The paired homeobox gene Uncx4.1 specifies pedicles, transverse processes and proximal ribs of the vertebral column

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Accepted 15 March 2000; published on WWW 10 May 2000

SUMMARY

The axial skeleton develops from the sclerotome, a mesenchymal cell mass derived from the ventral halves of the somites, segmentally repeated units located on either side of the neural tube. Cells from the medial part of the sclerotome form the axial perichondral tube, which gives rise to vertebral bodies and intervertebral discs; the lateral regions of the sclerotome will form the vertebral arches and ribs. Mesenchymal sclerotome cells condense and differentiate into chondrocytes to form a cartilaginous preskeleton that is later replaced by bone tissue. Uncx4.1 is a paired type homeodomain transcription factor expressed in a dynamic pattern in the somite and sclerotome. Here we show that mice homozygous for a targeted mutation of the Uncx4.1 gene die perinatally and exhibit severe malformations of the axial skeleton. Pedicles, transverse processes and proximal ribs, elements derived from the lateral sclerotome, are lacking along the entire length of the vertebral column. The mesenchymal anlagen for these elements are formed initially, but condensation and chondrogenesis do not occur. Hence, Uncx4.1 is required for the maintenance and differentiation of particular elements of the axial skeleton.

Key words: Skeleton, Vertebra, Chondrogenesis, Somite, Sox9, Scleraxis, Pax1, Pax9

INTRODUCTION

The vertebrate skeleton is a complex organ composed of multiple elements of different shapes and origins that supports the vertebrate body with stability and mobility. The components of the skeleton, mostly made up of cartilage and bone, are highly adapted to particular functions resulting in a great variety of shape and structure.

Three distinct embryonic lineages contribute to the formation of the vertebrate skeleton. The neural crest gives rise to the craniofacial skeleton, the lateral plate mesoderm generates the appendicular skeleton, and the sclerotome forms most of the axial skeleton. The vertebral column and ribs, which constitute the axial skeleton, have a metameric organization. The functional unit of the vertebral column, the vertebra is composed of the dorsal neural arch, which can be subdivided in the lateral pedicle and the dorsal lamina, the ventral vertebral body, which provides attachment for the intervertebral discs, and the ribs of the thoracic region. According to their axial level, the individual elements of the vertebra are highly adapted in shape and structure to perform particular functions.

The metameric organization of the vertebral column derives from the somites, segmentally repeated units in the paraxial mesoderm. Upon signals from the notochord the ventral half of the somite deep epithelializes and forms the mesenchymal sclerotome whereas the dorsal somite half remains epithelial, forming the dermomyotome, which will give rise to striated muscles and the dermis of the body. Some of the sclerotomal cells migrate medially and form the perichondral tube, an initially unsegmented structure, which will form the vertebral bodies and the intervertebral discs. In contrast, neural arches and ribs can be traced back to lateral sclerotomal regions. However, cell labeling experiments performed in the avian system suggested that there is no simple relation between the vertebrae and the somites. Rather, the vertebral body is made up of adjacent parts of two somites, whereas the neural arch derives from the caudal half of one somite (Keynes and Stern, 1988; Christ and Wilting, 1992; Yamaguchi, 1997; Gossler and Hrabe de Angelis, 1998).

Mesenchymal cells of the sclerotome first condense and differentiate into chondrocytes to form a cartilaginous preskeleton, which will later be replaced by bone, a process known as endochondrial ossification (Erlebacher et al., 1995; Karsenty, 1998). Thus, patterning of the individual skeletal elements occurs at the level of the mesenchymal preskeleton most likely mediated by differential proliferation and condensation of mesenchymal cells.

A large number of human genetic disorders and of mouse mutants as well as embryological manipulations in the chick embryo have shed some light on the genetic control of early somite patterning and differentiation, and on cell lineage decisions in chondrogenesis and osteogenesis. However, we are far from a comprehensive picture of the genetic control of
vertebral column development. Particularly, the nature of the molecular programs leading to regionalization and morphogenesis of elements of the vertebral column is just beginning to be unraveled (Theiler, 1988; Mundlos and Olsen, 1997a,b).

