Development 137, 3847-3856 (2010) doi:10.1242/dev.053421

Pitx2 defines alternate pathways acting through MyoD during limb and somitic myogenesis

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SUMMARY

The *MyoD* gene is part of the core regulatory network that governs skeletal myogenesis and acts as an essential determinant of the myogenic cell fate. Although generic regulatory networks converging on this gene have been described, the specific mechanisms leading to *MyoD* expression in muscles of different ontology remain misunderstood. We now show that the homeobox gene *Pitx2* is required for initial activation of the *MyoD* gene in limb muscle precursors through direct binding of Pitx2 to the *MyoD* core enhancer. Whereas *Myf5* and *Mrf4* are dispensable for limb muscle progenitor fate, inactivation of *Myf5* and *Mrf4* in *Pitx2* mutants results in a drastic decrease of limb *MyoD* expression. Thus, *Pitx2* and *Myf5* define parallel genetic pathways for limb myogenesis. We show a similar dependence on *Pitx2* and *Myf5(Mrf4)* in myotome, where *MyoD* expression is initially activated by *Myf5* and *Mrf4*. In their absence, *MyoD* expression is eventually rescued by a *Pax3*-dependent mechanism. We now provide evidence that *Pitx2* contributes to the rescue of *MyoD* expression and that it acts downstream of *Pax3*. We thus propose that myogenic differentiation of somite-derived muscle cells relies on two parallel genetic pathways, with the *Pitx2* pathway being of primary importance for limb myogenesis but the *Myf5* and *Mrf4* pathway predominating in myotome. Muscle-specific wiring of regulatory networks composed of similar transcription factors thus underlies development of distinct skeletal muscles.

KEY WORDS: Muscle, Differentiation, Regulatory networks, Mouse, Myod1

INTRODUCTION

Much that we have learned about muscle development has emphasized a general regulatory network driving myogenesis. The program for skeletal muscle development depends on a genetic network that is centred on a group of basic helix-loop-helix muscle regulatory factors (MRF) that control determination of myogenic progenitors and differentiation of myoblasts. The implication of this core genetic network in all skeletal muscles, together with the shared properties of MRFs, has led to a relatively simple view of muscle development. By contrast, different skeletal muscles clearly achieve different functions through intrinsic differences that are probably built into their developmental program. The molecular mechanisms that underlie the unique identity of different muscles remain elusive. We already have indications that two groups of body skeletal muscles have distinct mechanisms of formation although they both derive from somites. Indeed, most muscles of the trunk develop by growth, expansion and reorganization of the myotome (Cossu et al., 1996; Denetclaw et al., 1997; Tajbakhsh et al., 1996b), giving rise to back, intercostal and ventral body muscles (Christ et al., 1983; Christ and Brand-Saberi, 2002). By contrast, several muscle groups including limb, diaphragm, intrinsic tongue and pharynx muscles do not go through a myotomal intermediate. Instead, myogenic progenitor cells of the hypaxial dermomyotome undergo an epithelial-to-mesenchymal transition and migrate as single cells to their respective destinations (Bladt et al., 1995; Christ and Ordahl, 1995; Mackenzie et al., 1998; Noden, 1983; Ordahl and Williams, 1998). Finally, extra-ocular and branchiomeric muscles do not derive from somites, but rather from cranial mesoderm (Noden and Francis-West, 2006).

In spite of these developmental differences, all skeletal muscles rely on the regulatory network involving MRFs. Thus, *Myf5* is expressed at the onset of myogenesis in the mouse embryo (Ott et al., 1991) when, together with *Mrf4*, it determines the myogenic cell fate (Kassar-Duchossoy et al., 2004). *MyoD* is subsequently expressed and can direct cells into the myogenic program in the absence of *Myf5* and *Mrf4* (Braun et al., 1992). Myf5, Mrf4 and MyoD thus constitute the core regulatory network for myogenic determination. In their absence, precursor myoblast cells are lacking and skeletal muscles do not form (Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993). By contrast, myogenin functions as an essential differentiation factor, as inactivation of its gene prevents formation of functional muscle fibres in vivo without affecting myoblast determination (Hasty et al., 1993; Nabeshima et al., 1993). Although this core network has been implicated in all skeletal myogenesis, various studies on hierarchical interactions between MRFs and with upstream transcription factors such as Pax3 suggested that 'wiring' of the myogenesis network is different in trunk, limbs or head muscles (Bajard et al., 2006; Kablar et al., 1999; Kassar-Duchossoy et al., 2004; Sambasivan et al., 2009). In particular, different transcription factors were shown to modulate the core network in these muscles (Dastjerdi et al., 2007; Grifone et al., 2005; Grifone et al., 2007; Mankoo et al., 1999).

The *Pitx2* gene has previously been shown to be expressed during embryonic myogenesis (Diehl et al., 2006; Dong et al., 2006; L'Honoré et al., 2007; Shih et al., 2007b) of different skeletal muscle types, including extraocular, branchiomeric, trunk and limb muscles. Whereas *Pitx2* was suggested to regulate *MRF* transcription in extraocular and branchiomeric muscles (Diehl et al., 2006; Dong et al., 2006), it was also implicated in proliferation and survival of muscle progenitor cells in branchiomeric muscles

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Accepted 18 September 2010

(Dong et al., 2006; Shih et al., 2007a). During body muscle development, *Pitx2* is expressed in muscle progenitor cells and early differentiating cells and its expression decreases at terminal differentiation (L'Honoré et al., 2007; Shih et al., 2007b). However, the precise role of Pitx2 in those muscles is unknown.

Pitx2 belongs to the Pitx gene family that includes three members in vertebrates. This family encodes paired-related homeodomain transcription factors that play major roles in early patterning and organogenesis. *PITX2* mutations have been associated with human Axenfeld-Rieger syndrome type I, an autosomal dominant disorder that includes dental hypoplasia, anterior segment eye defects, craniofacial dysmorphologies and heart defects as cardinal features (Gage et al., 1999; Kioussi et al., 2002; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999; Semina et al., 1996). Pitx2 is an essential effector for left-right asymmetry, and it is essential for pituitary and craniofacial development (Gage et al., 1999; Lin et al., 1999). The mouse *Pitx2* gene produces three spliced isoforms (a, b and c) from two different promoters. Ablation of all three Pitx2 isoforms in the mouse embryo causes lethality at mid-gestation (E10.5-E13.5) with axial malformations, open body wall, heart malformations, laterality defects and arrest of multiple organ development.

In this report, we defined the role of *Pitx2* in both limb and myotome muscle development. We show that *Pitx2* is crucial for the onset of *MyoD* gene expression in limb muscle progenitors and that it acts on the *MyoD* core enhancer. This action is later compensated by a Myf5-dependent mechanism. By contrast, initiation of *MyoD* expression in myotome is not dependent on *Pitx2* but we show that *Pitx2* acts in a *Pax3*-dependent genetic pathway parallel to *Myf5* and *Mrf4* such that *Pitx2* rescues *MyoD*-mediated myogenesis in their absence. We propose that the joint actions of *Myf5*, *Mrf4* and *Pitx2* are crucial for control of *MyoD* expression in muscles with different contributions of each gene depending on muscle ontology.

MATERIALS AND METHODS

Animal handling and genotyping

All animal procedures were approved and conducted in accordance with IRCM Animal Ethics Review Committee regulations. *Pitx2*$^{-/-}$, *Myf5*^{nlacZnlacZ} and *Splotch* mice have been described previously (Gage et al., 1999; Tajbakhsh et al., 1996a; Underhill et al., 1995). Genotyping was carried out by PCR using DNA isolated from umbilical cord/amniotic membrane of embryos or adult tail sections.

