SUPPLEMENTAL DATA

PR assembly of a nuclear complex with AP-1, Stat3, and ErbB-2 via the integration of its rapid and genomic effects governs breast cancer growth and response to endocrine therapy

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Inventory of Supplemental Data

Supplemental Figure 1, related to Figures 1, 3 and 5
Supplemental Figure 2, related to Figure 2
Supplemental Figure 3, related to Figure 6
Supplemental Figure 4, related to Figure 8
Supplemental Figure 5 related to Figure 8
Supplemental Figure 6 related to Figure 8

Supplemental Table S1, related to Figure 7
Supplemental Table S2, related to Figure 7
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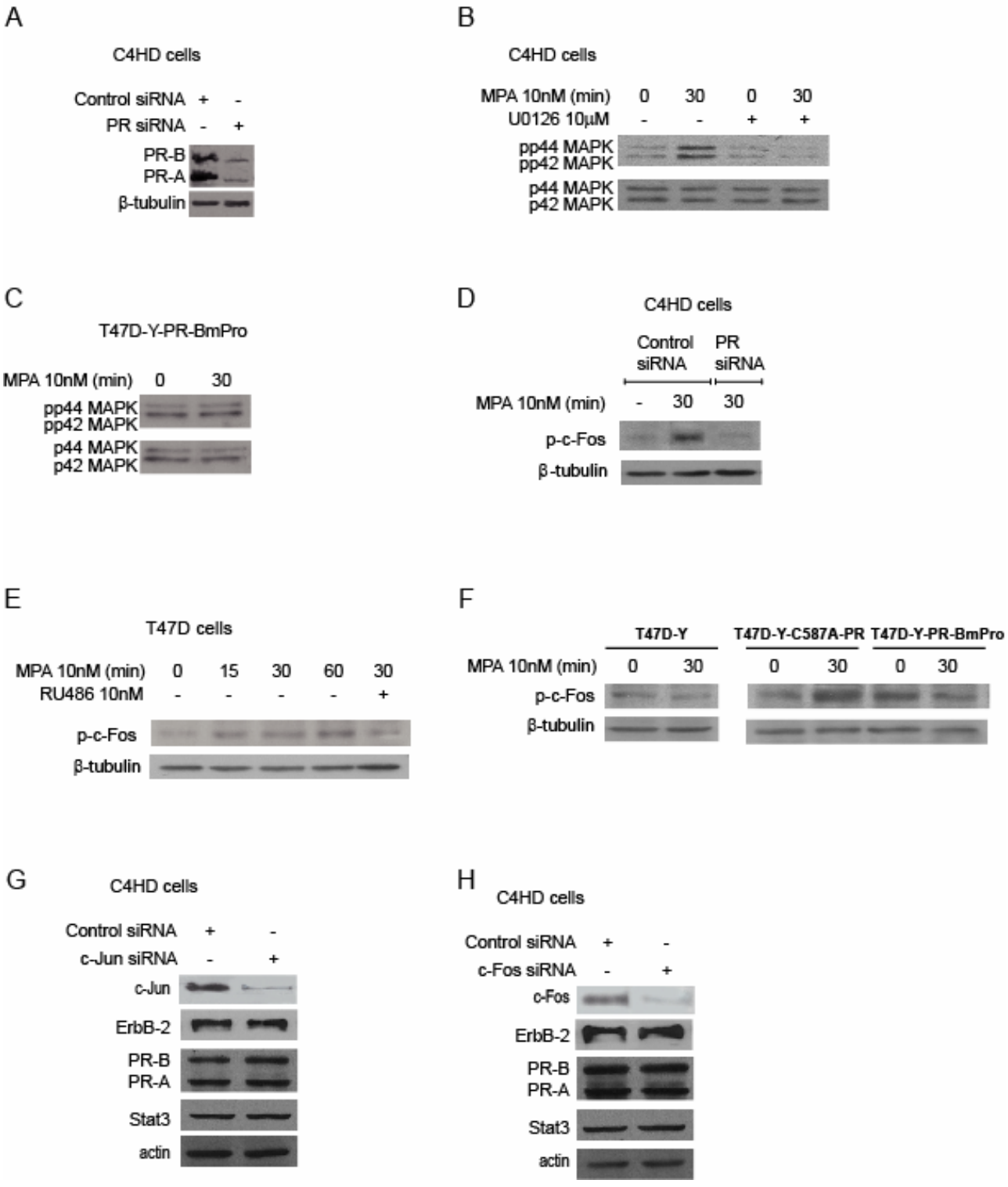
Supplemental Methods