Uncx4.1 encodes a paired-type homeobox transcription factor with a conspicuous expression pattern in the developing somite and sclerotome suggestive of a function in axial skeleton development (Rovescalli et al., 1996; Saito et al., 1996; Mansouri et al., 1997; Neidhardt et al., 1997). Uncx4.1 is first expressed in the entire caudal half of the newly formed somite. Later it is downregulated in the dermatome and myotome but is maintained in the sclerotome (Mansouri et al., 1997; Neidhardt et al., 1997). Uncx4.1 expression is lacking in Delta like-1 homozygous mutant embryos (Barrantes et al., 1999), and is partially absent in Lunatic fringe mutants (Evrard et al., 1998; Zhang and Gridley, 1998) suggesting that Uncx4.1 is a target of the Notch/Delta signaling pathway and may be required in the epithelialization and craniofacial patterning of somites. Additionally or alternatively, Uncx4.1 may play a role in the formation and/or differentiation of the sclerotome. Uncx4.1 expression is also detectable in the first branchial arch, condensing mesenchyme of the mesonephric and metanephric kidney, first digit of the forelimb and central nervous system (Mansouri et al., 1997; Neidhardt et al., 1997), suggesting control functions in multiple processes during embryogenesis.

To elucidate the role of Uncx4.1 in mouse development, we have generated a targeted mutation resulting in a deletion of the entire transcription unit of the gene. Mice homozygous for the mutant allele die shortly after birth due to severe skeletal malformations. The metameric organization of the vertebral column is unaffected including normal anteroposterior polarity of somites. However, mutants completely lack the vertebral elements derived from the lateral sclerotome, the pedicles, transverse processes and the proximal ribs. Histological and molecular analysis suggest that Uncx4.1 functions in the regionalization of the vertebra at the level of the mesenchymal precursor cells.

MATERIALS AND METHODS

Generation of Uncx4.1 mutant mice

To clone the mouse Uncx4.1 locus, a 129/Ola genomic cosmid library (obtained from the Resourcenzentrum Berlin) was screened using the mouse cDNA (Neidhardt et al., 1997) as a probe. A single cosmid clone was purified, a 15 kb SacI fragment harboring the entire Uncx4.1 transcription unit was subcloned and characterized by restriction mapping. To generate a targeting construct allowing conditional inactivation of the Uncx4.1 gene, a 14 kb genomic subfragment was modified as follows: a loxP site was introduced into a StuI site located approximately 2 kb 5' of the start of transcription, and a floxed neo cassette (pMC1-neo-polY) was inserted into a ClaI site located approx. 5 kb 3' of the second exon (Gu et al., 1993). The targeting vector was linearized at a unique SacI site and introduced into E14 ES cells (Hooper et al., 1987). 274 G418-resistant colonies were screened by Southern blot analysis. Three ES cell clones were correctly targeted at both ends, resulting in a targeting frequency of 1 in 91. Cre recombinase was transiently expressed in these targeted ES cell lines to mediate excision of loxP-flanked neo cassette 5 kb 3' to the second exon. The targeting vector was electroporated in ES cells and G418-resistant colonies analyzed by Southern blot analysis for homologous integration at the Uncx4.1 locus. Of 274 colonies screened, two showed the correct restriction fragment length polymorphism (RFLP) at the 5'-site, six at the 3'-site only. Three ES cell clones were correctly targeted at both ends resulting in a targeting frequency of 1 in 91. Two ES cell clones were chosen for further analysis. Cre recombinase was transiently expressed in these targeted ES cell lines to mediate excision of loxP-flanked sequences (Gu et al., 1993). 120 G418-sensitive colonies were screened by Southern blot analysis for excision events. 28 ES cell lines carrying the Uncx4.1(del1) allele with a complete deletion were identified, two of which were used for further experiments. Uncx4.1(del1+/+) ES cell lines of strain 129/Ola were microinjected into NMRI albino mouse blastocysts to generate chimeras. 10 male mice with high degree of chimerism were obtained and mated to NMRI females. Three chimeras gave germ line transmission. Six heterozygous males were crossed to NMRI females, heterozygous offspring intercrossed and embryos and newborns analyzed for phenotypic alterations.

Genotyping

Genotypic characterization of ES cells, embryos and adult mice was done by Southern blot analysis of BamH1-digested genomic DNA. DNA was derived from embryonic yolk sacs and from adult tails, and hybridized with probes distinguishing wild-type, Uncx4.1(neoflox) and partial and complete cre-mediated deletion alleles. The 5'-probe is a 0.8 kb BamH1-SacI fragment recognizing a 3.8 kb band in the wild type and a 1.6 kb band in all targeted alleles. The 3'-probe, a 1.3 kb KpnI-SacI fragment, detects a 6 kb fragment in the wild type and a 4.8 kb fragment in targeted alleles. The neo probe detects a 2.6 kb BamH1 fragment in the Uncx4.1(neoflox) allele, the internal probe a 2 kb fragment in all alleles except Uncx4.1(del1).