Whole-mount in situ hybridization and X-Gal staining, histology and immunohistochemistry

Mouse embryos were collected after natural overnight matings. Noon of the day on which a vaginal plug was detected was considered to be E0.5. Embryos were staged more precisely by counting the number of somites posterior to the forelimb bud and scoring that first somite as 13 (Lewandoski et al., 2000). For X-Gal staining, embryos dissected in PBS were fixed for 30-45 minutes (depending on stage) with 4% paraformaldehyde (PFA) in PBS, at 4°C. Embryos were then rinsed three times in PBS and stained with X-gal (1mg/mL) for 2-12 hours at 30°C. Embryos were rinsed in PBS and post-fixed overnight in 4% PFA. Whole-mount in situ hybridization was performed as described in the protocols from Dr Janet Rossant's laboratory (http://www.sickkids.ca/rossant/custom/protocols.asp) using digoxigenin-labelled riboprobes for *MyoD* and *myogenin* (plasmids kindly provided by S. Tajbakhsh).

Fluorescent co-immunohistochemistry and peroxidase immunohistochemistry were carried out as described previously (Lanctôt et al., 1999). The following primary antibodies were used: polyclonal anti-Pitx2, 1:200 (L'Honoré et al., 2007); goat anti-β-galactosidase, 1:400 (Sigma); monoclonal anti-Pax3, 1:50; monoclonal anti-myogenin, 1:50 (from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA52242, USA); monoclonal anti-MyoD, 1:100 (Pharmingen, Mississauga, ON, Canada); and polyclonal anti-Ki67, 1:100 (LabVision/Neomarkers, Fremont, CA, USA). Secondary antibodies were coupled to biotin (anti-rabbit BA1000, or anti-mouse BA2000, Vector Labs, Burlington, ON, Canada) and used at dilution of 1:150, or coupled to a fluorochrome, Alexa 488 or 555 (Molecular Probes/Invitrogen, Carlsbad, CA, USA) and used at dilution of 1:250. Strepatvidin was coupled to peroxidase HRP (NEL750, NEN Life Science, Bridgewater, NJ, USA) and used at 1:1000 or coupled to a fluorochrome, Alexa 488 or 555 (Molecular Probes/Invitrogen, Carlsbad, CA, USA) and used at 1:500. For immunohistochemistry, reactions were performed using diaminobenzidine (DAB, Sigma-Aldrich, St Louis, MO, USA) as peroxidase substrate (L'Honoré et al., 2007) and slides were counterstained with Methyl Green. For immunofluorescence, slides were mounted using Mowiol.

Plasmids and transfection assays

The *MyoD* CE and CE-mut luciferase reporter plasmids were constructed in pXP2 driven by the mouse *MyoD* proximal promoter (–285/+15 bp). The intact *MyoD* 258 bp CE was cloned upstream of this promoter and the CE-mut plasmids included mutations in either or both PRE as indicated in Fig. 4A. The following oligonucleotides were used for site directed mutagenesis of the PitxRE sites: PRE1fwd, CAGCAGCTGGTCACAAAGCCAGTGAATTCCCCAGAGTGCTC; PRE1rev, GAGCACTCTGGGGAATTCACTGGCTTTGTGACCAGCTGCTG; PRE2fwd, GTGAATTCCCCAGAGTGCTCGACTTCAAACCCGTGACTCACAACAC; and PRE2rev, GTGTTGTGAGTCACGGGTTTGAAGTCGAGCACTCTGGGGAATTCAC. The reporter plasmids were sequenced to ascertain the expected sequences.

CV-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum and penicillin/streptavidin antibiotics and maintained in a humidified incubator (37°C, 5% CO_2). For transfection, 40,000 cells were plated in 12-well-plates. Cells were transfected by the calcium phosphate co-precipitation method using a total of 1.5 μg of DNA (100 ng of CE-wt or CE-mut reporter plasmid, 0-50 ng of RSV-Pitx2 or empty RSV-expression vector, 50 ng of CMV-β-galactosidase as internal control and 1.3 μg of carrier DNA). Cells were harvested 24 hours later using transfection lysis buffer [0.1 M Tris (pH 8.0), 0.5% NP-40, 1 mM DTT]. Luciferase activity was assayed by injection of 100 μl of luciferine [0.5 mM in 100 mM Tris (pH 8.0)] to 100 μl of lysates using the Glomax luminometer (Promega, Madison, WI, USA). β-Gal activity was determined using the β-gal reporter gene Galacto-Star (TROPIX) assay system.

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed as described previously (Coulon et al., 2007) on either E12 hindlimbs, carefully dissected hindlimb muscles at E15 or E12 myotomes. Similar results were obtained using a Pitx2-specific antibody (L'Honoré et al., 2007) as well as an antibody that recognizes equally well the three Pitx factors. For ChIP analyses of the *MyoD* locus, the following primers were used in qPCR: CEfwd, CCAGTTAATCTCCCAGAGTGCTCA; CErev, TGAGCTAGAGAAACCGGAGAAAC; DRRfwd, AGGTGCTAGTTGGATCCGGTTT; DRRrev, CATTTCAGCTCCCTTGGCTAGTCT; PRfwd, ACTCCTATGCTTTGCCTGGTCT; and PRrev, ATACGCGGTAGCACTTGGCTAT. Enrichments were calculated relative to Q-PCR analyses of a control sequence within the *POMC* gene promoter that is not expressed in muscles or limbs: POMCfwd, TCGGAGTGGAATTACCTATGTGCG; and POMCrev, TGGTTTCACAAGATATCACACTTTCCC. The β-actin and GAPDH loci were also used as negative control and yielded similar results.

RESULTS

Having previously shown (L'Honoré et al., 2007) that Pitx2 is expressed before MyoD in Pax3-positive myogenic progenitors that have entered limb buds (Fig. 1A-C), we wanted to assess Pitx2 expression in relation to the first expressed myogenic bHLH factor

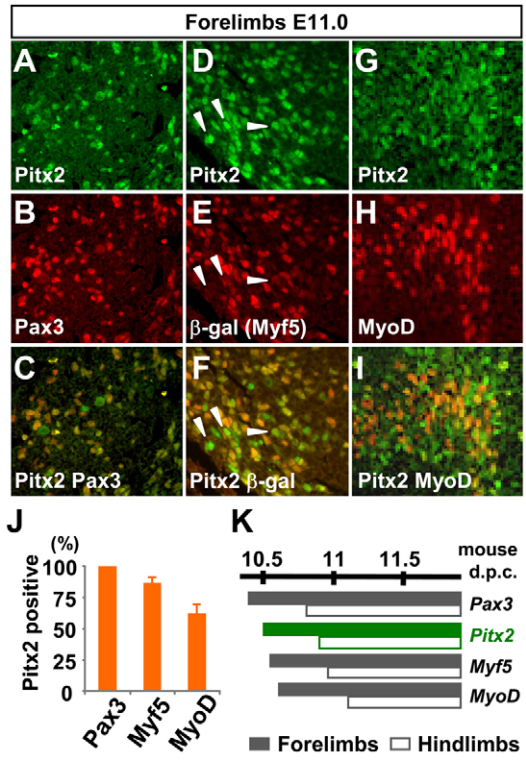

Fig. 1. Limb expression of Pitx2 precedes Myf5 and MyoD. (**A-I**) Expression of Pitx2 (green) (A,C,D,F,G,I), Pax3 (red) (B,C), β-galactosidase (Myf5, red) (E,F) and MyoD (red) (H,I) revealed by co-immunofluorescence on transverse sections of forelimbs from E11.0 *Myf5*$^{nLacZ/+}$ embryos. Arrowheads indicate Pitx2-positive cells that do not express Myf5 (β-galactosidase). This population represents 13±1% (*n*=6) of the total number of cells expressing Pitx2. (**J**) Quantitation of Pitx2-positive cells expressing the indicated myogenic markers. (**K**) Schematic illustration of *Pitx2*, *Myf5*, *MyoD* and *Pax3* expression in forelimb (filled boxes) and hindlimb (clear boxes) during mouse embryonic development.

