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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

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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 FGF8/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 FGF8/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 pIRES2-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 pIRES2-EGFP. *Sac*I-*Eco*RI 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-vivo* analysis *Lbx1* and *Lbx1*$^{S223A\ S227A\ S234A}$ were subcloned into pT2 Caggs *NLSmCherry*-IRES *EGFPcaax* using Gibson Assembly® (NEB) to generate pT2 Caggs *Lbx1*-IRES *EGFPcaax* and pT2 Caggs *Lbx1*$^{S223A\ S227A\ S234A}$–IRES *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 (34B2, Cell Signalling Technology; 1/1000), anti-Flag rabbit IgG monoclonal antibody (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 FGF8 was purchased from BD Biosciences.

For *in-vivo* 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-chicken (A-11039, Life Technnologies; 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 $MgCl_2$), then treated with 10U Shrimp alkaline phosphatate 37 C for 1 h. Immunoprecipitated Lbx1 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,

Table 1
All primers used for making Lbx1 expression vectors are listed below.

Eco/Flag-Lbx1-A	cccgaattcCTACTTATCGTCGTCATCCTTGTAATCatcgtccacctcgatttcctc
Myc-Lbx1-S	CCCGAGCTCACCATGGCATCAATGCAGAAGCTGATCTCAGAGGAGGACCTGatgacctccagctctaaagaca
s123a-S	aaagacaggcaGccaaaaaa
s123a-A	cgtcgtttttggCtgcctg
s129a-S	aaaaacgacggaagGcccgc
s129a-A	aaggctgtgcgggCcttccg
t131a-S	gaagtcccgcGcagccttca
t131a-A	tgaaggctgCgcgggacttc
s223a-S	tcggggcccattGctccca
s223a-A	gaaagactgggagCaatggg
s227a-S	tctcccagtcttGccccaag
s227a-A	ggctcttggggCaagactgg
s234a-S	agcctttccacagGccccat
s234a-A	cgaggatggggCctgtggaa
s247a-S	acgagttcGcagaggaggac
s247a-A	gtcctcctctgCgaactcgt
s247a-A2	ttcctcgtcctcctctgCgaact

