Six1 and Six4 homeoproteins are required for Pax3 and Mrf expression during myogenesis in the mouse embryo

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Summary
In mammals, Six5, Six4 and Six1 genes are co-expressed during mouse myogenesis. Six4 and Six5 single knockout (KO) mice have no developmental defects, while Six1 KO mice die at birth and show multiple organ developmental defects. We have generated Six1/Six4 double KO mice and show an aggravation of the phenotype previously reported for the single Six1 KO. Six1/Six4 double KO mice are characterized by severe craniofacial and rib defects, and general muscle hypoplasia. At the limb bud level, Six1 and Six4 homeogenes control early steps of myogenic cell delamination and migration from the somite through the control of Pax3 gene expression. Impaired in their migratory pathway, cells of the somitic ventrolateral dermomyotome are rerouted, lose their identity and die by apoptosis. At the interlimb level, epaxial Mef expression is abolished, while it is preserved in Pax3-deficient embryos. Within the myotome, absence of Six1 and Six4 impairs the expression of the myogenic regulatory factors myogenin and Myod1, and Mrf4 expression becomes undetectable. Myf5 expression is correctly initiated but becomes restricted to the caudal region of each somite. Early syndetomal expression of scleraxis is reduced in the Six1/Six4 embryo, while the myotomal expression of Fgfr4 and Fgf8 but not Fgf4 and Fgf6 is maintained. These results highlight the different roles played by Six proteins during skeletal myogenesis.

Key words: Six/sine oculis homeoproteins, Pax3, Myogenesis, Hypaxial lip, Migration, Myotome, Syndetome

Introduction
Six1 and Six4 genes belong to the sine oculis/Six gene family that includes six members in vertebrates (from Six1 to Six6), three members in Drosophila, four members in Caenorhabditis elegans and at least two members in basal metazoans (Kawakami et al., 2000; Pineda et al., 2000; Seo et al., 1999; Dozier et al., 2001; Bebenek et al., 2004). Originally, sine oculis was identified in Drosophila, where it belongs to a network of genes that drives eye development synergistically with eyeless (Pax orthologs), eyes absent and dachshund (Cheyette et al., 1994; Pignoni et al., 1997; Serikaku and O’Tousa, 1994). Absence of sine oculis leads to massive apoptosis of precursor cells in the developing compound eye (Cheyette et al., 1994). In Drosophila, two other sine oculis related genes have been identified: optix, which is expressed in the eye imaginal disc (Toy et al., 1998); and dSIX4, which is required for myoblast fusion (Kirby et al., 2001). In vertebrates embryos, Six1 and Six4 expression have been detected in overlapping territories: neural placodes, Rathke’s pouch, DRG, dermomyotome, myotome, limb bud mesenchyme and in myogenic migrating precursors (Esteve and Bovolenta, 1999; Oliver et al., 1995; Ozaki et al., 2001; Bessarab et al., 2004; Fougerousse et al., 2002; Laclef et al., 2003a; Schlosser and Ahrens, 2004). This co-expression may reflect co-regulation of both genes, which are only separated by 100 kb (Boucher et al., 1996). Six4+/− mouse present with no major developmental defects (Ozaki et al., 2004), while Six1+/− mouse neonates do not survive and have defects in many organs, including kidney (Xu et al., 2003), thymus, parotid glands and ear (Laclef et al., 2003b; Zheng et al., 2003; Ozaki et al., 2004), and rib, craniofacial and muscle deficiencies (Laclef et al., 2003a; Li et al., 2003). Six1 is also expressed at high levels in adult skeletal muscles where it participates, in synergy with Eya1, in the establishment of the fast/glycolytic phenotype of the myofiber (Grifone et al., 2004). With respect to muscle development, it has been shown that Six1−/− fetuses have a selective loss of muscles including diaphragm, proximal and distal forelimb muscles, distal ventral hindlimb muscles and abdominal muscles (Laclef et al., 2003a).

Back skeletal muscles are derived from somitic progenitors originating from the epaxial dermomyotome. At the interlimb level, the lateral myotome and dermomyotome produce the...
hypaxial muscles, including thoracic intercostal, abdominal and limb muscles (Buckingham, 2001). Extension of the lateral dermomyotome is under the control of Pax3 (Williams and Ordahl, 1994), and in Pax3-/- mouse most hypaxial migrating myogenic precursors are lacking (Tremblay et al., 1998). Except for tongue muscles, which have a somitic origin, head muscle progenitors originate from head paraxial mesoderm and migrate into the pharyngeal arches to give rise to head and neck muscles (Hacker et al., 1998). Limb muscles are formed by cells of the dermomyotome that delaminate from somites and migrate into the limb bud where they further proliferate before activating Mrf genes (Rees et al., 2003). Mrf activation, and more particularly Myf5, is under the control of different signaling pathways, depending on the position of the myogenic precursors cells in the embryo (Hadjhouchel et al., 2003; Tzahor et al., 2003). Delamination of myogenic precursors from the dermomyotome is under the control of the Mef tyrosine kinase receptor and scatter factor/hepatocyte growth factor (SF/HGF) produced by the limb mesenchyme (Bladt et al., 1995; Dietrich et al., 1999). Invasion of the limb by myogenic progenitors is also under the control of the Lbx1 homeogene (Alvarez et al., 2003). Both Lbx1 and Met expressions are under the control of Pax3, as delamination and myoblast migration into the limb bud is prevented in Pax3-/- mice (Bober et al., 1994; Epstein et al., 1996; Goulding et al., 1994). Pax3 expression is not impaired in Six1-/- embryos, allowing myoblast migration and limb muscle formation (Laclef et al., 2003a). We suggested that ontogenesis of most remaining axial and limb muscles present in Six1-/- embryos are under Six4 or Six5 control, as we detected Six5, Six4 and Pax3 expression in migrating myoblasts of Six1-/- embryos at the limb level (Laclef et al., 2003a) (data not shown).

The sclerotome, another somitic compartment, gives rise to the axial skeleton and ribs. Interactions between the incipient ribs and growing myotomes at the intercostal level might occur through Fgf and Pdgf molecules produced by the myotomes (Huang et al., 2003). In this context, inhibition of Fgf signaling causes deletion of developing ribs (Huang et al., 2003b). The syndetome, which is derived from the sclerotome, gives rise to the axial tendons. Scleraxis is one of the earliest genetic marker characterized for this somitic lineage (Schweitzer et al., 2001). Induction and individualization of the syndetome requires the dermomyotome/myotome contact involving Fgf signaling (Brent et al., 2005).

