

p38 MAP kinase regulates the expression of *XMyf5* and affects distinct myogenic programs during *Xenopus* development

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Abstract

The p38 MAPK signaling pathway is essential for skeletal muscle differentiation in tissue culture models. We demonstrate a novel role for p38 MAPK in myogenesis during early *Xenopus laevis* development. Interfering with p38 MAPK causes distinct defects in myogenesis. The initial expression of Myf5 is selectively blocked, while expression of MyoD is unaffected. Expression of a subset of muscle structural genes is reduced. Convergent extension movements are prevented and segmentation of the paraxial mesoderm is delayed, probably due to the failure of cells to withdraw from the cell cycle. Myotubes are properly formed; however, at later stages, they begin to degenerate, and the boundaries between somites disappear. Significant apoptotic cell death occurs in most parts of the somites. The ventral body wall muscle derived from migratory progenitor cells of the ventral somite region is poorly formed. Our data indicate that the developmental defects caused by p38 α -knockdown were mediated by the loss of *XMyf5* expression. Thus, this study identifies a specific intracellular pathway in which p38 MAPK and Myf5 proteins regulate a distinct myogenic program.

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Introduction

In mammals, it is well established that several factors secreted in the somite environment initiate the myogenic program characterized by the activation of two muscle regulatory factors (MRFs), Myf5 and MyoD (reviewed in Tajbakhsh and Buckingham, 2000). The secreted factors that control myogenesis in the somite include Sonic hedgehog (Shh), Wnt proteins, bone morphogenic factors (BMPs) and BMP antagonists such as Noggin (Buckingham and Cossu, 1997; Cossu and Borello, 1999; McGrew and Pourquie, 1998; Munsterberg et al., 1995). Although the MRFs are essential for the establishment of muscle-cell precursors and their differentiation, little is known about the intracellular signals activating their expression in the proper time and place during development.

In *Xenopus laevis*, the expression of *XMyf5* and *XMyoD* occurs during gastrulation in the presomitic mesoderm region

(dorsal–lateral marginal zone; DLMZ) that subsequently differentiates to myotomal muscle cells (Hopwood et al., 1991). The activation of these MRFs in the future muscle forming region occurs in response to mesoderm inducing factors like of the FGF, TGF β and Wnt families (Dale and Jones, 1999; Kimelman and Griffin, 2000; McDowell and Gurdon, 1999; Shi et al., 2002; Smith, 1995). Other crucial signaling molecules at these early gastrula stages are BMP antagonist proteins secreted from the Spemann organizer region. BMP antagonists dorsalize the DLMZ to muscle (Harland and Gerhart, 1997; Smith et al., 1993). Dorsalization of the DLMZ is necessary to preserve *XMyoD* expression in this region (Frank and Harland, 1991). BMP antagonism also induces the MAPK pathway, which increases MyoD protein levels and converts it into a potent differentiation factor (Zetser et al., 2001).

While the secreted ligands are known, the intracellular signaling proteins involved are still relatively unexplored. Cell culture studies suggest that the intracellular p38 MAPK pathway is involved in several aspects of myogenic determination and differentiation. The kinase activity of p38 MAPK is

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induced during the differentiation of certain myoblast cell lines (Cuenda and Cohen, 1999; Zetser et al., 1999). Inhibition of the pathway by different means completely abolishes *in vitro* differentiation (Wu et al., 2000; Zetser et al., 1999). Once activated, p38 stimulates the activities of MyoD/E47 heterodimers and MEF2 proteins, orchestrating the expression of many muscle-specific genes (Lluis et al., 2005). Interestingly, p38 activity facilitates MyoD and MEF2 binding to a subset of late-activated promoters (Penn et al., 2004). p38 participates in the transcription of this subset of genes by inducing chromatin modifications at their loci. p38 activity induces the recruitment of SWI-SNF remodeling complexes to muscle regulatory elements (Simone et al., 2004).

The studies of p38 function in myogenic cell lines are limited to specific stages of myogenesis, and are isolated from a real developmental context. Therefore, an animal model system that could study the involvement of p38 MAPK in early development of skeletal muscle is needed. p38 α null mice developed relatively normally (Adams et al., 2000; Tamura et al., 2000). The lack of any severe developmental phenotype might be attributed to the redundant function of other isoforms of p38, such as p38 β , in mice. Thus, double and/or triple knockout mice strains might be needed in order to analyze its function. In *X. laevis*, only two isoforms of p38 are known; p38 α (Mpk2) and p38 γ (SAPK3) (Perdiguero et al., 2003; Rouse et al., 1994). Cell culture studies have shown that p38 γ is probably not involved in the known functions of this signaling pathway in myogenesis (Cuenda and Cohen, 1999; Wu et al., 2000; Zetser et al., 1999). Therefore, in the present study, different approaches were used to inhibit p38 α in the developing *Xenopus* embryo. Interestingly, interference with the p38 pathway specifically prevented the expression of *XMyf5*, but not of *XMyoD*. Consequently, several defects in muscle development were observed. At gastrula–neurula stages, convergent extension cell movements occurring in paraxial mesoderm were impeded and the expression of distinct muscle-specific structural genes was reduced. At later neurula–early tailbud stages, a slight delay in segmentation occurred. By later tailbud stages, there was a general deterioration of the somite borders that was a result of myotube degeneration and massive apoptosis at the ventral (hypaxial) part of the trunk somites. The loss of migratory *pax3* expressing cells in the hypaxial somite led to poor body wall muscle formation. Our data indicate that the developmental defects caused by inhibition of p38 α were mediated by the loss of *XMyf5* expression. Therefore, this study delineates a specific intracellular pathway involving p38 MAPK and *XMyf5* proteins.

Materials and methods

Embryonic manipulation

Ovulation, *in vitro* fertilization, embryo culture and dissections were carried out as described (Re'em-Kalma et al., 1995). Embryos were staged according to (Nieuwkoop and Faber, 1967). Embryos were taken at different stages and analyzed for RT-PCR, Western blotting, immunohistochemistry and TUNEL assays. Ventral marginal zone (VMZ), dorsal–lateral marginal zone (DLMZ)

and dorsal marginal (DMZ) explants were removed at stage 10–10.25, and cultured to different stages as described (Bonstein et al., 1998). In some experiments, VMZ explants were treated with Noggin and the p38 inhibitor: SB 203580 (30 μ M).

Microinjections

Capped synthetic dominant negative MKK6 (DN MKK6) RNA (0.1 ng) and two morpholino antisense oligonucleotides, Xp38 α (5'GACGTAA-GATTGATTGGATGACATA3') and XMyf5 (5'AATGGCAGCTATC-TACCATCTCCAT3') were obtained (Gene Tools) along with a four nucleotide mismatch control p38 α morpholino (5'GACCTAACATTGATTCGAT-CACATA3') and injected (7–14 ng) into the marginal zone of one- or two-cell stage embryos in 0.3 \times MR containing 3% Ficol1. For rescue experiments, synthetic capped *XMyf5* RNA (Hopwood et al., 1991) was injected along with the p38 α morpholino into the marginal zone of one-cell stage embryos.

Western blot analysis

Protein extracts were prepared as described (Zetser et al., 2001). Equal amounts of extracted proteins were loaded (approximately 40 μ g), separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters by standard methods. CREB, phospho CREB, p38, phospho p38 were detected by polyclonal antibodies (Cell Signaling). Antibody binding was detected using the Pierce SuperSignal chemiluminescence detection system.

TUNEL staining

Embryos were fixed in MEMFA for 2 h, dehydrated, embedded in paraffin and sectioned. Transverse sections (5 μ m) were treated according to the supplied protocol (In situ Cell Death Detection Kit, Fluorescein, Roche Diagnostic, Mannheim, Germany). Nuclear counterstaining was performed using TO-PRO-3 iodide (Molecular Probes).