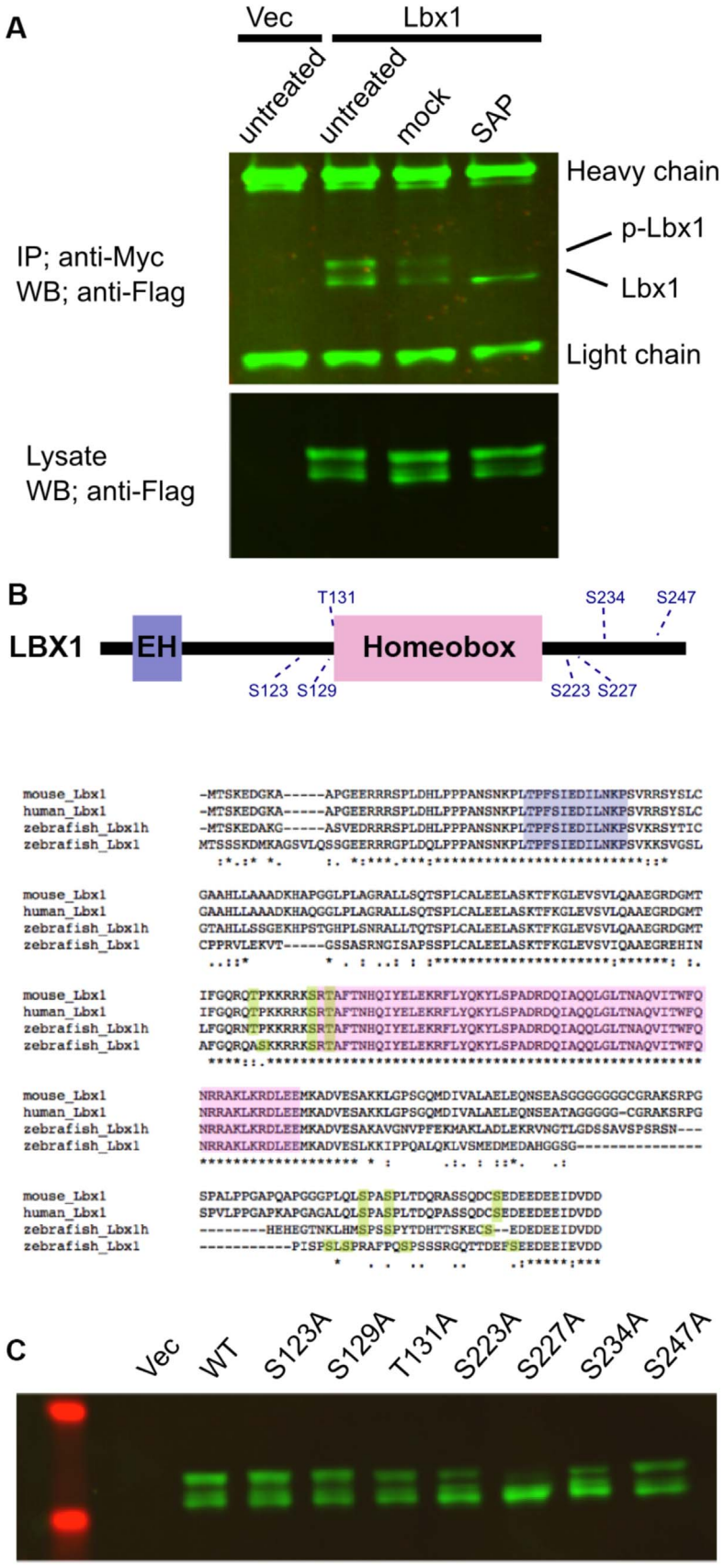

Fig. 1. LBX1 is phosphorylated. (a) Myc/Flag-tagged *Lbx1* were expressed in NIH3T3 cells. At 24 h post transfection, cells were lysed with lysis buffer. LBX1 was immunoprecipitated using anti-Myc antibody. Immunoprecipitates were washed and treated with shrimp alkaline phosphatase (SAP) at 37 C for 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-taged 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 $LBX1^{S227A}$ was severely reduced. Phosphorylation of $LBX1^{S223A}$ and $LBX1^{S234A}$ were moderately reduced.