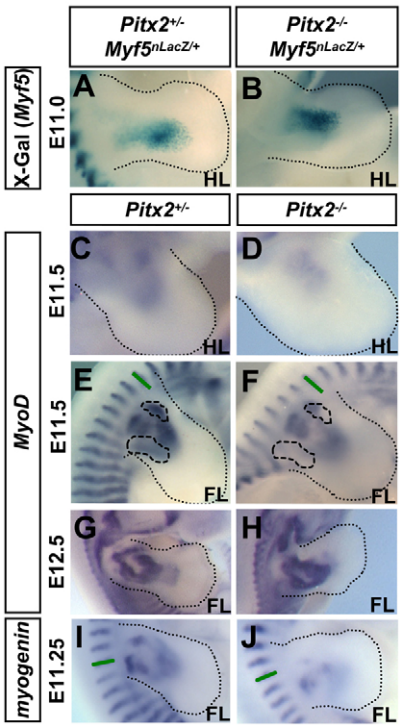

Fig. 2. *MyoD* and myogenin, but not *Myf5*, expression are deficient in limbs of *Pitx2* mutant embryos. (**A**,**B**) β-Galactosidase staining of E11.0 hindlimb buds from *Pitx2*$^{+/-}$;*Myf5*$^{nLacZ/+}$ (A) and *Pitx2*$^{-/-}$;*Myf5*$^{nLacZ/+}$ (B) embryos. (**C-J**) Whole-mount in situ hybridization at hindlimb (C,D) or forelimb level (E-J) of *Pitx2*$^{+/-}$;*Myf5*$^{nLacZ/+}$ (C,E,G,I) and *Pitx2*$^{-/-}$;*Myf5*$^{nLacZ/+}$ (D,F,H,J) embryos at E11.5 (C-F), E12.5 (G,H) or E11.25 (I,J), hybridized with *MyoD* (C-H) or myogenin (I,J) probes. Complementary data are presented in Fig. S1 in the supplementary material. Green bars in E,F,I,J indicate width of myotomes.

Myf5. Using *Myf5*^{nlacZ} heterozygous mice that carry a *lacZ* insertion into the *Myf5* locus (Tajbakhsh and Buckingham, 1994), we did co-labelling by immunofluorescence for Pitx2 and β-galactosidase (Myf5) in E11.0 forelimb buds. Whereas all Myf5-expressing cells co-express Pitx2 (Fig. 1D-F), some Pitx2-positive Myf5-negative cells (13±1%, *n*=6) are also observed (Fig. 1F, arrowheads). As the expression of MyoD is more restricted at this developmental stage (Fig. 1G-J) (L'Honoré et al., 2007), these data show that Pitx2 expression precedes activation of the limb myogenic program (Fig. 1K).

MyoD expression is decreased in *Pitx2*$^{-/-}$ limb buds

In order to assess a putative role of *Pitx2* in the myogenic program, we crossed the *Myf5*^{nlacZ} indicator allele into the *Pitx2*-null background. Expression of β-galactosidase (Myf5) in *Pitx2*$^{+/-}$ embryos is identical to *Pitx2*$^{+/+}$ embryos (data not shown), and thus heterozygote embryos were used throughout as control. Mice heterozygous for *Myf5*^{nlacZ} did not show significant differences in β-galactosidase (Myf5) expression in E11.0 *Pitx2*$^{-/-}$ forelimb (FL) and hindlimb (HL) buds compared with *Pitx2*$^{+/-}$ embryos (Fig. 2A,B; Fig. S1A,B in the supplementary material). The early expression of *Myf5* in limb progenitors thus appears to be largely independent of *Pitx2*. By contrast, the onset of hindlimb bud *MyoD* expression (Fig. 2C) is markedly decreased in *Pitx2*$^{-/-}$ embryos (Fig. 2D). A similar decrease was observed in FL buds at E10.5 (data not shown) and decreased *MyoD* transcripts are still observed in E11.5 FL and E12.5 HL (Fig. 2F, compare with 2E; see Fig. S1C,D in the supplementary material). However, *MyoD* expression is largely recovered in E12.5 FL buds (Fig. 2G,H). As myogenin expression is dependent on *MyoD* during limb myogenesis (Sassoon et al., 1989; Wright et al., 1989), we assessed its expression by in situ hybridization in *Pitx2*$^{-/-}$ embryos. We observed decreased myogenin transcripts, in agreement with their purported dependence on *MyoD* (Fig. 2J, compare with 2I). As shown for *MyoD* in E12.5 FL (Fig. 2G,H), similar patterns of *MyoD* and myogenin were observed in E13.0 FL and HL (see Fig. S1E,F in the supplementary material and data not shown), suggesting full recovery of their expression.

Delayed myogenic differentiation in *Pitx2*$^{-/-}$ limb buds

The decreased *MyoD* expression in *Pitx2* mutant limb buds may be due to altered progenitor number or delayed myogenic differentiation. In order to assess these possibilities, we analyzed myogenic precursors and their proliferation status by co-labelling for Pax3 and Ki67. The number of Pax3-positive cells is unaltered

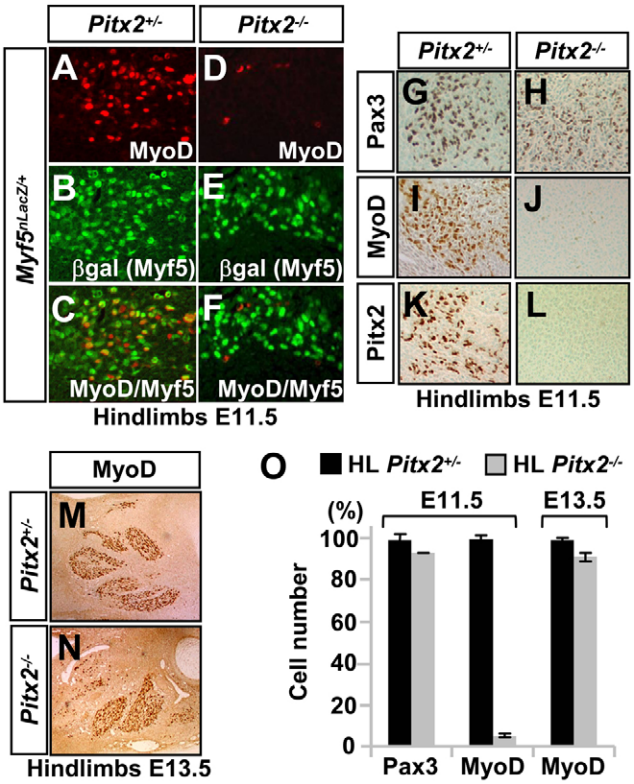

Fig. 3. Limb MyoD deficiency is transient in *Pitx2*$^{-/-}$ embryos. (**A-F**) Co-staining by immunofluorescence for MyoD (red) (A,C,D,F) or β-galactosidase (Myf5, green) (B,C,E,F) on transverse sections of hindlimb buds from E11.5 *Pitx2*$^{+/-}$;*Myf5*$^{nLacZ/+}$ (A-C) and *Pitx2*$^{-/-}$;*Myf5*$^{nLacZ/+}$ (D-F) embryos. (**G-N**) Immunohistochemical analyses of Pax3 (G,H), MyoD (I,J,M,N) or Pitx2 (K,L) on transverse sections of hindlimb buds from *Pitx2*$^{+/-}$ (G,I,K,M) and *Pitx2*$^{-/-}$ (H,J,L,N) embryos at E11.5 (G-L) and E13.5 (M,N). (**O**) Quantification of the number (±s.d.) of Pax3- or MyoD-positive cells in entire hindlimb buds from *Pitx2*$^{+/-}$ and *Pitx2*$^{-/-}$ embryos at E11.5 and E13.5. Fig. S2 in the supplementary material provides cell number and proliferation controls for these experiments.

