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# The Scaffold attachment factor b1 (Safb1) regulates myogenic differentiation by facilitating the transition of myogenic gene chromatin from a repressed to an activated state

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## ABSTRACT

The regulation of skeletal muscle gene expression during myogenesis is mediated by lineage-specific transcription factors in combination with numerous cofactors, many of which modify chromatin structure. However, the involvement of scaffolding proteins that organize chromatin and chromatin-associated regulatory proteins has not extensively been explored in myogenic differentiation. Here, we report that Scaffold attachment factor b1 (Safb1), primarily associated with transcriptional repression, functions as a positive regulator of myogenic differentiation. Knockdown of *Safb1* inhibited skeletal muscle marker gene expression and differentiation in cultured C2C12 myoblasts. In contrast, over-expression resulted in the premature expression of critical muscle structural proteins and formation of enlarged thickened myotubes. *Safb1* co-immunoprecipitated with MyoD and was co-localized on myogenic promoters. Upon *Safb1* knockdown, the repressive H3K27me3 histone mark and binding of the Polycomb histone methyltransferase Ezh2 persisted at differentiation-dependent gene promoters. In contrast, the appearance of histone marks and regulators associated with myogenic gene activation, such as myogenin and the SWI/SNF chromatin remodelling enzyme ATPase, Brg1, was blocked. These results indicate

that the scaffold protein *Safb1* contributes to the activation of skeletal muscle gene expression during myogenic differentiation by facilitating the transition of promoter sequences from a repressive chromatin structure to one that is transcriptionally permissive.

## INTRODUCTION

Skeletal muscle differentiation involves morphological and transcriptional dynamics that turn a non-differentiated population of mononucleated myoblasts into multinucleated striated muscle fibres through a multistep process regulated by the muscle regulatory factors (MRFs) MyoD, Myf5, Myf6/MRF4 and myogenin. MRFs collaborate with sequence-specific transcription factors such as the myocyte enhancer factor-2 (MEF2) family of activators, chromatin remodelling enzymes and histone-modifying enzymes to generate transcriptional regulatory networks that promote skeletal muscle differentiation (1–3). In addition to regulators that induce and drive differentiation, myogenic genes are marked prior to the onset of differentiation by the deposition of the variant histone H3.3 (4). Despite the complexity of myogenic gene regulation, there is increasing evidence that other factors that are not expressed in a tissue-specific manner play fundamental roles in the control of cell differentiation (5–10).

One class of such factors includes nuclear scaffold attachment proteins. SATB1 (special AT-rich sequence-binding

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protein 1) is a well-characterized protein associated with chromatin and the nuclear matrix (11) that forms a three-dimensional 'cage-like' network structure in mouse thymocyte nuclei (12). In the context of T-cell development, SATB1 regulates gene activation by folding chromatin into loop domains, tethering specialized DNA elements to a SATB1 network structure containing chromatin remodelling enzymes and specific transcription factors to the anchor sites and thereby regulating the status of histone modification and nucleosomal positioning over long distances of DNA (13,14). More recently, the scaffold attachment factor A (SAF-A) has been identified as a factor required for the recruitment of Xist RNA on the inactive X chromosome and the concomitant increase of the repressive histone mark H3K27me3 through a mechanism that involves the RNA- and DNA-binding properties of SAF-A (15).

Scaffold attachment factor b1 (Safb1) is a large multi-functional protein that participates in a variety of cellular processes. It is involved in higher order chromatin structure and in the partitioning of chromatin into distinct topologically independent loops (16,17). Safb1 contains different functional domains; it binds RNA via its central recognition RNA motif domain (18,19) and has been isolated in complexes with RNA-processing proteins and RNA pol II. Thus, it has been suggested to be part of a 'transcriptosome' complex. Safb1 also contains a nuclear localization domain a Glu/Arg, Ser/Lys and Gly-rich domain that mediates protein-protein interactions, and a SAF-Box, which is a homeodomain-like DNA-binding motif that interacts with AT-rich scaffold/matrix attachment regions (S/MARs) (20,21). Transcriptional repression seems to be the most prominent function of Safb1. Safb1 was initially reported to repress estrogen-dependent transcription, but recent studies suggest that it may function in a more widespread manner by mediating repression of immune regulators and apoptotic genes (22,23).

Here, we show that Safb1 is able to interact with MyoD to co-occupy skeletal muscle promoter regions before and during the course of C2C12 cell differentiation. Reduced levels of Safb1 led to inhibition of cell differentiation, with a concomitant reduction of myogenic marker gene expression. In addition, the changes in the local chromatin environment that are expected during differentiation did not occur. Reduction of Safb1 resulted in maintenance of a repressive chromatin structure at target promoters, including continued association with the Polycomb group protein Ezh2 and its associated histone modification H3K27me3. Binding of proteins associated with myogenic gene activation, including myogenin, the SWI/SNF chromatin remodelling enzyme ATPase, Brg1, and incorporation of acetylated H3Ac and H4Ac at promoters in cells induced to differentiate in the absence of Safb1 did not occur. Thus, we propose a novel role for Safb1 as a scaffold protein that facilitates the transition from a repressive chromatin environment to an active and transcriptionally permissive chromatin environment during myogenic differentiation.

## MATERIALS AND METHODS

### Tissue culture, plasmids and viral constructs

Mouse C2C12 myoblasts (ATCC collection 1772) were maintained in growth media (GM) composed of Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine (Gibco). Differentiation was induced once cells reached 80% confluence with media supplemented with 2% horse serum (Gibco) instead of 10% fetal bovine serum. C2C12 cells transduced with lentiviral particles expressing shRNAs against mouse Safb1 or non-specific sequences were grown and differentiated in presence of 2.5 mg/ml of puromycin (Invitrogen). Cells transduced with retroviral vectors expressing the human cDNA of SAFB1 were selected with 700 mg/ml of G418 (Gibco). Lentiviral packaging 293T and BOSC cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics.

Retroviruses expressing human SAFB1 were generated by transient transfection of BOSC cells with pBabe or pBabe-SAFB1, which was generated by subcloning the SAFB1 cDNA from pcDNA1-SAFB1 (24) using EcoRI and cloned into the EcoRI site of the pBabe vector. Forty-eight hours post-transfection, the supernatant containing viral particles was collected and used to transduce C2C12 cells. Lentiviruses expressing shRNA against Safb1 and control sequences were generated by transfecting pLenti-Safb1-1, pLenti-Safb1-2 and pLenti-Scrambled vectors in 293T cells. After 48 h of transfection, supernatants containing lentiviral particles were collected, used to transduce C2C12 cells and selected with 2.5 mg/ml of puromycin. Oligonucleotides used to generate shRNA-Safb1-1 were as follows: 5'-GATCCGGACATCAGTGTGCTGGATGAGTGTGCTGTCCTCATCCAGCACACTGATGTCCTTTTTGGAAA-3, and sh-RNA Safb1-2: 5'-GATCCGCTGCAGGAGCATGCTATAGAGTGTGCTGTCCTCTATAGCATGCTCCTGCAGCTTTTTGGAAA-3'.

