Genomic Antagonism between Retinoic Acid and Estrogen Signaling in Breast Cancer

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SUMMARY

Retinoic acid (RA) triggers antiproliferative effects in tumor cells, and therefore RA and its synthetic analogs have great potential as anticarcinogenic agents. Retinoic acid receptors (RARs) mediate RA effects by directly regulating gene expression. To define the genetic network regulated by RARs in breast cancer, we identified RAR genomic targets using chromatin immunoprecipitation and expression analysis. We found that RAR binding throughout the genome is highly coincident with estrogen receptor α (ERα) binding, resulting in a widespread crosstalk of RA and estrogen signaling to antagonistically regulate breast cancer-associated genes. ERα- and RAR-binding sites appear to be coevolved on a large scale throughout the human genome, often resulting in competitive binding activity at nearby or overlapping cis-regulatory elements. The highly coordinated intersection between these two critical nuclear hormone receptor signaling pathways provides a global mechanism for balancing gene expression output via local regulatory interactions dispersed throughout the genome.

INTRODUCTION

Retinoic acid (RA) plays a major role in physiological processes ranging from embryonic development to homeostasis of adult tissues and organs (Niederreither and Dolle, 2008). Importantly, RA inhibits the growth and survival of cancer cells at pharmacological doses. The potent anticarcinogenic activity of RA is generally thought to result from direct and indirect effects on gene expression. Therefore a comprehensive analysis of the genomic targets of RA action should provide a better understanding of the mechanism of RA action in the prevention and treatment of cancer, as well as providing a framework that can be extended to other RA functions in organ development and homeostasis.

Previous work has identified two subfamilies of nuclear receptors as major mediators of RA signaling, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Evans, 1988; Giguere et al., 1987; Mangelsdorf and Evans, 1995; Petkovich et al., 1987). RARs dimerize with RXRs; the resulting heterodimers function as transcription factors, thereby eliciting the transcriptional effects of RA signaling. However, little is known about the genomic targets and effects of the different isoforms of the RARs. There is similarly scant information about the mechanism or extent of crosstalk between RA signaling and other nuclear hormone signaling pathways in a cellular context.

In breast cancer cells, RA and retinoids have been previously shown to be associated with downregulation of several genes essential for proliferation and survival (Liu et al., 1998; Zhou et al., 1997). However, it has been unclear if such genes are directly or indirectly regulated by RARs since liganded RAR-RXR heterodimers are thought to function primarily as transcriptional activators while repression is thought to be primarily mediated by unliganded heterodimers interfering with basal transcription (Chambon, 1996; Glass and Rosenfeld, 2000; Hu and Lazar, 2000; Niederreither and Dolle, 2008). Also, RA-induced apoptosis in breast cancer cells has been shown to be associated with upregulated expression of a handful of proapoptotic genes (Donato and Noy, 2005; Donato et al., 2007). However, although several genes implicated in the negative regulation of breast cancer cell proliferation and survival have been identified as RA responsive, the knowledge of the mechanism of transcriptional regulation by RARs is fragmentary. Whether and how RA signaling intersects with estrogen signaling, which promotes proliferation, has not been investigated on a genomic scale.

We therefore analyzed the genomic actions of RA through RARα and RARγ, which exert antiproliferative and apoptotic effects of RA in the breast cancer cell line MCF-7. MCF-7 is the most commonly used experimental system for the study of estrogen receptor α (ERα)-positive breast tumors, affording us the opportunity to compare the genomic effects of RA and estrogen signaling (Levenson and Jordan, 1997). Estrogen signaling, in contrast to RA signaling, drives proliferation and promotes survival and has been extensively studied in this cell line. To uncover the transcriptional networks of RARα and RARγ, we have integrated genome-wide binding site mapping with gene expression profiling. We found that RA signaling regulates the expression of many genes that have been implicated in breast carcinogenesis and/or whose expression is indicative for the clinical outcome of breast cancer. Interestingly, we found
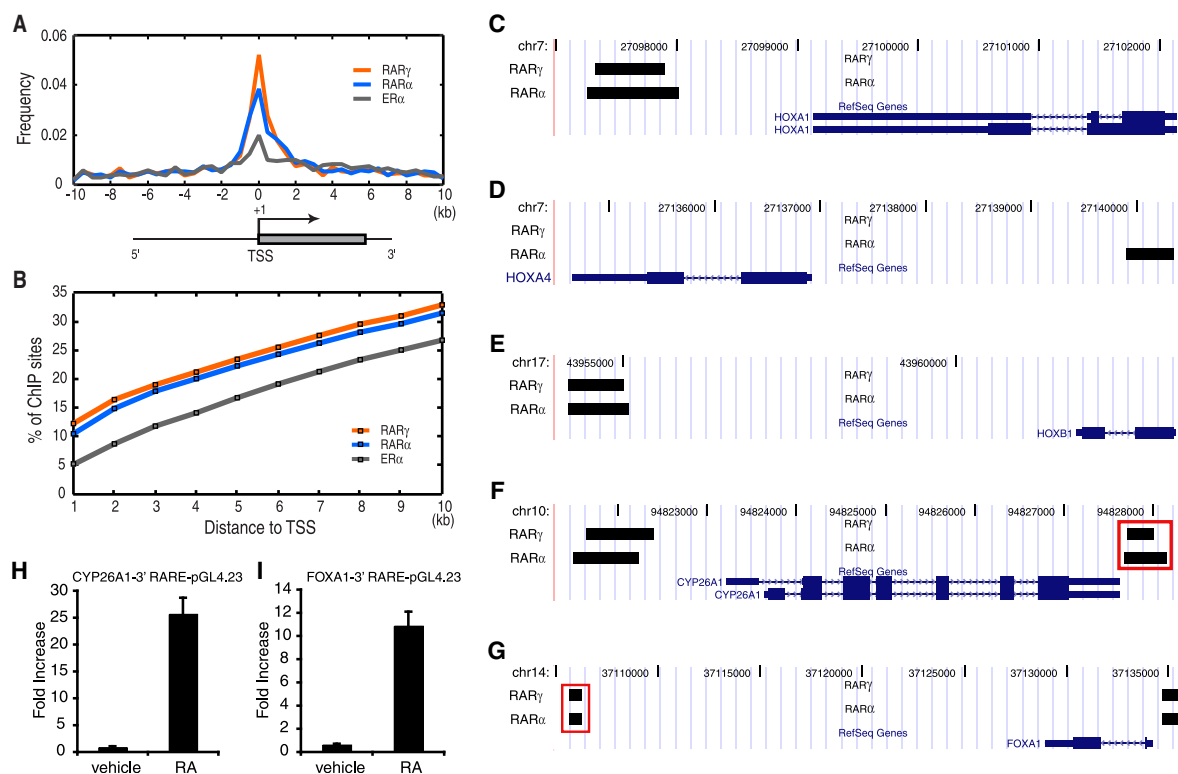
Figure 1. Genome-wide Identification of RARγ- and RARα-Binding Sites in MCF-7 Cells
(A) Distribution of RARγ- RARα-, and ERα-binding sites residing within 10 kb upstream or downstream to annotated transcription start sites (TSSs).
(B) Cumulative frequency of RARγ- and RARα-binding sites for each 1 kb interval within 10 kb upstream or downstream to known TSSs.
(C–G) Known RAR-binding sites identified by ChIP-chip analyses. Black bars depict binding regions for RARγ and RARα. Known promoter-proximal RAR-binding sites for *HOXA1*, *HOXA4*, *HOXB1*, *CYP26A1*, and *FOXA1* were identified by genome-wide mapping in MCF-7. In addition, novel RAR-binding sites 3′ to *FOXA1* (F) and *CYP26A1* (G) (denoted by red rectangles) were identified.
(H and I) Novel binding regions for *CYP26A1* (H) and *FOXA1* (I) enhance expression of reporter constructs upon RA treatment. Upon RA agonist treatment these constructs markedly enhanced firefly luciferase expression compared to the original pGL4.23 construct. Error bars represent standard deviation (SD).

that RARα/RARγ exhibit extensive colocalization of their genomic binding regions with ERα in the vicinity of genes that are antagonistically regulated by estrogen and RA.

