**Summary**
Retinoic acid (RA) is a vitamin A-derived, non-peptidic, small lipophilic molecule that acts as ligand for nuclear RA receptors (RARs), converting them from transcriptional repressors to activators. The distribution and levels of RA in embryonic tissues are tightly controlled by regulated synthesis through the action of specific retinol and retinaldehyde dehydrogenases and by degradation via specific cytochrome P450s (CYP26s). Recent studies indicate that RA action involves an interplay between diffusion (morphogen-like) gradients and the establishment of signalling boundaries due to RA metabolism, thereby allowing RA to finely control the differentiation and patterning of various stem/progenitor cell populations. Here, we provide an overview of the RA biosynthesis, degradation and signalling pathways and review the main functions of this molecule during embryogenesis.

**Key words:** Retinoids, Retinoic acid, Retinol dehydrogenase (RDH), Retinaldehyde dehydrogenase (RALDH), CYP26, Hindbrain, Forebrain

**Introduction**
Retinoic acid (RA) is derived from the liposoluble vitamin A (retinol). Vitamin A has long been known to be indispensable for vision, as its derivative retinaldehyde (Fig. 1) acts as a light-sensitive molecule, the isomerisation of which triggers the phototransduction process in photoreceptor cells of the retina (reviewed by Parker and Crouch, 2010). Many other functions have been assigned to this vitamin, and work in avian and rodent models has established that maternal vitamin A deficiency affects the establishment of photoreceptor cell populations in the retina, the development of the neural tube, and the differentiation of the hindbrain (reviewed by Gale et al., 1999; White et al., 2000; Wilson et al., 1953).

About 25 years ago, the molecular basis of vitamin A action was elucidated when it was shown that its acidic metabolite, RA, acts as a ligand for transcription factors of the retinoic acid receptor (RAR) nuclear receptor superfamily, switching them from potential repressors to transcriptional activators. Possible functions of RA during embryogenesis were first inferred by studying its teratogenic effects, i.e. how the administration of excess doses of RA, either globally or by local implantation using RA-impregnated beads, interferes with normal developmental processes. These studies have been performed in a wide range of species including amphibians, zebrafish, chick and rodents (e.g. Avantaggiato et al., 1996; Durston et al., 1989). Gene knockout studies then confirmed the crucial functions of RARs in mouse development (reviewed by Mark et al., 2009). Eventually, the enzymatic pathways that regulate embryonic RA synthesis from maternal retinol – or egg-stored retinoids – were characterised, and it was found that another regulatory step involved the triggering of RA catabolism by a subfamily of cytochrome P450 enzymes. Altogether, the differential and often dynamic expression patterns of a small number of specific synthesizing and metabolising enzymes allow precise control of RA distribution within embryonic cell populations. A wide range of vertebrate models (from zebrafish to mouse) and experimental tools (including, for instance, reporter transgenes that reveal regions of active RA signalling, and selective antagonists for RARs or RA-synthesizing/metabolising enzymes) have been successfully used to decipher retinoid functions at the cellular and molecular levels.

Here, we first provide an overview of the pathways and proteins that regulate or mediate RA signalling during development, many of which appear to be highly conserved throughout vertebrate species. We then review the main functions of retinoic acid signalling during early embryonic development, first referring to the developing hindbrain as a system that has been most extensively studied with respect to RA functions and for which recent studies have refined our knowledge of the control of RA activity during rhombomeric segmentation. We further review extensive work that, over the last few years, has investigated how RA acts on progenitor cell populations in structures as diverse as the embryonic forebrain, the branchial apparatus and foregut derivatives, the neural plate and the posterior mesoderm during embryonic axial elongation. Understanding these functions and the underlying molecular events is of great importance, as retinoids are widely used in therapy and in many protocols for differentiating primary cultures or cell lines [including embryonic stem (ES) cells] into specific lineages (see Box 1). Retinoids thus hold promise for future use in stem cell-based therapy and regenerative medicine. Ongoing research will also guide more conventional clinical approaches, especially in the context of cancer chemoprevention or treatment (reviewed by Tang and Gudas, 2011).

**The RA synthesis pathway**
The only source of retinoids in most animals is diet derived, as these compounds cannot be synthesized de novo. In mammals, the main circulating retinoid is retinol bound to a carrier protein, retinol-binding protein 4 (RBP4) (see Box 2). Retinol homeostasis involves several proteins and enzymes that regulate its dietary uptake in intestinal cells and its storage mainly in liver hepatocytes and stellate cells (reviewed by D’Ambrosio et al., 2011). Maternal retinol transferred transplacentally is the major retinoid source for embryos of placental species. By contrast, oivous species store vitamin A in the egg yolk and, according to the species, the main source can be retinol, retinaldehyde, or carotenoids such as beta-carotene (see Simoes-Costa et al., 2008). Work performed in zebrafish has demonstrated the importance of a beta-carotene cleavage enzyme (BCMO1) that acts tissue-specifically to generate embryonic retinaldehyde (Lampert et al., 2003).

Typically, in mammals, retinol-RBP4 is taken up by target tissues, and this uptake can be facilitated in some tissues by a transport mechanism (reviewed by Sjoö et al., 1999). Once inside the cell, retinol is converted into retinaldehyde by a number of enzymes that are regulated by RA. The main circulating retinoid is retinol bound to a carrier protein, retinol-binding protein 4 (RBP4) (see Box 2). Retinol homeostasis involves several proteins and enzymes that regulate its dietary uptake in intestinal cells and its storage mainly in liver hepatocytes and stellate cells (reviewed by D’Ambrosio et al., 2011). Maternal retinol transferred transplacentally is the major retinoid source for embryos of placental species. By contrast, oivous species store vitamin A in the egg yolk and, according to the species, the main source can be retinol, retinaldehyde, or carotenoids such as beta-carotene (see Simoes-Costa et al., 2008). Work performed in zebrafish has demonstrated the importance of a beta-carotene cleavage enzyme (BCMO1) that acts tissue-specifically to generate embryonic retinaldehyde (Lampert et al., 2003).

Typically, in mammals, retinol-RBP4 is taken up by target tissues, and this uptake can be facilitated in some tissues by a transmembrane protein that is the product of the RA-inducible gene.
STRA6 (Kawaguchi et al., 2007). Interestingly, STRA6 mutations constitute the only demonstrated cases of human mutations that affect a gene from the retinoid pathway and lead to a complex spectrum of developmental abnormalities (Pasutto et al., 2007). Other human mutations involving retinoid-binding proteins selectively affect visual function (e.g. Maw et al., 1997). Maternal RBP4 cannot cross the placenta; retinol thus diffuses across the yolk sac and placenta, wherezygotic RBP4 synthesis occurs (Ward et al., 1997). Retinyl esters may also be significant retinoid sources for the embryo (Quado et al., 2005).

Box 1. RA and stem cell differentiation

Unlike most other adult tissues, the nervous system of mammals has a limited ability to compensate for the loss of cells after lesions. Embryonic stem (ES) cells have long attracted attention as a potential source of cells that can be driven to differentiate into specific lineages for the development of cell therapy and pharmaceutical screens. The spontaneous differentiation of neuronal cells from ES cells during in vitro culture is rather limited. Therefore, various protocols to increase the differentiation of neuronal cell types have been established. Recently, it was found that the addition of RA to rapidly proliferating mouse ES cells, cultured in suspension as embryoid bodies, leads to the selective generation of neural progenitors with characteristics of radial glial cells found in the developing central nervous system (Bibel et al., 2004; Plachta et al., 2004). These conditions led to a highly uniform population of Pax6-positive cells which, when further cultured in vitro, could differentiate into neurons that established synaptic contacts and exhibited electrophysiological properties similar to those of forebrain pyramidal neurons. When transplanted into the neural tube of chicken embryos, the RA-induced embryoid bodies contributed to spinal cord motoneurons and interneurons. In the future, these cellular systems could be used for studying commitment and neuronal specification in vitro, for pharmacological assays and drug screening, and for the selective isolation of differentiated neuronal cells or committed progenitors that may be used as a source for cell and tissue grafts.