Embryo analysis and documentation

Embryos for histological analysis were fixed in Bouin's fixative. Paraffin sections were stained with haematoxylin and eosin. RNA in situ hybridization of whole-mount embryos using digoxigenin-labeled antisense riboprobes for Sox9, Myogenin (Edmondson and Olson, 1989), Pax1 (Deutsch et al., 1991), Pax9 (Neubüser et al., 1995) and Scleraxis (Cserjesi et al., 1995) were performed as described (Neidhardt et al., 1997), as was whole-mount immunohistochemistry with 2H3 anti-neurofilmant antibody (Swiatek and Gridley, 1993) (from Developmental Studies Hybridoma Bank). A Sox9 cDNA was isolated in a screen for genes expressed in a 9.5 d mouse embryo (L. N. and B. G. H., unpublished data). Skeletal preparations using Alizarin red for bone and Alcian blue for cartilage were done as described (Mallo and Brändlin, 1997). Whole-mount specimen were paraffin sectioned, or transferred into 80% glycerol prior to documentation. Samples were photographed on Kodak 64T slide film, slides scanned and photographs processed in Adobe Photoshop 4.0.

RESULTS

Targeted disruption of the Uncx4.1 gene

To elucidate the role of Uncx4.1 in mouse development, we have generated a targeted mutation deleting the entire transcription unit of the gene (Fig. 1A). To that purpose, a targeting vector was constructed to introduce a single loxP site 2 kb upstream of the transcription start, and a loxP-flanked neo cassette 5 kb 3' to the second exon. The targeting vector was electroporated in ES cells and G418-resistant colonies analyzed by Southern blot analysis for homologous integration at the Uncx4.1 locus. Of 274 colonies screened, two showed the correct restriction fragment length polymorphism (RFLP) at the 5'-site, six at the 3'-site only. Three ES cell clones were correctly targeted at both ends resulting in a targeting frequency of 1 in 91. Two ES cell clones were chosen for further analysis. Cre recombinase was transiently expressed in these targeted ES cell lines to mediate excision of loxP-flanked sequences (Gu et al., 1993). 120 G418-sensitive colonies were screened by Southern blot analysis for excision events. 28 ES cell lines carrying the Uncx4.1(del1) allele with a complete deletion were identified, two of which were used for further experiments (Fig. 1B). Multiple Uncx4.1(del1+/+) ES cell lines of

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strain 129/Ola were microinjected into NMRI albino mouse blastocysts to generate chimeras, three of which transmitted the mutation through the germline. F1 heterozygous males were crossed to NMRI females, heterozygous offspring intercrossed and embryos and newborns analyzed for phenotypic alterations. Wild-type and mutant alleles were identified by Southern blot analysis of BamHI RFLPs employing probes for the 5′, the 3′ and internal region. The Uncx4.1del allele represents a null mutation as confirmed by RFLP analysis and absence of Uncx4.1 mRNA in homozygous mutant embryos (Fig. 1B,C and data not shown).

Fig. 1. Targeted deletion of the Uncx4.1 gene. (A) Targeting scheme. Restriction map of the Uncx4.1 wild-type locus. The construct was first integrated into the endogenous locus by homologous recombination and loxP flanked sequences were subsequently deleted by cre recombinase treatment (cre). loxP sites are indicated by arrowheads. B, BamHI; C, ClaI; K, KpnI; N, NotI; S, SacI; neo, neomycin selection cassette. (B) Southern blot analysis of ES cell clones after transient expression of cre recombinase. ES5 and ES187 are ES cell clones, (-) carrying the Uncx4.1 neoflox allele, (+) after cre recombinase treatment deleting Uncx4.1 together with the neo gene. Genomic BamHI fragments indicating wild-type and mutant alleles were analysed simultaneously. (C) Southern blot analysis of DNA extracted from 16.5 d.p.c. embryos derived from intercrosses of Uncx4.1del/+ mice. Genotypes are indicated above each lane. Simultaneous detection of 5′, 3′ and internal RFLPs in BamHI-digested genomic DNA. Wt, wild type; mut, mutant; neo, neomycin gene.

Fig. 2. Histological analysis of Uncx4.1−/− newborn pups. (A) Photograph of newborn pups. The Uncx4.1 mutant appears short and stumpy. (B,C) Histological comparison of newborn Uncx4.1−/− pups and wild-type littermates. (B) Sagittal section. The mutant shows a massive kink in the thoracic region (arrows), the lung (l) is less inflated than in the control. (C) Parasagittal section, enlargement of the vertebral column in the lumbar region. Dorsal root ganglia (arrows) are fused in the mutant over the entire extension of the vertebral column.

