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Sin3 interacts with Foxk1 and regulates myogenic progenitors

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Abstract

We have previously reported Foxk1 as an important transcription factor in the myogenic progenitors. SWI-independent-3 (Sin3) has been identified as a Foxk1 binding candidate using a yeast two-hybrid screen. In the present study, we have identified the Foxk1 N-terminal (1–40) region as the Sin3 interacting domain (SID), and the PAH2 of Sin3 as the Foxk1 binding domain utilizing yeast two-hybrid and GST pull-down assays. Further studies revealed that knockdown of Sin3a or Sin3b results in cell cycle arrest and upregulation of cell cycle inhibitor genes. In summary, our present studies have shown that Foxk1 interacts with Sin3 through the SID and that Sin3 has an important role in the regulation of cell cycle kinetics of theMPC population. The results of these studies continue to define and assemble the networks that regulate the MPCs and muscle regeneration.

Keywords

Foxk1; Sin3; Myogenic progenitor; Cell cycle

Introduction

Adult skeletal muscle has a remarkable regenerative capacity and is able to restore its muscle architecture even after more than 80 % of the tissue is damaged. This regenerative capacity is due to a pool of myogenic progenitors (i.e., satellite cells) that are resident in adult skeletal muscle [1]. Myogenic progenitors or satellite cells are located between the basement membrane and the cell membrane of individual muscle fibers. Following injury or disease (muscular dystrophy, sarcopenia, etc.), satellite cells become activated, they proliferate, differentiate, and fuse to form new muscle fibers or they contribute to the repair of injured myofibers. Studies using genetic mouse models and molecular biological

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techniques have defined a number of satellite cell markers including CD29, CD34, C-met, integrin alpha7, m-cadherin, Foxk1, Pax3, Pax7, and Syndecan3/4 [2–4]. Additionally, a number of studies have uncovered signaling pathways that regulate the satellite cell, including the IGF signaling, Notch signaling, Tgf beta signaling, and Wnt signaling pathways [5, 6]. Despite these findings, the regulatory network for the myogenic progenitors or satellite cells remains incompletely defined.

The *forkhead* gene family has a broad role in stem cell biology, cell cycle kinetics, aging, cancer development, and cell differentiation [7–10]. We have previously identified Foxk1 as a regulator of the myogenic progenitor cell (MPC) population [11]. Using a gene disruption strategy, we produced Foxk1 null mice [12]. These Foxk1 null mice have a small body size, decreased number of satellite cells, and perturbed muscle regeneration. We have also shown that Foxk1 regulates *p21* gene expression, an important cell cycle dependent kinase inhibitor for the MPCs [12]. Moreover, all the defects associated with the Foxk1 null mouse model were rescued in mice that lacked both Foxk1 and p21 [13]. In addition, we have recently identified Fhl2 as a potent co-repressor of Foxk1. We have observed that Foxk1 and Fhl2 synergistically repress Foxo4 activity although the mechanism of Foxk1 in transcriptional repression is not clear [14].

The SWI-independent-3 (Sin3) complex is an important histone deacetylase 1 (HDAC1)-containing repression complex [15]. The essential role of the Sin3 complex in cellular regulation is evident as it is conserved from yeast to mammals [16, 17]. The components of Sin3 complex include Sin3a, Sin3b, HDAC1, HDAC2, Rbbp4, Rbbp7, Sap18, Sap30, and Sds3. Within the complex, Sin3 serves as a scaffold protein, providing a platform for other complex members to interact. The scaffold function of Sin3 is served by its multiple domains, including four paired amphipathic helix (PAH) domains, a HDAC interaction domain (HID), and a highly conserved region (HCR) domain [16]. PAH3 and HID domains play important roles in the association with these component members. In addition to these components of the Sin3 complex, Sin3 also interacts with a number of transcription factors through these domains, including the PAH1 and PAH2 domains. Sin3 interacting transcription factors include NRSF/REST which results in the repression of neuron-specific gene expression in non-neuronal cells through the binding to the Sin3 PAH1 domain [18]. Additionally, Mad1 recruits the Sin3 repression complex to inhibit Max–Myc activity through the interaction with the PAH2 domain [19, 20].

Previously, Sin3b was isolated as a Foxk1 interaction candidate using the yeast two-hybrid screen [21]. In the present study, we extend our studies and define the protein interaction between Foxk1 and Sin3, and identify a Sin3 interacting domain (SID) in the N-terminal of Foxk1. We also demonstrate that both Sin3a and Sin3b are important regulators of the cell cycle in myogenic progenitors.