in *Pitx2* mutant limb buds and their proliferation status is also unaffected (see Fig. S2D-F, compare with S2A-C in the supplementary material). Also consistent with the unaltered *Myf5* expression pattern (Fig. 2A,B), the number of Myf5 and Pax3 double positive cells was similar in Pitx2-deficient limb buds (see Fig. S2M-O, compare with S2G-I in the supplementary material). However, co-labelling of myogenic precursors with Myf5 and MyoD revealed almost no double positive cells in *Pitx2*$^{-/-}$ embryos (Fig. 3D-F) compared with their control siblings (Fig. 3A-C). These data are thus consistent with a delay of *MyoD* activation. In order to quantify this delay, immuno-peroxidase labelling was performed on both E11.5 and E13.5 HL buds. The number of Pax3-positive cells was similar for both *Pitx2*$^{-/-}$ and *Pitx2*$^{+/-}$ HL buds at E11.5 (Fig. 3G,H,O), indicating that the number of progenitors that migrated into the mutant limb buds is not dependent on Pitx2. This is consistent with the onset of *Pitx2* expression after migration of limb bud progenitors (L'Honoré et al., 2007). As expected, the number of MyoD expressing cells was considerably decreased in E11.5 *Pitx2*$^{-/-}$ HL buds (Fig. 3I,J,O). However, quantitation of MyoD-positive cells in E13.5 HL buds revealed a complete recovery of the number of MyoD-expressing cells (Fig. 3M-O). These results clearly indicate that the absence of Pitx2 causes a delay in MyoD expression that is fully recovered by E13.5.

Pitx2 acts directly on the *MyoD* core enhancer

We considered the possibility that *Pitx2* may act directly on the *MyoD* gene. Appropriate spatiotemporal expression of *MyoD* is reproduced by a 24 kb fragment of 5′ regulatory sequences that contains two enhancers called core enhancer (CE) (Faerman et al., 1995; Goldhamer et al., 1992; Goldhamer et al., 1995) and distal regulatory region (DRR) (Asakura et al., 1995; Tapscott et al., 1992). Although DRR activity is restricted to differentiated cells and maintains *MyoD* expression at foetal stages and postnatally (Asakura et al., 1995; Chen et al., 2002; Hughes et al., 1993; L'Honoré et al., 2003), genetic studies have ascribed initiation of *MyoD* expression to the CE (Chen and Goldhamer, 2004; Kablar et al., 1999). The highly conserved 258 bp CE element is located about 20 kb upstream of the *MyoD* gene and it recapitulates the skeletal muscle pattern of *MyoD* expression during embryonic development (Faerman et al., 1995; Goldhamer et al., 1992; Goldhamer et al., 1995).

Interestingly, targeted deletion of the *MyoD* CE leads to a delay of *MyoD* expression in limbs (Chen and Goldhamer, 2004) that is similar to that observed in *Pitx2*$^{-/-}$ embryos (Figs 2 and 3), suggesting that the *MyoD* CE may be a direct target of Pitx2. To assess this hypothesis, we analyzed the CE sequence (Goldhamer et al., 1995) and this revealed two well conserved putative Pitx binding sites (Fig. 4A). The sites (TAATCT and TAAGCT) are conserved between mouse and human (see Fig. S3A in the supplementary material) and correspond to sequences previously shown to bind and be activated in response to Pitx1 and Pitx2 (Tremblay et al., 2000); furthermore, the sites are in opposite orientation relative to each other, as often observed in pituitary Pitx target genes (Tremblay et al., 1998). We used gel retardation assays to verify that Pitx2 binds these two sites (PRE1 and PRE2) in vitro (see Fig. S3B in the supplementary material). Similar binding was observed at each site and a probe containing both sites showed cooperative binding. The in vivo occupancy of the CE was ascertained directly by chromatin immunoprecipitation (ChIP) using HL buds from E12 and E15 embryos and anti-Pitx2 antibody. Significant recruitment of Pitx factors was observed both in E12 and E15 buds at the CE but not at the DRR (Fig. 4B). Interestingly, recruitment was also observed at about –600 bp in the promoter region (PR), in agreement with the presence of a putative Pitx binding site at –615 bp. This site was tested by gel retardation and shown to bind Pitx2 (see Fig. S3B in the supplementary material). The CE may thus be a direct target of Pitx factor action and this possibility was tested directly in co-transfection experiments in CV1 cells. The reporters containing the CE inserted upstream of the proximal *MyoD* promoter exhibited dose-dependent activation in response to increasing amounts of Pitx2 and mutagenesis of the two putative Pitx-binding sites in CE prevented this activation (Fig. 4C). Linker-scanning mutagenesis of the CE provided an in-depth functional analysis of this element (Kucharczuk et al., 1999) but, surprisingly, it did not identify any essential sequence for *MyoD* limb expression. In particular, mutants LS-8 and LS-9 that respectively encompass the PRE1 and PRE2 did not affect limb activity. To assess the hypothesis that PRE1 and PRE2 may have redundant activities, we performed co-transfection experiments for Pitx2 activation of a wild-type or mutant CE reporter (Fig. 4D). Whereas mutagenesis of either site had a marginal effect, their combined mutation abrogated activation, which highlights their redundancy. These experiments support the

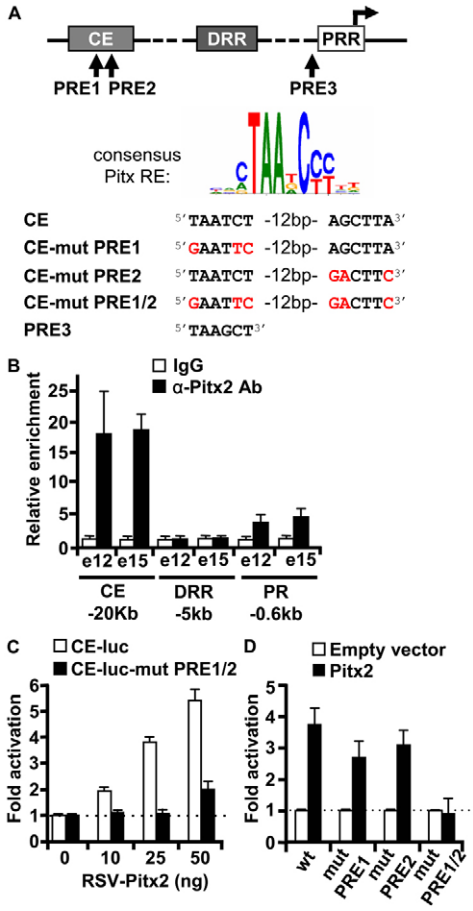

Fig. 4. Pitx2 directly regulates *MyoD* expression through binding of the core enhancer. (**A**) Schematic representation of *MyoD* gene regulatory regions comprising a minimal promoter called proximal regulatory region (PRR) (Tapscott et al., 1992) and two enhancers: the core enhancer (CE) (Goldhamer et al., 1992) and the distal regulatory region (DRR) (Tapscott et al., 1992). The 258 bp CE contains two putative consensus Pitx-binding sites (PRE1 and PRE2). A third putative Pitx binding site (PRE3) is present at –615 bp in the promoter region (PR) close to the PRR. The consensus PitxRE was obtained from the Genomatix database. Sequences of the three PRE are shown together with mutations used in transactivation assays (in red). (**B**) Chromatin immunoprecipitation (ChIP) analysis for the presence of Pitx2 at the indicated *MyoD* regulatory sequences were performed on chromatin isolated from hindlimb buds of wild-type E12 or E15 embryos. The histogram shows enrichments (±s.e.m., *n*=3) obtained at CE, DRR and PR relative to the *POMC* promoter used as reference. (**C**) Activation of CE by Pitx2. CV1 cells that do not express Pitx transcription factors were transfected with a luciferase reporter construct containing *MyoD* CE fused to PRR sequences (CE-luc). Dose response is shown for co-transfection of increasing amounts of Pitx2 expression plasmid as indicated. The double PRE1/2 mutations prevented Pitx2 activation. (**D**) Effect of single or joint PRE site mutation on reporter activation by Pitx2. Results are the average of three independent experiments, each performed in duplicate (±s.e.m.).