Enzymes that oxidize retinol to retinaldehyde (Fig. 1A) belong to two classes: the cytosolic alcohol dehydrogenases (ADHs) belonging to the medium-chain dehydrogenase/reductase family; and microsomal short-chain dehydrogenases/reductases (retinol dehydrogenases, RDHs) (reviewed by Pares et al., 2008). ADH5 (previously called ADH3) is ubiquitously expressed in the embryo and adult, whereas ADH1 and ADH7 (previously called ADH4) are tissue restricted (Ang et al., 1996). Null mouse mutants for Adh5 display reduced viability and growth defects, which can be rescued by dietary supplementation with retinol (Molotkov et al., 2002a). No obvious phenotype is associated with the loss of ADH1 and ADH7 when mice are maintained on a vitamin A-sufficient diet (Deltour et al., 1999b). However, when large doses of retinol are administered to Adh1–/– mutants, the mice are more sensitive to vitamin A embryotoxicity (Molotkov et al., 2002b). This suggests that ADH enzymes might have a role in controlling the removal of excess retinol, rather than participating in RA synthesis. RDHs are well known to act during the visual cycle (Parker and Crouch, 2010). Rdh3–/– mice are viable but suffer from a delay in dark adaptation, consistent with a role in regenerating 11-cis-retinaldehyde after photobleaching. By contrast, Rdh10, which displays specific expression domains during development (Cammas et al., 2007; Romand et al., 2008) (Table 1), plays an important role in RA synthesis, as its loss-of-function is lethal between embryonic day (E) 10.5 and E14.5 (Sandell et al., 2007). Rdh10 mutants (Fig. 1B,C) exhibit abnormalities characteristic of RA deficiency (Table 2), which can be partly rescued by maternal RA supplementation (Rhinn et al., 2011).

The next step in RA synthesis is the oxidation of retinaldehyde to RA (Fig. 1A), which is carried out by three retinaldehyde dehydrogenases (RALDHs): RALDH1, RALDH2 and RALDH3 (also known as ALDH1A1, ALDH1A2 and ALDH1A3). RALDHs display distinct and specific expression patterns that correlate with RA activity as detected by reporter transgenes (Table 1). Raldh2 is the earliest RALDH to be expressed, and is found in the primitive streak and mesodermal cells, and later in somitic and lateral mesoderm, posterior heart tube and rostral forebrain (Niederreither...
**Box 2. Additional components of the retinoid pathway**

Retinol-binding protein 4 (RBPA4). Mainly synthesized in the yolk sac and postnatally in liver. It binds to retinol and delivers retinol from the liver to peripheral tissues.

Transferrin (TR). A serum protein that associates with RBPA4-retinol to prevent retinol degradation by the kidney. The TR-RBPA4-retinol complex transports retinol in the circulation and delivers it to target tissues.

Ocular retinoid-binding proteins. These include retinol dehydrogenase-domain proteins 1 and 3 (RLBP1 and RBP3), which are produced by retinal cells and are involved in the isomerisation and/or shunting of retinol and retinyldehyde during the visual cycle.

Cytochrome P450 1B1 (CYP1B1). May catalyze the oxidation of retinol into retinaldehyde and RA. Human CYP1B1 mutations are a major cause of congenital glaucoma, a severely blinding disease.

Cellular retinol-binding proteins (CRBP-I and -II, also known as RBPA1 and 2). These proteins belong to the fatty acid-binding (FABP) family. They bind both all-trans-retinol and all-trans-retinyldehyde, and may function to control levels of intracellular retinol accumulation and esterification.

Cellular retinoic acid-binding proteins (CRABP-I and -II, also known as CRABP1 and 2). Bind all-trans-RA intracellularly. They solubilise and protect RA in the aqueous cytosol, although differential functions have been proposed, with CRABP-I presenting RA to metabolising (CYP26) enzymes, and CRABP-II favouring nuclear import and delivery of RA to RARs by direct protein-protein interactions.

Fatty acid-binding protein 5 (FABP5). Binds RA with a lower affinity than CRABPs, and may play a role in inducing a non-canonical RA signalling pathway. In cell lines, FABP5 favours RA binding to peroxisome proliferator-activated receptor β/γ (PPARβ/γ), which in turn can induce anti-apoptotic and proliferative responses when the FABP5 concentration exceeds that of CRABP-II.

et al., 1997). Raldh2−/− mouse mutants (Fig. 1D,E) die before midgestation from defective heart morphogenesis and exhibit numerous abnormalities (Table 2) (Niederreither et al., 1999; Niederreither et al., 2001; Niederreither et al., 2000). Some of these abnormalities can be rescued by transient maternal RA supplementation from E7.5 to E8.5-9.5 (e.g. Mic et al., 2002; Niederreither et al., 2003). RALDH2 is solely responsible for embryonic RA synthesis until E8.5, and thereafter RALDH1 and RALDH3 contribute to RA synthesis in the eyes and olfactory system. Raldh3−/− mice have defects in nasal and ocular development and die at birth due to respiratory distress (Fig. 1F,G) (Dupé et al., 2003). Raldh1−/− mutants, by contrast, are viable and show minor defects in the dorsal retina (Matt et al., 2005; Molotkov et al., 2006).

The RA degradation pathway

The distribution and levels of RA have to be tightly controlled during embryogenesis, and an important level of control is achieved through tissue-specific oxidative metabolism. Enzymes of the cytochrome P450 26 subfamily (CYP26A1, CYP26B1 and CYP26C1) catalyze reactions that convert RA into more polar metabolites, primarily 4-hydroxy-RA, which can be further oxidized to 4-oxo-RA (e.g. Chithalen et al., 2002) (Fig. 2A). Although 4-oxo-RA is able to bind RARs and interferes with embryonic patterning when administered exogenously (Pijnappel et al., 1993), both expression data and functional studies indicate that the role of CYP26-mediated RA metabolism is essentially to prevent inappropriate signalling in specific cell populations (reviewed by Pennimpede et al., 2010). CYP26 enzymes display differential expression patterns (Table 1) that are often complementary to the RALDH expression domains. Cyp26a1 and Cyp26c1 are the first to be expressed and are found in the rostralmost embryonic epiblast, and all three Cyp26 genes are expressed in a sequential manner during hindbrain development (MacLean et al., 2001; Siribut et al., 2005) (see below). Cyp26a1 is specifically expressed in tail bud tissues and Cyp26b1 in distal limb bud mesenchyme (Yashiro et al., 2004). After mid-gestation, each Cyp26 gene displays complex patterns of expression in several developing organs, including the retina, inner ear and dental epithelium (Abu-Abed et al., 2002; Romand et al., 2006; Tahayato et al., 2003).

Genetic ablation of Cyp26a1 and Cyp26b1 results in phenotypes reminiscent of RA-induced teratogenesis (Table 2). Cyp26a1−/− mice (Fig. 2B,C) display truncation of the posterior body region, abnormal hindbrain patterning and transformation of cervical vertebrae (Abu-Abed et al., 2001; Sakai et al., 2001). Cyp26b1−/− mutants (Fig. 2D-G) exhibit severe limb malformations and facial abnormalities (MacLean et al., 2009; Yashiro et al., 2004). Cyp26c1 loss-of-function embryos are viable, although compound inactivation of any other Cyp26 results in early embryonic patterning defects (Uehara et al., 2007; Uehara et al., 2009) (Fig. 2H,I). Disruption of the P450 oxidoreductase (POR), an enzyme required for P450 cytochrome activity, phenocopies many of these defects, underscoring the importance of CYP26 function during development (Ribes et al., 2007).

Collectively, these data show that CYP26 enzymes have major developmental functions, which are best described as preventing any detrimental (teratogenic) effect of endogenous RA in regions where it should not be allowed to signal. Interestingly, RA can control the expression of its own metabolising enzymes. RA treatments in vivo and in cultured cells lead to rapid upregulation of the Cyp26a1 gene, which contains two functional RA-response elements (RAREs) (Loudig et al., 2005). These data have been integrated into mathematical models indicating how RA might participate in autoregulatory negative-feedback loops by inducing expression of catabolising enzyme(s) (White et al., 2007) (see below).