Methods

DNA, RNA, and qPCR

Sin3a and Sin3b expression plasmids were provided by Dr. Ron DePinho. Additional plasmids were routinely constructed using PCR and verified by sequence analysis. RNA was

prepared with Tripure (Roche) and cDNA synthesis was performed with first-strand cDNA synthesis kit (Invitrogen) as previously described [22]. qPCR was performed with Sybergreen reagent (ABI system) using the HT7900 system (ABI system).

Yeast two-hybrid

The yeast two-hybrid assay was performed using the Match-maker Two-hybrid System (Clontech) as previously described [22]. Full length and deletion of Foxk1 constructs were cloned into the pGBKT7 vector. Full length and deletion of Sin3b (293) constructs were cloned into the pGADT7 vector.

Western blot and GST pull-down

Cell extracts were prepared as previously described [14]. Antibodies utilized for western blot analysis include anti-myc (clone 9E10, Santa Cruz) and anti-alpha tubulin (Sigma) sera. The proteins of Foxk1 deletions and Sin3 deletions were synthesized in vitro in the presence of ^{35}S-Methioine using the TNT system (Promega). *E. coli* BL21 cells harboring GST, GST-Foxk1 (1–40), or GST-Sin3b (293) were induced with IPTG, extracted with B-PER bacterial Protein Extraction Reagent (Pierce Biochemicals) and then purified with Glutathione-Sepharose 4B (GE Healthcare). GST fusion proteins bound to Sepharose beads were incubated with ^{35}S-Met-labeled protein product. The pull-down complex was washed with the bead binding buffer and then resuspended in the sample buffer, analyzed using a 4–20 % gel and imaged using the Typhoon phosphorImager (GE Healthcare) [14].

siRNA and cell cycle analysis

Sin3a siRNA oligonucleotides, Sin3b siRNA oligonucleotides, and RNA-Induced Silencing Complex free (RISC free) were purchased from Dharmacon. To identify the effective siRNA candidates, C2C12 myoblasts were transfected with Myc-Sin3a or Myc-Sin3b expression plasmids and siRNA oligonucleotides or RISC free control using lipofectamine reagent (Invitrogen) in 6-well plates. The following siRNA oligonucleotides were selected: Sin3a #3 (sense: 5′-ggauggaugaaguauauaauu-3′; antisense: 5′-puuauauacuucauccauccuu-3′), Sin3a #4 (sense: 5′-cagacuacguggagcgauauu-3′; antisense: 5′-puaucgcuccacguagucuguu-3′), Sin3b #2 (sense: 5′-caacaaugcuaucagcuauuu-3′; antisense: 5′-pauagcugauagcauuguuguu-3′), and Sin3b #4 (sense: 5′-cgacguaugucugaagguguu-3′; antisense: 5′-pcaccuucagacauacgucguu-3′). For cell cycle analysis, C2C12 myoblasts were transfected with the selected siRNA oligonucleotides or the control (RISC-free oligonucleotides) in 6-well plates. Cells were collected 48 h after transfection and prepared as previously described [14]. Cell cycle profiles were obtained and analyzed using a FACScan (BD) and processed using CellQuest software (BD) [14].

Statistics

Student's *t* tests were performed as previously described [23]. The significant difference is identified as $p < 0.05$. Data are presented as mean ± SEM.

Results

Foxk1 interacts with Sin3b (293)

Previous studies have reported Sin3b (293) as a potential Foxk1 binding candidate using the yeast two-hybrid screen [21]. To define the protein interaction domain between Foxk1 and Sin3b (293), we cloned the deletions of Foxk1 and Sin3b (293) into pGBKT7 and pGADT7 vectors, respectively, as shown in Fig. 1a, b. All of the constructs were tested to exclude autoactivation in the yeast two-hybrid assays. Each of the pGBKT7–Foxk1 constructs was cotransformed into the AH109 reporter strain with pGADT7–Sin3b (293). The protein interaction between the Foxk1 deletion constructs and Sin3b (293) was analyzed based on the yeast clone growth in the selection medium. As summarized in Fig. 1a, the Foxk1 N-terminal (1–40) domain was required for its interaction with Sin3b (293) and was defined as the SID. Utilizing a similar strategy, the yeast reporter strain AH109 was cotransformed with pGBT7–Foxk1 and pGADT7–Sin3b (293) deletion constructs to identify the Foxk1 interaction domain in Sin3b (293). We observed that the PAH2 domain in Sin3b harbors the Foxk1 binding domain, which is outlined in Fig. 1b.