Postnatal lethality and skeletal malformations in Uncx4.1−/− embryos
Mice heterozygous for the Uncx4.1del allele appear normal and are fertile. In contrast, no viable homozygotes were recovered from heterozygous intercrosses at 2 weeks of age (61 wild type, 145 heterozygotes of 206 offspring). However, homozygotes were observed at Mendelian ratio from embryonic day 10 to
18 post coitum (d.p.c.) as well as in newborn pups. Inspection of newborn litters revealed that homozygotes were indeed born, but died shortly after delivery.

Homozygous mutant pups were recognized by a shortened body axis resulting from a severe lordosis in the thoracic region of the vertebral column as revealed by histological analysis (Fig. 2A,B). The thoracic cavity seemed smaller and distorted, the lungs were hardly inflated. Perinatal lethality may therefore be caused by respiratory failure. Dorsal root ganglia appeared fused along the entire vertebral column (Fig. 2C). Inspection of isolated urogenital systems and histological analysis of 18.5 d.p.c. kidneys did not reveal gross morphological defects. Similarly, no obvious alterations were detected in the central nervous system of homozygotes (data not shown).

The histological analysis of newborn pups revealed severe skeletal abnormalities. To evaluate the nature and severity of these defects skeletal preparations of 18.5 d.p.c. mutant embryos (n=16) and wild-type littermates (n=15) were made (Fig. 3A,B). The skull and the appendicular skeleton appeared normal. The axial skeletons of Uncx4.1−/− mutants show a metameric organization, but are severely perturbed by scoliosis and lordosis. The rib cage is distorted with occasional fusion of ribs and sternal malformations. The thirteenth rib is invariably absent. The proximal ends of the ribs do not contact the vertebral bodies, but are weakly attached to the laminae or float freely. In the vertebral column, occasional fusion of adjacent laminae, particularly in the cervical and upper thoracic region, and split ossification centers of vertebral bodies were observed. In some cases, vertebral bodies and laminae were increased in width. Along the entire length of the vertebral column, the pedicles are strongly reduced or absent. Instead, vertebral bodies and laminae are connected by loose fibrous tissue. This observation was confirmed by inspection of individual vertebrae from the cervical, thoracic and lumbar regions (Fig. 3C-E). In addition, the proximal ribs and rib

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**Fig. 3.** Skeletal malformations in 18.5 d.p.c. Uncx4.1−/− embryos. (A,B) Whole skeleton preparations. (A) Lateral view. Note severe lordosis in the thoracic region, distorted rib cage due to unattached proximal ends of ribs (arrowhead) and the gap in the vertebral column in the mutant (arrow). (B) Ventral view. Note shortening of the trunk skeleton due to scoliosis, enlargement of vertebral bodies with occasional split ossification center (asterisk), fused ribs (arrow) and loss of thirteenth rib (arrowhead) in the mutant. (C,D) Individual vertebrae, ventral views. (C) Fourth cervical vertebra. Loss of pedicles (p) and transverse processes (tp), and slight increase in width of the lamina (l) in the mutant. (D) Fourth thoracic vertebra. Proximal ribs (pr), transverse processes and pedicles are absent, distal ribs are unattached in the mutant. (E) Fourth lumbar vertebra. Split ossification center of the vertebral body (vb) and unattached lamina due to loss of pedicles and transverse processes in the mutant. Wt, wild type; −/−, Uncx4.1/Uncx4.1 mutant.

**Fig. 4.** Analysis of formation of the cartilaginous preskeleton in whole 13.5 d.p.c. Uncx4.1−/− embryos by Alcian blue staining. The metameric pattern of the cartilage of ribs, vertebral bodies, intervertebral discs and laminae is normal in the mutant, the cartilage of the proximal ribs (arrows) and pedicles (arrow heads) is absent. Dr, distal rib; l, lamina; p, pedicle; vb, vertebral body; pr, proximal rib. (A,B) Lateral view; (C,D) dorsal view; (A,C) wild-type, (B,D) Uncx4.1/Uncx4.1 mutant.
joints are missing. The metameric pattern of intervertebral discs, vertebral bodies, laminae and distal ribs is preserved in Uncx4.1-/- mutants suggesting that Uncx4.1 is not essential and may only play a minor role in setting up or maintaining craniocaudal polarity in the somite.

