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Altered myogenesis in *Six1*-deficient mice

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SUMMARY

Six homeoproteins are expressed in several tissues, including muscle, during vertebrate embryogenesis, suggesting that they may be involved in diverse differentiation processes. To determine the functions of the *Six1* gene during myogenesis, we constructed *Six1*-deficient mice by replacing its first exon with the *lacZ* gene. Mice lacking *Six1* die at birth because of severe rib malformations and show extensive muscle hypoplasia affecting most of the body muscles in particular certain hypaxial muscles. *Six1*$^{-/-}$ embryos have impaired primary myogenesis, characterized, at E13.5, by a severe reduction and disorganisation of primary myofibers in most body muscles. While *Myf5, MyoD* and *myogenin* are correctly expressed in the somitic compartment in early *Six1*$^{-/-}$ embryos, by E11.5 *MyoD* and *myogenin* gene activation is reduced and delayed in limb buds. However, this is not the consequence of a reduced ability of myogenic precursor cells to migrate into the limb buds or of an abnormal apoptosis of myoblasts lacking *Six1*. It appears therefore that *Six1* plays a specific role in hypaxial muscle differentiation, distinct from those of other hypaxial determinants such as *Pax3, cMet, Lbx1* or *Mox2*.

Key words: Six/sine oculis homeoproteins, Myogenesis, MyoD, Myogenin, Myf5, Pax3

INTRODUCTION

Six genes constitute a large family of genes that are highly conserved within the animal kingdom. The Six homeoproteins are characterized by a Six domain (SD) and a Six-type homeodomain (HD), both of which are needed for specific DNA binding and cooperative interactions with co-factors. In mammals, six members of the *Six* family have so far been identified which can be divided into three subclasses designated, the Six1/2, Six3/6 and Six4/5 subfamilies (Seo et al., 1999). The Six4 protein was first identified as a factor binding specifically to the ARE sequence (Kawakami et al., 1996a; Kawakami et al., 1996b). It was subsequently demonstrated that Six1, Six2, Six4 and Six5 show similar binding specificity to the ARE/MEF3 site (consensus sequence TCAGGTTC) (Ohto et al., 1999; Spitz et al., 1998).

Studies in *Drosophila* have revealed that *sine oculis* (*so*), the first *Six* family gene identified, acts within a synergistic regulatory network that includes *eyeless* (*Pax* family), *eyes absent* (*Eya* family) and *dachshund* (*Dach* family), to trigger compound eye organogenesis. Subsequent genetic analyses revealed that direct interactions of *So* and *Eya* proteins underlie the functional synergy between these proteins in inducing ectopic eye development (Pignoni et al., 1997). However, the molecular basis for this cooperativity is not fully understood, and no direct target gene of *so* and *eya* has been identified in *Drosophila*. In contrast, we have previously shown that the Mef3 site, present in the 184 bp myogenin promoter, is needed to confer a pattern of *lacZ* reporter gene expression mimicking that of the endogenous myogenin gene during mouse embryogenesis (Spitz et al., 1998). Since Six1, Six4 and Six5 proteins specifically bind the Mef3 site and are present in the embryo when myogenin is activated, we proposed that Six homeoproteins could act as key regulators of myogenin activation. Indeed, misexpression of Six1 together with Eya2 can induce myogenic genes such as *MyoD*, *myogenin* and *myosin heavy chain* in chicken somite explants (Heanue et al., 1999). Taken together, these results strongly suggest that Six homeoproteins, acting in collaboration with an Eya co-activator, might directly transactivate skeletal muscle target genes. In further agreement with this idea, the Six1, Six4 and Six5 genes have all been shown to be expressed in somites during embryogenesis (Oliver, 1995; Ozaki, 2001; Fougerousse, 2002) (our unpublished data). However, mice lacking either Six4 or Six5 develop normally and show no muscle defects, suggesting the possibility of mutual compensation among Six homeoproteins (Klesert et al., 2000; Ozaki et al., 2001; Sarkar et al., 2000).

The skeletal body muscles of vertebrates are derived from somitic progenitors originating from the epithelial dermomyotome, which in turn gives rise to the myotome. The medial myotome produces epaxial muscles, which yield the intrinsic back muscles. The lateral myotome and the lateral portion of the dermomyotome produce the hypaxial muscles, which includes thoracic intercostal and abdominal muscles, limb muscles and superficial back muscles, as well as the diaphragm and the tip of the tongue (Ordahl and Le Douarin, 1992).

Markers of myogenic specification belong to the family of basic helix-loop-helix (bHLH) transcription factors composed of Myf5, MyoD, myogenin and Myf6 (MRF4). The different roles played in vivo by the myogenic regulatory factors (MRF) have been elucidated from gene targeting experiments. While mice lacking either Myf5 or MyoD have normal skeletal muscle (Braun et al., 1992; Rudnicki et al., 1992), mice lacking both Myf5 and MyoD exhibit a complete absence of myogenic cells (Rudnicki et al., 1993), thus indicating that Myf5 and MyoD have redundant functions (Rudnicki et al., 1993). Nonetheless, it is clear that Myf5 and MyoD have different roles in the determination of epaxial and hypaxial myogenic progenitors (Kablar et al., 1997). The development of hypaxial muscles in sites distant from the somites depends on a multistep process including specification of progenitors in the lateral dermomyotome, delamination, migration through different pathways towards correct sites, proliferation of the migrating precursor cells and then differentiation. These different steps are controlled by Pax3, the c-Met tyrosine kinase receptor, its ligand SF/HGF and the homeobox factor Lbx1 (Birchmeier and Brohmann, 2000). The homeobox factor Mox2 is also essential for normal limb muscle formation, although it is not required for the migration of myogenic precursors (Mankoo et al., 1999). Furthermore, in addition to the spatial distinction of the different myogenic compartments, two sequential waves of myofiber formation can be distinguished. In the mouse, a primary wave of muscle differentiation begins on about E12.5 and a secondary wave begins at approximately E15.5 (Kelly and Zacks, 1969). Mice lacking myogenin or both MyoD and Myf6 display a severe muscle hypoplasia resulting from defects of secondary myogenesis (Hasty et al., 1993; Nabeshima et al., 1993; Rawls et al., 1998; Valdez et al., 2000; Venuti et al., 1995), suggesting the existence of different myogenic populations dependent either on different thresholds of MRF, or dependent on myogenin alone or on both MyoD and Myf6 for normal differentiation.

Here, we describe the fetal and embryonic phenotype of *Six1*-deficient mice and demonstrate that *Six1* is required for primary myogenesis of most body muscles, particularly those of hypaxial origin. The Six1 phenotype is partially reminiscent of the myogenic alterations due to the *Pax3* mutation in *Splotch* embryos (Tremblay et al., 1998). However, we show that in contrast to *Pax3*, *Six1* is not required for delamination and migration of muscle precursor cells. Instead, *Six1* appears necessary for *MyoD* and *myogenin* activation in distal territories. Thus, the Six1 homeoprotein is required later than Pax3 during hypaxial muscle differentiation and plays a role distinct from those of other hypaxial determinants such as *cMet*, *Lbx1* and *Mox2*.

MATERIALS AND METHODS

Construction of *Six1* gene targeting vector

We have isolated one *Six1* genomic λFixII DNA clone from a 129Sv genomic library. We subcloned a *Not*I-*Sac*II 5′-genomic fragment and an *Sfi*I-*Asp*718 3′-genomic fragment into pBluescript KS+ (Stratagene). A 3′ DNA region (*Sfi*I-*Eco*RI, 2.3 kb fragment) was then ligated into a *Xba*I-*Eco*RI pPNT vector, leading to p3′*Six1*PNT vector. The 3′ fragment contains the last 24 nucleotides of the first exon, the first intron as well as the second *Six1* exon. A 5′ DNA region (*Spe*I-*Sac*II 3.5 kb fragment) was ligated to a *Spe*I-*Nco*I pKST-nls-*lacZ* vector. This 5′ genomic fragment possesses 3.5 kb DNA upstream of *Six1* transcription initiation site as well as 190 bp of 5′ non coding region. The translation initiation ATG is provided by the pKST-nls-*lacZ* vector. The 5′*Six1*-nlslacZ fragment was further cloned in p3′*Six1*PNT vector, leading to the final invalidation plasmid. This plasmid was linearized with *Not*I before electroporation in ES cells. Homologous recombination with this disruption vector should lead to the deletion of the first *Six1* exon including the first 178 amino acids (aa) coding for the Six domain and the homeodomain, which together are responsible for the specific DNA binding activity of Six1 protein.

ES cell screening and chimeric mouse production

DNA linearized by *Not*I digestion (35 μg) was electroporated (250V; 500 μF) into 1.5×10^7 MPI-II embryonic stem (ES) cells. ES cells were selected with 250 μg/ml of G418 48 hours after electroporation, and with 0.5 μg/ml ganciclovyr, 72 hours after electroporation. The DNA of 279 resistant clones was analysed by Southern blot after *Nco*I digestion. A 5′ *Not*I-*Spe*I fragment and a 3′ *Eco*RI-*Asp*718 fragments were used as external probes. Three independent homologous recombinant clones were identified. For the three recombinant clones, 10-12 cells were microinjected into C57BL6 blastocysts, which were further implanted into pseudopregnant mice. Chimaeric males were obtained for the three clones and yielded germline transmission. Heterozygous progenies were generated by backcrosses to C57BL6 and 129/SvJ females, and mice were genotyped by PCR analysis. The forward primer in exon1 was 5′GGGAGAACAGAAACCAAGT3′, and the reverse primer in the *lacZ* allele was 5′TCATCGCGAGCCATGCGG3′. All homozygous embryos were genotyped by Southern blot analysis as described above.

X-gal staining of mouse embryo

Embryos were staged, taking the appearance of the vaginal plug as embryonic day (E) 0.5. Embryos were dissected in PBS, fixed in 4% paraformaldehyde (PFA) for 3 hours at 4°C, washed twice in PBS, and then stained in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining solution (1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 2 mM $MgCl_2$ in PBS) at 37°C. Genotyping of the embryos was carried out by Southern blot using DNA extracted from the yolk sac. For section analysis, stained embryos were dehydrated in increasing concentrations of ethanol, cleared in xylene and embedded in paraffin. Transverse sections (10-20 μm thickness) were dewaxed in xylene and mounted in Eukitt.