RESULTS

Identification of Genomic RAR Target Sites
To define the RAR transcriptional network, we first mapped the genomic binding sites of RARα and RARγ. Because none of the commercially available isoform-specific antibodies were adequate to render high-quality chromatin immunoprecipitation (ChIP) (data not shown), we used bacterial artificial chromosome (BAC) transgenesis (Poser et al., 2008) to generate two transgenic MCF-7 lines, which stably express RARα and RARγ tagged with eGFP at their C termini at physiological levels (Figures S1A–S1E available online). A total of 7346 high-confidence RARα-binding sites and 3916 RARγ sites were identified by ChIP-chip analysis in the transgenic cells treated with synthetic receptor-selective agonists for 1 hr using a p value cut-off of 1e-4 (Table S1 and Figures S2A and S2B). Alternatively, we applied a p value cut-off of 1e-3 to identify lower-confidence binding regions (Table S2). We validated 40 randomly selected binding regions by ChIP-

qPCR (quantitative PCR) and found all of the tested regions significantly enriched compared to genomic input DNA (Figures S3A–S3C) indicating a very low number of false positives in the ChIP-chip experiments. The binding sites of the two isoforms showed a marked overlap as 3238 (82.7%) of the RARγ sites were found to be within 1 kilobase (kb) to RARα sites. Since RARα is expressed at a higher level than RARγ, the larger number of sites for RARα might reflect an increased binding probability due to the higher abundance of this transcription factor. Overall, the large proportion of RARα/RARγ common target sites indicates a high degree of functional redundancy as suggested by mouse knockout studies (Lohnes et al., 1994).

Identification of RAR-binding sites has previously focused on promoter and promoter-proximal regions of RA-regulated genes, and so far only a small number of direct targets are known (Balmer and Blomhoff, 2002; Niederreither and Dolle, 2008). However, we found only a small portion of RAR-binding sites mapped to promoter-proximal regions (Figures 1A and 1B). Hence, most RAR-binding sites were found in intronic or promoter-distal intergenic regions previously undefined as RAR-binding sites. Within the list of RAR-binding sites in promoter or in promoter-proximal regions, we confirmed a number of previously characterized
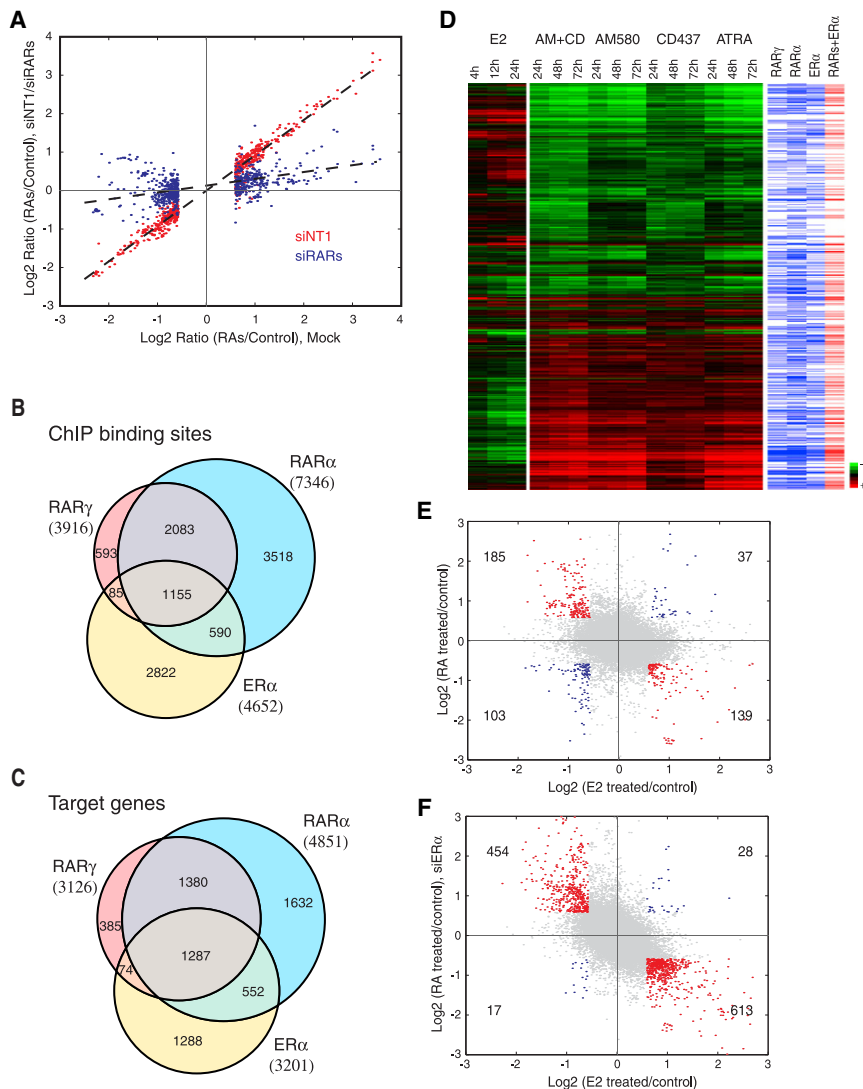
Figure 2. Colocalization of RARα-, RARγ-, and ERα-Binding Regions and Antagonistic Effects on Gene Expression between RA and Estrogen Signaling

(A) Transcriptional response of RAs in MCF-7 cells is mediated by RARs. x axis denotes Log2 transformed fold changes in gene expression after RA agonist treatment (100 nM AM580/CD437) relative to vehicle control (DMSO) treatment in mock RNAi experiments. Only genes with significant expression changes (1.5-fold change) are shown. y axis shows Log2 transformed fold changes in gene expression after RA treatment relative to vehicle control treatment in RARγ and RARα knockdown cells (siRARs, blue spots) and in RNAi control cells (siNT1, red spots).

(B) Venn diagram displaying shared regions bound by RARγ, RARα, and ERα. ERα-binding sites are based on the union of two recent genomic studies (Carroll et al., 2006; Hua et al., 2008).

(C) Venn diagram displaying shared putative target genes of RARγ, RARα, and ERα, as defined by the presence of at least one binding region within 50 kb to the TSSs.

(D) Comparison of time course gene expression profiles induced by estrogen and different RA agonist treatment for 1413 RA-regulated genes. Genes containing binding sites within 50 kb to the TSSs are denoted by blue (RARγ, RARα, or ERα) and red bars (RARs and ERα).

(E and F) Comparison of gene expression changes in response to estrogen and RA agonists. x axis shows Log2 transformed fold changes in gene expression after estrogen (10 nM E2) treatment relative to control (EtOH) treatment for 24 hr. y axis shows Log2 transformed fold changes in gene expression after RA agonist treatment (100 nM AM580 and 100 nM CD437) relative to vehicle control (DMSO) treatment for 72 hr in control-treated (siNT1) MCF-7 cells (E) and ERα-depleted (siER) MCF-7 cells (F). Genes with fold changes greater than 1.5 or less than −1.5 for both x and y axes are highlighted in red or blue, respectively.

functional RAR sites for known RA-inducible genes, including several HOX family genes (Figures 1C–1E), *CYP26A1* (Figure 1F), and *FOXA1* (Figure 1G) (Balmer and Blomhoff, 2002). Interestingly, we found additional novel RAR-binding sites nearby some of these genes, such as 3′ binding sites for *FOXA1* and *CYP26A1* (Figures 1F and 1G). We tested whether these sites could act as regulatory elements using a luciferase reporter assay, and both were able to drive the reporter gene expression in an RA agonist-dependent manner (Figures 1H and 1I).

RAR-Dependent Regulation of Gene Expression

To correlate the binding site data with the transcriptional effects of the RARs, we performed gene expression profiling after ligand treatment. Because the physiological ligand all-*trans* retinoic acid (ATRA) can elicit transcriptional effects independent from binding to RARs, e.g., through PPARδ (Schug et al., 2007), we generated expression profiles for ATRA and RAR-selective agonists AM580 (RARα-specific) and CD437 (RARγ-specific). Comparisons between these expression profiles showed a high

degree of correlation (Figure S4). CD437 and AM580 elicited similar transcriptional effects, consistent with the large overlap observed for the binding sites of RARα and RARγ.

To test whether the transcriptional response of the two selective agonists is mediated by RARs, we used RNAi to analyze gene expression changes upon RAR depletion in the presence and absence of the agonists. Knockdown of RARα and RARγ decreased or reverted most transcriptional changes caused by AM580 and CD437 (Figure 2A). This result demonstrates that both activation and repression of most genes in MCF-7 cells by RA agonists require RARs.

