

The concerted action of *Meox* homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites

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Summary

The paraxial mesoderm of the somites of the vertebrate embryo contains the precursors of the axial skeleton, skeletal muscles and dermis. The *Meox1* and *Meox2* homeobox genes are expressed in the somites and their derivatives during embryogenesis. Mice homozygous for a null mutation in *Meox1* display relatively mild defects in sclerotome derived vertebral and rib bones, whereas absence of *Meox2* function leads to defective differentiation and morphogenesis of the limb muscles. By contrast, mice carrying null mutations for both *Meox* genes display a dramatic and wide-ranging synthetic phenotype associated

with extremely disrupted somite morphogenesis, patterning and differentiation. Mutant animals lack an axial skeleton and skeletal muscles are severely deficient. Our results demonstrate that *Meox1* and *Meox2* genes function together and upstream of several genetic hierarchies that are required for the development of somites. In particular, our studies place *Meox* gene function upstream of *Pax* genes in the regulation of chondrogenic and myogenic differentiation of paraxial mesoderm.

Key words: Somite, Myogenesis, Chondrogenesis, Homeobox

Introduction

A key feature of the body plan of the vertebrate embryo is the presence of somites, which are transient segments of the paraxial mesoderm flanking the notochord and neural tube. Somites form as epithelial blocks of cells, which bud off in a highly coordinated fashion from the anterior end of the unsegmented presomitic mesoderm (PSM). The strict temporal and spatial regulation of somitogenesis is of fundamental importance, because segmentation of structures such as the peripheral spinal nerves, vertebrae, axial muscles and early blood vessels, develops according to the periodicity of somite segmentation. Each somite is subdivided into rostral and caudal compartments that differ in adhesive properties and gene expression, and this differentiation patterns the spinal nerves and ganglia and is also the mechanism that maintains borders between segments.

Experimental and gene-expression data strongly indicate that the generation of somite periodicity and the establishment of rostrocaudal polarity takes place before segment-border formation in the apparently homogenous PSM. Studies of knockout mice have confirmed that the Notch/Delta signaling pathway has a crucial role in establishing both temporal periodicity in the PSM and rostrocaudal polarity in somite primordia (reviewed by Pourquie, 2001; Saga and Takeda, 2001). Evidence of the molecular nature of the oscillator has emerged recently from studies that demonstrate lunatic fringe

implements periodic inhibition of Notch signalling to establish a negative feedback loop, which controls the cyclic expression of genes in the presomitic mesoderm (Dale et al., 2003).

The correct formation, patterning and differentiation of somites requires the activity of at least several genetic pathways. Wnt signals from the surface ectoderm are implicated in somite epithelialisation (Borycki et al., 2000), and the paraxis bHLH transcription factor is necessary for the formation of epithelial somites (Burgess et al., 1996; Johnson et al., 2001).

Mutations in the Notch pathway disrupt not only the patterning of the PSM but also anteroposterior polarity of somites (Barrantes et al., 1999). *Foxc1* and *Foxc2* are both required for the formation of segmented somites, and may function by interaction with the Notch signalling pathway in the anterior presomitic mesoderm (Kume et al., 2001). Whereas mutations in *Eph*, *Ephrin* and *cadherin* genes in mice have not revealed phenotypes – in contrast to zebrafish (Durbin et al., 1998) and frog embryos (Kim et al., 2000) – affecting somite boundaries, a dominant-negative *papc* (cadherin) molecule disrupts the epithelial organization of cells at the segmental borders between somites in transgenic mice (Rhee et al., 2003).

A large body of evidence, from both in vitro and in vivo experiments (reviewed by Brent and Tabin, 2002), indicates that antagonism between different signals from adjacent tissues is required to subdivide the somite into distinct compartments:

the ventral mesenchymal sclerotome that generates the chondrogenic axial skeleton, and the dorsal epithelial dermomyotome that forms the skeletal muscles of the trunk, limbs and tongue. Sonic hedgehog (SHH) and noggin are thought to be the ventralising signals for sclerotome induction, and WNT proteins are involved in establishment of the dorsal domain of the somite. BMP signals, originating in the lateral mesoderm, negatively regulate the spatial and temporal activation of somitic myogenesis (Reshef et al., 1998) and positively regulate lateral somitic cell fates (Pourquie et al., 1996; Tonegawa et al., 1997). It is likely that noggin-mediated antagonism of BMP signaling is required for both myotomal and sclerotomal development (McMahon et al., 1998). There is also evidence that SHH changes the competence of target somitic cells to respond to BMPs to induce chondrogenesis (Murtaugh et al., 1999).

The induction of *Pax1* and *Pax9* gene expression by SHH is necessary for vertebral and rib formation (Peters et al., 1999), and *Foxc2* is required for sclerotome proliferation (Winnier et al., 1997). Targeted mutagenesis of the MRF family of bHLH transcriptional activators (MYF5, MYOD1, myogenin, MRF4) in the mouse has revealed an essential, but different, role for members of this gene family in the formation of skeletal muscle (reviewed by Arnold and Braun, 2000), and *Pax3* and *Myf5* are required for the expression of *Myod1* in the trunk (Tajbakhsh et al., 1997). Long range signaling by SHH has a role in the induction of *Myf5* gene expression in the dorsal somite (Gustafsson et al., 2002).

We have previously described the isolation of the MEOX sub-family of homeobox transcription factors (Candia et al., 1992; Candia et al., 1996). Both *Meox1* and *Meox2* genes have characteristic expression in the somites of the paraxial mesoderm in vertebrate embryos. *Meox1* mutant mice display defects restricted to sclerotomal derivatives, the vertebrae and ribs are fused (S.S., B.M., C.W., V.P. and H.A., unpublished). By contrast, the *Meox2* mutation produces a phenotype that affects the development of the limb muscles (Mankoo et al., 1999). *Meox2* is required for the expression of *Pax3* RNA in migrating limb myoblasts; and also for the induction of *Myf5* gene expression, but not that of *Myod1*, in limb myoblasts. As each single *Meox* gene mutation affected only a subset of somitic derivatives, despite a largely overlapping expression pattern, this raised the possibility the two genes have overlapping functions and are capable of compensating for each others absence. To investigate the combined function of the MEOX subfamily of homeoproteins, we crossed mutations for both *Meox1* and *Meox2*. The complete absence of *Meox* gene activity resulted in unexpected and severe defects in somite development. The axial skeleton and most skeletal muscles were not formed. Somite epithelialisation and rostrocaudal somite patterning were also disrupted, as was the maintenance of somite boundaries. Both *Meox1* and *Meox2* genes were also required for the normal differentiation of cells derived from both the sclerotome and dermomyotome. We propose that the concerted activity of the two *Meox* genes is an essential component of the genetic circuitry that regulates somitogenesis.