Whole-mount skeletal staining

To stain cartilage, E18.5 fetuses were skinned and eviscerated prior to fixation. Embryos were fixed in 95% ethanol for 3 days, and then placed for 24 hours in Alcian Blue solution (15 mg Alcian Blue 8GX (Sigma) in 80 ml 95% ethanol and 20 ml glacial acetic acid) at 4°C. To stain bone, embryos were rinsed twice in 95% ethanol and placed for 2 days in 95% ethanol, prior to clearing in 1% KOH for 2 hours at 4°C, and counterstaining in Alizarin Red solution (5 mg Alizarin Red (Sigma) in 100 ml of 1% KOH) for 3 hours at 4°C. Clearing of embryos was completed in the following ratios of 1% KOH to glycerol: 80:20, 60:40, 40:60, 20:80.

Histology, immunohistochemistry and embryos extracts

E18.5 fetuses were snap frozen in isopentane (–30°C) cooled in liquid nitrogen and sliced into 14 μm sagittal cryostat sections. For histological staining, sections were fixed for 15 minutes in 4% PFA, and stained with Haematoxylin and Eosin, quickly dehydrated and mounted in Eukitt. For β-galactosidase detection, sections were fixed for 5 minutes in 1% formaldehyde (1× PBS; 5 mM EGTA; 2 mM $MgCl_2$; 0.02% NP40), incubated in X-gal staining solution at 37°C overnight, and then counterstained with Eosin, quickly dehydrated and mounted in Eukitt. For fast or slow myosin heavy chain (MHC) immunodetection, sections are dried for 30 minutes at room temperature, incubated overnight with 1/2000 antibody (MY32 and NOQ7.4.2.D; Sigma) in PBS, washed twice in PBS and treated according to the Vectastain ABC Kit protocol (Vector Laboratories). Immunostained sections were mounted in aqueous Vectashield (Vector Laboratories). Counting of the respective slow and fast myofibers, determination of the cross-section areas of dorsal intercostal muscles, ventral intercostal muscles, tibialis anterior, plantaris and median gestrocnemius muscles, and determination of individual fiber areas were performed using the computer-assisted morphometric measurements logiciel Image Tool 3.0 (http://ddsdx.uthscsa.edu/dig/download.html).

Fixed embryos were incubated overnight in 20% sucrose before being frozen in isopentane and sectioned (10 μm). Dried sections are incubated for 20 minutes in 1× PBS, 0.1% Triton X-100, blocked for 1 hour in saturation solution (1× PBS, 1.6% goat serum, 2% BSA, 0.1% Triton X-100), incubated overnight with primary antibodies in a saturation solution [Myf5 (Santa Cruz) 1/800, MyoD (DAKO) 1/20, myogenin (DAKO) 1/30, Pax3 1/2000, β-gal (Rockland) 1/500]. After three washes in PBT (1× PBS, 0.1% Tween 20), slides were incubated for 1 hour with secondary antibodies [1/200 mouse-FITC (Jackson Laboratories), 1/100 rabbit-FITC (DAKO), 1/500 rabbit-Texas red (Vector Laboratories)] and washed in PBT prior to mounting in Vectashield. Apoptosis was detected with the Fluorescein In Situ Cell Death Detection Kit, according to the protocole provided by the manufacturer, Roche.

Preparation of adult muscle nuclear extracts and total embryo extracts, as well as gel-mobility shift assays (GMSA) were performed as described previously (Spitz et al., 1998).

Whole-mount in situ hybridization

Embryos were collected and treated according to the protocol described by Jowett (Jowett and Lettice, 1994), and adapted for whole-mount in situ hybridization of mouse embryos. Embryos were dissected in PBS, fixed in 4% PFA for 3 hours at 4°C, washed twice in PBS, dehydrated in sequentially increasing concentrations of methanol in PBT (25%, 50%, 75%, 2× 100%) and stored at –20°C in 100% methanol. They were subsequently rehydrated following the reverse procedure up to the PBT stage. Embryos were bleached in 6% H_2O_2 in PBT for 1 hour, washed twice in PBT, treated with proteinase K solution (1 μg proteinase K per ml of 100 mM Tris, 50 mM EDTA) for 30 minutes at room temperature (RT), washed twice in PBT, refixed in 4% PFA + 0.2% glutaraldehyde for 30 minutes. After two washes in PBT, embryos were placed for at least 2 hours in hybridization buffer (50% formamide; 5× SSC; 0.5% Chaps; 0.1% Tween 20, 20 μg/ml yeast tRNA, heparin, pH adjusted to 4.5 with citric acid), before overnight hybridization with 1 μg digoxigenin (DIG)-labeled antisense RNA probe at 70°C. Embryos were then washed twice in hybridization buffer and twice in MABT (100 mM maleic acid pH 7.5; 150 mM NaCl; 0.1% Tween 20) at hybridization temperature. Following this they were incubated for 1 hour in MABT supplemented with 2% blocking powder (MABTB) at RT, and 2 hours in MABTB containing 20% goat serum (heated to 60°C before use), and overnight at 4°C in MABTB, 20% goat serum containing 1/2000 alkaline phosphatase anti-DIG Fab fragments (Boehringer). Embryos were then washed at least 5× 1 hour in PBT, 1% BSA, prior to incubation for 30 minutes in NTMT (100 mM Tris pH 9.5, 50 mM $MgCl_2$ and 0.1% Tween 20) and staining overnight in NTMT solution containing NBT/BCIP substrates (Gibco). Stained embryos were refixed in 4% PFA overnight, and transferred into 100% glycerol.

DIG-labeled antisense RNA probes were prepared from linearized plasmids with DIG RNA Labeling mixture (Boehringer) and T3 (*MyoD*) or T7 (*myogenin*) RNA polymerase according to the instructions provided by the manufacturer.

RESULTS

Generation of *Six1*-deficient mice

Inactivation of the *Six1* gene was achieved by replacing the coding sequence of the first exon with an ATG-nls-*lacZ* gene and a PGK-*neomycin* cassette (Fig. 1A). The deleted sequence (starting in the 5′ non coding region and extending to amino acid 178) codes for the N-terminal part of the Six1 protein, including the homeo- and Six-domains, both of which are involved in specific DNA binding. Male chimerae were interbred with 129SV and C57BL6 females to establish 129SV and C57BL6 mice strains carrying the *Six1* mutation. In both genetic backgrounds (F5 generation for C57BL6) heterozygous *Six1*$^{+/-}$ mutants are viable, fertile and appear normal. However, when heterozygous *Six1*$^{+/-}$ mutants are intercrossed, *Six1*$^{-/-}$ homozygous mice die at birth and have a characteristic phenotype (Fig. 1B). Southern blot analysis with 5′ and 3′ external probes was used to establish the genotype of these newborn pups (Fig. 1C). Immunohistochemistry with specific anti-Six1 antibodies failed to detect Six1 protein in *Six1*$^{-/-}$ animals (data not shown), and gel-mobility shift assays (GMSA) failed to detect Six1/DNA binding activity in total protein extracts from *Six1*$^{-/-}$ embryos (Fig. 1D), while Six4 and Six5 proteins accumulate normally. Since identical results were obtained when we compared 129SV and C57BL6-hybrid strains of *Six1*$^{-/-}$ embryos, the following results are presented without making distinctions for strain.

Ribs and sternum malformation in *Six1*$^{-/-}$ fetuses

Six1$^{-/-}$ newborns are easily recognized at birth by their abnormal external morphology, which suggests that absence of *Six1* could lead to skeletal malformations. Therefore, we performed skeletal Alcian Blue and Alizarin Red staining on E18.5 *Six1*$^{-/-}$ fetuses and found that the ribs and the sternum are severely disturbed (Fig. 2A-D). The abnormalities include rib bifurcation (arrowhead Fig. 2E-F), fusion of cartilage segments from adjacent ribs (arrows Fig. 2A,C; asterisk Fig. 2E-F), truncated distal ribs that fail to attach to the sternum, and disorganized ossification of the sternum. Rib and sternal defects have been observed in all *Six1*$^{-/-}$ fetuses examined (5/5), with only two fetuses showing one or two ribs left attached to the sternum (2/5). Thus, the rib defects are not the same in all *Six1*$^{-/-}$ fetuses and appear asymmetric, with the right side more strongly affected. These skeletal defects however are likely to be secondary because *Six1* is not expressed in the rib cartilage or sternum (see later, and data not shown) (Oliver et al., 1995).

