FGF2 induces breast cancer growth through ligand-independent activation and recruitment of ERα and PRBΔ4 isoform to *MYC* regulatory sequences

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Progression to hormone-independent growth leading to endocrine therapy resistance occurs in a high proportion of patients with estrogen receptor alpha (ERα) and progesterone receptors (PR) positive breast cancer. We and others have previously shown that estrogen- and progestin-induced tumor growth requires ERα and PR interaction at their target genes. Here, we show that fibroblast growth factor 2 (FGF2)-induces cell proliferation and tumor growth through hormone-independent ERα and PR activation and their interaction at the *MYC* enhancer and proximal promoter. MYC inhibitors, antiestrogens or antiprogestins reverted FGF2-induced effects. LC−MS/MS identified 700 canonical proteins recruited to *MYC* regulatory sequences after FGF2 stimulation, 397 of which required active ERα (ERα-dependent). We identified ERα-dependent proteins regulating transcription that, after FGF2 treatment, were recruited to the enhancer as well as proteins involved in transcription initiation that were recruited to the proximal promoter. Also, among the ERα-dependent and independent proteins detected at both sites, PR isoforms A and B as well as the novel protein product PRBΔ4 were found. PRBΔ4 lacks the hormone-binding domain and was able to induce reporter gene expression from estrogen-regulated elements and to increase cell proliferation when cells were stimulated with FGF2 but not by progestins. Analysis of the

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Cancer Genome Atlas data set revealed that *PRBΔ4* expression is associated with worse overall survival in luminal breast cancer patients. This discovery provides a new mechanism by which growth factor signaling can engage nonclassical hormone receptor isoforms such as PRBΔ4, which interacts with growth-factor activated ERα and PR to stimulate *MYC* gene expression and hence progression to endocrine resistance.

What's new?

Activation of estrogen and progesterone receptors (ERα and PR) may lead breast cancer down the path to hormone-independent growth and treatment resistance. Here, the authors investigated how fibroblast growth factor 2 (FGF2) promotes interaction between ERα and PR. They showed that FGF2 initiates interaction between ERα and PR at *MYC* regulatory regions, boosting MYC expression and increasing cell proliferation. They identified 700 proteins recruited to *MYC* regulatory regions following treatment with FGF2. Among these, 3 were progesterone receptor isoforms, including a novel form called PRBΔ4. These findings open a new path for researching these isoforms as potential therapeutic targets.

Introduction

Estrogen and progesterone receptors (ERα and PR, respectively) are prognostic and predictive markers expressed in 70% of breast cancers. Their expression levels drive therapeutic decisions, with positive tumors considered luminal breast cancers by clinical pathologists.[1] Both ERα and PR are ligand-activated transcription factors that regulate the expression of specific genes by binding to consensus-like sequences in DNA.[2] However, ligand-independent activation of both receptors has been proposed as a mechanism underlying the acquisition of hormone independence or hormone resistance.[3–6]

Carcinoma-associated fibroblasts (CAFs) produce growth factors, including fibroblast growth factor 2 (FGF2), which can stimulate neoplastic cell proliferation and induce hormone-independent (HI) tumor growth.[6,7] We previously demonstrated that PR activation mediates FGF2-induced *MYC* and *CCND1* transcription and that increased levels of these two proteins lead to HI cell proliferation.[3,6,8]

A physical interaction between ERα and PR was first described as a nongenomic mechanism of Src/ERK pathway activation.[9,10] Later, we demonstrated a direct crosstalk between both receptors at specific gene promoter regions which mediates progestin-dependent tumor growth.[11,12] These studies highlighted the role of ERα as a mediator of progestin-induced effects. Reciprocal evidence has also been reported by Daniel *et al.,*[13] who showed that PR regulates ERα-mediated functions. More recently, massive interaction of both receptors after global chromatin binding and gene expression analysis was described by Mohammed *et al.*[14] and Singhal *et al.,*[15] who proposed that PR drives ERα activity in breast cancer. Therefore, compelling evidence supports ERα/PR interaction at DNA regulatory regions in response to estrogen or progestin stimulation. However, ligand-independent ERα and PR activation in response to growth factor/receptor tyrosine kinase signaling remain an important driver of HI growth and endocrine resistance. To date, there is no information regarding ERα and PR interaction at gene promoters to mediate gene expression after growth factor-induced signaling.

The aim of the present study was to investigate if FGF2 stimulation induces ligand-independent ERα interaction with PR at DNA regulatory regions found in *MYC* gene and to define other interactors that may help to understand FGF2 signaling in a hormone receptor-dependent context. In this work, we demonstrate that ERα and the classical PR isoforms, PRA and PRB, interact at two *MYC* regulatory sequences and at *CCND1* and *pS2/TFF1* promoters. Focusing on *MYC* gene, we show that this interaction is necessary to induce *MYC* expression and HI cell proliferation. In addition, we reveal the participation of nonclassical PR protein isoforms, which until now had only been described at the mRNA level, together with other interactors at the enhancer or proximal promoter of *MYC*. The understanding of the interactions between PR isoforms and ERα in the context of their coregulatory proteins will open new veins for drug design to impede *MYC* transcription and thus tumor growth.

Materials and Methods

Animals

Two-month-old virgin female BALB/c mice were bred at IByME Animal Facility. Mouse experiments were approved by local IACUC authorities and complied with regulatory standards of animal ethics.[16]

Tumors

C4-HI mammary carcinomas from the MPA breast cancer model[17] were transplanted by subcutaneous (s.c.) injection into the inguinal flank of BALB/c mice. KJ-Pyr-9 treatments (10 mg/kg/day; intraperitoneal [IP]), or vehicle (10% glucose, 5% Tween-80 in 1× PBS) were initiated when tumors reached a size of approximately 50 mm². Mice were euthanized 1 hr post-BrdU (1.5 mg/mice) IP injection.

Cell lines

Human T47D and MCF-7 cells obtained from ATCC were validated by Genetica DNA Laboratories Inc. (Cincinnati, OH) by

short tandem repeat profiling. The PR−/ERα + T47D-Y cells were kind gifts of Dr. K. Horwitz[18] and were also validated. All cell lines were maintained in DMEM/F12 without phenol red (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin and 100 μg/ml streptomycin with 10% fetal bovine serum (FBS, Gibco, New York, NY). Steroid-stripped FBS (chFBS) was prepared as described previously.[6] Passages lower than 15 were used.

Plasmids
pSG5 empty vector and pSG5-PRB were kind gifts from Dr. K. Horwitz.[19] pSG5-PRBΔ4 construct, containing the total deletion of PRB exon 4, was generated by PB-L Productos Bio-Lógicos. All constructs were verified by sequencing.

Cell line transfection
T47D-Y cells were transiently or stably transfected to express PRBΔ4 isoform using Lipofectamine Reagent (Invitrogen, Carlsbad, CA) following manufacturer's instructions. For stable transfection, cells were cotransfected with a plasmid encoding the neomycin resistance gene (pcDNA3.1 neo) together with pSG5 or pSG5-PRBΔ4 constructs. Cells were cultured for 2 weeks in media supplemented with 400 μg/ml of the neomycin analog G418 (Invitrogen), and then expanded and maintained in the presence of 200 μg/ml G418.

Cell proliferation
Cell proliferation was evaluated by either [^{3}H]-thymidine uptake or cell counting.[6] Cells were plated with DMEM/F12 plus 10% FBS for 24 hr. After starving for 24 hr with 1% chFBS, the cultures were incubated with the experimental solutions.

Cell extracts and Western blot
Total cell extracts were prepared by using RIPA lysis buffer including protease inhibitors. Nuclear extracts (NEs) were obtained by using TEDGS10% buffer including protease inhibitors. Further details are provided in the Supporting Information Methods. Proteins were quantified, separated on discontinuous polyacrylamide gels and detected by Western Blot (WB), as previously described.[6] Co-immunoprecipitation (IP) was conducted as described previously.[12]

Immunohistochemistry, immunofluorescence and PLA assays
Sections of formalin-fixed, paraffin-embedded tissues were reacted with different antibodies using the avidin–biotin peroxidase complex technique (Vector Lab, Burlingame, CA) and counterstained with hematoxylin. Images were obtained using a Nikon Eclipse E800 microscope with ACT-2U software. Immunofluorescence was carried out on chamber slides, cultures fixed in 70% ethanol and processed as described previously.[12] The Duolink *in situ* PLA kit (Sigma-Aldrich, St. Louis, MO) was used to detect ERα/PR protein interaction. Further details are provided in the Supporting Information Methods. The valuation

of ERα/PR dimers was quantified as the area of dots in the nuclei using ImageJ software (NIH, Bethesda, MD).[20]

Reporter assay
Cells were transfected with PRE-Luc, ERE-Luc or different constructs of human *MYC* enhancer/promoter-Luc, together with Renilla expression plasmid. Luciferase activity was measured using the dual-luciferase reporter assay system (Promega). Further details are provided in the Supporting Information Methods.

