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vhnf1 integrates global RA patterning and local FGF signals to direct posterior hindbrain development in zebrafish

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Summary

The vertebrate hindbrain is transiently divided along the anterior-posterior axis into seven morphologically and molecularly distinct segments, or rhombomeres, that correspond to Hox expression domains. The establishment of a proper '*hox* code' is required for the development of unique rhombomere identities, including specification of neuronal fates. *valentino (val)*, the zebrafish ortholog of *mafB/Kreisler* (*Kr*), encodes a bZip transcription factor that is required cell autonomously for the development of rhombomere (r) 5 and r6 and for activation of Hox group 3 gene expression. Recent work has demonstrated that the expression of *val* itself depends on three factors: retinoic acid (RA) signals from the paraxial mesoderm; fibroblast growth factor (Fgf) signals from r4; and *variant hepatocyte nuclear factor 1* (*vhnf1*, also known as *tcf2*), a homeodomain transcription factor expressed posterior to the r4-5 boundary. We have investigated the interactions between these inputs onto *val* expression in the developing zebrafish hindbrain. We show that RA induces *val* expression via activation of *vhnf1* expression in the hindbrain. Fgf signals from r4, acting through the MapK pathway, then cooperate with Vhnf1 to activate *val* expression and subsequent r5 and r6 development. Additionally, *vhnf1* and *val* function as part of a multistep process required for the repression of r4 identity in the posterior hindbrain. *vhnf1* acts largely independently of *val* to repress the r4 '*hox* code' posterior to the r4-5 boundary and therefore to block acquisition of r4-specific neuronal fates in the posterior hindbrain. However, *vhnf1* is not able to repress all aspects of r4 identity equivalently. *val* is required downstream of *vhnf1* to repress r4-like cell-surface properties, as determined by an '*Eph-ephrin* code', by repressing *ephrin-B2a* expression in r5 and r6. The different requirements for *vhnf1* and *val* to repress *hoxb1a* and *ephrin-B2a*, respectively, demonstrate that not all aspects of an individual rhombomere's identity are regulated coordinately.

Key words: Rhombomere, Hindbrain, Retinoic acid, *vhnf1*, *valentino*, *mafB*, Fibroblast growth factor, *hox*

Introduction

The rhombomeres of the vertebrate hindbrain are a series of seven transient segments first distinguished by expression of molecular markers and later as morphological segments giving rise to distinct neuronal fates (Lumsden and Krumlauf, 1996; Moens and Prince, 2002). The acquisition of proper anteroposterior (AP) identity and subsequent neuronal development in the hindbrain is established by the Hox genes that are expressed in nested, rhombomere-restricted domains and that play a role in the specification of AP axis identities across metazoan phyla (Wilkinson et al., 1989). Although the functions of Hox genes in specifying rhombomere identity are well established, the genetic events leading to the deployment of Hox gene expression remain to be fully elucidated. This work focuses on the genetic hierarchy that establishes appropriate Hox gene expression in the caudal hindbrain, where specification of rhombomere (r) 5 and r6 identity involves the activation of Hox paralog group 3 (*hox3*) expression and the repression of *hox1* gene expression.

Previous work has shown that the transcription factors MafB and Krox20 are direct regulators of Hox gene expression in r5 and r6. *Kreisler* (*Kr*) and *valentino* (*val*), the mouse and zebrafish orthologs of *mafB*, respectively, are required for normal r5 and r6 development (Cordes and Barsh, 1994; McKay et al., 1994; Moens et al., 1996; Moens et al., 1998; Prince et al., 1998) and MafB has been shown to activate *hoxb3* and *hoxa3* expression directly in transgenic mice (Manzanares et al., 1997; Manzanares et al., 1999a; Manzanares et al., 2001; Manzanares et al., 2002). Krox20 is required for the maintenance of r3 and r5 identity (Schneider-Maunoury et al., 1997) and cooperates with Kr to regulate *hoxb3* directly in r5 (Manzanares et al., 2002). Furthermore, *hox3* genes are both necessary and sufficient for the development of somatic motoneurons in the hindbrain (Gaufo et al., 2003; Guidato et al., 2003).

Since *mafB/Kr/val* plays an important and conserved role in the control of r5 and r6 specification, significant effort has been made to understand how its expression is established in the

developing hindbrain. Several inputs onto *mafB/Kr/val* expression have been identified: retinoic acid (RA) signaling is necessary for acquisition of all hindbrain fates posterior to r3, including expression of *mafB/Kr/val*, as determined by pharmacologic and genetic disruption of RA production or activity (Dupe and Lumsden, 2001; Gavalas and Krumlauf, 2000; Linville et al., 2004; Wendling et al., 2001). Transplantation and genetic mosaic analyses have suggested that the relevant source of RA for posterior hindbrain patterning appears to be the trunk paraxial mesoderm (Begemann et al., 2001; Gould et al., 1998). Secondly, *variant hepatocyte nuclear factor1* (*vhnf1*; *tcf2* – Zebrafish Information Network), a homeodomain transcription factor expressed throughout the posterior hindbrain and anterior spinal cord, was identified in a genetic screen in the zebrafish as a positive regulator of *val* (Sun and Hopkins, 2001). Finally, work in zebrafish showed that r4 is a source of Fgf3 and Fgf8, which are required for the patterning of surrounding rhombomeres, including the initiation of *val* expression (Maves et al., 2002; Walshe et al., 2002). Wiellette and Sive (2003) have recently demonstrated that Fgfs and Vhnf1 synergize to drive the expression of both *val* and *krox20* in r5.

Specification of r5 and r6 identities requires not only the activation of r5- and r6-specific gene expression, but also the repression of r4-specific gene expression. Recent work in the zebrafish has shown that Vhnf1 represses *hoxb1a* in a *val*-independent manner (Wiellette and Sive, 2003), while other work in the mouse has shown that *hox3* genes, which are targets of MafB/Kr/Val, are required for *hoxb1* repression (Gaufo et al., 2003). The role of MafB/Kr/Val itself in the repression of *hoxb1* is controversial: while some posterior expansion of *hoxb1* expression in mouse *Kr* mutants and zebrafish *val* mutants has been reported (McKay et al., 1994; Prince et al., 1998), little expansion of a *Hoxb1*-r4 reporter transgene was observed in *Kr* mutants (Manzanares et al., 1999b).

We have investigated how global RA signals, local FGF signals and *vhnf1* expression are integrated to specify r5 and r6 in the developing zebrafish hindbrain. We show that RA signals are essential for the activation of *vhnf1* expression and that Vhnf1 acts downstream of RA signaling to drive *val* expression. Secondly, we show that Vhnf1 strictly requires r4-derived Fgf signals, probably through the Map kinase cascade, to initiate *val* expression. Vhnf1 therefore integrates local r4-Fgf signals with global positional information provided by RA to specify r5 and r6 identities. We have also investigated how Vhnf1 and Val contribute to the repression of r4-specific gene expression in the r5-6 territory. We find that repression of r4 gene expression in the r5-6 territory is initially established in a *vhnf1*-independent manner, but that *vhnf1* is then rapidly required to reinforce this restriction. By contrast, *val* plays a relatively minor and late role in the repression of *hox1*/r4-identity. Thus, specification of r5 and r6 identity is achieved through temporally and genetically distinct steps that establish and then maintain repression of r4 identity, as well as activating r5- and r6-specific gene expression. Furthermore, we find that different aspects of rhombomere identity, specifically those determining neuronal identity and those determining cell-surface character, are regulated independently. As a consequence, cells with very different '*hox* codes' can mix freely in genetic mosaics because they share an '*Eph-ephrin* code.'