We analyzed expression changes after treatment with all individual ligands and the combination of AM580 and CD437 in triplicates over a time course (0, 24, 48, 72 hr). We also compared the gene expression profiles upon ligand treatment in a gene expression time course aimed at identifying early-response direct targets (0, 4, 12, 24 hr). We observed a relatively small number of significant transcript changes in the 0–24 hr time course compared to the 0–72 hr time course. Overall, we
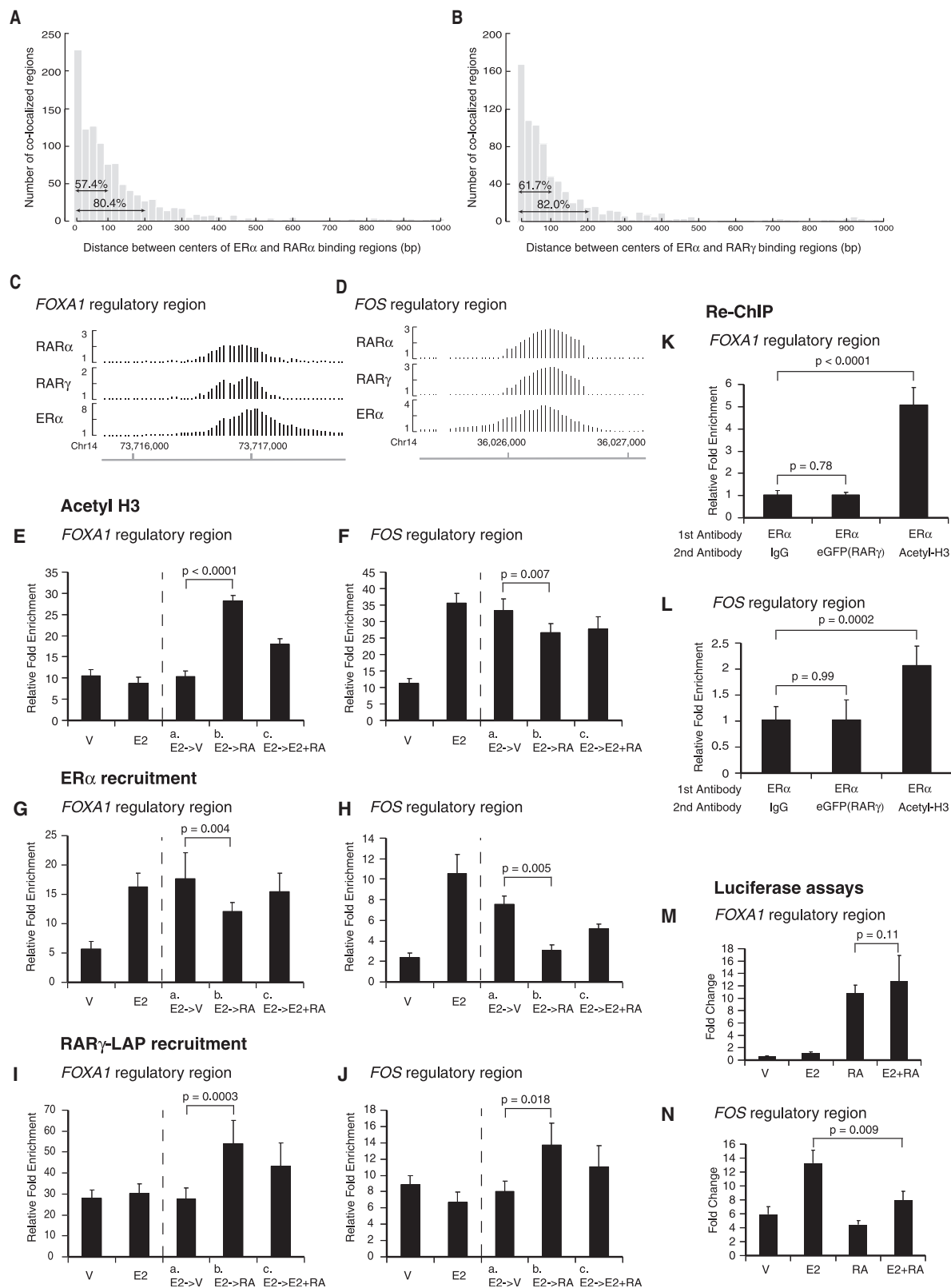
Figure 3. Antagonistic Actions of RARs and ERα Bound to Shared Regulatory Elements
(A and B) Distance between binding region centers of ERα and RARα (A) or RARγ (B).

identified a total of 1413 genes (Benjamini-Hochberg adjusted p ≤ 0.0005) (Table S3), which were significantly regulated by RA and RA agonists. Three hundred and six showed differential expression within the first 24 hr of ligand treatment. For a large proportion of transcripts differentially expressed in the 0–72 hr time course (46.5%) (hypergeometric test, p = 2.30e-140), we observed RAR-binding sites within 50 kb to the transcription start site (TSS) of the regulated gene, indicating that about half of the RA-regulated genes represent direct effects of liganded RAR rather than secondary effects. Previous work investigating the role of liganded RARs in the regulation of transcription has mainly focused on activation of expression, while the repressive function has been thought to be mediated mainly by unliganded RARs. However, downregulated transcripts constitute a large fraction (52.8%) of RA-dependent expression changes in MCF-7 cells, and we observed no marked bias of RAR binding toward ligand-activated or -repressed genes (52.5% and 41.2%, respectively). RAR regions are highly significantly enriched in both up- and downregulated genes (p = 4.03e-92 and p = 2.20e-50, respectively). Further, we demonstrate for six putative RAR direct target genes, which were significantly downregulated or upregulated by RA agonists, that neither RA-mediated repression nor RA-mediated activation require de novo protein synthesis (Figures S5A–S5F). Collectively, these findings support the hypothesis that both activation and repression involve binding of liganded RARs at target genes.

ERα- and RAR-Binding Regions Colocalize and Mediate Antagonistic Actions on Gene Expression

We and others have mapped ERα binding genome wide in MCF-7 cells (Carroll et al., 2006; Hua et al., 2008; Lin et al., 2007). When we compared RAR-binding regions with ERα regions, we found a marked colocalization. 39.3% of ERα regions were observed within 1 kb of RAR-binding regions (Figure 2B). At the gene level there was an even larger overlap; ERα and RARs share 59.8% of their putative target genes as defined by the presence of at least one binding region within 50 kb to the TSS (Figure 2C).

The extensive colocalization of RAR and ERα genomic binding sites suggested potential crosstalk of RA and estrogen signaling in the regulation of gene expression. To systematically identify transcripts that are differentially regulated by RA agonists and estrogen, we analyzed changes in gene expression after treatment with estrogen and compared these results with our RA

agonist data (Figures 2D and 2E). We found 139 genes downregulated by RA agonists to be upregulated by estrogen, while 185 estrogen-repressed genes were upregulated by RA agonists. A considerably smaller number of genes were upregulated (37) or downregulated (103) by both estrogen and RA agonists. Thus, the RA and estrogen signaling pathways appear to mainly antagonize each other. We further validated this result by testing the effects of RA agonists before and after the RNAi depletion of ERα. Knockdown of ERα increased the number of both upregulated and downregulated transcripts upon RA treatment (Figure 2F). We also analyzed the effects of single treatment with RA agonists and E2 or simultaneous cotreatment on the expression of nine individual target genes that were associated with unique RAR or ERα regions or ERα/RAR-binding regions (Figures S6A–S6C). RA agonists and E2 had an antagonistic effect on the expression levels of common target genes but not on unique targets of RARs or ERα, whose expression levels were affected by RA agonists or E2, respectively. Collectively, these findings indicate an extensive crosstalk of ERα and RARs to regulate gene expression. However, despite their opposing effects on the majority of target genes, ERα and RARα appear to activate each other. We observed ERα binding in the proximity of the *RARA* TSS (Figure S7A) and upregulated expression of *RARA* upon estrogen treatment. Likewise RARα bound near the TSS of *ESR1* (Figure S7B) and RA agonist treatment led to upregulated expression of *ESR1*. This crossregulation between the two antagonizing transcription factors presents an additional level of control for achieving a balanced regulation of gene expression by the two signaling pathways.

Antagonistic Actions of RARs and ERα Bound to Shared Regulatory Elements

In order to determine the major mechanism of the global ERα/RAR antagonism we performed a series of additional computational analyses and experiments. First, we analyzed the distance between the putative binding sites of ERα and RARs in overlapping binding regions. Using the center of a binding region as the putative binding site, we found that most ERα- and RAR-binding sites occur within 100 nt (Figures 3A and 3B). Considering the resolution limit of ChIP-chip, this finding indicates that most binding sites of ERα and RARs occur very close to each other, overlap, or are identical. This finding suggests competitive binding for the same genomic binding sites or steric hindrance

(C and D) Ratios of normalized ChIP versus input signal intensities for the putative *FOXA1* and *FOS* regulatory regions. Ratios were calculated from three replicates. Coordinates refer to UCSC hg16.

