

The tumour suppressor and chromatin-remodelling factor BRG1 antagonizes Myc activity and promotes cell differentiation in human cancer

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BRG1, a member of the SWI/SNF complex, is mutated in cancer, but it is unclear how it promotes tumorigenesis. We report that re-expression of BRG1 in lung cancer cells up-regulates lung-specific transcripts, restoring the gene expression signature of normal lung. Using cell lines from several cancer types we found that those lacking BRG1 do not respond to retinoic acid (RA) or glucocorticoids (GC), while restoration of BRG1 restores sensitivity. Conversely, in SH-SY5Y cells, a paradigm of RA-dependent differentiation, abrogation of BRG1 prevented the response to RA. Further, our data suggest an antagonistic functional connection between BRG1 and MYC, whereby, refractoriness to RA and GC by BRG1 inactivation involves deregulation of MYC activity. Mechanistically, some of these effects are mediated by BRG1 binding to MYC and MYC-target promoters. The BRG1-MYC antagonism was also evident in primary tumours. Finally, BRG1 restoration significantly dampened invasion and progression and decreased MYC in lung cancer cells orthotopically implanted in nude mice. Thus, BRG1 inactivation enables cancer cells to sustain undifferentiated gene expression programs and prevent its response to environmental stimuli.

INTRODUCTION

The BRG1 protein features a bromodomain and helicase/ATPase activity and is a central component of the SWI/SNF chromatin-remodelling complex (Peterson, 2002). The tumour

suppressor nature of the *BRG1* gene, also called *SMARCA4*, is strongly supported by several genetic and functional observations. Somatic and homozygous mutations at *BRG1* were first identified in a variety of cancer cell lines (Wong et al, 2000), and estimates from our recent data suggest the frequency of biallelic *BRG1* inactivation to be as high as 30% in cancer cell lines of lung origin (Medina et al, 2008). In addition to studies of cell lines, the absence of BRG1 protein and *BRG1* mutations have been observed in lung primary tumours (Reisman et al, 2003; Rodriguez-Nieto et al, 2011). The propensity of *Brg1* heterozygous mice to develop tumours also implies the involvement of BRG1 in cancer (Bultman et al, 2008). In addition, BRG1 is known to bind or regulate the activity of proteins involved in cancer development, such as BRCA1, beta-catenin, LKB1, FANCA and RB (reviewed in Medina & Sanchez-Cespedes, 2008). Genetic alterations of other components of the SWI/SNF complex, in particular somatic mutations at the *hSNF5* (also called *SMARCB1*), *ARID1A* (also called *SMARCF1*) and *BAF180*

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(also called as *PBRM1*) subunits, occur at a high frequency in several types of cancer (Wilson & Roberts, 2011), providing further evidence of the great importance of the complex in tumour development.

The SWI/SNF complex regulates transcription by remodelling chromatin structure, via the ATP-dependent disruption of DNA–histone interactions at the nucleosomes (Kwon et al, 1994). Through this activity, the complex controls a wide variety of important physiological and cellular processes, including tissue-specificity, inflammatory processes, immunological responses and early embryonic development (Hendricks et al, 2004; Kwon & Wagner, 2007). More recently it has been shown that Brg1-containing Swi/Snf complexes also serve to enhance somatic cell reprogramming (Singhal et al, 2010). The control exerted by BRG1 on some of these processes is related to its involvement in regulating hormone-responsive promoters. Specific components of the SWI/SNF complex bind to various nuclear receptors (NRs), for example estrogens, progesterone, corticoids, retinoic acid (RA) and vitamin D3 receptors (Chiba et al, 1994; Fryer & Archer, 1998; Hsiao et al, 2003; Johnson et al, 2008), leading to its recruitment to gene-specific promoters (Medina et al, 2005; Peterson et al, 1994).

We recently observed that *BRG1* inactivation and *MYC* amplification are mutually exclusive in lung cancer, suggesting a functional relationship between the *MYC* and *BRG1* proteins (Medina et al, 2008). A few publications have reported a biological connection between the SWI/SNF complex and *MYC*. The SWI/SNF complex is required to mediate gene transactivation of the *Myc* target gene *cad* (Pal et al, 2003), and recruitment of SWI/SNF to *MYC*-binding promoters depends on *MYC*–INI1 interaction (Cheng et al, 1999). Moreover, in mouse epithelial mammary cells, transcription of the *CEBPδ* gene is strongly induced in G(0) through a mechanism involving Brg1, while *CEBPδ* expression is repressed by cMyc in proliferating cells (Zhang et al, 2007).

We have a good understanding of the involvement of *BRG1* and the SWI/SNF complex in a variety of cellular processes. However, we lack specific knowledge about how the loss of *BRG1* contributes to tumour development. The results presented in this paper suggest that, through *BRG1* inactivation, the cancer cell abolishes the regulation of *MYC* activity and prevents the appropriate control of gene expression in response to the activation of NRs, thereby, promoting cell growth and maintaining an undifferentiated status.

RESULTS

Lack of activity and inability to suppress cell growth of *BRG1* mutations found in human tumours

We previously reported the common presence of mutations of *BRG1* in lung cancer cells and in lung primary tumours (Medina et al, 2008; Rodriguez-Nieto et al, 2011). Most of the mutations identified predicted truncated proteins and had no detectable *BRG1* protein. Among the few mutations identified in tumours that predict non-truncated proteins are a missense change at a highly conserved aminoacid (W764R) in the NCI-H2126 cell line

and an in-frame deletion (E668–Q758 del.) in the NCI-H1703 cell line (Medina et al, 2008). Both mutations affect the ATPase domain (Fig 1A). To ascertain the lack of function of these two mutations we first cloned them in the pcDNA4/TO expression vector). As expected, the E668–Q758del (henceforth called 668–758Δ) mutant (mut) produces a shorter protein than the wild-type (wt) and the W764R mut (Fig 1B). To determine the effect of these mutations on the activity of *BRG1* we took advantage of a previously described transcription reporter assay (Johnson et al, 2008). In this system, the transfection of a *BRG1* construct and an MMTV-luciferase reporter plasmid enables the contribution of *BRG1* to the hormone-dependent regulation of MMTV transcription to be assessed. As a model we used the human lung carcinoma cell line (NCI-H1299; henceforth referred to as H1299), which lacks *BRG1* due to a homozygous small intragenic deletion (Wong et al, 2000). The analysis revealed a 5–8 fold increase in MMTV-dependent transcription in the presence of the hormone when the cells were transfected with the wt *BRG1* compared with those transfected with either of the muts or with the empty vector (Fig 1C). This indicates a lack of function of all the *BRG1* muts tested.

We also compared the efficiency of the wt *BRG1* and the muts in suppressing cell growth in two *BRG1*-deficient lung cancer cells, NIH-H1299 and A549 (Medina et al, 2008). Overexpression of wt*BRG1*, but not of the two muts, significantly suppressed colony formation (Fig 1D).

Restoration of *BRG1* in lung cancer cells triggers a normal lung gene expression signature

To evaluate the tumour suppressor role of *BRG1* further we constructed H1299-derived isogenic cell lines that express stable and inducible wt and 668–758Δ *BRG1*. Ectopic *BRG1* protein was detectable 6 h after induction with doxycycline (dox; Fig 1E). Immunofluorescence confirmed the ectopic and nuclear *BRG1* expression in >90% of the cell population (Fig S1A of Supporting Information).