Loss of vertebral structures derived from the lateral sclerotomal mesenchyme

To gain insight into the morphogenetic failure underlying the skeletal defects observed in Uncx4.1-/- mutants, cartilage stainings of 13.5 d.p.c. embryos and histological analyses of 12.5 d.p.c. and 11.5 d.p.c. embryos were performed. In the wild type at 13.5 d.p.c., the axial skeleton consists of a cartilaginous template and ossification has not started yet. In the cervical and upper thoracic region, all vertebral elements are established. In the less mature lower trunk region, cartilaginous laminae are still to be formed. At this stage, the cartilaginous skeleton of Uncx4.1-/- mutants shows a regular metameric organization (Fig. 4A,B). However, the cartilaginous precursors of the pedicles and proximal ribs are missing (Fig. 4C,D). A slight lordosis of the vertebral column has developed, but no fusions or bifurcations of skeletal elements are detected \((n>10)\) indicating that the latter malformations, which were observed in newborn pups are secondary to the loss of pedicles and proximal ribs. In the wild type, cartilaginous condensations of pedicles are present at 12.5 d.p.c. In the mutant, these elements are absent whereas condensations of the intervertebral discs and vertebral bodies follow their normal metameric pattern (Fig. 5A,B). At 11.5 d.p.c. the precartilaginous mesenchymal condensations of the pedicles (and proximal ribs) are well established in the wild type, whereas in the mutant the mesenchyme is sparse (Fig. 5C). These data suggest that the mesenchyme from which the pedicles, transverse processes and proximal ribs are derived, is unable to proliferate and condense, whereas other components of the vertebral column are not affected.

Patterning, compartmentalization and cellular differentiation in somites of Uncx4.1-/- embryos

Craniocaudal polarity of somites is established early in somitogenesis under the control of the Delta/Notch signaling pathway. Mutations in components of this pathway lead to loss of somitic polarity and failure of epithelialization of mesenchymal cells from the unsegmented paraxial mesoderm (Barrantes et al., 1999). In the wild type, the polarity of the somite is transferred to adjacent tissues including the neural crest. Consequently, neural crest cells exclusively migrate through the less condensed anterior somitic half resulting in a metameric pattern of spinal nerves (Keynes and Stern, 1984). In Uncx4.1-/- embryos, the dorsal root ganglia fuse, and this can be observed from 11.5 d.p.c. onwards (Figs 2C, 5B,C). In situ hybridizations showing neurofilament revealed expanded and perturbed dorsal root ganglia with a failure to form distinct entities already at 10.5 d.p.c. (Fig. 6B). However, immunohistochemical detection of neurofilament at this stage showed a metameric pattern of spinal nerve trajectories in the Uncx4.1-/- mutant, though axon bundles appeared less constricted than in the wild type (Fig. 6A). Somite borders are

Fig. 5. Histological stainings of the anlagen of the axial skeleton at 11.5 and 12.5 d.p.c. (A) 12.5 d.p.c., sagittal section. Normal metameric pattern of cartilaginous precursors of vertebral bodies and intervertebral discs in both wild type and mutant. (B) 12.5 d.p.c., parasagittal section. Dorsal root ganglia are well separated in the wild type (arrowheads) but fused in the mutant. Cartilaginous condensations of pedicles are not observed in the mutant (arrows). (C) 11.5 d.p.c., parasagittal section. Mesenchymal anlagen of pedicles (arrows) are reduced in the mutant. Drg, dorsal root ganglia; wt, wild type; +/- , Uncx4.1/Uncx4.1.

Fig. 6. Analysis of anteroposterior somitic polarity as detected by neurofilament staining at 10.5 d.p.c. (A) Anti-neurofilament staining. Spinal nerves (arrows) traverse the anterior half of the somite only but axons appear less bundled in the mutant. (B) Neurofilament in situ hybridization at 10.5 d.p.c. Dorsal root ganglia are irregularly shaped in the mutant. wt, wild type; +/- , Uncx4.1/Uncx4.1.
well formed and the denser appearance of caudal sclerotome observed in the wild type is unchanged in the mutant. Expression of *Dll-1*, which marks the presomitic mesoderm as well as caudal somitic halves (Bettenhausen et al., 1995) was unaltered (data not shown). Hence, epithelialization and segmentation of somites appear normal in the mutant, whereas cranio-caudal polarity of the somite may be impaired to some degree.

The histological analysis clearly uncovered a requirement for *Uncx4.1* in lateral sclerotomal cells. To confirm and extend our histological analysis, we compared the expression of several marker genes for somite differentiation and chondrogenesis in 10-11.5 d.p.c. embryos. *Myogenin* is expressed in the myotomes in a repeating metameric pattern (Edmondson and Olson, 1989), *Pax1* in ventromedial sclerotome cells, initially with a rather uniform expression along the anteroposterior axis of the somite (Deutsch et al., 1991; Neubüser et al., 1995), *Pax9* in the ventrolateral sclerotome compartment with a strong upregulation in the posterior somite half (Neubüser et al., 1995). *Pax1* and *Pax9* synergize in the development of ventral vertebral elements (Peters et al., 1999). Expression of *myogenin* and *Pax1* is unchanged in the mutant (Fig. 7A-F). This suggests that the (dermo)myotome is not affected and medial sclerotome is not expanded at the expense of lateral sclerotomal fates in the absence of *Uncx4.1*. The *Pax9* expression domain is unaltered in the mutant, but the strong expression in the posterior somite half is lacking (Fig. 7G-L). Instead, a weak and uniform staining within the sclerotome with stronger staining at the somite borders is observed suggesting that the upregulation of *Pax9* in the posterior somite half depends at least partially on *Uncx4.1* expression.

