% tween 20 (PBS-T). Three rounds of panning were performed in order to enrich for PR-binding peptides. Originating from the enriched pools, two rounds of sequential plaque purification of individual phage were performed. ELISA assay was performed with an anti-M13 phage antibody on enriched pools and on both rounds of plaque purified individual phage to evaluate peptide binding to hPR-A versus negative control BSA. ELISAs were performed at each step for hPR-A bound to biotinylated PRE and directly to plastic to verify assay results.

Gene Silencing. For studies involving transient transfection of small interfering RNA (siRNA), validated Stealth siRNA directed against a control Luciferase sequence (siLuc), control med GC or high GC or two different regions of SRC2 (GRIP-1) were obtained from Invitrogen (see Supp. Table S4 for siRNA sequences). T47D:A18 cells were plated in the presence of 100 nM siLuc or siGRIP-1 using DharmaFECT-1 (Dharmacon, Lafayette, CO) as the transfection agent according to the manufacturer's recommendations. After 70 hr of knockdown, cells were treated with the appropriate cytokine/ligand for 2hr and harvested for qPCR analysis.

Supplemental Figure Legends

Figure 1. *A, B.* PR dimerization mutants were not able to induce the transcription of IκBα, while still maintaining the ability to repress IL-1β induction of cytokines. T47D:C42 cells expressing hPR-B, hPR-B-A604T, hPR-B-3D or empty vector (control) were treated with vehicle (v) or 10nM R5020 (R) in the presence or absence of 10ng/ml IL-1β (I) for 2 hr or 6 hr. mRNA levels of IκBα (*A*) or CCL20 (*B)* were quantitated using qPCR. Results were expressed as fold induction over vehicle treated T47D:C42 control cells. *C*. Expression of hPR-B in T47D:C42 stable cell lines. 25μg of Whole cell extracts of each T47D:C42 stable cell lines were resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for PR1294 or α-tubulin as a loading control.

Figure 2. Ligand-activated PR does not affect the phosphorylation of MAPKs, c-Jun or p65. T47D:A18 cells were seeded in 6 cm plates for 48 hr and serum-starved with 0.1% charcoal-stripped FBS for 24 hr. Cells were pretreated with vehicle (v), 10nM R5020 (R) or 100 nM RU486 (RU) for 45 min, then treated with or without 10ng/ml IL-1β or 50ng/ml TNFα for indicated time points (*A*) or 30min (*B*). After treatment, cells were harvested and 25μg whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for indicated antibodies.

Figure 3. Characterization of synthetic AF-2 specific blocking peptide LX23. *A*. LX23 is a PR-selective CBI. Mammalian two-hybrid assay in HepG2 cells measuring the interaction between LX23 and NRs. HepG2 cells were transfected with 2100ng 5XGal4-

TATA-Luc, 100 ng CMV-βgal, 400 ng pM-LX23 and 400 ng VP16-fusion of the indicated nuclear receptor. 24 hr after transfection, cells were treated with vehicle (v) or 100nM agonist: R5020 (R) for hPR-A and hPR-B, estradiol (E) for ERα and ERβ, and dexamethasone (D) for GR. 48h after transfection, luciferase and βgal assays were performed. LX23 showed no interaction with ER but interacted with GR in a ligand-dependent manner. The observed GR/LX23 interaction doesn't affect our analysis in this study because dexamethasone is required for GR/LX23 interaction. *B.* LX23 binds to the AF-2 coactivator-binding pocket. Hela cells were transfected with 2100ng 5XGal4-TATA-Luc, 100 ng CMV-βgal, 400 ng pM-LX23 and 400 ng pVP16-hPR-B or pVP16-hPR-B E907A/E911A. 24 hr after transfection, cells were treated with vehicle or 10 nM R5020. 48h after transfection, luciferase and βgal assays were performed. LX23 only weakly interacted with the AF-2 mutant hPR-B-E907A/E911A. *C.* LX23 is a potent inhibitor of PR-mediated transcription. T47D:A18 cells were transfected with 1400 ng 2XPRE-tk-Luc, 100 ng CMV-β-gal, 1500 ng total of Gal4-DBD alone plus increasing amount (0, 500, 1000, 1500 ng) of Gal4-DBD-LX23. 24 hr after transfection, cells were treated with vehicle or 10nM R5020. 48h after transfection, luciferase and βgal assays were performed. All of the results are expressed as normalized luciferase activity (normalized with β−gal for transfection efficiency). Results were expressed as fold induction of the vehicle, and data is presented as the mean ± SE of triplicate amplification reactions. Bwt: pVP-16-hPR-B, Bmut: pVP16-hPR-B E907A/E911A.

