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REVIEW

Hindbrain induction and patterning during early vertebrate development

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Abstract

The hindbrain is a key relay hub of the central nervous system (CNS), linking the bilaterally symmetric half-sides of lower and upper CNS centers via an extensive network of neural pathways. Dedicated neural assemblies within the hindbrain control many physiological processes, including respiration, blood pressure, motor coordination and different sensations. During early development, the hindbrain forms metameric segmented units known as rhombomeres along the antero-posterior (AP) axis of the nervous system. These compartmentalized units are highly conserved during vertebrate evolution and act as the template for adult brainstem structure and function. TALE and HOX homeodomain family transcription factors play a key role in the initial induction of the hindbrain and its specification into rhombomeric cell fate identities along the AP axis. Signaling pathways, such as canonical-Wnt, FGF and retinoic acid, play multiple roles to initially induce the hindbrain and regulate *Hox* gene-family expression to control rhombomeric identity. Additional transcription factors including Krox20, Kreisler and others act both upstream and downstream to *Hox* genes, modulating their expression and protein activity. In this review, we will examine the earliest embryonic signaling pathways that induce the hindbrain and subsequent rhombomeric segmentation via *Hox* and other gene expression. We will examine how these signaling pathways and transcription factors interact to activate downstream targets that organize the segmented AP pattern of the embryonic vertebrate hindbrain.

Keywords Hindbrain · Neural specification and patterning · Hox proteins · Meis and Pbx proteins · FGF, Wnt and retinoic acid signaling · Rhombomere patterning

Introduction

The central nervous system (CNS) has been a classic model system to study pattern formation during early vertebrate development. Vertebrate CNS morphology is strikingly conserved, from fish to mammals. Dorsal ectodermal cells on the outer surface of the embryo are induced to neural fates by neighboring dorsal mesoderm cells. This induced neural plate tissue subsequently thickens, elevating at the embryo's two lateral edges to form the neural folds. These two arising neural folds merge at the dorsal midline of the embryo, creating the neural tube. The neural tube is asymmetric; the wider, thicker walled anterior region forms the brain, while the narrower, posterior part forms the spinal cord.

The brain subdivides into three regions with distinct antero-posterior (AP) characteristics: the most anterior forebrain (telencephalon and diencephalon), the midbrain (mesencephalon) and most caudally, the hindbrain (rhombencephalon). These regions are the morphological basis of distinct functional units of the brain. In the forebrain, the telencephalon gives rise to the cerebral cortex, basal ganglia and hippocampus. The diencephalon gives rise to the thalamus, hypothalamus and pineal gland. In the lateral diencephalon regions, optic vesicles form the eye structure. The midbrain will generate centers of sensory and motor control, whereas the hindbrain gives rise to the cerebellum, pons and

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medulla [1]. The hindbrain controls crucial physiological processes such as motor activity, respiration, sleep and blood circulation. It also receives processes and transmits multiple sensory inputs including the auditory, precerebellar and vestibular systems. Different streams of neural crest cells are generated in the hindbrain to give rise to cranial sensory ganglia, Schwann cells, cardiac connective tissue and most of the cranial skeleton [2].

The embryonic hindbrain is divided into seven or eight segmented regions called rhombomeres (r). In the most rostral hindbrain, r1 borders the midbrain in a region called the midbrain–hindbrain boundary, whereas at the most caudal end, r7/r8 borders the most-anterior spinal cord. Each rhombomere has unique gene expression patterns that promote the region-specific fates, differentiation of neurons and production of distinct neural crest streams. Rhombomeric units are highly conserved during vertebrate evolution, acting as templates for the adult hindbrain structure and function, by giving rise to the pons, medulla oblongata and different cerebellar cell layers. The most caudal region of the central nervous system, the spinal cord, forms posterior to the hindbrain, extending to the rear of the body.

This elegant CNS morphogenesis is achieved in the vertebrate embryo through a series of inductive events. In amphibians, Mangold and Spemann in the 1920s found that grafting a dorsal mesodermal lip removed from the blastopore of an early gastrula stage embryo induced a secondary nervous system in the naïve ventral ectoderm of the transplanted host [3]. This region is called "the organizer", and the phenomenon of this experiment was termed "embryonic induction". These experiments were confirmed in other vertebrates in analogous fish and bird Spemann–Mangold organizer regions [4, 5]. More recent genetic studies suggest that a neural inducing organizer activity also exists in mammals. The mammalian organizer center may be composed of two regions acting in differing time periods. The earlier anterior visceral endoderm establishes initial embryonic AP polarity, while the later node region induces neural tissue [6].

Seminal experiments of Nieuwkoop, Eyal-Giladi, Toivonen and Saxen suggested that there are two inductive steps involved in neural induction and patterning [7–9]. In the first step, the "organizer" initially induces a general neural tissue having an anterior forebrain fate in a process called "activation". The second "transformation" step is a caudalizing-posterior induction, which re-specifies the "activated" neuro-ectoderm into more posterior CNS cell fates such as hindbrain and spinal cord [7].

Three different neural inducing proteins (noggin, chordin, follistatin) were initially identified and characterized in amphibians [10–12]. These proteins all induce anterior-pan neural tissue. While structurally different, these proteins share one common activity, the ability to inhibit BMP protein signaling activity [12–14] by blocking BMP ligand–receptor interactions [14–16]. Thus, BMP antagonism serves as the initial "activation" signal inducing ectoderm to rostral neural fates. Since BMP signaling actively drives ectoderm cells to epidermal/non-neural cell fates, BMP signaling inhibition suffices to convert ventral epidermal ectoderm to a more dorsal neural fate [17–19].

Parallel to the discovery of the "activation" signal of BMP antagonism, neural caudalizing "transformation" molecules were also identified. Using *Xenopus* and chick experimental embryology techniques, in addition to zebrafish and mouse genetics, three signaling pathways were identified that caudalize rostral neural tissue. These include the basic fibroblast growth factor (bFGF), retinoic acid (RA) and canonical Wnt signaling pathways [20–35]. For each pathway, multiple ligands, receptors and antagonists are expressed in different temporal windows and embryonic locales during the neural patterning process. The same pathways seem to have variant spatial and temporal roles in specifying multiple posterior cell fates in the developing vertebrate nervous system. These caudalizing factors induce an initial posterior neural "ground state", which undergoes fine-tuning into distinct locales such as hindbrain and spinal cord along the neural AP axis.

While the sequence of morphological events that lead to the partition of the CNS into sub-domains is well known, the genetic networks that govern the specification and connectivity of different cell types along the CNS to yield a functional nervous system are only partially resolved (reviewed in [36–39]). This review examines the conserved transcriptional networks and morphogens that orchestrate the intricate regulation of early hindbrain specification and patterning in different vertebrates. It will cover both the earliest stages of hindbrain development/induction, as well as later stages of rhombomere specification. By addressing the complex interactive dynamics between signaling pathways, the earliest activation of Hox and TALE homeodomain proteins, as well as the later expression of non-homeodomain transcription factors, this review provides a unique comprehensive synopsis of the central processes of hindbrain development in relevant vertebrate systems.

The transcription factor blueprint of the hindbrain

The *Hox* genes are one of the most ancient regulators of body formation in metazoans, being crucial for AP axis formation in the developing vertebrate embryo. Hox proteins partner up with another ancient family of homeodomain proteins, the TALE class proteins, Meis and Pbx. By their joint interactive activities during the earliest stages of neural development, Meis, Pbx and Hox proteins act to induce and specify the hindbrain. Vertebrates typically have

thirteen Hox gene paralog groups (PG) expressed along the AP axis. The most anterior extent of Hox expression along the AP axis is the hindbrain, and the PG1–4 group genes are regionally expressed in the hindbrain, and required for its formation. Acting with the Hox genes to induce the hindbrain are the Meis/Pbx proteins. *Meis*/*Pbx* expression can precede *Hox* gene expression. In some systems, Meis/Pbx was shown to be required for the earliest activation of *Hox* gene expression in the hindbrain. In addition, various dimers or trimer of Meis/Pbx/Hox proteins directly activate target genes in the developing hindbrain. This review will address how these transcription factors interact with signaling pathways to regulate the earliest formation of the hindbrain.