RNA preparation, qPCR and chromatin immunoprecipitation assays
The experiments were performed according to previously published protocols.[8] Further details are provided in the Supporting Information Methods and Table S1.

Biotinylated *MYC* promoter fragments
We delimited the DNA fragments based on previous studies[21,22] and considering also the presence of ERE and PRE sequences. Enhancer-*MYC* (442 bp) and Proximal-*MYC* (338 bp) doubly biotinylated fragments from human *MYC* regulatory sequences were synthesized by PCR using Supreme NZYTaq 2× Green Master Mix (NZYTech), using specific primers biotinylated at their 5′ ends (Supporting Information Table S1) and Enh-Luc or −2,320 bp-Luc plasmids[22] as a template. To remove free biotinylated primers after PCR, reactions were passed through NZYGelpure columns (NZYTech), and the size of the DNA-biotin fragments was confirmed in agarose gels.

DNA pull-down assays
Bead-immobilized DNAs were incubated with NEs and pulled-down proteins analyzed by SDS-Page and WB. Further details are provided in the Supporting Information Methods.

Mass spectrometry analysis and protein quantification
Tryptic digestion was performed according to Carvalhais *et al.*[23] with few modifications. Nano-liquid chromatography–tandem mass spectrometry (nanoLC-MS/MS) analysis was carried out with a QExactive Orbitrap (Thermo Fisher Scientific, Waltham, MA) coupled to an Ultimate 3000 HPLC (high-pressure liquid chromatography) system (Dionex, Sunnyvale, CA). Full details are provided in the Supporting Information Methods. The exponentially modified protein abundance index (emPAI) values[24] were used as an approximate label-free relative quantitation of all identified proteins. Proteins were considered recruited by FGF2 to either human *MYC* enhancer or promoter-biotin sequences if their emPAI value was ≥ two fold compared to the control cells. ERα-dependent proteins recruited after ligand-independent activation by FGF2 were considered if their emPAI value in FGF2-treated cells was ≥ two fold in control and FGF2 + ICI cells. ERα-independent proteins recruited after FGF2 stimulation were those with emPAI value in FGF2-treated cells ≥ two fold in control but < two fold in FGF2 + ICI-treated cells. Unchanged proteins after FGF2 treatment were those with

emPAI value in FGF2-treated cells <2 and >0.5 in control cells. Full details of Bioinformatic analysis are provided in Supporting Information Methods.

Human breast cancer samples

NEs from 11 PR+ breast cancer samples obtained from patients undergoing breast cancer surgery were selected because they showed extra PR bands in WB in between PRB and PRA (n = 4) or because no intermediate bands were observed (n = 7). RNA was extracted and qPCR was carried out to evaluate total *PR* and *PRBΔ4* (Supporting Information Methods). The study was approved by the Institutional Review Boards of the Hospital Magdalena V de Martínez, General Pacheco and from IByME-CONICET (2012-028).

In silico analysis of PRBΔ4 expression prognostic value among primary breast carcinomas

To evaluate the PRBΔ4 mRNA prognostic value, we analyzed the TCGA-BRCA RNA-Seq dataset (n = 1,092). Briefly, the clinical/follow-up and preprocessed *PRBΔ4* isoform (ENST00000 263463.9), *PGR* (ENSG00000082175) and *ESR1* (ENSG000000 91831) full-length expression levels (TOIL RSEM log2 TPM) data were downloaded from the GA4GH (TOIL) hub at UCSC Xena browser (https://xenabrowser.net/). The intrinsic subtype classification of primary breast carcinomas into luminal-like, basal-like, ERBB2-enriched and normal-like groups was performed using the 50-gene (PAM50) predictor bioclassifier R script[25] based on the transcriptomics profiles. Briefly, the 709 Luminal A/B out of 1,092 primary breast carcinomas were classified into low (n = 649) or high (n = 60) PRBΔ4 mRNA expression levels according to the threshold identified by the StepMiner one-step algorithm base on *PRBΔ4* isoform (http://genedesk.ucsd.edu/home/public/StepMiner/). These groups were then compared based on the overall survival (Kaplan–Meier curves and log-rank test) using the Survival R package. Finally, the *PRBΔ4* negative cases (n = 649) were grouped according to the *PGR* full-length expression base on StepMiner algorithm for their further survival analysis.

Statistical analysis

ANOVA and Tukey multiple post *t* test were used to evaluate differences of means of multiple samples, and Student's *t* test was used to compare means of two different groups. In all graphs, the mean ± SEM is shown, and experiments were repeated at least three times unless specified. Significant differences between control and treated cells are indicated with asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Results

ERα mediates FGF2-induced cell proliferation by increasing PR and MYC expression

We previously demonstrated that in T47D cells FGF2 activates PR which is needed for FGF2-induced cell proliferation.[6,8] This prompted us to investigate if PR activation is also needed in MCF-7 cells, whose growth is driven by ERα, as well as to determine whether these two hormone receptors are activated by FGF2. After

5 min of FGF2 treatment, we observed an increase in the phosphorylation (p) of ERα (pSer167 and pSer118) and PR (pSer294) in both cell lines (Fig. 1a). This was accompanied by ERK1/2 and AKT activation, as described previously in T47D cells[8] and now shown in MCF-7 cells (Supporting Information Fig. S1a). Inhibitors for ERK1/2 (PD98059), PI3K (LY294002) or FGFR (PD173074 and BGJ398), blocked FGF2-induced ERα phosphorylation, suggesting that signal transduction pathways activated by FGF2 through different FGFRs are responsible for the increased ERα phosphorylation (Supporting Information Fig. S1b).

Cell proliferation stimulated by FGF2 was blocked by the antiestrogen ICI 182.780 (ICI) or by the FGFR inhibitors (Fig. 1b). Genetic blockade using siRNA to target ERα had similar effects as those of ICI (Supporting Information Fig. S1c). FGF2 also increased *MYC* and *pS2/TFF1* mRNA, as well as MYC and PR protein levels; while antiestrogen treatment abolished these effects (Fig. 1c and 1d). Altogether, these results indicate that ERα mediates FGF2-induced transcription in both hormone-responsive cell lines and that MYC could be a potential target to be explored in FGF2-driven tumors.

MYC inhibition reverts hormone-independent breast cancer growth

To further evaluate the role of MYC in FGF2-induced cell proliferation and tumor growth, MYC inhibitors were tested. *In vitro*, the MYC inhibitor 10058-F4,[26] decreased FGF2-induced proliferation in T47D (Fig. 1e, top left) and MCF-7 cells (data not shown). *In vivo*, we used the C4-HI murine mammary tumor in which we previously showed that stromal FGF2 activates PR.[6] The MYC inhibitor KJ-Pyr-9[27] significantly decreased the proliferation index (BrdU uptake), increased apoptosis (Tunel; Fig. 1e, bottom and Supporting Information Fig. S1d) and thus inhibited tumor growth (Fig. 1e, top right). We confirmed that FGFR inhibitors also downregulated ERα and consequently its phosphorylation (Supporting Information Fig. S1e), indicating that both, ERα and PR, are involved in HI tumor growth.

Overall, these results indicate that FGF2 exerts estrogenic-like effects through ERα activation, which in turn induces PR and *MYC* expression to mediate cell proliferation, and pose MYC as a convergent therapeutic target.

