

# Genetic analysis of p38 MAP kinases in myogenesis: fundamental role of p38 $\alpha$ in abrogating myoblast proliferation

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The p38 mitogen-activated protein kinase (MAPK) pathway plays a critical role in skeletal muscle differentiation. However, the relative contribution of the four p38 MAPKs (p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$  and p38 $\delta$ ) to this process is unknown. Here we show that myoblasts lacking p38 $\alpha$ , but not those lacking p38 $\beta$  or p38 $\delta$ , are unable to differentiate and form multinucleated myotubes, whereas p38 $\gamma$ -deficient myoblasts exhibit an attenuated fusion capacity. The defective myogenesis in the absence of p38 $\alpha$  is caused by delayed cell-cycle exit and continuous proliferation in differentiation-promoting conditions. Indeed, activation of JNK/cJun was enhanced in p38 $\alpha$ -deficient myoblasts leading to increased cyclin D1 transcription, whereas inhibition of JNK activity rescued the proliferation phenotype. Thus, p38 $\alpha$  controls myogenesis by antagonizing the activation of the JNK proliferation-promoting pathway, before its direct effect on muscle differentiation-specific gene transcription. More importantly, in agreement with the defective myogenesis of cultured p38 $\alpha^{\Delta/\Delta}$  myoblasts, neonatal muscle deficient in p38 $\alpha$  shows cellular hyperproliferation and delayed maturation. This study provides novel evidence of a fundamental role of p38 $\alpha$  in muscle formation *in vitro* and *in vivo*.

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## Introduction

Regulation of skeletal muscle formation (myogenesis) is essential for normal development as well as in pathological conditions such as muscular dystrophies and inflammatory myopathies in which prominent muscle loss and regeneration take place. Myogenesis is a dynamic process in which mononucleated undifferentiated myoblasts first proliferate, then withdraw from the cell cycle, and finally differentiate and fuse to form the multinucleated mature muscle fibers in the animal. This process is controlled by the MyoD family of muscle-specific basic helix–loop–helix proteins, known as muscle regulatory factors (MRFs), which in concert with members of the ubiquitous E2A and myocyte enhancer factor-2 (MEF2) families, activate the differentiation program by inducing transcription of regulatory and structural muscle-specific genes (Sartorelli and Caretti, 2005; Tapscott, 2005). The association of the myogenic effector transcription factors to E boxes on muscle loci and also their transcriptional activities are controlled by intracellular signaling pathways in response to yet to be identified extracellular cues. A signaling pathway that plays a fundamental role in myogenesis involves p38 mitogen-activated protein kinase (MAPK) (Keren *et al*, 2006; Lluís *et al*, 2006). p38 kinase activity increases over the course of differentiation and is required for full myoblast differentiation and fusion. In mammals, there are four p38 MAPKs, p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$  and p38 $\delta$ , which are phosphorylated and activated by MAPK kinases MKK6/3 (Nebreda and Porras, 2000). Once activated, p38 MAPKs phosphorylate serine/threonine residues of their substrates, which include transcription factors as well as protein kinases. Functional analysis of p38 $\alpha$  and p38 $\beta$  MAPKs in different cellular processes, including myogenesis, has been facilitated by the availability of pyridinyl imidazole compounds, such as SB203580, which inhibit both p38 isoforms. Indeed, treatment with SB203580 prevents the fusion of immortalized myoblasts into myotubes as well as the induction of muscle-specific genes, demonstrating the requirement of p38 $\alpha/\beta$  in myogenesis (Cuenda and Cohen, 1999; Zetser *et al*, 1999; Li *et al*, 2000; Wu *et al*, 2000). The specific mechanisms by which p38 $\alpha/\beta$  impinges upon the muscle regulatory pathway have been described in recent papers. p38 $\alpha/\beta$  augments the transcriptional activity of MEF2A and MEF2C by direct phosphorylation, promotes MyoD/E-protein heterodimerization and targets chromatin-remodeling enzymes to muscle-specific loci (Zetser *et al*, 1999; Zhao *et al*, 1999; Wu *et al*, 2000; Simone *et al*, 2004; Lluís *et al*, 2005), thereby inducing transcription of muscle-specific genes. p38 $\alpha/\beta$  can also increase the stability of critical muscle-specific transcripts (Briata *et al*, 2005). Recent *in vivo* studies with SB203580 have further demonstrated that p38 signaling is a crucial determinant of myogenic differentiation during early embryonic myotome development in mouse and *Xenopus* (de Angelis *et al*, 2005; Keren *et al*, 2005). Because of the lack of p38 $\gamma$  and

p38 $\delta$  pharmacological inhibitors, the involvement of these kinases in myogenesis remains unclear.

Taken together, the p38 signaling pathway appears to control myoblast differentiation both *in vitro* and in embryonic models; however, the specific impact and relative contribution of the individual p38 family members to myogenesis remains unsolved. We have addressed this question through a genetic approach, by using primary myoblasts derived from skeletal muscle of neonatal mice deficient in p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$  and p38 $\delta$ , as well as by analyzing the phenotype of neonatal muscle. Our findings have allowed us to characterize for the first time the specific role of each p38 MAPK in skeletal myogenesis. From these studies, p38 $\alpha$  emerges as the critical p38 MAPK in this process.

## Results

### **Expression pattern of p38 MAPKs in primary myoblasts**

Myoblasts proliferate in culture as undifferentiated cells in growth medium (GM) characterized by high serum content; upon confluence and serum withdrawal (differentiation medium, DM), myoblasts differentiate into myocytes, which subsequently begin to fuse into multinucleated myotubes. We first aimed to analyze the expression and activity of p38 MAPKs in primary myoblasts. p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  transcripts and corresponding proteins were expressed both in GM and DM, as demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting analyses, respectively (Figure 1A and B) (antibody specificity is shown in Supplementary Figure 1). p38 $\alpha$  and p38 $\gamma$  were the most abundant isoforms, p38 $\gamma$  being upregulated during differentiation. C2C12-immortalized myoblastic cells were found to express p38 $\alpha$ , p38 $\beta$  and p38 $\gamma$ , but not p38 $\delta$ , mRNA (Figure 1A). Thus, primary myoblasts constitute a more complete myogenic model than C2C12 cells for studying the relative contribution of p38 kinases to myogenesis. p38 phosphorylation was low in non-confluent primary myoblasts in GM, being induced in nearly confluent cells in GM (this time point is referred to as DM 0 h; i.e., the time of transfer of almost confluent myoblasts from GM to DM), and continued to be elevated in DM (Figure 1C, top). As the single band detected by the anti-phospho-p38-antibody could represent the activated form of all four p38 kinases, analysis of isoform-specific p38 activities became pertinent. p38 $\alpha$  and p38 $\gamma$  kinase activities were induced in differentiating compared to proliferating non-confluent myoblasts (Figure 1D). However, we could not determine the activity of the p38 $\beta$  and p38 $\delta$  isoforms due to the inability of the corresponding antibodies to work in immunoprecipitation assays. At variance with the results in primary myoblasts, p38 phosphorylation in C2C12 cells was detected only after 12 h in DM (Figure 1C, bottom), indicating an advancement in the kinetics of p38 activation in primary myoblasts. Similarly, the expression of myogenin (a marker of early differentiation) was advanced in primary myoblasts compared to C2C12 cells (Figure 1E; compare DM 0 and 12 h), suggesting a correlation between the early activation of p38 and the precocious induction of muscle differentiation-specific genes in primary cells in high serum proliferating conditions. In agreement with this, the expression of late differentiation markers (muscle creatine kinase (MCK) and MRF4) was also

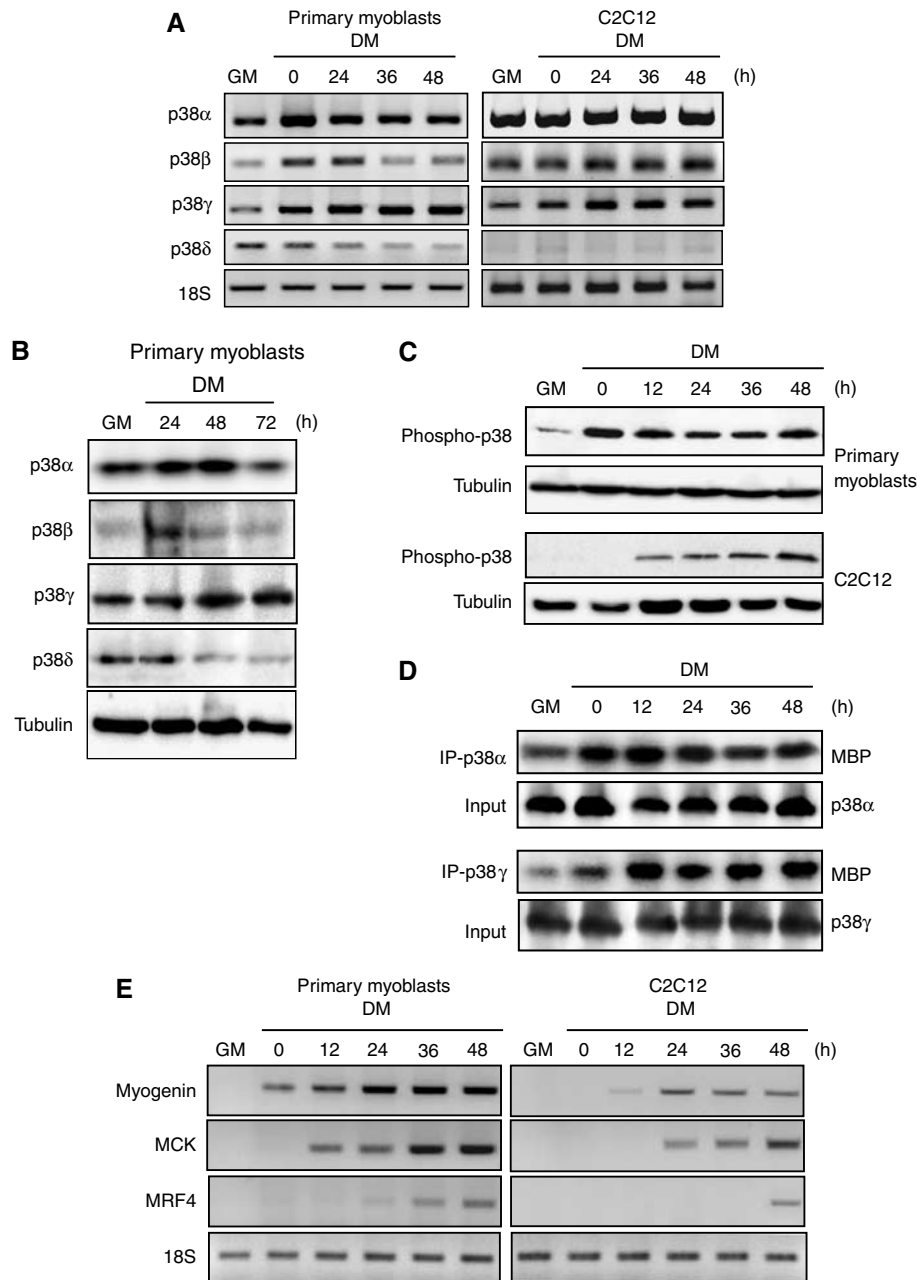
advanced in primary versus C2C12 differentiating myocytes (Figure 1E).

