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## Oncogenic targeting of BRM drives malignancy through C/EBP $\beta$ -dependent induction of $\alpha$ 5 integrin

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### Abstract

Integrin expression and activity are altered in tumors, and aberrant integrin signaling promotes malignancy. However, how integrins become altered in tumors remains poorly understood. We discovered that oncogenic activation of MEK signaling induces cell growth and survival, and promotes the malignant phenotype of mammary epithelial cells (MECs) by increasing  $\alpha$ 5 integrin expression. We determined that MEK activates c-Myc to reduce the transcription of the SWI/SNF chromatin remodeling enzyme Brahma (BRM). Our studies revealed that reduced BRM expression and/or activity drives the malignant behavior of MECs by epigenetically promoting C/EBP $\beta$  expression to directly induce  $\alpha$ 5 integrin transcription. Consistently, we could show that restoring BRM levels normalized the malignant behavior of transformed MECs in culture and *in vivo* by preventing C/EBP $\beta$ -dependent  $\alpha$ 5 integrin transcription. Our findings identify a novel mechanism whereby oncogenic signaling promotes malignant transformation by regulating transcription of a key chromatin remodeling molecule that regulates integrin-dependent stromal–epithelial interactions.

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## Keywords

$\alpha 5$  integrin; Brahma; breast cancer; C/EBP $\beta$ ; ECM–cell interactions; c-Myc

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## Introduction

Oncogenes induce malignancy by altering the levels and activity of cell cycle, stress response and apoptosis regulatory genes that promote aberrant cell growth and survival.<sup>1</sup> Oncogenic transformation is functionally linked to a perturbed tissue stroma characterized by increased deposition and remodeling of the extracellular matrix (ECM).<sup>2–6</sup> Tumors consistently show altered levels and activity of ECM receptors such as integrins, and elevated integrin signaling induces the malignant behavior of cells in culture and *in vivo*.<sup>7–9</sup> Consistently, interfering with integrin activity and signaling inhibits the malignant behavior of oncogenically transformed cells in culture and prevents malignant progression of oncogenically primed tissues *in vivo*.<sup>10–15</sup> These findings highlight critical links between oncogenic transformation and integrin-dependent adhesion. Nevertheless, the molecular mechanisms underlying this phenotypic relationship remain unclear.

$\alpha 5\beta 1$  integrin and its ligand fibronectin (FN) are frequently upregulated in primary and metastatic tumors, and high expressions of both  $\alpha 5\beta 1$  integrin and FN correlate with poor patient prognosis.<sup>16–18</sup>  $\alpha 5\beta 1$  integrin and FN are also often increased in oncogenically transformed cell lines.<sup>19–22</sup> Indeed, ErbB2-mediated transformation of mammary epithelial cells (MECs) is accompanied by an increase in  $\alpha 5$  integrin, and reducing  $\alpha 5$  integrin levels or activity decrease cell growth and induce apoptosis to temper the malignant behavior of breast cancer cells in culture.<sup>18,23</sup> These findings underscore the importance of  $\alpha 5\beta 1$  integrin–FN interactions in malignancy. Nevertheless, whether oncogenic signaling drives tumorigenesis by specifically increasing  $\alpha 5\beta 1$  integrin expression and/or activity and how, has yet to be determined.

Evidence suggests that many tumor-associated changes in integrin expression may be epigenetically mediated through mechanisms such as aberrant DNA methylation or histone modifications.<sup>24,25</sup> Here, we focused on the ATP-dependent SWI/ SNFs chromatin remodeling complex that shifts the position of histones, making the DNA more accessible to transcription factors and co-regulatory proteins. Consistent with their central role in modifying gene transcription, SWI/SNFs regulate levels of genes that control multiple pathways linked to cell cycle control, survival, adhesion and invasion.<sup>26,27</sup> We previously showed that loss of the SWI/SNF protein Brg1 in fibroblasts is associated with a concomitant increase in  $\alpha 5$  and  $\alpha v$  integrin protein, and that this is accompanied by an increase in cell adhesion.<sup>28</sup> Because recent data show that the SWI/SNF protein Brahma (BRM)/ SMARCA2 is frequently downregulated in solid tumors and its absence correlates with advanced stages of disease progression and poor patient prognosis,<sup>29–33</sup> we asked whether increased levels of  $\alpha 5$  integrin expressed by many epithelial tumors could be functionally linked to loss of BRM and, if so, whether this could be mediated by oncogenic transformation.

We found that oncogenic activation of extracellular signal-regulated kinase (ERK) represses transcription of the SWI/SNF chromatin remodeling enzyme BRM by increasing c-Myc expression. We determined that loss of BRM epigenetically induces *C/EBP $\beta$*  transcription, which then directly induces  $\alpha 5$  integrin transcription to promote the malignant behavior of MECs. Our findings represent the first direct evidence linking chromatin remodeling to  $\alpha 5$  integrin expression and demonstrate that oncogenes promote transformation by epigenetically controlling stromal–epithelial interactions.

## Results

### Oncogenic transformation is mediated by FN ligation of $\alpha 5\beta 1$ integrin

To explore whether altered integrin signaling associated with mammary tumors is directly mediated by oncogenic transformation and how, we profiled integrin expression in nonmalignant MCF10A (control, Ctrl) cells before and after amplification of the growth factor receptor-Ras (GFR-Ras) signaling by ectopic expression of Ha-Ras (AT1), ErbB2 and RasV12.<sup>3,34,35</sup> Consistent with a causal relationship between elevated GFR-Ras signaling and perturbed cell–ECM interactions, we found that when grown within a reconstituted basement membrane (rBM), Ha-Ras, ErbB2 and RasV12 MECs formed large, continuously proliferating Ki67 positive, disorganized colonies of which more than 80% lacked apical polarity and a central lumen (Figure 1a, Supplementary Table). By contrast, 80% of the non-transformed MCF10A MECs formed apical–basal polarized and growth-arrested acinar structures with a central lumen, containing random numbers of activated caspase-3-positive cells. Moreover, the acini-like structures assembled by the nonmalignant MECs were characterized by basally localized  $\beta 4$  integrin, apical GM130 and cell–cell associated  $\beta$ -catenin, consistent with normal acinar morphogenesis (Figure 1a). Immunostaining, fluorescence-activated cell sorting (data not shown), immunoblot (Figure 1b) and mRNA analysis (Figure 1c) revealed that the perturbed cell–ECM interactions induced by GFR-Ras-mediated transformation significantly increased levels of  $\alpha 5$  integrin, whereas no consistent protein or mRNA changes were detected for any of the other integrins examined (data not shown), including the major collagen receptor  $\alpha 2$  integrin. The functional contribution of increased  $\alpha 5\beta 1$  integrin levels to the malignant phenotype was revealed by studies in which inhibiting  $\alpha 5\beta 1$ -integrin–ligand interactions using specific function-blocking antibodies to  $\alpha 5$  integrin or FN, but not  $\alpha 2$  integrin, reverted the tumor phenotype toward that of a differentiated, nonmalignant mammary tissue. MCF10A-ErbB2 cells incubated with  $\alpha 5$  integrin or FN function-blocking antibodies formed growth-arrested, polarized acini with cleared lumens (Figures 1d and e). Likewise, reducing  $\alpha 5$  integrin activity in the EGFR-transformed HMT-3522 T4-2 breast cancer cells repressed their malignant behavior in a three-dimensional (3D) rBM assay as indicated by reduced cell growth, survival and invasion<sup>18</sup> (data not shown). Moreover, knocking down  $\alpha 5$  integrin in the IGF-1R-transformed MCF-7 MECs also significantly inhibited their anchorage-independent growth in a soft agar assay (Figure 1f, Supplementary Figure 1a). Ectopic expression of FN-ligated  $\alpha 5$  integrin induced the growth and survival and perturbed the morphogenesis of nonmalignant MECs (Figures 1g and h, Supplementary Figure 1b and c). These findings demonstrated that oncogenic transformation through GFR-Ras signaling consistently perturbs MEC–ECM interactions by specifically upregulating  $\alpha 5$  integrin.

## Oncogenic ERK increases c-Myc to induce $\alpha 5$ integrin expression by repressing BRM transcription

We next explored how GFR-Ras transformation induces  $\alpha 5$  integrin expression. GFR-Ras activity stimulates MEK-ERK, and chronically elevated MEK-ERK activity promotes malignant transformation of breast cancer cells in culture and *in vivo*.<sup>36,37</sup> Accordingly, we tested whether inhibiting MEK-ERK activity could normalize the malignant behavior of GFR-Ras-transformed MECs and if this was mediated through modulation of  $\alpha 5\beta 1$  integrin expression. We found that inhibiting MEK signaling in the EGFR-transformed HMT-3522 T4-2 MECs as well as the RasV12-transformed MCF10A MECs significantly inhibited their malignant behavior within rBM and in a soft agar assay, respectively (Figures 2a and b; Supplementary Table and Supplementary Figure 2a). We noted that when MEK activity was reduced in the ErbB2-transformed MECs, they assembled polarized, growth-arrested acinar-like structures with a central lumen when grown in rBM as indicated by a significant reduction in Ki67 staining, increased activated caspase-3-positive cells in the center of the colonies, and basally localized  $\beta 4$  integrin and apically localized GM130 (Figures 2d–g, Supplementary Table). Importantly, MEK inhibition also simultaneously reduced  $\alpha 5$  integrin levels in all GFR-Ras-transformed MECs (Figures 2c, h and i), implicating MEK signaling as a key regulator of  $\alpha 5$  integrin expression.