To test the hypothesis that Six4 acts in common with Six1 during myogenesis, we produced double Six1Six4 knockout (dKO) mice. We show here that Six1-/-Six4-/- embryos develop a more severe muscle phenotype than did the Six1-/- embryo. No muscle is detected in the limbs because of the downregulation of Pax3 in the ventral dermomyotomal lips of the somites from which hypaxial progenitors arise. There is no proliferation defects but most of these precursors migrate aberrantly, lose their myogenic identity and die by apoptosis. Epaxial and non migrating hypaxial musculature is affected by severely compromised expression of Mrf genes within the myotome. Our results finally suggest that the rib phenotype developed by Six1-/-Six4-/- fetuses could be the result of a loss of Mrf4 expression and a downregulation of Fgf production in the myotome.

Materials and methods
Construction of Six1-Six4 gene targeting vector
We isolated several Six4 genomic XFixII DNA clones from a 129Sv genomic library. We subcloned a 14 kb NolI-NolI genomic fragment comprising the entire Six4 gene into pBluescript KS+ (Stratagene). A 3’ 3.1 kb Nar-EcoRI fragment comprising the end of the first intron and the beginning of the second Six4 exon was then ligated in a plox2hygro plasmid leading to p3’Six4lox2hygro. A 5’ DNA region (NolI-Eco47III 4.2 kb fragment) was ligated in frame into a pEGFP expression vector (Clontech). This 5’ genomic fragment possesses 3.2 kb DNA upstream of Six4 transcription initiation sites as well as the first 144 amino acids. The 5’Six4-GFP fragment was further cloned in p3’Six4lox2hygro, leading to the final invalidation plasmid. Homologous recombination with this disruption vector was expected to lead to the deletion of the last 370 amino acids of the first Six4 exon, including most of the Six domain and the entire homeodomain, which, together, are responsible for the specific DNA-binding activity of Six4 protein.

ES cell screening and chimeric mouse production
Specific Six4 DNA fragment digested by NolI digestion (35 µg), to eliminate plasmid DNA sequences, was electroporated (250V; 500 F) into 1.5x107 Six1-lacZ embryonic stem cells (Laclef et al., 2003a). ES cells were selected with 150 µg/ml hygromycin 24 hours after electroporation. The DNA of 310 Six1-Six4 resistant clones was analyzed by Southern blot after Pst digestion. A 5’ fragment and a 3’ fragments were used as external probes. Ten to 12 cells of three Six1-Six4 independent recombinant ES clones were microinjected into C57BL6 blastocysts, which were further implanted into pseudopregnant mice. Heterozygous progenies were obtained by backcrosses to C57BL6 and 129/SVj females. All three clones were recombined on the Six1-lacZ allele, as F1 animals from the three clones were either wild type or heterozygous for both Six1 and Six4. F1 progeny was then crossed with ElaCre animals expressing the Cre recombinase ubiquitously under the control of E2A promoter (Holzenberger et al., 2000). Deletion of the PGK-hygroycin cassette was ascertained by Southern blot analysis. All homozygous embryos and fetuses have been genotyped by Southern blot analysis.

X-gal staining, whole-mount skeletal staining, histology and immunohistochemistry of the embryos were performed as described previously (Laclef et al., 2003a). Vibratome section (120 µm) were performed after inclusion of the embryos in 4% agarose. Sections were then mounted in Kaiser’s glycerol gelatin solution (Merck). Gel mobility shift assays were performed as described (Grifone et al., 2004).

Results
Generation of Six1Six4-deficient mice
Owing to the genetic proximity (about 100 kb) of Six1 and Six4 genes on chromosome 12, invalidation of the Six4 gene was achieved in ES cells already invalidated at the Six1 locus (Laclef et al., 2003a). Inactivation of the Six4 gene was achieved by replacing the Six domain and homeodomain of the Six4 protein, both of which are required for specific DNA binding, by the EGFP cDNA (Fig. 1A). To identify the recombination events at the Six4 locus that took place on the Six1 recombined and wild-type allele, we analyzed the DNA of the progeny of chimeric animals for both Six1 and Six4. In the three independent ES clones where recombination events at the Six4 locus were identified. F1 DNA analysis showed that all three recombination events occurred on the recombined Six1 allele, leading to a Six1lacZ/Six4gfp/Six1”Six4” genotype, hereafter referred to Six1lacZ-Six4gfp.
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5’ and 3’ external probes confirmed these events (Fig. 1B).

Six1+/–Six4+/– animals are viable and fertile, suggesting that one allele of Six1 and Six4 are sufficient for correct development, either on 129SV or C57black6N backgrounds (F8 generation). Indeed, Six1+/–Six4+/– animals die at birth and can be easily distinguished from their littermates. They are smaller, have an enlarged abdomen and present severe craniofacial malformations (Fig. 1C). In contrast to Six1–/–, Six1+/–Six4+/– are earless, have open eyelids, a very reduced maxillary and mandible, abnormally bent hindlimbs and forelimbs, and a general bent posture as if dorsal musculature is inadequate.

Skeletal malformations in Six1–/–Six4–/– newborn animals

Examination of the skeleton of E18.5 Six1–/–Six4–/– fetuses revealed a more severe phenotype than was observed in Six1–/– fetuses (Laclef et al., 2003b). This was most severe at the rib/sternum level, where the distal ribs were reduced to small protrusions (Fig. 2B,C). None of the six Six1–/–Six4–/– fetuses analyzed in this study, on the two genetic mutant backgrounds, shows rib attachment to the sternum (Fig. 2), whereas such attachments were observed occasionally in Six1–/– mutants (Laclef et al., 2003a). At the cranial level, the mandible bone is drastically shortened and the fetuses have small orbits with protruding eyes. Meckel cartilage is absent, jugal, nasal and premaxilla bones are absent, the palatal process of the maxilla is lacking, the squamosal bone is reduced, and the cartilages of the inner ear and the ectotympanic bone are lacking (Fig. 2D,E, and data not shown).

It thus appears that absence of Six4 in a Six1–/– background leads to a more severe craniofacial and rib phenotype. In both forelimbs and hindlimbs, a clinodactily (digit curvature) with a curved fifth digit was observed in all E18.5 fetuses analyzed (n=6, Fig. 2F,G).