Materials and methods

Zebrafish stocks, RNA in-situ hybridization, antibody staining and genetic mosaics

Zebrafish strains and mutant alleles used in this study were *AB for wild-type, *val*^{b337} (Moens et al., 1998), *vhnf1*^{hi2169} (Sun and Hopkins, 2001), *acerebellar* (*ace*) (Reifers et al., 1998) and *neckless* (*nls*) (Begemann et al., 2001).

Two color in RNA in-situ hybridization was performed essentially as described (Prince et al., 1998), except that in some cases BM-Purple (Roche) and Iodo-Nitrotetrazolium Violet (Sigma) were used as alkaline phosphatase substrates. For *cyp26b1* a probe was synthesized by linearizing IMAGE clone 3722563 (Invitrogen) with *Sal*I and transcribing with SP6. Whole-mount immunohistochemistry, using the 3A10 antibody to detect Mauthner cells, was performed as described (Hatta, 1992), except a biotinylated secondary antibody and the ABC kit (Vector Labs) were used prior to detection with FITC-Tyramide (Perkin-Elmer). Genetic mosaic analysis was performed essentially as described (Moens and Fritz, 1999).

Generation of Val-monoclonal antibody, immunoblot analysis and genotyping

A GST fusion protein, where GST was fused to the full open reading frame of Val, was used to generate monoclonal antibodies in the FHCRC Biologics Facility. Immunoblotting was performed with hybridoma supernatant (1:4) essentially as described (Waskiewicz et al., 2001), except that NuPAGE sample buffer and 10% NuPAGE gels (Invitrogen) were used. Three embryo equivalents were loaded per lane.

Genotyping of embryos with respect to *val* was performed as described (Moens et al., 1998). Genotyping for *nls* was performed similarly with the primers 5′-GCTCCCACAGTAAGTTCCTGACC-TA and 5′-GTTGTGGTCAGAATGGACAGACAGA, followed by digestion with excess *Pst*I, which cuts the mutant allele.

Morpholino injections, mRNA overexpression and pharmacological treatments

Embryos lacking both Fgf3 and Fgf8 function were generated as described (Maves et al., 2002). mRNA for injections was generated using the mMessage mMachine kit (Ambion) and the following plasmids linearized with the indicated enzymes and used at the given final concentration: pCS2+*vhnf1* (Sun and Hopkins, 2001), *Not*I, 50 ng/μl; pCS2+*fgf3* (Maves et al., 2002), *Not*I, 25 ng/μl; pSP64-T-caXMek (Umbhauer et al., 1995), *Sal*I, 20 ng/μl; noggin-GFP, *Not*I, 20 ng/μl (D. Kimelman, personal communication). For experiments involving the injection of more than one mRNA, total mRNA injected was normalized with *eGFP* mRNA.

Pharmacological treatments of dechorionated embryos were performed in agarose- (1.2% in embryo medium) coated dishes as follows: AGN193109 (Agarwal et al., 1996) 10 μM in 2% DMSO in embryo medium, all-trans RA (Sigma) at given final μM concentration in 0.1% ethanol in embryo medium.

Results

vhnf1 acts downstream of retinoic acid to drive *val* expression

Both retinoid signaling and the transcription factor Vhnf1 have been shown to be required for *val*/*mafB/Kr* expression in the posterior hindbrain (Gavalas and Krumlauf, 2000; Sun and Hopkins, 2001). Since the pattern and timing of *vhnf1* expression resembles that of other RA-controlled genes in the hindbrain, such as *hoxb1b* (Alexandre et al., 1996), we asked whether the requirement of RA for normal *val/mafB/Kr* expression might be indirectly mediated via the control of *vhnf1* expression by RA.

To determine if RA is sufficient for the induction of *vhnf1* expression, we treated embryos with all-trans RA from 8.2 hours postfertilization (hpf) (70% epiboly) to approximately 10.7 hpf (2-3 somite stage). Embryos treated with all-trans RA exhibited a dose-dependent expansion of *vhnf1* expression along the AP axis compared with solvent-treated controls (Fig. 1A,B and data not shown). To determine if *vhnf1* expression requires RA signaling, zebrafish embryos were treated with the pan-retinoic acid receptor antagonist AGN193109 (Agarwal et al., 1996; Johnson et al., 1995) beginning at 4.5 hpf, prior to the onset of gastrulation, until approximately the 3 somite stage (11 hpf). 10^{-5} M AGN193109, which blocks expression of *val* and other hindbrain markers caudal to r4 (Linville et al., 2004) resulted in a severe reduction or complete loss of neural expression of *vhnf1* in all treated embryos (n=103, Fig. 1D). Furthermore, *neckless (nls)* mutant embryos, which lack Raldh2, the final enzyme in the biosynthesis of all-trans RA (Begemann et al., 2001), exhibited reduced levels of *vhnf1* expression (n=51/51 $nls^{-/-}$ embryos vs. 5/98 wild-type siblings). The effect was most striking at the end of gastrulation, when nls^{-} embryos (Fig. 1F) had virtually no *vhnf1* expression compared with the robust *vhnf1* expression of their wild-type siblings (Fig. 1E). *vhnf1* expression in nls^{-} embryos partially recovered by early somite stages (11 hpf), consistent with previous studies suggesting that RA signaling is attenuated, but not fully blocked, in zebrafish *raldh2* mutants (Grandel et al., 2002).

To determine if *vhnf1* functions downstream of RA to activate *val/mafB/Kr*, we overexpressed *vhnf1* in combination with 10^{-5} M AGN193019 treatment to block RA signaling. Solvent (DMSO)-treated embryos had essentially normal *val* expression at 11.7 hpf (Fig. 1G), whereas only 2% of embryos treated with 10^{-5} M AGN193019 (n=63) had cells with strong *val* expression (Fig. 1H). Overexpression of *vhnf1* restored robust *val* expression in 88% of embryos (n=126, Fig. 1J), demonstrating that *vhnf1* functions downstream of RA in controlling *val* expression. Together, these data demonstrate that RA probably contributes to the specification of r5 and r6 by inducing *vhnf1* expression in the caudal hindbrain and therefore indirectly regulating *val*.

vhnf1 and Fgfs cooperate to drive *val* expression

Other work has shown that *val* expression in r5 and r6 also depends on *fgf3* and *fgf8*, which are strongly expressed in r4 (Maves et al., 2002; Walshe et al., 2002). Expression of *vhnf1* in the hindbrain does not require *fgf3* and *fgf8* expression in r4, and vice versa (data not shown and see below) (Wiellette and Sive, 2003). Therefore, we were interested in how Fgfs and *vhnf1* interact to drive *val* expression.

In order to determine if *vhnf1* requires Fgf signaling for its ability to drive *val*, we overexpressed *vhnf1* in wild-type (wt) embryos and embryos lacking Fgf3 and Fgf8 function. In wt embryos, overexpression of *vhnf1* by mRNA injection expanded the *val* expression domain anteriorly (Fig. 2A,B) to include the r2-r4 territories. By contrast, overexpression of *vhnf1* in embryos lacking Fgf3 and Fgf8 function did not drive *val* expression in either its endogenous domain of r5 and r6 or ectopically in more anterior rhombomeres (Fig. 2C). These data demonstrate that activation of *val* by Vhnf1 strictly depends on Fgf signaling.