Gene regulation by RA

RA acts by binding to RARs, which are members of the nuclear receptor superfamily (reviewed by Rochette-Egly and Germain, 2009). There are three RARs (RARα, RARβ and RARγ) that are conserved throughout vertebrates and that primarily bind all-trans-RA. RARs act in heterodimeric combinations with retinoid X receptors (RXRα, RXRβ and RXRγ). RXRs bind a stereoisomer, 9-cis-RA, which, unlike all-trans-RA, is not detected endogenously in embryos or adult tissues (Mic et al., 2003); therefore, it was suggested that RXRs act mainly as scaffolding proteins to facilitate DNA binding of the RAR-RXR complex (see Chawla et al., 2001). Three receptors (RARα, RXRα and RXRβ) have widespread expression patterns, whereas the others (RARβ, RARγ and RXRγ) show more complex, tissue-specific expression (reviewed by Döll, 2009) (Table 1). Thus, most tissues are potential targets of retinoid actions, although different heterodimeric complexes can transduce the RA signal. Gene knockout studies in mouse revealed a large degree of functional redundancy between RAR/RXR heterodimers, with developmental abnormalities usually occurring when two receptors are inactivated in combination, except in the case of RXRα mutants, which die in utero due to heart defects (reviewed by Mark et al., 2009) (Table 2).
In the nucleus, RAR/RXR dimers bind to DNA motifs known as RAREs. RAREs consist of a direct repeat of a core hexameric sequence 5′-(A/G)G(G/T)TCAG-3′ or of the more relaxed 5′-(A/G)G(T/G)T(C/G)A-3′ motif, separated by 1, 2 or 5 bp (see Balmer and Blomhoff, 2002). RAR/RXRs can bind RAREs even in the absence of ligand, thereby recruiting co-repressor complexes and maintaining target gene repression (Fig. 3). In the presence of ligand, a conformational change leads to the release of co-repressors and the recruitment of co-activator complexes. These regulate target gene expression.

Numerous RAR target genes have been identified (see Balmer and Blomhoff, 2002), including genes from within the retinoid pathway, such as Rarb, Crbp1/2 (Rbp1/2), Crabp1/2 and Cyp26a1 (see Box 1 and Table 3). Also, several members of the Hox gene family, including Hoxa1, Hoxb1, Hoxb4 and Hoxd4, harbour RAREs, the function of which has been demonstrated in vivo (reviewed by Marshall et al., 1996). The number of putative target genes is increasing rapidly through novel technologies. Recently, for example, Luijten et al. used rodent whole-embryo culture combined with RA treatments and performed microarray analysis to identify genes that were up- or downregulated by RA (Luijten et al., 2010). It should also be stressed that some of the effects of RA might involve binding to other nuclear receptors, such as PPARβ/γ (Schug et al., 2007).

### RA functions during development

#### RA signalling during hindbrain development

Segmentation and patterning of the hindbrain are regulated by RA

Numerous studies have focused on the embryonic hindbrain as an experimental paradigm for understanding RA regulatory effects. Hindbrain development involves the generation of seven to eight neuroepithelial compartments or rhombomeres (Fig. 4A), each with a distinct identity according to its anteroposterior (A-P) position (Kiecker and Lumsden, 2005). This segmentation underlies several events required for development of the brain stem, inner ear, branchial arches, and even the heart and large vessels, which are colonised by hindbrain-derived neural crest cells. Several Hox genes, according to their spatially restricted expression patterns, are required for the growth and/or positional identity of specific

| Gene | Main expression sites* | Main references‡ |
|------|------------------------|------------------|
| Rdh10 | Ventral neural plate (hindbrain, spinal cord); mid-hindbrain isthmus; optic vesicle, otocyst (ventral); nasal epithelium; dorsal somitic mesoderm, mesonephros, lateral plate mesoderm; foregut mesenchyme and endoderm, branchial pouches; proximal limb bud mesoderm | Blentic et al., 2003; Suzuki et al., 2000 (chick); Haselbeck et al., 1999; Li et al., 2000; Suzuki et al., 2000 (mouse) |
| Raldh1 | Ventral midbrain; optic vesicle, dorsal retina and lens; thymus primordium (3rd branchial pouch) (mouse); posterior foregut and midgut endoderm (chick); mesonephros | Blentic et al., 2003; Haselbeck et al., 1999; Swindell et al., 1999; Ribes et al., 2009; Ribes et al., 2006 (mouse) |
| Raldh2 | Primitive streak, node, posterior embryonic mesoderm; anterior most neural plate and optic vesicle (transient); pre-somite and somitic mesoderm, mesonephros, lateral plate mesoderm; posterior branchial arches and foregut mesenchyme; posterior heart tube | Blentic et al., 2003; Suzuki et al., 2000 (chick); Li et al., 2000; Mic et al., 2000; Suzuki et al., 2000 (mouse) |
| Raldh3 | Node (chick); head ectoderm, nasal epithelium, optic vesicle, dorsal and ventral retina; otocyst (dorsal); mid-hindbrain isthmus; Rathke’s pouch (pituitary anlage) | Blentic et al., 2003; Swindell et al., 1999 (chick); Fuji et al., 1997; MacLean et al., 2001; Sirbu et al., 2005 (mouse) |
| Cyp26a1 | Anteriormost epiblast and neural plate (transient); primitive streak and posterior mesoderm; (pre)rhombomeres 2 (mouse), 3 and rostral spinal cord (chick); posterior hindbrain and branchial arch mesenchyme; heart endocardium; caudal neural plate and tail bud; distal limb bud ectoderm | Reijntjes et al., 2003; MacLean et al., 2001; Sirbu et al., 2005 (mouse) |
| Cyp26b1 | (Pre)rhombomeres 3, 5, 2-4 (ventral) (mouse), 1, 4, 6 (chick); posterior (2nd-6th) branchial arch ectoderm/endordem; distal limb bud mesenchyme; mid-hindbrain isthmus; heart and vascular endothelia; tail bud (transient) | Reijntjes et al., 2003; MacLean et al., 2001; Sirbu et al., 2005 (mouse) |
| Cyp26c1 | Rostral head mesenchyme (transient); (pre)rhombomeres 2, 4 (chick), 2, 3, 5 (chick); hindbrain mesenchyme (facing r3, and post-otic); first branchial arch and pouch; otocyst (ventral) | Reijntjes et al., 2004 (chick); Sirbu et al., 2005; Tahayato et al., 2003 (mouse) |
| Rara | Widespread, weaker in forebrain neuroepithelium; up-regulated in rhombomeres 4, 7 and spinal cord | Ruberte et al., 1991; Ruberte et al., 1993 (mouse) |
| Rarb | Rhombomere 7 and spinal cord; head and branchial arch mesenchyme (except mandibular arch); foregut endoderm and mesenchyme; mesonephros, lateral plate mesoderm; proximal limb bud mesenchyme | Smith and Eichele, 1991 (chick); Dollé et al., 1989; Ruberte et al., 1991; Ruberte et al., 1993 (mouse) |
| Rarg | Primitive streak; frontonasal and 1st branchial arch mesenchyme; limb bud mesenchyme; trunk and caudal neural plate (transient); perisomatic and tail bud mesoderm; precartilaginous cell populations | Abu-Abed et al., 2003; Dollé et al., 1989; Ruberte et al., 1991; Ruberte et al., 1990 (mouse) |
| Rxra | Widespread/ubiquitous; up-regulated in posterior hindbrain and dorsal spinal cord (chick) | Hoover and Glover, 1998 (chick); Dollé et al., 1994 (mouse) |
| Rxrb | Widespread/ubiquitous | Dollé et al., 1994 (mouse) |
| Rxrg | Cranial and peripheral nervous system neural crest (chick); myotomes, developing muscle (mouse) | Rowe and Brickell, 1995 (chick); Dollé et al., 1994 (mouse) |

*The stages covered correspond to embryogenesis, roughly from the onset of gastrulation to somitogenesis/early organogenesis. Expression at earlier stages (pre-implantation stages for mammals, blastula/morula) or during later organogenesis (foetal development in mammals) is not summarised.

