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

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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 HP1 γ -LSD1 complex repressor complex, which is further anchored via binding of HP1 γ 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 progesterone regulation of breast cancer cell growth.

Keywords BRG1; chromatin remodeling; H1.2; hormone-dependent gene repression

Subject Categories Cancer; Chromatin, Epigenetics, Genomics & Functional Genomics; Transcription

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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 and 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 (Sera & Wolffe, 1998; Bustin *et al.*, 2005). The length, charge, and number of post-translational 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: (i) post-translational modification (PTM) of histones and (ii) 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 the 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

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complexes and to gene repression have not been defined (Ooi *et al*, 2006).

Hormone receptors (HRs) are ligand-regulated transcription factors, some of which have the capacity to bind their responsive elements in chromatin, thus operating as “pioneer” factors (Zaret & Carroll, 2011). In breast cancer cells, nucleosomal organization facilitates progesterone receptor (PR) binding and hormonal induction. PR association with histone tail modifiers and chromatin-remodeling complexes which interact with histones stabilizes PR binding (Ballaré *et al*, 2013a). A well-defined model system for studying these processes is the hormonal gene regulation of the mouse mammary tumor virus (MMTV), which is organized in positioned nucleosomes, with a single nucleosome containing the HREs and the NF1 binding site (Richard-Foy & Hager, 1987; Truss *et al*, 1995). We have reported that nucleosomes are in fact necessary for the synergistic function of PR and NF1 during activation (Chavez & Beato, 1997; Di Croce *et al*, 1999).

Incubation of T47D breast cancer cells containing a single copy of the MMTV with the progestin R5020 activates Src/Ras/Erk and the CDK2 cell signaling cascades, leading to activation of the MMTV promoter and progestin-induced cell proliferation (Migliaccio *et al*, 1998). Few minutes following hormone addition, two successive cycles of chromatin remodeling are carried out by a minimum of ten enzymes, which promote the displacement of H2A/H2B dimers and linker histone H1, 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 (Willmann & Beato, 1986; Schauer *et al*, 1989). In the absence of ligand, PR binds genomic sites and targets a repressive complex containing HP1 γ , 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 HP1 γ -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 physiological 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 has been linked to the repressive actions of progestins (Giangrande & McDonnell, 1999). However, all these mechanisms are a matter of debate as the 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 HP1 γ -LSD1 complex and BRG1. In contrast to what was observed during hormone activation (Vicent *et al*, 2009), only BRG1 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 (Ballaré *et al*, 2013b). For our study, we have focused on the set of 1,773 regulated mRNAs, which were found in expression microarrays as well as in RNA-seq experiments. Of these, 1,124 were up-regulated and 649 were down-regulated by hormone (fold change in each assay ≥ 1.4 , Figs 1A and EV1A). Box plots of the RPKM confirmed the

Figure 1. PR is recruited to hormone-repressed genes in breast cancer cells.

- Differentially expressed genes, as detected both in RNA-seq and microarray experiments (fold induction threshold ≥ 1.4).
- 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 $-/+5$ kb 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 min are 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), $***P = 2.40E-023$ (DOWN); right panel: PRBs 5 min: $**P = 4.64E-008$ (UP), $*P = 0.026$ (DOWN).
- 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.
- 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).
- 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- and down-regulated (window: -10 kb $+5$ kb around the gene) as well as for the proximal promoters of the down-regulated genes (window: -1 kb $+1$ kb around the TSS). Lower panel: normalized enrichment at summits (± 50 bp) of PRBS R5' using the same set as shown in upper panel. Data are represented as mean $\pm$ SD from three experiments performed in duplicate.