We hypothesized that if Fgfs and Vhnf1 synergize to drive *val* expression, then overexpression of *fgf3* and *vhnf1* together would drive broad ectopic expression of *val* in zebrafish embryos. We overexpressed *vhnf1* and *fgf3* either alone or together and assessed *val* expression by both in-situ hybridization and immunoblotting at the mid-gastrula stage (8.25 hpf, 80% epiboly), just prior to the normal onset of *val* expression. Overexpression of *fgf3* alone did not result in ectopic *val* expression (Fig. 2E), nor did it result in a significant anterior expansion of *vhnf1* expression (data not shown);

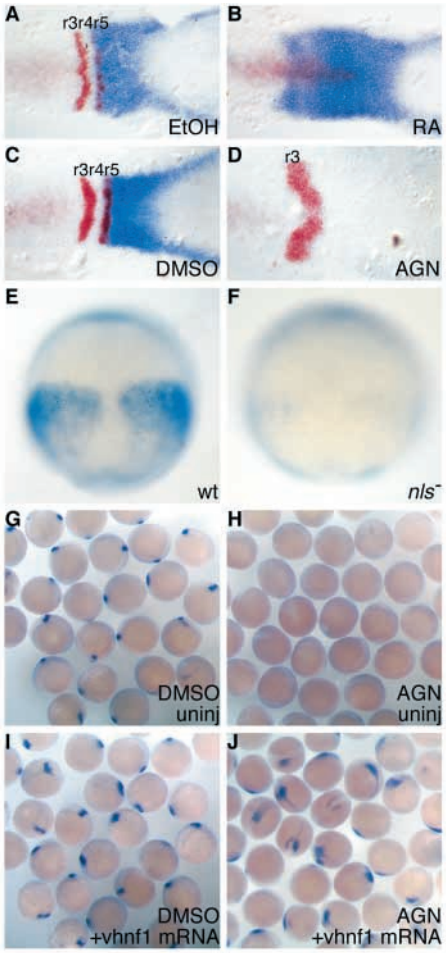

Fig. 1. *vhnf1* acts downstream of RA to activate *val*. (A,B) *vhnf1* (blue) is expressed posterior to the r4/5 boundary at about 10.7 hpf in control EtOH-treated embryos (A, *krox20* expression in r3 and r5 is in red), and is expanded in its AP extent in the hindbrain of embryos treated with 10^{-7} M all-trans RA (B). (C,D) Treating embryos with 10^{-5} M AGN193109, a pan-RAR antagonist, from 4.5-11 hpf blocks *vhnf1* expression and r5 specification (D) compared with DMSO-treated controls (C). (E,F) Wild-type embryos (E) exhibit robust *vhnf1* expression by 9.5-10 hpf compared with their nls^{-} siblings (F). (G-J) Embryos were treated with 2% DMSO or 10^{-5} M AGN193109, either alone or in combination with overexpression of *vhnf1* (35 pg mRNA) as shown. (G) DMSO-treated controls show normal *val* expression (blue) in r5 and r6 at approximately 11.7 hpf and this expression is inhibited by treatment with AGN 193109 (H). Overexpression of *vhnf1* causes a slight expansion of *val* expression in untreated embryos (I) and rescues *val* expression in AGN193109-treated embryos (J). A-F are shown as dorsal views with anterior to the left (A-D) or to the top (E,F).

overexpression of *vhnf1* alone mildly upregulated *val* (Fig. 2F). By contrast, overexpression of *fgf3* and *vhnf1* together caused a dramatic upregulation of *val* expression throughout the embryo (Fig. 2G), correlating with a dramatic increase in Val protein as assessed by immunoblotting (Fig. 2D′-G′). This result is specific and not simply the result of dorsalization by ectopic *fgf3,* since co-injection of *vhnf1* with *noggin*, a dorsalizing BMP antagonist (Furthauer et al., 1999; Zimmerman et al., 1996), did not induce robust *val* expression (Fig. 2H,I,H′,I′). Similarly, *wnt8-orf1* (Lekven et al., 2001), another classic posteriorizing factor, failed to cooperate with *vhnf1* to induce robust *val* expression (data not shown). Wiellette and Sive (Wiellette and Sive, 2003) obtained similar results using a combination of *vhnf1* overexpression and Fgf-bead implantation. Our data demonstrate that *vhnf1* and Fgf signaling are capable of globally activating *val* expression along the entirety of the anterior-posterior axis in both the epiblast and hypoblast.

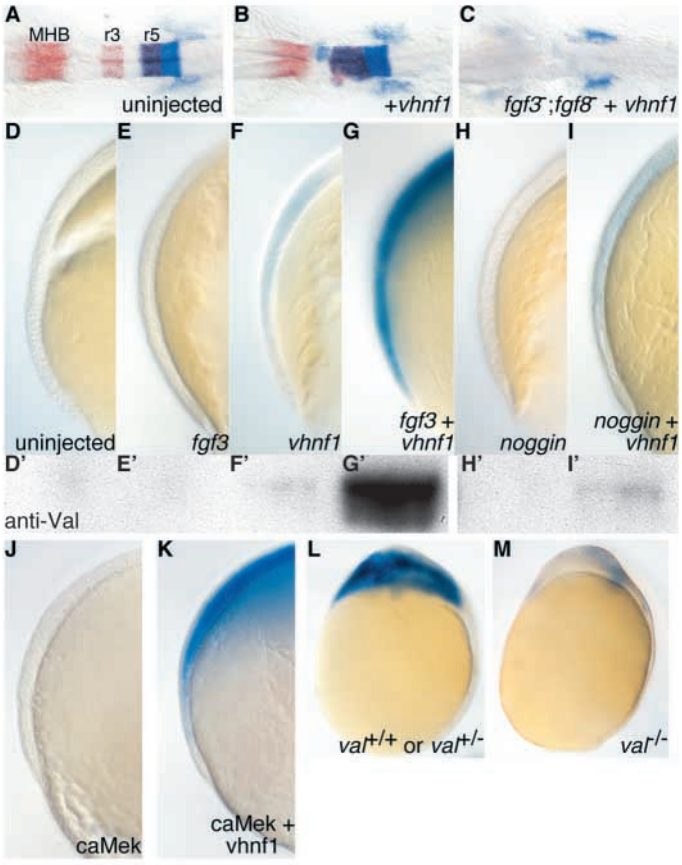

Fig. 2. *vhnf1* and Fgfs cooperate to drive *val* expression. (A-C) *vhnf1* requires Fgfs to drive *val* expression in the hindbrain. At 18 hpf *val* (blue) is normally expressed in r5 and r6 (A); *krox20* expression in r3 and r5 and *eng3* at the mid-hindbrain boundary (MHB) is in red. After injection of *vhnf1* mRNA (50 pg), *val* expression expands anteriorly to approximately the level of r2 (B). By contrast, *vhnf1* overexpression in *fgf3*$^{-}$;*fgf8*$^{-}$ embryos does not drive *val* expression (C). (D-I) *vhnf1* and *fgfs* cooperate to drive *val* expression. *val* (blue) is not normally expressed at 8.25 hpf (D), and injection of *fgf3* mRNA (25 pg) alone is not sufficient to induce *val* (E). *vhnf1* mRNA alone will induce a low level of *val* (F), while *vhnf1* and *fgf3* together induce *val* at a high level (G). Injection of *noggin-gfp* mRNA (20 pg) causes a dorsalization similar to *fgf3* but when injected alone (H) or with *vhnf1* mRNA (I) it does not induce *val*. (D′-I′) Val protein is upregulated similarly to transcript levels at 8.25 hpf following mRNA injection (as in D-I), as detected by anti-Val immunoblot of lysed embryos. (J,K) Overexpression of caMek mRNA (20 pg) alone does not induce *val* expression (J) but like *fgf3* can cooperate with *vhnf1* to do so (K). (L,M) Robust upregulation of *val* downstream of *fgf3* and *vhnf1* requires *val* autoregulation. 12 hpf *val*$^{+/+}$ and *val*$^{+/-}$ embryos expressing *vhnf1* and *fgf3* exhibit robust *val* expression (L), while little or no *val* is detected in *val*$^{-/-}$ embryos (M). A-C, are dorsal views with anterior to the left. D-M are optical cross sections near the dorsal midline.