(E and F) Histone 3 (H3) acetylation is antagonistically regulated by E2 and RA agonists at *FOXA1* and *FOS* regulatory regions. RARγ-LAP MCF-7 cells grown in medium with charcoal-stripped FBS were either treated with vehicle or E2 (10 nM) for 45 min. The medium of E2-treated cells was then changed with medium containing vehicle (a), or CD437 (100 nM) (b), or a mixture of E2 (10 nM) and CD437 (100 nM). RA denotes CD437. Relative fold enrichment was determined by ChIP-qPCR using a pan-specific antibody against Acetyl-H3.

(G–J) ERα and RARγ-LAP recruitment is antagonistically regulated by E2 and RA agonists at *FOXA1* and *FOS* regulatory regions. Relative fold enrichment was determined by ChIP-qPCR using an antibody against ERα or eGFP using the chromatin obtained from the experiment described above (E and F).

(K and L) *FOXA1* and *FOS* regulatory regions do not cobind ERα and RARγ-LAP. RARγ-LAP MCF-7 cells grown in medium with charcoal-stripped FBS were treated with E2 (10 nM) and CD437 (100 nM) for 2 hr. The first ChIP was performed with an antibody against ERα. Immunoprecipitated chromatin was eluted and a second ChIP was performed with IgG (negative control) or antibody against eGFP (targeting RARγ-LAP) or Acetyl-H3 (positive control). Relative enrichment was determined for the re-ChIPed chromatin by qPCR.

(M and N) ERα/RAR-binding region for *FOS* exhibits a differential response to estrogen and RA agonists. *FOS* and *FOXA1* regulatory regions (FOS_2, FOXA1_1, Table S9) cloned into Firefly luciferase vector pGL4.23 were cotransfected into MCF-7 cells with the Renilla luciferase vector pGL4.73 used to correct for transfection efficiency. All error bars represent SD.
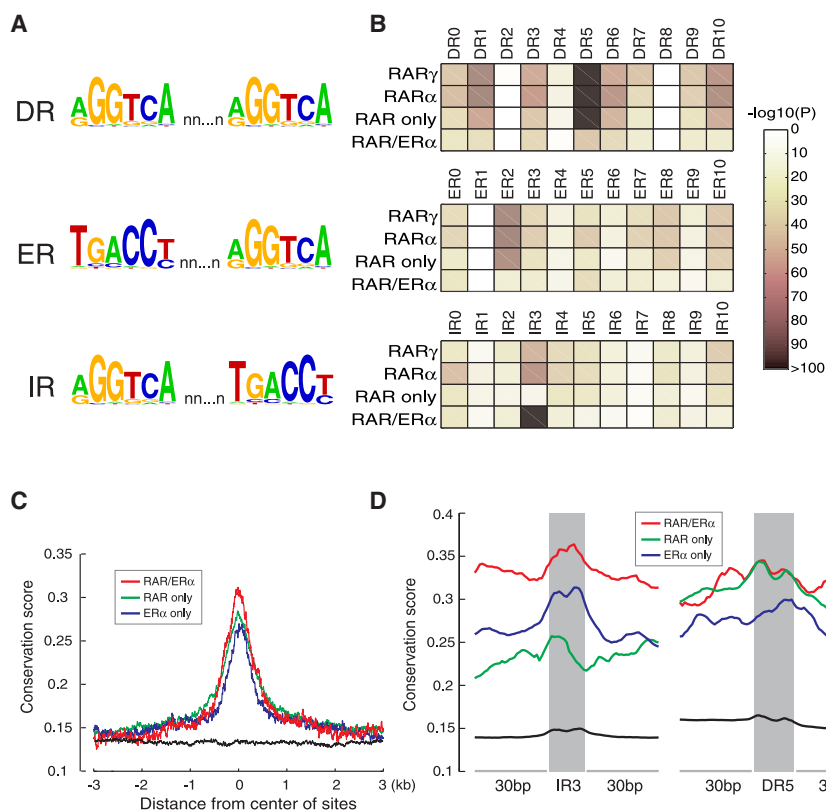
A

B

C

D

Figure 4. Enriched Hormone Response Elements and Evolutionary Conservation of RAR- and ERα-Binding Regions

(A) Canonical HREs are composed of two half-sites (PuGGTCA) separated by a variable-length spacer. Hormone response elements (HREs) can be configured as direct repeats (IR), everted repeats (ER), or inverted repeats (IR).

(B) Motif enrichment analysis for all HREs with spacer lengths from 0 to 10 in RARα- or RARγ-binding regions, RAR and ERα common regions (ERα/RAR), and RAR unique regions (RAR only).

(C) Conservation profiles of RAR and ERα common sites (depicted in red), RAR unique sites (depicted in green), and ERα unique sites (depicted in blue). The conservation profile of local genomic background is depicted in black.

(D) Conservation profiles of IR3 and DR5 motifs and 30 bp flanking regions in RAR and ERα common sites (depicted in red), RAR unique sites (depicted in green), and ERα unique sites (depicted in blue). The conservation profile of all predicted IR3 or DR5 motifs and 30 bp flanking regions in the human genome is depicted in black.

between close sites. By manual inspection of ERα- and RAR-binding events using normalized ChIP-chip intensities, we found that many overlapping regions have nearly identical peaks (Figures S8A–S8R). RARα as well as RARα-RXR dimers bind in vitro to synthetic and natural estrogen response elements (EREs) (IR3) by gel-shift assays (Klinge et al., 1997; Naar et al., 1991) and can compete with ERα for binding to EREs (Joyeux et al., 1996) (R.K., S.H., and K.P.W., data not shown). To test the model of competitive binding in vivo we chose two example elements where RARs and ERα overlap. The first was a putative regulatory element of *FOXA1*, whose gene expression is upregulated by RA agonists and downregulated by E2 (Figure 3C). The second was a putative regulatory element of *FOS*, whose gene expression is upregulated by E2 and downregulated by RA agonists (Figure 3D). We tested the effects of RA agonists and E2 treatment on H3 acetylation and ERα and RARγ recruitment at these two ERα/RAR-binding regions. E2 decreases and RA agonists increase H3 acetylation for the *FOXA1* element (Figure 3E), while opposite effects were observed for the *FOS* regulatory element (Figure 3F), indicating that the antagonistic effects of RA and E2 on gene expression are mediated through opposite effects on cofactor recruitment to common ERα/RAR sites. These opposite effects on H3 acetylation were also correlated with changes in ERα and RARγ recruitment. Upon initial E2 treatment, ERα recruitment was found to be increased for both regions, but subsequent treatment with RA agonists in absence of E2 led to a decrease in ERα binding and an increase in RARγ binding, which could be reverted by simultaneous cotreatment with E2 and RA agonists (Figures 3G–3J). This finding supports

a model of competitive binding of ERα and RARγ. To further corroborate this hypothesis, we tested whether ERα and RAR were cobound to the *FOXA1* and *FOS* elements by Re-ChIP and qPCR. Consistent with a competitive binding mechanism, no simultaneous cobinding of RAR and ERα at these regulatory elements was observed in vivo (Figures 3K and 3L).

Taking these results together, we propose that competition for the same binding element, overlapping elements, or very close elements presents one mechanism for the antagonistic regulation of genes with common ERα/RAR-binding regions. Such closely overlapping binding sites are found within the majority of ERα and RAR target genes (71%). In addition, it is notable that 557 out of the 1913 (29.1%) ERα and RAR common putative target genes do not contain colocalized ERα- and RAR-binding sites. Thus, while convergent regulation of common target genes by RA and estrogen signaling may occur predominantly through binding shared regulatory regions, it may in a substantial minority of cases also occur through independent regulatory regions via longer-range effects.

