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Brg1 controls the expression of *Pax7* to promote viability and proliferation of mouse primary myoblasts

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Abstract

Brg1 (Brahma-related gene 1) is a catalytic component of the evolutionarily conserved mammalian SWI/SNF ATP-dependent chromatin remodeling enzymes that disrupt histone-DNA contacts on the nucleosome. While the requirement for the SWI/SNF enzymes in cell differentiation has been extensively studied, its role in precursor cell proliferation and survival is not as well defined. Muscle satellite cells constitute the stem cell pool that sustains and regenerates myofibers in adult skeletal muscle. Here we show that deletion of Brg1 in primary mouse myoblasts derived from muscle satellite cells cultured *ex vivo* leads to a cell proliferation defect and apoptosis. We determined that Brg1 regulates cell proliferation and survival by controlling chromatin remodeling and activating transcription at the *Pax7* promoter, which is expressed during somite development and is required for controlling viability of the satellite cell population. Reintroduction of catalytically active Brg1 or of *Pax7* into Brg1-deficient satellite cells rescued the apoptotic phenotype and restored proliferation. These data demonstrate that Brg1 functions as a positive regulator for cellular proliferation and survival of primary myoblasts. Therefore the regulation of gene expression through Brg1-mediated chromatin remodeling is critical not just for skeletal muscle differentiation but for maintaining the myoblast population as well.

Keywords

Brg1; SWI/SNF; Pax7; myoblast; cell proliferation

Introduction

Mammalian SWI/SNF ATP-dependent chromatin remodeling enzymes disrupt histone-DNA contacts on the nucleosome using the energy released by ATP hydrolysis (Imbalzano et al., 1994; Kwon et al., 1994). These structural alterations result in increased or decreased chromatin accessibility for the binding of regulatory proteins that modulate replication, recombination, and transcription (Bartholomew, 2014; Clapier and Cairns, 2009; Mueller-Planitz et al., 2013). The SWI/SNF family is highly conserved in eukaryotes and its catalytic

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activity is provided by one of two mutually exclusive ATPase subunits called Brahma (Brm) or Brg1 (Khavari et al., 1993; Muchardt and Yaniv, 1993; Wang et al., 1996). The SWI/SNF complexes also include other proteins known as Brg1 and Brm-associated factors that may modulate the activities of Brm or Brg1 (Wang et al., 1996). Chromatin remodeling is an important feature for integrating different signal transduction pathways into specific transcriptional responses that will determine the cellular fate. In this regard, the SWI/SNF complex is associated with the control of cellular proliferation, differentiation, and the cell cycle (de la Serna et al., 2006; Ho and Crabtree, 2010; Wu, 2012). The role of the SWI/SNF complex in differentiation of different lineages has been studied extensively; its role in proliferation and cell survival of precursor cells is less well understood.

Previous studies have definitively shown that Brg1 is necessary for early embryogenesis (Bultman et al., 2000; Bultman et al., 2006; Ho et al., 2009; Kidder et al., 2009; Sumi-Ichinose et al., 1997) and for terminal differentiation of most tissues (de la Serna et al., 2006; Ho and Crabtree, 2010; Wu, 2012). In contrast, it appears that the requirement for Brg1 in the development of precursor cells or in the maintenance of differentiated cells is more variable. For instance, the development and proliferation of mouse keratinocyte precursors were not impacted by Brg1 depletion (Indra et al., 2005). Depletion of Brg1 in *Xenopus* did not impact neural induction or cell fate determination (Seo et al., 2005). However, others studying depletion of Brg1 in mouse neural stem cells reached the opposite conclusion because Brg1 was required for neural stem cell maintenance (Lessard et al., 2007; Matsumoto et al., 2006). Expression of a Brg1 protein mutated in the ATPase domain supported primitive erythropoiesis in the yolk sac and development of pro- and basophilic erythroblasts in the fetal liver but were deficient for definitive erythropoiesis (Bultman et al., 2005). Brg1 is required to complete the stages of T cell development (Chi et al., 2003; Gebuhr et al., 2003) but is not required survival of mature T lymphocytes (Gebuhr et al., 2003). Differentiation of precursor cells into osteoblasts using a cell culture model system proceeded normally in the presence of a dominant negative Brg1 (Cruzat et al., 2009). Brg1 conditional embryonic fibroblasts depleted for Brg1 *ex vivo* survived and proliferated as well as control cells (Bultman et al., 2000). *In vivo* electroporation resulting in short-term expression of an ATPase-deficient Brg1 in skeletal muscle resulted in inhibition of expression of the Myogenin regulatory protein (Ohkawa et al., 2007), but skeletal muscle-specific depletion of Myogenin post-myogenesis resulted in only modest effects (Knapp et al., 2006), suggesting that there may not be an absolute requirement for Brg1 in terminally differentiated skeletal muscle.