ERα and PR mediate MYC gene expression induced by FGF2

We focused on elucidating how ligand-independent activation of ERα by FGF2 regulates *MYC* transcription. *MYC* is an excellent candidate representative of hormone-controlled genes because its regulatory sequences include an upstream enhancer region implicated in E2/ERα induction in MCF-7 cells[22] and a proximal promoter that mediates progestin-induced PR/ERα regulation in T47D cells.[8,12,21] Both regulatory sequences have several estrogen and progesterone response elements (ERE and PRE, respectively; Fig. 2a). We used T47D cells because they have basal estrogen-independent expression of PR, allowing us to study the contribution of each receptor in a ligand-independent activation context. These cells were transfected with three different plasmids
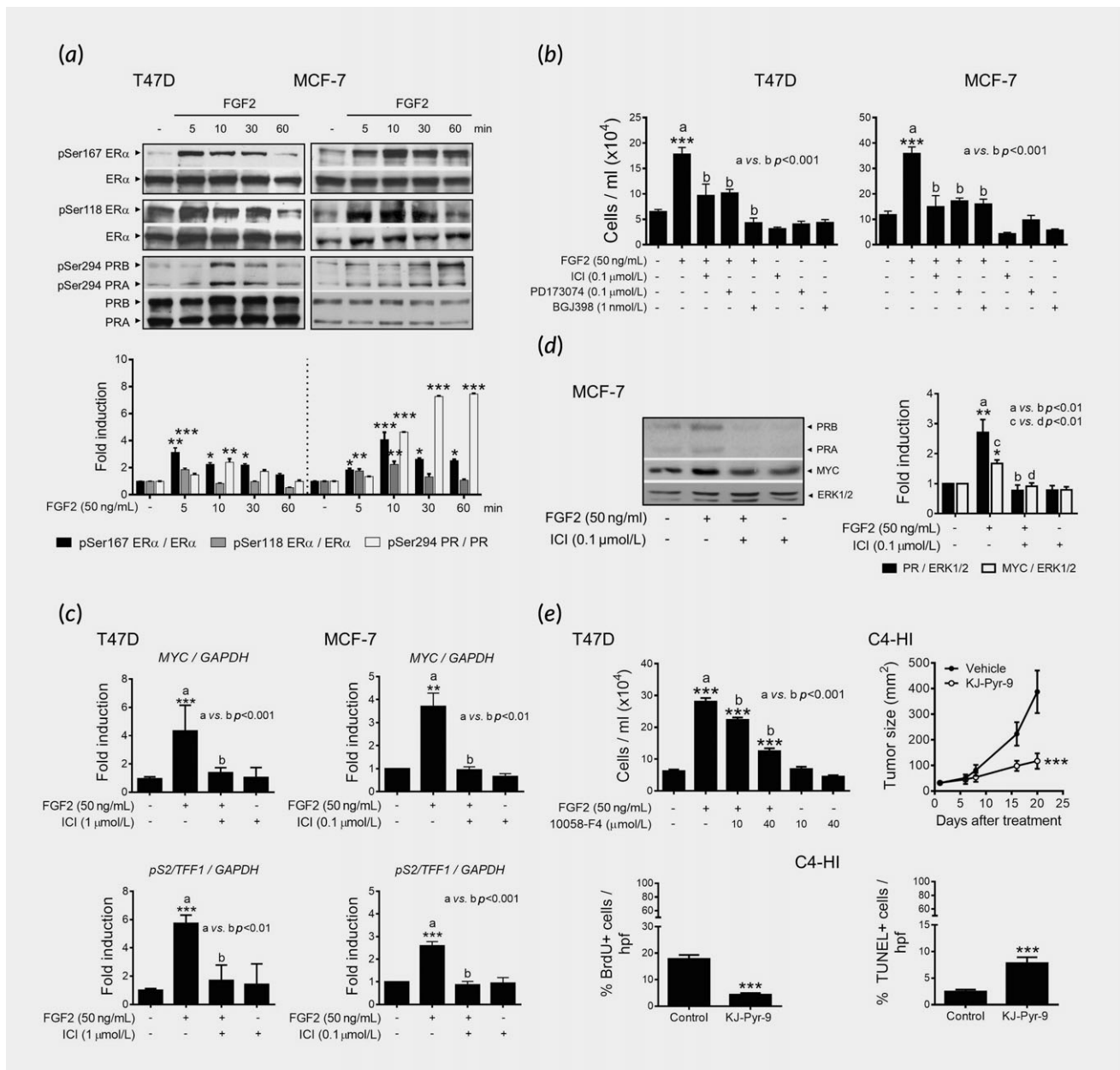
Figure 1. MYC is involved in FGF2-induced cell proliferation through ERα and PR activation. (*a*) Immunoblot analysis of total or phosphorylated (p) ERα and PR in cells incubated with or without FGF2 (50 ng/ml; top). The band intensity ratios relative to the corresponding value for time zero were plotted (bottom). (*b*) Cell counting assays. Cultures were starved and treated for 7 days with FGF2 with or without ICI 182.780 (ICI; antiestrogen) or two FGFR inhibitors (PD173074 or BGJ398). (*c*) *MYC* and *pS2/TFF1* mRNA expression relative to that of *GAPDH* evaluated by qPCR using cells treated for 1 h with FGF2 with or without ICI. (*d*) Immunoblot analysis of PR, MYC and ERK1/2 (loading control) expression after 24 h of FGF2 incubation with or without ICI (left). The band intensity ratios relative to the corresponding value for control cells were plotted (right). (*e*) Cell proliferation (top left) was evaluated as explained in Figure 1*b* in cells treated or not with the MYC inhibitor 10058-F4. Growth curves (top right) of C4-HI tumors treated with KJ-Pyr-9 or vehicle. Tumors were measured (length and width) and the mean size ± SEM of one representative experiment of two is shown. Proliferation index (BrdU uptake, bottom left) and apoptosis (Tunel, bottom right) were quantified and plotted (see Supporting Information Fig. S1*d* for representative images).

containing the distal enhancer, the proximal promoter, or the basal (−225 + 211 Kb; negative control) promoter of human *MYC*[22] coupled to Luciferase (Luc). FGF2, E2 or the synthetic progestin MPA, induced similar levels of Luc activity under the control of the distal enhancer or proximal promoter, while the basal promoter was not sensitive to the treatments. ICI inhibited

FGF2- or E2-induced Luc activity from both sites. The anti-progestin mifepristone (MFP) inhibited MPA-induced Luc activity in both regulatory sequences but could not inhibit the FGF2-induced effect at the proximal promoter (Fig. 2*b*). Collectively, our data showed that *MYC* enhancer and proximal promoter are both sensitive to E2, MPA and FGF2. ERα mediated
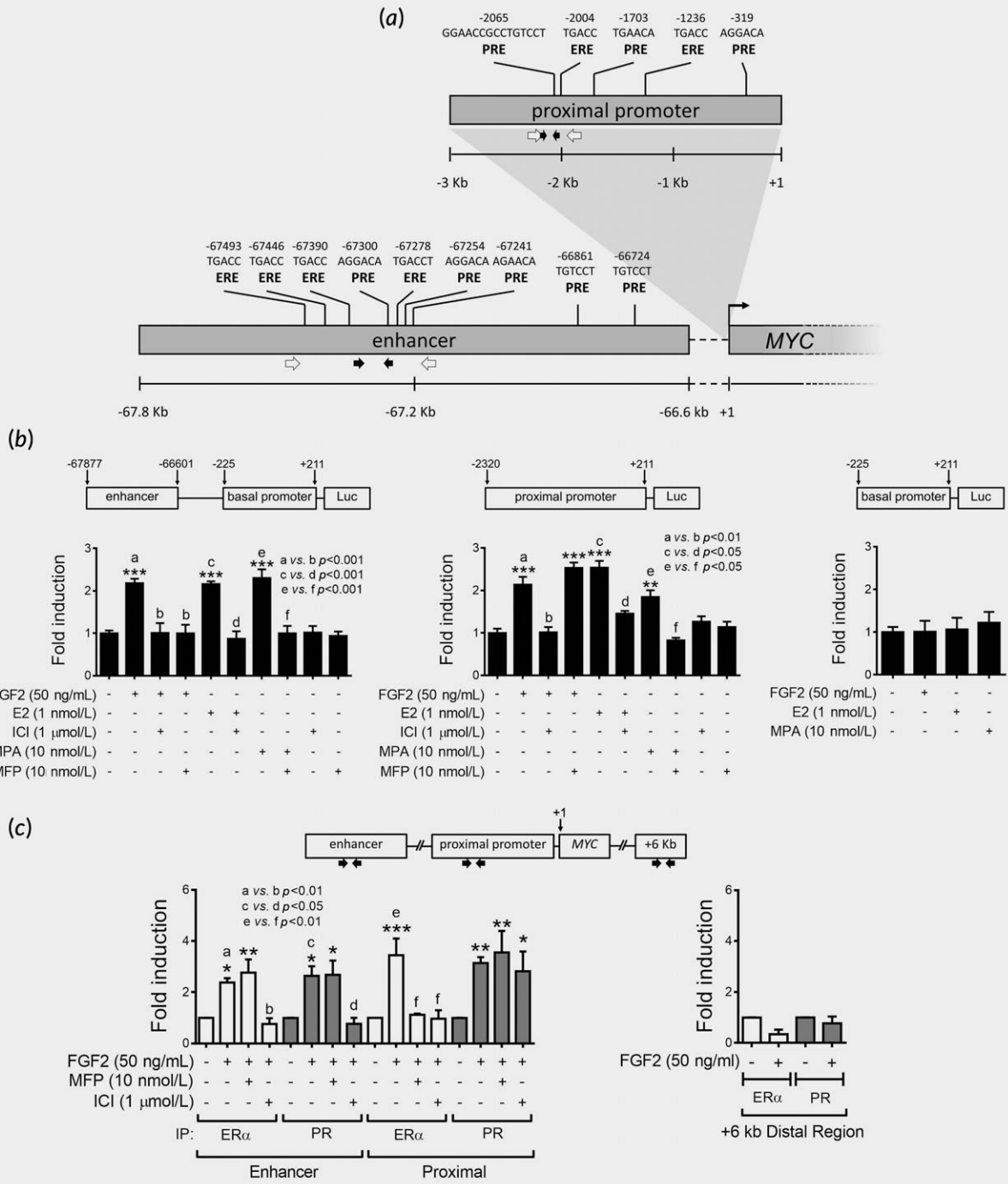
Figure 2. ERα and PR mediate *MYC* transcription after FGF2 stimulation in T47D cells. (*a*) Schematic representation of predicted half-ERE (estrogen response elements) or PRE (progesterone response elements) sites in the upstream proximal promoter and enhancer regions of human *MYC* gene relative to the transcription start site (TSS; black arrow at +1). Distance of the regulatory regions from the TSS, and primers used in ChIP/qPCR (black arrows) and pull-down (white arrows) assays described in this study are shown. (*b*) Luc reporter activity of *MYC* distal 67 Kb upstream enhancer (left), proximal promoter (middle) or basal promoter (negative control; right) after incubation with FGF2/E2/MPA with or without ICI or MFP for 24 h. (*c*) ChIP/qPCR studies using control or 10 min FGF2-treated cells together with ICI or MFP to detect the presence of ERα and PR on the *MYC* enhancer or proximal promoter regions. The +6 Kb distal regions were included as a negative control of receptor binding. Two representative experiments are shown (mean ± SEM).