Whole-mount *in situ* hybridization and immunohistochemistry

Whole-mount *in situ* hybridization was carried out with digoxigenin-labeled probes (Harland, 1991; Hemmati-Brivanlou et al., 1990; Zetser et al., 2001). Embryos were cultured to different stages and subsequently fixed for *in situ* hybridization. *XMyoD* (Frank and Harland, 1991), *XMyf5* (Hopwood et al., 1991), *pax3* (Martin and Harland, 2001) and *muscle actin* (Hemmati-Brivanlou et al., 1990) specific probes were used. Whole-mount immunostaining was performed as described (Brivanlou and Harland, 1989). Hybridoma supernatant of 12/101-mature muscle antigen (Kintner and Brockes, 1984) was diluted 1:1. Antibody against PCNA (proliferation cell nuclear antigen) was used at a dilution of 1:250 (DakoCytomation). For immunohistochemistry on sections, slides were deparaffinized and hydrated with a decreased alcohol gradient. Then sections were blocked with goat serum for 1 h following 12 h incubation with the primary antibody (12/101, PCNA). Slides were incubated with a secondary rhodamine HRP-conjugated antibody, counterstained with TO-PRO-3 and were visualized by confocal microscopy.

RT-PCR

RT-PCR was performed as described (Wilson and Melton, 1994), except that random hexamers (100 ng per reaction) were used for reverse transcription. Primers for EF1 α (Hemmati-Brivanlou and Melton, 1994), *XMyoD* (Nicolas et al., 1998), and *muscle actin* (Wilson and Melton, 1994) were described. Primers for *XMyf5*, *desmin*, *MHC*, *tropomyosin* are as follows: *tropomyosin* (F 5'TGG AGA TGG CGG AGA AGA AG3'; R 5'GCA GCA AGT GGC AGT CAC GA3'); *MHC* (F 5'AGG ACC TGG TGG ACA AAC TTC A3'; R 5'TAT TGG AGG CAA GAA AGA GTC TGA AG3'); *desmin* (F 5'CCA GGG TGA ACG AGC TGT AT3'; R 5'TCA ACA TCC CCT CTG AAA GC3'); *XMyf5* (F 5'CTA TTC AGA ATG GAG ATG GT3'; R 5'GTC TTGGAG ACT CTC AAT A3').

Results

p38 MAPK is active in the dorsal–lateral marginal zone during early muscle determination

To study the possible involvement of p38 MAPK in early development of skeletal muscle, we compared the phosphorylation status of one substrate, the CREB protein in the ventral marginal zone (VMZ) and the dorsal–lateral marginal zone (DLMZ) from gastrula through neurula stages. VMZ and DLMZ explants were dissected at early gastrula stages, and cultured until sibling embryos reached neurula stages. Protein extracts were isolated from explants and CREB was detected by Western analysis (Fig. 1A). Levels of phosphorylated CREB were higher in neurula stage DLMZ explants versus VMZ explants (compare lanes 1 to 2 and 3 to 4). CREB can serve as a substrate for other kinases, and therefore other approaches were taken to ascertain that p38 MAPK was involved. We analyzed the phosphorylation status of p38 MAPK itself (Fig. 1A, right panel). A battery of commercial anti phospho p38 antibodies were analyzed (not shown), and only one weakly identified the protein. Still, we could consistently show that the phosphorylated form of p38 was found in the DLMZ, and not in the VMZ (right panel). In other experiments, VMZ explants were isolated at early gastrula stages and treated with Noggin, and SB203580, a p38 α/β inhibitor (see Fig. 1B). Noggin dorsalization of VMZ explants induced the phosphorylation of CREB relative to control VMZ explants. Cotreatment of explants with Noggin

and SB203580 inhibited CREB phosphorylation, suggesting the involvement of p38 MAPK. Overall, these results indicate that p38 MAPK is active in the DLMZ during the early stages of muscle specification.

*Inhibition of p38 MAPK prevents the expression of *XMyf5* but not of *XMyoD**

The functional role of p38 MAPK in myogenesis was explored. Three different approaches were used to block p38 MAPK activity in explants and embryos. In the first, VMZ explants were isolated at early gastrula stages and dorsalized by Noggin treatment. A pharmacological inhibitor of p38 MAPK, SB203580 was added to these explants. In the second approach, embryos at the one-cell stage were injected with RNA encoding an inactive form of MKK6 (DN MKK6), the direct kinase of p38 MAPK. MKK6 expression was verified by Western analysis of extracts from the injected embryos (Fig. 2A2, lower panel). In the third approach, embryos were injected with a p38 α MAPK antisense morpholino (p38 α MO) or with a control morpholino (p38 α CMO). A substantial decrease of p38 protein levels in p38 α MO-injected embryos was observed by Western analysis (Fig. 2A3, lower panel). To confirm that p38 activity was also affected in these embryos, an *in vitro* kinase assay was performed (Supplementary Fig. S1). Kinase activity of p38 in extracts from p38 α MO-injected embryonic DLMZ explants was significantly lower than in control explants. Next, DLMZ explants were isolated from injected embryos at early gastrula stages and cultured until sibling embryos reached neurula stages. RNA was extracted and the expression of MRFs was analyzed by RT-PCR (Fig. 2A). Using all three p38 inhibitors, we found substantially lower levels of *XMyf5* transcripts in treated embryos relative to control embryos. The levels of *XMyf5* transcripts were not changed in p38 α CMO-injected, relative to control embryos. In contrast to *XMyf5*, levels of *XMyoD* transcripts were unchanged in these experiments, suggesting that p38 MAPK selectively affects *XMyf5* and not *XMyoD*. The levels of *muscle actin* were only marginally reduced in treated embryos relative to controls. Our experiments indicate that DN MKK6 and p38 α MO caused identical myogenic phenotypes, thus we chose to present p38 α MO knockdown experiments.

To determine if p38-knockdown affected early expression of *XMyf5*, embryos were injected into one-cell stage embryos with the p38 α MO. *XMyoD* and *XMyf5* expression was examined at late gastrula–early neurula stages (Fig. 2B). The expression of *XMyf5* is significantly reduced in the injected embryos (90% of injected embryos did not express detectable levels of *XMyf5*, $n = 40$; Fig. 2B, lower panels) in comparison to the uninjected control embryos (90% expressed *XMyf5*, $n = 62$). The expression of *XMyoD* was robust in both injected and uninjected control embryos (Fig. 2B, upper panels). To further analyze the expression of these MRFs during later stages of development, both blastomeres of two-cell stage embryos were injected with the p38 α MO, and embryos were grown to tailbud stages and examined for *XMyoD* and *XMyf5* expression (Fig. 2C). In uninjected controls, *XMyf5* expression

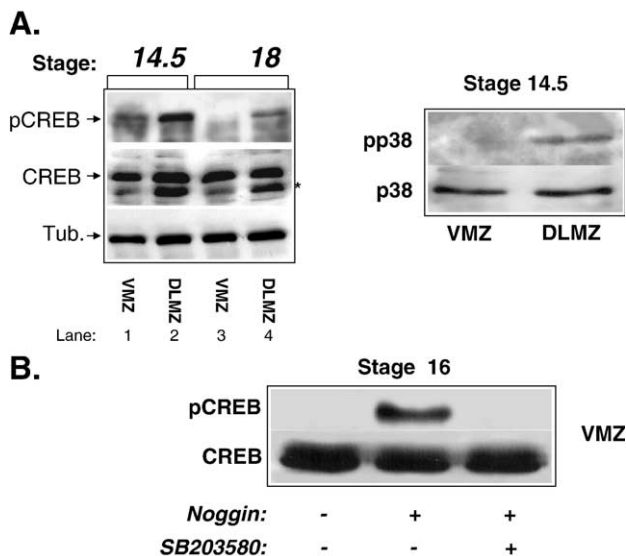


Fig. 1. p38 MAPK and its substrate CREB are phosphorylated in DLMZ but not in VMZ explants. (A) VMZ and DLMZ explants were dissected at stage 10.25, and cultured until sibling embryos reached later gastrula and neurula stages as indicated. Proteins were extracted from 18 explants and CREB, phospho CREB (pCREB), α tubulin, p38 and phospho p38 (pp38) were detected by Western analysis. The asterisk represents an unidentified cross-reacting protein. (B) VMZ explants were isolated at stage 10+, incubated with Noggin or SB203580 (30 μ M) and cultured until sibling embryos reached stage 16. Proteins were extracted from 18 explants. CREB and phospho CREB were detected by Western blotting.

