Phosphorylation of Lbx1 controls lateral myoblast migration into the limb

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ABSTRACT

The migration of limb myogenic precursors from limb level somites to their ultimate site of differentiation in the limb is a paradigmatic example of a set of dynamic and orchestrated migratory cell behaviours. The homeobox containing transcription factor ladybird homeobox 1 (Lbx1) is a central regulator of limb myoblast migration, null mutations of Lbx1 result in severe disruptions to limb muscle formation, particularly in the distal region of the limb in mice (Gross et al., 2000). As such Lbx1 has been hypothesized to control lateral migration of myoblasts into the distal limb anlage. It acts as a core regulator of the limb myoblast migration machinery, controlled by Pax3. A secondary role for Lbx1 in the differentiation and commitment of limb musculature has also been proposed (Brohmann et al., 2000; Uchiyama et al., 2000). Here we show that lateral migration, but not differentiation or commitment of limb myoblasts, is controlled by the phosphorylation of three adjacent serine residues of Lbx1. Electroporation of limb level somites in the chick embryo with a dephosphomimetic form of Lbx1 results in a specific defect in the lateral migration of limb myoblasts. Although the initial delamination and migration of myoblasts is unaffected, migration into the distal limb bud is severely disrupted. Interestingly, myoblasts undergo normal differentiation independent of their migratory status, suggesting that the differentiation potential of hypaxial muscle is not regulated by the phosphorylation state of Lbx1. Furthermore, we show that FGF8 and ERK mediated signal transduction, both critical regulators of the developing limb bud, have the capacity to induce the phosphorylation of Lbx1 at these residues. Overall, this suggests a mechanism whereby the phosphorylation of Lbx1, potentially through FGF8 and ERK signalling, controls the lateral migration of myoblasts into the distal limb bud.

1. Introduction

Limb musculature is exclusively derived from hypaxial limb level somites. Through a highly conserved developmental and genetic program, myoblasts in limb level somites undergo stereotypical delamination, long range lateral migration and subsequent differentiation (Birchmeier and Brohmann, 2000; Dietrich et al., 1999; Vasyutina and Birchmeier, 2006). Central to this delamination and migration are the factors Lbx1, Pax3 and c-Met (Bladt et al. 1995; Schmidt et al. 1995; Tajbakhsh et al. 1997). Pax3 transcriptionally controls expression of the c-Met gene by binding to its promoter. Subsequently (Epstein et al. 1996), the expression of cMET induces the epithelial to mesenchymal transition required for myoblast delamination from the dermomyotome of limb level somites. Consequently, null mutants for both Pax3 and cMet both exhibit a loss in limb musculature. Pax3 also controls the expression of Lbx1, although the exact mechanism by which this regulation occurs remains unknown (Mennerich et al., 1998).

Furthermore, while LBX1 and PAX3 are co-expressed in delaminating myoblasts (Daston et al., 1996; Dietrich, 1999), their corresponding knock-out phenotypes reveal important functional differences. While knock-out mice for either cMet or Pax3 show a complete absence of hypaxial limb muscle, mice that lack LBX1 exhibit a loss of limb musculature preferentially within the distal limb musculature (Brohmann et al., 2000; Gross et al., 2000). Furthermore, other hypaxial muscle that undergo ventral-ward migration form without apparent defects, suggesting a role of LBX1 specifically in lateral migration. Indeed, in Lbx1 mutants, limb bud myoblasts delaminate correctly but fail to migrate properly into the limb field (Brohmann et al., 2000; Gross et al., 2000). This has led to speculations that a pro-migratory signal originates from the limb anlage to regulate LBX1.
activity (Gross et al., 2000), while a secondary role for LBX1 controlling differentiation and commitment of limb musculature has also been proposed (Brohmann et al., 2000; Uchiyama et al., 2000). In this work we describe the requirement of LBX1 phosphorylation for myoblasts to migrate into the distal limb anlage. Furthermore, the FGFR/ERK pathway is capable of modulating LBX1 phosphorylation. As such a likely candidate for the speculated pro-migratory signals (Gross et al., 2000) controlling LBX1 activity is the FGFR/ERK pathway, which controls the LBX1 phosphorylation state.