Supplemental Figure 1

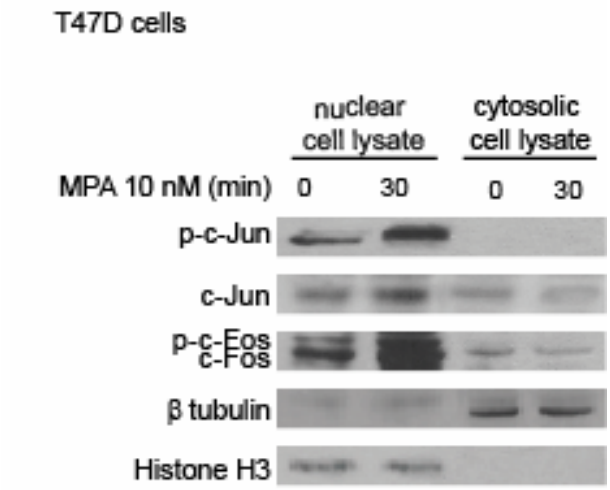


Supplemental Figure 1. Controls of siRNAs effects on PR, c-Jun, and c-Fos protein expression and of UO126 effects on basal p42/p44 MAPKs activation. Lack of p42/p44 activation in T47-Y-PR-BmPro cells. MPA induction of c-Fos phosphorylation studied with a phospho c-Fos antibody

Effects of siRNAs on PR expression. C4HD cells were transfected with PR siRNA or with control siRNA for 48 h. Sequence of PR siRNA is listed under "Supplemental Methods". Results shown were obtained by the use of siRNAs labeled as #1. **(A)** Cell lysates were analyzed by Western blots (WB) with a PR antibody and β-tubulin was used as loading control. Signal intensities of PR bands in the WBs were analyzed by densitometry and normalized to β-tubulin bands. Data analysis showed that siRNAs resulted in 75-80% knockdown of PR protein expression ($P < 0.001$). **(B)** Lack of effect of UO126 on basal p42/p44MAPKs activation. Cells were treated as indicated and WB was performed with a phospho (p)p42/p44 antibody. Filters were reprobed with a total p42/p44 antibody. **(C)** T47-Y-PR-BmPro cells were treated with MPA as indicated and WB was performed as in **(B)**. **(D)** to **(F)** MPA induction of c-Fos phosphorylation. Cells were treated with MPA or pretreated with RU486 and transfected with PR or control siRNAs before MPA stimulation. T47D-Y cells were transfected with either PR-BmPro or C587A-PR mutants and were then treated with MPA. WBs in **D** to **F** were performed with a phospho Thr 232 (p)-c-Fos antibody and filters were reprobed with a total c-Fos. **(G and H)** Effects of siRNAs on c-Jun **(G)** and c-Fos **(H)** expressions. C4HD cells were transfected with the respective siRNA or with control siRNA as in **(A)**. Sequence of siRNAs are listed under "Supplemental Methods". Cell lysates were analyzed by WBs with c-Jun or c-Fos antibodies. Blots were also revealed with ErbB-2, PR, and Stat3 antibodies as control of the specificity of siRNAs action and to demonstrate that silencing c-Jun or c-Fos expressions did not modify the levels of the other proteins interacting with AP-1 at nuclear level (see Figures 4 and 5 under "Results"). Actin was used as loading control. Signal intensities of c-

Jun and c-Fos bands in the WBs were analyzed as in **(A)** and normalized to actin bands. Data analysis showed that siRNAs resulted in 75-80% knockdown of specific protein ($P < 0.001$). No effects of c-Jun or c-Fos siRNAs were found on ErbB-2, PR-B, PR-A, or Stat3 expression. **Related to Figure 1. Panels G and H are also related to Figures 3 and 5.**