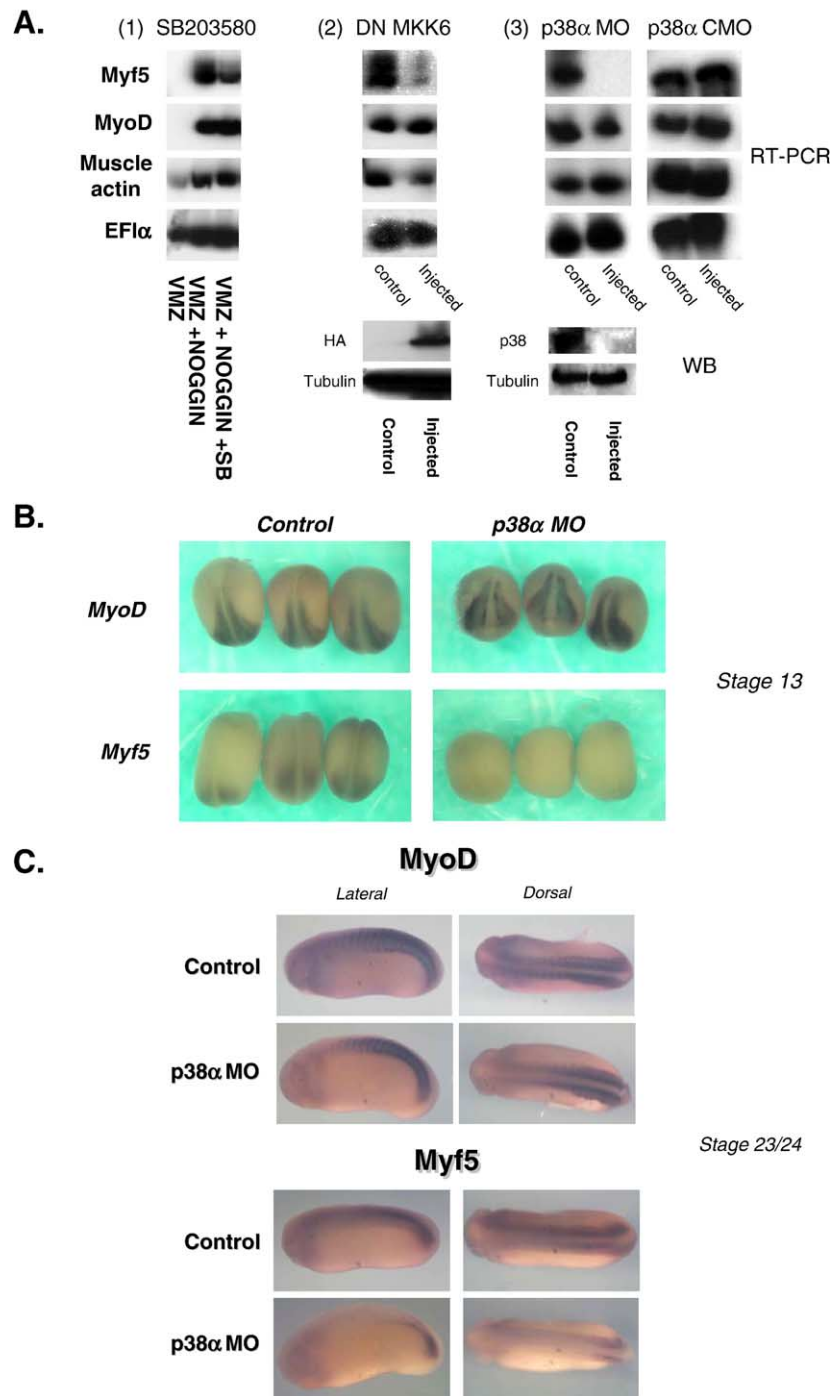


Fig. 2. Inhibition of p38 blocks the expression of *XMyf5* without affecting *XMyoD* expression. (A) 18 VMZ explants were dissected at stage 10.25 from control embryos and incubated with Noggin and SB (30 μ m) [1]. In parallel, embryos at the one-cell stage were injected with 0.5 ng of in vitro transcribed DN MKK6 mRNA [2], or with 7.5 ng of p38 α antisense morpholino (p38 α MO) [3], or with 7.5 ng of control antisense morpholino (p38 α CMO) [3]. At stage 10.25, 18 DLMZ explants were dissected from control and treated embryos. Explants were cultured until sibling embryos reached early neurula stages, and total RNA was isolated. RT PCR analysis was performed with primers to *XMyf5*, *XMyoD*, *muscle actin* and *EF1 α* serving as a control for quantifying RNA levels of different samples. Control-RT PCR was also performed (not shown). For Western analysis, DLMZ explants from p38 α MO, DN MKK6-injected and control embryos were cultured until sibling embryos reached later gastrula stages. Proteins were extracted from explants. Antibodies to HA were used to detect ectopic DN MKK6 protein, and antibodies to p38, to detect endogenous p38 α protein. (B, C) Expression patterns of the myogenic transcription factors *XMyf5* and *XMyoD* at different developmental stages. One-cell stage embryos were injected with p38 α MO. At stages 13 (B) and 23/24 (C), embryos were fixed and analyzed for the expression of *XMyoD* and *XMyf5* by whole-mount in situ hybridization. (B) 92% of control embryos were expressed *XMyoD* ($n = 51$) and 94% of the p38 α MO-injected embryos expressed *XMyoD* ($n = 35$). 90% of control embryos expressed for *XMyf5* ($n = 62$) and only 20% of the p38 α MO-injected embryos expressed *XMyf5* ($n = 40$). Dorsal view, anterior-top. (C) 93% of control embryos expressed *XMyoD* ($n = 30$) and 90% of p38 α MO-injected embryos expressed *XMyoD* ($n = 27$). 97% of control embryos expressed *XMyf5* in the somites ($n = 35$) and 16% of p38 α MO-injected embryos were stained for *XMyf5* in the somites ($n = 40$). Anterior-left.

appeared mainly in the lips of the somites while *XMyoD* was stained throughout the somites in a distinct segmental pattern (Martin and Harland, 2001). In most p38 α MO-injected embryos, *XMyf5* staining was specifically affected while *XMyoD* was not, relative to controls (Fig. 2C). Knockdown of p38 MAPK reduced anterior somitic staining of *XMyf5*, while it did not affect posterior presomitic staining. These results suggest that p38 MAPK is needed for the initial expression of *XMyf5* during gastrulation and also for later expression in the somites, but not in the tail. This observation indicates that p38 MAPK function may be limited to distinct myogenic programs in *Xenopus* development (Nicolas et al., 1998).

Knockdown of p38 MAPK prevents convergent extension of the paraxial mesoderm

Embryos depleted of p38 MAPK underwent cleavage and gastrulation normally, suggesting that p38 was not required for early embryonic development. However, as development continued, several defects became apparent. By stage 25, control embryos had developed an elongated shape, while treated embryos failed to completely elongate along the anterior–posterior axis (data not shown). The reduced embryo body length could be caused by a defect in mesoderm induction

or by a defect in convergent extension. The first possibility seemed unlikely, as we observed no major differences in the levels of *XMyoD* expression in p38-knockdown embryos relative to control embryos (Fig. 2). Furthermore, the early differentiation of paraxial mesoderm into muscle tissue appeared normal in treated embryos, as judged by the expression of *muscle actin* (Fig. 2A). We then examined convergent extension by comparing the ability of DLMZ explants to elongate. Knockdown of p38 MAPK activity significantly reduced elongation of DLMZ explants relative to control explants (Fig. 3A, upper panels). In p38-knockdown embryos, DMZ explants elongated in a normal manner, similar to control DMZ explants (Fig. 3A, lower panel). In later stages of development, notochord development also appears normal in p38-knockdown embryos (data not shown). Therefore, the loss of convergent extension appears to be specifically restricted to muscle-fated paraxial and not notochord-fated axial mesodermal regions of the embryo.