2. Materials and methods

2.1. Plasmids, antibodies and reagents

Expression vectors for Myc and Flag-tagged zebrafish (z) lbx1 were cloned into pIRE5-EGFP vector. Point mutations in lbx1 were generated using PCR-based site-directed mutagenesis. lbx1 point mutants were amplified by PCR and inserted into the pIRE5-EGFP. SaeI-EcoRI fragments of wild type (WT) lbx1 and lbx1 S223A S227A S234A were subcloned into pcDNA. The resulting plasmid construct pcDNA lbx1 and lbx1 S223A S227A S234A were used for immunohistochemistry. For in-vitro analysis lbx1 and lbx1 S223A S227A S234A were subcloned into pT2 Caggs-NLSmCherry-EGFPcaax and pT2 Caggs lbx1 S223A S227A S234A-EGFPcaax. See Table 1 for plasmids used in cloning and mutagenesis.

2.2. Antibodies and reagents

For in-vitro experiments the following antibodies and reagents were used: anti-Myc mouse IgG2a monoclonal antibody (9b11, Cell Signalling Technology; 1/2000), anti-PXSP rabbit IgG monoclonal antibody (ab150113, Abcam; 1/400), IRDye-680 and 800 secondary antibodies were purchased from LI-COR and conjugated anti-mouse antibody (ab150113, Abcam; 1/400), IRDye-800 conjugated anti-rabbit (A-31572, Life Technologies; 1:500), and Alexa Fluor 488 antibody (34B2, Cell Signalling Technology; 1/1000), anti-Flag rabbit IgG monoclonal antibody (f7425, Sigma; 1/1000), Alexa Fluor 488 conjugated anti-chicken (A-11039, Life Technnologies; 1:500), Alexa Fluor 488 conjugated anti-mouse (A-31572, Life Technologies; 1:500), and Alexa Fluor 488 conjugated anti-mouse antibody (ab150113, Abcam; 1/400) for 30 min, followed by counterstaining with Hoechst 333258. Western blotting. Anti-Flag and PXSP antibodies were diluted to 1:1000. Anti-Myc antibody was diluted to 1:2000. For in-vivo experiments the following antibodies and reagents were used: anti-GFP chicken polyclonal (ab13970, Abcam; 1/500), anti-myosin heavy chain (MyHC) chicken polyclonal (ab13970, Abcam; 1/500), anti-RFP rabbit polyclonal (f7425, Sigma; 1/1000) Alexa Fluor 488 conjugated anti-mouse antibody (ab150113, Abcam; 1/400), IRDye-700 and 800 secondary antibodies were purchased from LI-COR and diluted to 1:15000. PMA was purchased from Sigma. MG132, U0126 and SU5402 were purchased from calbiochem. Recombinant FGFR8 was purchased from BD Biosciences.

For in-vitro analysis the following antibodies were used: anti-GFP chicken polyclonal (ab13970, Abcam; 1/500), anti-RFP rabbit polyclonal (ab6234, Abcam; 1/500), anti-myosin heavy chain (MyHC) IgG2b mouse monoclonal (MF20, Developmental Studies Hybridoma Bank; 1/10). Secondary antibodies used are Alexa Fluor 488 conjugated anti-mouse (A-11039, Life Technologies; 1/500), Alexa Fluor 555 conjugated anti-rabbit (A-31572, Life Technologies; 1/500), and Alexa Fluor 647 conjugated anti-mouse (A-31571, Life Technologies; 1/500).

2.3. Cell line and transfection

NIH3T3 cell lines are maintained in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin. C2C12 cells are maintained in DMEM supplemented with 20% fetal calf serum and penicillin/streptomycin. For transfection, cells were subcultured and grown overnight, then transiently transfected with various expression construct using Lipofectamin 2000 according to manufacturer’s protocol.

2.4. Shrimp alkaline phosphatase treatment and Western blot

Transiently transfected cells were lysed with lysis buffer (20 mM Tris/HCl, pH7.5, 150 mM NaCl, 2 mM EGTA, 25 mM beta-glycerophosphate, 50 mM sodium fluoride, 10% glycerol, 1% Triton X-100, 1 mM sodium vanadate). Protease inhibitor cocktail (Roche) at 24hs post-transfection. After centrifugation, clarified cell lysates were subjected to immunoprecipitation or Western blotting. For shrimp alkaline phosphatase treatment (SAP), cell lysate was immunoprecipitated by rotating with 2 mg anti-Myc antibody (Cell Signalling Technology) and 10 ml protein G sepharose at 4 C overnight. The beads were washed with lysis and dephosphorylation buffer (50 mM Tris-HCl pH 9.0, 150 mM NaCl, 10 mM MgCl2), then treated with 10U Shrimp alkaline phosphatase 37 C for 1 h. Immunoprecipitated Lhx1 was solubilized with Laemmli’s SDS-PAGE sample buffer and subjected to Western blotting. Anti-Flag and PXSP antibodies were diluted to 1:1000. Anti-Myc antibody was diluted to 1:2000.