Muscle satellite cells are located under the basal lamina that surrounds each myofiber (Mauro, 1961). Satellite cells have the capability to proliferate and to differentiate in order to sustain basal physiological myofiber turnover and muscle regeneration (Brack and Rando, 2012; Chang and Rudnicki, 2014; Montarras et al., 2013; Motohashi and Asakura, 2014; Sambasivan and Tajbakhsh, 2015), highlighting the need for effective mechanisms to maintain the satellite cell pool. The Pax7 transcriptional regulator has been shown to have an important role in the proliferation of the muscle stem cell pool (Brack and Rando, 2012; Buckingham and Rigby, 2014; Chang and Rudnicki, 2014; Montarras et al., 2013; Motohashi and Asakura, 2014; Sambasivan and Tajbakhsh, 2015). *Pax7*^{-/-} mice die within

2–3 weeks after birth, presumably due to the abnormal development of neural crest derivatives (Mansouri et al., 1996). *Pax7* knockout mice have a diminished number of muscle satellite cells and were impaired for muscle regeneration, supporting the idea that *Pax7* is required for the propagation and function of the satellite cell population (Oustanina et al., 2004; Seale et al., 2000). Moreover, deletion of *Pax7* led to an extended G2/M phase of the cell cycle and the pool of satellite cells is progressively lost due to cell death (Relaix et al., 2006). The anti-apoptotic properties of *Pax7* cannot be compensated by the closely related *Pax3* protein, highlighting the importance of *Pax7* in promoting cell survival and in controlling the stem cell populations of adult tissues (Relaix et al., 2006). More recent tissue-specific analyses have provided additional evidence for *Pax7* function in the maintenance and regenerative capacity of satellite cells (Gunther et al., 2013; von Maltzahn et al., 2013). The onset of satellite cell differentiation leads to the down-regulation of *Pax7* and triggers the expression of Myogenin (Zammit et al., 2004). Consistent with these observations, overexpression or constitutive expression of *Pax7* inhibited or delayed the expression of Myogenin in cultured cells (Olguin and Olwin, 2004; Zammit et al., 2006), while Myogenin expression repressed *Pax7* expression, suggesting a reciprocal inhibition of regulators controlling satellite cell maintenance and differentiation (Olguin et al., 2007).

We investigated the function of the chromatin remodeling enzyme Brg1 in proliferating primary myoblasts derived from muscle satellite cells. We identified a critical role for Brg1 in directly regulating the transcriptional regulator *Pax7* and, consequently, a role in satellite cell proliferation and survival under non-differentiating conditions.

Materials and methods

Generation of plasmids

Full-length, FLAG-tagged wild type human Brg1 and a mutated version in the ATP binding site have been previously described (De la Serna et al., 2000; Khavari et al., 1993; Sif et al., 2001). Briefly, both the FLAG-tagged wildtype and ATP binding site mutant Brg1 alleles were excised from the pBluescript vector as a ClaI fragment, blunted, and cloned into the EcoRV site of pCR3.1 (Invitrogen). Plasmid EYFP-c1-Brg1 (a kind gift from Gordon Hager), encodes YFP fused to the N-terminus of Brg1 (Johnson et al., 2008). The NheI–BamHI fragment from this plasmid was cloned into AgeI–BamHI digested pCR3.1-Brg1 for both the wildtype and mutant Brg1 sequences to create YFP-Brg1 fusion proteins. The resulting plasmids were digested with AgeI and PmeI and the sequences encoding the YFP-Brg1 fusion proteins were cloned into AgeI–PmeI digested pBABE vector encoding mCherry flanked by Cre recombination sites. The murine *Pax7* coding region was PCR amplified from cDNA prepared from C57BL/6 primary myoblasts using the primers indicated in Table 1 and cloned into the pBABE retroviral vector.