‡Relevant references are quoted for two species (chick and mouse), with observations made only in one species indicated among the list of main expression sites.
Table 2. Loss-of-function phenotypes resulting from targeted inactivation of retinoid signalling pathway genes in mice

| Gene(s)* | Stage of lethality | Loss-of-function phenotype | Main references |
|----------|--------------------|---------------------------|-----------------|
| Adh1     | Viable             | Postnatal increase in vitamin A toxicity | Deltour et al., 1999a; Deltour et al., 1999b; Molotkov et al., 2002b |
| Adh5     | Postnatal          | Growth deficiency, vitamin A toxicity | Deltour et al., 1999b; Molotkov et al., 2002a; Molotkov et al., 2002b |
| Adh7     | Viable             | Sensitivity to vitamin A deficiency | Deltour et al., 1999a; Deltour et al., 1999b |
| Rdh5     | Viable             | Vision: delay in dark adaptation | Driessen et al., 2000 |
| Rdh10    | E10.5-14.5         | Small optic vesicles/eyes; abnormal hindbrain and posterior branchial arches; abnormal heart tube; small forelimb buds; defects in organogenesis (lumbar, gut, pancreas, kidney) | Cunningham et al., 2011; Sandell et al., 2007; Rhinn et al., 2011 |
| Raldh1   | Viable             | No abnormality reported | Fan et al., 2003; Matt et al., 2005 |
| Raldh2   | E9.5-10.5          | Hyposplastic optic vesicles; abnormal hindbrain ('anteriorisation'); lack of posterior branchial arches; impaired heart looping and chamber differentiation; truncation of body axis, asymmetry in somite formation; absence of limb buds; defects in organogenesis (lumbar, gut, pancreas, kidney) | Mic et al., 2002; Niederreither et al., 2001; Niederreither et al., 2000; Ribes et al., 2009; Ribes et al., 2006; Sirbu and Duester, 2006; Vermot et al., 2005 |
| Raldh3   | Neonatal           | Shortening of ventral retina; nasal abnormality (choanal atresia); altered GABAergic neuronal differentiation in forebrain basal ganglia | Chatzi et al., 2011; Dupé et al., 2003; Matt et al., 2005; Molotkov et al., 2006 |
| Cyp26a1  | Neonatal           | Abnormal hindbrain ('posteriorisation'); truncation of posterior body, sometimes with sirenomeilia ('mermaid-like tail'); vertebral transformations | Abu-Abed et al., 2001; Sakai et al., 2001 |
| Cyp26b1  | Neonatal           | Limb defects (abnormal distal skeleton and cartilage maturation); craniofacial abnormalities (reduced maxilla and mandible, cleft palate); gonadal abnormalities (premature meiosis, apoptosis of male germ cells) | Bowles et al., 2006; MacLean et al., 2009; MacLean et al., 2007; Yashiro et al., 2004 |
| Cyp26c1  | Viable             | No abnormality reported | Uehara et al., 2007 |
| Cyp26a1;Cyp26c1 | E9.5-10.5 | Reduced forebrain and midbrain, hindbrain expansion; deficiency in cranial neural crest | Uehara et al., 2007 |
| Rara     | Postnatal (variable) | Growth deficiency; vertebral transformations/abnormalities; malformed laryngeal cartilages; webbed digits (variable); male sterility (degeneration of testis germinal epithelium) | Ghyselinck et al., 1997; Lufkin et al., 1993 |
| Rarb     | Viable             | Growth deficiency; vertebral transformations/abnormalities; ocular abnormality (retrolenticular membrane); locomotor behavioural defects | Ghyselinck et al., 1997; Krezel et al., 1998 |
| Rarg     | Postnatal (variable) | Growth deficiency; vertebral transformations/abnormalities; malformed laryngeal and tracheal cartilages | Chapellier et al., 2002; Ghyselinck et al., 2007; Lohnes et al., 1993 |
| Rara;Rarb | Neonatal           | Abnormal hindbrain patterning ('anteriorisation'); absence/abnormality of posterior branchial arch derivatives (thymus, parathyroids) and salivary glands; heart outflow tract and large vessel abnormalities; severe laryngeal/tracheal abnormalities; lung hypoplasia, lack of oesophagotraheal separation; kidney and female genital tract abnormalities | Batourina et al., 2001; Dupé et al., 1999; Ghyselinck et al., 1997; Lohnes et al., 1994; Mendelsohn et al., 1994 |
| Rara;Rarg | E12.5 to neonatal  | Absence/abnormality of posterior branchial arch derivatives (thymus, parathyroids) and salivary glands; eye defects (retinal coloboma, absence of lens); heart outflow tract and large vessel abnormalities, myocardial hypoplasia; severe laryngeal/tracheal abnormalities; craniofacial and limb skeletal defects; kidney, male and female genital tract abnormalities | Ghyselinck et al., 1997; Lohnes et al., 1994; Mendelsohn et al., 1994; Wendling et al., 2001 |
| Rxra     | E13.5-16.5         | Heart outflow tract and large vessel abnormalities, myocardial hypoplasia; eye defects (shortening of ventral retinal abnormal cornea); placental defect (disorganisation of labyrinthine zone) | Gruber et al., 1996; Kastner et al., 1994; Merki et al., 2005; Sapin et al., 1997; Sucov et al., 1994 |
| Rxrb     | Partial perinatal lethality | Male sterility (abnormal Sertoli cells, impaired spermatozoid production) | Kastner et al., 1996 |
| Rrg      | Viable             | Behavioural and depression-like defects | Krezel et al., 1998; Krzyzosiak et al., 2010 |
| Rxra;Rxrb | E9.5-10.5          | Truncation of posterior body; abnormal nasal region and posterior branchial arches; abnormal heart tube; placental defect (absence of labyrinthine zone) | Wendling et al., 1999 |

*All phenotypes refer to homozygous germline mutants with gene disruptions generated in embryonic stem (ES) cells. Some examples of compound (double homozygous null) mutations leading to severe embryonic abnormalities are also given. The double mutations were generated through mouse intercrosses, except for Cyp26a1;Cyp26c1, for which the two neighbouring genes were deleted in ES cells.
rhombomeres (reviewed by Marshall et al., 1996; Rijli et al., 1998). Treatment of pregnant mice or rats with excess RA leads to teratogenic changes in the hindbrain (Morris, 1972). Interestingly, RA exposure at late gastrula/early neurula stages increases hindbrain size at the expense of other brain regions (Avantaggiato et al., 1996), whereas RA treatment at later stages specifically leads to a ‘posteriorisation’ of rhombomeres (r) 2-3 to an r4-r5 identity (see Marshall et al., 1996).

Evidence that endogenous retinoids are required for hindbrain patterning was found in vitamin A-deficient (VAD) quail embryos, in which the caudal hindbrain region (r4-r8) was misspecified into an enlarged r3, and more anterior rhombomeres expanded posteriorly (Fig. 4B) (Gale et al., 1999). Region-specific effects of RA deficiency have also been documented in Raldh2–/– mouse mutants, which lack rhombomeric segmentation and exhibit a severe reduction of the posterior hindbrain. Strikingly, the expression of genes normally restricted to r3-r4 [such as Hoxb1, Krox20 (Egr2)] spreads posteriorly in these mutants, whereas expression of r5-r7 determinants (Mafb, Hoxa4) is reduced or abolished (Fig. 4C) (Niederreither et al., 2000). These patterning defects have dramatic consequences for related developmental events, such as inner ear patterning, neural crest migration (leading to a lack of development of all branchial arches except for the first one), or neurite/cranial nerve differentiation. In zebrafish embryos, a lack of development of all branchial arches except for the first (b1, b2) is observed when at least two receptors are abolished (Fig. 4C) (Niederreither et al., 2000). These patterning abnormalities are found only when at least two receptors are inactivated in combination. Interestingly, such compound inactivations have different outcomes on hindbrain patterning.