2.5. Immunohistochemical analysis

C2C12 cells were transiently transfected by pcDNA Wt Lbx1 or Lbx1 S223A S227A S234A. At 24hrs post transfection, cells were fixed with 4% PFA at room temperature for 20 min, washed with PBS (-) and permealized with ice-cold 0.05% triton X-100/ PBS for 5 min, and blocked with 10% fetal calf serum and 1% BSA. Cells were incubated with anti-Myc mouse IgG2a monoclonal antibody (9b11, Cell Signalling Technology; 1/2000) 4 C overnight and then treated with Alexa Fluor 488 conjugated anti-mouse antibody (ab150113, Abcam; 1/400) for 30 min, followed by counterstaining with Hoechst 333258.

2.6. In-vivo electroporation and analysis of LBX1 and Lbx S223A S227A S234A

Each plasmid was co-electroporated with pT2 Caggs-nlsRFP as an electroporation control and pCaggs Transposase to allow for genomic integration and long term labelling of electroporated cells. Electroporation was performed as described (Scaal, Gros, Lesbros, 2008).

Table 1

<table>
<thead>
<tr>
<th>Plasmid</th>
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<tr>
<td>Eco/Flag-Lbx1-A</td>
<td>Control plasmid</td>
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<td>Myc-Lbx1-S</td>
<td>Myc-tagged Lbx1</td>
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<tr>
<td>s128a-S</td>
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Fig. 1. LBX1 is phosphorylated. (a) Myc/Flag-tagged Lbx1 were expressed in NIH3T3 cells. At 24 h post transfection, cells were lysed with lysis buffer. LBX1 was immunoprecipitated using anti-Myc antibody. Immunoprecipitates were washed and treated with shrimp alkaline phosphatase (SAP) at 37°C for 1 h, then subjected to Western blotting using anti-Flag antibody. Mobility shift of LBX1 by SAP treatment shows that LBX1 is phosphorylated in cells. (b) Seven phosphorylation sites were predicted from Scansite, as shown (green shade). The region shown by blue shade and pink shade show EH (Eps15) homology motif (a protein-protein interaction domain) and the DNA binding Homeobox domain respectively. (c) Myc-tagged LBX1 and points mutants were transiently transfected to NIH3T3 cells. At 24 h post transfection, cells were lysed with lysis buffer. Wild type (Wt) LBX1 and mutants are detected by Western blotting using anti-Myc antibody and IRdye800 conjugated anti-mouse IgG. Phosphorylation of LBX1S227A was severely reduced. Phosphorylation of LBX1S223A and LBX1S234A were moderately reduced.
due to phosphorylation, Lbx1 transfected lysates were immunoprecipitated using the myc tag and subjected to treatment with Shrimp Alkaline Phosphatase (SAP), which removes phosphorylation at all sites. As expected this resulted in a disappearance of the doublet, in favour of a single band at the lower molecular weight (Fig. 1a), thus confirming LBX1 to be a phosphoprotein.