*Scleraxis* is expressed in the entire sclerotome at 9.5 and 10.5 d.p.c. Subsequently, expression is confined to the precartilaginous mesenchymal anlagen, and is downregulated prior to the onset of chondrogenesis (Cserjesi et al., 1995). Gene targeting in combination with chimeric analysis has shown that *Scleraxis* is required for formation of chondrogenic lineages (Brown et al., 1999). At 11.5 d.p.c., *Scleraxis* expression marks the mesenchymal

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**Fig. 7.** Analysis of marker genes expressed during segmentation and sclerotome formation in 10-11.5 d.p.c. wild-type and *Uncx4.1*−/− embryos. (A-L) Whole-mount in situ hybridization with antisense riboprobes. The probes are indicated at the upper middle. Wild-type embryos are on the left, *Uncx4.1*−/− embryos on the right. *Myogenin* (A,B) and *Pax1* (C-F) expression is unaltered in the mutant. (A-D) 11.5 d.p.c. whole-mount embryos; (E,F) paraffin sections of whole-mount-stained 10.5 d.p.c. embryos, thoracic region. *Pax9* expression (G-L) is reduced in the posterior somite compartment. (G) 9.75 d.p.c. wild-type embryo, (H) 10.0 d.p.c. mutant embryo. (I,J) 1-somite-thick transverse sections through the cervical region of 10.5 d.p.c. embryos. The ventrolateral extent of the *Pax9* expression domain is unaltered. (K,L) Flat mounts of one half of a trunk between forelimb and hindlimb levels of 10.5 d.p.c. embryos. In the wild type (K), expression of *Pax9* is low in the anterior somite half (a) but high in the posterior half (open box, p). In the mutant (L), no upregulation in the posterior half is observed but strong staining at the somite borders is evident.
further supported by the expression of Sox9. Sox9 is expressed in condensed mesenchyme and the precursors of all cartilage (Wright et al., 1995). Recently, gene targeting proved that Sox9 is required for formation of cartilage from condensing mesenchyme (Bi et al., 1999). At 11.5 d.p.c., Sox9 expression is lacking solely in the anlagen of the pedicles and proximal ribs of the Uncx4.1-/- mutant suggesting that chondrogenesis is specifically missing in these elements of the vertebral column (Fig. 8B).

**DISCUSSION**

Our results show that Uncx4.1 is specifically required for the formation of vertebral elements derived from the lateral sclerotome. Along the entire vertebral column, the pedicles, proximal ribs and transverse processes are missing. Loss of these structures results in severe skeletal malformation leading to perinatal death. The mesenchymal anlagen of these elements are established, but do not condense or form a cartilaginous preskeleton. Uncx4.1 is not required or may only play a minor role in the establishment or maintenance of the craniocaudal polarity of the somite, nor does the loss of Uncx4.1 lead to gross morphological defects at other sites of expression. Similar results have been found by Mansouri et al. (2000) who have generated another mutation in Uncx4.1 using an alternative gene targeting strategy.

**Patterning the sclerotome**

Numerous embryological experiments have indicated the profound influence of the notochord in inducing sclerotomal cells from the ventral half of the newly formed somite. SHH, which is secreted by the notochord, has been shown to be the molecular signal both sufficient and required for the induction and maintenance of the sclerotome. SHH mutant embryos completely lack sclerotome-derived vertebral elements (Fan et al., 1995; Chiang et al., 1996). The present study in combination with a recent report on Pax1 and Pax9 double mutant embryos (Peters et al., 1999) extend our knowledge on sclerotome differentiation by suggesting genetic pathways involved in the regionalization of the vertebra.

