Hormone induced repression of genes requires BRG1-mediated H1.2 deposition at target promoters

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Hormone dependent chromatin remodeling in gene repression

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Running Title: Hormone-dependent chromatin remodeling in gene repression.

Abstract

Eukaryotic gene regulation is associated with changes in chromatin compaction that modulate access to DNA regulatory sequences relevant for transcriptional activation or repression. Although much is known about the mechanism of chromatin remodeling in hormonal gene activation, how repression is accomplished is much less understood. Here we report that in breast cancer cells, ligand-activated progesterone receptor (PR) is directly recruited to transcriptionally repressed genes involved in cell proliferation along with the kinases ERK1/2 and MSK1. PR recruits BRG1 associated with the HP1γ-LSD1.com repressor complex, which is further anchored *via* binding of HP1γ to the H3K9me3 signal deposited by SUV39H2. In contrast to what is observed during gene activation, only BRG1 and not the BAF complex is recruited to repressed promoters, likely due to local enrichment of the pioneer factor FOXA1. BRG1 participates in gene repression by interacting with H1.2, facilitating its deposition and stabilizing nucleosome positioning around the transcription start site. Our results uncover a mechanism of hormone-dependent transcriptional repression and a novel role for BRG1 in progestin regulation of breast cancer cell growth.

Highlights:

In this report we have discovered a new function of BRG1 during hormone-mediated gene repression. The ATPase BRG1 without the other subunits of the BAF complex is recruited by the hormone receptor to genomic sites marked by the pioneering factor FOXA1, and remodels the target chromatin by facilitating linker histone H1.2 deposition.

Introduction

Genomic DNA is packaged within the eukaryotic nucleus into highly organized chromatin. The basic structural unit of the chromatin is the nucleosome core particle, which consists of approximately 146 base pairs of DNA wrapped around a histone octamer containing two molecules each of core histones H2A, H2B, H3, and H4. The nucleosome core particle associated with linker histone H1 on linker DNA, constitutes the fundamental repeating unit of chromatin. Histone H1 interacts with both the nucleosome core as well as the linker DNA, and promotes the higher-order folding and compaction of chromatin. There are several histone H1 variants, all of which are generally considered transcriptional repressors (Bustin et al., 2005; Sera and Wolffe, 1998). The length, charge and number of posttranslational modification sites of the C-terminal tails vary between histone H1 variants, suggesting that individual H1 variants may play distinct roles in the regulation of higher-order chromatin structure (Clausell et al., 2009; Harshman et al., 2013).

At least one functional consequence of chromatin packaging is to restrict access of DNA-binding proteins that regulate transcription to the promoter. However, chromatin structure is very dynamic and undergoes extensive remodeling that leads to either activation or repression of transcription. Two highly conserved chromatin-remodeling mechanisms have been found in eukaryotic cells: 1) post-translational modification (PTM) of histones and 2) ATP-dependent chromatin remodeling, which is catalyzed by enzymatic complexes containing ATPases that use the energy of ATP hydrolysis to disrupt histone-DNA interaction. There are several ATP-dependent remodeling complexes, including the SWI/SNF complex in yeast and its homologues in other higher eukaryotes, BAF and PBAF, containing BRG1 and/or BRM ATPase subunits. These complexes have been shown to alter nucleosome structure and to facilitate transcription factor binding, thus promoting gene activation. Repression of genes often involves the establishment and maintenance of a chromatin conformation that prevents transcription and also requires ATP-dependent remodeling complexes (Holstege et al., 1998; Ooi et al., 2006). For instance, the BRG1-containing hSWI/SNF complex is required for repression of MYC target genes (Pal et al., 2003; Zhang et al., 2005) and has been found in co-repressor complexes associated with REST (Battaglioli et al., 2002). Despite this evidence, the molecular mechanisms by which SWI/SNF contributes to binding of transcriptional silencing complexes and to gene repression have not been defined (Ooi et al., 2006).

Nuclear receptors (NRs) are ligand-regulated transcription factors, some of which have the ability to access their binding sites in the chromatin context, thus behaving as “pioneer” factors (Zaret and Carroll, 2011). In breast cancer cells, the progesterone receptor (PR) binds preferentially to its cognate sites when these are organized in well-positioned nucleosomes, as a consequence of being part of a complex with protein kinases and other histone modifying enzymes (Ballare et al., 2013a). A well-characterized model system for studying these processes is the regulation of the expression of the Mouse Mammary Tumour Virus (MMTV) provirus. When integrated in mouse or human chromosomes, the MMTV promoter is organized into positioned nucleosomes, with a nucleosome covering the HREs and the binding site for NF1 (Richard-Foy and Hager, 1987; Truss et al., 1995) and we have shown that nucleosomes are required for the synergistic function of PR and NF1 (Chavez and Beato, 1997; Di Croce et al., 1999). Progestin administration to T47D breast cancer cells carrying a single copy of an MMTV reporter gene activates the Src/Ras/Erk and the CDK2 cascades via cell membrane attached PR, leading to progestin-induced cell proliferation and activation of the MMTV promoter (Migliaccio et al., 1998). Within minutes of hormone addition two consecutive cycles of chromatin remodeling catalyzed by at least ten enzymes lead to the eviction of linker histone H1 and core histone H2A/H2B dimers, creating a suitable platform for transcription (Vicent et al., 2009; Wright et al., 2012)

We have shown that steroid hormone receptors can interact with DNA in the absence of hormone (Schauer et al., 1989; Willmann and Beato, 1986). In the absence of ligand PR binds genomic sites and targets a repressive complex containing HP1γ, LSD1, HDAC1/2, CoREST, KDM5B and the RNA SRA (Steroid receptor RNA Activator) to 20% of hormone-inducible genes, keeping these genes silenced prior to hormone treatment (Vicent et al., 2013). Upon hormonal treatment the HP1γ-LSD1 complex is displaced as a result of rapid phosphorylation of histone H3 at serine 10 mediated by MSK1, which is recruited to the target sites by the activated PR (Vicent et al., 2013).

It is believed that down-regulation of genes that occurs concomitantly with the "classic" activation in response to agonistic steroid receptor ligands is a relatively minor mechanism and its physiologically relevance has been questioned. Several possibilities have been postulated: i) down-regulation occurs without interaction between the hormone receptor and a promoter or enhancer sequence of repressed genes, ii) down-regulation is mediated by the presence of negative responsive elements (Surjit et al., 2011); and iii) some repressive effects could be explained by squelching of certain components of the transcriptional machinery (Santos et al., 2011). In the case of progesterone, while PR_B isoform is supposed to function predominantly as an activator of progesterone-responsive genes, the PR_A isoform have been linked to the repressive actions of progestins (Giangrande and McDonnell, 1999). However, all these mecanismo are a matter of debate he molecular mechanism of repression is not clear.

Here we delineate the mechanism of gene down-regulation by progestins in T47D breast cancer cells. We found around 650 hormone-repressed genes, which are involved in relevant cell functions such as intracellular signaling, cell fate commitment and cell proliferation. In response to progestins, activated PR is directly recruited to the promoters of a subset of these genes in association with kinases, the HP1γ-LSD1.com complex and BRG1. In contrast to what was observed during hormone activation (Vicent et al., 2009), only BRG-1 and not the whole BAF complex is involved in repression. The basis for this specificity could be found in the enrichment of the pioneer factor FOXA1 in repressed genes prior to hormone exposure. We find that BRG1 contributes to remodeling of the target chromatin, interacts with H1.2 and facilitates its deposition compacting the chromatin structure around the TSS of the hormone-repressed genes.

Results

Progestins down-regulate expression of genes involved in cell growth and apoptosis

Treatment of T47DML breast cancer cells (a clone of T47D cells carrying a single copy of the MMTV-*luc* transgene integrated in their genome (Truss *et al*., 1995) with the synthetic progestin R5020 (10 nM) changes the expression levels of a large number of genes (Ballare *et al*., 2013b). For our study we have focused on the set of 1773 regulated mRNAs, which were found in expression microarrays as well as in RNA-seq experiments. Of these, 1124 were up-regulated and 649 were down-regulated by hormone (Fold change in each assay ≥1.4, Figure 1A and S1A). Box plots of the RPKM confirmed the expected significance of the hormone effects (Figure S1B). GO analysis revealed that down-regulated genes are associated with relevant cell functions including intracellular signaling cascades, cell proliferation, cell adhesion and cell fate commitment, thus complementing and reinforcing the pathways associated with the up-regulated genes (Figure S1C and Ballare *et al*., 2013b).

Since the levels of mRNA transcripts can be affected not only by the transcription rate but also by post-transcriptional processes, we performed iRNA-seq analysis, which focus on intron transcription and therefore is a better indication of the transcription rate (Madsen et al., 2015). After hormone exposure we found 1370 and 664 up- and down regulated genes, respectively (Figure 1A inset, ≥ 2.0-fold, p-value < 0.03, log CPM > 1). As the sensitivity of iRNAseq is more dependent on sequencing depth and also cannot detect transcription for genes with no introns or few short introns (Madsen et al., 2015), comparison of iRNA-seq data with exon transcripts revealed a minimum of 48-50.2 % overlapping between these two datasets. Thus, incubation of T47D cells with hormone promotes acute transcriptional changes in RNA levels.

Hormone-dependent down-regulation requires binding of PR to target genes

To test whether gene down-regulation by Pg in breast cancer cells requires PR binding to target genes, we used two sets of data: our previously published ChIP-seq of the PR in T47DML cells, which detected 25,000 PR binding sites (PRBs) after 60 min of hormone exposure and a new ChIP-seq experiment with the same PR antibody and higher coverage, which found 48,541 PRBs (see Material and Methods) (Figure S1D). Compared with non-regulated genes, PRBs density as well as the percentage of genes with PRBs was significantly higher in regulated genes, particularly in up-regulated but also in down-regulated genes (window: -5kb from the TSS to TTS) (Figure 1B, C and D and see Supplementary Table 1 as a summary of the genomic distribution of PRBs). These studies were performed with PRBs data detected after 5 and 30 min of hormone (Figure 1B). Interestingly, we found that the differences between the down-regulated and non-regulated genes was larger with the set of PRBs occupied at 5 min and located close to the TSS (Figure 1B right panel). In addition, PRBs are localized more closely to the TSS of up and down genes compared with random sites (Figure 1D). Importantly, comparing the same number of up and down-regulated genes we found similar enriched motifs around the PRBs, discarding the existence of specific negative responsive elements (Figure S1E). Thus, hormone-repressed genes are significantly enriched in PRBs, arguing for a direct effect of the hormone on the repression of these genes.

We next compared the kinetics of PR binding by analyzing ChIP-seq results after exposure to hormone for different times (Ballare et al., 2013a). The analysis showed that while the occupancy of PRBs associated with up-regulated genes peaked at 30-60 min and was maintained high until 6 hours of hormone exposure (Figure 1E, upper panel), occupancy of PRBs associated to down-regulated genes peaked at 30 min and decreased thereafter, reaching low values at 6 hours of hormone exposure (Figure 1E, middle panel). A similar tendency was observed when the analysis was limited to sites around the TSS of the down-regulated genes (Figure 1E, bottom panel and see quantification on the lower panel), suggesting a transient PR binding to down-regulated genes.

The phosphorylated receptor along with the kinases ERK and MSK1 participate in hormone-dependent gene repression

Next, we asked whether the PR recruited to the down-regulated genes is phosphorylated in S294 and S400, two modifications associated with the “active” form of the receptor (Weigel *et a*l., 1995; Zhang *et al*., 1994, 1995). For a quantitative analysis we selected three transcriptionally repressed genes exhibiting PRBs in their promoter region: Breast Carcinoma Amplified Sequence 1 (*BCAS1*), Keratin 23 (*KRT23*) and Insulin-Like Growth Factor Binding Protein 5 (*IGFBP5*) (Figure 2A and S2A). In RNA-seq assays performed after 1 and 6h of hormone exposure we found between 45-60% hormone-dependent reduction in the transcripts from these genes (Figure S2B). These results were validated by quantitative PCR (Figure 2B). ChIP experiments using specific antibodies for the two phosphorylated forms of PR after 5

and 30 min of hormone exposure showed significantly increased recruitment to *BCAS1, KRT23* and *IGFBP5* genes of both PRS294ph and PRS400ph (Figure 2C and S3A-C). By sequential ChIP (re-ChIP) we found that S294 and S400 are both associated simultaneously to the same DNA region in the *BCAS1* and *KRT23* PRBs (Figure S3D). Thus, the activated form of PR is recruited to hormone-repressed genes.

In the context of gene activation PR forms a ternary complex with the active form of the kinases ERK and MSK1, which are selectively recruited to the target HREs (Figure S3E and (Vicent *et a*l., 2006)). As ERK1/2 is the kinase responsible for the hormone-dependent S294 phosphorylation (Vicent et al., 2006), we asked whether ERK is also recruited to hormone-repressed genes. ChIP experiments confirmed the recruitment of ERK1/2 as well as MSK1 to the *BCAS1*, *KRT23* and *IGFBP5* gene promoters after hormone exposure (Figure 2D and data not shown). On the other hand, no increase in PR and ERK was detected in *TFAP2* and *BAZ2B,* two hormone-repressed genes lacking PRBs in the promoter as well as in *RABEPK* and *PELO*, two non-regulated genes (Figure S4A). Thus, the activated form of PR associated with the kinases ERK1/2 and MSK1 is recruited to both activated and repressed genes

The HP1γ-LSD1.com complex is recruited to repressed genes and needed for repression

We wondered whether the repressive complex composed of HP1γ, the LSD1 associated complex (LSD1.com) and the RNA SRA that contributes to basal repression of hormone-activated genes (Vicent *et al*., 2013) is also involved in hormone-mediated repression. ChIP experiments performed in cells incubated for 5 min with hormone showed recruitment to the promoter of the *BCAS1*, *KRT23* and *IGFBP5* not only of PR but also of LSD1 and HP1γ (Figure 2E). No recruitment of the the repressive complex was observed in the control genes (Figure S4A). Recruitment of the repressive complex was important for down-regulation, as depletion of either HP1γ or the RNA-SRA significantly reduced hormonal repression of these genes compared with control cells transfected with scrambled siRNAs (Figure 2F and S4B). No significant change in the basal levels of the repressed genes was observed after HP1γ or SRA knockdown (Figure S4C).