### RNA isolation and gene expression analysis

Total RNA was isolated from samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA from each sample was used to prepare cDNA using Superscript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye using the Fast SYBR green master mix on the ABI StepOne Plus Sequence Detection System (Applied Biosystems). Relative expression levels for each gene were determined using the Comparative Ct method (25) and normalized to the relative levels of EF1-alpha expression (26). One-tailed *t* tests were used for statistical analyses. Primer sequences used for real time PCR are as follows:

EF1alpha For: 5'-AGCTTCTCTGACTACCCTCCACTT-3', Rev: 5'-GACCGTTCTTCCACCACTGATT-3'; Myogenin For: 5'-CAAGTGTGCACATCTGTTCTAGTCTCT-3', Rev: 5'-GTATCATCAGCACAGGAGACC

TTGGT-3'; Skeletal Actin For: 5'-TTGTGCGCGACATCAAAGAGAAGC-3', Rev: 5'-GAAACGCTCATTGCCGATGGTGAT-3'; MyhIIB For: 5'-TCAATGAGATGGAGATCCAGCTGAAC-3', Rev: 5'-GTCCAGGTGCAGCTGTGTGTCCTTC-3'; MCK For: 5'-GCCGGGGATGAGGAGTCCTAC-3', Rev: 5'-GCAGTGCCGAGGCCAGAGTGA-3'; Alpha Sarcoglycan For: 5'-AGAGGCTGCTGCTGCTGATT-3', Rev: 5'-GAGGTGCCAAGGTGTCATAG-3'; Safb1 For: 5'-GAAGAAGCGGAACCTCGACTC-3', Rev: 5'-TCATCAGGATCCCCACCTTC A-3'; Dystrophin For: 5'-GTTTCATTGATGGAGACGGAA-3', Rev: 5'-GCACTTCAGCTTCTTCATCT-3'.

### Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays using cultured C2C12 cells and E9.5 dpc mouse embryos were performed as previously described (27,28). Briefly, cells, somite-enriched tissue or yolk sac were fixed with 1% formaldehyde to cross-link protein–DNA complexes and then lysed in a buffer containing 1% SDS, 10 mM EDTA, pH 8, and 50 mM Tris-HCl, pH 8, with protease inhibitors. After sonication, chromatin was pre-cleared for 1 h with protein A-agarose beads blocked with DNA salmon sperm, and lysates were incubated overnight with specific antibodies against Safb1 (A300-812A, Bethyl), MyoD (Santa Cruz Biotechnology, sc-304), Myogenin (Santa Cruz Biotechnology, sc-576), H3K27me3 (Millipore, 07-449), Ezh2 (Millipore, 05-1319), H4Ac (Millipore, 06-866), H3Ac (Millipore, 06-599), polyclonal rabbit antisera against Brg1 (29) or normal rabbit IgG (Santa Cruz Biotechnology, sc-3888). Cross-linking was reversed, and DNA was purified using MiniElute Spin Columns (QIAGEN, Hilden, Germany) and used as template for quantitative PCR using the Fast SYBR green master mix on the ABI StepOne Plus Sequence Detection System. Quantification was performed using the comparative Ct method and presented as fold differences relative to input and values obtained by normal rabbit IgG with the formula  $(2^{(Ct_{IgG}-Ct_{input})}) / (2^{(Ct_{Ab}-Ct_{input})})$  (25). Re-ChIP, or sequential ChIP, experiments were performed as described (30). Two-tailed *t* tests were used for statistical analyses. Primer sequences used for real time PCR were as follows: Myogenin promoter (−10 to −150), For: 5'-ACGCCAACTGCTGGGTGCCA-3'

Rev 5'-GAATCACATGTAATCCACTGGA-3'; MCK enhancer (−1050 to −1210), For: 5'-GACACCCGAGATGCCTGGTT-3', Rev: 5'-GATCCACCAGGGACAGGGTT-3'; Skeletal Actin promoter (−81 to −265), For: 5'-ACAGCGGTCAAAGCAGTG-3', Rev: 5'-CTCCCAACTGGCTCCAAGG-3'; MyhIIB promoter (−20 to −155), For: 5'-CACCCAAGCCGGGAGAAACAGCC-3', Rev: 5'-GAGGAAGGACAGGACAGAGGCACC-3';

IgH enhancer (11–158 of enhancer core sequence)

For: 5'-GCCGATCAGAACCAGAACACC-3', Rev: 5'-TGGTGGGGCTGGACAGAGTGTTTC-3'.

### Co-immunoprecipitation and western blots

Protein extracts from myoblasts and myotubes were obtained with RIPA modified buffer (50 mM Tris HCl

pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, supplemented with proteases inhibitors). For IPs, extracts were pre-cleared for 1 h with protein A (GE) at 4°C. Four milligrams of specific antibodies or rabbit or mouse control IgG were added to the extracts and incubated overnight at 4°C with rotation. Immunocomplexes were collected by adding protein A, and after extensively washing, 40 ml of loading buffer was added and the samples were boiled to elute the complexes.

Antibodies used for western blots were anti-Safb1 (Bethyl A300-812A), anti-Myogenin (BD-556358), anti-MHC (Hybridoma MF-20; Developmental Studies Hybridoma Bank, Dept. Biology, University of Iowa), anti-MyoD (Santa Cruz Biotechnology, sc-32758 and sc-304), anti-PI3K (Millipore 06-496), polyclonal rabbit antisera against Brg1 (29) and anti-β-actin (Santa Cruz Biotechnology, sc-81178). Where indicated, western blots were quantified using ImageJ software.

### Immunofluorescence

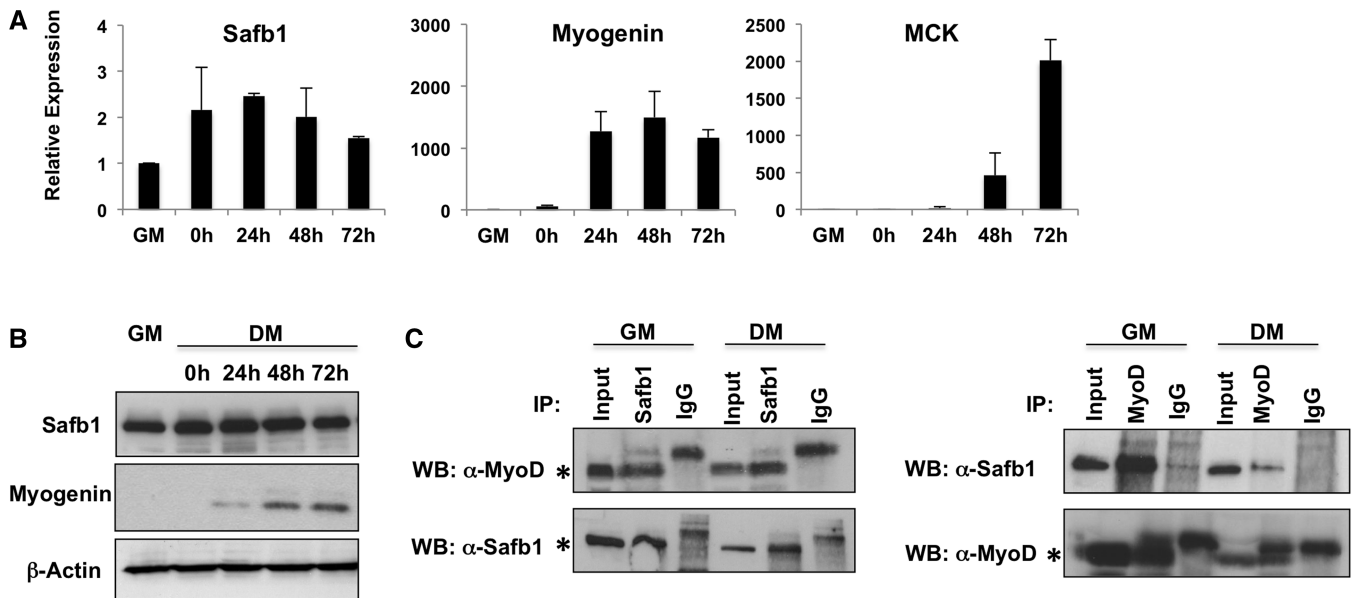
C2C12 cells were grown on coverslips treated with collagen type I (Sigma) in 24-well plates. Cells were fixed in methanol at −70°C for 10 min, blocked with 10% fetal bovine serum, 10% albumin and 1% TBS Tween and permeabilized with 0.05% Triton X-100. Subsequently, cells were incubated overnight at 4°C with anti-MHC primary antibody (Hybridoma MF-20; Developmental Studies Hybridoma Bank, Dept. Biology, University of Iowa). After three washes with phosphate-buffered saline, samples were incubated with secondary antibodies coupled with fluorescein isothiocyanate or carboxymethyl indocyanine-3 for 1 h at room temperature. Samples were washed and mounted in Vectashield-4',6-diamidino-2-phenylindole medium (Vector Laboratories). Immunostaining analysis was recorded using an Epifluorescence Zeiss Axioplan 2 microscope (Zeiss MicroImaging 9, Inc. New York, NY).