model that Pitx2 is directly recruited to the *MyoD* CE to activate *MyoD* expression in early development, notwithstanding a possible action through other Pitx sites. The model is further supported by equivalent delays of limb MyoD expression observed in *Pitx2*$^{-/-}$ (Fig. 2) and mice deleted of the *MyoD* CE (Chen and Goldhamer, 2004).

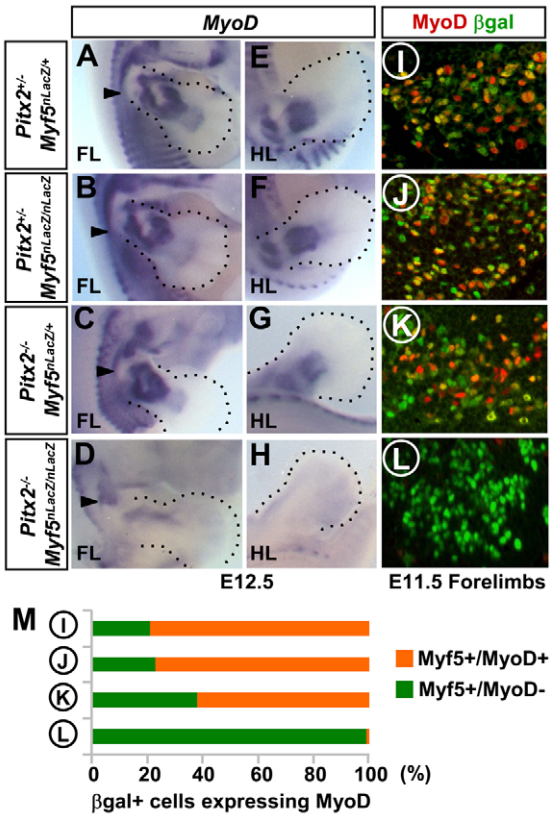

Fig. 5. *Myf5* compensates functionally for *MyoD* expression in *Pitx2*$^{-/-}$ limbs. (**A-H**) Dorsal views of forelimb (A-D) and hindlimb (E-H) buds from *Pitx2*$^{+/-}$;*Myf5*$^{nLacZ/+}$ (A,E), *Pitx2*$^{+/-}$;*Myf5*$^{nLacZ/LacZ}$ (B,F), *Pitx2*$^{-/-}$;*Myf5*$^{nLacZ/+}$ (C,G) and *Pitx2*$^{-/-}$;*Myf5*$^{nLacZ/LacZ}$ (D,H) embryos at E12.5 stained by whole-mount in situ hybridization for *MyoD* transcripts. (**I-L**) Co-staining by immunofluorescence for β-galactosidase (Myf5, green) or MyoD (red) on transverse sections of forelimb buds from E11.5 *Pitx2*$^{+/-}$;*Myf5*$^{nLacZ/+}$ (I), *Pitx2*$^{+/-}$;*Myf5*$^{nLacZ/nLacZ}$ (J) *Pitx2*$^{-/-}$;*Myf5*$^{nLacZ/+}$ (K) and *Pitx2*$^{-/-}$;*Myf5*$^{nLacZ/nLacZ}$ (L) embryos. (**M**) Quantification of the percentage of Myf5 (β-galactosidase)-positive cells co-expressing MyoD in forelimb buds from embryos analyzed in I-L. See also Fig. S3 in the supplementary material for controls of β-galactosidase (Myf5) expression.

Myf5 cooperates with *Pitx2* for limb *MyoD* expression

The delay in MyoD expression observed in *Pitx2*$^{-/-}$ limb buds thus appears to be due to the failure to activate the *MyoD* core enhancer. This delay is not compensated for by the related Pitx3 as the double *Pitx2*$^{-/-}$;*Pitx3*$^{-/-}$ embryos show similar myogenin expression patterns as the *Pitx2*$^{-/-}$ embryos (see Fig. S4 in the supplementary material). As *Myf5* is activated independently of *Pitx2* during early myogenesis, we then tested the possibility that *Myf5* may contribute to *MyoD* recovery in *Pitx2*$^{-/-}$ limbs. As previously reported (Tajbakhsh et al., 1997), *MyoD* expression is not affected in limb buds of *Myf5*$^{nlacZ/nlacZ}$ mutant embryos indicating that *Myf5* and *Mrf4* (which is inactivated in cis in this mutant) are dispensable for non-myotomal *MyoD* expression (Fig. 5B,F, compare with 5A,E). However, inactivation of *Myf5*(*Mrf4*) in the *Pitx2*$^{-/-}$ background resulted in almost complete loss of *MyoD* expression at E12.5 compared with *Pitx2*$^{-/-}$;*Myf5*$^{nlacZ/+}$ embryos (Fig. 5D,H, compare with 5C,G). As for the *Pitx2* knockout (Fig. 2A-D), the double *Pitx2* and *Myf5*(*Mrf4*) loss-of-function did not appear to

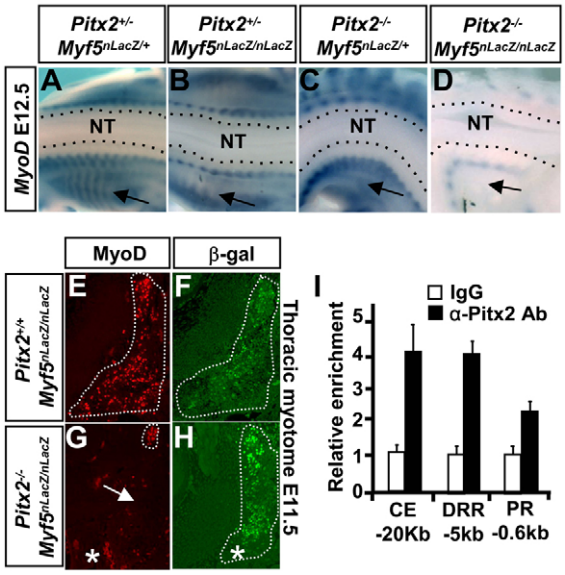

Fig. 6. *Pitx2* is sufficient for hypaxial myotome expression of *MyoD* in absence of *Myf5* and *Mrf4*. (**A-D**) Dorsal views of interlimb myotomes from *Pitx2*$^{+/-}$;*Myf5*$^{nLacZ/+}$ (A), *Pitx2*$^{+/-}$;*Myf5*$^{nLacZ/LacZ}$ (B), *Pitx2*$^{-/-}$;*Myf5*$^{nLacZ/+}$ (C) and *Pitx2*$^{-/-}$;*Myf5*$^{nLacZ/LacZ}$ (D) embryos at E12.5 stained by whole-mount in situ hybridization for *MyoD* transcripts. The black arrows indicate hypaxial myotome. See also Fig. S4A,B in the supplementary material. (**E-H**) Staining by immunofluorescence for MyoD (red, E,G) or β-galactosidase (Myf5, green, F,H) on transverse sections of thoracic somites from E11.5 *Pitx2*$^{+/+}$;*Myf5*$^{nLacZ/LacZ}$ (E,F) and *Pitx2*$^{-/-}$;*Myf5*$^{nLacZ/LacZ}$ (G,H) embryos. The white arrow indicates hypaxial myotome and the asterisk indicates non-specific staining of red blood cells. See also Fig. S6G,H in the supplementary material for myogenin expression. (**I**) Chromatin immunoprecipitation (ChIP) analysis for the presence of Pitx2 at the indicated *MyoD* regulatory sequences were performed on chromatin isolated from dissected myotome of wild-type E12 embryos. The histogram shows enrichments (±s.e.m., *n*=3) obtained at CE, DRR and PR relative to the *POMC* promoter used as reference.