To explore the generality of these observations, we performed RNA-seq experiments in cells transfected with siControl and siHP1γ. In siControl cells we found 888 and 581 genes up and down-regulated by hormone, respectively (1.5 fold change and p-value <0.05, Figure 2G). The proportion of genes affected by HP1γ knockdown was larger for down-regulated genes (35%) than for up-regulated genes (28.1%) and the magnitude of the effect was also significantly higher for down-regulated genes compared to up-regulated genes (*P*-value=0.007). Interestingly, 83.9% of the genes affected by siSRA in microarrays analysis were also affected by knockdown of HP1γ , suggesting that the HP1γ-LSD1.com/SRA complex fulfills a repressive function after exposure to hormone.

As H3K9me3 is important for the localization of HP1γ protein in the genome (Grewal and Jia, 2007), we asked whether it was also necessary for the recruitment of the HP1γ-complex to down-regulated target genes. We found increased H3K9me3 signal 5 min after hormone exposure in *BCAS1, KRT23* and *IGFBP5* genes (Figure 2E, right panel). To modify the levels of H3K9 trimethylation we used siRNA knockdown of several histone methyl transferases (HMTs) and found that siRNA against the HMT SUV39H2 significantly decreased both hormone-dependent H3K9me3 and HP1γ binding on the target promoters (Figure S4E-F) and interfered with the hormonal down-regulation (Figure S4D). Moreover, we observed hormone-dependent recruitment of SUV39H2 to this set of repressed genes (Figure S4G). No significant change in the basal levels of the repressed genes was observed after

SUV39H2 knockdown (Figure S4C). Thus, the HP1γ repressive complex associated with PR and the RNA SRA (Vicent et al., 2013) is recruited to hormone-repressed genes and is anchored by H3K9me3.

BRG1 but not the BAF complex is part of the HP1γ-LSD1.com complex involved in hormonal down-regulation

The ATP-dependent remodeling complex BAF has been shown to be needed for progestin gene activation and is recruited to PRBs *via* an interaction with PR (Vicent e*t a*l., 2009). We tested whether BRG1 (SNF2L/SMARCA4), one of the ATPases of the BAF complex, is also required for gene down-regulation. ChIPs experiments in cells exposed to hormone for 30 min showed a 2-3 fold-enrichment in BRG1 binding to *BCAS1*, KRT23 and *IGFBP5* gene promoters (Figure 3A). In the *BCAS1* gene, which showed a faster kinetics of PR binding compared to *KRT23,* recruitment of BRG1 is observed already after 1-2 min of hormone exposure (Figure S5A and B). To examine whether PR is required for genomic targeting of BRG1 to repressed genes we performed ChIPs assays in wild type T47DML as well in T47DY cells that express very low levels of both PR isoforms (Horwitz et al., 1995) but similar levels of BRG1 (Figure 3B, right panel). The hormone-dependent gene repression of *BCAS1*, KRT23 and *IGFBP5* as well as the recruitment of BRG1 was not observed in T47DY cells (Figure S5C and 3B left panel).

As in the presence of hormone we found simultaneous recruitment of the repressive complex and chromatin remodelers to the repressed genes (Figure 2), we explored whether these proteins could form a complex. Co-immunoprecipitation experiments using HP1γ specific antibodies showed that HP1γ associates with activated PR (Figure 3C upper panel) and with components of the LSD1.com complex, including LSD1, HDAC1, CoREST (Vicent et al., 2013). BRG1 interacts with the HP1γ repressive complex, especially after hormone exposure (Figure 3C, lower panel first row from the top). Unexpectedly, other subunits of the BAF complex, such as BAF170, BAF57 and BAF155, did not associate with HP1γ and were not recruited after hormone to repressed genes (Figure 3C, lower panel, second and third rows, Figure S6A and data not shown). In addition, we performed IP of LSD1 followed by mass spec. In the IPs of LSD1 from hormone-treated cell extracts we detected the core components of the LSD1.com repressive complex (LSD1, COREST, BRAF35, HDAC1 and HDAC2) as well as PR, HP1γ and BRG1 (Supplementary Table 2). Neither BAF170 nor BAF155 were detected in these assays.

To further validate the *in vivo* interaction between BRG1 and the HP1γ repressive complex, protein-fractionation of hormone-treated T47D cell lysates were carried out by FPLC with Superose 6 sizing column. Native BRG1 co-fractionated with BAF170 with an apparent molecular mass >1MDa (Figure 3D, upper panel), as previously reported for the SWI/SNF complex (Wang et al., 1996a; Wang et al., 1996b). However, BRG1 also eluted in a smaller complex with a molecular weight >669KDa (Figure 3D, upper panel) as reported previously (McKenna et al., 1998). IP of BRG1 containing fractions (Figure 3D, left) detected BAF170 and BAF155 in the heavy F1 fraction but not LSD1 or HDAC1, members of the LSD1.com repressor complex (Figure 3D, lower panel). Conversely, in the >0.67MDa F2 fraction we found BRG1 and members of the LSD1.com repressor complex but not with BAF (Figure 3D, lower panel). Thus, these results are compatible with the existence of a complex of BRG1 with the HP1γ-LSD1.com in T47D cells.

To explore whether other subunits of the BAF complex are required for hormone-dependent gene repression, T47DML cells were transfected with control siRNAs and with siRNAs against BRG1, or BRM, BRG1 and BRM (B/B), or BAF170, or BAF155 and treated with hormone (Figure 3E-G). We found 1.5 to 2-fold decrease in repression of *BCAS1*, *KRT23* and *IGFBP5* in BRG1-depleted cells compared to control cells (Figure 3E). In contrast, depletion of BAF 170 or BAF 155 did not affect

hormonal down-regulation of the same genes (Figure 3F), confirming our previous results (Figure 3C). No significant change in the basal levels of the repressed genes was observed after BAF155, 170 or BRG1 knockdown (Figure S4C).

BRG1 and BRM are highly homologous ATPases that can partly compensate for each other in T47D-MTVL cells. Depletion of BRM increases BRG1 levels and *viceversa* (Vicent *et al*., 2009). We therefore explored whether BRM is involved in hormone-dependent gene repression. We found that BRM is not part of the repressive complex and is not recruited to *KRT23* and *IGFBP5* genes after hormone (Figure S6B). In *BCAS1* BRM was only found after 5 and 60 min of hormone, thus exhibiting kinetics distinct from those of PR and BRG1 (Figure S6B compare with S5A and B). Unexpectedly, however depletion of BRM significantly affected hormone repression of *BCAS1*, *KRT23* and *IGFBP5* (Figure 3F and S6C). We therefore explored whether knockdown of BRM changed the levels or distribution of BRG1. By sequential co-IPs experiments with control and BRM siRNAs transfected cell extracts (Figure S6D) we found that depletion of BRM increased the amount of BRG1 in the BAF complex compared with siControl (Figure S6D, upper panel), while it reduced the BRG1 associated with HP1γ (lower panel). These findings provide a plausible explanation for the effect of BRM depletion on active repression (Figure 3F and S6C). The strongest effect on hormone repression was observed when both ATPases BRG1 and BRM were simultaneously depleted (Figure 3G, B/B), in agreement with a more effective reduction of the levels of BRG1 under these conditions. No significant change in the basal levels of the repressed genes was observed after BRM or B/B knockdown (Figure S4C). Moreover, in the fraction of BRG1 that binds HP1γ and is free of other BAF subunits we found the components of the LSD1.com repressive complex HDAC1, LSD1 and CoREST (Figure S6E, right panel, lane 5), confirming previous results (Figures S6A and 3D).

In order to confirm that BRG1 is the ATPase involved in hormone-dependent repression, T47DML cells were transfected with control empty plasmid or a plasmid expressing a catalytically inactive BRG1 carrying a point mutation K798R in the ATP binding domain (Khavari et al., 1993). The expression of the repressed genes *BCAS1*, *IGFBP5*, *KRT23* was significantly affected by the presence of the K798R BRG1 mutant compared with the control transfected cells (Figure S6F), showing that the catalytically dead BRG1 behaved as dominant negative in active repression. Thus, in contrast to what was observed during hormone activation, only the ATPase BRG1 and not the whole BAF complex is involved in hormone-dependent gene repression (Figures 3C, 3F and S6A).

To find out whether the effect of BRG1 on hormone-dependent genes is general we analyzed transcriptional regulation genome-wide. T47D-MTVL cells transfected with control siRNA or with siRNAs against BRG1 and BRM were exposed to hormone and the extracted RNA was analyzed using a SurePrint G3 Human gene expression 8x60K array (Agilent). In cells transfected with a control siRNA, 2,500 genes (11.8% of all genes in the array) were hormone-regulated (Figure 4A, left panel). From these, 1362 (54.4%) were dependent on BRG1 and BRM for their activity (Figure 4A, second panel from the left). Interestingly, in 848 genes (62.3% of B/B affected genes) BRG1/BRM was associated to gene repression (Figure 4A, third panel from the left). Moreover, 744 out of the 847 genes (87.7%) correspond to down-regulated genes in siControl condition, which are not down-regulated in siB/B treated cells (Figure 4A, right panel). In absolute values and using the same number of genes for each class, we found significantly more down than up-regulated genes that are dependent on BRG1 (146 *vs* 75, $P = 3.098 \times 10^{-5}$).

We confirmed the implication of BRG1 in hormone-dependent gene repression by RNA-seq experiments in cells transfected with siControl and siBRG1. We found that 44% of the down and 26% of the up-regulated genes (256 and 236, respectively) were significantly affected by BRG1 knockdown (Figure S7A). The repressed genes are significantly more affected than up-regulated genes (*P*-value= $3.59E^{-14}$).

Interestingly, 52.8% of the genes affected by siBRG1 were also affected by siHP1γ (Figure S7B), suggesting that both proteins are involved in the repression of the same genes.
Analysis of the Gene Ontology (GO) categories revealed that the ATPase-dependent genes were primarily implicated in regulation of RNA splicing and processing, regulation of initiation, elongation and termination of translation, regulation of stem cell differentiation, programmed cell death, cell proliferation and cell cycle (Figure S7C). To examine whether BRG1 regulates growth of tumor cells, we monitored progestin-dependent cell proliferation and apoptosis of cells transfected with BRG1/BRM siRNAs. Compared with cells transfected with an unrelated control siRNA, progestin-induced proliferation is reduced by BRG1/BRM knockdown (Figure 4B) (fold change: 2.7 ± 0.1 *vs* 1.5 ± 0.2 for control and BRG1/BRM-depleted cells, respectively). In addition, BRG1/BRM knockdown also decreased staurosporine-induced apoptosis in T47DML cells (Figure S7D). These results are in line with our previous findings with SRA knockdown and confirm that SRA and BRG1 are part of the same repressive complex (Figure S4B) and (Vicent et al., 2013).

Genome-wide analysis of BRG1 recruitment to chromatin
Next, we analyzed the genome-wide distribution of BRG1 using ChIP-seq. We identified 2228 and 6180 BRG1 peaks before and after hormone exposure (30 min), respectively. The BRG1 peaks were located mainly in introns (58,1%), promoters (31.4%) and in intergenic regions (30.1 %) (Figure 4C). The average BRG1 intensity profiles showed a distribution that peaks at the center of PR binding regions especially after hormone exposure (Figure 4D). A 52.3% of the BRG1 binding regions overlapped with PR after hormone (Figure 4D, inset). When similar experiments were performed using the subset of PRBs associated to regulated and non-regulated genes (window: -10kb from the TSS/+5kb from the TTS), a significant enrichment of BRG1 was observed in both up-regulated and down-regulated genes *vs* non-regulated genes (Figure 4E). The sites where PR will bind after hormone exposure are already weakly enriched in BRG1 prior to hormone, and hormone causes a significant accumulation of BRG1. These results confirm and generalize our finding that PR and BRG1 are recruited to genomic target regions of progestin-regulated genes.
To explore the existence of genomic regions where BRG1 can bind independently of other BAF subunits, we first analyzed published BRG1, BAF170 and BAF155 ChIP-seq data (Euskirchen et al., 2011). We found 2218, 505 and 411 regions where BRG1 can bind independently of BAF170, BAF155 or both, respectively (Figure S8A-C, Venn diagrams). Importantly, the BRG1 exclusive regions are associated with lower RNApol-II and poorly expressed genes, compared with BAF-associated regions, which support its repressive role (Figure S8A-C, right panels). We carried out similar experiments in T47D cells for BAF170 before and after hormone exposure for 30 min. We found 1662 BRG1 exclusive regions independent of BAF170 (Figure 4F, left panel, $P<1x10^{-5}$). A higher proportion of these were associated to repressed genes compared to BRG1-BAF170 bound regions or random regions, whereas sites with BRG1 and BAF170 were underrepresented in down-regulated genes (Figure 4F, right panel).