Severe muscle hypoplasia in *Six1*$^{-/-}$ newborns

Histological analysis of E18.5 fetuses revealed that *Six1*$^{-/-}$ mice have reduced muscle mass compared to control mice, especially in distal territories (Fig. 3). At the distal forelimb level, dorsal muscles are missing and ventral muscles are strongly reduced (Fig. 3A,B). At the distal hindlimb level, most ventral and intermediate muscles are lacking, whereas dorsal muscles are only slightly reduced (Fig. 3C,D). Several superficial back muscles such as the trapezius, the latissimus

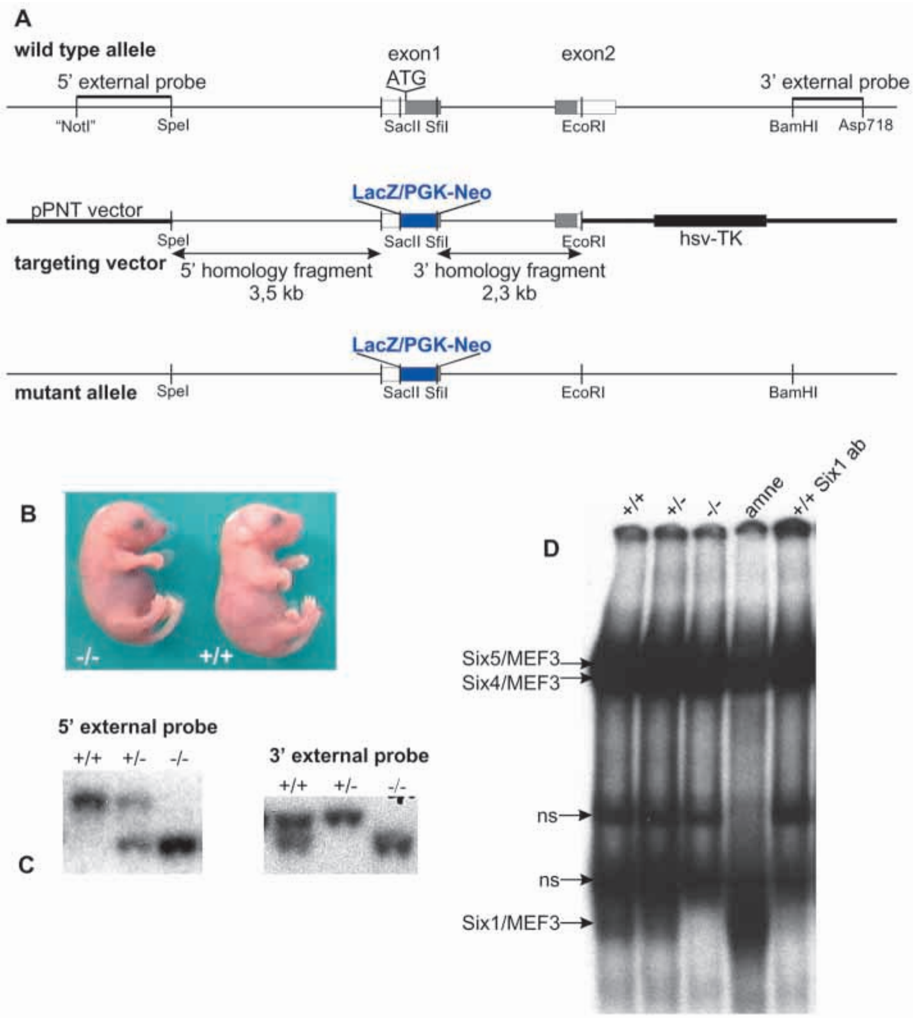

Fig. 1. Targeted disruption of the mouse *Six1* gene. (A) Schematic representation of the wild-type allele, targeting vector (pPNT) and disrupted allele. The deleted sequence (starting in the 5′ non coding region and extending to amino acid 178) codes for the N-terminal part of the Six1 protein, including the Six-domain and the Six-type homeodomain, both involved in specific DNA binding. The white and grey boxes represent the two exons of the *Six1* gene with the coding region in grey; the blue box represents the β-galactosidase reporter gene with the PGK-*neomycin* cassette downstream. The "NotI" site is a cloning site and thus is not present in the wild-type allele. (B) Phenotype of a newborn *Six1*$^{-/-}$ mouse (left) and wild-type littermate (right). (C) Southern blot analysis of genomic DNA digested with *Nco*I and hybridized with a 5′ external probe (left) and a 3′ external probe (right). (D) Gel-mobility shift assays performed with total protein extracts from E12.5 *Six1*$^{+/+}$, *Six1*$^{+/-}$ and *Six1*$^{-/-}$ embryos, and with adult muscle nuclear extracts (amne) using a *myogenin* MEF3 probe. Different DNA/protein complexes can be identified. The amount of Six4 and Six5 DNA binding activity is not diminished in *Six1*$^{-/-}$ extracts when compared to wild type, while no Six1 DNA binding activity is detected in *Six1*$^{-/-}$ extracts. Six1 ab: added Six1 antibodies are able to displace specifically the Six1/MEF3 complex. ns: non-specific protein/DNA interactions.

dorsi and the serratus dorsalis (Fig. 3J,K) are strikingly reduced or even absent. Back intercostal muscles are present and slightly reduced. In contrast, the diaphragm is devoid of skeletal muscle fibers and appears as a thin layer of connective tissue (Fig. 3N,O). At the head level, only the tongue and related muscles such as the genioglossus are markedly reduced, while head muscles such as the masseter appear correctly developed in *Six1*$^{-/-}$ fetuses (Fig. 3L,M). In contrast to muscles, the tendons and connective tissue appear to be correctly developed (Fig. 3E). Together, these results indicate

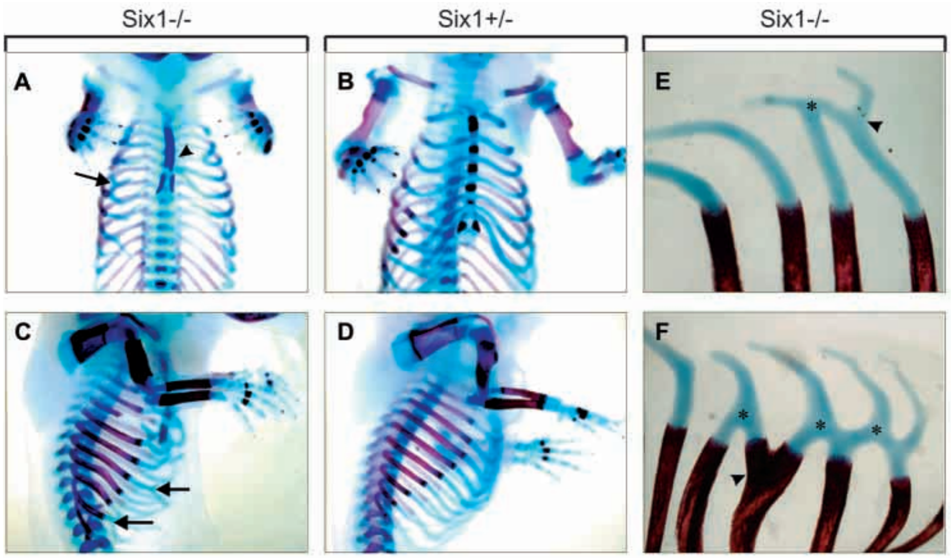

Fig. 2. Skeletal defects of *Six1*$^{-/-}$ fetuses revealed by Alizarin Red (skeleton) and Alcian Blue (cartilage) staining. (A,C,E,F) *Six1*$^{-/-}$ fetuses, (B,D) normal littermates. (A-B) Ventral view and (C-D) lateral view of the trunk skeleton showing malformations including rib bifurcation, fusion of rib cartilage from adjacent ribs (arrows), truncated distal rib segments with defects in the attachment to the sternum (here, only two ribs reach the sternum (arrowhead), and disorganized ossification of the sternum. (E-F) Magnification of adjacent rib fusion and branching in two different *Six1*$^{-/-}$ fetuses (anterior is left). (E) The cartilage segment of the fifth right rib splits up (arrowhead) before fusing with the forth right rib (asterisk). (F) The sixth to the ninth rib are fused (asterisk). The seventh rib forks at the distal part of the bone segment (arrowhead).

