

## Article

# Comparative expression profiling identifies differential roles for Myogenin and p38 $\alpha$ MAPK signaling in myogenesis

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**Skeletal muscle differentiation is mediated by a complex gene expression program requiring both the muscle-specific transcription factor Myogenin (Myog) and p38 $\alpha$  MAPK (p38 $\alpha$ ) signaling. However, the relative contribution of Myog and p38 $\alpha$  to the formation of mature myotubes remains unknown. Here, we have uncoupled the activity of Myog from that of p38 $\alpha$  to gain insight into the individual roles of these proteins in myogenesis. Comparative expression profiling confirmed that Myog activates the expression of genes involved in muscle function. Furthermore, we found that in the absence of p38 $\alpha$  signaling, Myog expression leads to the down-regulation of genes involved in cell cycle progression. Consistent with this, the expression of Myog is sufficient to induce cell cycle exit. Interestingly, p38 $\alpha$ -defective, Myog-expressing myoblasts fail to form multinucleated myotubes, suggesting an important role for p38 $\alpha$  in cell fusion. Through the analysis of p38 $\alpha$  up-regulated genes, the tetraspanin CD53 was identified as a candidate fusion protein, a role confirmed both *ex vivo* in primary myoblasts, and *in vivo* during myofiber regeneration in mice. Thus, our study has revealed an unexpected role for Myog in mediating cell cycle exit and has identified an essential role for p38 $\alpha$  in cell fusion through the up-regulation of CD53.**

**Keywords:** myogenin, p38 MAPK, myoblast fusion, miRNA, cell cycle exit, CD53

## Introduction

Skeletal muscle is one of several syncytial tissues in mammals (Chen et al., 2007; Rochlin et al., 2010; Abmayr and Pavlath, 2012). Multinucleated myofibers are derived from mesodermal precursors that originate in the somites (Charge and Rudnicki, 2004; Wang and Conboy, 2010; Aziz et al., 2012). Conversion of these mesodermal precursor cells to muscle is directed by a family of four myogenic basic helix–loop–helix transcription factors that have been termed muscle regulatory factors (MRFs): MyoD, Myf5, Myogenin (Myog), and MRF4 (Tapscott, 2005). The MRFs heterodimerize with constitutively expressed E-proteins (including E47, E12, E2-2, and HEB; Lassar et al., 1991) to permit binding to the E-Box DNA-binding element in the promoter of muscle genes (Murre et al., 1989). While these four MRFs can bind to an overlapping set of genomic loci (Neville et al., 1998; Spinner et al., 2002; Blais et al., 2005; Cao

et al., 2006), there appears to be non-overlapping functional roles for these related proteins in myogenesis (Wang and Jaenisch, 1997; Neville et al., 1998; Spinner et al., 2002; Cao et al., 2006).

Expression of MyoD, Myf5, and/or MRF4 within multipotent mesodermal precursors specifies the cells to become mononucleated myoblasts (Rudnicki et al., 1993; Kassam-Duchossoy et al., 2004). Expansion of this mononucleated myoblast population is driven by Myf5 expression that promotes cell proliferation (Ustanina et al., 2007). Terminal differentiation is then driven by expression of Myog which is essential for the formation of functional multinucleated myofibers (Hasty et al., 1993; Nabeshima et al., 1993). Though the temporally distinct expression patterns of the four different MRFs can partially explain their differential roles in myogenesis, genetic studies have demonstrated functional differences between family members. Indeed, while Myf5 up-regulation compensates for the loss of MyoD in knockout mice (Rudnicki et al., 1992, 1993), knock-in of Myog into the Myf5 locus in the Myf5/MyoD double knockout mice generated animals with reduced musculature that died at birth (Wang and Jaenisch, 1997). The mechanism through which Myog expression

led to the formation of a reduced number of healthy muscle fibers in these genetic studies remains unknown (Wang and Jaenisch, 1997).

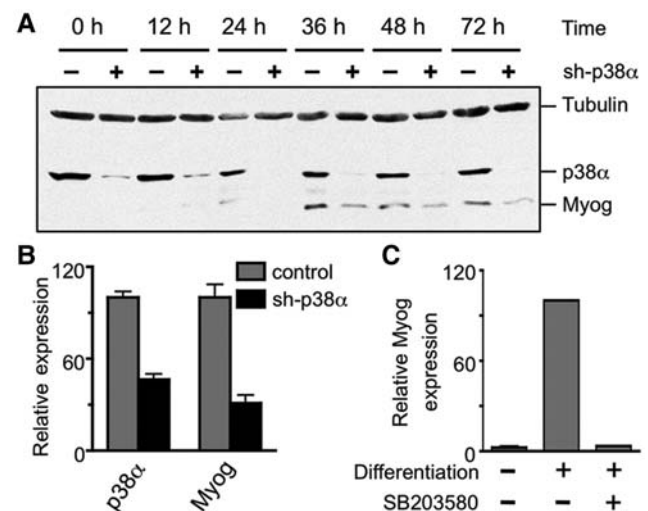
Among the signaling pathways that regulate myogenesis (Luo et al., 2005; Glass, 2010; Jang et al., 2012), the p38 MAPK signaling pathway is one of the most characterized (Zetser et al., 1999; Wu et al., 2000; Cabane et al., 2003; Keren et al., 2006; Perdiguero et al., 2007). Early in myogenesis, p38 $\gamma$  signaling is important in proliferating myoblasts where it prevents premature differentiation (Gillespie et al., 2009). Upon terminal differentiation, p38 $\alpha$  MAPK signaling is then activated where it represses Pax7 expression, mediates cell cycle withdrawal, and activates the expression of muscle-specific genes (Perdiguero et al., 2007; Palacios et al., 2010; Dilworth and Blais, 2011). While the complete list of proteins phosphorylated by p38 $\alpha$  MAPK signaling in differentiating myoblasts has not yet been established, this signaling pathway is well characterized for its role in regulating the myogenic gene expression program. Indeed, several different transcription factors have been shown to be directly phosphorylated by p38 $\alpha$ , including E47 (Lluis et al., 2005), BAF60c (Simone et al., 2004), and Mef2 (Zhao et al., 1999; Penn et al., 2004; Rampalli et al., 2007). These phosphorylation events have a profound effect on gene expression owing to the fact that they modulate the recruitment of chromatin remodeling enzymes to muscle-specific promoters (Simone et al., 2004; Rampalli et al., 2007; Aziz et al., 2010; Seenundun et al., 2010). Studies using myoblasts from mice with a muscle-specific knockout of the different p38 family members showed that p38 $\alpha$  (MAPK14) is the key isoform required for terminal differentiation (Perdiguero et al., 2007). Interestingly, characterizing p38 $\alpha$  function in myogenesis showed that expression of Myog is significantly reduced when the kinase is knocked-out (Perdiguero et al., 2007). Owing to the critical role of Myog in terminal muscle differentiation, we set out to examine the relative roles of Myog and p38 $\alpha$  signaling in the formation of multinucleated myotubes.