Homeodomain proteins

TALE class homeodomain proteins: Meis, Prep and Pbx proteins

The correct temporal and spatial expression of Hox paralogous group (PG1–4) proteins is crucial for establishing the initial segmentation of the hindbrain. Hindbrain induction is also dependent on Three-Amino acid Loop Extension (TALE) homeodomain proteins, which belong to the MEIS (Meis1–3 proteins), PREP and PBC (Pbx1–4 proteins) groups. TALE proteins are atypical homeodomain-containing transcription factors having three additional amino acids between the first and second helix of the homeodomain [40]. During hindbrain development in zebrafish and *Xenopus*, *meis*/*pbx* genes are activated very early, preceding *hox* gene expression in the presumptive neural plate. Meis/Pbx and Hox proteins interact at two distinct levels. Early in zebrafish and *Xenopus* development, Meis/Pbx proteins are required to initially activate *PG1–4 hox* gene expression in the hindbrain [41–44]. Later, Meis (Prep)/Pbx/Hox protein combinations bind target genes to activate their transcription. Some of these target genes are also *hox* and *meis* genes themselves, but non-*hox* gene targets also lie downstream to Meis/Pbx/Hox (Fig. 1) [45, 46].

Studies in *Xenopus* and zebrafish embryos revealed the requirement of Meis1 and Meis3 for proper hindbrain formation [41–44, 47–50]. The expression of *meis3* initiates early, at late gastrula stages in the presumptive hindbrain region. At later stages, its expression becomes localized to the r2–r4 region [42, 43, 47, 48]. In contrast, expression of the *meis1* and *meis2* genes is more general, expanding into more anterior neural regions than *meis3* in multiple vertebrate species, such as mouse, chick, *Xenopus* and zebrafish [43, 51–56]. In *Xenopus* and zebrafish, loss of Meis/Pbx function, obtained by either dominant-negative proteins, antisense oligonucleotide morpholino (MO) knockdown, or genetic mutation, triggers a loss of the entire hindbrain region, accompanied by a loss in expression of a many hindbrain markers, including *hox PG1-4* genes [41–44, 47]. This is concomitant with a posterior expansion and enlargement

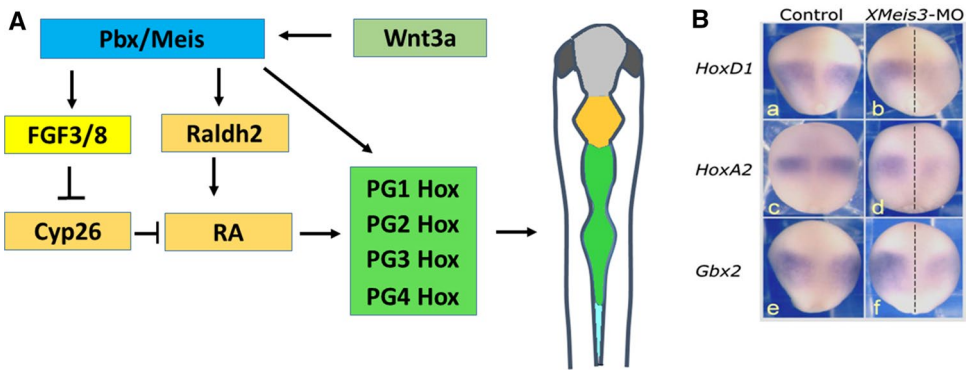

Fig. 1 Pbx/Meis proteins lie upstream of *Hox* gene expression in the early hindbrain. **A** Schematic representation of Pbx and Meis regulatory activity in the early hindbrain. Pbx/Meis proteins are induced in the neural plate by Wnt signaling prior to *Hox* gene expression. Pbx and Meis activate (individually and additively) the transcription of PG1–4 *Hox* genes, by binding to their Pbx/Meis-responsive elements. In parallel, Pbx/Meis also activate FGF and RA signaling to promote *Hox* gene expression. This is mediated by their binding to responsive elements in the *Raldh2* gene, the main RA-synthesizing enzyme, as well as by inducing *FGF* gene expression that in turn inhibits the RA-degrading enzyme Cyp26. Following the initial activation of *Hox* gene expression, Meis/Pbx synergize with HoxA1 protein to induce expression of other *Hox* genes (not shown). **B** Meis3 regulates early homeobox gene expression (from Elkouby et al. [44]). Meis3 knockdown inhibits early *HoxD1, HoxA2* and *Gbx2* gene expression. Embryos were injected into one blastomere at the two-cell stage with *Meis3*-MO (10–12.5 ng/blastomere; b, d, f). Gene expression was examined at late gastrula, stage 12.5. All embryos are viewed dorsally, and oriented with anterior at the top, posterior at the bottom. The dashed line in b, d, f indicates the dorsal midline; the *XMeis3*-MO-injected side is on the right. Early expression of *HoxD1, HoxA2* and *Gbx2* is inhibited on the *Meis3*-MO-injected side (100%, $n=16$; 88.2%, $n=34$; 66.4%, $n=33$, respectively). In neurula-stage Meis3-morphant sibling embryos, a typical inhibition of posterior neural cell types was observed

of more anterior forebrain structures. Meis3 also suffices for hindbrain induction, as its overexpression induces ectopic hindbrain formation while repressing forebrain formation in both *Xenopus* and zebrafish embryos and explants [42–44, 47, 48, 57]. In mouse and chick, Meis/Prep and Pbx proteins also are required for activation of hindbrain enhancers in the *hoxb1* and *hoxb2* genes [58–61].

Slightly later in zebrafish development, Meis3 synergizes with HoxB1b (HoxA1 in other vertebrates) protein to induce expression of various other hindbrain markers such as *hoxb1a* (*hoxb1*) and *krox20* [48]. Similarly, in *Xenopus*, HoxD1 and Meis3 co-expression enhances *hoxb3* and *krox20* expression [62]. In mice and zebrafish, the *krox20* promoter has a functional r3-specific Hox/Meis/Pbx binding site, although it does not appear to be a direct Meis3-target in *Xenopus* [44, 63, 64]. Moreover, *hoxd1* is a direct-target gene of Meis3 in *Xenopus* that acts downstream of Meis3 to induce hindbrain cell fates [44, 57, 62]. In *Xenopus*, the Zic1 and Pax3 transcription factors are required upstream of Meis3. Knockdown of Zic1 or Pax3 prevents early *meis3* gene expression, leading to a loss of hindbrain cell fates [57]. Ectopic Meis3 can rescue Zic1, but not Pax3 knockdown phenotypes.

Together with Meis proteins, the PBC family proteins are central for hindbrain induction. Pbx4 directly interacts with Meis1 and Meis3 proteins in zebrafish, and perturbation of either Pbx2 or Pbx4 activities eliminates r2–r6 cell fates in zebrafish [41, 43, 48, 65, 66]. *Pbx4* is expressed in the presumptive hindbrain region, where its early expression closely overlaps the *meis3* and *hoxb1b* (*hoxa1*) genes [48]. In *Xenopus*, Meis1 and Pbx1 proteins interact to regulate neural cell fate specification, where Pbx1 knockdown also disrupts hindbrain formation [50]. *Pbx1* is expressed in the presumptive forebrain, hindbrain and neural crest regions [53]. In *Xenopus*, Pbx1 and HoxD1 proteins strongly activate a heterologous mouse *hoxb1* enhancer, but Meis3 had no additive effect with either protein when tested separately or together [62, 67]. A Pbx protein partner that enhances Meis3 activity in *Xenopus* has not been identified. In chick and mice, Meis2 and Pbx proteins were found to synergize and to activate a *krox20* enhancer element in r3 [63]. Interestingly, in zebrafish, ectopic Meis1 expression rescued hindbrain formation in the absence of zygotic Pbx4 protein. This suggests a potential Pbx-independent mechanism of action, although maternal Pbx protein involvement was not fully ruled out (Fig. 1), [43].