Figure 1.

expected significance of the hormone effects (Fig EV1B). 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 (Fig EV1C and Ballaré *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 1,370 and 664 up- and down-regulated genes, respectively (Fig 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 Materials and Methods) (Fig EV1D). 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: -5 kb from the TSS to TTS) (Fig 1B–D and see Appendix Table S1 as a summary of the genomic distribution of PRBs). These studies were performed with PRBs data detected after 5 and 30 min of hormone (Fig 1B). Interestingly, we found that the differences between the down-regulated and non-regulated genes were larger with the set of PRBs occupied at 5 min and located close to the TSS (Fig 1B right panel). In addition,

PRBs are localized more closely to the TSS of up- and down-regulated genes compared with random sites (Fig 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 (Fig EV1E). 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 (Ballaré *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 h of hormone exposure (Fig 1E, upper panel), occupancy of PRBs associated to down-regulated genes peaked at 30 min and decreased thereafter, reaching low values at 6 h of hormone exposure (Fig 1E, middle panel). A similar tendency was observed when the analysis was limited to sites around the TSS of the down-regulated genes (Fig 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 (Zhang *et al*, 1994, 1995; Weigel *et al*, 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*) (Fig 2A and Appendix Fig S1A). In RNA-seq assays performed after 1 and 6 h of hormone exposure, we found between 45 and 60% hormone-dependent reduction in the transcripts from these genes (Appendix Fig S1B). These results were validated by quantitative PCR (Fig 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 (Fig 2C and Appendix Fig S2A–C). By sequential ChIP (re-ChIP), we found

Figure 2. Phosphorylated PR, ERK, and MSK1 kinases along with the HP1 γ -LSD1 complex 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 h 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 three experiments performed in duplicate. * P -value < 0.05 using Student's t -test.
- 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 $\pm$ SD of three experiments performed in duplicate. * P -value < 0.05 using Student's t -test.
- D ChIP assays with α -MSK1 and α -ERK2. Precipitated DNA was analyzed by PCR for the presence of sequences corresponding to the *BCAS1* and *KRT23* genes. Data are represented as mean $\pm$ SD from three experiments performed in duplicate. * P -value < 0.05 using Student's t -test.
- E T47D-MTVL cells were untreated (0) or treated for 5 min with 10 nM R5020 and subjected to ChIP assays with specific antibodies for α -PR, α -LSD1, α -HP1 γ , and H3K9me3. 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 three experiments performed in duplicate. * P -value < 0.05 using Student's t -test.
- F T47D-MTVL cells transfected with control or HP1 γ siRNAs were incubated with 10 nM R5020 for 6 h. 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 $\pm$ SD of three experiments performed in duplicate. The levels of HP1 γ and tubulin were analyzed by Western blotting using specific antibodies. * P -value < 0.05 using Student's t -test.
- G T47D-MTVL cells transfected with control or HP1 γ siRNAs were incubated with 10 nM R5020 for 6 h, and RNA-seq experiments were performed as described in the Materials and Methods section. The differentially expressed genes were considered when presented a fold change > 1.5 and P -value < 0.05 .

Source data are available online for this figure.

Figure 2.

that S294 and S400 are both associated simultaneously to the same DNA region in the *BCAS1* and *KRT23* PRBs (Appendix Fig S2D). 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 (Appendix Fig S2E and Vicent *et al*, 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 (Fig 2D and data not shown). On the other hand, no increase in PR and ERK was detected in *TFAP2* and *BAZZ2B*, two hormone-repressed genes lacking PRBs in the promoter as well as in *RABEPK* and *PELO*, two non-regulated genes (Fig EV2A). Thus, the activated form of PR associated with the kinases ERK1/2 and MSK1 is recruited to both activated and repressed genes.

The HP1 γ -LSD1 complex is recruited to repressed genes and needed for repression

We wondered whether the repressive complex composed of HP1 γ , the LSD1 associated complex (LSD1 complex), 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 HP1 γ (Fig 2E). No recruitment of the repressive complex was observed in the control genes (Fig EV2A). Recruitment of the repressive complex was important for down-regulation, as depletion of either HP1 γ or the RNA SRA significantly reduced hormonal repression of these genes compared with control cells transfected with scrambled siRNAs (Figs 2F and EV2B). No significant change in the basal levels of the repressed genes was observed after HP1 γ or SRA knockdown (Fig EV2C).