Supplemental Figure 2

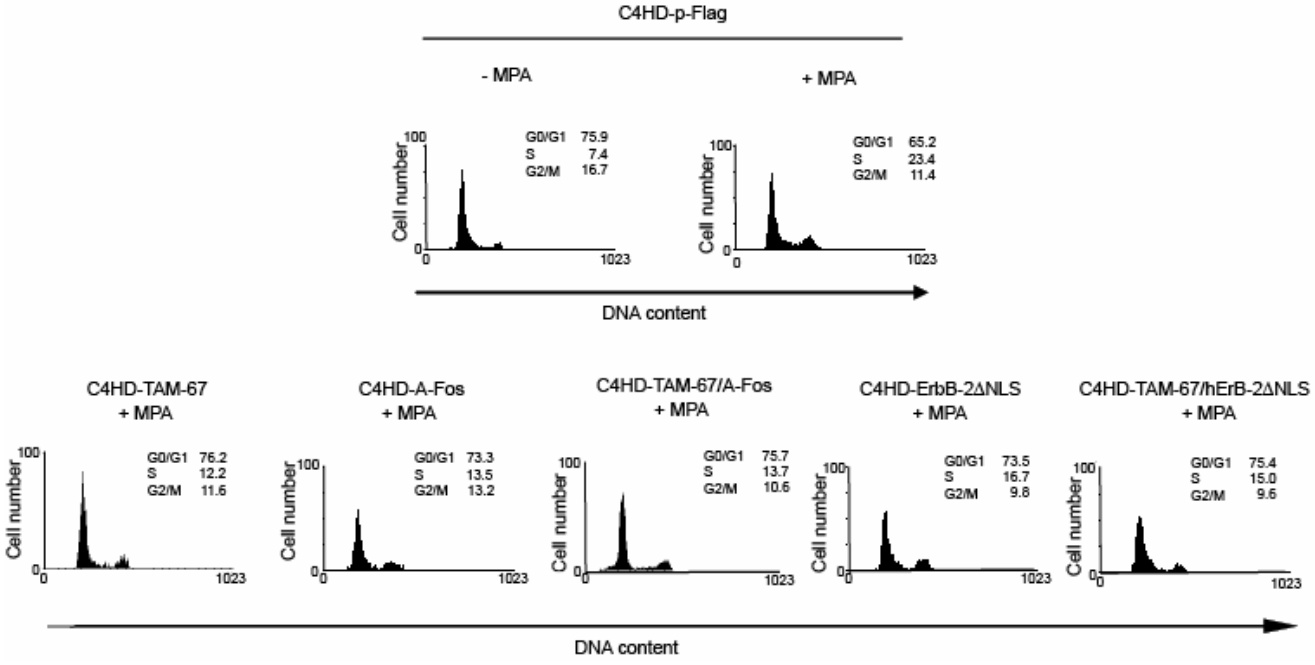


Supplemental Figure 2. MPA induced increase in the levels of c-Jun and c-Fos nuclear localization and phosphorylation levels

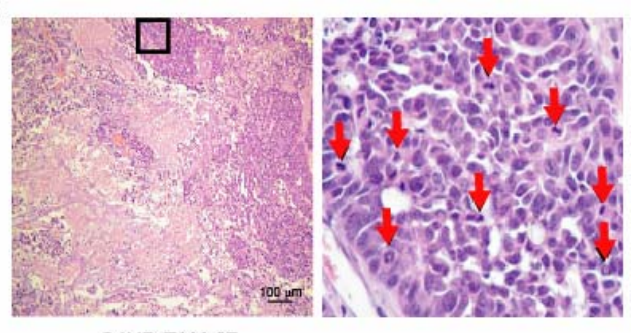
Nuclear and cytosolic protein extracts were analyzed by WB as described in Figure 1A. Histone H3 was used to control cellular fractionation efficiency. Data analysis showed that the increases in c-Jun and c-Fos phosphorylation in nuclear extracts from cells treated with MPA compared with the levels in untreated cells were significant ($P<0.001$). **Related to Figure 2.**