The defect in convergent extension could also be observed by detecting *XMyoD* expression in embryos (Fig. 2B). *XMyoD* is expressed symmetrically along the anterior–posterior axis, with its expression extending laterally at the posterior end (Fig. 2B). The *XMyoD* expression pattern is more laterally broadened, being positioned at a farther distance from the dorsal midline in p38 α MO-injected embryos compared to control embryos. The

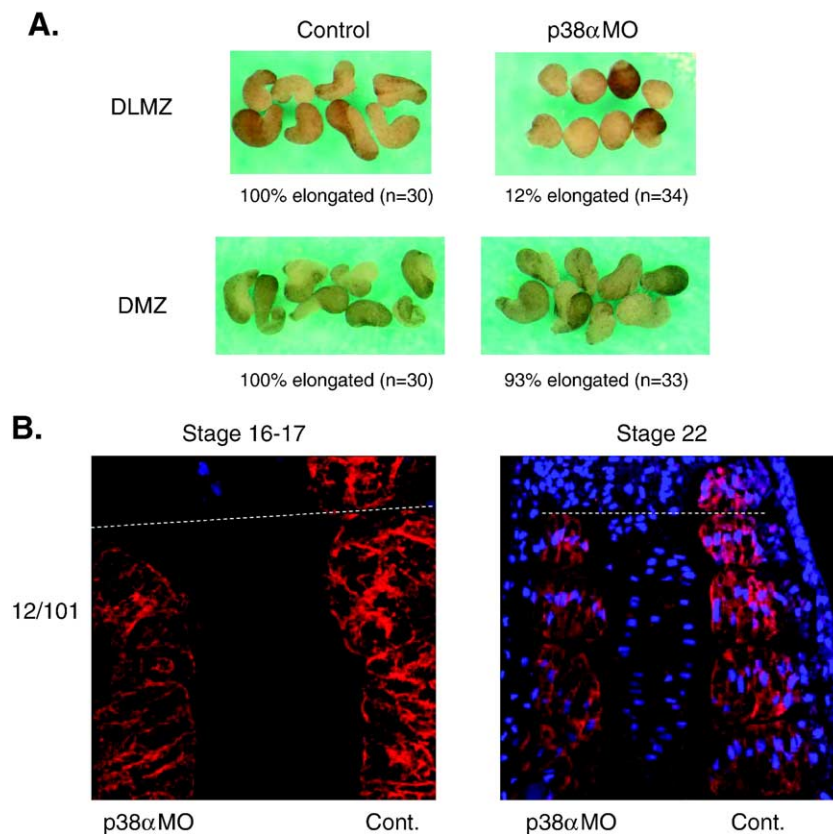


Fig. 3. Inhibition of p38 reduces convergent extension of presomitic mesoderm. (A) Embryos were injected with p38 α MO. DLMZ and DMZ explants were dissected at stage 10.25 and cultured until sibling embryos reached early neurula stages. (B) Longitudinal sections of embryos ($n = 5$) at stages 16 and 22, injected with p38 α MO in one blastomere at the two-cell stage, were immunostained, using the 12/101 antibody and viewed by confocal microscopy. Red staining represents presomitic and somitic mesoderm (12/101) and blue represents nuclei staining (TO-PRO3 iodide). Results shown are from one embryo. Similar results were obtained with all five embryos.

gap between the left and right symmetrical *XMyoD* expression blocks is wider and it appears that these cells have not undergone proper medial intercalation towards the dorsal midline during gastrulation. p38 α MO embryos were also about 15% shorter in length at these stages, in comparison to control embryos (Fig. 2B). Similar *XMyoD* expression patterns were seen in paraxial mesoderm in which convergent extension was inhibited by Ca⁺² channel blockers or by preventing cell cycle arrest (Leise and Mueller, 2004; Wallingford et al., 2002).

Another distinct consequence of p38-knockdown was observed at later neurula–tailbud stages, when somites begin to form. To view the forming somites, embryos were longitudinally-sectioned, and immunostained with the 12/101 antibody (Fig. 3B). At stage 16 (left panel), the mesoderm of the MO-treated side has not extended anteriorly as that of the control side. Additionally, one somite has formed on the control side, while in the MO-injected side, the presomitic mesoderm (PSM) has not segmented yet. Later, at stage 22, somites are formed on the p38 α MO-injected side, though in a delayed manner relative to the control side (right panel). The first somite of the MO-injected side is aligned with the second somite on the control side of the embryo. Overall, it appears that the process of PSM segmentation is normally initiated in the MO-injected side of the embryo, although the process is delayed relative to the uninjected control side.

Knockdown of p38 prevents cell cycle arrest

It is well established that mesoderm cells stop dividing with the onset of gastrulation through neurulation as the dorsal mesoderm delineates into axial and paraxial mesoderm (Hardcastle and Papalopulu, 2000; Saka and Smith, 2001). The paraxial mesoderm re-enters the cell cycle only after a somite has formed (Saka and Smith, 2001). Recently, it was shown that cell cycle withdrawal during these developmental stages is required for convergent extension and somitogenesis of the paraxial mesoderm (Leise and Mueller, 2004). Therefore, we analyzed whether the knockdown of p38 affected the cell cycle of paraxial mesoderm during early neurula stages. Two-cell stage embryos were unilaterally injected to one blastomere with p38 α MO; embryos were cultured to stage 15, longitudinally-sectioned, and dividing cells were detected by their positive immunostaining with antibodies to PCNA (Fig. 4). In agreement with previous studies, most of the cells within the paraxial mesoderm of the uninjected control side (right of the dashed line) did not stain for PCNA, suggesting that they were resting cells. In sharp contrast, most of the cells within the paraxial mesoderm on the p38 α MO-injected side (left of the dashed line) positively stained for PCNA. Additionally, the higher absolute number of nuclei in the MO-injected side relative to the control side indicates the differences in cell division between the two sides of the embryo (Fig. 4). Thus, we can conclude that p38 MAPK is most likely involved in cell cycle withdrawal in the paraxial mesoderm during gastrula to neurula stages. Cell proliferation caused by reduced p38 levels could explain the defects in convergent extension and the delay in somitogenesis (Leise and Mueller, 2004).

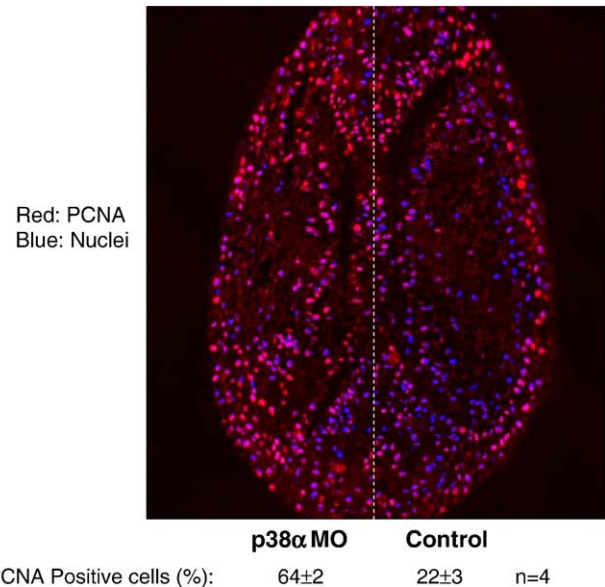


Fig. 4. Inhibition of p38 prevents cell cycle arrest in the presomitic mesoderm. Two-cell embryos were injected unilaterally into one blastomere with p38 α MO (7.5 ng). Embryos were cultured until late gastrula-early neurula stages, then fixed and longitudinally-sectioned. Proliferation cell nuclear antigen (PCNA) antibody was used to detect nuclei of proliferating cells (red staining). Nuclear counterstaining was done with TO-PRO3 iodide (blue staining). Section of one embryo is shown. Similar results were obtained with all other embryos ($n = 4$). Statistical analysis from the four embryos is presented.