The Foxk1 SID binds to the Sin3 PAH2 domain

The yeast two-hybrid studies revealed that the interacting regions included the Foxk1 SID and the Sin3 PAH2 domain. To further characterize the protein–protein interaction domains in vitro, we performed GST pull-down assays. As shown in Fig. 2b, the GST-Sin3b (293) protein was successfully purified. Foxk1 deletional proteins were efficiently synthesized in vitro in the presence of ^{35}S-Methione as shown in Fig. 2c (upper panel). Using the GST protein as the control (data not shown), GST-Sin3b (293) successfully pulled down Foxk1 deletion proteins containing the SID, but not Foxk1 (40–719), which lacks the SID, as shown in the Fig. 2c (lower panel). These studies (summarized in Fig. 2a) further confirmed the Foxk1–Sin3b interacting domains which were defined using the yeast two-hybrid assays. Using a similar strategy, we purified the GST-Foxk1 (1–40) protein as shown in Fig. 2e. As the PAH2 domains are conserved in Sin3a, Sin3b, and Sin3b (293), we included Sin3a and full length of Sin3b in the GST pull-down assays. All of the Sin3 proteins have been synthesized in vitro and labeled with ^{35}S-Methione, as shown in Fig. 2f (upper panel). As summarized in Fig. 2d, GST-Foxk1 (1–40) binds to all of the constructs of the Sin3a, Sin3b, Sin3b (293), and Sin3b (148–293), which harbor the PAH2 domain, but not Sin3b (1–171) (Fig. 2f). These results further establish that the Foxk1 SID interacts with the PAH2 domain of Sin3.

Knockdown of Sin3a results in cell cycle arrest

Our previous studies have demonstrated that Foxk1 plays an important role in the cell cycle regulation of MPCs [12]. Sin3a is reported as the ubiquitous transcriptional co-repressor and may have a specific functional role in selected lineages [24, 25]. To examine the functional role of Sin3a in the cell cycle regulation in MPCs, we utilized siRNA oligonucleotides to knockdown Sin3a. Myc-tagged Sin3a expression plasmid was cotransfected with the siRNA oligonucleotide pool, each oligonucleotide, or the RISC-free control. As shown in Fig. 3a, the transfection plasmid robustly expressed Myc-Sin3a. The siRNA oligonucleotide pool knocked down Sin3a almost completely and each oligonucleotide also knocked down Sin3a,

but #3 and #4 siRNA oligonucleotides were chosen for further studies as they were most effective (Fig. 3a). The siRNA oligonucleotides (#3 or #4) were transfected into the C2C12 myogenic cell line to examine the functional role of Sin3a and cell cycle regulation. As shown in Fig. 3b, the knockdown of Sin3a (siRNA #4) resulted in cell cycle arrest, which was further quantified in Fig. 3c. Sin3a siRNA #3 has the similar effect on the cell cycle kinetics (data not shown). To examine the gene expression profile in response to knockdown of Sin3a, we performed qPCR analysis (Fig. 3d). We observed that Sin3a was knocked down more than 60 % (compared to control) and Sin3b expression was not affected. These results support the hypothesis that the effect of siRNA treatment is due to the knockdown of Sin3a, but not Sin3b. Furthermore, we observed that the cell cycle inhibitor genes (*p21* and *p27*) were upregulated, but not *p57*. In addition, we observed an upregulation of Foxk1 expression with Sin3a knockdown.

Sin3b modulates the cell cycle progression

Previous reports have suggested that Sin3b plays an important role in the cell cycle exit, but is dispensable for cell cycle progression in MEFs [26, 27]. To examine the functional role of Sin3b in the myogenic progenitors, we knocked down Sin3b using specific siRNA oligonucleotides. As shown in Fig. 4a, both #2 and #4 siRNA oligonucleotides efficiently knocked down Sin3b using western blot analysis and were utilized in siRNA experiments. To examine the effect of Sin3b knockdown, we analyzed the cell cycle profile with siRNA treatment. Sin3b siRNA #4 treatments arrested the cell cycle in the G0/G1 phase as shown in Fig. 4b, c. We have also observed a similar effect with Sin3b siRNA #2 treatments (data not shown). We noted that the Sin3b knockdown-mediated cell cycle arrest was more modest compared with Sin3a (Fig. 3b, c). In addition, we performed qPCR analysis to examine the gene expression profile associated with Sin3 knockdown. We observed that *Sin3b* expression was knocked down approximately 60 % compared to the control using siRNA treatment, whereas the *Sin3a* expression was not affected. Further, we observed that the cell cycle inhibitor genes, *p21* and *p27*, were upregulated in response to Sin3b knockdown. Interestingly, *p57* was also upregulated in response to Sin3b knockdown, which supports the notion that Sin3b regulates *p57* gene expression through a Sin3a independent mechanism. In addition, we also observed that *Foxk1* expression was induced and similar to that observed with Sin3a knockdown.