Figure 4. SRC2 is not required for PR-mediated repression of the genes under investigation in this study. *A.* T47DA18 cells were transfected with stealth siRNA to

control (low GC or med GC) or SRC2 (si GRIP-1 116 or si GRIP-1 118). 70 hr after transfection, cells were treated with vehicle or 10nM R5020, in the presence or absence of 10ng/ml IL-1β for 2 hr, cells were harvested and mRNA levels of CCL2 or IL-8 were quantitated using qPCR. Results are expressed as fold induction over vehicle. *B.* T47D:A18 cells were transfected with stealth siRNA to control (med GC, si Luc) or SRC2 (si GRIP-1 116 or si GRIP-1 118). Cells were harvested 72 hr after transfection. 25μg of whole cell extract was resolved by SDS-PAGE, transferred to PVDF, and subjected to immunoblotting for GRIP-1, PR1294 or α-tubulin as a loading control. Using the same approach, we also ruled out the involvement of SRC-1 and SRC-3 in PR-mediated repression of the genes assessed in this study (data not shown).

Table 1. I-kappaB kinase/NF-kappaB genes identied from gene ontology analysis performed on R5020 regulated genes in hMEC cells.

Table 2. Inflammatory response genes identified from gene ontology analysis performed on R5020 regulated genes in hMEC cells.

Table 3. Correlation of expression of some genes significantly regulated by R5020 in hMEC cells that are part of IκB/NF-κB pathway (GO:0007249) and relapse-free survival in breast cancer patients

Table 4. Expression of cytokines/chemokines were induced by IL-1β or TNFα and repressed by cotreatment of R5020 in T47D:A18 cells. T47D:A18 cells were co-treated

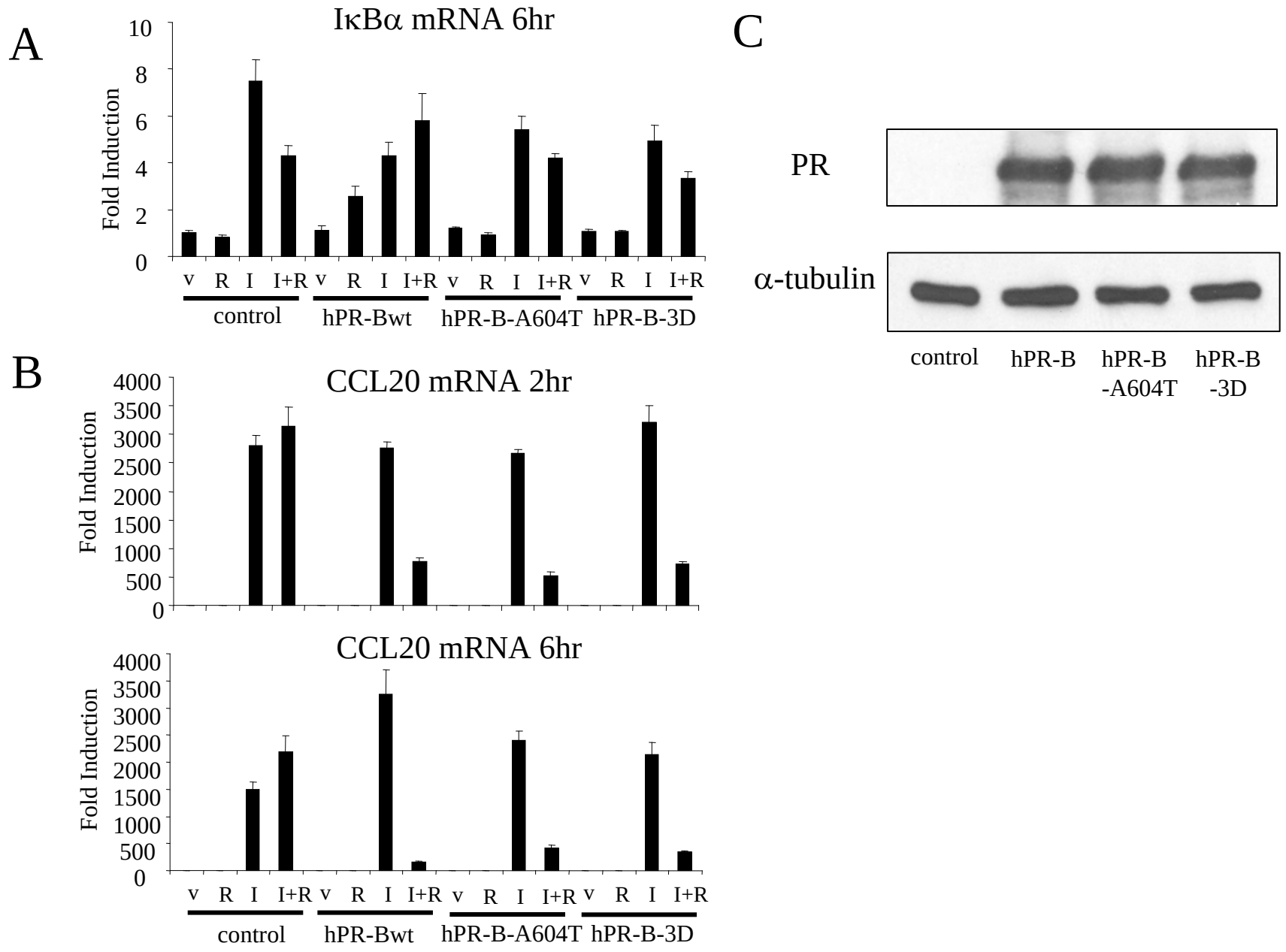
with vehicle (v) or 10nM R5020 (R) in the presence or absence of cytokines (10ng/ml IL-1β(I) or 50ng/ml TNFα(T)) for 6hr. mRNA levels of indicated genes were quantitated using qPCR. Results were expressed as fold induction over vehicle treated cells and data is presented as the mean ± SE.