Prior studies showed that Ras promotes fibroblast transformation by inhibiting expression of the SWI/SNF chromatin complex member BRM<sup>38</sup> and we found that loss of Brg-1 in fibroblasts, another member of the SWI/SNF complex, is accompanied by an increase in  $\alpha 5$  and  $\alpha v$  integrin protein.<sup>28</sup> We therefore asked whether GFR-Ras-ERK-mediated transformation was associated with decreased BRM or Brg-1 expression. We found that BRM but not Brg-1 (Figure 2j, Supplementary Figure 2b) was reduced in GFR-Ras-transformed MECs, negatively correlating with  $\alpha 5$  integrin expression. Treatment of ErbB2- and HMT-3522 T4-2-transformed MECs with the MEK inhibitor not only increased BRM levels but also simultaneously reduced  $\alpha 5$  integrin and inhibited their malignant behavior (Figure 2k, Supplementary Figure 2c). Importantly, we observed that ErbB2 transformation decreased BRM levels beyond those induced by proliferation (Figure 2l), suggesting that oncogenic ERK must regulate BRM expression through another mechanism. In this regard, ErbB2-transformed MECs express high levels of c-Myc that were reduced upon MEK inhibition (Figure 2m). c-Myc was identified to bind to the core promoter region of BRM by CHIP-seq experiments as part of the Encyclopedia of DNA Elements Project (ENCODE, via UCSC genome browser hg19; Supplementary Figure 2d). Furthermore, we determined that inhibiting c-Myc activity restored BRM mRNA expression (Figure 2n). These findings indicate that oncogenic ERK promotes the malignant phenotype of MECs by increasing  $\alpha 5$  integrin, suggesting this is likely mediated by c-Myc-dependent reduction of BRM expression.

## BRM regulates $\alpha 5$ integrin to modulate MEC malignant behavior in 3D and *in vivo*

To directly test the role of BRM in oncogenic transformation, we expressed wild-type BRM in ErbB2- and RasV12-transformed MCF10A cells to achieve levels expressed in nonmalignant MCF10A MECs and assayed for effects on integrin expression and malignant behavior. Re-expression of BRM in either ErbB2- or RasV12-transformed MECs

significantly reduced  $\alpha 5$  integrin expression to that of the non-transformed MCF10A MECs (Figure 3a) with little effect observed on levels of other integrins. Consistent with an association between FN-ligated  $\alpha 5$  integrin and the malignant phenotype, the ErbB2-transformed MCF10A MECs re-expressing BRM assembled differentiated acini in rBM that were growth arrested and polarized, as indicated by basal  $\beta 4$  integrin, apical GM130 (Figures 3b and d) and significantly reduced levels of Ki67-positive cells (Figure 3e). Re-expression of BRM in the ErbB2-transformed MECs also induced apoptosis in the cells within the core of the colony, as indicated by the appearance of activated caspase-3 staining (Figures 3b and f), such that the majority of the structures had cleared lumens (Figure 3c). Furthermore, when grown within rBM, the RasV12-transformed MCF10A MECs re-expressing BRM grew less (Supplementary Figure 3a), as indicated by reduced Ki67 (Figures 3g and i), were less invasive (Figures 3g and h) and showed a significant reduction in anchorage-independent growth and survival in a soft agar assay (Figures 3j and k). Moreover, re-expression of BRM in the RasV12-transformed MCF10A MECs repressed their tumorigenicity *in vivo*, as revealed by significantly reduced growth when injected into the mammary fat pad of immuno-compromised BalbC nu/nu mice. After 21 days, the tumors formed by the RasV12BRM cells showed a 70% reduction in volume and weight as compared with the RasV12 tumors (Figure 3l and Supplementary Figures 3b and c). These results demonstrate that BRM is a key regulator of GFR-Ras-induced malignancy and suggest that BRM may exert its tumor suppressor role by repressing the expression of  $\alpha 5$  integrin.

### Loss of BRM is associated with elevated $\alpha 5\beta 1$ integrin expression and activity

To directly explore the relationship between BRM and  $\alpha 5$  integrin, we manipulated BRM levels in nonmalignant MCF10A MECs and assayed for effects on  $\alpha 5$  integrin expression and function. BRM levels were silenced using an inducible BRM-specific small hairpin RNA (shRNA; BRMi) that achieved at least 80–90% knockdown when compared with MCF10A MECs infected with a scrambled shRNA (Ctrl; Figure 4a). Knockdown of BRM did not affect levels of its homologue Brg-1 (Figure 4a). Depletion of BRM, but not Brg-1, consistently increased  $\alpha 5$  integrin mRNA and protein levels in the MCF10A MECs (Figures 4b and c and Supplementary Figure 4). The elevated levels of  $\alpha 5$  integrin induced in the BRM knockout cells were functional, illustrated by increased cell surface localization as quantified by flow activated cytometry and increased adhesion to FN (Figures 4d and e). These findings confirm that the BRM SWI/ SNF chromatin remodeling molecule modulates levels of cellular  $\alpha 5$  integrin, and does so likely at the transcriptional level.

### BRM expression and activity enhance MEC tissue behavior by regulating FN- $\alpha 5\beta 1$ integrin interaction

We next explored functional links between BRM expression/activity and  $\alpha 5$  integrin-dependent mammary tissue behavior. MCF10A MECs in which BRM was knocked down using three independent shRNA-targeting sequences (BRMi) formed continuously growing, disorganized colonies as indicated by Ki67 staining and a 30% increased colony size (Figures 5a, Supplementary Figure 5). Loss of BRM expression resulted in loss of a central lumen in greater than 60% of the colonies in rBM (Figure 5b and Supplementary Figure 5e). By contrast, the MECs expressing a scrambled shRNA vector (Ctrl) assembled growth-

arrested, polarized acini with a central lumen that contained random activated caspase-3-positive cells. These structures also showed basally localized  $\beta 4$  integrin and deposited an endogenous laminin-5-containing basement membrane (BM) (Figure 5a). Interestingly, although the structures formed by the BRM knockdown MECs achieved basal polarity, as indicated by basal  $\beta 4$  integrin and laminin-5, they failed to achieve apical polarity, evaluated by GM130 (data not shown), and these aberrant structures expressed abundant  $\alpha 5$  integrin (data not shown) and deposited large quantities of FN (Figure 5a). These findings suggested that these cells are likely poised for transition to invasion. Indeed, FN is consistently downregulated in nonmalignant MECs during rBM-induced mammary morphogenesis, and its aberrant expression heralds malignant progression.<sup>39</sup>

We asked whether blocking either  $\alpha 5$  integrin-binding activity or interaction with its ligand, FN, would normalize the growth, survival and differentiation behavior of the BRM-silenced MECs in response to a 3D rBM. Consistently, we observed that inhibiting FN ligation of  $\alpha 5$  integrin, but not  $\alpha 2$  integrin ligand-binding activity, using function-blocking antibodies, normalized the aberrant phenotype induced by BRM silencing (Figures 5c–f). The colonies assembled by the MECs in which BRM was efficiently knocked down were essentially indistinguishable from the nonmalignant control MEC colonies such that they were able to form growth-arrested, polarized acini with a central lumen (Figures 5c–f). Moreover, we silenced BRM in malignant MCF7 cells and found that BRM silencing increased the ability to grow in soft agar, as well as increasing  $\alpha 5$  integrin expression (Supplementary Figure 6). These data directly link BRM to breast tumor progression through the regulation of  $\alpha 5$  integrin and FN.

Given that the ATPase activity of BRM is essential for its chromatin remodeling function,<sup>40</sup> we asked whether the tumor-suppressing action of BRM relied on its ATPase activity. We expressed a BRM construct containing a mutation in the ATP-binding site of its helicase-like domain (K749R) using a tetracycline-inducible system (Supplementary Figure 7). This mutant ATPase protein acts as a dominant negative by out-competing the binding of wild-type BRM with SWI/SNF complex members to compromise the chromatin remodeling activity.<sup>38,41</sup> Similar to the results obtained through BRM silencing, compromising BRM ATPase activity also perturbed rBM morphogenesis. K749R MCF10A MECs formed continuously growing colonies that contained abundant Ki67-positive cells (Figures 6a–c, f). K749R MEC colonies also exhibited an enhanced survival behavior, as evidenced by the absence of cleaved caspase-3 staining, which compromised luminal clearance (Figures 6d and e). Moreover, loss of BRM ATPase activity increased the expression of  $\alpha 5$  integrin (Figure 6g), and inhibiting FN ligation of  $\alpha 5\beta 1$  integrin reverted the tumor phenotype toward that exhibited by nonmalignant MCF10A MECs (data not shown). These findings implicate the chromatin remodeling activity of BRM as a novel tumor suppressor through its ability to regulate  $\alpha 5$  integrin and FN.