Hox proteins

PG1 Hox proteins are key factors controlling early hindbrain specification. The PG1 Hox proteins are homologs of the *Drosophila labial* gene and include the HoxA1, HoxB1 and HoxD1 proteins. In all vertebrates, expression of the *PG1 hox* genes precedes all other *hox* genes in the presumptive hindbrain region at early gastrula stages and persists through neurula stages [68–77]. PG1 proteins are essential for correct hindbrain induction and segmentation. Combined knockdown of HoxB1a (Mouse Hoxb1) and HoxB1b (Mouse Hoxa1) proteins in zebrafish, or *hoxA1* and *hoxB1* gene deletion in mouse embryos led to significant hindbrain perturbation [75, 76, 78, 79]. In *Xenopus* embryos, triple knockdown of all the PG1 genes, *hoxa1*, *hoxb1* and *hoxd1* caused a complete loss of r2–6, and the entire hindbrain resembled the Hox non-expressing r1 region [77]. This phenotype is reminiscent of Pbx2 loss-of-function in zebrafish embryos, arguing for cooperative function of Pbx2 and PG1 Hox proteins in the specification of r2–6 regions [66]. Supporting this assumption, HoxA1 hexapeptide mutant proteins that fail to interact with Pbx proteins cause severe hindbrain phenotypes in mice [80]. Moreover, the combined PG1 loss-of-function phenotype is synergistically stronger than that of each of the individual inhibitions, and in *Xenopus,* PG1 knockdown could be rescued by overexpression of HoxD1 protein alone. This suggests at least partial functional redundancy between PG1 members [77]. To gain insight into the transcriptional network regulated by HoxA1, microarray analysis was performed on the prospective r3–5 region of *hoxa1* null and wild-type mouse embryos [81]. Around 300 genes were differentially expressed between the samples. While many of these genes were previously identified to play a role in hindbrain development, new target genes were also found to be downregulated in *hoxa1*-nulls, such as *FGF receptor 3* (*fgfr3*), *zic1*, *hnf1b*, *foxd3* and *lhx5*, suggesting that HoxA1 protein acts in a genetic cascade upstream to many target-genes that control wider aspects of hindbrain development than previously thought. HoxA1 mutations in humans have brainstem function perturbations, and it was suggested that this could be related to improper embryonic hindbrain development [82].

Early PG1 protein expression is a prerequisite for the proper sequential expression of later, more posteriorly expressed *PG2–4 Hox* genes [62, 77]. In *PG*2 mutant mice, development of the r3/r4 region is disrupted with poor border formation between r2/r3. Cells in r2 express only *hoxa2,* the most anterior of all Hox genes, and *hoxa2* loss-of-function mutations cause r2 to r1 fate changes [83–87]. Notably, *Hox PG1* and *PG2* genes are both expressed in r4, but with temporal differences. Single *hoxb2* mutant mice had no hindbrain segmentation defects, but in *hoxa2*/*hoxb2* double-mutant mice, the r2/3 and r3/r4 borders were lost, suggesting that the action of both PG2 genes is synergistic [88]. Interestingly, recent sequence analysis of *hoxa2* in *fugu* uncovered the presence of two orthologues, *hoxa2a* and *hoxa2b*. Each orthologue contains distinct cis-regulatory elements to drive *hoxa2* expression in neural crest or rhombomeres, respectively. This study suggests that these

regulatory regions are conserved throughout vertebrate evolution to mediate differential *hoxa2* expression and activity during development [89]. A negative regulatory mechanism exists between PG3 and PG2 groups. Studies in mice and chick embryos showed that Hoxb3 protein directly binds to *hoxb1* regulatory regions and represses *hoxb1* expression posterior to r4 [90]. In multiple *PG3* mutants, r5/6 identity was disrupted and r4-specific *hoxb1* expression was ectopically activated in r5/6 [91]. In *PG4* mutants, in contrast, hindbrain development was normal [92].

A recent study compared the binding targets of HoxA1 proteins in zebrafish and mouse finding that they share many common hindbrain target genes. Many of these targets also shared occupancy with Meis and Pbx proteins [93]. HoxA1 also was found to bind enhancer regions of the *meis2* and *meis3* genes [94], suggesting that HoxA1 may be regulating the early expression of these *meis* genes. This coupled to studies in *Xenopus* showing Meis3 regulation of PG1 Hox gene expression suggests that there is a mutual co-dependence between Meis and Hox PG1 proteins to regulate the earliest stages of vertebrate hindbrain specification.

TALE and Hox proteins cross-talk in the hindbrain

As mentioned previously, during early development, Meis, Pbx and Hox proteins interact at two distinct levels. Initially, Meis–Prep/Pbx and PG1 Hox proteins may reciprocally co-activate each other's gene expression in the hindbrain. Later, Meis/Pbx/Hox protein combinations bind target genes to further activate *hox* gene expression in the hindbrain [42]. In zebrafish embryos, ChIP studies showed that Meis/Pbx proteins specifically bind the *hoxb1a* and *hoxb2a* promoters in their respective tissues of expression [95]. These promoters were also enriched for histone H4 acetylation. In embryos ectopically expressing dominant negative Pbx proteins, Meis/Pbx activity was inhibited and histone acetylation was highly reduced. Furthermore, Meis/Pbx protein complex formation removes histone deacetylase (HDAC) from Hox-regulated promoters. Additionally, Meis proteins recruit CBP/p300 histone acetylase to *hox* promoters. Thus, Meis proteins function as direct transcriptional activators of the *hoxb1a* target gene by controlling the accessibility of HDAC/CBP proteins to its promoter [95]. More recent studies in zebrafish embryos show that TALE protein complexes actively poise *hoxb1a* promoters for expression by chromatin modification, as early as blastula stages. Later expression of the *hoxb1a* promoter at gastrula stage is triggered by Hoxb1b protein binding to these TALE protein complexes [96]. Pbx–Hox and Meis–Prep binding sites have also been used to define a shared sequence syntax system for identifying functional hindbrain-specific enhancer elements in zebrafish [97]. In rhombomeric segments, *Hox* gene expression is positively controlled by auto- and cross-regulatory binding of Meis/Pbx/Hox proteins to enhancer elements. For example, the *hoxb1* gene enhancer that drives expression in r4 harbors distinct Meis/Pbx and Hox/Pbx binding sites. In mice, Hoxa1 together with Meis/Pbx proteins initially activates this enhancer, but later, Hoxb1 itself, together with Hoxb2, Meis3 and Pbx2/4 proteins, is required for expression maintenance in r4 [58, 65]. Interestingly, additional Hox proteins of the PG3 group, but not Pbx/Meis proteins, negatively regulate *hoxb1* such that it remains restricted to r4 [90, 91].

Many additional examples of such interdependent, cross-regulatory loops are known. For instance, *hoxd1*, *hoxb2* and *hoxa2* all require Meis/Pbx for their expression in zebrafish, *Xenopus*, chick and mouse [43, 44, 49, 59, 66, 98, 99]. In mice, *Hoxb2* expression is directly activated by HoxB1 in r4, and HoxB2 protein then drives *hoxb1* expression. Thus, HoxB2 indirectly controls its own expression via HoxB1 [59–61, 88, 100]. *Hoxa2* expression in r4 is also regulated by conserved vertebrate enhancer elements that bind Meis/Pbx and HoxB1/HoxA2 proteins [98, 99, 101]. In more anterior r2–r3, Meis3/Pbx proteins are required for the early neural expression of *hoxa2* in *Xenopus* [44]. Studies in mouse and chick suggest that r4 activation of *hoxa2* requires Meis3/Pbx proteins; however, it is still an open question for the r2 *hoxa2* enhancer regions, which contain Sox binding sites and perhaps is not regulated by Meis/Pbx proteins [98, 99]. In mouse and chick, more posteriorly, *hoxa3* expression in r5/6 is controlled by an element binding Meis/Pbx and HoxA3/HoxB3 proteins [102]. In r6/7, the expression of *hoxb4 and hoxd4* in mice is also regulated by Meis1/Pbx as well as Hoxb4/Hoxd4-responsive elements after initial induction by retinoic acid signaling [103–105]. Finally, adding an additional level of complexity, all rhombomeres fail to develop properly upon Meis3 knockdown in *Xenopus*, despite the fact that *meis3* expression is restricted to r2–4 [42, 44, 47]. The elimination of early r4 *fgf3* expression in Meis3-depleted embryos likely triggers these non-autonomous effects [57]. Similarly, r4-specific *fgf3* expression is eliminated in zebrafish *pbx/hox* mutants [66], and this triggers a loss of hindbrain cell fates from r2–r6. In *Xenopus* or zebrafish, any direct activation of Meis/Pbx/Hox enhancer sequences in more posterior rhombomeres could be mediated by more ubiquitously expressed Meis or Prep proteins (Fig. 1).