To explore the generality of these observations, we performed RNA-seq experiments in cells transfected with siControl and siHP1 γ .

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, Fig 2G). The proportion of genes affected by HP1 γ knockdown was larger for down-regulated genes (35%) than for up-regulated genes (28%), and the magnitude of the effect was also significantly higher for down-regulated genes compared to up-regulated genes (P -value = 0.007). The 96% of the non-regulated genes were not affected by siHP1 γ (Fig 2G). Interestingly, 83.9% of the genes affected by siSRA in microarrays analysis were also affected by knockdown of HP1 γ , suggesting that the HP1 γ -LSD1 complex/SRA complex fulfills a repressive function after exposure to hormone.

As H3K9me3 is important for the localization of HP1 γ protein in the genome (Grewal & Jia, 2007), we asked whether it was also necessary for the recruitment of the HP1 γ complex to down-regulated target genes. We found increased H3K9me3 signal 5 min after hormone exposure in *BCAS1*, *KRT23*, and *IGFBP5* genes (Fig 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 HP1 γ binding on the target promoters (Fig EV2E and F) and interfered with the hormonal down-regulation (Fig EV2D). Moreover, we observed hormone-dependent recruitment of SUV39H2 to this set of repressed genes (Fig EV2G). No significant change in the basal levels of the repressed genes was observed after SUV39H2 knockdown (Fig EV2C). Thus, the HP1 γ 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 HP1 γ -LSD1 complex involved in hormonal down-regulation

The ATP-dependent remodeling complex BAF has been shown to be needed for progesterin gene activation and is recruited to PRBs via an interaction with PR (Vicent *et al*, 2009). We tested whether BRG1 (SNF2L/SMARCA4), one of the ATPases of the BAF complex, is also

Figure 3. BRG1 interacts with the HP1 γ -LSD1 complex and is required for hormone-dependent active repression.

- T47D-MTVL cells were treated with R5020 as indicated and subjected to ChIP assays with α -BRG1 or control IgG. Precipitated DNA was analyzed by PCR for the presence of sequences corresponding to the *BCAS1*, *KRT23*, and *IGFBP5*. The histograms show the mean $\pm$ SD of three experiments performed in duplicate. * P -value < 0.05 using Student's t -test.
- T47D-MTVL or T47DY (PR $_{A}$ -/PR $_{B}$ -) cells were treated or not with hormone and subjected to ChIP assays with antibodies to BRG1. The histograms show the mean $\pm$ SD of three experiments performed in duplicate. * P -value < 0.05 using Student's t -test. The levels of PR and BRG1 were analyzed by Western blotting using specific antibodies (right panel).
- T47D-MTVL cells treated or not with hormone were lysed and immunoprecipitated either with α -HP1 γ -specific antibody or mouse IgG. The immunoprecipitates (IP) were analyzed by immunoblotting with specific antibodies for PRS294phos, PR, BRG1, BAF170, BAF57, KDM1, and HP1 γ .
- Protein fractionation of hormone-treated T47D cell lysates by FPLC with Superose 6 sizing column was analyzed by immunoblot with specific antibodies for BRG1 and BAF170 (upper panel). The BAF complex containing BRG1 and BAF170 as well as a smaller complex of BRG1 is 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).
- 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 $\pm$ SD of three experiments performed in duplicate. * P -value < 0.05 using Student's t -test. 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.
- Cells were transfected with siRNAs and treated with 10 nM R5020 as indicated, and the levels of repressed genes were measured by RT-PCR. The extent of knockdown was analyzed by Western blotting using specific antibodies (right panel). The histograms show the mean $\pm$ SD of three experiments performed in duplicate. * P -value < 0.05 using Student's t -test.
- 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 were measured by RT-PCR. The histograms show the mean $\pm$ SD of three experiments performed in duplicate. * P -value < 0.05 using Student's t -test. BC: *BCAS1*, KR: *KRT23* and IG: *IGFBP5* genes.