We then compared the gene expression profiles of the H1299tr-*BRG1*wt and H1299tr-*BRG1*mut cells without induction of *BRG1* expression *versus* cells induced at 6, 12 and 24 h. About 460 genes were differentially expressed (Table S1 of Supporting Information). Gene ontology analysis linked this gene expression signature to several functions, including organism and organ development (Fig S1B of Supporting Information). Manual examination of individual targets of this signature revealed RA, glucocorticoid (GC) receptor and *MYC*-targets (Fernandez et al, 2003; Table S2 of Supporting Information). Furthermore, many of the up-regulated transcripts are known to be expressed in the normal lung and some constitute lung tissue-specific markers (<http://biogps.gnf.org>; e.g. *ACP5*, *AQP1*, *CDH5* and *TIE1*; Fig S1C of Supporting Information). Expression changes at selected genes were confirmed by quantitative polymerase chain reaction (after reverse transcription) (RT-QPCR; Fig 1F) and verified in other *BRG1*-deficient lung cancer cell lines (NCI-H23 and A549), by transiently transfecting wt and mut *BRG1* (Figs S1D and E of Supporting Information). Our observations indicate that *BRG1* restoration partially restores the gene expression signature of the normal lung. To investigate

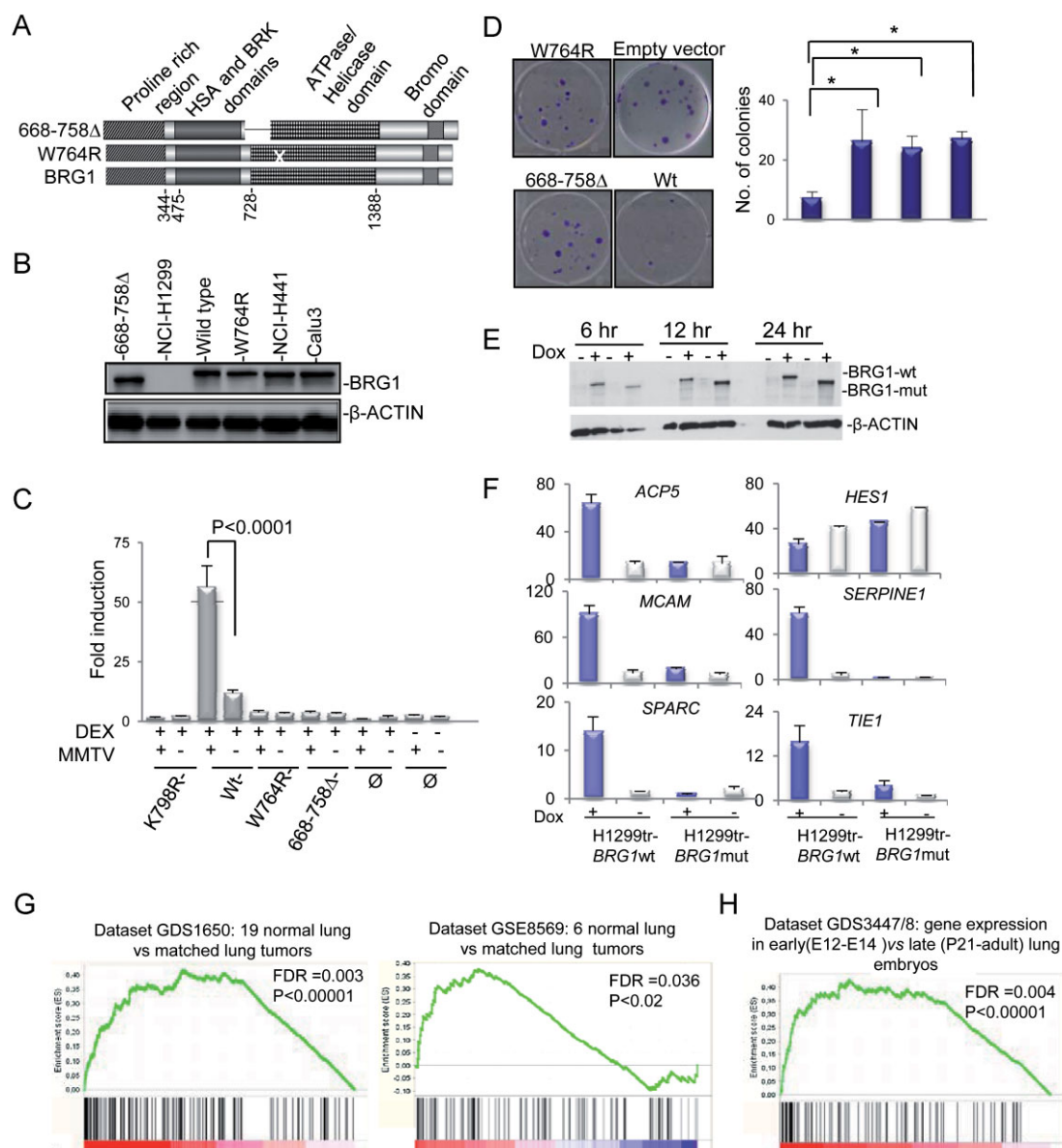


Figure 1. Wt BRG1 is required for cell growth arrest and cell differentiation.

- A.** Schematic representation of wtBRG1 and two mutations (668–758Δ and W764R) from human lung cancer cells.
- B.** Western blot shows expression of the three forms of cloned BRG1. The 668–758Δ in-frame deletion results in a shorter protein. The parental H1299 and two cell lines carrying wtBRG1 are also shown.
- C.** Restoration of wtBRG1 in H1299 cells potentiates the transcriptional activity of MMTV in cells exposed to DEX. Cells were transfected with MMTV-LTR-luciferase and pCMV renilla (internal control) along with the indicated BRG1 expression vectors. K798R is an inactive form of BRG1. Luciferase activity was assayed and normalized to renilla reporter. Data are from two-independent experiments.
- D.** In contrast to the muts and empty vector, wtBRG1 reduces colony formation in H1299 cells. On the right, crystal violet quantification of the assay. Error bars, SD of three replicates. $*p < 0.005$.
- E.** Western blot depicts the expression of the mut (668–758Δ) or wtBRG1 under a tet-repressor-controlled vector in the presence (+) or absence (–) of dox (2 ng/ul) at the indicated times. β-Actin, protein loading control.
- F.** RT-QPCR to verify the changes in expression of selected genes, relative to the housekeeping *GUSB*, upon induction of wtBRG1 and mutBRG1. Error bars, SD of three replicates.
- G, H.** Graphical representation of the ranked gene lists derived from the comparison (using GSEA) of the indicated datasets and the genes up-regulated in lung cancer cell lines (H1299tr-BRG1wt) upon BRG1-restoration (from Table S1 of Supporting Information). The genes up-regulated in the lung cancer cell line (H1299tr-BRG1wt) upon BRG1-restoration (dox +) are significantly similar to those up-regulated in normal lungs and in the lungs from late embryos. *p*-Values and false discovery rates (FDRs) are indicated. Error bars, SD of three replicates.

this further, we used Gene Set Enrichment Analysis (GSEA) to compare the wtBRG1 gene expression profile with two gene expression datasets, containing the analysis of normal and matched cancerous lungs, including one from our group (GSE8569; Angulo et al, 2008). We found that the gene expression signatures of normal lungs, in particular up-regulated genes, shared significant similarities with the wtBRG1 gene expression profile (Fig 1G), thus supporting our observations. Furthermore, the genes up-regulated when wtBRG1 was restored significantly matched the set of transcripts whose expression increased throughout the development of the embryonic lung (Fig 1H). Overall, these observations imply that *BRG1* inactivation in lung cancer prevents normal cell differentiation.

BRG1 is recruited to the E-boxes of MYC target genes

Restoration of wtBRG1 in lung cancer cells changed the expression of MYC-targets and MYC itself, which was slightly down-regulated. These observations, coupled with the previous

finding of a functional relationship between the SWI/SNF complex and MYC (Cheng et al, 1999; Pal et al, 2003), prompted us to investigate whether the BRG1 tumour suppressor function involves the control of MYC activity. First, we used chromatin immunoprecipitation (ChIP) to determine whether BRG1 was recruited to the MYC consensus regulatory elements, E-boxes, within the promoters regulated by BRG1 (Table S2 of Supporting Information). Chromatin of the H1299tr-BRG1wt and H1299tr-BRG1mut cells was precipitated using a BRG1 antibody or the IgG control (Fig S2A of Supporting Information) and DNA enrichment was measured by quantitative PCR using primer sets flanking the E-boxes of known MYC targets: *ACP5*, *AQP1*, *HES1* and *SERPINE1*. We found an at least 10-fold relative enrichment of PCR products after inducing wt BRG1, indicating that BRG1 is recruited to these gene promoters (Fig 2A). The mut form, which lacks residues involving the ATP binding site, showed little or no enrichment. To assess the specificity of BRG1 occupancy in the MYC-binding consensus sequences we also screened distant regions (>1000 bp from the E-boxes). The enrichment of BRG1