As RARs have partly redundant functions, hindbrain abnormalities are found only when at least two receptors are inactivated in combination. Interestingly, such compound inactivations have different outcomes on hindbrain patterning. Rara–/–;Rarg–/– mutants display severe malformations similar to those of Raldh2–/– mutants (Wendling et al., 2001) (Fig. 4C). Rara–/–;Rarb–/– mutants show a different phenotype, in which only r5-r7 have abnormal boundaries, and genes normally restricted to r5-r6 spread posteriorly at the expense of r7 markers (Fig. 4D) (Dupé et al., 1999). Exposure of cultured wild-type embryos to BMD493, a pan-RAR antagonist, at early somite stages phenocopied the Rara–/–;Rarg–/– hindbrain defects, whereas an earlier treatment at the beginning of gastrulation yielded an Rara–/–;Rarb–/–-like phenotype (Wendling et al., 2001). This indicates that RARα and/or RARγ mediates the early effects of RA on hindbrain patterning, whereas RARB functions later in development in setting up the size and caudal boundary of the r5-r6 territory.

Importantly, Cyp26 genes display differential, rhombomere-specific expression patterns. Cyp26a1 loss-of-function leads to subtle patterning defects, with an enlarged r4 and partial transformation of r3 to an r4-like identity (Abu-Abed et al., 2001; Sakai et al., 2001) (Fig. 4E). Hindbrain abnormalities were not observed in Cyp26b1–/– or Cyp26c1–/– mutants, although compound Cyp26a1;Cyp26c1 inactivation leads to severe defects (lack of segmentation and posteriorisation of the prospective r1-r4 region) (MacLean et al., 2009; Uehara et al., 2007) (Fig. 4F). As described below, CYP26 enzymes are likely to act in a concerted manner to control RA diffusion and maintain specific pre-rhombomeric territories in an RA-free state.

The hindbrain: an experimental paradigm to study RA morphogenetic gradients

The striking effects of both retinoid excess and retinoid deficiency on hindbrain patterning have stimulated research into the underlying mechanisms and modes of RA action. Almost 25 years ago, RA was identified as the first candidate vertebrate morphogen, following the discovery of uneven concentrations of RA across the developing chick limb bud (Thaller and Eichele, 1987). Morphogens are signalling molecules that act non-cell-
autonomously in a concentration-dependent fashion to assign positional identities to fields of cells (Wolpert, 2011). Over the last 10 years, several groups have tried to gain insights into how the spatial distribution of RA is regulated. Below, we discuss the ‘classical’ and newer aspects of this regulation and establishment of possible RA gradient(s), the main emerging concept being that several enzymatic activities (e.g. of RDHs and CYP26s) are required in addition to RALDHs to dynamically control and shape RA distributions within the embryo.

Does RA match the definition of a morphogen? As a small lipophilic molecule (Fig. 1) it is able to diffuse across cell membranes. Furthermore, it is soluble in water up to ~200 nM (Szuts and Harosi, 1991), i.e. within its physiological concentration range, so diffusion gradients might also occur in the extracellular space. Within the hindbrain, RA can control gene expression in a concentration-dependent manner, as demonstrated by concentration-dependent effects of RA treatments (e.g. Durston et al., 1989). Treatment of chick or zebrafish embryos with the RA antagonist BMS493 showed that more posterior rhombomere boundaries require progressively higher concentrations of RA for their correct positioning (Dupé and Lumsden, 2001; Maves and Kimmel, 2005). The RA source for hindbrain patterning is local and corresponds to presomitic mesoderm (PSM) and somites expressing Raldh2 near the caudal hindbrain (Fig. 5). These observations might support a role for RA acting as a diffusible morphogen in the pre-segmented hindbrain, perhaps diffusing from its mesodermal source via cell-cell contacts. Unlike other established morphogens [e.g. FGF8 or sonic hedgehog (SHH)], a gradient for RA has never been demonstrated, mainly owing to technical limitations. Recently, White et al. (White et al., 2007) visualised RA indirectly in zebrafish using a fluorescent marker associated with the control of RAREs (rare:yfp). In transgenic embryos, YFP expression was clearly graded from posterior to anterior regions of the hindbrain. This expression is lost in RA-deficient mutant embryos, but can be restored by the prior transplantation of cells overexpressing Raldh2 into somitic mesoderm. In most cases, the rescue spanned the width of the neural tube along a six-cell (70 μm) diameter (White et al., 2007).

In another zebrafish study, Maves and Kimmel (Maves and Kimmel, 2005) reported a sequential induction of RA target genes, and showed that posteriorly expressed genes do not require longer exposure, but require higher levels of RA for induction than anteriorly expressed genes. This suggests that the RA gradient would increase over time, and that the high concentrations needed to activate posterior genes are only reached later during hindbrain development. The authors proposed a model in which the hindbrain needs a temporally increasing source of RA to define rhombomere identities sequentially (Fig. 5A).

The observation that Cyp26 genes show rhombomeric-specific expression patterns drove the idea that degradation might be involved in shaping RA distribution. In a mouse study, Sirbu et al. correlated the dynamics of Hoxb1 expression, from its onset to its restriction in r4, with the dynamic expression of Cyp26 genes (Sirbu et al., 2005). They proposed a ‘shifting boundaries’ model in which the anterior boundaries of rhombomere-specific genes are fixed by the posterior limit of CYP26 activity at the time of their onset of expression. Hernandez et al. obtained consistent results in zebrafish, and elaborated a ‘gradient-free’ model, in which RA degradation by CYP26 enzymes determines progressively more posterior limits of RA-dependent gene expression in a stepwise manner (Hernandez et al., 2007). All three CYP26s would thus function to establish three sequential boundaries in RA responsiveness, i.e. pre-r3/r4, r4/r5 and r6/r7 (Fig. 5B).

As Cyp26a1 is under the control of RA signalling, a more subtle role for RA degradation has been proposed by White and colleagues (White et al., 2007; White and Schilling, 2008). Their model (Fig. 5C), in which CYP26A1 plays a central role in modulating RA levels dynamically, can reconcile the various
Observations described above. Using bead implantation, the authors showed that FGF signalling acts indirectly by inhibiting RA-dependent activation of Cyp26a1 expression. By computational analysis, they showed that RA gradients can be influenced by interacting feedback (RA signalling then inducing RA degradation) and feedforward (FGF signalling then repressing RA degradation) effects. The feedforward effects of FGFs couple the shape of the RA gradient to that of the FGF gradient. They argue that this makes the gradient stable to fluctuations in RA synthesis, but also over an expanding field of cells.

One aspect that should be addressed is regulation at the level of the source of RA. Studies in Xenopus proposed an alternative mode of RA gradient formation based on cooperation between RDH10 and RALDH2 (Strate et al., 2009). RDH10 produces retinaldehyde in the anterior cervical mesoderm and ventral hindbrain that diffuses posteriorly, where RALDH2 converts it into RA. Highest levels of RA would thus be produced at the anterior front of RALDH2 expression, where retinaldehyde concentration is highest, with decreasing concentrations observed posteriorly (Fig. 5D). This peak of RA would move posteriorly concomitant with the translocation of the RDH10 and RALDH2 expression domains, shifting the peak of the gradient from the hindbrain/spinal cord boundary to within the spinal cord. Further studies are required to address the significance of such cooperative effects in the hindbrain or in other morphogenetic fields. It was shown recently that Raldh2 compound mutant mice show reduced mesodermal or in other morphogenetic fields. It was shown recently that Raldh2 expression is under the transcriptional control of a ternary complex that includes Hox, Pbx and Meis proteins. Both Hoxa1;Pbx1;Pbx2 and Raldh2 expression (Vitobello et al., 2011). These authors show that HOXA1-PBX1/2-MEIS2 directly binds a regulatory element required to maintain normal Raldh2 expression. Thus, in the hindbrain, Hox proteins may regulate their own boundaries by controlling RALDH2 levels, thus contributing to shaping the RA gradient produced.

