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RESEARCH ARTICLE

Endothelial cell specification in the somite is compromised in Pax3-positive progenitors of *Foxc1/2* conditional mutants, with loss of forelimb myogenesis

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ABSTRACT

Pax3 and *Foxc2* have been shown genetically to mutually repress each other in the mouse somite. Perturbation of this balance in multipotent cells of the dermomyotome influences cell fate; upregulation of Foxc2 favours a vascular fate, whereas higher levels of Pax3 lead to myogenesis. Foxc1 has overlapping functions with Foxc2. In *Foxc1/2* double-mutant embryos, somitogenesis is severely affected, precluding analysis of somite derivatives. We have adopted a conditional approach whereby mutations in *Foxc1* and *Foxc2* genes were targeted to *Pax3*-expressing cells. Inclusion of a conditional reporter allele in the crosses made it possible to follow cells that had expressed *Pax3*. At the forelimb level, endothelial and myogenic cells migrate from adjacent somites into the limb bud. This population of endothelial cells is compromised in the double mutant, whereas excessive production of myogenic cells is observed in the trunk. However, strikingly, myogenic progenitors fail to enter the limbs, leading to the absence of skeletal muscle. Pax3-positive migratory myogenic progenitors, marked by expression of *Lbx1*, are specified in the somite at forelimb level, but endothelial progenitors are absent. The myogenic progenitors do not die, but differentiate prematurely adjacent to the somite. We conclude that the small proportion of somite-derived endothelial cells in the limb is required for the migration of myogenic limb progenitors.

KEY WORDS: Mouse embryo, Forelimb bud, Myogenic cells, Somite-derived endothelial cells, Pax3, Foxc1, Foxc2

INTRODUCTION

Foxc1 and Foxc2 transcription factors are important for tissue and organ development at different sites in the mouse embryo (Kume, 2009, 2010). They are expressed in paraxial mesoderm (Kume et al., 1998, 2000) and are required for somite maturation. In *Foxc1* or *Foxc2* single mutants, the ventral compartment of the somite, the sclerotome, is affected and its derivatives, the cartilage and bones of the ribs and vertebral column, are compromised. In the double mutant, somites fail to develop (Kume et al., 2001). The transcription factor Pax3, which plays an important role in myogenesis, is co-expressed with Foxc1/2 in paraxial mesoderm; however, it is subsequently confined to the dorsal compartment of the somite, the dermomyotome, whereas Foxc1/2 continue to be expressed at a high level in the sclerotome, as well as at a lower level in the dermomyotome where *Foxc2* transcripts are notably detectable in the hypaxial domain (Lagha et al., 2009). The dermomyotome is the source of all skeletal muscle in the trunk and limbs and also gives rise to other mesodermal derivatives, including vascular endothelial and smooth muscle cells (Buckingham and Mayeuf, 2012). Genetic experiments have shown reciprocal inhibition between *Pax3* and *Foxc2* with consequences for cell fate choices in the multipotent cells of the dermomyotome when this equilibrium is perturbed (Lagha et al., 2009). Thus, higher expression of *Foxc2*, relative to *Pax3*, promotes vascular derivatives whereas upregulation of *Pax3* promotes skeletal muscle at the expense of a vascular cell fate. The onset of skeletal myogenesis in the trunk results from delamination of Pax3-positive progenitors from the dermomyotome to form the underlying myotome (Buckingham and Mayeuf, 2012). At the limb level in the mouse embryo, bipotent progenitors (Kardon et al., 2002) marked by both Pax3 and Flk1 (Kdr – Mouse Genome Informatics; also known as VegfR2) (Mayeuf-Louchart et al., 2014) can give rise to endothelial (Pecam-1-positive) cells that migrate from the somites into the limb bud to form a subset of superficial blood vessels (Hutcheson et al., 2009), or to migrating myogenic progenitors that retain expression of Pax3 and contribute all the skeletal muscles of the limb (Buckingham and Mayeuf, 2012). Signalling pathways can potentially affect the balance between *Pax3* and *Foxc2*, as shown for Notch, which promotes *Foxc2* expression and the endothelial cell fate of dermomyotome progenitors that migrate into the forelimb (Mayeuf-Louchart et al., 2014).

Foxc1 has overlapping functions with *Foxc2* (Kume et al., 1998, 2001) and because it is also expressed in the somite, we have investigated the phenotype of *Foxc1/2* double mutants with respect to endothelial versus myogenic cells of the forelimb. Disruption of somitogenesis was avoided by targeting conditional mutations of both Foxc genes to *Pax3*-expressing cells. Whereas overproduction of myogenic cells was observed in the trunk, myogenic cells were absent from the limb resulting in no limb muscle formation. This striking result is discussed in the context of the loss of somite-derived endothelial cells observed in the double mutant.

RESULTS

Validation of double conditional *Foxc1/2* mutants

$Pax3^{Cre/+}$ mice (Engleka et al., 2005) were crossed with conditional $Foxc1^{flox/flox}$;$Foxc2^{flox/flox}$ mice (Sasman et al., 2012). In control and conditional mutant embryos, a $Rosa26^{flox\text{-}nLacZ}$ or $Rosa26^{tomato\text{-}floxGFP}$ reporter allele was also introduced into the

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crosses so that cells that express or had expressed *Pax3* could be followed. We observed a delay in recombination with the $Pax3^{Cre}$ allele, probably reflecting a delay in Cre recombinase accumulation, as indicated by GFP labelling of $Pax3^{Cre/+};Rosa26^{tomato\text{-}floxGFP/+}$ embryos (Fig. 1A) at embryonic day (E) 9.25, when recombination has occurred at forelimb level but is not detected in more posterior somites. By E10.5, recombination extends more posteriorly. To check the efficiency of recombination, the somites and forelimb region of E9.25 embryos were dissected to remove the neural tube and Pax3-positive neural crest cells (Fig. 1B). GFP-positive cells were isolated by flow cytometry. RT-qPCR analysis demonstrates that *Foxc1* transcripts are strongly reduced in $Pax3^{Cre/+};Foxc1^{flox/flox};Foxc2^{flox/+}$ ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/+}$) and $Pax3^{Cre/+};Foxc1^{flox/flox};Foxc2^{flox/flox}$ ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) embryos as well as transcripts of *Foxc2* in $Pax3^{Cre/+};Foxc1^{flox/+};Foxc2^{flox/flox}$ ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/\Delta}$) and $Pax3^{Cre/+};Foxc1^{flox/flox};Foxc2^{flox/flox}$ ($Pax3^{Cre/+}C1^{\Delta/\Delta}\,C2^{\Delta/\Delta}$) embryos (Fig. 1C).

