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A novel role for *lbx1* in *Xenopus* hypaxial myogenesis

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We have examined *lbx1* expression in early *X. laevis* tadpoles. In contrast to amniotes, *lbx1* is expressed in all of the myoblasts that contribute to the body wall musculature, as well as in a group of cells that migrate into the head. Despite this different expression, the function of *lbx1* appears to be conserved. Morpholino (MO) knockdown of *lbx1* causes a specific reduction of body wall muscles and hypoglossal muscles originating from the somites. Although myoblast migratory defects are observed in antisense MO injected tadpoles targeting *lbx1*, this results at least in part from a lack of myoblast proliferation in the hypaxial muscle domain. Conversely, overexpression of *lbx1* mRNA results in enlarged somites, an increase in cell proliferation, but a lack of differentiated muscle. The control of cell proliferation is linked to a strong downregulation of *myoD* expression in gain-of-function experiments. Co-injection of *myoD* mRNA with *lbx1* mRNA eliminates the overproliferation phenotype observed when *lbx1* is injected alone. The results indicate that a primary function of *lbx1* in hypaxial muscle development is to repress *myoD*, allowing myoblasts to proliferate before the eventual onset of terminal differentiation.

KEY WORDS: Hypaxial, Rectus abdominus, Rectus cervicus, Geniohyoideus, *lbx1*, *myoD*, *myf5*, *Xenopus laevis*, Cell proliferation, Myogenesis

INTRODUCTION

The ladybird homeobox (*lbx1*) gene is a member of the NK-class of homeobox transcription factors. *lbx1* and other NK-class genes are characterized by a homeobox DNA-binding domain and an engrailed homology domain (Jagla et al., 2001). In amniotes, *lbx1* is expressed in a subset of hypaxial myoblasts, which in the mouse migrate into the limbs, tongue, and diaphragm. By contrast, *lbx1* is completely excluded from inter-limb body wall hypaxial myoblasts (Dietrich, 1999; Dietrich et al., 1998). In addition to the difference in *lbx1* expression, these two types of hypaxial muscles are characterized by different modes of development. Body-wall muscles form as epithelial extensions of the dermomyotome, whereas *lbx1*-positive limb, tongue and diaphragm myoblasts undergo an epithelial to mesenchymal transition in the dermomyotome before undergoing long-range migration to their target sites (Dietrich, 1999). A null *lbx1* mutation in the mouse causes a specific lack of limb musculature attributed to migration defects, while hypaxial body wall muscles undergo normal development (Brohmann et al., 2000; Gross et al., 2000; Schafer and Braun, 1999).

The pre-metamorphic *X. laevis* tadpole does not contain limbs and the entire family of pipid frogs does not develop a tongue (McDiarmid and Altig, 1999), suggesting that early tadpoles might not express *lbx1*. However, these tadpoles do contain hypaxial muscles, which form the rectus abdominus muscles (Lynch, 1990; Martin and Harland, 2001; Ryke, 1953). These are homologous to the inter-limb body wall muscles of amniotes (McDiarmid and Altig, 1999). The temporal expression of muscle-specific markers in myoblasts that form the ventral body wall muscles is similar to that of amniote limb myoblasts (Martin and Harland, 2001). *pax3*, which is a marker of proliferative myoblasts, is expressed in cells that exit the somite and migrate ventrally. Later on, *pax3*-positive myoblasts are seen just ventral to differentiated muscle of the ventral body wall. The expression of *myf5*, a muscle regulatory factor (MRF), is similar to that of *pax3* in the hypaxial myoblasts. However, the MRF *myoD* is absent from hypaxial myoblasts as they exit the somite. Transcripts of *myoD* do not appear until cells begin to differentiate, and they overlap with 12/101 staining, a marker of differentiated skeletal muscle (Martin and Harland, 2001).

The expression of *myoD* has been shown to be associated with the terminal differentiation of skeletal muscle (Hopwood et al., 1989; Hopwood et al., 1992; Montarras et al., 1989; Tapscott et al., 1990; Weintraub et al., 1991). Indeed, *myoD* has been demonstrated to be an integral factor in cell cycle arrest during the terminal differentiation of skeletal muscle (Halevy et al., 1995). Although *Myf5* and *Myod1* have been shown to have partially redundant roles in mouse skeletal muscle formation, *Myf5* is expressed in proliferative myoblasts that have not exited the cell cycle (Hopwood et al., 1991; Martin and Harland, 2001; Montarras et al., 1991). For example, the inhibition of muscle differentiation in chick limb myoblasts by activated Notch results in a downregulation of *myoD*, but has no effect on *myf5* or *pax3* expression (Delfini et al., 2000).

The precise timing of the development of long-range hypaxial muscles requires a fine balance between proliferation and differentiation (Amthor et al., 1999; Amthor et al., 1998). The overlying ectoderm of somites provides a proliferative signal for myoblasts. When ectoderm is removed from chick somites, a transient upregulation of *myoD* is observed corresponding to a burst of muscle differentiation, but further muscle growth is halted. When ectoderm is removed from somites at limb and tongue levels, the premature differentiation prevents muscle precursors from migrating, and limb and tongue muscles do not form (Amthor et al., 1999).

Work on *lbx1* in hypaxial myogenesis has focused mainly cell migration, but *lbx1* has also been implicated in cell proliferation. Forced expression of *lbx1* in chick mesoderm and neural tube explants increases cell proliferation. In chick limb buds, *lbx1* increases *myoD* expression and the amount of differentiated muscle. This excess can be rescued by inhibiting cell proliferation (Mennerich and Braun, 2001). These results suggest that a role of *lbx1* in hypaxial myogenesis may be to expand the population of myoblast cells.

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We have examined the expression pattern of *lbx1* in *X. laevis* tadpoles and found that it is expressed in all of the myoblasts that will populate the rectus abdominus and the geniohyoideus, a hypoglossal muscle. We further demonstrate that *lbx1* controls myoblast proliferation through the downregulation of *myoD* and *p27*. Thus, muscle defects observed after *lbx1* loss of function probably result from the reduced capacity of myoblasts to proliferate.

MATERIALS AND METHODS

General methods

Xenopus laevis embryos were generated and cultured by standard methods (Sive et al., 2000). Embryos were allowed to develop in 0.3× Marc's modified Ringer (MMR) solution and staged according to the normal table (Nieuwkoop and Faber, 1967).

Isolation of *Xenopus laevis lbx1* clone

The primers TLBX-2 (5′-AGACGGCATGACGATTTTTGGC-3′) and TLBX-3 (5′-CCGAGATGGTGAGTACGGCTTC-3′) were designed from *Xenopus tropicalis* genomic sequence and used to screen an arrayed *X. laevis* cDNA library. A single clone of *lbx1* was found that contains the majority of the coding region. The primers Xlbx5up (5′-CCAAGTCAATGTATCAG-TCGCTGTG-3′) and Xlbx5down (5′-TCCTGACTGAGGGCTTGTT-TAGG-3′) were designed from *X. tropicalis* genomic sequence and used to amplify the 5′ end of the sequence from *X. laevis* genomic DNA.

Microinjection of DiI

A 0.25% stock solution of CellTracker CM-DiI (Molecular Probes) was made up in 100% ethanol. A working solution of 0.1% DiI was diluted in 3 M sucrose. Micropipettes were pulled and broken to a tip of ~20 μm, backfilled with DiI solution and pressure injected using a Picospritzer (General Valve). Tadpoles were immobilized while injecting and for viewing by immersion in 0.05% benzocaine solution.

Whole-mount in situ hybridization and antibody staining

Embryos were allowed to develop until the desired stage and then fixed for 2 hours in MEMFA. In situ hybridization was carried out with RNA probes labeled with digoxigenin-UTP using a multibasket technique (Sive et al., 2000). Differentiated skeletal muscle was visualized with the 12/101 monoclonal antibody (Kintner and Brockes, 1984). Immunohistochemistry used undiluted monoclonal hybridoma cell supernatant and a goat anti-mouse IgG conjugated to HRP or fluorescein (Jackson) as a secondary antibody at a 1:500 or 1:200 dilution, respectively. In cases where both in situ hybridization and 12/101 staining were carried out on embryos, in situ staining was performed first, followed immediately by immunohistochemistry. The anti-phosphohistone H3 antibody (Upstate Biotechnology) was used at a 1:1000 dilution in 2 mg/ml BSA in PBS plus 0.1% Triton X100. A goat anti-rabbit IgG secondary conjugated to HRP (BioRad) was used at a dilution of 1:1000.

Microinjection of MOs

Antisense and control MOs were ordered from Gene Tools. The following sequences were used: splice blocking, 5′-GAGTGAGGAACTTACCTTC-TGCTGC-3′; translation blocking, 5′-TCATCTTTGGAAGTCATAGTG-GGAC-3′; control, 5′-CCTCTTACCTCAGTTACAATTTATA-3′; and control zebrafish *lbx1* translation blocking, 5′-TTTAGAGCTGGAGGTC-ATCTCAGTC-3′. Stock solutions were resuspended at 50 mg/ml. Initially, 17, 33 and 57 ng of antisense MO were injected into one cell at the two-cell stage. An optimal dose of 33 ng was determined and used subsequently.

RT-PCR

RNA was extracted from whole tadpoles at stage 28 and used for RT-PCR (Wilson and Melton, 1994). Tadpoles used were untreated, injected with 33 ng of splice-MO into one out of two cells, or injected with 33 ng of MO into two out of two cells. The following primers were used: ef1α, U 5′-CAGATTGGTGCTGGATATGC-3′ and D 5′-ACTGCCTTGATGACTC-CTAG-3′, 268 nucleotides; *lbx1*-intron, U 5′-TCCTAAACAAGCCCTCA-GTCCG-3′ and D 5′-CCAACTCATAAATCTGGTGGTTCG-3′, 300 nucleotides (properly spliced). Twenty-one cycles were used to amplify for ef1α and 50 cycles for *lbx1* intron.

mRNA synthesis and microinjection

Synthetic mRNA was made using the mMessage mMachine SP6 kit (Ambion). A zebrafish *lbx1* I.M.A.G.E. EST (GenBank AL831789) was ordered from RZPD (Germany), subcloned from pSport to cs107, and digested with *Asc*I for mRNA synthesis. *Xenopus myoD* was synthesized from the p3 plasmid (Rupp et al., 1994). Mouse *Myf5* was synthesized from a full-length PCR insert in cs108 after being digested with *Asc*I. Nuclear β-galactosidase mRNA (pCS2-*Nls-NlacZ*, 100-200 pg) was co-injected with test mRNAs as a lineage tracer.

The synthesized mRNA was resuspended as a stock solution in DEPC-treated H_2O at a concentration of 1 mg/ml. Working solutions of *lbx1*, *myoD*, *myf5*, *myoD* + *lbx1* and *myf5* + *lbx1* were diluted to 0.1 mg/ml in DEPC-treated H_2O. Approximately 4 nl of each mRNA solution was injected into one cell at the two-cell stage. Injections were targeted to the mediolateral region of the embryo.