Table 5. Primers and siRNA sequences used.

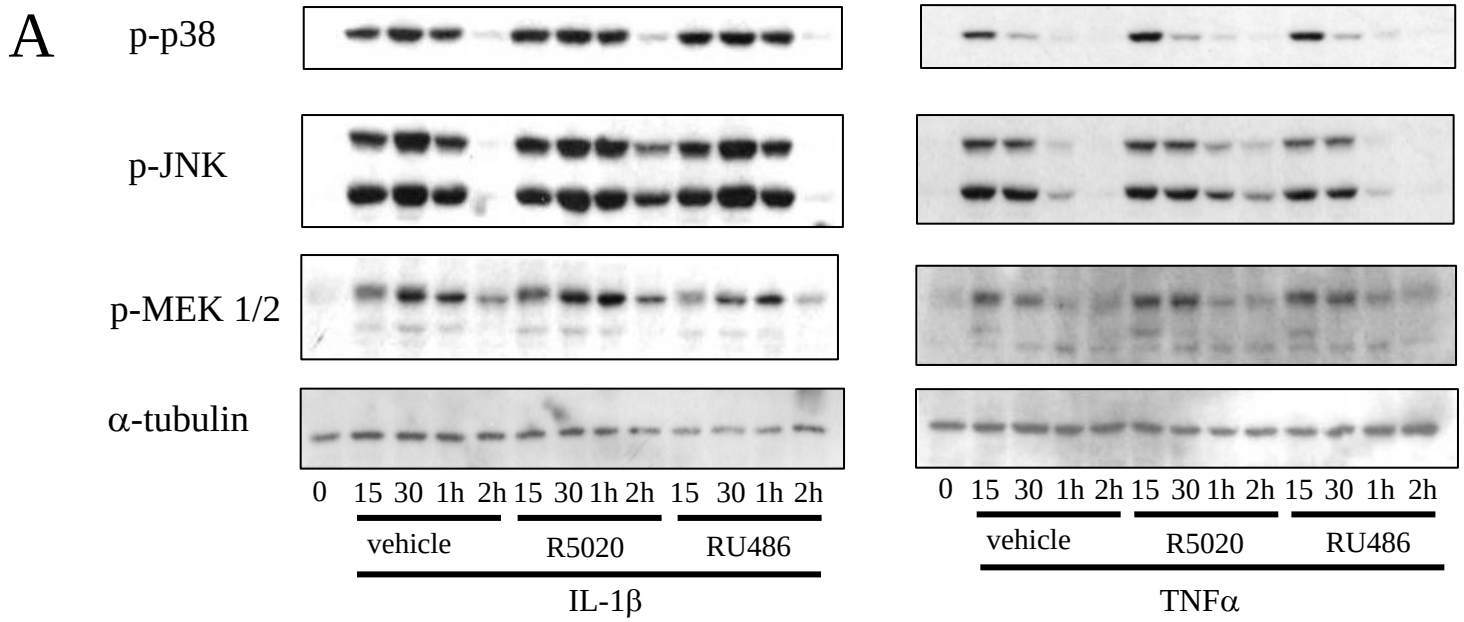
References

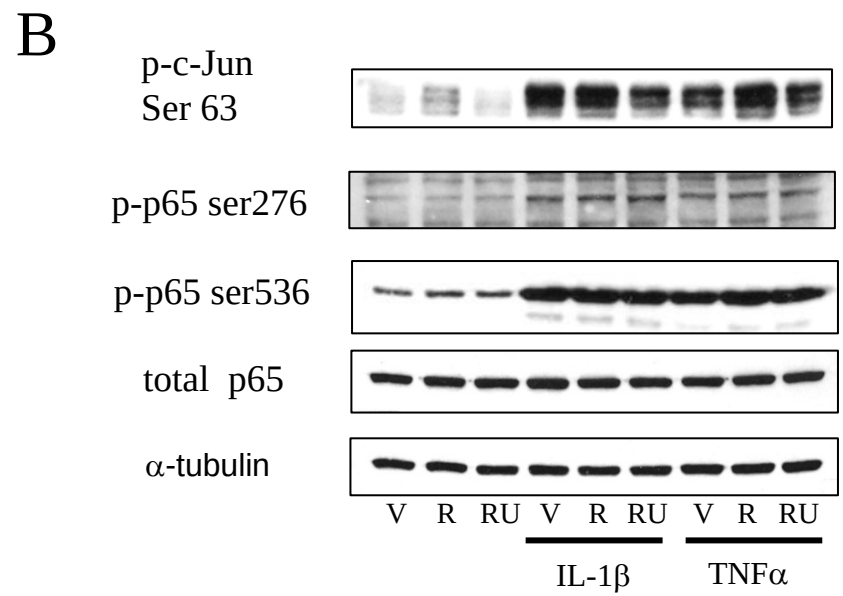
1. Chang C, Norris JD, Gron H, et al. Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Molecular and cellular biology 1999; 19: 8226-39.
2. Wade HE, Kobayashi S, Jansen MS, et al. Multimodal regulation of E2F1 by progestins. Molecular and cellular biology; in revision.
3. Wen DX, Xu YF, Mais DE, Goldman ME, McDonnell DP. The A and B isoforms of the human progesterone receptor operate through distinct signaling pathways within target cells. Molecular and cellular biology 1994; 14: 8356-64.
4. Backes C, Keller A, Kuentzer J, et al. GeneTrail--advanced gene set enrichment analysis. Nucleic Acids Res 2007; 35: W186-92.
5. Ho Sui SJ, Mortimer JR, Arenillas DJ, et al. oPOSSUM: identification of over-represented transcription factor binding sites in co-expressed genes. Nucleic Acids Res 2005; 33: 3154-64.
6. Benito M, Parker J, Du Q, et al. Adjustment of systematic microarray data biases. Bioinformatics 2004; 20: 105-14.
7. Norris JD, Paige LA, Christensen DJ, et al. Peptide antagonists of the human estrogen receptor. Science (New York, NY 1999; 285: 744-6.

Supplementary Figure 1.