Discussion

Skeletal muscle has a remarkable capacity for regeneration due to a progenitor cell population that is resident in adult skeletal muscle. While a number of transcription factors and signaling pathways have been shown to have an important role in the satellite cell population (also known as myogenic progenitors), the mechanistic regulation of these factors remain ill defined [2]. In the present study, we extended our studies of Foxk1 and defined the interaction domains for Foxk1 and Sin3b (293). We further examined the functional role for Sin3 proteins as regulators of the MPC population.

The Sin3 repression complex has been shown to play an important role in transcriptional repression from yeast to vertebrates [17]. The PAH domains of Sin3, specifically the PAH1

and PAH2 domain, have a critical role in the protein interaction between Sin3 and their interacting transcription factors. NMR and biochemical studies have defined the interaction between the PAH2 domain of Sin3 and the N-terminal of Mad1 [28, 29]. In this protein–protein interaction, PAH2 has a wedged helical bundle structure and forms a hydrophobic cleft. The SID of Mad1 forms an amphipathic alpha-helix structure and is bound in the hydrophobic pocket of the PAH2 domain [28, 29]. This PAH2-amphipathic alpha-helix interaction model has also been identified in other Sin3-interacting proteins including KLF11 family members, HBP1, and Pf1 [30–35]. In the present study, we defined the SID in the Foxk1 N-terminal region. The affinity between each SID and Sin3 PAH2 domain depends on the structure of the SID. Using the Gal4-UAS reporter system, Gal4-Foxk1 SID does not repress the transcription (data not shown), which might reflect the affinity between the Foxk1 SID and Sin3 PAH2 domain. Future studies will focus on the characterization of the additional factor(s) involved in the protein interaction between Foxk1 and Sin3 repression complex.

The functional role of Sin3 in the regulation of cell cycle kinetics has been well documented using biochemical and mouse genetic studies. Sin3 has been reported to interact with a number of signaling cascades resulting in cell cycle arrest [16]. For example, studies have demonstrated that Rb recruits the Sin3 repression complex and inhibits the E2F activator, thereby promoting cell cycle arrest [36]. Loss of Rb results in increased E2F activity. In addition, Mad–Max interacts with Sin3 through the Mad1 SID and opposes Myc–Max oncogenic activity [19, 20]. Sin3 also interacts selectively with signal cascades (e.g., SMRTER repressor) and modulates cell cycle progression [37]. Using siRNA techniques, Sin3 and SMRTER were shown to be required for the G2 phase cell cycle progression. Moreover, Sin3 binds to p53 thereby inhibiting p21 gene expression [38]. The dual functional roles for Sin3 as a regulator of cell cycle kinetics reflect the complexity and multiple functions of the Sin3 repression complex in cellular events. Our previous studies have shown that Foxk1 plays an important role in the cell cycle progression and represses p21 gene expression in MPCs. In the present study, the knockdown of both Sin3a and Sin3b resulted in the upregulation of p21 and p27, and cell cycle arrest. Our studies support the notion that the cyclin dependent kinase inhibitor, p21, is regulated, in part, by Sin3–Foxk1. Interestingly, Foxk1 gene expression is also upregulated with the knockdown of Sin3a or Sin3b. Therefore, we believe that Sin3 also has a dual role in the regulation of Foxk1. At the protein level, Foxk1 interacts with Sin3 and forms a potent transcriptional repression complex. Future studies will focus on the specific mechanism of the Sin3–Foxk1 repression of nodal pathways in the myogenic progenitors.

In vivo studies utilizing a gene disruption strategy for Sin3a and Sin3b provide further information regarding the functional roles of Sin3 in development, gene expression, cell cycle regulation, cell differentiation, and apoptosis. Studies using the Sin3A knockout mouse model have demonstrated its essential role in the cell cycle progression [24, 25]. The Sin3a null MEFs have reduced cellular proliferation and increased cell apoptosis. Consistent with these biochemical studies, loss of Sin3a resulted in the upregulation of Myc–Max target genes, including *Cyclin D2*, *Cyclin E*, and *E2F* target genes, including *Cyclin E*, *Cyclin D3*, *Cdc2a*, etc. The Sin3B null mice have a distinct phenotype compared with the Sin3A null

embryo [26]. The Sin3B null MEFs have normal cell cycle progression, but have a perturbation in cell cycle exit and cellular differentiation. Interestingly, the E2F target genes are upregulated in Sin3b null cells. In the present studies, the knockdown of Sin3b resulted in cell cycle arrest, although to a lesser degree compared with the Sin3a knockdown. This discrepancy may reflect a difference in the cell lineage difference (i.e., MEFs vs. MPCs). Alternatively, these differences may reflect a differential role for Sin3b during development where Sin3b may have a regulatory role of the cell cycle in the myogenic progenitors. Future studies will focus on the conditional knockout of Sin3 in the MPC population.