## Results

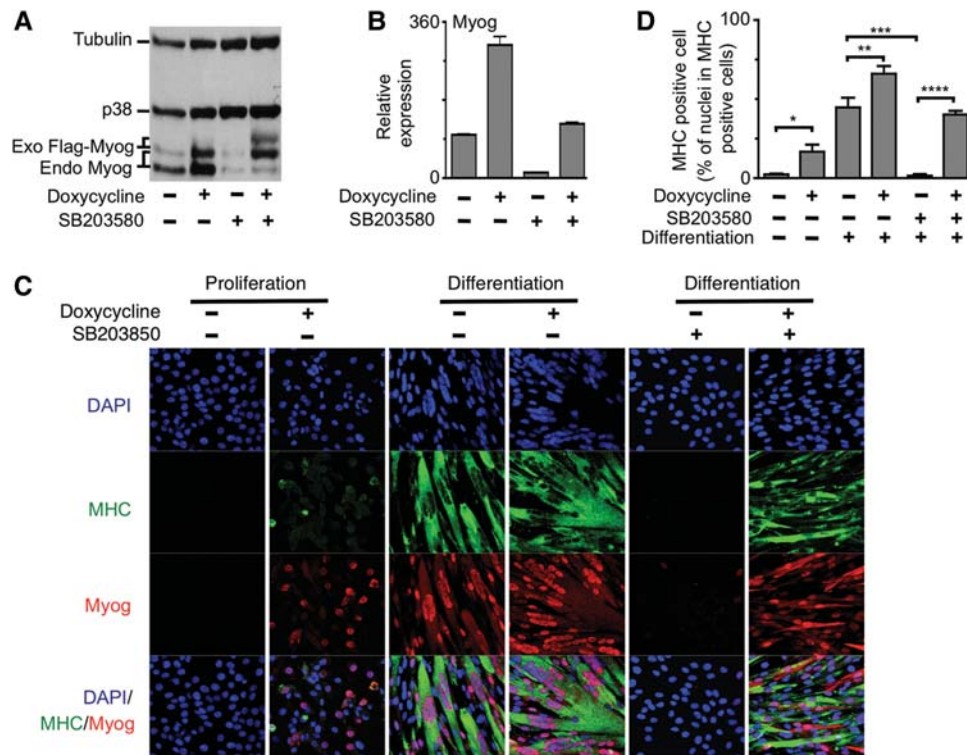
### *Myog can partially rescue the expression of muscle genes in the absence of p38 MAPK signaling*

To distinguish the roles of Myog and p38 $\alpha$  in terminal myogenesis, it was necessary to design a cell culture system that uncouples Myog expression from p38 $\alpha$  MAPK signaling. Initially, we validated the use of murine C2C12 myoblasts as a model system to study the relationship between p38 $\alpha$  signaling and Myog. Using shRNA mediated knock-down of p38 $\alpha$  (Figure 1A and B) or blocking of p38 $\alpha$  signaling using the small molecule inhibitor SB203580 (Figure 1C), we confirmed p38 $\alpha$  signaling is required for efficient expression of Myog in myoblasts undergoing differentiation. This result confirms that C2C12 cells recapitulate the p38 $\alpha$ -dependent activation of Myog previously shown in primary mouse myoblasts (Perdiguero et al., 2007) and serve as an appropriate model to study their relative contribution to myotubes formation. To uncouple the activity of Myog from that of p38 $\alpha$  in C2C12 myogenesis, we next generated a stable C2C12-derived cell line (C2i-Myog) that expresses a Doxycycline (Dox)-inducible cDNA encoding Flag-tagged Myog. In this system, the small molecule induction of exogenous Myog

combined with the pharmacological inhibition of p38 $\alpha$  signaling (SB) allows us to assess the relative contribution of these two pathways during myogenesis. As previously observed with the parental C2C12 cell line, treatment of C2i-Myog cells with SB resulted in a significant decrease in Myog at both the mRNA and protein levels (Figure 2A and B). However, the simultaneous addition of SB and Dox resulted in the expression of exogenous Flag-tagged Myog at levels comparable with those observed from the endogenous gene in the normal (untreated) differentiating myoblasts (Figure 2A and B). In addition, immunofluorescence analysis (Figure 2C, Col 3) confirmed that the C2i-Myog cell line is competent to differentiate as shown by the formation of multinucleated myotubes that express both Myog and the late marker of muscle differentiation myosin heavy chain (MHC). As expected, the blocking of p38 $\alpha$  signaling using SB prevented expression of both early (Myog) and late (MHC) markers of muscle differentiation (Figure 2B, C, Col 5, and D). In contrast, the late marker of differentiation MHC continues to be expressed under conditions where p38 $\alpha$  is inhibited but Myog expression is induced exogenously (SB + Dox; Figure 2C, Col 6, and D). Thus, exogenous expression of Myog in the absence of p38 $\alpha$  signaling can at least partially rescue the block in the myogenic gene expression program.



**Figure 1** Expression of Myogenin is modulated by p38 $\alpha$  signaling. (A and B) Knock-down of p38 $\alpha$  inhibits expression of Myog. (A) C2C12 cells transfected with a plasmid expressing a shRNA directed at p38 $\alpha$  (or scrambled control) were induced to differentiate for various lengths of time (as indicated). Whole cell protein extracts were subjected to western blot analysis using antibodies directed at tubulin, p38 $\alpha$ , or Myog. (B) RT-qPCR analysis was performed on total RNA isolated from cells differentiated for 48 h in the presence or absence of shRNA targeting p38 $\alpha$  (or a scrambled control). Values are expressed relative to the internal control DDX5 where the expression of p38 $\alpha$  or Myog at 48 h of differentiation was normalized to 100,  $n = 3$ . (C) The p38 $\alpha$ / $\beta$ -specific inhibitor SB blocks expression of Myog during myogenesis. C2C12 cells were differentiated for 48 h in the presence or absence of SB (10  $\mu$ M). Total RNA was extracted and subjected to RT-qPCR analysis. Values are expressed relative to the internal control DDX5 where the expression of Myog at 48 h of differentiation in the absence of SB was normalized to 100,  $n = 3$ .



**Figure 2** Exogenous expression of myogenin rescues the expression of MHC. C2C12 cells expressing a Dox-inducible cDNA encoding Flag-tagged Myog (C2i-Myog) were differentiated in the presence (or absence) of Dox and SB. (A) Total protein was extracted from cells and subjected to western blot using the antibodies indicated. (B) Total RNA was extracted from cells and subjected to RT-qPCR analysis using primers specific for Myog. Values are expressed relative to the internal control DDX5 where the expression in the absence of treatment was normalized to 100,  $n = 3$ . (C) C2i-Myog cells were maintained in the presence (or absence) of Dox and SB under conditions of proliferation or myogenic differentiation (48 h). Immunofluorescence analysis was then performed to examine expression of MHC, Myog and total DNA (DAPI). (D) Quantitative analysis of MHC-expressing cells after culturing in the presence (or absence) of Dox and/or SB as indicated. Values represent the mean percentage of nuclei in MHC positive cells  $\pm$  SEM from 10 different fields. \*\*\*\* $P < 0.0001$ , \*\*\* $P = 0.007$ , \*\* $P = 0.0171$ , \* $P = 0.029$ ,  $n = 3$ .

To examine the extent to which exogenous Myog expression rescued the p38 $\alpha$ -dependent block in signaling in myogenesis, we performed microarray analysis on RNA extracted from C2i-Myog cells that were differentiated under three conditions (Supplementary Table S1): normal (Control), inhibition of p38 $\alpha$  signaling (SB), and inhibition of p38 $\alpha$  signaling with exogenous Myog expression (SB + Dox). Comparative analysis of changes in gene expression (Table 1 and Supplementary Table S2) identified genes that require either p38 $\alpha$  signaling or Myog for normal expression levels. Using this approach, we found that 395 genes were down-regulated and 239 genes were up-regulated when p38 $\alpha$  signaling was inhibited in C2i-Myog cells (Table 1). Interestingly, transcript levels of 181 of the down-regulated genes and 101 of the up-regulated genes returned to normal when exogenous Myog expression was induced in the absence of p38 $\alpha$  signaling (SB + Dox). These rescued genes were termed Myog-dependent genes (Table 1). Genes whose expression was significantly altered in the presence of SB but not rescued by exogenous expression of Myog (SB + Dox) were termed p38 $\alpha$ -dependent genes (Table 1). The quality of our comparative expression analysis was confirmed by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

using RNA isolated from independent experiments (Supplementary Figure S1 and data not shown).