Non-homeodomain transcription regulators

Three early expressed transcription factor proteins, variant hepatocyte nuclear factor-1 (Vhnf1), Krox20, and the Mafb/Kreisler/Valentino proteins, are crucial for regulating the earliest expression of *hox* genes. These transcription factors are thus essential for the establishment of correct

rhombomeric AP identity in the early hindbrain. Genetic disruption of these proteins severely perturbs the formation of r3–r6. Vhnf1 is the earliest gene expressed in r5/r6. In zebrafish, the Vhnf1 protein induces both *krox20* (r5) and *mafb* (r5/6) expression, which subsequently determines correct rhombomeric identities. Loss of Vhnf1 reduces r5/6 domains while the *hoxb1a*-expressing r4 region is expanded [106–108]. In mice, a Vhnf1-binding site was identified in a regulatory element of the *kreisler* gene. Mutating this site results in loss of *kreisler* induction, indicating that Vhnf1 is essential for rhombomere-specific *kreisler* expression in the future r5/r6 domain [109] (Fig. 2).

Krox20 is expressed in rhombomeres 3 and 5, and is paramount for r3/r5 regional identity and cell fate, as directly evident by various knockout or knockdown experiments in all vertebrates [107, 110–112]. In addition to Vhnf1, induction of *krox20* is triggered by a positive input from Pbx/Meis and HoxB1 [63, 113–115]. In chick, mice and zebrafish, *krox20* promoter/enhancer sites have functional r3-specific Hoxb1/Meis/Pbx binding sites, although in *Xenopus* it does not appear to be a direct Meis3-target [44, 63, 64]. Following the upregulation of *krox20* via these multiple regulators, it acts in a positive auto-regulatory loop to maintain its own expression. A recent study revealed that one of the *krox20* regulatory regions is required not only to initiate its expression but also to enable the auto-regulatory binding site to function [116]. This region acts with additional cis-regulatory elements, some of which are positioned further away from the *krox20* loci. These multiple regulatory modes ensure a proper level of *krox20* auto-regulation in r3 and r5. Notably, another member of Egr family, Egr4, was recently identified in *Xenopus* to be expressed in the posterior hindbrain [117]. Knockdown experiments uncovered a role for Egr4 in *krox20* and *mafb* upregulation in r5 or r5/r6, respectively, indicating that in the frog, Egr4 mediates the effect of Vhnf1 to activate these two genes in the posterior hindbrain (Fig. 2).

Initially, Hoxb1 activates *krox20* by binding to its enhancer. Later, when Hoxb1 expression levels increase and are restricted to r4, it represses *krox20* expression. Reciprocally, Vhnf1 and Krox20 proteins repress *hoxb1* to limit its expression to r4. These dual-negative regulatory interactions lead to the establishment of well-defined rhombomeric identities [107, 108, 112]. Moreover, Krox20 protein directly activates the expression of the *ephA4* receptor gene in r3/r5, which in turn sharpens the rhombomeric borders by preventing cell intermixing between segments [118–120]. Interestingly, two negative regulators of *krox20* gene expression, Nab and Nlz (also known as Neurl1a), have been identified in zebrafish, mice and chick hindbrains [121–123]. These

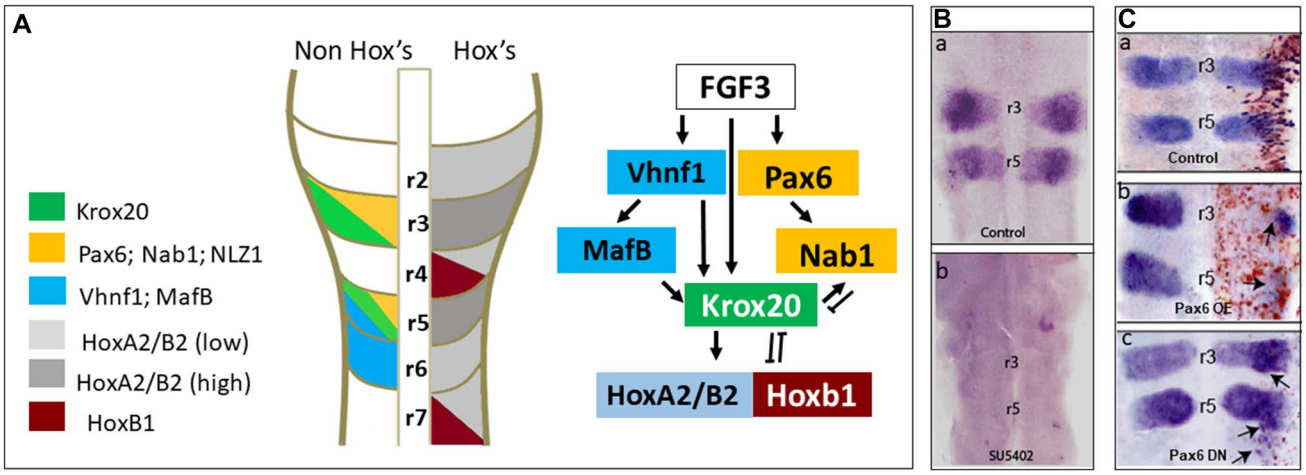

Fig. 2 Upstream and downstream regulation of Krox20 in the hindbrain. **A** Induction of *Krox20* gene expression in r3/r5 is initiated by FGF signaling. FGF induces Vhnf1 in r5/r6 that upregulates MafB, leading to Krox20 induction in r5. In r3, FGF induces *Krox20* expression in a Vhnf1-independent manner. Krox20 expression is restricted to the correct rhombomeres by Pax6, which is also induced by FGF. Pax6, which is expressed in r3/r5 is upregulated, and acts as a negative regulator of *Krox20* expression via the induction of Nab1. NLZ is another Krox20-negative regulator that is also co-expressed in r3/r5. In parallel, *Hoxb1* is expressed in r4 and represses *Krox20* expression in r4. Following the upregulation of Krox20 in r3/r5, Krox20 regulates *PG-2* Hox gene expression in r3/r5. It also induces the expression of its negative modulator Nab1. In addition, Krox20 represses *Hoxb1* expression in r3/r5, leading to accurate r3/r4/r5 identities. In early stages of development, Krox20 transiently activates *Hoxb1* (not shown). **B** FGF acts upstream of *Krox20* in the chick hindbrain. (a) *Krox20* is expressed in control embryos, but is missing in (b) hindbrains treated with the FGF receptor inhibitor SU5402 (from Weisinger et al. [142]). **C** Pax6 is a negative modulator of *Krox20* that restricts its expression to r3/r5 in the chick hindbrain. (a) Hindbrains electroporated with control plasmid show normal *Krox20* expression. (b) Overexpression (OE) of Pax6 leads to a reduction in *Krox20* expression (arrows). (c) Expression of a dominant-negative (DN) Pax6 results in the expansion of *Krox20* (arrows) to additional domains (from Kayam et al. [124])

factors co-localize with *krox20*, but repress its transcription, restricting its expression to the odd-numbered rhombomeres. Krox20 itself is involved in mediating its negative transcriptional regulation by acting as a SUMO ligase that simulates Nab protein activity [123]. Moreover, Hoxb1 was found to upregulate *nlz* and thus indirectly repress *krox20* expression [115]. The paired-rule gene *pax6* is also co-expressed with *krox20* in r3/r5 but negatively regulates it, leading to the stabilization of *krox20* expression borders in r3/r5 [124]. Pax6 protein negatively modulates *krox20* gene expression via its ability to induce *nab1* gene expression; nab1 protein subsequently binds to *krox20* regulatory elements to repress its expression (Fig. 2).