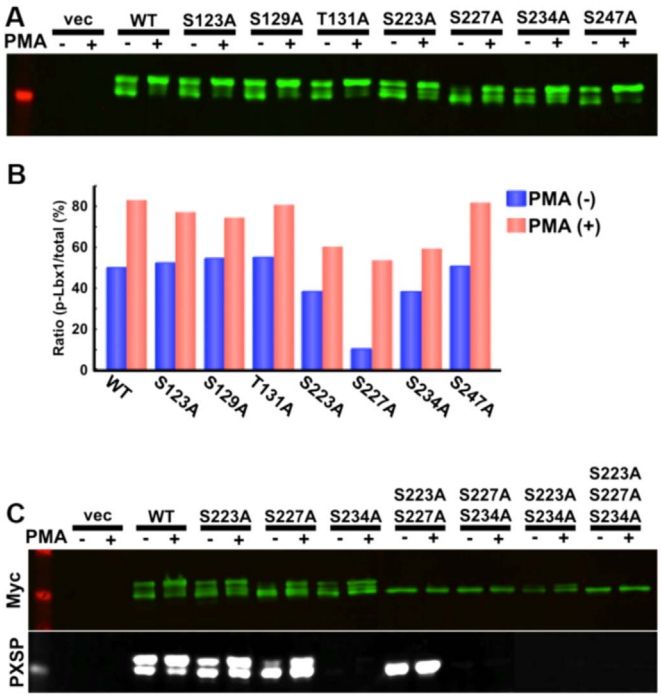

Fig. 2. LBX1 is phosphorylated at S223, S227, and S234. (a) Myc-taged *Lbx1* and points mutants were transiently transfected to NIH3T3 cells. At 24 h post transfection, cells were treated with 100 nM PMA for 30 min and lysed. Wild type (Wt) LBX1 and mutants are detected by Western blotting using anti-Myc-tag and IRdye800 conjugated anti-mouse IgG. Phosphorylation of wild type (Wt) LBX1 was upregulated by PMA and quantitated in **(b)**. Phosphorylation of S227A was also upregulated by PMA treatment, suggesting LBX1 is phosphorylated at multiple sites. **(c)** S223 and S234 were predicted to be ERK substrates and an anti-PXSP antibody was used to detect the phosphorylation at these sites. Myc-taged Lbx1 and points mutants were transiently transfected to NIH3T3 cells. At 24 h post transfection, cells were treated with 100 nM PMA for 30 min and lysed. Wild type (Wt) LBX1 and mutants are detected by Western blotting using anti-Myc and anti-Erk substrate (PXSP) antibody. IRdye800 conjugated anti-mouse IgG and IRdye700 conjugated anti-Rabbit IgG antibodies were used as secondary antibodies. Phosphorylation at S234 but not S223 was detected by anti-PXSP antibody, revealing that S234 alone is a likely to be an ERK substrate. Mobility difference between $LBX1^{S227A}$ and $LBX1^{S223A\ S227A}$ double mutant reveals that S223A is also phosphorylated. Note that the phosphorylations at S223 and S227 were both increased by PMA treatment.

and Marcelle, 2004). Antibody staining was performed as described (Marcelle, Ahlgren, and Bronner-Fraser, 1999). For observation, limb buds were dissected, cleared in 80% glycerol/H_2O and examined using an SP5 confocal microscope (Leica) with a 40x oil immersion lens. Quantifications of cell distribution along the PD axis were performed in Imaris 8.0 and Prism 7. Each channel was segmented out using either Imaris surface rendering, location of each surface was extracted using the Vantage module. Location data for all samples is imported into Prism and analysed using Kruskal-Wallis and uncorrected Dunn's test to generate P-values for each paired comparison.

3. Results

3.1. Lbx1 is a phosphoprotein in vitro

In-silico analysis of the LBX1 protein sequence predicted several highly conserved phosphorylation sites, which we speculated could be utilised to regulate its function during limb myoblast migration. To validate phospho regulation at these sites, we perform immune-precipitations of LBX1 *in-vitro* using a N-terminal Myc-tagged and C-terminal Flag-tagged version of *lbx1* over expressed in NIH3T3. Western blot analysis of this construct revealed a stereotypical doublet, typical for phosphoproteins, potentially representing phosphorylated and non-phosphorylated fractions. To confirm that doublet is indeed 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 zebrafish (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 $LBX1^{S227A}$ 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 $LBX1^{S223A}$ and $LBX1^{S234A}$ being reduced in a more modest manner compared with $Lbx1^{S227A}$. These result suggest that these adjacent sites represent the potential other phosphorylation sites detected in this analysis. Both $LBX1^{S223}$ and $LBX1^{S234}$ 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 $LBX1^{S234}$ but not on $LBX1^{S223}$. Furthermore, while the individual $LBX1^{S223A}$, $LBX1^{S227A}$ and $LBX1^{S234A}$ mutant proteins are still capable of being phosphorylated, double or triple mutants with LBX^{S227A} 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 $LBX1^{S223A\ S227A\ S234A}$ mutant proteins were assessed over a period of 6 h. We found that $LBX1^{S223A\ S227A\ S234A}$ does not exhibit any changes in protein stability, when compared to wildtype LBX1 (Fig. 3a). Furthermore, neither does the subcellular localization of $LBX1^{S223A\ S227A\ S234A}$ 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