#### *Myogenin expression down-regulates genes involved in cell cycle progression*

The role of Myog as a key regulator of muscle differentiation was confirmed as Gene Ontology (GO) analysis of Myog-induced genes showed an enrichment for factors involved in new protein synthesis, cell metabolism, and muscle contraction (Table 1 and Supplementary Table S2). A search of transcription factor binding sites within the promoter region ( $-1000$  to  $+1000$  bp) identified a highly significant enrichment for both the consensus Myog-binding sites (E-Box) and Sp1-binding sites (Table 1 and Supplementary Table S2), two promoter elements that have previously been shown to co-operate in the regulation of muscle-specific gene expression (Biesiada et al., 1999). Thus, as expected, our array analysis confirms that Myog acts as a transcriptional activator to mediate the expression of genes involved in muscle differentiation and function.

To our surprise, the transcriptional activator Myog was also responsible for the down-regulation of a significant number of genes. GO analysis of these down-regulated genes showed an extremely high enrichment of genes involved in cell cycle regulation

**Table 1** Summary of comparative expression profiling for differentiating myoblasts.

Classification	Number of genes	Enriched GO terms		Enriched transcription factor binding sites		
		GO—Biological processes	Adjusted <i>P</i> -value	Factor	DNA-binding element	Adjusted <i>P</i> -value
<b>Myog-dependent</b>						
Myog induced	181	tRNA aminoacylation for protein translation	2.34E-05	Sp1	GGGCGGR	1.79E-23
		Cellular amino acid and metabolic process	3.57E-03	AP4 (Ebox)	CAGCTG	2.44E-11
		Carboxylic acid transport	7.68E-03	MAZ	GGGAGRR	1.37E-07
		Muscle contraction	7.68E-03	NFAT	TGGAAA	1.69E-04
		Ribosome biogenesis	9.81E-03	LEF1	CTTTGT	7.00E-04
Myog repressed	101	M phase	2.20E-23	Sp1	GGGCGGR	4.04E-12
		Cell cycle phase	3.60E-22	E2F	NCSCGCSAAAN	9.54E-10
		Nuclear division	6.55E-21	NFY	GATTGGY	5.82E-09
		Mitosis	6.55E-21	E2F	TTTSGCGGMNR	2.82E-08
<b>p38-dependent</b>						
p38 $\alpha$ induced	214	Cell adhesion	1.19E-03	MEF2	YTATTTNR	3.14E-10
		Biological adhesion	1.19E-03	SRF	CCAWATAWGGMNMNG	1.19E-09
		Developmental process	4.21E-03	SRF	SCCAWATAWGGMNM	1.30E-09
		Extracellular structure organization	2.53E-02	MEF2	KCTAWAAATAGM	1.52E-09
		Skeletal system development	2.62E-02	FOXO4	TTGTTT	1.38E-08
p38 $\alpha$ repressed	138	Nitric oxide mediated signal transduction	4.85E-02	Unknown	ACTAYRNNNCCCR	1.31E-05
		Detoxification of copper ion	4.85E-02	CHOP	NNRTGCAATMCCC	2.19E-05
		Intracellular signaling cascade	8.86E-02	Unknown	CCAATNNSNNNGCG	2.87E-04
				Unknown	YGGCYRCGC	3.85E-04

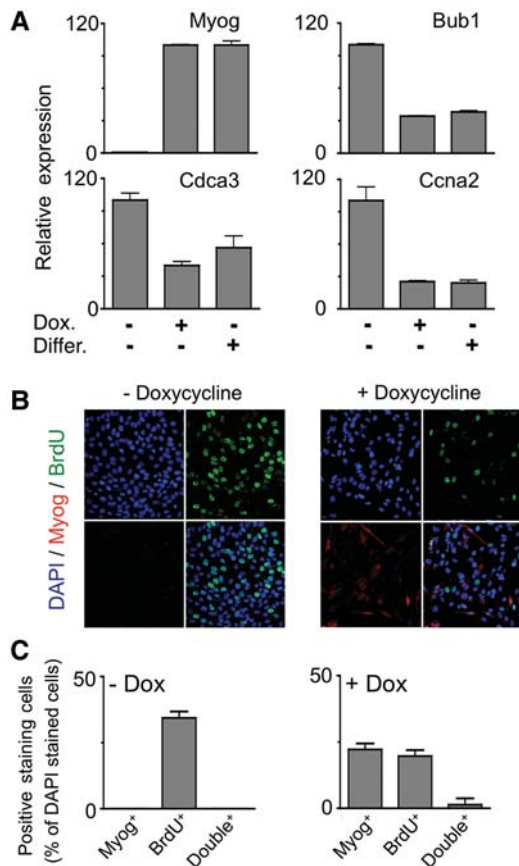
Microarray analysis was performed to examine the expression of genes in C2i-Myog cells expressing a Dox-inducible Flag-Myog protein in conditions where p38 $\alpha$  signaling was modulated using SB. Analyses of GO, or DNA-binding elements present in promoters, were performed to identify enrichments specific to the four different gene sets as outlined in Materials and methods (see Supplementary Table S2 for a complete list of regulated genes, GO terms, and binding elements).

(Table 1 and Supplementary Table S2). This result predicts that Myog would play a direct role in withdrawal of the cell cycle through a down-regulation of key factors involving myoblast proliferation. To test this possibility, we first examined cell cycle progression using BrdU pulse labeling experiments in differentiating C2C12 myoblasts. Immunostaining of the mixed population of differentiating myoblasts and undifferentiated ‘reserve’ cells, we observed that Myog-expressing cells rarely incorporated BrdU, suggesting that these cells no longer proliferated (Supplementary Figure S2A and B). In contrast, most of the BrdU-positive myoblasts demonstrated expression of MyoD, consistent with the previously described role for MyoD in activating genes involved in regulating cell proliferation (Zhang et al., 2010). Consistent with our prediction, these results would suggest that MyoD expression alone in differentiation conditions is insufficient to mediate cell cycle withdrawal, and instead it is the MyoD-dependent up-regulation of Myog that is responsible for initiating a gene expression program that leads to cell cycle exit during myogenesis.

To further examine the role of Myog in cell cycle exit, we performed BrdU pulse labeling experiments in C2i-Myog cells under proliferative conditions—a condition where MyoD is abundant but Myog is not normally expressed. In proliferating myoblasts induced to express Myog by Dox, we observed a 2–4-fold decrease in several key cell cycle genes including *Ccna2*, *Cdca3*, and *Bub1* (Figure 3A). Coincidentally, we observe a ~2-fold decrease in the percentage of BrdU-positive nuclei (Figure 3B and C). Importantly, cells that showed detectable expression of Myog protein did not proliferate as only 2% of Myog-positive nuclei also stained positive for BrdU. Taken together, these findings confirm a critical role for Myog in mediating terminal myogenesis through cell cycle exit and the synthesis of muscle-specific genes.