### **Consequences of absence of p38 MAPKs in myoblast differentiation**

To directly evaluate the contribution of p38 MAPKs (p38s) to muscle differentiation, we analyzed comparatively the expression of muscle differentiation gene products in p38s-deficient myoblast cultures (p38 $\alpha^{\Delta/\Delta}$ , p38 $\beta^{\Delta/\Delta}$ , p38 $\gamma^{\Delta/\Delta}$  and p38 $\delta^{\Delta/\Delta}$ ) and corresponding wild-type (WT) cells by quantitative RT-PCR (qRT-PCR), at different intervals in GM and DM. Absence of expression of each p38 isoform in the corresponding p38-deficient myoblasts was confirmed previously (Supplementary Figure 1B). A similar temporal expression pattern of differentiation markers was observed in WT myoblasts as in those deficient in p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  (Supplementary Figure 2A). By contrast, delayed kinetics and reduced expression of myogenin, MCK and myosin heavy chain (MHC-2X) were exhibited by p38 $\alpha$ -deficient myogenic cells (Figure 2A). Notably, the deficient myogenic differentiation of p38 $\alpha^{\Delta/\Delta}$  myoblasts was rescued by retroviral delivery of p38 $\alpha$  (Supplementary Figure 3A). These results support the conclusion that early and late muscle-specific gene expression is mediated not by all p38 MAPKs, but exclusively by p38 $\alpha$ .

### **Recruitment of the transcriptional machinery to muscle loci is reduced in p38 $\alpha$ -deficient myoblasts**

Recent reports have shown that the activity and engagement of MyoD/E47 and MEF2 transcription factors and chromatin-associated enzymes such as Brg1 and RNA polymerase II (Pol II) on muscle promoters can be regulated by SB203580 treatment and/or MKK6 overexpression in myogenic cell lines (Penn *et al*, 2004; Simone *et al*, 2004; Lluís *et al*, 2005). On the basis of the results shown so far in this study, we hypothesized that the p38 $\alpha$  isoform would mediate the recruitment of these chromatin-associated activities to muscle genes in primary myoblasts. Chromatin immunoprecipitation (ChIP) assays demonstrated that MyoD and MEF2 were specifically associated with the promoter regions of myogenin and MCK genes in WT cells (Figure 2B). Of note, binding of these myogenic transcription factors to the myogenin promoter was detected already in GM (DM 0 h) (Figure 2B, left), in agreement with the precocious expression of myogenin transcripts in primary myoblasts in high serum-rich medium (Figures 1E and 2A). However, the recruitment of these factors to the myogenin and MCK gene promoters was compromised in p38 $\alpha^{\Delta/\Delta}$  myoblasts. Furthermore, deficiency in p38 $\alpha$  also prevented the engagement of RNA Pol II on both muscle loci (Figure 2B). To directly demonstrate functional consequences of p38 $\alpha$  deficiency on muscle-specific transcription, promoter-reporter analyses using Myogenin-Luc, MCK-Luc and p4RE-tk-Luc (containing four multimerized E boxes) plasmids were performed. Luciferase activities from all three promoters were lower in p38 $\alpha^{\Delta/\Delta}$  myoblasts than in WT cells (Figure 2C); more importantly, these activities could be rescued by ectopic delivery of p38 $\alpha$ , confirming that the absence of p38 $\alpha$  is responsible for the transcriptional defect. These results extended to primary myoblasts the previously reported effect of pharmacological inhibition of p38 $\alpha/\beta$  on early and late muscle-specific gene transcription in myoblast cell lines, and demonstrated the



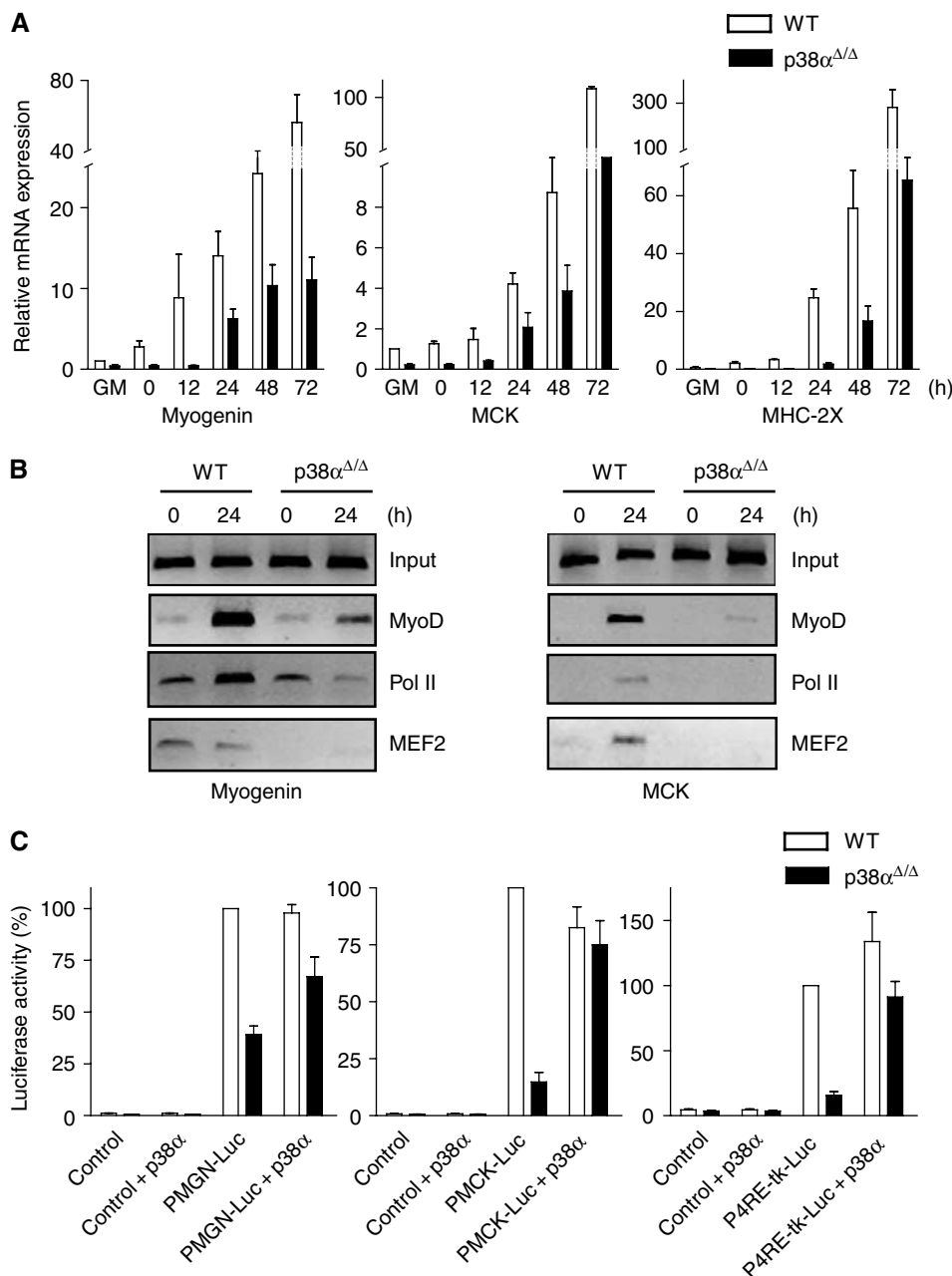
**Figure 1** Expression and activation pattern of p38 MAPKs in primary myoblasts. **(A)** Myoblasts were cultured in GM until subconfluence, and then shifted to DM for the indicated times (hours). Expression of p38 $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  mRNA was analyzed by RT-PCR. 18S expression was used as control. **(B)** Analysis of p38 MAPKs protein expression in primary myoblasts by Western blotting with p38 isoform-specific antibodies (see Supplementary Figure 1). **(C)** p38 phosphorylation in primary myoblasts (top) and C2C12 cells (bottom) was analyzed by Western blotting using a specific anti-phospho-p38 antibody. **(D)** p38 $\alpha$  and p38 $\gamma$  kinase assays in primary myoblasts. **(E)** Comparative analysis of muscle differentiation-specific gene markers in primary myoblasts and C2C12 cells by RT-PCR: myogenin (early marker); MCK and MRF4 (late markers).

specific and non-dispensable role of the p38 $\alpha$  kinase in this process.