Mafb/Kreisler/Valentino proteins are expressed in r5/r6, and are obligatory for their specification. In *mafb* mutants, r5/6 identity is lost and r4 is expanded [125, 126]. Mafb and Vhnf1 mutually regulate gene expression in a positive feedback loop. Mafb protein regulates *ephrinB2a* expression, which represses r4, and further promotes r5/6 identities [108, 127]. Vhnf1 together with Mafb and Krox20 also activates *hoxa3* and *hoxb3* expression, which is crucial to specify r5/6 identity [108, 128, 129]. In zebrafish embryos, *krox20* transcriptional activation also requires the Vhnf1 protein, which synergizes with FGF activity to induce *mafb/kreisler/valentino* and *krox20* gene expression. Vhnf1 protein in turn represses *hoxb1* gene expression, limiting its expression to r4, thus enabling formation of more posterior rhombomeric fates [107, 108]. Subsequently, Vhnf1 together with Mafb and Krox20 activate *hoxa3* and *hoxb3* expression in r5/6 to specify r5/6 identity [108, 128] (Fig. 2).

Signaling pathways induce the hindbrain

FGF signaling

FGF signaling plays a key role in hindbrain induction and patterning. Pioneer studies in the chick embryo revealed that caudal epiblast cells fated to give rise to hindbrain character are located adjacent and lateral to the anterior primitive streak of stage 4 embryos [130, 131]. As cells in and around the streak express different FGFs [132, 133], a possibility was raised that FGFs participate in the induction of the hindbrain. Exposure of neural plate explants from different stages and axial levels to FGFs confirmed that FGF signaling is required, although not sufficient, to induce cells of hindbrain character [134].

In zebrafish embryos, the *FGF3* and *FGF8* genes are expressed in the presumptive hindbrain primordia at 80–90% epiboly, before the onset of rhombomere segmentation [135, 136]. At segmentation initiation, *FGF3/8* transcripts are expressed in the central r4 region, with r3/r5 overlap. When morphological segments have formed, *FGF3* continues to be expressed in r4, but *FGF8* expression is extinguished, with newly shifted expression to the more anterior isthmus region. FGF3/8 activities appear to be functionally redundant since a strong hindbrain phenotype is only seen in zebrafish embryos co-injected with morpholino oligonucleotides (MO) to both genes. FGF3/8 knockdown severely inhibited formation of all rhombomeres, except r4. In FGF3/8 morphant embryos, initial *hoxB1* expression in r4 was normal but declined with time, suggesting that neighboring rhombomeres are necessary to maintain its expression. However, in *FGF3/8* morphants, neurons derived for r1–3 and r5–7 are disrupted, but r4-derived neurons formed fairly typically. The possibility that FGFs are acting redundantly in the hindbrain is also reinforced in mice, where otic-placode induction is severely perturbed in FGF3 null embryos, but hindbrain segmentation remains normal [137].

The expression of *FGF3* in hindbrain primordia is conserved in vertebrates [133, 135, 136, 138–141]. Yet, in contrast to the r4-restricted expression in *Xenopus* and zebrafish, in chick and mice *FGF3* is also evident in r2 and r6 [105, 120, 140–142]. It was suggested that early FGF3/8 signaling in r4 forms a primary hindbrain-inducing center since transplanted r4 cells or ectopic expression of FGF3/8 induces expression of r5/6-specific markers in chick and zebrafish embryos [135, 143]. In *Xenopus*, *FGF8* is also involved in hindbrain induction; one of its splice forms, *FGF8a,* mediates hindbrain specification since its knockdown severely perturbs formation of hindbrain and other posterior neural fates [25]. Noticeably, studies in chick and mice demonstrate that much after rhombomere specification takes place, *FGF3* is downregulated from r2/r4/r6 but maintained in all rhombomere boundaries [120, 144]. Rhombomere boundaries display unique cellular and molecular properties that are different from rhombomere bodies [145–149]. In chick, the expression of different boundary markers requires FGF3 [144]. Recent findings suggested that rhombomere boundaries serve as pools of neural-stem-like cells that express Sox2 and nestin and contribute neurons to the hindbrain at stages when rhombomere cells are actively differentiating [150]. Whether FGF signaling is also required for the development and/or maintenance of these neural stem cells in the hindbrain awaits further studies.

A tight cross-talk exists between different transcription factors and FGF signaling in the hindbrain. These interactions play critical early roles in hindbrain induction and segmental patterning. For instance, the activity of Meis proteins is required to induce *FGF3* expression as Meis3 knockdown in *Xenopus* resulted in elimination of early *FGF3* expression in r4 [57]. As a consequence, the entire hindbrain failed to form due to the loss in FGF signaling [42, 151], and Meis3 protein cannot induce hindbrain marker expression in the absence of FGF signaling [24, 151]. Moreover, in an attempt to screen for Hoxb1 target genes in zebrafish, a novel gene,

ppp1r14al, was identified that is induced by Hoxb1 in r4. Ppp1r14al in turn regulates *FGF3* expression in r4, indicating that it is also essential for the establishment of the earliest hindbrain signaling center in r4 [46]. The PG1–3 Hox groups, Vhnf1, Krox20 and Kreisler/MafB/Valentino proteins all act downstream of FGF signaling to induce hindbrain segments. For instance, FGF3 signal indirectly activates *hoxa2*, *hoxb2* and *hoxb3* expression via induction of *krox20* gene expression [128, 129, 132, 143, 152]. FGF signaling also up-regulates *vhnf1* gene expression, which in zebrafish controls caudal hindbrain specification by synergizing with FGF activity to induce *mafb*/*kreisler* and *krox20* expression. Upon FGF signaling activation, Mafb and Vhnf1 proteins are upregulated and bind to specific enhancer elements in the *krox20* promoter. This transcriptional regulation is required for initial *krox20* expression in r3/r5, but not for its later maintenance [107, 113]. In addition, Vhnf1 protein also appears to activate *fgf3* expression and to repress *hoxB1* gene expression, presumably to exclusively limit its expression to r4. Thus, in the hindbrain, the FGF-inducing center acts at a pivotal position, being downstream to Meis/Pbx/Vhnf1/Hox PG1 proteins, but upstream to PG2–3 Hox and Vhnf1/Krox*20*/Kreisler gene activity.

Studies in the chick hindbrain demonstrated that FGF signaling activates the MAP kinase signal transduction protein ERK1/2 that in turn induces the expression of the Ets-family transcription factor *pea3*. This signaling cascade is required for *krox20* induction in the early hindbrain [142, 153, 154]. FGF3 was also found to induce the expression of *pax6* in r3/r5, that in turn negatively regulates *krox20* expression, via the induction of *nab1* [124]. Thus, by regulating both *krox20* and *pax6,* which mutually repress each other, FGF3 acts as a guardian to sharply define rhombomere borders. Moreover, Sprouty4 protein, which is also induced throughout the hindbrain by FGF, acts in a negative-feedback loop to define sharp rhombomeric domains. Enhanced FGF signaling by *sprouty4* knockdown triggers premature and ectopic *krox20* gene expression fusing r3 with r5 that eliminate r4 cell fates [115]. Thus, a multitude of molecular interactions are modulated via the FGF pathway to provide an accurate positional identity of hindbrain rhombomeres.

Wnt signaling

Many Wnt ligands, such as Wnt-1, -3, -3a, -4, -8, -8b and -10, are expressed in the developing CNS [33, 155–161]. Their role in posterior neural development was first shown in *Wnt1* and *Wnt3a* knockout mouse embryos, in which midbrain, hindbrain and spinal cord structures were poorly developed [162–164]. *Wnt3* null mouse embryos had posterior truncations, with expanded expression of the *otx2* forebrain marker, and a concomitant loss of expression of the hindbrain *hoxb1* marker [165]. Similar results were seen in chick embryos implanted with beads soaked with a soluble form of the Frz receptor (mFrz8-CRD) that antagonizes endogenous Wnt ligand signaling. These embryos had an expansion of forebrain markers (*otx2 and pax6*) and a down-regulation of hindbrain (*gbx2*) markers [166]. In cultured chick neural explants, mFrz8-CRD also eliminated expression of the *gbx2*, *krox20* and *hoxb4* hindbrain markers [166, 167]. Similar results were also confirmed in *Xenopus* embryos, where expression of dominant-negative Wnt proteins that inhibit canonical Wnt activity anteriorized embryos [34]. Like *Wnt3*$^{-/-}$ mouse embryos, these embryos also exhibited neural tube closure defects and expression of the *xanf1* and *otx2* forebrain markers was posteriorly expanded, while expression of the hindbrain *krox20* marker was reduced [34]. Specific *Wnt3a*-MO targeting suggested a role in hindbrain development in both zebrafish and *Xenopus* embryos [44, 168–170]. Targeted mesodermal *Wnt3a* knockdown in *Xenopus* ablated hindbrain formation [44]. *Wnt3a* morphant embryos exhibited neural convergent and extension defects, having the typical caudal expansion of forebrain markers, with depletion of hindbrain markers. Moreover, during late gastrula stages, expression of homeoproteins that regulate early hindbrain induction such as *meis3*, *hoxd1* and *gbx2* was reduced [44]. *Wnt3a* MO pheno-copied the neural phenotypes of the general canonical Wnt inhibitor Dkk1 [44], further supporting a role for Wnt3a as the primary posterior neural inducer in vertebrates. In contrast, zebrafish mutated for both *Wnt1*/*Wnt10b* did not have abnormal hindbrain morphology [171, 172], while knockdown of *Wnt3a* or *Wnt8b* did affect hindbrain patterning in zebrafish [172], indicating a potential redundancy in the function of different Wnt ligands for normal hindbrain patterning.