Pax3 expression is not restricted to somites, but is also a feature of neural crest cells derived from the dorsal neural tube. Developmental defects have been described in *Foxc1* and/or *Foxc2* null mutants in derivatives of neural crest cells where these genes are also expressed (Kume et al., 1998; Seo and Kume, 2006). Similar defects are observed in the conditional mutants in which the cranial skeleton is affected (Fig. S1A). At the trunk level, formation of the axial skeleton is also affected (Fig. S1A), in keeping with the role of *Foxc1/2* in the development of derivatives of the sclerotome (Kume et al., 2001). However, the axial skeleton and ribs begin to form and somitogenesis takes place in the double conditional mutants (Fig. 2), in contrast to the *Foxc1/2* null mutant. At later stages, we observed truncation of the tail and loss of posterior somites (Fig. S1B), reflecting extensive *Pax3*-mediated recombination by E11.5.

Myogenic phenotypes of conditional *Foxc1/2* mutants

As predicted from the analysis of single *Foxc2* mutants (Lagha et al., 2009), excessive production of cells expressing the myogenic determination gene *Myod1* is observed in the trunk region of double conditional ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) mutants compared with control heterozygote ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) embryos at E11.5 (Fig. 2A). Quantification of MyoD-positive cells in interlimb somites indicates an increase of myogenic cell number in double conditional ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) mutants compared with control heterozygote ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) embryos at E10.5 (Fig. 2B). The epithelial structure of the dermomyotome is maintained, as shown on sections of interlimb somites (Fig. 2B). This is also the case at the forelimb level at E9.5 (Fig. 3A).

However, there is a striking absence of myogenic cells expressing *Myod1* in the forelimb of the double mutant (Fig. 2A; Fig. 4A). These cells are somewhat reduced in the absence of Foxc1 and are more severely reduced in the absence of Foxc2 (Fig. 4A). In the double conditional mutant, forelimb muscles are absent at E19.5 as shown by myosin staining on whole embryos and by Haematoxylin and Eosin staining on forelimb sections (Fig. 4B). Absence of skeletal muscle is due to a failure of migration of *Pax3*-expressing

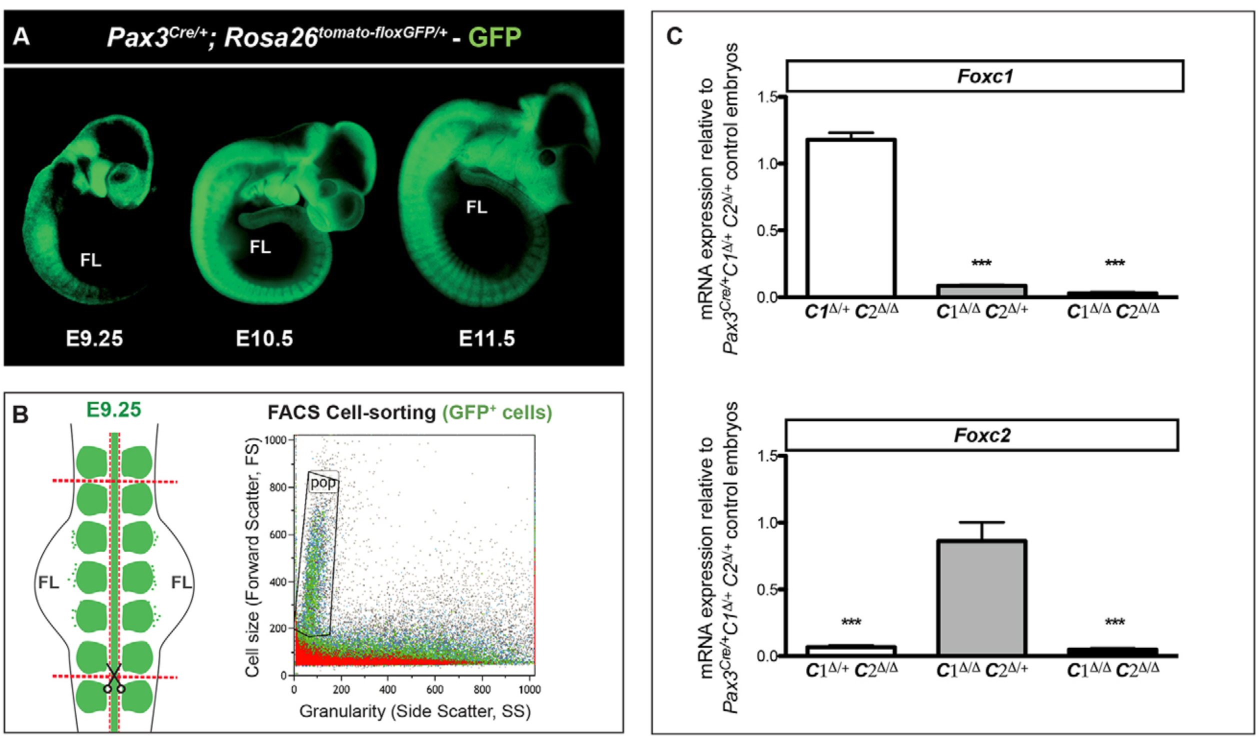

Fig. 1. Conditional mutation of *Foxc1* and *Foxc2* in Pax3-positive cells and their derivatives. (A) GFP fluorescence labelling of Pax3-positive derivatives in $Pax3^{Cre/+}; Rosa26^{tomato\text{-}floxGFP/+}$ embryos at E9.25 (left panel), E10.5 (central panel) and E11.5 (right panel). FL, forelimb. (B) Schematic representation of tissues extracted from $Pax3^{Cre/+};Rosa26^{tomato\text{-}floxGFP/+}$ embryos, at E9.25, in different *Foxc1/2* genetic backgrounds. The five pairs of somites at the level of the forelimbs and the two forelimbs were retained for FACS cell sorting, whereas the neural tube with Pax3-positive neural crest cells was removed. Fluorescent GFP cells (pop) were sorted by flow cytometry (FACS), as shown in the right-hand panel, with cell size and granularity as additional criteria. (C) RT-qPCR analysis of *Foxc1* and *Foxc2* transcripts on the cells isolated as shown in B, in conditional mutants for *Foxc1* ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/+}$), *Foxc2* ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/\Delta}$) and both genes ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$). *Gapdh* was used as the reference gene and the control ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) was taken as 1 (***$P<0.001$, $n=3,2,3$, from left to right). In these experiments, compensatory upregulation of *Foxc1* or *Foxc2* was not observed, as also reported for aortic arteries (Winnier et al., 1999). Error bars represent s.e.m.