#### **Consequences of absence of p38 MAPKs in myoblast fusion**

To directly investigate the contribution of p38 MAPKs to myoblast fusion, we examined the capacity of WT and p38-deficient myoblasts to form plurinucleated myotubes in DM. Differentiated WT myoblasts displayed a multinucleated morphology, which was similarly observed in cells deficient in p38 $\beta$ , p38 $\gamma$  and p38 $\delta$ , whereas p38 $\alpha$ -deficient myocytes

were primarily uninuclear, exhibiting a severe defect in their ability to form multinucleated cells, even after 48 h DM (Figure 3A; Supplementary Figure 4). Notably, this defect could be rescued by retroviral delivery of p38 $\alpha$  (Supplementary Figure 3B). Fusion was also impaired in SB203580-treated WT myoblasts in DM (not shown). Although myotube formation did occur in p38 $\gamma$ -deficient myoblasts, it was attenuated with respect to WT cells, as evidenced by the reduced fusion index and total number of myotubes formed (Figure 3B). From these results, we speculated that potential redundancies and/or compensatory

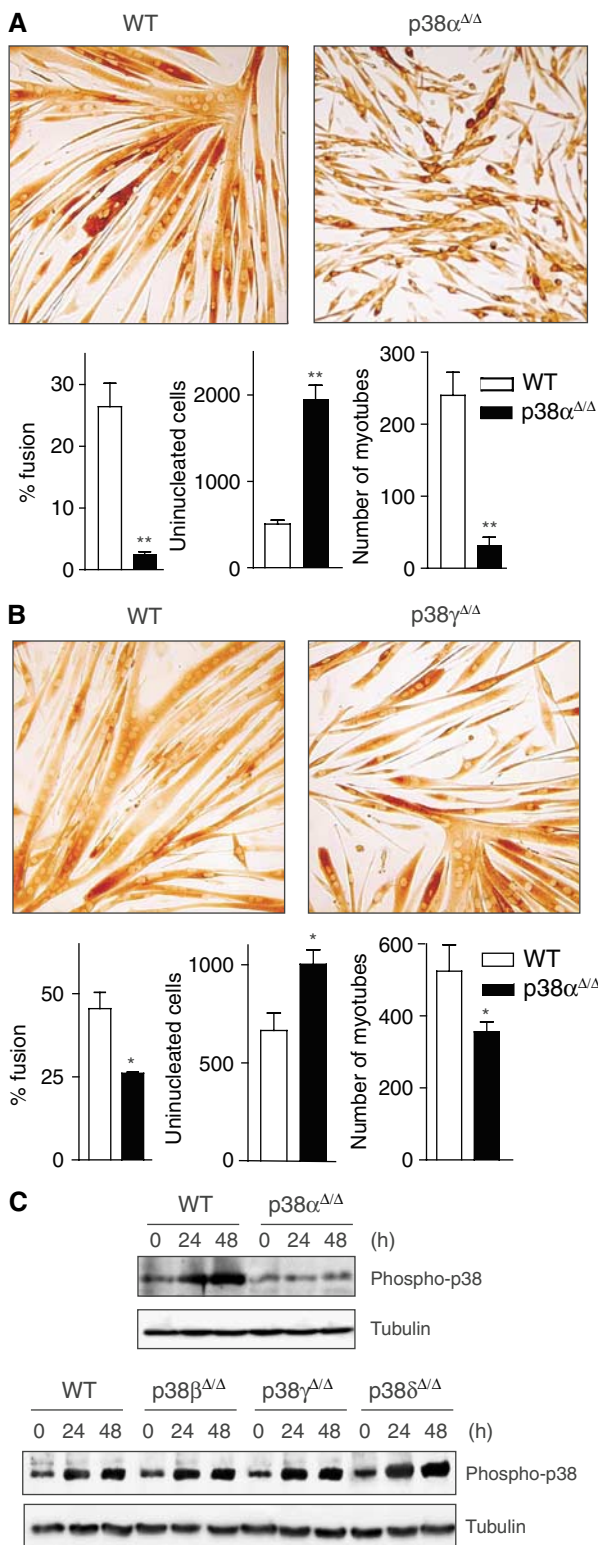


**Figure 2** p38 $\alpha$  deficiency reduces the expression of early and late muscle differentiation-specific gene products in differentiating primary myoblasts. WT (p38 $\alpha^{+/+}$ ) and p38 $\alpha$ -deficient (p38 $\alpha^{\Delta/\Delta}$ ) primary myoblasts were cultured as in Figure 1. (A) Comparative qRT-PCR mRNA analysis of myogenin (left), MCK (center) and MHC-2X (right). (B) ChIP analysis was performed using MyoD, RNA pol II and MEF2 antibodies, and subjected to PCR with primers corresponding to the myogenin and MCK promoter regions. (C) Myoblasts were transfected with p-Myogenin-Luc, pMCK-Luc, p4RE-tk-Luc vectors or an empty vector (control), in the absence or presence of a p38 $\alpha$  expression plasmid, and incubated in DM for 36 h. Luciferase activities are expressed relative to the activity found for WT myoblasts.

mechanisms might be occurring among the isoforms. Accordingly, we showed that the expression pattern of phosphorylated p38 during myogenesis was indistinguishable between WT myoblasts and myoblasts deficient in p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  (Figure 3C); indeed, no significant changes in the expression of the different p38 isoforms were observed in myoblasts deficient in p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  (not shown), in contrast, the levels of phosphorylated p38 were markedly reduced in p38 $\alpha^{\Delta/\Delta}$  myoblasts, which could be attributed to the diminished expression of p38 $\beta$  and p38 $\gamma$  in these cells (Supplementary Figure 1C).

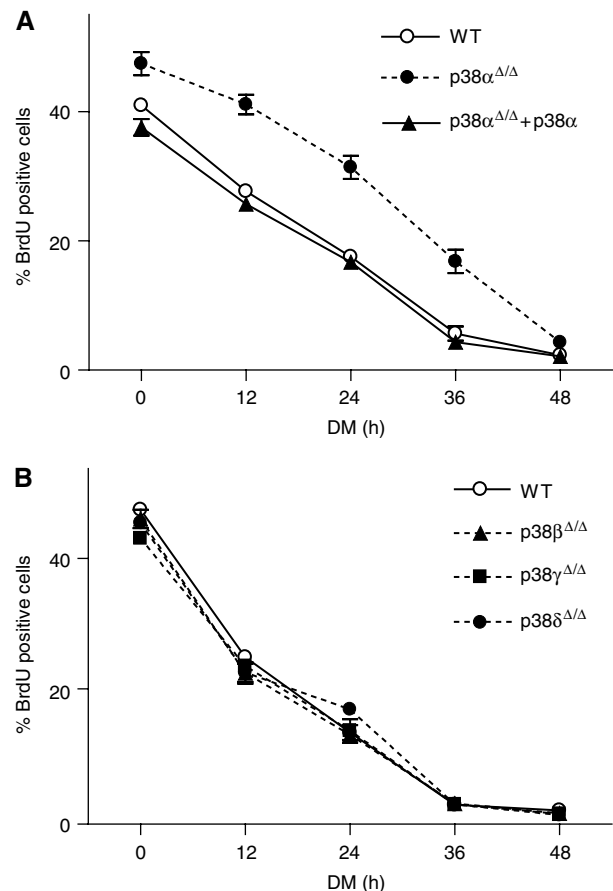
#### Delayed cell-cycle exit correlates with impaired differentiation of p38 $\alpha$ -deficient myoblasts

The simultaneous expression of myogenin and activation of p38 in nearly confluent primary myoblasts in GM (Figure 1C–E; DM 0 h), together with the reported implication of p38 in the proliferation of several cell types (Haq *et al*, 2002; Lee *et al*, 2002; Engel *et al*, 2005; Faust *et al*, 2005), suggested that the differentiation defect of p38 $\alpha$ -deficient myoblasts could be caused, at least in part, by alterations in cell-cycle exit. To test this hypothesis, we analyzed potential differences in the percentage of cells in S phase among the different p38-



**Figure 3** p38 $\alpha$  deficiency abrogates multinucleated myotube formation. WT and p38 $\alpha^{\Delta/\Delta}$  (A) and p38 $\gamma^{\Delta/\Delta}$  (B) myoblasts were switched to DM to induce myoblast fusion. Cells were immunostained for eMHC to define nuclei inside myotubes. Several parameters were analyzed: the percentage of nuclei within eMHC-positive cells (% fusion); the number of uninucleated cells; the number of myotubes. (C) p38 phosphorylation is reduced in p38 $\alpha^{\Delta/\Delta}$  myoblasts. Western blotting analysis of phospho-p38 levels in p38-deficient and WT myoblasts in DM (hours). (Top) WT versus p38 $\alpha^{\Delta/\Delta}$ , (bottom) WT versus p38 $\beta^{\Delta/\Delta}$ , p38 $\gamma^{\Delta/\Delta}$  and p38 $\delta^{\Delta/\Delta}$ .

deficient and WT cells by determining the incorporation of bromodeoxyuridine (BrdU) at different intervals after transfer to low-serum DM. As shown in Figure 4A, after 12 h in DM, 45% of p38 $\alpha$ -deficient myoblasts remained in S phase compared with 30% of WT; differences in S-phase myoblasts were still observed after 24 and 36 h in DM, with 32% and 18% BrdU-positive p38 $\alpha$ -deficient cells versus 10% and 3% BrdU-positive WT cells, respectively. In contrast, no significant alterations were observed in myoblasts deficient in p38 $\beta$ , p38 $\gamma$  or p38 $\delta$  kinases (Figure 4B). Notably, the proliferation phenotype of p38 $\alpha^{\Delta/\Delta}$  myoblasts in DM was rescued by retroviral delivery of p38 $\alpha$  (Figure 4A). Moreover, FACS analysis showed an increased population of p38 $\alpha$ -deficient myoblasts in G2/M and S phases of the cell cycle compared with WT myoblasts after 24 h in DM (Supplementary Figure 5A). Altogether, these experiments evidenced that p38 $\alpha^{\Delta/\Delta}$  (but not p38 $\beta^{\Delta/\Delta}$ , p38 $\gamma^{\Delta/\Delta}$  and p38 $\delta^{\Delta/\Delta}$ ) myogenic cells display continued proliferation under conditions of low serum that normally induce cell-cycle withdrawal and terminal differentiation of WT myoblasts, indicating that myoblasts deficient in p38 $\alpha$  have an impaired ability to exit the cell cycle. Furthermore, p38 $\alpha^{\Delta/\Delta}$  myoblasts also exhibited an enhanced proliferative potential in GM (Supplementary Figure 5B), supporting the notion that myogenic cells lacking p38 $\alpha$