Western blots and antibodies

Proliferating myoblasts were washed with PBS and solubilized with RIPA buffer (10 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 7.4, 150 mM NaCl, 2 mM ethylenediamine-tetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, and 10% glycerol) containing protease inhibitors (Complete Mini; Roche). 100 µg of each sample was prepared

for SDS-polyacrylamide gel electrophoresis by boiling in sample buffer. The resolved proteins were electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad). The proteins of interest were then detected with the specific polyclonal or monoclonal antibodies indicated, followed by species-appropriate peroxidase-conjugated antibodies (Pierce) and chemiluminescent detection (ECL PLUS; GE Healthcare). Rabbit anti-Brg1 antibody (H-88; sc-10768); goat anti-Pax-3/7 antibody (N-19; sc-7749); rabbit anti-Caspase-3 antibody (H-277; sc-7148) were purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-PI3 Kinase p85 antibody, N-SH2 domain (ABS233) was obtained from Millipore. Mouse anti-GAPDH-Peroxidase conjugated (G9295) was obtained from Sigma Aldrich.

Animal use and cell culture

Mice were housed in the University of Massachusetts Medical School animal care facility as specified by Institutional Animal Care and Use Committee guidelines. Satellite cells were isolated from total leg muscle from Brg1 conditional mice (Bultman et al., 2000) as described (Bischoff and Heintz, 1994). The cells were grown in culture on plates coated in 0.02% collagen (Advanced BioMatrix) in a 1:1 mix of DMEM and F-10 media supplemented with 20% fetal bovine serum, 2% Chick Embryo Extract (Sera Laboratories) and 25 ng/ml recombinant basic FGF (Millipore).

Retrovirus was produced as previously described (Morgenstern and Land, 1990). Cells were infected with retrovirus in the presence of 8 µg/ml polybrene and selected for 2 days with 1 µg/ml puromycin (Invitrogen). After 2 days of puromycin selection, cells were replated at low density and allowed to grow for one week under puromycin selection. mCherry expressing cells were transferred to collagen coated wells of 96 well plates. Media was changed every other day, and cells were transferred to a new collagen coated well every 5 days. Once there were sufficient cells to reach 50% confluence on a 60 mm dish, cells were tested for transgene expression by western blot. Adenovirus for expression of Cre-recombinase (Ad-Cre; University of Iowa Viral Vector Core) (Stec et al., 1999) was administered at a MOI of 50.

TUNEL staining

Cells were fixed in PBS, pH 7.4 containing 2% formaldehyde at 4 °C for 15 min, permeabilized with ice cold 70% ethanol, and washed 3 times with ice cold PBS. Incorporation of biotinylated dUTP onto the 3' ends of fragmented DNA was performed with terminal deoxynucleotidyl transferase. Briefly, cells were incubated in TdT equilibration buffer (2.5 mM Tris-HCl, pH 6.6, 0.2 M potassium cacodylate, 2.5 mM CoCl₂, 0.25 mg/mL bovine serum albumin (BSA)). The cell suspension was incubated at 37°C for 30 min with occasional gentle mixing. Then 0.5 U/µL of TdT enzyme and 40 pmol/µL biotinylated-dUTP were added (Roche Diagnostics Corp.). Cells were washed in PBS, collected by centrifugation, and incubated 30 min at RT in blocking solution containing 3% BSA and 20% normal goat serum in 1% (w/v) in 0.1 M Tris-buffered saline (TBS). Samples were subsequently treated for 1 h at RT with peroxidase-labeled anti-digoxigenin antibody followed by washing and developed with 0.05% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (DAB).