affect the number of limb muscle progenitors as assessed by β-galactosidase (Myf5) staining (Fig. 5I-L; see Fig. S5 in the supplementary material). The *Pitx2* and *Myf5*(*Mrf4*) double mutant is therefore specifically deficient in *MyoD* activation but not in formation or migration of precursors to the limb buds. In order to quantify the *Myf5* compensation of *Pitx2*, we counted the ratios of MyoD-positive cells relative to Myf5-positive cells (Fig. 5I-M). Whereas absence of Myf5 and Pitx2 resulted in complete loss of MyoD expression (L), the presence of one active *Myf5* allele prevented this loss in about 60% of cells (K). These results clearly suggest that *Myf5* is acting in parallel to *Pitx2* for control of *MyoD* expression during early limb bud myogenesis.

Pitx2 in myotome expression of *MyoD*

We have previously shown Pitx2 expression in myogenic cells of the myotome (L'Honoré et al., 2007). Myotome expression of *MyoD* (Fig. 2F and data not shown) and of myogenin (Fig. 2J) is not delayed in *Pitx2*$^{-/-}$ embryos, in contrast to limb muscle cells. We wanted to test the hypothesis that *Pitx2* and *Myf5/Mrf4* may also cooperate for *MyoD* expression in myotome. In *Myf5*$^{nlacZ/lacZ}$ mutant embryos where both Myf5 and Mrf4 are absent, *MyoD* expression in myotome is delayed by ~2 days. Recovery of *MyoD* expression, which is dependent on *Pax3*, appears by E11.5 and is complete by E12.5 (Tajbakhsh et al., 1997). Investigation of E12.5 *Pitx2*$^{-/-}$;*Myf5*$^{nlacZ/lacZ}$ embryos that are deficient in Pitx2, Myf5 and Mrf4 showed an almost complete loss of *MyoD* myotome expression (Fig. 6D) compared with *Myf5* (Fig. 6B) and *Pitx2* (Fig. 6C) mutants (also see Fig. S6A-D in the supplementary material). The remaining weak expression of MyoD in these double mutants was ascertained by immunohistofluorescence on E11.5 transverse thoracic sections. Staining for MyoD and myogenin revealed a small patch of positive cells in the most epaxial part of the myotome in *Pitx2*$^{-/-}$;*Myf5*$^{nlacZ/lacZ}$ embryos (Fig. 6G, compare with 6E; see Fig. S6G,H in the supplementary material). However, the loss of Pitx2 did not affect the appearance of myotome cells as revealed by β-galactosidase staining (Fig. 6F,H; see Fig. S6E-H in the supplementary material). *Pitx2*$^{-/-}$ embryos are characterized by severe trunk distortion (see Fig. S6C,D in the supplementary material). It is noteworthy that this distortion had no effect on *MyoD* expression in myotome of *Pitx2* mutants, clearly not supporting the idea of non cell-autonomous effects in the myotome of *Pitx2*$^{-/-}$;*Myf5*$^{nlacZ/lacZ}$ embryos. In view of the many apparent targets of Pitx2 recruitment/action at the *MyoD* locus revealed by ChIP in limb buds (Fig. 4B), we also ascertained the presence of Pitx2 at the same sites in E12 myotome. The experiments clearly indicated recruitment of Pitx2 at the CE, DRR and –600 bp regions of the *MyoD* gene (Fig. 6I). These data thus suggest multiple targets for Pitx2 action on the *MyoD* gene and, in particular, support the involvement of the DRR for myotome *MyoD* expression (Chen et al., 2002).

As *Pax3* was involved in recovery of *MyoD* expression in myotome of *Myf5/Mrf4* mutants (Kassar-Duchossoy et al., 2004; Tajbakhsh et al., 1997), we investigated a putative role of *Pax3* in myotome expression of *Pitx2*. We first excluded the possibility that Pax3 might act directly on PREs using gel retardation (see Fig. S3B in the supplementary material). The *Pax3* mutant *Splotch* mouse exhibits disorganized dermomyotome and it has slightly delayed myogenic differentiation. Analysis of these mutants at E11.5, when myogenesis resumes (as evidenced by myogenin expression, Fig. 7B,D), revealed a complete deficit of Pitx2 expression (Fig. 7A,C); this deficit is restricted to the myotome and is not observed in neighbouring mesenchyme, indicating that *Pitx2* is downstream of *Pax3* during myotome myogenesis. If myotome expression of *Pitx2* is downstream of *Pax3*, inactivation of both genes should have no greater effect than either mutation: this was indeed observed in myotome (Fig. 7E-G). These results indicate that *Pitx2* is a likely intermediate downstream of *Pax3* for *MyoD* recovery in *Myf5(Mrf4)* mutant myotomes. Interestingly, we observed a similar relationship between *Pax3* and *Pitx2* at shoulder girdle level. *MyoD* expression in a muscle opposite the forelimb is truncated in *Pax3*$^{Sp/Sp}$ embryos (Fig. 7H,I). This truncation is also observed in *Pitx2*$^{-/-}$ embryos (Fig. 7K,M) and the absence of both Pax3 and Pitx2 did not aggravate this truncation (Fig. 7H-J). As previously described, this truncation is not observed in *Myf5*$^{nlacZ/nlacZ}$ mutants (Fig. 7K,L) (Tajbakhsh et al., 1997), but we now provide evidence that *MyoD* expression in this muscle is also dependent on both *Pitx2* and *Myf5/Mrf4* pathways, as the double mutant is almost completely devoid of *MyoD* (Fig. 7K-N). These data are consistent with *Pax3* regulation of *Pitx2* expression in both myotome and shoulder girdle, and with a primary role of the *Pax3/Pitx2* pathway in the shoulder girdle.