Materials and methods

Generation and breeding of mutant mice

Details of the generation of the two *Meox1* mutations are to be

published separately (S.S., B.M., C.W., V.P. and H.A., unpublished). Molecular analysis of a transgenic line identified a recessive insertional mutation led to the conclusion that a DNA fragment consisting of 1.8 kb of the interferon α/β -inducible mouse Mx1 promoter, the 1.1 kb HTLV1 Tax cDNA, and part of intron 2 and non-coding exon 3 of the mouse β -globin gene, had inserted fortuitously in first intron of the *Meox1* gene and ending 31.2 kb downstream of the stop codon, deleting a total of 45.4 kb of DNA. The allele was given the full designation *Meox1*^{TgN2627ARN} and is here referred to as *Meox1*^{im} (insertional mutant). A knockout allele in *Meox1* was generated by established procedures in ES cells. Upon homologous recombination, the transcription start site, the entire exon 1, including the translational start codon, and 2.3 kb of the flanking intron 1 of *Meox1* were deleted. Chimaeric males from four independent clones transmitted the mutation and generated heterozygous animals. Homozygous animals derived from these clones had identical phenotypes. According to nomenclature rules, this allele carries the full designation *Meox1*^{im1BSM} and is here referred to as *Meox1*⁻. The targeted disruption of the *Meox2* gene (*Meox2*⁻) been described previously (Mankoo et al., 1999). The *Meox1*⁻ and *Meox2*⁻ alleles were kept on a mixed C57BL/6//129/Ola background and the *Meox1*^{im} allele on a mixed C57BL/6//C3H/HeJ background.

Determination of recombinant embryonic stem cells and genotyping of animals

Details on the molecular identification of the transgene insertion of *Meox1* and the determination of all mutant genotypes can be obtained on request.

Histology, in situ hybridisation and skeletal preparations

For histology, embryos or tissues were fixed in Bouin's fixative, dehydrated and embedded in paraffin wax. Serial sections (8 μ m) were stained with Haematoxylin and Eosin. For semi-thin sections the tissues were embedded in epoxy resin and sections were cut with a glass knife. Whole mount in situ hybridization was performed as previously described (Mankoo et al., 1999). For cryosectioning, embryos were postfixed in 4% paraformaldehyde, equilibrated in 30% sucrose and embedded in OCT. Skeletal preparations of newborn pups were produced using a combination of Alcian Blue and Alizarin Red staining.

Results

Abnormal development of the axial skeleton in the absence of *Meox* gene activity

To investigate the role of *Meox* genes during mammalian embryogenesis, we generated mice carrying null mutations at the loci of the known members of this family: *Meox1* and *Meox2*. Two null alleles of the *Meox1* locus were used. The *Meox1*^{im} allele was produced by the fortuitous insertion of an HTLV-1 *Tax* transgene into first intron of the *Meox1* locus that deleted the rest of the gene (S.S., B.M., C.W., V.P. and H.A., unpublished), while the *Meox1*⁻ allele was generated by targeted mutagenesis in embryonic stem (ES) cells, deleting exon 1. Animals homozygous for either of the two alleles did not express *Meox1* mRNA (data not shown) and have abnormalities of the axial skeleton characterised by the presence of hemi-vertebrae, as well as rib, vertebral and cranio-vertebral fusions, but no apparent defects in skeletal myogenesis (data not shown). Compound heterozygotes for the two *Meox1* mutant alleles (*Meox1*^{im/-}) had the same phenotype as the single mutant homozygotes (data not shown), supporting the conclusion that both mutations have produced null alleles at this locus. *Meox2*-deficient animals (*Meox2*^{-/-}) have no

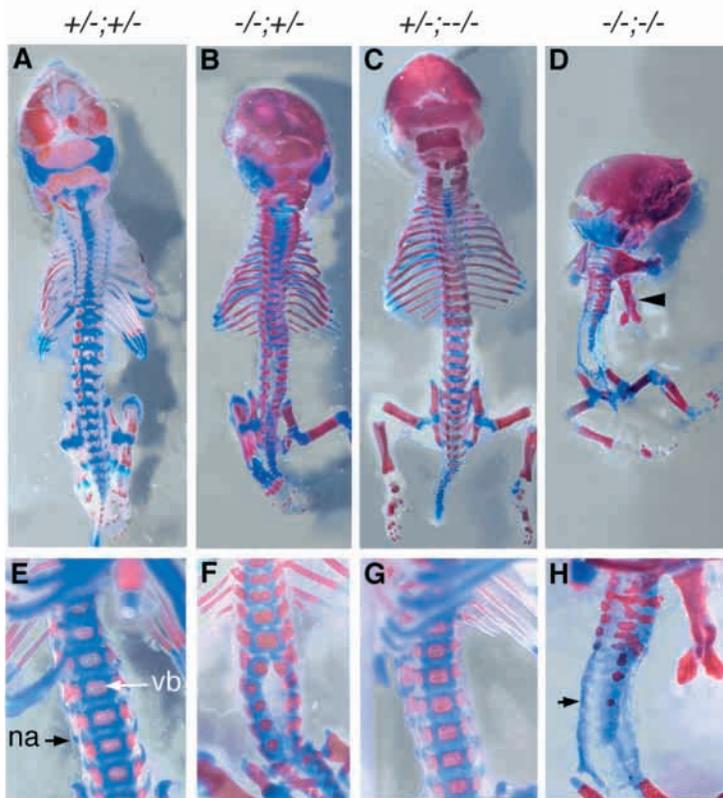


Fig. 1. *Meox1*;*Meox2* mutants have profound axial skeleton defects. Alcian Blue/Alizarin Red skeletal preparations of neonates. The forelimbs and shoulder girdle have been removed for ease of viewing. In contrast to control littermates (*Meox1*^{+/+};*Meox2*^{+/+} A and E, *n*>6), animals with mutations in both *Meox1* and *Meox2* genes have defects in the development of the axial skeleton (B-H, *n*>6). *Meox1*^{-/-};*Meox2*^{+/+} display rib fusions and deformations, and vertebral bodies at the lumbar level are split, and tail vertebrae are fused (B,F). *Meox1*^{+/+};*Meox2*^{-/-} are less severely affected, there are no rib defects and lumbar vertebrae appear normal, but tail vertebrae are malformed and fused (C,G). *Meox1*^{-/-};*Meox2*^{-/-} animals lack an axial skeleton (D,H), there are no ribs and, while ossified, deformed vertebrae are formed at the cervical and thoracic level; more posterior lumbar vertebrae are present only as cartilage condensations at the position expected of the neural arches (arrow in H) and tail vertebrae are completely absent. The sternum develops, albeit abnormally, in the absence of the ribs (arrowhead in D). Normally developed neural arches (na) and vertebral bodies (vb) are identified in E.