Gene expression analysis

RNA was purified from three independent biological replicates of proliferating primary myoblasts with TRIzol following the manufacturer's protocol (Invitrogen). The RNA samples (1 µg) were used as templates for cDNA synthesis with random primers and SuperScript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed with Fast SYBR green master mix on the ABI StepOne Plus Sequence Detection System using the primers listed in Table 1 and normalized to the levels of EF1-alpha expression (Ohkawa et al., 2006). One-tailed t tests were performed for statistical analyses using Kaleidagraph software.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays using cultured mouse primary myoblasts were performed in triplicate from three independent biological samples as described (Dacwag et al., 2007; Hernandez-Hernandez et al., 2013). Proliferating cell lysates were incubated overnight with polyclonal rabbit antisera against Brg1 (De la Serna et al., 2000), or normal rabbit IgG (Santa Cruz Biotechnology, sc-3888). Cross-linking was reversed, and DNA was purified using ChIP DNA Clean and Concentrator Columns (Zymo Research). qPCR was performed using Fast SYBR green master mix on the ABI StepOne Plus Sequence Detection System. Primers used are listed in Table 1. Quantification was done using the comparative Ct method to obtain the percent of total input DNA pulled down by each antibody. Unpaired Student's t tests (two-tailed) were performed using the Kaleidagraph software.

Restriction enzyme accessibility assay

Nuclei from three independent biological replicates of proliferating myoblasts were isolated by sucrose gradients. Briefly, the cells were trypsinized, resuspended in buffer containing 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT and homogenized with a dounce tissue homogenizer. Nuclei were separated from cytoplasm by centrifuging the samples 5 min at $218 \times g$ at 4 °C, resuspended in 3 ml of Buffer S1 (0.25 M sucrose, 10 mM MgCl₂), layered over 3 ml of Buffer S2 (0.35 M sucrose, 10 mM MgCl₂) and centrifuged at $1430 \times g$ at 4 °C. Restriction enzyme accessibility assays using cultured myoblasts derived from mouse satellite cells were performed as described (Ohkawa et al., 2012) using primers described in Table 1. Unpaired Student's t tests (two-tailed) were performed using the Kaleidagraph software.

Results and Discussion

Depletion of Brg1 leads to cell death in satellite cell derived myoblasts

In order to understand the role of Brg1 in the proliferation and maintenance of the satellite cell population, we isolated primary satellite cells from mice homozygous for a conditional Brg1 allele (Brg1 c/c) and propagated them in culture. In this model (Bultman et al., 2000), the gene is rendered nonfunctional upon infection of the cells with adenovirus encoding the Cre recombinase (Ad-Cre). As a control for our experiments, we isolated primary satellite cells from wild type C57BL/6 mice. A representative western blot showed a decrease in the expression of Brg1 in the Ad-Cre-infected Brg1 conditional myoblasts 3 days after plating

when compared to the C57BL/6 control cells. Depletion of Brg1 led to a decrease in the rate of myoblast proliferation (Fig. 1B). Moreover, a dramatic decrease in the myoblast population attached to the plate was observed upon Brg1 depletion (Fig. 1C). As early as one day after plating, approximately 10% of the Ad-Cre-infected Brg1 conditional cells detached from the plate, with approximately 95% of cells detached after 4 days of culture under proliferating conditions (Fig. 1C). In the case of control myoblasts derived from satellite cells from the wild type mice, ~5% of cells were detached from the plate upon Ad-Cre infection (Fig. 1C). Only 2–3% cell detachment was observed for the non-infected cells over the course of the experiment (Fig. 1C).