FGF2-induced activation of both regulatory sequences, while PR seemed to be transcriptionally necessary only at the enhancer region. These differences do not impede the inhibitory effect of MFP on FGF2-induced *MYC* transcription.[8]

Chromatin immunoprecipitation (ChIP) assays were used to confirm ERα and PR binding to enhancer and proximal *MYC* regulatory sites. The optimal incubation time with FGF2 was selected from a time course experiment in which the pioneer transcription factor FOXA1[28] was included (Supporting Information Fig. S2a). After 10 min of incubation, both ERα and PR were bound to both *MYC* regulatory sequences while FOXA1, as expected, was recruited earlier than both hormone receptors. ERα and PR were also recruited at the promoters of other two estrogen-regulated genes *pS2/TFF1* and *CCND1* after FGF2 activation (Supporting Information Figs. S2b and c).

Given that ICI or MFP blocked FGF2-induced *MYC* transcription in T47D cells (Fig. 1c and Ref. 8, respectively), we evaluated their effects on FGF2-induced ERα/PR binding at both *MYC* enhancer and proximal regulatory sequences. ICI inhibited the recruitment of ERα at both sites and of PR to the enhancer site. MFP did not alter ERα or PR binding at the enhancer site nor PR binding to the proximal site. However, it did inhibit ERα binding at the proximal region (Fig. 2c). Overall, these results show that FGF2 induces the ligand-independent recruitment of ERα and PR at the promoter of ERα/PR target genes. As expected, based on their different mechanisms of inhibition, ICI and MFP exert different effects on the hormone receptor recruitment pattern to block FGF2-induced *MYC* transcription.

FGF2 induces ERα interaction with PRA and PRB at the MYC enhancer and proximal promoter

The results presented above showed a close relationship between ERα and PR after FGF2 incubation. However, they did not provide evidence for a physical association between these receptors, or whether both receptors bind nearby regions at the same promoter sites, nor did they discriminate between the PR isoforms involved. To address these questions, we incubated MCF-7 and T47D cells with FGF2, and NEs were subjected to IP with an ERα antibody. Both PR isoforms could co-IP with ERα, an interaction which was increased by FGF2 stimulation (Fig. 3a). Moreover, the physical interaction was confirmed using *in situ* PLA assays (Supporting Information Fig. S3). These results suggested that ERα and both PR isoforms directly interact in the nuclei of FGF2-stimulated breast cancer cells.

To further evaluate protein–protein interactions (PPIs) with ERα at *MYC* regulatory sequences in FGF2-treated cells, we designed a pull-down assay in which NEs of T47D cells treated with FGF2, E2 and/or ICI were incubated with biotinylated DNAs corresponding to the enhancer and proximal *MYC* regulatory sequences (DNA-biotin; Fig. 3b). WB confirmed that ERα and PR were pulled-down with both sequences and were in agreement with the results presented in Figure 2 where both steroid receptors bind to *MYC* regulatory regions after FGF2 priming (Fig. 3c). FGF2 as well as E2 induced the binding of ERα, PRA and PRB to both sites (Fig. 3c, top). ICI blocked FGF2-induced ERα binding to both sites but only reduced PR binding to the enhancer sequence and it did not block PR binding to the proximal promoter (Fig. 3c, bottom). The antiestrogen increased PR binding to the proximal sites regardless of FGF2 presence, showing similar results as those presented in Figure 2c. However, PR binding by itself (without ERα) could not mediate proximal-Luc activation (Fig. 2b) or *MYC* expression after FGF2 stimulation. A schematic representation of the data obtained is presented in Figure 3d.

ERα-dependent and -independent PPIs at the MYC hormone-regulated sequences

After FGF2 treatment, a total of 1,303 proteins increased, 496 did not change and 57 decreased at the *MYC* enhancer and proximal sequences (raw data is depicted in Supporting Information Table S2, protein lists in Table S3 and protein distribution in Supporting Information Fig. S4). The ERα-dependent recruited proteins were defined as those that were increased with FGF2 compared to the control but reduced by FGF2 + ICI, while the ERα-independent FGF2-recruited proteins were considered as those increased by FGF2 but that did not change after FGF2 + ICI treatment. The protein distribution shows that FGF2 mediates a greater proportion of ERα-dependent recruited proteins in the enhancer, and ERα-independent enrichment in the proximal sequences (Supporting Information Fig. S4). Proteins representing the canonical identifications were considered for bioinformatic analysis (Supporting Information Table S3). Biological Processes significantly represented in each case are depicted in the Supporting Information Figure S5a.

To define the PPIs that could result from ligand-independent ERα and PR activation at the *MYC* enhancer and proximal sites, we focused the analysis considering only the proteins with DNA-binding activity, specifically involved in transcription, chromatin remodeling as well as proteasome degradation and splicing (associated with transcription regulation; Supporting Information Table S4) and propose an interaction network based on data extracted from IMEX Consortium. Network analysis shows a high degree of interaction between the proteins identified in the *MYC* enhancer and proximal promoter as well as between the ERα-dependent and -independent recruited proteins (Fig. 4). For validation, we selected TRIM28, which is enriched at the *MYC* enhancer in an ERα-dependent way, and STAT1, recruited to the *MYC* enhancer and proximal sequences in an ERα-dependent and independent manner, respectively. Co-IP assays of these two proteins with ERα after FGF2 activation confirmed our MS/MS findings (Supporting Information Fig. S6).