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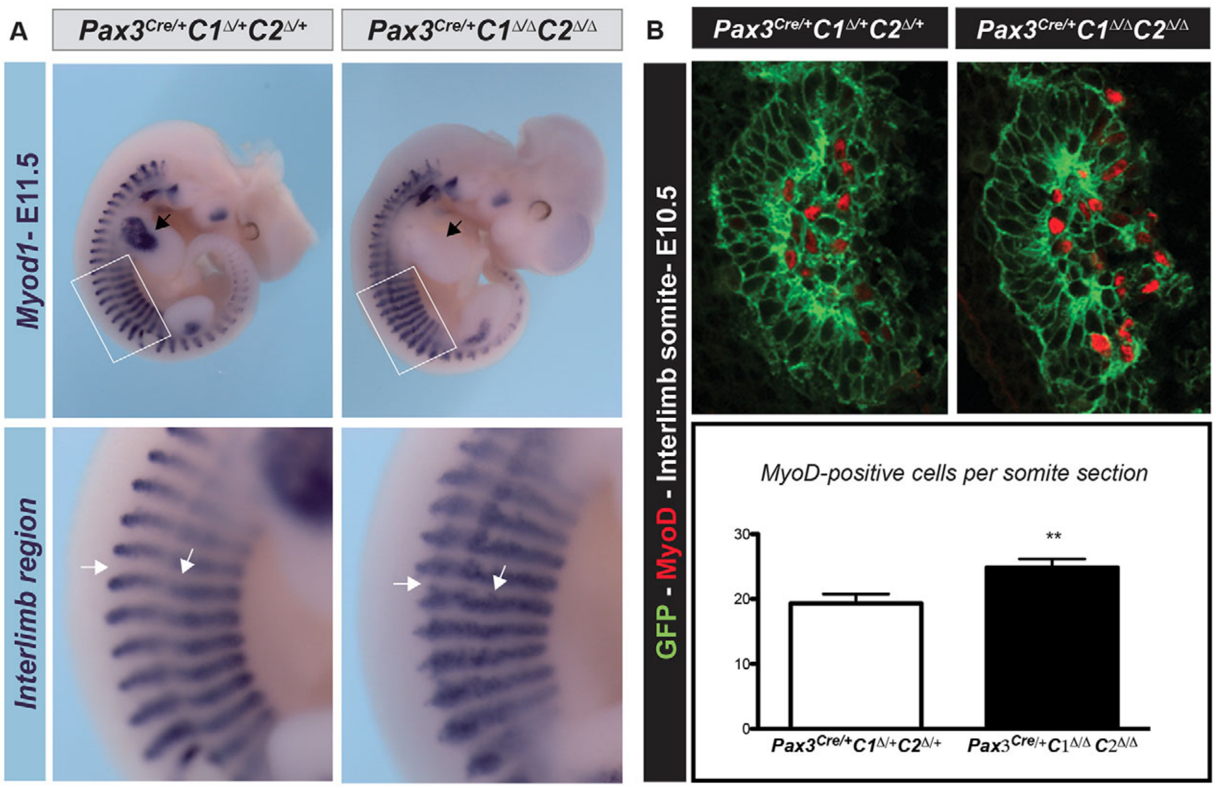

Fig. 2. Increased numbers of myogenic cells in the somites of *Foxc1/2* double conditional mutant embryos. (A) Whole-mount *in situ* hybridisation for transcripts of *Myod1* in heterozygote control $Pax3^{Cre/+}$;$Foxc1^{flox/+}$;$Foxc2^{flox/+}$ ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) and double conditional mutant $Pax3^{Cre/+}$;$Foxc1^{flox/flox}$;$Foxc2^{flox/flox}$ ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) embryos at E11.5. Whole embryos are shown in upper panels with lower panels showing higher magnification images of the interlimb region (indicated by boxes in upper panels) where excessive *Myod1*-expressing myogenic cells are present with dispersion around the somites in double conditional mutants but not in control embryos (white arrows). Black arrows in the upper panels indicate *Myod1*-expressing cells, which are absent in double conditional mutant embryos. (B) Immunostaining on sections (longitudinal view), at the interlimb level (upper panels) of $Pax3^{Cre/+}$;$Rosa26^{tomato\text{-}floxGFP/+}$ embryos on heterozygote control ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) and double conditional mutant ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) backgrounds at E10.5, with antibodies to GFP (green) and MyoD (red). The lower panel represents the quantification of MyoD-positive cells per somite section (**P<0.01, n>27, sections from three embryos for each genotype). Error bars represent s.e.m.

myogenic progenitor cells into the limb bud (Fig. 3B; Fig. 4C). In the genetic-tracing experiment shown in Fig. 4C, endothelial cells derived from *Pax3*-expressing progenitors in the somite would also normally be labelled in the limb bud (Hutcheson et al., 2009; Mayeuf-Louchart et al., 2014). However such labelling is not evident in the absence of Foxc1/2 and foci of residual β-galactosidase-positive cells correspond to neural crest cells that will contribute to the sympathetic nervous system, derived from dorsal root ganglia, marked by AP2α (Tfap2a – Mouse Genome Informatics), which are not affected in the *Foxc1/2* conditional mutants (Fig. S2).

Endothelial cells that derive from the somite

In the forelimb of the mouse embryo, only a small proportion of endothelial cells are derived from the somite (Hutcheson et al., 2009; Mayeuf-Louchart et al., 2014). In $Pax3^{Cre/+}$ embryos in the presence of a $Rosa26^{tomato\text{-}floxGFP}$ allele, such Pecam-1-positive endothelial cells can be distinguished (Fig. 5A). These cells are not detectable on the double conditional *Foxc1/2* mutant background and are reduced in the single mutants, notably in the absence of Foxc2. Quantification of GFP-positive cells that are positive for Pecam-1, after separation by flow cytometry, confirms the reduction in this cell population in the forelimb of *Foxc1/2* conditional mutants at E10.5 (Fig. 5B).

Before cells migrate from the somite to the forelimb, they co-express *Pax3* and *Flk1*, which encodes vascular growth factor receptor 2 (Mayeuf-Louchart et al., 2014). Subsequently, maintenance of *Pax3* expression and upregulation of the gene for the transcription factor Lbx1 characterises myogenic progenitor cells, whereas endothelial progenitors continue to express *Flk1* and rapidly activate markers of the endothelial phenotype such as Pecam1. It is therefore possible to distinguish these two progenitor types in the somite. GFP-positive cells isolated from somites at the forelimb level of $Pax3^{Cre/+}$;$Rosa26^{tomato\text{-}floxGFP/+}$ embryos at E9.25 were separated by flow cytometry and the ratio of *Lbx1* to *Flk1* transcripts quantified by RT-qPCR (Fig. 5C). On the double conditional *Foxc1/2* mutant background this ratio is significantly higher. This demonstrates that migratory myogenic progenitors are specified and that they accumulate at the expense of endothelial progenitors in the Pax3-positive population of cells that give rise to both cell types in the somite.