Complementing these loss-of-function studies, Wnt gain-of-function activity induces hindbrain neural cell fates. The *Xenopus* animal cap (AC) explant system has provided a great tool for examining the role of Wnt signaling in neural patterning. BMP4 antagonism in AC explants induces neural tissue. Such neuralized explants mimic the initial state of the newly induced embryonic neural plate. AC explants express pan-neural and anterior neural markers, and will develop as anterior forebrain/cement gland in the absence of additional caudalizing signals [10]. Neuralized AC explants overexpressing different Wnt ligands or downstream effectors, such as β-catenin or inducible constitutively active Tcf, robustly induced expression of hindbrain markers, while strongly repressing anterior neural marker expression [33, 35, 44, 173–175]. In addition, expression of the earliest hindbrain specifying homeoproteins, *meis3*, *hoxd1*, *hoxa2* and *gbx2*, along with caudalizing *FGF3* and *FGF8* genes, were also induced in gastrula-stage-neuralized AC explants overexpressing either Wnt3a or β-catenin [44, 176]. Chick forebrain explants were also caudally transformed by Wnt3a

added to the culture medium (supplemented with FGF), as evident by the induced expression of *gbx2* and *krox20*, instead of *otx2* [166]. Canonical Wnt induction of posterior neural cell fates was shown in both frogs and zebrafish to occur specifically during mid-late gastrula stages [35, 44, 169, 170].

Zebrafish embryos overexpressing a heat-shock protein (Hsp)–*Wnt8* driver-plasmid induced at gastrula stages had an anterior shift in hindbrain markers, with forebrain markers pushed to the anterior extremity [169]. Zebrafish *headless* mutants lacked midbrain, eye and forebrain tissues and weakly expressed *anf1*, *six3* and *rx3* forebrain markers. Concomitantly, *krox20* expression was expanded anteriorly [177]. *Headless* was identified as a point mutation in the Wnt downstream negative-effector *Tcf3* gene, and mutant TCF3 protein was unable to translocate to the nucleus or to bind DNA, thus causing a loss-of-function phenotype [177]. In cells where the canonical Wnt pathway is not activated, Tcf3 represses expression of Wnt-pathway target genes. The loss of Tcf3-mediated repression in the *headless* mutants reflects an overactive canonical Wnt pathway in the embryos [177]. *Xenopus* embryos overexpressing an inducible β-catenin protein activated at gastrula stages, or a CMV-promoter driving zygotic *Wnt3a* expression both induced caudalized embryos with ectopic expansion of hindbrain markers anteriorly, and down-regulation of anterior markers [35, 44]. This anterior transformation in morphology and gene expression pattern to more caudal fates was also evident in *Dkk1* null mouse embryos [178] and chick embryos implanted with Wnt3a-soaked beads [166].

The Wnt ligands that induce hindbrain from the paraxial mesoderm may vary between species. In *Xenopus*, this ligand is Wnt3a, since its specific knockdown inhibits hindbrain formation despite relatively normal *wnt8* mesodermal expression levels [44]. In zebrafish, paraxial mesoderm expressed Wnt8a protein is required for hindbrain formation [169]. In mice and chick, Wnt3 and Wnt8 ligands are also expressed during early development. Whether their hindbrain inducing effects are mediated via the mesoderm or directly in the neural plate still needs to be determined.

RA signaling

RA signaling is also seminal for hindbrain patterning. Yet, unlike FGF and Wnt, its availability is largely dependent on diet, as it is produced from vitamin A (reviewed in [179, 180]). The sensitivity of the hindbrain to small perturbations in RA levels has made it an excellent model to study how morphogen gradients govern pattern formation. Studies in frog [32, 74, 181], chick [104, 182], quail [183, 184], mouse [79, 185, 186], rat [187, 188] and zebrafish [189–192] have generated a vast amount of knowledge on the manner by which RA controls hindbrain regional identity. RA signaling is unique among morphogens as it relies not only on its source of production, but on its site of degradation, creating a posterior^{high}–anterior^{low} activity gradient.

Initially, the existence of a RA gradient was debatable since depletion of endogenous RA was rescued by applying uniform and high doses of RA throughout the embryo, which resulted in fairly normal hindbrain [180, 193–195]. These findings suggest that a RA gradient is generated not only through diffusion from its posterior source but also from its anterior inactivation. Indeed, there are two main groups of metabolic enzymes that coordinate RA levels. RALDHs convert retinaldehyde into RA, whereas CYP26s inactivate RA via oxidation [196–199]. RA is initially synthesized in the presomitic mesoderm by RALDH, and it diffuses into the adjacent hindbrain in a posterior^{high}–anterior^{low} gradient. CYP26 is expressed in the anterior hindbrain, where it leads to RA degradation, further reinforcing the decrease in RA signaling in the rostral embryo [193–195, 200–205]. RA transduces its effects by binding to RA receptors (RARs) and retinoid X receptor (RXRs) [206–210]. Unlike FGF and Wnt, RA uses a nuclear, rather than a membranal, receptor. Upon binding to RA, the receptors act as transcriptional regulators by directly binding specific RA-responsive elements (RAREs) that are positioned at the 3′ or 5′ ends of different hindbrain patterning genes, such as *hoxa1, hoxb1, hoxb4 and vhnf1* [79, 104, 108, 211–219]. For instance, in r6/r7, the initial expression of *hoxb4* and *hoxd4* is activated through RAREs [103–105]. Interestingly, the early transcription of the *hoxb1* gene is initiated by a conserved 3′ RARE, but later, when *hoxb1* expression is restricted to r4, its repression in r3 and r5 is mediated by a 5′ RARE [220, 221]. Furthermore, the *rarβ* gene itself contains a HoxB4/HoxD4-responsive element [105], providing a positive feed-forward loop to maintain RA levels and PG4 *Hox* expression in r6/7.

In addition to controlling the expression of hindbrain segmentation genes in a graded manner, RA regulates its own metabolism in the same manner. RA downregulates the expression of *Raldh* genes in a negative feedback loop, while upregulating the expression of *Cyp26* in a positive feedback loop [191, 194, 195, 202, 222–225]. Yet, RA also induces its own synthesis via a feed-forward mechanism. Experiments in mice and *Xenopus* embryos revealed that Hoxa1–Pbx1/2–Meis2 directly binds a specific regulatory element that is required for maintaining Raldh2 expression levels. As RA induces the expression of Hoxa1, this study revealed an indirect autoregulation of RA synthesis via Hoxa1 [226]. Moreover, RA positively regulates the expression of the intracellular RA-binding proteins, Crabps, which mediate RA transfer to Cyp26 to trigger its degradation [194]. Intriguingly, Crabps were also found to promote the delivery of RA to its receptors, thus eliciting RA signaling [191, 227, 228]. The duality of RA regulation on its

own metabolism requires future research to reveal how its autoregulation is controlled in the hindbrain.