Source data are available online for this figure.

Figure 3.

required for gene down-regulation. ChIP experiments in cells exposed to hormone for 30 min showed a 2- to 3-fold enrichment in BRG1 binding to *BCAS1*, *KRT23*, and *IGFBP5* gene promoters (Fig 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 (Appendix Fig S3A 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 (Fig 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 (Fig 3B left panel and Appendix Fig S3C).

As in the presence of hormone, we found simultaneous recruitment of the repressive complex and chromatin remodelers to the repressed genes (Fig 2), we explored whether these proteins could form a complex. Co-immunoprecipitation experiments using HP1 γ -specific antibodies showed that HP1 γ associates with activated PR (Fig 3C upper panel) and with components of the LSD1 complex, including LSD1, HDAC1, and CoREST (Vicent et al, 2013). BRG1 interacts with the HP1 γ repressive complex, especially after hormone exposure (Fig 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 HP1 γ and were not recruited after hormone to repressed genes (Fig 3C, lower panel, second and third rows, Fig EV3A and data not shown). Although the repressor complex that includes HP1 γ and LSD1 is preformed at uninduced conditions (Vicent et al, 2013), we have detected an increased binding between these two proteins in the presence of hormone (Fig 3C lower panel), probably because the presence of the activated PR, BRG1, or a still unknown factor stabilize HP1 γ –LSD1 interaction.

In addition, we performed IP of LSD1 followed by mass spectrometry. In the IPs of LSD1 from hormone-treated cell extracts, we detected the core components of the LSD1 complex repressive complex (LSD1, CoREST, BRAF35, HDAC1, and HDAC2) as well as PR, HP1 γ , and BRG1 (Appendix Table S2). Neither BAF170 nor BAF155 were detected in these assays.

To further validate the *in vivo* interaction between BRG1 and the HP1 γ repressive complex, protein fractionation of hormone-treated T47D cell lysates was carried out by FPLC with Superose 6 sizing column. Native BRG1 co-fractionated with BAF170 with an apparent molecular mass > 1 MDa (Fig 3D, upper panel), as previously reported for the SWI/SNF complex (Wang et al, 1996a,b). However, BRG1 also eluted in a smaller complex with a molecular weight > 669 kDa (Fig 3D, upper panel) as reported previously (McKenna et al, 1998). IP of BRG1 containing fractions (Fig 3D, left) detected BAF170 and BAF155 in the heavy F1 fraction but not LSD1 or HDAC1, members of the LSD1 complex repressor complex (Fig 3D, lower panel). Conversely, in the > 0.67 MDa F2 fraction, we found BRG1 and members of the LSD1 complex repressor complex but not with BAF (Fig 3D, lower panel). Thus, these results are compatible with the existence of a complex of BRG1 with the HP1 γ –LSD1 complex 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 (Fig 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 (Fig 3E). In contrast, depletion of BAF 170 or BAF 155 did not affect hormonal down-regulation of the same genes (Fig 3F), confirming our previous results (Fig 3C). No significant change in the basal levels of the repressed genes was observed after BAF155, BAF170, or BRG1 knockdown (Fig EV2C).