#### *The regulator of proliferation miR-20a is a direct target of myogenin*

To understand the mechanism by which the transcriptional activator Myog acts to down-regulate genes involved in cell cycle progression, we examined the promoter region of Myog-dependent genes identified by expression arrays. Remarkably, Myog-binding sites (E-boxes) are not enriched in Myog-repressed genes suggestive of an indirect regulatory mechanism. Instead they are enriched in binding sites for the transcriptional regulator of cell cycle E2F (see columns on the right-hand side of Table 1). Consistent with this, RT-qPCR analysis of different E2F family members show that exogenous expression of myogenin in proliferating myoblasts leads to a decreased expression of E2F1, E2F5, and E2F7 (Supplementary Figure S3A). While E2F3 transcript levels are unaffected, E2F3 protein levels decrease upon Myog expression (Supplementary Figure S3A and B). Interestingly, previous studies have shown that the miRNA miR-20a modulates cell cycle withdrawal through the targeting of E2F transcripts (O’Donnell et al., 2005; Sylvestre et al., 2007; Nagel et al., 2009). This suggested to us that the transcriptional activator Myog could increase the expression of miR-20a, which in turn leads to the down-regulation of E2F proteins. To test the hypothesis that Myog regulates the expression of miR-20a, we first determined whether Myog binds to the promoter of the miR-20a gene. Chromatin immunoprecipitation analysis confirms that Myog binds to the E-box containing (E1) region (Figure 4A) that has been shown to act as the transcriptional regulatory region for miR-20a (O’Donnell et al., 2005; Sylvestre et al., 2007; Nagel et al., 2009). In addition, miR-20a expression strongly correlates with Myog expression. Indeed, miR-20a is up-regulated during muscle differentiation (data not shown), and can be further up-regulated by over-expression of Myog (Figure 4B, Dox vs. Cont). Furthermore,



**Figure 3** Myog expression down-regulates genes involved in cell cycle progression leading to an inhibition of proliferation. **(A)** C2i-Myog cells were treated with Dox in conditions of either proliferation or differentiation as indicated. Total RNA was isolated from cells and RT-qPCR was performed on a subset of Myog-dependent genes. Values are expressed relative to the internal control DDX5 where the condition of maximal expression was normalized to 100,  $n = 3$ . **(B)** C2i-Myog cells were treated with Dox for 24 h in conditions of proliferation and subjected to a 2 h pulse of BrdU prior to fixing for immunofluorescence analysis. Cells were stained for Myog (red), BrdU (green), or DAPI (blue). **(C)** Quantitative analysis of Myog and BrdU co-staining proliferating C2i-Myog cells. Values represent the mean percentage of nuclei ( $\pm$  SEM from 10 different fields) that stain positive for Myog and/or BrdU,  $n = 3$ .

expression of miR-20a, which is repressed in the absence of p38 $\alpha$  signaling (Figure 4B, SB), can be recovered by the expression of exogenous Myog (Figure 4B, SB + Dox). Importantly, we also observe a 2-fold up-regulation of miR-20a expression in proliferating myoblasts that have been induced to express exogenous Myog (data not shown). This demonstrates that miR-20a is a direct transcriptional target of Myog.

To determine the importance of miR-20a expression to Myog-induced cell cycle exit, we used antagomir technology to knock-down the miRNA. Transfection of the miR-20a specific antagomir resulted in a 35% decrease in levels of the miRNA in C2i-Myog cells induced to express Myog (Figure 4C). Importantly, this inhibition of miR-20a function in proliferating myoblasts induced to express Myog resulted in a 45% increase

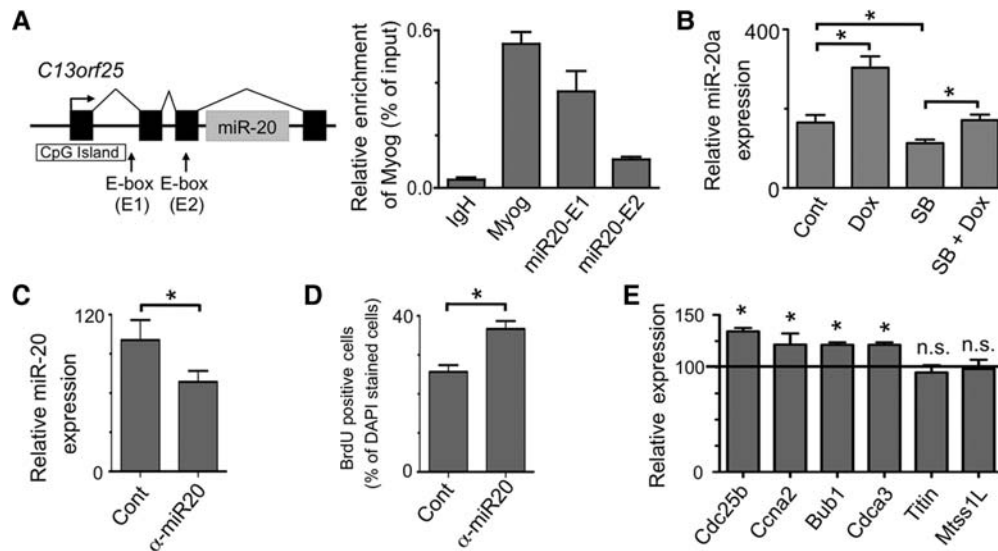
in the number of BrdU-positive cells (Figure 4D). This increase in the number of proliferating myoblasts is matched by a significant increase in the expression of several genes involved in cell cycle regulation (Figure 4E). Thus, one mechanism by which Myog facilitates cell cycle exit is through the up-regulation of miR-20a, a miRNA that is known to target E2F1, E2F2, and E2F3 to block cell cycle progression (O'Donnell et al., 2005; Sylvestre et al., 2007; Nagel et al., 2009).

#### *p38 $\alpha$ -dependent genes are required for myoblast fusion*

Having identified a role for Myog in promoting muscle differentiation and cell cycle exit, we next focused our attention on p38 $\alpha$  signaling in myogenesis. GO analysis of genes up-regulated by p38 $\alpha$  revealed a significant enrichment for factors involved in cell adhesion (Table 1 and Supplementary Table S2). Consistent with this result, we noticed that in the absence of p38 $\alpha$  signaling, myoblasts expressing Myog align efficiently but appear unable to form myotubes, suggesting a defect in cell fusion (Figure 2C, Col 6). To confirm this phenotype, we generated two cell lines expressing fluorescent markers at different cellular locations that allow direct visualization and quantification of cell fusion. The first cell line (C2i-Myog-YFP) expresses YFP exclusively in the cell membrane while the second cell line (C2i-Myog-RFP) expresses RFP only in the cytoplasm. As expected, under normal conditions of differentiation, C2i-Myog-YFP and C2i-Myog-RFP cells (mixed at a ratio of 1:1) fuse efficiently to generate multinucleated myotubes that are marked by both YFP and RFP (Figure 5A, Col 1 & Col 2, B and C). In contrast, while p38 $\alpha$  deficient myoblasts that express Myog can properly align, they remain marked exclusively by RFP or YFP, demonstrating a complete failure of cell fusion (Figure 5A, Col 4, and B). Thus, fusion to form myotubes requires p38 $\alpha$  signaling.

We reasoned that while p38 $\alpha$  could modulate myotube formation through direct phosphorylation of factors involved in cell fusion, p38 $\alpha$  is also known to modulate expression of specific genes through phosphorylation of transcriptional regulators (Simone et al., 2004; Rampalli et al., 2007; Aziz et al., 2010; Seenundun et al., 2010). This led us to examine our list of p38 $\alpha$ -dependent genes to determine whether it contains factors involved in cell fusion. Using Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005), we assessed the overlap between expression changes and genes known to play a role in myoblast fusion (Supplementary Figure S4). This analysis demonstrates that p38 $\alpha$ -dependent (SB treatment vs. control) genes ( $P = 0.00789$ ), but not Myog-dependent (SB + Dox treatment vs. SB treatment) genes ( $P = 0.21253$ ), are enriched for factors involved in cell fusion. These results demonstrate that p38 $\alpha$  signaling modulates the up-regulation of genes previously known to be involved in myoblast fusion.