Mutant *lbx1* construction

The 5′ end of the zebrafish *lbx1* EST (GenBank AL831789) was amplified with the primers LBXCLAI (5′-CCATCGATGGCGTATGAGGACTAA-AGTTCGGGTG-3′) and MLBXR (5′-GACTTCTTAACGGAGAGAGG-CTTGTTGG-3′). The 3′ end was amplified with the primers LBXECORI (5′-CGGAATTCCGCCTTGCATTTCAAGTTCTTCCGTG-3′) and MLBXF (5′-CCAACAAGCCTCTCTCCGTTAAGAAGTC-3′). The 5′ and 3′ amplified fragments were gel purified and mixed together, followed by amplification using the LBXCLAI and LBXECORI primers. The resulting product was digested with *Cla*I and *Eco*RI and gel purified. This was then inserted into the *Cla*I, *Eco*RI sites of pCS107.

Sectioning and counting nuclei

Tadpoles were embedded in 4% low melt agarose and sectioned using a vibratome (Oxford). The majority of sections were cut at a thickness of 100 μm, except for sections in which the anti-phosphohistone H3 antibody was used, which were cut at 200 μm. The anti-phosphohistone H3 stained sections were cleared and mounted in wells cut into Sylgard coated slides and visualized using a Zeiss Axioplan microscope. Stained nuclei in the immediate vicinity of the somite through the entire 200 μm sections were counted using the microscope, and not from subsequent photographs. Sections at approximately the level of the 3rd trunk somite were used for counting. *P* values were obtained using the paired Student's *t*-test. Values less than 0.01 were considered to be statistically significant.

RESULTS

Identification of hypaxial muscles in *X. laevis*

The 12/101 antibody, which marks differentiated skeletal muscle (Kintner and Brockes, 1984), was used to identify muscles of the *X. laevis* tadpole. At stage 37 (Fig. 1A), the hypaxial rectus abdominus and rectus cervicus muscles can be clearly distinguished from those of the epaxial myotome (McDiarmid and Altig, 1999; Ryke, 1953). By stage 45 (Fig. 1B), the cranial muscles can clearly be seen. The geniohyoideus muscles are considered to be of somite origin, and are present as a pair of muscles extending in an anterior-to-posterior manner near the mouth of the tadpole (Hammond, 1965; Hazelton, 1970).

Identification and expression of *X. laevis lbx1*

PCR primers designed from *Xenopus tropicalis* genomic sequence were used to screen an arrayed *X. laevis* cDNA library. A single clone of *lbx1* was found that contains the majority of the coding region minus the 5′ end. The 5′ end of the sequence was determined by PCR of *X. laevis* genomic DNA using sequence predictions from the *X. tropicalis* genome. *X. laevis lbx1* contains two conserved domains typical of the NK class of transcription

factors, including the engrailed homology domain (eh1) and homeodomain. These domains are identical to the corresponding regions of *X. tropicalis*, mouse and zebrafish *lbx1*. Overall, the amino acid identities are 94%, 79% and 63% between *X. laevis lbx1* and *X. tropicalis*, mouse and zebrafish *lbx1*, respectively, while the nucleic acid identities are 88%, 66% and 61%, respectively. The mRNA expression pattern of *lbx1* (Fig. 1C-H) is first established at stage 17 in the neural tissue, and at later stages defines a dorso-intermediate region of the neural tube, consistent with interneuron specific expression in the mouse (Gross et al., 2002). Expression of *lbx1* during gastrula and early neurula stages is not detectable by in situ hybridization (data not shown). Initiation of mesodermal expression can be seen at stage 26 in the ventrolateral region of anterior trunk somites (Fig. 1D, arrowhead). As development proceeds, expression expands posteriorly and ventrally, consistent with the expression of other genes that mark the developing hypaxial body wall (Martin and Harland, 2001). At stage 37/38, a thin stream of cells appears to be migrating into the head (Fig. 1G, arrow, Fig. 3C arrowheads). By stage 42, expression becomes very weak in the ventral domain of the body wall muscles (Fig. 1H, arrowheads) before it is eventually lost in this region.

The geniohyoideus muscles originate from anterior trunk somites

In order to determine the fate of *lbx1*-positive cells that appear to migrate into the head, DiI was injected into the ventrolateral domain of the anterior trunk somites (approximately trunk somites 1-3) (Fig. 2A). Labeled cells were visualized at stage 37 (Fig. 2B) and 45 (Fig. 2C). DiI-positive cells can be seen moving ventrally and anteriorly away from the site of injection (Fig. 2B, arrowhead) at stage 37. The path of migration is similar to tongue myoblasts in other organisms, where cells migrate in a thin stream around the hyoid arch and into the head (Mackenzie et al., 1998). At stage 45, the final location of labeled cells in the head can be seen as a thin band (Fig. 2C, arrows). Co-localization of DiI- and

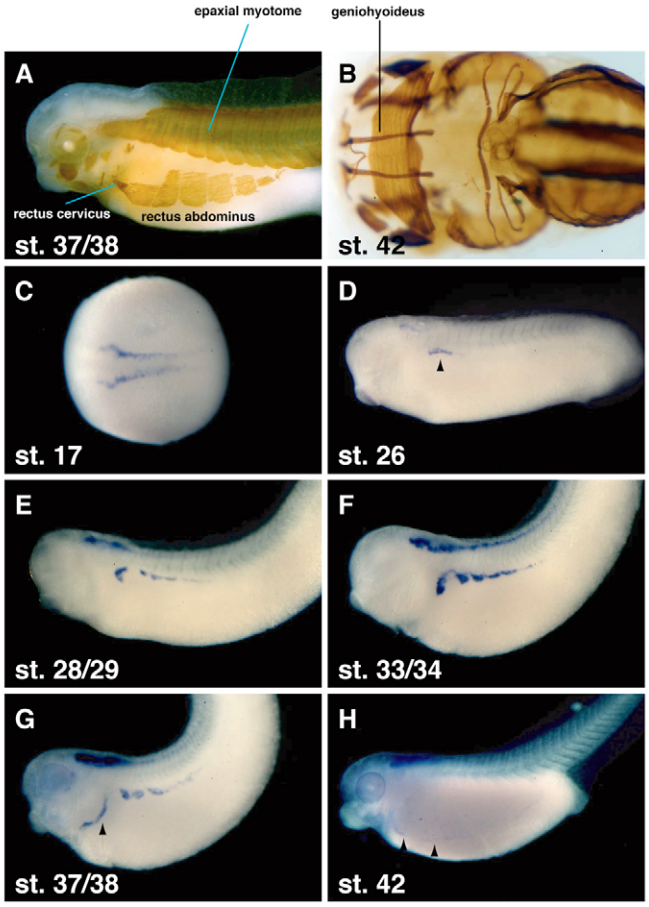

Fig. 1. *Xenopus* hypaxial muscles and *lbx1* expression. Stage 37 (**A**) and 45 (**B**) tadpoles were stained with the 12/101 antibody. A is a lateral view, while B is a ventral view with anterior to the left. The hypaxial muscles consist of the rectus abdominus, rectus cervicus and geniohyoideus. (C-H) Expression of *lbx1* from stage 17 to 42. (**C**) Neural expression precedes myoblast expression (stage 17, dorsal view). (**D**) Early myoblast expression can be seen at stage 26 (arrowhead). (**E**,**F**) Myoblast expression expands posteriorly and ventrally as development procedes. (**G**) At stage 37, *lbx1*-positive cells can be seen migrating around the hyoid (arrow). (**H**) By stage 42, very few *lbx1*-positive myoblasts are seen in the ventral body wall region (arrowheads).

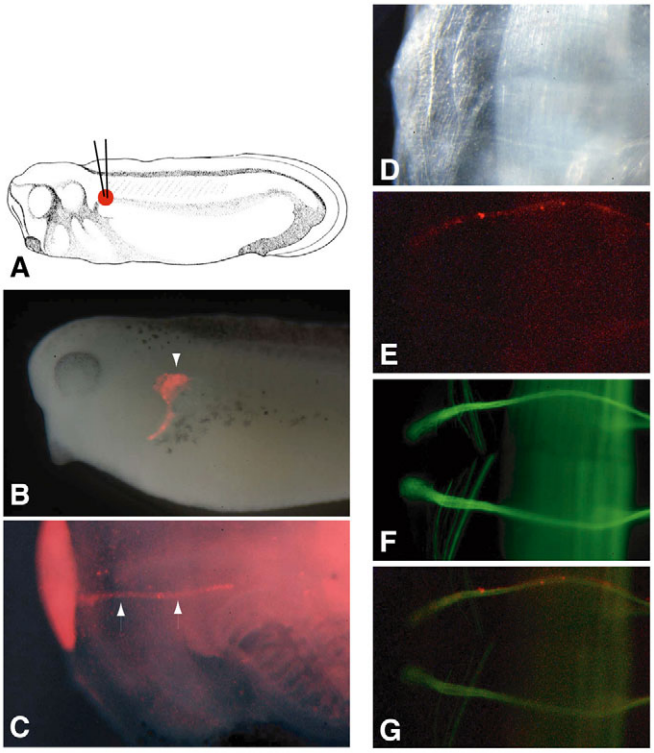

Fig. 2. The geniohyoideus is a hypaxial muscle and originates from the anterior trunk somites. DiI labeling (red) and 12/101 immunofluorescence (green) was used to track the migration of cells from the anterior trunk somites. (**A**) A schematic of the stage 28 injections [modified, with permission, from Niewkoop and Faber (Niewkoop and Faber, 1967)]. A live tadpole is visualized at stage 37 (**B**) and 45 (**C**). The original site of the injection is marked by an arrowhead, while labeled cells that have migrated into the head are marked with arrows. (C-G) Ventral views of the head. (**D**-**G**) The migratory population of cells coming from the anterior trunk somites co-localize with the geniohyoideus muscle at stage 45. A light-field image is shown in D, DiI labeling in E, 12/101 in F and the merge of the DiI and 12/101 images in G.

12/101-labeled cells indicates that the migrating population of cells coming from the anterior trunk somites gives rise to the geniohyoideus (Fig. 2D-G). The same population of cells was also found to give rise to the rectus cervicus muscle (data not shown). DiI-labeled cells only give rise to these muscles on the side of the tadpole in which the somites were labeled (control side not shown).

Conserved cell-type of *lbx1*-positive myoblasts in tadpole hypaxial muscle development

Rectus abdominus and other body wall muscles form as epithelial extensions of the dermomyotome in amniotes and fail to express *lbx1* (Dietrich, 1999). Because *lbx1* is expressed in precursors of the rectus abdominus in *X. laevis*, we wanted to determine whether these myoblasts migrate in a manner similar to *lbx1*-positive long-range myoblasts of amniotes. *lbx1*-positive myoblasts are present at the somite clefts of a stage 37 tadpole, in the posterior-most region of *lbx1* expression (Fig. 3A, arrows). This is also true for other hypaxial specific markers such as *tbx3* (Fig. 3B). In amniotes, the expression of hypaxial markers span the entire width of the somite in the body wall region as they extend as epithelia, whereas *lbx1*-positive zebrafish fin myoblasts emerge at somite clefts and migrate as mesenchymal cells (Neyt et al., 2000).