### **BRM inhibits the malignant phenotype by repressing C/EBP $\beta$ -transcription**

We next investigated the mechanism whereby BRM regulates  $\alpha 5$  integrin expression. BRM incorporates into the SWI/SNF chromatin remodeling complex to regulate the expression of multiple genes including transcription factors such as the C/EBPs.<sup>26,42</sup> C/EBP $\beta$  is a



transcriptional factor whose activity/expression increases following Ras-MEK-ERK activation, and this protein can bind to the  $\alpha 5$  integrin promoter to increase its expression (Supplementary Figure 8).<sup>43,44</sup> We observed that when BRM was silenced in the nonmalignant MCF10A MECs, C/EBP $\beta$  protein increased by more than 2.5-fold (Figure 7a). We also noted that loss of either BRM expression or its activity consistently increased C/EBP $\beta$  mRNA, implying transcriptional regulation by BRM (Figure 7b). Chromatin immunoprecipitation analysis indicated that there is a direct interaction between BRM protein and the C/EBP $\beta$  promoter that requires its ATPase-dependent helicase activity, suggesting the BRM SWI/SNF complex epigenetically regulates C/EBP $\beta$  transcription (Figure 7c). The functional implications of this relationship were revealed by further studies in which we could show that silencing of C/EBP $\beta$  prevented the malignant behavior of MECs induced by loss of BRM (Figures 7d and e). Thus, although reducing BRM levels in the nonmalignant MCF10A MECs increased their growth and survival, and disrupted their morphogenesis in a 3D rBM, simultaneous knockdown of C/EBP $\beta$  normalized this premalignant phenotype, as indicated by the assembly of growth-arrested, polarized acini with apoptosis-induced luminal clearance (Figures 7e–h). These findings indicate that BRM epigenetically regulates the expression of a key transcription factor C/EBP $\beta$  that regulates the expression of the malignant phenotype of MECs.

### BRM prevents C/EBP $\beta$ -dependent transcription of $\alpha 5$ integrin

We next explored functional links between BRM-regulated C/EBP $\beta$  and  $\alpha 5$  integrin expressions. Consistent with a relationship between BRM and C/EBP $\beta$ -mediated induction of  $\alpha 5$  integrin and mammary tumor behavior, immunoblot analysis revealed that shRNA-mediated knockdown of C/EBP $\beta$  significantly reduced  $\alpha 5$  integrin protein (Figure 8a), while also repressing the malignant phenotype (Figure 7e). Upon further examination, we noted that although loss of BRM increased  $\alpha 5$  integrin mRNA, simultaneously knocking down C/EBP $\beta$  prevented this increase, suggesting that loss of BRM increased  $\alpha 5$  integrin transcription via increased C/EBP $\beta$  (Figure 8b). Consistently, ErbB2- and RasV12-transformed MCF10A MECs showed a robust increase in both C/EBP $\beta$  and  $\alpha 5$  integrin, with the increased  $\alpha 5$  integrin mRNA level prevented by knocking down of C/EBP $\beta$  (Figure 8c). Indeed, chromatin immunoprecipitation assay confirmed that C/EBP $\beta$  directly binds to the  $\alpha 5$  integrin promoter (Figure 8d). These data indicate that oncogene-induced loss of BRM promotes the malignant phenotype of MECs by increasing C/EBP $\beta$  to directly induce  $\alpha 5$  integrin transcription.

## Discussion

Tumors arise in the context of an altered microenvironment characterized by ECM deposition, altered stromal–epithelial interactions and increased integrin expression.<sup>6</sup> In this study, we show that Ras/MAPK signaling increases the expression of c-Myc that, in turn, suppresses the transcription of the SWI/SNF chromatin remodeling enzyme BRM. The suppression of BRM leads to a de-repression of the transcription factor C/EBP $\beta$ , and thereby driving  $\alpha 5$  integrin expression. Our results represent the first evidence linking altered integrin expression to both oncogenic transformation and chromatin remodeling, identifying an epigenetic regulation for  $\alpha 5$  integrin dysregulation in breast cancer.



Our findings emphasize the importance of  $\alpha 5\beta 1$  integrin in cancer progression. Maschler et al. showed that Ras transformation of mouse MECs increased cell adhesion along with the expression of various integrin subunits including  $\alpha 5$ , and implicate the ERK/MAPK pathway in the acquisition of this phenotype.<sup>20</sup> More recently, it has been reported that ErbB2 transformation of MECs induces an increase in  $\alpha 5$  integrin expression and that  $\alpha 5$  integrin is necessary for ErbB2-mediated inhibition of luminal apoptosis.<sup>23</sup> In accordance with these reports, our findings describe that  $\alpha 5\beta 1$  integrin–FN interactions promote MEC growth and survival to drive a tumor phenotype, and blocking  $\alpha 5\beta 1$  integrin–FN interactions reverts the tumor phenotype toward that of nonmalignant mammary tissues. Together with previous reports, our work points out the importance of  $\alpha 5$  integrin in oncogenic transformation and in the acquisition of the transformed phenotype. Although the roles of integrins in activating multiple intracellular signaling pathways, such as Jnk, MAPK and PI3K, and influencing GFR signaling to enhance cell growth and survival, and contribute to the malignant phenotype of cancer are appreciated,<sup>9,45</sup> no functional data exist to clarify the mechanisms underlying these relationships to date. Here, we demonstrate a specific and novel link between GFR/Ras transformation, MEK-c-Myc signaling and the regulation of  $\alpha 5$  integrin through the chromatin remodeling ATPase BRM and the transcriptional factor C/EBP $\beta$ , providing, for the first time, a molecular mechanism that drives the alteration of  $\alpha 5$  integrin expression during oncogenic transformation.

Many signal transduction pathways and tumor suppressors, such as retinoblastoma (Rb), p53 and BRCA1, are functionally dependent on the SWI/SNF remodeling complex.<sup>46–48</sup> BRM loss or downregulation has been widely correlated with tumor progression and increased risk of malignancy.<sup>29–33,49</sup> Very little is known about the expression and role of BRM in breast cancer. One published report shows that BRM is lost in 15% of the breast cancer samples analyzed, without any correlation to the differentiation state.<sup>50</sup> Consistently, we observe that BRM expression is decreased in GFR/Ras-transformed MECs (Figure 2). Although inactivating mutations of many components of the SWI/SNF complex have been found in cancers, no genetic mutations have been reported for BRM.<sup>27</sup> We propose a new mechanism by which BRM is oncogenically silenced in cancer, through a MAPK-c-Myc-induced repression with potential widespread gene regulation, including genes involved in growth suppression (Figure 2).<sup>51</sup> Our work supports data from the literature describing BRM as a negative regulator of cell proliferation and cancer progression (Figure 3, Supplementary Figure 9).<sup>33,50,52</sup> The inhibition of BRM expression, as well as the abrogation of its enzymatic chromatin remodeling activity, perturbs 3D rBM-directed mammary morphogenesis via enhanced MEC survival and reducing  $\alpha 5$  integrin expression (Figures 4–6). Therefore, our study indicates that BRM-mediated  $\alpha 5$  integrin downregulation is facilitated by an epigenetic mechanism that involves ATPase-dependent chromatin remodeling. In addition, our data are consistent with previous reports that show that BRM can be reversibly suppressed in cancer, making it a potential target for cancer therapy.<sup>50,53</sup>

Furthermore, our findings show for the first time that BRM loss, as well the loss of its enzymatic activity, induces the upregulation of the transcription factor C/EBP $\beta$  (Figure 7). Although other C/EBP proteins are expressed in the mammary gland, C/EBP $\beta$  appears to have a critical role in mammary gland development and breast cancer.<sup>54</sup> The N-terminal region of C/EBP $\beta$  recruits the SWI/SNF nucleosome-remodeling complex, interacting with

BRM to activate silenced genes via chromatin remodeling and increase in transcription factor access to their binding sites.<sup>42</sup> C/EBP $\beta$  expression is dysregulated in human breast cancers, especially the most aggressive and poorly differentiated ones.<sup>55</sup> We found that BRM binds to the promoter of C/EBP $\beta$ , suggesting that it is able to interfere with the transcriptional apparatus and repress its activity. However, the BRM ATPase mutant does not bind to the C/EBP $\beta$  promoter and does not influence its transcription (Figure 7). These data suggest that the chromatin remodeling activity of BRM is involved in the regulation of C/EBP $\beta$ . We propose that when BRM is functionally active, it regulates its accessibility to the C/EBP $\beta$  promoter as well as of other factors, mediating the suppression of C/EBP $\beta$ .

Our findings emphasize the importance of examining the role of the SWI/SNF chromatin remodeling ATPase BRM as a critical regulator of normal tissue differentiation and homeostasis and illustrate the potential permissive role of BRM loss in tumor progression. Owing to the epigenetic function of the SWI/SNF complex, it is possible that there are many other relevant genes altered by BRM perturbation within our specific experiments. We chose to focus on the identification of regulators of stromal–epithelial interactions, discovering a significant upregulation of  $\alpha$ 5 integrin and C/EBP $\beta$  in the absence of BRM, and a strong reversion of the malignant phenotype upon silencing of C/EBP $\beta$ . Taken together, our data illustrate a novel and intriguing link between oncogenic transformation, epigenetic changes and cell–ECM interactions that controls tissue architecture. Based on the data presented, BRM loss may occur in breast cancers when GFR/Ras/ MAPK are dysregulated, leading to C/EBP $\beta$  upregulation and subsequent  $\alpha$ 5 integrin upregulation, contributing to disease progression (Figure 8e). One might speculate that this downregulation may be enhanced in the presence of ErbB2 or Myc gene amplifications. The presented findings provide the first evidence of a molecular mechanism to explain altered stromal–epithelial interaction. Understanding the mechanisms underlying the role of cell–ECM interactions in oncogene-driven transformation may lead to targetable pathways for the treatment of breast cancer.