3.2. Mutagenesis reveals three phosphorylation sites in the c-terminus of Lbx1

In-silico analysis using ScanSite (Obenauer et al., 2003) identified seven potential phosphorylation sites of LBX1 conserved between human, mouse and zebra fish (Fig. 1b). Six of the predicted phosphorylation sites are on Serine residues while one is on a Threonine residue (Fig. 1b). Sequential mutation of each of these sites to non-phosphorylatable Alanine residues followed by western blot analyses reveals that S227 is highly phosphorylated, while the two other adjacent Serines (S223 and S234) are moderately phosphorylated in vitro. None of the other Serine or Threonine residues were found to significantly contribute to phosphorylation (Fig. 1c). To identify if S227 is the only phosphorylation site in LBX1 we used a Phorbol-12-Myristate-13-Acetate (PMA) treatment. PMA is a highly potent activator of Protein Kinase C and as such results in the constitutive phosphorylation of all phosphorylatable residues. Although LBX1S227A undergoes the most severe reduction in phosphorylation, PMA treatment is still capable of forcing this protein to a phosphorylated state to approximately 50% of that occurs for similar treatments of WT LBX1, suggesting additional residues can also be phosphorylated (Fig. 2a). In line with this observation, of the seven identified potential phosphorylation sites three specific point mutations (S223A, S227A, and S234A) lead to a reduction of phosphorylated LBX1, albeit with the phosphorylation LBX1S223A and LBX1S234A being reduced in a more modest manner compared with Lbx1S227A. These result suggest that these adjacent sites represent the potential other phosphorylation sites detected in this analysis. Both LBX1S223 and LBX1S234 are predicted to be phosphorylated by ERK, based on the known consensus sequence (Fig. 2b). ERK phosphorylates PXSP motifs and the PXSP antibody detects phosphospecific sites within this predicted phosphorylation consensus site. Using a PXSP antibody we identified potentially ERK specific phosphorylation occurring on LBX1S234 but not on Lbx1S223. Furthermore, while the individual LBX1S223A, LBX1S227A and LBX1S234A mutant proteins are still capable of being phosphorylated, double or triple mutants with LbxS227A appear incapable of being phosphorylated, even after PMA treatment (Fig. 2c).

We wondered whether protein stability and sub-cellular localization of LBX1 were affected by the triple S > A mutation. Using proteasome inhibitor MG132, the stability of triple LBX1S223A, S227A, S234A mutant proteins were assessed over a period of 6 h. We found that LBX1S223A, S227A, S234A does not exhibit any changes in protein stability, when compared to wildtype LBX1 (Fig. 3a). Furthermore, neither does the subcellular localization of LBX1S223A, S227A and S234A alter, compared to wildtype LBX1 when assessed by immunofluorescence microscopy (Fig. 3b).

3.3. FGF signalling stimulates LBX1 phosphorylation through ERK in vitro

FGF8 and its downstream effector ERK are important signalling molecules that regulate formation of the distal limb anlage and induce phosphorylation of specific target proteins (Corson et al., 2003; Lewandoski et al., 2000; Borello et al., 2008; Suzuki-Hirano et al., 2010). Since we have established a potential role for ERK in the phosphorylation of LBX1 we set out to validate this and further interrogate potential upstream processes. If LBX1 phosphorylation is indeed regulated by signalling through FGF8 and/or ERK, this would provide insights into the pro-migratory factor first hypothesized by...
Gross et al. (2000) which was speculated to be important for lateral migration of myoblast into the distal limb. We transiently express myc-tagged LBX1 in NIH3T3 cells, and cells were treated with either 50 μM of the FGF antagonist SU5402 or 20 μM of the ERK inhibitor U0126 and subsequently with 125 ng/ml recombinant FGF8 for 30 min. We find that the ectopic addition of FGF8 has the capacity to increase phosphorylation of LBX1, which is inhibited by the co-incubation of FGF receptor inhibitor (SU5402) or ERK inhibitor (U0126) (Fig. 3c). These results reveal that LBX1 can be phosphorylated, at least in part, through the activation of the FGF8/ERK signalling pathway.

3.4. Over expression of the dephosphomimetic LBX1 inhibits intra-limb migration of myoblasts but not their initial emigration from the somites

