Restriction of retinoic acid activity by Cyp26b1 is required for proper timing and patterning of osteogenesis during zebrafish development

Kathrin Laue1,2, Martina Jänicke1,2, Nikki Plaster1,*, Carmen Sonntag1 and Matthias Hammerschmidt1,2,†

Skeletal syndromes are among the most common birth defects. Vertebrate skeletogenesis involves two major cell types: cartilage-forming chondrocytes and bone-forming osteoblasts. In vitro, both are under the control of retinoic acid (RA), but its exact in vivo effects remained elusive. Here, based on the positional cloning of the dolphin mutation, we have studied the role of the RA-oxidizing enzyme Cyp26b1 during cartilage and bone development in zebrafish. cyp26b1 is expressed in condensing chondrocytes as well as in osteoblasts and their precursors. cyp26b1 mutants and RA-treated wild-type fish display a reduction in midline cartilage and the hyperossification of facial and axial bones, leading to fusions of vertebral primordia, a defect not previously described in the context of RA signaling. Fusions of cervical vertebrae were also obtained by treating mouse fetuses with the specific Cyp26 inhibitor R115866. Together with data on the expression of osteoblast markers, our results indicate that temporal and spatial restriction of RA signaling by Cyp26 enzymes is required to attenuate osteoblast maturation and/or activity in vivo. cyp26b1 mutants may serve as a model to study the etiology of human vertebral disorders such as Klippel-Feil anomaly.

KEY WORDS: Cyp26b1, Retinoic acid, Bmp2, Cartilage, Bone, Chondrocyte, Osteoblast, Osteopontin, Osterix, Craniofacial development, Vertebra, Zebrafish

INTRODUCTION

Skeletal development is highly conserved in vertebrates and involves two main processes: skeletal patterning to define the shape and location of the different skeletal elements within the developing body, and differentiation of skeletogenic cells (Karsenty and Wagner, 2002; Mariani and Martin, 2003). Cartilage-forming chondrocytes and bone-forming osteoblasts share a common mesenchymal progenitor that derives from neural crest, sclerotome or lateral plate mesoderm (Olsen et al., 2000). Skeletogenesis is initiated when mesenchymal cells aggregate to form mesenchymal condensations. In most parts of the bony skeleton, including the vertebral column of mammals, but not of teleosts (Bird and Mabee, 2003; Elizondo et al., 2005; Fleming et al., 2004; Inohaya et al., 2007), a cartilaginous anlage serves as a template to model the future bone (endochondral ossification). In this case, cells within the condensation become chondrocytes, whereas cells at the periphery of the skeletal element form a structure called the perichondrium (Karsenty and Wagner, 2002). During ossification, chondrocytes in the core of the condensate become hypertrophic, a transition reflected in the switch from Col2a1 (encoding collagen type II) to Col10a1 (collagen type X) expression, while osteoblasts start to mature within the perichondrium (now also called the periosteum) and form ossification centers that eventually replace the cartilage (Colnot, 2005; Karsenty and Wagner, 2002). Alternatively, some skeletal elements, including parts of the craniofacial system, are generated by direct differentiation of mesenchymal cells into osteoblasts (intramembranous or dermal ossification). Maturing osteoblasts express the same marker genes as hypertrophic chondrocytes, including the transcription factor gene runx2 (also called cbfa1) (Flores et al., 2006; Flores et al., 2004), osteopontin (opn; also called sp1) (Kawasaki et al., 2004), which encodes a component of bone matrix (Alford and Hankenson, 2006), and, at least in zebrafish, col10a1 (Avaron et al., 2006), whereas the transcription factor Osterix (Osx; also called Sp7) is a specific marker and regulator of the osteoblast lineage (Nakashima et al., 2002).

A known signal regulating skeletogenic cell development is all-trans retinoic acid (RA) (Adams et al., 2007; Weston et al., 2003), a derivative of vitamin A that is required for multiple processes of vertebrate development (Niederreither and Dolle, 2008). RA is a diffusible lipophilic molecule that binds to nuclear receptors [retinoic acid receptors (RARs) and retinoid X receptors (RXRs)] to regulate the transcription of target genes. RA concentrations are determined by the balance between RA synthesis via retinaldehyde hydrogenases (Aldh1-3) and RA oxidation by cytochrome P450 enzymes of the Cyp26 class (Blomhoff and Blomhoff, 2006; Fujii et al., 1997; White et al., 1997). As in mammals, three different zebrafish cyp26 genes have been described: cyp26a1, cyp26b1 and cyp26c1 (formerly cyp26d1), which are expressed in distinct, but partially overlapping patterns (Abu-Abed et al., 2001; Emoto et al., 2005; Gu et al., 2005; Hernandez et al., 2007; Kudoh et al., 2002; MacLean et al., 2001; Tahayato et al., 2003; Zhao et al., 2005). The in vivo requirement for Cyp26 enzymes was revealed via Cyp26a1 and Cyp26b1 gene targeting in mouse (Abu-Abed et al., 2001; MacLean et al., 2007; Yashiro et al., 2004), and via cyp26a1 (giraffe) mutants (Emoto et al., 2005) and antisense-mediated knockdown of cyp26a1, cyp26b1 and cyp26c1 in zebrafish (Echeverri and Oates, 2007; Hernandez et al., 2007; Kudoh et al., 2002; Reijntjes et al., 2007; Shelton et al., 2006; White et al., 2007). Of the zebrafish reports, only one addressed the role of Cyp26 enzymes during skeletogenesis, claiming that Cyp26b1 is required for the patterning and migration of cranial neural crest (Reijntjes et al., 2007).