Loss of somitic boundaries occurs at tailbud stages in embryos injected with the p38 α antisense morpholino

Results presented in Fig. 3B suggest that the initial segmentation of somites in the MO-injected side of embryos occurs fairly normally, though delayed relative to the control-uninjected side. Next, we examined somite morphology and muscle fiber structure at later tailbud stages. In whole-mount staining of *muscle actin*, the segmental pattern of the somites is clearly detected in control embryos (Fig. 5A, upper panel). In contrast, somite boundaries cannot be detected in those embryos injected with the p38 α MO. Loss of somite boundaries could be the outcome of an abnormal structure of the myofibers. To view myofibers, longitudinal sections of the same stage embryos as above were immunostained with an antibody to skeletal muscle specific antigen, 12/101 (Fig. 5A, lower panel). Myofibers of control embryos extend from one somite boundary to the next in a parallel orientation, and their staining is intense and uniform. In p38-knockdown embryos, the intensity of staining is much weaker and boundaries between somites can no longer be detected (Fig. 5A, lower panel). This abnormal staining indicates that myotubes may be damaged in MO-injected embryos. To view myotubes at a better resolution, longitudinal sections were immunostained with muscle-specific antibodies (Fig. 5B). Myotube diameter and the intensity of staining are significantly higher in controls versus p38-knockdown embryos. Overall, these data indicate that myotubes are formed, though specific deficiencies may account for defects in myotube structure in p38-knockdown embryos. Since the expression of *XMyf5*, but not of *XMyoD* is reduced in the p38-knockdown embryos, we hypothesized that

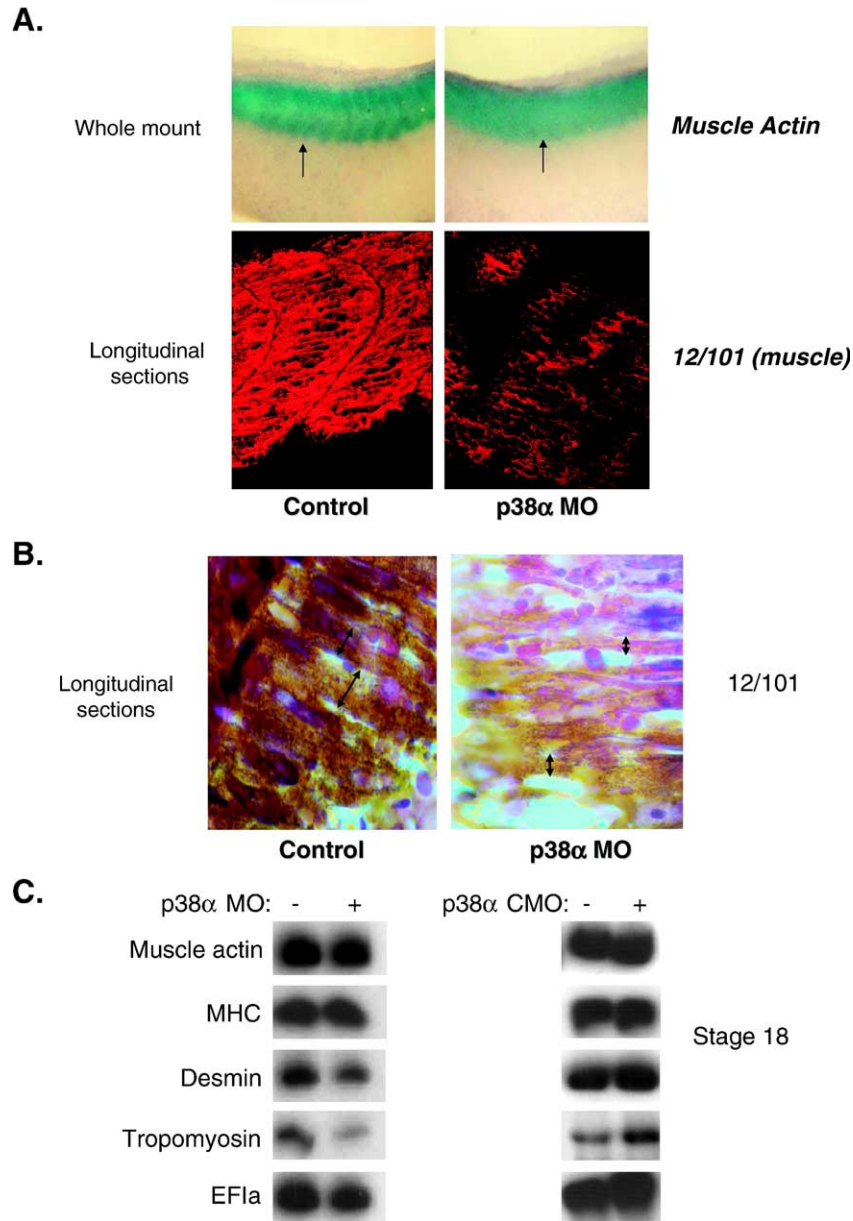


Fig. 5. Somite boundaries and myotube structure are disrupted in p38 α -knockdown embryos. (A) Embryos ($n = 15$) were injected with p38 α MO and cultured until they reached stage 25. Embryos were fixed and taken for whole-mount in situ hybridization for detecting *muscle actin* (upper panel). Somite patterning was observed on longitudinal sections immunostained with the 12/101 antibody and analyzed by confocal microscopy (lower panel) ($n = 7$). (B) Longitudinal sections of MO-injected and uninjected embryos (stage 25) were immunostained with the 12/101 antibody and analyzed under light microscopy ($n = 6$). Double arrows show myotube diameter. (C) 18 DLMZ explants were removed from each uninjected and p38 α MO-injected embryos and cultured until sibling embryos reached stage 18 and RNA was isolated. RT-PCR analysis was performed with primers to *MHC*, *desmin*, *tropomyosin*, *muscle actin* and *EF1a* serving as a control for quantifying RNA levels in the different samples. Control-RT PCR was performed (not shown). All injected embryos in panels A and B displayed similar phenotypes.

the expression of distinct muscle-specific structural genes could be affected in these embryos. Hence, the expression of several structural genes was compared between control and p38-knockdown embryos. Whereas, the expression of *muscle actin* and *myosin heavy chain* was comparable, the levels of *desmin* and *tropomyosin* were lower in p38-knockdown embryos, relative to controls (Fig. 5C). Expression levels of *tropomyosin* and *desmin* were not affected in embryos injected with the control morpholino. These results suggest that p38 MAPK is involved in the expression of distinct muscle structural genes. Lower expression of certain muscle structural

proteins may explain the defects in myotube structure observed in p38-knockdown embryos.

Cells in large parts of the somite undergo apoptotic cell death in p38 α MO-injected embryos

In other model systems, it was shown that defects in myoblast cell cycle withdrawal and abnormal expression of structural genes could lead to apoptosis and muscle degeneration (Walsh and Perlman, 1997). The relatively low myofiber-staining (Fig. 5) suggests that apoptotic cell death could be

occurring in the somites of p38 α MO-injected embryos. Therefore, we analyzed apoptotic cell death in the somites, at the same developmental stages (Fig. 6A). TUNEL staining of a transverse section shows that significant apoptosis occurs in the somites of the p38 α MO-injected half, but not in the control half of the embryo. Specifically, the majority of TUNEL-positive cells are concentrated in the ventral half of the somite,

while the dorsal part is unaffected (Fig. 6A). Interestingly, at tailbud stages, the expression of *XMyf5* is restricted to the dorso-medial and ventro-lateral lips of the somites (Martin and Harland, 2001), suggesting that apoptosis is not completely overlapping the regions of *XMyf5* expression.

To further analyze the consequences of the apoptotic process at these particular somitic regions, stage 31 embryos