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 vice versa (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 (Fig EV3B). In *BCAS1*, BRM was only found after 5 and 60 min of hormone, thus exhibiting kinetics distinct from those of PR and BRG1 (Fig EV3B compare with S5A and B). Unexpectedly, however depletion of BRM significantly affected hormone repression of *BCAS1*, *KRT23*, and *IGFBP5* (Figs 3F and EV3C). 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 (Fig EV3D), we found that depletion of BRM increased the amount of BRG1 in the BAF complex compared with siControl (Fig EV3D, upper panel), while it reduced the BRG1 associated with HP1 γ (lower panel). These findings provide a plausible explanation for the effect of BRM depletion on active repression (Figs 3F and EV3C). The strongest effect on hormone repression was observed when both ATPases BRG1 and BRM were simultaneously depleted (Fig 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 (Fig EV2C). Moreover, in the fraction of BRG1 that binds HP1 γ and is free of other BAF subunits, we found the components of the LSD1 complex repressive complex HDAC1, LSD1, and CoREST (Fig EV3E, right panel, lane 5), confirming previous results (Figs 3D and EV3A).

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*, and *KRT23* was significantly affected by the presence of the K798R BRG1 mutant compared with the control transfected cells (Fig EV3F), 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 (Figs 3C and F, and EV3A).

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 8 × 60 K array (Agilent). In cells transfected with a control siRNA, 2,500 genes (11.8% of all genes in the array) were hormone-regulated (Fig 4A, left panel). From these, 1,362 (54.4%) were dependent on BRG1 and BRM for their activity (Fig 4A, second panel from the left). Interestingly, in 848 genes (62.3% of B/B affected genes), BRG1/BRM was associated to gene repression (Fig 4A, third panel from the left). Moreover, 744 out of the 847

Figure 4.

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 1,359 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 $\pm$ SD ($n = 5$). * P -value < 0.05 using Student's t -test.
- 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 Genomewide 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; ** P -value < 0.01; *** P -value < 0.001

genes (87.7%) correspond to down-regulated genes in siControl condition, which are not down-regulated in siB/B-treated cells (Fig 4A, right panel). In absolute values and using the same number of genes for each class, we found significantly more down-regulated 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 (Fig EV4A). The repressed genes are significantly more affected than up-regulated genes (P -value = $3.59E^{-14}$). The 98% of the non-regulated genes were not affected by siBRG1 (Fig EV4A). Interestingly, 52.8% of the genes affected by siBRG1 were also affected by siHP1 γ (Fig EV4B), 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 the 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 (Fig EV4C). To examine whether BRG1 regulates growth of tumor cells, we monitored progesterin-dependent cell proliferation and apoptosis of cells transfected with BRG1/BRM siRNAs. Compared with cells transfected with an unrelated control siRNA, progesterin-induced proliferation is reduced by BRG1/BRM knockdown (Fig 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 (Fig EV4D). 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 (Fig EV2B) and (Vicent *et al*, 2013).

Genomewide analysis of BRG1 recruitment to chromatin

Next, we analyzed the genomewide distribution of BRG1 using ChIP-seq. We identified 2,228 and 6,180 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%) (Fig 4C). The average BRG1 intensity profiles showed a distribution that peaks at the center of PR binding regions especially after hormone exposure (Fig 4D). A 52.3% of the BRG1 binding regions overlapped with PR after hormone (Fig 4D,

inset). When similar experiments were performed using the subset of PRBs associated to regulated and non-regulated genes (window: -10 kb from the TSS/+5 kb from the TTS), a significant enrichment of BRG1 was observed in both up-regulated and down-regulated genes vs. non-regulated genes (Fig 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 progesterin-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 2,218, 505, and 411 regions where BRG1 can bind independently of BAF170, BAF155, or both, respectively (Appendix Fig S4A–C, Venn diagrams). Importantly, the BRG1 exclusive regions are associated with lower RNA pol II and poorly expressed genes, compared with BAF-associated regions, which support its repressive role (Appendix Fig S4A–C, right panels). We carried out similar experiments in T47D cells for BAF170 before and after hormone exposure for 30 min. We found 1,662 BRG1 exclusive regions independent of BAF170 (Fig 4F, left panel, $P < 1 \times 10^{-5}$). 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 (Fig 4F, right panel).