The phenotype of the transgenic mice was compared with a human condition connected to the deletion of 14q22. This region comprises the SIX1, SIX4 and SIX6 gene cluster of about 200 kb. We have previously found that some of the human symptoms correspond to what can be observed in Six1 homo- and heterozygous null mutant mice, such as hypotonia and hypoplastic kidneys, while others (such as anophthalmia and pituitary hypoplasia) were not observed in the Six1–/– null (Bennett et al., 1991; Gallardo et al., 1999; Laclef et al., 2003b; Lemyre et al., 1998; Xu et al., 2003). It has been shown that in these individuals the SIX6 gene was deleted, suggesting that anophthalmia and pituitary hypoplasia could be caused by SIX6 haploinsufficiency (Gallardo et al., 1999), which agrees with analysis of Six6 KO mice (Li et al., 2002). We identified hemizygosity for both SIX1 and SIX4 genes in a human 21-week-old fetus (Fig. 2H). This fetus had hypoplastic kidneys and clinodactily (Bennett et al., 1991), suggesting that Six1 and Six4 haploinsufficiency could be responsible for these developmental defects.

General muscle deficiencies in double Six1Six4 newborn animals

We have shown previously that Six1–/– fetuses present a severe but selective muscle hypoplasia, while Six4–/– fetuses are normal (Laclef et al., 2003a; Ozaki et al., 2001). As Six1 and Six4 are co-expressed during myogenesis, we suspected a compensatory role for Six4 in the genesis of the remaining muscles of Six1–/– fetuses. X-gal staining of Six1–/–Six4–/– E18.5 fetuses revealed a more severe muscle phenotype than observed previously in Six1–/– fetuses (Fig. 3A-L). All muscles of the distal forelimb and hindlimb are missing, as revealed by the absence of any fast or slow myosin positive fibers (Fig. 3I-
most ventral muscles are missing (see enlargement of Fig. 3G,H). Back muscle masses at the forelimb level and more rostrally are incorrectly shaped, while back muscles at the interlimb level and at the hindlimb level show less disorganization (Fig. 3E,F). This suggests that Six1 and Six4 are dispensable for the formation of remaining back muscle fibers because all of these muscles express lacZ (Fig. 3C-F), myosin heavy chains and desmin proteins (data not shown). Tendon formation did not appear altered in these remaining muscles (data not shown). Most of the head muscles, including the masseter, temporalis, pterygoid and extra-ocular muscles are present at E18.5 (Fig. 3A,B; data not shown), showing that Six1 and Six4 are not required for the formation of head muscles. However, while the mylohyoid is present, the genioglossus muscle is absent and intrinsic tongue muscle is reduced (data not shown) in dKO E18.5 fetuses.

**Primary myogenesis is severely disorganized in Six1−/−Six4−/− embryos**

A severe muscle hypoplasia was already apparent in embryonic forelimbs by E13.5, as revealed by β-gal staining (Fig. 3M-N) and desmin expression (Fig. 3S-T). There is a total absence of desmin-positive myogenic cells in limbs. There is also a disorganization of muscle masses at the back level, which is more obvious rostrally to the forelimb (compare Fig. 3T with 3V), and a total absence of myogenic cells in the abdominal region (Fig. 3U-V). In the head, most muscles are lacking at the eye level (Fig. 3O-P) and the tongue muscles are absent (Fig. 3Q-R), while other muscles like the masseter are present (Fig. 3Q-R). In the head, these defects seem transient, as most head muscles are present by E18.5.

**Severe myotomal disorganization in Six1−/−Six4−/− embryos**

Six1 and Six4 expression has been monitored by X-Gal staining and GFP expression at different embryonic stages ranging from E9.5 to E12.5 on wild-type and null backgrounds (Fig. 4). In E9.5-E10.5 Six1−/−Six4−/− embryos, Six1 and Six4 genes are still highly expressed in somites, suggesting that Six1 and Six4 proteins are not required for their own transcription in these structures. Conversely, their gene expression is severely reduced at the cranial level in the trigeminal placode and in the otic vesicle, where X-Gal staining becomes barely detectable at E10.5 (Fig. 4E-H). These results suggest either that in these cells Six1 and Six4 control positively their own transcription, or that apoptosis occurs in these placodal precursors because they require Six1 and Six4 to survive (see later). At E9.5, Six1 expression was greatly reduced in the otic vesicle of Six1−/−Six4−/− embryo (Fig. 4; compare A,C with B,D) probably reflecting a loss of Six1−/−Six4−/− cells through apoptosis (Ozaki et al., 2004; Zheng et al., 2003). At E9.5, the...
maxillary primordium and mandibular primordium of the first and second branchial arches appeared fused (compare Fig. 4C with 4D).

At E10.5, Six1 and Six4 gene expression within each somite is largely diffuse rostrocaudally and severely reduced ventrally (Fig. 4F-P). Vibratome sections through the dKO embryo show that β-gal-positive cells are located ventromedially at the hindlimb level and ventrolaterally beneath the ectoderm at interlimb or forelimb levels (Fig. 4L,N,P), instead of extending ventrally or migrating into limb buds as shown in control heterozygous embryos (Fig. 4K,M,O). These mislocated β-gal-positive cells do not express Pax3, desmin or Myf5, and are probably no longer myogenic precursor cells (see later).

Six1 gene expression in the DRG is not affected (Fig. 4M-P). At E11.5, β-gal-positive cells are dispersed in the Six1–/– Six4–/– somites when compared with the heterozygous (Fig. 4W-Z). Six1 and Six4 gene expression is not detected in the ventral and dorsal lips of the dermomyotome (Fig. 4Q,R,U,V). At E12.5, β-gal-positive cells also appear less abundant at this stage in the homozygote. It is also evident that most of cells are blocked in their migration pathway, and fail to enter the limb bud (Fig. 4L,N,P).
Mislocated cells in Six1\textsuperscript{−/−}Six4\textsuperscript{−/−} embryos die by apoptosis

Whether Six1 and Six4 are important for the proliferation of myogenic progenitors was first examined by the analysis of BrdU incorporation and phospho-histone H3 expression. No significant difference of proliferation was observed in the somites of E10-E11 dKO versus control embryos (data not shown). We next analyzed confocal sections to evaluate the total number of β-gal-positive cells in one somite of E10.5 heterozygous and dKO embryos. Analysis of seven serial sections at the hindlimb level showed that the number of β-gal-positive cells in the dKO (78±28) was significantly greater ($P$<0.0005, Student’s $t$-test) than in heterozygotes (32±14), which reflects their failure to migrate to the limb bud. At the abdominal level, we found 229 positive cells (±39) in a heterozygous and 302 in a dKO (±89) ($P$<0.025), showing no