We next compared all the canonical proteins identified in FGF2-treated cells (Supporting Information Table S3) with known ERα (1230) and PR (349) interactors which have been identified after steroid stimulation by others (Supporting Information Table S5). A total of 352 proteins (50.3%) recruited to both sequences by FGF2 stimulation were known ERα and/or PR ligand-dependent interacting proteins. We identified 348 novel
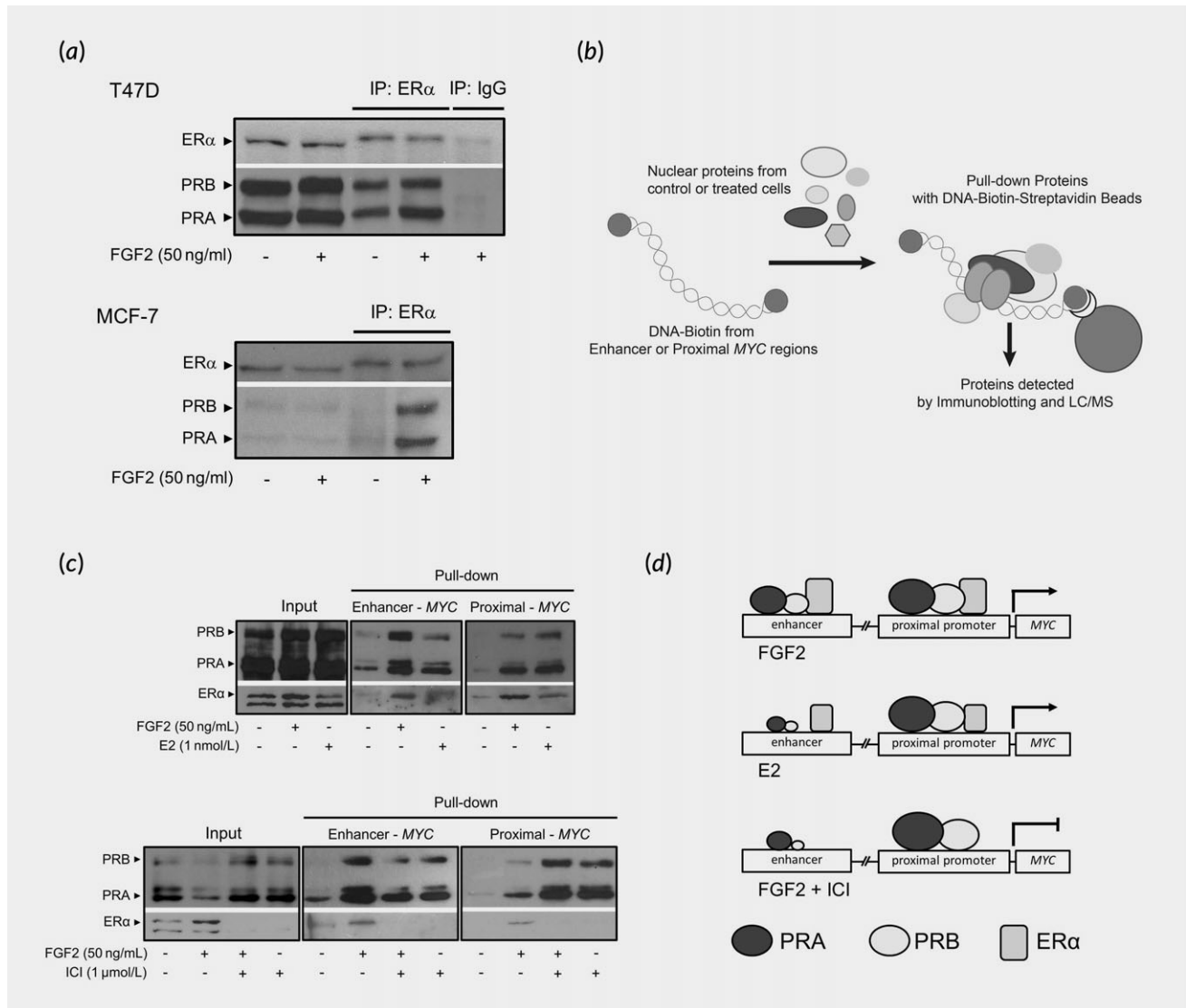
Figure 3. Nuclear interactions between PR isoforms and ERα after ligand-independent activation. (*a*) Co-IP of ERα with PR. NEs from control or 10 min FGF2-treated cells were incubated with ERα (HC-20) or IgG antibodies. IP proteins were analyzed by WB. Both PR isoforms (H-190 antibody) interact with ERα after FGF2 stimulation. (*b*) Schematic representation of the *in vitro* pull-down assay for the detection of protein complexes on *MYC* regulatory sequences. DNA-biotin was immobilized to streptavidin magnetic beads and was incubated with NEs to allow complex formation. After the samples were washed, bound proteins were identified by immunoblotting and nanoLC-MS/MS. (*c*) Immunoblot analysis of ERα (HC-20) and PR (H-190) after pull-down experiments with the enhancer or proximal regulatory sequences of *MYC* using NEs from control or 45-min FGF2-, E2- or FGF2 + ICI-treated T47D cells. Two representative experiments are shown (mean ± SEM). (*d*) Schematic representation of the obtained results.

possible ERα/PR interactors recruited after FGF2 treatment; 198 were ERα-dependent (156 in the enhancer and 67 in the proximal sequences). The processes "Chromatin assembly", "DNA templated transcription, elongation" and processes related to DNA replication and repair were more represented by the newly identified proteins. On the other hand, "chromatin remodeling" was more represented by the already known proteins. As expected, the proteins at the *MYC* proximal promoter region were those that most represented "positive regulation of RNA polymerase II transcriptional preinitiation complex assembly" and "positive regulation of DNA-templated transcription, initiation" (Supporting Information Fig. S5*b*).

Focusing on the proteins with DNA-binding activity and comparing our data to established ERα and PR interactors (Supporting information Table S4), several new ERα-dependent proteins recruited were identified (Supporting Information Fig. S7). The ERα-dependent upregulated proteins in both *MYC* sequences consisted of 19 histones, ERα, CTNNB1 and PSMC6 (Supporting Information Table S4). Fifty-five proteins were exclusively recruited to the *MYC* enhancer. Twenty-one of these proteins have not been described as either ERα or PR hormone-induced interactors. According to Uniprot annotation: SMARCA5, DNMT1, CNOT1, ARHGAP35 and CHD5 are transcription repressors, MLX regulates transcription of glycolytic genes including *MYC*,[29] while
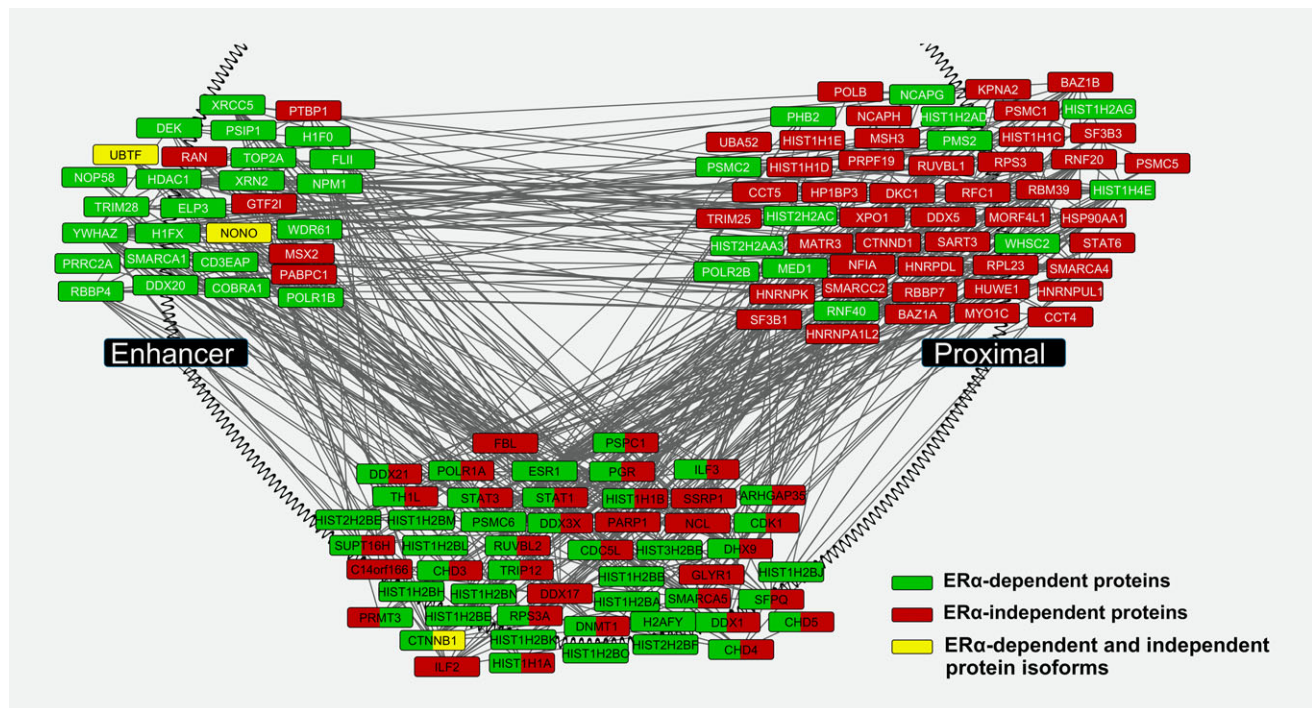
Figure 4. FGF2-induced ERα interactome at *MYC* regulatory sequences. PPIs network from proteins with DNA-binding activity (listed in Supporting Information Table S4), defined by IMEx database and visualized by Cytoscape. Protein colors: green and red for ERα-dependent and -independent proteins, respectively, considering ICI effects. Yellow stands for protein with different isoforms detected in enhancer and proximal promoter. [Color figure can be viewed at wileyonlinelibrary.com]