Interestingly, while expression of the RA-degrading enzyme *Cyp26a1* gene is tightly regulated by the levels of RA [194], two other members of the cyp26 family, cyp26b1 and cyp26c1, are not directly regulated by RA signaling and display an unexpected segmental expression with lower levels in r3/r5 than in r2/r4/r6 [193, 195, 229]. A recent study in zebrafish found that this segmental expression plays a key role in sharpening r3–r5 segmental gene expression [229]. During hindbrain segmentation, some r3/r5 (Krox20^{+}) cells are initially found in r4, but later switch into r4 (Hoxb1^{+}) identity. This study found that the *krox20*-intermingled cells are exposed to lower RA levels in r4 due to the elevated activity of cyp26b1 and cyp26c1, which results in downregulation of *krox20* and upregulation of *hoxb1*. The coupling between segmental gene expression and dynamic levels of RA provides the first evidence describing how a signal like RA that is thought to act mainly in a graded caudal–rostral axis orchestrates boundary sharpening via regulating levels of gene expression in alternating rhombomeres [230].

Visualization of the actual RA gradient in live embryos was needed to fully confirm its existence [231]. Initially, this challenge was tackled by generating transgenic mice or zebrafish embryos where GFP/LacZ expression was driven under the control of RAREs through in vivo injection of RA–GFP fused constructs [193, 210, 232, 233]. These strategies supported the shape of a RA gradient, which fitted well with the expression domains of *RALDH* and *CYP26* genes. However, the non-peptidic structure of RA, as well as the failure to observe the GFP signals at early stages, prevented full in vivo validation [194, 210, 234–236]. More recently, imaging tools were developed for this purpose. In one approach, genetically encoded probes for RA (GEPRs) were fused with different GFP variants and introduced into zebrafish. Each GERP displayed a different affinity to RA. Binding of RA to different GERPs led to conformational change that was converted into changes in fluorescence resonance energy transfer (FRET) [237]. This strategy confirmed the concentration gradient of RA in live embryos, where the local source and sink jointly establish the highest RA concentration in the mid-trunk and the lowest in the tail and head. A more recent technology utilized fluorescence lifetime imaging microscopy and phasor analysis to calculate the relative abundances of RA along the hindbrain [238]. This strategy, which is based on the endogenous fluorescence of RA, demonstrated that intracellular free RA forms an anteriorly declining gradient similar to that previously reported with FRET or RARE-GFP/LacZ [193, 237]. This sensitive technology also enabled visualization of random fluctuations in RA levels that can vary rapidly within one hindbrain position. This study suggested that individual cells can actively control the magnitude of random fluctuations of RA levels to preserve the required concentrations for hindbrain segmentation [231].

In general, limiting RA activity results in shortening or loss of the posterior hindbrain and caudal expansion of its anterior part. Yet, the severity of these phenotypes depends on the level in which RA signaling was modified. Complete vitamin A deficiency (VAD) was initially studied in quail embryos, causing a complete loss of r4–r8, and a posterior expansion of r3 [183]. This morphological distortion was combined with loss of posteriorly expressed genes, such as *hoxb1*, *FGF3*, and *mafB*, together with caudal expansion of the r3 stripe of *krox20*. In rats, VAD was accomplished by a gradual, rather than total reduction of vitamin A [187, 188]. These embryos displayed a correlation between titrated reduced doses of RA and a progressive expansion of anterior rhombomeres at the expense of posterior ones. Reduction of RA-signaling levels was also achieved by modulation of its metabolism. In *Xenopus*, ectopic Cyp26 protein anteriorized the posterior hindbrain in a dose-dependent manner [239]. Yet, only a partial duplication of anterior rhombomeres was observed in the posterior hindbrain. Similar intermediate effects were found in two zebrafish lines, *neckless* and *no-fin*, where the *raldh2* gene is mutated [200, 240]. Attenuation of RARs was another means to inhibit RA. This was performed by the generation of *RAR* null mice or zebrafish, or by expressing dominant negative *RARs* in mice, chick and frog [32, 74, 181, 183, 203, 241–243]. In most cases, expression of posterior genes was delayed and formation of the posterior rhombomeres was disrupted but not lost. Notably, stronger defects were observed upon combination of different *RAR* mutations or inhibitors [185–203]. Finally, the less severe hindbrain phenotypes were achieved by manipulating RA-binding sites on target genes. For example, mutations of RAREs in the 3′ ends of *hoxa1* and *hoxb1* delayed their initial rhombomeric expression, but this was fully restored later in development [78, 79, 213]. These studies demonstrate the complex level of regulation of RA signaling in the hindbrain, which combines different sources for RA synthesis and degradation, the activity of several RA receptor subtypes and the binding to RAREs on different hindbrain genes to deliver the correct patterning outcome.

Cross-talk between hindbrain signaling pathways: cooperative or antagonistic activities?

As mentioned, the role of RA, FGF and Wnt in regulating the expression of key hindbrain genes is well documented. Yet, there are marked differences between these morphogens in terms of their expression/activity patterns. Whereas RA signal spans the entire hindbrain in a gradient manner [180], expression of FGFs and Wnts is more limited:

FGF3/*FGF8* is expressed in r4 (zebrafish, frog) or r2/r4/r6 (chick and mice) [133, 135, 136, 139, 141]. *Wnt8a* and *Wnt8b* are expressed in r4 and r3/r5, respectively [244, 245]. The neural patterning of Wnt3a and Wnt8a ligands, as well as FGFs, is also expressed in the dorsal–lateral mesodermal regions similar to RA, where they are secreted to induce posterior cell fates in adjacent neural tissue (Fig. 3) [44]. How the localized FGF and Wnt signals and the graded RA pathway are integrated to govern the positional identity of the hindbrain is not well understood.

Signaling cooperativity

Interactions mediating the expression of all these signals have been demonstrated.

For example, in VAD-quail embryos or in mice carrying a null mutation for the *raldh2* gene, *FGF3* expression is lost [183, 246]. This observation indicated that the induction and/or maintenance of FGFs in hindbrain segments involve RA signaling. Moreover, *FGF3*-null mice have reduced *wnt8a* expression in r4, whereas excess FGF8 in hindbrain explants induces ectopic *wnt8a* expression. These

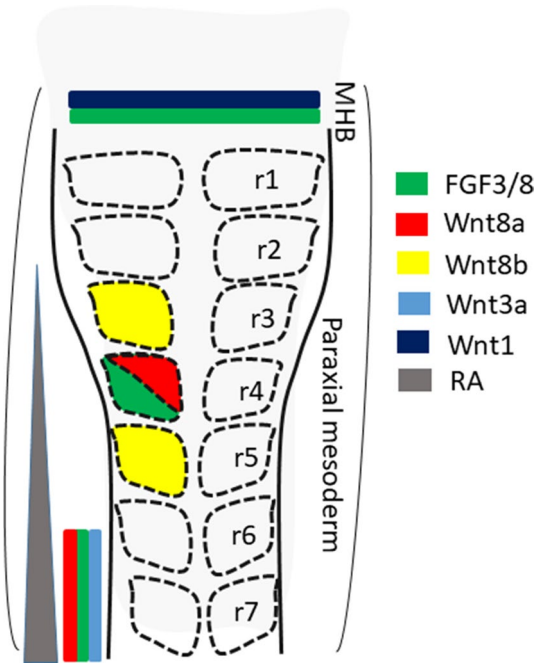

Fig. 3 A summary of the expression domains of different signaling factors in the hindbrain. The spatial organization of the multiple signaling factors triggering hindbrain induction and patterning without temporal separation is shown. Before rhombomere specification, FGF3/8 and canonical Wnt 3a/8a are expressed in the paraxial mesoderm adjacent to the caudal hindbrain. RA is also synthesized in the same domain and acts in a caudal–rostral gradient along the hindbrain. Slightly later, FGF3/8 and Wnt8a are expressed in r4, whereas Wnt8b is expressed in r3/r5. FGF3/8 and Wnt1/3a are also secreted from the mid–hindbrain boundary

results suggest that the *wnt8a* expression in the hindbrain is mediated by FGF signaling [247]. Further support for the Wnt–FGF cross-talk was shown by studying *sprouty1/2* genes. Sprouty proteins are negative regulators of the FGF signaling pathway and restrict *Wnt8a* to r4. Mice mutated for *Spry1/2* showed expansion of *Wnt8a* into additional rhombomeres, probably via releasing the negative regulation of FGF signaling [248]. However, no gross hindbrain malformations appeared in the *Spry1/2* mutants, thus the interactive roles of FGF and Wnt in regulating hindbrain induction and patterning are still unclear.