Fig. 4. X-Gal and GFP expression in heterozygous (A,C,E,G,I,K,M,O,Q,S,U,W,Y) and homozygous mutant animals (B,D,F,H,J,L,N,P,R,T,V,X,Z) at E9.5 (A−D), E10.5 (E−P), E11.5 (Q−V) and E12.5 embryos (W−Z), revealing Six4-GFP expression and Six1-\textit{lacZ} expression. (A−D) Six1 expression is detected in the head mesenchyme (asterisk in A), otic vesicle (white arrow in A), branchial arches (black arrow in A) and pharyngeal clefts (white arrows in C) in heterozygous embryos. In dKO embryos, Six1 expression at the head level is severely reduced (asterisk in B) and lost in the otic vesicle (white arrow in B); although mesodermal Six1 expression is still detected (D), most Six1 expression is lost in pharyngeal pouch endoderm and surface ectoderm in dKO embryos. Fusion between the first and second branchial arches is observed at this stage (white arrow in D). At the thoracic level, the nephrogenic chord expression of Six1 is detected in the heterozygote (black arrowhead in A) but not detected in the dKO embryo (black arrowhead in B). At the somitic level, Six1 is expressed at comparable levels in the heterozygous (A) and homozygous (B) animals. (E−P) Six1 is still highly expressed in the ventral otic vesicle (white arrow in E,G) and trigeminal ganglion (white arrowhead in E,G) in heterozygotes, but not in the dKO embryos (white arrow and white arrowhead, respectively, in F and H). (G,H) Enlargement of control (G) and KO (H) embryos at the head level. (LJ) Enlargement of control (I) and KO (J) embryo at the interlimb level. At the somitic interlimb level, Six1 expression is detected in the myotome (E,J). In the dKO, Six1 expression is more diffuse, while still expressed in myotomes (F,J). (K−P) Vibratome sections. At the hindlimb level (K,L), Six1 is detected in the nephrogenic chord (arrow in K) but is not in the dKO, while many β-gal-positive cells invade this ventral region of the embryo (L). Six1-positive cells enter the limb bud in the heterozygous embryos (K), but not in the dKO embryo, where somitic β-gal-positive cells are not confined to the somite but are found more ventromedially (L). At the interlimb level (M,N) Six1-positive cells are diffuse and do not extend ventrally in the dKO embryo (N), while they invade ventral region in the heterozygous embryos (M). Six1 expression in DRG is preserved, but a few β-gal-positive cells are also seen in the neural tube in the dKO (N,P; data not shown). At the forelimb level (O,P), Six1-positive cells are present in the limb bud in the heterozygous embryos (O), while in dKO embryos most cells accumulate less ventrally, and are not found in the limb bud, but are also detected beneath the ectoderm more medially (P). (Q−V) Six1 (Q, R) and Six4 (S−V) are mainly detected in limbs, and somites, where they are co-expressed in the DRG, myotomes, and ventral (black arrows) and dorsal dermomyotomal lips. In dKO, somites still express these two genes (R,T). Myotomes are disorganized, their ventral extensions are reduced (white double headed arrow), and GFP and β-gal are no longer detected in the ventral lips (black arrows). A population of GFP and β-gal-positive cells is found in the limbs of dKO embryos but they are not myogenic cells (Bonnin et al., 2005). (W−Z) Six1 is expressed at higher levels in heterozygous (W,Y) than in homozygous mutant embryos (X,Z). At the thoracic level, ventral extension of the dermomyotome, normally marked by Six1 expression, is lost in dKO embryos (white arrows). Posterior expression in the limbs is maintained.
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proliferation defects of Six1+/Six4+/ cells at this level. At the forelimb level, we found 55 positive cells (±19) in a heterozygous and 152 in a dKO (±39) (P<0.0005). These results combined with the observation of comparable levels of β-gal and GFP accumulation in heterozygous and dKO embryos (Fig. 4I,J,U,V) indicated that at least until E10.5, the absence of Six1 and Six4 in vivo does not prevent proliferation of the somitic myogenic precursors.

As Six1+/Six4+/ fetuses were smaller than their littermate without proliferation defect in somitic structures, at least between E10 and E11, we decided to examine the extent of apoptosis in the dKO embryos. In contrast to control heterozygous embryos, activated caspase 3-positive cells were detected at the ventral lip level of dKO embryos at E10.5 (Fig. 5A-D), and later at E12.5 in cells which were rerouted ventrally (Fig. 5E,F). These apoptotic events were not specific to the somitic compartment as caspase 3-positive cells were also found at E10.5 in branchial arches (Fig. 5G,H).

Impaired migration of myogenic precursors in Six1+/Six4+/ embryos is due to a decrease of Lbx1, Met and Pax3 expression

To further investigate the origin of limb muscle defects, the expression of primordial myogenic markers was examined at E10.5 in dKO and heterozygous embryos. In less mature caudal somites, Pax3 is expressed normally in the dorsal neural tube and the dermomyotome of Six1+/Six4+/ embryos (Fig. 6, part I, A-F). In more rostral somites, at the hindlimb level, the ventralmost expression of Pax3 is lower and no Pax3-positive cells have delaminated from the dermomyotome to invade the limb bud (Fig. 6, part I, G-J). At the thoracic level, Pax3 expression in the dorsal and ventral extensions of the dermomyotome is undetectable (Fig. 6, part I, M-N) and Pax3 expression is only detected in caudal and rostral lips, where it is conversely higher than in heterozygous embryos (Fig. 6, part I, K,L). At the forelimb level, Pax3 expression remains detectable in the caudal lips but is not detected in dorsal and ventral lips. Hence, Pax3 expression is also restricted to a central domain within the somites and no Pax3-positive cells are detected in the forelimb (Fig. 6, part I, O-R). More rostrally in occipital somites, Pax3 expression is severely reduced (Fig. 6, part I, A,B). In E9.5, dKO embryos, Pax3 expression is also restricted to the caudal lips of the dermomyotome of existing somites (data not shown).