1Max-Planck-Institute of Immunobiology, Stuebeweg 51, D-79108 Freiburg, Germany. 2Institute for Developmental Biology, University of Cologne, D-50923 Cologne, Germany.
*Present address: Department of Developmental and Cell Biology, UCI, Irvine, CA 92697, USA
†Author for correspondence (e-mail: mhammers@uni-koeln.de)

Accepted 1 October 2008
Knockout of Cyp26b1 in mouse causes severe limb defects that have been attributed to a combination of shifts in the proximodistal patterning of the limb bud and a retardation of chondrocyte maturation (Yashiro et al., 2004). This suggests that Cyp26b1 interferes with the reported role of RA in blocking chondrocyte specification from mesenchymal precursors (Weston et al., 2003). Other data suggest a later and seemingly opposing role for RA signaling in promoting hypertrophic maturation of chondrocytes and subsequent replacement by bone (Weston et al., 2003), although this has not yet been addressed genetically. Also, it has remained unclear to what extent this latter effect is due to interference with chondrocytes (Iwamoto et al., 1993; Weston et al., 2003) versus osteoblasts (Manji et al., 1998; Song et al., 2005) and with osteoblast maturation versus activity.

Here, we have studied the role of Cyp26b1 as an essential regulator of skeletal development in zebrafish. cyp26b1 is expressed in chondrogenic mesenchymal condensations as well as in osteoblast precursors of endochondral and intramembranous bones, including vertebrae. cyp26b1 mutants display multiple defects during chondro- and osteogenesis, all of which can be mimicked by treatment with RA. This indicates that in contrast to a recent report (Reijntjes et al., 2007), zebrafish Cyp26b1 acts by restricting retinoid signaling. The hyperossification of craniofacial bones and vertebrae of mutant animals is anticipated by an increase in osteopontin expression in osteoblasts. Comparing the axial defects of cyp26b1 mutants with those caused by transgenic overexpression of the Bone morphogenetic protein Bmp2, a well-known positive regulator of osteoblast maturation upstream of Runx2 and Osd (Ulsamer et al., 2008; Wu et al., 2007), we noticed striking differences in the pattern of hyperossification and in the molecular signature of the supernumerary osteoblasts. Together, these data suggest that RA inhibition by Cyp26 enzymes is required to restrict ossification in vivo, most likely by attenuating osteoblast activity.

MATERIALS AND METHODS

Zebrafish lines

Unless noted otherwise, the dol allele ti230g was used (Piotrowski et al., 1996). For temporally controlled bmp2b overexpression, the transgenic line Tg(hsp70:bmp2b) was used as described (Chocron et al., 2007). According to RT-PCR analyses, the transgene is activated within 10 minutes after the heat shock, with transcripts remaining detectable for ~24 hours.

Mapping and cloning of dol/cyp26b1

Genetic rough mapping of dol230g was carried out via bulk meiotic segregation analyses as described (Laue et al., 2008). For chromosomal walking, the BAC libraries CHORI-211 and DanioKey (RZPD) were screened by PCR. For identification of the mutant lesion and for genotyping, walking, the BAC libraries CHORI-211 and DanioKey (RZPD) were screened by PCR. For identification of the mutant lesion and for genotyping, primer sequences are available upon request.

Morpholino oligonucleotide (MO) injections

An MO against the cyp26b1 splice-donor junction between exon 3 and intron 3 (Cyp26b1-SDEx3 MO, 5′-AGCATATTCACCATTTTGC- TTCTCTGT-3′) was purchased from Gene Tools. For full knockdown, ~0.5 pmol, and for hypomorphs (Fig. 6E), 0.2 pmol, of MO was injected per embryo at the one-cell stage as described (Nasevicius and Ekker, 2000). aldh1a MO was used as described (Begemann et al., 2001).

Tissue labeling procedures

In situ hybridization and fluorescent double in situ hybridization were performed as described (Clay and Ramakrishnan, 2005; Hammerschmidt et al., 1996). For zebrafish opn and osx, cDNA fragments were amplified by RT-PCR with primer sequences obtained from the Ensembl database, and cloned into pCRII-TOPO (Invitrogen). For probe synthesis, plasmids were linearized with XhoI and transcribed with SP6 RNA polymerase. For cyp26b1, clone IMAGp998A011194Q1 was obtained from RZPD, linearized with KpnI, and transcribed with SP6 RNA polymerase. Probes for cyp26a1 and cyp26c1 (Hernandez et al., 2007), col2a1 (Yan et al., 1995), coll1a1 (Nica et al., 2006), dix2a (Akimenko et al., 1994) and sox9a (Wada et al., 2005) were synthesized as described.

Immunostainings were carried out using the Vectastain ABC Kit (produced by the University of Oregon), anti-GFP (1:200, Roche). For sectioning, embryos were embedded in paraffin.