elements were detectable at or posterior to the pelvic girdle (Fig. 1D,H). The occipital skull bones, which are somite derived, were hypoplastic, whereas other cranial bones were unaffected (not shown). These observations demonstrate that strong dosage dependent interactions between *Meox1* and *Meox2* are essential for the formation of the axial skeleton; each gene can compensate, to a differing extent, for the absence of the other.

skeletal defects but are characterised by deficiencies in limb myogenesis (Mankoo et al., 1999). Compound heterozygotes (*Meox1*^{+/im};*Meox2*^{+/+} or *Meox1*^{+/+};*Meox2*^{+/im}) displayed no abnormalities and were intercrossed to produce mice carrying various combinations of wild-type and mutant *Meox* alleles. Animals with a single or no wild-type *Meox* allele were born at the expected Mendelian ratio (of 1/8 and 1/16, respectively) but were severely malformed and died shortly after birth. More specifically, the trunks of such mutants were drastically reduced in length, while the skin was loose and cyanotic. The tail in double homozygous mutants (hereafter referred to as *Meox1*^{-/-};*Meox2*^{-/-}) was reduced to a rudimentary stump lacking skeletal elements (data not shown).

Skeletal preparations of animals carrying a single wild-type *Meox1* allele (*Meox1*^{+/+};*Meox2*^{-/-}) displayed defects affecting the axial skeleton; the ribs were normal, and vertebral defects were only apparent at posterior levels (Fig. 1C,G). Animals with a single wild-type *Meox2* allele (*Meox1*^{-/-};*Meox2*^{+/+}) were more seriously affected; ribs were present but with fusions, and vertebral bodies were split at the lumbar level, while posterior to the pelvic girdle poorly differentiated cartilaginous elements were seen in place of vertebrae (Fig. 1B,F). Skeletal preparations of double mutants (*Meox1*^{-/-};*Meox2*^{-/-}) revealed a striking phenotype, these animals lacked a normal vertebral column, which was largely replaced by two strips of fused cartilage, corresponding in position to the neural arches. There was no cartilage or bone present in the ventral midline the location of vertebral bodies; and, although centres of ossification were observed at the cervical and thoracic level of the axial skeleton, neither normal vertebrae nor ribs were observed (Fig. 1D). In addition, no skeletal or cartilaginous

Skeletal muscle defects in *Meox* mutants

In addition to the skeletal abnormalities, *Meox*-deficient animals had major defects in the development of the somite derived skeletal musculature. Thus, double homozygous mutants (*Meox1*^{-/-};*Meox2*^{-/-}) had a severe depletion of the pre-vertebral muscles of the head and neck, and also in the epaxial (paraspinal) and hypaxial muscles of the trunk (abdominal wall and intercostal) and limb (Fig. 2C,D). As a consequence, it was no longer possible to identify individual muscles in the mutants. Interestingly, the intrinsic muscles of the tongue, despite originating in somitic mesoderm, were relatively unaffected in the double mutants (Fig. 2A,C). Cranial muscles that do not originate in the somitic mesoderm were generally unaffected (data not shown), including the masseter and extra-ocular muscles that do express *Meox* genes during embryonic development (Candia et al., 1992). The failure of the epaxial skeletal musculature to develop normally was associated with the absence of the overlying brown adipose tissue (Fig. 2D).

Histological analysis reveals that somite formation and patterning require *Meox* genes

The rib, vertebral and occipital bone defects and the muscle abnormalities of mice lacking MEOX proteins indicate that the combined function of *Meox* genes is required for normal somitogenesis. To analyse the processes of somite patterning and differentiation in *Meox*-deficient embryos, we examined histological sections from E9.5-10.5 double mutants. Contrary to control embryos, in which newly formed somites appeared as well defined epithelial spheres that differentiated into a dorsal epithelial dermomyotome and a ventral mesenchymal sclerotome, the newly generated somites of mutant embryos

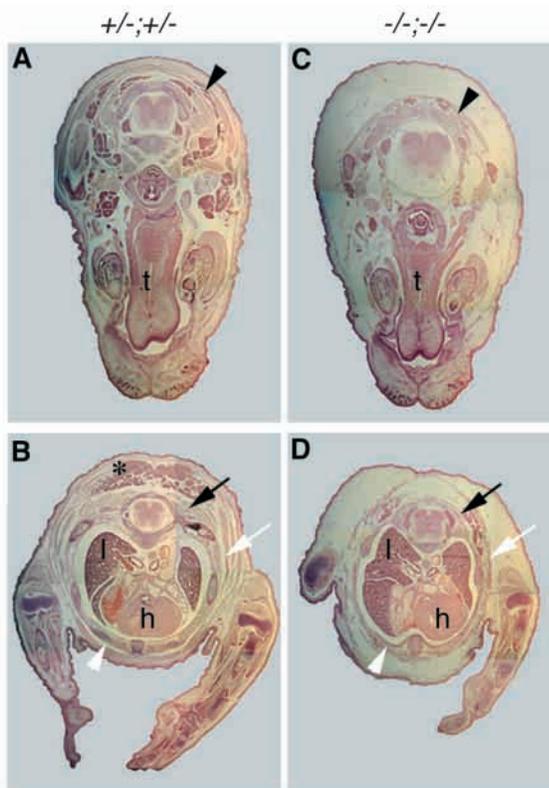


Fig. 2. Skeletal muscle abnormalities in *Meox* double mutants. Transverse paraffin wax-embedded sections of control (A,B) and mutant (C,D) foetuses at E16.5, at the level of the tongue (A,C) and forelimbs (B,D) demonstrating that most skeletal muscles are absent or reduced in size in the mutant. These include: the prevertebral muscles of the neck (black arrowhead in A and C), the epaxial (black arrow) muscles of the trunk and also the hypaxial muscles of the trunk including those of the abdominal wall (white arrows) and intercostal muscles (white arrowhead). The brown fat overlying the shoulder muscles in control foetuses (* in B) was also absent in mutants. t, tongue; l, lung; h, heart.

were irregular in shape and failed to epithelialise. The basal lamina that normally surrounds each somite and separates it from its neighbours was no longer detectable between somites, although it was clearly present both dorsally and ventrally to the somites of double mutant embryos (Fig. 3A,B). Moreover, no evidence of differentiation into morphologically identifiable compartments was observed in more mature somites of mutant embryos (Fig. 3C,D). In addition, the segmented organisation of the ventral sclerotome (which normally gives rise to the vertebral bodies, neural arches and pedicles of vertebrae), resulting from the alteration of anterior and posterior sclerotome halves (composed of loose and dense mesenchyme, respectively), was lost in mutants and replaced by a uniform unsegmented mesenchyme (Fig. 3E,F). The alteration of anterior and posterior properties of each somite patterns the dorsal root ganglia (DRGs) and spinal nerves. As a result of the *Meox* double mutation, DRGs, which normally differentiate in the anterior half of the sclerotome of each somite, were irregular in shape and size and fused together (Fig. 3G,H) and similarly spinal nerves were irregular in spacing and direction (data not shown).