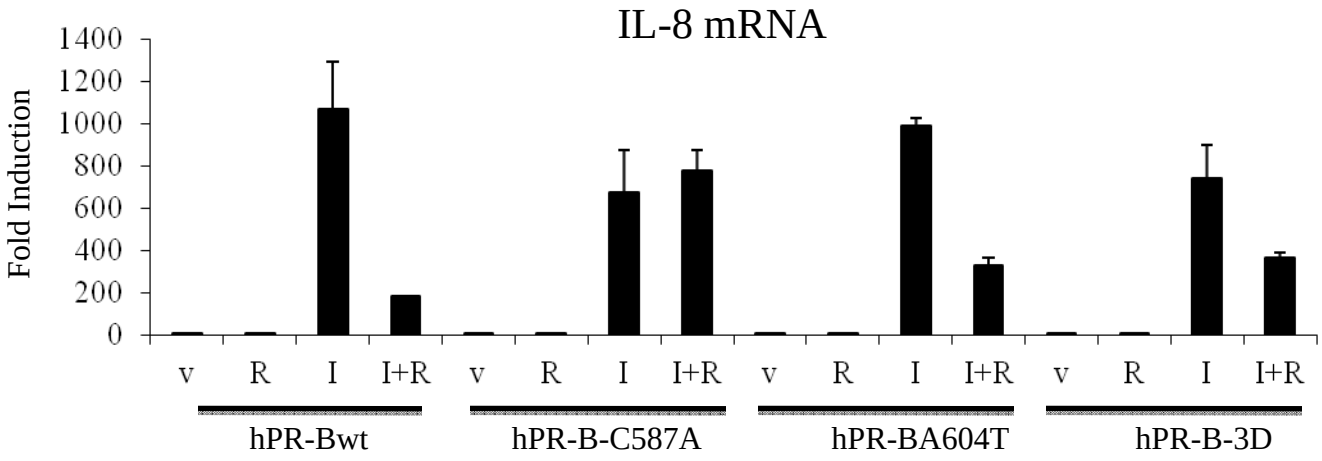
Supplementary Figure 2.





Supplementary Figure 3.

A



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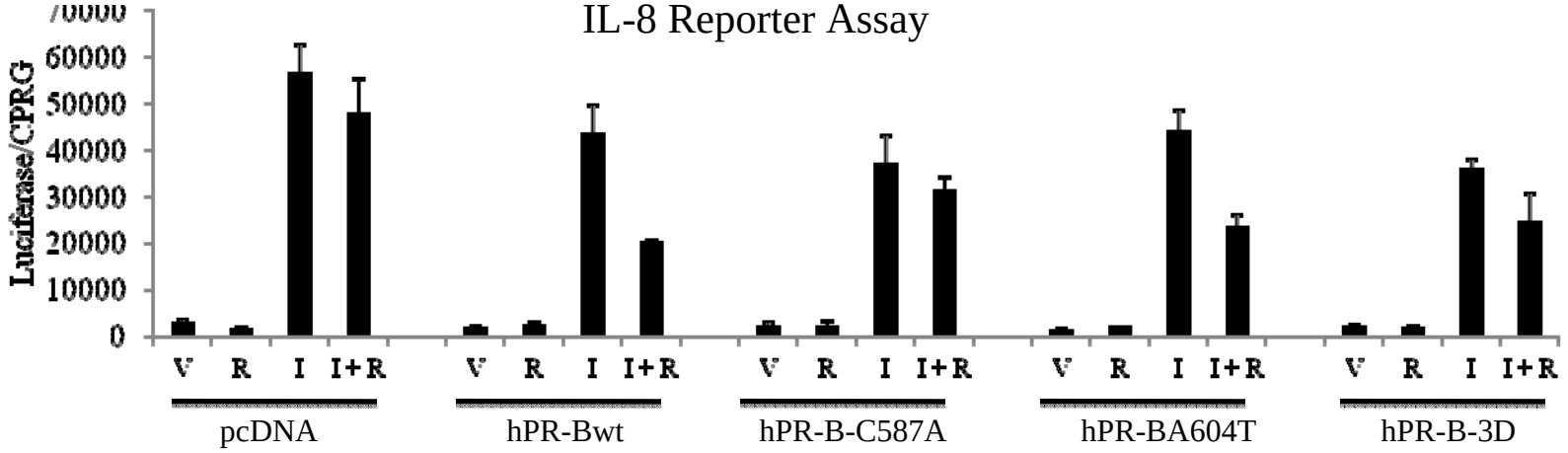
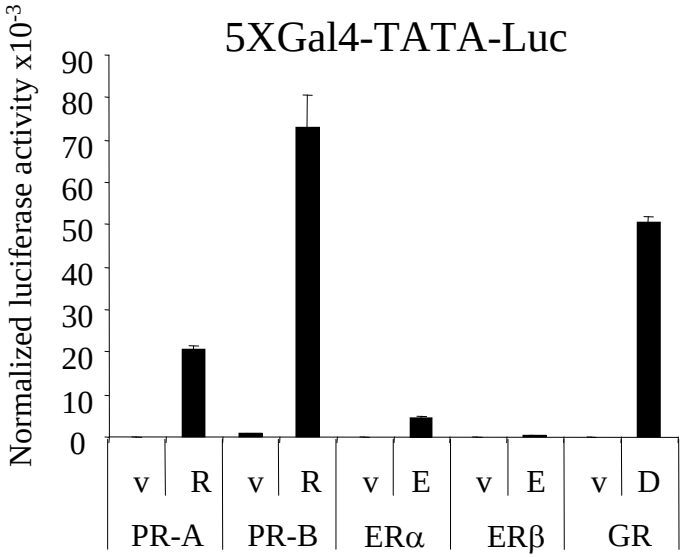


