Heregulin Co-opts PR Transcriptional Action Via Stat3 Role As a Coregulator to Drive Cancer Growth

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Accumulated findings have demonstrated the presence of bidirectional interactions between progesterone receptor (PR) and the ErbB family of receptor tyrosine kinases signaling pathways in breast cancer. We previously revealed signal transducer and activator of transcription 3 (Stat3) as a nodal convergence point between said signaling pathways proving that Stat3 is activated by one of the ErbBs' ligands, heregulin (HRG)β1 via ErbB2 and through the co-option of PR as a signaling molecule. Here, we found that HRGβ1 induced Stat3 recruitment to the promoters of the progestin-regulated cell cycle modulators Bcl-X_L and p21^{CIP1} and also stimulated Stat3 binding to the mouse mammary tumor virus promoter, which carries consensus progesterone response elements. Interestingly, HRGβ1-activated Stat3 displayed differential functions on PR activity depending on the promoter bound. Indeed, Stat3 was required for PR binding in bcl-X, p21^{CIP1}, and c-myc promoters while exerting a PR coactivator function on the mouse mammary tumor virus promoter. Stat3 also proved to be necessary for HRGβ1-induced in vivo tumor growth. Our results endow Stat3 a novel function as a coregulator of HRGβ1-activated PR to promote breast cancer growth. These findings underscore the importance of understanding the complex interactions between PR and other regulatory factors, such as Stat3, that contribute to determine the context-dependent transcriptional actions of PR. (*Molecular Endocrinology* 29: 1468–1485, 2015)

Progesterone receptor (PR) is a critical mediator of mammary gland development and contributes to breast cancer progression. The PR exists in 2 primary isoforms (PR-A and PR-B), differing structurally by the inclusion of an N-terminal segment unique to the full-length isoform, PR-B. PR action is context-dependent, that is, PR action differs in normal vs neoplastic tissue and according to hormone exposure, as well as organ site (for example, proliferative in the breast vs inhibitory in the uterus). In addition, PR isoform-specific activities (PR-A vs PR-B) overlap but can have very disparate activities within a given target tissue and at selected gene promoters. In its classical mechanism of action PR associates with specific progesterone response elements (PREs) on chro-

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Abbreviations: AcH4, acetyl-histone H4; CBP, CREB binding protein; ChIP, chromatin immunoprecipitation; ER, estrogen receptor; FoxA1, Forkhead box protein A1; GAS, gamma interferon-activated sequence; GFP, green fluorescent protein; GR, glucocorticoid receptor; hErbB2ΔNLS, ErbB2 NLD mutant; H3K4me1, monomethylated histone H3 lysine 4; HRG, heregulin; MMTV, mouse mammary tumor virus; NErbB2, nuclear ErbB2; NLD, nuclear localization domain; PR, progesterone receptor; PRE, progesterone response element; qPCR, quantitative PCR; RU486, mifepristone; siRNA, small interfering RNA; Sp1, specificity protein 1; Stat3, signal transducer and activator of transcription 3.

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matin. DNA-bound PR recruits transcriptional coactivators and associated cofactors, which modify the local chromatin structure and facilitate transcriptional activation, resulting in activation or repression of PR target genes (1). PR may also alter gene expression nonclassically, where the receptor tethers to other transcription factors bound to DNA, including activator protein 1 (AP-1), specificity protein 1 (Sp1), and signal transducer and activator of transcription 3 (Stat3) (2–4). In addition to its direct transcriptional effects, PR activates signal transduction pathways in breast cancer cells through a rapid or nongenomic mechanism (5, 6). PR transcriptional outcome is affected by complex interactions between PR and other regulatory factors which results in different PR cistromes (7). Indeed, promoter specificity is determined by interactions between PR and transcriptional coactivators and corepressors and by interactions with other members of the steroid receptor superfamily (8). Interactions with potential PR pioneer factors may lead to chromatin remodeling, allowing for efficient PR recruitment and subsequent target-gene transcription, as for other steroid hormone receptors (9–12). Promoter selectivity as well as PR subcellular localization and turnover are dictated by PR posttranslational modifications. Recent mechanistic studies have shown that PR is capable of driving breast cancer progression in both the absence and presence of progestin revealing that both PR isoforms display ligand dependent and independent activities (8, 13). Indeed, we and others have demonstrated that PR function is heavily influenced by cross-talk/input from peptide growth factor-initiated signal transduction pathways (14). We have found that heregulin (HRG)β1, one of the ligands of the ErbB family of receptor tyrosine kinases, induces PR transcriptional activation through a mechanism that requires functional ErbB2 (15). Another key determinant of PR action which may modify its kinetics lies in the dosing (cyclical vs continuous) and source (natural vs synthetic) of ligand (16). All of these levels of complexity of PR actions underscore PR as an outstanding therapeutic target. We have previously unraveled the role of Stat3 as the common downstream effector of both PR and HRGβ1/ErbBs signaling pathways (17, 18). We have shown that HRGβ1/ErbB2 activates Stat3 through the co-option of PR signaling to drive breast cancer growth (17). Furthermore, we observed Stat3 activity as a coactivator of PR (19) in the transcription of 2 endogenous genes involved in cell cycle regulation and modulated by progesterone: bcl-X (20) and $p21^{CIP1}$ (21).

Pioneering or licensing factors are proteins with an intrinsic ability to bind to condensed chromatin and prime specific genomic loci for subsequent receptor binding (22). Their recruitment to the chromatin is sequence specific and can be facilitated by an epigenetic signature dependent on histone methylation (23). Estrogen, androgen and glucocorticoid receptors (ER, AR, and GR) were shown to require pioneering factor activity (9–11) but PR showed direct nucleosome binding for optimal function when activated by the synthetic progestin R5020 in a recent report (24). Here, we explored whether HRGβ1-induced transcriptional activation of PR also results in the modulation of $Bcl\text{-}X_L$ and $p21^{CIP1}$ expressions and whether Stat3 is involved in HRGβ1-induced PR transcriptional actions in breast cancer. We found that HRGβ1 induced Stat3 recruitment to bcl-X and $p21^{CIP1}$ promoters. HRGβ1 also stimulated Stat3 binding to the mouse mammary tumor virus (MMTV) promoter, which carries consensus PREs. Interestingly, Stat3 acted as a licensing factor for PR binding in bcl-X and $p21^{CIP1}$ promoters but exerted a coactivator function on the MMTV promoter. These findings indicate that HRGβ1-induced Stat3 activation modifies PR function differentially depending on the promoter context, therefore highlighting the significance of fully understanding the determinants of context-dependent PR action.

Results

HRG induces $Bcl\text{-}X_L$ and $p21^{CIP1}$ expression in breast cancer via PR, Stat3, and ErbB2

Our previous findings revealed that HRGβ1 stimulates PR transcriptional activation in breast cancer cells (15). On the other hand, we recently demonstrated that progestin upregulates bcl-X and $p21^{CIP1}$ (19) gene expression in breast cancer via the assembly of a transcriptional complex in which Stat3 and PR physically associate and where Stat3 acts as PR coactivator. In light of these findings, here we firstly explored whether HRGβ1 transcriptional activation of PR also results in the modulation of $Bcl\text{-}X_L$ and $p21^{CIP1}$ expressions and secondly, whether Stat3 is involved in HRGβ1-induced PR transcriptional actions in breast cancer. We used C4HD murine mammary tumor cells (19) and T47D human breast cancer cells. C4HD cells express ER and PR, and overexpress membrane ErbB2. Both cellular models exhibit a potent proliferative response to HRGβ1, mediated by ErbB2 and PR, as previously found by us and others (15, 25). Here, we found that HRGβ1 significantly induced $Bcl\text{-}X_L$ protein expression in C4HD cells (Figure 1A). Consistent with previous findings in T47D and MCF7 cells (26, 27), we observed that HRGβ1 also upregulated $p21^{CIP1}$ protein levels in C4HD cells (Figure 1A). To assess PR involvement in HRGβ1 effects, we preincubated cells with the antiprogestin RU486 (or Mifepristone) or knocked down PR

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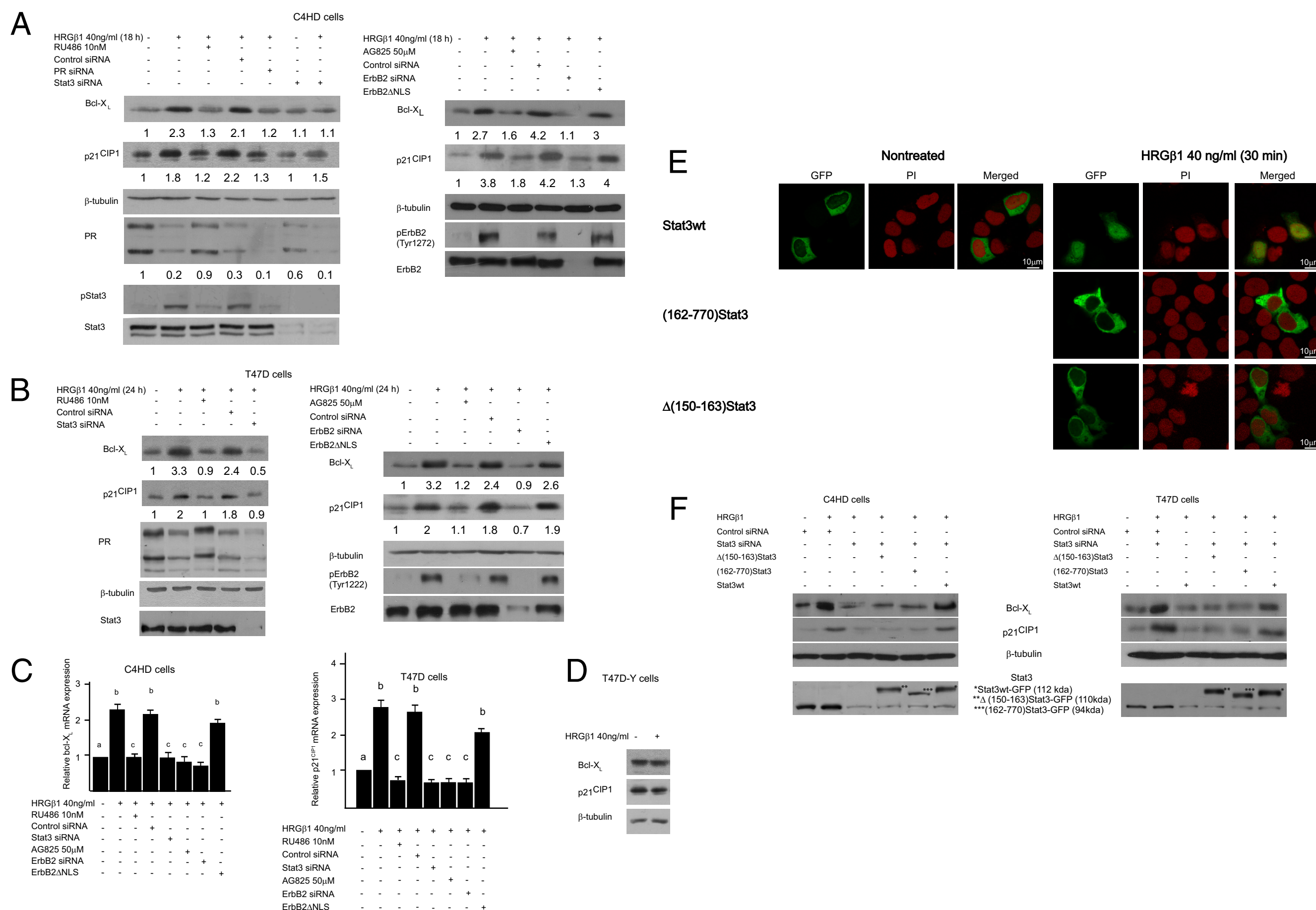