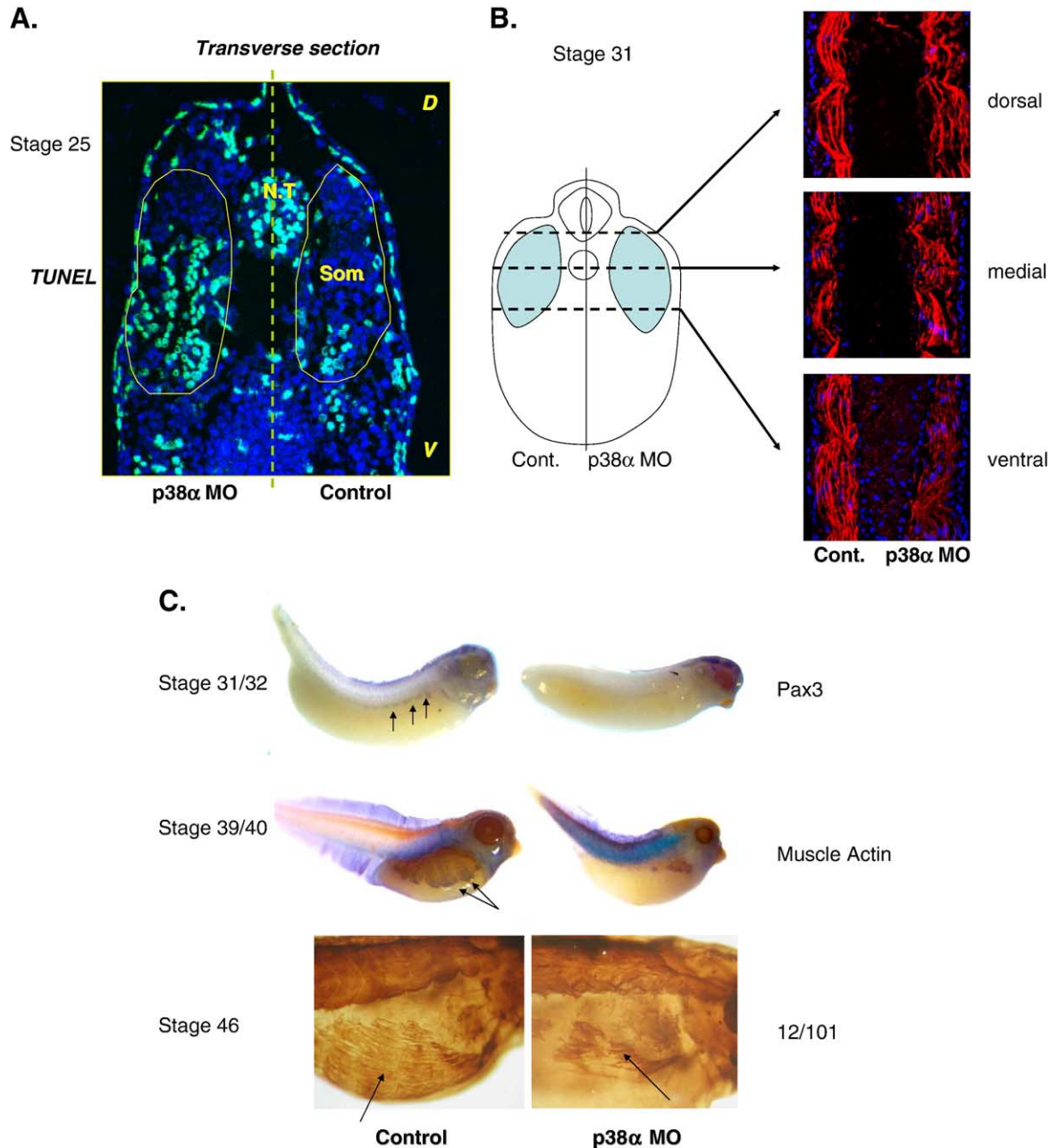


Fig. 6. The ventral portions of the somite undergo apoptotic cell death in tailbud p38-knockdown embryos. (A) Two-cell embryos were injected unilaterally into one blastomere with p38 α MO (7.5 ng). Embryos were cultured to stage 25. Four embryos were transversely-sectioned and TUNEL analysis was performed followed by TRO-PRO3 staining. One sectioned embryo is shown. Similar results were obtained with all other embryos ($n = 4$). Cells undergoing apoptosis are stained in light blue. Approximate somite borders are schematically presented. Som. is somite and N.T. is neural tube. (B) Two cell embryos ($n = 5$) were injected unilaterally with p38 α MO (7.5 ng). Embryos were cultured to stage 31. Injected embryos were longitudinally sectioned at different dorso-ventral levels of the somites and immunostained with the 12/101 antibody (red staining) followed by TRO-PRO3 staining (blue staining). One sectioned embryo is shown. Similar results were obtained with all other embryos ($n = 5$). (C) Embryos were injected with p38 α MO. Embryos were fixed and taken for whole-mount in situ hybridization to *pax3* and *muscle actin*. Arrows point at migrating muscle progenitor cells. Embryos were injected with p38 α MO and cultured to stage 46. Whole embryos were fixed, immunostained with the 12/101 antibody and analyzed by light microscopy. Arrows point at muscle cells (lower panel).

were longitudinally sectioned at different dorso-ventral levels of the somites and immunostained to detect myofibers (Fig. 6B). The severity of defects in myofibers is correlated with the degree of apoptosis in the different planes of the somite. Very weak muscle staining and disorganized muscle fibers are observed in the ventral plane of the somite (lower panel). The defects are much less pronounced in the medial and dorsal planes. Thus, the most severe effects of p38-knockdown are massive apoptotic cell death and muscle fiber degeneration, which occur in the ventral half of the somite where *XMyf5* is normally expressed.

The ventral body wall muscle is formed in *Xenopus* from migratory precursors originating in the ventral part of the somite (Martin and Harland, 2001). As muscle cells in this region of the somites undergo extensive apoptosis in p38-knockdown embryos, one would expect that muscle precursor migration to form the ventral body wall might be affected. The ventral migratory precursors can be identified as *pax3*-expressing cells (Martin and Harland, 2001). Expression of *pax3* is almost absent in the same region of embryos injected with p38 α MO, in comparison to controls (Fig. 6C). The migratory pathway of muscle cells from the hypaxial somite towards the ventral body plain was viewed by whole-mount staining of *muscle actin* in tadpole stage embryos (Fig. 6C). Clusters of muscle actin-expressing cells (marked by arrows in the figure) are found in between the somites and ventral region of control embryos. These clusters are almost completely absent in the embryos injected with p38 α MO, suggesting that the formation of migratory expressing *pax3* muscle precursors was prevented. At later stages when ventral muscle wall is already formed in wild type embryos (lower panel, control), very few myofibers are stained in MO-injected embryos. Therefore, the robust apoptosis and degeneration of muscle fibers in the ventral portion of the somite are the likely causes for absence of precursor cell migration and formation of ventral muscle wall in p38-knockdown embryos.

Exogenous expression of XMyf5 rescues the phenotype mediated by p38-knockdown

The observation that levels of *XMyf5*, but not of *XMyoD* were reduced in p38-knockdown embryos raised that possibility that the muscle phenotypes of these embryos were mediated by the absence of *XMyf5* expression. For investigating this possibility, we microinjected both blastomeres of two-cell stage embryos with an *XMyf5* antisense morpholino oligonucleotide (*XMyf5* MO). First, we examined convergent extension by comparing the ability of DLMZ explants to elongate. Knockdown of Myf5 significantly reduced elongation of DLMZ explants (Fig. 7A). Next, *XMyf5*-knockdown embryos were cultured to stage 30 and the somites were identified by whole-mount staining of *muscle actin* (Fig. 7B). *Muscle actin* expression levels were similar in both control and *XMyf5* MO-injected embryos, though the segmental pattern of the injected embryos was eliminated relative to controls. We also analyzed the

expression of several muscle-specific structural genes (Fig. 7C). The expression of *muscle actin* and *MHC* was unaffected, though expression of *desmin* and *tropomyosin* was reduced in embryos injected with *XMyf5* MO relative to control embryos. This expression profile is identical to that of embryos injected with p38 α MO (compare Fig. 7C to 5C). Finally, we analyzed apoptotic cell death in the somites of *XMyf5* MO-injected embryos by TUNEL staining of cross-sectioned embryos (Supplementary Fig. S2). Like in p38-knockdown embryos, significant apoptosis occurred in the somites of the *XMyf5* MO-injected half, but not in the control half of the embryos. Therefore, it appears that all aspects of the phenotype of *XMyf5*-knockdown analyzed are similar to that of p38-knockdown.

To further explore the involvement of Myf5 in the phenotype of p38-depleted embryos, we determined whether ectopic expression of *XMyf5* could restore normal development to these embryos (Fig. 7D). Injection of the p38 α MO prevented the segmental pattern of somites seen in control embryos (compare middle to left panel). Embryos, coinjected with *XMyf5*-encoding RNA and the p38 α MO showed a very distinct segmental pattern; they appeared identical to control embryos. Next, we analyzed whether the ectopic expression of *XMyf5* could restore the expression of specific skeletal muscle genes (Fig. 7D, lower panel). The repressed expression of *desmin* and *tropomyosin* in p38-knockdown embryos was restored in embryos injected with *XMyf5* mRNA. Therefore, we can conclude that *XMyf5* is a major downstream target of the p38 MAPK signaling pathway in muscle development.