Supplemental Figure 3

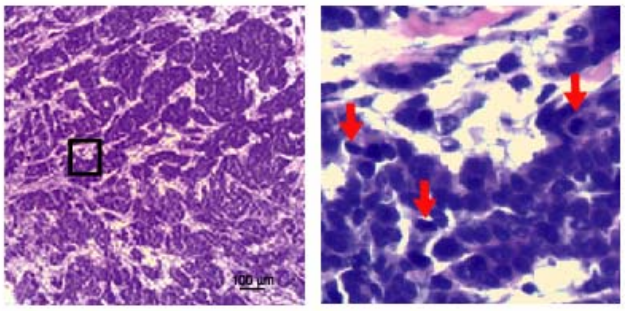
A



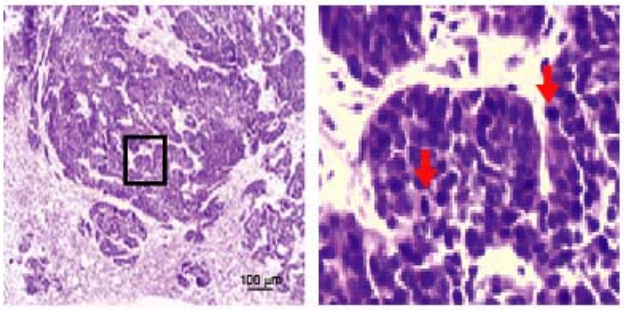
B



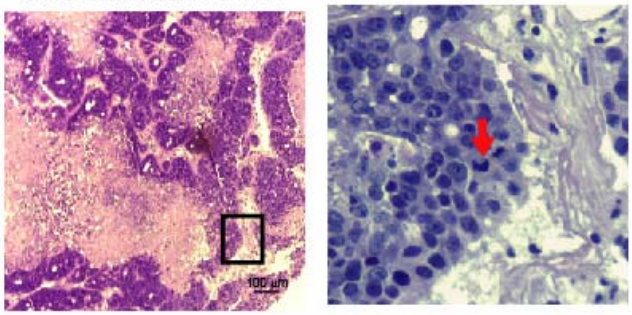
C4HD-TAM-67



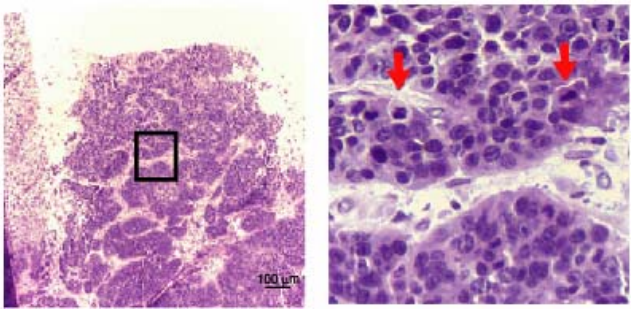
C4HD-A-Fos



C4HD-TAM-67/ErbB-2ΔNLS



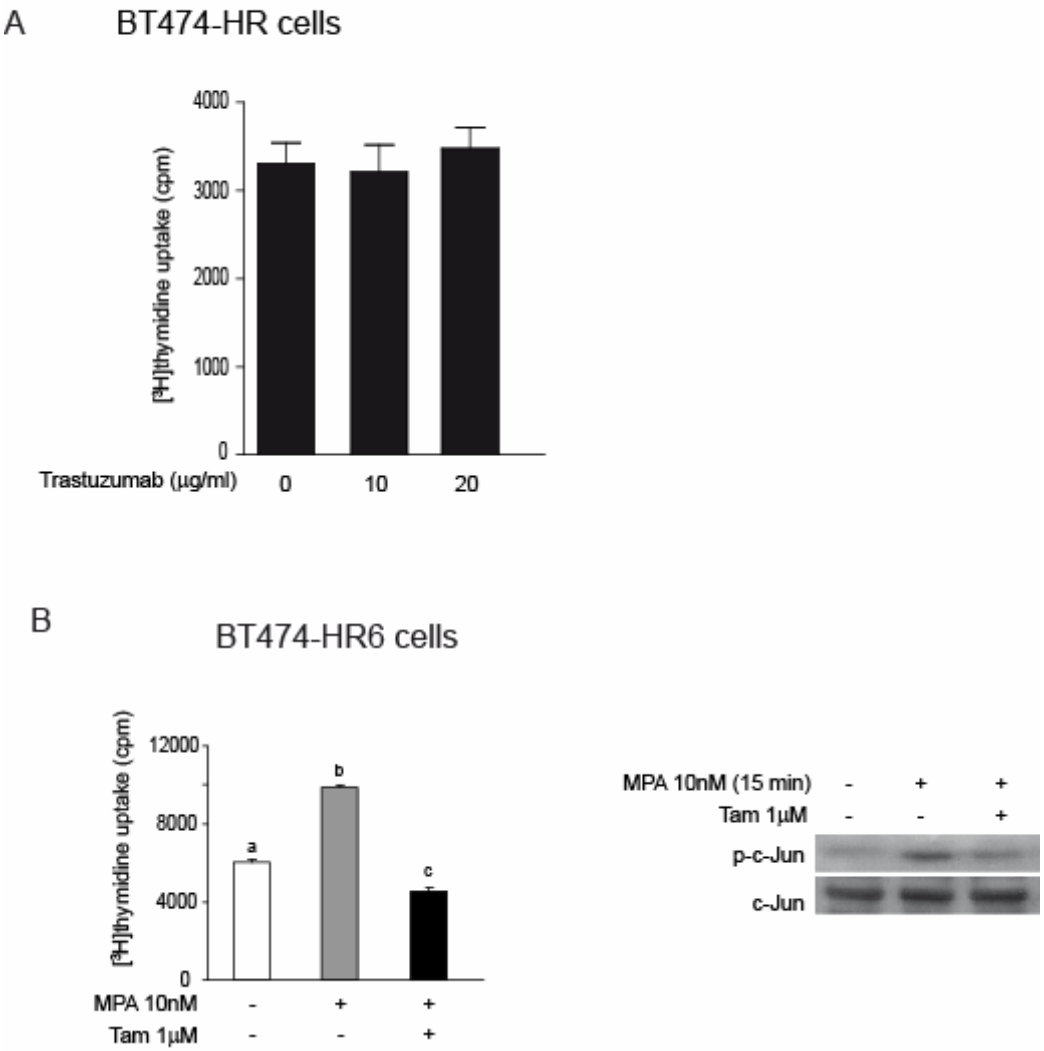
C4HD-hErbB-2ΔNLS



Supplemental Figure 3. The multimeric AP-1/Stat3/ErbB-2 transcriptional complex is involved in progestin-induced *in vitro* and *in vivo* breast cancer growth

(A) C4HD cells were transfected with the indicated expression vectors before MPA stimulation for 48h and were then stained with PI and analyzed for cell cycle distribution by flow cytometry. The experiments shown are representative of a total of three. **(B)** Histopathological analysis of tumors from the *in vivo* model. H&E staining of histological sections obtained from tumors excised at day 27. Mitoses are indicated with red arrows. Histopathological