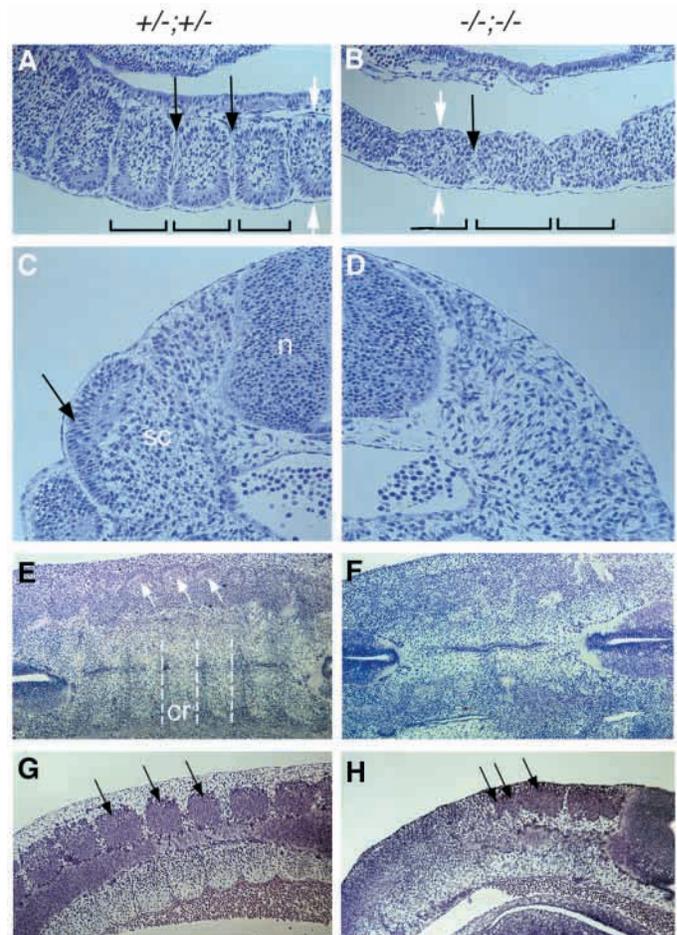
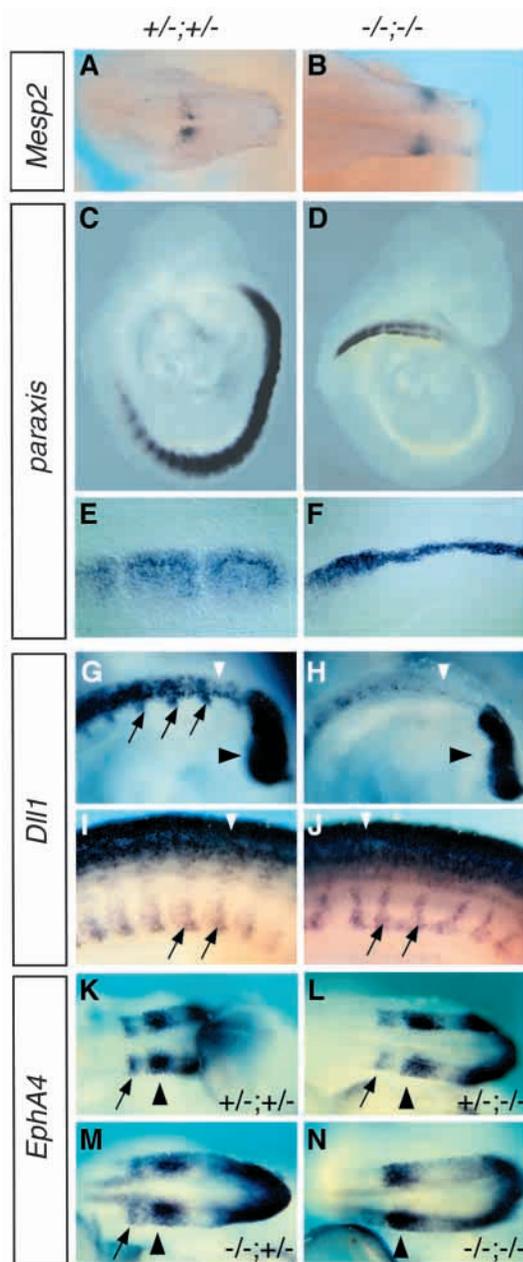


Fig. 3. Defective somitogenesis in the absence of *Meox* gene function. (A,B) Thin resin sagittal sections of the caudal region of E9.5 embryos. Unlike those of control *Meox1*^{+/-};*Meox2*^{+/-} embryos (A), newly formed somites from double mutant *Meox1*^{-/-};*Meox2*^{-/-} embryos (B) are irregularly shaped and sized (compare sizes of bars), not organized into epithelial spheres and the basal lamina that normally surrounds each somite is no longer evident between somites (black arrows), although it is present dorsal and ventral to somites (white arrows). (C,D) Transverse rostral sections of E10.5 embryos. The epithelial dermomyotome (arrow), characteristic of mature differentiated somites in controls (C), is absent in *Meox1*;*Meox2* double homozygous mutants (D). n, neural tube, sc, sclerotome. (E,F) Longitudinal sections of E10.5 embryos. The segmented organization of adjacent sclerotomes (dashed lines) in controls (E) is absent in double *Meox* mutants (F). Furthermore, the anteroposterior polarity of each sclerotome, consisting of a rostral half (r) and denser caudal half (c) is not apparent. The epithelial dermomyotome in controls (E, arrows) is again not evident in mutants (F). (G,H) Para-sagittal sections of control and mutant embryos at E10.5. In control embryos, the dorsal root ganglia (DRG) are regularly sized and shaped (G, arrows); by contrast, they are uneven in size and spacing and often fused in mutants (H, arrows).