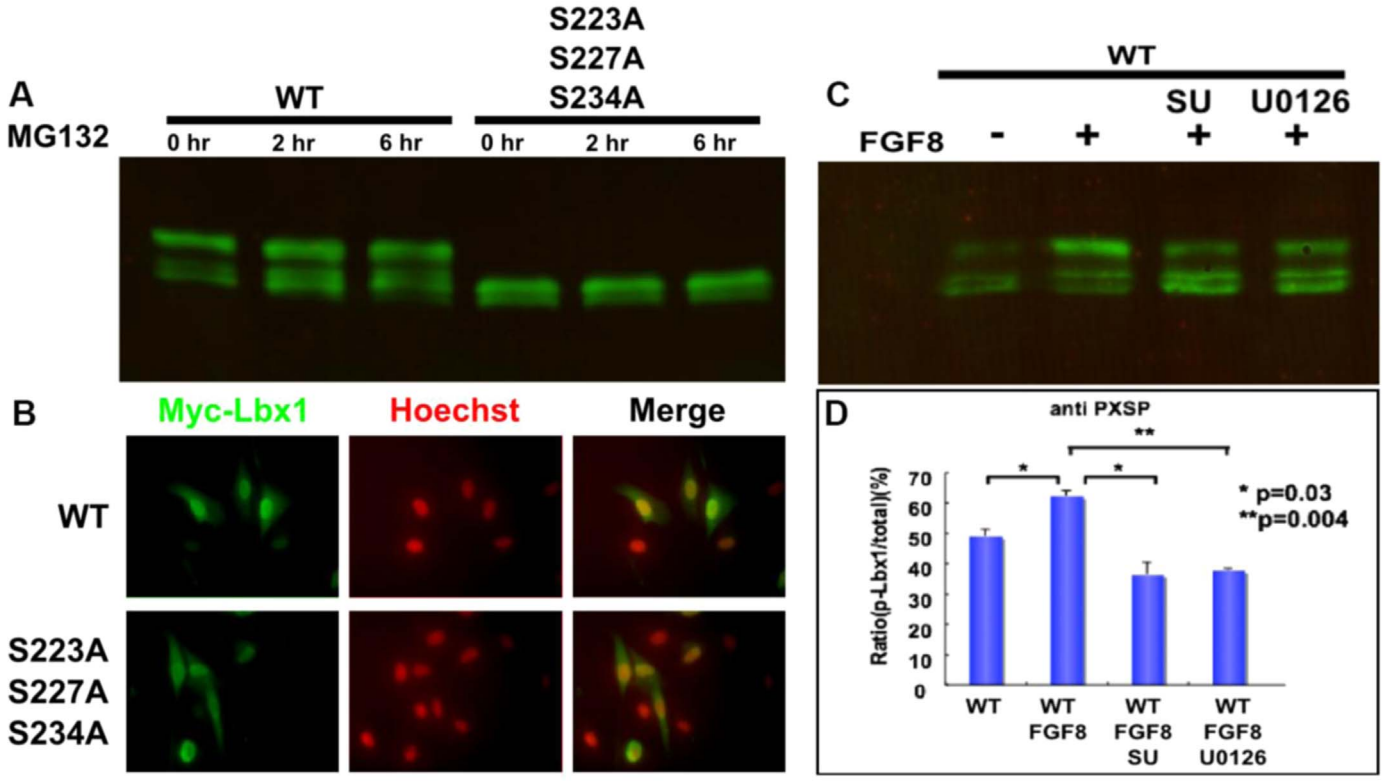

Fig. 3. Phosphorylation does not affect LBX1 degradation and localization. (a) Myc-taged *Lbx1* and *Lbx1*$^{S223A\ S227A\ S234A}$ were transiently transfected to NIH3T3 cells. At 24 h post transfection, cells were treated with 25 μM MG132 for 2 or 6 h and lysed with lysis buffer. Wild type (Wt) LBX1 and mutants are detected by Western blotting using anti-Myc tag. MG132 did not have any effect on the degradation of phosphorylated and unphosphorylated form of LBX1, suggesting that phosphorylation does not have any effect on the proteasome-mediated degradation of LBX1. **(b)** Myc-tagged Lbx1 and Lbx1$^{S223A\ S227A\ S234A}$ were transiently transfected to C2C12 cells. At 24 h post transfection, cells were fixed and stained with anti-Myc antibody to identify the intracellular localization of LBX1. Intracellular localization is similar between LBX1 and LBX1$^{S223A\ S227A\ S234A}$, suggesting LBX1 phosphorylation did not have any effect on the degradation nor the intracellular localization. **(c)** Myc-tagged Lbx1 was transiently transfected to NIH3T3. At 24 h post transfection, culture medium are replaced to serum free DMEM. After 4 h, cells were treated with 50 μM SU5402 or 20 μM U0126 for 2 h, followed by treatment with 125 ng/ml recombinant FGF8 for 30 min. Cells were lysed with lysis buffer and subjected to Western blotting. LBX1 phosphorylation was up-regulated by FGF8. This up-regulation was inhibited by FGF receptor inhibitor SU5402 or ERK inhibitor U0126.