Supplementary Figure 4.

A



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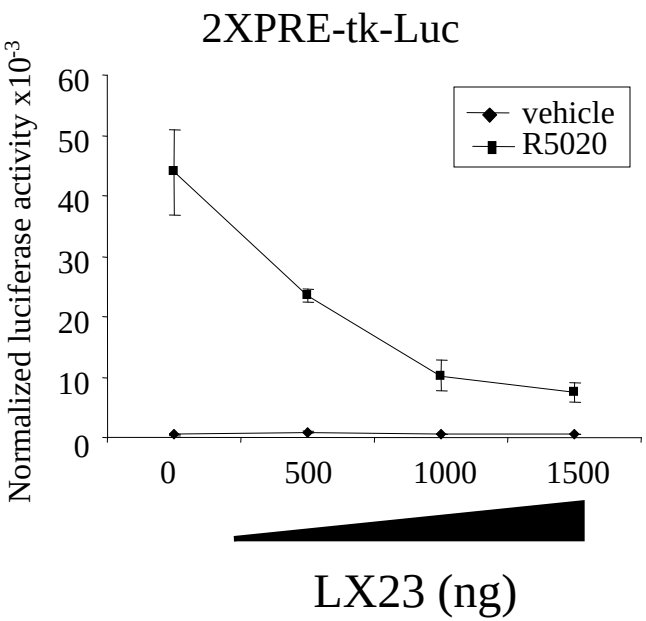

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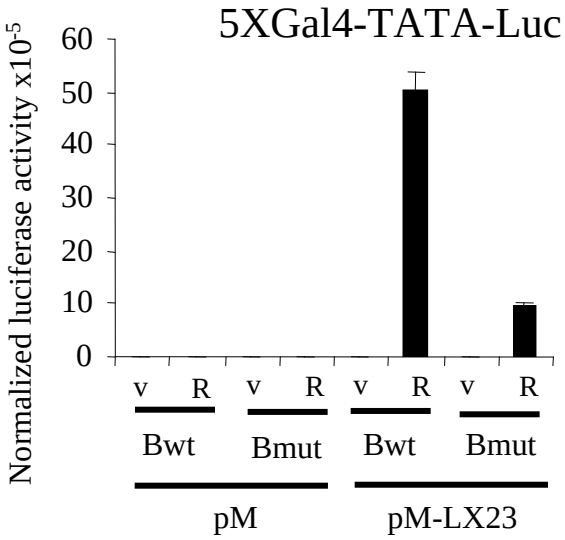

Supplementary Table S1.

Genes in GO:0007249 (I-kappaB kinase/NF-kappaB cascade) regulated by R5020 in hMEC cells

APOL3	RHOC
BCL10	RIPK2
BCL3	SLC20A1
BIRC2	SQSTM1
CASP1	TFG
F2R	TGM2
HMOX1	TLR4
IKBKE	TLR6
IL1B	TNFA
IRAK2	TNFSF10A
LITAF	TNFSF10
LY96	TRAF5
MALT1	TRAF6
MAPK37	TSPAN6
NEK6	VAPA
NKIRAS1	ZDHHC13
PLK2	

Supplementary Table S2.