## Materials and Methods

### Reagents, cell cultures and 3D morphogenesis assay

MCF10A cells were propagated and manipulated as previously described.<sup>56</sup> MCF10AT1 MECs were obtained from the Barbara Ann Karmanos Cancer Institute (Detroit, MI, USA) and the generation of MCF10A RasV12<sup>35</sup> and MCF10AerbB2 have been previously described.<sup>3</sup> The HMT-3522 S-1 and T4-2 MECs were grown and manipulated as described previously.<sup>57</sup> MCF7 cells were cultured according to manufacturer's recommendations (American Type Culture Collection, Manassas, VA, USA). rBM was purchased from BD Biosciences (San Jose, CA, USA).

### Vector constructs and ectopic gene expression

Four Myc tags were added to the C terminus of a full-length BRM construct and cloned into the pLVpuroTetO7mCMV tetracycline-inducible lentiviral vector and expressed bicistronically with EGFP. Full-length human  $\alpha$ 5 integrin was cloned into the lentiviral pLVpuroTetO7mCMV tetracycline-inducible vector and expressed bicistronically with mCherry. The shRNA for BRM, Brg1 and scrambled control were derived from siRNA

previously designed<sup>58</sup> and were cloned into the pLVrtTRKRAB-NeoR tetracycline-inducible vector and expressed bicistronically with EGFP. Additional BRM shRNAs were used to repeat and confirm our data. Full-length human BRM containing a single-point mutation in the ATPase-binding domain (K749R)<sup>38</sup> was cloned into the pRet puro Tet IRES EGFP tetracycline-inducible vector. The  $\alpha 5$  integrin and C/EBP $\beta$  shRNA were purchased from Sigma-Aldrich (St Louis, MO, USA; TRCN0000029653 and TRCN0000007441). The C/EBP $\beta$  shRNA was cloned into pLVU6shRNAi CMV mCherry vector. For shRNA sequences, see Supplementary Information. The preparation of virus, cell transduction and selection has been previously described.<sup>57</sup>

### Immunofluorescence and immunoblotting

3D cultures were analyzed and immunoblotting were performed as previously described.<sup>10,56,57</sup>

### Morphometric analysis, cell proliferation and apoptosis

Colony size in 3D rBM assays was analyzed at indicated times as previously described.<sup>56</sup> Cell proliferation and apoptosis were measured by calculating the percentage of Ki67 or caspase-3 labeled colonies.

### Real-time PCR

Random-primed cDNA was prepared from total isolated RNA (Trizol; Invitrogen, Carlsbad, CA, USA). Target cDNA sequences were quantified by real-time PCR using SYBR. For primer sequence, see Supplementary Information.

### Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were performed as described in Gilbert *et al.*<sup>57</sup> according to the manufacturer's directions (17–371, Millipore, Billerica, MA, USA). BRM/DNA and C/EBP $\beta$ /DNA fragments were immunoprecipitated using the rabbit polyclonal anti-BRM (ab15597, Abcam, Cambridge, MA, USA) and the anti-C/EBP $\beta$  (C-19, sc-150, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies, respectively. Rabbit IgG (02-6102, Invitrogen) was used as a negative control and anti-RNAPolymerase II (05-623B, Millipore) antibody as a positive control. For primer sequences, see Supplementary information.

### Function-blocking, MEK and c-Myc inhibition studies

To inhibit integrin function, cells were incubated with antibodies against  $\alpha 2$  integrin (MAB1988),  $\alpha 5$  integrin (MAB1956Z), FN (MAB88916; Millipore) or IgG isotype-matched control monoclonal antibodies (Jackson ImmunoResearch, West Grove, PA, USA; 2–20  $\mu$ g IgG/ml) at the time of cell embedment in 3D rBM assays. For the MEK inhibition studies, cells were incubated with U0126 (10  $\mu$ M, LC Laboratories, Woburn, MA, USA) at the time of cell embedment in 3D. For the c-Myc inhibition study, cells were incubated with 10058/F4 for 24 h (100  $\mu$ M, Millipore).

### Adhesion assay

Plates were coated with 0.05% Matrigel, 0.06 mg/ml collagen I, 10 g/ml collagen IV (BD Biosciences), 10 µg/ml FN, 10 µg/ml laminin I (Sigma, St Louis, MO, USA) or 1% bovine serum albumin (Amresco, Solon, OH) diluted in phosphate-buffered saline (Invitrogen), blocked (1 h room temperature; 0.1% bovine serum albumin), incubated with cells (30 min, 37 °C), washed (3 × phosphate-buffered saline) and incubated with 0.5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (4 h, room temperature; Sigma). Cells were lysed and quantified via spectrophotometry.

### Flow cytometry

Cells were isolated, nonspecific binding blocked (60 min, Dulbecco's phosphate-buffered saline, 0.1% bovine serum albumin) and incubated with saturating concentrations of primary monoclonal antibody (1 h), washed three times with phosphate-buffered saline and labeled with phycoerythrin-conjugated goat immunoglobulin. Stained cells were washed three times and data were acquired on a FACScan (Beckton Dickinson, San Jose, CA, USA). All manipulations were conducted at 4°C. A minimum of 20 000 gated events were collected to maximize statistical power. Histogram analysis was completed on FlowJo Analysis Software (Tree Star, Inc., Ashland, OR, USA).

### Soft agar assay and *in vivo* study

Anchorage-independent growth was assessed using a soft agar assay.<sup>57</sup> All *in vivo* experiments were performed in accordance with the guidelines of Laboratory Animal Research at the University of California, San Francisco. Five-week-old BalbC nu/nu mice were subcutaneously injected into the mammary fatpad ( $5 \times 10^6$  cells/injection, together with Matrigel), and palpable lesions were detected, measured and monitored biweekly for 3 weeks. At experiment termination, mice were killed and tumors were dissected and weighed.

### Statistical analysis

InStat software (Graphpad, LaJolla, CA, USA) was used to conduct the statistical analysis of our data. Unless otherwise stated, two-tailed Student's *t*-tests were used for significance testing and two-tailed Pearson tests were used for correlation analysis. Means are presented as  $\pm$  s.e.m. of three to five independent experiments and statistical significance was considered  $P < 0.05$ . Unless otherwise noted, sample size was  $n = 3$ .

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

We thank R Bainer for analyzing the BRM promoter, MP Marinkovich for the BM165 antibody and C Damsky for the AIIB2 antibody. This work was supported by DOD BC044791 (W81XWH-05-1-0330), and NIH/NCI R01 CA138818 (to VMW), NIH/NCI R01 CA085492 (to HLM and VMW), DOD BC062562 (to JKM), and NIEHS U01ES019458 (to ZW).