What happens to myogenic progenitors in the *Foxc1/2* conditional mutant?

Delamination and migration of Pax3-positive muscle progenitors into the limb bud depends on the Met tyrosine kinase receptor (Bladt et al., 1995). In the double conditional *Foxc1/2* mutant, the *Met* gene (also known as *c-Met*) is expressed normally in the hypaxial domain of somites (Fig. 6A). Transcripts are not detected in the forelimbs at E10.5, as expected from the absence of Pax3-positive myogenic progenitors. Lbx1, which activates the gene for the cytokine receptor Cxcr4 required for the migration of a subset of myogenic progenitors, is also expressed in these Pax3-positive

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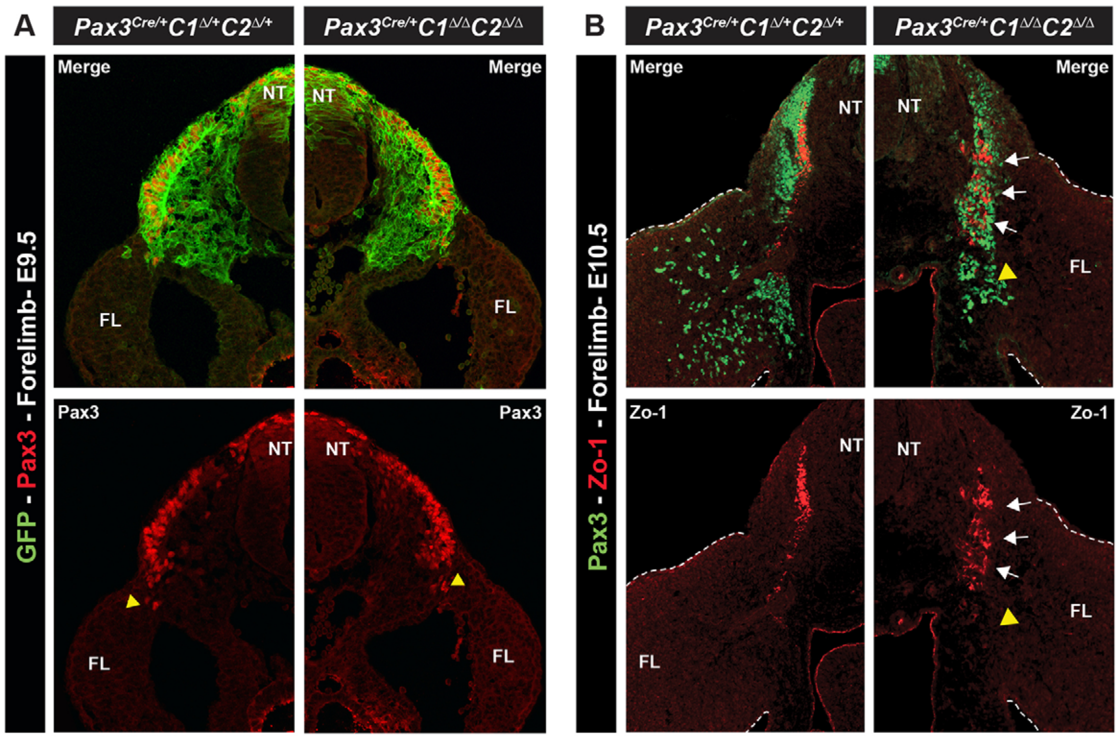

Fig. 3. Somite structure and Pax3-positive cells at the forelimb level in the double conditional *Foxc1/2* mutant. (A) Immunostaining on sections with antibodies to Pax3 (red) and GFP (green) of $Pax3^{Cre/+};Rosa26^{tomato\text{-}floxGFP/+}$ embryos on heterozygote control ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) and double conditional mutant ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) backgrounds, at the forelimb level at E9.5. At this stage, the structure of the somite of the double conditional mutant is similar to that of the control embryo and first migrating myogenic progenitors are detected (arrowheads). (B) Immunostaining on sections at the forelimb (FL) level of heterozygote control and double mutant embryos at E10.5, with antibodies to Pax3 (green) and Zo-1 (red), which marks the epithelial structure of the somite. The arrows point to the extension and structural disorganisation of dermomyotomal cells of the hypaxial somite adjacent to the FL in the mutant. The arrowheads point to myogenic progenitors that have lost the expression of the epithelial marker Zo-1. NT, neural tube.

cells in the somites at forelimb level. It is still present in *Foxc1/2* mutants at E9.25 (Fig. 5C) and at E10.5 (Fig. 6B), but not in the limbs where migration has failed to occur. By E10.5, Pax3- and Lbx1-positive cells have left the somite and accumulated in the adjacent region proximal to the forelimbs (Fig. 6B). Labelling with the epithelial marker Zo-1 (Tjp1 – Mouse Genome Informatics) reveals Pax3-positive cells that are Zo-1 positive in this region, where the hypaxial somite has broken down, but cells have failed to migrate into the limb. It also shows many cells that are Pax3 positive and Zo-1 negative, indicating that they had left the dermomyotome epithelium (Fig. 3B). At E9.5, when Pax3-positive cells delaminate and begin migrating into the forelimb, somites at the limb level in the double conditional mutant have a normal morphology (Fig. 3A) and we see no indication of cell death at this or later stages (data not shown).

At E10.5, a few myogenic progenitor cells in the forelimb begin to express the myogenic determination factor Myf5, as well as myogenic cells in the myotome of the somites where muscle is forming in control embryos (Fig. 6C). In the *Foxc1/2* conditional mutant, excess Myf5-positive cells are dispersed around the myotome, as seen for MyoD-positive cells (Fig. 2B); however, additional labelled cells are observed outside this structure, in the region immediately adjacent to the proximal forelimb (Fig. 6C). By E11.5 in the mutant, myogenic cells in this position express the myogenic differentiation factor myogenin and are labelled with a myosin heavy chain antibody that marks muscle fibres (Fig. 6D). Differentiated cells are not normally observed in this position. We therefore conclude that myogenic progenitors, which do not migrate into the limb, differentiate prematurely.

DISCUSSION

In conclusion, we show that deletion of *Foxc1* and *Foxc2* specifically in Pax3-positive cells affects cell fate choices in the dermomyotome of somites at forelimb level, promoting the myogenic cell fate at the expense of endothelial cells that migrate to the limb. However, despite this increase in myogenic cell fate specification, no myogenic cells are found in the forelimb. Loss of endothelial cell specification compromises myogenic cell migration. Instead of migrating into the forelimb, these cells undergo premature myogenic differentiation. We conclude that the small percentage of endothelial cells specified in the somite plays a crucial role in ensuring correct migration of myogenic cells into the limb.