We sought to determine whether the decrease in cell number was due to cell death. First we analyzed the expression of apoptotic markers. Ad-Cre-infection of the Brg1 conditional myoblasts led to an increase in TUNEL staining, which indicates DNA fragmentation that results from apoptotic signaling cascades (Fig. 2A). This result was confirmed by western blots showing the activation of Caspase-3; only the myoblasts depleted for Brg1 showed elevated levels of activated Caspase-3 (Fig. 2B). It is interesting that even though some expression of Brg1 could still be detected 2 days post-infection (Fig. 1A), Caspase-3 was activated and detected as early as 1 day post-infection (Fig. 2B). The results suggest that in some cells the expression of Brg1 diminished soon after infection, which led to the rapid activation of the apoptotic pathway. These data also suggest that Brg1 serves as an anti-apoptotic factor in primary myoblasts. This finding is consistent with prior developmental studies indicating that Brg1 represses apoptosis at specific stages of T cell development (Gebuhr et al., 2003) and in neural crest cell proliferation that is necessary for subsequent events during cardiovascular development (Li et al., 2013). More broadly, this conclusion is consistent with observations that Brg1 suppresses apoptosis in response to UV and other DNA damaging agents (Gong et al., 2008; Kothandapani et al., 2012; Liu et al., 2014; Ondrusova et al., 2013; Park et al., 2009; Saladi et al., 2013) and that Brg1 represses E2F1-induced apoptosis (Liu et al., 2004).

The catalytic activity of Brg1 is required to restore proliferation and to rescue apoptosis in myoblasts depleted for Brg1

We sought to investigate whether reintroduction of Brg1 or a catalytically inactive Brg1 into Brg1-depleted myoblasts would rescue the observed phenotypes. This experiment would demonstrate whether or not the ATPase activity of Brg1 was necessary to promote cell proliferation and prevent cell death. We generated a retrovirus that expresses mCherry flanked by Cre recombination sites upstream of a YFP-tagged wild-type Brg1 (WT-Brg1) or a YFP-tagged, ATPase deficient Brg1 mutant that contains a K-to-R point mutation at the ATP binding site. This mutant has previously been shown to act as a dominant negative (DN-Brg1) (De la Serna et al., 2000; de la Serna et al., 2005; Khavari et al., 1993). In the absence of Cre recombinase, only the mCherry is expressed. Upon introduction of Cre-recombinase, the conditional endogenous Brg1 alleles were deleted, the mCherry encoded by the retrovirus was deleted, and the YFP-Brg1 was expressed. The WT-Brg1 was able to rescue the proliferation phenotype in Brg1-depleted myoblasts, whereas the DN-Brg1 was not (Fig. 3A). Expression of both the WT-Brg1 and DN-Brg1 proteins was verified by western blot over the course of the experiment (Fig. 3B). Similarly, reintroduction of WT-

Brg1, but not DN-Brg1, rescued Brg1 deficient myoblasts from apoptosis as shown by a decrease in the expression of activated Caspase-3 (Fig. 3B). We conclude that Brg1 is required for myoblast proliferation and viability and that these functions require a functional ATPase domain. We postulate that the requirement for the catalytic activity indicates that the mechanism of Brg1 function involves alterations in gene expression via its ATP-dependent chromatin remodeling function.

Brg1 controls myoblast proliferation and survival by remodeling chromatin at the *Pax7* promoter

The decrease in proliferation and viability observed in the Brg1 depleted myoblasts suggested that expression of one or more genes required for the maintenance of myoblasts were perturbed. Pax7 is an important regulator of the proliferative state of muscle satellite cells (Gunther et al., 2013; Oustanina et al., 2004; Seale et al., 2000; von Maltzahn et al., 2013). Given the roles of Brg1 and Pax7 in myoblast proliferation, we tested the possibility that Brg1 might be regulating Pax7 mRNA and protein levels in proliferating satellite cell-derived myoblasts. We observed a decrease in *Pax7* expression in proliferating Brg1-depleted myoblasts compared to the controls (Fig. 4A, B). This phenotype was partially rescued when Brg1-depleted myoblasts were co-infected with a retrovirus constitutively expressing *Pax7* (Fig. 4C, D). These results suggest that a primary function of Brg1 in proliferating myoblasts is to facilitate the expression of *Pax7* to maintain myoblast proliferation and survival. Interestingly, expression of Pax7 in non-infected control myoblasts caused a small but reproducible increase in the cell proliferation rate compared to the controls infected with the empty vector pBABE (Fig. 4D). These data indicate that overexpression of Pax7 accelerates the cell proliferation rate of myoblasts, highlighting the importance of Pax7 in the proliferation of satellite cells.