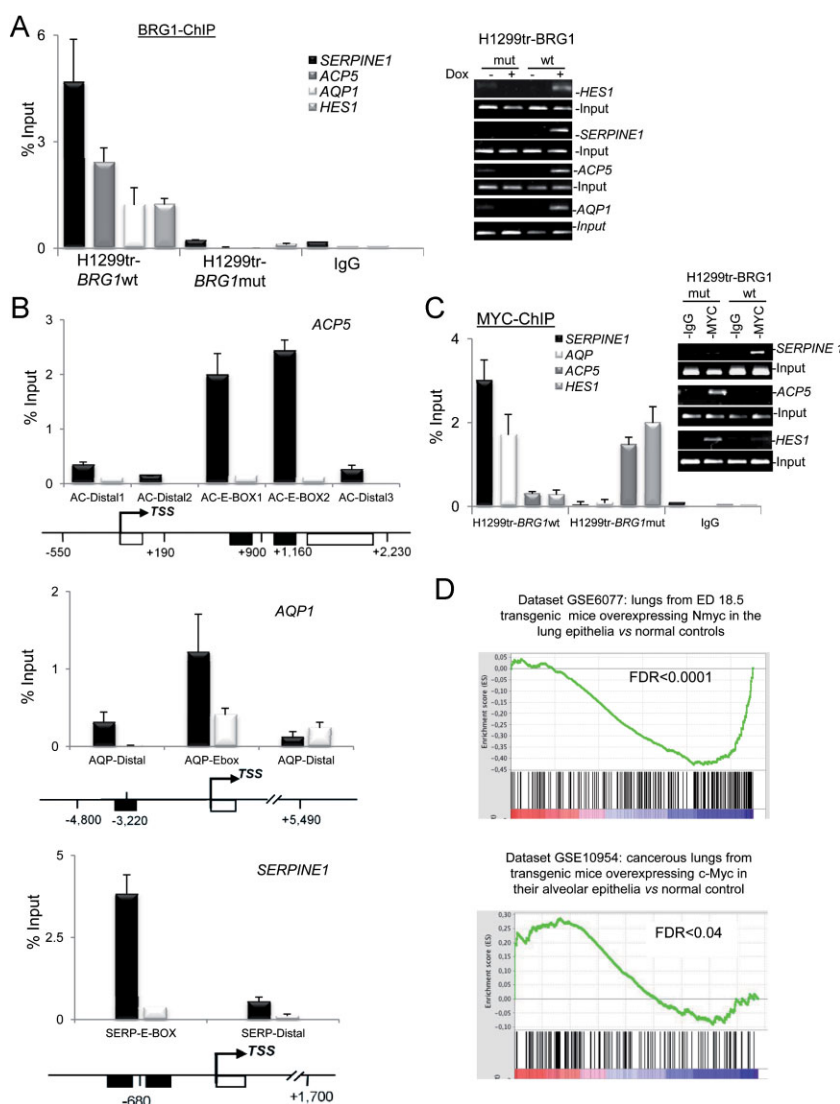


Figure 2. Simultaneous recruitment of BRG1 and MYC to E-boxes of MYC-targets.

- ChIP analysis of BRG1 levels followed by QPCR to determine DNA enrichment in the E-boxes of the indicated MYC-targets. The 2% agarose gel of a semiquantitative PCR is shown for comparison. Cells uninduced (–) or induced (+) for BRG1 expression (dox, 2 ng/ul).
- Promoter screening using QPCR of BRG1 immunoprecipitates. The bars represent the relative enrichment in the H1299tr-BRG1wt (black) and the H1299tr-BRG1mt (white) clones. The location of the E-boxes (black boxes) relative to the TSS is illustrated below each graph. Error bars, SD of three replicates.
- ChIP of MYC in H1299tr-BRG1wt or H1299tr-BRG1mut cells after induction of BRG1 expression followed by QPCR to determine MYC occupancy in the E-boxes of the indicated genes. The 2% agarose gel of a semiquantitative PCR is shown for comparison.
- Ranked gene lists derived from the GSEA comparison of the indicated datasets. Top, genes up-regulated in the lung cancer cell line (H1299tr-BRG1wt) upon BRG1-restoration (dox +) are significantly similar to the genes down-regulated in the lungs of *Nmyc*-overexpressing mice. Bottom, genes down-regulated in lung cancer cell lines (H1299tr-BRG1wt) upon BRG1-restoration are significantly similar to those up-regulated in the lungs of *cmyc*-overexpressing mice. Corresponding FDRs are indicated.

was greater in the E-boxes, indicating specific recruitment to these regions (Fig 2B). In addition to MYC targets, we evaluated and confirmed the occupancy of BRG1 in the promoters of other genes that are up-regulated by BRG1 (Fig S2B of Supporting Information).

Next, we performed ChIP to determine whether MYC was recruited to the E-boxes in a BRG1-dependent manner. Using the same primer sets we observed MYC occupancy in the promoter of *AQP1* and *SERPINE1* only upon induction of wtBRG1 expression (Fig 2C). This indicates that the co-occupancy of BRG1 and MYC in these promoters enables gene expression. Conversely, for *HES1* and *ACP5*, MYC recruitment occurred only in the absence of wtBRG1, suggesting that BRG1 prevents the access of MYC to these promoters. The occupancy of MYC will lead to an increase and the decrease in *HES1* and *ACP5* expression, respectively (Fig 1F). Interestingly, and supporting our observations, GSEA analysis of datasets from GEO concluded that gene expression profiles from lungs of embryos overexpressing *Nmyc* and from lung tumours developing in *c-myc* transgenic mice are significantly inversely correlated with gene expression after restoring BRG1 in lung cancer cells (Fig 2D). Although, more experimental evidence is required to draw definite conclusions and to understand the mechanisms involved, these data suggest an antagonistic function between the tumour suppressor BRG1 and the MYC oncogenes, that is consistent with the mutually exclusive presence of BRG1 inactivation and MYC amplification in lung cancer (Medina et al, 2008).

BRG1 is required for changes in cell morphology in response to nuclear receptor-mediated signals

Induction of wtBRG1 rendered expression changes in known transcriptional targets of GC and RA receptors (Table S2 of Supporting Information). Retinoids and corticoids are critical for lung embryonic development and normal lung function (Cole et al, 2004; Malpel et al, 2000). It is also well established that, in sensitive cells, retinoids mediate morphological changes and that these are associated with cell differentiation (Påhlman et al, 1984). We hypothesized that *BRG1* inactivation in cancer confers refractoriness on the action of these receptors. To test our hypothesis, we examined whether wtBRG1 triggers changes in the cell phenotype or growth in a GC- or RA-dependent manner. We first confirmed the expression of GC receptors in the H1299 parental cells and in the derived clones (Fig S3A of Supporting Information). The H1299 cells had previously described as expressing various types of RA receptors after treatment with retinoids (Choi et al, 2007). Here, we observed that treatment with all trans-RA induced expression of *RARB*, the level being higher in H1299tr-BRG1wt than in H1299tr-BRG1mut cells (Fig S3B of Supporting Information). After treating these cells with dexamethasone (DEX) and/or RA for 4 days, strong morphological changes became apparent in more than 90% of them, but only in the wtBRG1-expressing cells (Fig S3C of Supporting Information). Exposure to DEX induced remarkable changes in shape, from a round to a spread cell morphology, and a larger size with a larger volume of cytoplasm. On the other hand, exposure to RA also transformed

cell morphology towards an elongated appearance. To improve the determination of differences in cell morphology and growth we introduced genes encoding different fluorescent proteins. Equal numbers of H1299tr-BRG1wt and H1299tr-BRG1mut cells (expressing green and red fluorescent-labelled proteins, respectively), were co-cultured, and their relative growth and phenotypic changes followed in real time using fluorescence microscopy. Only the green cells (expressing wtBRG1) underwent morphological changes after exposure to RA and/or DEX (Fig 3A and Fig S3D of Supporting Information). The phenotypic changes induced by DEX treatment involved the re-distribution of actin and alpha-tubulin from a disorganized network to a fibre-like appearance, indicating the presence of filaments (Fig 3B and Fig S3E of Supporting Information). This recalls the actin filaments observed by others (Asp et al, 2002; Rosson et al, 2005). We also tested the effect of wtBRG1 and the influence of the different treatments on cell growth suppression. Induction of wtBRG1 significantly reduced cell growth compared with induction of mutBRG1 (Fig 3C). Collectively, these observations demonstrate the requirement of BRG1 to modulate morphological changes and to reduce cell growth in response to the activation of specific NRs.