To identify novel factors involved in myoblast fusion, we further queried our p38 $\alpha$ -dependent gene set. Reasoning that p38 $\alpha$  activity is up-regulated during myogenesis, we narrowed down our list of candidate genes to those whose expression increases during differentiation. Calculating the correlation coefficients between the expression profile of Myog and each of our p38 $\alpha$ -dependent genes across the time points of GSE11415 (Ma et al., 2008) allowed us to identify 112 p38 $\alpha$ -dependent genes whose expression is up-regulated during myogenesis (data not



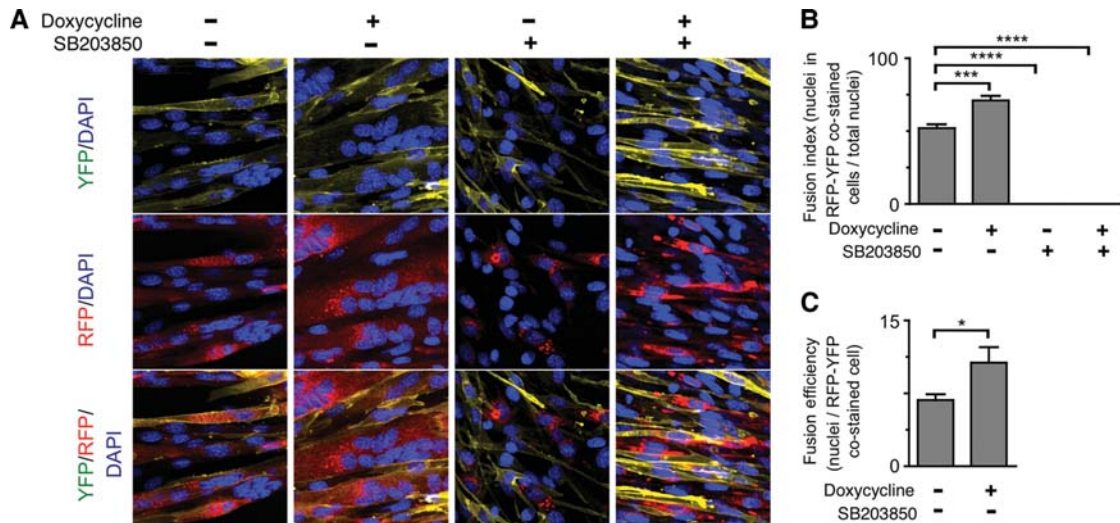
**Figure 4** Myog directly regulates the expression of miR-20a to modulate cell cycle progression. **(A)** Myog is targeted to the promoter of the miR-20a-containing miR19-72 transcript. A schematic representation of the miR-20a genomic locus (transcribed as a part of the miR17-92 cluster from an intron of the C13orf25 gene) is shown in the left panel. The positioning of amplicons E1 and E2 (containing an E-box) are indicated by arrows. ChIP was performed on proliferating C2i-Myog cells that were induced to express Myog by treatment with Dox. Chromatin immunoprecipitated with an anti-Myog antibody (Santa Cruz) was deproteinized, and analyzed by qPCR. The IgH enhancer (IgH) was used as a negative control, while the myogenin promoter (Myog) served as a positive control. The regulatory region of the miR19-72 cluster was then analyzed using primers that amplified E-box containing regions (miR20-E1 and miR20-E2) as previously defined (O'Donnell et al., 2005). **(B)** C2i-Myog cells were treated with (or absence) of Dox and SB under differentiation (48 h) conditions. Total RNA was then extracted and subjected to RT-qPCR analysis using primers specific for miR-20a or U6snoRNA (control). Values are normalized to the internal control U6snoRNA and expressed relative to the amount of miR-20a observed in proliferating C2i-Myog cells  $\pm$  SEM.  $*P < 0.05$ ,  $n = 3$ . **(C)** C2i-Myog cells were transfected with an antagomir specifically targeting miR-20a (or control sequence). Cells were treated with Dox for 24 h in proliferative conditions. Total RNA was then extracted and subjected to RT-qPCR as described in **B**. **(D)** Quantitative analysis of BrdU staining in antagomir-treated C2i-Myog cells treated with doxycycline in proliferative conditions. Values represent the mean percentage of nuclei ( $\pm$ SEM from 10 different fields) that stain positive for BrdU.  $*P < 0.05$ ,  $n = 3$ . **(E)** Expression analysis of Myog target genes in Dox-treated proliferating C2i-Myog cells after treatment with miR-20a targeting antagomir. Total RNA was isolated from cells and RT-qPCR was performed on a subset of Myog-dependent genes. Values are expressed relative to the internal control DDX5 where the condition of maximal expression was normalized to 100.  $*P < 0.05$ ,  $n = 3$ .

shown). This subset of p38 $\alpha$ -dependent genes was queried in the protein–protein interactions STRING database (Szklarczyk et al., 2011) to identify candidate proteins involved in fusion based on their ability to interact with proteins that have previously been shown to mediate myoblast fusion—guilt by association. Using this approach, we identified the tetraspanin CD53 as a potential fusion protein whose expression is regulated by p38 $\alpha$  signaling during myotube formation (Supplementary Figure S5).

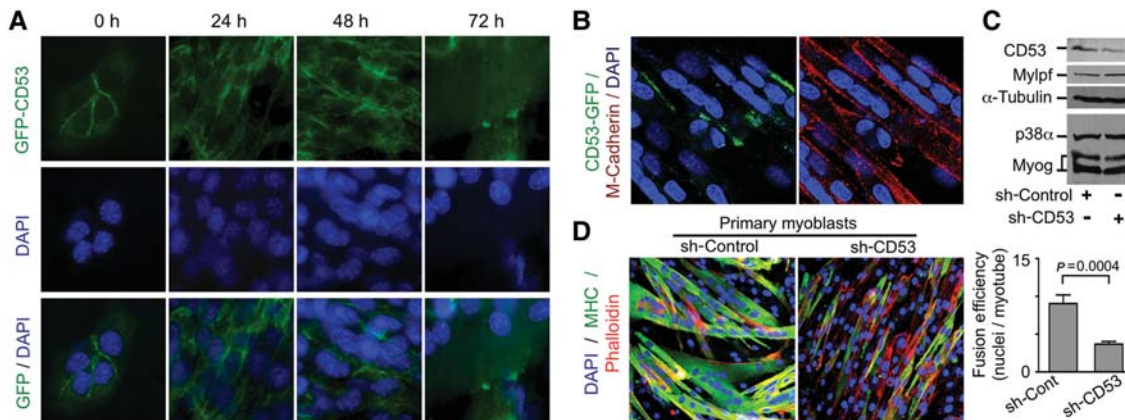
The potential of CD53 as a mediator of myoblast fusion was first evaluated by examining its cellular localization by microscopy. Interestingly, CD53 concentrates in localized microdomains of the plasma membrane (Figure 6A). This contrasts with the localization of other membrane proteins such as M-Cadherin (Figure 6B) and  $\beta$ 1-integrin (data not shown) that displayed a uniform coating of the myotube membrane. CD53 enrichment in the contact sites between cells was first seen in unfused C2C12 myoblasts at the beginning of the differentiation ( $t = 0$ ) and persisted in partially fused myotubes ( $t = 24$  and 48 h). By the end of the differentiation ( $t = 72$  h), CD53 was mostly concentrated in a few contact sites along the plasma membrane of now highly fused myotubes, perhaps reflecting nearing the end of the fusion

process and exhaustion of fusogenic cells. This temporal and spatial localization of CD53 is consistent with a role in myoblast fusion. The involvement of CD53 is also supported by actual fusion events in live cells, where differentiating myocytes were observed to fuse at CD53-enriched contact sites (Supplementary Movie).