Discussion

In this study, we have identified a unique role for the p38 MAPK signaling pathway in embryonic muscle cell formation. Knockdown of p38 affects very specific aspects of skeletal muscle formation and maintenance, but not the overall process of mesoderm or muscle cell induction. This result is quite surprising, since p38 levels are likely reduced in all tissues, while the effects are specific to muscle.

p38 MAPK and XMyf5 expression

In p38-knockdown embryos, *XMyf5* expression was highly reduced in late gastrula stage mesoderm, yet *XMyoD* expression was not altered. The expression of *XMyf5* initiates at early gastrula stages prior to the expression of *XMyoD*. *XMyf5* expression is restricted in two symmetrical dorso-lateral domains (Dosch et al., 1997), whereas early gastrula expression of *XMyoD* occurs in the entire marginal zone with the exception of the dorsal domain (Frank and Harland, 1991). Later in development, during somitogenesis, *XMyf5* expression is restricted to the posterior somites and the tail, while *XMyoD* is expressed in more mature anterior somites (Dosch et al., 1997). Their expression within the somites is also different; *XMyoD* is expressed throughout the myotome, while *XMyf5* expression is restricted to the most ventral and dorsal regions of the somite (Martin and Harland, 2001).

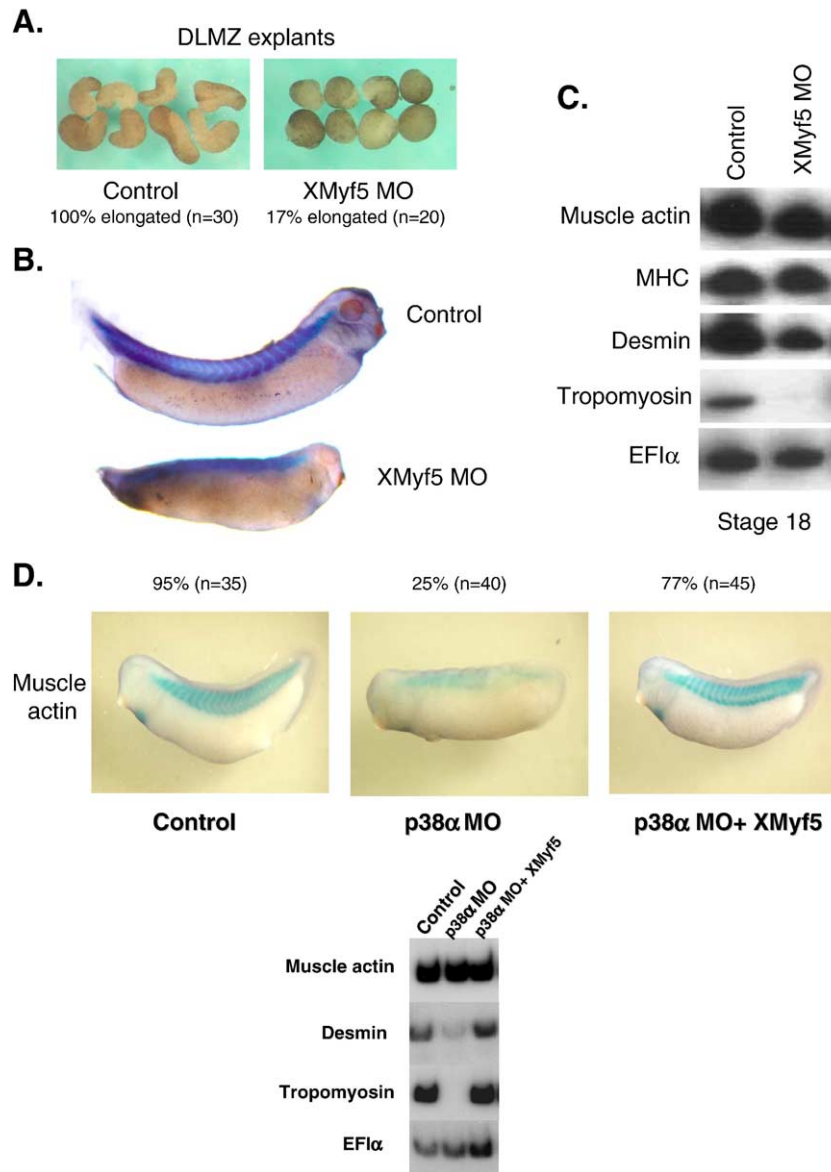


Fig. 7. Exogenous expression of *XMyf5* rescues the phenotype of p38-knockdown embryos. (A) Embryos were injected with *XMyf5* MO. DLMZ explants were dissected at stage 10.25 and cultured until sibling embryos reached early neurula stages. (B) Embryos were injected with *XMyf5* MO and cultured to tadpole stage. Embryos were fixed and taken for whole-mount in situ hybridization using a probe of *muscle actin*. 95% of control embryos displayed segmental phenotype ($n = 38$). 92% of MO-injected embryos lost the segmented phenotype ($n = 41$). (C) Embryos were injected with the *XMyf5* MO. 18 DLMZ explants were dissected at stages 10.25 and cultured until sibling embryos reached early neurula stages (stage 18). RNA was isolated and RT PCR analysis was performed with primers to *MHC*, *desmin*, *tropomyosin*, *muscle actin* and *EF1 α* serving as a control for quantifying RNA levels in each sample. Control-RT PCR reactions were performed (not shown). (D) Embryos were injected with *XMyf5*-encoding RNA (0.8 ng), and p38 α MO and cultured until control embryos reached stage 29/30. Embryos were fixed and analyzed by whole-mount in situ hybridization using the *muscle actin* probe (upper panel). Statistical analysis relates to the percentage of embryos displaying normal segmented phenotypes. Embryos were injected as described above. 18 DLMZ explants were dissected at stages 10.25 and cultured until sibling embryos reached early neurula stages (stage 18). RNA was isolated and RT PCR analysis was performed with primers to *desmin*, *tropomyosin*, *muscle actin* and *EF1 α* (lower panel).

These results emphasize the differences in the regulation of *XMyf5* and *XMyoD* expression. Moreover, in this work, we show that p38 MAPK controls the more anterior somite expression of *XMyf5*, but not its expression in the tail (Fig. 2C). Thus, this study identifies an intracellular pathway in which p38 MAPK modulates specific temporal and spatial expression of *XMyf5*.

Our observations strongly suggest that all of the myogenic phenotypes observed in p38-knockdown embryos could be attributed to the lack of *XMyf5* expression (Fig. 7). Supporting

our results, in zebrafish embryos, injection of *Myf5* MO caused a very similar phenotype. In these embryos, convergent extension was inhibited, somites were abnormally patterned, *desmin* expression was reduced, yet *MyoD* expression was normal (Chen and Tsai, 2002).

One obvious next goal would be to investigate how p38 MAPK regulates the expression of *XMyf5*. One approach is to study the possible cross-talk of p38 MAPK with other signaling molecules affecting the expression of *XMyf5*. Previous studies in *Xenopus* suggested that the *XMyf5* gene

was a direct target of canonical wnt signaling (Shi et al., 2002). It will be interesting to determine the epistatic relationship between p38 and wnt signaling in regulating *XMyf5* expression and subsequent muscle formation. *Myf5* expression has recently been demonstrated to be regulated by CREB protein in a wnt-dependent manner during mouse myogenesis (Chen et al., 2005). We show that p38 activity is required for CREB protein phosphorylation (Fig. 1). Thus, it is tempting to speculate that p38 and wnt-signaling pathways could converge and regulate the overall levels of activated CREB protein required for embryonic *Myf5* expression.

A second approach is to investigate the regulatory sequences of the *XMyf5* promoter. The *XMyf5* promoter region has been mapped in *X. laevis* and *tropicalis* (Polli and Amaya, 2002). Several transcriptional activators and repressors are involved in the precise temporal expression of *XMyf5*. Interestingly, a TCF binding site suggesting the involvement of the canonical wnt pathway was identified in distal regulatory sequences of *XMyf5* (Yang et al., 2002). However, the site which binds XTcf3 protein mediates repression of *XMyf5* transcription in the dorsal and ventral midline, but does not contribute to its activation. Thus, it cannot explain how the wnt pathway may activate the transcription of *XMyf5*. Other studies suggest that Vent1 protein functions to repress *XMyf5* expression in the ventral domain of the marginal zone (Polli and Amaya, 2002). *Vent1* expression in the marginal zone region is highly regulated during gastrulation (Gawantka et al., 1995), and perhaps p38 signaling represses the expression of the *Vent1* gene, thus enabling *XMyf5* expression only in the dorsal lateral mesoderm. T-box proteins have been shown to activate transcription of the *XMyf5* gene (Lin et al., 2003) and could be potential targets for p38 signaling. Identification of p38 responsive *cis*-elements in the *XMyf5* promoter would surely unravel the pathway connecting p38 signaling to early muscle development.