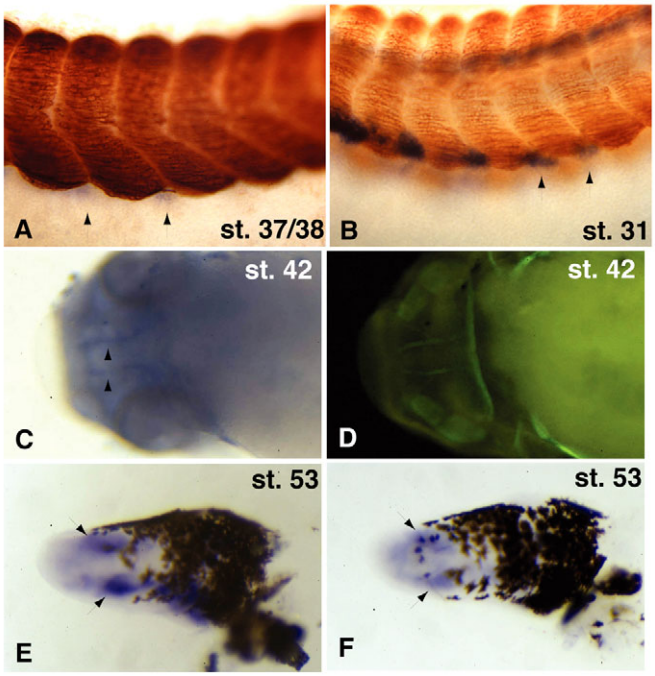

Fig. 3. Ventral body wall myoblasts appear to migrate as mesenchymal cells. (**A**) *lbx1*-expressing myoblasts of a stage 37/38 tadpole emerge at the somite cleft (arrows, most posterior somite with emerging myoblasts shown). (**B**) Another hypaxial myoblast-specific marker, *tbx3*, can be seen expressed in cells migrating out of the somite clefts in a stage 31 tadpole (arrows). Myotomes in A and B are labeled with 12/101 (brown). (**C**,**D**) *lbx1* expression is found in long-range type hypaxial myoblasts. *lbx1* expression in the geniohyoideus can be seen in a ventral view of a stage 42 tadpole (C, arrowheads), which co-localizes with 12/101 (D). (**E**,**F**) *lbx1* is also found in the migrating muscles of the hindlimb of a stage 53 tadpole (E, arrows), similar to *pax3* expression at the same stage (F, arrows) (limbs are shown from a lateral view, distal end towards the left).

If *lbx1* has a truly homologous role in *X. laevis* myoblast migration, we would expect it to be expressed in the long-range type migratory myoblasts such as those that migrate into the limbs and the head. Indeed, *lbx1*-positive myoblasts migrate into the head (Fig. 3C, arrowheads) in a region that co-localizes with 12/101 staining of the geniohyoideus muscles (Fig. 3D). In addition, *lbx1*-positive myoblasts are present in stage 53 limb buds (Fig. 3E, arrows) in a region similar to *pax3*-positive cells (Fig. 3F, arrows). Both genes are expressed in the dorsal and ventral regions, consistent with the development of limb muscle masses in other tetrapods (Uchiyama et al., 2000). Taken together, these results suggest conservation in the type of *lbx1*-positive myoblast migration in *X. laevis*, despite expression of *lbx1* in myoblasts that populate the body wall musculature.

Conserved function of *lbx1* in hypaxial muscle development

To determine whether *lbx1* is required for hypaxial muscle development, a splice-blocking antisense MO was used to inhibit *lbx1* function. Embryos injected into one cell at the two-cell stage or into both cells at the two-cell stage with 33 ng of MO into each cell were analyzed by RT-PCR. The results indicate a clear decrease in properly spliced transcript (Fig. 4A). Quantification of the radiolabeled bands showed that the one-cell injection caused a 85% decrease in radioactive signal from the properly spliced transcript compared with the control, while the two-cell injection led to a 91% decrease. Embryos were injected into one cell at the two-cell stage with either 33 ng of the splice blocking MO or 33 ng of a control MO and then stained with 12/101. Those injected with the splice blocking MO exhibit a severe decrease in body wall muscle when compared with the uninjected side at stages 37 (Fig. 4F,G) and 40 (Fig. 4H,I) (87% with defects, n=197). In addition to body wall muscle defects, anterior ventral views of stage 40 tadpoles show a loss of the geniohyoideus muscle on the injected side (Fig. 4O, arrow). By contrast, the control MO injected tadpoles are largely unaffected on the injected side at stage 37 and 40 (Fig. 4B-E,N, arrow) (16% with defects, n=85). Apart from the control MO, which is famously non-toxic, other MOs, such as one directed against zebrafish *lbx1* have never caused hypaxial muscle defects (data not shown). As a further control, a second MO was designed to block the translation of *lbx1*, in order to confirm the knockdown phenotype. When 33 ng of translation blocking MO was injected into one cell at the two-cell stage, the same loss of hypaxial muscle phenotype was seen at both stage 37 and 40 (Fig. 4J-M) (92% with defects, n=40). Later in development, tadpoles injected with either the splice or translation blocking MO exhibit some development of hypaxial muscles (Fig. 4I,M), but the amount of muscle is always significantly less than on the uninjected side (compare Fig. 4I,M with 4H,L). These results indicate that *lbx1* is required for the proper development of hypaxial muscles that are derived from *lbx1*-positive myoblasts, similar to mouse *lbx1* (Brohmann et al., 2000; Gross et al., 2000; Schafer and Braun, 1999).

The lack of hypaxial muscles observed in *lbx1* mutant mice is due to aberrant migration of myoblasts. The lack of body wall muscles in *Xenopus* tadpoles that have been injected with *lbx1*-splice MO can also be partially linked to abnormal migration of myoblasts. Fig. 4P-S shows stage 37 tadpoles that have been stained with *pax3*, a marker of proliferative myoblasts, and the 12/101 antibody, which marks differentiated skeletal muscle. Merged images showing both *pax3* and 12/101 staining (Fig. 4Q,S) indicate the position of *pax3*-positive hypaxial myoblasts relative

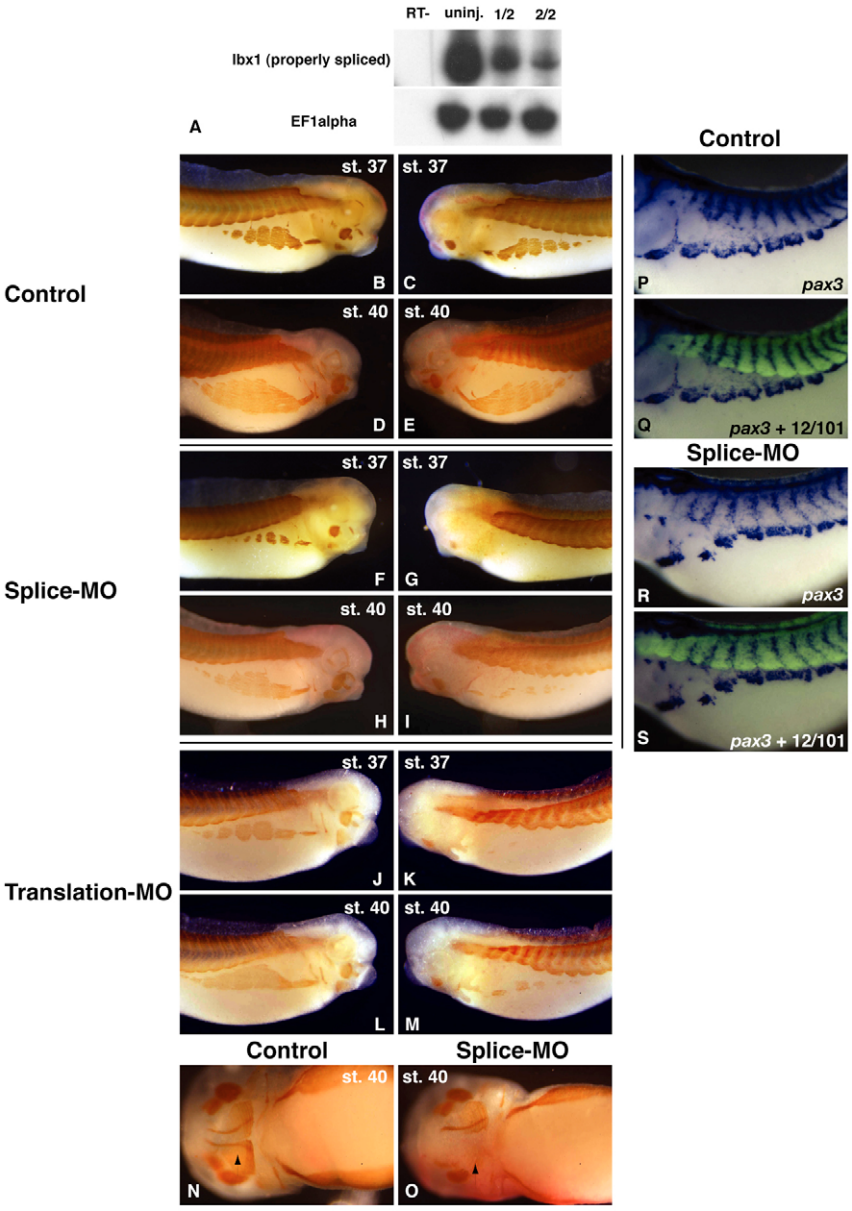

Fig. 4. *lbx1* is required for hypaxial muscle formation. RT-PCR was performed on tadpoles injected with *lbx1*-splice MO into one out of two cells or both cells at the two-cell stage. (**A**) A specific loss of the properly spliced *lbx1* transcript is seen. Embryos were injected into one cell at the two-cell stage with a control MO (**B**-**E**), the *lbx1*-splice blocking MO (**F**-**I**) or the *lbx1*-translation blocking MO (**J**-**M**) along with β-galactosidase mRNA as a lineage tracer (red). Tadpoles were stained with the 12/101 antibody (brown). The uninjected (B,D,F,H,J,L) and injected (C,E,G,I,K,M) sides of each tadpole are shown, where the uninjected sides are pictured with anterior towards the right. The control MO-injected tadpoles do not show a difference in the presence of rectus abdominus muscles on the injected side compared with the uninjected side at stage 37 (compare B with C) or stage 40 (D compared to E). Tadpoles injected with the *lbx1*-splice MO exhibit a loss of rectus abdominus and rectus cervicus muscles on the injected side at stage 37 (compare F with G). At stage 40, these muscles appear but are greatly reduced in the *lbx1*-splice MO-injected tadpoles (compare H with I). Similar results were observed for the lbx1-trans MO-injected tadpoles (J-M). (**N**,**O**) Stage 40 control injected (N) and *lbx1*-splice injected (O) embryos are shown from a ventral view, with the MO injected side downwards (embryos were injected into one cell at the two-cell stage). The geniohyoideus muscle is lost on the *lbx1*-splice injected side of the tadpole (O, arrow), whereas both are present in the control injected MO (N, arrow). Myoblast migration defects are observed in *lbx1*-splice injected tadpoles. (**P**-**S**) Tadpoles in P and R are stained for *pax3* mRNA (purple) and then merged with the 12/101 antibody (green, Q,S). A control tadpole is pictured in P and Q, which shows the movement of *pax3*-positive myoblasts away from the differentiated epaxial myotome. In *lbx1*-splice-injected tadpoles (R,S), a large number of *pax3*-positive myoblasts remain adjacent to the differentiated myotome.

to the somites from which they were derived (12/101, green). In control tadpoles, *pax3*-positive cells have moved ventrally away from the somites (Fig. 4Q). In MO injected tadpoles (Fig. 4R,S), anterior myoblasts have begun to migrate away from the somites, but more posterior cells remain adjacent to the ventrolateral edge of the somites (80% with migration defect, *n*=25).