## RESULTS

### Safb1 is present in a myogenic protein complex containing MyoD

Studies of the human xanthine oxidoreductase gene have previously shown that E boxes upstream of the transcription start site negatively regulate gene expression and that these sequences bind a complex of regulatory factors that includes Safb1, another scaffold protein, SAF-A, and transcriptional and chromatin regulatory proteins. Because the activation of myogenic genes requires E boxes that bind to the MyoD family of myogenic regulatory proteins, we hypothesized that myogenic gene activation might involve cooperation between MyoD and related factors and the Safb1 scaffold protein. First, we analysed the temporal expression pattern of Safb1 during a time course of differentiation in C2C12 myoblasts by real-time PCR and western blot analyses. Safb1 mRNA levels were elevated ~1.5- to 2-fold in confluent and in differentiating C2C12 cells (Figure 1A); however, we observed no significant changes in Safb1 protein





**Figure 1.** Safb1 co-immunoprecipitates with MyoD and Brg1 during C2C12 cell differentiation. (A and B) Total RNA and protein from proliferating myoblasts in growth media (GM), confluent myoblasts (time 0) and myoblasts exposed to differentiation media (DM) for up to 72 h were collected and analysed for Safb1 mRNA and protein levels. Myogenic markers of differentiation Myogenin and MCK were monitored as controls for C2C12 cell differentiation.  $\beta$ -actin was monitored as a loading control for the western blot. mRNA data are presented as the expression relative to EF-1 $\alpha$  levels. The expression in growth media was normalized to 1. Data represent the average of three independent analyses  $\pm$  SEM. *t*-test analysis of Safb1 mRNA levels indicated that only the 0h timepoint gave a *P* value > 0.05 relative to the value obtained in proliferating myoblasts. ImageJ quantification of the western blot indicated that Safb1 levels varied by no more than 15% from the level present in growth media through 48 h post-differentiation and that Safb1 levels were reduced by 40% in differentiated cells (72 h post-differentiation). (C) Whole cell protein extracts from myoblast and myotubes were collected to perform co-immunoprecipitation assays for Safb1 and MyoD. Asterisks indicate the MyoD or Safb1 specific bands. GM, growth media; DM, differentiation media; IP, immunoprecipitation; WB, western blot.

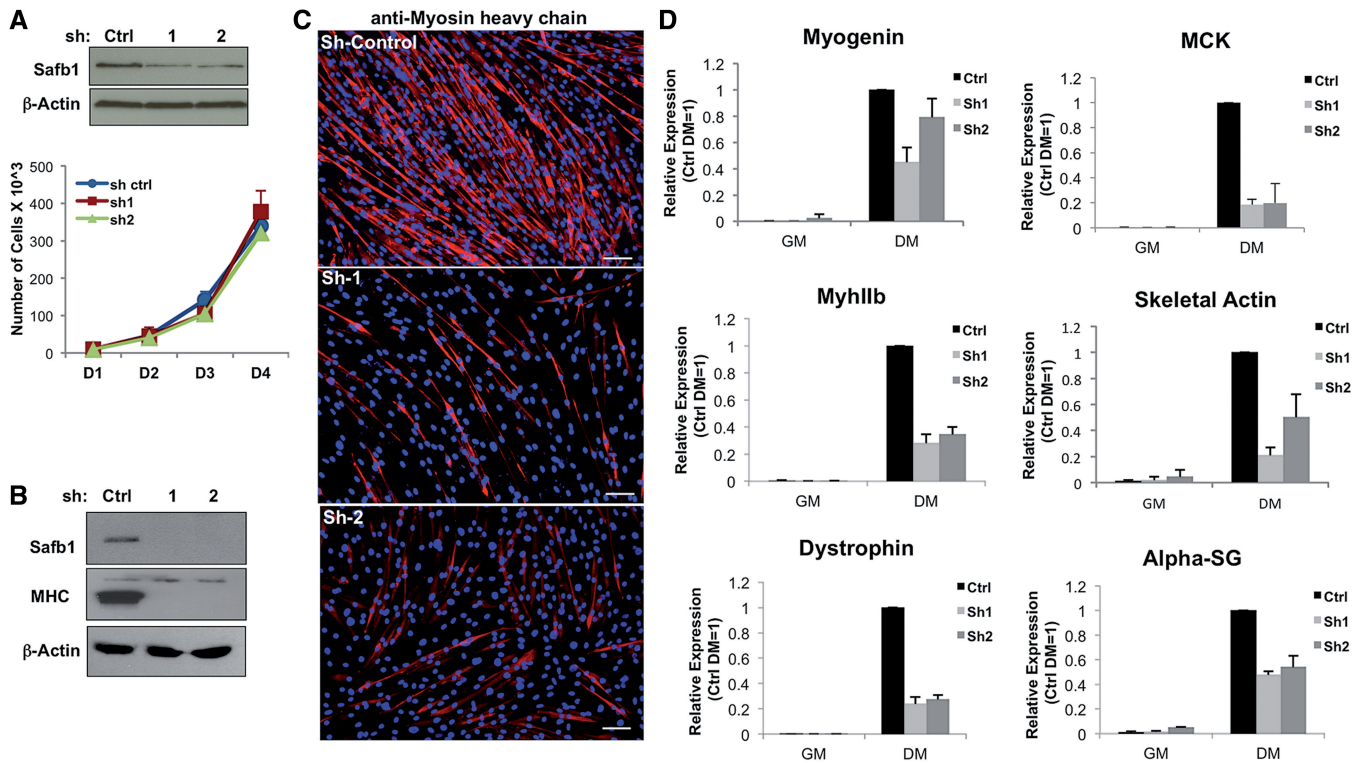
abundance as a consequence of cell differentiation (Figure 1B). Co-immunoprecipitation assays were performed using protein extracts from C2C12 cells to determine whether Safb1 could interact with MyoD. As shown in Figure 1C, association between endogenous Safb1 and endogenous MyoD was revealed when an anti-Safb1 antibody was used to immunoprecipitate protein extracts from both proliferating myoblasts (GM) and differentiated myotubes (DM). Additionally, the anti-MyoD antibody was able to immunoprecipitate Safb1 in extracts from both stages of differentiation (Figure 1C). These data suggest a role of Safb1 as a potential cofactor of MyoD during transcriptional regulation of skeletal muscle genes.

#### Safb1 is required for C2C12 cell differentiation and skeletal muscle gene expression

To better understand the function of Safb1 during skeletal myogenesis, we conducted knockdown experiments by transducing C2C12 cells with a lentivirus expressing specific shRNA against the mouse Safb1 gene or a scrambled sequence. First, we performed proliferation assays and determined that Safb1 knockdown cells proliferated at the same rate as the control cells (Figure 2A), suggesting that Safb1 deficiency did not affect the progression of myoblasts through the cell cycle. A western blot analysis confirmed knockdown of Safb1 in the proliferating myoblasts (Figure 2A). We next examined the effect of reduction of Safb1 protein

levels on myoblast differentiation; Figure 2B shows the extent of Safb1 knockdown in cells differentiated for 72 h. Additional western blot analysis to examine the expression of the skeletal muscle differentiation marker, Myosin Heavy Chain (MHC), revealed a significant block in the accumulation of MHC proteins compared with the expression of MHC in control cells expressing the scrambled sequence shRNA (Figure 2B). Furthermore, formation of myotubes in knockdown C2C12 cells was markedly reduced and was accompanied by reduction in the number of MHC-positive myotubes (Figure 2C) as shown by immunostaining experiments. Total RNA from knockdown cells was extracted to analyse gene expression of muscle-specific genes that are up-regulated as function of muscle differentiation. As shown in Figure 2D, expression of myogenin was modestly affected by expression of the shRNAs targeting Safb1 compared to the scrambled sequence control. However, transcription levels of muscle creatine kinase, myosin heavy chain IIb, skeletal actin, dystrophin and alpha-sarcoglycan, genes normally up-regulated at later times in differentiating myotubes, were markedly reduced in the knockdown cells. These results suggest an important role of Safb1 during transcriptional activation of skeletal muscle genes during myogenesis.