The increase of myogenic cells in the absence of Foxc1 and Foxc2 is also observed in interlimb somites where MyoD-positive cells extend beyond the normal limits of the myotome. This observation is similar to the phenotype observed in the presence of the gain-of-function $Pax3^{Pax3\text{-}FKHR}$ allele (Relaix et al., 2003) and is consistent with an upregulation of the Pax3-dependent myogenic pathway in the absence of Foxc1/2. In somites of double conditional mutants at forelimb level, analysis of the expression of Lbx1, which is an early marker of myogenic progenitors, shows that the proportion of Lbx1-positive cells is increased, compared with Flk1-positive cells (which include the endothelial population) (Mayeuf-Louchart et al., 2014). This therefore demonstrates promotion of the myogenic cell fate at the expense of the endothelial fate, which reflects the loss of Foxc1/2 in Pax3-expressing cells. At these early stages, mutant somites have a similar morphology to controls, so that delamination and migration of

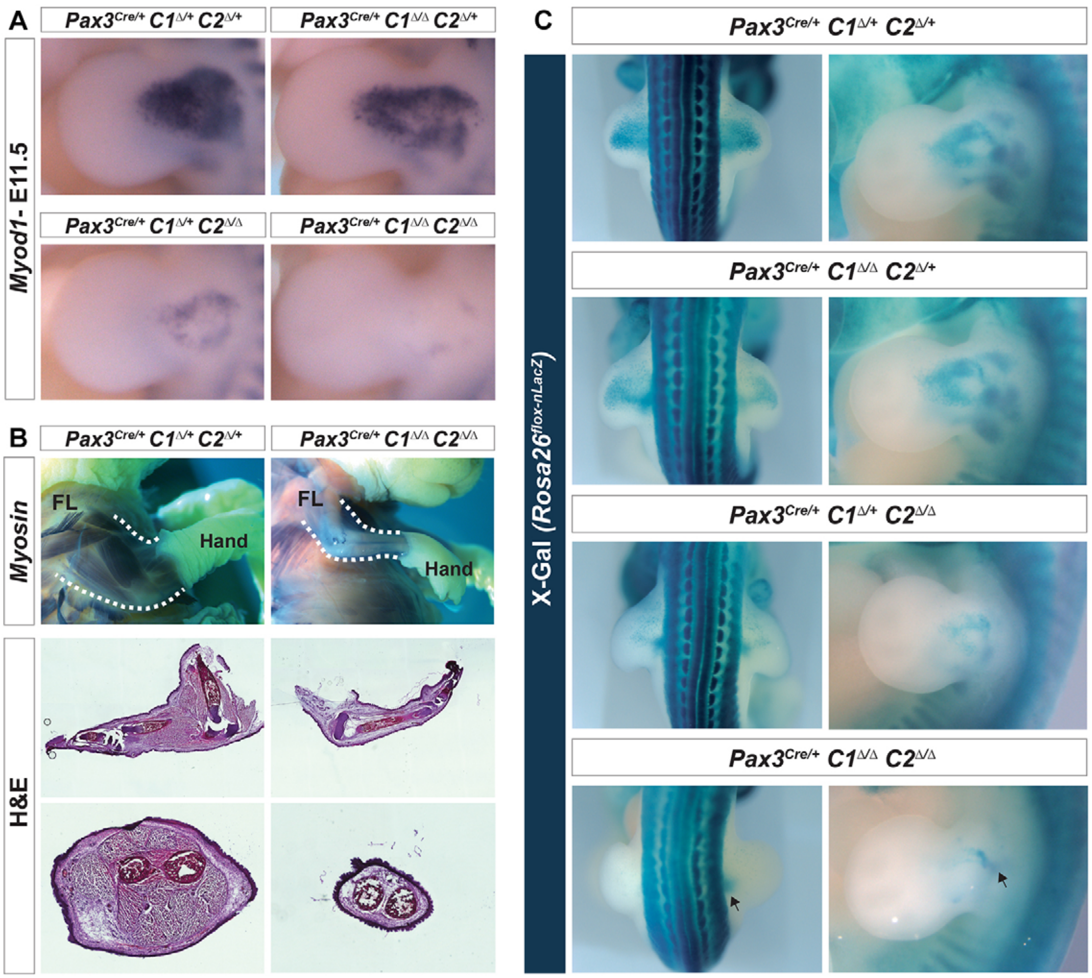

Fig. 4. Failure of cell migration from the somite to the forelimb in *Foxc1/2* mutant embryos. (A) Whole-mount *in situ* hybridisation for transcripts of *Myod1*, showing a lateral view of the forelimb of conditional $Pax3^{Cre/+}$;$Foxc1^{flox/+}$;$Foxc2^{flox/+}$ ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) heterozygote control, mutant *Foxc1* ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/+}$), mutant *Foxc2* ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/\Delta}$) and double mutant *Foxc1* and *Foxc2* ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) embryos at E11.5. (B) Whole-mount immunostaining with an antibody to myosin heavy chain of forelimbs (upper panels) and Haematoxylin and Eosin (H&E) staining on longitudinal forelimb sections (middle panels) and transversal sections (lower panels) of heterozygote control ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) and double conditional mutant ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) embryos, at E19.5, showing loss of skeletal muscles in the mutant forelimb (FL). (C) Genetic-tracing experiments with X-Gal staining of $Pax3^{Cre/+}$;$Rosa26^{flox-nLacZ/+}$ embryos on different *Foxc1/2* genetic backgrounds (as in A). Left panels represent dorsal views of forelimbs at E10.5 and right panels lateral views of forelimbs at E11.5. X-Gal staining from the $Rosa26^{flox-nLacZ}$ allele is reduced in $Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/\Delta}$ embryos and is reduced to a greater extent in double conditional mutants ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$). Black arrows indicate a neural crest-derived nerve (Fig. S2) in the double conditional mutant, which is normally hidden by myogenic cells.

myogenic progenitors is not prevented by somite disorganisation. The delay in Cre recombinase activity from the $Pax3^{Cre}$ allele avoids early loss of Foxc1/2, which affects somitogenesis, as seen in posterior somites of the double conditional mutant. The double mutant phenotype that we observed at forelimb level is more pronounced than that of the single *Foxc2* conditional mutant indicating the role of Foxc1, and indeed the *Foxc1* conditional mutant also shows a reduction of limb myogenesis although this is less pronounced than in the absence of Foxc2. We had previously observed a decrease in Pax3-positive cells in the forelimbs of *Foxc2* mutant embryos (Lagha et al., 2009), similar to that reported here for the conditional *Foxc2* mutant. We had proposed that it might be due to upregulation of *Pax7*, which perturbs myogenic progenitor cell proliferation in the limb (Relaix et al., 2004). However, the absence of all Pax3-positive cells in the forelimbs of the double conditional *Foxc1/2* mutants precludes this explanation.