Met expression is not affected in the most caudal somites of dKO embryos (Fig. 6, part II, A,B), but it declines more rostrally (Fig. 6, part II: A,B,E,F). In somites facing the hindlimb, Met is poorly detectable in the ventral dermomyotome and only a few Met-positive cells are detected in the limb (Fig. 6, part II, C,D). At the interlimb level, Met is mainly detected in ectodermal structures. The absence of Met in the ventral dermomyotome coincides with the downregulation of Pax3, while its downregulation in the epaxial domain is specific to the Six1/Six4 dKO. At the forelimb level, a weak Met expression is detected in the centre of the somite (Fig. 6, part II, H) where Pax3 was also restricted (Fig. 6, part I, R). This Met expression domain is not observed in heterozygous or wild-type embryos (Fig. 6, part II, G), probably because of a low Pax3 expression (Fig. 6, part I, Q). However, this level of Met appears insufficient to allow myoblast migration as no myogenic precursors are detected in the forelimb of dKO embryos.

Lbx1 expression is not detected at forelimb and hypoglossal chord levels in dKO embryos when compared with the control (Fig. 6, part III, A-D). Lbx1 is weekly detectable in three out of the six Lbx1-expressing somites facing the hindlimb bud (Fig. 6, part III, E,F), corresponding to sacral somites where

![Image](image-url)
Met and Pax3 are expressed ventrally. Lbx1 was not detected in occipital somites and at the forelimb level in E9.5 dKO embryos (data not shown).

Bandshift assays using a potential MEF3 site (TCAGGTTTTC) found in the human and mouse Lbx1 promoter (TCAGGTTgGC) as a probe together with nuclear extracts or in vitro synthesized Six1 protein, demonstrated a specific interaction of Six proteins with the Lbx1 promoter (Fig. 6, part III, G; data not shown). Hence, Lbx-1 may be under the control of Six proteins, at least in a population of myogenic migrating precursors. Nonetheless, the presence of a few Lbx-1 positive cells in the hindlimb suggests that some specific myogenic precursors can activate Lbx1 and Met in the absence of Six1 and Six4 homeogenes.

Finally, at hindlimb and interlimb levels, no major difference in Pax7 expression, another dermomyotomal marker, is
detected between control and dKO embryos (Fig. 6, part IV, C,D). However, at the forelimb level, Pax7 is slightly reduced in the ventral and dorsal dermomyotome in the dKO embryo (Fig. 6, part IV, I,J).

**Pax3 expression in the dermomyotome is under the control of Six1 and Six4**

We next performed double immunofluorescence on transverse sections of E10.5 embryos to detect both Pax3 and *lacZ* expression, the latest reflecting the presence of cells that turned on Six1 gene expression (Fig. 7). Caudally to the hindlimb in both heterozygous and homozygous embryos, Pax3 and Six1 are co-expressed in the entire epithelial dermomyotome (Fig. 7A-D). At the hindlimb level in heterozygous embryos, Pax3 and Six1 genes are co-expressed ventrally in a region where there was no lateral migration to the limb (Fig. 7G-H) and in some laminin- and desmin-positive myotomal cells (Fig. 7E-F,I-J). In caudal most somites facing the hindlimb of dKO embryos, Pax3 and Six1 genes are co-expressed ventrally in a region where there was no lateral migration to the limb (Fig. 7G-H) and in some laminin- and desmin-positive myotomal cells (Fig. 7K-L). In rostral most somites facing the hindlimb, Pax3 expression becomes lower ventrally, whereas many Six1-positive cells are still present (Fig. 7M,N). Without Six proteins, these cells lose Pax3 expression. They subsequently lose their identity and migrate medially instead of invading the bud or differentiating in the myotome (Fig. 7G,H,M,N), even when Met and Lbx1 are transiently activated (Fig. 6, parts II,III). Thus, Six proteins are required to activate Pax3 expression and to impose a myogenic fate to dermomyotomal cells of the somites. Interestingly, in the most caudal somites of dKO embryos, the weak level of Lbx1 observed in hypaxial cells expressing Pax3 suggests that Pax3 requires Six1 and Six4 to activate *Lbx1* gene to high level (Fig. 6, part III). At the thoracic level in dKO embryos, Pax3 expression is severely reduced and remains only detectable in a medial domain of the somite (Fig. 7O-R).

At the forelimb level in dKO embryos, the ventrolateral extension of Pax3 expression is severely reduced when compared with the control (Fig. 7S,U). Six1 is uniformly expressed dorsoventrally from the ectoderm to more internal mesenchyme structures, including the myotome (Fig. 7V) instead of being restricted to myotomal and migrating cells (Fig. 7T). In absence of Six1/Six4, cells are unable to express Pax3, lose their myogenic identity and are consequently dispersed and re-located beneath the ectoderm or more medially. Although laminin expression that delimits the myotome is robust in heterozygous mice (Fig. 7W), a few laminin-positive cells are detected in the dKO at the forelimb level (Fig. 7X), showing a severe reduction of the myotome at this rostral level when compared with caudal levels (Fig. 7K).