The expression of *myf5* is affected by *lbx1* manipulations

In addition to the loss of hypaxial muscle, the *lbx1*-splice MO also causes a specific loss of ventral epaxial musculature in the anterior trunk somites. A transverse section of a stage 40 tadpole that was injected with *lbx1*-splice MO in one cell at the two-cell stage illustrates this loss (Fig. 5A,B). After the initial onset of myogenesis in the epaxial domain of somites, the musculature of this region expands in the dorsal and ventral domains (Denetclaw et al., 1997; Denetclaw and Ordahl, 2000). This expansion is marked by *myf5* expression in proliferative myoblasts at the ventrolateral and dorsomedial lips of the dermomyotome. We examined *myf5* expression in injected tadpoles and found that it is specifically reduced in the ventrolateral region of anterior somites, but is expressed at normal levels in dorsal and posterior ventral domains (Fig. 5D, arrow) (96% with reduced *myf5* expression, *n*=26). Injection of a control MO has no effect on *myf5* expression (Fig. 5F) (*n*=6, one with slightly reduced *myf5* expression). The first eight trunk somites express *lbx1* in their ventrolateral domain (Fig. 1), which normally overlaps with *myf5* expression in this region (Fig. 5G-J, arrows). When *lbx1* mRNA is overexpressed in somites, a dramatic increase in *myf5* expression is observed, as well as an enlarged somite (Fig. 5K) (100% with increased *myf5* expression, *n*=13). Despite the increased size of the somite, there is a lack of differentiated muscle, as determined by 12/101 staining (Fig. 5L). The co-injection of *lbx1* mRNA with the *lbx1*-splice MO rescues the loss of *myf5* expression in the ventrolateral domain of the somite (Fig. 5M,N, compare with 5D), in addition to upregulating *myf5* in other regions of the somite targeted by the injection (100% rescue, *n*=7).

lbx1 controls myoblast proliferation

Because of the enlarged somites with a lack of differentiated muscle in *lbx1*-injected tadpoles, we investigated whether *lbx1* is controlling myoblast proliferation rather than directly upregulating *myf5* expression. An antibody to phosphorylated histone H3 (pH3) was used to visualize mitotic cells (Saka and Smith, 2001). In normal, unmanipulated tadpoles, an abundance of mitotic cells are found at the ventrolateral region of developing somites, where *lbx1*-positive myoblasts are normally present (Fig. 6A-D, compare Fig. 6B with Fig. 5G).

The *lbx1*-splice MO was injected into one cell at the two-cell stage and stage 31 tadpoles were stained with the pH3 (Fig. 6E) and 12/101 (Fig. 6F) antibodies. A specific loss of mitotic cells is seen in the ventrolateral region of the somite on the injected side, as well as a slight loss of muscle in this region. The opposite is found in *lbx1* mRNA-injected tadpoles (Fig. 6G,H), where a dramatic increase in mitotic cells is observed in the somitic region of injected tadpoles (Fig. 6G); this also corresponds to a lack of differentiated muscle (Fig. 6H). Injection of a control MO and β-galactosidase mRNA has no significant effect on the number of mitotic cells or the amount of differentiated muscle (Fig. 6I,J; results summarized in Fig. 10). At later stages in *lbx1* mRNA injected tadpoles, mitosis decreases, corresponding to the terminal differentiation of these cells (Fig. 6K,L). In some cases, the excess differentiated muscle crosses the midline of injected tadpoles (Fig. 6L). By stage 41, the amount of differentiated muscle on the *lbx1* mRNA-injected side of the tadpoles greatly exceeds the amount on the uninjected side, though ectopic muscle is never observed outside the somitic region. Fig. 6M,N shows transverse sections through a stage 41 tadpole at a trunk (Fig. 6M) and tail level (Fig. 6N). An increase in differentiated muscle is seen at both levels. This rules out the possibility that the increase in area of muscle in the section is due to distortion of the embryo (due to defective convergence and extension), and thus confirms that that increase in muscle mass is due to increased cell proliferation.

Lbx1 downregulates *myoD* and *p27*

In many cases, exit from the cell cycle and terminal differentiation of myoblasts can be caused by the expression of *myoD* (Delfini and Duprez, 2004; Halevy et al., 1995; Hopwood and Gurdon, 1990; Pownall et al., 2002; Weintraub et al., 1991). We therefore investigated whether *lbx1* may promote myoblast proliferation and lack of differentiation through the inhibition of *myoD* expression.

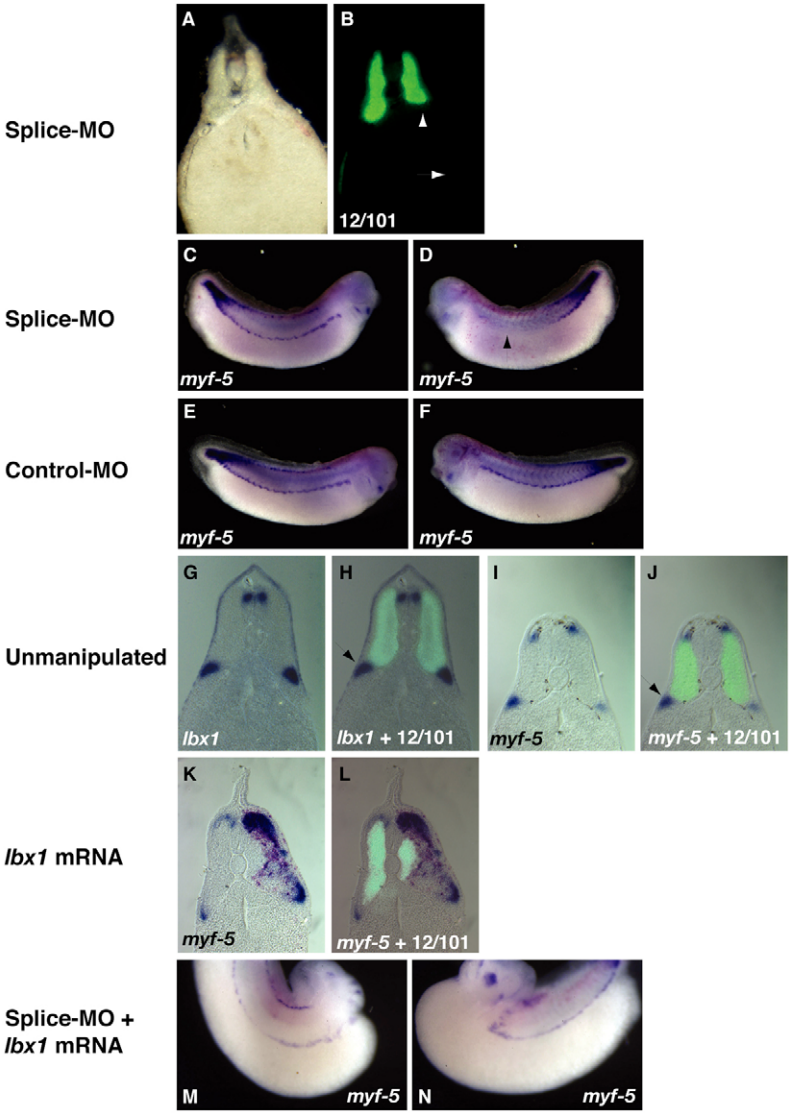

Fig. 5. *myf5* expression is affected by the gain or loss of *lbx1*. (**A**,**B**) A transverse section through an *lbx1*-splice-injected tadpole (left side uninjected, right injected) reveals not only a loss of ventral body wall muscles (B, arrow), but also a loss of a more dorsal domain of epaxial muscle (B, arrowhead). A corresponding loss of *myf5* expression is seen in stage 28 *lbx1*-splice injected tadpoles. (**C**,**D**) An *lbx1*-splice injected tadpole where C is the uninjected side and D is the injected side. (**E**,**F**) Control MO-injected tadpole where E is the uninjected side and F is the injected side. All of these panels are stained for *myf5* expression. A loss of *myf5* expression in the region that *lbx1* is normally expressed is seen in the *lbx1*-splice-injected tadpole on the injected side (D, arrow). The similar expression domains for *lbx1* and *myf5* in the hypaxial myoblasts can be seen in G-J. (**G**,**H**) A transverse section of a stage 28 tadpole at approximately the level of trunk somite 3. The section is stained for *lbx1* (G) and merged with 12/101 staining (H, green). (**I**,**J**) Similarly, a transverse section from a stage 28 tadpole at approximately the level of trunk somite 3. This section is stained for *myf5* (I) and merged with 12/101 staining (J, green). Both transcripts are found in the ventral hypaxial myoblast region of the somite (H,J, arrows). Overexpression of zebrafish *lbx1* causes an increase in *myf5* expression and larger somites, but a decrease in differentiated muscle. (**K**,**L**) A transverse section of a stage 28 tadpole, showing *myf5* staining (K) merged with 12/101 staining (L, green). Embryos were injected into one cell at the two-cell stage. The injected side is to the right. (**M**,**N**) Stage 29 tadpole co-injected with *lbx1*-splice MO and *lbx1* mRNA. The uninjected side is shown in M and injected side in N. The loss of *myf5* expression is rescued when *lbx1* mRNA is added back to *lbx1*-splice injected cells.