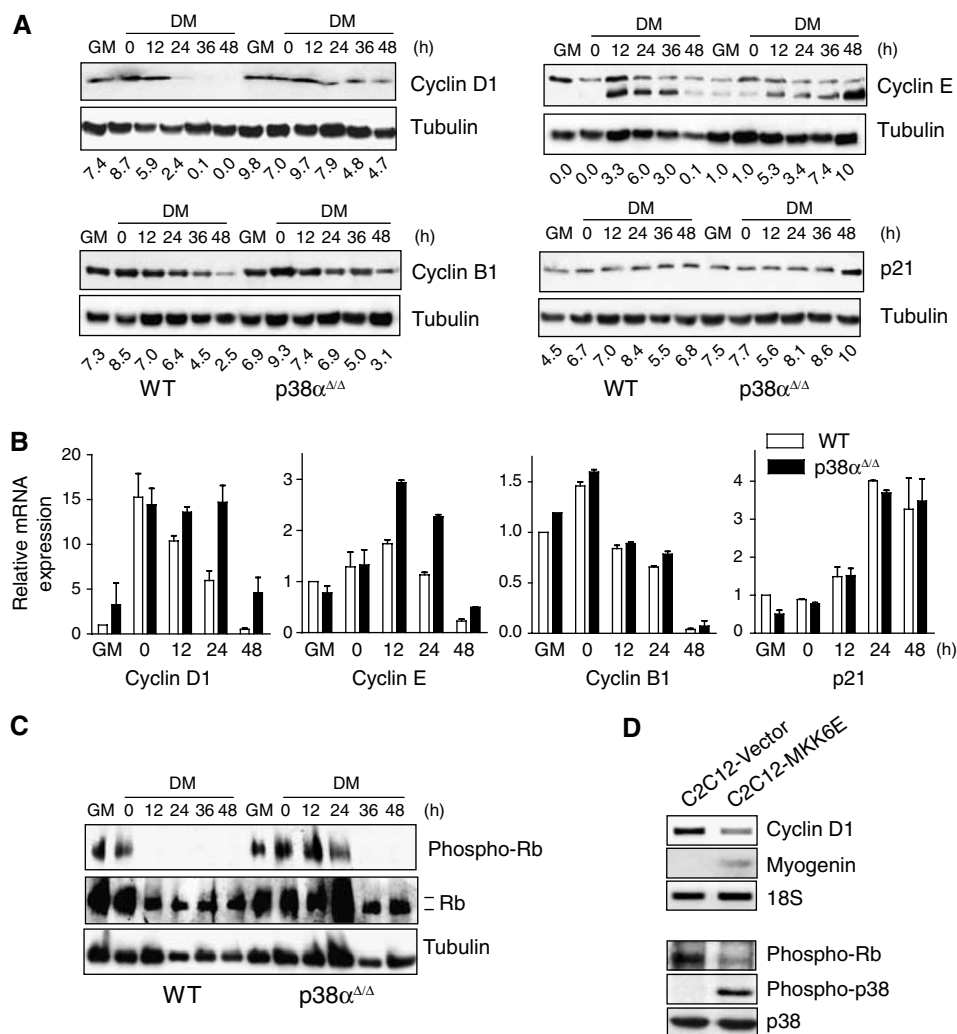
**Figure 4** Delayed cell-cycle exit of p38 $\alpha^{\Delta/\Delta}$  primary myoblasts. Myoblasts were cultured in GM and then shifted to DM for the indicated times (hours), and incubated for 1 h with BrdU. (A) WT, p38 $\alpha^{\Delta/\Delta}$  and p38 $\alpha^{\Delta/\Delta}$  myoblasts infected with a p38 $\alpha$ -expressing retrovirus. (B) WT and p38 $\beta^{\Delta/\Delta}$ , p38 $\gamma^{\Delta/\Delta}$  and p38 $\delta^{\Delta/\Delta}$ . Cells were fixed and immunostained against BrdU, and positive cells were quantified.

possess an increased propensity for self-renewal rather than progression through the differentiation program. Of note, the levels of Myf5 and MyoD were not reduced in p38 $\alpha^{\Delta/\Delta}$  cells in GM (and were even higher in DM) (Supplementary Figure 5C), suggesting that the delayed and reduced expression of differentiation-specific genes in p38 $\alpha^{\Delta/\Delta}$  cells cannot be ascribed to defects in expression of MRFs operating in proliferation and early differentiation stages.

### Altered expression of cell-cycle regulators in p38 $\alpha$ -deficient myoblasts

To directly investigate the causes of the enhanced proliferation of p38 $\alpha^{\Delta/\Delta}$  cells, we searched for potential differences in cell-cycle-associated proteins, whose expression is known to be modulated in myogenesis upon GM to DM transfer (Kitzmann and Fernandez, 2001). qRT-PCR and immunoblotting analyses showed that cyclin D1 mRNA and protein expression, respectively, decreased rapidly in WT myoblasts after transfer to DM, whereas they were still readily detected in p38 $\alpha$ -deficient myoblasts after 48 h in DM (Figure 5A and B). Potential regulation by p38 $\alpha$  deficiency of cyclin E was observed both at the mRNA and protein levels (Figure 5A

and B), but was less dramatic, and may therefore be due to secondary effects of changes in cyclin D1. Indeed, cyclin D1 is known to be essential for the induction of cyclin E in other cell types (Nurse, 1994; Sherr, 1994). The lack of any significant regulation of cyclin B1 and p21 in p38 $\alpha^{\Delta/\Delta}$  myoblasts also indicated that the effects of p38 $\alpha$  deficiency on cyclin D1 were specific. The pRb protein dephosphorylation is required for full cell-cycle exit and initiation of the myogenic program (Halevy *et al*, 1995). Importantly, myoblasts lacking p38 $\alpha$  contained a substantially higher level of hyperphosphorylated pRb than did WT control cells in DM, which was also maintained for longer periods of time, indicating that pRb dephosphorylation was delayed and defective in p38 $\alpha^{\Delta/\Delta}$  myoblasts (Figure 5C). If p38 $\alpha$  contributes causally to down-regulation of cyclin D1 levels and subsequent activation of myogenic differentiation, then its constitutive activation should be sufficient for advancing or even inducing both events in proliferation-promoting conditions. Indeed, C2C12 cells stably expressing a constitutively active form of MKK6—presenting high levels of activated p38 (Figure 5D)—exhibited a pronounced reduction of cyclin D1 levels and phosphorylated pRb coincident with induction of myogenin

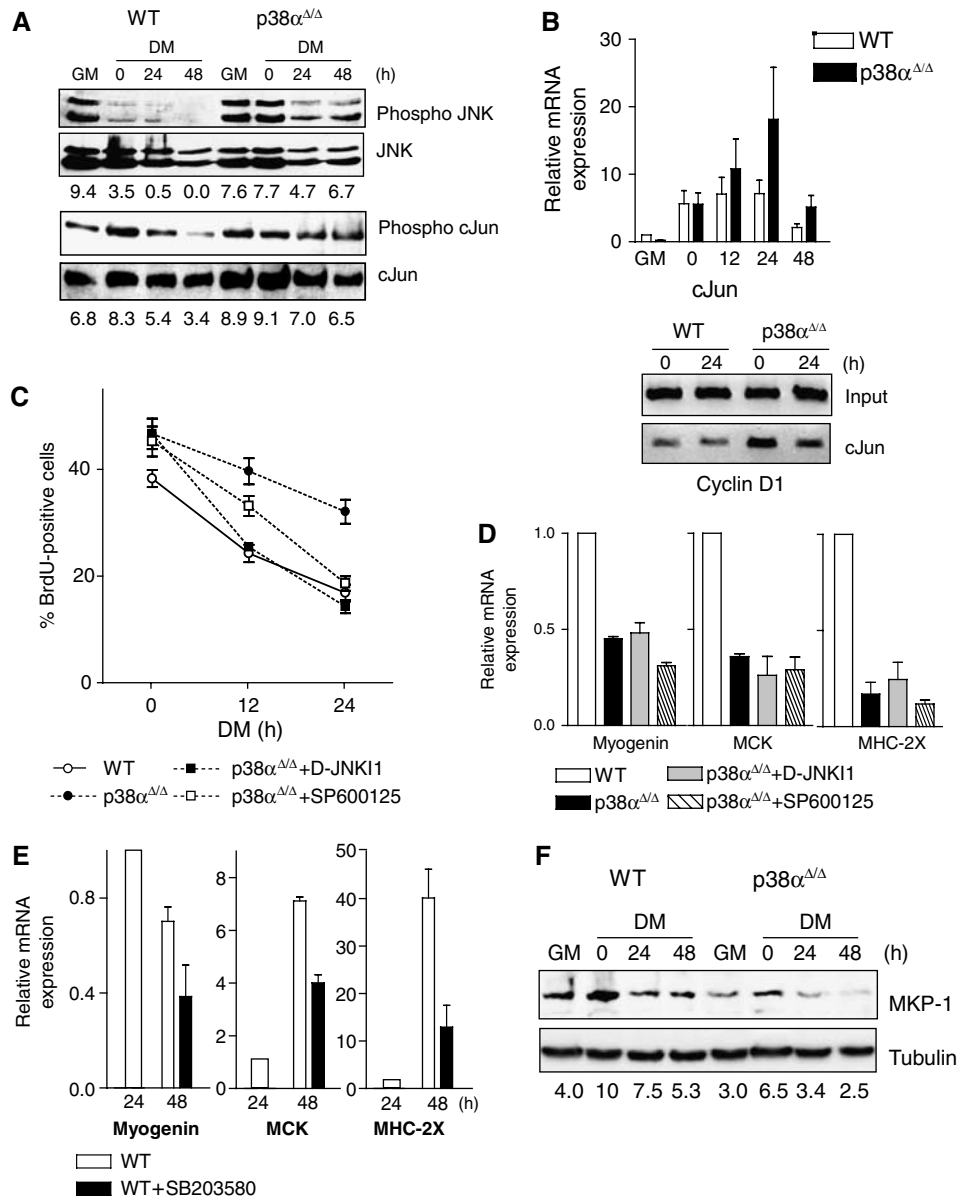


**Figure 5** Altered expression of cell-cycle regulators in p38 $\alpha^{\Delta/\Delta}$  myoblasts. WT and p38 $\alpha^{\Delta/\Delta}$  myoblasts were cultured as in Figure 4. Analysis of cyclin D1, cyclin E, cyclin B1 and p21 expression by Western blotting (A) and qRT-PCR (B). Quantification of the immunoblots by scanning densitometry (corrected by tubulin expression) is shown. (C) Analysis of phosphorylated and total Rb. (D) Overexpression of MKK6E in C2C12 cells regulates cyclin D1 and myogenin mRNA expression and pRb phosphorylation in GM. Confirmation of p38 phosphorylation by MKK6E is shown.

expression in proliferating conditions (Figure 5D). Together, these results suggest that p38 $\alpha$  activity is required to downregulate cyclin D1 expression and pRb hyperphosphorylation, leading to an irreversible block in G1-to-S progression and commencement of myogenic differentiation.