Uncx4.1 is responsible for the specification of pedicles, transverse processes and proximal ribs, elements derived form the lateral sclerotome. Pax1 is initially expressed in all sclerotomal cells before it becomes restricted to the posterior ventromedial compartment (Deutsch et al., 1991). Pax9, which is the most closely related member to Pax1 within the paired-box family of transcriptional regulators, is predominantly expressed in the posterior ventrolateral compartment of the sclerotome (Neubüser et al., 1995). Gene targeting as well as natural mutants have shown that Pax1 is required for the formation of vertebral bodies and intervertebral discs, particularly in the lumbar-sacral region (Wallin et al., 1994; Dietrich and Gruss, 1995; Wilms et al., 1998). Pax9 single mutants do not exhibit defects in the vertebral column (Peters et al., 1998). Double mutants of Pax1 and Pax9 completely lack vertebral bodies, intervertebral discs and proximal ribs, structures derived from the ventromedial and ventrolateral sclerotome. Neural arches and distal ribs are unaffected, however. Hence, formation of vertebral bodies and intervertebral discs requires Pax1 and Pax9, proximal rib formation requires Pax1, Pax9 and Uncx4.1, whereas for pedicle formation Uncx4.1 is required. Other factors may exist, possibly also of the paired homeobox family, which control the development of the laminae and distal ribs.

The expression domains of Pax1, Pax9 and Uncx4.1 initially overlap in the caudal sclerotome. However, they are controlled by distinct mechanisms. Uncx4.1 expression depends on the Delta/Notch signaling pathway (Barrantes et al., 1999), whereas Pax1 and Pax9 induction (and maintenance) is regulated by signals from the notochord and floor plate, most importantly SHH (Neubüser et al., 1995, Münsterberg et al., 1995; Chiang et al., 1996). In agreement with this, expression of Uncx4.1 is unchanged in the Pax1/Pax9 double mutant (Peters et al., 1999), and Pax1 expression is not affected by loss of Uncx4.1 expression. However, the loss of Pax9 upregulation in the posterior somite compartment in the Uncx4.1 mutant suggests that Pax1 and Pax9 expression are differently regulated after the initial induction. Maintenance of Pax1 expression may depend on the continuous presence of SHH signal (Münsterberg et al., 1995; Chiang et al., 1996), whereas the upregulation of Pax9 expression in the posterior somite half is under the control of Uncx4.1 and thus of the Notch/Delta signaling pathway. Hence, Pax9 may be a target of Uncx4.1 and mediate its effect on sclerotomal cells in the ventrolateral posterior somite region possibly the ones fated to contribute to proximal rib development.

Later, expression domains are confined to the mesenchymal anlagen of the vertebral elements controlled by either factor or a combination of factors. Thus, regional specification of the sclerotome may be the consequence of a refinement of the expression domains of these factors, followed by their autonomous control of the further development of specific skeletal elements.

**Cellular consequences of Uncx4.1 function**

Our results show that Uncx4.1 is specifically required for the
formation of vertebral elements derived from the lateral sclerotome, the pedicles, transverse processes and the proximal ribs. The mesenchymal anlagen of these elements are established, as shown by Scleraxis expression, but do not condense or form a cartilaginous preskeleton. Uncx4.1 may therefore be required for the maintenance and further differentiation of these structures. These functions may be mediated by the control of proliferation and condensation of the mesenchyme from which these elements develop and by the control of chondrogenesis. The latter is substantiated by the fact that Sox9 expression is lacking specifically in the primordia of the elements controlled by Uncx4.1. Thus, both Scleraxis and Sox9 may serve as general control factors of skeletal development, the former in the formation of the mesenchymal prepattern, the latter in the formation of the cartilaginous template for the vertebral column. Of course, a thorough analysis of cellular changes in the lateral sclerotomal regions in the Uncx4.1 mutant will be required to gain a more detailed picture of the genes directly dependent on Uncx4.1. The analysis of the Pax1/Pax9 double mutants may suggest a similar cellular program regulated by these two factors in the specification of the ventral sclerotomal structures (Peters et al., 1999). In the Pax1/Pax9 double mutant, mesenchymal condensations are not formed in the ventromedial and ventrolateral sclerotomal regions. Both factors are required to maintain a high proliferative rate of mesenchymal cells, which might be required to initiate and maintain the chondrogenic program. A reduction of mesenchymal cell proliferation leads (most likely secondarily) to increased apoptosis and affects the maintenance of Sox9 expression which is, however, required for the chondrogenic program (Bi et al., 1999). We have not detected Sox9 expression in the precursors of the lateral sclerotome in the Uncx4.1 mutant embryos, but we cannot exclude that it is initially expressed and not maintained, as is observed in the Pax1/Pax9 double mutant. It is tempting to speculate that Pax1, Pax9, Uncx4.1 and related transcription factor genes controlling the development of distal ribs and laminae might be directly regulated by the bHLH transcription factor Scleraxis and that, in turn, Sox9 is a direct target of all of them.