RESULTS

The phenotype of dolphin mutants is caused by Cyp26b1 loss-of-function

The dolphin mutant dolP230g, which was previously identified in a large-scale ENU mutagenesis screen, is characterized by a beak-like appearance of the jaw (Fig. 1A,B) (Piotrowski et al., 1996) and, in some genetic backgrounds, by shorter and malformed pectoral fins (Fig. 1C,D). Using bulk segregation linkage analysis, the dolphin locus was mapped to a 0.38 cm interval on linkage group 7 (Fig. 1E); this was followed by a BAC walk to construct a contig covering the interval (Fig. 1F). Newly designed markers (KL1-7) among three overlapping BACs within the contig showed no recombination in 4500 meioses (Fig. 1F).

The three BACs contained several genes, all of which were sequenced. Only in the cyp26b1 gene of mutants was a mutation found, comprising a GT→AT transition in the splice-donor site of the exon 3-intron 3 junction (Fig. 1H). Sequencing of independent cDNA clones revealed the use of a downstream GT as a novel splice-donor in mutants (50/50), but not in wild-type embryos of the same strain (0/50) (Fig. 1I,J). The resulting transcript carries an insertion of seven nucleotides, leading to a frame shift and premature termination of the protein. This C-terminal truncation removes most of the highly conserved cytochrome P450 domain, including the oxygen-, steroid- and heme-binding sites (Fig. 1G). Subsequently, a second cyp26b1 allele, sa0002, was identified by TILLING (Wienholds et al., 2003), with an AAG+TAG nonsense mutation at nucleotide position 135 of the coding region, causing an even more severe truncation of the protein after 45 amino acid residues (Fig. 1G) (http://www.sanger.ac.uk/cgi-bin/Projects/D_rerio/mutres/ tracking.pl). sa0002 failed to complement ti230g, and sa0002 mutants showed craniofacial and axial defects indistinguishable from those of ti230g mutants (see Fig. 1G). In contrast to full-length Cyp26b1, both truncated versions were completely inactive upon forced expression in early zebrafish embryos (see Fig. S1L in the supplementary material). Furthermore, the axial hyperossification of ti230g mutants could be rescued or converted to hypo-ossified phenotypes by temporally controlled reaplication of wild-type cyp26b1 (see Fig. S1M and S1N).
S1H in the supplementary material). Finally, the defects of dol mutants could be phenocopied in wild-type fish by injecting an antisense MO targeting the splice site affected in the ti230g allele (Fig. 4G,N; Fig. 5C; Fig. 6E,F; see Fig. S2 in the supplementary material). Together, this indicates that the defects of zebrafish dol mutants are caused by null mutations in the cyp26b1 gene.

cyp26b1 is expressed in cranial precartilage condensations, perichondrial cells and osteoblasts
cyp26b1 has been shown to be expressed in various regions of zebrafish embryos (Hernandez et al., 2007; Zhao et al., 2005). We have extended these studies, focusing on developing skeletal elements. At 26 hours post-fertilization (hpf), cyp26b1 was expressed in multiple domains in close proximity to postmigratory cranial neural crest (CNC) cells, but not in CNC itself, as revealed by double stainings with cyp26b1 and the CNC markers dlx2a or fli1a (Fig. 2A-D). cyp26b1 was prominently expressed in the forehead close to the CNC that, according to cell-tracing experiments, gives rise to chondrocytes of the neurocranial ethmoid plate (Eberhart et al., 2006; Wada et al., 2005) (Fig. 2A,B). In addition, cyp26b1 was expressed adjacent to postmigratory CNC that gives rise to the pharyngeal arches (Fig. 2C,D).

At 48 hpf, cyp26b1 was coexpressed with sox9a (Fig. 2H) in mesenchymal condensations that give rise to pharyngeal arches (Fig. 2F) and neurocranial cartilages (Fig. 2G). However, concomitant with the onset of col2a1 expression, which is a marker for specified chondrocytes (Yan et al., 1995), cyp26b1 expression ceased within the cartilaginous elements but remained strong in perichondrial cells (Fig. 2I-K). A similar transient expression in mesenchymal condensations and persistent expression in perichondria of the craniofacial skeletal elements has also been described for mouse Cyp26b1 (Abu-Abed et al., 2002). The perichondrium is a supposed source of osteoblasts. However, in contrast to widespread perichondrial expression of cyp26b1, expression of the osteoblast markers osterix (osx) (Fig. 2L), osteopontin (opn) (Fig. 2M) and col10a1 (Fig. 2N) (Avaron et al., 2006) was confined to those perichondrial domains undergoing endochondral ossification (Fig. 2O). In such col10a1-positive cells, cyp26b1 transcript levels were lower than in the adjacent col10a1-negative perichondrium (Fig. 2P,Q), suggesting that cyp26b1 expression decreases when osteoblasts differentiate and/or become active.