To directly test the role of CD53 in cell fusion, we used lentiviral-based shRNA to knock-down CD53 expression in C2C12 cells (Supplementary Figure S6A) and in primary mouse myoblasts (Figure 6C and D). Two shRNAs (that target different sequences in the mRNA) were used, each providing a decrease of  $\sim 70\%$  in CD53 protein levels (Figure 6C and data not shown). We found that knocking-down CD53 strongly impairs cell fusion in both primary myoblasts and C2C12 cells (Figure 6D and Supplementary Figure S6A). This defect in myoblast fusion is quantitated by the 2–3-fold decrease in the percentage of cells that are incorporated into multinucleated myoblasts (Supplementary Figure S7A). At the same time, the limited number of cells that did form myotubes showed a 3–4-fold lower number of fusion events per myotube (Figure 6D). To determine whether CD53 plays a direct role in myoblasts fusion, we



**Figure 5** The p38 $\alpha$  signaling pathway is required to mediate formation of multinucleated myotubes. **(A)** C2i-Myog cells were stably transfected with a cDNA encoding either mCherry (C2i-Myog-RFP) or the membrane targeted YFP-GL-GPI (C2i-Myog-YFP). C2i-Myog-RFP and C2i-Myog-YFP cells were then mixed and plated in culture at a ratio of 1:1. Transfected cells were then differentiated in the presence or absence of Dox and/or SB as indicated. **(B and C)** Quantitative analysis of myoblast fusion after treatment of cells in the presence (or absence) of Dox and/or SB. **(B)** Values for fusion index represent the mean percentage of total nuclei that are in myotubes (RFP-YFP co-stained cells)  $\pm$  SEM from 10 different fields. \*\*\*\* $P < 0.0001$ , \*\*\* $P = 0.0002$ ,  $n = 3$ . **(C)** Values for fusion efficiency represent the mean number of nuclei per myotubes (RFP-YFP co-stained cells)  $\pm$  SEM from 10 different fields (\* $P = 0.035$ ), for the two cell populations in which cell fusion occurs,  $n = 3$ .



**Figure 6** The tetraspanin protein CD53 is required for myoblast fusion *in vitro*. **(A and B)** CD53 localizes to cell–cell junctions. **(A)** Immunofluorescence analysis was performed to examine CD53 (GFP) localization in differentiating myoblasts. C2C12 cells were transiently transfected with the plasmid expressing CD53-GFP, and induced to differentiate for between 0 and 72 h. Cells were stained for total DNA (DAPI). **(B)** Confocal microscopy shows that while M-Cadherin (red) spread evenly throughout the cell membrane, CD53 (green) localizes to points of cell–cell contact. **(C and D)** Knock-down of CD53 blocks myoblast fusion. Primary mouse myoblast were infected with lentivirus expressing shRNA targeting CD53 (sh-CD53) or a non-targeted control (sh-Control) and induced to differentiate for 48 h. **(C)** Knock-down of CD53 does not cause a general block in muscle differentiation. Total protein was extracted from shRNA treated cells, and subjected to western blot analysis using antibodies indicated. **(D)** Immunofluorescence analysis was performed to examine the effect of CD53 knock-down on myoblast fusion. Cells were stained for MHC and total DNA (DAPI). Fusion efficiency (number of nuclei per myotube) was calculated from 10 fields of view under each condition. Values are presented as the mean  $\pm$  SEM,  $n = 6$ .

overexpressed CD53 in C2C12 cells. As expected for a direct facilitator of cell fusion, overexpression of CD53 in differentiating cells results in a highly significant increase in myoblast fusion events (Supplementary Figure S6B). Taken together these results validate CD53 as an important factor in promoting myotube formation *in vitro* and *ex vivo*.

While it is likely that CD53 directly modulates cell fusion, a lack of myoblast formation could also be caused by incomplete differentiation, or impaired cell adhesion (Junion et al., 2007). To determine whether CD53 knock-down affects muscle differentiation, we first examined the accumulation of muscle-specific proteins Myog and Mylpf in primary myoblasts. We found no difference

in the accumulation of these proteins upon CD53 knock-down suggesting that myogenesis is proceeding in the absence of this tetraspanin (Figure 6C). To further demonstrate that CD53 knock-down does not cause a block in myogenesis, gene expression arrays confirm that loss of the CD53 does not lead to a global change in gene expression during myoblast differentiation (Supplementary Figure S7B). Finally, RT-qPCR experiments demonstrate that CD53 knock-down does not affect the expression of several muscle differentiation markers including Myh3, Tnnt2, Desmin, SCGA, Cdh15, Myog, Ccna2, Ccnb1, or NCAM1 (data not shown). Together, these results show that loss of CD53 is not causing a general block in myogenesis. To determine the effect of CD53 knock-down on cell adhesion, we measured the ability of myoblasts to attach and spread-out on a matrix of differentiated C2C12 myotubes. We observed that CD53 knock-down has no significant effect on the ability of myoblasts to adhere to pre-plated myotubes (Supplementary Figure S7C and data not shown). Thus, the loss of CD53 in differentiating myoblasts does not impede the ability of these cells to adhere to adjacent myotubes. Therefore, loss of CD53 does not block cell differentiation or impair cell adhesion, which further supports the role of CD53 in promoting myoblast formation at the level of cell fusion.

Having demonstrated a role for CD53 in myoblast fusion *in vitro* and *ex vivo*, we next examined whether diminished levels of the tetraspanin protein would impede regeneration *in vivo* in mice subjected to cardiotoxin-induced muscle damage. Using lentivirus to infect the regenerating tibialis anterior (TA) muscle 48 h after cardiotoxin treatment, we achieved a 69% decrease in the expression of CD53 compared with control muscle (Supplementary Figure S7D). Analyzing the cross-sectional area of the newly formed fibers in the regenerating muscle, we found that decreased CD53 levels result in the formation of significantly smaller centrally nucleated fibers after 7 days (mean cross-sectional area of 181.2  $\mu\text{m}$  for sh-CD53 vs. 347.9  $\mu\text{m}$  for sh-Control;  $P$ -value =  $2.2\text{e}-16$ ) accompanied by a marked increase in the number of interstitial cells (Figure 7A). Importantly, after 21 days of regeneration, muscle fibers regenerated under conditions of reduced CD53 levels remain significantly smaller than the control (mean cross-sectional area of 268.5  $\mu\text{m}$  for sh-CD53 vs. 600.7  $\mu\text{m}$  for sh-Control;  $P$ -value =  $1.2\text{e}-13$ , Figure 7A). These results strongly suggest that the loss of CD53 in myogenic progenitors prevents these cells from fusing into the regenerating muscle, which ultimately result in smaller caliber fibers. If the observed small fiber phenotype is caused by a defect in cell fusion, one would expect to observe an increase in myogenic cells within the mononuclear interstitial cell population. As shown in Figure 7B, a significantly increased number of myogenic (MyoD<sup>+</sup>) cells is present in the interstitial space between myofibers of CD53 knock-down muscle compared with the control, confirming that CD53 is required for efficient formation of myofibers in regenerating muscle. In conclusion, experiments performed in defined environments (both *in vitro* using the C2C12 cell line and *ex vivo* using primary myoblasts) combined with studies carried out *in vivo* in the complex environment of muscle regeneration in mice demonstrate that CD53 is required for myoblast fusion.