In contrast, over-expression of SAFB1 in wild-type C2C12 cells induced formation of enlarged thicker myotubes after 72 h of differentiation (Figure 3A). Interestingly, the presence of MHC was detected



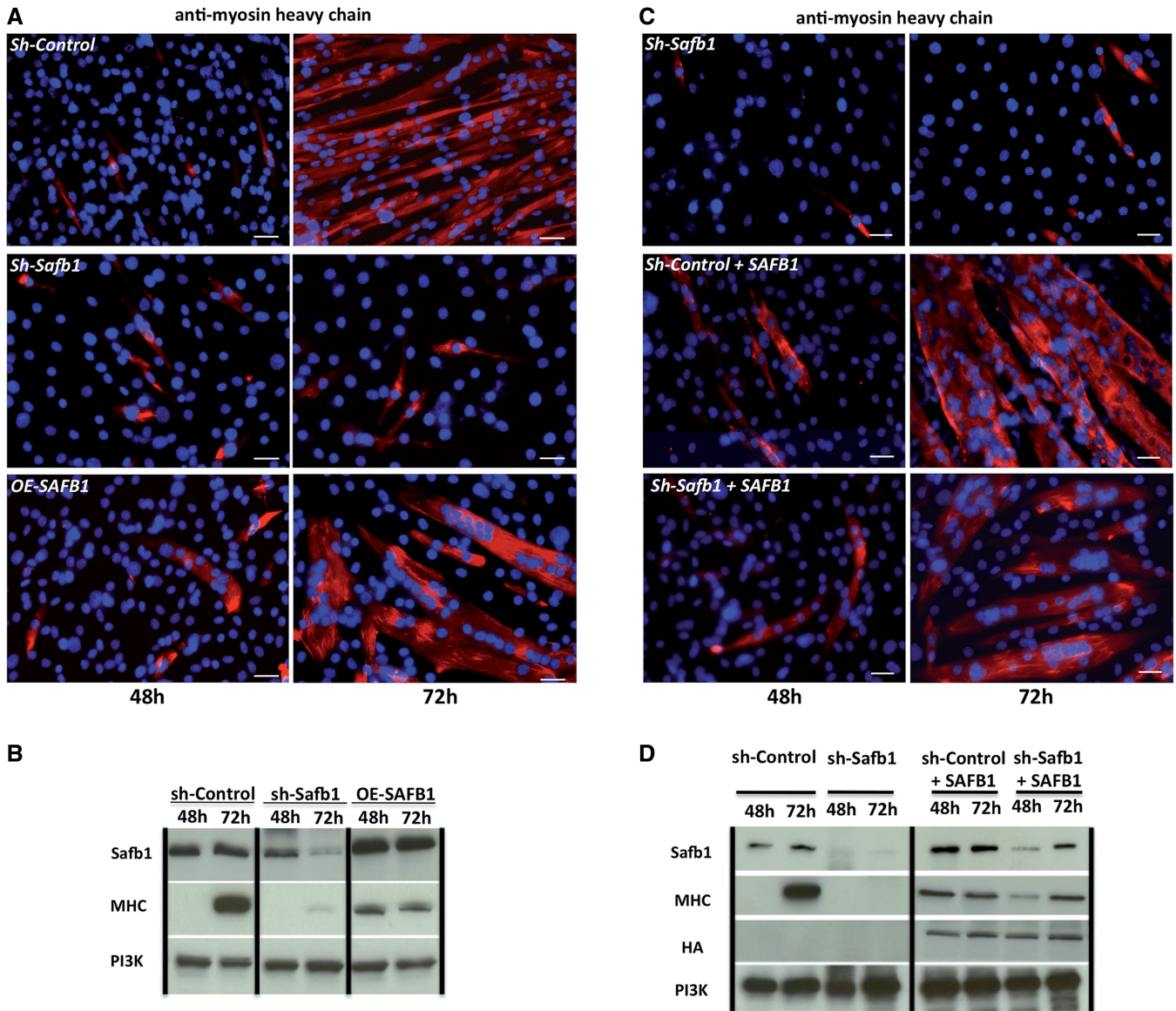
**Figure 2.** Safb1 knockdown blocks C2C12 cell differentiation and inhibits skeletal muscle genes expression. (A) shRNA-mediated knockdown of Safb1 in proliferating myoblasts does not affect proliferation. Two different sequences against Safb1 mRNA (sh1 and sh2) were used. Knockdown of Safb1 in proliferating myoblasts was confirmed by western blot. PI3K levels were monitored as a loading control. Cell counts were taken at 24 h intervals after seeding of equal numbers of cells. Data represent the average of three independent analyses  $\pm$  SEM. sh control cells (sh ctrl) express a scrambled sequence shRNA. (B) Confirmation of Safb1 knockdown in cells induced to differentiate for 72 h compared with cells expressing the control shRNA. MHC protein levels were also reduced in differentiated Safb1 knockdown cells.  $\beta$ -actin levels were monitored as a loading control. (C) Immunostaining indicates that the population of MHC-positive myotubes is markedly reduced in Safb1 knockdown C2C12 cells induced to differentiate. Nuclei were counterstained with DAPI. (D) Total RNA from C2C12 cells expressing shRNA against Safb1 was used to analyse expression levels of Myogenin, Muscle Creatine Kinase Myosin heavy chain IIb (Myh IIb), Skeletal Actin, Dystrophin and Alpha Sarcoglycan (alpha-SG) genes. Expression in differentiated sh-control (ctrl) cells was normalized to 1. Quantification represents the average of three independent experiments  $\pm$  SEM. *t* test analysis indicates that all values for mRNA levels in the differentiated knockdown cells are significantly reduced compared with the mRNA levels in control differentiated cells ( $P < 0.05$  for Sh2 KD of myogenin,  $P < 0.01$  for all other data points).

prematurely at 48 h post-differentiation in SAFB1 over-expressing cells (Figure 3B), even though MHC protein levels did not achieve the same level of induction as observed in control cells at 72 h. The reason for the lack of full MHC induction in these cells was not established. We further explored the specificity of our results by asking whether introduction of the SAFB1-expressing vector would rescue the block to MHC expression and differentiation caused by shRNA-mediated Safb1 knockdown. Expression of SAFB1 in control cells expressing the control shRNA resulted in enlarged myotubes (Figure 3C, middle panel), in agreement with the results obtained when SAFB1 was introduced into parental C2C12 cells (Figure 3A). Introduction of SAFB1 in cells expressing the shRNA against Safb1 rescued the expression of MHC and the formation of myotubes (Figure 3C, bottom panel), although the myotubes that formed were enlarged and thicker than myotubes that formed in the parental and control C2C12 cells. Consistent with this observation, Safb1 knockdown cells expressing SAFB1 showed both rescue of Safb1 expression as well as premature MHC expression (Figure 3D), as was observed in

SAFB1-expressing parental and control shRNA-expressing cells. These experiments further support the conclusion that Safb1 is a positive regulator of myogenesis.