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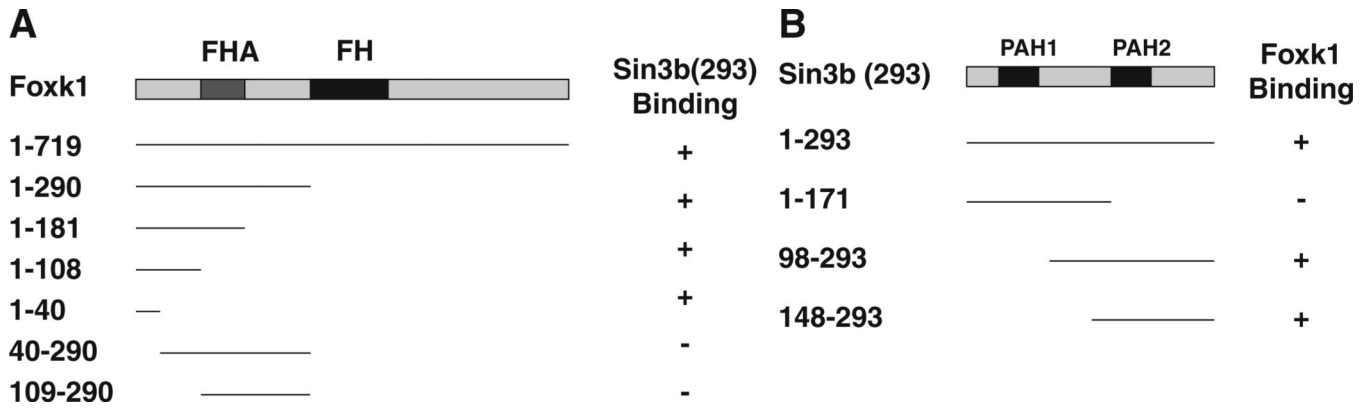


Fig. 1.

Characterization of the interaction between Foxk1 and Sin3b (293) using yeast two-hybrid assays. **a** Foxk1 N-terminal (1–40) domain is identified as the Sin3b-interacting domain (SID). All of the Foxk1 deletions were fused to the Gal4 DNA binding domain and used to analyze the interaction with Sin3b (293) using a yeast two-hybrid system. The protein interaction was determined by the yeast clone growth in the selection medium (+, positive; −, negative). FHA, *Forkhead* associated domain and FH, *Forkhead* domain. **b** The Sin3b PAH2 domain binds to Foxk1. Each of the Sin3b (293) deletions was fused to the Gal4 activation domain and used to analyze the interaction with Foxk1. The Sin3b (293) deletion containing the PAH1 domain did not bind to Foxk1, but the deletions harboring the PAH2 domain binds to Foxk1 (+, positive; −, negative; PAH, paired amphipathic helix domain)