Genes in GO:0006954(Inflammatory Response) regulated by R5020 in hMEC cells

ACVR1	HDAC9	TNFAIP6
ADRB2	IGFBP4	TPST1
AFAP1L2	IL10RB	UACA
AOX1	IL17C	ZFP36
APOL3	IL1A	
BCL6	IL1B	
BDKRB1	IL1RAP	
BLNK	IL1RN	
BMP2	IL23A	
BMPR1B	IL8	
C1S	IRAK2	
CARD18	ITGB6	
CD97	LY96	
CFB	NDST1	
CHST2	PARP4	
CLU	PLA2G7	
CX3CL1	PROCR	
CXCL1	PTX3	
CXCL10	RBM24	
CXCL11	RIPK2	
CXCL14	S1PR3	
CXCL2	SERPINF2	
CXCL5	SPN	
CXCL5	STAT3	
CXCL6	TGM2	
CXCR2	THBS1	
ELF3	TLR1	
FN1	TLR3	
FOS	TLR4	

Supplementary Table S3.

gene	v	R	I	I+R	T	T+R
IL-8	1.0 ± 0.5	0.9 ± 0.7	227.5 ± 23.4	21.1 ± 3.7	32.6 ± 4.4	2.2 ± 0.5
CCL2	1.0 ± 0.2	0.1 ± 0.1	190.5 ± 14.5	3.1 ± 1.5	256.6 ± 37.9	1.4 ± 0.2
CCL4	1.0 ± 0.4	0.3 ± 0.1	389.8 ± 27.3	160.9 ± 9.5	12.1 ± 1.7	25.0 ± 1.1
CCL5	1.0 ± 0.2	0.6 ± 0.1	44.4 ± 5.8	3.7 ± 0.4	13.8 ± 1.4	1.7 ± 0.2
CCL20	1.0 ± 0.2	1.1 ± 0.1	8719.3 ± 972.3	660.2 ± 69.0	3365.5 ± 401.8	44.8 ± 5.4
CXCL10	1.0 ± 0.2	2.1 ± 0.5	3019.3 ± 210.8	374.8 ± 33.6	1592.0 ± 260.6	75.3 ± 3.7
TNFα	1.0 ± 0.2	4.3 ± 0.5	217.8 ± 15.6	181.0 ± 20.1	1142.8 ± 152.0	335.5 ± 19.0

Supplementary Table S4.

Probe Set ID (133plus2.0)	GSE6532	GSE2034	GSE4922	Duke158*	Regulation by R5020 in hMEC	Gene symbol	Gene Title
221087_s_at	Positive	Positive			Down	APOL3	apolipoprotein L, 3
204908_s_at	Positive	Trend Pos		Positive	Down	BCL3	B-cell CLL/lymphoma 3
209970_x_at		Trend Pos	Trend Pos		Down	CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
211366_x_at	Trend Pos	Trend Pos			Down	CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
211367_s_at		Positive			Down	CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
211368_s_at	Positive	Trend Pos			Down	CASP1	caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
204549_at	Trend Pos			Trend Pos	Down	IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
214398_s_at				Trend Pos	Down	IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
205067_at	Positive	Trend Pos	Positive		Down	IL1B	interleukin 1, beta
39402_at	Positive	Trend Pos	Positive	Positive	Down	IL1B	interleukin 1, beta
209545_s_at	Trend Neg		Negative		Down	RIPK2	receptor-interacting serine-threonine kinase 2
201920_at	Trend Neg		Negative		Down	SLC20A1	solute carrier family 20 (phosphate transporter), member 1
201471_s_at				Negative	Down	SQSTM1	sequestosome 1
213112_s_at	Negative		Negative	Negative	Down	SQSTM1	sequestosome 1
244804_at				Negative	Down	SQSTM1	Sequestosome 1
217839_at		Negative	Negative		Up	TFG	TRK-fused gene
207446_at	Trend Pos	Positive	Positive	Trend Pos	Down	TLR6	toll-like receptor 6
209108_at	Trend Pos			Trend Pos	Down	TSPAN6	tetraspanin 6
209109_s_at	Trend Pos		Positive	Trend Pos	Down	TSPAN6	tetraspanin 6
219296_at	Negative	Negative	Trend Neg		Up	ZDHHC13	zinc finger, DHHC-type containing 13