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 seme 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-*lbx1*$^{S223A\ S227A\ S234A}$-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 *lbx1*$^{S223A\ S227A\ S234A}$ (n=6, Fig. 4a). In contrast at HH 27 the overexpression of *lbx1*$^{S223A\ S227A\ S234A}$, 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 *Lbx1*$^{S223A\ S227A\ S234A}$ 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 MF20^{+} limb 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^{+}/

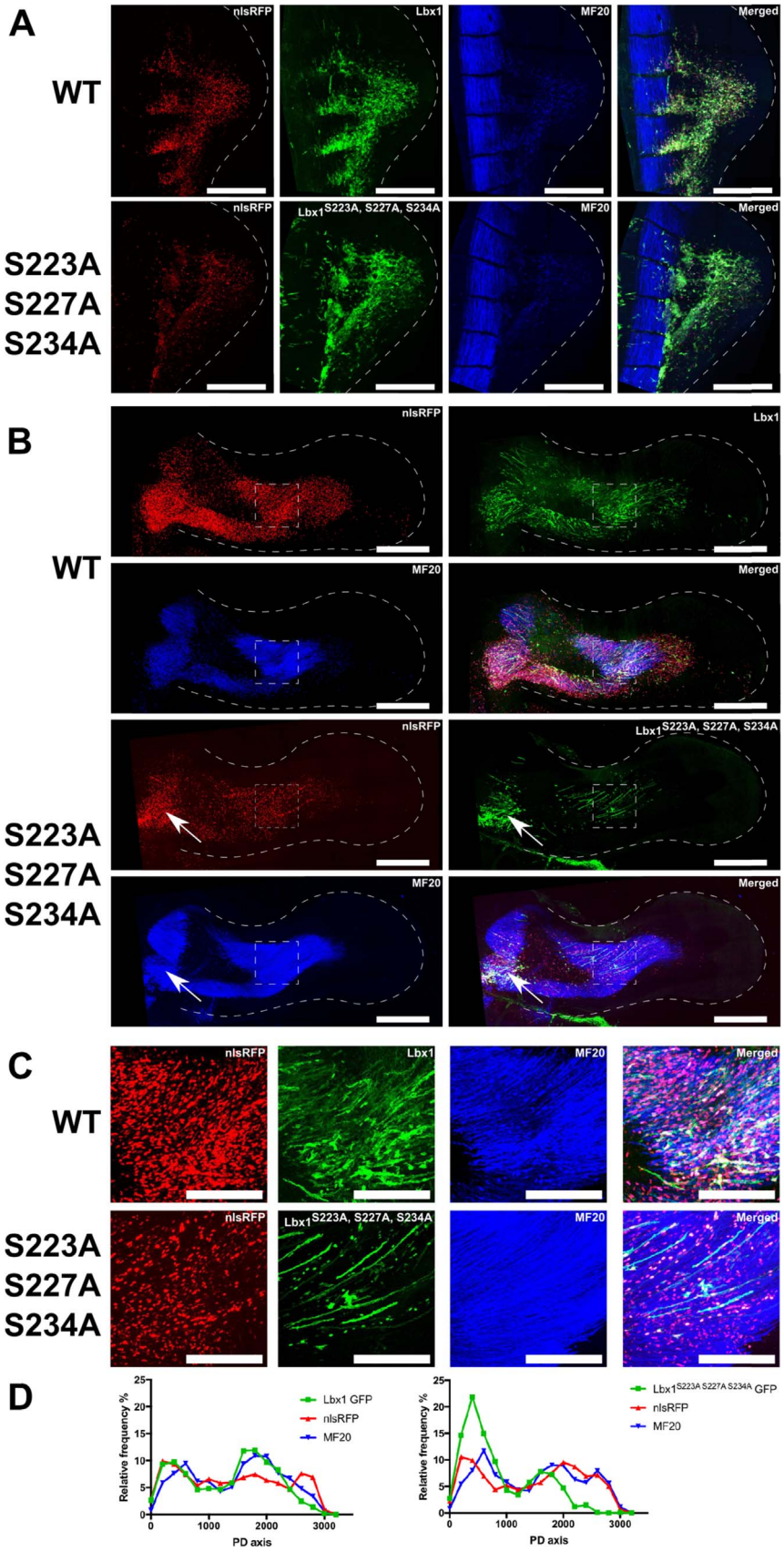

Fig. 4. LBX1 phosphorylation is essential for the migration of myogenic progenitors. pT2 Caggs-Lbx1-IRES-eGFPcaax or pT2 Caggs-Lbx1$^{S223A\ S227A\ S234A}$-IRES-eGFPcaax was electroporated with pT2 Caggs-RFP into the lateral region of limb level avian somites. Embryos were left to develop, and subsequently 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 LBX1$^{S223A\ S227A\ S234A}$. **(b)** At HH27 muscle progenitors that express LBX1$^{S223A\ S227A\ S234A}$ 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 Lbx1$^{S223A\ S227A\ S234A}$ electroporated myogenic progenitors. **(d)** Quantification of cell numbers from panel b. The overexpression of LBX1$^{S223A\ S227A\ S234A}$ 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 *lbx1,* GFP$^+$ cells are evenly distributed along the MF20$^+$ myogenic field. Only when *lbx1*$^{S223A\ S227A\ S234A}$ 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 *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 *lbx1* inhibits differentiation of hypaxial muscle by downregulation of *myoD* and *myf5*. *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 *in-vitro*, by FGF8 and ERK-mediated signalling. 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 *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 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 Lbx1 expression.

Taken together these data point towards a unique and specific role for LBX1 in the context of limb muscle development. 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 LBX1 phosphorylation state. Furthermore, somite specific over expression of an unphosphorylated-mimetic form of LBX1, but not wildtype LBX1 affects the lateral migration of myoblasts into the distal limb bud.