DISCUSSION

Many studies have supported the idea that one core regulatory MRF is sufficient to initiate myogenesis in skeletal muscles of various origins. Whereas *Myf5* and *Mrf4* are primarily involved in

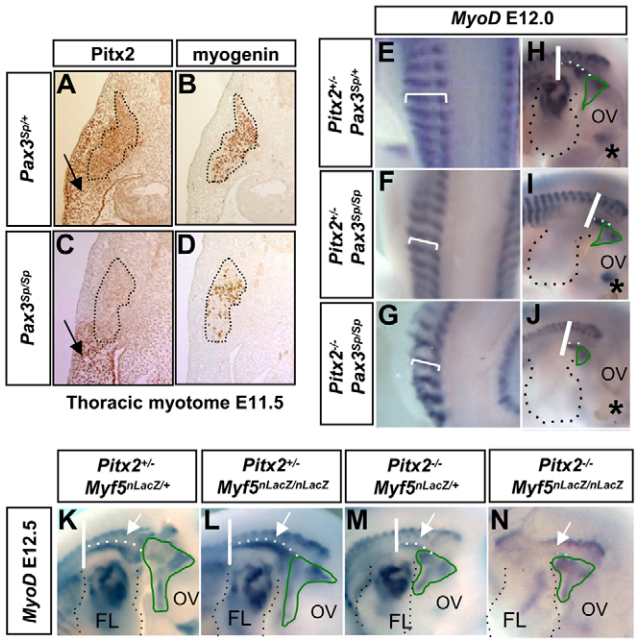

Fig. 7. Cooperation between *Pax3/Pitx2* and *Myf5/Mrf4* pathways for *MyoD* expression in myotome and shoulder girdle muscle. (**A-D**) Immunohistochemical analyses of Pitx2 and myogenin expression on transverse sections of thoracic somites from E11.5 $Pax3^{Sp/+}$ and $Pax3^{Sp/Sp}$ embryos. The black arrows indicate unaffected Pitx2 expression in mesenchyme, whereas dotted lines indicate myotomes. (**E-N**) Whole-mount in situ hybridization for *MyoD* performed on E12.0 (E-J) and E12.5 (K-N) embryos of the indicated genotypes. (E-G) Bracket indicates width of myotomes. (H-N) Forelimb buds are outlined with black dotted lines, whereas white dotted lines indicate shoulder girdle muscle; furthermore, the extent of this shoulder muscle is indicated by a white bar. Second branchial arch muscles affected by *Pitx2* mutation (Shih et al., 2007a) are outlined with green line. Asterisk indicates first branchial arch that is absent in *Pitx2* mutant (Dong et al., 2006). White arrows in K-N indicate presence (K-M) or absence (N) of shoulder muscle. OV, otic vesicle; FL, forelimb bud.

initial determination of the myogenic program, it appears that *MyoD* is the point of convergence for regulatory pathways that drive myogenic differentiation. Recent work has identified *Pitx2* as an upstream regulator for MRF expression in extra ocular muscles (Diehl et al., 2006) and *Pitx2* was also shown to cooperate with *Tbx1* for activation of the muscle fate in the pharyngeal arch (Dong et al., 2006; Shih et al., 2007a). The present work identifies a crucial role for *Pitx2* in control of somite-derived myogenesis, placing this gene either downstream of *Pax3* in myotome and in a parallel pathway to *Myf5* and *Mrf4* in limbs where *Pitx2* directly controls onset of *MyoD* expression. Taken collectively, these data support models that include *Pitx2*-dependent pathways in all skeletal muscles (Fig. 8A).

The present work documented a delay of limb bud *MyoD* expression in $Pitx2^{-/-}$ embryos. This delay is similar to the 1- to 2-day delay of *MyoD* activation in limb buds of *MyoD* CE-deleted mice. Accordingly, we show Pitx2 binding to the *MyoD* CE in embryonic limb buds by ChIP and binding site-dependent transcriptional activation by Pitx2. Thus, the *MyoD* core enhancer is the likely target for Pitx2 control of *MyoD* expression during limb myogenesis. CE-deleted mutant embryos are characterized by delayed *MyoD* expression not only in limbs but also in muscle precursors of the first branchial arch (Chen and Goldhamer, 2004). During branchiomeric development, *Pitx2* is expressed both in surface ectoderm and in the mesodermal core of the first arch, and interestingly conditional inactivation of *Pitx2* in pharyngeal mesoderm results in severe reduction of *MyoD* and myogenin expression (Dong et al., 2006). The CE may thus also constitute a cis target for *Pitx2*-dependent *MyoD* expression during branchiomeric myogenesis. In both $Pitx2^{-/-}$ and $CE^{loxP/loxP}$ mutants, the functional consequence of delayed *MyoD* expression was a delay in muscle differentiation as revealed by myogenin expression. This defect is reminiscent (somewhat less pronounced) of delayed limb and branchial arch differentiation observed in $MyoD^{-/-}$ embryos (Kablar et al., 1997). Collectively, these data suggest similar regulatory networks for Pitx2-dependent *MyoD* activation in *MyoD*-dependent lineages of the head and limb.

This similarity contrasts with Myf5/Mrf4 regulation of *MyoD*. Indeed, while *MyoD* expression is delayed in myotome of $Myf5^{nlacZ/nlacZ}$ mutant embryos, it is unaffected in limb buds and branchial arch-derived myogenic cells (Kablar et al., 1997; Tajbakhsh et al., 1997). Consistent with this, CE activity is not affected in $Myf5^{nlacZ/nlacZ}$ limb buds nor in branchial arch, suggesting that *Myf5* is dispensable for both CE activity and early *MyoD* expression in these lineages (Chen and Goldhamer, 2004).

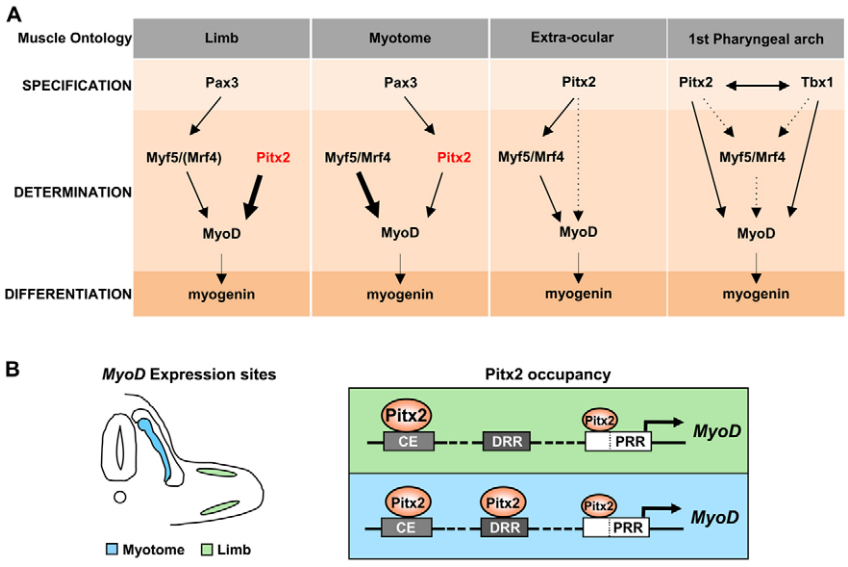

Fig. 8. MyoD gene regulation. (**A**) Gene networks controlling *MyoD* expression in limb, myotome, extra-ocular and pharyngeal arch muscles. *Pitx2* has a crucial role in initiation of *MyoD* expression in limb buds, but not in myotome, where *Myf5* and *Mrf4* play a predominant role. Nonetheless, *Pitx2*, *Myf5* and *Mrf4* are jointly contributing to *MyoD* expression in both limb and myotome, as revealed by their compound mutants. *Pax3* is required for *Pitx2* expression in myotome. In head (extra-ocular and 1st pharyngeal arch) muscles that do not express *Pax3*, *Pitx2* acts upstream of *Myf5 and Mrf4* (rather than in parallel), and in association with *Tbx1* in arches (Sambasivan et al., 2009). (**B**) Differential recruitment of Pitx2 to different regions of the *MyoD* gene depending on muscle ontology, in support of functional redundancy models (Frankel et al., 2010).

The present work nonetheless supports a role of *Myf5(Mrf4)* in limb *MyoD* expression and positioned these two genes in a parallel pathway relative to *Pitx2* (Fig. 8A).