Because of the prior work demonstrating the critical role of Pax7 on myoblast proliferation and maintenance, it was an obvious choice for a potential Brg1-regulated gene. qPCR confirmed that *Pax7* expression was indeed dependent on Brg1. We were somewhat surprised that reintroduction of Pax7 into Brg1-depleted myoblasts rescued both viability and proliferation. This result suggests that the requirement for Brg1 may be limited to the expression of *Pax7* and perhaps downstream Pax7 target genes, with modest contributions from other potential Brg1 target genes. It remains to be determined whether Brg1 regulates just one or a few genes in proliferating myoblasts or whether it is more widely required for gene expression, even if its contribution to the expression of many genes is not essential. Some precedent for the latter idea exists from an earlier study where it was determined that expression of a dominant negative Brg1 protein in MyoD reprogrammed fibroblasts inhibited differentiation and the expression of most myogenic genes. However, the extent of inhibition among the affected genes varied widely, with about one-third highly dependent on Brg1 and two-thirds showing only modest effects on gene expression in the presence of the catalytically inactive Brg1 (de la Serna et al., 2005).

We sought to further explore the mechanism of Brg1-dependent *Pax7* expression. Our data raised the possibility that Brg1 is capable of binding chromatin at the *Pax7* promoter. Chromatin immunoprecipitation (ChIP) experiments indicated that Brg1 was bound to the

Pax7 promoter in proliferating cells (Fig. 5A) as shown by a significant increase in pull-down compared to the IgG control. This interaction was abolished in the Brg1-depleted myoblasts. The IgH enhancer sequence was used as an additional specificity control; Brg1 binding was not observed at this sequence (Fig. 5A). Gene transcription is associated with alteration of nucleosome organization at regulatory sequences, which allows the binding of transcription factors to the chromatin and renders the chromatin more accessible to nuclease cleavage. Such changes are generally a consequence of ATP-dependent chromatin remodeling enzyme function. In order to determine whether or not Brg1 controls chromatin remodeling at the *Pax7* promoter, we identified several PvuII cleavage sites in the promoter region of *Pax7* (Fig. 5B) and assayed for changes in the accessibility of this enzyme upon Brg1 depletion. As shown in Figure 5C, a strong increase in PvuII accessibility was observed in the control cells at two nuclease sites (primer sets 1 and 2, Fig. 5B) in the promoter region of *Pax7* that are 347 and 637 bp upstream of the transcription start site, respectively. In contrast, depletion of Brg1 by Ad-Cre infection in the conditional myoblasts led to a decrease in nuclease accessibility at the *Pax7* promoter, resulting in a reduced level of PvuII cleavage (Fig. 5C). In contrast, PvuII sites located 6 kb upstream of the *Pax7* transcription start site (primer set 3) or within the *Pax7* coding region (primer set 4) showed no change in chromatin accessibility in any of the cells tested (Fig. 5C).

The data suggest a model where Brg1, as the enzymatic subunit of the chromatin remodeling SWI/SNF complex, functions as a co-activator at the *Pax7* promoter in proliferating myoblasts to drive *Pax7* expression. This would be consistent with previous findings indicating that Polycomb family members repress *Pax7* expression in satellite cells (Palacios et al., 2010) and the overall theme that Polycomb mediated repression is opposed by proteins of the Trithorax family, which includes Brg1 and related proteins (Kingston and Tamkun, 2014; Tamkun et al., 1992).