Histone H1.2 is loaded around the TSS of hormone-repressed genes
Since depletion of histone H1 and H2A/H2B have been reported on hormone up-regulated genes (Vicent *et al*., 2011; Vicent *et al.*, 2004) (Figure 5A, *MMTV*), we analyzed core and linker histones using ChIP in down-regulated genes. On the promoters of the repressed genes, *BCAS1* and *KRT23* hormone exposure did not affect significantly H2A and H4 content, but increased around 2-fold the H1 content (Figures 5A). Given that breast cancer cells depleted of individual histone H1 variants exhibit specific phenotypes (Sancho *et al.*, 2008), and that the only available

ChIP-quality antibodies are against histone H1.2 (Millan-Arino et al., 2014), we used this antibody for ChIP-seq experiments in naive cells and cells exposed to hormone. Analysis of the fold change in H1.2 reads around the TSS showed a significant enrichment in down-regulated genes compared with up-regulated or non-regulated genes (Figure 5B). Box plots of the fold change of the RNApol-II reads in the corresponding regions showed the inverse behavior (Figure 5B, right panel, DOWN *vs* other categories, $p<0.05$).

For analysing other somatic histone H1 variants we used T47DML-derived cell lines stably expressing physiological levels of HA-tagged versions of each of the five somatic H1 variants (H1.0, H1.2, H1.3, H1.4 and H1.5) (see 'Materials and Methods' section) (Sancho *et al*., 2008). ChIP-seq experiments with an anti-HA antibody showed a significant increase of the ratio hormone treated/ untreated (T0) over the promoters of down-regulated genes in cells lines expressing tagged H1.2 (Figure 5C; down *vs* non-regulated $p= 2.5x\ 10^{-68}$). No comparable changes were found for H1.4 (p=0.029), H1.0 (p= *n.s*.), H1.3 (p=0.012*)* and H1.5 (p= *n.s.*) (Figure 5C). Therefore, the effect of hormone on histone H1 accumulation around the TSS of hormone-repressed genes is accounted mainly by the H1.2 variant. H1.2 deposition is significantly increased in repressed genes, which presented PRBs in their promoters compared with those lacking PRBs (Figure 5D). These findings favor the concept that PR participates in targeting the machinery for deposition of histone H1 to the promoters of hormone-repressed genes.

To confirm that histone H1.2 is required for hormone dependent gene repression, we used doxycycline inducible shRNAs for the depletion of individual H1 variants in T47D cells (Sancho *et al*., 2008). Specific depletion of H1.2 by addition of doxycycline for 6 days reduced by around two-fold the hormone-dependent gene repression of *BCAS1*, *KRT23* and *IGFBP5* genes (Figure 5E). As a control, depletion of H1.3 did not significantly affect hormone-dependent repression (Figure 5E). Knockdown of H1.4 could not be performed, since the cells die by necrosis (Sancho *et al*., 2008).

BRG interacts with histone H1.2 and is required for its deposition and gene repression

Next, we want to address the mechanistic basis for the specificity for histone H1.2 during hormone-dependent active repression. As BRG1 is a key factor involved in histone H1 deposition (Figures 3, 4 and 6A) we hypothesized that BRG1 could in fact, interact selectively with H1.2. In co-IP experiments using hormone-treated cell extracts we found that H1.2 is associated with BRG1 (Figure 6A, left panel). This binding was specific for H1.2, as H1.3 and H1.5 failed to interact (Figure 6A, second and third row from the top). By using the reverse co-IP immunoprecipitating with the H1.2 antibody we confirmed the BRG1-H1.2 interaction (Figure 6A, middle panel). In fact, the association turned out to be hormone-dependent (Figure 6A, compare lanes 2 *vs* 3) and specific for BRG1, as BAF170 was not detected in this complex (Figure 6A, middle panel, second row). Therefore, the specificity of the active repression for H1.2 can be explained at least in part, by its preferential interaction with BRG1.Thus, BRG1 contributes to the repression through chromatin remodeling of target genes, mediated by its binding to FOXA1 and to the linker histone H1.2.

We next asked whether BRG1 is required for H1 deposition. To address this question, ChIP experiments using H1 antibodies were performed in cells transfected with control scramble siRNA or with BRG1/BRM siRNAs. In siControl cells we found 1.5 and 2.4 fold increase in H1 in the promoters of *BCAS1* and *KRT23* after 5 and 30 min of hormone exposure, respectively (Figure 6B, left and middle panels, siControl bars). In contrast, knockdown of BRG1/BRM inhibited hormone-dependent H1 loading to these two repressed genes (Figure 6B, siB/B bars). We extended our studies to other five hormone-repressed genes and observed a similar behavior

(Figure 6B, right panel). Thus, BRG1 is required for histone H1 loading to the promoters of hormone-repressed genes.

The BRG1-containing repressive complex enhances nucleosome occupancy and positioning around the TSS

We next asked whether BRG1-dependent H1 loading influences nucleosome positioning and dynamics around TSS. Wild type and BRG1/BRM-depleted T47DML cells treated or not with hormone were subjected to micrococcal nuclease (MNase) digestion of chromatin followed by high-throughput paired-end DNA sequencing (MNase-seq) (Figure S9A). The MNAse data showed the expected protection pattern around the TSS and gene body of previously reported genes (Buenrostro *et al*., 2013) coinciding with regions enriched in H2A.Z, H3K27ac and H3K4me3 (Figure S9A). To quantify the translational positioning of nucleosomes we define the nucleosome positioning score for a particular site as the fraction of nearby fragment midpoints (within 100 bp) that are within 15 bp of the site (Gaffney et al., 2012). The nucleosome positioning data presented a regularly spaced pattern around the TSS and gene body of the repressed genes *BCAS1*, *IGFBP5* and *PGR* (Figure 6C and S9B).

Next we calculated positioning scores for up- and down-regulated genes in a region between -800 +400 from the TSS and divided the nucleosome scores into quantiles (encompassing 5080 genes each) according to the magnitude of the hormonal effect. In the quantiles Q1 and Q4, clearly significant changes in the positioning scores were found in response to 30 min hormone exposure (Figure 6D, upper panel). In the first quartile Q1, which presented increased positioning scores after hormone, we found significantly more repressed than activated genes (154 down *vs* 103 up, Chi-square, $P<0.05$), while no significant differences where found in the Q4 quartile with decreased positioning scores (Figure 6D, lower panel). We then analyzed the effect of depleting BRG1 and BRM on the changes of nucleosome positioning. The observed increase in positioning after hormone in 154 repressed genes was not observed in BRG1/BRM depleted cells. In fact, we detected a significant reduction (Figure 6E). This subset of repressed genes is characterized by a significant dependence on BRG1 and BRM for their activity (38% affected *vs* 7% expected for random genes, Chi squared test: p-value= $2.2x10^{-16}$). Thus, hormone exposure increased nucleosome positioning around the TSS of repressed genes in an ATPase-dependent manner.

Next, we explored whether the increase in nucleosome positioning score in the down-regulated genes is accompanied by an increase in the nucleosome occupancy. To this end we calculated de number of nucleosome reads obtained after MNase digestion in the subset of 154 repressed genes before and after hormone exposure. We found an increase in nucleosome occupancy after hormone (Figure S10A, left) that was lost in BRG1/BRM depleted cells (Figure S10A, right). To explore whether this effect correlates with the extent of gene repression, down-regulated genes were divided in two subgroups according to the increase in nucleosome occupancy after hormone (Figure S10B). The group of genes with larger increased MNase reads around the TSS after hormone presented a significant stronger hormone-dependent gene repression compared to the genes with a weaker increase in MNase reads (HIGH and LOW, respectively in Figure S10B). Thus, the hormone, via the PR-associated BRG1, increased nucleosome positioning and occupancy around the TSS of the repressed genes.

BRG1-dependent gene repression directly affects the rate of transcription

As a proxy of transcription rates we used iRNA-seq that analyzes changes in levels of intron transcripts (Madsen et al., 2015). We found 599 and 263 genes up- and down-regulated, which overlap in iRNA-seq and microarray experiments. Notably,

the subset of down-regulated genes that was detected by iRNAseq were significantly more repressed by hormone (Figure S11A) and contained higher H1.2 that the set of genes selected using global RNA datasets (Figure S11B). These results indicate that the changes detected in target chromatin after hormone affect the rate of transcription.
Next, we asked whether BRG1 knockdown affected the levels of RNApol-II at the TSS in repressed genes. ChIPs experiments in the *BCAS1* gene in cells transfected with control siRNAs and exposed to hormone (30 min) showed 60-70% decrease in total and phosSer5 (initiation) RNApol-II compare to untreated cells (Figure S11C). Knockdown of BRG1/BRM significantly inhibited the hormone-dependent displacement of both total and initiating RNApol-II (Figure S11C, B/B bars). Thus, the ATPases BRG1 and BRM are necessary for the inhibition of transcription.

Down-regulated genes exhibit less accessible chromatin upon hormone exposure

The increase in H1.2 and nucleosome occupancy in repressed gene promoters suggests a more compact chromatin organization in these regions. We used a previously described DNAse I digestion assays on formaldehyde crosslinked chromatin (Di Stefano *et al*., 2014) to assess accessibility of DNA in chromatin. DNAse I digestion at the PRBs of the *BCAS1* gene promoter was significantly reduced in hormone-exposed cells compared with untreated cells (Figure 6F, left panel). This effect was not observed in another random region of the genome (Figure S12A). Similar studies were performed three other hormone repressed genes, *KRT23*, *IGFBP5* and *PGR,* by counting the reads obtained by DNAse-seq around PRBs in the absence and in the presence of hormone (Song and Crawford, 2010). While in up-regulated genes we detected an increase in DNAse cleavage after hormone, we found a significant decrease in the accessibility in the repressed genes (Figure 6G). Knockdown of BRG1/BRM abolished hormone-dependent decrease in DNAse I accessibility in *BCAS1* gene (Figure 6E, right panel). This effect was not due to a lower rate of transcription, since it was resitant to inihibition of transcription by either α-amanitin or DRB (Figure S12B, C and D). Moreover, the presence of α-amanitin or DRB did not affect the recruitment of PR and BRG1 (Figure S12E). Thus, the ATPases BRG1 and BRM are responsible for chromatin compaction around the TSS of the repressed genes likely via the deposition of H1.2.

The pioneer factor FOXA1 marks hormone-repressive promoters

One remaining question was how BRG1 and not BAF is recruited preferentially to repressed genes. Given that FOXA2 has been reported to interact with BRG1 (Li et al., 2012), we hypothesized that this behavior may also be shared by FOXA1, a close homolog involved in steroid receptor dynamics (Hurtado et al., 2011). A ChIP-seq analysis of FOXA1 binding in T47D cells showed enrichment at the promoters of hormone-repressed genes (Figure 7A, window: -/+3kb around TSS $p=5.1x10^{-5}$). In the absence of hormone, FOXA1 is significantly enriched at the PRBs and BRG1 sites in the repressed genes compared with up and non-regulated genes (Figure 7B and Figure S13A and B). Notably, in cells depleted of FOXA1, hormone repression of *BCAS1, KRT23 and IGFBP5* genes was compromised (Figure 7C) and BRG1 binding to the repressed genes was abolished (Figure 7D).
To assess the global transcriptional impact of FOXA1 in the hormonal response we performed RNA-seq analysis in cells transfected with siControl and siFOXA1. The percentage of genes affected by the siFOXA1 was greater for down than for up-regulated genes (68.5% *vs* 48.3%, which corresponds to 398 and 429 genes, respectively) (Figure S13C). Down-regulated genes are significantly more affected than up-regulated genes (*P*-value= $5.7E^{-12}$) and 80% of the repressed genes affected by siHP1γ where also affected by siFOXA1 (Figure S13D). Notably, 124 down-

regulated genes were affected by knockdown of HP1γ, BRG1 and FOXA1, supporting a common function of these three proteins in hormone-dependent gene repression (Figure S13D, right panel). By co-IP we found that FOXA1 and BRG1 interact in breast cancer cells particularly after hormone exposure (Figure 7E, compare lanes 3 *vs* 4, upper panel and 1 *vs* 2, lower panel). PR was also found associated to FOXA1 (Figure 7E). Thus, although we cannot rule out that other factors are involved, our data support a role of FOXA1 in targeting BRG1 to hormone repressed genes.

Discussion

In the present report we identify a subset of progestin target genes involved in proliferation, apoptosis and cell fate commitment in breast cancer cells that are actively repressed by hormone. Repression requires binding of activatied PR to target sites in the gene promoters similar to those involved in gene activation. In addition to ERK and MSK1 kinases, the activated PR recruits a repressive complex composed of HP1γ, histone demethylases, histone deacetylases, the SRA RNA and the ATPase BRG1. The BRG1 in the repressive complex increases linker histone H1.2 deposition and nucleosome occupancy, leading to chromatin compaction around the TSS that hinders RNA-pol-II loading and maintenance of PR binding (Figure 8).