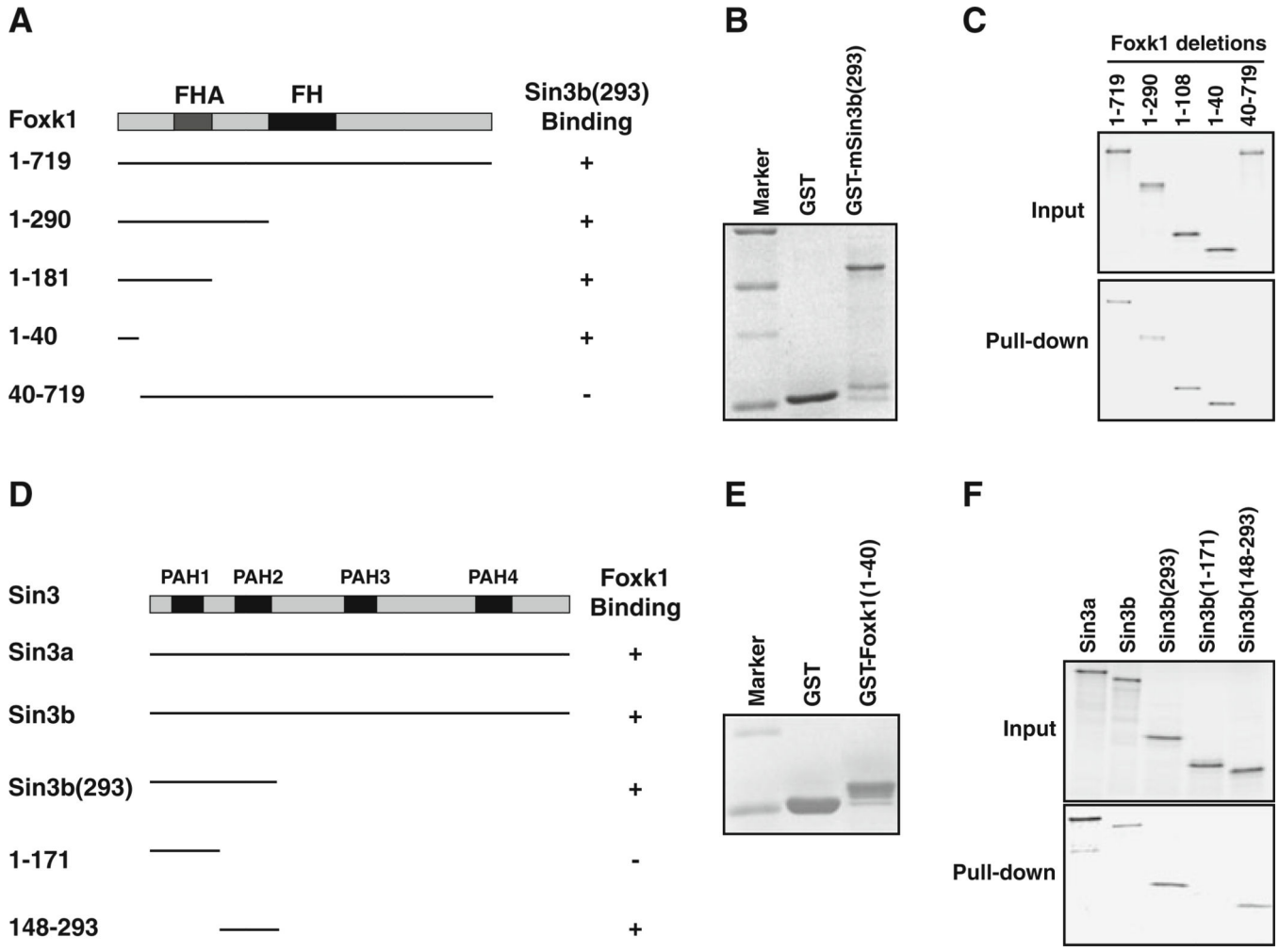


Fig. 2.

Foxk1 SID binds to the Sin3 PAH2 domain. **a** Schematic summary of the protein interaction between Sin3b (293) and Foxk1 deletions using GST pull-down assays. **b** Coomassie staining of purified GST or GST-Sin3b (293). **c** ^{35}S-labeled Foxk1 deletions were translated in vitro as input (*upper panel*) and pulled down using the GST-Sin3b (293) protein. The deletions harboring the Foxk1 SID were pulled down by GST-Sin3b (293), but the deletion of Foxk1 (40–719) lacking the SID did not interact (*lower panel*). **d** Schematic which summarizes the interaction between the Foxk1 SID and Sin3 constructs. **e** Coomassie staining of purified GST or GST-Foxk1 (1–40) proteins. **f** The in vitro synthesized ^{35}S-labeled Sin3 proteins were utilized in the GST pull-down assays as the input (*upper panel*). All of the Sin3 proteins harboring the PAH2 domain could bind to the Foxk1 SID, but Sin3b (1–171) which lacks the PAH2 domain could not bind to Foxk1