Figure 1. HRGβ1 induces Bcl-X_L and p21^{CIP1} expression via PR and Stat3. A, C4HD cells were treated with HRGβ1 or pretreated with RU486 or AG825 or transfected with PR, Stat3, or control siRNAs before HRGβ1 stimulation. Western blottings (WB) were performed with Bcl-X_L, p21^{CIP1}, PR, Stat3, pStat3, and pErbB2 antibodies, and membranes were reprobed with β-tubulin and total ErbB2 antibodies. Left panel, The WBs at the bottom show the effects of siRNAs on PR and Stat3 expression. Right panel, The WBs at the bottom show the effect of AG825 and of ErbB2 siRNAs on ErbB2 phosphorylation and expression, respectively. Bcl-X_L and p21^{CIP1} bands underwent densitometry, and values were normalized to β-tubulin protein bands setting the value of untreated cells as 1. B, T47D cells were treated and WBs were performed as in A. Bcl-X_L and p21^{CIP1} bands were quantified as described in A. C, Cells were transfected with ErbB-2, Stat3, and control siRNAs and were treated with HRGβ1. bcl-X_L and p21^{CIP1} mRNA expression levels were determined by reverse transcription real-time PCR (RT-qPCR). Fold change of mRNA levels upon HRGβ1 treatment was calculated by normalizing the absolute levels of bcl-X_L and p21^{CIP1} mRNA to GAPDH levels, used as an internal control, setting the value of untreated cells as 1. Data are presented as mean ± SEM. For b vs a and b vs c, $P < .001$, one-way ANOVA. D, T47D-Y cells were treated, and WBs were performed as in A. The experiments in A–C were repeated 3 times with similar results. E, Expression and function of (162–770)Stat3, Δ(150–163)Stat3, and Stat3wt vectors. Cells were transfected with Stat3 or control siRNAs and transfected with Stat3 NLD mutants (162–770)Stat3, Δ(150–163)Stat3, or with a plasmid encoding Stat3wt before HRGβ1 stimulation. GFP from (162–770)Stat3, Δ(150–163)Stat3, and Stat3wt was visualized by direct fluorescence imaging (green). Nuclei were stained with propidium iodide staining (red). F, Endogenous Stat3 expression was silenced by transfection with siRNAs, and expressions of (162–770)Stat3, Δ(150–162)Stat3, or Stat3wt were restored by cotransfection (2 μg/well) with the respective plasmids. WBs were performed with Bcl-X_L, p21^{CIP1}, and GFP antibodies and membranes were reprobed with Stat3 and β-tubulin antibodies. Expression of the mutant vectors is indicated with asterisks.

expression with PR small interfering RNAs (siRNAs), which inhibit expression of both PR isoforms. Our findings showed that HRGβ1 did not induce Bcl-X_L or p21^{CIP1} expression in the absence of PR expression or activation (Figure 1A), revealing that unliganded and HRGβ1-activated classical PR is involved in upregulation of both proteins. We also found that knockdown of Stat3 expression with siRNAs abrogated HRGβ1 induction of Bcl-X_L and p21^{CIP1} (Figure 1A). HRGβ1 treatment induced Stat3 tyrosine phosphorylation, which was blocked in the absence of PR expression or activation (Figure 1A) as previously shown by us (17). Supplemental Figure 1A shows that the Stat3 siRNAs used are specific to Stat3 and do not affect the protein levels of the closely related Stat family member Stat5. Other studies (28, 29) have described the participation of constitutively active Stat3 in the regulation of p21^{CIP1}, our present findings identify a novel role of Stat3 as mediator of HRGβ1 regulation of p21^{CIP1}.

Interestingly, we found that silencing Stat3 expression resulted in reduction of PR levels at both protein (Figure 1, A left panel and B left panel, and Supplemental Figure

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1A) and mRNA levels (Supplemental Figure 1B). To our knowledge there are no reports on Stat3 modulation of PR expression. However, chromatin immunoprecipitation (ChIP)-Sequencing data of Stat3 in breast cells obtained from ENCODE growing under basal conditions revealed a Stat3 binding peak located at −1737 to −1812 bp from the transcriptional start site of the *PgR* gene (Supplemental Figure 2). This Stat3 binding region was also significantly enriched in monomethylated histone H3 lysine 4 (H3K4me1), a marker of active enhancers. This suggests that PR could be a Stat3 target gene, which would account for the decrease in PR levels in cells in which we silenced Stat3 expression. In spite of the reduced levels of PR expression, HRGβ1 capacity to down-regulate PR (15) was not affected by silencing Stat3 expression (Figure 1A). Stat3 knockdown did not affect the protein levels of the PR-dependent genes mucin 1 (MUC1) (30), epidermal growth factor receptor (EGFR) (4), ErbB3 (31) (Supplemental Figure 3), or Stat5 (14) (Supplemental Figure 1A) indicating that the reduction of PR levels observed by inhibition of Stat3 expression is not responsible per se of HRGβ1 lack of regulation of Bcl-X_L and p21^{CIP1} in the presence of Stat3 siRNAs.

Next, we studied the role of ErbB2 in HRGβ1 action and found that blockade of ErbB2 kinase activity with the pharmacological inhibitor AG825 or knockdown of ErbB2 expression with siRNAs, inhibited Bcl-X_L and p21^{CIP1} protein up-regulation by HRGβ1 (Figure 1A, right panel).

We already revealed that in breast cancer cells stimulated with progestin, ErbB2 migrates to the nucleus where it assembles a transcriptional complex in which it functions as a coactivator of Stat3 to promote the expression of cyclin D1 and induce growth (3). Based on these findings, we next studied whether ErbB2 nuclear function participates in HRGβ1 regulation of Bcl-X_L and p21^{CIP1} expressions. To block the presence of nuclear ErbB2 (NErbB2), we transfected cells with a human ErbB2 nuclear localization domain (NLD) mutant (hErbB2ΔNLS), unable to translocate to the nucleus, and which we found functions as a dominant negative inhibitor of endogenous ErbB2 nuclear migration (3). Control of hErbB2ΔNLS function as dominant negative inhibitor of endogenous ErbB2 nuclear localization upon HRGβ1 stimulation is shown in Supplemental Figure 4. hErbB2ΔNLS retains its intrinsic tyrosine kinase activity and the capacity to activate classical ErbB2 cascades, including, as we already demonstrated, the activation of Stat3, and it does not affect endogenous ErbB2 signaling (3). Here, we found that transfection with hErbB2ΔNLS did not abrogate HRGβ1 ability to up-regulate Bcl-X_L or p21^{CIP1} (Figure 1A, right panel). Like in C4HD cells, HRGβ1 also up-regulated Bcl-X_L and p21^{CIP1} protein expression in T47D cells via PR, Stat3, and ErbB2 function as receptor tyrosine kinase, but independently of ErbB2 nuclear actions (Figure 1B). Bcl-X_L and p21^{CIP1} mRNA levels were also induced by HRGβ1 in both cell types via the same mechanisms than those involved in their regulation at protein level (Figure 1C). HRGβ1 treatment of the PR-null T47D cells (T47D-Y) had no effect neither on Bcl-X_L nor on p21^{CIP1} expression (Figure 1D), confirming the involvement of the classical PR.

In order to explore whether nuclear Stat3 function participates in HRGβ1 induction of Bcl-X_L and p21^{CIP1} expression, we used an RNAi reconstitution strategy. We transfected C4HD cells with Stat3 siRNAs specifically targeting Stat3 at its 3′-untranslated region, in combination with Stat3 NLD mutants, which are unable to translocate to the nucleus (Figure 1E), or with a plasmid encoding wild-type Stat3. Cotransfection of an expression vector encoding a Stat3 mutant protein, which lacks a portion of 162 amino acids from the N-terminal domain, including the 13 amino acids of the NLD (162–770)Stat3, was not capable of rescuing HRGβ1-induced Bcl-X_L or p21^{CIP1} expression (Figure 1F). Likewise, cotransfection of an expression vector which encodes a Stat3 mutant which only lacks the NLD Δ(150–163)Stat3, was ineffective to reconstitute HRGβ1-induced Bcl-X_L or p21^{CIP1} expressions (Figure 1F). Cotransfection of a wild-type Stat3 protein successfully enabled reconstitution of HRGβ1 effects on Bcl-X_L and p21^{CIP1} expression of (Figure 1F). Similar results were observed in T47D cells (Figure 1F). Controls of inhibition of Stat3 protein expression by the siRNAs used and for its reconstitution by transfection with all 3 plasmids are shown in Figure 1F, lower panel. These results show that nuclear Stat3 action mediates HRGβ1 induction of Bcl-X_L and p21^{CIP1} expressions. Together, our findings revealed that HRGβ1 up-regulates Bcl-X_L and p21^{CIP1} protein and mRNA expressions via the classical PR, ErbB2 canonical function as receptor tyrosine kinase, and Stat3 transcriptional function.