Comparison with the initial steps of hormone-dependent gene activation

We found that some early steps of hormonal gene regulation are shared between activation and gene repression, while others are not. In contrast to what has been reported for glucocorticoid receptor, which recognizes specific negative GREs near repressed genes (Surjit et al., 2011), the sequences of the PRBs of repressed genes are indistinguishable from the PRBs in hormone-activated genes (Ballare et al., 2013b). Moreover, as in hormone activated genes (Vicent et al., 2006), the PR that binds repressed genes is phosphorylated in S294 and S400 and associated with the activated kinases ERK and MSK1 . However, the location of PRBs and the kinetics of PR loading are different on repressed and activated genes. While the PRBs responsible for hormonal gene activation are preferentially located in enhancer regions at a distance from the induced genes, the PRBs involved in repression are close to the TSS of the target genes. In hormone activated genes PR binding increases continuously until 60 min and is maintained to 6 hours, while PR binding in repressed genes peaks at 30 min and decreases thereafter. This indicates that for mounting a repressive complex a shorter PR occupancy near the gene promoter is sufficient.

We previously reported that a repressor complex containing HP1γ (HP1-LSD1.com) and recruited by unliganded PR is involved in basal repression of genes that will be activated by hormone (Vicent et al., 2013). Notably, we now found that in response to hormone the ligand activated PR recruits a similar complex to repressed genes. There are also differences in the methyltransferases associated to the complexes recruited to activating and repressive PRBs. On activating PRBs PR recruits the ASCOM complex with the MLL2/3 methyltransferases that methylates histone H3K4 (Vicent et al., 2011), while in repressive PRBs PR recruits SUV39H2 that methylates histone H3K9 and stabilize HP1γ binding. No changes in either H3K27me3 or RING1b levels were observed after hormone exposure in this subset of actively repressed genes, pointing to a mechanism independent of the Polycomb complexes.

Another difference is the recruitment of CDK2 and PARP1, which are important for histone H1 displacement in hormone-activated genes (Wright et al., 2012), but are not found at repressive PRBs. Hormone activation also requires the histone acetyl

transferase PCAF that acetylates histone H3K14 anchoring the BAF complex (Vicent et al., 2009), but no changes in K14ac levels were detected after hormone exposure on repressive PRBs. Rather we observed deacetylation of K27 and K16 after hormone exposure in repressive PRBs, likely mediated by HDAC1 and HDAC2 present in the repressive complex (Vicent et al., 2013).
Finally, there are also important differences in the ATP-dependent complexes participating in hormone activation and repression. The NURF complex is important for histone H1 displacement in hormone-activated genes (Vicent et al., 2013), but we do not see a contribution to hormone repression (data not shown). Moreover, while the complete BAF complexes catalyzes histone H2A/H2B displacement, which is needed for gene activation (Vicent et al., 2013), only the ATPase BRG1 is involved in gene repression and catalyzes H1.2 deposition.

Only BRG1, the ATPase of the BAF complex is needed for repression
The chromatin remodeling complexes BAF and PBAF belong to the family of SWI/SNF and are composed of the BRG1 or BRM Snf2L ATPases and multiple additional subunits. Although full remodeling activity requires the complete BAF complex, BRG1 and BRM alone are sufficient to remodel nucleosomes *in vitro* (Phelan *et al*., 1999). During hormone-dependent gene activation PR associates with BAF and tethers it to the target chromatin, where it promotes the displacement of dimers of histones H2A and H2B (Vicent *et al*., 2009). This is in contrast with our present finding that in hormone repressed genes only the ATPase BRG1 but not of other subunits of BAF complex are recruited by PR to the promoter region. The ATPase activity of BRG1 but not other subunits of the BAF complex is needed for repression (Figure 3C-F).
BRG1 can be found at least in two large complexes, one associated with BAF and the other associated with HP1γ. We do not know how the distribution of BRG1 between BAF and the HP1γ complex is controlled. One possibility is differential acetylation of the ATPase. Acetylation of BRM by P300 has been reported to promote its displacement from the mSin3A/HDAC co-repressor complex (Zhang et al., 2010). Therefore, non-acetylated BRG1 could be associated with repressive complexes while its acetylated form could prefer the BAF or PBAF complexes. Other subunits of the PBAF complex including BAF180 and BRD7/9 also contain bromodomains, which can bind acetylated residues (Ho and Crabtree, 2010). We also found that BRG1 is phosphorylated in response to hormone (data not shown), and this could also influence its interactions.
The differences in the requirements for activating and repressing mechanisms could reflect features of the target chromatin prior to hormone exposure. Before hormone exposure, genes that will be activated by hormone are expected to be in a more 'closed' chromatin conformation, characterized, by presence of histone H1, low core histone acetylation and high H3K9me3, and can be occupied by unliganded PR associated with a HP1γ-containing repressive complex (Vicent *et al.*, 2013). Genes that will be repressed by hormone are expected to be initially in a more 'open' chromatin state, depleted of histone H1 with higher levels of core histone acetylation, kinases and H3K4me3, and occupied by RNA polymerase II and the basal transcriptional machinery (See Figure 8). It is possible that in order to activate silent genes the full remodeling activity of the NURF and BAF complexes are required, while to repress genes the remodeling capacity of the BRG1 ATPase may be sufficient, alone or in combination with SUV39H2 and H3K9 methylation. In support of this idea, gene activation in yeast requires most SWI/SNF subunits, while repression at the *SER3* gene is dependent primarily on the Snf2 ATPase (Martens and Winston, 2002). Finally, SWI/SNF recruitment can occur in two steps with a minimal complex containing BRG1 and/or BRM recruited first followed by recruitment of the entire SWI/SNF complex (Kadam *et al*., 2000). Thus, we can speculate that for

gene activation the two steps would be required, while during repression the first step would be sufficient. Notably, irrespective of whether the gene will be activated or repressed, BRG1-dependent chromatin remodeling events are required.

***How is BRG1 targeted to the promoters of repressed genes*?**
Given that BRG1 can be found in the BAF complex or associated with the repressing complex, the question arises as to how the targeting is controlled. Certainly PR is involved in targeting but cannot explain the distinction between activating and repressing PRBs. A possibility is that factors bound near repressive PRBs prior to hormone determine the nature of the recruited remodeling enzyme. In line with this idea, we found that the pioneer factor FOXA1 is enriched at the promoters of hormone repressed genes in the absence of hormone. Knockdown of FOXA1 compromised BRG1 recruitment and prevented hormone-dependent gene repression. Given that FOXA2 has been reported to interact with BRG1 (Li *et al*, Cell 2012), one can speculate that FOXA1 could contribute to BRG1 targeting, although we cannot exclude additional factors,

How does BRG1 mediate H1.2 selective deposition?
The major differences between histone H1.2 and other somatic H1 variants are found in the C-terminal tail, where unique post-translational modifications -including acetylation, phosphorylation and methylation- have been reported (Wisniewski *et al*., 2007). These differences may account for the selective binding of histone H1.2 to BRG1, but mutational studies will be required to address this possibility.
FRAP experiments with GFP tagged H1 somatic variants showed that H1.1 and H1.2 are the most mobile subtypes and exhibit low chromatin affinity and weak chromatin condensing activity (Th'ng *et al*., 2005), a behavior that was also observed *in vitro* (Clausell *et al*., 2009). H1.1 is not expressed in T47D cells, while H1.2 is expressed and enriched at chromosomal domains with low GC content and at lamina-associated domains rich in silenced genes (Millan-Arino *et al*., 2014). Depletion of H1.2 in T47D cells caused a general decrease in nucleosome spacing and cell cycle arrest in G1 (Sancho *et al*., 2008). Thus, in addition to its selective binding to BRG1 H1.2 exhibits specific properties in T47D cells.

A possible molecular mechanism of hormonal repression
One way genes can be actively repressed could be via chromatin compaction around the TSS. This can be obtained by localized deacetylation and methylation of core histones in specific residues. H3K9me3 and H3K27me3 are two marks that can be read by HP1 and PRC1 complex, respectively, promoting the formation of facultative heterochromatin and close chromatin structures, respectively. We found that the hormone-dependent recruitment of HP1γ to repressed promoters correlates with the accumulation of H1.2. Moreover, these two chromatin proteins have been reported to interact (Nielsen *et al*., 2001) possibly via the chromo domain of HP1 γ (Daujat *et al*., 2005). Moreover, the presence of HP1 γ and H1.2 in repressed genes depends on the ATPase activity of BRG1, which also promotes increased nucleosome occupancy. In fact, we found a significant overlapping between repressed genes dependent on BRG1 and genes exhibiting increased nucleosome-positioning score after hormone (27% *vs* 1% expected for random genes, Chi squared test p-value= 6.27×10^{-11}). Binding of histone H1.2 correlates with reduced accessibility to DNase I, shortening of the residence time of the PR, and decrease binding of RNA-pol-II, thus diminishing gene transcription.
The mechanism of active repression found in T47D cells can also be observed in MCF7 breast cancer cells, which express both ER and PR. In preliminary experiments we found that two genes, which are repressed after progestin exposure in MCF7 cells (Figure S14F-G, and (Ansquer *et al*., 2005) and exhibit PRBs close to

the TSS (Mohammed *et al*., 2015) showed hormone-dependent recruitment of PR along with BRG1 and histone H1 (Figure S14H). This finding suggests that the proposed mechanism of active repression operates also in a different luminal epithelial breast cancer cell line.
In summary, our results uncover a novel mechanism by which a significant number of functionally relevant genes are down-regulated by hormone in breast cancer cells. The factors involved in this process could be potential targets for the management of hormone-dependent cancers.

Materials and Methods

Cell Culture and hormone treatments

T47D-MTVL breast cancer cells carrying one stably integrated copy of the luciferase reporter gene driven by the MMTV promoter (Truss et al., 1995) were routinely grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For the experiments, cells were plated in RPMI medium without phenol red supplemented with 10% dextran-coated charcoal treated FBS (DCC/FBS) and 48 h later medium was replaced by fresh medium without serum. After 24h in serum-free conditions, cells were incubated with R5020 (10nM) for different times at 37ºC.
H1.2 and H1.3 knock-down cell lines as well as stable HA-tagged H1 variants cell lines were established from T47D-MTVL cells (Sancho et al., 2008). In order to induce depletion of the different H1 variants doxycycline (Sigma) was added at 2.5 μg/ml for 6 days, the cells were passaged at day 3 and then H1 levels was monitored by Western blot with specific antibodies (Figure 5E).

Chromatin Immunoprecipitation (ChIP) in cultured cells

ChIP assays were performed as described (Strutt and Paro, 1999) using anti-PR (Santa Cruz, H190); anti-HP1γ (Millipore, Mab 3450); anti-BRG1 [EPNCIR111A] (ab110641) from Abcam, anti-LSD1, PRS400, H1.2, H3K9me3, H4K16ac and anti-HA from Abcam; anti-MSK1, BAF170, FOXA1 and BAF155 from Santa Cruz; anti-H1 (AE4, Millipore); anti-BRM (Active Motif); anti-PRS294 (Novus) and anti H2A (gift from Dr Stefan Dimitrov).
Quantification of chromatin immunoprecipitation was performed by real time PCR using Roche Lightcycler (Roche). The fold enrichment of target sequence in the immunoprecipitated (IP) compared to input (Ref) fractions was calculated using the comparative Ct (the number of cycles required to reach a threshold concentration) method with the equation $2^{Ct(IP)-Ct(Ref.}$ Each of these values were corrected by the human β-globin gene and referred as relative abundance over time zero. Primers sequences are available on request.

RNA interference experiments

All siRNAs were transfected into the T47D-MTVL cells using Lipofectamine 2000 (Invitrogen). After 48 h the medium was replaced by fresh medium without serum. After 16 hs in serum-free conditions, cells were incubated with R5020 (10nM) or vehicle (ethanol) for different times at 37ºC. The down-regulation of BRG1, BRM and HP1γ expression was determined by Western blotting. The down-regulation of SRA was determined by measuring the RNA levels using Real Time-PCR. Primer sequences are available on request. BRG1, BRM, HP1γ siRNAs were purchased from Dharmacon (Thermo Scientific); SUV39H2, BAF155, BAF170 and FOXA1 siRNAs were purchased from Santa Cruz.

RNA extraction and RT-PCR

Total RNA was prepared and cDNA generated as previously described (Vicent et al., 2006). Quantification of LUC and GAPDH gene products was performed by real time

PCR. Each value calculated using the standard curve method was corrected by the human GAPDH and expressed as relative RNA abundance over time zero. Primer sequences for *BCAS1, KRT23*, *IGFBP5*, *BRM* and *BRG1* are available on request.

Coimmunoprecipitation assay

Cells were lysed and cell extracts (1 mg protein) were incubated overnight with protein A/G agarose beads previously coupled with 3μg of the corresponding antibodies or an unspecific control antibody. The immunoprecipitated proteins (IPs) were eluted by boiling in SDS-sample buffer. Inputs and IPs were analyzed by western blot using ERK2, PRS294, PR, BRG1, BAF170, BAF155, coREST, HDAC1, BAF57, KDM1 and HP1γ specific antibodies.

Cell proliferation assay

T47DMTVL cells transfected with control or BRG1 and BRM siRNAs were cultured as described above. Cells ($1x10^4$) were plated in a 96-well plate in the presence or absence of 10 nM R5020. The cell proliferation ELISA BrdU Colorimetric assay (Roche) was performed according to the manufacturer's instructions. Figure 4B shows the percentage increase of proliferation in the presence versus absence of R5020. The experiments were performed in quintuplicate.

DNAse I digestion analysis

Chromatin samples obtained as described before from two biological replicas were subjected to DNAse I digestion. Briefly, 2 ug of chromatin were treated with 0.15 and 0.4 Units of DNAse I (Roche) for 3 min at 37ºC in 1X DNAse incubation buffer. Control samples were incubated in the absence of DNAse I. Reactions were terminated by addition of 40 mM EDTA final concentration and the crosslinking was reversed by incubating the samples at 65ºC. After 6 hs, Proteinase K (40 ug/ml final concentration) was added to each reaction and incubated overnight at 37ºC. After careful phenol-chloroform extractions, the DNA was quantified and used as template for Real Time-PCR reactions using specific primers.