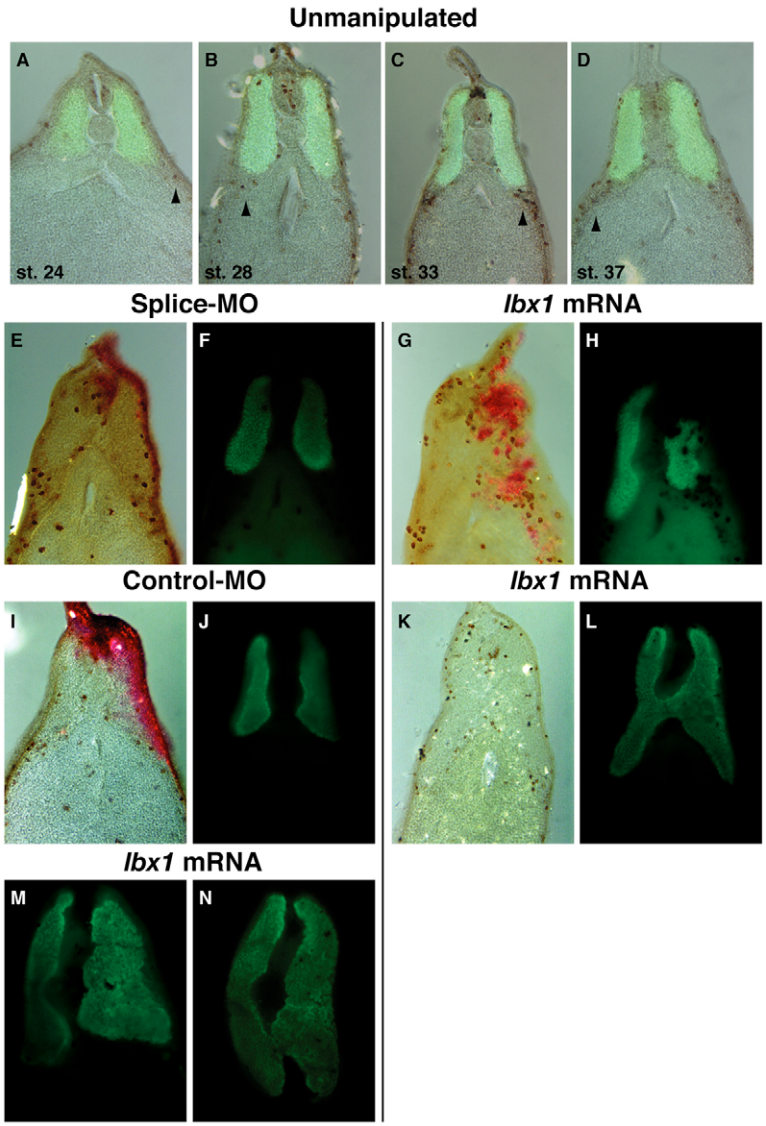

Fig. 6. Myoblast cell proliferation is controlled by *lbx1*. An anti-phospho histone H3 antibody (brown) in combination with 12/101 (green) shows cells proliferating in the hypaxial muscle region (**A**-**D**, arrows). A transverse section through a stage 24 tadpole, before *lbx1* expression is on in myoblasts, shows very little cell proliferation in the ventrolateral region of the somite (A, arrow). At stages 28 (B), 33 (C) and 37 (D), an increasing number of proliferating cells can be seen in the ventrolateral region of the somite (arrows). The remaining panels show tadpoles injected in one cell at the two-cell stage with either *lbx1*-splice MO (**E**,**F**), *lbx1* mRNA (G,H,K-N) or a control MO (I,J). All of these panels are transverse sections with the injected side on the right. Stage 33 tadpoles that have been injected with *lbx1*-splice MO show a decreased number of proliferating cells in the ventrolateral region of the somite (E). Consistent with the loss of proliferating cells in E, a slightly smaller differentiated muscle mass can be seen in the same section in F (12/101, green). (**G**) Stage 28 tadpoles injected with *lbx1* mRNA exhibit a dramatic increase in the number of proliferating cells in the region of the lineage tracer (red). (**H**) Active cell proliferation seen in G inhibits muscle differentiation (12/101, green). (**I**,**J**) Control injected tadpoles at stage 33 show no difference in cell proliferation or size of differentiated muscle. (**K**,**L**) By stage 37, tadpoles injected with *lbx1* mRNA have the same amount of cell proliferation, but the differentiated muscle mass on the injected side is much larger. (**M**,**N**) A stage 41 tadpole injected with *lbx1* mRNA has a larger differentiated muscle mass on the injected side in both the trunk (M) and tail (N).

One cell of each embryo was injected at the two-cell stage with *lbx1* mRNA and analyzed at various stages. At stage 22, a strong downregulation of *myoD* expression is observed on the injected side (Fig. 7A), while *pax3* expression is expanded (Fig. 7F). Transverse sections through stage 24 tadpoles show a complete loss of *myoD* expression on the injected side, despite the enlarged somite (Fig. 7B), again there is an expansion of *pax3* expression on the injected side (Fig. 7G). As late as stage 33, the same results are seen. In regions where *lbx1* is expressed (marked by red β-galactosidase staining), *myoD* expression is downregulated (Fig. 7C-E), while *pax3* is expanded (Fig. 7H-J) (100% with reduced *myoD* expression, n=9; 100% with increased *pax3* expression, n=10).

Despite the rapid proliferation and *pax3* expression in *lbx1*-injected cells, they do not have a hypaxial identity as shown by repression of endogenous *lbx1*. A stage 24 tadpole shows a loss of endogenous *lbx1* expression on the injected side (Fig. 7L) as opposed to the uninjected side (Fig. 7K). At stage 33, the targeted mis-expression of *lbx1* to posterior somites does not induce endogenous *lbx1* expression (Fig. 7M). Other markers expressed in hypaxial muscles, such as *tbx2* and *tbx3* show similar results (data not shown).

If *lbx1* normally represses *myoD* expression, the loss of *lbx1* function should result in a transient increase in *myoD*. This result is observed in stage 26 tadpoles that were injected with the *Lbx1*-splice MO into one cell at the two-cell stage. A transverse section shows a slight increase of *myoD* expression on the injected side in the ventrolateral region of the somite (Fig. 7N, arrow) (64% with increased *myoD* expression on injected side, n=11). In addition, the repression of *myoD* caused by the ectopic *lbx1* expression should coincide with the presence of ectopic *lbx1* mRNA. In a stage 31 tadpole, the presence of injected *lbx1* mRNA can be detected by in situ hybridization (Fig. 7O). At this stage, *myoD* is repressed on the injected side (Fig. 7P). By stage 41, ectopic *lbx1* mRNA can no longer be detected by in situ hybridization (Fig. 7R). This is also the stage at which a much larger differentiated myotome can be detected on the injected side (Fig. 6M,N). A corresponding increase in *myoD* expression is observed at this stage (Fig. 7S).

As previously mentioned, the expression of *myoD* can lead to exit from the cell cycle and terminal differentiation. In *Xenopus*, the cell cycle inhibitor *p27* is expressed in developing somites and has been shown to be required for the proper differentiation of the myotome (Vernon and Philpott, 2003). Normal expression is found along the lateral edge of the somite where active differentiation is occurring.

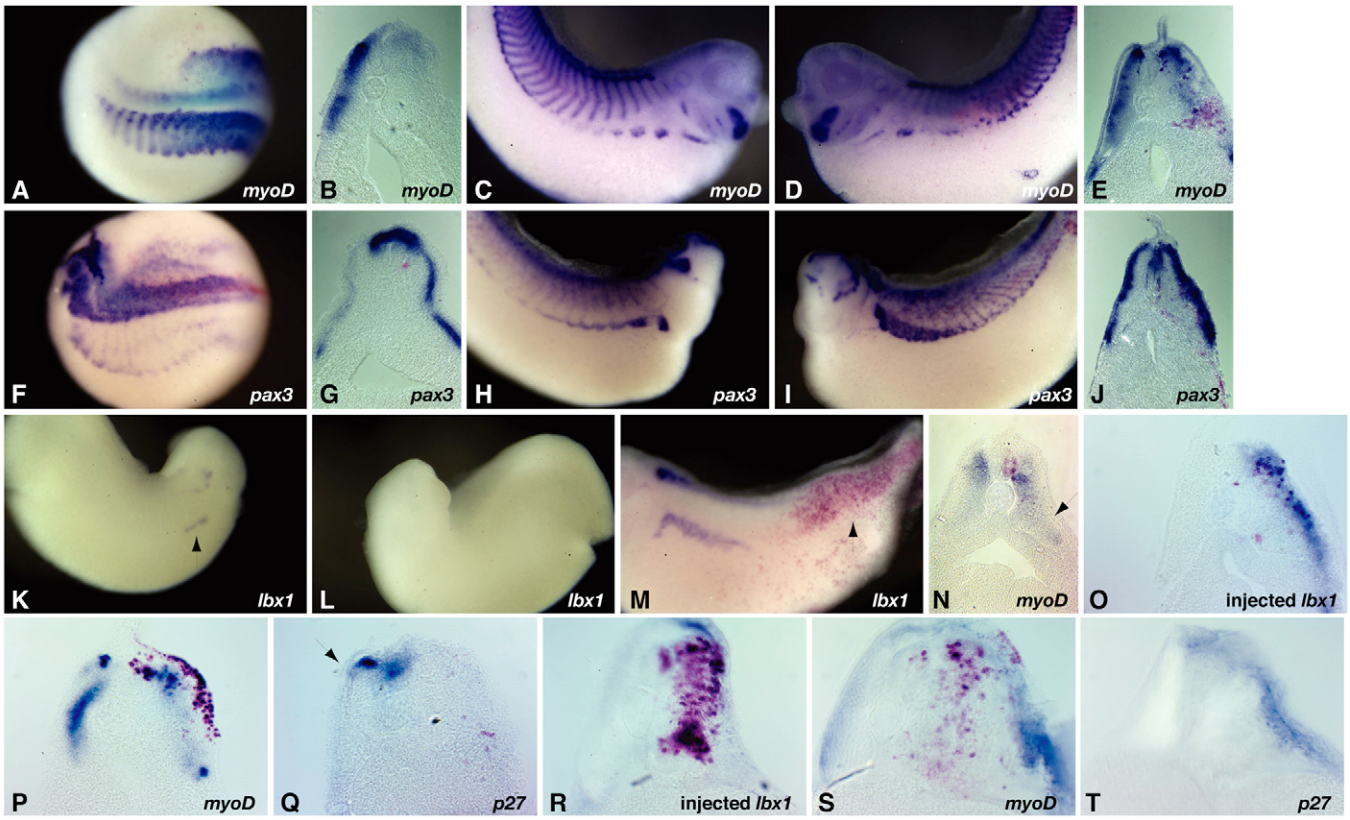

Fig. 7. Lbx1 downregulates *myoD*. Embryos were injected with *lbx1* mRNA in one cell at the two-cell stage and stained for *myoD* (**A**-**E**), *pax3* (**F**-**J**) or *lbx1* (**K**-**M**). Red staining is the β-galactosidase lineage tracer. At stage 22, *myoD* is downregulated on the injected side (A), while *pax3* expression is higher (F). These embryos are shown from a dorsal view, with the injected side on top. Transverse sections of stage 24 embryos show a complete loss of *myoD* expression on the injected side (right side), despite there being an enlarged somite (B), while *pax3* expression is higher on the injected side (right side) (G). At stage 33, expression of *myoD* is still downregulated in regions of the β-galactosidase lineage tracer (C-E). The uninjected side is shown in C, injected side in D, and a transverse section in E (injected side to the right). *pax3* expression is also still upregulated at stage 33 in regions of the lineage tracer (H,I). The uninjected side is shown in H, injected side in I, and a transverse section in J (injected side to the right). At stage 24, endogenous *lbx1* expression is lost on the injected side of the embryo (L), when compared with the uninjected side (K). At stage 33, ectopic *lbx1* expression in the posterior somites (red staining) fails to upregulate endogenous *lbx1* expression (M). (**N**) A stage 26, tadpole injected with *lbx1*-splice MO (right side) shows a mild upregulation of *myoD* in the ventrolateral domain of the somite (arrow). (**O**-**T**) Transverse sections of stage 31 (O-Q) and 41 (R-T) tadpoles injected with lbx1 into one cell at the two-cell stage were stained for injected lbx1 mRNA (O,R), *myoD* (P,S) and *p27* (Q,T, arrow in Q indicates region of strong expression on the uninjected side). When injected lbx1 mRNA is still present (O), *myoD* and *p27* are repressed on the injected side (P,Q). When the injected mRNA is no longer detectable (R), *myoD* and *p27* are upregulated on the injected side (S,T).