Role of Stat3 as a PR coregulator

To explore gene enhancers involved in the regulation of Bcl-X_L and p21^{CIP1} by HRGβ1, we first conducted reporter assays. Having observed that PR was involved in HRGβ1 effects, we tested the participation of the PREs present in the fourth promoter of bcl-X gene (bcl-XP4). On the other hand, we examined Sp1 binding sites at the proximal promoter of p21^{CIP1}, which previous findings, including ours, found to bind activated PR through tethering to Sp1 (19, 21). C4HD cells transfected with a reporter vector under the control of bcl-XP4, showed a

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significant increase in luciferase activity in the presence of HRGβ1 (Supplemental Figure 5A) which was blocked by RU486 pretreament (Supplemental Figure 5A). To further demonstrate that the bcl-XP4 promoter-driven luciferase activity was due to an interaction between HRGβ1-activated PR and the PRE region on DNA, C4HD cells were transfected with a reporter plasmid which contains a deletion of 95 nt spanning both PREs present in the murine bcl-X promoter (bcl-XP4ΔHRE-luc). In this condition, we detected no luciferase activity induction in the presence of HRGβ1 (Supplemental Figure 5A). By using the generic PRE reporter MMTV-Luc and as we previously reported (15), we observed a strong HRGβ1 induction of PR transcriptional activation which was abrogated by RU486 pretreatment (Supplemental Figure 5B). In addition, knockdown of Stat3 expression impaired HRGβ1 capacity to induce PR transcriptional activation both in bclX-P4 and in MMTV reporter constructs (Supplemental Figure 5, A and B). As regards the regulation of $p21^{CIP1}$ transcriptional activation, we observed that cells transfected with a $p21^{CIP1}$ reporter construct spanning nt −93 to −33 of the promoter (p21P93-S) containing the consensus Sp1 response elements, yielded a significant increase in luciferase activity in the presence of HRGβ1 (Supplemental Figure 5C). Intact Sp1 binding sites are required for HRGβ1 induction of $p21^{CIP1}$, because we detected no regulation of luciferase activity when cells were transfected with a mutant reporter vector carrying a point mutation in sp1–2 (p21P93-S-Luc-mut) (Supplemental Figure 5C). Consistent with the requirement of Stat3 in HRGβ1-induced $p21^{CIP1}$ expression, knockdown of Stat3 blocked HRGβ1 capacity to induce $p21^{CIP1}$ transcription (Supplemental Figure 5C). These results show that HRGβ1 induces $Bcl\text{-}X_L$ and $p21^{CIP1}$ promoters activation by a mechanism requiring PR and Stat3.

We next investigated the mechanism involved in HRGβ1 modulation of PR occupancy of the endogenous murine bcl-XP4, particularly focusing on Stat3 involvement. C4HD cells were treated with HRGβ1 and ChIP assays were performed with PR or Stat3 antibodies. We found that HRGβ1 treatment resulted in a recruitment of PR to the region of the bcl-XP4 containing the PREs. (Figure 2A). Next, we silenced Stat3 expression to explore whether it modulated PR loading at the PREs in said promoter. Notably, in absence of Stat3 expression, HRGβ1 was unable to induce PR recruitment to bcl-XP4 (Figure 2A). Reconstitution of wild-type Stat3 restored HRGβ1 capacity to induce PR loading to bcl-XP4. On the contrary, reconstitution of the expression of Stat3 mutants Δ(150–163)Stat3 or (162–770)Stat3 did not reestablish HRGβ1-stimulated PR binding to bcl-XP4 (Figure 2A). These findings suggest a functional role of nuclear Stat3 as mediator of PR recruitment to its response elements in bcl-XP4. In support of this possibility, our bioinformatics analysis identified Stat3 response elements (gamma interferon-activated sequence or GAS sites) in the murine bcl-XP4, located at position −133 relative to the transcription start site. To further assess Stat3 function, we explored HRGβ1 effects on Stat3 recruitment to the bcl-XP4 region containing the PREs. We found that HRGβ1 significantly stimulated Stat3 binding to this region of the bcl-XP4 promoter (Figure 2A). In C4HD cells in which we silenced endogenous Stat3 and restored the expression of Δ(150–163)Stat3 or (162–770)Stat3 mutant vectors, we detected no HRGβ1 stimulation of Stat3 loading, as expected due to said mutants inability to translocate to the nuclear compartment (Figure 2A). Consistent with our finding that NErbB2 function is not required for HRGβ1 induction of $Bcl\text{-}X_L$ expression, we found no ErbB2 recruitment to the PREs of bcl-XP4 in cells treated with HRGβ1 (Figure 2A). Histone acetylation positively correlates with active gene transcription (32). Accordingly, histone H4 acetylation was induced by HRGβ1 in the region of bcl-XP4 containing the PRE sites. Silencing of Stat3 expression abolished HRGβ1 effect on acetyl-histone H4 (AcH4), which was restored by reconstitution of wild-type Stat3 expression but not of any of the Stat3 mutants. In addition, our sequential ChIP studies (re-ChIPs) showed that HRGβ1 induced the simultaneous PR and Stat3 occupancy of this bcl-XP4 region (Figure 2B). Besides Stat3 role as a coregulator, the lack of PR recruitment to PREs at bcl-XP4 in cells where Stat3 expression was silenced, could be due to modifications in PR phosphorylation state and/or subcellular localization. To explore this possibility, we assessed HRGβ1 modulation of PR phosphorylation in cells transfected with Stat3 siRNAs. We previously found that HRGβ1 induces PR-B and PR-A phosphorylation on Ser294 in C4HD and T47D cells and that HRGβ1-activated p42/p44 MAPKs mediate said PR phosphorylation (15). It has also been demonstrated that progestin-activated MAPKs via EGFR, drive PR-B phosphorylation on Ser345 in T47D cells (4). Here, we show that HRGβ1 caused Ser345 phosphorylation in T47D cells (Figure 2C). Both residues have been found to mediate PR transcriptional activation (4). Therefore, we focused on these residues whose phosphorylation is induced via HRGβ1/ErbBs signaling (Figure 2C). Knockdown of Stat3 expression did not affect HRGβ1-stimulated phosphorylation of Ser294 or Ser345 in T47D cells (Figure 2C). Besides p42/p44 MAPKs-induced phosphorylation, multiple posttranslational modifications modulate PR localization and transcriptional activity. These modifications include phosphorylations

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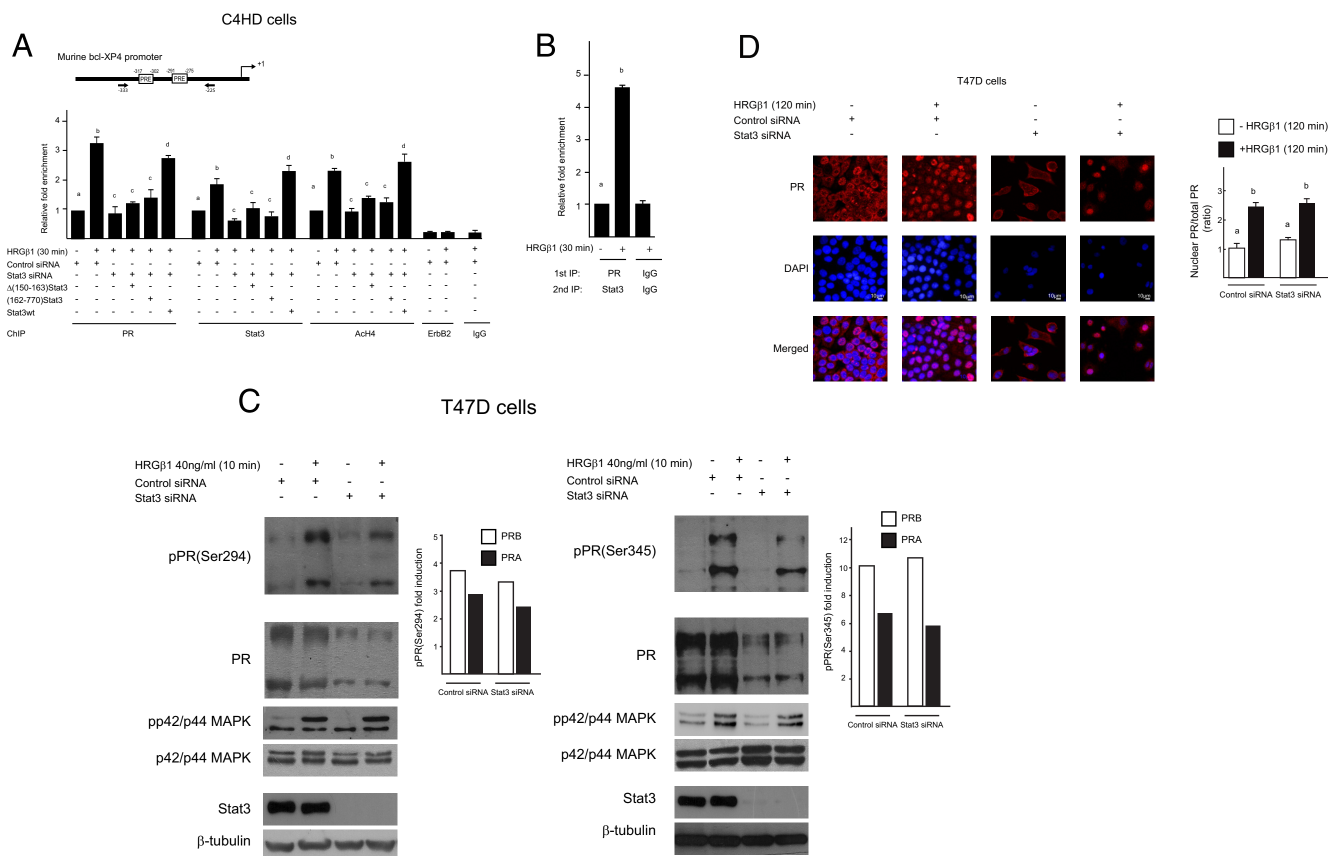

Figure 2. Stat3 acts as a coregulator of PR in bcl-X promoter. A, C4HD cells were transfected with Stat3 or control siRNAs and transfected with Stat3 NLD mutants (162–770)Stat3, Δ(150–163)Stat3, or with a plasmid encoding Stat3wt before HRGβ1 stimulation. Recruitment of PR, Stat3, AcH4, and ErbB2 to the murine bcl-XP4 promoter was analyzed by ChIP. Immunoprecipitated DNA was amplified by qPCR using primers (arrows) flanking the indicated PREs as already described (19). IgG was used as a negative control. 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 fold chromatin enrichment over untreated cells. For b vs a, b vs c, and d vs c, $P < .001$. B, Sequential ChIP. Chromatins from cells treated as described in A were first immunoprecipitated with a PR antibody and were then reimmunoprecipitated using a Stat3 antibody. qPCR and data analysis were performed as detailed in A. Results in A and B are mean ± SEM from 3 independent experiments. For b vs a, $P < .001$, one-way ANOVA. C, T47D cells were transfected with Stat3 or control siRNAs and treated with HRGβ1. WBs were performed with phospho antibodies, and membranes were reprobed with total PR, total p42/p44 MAPK, β-tubulin, and Stat3 antibodies. The bar plot on the right panel of the WBs represents the values obtained after densitometry of HRGβ1-induced pPR(Ser294) and pPR(Ser345) bands and normalization to PR protein bands. D, left panel, T47D cells were treated as indicated, and PR (red) was localized by immunofluorescence (IF) and confocal microscopy. Nuclei were stained with DAPI (blue). Right panel, Quantitative analysis of PR subcellular localization in confocal images of cells treated or untreated with HRGβ1 from the left panel. Fluorescence intensity ratio of nuclear PR vs total PR was calculated for 50 cells from each group. Data are presented as mean ± SD. For b vs a, $P < .001$, one-way ANOVA.

directed by cyclin-dependent kinase (CDK2) and casein kinase 2 (CK2), SUMOylation, and acetylation (33). As a broad readout of a possible perturbation of any of these PR modifications in cells lacking Stat3 expression, which could in turn modify PR actions, we explored PR subcellular localization. Quantitative analysis of PR subcellular localization in confocal images of cells treated or untreated with HRGβ1 showed that HRGβ1 induction of PR nuclear translocation was not affected by knockdown of Stat3 expression (Figure 2D).