Convergent extension movements of paraxial mesoderm are affected by p38 and XMyf5

One of the earliest effects of p38-knockdown was the failure of gastrula-stage DLMZ explants to efficiently elongate, suggesting that convergent extension movements, characteristic of these presomitic mesoderm cells were disrupted (Fig. 3). The typical cell cycle arrest observed in gastrula–neurula stage paraxial mesoderm cells was also lost (Fig. 4). Cell cycle withdrawal was demonstrated to be essential for proper convergent extension and somitogenesis in paraxial mesoderm (Leise and Mueller, 2004). In other cellular systems, p38 was shown to be an important regulator of cell cycle withdrawal (Ambrosino and Nebreda, 2001). Therefore, during these specific stages, p38 MAPK may function to arrest cell cycle via the cell cycle machinery. Cell cycle regulatory proteins, such as p27Xic1 and the Wee2 cdk inhibitors play a crucial role in early *Xenopus* muscle formation (Leise and Mueller, 2004; Vernon and Philpott, 2003). Depletion of the Wee2 protein in *Xenopus* embryos prevented cell cycle arrest and allowed continued proliferation

of paraxial mesoderm cells (Leise and Mueller, 2004). These embryos displayed poor convergent extension, elongation and somitogenesis. Hence, p38 and Wee2 knockdowns cause strikingly similar phenotypes. It will be interesting to investigate how the p38/*XMyf5* pathway communicates with proteins of the cell cycle machinery. The finding of this study that *XMyf5*-knockdown reduced DLMZ elongation indicates that p38 MAPK activity in convergent extension is mediated by *XMyf5*. The involvement of *XMyf5* in convergent extension movements could be mediated by its transcriptional activity. For example, activation of *p27Xic1* and/or *Wee2* transcription could link *XMyf5* to the prerequisite cell cycle exit, necessary for these cellular movements.

Segmentation is delayed in p38-knockdown embryos

In p38-knockdown embryos, segmentation of PSM occurred fairly normally, though in a delayed manner (Fig. 3B). This finding suggests that the segmentation clock which acts in the PSM behaves normally. Thus, cyclic expression of components of the *Notch* signaling pathway (Jen et al., 1997, 1999) is probably not affected by p38 MAPK. However, the delay in segmentation might be explained by high local concentrations of FGF8 at the anterior-most region of the PSM. FGF8 is expressed in the paraxial mesoderm in *Xenopus* (Monsoro-Burq et al., 2003). Normally, FGF8 is secreted at the posterior end of the PSM, and a gradient of this morphogen is created from the posterior end to the anterior boundary of the PSM (Pourquie, 2003). It was proposed that because of the progressive decrease of FGF8 expression during maturation of the PSM, when cells become located in the anterior PSM, they reach a threshold of FGF signaling allowing them to activate their segmentation program. Delay in segmentation could therefore be a result of an inhibitory concentration of FGF8 at the anterior PSM. Defected elongation of p38-knockdown embryos along the A–P axis could affect a FGF8 gradient, creating high levels of FGF8 concentrations at the anterior PSM and thus delaying segmentation.

Muscle degeneration in the ventral region of somites in p38-knockdown embryos

At tailbud stages, somite boundaries were blurred in p38-knockdown embryos. Myofiber staining was weaker and disorganized in comparison to controls; myotube diameters were also reduced. These results together with muscle staining at earlier neurula stages suggest that differentiation may occur normally, and the defects observed were in the maintenance of muscle fibers. Muscle degeneration could be a consequence of incomplete withdrawal from the cell cycle (Walsh and Perlman, 1997), and/or the improper expression of *desmin* and *tropomyosin*. Disruption of Desmin and Vimentin protein function in intermediate muscle filaments was shown to perturb intersomite junction (ISJ) organization in tadpole stage embryos (Cary and Klymkowsky, 1995). Desmin was shown to be associated with the periphery of the Z-disk and enriched at the ISJ where it functions to couple the myofibrils to the

sarcolemma (Cary and Klymkowsky, 1995). The specific function of Desmin at the ISJ may explain the limited effects of its reduced levels on myofiber structure. Still, the specific defects and lack of mechanical support may lead to the significant elevated levels of apoptosis observed in the ventral portions of the somite.

Interestingly, the most highly disorganized myofiber structure and significant apoptosis occur mainly in the ventral regions of the somites normally expressing *XMyf5*. However, apoptotic cell death occurs in other somitic areas located outside regions of *XMyf5* expression (Martin and Harland, 2001). The extensive apoptosis occurring throughout large regions of the somite could be a late and secondary effect of *XMyf5* expression loss that occurred in earlier developmental stages.

Is the Xenopus model universal?

A recent study analyzing the role of p38 in somite culture and mouse embryos came to the conclusion that p38 signaling affected *MEF2* transcription factors and muscle cell differentiation, but not the expression of *Myf5* (de Angelis et al., 2005). They suggest that p38 is not involved in the initial commitment of the myogenic lineage, and the conclusions made in this study are in agreement with several cell culture studies (Puri et al., 2000; Zetser et al., 1999). However, the cellular models can only undergo the terminal differentiation stage, and as such cannot really serve as model systems to study the possible affects of p38 on myogenic cell lineage commitment. Still, the question remains as to whether p38 regulates muscle differentiation in *Xenopus* embryos in a similar manner to other experimental systems. We cannot confirm if terminal differentiation was not effected in our system, since the elimination of *XMyf5* expression could mask downstream effects. This possibility can neither be excluded, since we did not address this question in the present study. Although, p38 regulated *XMyf5* expression, it could also modify the transcriptional activities of other MRF and *MEF2* proteins. If indeed terminal differentiation was also affected, then, how could the ectopic expression of *XMyf5* restore a normal muscle phenotype to the p38-knockdown embryos? One possible explanation is that over-expression of MRFs like *Myf5*, could compensate for the limited activities of *Myf5* or other MRFs. In contrast to our study, de Angelis et al. failed to observe that p38 inhibition reduced *Myf5* expression. There are several major differences between the experimental systems that could explain this discrepancy. First, their work uses the pharmacological inhibitor SB203580 as the sole means to inhibit p38 in explants and whole embryos, whereas we used several approaches to inhibit p38 activity. Disadvantages of pharmacological inhibitors are their toxicity and limited penetration, especially when working with three-dimensional explants and whole embryos. The effect of SB203580 on *Myf5* expression was weaker than the effect of other methods used in our present work (see Fig. 2A). Thus, one cannot exclude that limited tissue penetration could explain only partial inhibition. A second major difference is the developmental stages

analyzed; in this work, p38 inhibition was initiated from one-cell to gastrula stages in intact embryos and explants. In de Angelis et al., inhibition was initiated at the 20–25 somite stage (9.5 dpc). The initiation of *Myf5* expression occurs at very different developmental stages in mouse and *Xenopus*. Thus, differences in temporal *Myf5* expression could reflect changes in *Myf5* transcriptional regulation between the two systems. Future studies should uncover such differences, if they exist.

In conclusion, we suggest a model in which the loss of p38 activity in the embryo leads to a reduction of *XMyf5* expression. During gastrula–neurula stages, the loss of *XMyf5* protein prevents cell cycle arrest, which leads to reduced convergent extension in the paraxial mesoderm. At later neurula–early tailbud stages (stages 16–22), there is a slight delay in segmentation. By later tailbud stages (stage 25 and onwards), there is a general deterioration of the somite borders and structure. There is massive apoptosis in the ventral somites, with a concomitant loss of *pax3* expressing cells leading to poor body wall muscle formation. Future experiments should shed light on the upstream and downstream factors modifying p38 signaling and *XMyf5* expression in the developing *Xenopus* mesoderm.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ydbio.2005.09.020](https://doi.org/10.1016/j.ydbio.2005.09.020).

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