**Persistent activation of JNK/cJun underlies the continuous proliferation of p38 $\alpha$ -deficient myoblasts**  
Antagonistic effects of p38 and JNK signaling pathways in myoblast differentiation have been described (Meriane *et al*,

2000). However, the implication of JNK in this process is controversial (Meriane *et al*, 2000; Khurana and Dey, 2004). Within this context, we analyzed the activation of JNK in WT and p38 $\alpha$ -deficient myoblasts. JNK phosphorylation was high in WT myoblasts in GM, dropping as cells reached confluency (DM 0 h), and remained low after transfer to DM; in contrast, JNK activity continued to be elevated in p38 $\alpha$ -deficient myoblasts in DM, and, notably, it could be reduced by retroviral delivery of p38 $\alpha$  (Supplementary Figure 6A). This persistent JNK activity was translated into the phosphoryla-



**Figure 6** Increased JNK activity contributes to the enhanced proliferation of p38 $\alpha$ -deficient myoblasts. WT and p38 $\alpha^{\Delta\Delta}$  myoblasts were cultured as in Figure 5. (A) Phosphorylated JNK and Ser63-phosphorylated cJun were analyzed by Western blotting using anti-phospho-specific antibodies. Numbers show quantification of phospho-proteins normalized to total JNK and cJun levels. (B) qPCR analysis of the relative cJun mRNA levels (top). ChIP analysis was performed using an anti-cJun antibody, and PCR was performed with primers corresponding to the cyclin D1 promoter region (bottom). (C) p38 $\alpha^{\Delta\Delta}$  primary myoblasts were cultured in DM for the indicated hours, in the absence or presence of D-JNKI1 and SP600125 (JNK inhibitors), and incubated for 1 h with BrdU. BrdU-positive cells were quantified as in Figure 4. The effect of D-JNKI1 and SP600125 on JNK activation is shown in Supplementary Figure 6B. (D) Cells were cultured as in (C), and mRNA for the indicated myogenic markers was analyzed by qPCR. (E) p38 $\alpha$  activity is necessary for myogenic differentiation, independent of cell-cycle withdrawal. Myoblasts cultured in GM were shifted to DM for 24 h and then cultured for an additional 24 h period in DM alone or DM supplemented with SB203580. mRNA for the indicated myogenic markers was analyzed by qPCR. (F) Reduced MKP-1 levels in p38 $\alpha$ -deficient myoblasts. Myoblast extracts used in (A) were analyzed by Western blotting using anti-MKP-1 and anti-tubulin antibodies.

tion of its downstream substrate cJun on Ser63 (Figure 6A). Furthermore, cJun mRNA levels were also increased in p38 $\alpha^{\Delta/\Delta}$  myoblasts (Figure 6B), suggesting that p38 regulates cJun gene expression indirectly, possibly via JNK/AP-1-mediated transcription from the cJun promoter (Angel *et al*, 1988). Of note, ChIP experiments revealed an increased association of cJun to the cyclin D1 promoter in p38 $\alpha$ -deficient myoblasts in DM (Figure 6B), supporting the notion of a cJun-mediated transcriptional induction of the cyclin D1 gene in these cells. These results suggested the existence of crosstalk between JNK and p38 signaling pathways in myogenesis, which might underlie the proliferation phenotype of myoblasts lacking p38 $\alpha$ . To obtain evidence that persistent activation of JNK could be responsible for the continuous proliferation of p38 $\alpha$ -deficient myoblasts in differentiation-promoting conditions, BrdU incorporation in these cells was determined in DM in the absence or presence of the specific JNK inhibitor, D-JNKI1 (Borsello *et al*, 2003) (Supplementary Figure 6B). As shown in Figure 6C, the number of BrdU-positive p38 $\alpha$ -deficient myoblasts was reduced by D-JNKI1 treatment to the levels obtained with WT myoblasts. Similar inhibitory effects on myoblast proliferation were also observed with another JNK inhibitor (SP600125) (Figure 6C; Supplementary Figure 6B), thereby demonstrating that inhibition of JNK activation reduced the proliferation of p38 $\alpha$ -deficient myoblasts in differentiating conditions. Taken together, our results indicate that p38 $\alpha$  controls myoblast proliferation by downregulating JNK pathway activation. On the basis of an early study by Bennett and Tonks, showing that overexpression of the MAPK phosphatase-1 (MKP-1) in C2C12 cells modulated myogenesis (Bennett and Tonks, 1997), we hypothesized that the enhanced activation of JNK in p38 $\alpha$ -deficient proliferating myoblasts might involve deregulation of this phosphatase. As shown in Figure 6F, the expression of MKP-1 was, indeed, reduced in p38 $\alpha^{\Delta/\Delta}$  compared to WT myoblasts in DM, and this reduction could be significantly reversed by retroviral delivery of p38 $\alpha$  (Supplementary Figure 6C), suggesting that p38 $\alpha$  may regulate MKP-1 expression. These results further suggested that the downregulation of MKP-1 levels could, in turn, be partially responsible for the persistent phosphorylation of JNK in the absence of p38 $\alpha$ .

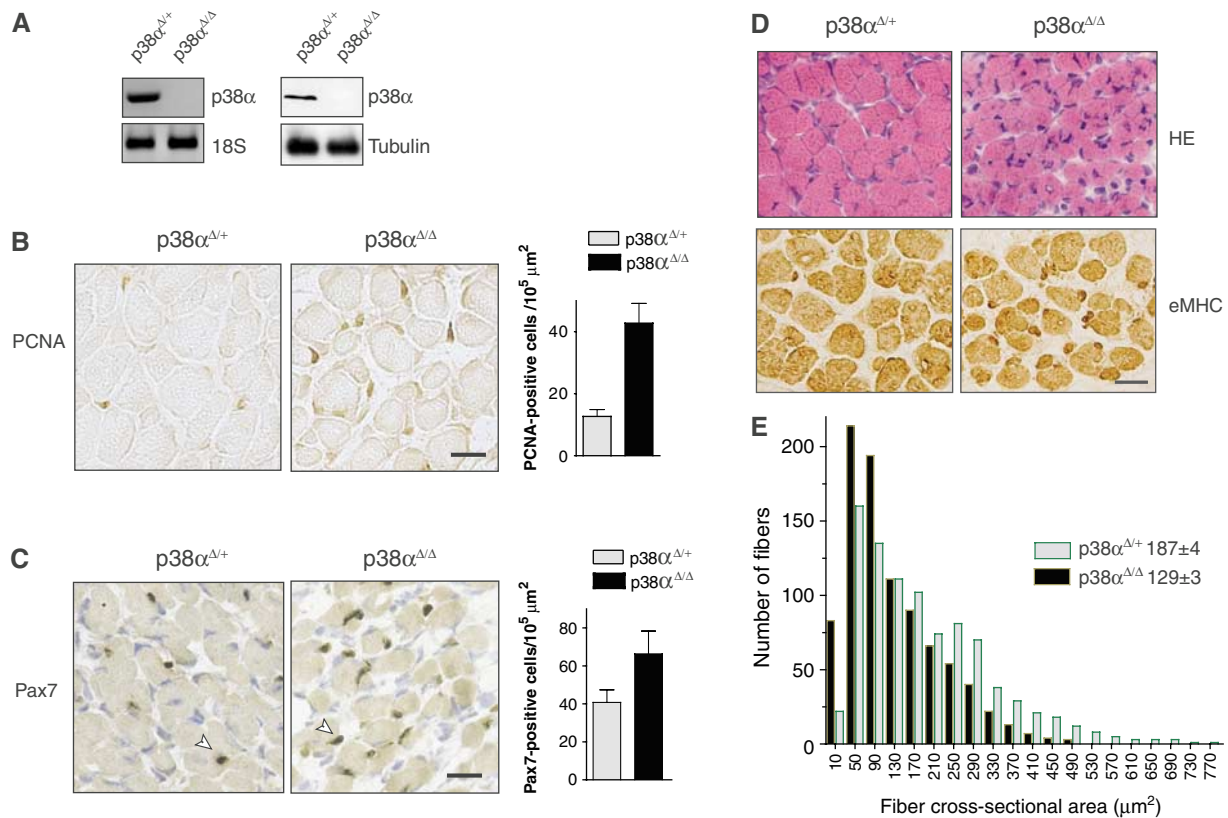
#### **p38 $\alpha$ regulates muscle-specific gene expression independently of cell-cycle exit**

The continuous myoblast proliferation in the absence of p38 $\alpha$  shown so far may suggest that the impaired muscle-specific gene expression in p38 $\alpha$ -deficient myoblasts is secondary to the primary proliferation defect. However, the expression of myogenin and MCK in myoblasts lacking p38 $\alpha$  was not rescued by inhibition of JNK activation (Figure 6D), suggesting that functional p38 $\alpha$  activity is necessary not only for cessation of proliferation but also for commencement of the muscle gene program. To investigate further whether p38 $\alpha$  can exert a direct effect on myoblast differentiation, independent of its antiproliferative function, p38 $\alpha/\beta$  activity was inhibited in WT myoblasts after cell-cycle exit and its consequences on muscle gene expression were analyzed subsequently. To this end, WT myoblasts were cultured for 24 h in DM to allow cell-cycle withdrawal, as shown by the absence of phosphorylated pRb (Supplementary Figure 6D); then,

cells were further incubated for an additional period of 24 h in DM in the absence or presence of SB203580 to evaluate the effect of p38 $\alpha$  inhibition exclusively on muscle-specific gene expression. As shown in Figure 6E, the late SB203580 cell treatment resulted in a reduction in the expression of myogenin, MCK and MHC mRNAs compared to myoblasts cultured for 48 h in DM in the absence of the inhibitor. These results, together with those shown in Figure 2B, uncouple two tightly linked functions of p38 $\alpha$  in myogenesis, and demonstrate that p38 $\alpha$  controls sequential steps in the myogenic pathway.