Epithelialization and segmentation of somites

Somites appear as simple epithelial spheres from the caudal end of the unsegmented paraxial mesoderm. They become quickly polarized along the dorsoventral and mediolateral axis by signals from neighboring tissues, namely Sonic Hedgehog from the notochord and floor plate, Wnt signals from the dorsal neural tube and the surface ectoderm, and BMPs from the lateral plate (reviewed in Gossler and Hrabe de Angelis, 1998). These signals compete in inducing and maintaining the mesenchymal sclerotome in the ventral half, and the epithelial dermomyotome in the dorsal half of the somite. Craniocaudal polarity of the somite as indicated by a condensed caudal and a less condensed cranial sclerotomal half is not controlled by environmental cues but is under the regime of the Delta/Notch signaling system which already operates in the presomitic mesoderm (Hrabe de Angelis et al., 1997; Barrantes et al., 1999). Loss of Delta-like 1, Notch1 or components of the intracellular signaling pathway results in severe somite disorganization compatible with a role of this signaling system both in establishing craniocaudal (anteroposterior) polarity as well as epithelialization of the somite. Uncx4.1 expression is found in the entire caudal half of a newly formed somite suggestive of a role in establishing or maintaining somitic polarity along the AP axis. In support of this, Uncx4.1 expression depends on the Delta/Notch signaling pathway as shown by complete loss of its expression in Dil-1 mutants and a clear reduction in mutants of other members of this pathway (Evvard et al., 1998; Zhang and Gridley, 1998; Barrantes et al., 1999). However, our analysis of Uncx4.1 mutant embryos clearly showed that Uncx4.1 is not required for establishing or maintaining the AP polarity of the somite. Histological and marker analysis as well as the conservation of the metameric organization of the vertebral column argue that Uncx4.1 has no major role in this process. Craniocaudal polarity of the somite is also imposed on adjacent structures including the neural crest (Keynes and Stern, 1984). Dorsal root ganglia and spinal nerves are located in the lateral less condensed cranial sclerotomes. In the Uncx4.1 mutant, the spinal nerves still traverse the cranial compartment, but the trajectories appear less bundled. Similarly, the dorsal root ganglia appear less tight in shape. The fusion of dorsal root ganglia in newborn pups is probably secondary to the loss of lateral vertebral elements in the mutant, however, the subtle alterations of neural crest trajectories in the 10.5 and 11.5 day mutant embryo may point to a requirement for Uncx4.1 in mediating somitic cranio-caudal polarity onto the neural crest. Alternatively, a reduction of cell proliferation in the lateral caudal sclerotome might affect cell density in the cranial compartment.

Uncx4.1 function in embryogenesis

Uncx4.1, a transcription factor gene of the paired homebox class shows a dynamic temporal-spatial expression pattern during mouse development. Outside the paraxial mesoderm, expression is found at various sites in the central nervous system, in the branchial arch region, in the first digit of the forelimb and particularly strong in the condensing mesenchyme of the mesonephric and metanephric kidneys (Mansouri et al., 1997; Neidhardt et al., 1997). We have not detected gross morphological defects at these sites of expression. Our gene targeting approach using cre-mediated excision of the entire Uncx4.1 transcription unit has generated an unambiguous null allele excluding the possibility that there is residual Uncx4.1 activity in the embryos. It is possible that our analysis of the Uncx4.1 mutant has missed subtle phenotypes in these tissues. Alternatively, Uncx4.1 may exert its functions in a redundant manner with other transcription factors. Interestingly, Uncx4.1 expression in the condensing nephrogenic mesenchyme overlaps with the expression of the paired class transcription factor Pax2. Pax2 is also expressed in the Wolffian duct and its derivatives, and is required for setting up the whole urogenital system, precluding the analysis of its specific requirement in the nephrogenic mesenchyme (Dressler et al., 1990; Torres et al., 1995). Although the development of the kidney tubules from the nephrogenic mesenchyme on one hand and of the mesenchymal skeleton from the sclerotome on the other hand seem very different, they may share parallels on the cellular level. It is suggestive that Uncx4.1 in conjunction with other transcription factors of the homeobox or paired class controls similar processes in the nephrogenic and the sclerotomal mesenchyme, namely the local control of cell proliferation and condensation.
We thank Moises Mallo and Bodo Christ for discussion and advice. Peter Gruss and Eric Olson for probes, Rudi Balling for probes and communication of unpublished data. Werner Müller for loxp plasmids, and Rolf Klemier and Davor Sotler for support. The 129/Ola cosmid genomic library was obtained from the Resourcenzentrum (RZPD) in Berlin. The anti-neurofilament antibody 2F13 developed by Tom M. Jessell and Jane Dodd was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa.

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