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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