We examined the expression of *p27* in *lbx1*-injected tadpoles. At stage 31, when injected *lbx1* is present and *myoD* is repressed, *p27* is also repressed compared to the uninjected side (Fig. 7Q, arrow indicates a region of strong expression on the uninjected side, which is absent on the injected side). At stage 41, when injected *lbx1* mRNA is no longer present and *myoD* is upregulated on the injected side, *p27* is also upregulated on the injected side, indicating that the cells that were overproliferating are now terminally differentiating (Fig. 7T, right side).

The engrailed repressor domain is required for *lbx1* function

PCR-based mutagenesis was used to delete a 12 amino acid stretch that spans the entire eh1 domain. The over-expression of this mutant construct resulted in a slight dominant-negative phenotype. Instead of the decrease in *myoD* expression and increase in *pax3* expression on the injected side observed after wild-type *lbx1* expression, the opposite results were obtained. A transverse section through a stage 24 embryo shows an increase of *myoD* expression on the injected side (Fig. 8A, arrow) (90% with increased *myoD* on injected side, n=20), whereas pax3 is downregulated on the injected side of a stage 31 tadpole (Fig. 8B, arrow) (78% with reduced *pax3* on injected side, n=9). The amount of cell proliferation does not appear to increase on the injected side of stage 29 tadpoles, as judged with the pH3 antibody (Fig. 8C,E). In addition, the myotome is abnormal on the injected sides of stage 29 tadpoles, having a shorter but wider shape (Fig. 8D,F, arrows).

Co-injection of *myoD* with *lbx1* represses myoblast proliferation

If *lbx1* is controlling myoblast proliferation through the downregulation of *myoD*, then the co-injection of *myoD* with *lbx1* should eliminate the overproliferation of myoblasts. In *Xenopus*, the overexpression of *myoD* or *myf5* in the somitic region causes an expansion of myotome through the recruitment of non-myogenic cell lineages, rather than an increase in the proliferation of myoblasts

(Ludolph et al., 1994). In *lbx1*-injected tadpoles, there is an increase of mitotic cells (Fig. 9A), a decrease of differentiated muscle (Fig. 9B) and an expansion of *pax3* expression (Fig. 9C). This is in contrast to the injection of *myoD* alone, which causes no change in mitotic cells (Fig. 9D), a large increase in differentiated muscle (Fig. 9E), and a slight decrease in *pax3* expression (Fig. 9F). *myf5* injection causes a slight expansion of the myotome (Fig. 9H), but has no effect on cell proliferation (Fig. 9G) or *pax3* expression (Fig. 9I). When *lbx1* is co-injected with *myoD*, a phenotype that is the same as *myoD* alone is observed. There is an increase in differentiated muscle (Fig. 9K) with no increase in cell proliferation (Fig. 9J). *pax3* expression is also decreased on the injected side of the tadpole (Fig. 9L). Thus, *lbx1* cannot promote myoblast proliferation in the presence of *myoD*. However, co-expression of *myf5* with *lbx1* produces a phenotype similar to *lbx1* alone, where a slightly smaller myotome is observed (Fig. 9N) but there is also an increase in cell proliferation (Fig. 9M) and *pax3* expression (Fig. 9O).

The summary of the numbers of phospho-histone H3 nuclei in the immediate somitic region of injected versus uninjected halves of tadpoles is shown in Fig. 10. Significantly more mitotic nuclei are observed on the injected sides of *lbx1* and *lbx1* + *myf5* mRNA-injected tadpoles, while significantly fewer are observed on the *lbx1*-splice MO injected sides of tadpoles (paired Student's *t*-test, $P<0.01$. Fig. 10, indicated with an asterisk). All other injections shown, including the control MO + β-galactosidase mRNA-injected tadpoles, do not show a significant difference in the amount of mitotic nuclei on the injected side.

Enlarged somites in *lbx1*-injected tadpoles are not the result of the recruitment of non-myogenic lineages

As previously described, the enlarged myotomes of *myoD*-injected tadpoles is the result of the recruitment of non-myogenic lineages, and is not due to increased cell proliferation (Ludolph et al., 1994). Transverse sections of stage 29 tadpoles that have been injected with *myoD* (Fig. 11A) or *myoD* + *lbx1* (Fig. 11B), and stained with the pH3 antibody exhibit larger somites on the injected side but no increase in cell proliferation. In situ hybridization for pax8, a pronephric marker, shows that pronephric tubules are lost on the *myoD* (91% absent, n=11) or *myoD* + *lbx1* (80% absent, n=10) injected sides of stage 31 tadpoles (Fig. 11H,J). The pronephric tubules are present on the uninjected sides (Fig. 11G,I, arrows). However, transverse sections through tadpoles injected with *lbx1* alone show normal expression of NCAM in the neural tube on the injected side (Fig. 11C,arrow), as well as pax8 in the pronephric tubules (Fig. 11D,arrow) (100% normal, n=8). Similarly, lateral views of a stage 31 tadpole injected with *lbx1* show normal expression of pax8 in the pronephric tubules on both the uninjected (Fig. 11E) and injected (Fig. 11F) sides (arrows). These results, combined with the cell proliferation results of Figs 6 and 9, indicate that the enlarged myotomes of *lbx1*-injected tadpoles are the result of increased myoblast proliferation and not from the recruitment of non-myogenic lineages.

DISCUSSION

The expression of *lbx1* in *X. laevis* is unusual for tetrapods

Expression of *lbx1* in myoblasts that will form the rectus abdominus muscles in *Xenopus laevis* is different from its expression in other tetrapods; it is only found in prospective limb and tongue muscles in the mouse and chicken. In amniotes, a very clear distinction is made between myoblasts that undergo long-range migration into the limbs and head, and those that extend as epithelia to form the inter-limb

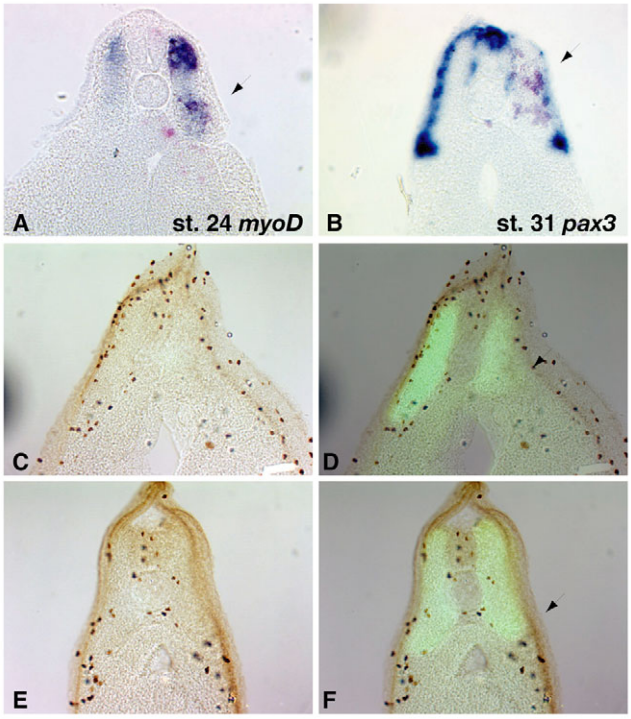

Fig. 8. The engrailed repressor domain of *lbx1* is necessary for its function. (A-F) mRNA from a mutant *lbx1* construct that lacks the engrailed homology domain was injected into one cell at the two-cell stage. The injected sides are on the right. Phenotypes opposite of wild-type *lbx1* overexpression were observed. At stage 24, a transverse section reveals an increase in *myoD* staining on the injected side (**A**, arrow), while a transverse section through a stage 31 tadpole exhibits a decrease in Pax3 staining (**B**, arrow). Transverse sections through stage 29 tadpoles (**C**-**F**) were stained with the pH3 antibody alone (C,E) or with 12/101 (D,F). No increase in the number of mitotic nuclei is observed on the injected sides (C,E), and 12/101 staining (D,F) indicates a slightly shorter, yet wider, differentiated myotome (arrows).

abdominal muscles (Dietrich, 1999). This distinction is based both on the mechanism by which these cells populate target sites (mesenchymal versus epithelial) and by the genes that they express. In amniotes, *lbx1* was the first molecular marker that was specific to the long-range type of migratory myoblasts (Jagla et al., 1995). The expression of *lbx1* in both limb, head and body wall muscles in *X. laevis* suggests that the mechanism of epithelial extension to form body wall muscles has been lost or significantly modified in this animal.

Conservation of *lbx1* function

The unique expression of *lbx1* in body wall muscle precursors raises the issue of whether or not *lbx1* plays a prominent role in the development of these muscles. The *lbx1* mutation in mouse causes a severe loss, but not depletion of muscles in limbs (Brohmann et al., 2000; Gross et al., 2000; Schafer and Braun, 1999). Likewise, knockdown of *lbx1* function in *X. laevis* using antisense MOs depletes body wall and hypoglossal muscles (Fig. 4). From these data, it appears that the long-range migration mechanism used in amniote and zebrafish limbs/fins is also used for body wall muscle development in *X. laevis* tadpoles. In support of this idea, in situ hybridization patterns show that body wall muscle precursors migrate as mesenchymal cells rather than as epithelial extensions (Fig. 3). Thus, *lbx1*-positive

myoblasts emerge from the somite clefts before moving on to populate the body wall muscle (Fig. 3A,B). This is similar to what is seen in the migration of zebrafish myoblasts into the fin, suggesting conservation in the type of migration (Neyt et al., 2000). Finally, *X. laevis lbx1* is also expressed in hypaxial myoblasts that are traditionally considered the long-range type, namely limb and hypoglossal myoblasts (Fig. 3C,E). Taken together, these data support the idea that body wall myoblasts in *X. laevis* are migrating in a manner similar to limb/fin and tongue myoblasts of amniotes and zebrafish.