analysis revealed that C4HD-TAM-67, C4HD-A-Fos and C4HD-hErbB-2ΔNLS tumors showed histological grade II, with 3-4 mitoses per 10 high-power fields (HPF), C4HD-TAM-67/hErbB-2ΔNLS tumors displayed histological grade II with 0-1 mitoses per 10 HPF, and tumors from control C4HD-pFlag cells showed histological grade III with over 8 mitoses per 10 HPF. **Related to Figure 6.**

Supplemental Figure 4

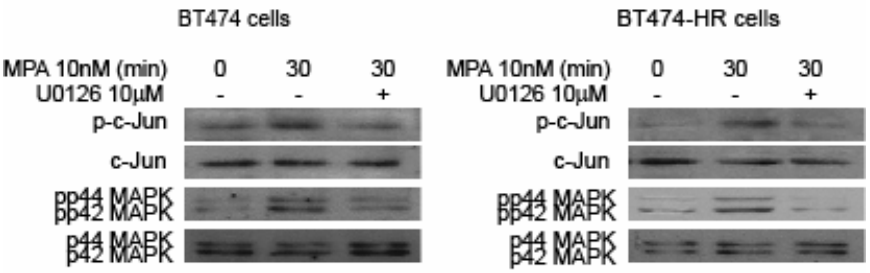


Supplemental Figure 4. BT474-HR clones response to trastuzumab and MPA and Tam

(A) Control of lack of response to trastuzumab in the BT474-HR clone selected in this study. Cells were treated with the indicated concentrations of trastuzumab and proliferation was evaluated as described in Figure 6. **(B)**

BT474-HR6 cells were treated as indicated and proliferation and c-Jun phosphorylation were studied as in Figures 6 and 1 respectively. **Related to Figure 8.**