BRG1 reduces MYC levels and orchestrates gene expression following RA and DEX treatments

To understand the molecular basis underlying these changes we tested for BRG1-dependent RA- and DEX-specific gene expression. We have shown that BRG1 occupies MYC-binding regions (Fig 2) and it has been reported that exposure to retinoids or corticoids reduces MYC levels (Doyle et al, 1989). We hypothesized that BRG1 is required for MYC down-regulation in response to RA and DEX. After treatment with either RA or DEX we observed a marked reduction of MYC protein only in the wt BRG1-expressing cells, indicating that this is a BRG1-dependent process (Fig 3D and Fig S3F of Supporting Information). We then tested for specific RA- or DEX-dependent modulation of transcription among 13 selected genes (Fig 3E). Overall, the transcripts could be classified as: (i) unrelated or moderately related to RA or DEX (*IL11* and *ACP5*), (ii) RA-dependent (*SPARC* and *TIE1*), (iii) DEX-dependent (*FBLIM1*, *MCAM*, *PCDH1*, *SERPINE 1*, *TAGLN* and *WNT7B*) or (iv) either RA- or DEX-dependent (*AQP1* and *MYC*). In the case of *HES1*, strong BRG1-independent over-expression was observed after RA or GC treatment. Interestingly, some DEX-dependent transcripts included *FBLIM1*, *SERPINE1* and *TAGLN*, which are related to the actin cytoskeleton (Ghosh & Vaughan, 2012; Goodman et al, 2003; Tu et al, 2003). These could contribute to the phenotypic changes induced by DEX.

To demonstrate further the requirement of BRG1 for mediating RA receptor-dependent transcription, we measured the expression of *CYP26A1*, a *bona fide* target RA receptor, carrying RA response elements (Rochette-Egly et al, 2000) in a panel of cancer cells including the parental H1299 cells. Cancer cells carrying *BRG1*-inactivating mutations were completely resistant to RA-induced *CYP26A1* expression, while cells with *MYC* amplification showed some RA-dependent induction of *CYP26A1* (Fig 3F). Intriguingly, the H1437 cells, which are wt for

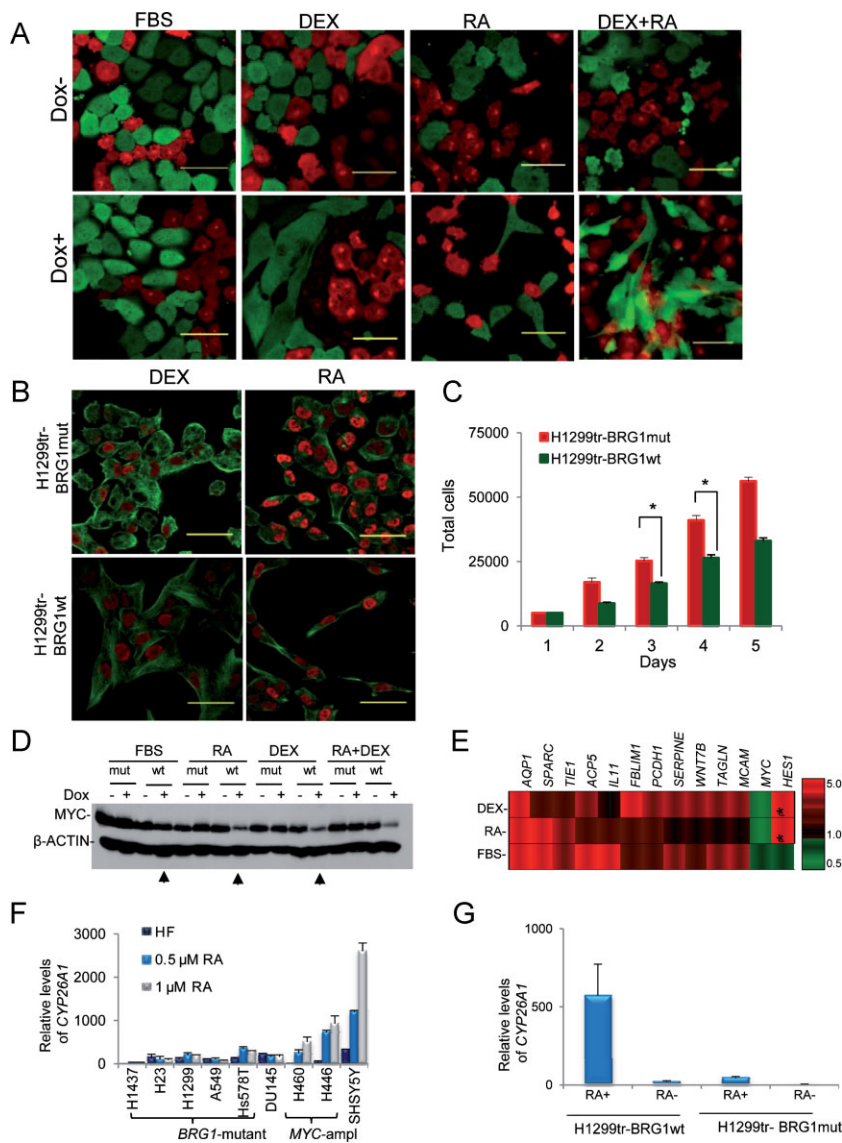


Figure 3. BRG1 wt is required for RA- and DEX-induced changes in cell morphology and gene expression.

- A.** Equal numbers of H1299tr-BRG1wt and H1299tr-BRG1mut cells (expressing green and red fluorescent-labelled proteins, respectively), were co-cultured under the indicated conditions. Phenotypic changes were followed in real time using fluorescence spectroscopy. Scale bar, 50 μ m.
- B.** Immunofluorescence of BRG1 (red) and alpha-tubulin (green) in the indicated cells. Cells were treated as in (A). The percentage of H1299tr-BRG1wt cells adopting the change in morphology after RA and DEX treatment was >90%. Scale bar, 50 μ m.
- C.** Number of cells expressing the GFP (green) and RFP (red) proteins after induction with dox. Cells were counted by flow cytometry. Error bars, SD of four replicates. * $p < 0.01$, two-tailed Student's *t*-test.
- D.** Reduction of MYC in cells expressing wtBRG1 after treatment with either RA or DEX or both (RA + DEX) for 24 h, or FBS. β -Actin, loading control. Arrows, lanes depicting the reduction of MYC protein.
- E.** Heat map of the expression levels, assessed by real time RT-QPCR, of the indicated genes and conditions (RA, DEX or FBS) in H1299tr-BRG1wt cells, relative to the levels of expression in H1299tr-BRG1mut, matched for each condition. Asterisks indicate changes of gene expression independent of BRG1.
- F.** mRNA levels of *CYP26A1*, relative to the control *GUSB*, in a panel of cancer cell lines, treated with the indicated RA concentration or with HF medium. The presence of *BRG1* mutations or *MYC* amplification in the cells is indicated. The lung cancer H1437 and the SH-SY5Y neuroblastoma cells are wt for *BRG1* and *MYC*.
- G.** mRNA levels of *CYP26A1* relative to *GUSB* in the clones following induction with (+) or without (–) dox, before (RA–) and after (RA+) RA treatment (0.5 μ M, 24 h). Error bars, SD of three replicates.