The over-expression of the dephosphomimetic form of 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 LBX1 binds to the required promoter regions but fails to induce promoter activation after the blocking of LBX1 phosphorylation, or alternatively DNA binding is affected and in doing so it sequesters other binding partners away from the promoter region. This is beyond the scope the current study, but can be addressed in future work.

The link between limb patterning and muscle development has previously been made through the action of SF/HGF (Scaal et al., 1999). Here we open the possibility for a second link between limb patterning and muscle development independent of SF/HGF. Apart from its important role in distal limb outgrowth, we suggest FGF8 has the capacity to regulate the phosphorylation of Lbx1 through the regulation of ERK. As such, the overexpression of an artificial form of Lbx1 that can no longer be phosphorylated specifically inhibits the migration into the distal limb field, but does not have the earlier delamination processes which occur outside of the reach of ERK signalling in the distal limb anlage.

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 LBX1 *in-vitro*. Over expression of the dephosphomimetic form *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 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 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 *cxcr4*. CXCR4 was identified as being expressed in migratory limb myoblasts through profiling of LBX1 positive myoblasts from transgenic mice in which GFP had been knocked into the *Lbx1* locus (Vasyutina et al., 2005). Further analyses revealed that *Cxcr4* is expressed on migrating limb myoblasts, while its ligand, *Sdf1* is expressed in the limb anlage. Mice that lack CXCR4 result in a phenotype where lateral migration of limb myoblast migration is affected, with distal limb musculature 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, LBX1 phosphorylation is induced through the action of ERK. This may alter the affinity of 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.

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