#### **Mice lacking p38 $\alpha$ exhibit increased myoblast proliferation and delayed myofiber growth and maturation in the neonatal period**

Our previous results in cultured muscle cells indicated that p38 $\alpha$  regulated different myogenic stages. To investigate whether p38 $\alpha$  could play similar functions *in vivo*, we analyzed limb muscles from neonatal p38 $\alpha^{\Delta/\Delta}$  and p38 $\alpha^{\Delta/+}$  mice. RT-PCR and Western blotting analyses confirmed the absence of p38 $\alpha$  mRNA and protein in skeletal muscle, respectively, of p38 $\alpha^{\Delta/\Delta}$  mice (Figure 7A). p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  protein levels were unaffected (not shown). To analyze the possible effect of p38 $\alpha$  inactivation on the cell cycle in skeletal muscle *in vivo*, we stained muscle sections of p38 $\alpha^{\Delta/\Delta}$  and p38 $\alpha^{\Delta/+}$  at neonatal stages with an antibody against proliferating cell nuclear antigen (PCNA). PCNA is expressed in replicating cells throughout S phase and thus allows detection of dividing cells. Among littermates, the number of PCNA-expressing cells was highest in p38 $\alpha^{\Delta/\Delta}$  mice (Figure 7B). Furthermore, the number of myoblasts, identified by the expression of Pax7, was also higher in p38 $\alpha^{\Delta/\Delta}$  neonatal muscle than in WT counterparts (Figure 7C). Histological analysis by hematoxylin/eosin (HE) staining showed that myofibers did form in p38 $\alpha^{\Delta/\Delta}$  muscle; however, the size of individual myofibers was smaller than in p38 $\alpha^{\Delta/+}$  muscle, as revealed by morphometric analysis at cross-sections (Figure 7D–E). These results suggested that p38 $\alpha$  plays a role in regulating myofiber growth *in vivo*, in agreement with the predominance of small myotubes and single-nucleated myocytes in p38 $\alpha^{\Delta/\Delta}$  cultures. Furthermore, the expression of embryonic MHC was more pronounced in small fibers of p38 $\alpha$ -deficient muscle (Figure 7D), demonstrating the existence of more immature myofibers in p38 $\alpha^{\Delta/\Delta}$  neonatal mice than in p38 $\alpha^{\Delta/+}$  counterparts. These results indicated that myofiber growth and maturation are delayed *in vivo*, in the absence of functional p38 $\alpha$ . In contrast with these results, p38 $\gamma^{\Delta/\Delta}$  neonatal muscle did not show any of these alterations (Supplementary Figure 7A), suggesting *in vivo* compensations of the observed phenotype *in vitro*. Similarly, no major differences were detected in muscles of p38 $\beta^{\Delta/\Delta}$  and p38 $\delta^{\Delta/\Delta}$  mice, respectively (not shown). To exclude a possible correlation of these observations with the presence of structural abnormalities and muscle damage secondary to the absence of p38 $\alpha$ , we labeled apoptotic cells with an antibody against the activated form of caspase-3 and performed transmission electron microscopy studies. No differences were observed between WT and p38 $\alpha^{\Delta/\Delta}$  neonatal mice in skeletal muscle apoptotic rates (Supplementary Figure 7B). Moreover, the ultrastructure of myofibrils and sarcomeres was preserved in p38 $\alpha^{\Delta/\Delta}$  neonatal muscle (Figure 8).



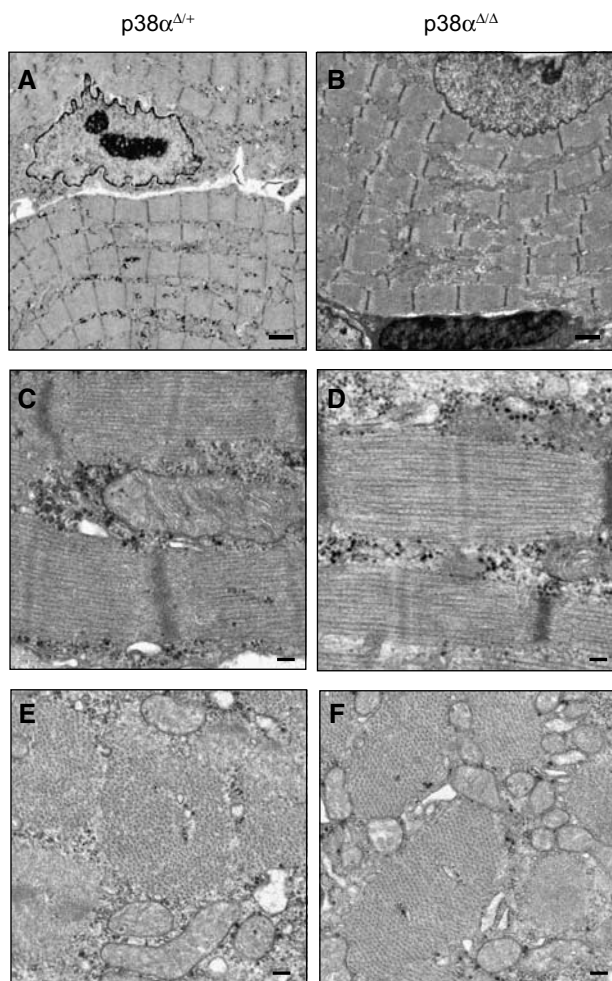
**Figure 7** Reduced fiber size and increased cell proliferation in p38<sup>Δ/Δ</sup> neonatal muscles. (A) p38<sup>α</sup> expression in neonatal muscles analyzed by RT-PCR (left) and Western blotting using an anti-p38<sup>α</sup>-specific antibody (right). (B, C) The number of PCNA- and Pax7-positive cells (arrowhead) was quantified on p38<sup>Δ/+</sup> and p38<sup>Δ/Δ</sup> neonatal muscle sections. Scale bar, 20 μm. (D) Top: HE staining showing reduced myofiber size in p38<sup>Δ/Δ</sup> muscle; bottom: presence of small immature myofibers stained strongly with an anti-embryonic MHC antibody. Scale bar, 20 μm. (E) Frequency histograms of myofiber size in p38<sup>Δ/+</sup> and p38<sup>Δ/Δ</sup> neonatal muscle. A Mann-Whitney non-parametric test was used for comparisons between groups. Data are means ± s.e.m. *P* < 0.001.

## Discussion

The p38 MAPK has emerged in the last years as a fundamental pathway in myogenesis. This conclusion has relied largely on studies performed in immortalized myogenic cell lines, by using pyridinyl imidazole inhibitors such as SB203580, which are inhibitors of both p38<sup>α</sup> and p38<sup>β</sup> kinases, and by overexpressing constitutively active and kinase-dead forms of components of the signaling pathway. Thus, the relative contribution of the four p38 MAPKs to myogenesis is unknown. In addition, no *in vivo* studies beyond the embryonic stages have been performed. Here, we demonstrate that p38 kinases play distinct roles in myogenesis, p38<sup>α</sup> being the crucial kinase. Myoblasts obtained from mice lacking p38<sup>α</sup> showed delayed cell-cycle exit and continued proliferation, as well as impaired myoblast differentiation and fusion. Moreover, skeletal muscle from neonatal mice deficient in p38<sup>α</sup> displayed increased myoblast proliferation, reduced myofiber growth and delayed maturation. In contrast, lack of the p38<sup>β</sup> and p38<sup>δ</sup> had no major phenotypic consequences in any of these models. p38<sup>γ</sup>-deficient myoblasts presented attenuated fusion *in vitro* although no major alteration was detected on neonatal muscle. In conclusion, p38<sup>α</sup> emerges as the central p38 MAPK in myogenesis *in vitro* and *in vivo*. We demonstrate a key role for p38<sup>α</sup> in controlling myoblast proliferation, preceding its direct regulation of the muscle-specific gene

program, by antagonizing the JNK/cJun pathway, probably via MKP-1. The function and mechanism of action of p38<sup>α</sup> in the regulation of myoblast proliferation constitute novel and previously unknown activities of the p38 pathway in skeletal myogenesis.

Proliferation and differentiation are mutually exclusive processes in myogenesis. Indeed, cessation of proliferation by downregulation of cyclin D1 and dephosphorylation of pRb is required for initiation of muscle-specific gene expression (Rao *et al*, 1994; Halevy *et al*, 1995; Skapek *et al*, 1995; Novitch *et al*, 1996, 1999; Puri *et al*, 2001; Guo *et al*, 2003; Huh *et al*, 2004). Thus, the impaired differentiation in the absence of p38<sup>α</sup> could be attributed to continuous myoblast proliferation, due to the persistence of cyclin D1 and hyperphosphorylated pRb in differentiation-promoting conditions, implying a negative regulation of cyclin D1 by p38<sup>α</sup>. Transcriptional and post-transcriptional downregulation of cyclin D1 by p38 was previously reported in other cell types, although the molecular mechanisms were not fully understood (Lavoie *et al*, 1996; Casanovas *et al*, 2000). We propose that the increased cyclin D1 expression in p38<sup>α</sup>-deficient myoblasts, which underlies their continued proliferation, may be caused by cJun-mediated transcriptional induction of the cyclin D1 promoter (see below). Two recent reports have shown that p38<sup>α</sup> inhibition enables proliferation of adult mammalian cardiomyocytes by promoting cytokinesis (Engel *et al*, 2005) and overrides fibroblast contact



**Figure 8** Muscle structure is not altered in  $p38\alpha^{\Delta/\Delta}$  neonatal muscles. Representative transmission electron micrographs of longitudinal and transverse  $p38\alpha^{\Delta/+}$  and  $p38\alpha^{\Delta/\Delta}$  sections showed no morphological abnormalities in myofibrils and muscle sarcomeres. (A, B) Longitudinal sections. Scale bar, 5  $\mu$ m. (C, D) Higher magnification of longitudinal sections. Scale bar, 500 nm. (E, F) Transversal sections. Scale bar, 500 nm.

inhibition by impaired accumulation of p27 (Faust *et al*, 2005). Interestingly, we observed no differences in the accumulation of p27 in differentiating myoblasts regardless of the presence or absence of p38 $\alpha$  (data not shown), suggesting the existence of different cell-type-dependent p38 $\alpha$ -controlled proliferation mechanisms. At variance with these reports, p38 $\alpha$  seemed dispensable for proliferation of B and T lymphocytes (Kim *et al*, 2005b). A recent study also implicated p38 $\alpha/\beta$  MAPKs in satellite cell activation (Jones *et al*, 2005); however, unspecific effects of the high concentration of SB203580 used in this study, by potential inhibition of additional signaling molecules besides p38 $\alpha/\beta$  (Davies *et al*, 2000), cannot be excluded.