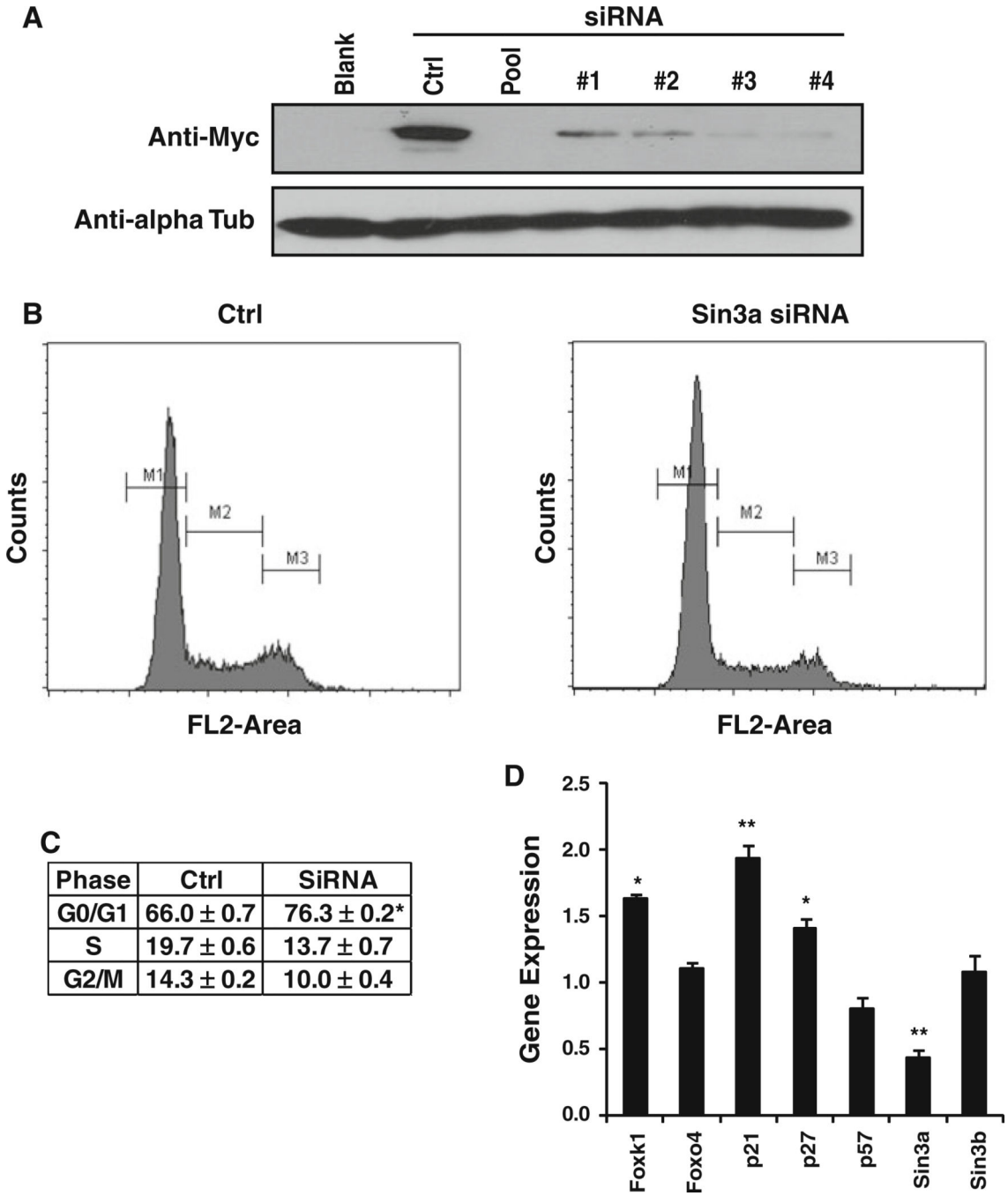
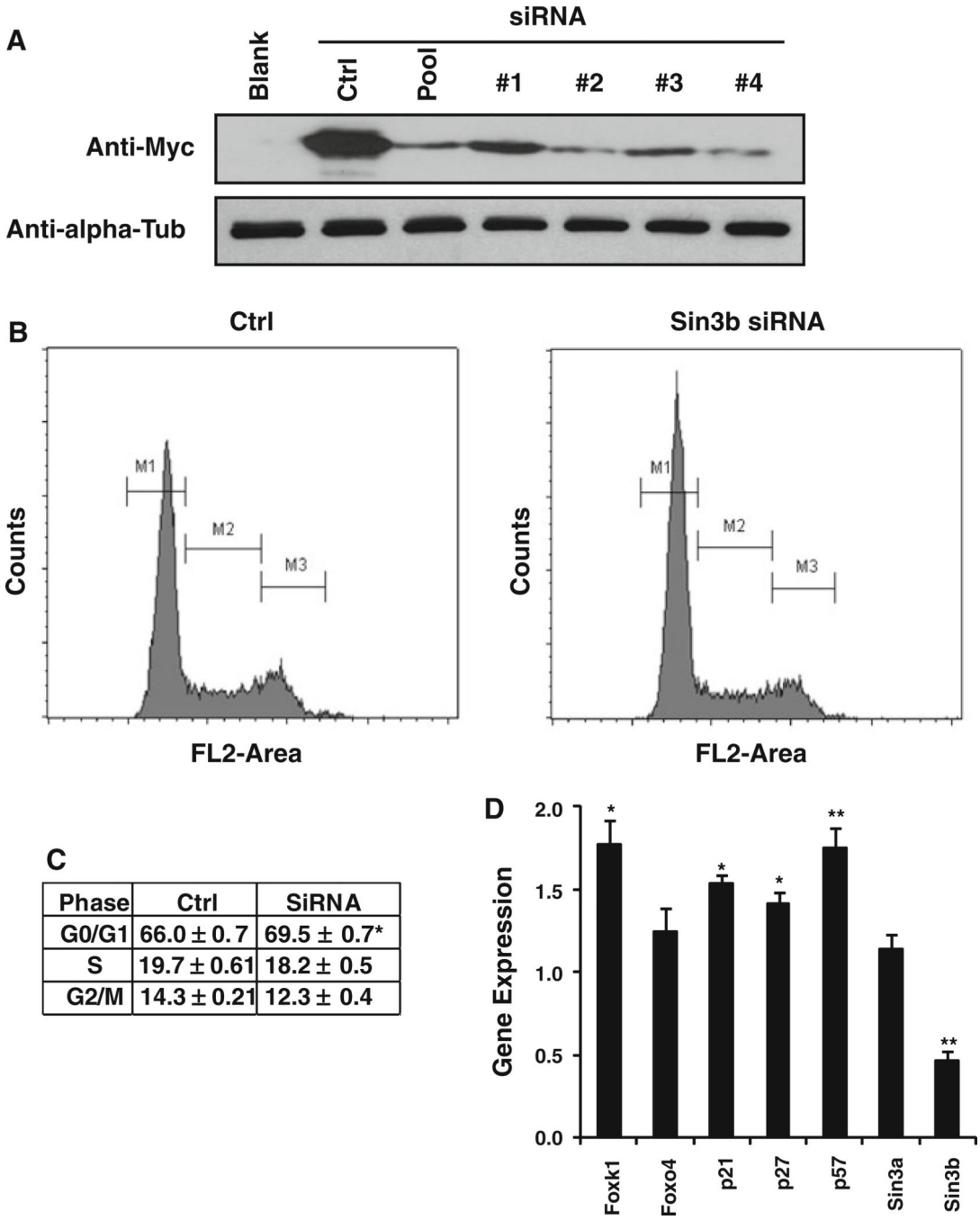