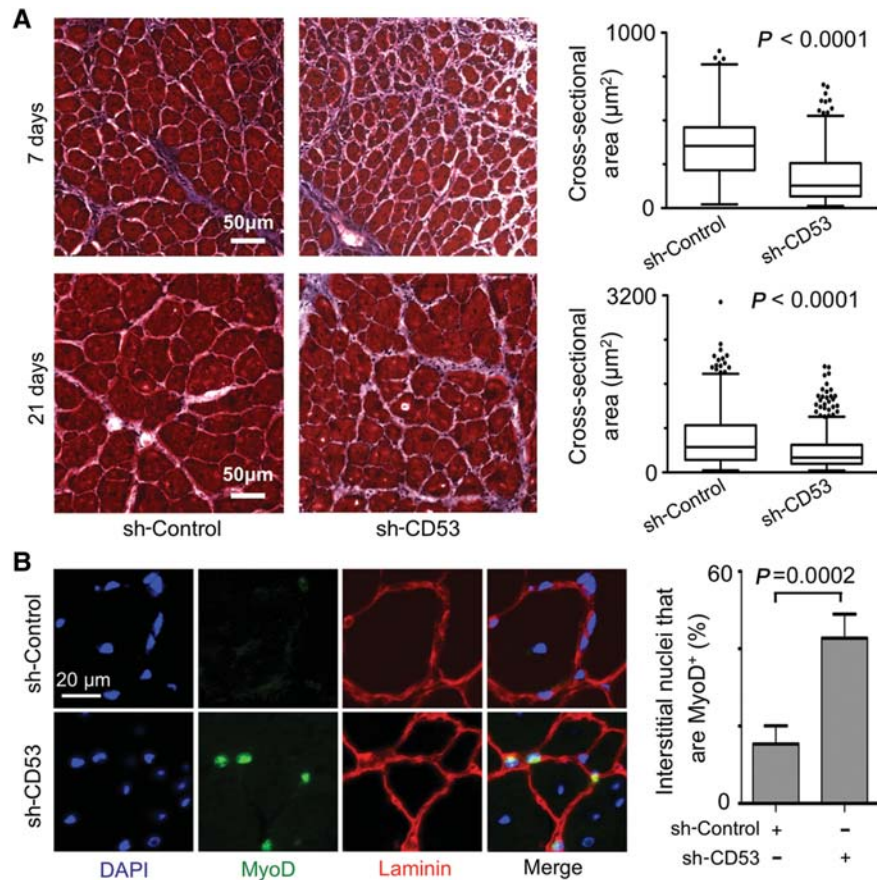
## Discussion

The importance of both Myog (Hasty et al., 1993; Nabeshima et al., 1993) and p38 $\alpha$  signaling (Zetser et al., 1999; Perdiguero et al., 2007) to the myogenic process has long been recognized. However, the individual contribution(s) of Myog and p38 $\alpha$  to the myogenic process has not been addressed. Here, we show that the transcriptional activator Myog up-regulates a subset of genes involved in differentiation and muscle cell function. In addition, Myog mediates cell cycle exit during terminal differentiation by directing the repression of an important subset of genes involved in cell proliferation. Interestingly, inhibition of cell cycle progression by Myog is mediated, at least in part, through the up-regulation of a specific microRNA, miR-20a. Thus, while genes involved in cell cycle exit and muscle function are p38 $\alpha$ -dependent (Cabane et al., 2003; Perdiguero et al., 2007), our results show that this function is mediated for a large part through the ability of p38 $\alpha$  to direct the proper developmental expression of Myog. In addition, we show that p38 $\alpha$  up-regulates genes involved in cell adhesion, extracellular structural organization, and cell fusion in a Myog-independent manner. Interestingly, study of the mechanism through which p38 $\alpha$  promotes cell fusion allowed us to identify a novel component of the myoblast fusion machinery, the tetraspanin CD53, which localizes to points of cell–cell contact to facilitate the formation of syncytial myotubes.

By uncoupling the functional dependence of Myog on p38 $\alpha$  signaling, we found that the ability of p38 $\alpha$  to modulate cell cycle exit depends on its ability to modulate Myog gene expression. Indeed, expression of Myog is sufficient to induce cell cycle exit through a down-regulation of genes involved in cell cycle progression. This down-regulation of genes involved in cell cycle progression does not occur through a direct repressive mechanism, but is instead mediated by the up-regulation of genes which act to inhibit cell cycle progression. Indeed, we show that Myog directly regulates the expression of miR-20a, a miRNA that is well characterized for its ability to reduce accumulation of E2F1 and E2F3 through the targeting of the 3'UTR of their mRNAs (O'Donnell et al., 2005; Sylvestre et al., 2007; Nagel et al., 2009). Interestingly, Myog expression also up-regulates the expression of LATS2 (data not shown, see Supplementary Table S2), a kinase that was recently implicated in targeting of the transcriptional repressor complex DREAM to E2F target genes to block cell cycle progression (Tschop et al., 2011). This suggests that Myog blocks proliferation through the activation of several factors that target E2F transcriptional activity to genes that modulate cell cycle progression. As previously observed (Andres and Walsh, 1996), our study demonstrates that a small number of Myog-expressing cells can complete a round of cell cycle. The appearance of these rare Myog/BrdU double-positive cells likely reflects our finding that Myog does not directly repress transcription of genes that drive cell cycle progression, but instead sets in motion the expression of factors that in turn mediate the repression of specific genes to induce cell cycle exit. Thus, we propose that the expression of Myog constitutes a 'point of no return' where the transcriptional factor initiates a gene expression program that commits the differentiating myoblast to exit cell cycle.

Previous studies have suggested that Myog can substitute for MyoD and Myf5 in the specification of skeletal muscle (Wang





**Figure 7** Knock-down of CD53 in regenerating TA muscle results in reduced muscle fiber size. **(A)** Cardiotoxin-damaged mouse TA muscle was infected with lentivirus expressing shRNA targeting CD53 (sh-CD53) or a non-targeted control (sh-Control) and allowed to regenerate for 7 or 21 days. Cross-sections were stained using Masson's Trichrome, and the cross-sectional area of the individual fibers was determined. Statistical analysis was performed on 600 fibers from each condition using a non-parametric (Wilcoxon) test. Values are presented as the median fiber area  $\pm$  SD,  $n = 6$ . See Supplementary Materials and methods for further details. **(B)** Knock-down of CD53 causes an accumulation of MyoD<sup>+</sup> myoblasts at the regenerating front of the damaged TA muscle. The border of the regenerating TA muscle (day 21) was analyzed by immunohistochemistry using antibodies recognizing MyoD, Laminin or total DNA (DAPI). MyoD positive nuclei were quantitated as a percentage of all DAPI staining nuclei in the interstitial space. Value represents the mean  $\pm$  SEM from 10 different fields,  $n = 3$ .

and Jaenisch, 1997). Indeed, ectopic expression of Myog from the Myf5 locus allows for the formation of healthy muscle fibers in MyoD/Myf5 double knockout mice (Wang and Jaenisch, 1997). However, it should be noted that while healthy muscle fibers are formed in these mice, their number is greatly reduced leading to postnatal death from insufficient musculature (Wang and Jaenisch, 1997). Thus, these studies suggest that Myog also has non-redundant functions with MyoD and Myf5. Our finding that Myog can block cell cycle progression in proliferating myoblasts is particularly interesting in this context since it represents a major functional difference between Myog and its related family members MyoD and Myf5. Indeed, MyoD and Myf5 play an opposing role of promoting myoblast proliferation (Ustanina et al., 2007; Zhang et al., 2010). As such, our results predict that precocious expression of Myog from the Myf5 locus would induce cell cycle exit and prevent expansion of muscle progenitors, leading to a greatly reduced population available to form myofibers. Therefore, the role of Myog we have discovered in blocking cell cycle progression is fully supported by the Myog knock-in mouse phenotype (Wang and Jaenisch, 1997), and

provides the first mechanistic explanation for the reduced musculature observed in these animals.