Migration defects in *lbx1* loss of function may be secondary to reduced myoblast proliferation

Several results support a role for *lbx1* in mouse hypaxial myoblast migration (Brohmann et al., 2000; Gross et al., 2000; Schafer and Braun, 1999; Uchiyama et al., 2000). Using antisense MOs to knockdown *lbx1*, we have observed a similar effect in *Xenopus*. However, we also found that *lbx1* plays a central role in myoblast proliferation, and that this may be its primary role.

A proper balance between proliferation and differentiation is needed for hypaxial myoblast migration (Amthor et al., 1999; Amthor et al., 1998). In the chick, reducing the proliferative capacity of myoblasts by removing the ectoderm at limb and tongue levels causes a lack of limb and tongue musculature (Amthor et al., 1999). Furthermore, in mouse *lbx1* mutants, some limb muscles form, in addition to tongue and diaphragm muscles (Brohmann et al., 2000; Gross et al., 2000; Schafer and Braun, 1999). The precursors to all of these muscles are normally *lbx1* positive. Although they form, these muscles are reduced in size, suggesting that myoblasts have not proliferated to the proper extent. If *lbx1* is only involved in the migration of myoblasts, one would expect there to be more severe defects in tongue and

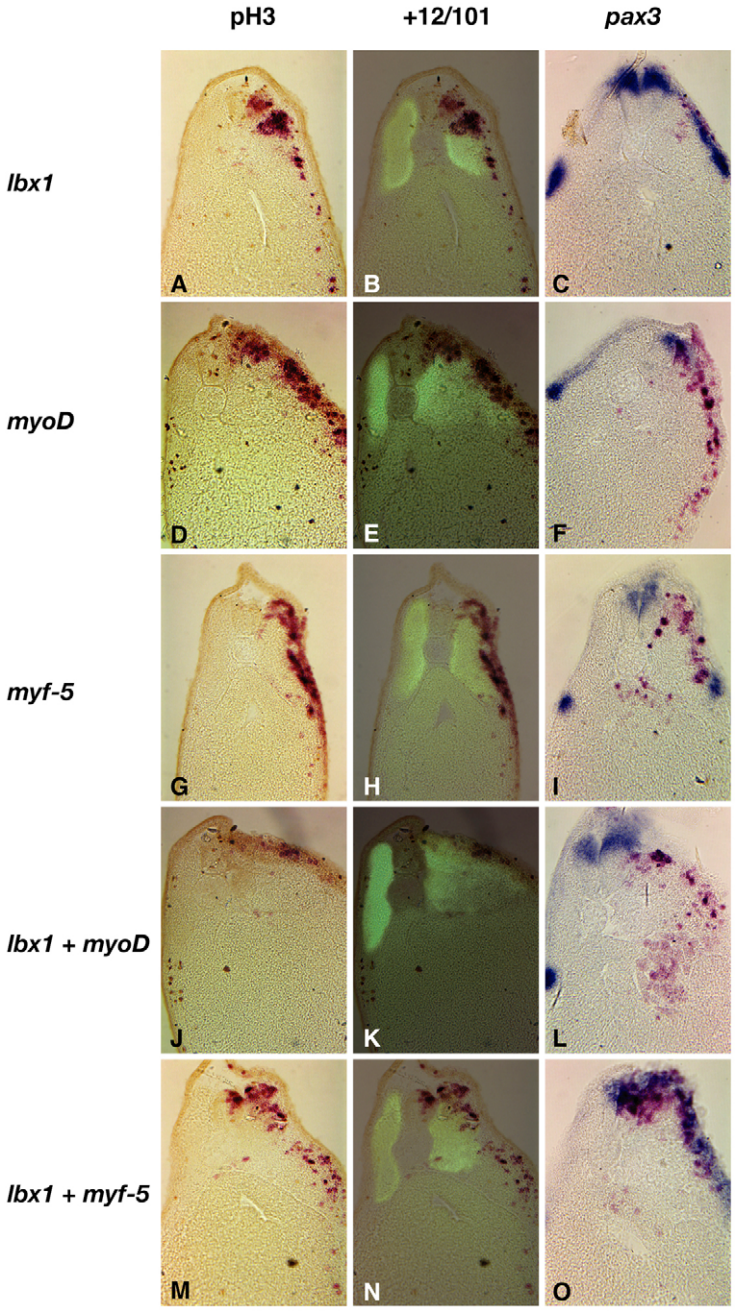

Fig. 9. Co-injection of *myoD* mRNA with *lbx1* mRNA eliminates overproliferation and *pax3* upregulation. (A-O) Embryos were injected into one cell at the two-cell stage with, *lbx1* (**A**-**C**), *myoD* (**D**-**F**), *myf5* (**G**-**I**), *lbx1* + *myoD* (**J**-**L**) and *lbx1* + *myf5* (**M**-**O**). Injected embryos were grown until stage 29, sectioned in the transverse plane, and analyzed with anti-phospho-histone H3 antibody (A,D,G,J,M), 12/101 (B,E,H,K,N) or *pax3* expression (C,F,I,L,O). The injected side of each tadpole is on the right, marked by β-galactosidase (red). Anti-phospho-histone H3 and 12/101 staining were performed on the same sections, while *pax3* staining was carried out on different tadpoles. Injection of *lbx1* causes an increase in proliferation (A), decrease in differentiated muscle (B) and increase in *pax3* (C). Injection of *myoD* causes no change in proliferation (D), an increase in differentiated muscle (E) and a decrease in *pax3* expression (F). Injection of *myf5* causes no significant change in cell proliferation (G), a slight increase in differentiated muscle (H) and no change in *pax3* expression (I). Injection of *lbx1* + *myoD* (J-L) produces a phenotype consistent with injection of *myoD* alone, while injection of *lbx1* + *myf5* (M-O) causes a phenotype like that of *lbx1* alone.

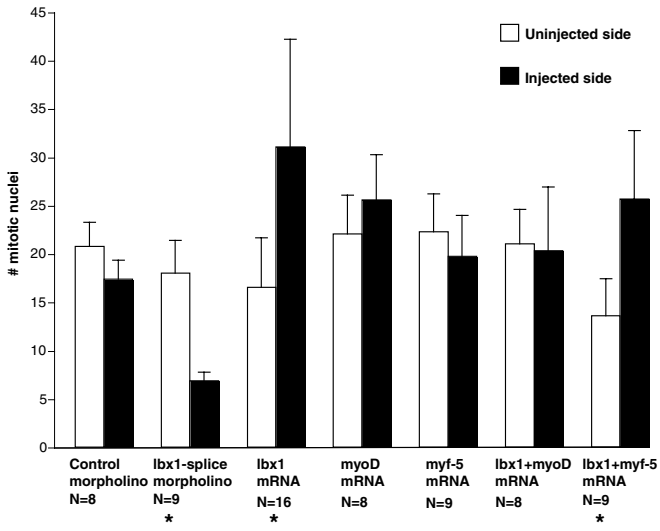

Fig. 10. Summary of cell proliferation data. The number of phospho-histone H3-positive nuclei in the immediate somitic area of the uninjected versus injected halves of manipulated tadpoles are represented. Injection of the *lbx1*-splice MO, *lbx1* mRNA or *lbx1* mRNA plus *myf5* mRNA caused a significant difference in the number of mitotic nuclei, indicated by an asterisk on the *x*-axis (paired Student's *t*-test, $P<0.01$). The control MO used is the zebrafish translation blocking MO.

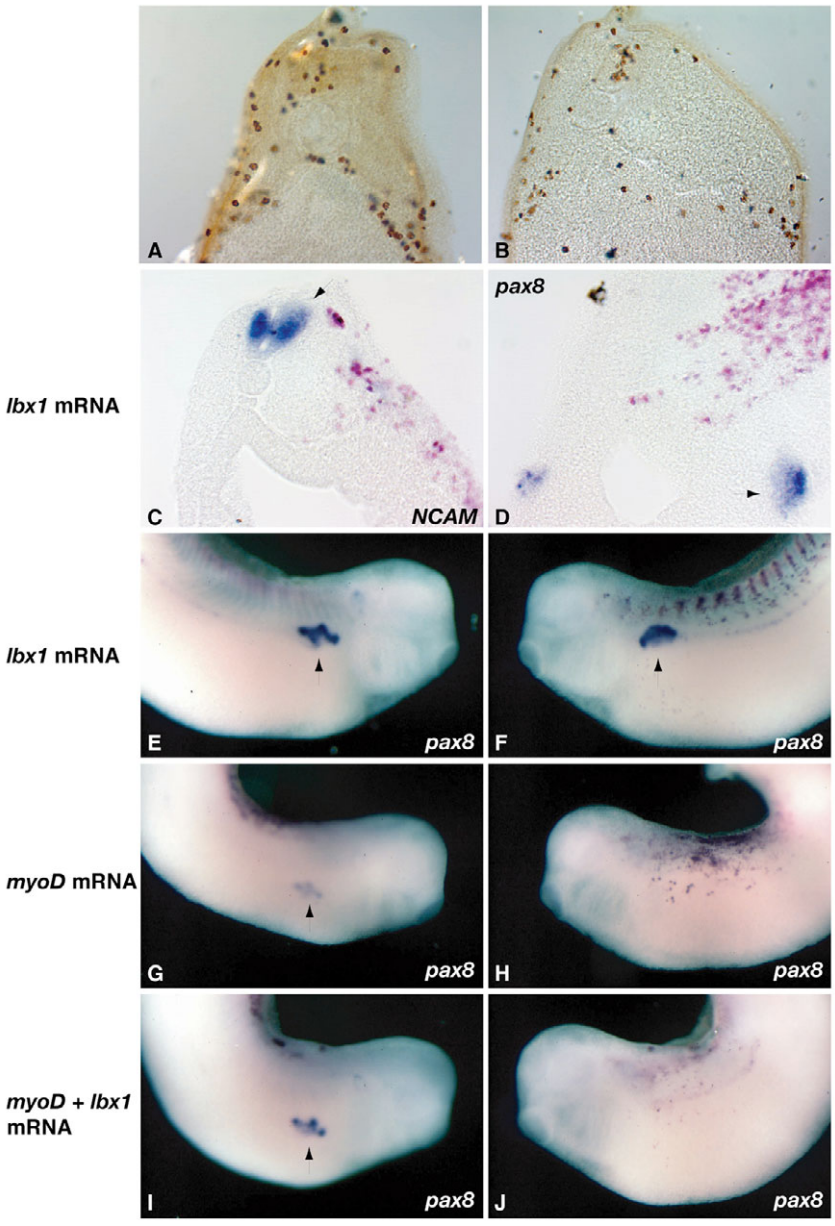

Fig. 11. Enlarged myotomes of *lbx1*-injected tadpoles is not the result of recruitment of non-myogenic lineages. (**A,B**) Injection of *myoD* (A) or *myoD* + *lbx1* (B) mRNA does not cause an increase in cell proliferation on the injected side (right side), as judged by pH3 staining on transverse sections of stage 29 tadpoles. Instead, non-myogenic lineages such as the pronephros are recruited to the myotome. (**C**,**D**) Transverse sections through stage 31 tadpoles indicate that both NCAM staining in the neural tube (C, arrow) and Pax8 staining in the pronephric tubules (D, arrow) are present on the injected side. (**E**,**F**) Lateral views of a tadpole injected with *lbx1* show the presence of pronephric tubules on both the uninjected (E) and injected (F) sides (arrows). (**G**-**J**) Stage 31 tadpoles injected with *myoD* alone (G,H) or *myoD* + *lbx1* (I,J), and stained with pax8, show normal pronephric tubules on the uninjected sides (G,I, arrows), but are lacking on the injected sides (H,J). However, tadpoles injected with *lbx1* alone do not show a lack of tissues surrounding the myotome, despite there being a larger somite.