The failure of myogenic progenitors to migrate into the forelimb does not appear to reflect an obvious deficit in these cells and indeed they express Pax3, which ensures myogenic, migratory and survival functions (Buckingham and Rigby, 2014). Rather, it is the absence of endothelial cell migration from the somite that correlates with this phenotype. In the *Foxc1/2* double mutant, we conclude that bipotent progenitor cells in the somite assume a myogenic, at the expense of an endothelial, cell fate, although remaining endothelial cells might also have a migratory defect (Hayashi and Kume, 2008; Hayashi et al., 2008) that prevents them entering the limb. In the chick embryo, it had been shown that endothelial and myogenic cells derived from the somite migrate independently and distribute to different locations (Huang et al., 2003). This is also indicated by genetic-tracing experiments in the mouse (Hutcheson et al., 2009; Mayeuf-Louchart et al., 2014). Furthermore, endothelial cells migrate from the mouse somite to the limb bud before myogenic progenitors (Tozer et al., 2007; Yvernogeau et al., 2012). Grafting of genetically marked mouse somites into the chick embryo at limb level demonstrated this clearly and showed that endothelial cell migration is independent of Pax3 whereas it depends on Flk1.

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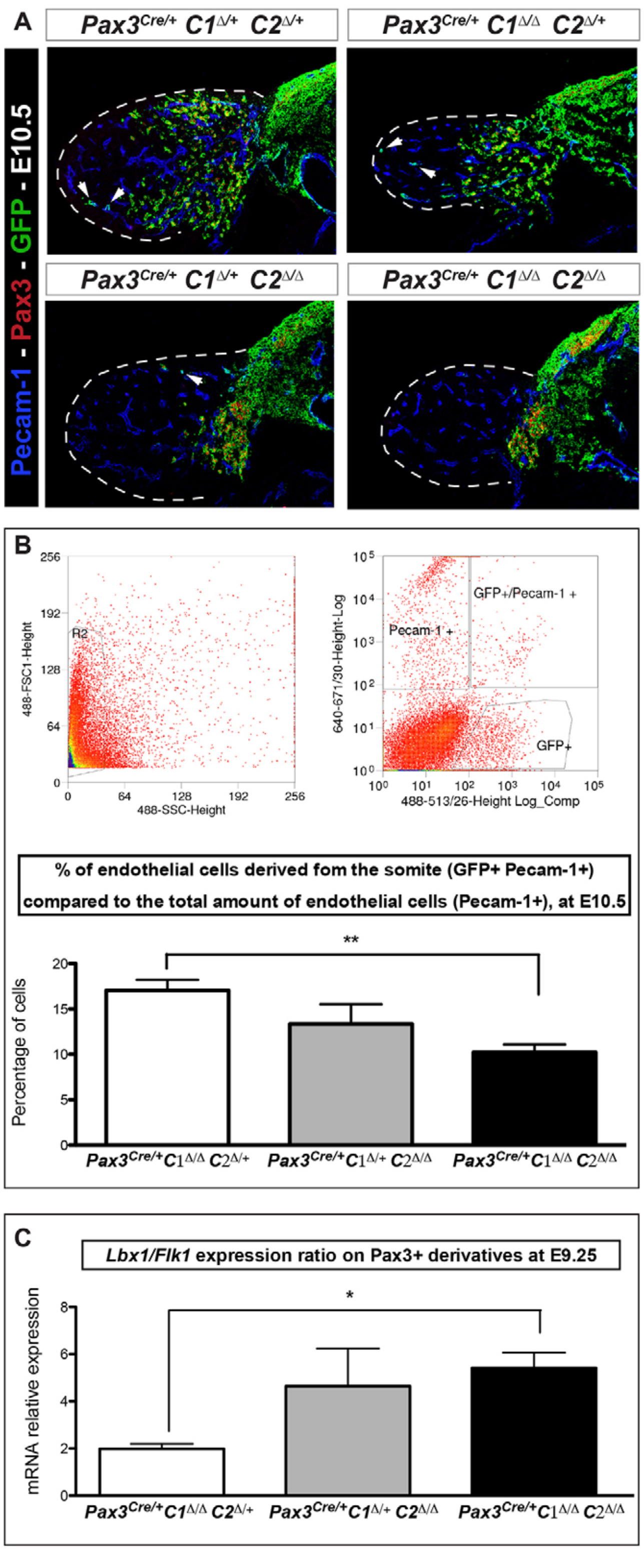

Fig. 5. Reduced somite-derived endothelial cells in the double conditional *Foxc1/Foxc2* mutant embryos. (A) Immunostaining on sections with antibodies to Pax3 (red), GFP (green) and the endothelial cell marker Pecam-1 (blue), at the forelimb level of *Pax3*$^{Cre/+}$;*Rosa26*$^{tomato\text{-}floxGFP/+}$ embryos on different *Foxc1/2* conditional mutant backgrounds at E10.5. In double conditional mutant embryos (*Pax3*$^{Cre/+}$*C1*$^{\Delta/\Delta}$*C2*$^{\Delta/\Delta}$), the total amount of Pecam-1-positive (red) cells in the forelimb is comparable to the heterozygote control (*Pax3*$^{Cre/+}$*C1*$^{\Delta/+}$*C2*$^{\Delta/+}$) whereas endothelial cells derived from the somite (GFP- and Pecam-1-positive, white arrowheads) are decreased. Dashed lines delineate the outline of the forelimb. (B) Quantification of the different populations of endothelial cells in the forelimbs at E10.5. Forelimbs were dissected and cell sorted by flow cytometry from *Pax3*$^{Cre/+}$;*Rosa26*$^{tomato\text{-}floxGFP/+}$ embryos, after immunostaining with Pecam-1 antibody on living cells. Three populations of cells were separated: non-endothelial cells that had expressed Pax3 (GFP$^+$), endothelial cells derived from Pax3-positive cells (GFP$^+$ Pecam-1$^+$) and other endothelial cells in the forelimb (Pecam-1$^+$) (upper panel). Quantification of the proportion of endothelial cells derived from the Pax3-positive cells of the somite compared with the total endothelial cell population in different *Foxc1/2* genetic backgrounds is represented in the lower panel. (**$P<0.01$, n=2,3,4, from left to right). (C) RT-qPCR analysis on GFP-positive cells isolated by flow cytometry from forelimbs and somites at forelimb level of *Pax3*$^{Cre/+}$;*Rosa26*$^{tomato\text{-}floxGFP/+}$ embryos at E9.25, on different *Foxc1/2* genetic backgrounds. The ratio of *Lbx1/Flk1* transcripts is increased in mutants for *Foxc2* (*Pax3*$^{Cre/+}$*C1*$^{\Delta/+}$*C2*$^{\Delta/\Delta}$) and double *Foxc1/2* mutants (*Pax3*$^{Cre/+}$*C1*$^{\Delta/\Delta}$*C2*$^{\Delta/\Delta}$) compared with mutants for *Foxc1* (*Pax3*$^{Cre/+}$*C1*$^{\Delta/\Delta}$*C2*$^{\Delta/+}$), indicating specification of migratory myogenic cells in greater numbers compared with endothelial cells. (*$P<0.05$, n=2,3,3, from left to right). Error bars represent s.e.m.