In addition to the perichondrium/periosteum, zebrafish cyp26b1 was expressed in various bone primordia that ossify in an intramembranous manner (Cubbage and Mabee, 1996; Elizondo et al., 2005). Examples include the opercle (see Fig. 3A,D) and cleithrum (see Fig. S4G in the supplementary material). At 72 and 120 hpf, the opercular bone matrix (Fig. 3C,F) was surrounded by osteoblasts coexpressing col10a1, osx and opn (Fig. 3B,E; see S4A-F in the supplementary material). Double in situ hybridization for cyp26b1 and col10a1 further revealed that, similar to in the perichondrium, cyp26b1 levels in col10a1-positive osteoblasts of the opercle (Fig. 3A,D) and cleithrum (see Fig. S4G in the supplementary material) were considerably weaker than in adjacent cells, which are most likely immature and/or less active osteoblasts.
Osteoblast expression of cyp26b1 was also found in the elements of the axial skeleton. The anterior part of the notochord, which becomes uniformly ossified (basioccipital articulatory process) (see Fig. 6A), was lined by a continuous layer of cyp26b1-positive cells (Fig. 3G), whereas in trunk and tail, where ossification of vertebral primordia occurs in a segmented manner, cyp26b1-positive cells displayed a corresponding metameric distribution (Fig. 3H,I). The same metameric pattern was obtained for the osteoblast markers opn and col10a1 (Fig. 3L-N). The position of such osteoblasts at intersomitic borders coincided with the anterior borders of forming vertebral bodies stained with Alizarin Red (Fig. 3O), suggesting that cells were localized within the intervertebral zones. Comparative expression analyses at different developmental time points further revealed a continuous decline in the number of axial cyp26b1-positive cells from 96-156 hpf, while the number of col10a1- and opn-positive cells increased (Fig. 3P), with transient coexpression of cyp26b1 and opn in the same cells at 144 hpf (Fig. 3J).

cyp26b1 mutants lack cartilaginous elements in the midline of the neurocranium and pharyngeal arches

Alcian Blue stainings of the cartilage of cyp26b1 mutants and morphants at 120 hpf revealed specific defects in midline elements of the visceral skeleton and the neurocranium, whereas most other cartilaginous elements appeared largely normal (Fig. 4E-G). The ceratohyals of the left and right second arches were fused in the midline (Fig. 4H,I), and the medial elements (basibranchial) of the posterior gill arches (p5, p6) were missing (Fig. 4J,K). In addition, the medial ethmoid plate of the neurocranium was missing or strongly reduced (Fig. 4L-N), very similar to the phenotype previously described for mutants in sonic hedgehog (Wada et al., 2005). To determine when this defect arises, we stained embryos for col2a1 and sox9a to label chondrocytes and their precursors. At 36 hpf, col2a1 is expressed in chondrocyte precursors of the trabeculae cranii (Fig. 4A), which are lateral structures of the neurocranium surrounding the ethmoid plate and extending further posteriorly (Schilling and Kimmel, 1997). At this stage, col2a1 expression in cyp26b1 mutants appeared normal (Fig. 4B). However, at 56 hpf, the col2a1 expression domains were shifted posteriorly and fused at the midline, anticipating the subsequent absence of the medial ethmoid (Fig. 4C,D).

cyp26b1 mutants display hyperossification of craniofacial bones and axial skeleton, leading to the fusion of vertebral bodies

As in higher vertebrates, the majority of the zebrafish craniofacial skeleton forms through endochondral ossification, starting at 6 days post-fertilization (dpf); for example, in restricted regions of the
ceratohyal and hyomandibula of the second pharyngeal arch. Intramembranous bones form even earlier, with mineralization of the opercle starting at 3 dpf (Cubbage and Mabee, 1996).

Staining cyp26b1 mutants or morphants for mineralized bone matrix with Alizarin Red, we observed hyperossification of both endochondral and intramembranous bones. At 192 hpf, the mineralized domain in the opercle of mutants and morphants was larger than in wild-type animals (Fig. 5A-C). Also, mutants displayed significantly stronger and more advanced endochondral mineralization of the ceratohyal (Fig. 5D-F).

In addition to craniofacial defects, cyp26b1 mutants and morphants exhibited severe abnormalities in the axial skeleton, which in teleost larvae is formed through ossification of the sheath around the notochord (perichordal ossification). Vertebral ossification starts at the level of the fourth vertebral body (centrum), from where it proceeds anteriorly and posteriorly (Bird and Mabee, 2003; Gavaia et al., 2006; Stemple, 2005). At 180 hpf, wild-type larvae exhibited six to eight Alizarin Red-positive centra, with centra 3 and 4 correspondingly broader than the others (Du et al., 2001) (Fig. 6C,F). In cross-sections, the mineralized perichondal sheath of mutants appeared broader and more strongly stained than in wild-type zebrafish, unsegmented ossification around the anterior part of the notochord gives rise to the basioccipital articulatory process, while metamic mineralization more posteriorly forms the vertebral column. Vertebral ossification starts at the level of the fourth vertebral body (centrum), from where it proceeds anteriorly and posteriorly (Bird and Mabee, 2003; Gavaia et al., 2006; Stemple, 2005). At 180 hpf, wild-type larvae exhibited six to eight Alizarin Red-positive centra, with centra 3 and 4 correspondingly broader than the others (Fig. 6A). By contrast, cyp26b1 mutants and morphants showed a complete fusion of Alizarin Red-positive vertebral bodies (O). Arrows in L-N point to positive cells, arrowheads to borders of somites 5-8. Numbers of perichordal cyp26b1-, col10a1- and opn-positive cells at different developmental time points. Ten fish were evaluated per condition; standard errors are indicated.