### Safb1 is present at myogenic regulatory regions

Having observed the endogenous interaction of Safb1 and MyoD and considering the previous reports describing the ability of Safb1 to interact with chromatin (23,31), we reasoned that Safb1 could be playing a direct role in transcriptional regulation of skeletal muscle genes during myogenesis. To test this hypothesis, we performed ChIP assays before and during differentiation of C2C12 cells. As shown in Figure 4A, Safb1 was able to bind to skeletal muscle gene regulatory sequences both in proliferating myoblasts as well as in differentiated myotubes (Figure 4A). As expected, binding of MyoD was found in muscle promoters both in proliferating myoblasts and in myotubes, with a significant enhancement of MyoD binding at the MCK gene (32). In contrast, myogenin protein was inducibly recruited to muscle regulatory regions upon cell differentiation, in agreement with prior studies (33–35). The IgH enhancer was used as a negative



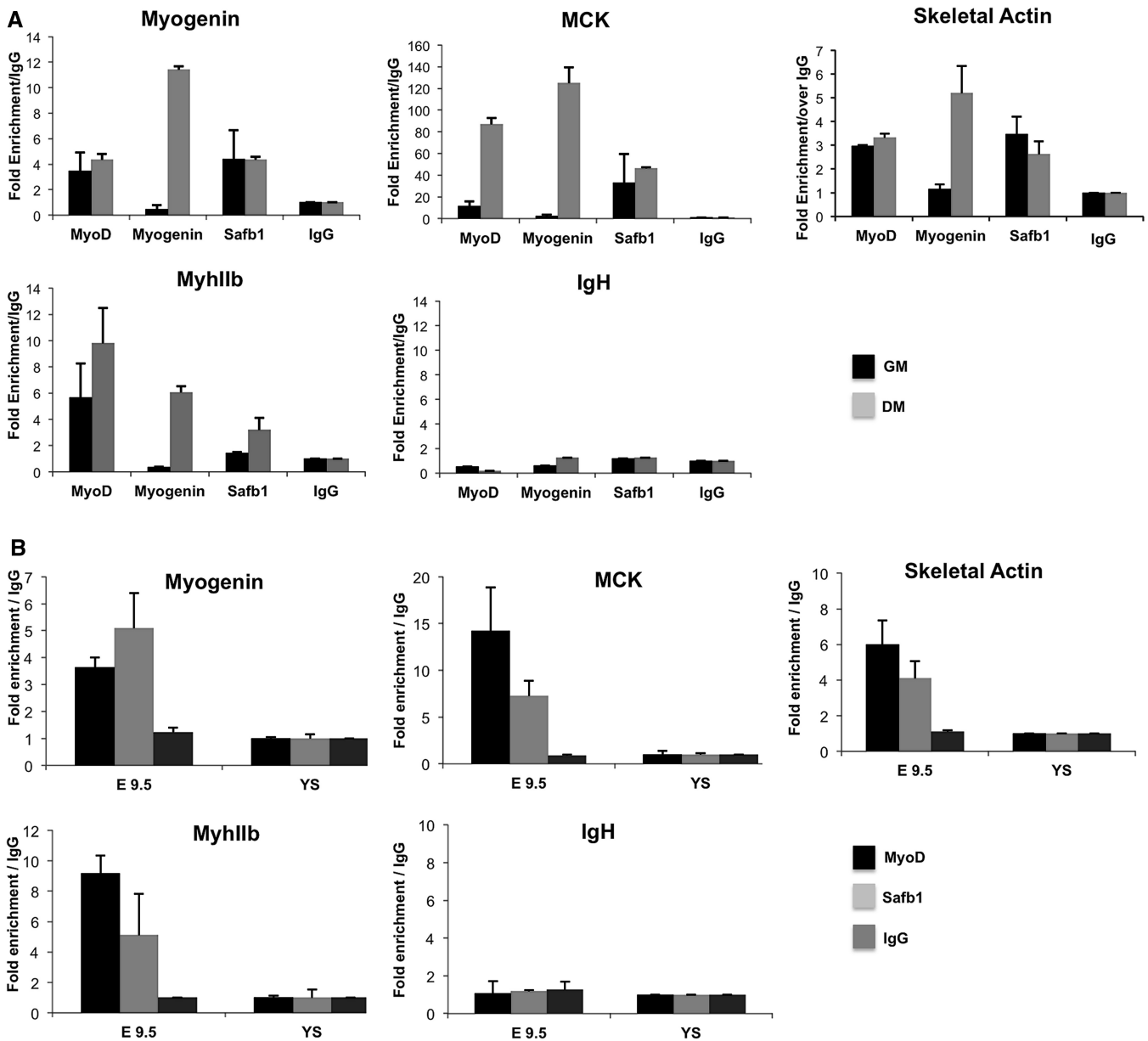
**Figure 3.** Over-expression of SAFB1 leads to formation of enlarged, thicker, MHC-positive myotubes. (A) C2C12 cells infected with the shRNA control vector, an shRNA against *Safb1* or a vector encoding SAFB1 were immunostained for MHC after 48 and 72 h of differentiation. Nuclei were counterstained with DAPI. (B) Western blot of protein extracts obtained from duplicate plates of cells in (A) showing *Safb1* and MHC levels. PI3K levels were monitored as a loading control. (C) Immunostaining indicates that exogenous expression of SAFB1 in *Safb1* knockdown cells rescues MHC expression and results in myotube formation similar to that observed in sh-control cells over-expressing SAFB1. Nuclei were counterstained with DAPI. (D) Rescue of *Safb1* expression after knockdown of endogenous *Safb1* (*shSafb1* + SAFB1), reestablishes MHC expression at 48 h of differentiation. HA signal indicates the presence of exogenous SAFB1, which is HA-tagged. PI3K levels were monitored as a loading control.

sequence control because it contains a consensus E box but does not bind to myogenic transcription factors in myoblasts or in differentiated cells (4,26,34,36–40). To confirm the physiological relevance of this observation, we looked for the presence of *Safb1* at muscle promoters in the context of presence of mouse embryonic development. Mouse embryos at 9.5 dpc were dissected, and tissue enriched in somites was used to perform ChIP analysis following the methodology previously described (27). As shown in Figure 4B, both *Safb1* and MyoD were enriched at the myogenin, muscle creatine kinase, skeletal actin and myosin heavy chain IIb regulatory sequences in tissue enriched in somites. Neither *Safb1* nor

MyoD was present at these myogenic promoters in yolk sac, the tissue used as negative control. These data suggest a role for *Safb1* as a cofactor that directly participates in the transcriptional machinery present at skeletal muscle loci during activation of skeletal muscle genes.

The co-IP and ChIP data suggest that *Safb1* and MyoD may co-occupy myogenic regulatory regions both before and after induction of differentiation. To address this question, sequential chromatin immunoprecipitation (ChIP-ReChIP) experiments were performed. ChIP-ReChIP revealed that MyoD is recruited to regulatory sequences of the myogenin, muscle creatine kinase and





**Figure 4.** Safb1 is recruited to regulatory sequences of skeletal muscle-specific genes before and during C2C12 cell differentiation and in somite-enriched tissue isolated from mouse embryos at E9.5. (A) ChIP analysis of skeletal muscle promoters Myogenin, Muscle Creatine Kinase, Myosin heavy chain IIb (Myh IIb) and IgH (as a negative control) in C2C12 myoblasts (GM) and myotubes differentiated for 72 h (DM). (B) ChIP analysis of skeletal muscle promoters in somite-enriched tissue obtained from mouse embryos at E9.5 dpc. Yolk sac (YS) from E9.5 embryos was used as negative control. Quantification represents the mean of three independent experiments  $\pm$  SEM. *t* test analyses indicate that all binding events except for myogenin binding to myogenic gene promoters in growth media conditions are statistically significant ( $P < 0.05$ ) compared with the IgG controls (panel A) and that all MyoD and Safb1 binding in E9.5 somite-enriched tissue is statistically significant ( $P < 0.01$ ) compared with binding in yolk sac (panel B).