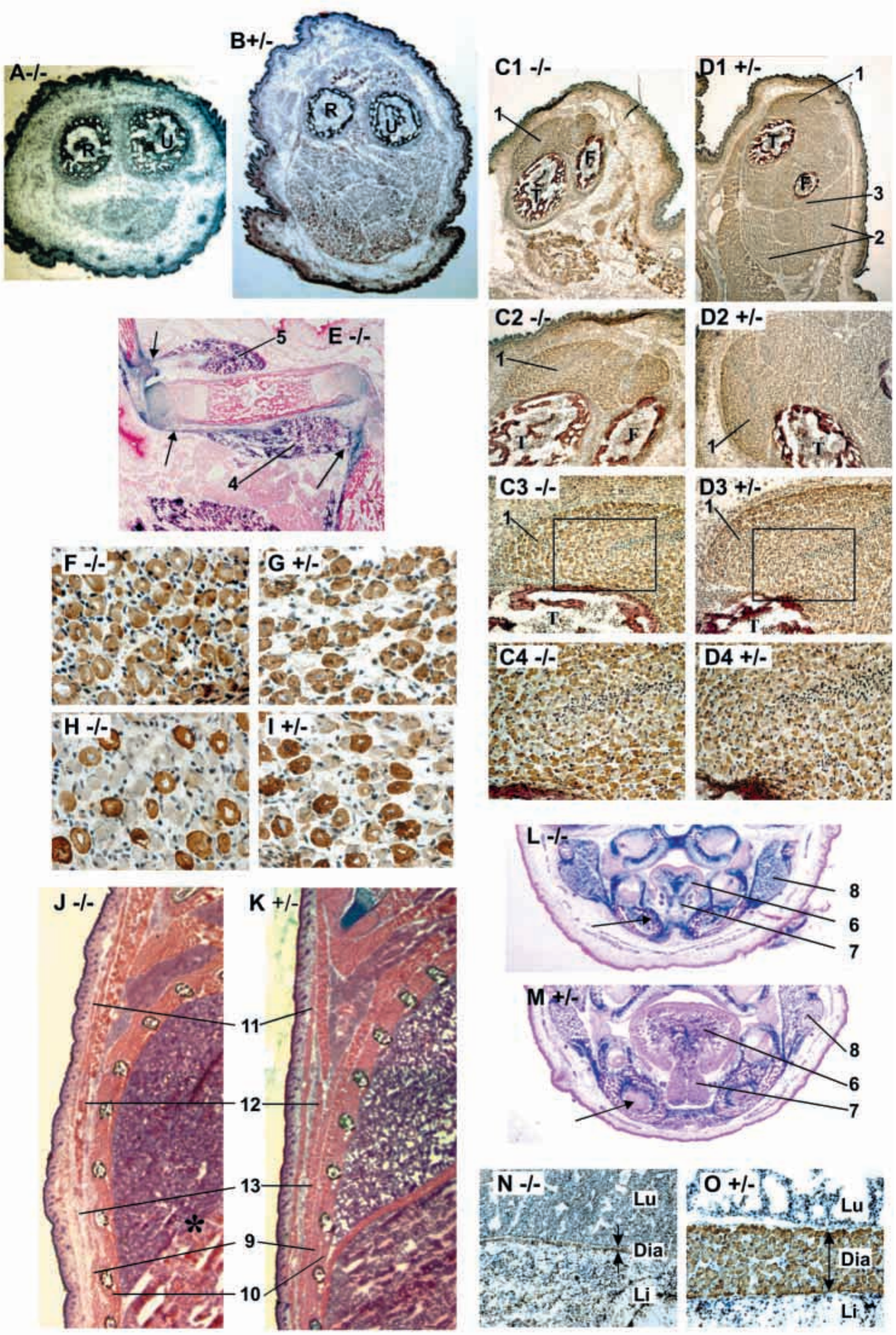

Fig. 3. Severe and selective muscular hypoplasia of *Six1*$^{-/-}$ fetuses. Histological sections of E18.5 *Six1*$^{-/-}$ (A,C,E,F,H,J,L,N) and *Six1*$^{+/-}$ (B,D,G,I,K,M,O) fetuses. (A,B) Haematoxylin-stained transverse sections at the distal forelimb level reveal a drastic reduction of the muscular masses in *Six1*$^{-/-}$, compared to normal littermates: most dorsal and ventral masses are missing in *Six1*$^{-/-}$ embryos; R, radius; U, ulna. (C,D) Transverse sections of distal hindlimb stained with Haematoxylin and fast MHC (MY32) antibody reveal an important hypoplasia and absence of most ventral and intermediate muscle masses in *Six1*$^{-/-}$ fetus (2, medial and lateral gastrocnemius; 3, soleus*)*, while most dorsal muscles are present but much smaller (1, tibialis anterior); T,- tibia; F, fibula. (C2-C4 and D2-D4) Higher magnifications of C1 and D1, respectively, at the tibialis anterior level. While the size of tibialis anterior is reduced by approximately 33% in *Six1*$^{-/-}$ fetuses, the density of the myogenic cells and their size are similar in *Six1*$^{-/-}$ and *Six1*$^{+/-}$ mice. As a result, the total number of myofibers is reduced by approximately 33%. (E) X-gal/Eosin-stained sagittal section at forelimb level of an E18.5 *Six1*$^{-/-}$ fetus: a strong hypoplasia characterizes both triceps brachii (4) and biceps brachii (5), but tendons (arrows) of these muscles seem correctly developed and attached. (F-I) Immunochemistry and Haematoxylin coloration of triceps muscle sections showing slow (F,G) and fast (H,I) MHC. Whereas this muscle is reduced in size in *Six1*$^{-/-}$ fetuses, slow and fast myofibers are present in equivalent relative proportions. (J-K) Haematoxylin/Eosin-stained sagittal sections at the thoracic level show that most superficial back muscles (11-13) are strikingly reduced and disorganized in *Six1*$^{-/-}$ fetuses, whereas intercostal muscles (9-10) are less affected. 9, intercostal interni, 10, intercostal externi, 11, spinotrapezius; 12, latissimus dorsi; 13, serratus dorsalis. (L,M) X-gal/Eosin-stained transverse sections at the head level. The tongue (6) is significantly reduced; the genioglossus muscle (7) is absent, but the masseter (8) seems correctly developed. Note also the Meckel's cartilage hypoplasia (arrows). (N,O) Fast myosin immunochemistry of diaphragm sections. In the *Six1*$^{-/-}$ embryo the diaphragm is reduced to a thin layer of connective tissue, without any detectable muscle fiber.

that absence of *Six1* leads to an extensive muscle hypoplasia affecting most of epaxial and hypaxial body muscles and in particular certain hypaxial muscle groups.

The smaller size of the muscles in *Six1*$^{-/-}$ fetuses is due to a reduced number of myofibers in *Six1*$^{-/-}$ fetuses. The decrease in the number of myofibers is very variable in different muscles. For example, the number of fibers is reduced by about 10% in dorsal intercostal muscles, 50% in ventral intercostal muscles, 33% in tibialis anterior and 100% in soleus, plantaris and medial gastrocnemius (Fig. 3C,D). Moreover, the reduction in myofiber number is not limited to a specific fiber type since the proportion of slow and fast fiber types is not altered (Fig. 3F-I). Slow myosin immunostaining can be used to distinguish primary fibers and to assess their number, even if a small percentage of the primary fibers do not express slow myosin at this stage. In all aforementioned muscles the number

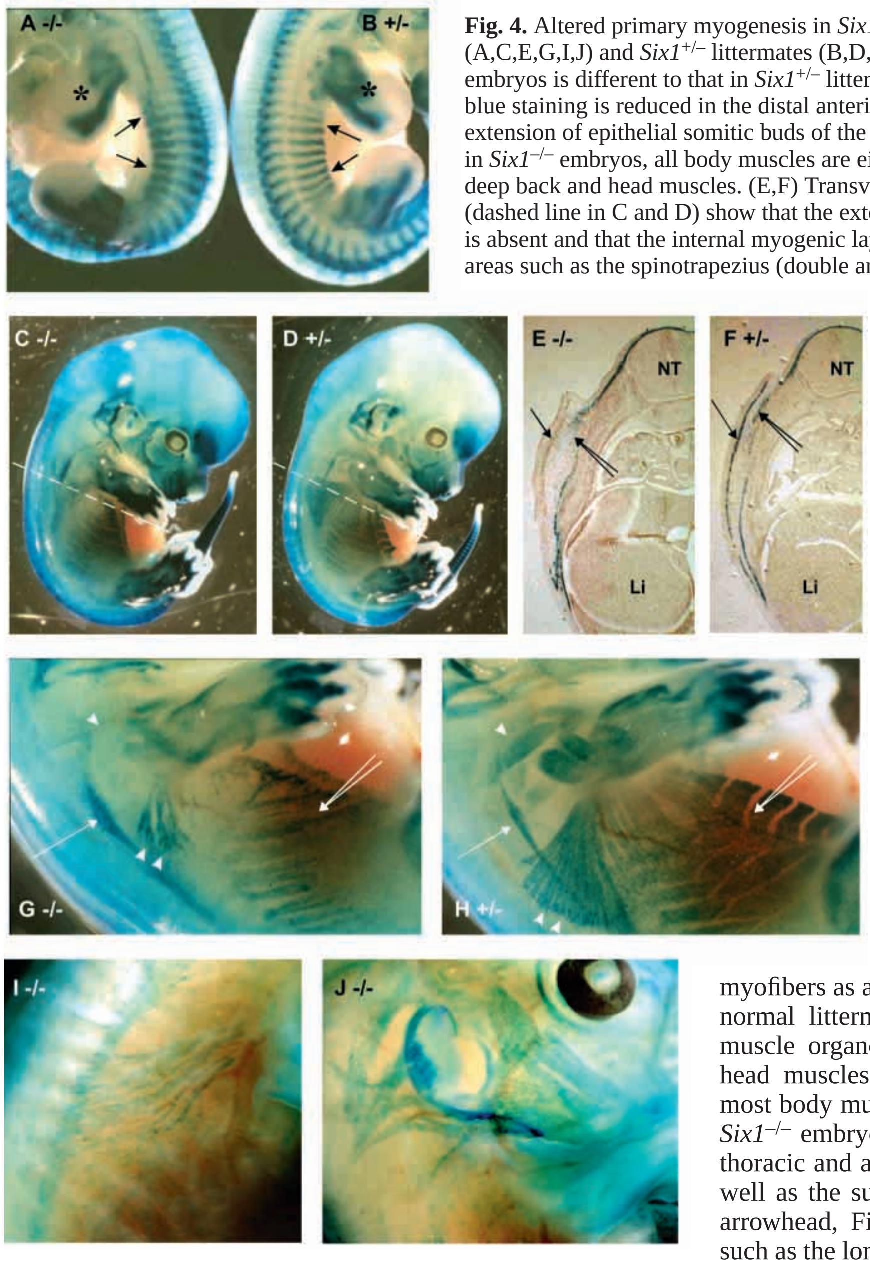

Fig. 4. Altered primary myogenesis in *Six1*$^{-/-}$ embryos. X-gal staining of *Six1*$^{-/-}$ embryos (A,C,E,G,I,J) and *Six1*$^{+/-}$ littermates (B,D,F,H). (A,B) At E12.5, the staining observed in *Six1*$^{-/-}$ embryos is different to that in *Six1*$^{+/-}$ littermates in few restricted areas: at the limb level the blue staining is reduced in the distal anterior part (*), and at the interlimb level the ventral extension of epithelial somitic buds of the dermomyotome is reduced (arrows). (C,D) At E13.5 in *Six1*$^{-/-}$ embryos, all body muscles are either absent or severely disorganized, except some deep back and head muscles. (E,F) Transverse sections of E13.5 embryos at the trunk level (dashed line in C and D) show that the external myogenic layer, the cutaneus maximus (arrow), is absent and that the internal myogenic layer is reduced and disorganized. Also specific muscle areas such as the spinotrapezius (double arrow) are missing. (G,H) Detail of E13.5 *Six1*$^{-/-}$ embryos. Note the absence of most muscles at the shoulder level (single arrowhead), the disorganization of the latissimus dorsi (double arrowhead), and the strong reduction of abdominal and thoracic muscles (double arrow), whereas the deep back muscle, longissimus dorsi, appears correctly developed (arrow). (I,J) Detail of E14.5 *Six1*$^{-/-}$ embryos at deep back muscle (I) and head (J) levels showing blue myotubes correctly shaped at head level, but reduced in number at the body level.

of slow primary fibers is reduced proportionately to the reduction in total fiber number. The number of primary myofibers directly influences the number of secondary myofibers, since primary myofibers serve as a scaffold for the subsequent formation of secondary myofibers. Therefore, these findings raise the possibility that muscle hypoplasia in *Six1*$^{-/-}$ fetuses results from defects in the formation of primary myofibers at early stages of embryogenesis.