MyoD regulation has been studied extensively in myotomal lineages and supported models in which *Myf5*, *Mrf4*, as well as *Pax3*, operate upstream of *MyoD* (Tajbakhsh and Cossu, 1997). In *Myf5*$^{nlacZ/nlacZ}$ mutant embryos, *MyoD* expression is delayed by ~2 days in myotome (Kablar et al., 1997; Tajbakhsh et al., 1997) and this delay appeared to be due to developmental arrest of myogenic progenitors along the dermomyotome (Tajbakhsh et al., 1996b). Using allelic series of *Myf5* mutants that differentially affect *Mrf4* expression together with *Mrf4* mutants, it has been shown that *Myf5*$^{nlacZ/nlacZ}$ and *Myf5;Mrf4* mutants exhibit the same delayed *MyoD* expression, supporting the conclusion that both *Mrf4* and *Myf5* act upstream of *MyoD* in myotomes (Kassar-Duchossoy et al., 2004). This dependence on *Myf5* and *Mrf4* was transient and the recovery of *MyoD* expression operates through a *Pax3*-dependent mechanism, although this may be mediated through a secondary factor (Tajbakhsh et al., 1997). We complemented this model by positioning *Pitx2* under control of *Pax3* (Fig. 8A). As the double mutant for *Pitx2* and *Myf5(Mrf4)* is deficient in myotome expression of *MyoD*, Pitx2 is a likely intermediate for *Pax3* control of *MyoD*. Thus, *Pitx2* defines a complementary pathway for activation of the myogenic pathway through *MyoD*.

Regulation of *MyoD* expression is complex: although it can be recapitulated by the complementary activities of the CE and DRR, various transgenic studies have concluded that both enhancers are dispensable either for *Myf5* regulation or *Pax3*-dependant expression of *MyoD* in myotomal lineages (Chen et al., 2002; Chen and Goldhamer, 2004; Kucharczuk et al., 1999). With the exception of E-boxes, the two enhancers lack cis-motifs known to regulate muscle genes during development. Taken together, these observations suggest a functional redundancy in cis-regulatory mechanisms controlling *MyoD* expression. Such redundancy of transcriptional control mechanisms appears to be a frequent feature of developmental regulatory pathways, as elegantly shown recently in *Drosophila* larvae (Frankel et al., 2010) and the action of Pitx2 on the *MyoD* gene is consistent with this model (Fig. 8B). Whereas the CE appears to be the predominant site of action for *MyoD* expression in limb buds, ChIP analyses also revealed Pitx2 recruitment in the –600 bp PR region, but not at the DRR. This proximal site, which is close to documented sites for Pax3 and FoxO3 (Hu et al., 2008), may thus also contribute to expression. Furthermore, similar ChIP analyses in myotome revealed Pitx2 at the three *MyoD* regulatory regions (CE, DRR and PR). Pitx2 recruitment to the DRR was unexpected as no consensus Pitx-binding site is present in this enhancer. Pitx2 may be indirectly recruited to the DRR through its interaction with another transcription factor. Serum response factor (SRF) is a likely candidate as the DRR contains a SRF-binding CArG element previously shown to be required for *MyoD* expression in skeletal myoblasts (L'Honoré et al., 2003) and Pitx2 has been shown to interact with SRF and increase its association with DNA (Shang et al., 2008).

In *Pitx2;Myf5(Mrf4)* mutants, progenitor cells are still present at E11.5-E12.5 in both myotome and limb, as revealed by β-galactosidase staining but most of these cells do not express MyoD or myogenin. This situation is reminiscent but somewhat less pronounced than in *Myf5;Mrf4;MyoD* triple mutants, where in absence of all myogenic bHLH, somitic progenitors cells fail to commit to the muscle lineage. Those progenitors have been reported to assume nonmuscle fates in trunk and limbs, being first integrated in cartilage primordia, but then to undergo apoptosis. This death occurs several days after their birth (E13.5) (Kablar et al., 1999; Kablar et al., 2003) and may be linked to their failure to commit. It is, thus, tempting to speculate that a similar mechanism occurs in *Pitx2;Myf5(Mrf4)* mutants: analysis of those mutants at E12.5 revealed a slight increase of apoptosis in myotome and limb compared to control. Unfortunately, the early lethality (E13) of *Pitx2;Myf5(Mrf4)* mutants did not allow us to perform analyses at later stages.

As reported for *Pax3;Myf5;Mrf4* mice (Kassar-Duchossoy et al., 2004; Tajbakhsh et al., 1997), we note an incomplete penetrance and residual *MyoD* and myogenin expression in *Pitx2;Myf5(Mrf4)* embryos, leading to sporadic myogenesis in the myotome (particularly in the most epaxial part) and in the limb (dorsal mass). This could be due to compensation by another factor. Likely candidates include: Pax7, which is expressed in precursors from E11.5 (Kassar-Duchossoy et al., 2005; Relaix et al., 2005); Tbx1, which is initially expressed in dorsal masses of the limbs (Dastjerdi et al., 2007) and reminiscent of residual *MyoD* expression observed in *Pitx2;Myf5(Mrf4)* mutants; and, finally, Pitx3, which is expressed after Pitx2 during embryonic myogenesis (L'Honoré et al., 2007). *Pitx3* does not compensate for loss of *Pitx2* towards myogenin or *MyoD* expression in limbs (see Fig. S4 in the supplementary material), but we cannot exclude the possibility that it could exert its action in absence of both *Pitx2* and *Myf5*. These seemingly *Pitx2*-independent myogenic cells may represent a subpopulation that is regulated by yet another alternate myogenic pathway.

The present work delineated differential requirements for *Pitx2* in migratory versus non-migratory somite-derived myogenic cells. These body myogenic cells share a crucial dependence on *Pax3* for their fate, in contrast to extra-ocular and branchial arch muscles (Sambasivan et al., 2009), which do not express *Pax3*. It is noteworthy that *Pitx2* is complementary to *Myf5* and *Mrf4* in *Pax3*-dependent body muscles, whereas it is genetically upstream of the MRF core regulatory network in *Pax3*-independent head muscles. Thus, all skeletal muscles include *Pitx2* in their genetic program. Different muscles have evolved the striking ability to co-opt selected elements of a core regulatory network together with a complementary genetic pathway in order to direct similar, yet distinct, muscle cell fates at different anatomical locations. Pitx transcription factors have been shown to interact physically and functionally with factors of multiple structural families, including POU-homeo factors (Szeto et al., 1996), bHLH factors (Poulin et al., 2000), Smads (Nudi et al., 2005), Tbox (Lamolet et al., 2001), Egr and nuclear receptors (Tremblay and Drouin, 1999), and LEF1 (Vadlamudi et al., 2005). In view of these multiple interactions, the implication of *Pitx2* in the myogenic program provides numerous alternative mechanisms to modulate unique muscle identities in limb, trunk, pharyngeal or ocular muscles.

Acknowledgements

We are grateful to Phil Gage and Sally Camper, who provided the *Pitx2* mutant mice; to Sharaghim Tajbakhsh and Margaret Buckingham (Institut Pasteur, Paris), who provided the *Myf5*^{nLacZ} mice; and to Alan Underhill (University of Alberta, Edmonton, Canada) for the *Splotch* mice. The help of Philippe Daubas for gel retardation is greatly appreciated. We thank Catherine Couture for plasmid constructions, Annie Vallée of the IRCM Histology Core for her precious help in tissue and section preparations, and Julie D'Amours and Isabelle Brisson for animal husbandry. The secretarial assistance of Lise Laroche was highly appreciated. This work was supported by a postdoctoral Fellowship from Fondation de la recherche médicale to A.L. and by grants to J.D. from the Canadian Institutes of Health Research (MT-15081).

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.053421/-/DC1

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