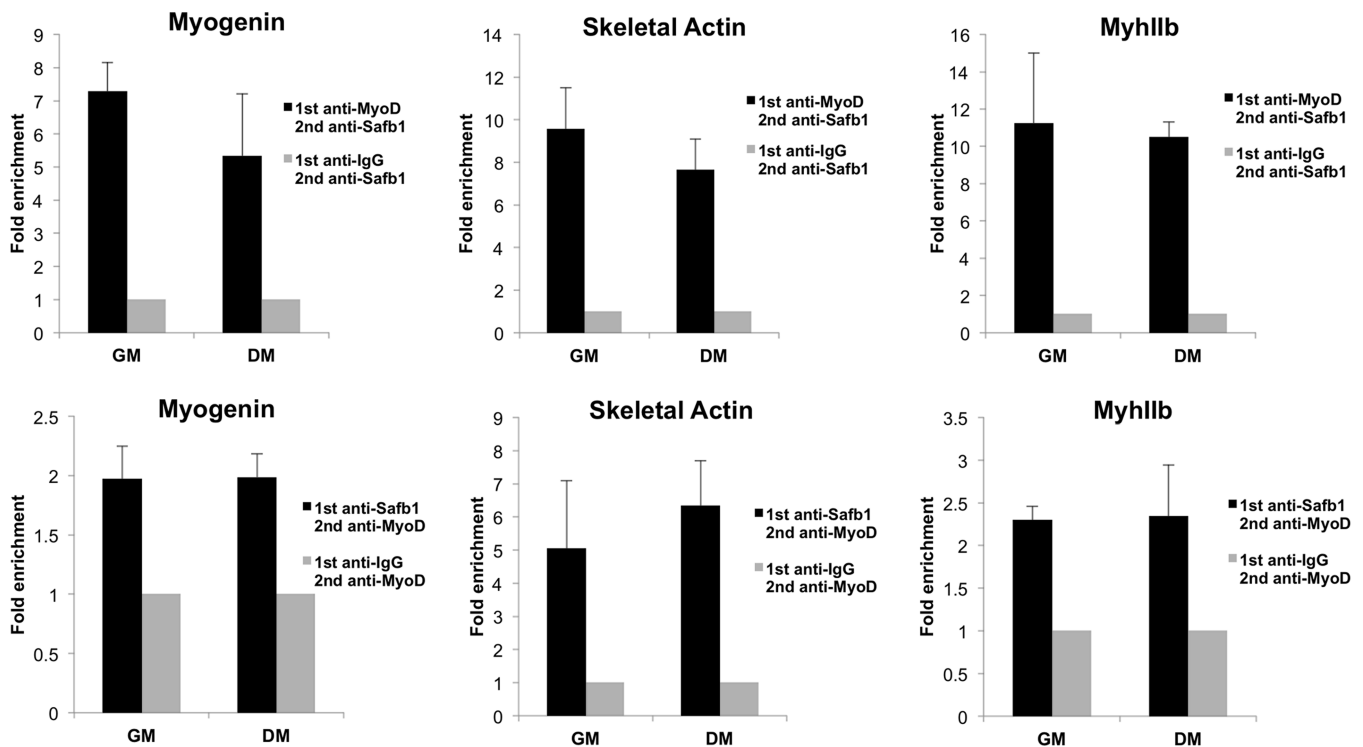
skeletal actin genes when we used chromatin previously precipitated with an antibody against Safb1 both in myoblasts and differentiated cells (Figure 5). Similarly, when MyoD was used as the first target for the immunoprecipitation, we were able to detect enrichment of Safb1 at the same sequences. Neither Safb1 nor MyoD was able to immunoprecipitate myogenic regulatory sequences after pull down with control anti-IgG. These data demonstrate that Safb1 and MyoD co-occupy the same sequences of myogenic genes before differentiation

and that this binding is sustained after the onset of the cellular differentiation process.

#### Knockdown of Safb1 favours maintenance of Polycomb-mediated repressive marks at the regulatory sequences of myogenic genes and prevents the transition to an activated chromatin state

We analysed the chromatin environment at myogenic promoters in myoblasts where Safb1 expression was





**Figure 5.** Safb1 and MyoD are co-localized at regulatory sequences of myogenic promoters in myoblasts prior to the activation of transcription. ReChIPs were performed by immunoprecipitating chromatin with an antibody against MyoD followed by a second immunoprecipitation using antibody against Safb1 (upper panel) or using Safb1 as the first immunoprecipitating antibody (lower panel) in undifferentiated myoblasts (GM) and 72 h differentiated myotubes (DM). ChIP values were normalized against the input and expressed as relative enrichment of the material precipitated by the indicated antibody. Data represent the mean of three independent experiments  $\pm$  SEM. *t* test analyses indicate there is no statistical difference when comparing MyoD/Safb1 ReChIP signals in growth media with the signals obtained in differentiation media. MyoD/Safb1 ReChIP signals relative to IgG controls were statistically significant ( $P < 0.05$ ).

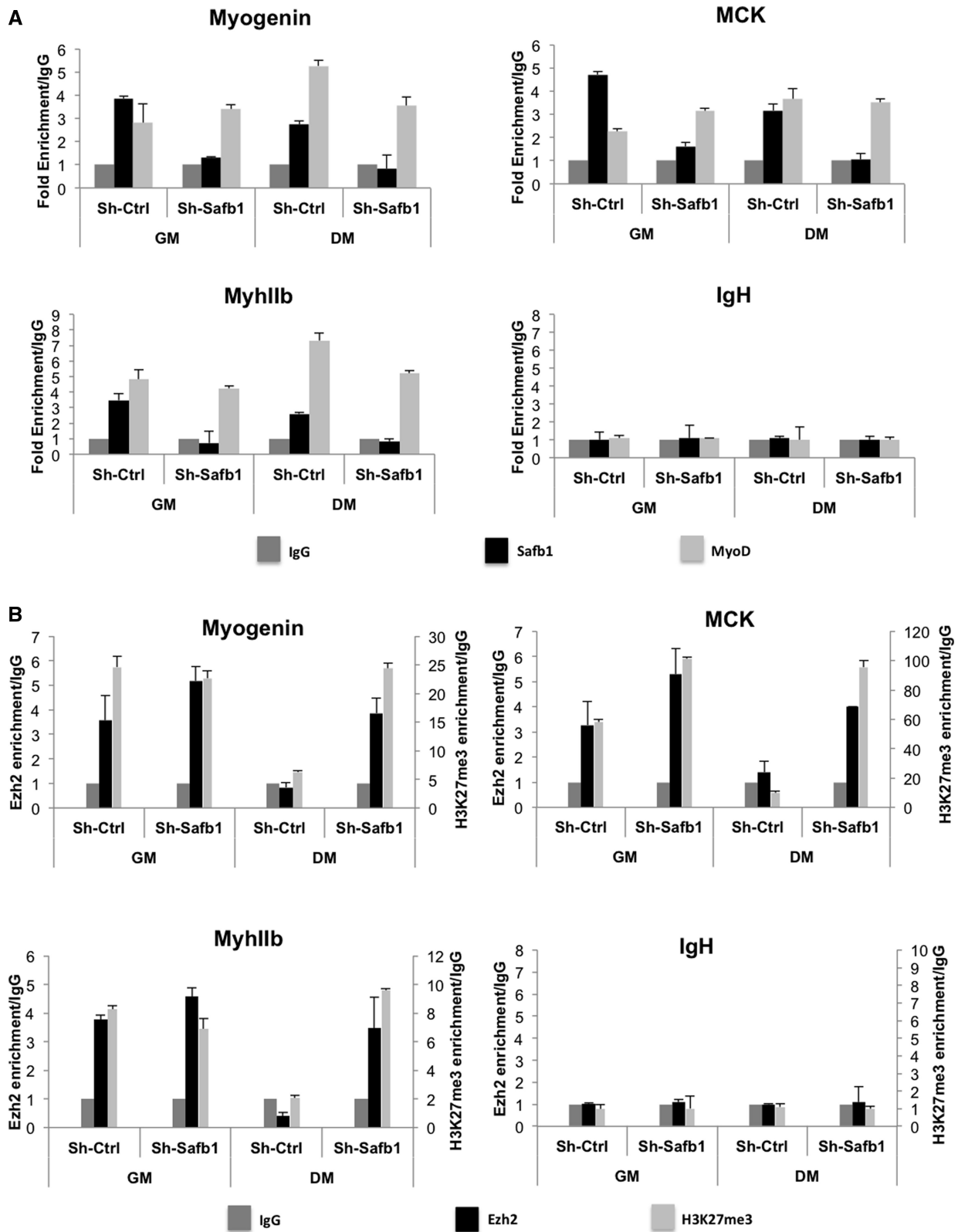
inhibited. As shown in Figure 6A, the knockdown of Safb1 resulted in minimal or no reduction in the recruitment of MyoD at skeletal muscle gene promoters, whereas recruitment of Safb1 was compromised, as expected. This result indicates that Safb1 is not required for the initial interaction of MyoD with target promoters in myoblasts and likely functions downstream of MyoD binding to myogenic loci. Because cells deficient for Safb1 exhibited reduced differentiation capability, we decided to analyse the recruitment of known transcriptional repressors and activators of skeletal muscle genes at muscle promoters before and after differentiation. The Polycomb group protein Ezh2 is responsible for tri-methylation of histone H3 lysine 27 (H3K27me3), a chromatin mark associated with repressed chromatin (41), and has been shown to maintain skeletal muscle genes in a repressed state prior to differentiation (42–44). As shown in Figure 6B, in control cells, both Ezh2 and H3K27me3 are present at target gene promoters in myoblasts but are not localized to these sequences in differentiated control cells. Knockdown of Safb1 results in roughly equivalent recruitment of the Ezh2 and H3K27me3 to myogenic promoters in myoblasts and in differentiated cells (Figure 6B). The data indicate that when Safb1 levels are reduced, the chromatin environment associated with Pc Group-mediated gene repression is maintained despite the onset of differentiation. These data are also consistent with the reduced transcriptional levels of these genes and the block of cell