Altered primary myogenesis of most body muscles in *Six1*$^{-/-}$ embryos

Staining for β-galactosidase of *Six1*$^{+/-}$ embryos between E8 and E13.5 revealed that this recombinant allele behaves as the endogenous one: *lacZ* expression recapitulates the spatiotemporal expression of *Six1* already published (Oliver, 1995) (C.L., E.S., J.D. and P.M., unpublished). β-galactosidase expression is missing in cells in the anterior region of the forelimb bud (asterisks Fig. 4A,B) of *Six1*$^{-/-}$ embryos and the ventral extension of dermomyotome at the interlimb level is significantly reduced (arrows Fig. 4A,B) compared with that of the heterozygotes. Primary myofiber formation begins at approximately E12.5 in the mouse by the fusion of embryonic myoblasts and is complete by E15.5. After E15.5, a second population of myoblasts begin to fuse to form secondary myofibers, using the primary myofibers as a scaffold. Major differences between *Six1*$^{-/-}$ and normal littermates appear between E12.5 and E13.5 while muscle organogenesis is progressing (Fig. 4A-H). Whereas head muscles appear correctly differentiated (Fig. 4C,D,J), most body muscles are strikingly reduced and disorganized in *Six1*$^{-/-}$ embryos, especially shoulder (arrowhead, Fig. 4G,H), thoracic and abdominal (double arrow, Fig. 4G,H) muscles as well as the superficial back muscle, latissimus dorsi (double arrowhead, Fig. 4G,H). However, some deep back muscles such as the longissimus dorsi seem correctly developed (arrow, Fig. 4G-H). Transverse sections at the interlimb level (Fig. 4E-F) show that the external myogenic layer (cutaneus maximus) is absent. In addition, the internal myogenic layer is reduced and disorganized, and specific muscle areas (spinotrapezius) are missing. Nevertheless, a few primary myofibers are present at shoulder level (Fig. 4I). These results clearly show that in the absence of *Six1*, the primary myogenesis of most body muscles is strikingly impaired. Therefore, it is reasonable to speculate that the reduced number of primary myofibers differentiated at this stage might be the cause of muscle hypoplasia in *Six1*$^{-/-}$ fetuses.

Normal specification of the myotomal cells in *Six1*$^{-/-}$ embryos

The abnormal primary myogenesis observed in *Six1*$^{-/-}$ embryos at E13.5 could be the consequence of: (1) an altered specification of the myogenic precursor cells in the somites; (2) a defect in the migration process of muscle progenitor cells

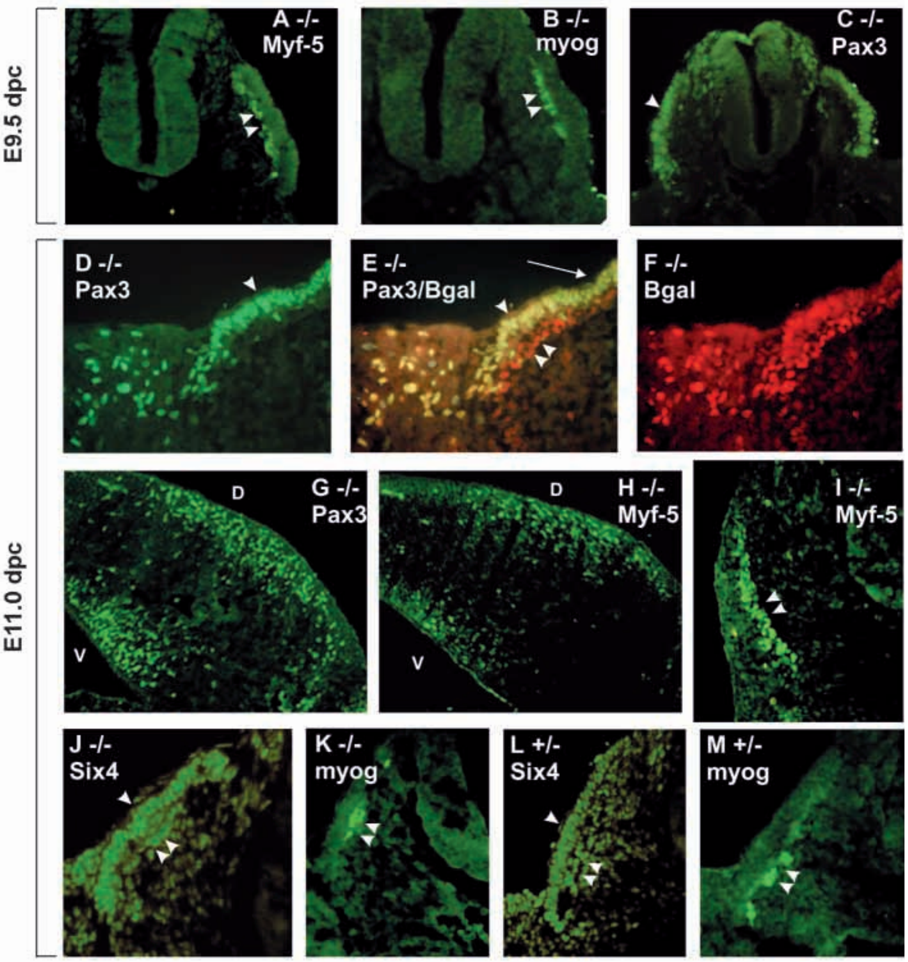

Fig. 5. *Six1* is not required for myotomal differentiation or myogenic precursor cell migration. Immunochemistry experiments performed on transverse sections of E9.5 (A-C) and E11 (D-M) embryos did not show any differences between *Six1*$^{-/-}$, *Six1*$^{+/-}$ and wild-type littermates. Presented here are only the results obtained with *Six1*$^{-/-}$ embryos (A-K), except for L and M which show the expression of Six4 and myogenin in *Six1*$^{+/-}$ embryos. D, dorsal; V, ventral. (A-C) Absence of Six1 does not impair early somitic differentiation: at E9.5 immunostaining with Myf5 (A), myogenin (B) and Pax3 (C) antibodies shows that Myf5 and myogenin accumulate correctly in the myotome (double arrowheads in A and B) and that Pax3 is normal in the dermomyotome (arrowhead in C). (D-F) Six1 does not control the expression of Pax3 in the dermomyotome and does not impair Pax3-dependent migration of hypaxial progenitor. (D) At E11, Pax3 expression is detected in the dermomyotome (arrowhead) and in migrating myogenic cells delaminating from the lateral edge of the dermomyotome at the forelimb level. (F) β-galactosidase immunostaining revealed Six1-expressing cells in the myotome, in the lateral part of the dermomyotome and in migrating cells. (E) Double staining for Pax3 and β-gal demonstrate that most of the Pax3-expressing cells in the lateral part of the dermomyotome, as well as most of the migrating precursors, also co-express Six1 (arrowhead), whereas differentiated myotomal cells express only Six1 (double arrowhead) and median dermomyotomal cells express only Pax3 (arrow). (G,H) Absence of Six1 does not impair Pax3-dependent migration into the limbs. Immunostaining with Pax3 antibodies shows that Pax3 accumulates correctly in migrating myogenic cells of both ventral and dorsal regions of the forelimb bud (G) at E11. (H) Six1 does not control the expression of Myf5 in limb buds: immunostaining with Myf5 antibodies shows that Myf5 can also be detected in dorsal and ventral myogenic regions of the forelimb bud at E11. (I-M) Six1 is not required for the activation of Myf5, Six4 and myogenin expression in the myotome. (I) Myf5 expression (double arrowheads) is not altered in *Six1*$^{-/-}$ myotome. (J,L) Six4 antibodies allow detection of Six4 accumulation in the dermomyotome (single arrowhead) and myotome (double arrowheads) in *Six1*$^{-/-}$ (J) and *Six1*$^{+/-}$ (L) embryos. Expression of Six4 in the myotome could compensate the absence of Six1 (K,M). Myogenin expression is detected with a specific antibody in both *Six1*$^{-/-}$ (K) and *Six1*$^{+/-}$ (M) embryos in the myotome (double arrowheads).

from somites towards distal territories; (3) a proliferative defect or abnormal apoptosis of migrating myogenic progenitors, or (4) an altered differentiation of the myogenic cells in distal territories.

To gain further insight into the role of Six1 during these different steps of myogenesis, we studied the expression of myogenic factors in early embryos. At E9.5, the expression of *Myf5* and *myogenin* is no different in the somites of *Six1*$^{-/-}$ embryos compared with those of normal littermates (Fig. 5A,B). At this stage, *Pax3* is also correctly expressed in the dermomyotome (Fig. 5C). Thus, absence of *Six1* does not impair early specification of myogenic cells in the somites.

Normal migration initiation of the myogenic precursor cells in *Six1*$^{-/-}$ embryos

Pax3 homeoprotein is required for the delamination and the migration of myogenic precursor cells that originate from the ventrolateral part of the dermomyotome (Bober et al., 1994; Daston et al., 1996; Goulding et al., 1994; Tremblay et al., 1998). In *Six1*$^{-/-}$ embryos, Pax3-expressing cells delaminate and migrate correctly into the limb buds at E11 (Fig. 5D,E,G). β-galactosidase immunostaining revealed cells in which *Six1* has been activated and interestingly, whereas Six1 and Pax3 are mainly expressed in complementary territories in somites (Pax3 in the dermomyotome and Six1 in the myotome), in the ventral lip and in delaminating cells Six1 and Pax3 are co-expressed (Fig. 5D-F). An appropriate number of migrating Pax3-expressing cells are also present in limb buds of *Six1*$^{-/-}$ embryos (Fig. 5G), indicating that initial steps of progenitor specification and proliferation are not affected by the absence of *Six1*. At this stage Myf5 expression begins normally in limb buds (Fig. 5H) and persists in somites of *Six1*$^{-/-}$ embryos (Fig. 5I). Together these results demonstrate that *Six1* is not required for *Pax3* and *Myf5* expression in somites or migrating cells, and that absence of *Six1* does not impair the initiation of myogenic precursor cell migration into the limb buds.