Fig. 3. cyp26b1 is expressed in osteoblasts and their precursors. Stainings of wild-type zebrafish at the stages indicated in the upper right corners and with the in situ RNA probes indicated in the lower right corners. (A-F) Confocal sections of double fluorescent in situ hybridizations and Alizarin Red stainings (alR), counterstained with DAPI (blue). Cells with weak cyp26b1 and strong col10a1 expression are indicated with white arrows, cells with strong cyp26b1 but absent col10a1 expression with red arrows, and cells with strong col10a1 but absent cyp26b1 expression, which most likely represent fully mature/active osteoblasts, with green arrows. See text for details. For single-channel images of B,E, see Fig. S4 in the supplementary material. (G-I) cyp26b1 displays uniform expression in perichordal cells in anterior regions of the notochord (n) (G, left panel shows longitudinal section; right panel shows transverse section; counterstained with Eosin), and metameric expression in the trunk (H,I; lateral views). In H, cyp26b1-positive cells are (still) underneath the notochord (arrowhead) and others are (already) in perichordal positions (arrows), whereas in I all cells are perichordal (arrows). Positive cells dorsal of the notochord in K are most likely represent ventral spinal cord neurons (scn). (J-O) col10a1 and opn show a similar expression pattern to cyp26b1 (L-N) and transient coexpression with cyp26b1 (J,K) at intersomitic borders, coincident with the anterior borders of the the Alizarin Red-positive vertebral bodies (O). Arrows in L-N point to positive cells, arrowheads to borders of somites 5-8.
siblings, whereas notochordal cells remained Alizarin Red-negative and normally vacuolated (Fig. 6G-J). Interestingly, cyp26b1 heterozygotes and wild-type embryos injected with lower amounts of cyp26b1 MO displayed an intermediate phenotype with distinct, but broader, centra in anterior regions and precocious centra mineralization in caudal regions of the notochord (Fig. 6, compare B,E with A,D). This suggests that Cyp26b1 is required to attenuate vertebral growth.

**Treatment with RA phenocopies, and inhibition of RA synthesis rescues, the craniofacial and axial defects of cyp26b1 mutants**

In mouse, there is evidence for a negative effect of Cyp26 enzymes on retinoid signaling (Fujii et al., 1997; Niederreither et al., 2002; White et al., 1997). However, according to a recent study, the phenotype of the cyp26b1 morphant zebrafish more closely resembles that of RA deficiency, suggesting that Cyp26 enzymes might generate, rather than metabolize, biologically active retinoids (Reijntjes et al., 2007). To test this, and to determine the crucial time window(s) of Cyp26b1 activity, we treated wild-type and cyp26b1 mutant embryos for various time intervals with all-trans RA (RA excess) or the competitive Aldh inhibitor 4-(diethylamino)benzaldehyde (DEAB; RA deficiency).

Strikingly, RA treatment of wild-type embryos from 24 to 50 hpf caused the same neurocranial phenotype as in cyp26b1 mutant embryos, characterized by the absence of the medial ethmoid plate at 120 hpf (Fig. 7A,B; compare with Fig. 4M) \((n=25/25)\). By contrast, RA treatments commencing after 48 hpf did not alter anterior neurocranial morphology (data not shown). Conversely, DEAB treatment of cyp26b1 mutants and morphants from 24 to 50 hpf caused the same craniofacial and axial defects as in cyp26b1 mutant embryos, characterized by the absence of the medial ethmoid plate at 120 hpf (Fig. 7A,B; compare with Fig. 4M) \((n=25/25)\). By contrast, RA treatments commencing after 48 hpf did not alter anterior neurocranial morphology (data not shown). Conversely, DEAB treatment of cyp26b1 mutants and morphants from 24 to 50 hpf caused the same craniofacial and axial defects as in cyp26b1 mutant embryos, characterized by the absence of the medial ethmoid plate at 120 hpf (Fig. 7A,B; compare with Fig. 4M) \((n=25/25)\).
hpf rescued the ethmoid phenotype (Fig. 7D) (*n=9/10), whereas the same treatment of wild-type embryos left neurocranial morphology intact (Fig. 7C) (*n=29/29) but caused a reduction of gill arches, reminiscent of the phenotype of aldh1a mutants (Begemann et al., 2001). Together, this indicates that the anterior neurocranial cartilage defects of cyp26b1 mutants are caused by RA excess during the second day of development.

Interference with bone development required significantly later treatments. Whereas RA treatment from 24 to 50 hpf had no effect on craniofacial ossification at 180 hpf (Fig. 7, compare F with E) (*n=45/45), treatment from 72 to 96 hpf and onwards caused hyperossification of both endochondral and intramembranous bones (Fig. 7G) (n=25/26), comparable to that seen in cyp26b1 mutants (Fig. 5B,E). Consistently, craniofacial ossification of mutants was significantly reduced upon DEAB treatment starting at 72 or 96 hpf (Fig. 7H). Similarly, treatment of wild-type larvae with RA or the specific Cyp26 inhibitor R115866 (Njar et al., 2006) from 96 hpf resulted in axial hyperossification at 180 hpf (Fig. 7K,L) (n=32/33 and 27/27), as in cyp26b1 mutants (Fig. 7S), whereas earlier RA treatments (48-72 hpf) had no effect (Fig. 7I) (n=40/40). Conversely, ossification of centra was blocked or significantly reduced when wild-type or cyp26b1 mutant larvae were treated with DEAB from 96 hpf (Fig. 7Q,R) (n=33/33). Intriguingly, a significant reduction in the axial hyperossification of cyp26b1 mutants (Fig. 7M) to wild-type levels was obtained upon injection of aldh1a MO (Begemann et al., 2001) (Fig. 7S,T) (n=9/12). Together, this indicates that Cyp26b1 is required at different developmental stages to regulate skeletal patterning and ossification of skeletal elements by inactivating RA. This anti-RA effect is consistent with the data obtained in mouse, but in contrast to the conclusions by Reijntjes et al. described above (Reijntjes et al., 2007).