Table 3. Examples of genes containing functional and/or evolutionarily conserved RA-response elements

| Gene Product | Gene Product |
|--------------|--------------|
| **Transcription factors** | **Product** |
| **Nuclear receptors** | **Product** |
| Nr2c1 | early embryonic nuclear receptor, TR2 |
| Rara | retinoic acid receptor α |
| Rabb | retinoic acid receptor β |
| Rarg | retinoic acid receptor γ |
| **Homeodomain** | **Product** |
| Hoxa1, Hoxb1, Hoxa4, Hoxb4, Hoxd4, Hoxb5 | caudal type homeobox 1 |
| Cdx1 | paired box gene 6 |
| Pdx1 | pancreatic and duodenal homeobox 1 |
| Pitx2 | paired-like homeodomain transcription factor 2 |
| Tlx2 | T-cell leukemia homeobox 2 |
| **Others** | **Product** |
| Cebpe | C/EBP epsilon |
| Egr1 | early growth response 1, Krx24 |
| Ets1 | E26 avian leukemia oncogene 1 |
| Foxa1 | hepatic nuclear factor 3α |
| Hnf1a | Tf1, LF-B1 |
| Neurog2 | neurogenin 2 |
| Olig2 | oligodendrocyte transcription factor 2 |
| Pouf1f1 | pituitary-specific transcription factor 1, Pit1/GHF1 |
| Pouf5f1 | Oct4 (RA-mediated repression) |
| Stat1 | signal transducer and activator of transcription 1 |
| **Proteins from the retinoid pathway** | **Product** |
| Adh7 | ADH7, formerly ADH3 or class IV ADH |
| Aldh1a1 | RALDH1 |
| Crapb2 | CRABP-II |
| Cyp26a1 | CRYP26A1 |
| Rbp1 | CRBP-I |
| **Adhesion/extracellular matrix proteins** | **Product** |
| Icam1 | intercellular adhesion molecule 1 |
| Itgb3 | β3 integrin |
| Lamb1-1 | laminin B1 |
| **Secreted proteins/signalling factors and hormones** | **Product** |
| Epo | erythropoietin |
| Fgf8 | fibroblast growth factor 8 |
| Ghr | growth hormone |
| Gnrh1 | gonadotropin releasing hormone 1 |
| Tshb | thyroid stimulating hormone β subunit |
| Lefty1 | left-right determination factor 1 |
| Mdk | midkine |
| Nodal | Nodal |
| Oct | octosin |
| Plat | tissue plasminogen activator, t-PA |
| Pth1r | PTH/PTH-RP receptor |
| Shh | sonic hedgehog |
| Prl1d1 | placental lactogen 1, chorionic somatomammotropin hormone 1 |
| **Membrane receptors** | **Product** |
| Il2ra | interleukin 2 receptor α |
| Ngrf | nerve growth factor receptor |
| Ptpa | platelet-activating factor receptor |
| **Neurotransmitter receptors** | **Product** |
| Adrb1 | adrenergic receptor β1 |
| Drd2 | dopamine D2 receptor |
| **Enzymes** | **Product** |
| Cd38 | ADP-ribosyl cyclase 1 |
| Cyp24a1 | 25-OH-vitamin D3-24-hydroxylase |
| Hsd17b1 | 17-beta hydroxysteroid dehydrogenase 1 |
| Mmp11 | matrix metallopeptidase 11, Stromelysin-3 |
| Pck1 | phosphoenolpyruvate carboxykinase 1 |
| Prkca | protein kinase Cα |
| Tgm2 | transglutaminase 2 |
| **Others** | **Product** |
| Afp | α-fetoprotein |
| Apoa1, Apoa2, Apoa3 | apolipoproteins A-1, A-2, C-3 |
| Cryab | αB-crystallin |
| Nes | nestin |
| Pcp2 | Purkinje cell protein 2 |
| Sftp3 | surfactant-associated protein B |
| Ucp1 | uncoupling protein 1 |

The list is organised according to the types of proteins encoded and is by no means exhaustive. For detailed lists of RA-responsive genes from which these data were mainly compiled, see Balmer and Blomhoff (Balmer and Blomhoff, 2002; Balmer and Blomhoff, 2005).
Fig. 4. Hindbrain abnormalities in animal models with altered RA signalling. (A) The hindbrain rhombomeric structure in a wild-type (WT) embryo, highlighting rhombomeres 2-7 (r2-r7) with adjacent somites (black) and otocyst (blue) also shown. Pre-segmental/segmental expression patterns of three key rhombomeric markers [Kreisler (MaFB), Hoxb1, Krox20] are depicted (red bars; dashed bars indicate patchy or ill-defined expression domains). (B-F) Alterations in hindbrain segmentation and molecular patterning are illustrated in various animal models with endogenous deficiency in RA signalling: (B) vitamin A-deficient (VAD) quail; (C) Raldh2−/−, as well as Rara−/-;Rarg−/- signalling mutant mice (lacking RARα and RARγ); (D) Rara−/-;Rarb−/- mice (lacking RARα and RARβ); (E) Cyp26a1−/- mice; and (F) Cyp26a1−/-;Cyp26c1−/- mice. Abnormal (enlarged, non-segmented and/or abnormally patterned) rhombomeres are indicated by quotation marks. Otocyst abnormalities were not determined (ND) in some models. SC, spinal cord.

RA functions during forebrain development

Whereas many studies have investigated RA functions in the developing hindbrain, other aspects of brain development remain less explored. Initial studies using the chick system in which an RAR/RXR antagonist was delivered by bead implantation (Schneider et al., 2001), or using the VAD quail model (Halilagic et al., 2003), indicated a role for RA in A-P patterning of the embryonic forebrain and cell survival in the telencephalon (the anteriormost forebrain derivative). Moreover, RA was suggested to function mutants only partly supported the avian experimental data. Using a RARE-containing reporter transgene it was found that Raldh2−/− mutants at E14.5 after maternal RA rescue, and reported no change in cell proliferation or in the overall organisation of the affected meninges, and found that all-trans-RA treatment improved cortical neurogenesis in the cerebral cortex, based on the observation that Raldh2−/− and to a lesser extent Raldh1−/− is expressed in the developing meningeal cell layer from E13.5 to postnatal stages. RA produced by meningeal cells could thus diffuse within the neuroepithelium, where it may influence progenitor cell proliferation or differentiation and/or radial migration along cortical layers. Meningeally produced RA may regulate neurogenesis in other brain regions at later developmental stages (Zhang et al., 2003). A new player came from the recent work of Siegenthaler et al. (Siegenthaler et al., 2009). RDH10, which acts upstream of RALDHs, is also expressed in the developing meninges (Romand et al., 2008) and, while studying Foxc1 mutant mice that exhibit defective forebrain meningeal formation, Siegenthaler et al. showed decreased Raldh2 and Rdh10 expression in the affected meninges, and found that all-trans-RA treatment improved cortical development both in vivo and in an explant culture system. Chatzi et al. (Chatzi et al., 2011) challenged these results and the hypothesis of a role for meningeal RA. These authors analysed Raldh2−/− mutants at E14.5 after maternal RA rescue, and reported no change in cell proliferation or in the overall organisation of the cortical layers, although the meningeal layers lacked RA activity as observed with a reporter transgene. This issue will need to be resolved by more in-depth studies and might require the generation of additional murine models with tissue-specific ablation of RA synthesis in meninges. Currently, the only unequivocal role of RA in the forebrain at the mid to late developmental stages relates to the differentiation of GABAergic striatal projection neurons and interneurons migrating to olfactory bulb and cortex, and involves RALDH3 activity in the LGE subventricular zone (Chatzi et al., 2011; Molotkova et al., 2007). Furthermore, it is becoming clear that endogenous RA functions are likely to persist in adult neuronal populations (see Box 3).
Raldh2 function in the branchial apparatus was uncovered in Raldh2–/– mutants rescued by stage-specific maternal RA. Analysis of an RA-responsive transgene revealed a more extended area of RA activity, extending up to the posterior edge of the 2nd arch. Furthermore, this activity was found in both mesenchymal and endodermal cell layers. RA levels are produced at the hindbrain/spinal cord boundary, and this peak moves posteriorly as development proceeds. The models shown in A-D are not mutually exclusive and together they are likely to account for the sequential regulatory effects of RA in pre-segmented and segmented hindbrain.