While Myog mediates the effects of p38 $\alpha$  on cell cycle exit, we have identified myoblast fusion as a process that is regulated by p38 $\alpha$  in a Myog-independent manner. Among the p38 $\alpha$ -dependent genes revealed in our comparative expression analysis, we have identified the tetraspanin protein CD53 as a membrane associated protein that is required for the formation of multinucleated myotubes. The importance of tetraspanin proteins in fusion of various syncytial cell-types has previously been suggested by genetic experiments. CD9 and CD81 (which belong to the same four-cysteine containing subfamily of tetraspanins as CD53) play an important role in oocyte-sperm fusion (Takeda et al., 2003). Furthermore, studies in C2C12 cells have shown that antibodies that block CD81 and CD9 function induce a delay in myoblast fusion (Tachibana and Hemler, 1999). We note that the role of the tetraspanin proteins CD9 and CD81 in myoblast fusion has been debated due to the fact that CD9/CD81 double null mice do not display a muscle-phenotype (Takeda et al., 2003). However, the formation of healthy muscle

in mice that lack CD9 and CD81 could be explained by compensatory effects due to the expression of other members of the tetraspanin family. To minimize compensatory effects in our study, we have used acute knock-down using shRNA, and these experiments unequivocally demonstrate a role for CD53 in myoblast fusion. Furthermore, we have shown that CD53 is necessary for efficient myoblast fusion using both the defined environment of cultured myoblasts and the complex environment of *in vivo* muscle regeneration. Therefore, our results clearly establish the tetraspanin protein CD53 as a novel component of the myoblast fusion machinery.

Functionally, the tetraspanin family of integral membrane proteins is known to self-associate, forming a ‘tetraspanin web’ that recruits additional interacting proteins at the cell surface (Charrin et al., 2009; Jegou et al., 2011). In the case of CD9, it was shown that the tetraspanin was not required for sperm–egg adhesion, but instead mediates the formation of fusion competent microdomains on the oocyte membrane that permit fertilization (Jegou et al., 2011). Our observation that CD53 displays a localization that is restricted to sites of cell–cell contact suggests that this related tetraspanin acts in a similar manner to establish microdomains within the myoblast membrane. Interestingly, studies in macrophages (another fusion competent cell type) showed that CD53 co-localizes with CD9 and CD81 in microdomains that are exploited by human immunodeficiency virus to generate membrane buds (Deneka et al., 2007). Based on these observations, we speculate that CD53 (in conjunction with additional tetraspanins) may be functioning to establish microdomains that regulate the complement of fusion proteins present at the cell–cell interface in differentiating myoblasts. Proteins known to interact with members of the tetraspanin family include several factors implicated in cell fusion (Charrin et al., 2009) such as integrins, adhesion molecules, metalloproteinases, as well as intracellular signaling molecules (activated protein kinase C alpha). The identification of proteins in the myoblast-specific CD53 tetraspanin web will be the focus of future studies.

In summary, we have developed a highly useful cell model system for skeletal myogenesis that permits the examination of steps in differentiation program beyond the loss of the critical myogenic transcription factor Myog. Exploitation of this system through the use of comparative expression profiling allowed us to identify genes that constitute the Myog- and p38 $\alpha$ -dependent muscle transcriptome. Analysis of these genes identified a role for Myog in mediating the p38 $\alpha$ -dependent exit of the cell cycle, while also identifying a Myog-independent role for p38 $\alpha$  signaling in mediating myoblast fusion. Furthermore, this system has permitted us to identify CD53 as a novel mediator of myoblast fusion.

## Materials and methods

### Cell culture

C2C12 cells (Yaffe and Saxel, 1977), and all derivative stable cell lines (see below), were maintained in proliferative conditions at a cell density of <70% confluency in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS). Near confluence, myoblasts were induced to differentiate through serum withdrawal using DMEM containing 1% horse serum, insulin (10  $\mu$ g/ml), and transferrin (10  $\mu$ g/ml) for 48 h. Where

indicated, expression of exogenous Flag-tagged mouse Myog was induced at the time of differentiation through the addition of 0.2  $\mu$ g/ml Dox (Sigma). Importantly, the use of Dox at this concentration did not significantly affect C2C12 differentiation (data not shown). Inhibition of p38 $\alpha$  signaling was established through the addition of 10  $\mu$ M SB (Calbiochem) at the time of differentiation and maintained for 48 h. Cells were incubated in various conditions including: Dox-treated (to control for effects of Dox—but also for potential effects from increased Myog expression); SB-treated (blocks p38 $\alpha$  signaling); and SB- and Dox-treated (blocks p38 $\alpha$  signaling while allowing expression of exogenous Flag-Myog). Pulse labeling of proliferating cells was performed by incubating cells for 2 h in the presence of BrdU.

Primary myoblasts were isolated as previously described (Joe et al., 2010). Briefly, hindlimb muscles were isolated from 6-week-old CD1 mice (Charles River). The tissue was then disrupted with forceps, and treated with collagenase B (1.5 U/ml) and dispase II (2.4 U/ml). Mononucleated cells were isolated from the homogenate by passing the population through a 40  $\mu$ m cell strainer. Primary myoblasts were then isolated as an integrin  $\alpha$ 7<sup>+</sup>/CD34<sup>+</sup> population by flow cytometry. Primary mouse myoblasts were grown in Ham’s F10 medium containing 20% FCS, and bFGF (2.5 ng/ml).

### Generation of stably transfected cell lines

The Tet-Repressor-expressing cell line C2i was generated by electroporation of the plasmid pPyCAGIP-TetR into C2C12 cells, and selection with puromycin until individual clones formed. Individual clones were screened by immunofluorescence to ensure they retained the potential to differentiate (express Myog and Myh3 while forming multinucleated myotubes) upon serum withdrawal. C2i-Myog cells, which express a doxycyclin-inducible cDNA encoding Flag-tagged mouse Myog, were generated by electroporating the plasmid pCDNA5/TO-FL-Myog into C2i cells, and selecting for hygromycin resistance until individual clones formed. Individual clones were then screened for Dox-inducible Myog expression as well as differentiation, as described above. C2i-Myog-YFP and C2i-Myog-RFP cells were generated by electroporating the plasmids pSNAP-GPI-GL-YFP or pSNAP-mCherry, respectively, into C2i-Myog cells, and selecting with G418 until individual clones formed. Again, clones were screened for cells that displayed proper localization of fluorescent proteins, and an ability to differentiate as described above.

### Fluorescent localization of CD53

Localization of CD53 in differentiating C2C12 cells was performed using the Split-GFP system (Cabantous and Waldo, 2006). Full-length mouse CD53 cDNA was PCR amplified and cloned into the plasmid pCM-mGFP-Cterm (Theranostech Inc.) that had been previously digested with *Xho*I and *Bam*HI. The cDNA coding for the resulting fusion protein (full-length CD53 with a tag of 16 a.a. from the C-terminus of GFP) was then amplified and cloned into the plasmid pPyCAGIH to generate pPyCAGIH-CD53\_GFP11. The cDNA encoding N-terminal portion of GFP (GFP1-10) was also amplified by PCR, and cloned into the plasmid pPyCAGIP.

A stable GFP1-10 expressing myoblast cell line (C2-GFP1-10) was generated by electroporation using the plasmid pPyCAGIP-GFP1-10. C2-GFP1-10 cells were then transiently transfected

with pPyCAGIH-CD53\_GFP11 using lipofectamine, and allowed to differentiate for 48 h.

#### Microarrays

Total RNA isolated from C2i-Myog cells treated under various conditions was labeled and hybridized to the Affymetrix GeneChip Mouse Gene 1.0 ST gene expression microarrays using standard conditions. Intensity values from biological triplicates were processed by variance stabilization and normalization (Huber et al., 2002) and summarized by RMA (Irizarry et al., 2003). Expression profiles of C2i-Myog cells treated with Dox, SB, or both were contrasted to vehicle treated cells or each other. Differentially expressed transcripts in each contrast with an adjusted  $P$ -value  $\leq 0.05$  where determined using limma (Smyth, 2004). GO analyses were performed with the R package GStats (Falcon and Gentleman, 2007). GSEA analyses were performed using the default parameters (Subramanian et al., 2005). Genes determined to be p38 $\alpha$ - or Myog-dependent were examined for their correlative expression compared with Myog in differentiating C2C12 cells (Ma et al., 2008). Microarray data are available at GEO under record number GSE25763.

#### Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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