One of the key downstream effectors of Fgf signaling is the Ras-Map kinase (MapK) signaling cascade (Powers et al., 2000). To determine if Fgf signaling through MapK synergizes with *vhnf1* to drive *val*, we coexpressed *vhnf1* with a constitutively active MapK/ERK kinase (caMek) mRNA (Umbhauer et al., 1995). caMek alone did not drive *val* expression (Fig. 2J) but cooperated with *vhnf1* to drive *val* expression in a manner similar to that of Fgf (Fig. 2K).

The normal expression of *val* in r5 and r6 is dependent on a positive autoregulatory loop (Giudicelli et al., 2003; Moens et al., 1998). In order to determine if the *vhnf1-fgf3* interaction we observed is dependent on this positive autoregulatory loop, we repeated the *vhnf1-fgf3* overexpression experiments in embryos from a *val*$^{+/-}$ intercross. We found that strong expression of *val* at 8.5 or 12 hpf correlated with wt or heterozygous genotype (94%, *n*=98, Fig. 2L), while nearly all embryos with little to no *val* expression were mutant (88%, *n*=51, 2M). These data suggest that *val* autoregulation is required for *vhnf1* and *fgfs* to drive robust *val* expression.

Vhnf1 controls some aspects of rhombomere identity independently of Val

The above data and that of others demonstrate that RA activates *vhnf1*, which in cooperation with Fgfs is both necessary and sufficient to drive expression of *val* in r5 and r6. To determine whether *vhnf1* performs all its functions in hindbrain development through its regulation of *val*, or whether the functions of *val* and *vhnf1* are in part separable, we carefully compared molecular and neuroanatomical markers of rhombomere identity in *val*$^{-}$ and *vhnf1*$^{-}$ embryos. If the principal role of *vhnf1* in specifying hindbrain fates is the initiation of *val* expression, *val*$^{-}$ and *vhnf1*$^{-}$ embryos should exhibit identical phenotypes.

Both *val* and *vhnf1* mutant embryos failed to develop a recognizable r5-r6 territory. This is exemplified by the loss of r5 *krox20* expression (Fig. 3A-C), of *hox3* gene expression and of r5-6 specific abducens motoneurons (data not shown) in both mutants. However, there are subtle differences in the two mutant phenotypes. For example, in *val* mutants the remnant of r5 *krox20* expression is always in the dorsalmost hindbrain (Fig. 3B), while *vhnf1* mutants often have fewer *krox20*-positive cells than do *val* mutants and many of the cells are located ventrally (Fig. 3C). Furthermore, the r5-6 region of *val* mutants is about the length of a single rhombomere (Moens et al., 1996), while that of *vhnf1* mutants is the same as in wild-type embryos (data not shown). This is unexpected if *vhnf1* performs all its functions through *val*.

A stark difference between *vhnf1*$^{-}$ and *val*$^{-}$ embryos became apparent when we examined the expression of r4 markers. The transcription factor *hoxb1a* is initially expressed posterior to the