differentiation observed upon Safb1 knockdown (Figure 2C and D).

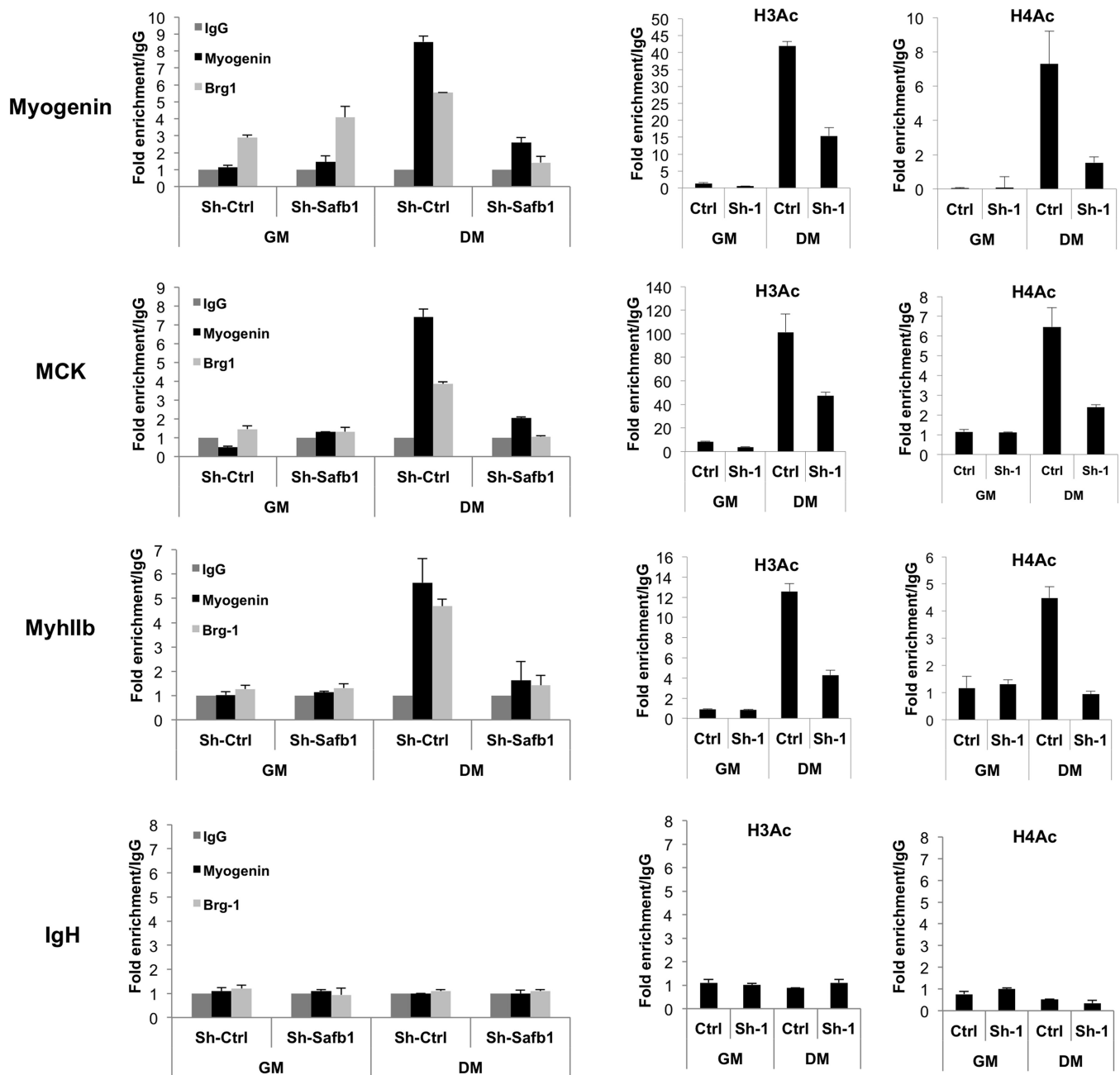
To further support the idea that myogenic target genes are maintained in a repressive chromatin environment when Safb1 levels were reduced, we performed additional ChIP experiments to examine the recruitment of factors and histone marks associated with transcriptionally active myogenic genes. Recruitment of the myogenic activator myogenin and the SWI/SNF ATPase Brg1, which is required for chromatin remodelling associated with myogenic gene activation (26,38,45,46), is normally enhanced upon differentiation but is compromised in Safb1 knockdown cells induced to differentiate (Figure 7). Similar results were observed for the incorporation of acetylated histones H3 and H4 (Figure 7), which are histone marks linked to myogenic gene activation as well as gene activation in general (47,48). These results are also entirely consistent with the observed inhibition of myogenic gene expression upon Safb1 knockdown (Figure 2C and D). We conclude that Safb1 is necessary to mediate the transition between the repressed chromatin and transcriptionally competent chromatin at myogenic promoters during myogenesis.