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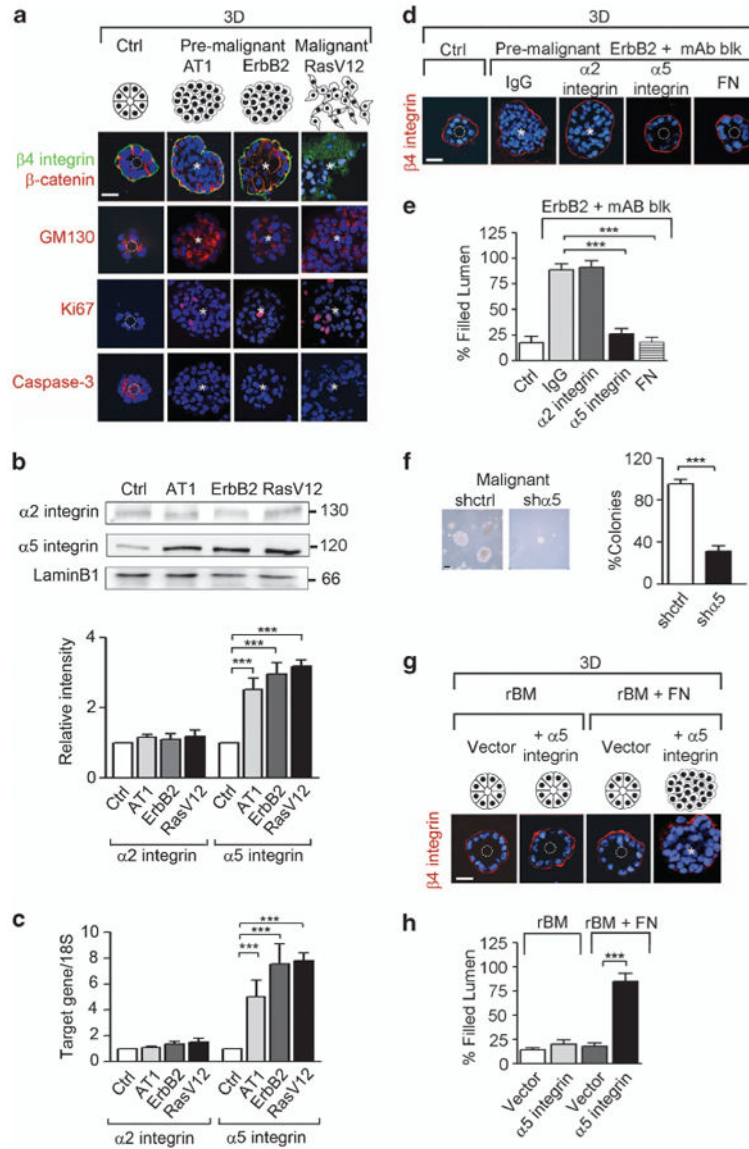
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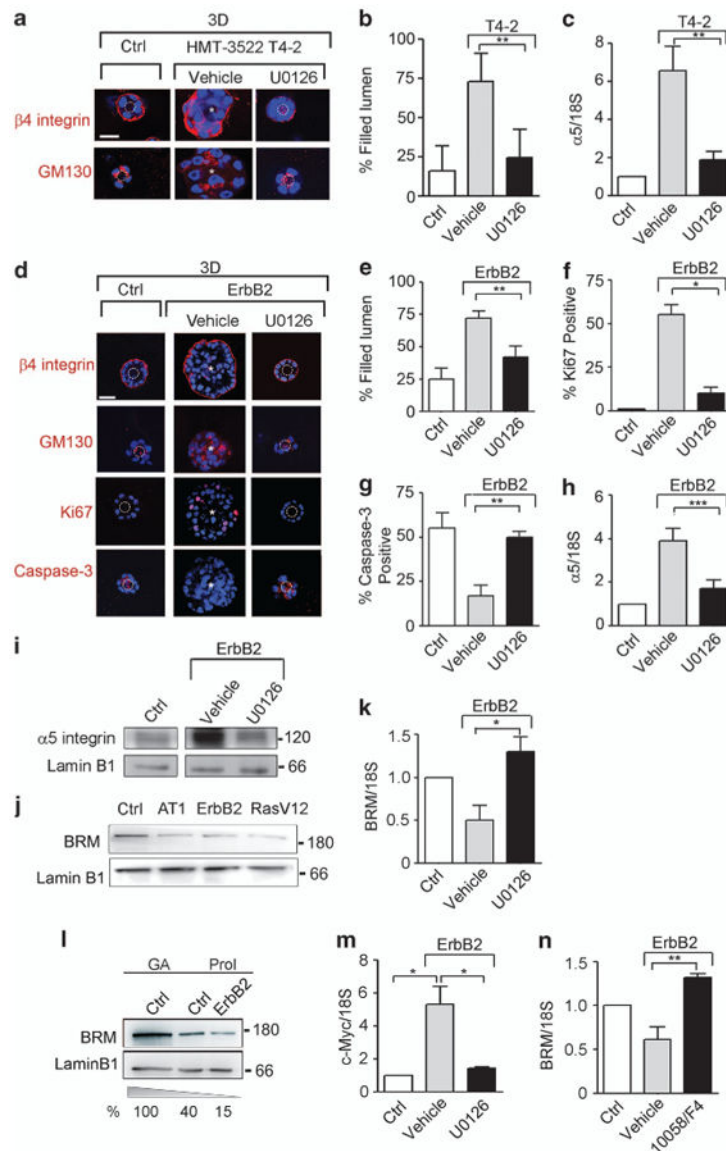
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**Figure 1.** Oncogenic transformation is mediated by fibronectin (FN) ligation of  $\alpha 5\beta 1$  integrin. **(a)** Schematic representation of the different morphologies observed in MCF10A (control, Ctrl), MCF10AT1 (AT1), MCF10A-ErbB2 (ErbB2) and MCF10A-RasV12 (RasV12) cells grown in reconstituted basement membrane (rBM) for 16 days. Lower panels: immunofluorescence staining for  $\beta 4$  integrin and  $\beta$ -catenin (green and red, respectively), the apical marker GM130 (red), the proliferative marker Ki67 (red) and the apoptotic marker cleaved caspase-3 (red); nuclei were visualized with 4'-6-diamidino-2-phenylindole (DAPI; blue). **(b)** Upper panels: cells in **a** were analyzed by immunoblot for the expression of the integrin subunits,  $\alpha 2$  and  $\alpha 5$ , and total lamin B1 (loading control). Lower panel: histogram demonstrating the ratio between integrin subunits expression to their internal control, lamin B1. **(c)** mRNA of  $\alpha 2$  and  $\alpha 5$  integrin from cells in **a** was analyzed by reverse transcription-PCR and normalized to 18S. **(d)** ErbB2 cells were treated with function-blocking antibodies

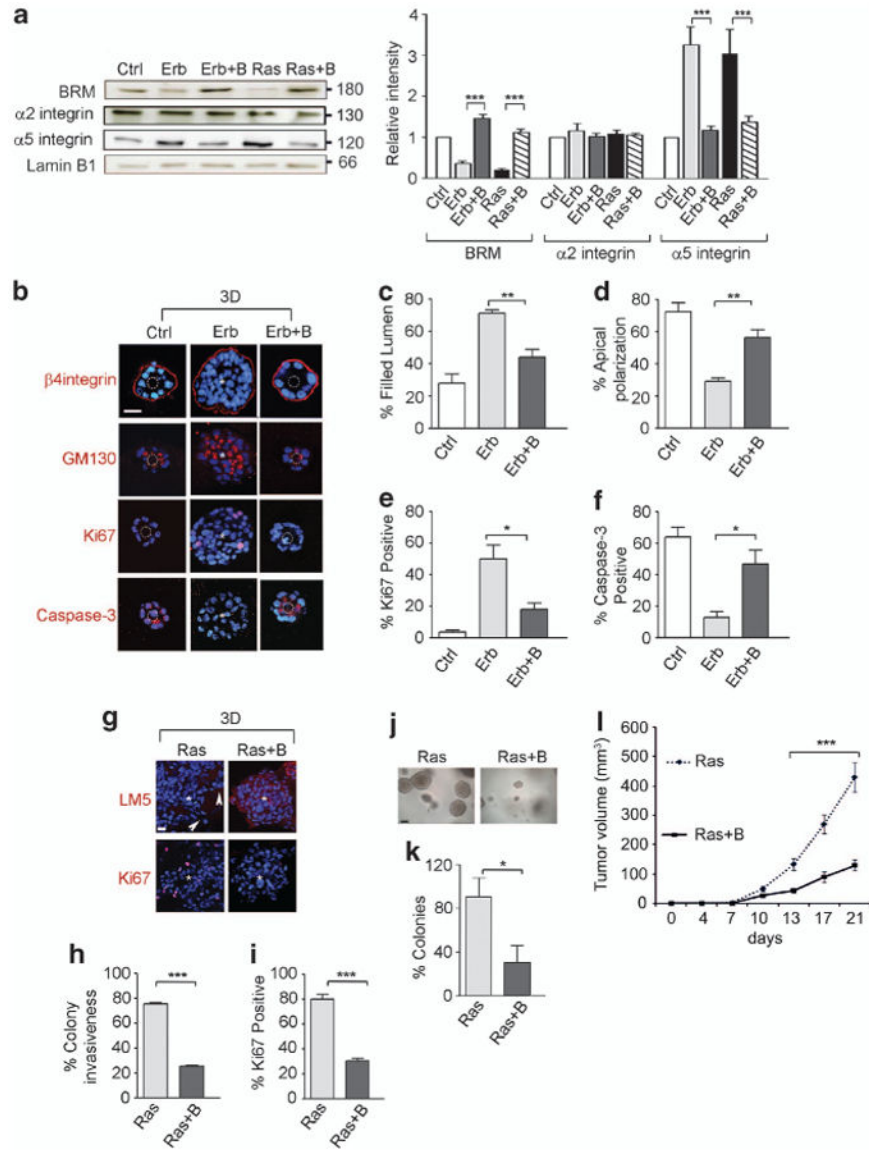
(mAb blk) against  $\alpha 2$  integrin,  $\alpha 5$  integrin and FN or isotype control IgG (IgG). The colonies were stained for  $\beta 4$  integrin (red) and nuclei were visualized with DAPI (blue). (e) Graph showing the percentage of filled lumens from **d**. (f) MCF7 cells were transduced with a lentiviral scrambled (shctrl) or a  $\alpha 5$  integrin knockdown constructs (sh $\alpha 5$ ). Phase-contrast images and percentage of tumor colonies greater than 30  $\mu\text{m}$  in diameter embedded within soft agar is shown. Scale bar, 60  $\mu\text{m}$ . (g)  $\alpha 5$  Integrin was ectopically expressed in MCF10A cells (+ $\alpha 5$  integrin). Cells were grown in 3D rBM, treated with fibronectin (+FN) and immunostained for  $\beta 4$  integrin (red); nuclei were visualized with DAPI (blue). (h) Graph showing the percentage of filled lumen colonies from **g**. In all pictures, white dashed circles indicate cleared lumens; asterisks indicate the absence of a lumen. Scale bar, 25  $\mu\text{m}$  ( $n > 50$  acini). Results are the mean  $\pm$  s.e.m. of three separate experiments (\*\*\*)  $P < 0.001$ .