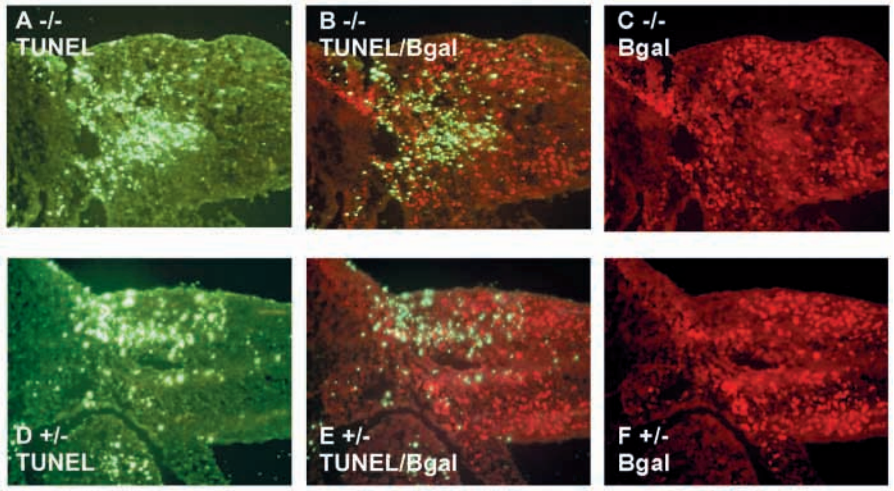

Fig. 6. Absence of *Six1* does not lead to an increase in apoptosis in Six1-expressing cells. TUNEL assays at the forelimb bud level of E11 *Six1*$^{-/-}$ and *Six1*$^{+/-}$ embryos (A,B,D,E). Six1-expressing cells are detected by an antibody against β-galactosidase (B,C,E,F). (A-C) Apoptosis in *Six1*$^{-/-}$ embryos (A) is not increased in the dorsal and ventral aspects of the limb bud where Six1-positive migrating myogenic cells are detected (C), as revealed by double staining (B). (D-F) In *Six1*$^{+/-}$ embryos, similarly no massive apoptosis is detected in ventral and dorsal regions of the limbs (D) where Six1-positive cells accumulate (F), as revealed by double staining (E).

Transcription of the *lacZ* gene inserted at the *Six1* locus is not affected in *Six1*$^{-/-}$ embryos, (Fig. 5E,F) showing that Six1 does not regulate its own transcription and that the inserted nls-*lacZ* gene and PGK-*neomycin* cassette do not impair transcription from the *Six1* locus. *Six1* is located between the *Six6* and *Six4* genes on chromosome 12 in the mouse (Gallardo et al., 1999). Six6 expression is restricted to developing retina, hypothalamic and pituitary regions (Jean et al., 1999). Six4, however, is a putative myogenic regulator despite the fact that knockout experiments did not lead to muscle defects (Ozaki et al., 2001; Spitz et al., 1998). Indeed, the Six4 expression pattern during mouse development is very similar to that of Six1 (Oliver et al., 1995; Ozaki et al., 2001), and our data indicate that Six4 expression in somites is not altered in *Six1*$^{-/-}$ embryos (Fig. 5J,L). While Six4 is mainly expressed in the dermomyotome (arrowheads Fig. 5J,L), in the myotome, Six4 appears to be colocalised with myogenin (double arrowheads Fig. 5J-M), suggesting that in these myotomal cells Six4 might compensate for the absence of Six1.

No increase of cell death within the myogenic progenitor population in *Six1*$^{-/-}$ embryos

Given that Pax3-expressing cells migrate correctly into the limb buds in *Six1*$^{-/-}$ embryos and that Pax3 is required for myoblast proliferation (Borycki et al., 1999), the *Six1*$^{-/-}$ myogenic precursor cells do not seem to be impaired in their proliferation potential at least until E11. To determine whether *Six1*$^{-/-}$ myogenic progenitors undergo apoptosis, we performed TUNEL staining on sections of E11 *Six1*$^{-/-}$ and *Six1*$^{+/-}$ embryos at the forelimb level (Fig. 6). These experiments did not provide evidence for an increase in cell death by apoptosis in *Six1*$^{-/-}$ embryos (Fig. 6A,B) compared with heterozygous littermates (Fig. 6D,E). Hence, the β-galactosidase-expressing cells that congregate into dorsal and ventral muscle masses in the limb bud are not stained strongly by the TUNEL reaction (Fig. 6B,C,E,F).

Severe alterations of *MyoD* and *myogenin* expression in limb buds of *Six1*$^{-/-}$ embryos

Whole-mount in situ hybridization experiments revealed that at E11.5, no *MyoD*- and *myogenin*-expressing cells are detected in *Six1*$^{-/-}$ limb buds (arrowheads Fig. 7A-F). Thus, while dispensable for *MyoD* and *myogenin* expression in somites, *Six1* is required for the proper activation of *MyoD* and *myogenin* genes in the limb buds. In addition, *MyoD* and *myogenin* staining revealed a strong disorganization of the ventral extension of the dermomyotome at the interlimb level (arrows Fig. 7C,D,G,H), indicating that *Six1* is necessary for the lateral expansion of the dermomyotomal cells in this region. In addition, while the expression of *Pax3*, *myf5* and *myogenin* is not altered in E9.5 embryos, the myotomal extension of *MyoD* expression is reduced in the epaxial compartment of E11.5 embryos (double arrowhead Fig. 7C,D), suggesting that *Six1* fulfils an important function in both ventral and dorsal lip expansion.

In E12.5 *Six1*$^{-/-}$ embryos, few *MyoD*-expressing cells are detected in forelimb buds (arrowhead Fig. 7I,K). At the hindlimb level, we observed a few *MyoD*-positive cells in the ventral region, while many *MyoD*-expressing cells were present in the dorsal region (arrowhead Fig. 7L). This result is in agreement with the absence of ventral muscle masses in the distal hindlimb (gastrocnemius, soleus) in E18.5 *Six1*$^{-/-}$ fetuses (Fig. 3C,D). *MyoD* in situ hybridization of E12.5 embryos also revealed a severe muscle hypoplasia at the shoulder level (arrowheads Fig. 7I,J), thus confirming the results obtained by β-galactosidase staining (Fig. 4G,H).

DISCUSSION

Disruption of the *Six1* gene leads to neonatal lethality due to the absence of diaphragm muscle and a thoracic cage deformity that could prevent correct breathing at birth. *Six1*$^{-/-}$ newborns clearly display a severe and selective muscle hypoplasia resulting from an impaired primary myogenesis of most body muscles. We conclude that the role of the *Six1* gene during myogenic differentiation, is as follows. (1) From E9.5 and thereafter, *Six1* is dispensable for the transcription of *MRFs* in somites. (2) At E11.5 dpc, *Six1* is needed for *MyoD* and *myogenin* activation in limb buds. (3) The delay of *MyoD* activation in limb buds is neither due to a delay of *Pax3*-dependent migration of hypaxial precursor cells, nor to a delay of *Myf5* activation in limb buds. (4) At E13.5 dpc, *Six1* is essential for proper primary myogenesis of most body muscles.

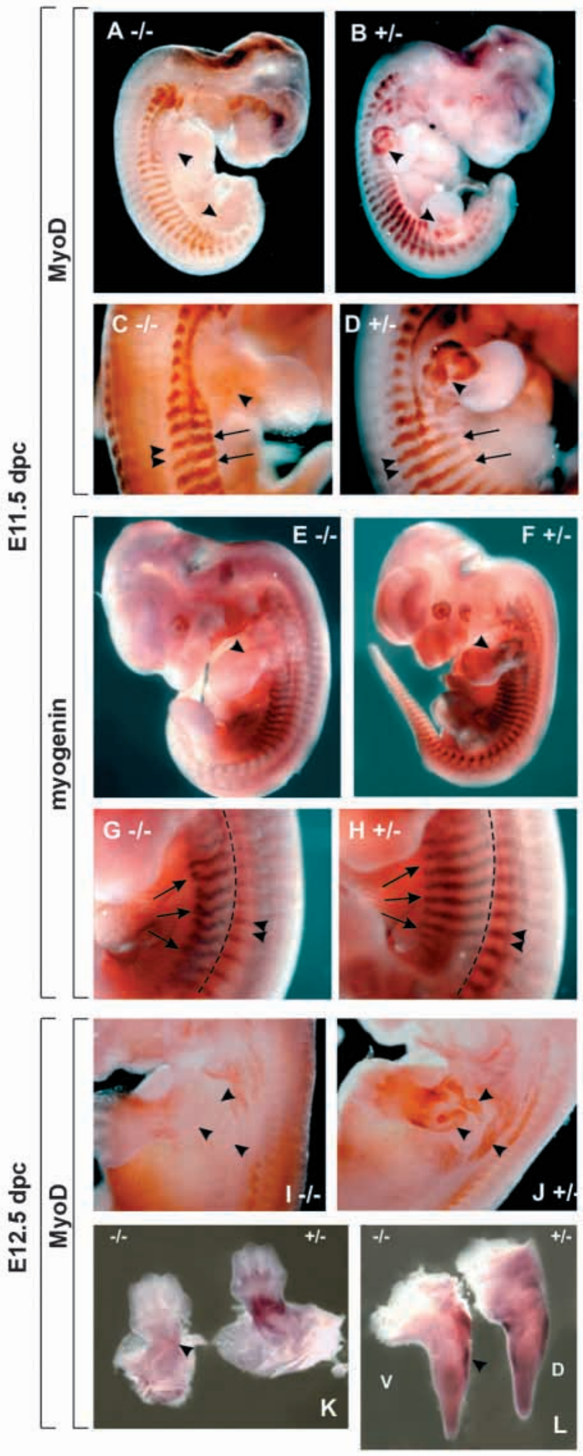

Fig. 7. *Six1* is needed for MyoD and myogenin expression in distal territories. Whole-mount in situ hybridization of *Six1*$^{-/-}$ embryos (A,C,E,G,I) and *Six1*$^{+/-}$ littermates (B,D,F,H,J) revealed that Six1-deficient mice fail to activate *MyoD* and *myogenin* genes in distal territories. (A-D) Hybridization with the *MyoD* mRNA probe shows the absence of *MyoD* expression in the limb buds (arrowheads) and the reduced ventrolateral extension of the dermomyotome (arrows), and the absence of *MyoD* expression in the epaxial most domain (double arrowheads). (E-H) Hybridization with the *myogenin* mRNA shows the absence of *myogenin* expression in the limb buds (arrowheads) and the altered organisation of the ventrolateral part of the dermomyotome (arrows). A broken line separates the epaxial and hypaxial myotome showing that *myogenin* expression is reduced in the epaxial most domain (double arrowheads). (I-J) At E12.5, hybridization with a *MyoD* mRNA probe reveals a decrease of *MyoD*-expressing cells at the shoulder level (arrowheads). (K-L) Detail of the *Six1*$^{-/-}$ (left) and *Six1*$^{+/-}$ (right) forelimbs (K) and hindlimbs (L) of E12.5 embryos showing that from this stage forelimb muscles are more affected than hindlimb muscles. (K) Dorsal view of forelimb buds shows a few *MyoD*-expressing cells in the *Six1*$^{-/-}$ forelimb (arrowhead). (L) Lateral view of hindlimb buds shows a few *MyoD*-expressing cells restricted to the dorsal region of *Six1*$^{-/-}$ hindlimb (arrowhead).