BRG1 and *MYC*, were completely resistant to RA. Restoration of wtBRG1 expression in the H1299tr-BRG1wt clones restored the ability of the cells to increase *CYP26A1* levels upon RA treatment (Fig 3G). These observations were also confirmed in the Hs578T and DU145 cell lines, derived from breast and prostate cancer, respectively, which are deficient in BRG1 due to inactivating mutations (Wong et al, 2000; Fig 4A). Conversely, we depleted BRG1 expression in the RA-responsive cancer cells SH-SY5Y and H446, derived from neuroblastoma and lung cancer, respectively (Geradts et al, 1993; Pählman et al, 1984). Cells were infected with a lentivirus carrying five different shRNAs against BRG1. The shRNAs#1 and #4, reduced BRG1 levels by up to 90% (Fig S4A of Supporting Information). The endogenous levels of BRM, another ATPase of the SWI/SNF complex with a highly similar sequence to that of BRG1 (Kwon & Wagner, 2007), were high and low in the H446 and SH-SY5Y cells, respectively, and were not affected by shRNAs#1 and #4 (Fig S4A and B of Supporting Information). In the SH-SY5Y cells, depletion of

BRG1 prevented down-regulation of MYC and up-regulation of *CYP26A1* (Fig 4B and C). It also impaired morphological differentiation and promoted cell growth upon RA-treatment (Fig 4D and Fig S4C of Supporting Information). These are established and characteristic features of RA-dependent cell differentiation in SH-SY5Y cells (Pählman et al, 1984). Again, in the H446 cells, depletion of BRG1 prevented the increase in *CYP26A1* upon RA treatment, although, levels of MYC remained constant probably because MYC is amplified in these cells (Fig 4C–E). On the other hand, the H446 cells do not undergo measurable RA-dependent morphological changes (Geradts et al, 1993). Here, we observed that depletion of *BRG1* in these cells triggered the appearance of a significant number of spherical cell masses (Fig S4D of Supporting Information). The H446 cells have two sub-lines, floating and surface-adherent cells, which probably represent two distinct histopathological subtypes (Calbo et al, 2011). We cannot confirm that the spherical cell masses correspond to the floating sub-line. It is

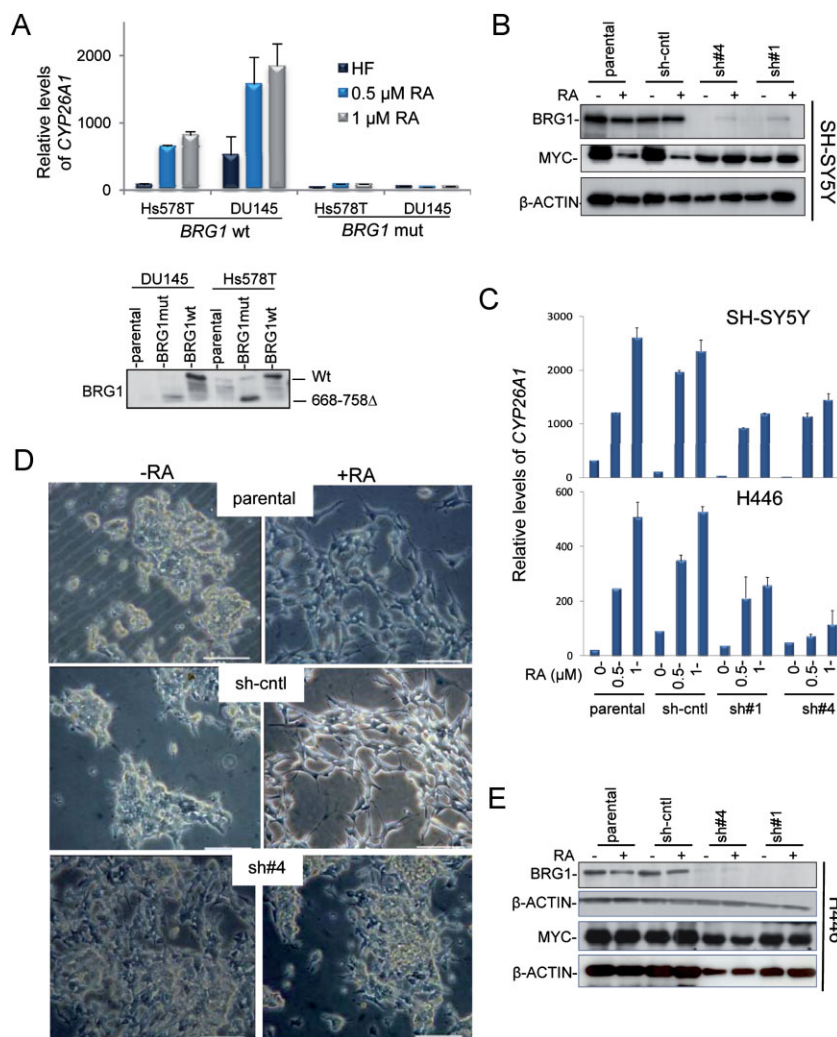


Figure 4. BRG1 requirement for a response to RA can be extended to other cancer types.

A. mRNA levels of *CYP26A1*, relative to the control *GUSB* in the Hs578T and DU145 from breast and prostate cancer, respectively, after reintroduction of wt and mut BRG1, treated with RA or with HF medium. Western blot, ectopic expression of wt and mut BRG1 in each cell line. Note that the parental Hs578T cells show endogenous but inactive BRG1 protein due to a homozygous mutation (p.Pro197Ser; Wong et al, 2000). Error bars, SD of three replicates.

B. Down-regulation of BRG1 protein levels, with two-independent shRNAs targeting BRG1 (sh#1 and sh#4) in the SH-SY5Y abolishes the ability to reduce MYC protein levels upon RA treatment. A scramble shRNAs (sh-cntl) was used as the control.

C. mRNA levels of *CYP26A1*, relative to the control *GUSB*, after depletion of BRG1 in the SH-SY5Y and H446 upon RA treatment (for 24 h).

D. Phase contrast images showing that depletion of BRG1 protein in the SH-SY5Y impedes the acquisition of cell differentiation-related morphological changes upon RA treatment. Scale bar, 100 μ m.

E. Down-regulation of BRG1 protein levels shows no changes in MYC in the H446 because these exhibit MYC-constitutive expression due to gene amplification.

also possible that these structures correspond to less differentiated cells.

BRG1 and MYC are dynamically recruited to the promoters of retinoid and corticoid receptor target genes to regulate gene transcription

Having demonstrated that BRG1 is required for conferring changes in cell morphology and for triggering specific patterns of gene expression in response to treatment with RA or DEX, we wondered whether these involved changes in the recruitment of BRG1 to gene promoters. To examine this we used ChIP, precipitating the chromatin with anti-BRG1 antibody. We observed a dynamic pattern of BRG1 binding to gene promoters by virtue of which recruitment was closely associated with increased gene expression following a given treatment or condition (Fig 5A and B). For example, *SPARC*, which is strongly up-regulated by RA but not by DEX, recruits BRG1 to its promoter mainly when BRG1 wt-expressing cells are exposed to RA while *SERPINE1*, with increased gene expression in response to DEX, recruits BRG1 upon treatment with DEX but not with RA. On the other hand, *AQP1*, a lung-specific transcript with a

very high level of expression in foetal bovine serum (FBS)-maintained cells but also after DEX and RA treatment, shows enrichment of BRG1 in its promoter under each condition. We next sought to examine the dynamics of MYC recruitment to its targets in response to RA and DEX and the requirement for wtBRG1. MYC was recruited to the *AQP1* and *SERPINE1* promoters in both, upon DEX and RA treatment (Fig 5B). The recruitment of MYC to its target promoters was more effective in the presence of wtBRG1. Collectively these findings indicate that BRG1 occupancy in gene promoters is dynamic and varies to adjust gene expression to the environment, and that co-occupancy of BRG1 and MYC in the promoter of *AQP1* and *SERPINE1* is required to enable the appropriate expression of these genes.