**Six homeoproteins control early Mrf genes expression**

We have previously shown that Six proteins were directly required for myogenin transactivation during embryogenesis (Spitz et al., 1998). Although no alteration of myogenin expression has been reported in *Six4*–/– embryos, we showed that Six1 was required for early expression of myogenin in limbs but not in the myotome (Laclef et al., 2003a). We show here that 90% of myogenin level was lost in the absence of Six1 gene expression (Fig. 7). Caudally to the hindlimb in both heterozygous and homozygous embryos, *lacZ* expression is similarly expressed in rostral somites of both heterozygous and dKO embryos, and is co-expressed with Six1 (compare C,D with A,B). At the hindlimb level in the dKO embryos, we checked desmin, laminin, Six1 and Pax3 expression in sacral somites facing the limb bud (M,N). Laminin and desmin expression is found in the myotomes in Six1-expressing cells on both wild-type and dKO sections (white arrows in I-L). At this hindlimb level, the myotome of dKO embryos is not disorganized. More rostrally, Pax3 expression is progressively lost ventrally in lumbar somites at the hindlimb level, while most cells are Six1 positive (compare G,H with M,N). At the thoracic level, Pax3 expression is restricted in the medial aspect of the dermomyotome of dKO embryos (Q), while Six1 gene expression is found in the myotomes (R). More rostrally, at the forelimb bud level, Six1-positive/Pax3-negative cells are disorganized and have lost their identity; they also fail to enter the limb bud in the double KO (compare S,T with U,V). At this level, the myotome of dKO embryos is disorganized, as revealed by the low laminin expression (white arrow in X), when compared with laminin expression in control embryos (white arrow in W).
Six1 and Six4 at E9.5 (Fig. 8, part II, A,B). A few specific cells can nevertheless bypass Six signaling to activate myogenin and probably give rise to the remaining epaxial somites of Six1–/–Six4–/– embryos, and more faintly in epaxial-rostral somites in dKO embryos. (Part II) Myogenin expression in E9.5 embryos in heterozygous (A) and dKO (B) embryos. A few positive myogenin-expressing cells are detected in the most rostral somites of dKO embryos (arrows in B). (Part III) Myogenin expression in E10.5 heterozygous (A,C,E) and dKO (B,D,F) embryos. Vibratome sections at the interlimb level (C,D) and forelimb level (E,F) show a strong decrease of myogenin expression that is faintly detected in the epaxial region of dKO embryos, hypaxial extension expression domain being lost. (Part IV) Myod1 expression in E10.5 heterozygous (A,C,E,G) and dKO (B,D,F,H) embryos. Most Myod1 expression is lost in Six1–/–Six4–/– embryos, and remaining expression can be visualized at the interlimb or forelimb levels in the central and hypaxial somites (F,H). (Part V) Myf5 expression in heterozygous (A,C,E,G) and in dKO (B,D,F,H) E10.5 embryos. Myf5 is mainly detected in caudal lips of homozygous dKO animals at interlimb levels (B, upper right), ventral and dorsal lip expression being lost (compare A with B). This expression loss is well detected on vibratome sections at hindlimb (C,D), interlimb (E,F) and forelimb (G,H) levels. In the dKO, at the forelimb level the myotome is formed only of a central region expressing Myf5 (H). In the top right-hand corners of A and B, magnification of interlimb Myf5 expression can be seen that is restricted to the caudal region of the somites in the dKO embryo (B). (Part VI) At E10.5, MRF4 expression is lost in Six1–/–Six4–/– embryos (B), when compared with control heterozygous expression (A). (Part VII) Myod1 expression in interlimb somites of dKO embryos is detected (B) with restricted ventral extension (B, and enlargement in the top right-hand corner) when compared with Myod1 expression in heterozygous embryos (A, enlargement in the top right-hand corner). Vibratome sections (C-H) showing a faint Myod1 expression at hindlimb level, with most dorsal and ventral extension being lost (C,D). At the interlimb level, Myod1 expression is restricted to the central region of the myotome, dorsal and ventral expression is lost (E,F). A low myotomal expression is detected at the forelimb level (G,H).

Myf5 expression in E9.25 (20 somites stage) in heterozygous (A) and dKO (B) embryos. Myf5 is expressed in the epaxial somites of heterozygous embryos, and more faintly in epaxial-rostral somites in dKO embryos. (Part I) Myf5 expression in E9.25 (20 somites stage) in heterozygous (A) and dKO (B) embryos. A few positive myogenin-expressing cells are detected in the most rostral somites of dKO embryos (arrows in B). (Part III) Myogenin expression in E10.5 heterozygous (A,C,E) and dKO (B,D,F) embryos. Vibratome sections at the interlimb level (C,D) and forelimb level (E,F) show a strong decrease of myogenin expression that is faintly detected in the epaxial region of dKO embryos, hypaxial extension expression domain being lost. (Part IV) Myod1 expression in E10.5 heterozygous (A,C,E,G) and dKO (B,D,F,H) embryos. Most Myod1 expression is lost in Six1–/–Six4–/– embryos, and remaining expression can be visualized at the interlimb or forelimb levels in the central and hypaxial somites (F,H). (Part V) Myf5 expression in heterozygous (A,C,E,G) and in dKO (B,D,F,H) E10.5 embryos. Myf5 is mainly detected in caudal lips of homozygous dKO animals at interlimb levels (B, upper right), ventral and dorsal lip expression being lost (compare A with B). This expression loss is well detected on vibratome sections at hindlimb (C,D), interlimb (E,F) and forelimb (G,H) levels. In the dKO, at the forelimb level the myotome is formed only of a central region expressing Myf5 (H). In the top right-hand corners of A and B, magnification of interlimb Myf5 expression can be seen that is restricted to the caudal region of the somites in the dKO embryo (B). (Part VI) At E10.5, MRF4 expression is lost in Six1–/–Six4–/– embryos (B), when compared with control heterozygous expression (A). (Part VII) Myod1 expression in interlimb somites of dKO embryos is detected (B) with restricted ventral extension (B, and enlargement in the top right-hand corner) when compared with Myod1 expression in heterozygous embryos (A, enlargement in the top right-hand corner). Vibratome sections (C-H) showing a faint Myod1 expression at hindlimb level, with most dorsal and ventral extension being lost (C,D). At the interlimb level, Myod1 expression is restricted to the central region of the myotome, dorsal and ventral expression is lost (E,F). A low myotomal expression is detected at the forelimb level (G,H).
Impaired myogenesis in Six1Six4 null embryos

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Fgf signaling is altered in Six1Six4 KO embryos

The hypothesis that rib defects observed in dKO fetuses was caused by an insufficient Fgf myotomal production was tested in dKO E10.5 embryos. Fgf4 expression is relatively reduced in the myotome of Six1–/–Six4–/– embryos, especially in more rostral somites, and Fgf6 expression seems more severely diminished than Fgf4 in dKO embryos (Fig. 9, parts I, II). Fgf8 expression is detected in the myotomes, AER, presomitic mesoderm and branchial arches of heterozygous embryos, and this expression is generally unaffected in Six1–/–Six4–/– embryos, except in the third and fourth branchial arches (Fig. 9, part III, A-E). Fgf4 is expressed by the proliferating myotomal cells (Marcell et al., 1995), and presumably in proliferating cells of dKO embryos as well (Fig. 9, part IV).

Finally, scleraxis expression in the syndetome has been shown to be controlled by Fgf signaling provided by the adjacent myotome (Brent and Tabin, 2004). Interestingly, no scleraxis expression is detected in the somites of Six1–/–Six4–/– embryos (Fig. 9, part V). Moreover Six1 and Six4 proteins are not produced by normal syndetomal cells (Fig. 9, part VI; data not shown). These results suggest that some specific Fgf molecules emanating from the somite are absent in dKO embryos, leading to the loss of early scleraxis expression.