### RA actions in foregut derivatives

RA signalling has numerous other functions during early embryogenesis. We briefly discuss how these functions may correlate with gradients of activity and/or diffusion between cell layers, first focusing on the branchial apparatus and then the lungs and pancreas. Details of RA functions in other organ systems can be found in other reviews (Duester, 2008; Niederreither and Dollé, 2008).

#### RA signalling in the branchial apparatus

Branchial arches are segmental structures that develop along the embryonic foregut endoderm. They are colonised by segmental mid/hindbrain neural crest streams and give rise to various derivatives including the hyoid bone, thyroid, parathyroids and thymus, and specific cardiac populations. RA is produced locally by RALDH2, which is expressed in the mesenchyme surrounding the foregut up to a rather sharp boundary at the level of the 4th-6th arches (Niederreither et al., 2003). Analysis of an RA-responsive transgene revealed a more extended area of RA activity, extending up to the posterior edge of the 2nd arch. Furthermore, this activity was found in both mesenchymal and endodermal cell layers. RALDH2 function in the branchial apparatus was uncovered in Raldh2–/– mutants rescued by stage-specific maternal RA supplementation (Niederreither et al., 2003). This supplementation partly rescued hindbrain and neural crest defects, unveiling an abnormal branchial phenotype in which all structures derived from the 3rd, 4th and 6th arches failed to develop, including endodermal pouches and aortic arch arteries. Consequently, many of the derivatives of these arches were missing or abnormal at foetal stages. Abnormalities of the aorta and large vessels, which are derived from the aortic arches, were also seen, with a lack of aortic trunk septation (persistent truncus arteriosus) incompatible with postnatal survival. Treatment of early somite stage wild-type embryos with a pan-RAR antagonist (BMS493) led to similar effects on posterior branchial arches (Wendling et al., 2000). Generation of a hypomorphic Raldh2 mutation revealed a particular susceptibility of the branchial region to diminished RA synthesis, as hypomorphic mutants displayed abnormalities that phenocopied the rescued Raldh2+/− mutants (Vermot et al., 2003). These abnormalities phenocopy a human condition, DiGeorge syndrome, which is caused by chromosomal deletions that affect the genes encoding the transcription factor T-box 1 (TBX1) and the adaptor protein CRKL. Analysis of murine Tbx1/Crkl loss-of-function models revealed locally increased RA signalling due to changes in the Raldh2 and Cyp26a1/Cyp26b1 expression domains (Guris et al., 2006), and a genetic interaction between Raldh2 and Tbx1 was recently demonstrated (Ryckebusch et al., 2010). Collectively, these data show that RA acts to pattern posterior branchial arches and their derivatives and implicate the retinoid pathway in the pathogenesis of DiGeorge syndrome.

Consistent molecular abnormalities have been observed in the branchial region of Raldh2 mutants (Niederreither et al., 2003; Vermot et al., 2003) and BMS493-treated cultured embryos (Wendling et al., 2000). Among the affected genes are Hoxa1 and Hoxb1, which are two RARE-containing genes that are also
Box 3. Retinoids in the adult brain
Retinoid functions are likely to persist through postnatal life. Mice mutant for RARβ and RXRγ, two receptors specifically expressed in striatal structures including the caudate putamen and nucleus accumbens (NAC), show reduced locomotor activity and impaired motor performance typical of abnormal striatal function. Importantly, Rarg−/− mice show increased despair behaviour and another key symptom of depression (anhedonia), which are both reversed by chronic antidepressant treatment. Adenovirus-mediated re-expression of RXRγ within the NAC also reversed these behaviours, clearly demonstrating a postnatal function for RXRγ signalling (Krzysziak et al., 2010). These findings suggest that altered retinoid signalling could contribute to diseases affecting the nigrostriatal system, such as Parkinson’s and Huntington’s diseases. RA may also act in the hippocampus, a key structure for memory processing and emotion. Neurogenesis occurs throughout life in the hippocampal granular zone, a site of high RA activity. Rarb−/− and Rarg−/− mice are deficient in spatial learning and memory, like vitamin A-deficient (VAD) rats, for which the deficits can be rescued by RA treatment (Bonnet et al., 2008). Altered retinoid signalling may also be involved in the degradation of hippocampal function in aging mice. Some pioneering studies suggest that retinoids might regulate the proliferation and/or differentiation of hippocampal stem cells into functional neurons. Adult neural stem cells are also found in the forebrain subventricular zone and in the olfactory bulb. In vitro, RA can increase neurogenesis by enhancing the proliferation and differentiation of adult forebrain neuroblasts, and in vivo it may regulate the proliferation of slowly dividing astrocytes in the subventricular zone (Haskell and LaMantia, 2005). Further characterisation of these functions might have important implications for the therapy or prevention of neurodegenerative diseases.

affected in the developing hindbrain. Other possible effectors of the RA-deficiency phenotype include Fgf genes, mainly Fgf8 and Fgf3; the expression of which was severely reduced in Raldh2 mutants. Neither the molecular nor the phenotypic studies provided clear evidence for an RA concentration gradient acting in the branchial region. They indicated, however, that the retinoid signal acts non-cell-autonomously: by analogy with the hindbrain model, it can be defined both as a ‘vertical’ signal travelling from mesenchyme to pharyngeal endoderm and as a ‘planar’ signal diffusing along the branchial region up to the level of the 3rd arch. Another parallel with the hindbrain is that RA action may be restricted by the activity of CYP26s, all of which are expressed in specific branchial/cervical cell populations (Table 1).

RA signalling during lung development
Region-specific retinoid signalling has other important functions in the development of foregut derivatives. Analysis of Raldh2−/− mutants and experiments performed on embryonic explants demonstrated that a lack of RA/RAR activity prevents induction and growth of the primary lung buds (Desai et al., 2006; Wang et al., 2006). The underlying molecular events include a lack of Fgf10 induction in the lung field (the region where primary lung buds are induced) caused by upregulation of TGFβ signalling, which has an inhibitory effect on Fgf10 expression (Chen et al., 2007; Chen et al., 2010).

RA signalling and pancreas development
It was demonstrated in zebrafish (see Alexia et al., 2009), Xenopus (Chen et al., 2004) and mouse (Martin et al., 2005; Molotkov et al., 2005) that RA is also required for pancreas development. Here, RA (produced mesodernally by RALDH2) acts by diffusing towards the endoderm, where most of the molecular abnormalities are observed under RA deficiency. The pancreas derives from two endodermal primordia known as the ventral and dorsal buds, and lack of RA specifically affects induction and growth of the dorsal pancreatic bud. One important role of RA is to downregulate SHH signalling, which has an inhibitory effect on pancreas induction. RA may also act at later stages of specification of pancreatic endocrine cell lineages (Martin et al., 2005; Ostrom et al., 2008). Recent work indicates that RA may act more globally to coordinate the position of endoderm-derived organs along the foregut and midgut A-P axis (Bayha et al., 2009), and that CYP26 enzymes may restrict the extent of RA signalling and set up the limit of the pancreatic field (Kinkel et al., 2009).