MYC down-regulation upon RA and DEX treatment requires a shift in BRG1 occupancy within the MYC locus

To establish what causes MYC down-regulation following restoration of wtBRG1 in lung cancer cells we examined whether BRG1 recruitment to the *MYC* locus took place and tested for possible changes after treatment with RA and DEX. To this end

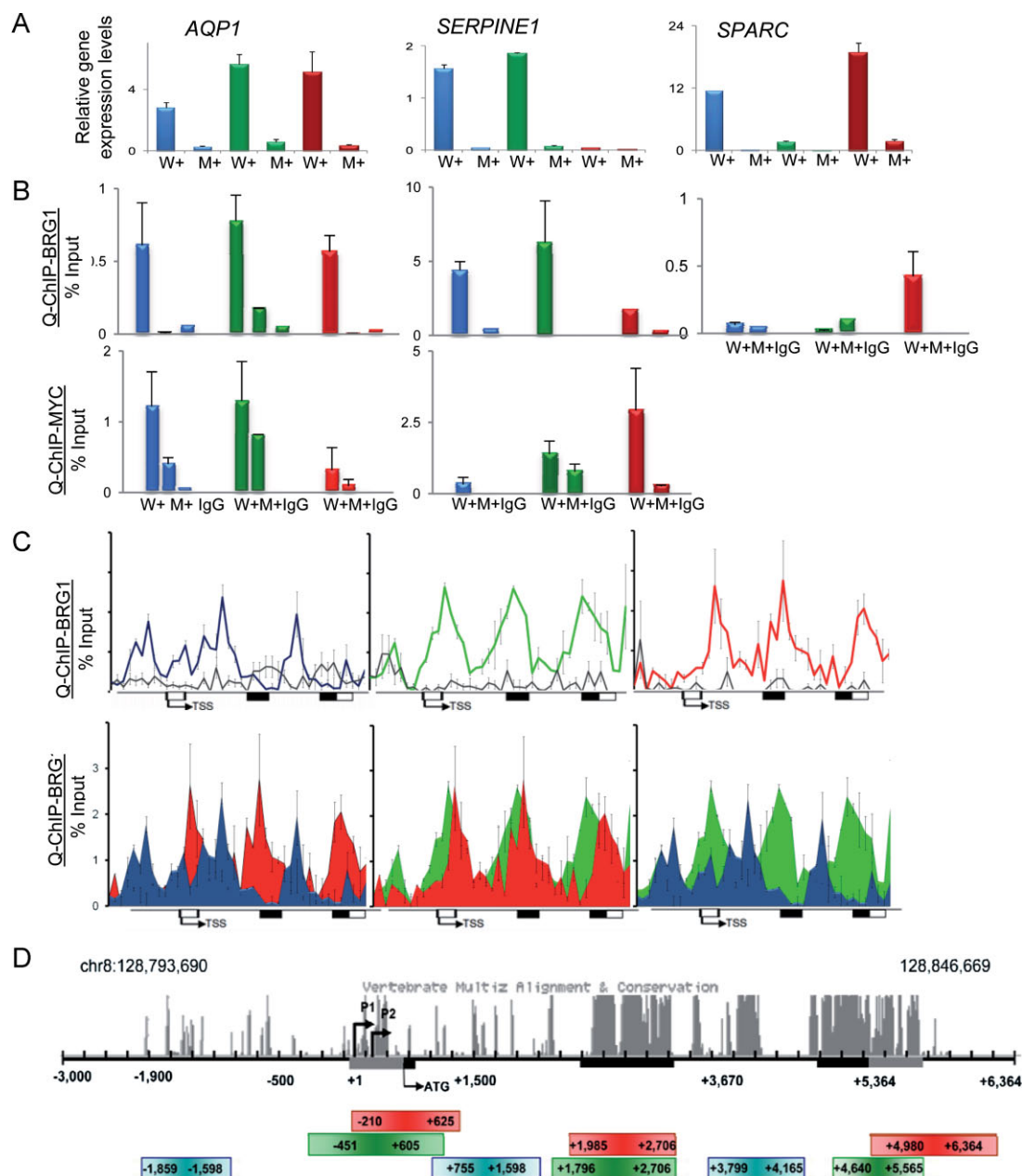


Figure 5. Gene expression and dynamic BRG1 and MYC recruitment to gene promoters upon RA and DEX treatment.

A. mRNA levels of the indicated transcripts relative to *GUSB* under each condition upon induction of BRG1 expression in the H1299tr-BRG1wt (W+) and H1299tr-BRG1mut (M+) cells with dox. Blue bars, FBS; red bars, RA treatment; green bars, DEX treatment.

B. Top panel: ChIP with BRG1 in W+ or M+ cells upon induction of BRG1 expression for each condition (blue, FBS; red, RA; green, DEX), followed by real-time QPCR to determine the enrichment of BRG1 in the indicated gene promoters, relative to the input. IgG, negative controls. Bottom panel: ChIP with MYC in the W+ or M+ cells, for each condition (blue, FBS; red, RA; green, DEX), followed by real-time QPCR to determine the enrichment of MYC in the indicated gene promoters relative to the input. IgG controls are also shown.

C. Relative enrichment of BRG1 at the *MYC* locus under different conditions in the indicated cells. Black lines, IgG controls. The lower panel depicts the paired comparisons of the BRG1 binding to the different regions of the *MYC* locus under each condition. The human *MYC* locus is represented under each graph. Black boxes, coding exons (data for the muts, Fig S5 of Supporting Information).

D. Analysis of mammalian conservation of the *MYC* locus. Highly conserved, non-coding sequences were identified at the P1 and P2 promoters, the untranslated region in exon 3 and in introns 1 and 2. Vertical lines, position of the 44 primer sets used in the analysis. The different-coloured boxes indicate the nucleotide position of regions that bind BRG1 with different treatments (blue, FBS; red, RA; green, DEX).

we again used ChIP analysis and subsequent QPCR of 44 different fragments spanning about 10 kb within the *MYC* locus (Fig 5C). We observed that before treatment with RA or DEX, wtBRG1 preferentially occupied the following regions of the *MYC* gene: -1860 to -1600, +755 to +1600 and +3800 to +4165 bp from the transcription start site (TSS). However, exposing the cells to RA or DEX localized BRG1 to the *MYC* TSS and coding exons, indicating that these sequences are associated to the BRG1-dependent down-regulation of *MYC* expression. Conversely, the mut BRG1 was not bound to the *MYC* locus under any of the conditions tested (Fig S5 of Supporting Information). Overall, the regions found to recruit BRG1 were those that are highly conserved among vertebrates, which implies that they have regulatory functions (Fig 5D). These data suggest that, through the inactivation of BRG1, tumour cells avoid the proper transcriptional repression of *MYC*, giving rise to constitutively high levels of this oncogene.

Restoration of BRG1 increases survival and reduces tumour invasiveness in nude mice

To investigate the tumorigenicity of the H1299tr-BRG1wt and H1299tr-BRG1mut cells *in vivo*, we examined their capacity to grow orthotopically and to form tumours in the lung parenchyma of nude mice after intrapulmonary injection. Animals were randomly assigned among six groups, each of 18–20 mice, orthotopically implanted with: H1299tr-BRG1mut treated with dox (group 1); H1299tr-BRG1wt treated with dox (group 2); H1299tr-BRG1wt untreated (group 3); H1299tr-BRG1mut treated with dox, RA and DEX (group 4); H1299tr-BRG1wt treated with dox, RA and DEX (group 5) and H1299tr-BRG1wt treated only with RA and DEX (group 6). All groups of mice developed orthotopic tumours that efficiently expressed mut or wt BRG1 when treated with dox (Fig 6A). Next, we compared overall survival of the different groups of mice. Log-rank (Mantel–Cox) tests of the plots revealed a significant survival benefit in mice expressing wtBRG1 (groups 2 and 5; mean \pm SD survival time of 125.8 ± 15.8 days) compared with groups 1 and 4 (70 ± 5.2 days; $p = 0.003$) and groups 3 and 6 (87 ± 6.5 days; $p = 0.026$; Fig 6B).

To investigate the characteristics of the tumours that arose in the distinct groups that could account for the differences in aggressiveness we examined the tumours macroscopically and histopathologically. The wtBRG1-expressing tumours had less capability of invasiveness, that is fewer multifocal lesions and less infiltration in adjacent normal tissues than in mutBRG1-tumours (Fig 6C and D and Fig S6A of Supporting Information). Interestingly, in some of the wtBRG1-expressing tumours cells had an elongated appearance, recalling the morphological changes acquired by the H1299tr-BRG1wt cells in culture after treatment with RA and DEX (Fig 6D). The treatment with RA and DEX did not provide additional significant survival advantages or further reduction in tumour invasiveness in the wtBRG1 group, although, there was a trend towards reduced invasiveness in the treated mice (data not shown).

We also tested for differences in the levels of *MYC* by RT-QPCR and immunostaining and of the RA-transcripts *CYP26A1* and *RARB* in the orthotopic tumours and confirmed that the

wtBRG1 tumours (from groups 2 and 5) had significantly more *CYP26A1* and *RARB* and less *MYC* compared with all the other groups (Fig 6E). It is interesting to note that the tumour levels of *CYP26A1*, *MYC* and *RARB* were higher in the wtBRG1-expressing mice, which received RA treatment, compared with the wtBRG1-expressing mice that did not receive RA-treatment (groups 2 and 5; Fig S6B of Supporting Information), although, the differences were not statistically significant. This was probably due to the presence of hormones in the blood of these animals. The endogenous levels of RA and GC were probably high in these mice in response to the injury caused by the experimental procedures and the tumour development. This also helps explain the lack of additional survival benefits in the RA-treated mice.