Together, our findings point to a role of Stat3 as a PR coregulator. To further investigate this possibility, we used an available genome-wide map of PR in T47D cells treated with progestin (7) in which we explored the enrichment of GAS sites located in the proximity of PR peaks, identified by ChIP-Seq in said study. Our analysis in T47D cells showed that 27% (1711 out of 6289) of the progestin-induced PR peaks containing the PRE motif defined by TRANSFAC also possess a GAS site within 500 bp (Figure 3A). Indeed, analysis of the distances between the PRE and GAS sites showed an increased frequency of GAS sites located within 500 bp relative to the PRE sequence (Figure 3B). We further verified this observation by using Spaced Motif Analysis Tool, which allowed us to find significantly enriched distances between the PRE and GAS sites (Figure 3C). Our findings indicated that there is a significant enrichment of GAS sites located on the opposite strand of the PRE (Figure 3C, left panel, significant enrichment is highlighted in red). Same analysis was performed for the binding motif of the tran-

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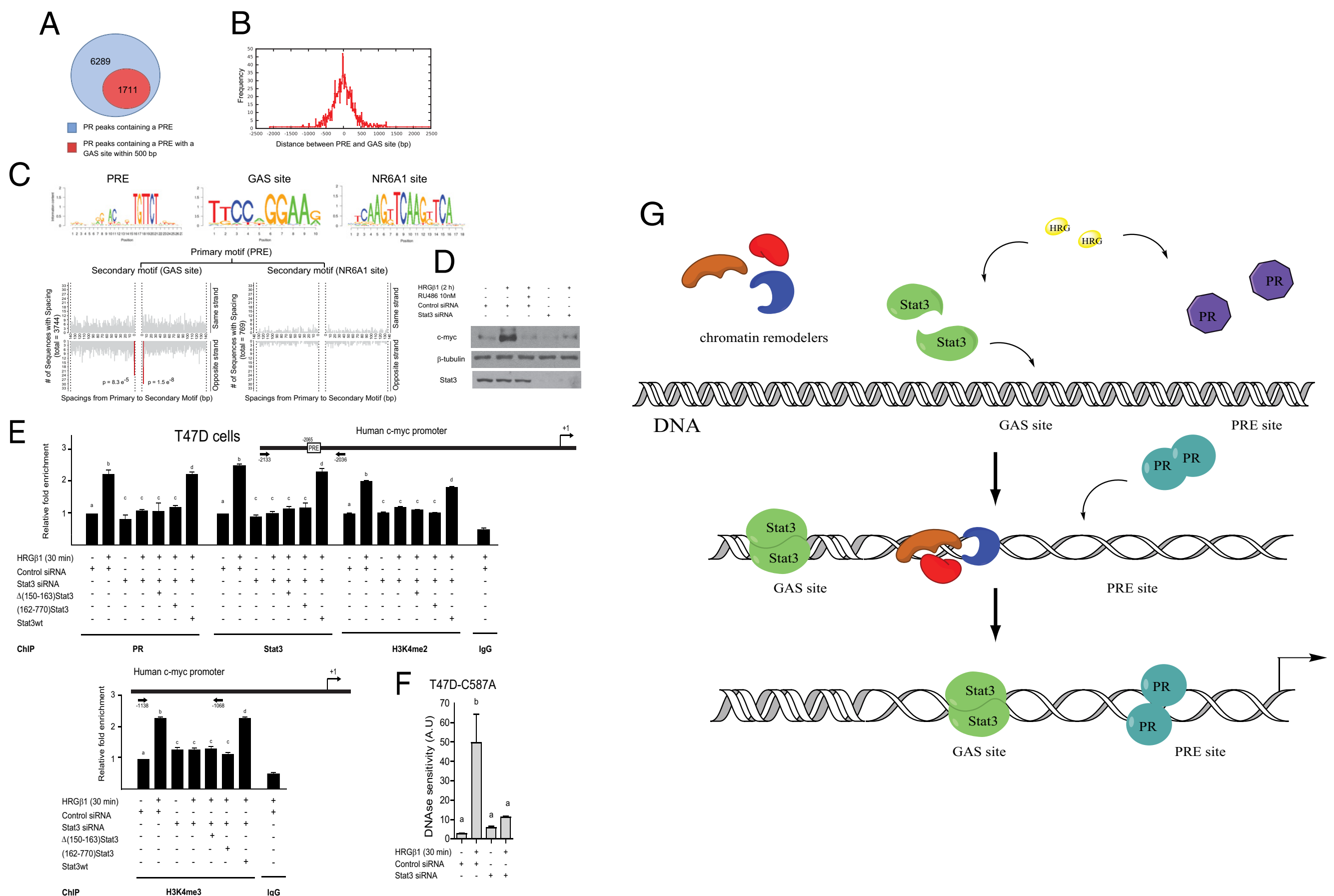

Figure 3. Association of PR and Stat3 binding sites. A, Venn diagram of the PR peaks containing a PRE, and the subgroup of those which also possess a GAS site within 500 bp. B, Analysis of frequency distribution of the distance between PREs closest to ChIP-Seq peaks summits present in biological triplicates and the closest GAS site determined by in silico analysis. C, Association between PRE and GAS sites. Spaced Motif Analysis Tool analysis for enrichment of distances between the closest GAS site and the PRE closest to peak summit. The transcription factor NRSA1 motif was used as a negative control. D, c-myc expression requires PR and Stat3. T47D cells were treated with HRGβ1 or pretreated with RU486 or transfected with Stat3 or control siRNAs before HRGβ1 stimulation. Western blottings were performed with c-myc and Stat3 antibodies, and membranes were reprobed with β-tubulin antibody. E, Nuclear Stat3 is required for PR binding to c-myc promoter. Recruitment of PR, Stat3, H3K4me2, and H3K4me3 to the human c-myc promoter was analyzed by ChIP in T47D cells treated with HRGβ1 for 30 minutes. Immunoprecipitated DNA was amplified by real-time PCR (qPCR) using primers (arrows) flanking the indicated PRE (upper panel) or spanning the proximal promoter region (lower panel). qPCR and data analysis were performed as detailed in Figure 2. For b vs a and d vs c, $P < .001$, one-way ANOVA. The results in this panel are mean ± SEM from 3 independent experiments. F, Stat3 enables chromatin accessibility at c-myc promoter. T47D-C587A cells were transfected with either an unspecific control siRNA or a siRNA targeting Stat3 and treated with HRGβ1 as indicated. DNAse assay was performed as indicated in Materials and Methods. The bars represent the mean and SD of 3 independent experiments (a vs b, $P < .05$, one-way ANOVA). G, Schematic representation of the mechanism proposed for HRGβ1-induced Stat3 and PR functional interaction at the chromatin.

scription factor NR6A1, which contains a similar base proportion and belongs to the nuclear hormone receptor family (34), and no significant enrichment was found (Figure 3C, right panel) supporting the specificity of our analysis. These series of findings provide further evidence of a functional link between PR and Stat3 transcriptional activity.

In the frame of these results, we decided to explore whether Stat3 fulfils a role as a PR coregulator in the transcription of c-myc, a well-acknowledged PR target gene involved in transducing PR proliferative effects in breast cancer (35), whose expression has also been found to be induced by HRGβ1 (36, 37). Functional PREs have been identified in the c-myc promoter (35). Moreover, Stat3 has been reported to modulate c-myc transcriptional activation (38, 39). Here, we found that HRGβ1 induces c-myc protein expression via PR and Stat3 in T47D cells (Figure 3D). Also, using bioinformatics we found a GAS site at −2056 bp relative to c-myc transcriptional start site. Our ChIP analysis in T47D cells using primers spanning the PRE site at position −2065 of the human c-myc promoter revealed that, as in bcl-XP4, HRGβ1 induced recruitment of PR to the c-myc promoter region containing the PRE and knockdown of Stat3 expression abrogated HRGβ1 effects (Figure 3E, left panel). Reconstitution of wild-type Stat3 but not of Δ(150–163)Stat3 or (162–770)Stat3 mutants in T47D, in which we silenced endogenous Stat3, restored HRGβ1 capacity to induce PR loading. We also found that HRGβ1 in-

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duced Stat3 binding to this region of the c-myc promoter (Figure 3E, left panel). As expected due to their inability to translocate to the nuclear compartment, we detected no loading of Stat3 mutant vectors Δ(150–163)Stat3 or (162–770)Stat3, in HRGβ1-stimulated T47D cells. H3K4me is a chromatin signature associated to regulatory elements (40). Although trimethylation (H3K4me3) is a mark of active promoters (41), dimethylation on lysine 4 (H3K4me2) is a hallmark of enhancers (41, 42). It has also been found that binding of Forkhead box protein A1 (FoxA1), a pioneer factor for ER in breast cancer cells, to its dependent enhancers increases H3K4me2. On the other hand, depletion of H3K4me2 decreases the binding to chromatin of FoxA1, as well as of pre-B-cell leukemia homeobox 1 (PBX1), another most recently identified ER pioneer factor (9, 43). Stimulation by HRGβ1 induced H3K4me3 deposition on c-myc proximal promoter (−1138 to −1068 bp) (Figure 3E, right panel). The enhancer-associated epigenetic mark H3K4me2 was also induced by HRGβ1 treatment in the PRE located at −2065 bp of c-myc (Figure 3E, left panel). Knockdown of Stat3 expression inhibited di- and trimethylation of H3K4, which was restored only in the presence of wild-type Stat3 but not in cells transfected with the Stat3 mutants incapable of translocating to the nucleus (Figure 3E). These results suggest that Stat3 is required for the impartment of both epigenetic signatures by HRGβ1 on c-myc gene.