Crosstalk between p38 and JNK signaling pathways has been previously described in different cell types (Nemoto *et al*, 1998; Zechner *et al*, 1998; Chen *et al*, 2000; Porras *et al*, 2004), through undefined mechanisms. In the context of skeletal myogenesis, two studies have suggested opposite roles for JNK activity in muscle differentiation (Meriane *et al*, 2000; Khurana and Dey, 2004); however, neither an involvement of

JNK in myoblast proliferation nor a crosstalk with the p38 pathway in myogenesis had ever been reported. Our results demonstrated that JNK activity increased and persisted in myoblasts lacking p38 $\alpha$  in differentiation-promoting conditions. Most importantly, JNK activation mediated the increased proliferation potential of p38 $\alpha$ -deficient myoblasts, as inhibition of JNK activation completely reversed the proliferation phenotype. Our data further showed that the enhanced activation of JNK in p38 $\alpha$ -deficient myoblasts translated into increased levels of its substrate phospho-cJun and subsequent induction of cJun/AP-1-mediated cJun gene transcription, which in turn led to increased recruitment of cJun to the cyclin D1 loci in differentiating myoblasts *in vivo*, presumably via the AP-1 sites on the cyclin D1 promoter (Bakiri *et al*, 2000). Thus, p38 $\alpha$  controls myoblast proliferation by antagonizing the proliferation-promoting function of JNK, constituting a novel mechanism whereby p38 $\alpha$  regulates myogenesis. This antagonism might be mediated, at least in part, by MKP-1, as suggested by the observed downregulated expression of this phosphatase in DM in the absence of p38 $\alpha$ , and its rescue by ectopic reconstitution of p38 $\alpha$  levels. Notably, regulation of MKP-1 expression and activity by p38 $\alpha$  could be direct, based on studies showing that phosphorylation and acetylation of histone H3 on MKP-1 chromatin in response to stress was dependent on SB203580 cell treatment (Li *et al*, 2001), and that catalytic activation of MKP-1 depended on its direct association with p38 (Hutter *et al*, 2000).

The studies of p38 function in either myogenic cell lines or primary myoblasts are limited to specific stages of myogenesis, and are isolated from the real developmental context. Thus, *in vivo* analysis in animal models to confirm the role of p38 becomes pertinent. Targeted inactivation of the mouse p38 $\alpha$  gene led to a lethal phenotype (Adams *et al*, 2000; Tamura *et al*, 2000); in one study, the placental defect could be rescued and at a very low frequency the mutant mice survived (Adams *et al*, 2000). Using an embryo-specific CRE line, we were able to show that mutant pups survived to term but died shortly thereafter (L Hui, unpublished observations). p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  and double p38 $\gamma/\delta$  knockout mice develop normally into adulthood, suggesting redundant functions among the isoforms (Beardmore *et al*, 2005; Sabio *et al*, 2005; Kim *et al*, 2005a). Recently, two studies reported alternative approaches for studying the role of p38 MAPK pathway in early myogenic development. Using a MEF2 transgenic reporter mouse, de Angelis *et al* (2005) found that SB203580 treatment blocked MEF2 activity and differentiation in the somites, whereas commitment to the myogenic lineage was not affected, as the expression of the Myf5 locus was not changed by p38 $\alpha/\beta$  inhibition. In addition, Keren *et al* (2005) showed that interference with the p38 pathway in early developing *Xenopus laevis* specifically prevented the expression of XMyf5—at variance with the results in mouse embryos—resulting in several defects in muscle development. Interestingly, we found no decrease in Myf5 expression in the absence of functional p38 $\alpha$  MAPK in mouse primary myoblasts (see Figure 4E). Whether the transcriptional control of Myf5 expression by p38 is specific of *Xenopus* mesoderm deserves further analysis. In our study, we investigated the impact of specific deletion of p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  on neonatal skeletal muscle. Although the ultrastructure of myofibrils and sarcomeres was preserved,

we found increased myoblast proliferation, reduced myofiber growth and delayed differentiation in myofibers of neonatal p38 $\alpha$ -deficient mice. No major differences were detected in p38 $\gamma$ -deficient muscle at the same neonatal stage, in spite of the reduced fusion index observed *in vitro*; similarly, no major phenotype was observed in muscles of p38 $\beta$ - and p38 $\delta$ -deficient neonatal mice, highlighting the fact that muscle p38 $\alpha$  deficiency is the only one that cannot be compensated *in vivo*.

This study unambiguously demonstrates that p38 $\alpha$  is the central p38 MAPK in myogenesis. Our results, together with previous studies, uncover two distinct roles of p38 $\alpha$  along the myogenic pathway: as a regulator of myoblast proliferation and a second role in controlling muscle-specific gene expression. These data also provide the first evidence for p38 $\alpha$  signaling in postnatal muscle development.

## Materials and methods

### Reagents

The p38 $\alpha$ / $\beta$  inhibitor SB203580, JNK inhibitor SP600125 and MEK inhibitor PD098059 (Calbiochem) were used at a final concentration of 10, 20 and 50  $\mu$ M, respectively. The JNK-specific inhibitor D-JNK11 (Borsello *et al*, 2003) was synthesized at the IMP, Vienna, and used at a final concentration of 10  $\mu$ M.

### Mice

Mice carrying the floxed p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  alleles (p38 $\alpha$  f/f, p38 $\beta$  f/f, p38 $\gamma$  f/f, p38 $\delta$  f/f, respectively) were generated by Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, USA). The p38 $\alpha$  f/f were described by Engel *et al* (2005); p38 $\beta$  f/f, p38 $\gamma$  f/f, p38 $\delta$  f/f will be described elsewhere. p38 $\alpha$ f/f mice were crossed to More-CRE knock-in mice. Heterozygous mice (More-cre/p38 $\alpha$ <sup>Δ/+</sup>) were crossed to the p38 $\alpha$  f/f to obtain p38 $\alpha$ <sup>Δ/Δ</sup> mice. The protamine-CRE transgenic line was used to generate the deleted allele in the germ line of the p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  mutant mice. The genetic background of this intercross was C57BL6/J  $\times$  129Sv. Genotyping primers are described in Supplementary data.

### Cell culture and isolation of primary myoblasts

The C2C12, C2C12/MKK6E and 293T cell lines were cultured as described in Supplementary data. Primary myoblasts were obtained from muscles of mice deficient in p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$ , p38 $\delta$  (p38 $\alpha$ <sup>Δ/Δ</sup>, p38 $\beta$ <sup>Δ/Δ</sup>, p38 $\gamma$ <sup>Δ/Δ</sup> and p38 $\delta$ <sup>Δ/Δ</sup>, respectively) and from their corresponding WT counterparts. Isolation and culture were performed as described in Supplementary data.

## References

- Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S, Valladares A, Perez L, Klein R, Nebreda AR (2000) Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. *Mol Cell* **6**: 109–116
- Angel P, Hattori K, Smeal T, Karin M (1988) The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell* **55**: 875–885
- Bakiri L, Lallemand D, Bossy-Wetzel E, Yaniv M (2000) Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. *EMBO J* **19**: 2056–2068
- Beardmore VA, Hinton HJ, Eftychi C, Apostolaki M, Armaka M, Darragh J, McIlrath J, Carr JM, Armit LJ, Clacher C, Malone L, Kollias G, Arthur JS (2005) Generation and characterization of p38beta (MAPK11) gene-targeted mice. *Mol Cell Biol* **25**: 10454–10464
- Bennett AM, Tonks NK (1997) Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases. *Science* **278**: 1288–1291

### Plasmid constructs, transfection and retroviral infection

The following plasmids were used: pEFmink-p38 $\alpha$ , pEFmink-p38 $\beta$ , pEFmink-p38 $\gamma$ , pEFmink-p38 $\delta$ , pMyogenin-Luc, pMCK-Luc and p4RE-TK-Luc, and MSCV-p38 $\alpha$  retroviral construct. 293T cells and primary myoblasts were transiently transfected by standard methods. Luciferase assay, and details of plasmids, transfection and infection procedures are described in Supplementary data.

### Proliferation and fusion assays

To detect S-phase cells, cultures were pulsed with BrdU (Sigma) for 1 h and then were processed and analyzed as described in Supplementary data. For fusion analysis, cells were immunostained with embryonic myosin heavy chain (eMHC) antibody as described in Supplementary data.

### RNA isolation, RT-PCR and qRT-PCR

RNA was analyzed by RT-PCR or qRT-PCR. DNA primers and details of the procedure are described in Supplementary data.

### Chromatin immunoprecipitation assays

Standard ChIP assays were performed using commercial antibodies against MyoD, MEF2, cJun (Santa Cruz) and RNA Pol II (Abcam). Details of ChIP analysis (including DNA primers and PCR conditions) are described in Supplementary data.

### Western blotting, immunoprecipitation and kinase assay

Preparation of cell lysates, Western blotting, immunoprecipitation and *in vitro* kinase reactions were performed as described in Supplementary data.