(5) *Six1*$^{-/-}$ fetuses show a reduced number of myofibers, but the remaining myofibers are properly differentiated into fast and slow types.

Six1 is not necessary for MRF activation in somite

Our results show that *Six1* is not needed for proper transcriptional activation of *Myf5*, *MyoD* and *myogenin* genes in the myotome. These results are in apparent conflict with our previous finding showing that the MEF3 binding site present in the 184 bp myogenin promoter was absolutely required for its expression in a transgenic mouse model (Spitz et al., 1998). Two different hypotheses could explain this apparent discrepancy. The first possibility that we favour involves a functional redundancy between Six1, Six4 and Six5. These proteins are co-expressed in somites, show similar binding specificities to the *myogenin* MEF3 site (Spitz et al., 1998) and are known to be able to activate the *myogenin* promoter in transient transfection assays (Ohto et al., 1999). The Six4 expression pattern is almost identical to that of Six1 (Ohto et al., 1999) and we show that Six4 is correctly expressed in somites of *Six1*$^{-/-}$ embryos at E11. Thus, Six4 might partially compensate for the absence of Six1 to activate *myogenin* in the myotomal cells. Conversely, the absence of muscle defects in mice lacking Six4 or Six5 may be due to partial genetic redundancy and compensation by Six1 (Klesert et al., 2000; Ozaki et al., 2001; Sarkar et al., 2000). Nevertheless, as *MyoD* and *myogenin* expression is delayed and reduced in limb buds of *Six1*$^{-/-}$ embryos, *Six4* and *Six5* cannot substitute for *Six1* functions in all myoblast populations. According to this hypothesis the selective muscle hypoplasia described in *Six1*$^{-/-}$ mice could result either from insufficient levels of Six4 and Six5 to compensate for Six1 in the affected myogenic precursor cells or from the existence of specific Six1 target genes.

The second possibility is that the endogenous *myogenin* gene does not behave as the 184 bp promoter fragment used in the transgenic study (Spitz et al., 1998). While this promoter fragment is efficient in recapitulating the embryonic expression of *myogenin* in a transgenic animal model, enhancer elements upstream of the 184 pb promoter have been characterized that are active during embryonic development (Cheng et al., 1993; Yee and Rigby, 1993). According to this second hypothesis, Six1 would not occupy the MEF3 site of the endogenous *myogenin* gene in *Six1*$^{-/-}$ embryos. Nevertheless, enhancers at the *myogenin* locus would override this absence and allow *myogenin* transcription in the somites. Absence of MEF3 site occupancy of the native *myogenin* locus could thus have a less severe repercussion than mutation of the MEF3 site on the 184

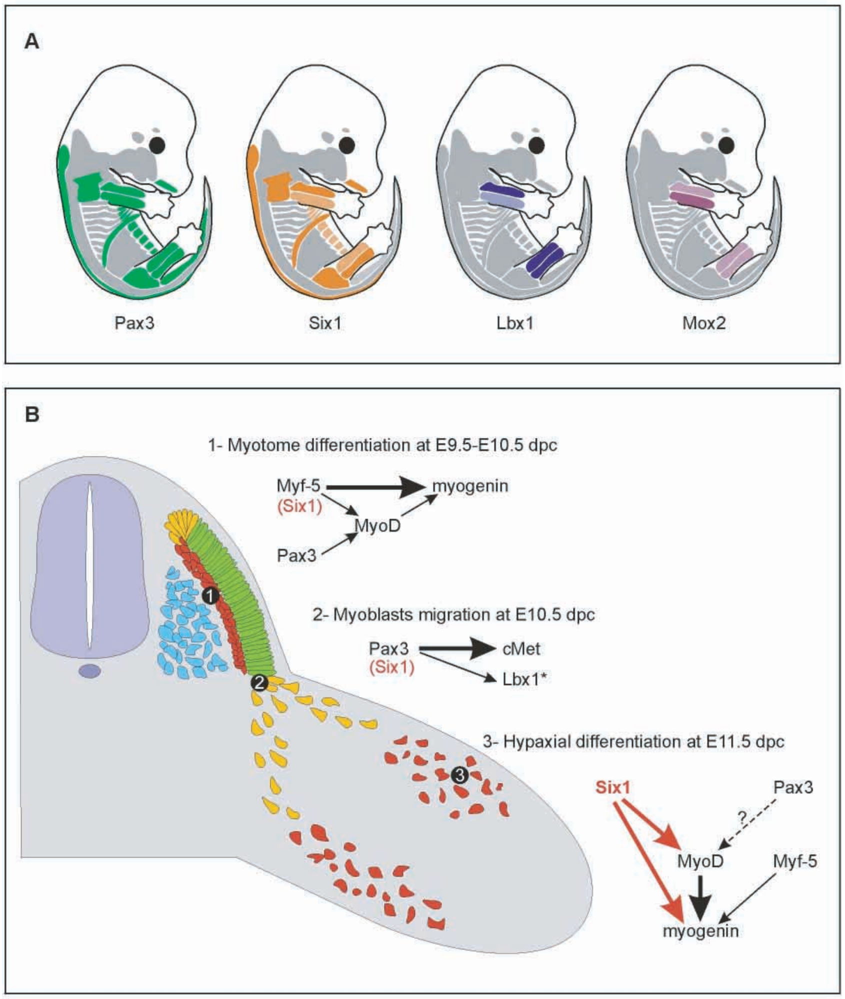

Fig. 8. (A) Schematic representation of the myogenic phenotypes of *Splotch* mutant (similar phenotype is described for *cMet*$^{-/-}$ and *Gab1*$^{-/-}$ mice), and for *Six1* and *Lbx1* knockout mice and *Mox2* (Bladt et al., 1995; Tremblay et al., 1998; Mankoo et al., 1999; Schafer and Braun, 1999; Brohmann et al., 2000; Gross et al., 2000; Sachs et al., 2000). Muscles not affected are in grey; green, orange, blue and purple muscles are missing (dark colours) or reduced (light colours) in *Splotch*, *Six1*$^{-/-}$, *Lbx1*$^{-/-}$ and *Mox2*$^{-/-}$ mice respectively. A thin layer in the most dorsal region represents superficial back muscles. At the limb level, upper muscle mass represents dorsal muscles, lower muscle mass represents ventral muscles.
(B) Schematic representation of the genetic mechanisms underlying myogenesis in the different myogenic compartments and at different times (adapted from Birchmeier and Brohmann, 2000). *Six1* is expressed but plays no role in somite differentiation (1) and in the migration of myogenic precursor cells (2) (red in parenthesis). Migration is controlled by Pax3, and Lbx1 is required for migration at occipital and limb level only (*).
(3) Instead *Six1* is required for *MyoD* and *myogenin* expression in limb buds (bold red).

bp fragment used in our previous transgenic investigations. Analysis of double *Six1/Six4* and *Six1/Six5* knockout mice will distinguish between these two hypotheses.

Six1 is needed for *MyoD* and *myogenin* activation in limb buds at E11.5

We have demonstrated that in *Six1*$^{-/-}$ mice, hypaxial progenitors are correctly specified in somites, migrate normally into the limb buds and do not undergo apoptosis. However, there is a failure of these cells to activate *MyoD* and *myogenin* at E11.5. These observations argue in favour of a direct role of *Six1* in *MyoD* and *myogenin* gene regulation, as previously suggested by misexpression experiments in chicken somite explants (Heanue et al., 1999). Accordingly, *Six1* could bind to enhancers of these genes that are specific for their transcriptional activation in limb buds and in the ventrolateral extension of the dermomyotome.

We have already demonstrated that Six homeoproteins can directly control *myogenin* expression (Spitz et al., 1998). Regulatory elements controlling expression of *MyoD* in different territories have been characterized (Goldhamer et al., 1995; Kablar et al., 1999) and consist of two regulatory regions upstream of the transcription start site: a core enhancer at –20 kb and a distal enhancer at –11 kb. The distal enhancer alone is not sufficient to drive transcription in embryonic limb buds at E11.0. However, at E12.0 this enhancer is functional, showing that the other regulatory elements present in the core enhancer are required to activate *MyoD* between E11.0 and E12.0 (Asakura et al., 1995). This is reminiscent of our observations in *Six1*$^{-/-}$ limb buds: while undetectable at E11.5, some *MyoD*-positive cells are present at E12.5, suggesting that *Six1* could control *MyoD* transcription through regulatory elements present in the distal enhancer. Careful analysis of this distal enhancer revealed the presence of a putative MEF3 site (box17), for which mutations lead to a reduced expression of *MyoD* (Kucharczuk et al., 1999). However, a delay of 1 day in *MyoD* activation in the limb buds of *Six1*$^{-/-}$ embryos is unlikely to be sufficient to impair primary myogenesis, or to provoke severe muscle hypoplasia in fetuses, since a delay of 2.5 days in myogenic differentiation in limb buds of *MyoD*$^{-/-}$ embryos does not lead to subsequent muscle alterations (Kablar et al., 1997). Therefore, it appears that *Six1* is not only involved in *MyoD* and *myogenin* gene activation in limb buds, but also acts at later steps of the myogenic differentiation process.