The temporal and spatial pattern of axial hyperossification caused by RA differs from that caused by Bmp2b

To gain insight into the cellular basis of RA-induced hyperossification, we compared the effects of RA application with those caused by excessive Bmp2, a well-known positive regulator of osteoblast maturation (Wu et al., 2007). For temporally controlled Bmp2 overexpression, we used transgenic fish that carry the bmp2b cDNA under the control of the heat-inducible hsp70 promoter (Chocron et al., 2007). Applying the heat shock at 50 hpf, when RA applications had no effect on centra ossification (Fig. 7J), transgenic bmp2b fish displayed a fusion of centra 3-6 (Fig. 7O). These are the first ossifying centra during normal development (see above). By contrast, Bmp2 applied at 96 or 144 hpf left the early-ossifying centra unaffected, causing fusions of the later ossifying, more anterior and more-posterior vertebral bodies (Fig. 7P) (data not shown), whereas centra 3-6 were fused upon RA application at these later stages (Fig. 7K; see Fig. S6A,B in the supplementary material). Strikingly, fusions of centra 3-6 were even obtained upon RA administration at 12 or 15 dpf (Fig. 7M,N; see Fig. S6C-F in the supplementary material). This indicates that Bmp2 affects osteoblast precursors at stages when they are not yet sensitive to RA, whereas RA can still affect osteoblasts long after they have become insensitive to Bmp2.

cyp26b1 mutant osteoblasts display increased expression of osteopontin

To directly compare the effects of Cyp26b1/RA and Bmp2b on osteoblasts, we stained the osteoblast markers col10a1, osx, opn and cyp26b1 itself. In craniofacial skeletal elements of cyp26b1 mutants, cyp26b1 expression was much stronger than in wild-type siblings (Fig. 8A). Similarly, osteoblasts of the opercle of mutants displayed stronger opn expression. At 72 hpf, the number of opn-positive cells appeared normal, with higher expression levels per cell (Fig. 8G). However, at 120 hpf, there were supernumerary cells in normally opn-negative subdomains of the opercle (Fig. 8C). By contrast, expression levels and patterns of osx and col10a1 appeared unaltered in mutants (Fig. 8D,E).

Similarly, in the axial skeleton, cyp26b1 mutants displayed a striking increase in cyp26b1- and opn-positive cells, with premature expression and an extension into more-posterior trunk regions (Fig. 8F,G; see Fig. S7 in the supplementary material), whereas the number of col10a1-positive cells was normal (Fig. 8H). Ectopic cyp26b1-positive cells were present ventral of the notochord, in contrast to their preferential perichondral localization in wild-type siblings (Fig. 8F). According to previous studies, axial osteoblasts stem from the sclerotome in ventral-most regions of the somites, from where they move dorsally towards the notochord (Inohaya et al., 2007; Morin-Kensicki and Eisen, 1997), consistent with the...
ventral-to-dorsal progression of centra ossification (Fig. 6K). Thus, the ventral cyp26b1-positive cells are possibly immature osteoblasts that express cyp26b1 precociously in the mutant.

By contrast, overexpression of Bmp2b left the number of cyp26b1-positive cells unaffected (Fig. 8I), suggesting that their increase in cyp26b1 mutants might primarily reflect the loss of a negative RA→Cyp26 feedback loop, whereas late DEAB treatment reverses the mutant phenotype and causes delayed ossification (H). (I-T) Alizarin Red-stained centra after treatment with RA (J,K,N), R115866 (L), DEAB (Q,R) or heat shock (hs; O,P) at indicated developmental stages, or after aldh1a MO injection (T); 180 hpf (I-L,O-T) or 360 hpf (M,N; vertebrae numbers indicated); lateral views. In I-P, the early-specific centra at the level of somites 3-6 are indicated by a bar. Inset in P shows precocious and unsegmented perichondral mineralization at somite levels 18-26 of the same bmp2b transgenic animal. See text for details.