Molecular analysis of epithelialisation and patterning of somites

To investigate the molecular mechanisms that underlie the morphological defects in somite patterning and differentiation observed in *Meox*-deficient animals, we analysed the expression of molecular markers of somitic cell lineages by in situ



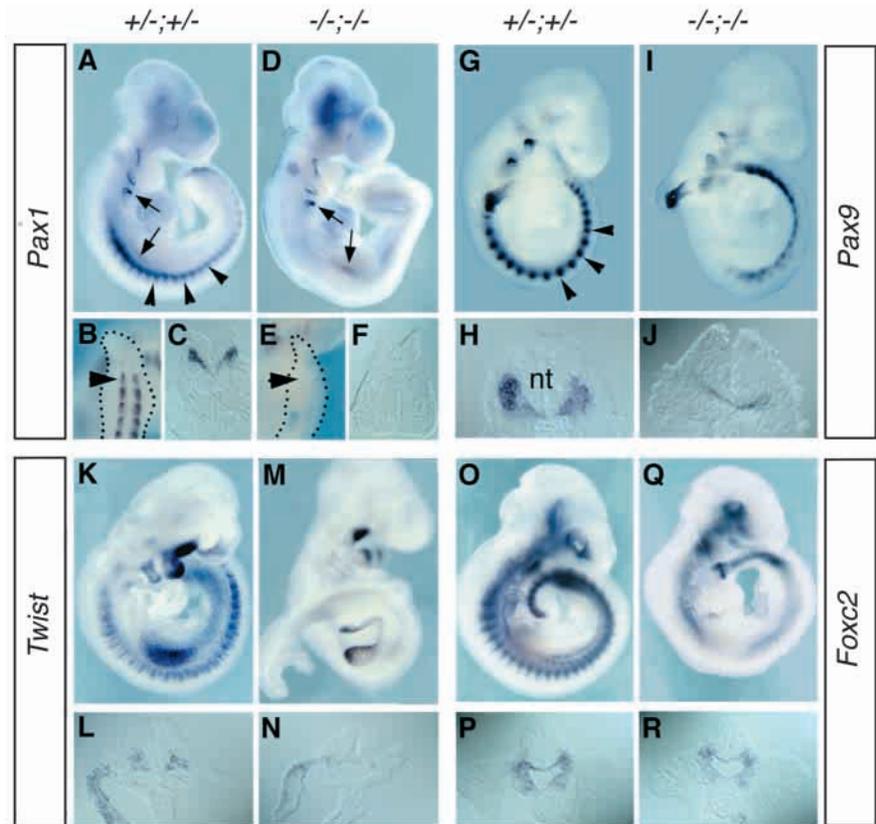
hybridisation. *Meox1* is expressed in the most rostral part of the pre-somitic mesoderm and throughout newly formed somites, while *Meox2* is first expressed concomitant with the formation of epithelial somites. The initial formation of somites is known to be dependent on the function of the Notch signalling pathway in the pre-somitic mesoderm to establish boundaries and anteroposterior somite patterning (Barrantes et al., 1999; Saga and Takeda, 2001). We examined the expression in the rostral pre-somitic mesoderm of members of the Notch signalling pathway in *Meox* double mutants. The pre-somitic mesoderm expression of neither *Mesp2* (Fig. 4A,B) nor *Lfng* (data not shown) was affected, indicating that *Meox* gene function is not required for the expression of these genes in the most anterior pre-somitic mesoderm when segmentation is specified; and supports the histological finding that segmental units are formed in the paraxial mesoderm of *Meox* double mutants.

Fig. 4. Somite epithelialisation and patterning requires both *Meox* genes. In situ hybridization analysis of *Mesp2* (A,B), *paraxis* (C-F) and *Dll1* (G-J) expression in control (A,C,E,G,I) and double *Meox1*^{-/-};*Meox2*^{-/-} mutant (B,D,F,H,J) embryos at E9.5. In A,B,E-N the caudal end of the embryo is towards the right. (A,B) Dorsal aspects of *Mesp2* expression in the rostral presomitic mesoderm. Control (A) and mutant (B) embryos have similar expression profiles. Expression of *paraxis* in whole-mount preparations (C,D) and para-sagittal sections (E,F). In controls (C,E), *paraxis* is expressed throughout the epithelial somites, but double mutant embryos (D,F) express *paraxis* in a dorsally restricted domain. Furthermore, this expression is not maintained in older, more rostral, somites. Expression of *Dll1* in caudal (G,H) and rostral (I,J) somites. In control embryos (G), *Dll1* is expressed at high levels in presomitic mesoderm (black arrowhead), the caudal halves of newly formed somites (arrows) and the neural tube (white arrowhead). In mutants (H), *Dll1* is expressed at high levels in the presomitic mesoderm (black arrowhead), but its expression in somites is virtually extinguished, whereas the neural tube expression remains (white arrowhead). (I) *Dll1* is expressed in the myotome (arrows) of anterior differentiated somites of control embryos, and in the neural tube (white arrowhead). In mutants (J), the myotome expression is fused ventrally (arrows). (K-N) In situ hybridization analysis of *Epha4*. (K) In control embryos, *Meox1*^{+/-};*Meox2*^{+/-}, dorsal views of *Epha4* expression show a broad stripe (arrowhead) in the most rostral presomitic mesoderm (in the next somite to form) and a narrow stripe of expression (arrow) in the rostral half of the most recently formed somite. In embryos with one wild-type *Meox* allele, *Meox1*^{+/-};*Meox2*^{-/-} (L) and *Meox1*^{-/-};*Meox2*^{+/-} (M), the rostral somite stripe of expression (arrow) is less refined. In *Meox1*^{-/-};*Meox2*^{-/-} mutants (N) the rostral half-stripe of *Epha4* expression is absent and only the posterior stripe (arrowhead) is visible.

The formation of somites emerging at the most anterior end of the pre-somitic mesoderm was also analysed using *paraxis* (Tcf15 – Mouse Genome Informatics), a gene that is normally expressed throughout the epithelial somites and in the epithelial dermomyotome of mature somites (Burgess et al., 1995) (Fig. 4C,E). In double *Meox* mutants, *paraxis* expression in newly formed somites, albeit segmental, was restricted to a narrow dorsal domain (Fig. 4D,F), similar to that seen with *Dll1* (not shown). No signal was detected in more anterior somites indicating that the dermomyotome-specific expression of *paraxis* was absent. These data suggest that *Meox1* and *Meox2* are important for epithelialisation events in the newly formed somites and of the dermomyotome. *Paraxis* is essential for somite epithelialisation (Burgess et al., 1996) and, therefore, the reduced *paraxis* expression could explain the absence of epithelialisation of newly formed mutant somites and the absence of an epithelial dermomyotome in older somites. *Paraxis* is also implicated in maintaining the anteroposterior polarity of somites (Barnes et al., 1997; Johnson et al., 2001), and the disruption of *paraxis* gene expression in the absence of *Meox* proteins could contribute to the observed defects in somite patterning.