**Figure 2.**

Oncogenic ERK increases c-Myc to induce  $\alpha 5$  integrin expression by repressing BRM transcription. **(a)** HMT-3522 S1 (control, Ctrl) and T4-2 cells treated with dimethyl sulfoxide (DMSO; vehicle) or with the MEK inhibitor, U0126, were grown in 3D rBM for 16 days, stained for  $\beta 4$  integrin (red) and GM130 (red); nuclei were visualized using 4'-6-diamidino-2-phenylindole (DAPI; blue). The percentage of filled lumen colonies is shown in **b**. **(c)**  $\alpha 5$  Integrin mRNA was quantified by reverse transcription (RT)-PCR and normalized to 18S. **(d)** MCF10A (Ctrl) and ErbB2 cells treated with DMSO (vehicle) or with the MEK inhibitor, U0126, were grown in 3D rBM for 16 days, stained for  $\beta 4$  integrin (red), GM130 (red), Ki67 (red) and cleaved caspase-3 (red); nuclei were visualized using DAPI (blue). **(e-g)** The percentage of filled lumen colonies **(e)**, Ki67 **(f)** and caspase-3 **(g)**-positive colonies is shown. **(h)**  $\alpha 5$  Integrin mRNA was quantified by RT-PCR and normalized to 18S. **(i)**  $\alpha 5$  Integrin protein was analyzed by immunoblot and laminB1 was used as loading control. **(j)**

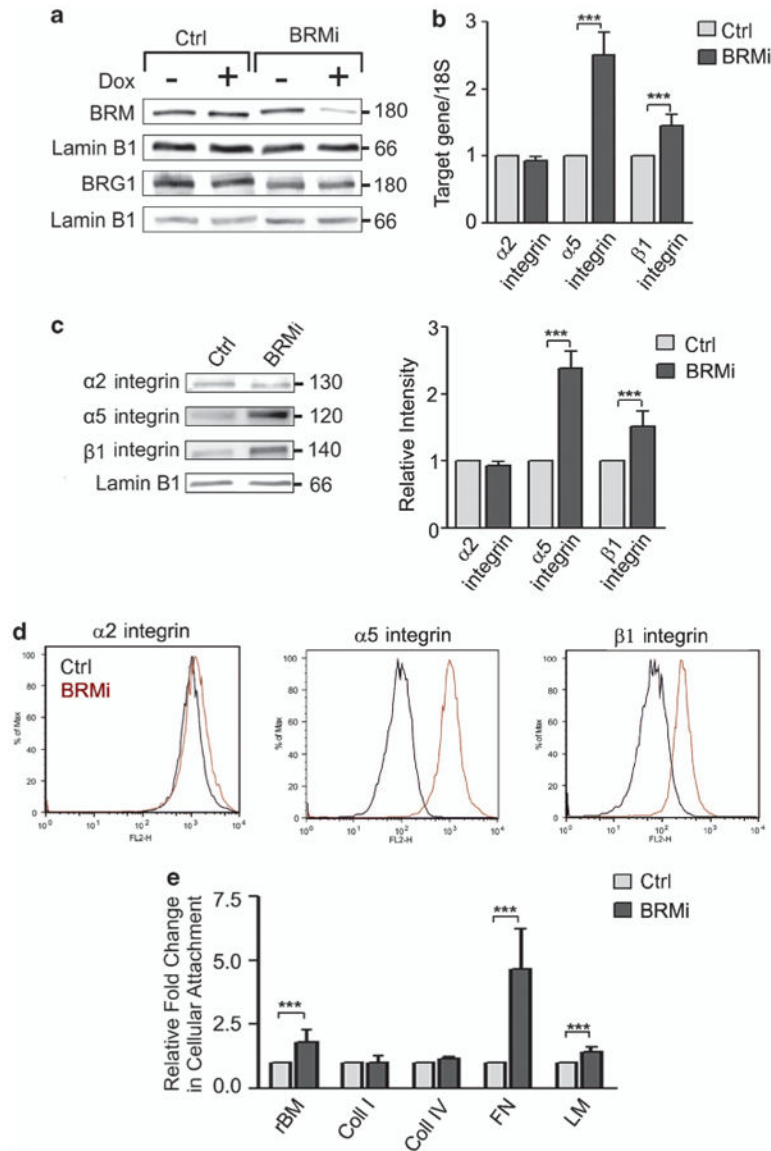
MCF10A (Ctrl), MCF10AT1 (AT1), MCF10A-ErbB2 (ErbB2) and MCF10A-RasV12 (RasV12) cells were analyzed by immunoblot for the expression of BRM and laminB1 (loading control). **(k)** Graph showing BRM mRNA quantification of cells treated as in **d** and normalized to 18S. **(l)** BRM protein expression was analyzed in serum starved, growth arrested (GA) MCF10A (Ctrl) and proliferating MCF10A and ErbB2 cells, and normalized to lamin B1 (%). **(m)** Graph showing c-Myc mRNA quantification of cells treated as in **d** and normalized to 18S. **(n)** MCF10A (Ctrl) and ErbB2 cells were treated with DMSO (vehicle) or with the c-Myc inhibitor, 10058/F4, and BRM mRNA was analyzed as described above. White dashed circles indicate cleared lumens; asterisks indicate the absence of a lumen. Scale bar, 25  $\mu$ m. In all histograms, results are the mean  $\pm$  s.e.m. of three separate experiments (\* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001).





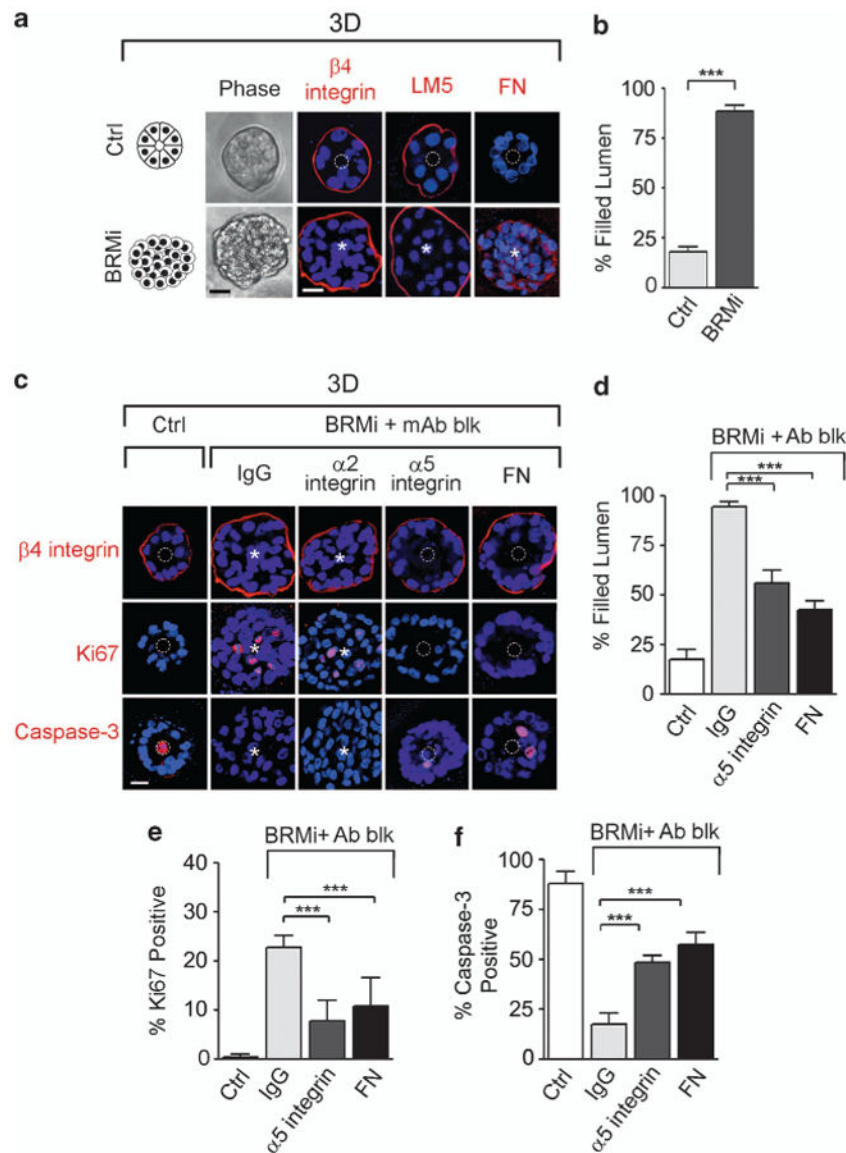
**Figure 3.** BRM regulates  $\alpha 5$  integrin to modulate MEC malignant behavior in 3D and *in vivo*. BRM was ectopically expressed in MCF10A-ErbB2 (Erb) and MCF10A-RasV12 (Ras) cells. (a) MCF10A (control, Ctrl), ErbB2 (Erb), ErbB2+BRM (Erb+B), RasV12 (Ras) and RasV12+BRM (Ras+B) cells were analyzed for BRM,  $\alpha 5$  integrin and  $\alpha 2$  integrin protein expression and normalized to the loading control, lamin B1. (b–i) Cells in a were grown in 3D rBM and stained for  $\beta 4$  integrin (red), GM130 (red), Ki67 (red), cleaved caspase-3 (red) and laminin5 (LM5, red); nuclei were visualized using 4'-6-diamidino-2-phenylindole (blue). White dashed circles indicate cleared lumens; asterisks indicate the absence of a lumen. Scale bar, 25  $\mu$ m. (c–f) Graphs demonstrating the percentage of filled lumens (c), apical polarized GM130 (d), Ki67 (e) and caspase-3 (f)-positive colonies from b. (h, i) Graph showing the percentage of invasive (h) and Ki67-positive (i) colonies from g. Arrowheads point out the invasive protrusions ( $n > 50$  acini per sample;  $*P < 0.05$ ,  $**P < 0.01$ ,