To determine whether genomic regions with shared ERα and RAR binding have regulatory potential when removed from their genomic context we used a simple luciferase reporter assay. We tested the responsiveness to RA agonists and E2 for seven ERα/RAR-binding regions as well as two unique RAR-binding regions. RAR-only regions were responsive to RA agonists but not to E2 (Figures S9A and S9B). Two of the ERα/RAR-binding regions (a *GREB1* element and the *FOS* element shown in Figures S9C and 3D, 3F, 3H, 3J, 3L, and 3N) mediated an antagonistic response to E2 and RA agonists, while the other elements were responsive to either E2 or RA agonists (including the *FOXA1* element shown in Figures 3C, 3E, 3G, 3I, 3K, and 3M and a *BTG2* element shown in Figure S9D) or did not show any
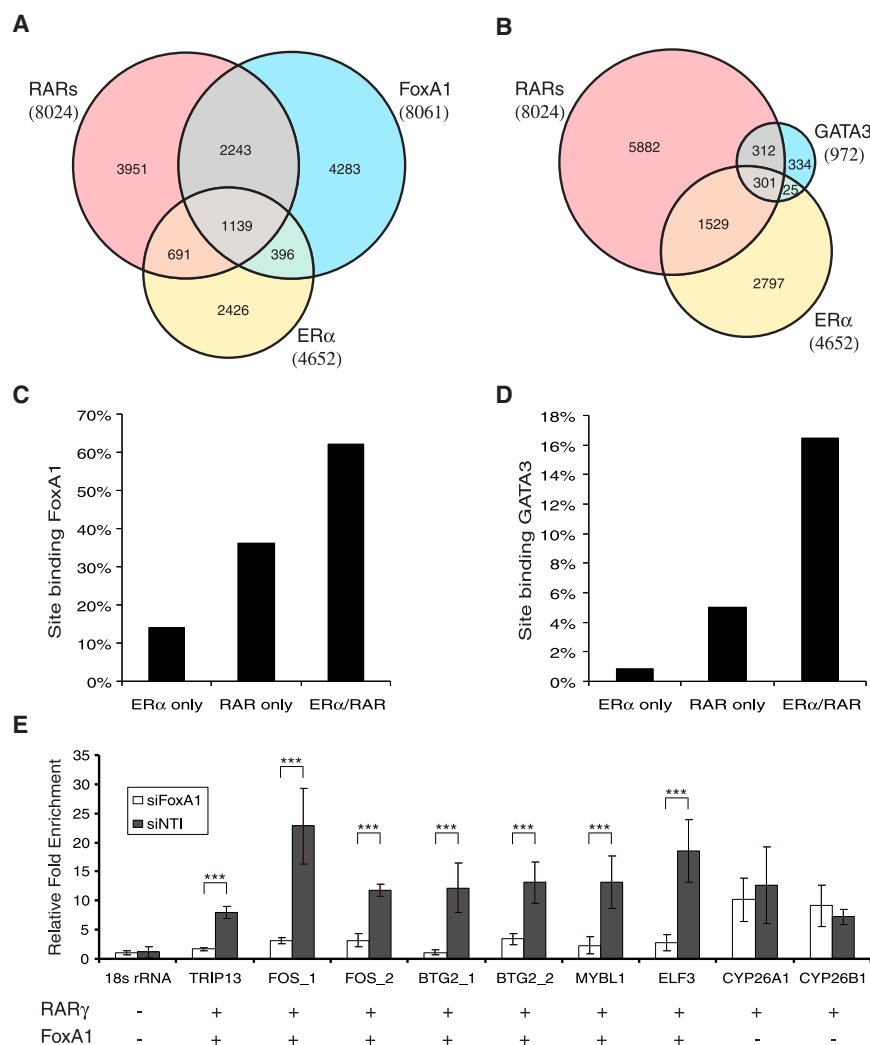
identified DR5, which is frequently present in known RAR-binding sites, as the most significantly enriched HRE in our in vivo RAR-binding regions (Figure 4B). We also noticed a significant enrichment for several other types of HREs. For some of them, e.g., DR0, DR1, DR10, IR0, and ER8, there is experimental evidence supporting their role as RAREs (Mangelsdorf et al., 1994). However, we also found HREs significantly enriched that have not been implicated as RAREs before, e.g., ER2.

Regions with colocalization of ERα and RAR binding showed a significant enrichment for the canonical estrogen response element (ERE) IR3 and several known RAREs su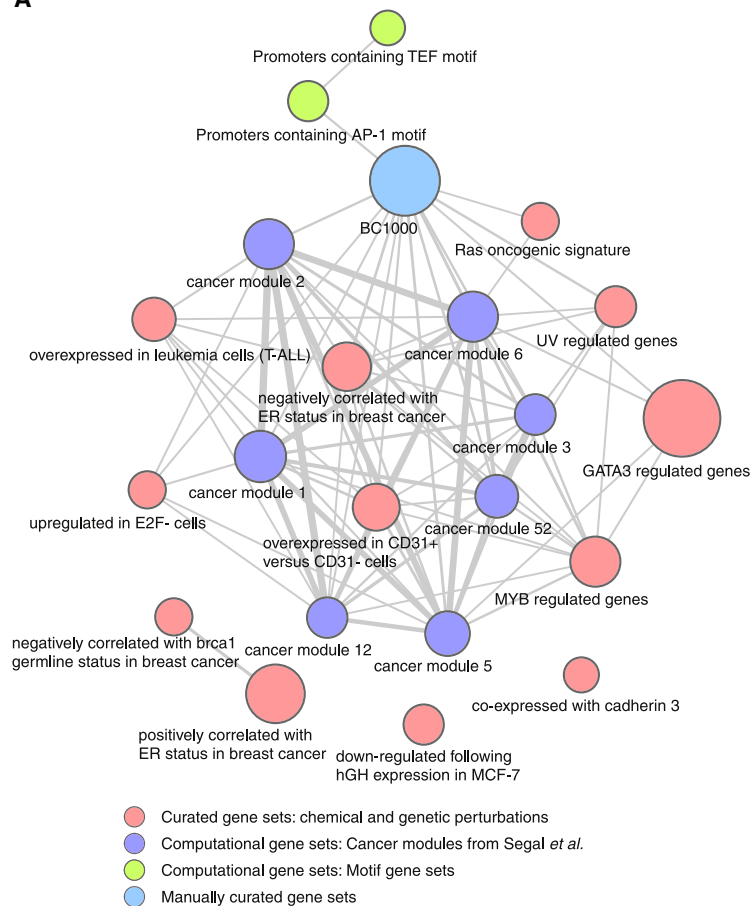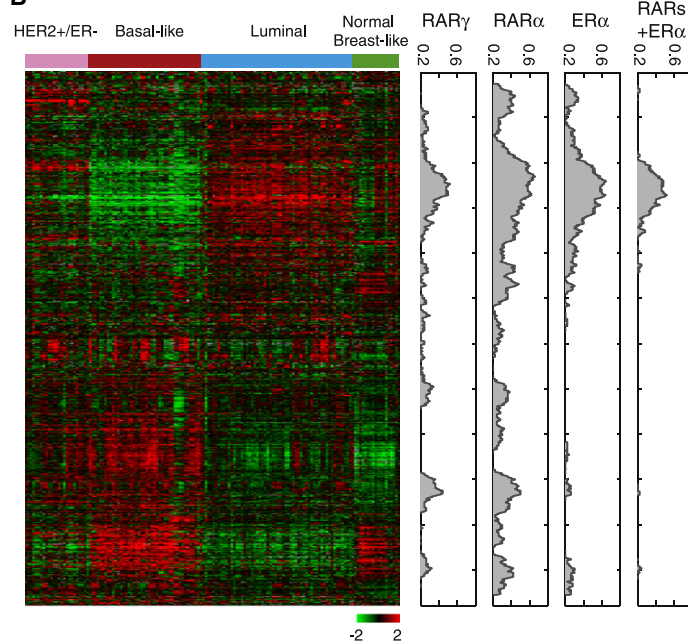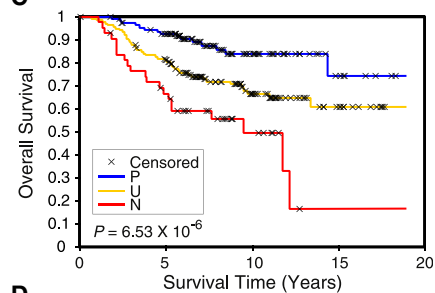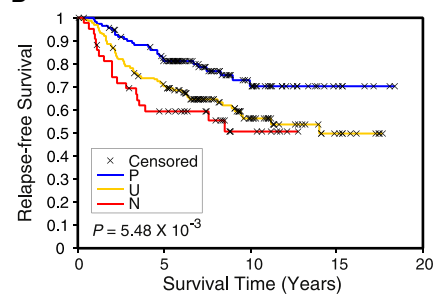ch as DR5, indicating that ERα and RARs may bind to canonical response elements in shared binding regions. However, when we compared the enrichment of IR3 and DR5 between unique and colocalizing ERα- and RAR-binding regions, respectively, we observed a reduced enrichment in the colocalizing regions (Figures S10A and S10B). This latter result indicates that an indirect binding mechanism may play an important role for the recruitment of both RARs and ERα to these elements.