We also examined the expression of *Dll1*, a member of the Notch signalling pathway, which is expressed in the pre-somitic mesoderm and the posterior half of newly formed somites and is required for epithelialisation of somites and establishment of their anteroposterior polarity (Hrabe de Angelis et al., 1997). In E9.5 *Meox1*;*Meox2* double mutants, expression of *Dll1* in the pre-somitic mesoderm was unaffected, but expression in the newly formed somites was greatly reduced and restricted

Fig. 5. Disrupted sclerotomal differentiation in *Meox1;Meox2* mutants. Whole-mount preparations of in situ hybridization analysis of markers expressed at E9.5 in the sclerotome: *Pax1* (A-F), *Pax9* (G-J), *Twist* (K-N) and *Foxc2* (O-R). Representative transverse cryosections of whole-mount preparations of control (C,H,L,P) and mutant (F,J,N,R) embryos are shown. (A-C) *Pax1* is expressed at high levels in the sclerotome of control embryos (arrowheads), but is not detected in the somites of *Meox* double mutants (D-F), although branchial arch and limb bud expression persists (arrows). The expression of *Pax1* is first seen shortly after epithelial somites form in control embryos (B,C; arrowhead), but is not induced in *Meox* double mutants (E,F; arrowhead). (G-J) *Pax9* is also expressed at high levels in the sclerotome of control embryos (G,H), especially in the caudal half-somites (arrowheads). By contrast, double *Meox* mutant embryos show greatly reduced *Pax9* expression, most evident in the caudal half-somites (I,J). The residual *Pax9* expression is restricted to the sclerotomal cells closest to the neural tube (J). (K-N) *Twist* RNA is detected throughout epithelial somites, and in the sclerotome and dermomyotome of differentiated somites (K,L). In *Meox* double mutants (M,N), *Twist* expression is greatly reduced in somites, while expression persists in branchial arches and limb buds. (O-R) *Foxc2* is expressed in control embryos in sclerotomal cells (O) preceding *Pax1* and *Pax9*, while its expression in the posterior half of somites of mutant embryos is reduced dramatically in the mutant (Q). The distribution of the *Foxc2* signal on sectional analysis is, however, similar in control (P) and mutant (R) embryos.



dorsally (Fig. 4G,H; data not shown). As visualised by *Dll1* expression, the myotomes of anterior somites in *Meox1; Meox2* double mutants were fused ventrally but separated dorsally (Fig. 4I,J). This analysis is consistent with a defect in epithelialisation of somites in double mutants. Furthermore, it indicates that in the absence of *Meox* proteins, the specification of the posterior somitic halves is not maintained resulting in somitic fusions and abnormal patterning of DRGs and spinal nerves.

To determine whether the observed abnormalities in somite polarity were a consequence only of defects in specification of the posterior half of somites, we examined the expression of *Epha4*. At E9.5, *Epha4* expression is located in two stripes: a broad posterior stripe at the most anterior border of the presomitic mesoderm and an anterior stripe in the anterior half of the most newly formed somite (Barrantes et al., 1999) (Fig. 4K). In all mutants, the posterior stripe of *Epha4* expression in the presomitic mesoderm was not altered. In mutants with only one wild-type *Meox* allele, the anterior stripe of expression was present but less refined than in controls (Fig. 4L,M); however, in the *Meox1; Meox2* double homozygotes, the expression corresponding to the anterior half of the prospective somite was ablated (Fig. 4N), indicating that patterning of the anterior half of the somite is also defective in the *Meox* mutants.

Sclerotome differentiation but not sclerotome specification is perturbed in mutant embryos

To further examine somitic differentiation in *Meox*-deficient animals, we analysed the expression of *Pax1* and *Pax9*, two

genes that are expressed in the sclerotome and are critical for normal axial skeleton development (Peters et al., 1999). In E9.5 *Meox1; Meox2* double mutants, *Pax1* mRNA was absent from the paraxial mesoderm throughout the anteroposterior axis, while branchial arch and limb bud expression were unaffected (Fig. 5A-F). In addition, *Pax9* expression was dramatically reduced, particularly in the posterior somite halves, resulting in a continuous, albeit anteriorly truncated, band of reduced expression along the anteroposterior axis of the mutant embryo (Fig. 5G-J), further supporting a defect in somite compartmentalisation.

Pax1 mutant animals are characterised by vertebral abnormalities that are milder compared with those observed in *Meox1; Meox2* double mutants (Wilm et al., 1998), whereas *Pax9*-deficient animals have normal axial skeletons (Peters et al., 1998). Although absence of both functional *Pax1* and *Pax9* genes in one animal results in a more severe defect of the axial skeleton (Peters et al., 1999), such a mutant is less severely affected compared with *Meox1;Meox2* double mutants. This suggests that additional, non PAX-mediated genetic pathways involved in sclerotome differentiation are disrupted in the *Meox* double mutants. This is supported by the observed loss, in the paraxial mesoderm of double mutant *Meox* animals, of expression of *Twist* (Fig. 5K-N), a gene that is implicated in myogenic and chondrogenic differentiation (Fuchtbauer, 1995; Spicer et al., 1996). To investigate whether the effect on *Pax1*, *Pax9* and *Twist* expression was due to a failure of sclerotome specification, we examined the expression of *Foxc2*, a gene that