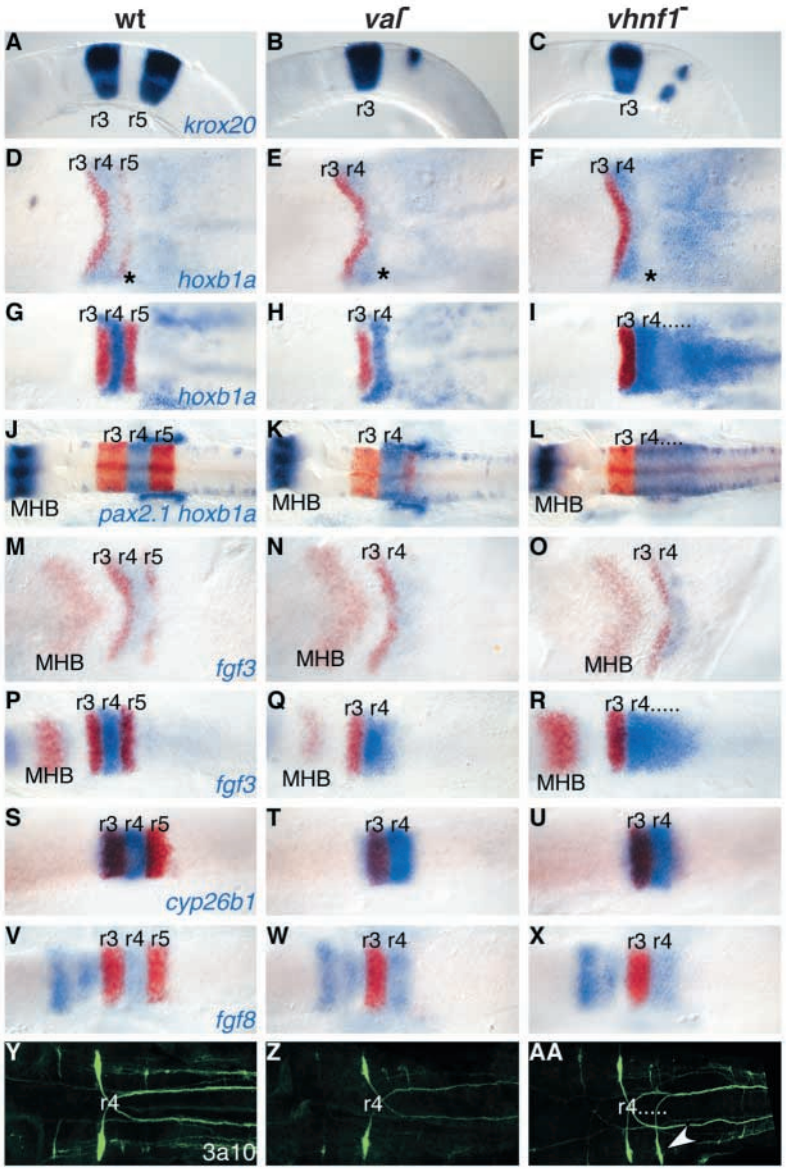

Fig. 3. Vhnf1 participates in a multistep process to repress r4 identity in r5 and r6. Wild-type (wt; left column), *val*– (middle column) and *vhnf1*– (right column). (A-C) *krox20* (blue) in r3 and r5 of wt (A), with a small dorsal stripe in the r5 territory of *val*– embryos (B) compared with the variable dorsal and ventral expression in *vhnf1*– embryos (C). (D-L) Repression of *hoxb1a* (blue) in r5 and r6 requires multiple genes. *hoxb1a* is initially downregulated posterior to r4 (asterisks) at 10.7 hpf in wt (D), *val*– (E) and *vhnf1*– (F) embryos. By 11.7 hpf *hoxb1a* expression is largely restricted to r4 in wt (G) and *val*– (H) embryos but is upregulated posterior to r4 in *vhnf1* mutants (I). At 18-20 hpf *hoxb1a* is clearly expanded in *vhnf1* mutants (L) and mildly expanded in *val* mutants (K) compared with wt (J). (M-R) *fgf3* (blue) expression is similar between wt (M), *val*– (N) and *vhnf1*– (O) embryos at 10.5 hpf. By 11.7 hpf *fgf3* is normally only highly expressed in r4 (P), while in *vhnf1* mutants (R) its expression expands posteriorly. (S-U) Expression of the RA-metabolizing enzyme *cyp26b1* is restricted to r4 and r3 at 12 hpf in wt (S) *val*– (T) and *vhnf1*– (U) embryos. Expression of *fgf8* is also limited to r4 and anterior by 12 hpf in wt (V) *val*– (W) and *vhnf1*– (X) embryos. (Y-AA) A single pair of Mauthner cells is present in r4 of wt (Y) and *val*– (Z) embryos, while supernumerary Mauthner cells are detected in 58% of *vhnf1*– embryos (AA; arrowhead). A-C are lateral views with anterior to the left and dorsal to the top. D-AA are dorsal views with anterior to the left. *krox20* expression in r3 and r5 is in red (D-X). The mid-hindbrain boundary (MHB) is marked by *pax2.1* (blue, J-L; red, M-O) or *en3* (red, P-R).

r3-4 boundary and is subsequently upregulated in r4 and downregulated posteriorly (Prince et al., 1998). Expression of *hoxb1a* at 10.5 hpf was essentially unchanged in *val*– and *vhnf1*– embryos compared with wt (Fig. 3D-F), with all embryos demonstrating an upregulation of *hoxb1a* in r4, and a slightly lower level of expression just posterior to r4. Shortly thereafter (11.7 hpf) *vhnf1*– embryos showed a pronounced posterior expansion and upregulation of *hoxb1a* expression (Fig. 3I) (Sun and Hopkins, 2001), compared with wt and *val*– embryos (Fig. 3G,H). At 18 hpf, expansion of *hoxb1a* expression in *vhnf1* mutants persisted in the domain that would normally adopt r5-6 fates (Fig. 3L). By contrast, the *hoxb1a* expression in *val*– embryos expanded only very weakly posterior to r4 (Prince et al., 1998) and in a more limited domain compared with that of *vhnf1*– embryos (compare Fig. 3K,L). Similarly, *fgf3* expression, which is normally restricted to r4 at the end of gastrulation (Maroon et al., 2002; Maves et al., 2002), was initially normal in *vhnf1*– embryos (Fig. 3M-O). However, *fgf3* expression expanded posteriorly over the subsequent few hours in *vhnf1* mutants compared with wt and *val* mutants (Fig. 3P-R).

While some r4 markers behave differently in *vhnf1* and *val* mutants, other r4 markers are unaffected in either mutant. *cyp26b1*, which encodes a retinoic acid-degrading enzyme, is expressed in r4 beginning at tailbud stage and expands to include r3 by 12 hpf (R.E.H. and C.B.M., unpublished). *fgf8* is expressed in a domain anterior to the r4-5 boundary at 12 hpf (Reifers et al., 1998). Surprisingly, neither marker was expanded posterior to r4 in either *val* or *vhnf1* mutants at a stage when *hoxb1a* was strongly expanded in *vhnf1* mutants (Fig. 3S-X).

Wiellette and Sive (Wiellette and Sive, 2003) reported that the Mauthner cell, a large identified reticulospinal neuron characteristic of r4, is duplicated in more posterior segments in *vhnf1* mutants. Our finding that the transformation to r4 identity in *vhnf1* mutants was incomplete based on marker gene expression led us to reexamine Mauthner cell specification. We found that only 58% of mutants had one or more supernumerary Mauthner cells, consistent with an incomplete transformation (n=19, Fig. 3AA). *val* mutants never had supernumerary Mauthner cells (n=19, Fig. 3Z), consistent with only a very weak anterior transformation.

Taken together, our marker analysis demonstrates that *vhnf1* function in hindbrain patterning is only partially executed through its activation of *val*. Both *vhnf1* and *val* are required for upregulation of r5 and r6 markers, but *vhnf1* functions largely independently of *val* to repress some aspects of r4

identity. However, even in the absence of *vhnf1* function the r5-6 territory appears to be specified properly initially, including transient restriction of r4-specific genes. *vhnf1,* but not *val*, is strongly required to maintain repression of some, but not all, r4 markers, and the partial transformation of the r5-6 region of *vhnf1* mutants to r4 identity correlates with a variable gain of r4-specific neurons.

vhnf1 and *val* mutant cells behave equivalently in genetic mosaic analysis

Individual rhombomeres have specific cellular surface characteristics and the involvement of individual genes in the acquisition of this aspect of rhombomere identity can be determined by genetic mosaic analysis. Previous work has shown that *val* is required cell autonomously for the acquisition of r5 and r6 identity (Moens et al., 1996). Similarly, *vhnf1* mutant cells were excluded from r5 and r6 of wild-type hosts (Fig. 4B, *n*=20, compare with control in Fig. 4A), only occasionally contributing to the r5-6 boundary of the host hindbrain. Conversely, wild-type cells typically formed compact clusters within the presumptive r5-6 of *vhnf1*$^{-}$ hosts (arrowheads in Fig. 4C, *n*=29) and the clusters of cells located in the presumptive r5 expressed *krox20*. These dense clusters were unilateral, consistent with a failure of the transplanted wild-type cells to make a characteristic division that requires single cells to insert themselves across the midline (Geldmacher-Voss et al., 2003; Kimmel et al., 1994). These data demonstrate that *vhnf1* is required cell autonomously for cells to acquire r5 and r6 identities.

Our marker analysis demonstrated that *vhnf1*$^{-}$ and *val*$^{-}$ embryos have unique molecular phenotypes: based on Hox gene expression, the r5-6 region of *vhnf1*$^{-}$ embryos is strongly transformed to r4 identity, while this region of *val* mutants has an indeterminate *hox*-less 'rX' identity. Therefore we anticipated that *vhnf1*$^{-}$ and *val*$^{-}$ cells would behave differently in genetic mosaics. Specifically, we predicted that while neither *val*$^{-}$ nor *vhnf1*$^{-}$ cells can contribute to r5 and r6 of wild-type hosts (as observed above), *val*$^{-}$ cells lying in the expanded r4-like domain of a *vhnf1* mutant host would adopt 'rX' identity and would therefore be unable to contribute. Similarly, we predicted that r4-like *vhnf1*$^{-}$ cells lying in 'rX' of a *val*$^{-}$ host would be excluded from this territory. However, we did not observe this to be the case. *vhnf1*$^{-}$ cells transplanted into a *val*$^{-}$ host embryo (Fig. 4D, *n*=15), and conversely *val*$^{-}$ cells transplanted into a *vhnf1*$^{-}$ host embryo (Fig. 4E, *n*=17), were able to contribute throughout the hindbrain, including the respective mis-specified r5-6 regions (brackets in Fig. 4D,E). These data suggest that, although the presumptive r5-6 regions of *vhnf1*$^{-}$ and *val*$^{-}$ embryos have distinct molecular identities at the level of *hoxb1a* and *fgf3* expression, the cellular surface characteristics of *vhnf1*$^{-}$ and *val*$^{-}$ cells in this region of the hindbrain are identical.