diaphragm muscles. If the starting population of hypaxial myoblasts has a reduced ability to proliferate, and some myoblasts differentiate at various points along the migratory path, than those sites farthest from the somite would exhibit the most severe defects. This is what it seen in mouse mutants, where the distal regions of limbs completely lack musculature, but the more proximal regions, as well as the diaphragm and tongue, have musculature, except in reduced sizes. This is also consistent with the lack of restriction of myoblast fates, such that muscle compartments are specified at the periphery and filled progressively by migrating myoblasts (Kardon et al., 2003).

The ability of myoblast cells to migrate may also require a critical mass of cells. The migration defects seen in *lbx1* knockdown *Xenopus* tadpoles occur early, and recover in an anterior to posterior progression as the pool of myoblasts expands. Thus, at the time that *pax3*-positive myoblasts originating from trunk somite 4 may be stuck adjacent to the somite, those originating from trunk somite 1 have migrated ventrally (Fig. 4R,S). This may be the result of those cells having had a longer time to proliferate through non-*lbx1* mediated means, such as *pax3*-dependent proliferation. This may also explain why in mice mutant for *lbx1*, tongue muscles, which originate from anterior somites, form, whereas hindlimb muscles, which originate from posterior somites, completely fail to form (Brohmann et al., 2000; Gross et al., 2000; Schafer and Braun, 1999).

Finally, evidence that *lbx1* is not directly involved in myoblast migration comes from the overexpression of *lbx1*. When *lbx1* is overexpressed, there is no increase in number of migratory cells. In fact, there are fewer hypaxial muscles in these tadpoles (data not shown).

lbx1-positive myoblasts contribute to epaxial musculature

At early tadpole stages, *Xenopus* contain two types of skeletal muscle in the trunk. In the dorsal region, there is the dorsalis trunci, representing the epaxial domain. The ventral region contains the rectus abdominus, which is the hypaxial component of the trunk musculature (McDiarmid and Altig, 1999). In *lbx1*-splice MO-injected tadpoles, the ventral component of anterior epaxial muscle is reduced, with a corresponding loss of *myf5* staining in this region (Fig. 5B,D). This indicates that *lbx1*-positive myoblasts contribute to the formation of epaxial musculature. This is the first example of a case where *lbx1*-positive myoblasts contribute to both epaxial and hypaxial muscle, suggesting that the developmental programs of these two muscle types are more similar than previously suggested. The role of *lbx1* in both types of muscle development also supports earlier arguments that the primary role of *lbx1* is to promote myoblast proliferation, and not migration.

In vivo evidence that *lbx1* controls myoblast proliferation

Previous work has shown that *lbx1* can promote the proliferation of myoblasts in tissue explants (Mennerich and Braun, 2001). Here, we show that this is also true in the context of the whole embryo. When ectopic *lbx1* mRNA was targeted to the somitic region, enlarged somites were formed. Initially, these enlarged somites contained a smaller amount of differentiated muscle (Fig. 6H). Later on, after the injected *lbx1* had been depleted, the enlarged somites differentiated into a large amount of muscle (Fig. 6M,N). This was the first indication that *lbx1* was controlling cell proliferation in the somite, as myoblasts can divide or differentiate, but not do both. However, *lbx1* lacks the ability to induce myoblasts, as injections targeted outside the somite did not result in the formation of ectopic myoblasts or muscle (data not shown). The presence of expanded *pax3* expression as well as a higher number of mitotic cells in *lbx1*-injected tadpoles indicates that *lbx1* is a major regulator of cell proliferation (Fig. 9A-C).

lbx1 controls myoblast proliferation by downregulating *myoD*

In the chick, *lbx1* overexpression in the limb region leads to an increase in *myoD* expression and limb muscle formation. This process requires cell proliferation, indicating that *lbx1* may be expanding the population of myoblasts (Mennerich and Braun, 2001). We have found that the increase in *myoD* expression in these experiments is the end result of a period of myoblast proliferation in which *myoD* is not expressed. The overexpression of *lbx1* in the somitic region strongly inhibits the expression of *myoD*, so we suggest that the increase in *myoD* expression in the chick experiments may follow a period of myoblast proliferation in which *myoD* is not expressed. This would fit with the putative role of Lbx1 protein as a transcriptional repressor. In addition to having a homeobox DNA-binding domain, it also has an engrailed homology region, which has been shown to mediate transcriptional repression (Jagla et al., 2001). Indeed, our results demonstrate that after the injected *lbx1* can no longer be detected by in situ hybridization, the intensity of *myoD* staining on the injected side is actually greater than the amount on the uninjected side (Fig. 7S), similar to what was seen in the chick experiments.

We found that while *lbx1* represses *myoD*, and causes proliferation; the proliferative effect can be reversed by restoring *myoD* expression with injected mRNA (Figs 9, 10). Thus the enlarged somites caused by *lbx1* expression are different in origin from enlarged somites caused by ectopic expression of *myoD* or *myf5*. The overexpression of *myoD* and *myf5* in *Xenopus* embryos has previously been shown to produce enlarged myotomes, as the result of the induction of myogenesis in cells that would otherwise have a different fate (Ludolph et al., 1994). We confirmed this observation by showing that overexpressed *myoD* causes no change in the number of mitotic cells, a slight decrease in *pax3* expression, and an increase in differentiated muscle (Fig. 9D-F). When we co-expressed *myoD* and *lbx1*, the same result was obtained, indicating that *lbx1* does not have the ability to control cell proliferation independent of *myoD* levels (Fig. 9J-L). This indicates that the control of cell proliferation by *lbx1* is achieved through the inactivation of *myoD* expression.

Further evidence that *lbx1* represses *myoD*

In addition to the gain-of-function experiments showing that *lbx1* inhibits *myoD* expression, we also find evidence in MO loss-of-function experiments that links *lbx1* to the inhibition of *myoD* transcription. If *lbx1* has a direct role in repressing *myoD*, loss of *lbx1* function should result in a transient upregulation of *myoD*. Stage 26 tadpoles that have been injected with the *lbx1*-splice MO exhibit an increase in *myoD* expression on the injected side (Fig. 7N), suggesting that the repression by endogenous *lbx1* has been relieved.

We have also shown that the putative repressor domain of *lbx1* is essential for its function. The removal of a 12 amino acid stretch that encompasses the entire eh1 domain changes the function of *lbx1*. When overexpressed, the mutant construct no longer inhibits *myoD*. Instead, a weak increase in *myoD* expression is seen on the injected side (Fig. 8A). Furthermore, rather than *pax3* being upregulated on

the injected side, it is inhibited (Fig. 8B). These results indicate that *lbx1* normally functions as a repressor and that the removal of the eh1 domain produces a weak dominant-negative effect.

The nature of the dominant negative activity of *lbx1^{eh-}*

The opposite activity of *lbx1^{eh-}* relative to wild-type *lbx1* indicates that it is acting as dominant negative. Contrasting with the *lbx1*-splice MO, the *lbx1^{eh-}* injections also have an effect outside of the normal *lbx1* expression domain. This result suggests that *lbx1^{eh-}* interferes with the normal function of a protein other than *lbx1*. This may include another NK-class transcription factor, or perhaps even *pax3*, which is expressed throughout the entire dorsoventral axis of the somite. *Pax3* contains both a homeobox DNA-binding domain as well as an eh1 repressor domain, similar to *lbx1*. The Lbx1^{eh-} protein may bind to homeobox-binding sites and prevent the binding of endogenous homeobox containing proteins. Owing to the lack of the eh1 domain, it would not be able to repress the target gene. A similar phenotype to *lbx1^{eh-}* overexpression is observed when the bagpipe homologue *koza* is overexpressed in *Xenopus* (Newman and Krieg, 2002). Bagpipe is a member of the NK-class of transcription factors. Importantly though, *koza* contains a non-conservative amino acid substitution in the eh1 domain. This may in effect cause it to be a natural dominant-negative protein.

A molecular link between *lbx1* and cell cycle control

The cell cycle inhibitor *p27* is expressed in developing *Xenopus* somites. When its function is reduced using antisense MOs, enlarged undifferentiated somites result, similar to *lbx1*-injected tadpoles (Vernon and Philpott, 2003). These results indicate that *p27* is a central player in the precise timing of myotome differentiation and that *lbx1* may inhibit its expression. We tested this by examining *p27* expression in *lbx1*-injected tadpoles. At stage 31, when injected *lbx1* mRNA is still present, *p27* expression is inhibited on the injected side (Fig. 7Q). At stage 41, when injected *lbx1* mRNA is no longer detectable, *p27* expression is enhanced on the injected side (Fig. 7T). These results are similar to *myoD* expression in the same tadpoles and indicate that cell-cycle inhibition is disrupted in *lbx1*-injected tadpoles.

Recruitment of non-myogenic lineages is not responsible for enlarged myotomes in *lbx1*-injected tadpoles

The enhanced cell proliferation seen in *lbx1*-injected tadpoles suggests that this is the cause of enlarged somites, but there may also be a recruitment of non-myogenic lineages similar to what is seen in *myoD*-injected tadpoles (Ludolph et al., 1994). We examined tissues surrounding the somite in *lbx1*-injected tadpoles and found that both the neural tube and pronephros form normally (Fig. 11C-F). However, the pronephros is absent in both *myoD* and *myoD* + *lbx1*-injected tadpoles (Fig. 11G-J). These results demonstrate that the enlarged somites in *lbx1*-injected tadpoles are the result of enhanced cell proliferation and not from the recruitment of non-myogenic lineages.

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