We examined the evolutionary conservation of regions bound by ERα and RAR among vertebrates. Both ERα- and RAR-binding regions showed relatively high sequence conservation as compared to genomic background (Figure 4C). Likewise, IR3 and DR5 elements were found to be conserved in these regions (Figure 4D), supporting their putative roles as functional cis-regulatory elements. Interestingly, colocalizing binding regions for ERα and RARs showed a slight but significantly higher conservation than ERα or RAR unique sites (p = 1.21e-10 and p = 2.29e-5, respectively), which may indicate a higher functional constraint for the shared cis-regulatory regions. We observed for these regions a markedly higher conservation for IR3 elements than for unique ERα- or RAR-binding regions,

response (data not shown). These results indicate that colocalized binding elements for ERα and RARs can sometimes be sufficient to cause the antagonizing effect of estrogen and RA on gene expression. However, there might be alternative mechanisms that require the integrated action of multiple cis-regulatory elements to differentially regulate expression by estrogen and RA. In this context, we note that a considerable proportion of putative direct ERα or RAR target genes (25.5% and 35.6% for ERα and RARs, respectively) contain more than one binding site within 50 kb to the TSSs. Also, RAR and/or ERα actions at common regions may require a specific chromatin status that cannot be recapitulated with reporter plasmids.

Enriched HREs and Evolutionary Conservation of RAR-Binding Sites

We searched all RAR-binding sites for the presence of putative retinoic acid response elements (RAREs). To perform an unbiased analysis, we calculated the enrichment for all canonical hormone response elements (HREs), i.e., two direct (DR), inverted (IR), and everted (ER) hexameric PuGGTCA repeats with half-site spacer lengths from 0 to 10 (Figure 4A). This analysis

A

Promoters containing TEF motif

Promoters containing AP-1 motif

BC1000

Ras oncogenic signature

cancer module 2

UV regulated genes

overexpressed in leukemia cells (T-ALL)

cancer module 6

negatively correlated with ER status in breast cancer

cancer module 3

GATA3 regulated genes

cancer module 1

upregulated in E2F- cells

cancer module 52

overexpressed in CD31+ versus CD31- cells

MYB regulated genes

negatively correlated with brca1 germline status in breast cancer

cancer module 12

cancer module 5

co-expressed with cadherin 3

positively correlated with ER status in breast cancer

down-regulated following hGH expression in MCF-7

- 🔴 Curated gene sets: chemical and genetic perturbations
- 🟣 Computational gene sets: Cancer modules from Segal *et al.*
- 🟢 Computational gene sets: Motif gene sets
- 🔵 Manually curated gene sets

B

HER2+/ER- Basal-like Luminal Normal Breast-like RARγ RARα ERα RARs +ERα

-2   2

C

Overall Survival

× Censored
P
U
N

$P = 6.53 \times 10^{-6}$

Survival Time (Years)

D

Relapse-free Survival

× Censored
P
U
N

$P = 5.48 \times 10^{-3}$

Survival Time (Years)

which may indicate a prevalent role of IR3 for the function of ERα/RAR-binding regions.

Transcription Factor Motifs in RAR-Binding Sites

To identify putative transcription factors that specifically facilitate the binding of RARs and/or coregulate transcriptional effects of RARs, we searched all RAR-binding regions for enrichment of known transcription factor motifs. This analysis identified a number of putative binding motifs from several transcription factor families (Figure S10C and Table S4). Significantly enriched motifs were found for AP-1, Forkhead, and GATA transcription factors, which all have been previously reported to be highly enriched in ERα-binding sites (Carroll et al., 2006; Hua et al., 2008). While motifs for these factors were significantly enriched when we considered all RAR sites, the most significant enrichment of Forkhead and GATA motifs was found in ERα/RAR common binding regions. For the AP-1 (Fos) motif, we observed a markedly higher enrichment in both unique RAR-binding regions and ERα/RAR common binding regions compared to unique ERα-binding regions (Figure S10C).

FoxA1 and GATA3 Binding Coincides with RAR and ERα Binding

FoxA1 and GATA3 are likely candidates for Forkhead and GATA family members that are binding to the motifs enriched in regions with RAR genomic binding. Transcription factors such as FoxA1 have been proposed to act as pioneering transcription factors that facilitate the binding of ERα to enhancer elements (Carroll et al., 2005; Laganiere et al., 2005; Lupien et al., 2008). GATA transcription factors have been shown to mediate long-range chromatin interactions (Ansel et al., 2006). In particular, GATA3 is an essential regulator of mammary luminal cell fate, is coexpressed with ERα in breast carcinomas, and is a strong predictor of breast cancer differentiation (Kouros-Mehr et al., 2008; Lacroix and Leclercq, 2004). To validate specific FoxA1 and GATA3 associations with the enriched Forkhead and GATA motifs in RAR-binding sites, we performed ChIP-chip analysis using a FoxA1-specific antibody or a GFP antibody with BAC-transgenic GATA3-eGFP cell line, respectively. We identified 8061 high-confidence FoxA1- and 972 GATA3-binding regions, which we typically found distal to TSSs (Figures S2C and S2D) as observed for ERα- and RAR-binding regions. These regions significantly colocalized with ERα- and RAR-binding regions (Figures 5A and 5B). Also, FoxA1- and GATA3-binding regions exhibited a marked colocalization (Figures S11A–S11D). As predicted by the motif enrichment analysis, FoxA1- and GATA3-binding sites showed the highest overlap with shared ERα/RAR elements (Figures 5C and 5D). Unique RAR-binding regions exhibited a lower frequency of FoxA1 and GATA3 binding, which further decreased for unique ERα-binding regions. These findings indicate that both FoxA1 and GATA3 may be bona fide coregulators for RARs and ERα and play in particular an important role for the function of shared ERα/RAR-binding elements.

FoxA1 Is Required for RAR Recruitment

FoxA1 was recently shown to facilitate ERα recruitment by inducing chromatin opening at ERα enhancers (Carroll et al., 2005; Lupien et al., 2008). To determine whether there is a similar role of FoxA1 for RAR recruitment, we quantified RARγ binding upon FoxA1 knockdown by quantitative PCR. For this analysis we selected from our ChIP-chip data seven sites that were found to bind both RARγ and FoxA1, two sites that bound RARγ but not FoxA1, and a negative control site that bound neither RARγ nor FoxA1. We found that FoxA1 depletion significantly decreased RARγ binding for all seven RARγ/FoxA1-binding sites tested, but not for the RARγ sites without FoxA1 binding (Figure 5E). Thus, FoxA1 is required for RAR recruitment to specific target sites. We also tested whether GATA3 is required for RAR binding and found that GATA3 depletion had no effect on RAR recruitment (data not shown), suggesting that GATA3 function may be compensated for by FoxA1 or other GATA family factors. To further investigate the role of FoxA1 and GATA3 in RA signaling, we profiled the effects of FoxA1 and GATA3 depletion on RA-regulated gene expression. Knockdown of FoxA1 had a significant effect (Figure S12A), while GATA3 depletion had only a minor effect on many RA-regulated transcripts (Figure S12B). Importantly, FoxA1 depletion affected the expression of genes with adjacent FoxA1/RAR regions but had typically no effect on RA-regulated genes adjacent to unique binding RAR regions.

Together, these findings indicate that the primary interaction of RARs with chromatin might utilize similar mechanisms as have been proposed for ERα. Importantly, the motif enrichment analysis predicts that HRE-independent recruitment requiring FoxA1, GATA3, and AP-1 may play a key role for the binding of ERα and RARs to shared binding elements. In this context it is worthy to note that *FOXA1*, *GATA3*, and *FOS* are also putative direct targets of ERα and RARs with overlapping binding regions (Figures S7C–S7E) that are antagonistically regulated by estrogen and RA. Whereas the expression of *FOS* and *GATA3* (Eeckhoute et al., 2007) is activated by estrogen and according to our data repressed by RA, we found the expression of *FOXA1* to be upregulated by RA and downregulated by estrogen. These

Figure 6. RAR Targets as Breast Cancer-Relevant Genes

(A) Network view of functional modules enriched in RA-regulated genes. Each node represents a functional module or set of biologically relevant genes (see also Table S7). The node size is proportional to the minus logarithm of the adjusted p value for testing the module enrichment of RA-regulated genes. Edge width correlates with the minus logarithm of the adjusted p value for testing the enrichment between functional modules.