Concomitant alterations in MYC and BRG1 are uncommon in human cancer

We previously reported the mutually exclusive presence of *BRG1* and *MYC* alterations in lung cancer cell lines (Medina et al, 2008). Here, we aimed to extend the analysis to more lung tumours and cell lines. To this end, we immunostained BRG1 and MYC as readouts for the presence of gene inactivation and amplification, respectively, in 122 lung primary tumours. We also gathered information from the Cancer Cell Line Project, in the Sanger database, on the *MYC*-family (including *MYCN*, *MYCL* and *MYC*) and *BRG1* status and extend our previous work to 67 lung cancer cell lines. Our current data suggest a lack of concomitant alterations at MYC and BRG1 (Fig 6F and G). Interestingly, among the cell lines reported in the Sanger database to be positive for *BRG1* mutations there is one neuroblastoma, a type of tumour that carry *MYCN* amplification at a very high frequency. Consistent with our findings in lung cancer, the *BRG1*-mut neuroblastoma cell line was among the few neuroblastoma cells negative for *MYC* alterations. Although, the results were not statistically significant, probably due to the small number of cells evaluated, this suggests that mutually exclusive BRG1 and MYC mutations can be extended to other tumour types and that BRG1 may play an important role in neuroblastomas that are negative for MYC amplification.

DISCUSSION

BRG1 is a *bona fide* tumour suppressor, that is frequently inactivated in lung tumours but whose biological function is not yet fully understood. We demonstrate that restoration of BRG1 activity in lung cancer cells induces global changes in gene expression to give a more normal lung gene expression signature. This suggests that *BRG1* inactivation contributes to cancer by maintaining undifferentiated gene expression programs. In this regard, the SWI/SNF complex has been linked to mammalian differentiation of muscle and neurons, among other cell types (De la Serna et al, 2001; Seo et al, 2005). Furthermore, the loss of *SNF5*, another tumour suppressor of the SWI/SNF complex, has been associated with activation of stem-associated programs (Wilson et al, 2010), which is compatible with our present observations.



- A. Representative western blot confirming the dox-dependent (+) induction of BRG1 in orthotopic tumours.
- B. Kaplan–Meier curves showing overall survival probability. Red line, $n = 16$; green line, $n = 19$; blue line, $n = 19$. p -Values are those associated with the log-rank (Mantel–Cox) test.
- C. Number of tumours and of analysable tumours showing infiltration and multifocal lesions. (+) and (–) denote dox treatment of the mice. Asterisks denote statistical significance (Chi-squared test) in H1299tr-BRG1mut (+) and H1299tr-BRG1wt (–) compared with H1299tr-BRGwt (+). * $p < 0.05$; ** $p < 0.01$.
- D. Representative H&E preparations of tumours arising in mice. Scale bars, 100 μ M. In the picture on the right the scale bar represents 25 μ M. Arrows indicate cells with elongated appearance.
- E. Top panel: mRNA levels of *CYP26A1* and *RARB*, relative to the control *GUSB*, in the orthotopic tumours from the indicated groups of mice. Error bars, SD of three replicates. p -Value associated with two-tailed Student's t -test. Bottom panel: Representative negative (left) and positive (right) immunostaining of MYC in the indicated tumours (original magnification, 200 \times).
- F. Representative immunostainings of BRG1 and MYC in lung primary tumours (original magnification, 100 \times).
- G. Mutually exclusive presence of MYC amplification and BRG1 inactivation (dark purple) in three different sets of tumours. Top panel, lung primary tumours for which alterations at MYC and BRG1 have been determined by immunostaining (see Material and Methods Section). In the middle panel, lung cancer cell lines for which information regarding MYC and BRG1 gene status has been extracted previously (Medina et al, 2008) and from Sanger cell line project (www.sanger.ac.uk/genetics/CGP/CellLines/). Lower panel, neuroblastoma cell lines. Data from Sanger cell line project. p -Value associated with Chi-squared test.
- H. Proposed model for BRG1 and MYC functional connection and cancer development. The presence of either BRG1-inactivating mutations or MYC amplification confer refractoriness on NR-mediated signalling and an increase in MYC levels. This prevents the expression of lung-specific transcripts. Upon BRG1 restoration or MYC depletion, the cells recover the ability to respond to NR and restore the normal lung gene signature. The levels of MYC can be regulated depending on the environmental stimuli (e.g. inflammation, tissue damage, etc.). In this scenario, the cells can modulate gene expression, down-regulate MYC and change morphology in response to RA, GC or others. The purple circle and the red rectangle represent MYC and BRG1 proteins, respectively.

SWI/SNF and MYC are known to be functionally related, since MYC physically interacts with the SWI/SNF component, INI1 (Cheng et al, 1999), and BRG1 is required to repress the MYC-target, *cad* (Pal et al, 2003). As further evidence of the relationship between the two cancer-related proteins, we previously described that MYC amplification and BRG1 inactivation in lung cancer cell lines are mutually exclusive (Medina et al, 2008). In the current study we have confirmed and extended these observations to lung primary tumours and neuroblastomas. Given that MYC is an oncogene and BRG1 is a tumour suppressor, we would expect the relationship to be antagonistic. Consistent with this, we report a tight functional connection whereby BRG1 down-regulates MYC expression and regulates the expression of MYC target genes. In tumours carrying BRG1 mutations this regulation is abolished, causing a lack of control of MYC activity. However, the relationship of BRG1 and MYC in the control of the expression of MYC target genes is complex and depends on the target gene. In some cases (*i.e.* AQP1 and SERPINE) the recruitment of BRG1 to the E-boxes enables MYC-mediated transcriptional activation. In contrast, the recruitment of BRG1 to the E-boxes of ACP5 and HES1 promoters prevents MYC transcriptional repression (ACP5) and MYC transcriptional activation (HES1).

We also observed that the gene expression signatures of embryonic lungs from *Nmyc*-overexpressing mice and of lung tumours from *c-myc* transgenic mice are the converse of those obtained by restoring BRG1 in lung cancer cells. Overall, these observations strongly suggest that the cell differentiation-related signatures induced by BRG1 restoration involve, among other factors, the control of MYC activity. Taken together, the genetic and functional observations suggest that, in cancer cells, BRG1 inactivation is, to some extent, tantamount to MYC amplification (Fig 6H).

We also show in this study that lung cancer cells lacking BRG1 do not respond to RA or GC treatment, while expression of wtBRG1 restores sensitivity to these compounds, as determined by changes in cell morphology and up-regulation of RA- and GC-receptor targets. Loss of the ability to respond to GC and RA, despite expressing NRs, has been reported in lung cancer (Kim et al, 1995; Waters et al, 2004). In addition to being critical to lung embryonic development and normal function (Cole et al, 2004; Malpel et al, 2000), RA and GC are important in lung cancer prevention. In this regard, RA deficiency generates lung tumours in mice (Saffiotti et al, 1967) while a failure to respond to GCs, which are involved in resolving inflammation of the lung epithelia, constitute a risk factor for lung cancer, especially in smokers (Shi et al, 2009). Our current work demonstrates that the acquired refractoriness to RA and GC signalling observed in lung cancer is achieved by BRG1 inactivation. Intriguingly, the H1437 cells, which are wt for BRG1 and MYC, showed resistance to RA, suggesting a defect in SWI/SNF activity that could be caused by gene alterations at other components of the complex (Wilson & Roberts, 2011). Steroid and retinoid receptors are known to be related to the SWI/SNF complex. The nucleosome-disrupting activity of the SWI/SNF complex is necessary to allow transcription by RA and GC receptors (Nagaich et al, 2004; Ostlund Farrants et al, 1997) and the BAF60c1 and BAF60c2

subunits of the SWI/SNF complex interact with RA receptors (Flajollet et al, 2007). Overall, our data strongly indicate that BRG1 inactivation in lung cancer affects the function of the SWI/SNF complex in mediating the response to RA and GC, thereby contributing to lung tumour development. Moreover, the association between BRG1 deficiency and lack of response to RA was confirmed in the neuroblastoma cells SH-SY5Y, a paradigm of RA-dependent differentiation (Påhlman et al, 1984), and in breast and prostate cancer cells, indicating that the observations can be extended to other tumour types. It is interesting to speculate that intrinsic resistance to estrogen- or androgen-antagonist treatments, in breast and prostate cancer patients, respectively, could also be attributed to BRG1-inactivating mutations.