The above results demonstrate that the phosphorylation status of LBX1 can be regulated by FGF8 and ERK, which are important signalling molecules in the distal limb anlage (Corson et al., 2003; Lewandoski et al., 2000). Since lbx1−/− mice have phenotypes that suggest the intra-limb migration of myoblasts is specifically disrupted (Gross et al., 2000), this led us to interrogate the effect of over-expressing either WT LBX1 or the dephosphomimetic form of LBX1 on the migration of myoblasts into the distal limb anlage. Even though S227 is the major site of phosphorylation, S223 and S234 seem to play secondary roles, as such the triple dephosphomimetic form of LBX1 was used. To interrogate the cell autonomous role of Lbx1 phosphorylation in myoblasts populating the embryonic limb we electroporated the ubiquitously expressing either a construct ubiquitously expressing WT LBX1 and GFP (caggs-lbx1-IRES-eGFPcaax) or a ubiquitously expressing the dephosphomimetic LBX1 and GFP (caggs-lbx1S223A S227A S234A-IRES-eGFPcaax) plasmids into the lateral region of limb level avian somites. These plasmids were co-electroporated with a ubiquitously expressing nuclear RFP plasmid (caggs-nlsRFP), which provides an internal electroporation control. This allows for precise control of the spatio-temporal expression and gene function analysis in a cell specific manner. It enabled the targeted overexpression of mutant and WT forms of LBX1 in the limb myoblasts as they delaminate and migrate into the limb bud. Embryos were fixed at Hamburger and Hamilton Stage (HH) 23 and HH 27, representing stages shortly after delamination and migration into the limb (HH 23), and the period during intra limb migration (HH27). Subsequent staining for the muscle marker MF20 allows us to determine the relative distribution of both GFP and RFP signals within the entire myogenic field. At HH 23 we find that delamination and early migration is not affected by the over-expression of either lbx1 or lbx1S223A S227A S234A (n=6, Fig. 4a). In contrast at HH 27 the overexpression of lbx1S223A S227A S234A, but not lbx1 results in a failure of migration into the distal limb anlage (n=7, Fig. 4a). A limited number of cells that over-express Lbx1S223A S227A S234A escape this inhibition and manage to migrate further distally into the limb myogenic field without any change in their myogenic potential, differentiating as MF20+ elongated myoblasts (n=7, Fig. 4c, Supplementary Fig. 1d). We have furthermore quantified the number of GFP+ and RFP+/GFP signals within the entire myogenic field (Fig. 4D, Supplementary Fig. 1a–c). Specifically, we compared the RFP+/GFP population to the MF20+ population and the GFP+ population to the RFP+/GFP population. The distribution of RFP+/GFP+ signal within the MB1 population is shown in Fig. 4D.
Fig. 4. LBX1 phosphorylation is essential for the migration of myogenic progenitors. pT2 Caggs-Lbx1-IRES-eGFPcaax or pT2 Caggs-Lbx1S223A S227A S234A-IRES-eGFPcaax was electroporated with pT2 Caggs-RFP into the lateral region of limb level avian somites. Embryos were left to develop, and subsequently analysed at HH23 and HH27 by staining for RFP, GFP, and MF20. (a) At HH23 the delamination of muscle progenitors is not affected by the expression of LBX1S223A S227A S234A. (b) At HH27 muscle progenitors that express Lbx1S223A S227A S234A fail to migrate into the limb field while the overexpression of Wild-type LBX1 does not. (c) Zoom from bounding boxes in panel b. No difference was observed in the capacity to differentiate of Lbx1 and Lbx1S223A S227A S234A electroporated myogenic progenitors. (d) Quantification of cell numbers from panel b. The overexpression of LBX1S223A S227A S234A results in a preferential localization of GFP signals in the proximal region of the limb bud. Scale bars: A-B 500 µm, C 200 µm.
GFP cells is even across the MF20+ myogenic field. Similarly, when overexpressing \textit{lbx1}, GFP+ cells are evenly distributed along the MF20+ myogenic field. Only when \textit{lbx1} \textit{S223A S257A S2344} is overexpressed do GFP+ cells preferentially localize proximally. Collectively, this data suggests that the phosphorylation status of Lbx1 within somite derived limb myoblasts regulates the migratory properties of cells within the limb field but does not affect their differentiation potential.

4. Discussion

Even though Lbx1 is expressed in all hypaxial muscle progenitors \textit{Lbx1/--} mice show specific defect in the limb, but not in other hypaxial muscles. Within the limb these defects are more severe in the distal than in the proximal limb, a fact that implicates Lbx1 activity in the control the lateral migration of myoblasts, an activity, these results suggest, is likely regulated by a signal(s) expressed in the distal limb anlage. Previous research in Xenopus suggests that over expression of \textit{lbx1} inhibits differentiation of hypaxial muscle by downregulation of \textit{myod} and \textit{myf5}. \textit{Lbx1} could thus provide a permissive state in which migration of myogenic progenitors can occur (Martin and Harland, 2006). However, hypaxial muscle in Xenopus, at pre limb bud stages (Martin and Harland, 2006), does not undergo lateral migration, and as such would unlikely to be regulated by Lbx1 in a similar manner as described above.