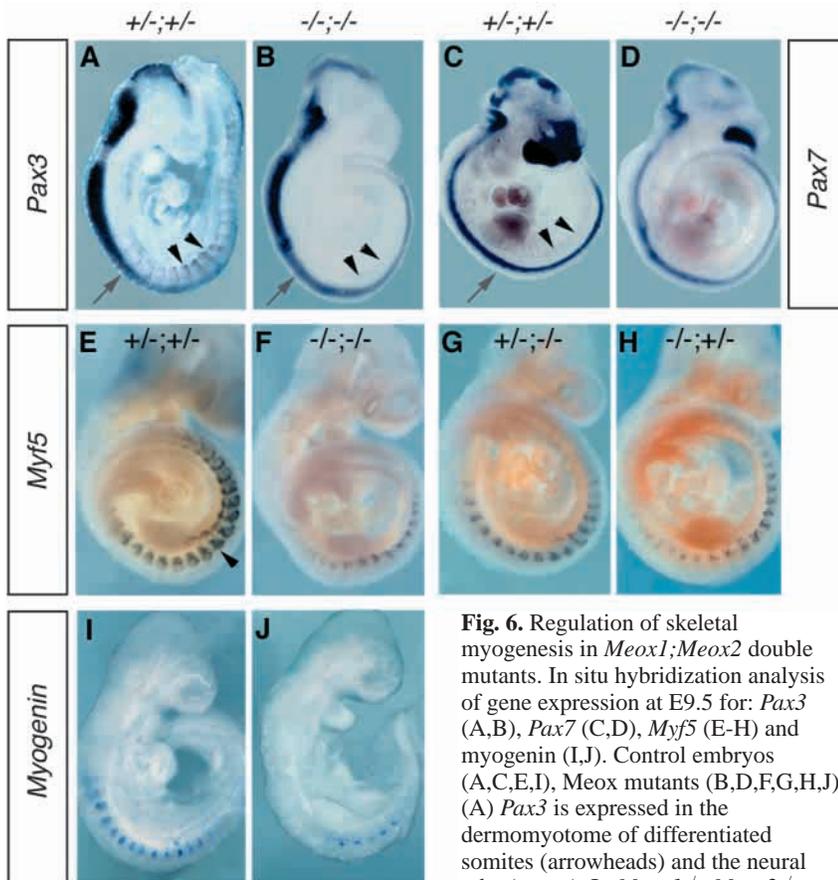


Fig. 6. Regulation of skeletal myogenesis in *Meox1;Meox2* double mutants. In situ hybridization analysis of gene expression at E9.5 for: *Pax3* (A,B), *Pax7* (C,D), *Myf5* (E-H) and myogenin (I,J). Control embryos (A,C,E,I), Meox mutants (B,D,F,G,H,J). (A) *Pax3* is expressed in the dermomyotome of differentiated somites (arrowheads) and the neural tube (arrow). In *Meox1^{-/-};Meox2^{-/-}*

embryos (B), however, *Pax3* is expressed at very reduced levels in the ventrolateral region of the dermomyotome of somites (arrowheads), while neural tube expression remains normal (arrow). The dermomyotome *Pax7* expression seen in control embryos (arrowheads) (C) is extinguished in Meox double mutants (D). *Myf5* mRNA is localised to the ventrolateral dermomyotome of control embryos (arrowhead, E). In double mutant embryos, *Myf5* expression is not detected in caudal somites, and only at reduced levels in rostral somites (F). *Myf5* expression is limited along the dorsoventral axis in mutants, compared with controls. Embryos with only one wild-type Meox allele have an intermediate phenotype; those with one *Meox1* allele (G), *Meox1^{+/-};Meox2^{-/-}*, were less severely affected than those with one *Meox2* allele (H), *Meox1^{-/-};Meox2^{+/-}*. (I,J) In the absence of Meox gene function, the expression of myogenin was reduced and limited to inter-limb somites (J).

is expressed in ventral somites and plays a key role in the proliferation of sclerotomal cells (Winnier et al., 1997). We observed that in the mutants uniformly low levels of *Foxc2* mRNA throughout the anteroposterior axis replaced the normal segmental pattern of expression (Fig. 5O-R). This presence of *Foxc2* transcripts indicates that sclerotome is specified in Meox double mutants; however it fails to differentiate further into anterior and posterior compartments, and their derivatives. Overall, multiple defects in sclerotome differentiation are likely to explain the profoundly severe defects in the formation and patterning of the axial skeleton of *Meox1; Meox2* double mutants.

Failure of the molecular programme for skeletal myogenesis in the absence of Meox genes

To study the mechanisms underlying the skeletal muscle defects, we examined the expression of essential regulators of myogenesis in the dermomyotome and myotome, such as *Pax3*

and *Myf5* (Maroto et al., 1997; Tajbakhsh et al., 1997). In E9.5 *Meox1; Meox2* double mutant embryos, the expression of *Pax3* was severely reduced in paraxial mesoderm with only a weak signal observed in the ventrolateral region of somites at the level of the forelimb bud (Fig. 6A,B). This signal is likely to correspond to the precursors of limb myoblasts that colonise the limbs of *Meox1; Meox2* mutants. This finding indicates that despite the failure of differentiation of an epithelial dermomyotome, at least a certain degree of myoblast specification takes place in Meox double mutants. A similar reduction was observed in the expression of *Pax7* (Fig. 6C,D), an additional marker of dermomyotome (Jostes et al., 1990). The dramatic attenuation of *Pax3* and *Pax7* expression in *Meox1; Meox2* mutants indicates that Meox genes function as crucial regulators of genetic pathways upstream of *Pax3* and *Pax7* gene activation.

We then examined the formation of myotome using *Myf5* and myogenin as molecular markers (Tajbakhsh et al., 1997; Smith et al., 1994). In *Meox1^{-/-}; Meox2^{-/-}* mutants, the most caudal and rostral somites did not express *Myf5*, although inter-limb somites showed low levels of *Myf5* expression that did not extend as far dorsally and ventrally as in control embryos (Fig. 6E,F). Interestingly, the expression of *Myf5* showed a dependency on Meox gene dose, which was not observed with any of the other markers of somite differentiation described above. Although embryos with one wild-type *Meox1* allele showed some loss of *Myf5* expression compared with controls (Fig. 6G), those embryos possessing only one wild-type *Meox2* allele showed a significant downregulation of *Myf5* expression (Fig. 6H). The expression of myogenin, which identifies differentiated myocytes, was affected in a manner that paralleled that of *Myf5*, low levels were

detected only in a ventral domain of inter-limb somites of Meox double mutants (Fig. 6I,J); and *Myogenin* expression levels also demonstrated a variation that was dependent on Meox gene dosage (data not shown). Overall, our analysis indicates that the combined action of *Meox1* and *Meox2* genes is a crucial regulator of genes involved in skeletal muscle specification and differentiation.

Discussion

Loss of Meox gene function impacts at several different levels of somitogenesis

The loss of *Meox1* and *Meox2* function from the somitic tissue generates a synthetic phenotype that could not be predicted from the phenotype of either single mutant, demonstrating a strong interaction between the two Meox genes during mammalian embryogenesis. Furthermore, the loss of Meox gene function impacts somite specification and development at

several different levels, providing further insight into the gene regulation hierarchies operating during crucial phases of paraxial mesoderm development. A number of gene pathways are affected, involving genes known to have essential functions in somite patterning and differentiation.