Cell sorting in the hindbrain is probably controlled by repulsive interactions between Ephs and Ephrins (Cooke et al., 2001; Mellitzer et al., 1999; Xu et al., 1999). Therefore, we determined whether the cell-surface properties of *val*$^{-}$ and *vhnf1*$^{-}$ cells observed in our mosaic analysis were reflected in *Eph* and *ephrin* expression patterns. In spite of marked differences in *hoxb1a* and *fgf3* expression in the two mutants, *Eph* and *ephrin* expression were very similar. In both *vhnf1*$^{-}$ and *val*$^{-}$ embryos, expression of *EphB4a* in r5 and r6 and of *EphA4* in r5 was greatly reduced (Fig. 4F-H and data not shown); this reflects changes in *krox20* and *hox3* expression, which were also similar in the two mutants. By contrast, *ephrin-B2a*, which is normally not expressed in r5 or r6, was expanded in both *val*$^{-}$ and *vhnf1*$^{-}$ embryos (Fig. 4I-K) (Cooke et al., 2001). The similar cell-sorting behavior of *vhnf1*$^{-}$ and *val*$^{-}$ cells in genetic mosaics would not have been predicted by the examination of Hox expression alone. These results demonstrate that rhombomere-specific neuronal identity as determined by a '*hox* code' can be unlinked from rhombomere-specific cell-surface properties as determined by an '*Eph-ephrin* code'.

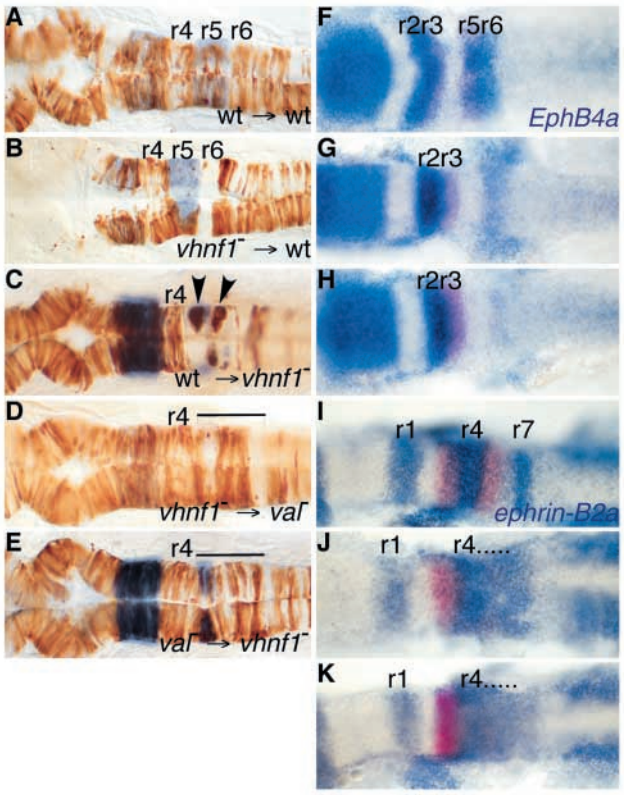

Fig. 4. *vhnf1* and *val* mutant cells behave equivalently in genetic mosaics. (A) Donor-derived cells (brown) from a wild-type embryo contribute throughout the hindbrain of a wild-type host embryo. *Krox20* expression in r3 and r5 is in blue. (B) *vhnf1*$^{-}$ cells are excluded from r5 and r6 of wild-type host embryos. (C) Wild-type cells form tight aggregates (arrowheads) in the r5-6 region of *vhnf1*$^{-}$ host embryos. These wild-type-derived cells express *krox20* when in the r5 region. (D) *vhnf1*$^{-}$ cells were able to contribute throughout the hindbrain of *val*$^{-}$ host embryos, including the presumptive r5-6 region (bracket), and conversely (E) *val*$^{-}$ cells contributed to the entire hindbrain of *vhnf1*$^{-}$ host embryos. Cell behaviors in genetic mosaics are consistent with similar changes in *Eph* and *ephrin* expression in *val* and *vhnf1* mutant embryos. (F,I) Wild-type (wt); (G,J) *val*$^{-}$; (H,K) *vhnf1*$^{-}$. (F-H) At 11.6 hpf, r5 and r6 expression of *EphB4a* (blue) is normally expressed in r2, r3, r5 and r6 of the hindbrain (F). The expression in r5 and r6 is strongly reduced in both *val*$^{-}$ (G) and *vhnf1*$^{-}$ (H) embryos. (I-K) *ephrin-B2a* (blue) is normally expressed in r1, r4 and r7 at 12 hpf (I). Its expression is upregulated posterior to r4 in *val*$^{-}$ (J) and *vhnf1*$^{-}$ (K) embryos. *krox20* expression in r3 and r5 is in purple (F-H) or red (I-K). Embryos are shown in dorsal views with anterior to the left.

Ectopically expressed *vhnf1* represses *hoxb1a* and r4-*ephrin-B2a* in a *val* dependent manner

The expansion of *hoxb1a* and *ephrin-B2a* in *vhnf1* mutants suggests that *vhnf1* normally represses the expression of these two genes. Since *ephrin-B2a* expression is significantly expanded in *val*⁻ embryos while *hoxb1a* is not, we hypothesized that *vhnf1* repression of *ephrin-B2a* requires *val* while *vhnf1* repression of *hoxb1a* does not. In order to test this hypothesis, we overexpressed *vhnf1* in embryos from an intercross of heterozygous *val* fish and then assessed the expression of *hoxb1a* and *ephrin-B2a* by in-situ hybridization.

Consistent with *vhnf1* playing a role in the repression of *hoxb1a* and *ephrin-B2a*, we observed that wild-type embryos injected with *vhnf1* mRNA showed repression of *hoxb1a* (88%, *n*=114) and *ephrin-B2a* (88%, *n*=91) in r4 at about 5-9 somites (11.5-13 hpf). Many of these embryos had nearly a complete loss of the r4 *hoxb1a* or *ephrin-B2a* domain, with only a few *hoxb1a* (Fig. 5B) or *ephrin-B2a* (Fig. 5F) positive cells remaining between two-fused *krox20* stripes. However, essentially no homozygous *val*⁻ embryos showed repression of *hoxb1a* (6%, *n*=51, Fig. 5D) or *ephrin-B2a* (0%, *n*=33, Fig. 5H) in r4 following overexpression of *vhnf1*.

These data demonstrate that, as predicted, *val* is required for the repression of *ephrin-B2a*. Furthermore, although *val* is not required to repress *hoxb1a* in the r5-6 region (Fig. 3H), it is required to repress *hoxb1a* when *vhnf1* is overexpressed in r4. This suggests that in addition to Val cooperating with Vhnf1 to repress *hoxb1a*, another factor, expressed in r5/6 but not in r4, may cooperate with Vhnf1 in the r5/6 region to repress *hoxb1a* in a manner that is partially redundant with *val*.