In order to define whether Stat3 facilitated PR engagement to DNA, we assessed chromatin accessibility at the PRE present in the c-myc promoter by using a DNAse I sensitivity assay. This method uses the capacity of the DNAse I enzyme to digest selectively the regions on the DNA associated with a more exposed chromatin structure. For this purpose, we took advantage of the T47D-Y-C587A cell line, which stably expresses a PR harboring a substitution of the cysteine 587 for alanine that renders the receptor unable to bind to DNA or to tether to other transcription factors bound to DNA (44). This particular feature of T47D-Y-C587A cells allowed us to disclose the effects of Stat3 on the chromatin structure at the c-myc PRE site, independently of PR binding at said PRE. The region of c-myc promoter containing the PRE displayed high sensitivity to DNAse I after HRGβ1 treatment (Figure 3F). Silencing of Stat3 decreased HRGβ1-stimulated DNAse I sensitivity, revealing Stat3 capacity to make the chromatin more accessible at this region and therefore to function as a coregulator to promote chromatin accessibility (Figure 3F). Taken together, these results fit into a model where HRGβ1 treatment induces Stat3 binding to a GAS element. We propose that this event is followed by recruitment of chromatin remodelers, which would then enable activated-PR accessibility to chromatin, as depicted in Figure 3G. The MMTV LTR has been widely used as a model system for PR-mediated chromatin remodeling. The studies on the MMTV promoter that have proposed PR primarily functions facilitating the generation of accessible chromatin for binding of auxiliary transcription factors, together with PR capacity to occupy nucleosomal DNA (45, 46), have characterized PR as a pioneer factor in said promoter context. However, specifically for GR-mediated transcription of the MMTV promoter, FoxA1 has been shown to increase accessibility of the promoter without the presence of hormone, facilitating GR binding and glucocorticoid dependent transcription (47). Given the requirement of Stat3 for MMTV transcription by HRGβ1-activated PR (Supplemental Figure 5B), we investigated PR and Stat3 recruitment to said promoter. We performed ChIP assays using T47D-Cat0 cells which derive from the human breast cancer cell line T47D and carry a stably integrated MMTV reporter construct (48). PR and Stat3 binding to the MMTV promoter was detected at 30 minutes after HRGβ1 stimulation (Figure 4A). CREB binding protein (CBP), p300, and AcH4 were also loaded in the presence of HRGβ1 indicating active gene transcription. p300 and CBP recruitment was previously described for progestin-activated PR in T47D-Cat0 cells (49). To further explore whether the regulation of HRGβ1-induced PR transcriptional activation observed in Supplemental Figure 5B could be due to Stat3 acting as a regulator of chromatin openness, we silenced Stat3 expression and performed ChIP assays. Notably, HRGβ1-induced PR loading to the MMTV promoter was independent of Stat3 expression (Figure 4B), which is in accordance with PR acting itself as a pioneer factor in the transcription of the MMTV promoter (24). Indeed, transfection of the Stat3 mutants unable to perform nuclear translocation did not affect HRGβ1-induced PR recruitment (Figure 4B). Remarkably, HRGβ1-stimulated acetylation of histones 3 and 4 was blocked when Stat3 expression was silenced or reconstituted with its mutant counterparts (Figure 4B). These results suggest that Stat3 is dispensable for PR recruitment to DNA but that it is required for PR transcriptional activation. As a matter of fact, PR transcriptional activation on the MMTV promoter is suppressed in the absence of Stat3 (Supplemental Figure 5B). We also observed PR and Stat3 simultaneous binding to the chromatinized MMTV promoter when T47D-Cat0 cells were treated with HRGβ1 (Figure 4C). Taken as a whole, these findings point to Stat3 function as a coactivator in this specific promoter context.

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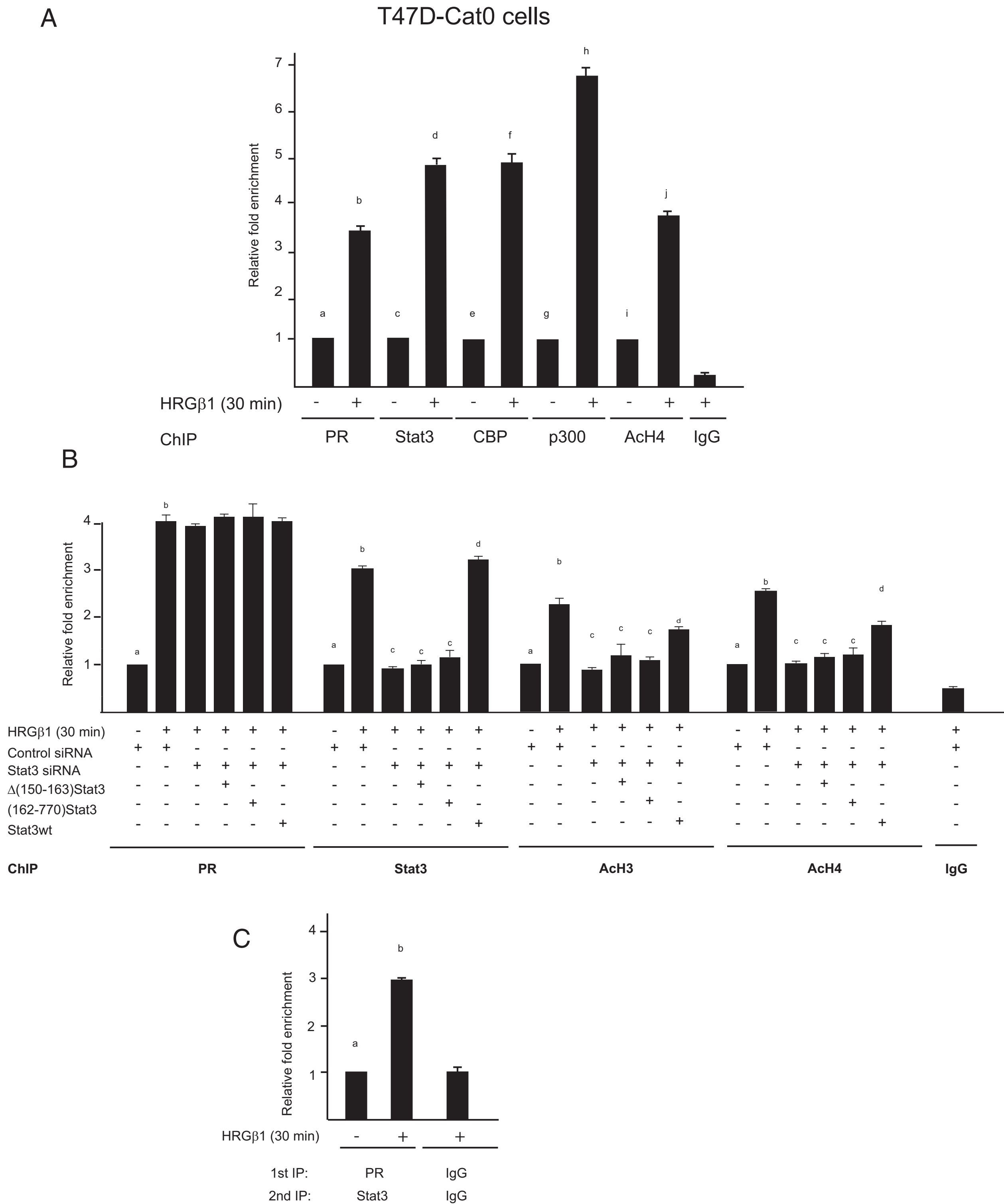

Figure 4. Stat3 is dispensable for HRGβ1-induced PR binding to the MMTV promoter. A, T47D-Cat0 cells were treated with HRGβ1, and recruitment of PR, Stat3, CBP, p300, and H4 acetylation levels (AcH4) to the MMTV promoter was analyzed by ChIP. Immunoprecipitated DNA was amplified by qPCR using primer pairs flanking the PREs of the stably transfected MMTV promoter as described before (19). qPCR and data analysis were performed as detailed above in Figure 2. For b vs a, d vs c, f vs e, h vs g, and j vs i, $P < .001$. B, T47D-Cat0 cells were treated as described in Figure 3, and recruitment of PR, Stat3, AcH3, and AcH4 to the MMTV promoter was analyzed by ChIP. Immunoprecipitated DNA was amplified by qPCR using primer pairs flanking the PREs of the stably transfected MMTV promoter as described before (19). IgG was used as a negative control. qPCR and data analysis were performed as detailed in Figure 3. For b vs a, b vs c, and d vs c, $P < .001$. C, Sequential ChIP. Chromatins from cells treated as described in A were first immunoprecipitated with a PR antibody and were then reimmunoprecipitated using a Stat3 antibody. qPCR and data analysis were performed as detailed in A. For b vs a, $P < .001$, one-way ANOVA. Results in A–C are mean ± SEM from 3 independent experiments.

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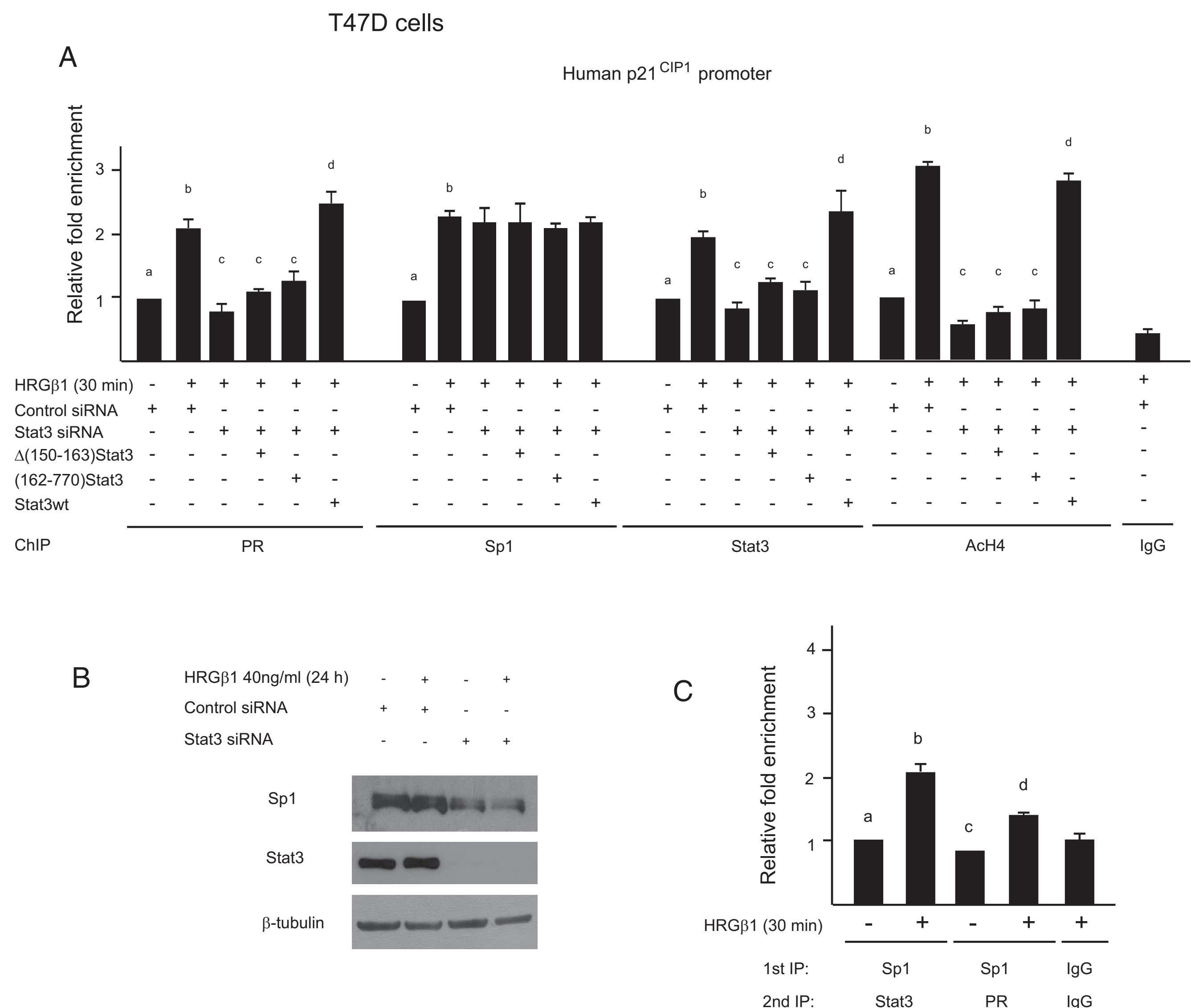