\*\*\* $P < 0.001$ ). **(j, k)** Phase-contrast images **(j)** and percentage of Ras and Ras + B tumor colonies greater than 30  $\mu\text{m}$  in diameter **(k)** embedded within soft agar. Scale bar, 60  $\mu\text{m}$ . **(l)** Ras and Ras + B cells were orthotopically injected into the mammary fatpad of Balb/c nu/nu mice. Graph showing tumor volumes measured twice a week for 21 days ( $n = 10$ , \*\*\* $P < 0.001$ ).



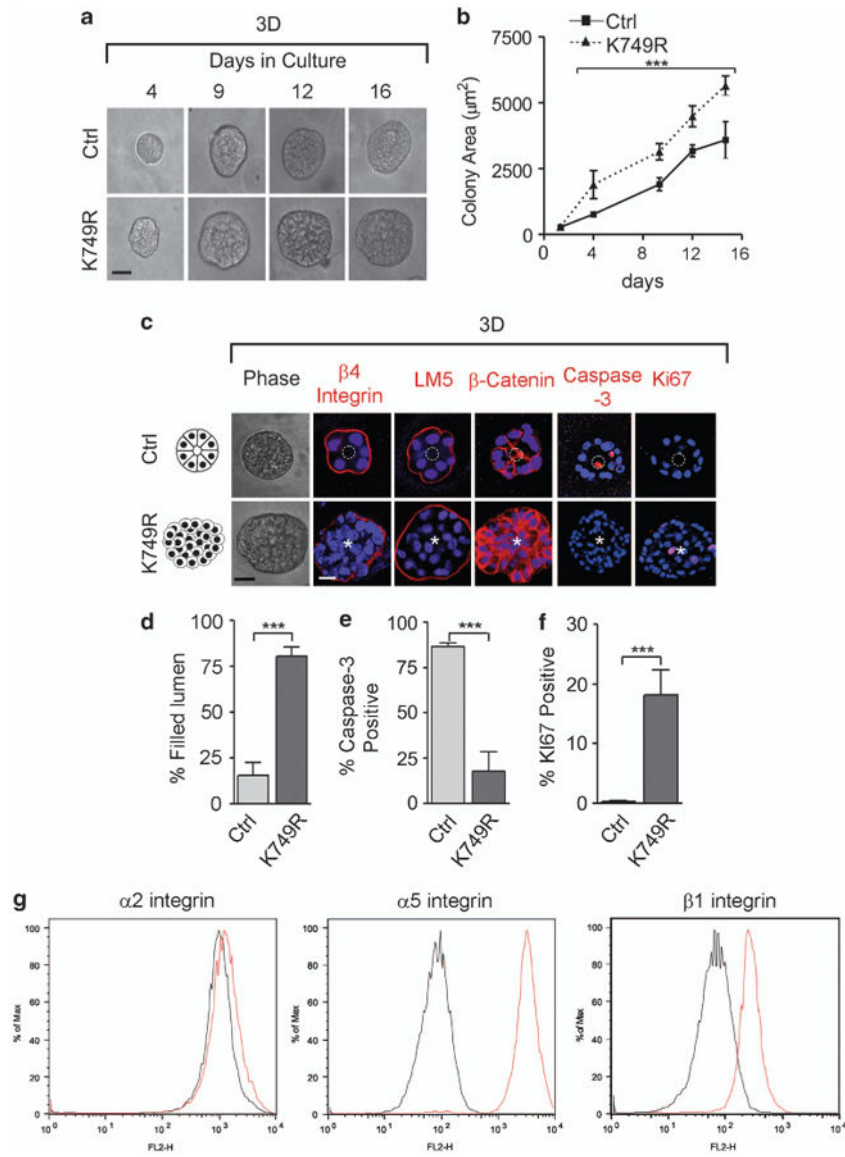
**Figure 4.**

Loss of BRM is associated with elevated  $\alpha 5 \beta 1$  integrin expression and activity. **(a)** MCF10A cells were transduced with inducible shRNA scrambled (control, Ctrl) or BRM (BRMi) knockdown constructs and induced with doxycycline (Dox +/-). Cell extracts were analyzed by immunoblot using BRM, Brg1 and laminB1 specific antibodies. **(b, c)** Cells in a were analyzed for mRNA (normalized to 18S) **(b)** and protein expression **(c)** of the integrin subunits  $\alpha 2$ ,  $\alpha 5$  and  $\beta 1$ . Right panel **(c)**: protein expression was normalized to the loading control, lamin B1. **(d)** Representative histogram plots of three separate fluorescence-activated cell sorting analyses of cell surface integrin  $\alpha 2$ ,  $\alpha 5$  and  $\beta 1$  in Ctrl and BRMi cells. **(e)** Ctrl and BRMi cells were plated on different extracellular matrix proteins for 24 h, fixed, stained and counted. Graph showing the ratio in cellular attachment between Ctrl and BRMi cells. In all histograms, results are the mean  $\pm$  s.e.m. of three separate experiments (\*\*\*) $P < 0.001$ .



**Figure 5.** Loss of BRM expression enhances MEC 3D behavior by regulating fibronectin- $\alpha 5\beta 1$  integrin interaction. MCF10A cells were transduced with inducible shRNA scrambled (control, Ctrl) or BRM (BRMi) knockdown constructs. **(a)** Left panels: phase-contrast pictures of Scrm and BRMi acini. Right panels: immunostaining (red) for integrin  $\beta 4$ , laminin-5 (LM5) and fibronectin (FN); nuclei were visualized with 4'-6-diamidino-2-phenylindole (DAPI; blue). **(b)** Colonies in **a** were scored for the presence of a filled lumen. **(c)** BRMi cells were treated with  $\alpha 2$  integrin,  $\alpha 5$  integrin, FN function-blocking antibodies (mAb blk) or isotype control IgG (IgG), and stained for  $\beta 4$  integrin (red), cleaved caspase-3 (red) and Ki67 (red); nuclei were visualized using DAPI (blue). **(d-f)** Graphs showing the percentage of filled lumens **(d)**, Ki67 **(e)** and caspase-3 **(f)**-positive colonies. White dashed circles indicate cleared lumens; asterisks indicate the absence of a lumen. Scale bar, 25  $\mu$ m.

Results are the mean  $\pm$  s.e.m. of five separate experiments ( $n > 50$  acini per sample; \*\*\* $P < 0.001$ ).