### Histology and immunohistochemistry

Hind limbs were obtained from p38 $\alpha$ <sup>Δ/Δ</sup>, p38 $\beta$ <sup>Δ/Δ</sup>, p38 $\gamma$ <sup>Δ/Δ</sup>, p38 $\delta$ <sup>Δ/Δ</sup> and corresponding p38<sup>Δ/+</sup> mice at postnatal day 2. Immunohistochemical and transmission electron microscopy analyses on muscle cryosections were performed as described in Supplementary data.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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- Borsello T, Clarke PG, Hirt L, Vercelli A, Repici M, Schorderet DF, Bogousslavsky J, Bonny C (2003) A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat Med* **9**: 1180–1186
- Briata P, Forcales SV, Ponassi M, Corte G, Chen CY, Karin M, Puri PL, Gherzi R (2005) p38-dependent phosphorylation of the mRNA decay-promoting factor KSRP controls the stability of select myogenic transcripts. *Mol Cell* **20**: 891–903
- Casanovas O, Miro F, Estanyol JM, Itarte E, Agell N, Bachs O (2000) Osmotic stress regulates the stability of cyclin D1 in a p38SAPK2-dependent manner. *J Biol Chem* **275**: 35091–35097
- Chen G, Hitomi M, Han J, Stacey DW (2000) The p38 pathway provides negative feedback for Ras proliferative signaling. *J Biol Chem* **275**: 38973–38980
- Cuenda A, Cohen P (1999) Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. *J Biol Chem* **274**: 4341–4346

- Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351**: 95–105
- de Angelis L, Zhao J, Andreucci JJ, Olson EN, Cossu G, McDermott JC (2005) Regulation of vertebrate myotome development by the p38 MAP kinase-MEF2 signaling pathway. *Dev Biol* **283**: 171–179
- Engel FB, Schebesta M, Duong MT, Lu G, Ren S, Madwed JB, Jiang H, Wang Y, Keating MT (2005) p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. *Genes Dev* **19**: 1175–1187
- Faust D, Dolado I, Cuadrado A, Oesch F, Weiss C, Nebreda AR, Dietrich C (2005) p38alpha MAPK is required for contact inhibition. *Oncogene* **24**: 7941–7945
- Guo CS, Degnin C, Fiddler TA, Stauffer D, Thayer MJ (2003) Regulation of MyoD activity and muscle cell differentiation by MDM2, pRb, and Sp1. *J Biol Chem* **278**: 22615–22622
- Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, Beach D, Lassar AB (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**: 1018–1021
- Haq R, Brenton JD, Takahashi M, Finan D, Finkielstein A, Damaraju S, Rottapel R, Zanke B (2002) Constitutive p38HOG mitogen-activated protein kinase activation induces permanent cell cycle arrest and senescence. *Cancer Res* **62**: 5076–5082
- Huh MS, Parker MH, Scime A, Parks R, Rudnicki MA (2004) Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation. *J Cell Biol* **166**: 865–876
- Hutter D, Chen P, Barnes J, Liu Y (2000) Catalytic activation of mitogen-activated protein (MAP) kinase phosphatase-1 by binding to p38 MAP kinase: critical role of the p38 C-terminal domain in its negative regulation. *Biochem J* **352** (Part 1): 155–163
- Jones NC, Tyner KJ, Nibarger L, Stanley HM, Cornelison DD, Fedorov YV, Olwin BB (2005) The p38alpha/beta MAPK functions as a molecular switch to activate the quiescent satellite cell. *J Cell Biol* **169**: 105–116
- Keren A, Bengal E, Frank D (2005) p38 MAP kinase regulates the expression of XMyf5 and affects distinct myogenic programs during *Xenopus* development. *Dev Biol* **288**: 73–86
- Keren A, Tamir Y, Bengal E (2006) The p38 MAPK signaling pathway: a major regulator of skeletal muscle development. *Mol Cell Endocrinol* **252**: 224–230
- Khurana A, Dey CS (2004) Involvement of c-Jun N-terminal kinase activities in skeletal muscle differentiation. *J Muscle Res Cell Motil* **25**: 645–655
- Kim HP, Wang X, Nakao A, Kim SI, Murase N, Choi ME, Ryter SW, Choi AM (2005a) Caveolin-1 expression by means of p38(beta) mitogen-activated protein kinase mediates the antiproliferative effect of carbon monoxide. *Proc Natl Acad Sci USA* **102**: 11319–11324
- Kim JM, White JM, Shaw AS, Sleckman BP (2005b) MAPK p38 alpha is dispensable for lymphocyte development and proliferation. *J Immunol* **174**: 1239–1244
- Kitzmann M, Fernandez A (2001) Crosstalk between cell cycle regulators and the myogenic factor MyoD in skeletal myoblasts. *Cell Mol Life Sci* **58**: 571–579
- Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J (1996) Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem* **271**: 20608–20616
- Lee J, Hong F, Kwon S, Kim SS, Kim DO, Kang HS, Lee SJ, Ha J (2002) Activation of p38 MAPK induces cell cycle arrest via inhibition of Raf/ERK pathway during muscle differentiation. *Biochem Biophys Res Commun* **298**: 765–771
- Li J, Gorospe M, Hutter D, Barnes J, Keyse SM, Liu Y (2001) Transcriptional induction of MKP-1 in response to stress is associated with histone H3 phosphorylation-acetylation. *Mol Cell Biol* **21**: 8213–8224
- Li Y, Jiang B, Ensign WY, Vogt PK, Han J (2000) Myogenic differentiation requires signalling through both phosphatidylinositol 3-kinase and p38 MAP kinase. *Cell Signal* **12**: 751–757
- Lluis F, Ballestar E, Suelves M, Esteller M, Munoz-Canoves P (2005) E47 phosphorylation by p38 MAPK promotes MyoD/E47 association and muscle-specific gene transcription. *EMBO J* **24**: 974–984
- Lluis F, Perdiguero E, Nebreda AR, Munoz-Canoves P (2006) Regulation of skeletal muscle gene expression by p38 MAP kinases. *Trends Cell Biol* **16**: 36–44
- Meriane M, Roux P, Primig M, Fort P, Gauthier-Rouviere C (2000) Critical activities of Rac1 and Cdc42Hs in skeletal myogenesis: antagonistic effects of JNK and p38 pathways. *Mol Biol Cell* **11**: 2513–2528
- Nebreda AR, Porras A (2000) p38 MAP kinases: beyond the stress response. *Trends Biochem Sci* **25**: 257–260
- Nemoto S, Sheng Z, Lin A (1998) Opposing effects of Jun kinase and p38 mitogen-activated protein kinases on cardiomyocyte hypertrophy. *Mol Cell Biol* **18**: 3518–3526
- Novitsch BG, Mulligan GJ, Jacks T, Lassar AB (1996) Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. *J Cell Biol* **135**: 441–456
- Novitsch BG, Spicer DB, Kim PS, Cheung WL, Lassar AB (1999) pRb is required for MEF2-dependent gene expression as well as cell cycle arrest during skeletal muscle differentiation. *Curr Biol* **9**: 449–459
- Nurse P (1994) Ordering S phase and M phase in the cell cycle. *Cell* **79**: 547–550
- Penn BH, Bergstrom DA, Dilworth FJ, Bengal E, Tapscott SJ (2004) A MyoD-generated feed-forward circuit temporally patterns gene expression during skeletal muscle differentiation. *Genes Dev* **18**: 2348–2353
- Porras A, Zuluaga S, Black E, Valladares A, Alvarez AM, Ambrosino C, Benito M, Nebreda AR (2004) P38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. *Mol Biol Cell* **15**: 922–933
- Puri PL, Iezzi S, Stiegler P, Chen TT, Schiltz RL, Muscat GE, Giordano A, Kedes L, Wang JY, Sartorelli V (2001) Class I histone deacetylases sequentially interact with MyoD and pRb during skeletal myogenesis. *Mol Cell* **8**: 885–897
- Rao SS, Chu C, Kohtz DS (1994) Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helix-loop-helix regulators. *Mol Cell Biol* **14**: 5259–5267
- Sabio G, Arthur JS, Kuma Y, Peggie M, Carr J, Murray-Tait V, Centeno F, Goedert M, Morrice NA, Cuenda A (2005) p38gamma regulates the localisation of SAP97 in the cytoskeleton by modulating its interaction with GKAP. *EMBO J* **24**: 1134–1145
- Sartorelli V, Caretti G (2005) Mechanisms underlying the transcriptional regulation of skeletal myogenesis. *Curr Opin Genet Dev* **15**: 528–535
- Sherr CJ (1994) G1 phase progression: cycling on cue. *Cell* **79**: 551–555
- Simone C, Forcales SV, Hill DA, Imbalzano AN, Latella L, Puri PL (2004) p38 pathway targets SWI-SNF chromatin-remodeling complex to muscle-specific loci. *Nat Genet* **36**: 738–743
- Skapek SX, Rhee J, Spicer DB, Lassar AB (1995) Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. *Science* **267**: 1022–1027
- Tamura K, Sudo T, Senftleben U, Dadak AM, Johnson R, Karin M (2000) Requirement for p38 alpha in erythropoietin expression: a role for stress kinases in erythropoiesis. *Cell* **102**: 221–231
- Tapscott SJ (2005) The circuitry of a master switch: MyoD and the regulation of skeletal muscle gene transcription. *Development* **132**: 2685–2695
- Wu Z, Woodring P, Bhakta K, Tamura K, Wen F, Feramisco JR, Karin M, Wang JY, Puri PL (2000) p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. *Mol Cell Biol* **20**: 3951–3964
- Zechner D, Craig R, Hanford DS, McDonough PM, Sabbadini RA, Glembotski CC (1998) MKK6 activates myocardial cell NF-kappaB and inhibits apoptosis in a p38 mitogen-activated protein kinase-dependent manner. *J Biol Chem* **273**: 8232–8239
- Zetser A, Greninger E, Bengal E (1999) p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation. Participation of the Mef2c transcription factor. *J Biol Chem* **274**: 5193–5200
- Zhao M, New L, Kravchenko VV, Kato Y, Gram H, Di Padova F, Olson EN, Ulevitch RJ, Han J (1999) Regulation of the MEF2 family of transcription factors by p38. *Mol Cell Biol* **19**: 21–30