## DISCUSSION

In this work, we showed that Safb1 is a component of the transcriptional machinery that is required for proper



**Figure 6.** shRNA-mediated knockdown of Safb1 does not alter MyoD recruitment to myogenic sequences but increases Polycomb repressive marks. Proliferating (GM) or 72 h differentiated (DM) C2C12 cells expressing control shRNA or shRNA against Safb1 were fixed and processed for ChIP assays using (A) antibodies against MyoD and Safb1 (top panel) or (B) antibodies against Ezh2 and H3K27me3 at the indicated gene regulatory sequences. H3K27me3 enrichment is presented on the right-hand y-axis in the bottom panel. Data represent the mean of three independent experiments  $\pm$  SEM. *t* test analyses indicate that when comparing binding in sh-Safb1-treated differentiated cells with sh-Control differentiated cells, the binding of Safb1 was significantly reduced ( $P < 0.01$ ), the binding of MyoD was not significantly reduced, except for the binding to the Myh11b promoter ( $P = 0.045$ ), and the binding of Ezh2 and H3K27me3 were significantly reduced ( $P < 0.01$  and  $P < 0.005$ , respectively).



**Figure 7.** Recruitment of transcriptional activators and associated chromatin marks is inhibited in Safb1 knockdown cells. Proliferating (GM) or 72 h differentiated (DM) C2C12 cells expressing control shRNA or shRNA against Safb1 were fixed and processed for ChIP assays using antibodies against Myogenin, Brg1, acetylated H3 or acetylated H4 at the indicated gene regulatory sequences. Data represent the mean of three independent experiments  $\pm$  SEM. *t* test analyses indicate that binding of myogenin and Brg1 and incorporation of acetylated H3 and H4 was significantly reduced in differentiated sh-Safb1-treated cells compared with binding/incorporation in differentiated sh-Control cells (myogenin  $P < 0.05$ , Brg1  $P < 0.002$ , acetylated H3  $P < 0.005$ , acetylated H4  $P < 0.002$ ).

expression of skeletal muscle genes during myogenesis. Together with MyoD, Safb1 binds to muscle regulatory sequences both in non-differentiated myoblasts and in differentiated myotubes, facilitating the transition from a repressive transcriptional state to an active chromatin environment to promote myogenic gene expression and formation of multinucleated myotubes in culture.

Experiments that revealed an association between Safb1 and MyoD in myoblast extracts and that indicated

co-occupancy of myogenic promoter sequences by these proteins prior to differentiation suggest a mechanism by which Safb1 could be localized to myogenic gene regulatory sequences. The observation that MyoD binding to myogenic genes in myoblasts was unaffected by Safb1 knockdown supports the idea that MyoD targets Safb1 to differentiation-dependent gene promoters. Such targeting could be the result of direct physical interactions between MyoD and Safb1 or could be mediated by

other proteins that are known to be present with MyoD, such as E proteins and Mef2 factors. To date, none of the experiments that we have performed using bacterially expressed or *in vitro* translated proteins support the hypothesis that MyoD and Safb1 directly interact (unpublished), but the technical limitations of such approaches do not exclude the possibility that direct interactions occur but are dependent on post-translation modifications to one or both proteins. Our data also indicate that initial MyoD binding to myogenic genes prior to differentiation is independent of Safb1. This finding is consistent with the idea that MyoD binding in myoblasts is a critical step in reprogramming the myoblast genome in preparation for differentiation (32). Based on the data, we predict that MyoD, or perhaps either MyoD or the related myogenic regulator Myf5, is required for Safb1 binding. Definitive demonstration of this prediction will require assessment of Safb1 binding to myogenic promoters in myoblasts where MyoD and/or Myf5 levels have been reduced.

Nuclear scaffold proteins facilitate protein-protein interactions as well as provide an additional mechanism for linking to chromatin by binding to AT-rich S/MARs in the genome (49–51). Thus, Safb1 may function in part to physically link MyoD-bound myogenic regulatory sequences to chromatin domains associated with gene repression or activation. Safb1 has been confirmed as a nuclear matrix protein (20), and its ability to link to the nuclear matrix may facilitate gene regulation, as has been indicated for many other nuclear matrix proteins. Furthermore, Safb1 has been implicated in organization of chromatin via its physical interaction with known ATP-dependent chromatin remodelling proteins such as Chd1 and Brg1 (31,52). Altogether, the involvement of Safb1 in the regulation of myogenic gene expression emphasizes the relevance of the local chromatin structure and raises the provocative possibility that higher order chromatin organization may also be involved (16,17).

The ChIP data presented in Figures 6 and 7 identify Safb1 as being necessary for myogenic promoters to transition from a repressed chromatin state to a transcriptionally active state. Reduced levels of Safb1 resulted in the maintenance of the Polycomb group histone methyltransferase Ezh2 and its repressive mark H3K27me3 on muscle promoters despite induction of cell differentiation. Ezh2 is a critical negative regulator of skeletal muscle differentiation (53), and recent work details how both the Ezh1 paralog protein as well as the UTX lysine demethylase contribute to myogenic gene activation by countering the repressive effects of Ezh2 and H3K27me3 (54–56). In the absence of removal of Ezh2 and H3K27me3 from myogenic gene promoters, the normal recruitment of activators, co-activators and modified histones associated with transcriptional activation did not occur. Determining whether Safb1 interacts directly with Ezh2 or other members of the PRC2 complex as a mechanism that helps target the enzyme to myogenic promoters prior to differentiation would be a future step in better understanding the potential inter-related functions of Ezh2 and Safb1. Similarly, a survey of Safb1-interacting proteins subsequent to differentiation

signalling may yield insight into the organization of co-activators that promote myogenic gene expression.

Exactly how Safb1 functions both pre- and post-differentiation remains to be determined. One possibility is that Safb1 serves as a scaffold for negative co-regulatory proteins prior to differentiation, and differentiation-induced expression of myogenin and/or induction or activation of other positively acting co-regulatory proteins displace the negative co-regulatory proteins and use Safb1 as a scaffold to help associate with target gene promoters. In this model, Safb1 could act as a co-repressor and a co-activator but would not necessarily change structurally when differentiation is induced. Other co-regulatory proteins have also been shown to affect cell differentiation both positively and negatively. For example, SWI/SNF chromatin remodelling enzymes are required for the differentiation of most tissue types (57,58) yet are reported to act as negative regulators of transcription at specific stages of some tissue differentiation events (59) and during ES cell differentiation (60–62). The arginine methyltransferase Prmt5, like Safb1, was initially characterized as a negative regulator of gene expression yet was subsequently shown to be required for both myogenic- and adipogenic-specific gene expression and differentiation (28,63,64). The functional activity of each of these regulators is likely the same regardless of context; positive or negative outcomes with regard to transcriptional regulation are almost certainly dependent on the local chromatin environment in which the regulator functions.

Another possibility is that Safb1 is structurally modified upon differentiation signalling, which may make Safb1 more likely to interact with different transcriptional co-activators. It has been recently shown that sumoylation of Safb1 lysines 231 and 294 by the SUMO E3 ligase PIAS1, modulates the repressor activity of Safb1 by increasing its ability to interact with HDAC3 *in vitro* (24). Because HDAC1 and HDAC2 are known to interact with MyoD at muscle gene promoters in the non-differentiated state to repress the transactivation potential of MyoD (26,65,66), it may be possible that the presence or absence of sumo groups contributes to the function of Safb1 in myogenesis. However, attempts to date to identify sumoylated Safb1 in C2C12 myoblasts or myotubes have not been successful (unpublished data). Similarly, it has been demonstrated that phosphorylation of Safb1 modulates Safb1 repressor activity at the hXOR promoter (31). Other as yet uncharacterized post-translational modifications could also contribute to Safb1 function during myogenesis.

In conclusion, our work demonstrates that Safb1 scaffold protein can function as a co-activator in a cellular differentiation process. The evidence suggests that Safb1 is targeted to myogenic gene regulatory sequences by MyoD prior to differentiation and that its presence facilitates the transition of myogenic gene regulatory sequences from a repressed chromatin environment to an active chromatin environment. Thus, the Safb1 scaffold protein represents a novel type of co-activator that facilitates the activation of myogenic genes during myogenic differentiation.



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