Histone H1.2 is loaded around the TSS of hormone-repressed genes

Since depletion of histone H1 and H2A/H2B has been reported on hormone up-regulated genes (Vicent *et al*, 2004, 2011) (Fig 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 twofold the H1 content (Fig 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 (Fig 5B). Box plots of the fold

Figure 5.

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 analyzed by PCR for the presence of sequences corresponding to the *BCAS1*, *KRT23*, and *MMTV* genes. Data are represented as mean $\pm$ SD from three experiments performed in duplicate. **P*-value < 0.05 using Student's *t*-test.
- 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. Central values represent the median, the bars the 25th and 75th percentile and the dashed lines the lower and upper whiskers. **P*-value < 0.05; ****P*-value < 0.001 using Wilcoxon rank sum test with continuity correction.
- 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. Central values represent the median, the bars the 25th and 75th percentile and the dashed lines the lower and upper whiskers. ****P*-values for H1.2 DOWN vs. NON: 2.5×10^{-68} , DOWN vs. UP: 8.3×10^{-72} , UP vs. NON: 2.7×10^{-6} using Student's *t*-test.
- D Hormone-dependent deposition of H1.2 was quantified in down-regulated genes, which presented PRBs (PRBs+) or not (PRBs–) in their promoter region. Central values represent the median, the bars the 25th and 75th percentile and the dashed lines the lower and upper whiskers. **P*-value < 0.05 using chi-square test.
- E T47D-MTVL/H1.2 and H1.3 knockdown cell lines (Sancho *et al*, 2008) treated or not for 6 days with DOX to induce specific H1 depletion were incubated for 6 h 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 (lower panel). Data are represented as mean $\pm$ SD from three experiments performed in duplicate. **P*-value < 0.05 using Student's *t*-test.

Source data are available online for this figure.

change of the RNA pol II reads in the corresponding regions showed the inverse behavior (Fig 5B, right panel, DOWN vs. other categories, *P* < 0.05).

For analyzing 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 (Fig 5C; down- vs. non-regulated *P* = 2.5×10^{-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.) (Fig 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 (Fig 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 twofold the hormone-dependent gene repression of *BCAS1*, *KRT23*, and *IGFBP5* genes (Fig 5E). As a control, depletion of H1.3 did not significantly affect hormone-dependent repression (Fig 5E). Knockdown of H1.4 could not be performed, since the cells die by necrosis (Sancho *et al*, 2008).

BRG1 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 gene repression (Fig 3), 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 (Fig 6A, left panel). This binding was specific for H1.2, as H1.3 and H1.5 failed to interact (Fig 6A, upper

panel). By using the reverse co-IP immunoprecipitating with the H1.2 antibody, we confirmed the BRG1–H1.2 interaction (Fig 6A, lower panel). In fact, the association turned out to be hormone-dependent (Fig 6A, lower panel, compare lanes 2 vs. 3) and specific for BRG1, as BAF170 was not detected in this complex (Fig 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 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 (Fig 6B, left and middle panels, siControl bars). In contrast, knockdown of BRG1/BRM inhibited hormone-dependent H1 loading to these two repressed genes (Fig 6B, siB/B bars). We extended our studies to other five hormone-repressed genes and observed a similar behavior (Fig 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) (Appendix Fig S5A). 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 (Appendix Fig S5A). 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

Figure 6.