RA and limb development
Many early studies have investigated the role of RA in embryonic limb bud patterning, triggered by reports of its ability to induce mirror-image digit duplications when applied locally in chick wing buds (Tickle et al., 1982) and of the measurement of differential endogenous concentrations along the limb A-P axis (Thaller and Eichele, 1987). Abnormalities in limb skeletal patterning were first reported for Rara−/−;Rarg−/− compound mutant mice (Lohnes et al., 1994), and it was later found that mutants for RA-synthesizing enzymes have hypoplastic or absent forelimb buds (Rdh10−/− or Raldh2−/− mutants, respectively) (Cunningham et al., 2011; Niederreither et al., 1999; Sandell et al., 2007). A detailed study of compound Raldh mutants showed, however, that RA is not necessary for hindlimb development (Zhao et al., 2009). It was found that RA deficiency affects the expression of several regulators of forelimb bud growth and patterning, including Fgf4/8 and Shh (Mie et al., 2004b; Niederreither et al., 2002). A subsequent study concluded that these effects are indirect, with RA acting outside of the limb bud and perhaps even before its induction: here, an RA-dependent inhibition of FGF8 signalling in the body axis near the forelimb field would create a permissive environment allowing limb bud induction (Zhao et al., 2009). Eventually, limb bud cells need to develop in an RA-protected environment, mainly through the sustained action of CYP26B1 (Probst et al., 2011; Yashiro et al., 2004). Interestingly, such a function might have arisen prior to tetrapod limb specialisation. Indeed, RA signalling is also required for the development of zebrafish pectoral fin buds, acting from gastrulation to early somite stages as a permissive signal for the proper induction and growth of the fin bud (Gibert et al., 2006; Grandel and Brand, 2011).

RA function during somitogenesis and neural tube differentiation
Many in-depth studies have investigated the functions of RA during elongation of the embryonic body axis, where it controls several events relating to mesodermal segmentation and neurogenesis in the caudal neural tube (the future spinal cord). Somites are segmented epithelial structures that are formed sequentially along the left and right paraxial mesoderm. They are the precursors of various tissues: their dorsal portion, the dermomyotome, will differentiate into muscle and dermis, whereas their ventral part, the sclerotome, gives rise to skeletal elements (vertebral column and ribs). Somite formation is a rhythmic process that relies on a ‘clock and wavefront’ mechanism, in which a molecular oscillator driven by Wnt and Notch signalling generates cyclic waves of gene expression that progress rostrally along the PSM (reviewed by Gibb et al., 2010). This oscillator interacts with a system of
signalling gradients to create a maturation or ‘determination’ front, which is displaced posteriorly as the embryonic axis elongates. PSM cells become competent to respond to the oscillations when they become located anterior to the determination front, initiating the programme of somite formation. Recent findings suggest that the mechanism controlling the position of this front involves two dynamic, antagonizing gradients: a caudal-to-rostral Wnt/FGF gradient and an opposing RA gradient (Fig. 6) (Diez del Corral et al., 2003) (reviewed by Aulehla and Pourquie, 2010; Pourquie, 2011). As demonstrated by reporter transgenes, at gastrulation stages RA activity becomes progressively excluded from the posteriormost PSM, correlating with the onset of Cyp26a1 expression in this region (Ribes et al., 2009; Sirbu and Duester, 2006). At later stages during axial elongation, Raldh2 is specifically expressed in the newly formed somites and posteriormost PSM. Analysis of VAD quail embryos and Raldh2–/– mouse mutants revealed that lack of RA signalling leads to an anterior expansion of the Fgf8 domain along the PSM and to the formation of smaller somites (Diez del Corral et al., 2003; Vermot et al., 2005). RA has the ability to repress posterior ectodermal expression of Fgf8 for a short period of time when the somitogenesis molecular clock initiates (Sirbu and Duester, 2006).

Another important role of RA is to control the bilateral symmetry of the left and right somitic columns. Raldh2–/– mouse embryos often exhibit fewer somites on one (usually the right) side (Vermot et al., 2005). This asymmetry in somite formation is caused by the desynchronisation of the molecular waves of expression along the left and right PSM. Similar findings were obtained by experimentally interfering with RA signalling in chick (Vermot and Pourquie, 2005) and zebrafish (Kawakami et al., 2005) embryos. Recently, it was shown that a mutation in mouse Rere (which encodes a chromatin-remodelling protein also known as atrophin) leads to an asymmetry in somite formation similar to that observed in Raldh2–/– embryos (Vilhais-Neto et al., 2010). These authors showed that RERE positively regulates RA signalling by forming a complex with the nuclear receptor NR2F2 (COUP-TFII), p300 (EP300) and RARs, thereby promoting transcriptional activation of target genes. Interestingly, NR2F2 was found to be asymmetrically expressed in the right PSM, in a domain coinciding with asymmetrical RA signalling as detected by a reporter transgene. This led to a revised model in which transient asymmetrical RA signalling acts to ‘buffer’ asymetrically expressed molecules that act as determinants of the embryonic left-right axis, shielding the PSM from the desynchronizing action of these signals (Vilhais-Neto et al., 2010).

Intimately linked to mesodermal differentiation during body elongation is the process of cell fate determination within the developing spinal cord. RA acts by diffusing from paraxial/somitic mesoderm to the adjacent neural plate to control several molecular events necessary for the specification of neuronal cell types in the prospective spinal cord (Diez del Corral et al., 2003; Molotkova et al., 2005; Novitch et al., 2003; Wilson et al., 2004). Crucial to this process is the previously described functional antagonism between RA and FGF signalling, the latter acting to maintain a proliferative ‘stem’ zone in the caudalmost neural plate (Fig. 6). High FGF levels have an inhibitory effect on Raldh2 expression, and the presence of CYP26A1 in the caudal stem zone further prevents any inappropriate RA signalling. Once the FGF signal is attenuated along the PSM, RA will trigger the onset of spinal cord differentiation and promotes the induction of genes involved in the determination of ventral neuronal cell types. Importantly, canonical Wnt signalling mediates the transition from FGF to RA signalling during axis elongation (Olivera-Martinez and Storey, 2007). These events have been reviewed in detail elsewhere (Diez del Corral and Storey, 2004; Wilson et al., 2009).

Conclusions
Although there has been tremendous progress in characterising the numerous developmental events regulated by the retinoid pathway, many gaps remain with respect to the underlying mechanisms of RA-mediated patterning. It is unclear, in particular, whether there is a unifying mechanism that explains the effects of retinoids on cell proliferation, survival and/or differentiation, or whether there might be different outcomes in various developing systems. Interpretation of experimental data are complicated by the fact that exposure to RA (in cultured cells, whole embryos or explants) may have different, sometimes opposite, effects depending on the concentration, stage or duration of exposure. Strategies that interfere with endogenous retinoid signalling (e.g. through genetic loss-of-function, morpholinos) appear more reliable than approaches using exogenous retinoids, including RAR/RXR antagonists that may lead to the forced repression of target gene loci. The issue of whether RA functions as a true morphogen is still under debate and, surprisingly, it seems that this question has been less satisfactorily addressed for RA than for other candidate morphogens. For instance, there have been few attempts to assess how this signal is transmitted along embryonic cell populations using real-time live imaging. Progress on this issue will require the generation of more suitable reporter systems using short-lived fluorescent proteins.
Information on the molecular targets of RA signalling is also fragmentary. Recent studies led to novel insights into the interplay between retinoid and other signalling pathways (such as FGFr, Hedgehog, TGFβ) in several developing systems. However, a better knowledge of the immediate early RA target genes is necessary to clarify whether there are common regulatory networks, rather than specific gene targets, for each system. Transcriptomic analyses have begun to yield valuable information, both in cell lines and embryonic tissues (e.g. Chen et al., 2007). Studies at the chromatin level have thus far been hindered by the lack of high-grade RAR antibodies, and so far are only available for cultured cell lines (Delacroix et al., 2010; Mahony et al., 2011). The next challenges will be to decipher the molecular events that are regulated in vivo by RA signalling, and to determine how these events correlate with effects on cell growth, determination and/or differentiation in specific progenitor populations. This research will remain of paramount importance as retinoids will not only continue to be used as ‘conventional’ drugs, but are also promising compounds for modulating cell behaviour in stem cell-based therapy.

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Competing interests statement

The authors declare no competing financial interests.

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Development 139 (5)

**DEVELOPMENT**

**PRIMER 857**

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