Grafting of somites from *Flk1* mutant mouse embryo resulted in the loss of endothelial cell migration and also of migrating myogenic cells, leading the authors to propose that prior migration of somite-derived endothelial cells is essential for myogenic cell migration into the limb (Yvernogeau et al., 2012). Interpretation of this experiment is complicated by the initial expression of Flk1 in Pax3-positive progenitors that can give rise to both myogenic and endothelial derivatives (Mayeuf-Louchart et al., 2014), with the possibility that this expression plays an early role in determining myogenic cell behaviour. The analysis of *Foxc1/2* conditional mutants reported here provides another line of evidence supporting the hypothesis that somite-derived endothelial cells, which in the mouse represent only a small proportion of all the endothelial cells in the limb, are crucial for myogenic cell migration. In their absence, vascularisation of the limb takes place but no skeletal muscle forms. It remains to be seen how this effect is mediated; presumably, it occurs via signalling to myogenic progenitors by endothelial cells, necessary for their migration. Foxc1 in mesenchymal cells in the developing cerebellum has been shown to directly activate the gene that encodes Sdf1α (Cxcl12 – Mouse Genome Informatics) (Zarbalis et al., 2012), the ligand of Cxcr4, with major consequences for radial glial cell organisation and neuronal migration via Cxcr4 signalling in the absence of Foxc1 (Haldipur et al., 2014). Cxcr4 is expressed on myogenic progenitor cells that migrate to the limb and, in its absence, migration of a subset of progenitors is compromised and some limb muscles are missing (Vasyutina et al., 2005). A minor loss of limb muscle is seen in the *Foxc1* mutant but an effect on Cxcr4 signalling cannot explain the loss of all migrating muscle progenitors in the double mutant. Furthermore, it is not clear whether somite-derived endothelial cells express Sdf1. As the trajectories appear to be different (Yvernogeau et al., 2012), it seems less likely that myogenic cells follow a trail laid by their endothelial sisters. It is more probable that the specification of endothelial cells that is Foxc dependent has an indirect effect on the environment of myogenic progenitors within the somite, which modifies their subsequent migratory behaviour.

MATERIALS AND METHODS

Mouse strains

Conditional mutants for *Foxc1* (*Foxc1*$^{flox/flox}$) and *Foxc2* (*Foxc2*$^{flox/flox}$) (Sasman et al., 2012) were crossed with *Pax3*$^{Cre/+}$ mice (Engleka et al., 2005) to generate *Foxc1* (*C1*$^{\Delta/\Delta}$; *C2*$^{\Delta/+}$), *Foxc2* (*C1*$^{\Delta/+}$; *C2*$^{\Delta/\Delta}$) and double (*C1*$^{\Delta\Delta}$; *C2*$^{\Delta/\Delta}$) conditional mutant embryos. *Rosa26*$^{flox\text{-}nLacZ/+}$ (gift from J. F. Nicolas, Institut Pasteur, Paris, France) or *Rosa26*$^{tomato\text{-}floxGFP/+}$ (Muzumdar et al., 2007) mice were crossed onto this genetic background to introduce a Cre reporter allele. Embryos were dated taking E0.5 as the day