Figure 5. Stat3 acts as a coregulator of PR in its tethering mechanism. A, T47D cells were treated as described in Figure 2 and recruitment of PR, Sp1, Stat3, and AcH4 to the p21^{CIP1} promoter was analyzed by ChIP. Immunoprecipitated DNA was amplified by qPCR using primers as already described (19). IgG was used as a negative control. qPCR and data analysis were performed as detailed in Figure 2. For b vs a, b vs c, and d vs c, $P < .001$, one-way ANOVA. B, T47D cells were treated with HRGβ1 and transfected with Stat3 or control siRNAs before HRGβ1 stimulation. Western blottings were performed with Sp1 and Stat3 antibodies, and membranes were reprobed with β-tubulin antibody. C, Chromatins from cells treated or not with HRGβ1 were first immunoprecipitated with a Sp1 antibody and were then reimmunoprecipitated using either a Stat3 or a PR antibody. qPCR and data analysis were performed as detailed in Figure 2. For b vs a and d vs c, $P < .001$. Results in A and C are mean ± SEM from 3 independent experiments.

Role of Stat3 as a coregulator in the PR tethering mechanism

Progestin-induced expression of the cell cycle molecule p21^{CIP1} occurs via an interaction between PR and Sp1 at Sp1 sites located in the proximal p21^{CIP1} promoter (21). Interestingly, we have shown in Supplemental Figure 5C that both PR and an intact Sp1 binding site are required for HRGβ1 transcriptional regulation of p21^{CIP1}, suggesting that HRGβ1-activated PR could be modulating p21^{CIP1} transcription through a nonclassical tethering mechanism. In addition, we have also found that Stat3 is also a requisite for HRGβ1-regulation of p21^{CIP1} transcription and expression (Figure 1, A–C). The latter results prompted us to assess whether Stat3 was also regulating the recruitment of HRGβ1-activated PR to p21^{CIP1} promoter, as detected in bcl-XP4 and c-myc promoters. By performing ChIP assays using T47D cells we found that Sp1 was enriched at the proximal Sp1 elements of the p21^{CIP1} promoter in HRGβ1-treated cells (Figure 5A) and that PR was also significantly bound in this condition. As regards Stat3, we have previously shown that, in T47D breast cancer cells, the synthetic progestin medroxyprogesterone acetate induced Stat3 recruitment to the promoter region spanning the Sp1 binding site of

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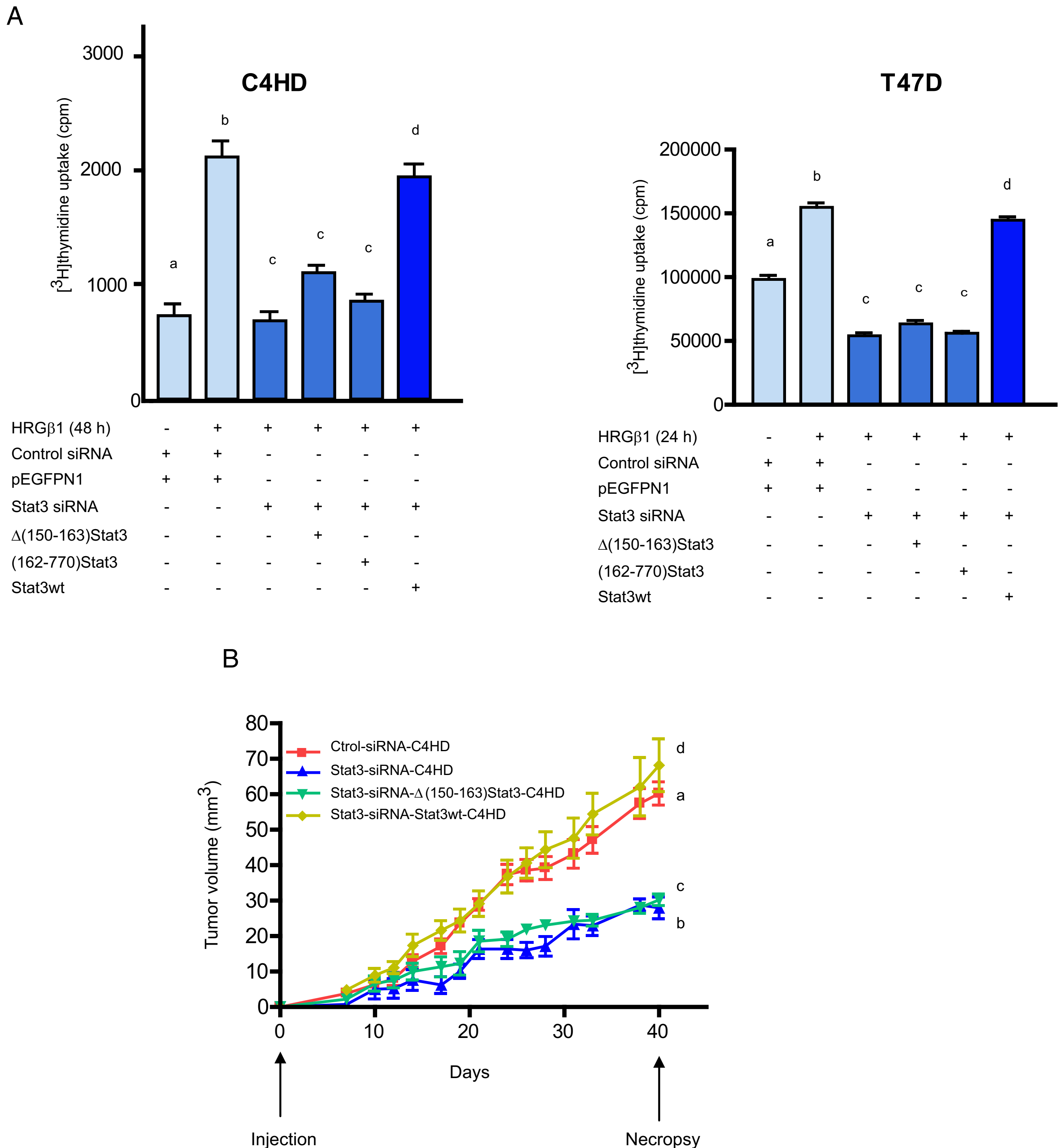

Figure 6. Stat3 nuclear signaling is essential in in vitro and in vivo HRGβ1-induced breast cancer cell proliferation. A, Endogenous Stat3 expression was silenced by transfection with siRNAs and expressions of (162–770)Stat3, Δ(150–162)Stat3, or Stat3wt were restored by cotransfection with the respective plasmids. C4HD (left panel) and T47D (right panel) cells were treated with HRGβ1 for 48 and 24 hours, respectively, and the incorporation of [^{3}H]thymidine was used as a measure of DNA synthesis. Data are presented as means ± SD ($P < .001$ for b vs a, b vs c, and d vs c). B, Cells (10^6) from each experimental group were inoculated sc into mice treated with HRGβ1, and tumor volume was calculated as described in Materials and Methods. Each point represents the mean volume ± SEM of 7 independent tumors for all experimental groups ($P < .001$ for a vs b, a vs c, and d vs b).

p21^{CIP1} promoter (19). Notably, HRGβ1 also stimulated a significant association of Stat3 to the Sp1 binding site in the p21^{CIP1} promoter (Figure 5A). When Stat3 expression was abolished using Stat3 siRNAs, HRGβ1 was unable to induce PR recruitment to the p21^{CIP1} promoter (Figure 5A). Reconstitution of the cytoplasmic function of Stat3

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by transfection with the mutant vectors Δ(150–163)Stat3 or (162–770)Stat3 did not rescue HRGβ1 capacity to induce PR binding to $p21^{CIP1}$ promoter either (Figure 5A). However, when cells were transfected with a wild-type Stat3 protein, PR associated to the Sp1 binding site in $p21^{CIP1}$ promoter upon HRGβ1 treatment and Stat3 loading was also significantly induced (Figure 5A). Although knockdown of Stat3 expression caused decreased levels of Sp1 expression (Figure 5B), Sp1 binding to its responsive element was unaffected in this condition (Figure 5A). This finding favors for the notion of PR-decreased binding to DNA (Figure 4A) due to Stat3-specific function as a PR coregulator licensing chromatin accessibility and not due to its reduced expression levels in the presence of Stat3 siRNAs (Figure 1A). Histone H4 acetylation was induced by HRGβ1 in $p21^{CIP1}$ promoter, was inhibited by the presence of Stat3 siRNAs and was restored by HRGβ1 treatment only in the presence of a wild-type Stat3 protein (Figure 5A). We next sought to examine whether PR, Sp1, and Stat3 were interacting simultaneously on the same Sp1 element of the proximal $p21^{CIP1}$ promoter, for which we performed sequential ChIP experiments. Chromatin derived from either HRGβ1 treated or untreated cells was immunoprecipitated with Sp1 antibodies followed by reimmunoprecipitation with Stat3 antibodies. The results shown in Figure 5C reveal that in the presence of HRGβ1, Stat3, and Sp1 simultaneously occupy the Sp1 element in the proximal $p21^{CIP1}$ promoter. Sequential ChIPs also evidence that HRGβ1-activated PR cobound with Sp1 to the Sp1 binding (Figure 5C). Together, these data indicate that Stat3 determines HRGβ1-activated PR tethering to Sp1, but it does not determine Sp1 recruitment to its binding site.