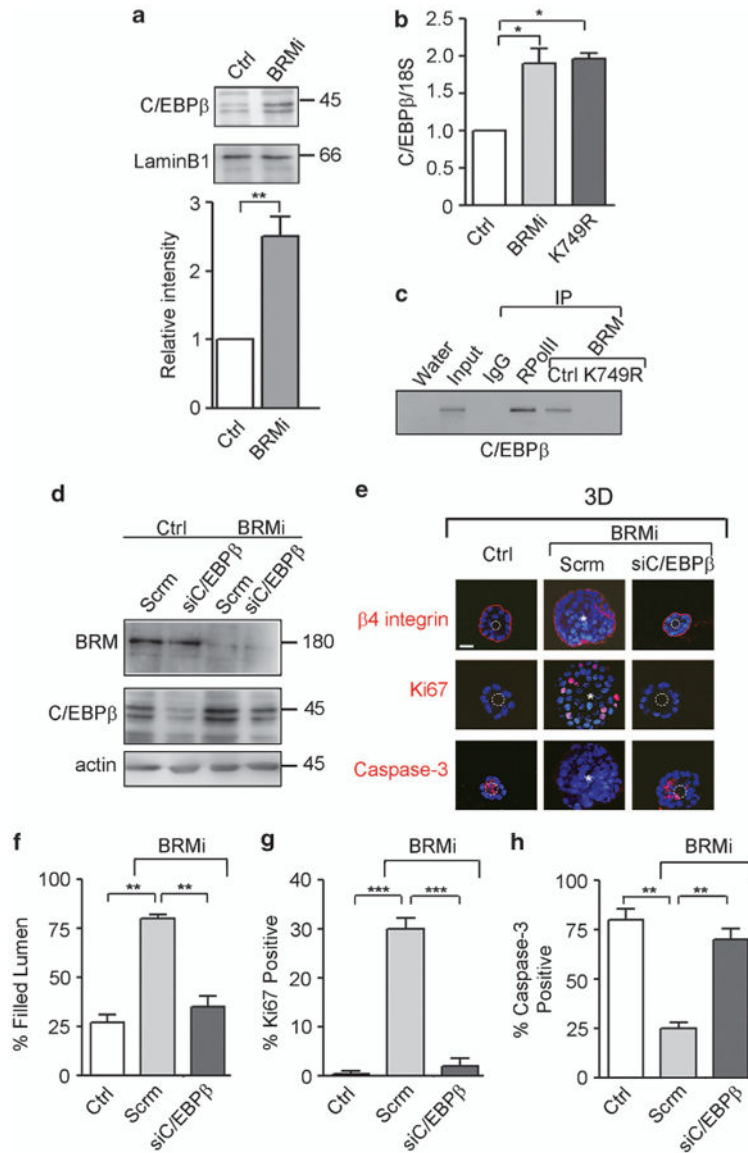


**Figure 6.**

Loss of Brm ATPase remodeling activity induces MEC survival in 3D. MCF10A cells were transduced with a retroviral control (Ctrl) and an ATPase-dead BRM mutant vector (K749R), and grown in 3D rBM for 16 days. **(a)** Phase-contrast pictures of Ctrl and K749R colonies. **(b)** Graph showing the colony size (depicted in area) of the cells from **a**. **(c)** Ctrl and K749R cells grown in 3D were imaged with phase-contrast microscopy (left panels) and stained for  $\beta 4$  integrin, laminin-5 (LM5),  $\beta$ -catenin, cleaved caspase-3, and Ki67; nuclei were visualized with 4'-6-diamidino-2-phenylindole (DAPI; blue). **(d)** Graph showing the percentage of colonies with a filled lumen, of **(e)** caspase-3 or **(f)** Ki67 positive colonies. White dashed circles indicate cleared lumens; asterisks indicate the absence of a lumen. Scale bar, 25  $\mu\text{m}$ . In all panels, results are the mean  $\pm$  s.e.m. of five separate experiments ( $n > 50$  acini per sample;  $***P < 0.001$ ). **(g)** Representative histogram plots of three separate

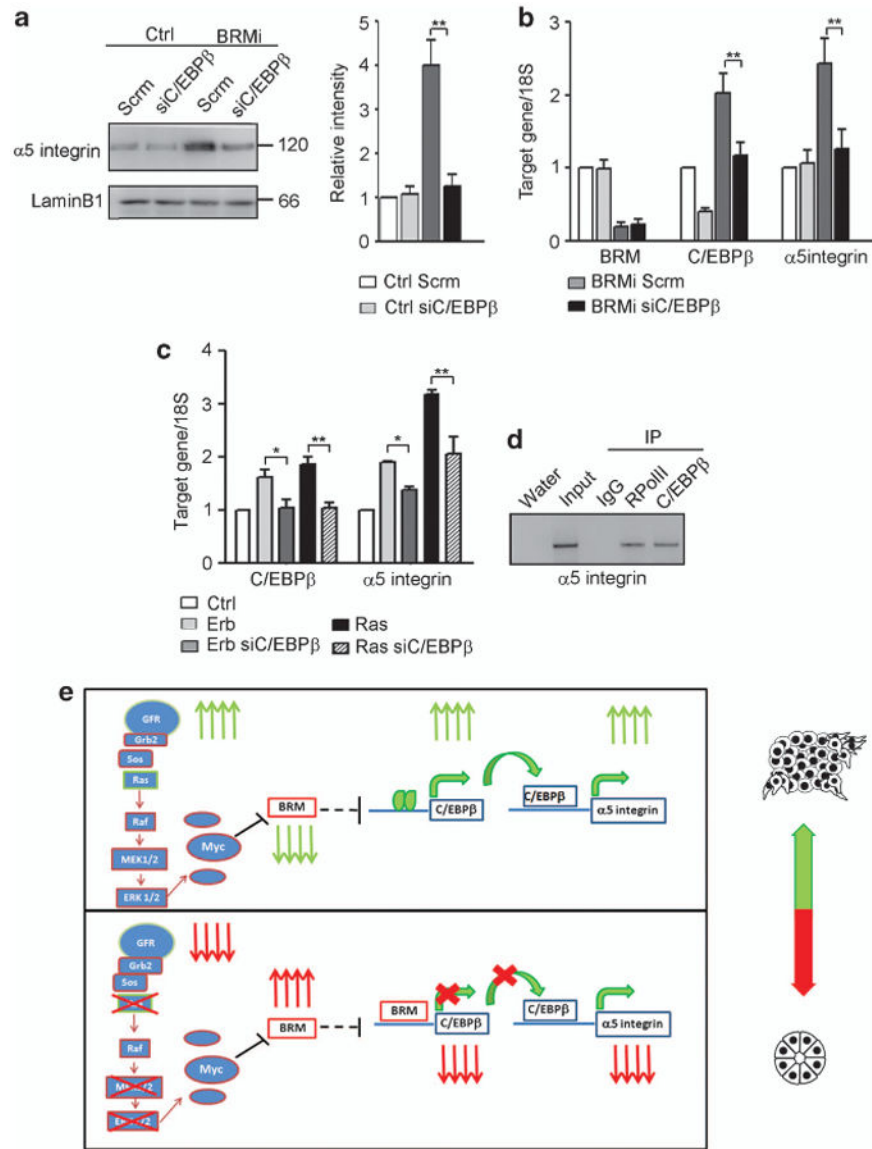


fluorescence-activated cell sorting analyses of cell surface integrins  $\alpha 2$ ,  $\alpha 5$  and  $\beta 1$  in Ctrl and K749R cells.



**Figure 7.** BRM inhibits the malignant phenotype by repressing C/EBP $\beta$  transcription. (a) Upper panels: lysates from MCF10A scrambled (control, Ctrl) and BRM silenced (BRMi) cells were analyzed by immunoblot for C/EBP $\beta$  and laminB1. Lower panel: graph showing C/EBP $\beta$  protein expression, normalized to the loading control, laminB1. (b) C/EBP $\beta$  mRNA was analyzed in MCF10A (Ctrl), BRM silenced and ATPase mutant (K749R) cells and normalized to 18S. (c) Representative gel of chromatin immunoprecipitation studies in Ctrl and K749R cells, revealing co-precipitation of BRM with C/EBP $\beta$  (RPolIII is RNA polymerase II antibody). (d) Ctrl and BRMi cells were transduced with a lentiviral scrambled (Scrm) or a C/EBP $\beta$  silencing vector (siC/EBP $\beta$ ) and analyzed for BRM, and C/EBP $\beta$  protein expression by immunoblotting. (e) Cells in d were grown in 3D rBM for 16 days, stained for  $\beta$ 4 integrin, Ki67 and cleaved caspase-3; nuclei were visualized with 4'-6-diamidino-2-phenylindole (DAPI; blue). (f) Graphs showing the percentage of colonies with

a filled lumen of (**g**) Ki67 or (**h**) caspase-3-positive colonies. White dashed circles indicate cleared lumens; asterisks indicate the absence of a lumen. Scale bar, 25  $\mu\text{m}$ . In all panels, results are the mean  $\pm$  s.e.m. of three separate experiments (\* $P < 0.05$ , \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).



**Figure 8.** BRM prevents C/EBPβ-dependent transcription of α5 integrin. (a) Left panel: Control (Ctrl) and BRMi cells were transduced with a lentiviral Scrm (Scrm) or a C/EBPβ silencing vector (siC/EBPβ) and analyzed for α5 integrin protein expression by immunoblotting. Right panel: graph showing α5 integrin protein expression, normalized to the loading control, laminB1. (b) mRNA of BRM, C/EBPβ and α5 integrin was analyzed by reverse transcription (RT)–PCR and normalized to 18S (\**P*<0.05, \*\**P*<0.01). (c) ErbB2 and RasV12 cells were transduced with a lentiviral Scrm (Erb, Ras) or a C/EBPβ silencing vector (Erb siC/EBPβ, Ras siC/EBPβ), analyzed for C/EBPβ and α5 integrin expression by RT–PCR and normalized to 18S. (d) Representative gel of chromatin immunoprecipitation studies in MCF10A cells revealing co-precipitation of C/EBPβ with the α5 integrin promoter (RPolII is RNA polymerase II antibody). In all panels, results are the mean ± s.e.m. of three separate experiments (\**P*<0.05, \*\**P*<0.01). (e) Upper panel: in the presence of oncogenic

transformation such as ErbB2 overexpression, when there is increased growth factor receptor (GFR) signaling, activated Ras-MEK-ERK signaling, expression of c-Myc is increased and represses BRM expression, leading to an increase in C/EBP $\beta$  expression/activity, thereby inducing  $\alpha$ 5 integrin by binding to the promoter. Lower panel: when the GFR and downstream Ras-MEK-ERK-c-Myc signaling pathways are suppressed or downregulated, BRM is upregulated, binds to the C/EBP $\beta$  promoter and represses the expression of C/EBP $\beta$ , causing  $\alpha$ 5 integrin downregulation.