Expression Arrays

RNA preparation and quantification of gene products from siControl and siB/B cells were performed as described (Vicent et al., 2006). Global gene expression assays were performed using Agilent Whole Human Gene Expression Microarrays 44K. Three independent samples were analyzed for each treatment. Genes were considered significantly regulated by hormones when expression changed **R**1.5-fold, relative to untreated samples (0 hr) and $p < 0.05$.

RNA-Seq

RNA was extracted from T47D-MTVL cells treated or not for 6hs with R5020 and submitted to massive sequencing using the Solexa Genome Analyzer. The sequence reads were aligned to the human genome reference (hg19), keeping only tags that mapped uniquely with up to two mismatches.

Micrococcal Nuclease (MNase)-seq

Mononucleosomal DNA from siControl and siB/B cells was prepared as described (Cappabianca et al., 1999). The obtained DNA was purified and subjected to deep sequencing using the Solexa Genome Analyzer.

ChIP-Seq

ChIP-DNA was purified and subjected to deep sequencing using the Solexa Genome Analyzer (Illumina, San Diego, CA). Single-ended sequences were trimmed to 50 bp and mapped to the human genome assembly hg19.

Extended bioinformatics methods for MNase-seq, ChIP-seq, RNA-seq and DNAse-seq experiments can be found in the Supplementary information.

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Figure Legends

Figure 1. **PR is recruited to hormone-repressed genes in breast cancer cells.**
(A) Differentially expressed genes, as detected both in RNA-seq and microarray experiments (fold induction threshold ≥1.4).
(B) Density of PRBS, defined as the total number of PR-peaks associated to each gene category (up-, down-, and non-regulated genes). PR-peaks were assigned to a gene when located from −5 Kb upstream of the TSS to the TTS (*left panel*) or to a window of -/+5Kb around the TSS (*right panel*). Peak density in the genome was calculated by considering random distribution of all PRBS in the whole genome. Data obtained after exposure to hormone for 5 and 30 minutes is shown. The p-values in comparison with the corresponding non-regulated genes are indicated. Left panel: for PRBs 5 min: *** P=1,04E-035 (UP), *** 2,40E-023 (DOWN); Right panel: PRBs 5 min: ** P=4,64E-008 (UP), * P=0.026 (DOWN).
(C) Heat Maps of the PRBS associated to up- and down-regulated genes. The log2 normalized signal enrichment centered on PRBs (T0 and R5') located from −5 Kb upstream of the TSS to the TTS of up- and down-regulated genes is shown (*upper panel*). *Lower panel:* The Average profile of PRBs at R5' associated to up-, down- and non-regulated genes is depicted.
(D) The graph shows the fraction of PRBs obtained after 5' of hormone as a function of the distance to a TSS of a gene up- or down-regulated (solid lines) compared to a random distribution (dashed lines).
(E) Kinetics of PR binding to up- and down-regulated genes. ChIP-seq data of PR obtained after 5, 30, 60 and 360 min of hormone exposure is plotted for up, down (window: -10kb +5kb around the gene) as well as for the proximal promoters of the down-regulated genes (window: -1kb +1kb around the TSS). *Lower Panel:* normalized enrichment at summits (+/-50bp) of PRBS R5' using the same set as shown in *upper panel*.

Figure 2. **Phosphorylated PR, ERK and MSK1 kinases along with the HP1γ-LSD1.com repressive complex are rapidly recruited to hormone-repressed genes in breast cancer cells.**
(A) Structure of *BCAS1*, *KRT23* and *IGFBP5* hormone-repressed genes. RNA-seq and PR ChIP-seq signals as well as the primers used in our study are shown.
(B) T47D-MTVL cells were untreated (0) or treated for 6 hours with 10 nM R5020, then the cells were lysed and total RNA was prepared, cDNA was generated and used as template for real time PCR with specific primers for *BCAS1*, *KRT23* and *IGFBP5* genes. The values were normalized with GAPDH and represent the mean and standard deviation from 3 experiments performed in duplicate. * *P*-value < 0.05.
(C) T47D-MTVL cells were untreated or treated for 5 and 30 min with hormone and submitted to ChIP assays with anti-PRS294phos antibody or IgG as control. The

histograms show the mean ± SD of 3 experiments performed in duplicate. * *P*-value < 0.05.
(D) ChIP assays with α-MSK1 and α-ERK2. Precipitated DNA was analysed by PCR for the presence of sequences corresponding to the *BCAS1* and *KRT23* genes. * *P*-value < 0.05.
(E) T47D-MTVL cells were untreated (0) or treated for 5 min with 10 nM R5020 and subjected to ChIP assays with α-PR, α-LSD1, α-HP1γ and H3K9me3 specific antibodies. The precipitated DNA fragments were subjected to PCR analysis to test for the presence of sequences corresponding to the *BCAS1*, *KRT23* and *IGFBP5*. The values represent the mean and standard deviation from 3 experiments performed in duplicate. * *P*-value < 0.05.
(F) T47D-MTVL cells transfected with control or HP1γ siRNAs, were incubated with 10 nM R5020 for 6h. RNA was extracted, and cDNA was generated and used as a template for real-time PCR with *BCAS1*, *KRT23*, *IGFBP5*, and GAPDH specific primers. The histograms show the mean ± SD of 3 experiments performed in duplicate. The levels of HP1γ and tubulin were analyzed by western blotting using specific antibodies. * *P*-value < 0.05.
(G) T47D-MTVL cells transfected with control or HP1γ siRNAs, were incubated with 10 nM R5020 for 6h and RNA-seq experiments were performed as described in methods section. The differentially expressed genes where considered when presented a fold change greater than 1.5 and *P*-value <0.05.

Figure 3 **BRG1 interacts with the HP1γ-LSD1.com complex and is required for hormone-dependent active repression.**
(A) T47D-MTVL cells were treated with R5020 as indicated and subjected to ChIP assays with α-BRG1 or control IgG. Precipitated DNA was analysed by PCR for the presence of sequences corresponding to the *BCAS1*, *KRT23* and *IGFBP5*. The histograms show the mean ± SD of 3 experiments performed in duplicate. * *P*-value < 0.05.
(B) T47D-MTVL or T47DY (PR_A-/PR_B-) cells were treated or not with hormone and subjected to ChIP assays with antibodies to BRG1. * *P*-value < 0.05. The levels of PR and BRG1 were analyzed by western blotting using specific antibodies (*right panel*).
(C) T47D-MTVL cells treated or not with hormone were lysed and immunoprecipitated either with α-HP1γ specific antibody or mouse IgG. The immunoprecipitates (IP) were analyzed by immunoblotting with PRS294phos, PR, BRG1, BAF170, BAF57, KDM1 and HP1γ specific antibodies.
D) Protein-fractionation of hormone-treated T47D cell lysates by FPLC with Superose 6 sizing column was analyzed by immunoblot with BRG1 and BAF170 specific antibodies (*upper panel*). The BAF complex containing BRG1 and BAF170 as well as a smaller complex of BRG1 are indicated (*upper panel*). Then, IP of BRG1 in the BRG1 eluting fractions from the Superose 6 was performed followed by western blot with BRG1, BAF170, BAF155, LSD1 and HDAC1 (*lower panel*). The scheme with the protocol is shown (*left panel*).
E) T47D-MTVL cells transfected with control or BRG1 siRNAs were treated with 10 nM R5020 as indicated; cDNA was generated and used as template for real time PCR with gene-specific primers. The histograms show the mean ± SD of 3 experiments performed in duplicate. * *P*-value < 0.05. The extent of BRG1 knockdown was monitored by RT-PCR (pink column) and western blot *(right panel)*. *BC: BCAS1*, *KR: KRT23* and *IG: IGFBP5* genes.
F) Cells were transfected with siRNAs and treated with 10 nM R5020 as indicated, and the levels of repressed genes was measured by RT-PCR. The extent of knockdown was analyzed by western blotting using specific antibodies *(lower panel)*. * *P*-value < 0.05.

(G) Cells were transfected with control or BRG1 and BRM (B/B) siRNAs and treated with 10 nM R5020 as indicated, and the levels of repressed genes was measured by RT-PCR. * *P*-value < 0.05. *BC: BCAS1*, *KR: KRT23* and *IG: IGFBP5* genes.

Figure 4 **BRG1 is recruited to hormone-repressed genes involved in cell proliferation and apoptosis.**
(A) T47D-MTVL cells transfected with control siRNA or siRNA against BRM and BRG1 (B/B) were induced or not with hormone, and the extracted RNA was hybridized to a gene expression array. Analysis of microarray data found 1359 genes regulated by hormone affected by B/B knockdown *(middle panel)*. In 62% of them (847 genes) B/B is associated with gene repression *(right panel)*.
(B) B/B knockdown reduces R5020-induced cell proliferation of T47D-MTVL cells. T47DMTVL cells were transfected with control or B/B siRNAs and treated with 10 nM R5020 as indicated, and the cell proliferation ELISA BrdU Colorimetric assay (Roche) was performed. The percentage of increase in proliferation is shown for each condition. Bars represent mean ± SD (n = 5). * *P*-value < 0.05; ** *P*-value < 0.01; *** *P*-value < 0.001
(C) Based on the analysis of ChIP-seq experiments performed for BRG1 (see materials and methods), the number as well as the distribution of binding regions is shown.
(D-E) Genome-wide distribution of BRG1 around all PRBs or PRBs associated to up, down or non-regulated genes (Panels D and E, respectively). The overlapping between PRBs and BRG1 is also shown (Panel D, inset).
(F) The overlapping of BRG1 with BAF170 binding regions is shown for T47D cells (left panel). More regions of BRG1 alone were found associated with hormone-repressed genes compared with BRG1-BAF170 and random regions (right panel). * *P*-value < 0.05.

Figure 5. **Linker histone H1.2 is enriched at the promoters of hormone-repressed genes.**
(A) ChIP assays with α-H2A, α-H4 and α-H1. Precipitated DNA was analysed by PCR for the presence of sequences corresponding to the *BCAS1, KRT23 and MMTV* genes. * *P*-value < 0.05.
(B) Left panel: ChIP-seq experiment using H1.2 specific antibody in untreated and cells exposed to hormone. The ratio between +R5020/T0 around the TSS (-475 +400) of the up, down and non-regulated genes is shown. Right panel: down-regulated genes presented an inverse trend for RNA pol II signal around the TSS (-475 +400) after hormone compared with Up and down-regulated genes. (*) *P*-value < 0.05; (***) *P*-value < 0.001.
(C) ChIP-seq experiments in T47D-MTVL cells expressing HA-tagged versions of the different H1 isoforms in untreated and cells exposed to hormone. The median corrected ratio between +R5020/T0 around the TSS (-475 +400) of the up, down and non-regulated genes is shown. A significant increase of the signal for H1.2 after hormone exposure was found in down-regulated genes compared with up and non-regulated genes. The median-corrected ratio represents the R5020/T0 ratio corrected by the median values obtained from the TSS of non-regulated genes for each isoform. (***) *P*-values for H1.2 DOWN *vs* NON: $2.5x10^{-68}$, *P*-value: DOWN *vs* UP: $8.3x10^{-72}$, UP *vs* NON: $2.7x10^{-6}$.
(D) Hormone-dependent deposition of H1.2 was quantified in down-regulated genes, which presented PRBs (PRBs+) or not (PRBs-) in their promoter region. (*) *P*-value < 0.05.
(E) T47D-MTVL/H1.2 and H1.3 knock-down cell lines (Sancho et al., 2008) treated or not for 6 days with DOX to induce specific H1 depletion, were incubated for 6h with 10 nM R5020 as indicated; cDNA was generated and used as template for real time

PCR with gene-specific primers. The extent of knockdown was analyzed by western blotting using specific antibodies (right panel). (*) *P*-value < 0.05.