We suggest that Brg1 may function as a co-activator for the CCAAT/Enhancer Binding Protein beta (C/EBP β) transcriptional activator protein. Recent evidence implicates C/EBP β as a direct regulator of *Pax7* expression in satellite cells and proliferating myoblasts (Fu et al., 2015; Marchildon et al., 2012). C/EBP β is expressed in *Pax7* expressing satellite cells and, like *Pax7* itself, is down-regulated upon differentiation signaling. A putative C/EBP β binding site was identified in the *Pax7* promoter; ChIP experiments demonstrated C/EBP β binding to promoter sequences containing this site and C/EBP β stimulated the expression of *Pax7* reporter genes. Knockdown of C/EBP β resulted in premature myogenic differentiation in culture, even in the absence of differentiation signaling, and increased muscle fiber cross-sectional area and enhanced muscle repair after injury in vivo (Marchildon et al., 2012). Thus C/EBP β is a critical regulator of *Pax7* in satellite cells and myoblasts.

Multiple examples of Brg1-mediated co-activation of C/EBP β exist. During bone differentiation, C/EBP β and Brg1-based SWI/SNF chromatin remodeling enzymes cooperate to positively and negatively regulate tissue-specific gene expression and to ultimately drive differentiation (Aguilar et al., 2014; Grandy et al., 2011; Villagra et al., 2006). Vitamin D regulated transcription involves C/EBP β and SWI/SNF enzymes (Christakos et al., 2007; Seth-Vollenweider et al., 2014; Zhong et al., 2009). Cooperativity between C/EBP β and Brg1 has also been reported in the regulation of mammary-specific

gene expression (Xu et al., 2007) and cooperativity between C/EBP β and other SWI/SNF subunits indicate a role in adipogenic and myeloid differentiation (Caramel et al., 2008; Kowenz-Leutz and Leutz, 1999; Kowenz-Leutz et al., 2010). Given the evidence listed here, it seems likely that the requirement for Brg1 in *Pax7* transcription in proliferating myoblasts is also dependent on C/EBP β , though the evidence does not preclude a role for Brg1 in facilitating the function of other components of the transcriptional machinery present at the *Pax7* promoter.

In conclusion, we report a direct and essential contribution for the Brg1 ATPase of the SWI/SNF chromatin remodeling enzyme in the proliferation and viability of satellite cell-derived myoblasts via a mechanism that promotes chromatin remodeling at and transcription of the *Pax7* gene. The data extend the requirement for Brg1 in myogenesis from a role in facilitating differentiation-specific gene expression and differentiation to maintenance of the myoblast population.

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Fig. 1. Brg1 is necessary for proliferation and survival of satellite cell derived primary myoblasts
 (A) Representative western blot showing Brg1 expression in C57BL/6 and Brg1 c/c myoblasts infected or not with Ad-Cre virus. GAPDH was used as an internal loading control. (B) Effect of Brg1 depletion upon Ad-Cre infection on myoblast survival. (C) Percentage of detached cells upon Ad-Cre infection and Brg1 depletion. Data are the mean $\pm$ S.E. from three independent experiments.

Fig. 2. Myoblasts depleted for Brg1 undergo apoptosis
(A) Representative light microscopy image of TUNEL stained C57BL/6 and Brg1 c/c myoblasts infected or not with Ad-Cre virus; arrowheads indicate apoptotic cells. (B) Representative western blot of activated Caspase-3 in myoblasts infected or not with Ad-Cre virus. PI3K levels were monitored as an internal loading control.

Fig. 3. Brg1 ATPase activity is required for myoblast proliferation and survival

Brg1 c/c myoblasts were infected with an inducible pBABE vector encoding wild type (WT) or ATPase deficient (DN) Brg1 and with the Ad-Cre virus. YFP expressing retroviral vector was used as a control. (A) Complementation of Brg1 depleted myoblasts with WT but not DN Brg1 restored proliferation and survival. Data are the mean $\pm$ S.E. from three independent experiments. (B) Representative western blots showing expression of exogenous WT and DN Brg1 in Ad-Cre infected Brg1 c/c myoblasts. Levels of activated Caspase-3 were also evaluated. PI3K levels were monitored as an internal loading control.