PURA specifically binds purine-rich elements in the *MYC* gene and promotes gene transcription.[30] In addition, ELP3, WDR61 and CDC5L also have transcriptional coactivator function. Among the remaining DNA binding proteins known to interact with ERα and/or PR we also found transcriptional repressors such as members of the NurD complex (HDAC1, RBBP7, CHD3 and CHD4) as well as TRIM28. Still, many transcriptional activators were also found, including NONO with its regulators PSPC1 and SFPQ, STAT3, STAT1, FLII, as well as coactivators known to play a role in *MYC* transcription (in addition to NONO, NPM1 and RUVBL2). CDK1 was also bound to the enhancer sequence. Specific histone variants and canonical isoforms are enriched in gene promoters,[31] this was even more evident in the proximal sequence, where 26/39 proteins were histones. Among the nonhistone proteins bound to the *MYC* proximal sequence, PRMT3 (ANM3_HUMAN) is an arginine methyltransferase whose expression and/or enzymatic activity is altered in breast tumors,[32] while PHB2 is a known selective ERα coregulator.[33] Therefore, among the known ERα interactors, positive and negative transcription regulators were identified, many of which can behave as both, depending on the context (Supporting Information Fig. S8). Regarding the proteins upregulated by FGF2 in an ERα-independent manner, 83% of these where uniquely localized in the proximal region which supports the idea that FGF2-induced transcription of *MYC* can be regulated at the proximal site, but ERα-dependent recruitment of proteins is needed at the enhancer.

PRBΔ4 isoform is activated by FGF2 and interacts with ERα at gene promoter

The nanoLC-MS/MS analysis (Supporting Information Table S2) identified three additional PR isoforms besides PRA and PRB: PRBΔ4, PRM and PRC (Supporting Information Fig. S9a). The recruitment of PRA, PRB and PRBΔ4 at both *MYC* enhancer and proximal sites was similarly increased by FGF2 but this was more relevant at the enhancer (Fig. 5a, left), where they were blocked by antiestrogen treatment (ERα-dependent interactions). Conversely, at the proximal promoter (Fig. 5a, right), ICI further increased their binding which is in agreement with the data shown in Figure 3c. Thus, PRA, PRB and PRBΔ4 binding to the enhancer sequence is ERα-dependent, but their binding to the proximal region is ERα-independent. PRM and PRC followed a similar trend but with lower emPAI values. Since PRBΔ4 lacks a complete ligand binding domain,[34] we determined whether FGF2 was able to activate PRBΔ4 isoform and thus render a proliferative advantage to PRBΔ4 expressing cells. The PR-negative T47D-Y cell subline was transfected with empty vector, PRB or PRBΔ4. WB identified the PRBΔ4 band in between PRB and PRA (Fig. 5b). As predicted, FGF2 but not MPA, induced PRBΔ4 phosphorylation at Ser294 (Supporting Information Fig. S10) and colocalization with pSer118 ERα (Fig. 5c and Supporting Information Fig. S9b). FGF2 activated ERE-Luc but not PRE-Luc (Fig. 5d) in PRBΔ4-transfected cells and induced the recruitment of PRBΔ4 isoform to *MYC* (Fig. 5e), *CCND1* and *pS2/TFF1* (Supporting Information Fig. S11) gene regulatory sequences in
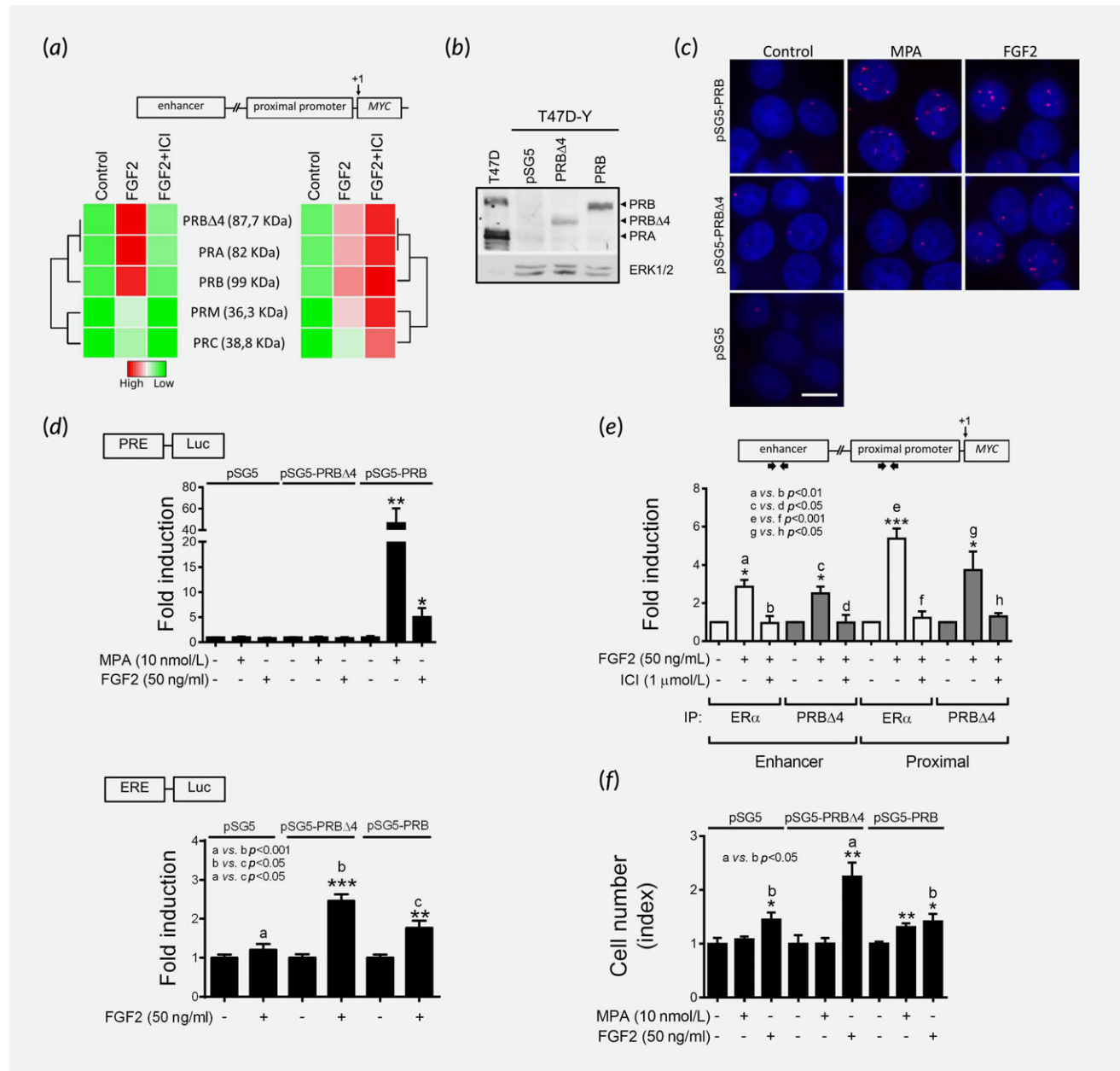
Figure 5. PRBΔ4 isoform actions after FGF2 treatment. (*a*) Heatmap showing the level of PR isoforms recruitment (emPAI values) to *MYC* regulatory sequences (left, enhancer; right, proximal promoter) in pull-down assays. Theoretical masses of the encoded proteins are given in kDa. (*b*) Immunoblot analysis of total PR (H-190 antibody) in ERα+/PR- T47D-Y cells transiently transfected with PRB, PRBΔ4 or empty vector. Extracts from T47D cells were used as a positive control of PRA and PRB expression, and ERK1/2 was used as a loading control. (*c*) Confocal images of immunofluorescence to detect pSer118 ERα/pSer294 PR interaction using *in situ* PLA assays after 30 min of FGF2 (50 ng/ml) or MPA (10 nmol/l) stimulation in transient T47D-Y transfected cells as in (*b*) (bar: 10 μm). Quantification of dots in nuclei is presented in Supporting Information Figure S9b. (*d*) T47D-Y cells transfected as in (*b*) together with PRE-Luc (top) or ERE-Luc (bottom) plasmids were treated for 24 h with experimental solutions and processed to measure luciferase activity. (*e*) ChIP/qPCR studies from stable PRBΔ4 transfected T47D-Y cells to detect the presence of ERα and PRBΔ4 on the *MYC* enhancer or proximal promoter regions after 30 min of FGF2 and ICI incubations. Two representative experiments are shown (mean ± SEM). (*f*) Cell counting assays from transiently transfected T47D-Y cells. Cultures were starved and treated for 7 days with FGF2 or MPA. [Color figure can be viewed at wileyonlinelibrary.com]

an ERα-dependent way, suggesting its role as a coactivator of ERα function. Moreover, FGF2 induced a slight increase in cell proliferation in the control cells; however, the increase was significantly higher in cells expressing the PRBΔ4 isoform (Fig. 5*f*).