Fig. 3.

Knockdown of Sin3a in myoblasts results in cell cycle arrest. **a** Identification of Sin3a siRNA oligonucleotides. Myc-Sin3a was cotransfected into the C2C12 myoblasts with the Sin3a siRNA oligonucleotides or control siRNA oligonucleotides. The expression was detected using anti-Myc serum and equal loading of the samples was verified using anti-alpha tubulin serum. All of the oligonucleotides successfully knocked down Sin3a (blank, no transfection; Ctrl, the RISC-free oligonucleotides; Pool, all of the four siRNA oligonucleotides). **b** Knockdown of Sin3a resulted in G0/G1 cell cycle arrest (Ctrl, RISC-

free oligonucleotides). The FACS profile is a representative profile with Sin3a siRNA #4 oligonucleotide knockdown. **c** Quantification of the cell cycle phases in **b**. Knockdown of Sin3a resulted in a significant increase of cells in the G0/G1 phase, (*$p < 0.01$; $n = 4$). **d** qPCR analysis was used to examine gene expression following Sin3a knockdown. Shown here is the ratio of the gene expression in the siRNA knockdown to that in the RISC-free control. The endogenous Sin3a siRNA was knocked down 60 % compared to control but Sin3b expression was unaffected. Note, increased expression of p21 and p27, which is consistent with cell cycle arrest, (*$p < 0.05$, **$p < 0.01$; $n = 6$)



Phase	Ctrl	SiRNA
G0/G1	66.0 ± 0. 7	69.5 ± 0.7*
S	19.7 ± 0.61	18.2 ± 0.5
G2/M	14.3 ± 0.21	12.3 ± 0.4

Fig. 4.

Sin3b is required for myogenic cell cycle progression. **a** Selection of Sin3b siRNA candidates. Each siRNA oligonucleotide or the pool was cotransfected with Myc-tagged Sin3b expression plasmid into C2C12 myogenic cells. The knockdown efficiency was evaluated based on the Myc-Sin3b expression level using western blot analysis (anti-Myc serum). Anti-alpha tubulin was used to demonstrate equal loading of samples (blank, no transfection; Ctrl, RISC-free oligonucleotides; pool, the combination of all of the four oligonucleotides). **b** Sin3b siRNA treatment modulates cell cycle progression.

Representative FACS profile is shown for RISC control and Sin3b siRNA #4 knockdown. **c** Quantification of the cell cycle phases in **b**. The G0/G1 phase is significantly increased with Sin3b knockdown, (*$p < 0.05$; $n = 4$). **d** Sin3b knockdown results in increased expression of *p21*, *p27*, *p57* and other selected transcripts in C2C12 myoblasts using qPCR. The Sin3a gene expression was not affected by the Sin3b knockdown, (*$p < 0.05$, **$p < 0.01$; $n = 6$)