Figure 6. **BRG1-dependent Histone H1.2 loading to repressed genes correlated with increased nucleosome positioning, decreased RNA-pol II and less accessible chromatin upon hormone exposure.**
(A) T47D-MTVL cells treated or not with hormone were lysed and immunoprecipitated either with α-BRG1 specific antibody (left panel) or H1.2 (right panel). The immunoprecipitates (IP) were analyzed by immunoblotting with BRG1, H1.2, H1.3, H1.5 and BAF170 specific antibodies.
(B) T47D-MTVL cells transfected with control or BRM and BRG1 (B/B) siRNAs were induced or not with hormone, and subjected to ChIP assays with α-H1. Precipitated DNA was analysed by PCR for the presence of sequences corresponding to the *BCAS1, KRT23, IGFBP5, VAMP1, CCDC173 and RABD3* genes. The histograms show the mean ± SD of 3 experiments performed in duplicate. * *P*-value < 0.05.
(C) T47DML cells treated or not with hormone were subjected to micrococcal nuclease (MNase) digestion of chromatin followed by high-throughput paired-end DNA sequencing (MNase-seq). Snapshot of the genome browser for the *BCAS1* gene is depicted. The expected MNase protection pattern around the TSS and gene body is observed and coincides with regions enriched in H2A.Z, H3K27ac and H3K4me3.
(D) T47D-MTVL cells treated or not with hormone were subjected to MNase-seq. To quantify the translational positioning of nucleosomes we calculated positioning mean scores for up and down-regulated genes in a region between -800 +400 from the TSS of all protein coding genes (Gaffney et al., 2012). Genes with nucleosome scores were divided into four quantiles (Q) according to the effect of the hormone. (***) P-value < 0.001. Lower panel: Percentage of genes found in quantiles Q1-Q4. In Q1, which presented increased positioning scores after hormone we found significantly more repressed than activated genes, while no significant differences where found in the Q4 with decreased positioning scores. * *P*-value < 0.05.
(E) T47D-MTVL cells transfected with control siRNA or siRNA against BRM and BRG1 (B/B) were induced or not with hormone, subjected to MNase-seq and the positioning scores were calculated as previously reported (Gaffney et al., 2012). The increase in positioning after hormone in repressed genes found in Q1 was not observed when the ATPases are missing. (***) P-value < 0.001.
(F) T47D-MTVL cells transfected with control or B/B siRNAs were treated or not with hormone and subjected to DNAse I accessibility assays (see Materials and Methods section). Each value corresponds to the mean ± SD of 3 experiments performed in duplicate. (**) *P*-value < 0.01.
(G) The reads obtained by DNAse-seq (Song and Crawford, 2010) in T47D-MTVL cells around PRBs in the absence and in the presence of hormone were quantified and expressed as signal/bp (to account for peak length variation) for up and down-regulated genes. Up-genes: *CORO2B*, *KCNH1*, *KLF15* and *ATP10A*; down-genes: *KRT23*, *BCAS1*, *IGFBP5* and *PGR*. (*) *P*-value <0.05, ** *P*-value <0.01.

Figure 7. **The pioneer factor FOXA1 marks repressed genes.**
(A) T47D-MTVL cells treated or not with hormone were subjected to ChIP-seq assay with α-FOXA1 (see materials and methods). The number of genes with FOXA1 peaks found in different windows around the TSS is shown. Proximal promoter window: -/+ 1Kb around TSS. (*) *P*-value <0.05, ** *P*-value <0.01 and *** *P*-value <0.001.
(B) FOXA1 is significantly enriched at PRBs localized in repressed genes compared with up- and non-regulated genes. Lower Panel: heat maps of the results shown in the upper panel. (***) *P*-value < 10^{-7}.

(C) Cells were transfected with control or FOXA1 siRNAs and treated with 10 nM R5020 as indicated, and the levels of repressed genes were measured by RT-PCR. * *P*-value <0.05. *BC: BCAS1*, *KR: KRT23* and *IG: IGFBP5* genes. The extent of knockdown was analyzed by western blotting using specific antibodies *(right panel)*.
(D) T47D-MTVL cells transfected with control or FOXA1 siRNAs were induced or not with hormone, and subjected to ChIP assays with α-BRG1 and IgG as control. Precipitated DNA was analyzed by PCR for the presence of sequences corresponding to the *BCAS1, KRT23 and IGFBP5* genes. The histograms show the mean ± SD of 3 experiments performed in duplicate. * *P*-value < 0.05.
(E) T47D-MTVL cells treated or not with hormone were lysed and immunoprecipitated either with α-FOXA1 specific antibody (upper panel) or BRG1 (lower panel). The immunoprecipitates (IP) were analyzed by immunoblotting with BRG1, PR and FOXA1 specific antibodies. The inespecific band in the control IgG of the FOXA1 IP, which does not coincide with PR_B or PR_A is also shown in a shorter exposition (right panel).

Figure 8. **Model of active repression induced by hormones**. At T=0 hormone-repressed genes are active with RNA pol II and an open chromatin configuration decorated by acetylated histones and FOXA1. Upon hormone exposure the HP1γ-LSD1.com complex interact with the ATPase BRG1 and is actively recruited to the target genes along with the kinases ERK, MSK1 and CDK2, responsible for PR phosphorylation in S294 and S400. Once bound to the chromatin the complex promotes histone deacetylation, demethylation and chromatin remodeling *via* BRG1, which increase nucleosome positioning and occupancy. This arrangement of nucleosomes constitutes a better platform for linker histone H1.2 binding, and thus close the target chromatin decreasing RNApol II loading and transcription. The factors involved in hormone-dependent repressive complex anchoring to target genomic regions as 1) PR-Hormone Responsive Element DNA binding, 2) FOXA1-BRG1 interaction and 3) HP1γ-H3K9me3 binding are highlighted.

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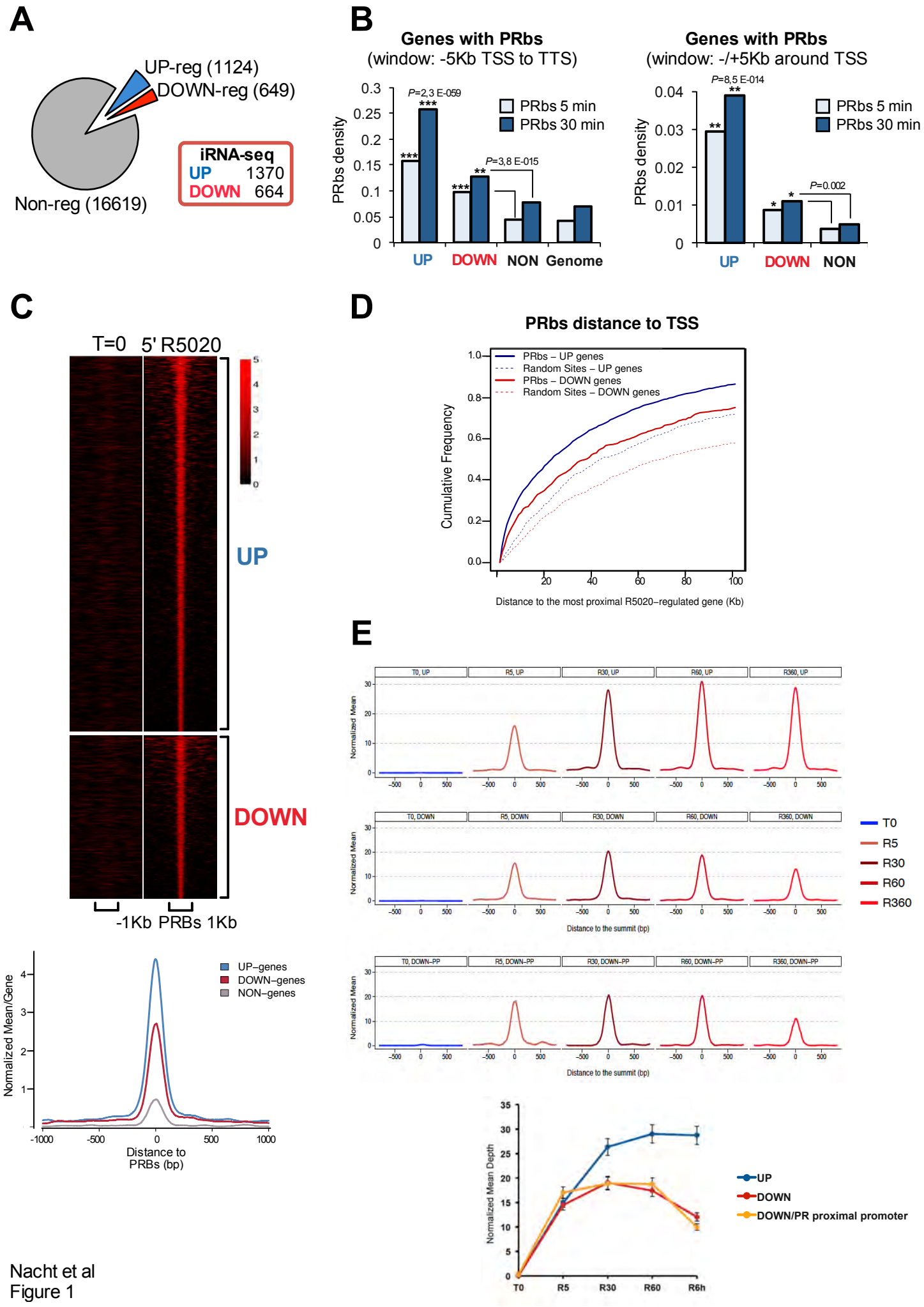
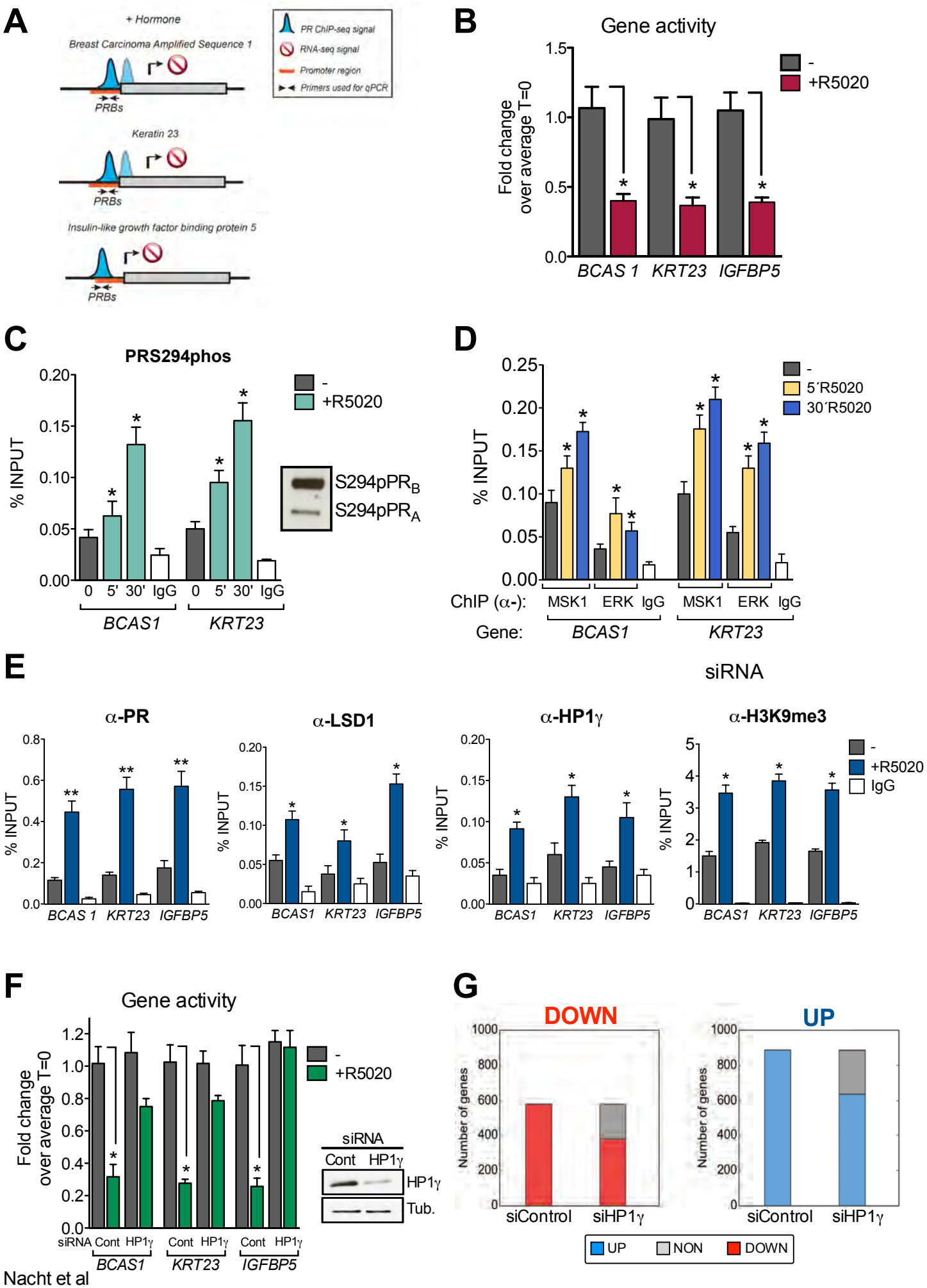