Additional evidence suggests that these morphogens cooperate to induce the hindbrain. In *Xenopus* embryos and explants, caudalizing canonical Wnt activity regulated early neural *FGF3/8* gene expression [35, 57]. Wnt3a protein activates expression of the hindbrain-specific *meis3* gene [44]. Meis3 protein then directly activates *FGF3/8* gene expression [57, 151]. In *Meis3* knockdown embryos, *FGF3* expression in r4 is eliminated and hindbrain cell fates are lost [57]. Ectopic FGF ligand expression can partially rescue Meis3 morphant phenotypes in *Xenopus* embryos [57]. Yet, neither canonical Wnt nor Meis3 protein activities efficiently caudalize the CNS, when downstream FGF signaling is compromised [24, 35, 151]. Moreover, Wnt activation of Meis3 protein modulates expression of RA direct target genes, such as *hoxd1* [30, 44, 62]. *Hoxd1* is a direct target of RA, Wnt and the Meis3 protein [30, 44, 62, 249, 250]. Meis3/RA act synergistically to optimize *hoxd1* gene expression to promote correct hindbrain formation [62].

Combined FGF and RA signaling activity was also found to induce hindbrain cell fate in the chick; exposure of caudal epiblast cells to FGFs led to the induction of *krox20* only when caudal paraxial mesoderm was also present [134]. This study suggested that a combination of FGF and paraxial mesoderm-caudalizing activity acts directly on epiblast cells to induce hindbrain character. This activity was later identified as RA [251]. The combined activity of RA and FGFs is also demonstrated in setting up the hindbrain–spinal cord border, as marked by *cdx1/4* expression. In zebrafish, loss of *cdx1/4* resulted in caudal expansion of hindbrain genes. This phenotype could only be rescued when both FGF and RA were inhibited, suggesting that both signals act together to coordinate the formation of the border between the hindbrain and spinal cord [252]. These observations show that CDX proteins modify the cell competence to respond to both FGFs and RA in the posterior neural tissues, including the hindbrain. Hence, both signals are required to define the precise hindbrain–spinal cord boundary.

At slightly later stages, rhombomere-derived FGFs are required for the specification of r3 and r5, as inhibition of FGF downregulates *krox20* expression in both segments [108, 135, 136, 142, 143]. Notably, a recent study in zebrafish elucidated a novel mechanism through which the

restriction of *krox20* expression to r3/5 is also mediated by different levels of RA in odd versus even segments. This is mediated by the higher expression and activity of Cyp26b in r4, so that Krox20 expressing cells that cross into the r4 territory are exposed to higher levels of RA than in r3/r5, which results in the downregulation of *krox20*, and the upregulation of *hoxb1* gene expression [229]. Yet, although defined RA levels are also necessary for *krox20* expression, RA activity is more limited than FGF3; attenuation of RA signaling results in loss of the more posterior r5 stripe of *krox20*, whereas it remains intact in r3 or even expands posteriorly, depending on the degree of RA modulation [108, 183, 188]. Nevertheless, synergism between RA and FGF is required to induce *krox20* through the induction of downstream mediators such as Vhnf1 [108].

From gastrulation stages and onwards, FGF and Wnt are also expressed in the posterior mesoderm, together with RA. All these signals were found to induce posterior and suppress anterior expression of genes involved in rhombomere specification, as detailed in the previous sections. The mesodermal FGFs and Wnts prevent the upregulation of *cyp26* in the posterior hindbrain [23, 194] and also maintain *raldh2* expression in this domain. RA, on the other hand, was found to induce the expression of *wnt1/3A* and the downstream FGF target gene, *pea3* [253]. In this way, posterior FGF and Wnt are involved in maintaining the hindbrain RA gradient, whereas RA positively regulates the expression/activity of these morphogens in more posterior domains. The tight connection of FGF and Wnt signaling with RA maintains stable and adaptable RA concentration levels along the hindbrain AP axis [180, 192].

Opposing signaling activities

One of the initial indications supporting a negative cross-talk between hindbrain signals came again from VAD-quail embryos, where the posterior hindbrain was abolished, but anterior regionalization occurred normally [183, 184]. The morphogen regulating patterning in the anterior hindbrain is the mid–hindbrain boundary (MHB)-derived FGF8 protein [212, 254–256]. Grafting of MHB or adding FGF8 beads into the posterior hindbrain induced ectopic expression of genes that are normally expressed anteriorly, together with downregulation of posterior genes, like *hoxb1*. These results were later confirmed by pharmacologic or genetic disruption of RA in other species, where the posterior hindbrain became anteriorized [179, 242, 257, 258]. Conversely, either inhibition of FGF8 or enhancement of RA activity led to the expansion of anterior *Hox* genes [256, 259]. Knockout of FGF8 also resulted in midbrain *Wnt1* expression expanding posteriorly into the isthmus and cerebellum [260]. These and other studies indicate that posterior RA and anterior FGF8 act in opposing manners to specify Hox-positive/negative domains to define the AP borders of the hindbrain.

Intriguingly, these antagonistic FGF8 and RA activities are similar to those seen in the posterior end of the embryo; FGF3/8 are expressed in neuro-mesodermal progenitor cells at the tail and act in a posterior^{high}–anterior^{low} gradient to inhibit their differentiation into mesoderm or ectoderm lineages. RA, which is produced in the more rostral paraxial mesoderm, acts in an opposite anterior^{high}–posterior^{low} gradient to trigger the differentiation of the progenitors and to induce Hox gene distribution [134, 261–263]. These observations strongly suggest that both signals act in the same direction to pattern the posterior hindbrain, but their interaction is antagonistic in the most anterior or posterior positions along the neural tube. Further support for this conclusion comes from a study where the distinct effects of FGF and RA were examined on different members of the HoxB group [214]. The anterior expression border of *hoxb4,* which normally lies in r6/7, was expanded anteriorly upon exogenous application of RA or by grafting posterior mesoderm adjacent to anterior rhombomeres. However, the expression pattern of the more caudal gene, *hoxb9*, was not modified upon manipulating RA signaling. Conversely, application or inhibition of FGF did not change the expression pattern of *hoxb4* but led to a dramatic expansion or loss of *hoxb9* gene expression, respectively.

The complex cross-talk between RA, FGF and Wnt is also evident in the positioning of the border between the hindbrain and the spinal cord, as marked by the anterior expression of *cdx1/4* at the level of somite 3. In zebrafish, loss of *cdx1/4* causes a caudal shift of the hindbrain–spinal cord border via posterior expansion of hindbrain genes (such as *hoxb4*), and caudalization of spinal cord markers, such as *hoxb8* [252, 264]. Hence, the spatial regulation of *cdx1/4* expression is crucial for positioning the hindbrain–spinal cord border region. Analyzing the regulatory role of RA and FGF revealed that in RA-deficient embryos, *cdx4* expression shifts dorsally while in FGF-deficient embryos *cdx4* expression shifts caudally. Yet, in embryos lacking both RA and FGF signals, the shift in *cdx4* axial expression is rescued and it is aligned at the level of somite 3, indicating that FGF negatively modulated RA activity in regulating *cdx4* expression [252, 265, 266]. Thus, for hindbrain patterning, the FGF and RA pathways have additive functions, but with respect to the axial position of the hindbrain/spinal cord border region, they may be antagonistic. In this way, FGF and RA interactions are different in each process: additive in hindbrain patterning, antagonistic in hindbrain size specification and epistatic in neural-mesodermal tissue alignment.

In Wnt-deficient embryos, *cdx4* expression shifts caudally, but the simultaneous loss of both Wnt and RA activities results in a more severe caudal shift of *cdx4* gene expression, in comparison to solely inhibiting Wnt

signaling, indicating that Wnt and RA act together to regulate *cdx4* axial positioning in the hindbrain–spinal cord border [170, 215, 267, 268]. These studies clearly demonstrate the multifaceted network of morphogen regulation that is differentially activated or inhibited along the hindbrain AP axis. In the future, it will be important to elucidate in greater detail the spatial and temporal dynamics of these regulatory signaling pathways to fully understand how their joint activities orchestrate rhombomere specification.

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