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 (upper panel) or H1.2 (lower panel). The immunoprecipitates (IP) were analyzed by immunoblotting with specific antibodies for BRG1, H1.2, H1.3, H1.5, and BAF170.
- 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 analyzed by PCR for the presence of sequences corresponding to the *BCAS1*, *KRT23*, *IGFBP5*, *VAMP1*, *CCDC173*, and *RABD3* genes. The histograms show the mean $\pm$ SD of three experiments performed in duplicate. **P*-value < 0.05 using Student's *t*-test.
- 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 and $+400$ 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 < 0.01, ****P*-value < 0.001 using Wilcoxon rank sum test with continuity correction. 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 were found in the Q4 with decreased positioning scores.
- 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. Central values represent the median, the bars the 25th and 75th percentile and the dashed lines the lower and upper whiskers. ****P*-value < 0.001 using Wilcoxon rank sum test with continuity correction.
- 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 $\pm$ SD of three experiments performed in duplicate. ***P*-value < 0.01 using Student's *t*-test.
- G The reads obtained by DNase-seq (Song & 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*. Central values represent the median, the bars the 25th and 75th percentile and the solid lines the lower and upper whiskers. ***P*-value < 0.05; ****P*-value < 0.01 using chi-square test.

Source data are available online for this figure.

TSS and gene body of the repressed genes *BCAS1*, *IGFBP5*, and *PGR* (Fig 6C and Appendix Fig S5B).

Next, we calculated positioning scores for up- and down-regulated genes in a region between -800 and $+400$ from the TSS and divided the nucleosome scores into quantiles (encompassing 5,080 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 (Fig 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-regulated vs. 103 up-regulated, chi-square, $P < 0.05$), while no significant differences were found in the Q4 quartile with decreased positioning scores (Fig 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 (Fig 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 = 2.2×10^{-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 the 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 (Appendix Fig S6A, left) that was lost in BRG1/BRM-depleted cells (Appendix Fig S6A, right). To explore whether this effect correlates with the extent of gene repression, down-regulated genes were divided into two subgroups according to the increase in nucleosome occupancy after hormone (Appendix Fig S6B). 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 Appendix Fig S6B). 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 iRNA-seq was significantly more repressed by hormone (Appendix Fig S7A) and contained higher H1.2 that the set of genes selected using global RNA datasets (Appendix Fig S7B). 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 RNA pol 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) RNA pol II compare to untreated cells (Appendix Fig S7C). Knockdown of BRG1/BRM significantly inhibited the hormone-dependent displacement of both total and initiating RNA pol II (Appendix Fig S7C, 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 cross-linked 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 (Fig 6F, left panel). This effect was not observed in another random region of the genome (Appendix Fig S8A). Similar studies were performed in 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 & Crawford, 2010). While in up-regulated genes, we detected an increase in DNase cleavage after hormone, and we found a significant decrease in the accessibility in the repressed genes (Fig 6G). Knockdown of BRG1/BRM abolished hormone-dependent decrease in DNase I accessibility in *BCAS1* gene (Fig 6E, right panel). This effect was not due to a lower rate of transcription, since it was resistant to inhibition of transcription by either α -amanitin or DRB (Appendix Fig S8B–D). Moreover, the presence of α -amanitin or DRB did not affect the recruitment of PR and BRG1 (Appendix Fig S8E). 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 (Fig 7A, window: $-/+3$ kb around TSS $P = 5.1 \times 10^{-5}$). 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 (Fig 7B and EV5A and B). Notably, in cells depleted of FOXA1, hormone repression of *BCAS1*, *KRT23*, and *IGFBP5* genes was compromised (Fig 7C) and BRG1 binding to the repressed genes was abolished (Fig 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-regulated than for up-regulated genes (68.5 vs. 48%, which corresponds to 398 and 429 genes, respectively) (Fig EV5C). Down-regulated genes are significantly more affected than up-regulated genes (P -value = 5.7×10^{-12}), and 80% of the repressed genes affected by siHP1 γ were also affected by siFOXA1 (Fig EV5D). The 96% of the non-regulated genes were not affected by siFOXA1 (Fig EV5C). Notably, 124 down-regulated genes were affected by knockdown of HP1 γ , BRG1, and FOXA1, supporting a common function of these three proteins in hormone-dependent gene repression (Fig EV5D, right panel). By co-IP, we found that FOXA1 and BRG1 interact in breast cancer cells particularly after hormone exposure (Fig 7E, compare lanes 3 vs. 4, upper panel and 1 vs. 2, lower panel). PR was also found associated to FOXA1 (Fig 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 progesterone target genes involved in proliferation, apoptosis, and cell fate commitment in breast cancer cells that are actively repressed by hormone. Repression requires binding of activated 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 HP1 γ , 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 (Fig 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