On the other hand, RA and GC treatment down-regulates MYC (Doyle et al, 1989; Medh et al, 2001). It is also known that the SWI/SNF complex is recruited to the MYC promoter to activate or repress its transcription in proliferating cells and during cell differentiation, respectively (Chi et al, 2003; De et al, 2011; Nagl et al, 2006). Here, we demonstrate that the reduction of MYC levels in response to RA and DEX is a BRG1-dependent phenomenon that implies a shift in BRG1 positioning within the MYC locus towards exonic regions, which in turn suggests that nucleosome repositioning takes place. In particular, the recruitment of BRG1 in exon 1 involves the P1 and P2 promoters, which are responsible for MYC transcription but are also required for the MYC-negative autoregulatory mechanism (Facchini et al, 1997). This implies that an inactive BRG1 dampens this autoregulatory mechanism, resulting in constitutive MYC expression. This would also explain previous reports of failures in the Myc-negative autoregulation control in many tumour-derived cell lines (Facchini et al, 1997). Furthermore, in growing cells, the BRG1-enriched regions within the MYC locus strongly overlap with those recently reported in HeLa cells, using ChIP-Seq to map the binding regions for components of the SWI/SNF complex (Euskirchen et al, 2011).

The mut form, which lacks residues involving the ATP binding site, showed little ability to bind to the MYC promoter or to other promoters. This indicates a potential disruption of protein interactions that could influence binding or the stability of binding. Although, it has recently been reported that some of the missense mutations of BRG1 found in human tumours are still able to interact with core members of the SWI/SNF complex and transactivate some known targets (Bartlett et al, 2010), the mutations tested here affect other BRG1 motifs and could behave differently.

Finally, BRG1 restoration significantly dampened invasion and progression of lung cancer cells orthotopically implanted in nude mice, further illustrating its tumour suppressor capacity. Moreover, the BRG1 wt-expressing tumours strongly mimicked the observations in cell culture of reduced MYC and increased RA-targets CYP26A1 and RARB. Taken together, the relatively high frequency of BRG1-inactivating mutations in lung cancer (Medina et al, 2008) and the great ability of BRG1 to suppress tumour growth and invasion highlight the role of BRG1 in lung cancer development and the value of investigating therapies aimed at restoring BRG1 activity or acting as BRG1-synthetic lethal.

The paper explained

PROBLEM:

Lung cancer is the main cause of death from cancer in most western countries, in part because of the low efficacy of most current therapies. In this regard, understanding the genetic architecture of lung malignancies has proved to be a reliable strategy to develop novel anticancer agents and foretell response to therapies. A large proportion of lung tumours endure genetic inactivation of BRG1, a component of a chromatin-remodelling complex. However, the role of BRG1 in cancer development is poorly understood, which hinders its potential use in clinical settings.

RESULTS:

This study demonstrates a causal role of BRG1 in the control of RA and GC-induced cell differentiation in lung cancer and in

other tumour types. This enables the cancer cell to sustain undifferentiated gene expression programs that affect the control of key cellular processes. Furthermore, it explains why lung cancer and other solid tumours are completely refractory to treatments based on these compounds that are effective therapies for some types of leukaemia.

IMPACT:

The presence of BRG1 mutations may be a marker for predicting resistance to retinoid- and GC-based therapies in cancer treatment.

In conclusion, our data provide convincing evidence that, through the inactivation of BRG1, cancer cells acquire the ability to prevent or revert cell differentiation and to avoid the response to gene expression programs triggered by NR activation. This occurs, at least in part, through the control of MYC. A well-established feature of many types of tumour cells, especially those of lung cancer, is their ability to grow independently of growth factors. This is achieved by constitutive activation of growth factor receptors (e.g. EGFR and MET) or signal transduction molecules (e.g. RAS and BRAF). As well as this ability, our findings indicate that cancer cells also become refractory to NR signalling, thereby, sustaining cell growth independently of the environment.

MATERIALS AND METHODS

Cell cultures and lung primary tumours

Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD), grown under recommended conditions and maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air. All cells tested negative for mycoplasma infection. Lung primary tumours from 122 patients were provided by the CNIO Tumour Bank Network and arranged in tissue microarrays, as previously described (Angulo et al, 2008). The study was approved by the relevant institutional review boards and ethics committees and informed consent was obtained from each patient.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as previously described (Medina et al, 2005). Briefly, preliminary fixation experiments were performed over a predetermined period. Cells were then fixed in 1% formaldehyde for 10 min and final conditions were chosen that yielded the best combination of *in vivo* fixed chromatin, high DNA recovery and small average size of chromatin fragments (an average length of 0.25–1 kb). Three-independent ChIP experiments were performed. QPCRs were

performed using SYBR Green Master Mix (Applied Biosystems, CA, USA). Relative enrichment was determined from a standard curve of serial dilutions of input samples. For semi-quantitative PCR, amplifications were performed with 30 cycles in a total volume of 25 µl and run in 2% agarose gels. QPCR was performed using Power SYBR Green Master Mix (Applied Biosystems). The sequences of primer sets used in each case are available upon request.

Treatments and luciferase assay

For treatment with DEX and all-trans-RA, cells were first depleted of FBS and subjected to a hormone-free (HF) medium by transfer to 10% charcoal–dextran-treated, heat-inactivated foetal bovine serum for 24 h before hormone treatment (Johnson et al, 2008). For DEX treatment, we treated cells for 24–72 h with 1 µM DEX before harvesting. For RA treatment, we treated cells for 12–72 h with 0.25, 0.50 or 1 µM of RA in DMSO or an equal volume of DMSO, as a vehicle control, before harvesting. For the luciferase transactivation assays we used the full-length MMTV long terminal repeat driving transcription of the luciferase gene, pRSV-GR and the human mut or wt BRG1 containing constructs, following the previously described procedures (Johnson et al, 2008). We also used the K798R, a mut form of the BRG1 gene that has been demonstrated to lack ATPase activity (Pattenden et al, 2002).

Orthotopic xenograft model

Athymic mice male *nu/nu*, aged 4–5 weeks, were maintained in a sterile environment. The experimental design was approved by the IDIBELL animal facility committee. H1299tr-BRG1mut and H1299tr-BRG1wt cells (2×10^6) were injected orthotopically into the lung. The control groups drank 1% sucrose and the treated groups drank 1% sucrose plus 2 mg/mL dox in water *ad libitum*. After allowing 20 days for tumours to develop, mice were randomized into four groups: mut or wt, and treated with vehicle or dox. After 10 days of dox induction, mice were also randomized for treatment with a combination of all-trans RA plus DEX (10 mg/kg/day of RA 3 times/week and 0.4 mg of DEX 3 times/week) or were untreated. Survival was followed for

225 days. Animals were sacrificed when they displayed serious respiratory difficulty, which was subsequently confirmed to be associated with lung tumour burden. For histological analysis of lung tumours, lungs were fixed and embedded in paraffin. Five-micrometer sections were stained with H&E, using standard protocols and examined by light microscopy in a blinded fashion. The tumour invasion of the parenchyma (infiltrating tumours) or the presence of multifocal lesions was evaluated by different researchers (EC and OAR), who assigned a score (absence, 0; presence, 1) to each tumour.

Accession numbers

Array data have been deposited in the Gene Expression Omnibus (GEO) under accession reference GSE35168.

For more detailed Materials and Methods see the Supporting Information.

Author contributions

OAR, SJ, GLH, AV and MSC conceived and designed the experiments; OAR, FS and PGX performed the experiments; EC contributed to the histopathological and immunohistochemical analysis and to the evaluation of the data; GGL and DP performed the bioinformatic analysis; OAR, SJ, GLH, AV and MSC analysed the data and wrote the paper.

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

For more information

<http://www.ncbi.nlm.nih.gov/sites/GDSbrowser>

<http://myccancergene.org/>

<http://AtlasGeneticsOncology.org/Genes/SMARCA4ID42333ch19p13.html>

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