Fig. 4. Pax7 expression is altered in Brg1 depleted myoblasts

(A) Depletion of Brg1 leads to a decrease in the expression of Pax7 mRNA. The individual mRNA levels were normalized to EF1 α levels. The normalized expression levels of the control cells were set as 1. Data are the mean $\pm$ S.E. from three independent experiments. * $p < 0.05$. (B) Representative western blot showing the expression of Pax7 in C57BL/6 and Brg1 c/c myoblasts infected with Ad-Cre virus. Samples were collected from day 1 to 4 after Ad-Cre infection. PI3K was used as an internal loading control. (C) Brg1 c/c myoblasts were infected with retrovirus encoding *Pax7* or the empty retroviral vector and infected or not with Ad-Cre. A representative western blot shows the increased levels of Pax7 in the cells infected with pBABE-*Pax7* compared to the pBABE empty vector. The levels of activated Caspase-3 were also evaluated, and PI3K was used as an internal loading control. (D) Proliferation assay on the cells described in (C). Data are the mean $\pm$ S.E. from three independent experiments.

Fig. 5. Brg1 binds to and remodels chromatin at the *Pax7* promoter in proliferating myoblasts (A) Chromatin immunoprecipitation assay of proliferating C57BL/6 and Brg1 c/c myoblasts infected or not with Ad-Cre virus. Brg1 binding to the *Pax7* promoter region was shown by a significant increase in binding compared to IgG. Brg1 binding to the *IgH* enhancer was used as a control. Depletion of Brg1 led to a significant decrease in binding. Data are the mean $\pm$ S.E. of three independent experiments. ** $p < 0.01$, comparing values for Brg1 c/c +/– Ad-Cre samples. (B) Schematic representation of the location of the PvuII sites and primers used for restriction enzyme accessibility assay (REAA). (C) REAA of C57BL/6 and Brg1 c/c myoblasts infected or not with Ad-Cre virus. The accessibility of PvuII sites in the *Pax7* promoter was increased as measured by REAA relative to PvuII sites far upstream or in the coding sequence of the *Pax7* gene. Depletion of Brg1 upon Ad-Cre infection led to a decrease in accessibility at promoter PvuII sites. All values are relative to the value for primer 1 in the uninfected C57BL/6 myoblasts, which was set to 1. Data are the mean $\pm$ S.E. from three independent experiments. * $p < 0.05$; ** $p < 0.01$.

Table 1

Primers used in this study

Primer name	Sequence 5'-3'	Use
<i>Pax7</i> F	GAGCTCCACCGCGGTGGCGGCCGCTCTAGC	Cloning <i>pax7</i>
<i>Pax7</i> R	GATCGAATTCTCTAGACTATTATTTGTCGTCATCATCC	Cloning <i>pax7</i>
<i>Pax7</i> LM1 F	GAGATCTGAATTCCTGCGCCTTGC	REAA
<i>Pax7</i> LM1 R	GATCGAGGGGAGGAGGGAGTCC	REAA
<i>Pax7</i> LM2 F	CCGGGAGATCTGAATTCCTGGGTAAG	REAA
<i>Pax7</i> LM2 R	TCCCCTCTCCCTCCCGAACTGG	REAA
<i>Pax7</i> LM3 F	CCGGGAGATCTGAATTCCTGGAGCAA	REAA
<i>Pax7</i> LM3 R	CTCTTTCTTGAACCCAGATAGCCTGCC	REAA
<i>Pax7</i> LM4 F	CCGGGAGATCTGAATTCCTGAGGGTG	REAA
<i>Pax7</i> LM4 R	GGCTGGACAGGATCTTCA	REAA
q <i>Pax7</i> F	GCAGCTGGAGGAGCTAGAGAAG	Gene expression
q <i>Pax7</i> R	GTCTCCTGGCTTGATGGAGTCG	Gene expression
q <i>EF1α</i> F	AGCTTCTCTGACTACCCTCCACTT	Gene expression
q <i>EF1α</i> R	GACCGTTCTTCCACCACTGATT	Gene expression
<i>Pax7</i> ChIP F	GTGGCGACAAGGAAGTTCAAACAAAC	ChIP
<i>Pax7</i> ChIP R	AAAGAAAGCCACTCCGCAACCTCTG	ChIP