We identified Lbx1 to be a phospho-protein, its phosphorylation being regulated, at least \textit{in-vitro}, by FGF8 and ERK-mediated signaling. Overexpression of a dephosphomimetic form of Lbx1 in the limb level somites of a chicken embryo results in disrupted migration into the distal limb anlage, instead myoblasts preferentially cluster in the proximal limb anlage. These observations correspond to those made in \textit{lbx1} knock-out mice which exhibit a disruption in ventralward migration, while in both cases the myogenic differentiation potential of these cells remained unaffected. In both wildtype and the unphosphorylated-mimetic \textit{Lbx1} overexpression, myoblasts underwent differentiation independent of the overexpression or its phosphorylation status. These data suggest that myoblast migration is not dependent on a permissive state, created by \textit{Lbx1} expression.

Taken together these data point towards a unique and specific role for \textit{Lbx1} in the context of limb muscle development. \textit{Lbx1} activity was hypothesized to be controlled by factors expressed within the distal limb anlage. Indeed, we found that FGF and its downstream effector ERK (Suzuki-Hirano et al., 2010), both expressed in the distal limb anlage, are capable of modulating \textit{Lbx1} phosphorylation state. Furthermore, somite specific over expression of an unphosphorylated-mimetic form of \textit{Lbx1}, but not wildtype \textit{Lbx1} affects the lateral migration of myoblasts into the distal limb bud. The over-expression of the dephosphomimetic form of \textit{Lbx1} results in a dominant negative effect on the lateral migration of myoblasts into the distal limb bud. The exact mechanism by which this dominant negative effect is achieved remains unclear. Two possible scenarios can be hypothesized, either the DNA binding domain of \textit{Lbx1} binds to the targeted promoter regions but fails to induce promoter activation after phosphorylation, or alternatively DNA binding is required promoter regions but fails to induce promoter activation after being regulated, at least \textit{in-vitro}, by FGF8 and ERK-mediated signaling. Overexpression of a dephosphomimetic form of \textit{Lbx1} leads to a defect specifically in lateral myoblast migration, not a recapitulation of the full mouse knockout phenotype. One possible mechanism might be that the ability of \textit{Lbx1} to bind and or regulate the promoters of specific target genes, required for lateral limb myoblast migration, is altered by phosphorylation. Such a model would explain why only lateral limb migration of limb myoblasts is disrupted in embryos overexpressing unphosphorylated-mimetic \textit{Lbx1} and not the initial the delamination and emigration of cells from the somite.

Intriguingly the phenotype we generate is strikingly similar to that exhibited by mouse embryos lacking the SDF1 receptor \textit{cxcr4}. \textit{Ccxr4} was identified as being expressed in migratory limb myoblasts through profiling of \textit{Lbx1} positive myoblasts from transgenic mice in which GFP had been knocked into the \textit{Lbx1} locus (Vasyutina et al., 2005). Further analyses revealed that \textit{Ccxr4} is expressed on migrating limb myoblasts, while its ligand, \textit{sdf}, is expressed in the limb anlage. Mice that lack \textit{Ccxr4} result in a phenotype where lateral migration of limb myoblast migration is affected, with distal limb muscleatur is more severely affected than proximal musculature (Vasyutina et al., 2005).

Collectively, these observations suggest a mechanism whereby once emigrating myoblasts come within range of FGF signalling originating form the distal limb anlage, \textit{Lbx1} phosphorylation is induced through the action of ERK. This may alter the affinity of \textit{Lbx1} for its target genes, which in turn control migration of somite derived myoblast through additional directed signalling within the limb field.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2017.08.025.

References


How could the phosphorylation status of Lbx1 affect its ability to regulate limb myoblast morphogenesis? Our results reveal no difference in protein stability or nuclear transport between phosphorylated or non-phosphorylated forms of \textit{Lbx1} \textit{in-vitro}. Over expression of the dephosphomimetic form \textit{in-vivo} leads to a defect specifically in lateral myoblast migration, not a recapitulation of the full mouse knockout phenotype. One possible mechanism might be that the ability of \textit{Lbx1} to bind and or regulate the promoters of specific target genes, required for lateral limb myoblast migration, is altered by phosphorylation. Such a model would explain why only lateral limb migration of limb myoblasts is disrupted in embryos overexpressing unphosphorylated-mimetic \textit{Lbx1} and not the initial the delamination and emigration of cells from the somite.