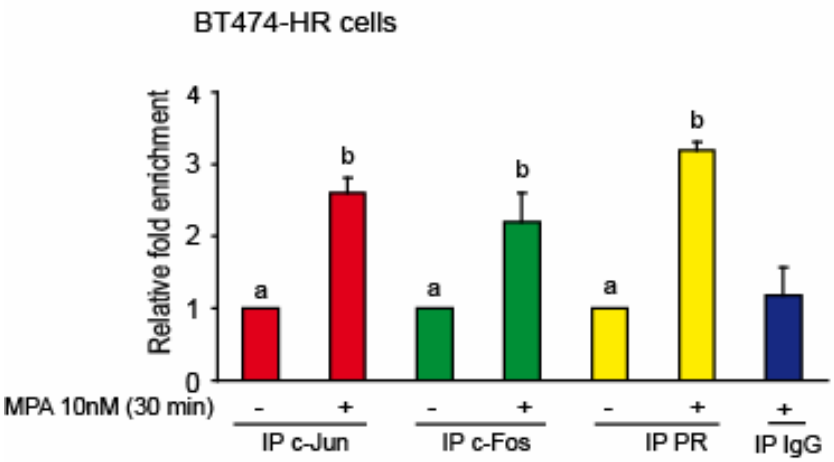
Supplemental Figure 5



Supplemental Figure 5. Inhibition of p42/p44MAPKs activity with UO126 in BT474-HR and BT474 cells blocks MPA-induced c-Jun phosphorylation

Cells were treated as indicated and WBs were performed as in Figure 1. **Related to Figure 8.**

Supplemental Figure 6



Supplemental Figure 6. Control of c-Fos corecruitment with c-Jun and PR to the cyclin D1 promoter to assemble the AP-1/PR complex upon MPA stimulation.

c-Jun, c-Fos, and PR recruitment to the cyclin D1 promoter was analyzed by ChIP, as in Figure 8. Immunoprecipitated DNA was amplified by qPCR using primers flanking the GAS and TRE sites. The arbitrary qPCR number obtained for each sample was normalized to the input, setting the value of the untreated sample as 1. Data are expressed as n-fold chromatin enrichment over untreated cells. For b vs. a: $P<0.001$. **Related to Figure 8.**

Supplemental Table S1 Study compliance with REMARK criteria

INTRODUCTION	1. The marker examined, study objectives, and hypotheses are stated in the Results section.
MATERIALS AND METHODS	Patients 2. The characteristics of study patients are detailed in Supplemental Table S2. Inclusion criteria are stated in the Methods and Supplemental Methods sections. 3. Treatment information is detailed in Supplemental Table S2. Specimen characteristics 4. Formalin-fixed paraffin-embedded material was used, which was stored at the Histopathology Department of Temuco Hospital, Chile, from 2001 to 2005 for construction into tissue microarrays. Assay methods 5. Details of protocols used for immunohistochemistry and immunofluorescence are provided under Methods and Supplemental Methods. Scoring of immunohistochemical and immunofluorescence markers was conducted blinded to survival time data, patient, and tumour characteristics. Study design 6. Patients were recruited retrospectively utilising anonymised archival material as specified in Methods and Supplemental Methods. The time period from which cases were recruited is also specified under Methods. 7. The clinical endpoint used is defined in Methods. 8. All candidate variables considered and included in statistical models are specified in the Methods section. 9. Sample size provided sufficient statistical power to address pre-specified hypotheses. Statistical analysis methods 10. The Methods section contains details on statistical methods used, model-building procedures and testing of assumptions. The multivariate analysis using the Cox multiple hazards model was conducted on samples of 99 and 85 patients and we carried out the adequate control of the confounding factors described under Methods in order to avoid increased bias and variability, unreliable confidence interval coverage, and problems with the model. Correlations between categorical variables were performed using the $\chi 2$-test or Fisher's exact test when the number of observations obtained for analysis was small. Specifically, Fisher's exact test was selected when the number of expected values was under five, because it uses the exact hypergeometric distribution to compute the p-value [1]. The $\chi 2$-test is basically an approximation of the results from the exact test, so few observations could potentially render erroneous results. 11. Details of how marker values were handled and cutpoints determined are provided in the Supplemental Methods section.
RESULTS	Data 12. The numbers of patients included within subgroups are detailed in the Results section. The number of events in the multivariate analysis is stated in the Results section. 13. Characteristics of study participants are detailed in Supplemental Table S2. Analysis and presentation 14. The relationship of p-c-Jun/PR nuclear colocalization to standard prognostic variables is detailed in the Results section (Table 1). 15. Univariate analyses for p-c-Jun/PR nuclear colocalization in the subgroups of patients are shown in Figure 7 and described in the text. 16. Multivariate analysis for p-c-Jun/PR nuclear colocalization including estimated effect size (hazard ratio) and confidence interval is described in the text. All variables included in the model were those that resulted statistically significant ($p<0.05$) in the log rank test, as described in Methods.
DISCUSSION	19. The study results were discussed in the context of our pre-specified hypothesis and other relevant studies. 20. The clinical value and implications for future research were stated. .