PRBΔ4 expression in human breast cancer

PRBΔ4 mRNA expression has already been detected in breast cancer tissues.[35,36] Using selected human breast tumor samples from our tumor bank,[37] we show that the samples in
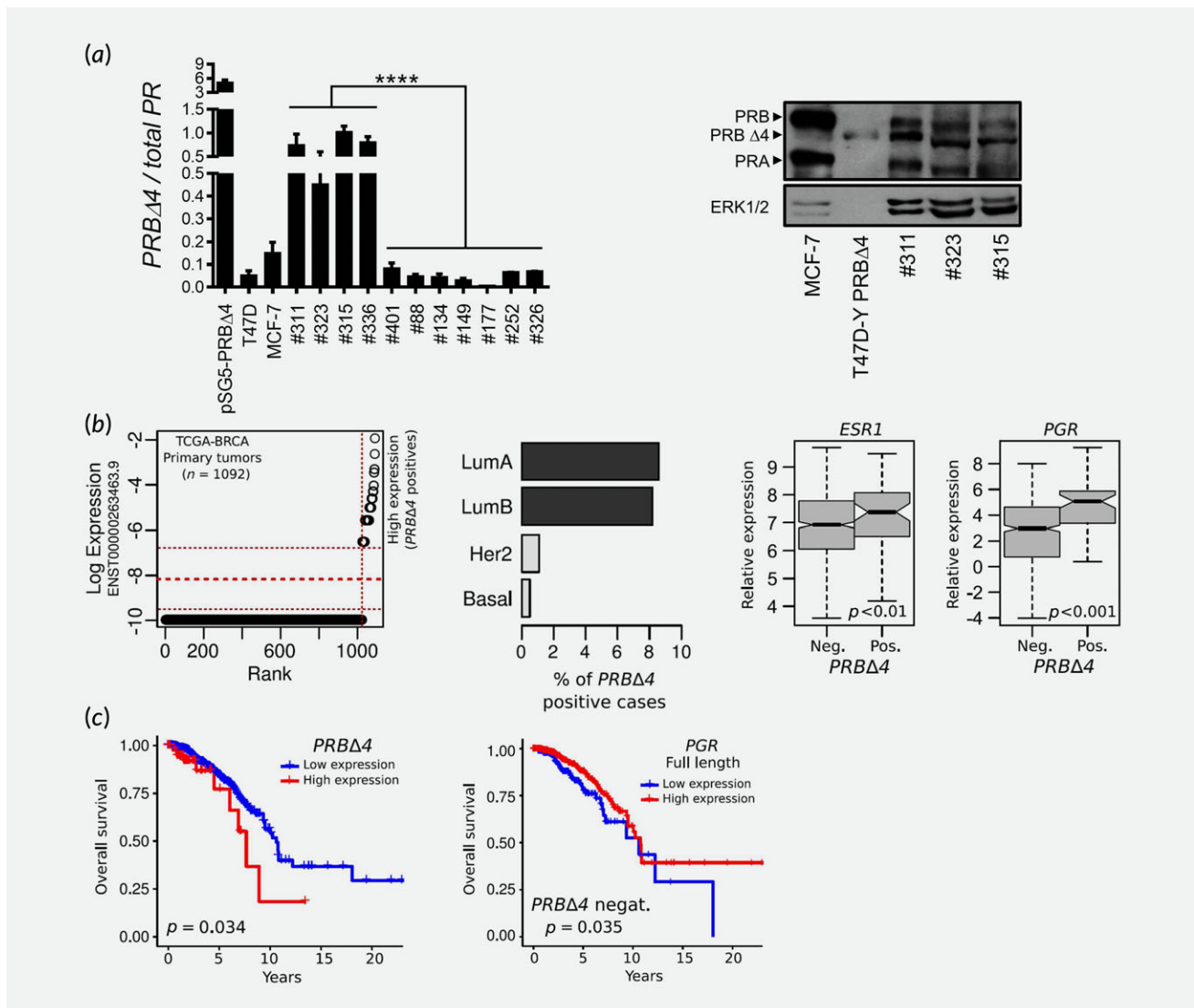
Figure 6. *PRBΔ4* isoform expression in breast cancer. (*a*) Left: Relation between *PRBΔ4* and total *PR* expression in samples in which an intermediate band between PRB and PRA was detected (*n* = 4) and in those with undetectable intermediate bands (*n* = 7). Right: Western blots of 3 representative cases showing a band compatible with the PRBΔ4 isoform. (*b*) *PRBΔ4* (ENST00000263463.9 transcript) expression (left) among 1,092 primary invasive breast carcinomas obtained from the TCGA database. Distribution of *PRBΔ4*-positive cases among breast carcinomas (BRCA) subtypes (middle). Boxplot of *ESR1* and *PGR* full-length mRNA expression (right) in negative and positive *PRBΔ4* LumA+LumB tumors samples from TCGA. (*c*) Overall survival curves of the *PRBΔ4* low and high expression cases (left) and *PGR* full length from *PRBΔ4* negative tumors (right) in LumA+LumB from the TCGA data set. [Color figure can be viewed at wileyonlinelibrary.com]

which the intermediate band between PRB and PRA coincides with the PRBΔ4 band of T47D-Y PRBΔ4 transfected cells (Fig. 6*a*, right), also expressed *PRBΔ4* mRNA (Fig. 6*a*, left), indicating that this novel variant may play a role in a subgroup of breast cancer patients. Lower *PRBΔ4* mRNA levels were observed in samples that did not express the intermediate band by western blot ($p < 0.001$).

TCGA analysis confirms that *PRBΔ4* mRNA is expressed only in a subgroup of tumors (8–9%), mainly luminal carcinomas (Fig. 6*b*, left and middle), and that *PRBΔ4* positive tumors express higher levels of *ESR1* and *PGR* full-length than the negative ones (Fig. 6*b*, right).

Interestingly, *PRBΔ4* mRNA expression in luminal tumors correlates with worse prognosis (Fig. 6*c*, left) while *PGR* full-length expression predicts a good outcome in *PRBΔ4*-negative patients (Fig. 6*c*, right).

Discussion

In this study, we propose that autocrine[38] or paracrine FGF2, probably produced by stromal CAFs,[6,39,40] activates FGFRs and downstream signaling pathways, which in turn activate ERα and classic and novel PR isoforms. These activated receptors form complexes that play key roles inducing the transcription of *MYC*, a master cancer gene. The *MYC* enhancer regulatory sequence is governed by
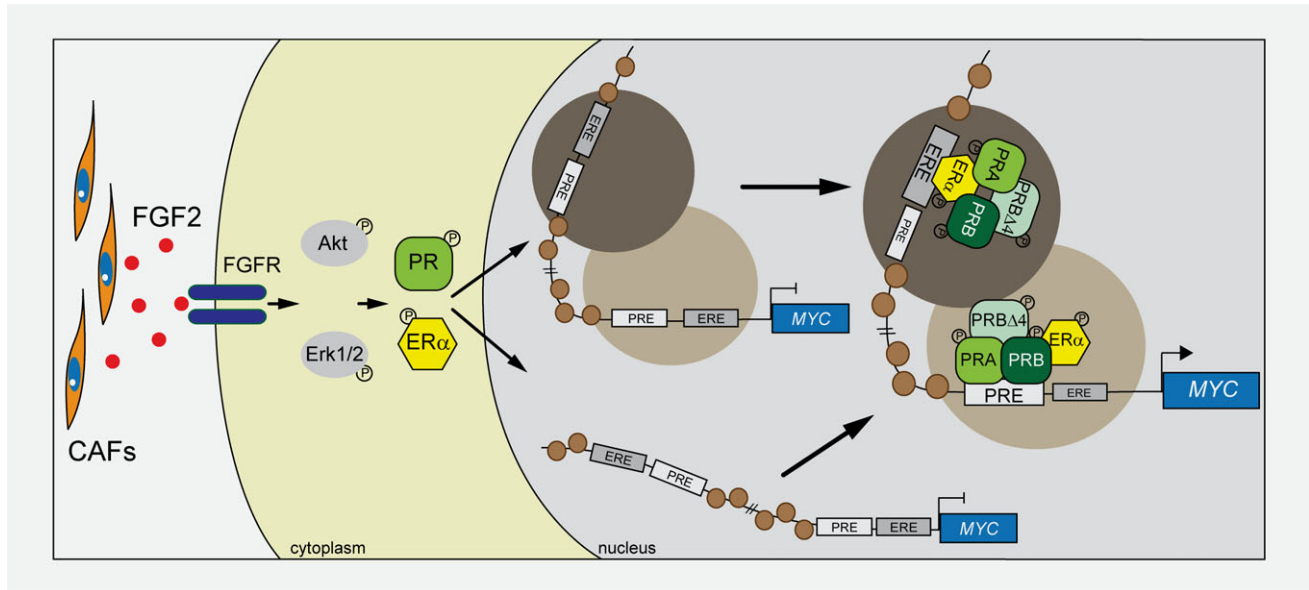
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Figure 7. Model of FGF2-induced *MYC* expression through regulatory sequences cooperation involving ERα and PR isoforms. See text for details. [Color figure can be viewed at wileyonlinelibrary.com]