Fig. 7. Retinoic acid (RA) deficiency reverts, whereas RA excess phenocopies, the skeletal alterations of cyp26b1 mutants, with a pattern of axial hyperossification different from that caused by Bmp2b overexpression. Genotypes are indicated in upper right corners, treatments in lower left corners. (A-D) Flat-mounts of Alcian Blue-stained neurocrania after treatment from 24-50 hpf; 120 hpf, dorsal views. Note the absence of the medial ethmoid plate (e) in the RA-treated wild-type zebrafish (B), and the recovery of this structure in the DEAB-treated cyp26b1 mutant (D). (E-H) Alizarin Red-stained heads after treatment from 24-50 hpf (F) or from 96-180 hpf (G,H); 180 hpf, dorsal views. Late (G), but not early (F), RA treatment phenocopies hyperossification of craniofacial bones, whereas late DEAB treatment reverses the mutant phenotype and causes delayed ossification (H). (I-T) Alizarin Red-stained centra after treatment with RA (J,K,N), R115866 (L), DEAB (Q,R) or heat shock (hs; O,P) at indicated developmental stages, or after aldh1a MO injection (T); 180 hpf (I-L,O-T) or 360 hpf (M,N; vertebrae numbers indicated); lateral views. In I-P, the early-specific centra at the level of somites 3-6 are indicated by a bar. Inset in P shows precocious and unsegmented perichondral mineralization at somite levels 18-26 of the same bmp2b transgenic animal. See text for details.

Inhibition of Cyp26 enzymes during mouse development leads to axial hyperossification and to fusion of cervical vertebrae

To study whether Cyp26 enzymes might have a similar role in restricting ossification during mammalian development, we treated mouse fetuses with the Cyp26 inhibitor R115866, starting at E13, shortly before the onset of vertebral ossification in untreated animals. At E18.5, treated mice often displayed fusions of neural arches of cervical vertebrae, particularly in C3-C5 (Fig. 9A,B) (n=4). However, no fusions were seen in thoracic, sacral or lumbar vertebrae, similar to the cervical restriction of fusions that is seen in several human vertebral disorders. Instead, in posterior regions, treated mice displayed ossification defects within vertebrae, including precocious fusions of neural arches with centra (Fig. 9C,D) (n=9). Also, the ribs were significantly thicker than in untreated embryos. In summary, this indicates that inhibition of Cyp26b1 causes similar shifts in the temporal and spatial pattern of ossification in mammals as in zebrafish.

DISCUSSION

Previous studies have shown that RA signaling plays multiple roles during skeletal patterning and the differentiation of skeletogenic cells. However, genetic evidence for the in vivo role of RA signaling and its inhibition during osteoblast development has thus far been missing. Here, we show that spatiotemporal restriction of RA
signaling by Cyp26b1 is required to attenuate osteoblast maturation/activity and ossification during zebrafish and mouse development. These studies reveal a previously unrecognized effect of unrestricted RA signaling on vertebral column formation, which could also be relevant in human congenital disorders with vertebral fusions. Furthermore, we demonstrate an earlier role of Cyp26b1 in skeletal patterning of the neurocranium, consistent with the palatal clefting caused by fetal exposure to teratogenic retinoid doses in human (Lammer et al., 1985; Young et al., 2000).

**Cyp26b1-dependent RA restriction is essential for the formation of craniofacial midline cartilages**

Zebrafish cyp26b1 mutants and wild-type embryos treated with RA during the second day of development display very specific deficiencies of cartilaginous elements in the midline of the neurocranium and pharyngeal arches (Fig. 4). Spatial restriction of these defects could be due to functional redundancy between the three Cyp26 paralogs in other craniofacial regions. Consistently, the cyp26b1 expression domain in the forehead, adjacent to the reported
location of precursors of the ethmoid plate, is one of the few cyp26b1-specific domains that lack cyp26a1 and cyp26c1 expression (Gu et al., 2005) (see Fig. S3A-C in the supplementary material).

What exactly excessive RA is doing to the midline cells remains elusive. In contrast to a recent report (Reijntjes et al., 2007), we found that the migration (see Fig. S5 in the supplementary material) and survival (our unpublished data) of CNC cells was unaffected in cyp26b1 mutants. In cell culture systems, RA can block chondrocyte specification (Weston et al., 2003). Accordingly, the limb malformations in Cyp26b1 mutant mice are due to chondrocyte apoptosis, which might result from such failed specification (Yashiro et al., 2004). In cyp26b1 mutant zebrafish, however, such a mechanism seems unlikely because we could not detect any increase in the number of TUNEL- or Acridine Orange-positive cells in the affected craniofacial domain between 24 and 96 hpf (our unpublished observations). In addition, cell proliferation rates, as determined via anti-phosphohistone H3 immunostaining, appeared unaltered (our unpublished observations). In this light, we currently favor the possibility that Cyp26b1 might interfere with early skeletal patterning; for example, by modulating a morphogenetic effect of an RA gradient to determine differential cell fates, as has recently been reported for Cyp26a1 during hindbrain patterning (White et al., 2007). Indeed, both in the neurocranium and the branchial skeleton, cyp26b1 is expressed medial to the RA-synthesizing enzyme Aldh1a1 (see Fig. S3D-G in the supplementary material). This suggests that the craniofacial system might be patterned by a mediolateral RA gradient, with lowest levels in medial positions. This would explain why in cyp26b1 mutants, only the medial-most cells are lost (Fig. 4J-M). Similarly, the altered neurocranial col2a1 expression pattern in cyp26b1 mutants at 50 hpf could indicate that medial positions (normally col2a1-negative) have acquired lateral (col2a1-positive) fates (Fig. 4C,D).