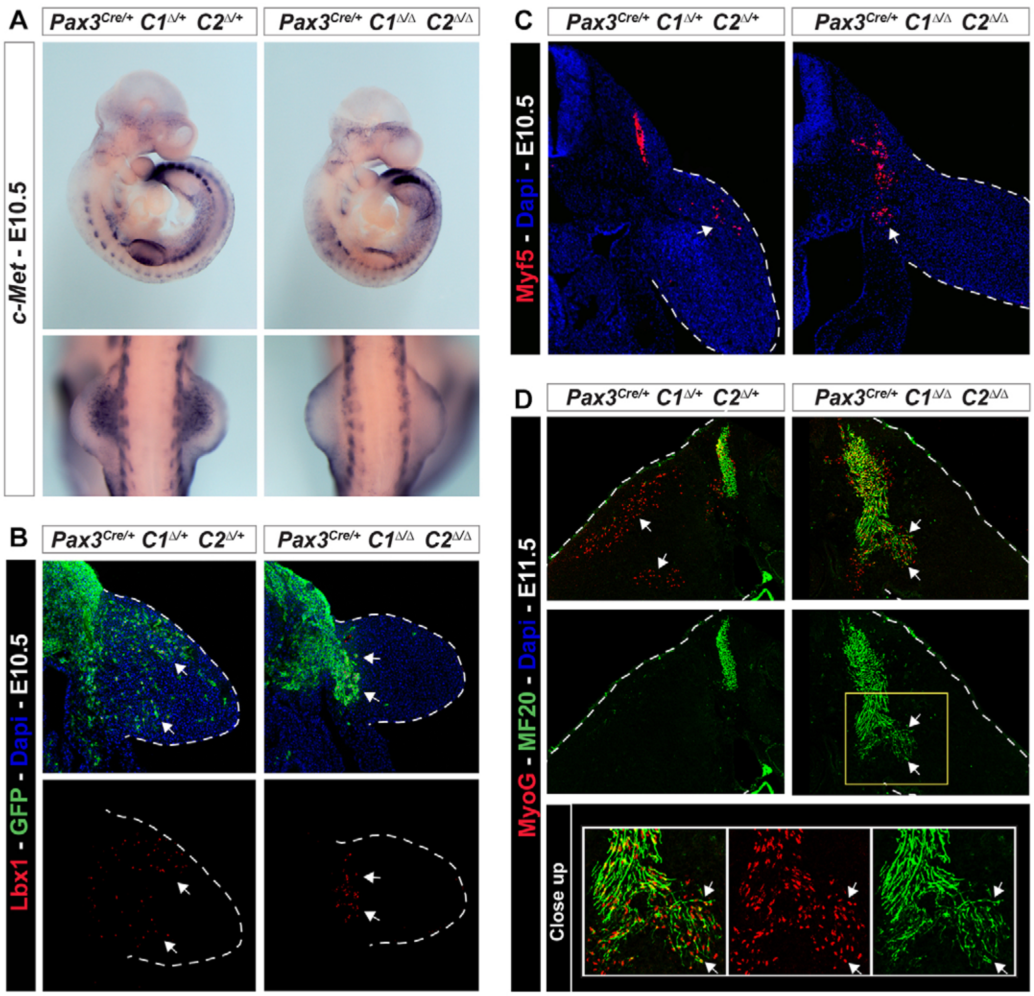

Fig. 6. Premature muscle differentiation of Pax3-positive cells that failed to migrate to the limb in the double conditional *Foxc1/2* mutant. (A) Whole-mount *in situ* hybridisation for transcripts of *Met* (*c-Met*) on control heterozygote $Pax3^{Cre/+}$;$Foxc1^{flox/+}$;$Foxc2^{flox/+}$ ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) and double conditional mutant $Pax3^{Cre/+}$;$Foxc1^{flox/flox}$;$Foxc2^{flox/flox}$ ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) embryos at E10.5. Lower panels represent dorsal views of forelimbs. *Met* expression is not detected in the proximal forelimbs of double conditional mutants compared with controls, but is expressed in hypaxial somites. Coloration of the limb ectoderm is due to trapping of the probe. (B) Immunostaining on DAPI-stained sections with antibodies to Lbx1 (red) and GFP (green) of $Pax3^{Cre/+}$;$Rosa26^{tomato\text{-}floxGFP/+}$ embryos on heterozygote control ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) and double conditional mutant ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) backgrounds, at the forelimb level at E10.5. Lbx1-positive cells (arrows) are present in the double conditional mutant but fail to enter the limb. In the lower panels Lbx1 staining alone is shown. (C) Immunostaining on DAPI-stained sections with a Myf5 (red) antibody at the forelimb level, in heterozygote control ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) or double conditional mutant ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) embryos at E10.5. Arrows show myogenic cells (Myf5-positive) that accumulate in the trunk adjacent to the forelimb in double conditional mutants whereas in control embryos Myf5-positive cells are found in the forelimb. (D) Immunostaining on sections with antibodies to the myogenic differentiation factor myogenin (MyoG) (red) and to myosin heavy chain (MF20) (green) at the forelimb level of control ($Pax3^{Cre/+}C1^{\Delta/+}C2^{\Delta/+}$) or double conditional mutant ($Pax3^{Cre/+}C1^{\Delta/\Delta}C2^{\Delta/\Delta}$) embryos at E11.5. Enlargements of the boxed area in the double mutant show differentiating cells, as well as muscle fibres, adjacent to the somite in the trunk of double conditional mutants (arrows), whereas in the control, differentiating myogenin-positive cells are found in the dorsal and ventral muscle masses of the forelimb, where myosin has not yet accumulated at this stage. Dashed lines delineate the outline of the forelimb.

after the vaginal plug and somite number was used for precise comparison. Double conditional heterozygotes $Pax3^{Cre/+}$;$Foxc1^{\Delta/+}$;$Foxc2^{\Delta/+}$ were used as controls in a number of experiments because their phenotypes were indistinguishable from $Pax3^{Cre/+}$;$Foxc1^{+/+}$;$Foxc2^{+/+}$ embryos. All animal procedures were carried out in the Institut Pasteur animal facility with approval by the French Ministry of Agriculture.

X-Gal staining, *in situ* hybridisation and immunostaining

X-Gal staining and whole-mount *in situ* hybridisation with digoxigenin-labelled probes were performed as described by Lagha et al. (2009). The *Met* probe was kindly provided by C. Birchmeier (Max Delbrück Centrum für Molekulare Medizin, Berlin, Germany), the *Myod1* probe was as described by Sassoon et al. (1989). Immunostaining of myosin heavy chain on a whole-mount embryo was carried out as previously described by Ouimette et al. (2010). Haematoxylin and Eosin histological staining and immunostainings were assessed on frozen sections (16 µm), after overnight incubation and 2 h of embryo fixation in PBS or 4% paraformaldehyde, respectively. Pax3, Pecam-1, GFP, Myf5, Lbx1 (gift of C. Birchmeier), MF20, ZO-1 and AP2α antibodies were used as previously described by Lagha et al. (2009) (see Table S1 for details). A confocal Zeiss LSM 700 laser scanning microscope was used for fluorescence image acquisition. Each experiment was performed on a minimum of three embryos per condition.

Skeletal preparation

Skeletal preparations were performed as described by Bensoussan-Trigano et al. (2011).

Microdissection and FACS-cell sorting of Pax3 derivatives

Embryos were microdissected in F-12 medium (Gibco). For E9.25 embryos, the forelimbs and their five adjacent somites were separated from the neural tube and delaminating neural crest cells, viewed by GFP fluorescence under a fluorescent binocular microscope. For E10.5 embryos, the forelimbs were recovered without somites. Tissues were conserved in F-12 medium during the time of the dissection and were then mechanically dissociated before enzymatic digestion in F-12, 0.1% collagenase D (Roche) at 37°C. Cells were re-suspended in F-12, 1% horse serum after 18 min of centrifugation (1800 rpm; 600 ***g***). After a 1-h incubation with Alexa Fluor 647 anti-mouse CD31 (Pecam-1) (BioLegend), followed by two rinses in PBS with 1% horse serum and two 18-min centrifugations (1800 rpm; 600 ***g***), cells were filtered with a 40-µm Cell Stainer Cap. A MoFlo Cytomation or Astrios (Beckman Coulter) apparatus was used for cell sorting, on the basis of size, granularity and GFP or Alexa 647 fluorescence. The number of cells was determined for each embryo.

RT-qPCR analysis

Sorted cells were recovered in 75 µl of RLT buffer and RNA was purified using the RNeasy Micro Kit (Qiagen). RT-qPCR analysis was performed as described by Lagha et al. (2009). See Table S2 for primer sequences.

Statistics

PRISM software was used for statistical analysis with the Student's *t*-test, preceded by the Fisher test. Data are presented as the mean and standard error of the mean (s.e.m.).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.M.-L. planned, performed and interpreted the experiments and contributed to writing the manuscript. T.K. provided the *Foxc1* and *Foxc2* conditional mutants. C.B. carried out histology and immunostaining. D.M. contributed to cell-sorting experiments and discussion, as well as helping with mouse maintenance. S.D.V. contributed to planning and interpreting experiments and advised on the complex genetic approaches. M.B. contributed to the planning and interpretation of the experiments and writing of the manuscript.

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Supplementary information

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