(B) Hierarchical clustering of 146-breast tumor set using the UNC Intrinsic gene set (Hu et al., 2006). The density profiles for RARγ, RARα, and ERα putative targets, as well as RAR and ERα common targets, were plotted. The density was calculated as the proportion of transcription factor putative targets in 50 neighbors for each gene in the cluster.

(C and D) RAR targets as prognostic indicators. Kaplan-Meier curves of overall survival (C) and relapse-free survival (D) among the 295 patients (van de Vijver et al., 2002) classified by RA signature values. The patient samples are grouped in three categories based on RA signature scores: P (positive RA score) (n = 73), N (negative RA score) (n = 74), and U (uncorrelated) (n = 148). p values were obtained from log-rank tests.

crossregulatory loops for putative common cofactors may be a key part of the transcriptional ERα/RAR circuitry mediating the antagonizing effects of estrogen and RA signaling in breast cancer cells.

ERα- and RAR-Dependent Gene Regulation in Breast Cancer

RAR-binding site mapping and expression profiling in MCF-7 cells revealed a marked antagonistic crosstalk between RA and estrogen signaling in this breast cancer model. Because estrogen signaling is a key pathway in breast carcinogenesis, we surmised that genes regulated by RA might also play a major role and thus have an important diagnostic and therapeutic value. Indeed, many putative RAR direct targets that are frequently also ERα direct targets have an established role in breast cancer or important cellular pathways (see Table S5 and Figures S7A–S7S and S13A–S13F).

To systematically validate the role of RAR-mediated transcriptional regulation in breast cancer, we analyzed the enrichment of RA-regulated genes in MCF-7 cells in functional modules (Subramanian et al., 2005) or gene signatures that have been previously associated with breast cancer. RA-regulated genes in MCF-7 cells are highly enriched in a number of breast cancer-relevant signatures (Figure 6A). Notably, the signature BC1000 comprised of 1347 manually curated genes implicated in breast cancer (Witt et al., 2006) is among the most enriched modules for RA-regulated genes. Two hundred and twenty-nine out of these 1347 (17.0%, multiple testing adjusted p = 3.03e-19) putative breast cancer genes are significantly regulated by RA in MCF-7 cells, and 108 out of those 229 RA targets have RAR-binding elements within 50 kb of the TSS (Figure 6A and Table S6). We also observed a very significant enrichment (multiple testing adjusted p = 5.32e-22) of the signature composed of genes regulated by GATA3 (Oh et al., 2006). RA-regulated genes in MCF-7 cells were found to be enriched in sets of genes that are both positively and negatively correlated with ER status in breast tumors (Table S7) (p = 1.24e-14 and p = 1.72e-10, respectively). Finally, we observed that RA-regulated genes are enriched in several cancer modules or functionally related genes that are conditionally activated or repressed in a variety of cancer types (Table S7) (Segal et al., 2004), indicating that RAR might act as a direct transcriptional regulator for a subset of genes within these cancer modules.

The meta-analysis described above suggests that many genes regulated by RA in MCF-7 cells are breast cancer relevant. We next analyzed the expression of putative direct RAR targets that were derived from our cell line experiments in breast tumor cells from patient samples. For this purpose, we analyzed gene expression profiles of 146 breast cancer patient samples (Hu et al., 2006). These expression profiles were previously used to classify breast tumors into distinct intrinsic subtypes (e.g., Luminal, Basal-like, HER2⁺/ER⁻) that differ in their clinical outcomes (Hu et al., 2006). When we analyzed RAR and ERα binding in the genes comprising these expression profiles, we observed that a gene cluster with high expression in Luminal type but low expression in more aggressive subtypes (Basal-like and HER2⁺/ER⁻) contains a high proportion of RAR targets as well as ERα targets (Figure 6B). This group of genes is characterized by high expression of ERα, its

putative coregulators (e.g., *FOXA1, GATA3*), and known direct targets (e.g., *TFF1*, *KRT18*). Interestingly, we also found gene clusters with high expression in Basal-like and HER2⁻/ER⁻ subtypes but low expression in Luminal and Normal-like types to contain a large fraction of genes that are putative direct targets of RARα and RARγ but not of ERα (Figure 6B).

Our observations that many RAR targets are breast cancer-relevant genes and are specifically expressed in different breast cancer subtypes suggested that these genes might possess a significant prognostic value. We therefore analyzed the clinical outcome for each tumor sample dependent on the expression of putative direct targets of RARs. For this analysis, we defined for each tumor sample an RA signature score, which measures the correlation between RA-dependent gene expression profiles in MCF-7 cells and the gene expression profile in a given tumor sample for putative RAR direct targets (See Experimental Procedures). We first examined the correlation between RA signature scores and clinical outcomes for a cohort of expression profiles from 295 breast tumor patients (van de Vijver et al., 2002). A total of 354 putative RAR direct targets were identified in this dataset. Kaplan-Meier survival analysis demonstrated a highly significant correlation between RA signatures and the patient overall survival (Log-rank test, p = 6.53e-6) and a significant correlation for relapse-free survival (Log-rank test, p = 5.48e-3) (Figures 6C and 6D). Positive RA signature scores indicated good prognosis while negative scores strongly indicated poor clinical outcomes. We also observed significant correlation between RA signature and standard clinical-pathologic indexes, such as tumor grade, tumor size, and ERα status (Table S8). These results were confirmed for two independent breast tumor cohorts (Figures S14A and S14B).

DISCUSSION

Comparing the overlap of different transcription factors can be a powerful means of inferring functional relationships, particularly when combined with expression data. Our results indicate that RARα- and RARγ-binding sites frequently overlap in the human genome, and comparisons of gene expression in response to isoform-specific agonists indicate considerable redundant function. Previous models of gene regulation by these crucial physiological and developmental regulators have been restricted by the focus on binding to promoters or promoter-proximal gene regions (Balmer and Blomhoff, 2002; Niederreither and Dolle, 2008). However, we found that the majority of RAR-binding sites occur distal to TSSs. These results are reminiscent of recent studies that have revealed a similar tendency for ERα to bind to distal elements (Carroll et al., 2006; Hua et al., 2008; Lin et al., 2007). The cofactor FoxA1 has been implicated in this recruitment of ERα to distal sites (Carroll et al., 2005; Lupien et al., 2008), and our results indicate that FoxA1 is similarly required for RAR recruitment to genomic binding sites. Likewise, GATA3 binding frequently coincides with ERα and RAR binding, although it is not strictly required for RAR recruitment. Strikingly, many of the genomic regions bound by RARs overlapped with those previously identified to bind ERα. Subsequent transcriptional analysis demonstrated that RARs and ERα tend to exhibit antagonistic effects on the transcription of target genes.
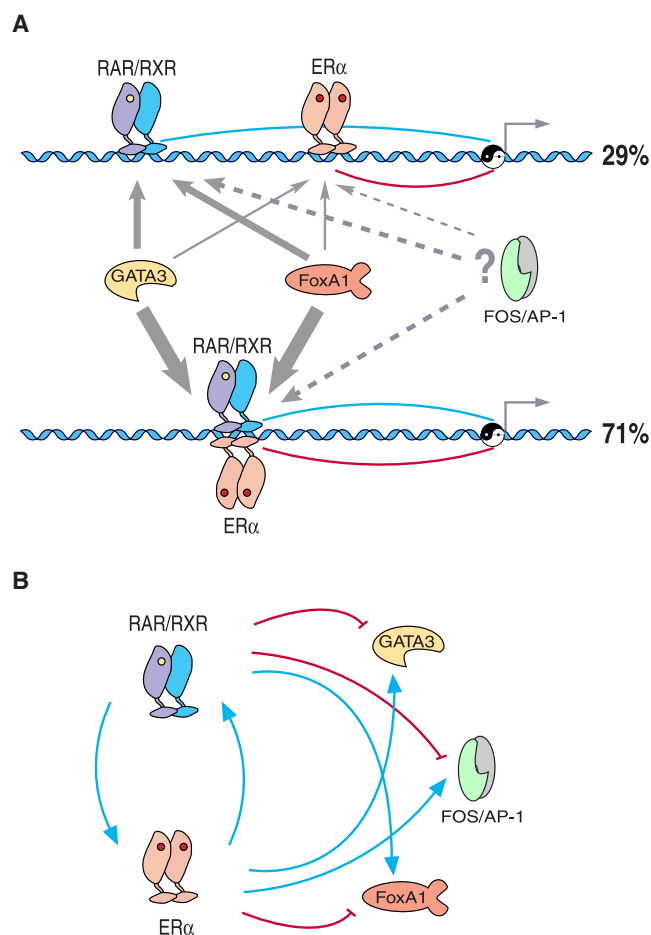
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Figure 7. A Model for the Antagonistic Regulation of Target Genes by RAR and ERα

(A) The antagonistic regulation of target genes by RAR and ERα can occur either through independent *cis*-regulatory elements or, as was most frequently found, through shared binding regions of ERα and RARs. FoxA1 and GATA3 may be essential for RAR- and/or ERα-mediated gene regulation. FoxA1 may act as an initial chromatin binding factor and facilitate further recruitment of the RAR/RXR heterodimer, ERα homodimer, and/or other cofactors. The line and arrow width indicates the frequency that FoxA1 or GATA3 participates in different types of RAR or ER regulatory regions. Motif enrichment analysis predicts a potential role for AP-1 in ERα and RAR recruitment to these sites.