Six1 is crucial for primary myogenesis of body muscles

Primary myogenesis is strikingly impaired in E13.5 *Six1*$^{-/-}$ embryos. Between E12.5 and E13.5 myoblasts fuse into multinucleated fibers and individual muscles adopt their characteristic shapes and positions (Baumeister et al., 1997). The morphogenesis characterizing primary myogenesis is altered in E13.5 *Six1*$^{-/-}$ embryos, even if early steps of myogenic determination have been correctly initiated in myotomal cells, showing that Six1 plays a crucial role in these morphogenetic events. Although a number of proteins are known to regulate events required for myogenesis in the early embryo, far less is known about the molecular factors needed during primary myogenesis. A delayed onset of primary myogenesis of hypaxial and epaxial muscles has been described in *MyoD*$^{-/-}$ and *Myf5*$^{-/-}$ mutants, respectively (Kablar et al., 1997). However, such a delay does not lead to subsequent muscle hypoplasia as found in *Six1* mutants. Although severe muscle hypoplasia is found in mice lacking either *myogenin* alone or both *MyoD* and *Myf6* (Hasty et al., 1993; Nabeshima et al., 1993; Rawls et al., 1998; Venuti et al., 1995), a reduction in the number of myofibers in these mutants results mainly from an altered secondary rather than primary myogenesis. While mice lacking NFATC3 also have reduced muscles as a consequence of altered primary myogenesis (Kegley et al., 2001), these mice are viable and do not show the profound and selective muscle hypoplasia observed in *Six1* knockout mice.

Myogenic phenotype of *Six1*$^{-/-}$ embryos is partly reminiscent of *Splotch* mutants

The muscle phenotype found in *Six1* knockout mice most closely resembles the myogenic defects described in *Splotch* mutants in which the *Pax3* gene is mutated (Fig. 8A). Pax3 is required for specification and migration initiation of hypaxial progenitors (Bober et al., 1994; Daston et al., 1996; Goulding et al., 1994; Tremblay et al., 1998). In *Splotch* mutants, the migration process is impaired and consequently no myoblasts reach the most distal regions. As a result, most of the hypaxial muscles, such as limb, tongue, diaphragm and the ventral thoracic and abdominal muscles fail to form (Tremblay et al., 1998). The similarity in the phenotypes caused by Six1 and Pax3 mutations suggest the possibility of a functional link between these two homeodomain transcription factors.

However, we demonstrate that Six1 and Pax3 are not needed for the same steps of hypaxial differentiation. *Pax3* and *Six1* do not regulate each other at the transcriptional level (this study and unpublished data) (Oliver et al., 1995). In addition, hypaxial precursor cells delaminate and migrate correctly in *Six1*$^{-/-}$ embryos, therefore allowing formation of some (albeit reduced) hypaxial muscle. Finally, we show that Six1 is needed for *MyoD* and *myogenin* gene activation in limb buds, indicating that Six1 function is restricted to distal-most myogenic territories and is necessary at a step occurring later than *Pax3*-dependent migration (Fig. 8B). In contrast to *Pax3* which is downregulated before *MyoD* and *Myf5* are turned on, *Six1* expression is maintained in differentiated myogenic cells.

It has been shown recently that the homeoprotein Six1 may be localised either in the cytoplasm or in the nucleus of myogenic cells during human embryogenesis (Fougerousse, 2002), suggesting that *Six1* activity may depend on environmental signals controlling Six1 protein translocation into the nucleus. In addition, Six proteins can recruit Eya co-factors to activate the transcription of their target genes, and Eya proteins may also be localized either in the cytoplasm or in the nucleus (Buller et al., 2001; Fan et al., 2000; Heanue et al., 1999; Ohto et al., 1999). The nuclear localisation of Six1 protein has been documented in adult skeletal muscles, where it controls expression of the muscle promoter of the *aldolase A* gene (Spitz et al., 1998; Spitz et al., 1997). Thus, it will be interesting to establish a correlation between the *Six1* nucleo-cytoplasmic shuttle, the wide expression of this protein, and the phenotype of the *Six1*$^{-/-}$ mice.

Specific myogenic features of *Six1*$^{-/-}$ mice compared with *cMet*-, *Gab1*-, *Lbx1*- and *Mox2*-deficient mice

Whether *Pax3* and *Six1* can cooperate to activate genes required for hypaxial lineage determination remains to be clarified. Interestingly, as in *Six1* knockout animals, mice lacking *c-Met*, *Gab1*, *Lbx1* or *Mox2* have, with certain important differences, impaired differentiation of the hypaxial lineage (Fig. 7).

The c-Met-tyrosine kinase receptor, whose expression is directly regulated by *Pax3* (Epstein et al., 1996), plays an essential role in the migration initiation of myogenic precursor cells (Bladt et al., 1995; Maina et al., 1996). Its specific ligand SF/HGF (scatter factor/hepatocyte growth factor) is expressed in limb mesenchyme and provides the signal for migration (Dietrich et al., 1999; Scaal et al., 1999) that is mediated by c-Met and subsequently relayed by intracellular signalling pathway requiring *Gab1* (Sachs et al., 2000). The myogenic phenotype of mice deficient for the *c-Met* gene is similar to the myogenic alteration described in *Splotch* mutants (Bladt et al., 1995). *Gab1*$^{-/-}$ embryos also display impaired migration of myogenic precursor cells into the limb anlagen, leading to lack of the diaphragm and extensor muscles of the forelimb (Sachs et al., 2000).

Pax3 is also necessary for *Lbx1* expression in myogenic precursor cells of the limb (Mennerich et al., 1998). *Lbx1* expression is restricted to the lateral part of the somites located at occipital, cervical and limb levels, where myogenic precursor cells delaminate and subsequently migrate over large distances along characteristic paths (Dietrich et al., 1998; Uchiyama et al., 2000). In *Lbx1*$^{-/-}$ embryos, precursor cells delaminate but fail to migrate laterally into the limb buds to form the dorsal muscle masses (Gross et al., 2000). At birth, inactivation of *Lbx1* leads to the lack of dorsal extensor muscles in forelimbs and to the absence of muscles in hindlimbs (Brohmann et al., 2000; Gross et al., 2000; Schafer and Braun, 1999). These muscular alterations differ from those of *Six1*$^{-/-}$ fetuses, in which forelimb muscles are more affected than hindlimb muscles. Moreover, in distal hindlimbs of *Six1*$^{-/-}$ fetuses the dorsal extensor muscles are reduced whereas most ventral flexor muscles are lacking. These results suggest that *Six1* and *Lbx1* genes have distinct functions during hypaxial muscle development, which could involve actions in complementary myogenic limb compartments.

Mox2 is another crucial gene controlling limb muscle development (Mankoo et al., 1999). In the distal forelimb of *Mox2*-deficient mice, several muscles of the flexor compartment are absent and the extensor muscles are severely reduced. In the hindlimb, although no specific muscle is absent, the overall muscle mass, in particular that of the gastrocnemius, is greatly reduced. These limb muscle alterations are similar to the phenotype of *Six1*$^{-/-}$ fetuses. However, whereas *Six1*$^{-/-}$ mice have no diaphragm, a very reduced tongue and disorganized body-wall muscles, *Mox2*$^{-/-}$ mice do not display such muscle defects (Mankoo et al., 1999).

Rib defects might be a consequence of the myogenic alterations

Rib and sternum defects are also important features of *Six1*$^{-/-}$ mutants. This skeletal phenotype is reminiscent of the rib defects initially reported in homozygous *Myf5* mutant mice

(Braun et al., 1992; Tajbakhsh et al., 1996). It has been shown more recently that these rib defects could result from the residual presence of the PGKneo cassette at the *Myf5* locus (Kaul et al., 2000). As a number of potential inductive signals expressed in myotome such as FGFs and PDGFα are absent in *Myf5* mutant mice (Grass et al., 1996; Tallquist et al., 2000), it has been proposed that the rib phenotype could result from secondary events resulting from myotome defects. This hypothesis was further supported by the generation of three different alleles of the *Myf6* gene, which is located 8 kb upstream of the *Myf5* gene on mouse chromosome 10 (Braun and Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995). Nevertheless, the generation of two other *Myf5* alleles, which do not produce any malformations of the ribs seems to rule out a direct involvement of the Myf5 and/or Myf6 proteins in the generation of the rib phenotype (Kaul et al., 2000). This does not necessarily mean, however, that cross-talk between different somitic layers is not required for rib formation, since the knockout of the *myogenin* gene and the mutation of the *Pax3* gene that reside on different chromosomes also result in a rib phenotype (Dickman et al., 1999; Hasty et al., 1993; Henderson et al., 1999; Nabeshima et al., 1993; Vivian et al., 2000).

In *Six1*$^{-/-}$ mice, only the sternal region of the ribs is affected. The distal rib primordium arises from the lateral portion of the somite (Olivera-Martinez et al., 2000), but its precise origin is still controversial. While some data demonstrate that both the proximal and the distal parts of the ribs originate from the sclerotomal mesenchyme (Huang et al., 2000), other results suggest that the sternal segment of the ribs originate from the ventrolateral part of the dermomyotome (Kato and Aoyama, 1998). The ventrolateral sclerotome marker, Mfh-1 (FoxC2), is closely associated with Pax3 in the somitic bud that invades the lateral plate mesoderm at the thoracic level, suggesting that interactions might occur between the incipient ribs and intercostal muscles during their migration and differentiation (Brent and Tabin, 2002; Sudo et al., 2001). In *Six1*$^{-/-}$ mice, the rib defects restricted to the distal segments are correlated with the muscle defects that are more severely affected in the ventral region than in the dorsal anlagen, suggesting that these skeletal defects are secondary to adjacent muscle defects.

Finally, it seems that in vertebrates the genetic markers of the hypaxial compartment are more diverse than initially suspected, and that different myogenic programmes can be activated, thereby leading to muscular diversity. Six1 appears as a new genetic marker whose function is unique for the building of specific body and limb muscles. Presently, no human pathology has been associated with *SIX1* mutations, but deletions in 14q (q22q23) overlapping the *SIX1* locus lead to multiple abnormalities including muscle hypotonia (Bennett et al., 1991; Gallardo et al., 1999; Lemyre et al., 1998), which may be due to *SIX1* haploinsufficiency.

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