**Cyp26b1-dependent RA restriction is required for proper spatiotemporal control of osteoblast biology and bone formation**

In Cyp26b1 mutant mice, possible defects during bone formation have only been marginally addressed (Yashiro et al., 2004). Also, expression of mouse Cyp26b1 in osteoblasts has not been described (Abu-Abed et al., 2002). Here, we show that zebrafish cyp26b1 transcripts colocalize at least transiently with oxs, col10a1 and opn, even in developing intramembranous bones that lack chondrocytes, strongly suggesting that cyp26b1 is expressed in osteoblasts. Our analyses further indicate that cyp26b1 expression levels are particularly high in immature and/or less active osteoblasts, whereas expression in fully differentiated and/or highly active osteoblasts is much lower, in line with its proposed role in attenuating osteoblast expression in fully differentiated and/or highly active osteoblasts is particularly high in immature and/or less active osteoblasts, whereas expression in fully differentiated and/or highly active osteoblasts is much lower, in line with its proposed role in attenuating osteoblast expression levels are often used to measure osteoblast activity because they increase in proportion to the amount of mineralized bone material, also in response to RA (Manji et al., 1998; Ohishi et al., 1995; Song et al., 2005). Second, cyp26b1 mutants displayed normal numbers of cells expressing other osteoblast markers, col10a1 and oxs. This is in striking contrast to the effect of Bmp2 overexpression, a well-studied positive regulator of osteoblast maturation, which caused hyperossification accompanied by an increase of col10a1-positive cells (Figs 7 and 8). Finally, axial osteoblasts were still sensitive to RA many days after they had arrived at their final perichordal destination and after they had become insensitive to Bmp2 (15 versus 4 dpf) (compare Fig. 7N with Fig. 3H and Fig. 7P).

In cell culture studies, RA and Bmp2 have also been shown to stimulate osteoclasts, which are bone-resorbing cells of the hematopoietic lineage (Cowan et al., 2005; Kaji et al., 1995). However, for several reasons, this does not seem relevant for the ossification defects of cyp26b1 mutants. First, we would expect a stimulation of osteoclasts to result in loss, rather than the observed gain, of bone. Second, according to histological stainings of the osteoclast marker enzyme tartrate-resistant acid phosphatase (TRAP; Acp5 – ZFIN), osteoclasts only become active long after...
the bone phenotype of cyp26b1 mutants has become apparent (14 dpf) (Gavaia et al., 2006; Witten et al., 2001). Third, knockdown of <i>pou.1</i> (Spil – ZFIN), which is required for specification of the entire myeloid lineage including osteoclasts (Rhodes et al., 2005; Zhao et al., 2007), did not affect axial ossification, although other myeloid derivatives, such as <i>mpx</i>-positive neutrophils, were completely absent (our unpublished results). This suggests that in contrast to Cyp26b1 and osteoclasts, osteoclasts are dispensable for bone formation during the larval stages we have investigated.

**Cyp26b1 and human disease: is <i>dolphin</i> a model for Klippel-Feil anomaly (KFA) or related syndromes?**

In higher vertebrates, such as birds and mammals, vertebra formation occurs via endochondral ossification, whereas in teleosts, vertebral bodies are formed through direct ossification in and around the notochordal sheath (perichordal centra). There has been some debate about the involvement of osteoblasts in fish axial skeleton development. According to one report, vertebral bodies in zebrafish arise by secretion of bone matrix from the notochord and without any involvement of osteoblasts (Fleming et al., 2004). By contrast, a more recent report claims that sclerotome-derived osteoblasts are present in intervertebral regions in Medaka (Inohaya et al., 2007).

Our data are consistent with the latter report and with the situation present in intervertebral regions in Medaka (Inohaya et al., 2007). Our data are consistent with the latter report and with the situation in higher vertebrates, pointing to the presence and activity of osteoblasts (Fleming et al., 2004). By contrast, according to one report, vertebral bodies in zebrafish are formed through direct ossification in and around the notochordal sheath (perichordal centra). There has been some debate about the involvement of osteoblasts in fish axial skeleton development. According to one report, vertebral bodies in zebrafish arise by secretion of bone matrix from the notochord and without any involvement of osteoblasts (Fleming et al., 2004). By contrast, a more recent report claims that sclerotome-derived osteoblasts are present in intervertebral regions in Medaka (Inohaya et al., 2007). Our data are consistent with the latter report and with the situation present in intervertebral regions in Medaka (Inohaya et al., 2007). Our data are consistent with the latter report and with the situation present in intervertebral regions in Medaka (Inohaya et al., 2007).

We thank Elisabeth Busch and Derek Stemple for providing the sa0002 allele; Lieven Meerpoel (Johnson and Johnson Pharmaceutical Research and Development) for free samples of R115866; Cecilia Moens, Charles Kimmel, Vicky Prince and Tom Schilling for zebrafish reagents; Caroline Jøhner and Norbert Jossik for their help with the mouse experiments; Tom Schilling, Shannon Fisher, Henry Roehl and Jochen Holzschuh for helpful discussions; and Kirsten Spoorenendonk and Stefan Schulte Merker for sharing unpublished data. Work in the laboratory of M.H. and the TILLING project leading to the isolation of the sa0002 allele were supported by the European Union (6th Framework Integrated Project ‘Zebrafish Models for Human Development and Disease’).

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/22/3775/DC1

**References**