Nacht et al
Figure 1



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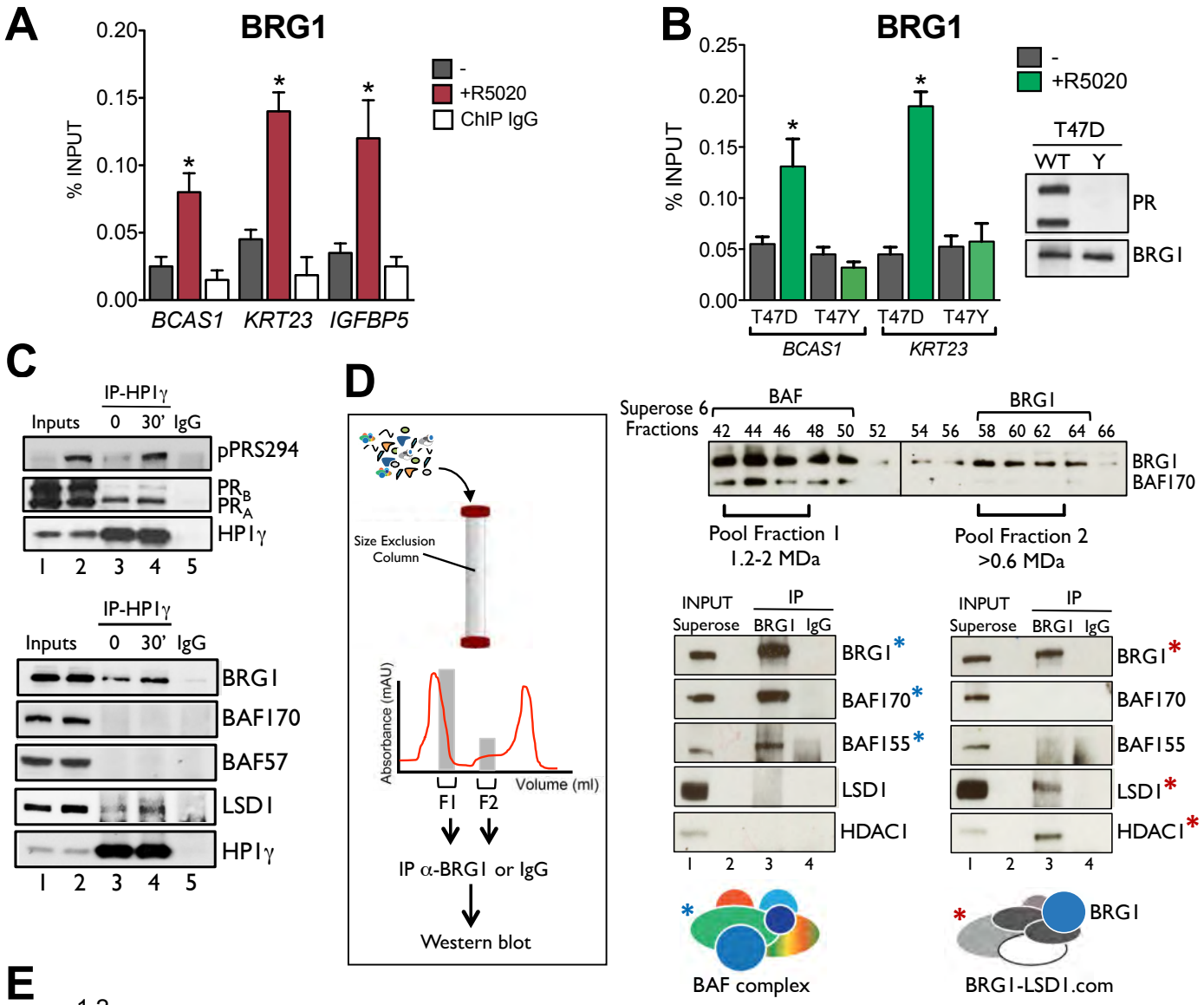

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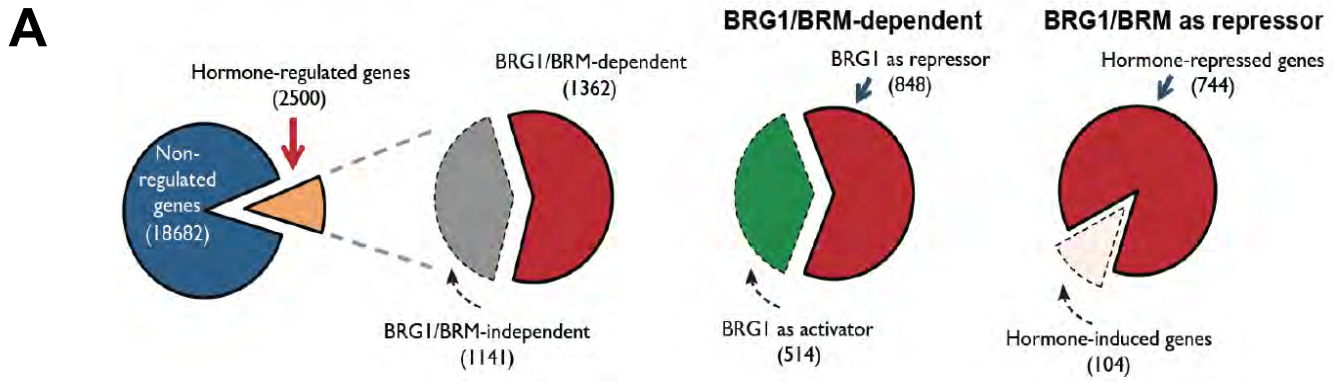
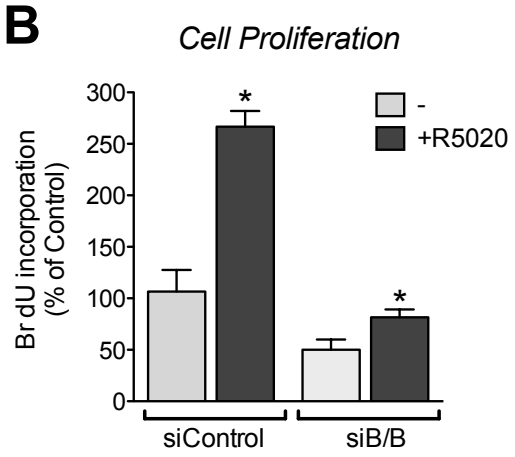
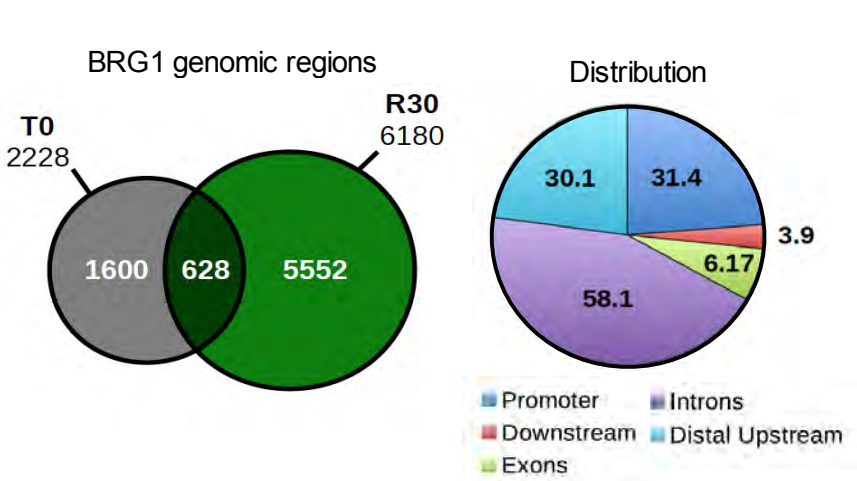
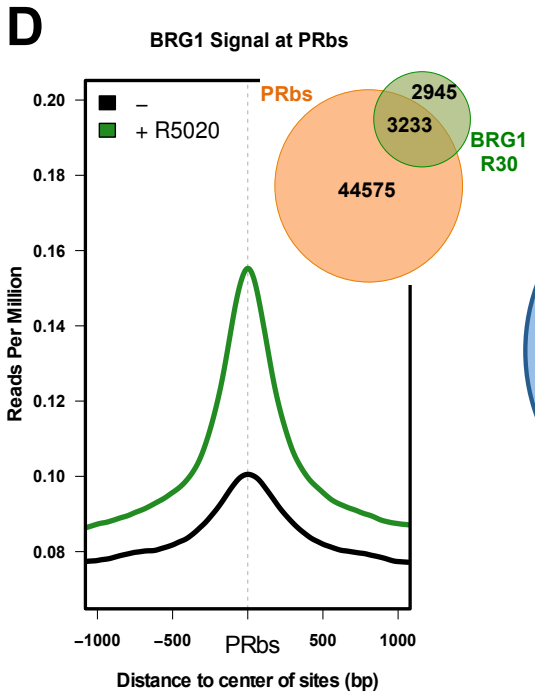
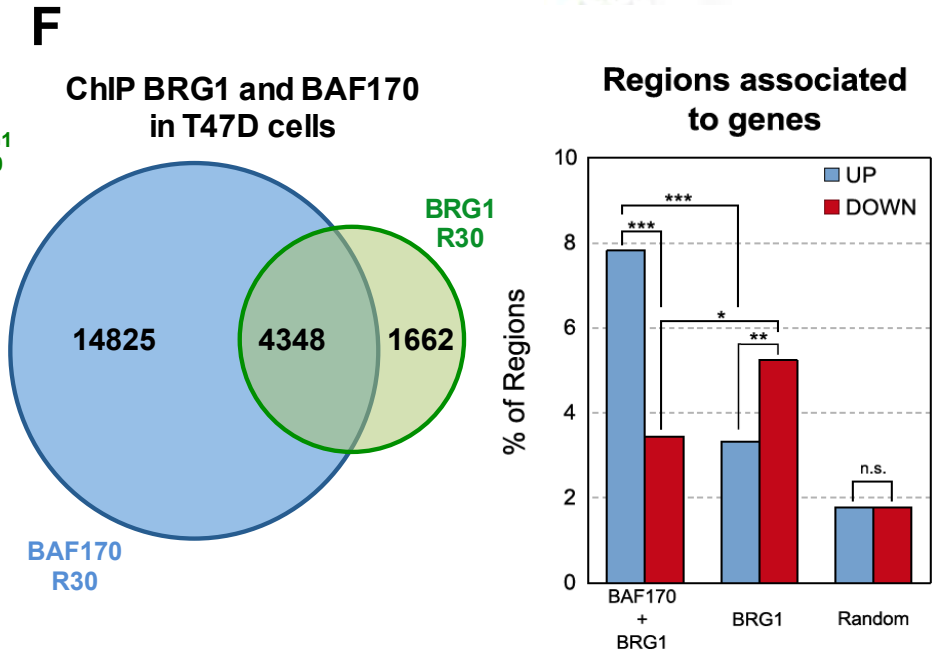
Figure 3