Multiple functions of the *Notch* pathway have been proposed (Barrantes et al., 1999; Takahashi et al., 2000) initially in the pre-somitic mesoderm (prior to any *Meox* gene function) to segment the mesoderm and establish rostrocaudal polarity of presumptive somites; and subsequently in nascent somites to regulate somite patterning and boundary formation. The polarity of the somites was disrupted in *Meox1*; *Meox2* mutants as a consequence of defects in patterning of both anterior and posterior halves, with an associated downregulation of *Dll1* and *Epha4* expression, a phenotype shared with the *Dll1* mutants. Our observations indicate that aspects of the phenotype of the *Meox1*; *Meox2* double mutants are partly (via *Dll1*) explained by a perturbation in the *Notch* signalling pathway. The irregular and different size of somites is similar to that observed in *Notch1* mutants (Conlon et al., 1995), which also supports our interpretation that the *Meox* genes are involved in the correct transition of cells from the presomitic mesoderm into somites.

The phenotype of *Meox1/Meox2* mutants resembles aspects of the *Foxc1/Foxc2* compound mutants (Kume et al., 2001); however, the effect of the *Foxc* mutations seems to impact at the anterior presomitic mesoderm prior to *Meox* gene expression, indicating that *Foxc* genes do not function directly to activate *Meox* expression.

The most dramatic aspect of the *Meox* double mutant phenotype is the severe loss of both ventral (vertebrae and ribs) and dorsal (skeletal muscles) somite derivatives. The sclerotomal defect in *Meox1*; *Meox2* mutants can be traced back to early defects in the specification and patterning of the ventral somite, as revealed by reduced expression of *Pax1*, *Pax9* and *Twist*. This interpretation is further supported by the abnormal dorsal restriction of paraxis expression in the newly formed somites. The absence of *Pax1* induction in the somite phenocopies the effect seen in the absence of hedgehog signalling in the mouse embryo (Zhang et al., 2001) and suggests that a function of *Meox* genes may be to mediate the response of somitic cells to hedgehog signals. The severe defects in skeletal muscles were also due to early defects in the patterning and differentiation of the myogenic derivatives of the dorsal somite, as revealed by alterations in *Pax3*, *Pax7*, *Twist*, *Myf5*, myogenin and paraxis expression.

Chondrogenesis and myogenesis require *Meox* activity

Our data indicate an essential requirement for *Meox* activity in both chondrogenesis and myogenesis in the somite, differentiation pathways that have been considered to be mutually exclusive. One mechanism by which this may occur is based on the requirement for Pax gene activity in chondrogenesis (*Pax1* and *Pax9*) (Peters et al., 1999) and myogenesis (*Pax3* and *Pax7*) (Tajbakhsh et al., 1997; Seale et al., 2000) and our observation that both *Meox* genes are co-expressed with all four of these Pax genes in somitic mesoderm. We have previously demonstrated that in migrating limb myoblasts, which express only *Meox2* and not *Meox1*, *Meox2* is upstream of *Pax3* (Mankoo et al., 1999). In the

present study, we have shown that the absence of *Meox* gene activity from somitic mesoderm disrupts the expression of all four somite-expressed Pax genes. Therefore, the differentiation of somite derivatives into cartilage and muscle requires the *Meox*-dependent expression of Pax gene function. Interestingly, we have also observed that *Meox* proteins can interact with Pax1 and Pax3, indicating that there may be cooperativity in the action of these proteins during somitogenesis (Stamatakis et al., 2001).

Possible mechanisms for *Meox* function

The patterning and differentiation of somites is governed by complex interacting signals that originate in adjacent tissues: neural tube, lateral plate mesoderm and surface ectoderm (Borycki and Emerson, 2000; Correia and Conlon, 2000; Gossler and Hrabe de Angelis, 1998). It is clear that the competition between antagonistic signals is largely responsible for the patterning of somites and the subsequent fate of the cells in the different somitic domains (reviewed by Brent and Tabin, 2002). These signals include SHH, noggin, WNT and BMP proteins. Signalling by WNT and SHH molecules, which have been shown to act at a distance greater than the length of a somite in vitro (Fan et al., 1997; Fan et al., 1995), appears to be responsible for the subdivision of the somite into dorsal and ventral subdomains respectively. Furthermore, *Sfrp2* is a SHH-inducible WNT antagonist that can block the dermomyotome-inducing properties of WNTs in explants (Lee et al., 2000) and, conversely, *GAS1* may function as a WNT-induced inhibitor of SHH activity in the dorsal somite (Lee et al., 2001). BMP signals can negatively regulate the spatial and temporal activation of somitic myogenesis (Reshef et al., 1998) and sclerotome induction by SHH (McMahon et al., 1998), and positively regulate lateral somite fates (Pourquie et al., 1996; Tonegawa et al., 1997). The suppression of BMP signals by noggin is probably required for both myotomal and sclerotomal development (McMahon et al., 1998). There is also evidence of interactions of these signals, for example, SHH regulates competence of cells to respond to BMP. In the absence of SHH, BMP signals result in lateral plate gene expression, but following prior exposure to SHH cells respond to BMP by inducing chondrogenesis in explant cultures (Murtaugh et al., 1999). As the dorsoventral and mediolateral subdivision of the somite is affected profoundly in the *Meox1/Meox2* mutants, it suggests that these genes may function to provide competence to the somitic cells to respond to one or more of these signals.

Whereas the formation of somite boundaries and the initial establishment of rostrocaudal polarity in the presomitic mesoderm are genetically separable (Nomura-Kitabayashi et al., 2002), and take place prior to the expression of both *Meox* genes, it is clear from our studies that the maintenance of boundaries and polarity in newly formed somites are not separate events and require the activity of both *Meox* genes. Evidence that interactions between compartments occur during somitogenesis is provided by the observed vertebral defects in *Myf5*;paraxis double mutants, which indicate an indirect role for *Myf5* in the development of the axial skeleton (A. Rawls, personal communication). Furthermore, the defects in the lateral sclerotome derivatives in *Pax3* mutant mice may result from a disruption in the interaction between *Pax3*-expressing dermomyotome and the non-expressing sclerotome (Henderson et al., 1999). Whereas the initiation of expression

of *Pax3* is independent of paraxis, the maintenance of *Pax3* expression in the dermomyotome requires paraxis (Wilson-Rawls et al., 1999). Therefore, in the dorsoventral dermomyotome paraxis may function as an intermediate in the regulation of *Pax3* expression by Meox genes.

The extreme nature of the *Meox1*; *Meox2* double mutant phenotype may be explained by one of two models: (1) The perturbation of a single early event in somite formation that results in failure of somitic cells to respond to one or more inductive signals from surrounding tissues; or (2) a synergistic perturbation of several somite patterning and differentiation pathways, with a compounding effect on the defects occurring in individual somite compartments. The expression pattern of the Meox genes is consistent with both hypotheses – which are not mutually exclusive, in any case – and much work will be required to resolve the complex combinatorial effect of these genes. Overall our studies demonstrate that Meox homeobox genes function in a co-ordinated manner to regulate critical processes that effect the development of somites.

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