ERα since PR isoforms recruitment depends mostly on ERα binding to DNA. On the other hand, in the *MYC* proximal regulatory sequences, PR binding is independent of the presence of ERα, although the interaction of both receptors is needed to induce gene transcription. It is possible that common interactors recruited to the enhancer and proximal-sensitive regions of *MYC* participate in promoting a physical interaction between both regions inducing FGF2-driven *MYC* transcription. A loop between the enhancer and the proximal promoter region of *MYC* gene has been demonstrated through 3C experiments in MCF-7 cells after estradiol stimulation.[41] However, the loop might be already established prior activated receptor binding,[42] or it may be the consequence of hormone or growth factor stimulation. This has been summarized in Figure 7. The fact that PRBΔ4 is activated by FGF2, but not by progestins, explains the advantage that PRBΔ4-expressing cells may have under a low hormone milieu. These results reveal that MYC inhibitors may thus be candidates to be used to potentiate endocrine therapy. Since progestins and other growth factors have been shown to activate ERα/PR,[12,43] the specificity of this effect may depend on the growth factor receptor repertoire of the cancer cells.

The development of MYC inhibitors has been delayed, perhaps because it is an undruggable target.[44] However, MYC inhibition strategies have been developed based on interrupting protein–protein interactions with MAX to abolish MYC-dependent transcriptional activity.[44] As a proof of concept, we demonstrated that MYC inhibitors blocked FGF2-induced cell proliferation *in vitro* in MCF-7 and T47D cells, this inhibition occurred even at concentrations lower than those reported to block cell proliferation in other cell lines, underscoring the exquisite sensitivity of these models.[45,46] For *in vivo* assays, we used the C4-HI murine model in which we have already demonstrated that its hormone-independent growth relies on stromal FGF2.[6] Interestingly, a

marked decrease in tumor growth was observed and although few data are available, this inhibition was more conspicuous than that observed in the MDA-MB-231 xenograft model.[27]

One of the major novelties of this study is the discovery of PR splice variants proteins that have previously only been observed at the mRNA levels. These novel isoforms were not detected in other interactome studies, probably because previous assays have been performed using natural ligands to activate hormone receptors.[14,47,48] Several PR isoforms have been predicted from the analysis of alternative splicing mechanisms.[36,49,50] PRM mRNA which contains exons 4–8 and retains an intronic sequence was described in human tissues and in T47D cells.[51] The PRC isoform results from an alternative translation site starting at methionine 595 and was also first described in T47D cells.[52] This form should retain the ability to bind the ligand, but not DNA. Other mRNA PR variants with different deleted exons were detected in breast cancer tissues;[34–36] among them, the variants with deletions of exon 4. However, how these mRNAs impact on PR signaling and if they were translated into proteins has been poorly investigated. Richer *et al.* built a Δ4 isoform in the background of the PRA receptor, and they showed that progestins did not induce binding to PRE sites nor activated gene reporters.[34] We show herein that PRBΔ4 variant, which has as an impaired ligand binding domain and lacks the nuclear localization signal,[34] may form part of transcriptional complexes at the promoters of key genes and most importantly, they play a role only in the ligand-independent activation of hormone receptors. Moreover, PR immunoreactive bands, compatible with the PRBΔ4 variant, were detected in nuclear extracts from selected human breast cancer samples which also showed *PRBΔ4* mRNA suggesting that these proteins are not just cell line artifacts. In line with our experimental data, *PRBΔ4* mRNA in luminal breast cancers correlated with worse prognosis in the TCGA database.

In luciferase experiments, FGF2 induced similar effects as progestins or estrogens using any of the two described *MYC* regulatory sequences. Whereas Wang *et al.*[22] showed that only the *MYC* enhancer is estrogen-responsive in MCF-7 cells, both sites proved to be responsive in T47D. The discrepancy between results may be attributed to the different cell lines tested. ERα activation and binding to particular sequences could be determined by the coordinated effects of key cofactors which can be cell-specific.[53] Alternatively, limited PR was available to interact with ERα after estradiol induction to mediate proximal *MYC* promoter activation in MCF-7 cells, whereas T47D cells have constitutively high levels of ERα and PR.

ICI blocked FGF2-induced ERα binding to both *MYC* regulatory sequences and binding of PR only to the enhancer, suggesting that in T47D cells, PR needs to be coupled with ERα at ERE sites at the enhancer, while at the proximal region, PR probably binds to PRE elements. However, as observed in luciferase experiments, in the absence of ERα this promoter is not transcriptionally active. The effect of MFP is less clear. As shown in previous studies, MFP activates PR, which binds to gene promoters.[54,55] Nevertheless, gene transcription is blocked due to recruitment of corepressors that turn off gene transcription.[56] In our experiments, MFP maintained ERα and PR binding at the enhancer region. ERα was not recruited at the proximal promoter, inhibiting gene transcription, which was likely due to the new conformation of a complex that was unable to bind ERα and other coactivators.

Notably, similar results were achieved using different experimental approaches. Pull-down and LC–MS/MS experiments faithfully reproduced the ERα and PR recruitment obtained by ChIP assays. In addition, the proteomic study allowed us to identify potential hormone receptor regulators. In this regard, most molecular studies performed in the PR field do not distinguish between both classical PR isoforms A and B. Here, we showed that although both isoforms are activated by FGF2 and are recruited to *MYC* enhancer and proximal regions, PRA is enriched in the enhancer, while both PRA and PRB were similarly recruited at the proximal promoter.

The proteomic studies indicate that several proteins participate in FGF2-induced *MYC* transcription that were not reported in studies in which ERα or PR were activated by agonists. Exclusive and shared interactors were recruited at the enhancer and proximal promoter of *MYC*, and protein distribution shows that FGF2 mediates a greater proportion of ERα-dependent recruited proteins in the enhancer and ERα-independent enrichment in the proximal sequences. It may be speculated that interacting partners of both sites may participate in inducing or maintaining the three-dimensional (3D) folded structure necessary for FGF2-induced *MYC* transcription. The detailed study of how these coregulators impact in maintaining this 3D structure will surely provide novel druggable targets that may better block FGF2-induced *MYC* transcription. TRIM28 has been associated with a metastatic signature, stemness and increased breast cancer growth.[57] STAT1 has been recently described as a SRC1-partner able to activate gene expression in endocrine-resistant cells,[58] while STAT3 is a well-known PR interactor.[59] We found that these proteins are FGF2-induced ERα-dependent interactors bound at the *MYC* enhancer. Interestingly, STAT1 and STAT3 were also bound to the proximal site in an ERα-independent way.

In summary, we report that FGF2 activates ERα and at least three PR isoforms, PRA, PRB and PRBΔ4, which interact forming complexes at the *MYC* enhancer and proximal promoter, being the former strictly ERα-dependent. PRBΔ4, which is activated by FGF2 but not by progestins, is related to a worse prognosis for luminal breast cancer patients. These novel findings will open new research lines evaluating the role of noncanonical PR isoforms in endocrine resistance and their use as possible therapeutic targets to be included together with classic endocrine therapies and MYC inhibitors to efficiently block luminal breast cancer growth.

Author contributions

SG, LAH and CL conceived and designed the study. SG, MR, TG, CPP, MAG, GS, GP, MFA, ACG, MFT, FLM and FA performed experiments. SG, AB, MA and LAH performed the bioinformatic analysis of the proteomic data. HG contributed with the human breast cancer samples, SG, LAH and CL wrote the manuscript, with contributions from BMJ and VN.

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