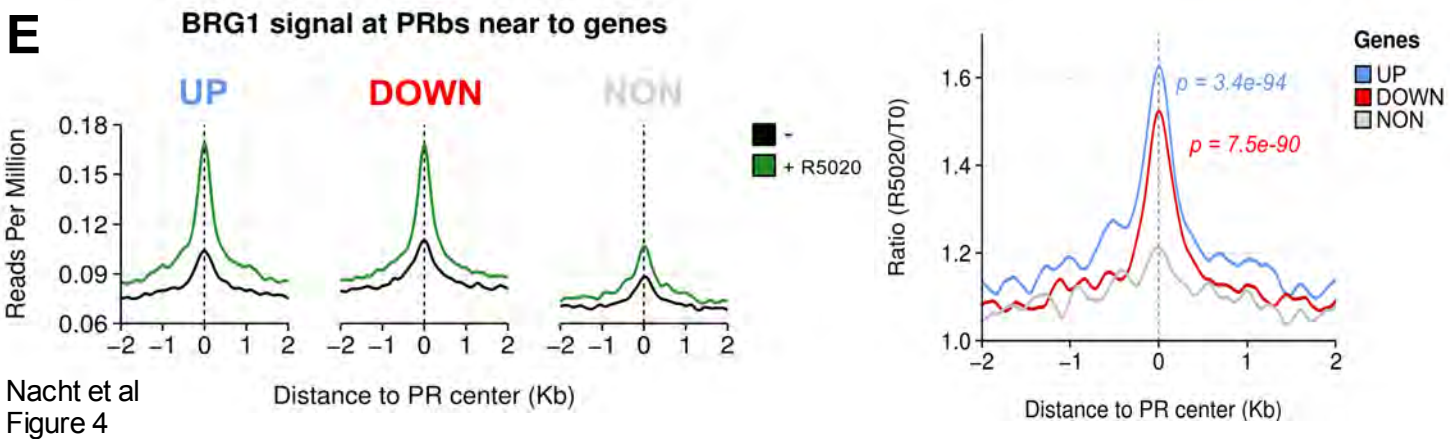




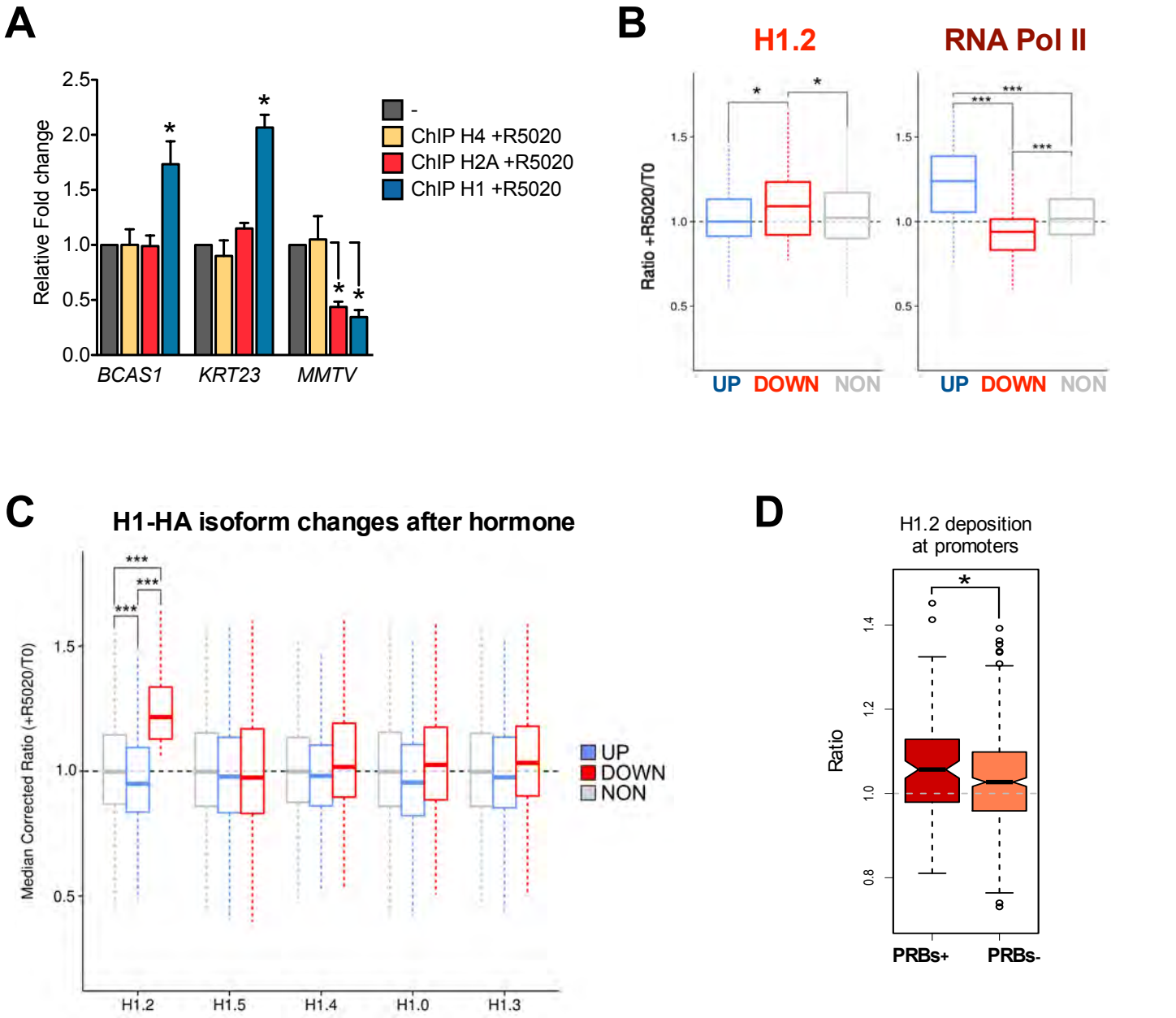




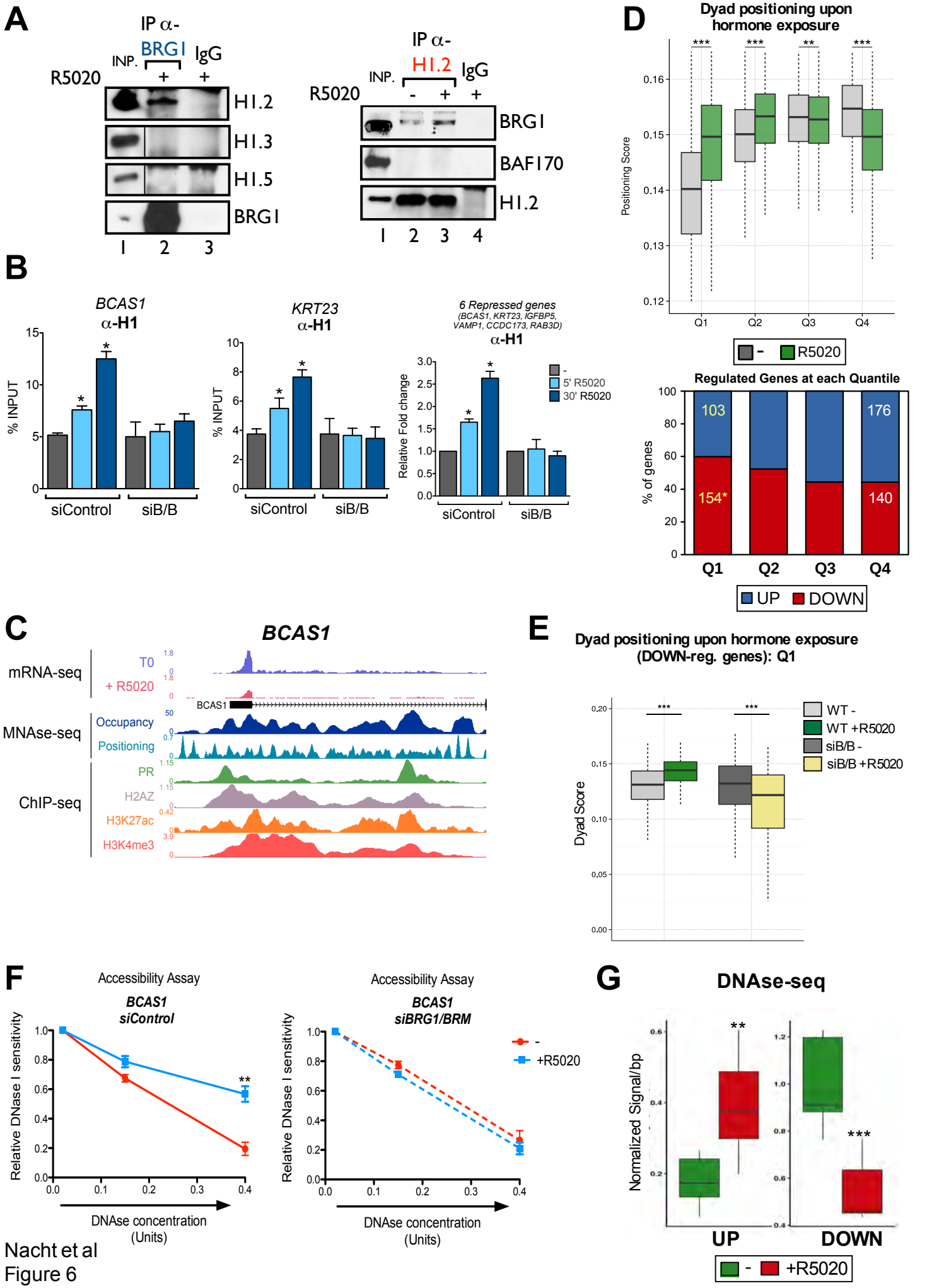




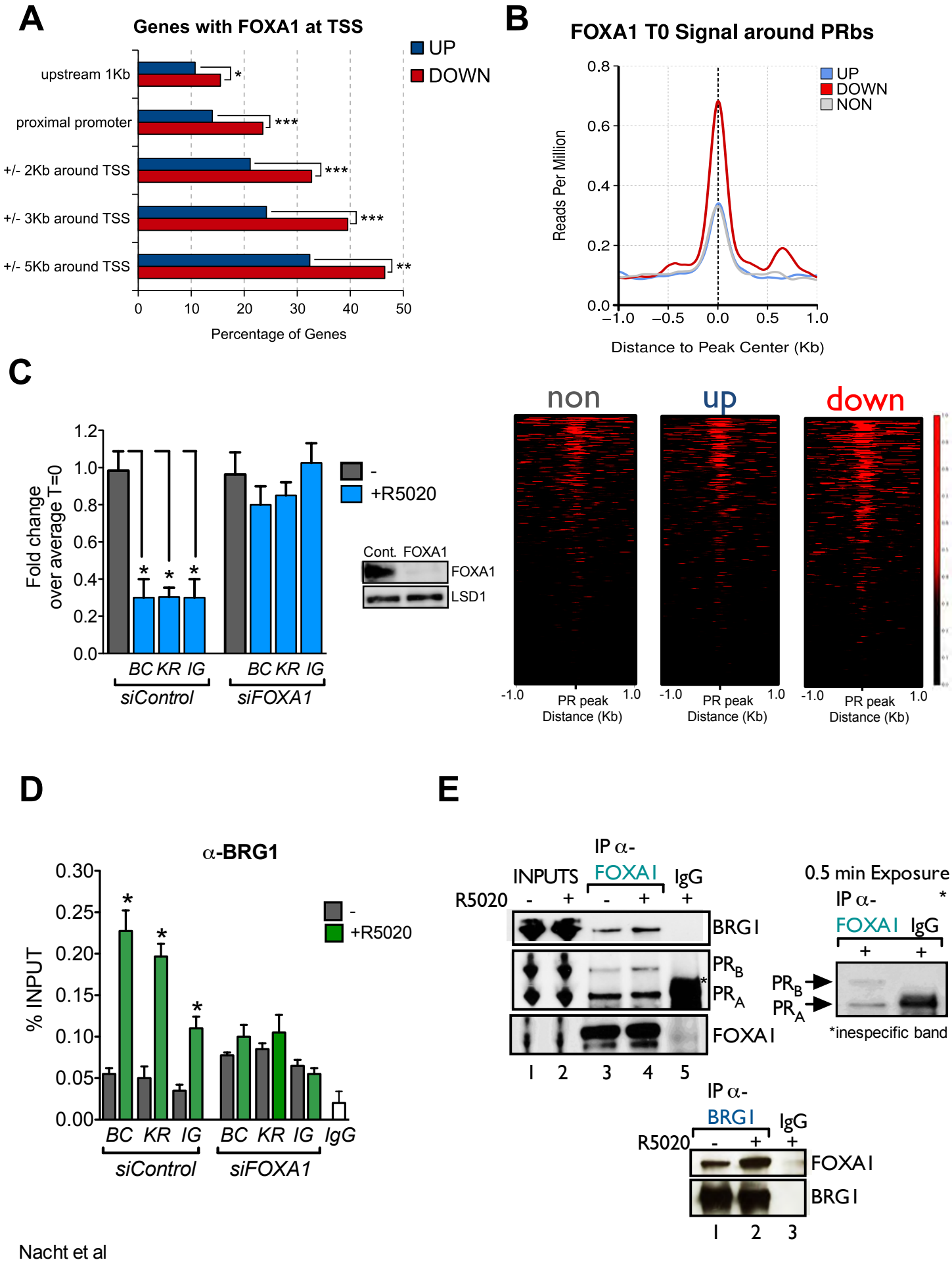
Nacht et al
Figure 4



Nacht et al
Figure 5



Nacht et al
Figure 6



Nacht et al
Figure 7

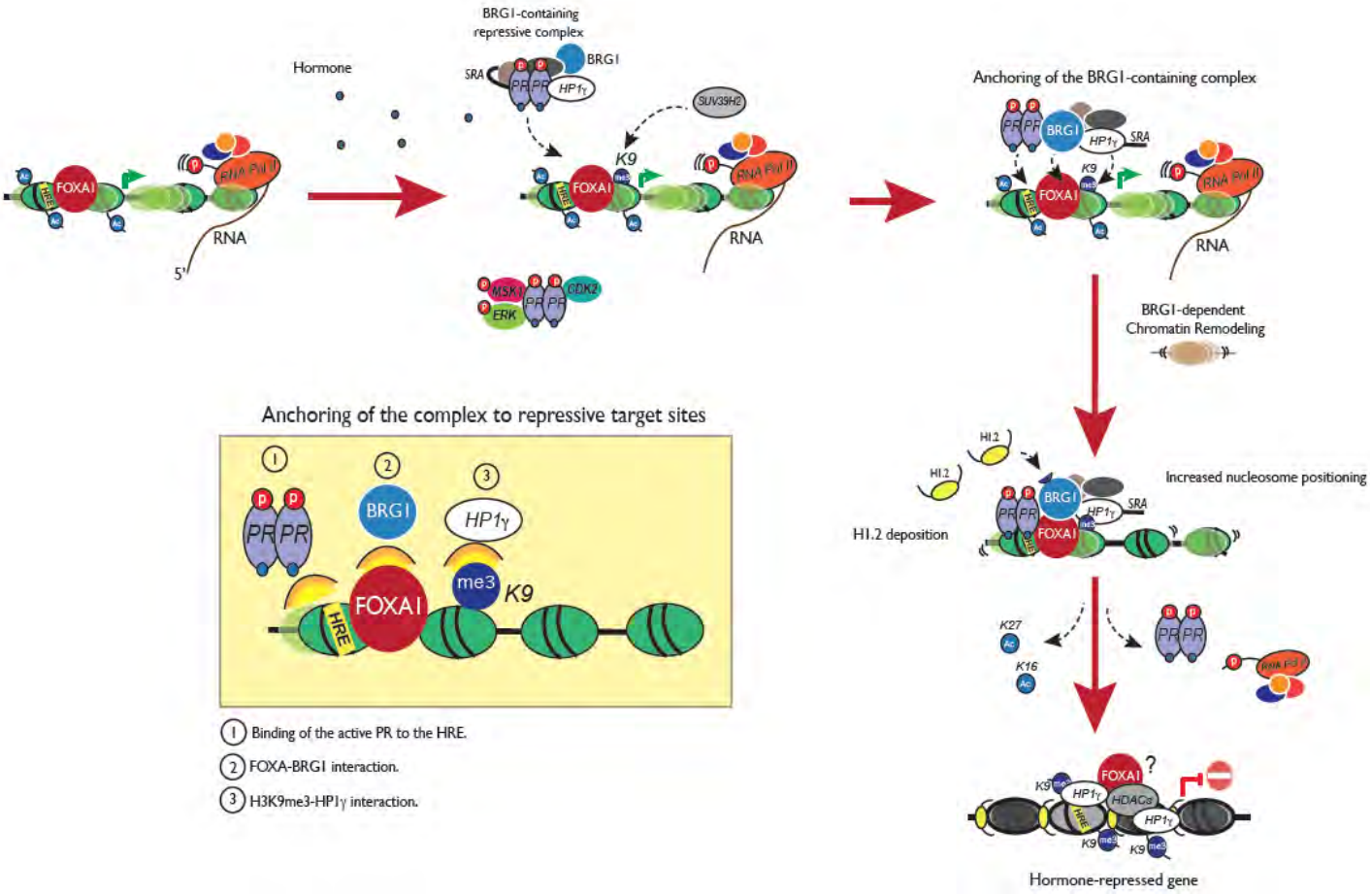


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Figure 8