(B) Transcriptional regulatory circuits composed of RAR, ERα, and their putative cofactors. The expression of *FOXA1*, *GATA3*, and *FOS* in MCF-7 cells is oppositely regulated by RAR and ERα upon RA or estrogen treatment. A negative feedback is achieved by positive crossregulation between the two antagonizing transcription factors RAR and ERα.

This can occur either through independent *cis*-regulatory elements or, more frequently, via shared binding regions of ERα and RARs (Figure 7A). In the specific instances we tested, ERα and RAR binding were mutually exclusive, indicating competitive binding of the two nuclear receptors to the same element or nearby *cis*-regulatory elements. Based on the known functions of their target genes in breast cancer, ERα and RARs appear to be "Yin and Yang" for the genetic regulation of proliferation and survival that are promoted by ERα and inhibited

by RARs. The finding that binding sites of RARs and ERα are coincident within the same enhancers or located in different enhancers for the same target genes, along with the finding that these two nuclear receptor signaling systems antagonistically regulate their target genes, indicates that these regulatory elements are coevolving to balance target gene expression. Interestingly, the ERα/RAR antagonism appears to regulate itself through crossregulatory loops between ERα, RARs, and their cofactors (Figure 7B). This balanced control of gene expression regulates fundamental cellular processes that when dysregulated can lead to cancer.

The identification of the genes regulated by RARs in breast cancer cells, and in particular the discovery of their extensive crosstalk with estrogen signaling, may benefit breast cancer diagnostics and therapeutic intervention. Specifically, RAR- and ERα-binding data can diagnostically differentiate tumor subtypes and patient outcome. Putative direct targets of ERα and RARs in MCF-7 cells are highly expressed in Luminal type breast tumors, indicating that their antagonistic effects may be relevant for primary ER-positive tumors. However, RARs appear to be important regulators of cancer-relevant genes that are not regulated by estrogen. We found such RAR targets to be expressed at high levels in Basal-like and HER2/ER⁻ tumors that are typically highly aggressive and associated with poor prognosis. Importantly, we demonstrated that in breast tumor samples, the expression of RAR targets identified in MCF-7 cells predicts a positive clinical outcome. Some of these genes may be targets for diagnosis and/or therapeutic intervention. Based on these findings, there is a strong rationale for the use of RA agonists in breast cancer treatment. However, success of RA-based therapies has been limited to treatment of acute promyelocytic leukemia (Altucci et al., 2007; Soprano et al., 2004), while clinical applications of RA in breast and other solid tumors have shown limited effects due to RA resistance (Freemantle et al., 2003; Schug et al., 2007). To harness the RA-mediated anticarcinogenic effects of RARs in breast cancer, this resistance must be overcome, perhaps via inhibition of FABP5 to block metabolism of RA into PPARδ agonists (Schug et al., 2008). Another potential approach would be the use of selective agonists or combination therapy with anti-estrogens in ER⁺/RAR⁺ patients. Alternatively, RA resistance could be bypassed by targeting RA-regulated genes and pathways that mediate the antineoplastic effects of RA in breast cancer; in this study, we have uncovered the framework of these effects.

EXPERIMENTAL PROCEDURES

Generation of BAC Transgenic MCF-7 Cell Lines

The BACs CTD-2343G9 (*RARA*), CTD-2644H7 (*RARG*), and RP11-1103A14 (*GATA3*) were obtained from Invitrogen. A LAP cassette was inserted as a C-terminal fusion using ET cloning; BAC DNA was extracted and transfected into MCF-7 breast cancer cells (ATCC HTB-22) for the generation of stable BAC transgenic cell lines as previously described (Poser et al., 2008).

Luciferase Reporter Assays

RAR- and ERα/RAR-binding regions were cloned into pGL4.23 (Promega). MCF-7 cells were transfected with pGL4.23 (containing the binding regions or the empty vector) and pGL4.73 using Lipofectamine LTX (Invitrogen), treated with agonists or vehicle, and assayed using the Dual-Glo Luciferase assay (Promega).

Reverse Transfection with siRNAs

Reverse transfection was carried out at a concentration of 50 nM of control siRNA or four siRNAs (ON-TARGET*plus* SMARTpool, Dharmacon) directed against the same target gene using Dharmafect 1 transfection reagent (Dharmacon).

Chromatin Immunoprecipitation Experiments

Cells at 80% confluency ($\sim$5 × 10^6 cells per ChIP) were subjected to ChIP as previously described, with the following antibodies: goat anti-GFP (raised against His-tagged full-length eGFP and affinity-purified with GST-tagged full-length eGFP), goat anti-FoxA1 (ab5089) from Abcam, anti-panH3ac (06-599) from Millipore, and anti-ERα (MC-20, sc-542x) and normal goat IgG (sc-2028) from Santa Cruz Biotechnologies.

For ChIP-qPCR assays, the fold enrichment of ChIPed DNA relative to input DNA at a given genomic site was determined by comparative C$_T$ ($\Delta\Delta$ C$_T$) method using StepOnePlus Real-Time PCR System and Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. An *ACTB* exonic region or *18S rRNA* genomic region was used for normalization. All primer sequences used for qPCR are described in Table S9.

For ChIP-chip, both ChIPed DNA and input DNA were subjected to linker-mediated PCR amplification and fragmentation and end-labeled with biotin using the GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix) as previously described. The resulting labeled samples were hybridized to Affymetrix GeneChip Human Tiling 2.0R Array Set following the Affymetrix Chromatin Immunoprecipitation Assay Protocol. Independent biological triplicates were performed for each transcription factor, as well as the control (input DNA).

qRT-PCR and Microarray Gene Expression Profiling Experiments

qRT-PCR was performed with cDNA generated from total RNA from MCF-7 cells treated with different agonists and/or transfected with siRNAs. Relative expression levels for specific genes were determined using StepOnePlus Real-Time PCR System and Power SYBR Green PCR Master Mix (Applied Biosystems). All primer sequences used for qRT-PCR are described in Table S9. For expression profiling, total RNA samples were labeled by direct incorporation of cyanine 3-labeled CTP using the Agilent Low RNA Input Linear Amplification Kit PLUS (One-Color) (Agilent Technologies) and hybridized to Agilent Human Genome Oligo Microarrays (4 × 44K) (Agilent Technologies). Hybridized microarrays were scanned using a GenePix 4000B scanner (Molecular Devices) at 5 μm resolution. All experiments were performed in triplicate.

Data Analysis

ChIP-chip tiling array data were normalized and analyzed with Affymetrix Tiling Analysis Software (TAS) as previously described (Bernstein et al., 2005) using p value cut-offs of 1e-4 (for detecting high-confidence binding regions) or 1e-3 (for detecting lower-confidence binding regions, Figures S15A–S15C). For the analysis of gene expression data the software package LIMMA (Smyth, 2004) was applied to detect significantly differentially expressed probes. The enrichment of known or predicted transcription factor binding motifs in ChIP-identified RAR- or FoxA1-binding regions was estimated by comparing the number of motifs in binding regions with the number of motifs in randomly selected genomic regions. Associations between RA-responsive genes and clinical and pathologic variables were examined using a Chi-Square contingency test with the JMP7 software package (SAS Institute Inc.). Patient sample data grouped into three RA signature categories were used to plot Kaplan-Meier survival curves for patient overall survival or relapse-free survival.

ACCESSION NUMBERS

Data are available in the Gene Expression Omnibus (GEO) database under GSE15244.

SUPPLEMENTAL DATA

Supplemental data include Supplemental Experimental Procedures, 15 figures, 9 tables, and Supplemental References and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00504-2.

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