Discussion

An important finding from this study is that the delamination and migration of the hypaxial precursors from the ventral lip to the limb buds are under the control of Six homeoproteins. In absence of Six1 and Six4, cells that normally give rise to the ventral lip of the dermomyotome lose Pax3 expression and lose their identity; some of them are misrouted dorsally and detected beneath the ectoderm, others are misrouted medially.

Met is the crucial factor necessary for myogenic cells of the VLL to undergo an epithelial to mesenchymal transition prior to migration in the limb bud (Bladt et al., 1995). In E10.5
SIX1+/−SIX4+/− embryos, Met expression is correct in the most caudal somites but severely reduced and highly diffuse more rostrally, thus completely preventing migration. As Met has been demonstrated to be directly controlled by Pax3 (Relaix et al., 2003), this caudorostral downregulation of Met in SIX1+/−SIX4+/− embryos may be a direct consequence of the loss of Pax3. This could be effectively the case for rostral somites where Pax3 expression is lost, but not for the most caudal somites facing the hindlimb, where Pax3 seems correctly activated, while Met is only faintly detected. These results coincide with previous results showing that Met activation in cells migrating from somite to limb was not entirely dependent on Pax3 (Mennerich et al., 1998). Thus, SIX1 and SIX4 control early steps of a genetic network involved in ventral lip formation and that coordinate the expression of a set of genes required for migration, including Pax3 and Met. Furthermore, while epaxial Met expression is not altered in splotch mice (Mennerich et al., 1998) and not increased in Pax3-FKHR mice (Relaix et al., 2003), it is abolished in all E10.5 SIX1−/+SIX4−/+ somites, even in caudal somites where Pax3 expression is not yet extinguished. This result suggests a direct control of Met by SIX homeoproteins, independently of Pax3. In the light of SIX1 metastatic properties (Ford et al., 1998; Yu et al., 2004), these results suggest that SIX1 could control the metastatic behavior of rhabdomyosarcoma cells through direct Met transactivation, as this proto-oncogene has been implicated in the development of several human cancers, including melanomas, breast cancer and rhabdomyosarcomas (Sharp et al., 2002).

Finally, our analysis provides strong evidence that SIX1 and SIX4 homeoproteins are also required for the activation of the Lbx1 gene in the hypaxial myogenic precursors. In E10.5 dKO embryos, Lbx1 expression is reduced but detectable in sacral somites facing the hindlimb where Pax3 is not affected by the lack of SIX homeoproteins. Lbx1 expression is completely impaired in more rostral somites and hypoglossal chord. So far, Lbx1 has been regarded as a Pax3 target because Lbx1 transcripts were not detectable in splotch mice (Dietrich et al., 1999). These conclusions were compromised by the fact that cells that would normally express Lbx1 are lost by the lack of Pax3 (Borycki et al., 1999). Furthermore Lbx1 expression is detectable in occipital somites of splotch embryos contrary to what observed in SIX1−/+SIX4−/+ embryos (Dietrich et al., 1999). These results suggest a direct control of Lbx1 by SIX proteins are further supported by bandshift assays demonstrating the capacity of SIX1, SIX4 and SIX5 homeoproteins to bind the potential MEF3 site identified in human and mouse Lbx1 promoter.

The absence of either SIX1 or SIX4 did not block Pax3 expression and cell migration into the limb bud (Laclef et al., 2003a; Ozaki et al., 2001). Therefore, the double knockout analysis clearly demonstrates the overlapping functions shared by SIX1 and SIX4 homeoproteins to activate the myogenic migration program in somites through the control of the expression of Pax3, Met and Lbx1 genes.

Absence of SIX1 and SIX4 homeoproteins impaired induction of Pax3 and Mrf5 leading to a severe trunk musculature hypoplasia

Epaxial myogenesis is more affected at the rostral level than at the interlimb and caudal levels in SIX1+/−SIX4−/+ embryos. This is observed from E10.5 and persisted throughout embryogenesis. Pax3, Mrfs and other myotomal-specific genes expression is more severely altered in rostral somites than in more caudal ones, giving rise to a more severe disorganization of back muscle masses at the shoulder level than at the interlimb and hip levels in dKO fetuses. This suggests that the regulatory myogenic pathways operating in rostral somites are distinct from those operating more caudally, and is reminiscent of the complex activation of the Mrf4/Myf5 locus in different precursor populations (Carvajal et al., 2001; Hadchouel et al., 2003). E13.5 SIX1+/−SIX4−/+ embryos develop more serious defects in the body musculature than splotch embryos, which are essentially affected in their most dorsal and ventral muscles (Tremblay et al., 1998). Thus, the digenesis of more profound thoracic and abdominal muscles in SIX1+/−SIX4−/+ embryos appears to rely on an impaired determination and differentiation of myotomal precursors. E10.5 SIX1+/−SIX4−/+ embryos, indeed, present a more severe decrease of Myf5 and Myod1 expression in the myotome than what observed in splotch embryo (Tajbakhsh et al., 1997), suggesting that SIX homeoproteins can activate both genes in subpopulations of myogenic precursors independently of Pax3. The genetic link between Myod1 expression and SIX1 during limb myogenesis (Laclef et al., 2003a) suggests a direct role for SIX1 and SIX4 in the transactivation of Myod1 in myogenic precursors, while it has been established that the activation of Myod1 by Pax3 is indirect (Relaix et al., 2003). It is also possible that impaired Myod1 expression is dependent upon the decrease of Myf5 expression and loss of Mrf4 expression (see below).

Most remaining myofibers in the dKO embryos arose from caudal dermomyotomal lips, as epaxial and hypaxial lip structure is lost. These dermomyotomal caudal lips precursors have been shown to contribute to myotome growth in its dorsoventral extent (Kahane et al., 1998), are known to express specifically Delta1, and are able to give rise to epaxial and hypaxial myocytes (Gros et al., 2004; Kahane et al., 1998).