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: $-/+1$ kb around TSS. * P -value < 0.05, ** P -value < 0.01 and *** P -value < 0.001 using chi-square test.
- 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. The histograms show the mean $\pm$ SD of three experiments performed in duplicate. * P -value < 0.05 using Student's t -test. 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 $\pm$ SD of three experiments performed in duplicate. * P -value < 0.05 using Student's t -test.
- 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 specific antibodies for BRG1, PR, and FOXA1. The non-specific band in the control IgG of the FOXA1 IP, which does not coincide with PR β or PR α , is also shown in a shorter exposition (right panel).

Source data are available online for this figure.

Figure 7.

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 HP1 γ -LSD1 complex interacts 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 RNA pol 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) HP1 γ -H3K9me3 binding are highlighted.

genes (Ballaré *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 h, 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 HP1 γ (HP1-LSD1 complex) 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 HP1 γ 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 (Fig 3C–F).

BRG1 can be found at least in two large complexes, one associated with BAF and the other associated with HP1 γ . We do not know how the distribution of BRG1 between BAF and the HP1 γ 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 & 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 the presence of histone H1, low core histone acetylation, and high H3K9me₃; and can be occupied by unliganded PR associated with a HP1 γ -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 Fig 8). It is possible that in order to activate silent genes, the full remodeling activity of the NURF and BAF complexes is 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 & 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*, 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 HP1 γ 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 (Appendix Fig S9F and 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 (Appendix Fig S9H). 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 μ g/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 24 h in serum-free conditions, cells were incubated with R5020 (10 nM) for different times at 37°C.

H1.2 and H1.3 knockdown 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 μ g/ml for 6 days, the cells were passaged at day 3, and then H1 levels were monitored by Western blot with specific antibodies (Fig 5E).

Chromatin immunoprecipitation (ChIP) in cultured cells

ChIP assays were performed as described (Strutt & Paro, 1999) using anti-PR (Santa Cruz, H190); anti-HP1 γ (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 was 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 h in serum-free conditions, cells were incubated with R5020 (10 nM) or vehicle (ethanol) for different times at 37°C. The down-regulation of BRG1, BRM, and HP1 γ 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, and HP1 γ 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 3 μ g 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 specific antibodies for ERK2, PRS294, PR, BRG1, BAF170, BAF155, coREST, HDAC1, BAF57, KDM1, and HP1 γ .

Cell proliferation assay

T47DMTVL cells transfected with control or BRG1 and BRM siRNAs were cultured as described above. Cells (1×10^4) 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 µg of chromatin were treated with 0.15 and 0.4 units of DNase I (Roche) for 3 min at 37°C in 1× DNase incubation buffer. Control samples were incubated in the absence of DNase I. Reactions were terminated by the addition of 40 mM EDTA final concentration, and the cross-linking was reversed by incubating the samples at 65°C. After 6 h, proteinase K (40 µg/ml final concentration) was added to each reaction and incubated overnight at 37°C. 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 1.5-fold, relative to untreated samples (0 h) and $P < 0.05$.

RNA-Seq

RNA was extracted from T47D-MTVL cells treated or not for 6 h 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 Appendix Supplementary Computational Procedures.

Database access codes

Raw sequencing reads in FASTQ format are available via Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) under accession number (GSE83785).

Expanded View for this article is available online.

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Author contributions

GPV and MB wrote the manuscript; GPV, MB, and ASN conceived or designed the experiments; ASN, AP, PS, RZ, and RHW performed the experiments; AP, DS, and JQ performed bioinformatics analyses.

Conflict of interest

The authors declare that they have no conflict of interest.

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