Supplemental Table S2
Clinicopathological characteristics of PR+ patients

Characteristic	Nº patients	%
Total number of patients	99	
Age (years)		
Mean	56	
SD	14.01	
Range	21-89	
Menopausal status		
Premenopausal	39	39.4
Postmenopausal	60	60.6
Tumor size		
T1	16	16.2
T2	58	58.6
T3	15	15.1
T4	10	10.1
Lymph node status		
N0	41	41.4
N1	27	27.3
N2	31	31.3
Distant metastasis		
M0	93	93.9
M1	6	6.1
Clinical stage		
I	12	12.3
II	49	50
III	31	31.6
IV	6	6.1
Not documented	1	
Tumor grade		
1	27	27.6
2	53	54.1
3	18	18.4
Not documented	1	
ER[a]		
positive	92	92.9
negative	7	7.1
ErbB-2		
positive	11	11.1
negative	88	88.9
Operation		
Breast conserving surgery	46	47.4
Mastectomy	51	52.6
Not documented	2	
Chemotherapy (Anthracycline-containing regimen)		
No	18	18.4
Yes	80	81.6
Not documented	1	
Endocrine therapy (Tamoxifen)		
No	13	13.3
Yes	85	86.7
Not documented	1	

[a]ER: Estrogen receptor. **Related to Figure 7.**

Supplemental Table S3

A. Comparison between nuclear p-c-Jun scores in the cohort of 85 patients treated with Tamoxifen displaying presence or absence of p-c-Jun/PR nuclear colocalization

p-c-Jun/PR nuclear colocalization	Number	p-c-Jun nuclear levels			
		Mean	Range	95% CI	*p Value*[a]
Negative	17	6.8	5-7	6.5-7.0	0.2
Positive	68	6.9	6-8	6.8-7.1	

B. Comparison between nuclear PR scores in the cohort of 85 patients treated with Tamoxifen displaying presence or absence of p-c-Jun/PR nuclear colocalization

p-c-Jun/PR nuclear colocalization	Number	PR nuclear levels			
		Mean	Range	95% CI	*p Value*[a]
Negative	17	5.9	3-7	5.3-6.4	0.1
Positive	68	6.4	3-8	6.1-6.7	

[a]Two sample t test with equal variances. **Related to Figure 7.**

Supplemental Table S4

Association between p-c-Jun and PR colocalization and membrane ErbB-2 overexpression in PR+ patients treated with Tamoxifen

	p-c-Jun and PR colocalization		
ErbB-2	(-) N=17	(+) N=68	*p* Value
-	14	67	0.02[a]
+	3	1	

[a] Fischer`s exact test. Scores of 3+ and only those of 2+ in which FISH confirmed ErbB-2 amplification were considered positive for membrane ErbB-2 overexpression. **Related to Figure 7.**

SUPPLEMENTAL METHODS

Antibodies

The following antibodies were used for Western blots: phospho-c-Jun (Ser 63/73), c-Jun (H-79), c-Fos (4), p-c-Fos (Thr 232), phospho-Stat3 (pStat3) (Tyr 705) (B-7), total Stat3 (C-20), phospho-p42/p44 MAPK (E-4), total p42 MAPK (C-14), ErbB-2 (C-18), and ERα (MC-20) all from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-Src (Tyr 416), phospho-ErbB2 (Tyr 1221/1222) and phospho-ErbB2 (Tyr 877) from Cell Signaling (Beverly, MA); v-Src (Ab-1) from Calbiochem EMD Millipore (Billerica, MA); cyclin D1, PR (clone hPRa7), and actin (clone ACTN05), from Neomarkers (Freemont, CA); β tubulin from Sigma-Aldrich; histone H3, from Abcam (Cambridge,MA); and horseradish peroxidase (HRP)-conjugated secondary antibody from Vector Laboratories (Burlingame, CA). The antibodies used for chromatin immunoprecipitation (ChIP) and sequential ChIP assays were the rabbit polyclonal c-Jun, c-fos, PR, Stat3, ErbB-2 and ERα antibodies (H-79, 4, H-190, C-20, C-18, and HC-20X, respectively, from Santa Cruz Biotechnology) and CBP/KAT3A (ab2832), p300 (clone RW128), acetyl histone H4 (06-866), and acetyl histone H3 (06-599) antibodies, from Millipore (Temecula, CA). Rabbit IgG (Sigma-Aldrich) was used as a negative control. Antibodies used for immunofluorescence and confocal microscopy in cell cultures were H-79 and 4 for c-Jun and c-Fos, respectively (Santa Cruz). PR was localized using the hPRa7 antibody (Neomarkers (Freemont, CA). Secondary antibody for c-Jun and c-Fos was a goat anti-rabbit IgG-Alexa 488 (Molecular Probes,