Myogenin expression is dramatically reduced in the myotome of SIX1−/+SIX4−/+ embryos at E9.5 and E10.5. Only a few positive cells are detected in the more central-epaxial part of the myotome. The comparative analysis of myogenin expression between SIX1−/+SIX4−/+ and splotch mutants embryos (Tajbakhsh et al., 1997) tends to demonstrate the dependence of myogenin activation by SIX proteins, as expected by our previous finding (Spitz et al., 1998). The observation that somitic expression of myogenin was preserved in SIX1−/+ or in SIX4−/+ embryos, and that limbs expression of myogenin was only delayed in SIX1−/+ embryos (Laclef et al., 2003a; Ozaki et al., 2001) probably reflected again the compensation mechanism that exists between SIX1 and SIX4. The remaining myogenin and β-gal-positive cells in the center of the myotomes suggest that a specific population of myogenic cells can activate myogenin and an alternative myogenic program in the absence of SIX proteins, as already proposed (Laclef et al., 2003a).

Surprisingly, Mrf4 expression is completely lost in SIX1−/+SIX4−/+ embryo. MRF4 has been recently identified as a key determination gene controlling the activation of Myod1 in the myotome, in parallel to Myf5 (Kassar-Duchossoy et al., 2004). The lack of Mrf4 in SIX1−/+SIX4−/+ embryo may thus participate in the downregulation of myotomal Myod1 expression. Myod1 expression has been shown to be under two
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complementary genetic pathways involving Myf5 and Pax3 (Tajbakhsh et al., 1997). Whether Myod1 activation by Six proteins follows the Myf5 or Pax3 network, or both, remains to be determined.

We have demonstrated that in the absence of Six1 and Six4 myogenic factors, the formation of the myotome is first compromised by a loss of Pax3 and second by an impaired activation of the Mrf proteins in the myogenic precursors already present in the myotome. It also shows that although impaired at multiple levels – absence of ventral and dorsal lips, and decrease of Mrf protein expression – primary myogenesis can nevertheless take place, mainly owing to contribution of caudal lips in which Pax3 is still expressed independently of Six1 and Six4.

Increased apoptosis in somites of Six1−/−Six4−/− embryos

Although there is some evidence that Six1 could control cell proliferation (Yu et al., 2004), proliferation deficiencies were not detected in the myogenic lineage of Six1−/−Six4−/− embryos. However, Six1 and Six4 appeared necessary to prevent cells from apoptosis and to induce myogenic differentiation through myogenin induction and the accompanying cell cycle withdrawal (Zhang et al., 1999). Cells in the branchial arches, in the DRG and in the ventrolateral dermomyotome of the dKO embryos were found to lose their identity and die by apoptosis. Interestingly, this phenotype has been reported in Drosophila, where absence of sine oculus does not prevent cell proliferation but induces apoptosis of those cells that are unable to progress in their differentiation (Cheyette et al., 1994). Interestingly, apoptosis has been also reported in Pax3−/− embryos at the somitic level (Borycki et al., 1999). It has been suggested that overexpression or misexpression of one protein of the Pax-Six-Eya-Dach network triggered a default apoptotic program (Clark et al., 2002). Apoptosis has also been detected in Six1−/− embryos in the metameric mesenchyme (Yu et al., 2003). This suggests that different cell types adopt the same strategy facing the absence of Six homeoproteins, or that Six proteins are important actors for cell survival.

Six1 and Six4 control Fgf production in the myotomes

Several Fgf molecules are produced by the somites (Karabagli et al., 2002). We find in fact that Fgf4 and Fgf6 ventral lateral somitic expression is greatly diminished in Six1−/−Six4−/− E10.5 embryos. We can hypothesize that as Fgf molecules produced by the ventrolateral myotome, i.e. Fgf6 and Fgf4, are lacking in E10.5 Six1−/−Six4−/− embryos, scleraxis transcription is delayed. In fact, while early scleraxis activation is inhibited, in E12.5 embryos, scleraxis expression becomes detectable (D. Duprez, personal communication). Our results support the hypothesis of Fgf signaling by the myotome is required to induce scleraxis and, hence, the syndetomal compartment, in agreement with recent findings (Brent et al., 2005). As axial tendon formation can take place during mouse embryogenesis even in the absence of scleraxis (Ronen Schweitzer, personal communication), the tendons observed at the axial level in E18.5 fetuses is not in conflict with a default early induction of scleraxis in Six1−/−Six4−/− embryos.

We already reported that Six1−/− mice had severe rib and skeletal craniofacial defects. Rib defects, as discussed already (Laclef et al., 2003a), are observed in several other KO that prevent axial myogenesis. Fgf and Pdgfa signaling is required for correct rib growth and both signaling pathways are diminished in the Myf5 KO that is devoid of early myotome and Mrf4 expression (Grass et al., 1996; Kassar-Duchossoy et al., 2004; Patapoutian et al., 1995; Tallquist et al., 2000), and one can hypothesize that it is the case for the other KO in which correct hypaxial myogenesis is impaired. Absence of early ventrolateral differentiated myotome producing Fgf signaling, as observed in Six1−/−Six4−/− embryos should preclude growing of the sternal region of the ribs (Evans, 2003; Huang et al., 2003). Six1 and Eya1 have been shown already to control Fgf3 and Fgf10 signaling during kidney and otic development (Xu et al., 1999; Zheng et al., 2003), suggesting that one signaling pathway controlled by the Pax-Six-Eya network may that of the Fgf signaling affecting different types of organogenesis.

Pax-Six-Eya genetic loop

The demonstration of an epistatic relationship between Six1/Six4 genes and Pax3 gene in myogenic precursors originating from the lateral dermomyotome of the somites is consistent with the genetic link characterized during early kidney development in the mouse embryo, where Pax2 expression has been shown to be markedly reduced in the metameric mesenchyme of Six1 mutant mice (Yu et al., 2003). The genetic hierarchy placing sine oculus downstream eyeless in Drosophila (Halder et al., 1998) does not seem to be conserved during myogenic development nor during the early organogenesis of kidney, as Six1 expression is not altered in the metameric mesenchyme of Pax2 mutant mice embryo (Xu et al., 2003). Interestingly, although Six1/Six4 control Pax3 expression in the lateral dermomyotome of the occipital, cervical, thoracic and lumbar somites, Pax3 is activated independently of Six proteins in the lateral dermomyotome of sacral and caudal somites, and in the anterior and posterior lips of the dermomyotome of all somites along the anteroposterior axis. Altogether, these results are reminiscent to the recent observation that Lbx1 gene is activated by Hox proteins in the somites facing the limb buds (Alvares et al., 2003), and suggest that Hox proteins that control the axial identity of somites (Burke, 2000) may, in cooperation with Six proteins, control Pax3, Lbx1 and, more generally, hypaxial myogenesis.

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