Stat3 nuclear signaling drives breast tumor growth induced by HRGβ1

We previously found that Stat3 was an absolute requirement for HRGβ1-induced proliferation of breast cancer cells (17). Because Stat3 function as a transcription factor is responsible for HRGβ1-induced regulation of $Bcl\text{-}X_L$ and $p21^{CIP1}$ (Figure 1F) we studied the relevance of Stat3 nuclear function in HRGβ1-induced breast cancer cell proliferation. As we have previously reported (17), HRGβ1 was able to induce a potent proliferative response on C4HD cells which was significantly inhibited when Stat3 expression was silenced with Stat3 siRNAs (Figure 6A, left panel). Cotransfection of Δ(150–163)Stat3 was not able to rescue HRGβ1-induced proliferation of C4HD cells (Figure 6A, left panel). Likewise, cotransfection of (162–770)Stat3 was not effective in reconstituting HRGβ1-induced proliferation (Figure 6A, left panel). Cotransfection of a wild-type Stat3 protein successfully enabled reconstitution of HRGβ1 effect in the proliferation of C4HD cells (Figure 6A, left panel). These results suggest that the nuclear function of Stat3 mediates HRGβ1-induced Stat3 regulation of in vitro breast cancer cell proliferation. Similar to C4HD cells, when T47D cells were treated with HRGβ1 for 24 hours (Figure 6A, right panel) we observed that both mutants were ineffective in rescuing HRGβ1-induced proliferation and transfection of the wild-type Stat3 protein restored HRGβ1 effect on proliferation. We then developed a preclinical model to address the effect of blockage of the nuclear function of Stat3 in HRGβ1-induced in vivo breast tumor growth. In this study, control-siRNA, Stat3-siRNA, Stat3-siRNA-Δ(150–163)Stat3, and Stat3-siRNA-Stat3wt C4HD cells were inoculated sc into mice treated with HRGβ1. Knockdown of Stat3 expression significantly inhibited HRGβ1-induced tumor growth (Figure 6B). Transfection of the Δ(150–163)Stat3 mutant did not rescue HRGβ1 ability to promote breast tumor growth, suggesting that Stat3 nuclear function is essential in HRGβ1-induced proliferation (Figure 6B). In accordance with the in vitro results observed in Figure 6A, transfection of the wild-type Stat3 protein completely restored HRGβ1-induced tumor growth (Figure 6B) indicating that the nuclear function of Stat3 is required for HRGβ1-induced tumor growth.

Taken together, our findings show that Stat3 functions as a coregulator of chromatin accessibility of HRGβ1-activated PR in the transcription of bcl-X, c-myc, and $p21^{CIP1}$ genes while acting as a coactivator in the transcription of the MMTV promoter.

Discussion

We have formerly shown that HRGβ1 treatment of C4HD and T47D breast cancer cells induces Stat3 tyrosine phosphorylation and transcriptional activation (17). Our present findings reveal that Stat3 plays an essential role in PR transcriptional activity by HRGβ1, suggesting that the direct transcriptional action of PR requires earlier events initiated by PR activation of cytoplasmic signaling. Given our previous demonstration of PR participation in Stat3 phosphorylation by HRGβ1 (17), the present result indicates that activated Stat3 is integrating the 2 modes of action of PR, PR rapid signaling and PR transcriptional effects. PR-initiated cytoplasmic signaling induces Stat3 activation, which modulates PR transcriptional activation. This pathway (PR-induced rapid Stat3 activation) provides a feed-forward regulatory loop which involves Stat3 recruitment in PR-regulated transcription. Another evidence of said feed-forward mechanism lies in PR and Stat3 coordinated regulation of protein ex-

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pression (Figure 1A). Stat3 is acting as a downstream effector of activated-PR to induce the transcription of bcl-X, c-myc, and $p21^{CIP}$ genes, important regulators of proliferation and apoptosis.

HRGβ1 induction of $Bcl\text{-}X_L$ through the ErbBs receptors has already been reported in cardiomyocytes (50) and in neurons (51). Our present findings demonstrate for the first time that HRGβ1 induces $Bcl\text{-}X_L$ in breast cancer cells through a mechanism requiring ErbB2, Stat3, and PR. The nuclear function of ErbB2 was not involved in HRGβ1 effect, because transfection of an ErbB2 mutant, which lacks the putative nuclear localization signal sequence, did not prevent $Bcl\text{-}X_L$ induction by HRGβ1. NErbB2 did not participate in HRGβ1-induced $p21^{CIP1}$ expression either. Interestingly, we previously found that medroxyprogesterone acetate-activated PR did assemble with ErbB2 and Stat3 at a GAS site in $p21^{CIP1}$ proximal promoter (52). The nature of PR activation (whether it is by progestins or whether it undergoes ligand-independent activation by growth factors, such as HRGβ1) could account for this difference. This is likely due to the differential availability for recruitment to the chromatin of proteins rapidly activated by either progestin or HRGβ1. Alternatively, the concerning responsive elements in DNA (PRE in the present work vs GAS in Ref. 52) may display differential chromatin structure. Indeed, chromatin landscape has been found to determine the accessibility of transcription factors to the DNA. The same arguments may explain the difference between the present findings compared with our previous work showing PR and ErbB2 acting as cofactors of Stat3 bound to its GAS at the cyclin D1 proximal promoter (3).

Stats have already been implicated in the regulation of human bcl-X gene transcription (53): Stat5 binds to bcl-X promoter in response to erythropoietin in erythroleukemia cells (54), Stat1 mediates the expression of the bcl-X gene upon LIF treatment of cardiac myoblasts (55), and Stat3 participation in $Bcl\text{-}X_L$ dysregulation is well acknowledged (56). However, no functional Stat3 binding sites were reported in the bcl-X proximal promoter. We have here reported that HRGβ1-activated Stat3 regulation on bcl-X transcription occurs via an intact PRE (Supplemental Figure 5A). Recently, ligand-activated PR was shown to regulate the transcription of human bcl-X by modulating the RNA polymerase II elongation process through binding to intragenic PREs (57). It remains to be determined whether unliganded PR exerts the latter regulation in the transcription of the murine bcl-X gene in spite of the divergence of the intronic sequences between both species.

A series of previous findings, and ours as well, has addressed the role of Stat3 as a modulator of the activity of the steroid hormone receptors glucocorticoid (58), androgen (59), estrogen (60), and progesterone (19). However, this is the first report of Stat3 regulation of a steroid hormone receptor which has been activated in a ligand-independent manner. Ligand-independent PR actions include scaffolding of growth factor pathways to enhance kinase signaling (17) and activation of progrowth and prosurvival transcriptional programs in breast cancer cells (61, 62). Recent reports have also implicated ligand-independent actions of PR in maintaining a basal repressive state in 20% of the hormone-inducible genes, keeping them silenced before hormone treatment (63) and in enhancing the proliferative responses to estradiol and IGF-1 via the assembly of a molecular scaffold composed by ER-α, the proline-, glutamate-, and leucine-rich protein 1 and insulin-like growth factor 1 receptor (IGF-1R) (8). The clarification of the coactivators and transcriptional complexes recruited by ligand independent-activated PR could provide tools for the design of alternative targets to inhibit PR-mediated transcription.

Pioneer factors are known for their capacity to open condensed chromatin, enabling subsequent binding of other transcription factors (22, 64). Based on the capacity of agonist-bound PR and GR to bind nucleosomal DNA, both were characterized as pioneering factors. However, the development of genome-wide techniques showed that after ligand activation, the vast majority of GR occupancy sites were targeted to preexisting remodeled chromatin, detected as DNAse I hypersensitive sites (11) raising the possibility that pioneer factors make chromatin accessible for steroid hormone receptors. Indeed, for ER and GR, nucleosome-depleted chromatin landscapes were found to be opened by pioneer factors such as FoxA1 and activator protein 1 (AP-1) (65, 66) which bound to chromatin regions carrying specific epigenetic modifications. Pioneer factors involvement in PR function remains, however, poorly known. Recently, Ballaré et al (24) showed that in T47D cells stably expressing the MMTV promoter, PR binds genomic regions exhibiting high sensitivity to DNAse I before stimulation with progestin, which increases after hormone stimulation due to PR action. A most exciting finding of this work was that these DNAse I hypersensitive sites exhibited high nucleosome presence and that progestin treatment results in remodeling of these nucleosomes, confirming the ability of PR to bind and remodel chromatin, thus acting as pioneer factor. This view favors for PR notion as a pioneer factor. The difference between the latter work and our current findings might reside in the stimulus used for PR activation, indeed, the present study focuses on HRGβ1 effects on PR function which may implicate differential PR behavior.

Interestingly, a member of the Stat family, Stat5, was found to act as a PR cofactor required for induction of the

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progestin-regulated gene receptor activator of nuclear factor kappa-B ligand (RANKL) in primary mouse mammary epithelial cells (67). The latter study suggests that Stat5a acts, at least in part, to facilitate binding of PR to specific enhancer sites at RANKL. Stat5a was also involved in the regulation of the PR target gene, 11β-hydroxysteroid dehydrogenase type 2, where PR tethered through Stat5a bound to distal regions of said promoter (68). In this study we evidence Stat3 requirement for HRGβ1-activated PR binding to DNA in bcl-XP4, p21^{CIP1}, and c-myc promoters. Interestingly, Stat3 was dispensable for PR recruitment to the MMTV promoter but was still necessary for MMTV transcriptional activation. These results suggest that Stat3 modifies gene transcription acting at 2 different levels and depending on the promoter context. On the one hand, Stat3 might enable PR accessibility to DNA and on the other it modifies transcriptional activation of DNA-bound PR. The former function may confer Stat3 a novel functional role as a potential pioneer factor for HRGβ1-activated PR, where Stat3 could be converting compacted into open chromatin making it permissive for the binding of another transcription factor (22, 64), such as PR. In addition, we have found an increased frequency of Stat3 binding sites (GAS sites) located within 500 bp relative to the PR-bound peaks (Figure 3B), which suggests that Stat3 could be guiding PR recruitment to the chromatin at the PRE sites. Preliminary data suggest that Stat5, another member of the Stat family of transcription factors, may be a putative pioneer factor for activated-PR in human breast cancer (33) and in normal mammary murine cells (67). As in our study, it has been revealed that there is significant enrichment of Stat5 consensus sites within PR-bound chromatin regions by performing an in silico analysis of a publicly available PR whole genome ChIP dataset (33).

Pioneer factors display passive and active roles. In the former, they perform previous binding to chromatin thereby reducing the number of additional factors that are needed to bind at a later time in order to create an active enhancer (12). In the latter, pioneer factors can directly facilitate other factors binding to nucleosomal DNA or open up the local chromatin facilitating other factors to bind (69). Given that Stat3 recruitment to DNA was not evident in untreated cells (Figures 2–5), we hypothesize that Stat3 might be actively participating in HRGβ1-activated PR loading to DNA by either opening the chromatin locally, positioning nucleosomes, enabling cooperative binding among other DNA-binding factors, or directly recruiting other chromatin remodelers which may in turn act as coactivators. Further research is required to discern Stat3 implication in these functions.