Eugene, OR) and for PR a rhodamine conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA

Reagents

Medroxyprogesterone acetate (MPA), 17-β estradiol (E2), RU486, U0126, and Tamoxifen were from Sigma-Aldrich (San Louis, MI).

siRNA sequences

siRNAs targeting c-Jun, c-Fos, PR and Stat3 were synthesized by Dharmacon, Inc. (Lafayatte, CO, USA) and their sequences were as follows:

c-Jun siRNA #1, 5'-GAACAGGUGGCACAGCUUA-3';

c-Jun siRNA #2, 5'-GAAACGACCUUCUACGACG-3';

c-Fos siRNA #1, 5'-GCGCAGAUCUGUCCGUCUC-3';

c-Fos siRNA #2, 5'-GGAUUUGACUGGAGGUCUG-3';

PR siRNA #1, 5'- AUAGGCGAGACUACAGACGUU -3';

PR siRNA #2, 5'-TCTGCCAGGTTTCCGGAACTT-3';

Stat3 siRNA #1, 5'-GGUCAAAUUUCCUGAGUUGUU-3';

and Stat3 siRNA #2, 5'-CCACGUUGGUGUUUCAUAAUU-3' .

Results shown in the manuscript were obtained by the use of c-Jun, c-Fos, PR and Stat3 siRNAs labeled as #1, but same results were obtained for each protein by silencing its expression with the siRNAs labeled as #2.

DNA sequence of primers used for quantitative ChIP

Mouse cyclin D1 amplicon containing one TRE site (-948) and two GAS sites (-971 and -874):

5'-TTCCGGTGGTCTGGTTCCT-3' and

5'-GAGACACGATAGGCTCCTTCCTAA-3'

Human cyclin D1 ampicon containing one TRE site (-954) and one GAS site (-984):

5'-GGAACCTTCGGTGGTCTTGTC-3' and

5'-GAATGGAAAGCTGAGAAACAGTGA-3'

DNA sequence of primers used for real-time quantitative RT-PCR

Mouse cyclin D1 cDNA:

5'-CGCCCTCCGTATCTTACT-3' and

5'-CGCACTTCTGCTCCTCAC-3'.

GAPDH cDNA: 5'-CCAGAACATCATCCCTGCAT-3' and

5'-GTTCAGCTCTGGGATGACCTT-3'.

Immunohistochemistry (IHC)

Antigen retrieval was performed in 10 mM sodium citrate buffer pH 6 for 20 min at 96-98ºC. Immunostainings were run with known positive and negative tumor controls and were blindly evaluated by two pathologists (PG and JCR). Estrogen (ER) and progesterone receptor (PR) were evaluated by IHC with clone 6F11 (Novocastra Laboratories, U.K) and clone hPRa2+hPRa3 (NeoMarkers, Freemont, CA), respectively, and scored as described [2]. Membrane ErbB-2 expression was studied using c-erb-B2 clone A0485 (dilution 1:300 overnight at 4ºC; Dako Carpinteria, CA) and ErbB-2 was

scored according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines [3]. Scores 2+ in which FISH confirmed ErbB-2 amplification, and 3+ were considered positive for membrane ErbB-2 overexpression.

Fluorescence in situ hybridization (FISH)

FISH was done according to PathVysion™ (Vysis, Inc, Downers Grove, IL) guidelines. ErbB-2 gene/chromosome 17 centromere signals ratio more than 2.2 was considered ErbB-2 amplification.

SUPPLEMENTAL REFERENCES

1. Upton G. **Fisher's exact test**. *Journal of the Royal Statistical Society, Series A* 1992; **155**:395-402. 2011.

2. Mohsin SK, Weiss H, Havighurst T, Clark GM, Berardo M, Roanh lD, To TV, Qian Z, Love RR, Allred DC: **Progesterone receptor by immunohistochemistry and clinical outcome in breast cancer: a validation study.** *Mod Pathol* 2004, **17:**1545-1554.

3. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van d, V, Wheeler TM, Hayes DF: **American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer.** *Arch Pathol Lab Med* 2007, **131**:18-43.