Discussion

Our data, together with that of others, suggest a model (Fig. 6) in which RA signaling activates *vhnf1* expression posterior to the r4-5 boundary. The expression of Vhnf1 is sufficient to repress the r4 '*hox* code' posterior to r4, including expression of *hoxb1a*, which specifies r4 neurons. Vhnf1 cooperates with Fgf signals from r4 to initiate the expression of *val* in r5 and r6. Subsequently Val activates the expression of *hox3* genes and others to specify r5-6-specific neuronal development. Furthermore, though Val is not strictly required to repress the majority of r4-specific *hox* gene expression in the r5-6 territory, it is required to both repress r4 and upregulate r5-6 cell-surface characteristics via an '*Eph-ephrin* code'. We discuss the data supporting aspects of this model below.

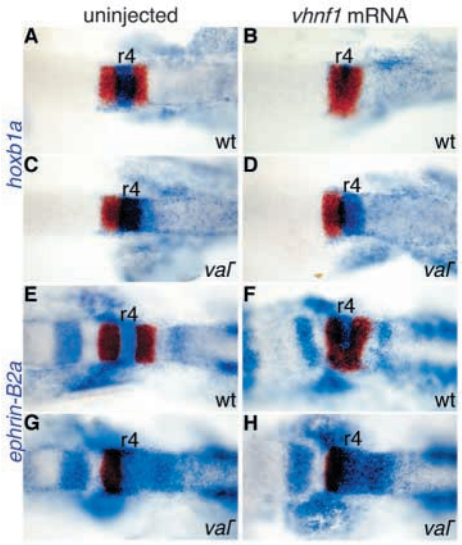

Fig. 5. When overexpressed, *vhnf1* requires *val* for its ability to repress both *hoxb1a* and *ephrin-B2a* in r4. Overexpression of *vhnf1* by mRNA injection (25 pg) represses *hoxb1a* (blue) in r4 of wild-type (B) but not *val* mutant embryos (D). Similarly, injection of *vhnf1* mRNA represses *ephrin-B2a* in r4 of wild-type (F) but not *val* mutant embryos (H). Dorsal views of 11.5-13 hpf embryos, anterior to the left, with *krox20* in red.

vhnf1 integrates global RA patterning with r4-derived Fgf signals

Previous work showed that *val/mafB/Kr* expression is reduced or lost after RAR antagonist treatment or genetic ablation of RA biosynthesis (Gavalas and Krumlauf, 2000; Linville et al., 2004). The data presented in Fig. 1 demonstrates that this is probably due to a primary effect of RA on *vhnf1* expression, since RA is both required and sufficient for *vhnf1* expression, and *vhnf1* can rescue *val/mafB/Kr* expression in embryos in which RA signaling is blocked.

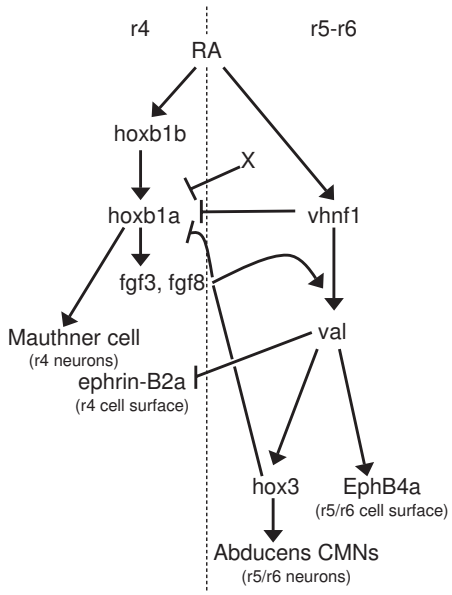

Fig. 6. A model for the functions of *vhnf1* and *val* in directing hindbrain development. RA activates *hoxb1b* and *hoxb1a* expression to initiate an r4 development program, including the specification of Mauthner neurons. An unknown factor, 'X', initially represses *hoxb1a* in r5 and r6, independent of *vhnf1*. RA activates *vhnf1* expression, which reinforces repression of *hoxb1a* expression in r5 and r6, possibly in cooperation with an unknown co-factor, which may or may not be the same 'X' above. Repression of *hoxb1a* by Vhnf1 blocks acquisition of r4 neuronal fates in r5 and r6, but Vhnf1 must act through Val to drive r5-6 neuronal development, including abducens cranial motor neurons (CMNs). Furthermore, Val is required for the acquisition of r5-6 cell-surface characteristics by both activating r5-6 *EphB4a* expression and repressing r4-like *ephrin-B2a* expression in r5 and r6. Finally, Val contributes to the maintenance of *hoxb1a* repression at later stages, possibly through activation of *hox* group 3 genes.

It remains unclear whether the activation of *vhnf1* by RA is direct, as has been determined for some Hox genes (Dupe et al., 1997; Gould et al., 1998; Marshall et al., 1994), or if it acts through a more indirect mechanism. Members of the steroid nuclear receptor superfamily, including RARs, can bind a DR1 motif upstream of murine *Vhnf1*, and the DR1 is required for full reporter activity in cultured cells, but it is unclear if this element mediates retinoic acid responsiveness (Power and Cereghini, 1996). Pbx proteins, which function as DNA-binding partners for Hox proteins, are also required for normal hindbrain expression of *vhnf1* (Waskiewicz et al., 2002). Since Pbx proteins are not known to be required for RA synthesis or to be regulated by RA, their requirement for *vhnf1* expression is likely to be either independent or downstream of RA signaling. A careful analysis of the *vhnf1* promoter will help elucidate which proteins directly regulate *vhnf1* in the hindbrain.

vhnf1 and Fgfs synergize to drive *val* expression

We have shown that *vhnf1* and Fgfs cooperate to drive *val* expression and specify r5 and r6 fates. Using Fgf-coated beads, Wiellette and Sive (Wiellette and Sive, 2003) demonstrated a similar synergy between Fgf and *vhnf1*. Our results support and extend their findings, by showing that unlike RA signals, Fgf signals are strictly required for *vhnf1* to induce *val* expression, since *vhnf1* cannot drive *val* expression in an embryo lacking *fgf3* and *fgf8*. Furthermore, we demonstrate that Fgf signaling through the MapK pathway is sufficient to cooperate with *vhnf1* in inducing *val* expression, and that robust upregulation of *val* by Vhnf1 and Fgfs requires a positive autoregulatory loop that is dependent on Val function.

The control of *val* expression by *vhnf1* is probably conserved across vertebrates. *vhnf1* is expressed in the hindbrain of mouse embryos in a similar domain to that in zebrafish, but its requirement for hindbrain development has not been determined because homozygous mutant mice have defects in visceral endoderm development and die prior to gastrulation (Coffinier et al., 1999). Inactivation of *vhnf1* in the embryonic tissues or CNS alone will be required to test the requirement for *vhnf1* in mammalian hindbrain patterning.