The differential function of Stat3 as a coactivator or as a potential pioneer factor may result in dissimilar postranslational modifications on PR. Certainly, PR modifications dramatically alter PR function, receptor localization and turnover and promoter selectivity, the latter being especially relevant in the context of the present work. In addition, it would be interesting to study the partners of HRGβ1-activated PR when bound to MMTV or to bcl-XP4 and c-myc, because they dictate, at least in part, promoter selectivity (61, 62). It remains to be determined whether additional DNA-binding transcription factors besides Stat3 are likely to be required to prime chromatin for recruitment of HRGβ1-activated PR in the promoters studied. Moreover, ChIP-Seq experiments to identify genomic patterns of HRGβ1-activated PR and Stat3 binding sites will be needed to determine the extent of PR-Stat3 interaction with other PR target genes. The strength of neighboring PREs and GAS sites may also contribute to determine whether Stat3 would function as a putative pioneer factor or as a coactivator, such that composite response elements composed of weak PREs neighboring GAS elements may require the pioneering factor activity of Stat3 whereas strong PRE sites may directly recruit PR to nucleosome-containing chromatin. Additionally, Stat3 may exert its pioneering activity in a selective manner depending on the specific chromatin compaction and epigenetic modifications present in each promoter.

In the present work, we have shown that the absence of Stat3 reduced PR protein levels (Figure 1A) did not modify HRGβ1-induced phosphorylation on residues 294 and 345 (Figure 2C) nor PR subcellular localization (Figure 2D). We therefore concluded that the pool of PR available to perform its function as a transcription factor is not essentially different as in the presence of Stat3. Assessment of other PR postranslational modifications such as acetylation, SUMOylation and ubiquitination both in presence and absence of HRGβ1-activated Stat3 would provide additional data on whether Stat3 is involved in the regulation of any of these modifications on PR, which might affect PR capacity to bind DNA and regulate transcription.

We have previously found that Stat3 acts as a coactivator of progestin-activated PR in the transcription of bcl-X and p21^{CIP1} (19). Interestingly, in the present work we found that ligand independently activated PR also couples with Stat3 to regulate bcl-X and p21^{CIP1} transcription. This result has therapeutic implications, because they indicate that in a PR-positive breast tumor in the absence of progestin or in the presence of low or subthreshold progestin concentrations, where unliganded PR-mediated transcription becomes important, that blockage of Stat3 results in the inhibition of PR function in the transcription of bcl-X and p21^{CIP1} and conse-

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quently, in the inhibition of cell growth. Furthermore, these results expand our knowledge on the elusive mechanisms of unliganded PR-mediated transcription. Finally, we found that Stat3 is a key player in HRGβ1-induced C4HD and T47D cell growth. Indeed, the nuclear function of Stat3 was shown to be essential for HRGβ1 regulation of tumor growth, underscoring the contribution of Stat3 as a transcriptional regulator over its function as a signaling molecule in the cytoplasm. These findings, together with previous in vitro results from our group, provide the first evidence that Stat3 participates in in vitro and in vivo HRGβ1-induced breast cancer growth, and are in line with the accumulating evidence demonstrating the involvement of Stat3 in the proliferation of breast cancer cells (70). The data here presented identifies a potential therapeutic intervention for PR-positive breast tumors consisting of targeting Stat3 function or PR/Stat3 interaction which will result in the inhibition of PR function.

Materials and Methods

Animals and tumors

Experiments were carried out with virgin female BALB/c mice raised at the Institute of Biology and Experimental Medicine of Buenos Aires. Animal studies were conducted as described previously (18) and in accordance with the highest standards of animal care as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institute of Biology and Experimental Medicine Animal Research Committee. The C4HD tumor line is of ductal origin, displays high levels of estrogen receptor and PR, overexpresses ErbB2 and ErbB3, exhibits low ErbB4 levels, and lacks EGF-R expression (71). This tumor line does not express glucocorticoid receptor or androgen receptor (71).

Cell cultures, treatments, and proliferation assays

Cell cultures, treatments and proliferations assays procedures are described under Supplemental Materials and Methods.

Antibodies and reagents

Antibodies and reagents used are detailed under Supplemental Materials and Methods.

Western blottings

Lysates were prepared from cells subjected to the different treatments, and proteins were subjected to SDS-PAGE as previously described (71). Membranes were immunoblotted with the antibodies detailed in each experiment. When phosphoprotein antibodies were used, filters were reprobed with total protein antibodies. Signal intensities of phospho-ErbB2 (pErbB2) bands were analyzed by densitometry and normalized to total protein bands. Similarly, signal intensities of Bcl-X_L, p21^{CIP1}, PR, Stat3, ErbB2, and green fluorescent protein (GFP) bands were normalized to β-tubulin bands. Data analysis showed a significant increase in pErbB2 levels by HRGβ1 treatment in comparison with untreated cells and a significant inhibition of HRGβ1-induced protein phosphorylation when using the pharmacological inhibitor of ErbB2 ($P < .001$). A similar data analysis showed that compared with control cells, the increase in Bcl-X_L and p21^{CIP1} levels by HRGβ1 treatment was significant, as was the inhibition of HRGβ1 effects by RU486, AG825, and Stat3 siRNAs ($P < .001$).

Plasmids and transient transfections

Plasmid sequences are detailed under Supplemental Materials and Methods. In experiments assessing HRGβ1 capacity to induce the transcriptional activation of PR, C4HD and T47D cells were transiently transfected for 48 hours with 500 ng of bcl-XP4-luc reporter plasmid or the deleted bcl-XP4ΔHRE-luc construct, or with 500 ng of p21P93-S-Luc or p21P93-S-Luc-mut and 10 ng of RL-CMV used to correct variations in transfection efficiency. As control, cells were transfected with 500 ng of either the empty vector pRc/CMV or pTATA-tk-Luc reporters. Cells were then starved for 24 hours and treated with HRGβ1 during 18 hours, or were left untreated. The FuGENE HD transfection reagent technique (Roche Biochemicals) was performed as described (71). Transfection efficiencies, evaluated using the pEGFP-N1 vector and determined by the percentage of cells that exhibited GFP 4 days after transfection, varied between 60% and 70%. Transfected cells were lysed and luciferase assays were carried out using the Dual-Luciferase Reporter Assay System (Promega) in accordance with manufacturer's instructions. Triplicate samples were analyzed for each datum point. Differences between experimental groups were analyzed by ANOVA followed by a Tukey test between groups.

siRNA transfections

siRNA sequences are detailed under Supplemental Materials and Methods. The transfection of siRNA duplexes was performed for 3 days by using DharmaFECT transfection reagent according to the manufacturer's directions. For reconstitution experiments in C4HD and T47D cells, the cotransfection of 50nM and 15nM Stat3 siRNA, respectively, with 2 μg of Stat3 expression vectors was performed by using DharmaFECT and FuGENE HD transfection reagents.

Immunofluorescence and confocal microscopy in cell cultures

ErbB2 was localized using a rabbit polyclonal (C-18) antibody (Santa Cruz Biotechnology, Inc). PR was localized using antibody anti-PR (clone hPRa7) (Neomarkers). Secondary antibodies were, for ErbB2, goat antirabbit IgG-rhodamine (Jackson ImmunoResearch) and donkey antimouse IgG Alexa Fluor 546 (Life Technologies) for PR. Negative controls were carried out using PBS instead of primary antibodies, or 5X competitive peptide (Santa Cruz Biotechnology, Inc) when ErbB2 was used. When cells were transfected with hErbB2ΔNLS, Stat3wt, (162–770)Stat3, or Δ(150–163)Stat3, GFP from these expression vectors was visualized by direct fluorescence imaging and nuclei were stained with propidium iodide (5 μg/mL). A detailed description of quantitative analysis of confocal images is provided in Supplemental Materials and Methods.

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ChIP and sequential ChIP assays

ChIP was performed as described elsewhere (71). Briefly, chromatin was sonicated to an average of about 500 bp. Sonicated chromatin was then immunoprecipitated using 4 μg of the indicated antibodies and rabbit IgG as control. The immunoprecipitate was collected using Protein A beads (Millipore), which were washed repeatedly to remove nonspecific DNA binding. Chromatin was eluted from the beads and cross-links were removed overnight at 65°C. DNA was then purified and quantified using real-time PCR. For sequential ChIP experiments, chromatin immunoprecipitates were eluted with dithiothreitol (DTT) and then subjected to a second round of immunoprecipitation with the indicated antibodies or with IgG.

RNA preparation and real-time quantitative RT-PCR

Total RNA was obtained and the P4 5′-leading exon mRNA levels were detected as we already described (19). Primers used are referred in Supplemental Materials and Methods.

Real-time quantitative PCR (qPCR)

ChIP DNA was amplified by real-time qPCR, performed with an ABI Prism 7500 sequence detector using SYBR green PCR master mix (Applied Biosystems). The primers designed to amplify the region of the murine bcl-XP4 promoter containing 2 PREs and the primers designed to amplify the integrated MMTV promoter were described before (19). Primers specific to the human $p21^{CIP1}$ promoter used to amplify the region containing the Sp1 response element were designed with Primer Express real-time PCR primer design software (Applied Biosystems) and were as follows: 5′CGCGAGGATGCGTGTTC3′ and 5′CATTCACCTGCCGCAGAAA3′. PCR was performed for 40 cycles with 15 seconds of denaturing at 95°C and annealing and extension at 60°C for 1 minute.

HRGβ1 pellet preparation and implantation

HRGβ1 pellet preparation and implantation is referred in Supplemental Materials and Methods.

In vivo inhibition of Stat3 expression

C4HD cells were transiently transfected with the siRNAs and expression vectors detailed in the plasmids and transient transfections section. The HRGβ1 inoculation and the analysis of the tumor parameters are detailed under Supplemental Materials and Methods.

DNAse I sensitivity assay

Cells were harvested in lysis buffer (10mM Tris-Cl [pH 7.5], 10mM NaCl, 3mM MgCl2, and 0.05% NP40) and incubated in ice for 10 minutes. Nuclei were isolated by centrifugation at 2000 rpm for 5 minutes at 4°C. Pellet was washed with digestion buffer (40mM Tris-Cl [pH 7.5], 10mM $MgCl_2$, and 1mM $CaCl_2$) and centrifuged at 2000 rpm for 5 minutes at 4°C. Pellets were resuspended in 200 μL, and each treatment was divided in 2 tubes (cut and uncut as input control). Samples were incubated or not with DNAse I (QR1; Promega) for 5 minutes at 37°C. Reaction was stopped by adding 50 μg of Proteinase K and incubating at 65°C for 2 hours. DNA was isolated by phenol extraction and ethanol precipitation. Sensitivity was measured by performing qPCR of 100 ng of DNA and calculated as $2^{\wedge(Ctcut\ -\ Ctuncut)}$.

Bioinformatic analysis

The description of the bioinformatic analysis is detailed under Supplemental Materials and Methods.

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