At this point it is unclear whether *vhnf1* acts directly or indirectly to regulate expression of *val*, and little is known about the direct regulation of the *val* locus. The classical *kreisler* mutation, an inversion approximately 30 kb upstream of the transcriptional start of *Kr/MafB*, disrupts a regulatory element that is specifically required for expression in r5 and r6 but not sites outside the hindbrain (Cordes and Barsh, 1994; Eichmann et al., 1997). This and other data (Hamada et al., 2003) suggest that elements controlling the hindbrain expression of *val/mafB/Kr* are rather distant from the gene. We have identified a consensus Hnf1-binding site (CTGTTAACATAACA) within a highly conserved island of homology (85% identity over 500 base pairs) approximately 22.5 kb upstream of the *MafB*/*Kr* gene of humans and mice (data not shown). However, we have as yet been unable to identify a corresponding island in the available genomic sequence from either *fugu* or zebrafish. Further analysis is necessary to determine if this potential Hnf1 binding site has any role in regulating hindbrain expression of *val/mafB/Kr*.

Although our data and that of Wiellette and Sive (Wiellette and Sive, 2003) show that Fgfs synergize with Vhnf1 to drive *val* expression, the mechanism underlying this effect is unclear. Our data suggest that Fgf signaling through the MapK cascade promotes Vhnf1 protein activity. We considered the possibility that MapKs may directly regulate Vhnf1 by phosphorylation. However, Vhnf1 is a poor substrate for Erk2 in an in-vitro assay and has only marginal consensus MapK phosphorylation sites (data not shown). More indirect mechanisms, in which MapK-signaling activates other proteins or expression of intermediate target genes, remain to be investigated.

Different aspects of rhombomere identity are regulated independently by Val and Vhnf1

The specification of r5 and r6 identities requires both the activation of r5- and r6-specific genes and the repression of r4-specific genes. Our data demonstrate that while *vhnf1* and *val* probably function in a linear pathway to activate r5- and r6-specific genes, the repression of r4-specific genes is more complex. Vhnf1 functions through Val to repress *ephrin-B2a* and thus repress r4-specific cell-surface properties, but it does not require Val to repress *hoxb1a* and thus repress r4-specific neuronal differentiation. As a result, *vhnf1* and *val* mutants have different '*hox* codes' and patterns of neuronal differentiation, but have similar '*Eph-ephrin* codes' and cell behaviors in genetic mosaics. These results show that different aspects of segment identity, in this case neuronal phenotype and cell-surface character, can be regulated independently.

vhnf1 and *val* participate in a multistep process to repress the r4 '*hox* code'

Wiellette and Sive (Wiellette and Sive, 2003) propose that a sweep of r4 identity from posterior to the r3/4 boundary is subsequently restricted to r4 by the expression of *vhnf1*. However, the expansion of r4 fates in *vhnf1* mutants is not complete, suggesting that multiple factors are required for the restriction of r4 fates.

Our analysis of r4 marker expression shows that *hoxb1a* expression is transiently downregulated posterior to r4 in *vhnf1* mutants, as in wt embryos. Thus, an unknown factor functions to repress the earliest *hoxb1a* expression in the presumptive r5/6 territory. After the onset of its expression, *vhnf1* rapidly becomes the primary repressor of *hoxb1a* expression. However, *vhnf1* does not repress all r4-specific gene expression as predicted by Wiellette and Sive (Wiellette and Sive, 2003), since *fgf8* and *cyp26b1* are restricted anterior to the r4/5 boundary even in *vhnf1* mutants. As a result, the expanded 'r4' territory in *vhnf1* mutants has different molecular identity from r4 proper. Consistent with this incomplete transformation, the duplication of r4-specific Mauthner cells in *vhnf1* mutants is not fully penetrant. Together, these results show that r4 is distinguished from the more posterior hindbrain by more than simply the expression of *vhnf1* posterior to the r4-5 boundary.

Once the repression of *hoxb1a* is strongly established by Vhnf1, the expression of *vhnf1* recedes from the hindbrain. This coincides with the period that *hoxb1a* expands slightly in *val*$^{-}$ embryos (Prince et al., 1998), suggesting that Val is required at later stages to maintain repression of *hoxb1a* in r5 and r6. Val may function to repress *hoxb1a* by activating *hox3* genes, which have been shown to be required for the maintained repression of *hoxb1* in the mouse (Gaufo et al., 2003).

val is required for the repression of the r4 '*Eph-ephrin*' code and the establishment of r5-6 cell adhesive properties

Wiellette and Sive (Wiellette and Sive, 2003) suggested that *vhnf1* may function non-autonomously through an unknown signal to specify the most anterior r5 fates, because they did not observe *vhnf1* expression extending to the r4-5 boundary. We have seen that the domain of *vhnf1* expression does include the entire r5 domain of *krox20* expression (Fig. 1A). Furthermore, our mosaic analysis demonstrates that *vhnf1* is required cell-autonomously for the acquisition of r5 and r6 fates (Fig. 4B,C), since *vhnf1*$^{-}$ cells are excluded from r5 and r6 of wild-type hosts.

In the absence of either *val* or *vhnf1*, cells in the r5-6 region acquire the same cell-surface properties as determined by reciprocally transplanting cells between the two mutants (Fig. 4D,E). This is in direct contrast to the distinct molecular phenotypes of the two mutants, including *hoxb1a* expression, but correlates with their similar patterns of *Eph* and *ephrin* expression. Cooke and colleagues (Cooke et al., 2001) demonstrated that cell sorting in *val* mosaics was attributable to repulsive signals between *val*$^{-}$ *ephrin*-expressing cells and wt *Eph*-expressing cells; the same mechanism probably explains the cell sorting we observed in *vhnf1* mosaics. The similar effects on *Eph* and *ephrin* expression in *val* and *vhnf1* mutants, and our observation that *vhnf1* requires *val* to repress *ephrin-B2a* when it is overexpressed, suggest that *vhnf1* functions through *val* to specify the cell-surface character of the r5-6 region, including repression of r4-specific adhesive character (i.e. *ephrin-B2a*).

Together, our data support a multistep model for the initial restriction of r4 identity and the specification of r5-6 development (Fig. 6). r4 identity is initially restricted by the repression of *hoxb1a* in the presumptive r5-6 region by an unknown, *vhnf1*-independent, mechanism. *vhnf1* expression is activated by RA up to the r4-5 boundary and strictly reinforces the restriction of *hoxb1a* to r4, thereby limiting the expression of *fgf3* and development of r4-specific Mauthner neurons. Vhnf1 also cooperates with Fgfs expressed in r4 to activate the expression of *val* and the r5-6 program of development. *val* subsequently drives the expression of r5-6 specific Hox genes and the development of r5-6-specific neurons. Although *val* is not strongly required for the repression of *hoxb1a* expression, it is required for the repression of r4-like cell-surface characteristics that drive cell sorting as mediated by *Eph* and *ephrin* expression. The different requirements for *vhnf1* and *val* in the specification of '*hox* code' and '*Eph-ephrin* code' demonstrate that the mechanisms that specify segmental neuronal identity and differential cell-surface characteristics between rhombomeres in the hindbrain can be independently regulated. Previous work showing that Eph and ephrin expression are regulated by *krox20* and *val/mafB/Kr* rather than by Hox genes (Cooke et al., 2001; Theil et al., 1998) had predicted that cell-surface characteristics could be regulated independently from other aspects of segment identity. However, due to cross-regulation between *krox20, val* and Hox genes, Hox expression and *Eph-ephrin* expression are generally coupled, so this prediction has not been tested. Our discovery of an instance in which Hox expression and *Eph-ephrin* expression are unlinked has allowed us to show directly that neuronal identity corresponds with '*hox* code', while cell sorting behaviors correspond with '*Eph-ephrin*' code.

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